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Screening and Characterization of bio-surfactant producing *Bacillus* spp. from milk samples

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Abstract
Biosurfactants are surface metabolites produced by many bacteria, amphiphilic in nature. Preliminary and specific tests were carried out for 23 biosurfactant producing *Bacillus* isolates to characterize up to species level. Preliminary tests like Gram's staining, bacterial spore staining, catalase, and oxidase confirmed the genus as *Bacillus* while specific tests helped to determine the species of *Bacillus*. As per the phenotypic identification of biosurfactant producing 23 isolates of *Bacillus* species from 6 milk samples (3 fresh and 3 spoiled), 13 isolates were identified as *Bacillus licheniformis* and 10 isolates as *Bacillus subtilis*. All the 23 isolates of *Bacillus* species when grown in sterile nutrient broth at 37^{0}C for 24 h for screening and confirmation of surfactant production, 10 isolates showed direct microscopic count (DMC) ranging from 7.0 to 7.5 log10/ml with spreading of butter oil of 1 to 2 mm., while 13 numbers had DMC between 8 and 8.6 log10 /ml while oil spreading diameter ranged from 3 to 5 mm, respectively. Among 23 isolates, B16 showed highest DMC of 8.6 log10/ml with higher oil spreading ability of 5 mm diameter. Isolate B16 identified as *B. licheniformis* phenotypically once showed higher DMC and oil spreading ability, that isolate was genotyped to confirm identity as *B. licheniformis* with accession number KY3233391.

Keywords: Biosurfactants, *Bacillus* spp., milk samples, specific test, phenotype

Introduction
Biosurfactants are surface-active compounds like synthetic surfactants but unlike the chemical surfactant, biosurfactant is synthesized by microbes like bacteria and fungi predominantly produced by bacteria. Biosurfactants are becoming an industrial reality over synthetic ones (Rashedi *et al.*, 2005). A large variety of *Bacillus subtilis*, as well as *Bacillus licheniformis* strains, produce lipopeptide biosurfactants which possess a high surfactant activity such as surface active properties and antibacterial activity having wide application in food and dairy

Materials and methods
Isolates of *Bacillus* spp. obtained from butter fat agar were subjected for preliminary identification tests like simple staining, Gram's staining, spore staining, catalase test, and oxidase test. To know the species of the isolates, they were subjected to specific tests such as pigmentation, motility, aerobic and anaerobic growth, acid from D-mannitol, acid from D-glucose, arginine dihydrolase, growth at 50^{0}C, haemolysis test, hydrolysis of starch, pH 8 and urea hydrolysis as per the procedure given by Harrigan (1998) and results were compared with the Bergey's manual of systematic bacteriology (Vos *et al.*, 2009) to identify the species of *Bacillus*.

Screening of isolates of *Bacillus* spp. for production of biosurfactants
1. Drop collapse assay
Butter oil was added at 40µl onto the clean glass slide and keep at 25 ^{o}C/1 h. Later transferred 50 µl of young cell-free supernatant of isolates of *Bacillus* spp. on to the surface of oil drop. The shape of the drop on the surface of oil was inspected visually after 1 to 2 min. Biosurfactant producing isolates produced collapsed drops while those that did not remain stable as given by Jazeh *et al.* (2012).

2. Slide Test
The procedure followed by Femiola *et al.* (2015) was adopted by transferring loopful of 18 h old culture of isolates of *Bacillus* spp. to drop of saline (0.85% Sodium chloride) on a grease free slide. The slide was slanted at 45^o and then observed the flow of wet preparation over the surface of the glass slide; flow indicated the production of biosurfactant by the isolate.

3. Oil spreading Technique
Oil spreading technique was performed as given by Sharma *et al*. (2014). The distilled water of 40 ml was taken in a petriplate. Butter oil of 10 µl was placed on the surface of the water. Cell-free supernatant of isolates of *Bacillus* spp. of 10 µl wasindustries (Sanjana *et al*., 2019).
Biosurfactants have the properties of reducing surface tension, emulsion stability, foaming ability and are usually non-toxic and biodegradable. Recently interest in biosurfactant has increased because of its diversity, flexibility in operation and more eco-friendly than synthetic surfactant (Sharma *et al.*, 2014). Biosurfactants are produced by industrial by-product like cheese whey from the dairy industry. They possess dairy and other industrial applications to avoid environmental pollution (Sanjana *et al*., 2017).

Literature regarding the characterization of biosurfactant producing *Bacillus* spp. from milk samples is not available. Hence literature pertaining to soil, petroleum industry waste samples from which *Bacillus* spp. were isolated and identified were also considered here. Oil spreading technique, drop collapse assay and slide test were the major screening tests to identify the production of biosurfactants from bacteria (Jazeh *et al*., 2012). Preliminary and specific tests were carried out to identify the surfactant-producing isolates of *Bacillus* spp. (Harrigan, 1998). The identification key for surfactant producing species of *Bacillus* was prepared by referring to Bergey's manual of systematic bacteriology (II edition) Volume 3 - the firmicutes edited by (Vos *et al*., 2009).

Transferred at the center of the oil layer. Biosurfactant displaced oil and formed clearance zone and the diameter of this clearing zone on the oil surface was measured and indicated as the surfactant activity also termed as the oil displacement activity.

Genotyping of best surfactant producer isolate of *Bacillus* spp.

Screening of 23 isolates of *Bacillus* spp. which were phenotyped by subjecting to preliminary and specific tests when subjected for surfactant activity one of the isolates was selected as the best based on DMC and oil displacement test. The isolate was further identified by 16s rRNA sequencing by outsourcing through Serene Biosciences, Molecular Diagnostics Research, Custom services and Training No. 30, II Cross, Marappa Garden, Benson Town Post, Bangalore -560 046.

Result and Discussion

Phenotypic identification of surfactant-producing isolates of *Bacillus* spp.

All the 23 biosurfactant producing *Bacillus* isolates obtained from butter fat agar were subjected for catalase, oxidase and motility tests to complete preliminary identification to confirm the genus. Once the genus of the isolate was validated, specific biochemical tests were carried out to fix the species. All the 23 isolates were motile as well as showed positive for catalase and oxidase tests. These preliminary tests confirmed the isolates up to genus *Bacillus* and further for speciation, the isolates were subjected for specific biochemical tests such as pigmentation, aerobic and anaerobic growth, acid from D-mannitol, acid from D-glucose, arginine dihydrolase, haemolytic test, growth at 50^{o}C, hydrolysis of starch, urea hydrolysis and pH 8. Out of 23 isolates, 13 numbers were identified as *Bacillus licheniformis* and 10 numbers were *Bacillus subtilis* (Table 1) after comparing the results with the developed key for identification by the Bergey's manual of systematic bacteriology (Vos *et al.,* 2009).

Bacillus subtilis produced red pigment with aerobic growth and lack of arginine dihydrolase and urease while *Bacillus licheniformis* did not produce red pigment, was anaerobic, had arginine dihydrolase and urease as major phenotypic characteristics. None of 23 isolates showed haemolysis on horse blood agar indicating the non- pathogenic nature of the isolates. Out of 23 biosurfactant producers, 13 isolates were identified as *Bacillus licheniformis* and 10 isolates as *Bacillus subtilis* based on phenotypic characteristics and were coded from B1 to B23 continuously.

A compatible study by Sharma *et al*. (2014) who isolated *Bacillus* spp. from soil sample on nutrient agar by streaking and obtained 4 numbers. The isolates were subjected for spore staining, Gram's staining, catalase, and oxidase as preliminary tests and specific tests as IMViC and sugar fermentation tests that revealed the identity as *Bacillus subtilis* which were used later for biosurfactant production

Intensity of biosurfactant of *Bacillus* spp. produced in sterile nutrient broth medium

A total of 23 biosurfactant producing isolates were grown in sterile nutrient broth at 37^{o}C for 24 h and determined the pH, DMC and oil spreading ability (Plate 1). Among B1 to B23 isolates, 10 isolates showed low DMC of 7.0 to 7.5 log10/ml with the low spreading of butter oil of 1 to 2 mm. Another 13 isolates had better DMC between 8 and 8.6 log10 /ml with good oil spreading ability which may be attributed to better biosurfactant production due to increased biomass(Table 2). Out of 13 isolates, B16 showed highest DMC of 8.6 log10/ml with higher oil spreading ability of 5 mm diameter and hence used in further studies for optimization of media, incubation condition and extraction of biosurfactant production with its application in the dairy industry.

A similar study conducted by Priya and Usharani (2009), who used oil spreading technique for selection of best biosurfactant producing isolates among *Bacillus subtilis* and *Pseudomonas aeruginosa* using vegetable oil, kerosene, petrol, and diesel. One of the *B. subtilis* among 4, BS3 produced the higher zone ranging between 6 and 20 mm while *P. aeruginosa* PS3 produced 8 to 22 mm in vegetable oil, kerosene, petrol, and diesel respectively On par with the present study, Singh (2012) also found major species of *Bacillus* that produced biosurfactant as *Bacillus subtilis* and *Bacillus licheniformis*. The species were isolated from nutrient agar, required screening tests for production of biosurfactant. Out of 20 isolates obtained from the soil samples, 6 showed biosurfactant production. The isolated strain L4 identified as *Bacillus* spp. produced more biosurfactant based on oil spreading method.

Jazeh *et al.* (2012) isolated biosurfactant producing *Bacillus* spp. from petroleum contaminated soil in South Korea and Iran. They observed that 110 strains were able to produce biosurfactant through screening tests in which 59 strains showed positive for blood haemolysis test while 46 strains showed positive for drop collapse test and remaining were positive for oil spreading technique.

Statistical analysis was done by using R.VERSION 3. 1. 3 (2015-03-09). A statistically significant difference ($p \leq 0.05$) occurred in DMC of 23 isolates of *Bacillus* spp. but no significant difference was shown in oil displacement ability. DMC of B16 isolate was more, phenotyped as *Bacillus licheniformis* and used in further studies for optimization of production of biosurfactant.

Genotypic identity of Isolate B16

The isolate B16 that produced good cell numbers as well as oil spreading ability which was earlier phenotyped as *Bacillus licheniformis* was genotyped by 16s rRNA sequencing to confirm the phenotypic identity. It was confirmed as the *Bacillus licheniformis* only by gene sequencing (NCBI blast result) with accession number KY3233391.

Similarly, Zhang *et al*. (2014), isolated 53 *Bacillus* spp. from the soil and phenotyped as *Bacillus licheniformis* and further

confirmed through PCR and gene sequencing as *B. licheniformis* only and further used for biosurfactant production.

Once the isolate B16 was selected through oil spreading ability, the isolate was genotyped to confirm the phenotypic identity. Preliminary and specific tests helped to identify B16 as *Bacillus licheniformis* and were confirmed as the same by 16s rRNA sequencing (NCBI blast result) with accession number KY3233391 (Fig. 1).

Chen *et al.* (2006) showed 22.72% of *Bacillus* spp. isolated from crude oil samples in Iran on nutrient agar. High incident rate of *Bacillus* spp. of 22.73% was observed in domestic oil-contaminated waste water when compared to other organisms isolated, may be a direct correlation to their ability to produce emulsifiers to degrade oil rather than just a coincidence (Tabatabaee *et al.*, 2005), While Jaysree *et al.* (2011) also found *Bacillus subtilis* and *Bacillus licheniformis* are most important biosurfactant producers among *Bacillus* spp.

Table 1: Phenotypic Identification of surfactant producing isolates of *Bacillus* spp.

Biochemical Characteristics		Isolate code	
		B1, B2, B6, B9, B10, B12, B13, B15, B16, B17, B18, B20, B22 (13 numbers)	**B3, B4, B5, B7, B8, B11, B14, B19, B21, B23 (10 numbers)**
	Aerobic growth	+	+
	Anaerobic growth	+	-
	Acid from Mannitol	+	+
	Acid from D - Glucose	+	+
Specific tests	Arginine dihydrolase	+	-
	Haemolytic test	-	-
	Growth at 50^0C	+	+
	Growth at pH 8.0	+	+
	Hydrolysis Of Starch	+	+
	Hydrolysis of Urea	+	-
Identity		*Bacillus licheniformis*	*Bacillus subtilis*

Note: All the 23 isolates were Gram positive, spore forming, motile rods with catalase and oxidase positive.

A. Clear zone of 2 mm

B. Clear zone of 5 mm

Plate 1: Intensity of biosurfactant of Bacillus spp. produced in sterile nutrient broth Medium

Table 2: Intensity of biosurfactant of *Bacillus* spp. produced in sterile nutrient broth medium

Isolate code	pH	DMC (log10 cfu/ml)	Oil spreading ability (mm)
B1	6.0	7.30^{b}	1^{a}
B4	6.0	7.55^{c}	$_{2}$d
B7	6.5	7.38^{d}	1^{a}
B8	6.0	7.50^{e}	2^{e}
B11	6.5	7.45^{f}	2^{f}
B13	6.0	7.00^{a}	$_{1}$a
B14	6.0	7.48^{g}	2^{g}
B15	6.0	7.10^{a}	1^{a}
B18	6.0	7.34^{h}	$_{1}$a
B21	6.0	7.50^{i}	2^{h}
B2	6.5	8.00^{j}	3^{b}
B3	6.5	8.08^{k}	3^{i}
B5	6.0	8.20^{l}	3^{j}
B6	6.0	8.34^{m}	4^{c}
B9	6.0	8.28^{n}	4^{c}
B10	6.0	8.06^{o}	$_{3}$k

B12	6.5	8.26^{p}	4^{v}
B16	6.5	8.60^{q}	5^{c}
B17	6.5	8.20^{r}	3^{l}
B19	6.0	8.30^{s}	4^{c}
B20	6.0	8.10^{t}	3^{b}
B22	6.0	8.25^{u}	3^{m}
B23	6.0	8.30^{v}	4^{c}
CD (p≤ 0.05)		**0.069**	**0.0009**

Note: Values are average of three trials.
Lower case alphabets as superscript indicate significant difference in DMC and Oil spreading ability.

1 cgctcaggacgaacgctggcggcgtgcttaatacatgcaagtcgagcggac
cgacgggag
61 cttgctccctttaggtcagcggcggacgggtgagtaacacgtgggtaacctg
cctgtaag 121
actgggataactccgggaaaccggggctaataccggatgcttgattgaaccgcatggttc
181
aatcataaaaggtggctttcagctaccacttacagatggacccgcggcgcattagctagt
241
tggtgaggtaacggctcaccaaggcgacgatgcgtagccgacctgagagggtgatcggc
c
301
acactgggactgagacacggcccagactcctacgggaggcagcagtagggaatcttcc
gc
361
aatggacgaaagtctgacggagcaacgccgcgtgagtgatgaaggttttcggatcgtaaa
421
actctgttgttagggaagaacaagtaccgttcgaatagggcggtaccttgacggtaccta
481
accagaaagccacggctaactacgtgccagcagccgcggtaatacgtaggtggcaagc
gt
541
tgtccggaattattgggcgtaaagcgcgcgcaggcggtttcttaagtctgatgtgaaagc
601
ccccggctcaaccggggagggtcattggaaactggggaacttgagtgcagaagaggag
ag
661
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g
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g
781
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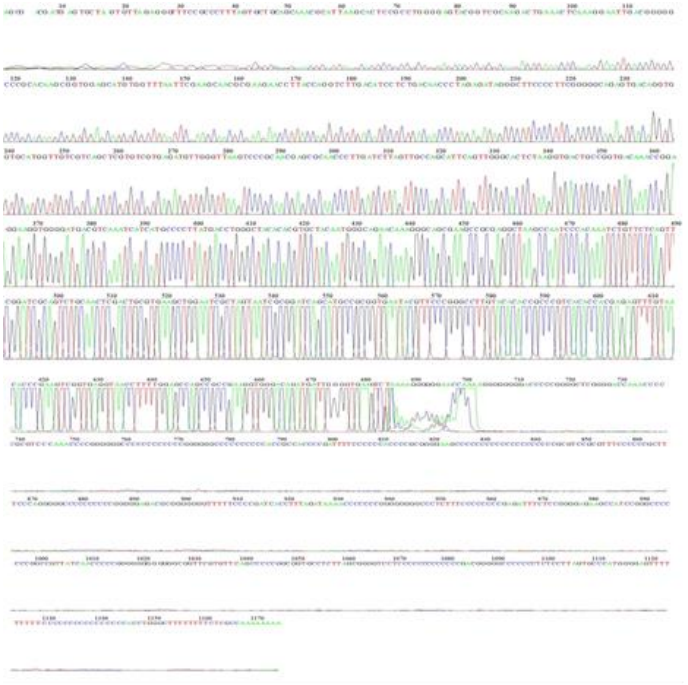

Fig 1: 16s rRNA sequences of Bacillus licheniformis B16

Conclusion

Among the 23 isolates of *Bacillus* spp., 13 were identified as *Bacillus licheniformis* and 10 as *Bacillus subtilis*, which were phenotyped by subjecting to preliminary and specific tests when subjected for surfactant activity. Among 23 isolates, B16 showed higher log DMC count of 8.60 with 5 mm as oil dispersing ability. *Bacillus licheniformis* isolate was selected as the best based on DMC and oil displacement test and the isolate was genotyped to confirm the phenotypic identity and was confirmed as the same

by 16s rRNA sequencing (NCBI blast result) with accession number KY3233391.

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