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Production of Biosurfactants by two different Isolates of *Bacillus* species

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Abstract : *To assess the potential of the two different isolates of Bacillus species (ST-1 and ST-2) isolated from oil contaminated soil and chicken farm to produce biosurfactant as the aim of this work. ST-1 was isolated from chicken farm and ST-2 was isolated from oil contaminated soil. 10% v/v and 20% v/v inoculums were used. The ability to produce biosurfactant was examined by oil spreading technique, emulsification activity and emulsification stability test using different types of oils (almond oil, olive oil, coconut oil and kerosene). Difference in the ability to emulsify oil were observed among the bacterial isolates. The best results were obtained with ST-2(20%) for all type of oils and the maximum emulsifying acivity was recorded on kerosene. The microbial biosurfactant was extracted and detected by Thin Layer Chromatography (TLC). Antifungal activity of bacterial isolates were also tested to know that both or any of these two strains posses the antifungal activity. The result show no antifungal activity in any of the two isolates.*

Key words: *Bacillus, biosurfactant, emulsification, substrate.*

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Introduction :

Biosurfactants are amphiphilic compounds produced by microorganisms with pronounced surface and emulsifying activities (Singh *et al.*, 2007). Biosurfactants can be divided into 4 groups base on their overall structures. They are glycolipids, phospholipids, lipoproteins or lipopeptides and polymeric (Healy *et al.*, 1996). Microbial surfactants comprise a diverse group of surface-active molecules which are categorized by their chemical composition and microbial origin. They include glycolipids, lipopeptides, polysaccharide–protein complexes, protein-like

substances, lipopolysaccharides, phospholipids, fatty acids and neutral lipids (van Hamme and Ward 1999). Therefore, it is reasonable to expect diverse properties and physiological functions of biosurfactants such as increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation (Singh and Cameotra, 2004).

Biosurfactants are microbial surface-active compounds. They acquired their importance from their use in several industrial fields such as pharmaceutical petroleum and food industries. Biosurfactants have many properties such as soaping, emulsifying, foaming, dispersing oil (Rahman and Gakpe 2008). They are widely used in industrial and environmental fields such as microbially enhanced oil recovery (MEOR), oil tanks cleaning and bioremediation of oil polluted water and soil (Desai and Banat, 1997). The potential applications of biosurfactants in industrial include emulsification and foaming for food processing, wetting and phase dispersion for cosmetics and textiles, or solubilization for agrochemicals (Lin, 1996). In addition, biosurfactants can be use in environmental applications such as bioremediation and dispersion of oil spills (Desai and Banat, 1997).

Most of the surfactants used today are chemically synthesized. Many recent studies are focused on the use of microorganisms in the production of biosurfactants (bioemulsifier). Biosurfactants are more active and less toxic than chemical surfactants which are difficult to remove or degrade from the environment (Desai and Banat, 1997). Biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil (Kosaric, 2001).

Several *Bacillus* species produce a lipopeptide biosurfactant; the most important one is surfactin which is produced from *Bacillus subtilis* (Nakano and Zuber, 1989). Surfactin is not ribosomally synthesized; it is synthesized by a multi functional enzyme system as that involved in the synthesis of the peptide antibiotics released from bacilli bacteria (Nakano and Zuber, 1989). Moreover *B. licheniformis* has the ability to produce many surface active lipopeptides (Cooper and Goldenberg, 1987).

Materials and Methods:

Sample collection : Soil sample was collected from two different areas in Patna. Soil sample 1 was collected from chicken farm near Sultanganj and soil sample 2 was collected from car repairing garage near Patna High Court.

Isolation : Direct isolation of the microorganisms was carried out using serial dilution on (up to 10-7) of soil samples in 0.85 % sterile saline. The spreading method was done on nutrient agar plates and incubation was done at 30°C for 24 hours. Morphologically distinct colonies were isolated and purified by replicating on the same solid medium to obtain pure cultures (Samadi *et al.*, 2007).

Identification : Various biochemical test were performed in order to identify and differentiate the two isolates of bacteria (ST-1 and ST-2) such as gram staining, IMViC, motility, gelatin, litmus milk test. (Nasr *et al.*, 2009).

Antifungal activity of bacterial isolates: Antifungal property was examined by dual culture technique. Agar blocks (5mm dia.) containing 5-day-old mycelia were placed in the middle of PDA containing Petri plates. It was incubation for 24 hrs at 28^0C. One loop of each bacterial culture was placed at 2.5 cm from the edge of fungal colony. Inhibition of mycelia growth was evaluated after incubation at 28 ^{0}C for 5 days (Olteanu et al., 2011).

Biosurfactant production: Bacterial strains were inoculated in 100 ml of nutrient broth in 250 ml of glass flask and incubated in shaker incubator, 200 rpm at 30^{0}C for 16-18 hours. 10%(v/v) and 20%(v/v) inoculum was inoculated in 100 ml of Mckeen medium in a 250 ml of glass flask and incubated in rotator shaker, 190 rpm at 30^{0}C for 96 hrs.(Phitnaree *et al*., 2008).

Extraction of biosurfactants: After 96 hours of cultivation, the bacterial cells were removed from 20ml culture broth by centrifugation at 5000 rpm for 10 mins. The pellets were obtained by centrifugation of the supernatant at 7000 rpm for 10mins (Phitnaree *et al*., 2008) and analyzed by TLC method. (Lee *et al*.,2006).

Oil spreading technique: 40 ml of distilled water was added to a large petridish and 50 ml of oil (sunflower oil, olive oil, kerosene) was added to the surface of water. 10 ml supernatant was added to the surface of oil. Occurrence of clear zone was an indication of biosurfactant productions. The diameter of clear zone on the oil surface was measured and compared to 10 ml of distilled water as negative control. (Nasr *et al*., 2009).

Emulsification activity : It was done by measuring the optical density at 540 nm using spectrophotometer (Patel and Desai, 1997) and measuring the emulsion stability after 24 hrs by calculating emulsification index. (Dehghan-Noudeh *et al*., 2005).

In first method: 2ml samples of cell free supernatant were added in the tubes containing 2 ml distilled water and the solution was mixed with 1ml of substrate (olive oil, almond oil, coconut oil and kerosene). After vigorous vortex for 2mins, the tubes were allowed to set for 1 hour to separate aqueous and oil phase. Before measuring the OD of the aqueous phase at 540 nm and comparison was done with the negative control (uninoculated broth). (Lee et al., 2006).

In the second method: 2ml samples of the cell free supernatant and 2ml of oil were added to the tubes and vortex at high speed for 2mins. The mixture was incubated at room temperature for 24 hours (Priya and Usharani, 2009). The emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixtures total height and multiply by 100 (Olteanu *et al*., 2011).

Results and Discussion:

Identification of bacterial isolates: The bacterial isolates were isolated from oil contaminated soil (ST-1) and chicken farm (ST-2). Staining technique, biochemical test were performed in order to identify bacterial isolates. The isolates were found to be gram-positive, rod shaped.

Table 1. Biochemical characterization of bacterial isolates

Characteristics	ST-1	ST2
Cell morphology	bacilli	bacilli
Gram reaction	+	+
Voges-prokauer	+	–
Litmus milk test	+	–
Gelatin	+	–
Citrate	+	–
Indole	+	+
Motility	+	–

On the basis of the morphological and biochemical characterization the isolates were identified as two different ***Bacillus* species.** Similar result was found by Nasr *et al*., (2006). He found similar biochemical characteristics of his isolated ***Bacillus* species**.

Antifungal properties: Inhibition of the growth of three fungal strains (***Aspergillus niger, Aspergillus flavus, Cirvularia***) by bacterial isolates was tested on PDA media. No inhibition zone was observed against these three fungal

strains by any of the strains. It is well known that the antifungal action of several bacilli is due mainly to the production of different lipopeptide, like iturin, surfactin, lichenysins, mycosubtilin etc. For this reason, the ability of the new bacterial isolates to produce compounds was checked. In the work carried out by Olteanu *et al*. (2011), he isolated six ***bacillus*** strains (B1, B2, BW, BS, OS15 and OS17), strains B1,B2,BW and OS17 inhibited the growth of the fungal species (***Fusarium*** spp., ***Sclerotium bataticola, Rhizoctonia solani*** and ***Pythium*** spp.).

Biosurfactant production: For biosurfactant biosynthesis, the bacterial isolates were cultivated on Mckeen medium containing 2.5% glucose at 30 ^{0}C in rotary shaker at 190rpm for 96 hrs. The bacterial growth was evaluated by measuring OD at 540 nm. Maximum growth level was found for ST-2(20%). According to the study of Olteanu et al. (2011), it was shown that glucose increased both the biomass accumulation and biosurfactant production. Maximum growth level was found for B1, BS, BW, and OS17 after 84 hours of incubation.

Oil spreading technique: In order to detect the biosurfactant biosynthesis, oil spread technique was used. As substrate, several types of oil were used (almond oil, olive oil, coconut oil and kerosene). The ability of displacing the oil and spreading in the water was observed among the two strains tested.

Table 2. Oil spreading technique for bacterial isolates tested

Bacterial isolates:	Zone formation (mm) Olive oil	Almond oil	Coconut oil	Kerosene
ST-1 :				
10%	3	6	8	17
20%	3	7	9	19
ST-2 :				
10%	4	7	10	13
20%	4	8	11	25

The best result was obtained with the ST-2 (20%) for all types of oils. But the maximum action was recorded on kerosene (zone formation of 25 mm). It was reported by Olteanu et al, (2011) research that several biosurfactants had the ability to emulsify crude oil and vegetable oils. The strains B2 and OS17 for all type of oils, but the maximum action was recorded on kerosene (zone formation of 28 mm and 30 mm, respectively).

Emulsification activity: Emulsification activity of the biosurfactant produced was tested by two methods:

The OD at 540 nm of supernatants with different substrates (almond oil, olive oil, coconut oil, kerosene) was measured using spectrophotometer. The highest emulsification activity was detected in ST-2 (20%) against all type of substrates and has maximum emulsification activity against kerosene which gave it an importance in bioremediation of oil pollution. According to Olteanu *et al*, (2011) research, the highest emulsification activity was detected in B2 and OS17 against all types of substrates. These bacterial strains have on increased emulsification activity against kerosene (0.391 nm and 0.441nm respectively).

Table 3. Emulsification activity of biosurfactant from bacterial isolates tested

Bacterial isolates:	Emulsification activity at 540 nm Olive oil	Almond oil	Coconut oil	Kerosene oil
ST-1:				
10%	1.107	1.105	0.837	0.328
20%	1.101	0.996	0.826	0.318
ST-2:				
10%	1.275	1.228	0.736	0.467
20%	1.282	1.239	0.748	0.478

The emulsification stability (E_{24}) is another characteristic of biosurfactant and it was evaluated against the same substrate. The highest E_{24} value

was observed in ST-2 (20%) (41% against kerosene). According to the study Olteanu *et al*. (2011), the measurement of emulsion stability of supernatants with the substrates, after 24 hours, the highest E24 value was observed in B2 (48.75% against kerosene).

Table 4. Emulsification stability test for bacterial isolates tested

Bacterial isolates:	Emulsification activity at 540 nm Olive oil	Almond oil	Coconut oil	Kerosene oil
ST-1:				
10%	27.0	29.7	32.0	36.0
20%	27.7	30.2	32.8	36.9
ST-2:				
10%	30.0	33.8	37.8	40.2
20%	30.7	34.5	38.6	41.1

Analysis of biosurfactant: Crude biosurfactants extracted were analyzed by thin layer chromatography method. Surfactin from bacterial isolates ST-1 and ST-2 was placed on silica gel plate and identified by red colour spot after spraying with ninhydrin reagent.ST-2 produced more biosurfactant than ST-1. According to the work carried out by Olteanu *et al*. (2011), the presence of lipopeptides observed as red spot after spraying with ninhydrin reagent, the compounds produced by B2 and OS17. Similar result were found by Fernandes *et al*. (2007) and Priya and Usharani *et al*. (2009).

Conclusion:

The result obtained in the study conducted allow several conclusions:

On the basis of staining technique and biochemical tests performed, the bacterial isolates were identified as two different ***Bacillus*** species.

No inhibition zone was found against the three fungal strains (***Aspergillus niger, Aspergillus flavus, Cirvularia)*** by any of the bacterial isolates. Hence both the bacterial isolates do not have antifungal activity.

The ability of *Bacillus* species for biosurfactant production by two different isolates of bacteria (ST-1 and ST-2) was screened by oil spreading technique, emulsification activity and emulsification stability (E_{24} value) . The best result was obtained with ST-2(20%) for all types of oils and the maximum reaction was recorded on Kerosene.

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References:

Cooper DG and Goldenberg BG (1987). Surface –active gents from two *Bacillus species*. Appl. Environ. Microbial. 53:224-229.

Dehghan-Noudeh G, Housaindokht M, Bazzaz BS, (2005). Isolation, characterization, and hemolytic activities of a lipopeptide biosurfactant produced by *Bacillus subtilis* ATCC 6633. J. Microbial., vol.43, nr.3 (pp. 272-276).

Desai JD and Banat IM (1997). Microbial production of biosurfactants and their commercial Potential. Microbial Mol Biol Rev 61:47-64.

Fernandes PAV, Arruda IR, Santos AFAB, Araujo AA, Maior AAS, Ximenes EA (2007). Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug-resistant bacteria Brazilian J. Microbial., 38: 704-709.

Healy MG, Devine CM, Murphy R (1996). Microbial production of biosurfactants. Resource Conserve Recycling 18:41–57.

Kosaric N (2001). Biosurfactants for soil bioremediation. Food Technol. Biotechnol. 39 (4) 295-304.

Lee SC, JU-Soon Y, Sun-Hee K, Soo-Yeol C, Cher-Won H, Woo-Hong J, Yong-Lark, (2006). Production and characterization of lipopeptide biosurfactant from *Bacillus subtilis* A8-8. J. Microbiol. Biotechnol 716-723.

Lin SC (1996). Biosurfactants: recents advances. J Chem Technol Biotechnol 66:109–120.

Nakano MM, Zuber P (1989). *srfA* is an operon required for surfactin production, competence development, and efficient sporulation in *Bacillus subtilis*. J Bacterial 173:1770–1778.

Nasr S, Soudi MR, Mehrnia MR, Sarrafzadeh MH (2009). Characterization of novel biosurfactant producing strains of *Bacillus* spp. isolated from petroleum contaminated soil. Iranian J. Microbial., 1 (2), 54-61.

Olteanu V, Sicuia O, Ciuca M, Cârstea DM, Voaides C, Câmpeanu G, Cornea CP (2011). Production of biosurfactants and antifungal compounds by new strains of *Bacillus* spp. isolated from different sources. Romanian Biotechnol. Let., vol.16, nr.1 (pp. 84-91).

Patel RM, Desai AJ (1997). Surface active properties of rhamnolipids from *Pseudomonas aeruginosa GS3.* J. Basic Microbial., vol.37, nr. 4 (pp. 281-286).

Phitnaree S, Vatcharin R, Furusako K, Aran HK (2008). Production and applications of biosurfactan from *Bacillus subtilis* MUV4. Songklanakarin J. Sci. Technol., 30 (1), 87-93.

Priya T, Usharani G (2009). Comparative study for biosurfactant production by using *Bacillus subtilis* and *Pseudomonas aeruginosa*. Botany Research International. 2 (4), 284-287.

Rahman KSM and Gakpe E (2008). Production, characterization and applications of biosurfactants- Review. Biotechnology 7: 360-370.

Samadi N, Abadian N, Akhavan A, Fazeli MR, Tahzibi A and Jamalifar H (2007). Biosurfactant production by the strain isolation from contamination soil. J Biol Sci 7:1266-1269.

Singh A, Hamme JD and Ward OP (2007). Surfactants in microbiology and biotechnology. Part 2. Application aspects. Biotechnol. Adv., 25: 99-121.

Singh P and Cameotra SS (2004). Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol., 22: 142-146.

Van Hamme J, and Ward OP (1999). Influence of chemical surfactants on the biodegradation of crude oil by a mixed bacterial culture. Can. J. Microbial. 45:130-137.