Screening and Optimization of Biosurfactant Production by *Bacillus subtilis* from Restaurant Wastewater Contaminated Soil

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Abstract: Biosurfactant production from *Bacillus subtilis* RT9(4)B isolated from restaurant wastewater contaminated soil was investigated. The isolated *Bacillus subtilis* RT9(4)B was screened for biosurfactant production using the emulsification index, oil displacement, zone of haemolysis, surface tension and a positive drop collapse method. Effects of carbon and nitrogen sources, pH, temperature and incubation periods on biosurfactant production were determined by optimization. The biosurfactant characterization was by Fourer Transform Infra-Red and Gas Chromatography Mass Spectrometry analyses. Preliminary assessment showed that the emulsification index was 56.41 ± 1.30 %, oil displacement, 6.02 ± 1.24 mm, zone of haemolysis, 5.12 ± 1.06 mm, surface tension, 29.46 ± 0.62 mN/m and a positive drop collapse test. At optimal conditions of temperature (40 ºC), pH (7), carbon and nitrogen sources (lactose, 20 g/l and urea, 1.5 g/l respectively), the surfactant reduced surface tension up to 11.10 ± 0.78 mN/m and the emulsification index rose to 95.51 ± 2.66%. The highest biosurfactant produced was 3.73 ± 0.19 g/L at 72 h. *Bacillus subtilis* RT9(4)B produced lipopeptide type biosurfactant containing hexadecanoic, octadecanoic acid, peptides, aliphatic, alkyl and esters.
Key word: *Bacillus subtilis*, biosurfactants, emulsification index, restaurant wastewater, surface tension

INTRODUCTION

Biosurfactant is one of the very important bio-based microbial products. It is a naturally produced surface active compound capable of reducing tensions between different phases (Danyelle *et al*., 2016). It is produced by a wide range of microorganisms including bacteria, fungi and algae. *Bacilli* form the dominant bacterial biosurfactant producers. *Bacillus* species have long history of use in biotechnology. Virtually, all species of *Bacilli* have been implicated in one bio-based production or the other irrespective of whether they are pathogenic or not. *Bacillus subtilis* is non-pathogenic and generally regarded as safe (GRAS) (Josh *et al*., 2013) and their use in biosurfactant production could offer some special advantages.

Production of surface-active agents by biological means has attracted attentions recently. Environmental consciousness and concern have driven minds back to bio-based products. There are many reasons for the preference of biosurfactant to chemically-synthesized surfactants. On the one hand, their production is environmentally friendly. Besides, their products are biodegradable and less toxic. Furthermore, biosurfactants are stable over a wide variety of environmental factor (Paraszkiewicz *et al*., 2019). However, production of biosurfactants is faced with bottlenecks relating to lack of industrial availability (Fenibo *et al*., 2019). Chemically synthesized surfactants are non-biodegradable and toxic to the environment (Mulligan *et al*., 2014). Production of biosurfactant is not cost-effective.

Lipopeptide as a type of biosurfactant is a powerful cyclic lipopeptide with surface activities (Plaza *et al*., 2015). It is made up of fatty acids and seven amino acids (Pecci *et al*., 2010). Lipopeptide share the other general biosurfactant properties of environmental friendliness, biodegradable, low toxic and non-hazardous (Jacques 2011). In addition, lipopolypeptide specifically have better foaming properties and higher selectivity than their synthetic counterparts (Jacques 2011) and are active at extreme environmental conditions (Pacwa-Płociniczak *et al*., 2011).

The study investigated the production, characterization, and optimisation of biosurfactant produced by *Bacillus subtilis* RT9(4)B isolated from soil contaminated with restaurant wastewater with a view to

getting biosurfactant that can conveniently replace chemically derived surfactants.

MATERIALS AND METHODS

Isolation of Bacillus species: Ten gram of restaurant wastewater contaminated soil sample was suspended in 90 ml of sterile distilled water contain in 100 ml Erlenmeyer flask and amended with 1 ml engine oil. The medium was incubated at 25˚C for 48 hours on a rotary shaker (Model S150, Bartoworld, scientific, 1995) at 150 rmp. After incubation, the medium was serially diluted by transferring 1 ml of the stock solution into 9 ml of sterile distilled water in a test tube using a sterile pipette. One ml from the dilutions (10^{-1} to 10^{-6}) was transferred aseptically to sterile Petri-dishes. The sterilized nutrient agar (Oxoid CM0929, Hanis, UK) was allowed to cool to 45-50^{o}C before pouring into the Petri-dishes. The inoculated plates were incubated at 30 °C for 48 hour. Pure cultures were obtained by sub-culturing and stored on Nutrient agar (Oxoid CM002, Hampshire, England) slants at 4 °C refrigeration.

Identification of bacterial isolates: The selected isolate *Bacillus subtilis* RT9(4)B was identified through 16S rRNA sequencing. The protocol was followed according to the manufacturer's (QIAquick® Gel Extraction kit, Qiagen, Hilden, Germany) manual. Standard primers 8F:5-AGAGTTTGATCCTGGCTCAG-3 and 1492R:5 GGCTACCTTGTTACGACTT-3 and 27F:AGAGTTTGATCMTGGC and 1492R:5-GGCTACCTTGTTACGACTT-3 were used (Sreethar *et al*., 2014).

Screening of the Bacillus isolates for biosurfactant production: Screening of *Bacillus* isolates for surfactin production were carried out using nutrient broth (30 mL) in 100 mL flask inoculated with 3 mL McFarland 0.5 standardized pure culture grown on Nutrient broth for 24 hours. The inoculated culture media were incubated at 30 °C on a rotary shaker at150 rpm for 72 hours. The cultures were centrifuge (Model 80-213, 2000) at 3000 rpm for 30 minutes to obtain cell free supernatant. The supernatants were collected and cells discarded. The various supernatants were used for emulsification stability, drop collapse and oil spread ability.

Screening of Bacillus species for biosurfactant production

Determination of blood haemolytic ability: Sterilized Blood agar base was allowed to cool for 45 ºC and 20 ml aseptically collected goat blood was added, mixed gently and poured on Petri dishes. A 24 h freshly grown cultures were point inoculated using wire loop at the centre of the blood agar plates. The plates were incubated at 30 ºC for 24 h. The area of clear zone around the colonies were measured using meter rule as reported by El-Shahawy (2014).

Emulsification stability test (EI24): Kerosene (2 ml) was added to the same amount of cell free supernatant obtained through centrifugation, vortexed for 2 minutes using an electronic vortex machine (Model XH-B, 2012), and allowed to stand for 24 hours. 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. Emulsification stability test of culture samples was measured after 24 h (Balogun and Fagade, 2010) and values obtained recorded.

Drop collapse assay: The method of Seema and Nakuleshwar (2012) was adopted for the drop collapse assay. Ten microliters of cell free broth was dropped in the centre of a vegetable oil (Grand Cereal, Jos, Nigeria) drop on a clean glass slide. After one minute the drops was examined visually. The destabilizations of cell free broth dropped indicated positive result while non-destabilized drop indicated negative result. Activity of collected supernatant was compared with water as control (Seema and Nakuleshwar, 2012).

Determination of oil spreading ability: A 20 ml distilled water were dispensed in Petri plates. One millilitre of crude oil was dropped in the centre of the plates containing the distilled water. This was followed by dropping 20 µl of the supernatant of the culture of *Bacillus* isolate

at the centre of the crude oil. Ring formation due to displacement of crude oil was measured using a meter rule and a 20 μl distilled water was used as control as documented by Hasham *et al.* (2012).

Surface tension measurement: Surface tension was measured using a KSV Sigma 702 tensiomenter. All measurements were made on the cell-free broth obtained by centrifuging the culture at 10000 rpm for 15 minutes. Ten millilitre of each cell free broth was 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 (Mulligan *et al*., 2014).

Determination of cell dry weight: The initial weight of sterile Petri dish was taken using electronic weighing balance. Residue from centrifuged culture broth was poured on the sterile Petri dish and dried in a hot air oven for 1h at 180ºC. After drying the final weight of the plate was measured and the weight of the cell dry weight calculated using the following formula:

Cell dry weights = Final weight of the plate after drying - initial weight of the empty plate (Mulligan *et al*., 2014).

Preparation of mineral salt medium (MSM) for optimization study: Mineral salts medium (MSM) was prepared as described by Atlas (2010). The trace element solution was prepared first by adding components (0.232 g H_3BO_3, 0.174 g $ZnSO_4.7H_2O$, 0.116 g $FeSO_4(NH_4)_2SO_4.6H_2O$, 0.096 g $CoSO_4.7H_2O$, 0.022 g $(NH_4)6Mo7O_{24}.4H_2O$, 8.0 mg $CuSO_4.5H_2O$, 8.0 mg $MnSO_4.4H_2O$) to 1.0l of distilled water. The solution was then mixed thoroughly. The trace element solution (5.0 ml), was mixed with 12.5 g K_2HPO_4, 3.8 g KH_2PO_4, 1.0 g $(NH4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$ and 1000ml of distilled water. The solution was mixed thoroughly, gently heated to boil. The pH of the medium was adjusted to 7.0 using 1 M HCl and 1 % NaOH. The mixture was then autoclaved at 121 ºC for 15 min and cooled to 45 - 50 °C.

Determination of the effect of pH on biosurfactant production: This was done using fifty millilitres of sterile 40 g/l glucose mineral salt medium (Atlas, 2010) with varying initial medium pH from 6.0 ~ 10.0. The medium pH was adjusted using 1 M HCl and 1 % NaOH and then followed by inoculation with 3 mL of overnight nutrient broth culture (McFarland 0.5 standardized pure culture grown on Nutrient broth for 24 hours to obtain 1 x 10^8 CFU/ml). The media were incubated in an incubator shaker (Series F 200, England) for 72 h at 150 rpm.

Influence of incubation temperature on biosurfactant production: This was carried out using fifty millilitres of sterile 40 g/l glucose MSM (pH adjusted to 7.0). The fermentation medium was inoculated and incubated as described previously in screening for bacterial biosurfactant production. The rotary shaker (Bartoworld, Scientific, Model S150, 1995) temperature was regulated at different (25 - 45 ºC) interval for 72 h. After which the biosurfactant production was determined.

Determination of the effect of incubation time on biosurfactant production: In 250 ml Erlenmeyer flasks, 50 mL sterile 40 g/l glucose mineral salt medium were dispensed. The media were inoculated with 3 ml overnight culture of the strains under study. The fermentation media were previously adjusted to pH 7 before inoculation. The flasks were incubated at 30 ºC under shaking condition at 150 rpm for 120 h and biosurfactant production taking at 24 h interval.

Effect of different carbon sources on biosurfactant production: Effects of glucose, lactose, dextrose, and soluble starch at concentrations 10, 20, 30, 40, and 50 g/l were examined on biosurfactant production by *Bacillus subtilis*. To 1000 ml mineral salt medium, the carbon sources (glucose, lactose, dextrose and soluble starch) were added individually, the fermentation medium pH was adjusted to 7.0. This was then followed by inoculation with 3 ml of

overnight nutrient broth culture (McFarland 0.5 standardized pure culture grown on nutrient broth for 24 hours to obtain 1 x 10^8 CFU/ml) and incubated under shaking (Orbital Shaker, Series F200, England) condition at 150 rpm at 30 ºC for 72 h. After which biosurfactant production was determined.

Effect of different nitrogen sources on biosurfactant production: Effect of different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 g/l) of yeast extract, urea and proteose peptone on biosurfactant production were determined. To a 1000 ml mineral salt medium, varying concentrations of these nitrogen sources were added individually. The fermentation medium pH was adjusted to 7. The medium was inoculated with 3 ml overnight nutrient broth culture and was incubated under shaking condition at 150 rpm, 30 ºC for 72 h. After which biosurfactant production was determined.

Extraction of biosurfactant: To extract the crude biosurfactant, the culture supernatants was centrifuged at 10000 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 stand for 24 hours at 4ºC to precipitate. Equal volume of chloroform: methanol (2:1) was added, mixture was shaken vigorously and left to stand overnight. The resulting white coloured sediments were collected the following day (Anitha *et al*., 2015) for characterization.

Preliminary separation by column chromatography: The Column was loaded with 50 grams of slurry of silica gel. Aliquot of biosurfactant was fractionated using varying ratios (13:20 v/ v, 20:15 v/v, 15:25 v/v, 20:30 v/v and 10:15 v/v) of established solvent system (Chloroform and methanol). About 2 ml of eluent were collected at 10 minutes' interval. A total of 32 different fractions were collected for further purification.

Thin layer chromatography of partially purified fractions: Aluminium TLC sheets covered with silica gel prepared commercially were used. The plates were cut to fit 5 × 5 cm size. Drop of eluents were placed at distance of 0.5 cm from the bottom of the TLC plate. The plate was then placed in a chromatographic tank containing mixture of 15:25 ratio of Chloroform and methanol earlier determined as the best resolution. The plates were spread using sulfuric acid. Fractions with the same retention factor were pooled together (Mulligan *et al*., 2014).

Structural characterization and identification of biosurfactant: Structural classification of the biosurfactant was carried out using fourer transform infra-red (FTIR) and gas chromatography mass spectroscopy (GC-MS). The FTIR instrument (Buck scientific M530 USA) was equipped with a detector of deuterated triglycine sulphate and beam splitter of potassium bromide. The software of the Gram A1 was used to obtain the spectra. One milligram of the biosurfactant sample was mixed thoroughly with 100 mg of homogenized porcelain-milled Potassium bromide (KBr). During measurement, FTIR spectra was obtained at frequency regions of 4,000 – 600 cm^{-1} and co-added at 32 scans and at 4 cm^{-1} resolution. The FTIR spectra were displayed as transmitter values (Jain *et al*., 2012).

Ten milligrams (10 mg) of biosurfactant was mixed with 5 % HCl-methanol reagent. The reaction was stopped with addition 1 mL of sterile H_2O. The samples were recovered with methanol and 1 mL of samples were injected into a gas chromatograph (30 m × 0.25 mm ID × 0.25 mm). The carrier gas was Helium at a flow rate of 1.5 $mLmin^{-1}$ and the working temperature of the GC injector was 260 ˚C. The gradient temperature was set as range from 60 to 260 ºC at a speed of 5 ºC min^{-1}, through an isothermal phase of 10 min at the end of the analysis. The electron impact ion source was sustained at 200 ˚C. Mass spectra were recorded at 70 keV. The mass spectra were obtained with a m/z range: 40–700 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 library and the target mass spectra obtained from sample were compared with mass spectra obtained from the library as recently reported by Parthipan *et al*. (2017).

Statistical analysis of data obtained: The data obtained were presented in graphs, tables and charts. Data from biosurfactant production screening were statistically interpreted using Chi-square and Analysis of Variance (ANOVA). Optimization studies data were also analysed using analysis of variance (ANOVA) at 99 % confidence level. Means were separated using Duncan test. The means were compared using one-way ANOVA to indicate any significant difference among parameters and the variables.

RESULTS AND DISCUSSION

Preliminary screening of *Bacillus subtilis* for biosurfactant production

Bacillus subtilis was subjected to four preliminary screening assays in order to determine their biosurfactant production potential. These include haemolytic activity, oil collapse, emulsification index and oil spreading assays. This preliminary characterization showed on Table 1 indicated that *Bacillus subtilis* RT9(4)B among other *Bacillus* species had the highest β- haemolysis with 5.12 ± 1.06 mm zone of lyses. The initial emulsification index (E_{24}) was 56.41 ± 1.30 %. *Bacillus subtilis* displaced crude oil by 6.02 ± 1.24 mm with a positive drop collapse test. In previous reports, one or more screening assay is used as index for biosurfactant production (Kiran *et al*., 2010). Hence, Antoniou *et al.* (2015) used drop collapse test for preliminary determination of biosurfactant production from marine hydrocarbon-degrading bacteria using crude oil as carbon substrate. The tests in the present study have been applied by Anaukwu *et al.* (2015) and Sidkey *et al*. (2016).

β-haemolysis has been used as an attribute for biosurfactant producing potential by microorganisms. Consequently, El-Shahawy (2014) reported that the ability to haemolyse blood is a characteristic used in identifying biosurfactant production by microbes. In addition, Carrillo *et al*. (1996) have linked blood haemolysis with biosurfactant production and Eduardo *et al*. (2015) had also reported β-haemolysis for *Bacillus subtilis*. In line with the above, Roy *et al*. (2014) quantified the zone of clearing and noted a direct relationship between the zone of clearing and medium biosurfactant content. The β-haemolytic zone of clearing obtained in the present study of 5.12 ± 1.06 mm was lower than 8.7 mm previously obtained (Akintokum *et al*., 2017) for a related bacterium.

Table 1: Blood haemolysis of bacterial isolates

Isolate codes	Haemolysis	Zone of Haemolysis (mm)
RT9(4)B	Beta	5.12 ± 1.06^{a}
RT6(4)B	Beta	1.70 ± 2.57^{c}
RT1(3)A	Gamma	0.00 ± 0.00^{d}
RT8(3)C	Beta	2.82 ± 3.10^{b}
RT10(5)C	Apha	0.00 ± 0.00^{d}
RT7(4)B	Beta	2.55 ± 2.80^{b}
RT3(3)A	Gamma	0.00 ± 0.00^{d}
RT9(4)A	Beta	5.57 ± 0.55^{a}
RT4(4)C	Gamma	0.00 ± 0.00^{d}

Means with different superscripted alphabets along the columns are significantly different. Values are expressed as means±SE (Standard error of means). **Significant difference level at 0.01.

Table 2: Biosurfactant production parameters of the bacterial isolates

Isolate codes	Drop collapse	Oil spreading (mm)	Emulsification index (%)
RT9(4)B	+	6.02 ± 1.24[a]	56.41 ± 1.30[a]
RT6(4)B	-	0.00 ± 0.00[d]	0.00 ± 0.00[f]
RT1(3)A	-	0.00 ± 0.00[d]	0.00 ± 0.00[f]
RT8(3)C	+	2.62 ± 2.88[cb]	33.22 ± 3.40[d]
RT10(5)C	-	0.00 ± 0.00[d]	0.00 ± 0.00[f]
RT7(4)B	+	4.62 ± 0.4[ab]	48.95 ± 1.22[c]
RT3(3)A	-	0.00 ± 0.00[d]	0.00 ± 0.00[f]
RT9(4)A	+	5.87 ± 0.61[a]	51.25 ± 1.62[b]
RT4(4)C	-	0.00 ± 0.00[d]	0.00 ± 0.00[de]

Means with different superscripted alphabets along the columns are significantly different. Values are expressed as means±SE (Standard error of means). **Significant difference level at 0.01.

Table 3: Effect of different initial medium pH, temperature and incubation time on growth and biosurfactant production by *Bacillus subtilis* RT9(4)B.

Treatment	Variable	Emulsification index (%)	Surface tension (mN/m)	Cell dry weight (g/L)
pH	6	32.39 ± 1.97[d]	65.53 ± 1.74[a]	0.32 ± 0.08[b]
	7	49.13 ± 0.54[a]	55.16 ± 1.02[c]	0.53 ± 0.04[a]
	8	42.00 ± 0.36[b]	59.20 ± 1.61[b]	0.36 ± 0.06[b]
	9	34.83 ± 1.39[c]	63.81 ± 3.02[a]	0.30 ± 0.07[b]
	10	29.81 ± 1.00[e]	66.68 ± 1.51[a]	0.09 ± 0.10[c]
Temperature (ºC)	25	37.41 ± 0.66[d]	58.61 ± 3.00[a]	0.47 ± 0.13[cd]
	30	43.82 ± 1.11[c]	55.30 ± 0.97[ab]	0.83 ± 0.04[bc]
	35	60.47 ± 1.08[b]	39.97 ± 4.81[c]	1.16 ± 0.10[b]
	40	63.34 ± 0.73[a]	31.84 ± 1.68[d]	1.54 ± 0.09[a]
	45	56.13 ± 2.31[c]	42.80 ± 2.86[b]	1.15 ± 0.23[d]
Incubation Period (h)	24	35.73 ± 0.86[a]	48.12 ± 0.86[a]	0.13 ± 0.03[c]
	48	54.21 ± 1.09[b]	35.59 ± 2.18[b]	0.61 ± 0.31[b]
	72	84.44 ± 0.21[a]	31.94 ± 1.20[c]	1.03 ± 0.19[a]
	96	85.51 ± 2.66[a]	30.10 ± 0.78[cd]	0.70 ± 0.02[b]
	120	84.19 ± 1.03[a]	29.36 ± 0.82[d]	0.80 ± 0.02[ab]

Means with different superscripted alphabets along the same column for each test parameter are significantly different. Values are expressed as means ± SE (Standard error of means). **Significant difference level at 0.01.

In furtherance to the determination of the biosurfactant production ability of the *Bacillus* species, culture broth of *Bacillus subtilis* RT9(4)B tested positive for drop collapse (Table 2). Theoretically, in the presence of surfactant, a drop of culture supernatant spreads over oil surface as the interfacial tension between the droplet and oil surface is reduced (Batiata *et al*., 2006). The result of oil spreading technique indicated that the organism had oil spreading up to 6.02 mm (Table 2). Chandran and Das (2010) reported oil displacement to be a function of surfactant presence in the broth and that the diameter of the oil displacement is directly proportional to the activity of the surfactant. The oil spreading result of the present study is higher than that reported Nur and Mohammed (2015) for a related *Bacilli*, probably signifying more biosurfactant activity.

Emulsification index (EI24) activity of the organism was also determined as a preliminary test for the detection of biosurfactant producing potential (Table 2). Although not all bio-emulsifiers are biosurfactant producer, percentage emulsification has a direct correlation with biosurfactant production as high percent EI24 translates to high biosurfactant activity. The preliminary EI24 (56.41 ± 1.30 %) in this study is lower than that presented by

Ainon (2013) (EI24, 68 %) but higher than 50% obtained by Al-Wahabi *et al*. (2014) with related species of bacteria. The ability of the present organism to emulsify kerosene implies that it can be applied in various emulsion industries. The *Bacillus subtilis* RT9(4)B showed excellent biosurfactant production potential among other *Bacillus* species during the preliminary screenings. This necessitated the used of the isolate for further study.

Biosurfactant production by *Bacillus subtilis* RT9(4)B under varying pH, temperature and incubation periods

Effects of growth parameters on biosurfactant production by *Bacillus subtilis* isolated from soil contaminated with restaurant effluent was determined with a view to finding the optimum conditions for growth and biosurfactant production. Table 3 showed the effects of pH, temperature and incubation periods on biosurfactant production by *Bacillus subtilis*. The optimum pH for the *Bacillus subtilis* was 7.0.

Subsequent increase in pH led to a significant reduction in biosurfactant production. To grow and/or to produce metabolite, whether primary or secondary, a definite pH requirement is necessary. As the initial medium pH increased, both bacterial growth and biosurfactant production decreased. At this optimum pH (pH 7.0) the cell dry weight of 0.53 ± 0.04 g/l were obtained in the present study. Few related studies also obtained maximal biosurfactant at pH 7.0 (Husam and Ahmed, 2013).

Also, the effect of temperature on biosurfactant production is presented in Table 3. Maximum biosurfactant production was observed at 40 ºC. Similarly, the effect of different incubation periods on biosurfactant production potential of *Bacillus subtilis* is shown in Table 3. There is a strong dependence of microorganisms on some of the physicochemical parameters for secondary metabolite biosynthesis. In this study, biosurfactant production at 40 ºC indicated the highest EI24 activity of 63.34 ± 0.73 %, cell dry weight of 1.54 ± 0.09 g/l and reduction of inter surface of 31.84 ± 1.68 mN/m as shown in Table 3. Therefore, 40 ºC was recorded as the optimum temperature for biosurfactant production. This observation implies that the organism is moderately thermophilic and thus required high temperature for biosurfactant production. In a similar assessment, Dhail (2012) obtained optimum temperature range of 30 to 40 ºC while, Antoniou *et al*. (2015) obtained a non-temperature dependent biosurfactant.

The influence of incubation period on the bacterial growth and biosurfactant production was determined through a time course-study. The findings showed that increased incubation period increased biosurfactant production by *Bacillus subtilis*. Highest biosurfactant production was obtained at 96 h with a slight decline at 120 h (Table 3). This result could imply that using *B. subtilis* and optimizing substrates is required for optimal biosurfactant harvest. Previous study by Husam and Ahmed (2013) revealed that four days were needed for maximal biosurfactant production while an elevated increase up to nine days were obtained by Khopade *et al*. (2012). These biosurfactant production assessments were indirect as emulsification indices were measured rather than biosurfactant itself.

Biosurfactant production by *Bacillus subtilis* RT9(4)B under different concentrations of carbon and nitrogen sources

The effects of glucose, lactose, dextrose, and soluble starch at concentrations 10, 20, 30, 40, and 50 g/l were examined on biosurfactant production by *Bacillus subtilis*. Among the carbon sources, 20 g lactose was the most suitable for *Bacillus subtilis* biosurfactant production, followed by 50 g glucose (Table 4). The bacterium gave the highest biosurfactant production based on result of emulsification index, surface tension and cell dry weight of 85.65 ± 0.98 %, 27.76 ± 1.23 mN/m and 1.53 ± 0.00 g/l respectively at 20 g/l lactose (Table 4).

Consequently, 20 g/l lactose was recorded as the obtained optimal carbon source in this

study. While Antoniou *et al*. (2015), obtained biosurfactant independent of culture biomass quantity and carbon substrate using marine hydrocarbon-degrading bacteria. This study revealed that carbon source determines biosurfactant production. The study supported the reports of Amalesh *et al*. (2012) on lactose carbon substrate and Raza *et al*. (2007) on the dependence of biosurfactant production on carbon source.

The effect of the nitrogen source concentrations examined on biosurfactant production potential of *Bacillus subtilis* showed that 1.5 g urea gave the highest values followed by yeast extract (Table 5). Emulsification index, reduction of medium surface tension and cell dry weight value were 86.35 ± 1.02 %, 28.46 ± 0.62 mN/m, 2.05 ± 0.25 g/l respectively at 1.5 g/l urea. These findings were similar to report of Agarry *et al*. (2015), in which urea at 1.5 g/l concentration for biosurfactant production by *Bacillus subtilis*. This present work showed that an increase in urea concentration above 1.5 g/l reduced biosurfactant production.

Growth of *Bacillus subtilis* RT9(4)B under optimal medium condition

In Figure 1, 20 g of lactose and 1.5 g of urea were used as the optimum concentrations of carbon and nitrogen sources for biosurfactant production by *Bacillus subtilis* RT9(4)B. Following application of optimized conditions including pH 7, temperature 40 ^{o}C and carbon and incubation period at 96 h, maximum production was obtained as indicated by emulsification index (95.51 ± 2.66%) and surface tension (11.10±0.78 mN/m) reduction values. Overall, biosurfactant produced by *Bacillus subtilis* was 3.73 ± 0.19 g/l at 72 h. The optimisation led to approximately 13.2% reduction in surface tension (30.70 ± 1.42 to 27.10 ± 0.78 mN/m) and a 69.3% rise in the emulsification index (56.41±1.30 to 95.51 ± 2.66%) from the preliminary test values. The present optimisation result is related to the work of Nitschke and Pastore (2004); Sharma *et al*. (2015) who reported biosurfactant maximum yield of 3.2 g/l at 72 hours. This is in agreement with the study of Danashekar and Natarajan (2011) who found a highest EI24 values of, 75, 66.6 and 70% with Petrol, Kerosene and Diesel respectively in the culture samples. A basic advantage observed in this study is that culture conditions could be optimised leading to maximum biosurfactant yield. Consequently, *Bacillus subtilis* of the present study is a good candidate for biosurfactant production.

FT-IR profile of *Bacillus subtilis* RT9(4)B biosurfactant

The FTIR profile of *Bacillus subtilis* surfactant is presented in Table 6. Peaks, transmission and functional groups of the associated compounds. The notable functional groups were alkyl (CH_2- and –CH_3 chains), aliphatic, carbonyl, esters and peptides. The FT-IR findings of the present investigation is similar to that obtained by Faria *et al*. (2011) and Ibrahim *et al*. (2013) based on their lipopeptide reports.

GC - MS profile of biosurfactant produced by *Bacillus subtilis* RT9(4)B

The GC-MS profile showed that the compound produced by *Bacillus subtilis* was a lipopeptide derivative. The findings revealed the presence of 25 major peak (Table 7). The major compounds identified included Oleic acid (30.95%), Octadecanoic acid (25.90%), Cyclododecanol, 1-aminomethyl- (18.28%), Trimyristin (9.00%), Methyl stearate (7.79%), Stearic acid hydrazide (3.52%) and n-Hexadecanoic acid (1.09%).

Other compounds were present in relatively lower amounts. Combining the FT-IR and GC-MS findings, the functional groups including peptide presence and the numerous fatty acids preclude a lipopeptide-type biosurfactant. The characterisation profile of the present study is similar to the finding obtained by Donio *et al*. (2013) and Ibrahim *et al*. (2013) which was a confirmation of the presence of the identified compounds.

Table 4: Effect of different Carbon sources concentrations on growth and biosurfactant production by *Bacillus subtilis* RT9(4)B.

Treatments	Concentrations (g/l)	Emulsification index (%)	Surface tension (mN/m)	Biomass (g/l)
Soluble starch	10	28.04 ± 0.74^{e}	67.59 ± 1.92^{a}	0.07 ± 2.69
	20	32.83 ± 1.91^{d}	55.33 ± 0.59^{b}	0.07 ± 0.04
	30	42.77 ± 1.19^{c}	44.85 ± 1.04^{c}	0.07 ± 0.09
	40	46.59 ± 1.39^{b}	46.51 ± 2.76^{c}	0.08 ± 0.04
	50	54.07 ± 1.71^{a}	40.74 ± 3.30^{d}	0.08 ± 0.46
Glucose	10	55.87 ± 2.12^{c}	45.62 ± 0.87^{c}	0.11 ± 0.04^{bc}
	20	45.47 ± 1.52^{d}	57.04 ± 0.96^{a}	0.08 ± 0.10^{cd}
	30	44.16 ± 2.19^{d}	50.85 ± 0.42^{b}	0.07 ± 0.17^{d}
	40	63.38 ± 1.60^{b}	31.20 ± 3.52^{d}	0.29 ± 0.11^{b}
	50	77.79 ± 0.73^{a}	28.38 ± 2.06^{e}	0.59 ± 0.22^{a}
Lactose	10	76.52 ± 0.56^{b}	30.70 ± 1.42^{d}	0.96 ± 0.12^{b}
	20	85.65 ± 0.98^{a}	30.76 ± 1.72^{d}	1.53 ± 0.00^{a}
	30	76.23 ± 0.97^{b}	34.40 ± 1.20^{c}	0.52 ± 0.17^{c}
	40	65.97 ± 0.74^{c}	40.38 ± 0.59^{b}	1.52 ± 0.08^{a}
	50	51.00 ± 1.29^{d}	45.62 ± 1.58^{a}	1.44 ± 0.21^{a}
Mannose	10	66.70 ± 0.64^{a}	34.39 ± 0.82^{d}	0.12 ± 0.03^{c}
	20	51.76 ± 1.35^{b}	41.06 ± 0.60^{c}	0.48 ± 0.03^{a}
	30	50.47 ± 1.09^{b}	41.36 ± 1.15^{c}	0.17 ± 0.05^{c}
	40	42.24 ± 1.44^{c}	56.75 ± 0.59^{b}	0.30 ± 0.10^{b}
	50	26.71 ± 1.06^{d}	61.37 ± 0.82^{a}	0.57 ± 0.06^{a}

Means with different superscripted alphabets along the same column for each test parameter are significantly different. Values are expressed as means ± SE (Standard error of means). **Significant difference level at 0.01.

Table 5: Effect of different nitrogen source concentrations on growth and biosurfactant production by *Bacillus subtilis* RT9(4)B

Treatments	Concentration (g/l)	Emulsification index (%)	Surface tension (mN/m)	Cell dry weight (g/l)
Yeast extract	0.5	39.61 ± 1.13^{e}	62.61 ± 1.31^{a}	0.77 ± 0.06^{d}
	1.0	78.51 ± 0.99^{a}	27.25 ± 0.95^{d}	1.65 ± 0.09^{a}
	1.5	60.10 ± 0.09^{c}	31.58 ± 1.48^{c}	1.14 ± 0.08^{c}
	2.0	63.89 ± 0.51^{b}	30.80 ± 1.20^{c}	1.38 ± 0.08^{b}
	2.5	42.00 ± 0.77^{d}	51.51 ± 1.23^{b}	1.14 ± 0.03^{c}
Urea	0.5	58.91 ± 1.92^{e}	42.62 ± 1.92^{a}	0.66 ± 0.07^{c}
	1.0	75.31 ± 0.90^{c}	33.40 ± 1.15^{b}	1.27 ± 0.11^{b}
	1.5	86.35 ± 1.02^{a}	28.46 ± 0.62^{d}	2.05 ± 0.25^{a}
	2.0	81.05 ± 1.49^{b}	30.67 ± 1.51^{c}	1.43 ± 0.09^{b}
	2.5	69.72 ± 0.44^{d}	35.12 ± 1.86^{b}	0.80 ± 0.02^{c}
Peptone	0.5	55.73 ± 0.86^{b}	35.95 ± 2.18^{c}	0.13 ± 0.03^{a}
	1.0	64.21 ± 1.09^{a}	28.12 ± 0.86^{d}	0.08 ± 0.03^{b}
	1.5	44.44 ± 0.21^{c}	56.94 ± 1.20^{a}	0.08 ± 0.03^{c}
	2.0	43.84 ± 1.14^{c}	55.10 ± 0.78^{ab}	0.06 ± 0.02^{e}
	2.5	44.19 ± 1.03^{c}	54.36 ± 0.82^{b}	0.07 ± 0.02^{d}

Means with different superscripted alphabets along the same column for each test parameter are significantly different. Values are expressed as means ± SE (Standard error of means). **Significant difference level at 0.01.

Table 6: The FTIR profile of *Bacillus subtilis* RT9(4)B biosurfactant

S/No.	Peaks	Transmission (%)	Functional group
1	3490.006	35	Peptides
2	3001.030	65	Aliphatic
3	2065.125	80	Aliphatic
4	1642.420	45	Carbonyl
5	1432.404	65	Alkyl
6	1252.720	75	Alkyl
7	1112.254	15	Esters
8	913.529	20	CH_2

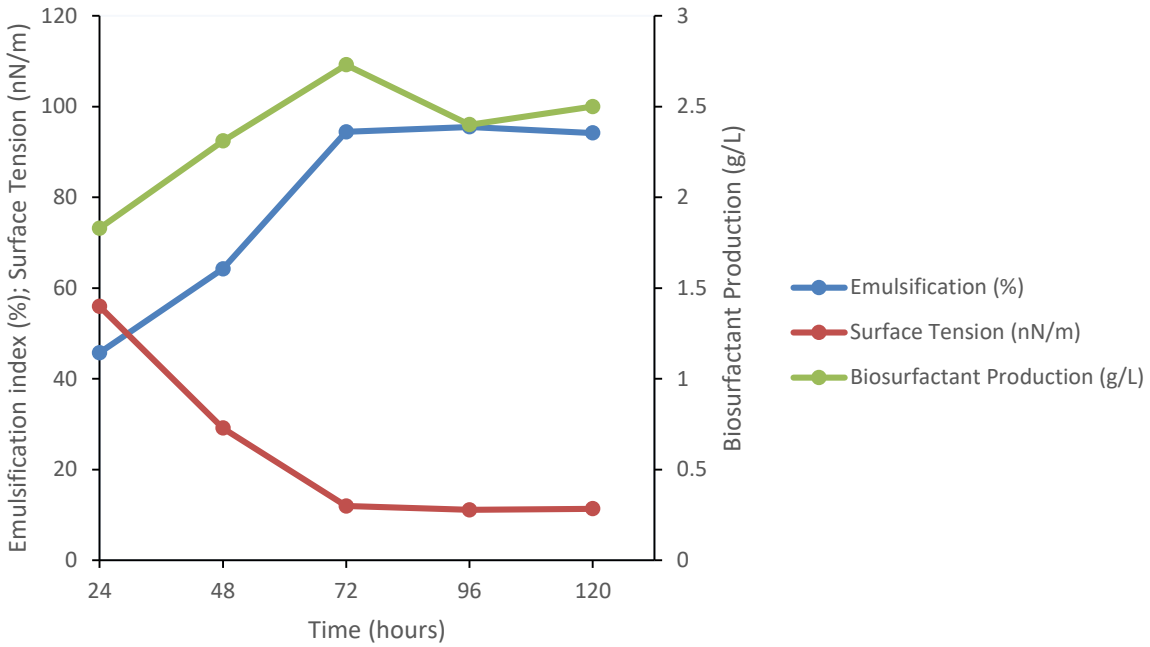

Figure 1: The emulsification index, surface tension and biosurfactant production profiles of *Bacillus subtilis* RT9(4)B grown in optimised medium.

Table 7: GC-MS Profile of Surfactin Produced by *Bacillus subtilis* RT9(4)B

No.	Retention time	Peak area (%)	Name of compounds
1	5.359	0.70	Tert-Butyl isopropyl disulfide, perfluoro
2	9.585	0.73	Cyclotetrasiloxane, octamethyl-
3	15.594	0.07	Plumbane, diethyldimethyl-
4	16.524	0.09	Cyclopentasiloxane, decamethyl-
5	18.540	0.04	Cyclotrisiloxane, hexamethyl-
6	33.544	0.08	Undecanoic acid, 10-methyl-, methyl ester
7	37.421	0.33	Dodecanoic acid
8	37.963	0.03	Nonanedioic acid, dimethyl ester
9	41.530	0.08	Tridecanoic acid, 12-methyl-, methyl ester
10	44.515	0.25	Tetradecanoic acid
11	48.818	0.25	Pentadecanoic acid, 14-methyl-, methyl ester
12	51.804	1.09	n-Hexadecanoic acid
13	54.362	0.18	9-Octadecenoic acid, methyl ester, (E)-
14	54.983	7.79	Methyl stearate
15	56.960	30.95	Oleic Acid
16	57.464	35.90	Octadecanoic acid
17	58.084	0.03	2,7-Octadien-1-ol
18	59.092	0.12	Pentanoic acid, propyl ester
19	61.883	0.03	9-Decenoic acid
20	62.310	0.06	Trimyristin
21	64.481	3.52	Stearic acid hydrazide
22	64.830	0.13	Undecane, 5,6-dimethyl-
23	65.140	0.25	Tridecane
24	65.838	9.00	Trimyristin67.040 18.28
25	67.040	18.28	Cyclododecanol, 1-aminomethyl-

CONCLUSION

This study revealed that soil contaminated with restaurant wastewater is a good source of biosurfactant producing *Bacillus* species. At optimal conditions of temperature (40 ºC), pH (7), carbon and nitrogen sources (lactose, 20 g/l and urea, 1.5 g/l respectively), there was an approximately 23.2% reduction in surface tension (14.46 ± 0.62 to 11.10 ± 0.78 mN/m) and a 69.3% rise in the emulsification index (56.41±1.30 to 95.51 ± 2.66%). The highest biosurfactant produced was 3.73 ± 0.19 g/l at 72 h. Furthermore, the *Bacillus subtilis* RT9(4)B produced lipopeptide type biosurfactant and therefore a potential xxcandidate for biosurfactant production of industrial application.

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