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Full Length Research Paper

Investigation of cellular hydrophobicity and surface activity effects of biosynthesed biosurfactant from broth media of PTCC 1561

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Biosurfactants as surface active molecules that are synthesized by microorganisms. These substances include many advantages in comparison with chemical surfactants. For instance they have lower toxicity, higher biodegradability, better environmental compatibility, higher foaming activity, and specific activity at extreme temperatures, pH ranges, and the ability to be synthesized from renewable feed stocksin. In this study, the production of biosurfactant, produced by PTCC 1561 was studied. This bactrium was grown in a nutrient broth medium and the production of biosurfactant was evaluated by the surface tension and emulisification index (E24), each 24 h. The production of biosurfactant was studied in different conditions, including time of incubation, temperature, aeration rate and presence of several additives containing mineral salts and hydrocarbons. Finally, the optimum condition for production of the biosurfactant was determined and the biosurfactant identity was investigated using chemical and spectroscopy methods. The maximum biosurfactant production by *Pseudomonas aeruginosa* PTCC 1561, was exhibited when it was grown in Brain Hearth Broth medium containing Fecl3, ZnSO4, FeSO4, starch and olive oil incubated in a 200 rpm shaker incubator at 37°C for 24 h . The structure of produced biosurfactant sugar-lipid was confirmed by chemical and spectroscopy methods.

Key words: Biosurfactant, emulsification index, surface tension.

INTRODUCTION

Surfactants are amphiphilic molecules which tend to lower the interfacial tension. They find applications in an extremely wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization. Chemically produced surfactants have increasingly been replaced by biotechnologically based compounds either enzymatic or microbial synthesis. Biosurfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surface or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively. *Pseudomonas* species is well known for its ability to produce rhamnolipid biosurfactant, with potential surface active properties, when grown on di erent carbon substrates and therefore, its a promising candidate for the large scale production of biosurfactants (Lin et al., 1998).

Rhamnolipids actually seem to play multiple roles. First, since they display potent surface tension reducing and emulsifying activities. These molecules are considered surfactants and as a result, have been mostly studied for their ability to solubilize and promote the uptake of hydrophobic substrates especially hydrocarbons such as n-alkanes. Another mechanism through which

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rhamnolipids enhance the biodegradation of poorly soluble molecules is by causing the cell surface to become more hydrophobic. Rhamnolipids display antibacterial activity mostly against Gram-positives and also a few Gram-negatives. Furthermore, antiviral, antifungal, mycoplasmacidal, algicidal, zoosporcidal, and antiamoebal activities have been reported (Al-Tahhan et al., 200; Itoh et al., 1971; Desai and Banat, 1997; Aranda et al., 2007).

MATERIALS AND METHODS

Test microorganism

The bacterium, *Pseudomonas aeruginosa* PTCC 1561, was purchased from the Persian Culture Type Collection, Tehran, Iran.

Hemolytic activity

P. aeruginosa PTCC 1561 was inoculated on blood agar plates (Merck) containing 5% (v/v) blood and incubated at 37°C for 48 h. Haemolysis activity was detected as the presence of a definite clear zone around a colony (Abu-Ruwaida et al., 1991; Dehghan Noudeh et al, 2005).

Foam forming activity

For foam forming activity measurement, *P. aeruginosa* PTCC 1561 was grown in 250 ml Erlenmeyer flasks, each containing 50 ml of nutrient broth (Merck at pH 7.4) medium. The flasks were incubated C in a 200 rpm shaker incubator at 37°C. Samples were drowning each 24 h and then 5 ml of supernatant in a graduated tube vortexed at high speed for 1 min. Foam activity was detected as duration of foam stability, and foam height was determined in a graduated cylinder (Dehghan et al., 2003).

Bacterial cultivation

First *P. aeruginosa* PTCC 1561 was grown in 250 ml conical flasks, containing 50ml nutrient broth medium. The flasks were incubated in a 200 rpm shaker incubator at 37°C. For analysing foam forming activity, surface activity, emulsification index and biomass, flasks were examined every 24 h. This process was carried out up to 120 h (Patel and Desai, 1997; Cooper et al, 1979).

Biomass separation

Microbial cells were removed from medium by centrifugation (12000 rpm, 4°C for 20 m) and biomass was measured by washing the pelleted cells with normal saline and then re-centrifuged at 12000 rpm. The cells were then dried overnight at 65°C and biomass weight was determined (Cha, 2008).

Surface activity measurement

Biosurfactant concentration is expressed in terms of critical micellar dilution (CMD) and it was estimated by measuring the surface tension (mN/m) for different concentrations using a duNouy Tensiometer (Model K 100 C). All measurements were made on cell free broth. CMD-1 and CMD-2 measurements were performed by measuring the surface tension of 10-times and 100-times diluted cell free broth. Negative controls contained sterile culture medium plus *P. aeruginosa* PTCC 1561 (an inoculum), at zero time (Cha, 2008; Wei et al., 2005; Carrillo, 1996).

Emulsification index measurement

Emulsification activity was measured by adding 5 ml of mineral oil to 5 ml of supernatant in a graduated tube and vortexed vigorously. The test tube was then detained for 24 h and the emulsification index (E24%) was determined using the following equation (Wei, 2005; Cooper and Goldenberg, 1987; Lin et al., 2005):

$$E_{24}\ (\%) = \frac{\text{Height of emulsified zone}}{\text{Height of total liquid mixture}} \times 100$$

Growth conditions

2 *P. aeruginosa* PTCC 1561 was grown in 250 ml conical flasks containing 50 ml brain hearth broth medium (Merck). The flasks were incubated in a 200 rpm shaker incubator at at 37°C. Samples were examined every 24 h to analyse the foam forming activity, and emulsification index. This process was carried out up to 120 h (Dehghan et al., 2003). In order to make maximum production of biosurfactant, *P. aeruginosa* PTCC 1561 was grown in brain hearth broth (BHB) medium containing different cations including Mn2+(0.2w/v), Fe2+(0.4 w/v), Fe3+(0.2w/v), Mg2+(0.4 w/v), Zn2+(0.1 w/v) and the mixture of these ions were added individually to each conical flasks (32,8,33,30). Also, Starch (0.1 w/v), olive oil (0.1v/v), and paraffin oil (0.1 v/v) were separately added to each flask. Three conical flasks were prepared for each additive and they were incubated in a 200 rpm shaker incubator at 37°C for 24 h. Flasks were then examined to analyse the emulsification index and foam stability as it was explained previously to select the best additives for biosurfactant production (Koch et al., 1948; Mercade et al., 1993).

According to previous reports, *P. aeruginosa* strains can grow and produce biosurfactants at temperatures between 30 - 40°C. To obtain the optimium temperature for the producing of biosurfactant by our selected strain *P. aeruginosa* PTCC 1561, four different temperatures were applied and bacteria were incubated at these selected temperatures. The strains were inoculated into 50 ml of brain hearth broth medium and were incubated in 200 rpm for 24 h separately at 30, 33, 37 and 40°C. In order to evaluate E24% of the emulsion, the cultures were then taken from the shaker incubator (Rocha et al., 2007; Thaniyavarn et al., 2006).

The biosurfactant production of *P. aeruginosa* has been demonstrated under aerobic conditions, Therefore the aeration rate plays an important role in biosurfactant production. To obtain the optimum condition for cultivation, five rates were selected as it has been showm in different studies. The strains were then inoculated into conical flasks containing 50 ml of brain hearth broth medium and were incubated with four different rates of 150, 200, 250, 300 and 350 rpm at 37°C for 24 h. The medium were then taken from shaker and the supernatant was evaluated by emulsion index in order to gain the optimum production time (Lima et al., 2008; Pornsunthorntawee et al., 2008).

Determination of cellular hydrophobicity

To determine the cellular hydrophobicity of tested strain(s), collected cells from the precultures were rinsed twice with sterile dd water and the rinsed cells were suspended in PUM buffer (K2HPO4.3H2O 22.2 g/l, KH2PO4 7.26 g/l, urea 1.8 g/l,

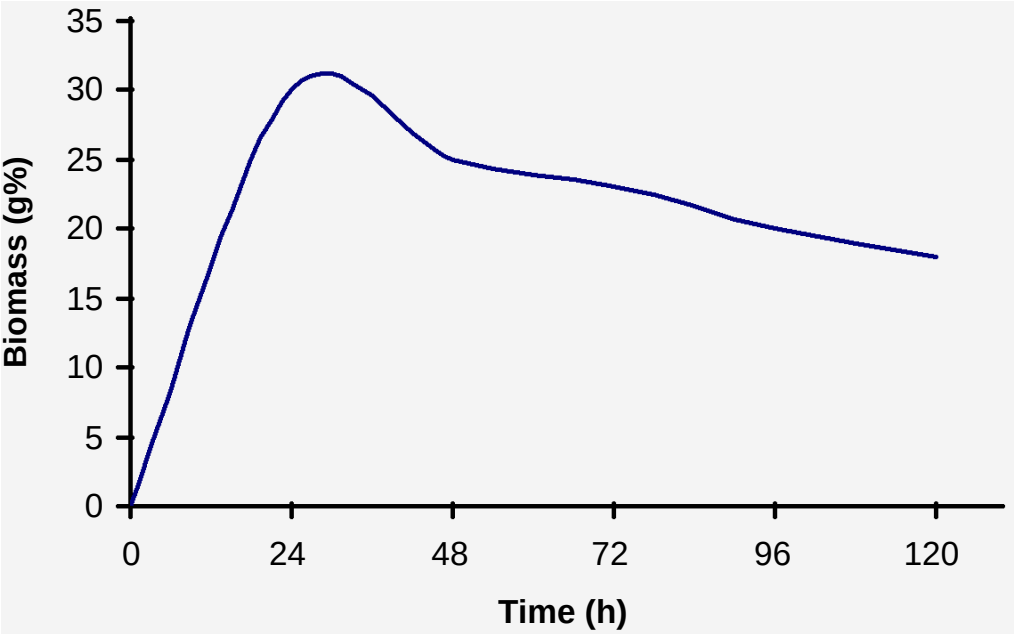

Figure 1. Cell growth and biosurfactant production by *P. aeruginosa* PTCC 1561 (200 rpm, 37°c) against time (Mean ± SD, n = 5).

MgSO4.7H2O 0.2 g/l) to make a final volume of 1.2 ml and an optical absorbance (at 400 nm) of 9.0 ± 1.0 (that is, a cell concentration of 1.3 ± 0.3 mg/l). Various volumes (0–0.2 ml) of examined hydrocarbons mineral oil were added into the cell suspensions, and were then incubated at 30°C for 10 min. The mixtures were subsequently agitated thoroughly with vortex mixer for 120 s. Then, the mixtures were allowed to stand for 15 min to separate the hydrocarbon and aqueous phases. Samples were taken carefully from the aqueous phase and the absorbance at 400 nm (OD400) of the samples was measured using a spectrophotometer. Cellular hydrophobicity is defined as follows (Lin et al., 2006):

$$\text{Hydrophobicity}(\%) = \left(1 - \frac{OD_{400} \text{ of aqueous phase of culture after mixing with hydrocarbon}}{OD_{400} \text{ of initial culture prior to hydrocarbon addition}}\right) \times 100$$

Biosurfactant isolation

For biosurfactant isolation, *P. aeruginosa* PTCC 1561 was grown in brain hearth broth medium including olive oil, Fe^{2+}, Fe^{3+} and Zn^{2+} at 37°C for 24 h with agitation speed of 200 rpm. Cells were removed by centrifugation at 9000 rpm and the biosurfactant was recovered by acidifying the supernatant to pH 2.0 with 6 M HCl, and the precipitate was collected by centrifugation at 12000 rpm for 20 min. It was then dissolved in Tris-HCl and extracted three times with chloroform:ethanol (2:1). The organic solvent layer was evaporated under vacuum on a rotary evaporator to dryness, and used as the extract for further analysis (Cha et al., 2008; Thaniyavarn et al., 2006; Pornsunthorntawee et al., 2008).

Characterization of biosurfact

The biosurfactant was hydrolysed with 6 M HCl at 110°C for 20 h and the lipid moiety was subsequently separated by extraction with chloroform. Several drops of bromine water were added to the extract. All carbohydrates are converted to furfural or its derivatives such as sulfuric acid in presence of concentrated acids. This product makes a violet complex with alcoholic solution of -naftol. 2 ml of a sample solution is placed in a test tube. Two drops of the Molisch reagent (a solution of -napthol in 95% ethanol) is added. The solution is then poured slowly into a tube containing two ml of concentrated sulfuric acid so that two layers formed. A positive test is indicated by the formation of a violet purple product at the interface of the two layers. Infrared spectroscopy was used to confirm the exact structure of the biosurfactant obtained from *P. aeruginosa* PTCC 1595. IR spectra were collected between 400 and 4000 wave numbers (cm- 1) (Wagner and Lang, 1988; Ariabarzin et al., 2009; Heyd et al., 2008; Park et al., 1998).

RESULTS

In the present study, these parameters were evaluated as potential predictors of producing surfactant in bacteria *P. aeruginosa* PTCC 1561 was isolated from nutrient agar cultures and tested by hemolytic method. This strain showed hemolytic activity (Dehghan et al., 2003) (unpublished data). Figure 1 shows the growth of *P. aeruginosa* PTCC 1561 on nutrient broth medium against time. Reduction of surface tension is considered a selection criterion for biosurfactant producing the microorganism in liquid medium.

Therefore, *P. aeruginosa* PTCC 1561 was cultured in liquid medium (nutrient broth) and bioemulsifier production, as evident from surface tension. Since reductions was measured in the surface tension in the broth, the maximum reduction was 35.52 mN/m achieved in the 24th hour of fermentation. CMD^{-1} and CMD^{-2} values followed a similar pattern as surface tension

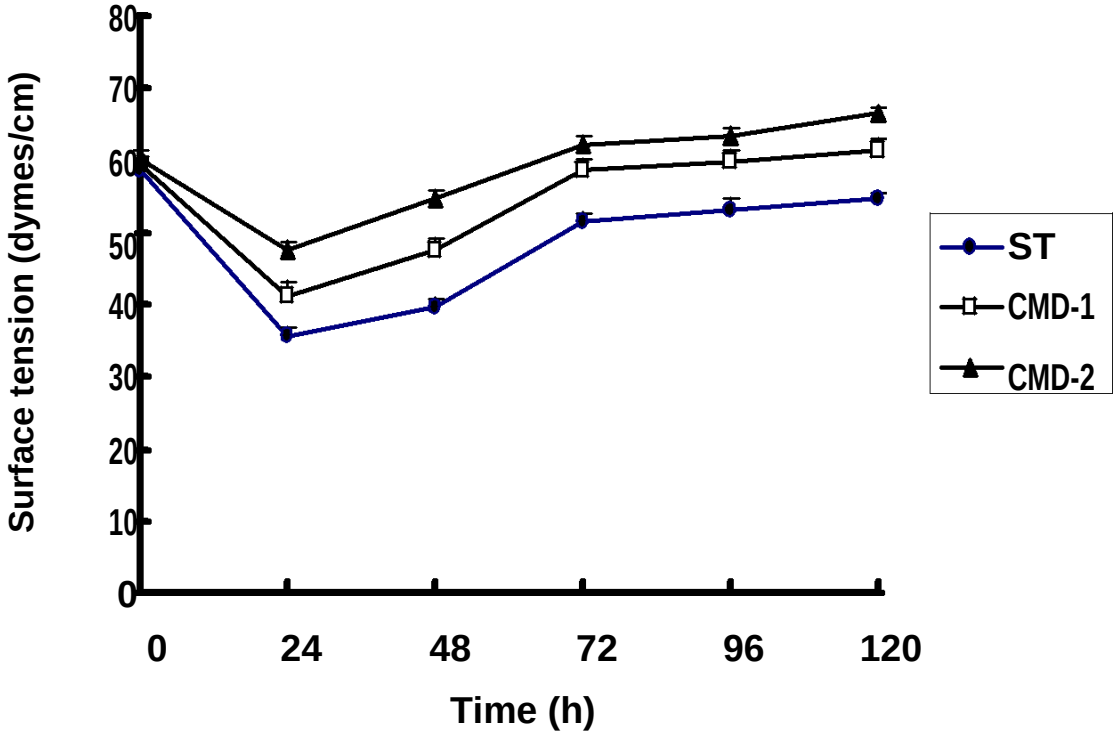

Figure 2. Surface activity profile of *P. aeruginosa* PTCC 1595 (37˚C, 200 rpm) (Mean ± SD, n = 5) against time. ST: Surface Tension. CMD^{-1}: Critical micelle dilution (10 times diluted) CMD^{-2}: Critical micelle dilution (100 time diluted).

Table 1. Emulsification index (E24%), foam stability and foam height results for supernatant of *P. aeruginosa* PTCC 1561 grown in nutrient broth medium (37˚C, 200 rpm) (n = 5).

Time (h)	0 Mean ± SD	24 Mean ± SD	48 Mean ± SD	72 Mean ± SD	96 Mean ± SD	120 Mean ± SD
Emulsification index (%)	50 ± 0.33	55 ± 0.48	48 ± 0.52	37 ± 0.46	19 ± 0.31	16 ± 0.28
Foam Height (mm)	50 ± 1.02	100 ± 1.52	100 ± 1.10	94 ± 0.54	71 ± 0.52	59 ± 1.12
Foam stability (h)	12 ± 0.41	72 ± 0.53	48 ± 0.43	33 ± 0.61	18 ± 0.83	15 ± 0.43

Table 2. Emulsification index percentage (E24%), foam stability and foam height results for supernatant of *P. aeruginosa* PTCC 1561 grown in brain hearth broth medium (37˚C, 200 rpm) (n = 5).

Time (h)	0 Mean ± SD	24 Mean ± SD	48 Mean ± SD	72 Mean ± SD	96 Mean ± SD	120 Mean ± SD
Emulsification index (%)	3.00 ± 0.22	14.00 ± 0.29	8.31 ± 0.32	3.73 ± 0.21	2.25 ± 0.19	1.82 ± 0.18
Foam height (mm)	11.00 ± 0.56	42.00 ± 0.64	37.00 ± 0.68	35.0 ± 0.61	31.00 ± 0.59	27.00 ± 0.41
Foam stability (h)	1.53 ± 0.11	24.00 ± 0.17	21.00 ± 0.19	5.02 ± 0.16	1.17 ± 0.09	0.53 ± 0.06

lowered (Figure 2). Table 1 shows emulsification index and foam activity of *P. aeruginosa* PTCC 1561 supernatant in nutrient broth. Emulsification index values followed a similar pattern as surface tension was lowering. *P aeruginosa* PTCC 1561 showed better foam forming activity and emulsification index after cultivation in brain hearth medium. As shown in Table 2, incubation of *P. aeruginosa* PTCC 1561 in BHB medium caused improvement in emulsification index. The production yield improved by addition of salts (Figure 3).

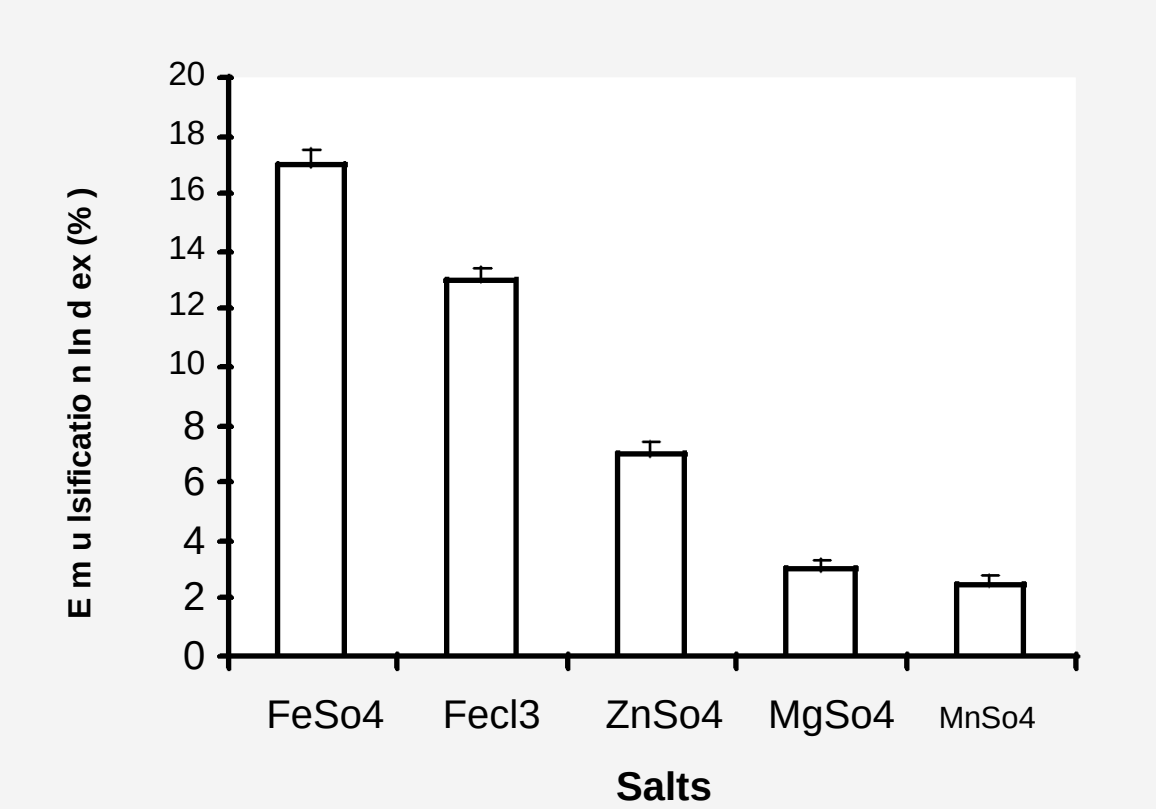

Figure 3. Emulsification index (E24%) of *Pseudomonas aeruginosa* PTCC 1561 examined with different salts (200 rpm, 24 h, 37˚C) (Mean ± SD, n = 5).

Adding of FeSO4, FeCl3 and ZnSO4. cations of Fe^{2+}, Fe^{3+} and Zn^{2+} caused enhancement of the yield, while Fe^{2+} caused a larger emulsification index increasing than Fe^{3+} and this caused a larger emulsification index increasing than Zn^{2+}. Based on emulsification index studies, it can be concluded that when Fe^{2+}, Fe^{3+} and Zn^{2+} were added together to the brain hearth broth medium, the best yield bioemulsifier was obtained (Figure 3). As results, applying starch to the medium caused improvement in emulsification index. Adding liquid paraffin oil had a negative affect on emulsified layer. But olive oil obviously increased emulsification index (Figure 4). Biochemical reactions and IR spectroscopy were carried out and the structure of the biosurfactant obtianed from the *P. aeruginosa* PTCC was confirm.

DISCUSSION

Biomass growth started from the first hour of fermentation and reached its maximum within 24 h after incubation followed by a reduction until 120 h throughout the fermentation. Hence it is supposed that maximal cell growth was achieved at 24th h of fermentation. In similar studies on *Pseudomonas* strains maximum growth was in 24 h after cultivation for *P. aeruginosa* APO2-1 and *P. aeruginosa* RhlA (Perfumo et al., 2006; Chavez et al., 2005). In another study, this factor was reported in 22 h for P. alcaligenes PA-10 (Hickey et al., 2007). As it is shown on Figure 2, it could be suggested that biosurfactant production started from the first hour of incubation and continued for the next 24 hours. CMD values were minimal at this period. Based on similar studies, with the maximum poroduction of biosurfactant, surface tension lowered to 28 mN/m for *P. aeruginosa* APO2-1, 30.1 mN/m for *P. aeruginosa* YPJ-80, 31.3 mN/m for *P. aeruginosa* MTCC 2297, and 30 mN/m for *P. aeruginosa* Bs20 (Perfumo et al., 2006; Park et al., 1998).

According to the growth curve of microorganism and the surface tension profile of supernatant and CMD values, it can be expected that the lowest the surface tension, the highest biosurfactant production by the microorganism. Hence this period was chosen as optimum for biosurfactant production for further experiments. As it was expected, increasing of incubation time causes improvement in emulsion index and this increase continued till 24 h of incubation, followed by a reduction during the time, and reached to the minimal level at 120 h. The data displayed that produced foam had the best

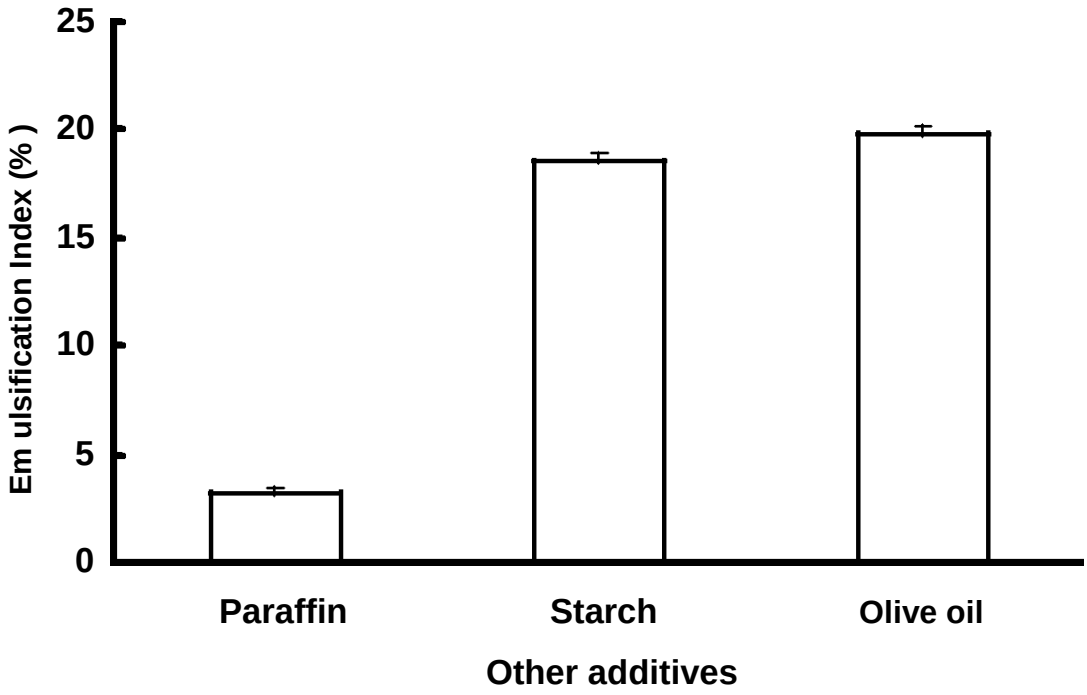

Figure 4. Emulsification index (E24%) of *P. aeruginosa* PTCC 1561 examined with different hydrocarbons (200 rpm, 24 h, 37˚C) (Mean ± SD, n = 5).

quality containing stability and height in this time. Results also showed that there is a rational correlation between surface activity (Figure 2), emulsification index and foam activity (Table 1). Emulsification index values followed a similar pattern as surface tension exhibited lowering while the supernatant surface tension was at lowest level, emulsification index raised to maximum. The optimum incubation time was 24 h for biosurfactant production, because emulsification index is an important parameter of surface active agents through the course of producing a stable emulsion. In other studies E24% has been reported 71, 64.2, 73.3 and 62.5% for *P. aeruginosa* APO2-1, *P. aeruginosa* YPJ -80 *P. aeruginosa* MTCC 2297 and *P. aeruginosa* Bs20 (Perfumo et al., 2006; Park et al., 1998) the best condition.

According to data obtained in this study, when *P. aeruginosa* TCC 1561 was grown in the nutrient broth medium, the production of the biosurfactant was relatively poor. The surface activities could be improved and a larger emulsification index achieved by growing the microorganism in BHB medium. Hence it can be concluded that incubation of the microorganism in BHB medium at 200 rpm has positive affects on the improvement of biosurfactant production yield. It is conclusive that the optimum conditions for biosurfactant production is a brain hearth broth containing starch, FeSO4 , FeCl3, ZnSO4 and olive oil incubated in a 200 rpm shaker incubator at 37°C for 24h.

As shown in Figure 3, applying FeCl3, ZnSO4 and FeSO4 to the nutrient broth medium caused an improvement in emulsification index. But MnSo4 and MgSO4 destroyed the emulsified layer between two phases and had negative affect on emulsification index. Applying starch to the medium, improved the emulsification index properties. Adding liquid paraffin had negative affect on emulsified layer, but olive oil increased emulsification index (Figure 4) . In similar studies, olive oil with fish oil have introduced the best additives for *P. aeruginosa* BYK-2(KCTC 18012P) and diesel oil with crude oil have been shown the most suitable carbon sources for *P. aeruginosa* T1 (Jeong et al., 2004; Lima et al., 2008). In several other studies, crude oil, olive oil, and palm oil have been mentioned as the best additives to culture media for *P. aeruginosa* IFO 3924, *P. aeruginosa* AT10, and *P. aeruginosa* IGB 83 (Abalos et al, 2004; Chavez and Martinez, 2001; Marsudi et al., 2008).

Determination of hydrophobicity percent in supernatant

The cell surface hydrophobicity of *P. aeruginosa* PTCC 1561 was studied and two different culture medias were used, icluding brain hearth broth and nutrient broth.

Hydrophobicities of *P. aeruginosa* PTCC1561 have been shown on Table 3. According to the data obtained in this study, *P. aeruginosa* PTCC 1561 possessed a higher cellular hydrophobicity in brain hearth broth medium thannutrient broth medium. Also emulsification index values of supernatants followed a similar pattern as

Table 3. Hydrophobicity percent and emulsification index in two nutrient broth and brain hearth broth media (n=5).

Medium	Hydrophobicity (%)	Emulsification index (%)
	Mean ± SD	Mean ± SD
Nutrient broth	11.82 ± 0.86	12.06 ± 0.92
Brain hearth broth	33.52 ± 1.18	51.25 ± 1.32

hydrophobicity. Based on several studies, it has been demonstrated that there is increasing cell surface hydrophobicity and as a result increasing in bacterial adhesion to hydrocarbons in culture media if the biosurfactant production in *P. aeruginosa* APO2-1 is in its highest level (Perfumo et al., 2006).

In previous studies on different *P. aeruginosa* species, the aeration rate were 180, 200 and 250 rpm for *P. aeruginosa* BYK-2(KCTC 18012P), *P. aeruginosa* T1, and *P. aeruginosa* S2 (Chen et al., 2007; Hasanuzzaman et al., 2004; Jeong et al., 2004). Optimum temperatures vary from 30 to 40°C for different *P. aeruginosa* strains. As the results showed in this research, culture of *P. aeruginosa* PTCC 1561 had better findings in emulsification index at 37°C (data not shown). Since there was correlation between emulsification index and biosurfactant production it was confirmed that the optimum temperature for biosurfactant production by *P. aeruginosa* PTCC 1561 was 37°C. In previous studies based on rhamnolipid production, incubation temperatures were 29 and 30ºC for *P. aeruginosa* RhlA and *P. aeruginosa* T1 (Chavez et al., 2005; Hasanuzzaman et al., 2004). Also the best temperature was 37ºC for both *P. aeruginosa* MTCC 2297 and *P. aeruginosa* S2 (Chen et al., 2007; George and Jayachandran, 2007).

The aeration rate plays an important role in biosurfactant production. The best conditions for rham-nolipid production is 200 rpm and the flasks should contain 50ml of medium (1/5 of whole flask volume) in order to maximize aeration. Incubation of *P. aeruginosa* at 200 rpm caused an improvement in emulsi-fication index due to more biosurfactant production. In previous studies on different *P. aeruginosa* species the aeration rate were 180, 200 and 250 rpm for *P. aeruginosa* BYK-2(KCTC 18012P), *P. aeruginosa* T1, and *P. aeruginosa* S2 (Chen et al., 2007; Hasanuzzaman et al., 2004; Jeong et al., 2004). Optimum temperatures for different *P. aeruginosa* strains were vary from 30 to 40ºC. In this study, *P. aeruginosa* PTCC 1561 culture was shown better findings in emulsification index at 37ºC. According to correlation between emulsification index and biosurfactant pro-duction it was demostrated that the optimum temperature for biosurfactant production by *P. aeruginosa* PTCC 1561 was 37ºC. In previous studies based on rhamnolipid production, incubation temperatures were 29 and 30ºC to *P. aeruginosa* RhlA and *P. aeruginosa* T1 (Chavez et al., 2005; Hasanuzzaman et al., 2004). Also the best temperature was 37ºC for both *P. aeruginosa* MTCC 2297 and *P. aeruginosa* S2 (Chen et al., 2007; Chavez et al., 2005). In another study, it was observed increasing in cell surface hydrophobicity if there was increasing in equivalent alkane carbon number (as carbon source for culture media of *B. subtilis* T 894 and *B. subtilis* Ro 662) (Noha et al., 2007). Also in another investigation, adsorption of rhamnolipids on *P. aeruginosa* CCTCC AB93066 and *P. aeruginosa* CCTCC AB93072 caused increasing in cell surface hydrophobicity (Zhong et al., 2008).

Characterization of bioemulsifier

It was shown Bromine water reaction was negative, that indicates the fatty acid chain was saturated. This test is for identification of carbohydrates from other substances and it is positive for all carbohydrates. Positive result of this test for present study, was a reason for sugar moiety in biosurfactant. The IR spectrum of the sample (Figure 5), showed the characteristic absorption bands at 3368 cm-1 resulting from hydroxyl groups, 2923 cm- 1 from long chain -CH2, 1732 cm-1 shows acid carbonyl group, 1637 cm-1 related with carboccylic acid moiety, 1456 cm-1 from -CH3, 1117 cm-1 from -CH2 and 1050 cm-1 is for C-O-C group.

These results indicate that the product has sugar-lipid structure. Therefore this biosurfactant with various activities such as detergent and solubilizing properties can be used as an interesting compound in the industries. Due to its low toxicity to the membranes, it would be reconsidered a suitable surfactant in drug formulations.

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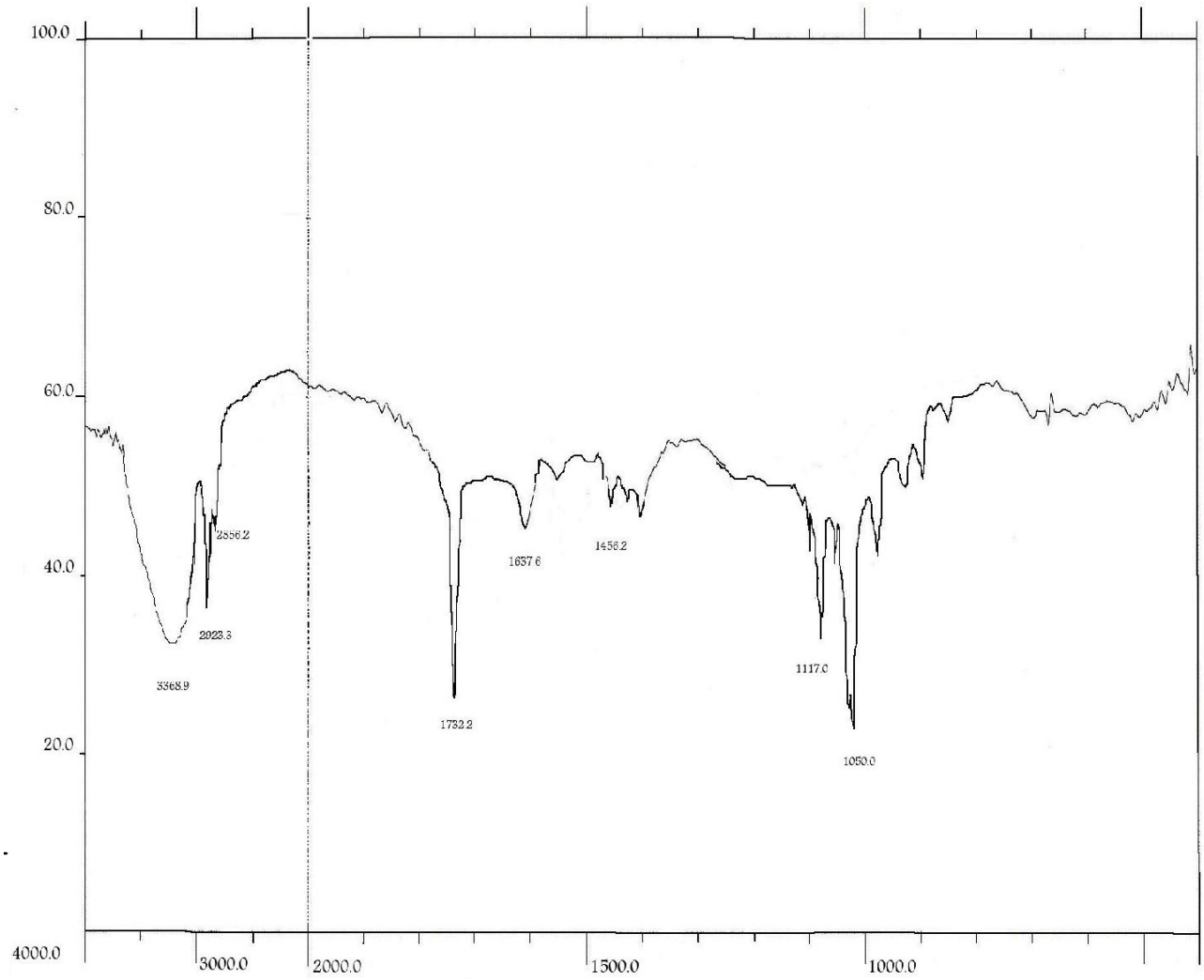

Figure 5. IR spectrum of the produced biosurfactant by *Pseudomonas aeruginosa* PTCC 1561.

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