

Development of low-cost culture media for effective biosurfactant production

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ABSTRACT

In this work, biosurfactant production by Pseudomonas aeruginosa and Bacillus subtilis strains was optimized using low-cost substrates. The highest biosurfactant production (3.2 g/L) by the P. aeruginosa strain was obtained using a culture medium containing corn steep liquor (CSL) (10% (v/v)) and molasses (10% (w/v)), whereas the best biosurfactant production by the B. subtilis isolate (1.3 g/L) was obtained using a culture medium consisting of 10% (v/v) of CSL. Subsequently, for the B. subtilis strain, the effect of different metals (iron, manganese and magnesium) on biosurfactant production was evaluated. When the culture medium CSL 10% was supplemented with the optimum concentration of those metals simultaneously, the biosurfactant production was increased up to 4.8 g/L. The biosurfactant produced by the P. aeruginosa strain was characterized as a mixture of eight different rhamnolipid congeners, being the most abundant the mono-rhamnolipid Rha-C₁₀-C₁₀, and the biosurfactant produced by the B. subtilis isolate consisted of a mixture of C₁₃-, C₁₄- and C₁₅-surfactin. Both biosurfactants exhibited a good performance in oil recovery assays when compared with chemical surfactants, suggesting their potential use as an alternative to traditional chemical surfactants in enhanced oil recovery or bioremediation.

1. INTRODUCTION

Surfactants are an important class of chemical compounds widely used in many of the everyday products we use. Most conventional surfactants available nowadays are chemically synthesized from petrochemical resources, meaning that they are expensive and hazardous to the environment due to their recalcitrant nature (Rebello et al., 2014).

In the recent years, an increase in environmental awareness has led to much more interest in the use of renewable-based, biodegradable and more environmentally friendly surfactants. The market

for these “green” alternatives to synthetic surfactants was 344 kilo tons in 2013, and it is expected to reach 462 kilo tons and 2308 million USD by 2020 (Grand View Research, 2014).

Biosurfactants, surface-active compounds synthesized by a variety of microorganisms (bacteria, yeasts and filamentous fungi), are attracting a pronounced interest owing to their potential advantages over their chemical counterparts; namely biosurfactants exhibit a similar or better performance and have less impact on the environment when compared with chemical surfactants, due to their lower toxicity and higher biodegradability. Furthermore, biosurfactants exhibit useful properties that include high selectivity, low critical micelle concentrations (*cmc*), effectiveness at extreme temperatures, pHs and salinities, and can be produced from renewable resources (Pereira et al., 2013; Gudiña et al., 2015a,b).

However, the application of biosurfactants depends on whether they can be produced economically at large-scale. Nowadays, a limited number of biosurfactants are produced at an industrial scale, mainly due to their high production costs, resulting from their relatively low productivities and the high prices of the culture media used (Henkel et al., 2012). As the culture medium can account for up to 30-50% of the overall production costs of biosurfactants, the replacement of expensive synthetic media by cheaper agro-industrial wastes and by-products can contribute to reduce their production costs and increase their competitiveness (Gudiña et al., 2015a,b).

Examples of these inexpensive agro-industrial wastes and by-products are molasses and Corn Steep Liquor (CSL). Molasses is a low-cost by-product generated during the crystallization of sugar from liquid extracts of sugarcane or sugar beet. It contains a high concentration of carbohydrates (usually about 50%), as well as other valuable compounds such as vitamins. CSL is a liquid by-product generated by the corn wet milling industry. It is rich in vitamins, minerals, amino acids and proteins, being an important source of nitrogen for many biotechnological processes (Henkel et al., 2012). The high nutritional content of both substrates, together with their availability and low price, makes them useful products to be used as culture medium or nutrient supplements for microorganisms in diverse industrial fermentation processes.

The aim of this work was to optimize biosurfactant production by *Pseudomonas aeruginosa* and *Bacillus subtilis* strains using low-cost substrates. The biosurfactants produced by both strains were characterized, and their applicability in oil recovery was evaluated and compared with two chemical surfactants.

2. RESULTS AND DISCUSSION

2.1. Study of biosurfactant production by *P. aeruginosa* #112

Biosurfactant production by *P. aeruginosa* #112 was evaluated using CSL dissolved in demineralized water at different concentrations (5%, 10% and 15% (v/v)) as culture medium, either alone or

supplemented with glucose (10 g/L) or molasses (10% (w/v)). The cultures were performed at 37°C and 180 rpm. The best results regarding biosurfactant production were obtained using a medium containing CSL (10% (v/v)) and molasses (10% (w/v)) (medium CSLM). The surface tension was reduced from 50.0 ± 0.5 mN/m up to 31.6 ± 0.7 mN/m after 144 hours of growth, with emulsifying indexes around 62%. The amount of biosurfactant recovered at the end of the fermentation was 3194 ± 245 mg/L, with a *cmc* of 50 mg/L. The mass spectrometry analysis of the biosurfactant produced by *P. aeruginosa* #112 in the medium CSLM revealed a mixture of eight rhamnolipid congeners, being the most abundant the mono-rhamnolipid Rha-C₁₀-C₁₀.

This culture medium, containing a combination of two low-cost agro-industrial by-products, without addition of salts, micronutrients or other supplements that would raise the price of the production process (the estimated price of CSLM is 0.024€/L), and without the addition of water-immiscible substrates, which makes easier the fermentation and recovery processes, is a promising alternative to reduce the production costs of rhamnolipids.

2.2. Study of biosurfactant production by *B. subtilis* #573

In order to establish the optimum CSL concentration for biosurfactant production by *B. subtilis* #573, different culture media prepared by dissolving CSL in demineralized water at different concentrations (5%, 10% and 15% (v/v)) were evaluated. The cultures were incubated at 37°C and 200 rpm, and were maintained until the maximum biosurfactant production was achieved in each case. The lowest surface tension value (30.7 ± 0.4 mN/m) and the highest emulsifying index ($55.0 \pm 2.0\%$) were obtained with the medium containing 10% of CSL after 48 hours of growth. The amount of biosurfactant produced with this medium was 1311 ± 109 mg/L, with a *cmc* of 160 mg/L. This biosurfactant was identified as a mixture of C₁₃-, C₁₄-, and C₁₅-surfactin. The estimated price of the medium CSL 10% is 0.004€/L. Therefore, the use of CSL as an alternative culture medium would greatly decrease the production cost of this biosurfactant at an industrial scale.

Furthermore, the effect of different metals (iron, manganese and magnesium) on biosurfactant production by *B. subtilis* #573 was evaluated using the medium CSL 10%, since these metals are co-factors of enzymes involved in the synthesis of surfactin. The best result was obtained when the medium CSL 10% was supplemented with a combination of the three metals tested (FeSO₄, 2.0 mM, MgSO₄, 0.8 mM and MnSO₄, 0.2 mM). After 72 hours of growth, the surface tension of the culture medium was reduced from 52.8 ± 0.3 mN/m up to 29.1 ± 0.6 mN/m, the emulsifying activity was $59.5 \pm 0.9\%$, and the amount of biosurfactant produced was 4829 ± 193 mg/L, which is almost four times the amount of biosurfactant produced in the medium CSL 10% without supplements.

2.3. Application of biosurfactants in the removal of crude oil from sand

The applicability of the biosurfactants produced by *P. aeruginosa* #112 and *B. subtilis* #573 (in the media CSLM and CSL 10%, respectively) in oil recovery was evaluated and compared with the chemical surfactants Enordet and Petrostep. The assays were performed using an artificially contaminated sand containing 10% (w/w) of Arabian Light crude oil.

The biosurfactant produced by *P. aeruginosa* #112 and the chemical surfactant Enordet exhibited a similar performance in oil recovery; at a concentration of 5 mg/mL, the percentages of oil recovered after 24 hours were $55.0 \pm 3.4\%$ and $54.4 \pm 5.9\%$, respectively. However, the chemical surfactant Petrostep exhibited a lower efficiency, recovering $30.5 \pm 2.5\%$ of oil at the same concentration; and the biosurfactant produced by *B. subtilis* #573 showed the worst result, recovering $25.1 \pm 1.7\%$ of oil.

The cell-free supernatant obtained after growing *P. aeruginosa* #112 in the medium CSLM for 144 hours was also evaluated for oil recovery. The percentage of oil recovered in this case ($64.2 \pm 3.5\%$) was higher than the obtained with the highest biosurfactant concentration tested (5 mg/mL), although the biosurfactant concentration in the cell-free supernatant was about 3.2 mg/ml, meaning that other compounds present in the cell-free supernatant can also enhance the oil recovery. The possibility of using the cell-free supernatants without further processing steps can contribute to increase the use of biosurfactants in applications such as enhanced oil recovery or bioremediation, as their purification represents a relevant portion of the overall production costs.

3. CONCLUSIONS

Low-cost culture media were developed using two agro-industrial by-products (CSL and molasses) for biosurfactant production. A medium containing CSL (10% (v/v)) and molasses (10% (w/v)) proved to be the best for biosurfactant production by *P. aeruginosa* #112, whereas a medium consisting of CLS (10% (v/v)) was the best for biosurfactant production by *B. subtilis* #573. The amounts of biosurfactant produced with these media were 3.2 and 1.3 g/L, respectively. Additionally, in the case of *B. subtilis* #573, the amount of biosurfactant produced was increased about 4 times using the medium CSL 10% supplemented with the appropriate concentration of iron, magnesium and manganese, thus representing a higher impact on the production cost. The biosurfactant produced by *P. aeruginosa* #112 was characterized as a mixture of eight different rhamnolipid congeners, whereas the biosurfactant produced by *B. subtilis* #573 was a mixture of C₁₃-, C₁₄- and C₁₅-surfactin. The rhamnolipid mixture exhibited a better performance in oil recovery assays when compared with the chemical surfactants Enordet and Petrostep, making it a promising candidate for applications in the oil industry or bioremediation processes.

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