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ORIGINAL PAPER

Agricultural Feedstock Supplemented with Manganese for Biosurfactant Production by *Bacillus subtilis*

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Abstract The greatest challenge in producing biomolecules at industrial scale is cost. In order to provide cheaper sources, the present study describes the production of biosurfactant using a low-cost medium supplemented with manganese. The feedstock used to produce biosurfactant was crude glycerol, a by-product of biodiesel production. Results showed that 5% (v/v) of glycerol and 0.05 mM of manganese was the best combination to produce biosurfactant. The produced biosurfactant was able to reduce surface tension and showed emulsification activity in diesel fuel. The main functional groups of the biosurfactant were identified by ^{1}H NMR and FTIR spectra. We identified the molecule as surfactin based on comparison with surfactin standard spectra described in the literature. This study showed conversion of low-value glycerol into value-added products as biosurfactant. The use of a by-product as a carbon source for biosurfactant production is a possible strategy for reducing production costs. In addition, biosurfactant production by *Bacillus subtilis* can be considered safe and commercially viable, because it is a non-pathogenic bacterium.

Keywords Fermentation · Bioproducts · Agriculture · Glycerol · Biodiesel · Biofuels

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Introduction

Biosurfactants are complex biomolecules produced by bacteria and fungi. They have important properties including solubility, low critical micelle concentration (CMC) and surface tension reduction [1].

The industrial-scale production of biosurfactants must overcome some challenges, such as low yield, expensive substrates and downstream processing operations that increase production costs. The commercially available biosurfactant surfactin is valued at approximately $15.3/1 mg. On the other hand, the cost of chemical surfactants is around one dollar/lb, to put it in perspective [2]. Therefore, potential substrates for biosurfactant production have been sought from agro-industrial crops and residues, to provide cheaper and renewable sources for production at industrial scale.

Biodiesel is obtained from triglycerides by a transesterification reaction with methanol. The main by-product from biodiesel production is glycerol. The world biodiesel market might reach 37 billion gallons by 2016 [3]. Consequently, crude glycerol will be increasingly available. Therefore, conversion of this low-value glycerol into value-added products have attracted attention.

The biosurfactant produced by *Bacillus subtilis* strains consists of a long-chain fatty acid linked in a short peptide moiety composed of seven amino acids. There are natural variations of the surfactin chemical structure [4]. The peptide sequence may change due to substitutions of amino acid in the peptide ring [5–7]. In addition, the length of the fatty acid chain can vary between 13 and 15 carbons [8–10] or least common homologous with 12 and 16 carbons [9, 11, 12]. These homologous can exhibit different properties and activities [13, 14].

The species *B. subtilis* is able to grow in many alternative carbon sources, including agricultural waste and by-products [15]. The supplementation of the medium with metallic ions may induce overproduction of surfactin. Manganese and iron salts added to the culture medium enhances both the biomass and surfactin concentration [16, 17].

This article describes the production of surfactin by *B. subtilis* in a medium with glycerol, a low-cost carbon source, supplemented with manganese salts. The properties of the crude surfactin such as surface tension and emulsification activity were tested. Chemical characterisation of the purified surfactin was obtained using Infrared spectra and ^{1}H NMR spectra.

Materials and Methods

Growth Conditions of *Bacillus subtilis*

Bacillus subtilis ATCC 6633 was grown in Erlenmeyer flasks with 50 mL of nutrient broth and 5% of glycerol. The medium was incubated on a rotary shaker at 180 rpm and 35 °C for 24 h. Afterwards, the culture medium and the cells were separated by centrifugation for 20 min at 1500×*g* using a K-24 centrifuge. The cells were washed with sterile sodium chloride solution 0.85% (w/v). The inoculum was adjusted by measuring the optical density of 0.35×10^{-1} (1.66 g L^{-1}) at 600 nm and 1 mL was used to inoculate the production medium.

Biosurfactant Production

The culture medium for biosurfactant production was composed of 50 mL of Bushnell-Haas medium. Factors such as glycerol concentration (5, 7 and 9% v/v) and manganese sulfate ($MnSO_4H_2O$ 99.6%—Mallinckrodt) concentration were tested to allow higher productivity of biosurfactant. The $MnSO_4$ was added to the medium to obtain concentrations of 0.01 and 0.05 mM. Bacterial growth was monitored by measuring the optical density at 600 nm (Hach DR/2500 Spectrophotometer). A calibration curve was built to relate the absorbance with cell dry weight. The pH of the production medium was measured after fermentation using a Digimed DMPH-2 pH meter. The flasks were incubated on a rotary shaker at 180 rpm and 35 °C for 72 h. The production of dry crude biosurfactant was calculated using optimum conditions for biosurfactant production.

Biosurfactant Extraction

Bacillus subtilis cells were removed from culture medium by centrifugation at 1500×*g* for 20 min using a K-24 centrifuge. The cell-free supernatant was subjected to acid precipitation, according Cooper et al. [18]. by the addition of 6 M HCl until the pH reached pH 2.0 and was stored at 4 °C overnight. Crude biosurfactant was recovered by centrifugation at 1500×*g* for 20 min.

Surface Tension Measurement

Surface tension measurements were performed using a Krüss K6 Tensiometer equipped with a Du Noüy platinum ring. The crude biosurfactant was dissolved in a phosphate buffer pH 7. The surface tension was plotted against concentration of crude biosurfactant to determine the critical micelle concentration (CMC).

Emulsification Index (E_{24})

The ability of the biosurfactant to emulsify liquid such as water and oil fuel was tested. Emulsifying activity was determined by the addition of 2 mL of diesel fuel and the same volume of biosurfactant solution at different concentrations in test tubes. The tubes were then vortexed at maximum speed for 2 min, and the emulsions produced were allowed to settle for 24 h at room temperature. The emulsification index (E_{24}) was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) [19].

Purification of the Biosurfactant

Crude biosurfactant was purified by column chromatography filled with silica gel 0.03–0.2 mm, 60 A (Acros Organics). The silica was suspended in chloroform/methanol (2:1). The crude biosurfactant (0.5 g) was dissolved in chloroform/methanol (2:1). The column was eluted using solutions with increasing polarities. Chloroform/methanol/ammonium hydroxide solution at a concentration of 28% (v/v) (80:20:4) (v/v/v), chloroform/methanol/ammonium hydroxide 28% (75:25:4) (v/v/v) and chloroform/methanol/ammonium hydroxide 28% (65:35:5) [5]. Fractions were collected and the presence of biosurfactant was detected by thin layer chromatography with ninhydrin solution. The fractions containing the biosurfactant were placed in a flask and the solvent was evaporated under vacuum by a rotary evaporator. The purified biosurfactant was analysed by FT-IR and NMR spectroscopy.

Characterisation with FT-IR Spectroscopy and Nuclear Magnetic Resonance (NMR)

Fourier transform infrared spectroscopy FT-IR was used to determine the chemical nature of the biosurfactant. The main functional groups of biosurfactant were obtained using an FT-IR spectrometer Shimadzu 8300.

The ^{1}H NMR spectra of biosurfactant were recorded on a Bruker 600 MHz spectrometer at room temperature operating, 64 scans (with tetramethylsilane as internal standard). Thirty milligrams of purified biosurfactant were dissolved in 0.5 mL deuterochloroform ($CDCl_3$). The assignment of the peaks in the ^{1}H NMR spectra was done according to the literature [8, 9, 17, 20].

Results and Discussion

Effect of Glycerol on Biosurfactant Production

Bacillus subtilis growth in the medium with different concentrations of glycerol is shown in Fig. 1. *Bacillus subtilis* growth was reported in cell dry weight (g L^{-1}) and the pH of the medium was measured after 48 h of incubation at 35 °C. The medium with 5% (v/v) glycerol supported the best *B. subtilis* growth and the pH of the medium was around 6. Higher levels of crude glycerol affected growth and the pH of the medium negatively. The medium with 7 and 9% (v/v) of glycerol strongly inhibited the *B. subtilis* growth. The pH of the medium decreased to around 4 and 5.

Glycerol is a molecule with a strong influence on the osmotic pressure within cells. High levels of glycerol in the medium can cause intracellular modifications in order to guarantee the bacterial adaptation exposed to unfavourable conditions [21]. Hence, in this study the concentration of glycerol greater than 5% in the medium prevented the bacterial growth.

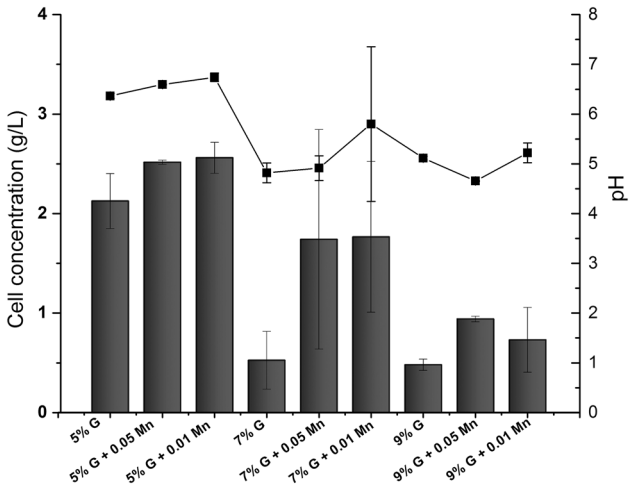

Fig. 1 *Bacillus subtilis* growth in medium with different concentrations of glycerol and manganese (*grey bar*). Measurement of pH value of medium after 72 h (*filled squared with solid line*). *Error bars* values of three independent experiments

The results showed that the addition of 0.01 or 0.05 mM of $MnSO_4$ in the medium increased microbial growth in all glycerol concentrations, especially in samples with 5% of glycerol. The cell dry weight reached 2.13 g L^{-1} in medium with glycerol 5% (v/v) while the samples with 0.01 and 0.05 mM of $MnSO_4$ reached 2.56 and 2.52 g L^{-1}, respectively. Therefore, there was no difference in the *B. subtilis* growth between 0.01 and 0.05 mM of $MnSO_4$.

Crude Biosurfactant Production

High glycerol concentrations (9% v/v) did not allow growth of the *B. subtilis*. Consequently, it was not possible to recovery any biosurfactant. At the concentrations of 7 and 5% (v/v), the amount of crude biosurfactant produced was 26 and 146 mg/L, respectively. Sousa et al. [22] also used 5% of glycerol in the medium to produce biosurfactant with different strains of *Bacillus*. The acid pH value of the medium at 7 and 9% of glycerol probably interfered with the recovery of biosurfactant because the biosurfactant is not soluble under acidic conditions [15].

The amount of dry crude biosurfactant was highest in the medium containing $MnSO_4$. The medium at 5% of glycerol supplemented with 0.01 and 0.05 mM of $MnSO_4$ produced 740 and 793 mg L^{-1} of crude biosurfactant, respectively. The manganese plays an important role in the surfactin production, because it improves nitrogen metabolism as it promotes synthesis of free amino acid required for surfactin production [23, 24].

Although manganese salts improve the surfactin production, the combination of glycerol and manganese can promote biofilm-associated sporulation [25]. For this, it is important to know which concentration of glycerol and manganese is the best for biosurfactant production. In this study, the addition of 0.05 mM of $MnSO_4$ improved the biosurfactant production significantly.

The medium with 5% glycerol supplemented with 0.01 and 0.05 mM $MnSO_4$ achieved superior production when compared with production from a synthetic medium. Al-Wahaibi et al. [26] used *B. subtilis* to produce biosurfactant in minimal medium with different sources of carbon. The yield in minimal medium with glucose and molasses was 300 and 500 mg L^{-1} of biosurfactant, respectively. Liu et al. [14] obtained 692 mg L^{-1} of biosurfactant from LB medium. In this work, the use of a low cost co-product as a source of carbon achieved a maximum production of 793 mg L^{-1}. Therefore, the use of this carbon source might be able to reduce the production costs of the biosurfactant.

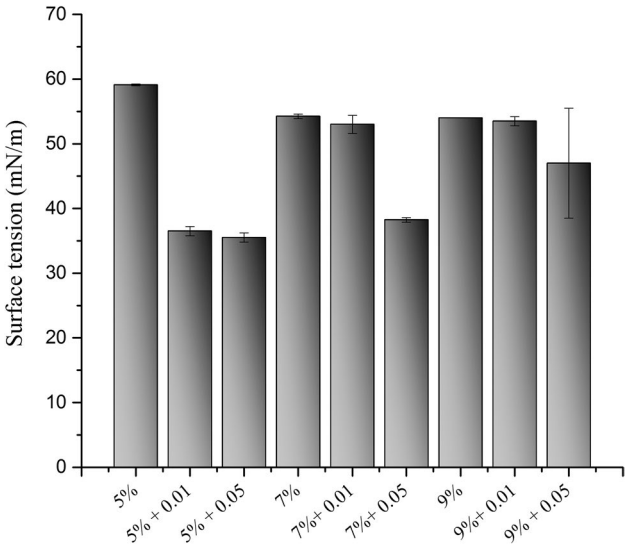

Fig. 2 Surface tension values (mN m^{-1}) obtained in the medium at 5, 7 and 9% (v/v) glycerol after 72 h of incubation. The medium was supplemented with 0.01 and 0.05 mM $MnSO_4$

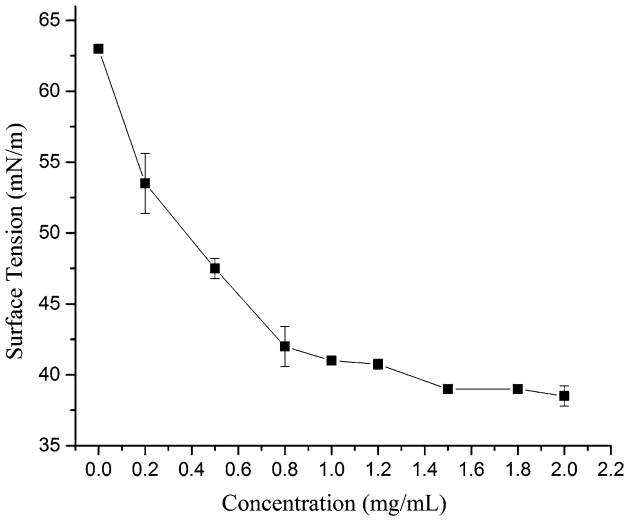

Fig. 3 Surface tension plotted against concentration of crude biosurfactant solution

Surface Tension and Critical Micelle Concentration (CMC)

Surface tension values (mN/m) of the medium at 5, 7 and 9% (v/v) glycerol are shown in Fig. 2. The media with 5% glycerol supplemented with 0.01 and 0.05 mM $MnSO_4$ were able to reduce the medium surface tension by 39 and 38%, respectively.

The surface tension values at different concentrations of crude biosurfactant are shown in Fig. 3. The crude biosurfactant solution at 0.8 mg mL^{-1} was able to reduce the buffer surface tension from 63 to 42 mN m^{-1}. Crude biosurfactant solution at 1.5 mg mL^{-1} (1.5 g L^{-1}) reduced the surface tension to 39 mN m^{-1}. The surface tension reduction and CMC value found by Abdel-Mawgoud et al. [27] were 36 mN m^{-1} and 15.3 mg L^{-1}, respectively. These authors suggest that variations in CMC values depends on the purity of the surfactin. The CMC values determined in the present study were from a crude biosurfactant. Thus, it may explain the high value of CMC found in this study. In addition, Liu et al. [28] proposed that the number of carbons of the fatty acids chain influence in the CMC and solubility of the surfactin.

Emulsifying Index (E_{24})

The emulsifying power is another important property of the biosurfactants. The emulsifying index of the crude biosurfactant solution against diesel fuel increased from 23.6 to 33.7%, according to the biosurfactant concentration. The highest emulsifying index was 37.7% at 1.5 mg mL^{-1} of crude biosurfactant solution. Interestingly, concentrations higher than 1.5 did not guarantee high emulsifying index. The crude biosurfactant solution showed properties such as emulsifying power and surface tension reduction that could improve oil recovery processes.

Purification and Chemical Characterisation of the Biosurfactant

The purified biosurfactant obtained from column chromatography showed a retention factor of 0.5. The same value of *Rf* was reported by Cho et al. [29]. The IR spectra of

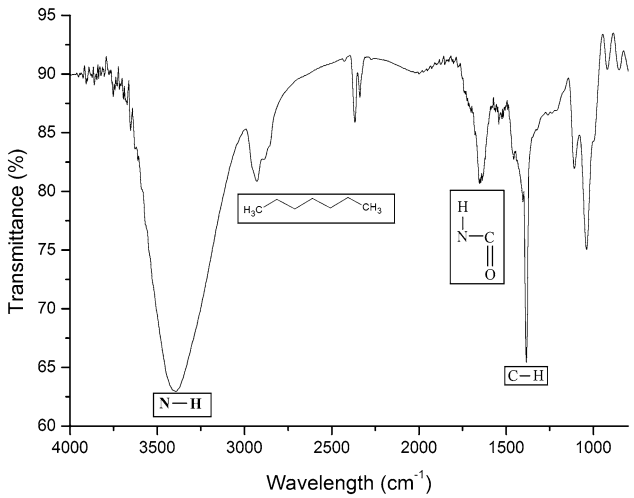

Fig. 4 Infrared spectra of the biosurfactant produced by *B. subtilis*

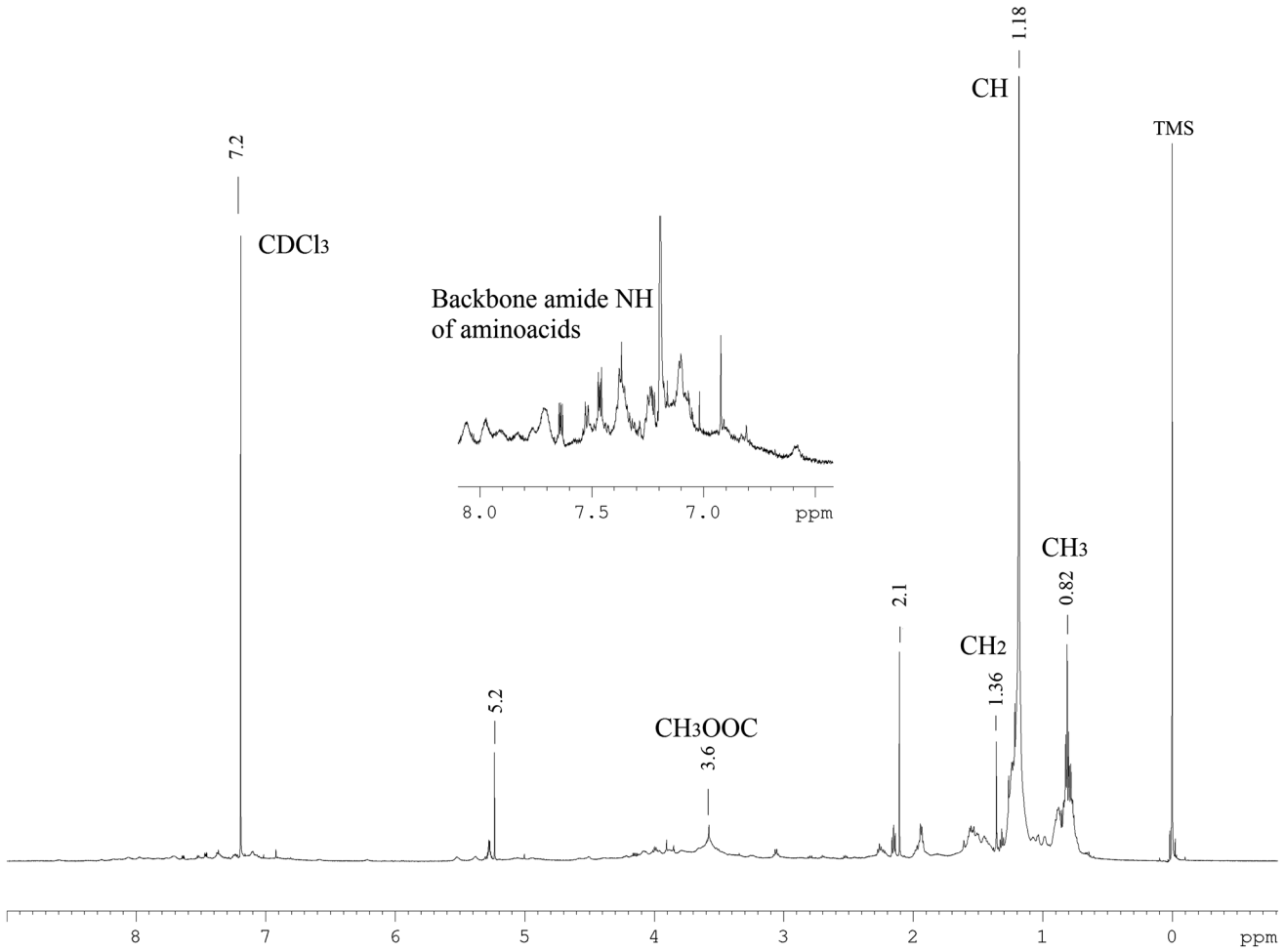

Fig. 5 Nuclear magnetic resonance (^{1}H NMR) spectra of the purified biosurfactant obtained in $CDCl_3$ at 25 °C

the biosurfactant indicates the presence of a peptide component at 3398 cm^{-1} resulting from N–H stretching mode as shown in Fig. 4. Bands at 2933 and 1382 cm^{-1} indicated the presence of an aliphatic chain. The absorbance around 1650 cm^{-1} belonged to C=O stretching vibration of the amide I region [30]. The peak at 1109 is because of C–O–C vibrations in esters [30, 31].

Figure 5 shows the ^{1}H NMR spectra as well as their assignments. The assigned peaks in ^{1}H NMR spectra showed similarity among the surfactin spectra described in other studies [5–8, 17–20, 32].

Backbone-amide-NH groups are in the region from δ = 7.7 to 7.0 ppm. Signals around δ = 5.2 indicated Hα from amino acids, which comprise the hydrophilic moiety. The peaks at δ = 2.1–0.82 ppm confirmed the presence of a long aliphatic chain, the hydrophobic moiety. A methyl ester proton (CH_3OOC) at δ = 3.6 ppm was observed. Distinct regions identified by IR and ^{1}H NMR spectra presented evidence that the molecule in the study is the biosurfactant, surfactin.

Conclusion

The crude glycerol from a biodiesel refinery can be a low-cost feedstock for biosurfactant production. The production is superior when compared with similar studies using glucose or LB medium as carbon source. In addition, biosurfactant production by *B. subtilis* can be considered as a safe molecule, because this member of the genus *Bacillus* is non-pathogenic. Other benefits of the process include the sustainable use of glycerol and the reduction in production costs of a highly useful product. Also, the biotechnological valorisation of crude glycerol makes biodiesel production more sustainable and economically attractive.

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Compliance with Ethical Standards

Conflict of interest The authors have declared no conflict of interest.

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