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Biosurfactant Production by *Rhodococcus Erythropolis* Sp. SB-1A Isolated from North Atlantic Ocean: Study on the Influence of Environmental Conditions

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Abstract

Biosurfactant production by *Rhodococcus erythropolis* SB-1A was studied based on Atlas oil agar medium in a batch reactor. The strain was isolated from a water sample collected from Northern Atlantic Ocean. Several parameters including carbon source (n-hexadecane, 0.5, 2, 3.5 and 5 v/v%), nitrogen source (NH_4NO_3,0.4, 0.7, 1 and 1.3 g/L), pH (5,6, 7 and 8) and salinity (NaCl, 13, 26, 39 and 52 g/L) were analyzed to optimize cultural and environmental conditions for biosurfactant production. Surface active properties of biosurfactants in the cell-free broth were monitored periodically. Results showed that the crude biosurfactants in the media cultivated under experimentally defined conditions reduced surface tension by 40 dynes/cm within 10 hours of cultivation. The research outputs demonstrated the possibility of using biosurfactants produced by *Rhodococcus erythropolis* SB-1A for remedying hydrocarbon contamination in North Atlantic Canada and beyond.

Keywords: *Rhodococcus erythropolis*, biosurfactant production, n-hexadecane, surface tension

Introduction

Biosurfactants, surface-active biomolecules produced by microorganisms, are a superior alternative for chemical surfactants due to their unique properties such as lower ecotoxicity, higher biodegradability and greater stability(Geys et al., 2014; Mukherjee et al., 2006).
In the past few decades, biosurfactants have shown great potential in environmental bioremediation, specifically, in desorption, solubilization and biodegradation of hydrophobic organic contaminants in the environments (Ivshina et al., 1998; Kanga et al., 1997; Kuyukina et al., 2005).

Many members of the genus *Rhodococcus* are known to be effective biosurfactant producers. *Rhodococcus* species can naturally persist and grow in various temperate and extreme environments especially in hydrocarbon-contaminated soils and waters(Kuyukina and Ivshina, 2010). Biosurfactants are considered as a by-product promoting the biodegradation of hydrocarbon by enhancing the adherence of genus *Rhodococcus* to hydrophobic phases (Neu, 1996); providing easy access to enter microbial cells by reducing the interfacial tension between the phases(Fiechter, 1992); increasing the microbial attack by hydrocarbon dispersion(Finnerty, 1994).

Different trehalose containing glycolipids are known to be produced throughout *Rhodococcus* genus such as *R. erythropolis*, "*R. longus*," *R. opacus*, and *R. ruber*(Franzetti et al., 2010). Among *Rhodococci R. erythropolis* was reported to have unique bioconversion and biodegradation ability due to its diversified enzyme system(De Carvalho and Da Fonseca, 2005). R. erythropolis can also generate Mycolate-containing glycolipids in term of bioflocculants working on a variety of suspended solids(Kurane et al., 1994; Kurane et al., 1995; Kurane and Tomizuka, 1992).

The influence of environmental conditions(e.g. carbon source, nitrogen source, pH) on the biosurfactant production has been intensively investigated(Bicca et al., 1999). Studies on the conditional requirements of *rhodococci* can acquire worthy information on microbial metabolism that allows the conditional parameters to be adjusted to meet the target of biotechnology (Pacheco et al., 2010).

This study aims to learn effects of carbon source, nitrogen source, pH and salinity on the biosurfactant production by a *Rhodococcus erythropolis* strain isolated from Northern Atlantic Ocean based on one-factor-at-a-time experiment; Meanwhile, to enhance the production efficiency as a guide for further up-scaling production.

Materials and Methods

Cultivation of *Rhodococcus erythropolis* SB-1A

R. erythropolis sp. SB-1A isolated from a water sample collected from Northern Atlantic Ocean was selected during the study. The bacterium was maintained in NBS mineral salt medium plate with the composition per litre as following : Nutrient Broth Broth,25 g; Agar, 15 g; NaCl, 22 g (Zhou et al., 2005).

The bacterium was grown in revised Atlas oil agar medium (Atlas, 2004). The mineral composition consisted of per litre: KH_2PO_4 :3.4 g ; K_2HPO_4: 4.4 g ; $MgSO_4.7H_2O$: 0.2 g ; $FeCL_3$: 0.05 g ; $CaCl_2.2H_2O$: 0.05 g. Glucose was added in 1g/L as inorganic carbon source to stimulate the cell growth in early phase. In order to evaluate the effect of organic carbon source on biosurfactant production, n-Hexadecane(0.5%, 2%, 3.5% and 5%, v/v) was added into the medium. The effect of salinity was studied by adding NaCl per litre of 13, 26, 39 and 52 g. The initial pH was adjusted to 6.5, but in order to investigate the effect of medium acidity-alkalinity on the biosurfactant production pH was adjusted to 6, 7, 8 and 9 by KH_2PO_4 and K_2HPO_4. NH_4NO_3 per litre of 0.4, 0.7, 1.0 and 1.3 g was added to investigate the influence of nitrogen source on biosurfactant production. All chemicals were analytical grade reagents unless specified. Sterile cultures (in flasks of 125 ml with 15 ml of liquid medium) were inoculated with 1.5% volume aliquot of a overnight preculture grown for 48 hours, and incubated at 30 ℃, 200 rpm in a rotary shaker for 96 hours.

Measurement of biosurfactant activity

Flasks were sacrificed periodically to collect culture samples followed by centrifugation at 10,000 rpm for 15 min to remove the cells. 10 ml of the cell free broth supernatant was submitted to surface tension measurements. The surface tension was determined in triplicate with a surface tensiometer (DuNouyTensiometer, Interfacial, CSC Scientific) at 25 ℃.

Results and Discussion

Isolation and identification of selected biosurfactant-producing bacterial strain

In the previous study, 55 strains were screened and isolated from Northern Atlantic Ocean water samples. The isolate SB-1A was selected with low reduced surface tension and high CMC^{-1} indicating that it could produce biosurfactants effectively. This purified isolate strain SB-1A was then subjected to 16S ribosomal DNA (rDNA) sequencing by universal bacterial primers F27 and R926 (position in *Escherichia Coli* 8-27 and 926-907, respectively). The obtained DNA sequence was matched with Basic Local Alignment Search Tool (BLAST) database. The identification result demonstrated that the isolated strain SB-1A belonged to *Rhodococcus erythropolis* with 99 Max identity%.

Effect of factors on surface tension reduction

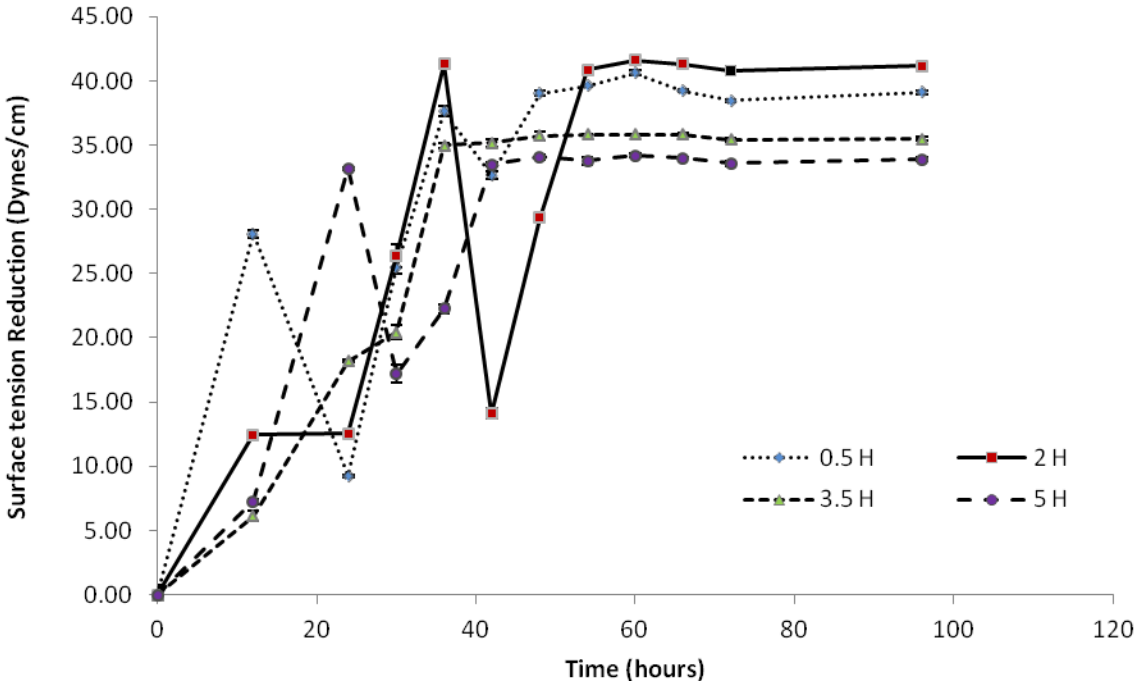

Figure 1. Effect of carbon source on surface tension reduction. Medium(g/L):KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.05; Glucose, 1; NaCl, 26; NH_4NO_3, 1; pH= 6.5.

The use of n-hexadecane as carbon source in biosurfactant production has been widely recognized as an effective biosurfactant inducer. In our previous study, n-hexadecane was used as sole carbon source, and the cell growth was found to be very slow. Glucose was then added as

supplemental carbon source and significantly stimulate the cell growth in early phase. In this study, glucose (1g/L) was used along with n-hexadecane. It has been found by many studies that due to the biosurfactant production and accumulation during the growth of biosurfactant producers, the surface tension declines between the logarithm phase and stationary phases (Toledo et al., 2008). In Fig. 1 surface tension declined after 12 hours in all the four levels of n-hexadecane and remained stable after 60 hours. Final surface tension reduction of lower level (2% and 0.5) n-hexadecane were close to 40 dynes/cm higher than higher lever (3.5% and 5%) n-hexadecane below 35 dynes/cm. This may indicate that redundant n-hexadecane can inhibit the biosurfactant production by reducing the interaction between air and the culture below it.

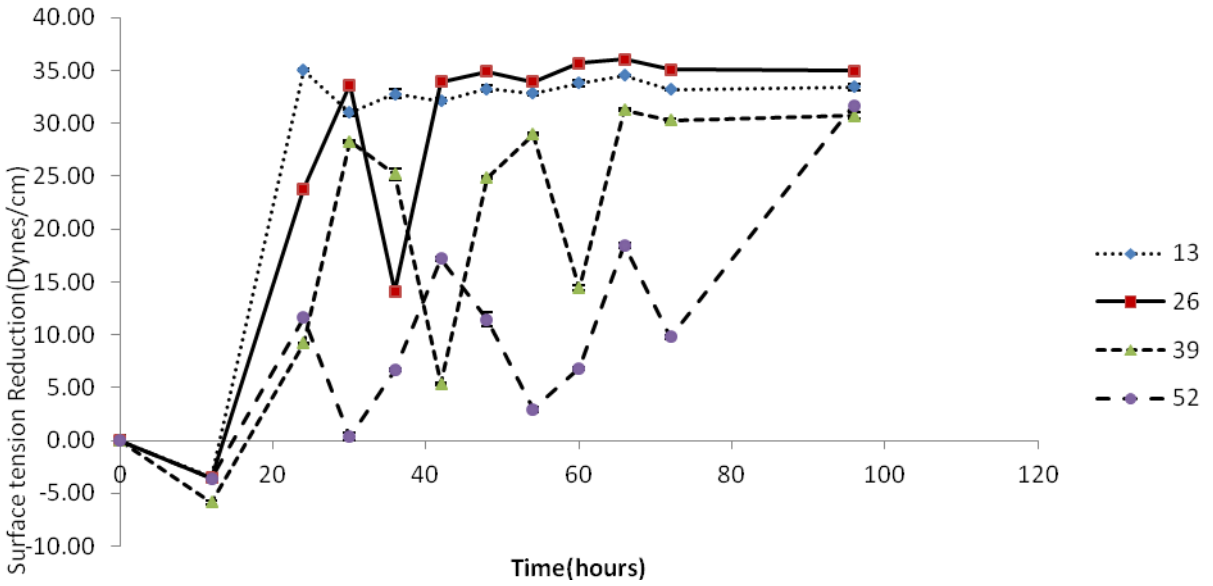

Figure 2. Effect of salinity on surface tension reduction. Medium(g/L):KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4$ ·$7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.05; Glucose, 1; n-hexadecane, 3.5 v/v%; NH_4NO_3, 1; pH= 6.5.

Salinity is one of the critical factor in the production of biosurfactant especially for those producer isolated from salty environments. Biosurfactants produced by *Bacillus mycoides* isolated from an Iranian oil field was enhanced with high salinity of 55.05 g/L total salt concentration while whereas low salinity had negative effect on biosurfactant production and cell growth (Najafi et al., 2010). However, effect of salinity on biosurfactant production by *Rhodococcus* was seldom studied. Salinity was in term of NaCl in this study, and the concentration was set from 13 to 52 g/L given that the isolate was from marine environment. As for lower salinity of 13 and 26 g/L, the surface tension was reduced by around 35 dynes/cm rapidly in 40 hours; By contrast, surface tension of 39 g/L salinity fluctuated before 70 hours and was ultimately reduced by 30 dynes/cm. The biosurfactant production was significantly inhibited under 52 g/L salinity. The surface tension was reduced by 30 dynes/cm after 100 hours but such tendency may not be maintained. The study indicated that the biosurfactant production by this *Rhodococcus* isolate was adapted to salinity similar to seawater of which the salinity is close to 35 ppt equivalent to NaCl concentration of 26g/L.

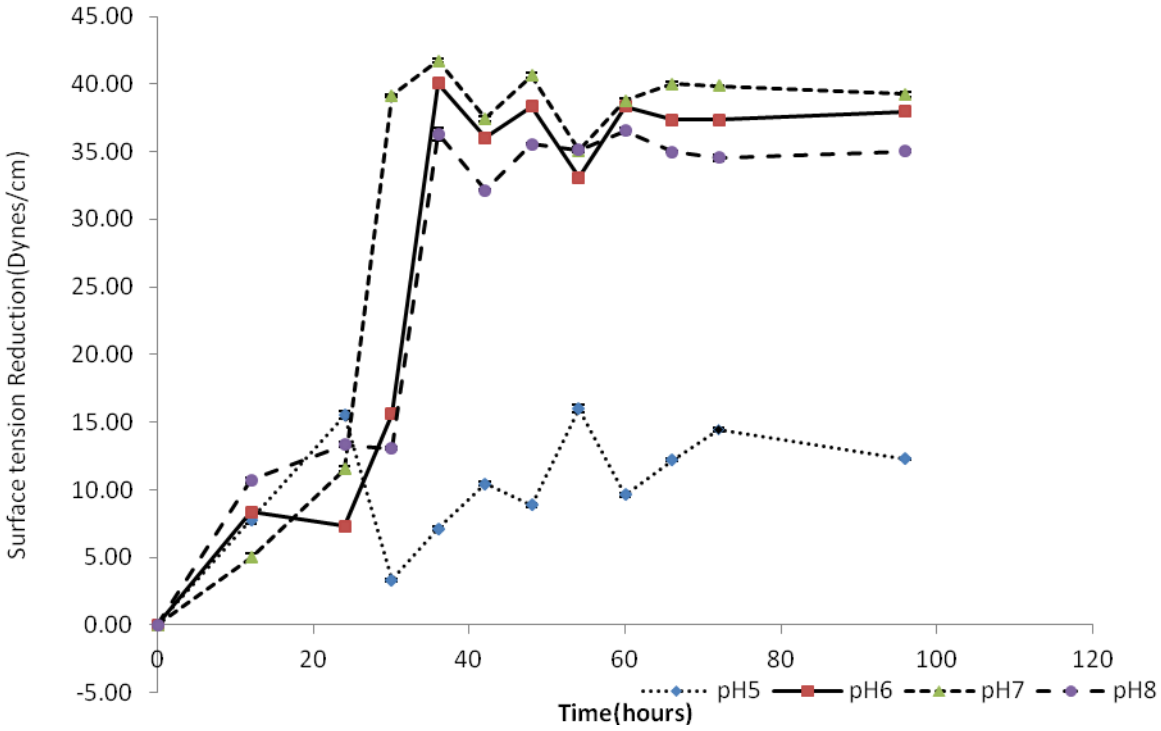

Figure 3. Effect of pH on surface tension reduction. Medium(g/L):$MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.05; Glucose, 1; n-hexadecane, 3.5 v/v%; NH_4NO_3, 1; NaCl, 26.

pH is an important parameter that may need periodical monitoring and adjustion in real biotechnology processes. The effect of pH on biosurfactant production varied among differnet biosurfactant producers but only a few studies have investigated the effect pH on *Rhodococcus* species. The surface tension of the biosurfactant by *Pseudomonas aeruginosa* isolated from oil-contaminated soil was found stable at a large range of pH between 2 and 10(Saikia et al., 2012). In our study, pH was controlled by Potassium Phosphate buffer along with NaOH or HCl solution end-point adjustion. No significant difference was observed between pH 6, 7 and 8; Although higher surface tension reduction was achieved when pH was 7, they have all reduced surface tension by 35 dynes/cm. The maximum surface tension reduction of 15 dynes/cm at pH 5 indicates the production favors non-acid condition and this could be a threshold of pH value setup for large scale production. Similar findings were reported in Rhamnolipid production by *Pseudomonas aeruginosa* isolate with better biosurfactant stability at high pH values up to 13(Abdel-Mawgoud et al., 2009).

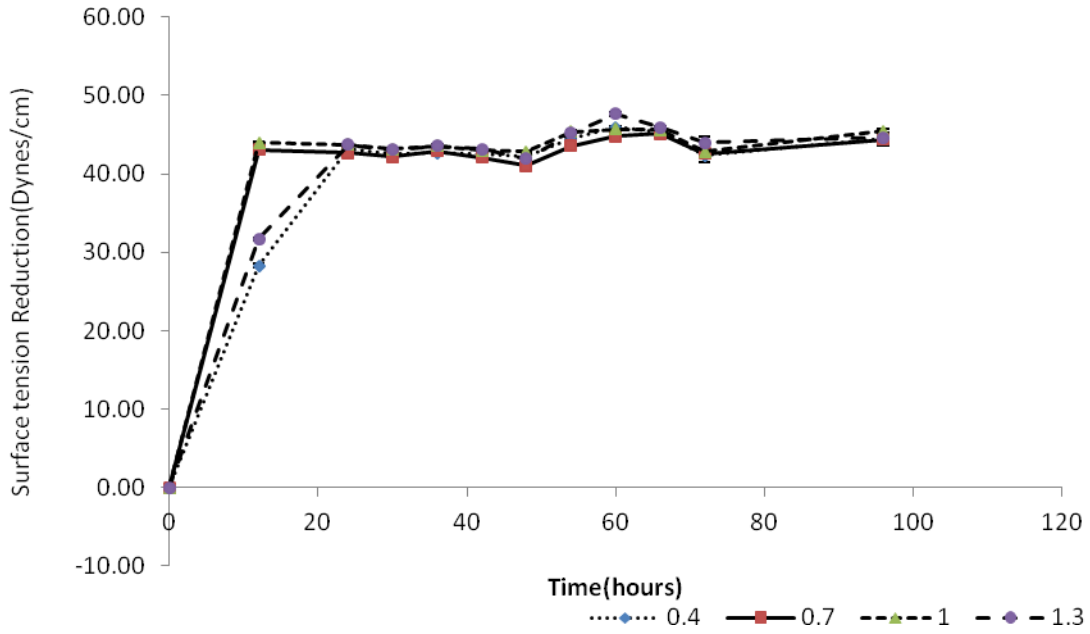

Figure 4. Effect of nitrogen source on surface tension reduction. Medium(g/L): $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.05; Glucose, 1; n-hexadecane, 3.5 v/v%; NaCl, 26; pH= 7.

To overcome the complex environment in the culture medium, only one nitrogen source was adopted. NH_4NO_3 as inorganic nitrogen has been found reaching the highest CMC^{-1} in biosurfactant production by *Pseudomonas aeruginosa* among KNO_3, $NaNO_2$, NH_4NO_3, $NaNO_3$ and beef extract (Cha et al., 2008). Biosurfactant production by a *Rhodococcus erythropolis* isolate grown on Various Carbon Sources using NH_4NO_3 as nitrogen source achieved high yield and emulsification index (Gogotov and Khodakov, 2008). However, the production of biosurfactant has been reported for some biosurfactant producers to yield high product only under limiting concentrations of nitrogen source (Chayabutra et al., 2001; Patel and Desai, 1997). In Fig.4 the effect of NH_4NO_3 of low concentrations on the time course of surface tension reduction were investigated. No significant differences were observed within the range between 0.4 and 1.3 g/L of NH_4NO_3l, although the surface tension declined earlier under the concentration of 0.7 and 1.0 g/L NH_4NO_3. Overall, the nitrogen in the above levels may not be a critical parameter.

Conclusions

The production of biosurfactants by the strain can be detected in the simple method by measuring surface tension reduction and enhanced based on the experimentally defined levels of carbon source, salinity, pH and nitrogen source with a surface tension reduction of 40 dynes/cm after 10 hours.

It was noted in previous study that the strain was adapted to the cold marine environments of northern Atlantic Canada and the cell free culture remained clear at 4 ℃ (Cai et al., 2014). This indicates that the produced biosurfactants may be effective under low temperature. This study demonstrated the possibility of applying biosurfactants produced by *Rhodococcus erythropolis*

strain SB-1A in enhancing bioremediation of petroleum hydrocarbon contaminated sites and offshore oil spill control in cold areas such as North Atlantic Canada and beyond.

Acknowledgement

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