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Characterization of Biosurfactant Produced by a Novel Thermophillic Strain (*Geobacillus thermoleovorans* JQ 912239)

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ABSTRACT

Ten thermophillic bacterial isolates were screened for their ability to produce biosurfactant depending on the emulsification activity, emulsification index and surface tension. The results showed that *Geobacillus thermoleovorans* (JQ 912239) was the most efficient one for biosurfactant production. The chemical composition of partial purified biosurfactant revealed that it consists of 37.7% lipids, 26.2% carbohydrate and 10.7% protein. The partial and/or purified biosurfactant was subjected to Fourier Transform Infrared Spectroscopy (FTIR), High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) and Nuclear Magnetic Resonance (NMR) to complete the chemical characterization. The results of the FTIR confirmed that the biosurfactant contains the lipid, carbohydrate and protein. The HPLC analysis revealed that the lipid fraction consists of palmitic acid, stearic acid and oleic acid, the carbohydrate part consists of xylose, mannose and maltose and the protein part consists of three amino acids (aspartic acid, glutamic acid and glutamine). The lipid fraction was further analyzed using GC and NMR. GC analysis indicated that it contains palmitic acid methyl ester (c16:0), stearic acid (c18:0) and oleic acid (c18:1n9c) however, purified biosurfactant contains a high percentage of palmitic acid methyl ester. The NMR analysis for purified biosurfactant detected that the main component of lipid fraction is triglycerides.

Key words: Thermophillic bacteria, *Geobacillus thermoleovorans,* biosurfactant, FTIR, GC, HPLC, HNMR

INTRODUCTION

Petroleum is a complex mixture of hydrocarbons, organic solvents and heavy metals. Bacteria have designed strategic approaches to overcome the harsh effects of organic solvents and heavy metals in contaminated soil by producing bioemulsifiers which can reduce the surface tension, interfacial tension of bacteria and increase the cell surface hydrophobicity of bacteria, thereby enhancing the dispersal, emulsification and degradation of hydrocarbon pollutants in the contaminated site (Al-Tahhan *et al*., 2000). The terms biosurfactant and bioemulsifier have often been used interchangeably to describe surface active biomolecules (Uzoigew *et al*., 2015), that are synthesized by microorganisms (Luna-Velasco *et al*., 2007) such as bacteria, fungi and yeast (Priya and Usharani, 2009). Biosurfactant are amphiphilic compounds contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual

molecules at the surface and interface respectively, which either adhere to cell surfaces or are excreted extracellularly in the growth medium (Mulligan, 2005). Biosurfactant have several advantages over the chemical surfactants, such as lower toxicity, higher biodegradability and structural diversity (Lazarkevich *et al*., 2015). Moreover, some biosurfactant have a potential as biologically active compounds and have applicability in the medical field, higher foaming, high selectivity and specific activity at extreme temperatures, pH and salinity and the ability to be synthesized from renewable feedstocks (Desai and Banat, 1997). Biosurfactants are mainly classified according to their molecular weight into low molecular-weight biosurfactant which include generally glycolipids or lipopeptides (Ron and Rosenberg, 2001; Yin *et al*., 2009). And High-molecular weight biosurfactant: These are usually referred to as bioemulsans (Salihu *et al*., 2009). The large number of bacterial species from different genera produce exo-cellular polymeric surfactant composed of polysaccharides, proteins, lipopolysaccharides, lipo-proteins or complex mixtures of these biopolymers (Ron and Rosenberg, 2001). Biosurfactant usually exhibit emulsifying capacity but bioemulsifier do not necessarily reduce the surface tension (Batista *et al*., 2010). Biosurfactant are a group of natural products of interest for biotechnological and industrial applications in dairy, food, beverage, cosmetics, detergent, textile, paint, mining, petroleum, paper pulp and pharmaceutical industries (Bodour and Maier, 2002). Many researchers elucidated on the wide range of applications of biosurfactants in medicine. They have strong antibacterial, antifungal and antivirus activity (Gharaei-Fathabad, 2011). Also other activity such anti-cancer activity, immunological adjuvants and gene delivery (Fakruddin, 2012).

According to the great importance of biosurfactants and due to limitation of studies about their production by thermophilic bacteria, this research aimed to characterize the biosurfactant produced by a novel thermophilic strain (*G. thermoleovorans* (JQ 912239)).

MATERIALS AND METHODS

Bacteria: Ten thermophillic bacterial isolates used in this study were isolated from hydrocarbon contaminated soil in Iraq. These isolates showed good ability to utilize crude oil and aromatic compounds and considered as a novel group of aromatic hydrocarbon degrading extreme thermophillic bacteria. All these isolates belonged to the family Bacillaceae and four of them were molecularly identified and reported for the first time as carbazole utilizing thermophilic bacteria one of them *Geobacillus thermoleovorans* (JQ 912239) and three belonged to *Anoxybacillus* sp. The all isolates were obtained from a previous study (AL-Jailawi *et al*., 2013) and activated by taking pure colonies in Luria Bertani (LB) broth medium.

Screening for biosurfactant producing bacteria: To detect the ability of the ten bacterial isolates for biosurfactant production, 50 mL of Mineral Salt Medium (MSM) (Arutchelvi *et al.,* 2009) was dispensed in 250 mL erlenmeyer flasks. The flasks were sterilized and then 1% of crude oil (sterilized by tyndallization) was added as a sole source of carbon and energy. The flasks were inoculated with 1% of fresh bacterial growth (18 h) and incubated under shaking (180 rpm) at 55°C for 7 days. Then the cultures were centrifuged at 4°C, 10000 rpm, for 15 min. Biosurfactant was investigated in cell-free supernatant using three screening methods: Emulsification Activity (EA) (Sifour *et al*., 2007), emulsification index (E24%) (Tabatabaee *et al*., 2005) and surface tension which was measured by tensiometer (Krüss GmbH, Hamburg, Germany) using the du Nouy ring method.

Extraction and purification of biosurfactant: Optimum conditions to produce biosurfactant by *G. thermoleovorans* JQ 912239 (the most efficient bacterial strain) were determined by growing this bacterium in MSM (pH 7) containing 1% crude oil as sole carbon source and 0.3% ammonium chloride as a nitrogen source at 60ºC with shaking (200 rpm) for 10 days (Al-Jailawi *et al*., 2015). Biosurfactant was extracted according to Jara *et al.* (2013) by precipitating the metabolic cell-free liquid with acetone 1:1 (v/v) and allowed to stand for 24 h at 4°C and then it was centrifuged (4000 rpm) for 15 min, at 5°C. The supernatant was discarded and the isolated biosurfactant was submitted to dialysis against deionized water for 72 h, at 5°C and subjected to change every 3 h. The crude extract was purified by silica gel column chromatography with following dimension (2.5×40) cm, filled with silica 60 gel, which was eluted with a sequence of hexane, chloroform, ethyl acetate and methanol. All fractions of elution steps were collected and tested for biosurfactant activity.

Chemical characterization of biosurfactant: The total carbohydrate, lipid and protein content in the biosurfactant were estimated by phenol-sulfuric acid method (DuBois *et al.,* 1956), Kaufmann and Brown (2008) and Bradford (1976) method, respectively.

The FT-IR characterization of isolated biosurfactant was done by weighting 2 mg and powdered it with KBr and then pressed for 30 sec to result a translucent KBr pellet. The biosurfactant was characterized using a fourier transform infrared spectrophotometer in a dry atmosphere. Absorption spectra were plotted using a built-in plotter. The biosurfactant was prepared for FT-IR spectra measurement in the frequency range of 400-4000 wave numbers (cm^{-1}) with resolution of 2 cm, 50 scans. Biosurfactant was further analyzed by HPLC using specified column. The fatty acid detected by C-8DB column with using mobile phase acetonitrile: tetrahydrofuran (THF): 0.1% of phosphoric acid in THF (50.4: 21.6: 28 v/v) and flow rate 1.5 mL min^{-1} at 40ºC and UV detector at 215 nm. While the carbohydrate analyzed by anion exchange shimpack A1 column, 3 μm particle size (50×4.6 mm I.D), the mobile system used (15 mM) NaOH spiked with 1 mM barium with refractive index detector was run at flow rate of 1.5 mL min^{-1}. Then the protein fraction was determined by shimpack XR-ODS column (50×4.6 mm I.D), 3 μm particle size, gradient were formed between two degassed solvent systems (solvent A: 5% methanol in 0.1 N sodium acetate buffer and solvent B: methanol, linear gradient from 0-20 min) with flow rate of 1 mL min^{-1} and UV detector at 254 nm. Also standard solutions for sugars, fatty acids and proteins were analyzed by HPLC. The lipid fraction was subjected to analysis by GC, it was performed using helium as carrier gas on a Shimadzu 17-A GC equipped with an fused silica capillary column (30 m×0.25 mm, 0.25 μm film thickness).

Noise-decoupled 1H NMR spectrum was used to analyze biosurfactant, by dissolving it in CDCL3 and the mixture was centrifuged (4000 rpm, 25ºC) for 15 min, then tested with Bruker Avance-II 500 spectrometer, Switzerland at 500 MHz.

RESULTS AND DISCUSSION

Screening for biosurfactant-producing bacteria: The ten thermophillic bacteria isolated from oil contaminated soils in Iraq (AL-Jailawi *et al*., 2013) were screened for biosurfactant production using three screening methods. The results presented in Fig. 1 show that the Emulsification Activity (EA) ranged between 0.1-0.34, with the maximum EA (0.34) was recorded by *Geobacillus thermoleovorans* Ir1 (JQ912239). The measurement of EA is an indicator for the activity of biosurfactant production (Jazeh *et al*., 2012).

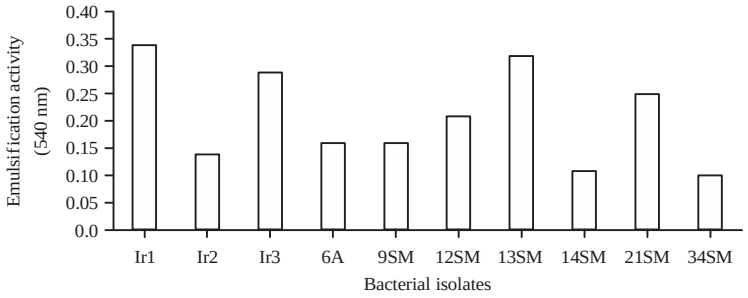

Fig. 1: Emulsification activities of the ten thermophillic bacterial isolates, cultured in mineral salt medium (pH 7) containing 1% crude oil, at 55 °C in shaker incubator (180 rpm) for 7 days. Ir1: *Geobacillus thermoleovorans* (JQ912239), Ir2: *A. rupiensis* (JQ912240), Ir3: *A. rupiensis* (JQ912241), 6A: *Anoxybacillus* sp.

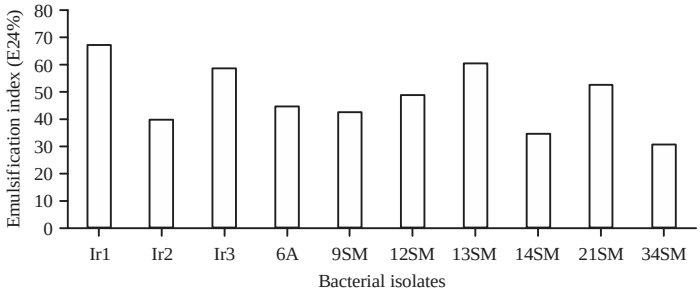

Fig. 2: Emulsification index (E24%) of the ten thermophillic bacterial isolates, cultured in mineral salt medium (pH 7) containing 1% crude oil, at 55°C in shaker incubator (180 rpm) for 7 days. Ir1: *Geobacillus thermoleovorans* (JQ912239), Ir2: *A. rupiensis* (JQ 912240), Ir3: *A. rupiensis* (JQ 912241), 6A: *Anoxybacillus* sp.

The emulsification activity of Yansan bioemulsifier of *Y. lipolytica* was 2.63 when using toulen as a carbon source (Amaral *et al*., 2006). On the other hand, Camargo de Morais *et al*. (2006) noticed that the EA of bioemulsifier produced by *P. citrinum* was 0.20 when using olive oil.

The results indicated in Fig. 2 show that all isolates were able to produce biosurfactant with variable emulsification index (E24%), the strain *G. thermoleovorans* Ir1 (JQ912239) showed the highest E24% (68%).

Bacteria generally prefer to metabolize substrates present in the aqueous phase; they also can take up the substrate if they are in close contact with the insoluble phase of the hydrocarbons (Abbasnezhad, 2009).

Priya and Usharani (2009) indicated that the highest E24% value (60%) was observed with *B. subtilis* when using vegetable oil as carbon source. While, Kalyani *et al*. (2014) found that biosurfactant producing by *Actinomycetes* has emulsification index 57.31% when grown in medium containing olive oil as the sole source of carbon.

The results illustrated in Fig. 3 indicate that *G. thermoleovorans* Ir1 (JQ912239) was given the highest reduction of surface tension (53 mN m^{-1}).

Zheng *et al*. (2012) reported that *G. pallidus* does not reduce the surface tension below 40 mN m^{-1} when growing on media supplemented with different hydrocarbons. Mulligan (2005)

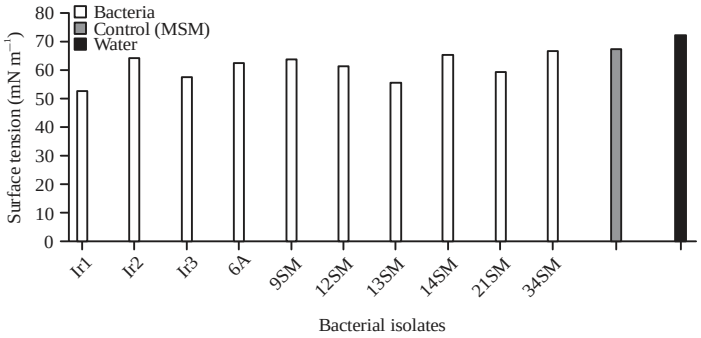

Fig. 3: Reduction in surface tension (mN m^{-1}) of the ten thermophillic bacterial isolates, cultured in mineral salt medium (pH 7) containing 1% crude oil, at 55°C in shaker incubator (180 rpm) for 7 days. Ir1: *Geobacillus thermoleovorans* (JQ912239), Ir2: *A. rupiensis* (JQ912240), Ir3: *A. rupiensis* (JQ912241), 6A: *Anoxybacillus* sp.

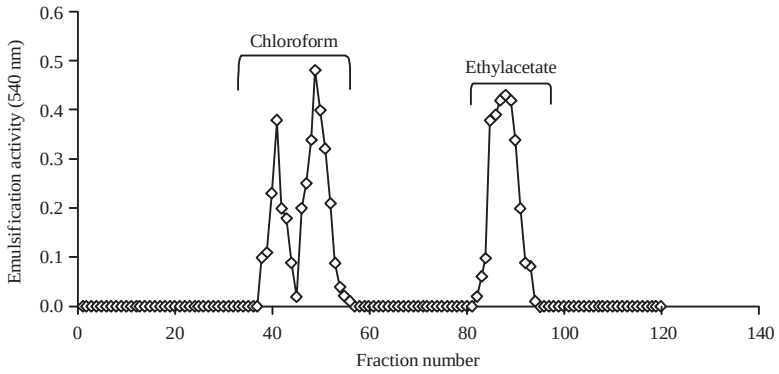

Fig. 4: Silica gel column chromatography with sequential elution system and flow rate 30 mL h^{-1}, fraction volume 3 mL per tube

reported that the low molecular weight biosurfactant are able to reduce the surface tension below 40 mN m^{-1} while the high molecular weight bioemulsifiers can form and stabilize emulsions without remarkable surface tension reduction (Batista *et al*., 2006).

The above results showed that all isolates were able to produce biosurfactant compounds and *G. thermoleovorans* Ir1 (JQ912239) was the most efficient one. Thus, this isolate was selected for subsequent study. According to the result of the surface tention it can be concluded that biosurfactant produced by this bacterium has a high molecular weight.

Purification of biosurfactant: *Geobacillus thermoleovorans* (JQ912239) was grown under the optimal conditions for biosurfactant production (Al-Jailawi *et al.,* 2015), after that the biosurfactant was extracted then purified by silica gel column chromatography. All eluted fractions were collected and measured the emulsification activity for each one. The results (Fig. 4), showed three peaks appear, two of them appeared when eluted with chloroform and the third one appeared with ethyl acetate. Results also indicated that the second peak gave the higher emulsification activity (EA= 0.48) while the third peak, which eluted with ethyl acetate gave EA = 0.43.

Silica gel column chromatography was used in several studies to purify biosurfactant compounds, Zhao *et al.* (2013) used silica gel column chromatography to purify rhaminolid produced

by *P. aeruginosa.* Thanomsub *et al.* (2007) also used silica gel column chromatography to purify the rhamnolipids produced by *P. aeruginosa* B189 with sequential washing by hexane, chloroform, ethyl acetate and methanol.

Characterization of biosurfactant: Biosurfactant from *G. thermoleovorans* (JQ 912239) varied in their content of lipid, carbohydrate and protein. The partially purified biosurfactant consists of 37.7% lipid(the main fraction), 26.2% carbohydrate and 10.7% protein.

These results were in accordance with Xue and Liu (2009), who showed that the bioemulsifier produced by *G. thermoleovorans* 5366T was contained the high ratio of lipid (35.8%) then carbohydrate (29.4%) and protein (15.8%).

The IR spectrum of partial purified biosurfactant produced by this bacterium (Fig. 5) showed the broad band at 3282 cm^{-1} and another band at 2922 cm^{-1} this may be attributed to the O-H groups of polysaccharide. This result was in accordance with Singh *et al.* (2011), who demonstrated the presence of a broadly stretching intense peak at around 3428 cm^{-1} and a weak C-H band at around 2928 cm^{-1} which is characteristic of hydroxyl groups of hetropolysaccharides of biosurfactant produced by *B. licheniformis*. The results (Fig. 5) also showed a strong band at 1654, 1537 and 1432 cm^{-1}, those may be attributed to the C = O, N-H and C-N respectively. In the study of Beech *et al.* (1999) they demonstrated a band at 1655 cm^{-1} which represented C-O stretching of carboxyl group and/or protein related band of amide I, also a band at 1427 cm^{-1}, which belonged to the protein group. In addition, FTIR spectrum (Fig. 5) indicated a band at 1000 cm^{-1} belonged to the polysaccharide. It was revealed that the region from 1200-950 cm^{-1} is associated with (C-O-C) stretching of polysaccharides (Dean *et al*., 2010).

The HPLC results revealed that there were many peaks of fatty acid component for partial purified biosurfactant, , the first peak was palmitic acid (34.1 μg mL^{-1}), the second peak was stearic acid (35.79 μg mL^{-1}), the third peak was oleic acid (Fig. 6). While the results of carbohydrate content showed three peaks. The first peak revealed xylose (20.5 μg mL^{-1}), the second peak was mannose (41.7 μg mL^{-1}) and the third one was maltose (21.84 μg mL^{-1}) (Fig. 7). The results

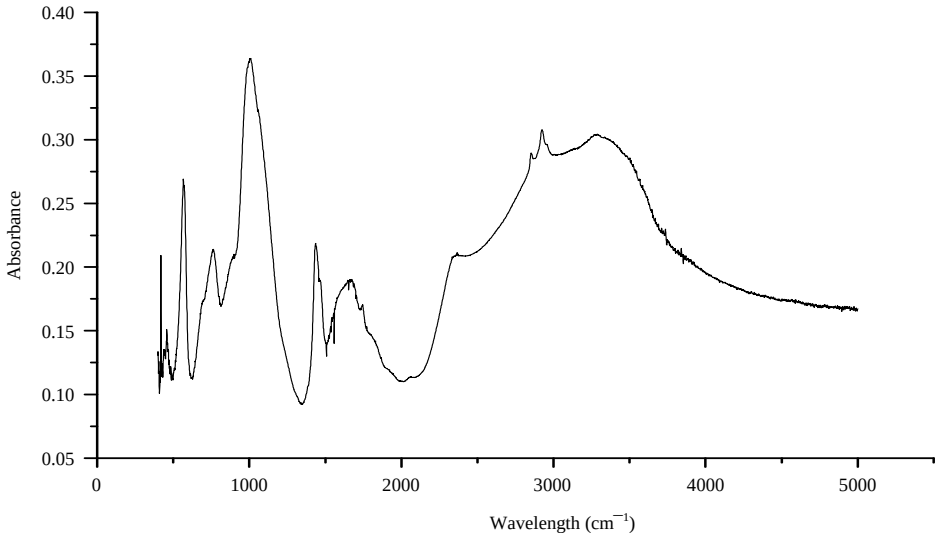

Fig. 5: FTIR spectrum of partial purified biosurfactant produced from *Geobacillus thermoleovorans* (JQ 912239), 50 scan for each spectrum

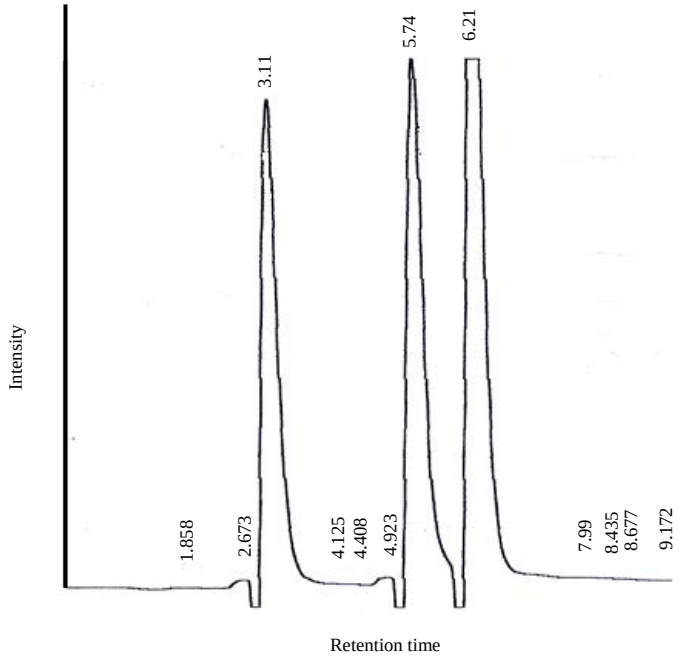

Fig. 6: HPLC analysis of fatty acids components of partial purified biosurfactant produce by *Geobacillus thermoleovorans* (JQ912239), equipped with binary delivery pump model LC-10A shimadzu, the eluted peak was monitored by SPD 10A VP detector

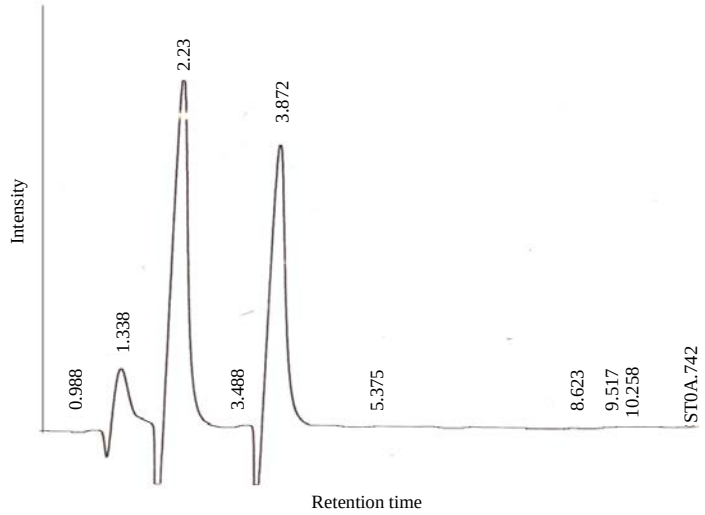

Fig. 7: HPLC analysis of the carbohydrate components of partial purified biosurfactant produce by *Geobacillus thermoleovorans* (JQ912239), equipped with binary delivery pump model LC-10A

also showed three peaks for amino acids, the first one was aspartic acid with concentration 11.55 μg mL^{-1} while the second was glutamic acid (20.60 μg mL^{-1}) and glutamine with concentration 15.7 μg mL^{-1} as shown in Fig. 8.

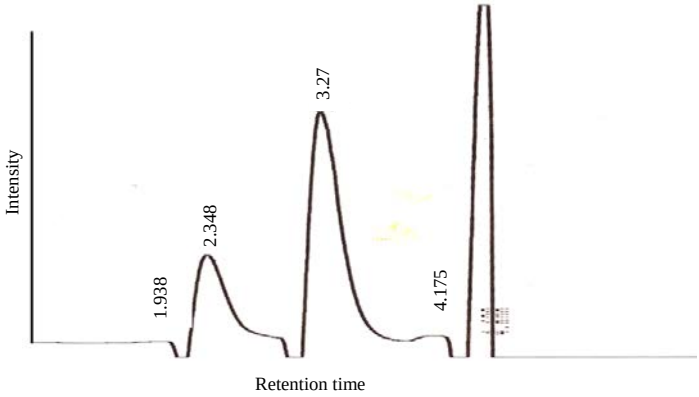

Fig. 8: HPLC analysis of the amino acid components of partial purified biosurfactant produce by *Geobacillus thermoleovorans* (JQ912239), with flow rate 1 mL min^{-1}

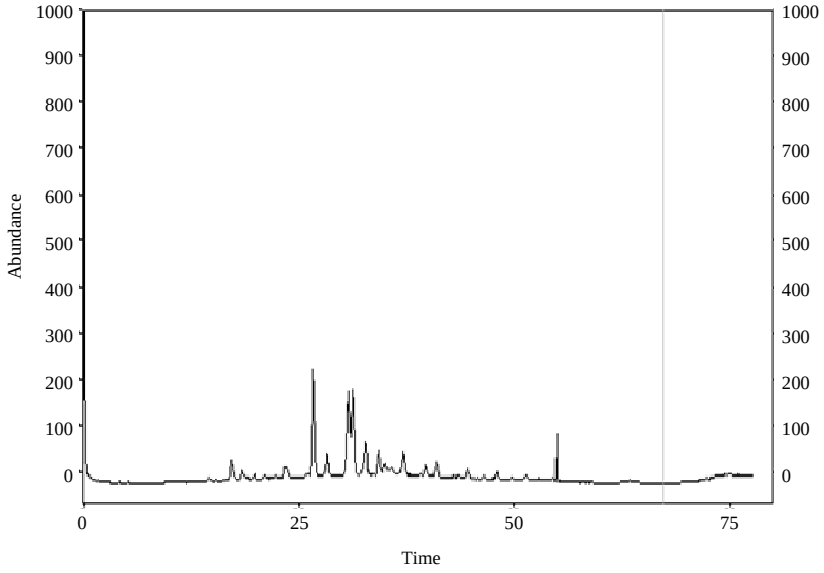

Fig. 9: GC analysis of fatty acid sample of partial purified biosurfactant produce by *Geobacillus thermoleovorans* (JQ912239) using helium as a carrier gas on a Shimadzu 17-A GC

Xue and Liu (2009) mentioned that the carbohydrate in the bioemulsifier produced by *G. thermoleovorans* 5366T mainly was D-mannose and main amino acids were glutamic acid, aspartic acid and alanine. While Adamu *et al*. (2015) revealed that the biosurfactant produced by *Bacillus sphaericus* EN3 was phospholipid and made up of palmitic acid, leucine, alanine, serine and arginine. Similarly, *Bacillus azotoformans* EN16 produced phospholipid with the following components: glutamine, stearic acid, oleic acid glycine, valine and arginine.

Gas chromatography analysis for lipid fraction of partial and purified biosurfactant demonstrated many peaks of fatty acids. The result (Fig. 9) showed that partial purified biosurfactant consists of a high percentage (58.9%) of palmitic acid methyl ester (C16:0), stearic

acid (C18:0), oleic acid (C18:1n9C) also there are many other fatty acids with less percentage. In comparison, results of purified biosurfactant also showed that it consists mainly (high percentage) of palmitic acid methyl ester (C16-0). Its ratio in the three peaks (after purification by silica gel) was 23, 44.4 and 30.3%, respectively.

Xue and Liu (2009) analyzed the lipid part of biosurfactant from *G. thermoleovorans* 5366T by gas chromatograph and mentioned that the main components were hexadecanoic acid, octadecenoic acid, octadecanoic acid. While Sharma *et al*. (2015) found by using gas chromatography and mass spectroscopy that the fatty acid produced by *Enterococcus faecium* was hexadecanoic acid. GC-MS analysis of lipidic fraction of bioemulsifier of *Aeribacillus pallidus* YM-1showed that hexadecanoic acid and octadecanoic acid were the major fatty acids that account for 94.4% of total fatty acids. Other fatty acids determined at lower extent were dodecanoic acid and tetradecanoic acid (Zheng *et al*., 2012).

The NMR spectrum (Fig. 10) showed many signals for the biosurfactant produced by *G. thermoleovorans* (JQ 912239) and the main signal was attributed to the triglycerides. When compared the results (NMR spectra) of partial and purified (by silica gel) biosurfactant, it was revealed that all purified peaks contain only triglycerides without the other compounds and the formula of triglyceride is $H_2C = \underset{H}{C}–\underset{H_2}{C}$ ~~~~~~~~~~ $\underset{C}{H_2}$ –COOH as shown in Fig. 11.

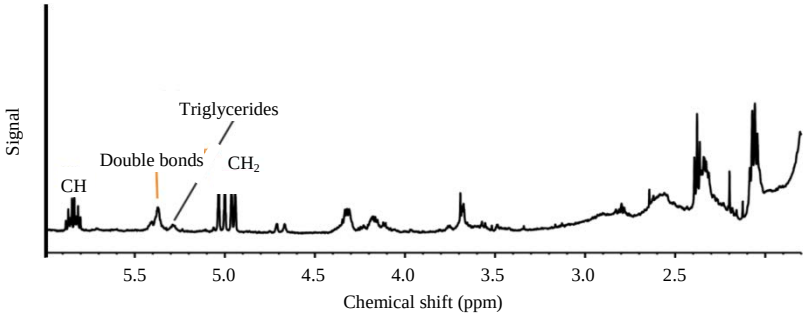

Fig. 10: Selective region of H-NMR spectra of partial purified biosurfactant produced by *Geobacillus thermoleovorans* (JQ 912239), in $CDCL_3$ at 298K, at 500 MHz

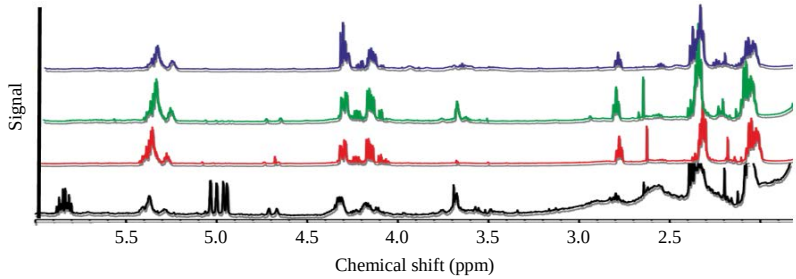

Fig. 11: Overlap of selective region of H-NMR spectra of biosurfactant produce by *Geobacillus thermoleovorans* (JQ912239). (black color) partial purified, (red color) purified band 1, (green color) purified band 2 and (blue color) purified band 3, in $CDCL_3$ at 298K, 500 MHz

Singh *et al.* (2011) analyzed exopolysaccharide produced by *B. licheniformis* using H NMR spectrum, they noticed seventeen anomeric signals, depicting complex and heterogeneous nature. While Amaral *et al.* (2006) indicated that the H NMR signals for bioemulsifier produce by *Y. lipolytica* attributed to polysaccharide at 3.2-4.4 ppm and the rest of the spectrum referred the presence of low levels of protein. Gudina *et al.* (2015) used NMR to detected the bioemulsifier produced by *Paenibacillus* sp. as a low molecular weight oligosaccharide-lipid complex in which the fatty acids and oligosaccharides were structurally associated involving either covalent or non-covalent bonds.

CONCLUSION

The chemical analysis of biosurfactant produced by a novel thermophillic *G. Thermoleovorans* (JQ912239) revealed that it consists of lipid (37.7%), carbohydrate (26.2%) and protein (10.7%). The emulsification activity may be attributed to the lipid and especially to palmitic acid methyl ester (triglycerides) which is the mainly consist of the lipid (main) fraction. This biosurfactant has a high molecular weight and might be new, therefore further studies needed to complete its characterization and investigate its applications.

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