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PRODUCTION AND CHARACTERIZATION OF BIOSURFACTANT BY *BACILLUS SUBTILIS* ISOLATED FROM SOIL SAMPLES

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ABSTRACT

This study investigated the capability of a biosurfactant produced by a novel strain of *Bacillus subtilis* to enhance the biodegradation rates and bioavailability of organic contaminants isolated from soil sample. 10 isolates of *Bacillus subtilis* were isolated from three different oil contaminated soil samples. All isolates showed α – haemolysis in haemolytic test. In oil displacement test, isolates BS1, BS2, BS3, BS4, BS5, BS7 and BS8 showed zone of diameter 25mm, 13mm, 18mm, 16mm, 9mm, 11mm and 18mm respectively. The E24 % of BS1, BS2, BS3, BS4, BS5, BS7 and BS8 were 38.46%, 15.38%, 35.71%, 30.76%, 15.38%, 14.28% and 23.07% respectively. Drop collapse test were given positive by isolates BS1, BS2, BS3, BS4, BS7 and BS8. The emulsification activity of produced biosurfactant is calculated by emulsification index (E24). E24% of biosurfactant produced by BS1, BS2, BS3, BS4 and BS8 are 34.21%, 19.44%, 32.43%, 31.57% and 16.66% respectively. BS1, BS2 and BS4 showed maximum biosurfactant activity and highest dry weight found in isolate BS1 (0.05 gm/100ml). Overall, the results indicated the potential use of *B. subtilis* in environmental remediation processes.

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INTRODUCTION

Surface active substances or surfactants are amphiphilic molecule that tends to lower surface tension of fluids. Also, they have the ability to orient themselves at interface of two immiscible compounds. Surfactants, owing to their surface activity find many industrial applications such as detergents, emulsifiers, de-emulsifiers, dispersants, wetting agents, foam retardant, stabilizers, gelling agent etc (Padmanabhan et al., 2014). Biologically synthesized surface active agents are known as biosurfactants (Shah et al., 2016). Biosurfactants are amphiphilic compound that have hydrophilic and hydrophobic domains. The hydrophilic domains are usually consisting of carbohydrates, amino acids and phosphate groups. Hydrophobic domains are usually made up of long chain fatty acids (Suganya, 2013). It is produced extracellularly or as part of the cell membranes by a variety of yeast, bacteria and filamentous fungi from various substances including sugars, oils and wastes (Femi-Ola et al., 2015).

These are able to reduce the surface and interfacial tensions and have the capacity to mix two immiscible solutions (Ali Diab and Shereen Gamal El Din, 2013). Biosurfactants comprise the properties of dropping surface tension, stabilizing emulsions, promoting foaming and are usually non-toxic and biodegradable. Recently interest in biosurfactant has increased because of its diversity, flexibility in operation, and more ecofriendly than chemical surfactant. Furthermore possibility of their production on large scale, selectivity, performance under intense conditions and their future applications in environmental fortification also these have been increasingly attracting the attention of the scientific and industrial community. These molecules have a potential to be used in a variety of industries like cosmetics, pharmaceuticals, humectants, food preservatives and detergents (Ali Diab and Shereen Gamal El Din, 2013). The most active biosurfactants can lower the surface tension of water from 72 to 30 mN·m⁻¹ and the interfacial tension between water and *n*-hexadecane from 40 to 1 mN·m⁻¹. Biosurfactant activities depend on the

concentration of the surface-active compounds until the critical micelle concentration (CMC) is obtained. At concentrations above the CMC, biosurfactant molecules associate to form micelles, bilayers and vesicles. Micelle formation enables biosurfactants to reduce the surface and interfacial tension and increase the solubility and bioavailability of hydrophobic organic compounds. The CMC is commonly used to measure the efficiency of surfactant. Efficient biosurfactants have a low CMC, which means that less biosurfactant is required to decrease the surface tension. Micelle formation has a significant role in microemulsion formation. Microemulsions are clear and stable liquid mixtures of water and oil domains separated by monolayer or aggregates of biosurfactants. Microemulsions are formed when one liquid phase is dispersed as droplets in another liquid phase, for example oil dispersed in water (direct microemulsion) or water dispersed in oil (reversed microemulsion). The biosurfactant effectiveness is determined by measuring its ability to change surface and interfacial tensions, stabilization of emulsions and by studying its hydrophilic-lipophilic balance (HLB). The HLB value is a measure to indicate whether a biosurfactant is related to water-in-oil or oil-in-water emulsion. This factor can be used to determine the suitable applicability of biosurfactants. Emulsifiers with low HLB are lipophilic and stabilize water-in-oil emulsification, whereas emulsifiers with high HLB have the opposite effect and confer better water solubility (Deshmukh *et al.*, 2014).

Nowadays, biosurfactants are produced using co- and by-products of different technologies as a carbon source for microorganisms (molasses, glycerol, whey, frying oil, animal fat, soapstock and starch-rich wastes e.g. potato wastes) (Juliana *et al.*, 2016). Several factors affect production of biosurfactants, such as the nature of carbon and nitrogen sources used, as well as the presence of phosphorus, iron, manganese and magnesium. In addition, other factors such as pH, temperature, agitation and operation mode are extremely important to quantity and quality of produced biosurfactant (Sidkey *et al.*, 2014). Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon (C_xH_y), microorganisms facilitate their diffusion into the cell by producing a variety of substances, the biosurfactants. Some bacteria and yeasts excrete ionic surfactants which emulsify the C_xH_y substrate in the growth medium. Some examples of this group of biosurfactants are rhamnolipids which are produced by different *Pseudomonas* sp., or the sophorolipids which are produced by several *Torulopsis* sp.. Some other microorganisms are capable of changing the structure of their cell wall, which they achieve by synthesizing lipopolysaccharides or nonionic surfactants in their cell wall. Examples of this group are: *Candida lipolytica* and *C. tropicalis* which produce cell wall-bound lipopolysaccharides when growing on *n*-alkanes and *Rhodococcus erythropolis*, and many *Mycobacterium* sp. and *Arthrobacter* sp. which synthesize nonionic trehalose corynomycolates. There are lipopolysaccharides, such as Emulsan, synthesized by *Acinetobacter* sp. and lipoproteins or lipopeptides, such as Surfactin and Subtilisin, produced by *Bacillus subtilis*. Other effective biosurfactants are: (i) Mycolates and Corynomycolates which are produced by *Rhodococcus* sp., *Corynebacteria* sp., *Mycobacteria* sp., and *Nocardia* sp. and (ii) ornithinlipids, which are produced by *Pseudomonas rubescens*, *Gluconobacter cerinus*, and *Thiobacillus ferrooxidans*.

In the present study, *Bacillus subtilis* used for the production of biosurfactants. *Bacillus* (genus *Bacillus*), any of a group of rod-shaped, gram-positive, aerobic or (under some conditions) anaerobic bacteria widely found in soil and water. Bacteria of the gram-positive genus *Bacillus* produce a number of cyclic lipopeptides, many of which have appreciable antibacterial or antifungal properties. There is considerable structural diversity as a consequence of differences in the nature of the fatty acid component, for example in chain-length (C_6 - C_{18}) and often the presence of hydroxyl groups and/or *iso*- or *anteiso*-methyl branches, as well as in the type, number and configuration of the amino acids in the peptide chain. For example, various strains of *B. subtilis* produce more than twenty different molecules with antibiotic activity including many lipopeptides. Surfactin (illustrated) in addition to its antibiotic properties is one of the most powerful biosurfactants. *B. subtilis* produces two further families of lipopeptide antibiotics, the *iturins* (bacillomycins, iturins and mycosubtilins) and fengycins (plipastatins) (Deshmukh *et al.*, 2014).

MATERIALS AND METHODS

Sample collection

Oil contaminated soil samples were collected from garrages at Shatabdi square, Kukde layout and Ravi nagar in Nagpur. The samples were collected in plastic bags and taken to the laboratory.

Isolation of *Bacillus subtilis* from soil samples

The samples were serially diluted in distilled water and 100 μ l from the final three dilutions of all the samples were spreaded on HiCrome Bacillus Agar plates. Plates were incubated at 37 °C for 24 hours. After 24 hours plates were observed for colonies.

Identification of *Bacillus subtilis*

The isolated colonies were identified on the basis of morphology by performing Gram staining and motility, biochemical by testing sugar fermentation using Glucose, Lactose, Mannitol, Maltose, Sucrose, IMViC Test, Catalase test, Oxidase test, Triple Sugar Iron (TSI) test, Urease test and cultural characteristics by inoculating bacteria on Hichrome Bacillus Agar and Bacillus Differentiation Agar.

Screening of isolated *Bacillus subtilis* for Biosurfactant activity

All the isolates were inoculated in 10 ml nutrient broth and incubated at 37°C for 4 days. Followed by incubation all the tubes should be subjected to centrifugation at 5000 rpm for 30 min. Screening of surfactant producing *Bacillus* should be done by adopting the following tests (Juliana *et al.*, 2016).

- 1. Haemolytic activity:** Isolates were screened on blood agar plates containing 5% (v/v) sheep blood and incubated at 37°C for 48 h. Hemolytic activity was detected by presence of a clear zone around bacterial colonies (Suganya, 2013).
- 2. Oil displacement test:** 50 mL of distilled water should be added to the Petriplate followed by addition of 100 μ L of crude oil to the surface of water. Then 10 μ L of cell free culture broth should be dropped on the crude oil surface.

The diameter of clear zone on the oil surface was measured and compared to 10 μ L of distilled water as negative control (Rabah and Bello, 2015).

3. **Emulsification assay method:** The emulsifying capacity was evaluated by an emulsification index (E24). The E24 of culture samples were determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, the cell-free broth obtained after incubation period and centrifuge at 4000 rpm for 4 min, after that vortex the mixture at high speed for 2 min and allowed to stand for 24h. The E24 index is given as percentage of the height of emulsified layer (mm) divided by the total height of the liquid column (mm). The percentage of emulsification index calculated by using the following equation (Sidkey *et al.*, 2014):

$$E24 = \frac{\text{Height of emulsion formed} \times 100}{\text{Total height of the solution}}$$

4. **Drop collapse method:** A drop of the culture supernatant was placed carefully on an oil coated glass slide and observed after one minute. If the drop of supernatant collapsed and spread on the oil coated surface, it signifies the presence of biosurfactant (positive). But if the drop remains after one minute, it was documented as negative. This test was simultaneously carried out on distilled water as control (Femi-Ola *et al.*, 2015).

Production of Biosurfactant

Each of the most active purified bacterial isolates was inoculated in 100ml autoclaved nutrient broth containing 1% (v/v) kerosene oil. The broths were then incubated at 37 °C for 48-72 hr. At the end of the incubation period the medium were centrifuged at 6000 rpm for 30 minutes for the removal of cells. The cell free broth (supernatant) was tested for the production and activity of biosurfactant by using ODA test and emulsification test (Ali Diab and Shereen Gamal El Din, 2013; Priya and Usharani, 2009; Sharma *et al.*, 2014).

Extraction of Biosurfactant

After incubation the bacterial cells were removed by centrifugation at 5000 rpm, 4°C for 20 minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2, using 1M H₂SO₄ and the acidified supernatant was left overnight at 4 °C for the complete precipitation of the biosurfactant. After centrifugation, the precipitate was dissolved in a 0.1M NaHCO₃ solution, followed by the biosurfactant extraction step with a solvent having a (2:1) CHCl₃-CH₃OH ratio at room temperature (25-27°C). The organic phase was transferred to a petriplate and left overnight for evaporation. White coloured sediments were obtained as a result i.e., the biosurfactant. Then sterile plates were taken and its weight was measured. Then the sediment was poured on the plates and their weight was measured. The dry weight was calculated using following formula: Dry weight of biosurfactant = weight of the plate after drying - weight of empty plate (Ali Diab and Shereen Gamal El Din, 2013; Sharma *et al.*, 2014).

Characterization of Biosurfactant Thin Layer Chromatography

Preliminary characterization of the biosurfactant was done by TLC method. A portion of the crude biosurfactant was

separated on a silica gel plate using CHCl₃:CH₃OH:H₂O (70:10:0.5, v/v/v) as developing solvent system with different color developing reagents. Ninhydrin reagent (0.5 g ninhydrin in 100 mL anhydrous acetone) was sprayed to detect lipopeptide biosurfactant as red spots (Priya and Usharani, 2009).

Emulsification activity

E24 index is defined as percentage of the height of emulsified layer divided by the height of liquid column. In this method, 2 ml solution of produced biosurfactant and 2 ml of kerosene oil were added to a screw cap tubes and vortex at high speed for 2 min. The mixtures were incubated at room temperature for 24 hours. The emulsification index (E24) was calculated by (Sharma *et al.*, 2014; Ghayyomi Jazeh *et al.*, 2012):

$$E24 = \frac{\text{Height of emulsion formed} \times 100}{\text{Total height of the solution}}$$

RESULTS AND DISCUSSION

Isolation of *Bacillus subtilis* from soil samples

On HiCrome Bacillus agar plates after incubation of 24 hours green and blue color colonies were obtained. The green colored colonies were streaked on HiCrome Bacillus agar slants and from these slants the isolates were further inoculated on nutrient agar slants. Total 10 isolates of *Bacillus subtilis* were obtained from three soil samples.

Identification of *Bacillus subtilis*

MORPHOLOGICAL CHARACTERISTICS

The results of morphological characteristics of all the isolates of *B. subtilis* are given in the following Table 1.

BIOCHEMICAL CHARACTERISTICS

The results biochemical characteristics of all the isolates of *B. subtilis* are given in the following Table 2 and 3.

CULTURAL CHARACTERISTICS

The results of cultural characteristics of all the isolates of *B. subtilis* on HiCrome Bacillus agar and Bacillus differentiation agar are given in the following Table 4 and Fig. 1-6. On the basis of morphological, biochemical and cultural characteristics it was confirmed that all the 10 isolates are of *Bacillus subtilis*.

Screening of isolated *Bacillus subtilis* for Biosurfactant activity

All isolated *Bacillus subtilis* were screened for confirmation of biosurfactant producing ability in which isolates BS1, BS2, BS3, BS4, BS5 BS7 and BS8 showed good results in the following tests:

- **Haemolytic activity:** All 10 isolates of *Bacillus subtilis* showed α -haemolysis on blood agar.
- **Oil displacement assay (ODA):** The isolates which had the ability to displace oil are shown in the following Table 5.

Table No. 1: Morphological characteristics of *Bacillus subtilis*

Bacterial isolates	Gram Staining	Endospore staining	Motility
BS1	Gram Positive, rods	Positive	Motile
BS2	Gram Positive, rods	Positive	Motile
BS3	Gram Positive, rods	Positive	Motile
BS4	Gram Positive, rods	Positive	Motile
BS5	Gram Positive, rods	Positive	Motile
BS6	Gram Positive, rods	Positive	Motile
BS7	Gram Positive, rods	Positive	Motile
BS8	Gram Positive, rods	Positive	Motile
BS9	Gram Positive, rods	Positive	Motile
BS10	Gram Positive, rods	Positive	Motile

*BS – *Bacillus subtilis***Table No. 2: Biochemical characteristics of *Bacillus subtili***

Bacterial isolates	Indole	MR	VP	Citrate	Urease	Catalase	TSI		
							Acid	Gas	H ₂ S
BS1	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
BS2	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
BS3	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
BS4	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
BS5	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
BS6	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
BS7	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
BS8	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
BS9	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
BS10	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve

Table No. 3: Sugar fermentation test of *Bacillus subtilis*

Bacterial isolates	Dextrose		Sucrose		Mannitol		Lactose	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
BS1	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BS2	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BS3	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BS4	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BS5	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BS6	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BS7	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BS8	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BS9	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BS10	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

Table No.4: Cultural characteristics of *B. subtilis* on HiCrome Bacillus agar and Bacillus differentiation agar

Bacterial isolates	HiCrome Bacillus agar		Bacillus differentiation agar	
	Shape, Margin	Colour	Shape, Margin	Colour
BS1	Circular, smooth	Green	Circular, smooth	Yellow
BS2	Circular, smooth	Green	Circular, smooth	Yellow
BS3	Circular, smooth	Green	Circular, smooth	Yellow
BS4	Circular, smooth	Green	Circular, smooth	Yellow
BS5	Circular, smooth	Green	Circular, smooth	Yellow
BS6	Circular, smooth	Green	Circular, smooth	Yellow
BS7	Circular, smooth	Green	Circular, smooth	Yellow
BS8	Circular, smooth	Green	Circular, smooth	Yellow
BS9	Circular, smooth	Green	Circular, smooth	Yellow
BS10	Circular, smooth	Green	Circular, smooth	Yellow

Table No. 5: Observations and results of ODA test

Bacterial isolates	Diameter of clear zone (in mm)	Inference
BS 1	25	POSITIVE
BS 2	13	POSITIVE
BS 3	18	POSITIVE
BS 4	16	POSITIVE
BS 5	9	POSITIVE
BS 6	-	NEGATIVE
BS 7	11	POSITIVE
BS 8	18	POSITIVE
BS 9	-	NEGATIVE
BS 10	-	NEGATIVE

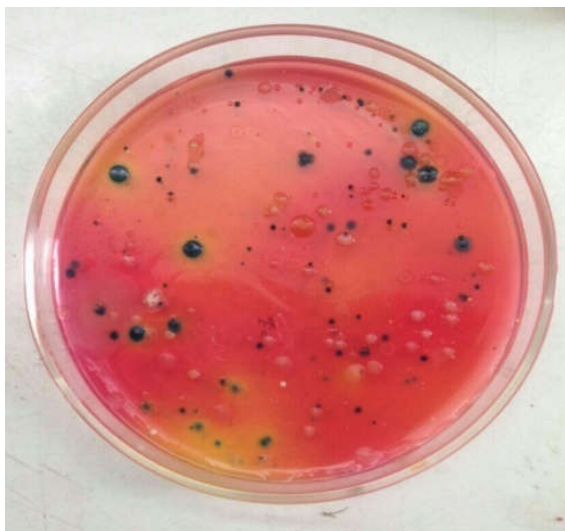


Figure No.1: Isolated colonies of *Bacillus* species on HiCrome *Bacillus* Agar



(A)



(B)

Figure No.3 : A] Nutrient broth before incubation
B] Nutrient broth after incubation

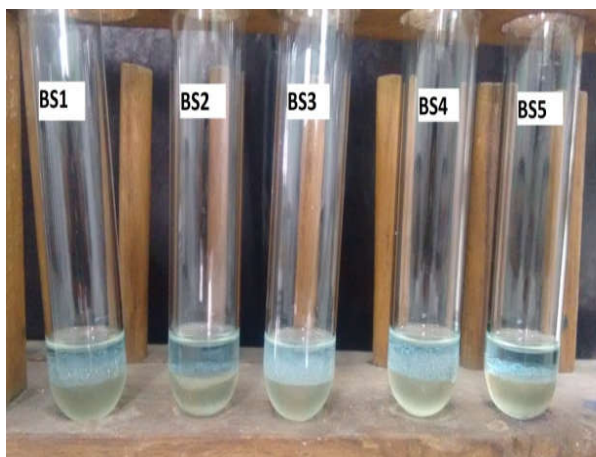


Figure No.2: Results of emulsification assay method

- **Emulsification assay method:** The isolates which had ability to emulsification are shown in the following Table 6.
- **Drop collapse method:** The results of drop collapse method are given in the following Table 7.

Production of Biosurfactant

Isolates BS1, BS2, BS3, BS4 and BS8 were used for production of biosurfactant. Production was done in 100ml

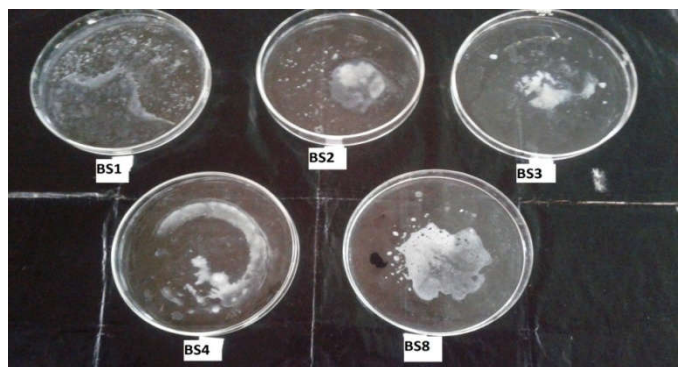


Figure No.4 : Petriplates containing dry biosurfactant

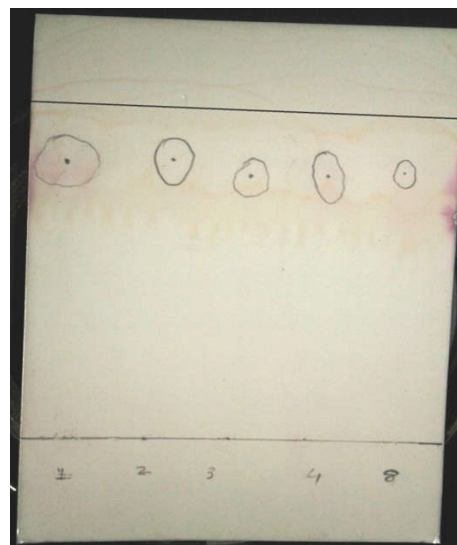


Figure No.5 : Results of TLC

nutrient broth containing 1% (v/v) kerosene oil. After incubation of 48-72 hr, broth was centrifuged to remove cells and supernatant was tested for biosurfactant production by ODA test and emulsification test. All isolates supernatant had given ODA test and emulsification test positive.

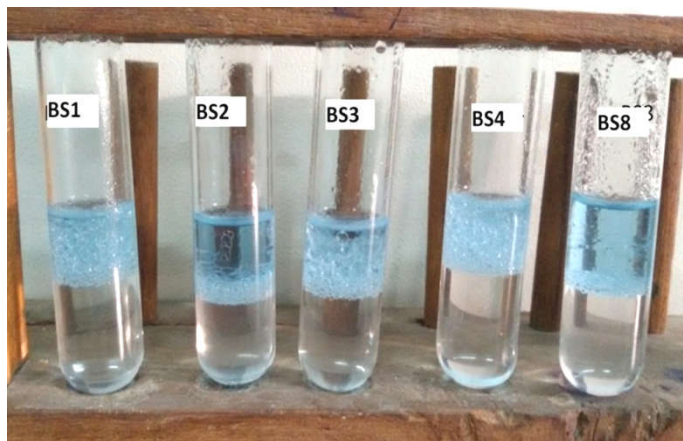


Figure No.6 : Emulsification activity of produced biosurfactants

Table No. 6: Observations and results of Emulsification assay method

Bacterial isolates	Height of emulsion (in cm)	Total height of solution (in cm)	E24%
BS1	0.5	1.3	38.46%
BS 2	0.2	1.3	15.38%
BS 3	0.5	1.4	35.71%
BS 4	0.4	1.3	30.76%
BS 5	0.2	1.3	15.38%
BS 6	-	1.4	-
BS 7	0.2	1.4	14.28%
BS 8	0.3	1.3	23.07%
BS 9	-	1.4	-
BS 10	-	1.5	-

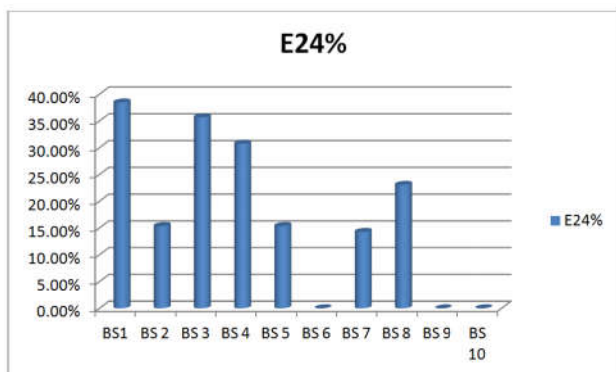


Figure No.7 : Emulsification assay of isolates

Table No. 7 : Results of drop collapse method

Bacterial isolates	Results of drop collapse method
BS1	POSITIVE
BS 2	POSITIVE
BS 3	POSITIVE
BS 4	POSITIVE
BS 5	NEGATIVE
BS 6	NEGATIVE
BS 7	POSITIVE
BS 8	POSITIVE
BS 9	NEGATIVE
BS 10	NEGATIVE

Extraction of Biosurfactant

White coloured sediments were obtained after extraction i.e., the biosurfactant. The dry weight of biosurfactant was calculated by using following formula: Dry weight of

biosurfactant = weight of the plate after drying - weight of empty plate (Table 8).

Table No. 8 : Dry weight of biosurfactants produced by isolates

Bacterial isolates	weight of empty plate (in gm)	weight of the plate after drying (in gm)	Dry weight of biosurfactant (in gm/100ml)
BS1	103.82	103.87	0.05
BS2	87.49	87.51	0.02
BS3	103.36	103.40	0.04
BS4	109.85	109.89	0.04
BS8	98.28	98.31	0.03

Figure No. 8 :Dry weight of biosurfactants produced by isolates

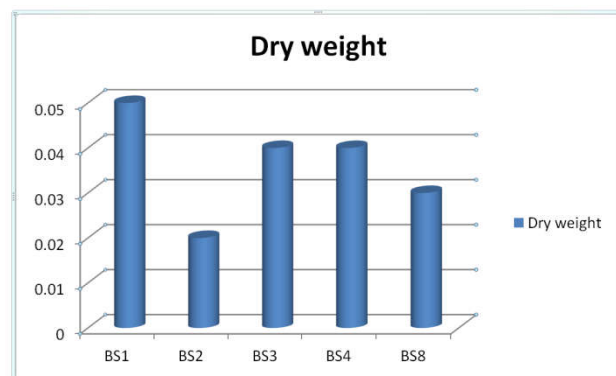


Table No. 9 : Rf values of produced biosurfactants

Bacterial isolates	Distance moved by solvent (in cm)	Distance moved by solute (in cm)	Rf
BS1	7.2	5.3	0.73
BS2	7.2	5.3	0.73
BS3	7.2	5.1	0.70
BS4	7.2	5.2	0.72
BS8	7.2	5.2	0.72

Table No. 10 : Emulsification index of produced biosurfactants

Bacterial isolates	Height of emulsion (in cm)	Total height of solution (in cm)	E24%
BS1	1.3	3.8	34.21%
BS 2	0.7	3.6	19.44%
BS 3	1.2	3.7	32.43%
BS 4	1.2	3.8	31.57%
BS 8	0.6	3.6	16.66%

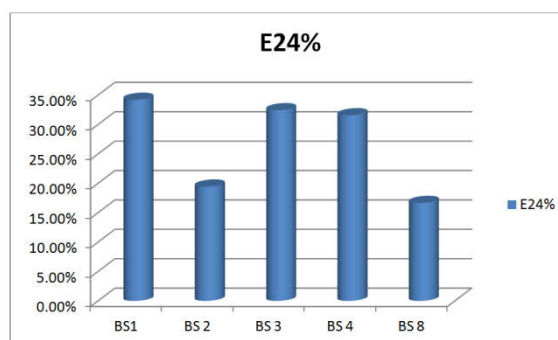


Figure No.9 : Emulsification index of produced biosurfactants

Characterization of Biosurfactant

- **Thin Layer Chromatography:** After spraying ninhydrin, red spots were observed which indicate that all five biosurfactant were lipopeptide biosurfactant (Table 9).

- **Emulsification activity:** Emulsification index of the produced biosurfactant are given in the following Table 10.

In present study, 10 isolates of *Bacillus subtilis* were isolated from three different oil contaminated soil samples. All isolates were Gram positive rods, motile and endospore forming and on the basis of morphological, biochemical, and cultural characteristics it was confirmed that all the isolates are of *Bacillus subtilis*. Then biosurfactant producing *Bacillus subtilis* were screened by haemolytic activity, oil displacement test, emulsification assay method and drop collapse method. All isolates showed α – haemolysis in haemolytic test. In oil displacement test, isolates BS1, BS2, BS3, BS4, BS5, BS7 and BS8 showed zone of diameter 25mm, 13mm, 18mm, 16mm, 9mm, 11mm and 18mm respectively [Table No.7]. The E24 % of BS1, BS2, BS3, BS4, BS5, BS7 and BS8 were 38.46%, 15.38%, 35.71%, 30.76%, 15.38%, 14.28% and 23.07% respectively [Table No.6]. Lastly, drop collapse test were given positive by isolates BS1, BS2, BS3, BS4, BS7 and BS8 [Table No.7].

The isolates BS1, BS2, BS3, BS4 and BS8 of *Bacillus subtilis* were used for production of biosurfactant in nutrient broth containing 1% (v/v) kerosene oil. After incubation, the cell free broths were tested for production by ODA and emulsification test. All isolates supernatant had given ODA test and emulsification test positive. Extraction of biosurfactant was done by acid precipitation and solvent extraction method with chloroform: methanol (2:1). Amount of biosurfactant obtained after production by isolates BS1, BS2, BS3, BS4 and BS8 were 0.05 gm/100ml, 0.02 gm/100ml, 0.04gm/100ml, 0.04 gm/100ml and 0.03 gm/100ml respectively. Biosurfactants were characterized using thin layer chromatography and found to be lipopeptides, as red spots were observed after spraying ninhydrin reagent on silica gel plate. Rf value of biosurfactant produced by isolates BS1, BS2, BS3, BS4 and BS8 are 0.73, 0.73, 0.70, 0.72 and 0.72 respectively. The emulsification activity of produced biosurfactant is calculated by emulsification index (E24). E24% of biosurfactant produced by BS1, BS2, BS3, BS4 and BS8 is 34.21%, 19.44%, 32.43%, 31.57% and 16.66% respectively.

Screening of biosurfactant producing *Bacillus subtilis* was done by haemolytic activity, oil displacement test, emulsification assay method and drop collapse method. These methods were also followed by Anitha *et al.* (2015), Olteanu Violeta *et al.* (2011), Suresh Chander *et al.* (2012), and Okore Chioma *et al.* (2013). In this study, all isolates showed α – haemolysis in haemolytic test. Okore Chioma *et al.* (2013) found that out of 9 isolates of both *Bacillus* and *Pseudomonas* 7 isolates showed α – haemolysis and 2 isolates showed β – haemolysis. β – haemolysis is one of the characteristics also of biosurfactant producing microorganisms. In this study, the zone produced by BS1, BS2, BS3, BS4, BS5, BS7 and BS8 isolates of *Bacillus subtilis* in ODA test had diameter of 25mm, 13mm, 18mm, 16mm, 9mm, 11mm and 18mm respectively [Table No.7]. Similarly, Olteanu violeta *et al.* (2011) obtained best results of zone diameter, 28 mm and 30 mm for B2 and OS17 strains of *Bacillus subtilis* respectively on kerosene (Olteanu Violeta *et al.*, 2011). In studies done by, Suganya (2013), and Suresh Chander *et al.* (2012) *Bacillus subtilis* showed zone of 17mm, 3.2 mm, on kerosene oil and 2.1 cm on gingelly oil, respectively. In study of Anayata

Sharma *et al.* (2014), strain B1 and B2 of *Bacillus* showed zone of 1cm and 2cm respectively. In this study, out of 10 isolates of *Bacillus subtilis* seven showed emulsification activity against kerosene oil. The E24 % of BS1, BS2, BS3, BS4, BS5, BS7 and BS8 were 38.46%, 15.38%, 35.71%, 30.76%, 15.38%, 14.28% and 23.07% respectively [Table No.6]. In the study of Femi-Ola *et al.* (2015) *Bacillus* showed emulsification capacity 51.61%. *Bacillus subtilis* MTCC441 in Suresh Chander *et al.* (2012) study also showed emulsification activity against kerosene oil. In this study, drop collapse test were given positive by isolates BS1, BS2, BS3, BS4, BS7 and BS8. Femi-Ola *et al.* (2015) also found drop collapse test positive for *Bacillus spp.*

In the present study, production of biosurfactant was done in nutrient broth containing 1% (v/v) kerosene oil and after incubation cell free broths were tested for production by ODA and emulsification test. Similar was followed by Anayata Sharma *et al.* (2014), Priya *et al.* (2009) and Ali Diab *et al.* (2013). And also, ODA and emulsification test were positive indicating that biosurfactant was produced. Extraction of biosurfactant was done by acid precipitation and solvent extraction method with chloroform: methanol (2:1). Similarly, Anayata Sharma *et al.* (2014), J.Anitha *et al.* (2015), Arutchelvi *et al.* (2009), Dhouha Ghribi *et al.* (2011), Priya *et al.* (2009), Olteanu Violeta *et al.* (2011), Suganya (2013), and Ali Diab *et al.* (2013) also used these methods for extraction of biosurfactant. In this study, the amount of biosurfactant produced by BS1, BS2, BS3, BS4 and BS8 are 0.05 gm/100ml, 0.02 gm/100ml, 0.04gm/100ml, 0.04gm/100ml and 0.03 gm/100ml respectively [Table No.8]. Similarly, in the study of Anayata Sharma *et al.* (2014) the amount of biosurfactant produced by isolates B1, B2 and B3 of *Bacillus spp.* were 0.125g, 0.105g and 0.14g respectively. In study of Arutchelvi *et al.* (2009), *Bacillus subtilis* was capable of producing 0.5 g L⁻¹ of crude biosurfactant (dry weight) in the mineral salt medium. After characterization of biosurfactants, produced by isolates BS1, BS2, BS3, BS4 and BS8 by TLC, it was found that they were lipopeptide in nature. Similar result was obtained by Anitha *et al.* (2015), Priya *et al.* (2009), Olteanu Violeta *et al.* (2011), Samadhan Waghmode *et al.* (2014) and Okore Chioma *et al.* (2013) in their studies. Rf values of biosurfactant produced by isolates BS1, BS2, BS3, BS4 and BS8 on kerosene are 0.73, 0.73, 0.70, 0.72 and 0.72 respectively. Priya *et al.* (2009) obtained Rf value of 0.51 for biosurfactant produced by *Bacillus subtilis* on kerosene. The Rf value of biosurfactant produced by *Bacillus subtilis* on substrates coconut waste, soyabean waste, basal medium, and sesame waste in Samadhan Waghmode *et al.* (2014) study were 0.33, 0.25, 0.21 and 0.22 respectively.

The emulsification activity of produced biosurfactant is calculated by emulsification index (E24).E24% of biosurfactant produced by BS1, BS2, BS3, BS4 and BS8 are 34.21%, 19.44%, 32.43%, 31.57% and 16.66% respectively [Table No.10]. This test was also followed by Anayata Sharma *et al.* (2014), Anitha *et al.* (2015), Juliana F.F. Secato *et al.* (2016), Olteanu Violeta *et al.* (2011), and Ali Diab *et al.* (2013). Olteanu Violeta *et al.* (2011) strains B2 and OS17 of *Bacillus subtilis* had showed best emulsification activity against kerosene oil. E24% of B2 and OS17 against kerosene oil was 48.75% and 51.25%, respectively. In study of Juliana F.F. Secato *et al.* (2016), the biosurfactant produced using 10% of inoculum showed emulsification index of 75% and 83% for soy oil and motor oil, respectively.

Conclusion

This study concludes that *Bacillus subtilis* is the potent producer of lipopeptide biosurfactant and can be used as a culture for large scale production of biosurfactant. These biosurfactant are also a potent emulsifier. So, that it has wide applications in oil, food, pharmaceuticals and cosmetic industries. It can also be used in bioremediation and microbial enhanced oil recovery.

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