Songklanakarin J. Sci. Technol.
45 (4), 525–533, Jul. – Aug. 2023

http://www.sjst.psu.ac.th

Original Article

Optimization of biosurfactant production by *Bacillus thuringiensis* c25 isolated from palm oil contaminated soil

Innocent Okonkwo Ogbonna[1], Walter Chinaka John[2], Grace Mwuese Gberikon[1], and Charles Chidozie Iheukwumere[3]

[1] *Department of Microbiology, College of Science, University of Agriculture, Makurdi, Nigeria*

[2] *Department of Science Laboratory Technology, Federal College of Forestry, Jos, Nigeria*

[3] *Department of Botany, College of Science, University of Agriculture, Makurdi, Nigeria*

Received: 14 April 2021; Revised: 6 July 2023; Accepted: 19 July 2023

Abstract

Biosurfactants of microbial origin have wide applicability because of their surface-active potentials. *Bacillus thuringiensis* c25 isolated from palm oil contaminated soil was used in the production of biosurfactant and the product was screened using emulsification stability test (E_{24}), drop-collapse, oil spreading, blood hemolysis and surface tension tests. The effects of incubation period, temperature, pH, and carbon source on biosurfactant production by *B. thuringiensis* c25 were investigated with a view to optimizing the production process. The biosurfactant produced was characterized using Fourier Transform Infrared Spectroscopy and Gas Chromatography-Mass Spectrometry. Initial assay showed emulsification index of 57.24 ± 0.25 %, oil displacement of 6.17 ± 0.21 mm and a positive drop-collapse test. Growth of *B. thuringiensis* at the optimal conditions of temperature 35 ºC, pH 7.0, glucose 10 g/L and yeast extract 1.5 g/L, reduced surface tension to 27.71 ± 0.88 mN/m and increased emulsification index to 84.78 ± 0.44%. Maximum biosurfactant production of 2.70 ± 0.04 g/L was obtained at 72 h. The characterization showed a lipopeptide biosurfactant type. Consequently, the *B. thuringiensis* of the present study is a good biosurfactant producer.

Keywords: *B. Thuringiensis*, biosurfactants, emulsification index, surface tension, palm oil

1. Introduction

The biosurfactants produced by *B. thuringiensis* are of lipopeptide class (Jacques, 2011). Kurstakins are the only cationic lipopeptide group and have been synthesized by *B. thuringiensis*. They are useful in heavy metal removal from contaminated soil and water, and effectively enhance phyto extraction (Awashti, Kumar, Makkar, & Cameotra, 1999). Biosurfactants in general have some environment related advantages over chemical surfactants. Chen, Wei, and Chang (2007), and Elazzazya, Abdelmoneima, and Almaghrabi, (2015) have enumerated some of these advantages to include environmental friendliness, bio degradability, simple and cheap substrate production, and capacity to perform even in extremes of environments. Biosurfactants have proven stability over a wide range of environmental factors (Fenibo, Ijoma, Selvarajan, & Chikere, 2019; Jacques, 2011; Paraszkiewicz, Moryl, Plaza, Bhagat, Satpute, & Bernat, 2019). While for chemical surfactants toxicity and non-degradability are contending issues (Hamme, Singh, & Ward, 2006), such issues more or less do not exist with surfactants of microbial origins.

*Corresponding author
Email address: jowaltee@gmail.com

Biosurfactant production lacks competitiveness over chemically-derived surfactants because of the scale of production. Microbial product quantity yield is usually poor *de novo* (Mulligan, Sharma, & Mudhoo, 2014). Poor yield, costly substrate, and complex cultivation processes limit the commercialization of biosurfactants (Sharma, Verma, Dhull, Maiti and Pandey, 2022). Most microbial bio-based product researchers are therefore faced with the challenges of optimization. Growth culture medium manipulation has been a very vital optimization approach addressed by several researchers. Hence, Makkar, Cameotra, and Banat (2011), Mulligan *et al*. (2014), and Sidkey, Mohamed, and Elkhouly (2014) recorded pH, temperature, agitation, oxygen level, and incubation time as possible factors affecting cell biomass. Sharma, Tiwari, and Pandey (2022) summarized the various optimization protocol and design techniques for maximum biosurfactant production and noted that Response Surface Methodology is the most accurate in dealing with factor optimization. They further noted that the key factors that researchers target during optimization for maximum biosurfactant production are temperature, pH, carbon to nitrogen ratio, and salinity. It is however important to mention that one-factor-at-a-time optimization still remains a useful approach in biotechnological optimization.

Wang, Nie, Tang, Wu, and Wu (2013) noted that the mechanism of biosurfactant production could be determined by the carbon chain length of the alkane carbon source. Culture environment parameter manipulations may not always lead to an optimal product yield. Therefore, optimization of biosurfactant production by *Bacilli* can best be done on a species-by-species basis. The range of different habitats where biosurfactant synthesizers are found may not have been fully and efficiently examined.

Nigeria is one of the largest palm oil producers in the world. A lot of palm oil contamination is seen in the palm oil processing, distribution, and market outlets. This causes pronounced pollution that could harm both land and aquatic biota. Utilization of the palm oil contaminated soil for producing valuable products is therefore indispensable. Furthermore, optimization of biosurfactant production will not lack merit, since at present the biosurfactant market is very minor compared to the chemical counterparts. The present study aimed to investigate the production and optimization of biosurfactant by *B. thuringiensis* c25 isolated from palm oil contaminated soil, a novel environment for biosurfactant production

2. Materials and Methods

2.1 *Bacillus thuringiensis* c25

The isolate from palm oil contaminated soil was characterized and identified (John, 2020; John, Ogbonna, Gberikon, and Iheukwumere, 2020). The organism with isolation code PO4(3)A, PCR code BS14, identified as *B. thuringiensis* strain c25, was deposited in the GenBank with CP022345 accession code. It is maintained in our laboratory on Nutrient Agar (Oxoid CM002, Hampshire, England) slants at 4 °C. For use, the organism was activated on the same Nutrient and Mannitol Egg Yolk Polymyxin Agar (Oxoid CM0929, Hanis, UK) and incubated at 30 °C for 24 h.

2.2 Cultivation of *B. thuringiensis*

This was carried out as described by Ainon (2013). The sterilized mineral salt medium (MSM) (Atlas, 2010) was distributed into three sterile 250 mL conical flasks. A 3 mL aliquot (McFarland 0.5 standardized pure culture grown on Nutrient broth for 24 h to obtain 1 x 10^8 CFU/mL) of seed culture containing *B. thuringiensis* was aseptically inoculated into each flask. The conical flasks containing the media were incubated on a rotary shaker at agitation speed of 150 rpm for 5 days at temperature of 30 ºC. The negative control was MSM without inoculation.

Biomass concentration of the fermentation broth of *B. thuringiensis* was measured at 24 hourly intervals using a spectrophotometer (OD 600 nm) and a distilled water blank. The reading was converted to g/L using a standard curve.

2.3 Screening of *B. thuringiensis* for biosurfactant production

2.3.1 Emulsification Stability Test (E_{24})

Emulsification Stability Test (E_{24}) was carried out using the method described by Balogun and Fagade (2010) as modified by John, Ogbonna, Gberikon, and Iheukwumere (2021). Kerosene (2 mL) was added to the same amount of cell free supernatant, vortexed for two minutes (electronic vortex machine, Model XH-B, 2012), and allowed to stand for 24 h before measurement. The height of emulsion formed was measured. The E_{24} index was given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) x 100.

2.3.2 Drop-collapse Test

This test was performed in accordance with the protocol in John *et al*. (2021). An aliquot of cell free broth was dropped in the center of Grand Cereal vegetable oil, placed on a grease-free clean glass slide according to the method of Seema and Nakuleshwar (2012). The drop was examined visually after one (1) minute. The destabilization of cell free broth dropped indicated positive result. Activity of collected supernatant was compared with water (which was used to replace culture supernatant) as control.

2.3.3 Oil spreading test

The method described by Hasham, Mohamed, and Mohamed (2012) was followed in the determination of oil spread ability of the culture filtrate. Distilled water (20 mL) was dispensed in Petri plate and crude oil (1 mL) was dropped in the center of the plate. This was followed by dropping 20 µL of the supernatant of the culture of *B. thuringiensis* at the center of the crude oil. Ring formation due to displacement of the oil was measured using a meter rule. The control was distilled water (20 μL).

2.3.4 Determination of blood hemolysis test

A sterile Blood Agar Base (LAB M Bury, England) was used with goat blood (20 mL) gently mixed and poured

onto Petri dishes. Fresh *B. thuringiensis* culture (24 h) was point inoculated using wire loop at the center of the plates followed by incubation at 30 ºC for 24 h. The areas of clear zones around the colonies were measured using a meter rule.

2.3.5 Determination of surface tension

Surface tension was measured using a KSV Sigma 702 Tensiometer. All measurements were made on the cell-free broth obtained by centrifuging the culture at 10000 rpm for 15 minutes. Ten milliliters of each cell free broth were transferred into a clean 20 mL beaker and placed onto the tensiometer platform. A platinum wire ring was submerged into the solution and then slowly pulled through the liquid-air interface, to measure the surface tension (mN/m). Between each measurement, the platinum wire ring was rinsed with water and flamed with Bunsen burner (John *et al*., 2021).

2.4 Effect of incubation time on biosurfactant production

In 250 mL Erlenmeyer flasks, 50 mL sterile 40 g/L glucose mineral salt medium (Atlas, 2010) were dispensed. The media were inoculated with 3 mL overnight culture of *B. thuringiensis* with pH adjusted to 7 before inoculation. The conical flasks containing the media were incubated on a rotary shaker at agitation speed of 150 rpm for 5 days at temperature of 30 ºC.

2.5 Influence of incubation temperature on biosurfactant production

This was carried out using fifty milliliters of sterile 40 g/L glucose mineral salt medium (pH 7.0). The fermentation medium was inoculated with 3 mL of overnight nutrient broth culture. Cultures were incubated under shaking in a rotary shaker (Orbital Shaker, Series F200, England) at 150 rpm for 72 h. The shaker temperatures 25, 30, 35, 40 and 45 ºC, were used.

2.6 Influence of pH on biosurfactant production

This was done using fifty milliliters of sterile 40 g/L glucose mineral salt medium with varied initial medium pH from 6.0 ~ 10.0. The medium pH was adjusted using 5 M HCl and 1 % NaOH and inoculated with 3 mL overnight culture of *B. thuringiensis* of the present study. The conical flasks containing the media were incubated on a rotary shaker at agitation speed of 150 rpm for 5 days at temperature of 30 ºC.

2.7 Effect of different carbon sources on biosurfactant production

Varying concentrations of different carbon sources were used to determine the effect of carbon source choice on biosurfactant producing *Bacillus* species (John *et al*., 2021). The concentrations tested were 10, 20, 30, 40, and 50 g/L. Glucose, lactose, dextrose, and soluble starch were used. To 1000 mL mineral salt medium, each of the mentioned carbon sources was added individually, the fermentation medium pH was adjusted to 7 using 5 M HCl and 1 % NaOH. This was then followed by inoculation of the medium with 3 mL (1 x 10^8 CFU/mL) overnight nutrient broth culture and incubated under shaking at 150 rpm at 30 ºC for 72 hours. The cells were harvested by centrifugation at 5,000 rpm for 10 minutes and supernatant was used to detect presence of biosurfactant using emulsification stability technique, surface tension assays, and cell dry weight.

2.8 Extraction of biosurfactant from *B. thuringiensis* fermentation broth

To extract the crude biosurfactant, the *B. thuringiensis* culture supernatant was centrifuged at 10,000 rpm for 20 minutes at 4 ºC. Then, the pH of the supernatant was reduced to 2.0 using 0.5M HCl after the supernatant was collected. The collected supernatant was allowed to stand for 24 hours at 4ºC for precipitate to settle, and the biosurfactant was mixed with methanol (2:1), centrifuged at 5,000 rpm for 20 min, and collected thereafter (Anitha, Jeyanthi, & Ganesh, 2015).

2.9 Structural identification of *B. thuringiensis* biosurfactant

2.9.1 Fourier transform infrared spectroscopy (FT-IR) analysis

The nature of chemical bonds and functional groups in the *B. thuringiensis* biosurfactant was determined using Fourier Transform Infrared Spectroscopy (Bulk Scientific M530, USA). The instrument was equipped with a detector of deuterated triglycine sulphate and beam splitter of potassium bromide. One milligram of the biosurfactant sample was mixed thoroughly with 100 mg of homogenized porcelain-milled potassium bromide (KBr). The pellets were immediately introduced into the sample holder and FTIR spectra were taken. The software of Gram A1 was used to obtain the spectra. FTIR spectra were obtained over the wavenumbers 4000 – 600 cm^{-1} with 32 scans per spectrum and 4 cm^{-1} resolution. The FTIR spectra are displayed as transmittivity values.

2.9.2 Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was carried out using Agilent GCMSD 7890 B (Agilent Technologies, USA) device. Ten milligrams (10 mg) of biosurfactant was mixed with 5% HCl-methanol reagent and the reaction was stopped by adding 1 mL of sterile water, the biosurfactant was recovered with methanol, and 1 mL samples were injected into the gas chromatograph. Carrier gas used was Helium, the flow rate was set as 1.5 mL min^{-1} and the working temperature of the GC injector ranged from 240 to 260 ºC. The temperature ramp was run from 60 to 260 ºC at a speed of 5 ºC min^{-1}, with an isothermal phase of 10 min at the end of the analysis. Mass spectra were obtained at 70 keV. The mass spectra were obtained over the m/z range 40–700 in ultra-high-resolution mode with an acquisition speed of 6 spectra/second. The identification of components was done in scan mode by using NIST11 and Wiley8 libraries, and the target mass spectra obtained from sample were compared with mass spectra obtained from the library (Parthipan *et al*., 2017).

2.10 Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura and Nei model (1993). The tree with the highest log likelihood (-5130.89) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology for superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). This analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1564 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2021)

2.11 Analysis of data

The results obtained are presented in graphs, tables and charts. Results were statistically assessed using Analysis of Variance (ANOVA) at 99 % confidence level. Means were separated using Duncan's test.

3. Results and Discussion

Different screening methods have been suggested for studying biosurfactant production efficiency (Okore *et al*., 2017). The present study utilized four measurements namely hemolytic activity, drop-collapse test, oil spreading, and emulsification index (E_{24}). These methods have been used by Anitha *et al*. (2015) in the study of production and characterization of biosurfactant by *Bacillus* sp.

3.1 Preliminary screening of *Bacillus thuringiensis* ability to produce biosurfactant

Results of hemolytic activity indicated that *B. thuringiensis* exhibited β-hemolysis with 4.60 ± 0.61 mm zone of lyses. The drop-collapse test was positive. The supernatant from *B. thuringiensis* exhibited spreading capacity of crude oil by 6.02 ± 1.24 mm. The initial emulsification index (E_{24}) revealed that emulsion was formed when tested with kerosene (E_{24} = 56.41 ± 1.30%). *B. thuringiensis* of the present study was β-hemolytic with a zone of clearing of 5.12 ± 1.06 mm.

According to El-Sheshtawy, Aiad, Osman, Abo-Elnasr, and Kobisy (2013), β-hemolysis is a rapid method for screening biosurfactant production. The result of the drop-collapse activity of the *B. thuringiensis* was positive suggesting a probable hydrophobic supernatant. In previous and independent studies, Jain, Mody, Mishra, and Jha (2013) and Viramontes-Ranos *et al*. (2010) also obtained positive drop collapse preliminary test results from some biosurfactant-producing bacteria. Oil spreading technique indicating biosurfactant activity by diameter of the clearing zone on the crude oil surface showed that the *B. thuringiensis* species had oil displacement diameter of 6.18 ± 2.65 mm, which was not remarkably lower than the 6.33 ± 0.33 mm obtained by Akintokun, Adebajo, and Akinremi (2017) for a related *Bacillus* species.

It was assumed that if the cell free culture broth used in present study contained biosurfactant, it would emulsify the kerosene present in the test solution. Results showed that *B. thuringiensis* had emulsification index of 57.24 ± 0.25 % before optimization. This result was lower than those obtained by Akintokun *et al*. (2017) and Sidkey *et al*. (2014) who reported 69.23% and 66 achieved with *Bacillus clausis* and *Bacillus cereus* respectively. Supernatant obtained from the preliminary screening showed excellent emulsification index, oil displacement, positive drop collapse and blood hemolysis results. Consequently, *B. thuringiensis* was further studied for the effects of environmental conditions on biosurfactant production.

3.2 Effect of incubation time

One-factor-at-a-time was employed in the optimization of the biosurfactant production. Biosurfactant production was directly proportional to incubation time. The highest biosurfactant production and bacterial growth were obtained at 120 h (Table 1). Surface tension of culture broth dropped rapidly after inoculation, reaching its lowest value (27.47±0.86 mN/m) after 120 h of growth. The highest emulsification index (81.10 ± 0.93 %) was also obtained after 120 h (5 days). Khopade *et al*. (2012) showed maximum emulsification after 9 days, which is a little longer than the five days obtained in the present study. Biomass content increased till after 72 h of incubation. There was a decrease in biomass after 72 h probably owing to limiting medium nutrients or production of growth inhibiting metabolites. At stationary phase, there could be substrate deprivation or limitation and the production of biosurfactants (Sharama *et al*., 2022).

3.3 Effect of temperature

The temperature range from 25 to 45 ºC was used in the present study because under ideal conditions most *Bacilli* grow best at 30 – 45 °C temperatures, although some thermophilic species can grow at temperatures up to 65 °C or even more (Grutsch, Niamma, Pittsley and McKillip (2018). Table 2 shows that *Bt* produced biosurfactant at all tested temperatures. However, maximum cell growth and biosurfactant production were obtained at incubation temperature of 35 °C. *B. thuringiensis* gave emulsification index of 64.28 ± 1.27 %, surface tension of 31.77 ± 7.56 mN/m and cell dry weight of 1.91 ± 0.04 g/L at 35 ºC. The present temperature (35 ºC) corroborates that of El-Sersy (2012) for *Bacillus subtilis* biosurfactant production, however, the temperature could be higher or lower depending on the cultivation conditions and the microorganism in question.

3.4 Effect of initial medium pH

Results presented in Table 3 show that *B. thuringiensis* grew at pH ranging from 6 to 10 with the highest biosurfactant production and cell growth occurring at pH 7 with relative decrease at other pH values. The emulsification activity of 60.73 ± 0.67% and surface tension reduction of 32.82 ± 1.27 mN/m at pH 7 in the present study is similar to that obtained by Anna and Parthasarathi (2014), for *Bacillus*

Table 1. Effect of incubation time on growth and biosurfactant production by *Bacillus thuringiensis*

Incubation period (h)	Emulsification index (%)	Surface tension (mN/m)	Cell dry weight (g/L)
24	28.01 ± 0.81^{e}	65.82 ± 4.97^{a}	1.06 ± 0.05^{c}
48	40.70 ± 0.69^{d}	53.76 ± 1.48^{b}	1.63 ± 0.33^{b}
72	72.68 ± 0.67^{c}	31.42 ± 0.59^{c}	2.00 ± 0.04^{a}
96	74.78 ± 0.44^{b}	29.71 ± 0.88^{c}	1.18 ± 0.17^{c}
120	81.10 ± 0.93^{a}	27.47 ± 0.86^{c}	1.13 ± 0.14^{c}

Means with different superscripts in a column are significantly different. Values are expressed as mean ± SE (Standard error of the mean). **Significant difference level at 0.01

Table 2. Effects of temperature on growth and biosurfactant production by *Bacillus thuringiensis*

Temperature (ºC)	Emulsification index (%)	Surface tension (mN/m)	Cell dry weight (g/L)
25	50.07 ± 0.68^{c}	60.17 ± 1.73^{a}	1.01 ± 0.09^{b}
30	47.52 ± 1.00^{d}	55.58 ± 3.62^{a}	0.64 ± 0.10^{c}
35	64.28 ± 1.27^{a}	31.77 ± 7.56^{c}	1.91 ± 0.04^{a}
40	57.21 ± 1.60^{b}	39.88 ± 1.97^{b}	0.70 ± 0.07^{c}
45	50.64 ± 0.82^{c}	39.37 ± 2.09^{b}	1.05 ± 0.11^{b}

Means with different superscripts in a column are significantly different. Values are expressed as mean ± SE. **Significant difference level at 0.01

cereus KBSB1. Some biosurfactants are produced at an acidic pH. For instance, Sharma, Verma, Dhull, Maiti, and Pandey (2022) produced rhamnolipid biosurfactant by *Pseudomonas aeruginosa* at an optimal pH of 4.0. This implies that the production pH may either be species dependent or substrate dependent. The pH range 6-10 was used in the present study because Grutsch *et al*. (2018) reported that under ideal conditions most *Bacilli* grow best at pH 7.

Table 3. Effects of pH on growth and biosurfactant production by *Bacillus thuringiensis*

pH	Emulsification index (%)	Surface tension (mN/m)	Cell dry weight (g/L)
6	50.24 ± 1.73b	54.79 ± 1.53ab	1.01 ± 0.09b
7	60.73 ± 0.67a	32.82 ± 1.27d	1.64 ± 0.10a
8	51.55 ± 0.71b	43.46 ± 1.38c	0.91 ± 0.04b
9	42.19 ± 1.80c	55.99 ± 1.65a	0.70 ± 0.07c
10	39.13 ± 1.18d	52.68 ± 2.36b	0.45 ± 0.11d

Means with different superscripts in a column are significantly different. Values are expressed as mean ± SE. **Significant difference level at 0.01

3.5 Effect of carbon sources

According to Wang *et al*. (2013), the mechanisms of biosurfactant production and cell surface hydrophobicity can be altered according to the chain length of the alkanes used as carbon source in biosurfactant production. In the current study, the carbon sources tested were soluble starch, mannose, lactose and glucose. *B. thuringiensis* c25 in this study produced maximum biosurfactant in the presence of a limited concentration of glucose (10 g/L) and gave absorbance of 2.21 ± 0.19 nm, EI (78.41 ±1.15 %) and reduced surface tension of the culture medium from 72 to 16.42 ± 1.10 dyne/cm (Table 4). This is contrary to the work of Tomar and Srinikethan (2016) who showed an emulsification index of 73% using *Bacillus* species under optimized condition (20 g/L) to produce the most biosurfactant. However, lactose, mannose and soluble starch gave levels of biosurfactant and cell growth comparable to those with glucose. Growth and biosurfactant

Table 4. Effects of carbon source and concentration on growth and biosurfactant production by *Bacillus thuringiensis*

Substrate	Concentration (g/L)	Emulsification index (%)	Surface tension (mN/m)	Biomass (nm)
Soluble starch	10	45.78 ± 1.16^{c}	52.44 ± 1.28^{b}	1.95 ± 0.05^{b}
	20	74.85 ± 2.09^{a}	31.58 ± 0.37^{c}	2.35 ± 0.06^{a}
	30	70.27 ± 1.28^{b}	34.35 ± 1.45^{c}	2.38 ± 0.13^{a}
	40	46.75 ± 0.92^{c}	55.70 ± 3.78^{b}	1.86 ± 0.04^{b}
	50	26.81 ± 4.52^{d}	67.77 ± 1.28^{a}	1.47 ± 0.08^{c}
Glucose	10	78.41 ± 1.15^{a}	28.42 ± 1.10^{d}	2.21 ± 0.19^{a}
	20	65.54 ± 1.06^{b}	31.27 ± 1.32^{c}	1.22 ± 0.03^{bc}
	30	60.05 ± 0.09^{c}	33.74 ± 2.27^{c}	0.98 ± 0.13^{cd}
	40	59.86 ± 0.36^{c}	41.97 ± 1.28^{b}	1.26 ± 0.19^{b}
	50	35.71 ± 2.51^{d}	58.88 ± 1.59^{a}	0.82 ± 0.07^{d}
Lactose	10	71.42 ± 1.28^{a}	32.20 ± 1.16^{d}	0.97 ± 0.04^{b}
	20	65.30 ± 1.68^{b}	31.01 ± 0.38^{c}	0.91 ± 0.03^{b}
	30	50.06 ± 0.05^{c}	40.77 ± 0.85^{b}	0.97 ± 0.14^{b}
	40	45.03 ± 1.09^{d}	56.03 ± 1.43^{a}	1.63 ± 0.10^{a}
	50	42.64 ± 1.54^{d}	55.17 ± 1.48^{a}	1.57 ± 0.06^{a}
Mannose	10	53.55 ± 0.19^{a}	39.36 ± 1.23^{e}	0.69 ± 0.02^{e}
	20	39.60 ± 1.15^{b}	58.04 ± 0.15^{d}	1.16 ± 0.04^{d}
	30	40.18 ± 1.50^{b}	59.55 ± 0.76^{c}	1.37 ± 0.06^{c}
	40	26.20 ± 0.95^{c}	62.82 ± 0.61^{b}	1.75 ± 0.04^{b}
	50	21.93 ± 1.60^{d}	67.13 ± 0.24^{a}	2.05 ± 0.09^{a}

Means with different superscripts in a column are significantly different. Values are expressed as mean ± SE (Standard error of means). **Significant difference level at 0.01

production decreased remarkably on increasing glucose concentration up to 50 g/L.

3.6 *B. thuringiensis* biosurfactant production in optimized medium

In order to determine the performance of *B. thuringiensis* under optimized cultivation conditions, the organism was grown combining all the best culture variables earlier determined. Results in Figure 1 present biosurfactant production at optimal culture conditions of temperature (35 °C), pH (7), glucose (10 g/L) and yeast extract (1.5 g/L) at 120 h. The results show an increase in biosurfactant production with incubation time, but with a drop after 96 h. The highest emulsification index and surface tension were 84.78 ± 0.44% and 14.71 ± 0.88 mN/m at 96 h, respectively. The results also revealed that *B. thuringiensis* had an estimated biosurfactant production of 2.70 ± 0.04 g/L at 72 h. The percentage increase in E_{24} following optimization is 48.11%. The observations in the present study imply that the organism could be optimized showing potential suitability for biosurfactant biotechnology. Husam and Ahmed (2013) demonstrated a similar result with a related species. Sharma, Verma, Dhull, Maiti, and Pandey (2022) who worked on simultaneous production of rhamnolipid biosurfactant and degradation of waste cooking oil by *Pseudomonas aeruginosa* P7815 in batch and fed-batch bioreactors obtained maximum biosurfactant value of 16 g/L. Although this is comparatively higher than that of the present study, the organisms and culture conditions were different. Considering other indices for determining biosurfactant suitability, ST reduction in the present study is one of the best reductions recorded.

3.7 Phylogenetic tree

The phylogenetic tree of the isolated *Bacilli* shows relatedness of the strains to each other (Figure 2). The *B. thuringiensis* of the present study was closely related to *Bacillus cereus* K3. The sequenced genes obtained from the various isolates showed multiple alignment in the phylogenetic tree based on amino acid sequences of *sfpAA*.

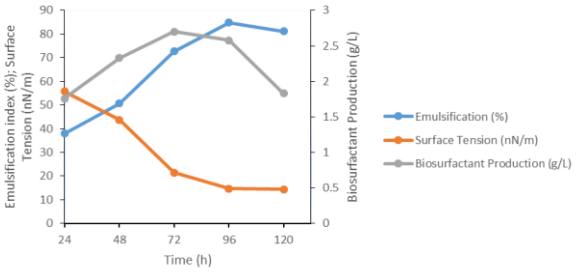

Figure 1. Biosurfactant production by *Bacillus thuringiensis* grown in optimized medium

3.8 FTIR characterization of biosurfactant produced by *Bacillus thuringiensis* c25

The FTIR characterization of biosurfactant produced by *B. thuringiensis* showed that thirteen peaks were eluted (Table 5). Amines, peptides, alkyl, aliphatic and esters (which are derivatives of proteins and lipids) were eluted, whereas the peaks 2, 4 and 5 were peptides, and peaks 6, 7, 12 and 13 were alkyl groups. Similarly, peaks 1, 4 and 8 were either amine or amino groups and peaks 9 and 10 were fatty acids or esters.

The FTIR characterization presented implies that the biosurfactant could only be a lipopeptide-type. Lipopeptide-type biosurfactant production has been reported previously for related microorganisms (Liu, Liu, Ju, Li, & Yu, 2016; Parthipan *et al*., 2017).

3.9 GC-MS Analysis of *Bacillus thuringiensis* c25 biosurfactant

Table 6 shows the GC-MS results on the biosurfactant produced by *B. thuringiensis*. The results reveal 33 peaks attributed to 26 different compounds. The

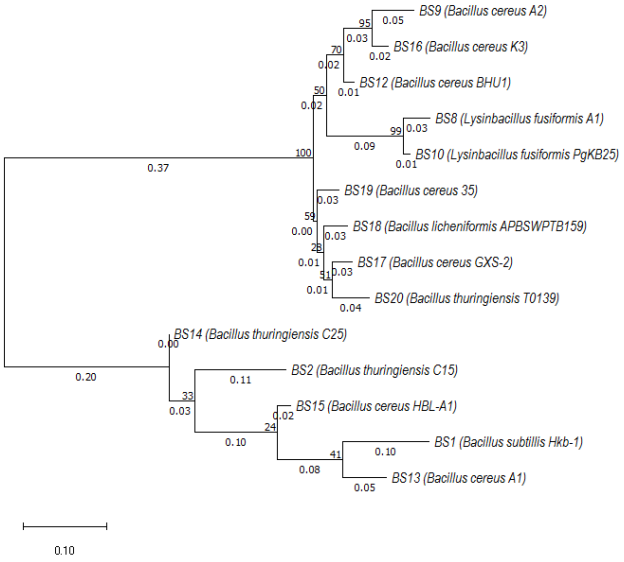

Figure 2. Phylogenetic tree of the *Bacillus* isolate

Table 5. FTIR profile of *Bacillus thuringiensis* surfactant

S/No.	Peaks	Transmission (%)	Functional groups
1	3666.676	40	Amine
2	3662.213	30	Peptides
3	3390.522	35	Peptides
4	3281.122	40	Amino group
5	3034.168	30	Peptide
6	2075.569	65	Alkyl
7	1506.999	60	Alkyl
8	1445.581	55	Amino group
9	1290.027	75	Esters
10	1187.567	75	Esters
11	1038.727	85	Aliphatic
12	907.501	80	Alkyl
13	755.206	75	Alkyl

predominant fatty acids eluted were 9-Octadecenoic acid (42.44%), Octadecenoic acid (34.11%) and Methyl stearate (5. 67%). Combining the peptides obtained from the FT-IR and the lipid components of the GC-MS results, one could infer that the surfactant so produced is of lipopeptide type. Octadecenoic acid reported as a major fatty acid in the present study has been reported previously (Kiran, Thomas, Selvin, Sabarathnam, & Lipton, 2010).

4. Conclusions

B. thuringiensis exhibited high amounts of emulsification from initial 57.24 ± 0.25 %, to a final 84.78±0.44%. The optimum growth conditions for the isolate to produce maximum biosurfactant (2.70 ± 0.04 g/L) were pH 7.0, temperature 35 ºC at 72 h incubation and 10 g/L glucose. The biosurfactant was composed of lipid and protein with the predominant fatty acids eluted being 9-Octadecenoic acid (42.44%), Octadecenoic acid (34.11%) and Methyl stearate (5. 67%). *B. thuringiensis* c25 is a good bacterium for producing biosurfactant.

References

Ainon, H., Noramiza, S., & Shahidan, R. (2013). Screening and optimization of biosurfactant production by the hydrocarbon-degrading bacteria. *Sains Malaysiana Journal*, *42*(5), 615–623.

Akintokun, A. K. 1., Adebajo, S. O. 1., & Akinremi, C. A. (2017). Potential biosurfactant-producing bacteria from pharmaceutical wastewater using simple screening methods in south-west, Nigeria. *Applied Environmental Research, 39*(2), 41-54.

Anitha, J., Jeyanthi, V., & Ganesh, P. (2015). Production and characterization of biosurfactant by *bacillus* and its

Table 6. GC-MS profile of biosurfactant produced by *Bacillus thuringiensis*

Peak	Retention time	Area (%)	Name of compound
1	5.669	1.89	Sulfuric acid, dimethyl ester
2	9.585	0.12	Benzoic acid, 4-methyl-2-trimethyl silyloxy-, trimethylsilyl ester
3	37.537	0.14	Dodecanoic acid
4	44.632	0.14	11-Bromoundecanoic acid
5	48.819	0.14	Hexadecanoic acid, methyl ester
6	51.842	0.44	n-Hexadecanoic acid
7	54.169	0.07	13-Docosenoic acid, methyl ester
8	54.362	0.10	11-Octadecenoic acid, methyl ester
9	54.983	5.67	Methyl stearate
10	57.309	42.44	9-Octadecenoic acid
11	57.619	34.11	Octadecanoic acid
12	58.239	0.34	2-Methyl-Z, Z-3,13-octadecadienol
13	58.627	0.09	Cyclohexanebutanoic acid
14	59.092	0.14	Oleic Acid
15	60.139	0.07	Oleic Acid
16	61.031	0.05	1-hexadecanesulfonamide, N-(2-amin oethyl)-
17	61.845	0.32	Oleic Acid
18	62.349	0.20	cis-11-Hexadecenal
19	62.581	0.26	Dodecanoic acid, 1-methylethyl ester
20	62.930	0.48	Oleic Acid
21	63.240	0.24	15-Hydroxypentadecanoic acid
22	63.551	0.61	Undecylenic acid
23	63.783	0.39	Stearic acid hydrazide
24	64.016	0.47	Methoxyacetic acid, tetradecyl ester
25	64.520	1.84	Methoxyacetic acid, tetradecyl ester
26	64.791	1.85	3-(Prop-2-enoyloxy) dodecane
27	64.946	0.92	Carbonic acid, dodecyl vinyl ester
28	65.140	1.44	Methoxyacetic acid, tridecyl ester
29	65.295	1.33	Methoxyacetic acid, tridecyl ester
30	65.566	0.72	Dodecane, 3-methyl-
31	66.070	2.32	15-Hydroxypentadecanoic acid
32	66.962	0.55	Oleic Acid
33	67.311	0.11	Pentadecanoic acid

applicability in enhanced oil recovery. *International Journal of Advanced Research in Biological Science, 2*(5), 7–16.

Anna, J. P. & Parthasarathi, R. (2014). Optimization and production of biosurfactant from *bacillus cereus* KBSB1. *International Journal of Advanced Research in Biological Science, 1*(5), 120-139.

Atlas, R. M. (2010). *Handbook of microbiological media* (4th ed.). New York, NY: Taylor and Francis Group.

Awashti, N., Kumar, A., Makkar, R., & Cameotra, S. (1999). Enhanced biodegradation of endosulfan, a chlorinated pesticide in presence of a biosurfactant. *Journal of Environmental Science and Health B, 34*, 793-803. Retrieved from https://doi.org/10.1080/03601239909373226

Balogun, S. A., & Fagade, O. E. (2010). Emulsifying bacteria in produce water from Niger Delta, Nigeria. *African Journal of Microbiological Research, 4*(9), 730-734.

Chen, S. Y., Wei, Y. H., & Chang, J. S. (2007). Repeated pH stat fed batch fermentation for rhamnolipid production with indigenous *pseudomonas aeruginoisa* 52. *Applied Microbiology and Biotechnology, 76*(1), 67-74. doi:10.1007/s00253-007-0980-2

Elazzazya, A. M., Abdelmoneima, T. S., & Almaghrabi, O. A. (2015). Isolation and characterization of bio surfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia. *Saudi Journal of Biological Science, 22*(4), 466–475. doi:10.1016/j.sjbs.2014.11.018

El-Sersy, N. (2012). A plackett-burman design to optimize biosurfactant production by marine *bacillus subtilis* N10. *Romanian Biotechnology Letters, 17*(2), 7049–7064.

El-Sheshtawy, H. S., Aiad, I., Osman, M. E., Abo-Elnasr, A. A., & Kobisy, A. S. (2013). Comparative study of biosurfactants production by *bacillus licheniformis* and *candida albicans* for microbial enhanced oil recovery. *Journal Applied Science Research, 9*(8), 4877-4883.

Fagade, O. E., & Okolie, B. I., Balogun, S. (2009). Effects of carbon and nitrogen sources on biosurfactant producing *bacillus* species isolates. *Nigerian Journal of Microbiology, 23*, 1915-1921.

Fenibo, E. O., Ijoma, G. N., Selvarajan, R., & Chikere, C. B. (2019). Microbial surfactants: The next generation multifunctional biomolecules for application in the petroleum industry and its associated environmental remediation. *Microorganisms, 7*(11), doi:10.3390/microorganisms7110581

Grutsch, A. A., Niamma, P. S., Pittsley R. H., & McKillip, J. L. (2018). Chapter 7- *Bacillus* spp. as pathogens in the dairy industry, In A. M. Holban, & A. M. Grumezescu (Ed.), *Handbook of Food Engineering, Foodborne Diseases*, 193 – 211. Cambridge, MA: Academic Press.

Hamme, J. D., Singh, A., & Ward, O. P. (2006). Physiological aspects. Part 1 in series of papers devoted to surfactants in microbiology and biotechnology. *Biotechnology Advances*, 24(6), 604-620. Retrieved from https://doi.org/10.1016/j.biotechadv.2006.08.001

Hasham, M. M, Mohamed A. F., & Mohamed, N. H. (2012). Production of biosurfactant from certain *candida* strains under special conditions. *Researcher, 4*(7), 39-55.

Husam, S. A., & Ahmed, I. M., (2013). Effect of different environmental and nutritional factors on biosurfactant production from *azotobacter chrococcum*. *International Journal of Advances in Pharmacy Biology and Chemistry, 2*(3), 477–481.

Jacques, P. (2011). Surfactin and other lipopeptides from *bacillus* spp. *Microbiological Monographs*, *20*, 58–83.

Jain, R. M., Mody, K., Mishra, A., & Jha, B. (2013). Production and structural characterization of biosurfactant produced by an alkaliphilic bacterium, *klebsiella* sp.: evaluation of different carbon sources. *Colloids Surf B Biointerfaces, 108*, 199–204. doi:101016/j.colsurb.2013.03.0002.

John, W. C. (2020). Isolation and characterization of biosurfactant-producing *bacillus* species from different contaminated soils and optimization of biosurfactant production by the isolates. (Doctoral thesis, Federal University of Agriculture, Makurdi, Nigeria).

John, W. C., Ogbonna, I. O., Gberikon, G. M., & Iheukwumere, C. C. (2020). Screening and characterization of biosurfactant-producing bacillus species isolated from contaminated soils in Makurdi metropolis. *Nigerian Journal of Biotechnology, 37* (2), 165-176.

John, W. C., Ogbonna, I. O., Gberikon, G. M. & Iheukwumere, C. C. (2021). Evaluation of biosurfactant production potential of *lysinibacillus fusiformis* MK559526 isolated from automobile-mechanic-workshop soil. *Brazilian Journal of Microbiology*, *52*, 663 – 674. Retrieved from https://doi.org/10.1007/s42770-021-00432-3

Khopade, A., Ren, B., Liu, X., Mahadik, K., Zhang, L., & Kokare, C. (2012). Production and characterization of biosurfactant from marine *streptomyces* species B3. *Journal of Colloid Interface Science, 367*, 311-318. doi:10.1016/j.jcis.2011.11.009

Kiran, S. K. G., Thomas, T. A., Selvin, J., Sabarathnam, B., & Lipton, A. P. (2010). Optimization and characterization of a new lipopeptide biosurfactant produced by marine *brevibacterium aureum* MSA13 in solid state culture. *Bioresource Technology, 101*, 2389-2396. doi:101016/j.biortech.2009.11.023

Liu, B., Liu, J., Ju, M., Li, X., & Yu, Q. (2016). Purification and characterization of biosurfactant produced by *bacillus licheniformis* y-1 and its application in remediation of petroleum contaminated soil. *Marine Pollution Bulletin, 107*(1), 46–51. Retrieved from https://doi.org/10.1016/j.marpolbul.2016.04.025

Makkar, R. S., Cameotra, S. S., & Banat, I. M., (2011). Advances in utilization of renewable substrates for biosurfactant production. *Journal of Applied Microbiology and Biotechnology, 1*, 1–19. doi:101186/2191-0855-1-5

Mulligan, C. N., Sharma, S. K., & Mudhoo, A. (2014). *Biosurfactants. Research Trends and Applications.* London, England: Boca Raton.

Okore, C. C., Nwaehiri, L. U., Mbanefo, O. N., Ogbulie, T. E., Ugenyi, A. U., Ogbuka, I. B., . . . Okwujiako, I. A. (2017). Study on microbial diversity of biosurfactant producing bacteria from contaminated environmental samples. *International Journal of Advanced Research*, 5(6), 1387-1396. doi:102 1474/JAR01/4548

Paraszkiewicz, K., Moryl, M., Plaza, G., Bhagat, D., Satpute, S., & Bernat, P. (2019). Surfactants of microbial origin as antibiofilm agents. *International Journal of Environmental Health Research*, 11, 1-20. Retrieved from https://doi.org/10.1080/09603123. 2019.1664729

Parthipan, P., Preetham, E., Machuca, L. L., Rahman, P. K. S. M., Murugan, K., & Rajasekar, A. (2017). Biosurfactant and degradative enzymes mediated crude oil degradation by bacterium *bacillus subtilis* A1. *Frontiers in Microbiology*, *8*, 1–14. doi: 103389/fmicb.2017.00193

Seema, D., & Nakuleshwar, D. J. (2012). Isolation of biosurfactant-producing marine bacteria. *African Journal of Environmental Science and Technology*, *6*(6), 263-266.

Sharma, S., Verma, R., Dhull, S., Maiti, S. K., & Pandey, L. M. (2022). Biodegradation of waste cooking oil and simultaneous production of rhamnolipid bio surfactant by *pseudomonas aeruginosa* P7815 in batch and fed-batch bioreactors. *Bioprocess Biosystem Engineering*, *45*, 309 – 319. Retrieved from https://doi.org/10.1007/s00449-021-026610-0

Sharma, S., Tiwari, P., & Pandey, L. (2022). Optimization of culture conditions for the production of biosurfactants. In Pandey, L. & Tiwari, P. (Eds.), *Microbial enhanced oil recovery*. *Green Energy and Technology*. Singapore: Springer. Retrieved from https://doi.org/10.1007/978-981-16-5465-7_7

Sidkey, N. M., Mohamed, H. F., & Elkhouly, I. H. (2016). Evaluation of different screening methods for biosurfactant producers isolated from contaminated Egyptian samples grown on industrial olive oil processing waste. *British Microbiology Research Journal*, *17*(4), 1-19. doi:10.8734/BMRI/2016/ 28437

Tamura K., & Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, *10*, 512-526.

Tamura K., Stecher G., & Kumar S. (2021). MEGA 11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*. Retrieved from https://doi.org/10.1093/molbev/msab120.

Tomar, G. S. & Srinikethan, G. (2016). Studies on production of biosurfactant from *pseudomonas aeruginosa* (MTCC7815) and its application in microbial enhanced oil recovery. *Research Journal of Chemical Environment Science*, *4*, 84–91.

Viramontes-Ranos, S. R., Portillo-Ruiz, M. C., Ballinas-Casarrubias, M. L. Torres- Muñoz, J. V. Rivera-Chavira, B. E. & Nevárez- Moorillón, G, V. (2010). Selection of biosurfactant/bioemulsifier-producing bacteria from hydrocarbon contaminated soil. *Brazilian Journal of Microbiology*, *41*, 668-675.

Wang, X. B., Nie, Y., Tang, Y. Q., Wu, G., & Wu, X. L. (2013). N-alkane chain length alters *dietzia* sp. strain DQ12-45-1b biosurfactant production and cell surface activity. *Applied and Environmental Microbiology*, *79*, 400–402. doi:101128/AEM. 02497-12