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Abstract: In this study, a novel biopolymer in the form of extracellular polysaccharide (EPS) produced by Pedobacter sp. strain MCC-Z was extracted, purified and characterized, and its surface and emulsifying properties were evaluated. The purified bioemulsifier, namely Pdb-Z, showed high emulsifying activity (E24%= 64%) and reduced the surface tension of water up to 41 mN/m with a critical micelle concentration value of 2.6 mg/mL. The chemical characterization of Pdb-Z was performed using 1HNMR, FT-IR, HPLC/MS/MS and GC/MS. Pdb-Z was found to contain 67% of carbohydrates, consisting mainly of galactose and minor quantities of talose, 30% of lipids, being pentadecanoic acid the major lipidic constituent, and 3% of proteins. The bioemulsifier was a glycolipids-protein complex with an estimated molecular mass of 106 Da. Furthermore, Pdb-Z emulsified pure aliphatic and aromatic hydrocarbons as well as diesel more efficiently than commercial synthetic surfactants, used for comparison. Our results suggest Pdb-Z has interesting properties for applications in remediation of hydrocarbon-contaminated environments and bioremediation processes.

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Chemical characterization and surface properties of a new bioemulsifier produced by *Pedobacter* sp.strain MCC-Z

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1.Introduction

Many prokaryotic and eukaryotic microorganisms synthesize a wide range of surface active compounds (SACs) which exhibit surface activities at interfaces, including the ability to lower surface and interfacial tension of liquids and to form micelles and microemulsions between different phases [1,2,3]. They exist in a wide variety of structurally different amphiphilic molecules containing both hydrophilic and hydrophobic units. Neu [4] divided microbial surface active compounds into low molecular weight SACs, also namely biosurfactants, and high molecular weight SACs including amphiphilic and polyphilic polymers. The biosurfactants lower the surface and interfacial tension, whereas the high molecular weight SACs, also called bioemulsifiers, are more effective in stabilizing oil-in-water emulsions [5]. The most extensively studied bioemulsifiers are emulsan, a lipopolysaccharide isolated from *Acinetobacter calcoaceticus* RAG-1 (now *Acinetobacter venetianus* RAG-1) [6,7], and alasan, a complex consisting of an anionic polysaccharide and proteins produced by *Acinetobacter radioresistens* KA53 [8]. Many SACs have the advantages of higher biodegradability, selectivity, biocompatibility and lower toxicity over chemically synthesized surfactants as well as higher specific activity at extreme temperatures, pH level, and salinity [9,10,11]. Thanks to all these superior characteristics, microbial SACs found applications in agriculture, environmental, food, cosmetic, chemical fields and medicine [12,13]. The main applications of SACs in environmental remediation include the bioremediation technologies to improve the biodegradation rate of organic compounds and soil washing. In a previous study [14], several

environmental strains were isolated from soil and a screening was performed for the ability to produce biopolymers with strong superficial and interfacial properties [15]. *Pedobacter* sp. strain MCC-Z produces an extracellular bioemulsifier in mineral salts medium with glucose as the only carbon source. In the present study, the crude bioemulsifier (BIO-Z) was purified (Pdb-Z), chemically characterized and its surface and emulsifying properties were evaluated.

2.Materials and Methods

2.1 Bacterial strain

Pedobacter sp. strain MCC-Z was isolated from a farm soil located in Piana di Monte Verna (CE), Italy [14]. The analysis of the 16S rRNA gene sequence (GeneBank accession number JF279930) assigned the strain MCC-Z to the genus *Pedobacter* a Gram negative, non-flagellated heterotrophic bacterium, non-pathogen, non-spore-forming, rod shaped, and producing pink colored colonies [15].

2.2 Media and culture conditions

A preculture was prepared by inoculating the strain MCC-Z into 10 mL of mineral salts medium (MSM) [16] amended with 1% (w/v) glucose as the only carbon source in a 50-mL flask. The preculture was incubated with shaking at 120 rpm for 72 h at 30°C. Then, 4.5 mL was used to inoculate 450 mL of MSM-0.5% glucose in a 1.5 L flask and the culture was incubated under the same conditions for 4 days.

2.3 Extraction and purification of bioemulsifier

The culture supernatant containing the crude bioemulsifier BIO-Z was separated from the cells by centrifugation at 9,000 x g at 25°C for 35 min. The supernatant was filtered through a 0.45-µm Millipore membrane (Milford, MA, USA), and the filtrate was dialyzed using 12 kDa cut

off dialysis membrane (Sigma-Aldrich, Steinheim, Germany) in order to separate molecules less than 12 kDa. The dialyzed was concentrated by evaporation and the concentrate was extracted with hexane (4:1, v/v) in a separatory funnel at 25°C. The white emulsion was separated from the water phase and then washed four times with additional water. Hexane was removed by rotary evaporation at 50°C under reduced pressure and the residue was then freeze-dried and weighed. For purification, the crude water-soluble bioemulsifier was applied to a Sephadex GC-25 (Pharmacia, Uppsala, Sweden). The column was pre-equilibrated and eluted with deionized water with the flow rate maintained at 1.0 mL/min. Fractions having major emulsifying activity (64%) were concentrated and lyophilized for further characterizations.

2.4 Emulsifying activity and surface tension measurements

The emulsification index (E_{24}%) was employed to quantify the emulsifying activity and the standard emulsification assay was conducted as previously described by Cooper and Goldenberg [17]. Two millilitres of cell-free supernatant was vigorously mixed with equal volume of hexane using a vortex mixer for 2 minutes into glass test tubes (105x15 mm). After that, the mixture was allowed to settle at room temperature for 24 h. Emulsification index (E_{24}%) was calculated as the ratio of the height occupied by the emulsion to the total liquid height. All measurements were mean values from three independent experiments.

The hydrocarbon substrate specificity of Pdb-Z was determined by the emulsification assay as described above, except that the standard hydrocarbon substrate, hexane, was substituted by other pure hydrocarbons (*n*-hexadecane, *iso*-octane, cyclohexane, toluene, xylene) or diesel fuel. The emulsification assay was performed on Pdb-Z samples diluted in distilled water at different concentrations (0.25-0.5-0.75-1 mg/mL). The synthetic surfactants Tween 20, Tween 80 and Triton X-100 (Sigma Aldrich) at 0.75% w/v were used as reference compounds. All measurements were mean values from three independent experiments.

Surface tension measurements were performed by the du Nöuy ring method using a 3S tensiometer (GBX, Romans sur Isère, France) on Pdb-Z solubilized in Ultra pure MilliQ water at concentrations ranging from 0 to 5.0 mg/mL. All determinations were performed in three replicates in two separate experiments.

2.5 Chemical characterization

2.5.1 Composition of bioemulsifier

The lipid content of Pdb-Z was determined by GC-MS analysis as described by Di Luccia et al. [18]. After an alkaline digestion, the extracted mixture of species was directly analysed by GC-MS as TMS derivates. For lipid analysis the oven temperature was increased from 25°C to 90°C in 1 min and held at 90°C for 1 min before increasing to 140°C at 25°C/min, to 200°C at 5°C/min and finally to 300°C at 10°C/min. Each species was univocally identified on the basis of retention times and electron impact fragmentation spectra (NIST library).

Neutral sugars were determined by GC-MS analysis after hydrolysis with methanolic-HCl at 80°C for 16 h. After neutralization by adding Ag_2CO_3, the re-N-acetylation was achieved with 50 µL acetic anhydride and incubating at room temperature overnight. The trimethylsilylation was carried out in 500 µL SIGMA-SIL-A at 80°C for 20 min. The sample was dried down under nitrogen, dissolved in 50 µL hexane, and centrifuged to remove the excess of solid reagents. The hexane supernatant was used for the GC-MS analysis. GC-MS analyses were performed on a Agilent 7890 GC/5975 MS system (Agilent technologies) equipped with DB-5MS fused silica capillary column (30 m, 0.25 mm ID, 0.25 µm ft) from J&W.

Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad). After the addition of 2x Laemmli buffer (Sigma-Aldrich), the samples were boiled at 100°C for 5 min and resolved by SDS-polyacrylamide gel electrophoresis. Furthermore analysis was performed on the Coomassie blue-stained spots excised from the gels. The spots were selected for mass spectral identification by the merging of images analyses. Proteins excised from the gel were

reduced, alkylated, and *in situ* digested with trypsin as described by Di Luccia et al.[18]. The peptide mixtures were analysed using a CHIP MS 6520 QTOF equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies). The sample was then fractionated on a C18 reverse-phase capillary column (75 µmx43mm in the Agilent Technologies chip) at flow rate of 400 nL/min with a linear gradient of eluent B (0.1 formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 60% in 50 min. Spectra were scanned over the range 400-2000 m/z. Analysis software and MS/MS spectra were compared with non-redundant protein databases (NCBInr 20090924, 9760158 sequences) and UniprotSwissprot (2011, 167910 sequences), with the taxonomy restriction to *Homo sapiens*, using MASCOT 2.1 software (Matrix Science, Boston, USA).

2.5.2 Fourier transform infrared spectroscopy

The infrared spectrum of the dried Pdb-Z was recorded on a Fourier Transformed Infrared spectrometer (IR Affinity-1 with ATR Miracle 10 Shimadzu) in the 4600 to 400 cm^{-1} spectral region with 45 accumulated scans and resolution of 8 cm^{-1}.

2.5.3 ^{1}H Nuclear Magnetic Resonance analysis and Hydrodinamic radius study

The ^{1}HNMR spectra of Pdb-Z were obtained at 600 MHz in D_2O and DMSO-d6 solution on a Brucker Avance 600 Hz, equipped with a 5 mm inverse broadband probe with z-axis gradients. All data were processed with TopSpin. ^{1}HNMR diffusion experiments were performed using the LED sequence with bipolar gradients [19]. The attenuation measured with this sequence is given by:

$$I/I_0 = -\exp\left[D (\gamma_H \delta \; G \; 2/\pi)^2 (\Delta - \delta/3 - \tau/2) \right] \qquad (1)$$

where I/I_0 is the normalized signal intensity, D is the diffusion coefficient, δ is the duration of the gradient pulse, γ_H is the gyromagnetic ratio of ^{1}H, G is the gradient strength, Δ is the diffusion time and τ is the eddy current delay. Typical acquisition

parameters were: recycle delay time between diffusion experiments 5 s; Δ, 1 s (DMSO) or 3 s (D_2O); δ, 4 ms; τ, 5 ms. For the DMSO sample, hydrodynamic radius (R_h) of an equivalent spherical particle was calculated using the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi (R_h} \quad (2)$$

Where η is the macroscopic viscosity value of the solvent, T the absolute temperature and k_B the Boltzmann constant. In the case of the D_2O sample, we calculated the hydrodynamic radius using dioxane as internal standard [20]. The Rh of the particle is calculated through the following relationship:

$$R_{h,eff}^{part} = R_h^{dioxane} (D_{dioxane}/D_{part}) \quad (3)$$

where $R_h^{dioxane}$ is the hydrodynamic radius of dioxane (0.21 nm) and $D_{dioxane}$ and D_{part} are the measured diffusion coefficients for dioxane and the particle, respectively.

3.Results and discussion

3.1 Organisms and culture conditions

In a previous study, the optimization of the culture conditions (incubation time, media, glucose concentration) was performed to improve the bioemulsifier production by a soil bacterium belonging to the Bacteroidetes phylum, *Pedobacter* sp. MCC-Z [15]. The maximum emulsifying activity was observed at the end of the exponential phase (3 days after inoculation) and remained constant for 48 h, indicating no further production of the bioemulsifier during the stationary phase. In particular, the emulsification index E_{24}% was equal to 64% at 92 h after the inoculation in MSM with 0.5% glucose as the only carbon source. The yield of the crude bioemulsifier BIO-Z was 0.1 – 0.15 g/L in MSM-glucose medium. The crude

bioemulsifier BIO-Z maintained its emulsification properties over a wide range of pH (3-11), at high salinity (20% NaCl), and during exposure to elevated temperatures (up to 80°C). Moreover, the water-in-oil emulsions formed by BIO-Z showed a good stability at room temperature, maintaining 62% of the original emulsion volume over a period of 16 weeks [15].

3.2 Purification and emulsifying activity

The dialyzed solution of the crude bioemulsifier BIO-Z was separated by gel filtration chromatography on Sephadex GC-25. Four fractions separated from the column, named as fraction I - IV, were tested for their emulsifying activity. The fractions I and IV possessed no emulsifying activity, whereas the fractions II and III showed an E_{24}% approximately equal to 64%. Respective recovery rates for the fractions were found to be 64.5% and 25.7%. The fractions II and III, namely Pdb-Z, were collected, concentrated and further characterized. An established criterion for emulsion-stabilizing capacity is the ability of an emulsifier to maintain at least 50% of the original emulsion volume 24 h after its formation [21]. Therefore our results indicate that Pdb-Z is an efficient bioemulsifier.

3.3 Chemical composition

Pdb-Z contained 67% of carbohydrates and was composed of galactose, xylose, N-acetyl glucosamine, galacturonic acid and talose monomer units. In terms of peak area, galactose was present as major constituent. Therefore, Pdb-Z can be considered as galactan polymers. Pdb-Z showed a lipid content of about 30% being pentadecanoic acid the major constituent and 12-methil-tridecanoic acid and adipic acid the minor constituents. The weight percentages of monosaccharides and lipids of Pdb-Z are shown in Table 1. SDS-PAGE analysis of Pdb-Z indicated the existence of two major proteins with apparent molecular masses of 23 and 8 kDa. The N-terminal amino acid sequences of the two proteins were determined. The first amino acids of the 23-kDa and 8-kDa proteins showed high similarity to two proteins from *Pedobacter*

agri: the first one (Accession number WP_010600298) containing an outer membrane protein β-barrel domain and OmpA domain and the second one (Accession number WP_010601798) containing a conserved domain of putative periplasmatic proteins. Recently, the secretion of OmpA-like proteins with emulsifying activity has been demonstrated to be a general property of the oil degrading *Acinetobacter* strains [22] being the activity due to hydrophobic amino acids in a β-barrel region [23]. Overall, our results suggested Pdb-Z is a complex where glycolipids are bound to two proteins. Moreover, the first evidence is provided of an involvement of OmpA-like proteins in the emulsifying activity by bacteria other than *Proteobacteria.*

3.4 FT-IR and ^{1}H Nuclear Magnetic Resonance spectroscopy

Pdb-Z was submitted to FT-IR and ^{1}HNMR analyses for identification of the main functional groups present in the bioemulsifier. In the FT-IR spectrum (Figure 1), the presence of a large broad band at 3300 cm^{-1}, assigned to O-H stretching, was indicative of significant water and O-H content, typical of polysaccharides. The spectrum also showed a band at 1654 cm^{-1} (υ C=O, amide) and another intense band at 1060 cm^{-1} (υ C-O-C, ethers). The attribution of the carbonyl band to an amide group was supported by the presence of bands at 1550 cm^{-1} (υ N-H, amide). Considering the carbohydrate structure, a small band at 900 cm^{-1} was seen in the spectrum. This band is related to anomers in polysaccharides since the region between 950 and 700 cm^{-1} is strongly dependent on the anomeric carbon [24]. Other important absorption bands in FT-IR spectrum of Pdb-Z were the one at 1740 cm^{-1}, assigned to C=O stretching of acetyl ester bonds, and two bands at 2970 and 2880 cm^{-1}, assigned to C-H asymmetric stretch of CH_2 and CH_3 groups, respectively [25]. Overall, the FT-IR spectrum suggested Pdb-Z is predominantly a polysaccharide although proteins are also present. Proton NMR study in DMSO and D_2O (Figure 2) also confirmed the presence of carboxyl, alkyl, methyl and keto groups (5.01 ppm, 4.88 ppm, 4.975 ppm, either/ester at 3.477 ppm, alkanes at 1.626 ppm, 1.241 ppm and 0.853 ppm). Proton NMR confirmed the presence of sugars and aliphatic chains whereas no signals

associated to proteins were evident. These results supported the previous conclusion of GC/MS analysis of Pdb-Z.

3.5 Hydrodinamic behaviour

In order to get insights into the dimension of the molecules constituting Pdb-Z, we conducted a diffusion study using NMR. DMSO is known to break the inter- and intra-molecular hydrogen bonds of polysaccharides, leading to the dispersion of aggregates and making it possible to study individual polymer chains. Variable-gradient ^{1}H-NMR experiments allowed measuring the R_h of the different components of the mixture through the measurement of D, the diffusion coefficient. In Table 2, the apparent D values for different signals are reported, together with the calculated R_h using the equation (2). From a hydrodynamic point of view, the mixture appears to be heterogeneous. R_h values smaller than 0.8 nm are typical of small molecules. In the sugar region, we find two values of around 5 nm, whereas larger values are observed for two signals in the aliphatic region (in the 11-13 nm range).

A different situation was observed in D_2O. The measured D for all the signals in the spectrum appeared to be homogeneous. Table 3 shows the measured D for the bioemulsifier (D^{part}) and dioxane (D^{diox}), used as internal standard [19], and the calculated R_h for the bioemulsifier, using then equation (3). A second measurement was performed on a sample diluted 1:10, in order to determine the effect of the concentration on particle dimensions. The behavior in D_2O differs from that in DMSO in two main aspects: i) the signals present a homogeneous D; ii) the particle size is largely increased, giving R_h values in the order of 20 nm. Such large particles display very broad/not detectable signals for the core, and only sugar and aliphatic chains that are mobile tails can be detected. The calculated R_h values are similar to those found for other carbohydrate-containing polymers like cellulose fibers [26], amylose [27], glycogen [28] and amylopectins [29]. Dinadayala and coworkers [30] reported for rabbit glycogen the values of the hydrodynamic radius and molecular mass of about 23 nm and $7x10^6$ Da, respectively. Then, we

can suppose that Pdb-Z has an molecular weight approximately equal to 10^6 Da. At the concentrations used in this study, the aggregation state of the molecules did not change significantly.

3.6 Surface properties of Pdb-Z

In order to evaluate the surface properties of Pdb-Z, the surface tension of increasing Pdb-Z concentrations was determined. Concentrations from 0 to 2 mg/mL reduced the surface tension from 73.7 ±0.1 to 41.4±0.6 mN/m (n=6) whereas no further decrease was observed when the concentrations were increased up to 5 mg/mL. A plot of surface tension versus the log of Pdb-Z concentration is presented in Figure 3. The CMC value for Pdb-Z, calculated as the intersection between two regression lines describing the curve, was equal to 2.6 mg/mL. The CMC is the concentration above which Pdb-Z reaches the saturation forming supra-molecular aggregates. In order to compare Pdb-Z performance with well-characterized high molecular weight SACs, the surface tension was used as a measure of Pdb-Z effectivity whereas the CMC value was used as a measurement of its efficiency as previously established by Neu [4]. Pdb-Z exhibited comparable ability to reduce the surface tension with Alasan by *A. radioresistens* [31] and superior performance as compared with Emulsan by *A. venetianus* RAG-1 [7] even though a higher CMC value was obtained for Pdb-Z in both cases. Neverthless, Pdb-Z presents CMC values comparable with Arabic gum (1.7 mg/mL) [32], a commercial emulsifier extensively used in food industry, indicating similar efficiency. Recently, Gutiérrez et al. [33] have characterized the emulsifying properties of a glycoprotein extract produced by a marine bacterium belonging to the Bacteroidetes phylum, *Flexibacter* sp. strain TG382. However, its surface properties has not been determined yet.

3.7 Emulsifying activity with various hydrophobic substrates

The substrate specificity of Pdb-Z was evaluated on different hydrophobic substrates and results are presented in Table 4. Pdb-Z formed stable emulsions with alkanes, aromatic hydrocarbons and diesel. The highest emulsifying activity was obtained with aromatic compounds, xylene (68%) and toluene (66%). Among aliphatic compounds, *iso*-octane served as the best substrate whereas the lowest E_{24}% value (21%) was obtained with *n*-hexadecane and cyclohexane at Pdb-Z concentration of 0.25 mg/mL. The broad range of substrate specificity of the bioemulsifier Pdb-Z suggests that it could be a good candidate for application in hydrocarbon remediation and oil recovery [34]. Synthetic commercial surfactants Tween 20, Tween 80 and Triton X-100 at 0.75 mg/mL were used to evaluate Pdb-Z performance as hydrocarbon emulsifier. Pdb-Z exhibited higher emulsifying activity on all hydrophobic substrates, except diesel fuel (40±1.5), in comparison with the synthetic surfactants (Table 4). Furthermore, the emulsions formed by Pdb-Z remained stable for four months at room temperature. Comparable extended stability has been previously observed for the glycoprotein bioemulsifiers produced by a marine *Antarctobacter* [35].

4.Conclusion

In this study, a new polymeric emulsifying agent was extracted and purified from *Pedobacter* sp. strain MCC-Z, belonging to the phylum Bacteroidetes, and its chemical composition, surface properties and emulsifying activity were defined. Pdb-Z was identified as a glicolipids-protein complex with an mass molecular of approximately $7x10^6$ Da. The high molecular weight, the stable physicochemical behaviour and the ability of Pdb-Z to form stable emulsions with several hydrocarbons and oils suggests potential applications in a variety of industrial sectors, such as food and cosmetics for emulsion formulation, as well as in environmental areas, such as oil-removal from tanks and ducts. In future, the structure-function relationship of this macromolecule will be studied for further applications.

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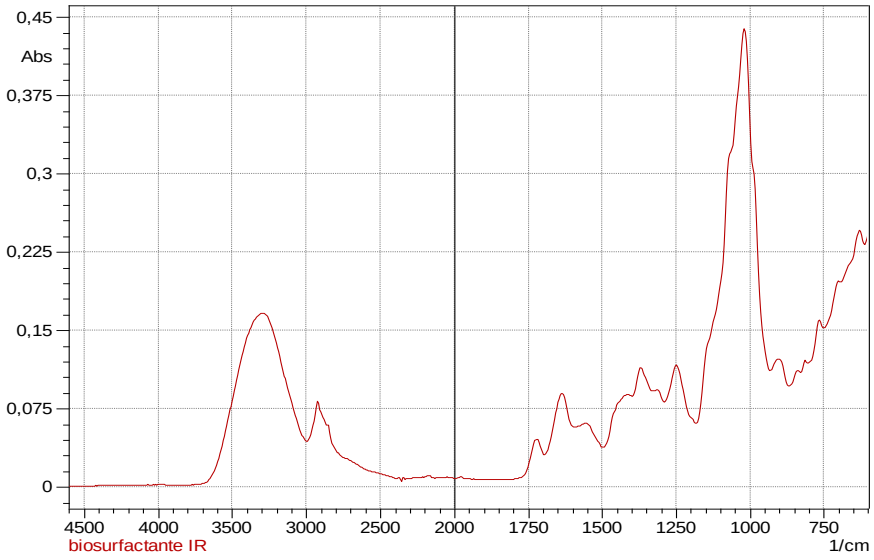
Figure 1: FT-IR spectrum of the bioemulsifier Pdb-Z produced of *Pedobacter* sp. strain MCC-Z

Figure 2: ^{1}H spectra NMR in (A) D_2O and (B) DMSO of Pdb-Z.

Figure 3: The surface tension of Pdb-Z solutions at different concentrations. The values are averages based on three replicates in two separate experiments (n=6) ± the standard deviation.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3500
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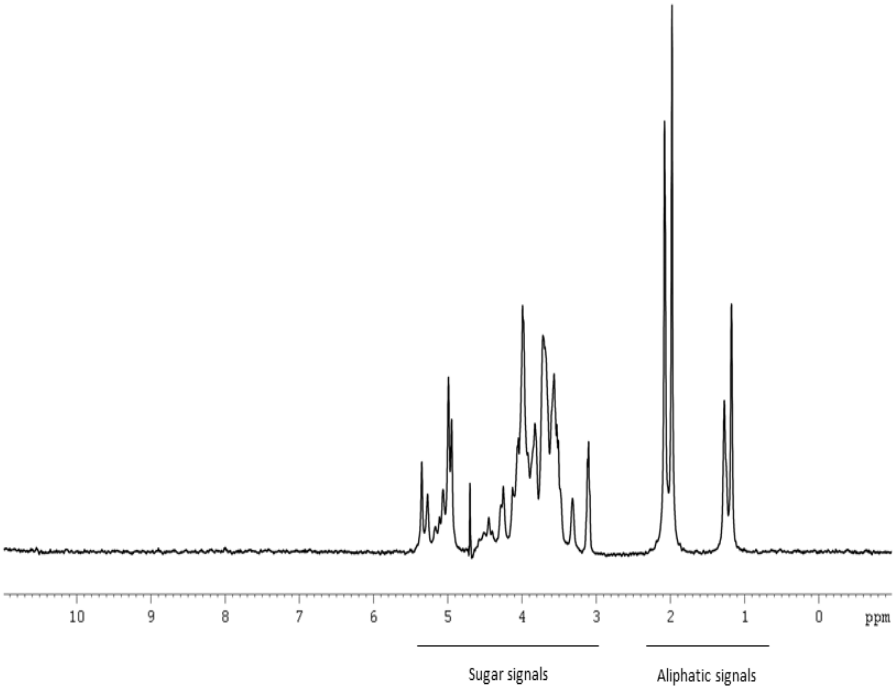
1500
1250
1000
750
1/cm
biosurfactante IR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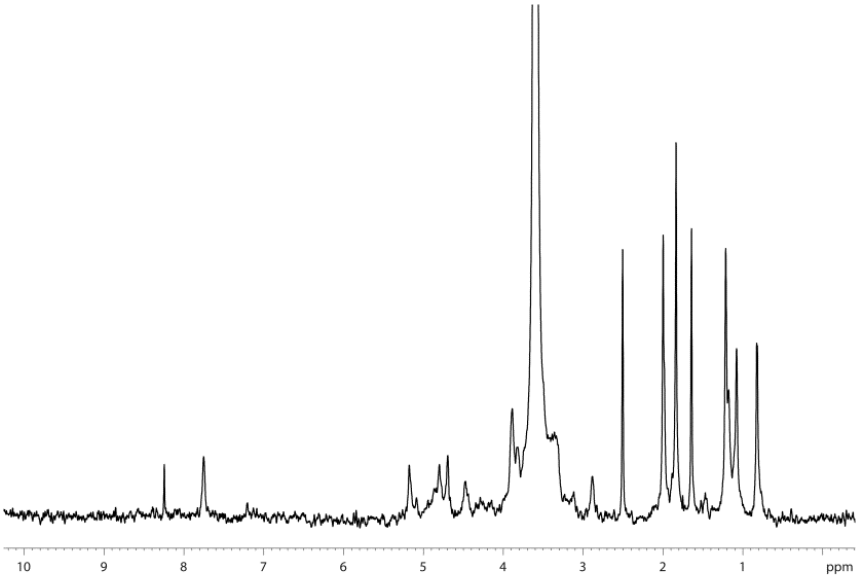
9
8
7
6
5
4
3
2
1
0
ppm
Sugar signals
Aliphatic signals

A


10
9
8
7
6
5
4
3
2
1
ppm

B

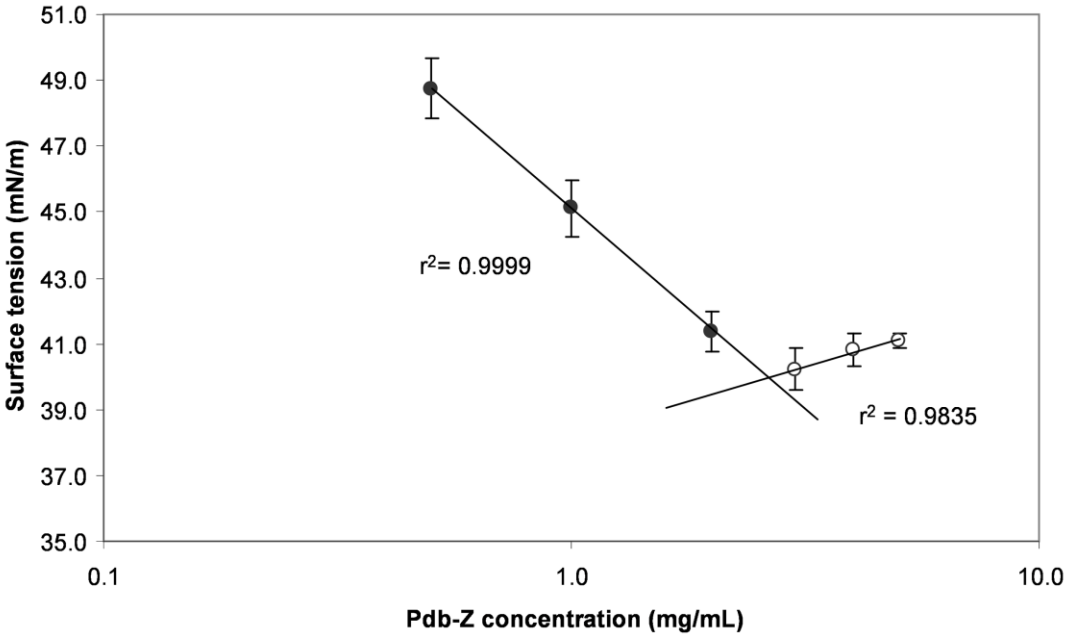

51.0
49.0
47.0
45.0
43.0
41.0
39.0
37.0
35.0
Surface tension (mN/m)
r2= 0.9999
r2 = 0.9835
0.1
1.0
10.0
Pdb-Z concentration (mg/mL)

Table 1: Monosaccharide and fatty acids composition of Pdb-Z produced by *Pedobacter* sp. strain MCC-Z.

Compound	Monosaccharides	
	Molecular formula	Wt%
Xylose	$C_5H_{10}O_5$	11.54
Galactose	$C_6H_{12}O_6$	51.17
N-acetylclucosamine	$C_8H_{15}NO_6$	4.73
Talose	$C_6H_{12}O_6$	0.14
Galacturonic acid	$C_6H_{10}O_7$	0.96
	Fatty acids	
	Molecular formula	Wt%
12-methyl-tridecanoic acid	$C_{14}H_{28}O_2$	2.43
Pentadecanoic acid	$C_{15}H_{30}O_2$	10.97
2-methoxy-myristic acid	$C_{14}H_{28}O_2$	6.07
Palmitic acid	$C_{16}H_{32}O_2$	4.47
Stearic acid	$C_{18}H_{32}O_2$	2.54
3-hydroxy-stearic acid	$C_{18}H_{36}O_3$	4.75
Adipic acid	$C_6H_{10}O_4$	0.21

Table 2: Self-diffusion coefficients, D, measured for Pdb-Z and the calculated hydrodynamic radius (R_h) for different signals observed in the DMSO spectrum.

δ (ppm)	$D/10^{-10}$ (m^2s^{-1})	R_h (nm)
8.25	6.19	0.19
7.75	0.14	8.34
5.18	0.22	5.30
4.80	1.93	0.60
4.70	0.23	5.09
3.89	0.69	1.68
3.60	6.22	0.19
2.00	0.10	10.99
1.84	0.09	13.43
1.64	4.80	0.24
1.22	1.70	0.68

Table 3: Self-diffusion coefficients, D, measured at 300K in D_2O for the bioemulsifier (D^{part}) and dioxane (D^{diox}), and the calculated hydrodynamic radius (R_h) at two different concentrations.

Dilution	$D^{part}/10^{-10}(m^2s^{-1})$	$D^{diox}/10^{-10}(m^2s^{-1})$	R_h (nm)
1:1	0.088	9.77	23.5
1:10	0.096	9.88	21.8

Table 4: Hydrocarbon substrate specificity of Pdb-Z and commercial synthetic surfactants[a].

Hydrocarbon/oil	Emulsifying index (E_{24}%)						
	Pdb-Z(mg/mL)				Tween-20	Tween-80	Triton X-100
	0.25	0.5	0.75	1.0	0.75 (mg/mL)		
n-Hexane	64.2±1.8	65.4±1.9	66.2±1.5	66.1±2.4	53.3±1.8	55.2±2.3	58.5±1.4
n-Hexadecane	21.4±1.9	21.4±1.8	64.1±2.5	64.5±2.5	57.2±1.4	59.4±1.6	60.1±2.1
Cyclohexane	21.2±1.6	62.6±1.8	62.3±2.4	62.3±2.4	48.2±2.1	44.1±1.9	48.0±1.8
iso-octane	40.4±1.9	62.0±2.2	62.6±2.2	62.1±2.0	58.1±2.4	54.0±1.8	54.3±1.9
Toluene	66.3±1.7	66.5±1.9	66.4±2.1	66.1±1.8	28.4±1.5	32.3±1.8	35.0±1.6
Xylene	68.2±2.0	68.5±1.5	68.3±1.5	68.4±1.7	62.3±2.5	59.3±1.6	61.7±1.7
Diesel fuel	28.5±2.0	28.1±2.4	44.4±1.5	44.1±1.7	61.0±2.6	59.6±1.9	63.7±1.5

[a] The values are the means±S.D.(n=3).

***Reviewer Suggestions**

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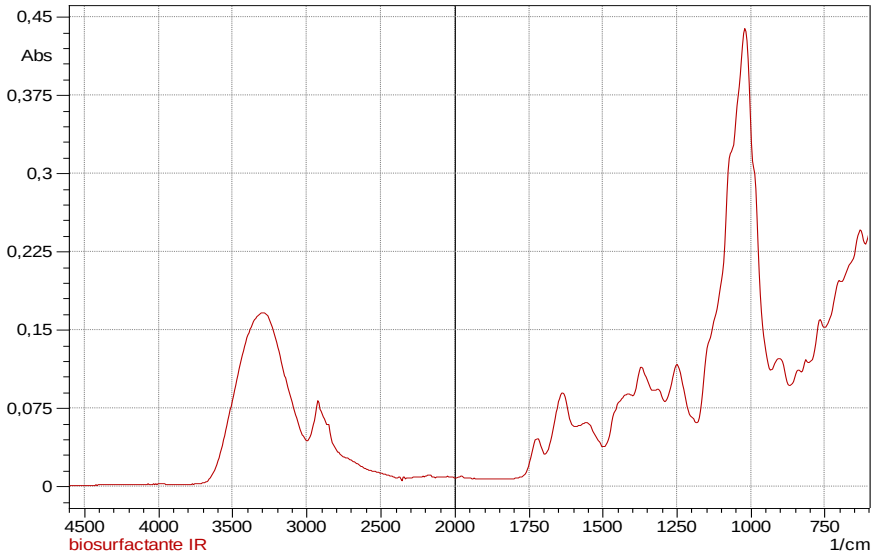
Figure(s)



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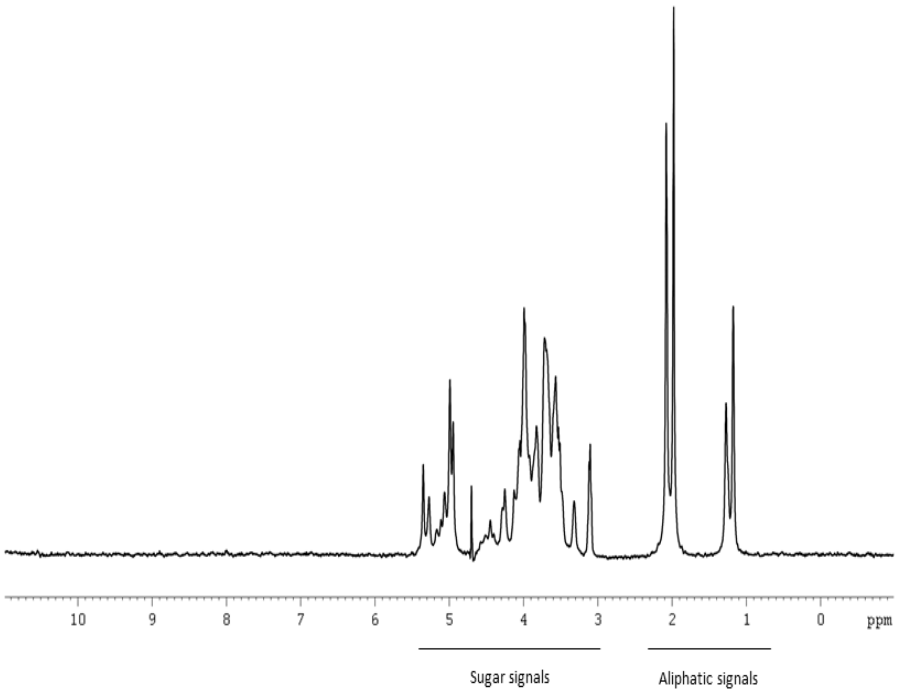

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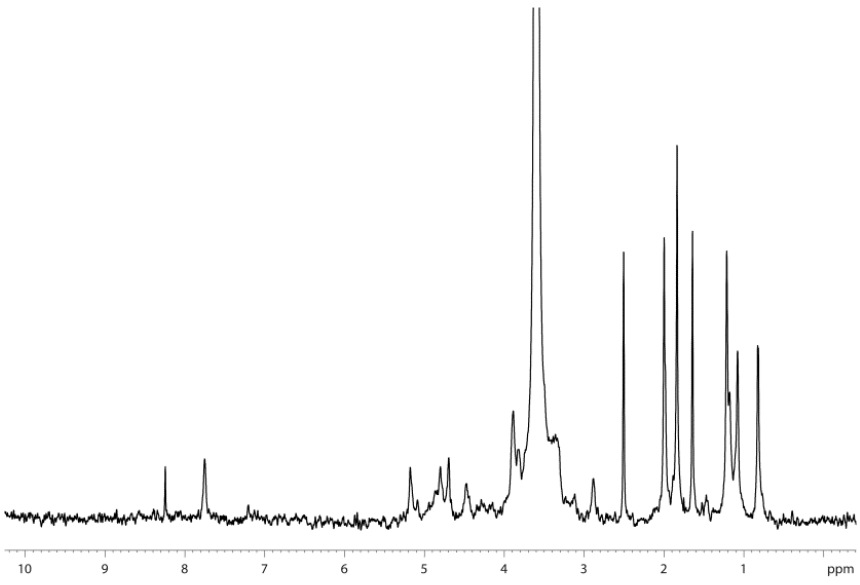

B

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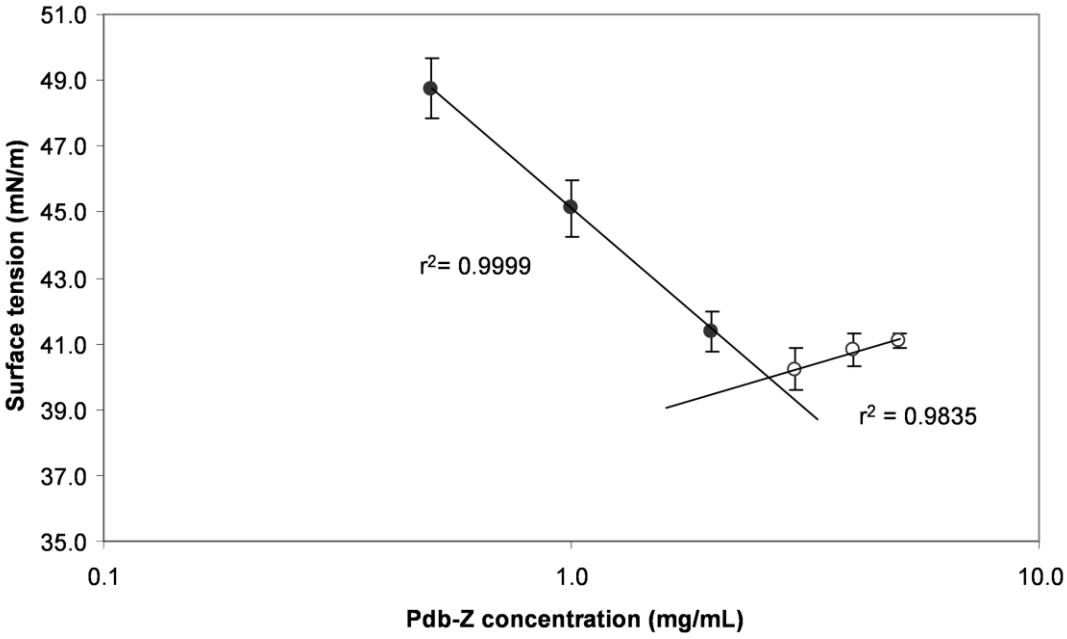

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