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TITLE OF THE PHD THESIS

APPLICATION OF INTEGRATED BIOCHEMICAL PROCESSES FOR THE VALORISATION OF SHEEP DAIRY BIORESIDUES IN THE FRAMEWORK OF THE WASTE BIOREFINERY CONCEPT

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ABSTRACT

The efficient recovery of materials/energy from biowastes needs to be intensified as much as possible, both in quantitative and qualitative terms. The development of integrated waste biorefineries could significantly contribute to the transition from an unsustainable fossil-based economic model to a bio-based one.

The concept of biorefinery is not totally new, and its traditional meaning has evolved driven mainly by two pivotal needs, environmental and economic sustainability.

The flexible integration of different processes aimed at producing a mix of biofuels and bioproducts, known as cascade approach, supports economic sustainability, since it makes possible hitting the market with an appropriate mix of products characterised either by significant market size or high added value. Such a flexible integration also has a high environmental value. As the number of usable and marketable outputs increases, this would logically correspond to less waste production, thus approaching the zero-waste concept.

The improvement in environmental sustainability is also the main element governing the desirable transition towards the deployment of waste biorefineries as new generation of biorefineries.

Indeed, the use of residual biomass would entail further environmental and economic benefits, such as the environmentally sound management of residues through valorisation and the reduction of production costs, since waste biomass is a widely available and inexpensive feedstock. Furthermore, the use of residual waste biomass would also contribute to the reduction of CO_2 emissions, considering that it is a renewable source for biofuel and bioproducts production, in contrast with fossil sources.

The present PhD thesis presents and describes a study which, framed in the above-described context and consistent with it, aims at a multi-step valorisation of sheep cheese whey (SCW), the primary waste product of the sheep dairy supply chain.

The proposed valorisation approach is based mainly on the high SCW lactose content, which is well suited to be converted through dark fermentation (DF) and anaerobic digestion (AD) into marketable gaseous, such as biohydrogen and biomethane, and soluble products, such as organic acids (OA).

The results attained during the experimental activities suggest that valorisation of sheep cheese whey would be possible by applying a multi-stage process aimed at energy and/or material recovery according to the request coming from each specific context.

Dark fermentation represents the core of the multi-stage process since can convert the lactose into shares of biohydrogen and organic acids as a function of the process operating parameters adopted.

In the framework of the present thesis, a maximum lactic acid yield was attained of 69 g_{HLa} L_{SCW}^{-1} when the operating pH was set at 6.0 and the fermentation time at 45 h.

A maximum yield of 5 L_{H2} L_{SCW}^{-1} was observed by adopting the same value of operating pH, but extending the fermentation time up to 168 h; furthermore, promising yields of marketable VFAs were attained.

The pool of organic acids obtainable through CW DF also proved to be suitable precursors for polyhydroxyalkanoates (PHA) production, even in the absence of specific inoculum and extra nutrients. The high nutrient content of sheep cheese whey made possible the selection of PHA-storing biomass without extra nitrogen supply, but on the other hand, it could also represent a limiting factor for PHA accumulation.

An overall yield 11-19 g PHA per litre of sheep cheese whey was obtained in function of the adopted pH in the fermentation stage. The adopted pH during the fermentation stage also affected, besides hydrogen and VFAs yields, the quality of the biopolymer produced in terms of HV fraction.

Dark fermentation is known to be suited to be performed as the first step in a double-stage CW methanization process, though the overall specific energy recovery observed in the present study resulted in being slightly lower than what obtained through single-stage AD, 0.81 vs 0.91 MJ L_{scw}^{-1} respectively. However, the two-stage approach may still be attractive in terms of process stability.

Furthermore, another possible valorisation route could be oriented toward the direct extraction of the valuable organic acids contained in the DF effluents through different systems, such as membrane extraction. The results obtained in the framework of this thesis showed that co-production of hydrogen and the recovery of a valuable organic acid like butyric acid is possible through DF of cheese whey and extraction with silicone membrane.

The results attained during the three years activity are promising and showed the inherent potential of the dairy waste to produce high-value products through a waste biorefinery approach.

The implementation of such integrated systems aimed at energy and material recovery from dairy wastes could support the dairy supply chain promoting environmentally sound practices, implementing circular bioeconomy concepts and creating new economic opportunities in rural areas.

SOMMARIO

Al giorno d'oggi, l'efficiente recupero di materiali e/o energia a partire da rifiuti di natura organica deve essere intensificato il più possibile, sia da un punto di vista quantitativo che qualitativo. A tal riguardo, lo sviluppo di bioraffinerie integrate di rifiuti potrebbe contribuire in modo significativo alla auspicata transizione dall'attuale modello economico basato su fonti fossili non rinnovabili ad uno basato su fonti biologiche e rinnovabili.

Il concetto di bioraffineria non è del tutto nuovo e il suo significato tradizionale si è evoluto nel tempo guidato principalmente da due esigenze fondamentali: da una parte la sostenibilità ambientale e dall'altra quella economica.

L'integrazione flessibile di diversi processi orientati a produrre un mix di biocarburanti e bioprodotti, noto come approccio a cascata, supporta la sostenibilità economica di una bioraffineria, in quanto finalizzato a ottenere i vantaggi di proporsi sul mercato con un mix adeguato di prodotti caratterizzati da significative fette di mercato (tipiche dei biocarburanti) oppure da prodotti che occupano nicchie di mercato ma caratterizzati da un elevato valore aggiunto. Un'integrazione così flessibile possiede altresì un alto valore ambientale poiché all'aumentare del numero di prodotti utilizzabili e commercializzabili corrisponde teoricamente ad una minore produzione di rifiuti, avvicinandosi così al concetto di rifiuto zero.

Il miglioramento della sostenibilità ambientale è anche l'aspetto principale che promuove la diffusione di bioraffinerie di rifiuti come nuova generazione di bioraffinerie.

In effetti, l'utilizzo di biomassa residuale comporterebbe ulteriori vantaggi di natura sia ambientale che economica, come la gestione ecologicamente corretta dei residui attraverso la valorizzazione e la riduzione dei costi di produzione, poiché la biomassa di scarto è una materia prima disponibile ed economica. Inoltre, l'utilizzo della biomassa residuale contribuirebbe anche alla riduzione delle emissioni di gas serra quali la CO_2, considerato che la biomassa può essere vista come una fonte rinnovabile per la produzione di biocarburanti e bioprodotti, a differenza delle fonti fossili.

La presente tesi di dottorato presenta e descrive uno studio che, inquadrato nel contesto sopra descritto e coerentemente con esso, mira a una valorizzazione in più fasi del siero di latte ovino (SCW), considerato il principale prodotto di scarto nella filiera lattiero caseario ovina.

L'approccio di valorizzazione proposto si basa principalmente sul suo elevato contenuto di lattosio, il quale risulta essere particolarmente adatto ad essere convertito attraverso processi di *Dark Fermentation* (DF) e di digestione anaerobica (AD) in prodotti gassosi commercializzabili, come bioidrogeno e biometano, e prodotti solubili, come acidi organici (OA).

I risultati ottenuti nel corso delle attività sperimentali di questa tesi suggeriscono che la valorizzazione del siero di latte ovino sia possibile applicando un processo multifase finalizzato al recupero di energia e/o materiali in base alla richiesta proveniente da ogni specifico contesto.

La *Dark Fermentation* rappresenta il nucleo del processo a più stadi poiché può convertire il lattosio in quote di bioidrogeno e acidi organici in funzione dei parametri operativi adottati.

Nel quadro della presente tesi, è stata ottenuta una resa massima di acido lattico pari a 69 g_{HLa} L_{SCW}^{-1} fissando il pH operativo a 6 e il tempo di fermentazione a 45 ore.

Inoltre, è stata ottenuta una resa massima di 5 L_{H2} L_{SCW}^{-1} adottando lo stesso valore di pH operativo, ma estendendo il tempo di fermentazione fino a 168 h. Allo stesso tempo sono stati raggiunti rendimenti promettenti in termini di acidi organici volatili (VFA) che potrebbero trovare una collocazione sul mercato oppure essere utilizzati per ulteriori processi di valorizzazione.

Lo stesso pool di acidi organici ottenuto si è infatti rivelato particolarmente adatto per la produzione di un particolare tipo di biopolimeri, i poliidrossialcanoati (PHA), anche in assenza di uno specifico inoculo e di nutrienti aggiuntivi. Tuttavia, mentre da una parte, l'alto contenuto di nutrienti del siero di latte ovino ha reso possibile la selezione di una biomassa in grado di immagazzinare al suo interno PHA senza ulteriore apporto di azoto, dall'altra esso potrebbe rappresentare un fattore limitante per l'accumulo di PHA nella fase successiva. Complessivamente è stata ottenuta una resa di 11-19 g di PHA per litro di siero di latte ovino in funzione del pH adottato nel corso della fase fermentazione. Il pH adottato durante la fase di fermentazione ha influenzato, oltre alle rese di bioidrogeno e VFA, anche la qualità stessa del biopolimero prodotto in termini di rapporto tra la frazione di HB e quella di HV.

Contestualmente, è inoltre noto come una fase di fermentazione sia particolarmente adatta per essere eseguita come il primo passo di un processo di metanizzazione in doppio stadio del siero di latte ovino. Tuttavia, nell'ambito di questo lavoro, il recupero specifico di energia complessivo osservato è risultato essere ad essere leggermente inferiore a quello ottenuto mediante digestione anaerobica in singolo stadio, 0,81 vs 0,91 MJ L_{SCW}^{-1} rispettivamente. Nonostante questo, l'approccio di digestione anaerobica in due fasi potrebbe essere ancora interessante, soprattutto in termini di stabilità del processo.

In aggiunta alle prove procedenti, nel corso di questo lavoro è stata investigata anche la possibilità di estrarre gli acidi organici prodotti nel corso della fermentazione del siero (in questo caso bovino) attraverso l'utilizzo di una membrana in silicone. La membrana si è rivelata essere particolarmente selettiva nei confronti dei diversi acidi presenti e, nelle condizioni valutate, è stato possibile estrarre acido butirrico direttamente dal reattore di fermentazione oppure a valle dello stesso.

Nel loro complesso, i risultati ottenuti durante i tre anni di attività possono essere considerati promettenti e hanno mostrato il potenziale intrinseco dei rifiuti lattiero-caseari nella produzione di composti di alto valore attraverso un approccio fondato sul concetto di bioraffineria per rifiuti.

In una visione più ampia, l'implementazione di sistemi integrati finalizzati al recupero di energia e materiali dai rifiuti lattiero-caseari potrebbe sostenere l'intera filiera lattiero casearia ovina promuovendo pratiche ecocompatibili e implementando i concetti propri di una bioeconomia circolare che possono portare a nuove opportunità economiche nelle aree rurali.

CONTENTS

LIST OF ABBREVIATIONS

AD	Anaerobic digestion
AS	Activated sludge
AS	Activated sludge
BBI-JU	Bio-Based Industries Joint Undertaking
BCI	Biorefinery complex index
BCP	Biorefinery complexity profile
BCW	Bovine cheese whey
BES	Bioelectrochemical systems
BMP	Biomethane potential
BSA	Bovine serum albumin
CAGR	Compound annual growth rate
CHP	Combined heat and power
CW	Cheese whey
DF	Dark fermentation
DO	Dissolved oxygen
DOC	Dissolved organic carbon
EEA	European Environmental Agency
EtOH	Ethanol
EU	European Union
FAO	Food and Agriculture Organisation of the United Nation
FBCW	Fermented bovine cheese whey
FCI	Feature complexity index
FID	Flame ionisation detector
FSCW	Fermented sheep cheese whey
FSSc	Fermented Scotta
GHG	Greenhouses gases
HAc	Acetic acid
HBu	Butyric acid
HLa	Lactic acid
HPB	Hydrogen producing bacteria
HPr	Propionic acid
HPY	Hydrogen production yield
HRT	Hydraulic retention time
HVa	Valeric acid
LAB	Lactic acid bacteria
LCA	Life cycle assessment
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
MMC	Mixed microbial cultures
PE	Polyethylene
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PLA	Polylactic acid
PMC	Pure microbial culture
PVC	Polyvinyl chloride
SBR	Sequencing batch reactor
sCarb	Soluble carbohydrates
SCW	Sheep cheese whey
SDG	Sustainable development goals
SER	Specific energy recovery
sProte	Soluble proteins
SRT	Sludge retention time
SSc	Scotta
TCD	thermal conductivity detector
TEE	Technical, economic and environmental (assessment)
TOC	Total organic carbon
TRL	Technology readiness level
TS	Total solid
VFA	Volatile fatty acids
VS	Volatile solid
WWTP	Wastewater treatment plant

LIST OF PUBLICATIONS

Papers on journals

I. **Control of fermentation duration and pH to orient biochemicals and biofuels production from cheese whey.** Asunis F., De Gioannis G., Isipato M., Muntoni A., Polettini A., Pomi R., Rossi A., Spiga D. – *Bioresource Technology* (2019), Vol. 289. Doi: https://doi.org/10.1016/j.biortech.2019.121722.

Conference proceedings

II. **Dark fermentation of sheep cheese whey: biochemicals and biofuels production as a function of fermentation time and pH**. Asunis F., Boni M. R., De Gioannis G., Isipato M., Muntoni A., Polettini A., Pomi R., Rossi A., Spiga D. – *VENICE 2018 - 7th International Symposium on Energy from Biomass and Waste* – 15-18 October 2018 – Venice, Italy.

III. **Valorisation of ovine cheese whey through PHA production.** Asunis F., Brundu A.P., Carucci A., Cocco F.G., De Gioannis G., Isipato M., Muntoni A., Spiga D. – *CEST 2019 16th International Conference on Environmental Science & Technology* – 4-7 September 2019 – Rhodes, Greece. Available here.

IV. **Three-stage process for hydrogen and PHA production from sheep cheese whey**. Asunis F., Boni M. R., Brundu A.P., Cappai G., Carucci A., Cocco F. G., De Gioannis G., Isipato M., Muntoni A., Polettini A., Pomi R., Rossi A., Spiga D. – *SARDINIA 2019 17th international waste management and landfill symposium* – 30 September – 4 October 2019– Santa Margherita di Pula, Italy.

GENERAL INTRODUCTION

In the last decade, the European Commission has sent clear policy signals concerning the importance it attaches to the European bioeconomy. A circular bioeconomy model covers the use of renewable biological resources - biowastes included - and their conversion into food, feed, bio-based products and biofuels by a range of technologies (European Commission, 2012).

A significant recent development has been the adoption in 2018 of the updated Bioeconomy Strategy, which aims at accelerating the deployment of a sustainable European bioeconomy to maximise its contribution to the 2030 agenda and its sustainable development goals, along with the EU's commitments under the Paris agreement (European Commission, 2018a, 2017a).

In this framework, efficient reuse or recovery of materials and energy from biowastes needs to be intensified as much as possible, both in quantitative and qualitative terms. The development of integrated waste biorefineries could significantly contribute the transition from an unsustainable fossil-based economic model to a bio-based one.

The biorefinery concept is not entirely new, and its traditional meaning has evolved driven by the crucial needs of environmental and economic sustainability (Muntoni, 2019).

The flexible integration of different processes aimed at producing a mix of biofuels and bioproducts is known as cascade approach, which can be traditional or inverse, and it supports the economic sustainability since it makes possible hitting the market with an appropriate mix of products characterised either by significant market size - typical of biofuels - or high added value.

Such a flexible integration also has a high environmental value. As the number of usable and marketable outputs increases, this would logically correspond to less waste production, thus approaching the zero-waste concept (Muntoni, 2019).

The improvement in environmental sustainability is also the main element governing the desirable transition towards the deployment of a new generation of biorefineries: waste biorefineries.

Indeed, the use of residual biomass would entail further environmental and economic benefits, such as the environmentally sound management of residues through valorisation and the reduction of production costs, since waste biomass is a widely available and inexpensive feedstock. Furthermore, the use of residual waste biomass would also contribute to the reduction of CO_2 emissions, considering that it is a renewable source for biofuel and bioproducts production, in contrast with fossil sources.

The present PhD thesis presents and describes a study which, framed in the above-described context and consistent with it, aims at a multi-step valorisation of sheep cheese whey (SCW), the primary waste product of the sheep dairy supply chain.

The reasons behind the study have a dual nature, one is socio-economic, the other is about technology development.

On the one hand, the EU milk sheep sector, with a production of 2.9 million tonnes in 2016, is recognised as a crucial sector in southern European countries, such as Greece, Italy, Spain, France (European Commission and Eurostat, 2015). Sheep grazing on meadows in the countryside are considered as part of the landscape as well a source of employment in disadvantaged agricultural areas. The high-quality traditional dairy products are recognised as the result of sustainable and multifunctional form of agriculture that contributes to preserving the environment and social cohesion in rural areas (Rossi, 2016). Nevertheless, the sheep dairy supply chain is recently experiencing painful economic difficulties, i.e. price volatility in a global and hypercompetitive market, low remuneration of the raw material, the small size of dairy farm, ages of the farmers and low generational turnover.

On the other hand, the high specific production (around 0.9 L per L of processed milk) and chemical composition make SCW an outstanding substrate for a multi-step biological valorisation aimed at recovering either biofuels and valuable bioproducts.

SCW is characterised by a high TOC (32 g L^{-1}), mainly composed of soluble carbohydrates, mostly in the form of lactose (46 g L^{-1}) (Asunis et al., 2019). The other main components of SCW derived from the raw milk involved: sheep milk proteins (5.5 g/100 g) and fats (5.9 g/100 g) are generally higher compared to cow milk (3.4 and 3.3 g/100g, respectively) (Balthazar et al., 2017). Additionally, significant amounts of other valuable components, such as citric acid, vitamins and minerals are also present in the composition of SCW.

Despite these exciting features for valorisation, the use of this specific substrate is hardly reported in the literature. The causes could be ascribed to the small size of European sheep dairy industry compared to the cow one (only 3% of the total European milk production is from sheep) and to its lack of technical and innovative development during the last decades.

The goals of the present thesis are thus meant to support the modernisation and strengthening of the sheep dairy industry processes, through the creation of new value chains as well as greener and more cost-effective production processes. At the same time, the utilisation of waste streams for production of value-added products not only improves the economics of such products but also provides industry with a strategy to overcome disposal problems of cheese whey.

More in detail, the proposed valorisation approach is based mainly on the high SCW lactose content (around 39-60 g L^{-1}, Prazeres et al. (2012)), which is well suited to be converted through dark fermentation (DF) and anaerobic digestion (AD) into marketable gaseous, such as biohydrogen and biomethane, and soluble products, such as organic acids.

While many studies have already addressed the issue of biohydrogen production from cheese whey (Akhlaghi et al., 2017a; Blanco et al., 2019; Colombo et al., 2019; De Gioannis et al., 2014; Ferreira Rosa et

al., 2014b; Montecchio et al., 2018; Perna et al., 2013), further efforts are still required to achieve the goal of a significant and steady hydrogen production.

Furthermore, the possibility of recovering, through proper optimisation of the operating parameters, other valuable products along with hydrogen has been mostly overlooked.

Indeed, during fermentation only 30 - 40% of the organic substrate is utilised for biogas (H_2 + CO_2) production, while the remaining 60 - 70% is converted into a range of soluble metabolites, the nature of which depends on the specific metabolic pathways prevailing (Sarma et al., 2015). The exploitation of such metabolites may involve direct separation and commercialisation of lactic acid or specific short-chain fatty acids, which are polyhydroxyalkanoates (PHA) precursors.

For the best of our knowledge, no studies are reported for PHA production from SCW.

PHA, produced and accumulated by different bacterial genera from various carbon sources under the form of granules into the bacterial cells as carbon and energy storage, are of interest due to their comparable properties to petroleum-based plastics (Amaro et al., 2019). However, the consolidation on the market for PHAs is currently limited by the high production costs due to the use of pure culture or either genetically modified bacteria and expensive feedstock (Johnson et al., 2010). Feedstock could account for about 40% of the total production cost (Amaro et al., 2019; Choi and Lee, 1999). The use of biowaste or a low-cost by-product as a starting substrate could overcome those problems and make the PHA competitive with the fossil-based plastic (Amaro et al., 2019). Moreover, combining the use of a biowaste carbon source with mixed microbial cultures (MMC), which do not require sterile conditions and expensive feedstocks, is interesting because it could further reduce production costs and make the process more sustainable, both economically and environmentally. Various authors have used different cheese whey to produce PHA by using selected MMC, such as raw cheese whey (Colombo et al., 2016; Valentino et al., 2017), second cheese whey and concentrated cheese whey powder (Colombo et al., 2019), cheese whey powder (Duque et al., 2014) and whey permeate (Carletto, 2014).

The present PhD thesis is organised according to the following different chapters.

The first chapter provides a general overview of the biorefinery concept, highlighting its links with the concept of circular bioeconomy and giving some key point about biorefineries definition and classification.

The second chapter presents a general overview about the dairy supply chain, with more emphasis on the sheep milk sector, and the generated residues, the latter in terms of management and innovative treatment and valorisation technologies.

The third chapter provides some theoretical fundamentals about the main biological processes studied in this thesis, namely dark fermentation, anaerobic digestion and PHA production.

The following chapters present and discuss the results of the experimental activity carried out during the three years of PhD activity. The experimental activity involves the processes of dark fermentation (chapter

5), biological PHA production (chapter 6), anaerobic digestion (chapter 7) as well as the extraction and the recovery of VFA (chapter 8).

The final chapter provides some general conclusions and recommendations for future research, according to the author's point of view.

1 THE ROLE OF ORGANIC WASTE IN A CIRCULAR BIOECONOMY

1.1 Production and issues associated with biowastes

Nowadays, humanity is facing the rising generation of wastes, as a result of the linear economy (take, use and dispose) and the growing urban population. In 2016, the total waste generated among the 28 members of the European Union (EU) by all economic activities and households amounted to 2 538 million tonnes; this was the highest production recorded for the EU-28 during the period 2004-2016 and corresponds roughly to 5 tonnes of waste generated per EU inhabitant.

In the wide variety of waste produced, an important role is played by those to which the term bio can be associated in various ways. This typology of residues is peculiar for origin, quantity produced, biochemical characteristics, environmental impacts associated with a possible inappropriate management, valorisation opportunities.

The lack of standardised definitions makes it difficult to assess the produced volumes of this category of waste.

Biowaste, as defined in the EU Waste Framework Directive (2008/98/EC), includes garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises as well as comparable waste from food processing plants (European Parliament and Council, 2008). Between 118 and 138 million tonnes of biowaste are generated annually, and this corresponds to approximately 300 kg of biowaste produced per EU citizen per year (EU-JRC-IES, 2011).

Incidentally, European definition of biowaste should not be confused with the broader term “biodegradable waste” as defined in the Landfill Directive (1999/31/EC), which also covers other biodegradable materials such as wood, paper, cardboard, sewage sludge, natural textiles. The amount of biodegradable waste exceeds 300 million tonnes generated every year in the EU (European Commission, 2012).

In the framework of biowaste production, food waste is estimated to be around 90-100 Mt year^{-1} with an estimated management cost of 143 billion euros (European Commission, 2008; FUSION, 2016). Food waste is composed of raw or cooked food materials and includes food loss before, during or after meal preparation in the household, as well as food discarded in the process of manufacturing, distribution, retail and food service activities. The global food and drink industry generates food supply chain waste on a multi-tonne scale every year, whose characteristics can vary widely from activity to activity. More in detail, the food supply chain waste could include brewer’s spent grain, grapes, olive pomace, surplus cheese whey, spent coffee grounds, tomato pomace, citrus peels as well a wide range of agro-industrial wastewater. Fava and co-workers estimated that the whole food European sector generates about 250 Mt year^{-1} of by-products

and residues (Fava et al., 2015). Table 1.1 presents a synthetic and partial picture of the production of some of the above-mentioned biodegradable wastes in Europe.

In the present thesis, the term biowaste will be used from now on, for example when dealing with the topic of waste bio-refineries, according to a rather broad meaning, encompassing any biodegradable organic waste, i.e. agricultural, organic household, forestry, food processing wastes and biosludge from Waste Water Treatment Plant (WWTP).

As mentioned above, improper management of solid and liquid biowaste has severe environmental repercussions.

The fate of a considerable share of the residues produced by the food supply chain (such as dairies or wineries) is, sometimes, even difficult to know. Only about 25% (about of 30 million tons per year in Europe) of solid food waste is effectively recycled into high-quality compost and stabilised digestate (Siebert, 2015), while a large share is still landfilled, leading to the generation of leachate and the release of uncontrolled greenhouse gases (GHG). The release of leachate into the environment can contaminate surface and groundwater and contribute to eutrophication problems. The production of greenhouse gases is considered the main environmental threat related to biowaste management. In general, in terms of GHG emissions, the waste sector is the fourth largest in EU, after energy, agriculture and industrial activities. The attention given to CO_2 emission is deserved in relation to the large quantities produced. However, methane is much more powerful as a greenhouse gas. It was estimated that methane emission from uncontrolled biowaste disposal in open dumps or sanitary landfills contributed for some 3% of total greenhouse gas emissions in the EU-28+ISL in 2017 (European Environmental Agency, 2018).

Moreover, landfilling leads to the diversion of carbon and nutrients in the biowaste away from ecosystems, making it unavailable for reuse. Inadequate management of food waste also has ethical implications; according to FAO (Food and Agriculture Organization of the United Nations - FAO, 2013), one-third of food produced for human consumption across the world is wasted or lost and adds 3 300 million tonnes of CO_2 equivalent GHG emitted to the planet atmosphere. Only the United States and China emit more. Finally, unmanaged biowaste poses local scale problems such as unpleasant odours, attraction of insects, rodents and other disease vectors.

The other side of the coin is represented by the numerous and exciting opportunities for valorisation that characterise biowaste. The adoption of the European Circular Economy Package by the EU Commission in December 2015 has opened the pathway for a resource-efficient society and sustainable recycling industry across Europe. In such a new context, biowaste is seen as a valuable biological resource and the biowaste management sector may play a pivotal role. The research work that is the subject of this doctoral thesis aims to bring a small contribution to this new deal.

Table 1.1. *Production of biodegradable wastes from various sources in Europe.*

Sector	Biodegradable waste	Production (Mt y^{-1})	Reference
Agricultural	Crop residue	122	Searle and Malins (2013)
Brewery	Brewer's spent grain	3.4	Pfaltzgraff et al. (2013)
Forestry	Forestry residue	40	Searle and Malins (2013)
Dairy industry	Cheese whey	13	Mollea et al. (2013)
Food	Food waste	88	Bio Intelligence Service (2010); European Commission (2008)
Olive mill	Olive mill wastewater Olive pomace	1.2÷30 4÷10	Pfaltzgraff et al. (2013); Scoma et al. (2014) Cristóbal et al. (2018); Scoma et al. (2014)
Orange industry	Orange peels	4.1	Cristóbal et al. (2018)
Potato industry	Potato peels	2.3	Cristóbal et al. (2018)
Pulp and paper industry	Pulping liquors, wood wastes, sludge, rejects	11	Monte et al. (2009)
Sugar beets	Sugar beets molasses Sugar beets pulp	0.3÷0.4 0.5÷1.5	Scoma et al. (2014) Scoma et al. (2014)
Tomato industry	Tomato pomace	0.1÷4	Cristóbal et al. (2018); Scoma et al. (2014)
Wine-making	Winery wastewater	11÷18	Scoma et al. (2014)
	Grape pomace	1.3÷2.8	Scoma et al. (2014)

1.2 Toward a European circular bioeconomy

The concepts of both the circular economy and bioeconomy have been introduced during the last decade in EU in response to concerns about the long-term viability of the prevailing resource-intensive economic model and its effect on the environment. Although different in origin, they both aim to contribute to strategic and operational EU policy objectives, for living healthy within the ecological limits of the planet (United Nation, 2015).

1.2.1 The EU action plan for the circular economy

In 2015, the EU set a concrete and ambitious action plan to support the transition toward a circular economy. The communication "Closing the loop – An EU action plan for the Circular Economy" defined the circular economy as economic system in which *"...the value of products, materials and resources is maintained in the economy for as long as possible, and the generation of waste minimised*" (European Commission, 2015). The EU action plan for the Circular Economy focuses on actions related to production, consumption, waste management, stimulation markets for secondary raw materials and water reuse. The priority areas of the action plan are plastics, food waste, critical raw materials, construction and demolition waste, and biomass and bio-based products. The action plan also focused on the need for innovation and investment in several and interconnected sectors as well as the need for continuous monitoring of the state of circular economy implementation.

The implementation of an EU circular economy is a relevant part of the EU continuous effort to transform Europe's economy into a more sustainable one. A circular economy seeks to increase the proportion of renewable or recyclable resources and reduce the consumption of raw materials and energy in the economy, while, at the same time, protecting the environment through cutting emissions and minimising material losses (European Environmental Agency, 2018). In 2019, the European Commission adopted a comprehensive report on the implementation of the Circular Economy Action Plan (European Commission, 2019). The EU Monitoring Framework for the Circular Economy shows that the transition has helped put the EU back on a path of job creation. In 2016, sectors relevant to the circular economy employed more than four million workers, a 6% increase compared to 2012 (European Commission, 2019). In the next years, additional jobs are expected to be created to meet the expected demand generated by fully functioning markets for secondary raw materials (European Commission, 2018b). Circularity has also opened up new business opportunities, given rise to new business models and developed new markets, domestically and outside the EU. In 2016, circular activities such as repair, reuse or recycling generated almost €147 billion in value. In Europe, recycling of municipal waste during the period 2008-2016 has increased and the contribution of recycled materials to the overall materials demand shows continuous improvement. However, on average, recycled materials only meet less than 12 % of the EU demand for materials.

The action plan promoted for the first time a systemic approach across the entire value chains of waste management. With regards of wastes, proper waste management (biowaste included) is an essential block of a circular economy, and it determines how the EU waste hierarchy is put into practice (European Commission, 2015; European Environmental Agency, 2018). The waste legislative framework was revised in 2018 with the aim of modernising waste management systems in the EU. In particular, much emphasis has been given to transforming wastes to resources. European commission pointed out the role of waste-to-energy in the circular economy intending to avoid unnecessary loss of valuable resource through landfilling and incineration (European Commission, 2017b). Moreover, EU members have been encouraged to identify technologies for efficient energy and material-efficient, to make better use of economic instruments and improve planning to avoid incineration overcapacity.

Nowadays, technologies such as composting or anaerobic digestion (AD) for biowaste management are seen as an excellent example of appropriate biowaste management in line with the circular economy. Combining AD and composting improves the environmental performance of the biowaste management with recovery of energy, in the form of biogas, and material, in the form of a composted digestate that could be used as fertiliser. For further details about the AD process see 3.4.2. Nevertheless, biowaste represents strategic biomass that can be used for new applications targeting bioproducts whose value exceeds that of biogas and compost.

1.2.2 The adoption of a European bioeconomy strategy

According to the European Commission, bioeconomy includes the production of renewable biological resources and the conversions of those resources and waste stream into value-added products, such as food, feed, bio-based products and bioenergy (European Commission, 2012). In early 2000, EU adopted a precise Bioeconomy Strategy with the primary goal to open the road to a more innovative, resource efficient and competitive society that reconciles food security with the sustainable use of renewable resources for industrial purposes, while ensuring environmental protection (European Commission, 2012). The strategy concerns agriculture, forestry, fisheries, food and pulp and paper production, as well as parts of chemical, biotechnological, energy and biowaste industries. In 2015, the Bioeconomy sector employed around 18 million persons in EU-28 and generated € 620 billion of value-added, representing 4.2% of the EU-28 GDP (Ronzon and M'Barek, 2018). The Bioeconomy strategy identified three fundamental areas of action: investment in research, innovation and skills; reinforced policy interaction and stakeholder engagement; enhancement of markets and competitiveness in bioeconomy (European Commission, 2018a).

The Bioeconomy Strategy has been recently updated in order to accelerate the deployment of a sustainable European Bioeconomy with more emphasis on its contribution to EU's priorities and objectives on Circular Economy, Industrial Policy, Energy Union a and Common Agricultural Policy, as well as global sustainability objectives such as the Paris Agreement and the United Nations Sustainable Development Goals (European Commission, 2018a). In the last decade, the EU Bioeconomy Strategy raised Member States' awareness of the opportunities that bioeconomy offers for development. As a result, national strategies and other policy initiatives dedicated to the bioeconomy have adopted or are under development in many of the Member States of the EU. The governments in France, Italy, Austria, Latvia, Norway, Spain, Finland, Ireland have already dedicated bioeconomy strategy at national level (IEA Bioenergy Task42, 2018).

However, specific features of national bioeconomies can be observed across the EU, showing that there is not a unique approach to the bioeconomy concept, but this approach must be adapted to the local contexts. For instance, the bioeconomy in Finland and Sweden is more oriented towards the forest-based sector, with the manufacture of paper (25% and 20% of their bioeconomy value-added, respectively). The manufacture of bio-based textiles generates 14% and 16% of the bioeconomy value in Italy and Portugal. The manufacture of bio-based chemicals, pharmaceuticals, plastics and rubber generates 36% and 35% of bioeconomy value-added of Ireland and Denmark respectively (European Commission, 2018a).

In 2015, the highest value-added annual growth occurred in the manufacture of bio-based chemicals (excl. biofuels) (+26%), the production of bioelectricity (+15%) and the manufacture of rubber and bio-based plastics (+13%), generating altogether an additional € 3.5 billion of value-added compared to the year before. From 2009 to 2015, European employment has increased in biobased chemicals, bio-based pharmaceuticals, bio-based plastics and bio-based rubber involving approximately 300 000 people (European Commission, 2018a). Nowadays, with focus on Italy situation, bioeconomy represents the 10% of the production and the

7.7% of employment based on the total economic production in 2018, this means 328 millions of euros and 2 millions of people (Intesa Sanpaolo and Federchimic Assobiotec, 2019). Italy is in third place for bioeconomy value after Germany and France (403 and 248 million euros, respectively) and before Spain and UK (221 and 190 million euros) (Intesa Sanpaolo and Federchimica Assobiotec, 2019).

The transition to a bioeconomy would critically require knowledge from scientific research, and appropriate bio-based technologies need to be developed. In this framework, bioeconomy-related R&I has become a priority for most European regions, and the deployment of various biorefining strategies is expected to make a more significant contribution to achieving EU policy priorities in the future (Biobased Industries Consortium, 2018).

As described better in the following paragraph, the biorefinery concept is an essential building block to establish a dynamic bioeconomy. Biorefinery concept addresses some of the most relevant aspects of the bioeconomy strategy providing the technology to fulfil all those ambitious objectives.

In the EU, there are several financial opportunities to promote initiatives in the framework of bioeconomy. The European Structural Investment Funds makes available part of the overall € 450 billion budget for the period 2014-2020 to promote the bioeconomy development. Dedicated EU funding for the bioeconomy under the EU R&D Framework Programme has more than doubled: from € 1.9 billion in FP7 to € 4.5 billion in Horizon2020. The substantial investments in bioeconomy related R&I is already generating relevant multi-disciplinary and cross-sectoral scientific knowledge and promising technologies and innovations are emerging. Substantial private funding has also been mobilised through the Bio-Based Industries Joint Undertaking (BBI-JU), a public-private partnership which is leveraging € 2.7 billion of private investment through € 1 billion EU investment. The BBI-JU aims to establish a European bio-based industry sector that creates new markets and value chains, and that develops advanced and sustainable bio-based products, technologies, materials and biofuels from renewable natural resources from land and sea, waste and industrial side-streams (European Commission, 2017a).

1.3 The biorefinery concept

The Circular Economy Action Plan, the Bioeconomy Strategy, as well as the waste hierarchy directive, represent the policies addresses by the EU for the next future. From a technical point of view, biorefineries can be considered as one of the technologies suited to achieve those ambitious objectives, being able to close the loops of organic streams and aiming at a valorisation approach characterised by multiple outputs in line with a cascading recovery of resources. Therefore, biorefineries are the key to implement a future knowledge-driven and environmentally sound biobased economy.

The biorefinery concept itself is not new (e.g. production of vegetable oils, paper production, starch production), but advanced biorefinery concepts have evolved during the previous decades driven by

environmental and economic sustainability factors as well as political inputs and technology development (Muntoni, 2019).

1.3.1 Definition and classification of biorefineries

Several definitions of biorefinery have been elaborated during the last decades, with the definition of IEA Bioenergy Task 42 as one of the most cited. The IEA Bioenergy Task 42 defines biorefinery as "*sustainable processing of biomass into a spectrum of biobased products (food, feed, chemicals, materials) and bioenergy (biofuels, power and/or heat*)" (IEA Bioenergy Task42, 2012). This definition includes a heterogeneity of inputs and outputs as well as different possible configurations making challenging to provide a univocal classification for biorefineries. According to the literature, biorefineries could be classified by systems or model, the status of technological implementation, size, feedstocks, platforms, processes and products. The most common and accepted classification is by IEA Bioenergy – Task 42 Biorefining, according to which biorefinery facilities can be categorised based on four features, i.e. feedstock materials, platforms, conversion processes and generated products (Table 1.2). Figure 1.1 illustrates a schematic representation of the biorefinery classification system and associated elements, as developed in Task 42 (Cherubini and Jungmeier, 2009).

1.3.1.1 Feedstock

Feedstock is the raw organic material that is converted into marketable products. Agricultural, forestry, agro-industrial and aquaculture sector can supply renewable feedstocks. According to their origin, feedstocks could be divided into dedicated crops and organics residues. Dedicated crops are plants that do not produce food and are cultivated mainly for energy production such as sugar, starch, lignocellulosic and oil crops. Organic residues are bioproducts obtained from processing biomass such as lignocellulosic residues and agroindustrial organic residues, as well as the biodegradable fractions found in municipal organic waste. Among the main aspects to consider when selecting a feedstock there are its availability during the year, price, technology involved for its pretreatment and subsequent conversion and the required platform.

1.3.1.2 Platforms

The feedstocks could be processed into different platforms which are defined as intermediate products from biomass feedstocks towards products or linkage between different biorefinery concept or final products. The platforms might represent mixtures of compounds or more isolated compounds. The possible platforms include syngas platform, biogas platform, C5/C6 sugar platform, carboxylates platform, plant-based oil and algae oil platform, organic solutions platform, lignin platform and pyrolysis oil platform.

1.3.1.3 Processes

These platforms can convert the feedstock into a wide range of marketable products using combinations of different processes. The classification of the technological processes identifies four main subgroups: biochemical (e.g. fermentation, aerobic conversion, AD, enzymatic processes), thermochemical (e.g. combustion, gasification, pyrolysis), chemical (e.g. catalysis, pulping, esterification, electrolysis, steam reforming, hydrolysis) and mechanical/physical (e.g. extraction, fibre separation fractionation, pressing, size reduction). Various pretreatments or post-treatments could be required in addition to the conversion process.

1.3.1.4 Products

The products from the transformed feedstock can be classified into energy products (biodiesel, bioethanol, biogas, synthetic biofuels, electricity/heat) and non-energetic products (food, animal feed, biofertilizer, biomaterial, chemicals and building blocks, polymers & resins). According to those definitions, biorefineries could be subdivided into energy-driven and material-driven biorefineries. Some products can be classified simultaneously as biofuels or biochemicals depending on the use. As an example, bioethanol could be used as biofuels for truck transportation or as a building block for plastic production.

Table 1.2. *Classification of different biorefinery, as proposed by IEA bioenergy Task 42.*

Feedstocks	**dedicated crops**	oil crops; sugar crops; starch & LC crops; grasses; aquatic biomass
	organic residues	LC residues; oil-based residues; organic industrial and municipal waste
Platforms		C5 sugars; C6 sugars; oils; biogas; syngas; organic juice; pyrolytic liquid; lignin; electricity/heat
Processes	**thermochemical**	combustion; gasification; hydrothermal upgrading; pyrolysis
	biochemical	fermentation; AD; aerobic conversion; enzymatic processes
	chemical	catalysis; pulping; esterification; hydrolysis; steam reforming; electrolysis
	mechanical/physical	extraction; fibre separation; mech. fractionation; pressing; separation
Products	**energy products**	biodiesel; bioethanol; biogas; synthetic biofuels; electricity/heat
	material products	food; animal feed; fertilisers; biomaterials; chemicals & building blocks; polymers & resins

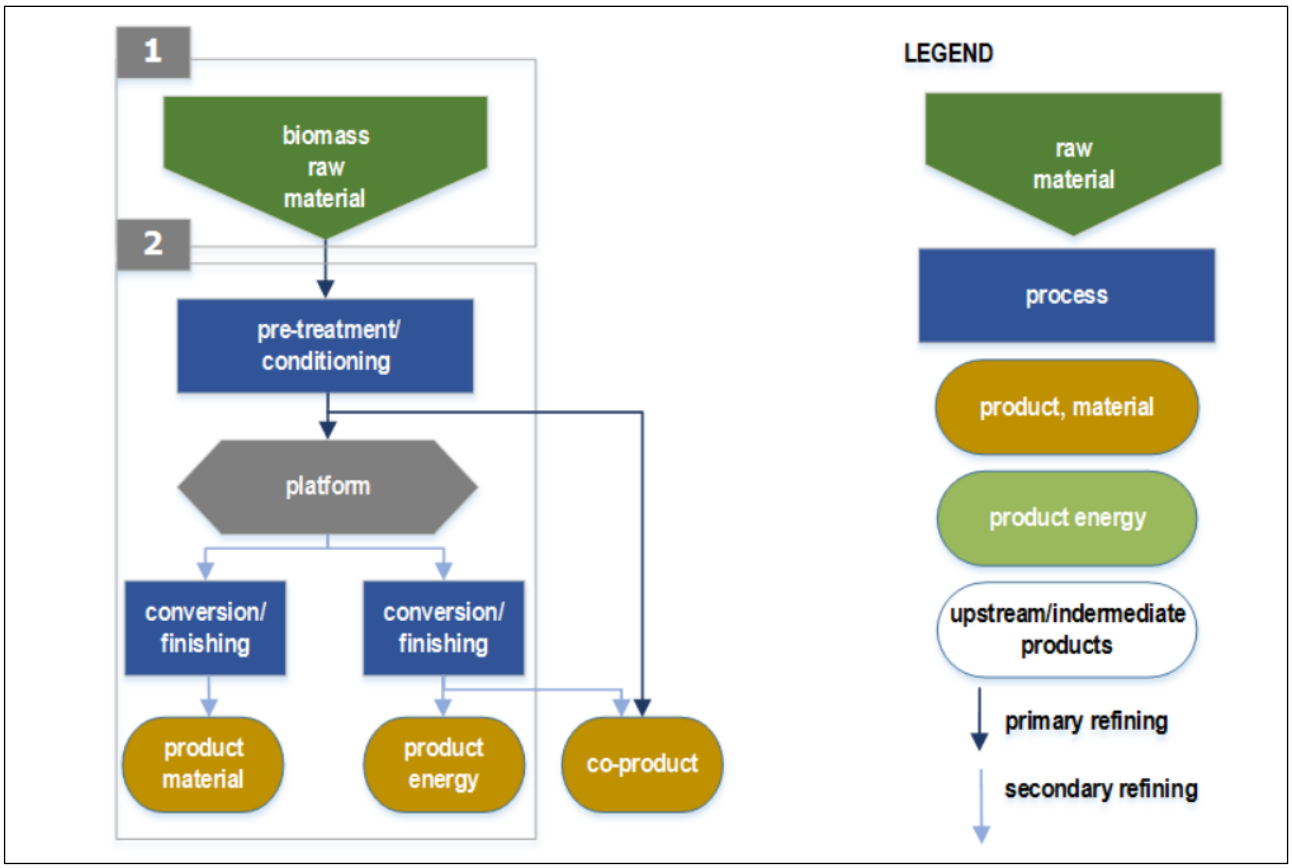


Figure 1.1. *Schematic representation of a biorefinery and associated element as described by IEA Bioenergy Task 42 (Cherubini and Jungmeier, 2009).*

1.3.2 Cascading recovery of resources

Despite the presence of numerous definitions and classification of biorefineries in literature, the principal and well-recognised feature of a biorefinery is the number and the type of obtained products. The output products are chosen considering not only the feedstock composition but also the product market demand. This kind of flexibility allows to change production cycles over time and protect the biorefinery against economic recession and seasonal demand cycles. The principle of processing feedstock for maximum yields and profit is usually referred as the cascade principle. The cascade-use of the feedstock for the production of biobased materials and energy in closed-loop process designs is the core principle that is addressed by different biorefinery pathways (IEA Bioenergy Task42, 2019). Biorefineries act in an analogous way to oil refinery, in which crude oil is processed to in a multi-step process obtain fuels and other products, which account for the 85% and 15% by mass of the initial input respectively. This multistep process could be oriented according to different outputs: biorefineries can produce energy in various forms, such as biofuels at large volumes and lower sale prices (inverse cascade) or molecules for chemistry, cosmetics or medicinal applications with low volumes but higher market prices (direct cascade). The selection of cascading type is site-specific, and it depends on existing specific strategies, priorities and other boundary condition, such as local availability of natural resources or energy supply policies. For countries that based its energy

consumption on the importation of fossil fuels (like European countries), inverse cascading could be more attractive. In this case, the deployment of biorefineries strategies will help to reduce the dependency on the fossil fuel and at the same time reducing greenhouse gases emission and stimulating regional and rural development (Aristizábal M and Cardona Alzate, 2018; Moncada et al., 2013).

Besides, the increase in the number of usable and marketable output would correspond to less waste production, thus approaching the theoretically zero-waste concept (Muntoni, 2019). As highlighted by several authors, the maximum use of the raw feedstock and the minimum production of residues is one of the sustainability challenges addressed to any biorefinery (Aristizábal M and Cardona Alzate, 2018; Moncada et al., 2013; Muntoni, 2019). From this point of view, a cascading recovery of resource could be the right approach to meet the environmental and economic sustainability of biorefineries.

1.3.3 Technical, economic and environmental assessment of biorefinery schemes

However, at the current state, biorefineries face some crucial challenges, which can be technical, economic and environmental. Unlike oil refineries, which can rely on mature technologies (optimised and highly predictable), the maturity level of technologies used in biorefinery is often considered a critical issue. Appropriate implementation of industrial biorefineries requires mature technologies, starting with R&D tasks, followed by pilot-plant tasks, demonstrations, and deployment strategies. Currently, different biorefinery concepts are under development showing various stages of development (TRL, Technology Readiness Level). Table 1.3 gives some examples, according to the feedstock used and the assigned TRL.

Table 1.3. *Overview of the main biorefinery approaches a function of feedstock and TRL, according to IEA Bioenergy Task42 (2019).*

Concept	Feedstock	TRL
Conventional biorefineries	starch (corn, wheat, cassava) and sugar crops (sugarcane, sugar beet), wood	9
Whole crop biorefineries	whole crop (including straw) cereals such as rye, wheat and maize	7-8
Oleochemical biorefineries	oil crops	7-9
Lignocellulosic feedstock biorefineries	lignocellulosic-rich biomass: e.g., straw, chaff, reed, miscanthus, wood	6-8
Green biorefineries	wet biomass: green crops and leaves, such as grass, sugar beet leaf	5-6
Marine biorefineries	aquatic biomass: microalgae and macroalgae	5-6

The implementation of biorefinery schemes requires reliable processing of various feedstocks able to prove environmentally superior products compared to their conventional counterparts and economically profitable production chains (IEA Bioenergy Task42, 2019). The characteristics and composition of feedstock should be well known to obtain optimal conversion, its optimal availability and economic benefit. The

feedstock heterogeneity is often considered as another challenge since it may require the use of different pre-treatment/valorisation processes. Another crucial element is that an integrated feedstock supply system is required to provide feedstock sustainably at a reasonable cost. In line with environmental sustainability, a biorefinery system should aim to waste minimisation, including the use of chemicals and energy. The biorefineries face challenges related to land-use change and its impact on the emission of pollutants and food security. When agricultural lands are used to obtain energy crops, the food supply and biodiversity can be affected leading to the well-know "Food-versus-energy dilemma" (Muntoni, 2019). The definition of biorefinery implies that the provided products and energy carriers demonstrate reduced environmental impacts compared to conventional products. Paragraph 1.4.2 gives a remarkable example about this aspect.

The large number of possible bio-based products theoretically obtainable within a biorefinery scheme must meet the quality and price requirement of the market. However, the economic feasibility of those products is currently still uncertain as their fossil counterparts are offered on the market at much lower cost. Besides, the integration of bioproducts in current value chains is an economic challenge in the biorefineries industrialisation. In this sense, support from the government and market pull initiative are an essential factor in determining the rate of development of biorefineries. Furthermore, social perception is also important. In the case of bioplastics production, such as Polyhydroxyalkanoates (PHA) for example, the high production cost compared with conventional fossil-based plastics could be compensated and justified by the increasingly favourable inclination of the customers to buy biodegradable e biobased plastic.

In addition, identification and optimisation of site-adapted biorefinery technologies and recycling paths from the various potentially available raw materials and conversion paths, as well as the implementation of a continuous improvement process, will potentially stimulate an accelerated market diffusion of the various biorefinery scheme. In the scientific literature, an increased interest in technical, economic and environmental (TEE) assessment of new biorefineries scheme has been observed recently but there is a lack of standardisation among different studies which are leading to poor comparability and inconsistency (Aristizábal M and Cardona Alzate, 2018; Cristóbal et al., 2018; IEA Bioenergy Task42, 2019; Moncada B et al., 2016; Moncada et al., 2013). The "Biorefinery fact sheets" consists of a brief description of the specific biorefinery scheme, including information regarding mass and energy balances as well as economic and environmental aspects (IEA Bioenergy Task42, 2019). Providing such a format enables an improved understanding of the value chains and allows the comparison of the different biorefinery concepts. Recently, IEA Bioenergy Task42 described a list of available biorefinery concepts and their environmental performance and economic feasibility based on available generic data in an "open access" approach, concerning assessment methodology and primary data origin to enable a strong knowledge-based community within the biorefinery sector (IEA Bioenergy Task42, 2019). More information about biorefineries classification, design and assessment could be found in a recent review by Aristizábal-Marulanda and Cardona Alzate (2018).

1.3.4 State of the art of biorefinery concept implementation in Europe

Although some biorefineries schemes, such as the first generation biorefineries for bioethanol production, are well-established technologies, others are still in the research-and-development, pilot or small-scale demonstration phase. Different attempts to quantify the state of art of European biorefineries have been made in the last years in order to support the transition towards a European circular bioeconomy.

In 2017, Bio-Based Industries Consortium identified 224 biorefineries across Europe (Figure 1.2). The picture distinguishes between "Sugar-/starch based biorefineries", producing bioethanol and other chemicals (63 plants), "Oil-/fat-based biorefineries - biodiesel" (64) and "Oil-/fat-based biorefineries - oleochemistry" (54), "Wood-based biorefineries" (25) excluding those that produce pulp for paper only, "Lignocellulose other than wood" (5) and finally "Biowaste-based biorefineries" (13).

In another study, 803 biorefineries have been identified in the EU, of which 507 produce bio-based chemicals, 363 liquid biofuels and 141 bio-based composites and fibres (multi-product facilities are counted more than once) (Parisi, 2018). Among those facilities, 177 are reported as integrated biorefineries that combine the production of bio-based products and energy (Parisi, 2018). This report shows that the location of most biorefineries corresponds to chemical clusters and ports, and the highest concentration of biorefineries is located in the central part of the EU, Belgium, and the Netherlands in particular. The Biowanze plant (Wanze, Belgium) is a clear example of first-generation biorefinery for the production of bioethanol at commercial scale. The plant has the capacity to produce 300 000 m^3 of bioethanol per year using agricultural and forestry biomass (wheat and sugar syrup) as feedstock and through the C5/C6 sugar platform. Other bioproducts include gluten and soluble protein concentrate. The Eastern part of the EU shows a lower number of biorefineries suggesting the presence of an untapped potential. Agricultural resources are the feedstock used by most biorefineries in all EU countries with the exception of Finland, Sweden and Portugal where the use of forestry feedstock is more predominant. Moreover, the report points out that marine and waste resources are relevant in some countries but not yet highly exploited within a biorefinery scheme.

Coming to the Italian situation, In Sardinia (Italy), Matrica (a joint venture between Novamont and ENI-Versalis) with the project FIRST2RUN is another clear example of the progressive transition of the European economy. The FIRST2RUN project aimed at demonstrating the techno, economic and environmental sustainability at industrial scale of a first-of-kind value chain where low input and underutilised oil crops (cardoon) grown in arid or marginal lands and not in competition with food or feed, are valorised for the extraction of vegetable oils. Those vegetable oils can be further converted into bio-monomers as building blocks for high added value bioproducts, biolubricants, cosmetics, bioplastics, additives through the integration of chemical and biotechnological processes. In Italy, they are worth to be mentioned the case of GFBiochemicals, aimed to scale-up a process for levulinic acid production directly from biomass, the project to reconvert the former chemical plant of Gela in a modern biorefinery promoted ENI, and the case of Bio-on, an industrial plant for the production of biopolymers (PHA) from agricultural waste.

A remarkable number of European projects based on biorefining are currently underway, and the number of biorefineries is expected to increase in the very next years.

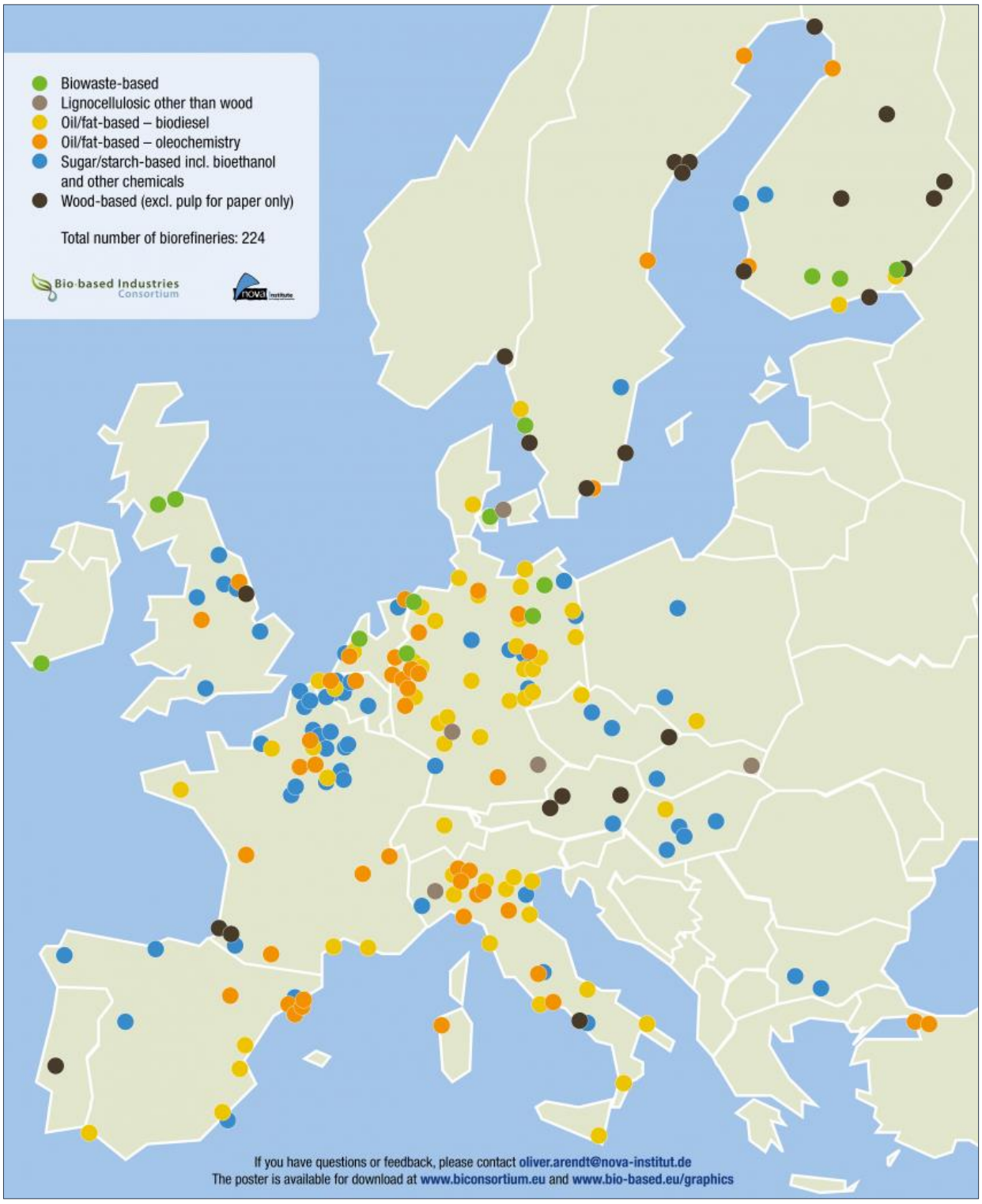


Figure 1.2. *Biorefineries installations in Europe in 2017 according to Biobased Industries Consortium.*

1.4 Waste biorefineries

As seen in the previous paragraph, the biorefinery concept is very case-specific, and its application depends on various and interconnected factors, such as availability and composition of the local biomass, the feasibility of the processes available for its conversion, local and political needs and market trends. For those reasons, biorefineries may have different production goals. Specific case biorefineries may be conceived for the specific type of products like the energy-producing biorefineries (bioethanol, biomethane). Furthermore, a biorefinery scheme can be used as an alternative biodegradable waste disposal method when efficient disposal is not suitable or with the purpose of fully valorise wastes, going beyond mere treatment.

Biorefining is an emerging concept in the field of biomass waste management supported by the idea that all kind of biomass-derived material can be converted into a different type of biofuels and chemical through a multi-step valorisation process. This consideration led to the definition of a new generation of biorefineries: waste biorefineries. The concept of a waste biorefinery involves the use of biowaste as a primary feedstock input for integrated processes aimed at converting the waste into bioenergy e/o biomaterials. The concept is recently raising a growing interest, as demonstrated by the increasing trend of scientific paper published in the field (Fava et al., 2015; Mohan et al., 2016; Muntoni, 2019; Vea et al., 2018).

1.4.1 Advantages of implementing waste biorefineries

As discussed in the previous paragraphs, the concepts of bioeconomy and circular economy have been introduced in the EU in response to concerns about the long-term sustainability of the prevailing resource-intensive economic model on the environment. Both the EU Circular Economy Action Plan and Bioeconomy Strategy have food waste, biomass and bio-based products as targets and aim to valorise biological residues. According to those considerations, the development of environmentally-sound biowaste management based on the concept of waste biorefinery may represent the link between bioeconomy and circular economy. The advantages of using this approach are several and multidisciplinary and, according to the biorefinery concept, can cover both environmental and economic aspects.

From the environmental point of view, a waste biorefinery approach will avoid the negative effect that can occur due to improper biowaste management, such as GHG and impacts on water and soil. This approach will contribute to control climate changes: the reduction of GHG emissions can be ascribed not only to avoiding the emissions related to the disposal of biowaste, but also to the fact that new products or energy are generated from a renewable source instead of from fossil fuels. Montazeri et al. (2016) carried out a meta-analysis of 86 Life cycle analysis (LCA) covering 34 bioproducts and found that in most of the cases a GHG reduction was achieved as compared to the conventional chemical production. Furthermore, the use of biowaste may also limit the need for importing external energy sources and avoiding the emission associated with transportation, considering that a waste biorefinery could represent a local feedstock. Other positive

aspects could be the prevention of uncontrolled discharging of biowaste in the environment. Those aspects are entirely consistent with the principles of prevention and recycling indicated in the EU waste directive.

Numerous sectors (e.g. the agricultural, forest-based, chemical and energy sectors) are starting to recognise the economic potential of biodegradable residue making available a wide range of opportunities to generate new products for the market. Innovation is expected to support the development of the market for bio-based products by stimulating industrial symbiosis between different biowaste producer (the waste produced by an industrial activity may be turned into the feedstock for another one)(European Commission, 2018a). The above considerations are entirely consistent with the aim of circular economy to keep the value of products, materials and resources in the economy as long as possible. At the same time, a biological waste stream could be converted in high-value product, as promoted by EU Bioeconomy strategy.

1.4.2 Promising platforms and processes

Following the definition of biorefinery given by IEA, the selected feedstocks could be processed into different platforms which are key intermediates that link the feedstocks and the final products. Considering the characteristics of biowastes as a feedstock, sugar and carboxylate platforms seems to be the most suitable and promising. Sugar platforms involves hydrolysis of complex carbohydrates (starch, hemicellulose) to obtained simpler sugar as glucose. Those simpler sugars act as input for biological fermentation processes, providing access to a variety of important chemical within the carboxylate platform. The bioconversion of the organic feedstock to short-chain carboxylates can be performed following different pathways by selected pure mixed cultures (PMC) or mixed microbial cultures (MMC) in engineered systems under anaerobic conditions (Agler et al., 2011). Carboxylates are dissociated organic acids characterised by the presence of at least one carboxyl group. The number of chemical building blocks obtainable by a carboxylate platform is considerable, and some of them have a high potential for market growth.

Furthermore, some fermentation pathways, such as dark fermentation (DF), involve the production of hydrogen, a promising biofuel, and alcohols in addition to generic carboxylates. The production of one or the other products in DF strongly depends on the process parameters applied, making it very flexible to different conditions and targeted final products. Additionally, the use of MMC in the DF process do not require sterile conditions, and this is a useful aspect when complex feedstock as biowaste are treated. The use of PMC for the treatment of biowaste may require a sterilisation step, that could strongly affect the economy of the process.

For those reasons, DF is believed to play a crucial role in a waste biorefinery scheme, acting as a combined sugar-carboxylates platform. However, this platform must be integrated with other processes for further downstream valorisation activities, according to the cascading recovery concept. Table 1.4 suggests some possible conversion processes that could be integrated within a waste biorefinery scheme. The table also reported an estimation of the TRL associated with each process. As for the biochemical process, it can be

noticed that AD and composting are well-established technologies, and they can be successfully used to obtain biogas and compost, while fermentation processes have a wider TRL, because they are extensively used in food and chemical industry, though most of the time using PMC and targeting a single product (i.e., ethanol fermentation, lactic fermentation). Other fermentative processes, such DF by MMC, are still in its infancy and mostly studied at the laboratory scale, though many efforts have been made recently in the field of MMC (see Chapter 3).

Table 1.4. *Possible conversion processes within a waste biorefinery based on the carboxylate platform (adapted from Aristizábal M and Cardona Alzate (2018).*

Processes	Technique	Description	TRL
Biochemical	fermentation	it uses microorganisms (PMC or MMC) to transform a substrate in recoverable products (as alcohols and organic acids)	5-9
	anaerobic digestion	the biowaste is submitted to bacterial decomposition in the absence of oxygen at 30–65 °C. The main product obtained is biogas (i.e., CH_4 and CO_2)	8-9
	aerobic treatment	the biowaste is submitted to bacterial decomposition in the presence of oxygen. this technique is applied to sewage treatment.	9
	enzymatic process	uses enzymes to transform a substrate in recoverable products (as alcohols and organic acids) at low temperature and low reaction rate	5-9
Chemical	catalytic processes	a reaction where intervenes a reagent or more, which has the presence of a catalyst.	8-9
	pulping	the process of making pulp, especially from lignocellulosic material	8-9
	esterification/ transesterification	these reactions are the most common for the biodiesel production using KOH or NaOH as a catalyst. The vegetable oils may be converted in methyl or ethyl esters of fatty acids	8-9
	hydrogenation	reaction between H_2 and other compound or element to produce biomolecules, usually in the presence of a catalyst.	8-9
	hydrolysis	this technique can use acids, alkalis, steam or enzymes to degrade the biowaste in its simple sugars	7-9
	oxidation	oxygen combination with other elements. Process where there is an apparent loss of electrons or ions	8-9
Mechanical	pretreatment	process where occurs the conditioning of biowaste for later to be transformed in added-value products. An example is milling, process for the particle size reduction of biowaste	9
	separation	method to achieve the separation of a chemical from a mixture, namely the purification of an interesting product.	6-9
	distillation	separation process of a component from a liquid mixture by selective evaporation and condensation	6-9
Bioelectrochemical (BES)	MFC, MEC	biological reactions are coupled to reaction at solid electrodes to produce electric power or valuable chemicals	5

A remarkable example of one of the possible waste biorefinery scheme has been presented in the recent document drafted by IEA Bioenergy task 42, based on the valorisation of mixed food waste from the domestic

sector (IEA Bioenergy Task42, 2019). This example includes many of the concept expressed in the previous paragraphs, showing the potential associated within millions of tonnes of biowaste produced every year in EU and around the world; food waste is the feedstock for Polylactic Acid (PLA) production according to the scheme presented in Figure 1.3. The PLA is a promising biodegradable and biobased polymer, and it is among the candidates to replace fossil-based plastics. The food waste is pretreated with a fungal hydrolysis step in order to extract sugars. Hydrolysis is followed by a lactic fermentation step using *Lactobacillus sp.*(PMC), and after an extraction process, lactic acid is obtained from the fermentation broth. The lactic acid downstream process involves lactide synthesis and then polymerisation of the lactide to obtain PLA as a final product. Lactic acid and lactide are intermediate products which could be even sold directly on the market. The remaining residual solid phase is used as animal feed as it contains valuable carbohydrates, proteins and lipids.

This biorefinery scheme focuses more on material production (high-value PLA) than the recovery of energy and represents a clear example of the application of the direct cascade principle. The bioconversion process was developed in laboratory scale and simulated for technical, environmental and technical feasibility. An input of 83 000 tonnes per year of food waste such as kitchen waste, whey, coffee mucilage and brewers' spent grains can produce 10 624 t of PLA, 12 118 t of lipid and 64 657 t of animal feed fraction. The environmental assessment based on GHG emissions and cumulated energy demand indicates a significantly better performance as compared to the fossil-feedstock reference (Figure 1.3d). In this case, the reference system was the production of polypropylene from crude oil, recognised as the closest fossil-based polymer to PLA in terms of characteristics. The economic assessment, based on cost and revenues comparison and sensitivity analysis, indicates that the prices of the high-value PLA significantly affect the economic performance of the biorefinery case study. The possible price is, in turn, strongly dependent on the attainable purity of the precursor lactic acid and the associated suitability for the designed uses (i.e. in food industry and pharmaceutical). The biorefinery case study assumes the production of a technical polymer grade while a medical-grade polymer may be required for pharmaceutical applications. In this case, further purification steps may be required, thus affecting the economic sustainability of the process.

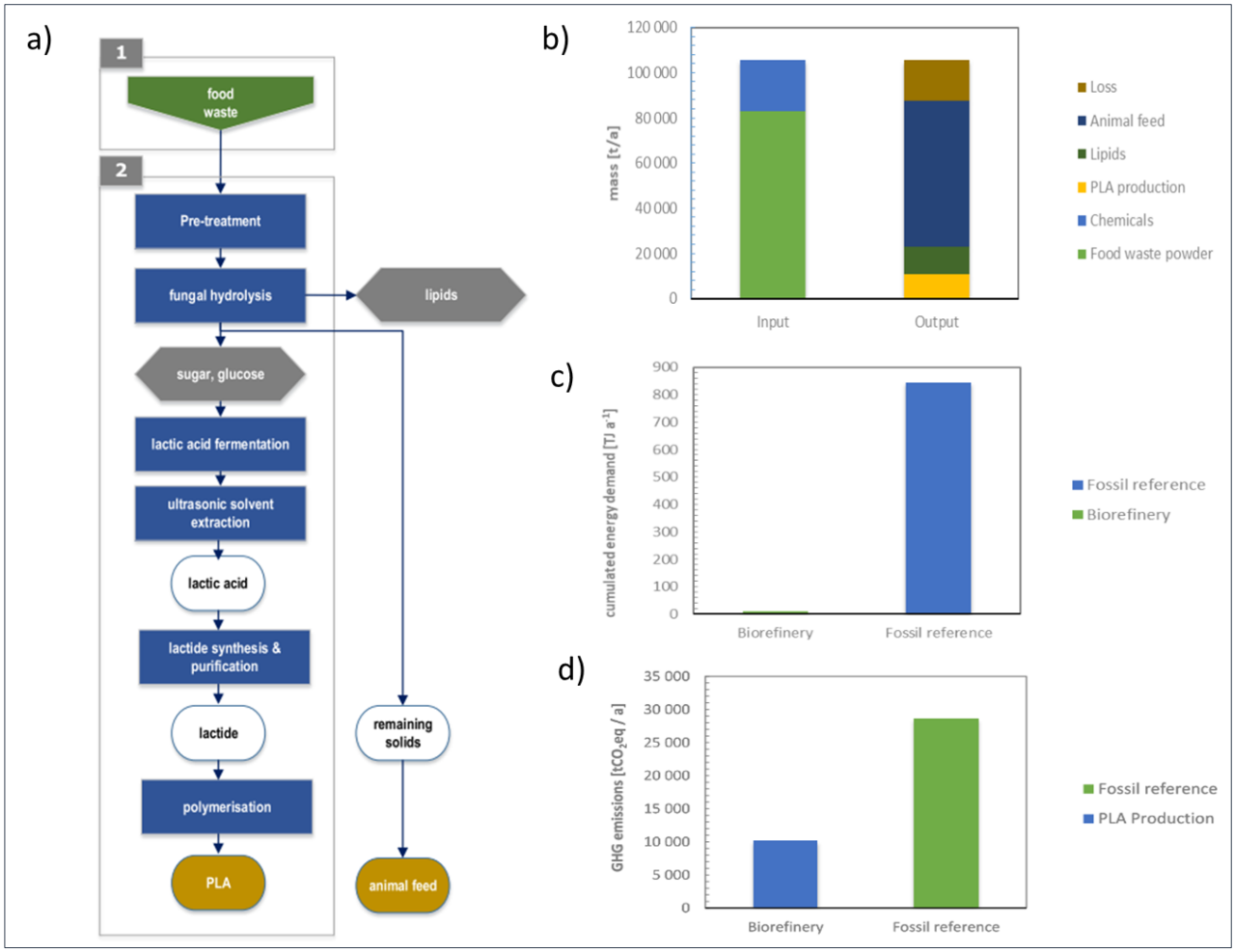


Figure 1.3. *Case study for food waste valorisation through production of biopolymer PLA, as presented by IEA Bioenergy Task42, 2019: process pathway (a); mass balance (b); cumulated energy demand (c) and GHG emission (d) of the proposed biorefinery as compared to reference plant.*

1.4.3 Marketable bioproducts and biofuels from waste biorefineries

The economic potential associated with waste biorefinery refers to the production of high-value products from biowaste, which is considered a renewable resource for new materials. Generally speaking, most of the common materials or fuels currently used have their bio-based counterpart. Since the technical feasibility for some of the processes necessary to produce those bio-based material has yet to be demonstrated, it is considered useful to think of the most promising bioproducts in terms of market demand and price. The adverse effect on the environment and the actual regulatory framework will make obligatory the shift in the next future from fossil-based production methods to bio-based production methods. The study of the evolution of the market is a crucial aspect in order to address not only the production cycles toward one product instead of another but also the efforts made by researchers and R&D systems to develop proper technologies. Research on new processes and demonstrations will open the way for improving the economic sustainability of waste biorefinery (Fava et al., 2015).

The next section gives an overview of some of the possible marketable bioproducts theoretically obtainable within a waste biorefinery (Table 1.5). The market demand of each bio-product has the potential to quickly increase in the next future, attracting the interest of companies operating in the sector. Most of them are currently produced from fossil fuels via chemical synthesis, and they have a well-consolidate market. However, they can also be produced biologically via microbial fermentation, but the production price is generally higher than chemical synthesis, making the bio-based production methods not competitive with the fossil-based production methods. Among the possible reasons, there are the lower manufacturing cost of oil-based methods and the currently lower efficiency of bio-based production methods. This aspect hinds the diffusion of bio-based solution and for this reason, researchers are orienting their efforts on reducing the production cost by the use of cheap feedstocks as biowaste, process optimisation and deep understanding of microorganism involved in biochemical pathway (i.e. engineered microbial consortia). More detailed information about some of the biological processes or bio-based production methods involved within a waste biorefinery will be given in Chapter 3.

1.4.3.1 Organic acids

Fermentation has been used widely by the chemical industry to produce short-chain fatty acids such as lactic, acetic, propionic acid and butyric acid. Each of them is a useful chemical or feedstock chemical widely used in the industrial, food and beverage, animal feed or pharmaceutical sector. Their industrial production is generally carried out by PMC able to guarantee high production yield and obtaining pure products that facilitate the downstream processes. The main cost is usually associated with feedstocks, which usually are refined and expensive sugars. The use of MMC-based fermentation to obtain those products from cheap substrate as biowastes has been largely ignored in the last decades, but it is recently gaining interest as research topic (see Chapter 3) (Agler et al., 2011; Jiang et al., 2017; Kleerebezem and van Loosdrecht, 2007; Sabra and Zeng, 2014).

An example of carboxylate acid is lactic acid. Lactic acid is a bulk chemical used in several application sectors, such as in the chemical industry as an intermediate for lactate esters manufacturing. Lactic acid is widely used also in the food and beverage sector, as well as in the pharmaceutical and personal care sector. The global lactic acid production is expected to grow from 0.7 Mt in 2013 to 1.9 Mt in 2020 with a market size worth of 8.7 billion of $ by 2025 and a compound annual growth rate (CAGR) of 18.7% (Gran View Research[1]). The main driver in its production increase is the need for PLA. Most of the commercial lactic acid is produced by bacterial fermentation of carbohydrates from corn, sugarcane, molasses and other crops.

The total global market for acetic, butyric and propionic acids is well consolidated, and the demand will be 18.5 Mt in 2020. The market demand is supplied by fossil-production method for about 90%.

[1] https://www.grandviewresearch.com/press-release/global-lactic-acid-and-poly-lactic-acid-market

Acetic acid is an important building block widely used in chemical industries to manufacture plastics, synthetic fibres and pesticides and in food and beverage industries as component of flavours, acidity regulators and preservatives. The acetic acid market demand is expected to reach 18.3 Mt by 2023, with a CAGR of 4.27% (Atasoy et al., 2018).

Propionic acid is mostly used in the form of calcium and sodium salts, and it could be used as a preservative for food (bread and other baked goods) and animal feed (directly or as its sodium salt). Moreover, propionic acid is a critical element in the manufacturing of vitamin E. Global demand is expected to reach 4.7 Mt by 2020, with growing at a CAGAR of 2.7%. Propionic acid is currently mainly synthesised by petrochemical route, and hence its production is vulnerable to price fluctuations of propane and natural gas.

Butyric acid is used in the animal feeding sector as a supplement and an antibiotic. It is largely recognised as an efficient energy source for animals, especially for swine and poultry. The total global demand for butyric acid is expected to reach 0.1 Mt by 2020, with a CAGR of 15.1%. The bio-based butyric acid production is growing due the fact that it is approved as a food flavouring agent (taste and aroma additive) by the United States Food and Drug Administration (Atasoy et al., 2018).

1.4.3.2 Biopolymers

Bioplastics for a circular economy has been considered among the top 10 emerging technologies for 2019 by World Economic Forum (World Economic Forum (WEF), 2019). There two main advantages associated to the use of some bioplastics instead of traditional plastics: the first advantage consists of replacing fossil carbon with renewable carbon from biomass (biowaste included) in the biopolymer production process; the second advantage is that some biopolymers are also biodegradable (under specific conditions) and can therefore contribute to the reduction of the problems related to the plastic waste which is not collected and sent to recycling but discharged into the environment leading to the presence of microplastics (European Bioplastics, 2018; Nova Institute, 2019; World Economic Forum (WEF), 2019). The suffix “bio” in biopolymer or bioplastic could be referred to the bio-based feature or the biodegradable feature or even both. It is worth mentioning that some biopolymers can be bio-based but non-biodegradable or vice versa.

Even though bioplastic currently represents about 1% of the 335 Mt of plastic produced annually worldwide, the biopolymers market demand is continuously growing due to the increased awareness of environmental solutions by the costumers, political support to circular bioeconomy and new applications and products. The global bioplastics production capacity is expected to increase from 2.11 Mt in 2018 to approximately 2.62 Mt in 2023 (European bioplastics). The capacities and production of bio-based polymers will continue to grow with an expected CAGR of about 4% until 2023, almost at the same rate as petrochemical polymers and plastics (Nova Institute, 2019). European states share 18% of the worldwide bio-based production capacity, but this figure is expected to rise to 25% until 2023 (European Bioplastics, 2018; Nova Institute, 2019).

Innovative biopolymers such as PLA and PHA are among the main drivers of this growth. According to a recent report, PLA has just left the embryonic stage for the early-growth stage of the S-curve[2], while PHA is currently still considered a phase behind that (Nova Institute, 2019).

Lactic acid polymerisation produces PLA, a biodegradable polymer which is primarily useful in food packaging, for rigid containers, disposable products and shrink wrap. PLA production is expected to remain the key driving force for global lactic acid demand. The PLA market size was estimated at USD 0.9 billion in 2017, and the European demand for PLA could potentially reach 0.6 Mt per year in 2025 (IEA Bioenergy Task42, 2012). NatureWorks (U.S.A) was the primary producer in 2018 with a production of 150 kt per year. Corbion (Netherland) and Total (France) produced a joint venture for a PLA plant in Thailand with a capacity of 75 kt year, while Synbra (Netherland) produces 50 kilotons per year.

PHAs are an important polymer family that has been in development for a while and that finally entered the market at commercial scale, with production capacities estimated to quadruple in the next five years (0.3 Mt in 2016) (European Bioplastic). These polyesters feature a wide array of physical and mechanical properties depending on their chemical composition. Generally, about 150 PHA monomers have been identified, and different types of PHA monomers offer distinctly different material properties and, in turn, engineering applications (Rodriguez-Perez et al., 2018; Valentino et al., 2017). Polyhydroxybutyrate (PHB), the most common among PHA, bacteria, has similar properties to polypropylene (Kleerebezem and van Loosdrecht, 2007). Nowadays, industrial PHA production is based on the use of sugar and glucose extracted from plant source for microbial fermentation by PMC. According to several authors, at the current state, the diffusion of PHA is hindered by high production cost related to the feedstock and downstream processes (Fava et al., 2015; Rodriguez-Perez et al., 2018; Valentino et al., 2017). Nevertheless, in 2018, Bio-on inaugurated an industrial plant in Bologna (Italy) claiming to be the first in the world to produce PHA industrially and cost-effectively.

Currently, PLA and PHA represent only a limited market in bioplastic production (10-20%) (European Bioplastics, 2018; Nova Institute, 2019). The 90% of the plastic market is made of only 5 polymers (polyethene (PE), polyethene terephthalate (PET), polypropylene, polyvinyl chloride (PVC) and polystyrene). The plastic market offers the Bio-PE and Bio-PET as a bio-based solution (but not biodegradable). In this case, what makes them bio-based is the fact that one of the precursors, ethylene, can be produced from bioethanol obtained through microbial fermentation of renewable resource.

On the one hand, the polymerisation of ethylene results in the production of Bio-PE that could be used to produce Bio-PE bottle fully biobased (Breskem (Brazil) share 52% of bio-PE market).

[2] S-curve refers to the behaviour of a product and/or business when enters in the market. At first, the growth is slow, and then it develops more rapidly, as consumers begin to warm up to the product. As the business expands, that growth continues and then eventually, a host of factors, both internal and external, cause the growth rate to decline and then gradually, they taper off.

On the other hand, the Bio-PET requires two ingredients that are the monoethylene glycol (produced from ethylene via ethylene oxide) and the terephthalic acid. The main bottleneck for obtaining a 100% biobased Bio-PET is that the precursors of terephthalic acid, the paraxylene, is still produced from crude oil. However, paraxylene could be produced even from isobutanol, which in turn could be produced from renewable resource via microbial fermentation. When a reliable microbial fermentation process for isobutanol will be available, it will be possible to obtain 100% bio-PET. Some companies (Coca Cola[3], Nestlè & Danone[4]) are investing in this field of research.

1.4.3.3 Biofuels

Biofuels are liquid or gaseous energy carrier. They have been indicated by EU's policy as a renewable alternative to fossil fuels for the EU's transport sector, helping to reduce GHG emissions and improve the EU's security of supply. By 2020, the EU aims to have 10% of the transport fuel of each EU country, coming from renewable sources such as biofuels (European Parliament and Council, 2015). Fuel suppliers are also required to reduce the GHG intensity of the EU fuel mix by 6% by 2020 in comparison to 2010 (European Parliament and Council, 2009). In this framework, the characteristics of a promising and suitable renewable biofuel are the potential to replace a significant portion of fossil fuels without affecting global food supplies; net positive energy balance; minimal negative environmental impact (Kleerebezem and van Loosdrecht, 2007). Biofuels production has been often linked to the so-called energy-vs-food dilemma, i.e. the use of agricultural land for energy production instead of food production, and to several other environmental pressures that may, directly and indirectly, impact biodiversity and the provision of ecosystem services (Correa et al., 2019). The most common biofuels that could be produced within the waste biorefinery are bioethanol, biobutanol, biomethane and biohydrogen. The biodiesel and synthetic gas are worth to be mentioned among them with the difference that they are not produced by microbial fermentation but by catalytic trans-esterification and gasification, respectively.

Bioethanol is one of the most common liquid biofuels in the transportation sector because it can replace petrol in modest percentages for use in ordinary spark-ignition engines (stationary or in vehicles), or that can be used at higher blend levels (usually up to 85% ethanol, or 100% in Brazil) in slightly modified engines, such as those provided in "flex-fuel" vehicles (REN21, 2019). Ethanol is also used in the chemical and beverage industries. Global bioethanol market is expected to grow significantly due to the rising demand for a cleaner and renewable source of energy. The global bioethanol market was valued at $5,652 million in 2015 and is expected to reach $9,544 million by 2022, growing at a CAGR of 7.6% from 2016 to 2022 (link). Global ethanol production increased by nearly 7% in 2018 (from 104 billion litres to 112 billion litres), and production in the

[3] https://www.coca-colacompany.com/plantbottle-technology
[4] https://www.nestle.it/media/pressreleases/naturall-bottle-alliance

United States and Brazil accounted for 83% (REN21, 2019). Current bioethanol production is based on sugarcane, sugar bet, wheat and other lignocellulosic materials.

Biomethane is probably the most common biofuels associated with biowaste since the use of AD for biowaste management could be considered as one of the most popular waste-to-energy conversion technology available. AD process produces a biogas that can be upgraded to biomethane for energy production or even as a transport fuel. In 2017, 17 783 biogas plant and 540 biomethane installations were in operation in Europe. The total Installed Electric Capacity in Europe continued to increase in 2017, growing by 5% to reach a total of 10 532 MW, while the electricity produced from biogas amounted to a European total of 65 179 GWh. Biomethane production also rose to a total of 19 352 GWh. In 2017, 15 European countries produced biomethane for direct industrial uses and injection into the existing gas grid and by the end of 2018 three new countries (Belgium, Estonia and Ireland) connected biomethane facilities to national gas grids for the first time (European Biogas Association (EBA), 2018; REN21, 2019). Among European countries, Germany has a higher number of biogas plant with around 9 700 operative plants in 2017, mainly used for industrial purpose. Global biomethane use for transport is concentrated in the United States and the EU. US biomethane consumption grew more than seven-fold between 2014 and 2017 and then increased another 13% in 2018 to some 22 petajoules (PJ). In Europe, the other globally significant market for biomethane for transport, consumption increased 13% in 2017, to 7.8 PJ (REN21, 2019). Production and use were concentrated in Sweden (5.2 PJ), where methane production from food wastes is encouraged as part of a sustainable waste reduction policy and where the use of biomethane in transport fuel is prioritised over its use for electricity production or injection into gas grids (IEA Bioenergy Task37, 2019). The next-largest European users of transport biomethane in 2017 were Germany (1.6 PJ), Norway (0.42 PJ) and the Netherlands (0.23 PJ) (European Biogas Association (EBA), 2018). However, despite all of those encouraging trends, it is worth to emphasise that the economic added value of the biogas produced is limited, mainly because it has to compete with natural gas (average price around 0.4-0.5 € kg^{-1}). Consequently, companies in the future will be most likely oriented towards the development of alternative processes that yield higher-value end-products, according to the cascade principle explained before (Kleerebezem et al., 2015).

It is the case of hydrogen, which is considered a promising biofuel because its combustion produces only vapour instead of greenhouse gases. The main driving force for investigating the production of hydrogen instead of methane within the waste biorefinery framework, is the higher economic value of hydrogen, owing to its more extensive range of applications in the chemical industry. Hydrogen can be extracted from fossil fuels and biomass, or water, or a mix of both. Natural gas accounts for around 75% of the annual global dedicated hydrogen production of around 70 Mt of hydrogen. The second source is represented by coal that accounts for around 23% while the rest is represented by oil and electricity (IEA, 2019). The most adopted method for hydrogen production are currently steam reforming (using water as an oxidant and a source of hydrogen) and gasification (where the raw material, such as coal or biomass, is converted into a synthesis

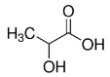
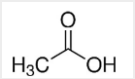
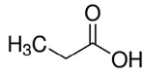
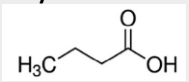
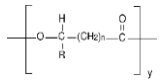
gas that is then transformed into hydrogen and CO_2) while electrolysis (where hydrogen is produced by splitting water into hydrogen and oxygen) still plays a minor role (IEA, 2019). The biological production through MMC-fermentation has been an emerging technology during the last decades, but at the current state, the technology is not yet fully developed, and no industrial applications are available. The price of hydrogen is affected mainly by the process adopted for its production, ranging around 1-5 € kg^{-1} and, in the near term, hydrogen production from fossil fuels will remain the most cost-competitive option in most cases (IEA, 2019).

In this framework, and taking into consideration the strong incentives for biomethane production in several European countries, a possible simplified and readily applicable waste biorefinery scheme could consist in combining fermentative H_2 and CO_2 production to CH_4 and CO_2 production via two-stage AD (De Gioannis et al., 2017). In this case, both mixtures should be refined to recover biohydrogen and biomethane, which could be then used individually or as a mixture (hythane). Hythane refers to the mixture of biogases, containing and methane and 10-25% H_2 by volume. Hythane has been recognised as a cost-effective biogas energy produced through an AD process using biowaste.

A more long-term solution may be represented by the use of innovative bioelectrochemical systems (BES) that can directly produce bioenergy by using MMC in Microbial Fuel Cell (MFC) or Microbial Electrolysis Cell (MEC). Those systems produce bioelectricity and biohydrogen, respectively. Among BES systems, microbial electrosynthesis (MES) is another promising bioprocess since it can recycle the CO_2 produced by other processes and covert it to SCFA for downstream applications (Vassilev et al., 2018; Batlle-Vilanova et al. 2017). However, BES is still in early lab stage and years of research are necessary to make such technologies competitive at industrial scale.

Other marketable bioproducts within the waste biorefinery can include biofertilizer made by the anaerobic digestates that could be rich in nutrients, such as nitrogen and phosphorous. Some authors suggest even the possibility to use carbon dioxide in the beverage industry or as a carbon source for further biological valorisation (Batlle-Vilanova et al., 2016; Irfan et al., 2019; Vassilev et al., 2018).

Table 1.5. *Overview of some of the main marketable products obtainable from waste biorefineries.*

Product	*Compound name*	*Market price (€ kg^{-1})*	*Market size (Mt y^{-1})*	*Applications*	*Production methods*	*References*
Biochemical	**Lactic acid** 	1-2	1.2 (2016) 1.9 (fcst for 2020)	industrial (acrylic acid, propylene glycol, acetaldehyde); food and beverage; personal care; pharmaceutical; PLA synthesis	microbial fermentation or chemical synthesis	Singhvi et al. (2018) Gran View Research, Inc.
	Acetic acid 	0.4-0.8	18 (fcst for 2023)	vinyl acetate monomer VAM (polymers, adhesives, dyes); food additive; vinegar; solvent; ester production; chemicals	chemical synthesis (carboxylation of methanol) and microbial fermentation (oxidative and anaerobic)	Atasoy et al. (2018) Gran View Research, Inc.
	Propionic acid 	0.3 2-2.5	0.40 (2013) 0.47 (fcst for 2020)	esters used food industry as aroma additive, food additive, flavouring agent (calcium sodium propionate); pharmaceuticals; animal feed supplement; Fishing bait additive; herbicides	chemical synthesis (ethylene hydroformylation, carboxylation of ethylene, direct oxidation of hydrocarbons), by-product of acetic acid manufacturing, microbial fermentation	Atasoy et al. (2018) Gran View Research, Inc.
	Butyric acid 	1.5-1.6	0.15 (fcs for 2020)	animal and human food additive; chemical intermediate; solvent; flavouring agent	chemical synthesis (oxidation of butyraldehyde), microbial fermentation	Atasoy et al. (2018)
	Ethanol	0.7-1.5 ($)	112 x 10^9 L (2017)	chemical (Solvent); transport as biofuels; beverage industries	microbial fermentation	REN21 (2019)
Biomaterial	**PLA** 	2.2-5.2	0.21 (2018)	packaging; textile; agriculture transportation; others	polymerization of lactic acid	Gran View Research, Inc.
	PHA 	3-3.5	0.03 (2018)	food packaging; others	biological production through microbial fermentation	European Bioplastic
Bioenergy	**Methane** 	0.2-0.5	65,179 GWh (2018, only Europe)	energy production	chemical (Sebastier and Fischer-Tropsch process) or biological (anaerobic digestion)	Kleerebezem et al., (2015)
	Hydrogen	1-6	70	energy production	steam reforming, gasification, electrolysis and microbial fermentation (process adopted affects the price)	IEA (2019) Kleerebezem et al. (2015)
	Bioelectricity	48.9 € MWh^{-1}		energy production	bioelectrochemical systems	

fcst: forecast

1.5 Conclusions

The waste biorefineries are candidate to receive higher interest in the next future, driven by the actual framework aimed to the transition from a linear, fossil and unsustainable economic model toward a circular, bio-based and sustainable economic model. The deployment of different waste biorefining strategies could represent the technical answer to achieve the ambitious objective set by EU for a sustainable future acting as a link between the some of the central core strategies of EU, i.e. Waste Hierarchy directive, Circular Economy Action Plan and European Bioeconomy strategy.

As the application of a biorefinery scheme is site-specific, and it depends on various and interconnected factors, specific studies are necessary to understand the feasibility to use such a biorefinery scheme in biowaste management. The implementation of this concept requires technical, environmental and economic assessment. With more focus with the technical aspects, research should be oriented on the application of different technologies (both innovative or well-established) in a integrate systems adapted for the specific waste stream considered and the local contest.

The following chapter outlines the dairy sector, which is considered among the most crucial European agri-food sector, with the focus on the valorisation of its primary residue, the cheese whey.

2 DAIRY INDUSTRY AND THE MANAGEMENT OF RELATED BIORESIDUES

2.1 The EU dairy sector

2.1.1 EU production of milk and dairy products

The dairy sector plays a significant role in the European economy considering that it is the second biggest agricultural sector in terms of output value after the vegetable and horticultural plant sector and before cereals (Augere-Granier, 2018). Milk production accounts for 15% of total EU agricultural revenue. All the EU member states produce milk but farms and herd sizes, yields and types of farming as well the obtained dairy products vary widely across Europe, from free-range farming in rural areas in southern Europe to large specialised dairy farms in the north-west and centre of Europe.

In 2017, the production of raw milk on the EU's farms was 170.1 million tonnes (Eurostat, 2018). European milk production showed a positive trend in the last years, considering that the production in 2013 was 159 million tonnes. The EU had the most significant production of milk in absolute terms and relative to population size among G20 members in 2016. The milk delivered to the dairies was 158.6 million tonnes, most of which was cow milk (96% of the total) while only a small part was from sheep and goat milk (3% of the total). Among the EU members, the primary producers of cow milk are Germany, France, the United Kingdom, the Netherlands, Poland and Italy that together provided around the 70% of cow milk collected by dairies. The production of sheep and goat milk is mainly located in in the countries bordering the Mediterranean Sea with Greece, Spain, France and Italy as primary producers (Figure 2.2).

Around 12 000 processing plants are employing 300 000 people in the EU. The dairy sector is predominantly organised in cooperatives, which hold a 55% market share. These cooperatives can be as large as a world-leading multinational companies or as small as SMEs or micro-enterprises. As showed in Figure 2.1, the raw milk delivered to dairies is processed into a wide spectrum of products such as cheese, butter, cream, drinking milk, acidified milk and powder products. The cheese and butter production used 66% of the total milk delivered to dairies. In 2017, the cheese production was around 10.2 million tonnes, and the main producers were Germany (21.8% of the EU total), France (18.8%) and Italy (12.4%). The production of vast volumes of added-value products, especially for exports, shows the more significant market orientation of the milk sector nowadays. For example, cheese production increased by 26 % between 2003 and 2013 and cheese exports rose by 69 % (European Commission and Eurostat, 2015). The EU is the world's biggest exporter of cheese and, more generally, one of the world's top three players for dairy exports, along with New Zealand and the United States (European Commission and Eurostat, 2015).

The EU's dairy policy, which is part of the agricultural common policy, consists of a range of instruments designed to support farmers and address market imbalances. In particular, it includes a collective market

organisation, public interventions and private storage provisions, direct payments and rural development measures. Exceptional measures can be mobilised in cases of severe market disturbance.

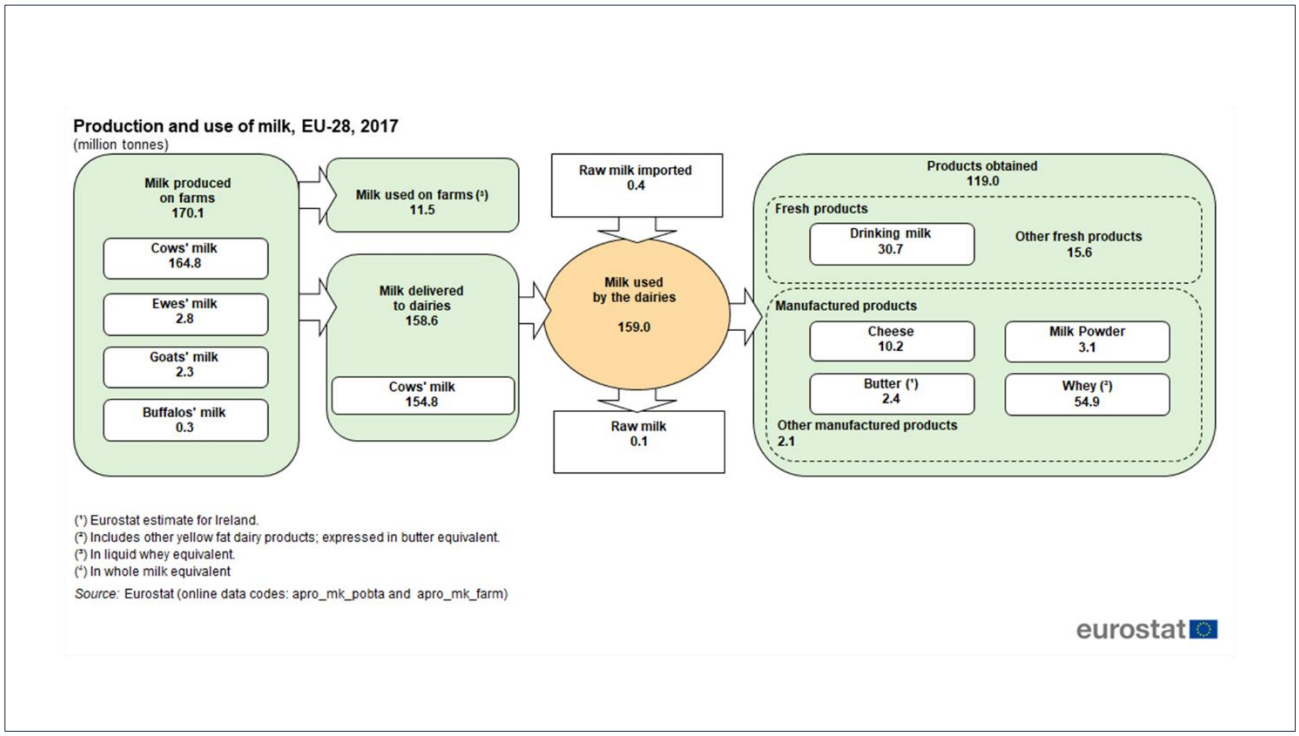


Figure 2.1. *Production and use of milk across the EU-28 in 2017 (source: Eurostat (apro_mk_pobta)).*

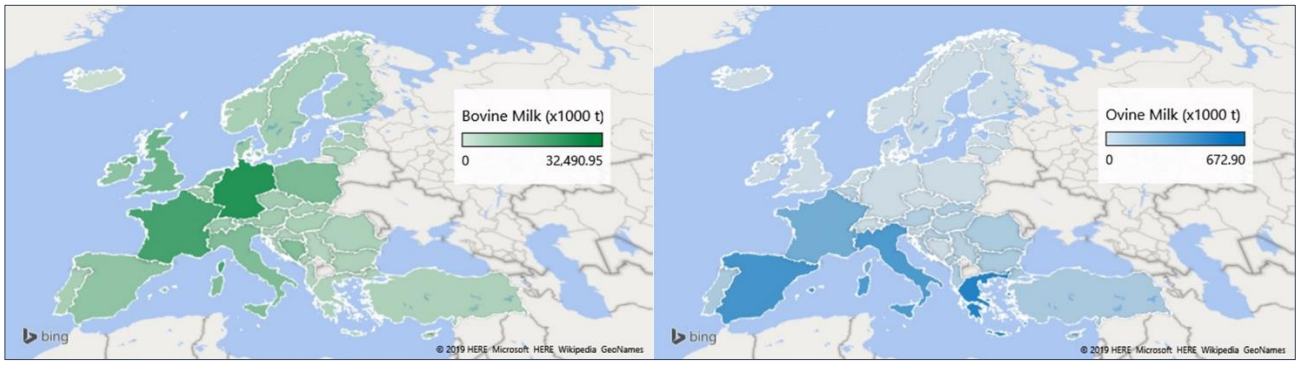


Figure 2.2. *Production of cow (left) and sheep (right) milk in Europe in 2017 (elaborated form Eurostat, source: Eurostat (apro_mk_pobta)).*

2.1.2 Scenarios for the EU dairy sector

2.1.2.1 Main current challenges for the EU dairy sector

Nowadays, the EU dairy sector is facing several challenges, among which price volatility is considered a significant challenge. Others recognised challenges for EU dairy sector involve the dairy sector structure, concerns related to climate changes, unfair trading practises and consumer requirements (Augere-Granier, 2018).

The reforms carried out by the Common Agricultural Policy (including the decrease in intervention prices introduced in 2013 and the end of milk quotas in 2015) have opened the dairy sector to the international market, making EU milk price more susceptible to international price developments and exposing EU farmers to more international competition. Raw milk price formation has changed completely since 2007, from a stable annual seasonal pattern of small price differences of 10 % to 15 % to multiannual cycles oscillating between 40 and 25 cents per litre. These significant price variations make it challenging to run a sustainable business (especially when farmers have planned investments based on higher average milk prices).

Most of the EU dairy sector is highly specialised in a single dairy output, and this can offer some advantages in terms of production cost and revenue, but it can become a substantial threat as it increases farmers' vulnerability to income shocks. Dairy farms with a more varied output are less vulnerable (this consideration is similar to the concept of biorefinery seen in the previous chapter, see 1.3.2). In addition, small dairy farms are numerous across the EU, and this makes them even more vulnerable to market fluctuations considering that their size probably means that they do not have the resources to buffer economic shocks. Another concern is related to the age of dairy producers: there are relatively few young farmers. In 2016, a third of farm managers in the EU were over 65, and only 11 % were under 40.

Climate changes, in particular, extreme weather conditions can strongly affect agriculture and in turn, the EU dairy sector. With climate change developing, lack of forage and grazing, animal heat stress and spreading of diseases are likely to become an increasingly frequent problem among farmer (Augere-Granier, 2018).

Unfair trading practices towards dairy farmers are commonly reported in the dairy sector. Dairy farmers usually occupy a weak position in the food supply chain in comparison with the dairy processing industry and the retail sector, which are the large operators in the chain. The price transmission along the supply chain is considered uneven, and market shocks are often fully transmitted to farmers, while price fluctuations are much more limited for processors and consumers.

In conclusion, consumer and society requirements constitute an additional challenge for the sector as some of these requirements can appear opposing: milk farmers are asked to produce quality milk that meets high environmental standards and animal welfare requirements, while the market demands cheap products.

2.1.2.2 Innovation for a more resilient and sustainable EU dairy sector

Under the several challenges above mentioned, the EU dairy sector must become more resilient and sustainable, both economically and environmentally. From an economic point of view, it is necessary to lower production costs to improve competitiveness, while also increasing the economic resilience of dairy farms confronted with high price volatility and market uncertainty.

At the same time, the dairy sector must be more efficient in the use of natural resources such as water and feed and do more to control the environmental impact of the dairy supply chain (reducing GHG emissions, water pollution). Resilient dairy farming also means taking good care of herds and meeting health

requirements (the second-worst animal welfare problem in Europe now is the poor welfare of dairy cows because of leg disorders, mastitis and reproductive problems). A recent EIP-AGRI report indicates three key areas through which to achieve robust and resilient production systems: (i) at the level of the cow, genetics and precision livestock farming (PLF) are areas with a high potential to enhance robustness and resilience; (ii) at the level of the farm, the report looks at ways to increase the capacity of a farm to absorb impacts caused by changes in environmental, social or economic conditions; (iii) for the dairy sector, the report identifies the essential role of information, communication and dialogue between farmers and consumers, which requires better knowledge and understanding of dairy processes and better ways to benchmark it, as well as proper translation of this in a way that the final consumer can understand and appreciate.

In line with those considerations, the European Dairy Association has recently declared the aim to move dairies towards a full circularity with continuous improvements not only in its economic performances but also in its environmental sustainability. European Dairy Association candidate itself as an essential participant of the global sustainability agenda setting ambitious objectives such as being a zero-waste industry with absolute circularity and virtuous water/energy cycle, have a small environmental footprint, being a net contributor to clean energy and biodiversity (European Dairy Association, 2018). This aim and other activities demonstrate that dairy industry is acutely aware of the challenge that it is going to face up in the next future.

Most of the considerations above are contained in "The EU dairy sector – Main features, challenges and prospects" prepared for, and addressed to, the members and staff of the European Parliament by the European Parliamentary Research Service.

2.1.3 The case of the Sardinia sheep milk supply chain

Across EU, there are specific European areas in which, despite the small size in terms of overall European produced milk, activities like livestock farming, especially dairy farming, are considered key activities not only from an economic perspective but also from a social and environmentally point of view. The case of the sheep milk supply chain in the region of Sardinia (Italy) is an excellent example of this consideration.

Sardina is the third region in European territory by the number of ovine heads (sheep and goats), with around 3 million animals, after the oriental region of Turkey and the Extremadura region in Spain. It is one of the areas with a higher density of sheep in the world. In 2017, the production of sheep milk in Sardinia was around 330 000 tonnes, which represent 71% of the Italian production and 16% of the European one. This also includes a small fraction (6%) of goat milk. The sector is structured in 12 267 small size dairy farmers (less than 300 animals per herd) and 41 dairy processing plant (but with 45% of total production concentrated in only 5 plants). The Sardinian sheep milk production is destined for cheese production, manufactured both in semi-artisanal and industrial manner. The Sardinian production of cheese was 47 000 tonnes in 2017 composed by three Protected Designation of Origin (PDO) cheeses ("Pecorino Romano", "Fiore Sardo", "Pecorino Sardo") and several minor productions, all strong linked with the local traditions and natural

resources (Vagnoni et al., 2017). Among them, Pecorino Romano PDO, which represents more than 90% of the total Sardinian PDO cheese production, is also one of the most exported Italian cheese and it is in large part sold in US as grating cheese type.

The role of the sheep supply chain goes beyond the economic one. Sheep grazing on meadows in the countryside, which are considered as part of the landscape, and the high-quality traditionally dairy products are believed to contributes to the sustainable development of rural areas by delivering public goods (landscape, heritage) and by having a positive impact on the local economy, helping to preserve social cohesion in rural communities, notably via synergies with tourism (Rossi, 2016).

Nevertheless, the Sardinian sheep dairy supply chain is experiencing most of the challenges mentioned in the previous paragraph, often amplified by the peculiarity of its system. Sardinia is classified as disadvantaged areas. Some part of the region has a low level of gross domestic product per capita, and they are considered fragile concerning milk production and with poor infrastructure (Soldi, 2016). Besides, remoteness (insularity) or mountainous conditions are geographical challenges, and the systems show lack of dynamism across the region due to the limited number of young farm manager compared to the older and the high dependency on external markets. The fluctuating dynamics of the Pecorino Romano PDO international price and the dominant role played by few industries represent structural limitations (Vagnoni et al., 2017). In February 2019, this context led Sardinian dairy farmers to pour milk on the roads to protest for milk prices falling under the cost of production.

All those aspects contributed to the common opinion that the Sardinian sheep milk sector needs an effective innovation process to tackle the deep structural crisis of the sector. According to the author of this work, such an innovation should be based on an integration of both economic and environmental aspects.

Recently some authors evaluated the environmental profile of the Sardinia sheep milk through Life Cycle Assessments (Mondello et al., 2018; Vagnoni et al., 2017). With the aim of an environmental profile improvement, the authors indicate the enteric feedstock fermentation and feed supply chain optimisation as clear priorities for reducing GHG emissions. Moreover, they also pointed out that a highly efficient and more green-energy based power supply, the use of less pollutants cleaning agents, as well as the adoption of a more cleaner wastewater management during the cheese production in dairies are key improvements that may represent further important step towards a more eco-sustainable dairy system (Mondello et al., 2018; Vagnoni et al., 2017). Currently, the EU project "SheepToShip LIFE" is underway and aims is to address the environmental benefits and implication of an eco-sustainable sheep supply chain using Sardinia as a case study that can be transferred in other similar Mediterranean areas.

In the Sardinia region, there is also a dairy supply chain based on bovine milk. This supply chain is in a small area, and it is managed by the cooperative "Arborea 3A". For its specific characteristic and the above consideration, this work will focus mostly on the sheep dairy supply chain.

2.2 Type of bioresidues produced during dairy products processing

Among the agroindustrial activities, dairy industries are well known as one of the main source of industrial effluent generation in Europe (Demirel et al., 2005). The cheese production process results in the generation of two main different waste stream: bioresidues, as cheese whey (CW) and second cheese whey, and dairy wastewater (Carvalho et al., 2013; Slavov, 2017). The production process strongly influences the composition of dairy waste streams, the kind of milk used (cow, sheep, goat, buffalo), the amount of water and detergents or sanitising agent used (Shete and Shinkar, 2013).

Cheese whey (CW) is a green-yellowish liquid resulting from the precipitation and removal of milk casein in cheese-making processes. CW is the most important by-product of the cheese-making process with a high specific production of 0.8-0.9 L per litre of processed milk, depending on the cheese yield and type of processed milk. For example, cow and goat milk have a lower yield of cheese compared to sheep milk (9.86, 9.84 and 14.78 kg of cheese per 100 kg of milk respectively) (Carvalho et al., 2013). In the EU, the overall production of CW can be estimated at 127-143 million tonnes per year, according to recent data on milk production[5] (Eurostat, 2018). Part of CW from cheese production can be further processed to obtain cottage cheese, curd cheese or Ricotta. This further step leads to the generation of the so-called second cheese whey, which can be found in the literature with different names, such as cottage CW or ricotta CW. In addition, specific industries are specialised in using CW to recover the whey protein from through membrane processes generating deproteinized CW or CW permeate (Carvalho et al., 2013).

Dairies are water-consuming activities, and water is used in every step of the technological line, including the cleaning and washing of tanks and equipment, disinfection, heating and cooling. A considerable amount of diary wastewater are produced, which can be classified into processing water, cleaning water and sanitary water (Ahmad et al., 2019; Slavov, 2017). The total amount of wastewater generated for a litre of milk processed is estimated in around 2.5 L with considerable fluctuation over the time, and the characteristics largely depend on the factory size, applied technology (Slavov, 2017). Dairy wastewater could include milk and cheese whey lost during the cheese-making process or culture starter[6] used in manufacturing.

2.3 Composition and characteristics of dairy bioresidues

CW is considered the main pollutant waste stream generated in dairy industries, due to its high volumetric and organic load (Carvalho et al., 2013; Prazeres et al., 2012; Ryan and Walsh, 2016; Slavov, 2017).

CW accounts for 80–90 % of the milk volume, retains 55% of milk nutrients (vitamins, minerals) and 20% of milk proteins, and is characterized by a COD and BOD concentration of 50–102 and 27–60 g L^{-1},

[5] Asssuming a CW yield of 0.8-0.9 litres of CW per litre of raw milk

[6] Starter culture are selected culture added to the milk during the cheese-making process in order to perform the fermentation and they influence the type of the produced cheese.

respectively, 90% of which in form of lactose (Carvalho et al., 2013; Ryan and Walsh, 2016). CW is also characterised by the presence of salts such as NaCl, KCl and calcium salts (0.46 – 10%), a pH of 3.8 – 6.5, depending on the whey type (acidic or sweet) and low alkalinity (Prazeres et al., 2012). Second cheese whey retains about 60% of the dry matter contained in the CW and is characterised by a lower protein concentration and higher salinity deriving from the second flocculation and addition of salts in the cottage cheese manufacturing (Carvalho et al., 2013). Since sheep milk has a high nutritional value and high concentrations of proteins, fats, minerals, and vitamins, as compared to the milk of other domestic species, those characteristics should be reflected in the CW. For instance, it is reasonable to find higher concentrations of proteins in sheep CW than in bovine CW (concentration in the milk of 5.5 and 3.4 g/100g respectively) (Balthazar et al., 2017). Sheep whey has a ratio of total nitrogen/dry matter much higher than the one existing in bovine whey, doubling the content in soluble proteins (Carvalho et al., 2013).

Further details about dairy wastewater characterisation are reported in (Carvalho et al., 2013) while a comprehensive review on physicochemical characteristics of sheep milk can be founded in (Balthazar et al., 2017).

2.4 Environmental issues related to dairy residues management

The main concern about the effect of dairy biowaste is related to its high organic content because, in the case that dairy biowaste is discharged without proper treatment, it can have serious adverse effects on the environmental.

On the one hand, when effluents from dairy waste are discharged into water bodies, the high amount of biodegradable organic matter is quickly consumed resulting in dissolved oxygen depletion and consequent rising of the eutrophication (Ahmad et al., 2019). The CW could be also toxic for aquatic animals (Ahmad et al., 2019). The discharge into the soil could affect its physical and chemical characteristics, resulting in decreased crop yield and oxygen availability. In addition, the presence of nitrogen compound as ammonia or nitrate could also contaminate groundwater.

On the other hand, if the dairy waste streams are discharged into the sewage systems, they may cause severe problems in terms of organic load for the biological treatment units in WWTPs (Ahmad et al., 2019). An example, they can alter the settleability of sludge, excessive depletion of the oxygen in the aerobic tank and general minor efficiency of the process. According to an estimation, about 40 L of untreated milk whey generates an organic load equal to what produced daily by 250 000 people (De Jesus et al., 2015).

From the previous statements, it is obvious that dairy bioresidues cannot be directly discharged to the environment without adequate treatment or valorisation. Disposal of whey by dumping in water bodies is now prohibited in most dairy producing nations by strict environmental legislation (Ryan and Walsh, 2016).

Table 2.1. *Composition of cheese whey, as reviewed in Carvalho et al. (2013).*

Lactose	Proteins	Fats	Minerals	BOD_5	COD	TS	TSS	TVS	TN	TKN	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	TP	$P\text{-}PO_4$	Reference
–	–	–	–	–	74.5± 0.4	–	9.4±0.5	–	–	0.146	–	–	0.124	–	Erguè et al. (2001)
50	–	–	–	–	74.2	66.83	22.15	–	–	1.49	0.17	–	–	–	Ghaly and Kamal (2004)
–	–	–	–	–	73–86	–	20–22	–	0.9–1.2	–	0.06-0.15	7–10	0.42-0.54	0.34-0.43	Farizoglu et al. (2007)
49.2	–	–	–	–	102.1	70.9	–	–	1.76	–	–	–	–	–	Ferchichi et al., 2005
–	–	–	–	35.5–46.0	60.3-66.7	–	4.1–10.0	–	–	–	–	–	–	–	Blonskaja and Vaalu, (2006)
45.9±0.88	2.71±0.05	9.44±1.14	–	37.7±2.8	68.6±3.3	5.93±0.38	1.35±0.06	5.61±0.36	–	1.12±0.01	–	–	$0.5\pm1.8\times10^{-3}$	–	Saddoud et al. (2007)
43.92	1.42	0.00	6.1		100	–	–	–	–	–	–	–	–	–	Yorgun et al. (2008)
–	–	0.99	–	29.5	73.4	–	7.2	–	–	–	–	–	–	–	Janczukowicz et al. (2008)
–	125±2[(a)]	0.9±0.5[(b)]	–	40±2.55	60±10	59±0.5	1.5±0.23	–	–	–	–	–	–	–	Gannoun et al. (2008)
42.6	–	–	–	–	86.3	–	6.9	–	0.2	–	–	–	–	–	Azbar et al. (2009a)
50–60	–	–	–	27–36	50–70	55–65	10–15	–	–	0.01–0.02	–	–	–	–	Ebrahimi et al. (2010)

[(a)] *as g* kg^{-1}

[(b)] *as (%)*

\- *not repored*

2.5 Dairy waste current management strategies: state of the art

The management of residues has always been a matter of concern for the dairy industry. Those residues, CW in particular, are often considered as an undesirable by-product having a limited interest and challenging to manage. In the past, the discharge of those residues into rivers was a common practice, but nowadays in Europe this practice has greatly diminished through the application of more stringent control measures and environmental legislation. Dairy industries have been forced either to treat or to acquire treatment facilities which compromise their production costs (Kasmi, 2018). According to Decision 97/80/EC, CW is a by-product obtained during the manufacture of cheese or casein. Nowadays, CW is mostly lost (Bosco et al., 2018; Chatzipaschali and Stamatis, 2012; Gonzfilez Siso, 1996; Kasmi, 2018; Slavov, 2017). Despite the environmental concerns, a share of dairy effluents, including about 50% of the whey produced worldwide, is currently released into the receptor without any treatment (Bosco et al., 2018; Slavov, 2017). The CW management strongly depends on the dairy size, while in small dairies it is common to collect the CW and provided as animal feed, in larger dairies the CW is treated with a dedicated WWTP (Valta et al., 2017). The costs associated to valorisation technologies are generally not tolerable to small and medium factories, so biological or physicochemical treatments constitute a viable and the most attractive alternative (Bosco et al., 2018; Prazeres et al., 2012). A comprehensive description of the value-added products that can be obtained from the valorisation of dairy bioresidues is given by Mollea et al. (2013), while Prazeres et al. (2012) give a clear overview of the state of the art of the different processes and technical constraints.

2.5.1 Animal feed and land spreading

In the past, direct use of CW as feed for farm animals or land spreading has been the most applied solution for CW management.

The use of CW as animal feed is a common practice and seems to be the most economically feasible option, since pig farms are often located close to the cheese manufacturing units which in turns makes daily whey transport easy, even in small quantities (Valta et al., 2017). It is recommended to use CW directly in a place where it is produced (Carlos et al., 2016). In the case of farms far away from the dairy this option may be unfeasible since it involves further transport costs and the increased risk of CW acidification (conversion of lactose into lactic acid). Furthermore, excessive lactose and minerals, however, can cause issues for farm animals that necessitate a limit in untreated whey use as an animal feed (Akhlaghi et al., 2017a; Ryan and Walsh, 2016) and for those reasons, often dilution is mandatory (Carlos et al., 2016; Chatzipaschali and Stamatis, 2012; CRA Consiglio per la ricerca e la sperimentazione in agricoltura, 2005). For instance, the production disciplinary of “Parma ham” says that the maximum amount of CW per day for pig is 15 litres.

As mentioned before, the high salt content and organic load associated with the CW are dangerous for soils and water body because of desertification and eutrophication. The use of CW as agricultural fertiliser

has incessantly been reduced because over the years, facilitated by some restrictions by law. The application of large quantities of whey without pretreatment leaves a high saline deposit in the soil, resulting in decreased crop yield and oxygen availability (Ahmad et al., 2019; Chatzipaschali and Stamatis, 2012; Ryan and Walsh, 2016).

Both uses have difficulties concerning volumes and high transportation costs that make these solutions impractical for the amounts of whey being created today (Ryan and Walsh, 2016).

2.5.2 Recovery of proteins and lactose

Advances in processing technology, including ultrafiltration, microfiltration, reverse osmosis, and ion-exchange, have resulted in developing several different finished whey products: whey protein concentrates (WPC) and whey protein isolate (WPI). Whey proteins have functional properties essential in food application. The recovery of protein from whey is performed by ultrafiltration generating a whey permeate as a side waste stream. Such permeate conserve about 80% of the lactose contained in the original whey coupled with a low concentration of proteins and fats and a high salinity (Bosco et al., 2018). Lactose can be recovered from CW and whey permeate by crystallisation (Ryan and Walsh, 2016). Lactose is used widely within the food and confectionery industries, bakery industries and in the preparation of infant formula (Ryan and Walsh, 2016). It can use for the direct product of various other compounds, such as lactulose, lactitol, hydrolysate lactose and galactooligosaccharides (Ryan and Walsh, 2016).

2.5.3 Physical-chemical treatments

Destruction and reduction of milk fat and protein colloids in the dairy wastewater can be achieved by physico-chemical treatments (Ahmad et al., 2019). One of the most used is the coagulation (flocculation) aimed at reducing the suspended and colloidal particles responsible for turbidity of water and helps in the reduction of organic substance responsible for COD and BOD contents (Ahmad et al., 2019; Carvalho et al., 2013). Since the reagent costs are high, and the soluble COD removal is inadequate in physical-chemical treatment processes, biological processes are usually preferred (Ahmad et al., 2019).

2.5.4 Aerobic treatments

Biological aerobic treatments include trickling filters, aerated lagoon, activated sludge process. The aerobic method is among the most used in the dairy industry, but it has reduced efficiency, especially if compared to anaerobic processes (Ahmad et al., 2019). This is due mostly to the rapid acidification (due to low water buffer capacity) and filamentous growth (high level of lactose). Trickling filters usually results in high-quality final effluents, but its use is limited in high strength effluents (more than 0.3 kg_{BOD5} m^{-3}), due to problem of heavy fouling (Ahmad et al., 2019). The use of aerated sequencing batch reactor (SBR) is to be preferred because of its different loading capabilities and effluent flexibility. Also, the activated sludge is a

typical process in the dairy industry, often coupled with aerobic sewage stabilisation (filter presses and centrifuge) for the use of sludge as fertiliser. However, from the energetic point of view, the traditional activated sludge process is not economically sustainable due to the high organic load of dairy effluent, and the consequent vast quantity of oxygen required for aeration and excess sludge produced. Sludge treatment consumes an average of about 900 kWh d^{-1} electric energy, including 100 kWh d^{-1} for dewatering (using a filter press) and 800 kWh d^{-1} for aerobic stabilization, accounting for 30% of the total energy required for aerobic treatment of dairy effluents (Dąbrowski et al., 2017).

2.5.5 Anaerobic digestion

AD, in which organic substrates are converted to methane (CH_4) is a well-established process to exploit the energy content of CW (De Gioannis et al., 2017; Traversi et al., 2013). Due to its high organic load and low alkalinity, AD of CW may result in an accumulation of volatile fatty acids (VFA) during lactose fermentation, leading to a consequent acidification and inhibition of the methanogenic activity, affecting the CH_4 yield as well as the stability of the process (De Gioannis et al., 2014; Hagen et al., 2014; Humberto et al., 2017; Prazeres et al., 2012; Traversi et al., 2013). Due to this issue, low biomethane potentials (BMP) ranging from 0.27 to 0.6 L CH_4 g_{VS}^{-1} have been reported by anaerobic digestion of CW under mesophilic conditions (35–37 °C) (Escalante et al., 2017; Labatut et al., 2011; Vivekanand et al., 2018), whilst in continuous applications, long HRT values (above 5 days) are typically applied to avoid process instability (Table 2.2).

In AD, external alkali addition (e.g., lime, bicarbonate, or hydroxide) or appropriate dilution is generally required to mitigate acidification, but both strategies would increase the operation costs, and the volumes to be treated. A more sustainable option is co-digestion of CW with substrates characterised by high buffering capacity, such as sewage sludge (Carrieri et al., 1993), dairy manure (Kavacik and Topaloglu, 2010; Rico et al., 2015; Vivekanand et al., 2018), poultry manure (Gelegenis et al., 2007), and cattle slurry (Comino et al., 2012), or fish ensilage (Vivekanand et al., 2018), although results from literature are controversial. Labatut et al. (2011) reported that co-digestion of CW with dairy manure, in proportion 10:90 and 25:75, resulted in a lower CH_4 yield (238-252 L kg^{-1} VS) than raw CW (424 L kg^{-1} VS). Vivekanand et al. (2018) also reported a decreased CH_4 yield when blending CW with cattle manure, fish ensilage, and both. On the other hand, Comino et al. (2012) obtained the highest CH_4 yield of 343 L CH_4 kg^{-1} VS co-digesting CW and cattle slurry in proportion 50:50 at 35°C and HRT of 42 days. Hublin and Zelić (2013) reported a maximum CH_4 yield of 15.7 L L^{-1} reactor by co-digestion of CW and cow manure at 55°C, with an optimum mixing ratio of 10:90, with the addition of 5 g $NaHCO_3$ L^{-1} for alkalinity control. In co-digestion processes, not only the maximum CH_4 yield but also the process stability is affected by the mixing ratio of the various substrates. When co-digesting CW and diluted poultry manure in a CSTR, Gelegenis et al. (2007) reported an increasing CH_4 yield for CW concentrations up to 35%, but the process became unstable when the CW fraction exceeded 50% (based on VS). However, when co-digesting CW and the screened liquid fraction of dairy manure, Rico et al. (2015)

reported an increase of CH_4 yield from 339 to 392 L CH_4 kg_{VS}^{-1} increasing the CW fraction from 15 to 85%, at 35°C and HRT of 15.6 days, without instability issues.

A two-step process, where hydrolysis-acetogenesis and methanogenesis are carried out in two different reactors, is another strategy to avoid process instability (Fernández et al., 2015), as well as increase COD removal, although it would result in a higher footprint of the plant, as well as increasing investment and operation costs. A two-step process would allow to recover H_2 in the acidogenic reactor, which could be used as fuel, alone or in combination with the CH_4 produced in the methanogenesis reactor (hytane), or circulated to the methanogenesis reactor to increase the overall CH_4 yield. Another advantage of two-step AD processes is the possibility of operating the methanogenic reactor at lower HRT (< 5 d) than one-step processes. Yilmazer and Yenigün (1999) and Saddoud et al. (2007) reported a biogas yield of 550 and 300 L kg^{-1} $COD_{removed}$, respectively, with COD removals above 90%, in a two-step AD process with 4 d HRT in the methanogenic reactor. With an HRT of 4.4 d, Antonopoulou et al. (2008) obtained a CH_4 yield of 3,270 L CH_4 kg^{-1} VSS, substantially higher than the 147 L kg_{VSS}^{-1} obtained by Venetsaneas et al. (2009) with 20 d HRT. However, Fernandez et al. (2015) compared one-step and two-step AD of CW under thermophilic conditions (55 °C), reporting a maximum yield of 349 L_{CH4} $kg_{CODfeed}^{-1}$ in the one-step AD, at 8.3 days HRT, whereas the two-step process was inhibited at HRT lower than 12.5 days. This suggests that two-steps processes may not be optimal for thermophilic AD.

The implementation of AD is not so common within the dairies industries, but recent examples of full-scale applications are increasing over Europe, favoured by the increasing interest in bioenergies. Valbio is one of the most active company in providing AD systems to dairy industries through its patented technology (Valbio Methcore®, Valbio) based on UASB technology. The company has commissioned more than 10 full-scale plants for dairy companies mostly located in France, Canada and Bulgaria. Those full-scale plants are designed to treat 0.3-10.5 million litres of whey per year with the production of 0.3-3.5 MWh y^{-1} and COD removal higher than 90%. Dairygold Co-Operative Society Limited recently installed the world's largest above-ground anaerobic digester (ADI/BVF®, Evoqua) in Mitchelstown, Co. Cork (Ireland). The low-rate anaerobic digester was designed to treat at full load operation 5500 m^3 d^{-1} of wastewater containing powdered milk, cheese waste and cheese whey meeting the strict discharge limits and contributing to satisfy the dairy energy needs. First Milk's Lake District creamery (Cumbria, UK) was in 2016 the first dairy processing site to feed upgraded biomethane generated entirely from cheese process residues to the national gas grid. The continuous stirred reactor, installed and operated by Clearfleaul, was designed to treat 1650 m^3 d^{-1} of dairy wastewater and whey producing 5.4 MWh of renewable bioenergy. This project is believed to open a pathway and soon replicated by other big dairy industries.

Table 2.2. *Overview of laboratory studies on continuous methane production from cheese whey, as sole substrate or in co-digestion, in one- or two-stage AD bioprocesses at different temperature, pH and hydraulic retention time (HRT).*

Processes	Substrate	Inoculum	Reactor[a]	T (°C)	pH	HRT (d)	Methane yield	
One-stage AD	bovine CW	granular anaerobic cultures	UASB	35	-	2 - 4.95 h	420 L kg_{COD}^{-1}	9
	85% bovine CW 15% liquid fraction of dairy manure (v/v)	-	CSTR	35	6.4 - 7.1	15.6	392 L_{CH4} kg_{VSin}^{-1}	n
	50% bovine CW 50% cattle slurry (v/v)	-	CSTR	35	6.9 - 8.7	42	343 L_{CH4} kg_{VS}^{-1}	n
	2 L bovine CW + 1 kg dairy manure + 1 L water	-	CSTR	34	6.5 - 7.5	5	0.9 L_{CH4} L^{-3} d^{-1}	n
Two-stage AD	CW powder	anaerobic sludge	UFAF	-	-	4	550 L_{biogas} $kg_{CODremoved}^{-1}$	9
	diluted bovine CW	anaerobic sludge	CSTR	37	7.3 - 8.5	4	300 L_{biogas} $kg_{CODremoved}^{-1}$	9
	CW	anaerobic sludge	CSTR	35	7.7	20	147 L_{CH4} kg_{VSS}^{-1}	9
	deproteized CW	-	SBR	55		25	349 L_{CH4} $kg_{CODfeed}^{-1}$	n
	sheep and goat CW (from Feta cheese)	-	PABR	35	8.0	4.4	3,270 L_{CH4} kg_{VSS}^{-1}	9

[a] *BMP, biomethane potential; CSTR, continuously stirred tank reactor; PABR, periodic anaerobic baffled reactor; SBR, sequence batch reactor; UFAF, up-flow anaerobic filter; UASB, upflow anaerobic sludge blanket.*

- not reported

2.6 Production and management of dairy bioresidues in the Sardinia region

With a focus on the Sardinian case, the last data reported[7] a production of approximately 330 000 tonnes of milk of ovine origin, 94% of which is sheep milk and the 6% is goat milk. Assuming a specific production of 0.85 litre of CW per litre of milk (Carvalho et al., 2013), the whole Sardinia sheep milk industry generates 280 000 tonnes of CW per year. The majority of the cheese manufacturing units use part of the CW for the production of ricotta cheese, with a yield of 0.07 kilos of Ricotta cheese per litre of CW. Based on the last data about ricotta cheese production in Sardinia (11 000 tonnes in 2010), it can be assumed that more than half of the CW produced is used for the production of ricotta cheese, resulting in approximately 140 000 tonnes of second CW, also known as Scotta. Figure 2.3 reports the Sankey diagram regarding the fate of the sheep milk in Sardinia, according to the considerations mentioned above. Overall, the Sardinia sheep supply chain generates 270 000 tonnes of bioresidues per year.

[7] according to the last report available by Sardinia region, available at this link.

From the data available in the literature and from some interviews with the players of the sector, emerged that the most applied solution for CW in Sardinia is the use as animal feed. The use of CW or Scotta as animal feed most of the time do not represent a source of income for the dairy. Most of the time, the dairy must pay the cost associated with transport. For the best of the author's knowledge, there are only two AD plants that currently treat CW and Scotta for biogas production but in co-digestion with other substrates. It is also worth to mention that the illegal discharge of the CW in the sewage system and rivers cannot be excluded, especially by the small size dairies. The quantification of this phenomena is hard and probably should require more specific studies. In the past, some cases of uncontrolled discharge of dairy residues in the sewage systems have been reported[8].

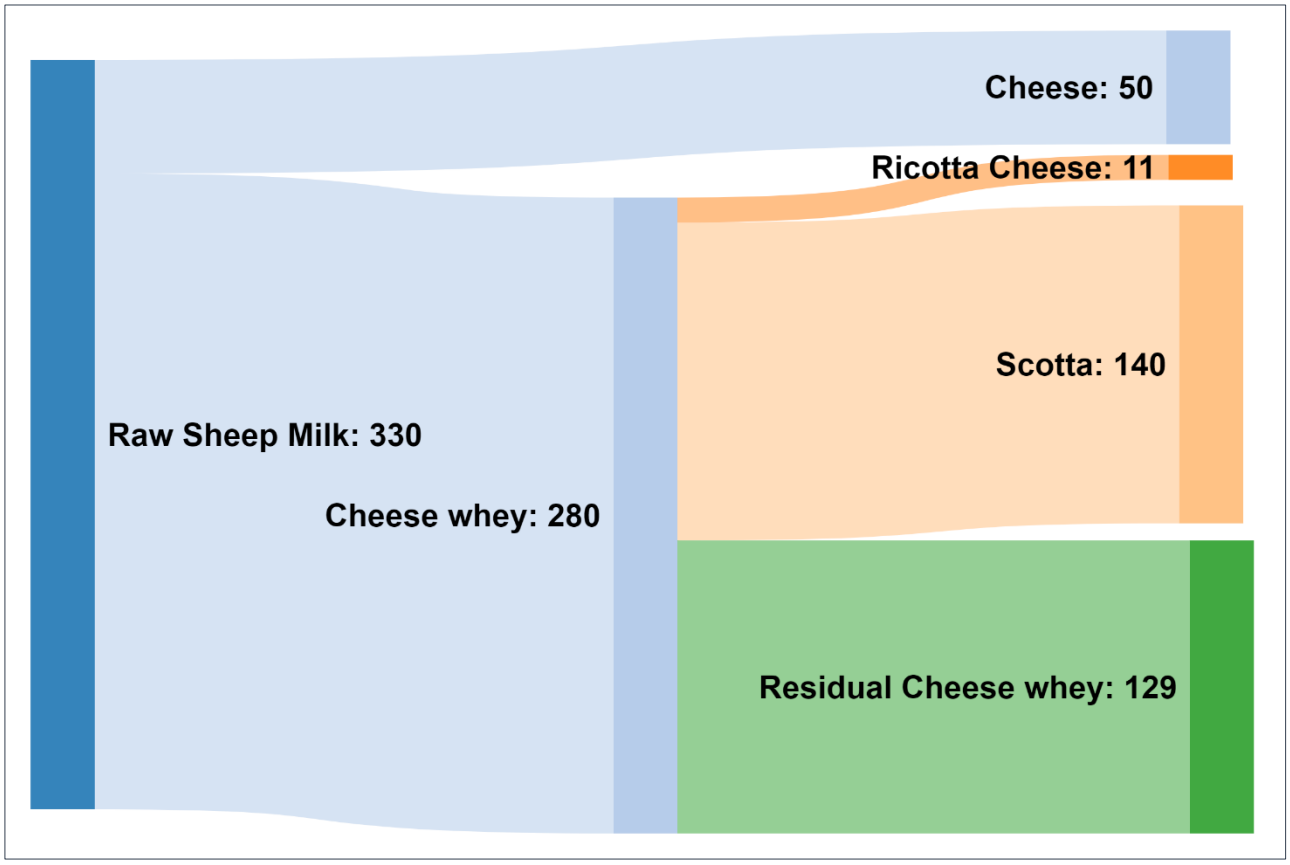


Figure 2.3. *Sankey diagram for the sheep dairy supply chain in Sardinia. Elaboration made by using last production data available (2017) and a conversion yield of 15% and 7% for Cheese and Ricotta, respectively. Data are expressed in kilotonnes.*

[8] https://www.sardiniapost.it/cronaca/abbanoa-lotta-gli-scarichi-anomali-nei-depuratori-due-casi-due-giorni/

2.7 Promising technologies for cheese whey valorisation within a waste biorefinery approach

Over time, considerable efforts to solve the issues related to CW management have been made. Firstly, the CW was considered a severe hazard for the environments and those efforts were oriented on its treatment with limited cases of valorisation. In the perspective of an upcoming implementation of a more sustainable economic growth model based on circular economy, efforts to look for efficient reuse or recovery of materials/energy from any valuable waste stream originated by the production cycles need to be intensified (Asquer et al., 2017). In line with this, the potential associated with CW is gradually emerging, especially for biotechnological processes. It is a common perception that innovative treatments are still a priority to deal with the high organic load of raw CW and its valorisation is advantageous both for the environment and for a sustainable bioeconomy (Bosco et al., 2018; Mollea et al., 2013; Prazeres et al., 2012; Ryan and Walsh, 2016).

The following paragraphs present an overview of promising technologies for CW valorisation. Much emphasis is given on the technologies feasible to fit within the waste biorefinery approach. For the fundamentals of most of the biological processes quoted in the following paragraphs, see chapter 3. For further details, the review of (Mollea et al., 2013) and (Ryan and Walsh, 2016) are recommended.

2.7.1 Fermentative processes

Lactic, acetic, propionic, butyric and other organic acids can be produced from lactose/whey fermentation. As seen previously, organic acids are products with a high value on the market of chemicals, and its production could represent an extra source of income for dairy industry.

Fermentative processes for production of lactic acid, hydrogen plus a pool of VFA and ethanol are described below. Bioconversion of CW through fermentative processes could be optimized to obtain other biochemicals. Some examples are the production of succinic and citric acid from whey (Ahmad et al., 2019; Ryan and Walsh, 2016).

2.7.1.1 Hydrogen and VFA production through dark fermentation

DF is a promising approach for CW valorisation due to its high carbohydrate content, mainly in the form of lactose, which can be converted to biohydrogen and VFAs (Akhlaghi et al., 2017a; De Gioannis et al., 2013). Several different inocula, including anaerobic sludge, activated sludge, compost, and pure cultures, with or without pretreatment, have been proposed for DF of CW (Antonopoulou et al., 2008; Davila-Vazquez et al., 2009, 2008; Venetsaneas et al., 2009; Yang et al., 2007). However, several studies relied on the indigenous biomass of CW, without inoculation, reporting H_2 yields of the same order of magnitude of those obtained in

the studies with addition of an external inoculum (Antonopoulou et al., 2008; De Gioannis et al., 2014; Fernández et al., 2015; Montecchio et al., 2018; Venetsaneas et al., 2009).

De Gioannis et al. (2014) compared DF of CW with (pretreated activated sludge) and without inoculum, in batch, obtaining a similar yield of 160-170 L_{H2} g_{TOC}^{-1} at pH 6-6.5, suggesting that addition of an external inoculum may not be required for starting up DF of CW. Enriched inocula, e.g. fermented CW, can be used to accelerate start-up, but this approach may also favour the development of competing microorganisms, which could decrease the H_2 yield. Perna et al. (2013) used fermented CW as inoculum in a packed bed reactor (PBR), obtaining a yield of only 0.7 mol_{H2} $mol_{lactose}^{-1}$ with a relatively high production of acetic acid (10 g L^{-1}), which suggests the development of homoacetogenic, H_2-consuming microorganisms. Among studies with pure cultures, both *Clostridium Saccharoperbutyacetonicum* (Ferchichi et al., 2005) and *Escherichia coli* (Rosales-Colunga et al., 2009) yielded 2.7 mol_{H2} $mol_{lactose}^{-1}$ from diluted CW and CW powder, respectively, in the same ranges of yields obtained with mixed cultures. Thus, the utilisation of PMC, which would result in higher operating costs in full-scale plants, does not seem a cost-effective approach for CW fermentation.

Various CW-based substrates have been used for DF experiments. Raw CW can be easily degraded by the indigenous bacteria, even at 4 °C, making difficult its storage (Tribst et al., 2019). Thus, many studies used re-hydrated CW powder, adjusting the water content to have a similar composition of the raw CW. Addition of bicarbonate was proposed for preventing acidification (Perna et al., 2013), although co-digestion with an alkaline substrate, such as manure (Ghimire et al., 2017) is a more sustainable approach. Dilution of CW was shown also to prevent a quick acidification of the fermentation broth, thus virtually increasing the H_2 yields, but this would drastically increase the quantity of wastewater in full-scale plant. Furthermore, dilution of CW would reduce the concentrations of micro and macro nutrients available for the microorganisms. Yields above 3 mol H_2 mol^{-1} lactose, and acetic and isobutyric acid concentrations above 5 g L^{-1}, were obtained supplementing CW with micronutrients such as calcium (Azbar et al., 2009b), whereas yields below 2 mol H_2 mol^{-1} lactose, as well as low VFA concentrations, were obtained from deproteinated or ultrafiltered CW (Fernández et al., 2015; Montecchio et al., 2018). This was likely due to the lack of nitrogen for microbial growth, and since the detrimental effect of nitrogen shortage could increase in long-term operation, addition of a protein recovery step before DF of CW is discouraged.

Bioreactors characterised by high biomass retention, such as fluidized bed reactors (Ferreira Rosa et al., 2014a, 2014b; Ottaviano et al., 2017), or sequence batch reactors (Fernández et al., 2015) can be advantageous for DF of CW, compared to CSTRs, since much lower HRTs can be applied. However, HRTs below 4 h may decrease the hydrogen yield (Ferreira Rosa et al., 2014a). Among the operation parameters, pH has the strongest impact on both H_2 yields and VFA production spectrum. An optimum pH between 5.5 and 6.5 for H_2 production from CW under mesophilic conditions has been reported in various studies (Asunis et al., 2019; Azbar et al., 2009c; Davila-Vazquez et al., 2008; De Gioannis et al., 2014; Ferchichi et al., 2005). An optimum pH of 4.5 was reported under thermophilic conditions (Azbar et al., 2009a), and (Ottaviano et

al., 2017) obtained a remarkable yield of 3.67 mol_{H2} $mol_{lactose}^{-1}$ from diluted CW in a thermophilic (55 °C) FBR operated at pH 4-4.5 and 4 hours HRT.

Table 2.3. *Summary of studies on continuous hydrogen and VFA production from several cheese whey-based substrates at different temperature, pH and hydraulic retention time (HRT).*

Substrates	Inoculum	Reactor[a]	T (°C)	pH	HRT (h)	H_2 yield	VFA[b] yield ($g\ L^{-1}$)	Reference
Dry whey permeate powder ($14\ g_{COD}\ L^{-1}\ d^{-1}$)	anaerobic sludge	CSTR	35		24	$3.2\ mM\ g_{COD}^{-1}$	HAc: 2.10 HPr: 0.08 HBu: 0.77 HCa: 1.18	Yang et al., 2007
CW	none	CSTR	35	5.2	24	$0.041\ m_{H2}^3\ kg_{CODadded}^{-1}$ (or 0.9 mol mol $_{glucose\ consumed}^{-1}$ or $2.49\ L\ L_{CW}^{-1}$)	HAc: 9.39 HBu 7.20	Antonopoulou et al., 2008
CW powder (OLR 92.4-184.4 $g_{lactose}\ L^{-1}\ d^{-1}$)	pretreated anaerobic granular sludge	CSTR	37	5.9	4-10	$2.8\ mol_{H2}\ mol_{lactose}^{-1}$	HAc: 4.50 HPr: 6.20 HBu: 10.60	Davila-Vazquez et al., 2009
CW (OLR 30 $g_{COD}\ L^{-1}\ d^{-1}$)	none	CSTR	35	5-6	24	$0.78\ mol_{H2}\ mol_{glucose\ consumed}^{-1}$	HAc: 9.2 HBu: 14.5	Venetsaneas et al 2009
CW powder supplemented with sodium bicarbonate (OLR 22-37 $g_{COD}\ L^{-1}\ d^{-1}$)	fermented cheese whey	PBR	30		24	$0.668\ mol_{H2}\ mol_{lactose}$	HAc: 10 HBu: 2	Perna et al., 2013
Cheese whey powder supplemented with medium (5 $g_{COD}\ L^{-1}$, OLR 30-120 $g_{COD}\ L^{-1}\ d^{-1}$)	pretreated anaerobic granular sludge	AFBR	30	4-4.5	1-4	$1.33\ mol_{H2}\ mol_{lactose}^{-1}$	HAc: 0.21 mol $mol_{lactose}^{-1}$ HBu: 0.41 mol $mol_{lactose}^{-1}$ HPr: 0.37 mol $mol_{lactose}^{-1}$	Ferreira Rosa et al., 2014a
Cheese whey powder supplemented with medium (5 $g_{COD}\ L^{-1}$,	pretreated anaerobic granular sludge	AFBR	30	4-4.5	6	$1.27\ mol_{H2}\ mol_{lactose}^{-1}$	-	Ferreira Rosa et al., 2014b
Deproteinized cheese whey (OLR 12.7-25.3 $g_{COD}\ L^{-1}\ d^{-1}$)	none	SBR	35	4.5-5.5	1.5-3.0	$12\ L_{H2}\ kg_{COD}^{-1}$	HAc: 2.34-3.41 HPr: 1.0 HBu: 0.5	Fernandez et al., 2015

Cheese whey + buffalo manure (OLR 0.7-2.6 g_{VS} L^{-1} d^{-1})	pretreated anaerobic sludge	CSTR	55		192-288	5.88 mmol $_{H2}$ g_{VS}	HAc: 4.18 mmol g_{VS}^{-1} HBu: 14.12 mmol g_{VS}^{-1} HPr: 0.51 mmol g_{VS}^{-1}	Ghimire et al., 2017
CW powder solution (4.9 $g_{lactose}L^{-1}$)	pretreated anaerobic granular sludge	AFBR	55	4-4.5	4	3.67 mol_{H2} $mol_{lactose}$	HAc: 0.46 HBu: 0.67	Ottaviano et al., 2017
Cheese whey (OLR: 29 g_{COD} $L^{-1}d^{-1}$)	kitchen waste compost	CSTR	30	5.5	24	0.9 mol $_{H2}$ $mole_{lactose\ consumed}^{-1}$	HAc: 3.0 HBu: 1.6	Castello et al., 2018
Ultrafiltered cheese whey	none	CSTR	36	5.5	6-12	1.33 – 1.84 mol $_{H2}$ $mol_{lactose}^{-1}$		Montecchio et al., 2018
CW powder	acclimated anaerobic sludge	CFSTR	30	4.5-7	1	n.a.	HAc: 3.5-12 HBu: 2-3 HPr: 2-3	Gouveia et al., 2017

[a] AFBR, anaerobic fluidized bed reactor; CFSTR, continuous flow stirred tank reactor; CSTR, continuously stirred tank reactor; PBR, packed bed reactor; SBR, sequence batch reactor.

[b] HAc, acetic acid; HBu, butyric acid; HCa, caproic acid; HIBu, Isobutyric acid; HPr, propionic acid; TVFAs, total volatile fatty acids

2.7.1.2 Lactic acid production through dark fermentation

Lactic acid is among the most important fermentation products from an economic point of view, and CW has been proposed as an alternative feedstock due to its high amounts of lactose. CW effluents have been used in fermentation processes to produce lactic acid (Arasaratnam et al., 1996; Büyükkileci and Harsa, 2004; Krischke et al., 1991; Luongo et al., 2019; Mostafa, 1996; Panesar et al., 2010; Tuli et al., 1985). Since lactic acid bacteria (LAB) have limited potential to biosynthesise amino acids, nucleotides and vitamins, supplementation of nutrients such as nitrogen is often required in industrial fermentation (Mazzoli et al., 2014; Prazeres et al., 2012). Raw CW can be effectively used as substrate for lactic acid production without extra nutrient supply, although enzymatic hydrolysis might be necessary to release the nitrogen from whey proteins. Xu et al. (2018) reported D-lactic acid production from hydrolysed CW powder by *Lactobacillus bulgaricus* in non-sterile conditions and without the addition of extra nutrients, with a productivity of 2.36 g L^{-1} d^{-1}, which could be further enhanced by addition of a small amount of yeast extract. Secchi et al. (2012) reported the use of ovine scotta for lactic acid production with yields up to 92% and productivity of 2 g L^{-1} h^{-1}, comparable to those obtained on ovine CW. The authors reported that the use of MMC for scotta bioconversion reduced the need for nutritional supplements, with no detrimental effects on the productive parameters compared to PMC. In addition, they reported that the use of PMC (*L. casei* and *S. thermophilus*) was proposed for the production of optically pure L-lactic acid that represents a product with higher added value as compared to the D-form. The lactic acid yield can be further improved by continuous extraction of the produced lactic acid since its accumulation inhibits the microorganisms. (Taleghani et al. (2018) reported a lactic acid production rate of 6.1 g L^{-1} h^{-1} in a fermentative reactor with integrated membrane extraction system, to be compared to 3.4 g L^{-1} h^{-1} obtained in the control reactor without membrane extraction.

Nowadays, no full-scale application (TRL 8-9) are reported for lactic acid production directly from CW. Nevertheless, the project *AgriChemWhey*, founded in the framework of BBI-JU, aims to build a first-of-a-kind, industrial-scale bio-refinery, which will convert dairy residues (excess whey permeate and delactosed whey permeate) into cost-competitive and sustainable lactic acid. Previously, the EU-funded project *WHETLAC* aimed to produce lactic acid from the residual whey permeate and confirmed from a technical point of view the possibility to obtain pure lactic acid (purity grade above 80-90%) by using immobilised fermentative bacteria in polyvinyl alcohol gel particles combined with a purification step by supercritical fluids. However the main conclusion was that the final price for lactic acid produced would be still 2 times higher when compared with similar marketable products; nevertheless, this technology could represent a reliable option for whey processing if compared with other emerging technologies (European Commission and CORDIS, 2009). The authors also highlighted that the dimension and fragmentation of cheese SMEs is a limiting factor for the deployment of this technology despite the fact it may be applied to most of the whey types produced.

2.7.1.3 Bioethanol production through dark fermentation

Fermentation of CW into ethanol is currently hardly competitive with the established processes based on sugar cane, corn starch or lignocellulosic biomass as raw material (Guimarães et al., 2010). Solventogenic fermentation of CW has been attempted with yeast such as *Saccharomyces cerevisiae* (Staniszewski et al., 2007), but low ethanol yield was obtained due to low lactose conversion and product inhibition. Conversely, *Kluyveromyces marxianus* yeast was shown to hydrolyse lactose, form biofilm and tolerate ethanol, and is thus a potential candidate for CW conversion into bioethanol (Joshi et al., 2011; Lane and Morrissey, 2010). Continuous fermentation is potentially superior than batch process, as it improves ethanol production, and reduces the fermentation time (Gabardo et al., 2014). Several techniques have been proposed to retain the microorganisms into the bioreactor, including cell immobilisation (Dahiya and Vij, 2012), cell recycle (Santos et al., 2016), and membrane retention (Wei et al., 2014). Christensen et al. (2011) obtained continuous ethanol production from CW, with a rate of 2.5–4.5 g L^{-1} h^{-1}, using a pure culture of *K. marxianus* immobilised in Ca-alginate.

The ethanol yield strictly depends on operation parameters such as substrate concentration, pH, and temperature. Using a continuous FBR with Ca-alginate immobilized-cells, Gabardo et al. (2014) obtained the highest ethanol productivity of 6.01 g $L^{-1}h^{-1}$ from CW permeate at a concentration of 150 g L^{-1} although the highest ethanol yield was obtained at 90 g L^{-1} concentration. Dragone et al. (2011) reported that a lactose concentration of 200 g L^{-1} and a temperature of 35 °C were optimal for ethanol production (81 g L^{-1} in 44 h) from CW powder by *K. fragilis*. Using the response surface methodology, Diniz et al. (2014) reported that temperatures between 33.3 and 38.5°C, pH between 4.7 and 5.7, lactose concentrations between 50 and 108 g L^{-1} and biomass concentrations between 2.4 and 3.3 (optical density at 600 nm) are optimal for ethanol production from CW by *K. marxianus*, with yields above 90% of the theoretical value.

It is worth to mention that examples of industrial application of a whey-to-biofuels bioprocess is based precisely on bioethanol production. Some examples of industrial-scale plants are located in Ireland, in New Zealand, in USA, in Denmark and Germany. In Ireland the factory of the Carbery Group, based in the County Cork, is the largest single cheese-producing facility and started the operation of an industrial-scale whey-to-ethanol plant in 1978, being the pioneer; in addition to producing a fine range of cheese, the company produces high-quality ethanol. Since 2005, the company has also been supplying fuel ethanol to a petrol company in Ireland. The Carbery process was later adopted by plants in New Zealand and the United States (Ling, 2008). In New Zealand, where half of the whey produced is converted to ethanol, Anchor Ethanol operates three whey-to-ethanol plants; the feedstock is deproteinated whey, concentrated from 4% to 8% lactose by reverse osmosis and fermented for about 24 h using *Kluyveromyces species*, attaining an ethanol titre of about 4%, followed by distillation and water removal to different ethanol grades.

2.7.2 Biopolymers production

The CW (first or second) has also become an exciting area of investigation because it could be used as raw material for bioplastics production (Ryan and Walsh, 2016b). Some of its principal constituents, like lactose or proteins, could be converted into a wide range of biopolymers, such as PLA, PHA and other bioplastics made from whey proteins. For instance, one of the main aims of the above mentioned Agrichemwhey project is the production of lactic acid for the subsequent conversion into PLA.

In the last two decades, a large number of studies were related to the production of PHA from CW using PMC of wild type microorganisms or recombinant ones. However, the efficient use of whey as a carbon source for PHA production is still hindered by numerous issues, including whey pre-treatments and PHA producing strain choice. PHA production from CW has been reported from microorganisms able to synthesize polymers from lactose, such as *Thermus thermophilus* (Pantazaki et al., 2009), *Pseudomonas hydrogenovora* (Koller et al., 2008), and *Bacillus megaterium* (Das et al., 2018) or engineered *Cupriavidus necator* (Povolo et al., 2010), the latter expressly designed to growth on lactose. While genetic engineering is a highly versatile and promising tool for enhancing PHA production from whey, the use of genetically engineered strains requires more controlled production plants. The use of other species of microorganism capable of higher PHA yields but unable to growth on lactose, in some cases, can be bypassed by chemically or enzymatically converting whey lactose into glucose and galactose prior to fermentation (Amaro et al., 2019). In this case, well known microorganisms like *Cupravida Necator*, may be able to accumulate up to 80% of its dry weigh, growing on the glucose. However, the pretreatment step, add to the final cost of PHAs and thus, from an industrial point of view, should be avoided.

Although higher PHA accumulation can be attained with PMC, MMC can be adapted to produce PHA from complex substrates, such as dairy biowastes with the advantage of do not require sterilisation. MMC has been used to directly produce PHAs from cheese whey lactose or after the first digestion of lactose by a different MMC (Carletto, 2014; Colombo et al., 2019, 2016; Duque et al., 2014; Gouveia et al., 2017; Valentino et al., 2015b). Although nutrient supplementation is commonly reported in literature for selecting PHA-storing MMC with good storage capacity (Oliveira et al., 2018), the high N and P contained in dairy biowaste might reduce, or even eliminate, the need for addition of external nutrients (Colombo et al., 2016). PHA production from fermented CW by mixed cultures resulted in storage yields of 0.7–0.8 $Cmol_{PHA}$ $Cmol_{substrate}^{-1}$, with PHA contents between 65-75% (Colombo et al., 2016; Duque et al., 2014; Oliveira et al., 2017; Valentino et al., 2015a). The PHA composition (PHB/PHV fraction) depends on the carboxylic acid present in CW fermentate: the highest the concentration of acetic and butyric acid, the highest is the PHB fraction, whereas high concentrations of propionic acid result in accumulation of PHV. PHV fraction up to 40% has been reported from fermented CW (Table 2.4). Fermented CW has also been used as a substrate for PHA production by phototrophic mixed culture (PHA content of 20-25% and yield of 0.6 $Cmol_{PHA}$ $Cmol_{substrate}^{-1}$) using light intensities comparable with those naturally obtained in sunny regions (Fradinho et al., 2019).

The interest in PHA production from CW is emerging even in real-scale application. The project YPACK is an EU-funded project aims to scale up and commercially validate two innovative food packaging solutions based on PHA produced from CW and almonds shells. Similarly, the WHEYPACK project aims to demonstrate the environmental and socio-economic benefits of a biodegradable food packaging material with a lower environmental impact through the reduction of GHG emissions in comparison with current petrol-based food packaging materials. The biodegradable food packaging material selected is PHB that will be obtained from a by-product (whey) that comes from the cheese industries; PHB will be produced using a process of microbial fermentation.

Table 2.4. *Summary of recent studies focusing on PHA production from cheese whey.*

Substrate	Fermentation yield (g_{COD} g_{COD}^{-1})	Fermentation products (PHA precursors) HLa/HAc/HBu/HPr/HVa/HCa/EtOH on $\%COD_{OA}$	Max PHA content (as g_{PHA} kg_{VSS}^{-1})	PHA storage yield ($g_{COD\text{-}PHA}$ $g_{COD\text{-}OA}^{-1}$)	Productivity (g_{PHA} L^{-1} d^{-1})	Polymer composition (%HB:%HV)	Reference
Second cheese whey	n.a.	HAc:50-55 HBu:18.6-36.3 as total OA	620 ± 450	0.84 ± 0.01	n.a.	100:0	Colombo et al., 2018
Concentrated whey permeate	n.a.	HAc:45-48 HBu:43-50 as total OA	551 ± 13	0.82 ± 0.11	n.a.	100:0	Colombo et al., 2018
Sweet cheese whey powder	0.64 ± 0.05	0/46/44/4/5/0/0	430	0.85 ± 0.12	0.20	89:11	Oliveira et al., 2018
Cheese whey	0.4 ± 0.0	58/16/26/0/0/0/0	659 ± 46	0.6 ± 0.0	10.9 ± 0.8	100:0	Colombo et al., 2016
Sterilised cheese whey	0.6 ± 0.1	6/58/13/19/4/0/0	814 ± 57	0.7 ± 0.1	28.2 ± 2.0	60:40	Colombo et al., 2016
Filtered whey permeate	0.5	0/44/50/2/1/3/0	530-630	0.41-0.63	n.a.	85:15	Valentino et al., 2015
Cheese whey	0.7 ± 0.2	1/58/22/6/4/0/9	650	0.7 ± 0.1	13.4	81:19	Duque et al., 2014

2.7.3 Bioelectrochemical systems (BES)

BES can be implemented to recover the energy contained in organic compounds contained in CW as electricity in MFC, or for synthesis of H_2 or other compounds in MEC. Antonopoulou et al. (2010) were the first to test CW, although 100 times diluted (0.73 g_{COD} L^{-1}) and amended with nutrients, as substrate for MFC, producing a maximum power density of 18.4 mW m^{-2} and coulombic efficiency (CE) of only 1.9%, due to the presence of undesired microorganisms in the CW. To address such an issue, Stamatelatou et al. (2011) filter-sterilised CW before 100 times dilution, obtaining power production up to 40 mW m^{-2}. The effect of COD concentration (0.35-6.7 g L^{-1}) was investigated by Tremouli et al. (2013), who reported the highest power production (46 mW m^{-2}) and CE (11.3%) from diluted CW at 6.7 g L^{-1} COD concentration, with 95% COD removal. Ghasemi et al. (2017) compared CW (50 g L^{-1} of lactose) and concentrated CW (100 g L^{-1} of lactose) as substrate in a cube-shaped, two-chamber MFC, reporting a higher power density (288 mW m^{-2}) from CW than concentrated CW (188 mW m^{-2}). Since carboxylic acids are favourable substrates for exoelectrogenic microorganisms, Wenzel et al. (2017) proposed fermented CW as substrate for a single chamber MFC, obtaining a dramatically higher power production (439 mW m^{-2}) than a control reactor fed with raw CW (0.34 mW m^{-2}). Indeed, exoelectrogenic microorganisms were enriched in the MFC fed with fermented CW, due to the high concentration of VFAs, whereas the high lactose and lactic acid concentrations of the raw CW resulted in a prevalence of fermentative microorganisms.

Both CW and fermented CW, as well as digested CW, have been used as substrate for H_2 production in MEC. Diluted CW (2 g L^{-1} COD), amended with a phosphate buffer solution, was used as substrate for H_2 production in a MEC, resulting in a production of 0.8 L H_2 L^{-1} d^{-1}, with energy recovery up to 71%. Rago et al. (2017). The CE above 100% obtained in this study was attributed to H_2 recycling by homoacetogenic bacteria. Moreno et al. (2015) combined DF and MEC for two-stage H_2 production from CW, obtaining an H_2 production of 0.5 L L^{-1} d^{-1} from filtered, eight times diluted fermented CW, supplemented with acetate, in a MEC. However, a rapid decrease in the MEC performance occurred, probably due to the lack of nutrients of the diluted substrate. Rivera et al. (2017) compared raw CW, fermented CW and digested CW for H_2 production in a single-chamber MEC. An H_2 production of 61 and 48 mL H_2 g^{-1} $COD_{removed}$ was obtained from digested and fermented CW, with a CE of 93 and 32%, respectively, whereas a negligible H_2 production (CE 1%) was obtained from raw CW (Rivera et al., 2017). However, the results of this study were influenced by the different composition of the substrates, which were not normalised in terms of COD concentration.

The results suggest that fermented CW, rather than raw CW, should be used as a substrate for efficient energy recovery in METs. METs can also be seen as a final polishing step after the AD process. Filtration and dilution should be avoided, since it may result in a lack of nutrients which can hinder the electrogenic activity. Nutrient supplementation should be minimised to avoid high operation costs in full-scale applications.

2.7.4 Other biological processes

Other biotechnological alternatives for the utilisation of dairy residues could be the production of exopolysaccharides, biosurfactants, bacteriocin, single-cell protein, single-cell oils or enzymes (De Jesus et al., 2015; Mollea et al., 2013). Most of those compounds have been studied recently, but more studies are necessary to develop microbial strains to obtain maximum yields at a cost that make the processes viable for scaling to industrial levels (De Jesus et al., 2015).

2.8 Conclusions

The European dairy sector could be considered the economic backbone of rural Europe considering its importance in terms of production, profit and employees. However, the EU dairy sector is currently experiencing some economic and environmental challenges, which are even amplified in some specific areas, e.g. the Italian region of Sardinia. It is a common perspective that the sector must become more resilient and sustainable, both economically and environmentally.

Dairy sector is well known to produce a considerable amount of bioresidues, principally cheese whey, and its management is often a crucial aspect for the environmental and economic sustainability of the dairies. In the last decade, dairies started recognising the potential of those residues from the point of view of valorisation. Some promising technologies have the potential to exploit the favourable characteristic of those residues adding benefit to the whole supply chain.

3 FUNDAMENTALS OF MIXED CULTURE BIOTECHNOLOGY FOR CHEMICALS AND ENERGY RECOVERY FROM BIOWASTES

As extensively discussed in the previous chapters, the concept of waste biorefinery involves the conversion of biowaste into a spectrum of bioproducts and bioenergy through various processes, among which biochemicals processes are believed to play a key role. More in detail, the concept of waste biorefinery entails different features that historically belong to two different fields: the biowaste treatment is typical of environmental biotechnology aimed at minimising effluent substrate concentrations, while product formation is typical of industrial biotechnology, aimed at maximising productivity and yields. Those two different goals strongly influence the choice of microorganisms, substrates and process operations.

With a focus on microorganism involved, while MMC based technology has been widely used in environmental technology (mostly with activated sludge process and AD), the use of PMC, carefully selected in the laboratory or created ex-novo by genetic engineering, traditionally belongs to the field of industrial biotechnology. Nowadays, many bioproducts such as organic acids and biopolymers but also amino acids, antibiotics and enzymes are produced by pure culture (Jiang et al., 2017). For instance, the industrial production of PHA employs genetically modified *Escherichia Coli* and *Alkaligenes* species (Kleerebezem and van Loosdrecht, 2007) while the production of lactic acid involves bacteria belonging to the genera of *Lactobacillus, Lactococcus, Streptococcus, Bacillus and enterococcus* (Miller et al., 2011).

Nowadays, various authors proposed the use of MMC as an attractive option and alternative to traditional PMC based biotechnology for the valorisation of biowastes within the waste biorefinery concept (Agler et al., 2011; Kleerebezem and van Loosdrecht, 2007; Sabra and Zeng, 2014). The interest in MMC-based technology is evolving driven by the pivotal aspects of environmental, economic and technical assessments of the biorefinery concept (see 1.3.3). The use of biowaste as a substrate for biochemicals production is environmentally friendly but, often even more important from the industrial point of view, represents a huge opportunity to make the process economically more competitive on the market. At the same time, since the use of PMC can hardly be adapted to a complex and unsterile substrate as biowastes, the use of MMC seems the best solution from a technical point of view.

As a clarifying example, the high interest in PHA production with MMC by both academia and industries is currently driven by those considerations. The biological production of PHA is still not considered commercially competitive because the current price of 2.2-5 $ kg^{-1} is still higher

than the conventional petroleum-based polymer, which typically cost less than 1.0 $ kg^{-1} (Valentino et al., 2017). It has been estimated that around 40-50% of the total production cost can be ascribed to the raw materials (sugar and glucose extracted from plant source) (Rodriguez-Perez et al., 2018; Valentino et al., 2017). Therefore, the reduction of production costs by applying different strategies, i.e. using biowaste as a cheap carbon source, is one of the main goals in research focused on PHA production.

In the next paragraphs, the use of MMC in biotechnology will be discussed with more emphasis on the technical and theoretical aspects than to the economic and environmental. Some biological processes will be briefly discussed: dark fermentation for hydrogen and organic acid production and 3-step process for PHA production. Those examples will also give an idea of the potential associated with the use of MMC within the waste biorefinery and will give some theoretical fundamentals about the biological processes studied in the following chapters.

3.1 Implementation of MMC in waste biorefineries

The term MMC is referred to microbial community composed of several species and strains. Based on ecological selection principles, a biological process based on MMC can be established and oriented towards a narrow product spectrum by manipulating the operation of the bioprocess or by varying the source of the natural inoculum. The use of MMC-based technology offers some attractive advantages compared to the use of PMC.

The simultaneous presence of several and closely interconnected bacterial species and strains represent the main advantages of an MMC-based process because of its robustness and stability. Robustness is a property that allows a biological system to maintain its function against internal and external perturbation, i.e. accumulation of intermediates and inhibitors, change in the substrate composition, contaminations and infections, and, more generally, every environmental change (Sabra and Zeng, 2014). In a stable MMC-based system, accumulation of intermediates and inhibitor is generally avoided because such a system can perform multistep transformation so that other species consume intermediates and inhibitors within the microbial community. Furthermore, MMC can handle the variations in biowaste composition because alternative metabolic pathways are available among different members of the microbial community leading to the so-called community flexibility, i.e., the ability of a system to shift the flow of electrons and carbon to the same products through alternative pathways (Sabra and Zeng, 2014). Even in the case of contamination, since MMC have a broader genetic base of resistance to phage, failures are less common than in PMC-based processes, often because if

one strain is wiped out, a second or third phage resistant strain in the inoculum may will take over and continue the fermentation. For those reasons, MMC can be used in an open unsterile biological process adding further saving in the production cost compared to PMC-based process (that require strong and expensive sterilization of the substrate).

Another interesting point is that, while PMC-based fermentation depends on bacteria that can be cultivated in the laboratory and that represent only about 1% of the total diversity that exists in nature, cultivating MMC could promote the growth of some bacteria that are "unculturable" in a traditional way (Sabra and Zeng, 2014). Some authors report the fact that many cultured bacterial isolates are lost and no longer viable once their bacterial associations are entirely removed (Sabra and Zeng, 2014). Furthermore, MMC processes can help find new substances of industrial interest because several secondary metabolites are produced within the biological community. Moreover, metabolites generated by mixed consortia often complement each other and work to the exclusion of unwanted microorganisms and therefore lead to a better process stability.

3.2 MMC fermentation

In engineered systems under anaerobic conditions, an MMC-based process can convert biowaste to a wide range of valuable chemicals as intermediates and end-products such as hydrogen, SCFA and methane. The process entails the cascade bioreaction consisting in different steps of hydrolysis and fermentation with different group of functional microorganisms including fermentative bacteria, acetogens, homoacetogens and hydrogenotrophic and acetoclastic methanogens involved (Agler et al., 2011; Kleerebezem and van Loosdrecht, 2007). This chain is regulated by a syntrophic mechanism between several microorganism species that cooperate.

Figure 3.1 gives an overview of the biochemicals reaction that can occur within an MMC-based process. In this scheme, the process is divided into hydrolysis and subsequent conversion by primary fermentation and secondary fermentation, according to Agler et al. (2011). This schematic representation emphasises the role of intermediates and the possible interconnection of different biochemicals processes; each of them may occur in the same bioreactor or different systems. With this point of view, an MMC fermentation act as a platform, the carboxylate platform (Agler et al., 2011). Table 3.1 and Table 3.2 show the possible bioreactions involved in MMC fermentations.

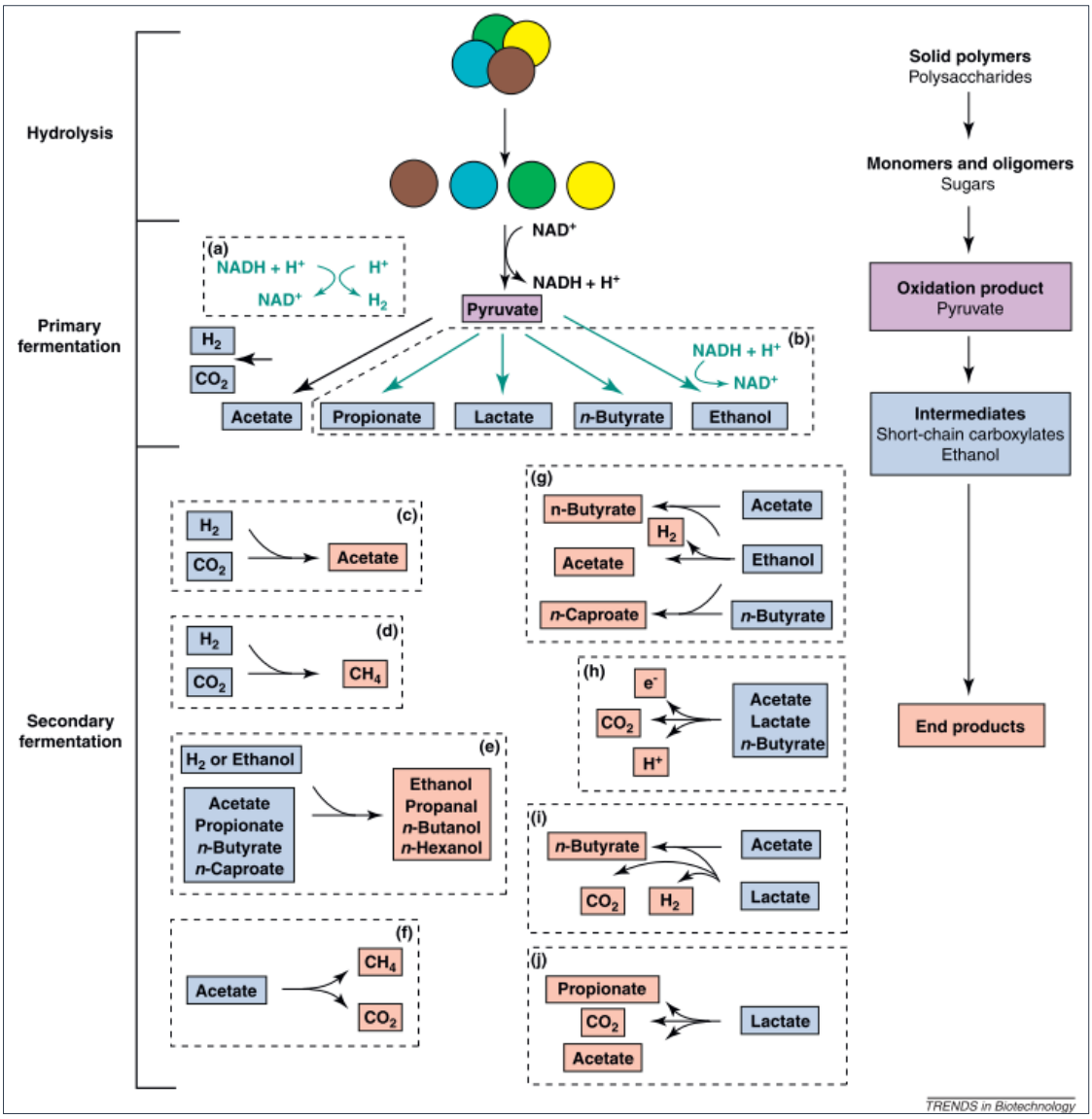


Figure 3.1. *Hydrolysis and subsequent conversion by primary and secondary fermentation reactions carried out by undefined mixed cultures, as described in Agler et al. (2011).*

3.2.1 Hydrolysis

The fermentation of biowastes from MMC requires a hydrolysis step to broke complex polymer, such as polysaccharides, into monomer and oligomers (Agler et al., 2011; Chandra et al., 2018). The capacity of the MMC to effectively hydrolyse the substrates is related to the specific hydrolytic bacteria present in the inoculum used and to the specific operative conditions. Since the MMC may not have the right enzymes, a specific pretreatment through the addition of enzymes or chemicals is a common practice in the literature. For example, the use of *β-galactosidase* enzyme from *Aspergillus oryzae* has been reported to facilitate hydrolysis of lactose into more fermentable sugars (glucose and galactose) for cheese whey (CW) fermentation by MMC consisted in anaerobic digestate (Colombo et al., 2019). In the specific

case of lactose-rich substrate, the capacity of the culture to hydrolyse lactose in glucose and galactose is specific of some strains belonging to lactic acid bacteria (see Table 3.1). In the case of lignocellulosic biowaste, several methods including acid-based methods, hydrothermal processing, mild alkaline methods, oxidative methods, steam explosion and ionic liquid solvent have been proposed to remove lignin and hemicellulose, which are resistant to microbial degradation, and release the cellulose (Jönsson and Martín, 2016). Interestingly, Yang et al. (2009) reported an efficient degradation of lignocellulosic plant biomass, without pre-treatment by the thermophilic anaerobe *Anaerocellum thermophilum DSM 6725.* Numerous efforts are being made to improve the performance of the hydrolysis step since it is considered the rate-limiting step of the whole fermentation process.

3.2.2 Primary fermentation

Primary fermentation reactions convert the sugars contained in the biowaste to carboxylates, such as acetate, propionate, lactate and butyrate, and biogas composed by hydrogen and carbon dioxide. As seen previously, each of those compounds can be valuable chemicals. The main biochemicals reactions involved in MMC fermentation of glucose are shown in Table 3.1.

Once the extracellular glucose is transported into the cytoplasm by phosphotransferase system, glucose is mainly converted to pyruvate through glycolysis via the Embden-Meyerhof (EM) pathway. The incidence of other pathways, as Entner-Doudoroff (ED) pathway and the pentose phosphate (PP) pathway, is usually relatively low. The oxidation of glucose to pyruvate result results in production of nicotinamide adenine dinucleotide (NADH) and H+. All equivalents must be reoxidized via H+ reduction by NADH oxidation (Figure 3.1*a*) or by NADH oxidation via reduction of pyruvate or its oxidised organic derivatives (Acetil-CoA), depending upon the hydrogen partial pressure (Figure 3.1*b*). If acetyl-CoA is then converted to acetate, NADH and reduced ferredoxin are used to convert H^+ to H_2 through a metalloenzyme called hydrogenase, yielding the theoretical maximum of 4 mol H_2 mol^{-1} glucose. At increasing hydrogen partial pressures, the flow of electrons from NADH shifts from H_2, acetate and CO_2 production towards formation of increasingly reduced fermentation products, such as butyrate and propionate, or alcohols such as ethanol, and only the remaining NADH and ferredoxin is used for H_2 production. The measured hydrogen production per mole of glucose in MMC fermentation is much lower than the maximum theoretical yields and will typically not exceed two moles since as part of the substrate is utilised for biomass production, and the degradation of the substrate might follow other biochemical pathways with lower hydrogen yield or without hydrogen production.

Among them, the butyric pathway is the second-most common pathway. The butyric pathway yields 2 mols of hydrogen per mole of glucose, and it is controlled by two main enzyme group, phosphotransbutyrylase and butyrate kinase and butyryl-CoA/acetate CoA-transferase. The butyric pathway is more favoured in order to avoid the accumulation of inhibitory reducing equivalent when H_2 partial pressure exceeds 60 Pa (Dai et al., 2017). Some authors proposed the molar ratio of butyric to acetic acid as a quantitative indicator of the H_2 yield associated with microbial metabolic pathways, with higher than 2 indicated for efficient H_2 production by anaerobic consortia (Ghimire, 2015).

Propionate is one of the reduced products of primary fermentation at elevated levels of hydrogen and results in a consumption of hydrogen. Two main pathways of propionate formed from pyruvate are the methylmalonyl-CoA pathway and acryloyl-CoA pathway (Stams et al., 1998).

With a focus on hydrogen production, the main bacteria involved in hydrogen production are facultative anaerobes, such as *Escherichia coli* and *Enterobacteriaceae,* or strict anaerobic microorganism belonging to *Clostridum sp*. Among them, *Clostridium butyricum* is responsible for the production of acetate and butyrate while *Clostridium articum* or *Clostridium propionicum* produce propionic acid. The presence of other species communities such as *Bacillus spp*. and *Lactobacillus spp.* can lower the H_2 yield by diverting the pathway to lactic fermentation.

Lactate fermentation dominates primary fermentation in undefined mixed cultures when high concentrations of easily degradable substrate are available because the lactate pathway enables rapid disposal of reducing equivalents (Agler et al., 2011). It is a common metabolic pathway when biowaste is rich in lactose, a disaccharide composed by one glucose and one galactose molecule, such a dairy wastewater or residue (cheese whey). As previously mentioned, the presence of lactose requires its hydrolysis in two monosaccharides, i.e. glucose and galactose, by enzymes such as *β-galactosidase*, produced by lactic acid bacteria (LAB). Homolactic fermentation produces lactate as a single end product via the Embden-Meyerhof-Parnas (EMP) pathway, according to which 2 mol of pyruvate are produced from glycolysis of glucose and then reduced to lactate, resulting in a yield of 4 mol lactic acid mol^{-1} lactose (Castillo Martinez et al., 2013; Sikora et al., 2013). In heterolactic fermentation, 1 mol of pyruvate is converted to lactate while the other mole is converted to ethanol (or acetate) and carbon dioxide via the phosphoketolase pathway, reducing the lactic acid yield (Castillo Martinez et al., 2013; Sikora et al., 2013). Fermentation pathway and the lactic acid (L- or D-) produced depends on the genus of lactic acid bacteria involved, as well as operating condition, in particular pH (Mazzoli et al., 2014; Miller et al., 2011; Xu et al., 2018).

Table 3.1. *Overview of the primary fermentation reactions in an MMC-based processes.*

Reaction	Typical Microbe	Conversion reactions
Glycolysis		glucose + $2NAD^+$ → 2 $pyruvate^-$ + 2NADH + $4H^+$ +2ATP
Acetate pathway	*Clostridium pasteurianum*	glucose → 2acetate + $4H_2$ + $2H^+$ + $2CO_2$
Butyrate pathway	*Clostridium butyricum*	glucose → butyrate + $2H_2$ + H^+ + $2CO_2$
Propionate pathway	*Clostrium acetobutylicum*	glucose + $2H_2$ → 2propionate + $2H^+$
Ethanol pathway		glucose → 2ethanol + $2CO_2$
Lactose hydrolysis	*Bacillus and Lactobacillus sp.*	lactose → glucose + galactose
Homolactic	*Streptococcus, Lactobacillus*	glucose → 2$lactate^-$ + $2H^+$
Heterolactic	*Leuconostac, Lactobacillus*	glucose → $lactate^-$ + $2H^+$ + CO_2 + acetate glucose → $lactate^-$ + $2H^+$ + CO_2 + ethanol

3.2.3 Secondary fermentation

The products of primary fermentation, such as lactate or acetate, are themselves valuable products when separated from the culture broth, but often they are substrates for further fermentation in the same MMC through secondary fermentation reactions (Figure 3.1) or in separate bioprocesses. Among the various stepwise bioreactions that can occur there are (Figure 3.1): autotrophic homoacetogenesis (c); hydrogenotrophic (d) and acetoclastic methanogenesis (f); carboxylate reduction to alcohols with hydrogen or ethanol (solventogenesis) (e); chain elongation of carboxylates with ethanol (g); bioelectrochemical reactions (h); lactate oxidation to n-butyrate (acetate and H+ as electron acceptor) (i) and lactate reduction to propionate (oxidation to acetate for energy conservation) (j).

3.1.1.1 Autotrophic homoacetogenesis

Homoacetogenesis is a critical hydrogen-consuming pathway within MMC fermentation, and it is often considered among the main causes for the decrease of H_2 yield in DF tests. Homoacetogens may oxidise or synthesise acetate depending on the external H_2 concentration. They compete with hydrogenotrophic methanogens for H_2 at low pH, and homoacetogenesis is favoured at high H_2 partial pressure (>500 PA). *Butyribacterium*, *Clostridium*, *Eubacterium*, *Peptostreptococcus*, and *Sporomusa* are commonly reported homoacetogens and belonging to strict anaerobes, fast-growing with some being spore-forming microorganisms which are

phylogenetically diverse and very versatile. For further details about homoacetogenesis during hydrogen production in by MMC, the lecture of Saady (2013) is recommended.

3.1.1.2 Hydrogenotrophic and acetoclastic methanogenesis

Methanogenesis represent the last step in the conventional process of AD. The main biogas product in AD is methane, which represent the compound with the lowest free energy content upon oxidation to carbon dioxide. Hydrogenotrophic methanogens produce CH_4 by reducing CO_2 using H_2 as the electron donor (Bundhoo and Mohee, 2016). Acetoclastic methanogens use Acetate for both electron donor and acceptor. The former reaction is carried out by *Methanosarcinaceae* and *Methanosaetaceae*, while *Methanomicrobiales* and *Methanobacteriales* perform the latter one (Karakashev et al., 2006).

3.1.1.3 Solventogenesis

The solventogenesis consist of carboxylate biological reduction of carboxylates, such as acetate, n-butyrate and n-caproate, to the corresponding alcohols using molecular hydrogen or ethanol. Alternatively, acetate can also be reduced to ethanol with an artificial mediator and a mixed culture at the cathode of a BES, where electrons donated from the cathode provide the required reducing power.

3.1.1.4 *Chain elongation of carboxylates*

An MMC that is capable of reducing acetate to ethanol (Figure 3.1e) can also produce n-butyrate by further reaction of ethanol with acetate (Figure 3.1g). Thus, these two secondary fermentation processes allow one MMC to convert acetate and hydrogen to n-butyrate by elongation of the acetate carbon chain.

3.1.1.5 Bioelectrochemical reactions

BES (Bioelectrochemical systems) are innovative systems in which biological reactions are coupled to reactions at solid electrodes to produce electric power or valuable chemicals. BES can be implemented to recover the energy contained in organic compounds contained in biowaste as electricity in microbial fuel cells (MFCs), or for synthesis of H_2 or other compounds in microbial electrolysis cells (MECs). In MFCs, specific microorganisms called exoelectrogens oxidise the organic substrate and transfer the electrons to an anode electrode. Electrons then flow to a cathode electrode through an external circuit, producing electric power, and combine to an electron acceptor, such as oxygen, closing the circuit (Logan et al., 2006). In MEC, protons

resulting from substrate oxidation are the final electron acceptor, producing H_2, if enough energy is provided to drive the reaction (Rago et al., 2016).

3.1.1.6 Lactate oxidation and reduction

The overtaking of lactate pathway over the other metabolic pathway can lead to an accumulation of lactic acid that can be oxidised and reduced by secondary fermentation reactions to other carboxylates, such as n-butyrate and propionate. For example, lactate oxidation to n-butyrate is catalysed by *Clostridium acetobutylicum* using acetate and H^+ as electron acceptors. The lactate oxidation and the acetate reduction have to be coupled in order to make the reaction energetically feasible (Agler et al., 2011). Another pathway is lactate reduction to propionate, which is catalysed by *Selenomonas ruminantium*. In this case, energy is stored as ATP during acetate production while it is coupled with lactate reduction. Overall it results in the conversion of three molecules of lactate into one of acetate and two of propionate. Those phenomena are common in the gut (Agler et al., 2011) and they have been pointed out in some studies involved DF of molasses or cheese whey (Fuess et al., 2019, 2018).

Table 3.2. *Overview of some of the possible secondary fermentation reactions in an MMC-based process (adapted from Agler et al. (2011)).*

Reaction	Typical Microbe	Conversion reactions
Autotrophic homoacetogenesis	*Acetobacterium woodii* *Clostridium aceticum*	$4H_2 + CO_2 \rightarrow$ acetate- + $H^+ + 2H_2O$
Aceticlastic methanogenesis	*Methanosaeta soehngenii*	acetate$^-$ + $H^+ \rightarrow CH_4 + CO_2$
Hydrogenotrophic methanogenesis	*Methanospirillum hungatei*	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$
Carboxylate reduction with molecular hydrogen	Undefined mixed culture	acetate- + H+ +$H_2 \rightarrow$ ethanol + H_2O propionate$^-$ $H^+ + 2H_2 \rightarrow$ propanol + H_2O n-butyrate$^-$ + $H^+ + 2H_2 \rightarrow$ n-butanol + H_2O n-caproate$^-$ $H^+ + 2H_2 \rightarrow$ n-hexanol + H_2O
Propionate reduction with ethanol	Undefined mixed culture	ethanol + $H_2O \rightarrow$ acetate$^-$ + $H^+ + 2H_2$ propionate$^-$ + $H^+ + 2H_2 \rightarrow$ propanol + H_2O
Chain elongation of Acetate	*Clostridium kluyveri*	ethanol + $H_2O \rightarrow$ acetate$^-$ +$H^+ + 2H_2$ ethanol + acetate $\rightarrow$ n-butyrate$^-$ + H_2O
Chain elongation of n-butyrate	*Clostridium kluyveri*	ethanol + $H_2O \rightarrow$ acetate$^-$ + $H^+ + 2H_2$ ethanol + n-butyrate$^-$ $\rightarrow$ n-caproate$^-$ + H_2O
Lactate oxidation to n-butyrate	*Clostridium acetobutylicum*	2acetate$^-$ + $H^+ + 2H_2 \rightarrow$ n-butyrate$^-$ + $2H_2O$ 2lactate$^-$ + $H^+ \rightarrow$ n-butyrate$^-$ + $2CO_2 + 2H_2$
Lactate reduction to propionate	Selemonas ruminantium	lactate$^-$ + $H_2O \rightarrow$ acetate$^-$ + $CO_2 + 2H_2$ lactate$^-$ + $H_2 \rightarrow$ propionate$^-$ + H_2O
Chain elongation of Acetate	*Clostridium kluyveri*	ethanol + $H_2O \rightarrow$ acetate$^-$ +$H^+ + 2H_2$ ethanol + acetate $\rightarrow$ n-butyrate$^-$ + H_2O

3.3 Principles of PHA biosynthesis

PHAs are polyester produced by different bacterial genera as intracellular storage materials. The production of PHA plays a pivotal role in the long-term survival of bacteria under nutrient-limiting condition by acting as carbon and energy source reserves. Metabolism PHA storage in MMC occurs in systems where electron donor and acceptor availability are separated (e.g. anaerobic/aerobic dynamics) or because the substrate is not continuously available for the microorganisms. Nowadays, more than 90 microbial species are known to produce PHA and about 150 PHA monomers have been identified (Kumar et al., 2019; Valentino et al., 2017). To date, *Cupriavidus necator* is the most extensively studied microorganism for the cost-effective production of PHA. Numerous other strains such as Bacillus cereus SPV, *Sinorhizobium meliloti*, *Azotobacter chroococcum* G-3, *Pseudomonas putida KT2440* and *Metylobacterium sp V49* also are gaining attention for the PHA production (Kunasundari and Sudesh, 2011). Among the PHA, the most common are poly(3-hydroxybutyrate), poly(3-hydroxyvalerate) and poly(3-

hydroxybutyrate-co-3-hydroxyval-erate), usually reported as P(3HB) or PHB, P(3HV) or PHV and P(3HB-co-3HV), respectively.

Research towards a thorough understanding of native pathways for PHA biosynthesis is still in progress (Lu et al., 2009). The PHA biosynthesis pathway is closely linked to different metabolic pathways with which it shares different intermediates, especially acetyl-coA (Lu et al., 2009; Tan et al., 2014). In some PHA-producing bacteria, such as *Cupriavidus necator*, *Chromatium vinosum*, and *Pseudomonas aeruginosa*, the metabolic flux from acetyl-CoA to PHA is strongly influenced by nutrient conditions (Steinbüchel and Hein, 2001). Under balanced nutrient conditions, the ratio between the carbon source and the essential nutrients, such as nitrogen and phosphorus, is suitable to sustain the active growth of microorganism in non-limiting condition. In this case, the production of high amounts of coenzyme A from Krebs Cycle blocks PHA synthesis by inhibiting *β-ketothiolase* (PhaA) so that acetyl-CoA is directed into the Krebs Cycle for energy production and cell growth (Figure 3.2). Conversely, under unbalanced nutrient conditions, i.e., when an essential nutrient such as nitrogen and phosphorus is limiting in the presence of excess carbon, coenzyme A levels are non-inhibitory allowing acetyl-CoA to be directed towards PHA synthetic pathways for PHA accumulation (Jung and Lee, 2000). This metabolic regulation strategy, in turn, enables the PHA-accumulating microbes to maximise nutrient resources in their adaptation to environmental conditions.

The PHA biosynthesis can occur from different carbon sources, such as sugar or volatile fatty acids (VFA), and thus, through different metabolic pathways, i.e. acetyl-CoA to 3-hydroxybutyryl-CoA from sugars, ex-novo fatty acids synthesis from sugars and fatty acid degradation. The acetyl-CoA remains the crucial intermediate for both sugars and VFA as a carbon source. In the first case glucose is metabolised to produce pyruvate which is then converted to acetyl-CoA, while in the second case, VFA can be catabolized into acetyl-CoA by the enzymatic activity of the β-oxidation pathway (Lu et al., 2009). With focus to PHB production (the most studied), the PHB biosynthesis involves a further series of enzymatic reaction that can be schematized in 3 steps: the first step involve the catalytic condensation of two acetyl-CoA molecules by *β-ketothiolase* (PhA) to form acetoacetyl-CoA; subsequently, *acetoacetyl-CoA reductase* (PhB), which depends on NADPH, catalyses the reduction of acetoacetyl-CoA to the (R)-isomer of 3-hydroxybutyril-CoA; finally, *PHA synthase* (PhaC) catalyses the polymerisation of 3-hydroxybutyril-CoA into 3-hydroxybutyrate (3HB) which is the monomer of P(3HB) (or just PHB) (Kumar et al., 2019; Lu et al., 2009). The type of VFA influences the PHA synthesis pathway involved that in turn determines the type of PHA monomers. For example, VFA with odd versus even chain lengths influences the balance of hydroxybutyrate (3HB) and hydroxyvalerate (3HV)

in the PHA copolymer. While even VFA such as acetate and butyrate are activated directly to acetyl-CoA, odd VFA such as propionate and valerate are activated to propionyl-CoA. The latter is then reduced to (R)-isomer of 3-hydroxyvaleryl-CoA which is in this case polymerised into P(3HV). Besides, a portion of propionyl-CoA can be converted to acetyl-CoA through decarboxylation (Pardelha et al., 2014). The intracellular PHA content then inhibits the flux of Acetyl-CoA and Propionyl-CoA production (Pardelha et al., 2014). More complex VFA can be converted but they have to pass through β-oxidation pathway to be converted to acetyl-CoA and propionyl-CoA but this led to higher energy consumption. The obtained PHA chain is aggregated in granules that are surrounded by specific lipids and proteins (Lu et al., 2009).

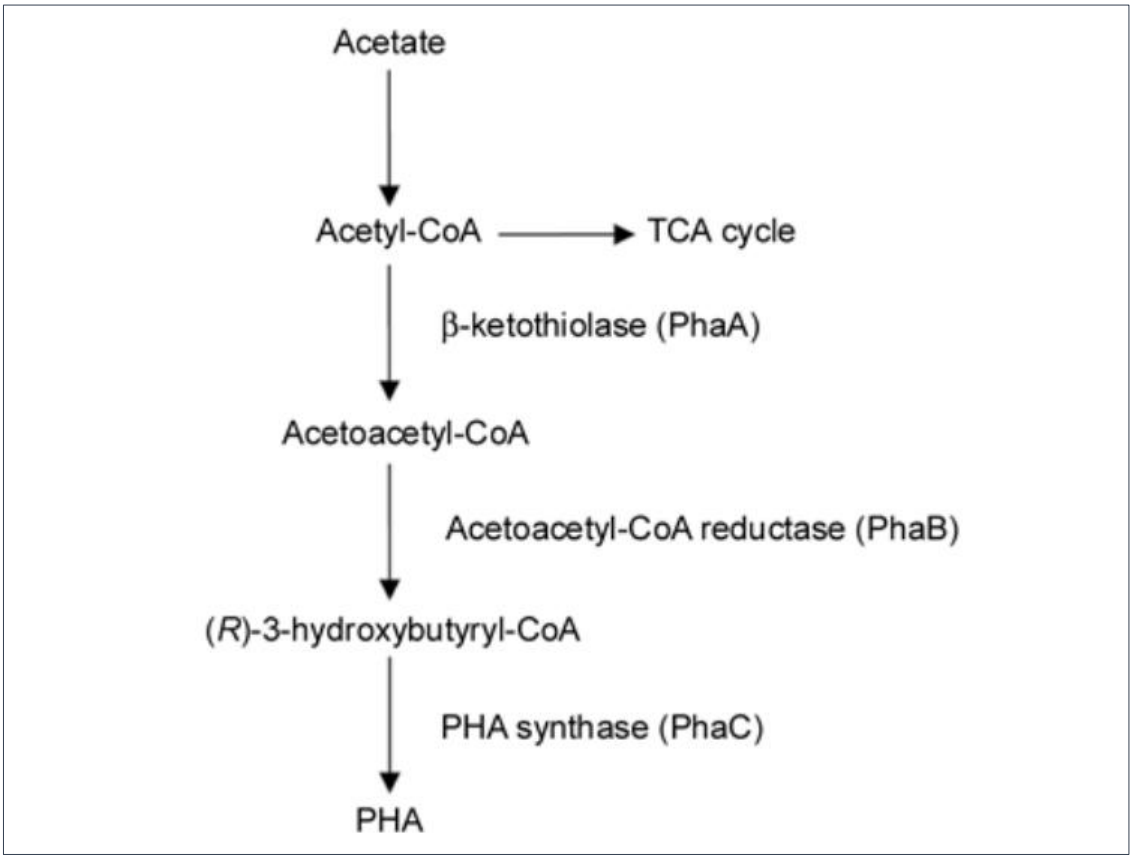


Figure 3.2. *Main metabolic pathway for the PHA biosynthesis.*

3.4 Strategies for orienting MMC processes toward the production of valuable bioproducts

The previous paragraphs emphasis that several routes are available in the MMC-based process under anaerobic conditions. Changes in the operational condition push the microbial

community to adapt to the selective pressure of the systems, and therefore, the output of the process could be controlled (Kleerebezem and van Loosdrecht, 2007). There is a tight relationship between the operational conditions and the products obtained. The possibility to orient the process to useful products is the main reason behind the increased interest in MMC-based process and may represent the key technology to treat biowaste within the biorefinery concept.

3.4.1 Dark fermentation for hydrogen production

During MMC fermentation, hydrogen is produced by fermentative microorganism as a way to dispose of the electrons resulting from oxidation of organic compound during their catabolism. In this work, an MMC-based fermentation oriented towards the production of hydrogen will be referred as “dark fermentation” (DF).

A primary limitation of the fermentative hydrogen production process is that no generally accepted selection strategy for favouring the most favourable fermentative hydrogen route is available in the literature. DF has proved to be a very sensitive process dependant on multiple factors that are also strictly interrelated and mutually interactive (Akhlaghi et al., 2017b). Among those multiple factors, there are substrate characteristics, organic loading rate, inoculum type, inoculum pre-treatment and selection methods, inoculum-to-substrate ratio, reactor type and operation regime, temperature, pH, hydraulic and cell residence time (HRT and SRT). Strategies which were applied to minimise H_2 consumption and increase H_2 yield included inoculum pretreatment, such as heat, acid and alkaline, ultrasonic treatments, as well the addition of chemical inhibitors such as BESA, chloroform, acetylene and long fatty acids. With regards to operational parameters, the most applied strategies include maintaining low pH, sparging with inert gas, increased stirring, decreasing the headspace pressure in the reactor, removing dissolved gases by immersed membrane, reducing the CO_2 partial pressure by a chemical CO_2 scavenger, organic loading rate shock or optimization, and operating at short HRT (Saady, 2013).

While DF process consists mainly of hydrolysis and fermentation (acidogenesis), the AD process also includes the step of acetogenesis (conversion of VFA into acetate) and methanogenesis. AD process requires longer HRT (>7 days) and higher pH (6.5-7.5) than the conventional DF.

3.4.2 The 3-step process for PHA production

MMC for PHA production has been proposed since they are more straightforward and less costly than the process with PMC since sterile conditions and infrastructure for an axenic bioprocess

are not required (Valentino et al., 2017). The PHAs production process can be achieved with various configurations, among which the so-called “3-step process” is one of the most used, mainly when MMC and biowaste are used. Different configurations could be used depending on the type of biowaste, flows and concentrations, or the already existing infrastructure. Despite the culture or the feedstock used, the PHA production process could be subdivided in pre-treatment or acidogenic step (Step I), culture growth/acclimation or culture enrichment (Step II) and PHA production/accumulation (Step III).

3.1.1.7 Step I

The step I is used only when a waste carbon source is used, and it has the goal to convert a complex organic biowaste, usually rich in carbohydrates, in a more chemically uniform influent for the following step and rich of VFA, which are considered PHA precursors. This step is usually reported as the acidogenic step since it aims to maximise the VFA content by acidogenic fermentation. VFA, such as acetate, propionate, valerate, lactate, are particularly recommended for MMC PHA production since they are readily made available and they are efficiently converted into PHA (Valentino et al., 2017). Methanogenic activity or other VFA-consuming pathway should be avoided adopting different strategies, i.e. low sludge retention time (SRT), low temperature and low pH. Besides, operating parameters of fermentation should be chosen carefully to achieve the goal of maximising the VFA conversion yields and thus targeting the specific VFA composition. Moreover, operating parameters of fermentation (hydraulic retention time (HRT), SRT, pH, temperature, OLR) have to be tuned in order to maximise the VFA conversion yield and to specify the VFA composition. The nutrient should be balanced considering also the following step, especially in terms of C/N ratio.

The step I is not required when the feedstock used is already rich in readily biodegradable organic carbon, such as methanol in pulp and paper foul condensate, a mixture of ethanol, glycerol and hardwood spent sulphite liquor from pulp industry. In those cases, it has been shown that the substrate could be used directly for both step II and step III. Another case in which the step I is not required is when the selected feedstock is a synthetic mixture of VFA.

Acidogenic fermentations are usually carried out in continuous flow in order to have steady acclimatised condition and reach high conversion rate and yields. Typical reactor configurations include CFSTR, UASB, biofilms reactors and packed-bed biofilm reactors.

The production of PHA from VFA of DF effluents in the bio-H_2 reactor is being paid attention recently. In this case, the target of maximising VFA yields may not correspond to the maximising of H2 yield but can remain a promising option (Yoon et al., 2019).

3.1.1.8 Step II

Step II has the goal of achieving enough active biomass capable of producing and accumulating the maximum PHA. In the case of MMC processes, step II is required for the selection and enrichment of a strain capable of PHA production from a mixed consortium used as inoculum, usually an Activated Sludge (AS) from WWTP. The culture enrichment is possible by applying the so-called feast/famine regime, which consists of alternating periods of presence (feast) and absence (famine) of the carbon source under fully aerobic conditions (Rodriguez-Perez et al., 2018; Valentino et al., 2017). This regime put selective pressure on the microbial community, in which microorganism have to adapt themselves to survive in cyclical and protracted phases of lack of carbon and compete on the short occasion when the carbon is available. In such a situation, those populations of species of bacteria that can assimilate carbon source mostly rapidly have a crucial competitive advance over other species. In this case, the rapid assimilation of the external organic substrate results in intracellular storage of PHA, which can be used as an internal organic substrate during famine period for both energy and growth. The shift of the metabolic pathway from a growth response to a storage response is a common competitive strategy of many species of bacteria.

For this reason, the population of species of bacteria expressing the PHA-storing phenotype become enriched in presence over time in dynamically fed bioprocesses. The most successful method for the selection is the use of the feast and famine regime established by using aerobic SBR. The selection of a PHA-storing MMC using biowaste from AS is commonly reported as a good strategy for studies involving PHA production. Feast and famine environments have been also established under alternating aerobic, anoxic, and anaerobic conditions, making it technically feasible to consider opportunities for integrating the ideas of biomass selection and production to a wide range of existing infrastructures for wastewater treatment, including even municipal wastewater treatment.

The performance of the biomass enrichment depends on several factors such as the sludge retention time (SRT), organic loading rate (OLR), feast-to-famine ratio and frequency, temperature, and substrate composition (Kleerebezem and van Loosdrecht, 2007; Reis et al., 2003). Even though a comprehensive multi-parametric model to predict the performance of the selection step has not yet been established, rules of thumb are increasingly applied with replicated success suggesting for robustness in practical engineering and implementation of biomass production for MMC PHA. As an example, it is largely recognized that a low feast-to-famine ratio plays a central role in order to obtain the selection of an MMC with significant PHA

storage capacity. Generally, a feast-to-famine ratio lower than 0.3 is reported to be optimal for this scope while higher ratios are associated with instability in the process (Lorini et al., 2020; Valentino et al., 2013).

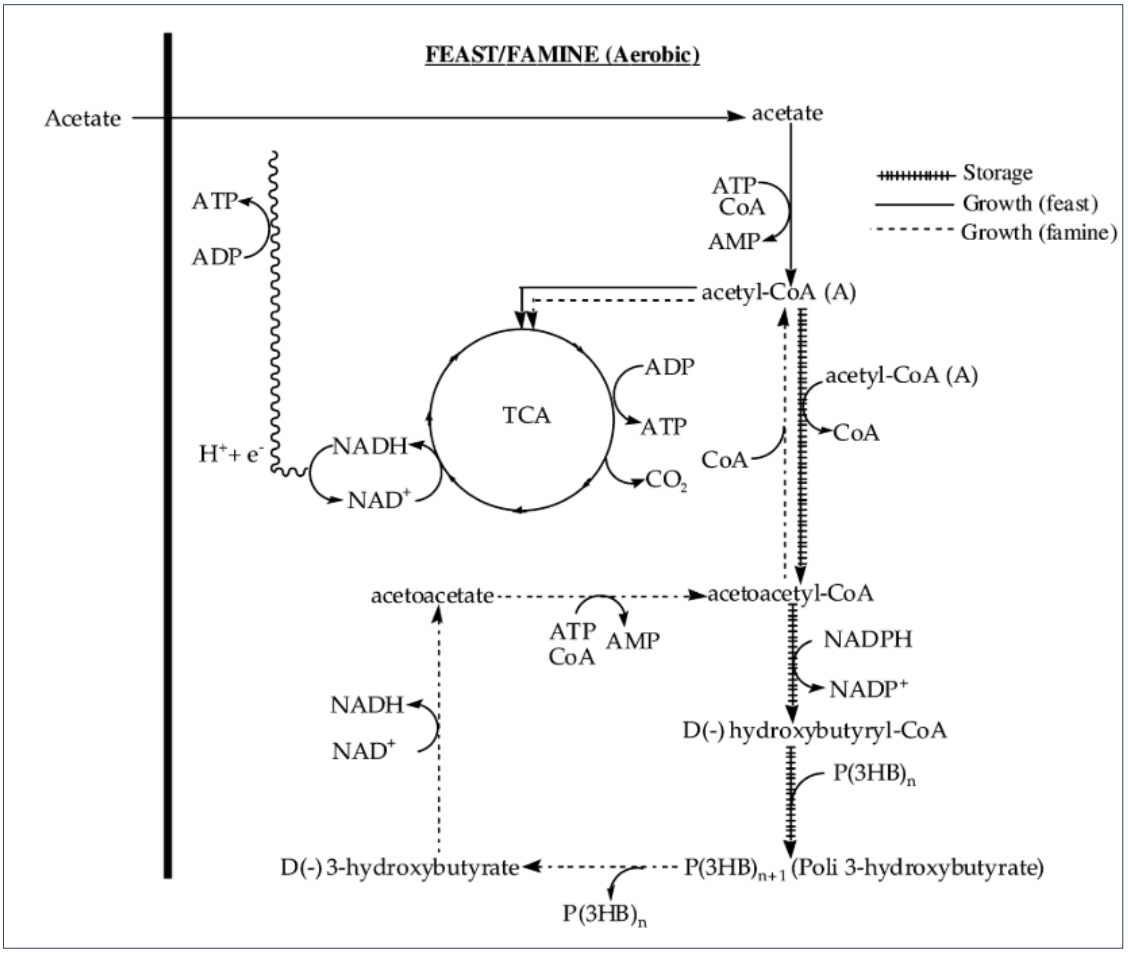


***Figure 3.3**. Possible metabolic pathways for acetate consumption under feast/famine condition, from Reis et al. (2003).*

3.1.1.9 Step III

Step III aims to achieve maximum PHA production with the selected culture. The carbon source used in the culture is mainly stored as PHA in microorganisms. This phase can be carried out in the same reactor of step II or a different reactor. Most of the lab-scale studies present in literature are in batch mode while some pilot-case studies are reported to use a continuous process (Rodriguez-Perez et al., 2018).

The most adopted feed strategy is the pulse-wise feeding strategy. This strategy consists of a repeated pulse feed until the maximum storage capacity of the culture. Adopting this strategy, the PHA content, i.e. the amount of PHA in the cell, may range between 40 and 80%. Higher is

the PHA content, more PHA per unit of biomass is produced, and the extraction is more straightforward, thus reducing the costs. PHA yields strongly varied from 0.02 g_{PHA} $g_{substrate}^{-1}$ to 0.9 g_{PHA} $g_{substrate}^{-1}$ (Rodriguez-Perez et al., 2018). Interestingly, around 60% of the studies reviewed by (Rodriguez-Perez et al., 2018) reported value lower than 0.5 g_{PHA} $g_{substrate}^{-1}$. About pilot-scale studies, they usually report lower yields with PHA content of 24-35% and Yields lower than 0.4 g_{PHA} $g_{substrate}^{-1}$.

3.5 Conclusions

Mixed culture biotechnology involves fundamental challenges because MMC are complex and not completely characterized. So far, the mechanisms that control open communities of microorganisms is not fully understood, which makes the design of such processes a problematic task (Regueira et al., 2018). Except for traditional MMC processes such as anaerobic digestion with the goal of biogas production, processes for optimal product formation using mixed culture are still under development (Sabra and Zeng, 2014). Nevertheless, there is a rapidly growing interest in engineered synthetic consortia for biotechnology application inspired by the powerful features of MMC (Jiang et al., 2017). Presently, there are only a few exceptions where mixed culture-based bioprocesses are used industrially. However, with the development of industrial biotechnology, it is perhaps time to reappraise the potential of mixed culture systems. (Sabra and Zeng, 2014). In line with this, a waste biorefinery based on MMC seems to represent the link between industrial biotechnology and environmental biotechnology.

4 SCOPE OF THE PhD THESIS

The efficient reuse or recovery of materials or energy from any valuable waste stream originated by the production cycles need to be intensified to foster the implementation of a more sustainable economic growth model based on the circular bioeconomy principles. This concept entails that resources recovery from wastes has to be enhanced as much as possible, both quantitively and qualitatively.

As far as biodegradable wastes are concerned, the definition of waste biorefinery fully includes such ambitious valorisation options. If the reasons behind the concept of waste biorefinery are mostly environmental in nature, it is equally true that the applicative perspectives of each technical proposal also pass for its proved economic sustainability.

In turn, economic feasibility depends on the possibility of integrating different processes aimed at producing a mix of biofuels and bioproducts according to a cascade approach, traditional or inverse, to enter the market with an appropriate mix of products characterised either by significant market size or high added value. Such a flexible integration also has a high environmental value; as the number of usable and marketable outputs increases, this would logically correspond to less waste production, thus approaching the zero-waste concept.

The present research work aims at studying a multi-step valorisation process of sheep cheese whey to tackle the environmental problems related to the management of this residue. More in general, the present thesis also aims to contribute to foster the modernisation and strengthening of the dairy industry processes, through the creation of new value chains as well as greener and more cost-effective production processes.

More in detail, the proposed valorisation approach is mostly based on the high lactose content of the sheep cheese whey (SCW), which is well suited to be converted through biochemical processes into marketable bioproducts.

In the treatment scheme which was studied, dark fermentation (DF) plays a pivotal role, considering that allows the conversion of the high organic content of cheese whey into a pool of biochemicals (lactic acid, VFA) and biofuels (H_2, CH_4), with different yields in function of the process parameters adopted and thus, opening the path to different further processes. The exploitation of fermented cheese whey may involve direct separation and commercialisation, e.g. of lactic acid or specific VFA, as well as use as polyhydroxyalkanoates (PHA) precursors, or further energy recovery through methanization.

In this framework, the pursuit of the main objective of the research activity was organised according to the following specific ones which, in turn, drove the development of the experimental activity:

- Studying the role exerted by some of the main operating parameters (pH, fermentation time) on the evolution of the DF of SCW, in particular in terms of type and yields of soluble and gaseous by-products;
- Evaluating the possibility of using the SCW DF outflow, rich in VFA, either for the selection of PHA accumulating biomass or in the following PHA production phase;
- Estimating the overall energy recovery achievable through the combination of SCW DF and anaerobic digestion (AD) (combined recovery of H_2 and CH_4);
- Assessing the possibility of recovering VFA from the SCW DF broth using innovative silicone membranes;
- Comparing different SCW valorisation schemes to define the most promising option.

Given the relatively limited number of studies documented in the scientific literature on multi-step valorisation of sheep dairy bioresidues, the present study is believed to contribute to opening up the path to further research aimed at exploring innovative management and valorisation strategies for the bioresidues produced within the sheep dairy supply chain.

Although the thesis focused on sheep cheese whey, the results obtained may foster the implementation of a “dairy waste biorefinery” approach to a broader context. In this respect, it is worth to emphasise that some of the experimental activities presented in the present manuscript were performed using bovine cheese whey.

5 CONTROL OF FERMENTATION DURATION AND pH TO ORIENT BIOCHEMICALS AND BIOFUELS PRODUCTION FROM SHEEP DAIRY RESIDUES

5.1 Introduction

As discussed previously, Dark Fermentation (DF) plays a central role within the waste biorefinery framework since it can act as a platform to convert a complex substrate in biohydrogen and a broad spectrum of intermediates, such as VFA, that could be exploited differently. The exploitation of such metabolites may involve direct separation and commercialisation or further processing of the fermentation effluent. DF could be coupled with a range of different processes, aimed, e.g. at biopolymer production (Colombo et al., 2016), electricity or further hydrogen production in microbial electrochemical systems (Moreno et al., 2015), methanogenesis (Fernández et al., 2015), or others.

It is acknowledged that DF is a complex process strongly depending on numerous and interconnected factors such as substrate composition, concentration and pre-treatment methods, presence/type of inoculum and inoculum pre-treatment, inoculum-to-substrate ratio, reactor type and operation regime, applied operating conditions (e.g. pH, HRT, SRT, temperature, organic loading rate, etc.) (De Gioannis et al., 2013). Operating pH and fermentation time, in particular, are known to govern the production yields of liquid and gaseous bioproducts by influencing the activity of enzymes, the degree of substrate hydrolysis, and the predominant metabolic pathways (Akhlaghi et al., 2017c). For these reasons, optimising the operating pH and process duration appears to be worth studying to adjust the type and yield of biochemicals and biofuels produced from CW.

To this aim, in the present chapter, batch fermentation tests were performed on raw sheep cheese whey (SCW) adopting different operating pHs and relating type and production yields of observed gaseous and liquid byproducts to the duration of the fermentation process. No inoculum was added prior the fermentation, nor any pre-treatment of the substrate was performed. Full-scale implementation of a CW DF treatment based on the indigenous MMC (mostly LAB) which are found in CW could lead to various advantages, i.e. no need for substrate sterilisation, no added costs for dedicated inoculum, and no energy consumption for inoculum/substrate pre-treatments. All of those can make, in turn, CW an even more attractive substrate and the process relatively more straightforward to implement. The fermentation of

raw CW making exclusive use of its indigenous biomass is not conventional in the literature, and the peculiar features of the process when operated under such conditions have never been pointed out before. Given the limited number of studies documented in the scientific literature on the combined biochemical and biofuel production from SCW, the present study is believed to open up the path to further research aimed at exploring innovative SCW management and valorisation strategies. Furthermore, several dairy plants use SCW for used for ricotta cheese production with the production of a second cheese whey commonly name Scotta (SSc). For this reason, the DF of SSc was briefly evaluated.

5.2 Materials and methods

5.2.1 Substrate

Samples of fresh raw sheep cheese whey (SCW) and scotta (SSc) were collected at a medium-size dairy industry located in Sardinia (Italy) which processes ovine milk for the production of pecorino cheese. SCW and SSc were sampled immediately after the cheese production process, transported to the laboratory in about 1 hour, divided into 1 L-bottles and stored at -15 °C until use to prevent biological degradation. Before the onset of the fermentation tests, the required amount of sample was thawed at room temperature for about 8 hours. The thawed SCW and SSc were directly used as the feed material in batch fermentation experiments without any additional external inoculum. The choice of freeze storage of the SCW and SSc samples was based on the evidence from previous studies (e.g. Tribst et al. (2019)) that freezing at -18 °C and subsequent thawing at 25 °C in 1 L containers did not alter the total number of microorganisms. The main characterisation parameters for the SCW and SSc samples analysed after thawing are reported in Table 5.1.

Table 5.1. *Main characterisation parameters of SCW (average value ± standard deviation).*

Parameter	Unit of measure	Cheese whey	Scotta
pH	-	6.16 ± 0.60	6.13 ± 0.03
Total solids (TS)	%	7.62 ± 0.30	6.01 ± 0.45
Volatile solids (VS)	%	7.05 ± 0.30	4.99 ± 0.40
Soluble carbohydrates (sCarb)*	g L^{-1}	46.5 ± 4.4	53.6 ± 4.4
Total organic carbon (TOC)	g L^{-1}	32.0 ± 1.6	30.5 ± 0.1
Soluble organic carbon (DOC)	g L^{-1}	26.8 ± 2.2	28.3 ± 0.1
Soluble proteins (sProt)**	g L^{-1}	10.7 ± 1.5	5.6 ± 0.4
Ammonia	mg L^{-1}	400 ± 100	500 ± 20
Fe	mg L^{-1}	0.6 ± 0.1	-
Mg	mg L^{-1}	87 ± 16	-
K	mg L^{-1}	1149 ± 168	-
Na	mg L^{-1}	578 ± 80	-
Ca	mg L^{-1}	335 ± 58	-

** expressed as lactose*

*** expressed as bovine serum albumin (BSA)*

- not measured

5.2.2 Experimental setup

The batch fermentation tests were carried out at 39 ± 1 °C using a 2-L glass reactor (BIOFLO 110 - New Brunswick Scientific; BioCommand Lite software; working volume = 1.8 L). The reactor was supplied with a mechanical stirring device (stirring rate = 150 rpm) and an automatic pH control software continuously controlling the addition of a 5 M NaOH solution. Gas production was measured using a eudiometer adopting the volume displacement principle. The measured gas volume was converted to standard temperature and pressure conditions (T = 273.15 K, P = 105 Pa). The reactor was covered with a black plastic film to prevent photofermentative reactions and initially flushed with N_2 gas to drive off air from the headspace. Six operating pHs (5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) were adopted during the tests on SCW, while an operating pH of 6 was adopted during the test on SSc. An additional test was performed on SCW without continuous control of the operating pH (UCpH). All the fermentative tests were run at least in duplicate, and the results will be reported as average values. Each test was stopped once any variation in metabolite concentration or appreciable gas production could be no longer detected. The resulting fermented SCW (FSCW) were stored at -15°C in 1-L bottles before the utilisation in the further valorisation processes (see Chapter 6).

5.2.3 Analytical methods

The concentration of total solids (TS), volatile solids (VS), total organic carbon (TOC) and soluble carbohydrates (sCarb, on 0.45-µm filtered samples) were measured on samples immediately before use according to the analytical methods reported in our previous paper (De Gioannis et al., 2014). The soluble protein (sProt) content was determined spectrophotometrically at 750 nm by the alkaline copper method as described by (Lowry et al., 1951), using bovine serum albumin (BSA) as the standard. All the spectrophotometric analyses were performed with a HITACHI U-200 spectrophotometer. The concentration of Fe, Mg, K, Na, Ca was determined on 0.45-µm filtered samples using an inductively coupled plasma-optical emission spectrometer (ICP-OES, Optima 7000DV, Perkin Elmer, MA, USA). The concentration of lactic acid (HLa) was analysed using a Dionex high-pressure liquid chromatography System UVD170U equipped with an Acclaim Organic Acid column. All analyses were conducted with isocratic elution (H_2PO_4 0.2% + sodium sulphate 100 mM at 0.9 mL min^{-1}). The concentration of VFAs (acetic [HAc], propionic [HPr], butyric + iso-butyric [HBu], valeric + iso-valeric [HVa], hexanoic + iso-hexanoic [HHex], heptanoic [HHep]) and ethanol [EtOH]) was determined using a gas chromatograph with flame-ionization detection (model 7890B, Agilent Technology) equipped with a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology). The samples were filtered using a 0.45 µm membrane and then acidified with concentrated H_3PO_4 (pH < 3). The injection volume was 0.6 µL. The temperatures of the injector and the detector were 230 °C and 300 °C, respectively. The oven temperature was initially set at 60 °C (3-min holding time), followed by a ramp of 10 °C min^{-1} up to 160 °C. Helium gas (1.6 mL min^{-1}, splitless) was used as the carrier gas.

The biogas was sampled periodically from the reactor headspace with a 1-mL gastight syringe and injected through a valve in a gas chromatograph (model 7890B, Agilent Technology) equipped with a thermal conductivity detector (TCD) and two stainless columns packed with HayeSep N (80/100 mesh) and Shincarbon ST (50/80 mesh) connected in series. The operating temperatures of the valve and the TCD were 90 °C and 200 °C, respectively, and He was the carrier gas at a constant pressure of 8 psi in the HayeSep N column and 25 psi in the Shincarbon ST column (at 70 °C). The oven temperature was set initially at 70 °C (3-min holding time), followed by a ramp of 10 °C min-1 up to 160 °C (3-min holding time).

All analyses were run in triplicate and results are presented as average values of the replicates and the associated standard deviation.

5.2.4 Kinetic models

A first-order kinetic model (see Eq. 1) was used to describe the time evolution of the carbohydrates degradation process.

$$\textit{Eq. 1: } \frac{C}{C_0} = a + b * exp(-k * t)$$

where C_0 and C are the carbohydrates concentration at time 0 and t, while a, b and k are the kinetic constants, specifically, k is the rate constant and a + b = 1.

The modified Gompertz equation was used to calculate the kinetic parameters for the H_2 production process, according to Eq. 2 (Lay et al., 1999):

$$\textit{Eq. 2: } HPY(t) = HPY_{max} * exp\left\{-exp\left[\frac{R_{max} \cdot e}{HPY_{max}}(-t) + 1\right]\right\}$$

where HPY is the cumulative H_2 production yield at time t, HPY_{max} is the maximum theoretical H_2 production yield, R_{max} is the maximum H_2 production rate, λ is the lag phase duration, t is the time, and "e" is the Neperian number.

The experimental data were fitted through Eq. 1 and Eq. 2 using the TableCurve 2D® software (v. 5.01, Systat Software Inc.) through least-squares non-linear regression. The coefficient of determination R^2 was used to evaluate the quality of data fitting for each experimental dataset. The time required for H_2 production to attain 95% of the maximum production yield, referred to as t95(H_2), was derived from the Gompertz equation as follows (Eq. 3).

$$\textit{Eq. 3: } t_{95(H2)} = \frac{HPY_{max}}{R_{max} \cdot e}\left(1 - ln\left(-ln0.95\right)\right) + \lambda$$

This parameter provides a measure of how fast the maximum H_2 production is achieved and proves useful to compare, from a kinetic viewpoint, experimental conditions with different associated H_2 generation yields.

5.3 Results and discussion

5.3.1 Dark fermentation of sheep cheese whey

The main characteristics of the SCW reported in Table 5.1 indicate that the organic content was largely associated to carbohydrates, with a concentration of 46 g L^{-1}, which corresponds to 73% of total DOC assuming that carbohydrates were only present as lactose ($C_{12}H_{24}O_{11}$). Soluble proteins were measured at a concentration of 11 g_{BSA} L^{-1}, accounting for 18% of total DOC, assuming an average C content of 0.46 g g_{BSA}^{-1} (Rouwenhorst et al., 1991). This value is higher than usually observed for cow CW (Carvalho et al., 2013).

5.1.1.1 *Primary fermentation: carbohydrates consumption and lactic acid production*

Substrate degradation during the fermentation tests was evaluated by observing the evolution of the normalised concentration (C/C_0) of soluble carbohydrates over time. The results are depicted in Figure 5.1. , where the solid lines represent the first-order model curves derived from Eq. 1. For all the experiments run at controlled pH conditions, the C/C_0 values decreased rapidly over time and the degradation kinetics was described with a high goodness of fit (R^2 > 0.97) by Eq. 1, as also observed by (Akhlaghi et al., 2019, 2017c; De Gioannis et al., 2014). The uncontrolled test (UCpH) was also found to be described by first-order-type kinetics (although with a slightly lower correlation – R^2 = 0.90), but the carbohydrates consumption rate and final consumption yield were considerably lower than for the other tests. In particular, the occurrence of inhibitory effects on carbohydrates degradation for the UCpH run was evident after 30 hours of fermentation, with the consumption yield levelling off after ~60 h and reaching a final value of 45%. Similar inhibition conditions of carbohydrates degradation in uncontrolled pH experiments were also observed in Tang et al. (2016) and most likely result from acid accumulation in the fermentation broth with an associated sharp pH decrease. In our experiments performed under uncontrolled pH conditions, pH dropped down significantly over time attaining a final value of 3.78.

In the controlled-pH tests, the operating pH was not found to affect the final carbohydrates removal, which was always rather high; conversely, it significantly influenced the degradation rate. The carbohydrates concentration was always reduced by more than 93% (up to 99%) of the original value, indicating a virtually complete removal of such species during the fermentation process.

The carbohydrates degradation kinetics was observed to be strongly dependent on pH, as clearly indicated by the trends of k and t95(carb) (see Figure 5.2). More specifically, both parameters were exponentially correlated with pH, with an almost tenfold increase in k from 0.015 h^{-1} at pH 5.0 to 0.176 h^{-1} at pH 7.5, and a decrease in t95(carb) from 395 h at pH 5.0 to 74 h at pH 7.5. Other authors showed similar effects of pH on the carbohydrates degradation rate (Infantes et al., 2011; Tang et al., 2016). Possible causes for the observed influence of pH on carbohydrates consumption kinetics are well known in the literature and include: 1) the increased enzymatic activity of biomass at higher pH conditions (Tang et al., 2016) 2) the decreased energy utilization yield by the biomass at low pHs, caused by undissociated acids crossing the cell membrane causing the need of an excess of metabolic energy to excrete the excess of protons released inside the cell (Infantes et al., 2011; Rodríguez et al., 2006); 3) the

changes in the degree of nutrient transport to the microbial cells (Panesar et al., 2007). Although it would not be possible, from the characterisation performed in the present study, to single out the individual contribution of the mechanisms mentioned above, the experimental results clearly show that pH had a well-defined and univocal effect on the substrate degradation rate.

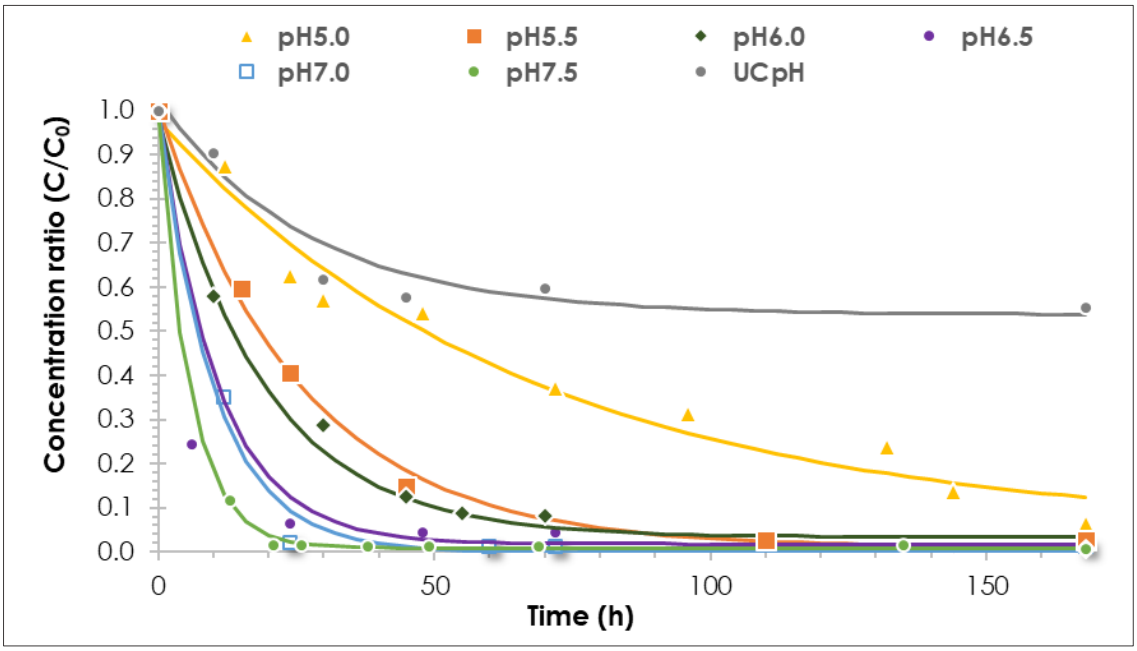


Figure 5.1. *Time evolution of soluble carbohydrates (as normalised concentration) as a function of pH.*

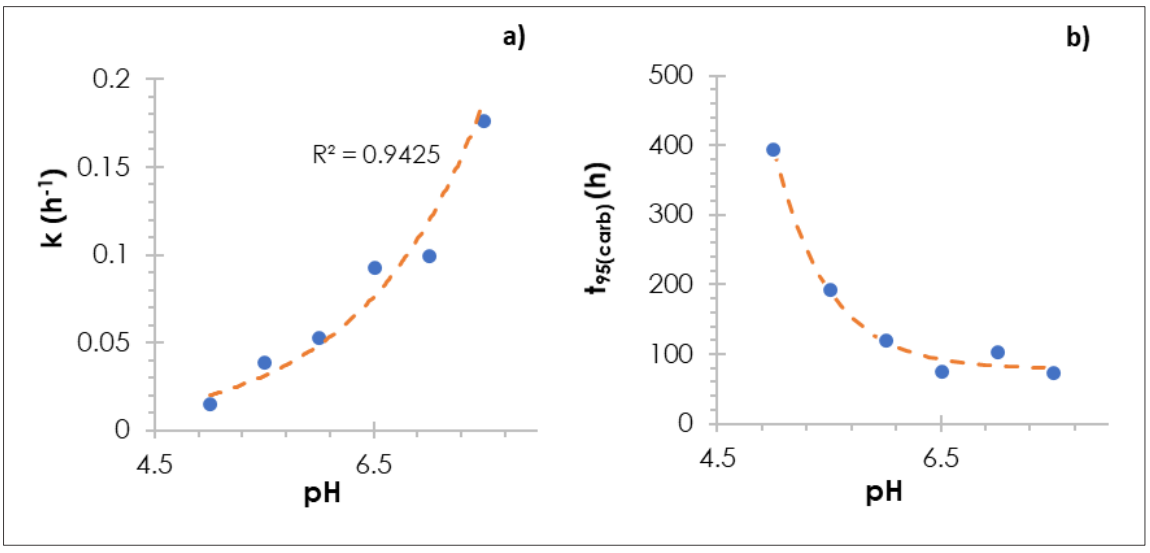


Figure 5.2. *Carbohydrates degradation kinetics: dependence of k (a) and t_{95}(carb) (b) on the operating pH.*

The time evolution of the metabolic products as a function of the operating pH is presented in Figure 5.4, which shows some distinguishing features of the fermentation process. For all the

controlled-pH experiments, the process was clearly governed by two consecutive substrate degradation stages, i.e. primary and secondary fermentation, involving carbohydrates conversion into lactic acid, followed by lactic acid transformation into VFA (mainly, acetic, propionic and butyric acids). Compared to such metabolic products, other species including either higher-molecular-weight VFA or ethanol were always detected at negligible concentrations. The UCpH test showed some initial HLa production, although at a much lower level than for the other experiments. In this case HLa production also displayed very slow kinetics, with a plateau of 5.4 $mmol_{HLa}$ g_{TOCi}^{-1} (corresponding to ~15 g L^{-1}) attained after approximately 60 h from the beginning of the process, mirroring the trend observed for carbohydrates degradation and confirming the occurrence of inhibitory effects on fermentation likely caused by the adverse pH environment (Panesar et al., 2007).

The maximum HLa concentration for controlled-pH tests was found to range from 15 to 24 mmol g_{TOCi}^{-1} (equals to 43-65 g L^{-1}) depending on the operating pH, while the peak production was attained after 12-96 h from the beginning of the experiments (Figure 5.4). The HLa production observed in the first stage of the fermentation process is most probably related to the presence of lactic acid bacteria (LAB) in SCW, as they are added as starter cultures during the cheese-making process (Sikora et al., 2013). As mentioned in Chapter 3, LAB can catabolize sugars (both mono- and di-saccharides) according to different metabolic pathways. The fermentation of sugar is preceded by a hydrolysis step, like the following:

lactose $\rightarrow$ *glucose + galactose*

The hydrolysis is carried out employing the lactase enzyme, such as *β-galactosidase,* that converts the lactose disaccharide into its monosaccharide components, glucose and galactose (Prazeres et al., 2012). The ability to effectively degrade lactose depends on the capacity to hydrolyse it and therefore to the capacity to produce the specific enzymes, which is associated only to specific strains of bacteria. This consideration is not trivial since it suggests that the indigenous microflora (dairy starters) was able to hydrolyse lactose and degrade it efficiently. The capacity of hydrolysing lactose from other mixed culture used as inoculum is not obvious and may limit the following steps.

Lactic fermentation can be converted lactose into only lactic acid (homolactic pathway) or lactic acid and other compounds, such as ethanol or acetate, (heterolactic pathway). The expected lactate production yield for the two pathway is 4 and 2 mol per mol of lactose consumed, respectively. The reactions involved are the following:

glucose $\rightarrow$ *2 lactate*

glucose $\rightarrow$ *lactate + CO_2 + ethanol*

glucose $\rightarrow$ *lactate + CO_2 + acetate*

The presence of lactic acid in high amount (equivalent to ~65 g L^{-1} after 45 h) opens the possibility to use SCW as cheap feedstock for commercial production of this valuable bioproducts (see Chapter 1). If the fermentation process is oriented towards HLa production, the heterolactic fermentation would be less favourable over the homolactic pathway in terms of lactate recovery yields due to the lower lactate productivity and the need for HLa separation and purification from the other metabolites (Mazzoli et al., 2014). The onset of either type of fermentation is governed by the nature of LAB present (Panesar et al., 2007), substrate or nutrient limitation factors (Bernárdez et al., 2008), as well as key operating parameters such as temperature and pH (Panesar et al., 2007). In the present study, the fact that metabolites other than HLa displayed extremely low or undetectable concentrations and no appreciable biogas production was observed indicated that the first fermentation stage involving lactate production was mainly associated to the homolactic pathway.

The metabolic reactions, therefore, included lactose hydrolysis followed by homolactic fermentation of glucose. This assumption was further confirmed by the fact that the calculated yield of lactose conversion to HLa was around 4 mol_{HLa} $mol_{lactose\ consumed}^{-1}$ for all tests, with some exception for the run at pH 7.0, which displayed a slightly lower yield of 3.2. The high lactic yield is believed to be a very distinguishing feature of the fermentation process tested, considering that no specific effort was made in the selection of the microbial community of the fermentation system. The formation of HLa as the primary metabolic product of CW has been documented by several literature studies, which mainly involved the use of whey powder or whey permeate inoculated with pure cultures (mostly, *Lactobacilli*) (Büyükkileci and Harsa, 2004; Göksungur et al., 2005; Kim et al., 2006; Negi et al., 2016; Tang et al., 2016). Conversely, when whey powder or whey permeate was inoculated with different types of residual biomass previously acclimated under anaerobic conditions, multiple metabolic pathways were observed to overlap, likely due to the concomitant presence of different microbial species, and other products (VFA and alcohols) turned out to form together with lactate at comparable concentrations (Gomes et al., 2015; Vasmara and Marchetti, 2017). However, it has been suggested that drying/osmotic pre-treatments of CW cause stress factors that may lead to damages to the cell membrane and inactivation of most of the LAB strains (Gomes et al., 2015). To this regard, fermentation tests conducted on non-pretreated CW (Pagliano et al., 2018) indicated a more important role of the

autochthonous LAB in the system, with a prevalence of lactate production over other metabolic routes. The fact that in the present study no preliminary treatment was applied to CW and no external inoculum was added, caused the fermentation process to be initially governed by the indigenous biomass in CW, which arguably comprised a significant portion of homolactic species. The absence of metabolic pathways overlapping with homolactic fermentation may have also resulted from the antimicrobial activity displayed by LAB that has been widely reported in the literature (Cabrol et al., 2017). While there are multiple mechanisms through which LAB can exert antimicrobial activity, it is likely that under the fermentation conditions tested in our experiments the excretion of bacteriocins by LAB may have inhibited the activity of other microorganisms (including hydrogen-producing bacteria(HPB)) during this stage (Jo et al., 2007; Noike et al., 2002). The experimental results also indicate that the microbial community tended to change over the fermentation time. At some point, the depletion of the carbohydrates converted by LAB into HLa became a limiting factor for their metabolism, so that different microbial species took over during the second fermentation stage, and a range of metabolic products was found to appear.

5.1.1.2 Secondary fermentation: hydrogen and organic acids production

The second stage of the fermentation process started when HLa production peaked (Figure 5.4) and was dominated by lactate-consuming pathways with an accompanied production of VFA, H_2 and CO_2. The soluble metabolic products detected mainly included short-chain fatty acids (acetic, propionic and butyric acids), while medium-chain fatty acids including valeric, hexanoic and heptanoic acids were below the analytical detection limit (10 ppm).

Different microbial pathways involving the transformation of lactate into a range of metabolic products are known from the literature and include the elementary reactions reported as following (and in Chapter 3) (Agler et al., 2011; García-Depraect et al., 2019; Thauer et al., 1977). Each of these equations can be combined with the others, and further fermentations reactions could be taken into account, such as autotrophic homoacetogenesis. The latter could be identified as a possible candidate to explain H_2 consumption during fermentation (Akhlaghi et al., 2017c; De Gioannis et al., 2014).

$$lactate + H_2O \rightarrow acetate + CO_2 + 2H_2$$

$$lactate + H_2O \rightarrow 0.5\ butyrate + CO_2 + H_2$$

$$lactate + H_2 \rightarrow propionate + 2H_2O$$

$$4H_2 + 2CO_2 \rightarrow acetate + 2H_2O$$

Given the fact that the main soluble metabolites were found to be present in the fermentation system in different proportions depending on the operating pH adopted, in our recent study, a specific investigation of the prevalent metabolic pathways was conducted by taking into account the possible biochemical reactions involving the species of concern (Asunis et al., 2019). Briefly, a system of 6 linear equations was set-up, expressing the mass balance conditions for HLa, HAc, HPr, HBu, H_2 and CO_2 in 4 unknowns (X_i) representing the contribution of reactions mentioned above. The solutions were found through a least-squares approach, and the results are reported in terms of values of the coefficient X_i as a function of pH and fermentation time. For further details about the mathematical modelling and results, see (Asunis et al., 2019).

In general terms, we found that changes in the operating pH caused a shift from one fermentation pathway to another, as indicated by different metabolic products becoming prevalent at different pH conditions. Homoacetogenesis turned out to provide a negligible contribution to the fermentation process when compared to the other metabolic pathways, which may be considered as a positive feature when the target metabolic product is H_2. As for the other reactions, more acidic pHs (up to 6.0) were found to favour lactate conversion into butyrate, with the lactate oxidation reaction yielding by far the most relevant contribution to the degradation process, and lactate reduction to propionate overlapping with the former yet at remarkably lower levels (in the order of 30-40%). No appreciable acetate production was detected at pH values of up to 6.0. As the operating pH increased, the fermentation process became governed by a broader set of metabolic pathways overlapping with each other, so that all three metabolites acetate, propionate and butyrate were present at detectable concentrations in the fermentation liquid. The results we obtained indicate that, while acetate production did not vary significantly as pH increased from 6.5 to 7.5, propionate production gradually tended to increase and overcome butyrate fermentation.

As seen previously, the reaction of lactate oxidation and reduction was accompanied by H_2 and CO_2 production, with no traces of methane in any test. The H_2 content in biogas was found to be always higher than 45% vol., and to increase with the operating pH up to 65% vol. (pH = 7.5) as a consequence of the increased CO_2 solubility in the liquid phase.

The HPY measured in the experiments expressed per unit of initial TOC are shown in Figure 5.3, along with the Gompertz curves derived by fitting the experimental data points with Eq. 2. The values of the kinetic parameters of the Gompertz equation are reported in Table 5.2. The data for the UCpH test is not reported, since no appreciable biogas production was observed during the fermentation process, due to the biomass above-mentioned inhibition effect. The

estimated HPY_{max} proved to be a non-monotonic function of pH, with a maximum of 162.7 L_{H2} kg_{TOCi}^{-1} at pH 6.0 and a minimum of 68.1 L_{H2} kg_{TOCi}^{-1} at pH 7.5. The observed HPY was a combined effect of the nature of the metabolic pathways governing the fermentation process in the hydrogenogenic stage and the conversion yield of the original substrate into lactate. The pH 6.0 condition combined the highest lactate production during primary fermentation with favourable metabolic pathways for H_2 generation (with a prevalence of butyrate fermentation along with some detectable contribution of acetate fermentation). For the tests at higher operating pHs, despite the high observed substrate conversion into HLa (max production of 17-21 $mmol_{HLa}$ g_{TOCi}^{-1}), propionic fermentation became relevant over the other reactions implying lower net HPYs due to the fact that in the lactate reduction to propionate, 1 mole of H_2 is consumed for each mole of propionate generated. On the other hand, the experiments at pHs 5.0 and 5.5, although displaying no relevant H_2-consuming pathways, showed a lower carbohydrates conversion into lactate during the primary fermentation.

In order to compare the results obtained in the present study with those reported in the literature, the measured HPY values were expressed per unit mass of lactose or hexose consumed, under the assumption that carbohydrates in CW were present in the form of lactose only and assuming a 2:1 carbon equivalence between lactose and glucose on a molar basis. The minimum yield (0.66 mol_{H2} $mol_{lactose}^{-1}$, or 0.33 mol_{H2} $mol_{glucose\ eq.}^{-1}$) was attained at pH 7.5, while the maximum value (1.54 mol_{H2} $mol_{lactose}^{-1}$, or 0.77 mol_{H2} $mol_{glucose\ eq.}^{-1}$) was displayed at pH 6.0. This figure is comparable to what observed by (Ferreira Rosa et al., 2014b) who worked on inoculated CW, and higher than what reported by (Akhlaghi et al., 2017c) who estimated a specific HPY of around 110 L_{H2} kg_{TOCi}^{-1} for CW fermentation tests performed at pH 5.5 without inoculum addition.

It is also interesting to point out that the rate of H_2 production was apparently not directly related to the achieved yield, as indicated by the values of t95-H_2 reported in Table 5.2.

It is tempting to hypothesise that the sharp shift from the primary to secondary fermentation, governed by, respectively, homolactic fermentation and a combination of the butyric, propionic and acetic pathways was caused by the intrinsic characteristics and composition of the original substrate as well as the existence of fermentation conditions favouring the growth of specialized biomass. As indicated in the previous section, the indigenous biomass present in CW was believed to be responsible for the onset of the homolactic pathway observed during the first stage. The interaction between hydrogen-producing bacteria (HPB) and LAB has been widely reported in the literature, with controversial effects of the two having been identified by different authors. According to the evidence from our experiments, the detrimental effect of

LAB on HPB reported in the literature (Noike et al., 2002) was likely to have occurred in the first fermentation stage due to the inhibitory effect exerted by the former. On the other hand, it may also be confirmed that, as reported by other investigations (Baghchehsaraee et al., 2009; Blanco et al., 2019; Cabrol et al., 2017; Chojnacka et al., 2011), some form of symbiosis exists between LAB and HPB. Blanco et al. (2019) proposed a trophic interaction between LAB and HPB as being capable of fermenting lactate and acetate (referred to as lactate cross-feeding). Based on the present study, this should be interpreted in the sense that the carbohydrates, once degraded during homolactic fermentation, become limiting for the LAB, while the lactate they produce is made available for use by HPB for H_2 production. To this regard, other authors (Fuess et al., 2018) have suggested that lactate can be utilised as the carbon source by several acidogenic biomass types, including both HPB and non-HPB. It should be mentioned that no evidence could be derived from our study of the existence of a threshold in lactate concentration identified by some authors (Baghchehsaraee et al., 2009) as being capable of fostering hydrogenogenesis by causing a shift in the metabolic reactions. Nor could we confirm the finding that H_2 production is suppressed in the presence of lactate as the only carbon source for HPB (Baghchehsaraee et al., 2009).

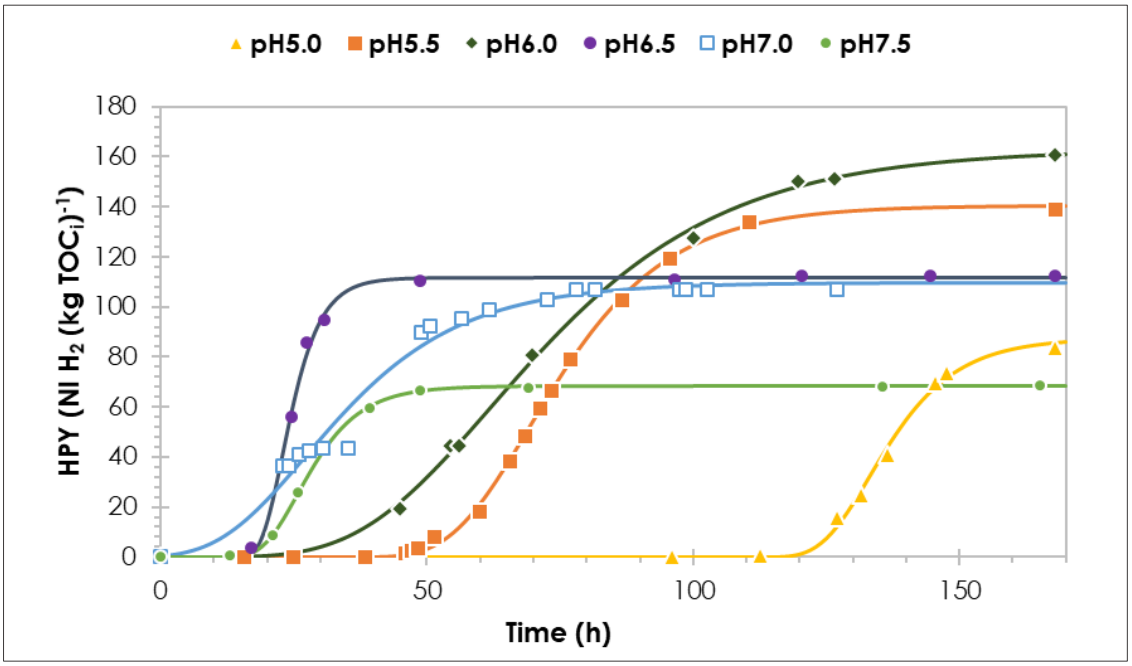


Figure 5.3. *Cumulative H_2 production yields as a function of pH during dark fermentation tests using raw sheep cheese whey as a substrate.*

In summary, the conclusion we can derive from our experiments is that the fermentation process involved a sequence of lactate production and lactate utilisation in a syntrophic system where the product of a given phase was the substrate for the subsequent stage. A similar finding has been previously documented by other authors (García-Depraect and León-Becerril, 2018; Ohnishi et al., 2010). The coexistence of LAB and HPB as well as the simultaneous presence of lactate and H_2 often observed in continuous fermentation systems (Chojnacka et al., 2011) should be interpreted in light of these considerations. It should also be mentioned that, as no specific microbial analysis of the digestate was performed, it was not possible to identify the biomass strains acting in either phase of the fermentation process, so that the postulated hydrogenogenic capability of some LAB strains (Cabrol et al., 2017) could not be assessed nor excluded either.

Table 5.2. *Hydrogen production kinetic parameters and related statistics for raw cheese whey dark fermentation tests.*

		HPY_{max}	R_m	λ	$t_{95\text{-}H2}$
		L H_2 kg TOC_i^{-1}	L H_2 kg TOC_i^{-1} h^{-1}	h	h
		pH 5.0			
R^2 = 0.996	Value	87.4	3.6	124.1	159.9
	Lower 95% conf. limit	80.2	2.9	146.9	
	Upper 95% conf. limit	94.7	4.2	122.0	
		pH 5.5			
R^2 = 0.999	Value	140.4	3.6	54.6	112.2
	Lower 95% conf. limit	137.8	3.4	53.8	
	Upper 95% conf. limit	143.0	3.7	55.4	
		pH 6.0			
R^2 = 0.998	Value	162.7	2.4	37.3	135.1
	Lower 95% conf. limit	156.2	2.2	34.4	
	Upper 95% conf. limit	169.3	2.7	40.3	
		pH 6.5			
R^2 = 0.998	Value	111.6	10.3	18.8	34.6
	Lower 95% conf. limit	109.3	8.4	17.5	
	Upper 95% conf. limit	113.9	12.3	20.2	
		pH 7.0			
R^2 = 0.979	Value	105.6	2.5	10.9	75.3
	Lower 95% conf. limit	103.7	1.9	6.2	
	Upper 95% conf. limit	115.5	3.1	15.6	
		pH 7.5			
R^2 = 0.999	Value	68.1	3.8	19.1	45.1
	Lower 95% conf. limit	67.5	3.6	18.6	
	Upper 95% conf. limit	68.8	4.1	19.5	

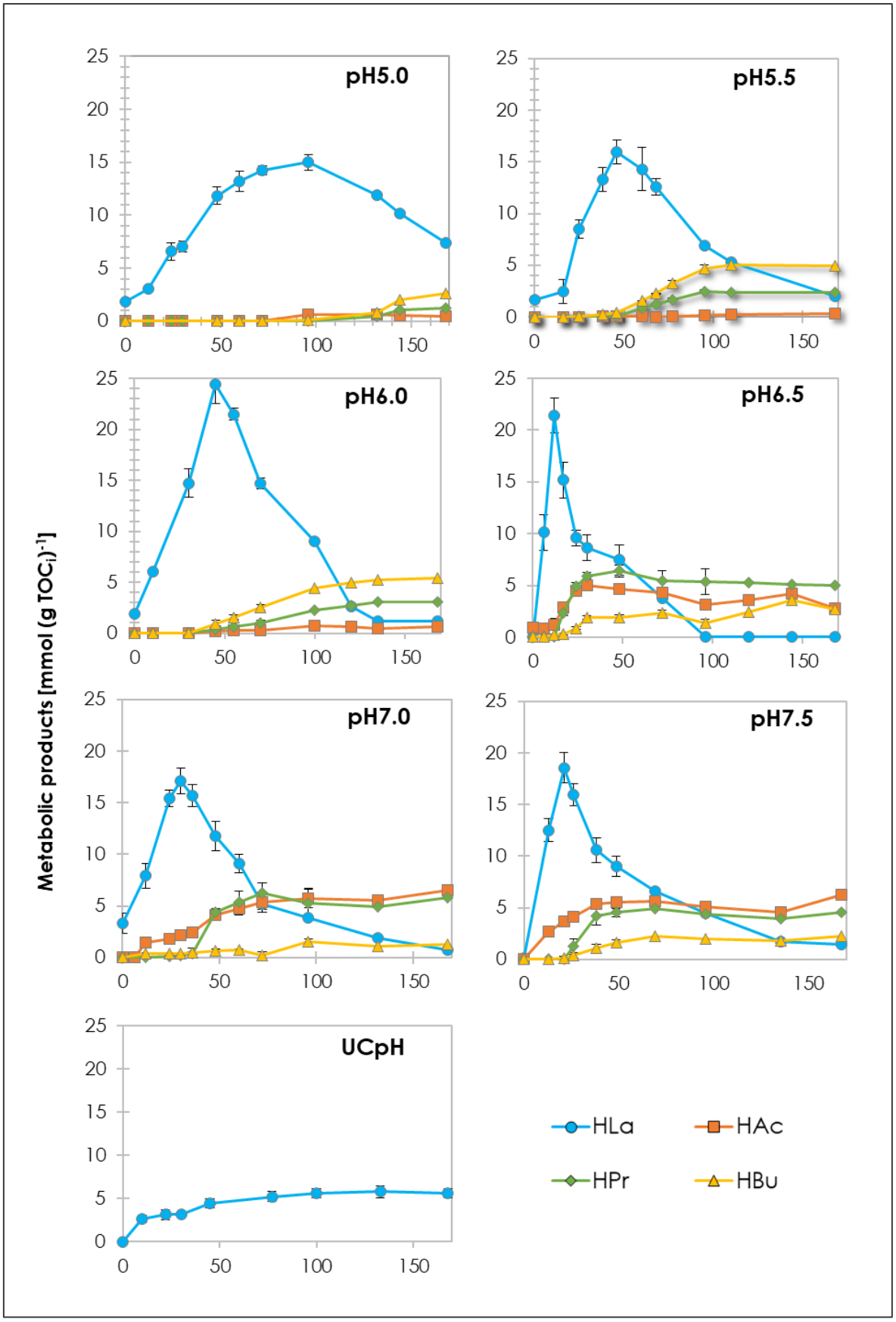


Figure 5.4. *Time evolution of metabolic products as a function of pH during dark fermentation of raw SCW.*

5.1.1.3 Impact of the operating pH on whey protein fate

In the perspective of further valorisation, it is worth to investigate the fate of the other main compound of SCW, the proteins, during the DF test considering that SCW proteins represent the primary nitrogen source in the medium. The microbial community could use proteins during fermentation and, as no nitrogen (N) or phosphorus (P) were supplemented prior the fermentation stage, proteins were necessarily hydrolysed to release N and P in order to support the cell growth. Optimal cell growth should be guaranteed by a non-limiting ratio between carbon and nitrogen sources. The initial carbon-to-nitrogen ratio (C/N), calculated considering the soluble organic carbon and nitrogen, the latter deriving from whey proteins (around 10 g_{BSA} L^{-1}) and initial ammonia (0.4 g L^{-1}), equals to 19 (or C:N=100:5.2). Similar consideration could be founded in (Duque et al., 2014), which performed an acidogenic fermentation of CW powder with an Anaerobic Moving Bed Reactor without supply extra nutrients (initial C: N= 100:5.4). Furthermore, it can be assumed that SCW contains further components (such as phosphorous, minerals and vitamins) which could be used for the culture metabolism, as similarly reported by other authors (Colombo et al., 2019; Duque et al., 2014; Oliveira et al., 2018).

The results obtained showed that protein removal during fermentation is different in function of the adopted pH with a maximum removal of around 60% at pH 6.5 and 7. Similar values are reported in (Gouveia et al., 2017). Proteins hydrolysis, which depends mainly on the capacity of the different microorganisms as well as their acclimation, is slower than that of carbohydrates. Extracellular proteases could hydrolyse proteins into peptides. Proteases, naturally present in the milk, have different optimum pH in terms of production and activity. Then peptides are broken down by peptidases to amino acids. Different pathways degrade amino acids to various end products, including organic acids, ammonia, CO_2, and small amounts of hydrogen and sulphur-containing compounds (Hassan and Nelson, 2012). Besides, it cannot be excluded that there may have been chemical hydrolysis of proteins during the test performed, also considering the effect of pH, temperature and fermentation.

Moreover, the protein degradation could generate a high concentration of ammonia which could be at the same time inhibitory for hydrogen production and further biological valorisation. The residual ammonia concentration in the fermentation outflow should be considered as the results of ammonia released from proteins minus the biomass ammonia uptake during the fermentation. The lower ammonia concentration founded for the tests carried out at pH 5.5 and 6 could be ascribed to a higher biomass growth as also suggested by the lower DOC/TOC Cmolar ratio founded at the end of the test conducted at pH 6 (0.76) compared to the one at pH of 7.5 (0.84) (see Table 6.1, Chapter 5). Further investigation about the fate of whey proteins during the dark fermentation is necessary concerning the following valorisation step, as pointed out also by (Gouveia et al., 2017).

5.3.2 Dark fermentation of sheep Scotta

In addition to the test performed with raw SCW, another further option regarding the use of sheep Scotta (SSc) was evaluated, though less in detail. Scotta is the main by-product in ricotta cheese-making process, and it is widely produced in southern Europe and particularly in Italy (Secchi et al., 2012). It is obtained after the flocculation of whey proteins and their separation as ricotta cheese induced by thermal treatment of cheese-whey at 85–90°C for about 20 min. Compared to the sheep cheese whey, the resulting sheep scotta has lower proteins content, 10 and 5 g_{BSA} L^{-1}, respectively (see Table 5.1). The dark fermentation test performed with sheep Scotta were performed with the same experimental setup adopted for SCW and adopting a fermentation pH of 6, as we saw that represents the optimum for both lactic acid and hydrogen production. With a focus on lactic acid production, the maximum HLa yield, obtained after 72 hours, was 23 $mmol_{HLA}$ g_{TOCi}^{-1}. The obtained yield is equal to 3.60 mol lactic acid per mol of lactose consumed, lower than what reported for SCW at pH of 6 after 45 hours (3.95 mol_{HLA} $mol_{lactose}$). The similar results obtained with the two substrates showed that the thermal pretreatment of SCW to produce Ricotta did not affect significantly the microorganism naturally present in the SCW and derived from the cheese-making process and, despite the lower content of protein (nitrogen source) of SSs with respect to SCW, the biomass was still able to perform the homolactic fermentation.

With a focus on hydrogen production, the HPY was 147 LH2 kgTOCi-1 (equivalent to 0.51 molH2 molglucose eq.-1 or 0.97 molH2 mollactose-1), slightly lower compared with what reported for SCW for the same pH (160 LH2 kgTOCi-1) but of the same magnitude (Figure 5.5). Further differences can be noted in terms of kinetics, with the test performed with Scotta resulting in more extended lag phase compared to SCW (90 and 37 hours, respectively).

The results are preliminary and further studies are indeed necessary. The similar performance in terms of lactic acid and biohydrogen yields obtained with SSCs may be advantageous in terms of processes optimisation. Indeed, dairy plants can decide to orient part of SCW for ricotta production without compromising the possibility to recover other bioproducts from SSCs still. The choice of Ricotta cheese production may be affected by the seasonality of milk production and by the market demand of Ricotta cheese or lactic acid. With regards to the Sardinia region, only the 50-60% of the SCW produced yearly is converted into Ricotta cheese (~7000 t per year[9]) with an average price of 4-5 € kg^{-1} (ISMEA and Laore Sardegna, 2015). At the same time, producing lactic acid or biohydrogen from SSCs instead of SCW results in comparable yields but in lower productivities according to the results obtained that can affect the feasibility of the process. For instance, maximum lactic acid productivity for SSCs was 36% less than for FFCW (21.6 and 35.3 g_{HLa} L^{-1} d^{-1} respectively, at pH of 6).

[9] https://www.agricolae.eu/wp-content/uploads/2019/06/Conmparto_Ovino_Sardegna.pdf

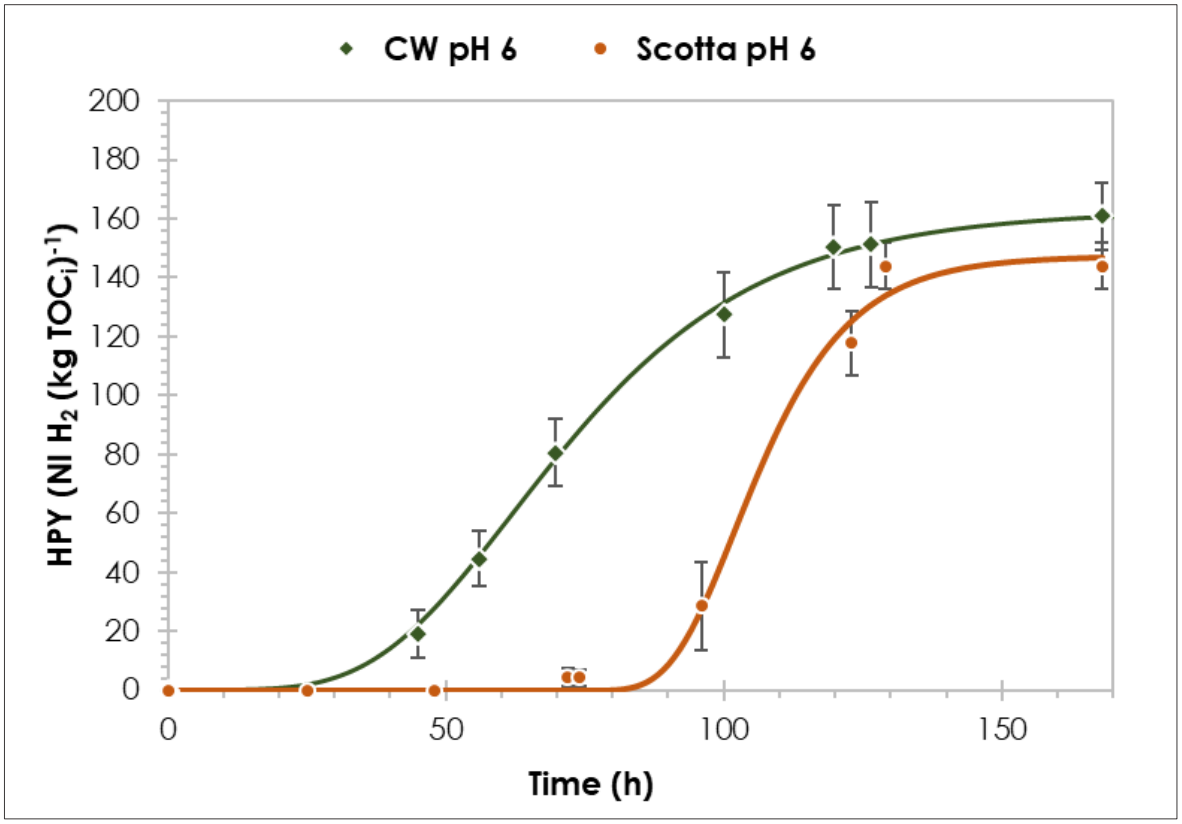


Figure 5.5. *Cumulative H_2 production yields as a function of pH during dark fermentation tests using raw sheep cheese whey and scotta as substrate.*

Table 5.3. *Hydrogen production kinetic parameters and related statistics for dark fermentation of Scotta.*

		HPY_{max}	R_m	λ	$t_{95\text{-}H2}$
		L H_2 kg TOC_i^{-1}	L H_2 kg TOC_i^{-1} h^{-1}	h	h
Scotta pH 6.0					
R^2 = 0.993	Value	147.2	12.7	90.3	136.6
	Lower 95% conf. limit	132.2	8.5	85.66	
	Upper 95% conf. limit	162.3	17.0	94.2	

5.4 Possible strategies for SCW valorisation

The evolution of the dark fermentative process, according to two separate stages, giving specific fermentation products may give rise to different SCW exploitation strategies to be implemented by arranging the fermentation conditions. More specifically, the experiments performed suggests two alternative strategies oriented to lactic acid production or biohydrogen and VFA production.

5.4.1 Production of lactic acid

If the product of interest is the lactic acid, the results obtained suggested that ~23 $mmol_{HLa}\ g_{TOCi}^{-1}$ (equivalent to ~ 69 $g_{HLa}\ L_{SCW}^{-1}$) could be obtained at pH = 6.0 by stopping the fermentation process after 45 hours (Table 5.4). Increasing the operating pH to 6.5-7.5 would reduce HLa production by some 10% (18-21 $mmol_{HLa}\ g_{TOCi}^{-1}$) (52-60 $g_{HLa}\ L_{SCW}^{-1}$) while allowing for the reduction of the fermentation time to 12-30 hours.

Lactic acid production by MMC from CW is interesting because it may contribute to reducing production costs compared to the traditional industrial production since no added costs for dedicated inoculum are required. On the one hand, the use of a cheap substrate, like CW, was already proposed as an alternative feedstock for HLa production, driven by its lower value and its renewability features (Luongo et al., 2019; Mazzoli et al., 2014; Secchi et al., 2012). In particular, as assessed previously, the main driver for lactic production nowadays is its polymerisation to produce PLA and it is essential to reduce the costs associated with the fermentative production of HLa, which should be at or below $ 0.8 per kilogram of lactic acid, in order to ensure that PLA can be competitive with fossil-fuel-based plastics (Mazzoli et al., 2014).

On the other hand, the use of MMC for the production of useful quantities of HLa is uncommon in literature. Recently, a repeated-batch fermentation of CW for semi-continuous lactic acid production using MMC at uncontrolled pH have been proposed (Luongo et al., 2019). The authors reported a maximum concentration of 20.1 $g_{HLA}\ L^{-1}$ and a maximum yield of 0.37 $g_{COD\text{-}LA}\ g_{COD\text{-}CW}^{-1}$ by using an HRT of 2 days and an MMC composed by a pretreated anaerobic digestate from the full-scale WWTP of the same facility where the CW were produced (Luongo et al., 2019). The approach adopted by Luongo et al. (2019) is the most recent and similar to this thesis work, except for the operative pH (uncontrolled VS controlled) and the origin of MMC involved (anaerobic digestate VS autochthonous microflora). The authors claim that adopted conditions represent a fascinating starting point for the industrial application of the process, due to the more realistic adopted condition with respect to previous studies.

On the industrial scale, the main bottleneck is the reduction of the chemical required for the neutralisation of the produced lactic acid, typically done using calcium hydroxide or calcium carbonate, which is added to the fermentation medium in order to avoid biomass inhibition. Besides, recovery of the lactic acid from the calcium lactate salt relies on strong acids such as sulfuric acid, which accounts for up to 50% of the overall lactic acid production costs incurred in the purification process (Miller et al., 2011). The stoichiometry of the neutralisation and acidulation, shown below, demonstrates the amounts of calcium and sulfuric acid required to produce and recover lactic acid from the bacterial process (Miller et al., 2011).

Neutralisation: $lactate^- + H^+ + 0.5Ca(OH)_2 \rightarrow 0.5calcium(lactate)_2 + H_2O$

Acidulation: $0.5calcium(lactate)_2 + 0.5H_2SO_4 \rightarrow lactic\ acid + 0.5CaSO_4$

The process produces a half mole of calcium sulphate (gypsum) for every mol of lactic acid. Gypsum is typically considered a waste stream that often implies a disposal cost, although it may be used in agriculture as a soil amendment. Academic and industrial research efforts to improve lactic acid economics are often oriented through the elimination of neutralisation and acidulation by replacing with other separation strategies, such as solvent extraction, or improvement of pH tolerance in LAB. The latter point is related to the differences in terms of pH between the optimal condition for fermentation (pH of 5-7) and recovery, considering that the pK_a of lactic acid is well above (3.86).

According to the results obtained in this study, a fermentation process carried out at pH 6 results in the production of around 23 $mmol_{HLa}$ g_{TOCl}^{-1} that means that are required 23 $mmol_{NaOH}$ g_{TOCl}^{-1} (assuming a HLa-NaOH ratio of 1). In other words, it means that for every 100 litres of processed SCW, 2.8 kg of NaOH are required to neutralise the 6.6 kg of lactic acid produced.

Another consideration is regarding the pH conditions: as we saw previously, higher pH values lead to faster process, especially in terms of carbohydrates consumption, compared to more acidic pH conditions. Unfortunately, higher pH conditions also lead to overlapping of heterolactic pathway to the homolactic one, and this implies lower lactic acid yields. This point could be overcome by using specific enzymes for the lactose hydrolysis, which is a common practice on an industrial scale (and also another of the cost items). For instance, (Xu et al., 2018) reported the use of neutral proteases to hydrolyses cheese whey powder and produce lactic acid in high concentration (up to 70 g L^{-1}) in batch fermentation with *Lactobacillus bulgaricus CGMCC 1*. In this study, results suggest that LAB had no particular difficulty in hydrolysing lactose, but further studies may be necessary to evaluate if further improvements are possible. i.e. the addition of some natural proteases.

5.4.2 Combined production of hydrogen and VFA

If the product of interest is the biohydrogen, the fermentation process would need to be oriented to attain completion of the second stage in order to provide a maximum HPY of 162.1 L_{H2} kg_{TOCi}^{-1} (5.2 L_{H2} L_{SCW}^{-1}) at pH 6.0 over a fermentation time of 168 hours (Table 5.4); these operating conditions would also imply the concomitant production of HBu (4.9 mmol g_{TOCi}^{-1} or 13.8 g_{HBu} L_{SCW}^{-1}) and HPr (2.8 mmol g_{TOCi} L_{SCW}^{-1} or 6.8 g_{HPr} L_{SCW}^{-1}).

The VFA obtained during biohydrogen production are valuable biochemicals, but nowadays the selective removal is a challenging task (see Chapter 8) considering the relative yield and their simultaneous presence in the fermentation broth. For this reason, the significant potential associated with this effluent is its further valorisation, such as PHA production or AD. In particular, those two processes are the most readily applicable at the current development stage. With a focus on PHA production, the fermentation products yield is among the critical parameter since VFA (acetic, butyric, propionic but also lactic acid) are considered PHA precursors. In this case, considering that the target product is a pool of different VFA, it is common practice in the

literature to express the fermentation product yields as the sum of the moles of carbon contained in the pool of VFA.

According to experimental results obtained, the fermentation products yield ($Y_{OA/SCW}$) was in the range of 0.5 - 0.6 $Cmol_{VFA}$ $Cmmol_{SCW}^{-1}$, except for the test performed without pH control (0.3 $Cmol_{VFA}$ $Cmmol_{SCW}^{-1}$). As a comparison, Colombo et al. (2016) reported similar yields using raw and sterilized CW in mesophilic batch fermentation using autochthonous lactic bacteria and heat-shocked digestate as inoculum (0.4 and 0.6 $Cmol_{VFA}$ $Cmol_{soluble\ substrates}^{-1}$) and Duque et al. (2014) reported slightly higher yield of 0.64 $gCOD_{VFA}$ $gCOD_{soluble\ substrates}^{-1}$ using CW powder in an anaerobic membrane bioreactor. The next chapter extensively discusses the case of PHA production from SCW.

In conclusion, a summary of the obtained yields with different operational parameters for each substrate is given in Table 5.4.

Table 5.4 *Bioproduct yields obtainable according to the experimental results obtained in this chapter.*

Product of interest	Operational mode	Max yield ($g\ L_{SCW}^{-1}$ or $L\ L_{SCW}^{-1}$)
Sheep cheese whey		
Lactic acid	Uncontrolled pH, 60 h	15 $g\ L_{SCW}^{-1}$
Lactic acid	pH 6, 45 h	65 $g\ L_{SCW}^{-1}$
Biohydrogen and pool of VFA	pH 6, 168 h	5.38 $L_{H2}\ L_{SCW}^{-1}$ or 5.2 $kJ_{H2}\ L_{SCW}^{-1}$ 0.53 $Cmol_{VFA}\ Cmol_{SCW}^{-1}$
Biohydrogen and pool of VFA	pH 7.5, 168 h	2.05 $L_{H2}\ L_{SCW}^{-1}$ 0.59 $Cmol_{VFA}\ Cmol_{SCW}^{-1}$
Scotta		
Lactic acid	pH 6, 72 h	62 $g\ L_{SSc}^{-1}$
Biohydrogen and pool of VFA	pH 6, 168 h	4.40 $L_{H2}\ L_{SSc}^{-1}$ or 4.4 $kJ_{H2}\ L_{SSc}^{-1}$

5.5 Conclusions

SCW fermentation using the indigenous biomass displayed two subsequent degradation stages: carbohydrates conversion into lactate followed by lactate degradation to VFA and H_2. Dark fermentation process confirmed to have a crucial role to orient the biorefining pathway towards different biochemical routes. Operating pH largely affected the substrate degradation yield and kinetics, and careful pH control proved essential to foster lactate production and prevent the inhibitory effects of acidic conditions.

Different CW exploitation strategies may be arranged by adjusting the operating pH and controlling the fermentation time. Among them, the most promising strategies within the waste biorefinery framework seems to be the production of lactic acid and the co-production of biohydrogen and VFA. When the product of interest is lactic acid, the maximum yield attained was 69 $g_{HLa}\ L_{SCW}^{-1}$ setting the operative pH at the value of 6.0 and the fermentation time at 45 h. If the product of interest is biohydrogen, the fermentation process

may be set to an operative pH of 6 and a fermentation time of 168 h to obtain the maximum yield of 5 L_{H2} L_{SCW}^{-1}. Along the H_2, the fermentation showed promising fermentation products yields, mostly VFA. Considering that nowadays, the selective recovery of each of them is still challenging, the most feasible options for the pool of VFA obtained is further valorisation with PHA production or anaerobic digestion. Furthermore, preliminary results obtained with Scotta fermentation, suggest that it is possible to obtain findings similar to SCW, opening the possibility to valorise even the Scotta in some dairy plants.

In conclusion, the results obtained open a path toward innovative valorisation of SCW. Further studies should be oriented in the optimisation of the lactic acid production, i.e. assessing different operational mode or evaluating the use of enzymes to foster hydrolysis process, or in alternative the optimisation of the co-production of hydrogen and a pool of VFA, i.e. use of continuous process. The downstream process is another field of study that should be pursued to make the process economically sustainable.

Part of the results of the presented work in this chapter have been published in *Bioresource Technology (2019)* as "**Control of fermentation duration and pH to orient biochemicals and biofuels production from cheese whey"** (https://doi.org/10.1016/j.biortech.2019.121722) and presented at *VENICE 2018 - 7th International Symposium on Energy from Biomass and Waste* as oral presentation "**Dark fermentation of sheep cheese whey: biochemicals and biofuels production as a function of fermentation time and pH"**.

6 POLYHYDROXYALKANOATES PRODUCTION FROM FERMENTED SHEEP CHEESE WHEY

6.1 Introduction

Fermentative process are valuable tools to convert a complex substrate into simpler compounds which can be used for further valorisation, according to a multi-step biological valorisation aimed at recovering either biofuels and biochemicals. In the previous chapter, dark fermentation was used to convert the high initial carbon and carbohydrates content into a gaseous product, such as hydrogen, and a pool of soluble products, such as VFA. The presence of VFA in the fermentation broth acquires further importance since they are considered precursors of valuable biopolymers: the polyhydroxyalkanoates (PHA).

PHA, produced and accumulated by different bacterial genera from various carbon sources under the form of granules into the bacterial cells as carbon and energy storage, are of interest due to their comparable properties to petroleum-based plastics (Amaro et al., 2019). However, the consolidation on the market for PHA is currently limited by the high production costs due to the use of pure culture or either genetically modified bacteria and expensive feedstock (Johnson et al., 2010). The feedstock could account for about 40% of the total production cost (Choi and Lee, 1999). The use of biowaste or a low-cost by-product as a starting substrate could overcome those problems and make the PHA competitive with the fossil-based plastic (Amaro et al., 2019). Moreover, combining the use of a waste carbon source with MMC, which do not require sterile conditions and expensive feedstocks, is interesting because it could further reduce production costs and make the process more sustainable, both economically and environmentally. Various authors have used different cheese whey to produce PHA by using selected MMC, such as raw cheese whey (Colombo et al., 2016; Valentino et al., 2015b), second cheese whey and concentrated cheese whey powder (Colombo et al., 2019), cheese whey powder (Duque et al., 2014) and whey permeate (Carletto, 2014). For the best of our knowledge, no studies are reported for PHA production from sheep cheese whey (SCW).

The PHAs production from biowaste or by-product feedstocks by MMC can be achieved with various configurations, among which the so-called “three-stage process” is the most used (see Paragraph 3.4). In this chapter, this configuration has been proposed for the further valorisation of the SCW (Figure 6.1). The fermented SCW (FSCW) obtained in the previous DF fermentation tests (which act as stage I) is rich in VFA, and it can be used for selection and enrichment of PHA-storing microorganisms (from MMC) in anaerobic sequencing batch reactor (SBR) operated under a feast and famine regime (stage II). The same VFA-rich stream can be also used as a feed for the selected PHA-storing microorganisms in a batch accumulation reactor to assess the culture’s maximum PHA storage capacity (stage III).

The main aim of the present chapter was to evaluate the feasibility and the limitation of using the DF effluent for PHA production from SCW. Furthermore, the influence of the operating pH adopted during the

fermentation phase was assessed in terms of onset of specific metabolic pathways, type of acid produced and, in turn, PHA composition and accumulation yield.

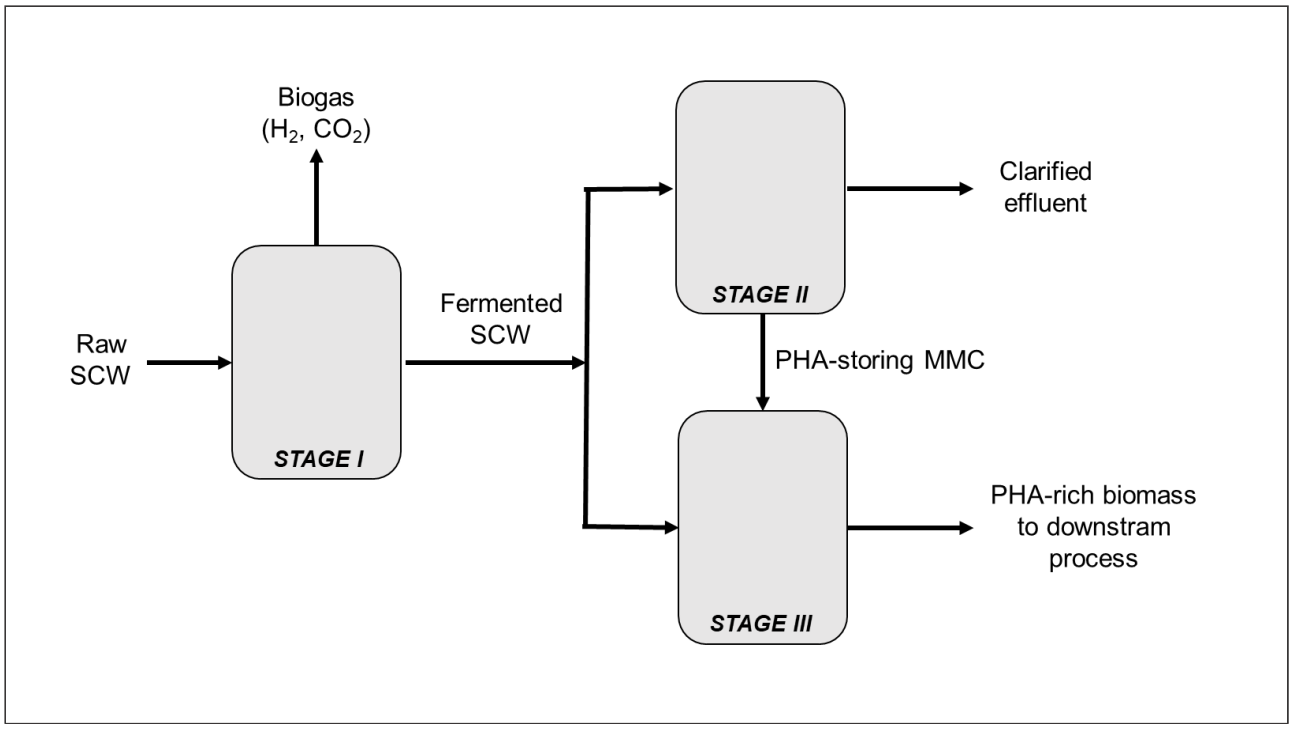


Figure 6.1. *Three-stage process for PHA production by MMC: dark fermentation (stage I), selection and enrichment of PHA-storing MMC (stage II) and PHA accumulation (stage III).*

6.2 Materials and methods

6.2.1 Experimental set-up

The supernatant of FSCW obtained in the previous DF tests were used as a substrate for the selection and enrichment of a PHA-storing MMC (stage II) and PHA accumulation test (stage III), as presented in Figure 6.1. For further details about the dark fermentation tests, see Chapter 5. Before the tests, the required amount of sample was thawed at room temperature for about 8 hours similarly to what was done for the raw SCW in the previous chapter.

A summary of the performance parameters obtained in the previous chapter for the DF of raw SCW and the main characterisation parameters of the resulting FSCW is presented in Table 6.1. Only the FSCW obtained at pH 5.5, 6, 7, 7.5 were used for the following stages of selection and PHA production (stages II and III).

Table 6.1 *Summary of the performance parameters obtained with dark fermentation of raw SCW and characterisation of the resulting FSCW as a function of the operating pH adopted.*

Parameter	Uncontrolled pH	pH 5.5	pH 6	pH 6.5	pH 7	pH 7.5
Fermentative Performance parameters						
$Y_{VFA/SCW}$ ($Cmmol_{VFA}$ $Cmmol_{SCW}^{-1}$)	0.26	0.53	0.53	0.48	0.58	0.59
TOC removal (%)	3	26	26	26	26	26
Carbohydrates removal (%)	45	93	97	99	99	99
Protein removal (%)	n.d.	23	32	58	60	24
Fermented sheep cheese whey (FSCW) characterisation						
TOC ($g\ L^{-1}$)	32.54	21.86	19.57	15.94	26.52	24.70
DOC ($g\ L^{-1}$)	26.14	17.64	15.35	12.91	22.06	19.05
Total VFA ($Cmol\ L^{-1}$)	0.67	1.08	1.05	0.79	1.10	1.03
VFA composition (%, Cmol basis) Ac / La / Pr / Bu / Va	0/100/0/0/0	2/18/21/59/1	4/10/25/60/2	18/0/47/34/1	36/1/48/14/2	31/11/35/23/0
Ammonia ($g\ L^{-1}$)	0.45	0.26	0.26	0.51	0.70	0.76
Soluble proteins ($g\ L^{-1}$)	9.60	4.08	3.65	1.75	2.30	6.45
C/N ($Cmol\ Nmol^{-1}$)	15	25	23	22	20	14

6.2.2 Selection and enrichment of PHA-storing MMC (stage II)

The enrichment in PHA-producing bacteria was performed in SBR with a working volume of 4-L, applying a feast and famine regime (Duque et al., 2014). The latter consists of alternating periods of presence (feast) and absence (famine) of the carbon source under fully aerobic conditions. The SBR cycle length was of 12 h, consisting of four discrete phases: (i) influent filling (4 min), (ii) aeration (675 min), (iii) settling (40 min), and (iv) withdrawal of the exhausted effluent (5 min). The SBR was inoculated with fresh activated sludge (AS) sampled from an aerobic tank of a municipal wastewater treatment plant and immediately submitted to the selection reactor. The initial TS content of the AS was 8 $g_{TS}\ L^{-1}$. The hydraulic retention time (HRT) and sludge retention time (SRT) were kept at 1 and 4 days, respectively. The SBR was operated at 25 °C, and the operative pH was kept in the range 7-9, automatically controlled by adding HCl or NaOH. Aeration and agitation were provided by supplying air at 200 NL h^{-1} and stirring set at 200 rpm. Pumping, aeration and stirring were automatically controlled by the Dia-Net software (DIAFERM - Diachrom SA). The selection stage was

monitored by determining the duration of both feast and famine phases achievable by using the dissolved oxygen (DO) concentration measured by a polarographic probe (InPro 6800, Mettler Toledo) in the selection media. Three different culture enrichments were performed and, for each new enrichment, fresh AS was collected from the wastewater treatment plant and immediately subjected to the SBR as the inoculum.

In the first cycle, the SBR was operated with a synthetic medium for 167 cycles, composed by CH_3COONa (1.395 g L^{-1}), NH_4Cl (0.214 g L^{-1}), KH_2PO_4 (0.054 g L^{-1}) (Colombo et al., 2016). A nutrient solution was added to the mineral medium (Silva et al., 2017). The organic load rate (OLR) was equal to 40 Cmmol L^{-1} $d^{-1,}$ and the C/N molar ratio was 100/10. In the following, the second and the third cycles were performed considering that the FSCW obtained at pH = 5.5 and pH 6 showed the prevalence of butyrate among the total VFA, while in the FSCW obtained at pH = 7 and 7.5 there was a prevalence of propionate and acetate. Since acetic, butyric and lactic acid were considered HB precursors while valeric and propionic acid were considered as HV precursors and the type of VFA plays a pivotal role in terms of physical characteristic of the obtained polymers, the SBR was operated firstly with: FSCW at pH = 6 (FSCW-6) for 43 cycles and FSCW-5.5 for another 23 cycles. After that, the SBR was fed with FSCW-7.5 (for the first 60 cycles), FSCW-7 (for 52 cycles) and FSCW-6.5 (for the last 34 cycles). The OLR during the enrichment phases was equal to 40.6 ± 6.7 Cmmol L^{-1} d^{-1} (FSCW-6 and FSCW-5.5) and 40.0 ± 1.6 Cmmol L^{-1} d^{-1} (FSCW-7.5, FSCW-7 and FSCW-6.5), respectively. FSCW was diluted with deionised water to obtain the target OLR. Allylthiourea (20 mg L^{-1}) was also added during the enrichments to inhibit nitrification (Colombo et al., 2016; Duque et al., 2014).

6.2.3 PHA accumulation (stage III)

PHA accumulation tests were performed in a fed-batch reactor with 1-L of working volume. The PHA accumulation tests consisted in feeding the synthetic medium or each FSCW from the stage I to 500 mL of enriched culture from the SBR of stage II (at least 3 SRT from the beginning of the selection). In stage III, the synthetic medium was prepared as described before, but without adding nitrogen (NH_4Cl) and phosphorus (KH_2PO_4) to ensure nutrient limiting conditions. Air was supplied through a ceramic diffuser, and the DO was continuously acquired by a polarographic probe (InPro 6800, Mettler Toledo). The PHA accumulation experiments were carried out by feeding the substrate adopting a pulse-wise method controlled by DO. Total dosed C was calculated, considering that the ratio of the carbon to the microorganism had to be the same as that inside the selection reactor (stage II) (Colombo et al., 2016). The accumulation tests were stopped when no DO variation followed the substrate feeding. The tests were carried out without pH control and at room temperature (25°C) with a mixing provided by magnetic stirring (300 rpm). For each substrate, the accumulation test was performed in duplicate.

6.2.4 Analytical methods

The total solids (TS), volatile solids (VS) and volatile suspended solids (VSS) were measured according to standard methods (APHA, 2012). The total organic carbon concentration (TOC) and its dissolved fraction (on 0.45 µm filtered samples, DOC) were measured using a Shimadzu TOC analyser (TOC-VCSN, Shimadzu, Japan).

Total soluble carbohydrates (on 0.45 µm filtered samples) were analysed spectrophotometrically at 490 nm according to colourimetric phenol-sulphuric acid method (Dubois et al., 1956). Lactose standards (0-100 mg L^{-1}) were used for the determination of the SCW total soluble carbohydrates.

The soluble protein content was determined spectrophotometrically at 750 nm by the alkaline copper method as described by Lowry et al. (1951), using bovine serum albumin (BSA) (0-100 mg L^{-1}) as standards. Soluble ammonia was measured on filtered samples using the Nessler spectrophotometric method at 420 nm.

All the spectrophotometric analyses were performed with a HITACHI U-200 spectrophotometer.

The gas produced during the fermentation tests was sampled periodically from the reactor headspace with a 1 mL gastight syringe and injected through a valve in a gas chromatograph (model 7890B, Agilent Technology) equipped with a thermal conductivity detector (TCD) and two stainless columns packed with HayeSep N (80/100 mesh) and Shincarbon ST (50/80 mesh) connected in series. The operating temperatures of the valve and the TCD were 90 °C and 200 °C, respectively, and He was the carrier gas at a constant pressure of 8 psi in the HayeSep N column and 25 psi in the Shincarbon ST column (at 70 °C). The oven temperature was set initially at 70 °C (3-min holding time), followed by a ramp of 10 °C min^{-1} up to 160 °C (3-min holding time). The concentration of lactic acid (HLa) was analysed on filtered samples (0.45 µm) using a Dionex high-pressure liquid chromatography system UVD170U equipped with an Acclaim Organic Acid column, with isocratic elution (H_2PO_4 0.2% + sodium sulphate 100 mM) at 0.9 mL min^{-1}). The concentration of volatile fatty acids (VFA) (acetic [HAc], propionic [HPr], butyric + isobutyric [HBu] and valeric + isovaleric [HVa]) was determined using a gas chromatograph with flame-ionization detection (model 7890B, Agilent Technology) equipped with a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology). The samples were filtered using a 0.45 µm membrane and then acidified with concentrated H_3PO_4 (pH<3). The injection volume was 0.6 µL. The temperatures of the injector and the detector were 230 °C and 300 °C, respectively. The oven temperature was initially set at 60 °C (3-min holding time), followed by a ramp of 10 °C min^{-1} up to 160 °C. He (1.6 mL min^{-1}, splitless) was used as the carrier gas.

For PHA determination, the mixed liquor samples (5 mL) from stage II and stage III were treated immediately with 1 mL of a NaClO solution (7% active Cl_2) according to Silva et al. (2017) and stored at -4°C for the following analysis. The PHA was then extracted, hydrolysed and determined by gas chromatography using a method adapted from Serafim et al. (2004). Firstly, the unfrozen sample was centrifuged (11 000 rpm, 15 min), and the supernatant was discharged. Then the pellet was resuspended with 2 mL of deionised water and centrifuged again (11 000 rpm, 15 min). Afterwards, the pellet recovered from previous procedure was

incubated for methanolysis in a 20% vol. sulphuric acid in methanol solution (1 mL) and extracted with chloroform (1 mL). The mixture was digested at 100°C for 3.5 h and then the organic phase (methylated monomers dissolved in chloroform) was extracted and injected (1 µL) into a gas chromatograph equipped with a flame ionization detector (model 7890B, Agilent Technology) and a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology) using helium as carrier gas at constant pressure (14.5 psi). The temperature of the injector and the detector were 280ºC and 230ºC respectively. The oven temperature was initially set at 40°C, followed by a ramp of 20°C min-1 until 100°C, then 3°C min-1 until 175 and then 20°C until a final temperature of 220°C (4 minutes holding time) for ensuring cleaning of the column after each injection. The 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) concentrations were quantified using a commercial polymer PHBV (88%/12%) (Sigma-Aldrich, CAS number 80181-31-3). Benzoic acid (50 mg L^{-1}) was used as internal standard and was added prior the methanolysis step.

6.2.5 Calculations

The fermentation yields $Y_{OA/SCW}$ were calculated as the ratio between the amount of the produced VFA (expressed in terms of Cmmol) and the initial TOC amount of SCW. The VFA concentrations, (including the HLa), were expressed as $Cmmol_{VFA}$ L^{-1}.

The PHA content in cells was calculated by dividing the measured PHA concentration by the biomass concentration [PHA = g_{PHA} kg_{VSS}^{-1})] and considering VSS to be constituted by both active biomass (X) and PHA (Duque et al., 2014). PHA and X were expressed in terms of Cmmol. For calculating X in Cmmol (44.2 $Cmmol_X$ g_X^{-1}), the chemical formula $C_5H_7NO_2$ was considered (Valentino et al., 2014). The specific VFA uptake rate ($-q_{OA}$) and the specific PHA accumulation rate (q_{PHA}) were determined from the linear regression of the experimental data of VFA, PHA and X specific concentrations, respectively, plotted over time. The storage yields $Y_{PHA/OA}$ was calculated as the ratio between q_{PHA} and q_{VFA}.

The C/N molar ratio was estimated by considering the total nitrogen as the sum of both the contributes from soluble proteins, adopting a conversion factor of 6.25 (Mariotti et al., 2008), and ammonia in the filtered samples (0.45 µm).

In the accumulation tests, the specific rates and yields were calculated as described before, for each pulse. In order to compare different accumulation tests, the first three pulses average values of each parameter were considered (Colombo et al., 2019, 2016).

Moreover, the total process yield ($Y_{PHA/SCW}$) was calculated as a product of the fermentation yield ($Y_{VFA/SCW}$) and the storage yield ($Y_{PHA/VFA}$), expressed as $Cmol_{PHA}$ $Cmol_{SCW}^{-1}$.

6.3 Results and discussion

6.3.1 Dark fermentation of sheep cheese whey

In the first stage of the proposed multi-stage valorisation scheme, the initial high organic carbon of SCW was converted through DF into an appreciable amount of biohydrogen and a pool of VFA, which are considered PHA precursors. A summary of the performance parameters obtained during the fermentation tests is presented in Table 6.1, and a more detailed discussion about this stage is presented in Chapter 5.

To summarise the results obtained previously, it has been confirmed that pH control is necessary to obtain an appreciable amount of gaseous product (i.e., biohydrogen) from raw SCW making exclusive use of its indigenous biomass. Moreover, the fermentation soluble products yield ($Y_{OA/SCW}$) was in the range of 0.5 - 0.6 $Cmol_{VFA}$ $Cmmol_{SCW}^{-1}$, except for the test performed without pH control (0.3 $Cmol_{VFA}$ $Cmmol_{SCW}^{-1}$). As a comparison, Colombo et al. (2016) reported similar yields using raw and sterilized CW in mesophilic batch fermentation using autochthonous lactic bacteria and heat-shocked digestate as inoculum (0.4 and 0.6 $Cmol_{VFA}$ $Cmol_{soluble\ substrates}^{-1}$) and Duque et al. (2014) reported slightly higher yield of 0.64 $gCOD_{VFA}$ $gCOD_{soluble\ substrates}^{-1}$ using CW powder in an anaerobic membrane bioreactor.

The difference in the operating pH did not strongly influence the total amount of produced organic acids, but largely affected the composition of the obtained VFA pool. In the perspective of the subsequent PHA production, the type of VFA plays a pivotal role in terms of the physical characteristic of the obtained polymers, such as crystallinity, brittleness and flexibility. Therefore, as pointed out also in other studies (Colombo et al., 2016; Gouveia et al., 2017), the possibility to influence the composition of PHA by controlling, through the proper choice of the operating pH, the fermentative metabolic pathways and, in turn, the type of VFA to be fed to the PHA storing biomass, is a promising strategy. The primary metabolites obtained in this study were butyrate, acetate, propionate and lactate. FSCW obtained with the test performed at pH = 5.5 and 6 showed the highest presence of butyrate, about the 60% $Cmol_{VFA}$ basis of the total VFA. The acetate presence increased with the adopted pH, and it was higher in FSCW-7 and FSCW-7.5 (31 – 36 % $Cmol_{VFA}$ basis of the total VFA). The propionate presence increased with the operative pH as well, and it was in the range of 35 - 48 % $Cmol_{VFA}$ basis of the total VFA in the tests performed at pH = 6.5, 7 and 7.5. The lactate presence at the end of the tests was low in all the FSCW, except for the test without pH control, which was the only organic acid produced.

The C/N ratio in the fermentation outflow, calculated considering the soluble organic carbon and nitrogen, the latter deriving from proteins and ammonia, was higher for FSCW-5.5 and FSCW-6 (C/N = 23 - 25) than for FSCW-6.5 and FSCW-7 (C/N = 20-22) and for FSCW-7.5 (C/N = 14) (Table 6.1). The differences in terms of C/N values derive from the different TOC and proteins removal efficiencies observed for the different operating pH values. The attention given to the C/N observed for the fermentation outflows is deserved concerning the effects that it exerts on the following phases. Regarding the protein removal, the fermentation tests carried

out at operating pH close to neutrality (6.5 and 7) showed the highest protein removal of 61 - 62%. The soluble ammonia concentration at the end of the fermentation tests increased with the adopted pH, reaching its higher value in FSCW-7.5 (0.7 g L^{-1}). In this respect, the pH confirms to be the pivotal operating parameter in fermentation processes.

In the perspective of a multistage, multiproduct process and considering the composition of the total VFA and the energy recovery in the form of biohydrogen (see Chapter 5), the occurrence of two prevalent metabolic pathway can be noted during the fermentation tests. On the one hand, the butyric pathway is expressed at acidic pH = 5.5, and 6 (~ 60% of total VFA) coupled with the higher specific hydrogen production yields. On the other hand, propionic and acetic acid becoming prevalent at higher pH conditions (more than 60% of the total VFA) but with a decrease of the $Y_{H2/SCW}$ by 30-60%. The possibility to affect the balance between energy and material recover by altering the fermentation pH is an exciting strategy considering the benefits achievable in terms of biorefining process flexibility.

6.3.2 PHA production

6.3.2.1 Acetate as substrate

A feast to famine ratios (calculated as the ratio between the lengths in hours of the two phases, F/F) in the range of 0.1 - 0.3 were observed using acetate as a synthetic medium; this trend was in line with values indicated by Valentino et al. (2017) for obtaining a good selection of PHA-storing bacteria. The average biomass concentration observed was 0.9 g_{VSS} L^{-1} (Figure 6.2a). The maximum PHA content observed at the end of the accumulation tests was 516 g_{PHA} $kg_{VSS}^{-1,}$ and the polymer was composed, as expected, entirely by HB. The storage yield was 0.60 $Cmol_{PHA}$ $Cmol_{acetate}^{-1}$. A typical accumulation test with acetate is presented in Figure 6.2b. The obtained performance parameters, in terms of PHA content and yield, were in line with what reported in literature (Valentino et al., 2017) confirming the possibility to select a PHA-storing MMC from the AS used in this study. The performance parameters obtained using the synthetic solution of acetate can be used as a benchmark for the results obtained with FSCW.

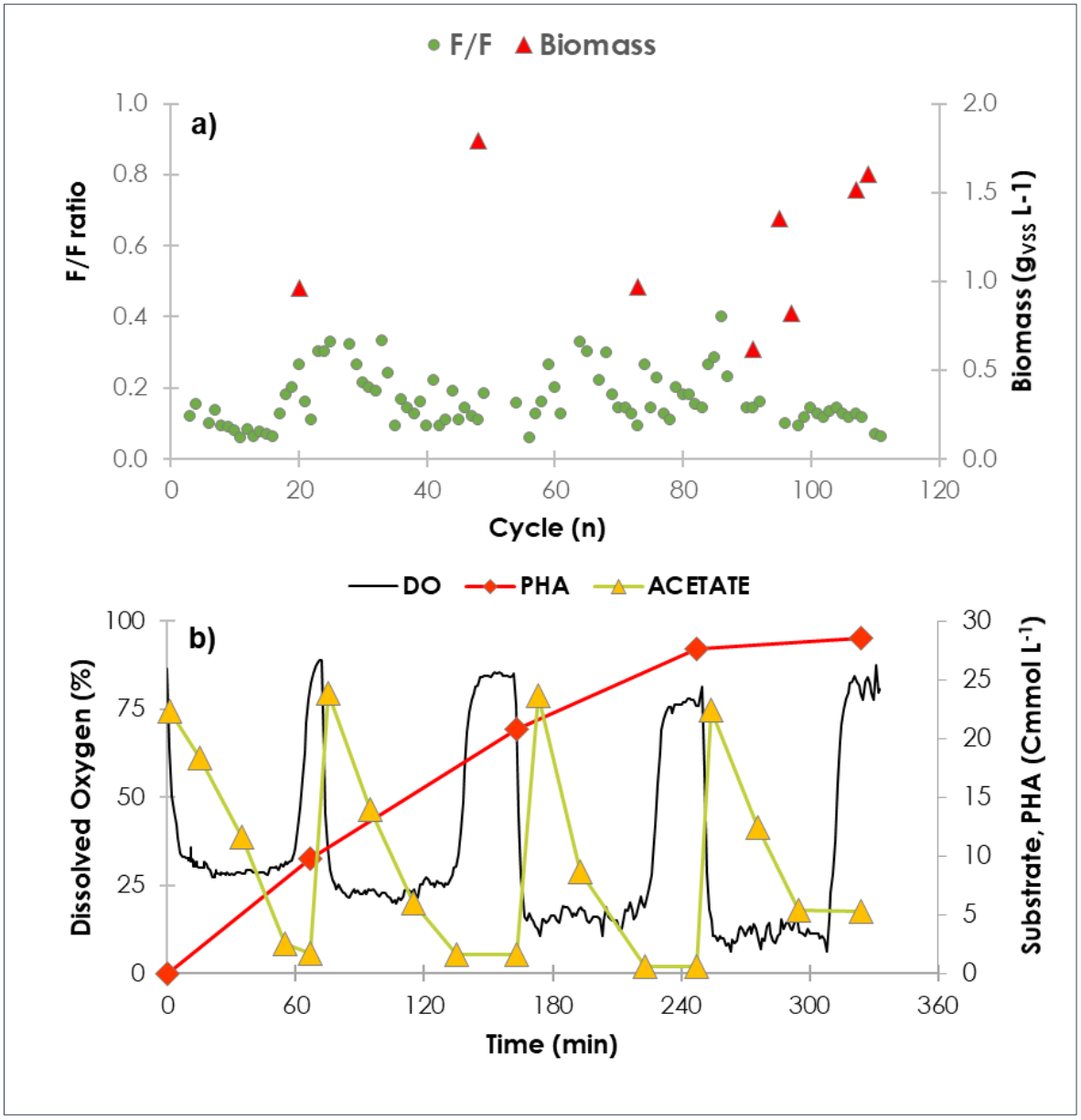


Figure 6.2. *Production of PHA with acetate as a substrate: a) feast and famine ratio (F/F) and biomass concentration during the selection stage; b) dissolved oxygen (DO), acetate consumption and PHA accumulation trend during a typical accumulation test. The grey dotted line indicates the fed pulses.*

The selection and accumulation stages with the fermented substrates were performed accordingly to the fact that the FSCW obtained at pH = 5.5 and 6 showed the prevalence of butyrate among the total VFA and the highest specific hydrogen production yields, while in the FSCW obtained at pH = 7.5, 7 and 6.5 there is a prevalence of propionate and acetate but with a decrease of the YH2/SCW by 30-60%. As mentioned before, the balance between energy and material recover plays a pivotal role in terms of flexible biorefining process, therefore the SBR was operated firstly with FSCW-6 and FSCW-5.5 (i.e., butyrate prevalence and high H2 yields) and then with FSCW-7.5, FSCW-7 and FSCW-6.5 (i.e., propionate/acetate prevalence and lower H2 production yields). Regarding the use of FSCW-6 and FSCW-5.5 as the substrates, the obtained F/F ratio was 0.16 ± 0.08, and the biomass concentration was in the range 0.9 - 1.8 g L-1 during the selection stage (Figure 6.3a). After 20 feeding cycles, it was possible to establish a suitable feast and famine regime in order to keep

the culture well selected in terms of PHA-accumulating bacteria. In the accumulation tests, the FSCW-6 exhibited slightly higher performance parameters compared to FSCW-5.5 in terms of PHA content (347 vs 302 g_{PHA} kg_{VSS}^{-1}), HV fraction in the obtained polymer (34% vs 24%) and $Y_{PHA/VFA}$ (0.44 vs 0.32 $Cmol_{PHA}$ $Cmol_{VFA}^{-1}$). A typical accumulation test with FSCW-6 is reported in Figure 6.3b.

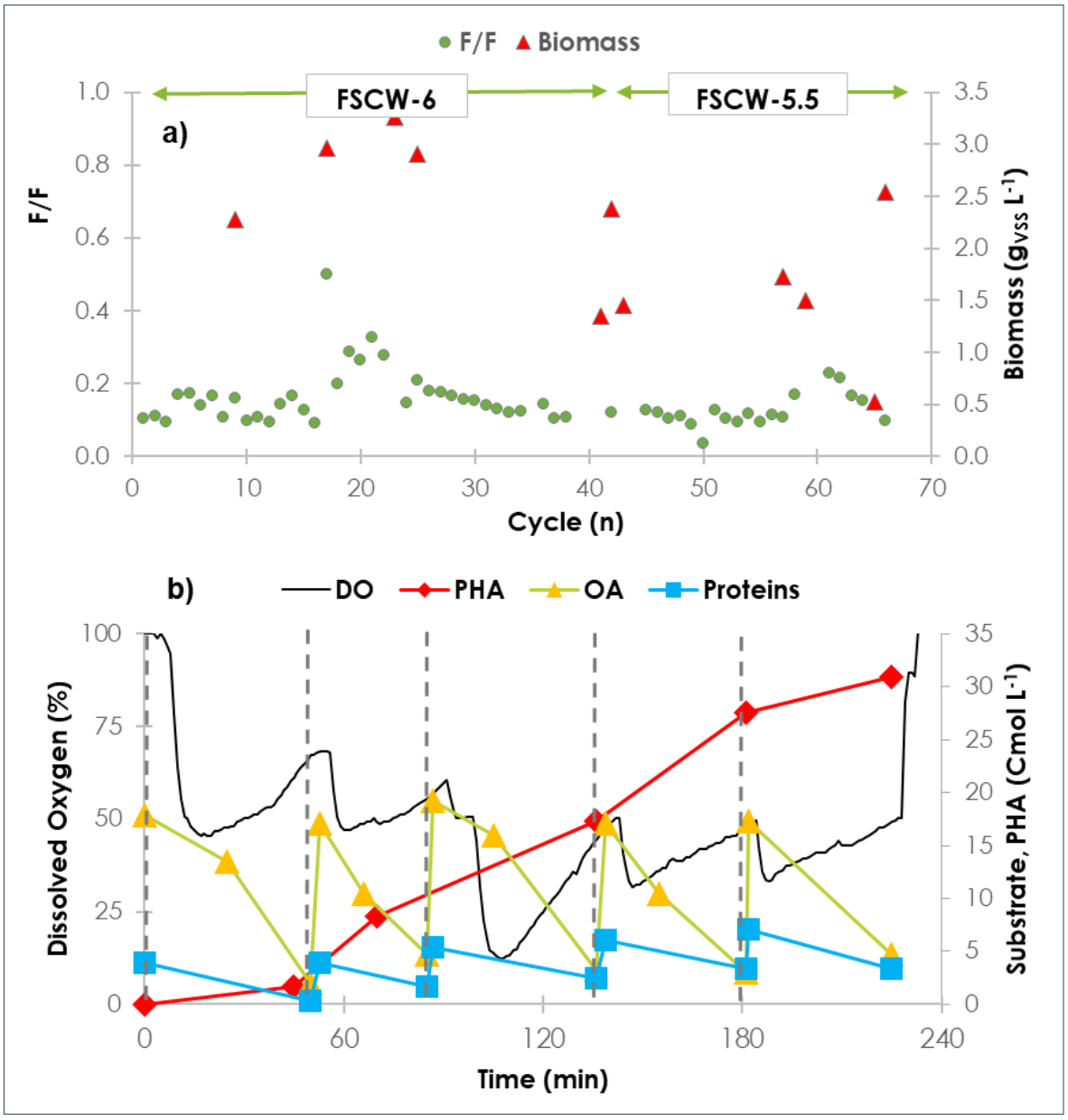


Figure 6.3. *Production of PHA with FSCW-6 and FSCW-5.5 as substrates: a) feast and famine ratio (F/F) and biomass concentration during the selection stage; b) dissolved oxygen (DO), organic acids (VFA) and proteins consumption and PHA accumulation trend during a typical accumulation test with FSCW-6. The grey dotted line indicates a pulse fed.*

Concerning the use of FSCW-7.5, FSCW-7 and FSCW-6.5 as the substrates, the obtained F/F ratio was 0.18 ± 0.08, and the biomass concentration was 1.01 ± 0.24 g L^{-1} during the selection phase (Figure 6.4a). The PHA content at the end of the tests performed with FSCW-7.5 and FSCW-7 were 354 and 317 gPHA kg_{VSS}^{-1}

respectively, but those tests were affected by an initial PHA content of 213 and 115 g_{PHA} kg_{VSS}^{-1}, respectively. A typical accumulation test with FSCW-7 is reported in Figure 6.4b. The test performed with FSCW-6.5 showed a low PHA content (179 g_{PHA} kg_{VSS}^{-1}), with an HV fraction of 32%.

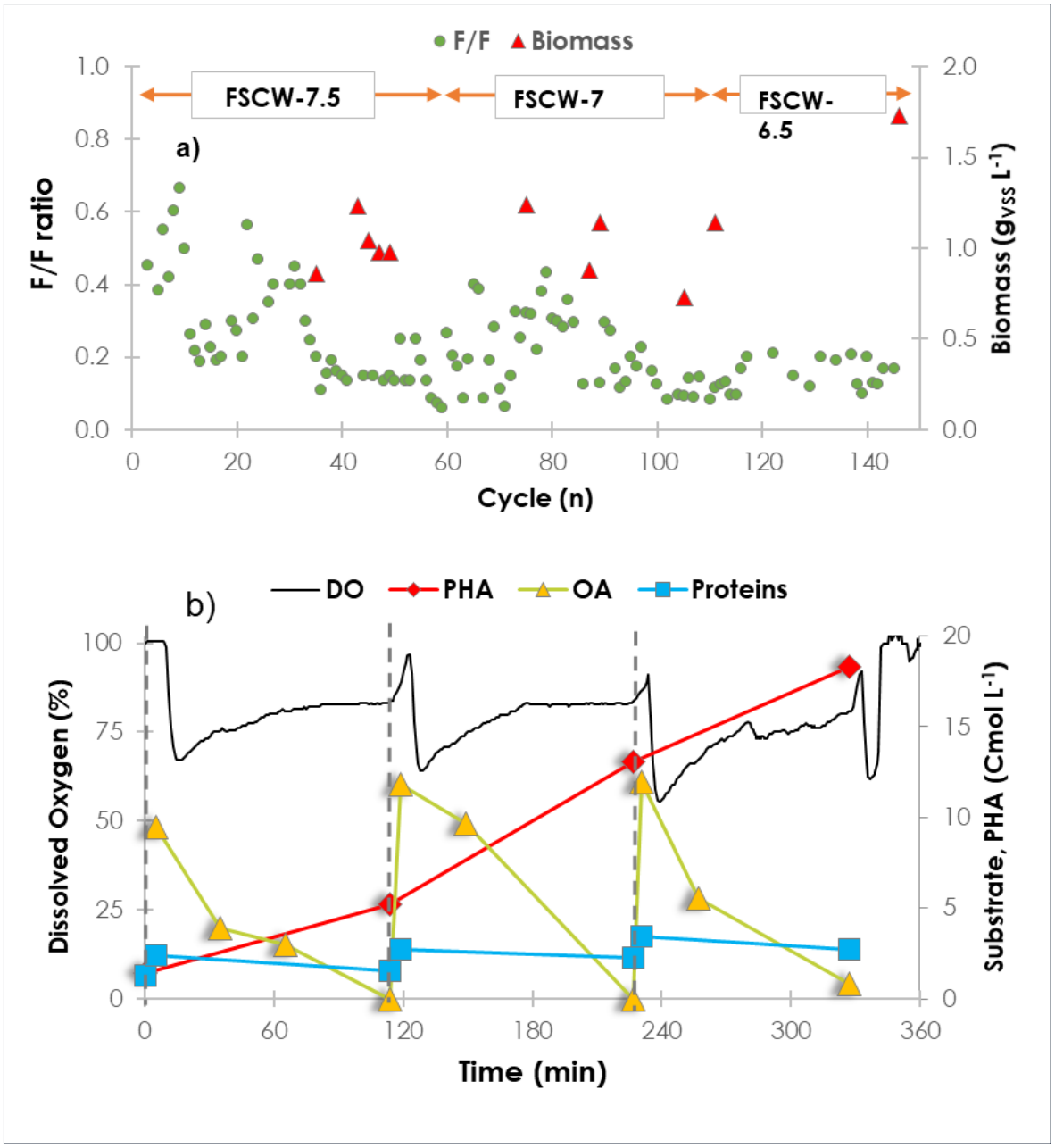


Figure 6.4. *Production of PHA with FSCW-7.5, FSCW-7 and FSCW-6 as substrates: a) Feast and famine (F/F) and biomass during the selection stage with FSCW-7.5, FSCW-7 and FSCW-6.5 as substrates. b) Dissolved oxygen (DO), organic acids (VFA) and proteins consumption and PHA accumulation trend during a typical accumulation test with FSCW-7. The grey dotted line indicates a pulse fed.*

The selection stages were carried out without nutrient supply during all the tests, and this brings us to some interesting considerations. It is reasonable to assume that the cellular growth was supported by both the ammonia and protein nitrogen content in the FSCW since a significative uptake was observed during the selection tests. The proteins consumption was in the range of 27 - 58 mg_{PROTE} $cycle^{-1}$, considering all the tests,

suggesting that the biomass was able to use cheese whey proteins as nitrogen and carbon source. However, the nitrogen availability in non-readily biodegradable protein-rich substrates such as CW is questionable and matter of discussion, since it may be limited by the culture's metabolising capacity (Oliveira et al., 2018). For this reason, the addition of a readily bioavailable nitrogen source (often in the form of ammonium salts) is a practice widely reported in literature; so further study is needed to clarify this point better.

As extensively mentioned previously, sheep milk is characterised by high protein and fats content, as well micronutrients like potassium, calcium and phosphorous (Balthazar et al., 2017), therefore the selection stage benefits of the peculiar characteristics of the SCW in terms of nutrient amounts. On the other hand, this aspect may have influenced the accumulation stage. The PHA content obtained for FSCW in the range of 30 - 35% (as $g_{PHA}\ g_{VSS}^{-1}$) is interesting since a PHA content slightly above 400 $g_{PHA}\ kg_{VSS}^{-1}$ is reported as a starting point to consider PHA recovery and commercialisation (Valentino et al., 2017). On the other hand, literature about PHA production from CW reported even higher value, although with nutrient supply in the selection phase (55 - 62%, Colombo et al., 2019; Duque et al., 2014). Regarding the influence of nutrient supply, Oliveira et al. (2018) reported at maximum PHA content of 43% during accumulation test with fermented CW (biomass selected with nitrogen supply). In the same study, the nitrogen supply was gradually decreased up to force the biomass to use only whey proteins (around 1.3 g L^{-1}) as nitrogen source, resulting in a drastically decreasing of storage capacity of the selected culture with low amounts of PHA being produced during the selection cycles (5 wt.%).

The performance parameters obtained during the PHA accumulations assays are presented in Table 6.2. The specific PHA uptake rate (q_{PHA}) was higher for FSCW-5.5 and FSCW-6 compared to what obtained for FSCW-7.5, FSCW-7 and FSCW-6.5. The observed variability in q_{PHA} accumulation kinetics is largely determined by population dynamics induced by the feast and famine culture enrichment strategy. The accumulation performed with the biomass selected during the third enrichment phase (i.e., using FSCW-7.5, FSCW-7 and FSCW-6.5) were affected by the high ammonia and protein present in these fermented substrates, as demonstrated by the higher specific ammonia and protein uptakes ($-q_{NH4}$ and $-q_{PROTE}$). Furthermore, during the accumulation assays, an increase of protein concentration in the medium was noticed probably due to the pulse-wise feeding operational strategy adopted in this study, that may lead to a decrease of the C/N ratio to value lower than 10, which are favourable for the growth. When substrates with an initial low C/N ratio (i.e., FSCW-7.5, C/N=14) are used, this mechanism is faster, and the occurrence of growth-response instead of PHA-response is favoured. For this consideration, the feeding strategy adopted in this study could represent a further limiting factor when a protein-rich substrate as SCW is used since it increases the risk of growth-response overtaking storage-response.

Regarding polymer composition, the copolymer produced was always a copolymer made by HB and HV. Interestingly, the HV fraction obtained FSCW-5.5 (23%) was similar to what was obtained with FSCW-7.5 (24%

HV) but showed a clear difference with FSCW-6 (34 %). Small changes in the operating fermentative pHs could lead to greater changes in the polymer quality and composition.

Table 6.2. *Overview of the PHA-accumulation assays with the MMC selected using the different substrates.*

Parameter	Acetate	FSCW-5.5	FSCW-6	FSCW-6.5	FSCW-7	FSCW-7.5
PHA_{max} (g_{PHA} kg_{VSS}^{-1})	516±34	302±46	347±6	179±87	337±33	354±9
PHA composition (wt.%HB/wt.%HV)	100:0	78:23	66:34	68:32	63:37	76:24
$-q_{VFA}$ ($Cmol_{VFA}$ $Cmol_X$ h^{-1})	0.69±0.08	0.34±0.19	0.38±0.01	0.11±0.04	0.19±0.01	0.23±0.04
q_{PHA} ($Cmol_{PHA}$ $Cmol_X$ h^{-1})	0.41±0.12	0.11±0.02	0.17±0.01	0.04±0.01	0.08±0.01	0.10±0.01
$Y_{PHA/VFA}$ ($Cmol_{PHA}$ $Cmol_{VFA}^{-1}$)	0.60	0.32	0.44	0.35	0.49	0.43
$-q_{NH4}$ (Nmol $Cmol_X^{-1}$ h^{-1})	n.a.	0.004±0.000	0.005±0.004	0.005±0.002	0.008±0.004	0.014±0.002
$-q_{PROTE}$ ($Cmol_{PROTE}$ $Cmol_X^{-1}$ h^{-1})	n.a.	0.04±0.02	0.05±0.04	0.01±0.02	0.02±0.04	0.08±0.01
$Y_{PHA/SCW}$ ($Cmol_{PHA}$ $Cmol_{SCW}^{-1}$)	n.a.	0.17	0.23	0.17	0.28	0.25

6.4 Combining PHA production with energy recovery

The cascading principle aims to maximise the recovery of resource from the initial raw feedstock. Considering the experimental results obtained in this chapter, the overall carbon recovery, calculated considering the carbon amount in the final product, the PHA, and the initial carbon content in the SCW, is in the range of 17-28% (Table 6.3). On the one hand, the highest overall PHA yield (and carbon recovery) were obtained adopting an operative pH of 7, as a result of the highest $Y_{VFA/SCW}$ and $Y_{PHA/VFA}$ and despite the limitation about the pulse-wise feeding strategy explained before. On the other hand, adopting an operative pH of 6 lead to a reduction of 20% in the PHA yield but an increase of 70% in the H_2 yield. Such flexibility it is an interesting feature of an integrated process-oriented on simultaneously recovery of PHA and energy in the form of hydrogen.

In a biorefinery scheme, the process should be oriented for the maximum recovery of resources, and the values briefly obtained here shows that there is room for further recovery. With this aim, the 3-step process here proposed may be integrated with a further step of anaerobic digestion in order to improve the global energy recovery. The residual sludge from the fermentation stage may represent a suitable substrate for

anaerobic process since it contains the residual acidogenic biomass and other non-fermented compounds. The integration of the 3-step process with an AD step may be beneficial also because of the potential of the digestate as a fertiliser in agriculture. In this case, SCW can be effectively valorized with a multistep process-oriented to a multiproduct production, i.e. PHA, hydrogen, methane, biofertilizer.

Table 6.3. *Summary of product yield (H_2 and PHA) obtained applying the 3-stage process to SCW valorisation.*

Operative pH adopted in stage I	Max H_2 yield (L_{H2} L_{SCW}^{-1})	Max PHA yield (g_{PHA} L_{SCW}^{-1})	Carbon recovery %Ci[(a)]
pH 5.5	4.51	11.44	17
pH 6	5.38	16.05	23
pH 6.5	3.35	11.33	17
pH 7	3.17	19.17	28
pH 7.5	2.04	17.11	25

[(a)] Calculated as (%Ci) considering the final C content of PHA (assumed equals to 46% and 50% for HB and HV respectively)

6.5 Conclusions

The valorisation of sheep cheese whey is possible by applying a three-stage process for both energy and material recovery. The first stage of dark fermentation can convert the sheep cheese whey into biohydrogen and organic acids, which represent ideal precursors for PHA, even in the absence of specific inoculum and extra nutrients. The high nutrient content of sheep cheese whey (whey protein, fats and minerals) on the one hand suggests that is possible to select a PHA-storing MMC without extra nitrogen supply, but on the other hand, could be a limiting factor for PHA accumulation. The adopted pH during the fermentation stage affected the hydrogen yield and the quality of the polymer produced in terms of HV fraction.

The overall PHA yield was in the range of 11-19 g_{PHA} L_{SCW}^{-1} with the maximum yield obtained by setting the fermentative operative pH at the value of 7.

Despite the results are promising, considering all the aspects explained before, further studies are necessary in order to optimise the process for this specific substrate in terms of selection of biomass with improved PHA storing capacity and consequent PHA accumulation performance. The integration of the above proposed 3 step process with an anaerobic digestion step seems to be a promising strategy for further valorisation and improved energy recovery.

Partial results of the presented work in this chapter have been presented at *CEST 2019 16th International Conference on Environmental Science & Technology* as oral presentation "**Valorisation of ovine cheese whey through PHA production"** and at *SARDINIA 2019 17th international waste management and landfill symposium* as oral presentation " **Three-stage process for hydrogen and PHA production from sheep cheese whey".**

7 ENERGY RECOVERY FROM ONE- AND TWO-STAGE ANAEROBIC DIGESTION OF SHEEP CHEESE WHEY

7.1 Introduction

Anaerobic digestion (AD) is a mature technology that is widely applied for biowaste valorisation and represents the typical example of energy recovery (Kleerebezem et al., 2015). Through AD, organic matter is converted into biogas, mainly composed of methane and carbon dioxide. Biogas can directly be used for electricity or heat production or upgraded to biomethane to reach the same quality of natural gas.

Although AD is a well-consolidated technology, the implementation of AD processes within dairies industries still represents a technical challenge which has limited its diffusion on a real scale in the last decades (see Chapter 3). Due to its high organic load and low alkalinity, AD of CW may result in an excess of non-buffered acidification during lactose fermentation and a consequent inhibition of the methanogenic activity, which in turn leads to a VFA accumulation, affecting the CH_4 yield as well as the stability of the process (De Gioannis et al., 2014; Hagen et al., 2014; Humberto et al., 2017; Prazeres et al., 2012; Traversi et al., 2013). Due to this issue, low biomethane yields ranging from 0.27 to 0.6 L CH_4 g^{-1} VS have been reported by anaerobic digestion of CW under mesophilic conditions (Escalante et al., 2017; Labatut et al., 2011; Vivekanand et al., 2018), whilst in continuous applications, long HRT values (above 5 days) are typically applied to avoid process instability (see Chapter 2). External alkali addition (e.g., lime, bicarbonate, or hydroxide) or appropriate dilution is generally required to mitigate acidification, but both strategies would increase the operation costs, and/or the volumes to be treated. A more sustainable option is co-digestion of CW with substrates characterised by high buffering capacity, such as sewage sludge (Carrieri et al., 1993), dairy manure (Kavacik and Topaloglu, 2010; Rico et al., 2015; Vivekanand et al., 2018), poultry manure (Gelegenis et al., 2007), and cattle slurry (Comino et al., 2012), or fish ensilage (Vivekanand et al., 2018), although results from literature are controversial.

A two-stage process, where hydrolysis-acetogenesis and methanogenesis are carried out in two different reactors, is another strategy to avoid process instability (Fernández et al., 2015), as well as increase COD removal, although it would result in a higher footprint of the plant, as well as increasing investment and operation costs. The two-stage AD process offers the possibility of operating the methanogenic reactor at lower HRT (< 5 d) than one-step process. Yilmazer and Yenigün (1999) and Saddoud et al. (2007) reported a biogas yield of 0.550 and 0.300 L g^{-1} $COD_{removed}$, respectively, with COD removals above 90%, in a two-stage AD process with HRT = 4 d in the methanogenic reactor.

Among all the biochemical processes studied in this thesis, one- or two-stage AD of CW may represent the most suitable application in the short-term period for energy recovery within dairy industries. One the one hand, AD is well-established technology and allow the recovery of energy in the form of biogas or

biomethane. The recovered energy from CW can be used in situ for the dairies processes or fed to the national gas grid (as biomethane). On the other hand, the two-stage process would allow to recover H_2 in the first reactor, opening the possibility to further options. Hydrogen could be circulated into the methanogenesis reactor or used to reduce biologically the CO_2 to increase the overall CH_4 yield. Alternatively, hydrogen can be used as biofuel, alone or in combination with the CH_4 produced in the methanogenesis reactor to produce biohythane, a gaseous fuel having a composition of H_2, CH_4 and CO_2 gases in the ranges 5–10%, 50–60%, and 35–45%, respectively. Biohythane has been proposed as a greener alternative to natural gas since can be produced from renewable source. Furthermore, when tested on internal combustion engine, biohythane showed a more significant reduction in emission of pollutants like oxides of nitrogen (NO_X) into the atmosphere (Mishra et al., 2017).

In this framework, the objective of the present chapter was to compare one- and two-stage AD of sheep cheese whey (SCW) aimed at recovering CH_4 and H_2 + CH_4, respectively. Biochemical Methane Potential tests (BMP) were performed under mesophilic conditions to evaluate the performances in terms of H_2 and CH_4 yields and the overall energy recovery achievable with SCW.

7.2 Materials and method

7.2.1 Substrates and inoculum

Raw SCW and the effluent of fermentation tests (FSCW) conducted at different operating pH (i.e.: 5, 5.5, 6, 6.5, 7, 7.5) were used as the substrate for the BMP test. Before the BMP tests, the required amount of sample was thawed at room temperature for about 8 hours. The inocula used for BMP assays was collected from a WWTP and acclimated with sodium acetate for one month. Specific methanogenic activity (SMA) tests were conducted in triplicate using 4 g_{COD}/L of sodium acetate, in order to determine the activity of the acclimated microflora. The final mesophilic methanogenic sludge had a TS = 2.03% and VS = 0.83%TS. Previous to the BMP tests, the inoculum was degassed for five days at 39 °C.

7.2.2 Experimental set-up

5.1.1.4 BMP assays

BMP assays were conducted in 120 mL serum bottles with a working volume of 60 mL and using 30 mL of inoculum in each bottle. Different food to microorganisms ratios (F/M) expressed in terms of volatile solids ($VS_{substrate}$:$VS_{inoculum}$) were used for BMP of SCW, in order to assess possible inhibitive conditions. The ratios adopted were equal to 0.25, 0.5, 1 and 2 respectively. For the BMP assays using FSCW as the substrate, a ratio of 0.5 $VS_{substrate}$:$VS_{inoculum}$ was adopted. Nutrients (NH_4Cl 26.6 g L^{-1}, KH_2PO_4 10 g L^{-1}, $MgCl_2{\cdot}6H_2O$ 6 g L^{-1}, $CaCl_2$ 2.27 g L^{-1}) and trace elements ($Fe_4Cl_2{\cdot}4H_2O$ 2 g L^{-1}, $CoCl_2$ 0.27 g L^{-1}, $MnCl_2{\cdot}6H_2O$ 0.12 g L^{-1}, $NiCl_2{\cdot}6H_2O$ 0.1

g L^{-1}, $ZnCl_2$ 0.05 g L^{-1}, H_3BO_3 0.05 g L^{-1}, $CuCl_2$ $2H_2O$ 0.04 g L^{-1}) were added in the amount of 1.2 mL for each BMP bottle to prevent deficiency during the tests according to (Le Hyaric et al., 2012). Sodium bicarbonate (3.3 g $NaHCO_3$ L^{-1} of liquid phase) was added as a buffer. The liquid volume of serum bottles was then adjusted to 60 mL with distilled water. The bottles were sealed with gas-tight rubber stoppers and flushed with N_2 for 3 min to achieve anaerobic conditions. Finally, the bottles were placed in incubator shaker (100 rpm) at a temperature of 37°C for 30 days. The assays containing only inoculum and water were used as controls to differentiate inoculum methane production from the substrate methane production. All experiments were conducted in duplicate.

Gas production was measured using the syringe method (Filer et al., 2019) and the measured gas volume was converted standard temperature and pressure conditions (T = 273.15 K, P = 10^5 Pa).

7.2.3 Analytical methods

The concentration of total solids (TS), volatile solids (VS), total organic carbon (TOC) and soluble carbohydrates (sCarb, on 0.45-µm filtered samples) were measured on samples immediately before use according to the analytical methods reported in (De Gioannis et al. (2014). All the spectrophotometric analyses were performed with a HITACHI U-200 spectrophotometer. The concentration of Fe, Mg, K, Na, Ca was determined on 0.45-µm filtered samples using an inductively coupled plasma-optical emission spectrometer (ICP-OES, Optima 7000DV, Perkin Elmer, MA, USA). The concentration of lactic acid (HLa) was analysed using a Dionex high-pressure liquid chromatography System UVD170U equipped with an Acclaim Organic Acid column. All analyses were conducted with isocratic elution (H_2PO_4 0.2% + sodium sulphate 100 mM at 0.9 mL min^{-1}). The concentration of VFAs (acetic [HAc], propionic [HPr], butyric + iso-butyric [HBu], valeric + iso-valeric [HVa], hexanoic + iso-hexanoic [HHex], heptanoic [HHep]) and ethanol [EtOH]) was determined using a gas chromatograph with flame-ionization detection (model 7890B, Agilent Technology) equipped with a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology). The samples were filtered using a 0.45 µm membrane and then acidified with concentrated H_3PO_4 (pH < 3). The injection volume was 0.6 µL. The temperatures of the injector and the detector were 230 °C and 300 °C, respectively. The oven temperature was initially set at 60 °C (3-min holding time), followed by a ramp of 10 °C min^{-1} up to 160 °C. Helium gas (1.6 mL min^{-1}, splitless) was used as the carrier gas.

The biogas was sampled periodically from the reactor headspace with a 1-mL gastight syringe and injected through a valve in a gas chromatograph (model 7890B, Agilent Technology) equipped with a thermal conductivity detector (TCD) and two stainless columns packed with HayeSep N (80/100 mesh) and Shincarbon ST (50/80 mesh) connected in series. The operating temperatures of the valve and the TCD were 90 °C and 200 °C, respectively, and He was the carrier gas at a constant pressure of 8 psi in the HayeSep N column and 25 psi in the Shincarbon ST column (at 70 °C). The oven temperature was set initially at 70 °C (3-min holding time), followed by a ramp of 10 °C min-1 up to 160 °C (3-min holding time).

All analyses were run in triplicate and results are presented as average values of the replicates and the associated standard deviation.

7.2.4 Kinetic models

The modified Gompertz equation was used to calculate the kinetic parameters for the CH_4 production process, according to Eq. 4 (Lay et al., 1999):

$$\textit{Eq. 4: } BMP(t) = BMP_{max} * exp\left\{-exp\left[\frac{R_{max} \cdot e}{BMP_{max}}(-t) + 1\right]\right\}$$

where BMP is the cumulative CH_4 production yield at time t, BMP_{max} is the maximum theoretical CH_4 production yield expressed per unit of TOC feed, R_{max} is the maximum CH_4 production rate, λ is the lag phase duration, t is the time, and "e" is the Neperian number.

The experimental data were fitted through Eq. 4 using the TableCurve 2D® software (v. 5.01, Systat Software Inc.) through least-squares non-linear regression. The coefficient of determination R^2 was used to evaluate the quality of data fitting for each experimental dataset. The time required for CH_4 production to attain 95% of the maximum production yield, referred to as $t95(CH_4)$, was derived from the Gompertz equation as follows (Eq. 5).

$$\textit{Eq. 5: } t_{95(CH4)} = \frac{BMP_{max}}{R_{max} \cdot e}\left(1 - ln\left(-ln0.95\right)\right) + \lambda$$

This parameter provides a measure of how fast the maximum CH_4 production is achieved and proves useful to compare, from a kinetic viewpoint, experimental conditions with different associated CH_4 generation yields.

7.2.5 Calculations

The BMP was expressed as litre methane produced per unit of mass of TOC added while hydrogen production yield (HPY) was expressed as a litre of hydrogen produced per unit of initial TOC present in SCW. In order to compare the performance with literature, BMP and HPY were expressed also per unit of mass of VS.

The theoretical BMP was calculated assuming a theoretical yield of 1026 L_{CH4} kg_{TOC}^{-1}, assuming that all the organic carbon is converted to biogas (55% methane) via the acetogenic pathway.

The specific gas production was converted to specific energy recovery (SER) considering one litre of initial SCW fed to the two configurations (one-stage AD and two-stage AD). The SER was calculated by considering the lower heating value of H_2 and CH_4, equal to 12.74 MJ Nm^{-3} and 35.16 MJ Nm^{-3}, respectively (De Gioannis et al., 2017). The overall SER of two-stage AD (H_2+CH_4) was calculated considering the TOC removal occurred in the first stage in order to compare the performance between the two stages

7.3 Results and discussion

7.3.1 BMP of raw SCW

The BMP of raw SCW were assayed using different F/M ratios (0.25, 0.5, 1, 2) to detect possible inhibitions (Figure 7.1). The BMPs were in the range of 814-909 L_{CH4} kg_{TOC}^{-1} (equals to 357-487 L_{CH4} kg_{VS}^{-1}) with the exception of the test performed with the F/M of 2, which was 413 L_{CH4} kg_{TOCi}^{-1} (244 L_{CH4} kg_{VS}^{-1}). All the tests showed BMP higher than the 87% of the theoretical one, except for test with the F/M of 2 (43% of the theoretical BMP).

Kinetic parameters and related statistics for raw SCW are presented in Table 7.1. Results suggested that no inhibition occurred adopting F/M of 0.25-1 whereas F/M of 2 results in 55% less biomethane production compared to the highest BMP reported at F/M of 0.25. Inhibition can be caused by acid accumulation as suggested by the lower pH value (7.7) measured after 30 days of test compared to the others (always higher than 8).

The results obtained are in the range of what reported in literature. BMP ranging from 270 to 600 L_{CH4} kg_{VS}^{-1} have been reported by AD using CW as substrate under mesophilic conditions (De Gioannis et al., 2014; Hagen et al., 2014; Humberto et al., 2017; Prazeres et al., 2012; Traversi et al., 2013). The maximum specific energy recovery for raw SCW was calculated in the range of 29-32 kJ L_{SCW}^{-1} (or 0.008-0.009 kWh L_{SCW}^{-1}).

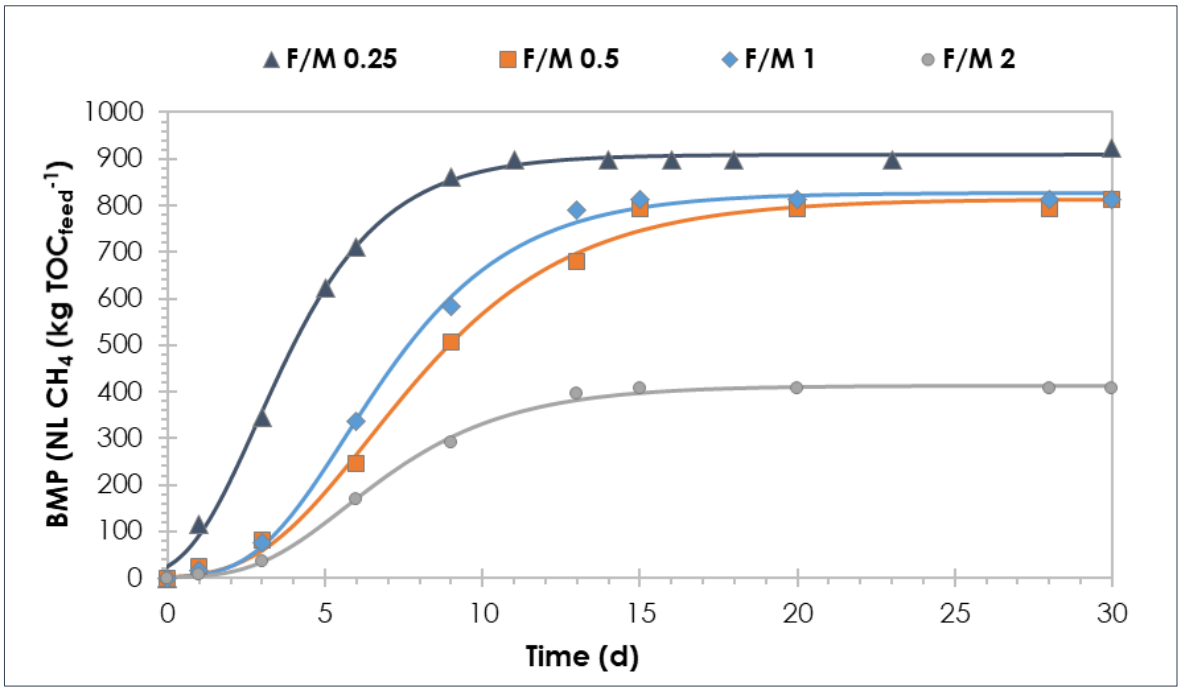


Figure 7.1. *BMP test on raw sheep cheese whey as a function of different F/M ratios.*

7.3.2 BMP of fermented SCW

The BMP of FSCW were assayed to investigate the possibility to couple a DF step with an AD step. The BMP obtained for FSCW obtained at different operating pH values, were in the range of 851-973 L_{CH4} kg_{TOC}^{-1} (equals to 524-603 L_{CH4} kg_{VS}^{-1}) with the exception of FSCW-7.5 which showed the lowest BMP of 379 L_{CH4} kg_{TOC}^{-1} (286 L_{CH4} kg_{VS}^{-1}) (Figure 7.2). BMP (FSCW-7.5 excluded) values are higher than those obtained for raw SCW with F/M of 0.5 (+ 5-19%).

The highest BMP was found using FSCW-5 as a substrate, and it was characterised also by the most extended lag phase of 11 days. A long lag phase could indicate that hydrolysis was the rate-limiting step in the AD process (Filer et al., 2019). In this specific case, FSCW-5 was characterised by a higher concentration of lactic acid (24 g L^{-1}, 48% of the total organic acids, as Cmol) due to the incomplete secondary fermentation of lactic acid into VFA (see Chapter 5). The BMP of FSCW-7 and FSCW-7.5 reached 70% of the theoretical value while the other reached valued higher than 90%. The lower R_{max} founded for FSCW-7 and FSCW-7.5 may be correlated with the presence of high amount of ammonia (700 and 760 mg L^{-1}, respectively) in those substrates. Despite ammonium is an essential nutrient for bacterial growth and ammonia concentration below 200 mg L^{-1} are suggested to be beneficial for the anaerobic digestion process(Chen et al., 2014), undesirably high concentration may inhibit methanogenesis ((Chen et al., 2014; Tian et al., 2019; Yenigün and Demirel, 2013). A wide range of ammonia concentration has been documented with the inhibitory total ammonia nitrogen concentration ranging from 1.7-14 N-NH_3 g L^{-1} (Chen et al., 2008). Furthermore, during BMP assays even higher ammonia concentration may have been reached due the breakdown of the whey proteins available in FSCW (in the range of 2-6 g L^{-1}).

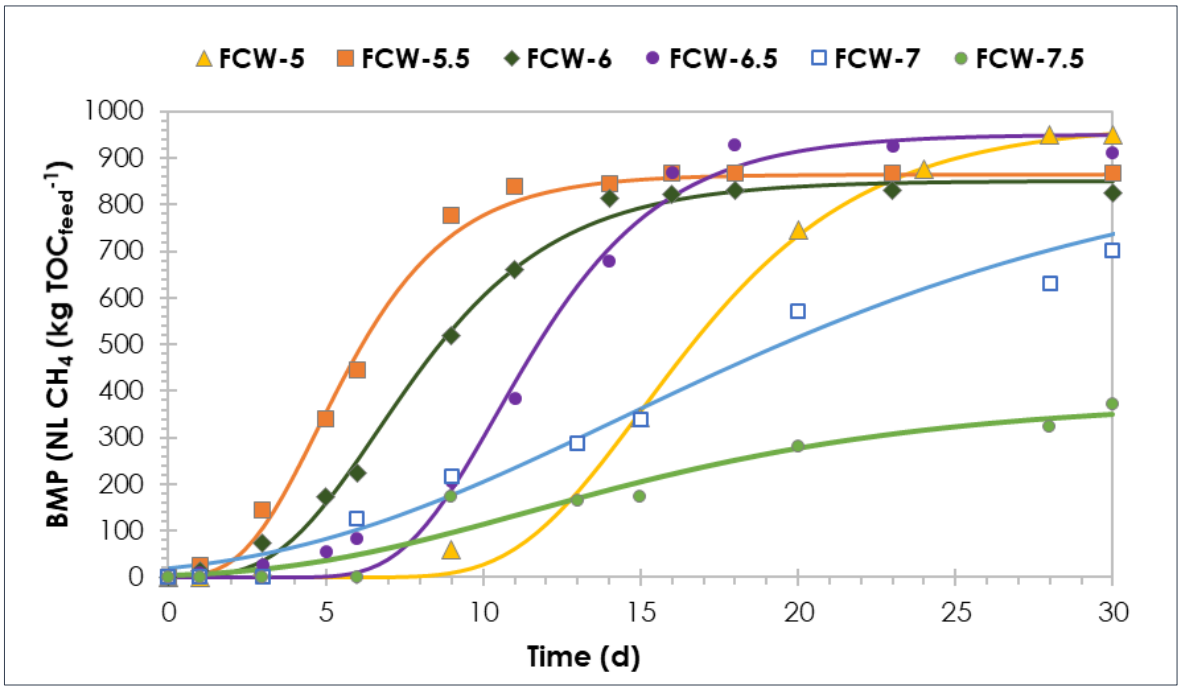


Figure 7.2. *BMP tests on fermented sheep cheese whey at different operating pH.*

Table 7.1. *Biomethane production kinetic parameters and related statistics for raw SCW and FSCW BMP assays.*

Substrates	BMP (L_{CH4} $kg_{TOCfeed}^{-1}$)	R_m (L_{CH4} $kg_{TOCfeed}^{-1}$ d^{-1})	λ d	R^2	t95 d
Raw SCW (F/M 0.25)	909.2	150.5	0.6	0.99	9.7
Raw SCW (F/M 0.5)	814.3	85.0	2.9	0.99	16.9
Raw SCW (F/M 1)	826.4	105.2	2.8	0.99	14.5
Raw SCW (F/M 2)	413.0	52.8	2.8	0.99	14.2
FSCW 5	973.3	90.6	11.1	0.99	26.8
FSCW 5.5	866.1	135.4	2.4	0.99	11.8
FSCW 6	851.0	99.3	3.47	0.99	16.7
FSCW 6.5	952.3	118.5	7.46	0.99	19.4
FSCW 7	923.1	32.25	3.78	0.97	35.2
FSCW 7.5	379.8	18.69	3.94	0.95	32.2

7.3.3 Overall specific energy recovery from SCW

Considering a combination of DF and AD (i.e., two-stage AD), the overall specific energy recovery starting from raw SCW was calculated in the range of 12-27 kJ L_{SCW}^{-1} (or 0.003-0.008 kWh L_{SCW}^{-1}) depending of the adopted pH value in the first stage (see Table 7.2). The TOC removal influences the energy recovery during the DF stage which was in the range of 22-26%. Despite the production of hydrogen during the DF stage, the overall energy recovery for a two-stage scenario is 7-15 % lower compared to a single AD stage (SER of 29.3 with F/M of 0.5). The use of a two-stage process may be still competitive in terms of process stability, but further studies are indeed required since BMP assays did not give this kind of information. In terms of carbon recovery, the biomethane produced in the two configurations accounted for the 27-40 % of the initial carbon content in the SCW. The two-stage configuration is characterised by lower carbon recovery compared to the one-stage.

Table 7.2. *Overall TOC removal and SER for SCW according to different process configurations: one- and a two-stage AD.*

Configuration	Carbon recovery as methane[(a)] (%Ci	SER stage I ($kJ_{H2}\ L_{SCW}^{-1}$)	SER stage II[(b)] ($kJ_{CH4}\ L_{SCW}^{-1}$)	Overall SER ($kJ_{H2+CH4}\ L_{SCW}^{-1}$)
Single-stage AD				
AD (F/M 0.25)	40	n.a.	1023	1023
AD (F/M 0.5)	36	n.a.	916	916
AD (F/M 1)	36	n.a.	930	930
AD (F/M 2)	18	n.a.	465	465
Two-stage DF+AD				
DF (pH 5) + AD (F/M 0.5)	32	36	816	852
DF (pH 5.5) + AD (F/M 0.5)	28	57	721	778
DF (pH 6) + AD (F/M 0.5)	27	67	708	775
DF (pH 6.5) + AD (F/M 0.5)	31	46	794	839
DF (pH 7) + AD (F/M 0.5)	31	43	808	851
DF (pH 7.5) + AD (F/M 0.5)	32	28	336	364

[(a)] Calculated as (%Ci) considering the C content of cumulated methane.
[(b)] Calculated considering the TOC removal during the first stage of DF (see Chapter 5, Table 1.1).
n.a. Not applicable

7.4 Conclusions

The results obtained in this chapter confirmed that the AD process might be used to exploit the energy content of SCW. One- and two-stage AD of SCW aimed at recovering methane or hydrogen plus methane were compared in terms of overall energy recovery.

The results showed that in a single-stage AD process, the specific energy recovery is 29-32 kJ per litre of SCW. Despite in literature a two-stage process (DF+AD) is widely reported as a possible solution for improvement in energy recovery, the results obtained in this study showed that the specific energy recovery is even less. The specific energy recovery in a two-stage process is 11.7-27.3 kJ per litre of SCW. A two-stage approach may still be competitive, considering the benefits in terms of process stability. Further studies are indeed necessary.

8 EXTRACTION OF VOLATILE FATTY ACIDS DURING DARK FERMENTATION OF CHEESE WHEY BY USING SILICONE MEMBRANES

8.1 Introduction

As pointed out in the previous chapters, DF is a promising process for the co-production of biohydrogen and a mixture of organic acids, mostly VFA, i.e. acetic, butyric, propionic acid. VFA can be used in chemical industry, as building block chemicals or also as a feedstock for further biological valorisation such as polyhydroxyalkanoates (PHA) production (Kleerebezem et al., 2015; Valentino et al., 2017). Recently, VFA production and recovery from renewable source by biological processes are getting more attention since the VFA production is traditionally based on non-renewable petrochemical sources (Atasoy et al., 2018).

The commercialisation of the VFA produced by DF requires cost-effective separation and recovery methods, which are nowadays considered a big challenge (Outram and Zhang, 2018). Separation and recovery methods can include in situ or ex-situ product recovery. In situ recovery is desirable since the continuous extraction of VFA from the fermentation broth has the further benefit of avoiding VFA accumulation and consequent inhibition of fermentative microorganisms. Moreover, it has been shown from several authors that VFA accumulation can significantly decrease the hydrogen yield in dark fermentation, with the butyric acid that has a more significant inhibitory effect than acetic on hydrogen-producing bacteria when the concentration exceeds 50 mM (Van Ginkel and Logan, 2005; Zhang et al., 2012). It is reasonable to hypothesize that the continuous extraction of butyric acids may lead to an improved biohydrogen production yields (Jones et al., 2017).

Several methods have been proposed for VFA recovery from DF effluents, i.e. nanofiltration, liquid-liquid extraction, adsorption, electrodialysis and membrane extraction. Among them, the use of membranes is well knowns in situ recovery method. The membrane extraction systems usually consist of two membranes, whereas the first membrane removes the acids from the fermentation broths with the use of a solvent, while the second is in contact with a sodium hydroxide solution (Outram and Zhang, 2018). The use of sodium hydroxide allows the recovery of the VFA in the form of sodium salt in the aqueous phase. Then, the use of a mineral acid in the downstream process is required to achieve the recovery of the products in the free acid form. This last step requires the use of another chemical and produce a mineral salt that will need to be disposed of in most of the cases (Outram and Zhang, 2018).

Among them, membrane extraction by silicone membrane and water as extractant has recently been proposed as a cost-effective solution (Outram and Zhang, 2018). Silicone is known to be permeable to organic substances and resistant to fouling problems. The use of water as an extractant avoid the use of expensive and non-environmentally friendly chemicals, making the process solvent-free.

In this chapter, the extraction of organic acids by silicone membrane will be evaluated during the DF of bovine cheese whey (BCW). Different operational conditions will be adopted, i.e. in-line and off-line separation, batch and continuous fermentations. The work here presented aimed to evaluate the feasibility to extract VFA from a real feedstock and to assess how the extraction affects the fermentation, especially in terms of bioproducts production (hydrogen and VFA) yields.

8.2 Materials and method

8.2.1 Design of experiments

The design of experiments involving extraction of organic acids from dark fermentan effluents of BCW is presented in Table 8.1. The following discussion was divided into three parts to facilitate understanding of the experiment: off-line extraction (I), in-line extraction in batch mode (II), in-line extraction in continuous mode (III).

Table 8.1. *Design of experiments involving extraction of organic acids from dark fermentation effluents of BCW.*

Experiments	Fermentation Operational mode	Conditions evaluated
I. Off-line extraction	UASB reactor in batch mode	substrate: BCW; acidified and unacidified
II. In-line extraction	UASB reactor in batch mode	substrate: BCW; extraction versus no extraction; pH 5 versus pH 4.5;
III. In-line extraction	UASB reactor in continuous mode	substrate: BCW; extraction versus no-extraction

8.2.2 Source of inoculum and pretreatment

In this study, the inoculum was digested sludge (DS) from a plant treating dairy effluents (Dairygold, Ireland). The DS had a total solid concentration of 66.0 g L^{-1} and a volatile solids concentration of 49.8 g L^{-1}. Heat pretreatment was done by heating thin tubes containing 5 mL of sludge in a heating plate at 90 °C for 15 minutes. The choice of DS as inoculum derived from previous screening tests which showed higher hydrogen yields (0.92 mol_{H2} $mol_{glucose\ eq.}^{-1}$) compared to activated sludge, heat-treated activated sludge and un-treated digested sludge (0.02, 0.17 and 0.65 mol_{H2} $mol_{glucose\ eq.}^{-1}$, respectively).

8.2.3 Cheese whey composition

Bovine cheese whey (BCW) from milk processing was collected from a dairy industry (Dairygold, Mitchelstown, Ireland). The composition at the moment of collection was reported in Table 8.2. The BCW was stored at -20 °C in 1 L bottles and defrost at 4 °C 48 hours before the utilisation, in order to minimise changes in its composition.

Table 8.2. *Main characterisation parameters of BCW (average value ± standard deviation).*

Parameter	Unit of measure	Bovine cheese whey
pH	-	6.42 ± 0.60
Total solids (TS)	g L^{-1}	69.98 ± 1.94
Volatile solids (VS)	g L^{-1}	64.04 ± 1.7
Soluble carbohydrates (sCarb)*	g L^{-1}	41.71 ± 0.91
Total COD	g L^{-1}	66.96 ± 4.80
Total DOC	g L^{-1}	20.82 ± 1.08
Soluble carbohydrates (sCarb)*	g L^{-1}	41.71 ± 0.91
Soluble proteins	g L^{-1}	2.3 ± 0.01
Lactate	mg L^{-1}	926 ± 1.5
Acetate	mg L^{-1}	262.14 ± 4.5
Propionate	mg L^{-1}	82.5 ± 1.8

8.2.4 Experimental set-ups

8.2.4.1 Off-line extraction of organic acids from fermented BCW

The experimental set-up adopted for the off-line extraction experiments is presented in Figure 8.1. Fermented BCW (FBCW) from previous batch test was used to assess the performance of organic acids extraction by using a silicone membrane. The FBCW was obtained from a batch DF assay (pH 5, 25°C, fermentation time of 7 days) and it was centrifuged (6000 rpm for 15 minutes) prior to the experiment. One glass bottle (fed tank) was filled with 600 mL of centrifuged FBCW and connected with the silicone membrane through a peristaltic pump operating at 10 mL min^{-1} (Masterflex L/S pump). The silicone membrane (internal diameter 2 mm, wall thickness 1 mm, length 3.5 m) was placed inside a second glass bottle filled with 600 mL of deionised water. Both bottles were mixed using a magnetic stirrer. The FBCW bottle and the tubes were flushed with nitrogen prior to the beginning of the experiment. The experiment takes 35 days at room temperature (25°C). At the 20^{th} day, the FBCW pH was corrected to the value of 3.2 using 2M HCl, and the deionised water of the draw solution was substituted with a fresh one.

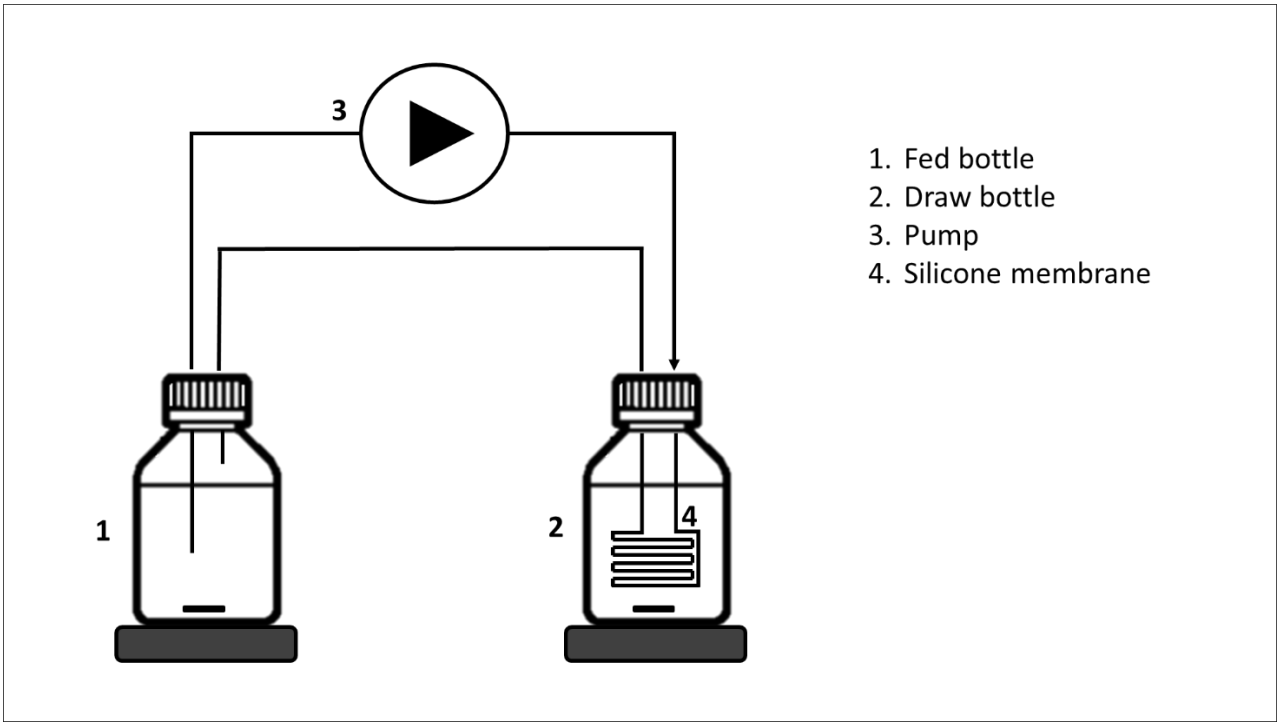


Figure 8.1. *Experimental set-up adopted for the off-line extraction of organic acids from fermented BCW.*

8.2.4.2 In-line extraction of organic acids from batch fermentation of BCW

The experimental set-up adopted for the in-line extraction from batch fermentation is presented in Figure 8.2. An upflow sludge blanket reactor (UASB) (working volume 800 mL) was used for this experiment. The temperature was kept at 37°C. Two different operative pH were adopted, 5 and 4.5, respectively and they were controlled by adding NaOH (3M).

The UASB reactor was fed with raw BCW and inoculated with heat-treated DS (4% in volume). The upflow velocity was set to be 1 m h^{-1} through a recirculation pump set at 21 mL min^{-1} (Masterflex L/S). The choice of this upflow velocity is derived from previous tests, aimed at targeting the optimum upflow velocity for hydrogen production in the range of 0.1-2 m h^{-1}. In detail, the upflow velocities of 1 and 2 m h^{-1} resulted in the hydrogen yield of 1 mol_{H2} $mol_{glucose\ eq.}^{-1}$, higher than the yield obtained at upflow velocities of 0.1 and 0.5 m h^{-1}. Thus, an upflow velocity of 1 m h^{-1} was selected considering that it is less energy demanding than 2 m h^{-1}.

The UASB reactor was connected with a silicone membrane (internal diameter 2 mm, wall thickness 1 mm, length 3.5 m) submerged in a glass bottle filled with 800 mL of deionised water, called draw bottle. The volume of the produced gas was collected from both the headspace UASB reactor and the draw bottle in 1 L gas bag and measured by the water displacement measurement method. The draw bottle was mixed using a magnetic stirrer.

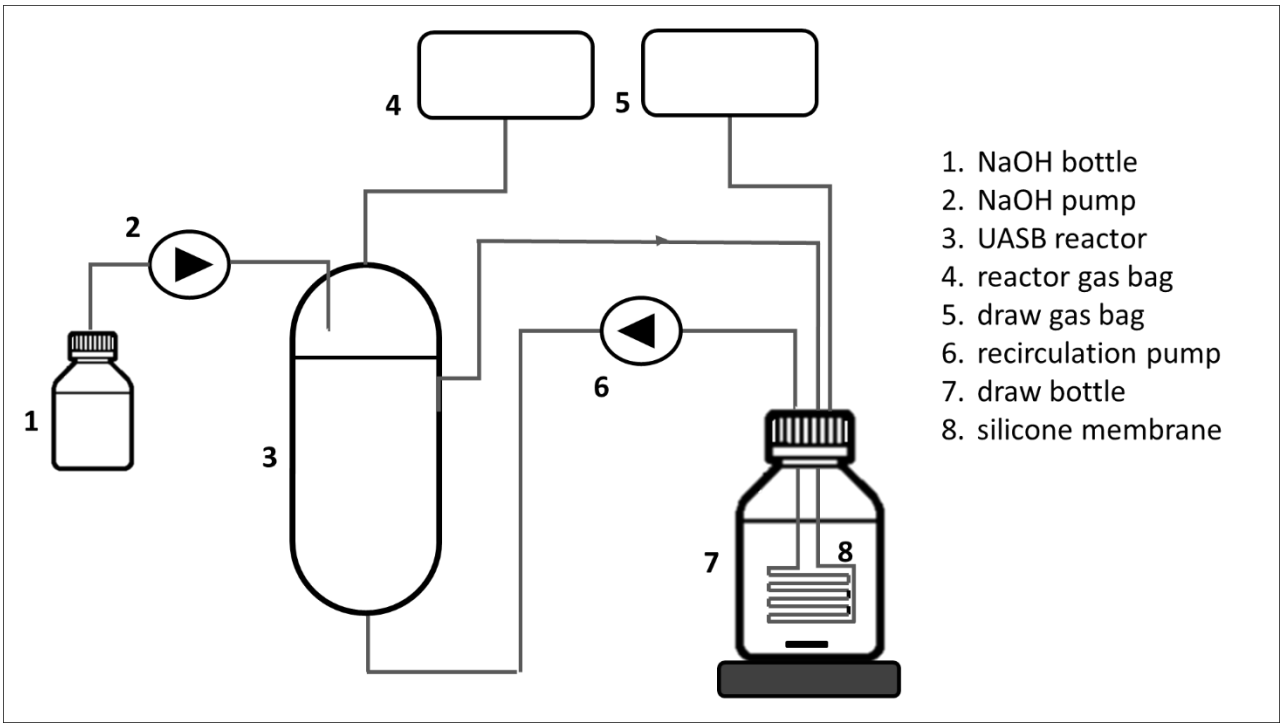


Figure 8.2. *Experimental set-up adopted for the in-line extraction of organic acids from batch fermentation of BCW.*

8.2.4.3 In-line extraction of organic acid from continuous fermentation of BCW

The experimental set-up adopted for the in-line extraction experiment from continuous DF is presented in Figure 8.3. An UASB reactor (working volume 800 mL) was used for this experiment. The temperature was kept at 37°C. Two different operative pH were adopted (5 and 4.5, respectively) controlled by adding NaOH (3 M). The reactor was fed with raw BCW and inoculated with heat-treated digested sludge (4% in volume). The reactor was started in batch mode and then shifted in continuous mode after 5 days. The raw BCW was kept at the temperature of 4° to prevent microbial activity. The upflow velocity was set to 1 m h^{-1} through a recirculation pump (Masterflex L/S), and the HRT was 1 day. The reactor was connected with a silicone membrane (internal diameter 2 mm, wall thickness 1 mm, length 3.5 m) submerged in a glass bottle filled with 800 mL of deionised water. The composition and the volume of the produced gas from UASB reactor and draw bottle was measured by using a continuous flowmeter and gas analyser (BlueSens). The draw bottle was mixed using a magnetic stirrer.

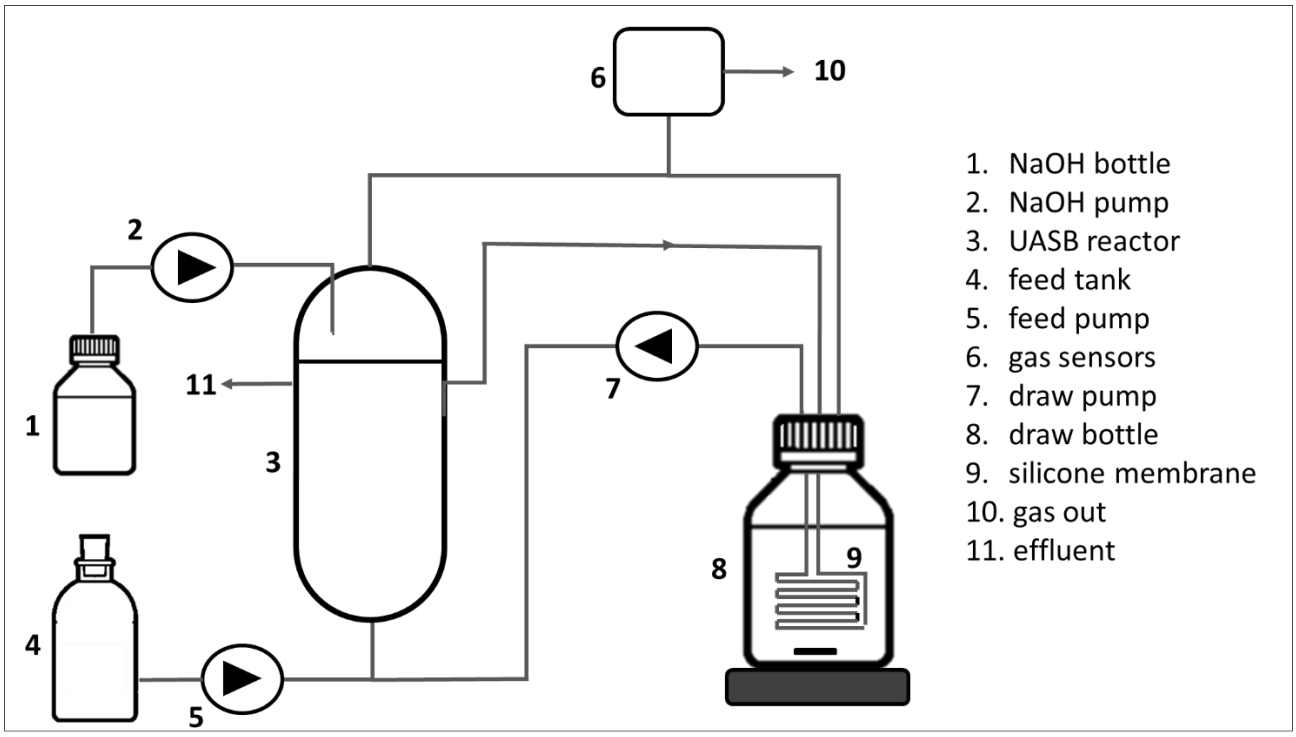


Figure 8.3. *Experimental set-up adopted for the in-line extraction of organic acids from continuous fermentation of BCW.*

8.2.5 Analytical methods

TS and VS were measured according to the APHA procedures (APHA, 1998). pH was measured with a pH probe (SlimTrode, Hamilton, Switzerland) connected to a pH controller (Cole Parmer 300, UK).

The concentration of sugars (lactose, glucose and galactose), organic acids (lactic, acetic, propionic, butyric, valeric and caproic acid) and ethanol were analysed by using liquid chromatography (Agilent Technology) equipped with a refractive index detector (RID) and an Hi-Plex H column. All analyses were conducted with isocratic elution (H_2PO_4 0.2% at 0.6 mL min^{-1}). The samples were centrifuged (6000 rpm, 10 minutes) and filtered using a 0.2 µm membrane.

Biogas production from the UASB batch experiments and the associated draw bottles was collected in 5 L gas bags and measured using the water displacement method. The biogas composition was analysed using a gas chromatograph (model 7890A, Agilent Technology) equipped with a thermal conductivity detector (TCD). Argon was the carried gas and oven, injector and detector were kept at 90, 90 and 200°C respectively. Biogas production and composition from the continuous experiments were measured continuously by using a gas sensor for CO_2 and H_2.

8.2.6 Calculations and kinetic models

The flux, J, is defined as the rate of mass transfer across the membrane (g m^{-2} h^{-1}) calculated by

$$J_i = \frac{1}{A}\frac{\Delta m_i}{\Delta t}$$

where Δm is the change in mass of species i (g), A is the membrane surface area for mass transfer (m^2), and Δt is the change in time (h). The average flux over the total experiment duration was used for the fermentation membrane extraction experiments.

The degree of extraction (E%) was calculated as follow:

$$E\% = \frac{m_i^{draw}}{m_i^{reactor} + m_i^{draw}}$$

where m_i is the mass of the compound *i* in the draw or in the reactor at the end of the experimentation.

The modified Gompertz equation was used to calculate the kinetic parameters for the H_2 production process and it was calculated as reported in Chapter 5.

8.3 Results and discussion

8.3.1 PART I: Off-line extraction of organic acids from fermented BCW

The extraction performance by using a silicone membrane was first evaluated using the FBCW obtained from previous batch DF tests. The tests involved the use of raw BCW as a substrate and pretreated DS as inoculum. The DF test was carried out in pH-controlled conditions (pH 5) and resulted in a hydrogen yield of 1.0 mol_{H2} $mol_{glucose}^{-1}$. The main metabolites present in the resulting FBCW were butyric (26 g L^{-1}), propionic (10 g L^{-1}), acetic (7 g L^{-1}) acid and a small amount of ethanol (1 g L^{-1}). The pH of the FBCW at the beginning of the test was 6.7 and did not change during the first part of the experiment (20 days). The concentration of some acids slightly decreased in the FBCW during the 20 days, but no organic acids were detected in the draw solution (Figure 8.4). Those changes may be associated with some residual microbial activity, considering that no effort was made to sterilise the FBCW. The pH in the draw solution decreased from the initial value of 5.5 to 3.9, suggesting that some H^+ were migrating through the silicone tube and considering that the deionised water has reduced or no buffer capacity. VFA can be present in an undissociated and dissociated form in function of the solution pH (Figure 8.5). The ratio between the dissociated and undissociated form is related to the pK_a value of each VFA. For instance, at pH of 6.7 the butyric acid (pk_a= 4.8) is almost all present in the dissociated form. However, the undissociated form, or free-form, is suggested to facilitate the migration through the silicone membrane (Outram and Zhang, 2018; Plácido and Zhang, 2018; Yesil et al., 2014). When the pH was adjusted to 3.4 on the day 20th, it was observed that the concentration of VFA in the FBCW decreased over time and simultaneously increased in the draw solution (Figure 8.4). The VFA (in the undissociated form) could successively diffuse through the non-porous silicone membrane, confirming the results obtained by Outram and Zhang (2018). During this second phase, the pH of the FBCW did not vary significantly. The butyric acid concentration in the FBCW (96% of which is in free-

form) decreased from 23 to 12 g L^{-1} in the following 15 days while increased up to 11 g L^{-1} in the draw solution. The fact that butyric acid was equally distributed between the draw solution and the FBCW (E% = 47%) suggests that it was near the equilibrium conditions. In the adopted batch conditions, the diffusion rate varies over time and significantly slowed down after 8 days since the driving force for mass transfer was provided by concentration gradient. The butyric acid showed a faster rate of mass transfer compared to acetic and propionic acid, which concentrations slowly increased in the draw up to 1 and 2 g L^{-1}, respectively. The total flow through the membrane was calculated equal to 0.257 g m^{-2} h^{-1} and was mainly represented by butyric acid (0.200 g m^{-2} h^{-1}) followed by propionic (0.042 g m^{-2} h^{-1}) and acetic acid (0.012 g m^{-2} h^{-1}). The overall mass transfer coefficient K_{OV} for butyric acid was 16.4 µm s^{-1}. The differences in the compound flow is related to the different affinity with the silicone membrane since bigger molecules (butyric) are more hydrophobic due to the longer chain length compared to smaller molecules (acetic). This consideration opens the possibility for a selective VFA recovery with a non-porous membrane. Based on the different hydrophobicity, the order of the selectivity is HBu > HPr > HAc, as stated by (Outram and Zhang, 2018) and confirmed by this study.

The results obtained in this first experiment suggest that it is possible to selectively recover the VFA present in the FBCW by using a silicone membrane and water as extractant. The main bottleneck is the requirement to acidify the FBCW to allow the presence of VFA in free-form and thus maximise the mass flow. This point may be feasible in a process scheme with off-line extraction after the DF.

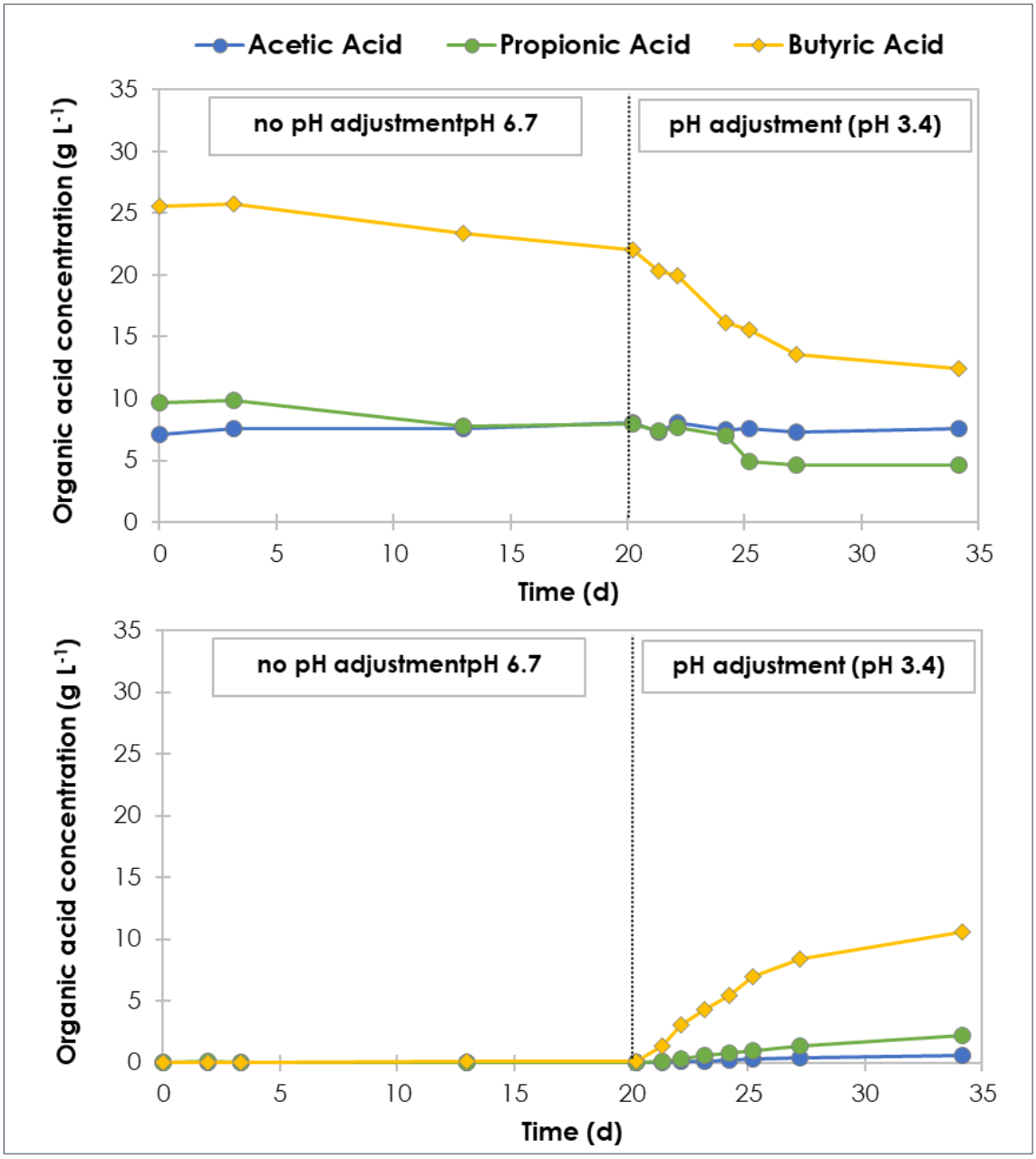


Figure 8.4. *Organic acids extraction test using silicone membrane: profile of organic acids in the fermented cheese whey (top figure) and in the extractant solution (bottom figure) as observed over time.*

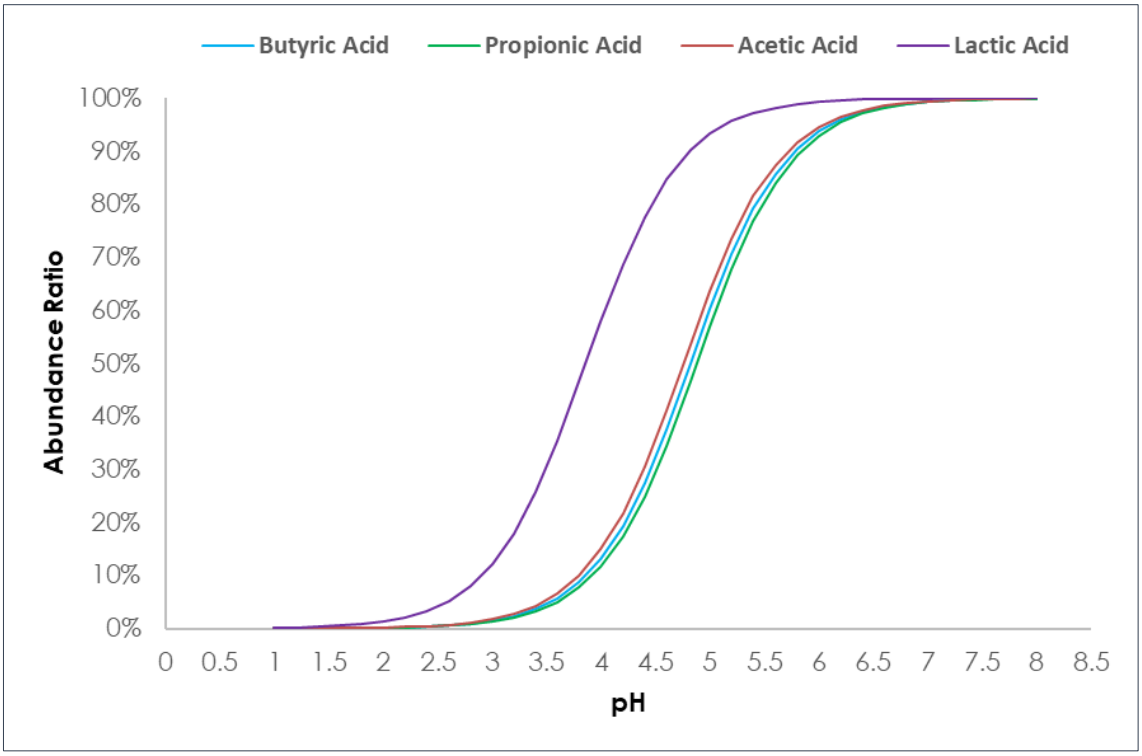


Figure 8.5. *Relationship between solution pH and dissociation status of organic acids.*

8.3.2 PART II: In-line extraction of organic acids from batch fermentation of BCW

The second part of the experiment aimed at coupling the extraction system with a batch DF test. Three different operative conditions were evaluated: operative pH 5 without extraction; operative pH of 5 coupled with extraction; operative pH 4.5 coupled with extraction.

The DF results in a consumption of soluble carbohydrates present in the BCW, mostly in the form of lactose, with the production of biogas (H_2 and CO_2) and a wide range of organic acids. The conversion of carbohydrates was up to 98% in all the tests despite the conditions adopted.

The evolution of the primary metabolites is presented in Figure 8.6 (a, c, e). A high amount of lactic acid characterised all the tests in the first days of the experiment (maximum values in the range 15-20 g L^{-1}) showing the occurrence of lactic fermentation among the possible biochemical pathways. Butyric acid was the other main metabolites found in the fermentation broth with a final concentration in the range of 10-20 g L^{-1}. While the lactic fermentation does not result in biohydrogen production, the production of butyric acid and acetic acid is associated with the production of biohydrogen (2 and 4 mol_{H2} $mol_{glucose}^{-1}$, respectively).

The hydrogen production yields for the three different configurations were presented in Figure 8.6b. The maximum hydrogen yields were 0.96 and 0.88 mol_{H2} $mol_{glucose}^{-1}$ obtained for the test performed at operative pH 5. The minimum hydrogen yield was found for the test performed at an operative pH 4.5, and it was equal to 0.49 mol_{H2} $mol_{glucose}^{-1}$ showing the detrimental effect of low pH values (i.e. < 4.5) on the dark fermentative

process. Despite the lower yield, the fermentation carried out at pH of 4.5 showed a faster production rate compared to test performed at a pH = 5 (Table 8.3).

Furthermore, the obtained yield obtained at pH=5 is slightly higher to what reported for DF of sheep cheese whey at pH of 6 (0.77 mol_{H2} $mol_{glucose}^{-1}$) in the previous chapter (Chapter 5 or Asunis et al. (2019)) and in line with the values reported in literature in similar conditions (Akhlaghi et al., 2017c; Antonopoulou et al., 2008; Ferreira Rosa et al., 2014b; Venetsaneas et al., 2009).

As clearly shown in Figure 8.6 (d, f), the concentration of metabolites increases in the draw solution over time. Moreover, in this test, the butyric acid exhibited a faster mass transfer rate compared to propionic and acetic acid. The final concentration in the draw solution of propionic acid did not exceed the value of 240 mg L^{-1} and no acetic acid was found in the draw solution. The silicone rubber membrane has proven to be selective since no lactic acid or other organic compound were found in the draw solution except for a small amount of ethanol (always below 200 mg L^{-1}). With a focus on butyric acid, the degree of extraction was more than doubled when the fermentation was carried out at pH = 4.5 instead of 5 (10 and 26% respectively). The butyric acid represents 78-85% of the total metabolites that crossed the membrane. In this case, the total flow was calculated to be 0.043 and 0.112 g m^{-2} h^{-1} for the butyric acid in the two cases. The pH is confirmed to be pivotal in the VFA diffusion through the membrane since the total flow for butyric acid in the previous off-line extraction test (at pH 3.3) was significantly higher (0.200 g m^{-2} h^{-1}) than the total flow obtained for the batch in-line extraction test. It is necessary to highlight the fact that at pH 5 only the 40% of the butyric acid is present in free form while decreasing the pH to the value of 4.5 the free-form fraction increase up to 68%. Small changes in operative pH may lead to higher VFA recovery.

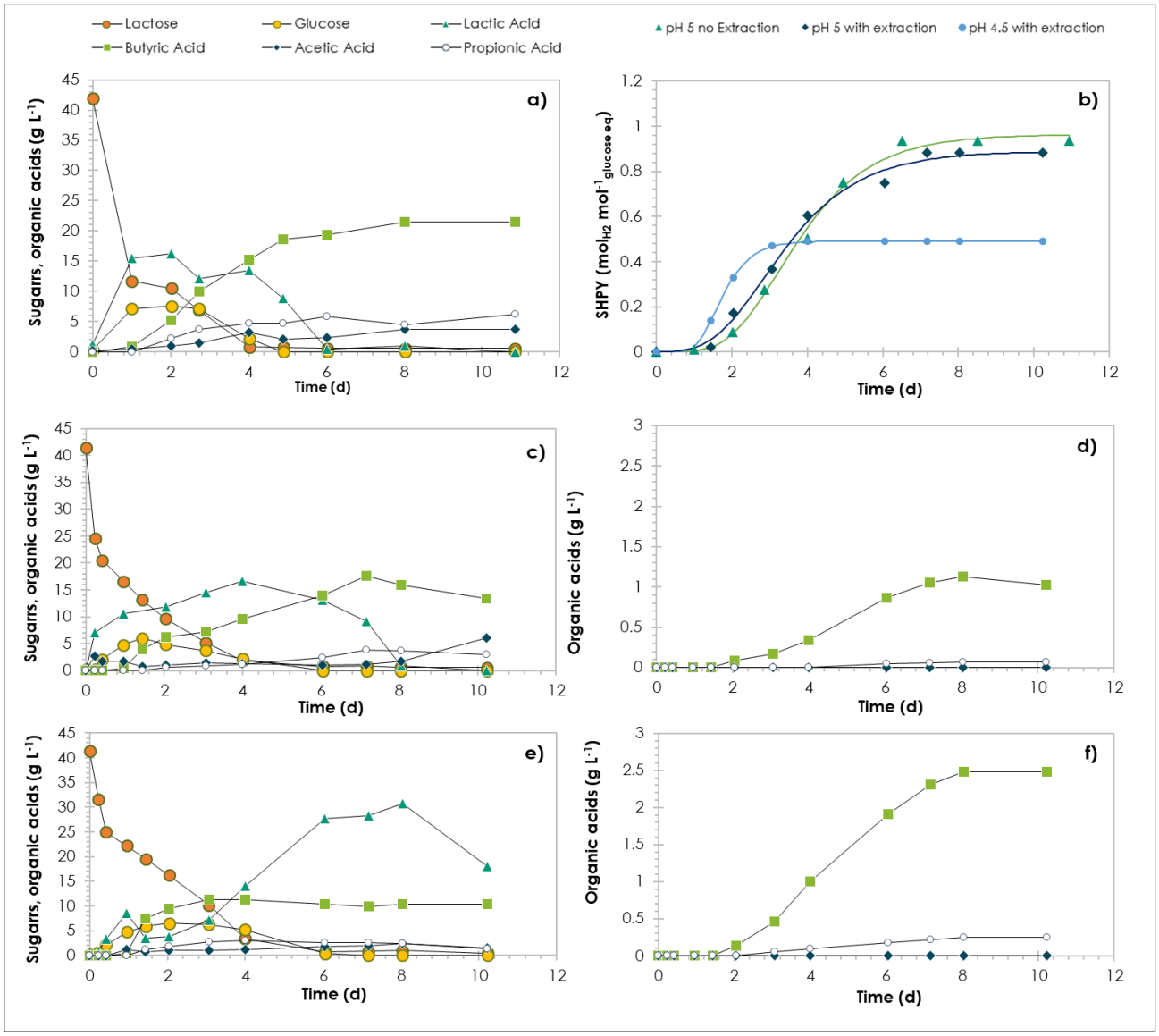


Figure 8.6. *Batch dark fermentation of BCW coupled with in-line extraction of organic acids: time evolution of lactose and organic acids for DF at pH 5 without extraction unit (a); cumulative H_2 production yields obtained for the batch tests at different conditions (pH 5 and 4.5) (b), the solid line represent the Gompertz curve derived by fitting the experimental data points; time evolution of sugars and organic acids in the reactor (c) and in the draw solution (d) for the DF at pH 5 with extraction unit; time evolution of metabolic products in the reactor (e) and in the draw solution (f) for the DF at pH 4.5 with extraction unit.*

Table 8.3. *Hydrogen production kinetic parameters and related statistics for cheese whey dark fermentation tests.*

		HPY_{max}	R_m	λ	$t_{95\text{-}H2}$
		$mol_{H2}\ mol_{glucose\ eq.}^{-1}$	$L_{H2}\ kg_{TOCi}^{-1}\ h^{-1}$	d	d
Cheese whey dark fermentation without extraction at pH 5					
$R^2 = 0.995$	Value	0.963	0.267	1.90	7.2
	Lower 95% conf. limit	0.904	0.211	1.53	
	Upper 95% conf. limit	1.022	0.323	2.26	
Cheese whey dark fermentation coupled with extraction at pH 5					
$R^2 = 0.993$	Value	0.888	0.238	1.47	7
	Lower 95% conf. limit	0.830	0.180	1.06	
	Upper 95% conf. limit	0.945	0.297	1.88	
Cheese whey dark fermentation coupled with extraction at pH 4.5					
$R^2 = 0.999$	Value	0.491	0.353	1.048	3.1
	Lower 95% conf. limit	0.488	0.337	1.017	
	Upper 95% conf. limit	0.494	0.369	1.080	

In addition to soluble compounds, the silicone rubber membrane also allows the migration of the gaseous compound produced during the fermentation, H_2 and CO_2. The permeability of the membrane to the gas made necessary to collect the gas from both the headspace reactor and draw solution. The migration of gas through the membrane was expected since previous authors suggest the use of silicone membrane to separate the biogas from the DF broth. Liang et al. (2002) reported that the use of silicone membrane reduces hydrogen partial pressure in the fermentation reactor and improves the hydrogen evolution rate by 10% and the hydrogen yield by 15%. This is due because H_2 and CO_2 removal from the fermentation broth may promote the bacteria's production of more hydrogen in thermodynamic equilibrium. In this study, the obtained results suggested that the presence of the extraction unit did not affect the fermentation process, as clearly shown from the analysis of the kinetic parameters in Table 8.3. The reason may be ascribed to the flow adopted for the recirculation (20 mL min^{-1}, necessary to obtain an upflow velocity of 1 m h^{-1}) lower than the flow adopted by Liang and co-worker (90-332 mL min^{-1}). Mass transfer coefficient of CO_2 and H_2 are directly proportional to the cross-flow velocity, and the values for CO_2 exceed those for H_2.

The diffusion of the gas and the volatile compounds could be improved by increasing the flow through the recirculation tubes, but this will also affect the upflow velocity of the reactor. Increasing upflow velocity of the reactors may have as a consequence an higher cost, but could also improve the overall fermentation process.

Interestingly, the batch DF test performed at the pH = 5 without extraction consumed more NaOH (51 g) than the test at pH = 5 with the extraction (30 g) to control the pH. The lower NaOH consumption may be advantageous in the perspective of real scale application.

The results showed that the in-line extraction of VFA from the fermentation broth is possible and did not affect the hydrogen production yields opening the possibility for a multi-product valorisation of BCW. Indeed,

the adopted condition allows the recovery of butyric acid, which is a valuable compound in the food industry as an additive or in the chemical industry as an intermediate. Decreasing the operative pH of the fermentation from 5 to 4.5 results in higher recovery of butyric acid (degree extraction from 10 to 26%) but halved the hydrogen yield (from 0.9 to 0.5 mol_{H2} $mol_{glucose}^{-1}$). An equilibrium between the optimal pH for the extraction and the biohydrogen production through DF is required. The adopted pH value plays a crucial role in the DF process, and the optimum pH widely ranged in 4.5-6 in function of substrates, inoculum and operational condition. However, some Authors investigated the possibility to perform DF in very acidic condition. (Mota et al., 2018) reported a stable, long-term production of hydrogen (average yield of 2.7 mol_{H2} $mol_{sucrose}^{-1}$) in DF of sucrose at pH of 2.7.

8.3.3 PART III: In-line extraction of organic acids from continuous fermentation of BCW

The third and last part of the experimental phase aimed at coupling the extraction system with the UASB reactor run in continuous mode. Two reactors were run in parallel, one of which was equipped with the extraction system. Both the reactors were fed with the same BCW from the fed tank at 4°C.

8.3.3.1 Evolution of the inflow over time

Despite the effort to keep the fed at 4°C to prevent microbiological activity, some changes in the fed were noticed. During the tests (42 days), the average pH of the influent was 4.47 ± 0.28, with the minimum value measured of 4.12 during the 23rd day (Figure 8.7). The freezing and the subsequent thawing did not significantly alter the total number of microorganisms (Tribst et al., 2019), thus keeping the fed at 4°C may lead the growth of some psychrophilic microorganisms present in the raw BCW. It is reasonable to assume that the fermentation of carbohydrates started inside the fed tank, altering the composition of the influent in terms of carbohydrate and lactic acid content. Partial fermentation of whey in the inlet was also reported in the work of Castelló et al. (2009), in which unsterilized whey was used as substrate. The concentration of total carbohydrates (lactose, glucose and galactose) and total organic acid (lactic and acetic acid) measured in the influent during this work is reported in Figure 8.7. The main organic acids found in the influent were lactic and acetic acid, suggesting the occurrence of a heterolactic pathway. The overall organic loading rate (OLR) was 16.6 ± 4.6 g_C L^{-1} d^{-1} when calculated considering the soluble organic carbon and 17.2 ± 8.6 g_{sCarbo} L^{-1} d^{-1} (equivalent to 20 $g_{sCarbo\text{-}COD}$ L^{-1} d^{-1}) when calculated considering the soluble carbohydrates (sCarbo). In this study, the variability of the fed composition can be considered a good representation of a real-scale application.

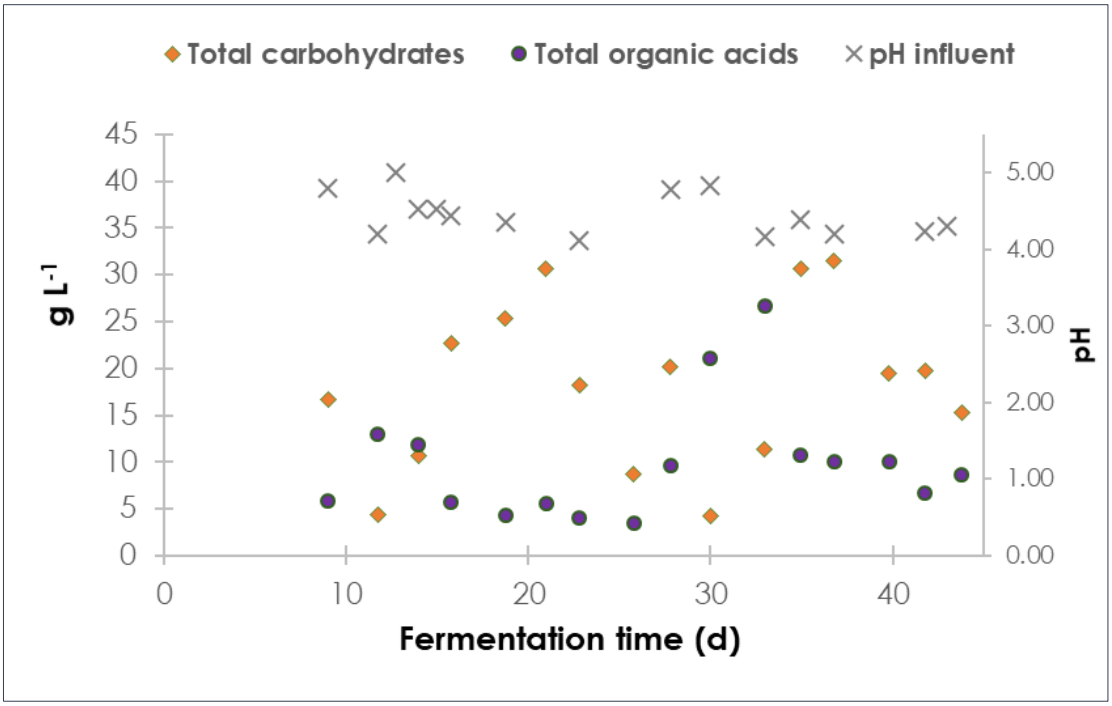


Figure 8.7. *Total organic acids, total carbohydrates and pH measured in the influent during the first 43 days of the III experimental phase (extraction vs. no extraction).*

8.3.3.2 Impact of extraction on dark fermentation of cheese whey

In the UASB reactor without the extraction unit, the carbohydrates were converted into a mix of organic acid with the production of hydrogen. The main metabolites were butyric acid, lactic and acetic acid. The butyric acid was confirmed to be the primary metabolite with an average concentration of 8.1 ± 3.6 g L^{-1} and a maximum value of 13.4 g L^{-1} during the 16th day. The presence of butyric acid and lactic acid suggest the overlapping of different metabolic pathways: lactic fermentation converts soluble carbohydrates into lactic acid while the butyric pathway directly converts carbohydrates in butyric acid and hydrogen (2 mol_{H2} $mol_{glucose}^{-1}$). The presence of acetate can be correlated with a heterolactic fermentation or with the production of hydrogen. In the first case, the glucose derived from lactose hydrolysis is converted into one mol of lactate and one mol of acetate while in the second case the glucose is converted into two mol of acetate with the production of 4 mol of hydrogen. The direct conversion of glucose into butyric acid and hydrogen is not the only possible metabolic pathway that explains the presence of such a metabolite. Indeed, lactate can be oxidised to butyrate in presence of acetate with a mechanism also referred as lactate cross-feeding (Blanco et al., 2019). According to this metabolic pathway, the consumption of one mol of lactate produces a mol of hydrogen. For instance, Blanco et al. (2019) estimated that hydrogen production from lactate and acetate could explain 75% of the total hydrogen volume produced during DF of synthetic cheese whey. Besides, since no propionic acid were detected during this test, the homoacetogenic pathway (i.e. hydrogen consumption) can be excluded. The average hydrogen production yield for the reactor without

extraction was 0.63 ± 0.37 mol_{H2} $mol_{glucose\ eq.\ consumed}^{-1}$, lower than the value of 0.88-0.96 mol_{H2} $mol_{glucose\ eq.}^{-1}$ reported in the previous paragraph with the UASB reactor run in batch mode. The average hydrogen productivity was 1.1 ± 0.6 L_{H2} L^{-1} d^{-1} with a peak of 1.7 L_{H2} L^{-1} d^{-1} in correspondence of the maximum butyric acid concentration in the reactor. No methane was detected among the biogas produced due to the combined effect of short HRT (1 d) and low pH (5) on methanogenic activity.

The UASB reactor equipped with the extraction unit showed similar performance compared to the UASB reactor without extraction. Figure 8.8 showed the evolution of soluble carbohydrates and total organic acids during the test. The average concentration of butyric acid in the reactor equipped with the extraction was 5.4 ± 2.9 g L^{-1} with a maximum value of 10.6 g L^{-1} during the 30th day. Also, in this case, the production of butyric acid was accompanied by hydrogen production. The average hydrogen production yield was lower compared to the reactor without extraction, and it was 0.58 ± 0.38 mol_{H2} $mol_{glucose\ eq.\ consumed}^{-1}$. The average hydrogen productivity was 0.9 ± 0.5 L_{H2} L^{-1} d^{-1} with a peak of 1.9 L_{H2} L^{-1} d^{-1} during the 36th day.

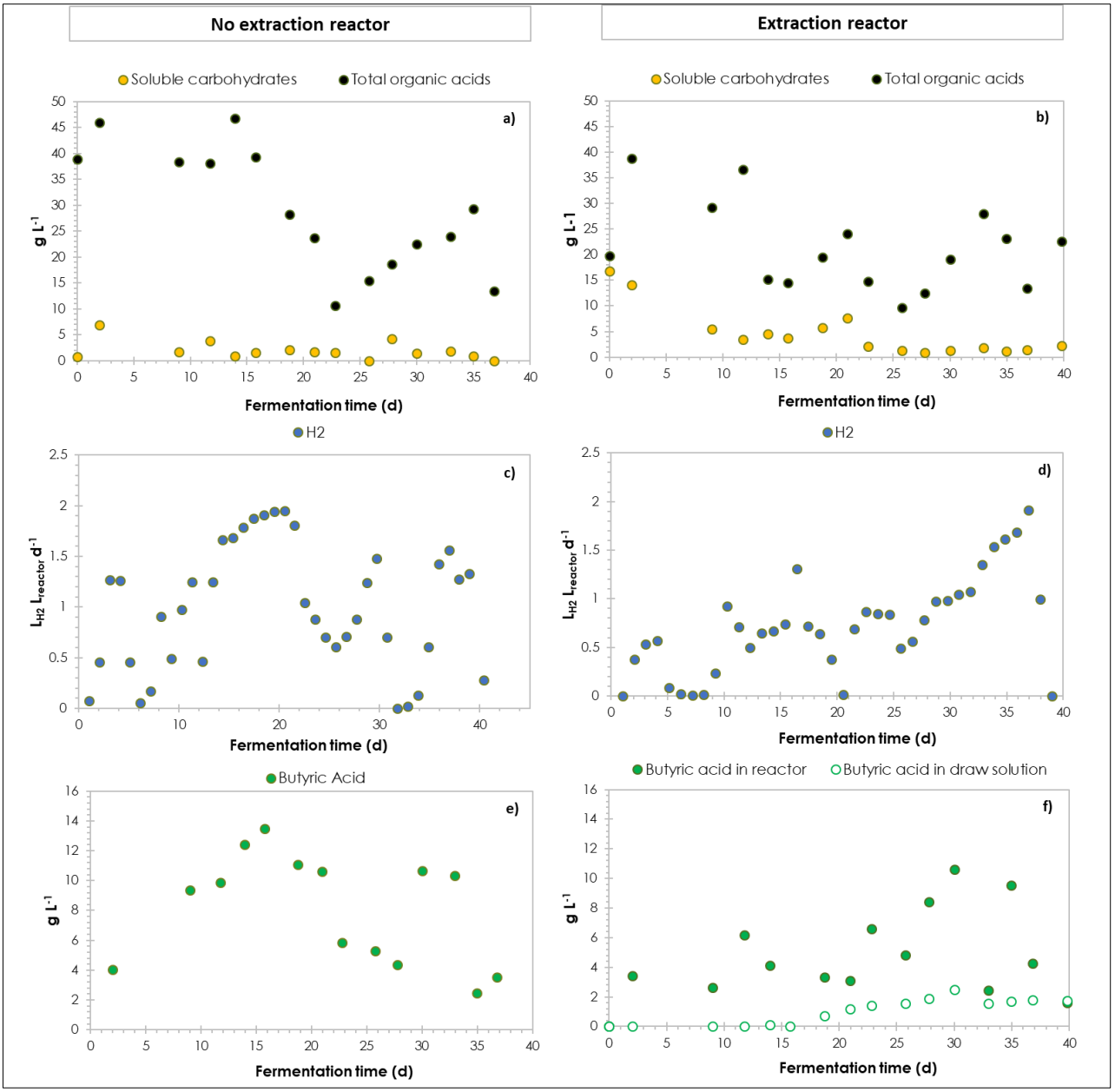


Figure 8.8. *Evolution of soluble carbohydrates, total organic acid, hydrogen productivities and butyric acid concentration for the reactor without extraction (left column) and the reactor with extraction (right column). For the latter, the butyric concentration in the draw solution is also reported.*

From the point of view of hydrogen production, the comparison between the two UASBS reactors suggests that the presence of the extraction unit did not affect significantly the fermentative process confirming the results obtained with the tests performed in batch mode. The use of UASB reactor for DF of cheese whey substrates is not common in literature. Castelló et al. (2009) reported that UASB reactor (HRT 12 h, OLR 20 g_{COD} L^{-1} d^{-1}, 30°C) can be used for hydrogen production from unsterilized whey but with a lower hydrogen productivity (average 0.122 L_{H2} L^{-1} d^{-1}) due to the difficult to suppress the methanogenic and homoacetogenic activity. Recently, the use of anaerobic structured-bed reactor (ASTBR) has been proposed as a novel reactor configuration for dark fermentation of cheese whey (Blanco et al., 2019). The ASTBR (HRT

1 d, OLR 24 g L^{-1} d^{-1}, 25°C) had an average volumetric productivity of 1.6 ± 0.7 L_{H2} L^{-1} d^{-1} and average hydrogen yield of 1.4 ± 0.7 mol_{H2} $mol_{lactose\ consumed}^{-1}$ equivalent to 0.7 mol_{H2} $mol_{glucose\ consumed}^{-1}$ (Blanco et al., 2019). The latter value is slightly lower than the value obtained in this study. The use of an anaerobic fluidized bed reactor (OLR 5 g_{COD} L^{-1} d^{-1}, 30°C) to treat cheese whey powder has been reported by other author with hydrogen yields (0.6-0.7 mol_{H2} $mol_{glucose}^{-1}$) in line with those reported in this study (Ferreira Rosa et al., 2014a, 2014b). The highest yield for hydrogen production from cheese whey through anaerobic fluidised bed reactor has been reported in the work of (Ottaviano et al., 2017) and it was equal to 1.8 mol_{H2} $mol_{glucose\ eq.}^{-1.}$

8.3.3.3 Recovery of butyric acid through the silicone membrane

The silicone membrane proved to be selective with respect to the VFA produced during the dark fermentation test. Indeed, more than 90% of the VFA founded in the draw solution was butyric acid. The evolution of butyric acid over time in the draw solution and the reactor is presented in Figure 8.8f. The concentration of butyric acid increased over time reaching the maximum value of 2.5 g L^{-1} on the 30th day, the same day in which the highest concentration value has been measured in the reactor (10.6 g L^{-1}). As seen previously, VFA can migrate through the silicone membrane only in the undissociated form. The pK_a for butyric acid is 4.8, and this means that at the operative pH of 5, only the 40% is present in undissociated form, strongly limiting the amount of butyric acid that can cross the membrane. Indeed, the highest measured concentration for butyric acid in the reactor (day 30) corresponds to 4.24 g L^{-1} of butyric acid in undissociated form. In this case, the equilibrium concentration between the reactor and the draw solution is equal to 2.1 g L^{-1}, which is close to the highest concentration measured in the draw solution during the 30th day. The fact that the day after (day 31) the concentration of butyric acid in the draw solution slightly decreased is probably associated with the decreased concentration of undissociated butyric acid in the reactor. The migration is driven by a gradient concentration of undissociated butyric acid, so also the migration from the draw solution to the reactor is possible. The other VFA able to cross the membrane was acetic acid, but in meagre amount (always lower than 180 mg L^{-1}) compared to butyric acid. This can be explained considering that concentration in the reactor widely ranges between 0.7-9.0 g L^{-1} and because the selectivity of the membrane is based on hydrophobicity and smaller molecules like acetic acid are less hydrophobic than butyric acid.

8.4 Conclusions

The results showed that co-production of hydrogen and the recovery of a valuable organic acid like butyric acid is possible through DF of cheese whey and extraction with silicone membrane. The produced VFA could be effectively and selectively extracted from the fermentation broth by using a silicone membrane. In an off-line application, adjustment of pH to lower value is necessary to maximise the total flow through the silicone membrane and the consequent total VFA recovery (E% = 34).

In general, the presence of the extraction unit did not strongly affect the biohydrogen yield when coupling batch DF with an in-line extraction. In this case, the operative pH plays an even more crucial role in the hydrogen production yield and butyric acid recovery compared to off-line application. On the one hand, optimum biohydrogen yields (~0.8-0.9 mol_{H2} $mol_{glucose}^{-1}$) are obtained at pH of 5 in batch DF of BCW. On the other hand, at pH of 5 only 40% of the butyric acid (pK_a 4.8) can effectively cross the membrane, reducing the maximum recovery potential. Decreasing the operative pH from 5 to 4.5 lead to higher recovery of butyric acids (from 10 to 26%) but halved the hydrogen yield (from 0.9 to 0.5 mol_{H2} $mol_{glucose}^{-1}$).

Coupling the silicone membrane system with continuous DF of CW at pH 5 results in promising hydrogen productivity (0.9 ± 0.5 L_{H2} L^{-1} d^{-1}) and yield (0.58 mol_{H2} $mol_{glucose\ eq.\ consumed}^{-1}$) though slightly lower than the continuous DF without extraction (1.1 ± 0.6 L_{H2} L^{-1} d^{-1} and 0.62 mol_{H2} $mol_{glucose\ eq.\ consumed}^{-1}$, respectively).

Further studies are required with the aim of optimisation of the extraction system and better understanding of the different affinity of VFA for the silicone at different operational condition (pH, temperature, cross flows). In addition, the final fermented cheese whey, in both batch and continuous experiments, after the extraction still contains organic acid and other valuable compound that could be further valorised in integrated systems such as anaerobic digestion or PHA production.

The research work presented in this chapter has been performed as a visiting PhD student at the National University of Ireland - Galway in the framework of the activities of **SFI Research Professorship investigating Innovative Energy Technologies for Biofuels, Bioenergy & a Sustainable Irish Bioeconomy (IETSBIO3).**

9 FINAL DISCUSSION AND FUTURE PERSPECTIVES

9.1 Introduction

The interest raised by the concept of waste biorefinery is expected to increase in the upcoming years since the possibility of implementing different valorization schemes can support the ambitious transition from a linear, fossil-based and environmentally unsustainable economic model toward a circular, bio-based and sustainable economic one, as also set by the EU for the next future.

The potential inherent a waste biorefinery is case-specific, mostly depending on technical, economic and environmental considerations. A modern and flexible waste biorefinery should be tailored on the local necessities and requirements, i.e. environmental issues associated to industrial processes or biowaste management, energy and material supply, competitiveness of local activities and job market.

In some EU regions such as Sardinia (Italy), the sheep dairy sector is largely recognised as critical and needs an effective innovation process to tackle the deep structural crisis that is experiencing during the last years. Such an innovation may be based on a proper integration of both economic and environmental aspects.

Management of sheep cheese whey (SCW) has always been a matter of concern for diaries, both economically and environmentally.

In the perspective of a SCW management strategy based on valorisation, the results presented in this thesis show that SCW may be an outstanding substrate for a combination of biochemical processes based on dark fermentation as pivotal step. Since several high-value bioproducts such as hydrogen or PHA can be recovered from SCW, this final chapter presents and discuss some possible biorefinery schemes based on the product yields obtained and consistent with the actual EU policies framework and the evolving bioproducts market demand. In this respect, the Biorefinery Complexity Index (BCI) and the Biorefinery complexity profile (BCP) as proposed by IEA can be used to compare the different biorefinery options in terms of development potential and overall TRLs.

Finally, a specific proposal for the Sardinian sheep dairy sector is presented and discussed.

9.2 Resource recovery from SCW through a fermentation-based biorefinery approach

Assumed that, as already underlined, dark fermentation always plays a pivotal role due to the ability to hydrolyse the remarkable sugar content of SCW and convert it into organic acids by using a mixed microbial culture, two main biorefinery scenarios have been considered: (1) energy-driven biorefineries aimed at recovering renewable energy in the form of biofuels, like methane and hydrogen; (2) material-driven biorefineries aimed at recovering high value biochemicals such as lactic acid or biopolymer like PLA or PHA.

Each scenario may have distinct process pathways, characterised by different levels of complexity and TRLs and driven by choice of the specific final product. The decision of the final product can be influenced by both the actual and forecasted market demand and prices. Due to the current low price of fossil fuels, the market price of transportation biofuels is lower compared to the chemicals and materials; however, the market volume is significantly higher. Among the other framework conditions that should be considered there are also the regulatory conditions, i.e. the renewable energy directive and the single-use plastic directive.

The graphic summaries of the different options reported in the following were developed according to what suggested by the IEA Bioenergy Task 42 and described in Cherubini and Jungmeier (2009).

Further details about biorefinery classification and marketable bioproduct are reported in Chapter 1.

9.2.1 Energy-driven biorefineries

Figure 9.1 illustrates some possible process pathways within the first scenario aimed at the recovery of energy from SCW.

The least complicated option includes the traditional single-stage AD for biogas production (Figure 9.1a), which can be combusted in a combined heat and power (CHP) station to produce electricity and heat for the same dairy plant or for directly supplying the national electricity grid or district heating systems. In the case of upgrade of biogas to biomethane (Figure 9.1b), the latter can be fed directly to the natural gas grid or even used a transportation biofuel within the same dairy supply chain, i.e. milk deliveries to the dairies performed with biomethane-powered trucks. The specific production of biomethane achievable is 26-29 litre of CH_4 per litre of SCW (equals to 0.9-1.0 MJ L_{SCW}^{-1}). Single-stage AD is a well-established technology, and the number of real-scale applications to the dairy industry residues are increasing. The case of the First Milk's Lake District creamery reported in Chapter 3 is a remarkable example of full scale renewable bioenergy production from dairy wastes (TRL of 9).

However, in one-stage AD, high CH_4 yields and process stability may be challenging to achieve and maintain due to excessive acidification which may occur during lactose fermentation. A well-known strategy aimed at limiting the risks of inhibition of the methanogenic bacteria consists in splitting the anaerobic digestion process into two phases to be performed in two different optimised reactors (hydrolysis and dark fermentation in the first reactor and methanogenesis in the second one). While the two-stage AD has been applied so far aiming at an optimised methane production, the present study suggests that a proper selection of the first stage could make possible also the combined recovery of biohydrogen and biomethane (2-5 L_{H2} L_{SCW}^{-1} + 20-23 L_{CH4} L_{SCW}^{-1}). The overall energy recovery considering both biohydrogen and biomethane would be 0.7-0.9 MJ L_{SCW}^{-1}, lower than that observed for the one-stage AD process. However, it is worth to underline that the issue deserves more studies since methane production was assessed performing simple BMP tests and, moreover, other studies pointed out the possibility to increase by 10-15% the overall energy recovery

achievable with a two-stage process as compared to the single-stage AD (De Gioannis et al., 2017). Ultimately, it is reasonable to admit that the methodology adopted in this study to compare the performance of the single and double-stage systems was not adequate and that, conversely, the two-stage process can lead to greater energy recovery, either in the form of a more stable and high production of biomethane or by virtue of the combined production of biohydrogen and biomethane (Figure 9.1c).

As regards the use of the recovered biohydrogen, it could be used as it is, or as a source of reducing equivalents to convert to further biomethane the CO_2 deriving from the possible upgrade of the biogas, or mixed with methane to produce hythane (Figure 9.1d). The direct use of hydrogen as a biofuel (Figure 9.1e) may represent a long-term solution since the time horizon of extensive implementation of the use of hydrogen as an energy carrier is still difficult to predict. The use of biohythane may represent a possible middle-term solution. Some studies suggest that biohythane is a more efficient fuel and source of less emissions (NOx) as compared to methane (Porpatham et al., 2007).

Finally, the use of liquid digestate as soil fertiliser is worth to be considered.

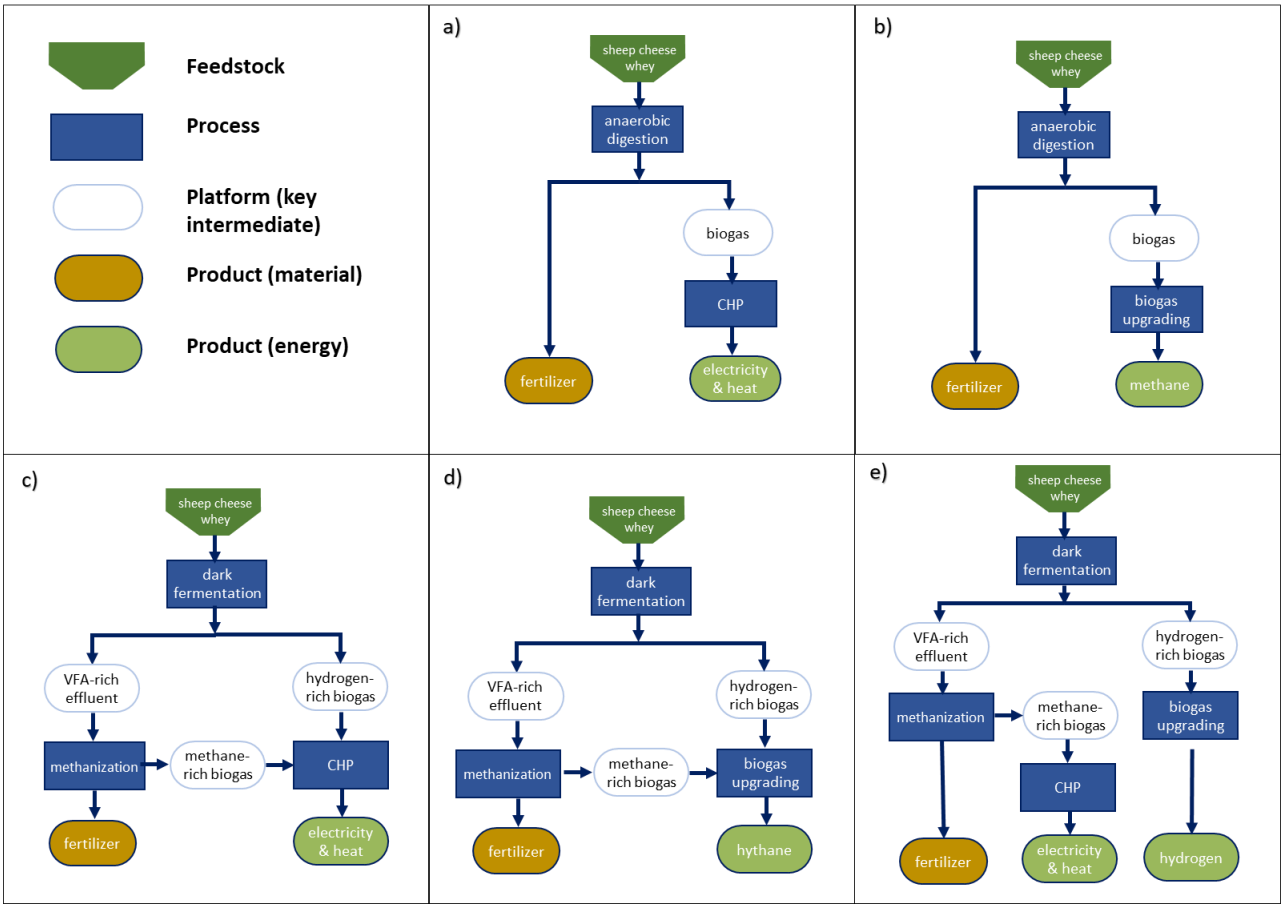


Figure 9.1. *Process pathways for the fermentation-based scenario aimed mainly at energy recovery from sheep cheese whey: one-stage AD for the production of biogas (a) or methane (b) and additional fertilizer; two-stage AD for the production of biogas (c), hythane (d) or hydrogen (e) and additional fertilizer.*

9.2.2 Material-driven biorefineries

9.2.2.1 Recovery of lactic acid and PLA biopolymer

Figure 9.2 illustrates some possible process pathways within the second scenario aimed at the recovery of high value chemicals and bioproduct such a lactic acid and biopolymer PLA from SCW.

The DF stage is managed so to optimise the lactic acid production, in particular by adopting an operating pH of 6 and a fermentation time of 45 h. No pre-treatment of SCW is required, and no inoculum is necessary since it can be assumed that SCW has enough microorganism as well as nutrients to allow the hydrolysis of the lactose and subsequent fermentation to lactic acid. After an extraction process (neutralisation and acidulation or solvent extraction), lactic acid, which is a high value marketable product, is obtained from the fermentation broth (Figure 9.2f). Alternatively, the lactic acid may be further processed to produce lactide, which is then polymerised to obtain PLA (Figure 9.2h).

During the process, some side-streams are produced. The residual biomass from the fermentation stage can be digested in an AD reactor to produce biogas, which can be used to produce electricity&heat in a CHP plant (Figure 9.2g and Figure 9.2h). During the lactic acid separation with calcium carbonate, gypsum is produced and need to be disposed.

Both lactic acid and PLA are characterized by a remarkable market demand, as discussed in Chapter 1. The BBI-JU project *AgriChemWhey* aims to build a flagship biorefinery (TRL 8) for the production of cost-competitive and sustainable lactic acid from dairy residues. The project also aims to encourage industrial symbiosis inspiring the creation of new value chains and valorising side streams with local partners, i.e. the use of gypsum, calcium phosphate and fermentation residues for agriculture and human nutrition.

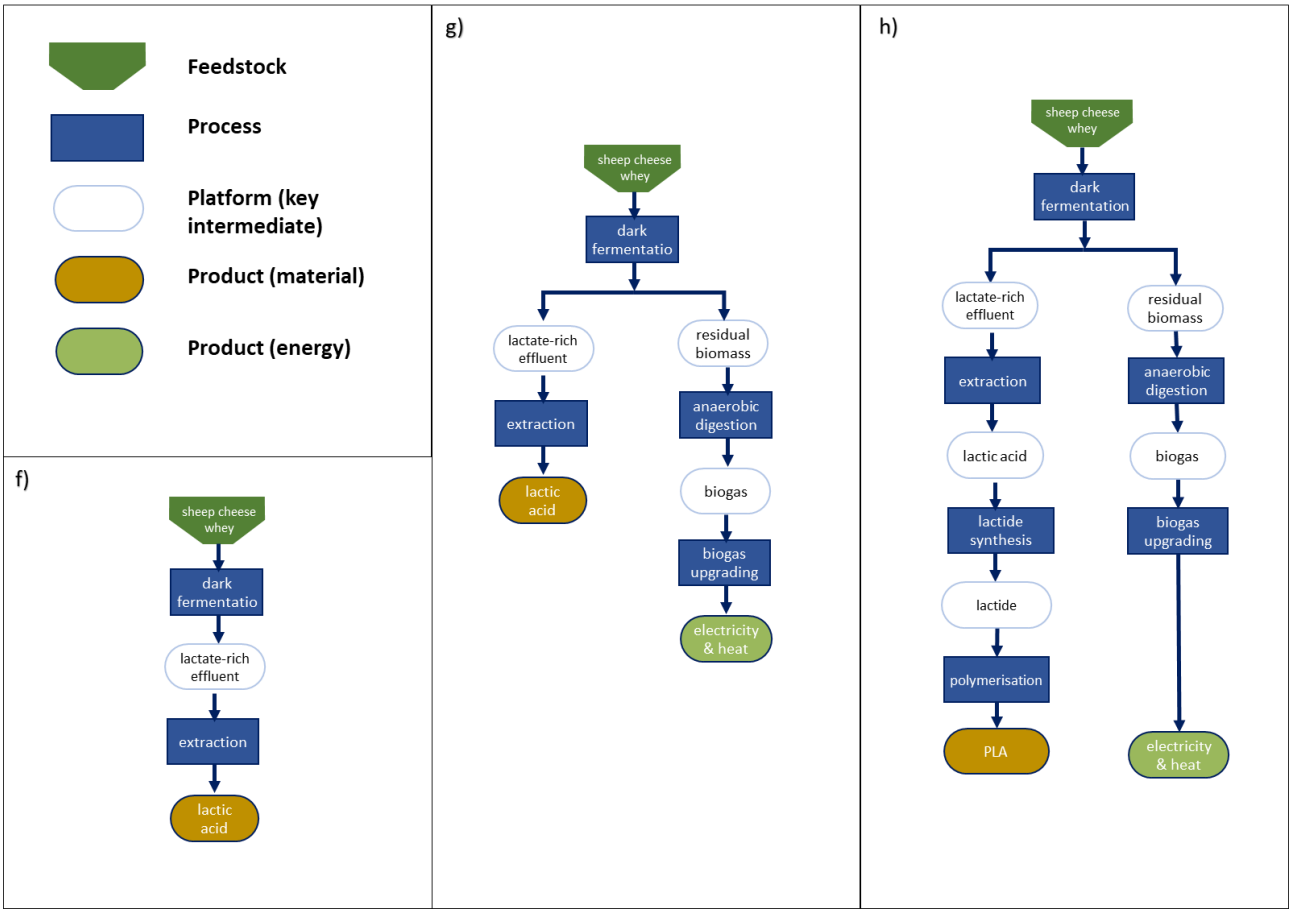


Figure 9.2. *Process pathways for the fermentation-based scenario aimed mainly at material recovery from sheep cheese whey: production of lactic acid (f); production of lactic acid, additional energy (g); production of PLA biopolymer, additional energy (h).*

9.2.2.2 Recovery of PHA biopolymer

Figure 9.3 illustrates some possible process pathways within the second scenario aimed at the recovery of biopolymer PHA from SCW.

In this case, the DF stage is oriented to producing a pool of VFA, which are PHA precursors (Figure 9.3l). The composition of the final PHA polymer can be addressed by proper operation of the fermentation stage. Indeed, the presence of propionic acid affects the HV content in the final polymer. The different PHA composition results in different applications and uses and, in turn, different market values.

After extraction, PHA can be prepared and sold as raw materials. Specific tailored-solutions can be investigated within the same dairy industry supply chain, i.e. the use of PHA-based packaging for dairy products, production of PHA-based fertiliser for improving the characteristic of the soil used for the sheep meadows. As an example, the European funded project WHEYPACK aimed to demonstrate the application of PHA-based food packaging to dairy products and demonstrate that the manufacturing of a PHA-based food packaging from whey has a lower carbon footprint than current manufacturing processes of PP-based ones. Similarly, the EU-funded project YPACK aims to scale up and validate novel PHA-based food packaging

solutions with active properties and a passive barrier to reduce food waste by prolonging food shelf life. As already mentioned for PLA, also PHA is currently characterised by an increasing demand, which is driving the applied research in the topic of PHA production from biowaste (TRL 5-6).

Within the proposed scenario, PHA production can be accompanied by energy recovery phases, both through anaerobic digestion of the residual fermentative biomass, and through the recovery of biohydrogen during the fermentation stage (Figure 9.3m). The recovered energy may be used to sustain the energy needs of the dairy system or as transportation fuels, the latter requiring biogas upgrading to biomethane.

Finally, the digestate from AD can be used as soil fertiliser.

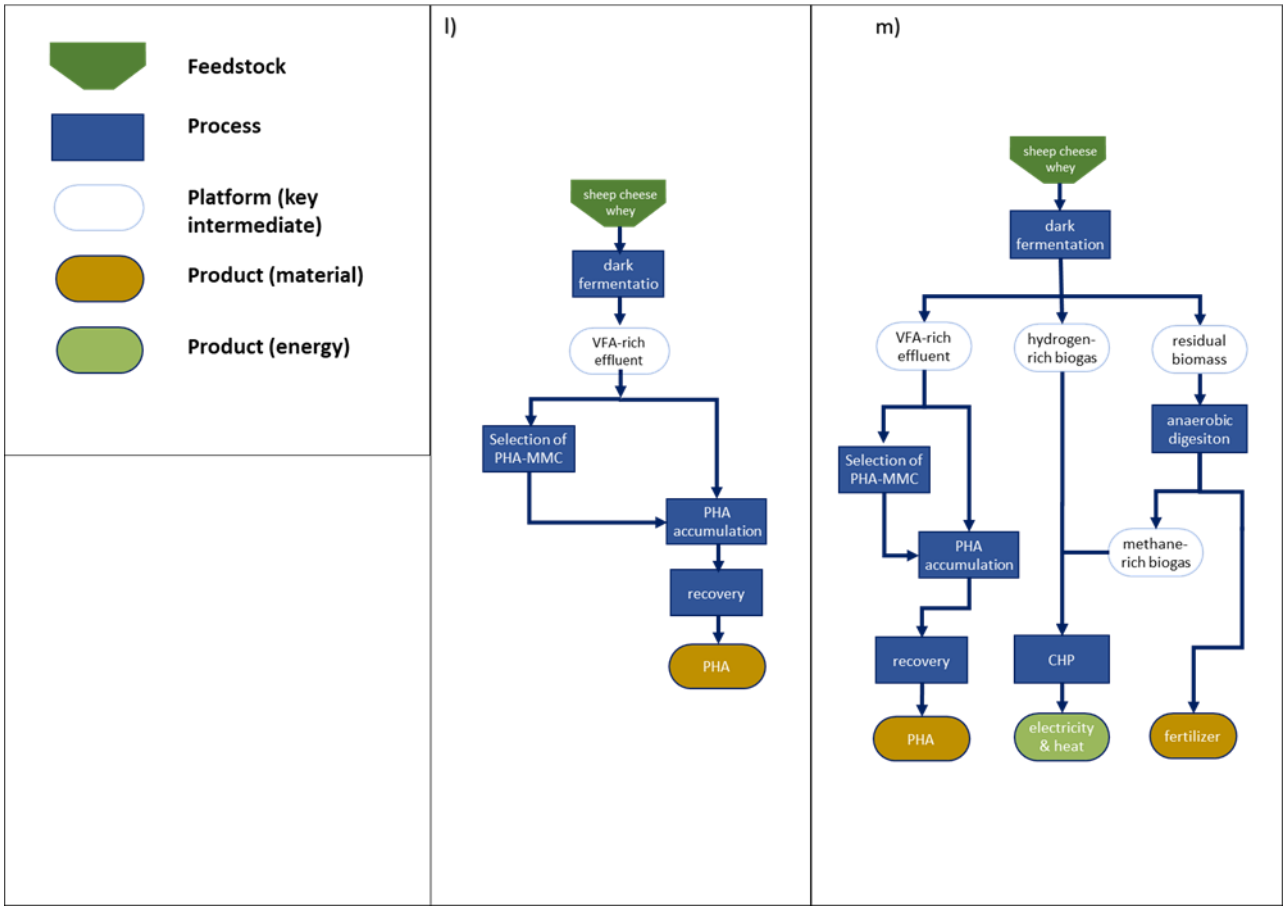


Figure 9.3. *Process pathways for the fermentation-based scenario aimed mainly at material recovery from sheep cheese whey: production of PHA biopolymer PHA (l); production of PHA biopolymer and additional energy (m).*

9.2.3 Comparison of the proposed options

The calculation of the BCI for each of the above described biorefinery options might provide a realistic comparison of in terms of development potential and overall TRLs.

BCI is calculated on the basis of four features defined by IEA for a biorefinery system, i.e. platforms, feedstock, products and processes.

Based on the technology readiness level (TRL), the Feature Complexity (FC) for each feature was calculated as assed from literature data. TRL ranges from 1 (basic principles observed) to 9 (actual system proven in operational environment). With the number of features and the FC of each single feature the Feature Complexity Index (FCI) for each of the four features is calculated. The BCI is the sum of the four FCIs.

The calculation formulas of the BCI are (Cherubini and Jungmeier, 2009; Jungmeier, 2014):

$$BCI = NF_{platforms} \cdot FC_{platforms} + NF_{feedstocks} \cdot FC_{feedstocks} + NF_{products} \cdot FC_{products}$$

$$+ NF_{processes} \cdot FC_{processes}$$

with

$$FCI_i = \sum_{i=1}^{m} NF_{ij}$$

and

$$FC_i = 10 - TRL_i$$

where BIC is the Biorefinery Complexity Index, NF_i is the number of features, FC_i is the feature complexity, *i* is the index for the four features (platform, feedstock, products, processes), FCI_i is the feature complexity index, and TRL is the technology readiness level of feature assessed between 1 and 9.

In addition, IEA proposed the Biorefinery Complexity Profile (BCP), which is a compact format to present the complexity of a biorefinery by giving the BCI and the four FCIs of the composing features. BCP is presented as:

$$BCP = (FCI_{platforms} / FCI_{feedstocks} / FCI_{products} / FCI_{processes})$$

In general terms, the higher the BCI, the more beyond "state of the art" is the biorefinery, as well as the lower is each FCI, the more the feature is applicable on real scale. As a benchmark to compare the complexity of other current and future biorefinery systems, the BCI of a biorefinery producing biodiesel from vegetable oil with (BCP 8 (1/1/3/3)) can be adopted as a reference of a fully deployed biorefinery (de Jong and Jungmeier, 2015; Jungmeier, 2014).

A graphic representation of BCI associated to each fermentation-based biorefinery options proposed for the valorisation of SCW is presented in Figure 9.4. The different BCI have been calculated according to Jungmeier (2014) and entirely reported in Supplementary.

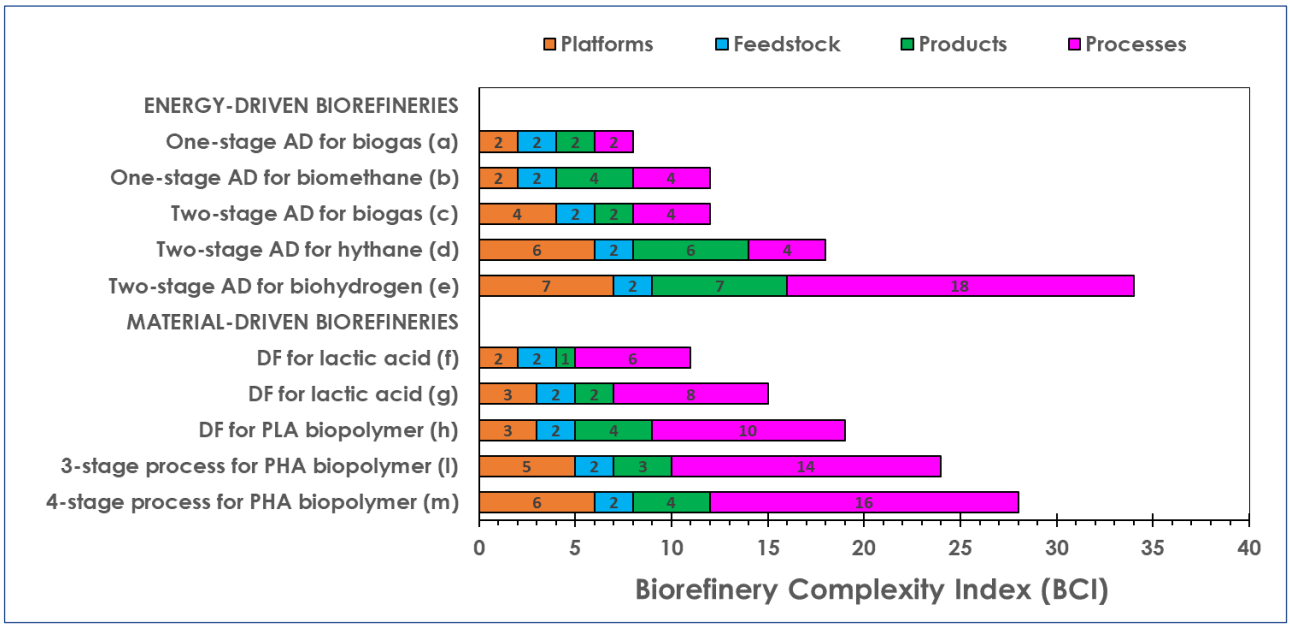


Figure 9.4. *BCI associated to the fermentation-based biorefinery options proposed for the valorisation of sheep cheese whey.*

As a clarifying example, the BCP in the case of one-stage AD from SCW (Figure 9.1a). The biorefinery scheme has 2 platforms, 1 feedstock, 2 final products and 2 processes:

- platforms: biogas (*TRL 9*); electricity&heat (*TRL 9*) *NF=2*
- feedstock: sheep cheese whey (*TRL 8*) *NF=1*
- products: electricity&heat (*TRL 9*); fertilizer (*TRL 9*) *NF=2*
- processes: anaerobic digestion (*TRL 9*); biogas combustion (*TRL 9*) *NF=2*

the FCI for each feature are:

- $FCI_{platforms} = (2*(10-9)) = 2*1 = 2$
- $FCI_{feedstock} = (1*(10-8)) = 1*2 = 2$
- $FCI_{products} = (2*(10-9)) = 2*1 = 2$
- $FCI_{processes} = (2*(10-9)) = 2*1 = 2$

The BCI is 8 as the sum of each FCI for platform, feedstock, product and processes and the resulting BCP is 8(2/2/2/2). As expected, the BCI is low since it refers to a well-established technology.

Considering the fact that the one-stage AD has the same BCI of the previously mentioned biorefinery producing biodiesel from vegetable oil, the BCI of the other biorefinery options to be applied to SCW can be compared to the one-stage SCW AD in terms of normalised BCI. Higher the normalised BCI, more complex is the biorefinery scheme and far from real-scale commercial application.

Table 9.1 summarizes the proposed biorefinery options, the process involved, the product yields, the BCP and normalized BCI. The table highlights the broad spectrum of bioproducts obtainable from SCW and helps the relative comparison of the different biorefinery concepts and their development potential.

***Table 9.1**. Summary of the fermentation-based biorefinery options proposed for the valorisation of sheep cheese whey.*

	Biorefinery option	Processes	Energy output as MJ L_{SCW}^{-1}	Material output as g L_{SCW}^{-1}	Biorefinery complexity profile BCP[(1)]	Normalised biorefinery complexity index	Carbon recovery [(2)] %
	Energy-driven biorefineries (Figure 9.1)						
a	**one-stage AD for biogas**	*AD, biogas combustion*	biogas[(3)]: 0.92-1.02	fertilizer: *n.d.*	8 (2/2/2/2)	*1.00*	*36-40*
b	**one-stage AD for methane**	*AD, biogas upgrading*	methane: *0.92-1.02*	fertilizer: *n.d.*	12 (2/2/4/4)	*1.50*	*36-40*
c	**Two-stage AD for biogas**	*DF, methanization, biogas combustion*	biogas[(4)]: *0.76-0.85*	fertilizer: *n.d.*	12 (4/2/2/4)	*1.50*	*27-32*
d	**Two-stage AD for hythane**	*DF, methanization, biogas upgrading*	hythane[(4)]: *0.76-0.85*	fertilizer: *n.d.*	18 (6/2/6/4)	*2.25*	*27-32*
e	**Two-stage AD for hydrogen**	*DF, methanization, biogas combustion, biogas upgrading*	methane: *0.71-0.82* hydrogen: *0.04-0.07*	fertilizer: *n.d.*	34 (7/2/7/18)	*4.25*	*27-32*
	Material-driven biorefineries – lactic acid and PLA biopolymer (Figure 9.2)						
f	**DF for lactic acid**	*DF, extraction*	none	lactic acid: 69	11 (2/2/1/6)	*1.88*	*86*
g	**DF for lactic acid**	*DF, extraction, AD, biogas combustion*	biogas[(5)]: 0.11-0.23	lactic acid: *69* fertilizer: *n.d.*	15 (3/2/2/8)	*1.38*	*86*
h	**DF for PLA biopolymer**	*DF, extraction, chemical synthesis, polymerisation, AD, biogas combustion*	biogas[(5)]: 0.11-0.23	PLA[(6)]: *29*	17 (3/2/4/10)	*2.38*	*45*
	Material-driven biorefineries – PHA biopolymer (Figure 9.3)						
i	**3-stage process for PHA biopolymer**	*DF, selection stage, accumulation stage, extraction*	none	PHA: *11-19*	24 (5/2/3/14)	*3.00*	*17-26*
m	**4-stage process for PHA biopolymer**	*DF, selection stage, accumulation stage, extraction, AD, biogas combustion*	biogas[(c)]: 0.11-0.23	PHA: *11-19* fertilizer: *n.d.*	28 (6/2/4/16)	*3.50*	*40-50*

Abbreviations: AD anaerobic digestion; DF dark fermentation.[(1)] BCP is presented as BCI ($FCI_{platforms}$/ $FCI_{feedstock}$/ $FCI_{products}$/ $FCI_{processes}$); [(2)] Calculated as the ratio between the carbon in the outputs and the initial carbon in the SCW (32 $g_C L^{-1}$). Only the products of interest have been considered. The carbon dioxide was not considered; [(3)] based on the lower heating value of methane; [(4)] based on the sum of lower heating value of hydrogen and methane; [(5)] estimated from the theoretical BMP value of the residues from the fermentation stage(4.48g_{TOC} L_{SCW}^{-1}) [(6)] for conversion yields, see supplementary data.

While a BCI of 8-9 is usually associated with commercial biorefineries, BCI in the range of 16-35 are associated with biorefineries under development. The lowest BCI is associated with one-stage AD (BCI 8) and this is also confirmed by the real case applications. The upgrade of biogas to methane increase the BCI of the biorefinery (BCI of 12) since it adds further steps in the process, i.e. the cleaning of the biogas from hydrogen

sulphide, oxygen and nitrogen, ammonia and water. In terms of normalised BCI, this means that one-stage AD with the upgrade to methane is "1.5 more complex" than one-stage AD without.

The upgrade to methane is mandatory when the target is to produce biofuel for transportation or for the national grid. Currently, the case of First Milk's District (UK) is the only case within the dairy sector aimed at supplying methane to the national grid. It can be expected that this can be replicated by other plants and dairy company, also driven by some national strategies in terms of renewable energy (see Chapter 1).

The production of lactic acid form SCW has a BCI of 11 while the production of PLA from the lactic acid produced via fermentation has a BCI of 17. In terms of normalised BCI, they have a normalised BCI of 1.88 and 3.00 respectively. This is mostly due the fact that the technology for polymerisation of lactic acid into PLA has already a high associated TRL. As a case of study, recently Total Corbion PLA, joint venture between Total and Corbion, inaugurated a production plant of 75 000 tons of PLA per year in Rayong (Thailand) using renewable, non-GMO sugarcane sourced locally as a feedstock. The novelty of the option proposed in this chapter associated to the use of biowaste, the SCW, as a feedstock that requires improvements and optimisation for MMC fermentation.

Things are slightly different for PHA production through the so-called 3-stage process since each stage of the process (acidogenic fermentation, selection and accumulation) needs further improvements. Currently, PHA production from biowaste is mostly associated to pilot plants (TRL 5-7), i.e. the case of the EU-funded project SMART-plant or the EuroPHA project.

It is expected that BCI for PLA and PHA will quickly decrease in the next years, driven by the rising market demand for bio-based product to replace the fossil-based plastics as well as EU objective for a circular bioeconomy.

The most "innovative" biorefinery options proposed is the biological production of hydrogen (BCI 34). The use of hydrogen as a clean biofuel has attracted much interest in the last decades, and recently some real and pilot cases have been reported. In Germany, the first hydrogen-fuel train has been launched last year with the aim to decrease the pollution of diesel and promoting the use of renewable energy. Indeed, hydrogen trains are equipped with fuel cells that produce electricity through a combination of hydrogen and oxygen, a process that leaves steam and water as the only emissions. The point is that the hydrogen-based application at the current state uses hydrogen produced by other sources than biological source. As mentioned before, biological production of hydrogen from biowastes is far away to see the light of real scale application. The technology is still in its infancy, mostly driven by laboratory-scale experiments (TRL 4-5). Considering the normalised BCI, the fermentative hydrogen production from SCW at the current state is 4.25 more complex than a "simple" one-stage AD, which is already commercialised.

Table 9.1 reports also the carbon recovery (C_i%) as an element of comparison. The C_i% has been calculated to give an overview of the amount of recoverable carbon with each configuration. Only the carbon of the target bioproduct has been considered, i.e. methane, lactic acid, PLA, PHA. Although there are possibilities that make carbon dioxide a useful bioproduct in the next future (i.e. as a feedstock for algae-based biorefineries), carbon dioxide has not been considered.

The calculated value widely ranges between 17 and 86, according to the solution adopted.

It is crucial to consider carbon recovery from the point of view of climate changes. The recovered carbon from SCW as a PLA-based or PHA-based food packaging contributes to reducing GHG since no fossil-based plastic is necessary. It is also worth to mention that, the case of methane combustion results in carbon dioxide emissions but since the methane derived from renewable source, those emissions are considered neutral.

To conclude, the results might become relevant for sheep dairy industry, decision-makers and investors as additional information to assist them in their strategies to implement the most promising biorefinery systems by minimising technical and economic risks.

9.3 Sheep Dairy biorefinery: a proposed scheme for the Sardinia sheep dairy supply chain

Since, as stated above, the sheep milk and dairy sector is of particular importance for several European rural areas including Sardinia Italian region, it is interesting to propose, on the basis of the results obtained and considerations previously reported on the possible SCW biorefinery scenarios, a hypothesis of valorisation scheme which may foster the creation of new value-chain inside the sheep dairy supply chain and, in turn, the economical development of the contexts under concern.

Considering the context represented by the Sardinia, a medium-large dairy sheep industry processes 5.9 $x10^6$ L of sheep milk in order to produce 498 t of dairy products (mostly Pecorino Romano) every year (Vagnoni et al., 2017). Assuming a specific production of 0.9 L_{SCW} $L_{sheep\ milk}^{-1}$ (Carvalho et al., 2013), the considered sheep milk industry generates 5.3 $x10^6$ L of SCW. Taking into consideration the results obtained in this study, a multi-step valorisation process of the produced SCW may be applied and oriented to the production of biohydrogen and biopolymers PHA. Such a process scheme would convert the high initial carbon content in the SCW into carbon dioxide, soluble metabolic compounds and non-soluble compounds as well biomass; in particular, provided the adoption of an operating pH = 6 during the fermentation stage, it could be theoretically possible to produce 28.8 x 10^6 L_{H2} per year from SCW with the production of 85-102 MJ of energy per year in the form of hydrogen. The soluble metabolites produced during the fermentation stage could be further processed to obtain 81 t of biopolymers, composed by HB (66%) and HV (33%). The overall carbon recovery from the raw SCW in the case of PHA production would be 0.23 $Cmmol_{PHA}$ $Cmmol_{SCW}^{-1}$.

In a real scale scenario and in order to tackle the market demand, PHAs with different monomers composition according have to be produced and at the lowest cost. In this respect, the use of SCW as substrate could be a feasible solution since feedstock availability would be certain and steady and, according to the test performed in the present study, no external inoculum would be necessary. Proper control of the operating pH would allow to obtain the required VFA profile necessary for the desired PHA composition, though the associated H_2 production would be affected.

Taking into consideration that the market price of PHA is around 3.4 € kg^{-1} (Colombo et al., 2019), a sheep dairy biorefinery could theoretically benefit from an extra revenue of 2 400 € t_{PHA}^{-1}, considering a production cost of 1 000 € t_{PHA}^{-1} (Colombo et al., 2019), and from the fact that the cost of substrate would be null. As for the energy recovery, it worth to point out that, as mentioned in the previous paragraph, besides the hydrogen production achievable during the fermentation stage, further energy could be obtained by feeding the residues from stage I and stage II to an anaerobic digestion stage in order to obtain methane.

Table 9.2. *Relevant data in the perspective of a dairy biorefinery based on the feedstock coming from a medium-large dairy sheep activity located in Sardinia (elaborated from Vagnoni et al. (2017)).*

Parameter	Value
Milk processed (L y^{-1})	5 953 871
Sheep cheese whey produced (L y^{-1})	5 358 484
Energy consumption, dairy plant (kW y^{-1})	593 669
Energy recovery through H_2 production* (kW y^{-1})	85 523-102 021
Dairy Products, total (t y^{-1})	498
PHA, total (t y^{-1})	61-81
PHA composition* HB:HV	66:34 or 78:33

** pH = 5.5 or 6.0 in the fermentation stage*

Currently, PHA production plants which use biowaste as a feedstock are relatively few across the world. The company Bio-on inaugurated a PHA production plant in Italy while other two companies, Hydal biotechnology and Full Cycle Bioplastic are planning to realize new PHA in the next future (Brigham and Riedel, 2019).

It is hard to define a reasonable plant size for PHA production, since it would be strongly dependent on the planned destination of the product. If the PHA is to be sold as a raw PHA pellet for further transformation, the selling price will be lower as compared to a situation in which the PHA pellet is processed on site and

transformed for a specific use, i.e. biofertilizer, biomedical uses. In the worst case a plant capacity up to 5 000-10 000 t y^{-1} would be required while in the best case even a size of 500-1000 t y^{-1} could be reasonable.

Assuming the yields obtained in this study, a PHA production plant with a capacity of 1000 t y^{-1} requires between 52 and 87 milions of litres of sheep cheese whey, coming from 61-102 milions litres of sheep milk.

This means that the PHA plant may process the 22-30% of the whole sheep cheese whey produced in Sardinia every year.

The largest dairy processing plant in Sardinia processes around 40 milions litres of sheep milk per year (F.lli Pinna) followed by other dairies with a capacity between 10 and 22 milions litres of sheep milk per year (Argiolas formaggi, CAO formaggi, Central). The remarkable flow of SCW which would be required in order to feed the hypothesized plant excludes the possibility of a single dairy processing plant as source of feedstock, but entails the development of a supply chain that covers a large part of the regional territory.

In this respect, the problem represented by an early fermentation of SCW during collection and transport should be faced.

It is worth to mention and highlight the fact that the PHA yield obtained in the present study are preliminary and there is room for further improvements. In the case of an improvement of PHA yield from 0.32-0.44 to the value of 0.8 Cmol $_{PHA}$ $Cmol_{OA}^{-1}$ (as reported in Colombo et al. (2017)), the amount of required SCW would be reduced to 32 milions of litres, close to the maximum amount of SCW produced by the single dairy plant of F.lli Pinna. Achieving a better conversion efficiency would be difficult, therefore a PHA yield of 0.8 would represent the best result in a scenario where a PHA production plant is associated to the dairy industry.

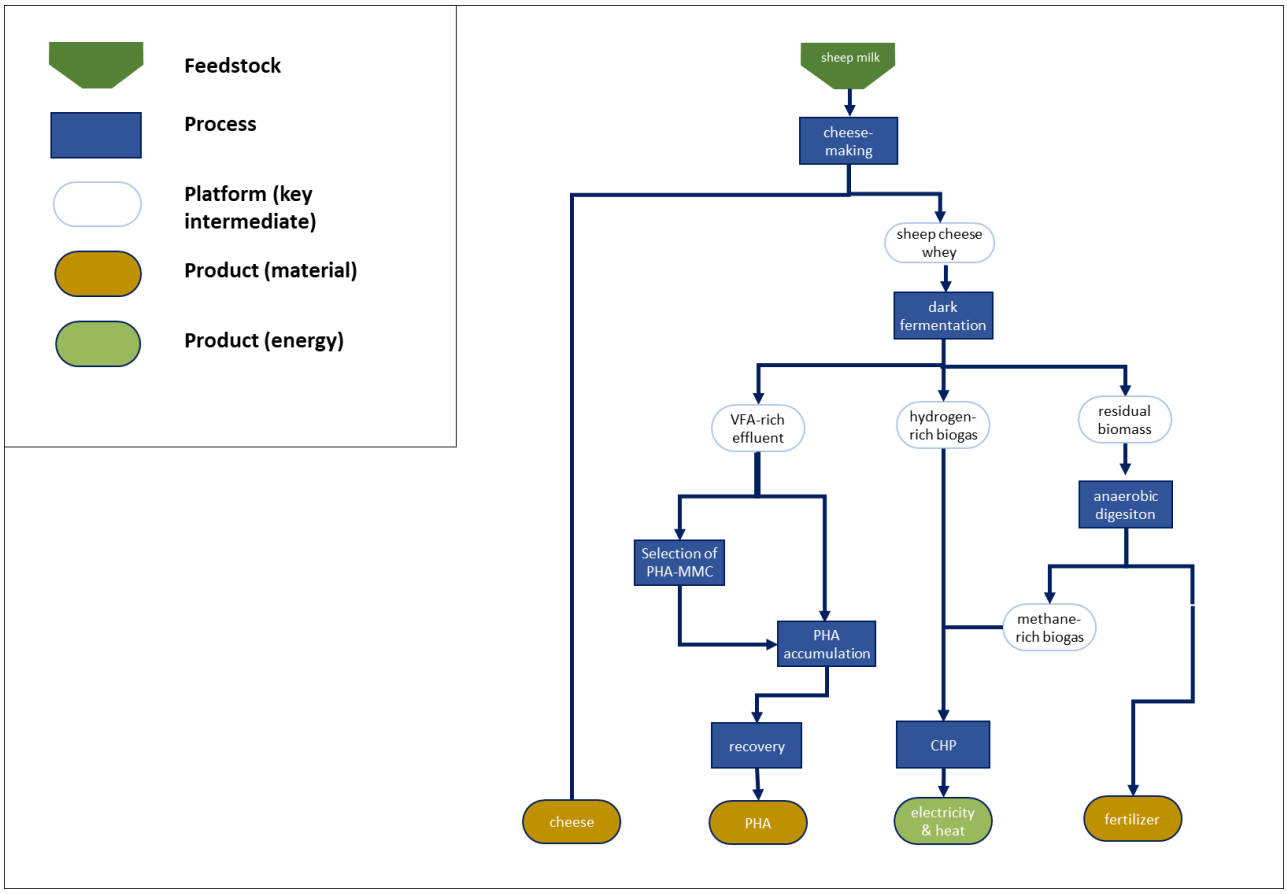


Figure 9.5. *Proposed process pathways for a dairy biorefinery aimed at the valorisation of sheep cheese whey.*

9.4 Conclusion and future perspectives

Although the results obtained in the present study could be further improved by assessing the very best operating conditions for recovering/producing each of the considered biproducts (lactic acid, pool of VFA, PHA, hydrogen and methane), the present study pointed out the possible feasibility of a dairy waste biorefinery approach.

In this respect, sheep cheese whey confirms to be an outstanding substrate to be biorefined through integrated biochemical processes.

The implementation of an integrated system aimed at energy and material recovery from dairy wastes could strongly support the dairy supply chain, promoting the circular bioeconomy and creating new economic opportunities in rural areas.

This experimental work carried out during the PhD three years addressed different processes that could fit well different SCW biorefinery scenarios, the latter spanning from almost ready applications to innovative solutions that need further studies.

Full scale implementation of energy recovery scenarios, methane recovery in particular, is obviously more plausible and could become a best available technology (BAT) for the sheep dairy sector, provided that the

optimisation necessary to maximise the extent of recovery with reference to the peculiar characteristics of SCW is achieved.

More innovative solution based on PLA and PHA production requires even more efforts in terms of optimization of the MMC dark fermentation stage with the aim to improve production yields (lactic or VFA) and kinetics. In this respect, production kinetics may be improved with the use of specific enzymes or chemicals in order to foster the lactose hydrolysis and improve productivities. Furthermore, also the type of adopted process (batch, fed-batch, continuous) requires a in depth assessment according to the target product. With focus on PHA production, fermentation should be optimized with regards to the composition of the VFAs pool to be fed to the stages of selection and accumulation and the C/N ratio. Moreover, the PHAs accumulation stage represent the main bottleneck of the whole process; the possibility to adopt different feed strategy should be pursued in order to improve the accumulation yields.

Finally, since the present study focused mostly on sheep cheese whey, further studies on scotta valorization are worth to be performed in order to verify the considered processes with a different substrate and to give the whole picture of the opportunities related to the dairy residues.

Driven by environmental and economic issues, the “dairy waste biorefinery” concept has the potential to exploit the un-tapped potential stored in millions of litres of sheep cheese whey produced yearly.

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SUPLEMENTARY DATA

I. PLA synthesis: conversion yields

Lactic acid recovery: *84% by weight*

Conversion yield from lactic acid to lactide: *73% by weight*

Recovery yield of lactide during purification: *93% by weight*

Conversion yield from lactide to PLA: *74% by weight*

Recovery yield of PLA during purification: *100%*

Carbon content in PLA: *0.5* g_C g_{PLA}^{-1} *(a*ssuming PM = 60 g mol^{-1} and a general formula of $C_3H_4O_2$)

II. Calculation of biorefinery complexity index (BCI)

Some general remarks:

- More detail definition and calculation about BCI and BCP can be found in the reports made by IEA Bioenergy Task 42 (Cherubini and Jungmeier, 2009; IEA Bioenergy Task42, 2019; Jungmeier, 2014)
- For simplification electricity and heat are combined into one platform "electricity&heat". It is considered as a platform when the energy is produced from process residues or directly from biomass feedstock. The produced energy can be used within the plant or for external uses. The energy&heat covered by external supply or other biofuels is not considered as a platform.
- For all the biorefinery options, SCW has been considered as the feedstock with a *TRL 8* (*FC 1*).

Energy-driven biorefinery

a) One-stage AD for biogas:

a. platforms: biogas (*TRL 9*); electricity&heat (*TRL 9*) *NF=2*

b. products: electricity&heat (*TRL 9*); fertilizer (*TRL 9*) *NF=2*

c. processes: anaerobic digestion (*TRL 9*); biogas combustion (*TRL 9*) *NF=2*

*BCI = (2*1) + (1*2) + (2*1) + (2*1) = 8*

BCP = 8 (2/2/2/2)

b) One-stage AD for biomethane:

a. platforms: biogas (*TRL 9*); electricity&heat (*TRL 9*). *NF = 2*

b. products: biomethane (*TRL 8*); electricity&heat (*TRL 9*); fertilizer (*TRL 9*). *NF =1 + 2*

c. processes: anaerobic digestion (*TRL 9*); biogas combustion (*TRL 9*); biogas upgrading to biomethane (*TRL 8*). *NF = 2 + 1*

*BCI = (2*1) + (1*2) + (1*2+2*1) + (2*1+1*2) = 12*

BCP = 12 (2/2/4/2)

c) Two-stage AD for biogas:

a. platforms: carboxylate (*TRL 8*); biogas (*TRL 9*); electricity&heat (*TRL 9*). *NF = 1 + 2*

b. products: electricity&heat (*TRL 9*); fertilizer (*TRL 9*). *NF =2*

c. processes: dark fermentation (*TRL 8*); anaerobic digestion (*TRL 9*); biogas upgrading (*TRL 9*). *NF = 1 + 2*

*BCI = (1*2+2*1) + (1*2) + (2*1) + (1*2+2*1) = 12*

BCP = 12 (4/2/2/4)

d) Two-stage AD for hythane:

a. platforms: carboxylate (*TRL 5*); biogas (*TRL 9*). *NF = 1 + 1*

b. products: biohythane (*TRL 5*); fertilizer (*TRL 9*). *NF =1 + 1*

c. processes: dark fermentation (*TRL 5*); anaerobic digestion (*TRL 9*); biogas upgrade to biohythane (*TRL 8*); *NF = 1 + 1 + 1*

*BCI = (1*5 + 1*1) + (1*2) + (1*5 + 1*1) + (1*5 + 1*1 + 1*2) = 18*

BCP = 18 (6/2/6/4)

e) Two-stage AD for hydrogen:

a. platforms: carboxylate (*TRL 5*); biogas (*TRL 9*); electricity&heat (*TRL 9*). *NF = 1 + 2*

b. products: hydrogen (*TRL 5*); electricity&heat (*TRL 9*); fertilizer (*TRL 9*). *NF =1 + 2*

c. processes: dark fermentation (*TRL 4*); anaerobic digestion (*TRL 9*); biogas combustion (*TRL 9*); biogas upgrade to biohydrogen (*TRL 5*), hydrogen storage (*TRL 5*) *NF = 1 +2 + 2*

*BCI = (1*5 + 2*1) + (1*2) + (1*5 + 2*1) + (1*6 + 2*1 + 2*5) = 34*

BCP = 34 (7/2/7/18)

Material-driven biorefinery

f) DF for lactic acid:

a. platforms: sugar (*TRL 8*). *NF = 1*

b. products: lactic acid (*TRL 9*). *NF =1*

c. processes: dark fermentation (*TRL 8*); separation (*TRL 8*); extraction (*TRL 8*). *NF = 3*

*BCI = (1*2) + (1*2) + (1*1) + (3*2+2*1) = 11*

BCP = 11 (2/2/1/6)

g) DF for lactic acid and energy:

a. platforms: sugar (*TRL 8*); electricity&heat (*TRL 9*). *NF = 1+1*

b. products: lactic acid (*TRL 9*); electricity&heat (*TRL 9*). *NF =2*

c. processes: dark fermentation (*TRL 8*); separation (*TRL 8*); extraction (*TRL 8*); anaerobic digestion (*TRL 9*); biogas combustion (*TRL 9*). *NF = 3 + 2*

*BCI = (1*2+1*2) + (1*2) + (2*1) + (3*2+2*1) = 15*

BCP = 15 (3/2/2/8)

h) DF for PLA biopolymer:

a. platforms: sugar (*TRL 8*); electricity&heat (*TRL 9*). *NF = 1+1*

b. products: lactic acid (*TRL 9*); lactide (*TRL 9*); PLA (*TRL 9*); electricity&heat (*TRL 9*).*NF =4*

c. processes: dark fermentation (*TRL 8*); lactide synthesis (*TRL 9*); polymerization (*TRL 9*); extraction (*TRL 8*); purification (*TRL 8*); anaerobic digestion (*TRL 9*); biogas combustion (*TRL 9*). *NF = 3 + 2*

*BCI = (1*2+1*1) + (1*2) + (4*1) + (3*2+4*1) = 19*

BCP = 19 (3/2/4/10)

i) 3-stage process for PHA biopolymer:

a. platforms: sugar (*TRL 5*). *NF = 1*

b. products: PHA (*TRL 7*). *NF =1*

c. processes: dark fermentation (*TRL* 6); selection stage (*TRL 6*); accumulation stage (*TRL 6*); extraction (*TRL 8*). *NF = 3 + 1*

*BCI = (1*5) + (1*2) + (1*3) + (3*4+1*2) = 24*

BCP = 24 (5/2/3/14)

j) 4 -stage process for PHA biopolymer:

a. platforms: sugar (*TRL 8*); electricity&heat (*TRL 9*). *NF = 1*

b. products: PHA (*TRL 7*); electricity&heat (*TRL 9*). *NF =1*

c. processes: dark fermentation (*TRL* 6); selection stage (*TRL 6*); accumulation stage (*TRL 6*); extraction (*TRL 8*); anaerobic digestion (*TRL 9*); biogas combustion (*TRL* 9). *NF = 3 + 1*

*BCI = (1*2+1*1) + (1*2) + (1*3+1*1) + (3*3+1*2+2*1) = 28*

BCP = 28 (6/2/4/16)

LIST OF TABLES

LIST OF FIGURES

REFERENCES

Agler, M.T., Wrenn, B.A., Zinder, S.H., Angenent, L.T., 2011. Waste to bioproduct conversion with undefined mixed cultures: The carboxylate platform. Trends Biotechnol. 29, 70–78. https://doi.org/10.1016/j.tibtech.2010.11.006

Ahmad, T., Aadil, R.M., Ahmed, H., Rahman, U. ur, Soares, B.C.V., Souza, S.L.Q., Pimentel, T.C., Scudino, H., Guimarães, J.T., Esmerino, E.A., Freitas, M.Q., Almada, R.B., Vendramel, S.M.R., Silva, M.C., Cruz, A.G., 2019. Treatment and utilization of dairy industrial waste: A review. Trends Food Sci. Technol. https://doi.org/10.1016/j.tifs.2019.04.003

Akhlaghi, M., Boni, M.R., De Gioannis, G., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., 2017a. A parametric response surface study of fermentative hydrogen production from cheese whey. Bioresour. Technol. 244, 473–483. https://doi.org/10.1016/j.biortech.2017.07.158

Akhlaghi, M., Boni, M.R., De Gioannis, G., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., 2017b. A parametric response surface study of fermentative hydrogen production from cheese whey. Bioresour. Technol. 244, 473–483. https://doi.org/10.1016/j.biortech.2017.07.158

Akhlaghi, M., Boni, M.R., De Gioannis, G., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., 2017c. A parametric response surface study of fermentative hydrogen production from cheese whey. Bioresour. Technol. 244, 473–483. https://doi.org/10.1016/j.biortech.2017.07.158

Akhlaghi, M., Boni, M.R., Polettini, A., Pomi, R., Rossi, A., De Gioannis, G., Muntoni, A., Spiga, D., 2019. Fermentative H2 production from food waste: Parametric analysis of factor effects. Bioresour. Technol. 276, 349–360. https://doi.org/10.1016/j.biortech.2019.01.012

Amaro, T.M.M.M., Rosa, D., Comi, G., Iacumin, L., 2019. Prospects for the Use of Whey for Polyhydroxyalkanoate (PHA) Production. Front. Microbiol. 10, 1–12. https://doi.org/10.3389/fmicb.2019.00992

Antonopoulou, G., Stamatelatou, K., Bebelis, S., Lyberatos, G., 2010. Electricity generation from synthetic substrates and cheese whey using a two chamber microbial fuel cell. Biochem. Eng. J. 50, 10–15. https://doi.org/10.1016/j.bej.2010.02.008

Antonopoulou, G., Stamatelatou, K., Venetsaneas, N., Kornaros, M., Lyberatos, G., 2008. Biohydrogen and Methane Production from Cheese Whey in a Two-Stage Anaerobic Process. Ind. Eng. Chem. Res. 5227–5233. https://doi.org/10.1021/ie071622x

Arasaratnam, V., Appadural, S., Balasubramaniam, K., 1996. Supplementation of whey with glucose and different nitrogen sources for lactic acid production by Lactobacillus delbrueckii.

Aristizábal M, V., Cardona Alzate, C.A., 2018. Methods for designing and assessing biorefineries: Review. Biofuels, Bioprod. Biorefining. https://doi.org/10.1002/bbb.1961

Asquer, C., Cappai, G., De Gioannis, G., Muntoni, A., Piredda, M., Spiga, D., 2017. Biomass ash reutilisation as

an additive in the composting process of organic fraction of municipal solid waste. Waste Manag. 69, 127–135. https://doi.org/10.1016/j.wasman.2017.08.009

Asunis, F., De Gioannis, G., Isipato, M., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., 2019. Control of fermentation duration and ph to orient biochemicals and biofuels production from cheese whey. Bioresour. Technol. 289, 121722. https://doi.org/10.1016/j.biortech.2019.121722

Atasoy, M., Owusu-Agyeman, I., Plaza, E., Cetecioglu, Z., 2018. Bio-based volatile fatty acid production and recovery from waste streams: Current status and future challenges, Bioresource Technology. Elsevier. https://doi.org/10.1016/j.biortech.2018.07.042

Augere-Granier, M.-L., 2018. The EU dairy sector.

Azbar, N., Dokgöz, F.T., Keskin, T., Eltem, R., Korkmaz, K.S., Gezgin, Y., Akbal, Z., Öncel, S., Dalay, M.C., Gönen, Ç., Tutuk, F., 2009a. Comparative evaluation of bio-hydrogen production from cheese whey wastewater under thermophilic and mesophilic anaerobic conditions. Int. J. Green Energy 6, 192–200. https://doi.org/10.1080/15435070902785027

Azbar, N., Dökgoz, F.T.Ç., Peker, Z., 2009b. Optimization of basal medium for fermentative hydrogen production from cheese whey wastewater. Int. J. Green Energy 6, 371–380. https://doi.org/10.1080/15435070903107049

Azbar, N., Tuba Ç Etinkaya Dokgöz, F., Keskin, T., Korkmaz, K.S., Syed, H.M., Çetinkaya Dokgöz, F.T., Keskin, T., Korkmaz, K.S., Syed, H.M., 2009c. Continuous fermentative hydrogen production from cheese whey wastewater under thermophilic anaerobic conditions. Int. J. Hydrogen Energy 34, 7441–7447. https://doi.org/10.1016/j.ijhydene.2009.04.032

Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A., 2009. Effect of extrinsic lactic acid on fermentative hydrogen production. Int. J. Hydrogen Energy 34, 2573–2579. https://doi.org/10.1016/j.ijhydene.2009.01.010

Balthazar, C.F., Pimentel, T.C., Ferrão, L.L., Almada, C.N., Santillo, A., Albenzio, M., Mollakhalili, N., Mortazavian, A.M., Nascimento, J.S., Silva, M.C., Freitas, M.Q., Sant'Ana, A.S., Granato, D., Cruz, A.G., 2017. Sheep Milk: Physicochemical Characteristics and Relevance for Functional Food Development. Compr. Rev. Food Sci. Food Saf. 16, 247–262. https://doi.org/10.1111/1541-4337.12250

Batlle-Vilanova, P., Puig, S., Gonzalez-Olmos, R., Balaguer, M.D., Colprim, J., 2016. Continuous acetate production through microbial electrosynthesis from CO2with microbial mixed culture. J. Chem. Technol. Biotechnol. 91, 921–927. https://doi.org/10.1002/jctb.4657

Bernárdez, P.F., Amado, I.R., Castro, L.P., Guerra, N.P., 2008. Production of a potentially probiotic culture of Lactobacillus casei subsp. casei CECT 4043 in whey. Int. Dairy J. 18, 1057–1065. https://doi.org/10.1016/J.IDAIRYJ.2008.05.004

Bio Intelligence Service, 2010. Preparatory Study on Food Waste Across EU 27, Technical Report. https://doi.org/10.2779/85947

Biobased Industries Consortium, 2018. Bioeconomy and the UN Sustainable Development Goals A view from the Bio-based Industries Consortium-July 2018 1–5.

Blanco, V.M.C., Oliveira, G.H.D., Zaiat, M., 2019. Dark fermentative biohydrogen production from synthetic cheese whey in an anaerobic structured-bed reactor: Performance evaluation and kinetic modeling. Renew. Energy 139, 1310–1319. https://doi.org/10.1016/j.renene.2019.03.029

Blonskaja, V., Vaalu, T., 2006. Investigation of different schemes for anaerobic treatment of food industry wastes in Estonia. Proc. Est. Acad. Sci. Chem 55, 14–28.

Bosco, F., Carletto, R.A., Marmo, L., 2018. An integrated cheese whey valorization process. Chem. Eng. Trans. 64, 379–384. https://doi.org/10.3303/CET1864064

Brigham, C.J., Riedel, S.L., 2019. The potential of polyhydroxyalkanoate production from food wastes. Appl. Food Biotechnol. 6, 7–18. https://doi.org/10.22037/afb.v6i1.22542

Bundhoo, M.A.Z.Z., Mohee, R., 2016. Inhibition of dark fermentative bio-hydrogen production: A review. Int. J. Hydrogen Energy 41, 6713–6733. https://doi.org/10.1016/j.ijhydene.2016.03.057

Büyükkileci, A.O., Harsa, S., 2004. Batch production of L(+) lactic acid from whey by Lactobacillus casei (NRRL B-441). J. Chem. Technol. Biotechnol. 79, 1036–1040. https://doi.org/10.1002/jctb.1094

Cabrol, L., Marone, A., Tapia-Venegas, E., Steyer, J.P., Ruiz-Filippi, G., Trably, E., 2017. Microbial ecology of fermentative hydrogen producing bioprocesses: Useful insights for driving the ecosystem function. FEMS Microbiol. Rev. 41, 158–181. https://doi.org/10.1093/femsre/fuw043

Carletto, R.A., 2014. Studio della produzione di Poliidrossialcanoati da siero di latte. Politecnico di Torino.

Carlos, J., Arévalo, S., Vázquez, H.C., Amr, E.S., Mohammed, K., Cedeño, C.B., Alicia, M., Vazquez, C., Carlos, A.M., Tecnológico, I., Zúñiga, D.T. De, De, K., Miguel, S., Ext, C.P.F., 2016. The Use of Sweet Whey for Weaning Pigs Faculty of Veterinary Medicine , Department of Internal Medicine Cellular and Molecular Biology Department , Guadalajara University , 16, 52–56. https://doi.org/10.5829/idosi.gv.2016.16.01.101203

Carrieri, C., Di Pinto, A.C., Rozzi, A., Santori, M., 1993. Anaerobic co-digestion of sewage sludge and concentrated soluble wastewaters. Water Sci. Technol. 28, 187–197. https://doi.org/10.2166/wst.1993.0102

Carvalho, F., Prazeres, A.R., Rivas, J., 2013. Cheese whey wastewater: Characterization and treatment. Sci. Total Environ. 445, 385–396. https://doi.org/10.1016/j.scitotenv.2012.12.038

Castelló, E., García y Santos, C., Iglesias, T., Paolino, G., Wenzel, J., Borzacconi, L., Etchebehere, C., 2009. Feasibility of biohydrogen production from cheese whey using a UASB reactor: Links between microbial community and reactor performance. Int. J. Hydrogen Energy 34, 5674–5682. https://doi.org/10.1016/j.ijhydene.2009.05.060

Castillo Martinez, F.A., Balciunas, E.M., Salgado, J.M., Domínguez González, J.M., Converti, A., Oliveira, R.P. de S., 2013. Lactic acid properties, applications and production: A review. Trends Food Sci. Technol. 30,

70–83. https://doi.org/10.1016/j.tifs.2012.11.007

Chandra, R., Castillo-Zacarias, C., Delgado, P., Parra-Saldívar, R., 2018. A biorefinery approach for dairy wastewater treatment and product recovery towards establishing a biorefinery complexity index. J. Clean. Prod. 183, 1184–1196. https://doi.org/10.1016/j.jclepro.2018.02.124

Chatzipaschali, A.A., Stamatis, A.G., 2012. Biotechnological utilization with a focus on anaerobic treatment of cheese whey: Current status and prospects. Energies 5, 3492–3525. https://doi.org/10.3390/en5093492

Chen, J.L., Ortiz, R., Steele, T.W.J., Stuckey, D.C., 2014. Toxicants inhibiting anaerobic digestion: A review. Biotechnol. Adv. 32, 1523–1534. https://doi.org/10.1016/j.biotechadv.2014.10.005

Chen, Y., Cheng, J.J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: A review. Bioresour. Technol. 99, 4044–4064. https://doi.org/10.1016/j.biortech.2007.01.057

Cherubini, F., Jungmeier, G., 2009. Toward a common classification approach for biorefinery systems. Biofuels, Bioprod. Biorefining. https://doi.org/10.1002/bbb

Choi, J., Lee, S.Y., 1999. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl. Microbiol. Biotechnol. 51, 13–21. https://doi.org/10.1007/s002530051357

Chojnacka, A., Błaszczyk, M.K., Szczęsny, P., Nowak, K., Sumińska, M., Tomczyk-Żak, K., Zielenkiewicz, U., Sikora, A., 2011. Comparative analysis of hydrogen-producing bacterial biofilms and granular sludge formed in continuous cultures of fermentative bacteria. Bioresour. Technol. 102, 10057–10064. https://doi.org/10.1016/J.BIORTECH.2011.08.063

Christensen, A.D., Kádár, Z., Oleskowicz-Popiel, P., Thomsen, M.H., 2011. Production of bioethanol from organic whey using Kluyveromyces marxianus. J. Ind. Microbiol. Biotechnol. 38, 283–289. https://doi.org/10.1007/s10295-010-0771-0

Colombo, B., Favini, F., Scaglia, B., Sciarria, T.P., D'Imporzano, G., Pognani, M., Alekseeva, A., Eisele, G., Cosentino, C., Adani, F., 2017. Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture. Biotechnol. Biofuels 10, 201. https://doi.org/10.1186/s13068-017-0888-8

Colombo, B., Pepè Sciarria, T., Reis, M., Scaglia, B., Adani, F., Sciarria, T.P., Reis, M., Scaglia, B., Adani, F., 2016. Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture. Bioresour. Technol. 218, 692–699. https://doi.org/10.1016/j.biortech.2016.07.024

Colombo, B., Villegas Calvo, M., Pepè Sciarria, T., Scaglia, B., Savio Kizito, S., D'Imporzano, G., Adani, F., 2019. Biohydrogen and polyhydroxyalkanoates (PHA) as products of a two-steps bioprocess from deproteinized dairy wastes. Waste Manag. 95, 22–31. https://doi.org/10.1016/j.wasman.2019.05.052

Comino, E., Riggio, V.A., Rosso, M., 2012. Biogas production by anaerobic co-digestion of cattle slurry and cheese whey. Bioresour. Technol. 114, 46–53. https://doi.org/10.1016/J.BIORTECH.2012.02.090

Correa, D.F., Beyer, H.L., Fargione, J.E., Hill, J.D., Possingham, H.P., Thomas-Hall, S.R., Schenk, P.M., 2019.

Towards the implementation of sustainable biofuel production systems. Renew. Sustain. Energy Rev. https://doi.org/10.1016/j.rser.2019.03.005

CRA Consiglio per la ricerca e la sperimentazione in agricoltura, 2005. Alimenti per il suoino biologico - Manuale pratico.

Cristóbal, J., Caldeira, C., Corrado, S., Sala, S., 2018. Techno-economic and profitability analysis of food waste biorefineries at European level. Bioresour. Technol. 259, 244–252. https://doi.org/10.1016/j.biortech.2018.03.016

Dąbrowski, W., Żyłka, R., Malinowski, P., 2017. Evaluation of energy consumption during aerobic sewage sludge treatment in dairy wastewater treatment plant. Environ. Res. 153, 135–139. https://doi.org/10.1016/j.envres.2016.12.001

Dahiya, M., Vij, S., 2012. Comparative Analysis of Bioethanol Production from Whey by different strains of Immobilized Thermotolerant Yeast. Int. J. Sci. Res. Publ. 2.

Dai, K., Wen, J.L., Zhang, F., Zeng, R.J., 2017. Valuable biochemical production in mixed culture fermentation: fundamentals and process coupling. Appl. Microbiol. Biotechnol. 101, 6575–6586. https://doi.org/10.1007/s00253-017-8441-z

Das, S., Majumder, A., Shukla, V., Suhazsini, · Priya, Radha, · P, 2018. Biosynthesis of Poly(3-hydroxybutyrate) from Cheese Whey by Bacillus megaterium NCIM 5472. J. Polym. Environ. 0. https://doi.org/10.1007/s10924-018-1288-2

Davila-Vazquez, G., Alatriste-Mondragón, F., de León-Rodríguez, A., Razo-Flores, E., 2008. Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: Influence of initial substrate concentration and pH. Int. J. Hydrogen Energy 33, 4989–4997. https://doi.org/10.1016/j.ijhydene.2008.06.065

Davila-Vazquez, G., Cota-Navarro, C.B., Rosales-Colunga, L.M., de León-Rodríguez, A., Razo-Flores, E., 2009. Continuous biohydrogen production using cheese whey: Improving the hydrogen production rate. Int. J. Hydrogen Energy 34, 4296–4304. https://doi.org/10.1016/j.ijhydene.2009.02.063

De Gioannis, G., Friargiu, M., Massi, E., Muntoni, A., Polettini, A., Pomi, R., Spiga, D., 2014. Biohydrogen production from dark fermentation of cheese whey: Influence of pH. Int. J. Hydrogen Energy 39, 20930–20941. https://doi.org/10.1016/j.ijhydene.2014.10.046

De Gioannis, G., Muntoni, A., Polettini, A., Pomi, R., 2013. A review of dark fermentative hydrogen production from biodegradable municipal waste fractions. Waste Manag. 33, 1345–1361. https://doi.org/10.1016/j.wasman.2013.02.019

De Gioannis, G., Muntoni, A., Polettini, A., Pomi, R., Spiga, D., 2017. Energy recovery from one- and two-stage anaerobic digestion of food waste. Waste Manag. https://doi.org/10.1016/j.wasman.2017.06.013

De Jesus, C.-S.A., Elba Ruth, V.-G., Daniel, S.-F.R., Sharma, A., 2015. Biotechnological Alternatives for the Utilization of Dairy Industry Waste Products. Adv. Biosci. Biotechnol. 06, 223–235.

https://doi.org/10.4236/abb.2015.63022

de Jong, E., Jungmeier, G., 2015. Biorefinery Concepts in Comparison to Petrochemical Refineries, Industrial Biorefineries and White Biotechnology. https://doi.org/10.1016/B978-0-444-63453-5.00001-X

Demirel, B., Yenigun, O., Onay, T.T., 2005. Anaerobic treatment of dairy wastewaters: A review. Process Biochem. 40, 2583–2595. https://doi.org/10.1016/j.procbio.2004.12.015

Diniz, R.H.S., Rodrigues, M.Q.R.B., Fietto, L.G., Passos, F.M.L., Silveira, W.B., 2014. Optimizing and validating the production of ethanol from cheese whey permeate by Kluyveromyces marxianus UFV-3. Biocatal. Agric. Biotechnol. 3, 111–117. https://doi.org/10.1016/j.bcab.2013.09.002

Dragone, G., Mussatto, S.I., Almeida e Silva, J.B., Teixeira, J.A., 2011. Optimal fermentation conditions for maximizing the ethanol production by Kluyveromyces fragilis from cheese whey powder. Biomass and Bioenergy 35, 1977–1982. https://doi.org/10.1016/J.BIOMBIOE.2011.01.045

Duque, A.F., Oliveira, C.S.S., Carmo, I.T.D., Gouveia, A.R., Pardelha, F., Ramos, A.M., Reis, M.A.M., 2014. Response of a three-stage process for PHA production by mixed microbial cultures to feedstock shift: impact on polymer composition. N. Biotechnol. 31, 276–288. https://doi.org/10.1016/j.nbt.2013.10.010

Ebrahimi, A., Najafpour, G.D., Mohammadi, M., Hashemiyeh, B., 2010. Biological treatment of whey in an UASFF bioreacotr following a three-satge RBC. Chem. Ind. Chem. Eng. Q. 16, 175–182. https://doi.org/10.2298/CICEQ100315025E

Erguè, T.H., Tezel, U., Guè, E., Demirer, G.N., Management, W., May, O., 2001. Anaerobic biotransformation and methane generation potential of cheese whey in batch and UASB reactors. Waste Manag. 21, 643–650. https://doi.org/10.1016/S0956-053X(00)00114-8

Escalante, H., Castro, L., Amaya, M.P., Jaimes, L., Jaimes-Estévez, J., 2017. Anaerobic digestion of cheese whey : Energetic and nutritional potential for the dairy sector in developing countries. Waste Manag. 71, 711–718. https://doi.org/10.1016/j.wasman.2017.09.026

EU-JRC-IES, 2011. Supporting Environmentally Sound Decisions for Waste Management – A technical guide to Life Cycle Thinking (LCT) and Life Cycle Assessment (LCA) for waste experts and LCA practitioners. https://doi.org/10.2788/53942

European Biogas Association (EBA), 2018. European Biogas Association Annual Report 2018.

European Bioplastics, 2018. Global production capacities of bioplastics 2018-2023.

European Commission, 2019. REPORT on the implementation of the Circular Economy Action Plan 10, 145–145. https://doi.org/10.1259/arr.1905.0091

European Commission, 2018a. A sustainable Bioeconomy for Europe: strengthening the connection between economy, society and the environment Updated Bioeconomy Strategy. https://doi.org/10.2777/478385

European Commission, 2018b. Impacts of circular economy policies on the labour market.

European Commission, 2017a. Bioeconomy Strategy review. A Commission Staff working document.

European Commission, 2017b. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions - The role of waste-to-energy in the circular economy, COM/2017/0034 final. https://doi.org/10.1002/bdd.453

European Commission, 2015. Closing the loop - An EU action plan for the Circular Economy. https://doi.org/10.1017/CBO9781107415324.004

European Commission, 2012. Innovating for Sustainable Growth: A Bioeconomy for Europe, Industrial Biotechnology. https://doi.org/10.1089/ind.2012.1508

European Commission, 2008. Communication from the commission to the council and the european parliament; on future steps in bio-waste management in the European Union,{SEC(2010) 577}.

European Commission, CORDIS, 2009. Final Report Summary -WHETLAC Transformation of the residual whey permeate from the cheese manufacture: lactic acid production 5–11.

European Commission, Eurostat, 2015. Milk and milk product statistics – Statistics Explained [WWW Document]. URL index.php/Milk_and_milk_product_statistics http://ec.europa.eu/eurostat/statistics-explained/

European Dairy Association, 2018. Sustainability Factsheet - Dairy as an important actor for climate and the environment.

European Environmental Agency, 2018. The circular economy and the bioeconomy - Partners in sustainability. https://doi.org/10.2800/02937

European Parliament and Council, 2015. DIRECTIVE 2009/28/EC -on the promotion of the use of energy from renewable sources, Official Journal of the European Communities.

European Parliament and Council, 2009. DIRECTIVE 2009/30/EC on fuel quality.

European Parliament and Council, 2008. Directive 2008/98/EC on waste and repealing certain directives. Off. J. Eur. Union 312, 3–30. https://doi.org/2008/98/EC.; 32008L0098

Eurostat, 2018. Agriculture, forestry and fishery statistics - 2018 Edition.

Farizoglu, B., Keskinler, B., Yildiz, E., Nuhoglu, A., 2007. Simultaneous removal of C, N, P from cheese whey by jet loop membrane bioreactor (JLMBR). J. Hazard. Mater. 146, 399–407. https://doi.org/10.1016/j.jhazmat.2006.12.051

Fava, F., Totaro, G., Diels, L., Reis, M., Duarte, J., Carioca, O.B., Poggi-Varaldo, H.M., Ferreira, B.S., 2015. Biowaste biorefinery in Europe: Opportunities and research & development needs. N. Biotechnol. 32, 100–108. https://doi.org/10.1016/j.nbt.2013.11.003

Ferchichi, M., Crabbe, E., Gil, G.H., Hintz, W., Almadidy, A., Saddoud, A., Hassaïri, I., Sayadi, S., Farizoglu, B., Keskinler, B., Yildiz, E., Nuhoglu, A., Ghaly, A.E., Kamal, M.A., Ergu, T.H., Tezel, U., Gu, E., Demirer, G.N., Yorgun, M.S., Balcioglu, I.A., Saygin, O., Blonskaja, V., Vaalu, T., 2005. Influence of initial pH on hydrogen production from cheese whey. J. Biotechnol. 120, 402–409.

https://doi.org/10.1016/j.jbiotec.2005.05.017

Fernández, C., Cuetos, M.J., Martínez, E.J., Gómez, X., 2015. Thermophilic anaerobic digestion of cheese whey: Coupling H2 and CH4 production. Biomass and Bioenergy 81, 55–62. https://doi.org/10.1016/j.biombioe.2015.05.024

Ferreira Rosa, P.R., Santos, S.C., Sakamoto, I.K., Varesche, M.B.A., Silva, E.L., 2014a. Hydrogen production from cheese whey with ethanol-type fermentation: Effect of hydraulic retention time on the microbial community composition. Bioresour. Technol. 161, 10–19. https://doi.org/10.1016/j.biortech.2014.03.020

Ferreira Rosa, P.R., Santos, S.C., Silva, E.L., 2014b. Different ratios of carbon sources in the fermentation of cheese whey and glucose as substrates for hydrogen and ethanol production in continuous reactors. Int. J. Hydrogen Energy 39, 1288–1296. https://doi.org/10.1016/j.ijhydene.2013.11.011

Filer, J., Ding, H.H., Chang, S., 2019. Biochemical methane potential (BMP) assay method for anaerobic digestion research. Water (Switzerland) 11. https://doi.org/10.3390/w11050921

Food and Agriculture Organization of the United Nations (FAO), 2013. Food wastage footprint, Food and Agriculture Organization of the United Nations (FAO).

Fradinho, J.C., Oehmen, A., Reis, M.A.M., 2019. Improving polyhydroxyalkanoates production in phototrophic mixed cultures by optimizing accumulator reactor operating conditions. Int. J. Biol. Macromol. 126, 1085–1092. https://doi.org/10.1016/j.ijbiomac.2018.12.270

Fuess, L.T., Ferraz, A.D.N., Machado, C.B., Zaiat, M., 2018. Temporal dynamics and metabolic correlation between lactate-producing and hydrogen-producing bacteria in sugarcane vinasse dark fermentation: The key role of lactate. Bioresour. Technol. 247, 426–433. https://doi.org/10.1016/j.biortech.2017.09.121

Fuess, L.T., Zaiat, M., do Nascimento, C.A.O., 2019. Novel insights on the versatility of biohydrogen production from sugarcane vinasse via thermophilic dark fermentation: Impacts of pH-driven operating strategies on acidogenesis metabolite profiles. Bioresour. Technol. 286, 121379. https://doi.org/10.1016/j.biortech.2019.121379

FUSION, 2016. Estimates of European food waste levels., FUSIONS.

Gabardo, S., Rech, R., Rosa, C.A., Ayub, M.A.Z., 2014. Dynamics of ethanol production from whey and whey permeate by immobilized strains of Kluyveromyces marxianus in batch and continuous bioreactors. Renew. Energy 69, 89–96. https://doi.org/10.1016/J.RENENE.2014.03.023

Gannoun, H., Khelifi, E., Bouallagui, H., Touhami, Y., Hamdi, M., 2008. Ecological clarification of cheese whey prior to anaerobic digestion in upflow anaerobic filter. Bioresour. Technol. 99, 6105–6111. https://doi.org/10.1016/j.biortech.2007.12.037

García-Depraect, O., León-Becerril, E., 2018. Fermentative biohydrogen production from tequila vinasse via the lactate-acetate pathway: Operational performance, kinetic analysis and microbial ecology. Fuel 234,

151–160. https://doi.org/10.1016/j.fuel.2018.06.126

García-Depraect, O., Rene, E.R., Diaz-Cruces, V.F., León-Becerril, E., 2019. Effect of process parameters on enhanced biohydrogen production from tequila vinasse via the lactate-acetate pathway. Bioresour. Technol. 273, 618–626. https://doi.org/10.1016/j.biortech.2018.11.056

Gelegenis, J., Georgakakis, D., Angelidaki, I., Mavris, V., 2007. Optimization of biogas production by co-digesting whey with diluted poultry manure. Renew. Energy 32, 2147–2160. https://doi.org/10.1016/j.renene.2006.11.015

Ghaly, A.E., Kamal, M.A., 2004. Submerged yeast fermentation of acid cheese whey for protein production and pollution potential reduction. Water Res. 38, 631–644. https://doi.org/10.1016/j.watres.2003.10.019

Ghasemi, M., Ahmad, A., Jafary, T., Azad, A.K., Kakooei, S., Wan Daud, W.R., Sedighi, M., 2017. Assessment of immobilized cell reactor and microbial fuel cell for simultaneous cheese whey treatment and lactic acid/electricity production. Int. J. Hydrogen Energy 42, 9107–9115. https://doi.org/10.1016/j.ijhydene.2016.04.136

Ghimire, A., 2015. Dark fermentative biohydrogen production from organic waste and application of by-products in a biorefinery concept. PhD thesis. Universitè Paris-est; Università degli studi di Cassino; UNESCO-IHE.

Ghimire, A., Luongo, V., Frunzo, L., Pirozzi, F., Lens, P.N.L., Esposito, G., 2017. Continuous biohydrogen production by thermophilic dark fermentation of cheese whey: Use of buffalo manure as buffering agent. Int. J. Hydrogen Energy 42, 4861–4869. https://doi.org/10.1016/J.IJHYDENE.2016.11.185

Göksungur, Y., Gündüz, M., Harsa, Ş., 2005. Optimization of lactic acid production from whey by L casei NRRL B-441 immobilized in chitosan stabilized Ca-alginate beads. J. Chem. Technol. Biotechnol. 80, 1282–1290. https://doi.org/10.1002/jctb.1326

Gomes, B.C., Rosa, P.R.F.R.F.R.F., Etchebehere, C., Silva, E.L., AmâncioVaresche, M.B., Am?ncioVaresche, M.B., Rú, P., Rosa, F., Etchebehere, C., Silva, E.L., Am, M.B., Anciovaresche, ^, Rosa, P.R.F.R.F.R.F., Etchebehere, C., Silva, E.L., AmâncioVaresche, M.B., 2015. Role of homo-and heterofermentative lactic acid bacteria on hydrogen-producing reactors operated with cheese whey wastewater. Int. J. Hydrogen Energy 40, 8650–8660. https://doi.org/10.1016/j.ijhydene.2015.05.035

Gonzfilez Siso, M.I., 1996. THE BIOTECHNOLOGICAL UTILIZATION OF CHEESE WHEY: A REVIEW. Bioresour. Technol. 57, 1–11.

Gouveia, A.R., Freitas, E.B., Galinha, C.F., Carvalho, G., Duque, A.F., Reis, M.A.M., 2017. Dynamic change of pH in acidogenic fermentation of cheese whey towards polyhydroxyalkanoates production: Impact on performance and microbial population. N. Biotechnol. 37, 108–116. https://doi.org/10.1016/j.nbt.2016.07.001

Guimarães, P.M.R., Teixeira, J.A., Domingues, L.L.L.L.L.L.L.L., Guimar??es, P.M.R., Teixeira, J.A., Domingues,

L.L.L.L.L.L.L.L., Guimarães, P.M.R., Teixeira, J.A., Domingues, L.L.L.L.L.L.L.L., 2010. Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey, Biotechnology Advances. https://doi.org/10.1016/j.biotechadv.2010.02.002

Hagen, L.H., Vivekanand, V., Linjordet, R., Pope, P.B., Eijsink, V.G.H., Horn, S.J., 2014. Microbial community structure and dynamics during co-digestion of whey permeate and cow manure in continuous stirred tank reactor systems. Bioresour. Technol. 171, 350–359. https://doi.org/10.1016/j.biortech.2014.08.095

Hassan, A.N., Nelson, B.K., 2012. Invited review: Anaerobic fermentation of dairy food wastewater. J. Dairy Sci. 95, 6188–6203. https://doi.org/10.3168/jds.2012-5732

Hublin, A., Zelić, B., 2013. Modelling of the whey and cow manure co-digestion process, in: Waste Management and Research. pp. 353–360. https://doi.org/10.1177/0734242X12455088

Humberto, E.-H., Liliana, C.-M., Veronique, B., Jaime, J.-E., 2017. Feasibility of the anaerobic digestion of cheese whey in a Plug Flow Reactor (PFR) under local conditions 265–278.

IEA, 2019. The future of Hydrogen - Seizing today's opportunities [WWW Document]. URL https://webstore.iea.org/download/direct/2803?fileName=The_Future_of_Hydrogen.pdf (accessed 7.9.19).

IEA Bioenergy Task37, 2019. Country Report: Sweden.

IEA Bioenergy Task42, 2019. Technical, Economic and Environmental Assessment of Biorefinery Concepts Developing a practical approach for characterisation. IEA Bioenergy.

IEA Bioenergy Task42, 2018. Bioeconomy and biorefining strategies in the EU Member States and beyond - Reference Year 2018.

IEA Bioenergy Task42, 2012. Bio-based Chemicals - Value Added Products from Biorefineries. https://doi.org/10.1007/978-3-319-07593-8_30

Infantes, D., González del Campo, A., Villaseñor, J., Fernández, F.J., 2011. Influence of pH, temperature and volatile fatty acids on hydrogen production by acidogenic fermentation. Int. J. Hydrogen Energy 36, 15595–15601. https://doi.org/10.1016/j.ijhydene.2011.09.061

Intesa Sanpaolo, Federchimic Assobiotec, 2019. La bioeconomia in Europa.

Irfan, M., Bai, Y., Zhou, L., Kazmi, M., Yuan, S., Maurice Mbadinga, S., Yang, S.Z., Liu, J.F., Sand, W., Gu, J.D., Mu, B.Z., 2019. Direct microbial transformation of carbon dioxide to value-added chemicals: A comprehensive analysis and application potentials. Bioresour. Technol. 288, 121401. https://doi.org/10.1016/j.biortech.2019.121401

ISMEA, Laore Sardegna, 2015. Report trimestale 15/2015.

Janczukowicz, W., Zieliński, M., Debowski, M., 2008. Biodegradability evaluation of dairy effluents originated in selected sections of dairy production. Bioresour. Technol. 99, 4199–4205. https://doi.org/10.1016/j.biortech.2007.08.077

Jiang, L.L., Zhou, J.J., Quan, C.S., Xiu, Z.L., 2017. Advances in industrial microbiome based on microbial consortium for biorefinery. Bioresour. Bioprocess. 4. https://doi.org/10.1186/s40643-017-0141-0

Jo, J.H., Jeon, C.O., Lee, D.S., Park, J.M., 2007. Process stability and microbial community structure in anaerobic hydrogen-producing microflora from food waste containing kimchi. J. Biotechnol. 131, 300–308. https://doi.org/10.1016/J.JBIOTEC.2007.07.492

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M., 2010. Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. Water Res. 44, 2141–2152. https://doi.org/10.1016/j.watres.2009.12.031

Jones, R.J., Massanet-Nicolau, J., Mulder, M.J.J.J., Premier, G., Dinsdale, R., Guwy, A., Jon Jones, R., Massanet-Nicolau, J., Mulder, M.J.J.J., Premier, G., Dinsdale, R., Guwy, A., 2017. Increased biohydrogen yields, volatile fatty acid production and substrate utilisation rates via the electrodialysis of a continually fed sucrose fermenter. Bioresour. Technol. 229, 46–52. https://doi.org/10.1016/j.biortech.2017.01.015

Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. Bioresour. Technol. 199, 103–112. https://doi.org/10.1016/j.biortech.2015.10.009

Joshi, Y., Senatore, B., Poletto, M., 2011. Kluyveromyces marxianus biofilm in cheese wheey fermentation for bioethanol production. Chem. Eng. Trans. 24, 493–498. https://doi.org/https://doi.org/10.3303/CET1124083

Jung, Y.M., Lee, Y.H., 2000. Utilization of oxidative pressure for enhanced production of poly-β-hydroxybutyrate and poly(3-hydroxybutyrate-3-hydroxyvalerate) in Ralstonia eutropha. J. Biosci. Bioeng. 90, 266–270. https://doi.org/10.1016/S1389-1723(00)80080-8

Jungmeier, G., 2014. The Biorefinery Complexity Index. IEA-Bioenergy Task 42 36.

Karakashev, D., Batstone, D.J., Trably, E., Angelidaki, I., 2006. Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae. Appl. Environ. Microbiol. 72, 5138–5141. https://doi.org/10.1128/AEM.00489-06

Kasmi, M., 2018. Biological Processes as Promoting Way for Both Treatment and Valorization of Dairy Industry Effluents. Waste and Biomass Valorization. https://doi.org/10.1007/s12649-016-9795-7

Kavacik, B., Topaloglu, B., 2010. Biogas production from co-digestion of a mixture of cheese whey and dairy manure. Biomass and Bioenergy 34, 1321–1329. https://doi.org/10.1016/j.biombioe.2010.04.006

Kim, H.O.K., Wee, Y.J., Kim, J.N., Yun, J.S., Ryu, H.W., 2006. Production of lactic acid from cheese whey by batch and repeated batch cultures of Lactobacillus sp. RKY2, in: Applied Biochemistry and Biotechnology. Humana Press, pp. 694–704. https://doi.org/10.1385/ABAB:131:1:694

Kleerebezem, R., Joosse, B., Rozendal, R., Van Loosdrecht, M.C.M., 2015. Anaerobic digestion without biogas? Rev. Environ. Sci. Biotechnol. 14, 787–801. https://doi.org/10.1007/s11157-015-9374-6

Kleerebezem, R., van Loosdrecht, M.C., 2007. Mixed culture biotechnology for bioenergy production. Curr.

Opin. Biotechnol. https://doi.org/10.1016/j.copbio.2007.05.001

Koller, M., Bona, R., Chiellini, E., Fernandes, E.G., Horvat, P., Kutschera, C., Hesse, P., Braunegg, G., 2008. Polyhydroxyalkanoate production from whey by Pseudomonas hydrogenovora. Bioresour. Technol. 99, 4854–4863. https://doi.org/10.1016/j.biortech.2007.09.049

Krischke, W., Schröder, M., Trösch, W., 1991. Continuous production of L-lactic acid from whey permeate by immobilized Lactobacillus casei subsp. casei. Appl. Microbiol. Biotechnol. 34, 573–578. https://doi.org/10.1007/BF00167901

Kumar, G., Ponnusamy, V.K., Bhosale, R.R., Shobana, S., Yoon, J.J., Bhatia, S.K., Rajesh Banu, J., Kim, S.H., 2019. A review on the conversion of volatile fatty acids to polyhydroxyalkanoates using dark fermentative effluents from hydrogen production. Bioresour. Technol. 287, 121427. https://doi.org/10.1016/j.biortech.2019.121427

Kunasundari, B., Sudesh, K., 2011. Isolation and recovery of microbial polyhydroxyalkanoates. Express Polym. Lett. 5, 620–634. https://doi.org/10.3144/expresspolymlett.2011.60

Labatut, R.A., Angenent, L.T., Scott, N.R., 2011. Biochemical methane potential and biodegradability of complex organic substrates. Bioresour. Technol. 102, 2255–2264. https://doi.org/10.1016/j.biortech.2010.10.035

Lane, M.M., Morrissey, J.P., 2010. Kluyveromyces marxianus: A yeast emerging from its sister's shadow. Fungal Biol. Rev. 24, 17–26. https://doi.org/10.1016/j.fbr.2010.01.001

Lay, J.-J., Lee, Y.-J., Noike, T., 1999. Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Res. 33, 2579–2586. https://doi.org/10.1016/S0043-1354(98)00483-7

Le Hyaric, R., Benbelkacem, H., Bollon, J., Bayard, R., Escudié, R., Buffière, P., 2012. Influence of moisture content on the specific methanogenic activity of dry mesophilic municipal solid waste digestate. J. Chem. Technol. Biotechnol. 87, 1032–1035. https://doi.org/10.1002/jctb.2722

Liang, T.M., Cheng, S.S., Wu, K.L., 2002. Behavioral study on hydrogen fermentation reactor installed with silicone rubber membrane. Int. J. Hydrogen Energy 27, 1157–1165. https://doi.org/10.1016/S0360-3199(02)00099-X

Logan, B.E., Hamelers, B., Rozendal, R., Schröder, U., Keller, J., Freguia, S., Aelterman, P., Verstraete, W., Rabaey, K., 2006. Microbial fuel cells: Methodology and technology. Environ. Sci. Technol. 40, 5181–5192. https://doi.org/10.1021/es0605016

Lorini, L., di Re, F., Majone, M., Valentino, F., 2020. High rate selection of PHA accumulating mixed cultures in Sequencing Batch Reactors with uncoupled carbon and nitrogen feeding. N. Biotechnol. 113391. https://doi.org/10.1016/j.icarus.2019.113391

Lowry, O.H., Rosebrough, N.J., Farr, L., Randall, R.J., 1951. Protein Measuremente with the folin phenol reagent. Anal. Biochem. 217, 220–230. https://doi.org/10.1016/0304-3894(92)87011-4

Lu, J., Tappel, R.C., Nomura, C.T., 2009. Mini-review: Biosynthesis of poly(hydroxyalkanoates). Polym. Rev.

49, 226–248. https://doi.org/10.1080/15583720903048243

Luongo, V., Policastro, G., Ghimire, A., Pirozzi, F., Fabbricino, M., 2019. Repeated-Batch Fermentation of Cheese Whey for Semi-Continuous Lactic Acid Production Using Mixed Cultures at Uncontrolled pH. Sustainability 11, 3330. https://doi.org/10.3390/su11123330

Mazzoli, R., Bosco, F., Mizrahi, I., Bayer, E.A., Pessione, E., 2014. Towards lactic acid bacteria-based biorefineries. Biotechnol. Adv. 32, 1216–1236. https://doi.org/10.1016/j.biotechadv.2014.07.005

Miller, C., Fosmer, A., Rush, B., McMullin, T., Beacom, D., Suominen, P., 2011. 3.17 - Industrial Production of Lactic Acid, Comprehensive Biotechnology. https://doi.org/10.1016/B978-0-08-088504-9.00177-X

Mishra, P., Balachandar, G., Das, D., 2017. Improvement in biohythane production using organic solid waste and distillery effluent. Waste Manag. 66, 70–78. https://doi.org/10.1016/j.wasman.2017.04.040

Mohan, S.V., Butti, S.K., Amulya, K., Dahiya, S., Modestra, J.A., 2016. Waste Biorefinery: A New Paradigm for a Sustainable Bioelectro Economy. Trends Biotechnol. https://doi.org/10.1016/j.tibtech.2016.06.006

Mollea, C., Marmo, L., Bosco, F., 2013. Valorisation of Cheese Whey, a By-Product from the Dairy Industry. Food Ind. https://doi.org/10.5772/53159

Moncada B, J., Aristizábal M, V., Cardona A, C.A., 2016. Design strategies for sustainable biorefineries. Biochem. Eng. J. 116, 122–134. https://doi.org/10.1016/j.bej.2016.06.009

Moncada, J., El-Halwagi, M.M., Cardona, C.A., 2013. Techno-economic analysis for a sugarcane biorefinery: Colombian case. Bioresour. Technol. 135, 533–543. https://doi.org/10.1016/J.BIORTECH.2012.08.137

Mondello, G., Salomone, R., Neri, E., Patrizi, N., Bastianoni, S., Lanuzza, F., 2018. Environmental hot-spots and improvement scenarios for Tuscan “Pecorino” cheese using Life Cycle Assessment. J. Clean. Prod. 195, 810–820. https://doi.org/10.1016/J.JCLEPRO.2018.05.078

Montazeri, M., Zaimes, G.G., Khanna, V., Eckelman, M.J., 2016. Meta-Analysis of Life Cycle Energy and Greenhouse Gas Emissions for Priority Biobased Chemicals. ACS Sustain. Chem. Eng. 4, 6443–6454. https://doi.org/10.1021/acssuschemeng.6b01217

Monte, M.C.C., Fuente, E., Blanco, A., Negro, C., 2009. Waste management from pulp and paper production in the European Union. Waste Manag. 29, 293–308. https://doi.org/10.1016/j.wasman.2008.02.002

Montecchio, D., Yuan, Y., Malpei, F., 2018. Hydrogen production dynamic during cheese whey Dark Fermentation: New insights from modelization. Int. J. Hydrogen Energy 43, 17588–17601. https://doi.org/10.1016/j.ijhydene.2018.07.146

Moreno, R., Escapa, A., Cara, J., Carracedo, B., Gómez, X., 2015. A two-stage process for hydrogen production from cheese whey: Integration of dark fermentation and biocatalyzed electrolysis. Int. J. Hydrogen Energy 40, 168–175. https://doi.org/10.1016/j.ijhydene.2014.10.120

Mostafa, N.A., 1996. Production of lactic acid from whey withagar immobilized cells in a continuous packed tubular reactor. Energy Convers. Manag. 37, 253–260. https://doi.org/10.1016/0196-8904(95)00184-0

Mota, V.T., Ferraz Júnior, A.D.N., Trably, E., Zaiat, M., 2018. Biohydrogen production at pH below 3.0: Is it

possible? Water Res. 128, 350–361. https://doi.org/10.1016/j.watres.2017.10.060

Muntoni, A., 2019. Waste biorefineries : opportunities and perspectives. Detritus 05, 1–2.

Negi, S., Pandey, A.K., Vats, S., Prasad, D., 2016. AN EFFICIENT PROCESS DEVELOPMENT FOR BIOETHANOL PRODUCTION FROM READILY AVAILABLE LIGNOCELLULOSIC BIOMASS WASTE 14–17.

Noike, T., Takabatake, H., Mizuno, O., Ohba, M., 2002. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int. J. Hydrogen Energy 27, 1367–1371. https://doi.org/10.1016/S0360-3199(02)00120-9

Nova Institute, 2019. Biobased Building Blocks and Polymers - Global Capacities, Production, and Applications–Status Quo and Trends 2018-2023.

Ohnishi, A., Bando, Y., Fujimoto, N., Suzuki, M., 2010. Development of a simple bio-hydrogen production system through dark fermentation by using unique microflora. https://doi.org/10.1016/j.ijhydene.2010.05.113

Oliveira, C., Silva, M., Silva, C.E., Carvalho, G., Reis, M.A.M., 2018. Assessment of Protein-Rich Cheese Whey Waste Stream as a Nutrients Source for Low-Cost Mixed Microbial PHA Production. Appl. Sci. 8, 1817. https://doi.org/10.3390/app8101817

Oliveira, C.S.S., Silva, C.E., Carvalho, G., Reis, M.A.M., 2017. Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: Feast and famine regime and uncoupled carbon and nitrogen availabilities. N. Biotechnol. 37, 69–79. https://doi.org/10.1016/j.nbt.2016.10.008

Ottaviano, L.M., Ramos, L.R., Botta, L.S., Amâncio Varesche, M.B., Silva, E.L., 2017. Continuous thermophilic hydrogen production from cheese whey powder solution in an anaerobic fluidized bed reactor: Effect of hydraulic retention time and initial substrate concentration. Int. J. Hydrogen Energy 42, 4848–4860. https://doi.org/10.1016/j.ijhydene.2016.11.168

Outram, V., Zhang, Y., 2018. Solvent-free membrane extraction of volatile fatty acids from acidogenic fermentation. Bioresour. Technol. 270, 400–408. https://doi.org/10.1016/j.biortech.2018.09.057

Pagliano, G., Ventorino, V., Panico, A., Romano, I., Robertiello, A., Pirozzi, F., Pepe, O., 2018. The effect of bacterial and archaeal populations on anaerobic process fed with mozzarella cheese whey and buttermilk. J. Environ. Manage. 217, 110–122. https://doi.org/10.1016/j.jenvman.2018.03.085

Panesar, P.S., KENNEDY, J.F., GANDHI, D.N., Bunko, K., 2007. Bioutilisation of whey for lactic acid production. Food Chem. 105, 1–14.

Panesar, P.S., Kennedy, J.F., Knill, C.J., Kosseva, M., 2010. Production of L(+) lactic acid using Lactobacillus casei from whey. Brazilian Arch. Biol. Technol. 53, 219–226. https://doi.org/10.1590/S1516-89132010000100027

Pantazaki, A.A., Papaneophytou, C.P., Pritsa, A.G., Liakopoulou-Kyriakides, M., Kyriakidis, D.A., 2009. Production of polyhydroxyalkanoates from whey by Thermus thermophilus HB8. Process Biochem. 44, 847–853. https://doi.org/10.1016/j.procbio.2009.04.002

Pardelha, F., Albuquerque, M.G., Reis, M.A.M., Oliveira, R., Dias, J.M., 2014. Dynamic metabolic modelling of volatile fatty acids conversion to polyhydroxyalkanoates by a mixed microbial culture. N. Biotechnol. 31, 335–344. https://doi.org/10.1016/j.nbt.2013.06.008

Parisi, C., 2018. Research Brief: Biorefineries distribution in the EU, European Commission - Joint Research Centre. European Commission - Joint Research Centre. https://doi.org/10.2760/126478

Perna, V., Castelló, E., Wenzel, J., Zampol, C., Fontes Lima, D.M., Borzacconi, L., Varesche, M.B., Zaiat, M., Etchebehere, C., 2013. Hydrogen production in an upflow anaerobic packed bed reactor used to treat cheese whey. Int. J. Hydrogen Energy 38, 54–62. https://doi.org/10.1016/j.ijhydene.2012.10.022

Pfaltzgraff, L.A., De Bruyn, M., Cooper, E.C., Budarin, V., Clark, J.H., 2013. Food waste biomass: A resource for high-value chemicals. Green Chem. 15, 307–314. https://doi.org/10.1039/c2gc36978h

Plácido, J., Zhang, Y., 2018. Evaluation of Esterification and Membrane Based Solvent Extraction as Methods for the Recovery of Short Chain Volatile Fatty Acids from Slaughterhouse Blood Anaerobic Mixed Fermentation. Waste and Biomass Valorization 9, 1767–1777. https://doi.org/10.1007/s12649-017-9952-7

Porpatham, E., Ramesh, A., Nagalingam, B., 2007. Effect of hydrogen addition on the performance of a biogas fuelled spark ignition engine. Int. J. Hydrogen Energy 32, 2057–2065. https://doi.org/10.1016/j.ijhydene.2006.09.001

Povolo, S., Toffano, P., Basaglia, M., Casella, S., 2010. Polyhydroxyalkanoates production by engineered Cupriavidus necator from waste material containing lactose. Bioresour. Technol. 101, 7902–7907. https://doi.org/10.1016/j.biortech.2010.05.029

Prazeres, A.R., Carvalho, F., Rivas, J., 2012. Cheese whey management: A review. J. Environ. Manage. 110, 48–68. https://doi.org/10.1016/j.jenvman.2012.05.018

Rago, L., Baeza, J.A., Guisasola, A., 2016. Bioelectrochemical hydrogen production with cheese whey as sole substrate. J. Chem. Technol. Biotechnol. 92, 173–179. https://doi.org/10.1002/jctb.4987

Regueira, A., González-Cabaleiro, R., Ofiţeru, I.D., Rodríguez, J., Lema, J.M., 2018. Electron bifurcation mechanism and homoacetogenesis explain products yields in mixed culture anaerobic fermentations. Water Res. 141, 349–356. https://doi.org/10.1016/j.watres.2018.05.013

Reis, M.A.M., Serafim, L.S., Lemos, P.C., Ramos, A.M., Aguiar, F.R., Van Loosdrecht, M.C.M., 2003. Production of polyhydroxyalkanoates by mixed microbial cultures. Bioprocess Biosyst. Eng. 25, 377–385. https://doi.org/10.1007/s00449-003-0322-4

REN21, 2019. Renewbles in Cities - 2019 Global Status Report.

Rico, C., Muñoz, N., Rico, J.L., 2015. Anaerobic co-digestion of cheese whey and the screened liquid fraction of dairy manure in a single continuously stirred tank reactor process: Limits in co-substrate ratios and organic loading rate. Bioresour. Technol. 189, 327–333. https://doi.org/10.1016/j.biortech.2015.04.032

Rivera, I., Bakonyi, P., Cuautle-Marín, M.A., Buitrón, G., 2017. Evaluation of various cheese whey treatment scenarios in single-chamber microbial electrolysis cells for improved biohydrogen production. Chemosphere. https://doi.org/10.1016/j.chemosphere.2017.01.128

Rodriguez-Perez, S., Serrano, A., Pantión, A.A., Alonso-Fariñas, B., 2018. Challenges of scaling-up PHA production from waste streams. A review. J. Environ. Manage. 205, 215–230. https://doi.org/10.1016/j.jenvman.2017.09.083

Rodríguez, J., Kleerebezem, R., Lema, J.M., van Loosdrecht, M.C.M., 2006. Modeling product formation in anaerobic mixed culture fermentations. Biotechnol. Bioeng. 93, 592–606. https://doi.org/10.1002/bit.20765

Ronzon, T., M'Barek, R., 2018. Socioeconomic indicators to monitor the EU's bioeconomy in transition. Sustain. 10. https://doi.org/10.3390/su10061745

Rosales-Colunga, L.M., Razo-Flores, E., Ordoñ Ez, L.G., Alatriste-Mondragón, F., De León-Rodríguez, A., Lomas, C., Ordoñez, L.G., Alatriste-Mondragón, F., De León-Rodríguez, A., 2009. Hydrogen production by Escherichia coli ΔhycA ΔlacI using cheese whey as substrate. Int. J. Hydrogen Energy 35, 491–499. https://doi.org/10.1016/j.ijhydene.2009.10.097

Rossi, R., 2016. The sheep and goat sector in the EU Main features, challenges and prospects.

Rouwenhorst, R.J., Frank Jzn, J., Scheffers, W.A., van Dijken, J.P., 1991. Determination of protein concentration by total organic carbon analysis. J. Biochem. Biophys. Methods 22, 119–128. https://doi.org/10.1016/0165-022X(91)90024-Q

Ryan, M.P., Walsh, G., 2016. The biotechnological potential of whey. Rev. Environ. Sci. Biotechnol. 1–20. https://doi.org/10.1007/s11157-016-9402-1

Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. Int. J. Hydrogen Energy. https://doi.org/10.1016/j.ijhydene.2013.07.122

Sabra, W., Zeng, A.P., 2014. Mixed microbial cultures for industrial biotechnology: Success, chance, and challenges, Industrial Biocatalysis.

Saddoud, A., Hassaïri, I., Sayadi, S., 2007. Anaerobic membrane reactor with phase separation for the treatment of cheese whey. Bioresour. Technol. 98, 2102–2108. https://doi.org/10.1016/j.biortech.2006.08.013

Santos, S.C., de Sousa, A.S., Dionísio, S.R., Tramontina, R., Ruller, R., Squina, F.M., Vaz Rossell, C.E., da Costa, A.C., Ienczak, J.L., 2016. Bioethanol production by recycled Scheffersomyces stipitis in sequential batch fermentations with high cell density using xylose and glucose mixture. Bioresour. Technol. 219, 319–329. https://doi.org/10.1016/J.BIORTECH.2016.07.102

Sarma, S.J., Pachapur, V., Brar, S.K., Le Bihan, Y., Buelna, G., 2015. Hydrogen biorefinery: Potential utilization of the liquid waste from fermentative hydrogen production. Renew. Sustain. Energy Rev. 50, 942–951. https://doi.org/10.1016/j.rser.2015.04.191

Scoma, A., Rebecchi, S., Bertin, L., Fava, F., 2014. High impact biowastes from South European agro-industries as feedstock for second-generation biorefineries. Crit. Rev. Biotechnol. 8551, 1–15. https://doi.org/10.3109/07388551.2014.947238

Searle, S., Malins, C., 2013. Availability of cellulosic residues and wastes in the EU - International Council on Clean Transportation. Int. Counc. Clean Transp. White Pap.

Secchi, N., Giunta, D., Pretti, L., García, M.R., Roggio, T., Mannazzu, I., Catzeddu, P., 2012. Bioconversion of ovine scotta into lactic acid with pure and mixed cultures of lactic acid bacteria. J. Ind. Microbiol. Biotechnol. 39, 175–181. https://doi.org/10.1007/s10295-011-1013-9

Shete, B.S., Shinkar, N.P., 2013. Dairy Industry Wastewater Sources, Characteristics & its Effects on Environment. Int. J. Curr. Eng. Technol. ISSN 2277 - 4106 3, 1611–1615. https://doi.org/10.1075/target.18.2.05shi

Siebert, S., 2015. Bio - Waste Recycling in Europe Against the Backdrop of the Circular Economy Package.

Sikora, A., Błaszczyk, M., Jurkowski, M., Zielenkiewicz, U., 2013. Lactic acid bacteria in hydrogen-producing consortia: on purpose or by coincidence? Lact. Acid Bact. Food, Heal. Livest. Purp. 487–514. https://doi.org/10.5772/50364

Singhvi, M., Zendo, T., Sonomoto, K., 2018. Free lactic acid production under acidic conditions by lactic acid bacteria strains: challenges and future prospects. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-018-9092-4

Slavov, A.K., 2017. General characteristics and treatment possibilities of dairy wastewater -a review. Food Technol. Biotechnol. 55, 14–28. https://doi.org/10.17113/ft b.55.01.17.4520

Soldi, R., 2016. Mapping of the disadvantaged areas for milk production in Europe. https://doi.org/10.2863/727410

Stamatelatou, K., Antonopoulou, G., Tremouli, A., Lyberatos, G., 2011. Production of gaseous biofuels and electricity from cheese whey. Ind. Eng. Chem. Res. 50, 639–644. https://doi.org/10.1021/ie1002262

Stams, A.J.M., Dijkema, C., Plugge, C.M., Lens, P., 1998. Contribution of 13C-NMR spectroscopy to the elucidation of pathways of propionate formation and degradation in methanogenic environments. Biodegradation 9, 463–473. https://doi.org/10.1023/A:1008342130938

Staniszewski, M., Kujawski, W., Lewandowska, M., 2007. Ethanol production from whey in bioreactor with co-immobilized enzyme and yeast cells followed by pervaporative recovery of product – Kinetic model predictions. J. Food Eng. 82, 618–625. https://doi.org/10.1016/J.JFOODENG.2007.03.031

Steinbüchel, A., Hein, S., 2001. Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. Adv. Biochem. Eng. Biotechnol. 71, 81–123. https://doi.org/10.1007/3-540-40021-4_3

Taleghani, H.G., Ghoreyshi, A.A., Najafpour, G.D., 2018. Thin film composite nanofiltration membrane for lactic acid production in membrane bioreactor. Biochem. Eng. J. 132, 152–160.

https://doi.org/10.1016/j.bej.2018.01.020

Tan, G.-Y.A., Chen, C.L., Li, L., Ge, L., Wang, L., Razaad, I.M.N., Li, Y., Zhao, L., Mo, Y., Wang, J.Y., 2014. Start a research on biopolymer polyhydroxyalkanoate (PHA): A review. Polymers (Basel). 6, 706–754. https://doi.org/10.3390/polym6030706

Tang, J., Wang, X.C., Hu, Y., Zhang, Y., Li, Y., 2016. Effect of pH on lactic acid production from acidogenic fermentation of food waste with different types of inocula. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2016.11.111

Thauer, R.K., Jungermann, K., Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41, 100–80.

Tian, H., Mancini, E., Treu, L., Angelidaki, I., Fotidis, I.A., 2019. Bioaugmentation strategy for overcoming ammonia inhibition during biomethanation of a protein-rich substrate. Chemosphere 231, 415–422. https://doi.org/10.1016/j.chemosphere.2019.05.140

Traversi, D., Bonetta, S., Degan, R., Villa, S., Porfido, A., Bellero, M., Carraro, E., Gilli, G., 2013. Environmental Advances Due to the Integration of Food Industries and Anaerobic Digestion for Biogas Production: Perspectives of the Italian Milk and Dairy Product Sector. Bioenerg. Res. https://doi.org/10.1007/s12155-013-9341-4

Tremouli, A., Antonopoulou, G., Bebelis, S., Lyberatos, G., 2013. Operation and characterization of a microbial fuel cell fed with pretreated cheese whey at different organic loads. Bioresour. Technol. 131, 380–389. https://doi.org/10.1016/j.biortech.2012.12.173

Tribst, A.A.L., Falcade, L.T.P., de Oliveira, M.M., 2019. Strategies for raw sheep milk storage in smallholdings: Effect of freezing or long-term refrigerated storage on microbial growth. J. Dairy Sci. 102, 4960–4971. https://doi.org/10.3168/jds.2018-15715

Tuli, A., Sethi, R.P., Khanna, P.K., Marwaha, S.S., Kennedy, J.F., 1985. Lactic acid production from whey permeate by immobilized Lactobacillus casei. Enzyme Microb. Technol. 7, 164–168. https://doi.org/10.1016/0141-0229(85)90058-4

United Nation, 2015. Transformin our world: The 2030 agenda for sustainable development. Undp.

Vagnoni, E., Franca, A., Porqueddu, C., Duce, P., 2017. Environmental profile of Sardinian sheep milk cheese supply chain: A comparison between two contrasting dairy systems. J. Clean. Prod. 165, 1078–1089. https://doi.org/10.1016/j.jclepro.2017.07.115

Valentino, F., Brusca, A.A., Beccari, M., Nuzzo, A., Zanaroli, G., Majone, M., 2013. Start up of biological sequencing batch reactor (SBR) and short-term biomass acclimation for polyhydroxyalkanoates production. J. Chem. Technol. Biotechnol. 88, 261–270. https://doi.org/10.1002/jctb.3824

Valentino, F., Karabegovic, L., Majone, M., Morgan-Sagastume, F., Werker, A., 2015a. Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels. Water Res. 77, 49–63.

https://doi.org/10.1016/j.watres.2015.03.016

Valentino, F., Morgan-Sagastume, F., Campanari, S., Villano, M., Werker, A., Majone, M., 2017. Carbon recovery from wastewater through bioconversion into biodegradable polymers. N. Biotechnol. 37, 9–23. https://doi.org/10.1016/j.nbt.2016.05.007

Valentino, F., Riccardi, C., Campanari, S., Pomata, D., Majone, M., 2015b. Fate of β-hexachlorocyclohexane in the mixed microbial cultures (MMCs) three-stage polyhydroxyalkanoates (PHA) production process from cheese whey. Bioresour. Technol. 192, 304–311. https://doi.org/10.1016/j.biortech.2015.05.083

Valta, K., Damala, P., Angeli, E., Antonopoulou, G., Malamis, D., Haralambous, K.J., 2017. Current Treatment Technologies of Cheese Whey and Wastewater by Greek Cheese Manufacturing Units and Potential Valorisation Opportunities. Waste and Biomass Valorization 8, 1649–1663. https://doi.org/10.1007/s12649-017-9862-8

Van Ginkel, S., Logan, B.E., 2005. Inhibition of biohydrogen production by undissociated acetic and butyric acids. Environ. Sci. Technol. 39, 9351–9356. https://doi.org/10.1021/es0510515

Vasmara, C., Marchetti, R., 2017. Initial pH influences in-batch hydrogen production from scotta permeate. Int. J. Hydrogen Energy 42, 14400–14408. https://doi.org/10.1016/j.ijhydene.2017.04.067

Vassilev, I., Hernandez, P.A., Batlle-Vilanova, P., Freguia, S., Krömer, J.O., Keller, J., Ledezma, P., Virdis, B., 2018. Microbial Electrosynthesis of Isobutyric, Butyric, Caproic Acids, and Corresponding Alcohols from Carbon Dioxide. ACS Sustain. Chem. Eng. 6, 8485–8493. https://doi.org/10.1021/acssuschemeng.8b00739

Vea, E.B., Romeo, D., Thomsen, M., 2018. Biowaste Valorisation in a Future Circular Bioeconomy. Procedia CIRP 69, 591–596. https://doi.org/10.1016/j.procir.2017.11.062

Venetsaneas, N., Antonopoulou, G., Stamatelatou, K., Kornaros, M., Lyberatos, G., 2009. Using cheese whey for hydrogen and methane generation in a two-stage continuous process with alternative pH controlling approaches. Bioresour. Technol. 100, 3713–3717. https://doi.org/10.1016/j.biortech.2009.01.025

Vivekanand, V., Mulat, D.G., Eijsink, V.G.H., Horn, S.J., 2018. Synergistic effects of anaerobic co-digestion of whey, manure and fish ensilage. Bioresour. Technol. 249, 35–41. https://doi.org/10.1016/J.BIORTECH.2017.09.169

Wenzel, J., Fuentes, L., Cabezas, A., Etchebehere, C., 2017. Microbial fuel cell coupled to biohydrogen reactor: a feasible technology to increase energy yield from cheese whey. Bioprocess Biosyst. Eng. 40, 807–819. https://doi.org/10.1007/s00449-017-1746-6

World Economic Forum (WEF), 2019. Top 10 Emerging Technologies 2019. World Econ. Forum Annu. Meet. 2019 4–15.

Xu, Q., Ouyang, J., Liu, P., Liu, J., Qian, Z., Zheng, Z., 2018. Valorization of dairy waste for enhanced D-lactic acid production at low cost. Process Biochem. 71, 18–22. https://doi.org/10.1016/j.procbio.2018.05.014

Yang, P., Zhang, R., McGarvey, J.A., Benemann, J.R., 2007. Biohydrogen production from cheese processing wastewater by anaerobic fermentation using mixed microbial communities. Int. J. Hydrogen Energy 32, 4761–4771. https://doi.org/10.1016/j.ijhydene.2007.07.038

Yang, S.J., Kataeva, I., Hamilton-Brehm, S.D., Engle, N.L., Tschaplinski, T.J., Doeppke, C., Davis, M., Westpheling, J., Adams, M.W.W., 2009. Efficient degradation of lignocellulosic plant biomass, without pretreatment, by the thermophilic anaerobe 'Anaerocellum thermophilum' DSM 6725. Appl. Environ. Microbiol. 75, 4762–4769. https://doi.org/10.1128/AEM.00236-09

Yenigün, O., Demirel, B., 2013. Ammonia inhibition in anaerobic digestion: A review. Process Biochem. 48, 901–911. https://doi.org/10.1016/j.procbio.2013.04.012

Yesil, H., Tugtas, A.E., Bayrakdar, A., Calli, B., 2014. Anaerobic fermentation of organic solid wastes: Volatile fatty acid production and separation. Water Sci. Technol. 69, 2132–2138. https://doi.org/10.2166/wst.2014.132

Yilmazer, G., Yenigün, O., 1999. Two-phase anaerobic treatment of cheese whey. Water Sci. Technol. 40, 289–295. https://doi.org/10.1016/S0273-1223(99)00397-2

Yoon, J.J., Bhatia, S.K., Banu, J.R., Kumar, G., Ponnusamy, V.K., Bhosale, R.R., Shobana, S., Yoon, J.J., Bhatia, S.K., Rajesh Banu, J., Kim, S.H., 2019. A review on the conversion of volatile fatty acids to polyhydroxyalkanoates using dark fermentative effluents from hydrogen production. Bioresour. Technol. 287, 121427. https://doi.org/10.1016/j.biortech.2019.121427

Yorgun, M.S., Balcioglu, I.A., Saygin, O., 2008. Performance comparison of ultrafiltration, nanofiltration and reverse osmosis on whey treatment. Desalination 229, 204–216. https://doi.org/10.1016/j.desal.2007.09.008

Zhang, S., Kim, T.H., Lee, Y., Hwang, S.J., 2012. Effects of VFAs concentration on bio-hydrogen production with clostridium bifermentans 3AT-ma. Energy Procedia 14, 518–523. https://doi.org/10.1016/j.egypro.2011.12.968