Isolation, Fermentation Optimization and Performance Studies of a Novel Biosurfactant Producing Strain *Bacillus amyloliquefaciens*

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In this research, biosurfactant-producing bacteria were isolated from the outlet sludge of a canteen and one promising strain was identified through 16S rDNA sequence as *Bacillus amyloliquefaciens*. This strain can utilize water-soluble carbon source and the FT-IR analysis indicated the biosurfactant was probably glycolipids. Further factors (fermentation time, temperature, carbon source, nitrogen source, ion concentration) affecting the biosurfactant production were determined. The optimum fermentation conditions and nutrient concentration were as follows: fermentation time 48 h, temperature 30 °C, fermentation pH 6, glucose concentration 30 g L^{-1}, peptone concentration 4 g L^{-1}, total phosphorus concentration 8 g L^{-1}, sodium chloride concentration 2 g L^{-1}, magnesium sulfate concentration 0.25 g L^{-1}, and calcium chloride concentration 0.04 g L^{-1}. In such conditions, the surface tension of the solution was 28 mN m^{-1}. At the same time, the formation of biosurfactant and nano-silica complex system was discussed to improve the biosurfactant performance and provide the basis for expanding the application scope of biosurfactant.

Key words:
biosurfactant, *Bacillus amyloliquefaciens*, nano-silica

Introduction

Petroleum hydrocarbon is the main source of energy in the world, as well as the main pollutant of soil and water. Accidental spills, uncontrolled landfills or improper storage and leaking underground storage tanks can all lead to contamination[1]. Compared with physicochemical methods, bioremediation has gained increasing interest in dealing with petroleum hydrocarbons, as it is environmentally friendly, cost-effective, efficient, while the end products of this process are mostly not harmful to environment[2]. Bioremediation refers to the complete elimination or conversion of toxic recalcitrant compounds into non-toxic forms by a bacterium or a microbial consortium[3]. However, microbial biodegradation is usually impeded by the limited low water solubility of petroleum hydrocarbon and limited capacity of bacteria to access and degrade the substrate[4]. The bioavailability of hydrocarbons can be enhanced by adding biosurfactant to the contaminated ecosystems to facilitate the uptake of bacteria[5].

Also, the biosurfactant can help the oil recovery process during water flooding in the oil field. Biosurfactants are a diverse group of surface-active chemical compounds with unique biochemical properties and are produced by a wide variety of microorganisms[6]. Based on the chemical composition and microbial origin, biosurfactants are classified as glycolipids, lipopeptides, fatty acids, polymeric type and particulate biosurfactants[7]. The most commonly isolated biosurfactants are glycolipids and lipopeptides, such as rhamnolipids[8], sophorolipids[9], surfactin and iturin[10]. Lipopeptides are effective biosurfactants which are usually produced by *Bacillus* strains.

Biosurfactants are amphiphilic compounds containing both hydrophilic and hydrophobic moieties in their structure and can reduce both surface and intersurface tensions which make them useful in emulsification processes[6]. They can form micelles to facilitate the process of emulsification of hydrocarbons in aqueous phase and enhance their availability for microbial uptake and degradation[11], the enhancement of biosurfactants is probably due to the increase in cell surface hydrophobicity that allows direct contact between the cell and the hydrocarbons[12]. Hence, they have potential for application in the degradation of hydrocarbon pollutants.

Compared with chemically synthesized surfactants, biosurfactants are of better environmental compatibility, higher foaming activity, lower toxicity, higher selectivity, biodegradability and specific activity in extreme conditions (temperatures, pH, and salinities)[13,14]. These attributes make biosurfactants promising alternatives to chemical surfactants

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most of which are costly, cause decreased microbial activity and do not function well under extreme conditions, thus raising serious environmental concern[15].

Research in the area of biosurfactants has attracted much attention due to their potential use in different areas, such as the food, agricultural and pharmaceutical industries, enhanced oil recovery and remediation of oil spills, petro-chemistry, paper and pulp industry[16,17]. Apart from this, Durgesh Narain Singh[8] reported a strain that produced copious amounts of biosurfactant when coal was added to the medium, which may be useful in the coal bed for *in situ* biotransformation of coal into methane. Biosurfactants could be useful in a variety of biotechnological and industrial processes, particularly in the oil industry[18]. A reduction of toxicity in crude oil by using biosurfactant-producing bacteria may also support the growth of other hydrocarbon-degrading bacterial strains to augment the process of oil degradation[19].

The purposes of this study were to isolate a strain that could produce a biosurfactant from a carbon source and investigate the cultural factors affecting the production of the extracellular biosurfactants to find the optimal composition of the growth medium and optimum culture conditions for the production in flask. The characteristics of the extracted biosurfactant have been studied. In addition, when the surfactant is used in the petrochemical industry, it can be compounded with other reagents and compounds, and the performance of the surfactant is greatly improved[20]. Therefore, in this paper the formation of biosurfactants and nano-silica complex was discussed to improve the biosurfactant performance and provide the basis for expanding their scope of application.

Materials and methods

Materials

All chemicals of analytical reagent grade were purchased from Beijing Chemical Factory (Beijing, China). All the biochemical reagents were purchased from Beijing Biological Technology Factory (Beijing, China). The strains were stored in a tube slant culture at 4 °C. General biological equipment was purchased from Shanghai Precision Instrument Co., LTD (Shanghai, China).

Culture media and microorganism culture

The composition of blue agar culture medium was as follows (per liter): cetyltrimethylammonium bromide (CTAB) 5.00 g, methylene blue 0.02 g, beef extract 5.00 g, peptone 10.00 g, NaCl 5.00 g, agar 20.00 g. Fermentation/enrichment medium contained (per liter): glucose 20.00 g, peptone 4.00 g, KH_2PO_4 5.00 g, K_2HPO_4 5.00 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, NaCl 2.00 g, $CaC1_2$ 0.08 g. Starch agar medium was composed of (per liter): peptone 10.00 g, beef extract 5.00 g, starch 2.00 g, agar 20.00 g. The composition of peptone water medium was as follows (per liter): peptone 10.00 g, NaCl 5.00 g, pH 7.6. Citrate medium was composed of (per liter): $NH_4H_2PO_4$ 1.00 g, K_2HPO_4 1.00 g, NaCl 5.00 g, $MgSO_4$ 0.20 g, sodium citrate 2.00 g, agar 20.00 g, 1 % (w/w) bromine thymol blue ethanol solution 10 mL, pH 6.8.

Isolation of hydrocarbon-degrading microorganisms

1 g canteen outfall sludge was added to 99 mL stilled water as 10^{-1} dilution, then 1 mL solution was diluted in 10 times until 10^5 times. 5 mL dilution was added to a 100 mL flask containing 50 mL enrichment culture medium, and cultured at 30 °C, 150 rpm for 2 d.

The dilution was smeared on the blue agar culture medium and cultured in the incubator at 30 °C for about 6 days, the strains with blue circle produced around the colonies were isolated and purified, and stored in the slant culture at 4 °C.

Isolation of biosurfactant-producing microorganisms

Each isolated strain was first activated and incubated into the 250 mL flask with 50 mL fermentation culture medium at the amount of 10 %, then incubated at 28 °C, 150 rpm for 2 days.

The biosurfactant produced was measured by the oil spreading method, and the diameter of the clear zone on the oil surface was related to the concentration of biosurfactant[21].

For the oil spreading experiment, 60 mL of distilled water was added to a large petri dish, followed by addition of 8 mL of liquid paraffin to the surface of the water to form an oil membrane. The fermentation liquor (1 mL) was added to the surface of oil membrane. The diameter of the clear zone on the oil surface was measured, and strains with diameter of the clear zone larger than 3 cm were chosen for next research.

Identification of biosurfactant-producing strain

For 16S rDNA gene amplification, the genomic DNA of optimum strain was extracted using Andybio DNA kit following the manufacturer's instructions.

The 16S rDNA was amplified using the universal primers. PCR amplification was performed under the following conditions: 4 min at 94 °C;

35 cycles of 30 sec at 94 °C, 30 sec at 52 °C, and 2 min at 72 °C, plus an additional 10 min cycle at 72 °C. The automatic sequence was carried out by Beijing Sun Biotech Co., Ltd. The 16S rDNA sequence was checked in GeneBank.

Optimum culture conditions for biosurfactant production

Experiments for optimum fermentation time

As the diameter of exclusive circle of biosurfactant was related to the concentration and activity of biosurfactant, a loop of slant culture was inoculated to the 250 mL flask with 50 mL fermentation culture medium, the fermentation experiment was carried out at 30 °C, 160 rpm, with an initial pH of 6. The diameter of exclusive circle was measured every 4 hours and the curve of the exclusive circle-time relationship was made.

Experiments for optimum culture conditions

To study the effect of pH on biosurfactant production, pH of the fermentation medium was adjusted to 4, 5, 6, 7, 8, 9, and cultured in the same conditions as in 2.6.1. Similarly, the temperature was adjusted to 25 °C – 40 °C to determine the effect of temperature on biosurfactant production.

Different carbon source may have a different effect on biosurfactant production; glucose, lactose, liquid paraffin and soluble starch (all at 20 g L^{-1}) were chosen as carbon source, while the other constituents remained the same to determine the best carbon source, and a set of 1 %, 2 %, 3 %, 4 %, 5 % of the concentration of the best carbon source was arranged to detect the optimum carbon source concentration. Similarly, based on the fermentation medium, NH_4Cl, peptone, yeast extract, NH_4NO_3, urea, $NaNO_3$ (4 g L^{-1}) was added as nitrogen source in the fermentation medium to determine the optimum nitrogen source. In addition, the ratio of KH_2PO_4 to K_2HPO_4 (w/w) was 1:1, the total phosphate concentration was set as 0.6 g L^{-1} to 1.4 g L^{-1} to determine the optimum phosphate concentration. The mass fraction of NaCl was set as 0 g L^{-1} to 0.5 g L^{-1}; and concentration of Ca^{2+}, Mg^{2+} was set as 0, 0.04 g L^{-1}, 0.08 g L^{-1}, 0.12 g L^{-1}, 0.16 g L^{-1}, 0.2 g L^{-1} and 0, 0.25 g L^{-1}, 0.5 g L^{-1}, 0.75 g L^{-1}, 1 g L^{-1} to determine the optimal fermentation conditions.

Preliminary characterization of biosurfactant

The fermentation liquor was centrifuged at 10000 rpm for 20 minutes, supernatant was adjusted to pH 2 by concentrated hydrochloric acid, stored overnight at 4 °C; then centralized again at 10000 rpm for 20 minutes, sediment was collected, washed and diluted with hydrochloric acid of pH 2, and then adjusted to pH 7 by 1 mol L^{-1} NaOH solution, crude biosurfactant was obtained after drying at 60 °C. Crude biosurfactant was extracted by CH_2Cl_2, after extraction CH_2Cl_2 was removed by rotary evaporation, pure biosurfactant was obtained after drying at 60 °C.

Fourier transform infrared spectroscopy (Nicolet 5700, Thermo-Electron Co. Ltd, Madison, WI USA) was used for the analysis of the component of purified biosurfactant. About 2 mg of dried biosurfactant was milled with 200 mg of KBr to form a very fine powder. This powder was then compressed into a thin pellet which could be analyzed by FT-IR spectra measurement in the wave number range of 4000–400 cm^{-1}.

The composite of biosurfactant and nano SiO_2

A certain concentration of biosurfactant solution was prepared. For the oil spreading experiment, the diameter of the clear zone on the oil surface was measured. A certain quantity of nano-silica (particle diameter: 15±5 nm) was added in the biosurfactant solution and the solution was mixed in an ultrasonic generator for 40 minutes to form the mixture. The diameter of the clear zone on the oil surface was then measured.

Analytical methods

The pH was measured by a pH meter (PHS-3B, Shanghai Precision & Scientific Instrument Co. Ltd, Shanghai, China). Surface tension was measured by a surface tensiometer (Shanghai Precision Instrument Co. Ltd.), and component of biosurfactant was analyzed by Fourier transform infrared spectroscopy (Nicolet 5700, Thermo-Electron Co. Ltd, Madison, WI USA). The morphology of nanoparticles was observed by TEM (JEM-2010, Japan Hitachi Ltd., Japan). The water-oil interfacial tension was measured by interfacial tension measurement (QJZY-1, Shanghai Xuan Ping Scientific Instrument Co. Ltd., China). All experiments were carried out four times.

Results and discussion

Isolation of hydrocarbon-degrading microorganisms

The canteen outfall sludge was diluted, and after enrichment the dilution was smeared on the blue agar culture medium and cultured in the incubator. As a result, 9 strains with blue circle produced around the colonies were isolated and purified, and stored in the slant culture at 4 °C.

Isolation of biosurfactant-producing microorganisms

Each isolated strain was first activated and then incubated in the fermentation culture medium. After fermentation, the biosurfactant produced was measured by the oil spreading method as mentioned earlier. The diameter of the clear zone on the oil surface was measured, and the results are shown in Table 1.

Table 1 – *Diameter of the clear zone on the oil surface of different isolated strains*

Strain	1	2	3	4	5	6	7	8	9
Diameter/cm	3.7	3.1	2.5	2.4	2.4	2.0	1	0	0

As shown in Table 1, strain 1 has the largest diameter of oil spreading circle, while strain 8 and strain 9 did not produce biosurfactant at all, strain 1 was chosen as the optimum biosurfactant-producing microorganism. The diameter of the clear zone on the oil surface was 3.7 cm. In other literature[22], the diameter of the clear zone was 3.4 cm. The biosurfactant production by bacterial strain 1 was slightly higher. Also the optimal fermentation conditions for biosurfactant production were studied.

Identification of biosurfactant-producing strain

The identification of strain 1 was carried out through 16S rDNA sequence by Beijing Sun Biotech Co. Ltd., which showed that strain 1 was 100 % identical to *Bacillus amyloliquefaciens* and the Accession No. was HQ824986.1. The phylogenetic neighbor-joining tree is shown in Figure 1.

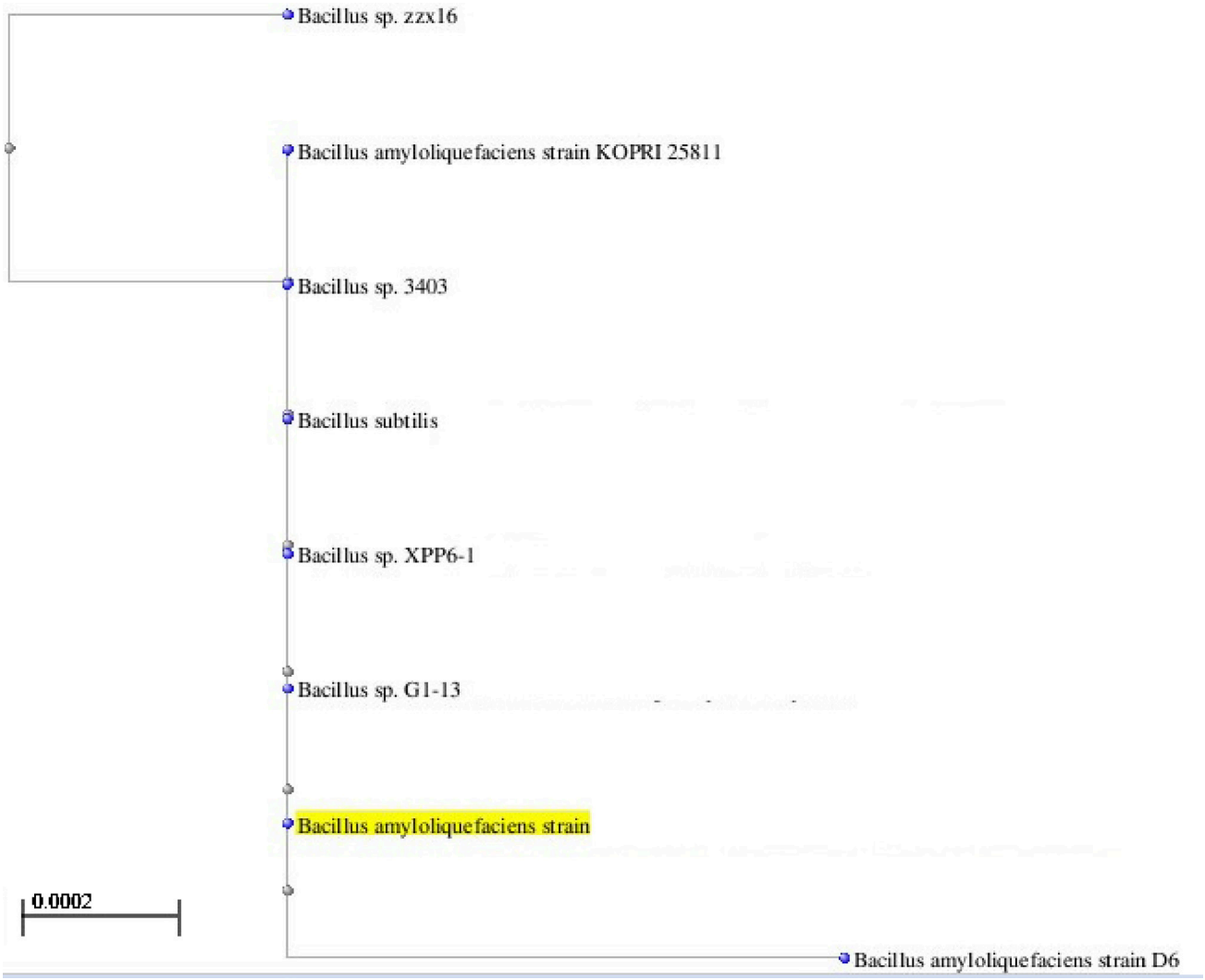

Fig. 1 – *Phylogenetic neighbor-joining tree of the strain*

Optimal cultural conditions for biosurfactant production of strain 1

Effect of fermentation time on biosurfactant production

A loop of slant culture was inoculated to the 250 mL flask with 50 mL fermentation culture medium, the fermentation experiment was carried out at 30 °C, 160 rpm, with an initial pH of 6. The exclusive circle was measured every 4 hours and the curve of the exclusive circle-time relationship was made as shown in Figure 2.

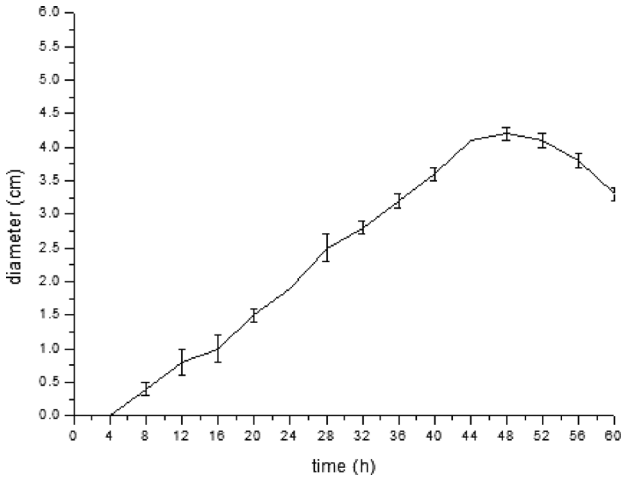

Fig. 2 – *Effect of fermentation time on biosurfactant production*

As shown in Figure 2, the biosurfactant produced by strain 1 was a primary metabolite, the diameter of explosive circle reached the max at 48 h, and the produced biosurfactant might have been consumed as nutrient as the fermentation went on, the optimum fermentation time was 48 h.

Effect of pH on biosurfactant production

The pH of solution has an important influence on the growth of microorganism. In the process of microorganism growth, the pH may cause changes of cell membrane, and thereby change the permeability and electric charge of the membrane. At the same time, pH may also cause changes in the degree of ionization of various ionic substances required for microorganism growth, and thus affect both the absorption of nutrients and the growth and reproduction of microorganisms. On the other hand, the activity of various enzymes involved in the microbial physiological and biochemical activity is influenced by the pH of the intracellular environment. Therefore, pH for bacterial growth played a pivotal role.

As microorganisms can generally grow well in the range of pH 4–9, the fermentation were carried out with an initial pH of 4 to 9, inoculation amount of 10 %, and cultured at 30 °C, 160 rpm, 48 hours. The results are shown in Figure 3(a). The biosurfactant production reached maximum at pH 6, indicating that the optimum fermentation initial pH was 6.

Effect of temperature on biosurfactant production

Temperature is an important survival factor of microorganisms. Temperature affects the activity of the enzyme and changes the rate of enzymatic reaction. Within the appropriate temperature range, if the temperature is increased, the enzymatic reaction rate will increase and lead to a corresponding increase in the rate of metabolism and growth rate of the microorganisms. At appropriate temperature, microorganisms can obtain the fastest growth and reproduction rate.

Strain 1 was inoculated in the fermentation medium with the inoculation amount of 10 %, pH of 6.0. The temperature gradient ranged from 25 °C to 40 °C. After fermentation at 160 rpm for 48 h, the diameter of explosive circle was measured. The results are shown in Figure 3(b). In the range of 25–30 °C, biosurfactant production increased as the temperature increased. However, above 30 °C, although *Bacillus amyloliquefaciens* was thermophilic, the biosurfactant production decreased apparently; thus, the optimum fermentation temperature was 30 °C.

Effect of carbon source on biosurfactant production

Carbon is one of the most essential elements forming functional compounds, to all heterotrophic microorganism, the carbon source also provides energy, the proper carbon source can assimilate the growth and metabolism of microbes.

To determine the best carbon source for biosurfactant production, the carbon source of the fermentation medium was replaced by lactose, liquid paraffin, and soluble starch (all at 20 g L^{-1}), respectively. Fermentation was carried out at 30 °C, 160 rpm, initial pH of 6 for 48 hours. The results showed that fermentation with glucose as carbon source had the largest diameter of the explosive circle, and was easier to utilize compared with other alternatives. The diameters of the explosive circle of fermentation with liquid paraffin and soluble starch as carbon source were the same, and only a slightly smaller than with glucose, indicating that liquid paraffin and soluble starch could be the alternative carbon source, and might be used in the biodegrading aspect.

Concentration of carbon source can affect the osmotic pressure, extracellular hypertonic solution can lead to plasmolysis, while hypotonic solution makes cells swell; thus, only isotonic solution is suitable for microbe growth and metabolism.

In order to assure the optimum carbon source concentration for biosurfactant production, concentration of glucose in the fermentation medium was

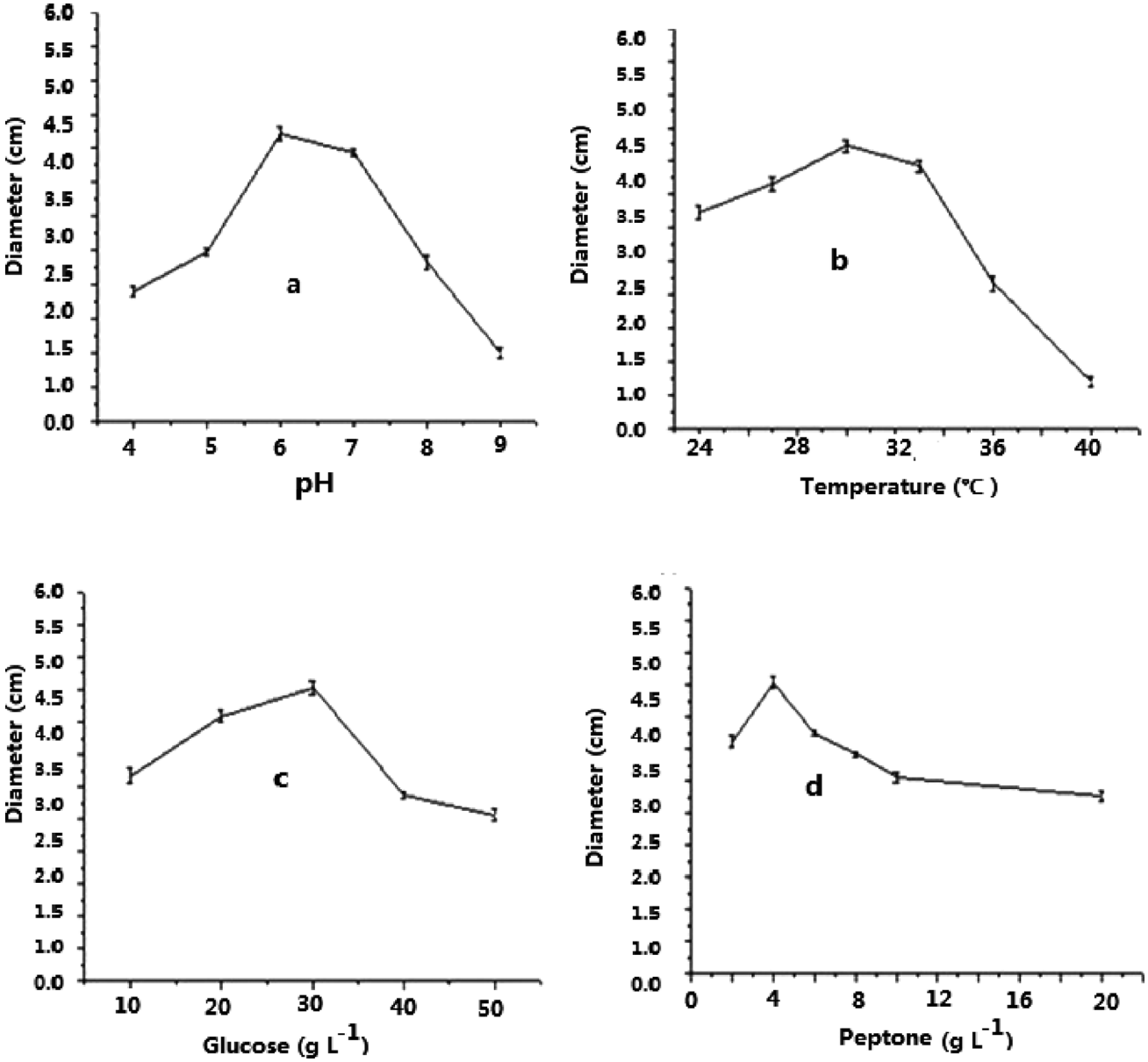

Fig. 3 – *Effect of pH, temperature, carbon source and nitrogen source on the biosurfactant production*

adjusted to 10 g L^{-1}, 20 g L^{-1}, 30 g L^{-1}, 40 g L^{-1}, 50 g L^{-1} with initial pH of 6 and cultured at 30 °C, 160 rpm for 48 hours. As the results show in Figure 3(c), in the range of 10–30 g L^{-1}, the diameter of explosive circle increased with higher concentrations, but when above 30 g L^{-1}, due to the viscosity, it became unsuitable for microbe growth and biosurfactant production, and biosurfactant production was limited. The suitable glucose concentration was 20–30 g L^{-1}.

Effect of nitrogen source and phosphate on biosurfactant production

Nitrogen is one of the main elements in the composition of protein and nucleic acid, and makes up 12–15 % of cell dry weight. To determine the best nitrogen source for biosurfactant production, the nitrogen source of the fermentation medium was replaced with $NaNO_3$, urea, yeast extract, peptone, NH_4Cl (4 g L^{-1}), respectively, glucose concentration was 30 g L^{-1} with initial pH of 6, cultured at 30 °C, 160 rpm, 48 hours. The results indicated that ionized nitrogen source is not easily utilized compared to protein, and peptone was the most favorable nitrogen source. Different carbon-nitrogen ratios have different effects on microbes, and in order to produce biosurfactant, a proper carbon-nitrogen ratio is important. Fermentation was carried out with initial pH of 6, glucose 30 g L^{-1}, 30 °C, 160 rpm, the concentration of peptone ranged from 0.2 % to 1 %, after 48 h, the diameter of explosive circle was measured, and the results are shown in Figure 3(d). In the low concentration, biosurfactant production increased with higher concentrations, but above 4 g L^{-1}, it became

unsuitable for microbe growth and biosurfactant production due to the viscosity limitation. The optimum peptone concentration was 4 g L^{-1}.

Phosphate exists in many compounds, enzymes, DNA and so on, microbes mainly obtain phosphate from inorganic phosphate. A lack of phosphate may lead to low activity of enzymes, which affects the physiological function of microbes. In order to determine the optimum phosphate concentration the ratio of KH_2PO_4 to K_2HPO_4 (w/w) was 1:1, the total phosphate concentration was set from 6 g L^{-1} to 14 g L^{-1}, with an initial pH of 6, glucose 30 g L^{-1}, peptone 4 g L^{-1}, after fermentation at 30 °C, 160 rpm for 48 h, the diameter of explosive circle was measured, and the results are shown in Figure 4(a). The diameter of explosive circle reached maximum when the concentration was 8 g L^{-1}, high concentration of K^+ could have an influence on the osmotic pressure, and lead to the low production of biosurfactant. The optimum phosphate concentration was 8 g L^{-1}.

Effect of concentration of metallic ions on biosurfactant production

Na^+ acts as a regulating substance and maintains the osmotic pressure, proper concentration of Na^+ should be achieved in the fermentation medium.

In order to determine the optimum salt concentration for biosurfactant production, salt concentrations ranged from 0 g L^{-1} to 5 g L^{-1}, with initial pH of 6, glucose 30 g L^{-1}, peptone 4 g L^{-1}, total phosphate concentration of 8 g L^{-1}, after fermentation at 30 °C, 160 rpm for 48 h, the diameter of explosive circle was measured, and the results are shown in Figure 4(b). NaCl could promote the synthetizing of biosurfactant, the diameter doubled when concentration of NaCl was 2 g L^{-1} compared with no addition of NaCl. However, high concentration of NaCl could have caused the high osmotic pressure and thus reduced the production of biosurfactant. The optimum concentration of NaCl was 2 g L^{-1}.

In the growth process and metabolism of microorganism, certain trace elements act as regulators in

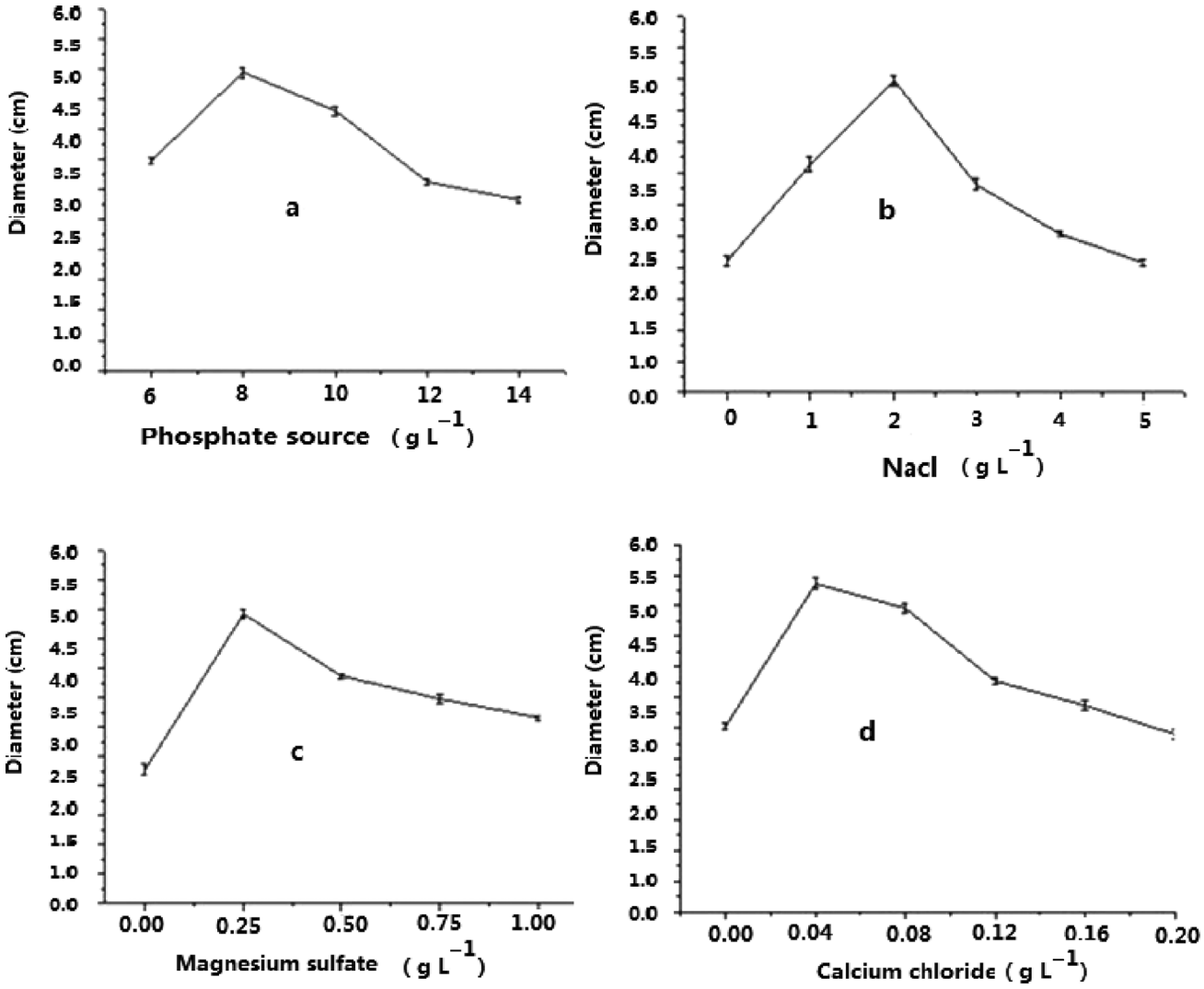

Fig. 4 – *Effect of phosphate, sodium, magnesium and calcium on the biosurfactant production*

the synthesis of physiologically active materials, Mg^{2+} is cofactor and activator of a variety of enzymes, involved in many metabolic processes, such as oxidative phosphorylation, glycolysis, cell reproduction, nucleic acid metabolism and protein synthesis. Experiments were conducted to determine the optimum concentration of Mg^{2+} for microbe growth and biosurfactant production. Fermentation medium with an initial pH of 6, glucose 30 g L^{-1}, peptone 4 g L^{-1}, NaCl 2 g L^{-1}, total phosphate concentration of 8 g L^{-1}, concentration of Mg^{2+} was set from 0 to 1 g L^{-1}, after fermentation at 30 °C, 160 rpm for 48 h, and the results are shown in Figure 4(c). A certain concentration of Mg^{2+} can advance the synthesis of biosurfactant, but a high concentration of SO_4^{2-} leads to sediment with Ca^{2+}, and decreases the production of biosurfactant. The optimum concentration of $MgSO_4$ was 0.25 g L^{-1}.

Ca^{2+} can lower the permeability of cytoplasm, adjust the pH value and alleviate the damage caused by other basic ions. Fermentation was carried out to determine the optimum Ca^{2+} density for biosurfactant production, fermentation medium was composed of glucose 30 g L^{-1}, peptone 4 g L^{-1}, NaCl 2 g L^{-1}, total phosphate concentration of 8 g L^{-1}, $MgSO_4$ 0.25 g L^{-1}, initial pH was 6, cultured at 30 °C, 160 rpm for 48 h with a range of $CaCl_2$ from 0 to 0.2 g L^{-1}; the results are shown in Figure 4(d). A larger amount of $CaCl_2$ can promote the production of biosurfactant, but a high concentration of $CaCl_2$ can lead to sediment with $MgSO_4$, the optimum concentration was 0.04 g L^{-1}. As shown in the results, optimum pH was 6–7, in this range, pH had less effect on biosurfactant production, and this was in agreement with a former report[23]. Glucose was also the optimal carbon source, while diameters of explosive circles with liquid paraffin and soluble starch as carbon source were the same, and only slightly smaller than with glucose, indicating that they could be used as alternative carbon sources.

In general, $MgSO_4$ and phosphate addition was important for the biosurfactant production. Phosphate was a source of K^+ and PO_4^{3-} and it can also act as a buffer, and this result matched the description in the other report[24]. $CaCl_2$ and NaCl were also important for biosurfactant production in our research.

Analysis of the component in the biosurfactant and measurement of surface tension

The infrared spectrum was employed as a convenient way to analyze the component in the supernatant of fermentation broth. The results are shown in Figure 5.

Infrared spectrum of the biosurfactant disclosed a broad stretching peak at 3420 cm^{-1}, which was characteristic of hydroxyl groups. Absorption around 2930 cm^{-1} was assigned to the symmetric

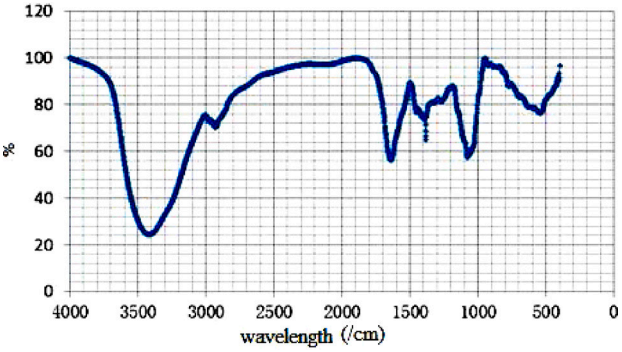

Fig. 5 – *FT-IR spectra of the dried biosurfactant*

stretch of CH_2 and CH_3 groups of aliphatic chains. The absorption peak at 1640 cm^{-1} showed the existence of C=O amide. The absorption around 1080 cm^{-1} was stretching of C-O-C, which could be related to the presence of glycolipids substances in the biosurfactant.

Biosurfactant production was evaluated for surface tension reduction[23,24], surface tension was measured by a surface tensiometer, and the surface tension could be reduced to 28 mN m^{-1}. Former reports showed that surface tension could be reduced to 28 mN m^{-1} [25] and 27 mN m^{-1} [26], indicating that strain 1 was of great value in biosurfactant research and production. More research should be conducted.

The complex system of nano-SiO_2-biosurfactant

A quantity of nano-silica was added to the surfactant solution, after ultrasonic vibration treatment to obtain the nano-silica composite with biosurfactant. In order to improve the application effect of surfactant, nano-silica particles with a surfactant complex were formed. The diameter of oil spreading circle was measured. Among them, the added mass of nano-silica was different. The diameters of oil spreading circle are also shown in Table 2. When the nano-silica compound was combined with the biosurfactant, the surface-active property could be greatly improved. In the oil-water interface layer, the hydrophilic group of surfactant was in the molecule of the aqueous phase, and the lipophilic group was in the oil phase, forming a certain intensity of water interfacial membrane. The nanoparticles do not have such an amphiphilic structure, but also have the ability to stabilize the structure. The nano-powder has high surface energy which can absorb some of the molecules in solution, thereby changing the wettability of the surface and reducing surface tension. The optimum surface-active performance was obtained when the mass ratio of surfactant to nano-particles was 7:5.

At the same time, after studying the oil spreading performance of nano-silica, the ability of the complex system of nano-SiO_2-biosurfactant to reduce the interfacial tension between oil and water was investigated

Table 2 – *Diameter of the clear zone on the oil surface after biosurfactant and biosurfactant nano-treatment*

Bio-surfactant	0.10 %	0.20 %	0.30 %	0.40 %	0.50 %
Diameter/cm	2.2	2.7	4	4.3	5
Bio-surfactant	0.60 %	0.70 %	0.80 %	0.90 %	1.00 %
Diameter/cm	5.8	6.2	6	6	6
Bio-surfactant+Nano	0.7 %+0.1 %	0.7 %+0.2 %	0.7 %+0.3 %	0.7 %+0.4 %	0.7 %+0.5 %
Diameter/cm	6.8	6.8	7.5	8.5	9
Bio-surfactant+Nano	0.7 %+0.6 %	0.7 %+0.7 %	0.7 %+0.8 %	0.7 %+0.9 %	0.7 %+1.0 %
Diameter/cm	8.6	7.9	7.5	7.6	7.2

and the result is shown in Figure 6. The mass ratio of surfactant to nano-particles was 7:5 according to the former research. The concentration of surfactant was 0.7 %. The interfacial tension rapidly declined with time, and the biosurfactant was preferentially adsorbed. The reason was that the nano-particle molecule was relatively larger than the biosurfactant molecule. The diffusion rate of nano-particle was slower. However, later the nano-particle was absorbed by the oil and water interface and then the interfacial tension was reduced. This indicates that nano-silica can effectively reduce the water-oil interfacial tension.

TEM analysis of the nano-system was carried out at the same time. The solution was dropped on the carbon film and after drying the sample was observed by TEM. The observation is shown in Figure 7. It was shown that the system with nano-silica formed a stable particle structure. The nano-particles and the surfactant formed a stable system, which gave the oil-water interface membrane large intensity. So, the oil droplets in the drawing process could achieve a low interfacial tension value, and maintain it for certain period of time.

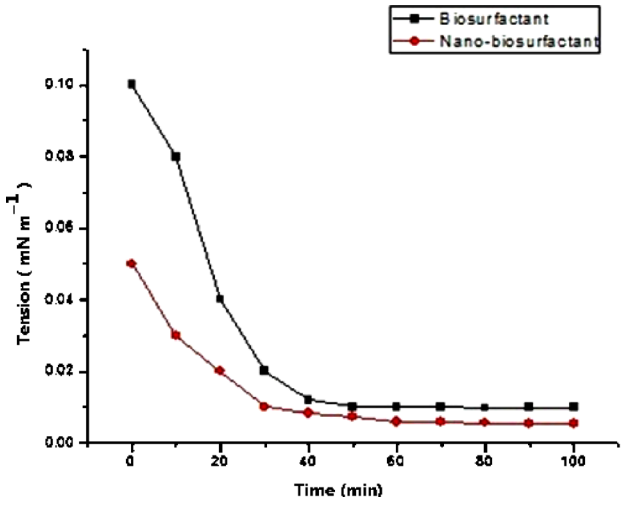

Fig. 6 – *Interface tension after the treatment of biosurfactant and nano-SiO_2 – biosurfactant*

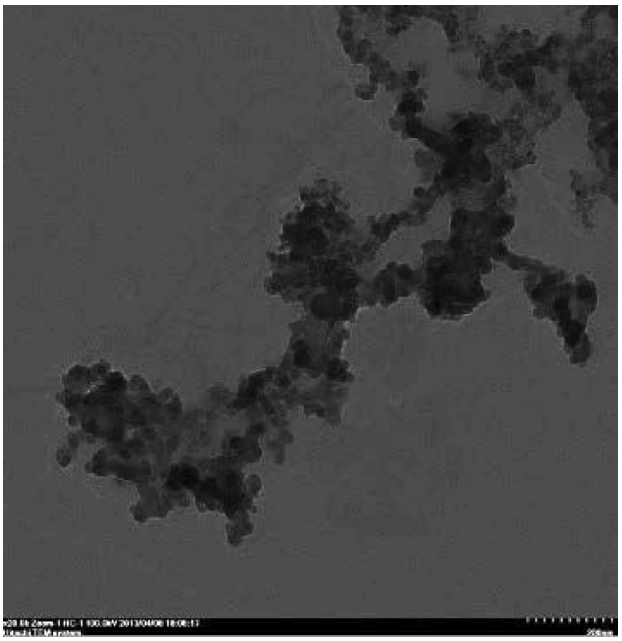

Fig. 7 – *TEM observation of nano-SiO_2-biosurfactant*

Conclusions

An efficient biosurfactant-producing strain was isolated from canteen outfalls screened sludge. Fermentation experiments showed the biosurfactant was a primary metabolite and might be consumed as a nutrient in the growth process, the maximum production was reached at 48 hours. FT-IR analysis indicated the biosurfactant could be polysaccharide or polysaccharide-like. Identification of the 16S rDNA indicated this strain was 100 % identical to *Bacillus amyloliquefaciens* and the Accession No. was HQ824986.1. The optimum fermentation medium for biosurfactant production was glucose 30 g L^{-1}, peptone 4 g L^{-1}, NaCl 2 g L^{-1}, total phosphate concentration of 8 g L^{-1}, $MgSO_4$ 0.25 g L^{-1}, $CaCl_2$ 0.04 g L^{-1}, initial pH was 6, culture condition was 30 °C, 160 rpm for 48 hours. Surface tension could be reduced to 28 mN m^{-1}, suggesting this strain was valuable in biosurfactant production and research. The nano-particles and the surfactant formed a stable system,

which gave the oil-water interface membrane large intensity, so that the oil droplets in the drawing process could achieve a low interfacial tension value, and maintain it for a certain period of time.

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References

1. *Trindade, P. V., Sobral, L. G., Rizzo, A. C., Leite, S. G., Soriano, A. U.,* Bioremediation of a weathered and a recently oil-contaminated soil from Brazil: A comparison study, Chemosphere **58** (2005) 515. doi: http://dx.doi.org/10.1016/j.chemosphere.2004.09.021
2. *Millioli, V. S., Servulo, E. L. C., Sobral, L. G. S., De Carvalho, D. D.,* Bioremediation of crude oil-bearing soil: evaluating the effect of rhamnolipids addition to soil toxicity and to crude oil biodegradation efficiency, Global NEST J. **11** (2009) 181.
3. *Das, P., Mukherjee, S., Sen, R.,* Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin, Chemosphere **72** (2008) 1229. doi: http://dx.doi.org/10.1016/j.chemosphere.2008.05.015
4. *Barathi, S., Vasudevan, N.,* Utilization of petroleum hydrocarbons by Pseudomonas fluorescens isolated from a petroleum-contaminated soil, Environ. Int. **26** (2001) 413. doi: http://dx.doi.org/10.1016/S0160-4120(01)00021-6
5. *Mulligan, C. N.,* Environmental applications of biosurfactants, Environ. Pollut. **133** (2005) 183. doi: http://dx.doi.org/10.1016/j.envpol.2004.06.009
6. *Mehdi, H., Emtiazi, G.,* Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation, Int. Biodeter. Biodegr. **62** (2008) 170. doi: http://dx.doi.org/10.1016/j.ibiod.2008.01.004
7. *Gautam, K. K., Tiagi, V. K.,* Microbial surfactants: a review, J. Oleo Sci. **55** (2006) 155. doi: http://dx.doi.org/10.5650/jos.55.155
8. *Singh, D. N., Tripathi, A. K.,* Coal induced production of a rhamnolipid biosurfactant by *Pseudomonas stutzeri*, isolated from the formation water of Jharia coalbed, Bioresour. Technol. **128** (2013) 215. doi: http://dx.doi.org/10.1016/j.biortech.2012.10.127
9. *Daverey, A., Pakshirajan, K.,* Production, Characterization, and properties of sophorolipids from the yeast *Candida bombicola* using a low-cost fermentative medium, Appl. Biochem. Biotechnol. **158** (2008) 663. doi: http://dx.doi.org/10.1007/s12010-008-8449-z
10. *Ahimou, F., Jacques, P., Deleu, M.,* Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity, Enzyme Microb. Technol. **27** (2000) 749. doi: http://dx.doi.org/10.1016/S0141-0229(00)00295-7
11. *Noudeh, G. D., Housaindokht, M., Bazzaz, B. S. F.,* Isolation, characterization and investigation of surface and hemolytic activities of a lipopeptide biosurfactant produced by Bacillus subtilis ATCC 6633, J. Microbiol. **43** (2005) 272.
12. *Luis, Y., Mara, E., Corbella, M., Turiegano, U. K., Antonio, P., Fernando, R.,* Characterization of bacterial strains able to grow on high molecular mass residues from crude oil processing, FEMS Microbiol. Ecol. **32** (2000) 69. doi: http://dx.doi.org/10.1111/j.1574-6941.2000.tb00700.x
13. *Radhika, C., Yao, J., Choi, M. M. F., Masakorala, K., Chan, Y.,* An efficient biosurfactant-producing and crude-oil emulsifying bacterium *Bacillus methylotrophicus* USTBa isolated from petroleum reservoir, Biochem. Eng. J. **74** (2013) 46. doi: http://dx.doi.org/10.1016/j.bej.2013.02.018
14. *Desai, J. D., Banat, I. M.,* Microbial production of surfactants and their commercial potential, Microbiol. Mol. Biol. Rev. **61** (1997) 47.
15. *Dastgheib, S. M. M., Amoozegar, M. A., Elahi, E., Asad, S., Banat, I. M.,* Bioemulsifier production by a halothermophilic Bacillus strain with potential applications in microbially enhanced oil recovery, Biotechnol. Lett. **30** (2008) 263. doi: http://dx.doi.org/10.1007/s10529-007-9530-3
16. *Khopade, A., Biao, R., Liu, X., Mahadik, K., Zhang, L., Kokare, C.,* Production and stability studies of the biosurfactant isolated from marine *Nocardiopsis* sp. B4, Desalination **285** (2012) 198. doi: http://dx.doi.org/10.1016/j.desal.2011.10.002
17. *Satpute, S. K., Banat, I. M., Dhakephalkar, P. K., Banpurkar, A. G., Chopade, B. A.,* Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms, Biotechnol. Adv. **28** (2010) 436. doi: http://dx.doi.org/10.1016/j.biotechadv.2010.02.006
18. *Bharali, P., Das, S., Konwar, B. K., Thakur, A. J.,* Crude biosurfactant from thermophilic *Alcaligenes faecalis*: Feasibility in petro-spill bioremediation, Int. Biodeter. Biodegr. **65** (2011) 682. doi: http://dx.doi.org/10.1016/j.ibiod.2011.04.001
19. *Kumari, B., Singh, S. N., Singh, D. P.,* Characterization of two biosurfactant producing strains in crude oil degradation, Process Biochem. **47** (2012) 2463. doi: http://dx.doi.org/10.1016/j.procbio.2012.10.010
20. *Zhu, H., Xia, J., Shun, Z., Zhang, J., Zhang, Y., Wang, F.,* Application of nanometer-silicon dioxide in tertiary oil recovery, Acta Petrolei Sinica **27** (2006) 96.
21. *Youssef, N. H., Duncan, K. E., Nagle, D. P., Savage, K. N., Knapp, R. M., McInerney, M. J.,* Comparison of methods to detect biosurfactant production by diverse microorganisms, J. Microbiol. Meth. **56** (2004) 339. doi: http://dx.doi.org/10.1016/j.mimet.2003.11.001
22. *Li, T., Yang, X.,* Screening of Bio-surfactant Bacteria Strain and Optimization of Fermentation Conditions, Natural Science Journal of Hainan University **27** (2009) 241.
23. *Najafi, A. R., Rahimpour, M. R., Jahanmiri, A. H., Roostaazad, R., Arabian, D., Ghobadi, Z.,* Enhancing biosurfactant production from an indigenous strain of Bacillus mycoides by optimizing the growth conditions using a response surface methodology, Chem. Eng. J. **163** (2010) 188. doi: http://dx.doi.org/10.1016/j.cej.2010.06.044
24. *Wan, N. W. M., Jamal, P., Alam, M. Z.,* Utilization of sludge palm oil as a novel substrate for biosurfactant production, Bioresour. Technol. **101 (**2010) 9241. doi: http://dx.doi.org/10.1016/j.biortech.2010.07.024
25. *Chandankere, R., Yao, J., Choi, M. M. F., Masakorala, K., Chan, Y.,* An efficient biosurfactant-producing and crude-oil emulsifying bacterium *Bacillus methylotrophicus* USTBa isolated from petroleum reservoir, Biochem. Eng. J. **74** (2013) 46. doi: http://dx.doi.org/10.1016/j.bej.2013.02.018
26. *Al-Bahry, S. N., Elshafie, A. E., Al-Wahaibi, Y. M., Al-Bemani, A. S., Joshi, S. J., Al-Lawati, A.,* Isolation and characterization of biosurfactant/biopolymer producing spore forming bacteria from oil contaminated sites and oil field of Oman, APCBEE Procedia **5** (2013) 242. doi: http://dx.doi.org/10.1016/j.apcbee.2013.05.042