Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2017, 9(8):6-12
(http://www.derpharmachemica.com/archive.html)

Screening of Some *Bacilli* Strains for their Abilities to Produce Biosurfactants

Mona F Ghazal[1*], Lobna A Moussa[1], Hussein E Makboul[2], Sayed A Fayed[3]

[1]Department of Agriculture and Microbiology, Soils, Water and Environment Research Institute, Giza, Egypt
[2]Department of Agriculture and Microbiology, Cairo University, Giza, Egypt
[3]Department of Biochemistry, Cairo University, Giza, Egypt

ABSTRACT

Biosurfactants are valuable microbial amphiphilic molecules (consisting of molecules having a polar water-soluble group attached to a water-insoluble hydrocarbon chain) with effective surface-active and biological properties applicable to several industries and processes. In recent years, natural biosurfactants had attracted attention because of their low toxicity, biodegradability and ecological acceptability. Two Bacilli species were tested for their abilities to produce biosurfactants by measuring their emulsification activity, emulsification index, oil displacement test, drop collapse and spreading over the blood agar plates. Also, the effects of different carbon and nitrogen sources, as well as, pH and inoculum size were examined as factors affecting the surfactants biosynthesis. Results showed that both Bacilli isolates can produce biosurfactant by using waste frying oil as a carbon source and peptone as a nitrogen source. There was an effect for different factors used in the production of biosurfactants showed by two isolates. Both Bacilli isolated were identified by 16s rRNA as Bacillus subtilis and Bacillus megaterium.

Keywords: Microbial biosurfactant, Emulsification, *Bacilli* isolates

INTRODUCTION

The surface-active compounds (surfactants) are commonly used in many industries, they are chemically synthesized; they are widely used in almost every sector of recent industry [1]. Most of these surfactants are petroleum based and are chemically synthesized. However, the leading trend towards using environmental friendly technologies has enhanced the search for biodegradable compounds of natural origin. Microorganisms are being increasingly investigated as cell factories for the production of various chemicals and materials from renewable resources due to their growing concerns in limited fossil resources. Microbial fermentation products derived from readily available, renewable, and inexpensive raw materials will competitively replace some of the products traditionally derived from non-renewable resources [2]. Biosurfactants are therefore the natural choice for such processes as they possess a host of advantages over synthetic surfactants, such as lower toxicity, biodegradability, specificity of action, simplicity of preparation, extensive applicability and effectiveness at a wide range of pH and temperature values [3]. The expansion in environmental carefulness has led to serious consideration of biological surfactants as the most promising alternative to existing product [4]. Moreover, they can be used as moistening agents, dispersing agents, emulsifiers, foaming agents, beneficial food elements and detergents in many industrial regions such as: organic chemicals, pharmaceutical, cosmetics, beverages and foods, metallurgy, mining, petroleum, petrochemicals, biological control and management and many others [5]. Biosurfactants have also many advantages rather than those of synthetic ones. They have the abilities to reduce superficial and interfacial tension reduction between solids, liquids and gases. Interest in their potential applications by various industries has recently increased significantly, particularly because of their environmental friendly nature and sustainability also, bioavailability, structural diversity, specific activity at extreme salinity, temperatures and pH [6,7].

The Gram-positive *Bacillus* species are non-pathogenic and free of exotoxins and endotoxins, safely used in foods. They are widely used for the production of industrially important biochemical. Because their efficient genetic manipulation and highly developed systems-level, they are considering as promising candidates as heterologous hosts [8].

In the present work, *Bacillus* isolates *viz*. *Bacillus* MG13 and *Bacillus* MG20 are tested for their ability to produce biosurfactants under the effect of different carbon and nitrogen sources. As well as, pH and inoculum size were examined as factors affecting the surfactants biosynthesis.

MATERIALS AND METHODS

Biosurfactants production

Seed inoculum preparation

A loop for each bacterial isolate was inoculated into 50 ml of nutrient broth medium in a 250 ml conical flask and incubated on a rotary shaker at 28°C; 200 rpm for 16-18 h up to O.D. became 0.100 at 600 nm. The biosurfactant production was performed in 250 ml conical flasks containing 100 ml of the modified Mckeen medium inoculated with 10% (v/v) from each bacterial isolate. The modified Mckeen medium composed of 2.5% of soy bean or petroleum oil, 0.25% monosodium glutamate, 0.3% yeast extract, 0.1% $MgSO_4$. $7H_2O$, 0.1% K_2HPO_4, 0.05% KCl and 0.1% (v/v) of trace elements solution (g/100 ml distilled water): 0.64 g $MgSO_4$. $7H_2O$, 0.16 g $CuSO_4.5H_2O$, 0.015 g $FeSO_4.7\ H_2O$, pH 7.0) [9]. The flasks were incubated on a rotary shaker at 28°C, with 150 rpm for 96 h.

Biosurfactant activity tests

Biosurfactants produced by each isolate was purified from the culture supernatants prepared by centrifuging the culture broth at 12000 rpm for 30 min at 4°C. The emulsification activity of the supernatant of bacterial isolates as well as the pure aqueous solution of biosurfactants (0.1 g biosurfactant in 50 ml distilled water) was tested by using two methods.

Emulsification activity test

Two ml of cell free supernatant were added into screw-capped tubes containing 2 ml of distilled water. The solution was mixed with 1 ml of a substrate (soybean or diesel oils), then after a vigorous vortex for 2 min, the tubes were allowed to stand for 1 h to separate aqueous and oil phases, before measuring the absorbance at 540 nm [10]. Aqueous phase was removed carefully and O.D. at 540 nm was measured and compared with un-inoculated broth used as negative control. Emulsification activity was defined as the measured optical density at 540 nm. Assays were carried out in triplicates.

Emulsification index (E_{24}%) test

Two ml of cell free supernatant and 2 ml of diesel oils were added to a screw cap tubes and vortex at high speed for 2 min to be a mixture. The mixtures were incubated at room temperature for 24 h. The emulsion index (E_{24}%) was then calculated from the ratio of the height of the emulsion zone to the total height of the oil, emulsion and aqueous zones [11].

Screening assays for potential biosurfactants producing isolates

Both tested *Bacilli* isolates were assayed qualitatively using different methods namely oil displacement test, drop collapse and hemolytic activity.

Oil displacement test

In the oil spreading technique developed by Morikawa et al. [12], 30 ml of distilled water was poured in a Petri dish to which 1 ml of coconut/sesame oil was added to the center. 20 μl of the culture supernatant obtained from the bacterial broth was added on top of the oil layer. The Petri dishes were closely observed for a zone of displacement in the oil, and the diameter of displacement was measured.

Drop collapse test

In order to test whether produced biosurfactant was able to decrease the surface tension between water and hydrophobic surfaces, the ability to collapse a droplet of water was tested as follows: 25 µl of extracted biosurfactant was pipetted as a droplet onto parafilm; the flattening of the droplet and the spreading of the droplet on the parafilm surface was followed over seconds or minutes. Subsequently, methylene blue (which had no influence on the shape of the droplets) was added to the water stain and allowed to dry and the diameter of the dried droplet was recorded [13].

Hemolytic activity

Isolates were screened on blood agar plates containing 5% (v/v) sheep blood and incubated at 37°C for 48 h. Hemolytic activity was detected as the presence of a clear zone around bacterial colonies [14].
All the experiments were done in triplicate.

Optimization of some factors for proper biosurfactant production

Effect of using different carbon sources on production of biosurfactant

Mineral Salt Medium (MSM) medium was supplemented by different organic carbon sources; there were waste frying oil, diesel oil, kerosene and petrol by 2% (v/v) concentrations. Each Erlenmeyer 250 ml flask containing 50 ml of MSM medium supplied with one carbon source, then inoculated with 10 ml broth of each of the isolated *Bacilli* (OD 1.0 at 600 nm) and incubated on a rotary shaker operated at 150 rpm for 72 h The cells were removed by centrifugation rpm and supernatant was used for emulsification activity and emulsification index (E24%) [14].

Effect of different nitrogen sources

The effect of nitrogen sources on biosurfactant production was investigated by adding 2% of NH_4NO_3, NH_4Cl, urea and peptone to mineral salt medium. The incubation was carried out on a shaker with 150 rpm at 30°C for 3 days. The cells were removed by centrifugation and supernatant was used for emulsification activity and emulsification index (E24%).

Effect of inoculum size on biosurfactants production

In the production of the biosurfactants using the studied bacterial strains, the inoculum size inoculated to the production medium was adjusted to furthermore inoculum size concentration from 2.5 to 10% (v/v).

Effect of pH

In 250 ml Erlenmeyer flasks containing 50 ml sterile Makeen medium supplied by the best sources of both carbon and nitrogen that were obtained in above studies.

The medium was adjusted at different PH values at 5, 7 and 9 and it was inoculated with starter culture by 10% bacterial inoculum concentration and incubated for 3 days at 30°C. The cells were removed by centrifugation and supernatant was used for estimation of emulsification activity and emulsification Index (E24%).

Identification of *Bacillus* isolates

PCR amplification for the 16S r RNA gene for the different bacteria

16S rRNA primers were used for amplification of the conserved region 16S rRNA gene according to Youssef et al. [15] based on *E. coli* genome. Primers were supplied by MWG, Germany. PCR amplifications were carried out in a total volume 25 µl containing 2.5 × 10 µl buffer, 2 µl 25 mM $MgCl_2$, 2 µl 2.5 mM dNTPs, 1 µl 10 pmol of each 27F and 1492R primers (Table 1), 1 µl 50 ng of genomic DNA and 0.2 µl TaqDNA polymerase (5 units/µl). PCR amplification was performed in a thermal cycler (Eppendorf) programmed for one cycle at 95°C for 5 min. Then 34 cycles were performed as follows: 1 min at 95°C for denaturation, 1 min at 55°C for annealing and 2 min at 72°C for elongation. Reaction mixture was then incubated at 72°C for 10 min for final extension and stored at 4°C.

DNA sequence and sequence analysis of 16S r RNA amplicones

The amplified PCR amplicones of the 16S r RNA gene (1500bp) were cut from the agarose gel and purified using the agrose DNA purification kit (Qiagene, Germany). The purified fragments were subjected to DNA sequencing using the automated DNA sequencer (Macrogen Company, Korea). The sequence analysis was performed using NCBI database and the obtained DNA nucleotide sequences were submitted to gene bank to get the accession numbers.

RESULTS AND DISCUSSION

Biosurfactant activity and emulsification index (E24%)

Results in Table 1 and Figure 1 showed that both *Bacilli* strains were able to produce biosurfactants due to its emulsification activity and emulsification index. The emulsification activity and emulsification Index observed were 1.980, 53% respectively in case of *Bacillus* MG20 while they were 1.850 and 49% respectively in case of *Bacillus* MG13. Biosurfactants are extracellular macromolecules produced by bacteria, yeast, and fungi and, in particular, by natural and recombinant bacteria when grown on different carbon sources. Specifically, the *Bacillus* species is well known for its ability to produce lipopeptides type biosurfactants with potential surface-active properties when grown on different carbon substrates [16]. Among the potential biosurfactant producing microbes, *Bacillus* species are known to produce cyclic lipopeptides including surfactants, iturins, fengycins and lichenysins as the major classes of biosurfactants [14].

Table 1: Emulsification activity and emulsification index of *Bacillus* strains

Bacterial strain	Emulsification activity (OD at 540 nm)	Emulsification index (E24%)
Bacillus MG20	1.95	48
Bacillus MG13	1.85	49

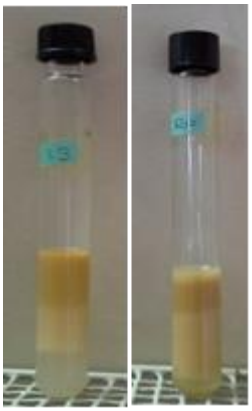

Figure 1: Emulsification index of *Bacilli* isolates

Screening assays for potential biosurfactants producing isolates

The screening of biosurfactants producing bacteria was carried out using hemolytic activity, oil collapse and oil spreading techniques. Selection of these methods was due to their strong advantages including simplicity, low cost, quick implementation and use of relatively common equipment that is accessible in almost every microbiological laboratory; however, as expected, these methods are not perfect or flawless [14].

Oil displacement technique

To the concentration of the biosurfactant produced by the both *Bacillus* strains, the petri dishes were closely observed for a zone of displacement in the oil, and the diameter of displacement was measured in mm. Both samples had significantly displaced the oil layer and started to spread in the water, showing a zone of displacement *Bacillus* MG13 the largest displacement diameter value 7.5 cm while displacement had observed by *Bacillus* MG20 was 5 cm. Using oil spread test, comparatively high abundances of surfactant-producing bacterial [16].

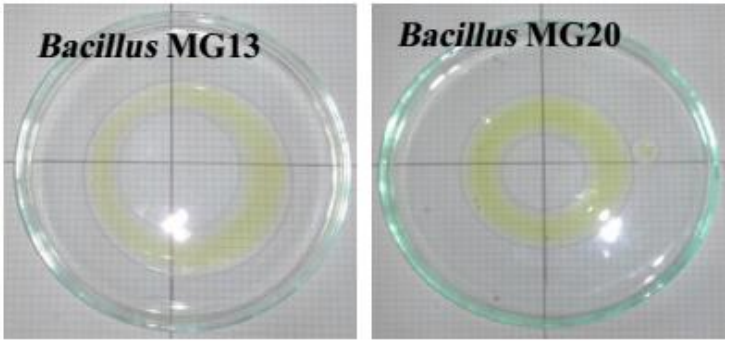

Figure 2: Oil displacement test of the biosurfactant produced by *Bacillus* isolates

Drop collapse

The drop collapse method depends on the principle that a drop of liquid containing a biosurfactant collapses and spreads over the oily surface. There is a direct relationship between the diameter of the sample and concentration of the biosurfactant and in contrast, the drop lacking biosurfactant remains beaded due to the hydrophobicity of the oil surface that cause aggregation of droplets [13]. In drop collapse assay, no activity was detected for distilled water as predicted. The biosurfactant droplets do result in a collapsed droplet (Figure 3); indicating their effects on reduction of surface tension, in this study the isolate *Bacillus* MG13 showed biggest drop collapse than other isolates (Figure 3).

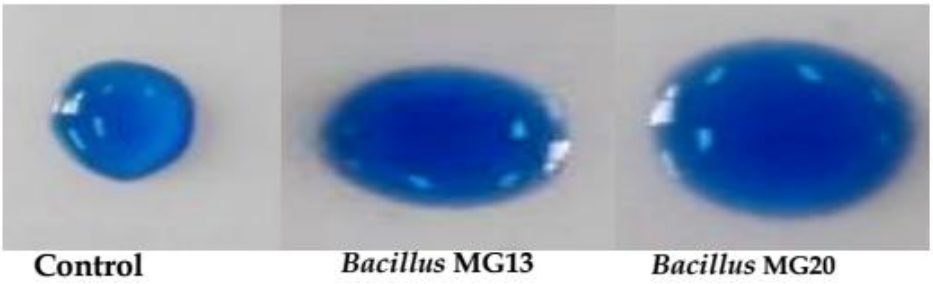

Figure 3: Drop collapse assay: Collapsed droplets

The biosurfactants activity showed that we had used real surfactant preparations, since the force or interfacial tension between the drop containing the surfactant and the Para film surface was reduced and resulted in the spread of the drop [17,18] also showed that surface tension was reduced by biosurfactants.

Hemolytic activity

Accordingly, in the present study, *Bacillus* strains displayed excellent hemolytic activity (Figure 4) [19] scored the hemolytic activity. The hemolytic strains to lower the surface tension. Hemolytic activity appears to be a good screening criterion in the search for biosurfactants produced bacteria [20]. Lysis of blood agar has been recommended as a method to screen for biosurfactant activity. This method is useful in predicting the promising strains regarding biosurfactant production. Since, in most cases, the degree of lysis of RBC is directly proportional to the concentration of biosurfactant production [16]. Hemolysis was included in this study since it is widely used to screen biosurfactant production and in some cases, it is the sole method used [21].

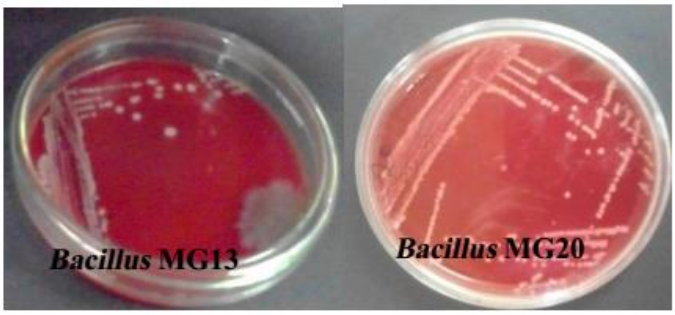

Figure 4: Hemolytic activity of *Bacilli* isolates

The biosurfactant produced by *B. subtilis*, surfactin, lysed red blood cells. Blood agar lysis has been used to quantify surfactin [22]. However, in some cases hemolytic assay excluded many good biosurfactant producers [16]; Hence in the present investigation the oil displacement assay and dropcollapse test with crude oil were also done to confirm biosurfactant production.

Effect of carbon sources

In order to reach over production of biosurfactants, nutritional requirements of *Bacilli* isolates biosurfactants producing strain and growth parameters were studied; different carbon sources could be used in the medium for biosurfactants production. The effect of using different carbon source showed in Figure 5.

The emulsification activity and emulsification index (E24%) of the produced biosurfactant was tested with different hydrocarbon and all the hydrocarbons were emulsified efficiently. Culture medium exhibited excellent surface activity on each carbon source tested and biosurfactant production was observed even after the stationary phase. Among the carbon source tested, waste frying oil shows the highest emulsification activity of (3.8) achieved by *Bacillus* MG20, while diesel oil showed the lowest activity of (0.648) which was also achieved by *Bacillus* MG20.

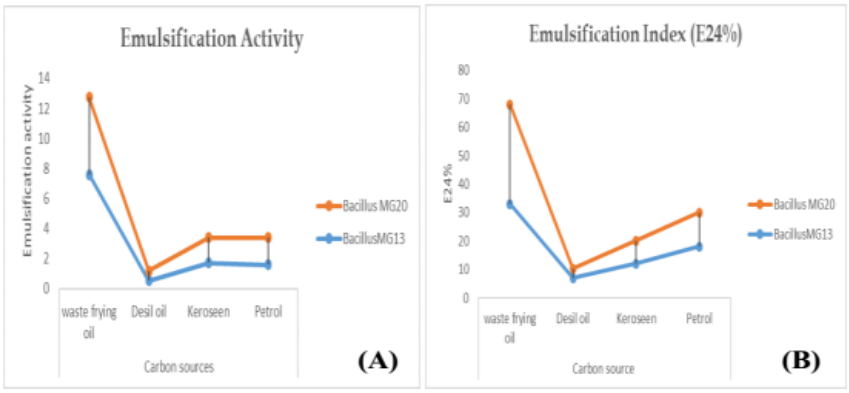

Figure 5: Effect of using different carbon sources on biosurfactant production by *Bacillus* isolates. (A) Emulsification activity; (B) Emulsification index

Waste frying oil is a raw material associated with vegetable industry is residual cooking or frying oil which is a major source of nutrient rich low cost fermentative waste. Large quantities of cooking oil are generated in restaurants worldwide. It has been estimated that on average100 billion oil waste/week is produced in United States alone [23]. There are few reports, which utilized the vast potential of these frying oils for biosurfactant production. The concluded that selected *Bacillus* strain has the potential to produce biosurfactant from waste frying soybean oil. Thus, studies have been carried out to explore possibilities of using low-cost and renewable agro-industrial wastes including distillery wastes, plant oils, oil wastes, starchy substances, and whey as substrates for cost effective production of biosurfactant [24]. The quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate [25].

Effect of nitrogen sources

Nitrogen plays an important role in the production of surface-active compounds by microorganisms [26]. Since medium constituents other than carbon sources also affect the production of biosurfactants [27]. In the present study, peptone shows the highest emulsification activity which achieved by *Bacillus* MG20 was followed by yeast extract by *Bacillus* MG20. The lowest emulsification activity in organic nitrogen sources was observed in Prommachan and Cthesis [28] reported that peptone increase growth and biosurfactants production. Also different inorganic nitrogen sources affected biosurfactant production by *Bacillus* isolates, while ammonium nitrate showed higher emulsification activity and emulsification index than ammonium chloride that achieved by *Bacillus* MG13 (Figure 6). Ammonium nitrate was a good substrate for the growth with good productivity. These results are in agreement report of Fagade et al. [29] in which ammonium salt was observed as a preferable nitrogen source for the biosurfactant production.

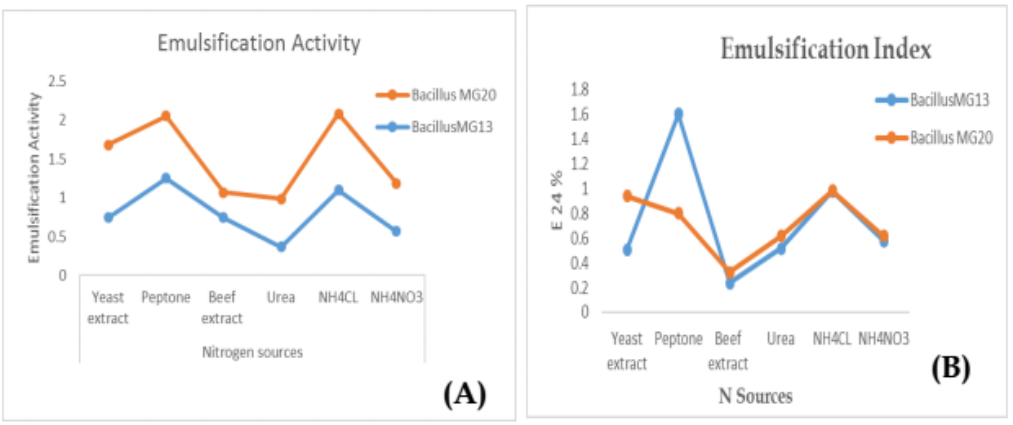

Figure 6: Effect of using different nitrogen sources on biosurfactant production by both *Bacilli* isolates. (A) Emulsification activity; (B) Emulsification index

Inoculum size

The data provided in Figure 7 indicated that, there were a gradual increase in emulsification activity and emulsification index and upon increasing the inoculum size up to 10% v/v by both *Bacillus* isolates, but emulsification activity of *Bacillus* MG13 was (1.28) and higher than *Bacillus* MG20, emulsification index reported by *Bacillus* MG20 was higher than Bacillus MG13, these results were agreed with [30]. It was observed that as inoculum density increases emulsification activity. In another study by Chander Suresh et al. [31] the production medium was seeded with *B. subtilis* MTCC441 led to good biosurfactant production.

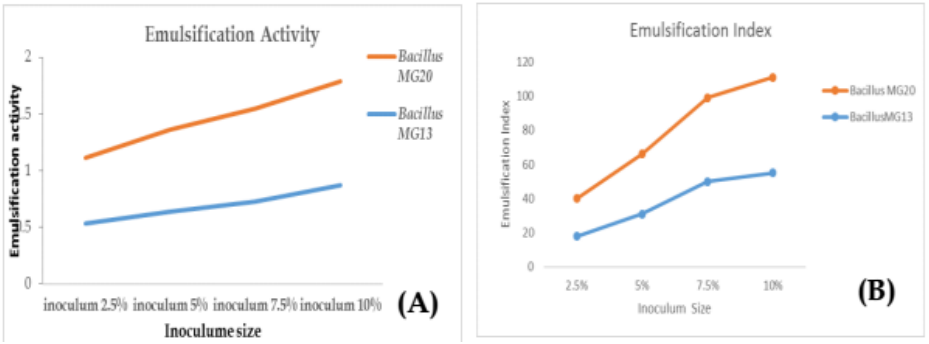

Figure 7: Effect of using different inoculum size on biosurfactant production by both *Bacilli* isolates. (A) Emulsification activity; (B) Emulsification index

Effect of pH

The important characteristics of most organisms are their strong dependence on the pH for cell growth and production of metabolites. In this study, biosurfactant production maintained increased values by increasing pH (Figure 8) by two *Bacillus* isolates.

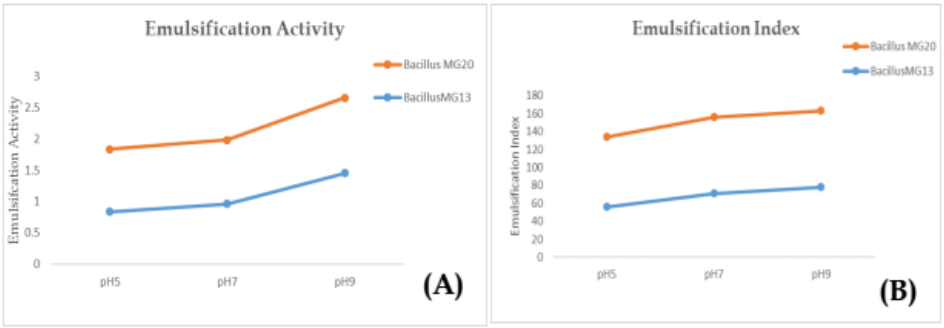

Figure 8: Effect of using different pH degrees on biosurfactant production by *Bacilli* isolates. (A) Emulsification activity; (B) Emulsification index

The highest emulsification activities and emulsification index achieved by of isolate *Bacillus* MG13 in pH 9 and the lowest emulsification activities and emulsification index reported by *Bacillus* MG8 in pH 5. Similar observation was reported [32].

Identification of *Bacillus* isolates

The most powerful new method for screening of microbial diversity for complex environmental samples is based on the cloning and sequencing of 16S rRNA gene [33]. The molecular approach has been used for bacterial phylogeny and is of great importance for definition and identification of species [34]. The positive result obtained from the above confirmatory assays confirmed the biosurfactant production by both strain. Based on the 16S rRNA gene sequences and using the Gene Bank BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). This study demonstrated that the 16S rRNA sequence of the *B. subtilis* MG13 was 100% identical to the type strain KP196795 of South Africa by 100%. Also, isolate *B. megaterium* MG20 was 100% identical to the strain type EU620419 of India. This is based on sequence alignments and the phylogenetic dendogram as shown in Figures 9 and 10.

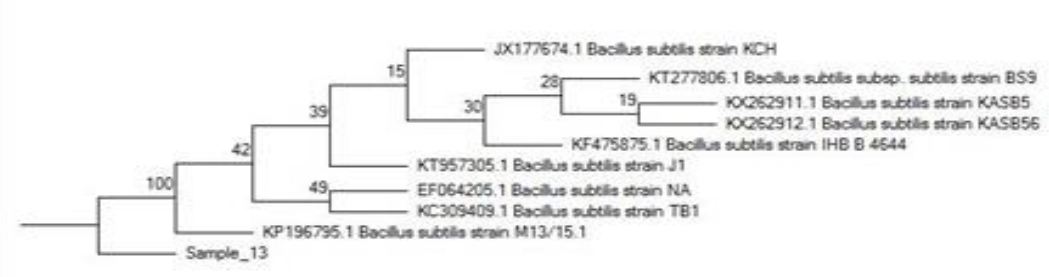

Figure 9: Phylogenetic dendrogram based up on 16S rRNA sequence (1500 bp) of *Bacillus subtilis* MG13strain compared with the sequence of standard strains obtained from the Gene Bank database

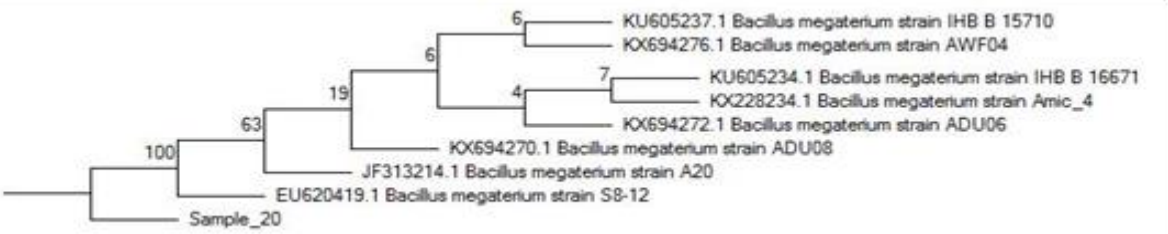

Figure 10: Phylogenetic dendrogram based up on 16S rRNA sequence (1500 bp) of *Bacillus megaterium* MG20 strain compared with the sequence of standard strains obtained from the Gene Bank database

CONCLUSION

Conclusion of the present study, both *Bacillus* strains can produce biosurfactants using different carbon and nitrogen sources. Environmental factors and growth conditions such as temperature, pH and inoculum size also nutrients in growth medium like source of carbon and nitrogen affect biosurfactants production through their effect on cellular growth and emulsification activity. Both Bacilli isolates *Bacillus* MG13 and *Bacillus* MG20 are identified as *B. subtilis* and *B. megaterium* respectively according to 16S rRNA analysis and Gene bank data.

REFERENCES

[1] N. Samadi, M.R. Fazeli, N. Abadian, A. Akhavan, A. Tahzibi, H. Jamalifar *J. Biol. Sci*., **2007**, 7, 1266-1269.
[2] I.K. Kim, A. Roldão, V. Siewers, *J. Nielsen, FEMS Yeast Res*., **2012**, 12, 228-248.
[3] D.P. Sachdev, S.S. Cameotra, *Appl. Microbiol. Biotechnol*., **2013**, 97, 1005-1016.
[4] M. Henkel, M.M. Muller, J.H. Kugler, R.B. Lovaglio, *J. Contiero, C. Syldatk, Process Biochem.*, **2012**, 47,1207-1226.
[5] N. Vedaraman, N. Venkatesh. *Brazi. J. Chem. Eng*., **2011**, 28, 175-180.
[6] C.N. Mullingan, *Curr. Opin. Coll. Inter. Sci.*, **2009**, 14, 372-378.
[7] S. Datta, S. Sahooand, B. Dipa, *J. Adv. Sci. Res*. **2011**,2, 32–36.
[8] I. G. De Jong, K. Beilharz, O. P. Kuipers, J. W. Veening, *J. Vis. Exp*., **2011**, 53, 3145-3151.
[9] S.R. Phitnaree, K.F. Vatcharin, H.K. Aran, *J. Sci. Technol*., **2008**, 30, 87-93.
[10] S.K., Satpute, B.D., Bhawsar, *Ind. J. Marine Sci*., **2008**, 37, 243-250.
[11] M.S., Yeh, Y.H. Wei, J.S Chang, *Biotechnol. Prog*., **2005**, 21, 1329-1334.
[12] M. Morikawa, Y. Hirata, T. Imanaka, *Biochimica et Biophysica Acta*., **2000**, 1488, 211-218.
[13] T. Tugrul, E. *World J. Microbiol. Biotechnol*., **2005**, 21, 851-853.
[14] G.A. Plaza, I. Zjawionyand, I.M. Banat, *J. Petroleum Sci. Eng*., **2006**, 50, 71-77.
[15] K.H. Wilson, R.B. Blitchington, R.C. Greene, *J. Mlin. Microbiol*., **1990**, 28, 1942-1946.
[16] N.H. Youssef, K.E. D.P. Duncan, K.N. Nagle Savage, R.M. Knapp, M.J. McInerney, *Microbiol. Methods*. **2004**, 56, 334-339.
[17] H. Ghojavand, F. Vahabzadeh, E. Roayaei, A.K. Shahraki, *J. Colloid Interface Sci*., **2008**, 324, 172-176.
[18] E. Walencka, S. Rozalska, B. Sadowska, B. Rozalska, *Folia Microbiol.*, **2008**, 53, 61-66.
[19] L. Rodrigues, J.A. Teixeira, H.C. Mei, R. *A Colloid surf. B. Biointerfaces*., **2006**, 53, 105-112.
[20] P. Carrillo, C. Mardaraz, S. Pitta-Alvarez, A. Giulietti, *World J. Microb. Biot*., **1996,** 12, 82-84.
[21] H. Yonebayashi, S. Yoshida, K. Ono, H. Enomoto, *Sekiyu Gakkaishi*., **2000**, 43, 59-69.
[22] A. Moran, M. Alejandraand, F. *Martinez, Biotechnol. Lett*., **2002**, 24, 177-180.
[23] V. Shah, M. Jurjevic, D. Badia, *Biotechnol. Prog*., **2007**, 23, 512-515.
[24] E. Montoneri, P. Savarino, S. Bottigliengo, V. Boffa, A.B. Prevot, D. Fabbri, E. Pramauro, *Fresenius Environ. Bull*., **2009**, 18, 219-223.
[25] K.S.M. Rahman, E. *Gakpe, Biotechnol*., **2008**, 7, 360-370.
[26] R.S. Makkar, S.S. Cameotra, *J. Surfact. Deterg*., **2002**, 5, 11-17.
[27] H.M. Abushady, A.S. Bashandy, N.H. Aziz, H.M.M. Ibrahim. *Inter. J. Agric. Biol*., **2005**, 7, 337-344.
[28] O. Prommachan,. M.S. Cthesis, Prince of Songkla University, (Songkhla, Thailand. **2002**).
[29] O.E. Fagade, B.I. Okolie, S. Balogun, *Nigerian J. Microbiol*., **2009**, 23, 1915-1921.
[30] I. Dhouha Ghribi, S. Ellouze-Chaabouni, *Biotechnol. Res. Inter*., **2011**, 2011, 4061-4067.
[31] C.R. Chander Suresh, T. Lohitnathl, D.J. Mukesh Kumar, P.T. Kalaichelvan, *Adv. Appl. Sci. Res*., **2012**, 3, 1827-1831.
[32] A.R, Najafi, M.R. Rahimpour, A.H. Jahanmir, R. Roostaazad, D. Arabian, Z. Ghobadi, *Chem. Eng. J*., **2010**, 163, 188-194.
[33] J.L. Balcázar, D. Vendrell, I. de Blas, I. Ruiz-Zarzuela, O. Gironés, J.L. *Múzquiz, Veterinary Microbiol*., **2007**, 122, 373-380.
[34] S. Mignard, J.P. Flandrois, *Microbiol. Methods.*, **2006**, 67, 574-581.