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ORIGINAL ARTICLE

Antibacterial Activity of Biosurfactant Produced by *Bacillus subtilis* LB3

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ABSTRACT
Biosurfactants (Bs) are amphiphilic molecules with hydrophilic and hydrophobic moieties which facilitate their easy diffusion and hence have various applications in different fields. Among 21 strains isolated from oil industrysoil.12 were selected based on biochemical characterization for screening of BS production by using hemolytic assay, lipase assay, oil displacement test, emulsification index and surface tension measurement. The isolate LB3 has shown significant reduction in surface tension (36.8 mN/m), emulsification index (70.83%), oil displacement (13.84 cm²), lipase activity (13 mm) and hemolysis activity (15 mm) hence was identified at molecular levels as Bacillus subtilis LB3. BS production from selected isolate was further tested in different media like LB, NB. MSM and MSMG. From which MSMG was found to be suitable medium based on surface tension reduction 25 mN/m antibacterial activity against K. pneumoniae, V. harveyi and V. parahaemolyticus. Production of BS was carried out in MSMG medium and purified by acid precipitation, solvent extraction and silica gel column chromatography. Purified BS was characterized by FTIR as lipopeptide. The BS has shown 2 µg/ml and 0.2 µg/ml MBC against V. parahaemolyticus and K. pneumoniae respectively as per visual observations. The produced BS from Bacillus subtilis LB3 has shown significant surface activity and also antibacterial activity hence can act as potential candidate for biomedical application.
Keywords*: Biosurfactant, Bacillus subtilis LB3, MSM, glucose, Antibacterial activity, MBC.*

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INTRODUCTION

Bacteria are the ancient and most wide spreaded organisms on earth. They are varied, versatile, and are commensal to all mammals. They can be both crucial and detrimental to health, depending on host interactions. Although certain microbes can be mutualistic, a large number of bacteria are pathogenic and can cause a multiple life-threatening infectious diseases [1]. The discovery of penicillin nearly 90 years ago revolutionized the treatment of bacterial disease. Since that time, numerous other antibiotics have been discovered from bacteria and fungi, or developed by chemical synthesis and have become effective chemotherapeutic options. The improper and misuse of antibiotics has resulted in the widespread development of resistance in many bacterial species[2]. Simultaneously, the rate of discovery of antimicrobial agents has steadily decreased in last few decades while antibiotic resistance among bacterial species has increased rapidly, creating multi-resistant organisms. The diseases caused by these organisms have become difficult to manage with the current antibiotic treatment regimens[3, 4]. The development of alternative treatment methods is crucial and considered by WHO to be perhaps the biggest challenge facing medical science [5].

BS's are amphipathic molecules with a hydrophilic and hydrophobic domain which facilitates the uptake of hydrocarbons into cells. Because of these traits, BS's accumulate at interfaces, lowering the surface tension and thereby enhancing the solubility of poorly soluble compounds in water [6].From the past few decades microbially produced BS have gained more attention due to their several advantages over their chemical counterparts such as biodegradability, lower toxicity, ecological acceptability, bioavailability, specificity and ability to be produced from renewable and cheaper substrates [7, 8]. Due to structure activity related properties of BS interests has been focused on biomedical applications of BS's such as

antiviral, antimicrobial, antitumor and antibiofilm properties [9, 10]. Various microbes are known to produce different kinds of BS among which lipopeptide produced by Bacillus sp are well known BS for their extensive applications in agriculture, environmental and biomedical applications. Since, products from Bacillus sp are in the GRAS list (generally regarded as safe) of US Food and Drug Administration (USFDA) and hence are regarded harmless [11]. The study was designed to isolate, and screen BS producing Bacillus sp and antibacterial activity of produced BS.

MATERIAL AND METHODS

Sample collection

Soil sample from oil industry was collected from Nanded district (Lat 19.159500'N and 77.310900 'E) Maharashtra, India by composite random sampling method. Sample was collected in sterile polythene bag and stored at 4^{o}C till further use.

Pretreatment of soil sample for isolation of bacillus sp

One gm of soil sample was suspended in 10ml of sterile physiological saline and pretreated with heat and antibiotics (nalidixic acid, streptomycin and tetracycline 50µg/ml) both individually and in different combinations for isolation of Bacillus sp. Pretreated samples were serially diluted and 0.1 ml of 10^{-4}-10^{-8} dilutions were spread on Luria-Berttannii (LB), Bacillus isolation (BIA) and Bacillus differentiation agar (BDA) plates and incubated at 37^{0}C until individual colonies were observed. After incubation, well isolated morphologically distinct colonies were selected for screening.

Biochemical identification for Bacillus sp

All isolates were identified for Bacillus sp by morphological and biochemical characterization. The morphological and biochemical characterization was done by the standard criteria given in Bergey's manual of determinative bacteriology [12].

Screening of isolates for BS production potential

BS production ability of all selected isolates was tested by hemolytic assay[13] and lipase activity[14]. Further screening was carried out by oil displacement, emulsification index and reduction in surface tension.

To perform further screening isolates were grown in MSM (g/l: $NaNO_3$-2.5, NaCl-1.0, KCl-1.0, $MgSO_4$-0.4, $CaCl_2$-0.01 and phosphoric acid (85%)-10ml. After autoclaving 1ml/l of sterile trace solution (g/l: $FeSO_4$-0.5, $ZnSO_4$-1.5, $MnSO_4$-1.5, H_3BO_3-0.3, $CuSO_4$-0.15, Na_2MoO_4-1.0)) at 37□ for 96 h. After incubation cells were separated by centrifugation and cell free broth was used as a source of crude BS. All the tests were performed in triplicate by using SDS (1%) as positive and distilled water as a negative control.

Oil displacement test

Crude oil (1000µl) was layered onto the surface of 40ml of distilled water in a petriplate to form a thin layer. 10µl of cell free supernatant and 10µl of each control (negative control: distilled water, positive control: 1% SDS and MSM uninoculated broth as blank) were added to the center of the formed oil layer. Presence of BS in the cell free supernatant is determined by the displacementof oil forming clear zone and diameter of this clearing zone indicates the surfactant activity[15]. Area displaced in presence of BS was calculated by using formula

$$\text{Area} = \pi r^2$$

Where, r -radius and πr- circumference of circular area.

Emulsification index (E_{24})

E_{24} was measured using the method described by Foukia et al., [16]. BS activity was measured by adding 1ml of soyabean oil to 1ml of cell-free supernatant. The mixture was vortexed at high speed for 2 min. The height of emulsion was measured by taking the layer formed in between aqueous and oil layer after 24h. Emulsions formed by the isolates were compared to those formed by distilled water as negative control and 1%SDS as a positive control. E_{24} was determined using the following formula.

$$\text{Emulsification index (\%)} = (\text{Height of emulsification layer} \div \text{Total Height}) \times 100$$

Surface tension measurement

Production of BS production was measured in terms of reduction in surface tension. Surface tension measurement was carried out by drop weight method using stalagnometer [17]. Initially surface tension of water and 1% SDS was determined at 30°C temperature. Surface tension was calculated by using formula

$$\sigma = \sigma_{H2O} \times m + m_{H2O}$$

Where, σ- Surface tension, σ_{H2O}- Surface tension of water (72.8mN/m), m- mass of water in presence of surfactant and m_{H2O}- mass of water in absence of surfactant.

Antimicrobial activity of BS produced by LB3

Antimicrobial activity of crude BS of LB3 was evaluated using agar well diffusion method [18]. Test organisms used for study were*Klebsiella pnemoniae* (MTCC 2453)*, Vibrio parahaemolyticus* (MTCC 451) and*Vibrio harveyi* (MTCC 7954). Test cultures were grown overnight in Mueller-Hinton broth (MHB) and the $O.D_{600}$ was adjusted to 0.5. The test cultures (0.1ml) were spreaded on the surface of solidified sterile MH agar medium by using alcohol sterilized glass spreader. Wells were punctured at the centre of agar plate using sterile borer (4.0 mm) to which 30µl of crude BS was added. Streptomycin (50µg/ml) and distilled water were used as positive and negative controls respectively. All the plates were incubated at 37□ for 24h. The presence of clear zone around the well was considered as antimicrobial activity of BS.

Selection of potential isolate

Based on BS production potential and antimicrobial activity studies isolate LB3 was selected as potential producer strain.

Identification of potential BS producer strain

Isolate LB3 showing maximum BS production and antimicrobial activity against test pathogens was identified at molecular level by 16S rRNA sequencing. The DNA was isolated from the LB3 cells by using spin column kit (HiMedia, India Bacterial 16S rRNA gene (1500bp)[19] and was amplified using polymerase chain reaction in a thermal cycler. The amplification conditions were 94^0C for3min, 52^0C for45s,72^0C for1minand final extension step of 72oC for3min. The PCR product was purified by Exonuclease I-Shrimp Alkaline Phosphatase (Exo-SAP) [20]and sequenced by using ABI 3730x1 Genetic Analyzer.Purified amplicons were sequenced by Sanger methodin ABI 3500xL genetic analyzer (Life Technologies, USA).Sequencing files (.ab1) were edited using CHROMASLITE(version 1.5) and further analyzed by Basic Local Alignment Search Tool(BLAST) with closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database that finds regions of local similarity between sequences[21]. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches [22]. The BLAST algorithms used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. Multiple alignments of these sequences were carried out by ClustalW 1.83 version of EBI (www.ebi.ac.uk/cgi-bin/ClustalW/) with 0.5 transition weight. The evolutionary distances were computed using maximum composite likelihood method[23] and all positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons. The phylogenetic tree was constructed in MEGA7 version (www.megasoftware.com) using neighbor joining (NJ), minimum evolution (ME) and up weighted pair group method with arithmetic mean algorithms (UPGMA)[24]. The tree was constructed by using Kimura 2 parameter model[25]. The sequence data of the representative strain was deposited in GenBank under the accession number **MN994288.**

Effect of different media composition on BS production and antibacterial activity by strain LB3

BS production by strain LB3 was assessed in presence of NB, LB broth, MSM (supplemented with 2% soyabean oil)and MSMG (MSM supplemented with 1% glucose) at pH 7.0 with 2% inoculum. All flaskswere incubated on a rotary shaking incubator at 37^0C and 120rpm.The medium showing maximum reduction in surface tension and promising antibacterial activity was selected and used for production.

Production and purification of BS

Active culture of strain LB3 was inoculated in 500 ml MSMG(g/l): $NaNO_3$-2.5, NaCl-1.0, KCl-1.0, $MgSO_4$-0.4, $CaCl_2$-0.01, glucose-1% and phosphoric acid (85%)-10 ml. After autoclaving 1 ml/l of sterile trace solution (g/l: $FeSO_4$-0.5, $ZnSO_4$-1.5, $MnSO_4$-1.5, H_3BO_3-0.3, $CuSO_4$-0.15, Na_2MoO_4-1.0) was added and pH was adjusted to 7.0, incubated at 37□, 120rpm for 96h.The bacterial cells were removed by centrifugation at 10,000rpm for 20min. The collected supernatant was acidified to pH 2.0 using 6N HCl for 24h at 4□.Pellets were collected by centrifugation, suspended in buffer of pH 7.0 and extracted with equal volumes of ethyl acetatein a separating funnel;organic phase was collectedand evaporated at 40□. The partially purified BS was further purified by silica gel column chromatography. Dried extract of BS was loaded on silica gel column (80-120 mesh) and fractions were collected in gradients of chloroform and methanol. All collected fractions were analyzed for BS activity by emulsification index and antimicrobial activity against *Klebsiella pneumoniae*; fractions showing higher activity were pooled together, evaporated and used for further analysis.

Characterization of purified BS by FTIR

To determine the chemical structure and components of the extracted BS, FTIR spectroscopic analysis (Shimadzu, Japan) was performed. Samples were prepared by homogeneous dispersal of 1mg of BS sample in pellets of potassium bromide. Infra-red (IR) absorption spectrum was obtained using a built-in plotter. The IR spectrum was obtained in the range of 450-3500cm^{-1} with a resolution of 4cm^{-1}[26].

Minimum bactericidal concentration(MBC) determination

The MBC value of a particular antimicrobial agent is either equal or greater than its MIC value for a particular microbial strain. A standard method namely, broth micro dilution susceptibility assay was used for the determination of MBC for the test organisms *viz, Klebsiella pneumoniae* and *Vibrio harveyi*. MBC was determined by using alamer blue dye as an MBC. 2000µg/ml to 0.02µg/ml dilutions were prepared by serial dilution method for BS which was dissolved in DMSO. 100µl of respective culture was added to wells and incubated at 37℃ for 24h. After incubation, 100µl of 1mg/ml alamer blue dye was added to each well and incubated at room temperature for 4h or until pink color development. The concentration showing development of pink color was considered as MBC.

RESULTS AND DISCUSSION

Isolation and screeningof BS producing Bacillus sp

In natural environments, microbes occur almost always in a mixed population composed of different strains and species. For isolation of pure culture of defined organism out of such mixed population, enrichment cultures with hydrophobic substrates or pretreatment of collected samples before isolation are very promising for isolation of BS producing microbes [27]. From pretreated oil industry sample 21 colonies were selected, 4 from nalidixic acid treatment (each 2 on LB and BIA), 8 from heat treatment (2 on BIA, 5 on LB and 1 on BDA), 6 from antibiotic treatment (2 on BIA, 3 on LB and 1 on BDA) and 3 from combined antibiotics and heat treated sample (1 on each BIA, LB and BDA) (Table-1; Fig. 1).

Table.1: Isolation of bacteria from oil industry soil

Media used / Pre-Treatments	Bacillus differentiation agar	Luria-Berttannii Broth	Bacillus isolation agar
Nalidixic acid	-	2	2
Heat treatment	2	5	1
Combined antibiotic treatment	2	3	1
Antibiotic + Heat treatment	1	1	1

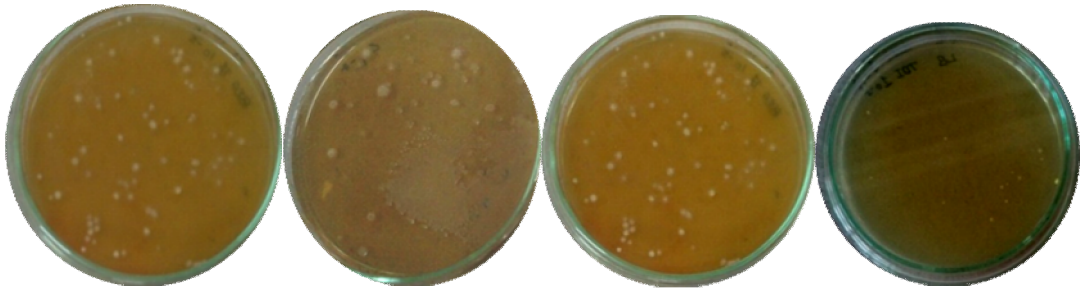


Fig.1: Isolation of bacteria from different soil samples.

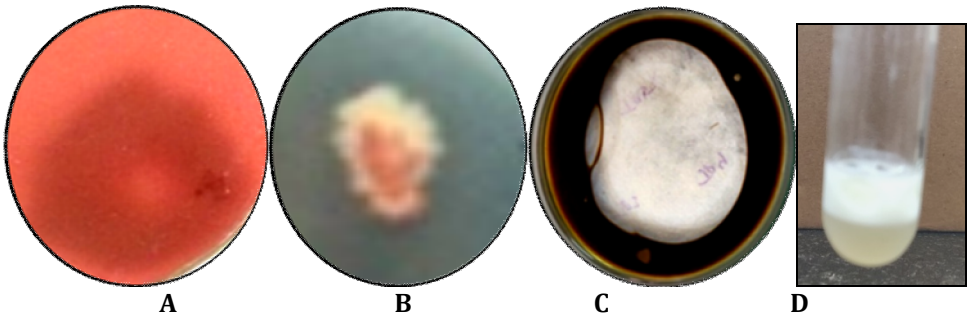
All the isolates were tested morphologically and biochemically for Bacillus sp. Among the 21 isolates screened 12 isolates were gram positive, motile rods which showed endospore formation. They were catalse positive with the ability to reduce nitrate. They showed negative tests for indole and methyl red while positive test for vogus prosker. These isolates has shown ability to utilize carbohydrates like arabinose, glucose, xylose, mannitol and maltose while were unable to utilize lactose (data not shown). Bacillus sp are the predominant flora of soil because of their ability to produce endospore and certain antimicrobial compounds. The potential of Bacillus sp to synthesize wide variety of bioactive metabolites makes them potentially applicable in wide range of clinical and pharmaceutical field [28].*B. amyloliquefaciens, B. subtilis*, and *B. atrophaeus* are well known potential producers of secondary metabolites, particularly cyclic lipopeptides such as iturin, fengycin, surfactin, bacilliomycin, etc which represents excellent surface properties due to which they became major candidates for agricultural, pharmaceutical and biotechnology industries [29].Nayarisseri et al., [30] has reported isolation of BS producing *Bacillus subtilis* ANSKLAB03 from chilika lake. Chittepu [14] reported the isolation of BSproducing bacteria from soil contaminated from groundnut oil cake dumping site while isolation of BS producing Bacillus sp was reported from diverse habitats such as hot springs, ocean, oil wells and petrol pump soilsby Joshi et al,[11].

BS's are diverse group of biomolecules, like glycolipids, lipopeptides, lipoproteins, lipopolysaccharides or phospholipids. Therefore, most methods for a general screening of BS producing microorganisms are based on physical effects of surfactants or their interference with hydrophobic interfaces [31]. Sidkey *et al*., [32] has reported that qualitative tests like hemolytic and lipolytic assays are sensitive and easy to use but should only be used for isolation of BS producing organisms because more than one method should be used for efficient BS producer. Although, qualitative test (oil spread method, emulsification index and reduction in surface tension) can be used during both screening and selection of efficient BS producer because they are more reliable and accurate [33]. Hence, BS production ability of selected 12 isolates was determined by using combination of different screening methods. All 12 isolates has shown lipid hydrolysis in the range of 10-13 mm whereas haemolysis activity was found in range of 10-15 mm. All the selected isolates has shown significant oil displacement activity (0.5-13.84 cm^2), reduction in surface tension of water (48.95-36.8mN/m) and emulsification index (39.33-70.83%) as shown in table -2.

Table.2: Screening of isolates for BS production

Isolates	Surface Tension (mN/m)	Emulsification Index (%)	Oil Displacement (cm^2)	Lipase activity (mm)	Hemolysis (mm)
LB1	39.19	45.33	19.6	10	11
LB2	39.90	43.75	21.22	10	10
LB3	**36.8**	**70.83**	**13.84**	**13**	**15**
LB6	42.64	43.66	19.6	11	10
LB9	48.95	44.84	13.84	12	13
LB18	40.82	39.33	13.84	10	10
LB19	40.64	40.77	10.47	10	10
LB20	41.61	43.22	15.62	10	10
BDA1	42.54	44.90	12.56	10	13
BDA4	42.64	43.5	10.21	10	10
BDA5	39.17	55.25	18.154	10	13
BIA9	39.14	43.88	18.266	10	13
SDS	21.14	50	28.26	-	-

During the screening it was found that isolate LB3 has highest potential for BS production based on all screening tests used. Since, LB3 has shownhighest lipase (13 mm) and haemolysis activity (15 mm) with significantreduction in surface tension (36.8mN/m), emulsification index (70.83%) andmaximum area displacement (13.84cm^2) (Fig. 2). Hence, LB3 was selected as a potential BS producer strain and was identified at molecular levels.



A B C D

Fig. 2: Screening of LB3 for BS production A) hemolysis activityB) lipolysis activityC) oil displacement test D) emulsification assay.

Identification of isolate LB3 at molecular levels

The 1200bp sequence obtained from 16S rRNA sequencing has been submitted to NCBI GenBank with an accession number**MN994288**. The sequence showed 100% homology with *Bacillus subtilis* strain DSM10. From the homology analysis it was confirmed that LB3 strain was closely associated with *Bacillus subtilis* (Fig. 3). Hence, the strain LB3 was identified as *Bacillus subtilis.*

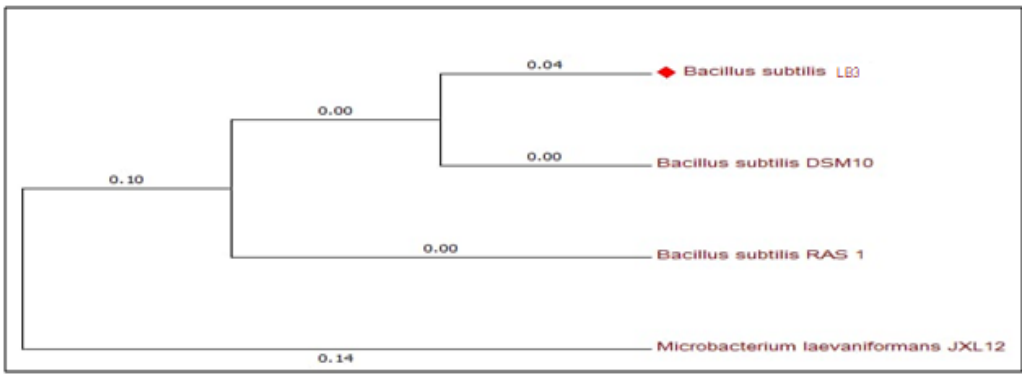


Fig.3: Neighbor joining tree based on 16S gene sequencing showing phylogenetic relationship between *Bacillus subtilis* (MN994288) and related members of the genus *Bacillus*.

Effect of different media composition on BS production and its antimicrobial activity

Effects of LB, NB, MSM and MSMG were studied on BS production and its antimicrobial activity.In presence of MSMG media reduction in surface tension was recorded as 25mN/m from 72.8mN/mand wasselectedas most suitable media for BS productionby *Bacillus subtilis* LB3. It was found that crude extract from MSMG media was also showing maximum antibacterial activity against *K. pnemoniae*(31 mm) and *V. parahaemolyticus* (23 mm) as compared to LB and NB medium (Table- 3). Hence, MSMG was selected as production medium.

Table3: effect of different media composition and glucose supplementation on BS production antimicrobial activity by LB3 and

Media used	Surface tension (mN/m)	Zone of inhibition (mm)		
		K. pneumoniae	*V. harveyi*	*V. parahemolyticus*
LB	45.39	19	10	-
NB	46.94	21	-	13
MSM	33.8	28	-	10
MSMG	25	31	-	23

Glucose is the typical example of the carbon source which can easily be metabolized by microorganisms through the glycolysis pathway for the generation of energy and is commonly reported to give higher yield of product [33]. Glucose is broken down to glucose-6-phosphate (G6P) via glycolytic pathway and this intermediate acts as the major precursors of carbohydrates found in the hydrophilic part of a BS. A series of enzymes are utilized to catalyze G6P on route to synthesizes various forms of hydrophilic moieties in the BS; trehalose, sophorose, rhamnose, mannose, and polysaccharide. For the formation of the hydrophobic moiety (lipid), glucose is oxidized to pyruvate which then transformed into acetyl-CoA that synthesizes malonyl-CoA when combined with oxaloacetate. Oxaloacetate will then be converted into fatty acid (precursors) for lipid production[34]. Similar results were reported by Gharibi *et al.,* (2012) they reported enhancement in lipopeptide production in presence of 6g/l glucose by *Bacillus subtilis* SPB1 strain. Heryani *et al.,* [35] observed that *Bacillus subtilis* BMN14 has shown maximum yield of BS in MSM supplemented with 1% glucose.

Production and purification of BS

After incubation of *Bacillus subtilis*LB3 in MSMG media produced BS was extracted as described earlier and the collected fractions were assessed for their BS productionby emulsification index and antimicrobial activity was tested (Fig.5) against *K. pnemoniae*. The fractions 11-15 showing highest emulsification index and antibacterial activity were pooled together, evaporated to dryness, dissolved in DMSO and used for further studies.

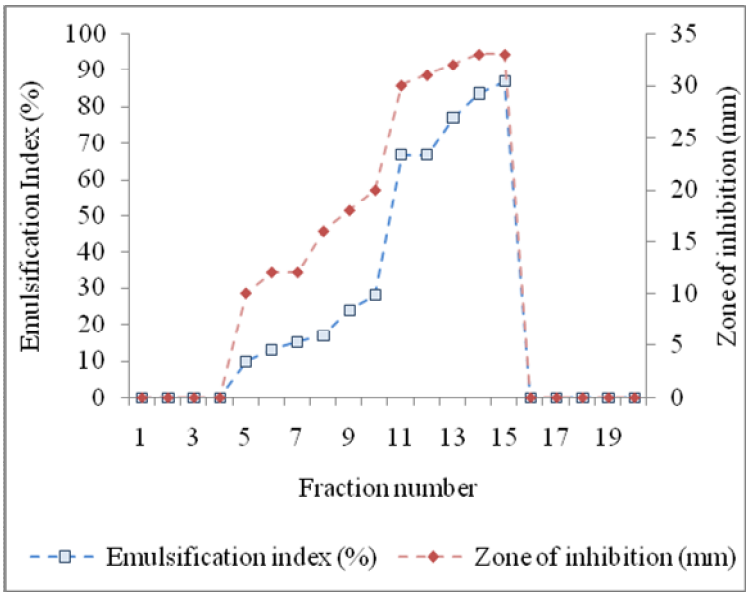


Fig.5: Purification profile of biosurfactant in terms of emulsification index and antibacterial activity against *K. pneumoniae*

Characterization of purified BS

FTIR spectrum of purified BS was studied to determine partial chemical structure of compound (Fig. 6). A sharp absorption peak at wave number 3026 was observed indicating the presence of C-H, N-H stretch, and it is a characteristic of carbon-containing compounds with amino groups [36]. Other absorbance peaks at 2347 is due to the presence of C-O bond while a sharp peak at 1227 indicates presence of C-O, N-H, bond indicating presence of carbonyl amide group. A broad and sharp peak at 760 is due to presence of alkane compounds. This characterization confirms the presence of lipopeptide compound. Similar results were reported by Thivaharan and Vytla, [37]. Jemil *et al.,* [38] reported the lipopeptide BS from *Bacillus methylotrophicus* DCS1 with similar characteristic peaks.

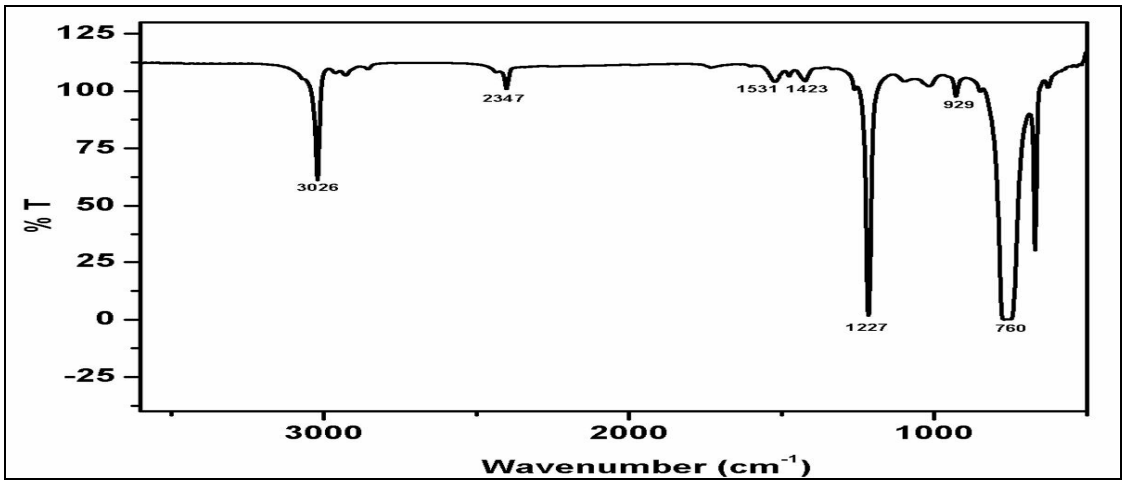


Fig.6: FTIR characterization of purified biosurfactant

MBC determination of BS from *Bacillus subtilis* LB3

MBC is the minimum concentration or dose of an antimicrobial agent at which it becomes lethal. The microbial culture when treated with an antimicrobial agent at or above its MBC cannot be revived even if transferred to an antimicrobial-free growth-supporting medium [39,40].By using alamer blue dye micro dilution assay MBC of the purified BS was determined. The change of blue to pink color indicated the minimum concentration required for inhibition of respective test pathogen. It was observed that BS at2µg/ml against *V. harveyi* and 0.2µg/ml against *K.pneumoniae*was most effective MBCs determined by visual observations. Resazurin is an active ingredient in MABA (alamer blue) and is permeable, blue and nonfluoroscent but after entry into viable cells resazurin is continuously reduced to resorufin a highly

fluorescent red colored compound by cells [41]. Jemil *et al*., [38] reported significant antimicrobial activity of DCS1 (2mg/ml) lipopeptides from *Bacillus methylotrophicus* DCS1 against *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Micrococcus luteus* (ATCC 4698) *Klebsiella pneumoniae* (ATCC13883), *Escherichia coli* (ATCC25922), *Salmonella typhimurium* (ATCC 19430), *Salmonella enterica* (ATCC 27853) and *Enterobacterium sp.* Antibacterial activity of BS produced by *B. subtilis* C19 was demonstrated by Yuliani et al., [42] against pathogens like *Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica Typhi, Listeria monocytogenes, and Candida albicans*).

CONCLUSION

The present study revealed the significance of lipopeptide BS produced from *Bacillus subtilis* LB3 isolated from oil contaminated soil as a potential antibacterial agent. The produced BS can also be exploited for various applications in biomedical and environmental fields due to its great surface tension reduction activity.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest among all authors.

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This data has been neither published nor submitted for publication, in whole or in part, either in a serial, professional journal or as a part in a book which is formally published and made available to the public.

REFERENCES

1. Cheesman, M.J., Ilanko, A., Blonk, B. & Cock, I.E. (2017). Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution?. Pharmacognosy. reviews., 11(22):57.
2. Ventola, C.L. (2015). The antibiotic resistance crisis: Part 1: Causes and threats. P. T., 40:277–83.
3. Powers, J.H. (2004). Antimicrobial drug development – The past, the present, and the future. Clin. Microbiol. Infect., 10(4):23–31.
4. Levy, S.B. (2000). Antibiotic and antiseptic resistance: Impact on public health. Pediatr. Infect. Dis. J., 19(10):S120–2.
5. WHO. Antimicrobial Resistance. World Health Organization.(2016). Available from: http://www.who.int /mediacentre/factsheets/fs194/en/
6. Kuiper, I., Lagendijk, E.L., Pickford. R., Derrick. J.P., Lamers, G.E., Thomas-Oates, J.E., Lugtenberg, B.J. & Bloemberg, G.V. (2004). Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. Mol. microbiol., 51(1):97-113.
7. Makkar, R.S. & Cameotra, S.S. (1999). Biosurfactant production by microorganisms on unconventional carbon sources. J. S. D., 2(2):237-41.
8. Sánchez, M., Teruel, J.A., Espuny, M,J., Marqués, A., Aranda, F,J., Manresa, Á. & Ortiz, A. (2006). Modulation of the physical properties of dielaidoylphosphatidylethanolamine membranes by a dirhamnolipid biosurfactant produced by *Pseudomonas aeruginosa*. Chem.Phys.Lipids., 142(1-2):118-27.
9. Fracchia, L., Banat, J.J., Cavallo, M. & Banat, I.M. (2015). Potential therapeutic applications of microbial surface-activecompounds. AIMS Bioeng., 2(3):144-62.
10. Callaghan, B., Lydon, H., Roelants, S.L., Van Bogaert, I.N., Marchant, R. & Banat, I.M. Mitchell CA. (2016). Lactonic Sophorolipids increase tumor burden in Apcmin+/-mice. PloS. one., 11(6).
11. Joshi, S.J., Suthar, H., Yadav, A.K., Hingurao, K. & Nerurkar, A. (2013). Occurrence of biosurfactant producing Bacillus spp. in diverse habitats. ISRN. biotechnol. 29;2013.
12. Bergey, D.H. & Holt, J.G. (1994). Bergey's manual of systemic bacteriology. Noel. R. Krieg (ed.) Editorial Williams &Wilkins Baltimore. London. 8:4-6.
13. Priyam, V. & Dinesh, S. (2018). Review on biosurfactant production and its applications. Int. J. Curr. Microbiol. Appl. Sci. *7*(8): 4228-4241.
14. Chittepu, O.R. (2019). Isolation and characterization of biosurfactant producing bacteria from groundnut oil cake dumping site for the control of foodborne pathogens. Grain & Oil. Sci. Technol. 2(1):15-20.
15. Satpute, S.K., Bhawsar, B.D., Dhakephalkar, P.K.& Chopade, B.A. (2008). Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Int. J. Mol. Sci., 37(2008):243.
16. Foukia, E. M., Mostafa, M.A.E. & Maysa, E.M. (2016). Optimization of biosurfactant production by *Bacillus brevis* using response surface methodology. Biotechnol. Rep. 9:31-37.

17. Dilmohamud, B.A., Seeneevassen, J., Rughooputh, S.D. & Ramasami, P. (2005). Surface tension and related thermodynamic parameters of alcohols using the Traube stalagmometer. Eur. J. Phys., 26(6):1079.
18. Garg, M. & Chatterjee, M. (2018). Isolation, characterization and antibacterial effect of biosurfactant from *Candida parapsilosis*. Biotechnol. Rep. 18:e00251.
19. Clarridge, JE. (2004) 3rd. Impact of 16S rRNA gene sequence analysis for identification ofbacteria on clinical microbiology and infectious diseases. Clin.Microbiol. Rev., 17(4):840-62.
20. Darby, A.C., Chandler, S.M., Welburn, S.C. & Douglas, A.E. (2005). Aphid-symbiotic bacteria cultured in insect cell lines. Appl. Environ. Microbiol., 71(8):4833-9.
21. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignmentsearch tool. J. Mol. Biol., 215(3):403-10. PubMed PMID: 2231712.
22. Gertz, E.M. (2005).BLAST Scoring Parameters, 16 March 2005.
23. Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences. 2004 Jul 27;101(30):11030-5.
24. Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol.Biol.Evol. 24(8):1596-9.
25. Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol.Evol., 16(2):111-20.
26. de Freitas Ferreira, J., Vieira, E.A. & Nitschke, M. (2019). The antibacterial activity of rhamnolipid biosurfactant is pH dependent. Food Res.Int., 116:737-44.
27. Walter, V., Syldatk, C. & Hausmann, R. (2010). Screening concepts for the isolation of biosurfactant producing microorganisms. In Biosurfactants. (pp. 1-13). Springer, New York, NY.
28. Amin, M., Rakhisi, Z. & Zarei, A.A. (2015). Isolation and identification of Bacillus species from soil and evaluation of their antibacterial properties.J. Clin. Microbiol. Infect., 2(1):23233-23233.10.17795/ajcmi-23233
29. Harwood, C.R., Mouillon, J.M., Pohl, S. & Arnau, J. (2018). Secondary metabolite production and the safety of industrially important members of the *Bacillus subtilis* group. FEMS Microbial. Rev., 42(6):721-38.
30. Nayarisseri, A., Singh, P.&Singh, S.K. (2018). Screening, isolation and characterization of biosurfactant producing *Bacillus subtilis* strain ANSKLAB03. Bioinfo. 14(6):304.
31. Walter, V, Syldatk, C. & Hausmann, R. (2013). Screening Concepts for the Isolation of Biosurfactant Producing Microorganisms. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Biosci. 2000-2013. Available from: https://www.ncbi.nlm.nih.gov/books/NBK6189/
32. Sidkey, N.M., Mohamed, H.F, & Elkhouly, H.I. (2016). Evaluation of different screening methods for biosurfactant producers isolated from contaminated Egyptian samples grown on industrial olive oil processing waste. Microbiol. Res. J. Int., 1-9.
33. Mounira, A. & Abdelhadi, G. (2015). Assessment of four different methods for selecting biosurfactant producing extremely halophilic bacteria. Afr. J. Biotechnol. 14(21):1764-1772:27. DOI: 10.5897/AJB2015.14611
34. Ghribi, D., Abdelkefi-Mesrati, L., Mnif, I., Kammoun, R., Ayadi, I., Saadaoui, I., Maktouf, S. & Chaabouni-Ellouze, S. (2012). Investigation of antimicrobial activity and statistical optimization of *Bacillus subtilis* SPB1 biosurfactant production in solid-state fermentation. BioMed. Res. Int., 24;2012.
35. Nurfarahin, A,H., Mohamed, M.S., Phang, L.Y. (2018). Culture Medium Development for Microbial-Derived Surfactants Production—An Overview. ***Mol.,*** 23(5):1049. https://doi.org/10.3390/molecules23051049
36. Heryani, H. & Putra, M.D. (2017). Kinetic study and modeling of biosurfactant production using Bacillus sp. Elec. J. Biotechnol. 27:49-54.
37. Thivaharan, V. and Vytla, R.M. (2013). Production of a Lipopeptide Biosurfactant by a Novel *Bacillus* sp. and Its Applicability to Enhanced Oil Recovery.Int. Schol. Res. Not. 2013. https://doi.org/10.1155/2013/621519
38. Kong, J. & Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. Acta. Biochem. Biophys. Sinica., 39:549–59.
39. Jemil, N., Ayed, H.B., Manresa, A., Nasri, M. & Hmidet, N. (2017). Antioxidant properties, antimicrobial and anti-adhesive activities of DCS1 lipopeptides from Bacillus methylotrophicus DCS1. BMC. Microbiol., 17(1):144.
40. Celiktas, O.Y., Kocabas, E.H., Bedir, E., Sukan, F.V., Ozek, T. & Baser, K.H. (2007). Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis*, depending on location and seasonal variations. Food Chem. 100(2):553-9.
41. Kuete, V., Nguemeving, J.R., Beng, V.P., Azebaze, A.G., Etoa, F.X., Meyer, M., Bodo, B. & Nkengfack, A.E. (2007). Antimicrobial activity of the methanolic extracts and compounds from Vismia laurentii De Wild (Guttiferae). J. Ethnopharmacol., 109(3):372-9.
42. e Silva, A.D., Oliveira, H.C., Silva, J.F., Sangalli-Leite, F., Scorzoni, L., Fusco-Almeida, A.M. & Mendes-Giannini, M.J. (2013). Microplate alamar Blue assay for Paracoccidioides susceptibility testing. J. Clin. Microbiol., 51(4):1250-2.
43. Yuliani, H., Perdani, M.S., Savitri, I., Manurung, M., Sahlan, M., Wijanarko, A. and Hermansyah, H., (2018). Antimicrobial activity of biosurfactant derived from *Bacillus subtilis* C19. Energy. Procedia,, 153:274-278.