Potential of *Enterococcus Faecium* LM5.2 for Lipopeptide Biosurfactant Production and Its Effect on the Growth of Maize (*Zea Mays* L.)

Lalit K. Chaurasia
Sikkim University
Ranjan K. Tirwa
Sikkim University
Buddhiman Tamang (bmtamang3@gmail.com)
Sikkim University https://orcid.org/0000-0001-8575-2129

Research Article

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Abstract

The lipopeptide biosurfactants' chemical characteristics from the lactic acid bacteria isolated from milk and milk products were studied and their effect on maize plant growth. The oil displacement test was performed as a primary screening method to select the BS producing bacteria. *Enterococcus faecium* LM5.2 had the maximum emulsification index of 45.1±3 and reduced the surface tension to 32.98 ± 0.23% among all the isolates. *E. faecium* LM5.2 efficiently produced 945.26 ± 4.62 mg/l biosurfactants within 48 hours in MRS broth under the optimum conditions. The confirmation of the identity of the isolate LM5.2 was done with physiochemical tests and 16S rRNA gene sequencing. The molecular phylogenetic relationship was evaluated by the Neighbour-Joining phylogenetic method. The biosurfactant was purified by TLC and identified as lipopeptide-like iturines and surfactins based on R_f values. Mass spectroscopy, NMR, and FTIR analysis also confirmed the biosurfactant's identity as the derivatives of iturin and surfactin. Both the biosurfactant and its producer bacterium were evaluated for their plant growth-promoting activity, and it was found that the biosurfactant and the bacterium could enhance plant growth. To the best of our knowledge, this is the first report of lipopeptide biosurfactant production from *Enterococcus faecium*. Moreover, the study also showed that the biosurfactant and biosurfactant producing *E. faecium* LM5.2 could be an eco-friendly plant growth-promoting agent.

Introduction

Sikkim is a small state situated in the North-East Himalayan region of India. It is well known for its unique food culture and traditional fermentation practices. Therefore, lactic acid bacteria (LAB) isolated from traditional fermented food products are the potential source to find new bioactive lipopeptides, glycolipids and other surface-active molecules (Fracchia et al. 2010). These surface-active molecules possess the ability to reduce the surface tension or interfacial tension of polar and non-polar phases of gas-liquid, liquid-liquid or solid-liquid medium. These molecules efficiently emulsify the two immiscible liquid phases (Fracchia et al.).

Many microorganisms produce different types of biosurfactants (BSs), which vary in structure and function. These BSs affect microbial activities such as microbial motility (Arutchelvi et al. 2008), biofilm formation (Hamme et al. 2006) and quorum sensing (Ron and Rosenberg 2001; Arutchelvi et al. 2008). Most of the BSs are very stable against the broad range of variation in pH, temperature, salt concentration and other environmental factors(Nitschke and Costa 2007). Due to the surface activity and foaming property (Desai and Banat 1997; Jagtap et al. 2010), BSs are used in the formulations of many commercial products such as cosmetics (Salek and Euston 2019), pharmaceutics (Roy 2018) and the agriculture sector (Sachdev and Cameotra 2013). Natural BS can be used as an eco-friendly alternative to chemical surfactant in mouth-wash formulations (Farias et al. 2019). However, the most important role of BSs is in extracting petroleum products (Desai and Banat 1997; Jagtap et al. 2010). BS is also very useful in enhancing oil extraction, cleaning the oil tanks and oil spills. Remediation of petroleum contaminated soil (Jimoh and Lin 2019) and heavy metal contaminated soil (Jimoh and Lin 2019) are other applications of BS.

In agriculture, BS has been reported to have antimicrobial activity against many plant pathogens and can be used for biological control in the agriculture field (Sachdev and Cameotra 2013). The most studied example is the rhizobacterial *Pseudomonas* sps. and *Bacillus* sp. which produces rhamnolipids and surfactin, respectively, inhibiting the growth of soft rot causing *Pectobacterium* sp. and *Dickey* sp. (Krzyzanowska et al. 2012). Rhamnolipid of *Pseudomonas* also stimulate the plant defence system (or immune system) and protects them from many pathogens (Vatsa et al. 2010). The BS plays a vital role in cell communication between rhizobacteria and plant root hairs (Dusane et al. 2010). BSs also increase the heavy soil's wettability by hydrophilisation and help the fertilisers evenly distribute in the soil (Okoliegbe and Agarry 2012).

LAB has been recognised as a safe (GRAS) status for use in the food and agriculture industry (Wessels et al. 2004). The present work was focused on the characterisation of the biosurfactant from LAB isolated from Sikkim, India's milk and milk products. Besides, the plant growth-promoting property of the BS and the BS producing bacterium was also determined in the maize plant under *in-vivo* condition.

Material And Methods

Sample collection and bacteria isolation

Five raw cow milk and five homemade *dahi* samples were collected from the local market of Gangtok Sikkim, India, and processed for bacterial isolation on De Man, Rogosa and Sharpe (MRS) agar media with 0.1 % $CaCO_3$. Bacteria were isolated by serial dilution method at 10^{-4}concentration of the pure milk/*dahi* samples (Ghatani and Tamang 2017).

Screening for biosurfactant (BS)production

Pure isolates were cultured in MRS broth at 37 ˚C for 48 h. The culture broth was centrifuged at 8000 rpm for 20 min to obtain the cell-free supernatant used for screening the BS. A qualitative (Oil displacement test)(Youssef et al. 2004) and quantitative screening techniques. i.e. estimation of Emulsification Index (Satpute et al. 2008) and surface tension measurement by Du Noüy ring method (Gudiña et al. 2010) was used to select efficient biosurfactant producing isolates.

Oil displacement test

A thin layer of 20µl of engine oil (Servo2T Supreme, India) was made on 30 ml distilled water in 100 mm glass petri plate, and 10 µl of cell-free supernatant was added gently to the oil film. The displacement of oil showed the presence of BS in the media(Youssef et al. 2004).

Estimation of Emulsification Index (EI_{24}): 5 ml of each sunflower oil and culture supernatant was taken in a test tube and vortexed for 5 min. The tubes were kept undisturbed overnight. After 24 hours, the total length of liquid in the media and the emulsified solution's length was measured. EI_{24} was calculated using

the formula mentioned by Satpute et al (Satpute et al. 2008).

Measurement of surface tension

DuNoüy platinum ring method is the most accurate method for measuring surface tension. The surface tension of the 48 hours culture supernatant was measured with Kruss GmbH Hamburg tensiometer's help at 25˚C(Gudiña et al. 2010; Jazeh et al 2012).

Production and extraction of biosurfactant: Lactic acid bacteria were cultured in MRS broth for 48 hours at 25˚C in a shaker incubator at 120 RPM. The culture broth was centrifuged at 12,000 rpm at 4˚C, and the supernatant was acidified with 5M HCl by adjusting to pH 2. The supernatant was kept at 4˚C overnight to allow the biosurfactant to settle down at the flask's bottom. After harvesting the precipitate by centrifugation (12,000 rpm for 20 min and 4˚C), it was suspended in $CHCl_3$:CH_3OH (2:1) solution and centrifuged again. Then the surfactant was collected from the interphase of $CHCl_3$ and CH_3OH. Collected biosurfactant was suspended in distilled water with adjustment of pH to 7. Finally, the biosurfactant was lyophilized for further chemical analysis (Chander et al. 2012).

Bacterial identification

Phenotypic identification of BS producing bacteria was made by microscopic observations, Gram staining, growth at different conditions and sugar fermentation tests according to the “Bergey’s manual of systematic bacteriology” (Vos et al. 2009). The genotypic characterisation was done by 16S rRNA gene sequencing. Bacterial genomic DNA was isolated by a modified phenol-chloroform method (Sambrook et al. 2006). Full-length 16s rRNA gene region was amplified by 27F and 1492R primers with standard PCR protocol (Sachdev and Cameotra 2013; Bee et al. 2019). PCR product was subjected to Sanger sequencing, and the forward and reverse sequences were aligned by Codon Code Aligner 7.1.2 software. The identity of bacterial isolates was confirmed by the BLAST tool from the NCBI nr/nt database. Partial sequence data were deposited in the GenBank nucleotide sequencing data library(Bento et al. 2005).

Purification of biosurfactant:100µg lyophilized biosurfactant was dissolved in 1ml methanol and applied on analytical Silica gel $60F_{254}$ plates (Merck, Germany). The chromatogram was developed in duplicate with mobile phase Chloroform: Methanol: Ammonia water::60:35:5. One plate was treated with ninhydrin solution, and another plate was treated with hydrochloric acid. Biosurfactant was purified by preparative Thin Layer Chromatography (TLC) with the mobile phase chloroform: methanol: ammonia water::65:35:5 on HF_{254} silica gel plate (Merck, Germany). The bands were observed under UV light at 254 nm (Varadavenkatesan and Murty 2013; Antonious et al. 2015).

Characterisation of biosurfactant

Chemical characteristics of isolated BS were determined by mass-spectroscopy (MS), infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) spectrometry.

LC-MS

The various biosurfactant fractions were isolated from the purified BS by LC-MS (Thermo Finnigan LCQ Advantage Max Ion Trap Mass Spectrometer Hyphenated with Thermo Finnigan Surveyor HPLC system). BS samples were dissolved in HPLC grade methanol (Merck, Germany) at the concentration of 2mg/ml, and 2µl was injected into the C18 column(5µl, 4.6 X 250mm) with the flow rate of 0.20 ml/min. The mobile phase was acetonitrile/water (with 0.1% TFA) gradient (10–90%). ESI-MS was obtained in positive mode with the scanning range of 50 to 2000^{Th}(Antonious et al. 2015).

FT-IR

FT-IR spectroscopy was used to elucidate the chemical bonds and functional groups present in the BS structure (Pornsunthorntawee et al. 2008).BS sample was analysed in Alpha FTIR spectrophotometer(Bruker, Germany), equipped with Opus graph plotter(Pornsunthorntawee et al. 2008). The lyophilized dry biosurfactant sample was mixed with potassium bromide (KBr) and compressed into a tablet form (Yalcin E 2010), scanned under 700 to 4000cm^{-1} with the resolution of 4cm^{-1}.

NMR

TLC purified BS sample was dissolved in deuterated water (D_2O) in the concentration of 50µg/ml, and ^{1}H-NMR spectroscopy was done to determine the structural characteristics of the BSwith the instrument ‘Bruker AFCEND-Germany spectrophotometer’. The scanning was done at the frequency of 400mHz (Makkar and Cameotra 1999). The spectrograph was analysed with ‘Mesternova’ software.

Effect of Biosurfactant on seedlings germination

First, the seeds were washed with 3% sodium hypochlorite to make them free from undesired fungal spores. Five different sets of three petri plates were prepared with 10 ml of 0, 150, 300, 450 and 600µg/ mlBS solutions soaked in filter papers and incubated in the dark. In each plate, five healthy maize seeds were incubated, and the growth of the seedlings was observed for one week. The observation was taken at a regular interval of 24 h.

In-vitro effect of biosurfactant on plant growth

The soil samples were collected from different agriculture sites of Sumbuk village, situated in South Sikkim, India. The soil was sterilised by autoclaving at 121^{o}C for 20 min twice. Two sets of five pots were prepared and marked as lactic acid bacteria, and control treatment, respectively and each pot was filled with 1.25 kg of soil. In parallel, broth culture of *E. faecium* LM5.2 was prepared with the cell density of 10^8 cells/ml by adjusting the optical density 0.08 at the

wavelength of 600 nm with UV-Visible spectrophotometer (PerkinElmer Lambda 25) (Bhuvaneswari et al. 1980; Bai et al. 2003). A set of 5 pots was inoculated with 20 ml of culture broth with the initial population of LAB in each pot was about 8x 10^5cells per kg soil. An equal amount of sterilised MRS broth was added to the control pots. The pots were incubated for one week at room temperature in undisturbed condition. For the first week, the observation for the seedlings development was made every 24 hours. Later, the plant length, number of leaves, length of leaves, and the plant's width were measured after every week for one month. After one month, the plants were removed from the soil carefully without damaging the roots, and fresh weight, dry weight and moisture content were recorded (Bhuyan-pawar et al. 2015).

Effect of Biosurfactant on rhizosphere microflora

After harvesting the plants, each potting soil's microbial loads were analysed using the serial dilution method. Mycorrhizal spore was extracted from each set of 100g soil by the sieving method. Mycorrhiza spores were counted manually with the help of a zoom stereomicroscope.

Statistical analysis:

All the data were replicated at least thrice to obtain the mean and the standard deviation. The initial screening of biosurfactant was analysed by One way ANOVA followed by Bonferroni post hoc analysis to determine the significant differences ($p < 0.05$) among the means of different isolates. Student's two-sample t-test analysed differences in the means of the effect of plant growth. All the analysis were performed in RStudio 3.6.3.

Results

Fifty discrete circular-shaped colonies, with a clear zone in calcium carbonate, supplemented MRS agar were selected as lactic acid bacteria and were screened for biosurfactant production after obtaining each isolate's pure culture.

Screening for biosurfactant production

Out of 50 isolates, only ten isolates showed an oil displacement zone of 0.5 cm to 3 cm in diameter (Table 1). Isolates that showed a diameter less than 0.5 cm were not considered for further analyses. The isolate LM5.2 showed the maximum emulsification index with a value of 45.1 ± 0.89. Besides, isolates CLS6.1 and LM5.8 also showed a remarkable emulsification index compared to other isolates (Table 1). The isolate LM5.2 reduced the surface tension to 32.98 ± 0.23% as determined by the Du Noüy platinum ring method. The isolates CLS6.1, LM5.1 and LM5.3 also reduced the surface tension to 32.56 ± 0.42, 31.68 ± 0.76 and 31.68 ± 0.62, respectively. The maximum biosurfactant production was found in LM5.2 culture, which was 945.26 ± 4.62 mg/ml, which was statistically significant ($p < 0.05$). Hence, based on the oil displacement test and the quantity of biosurfactant produced, isolate LM5.2 was selected for identification and further analyses.

Table 1
Screening and production of biosurfactant produced by different LAB isolates from raw cow milk and homemade dahi

S. No	Isolate	Source	Oil displacement zone (cm)	Emulsification Index (%)	Surface tension reduced (%)	Surfactant produced (mg/L)
1	LM1.8	Milk	1	37.89 ± 0.75^{ab}	30.44 ± 0.5^{bc}	694.82 ± 9.83^{a}
2	LM5.1	Milk	1.5	38.51 ± 1.47^{ab}	31.68 ± 0.76^{abc}	736.00 ± 8.01^{b}
3	LM5.2	Milk	3	**45.1 ± 0.98^{a}**	**32.98 ± 0.23^{a}**	**1050.60 ± 4.62^{c}**
4	LM5.3	Milk	3	40.2 ± 0.98^{ab}	31.68 ± 0.62^{abc}	453.43 ± 3.54^{d}
5	LM5.3.4	Milk	3	37.41 ± 1.30^{ab}	29.70 ± 0.39^{c}	384.80 ± 2.11^{e}
6	LM5.8	Milk	1	41.7 ± 1.79^{ab}	31.46 ± 0.75^{abc}	258.47 ± 4.28^{f}
7	LM5.9	Milk	3	38.09 ± 0.95^{ab}	30.05 ± 0.41^{c}	166.00 ± 8.86^{g}
8	LM5.10	Milk	2	41.18 ± 0.00^{ab}	30.11 ± 0.28^{bc}	635.78 ± 4.27^{h}
9	CL6.1	Curd	1	44.65 ± 0.53^{a}	32.56 ± 0.42^{ab}	766.60 ± 5.12^{i}
10	CLS7	Curd	2	36.19 ± 0.95^{b}	30.60 ± 0.60^{abc}	563.58 ± 2.73^{j}

Data represents the mean ± SD, Standard deviation of three independent replication. Means with different superscripts along the column are statistically significant at $p < 0.05$.

Identification of bacteria

The isolate LM5.2 was found to be a Gram-positive, coccus, arranged pairs or chains, catalase-negative, KOH-test negative and did not produce CO_2 from glucose. Based on physiological and biochemical tests (Table 2), this bacterium was tentatively identified as *Enterococcus sp*. The 16S rRNA sequence was searched for the similarity with the NCBI data bank with the BLAST tool's help and confirmed its species as *Enterococcus faecium*. The sequence was submitted in NCBI GeneBank with the accession number MH733938. Molecular Evolutionary Genetic Analyses (MEGA) tool version 10.05 was used to construct the consensus neighbour-joining tree (Fig. 1).

Table 2. Phenotypic characteristics of biosurfactant producing *Enterococcus faecium* LM5.2 from cow raw milk

Microscopy			Growth in/ at						Sugar Fermentation					
			NaCl conc.		Temperature			pH						
Cell Size	Cell Morphology	Cell arrangment	6.5%	18%	10°C	20°C to 45°C	65°C	3.6 to 9.6	Cellobiose	Lactose	Rhamnose	Glactose	Arabinose	Ribos
0.5 -2µm	Coccus	Single / paired	+	-	-	+	-	+	+	+	-	+	-	-

TLC Analysis

The thin layer chromatogram of the biosurfactant showed the spots at the R_f values of 0.26, 0.41 and 0.51under UV_{254}, which indicated that the BS contains conjugated double bonds in its structure. Upon ninhydrin treatment, pink colour spots developed, which indicated the lipopeptide nature of the BS.

Mass spectroscopy

The biosurfactant of *Enterococcus faecium*LM5.2 showed the HPLC-MS peaks for m/z 1054, which corresponds to the surfactin linear chain of Glu- Leu^2- Val- Asp-Leu^2 with C14(Ma et al. 2016). The peak at m/z 985 corresponds to the same amino acid chain with the 12 CH group in the hydrophobic moiety. The peak at m/z 906 indicated the presence of β-hydroxyl fatty acid chain associated with Glu- Leu^2- Val- Asp- Leu amino acid chain of surfactin(Ma et al. 2016). The peak at m/z 832.52 corresponds to sodium ion associated with C_7 fatty acid and Glu-Leu^2- Val- Asp- Leu^2 amino acid chain (Ma et al. 2016). The next peak at 685 also showed the precursor of surfactin amino acid chain [H]-Leu^2- Val- Asp- Leu^2- [OH] (Ma et al. 2016), which is the characteristic feature of surfactin. Peaks at m/z 758 showed the impurities of di-rhamnolipid (Sen et al. 2017). A strong peak of m/z at 610 could not be identified.

FTIR

The biosurfactant's spectroscopy showed the absorbance at 944 cm^{-1}, representing CH = CH bonds in the structure (Silverstein et al. 2005). The sharp medium peak at 1026 cm^{-1} showed the C-O bond stretch, and minor peaks at 1046 cm^{-1} indicated the presence of alkanes of the fatty acid chain(Silverstein et al. 2005). Weak peaks at 1108 cm^{-1} show unsaturated alcohol or phenol group present in the structure(Silverstein et al. 2005). A weak peak at 1458 cm^{-1} could be because of lactones, which is the characteristic of surfactin. Weak absorbance at 1542 cm^{-1} shows the N-H bend of amide that can be present in the peptide chain of the biosurfactant. CO-N group of the peptide is associated with a medium height peak at 1646 cm^{-1}(Silverstein et al. 2005). A weak broad peak at 2512 and 2849cm^{-1} corresponds to the lipopeptide biosurfactant's carboxyl group, while small peaks at 2915 cm^{-1} indicate alkanes (C-H) stretch in the structure (Silverstein et al. 2005).

NMR

The BS isolated from *Enterococcus faecium* LM5.2 showed a duplet peak at 0.8 ppm, indicating the presence of a terminal primary alkyl group of the fatty acid (Fig. 4). A strong of triplet peaks at 1.20, 1.25 and 1.26 ppm, associated with peaks at 1.47 and 1.66 ppm, showed a secondary alkyl group of the hydrocarbon chain. A small duplet peak at 1.84 and 1.85 ppm indicated the non-saturated hydrocarbon chain of fatty acid. The peak at 2.10 and 2.32 ppm showed $–CH_2\text{-}COO^-$, duplet at 3.2 ppm corresponds to –O-CH- group present in the structure, and a sharp, strong peak at 4.35 ppm showed a terminal amino group of the amino acid(Balan et al. 2017). The peak at 8.1 and 8.3 ppm indicated terminal amide that is indicative of the presence of amino acid in the BS (Tiwary and Dubey 2018).

Effect of biosurfactant on seedlings germination: The seeds germinated in petri plates with the treatment of different concentration of BS showed maximum growth at 450 µg/ml with the mean length of 4.3 cm on the sixth day, while the mean length of the control set of seedlings was 2.4 cm under the same conditions (Fig. 5). The two way ANOVA analyses followed by multiple pairwise comparisons with Bonferroni adjustment showed that the effect of BS at 450 µg/ml had a significant effect ($p < 0.05$) on the germination and growth of the seedlings.

Effect of biosurfactant producing *E. faecium* LM5.2on the potted plants

Enterococcus faecium LM5.2 imparts a significant effect on different aspects of maize growth, including seed germination, plant height, leaf development, leaf growth, weight and moisture content of the plant. Plant growth was estimated in terms of the number of days for seed germination, plant height, number of leaves developed and the length of leaves for one month (Fig. 6, supplementary table 1). The germination of seedling in the *E. faecium* LM5.2 took 5 ± 0.4 days, while it took 8 ± 0.8 days in the control pots, which was statistically significant ($p < 0.05$). The mean plant height in treated soil was observed about 28.82 ± 1.6 cm, while the control plants showed the mean height was 15.48 ± 2.2 cm (significant at $p < 0.05$). The average length of leaves in one month in a treated set of plants was 15.91 ± 1.99 cm; on the other hand, the average leave length of controlled plants was 8.64 ± 1.10 cm. The fresh weight and moisture content of the treated plant set was 0.989 ± 1.79 g and 61.69 ± 0.06%, respectively, while the fresh weight and moisture content of control plants was 0.667 ± 3.65g and 52.82 ± 0.07%. No significant difference was observed in the number of leaves.

Effect of *E. faecium* LM5.2 on soil microbial count

The total mycorrhizal spores per 100 g soil in the treated and controlled pots were 1424.44 ± 551.26 and 275.56 ± 38.23 respectively, which may be directly responsible for the enhanced plant growth in the treated soil. The total microbial count in the treated soil was 3.32 x 10^8cfu/g, while the control set of soil samples had 8.9 x 10^6cfu/g.

Discussion

This work discusses the characterisation of BS from *Enterococcus faecium* LM5.2 with its application in agriculture. BS production was screened by the qualitative as well as quantitative parameters. BSs were purified, and different spectroscopic methods evaluated their chemical nature.

Milk and milk products are the rich sources of lipids/fats and lactose, which provides ideal conditions for the growth of BS producing lactic acid bacteria (Yilmaz et al. 2009; Augustin and Hippolyte 2012). Many reports are available on the BS produced by *Bacillu*s and *Pseudomonas* species (Yin, Hua Qiang, Jing Jia, Yan Ye, Jinshao Peng, Hui Qin, Huaming Zhang, Na He 2009; Saharan et al. 2011; Joshi et al. 2013; Chaurasia et al. 2020) while very few are available on LAB as a potential source of BS (Gudiña et al. 2010; Augustin and Hippolyte 2012; Sharma et al. 2015). These reports have mentioned LAB as an efficient source of rhamnolipids and other glycolipid biosurfactants (Velraeds et al. 1996; Sharma et al. 2015). To our knowledge, this is the first report that mentions *Enterococcus faecium* as a potential source of lipopeptide surfactant. For primary screening oil displacement method was used; as it is easy, fast and cheap. EI_{24} is used as a quantitative screening method for evaluating the emulsification efficiency of biosurfactant (Noudeh et al. 2010), which was performed with sunflower oil. Mineral oil produces lots of foam during the mixing process, making it difficult to measure the emulsification zone correctly. While the sunflower oil (or other vegetable oil) does not pose a foaming problem, the emulsification zone can be measured accurately.

Moreover, the measurement of surface tension helps us to identify the best quality biosurfactant produced among our isolate. In our screening, out of 50 isolates, ten were found the efficient biosurfactant producer, which showed that LAB could provide some novel biosurfactant with different applications in the food and pharma industry. TLC showed the spot at R_f value 4.1, which corresponds to iturin (Zeriouh et al. 2011). The next spot was at R_f value 5.1, which may be the surfactin's derivative (Fernandes et al. 2007). The R_f value at 2.6 can be fengycin (Dlamini 2017). MS spectrograph showed the series of m/z values of 1054, 980, 906, 832, 758, 684 and 610, which is actually obtained by repeated subtraction of 74 from 1054. The values m/z 1054, 906, 832 and 685 belonged to different sodium-associated surfactin A derivatives (Ma et al. 2016). The values in the range of m/z 1050 to 1058 has also been reported for sodium associated surfactin A (Pecci et al. 2010; Ma et al. 2016; Chaurasia et al. 2020). FTIR spectra showed CH=CH, CO-N and N-H, which indicated peptidyl group in the BS. Peaks of alkanes and alkenes were also found, which suggested the lipid hydrocarbon chain present in the structure. NMR analyses also support the presence of similar structural properties. All these pieces of evidence prove that the *Enterococcus faecium* LM5.2 is producing lipopeptide biosurfactant. To the best of our knowledge, this is the first report of a lipopeptide biosurfactant from *E. faecium.*

Corn plant variety 'Vivek Maize Hybrid 53' was selected for our experiment as this variety is suitable for cultivation in the hilly region like Sikkim. The BS from *Enterococcus faecium* LM5.2 showed a remarkable effect on the germination of the seedlings under *in-vitro* condition. Similarly, significant (p<0.05) growth enhancement by BS producing bacterium was also observed in the potted plant experiment. LAB, including *E. faecium*, produces organic acids in the soil to help in solubilizing the phosphorus and other essential minerals for the plant (Alaylar et al. 2018). Simultaneously, the biosurfactant reduces the surface tension and increases the bioavailability of the nutrients to the plant (Sachdev and Cameotra 2013). Sachdev and Cameotra have also reported that BS increases the bio-adsorption of the water and nutrients from the soil to the roots that result in the plants' fast growth (Sachdev and Cameotra 2013). These bacteria also support the mycorrhizal growth in the rhizosphere, which may be another reason for the relatively better plant growth than the control ones (Frey-Klett et al. 2007; Gamalero et al. 2009). Sikkim is an organic state where chemical fertilisers and pesticides have been completely banned since 2016. These GRAS bacterial isolates, the potent producer of BS, can be used as a biofertiliser for crop improvement to boost the region's organic agriculture.

Conclusion

This study showed that the *E. faecium* LM5.2 isolated from raw cow milk is the potent source of lipopeptide BS, specially surfactin. Though purified BS fastened the seed germination, BS producing *E. faecium* LM5.2 also showed good seed germination and growth of maize plants in the *in vivo* condition. This study indicated that the BS producing *E. faecium* LM5.2 has the potential of a biofertiliser for crop improvement to boost organic agriculture.

Abbreviations

BS Biosurfactants

EI_{24} Emulsification index

TLC Thin Layer chromatography

ESI & APCI MS Electrospray ionisation and atmospheric pressure chemical ionisation mass spectrometry

m/z mass/charge

FTIR Fourier Transform Infrared Spectroscopy

H^1NMR Proton Nuclear Magnetic Resonance

Ppm Parts per million

Declarations

Conflict of interest and ethical approval: The authors declare that they have no conflict of interest. This article does not contain any study with human participants and animals performed by any of the authors.

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Figures

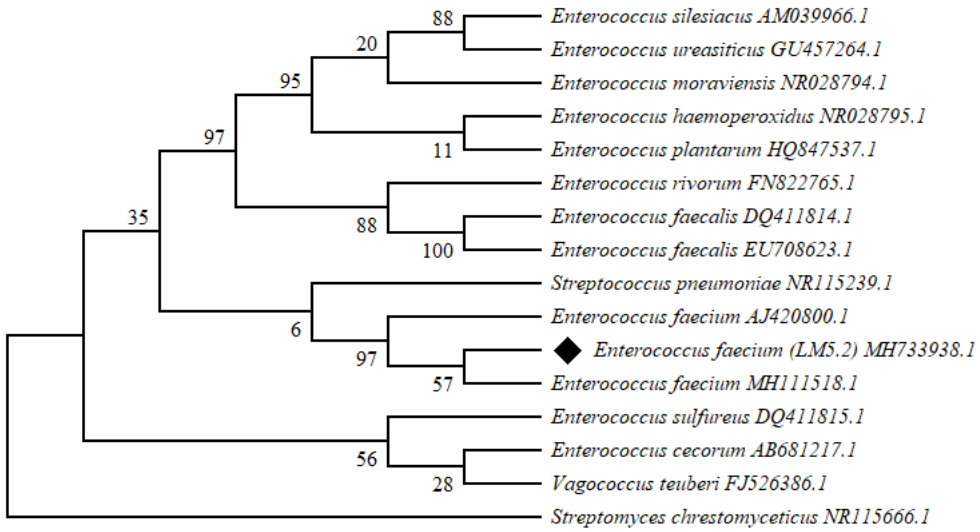

Figure 1

Molecular Phylogenetic analysis of Biosurfactant producing Enterococcus faecium LM5.2 by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar 2000). The tree with the highest log likelihood (-3876.16) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (16 categories (+G, parameter = 0.1735)). This analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 1229 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2016).

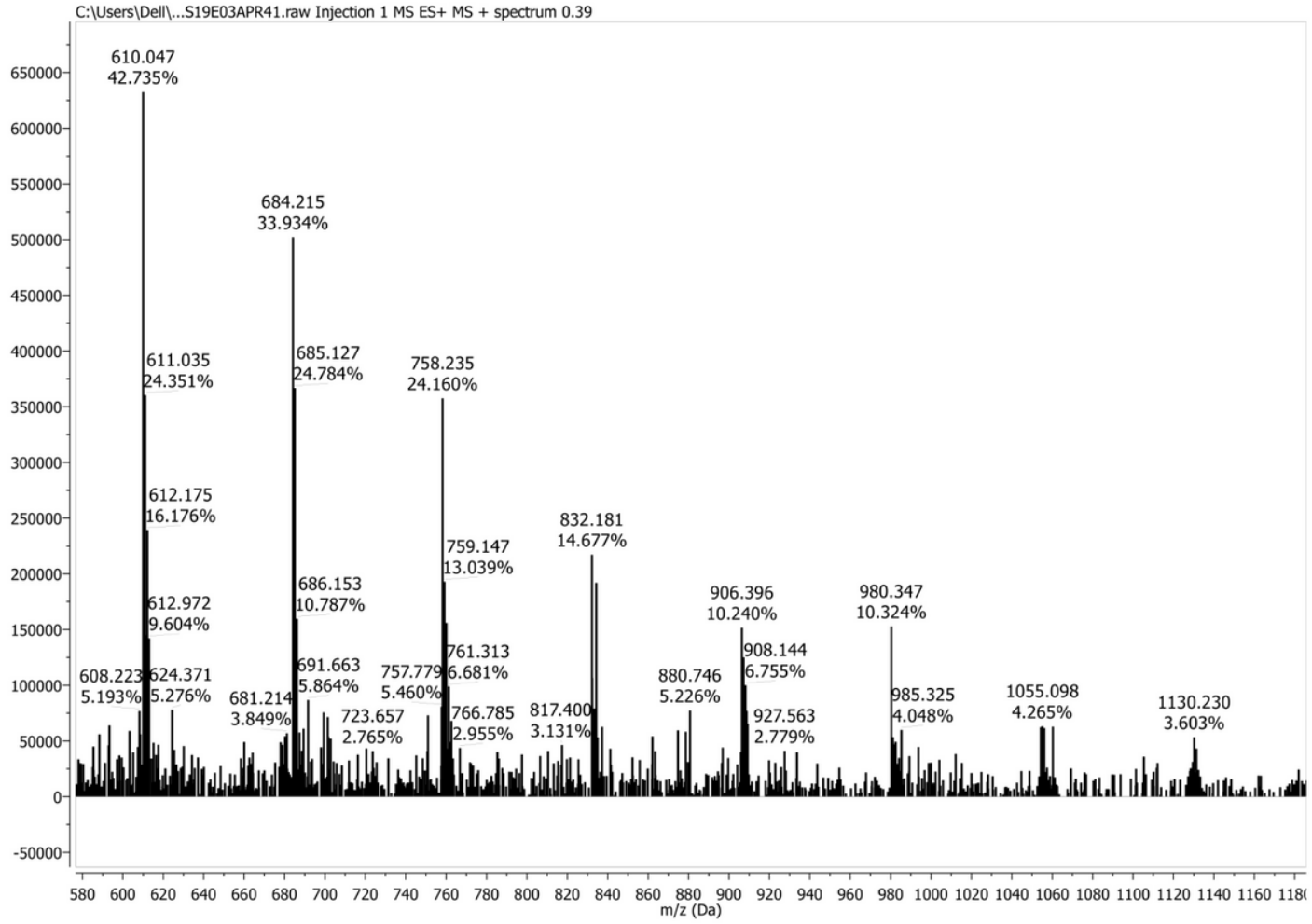

Figure 2

ES-MS spectroscopy of TLC purified biosurfactant from Enterococcus faecium LM5.2 with the peaks at m/z 1055, 985, 906, 832 and 685 for different derivatives of surfactins.

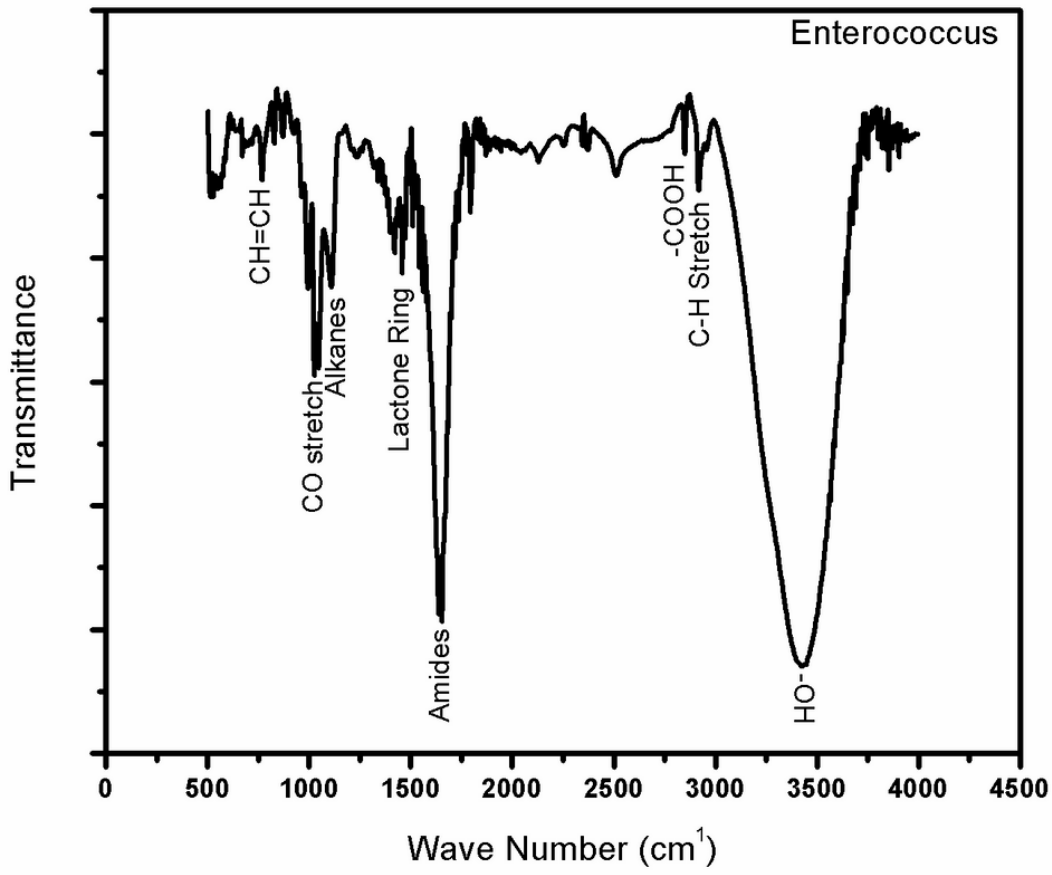

Figure 3

FTIR spectrum of Enterococcus faecium LM5.2 biosurfactant showing absorbance peaks for different lipopeptide functional groups and bonding.

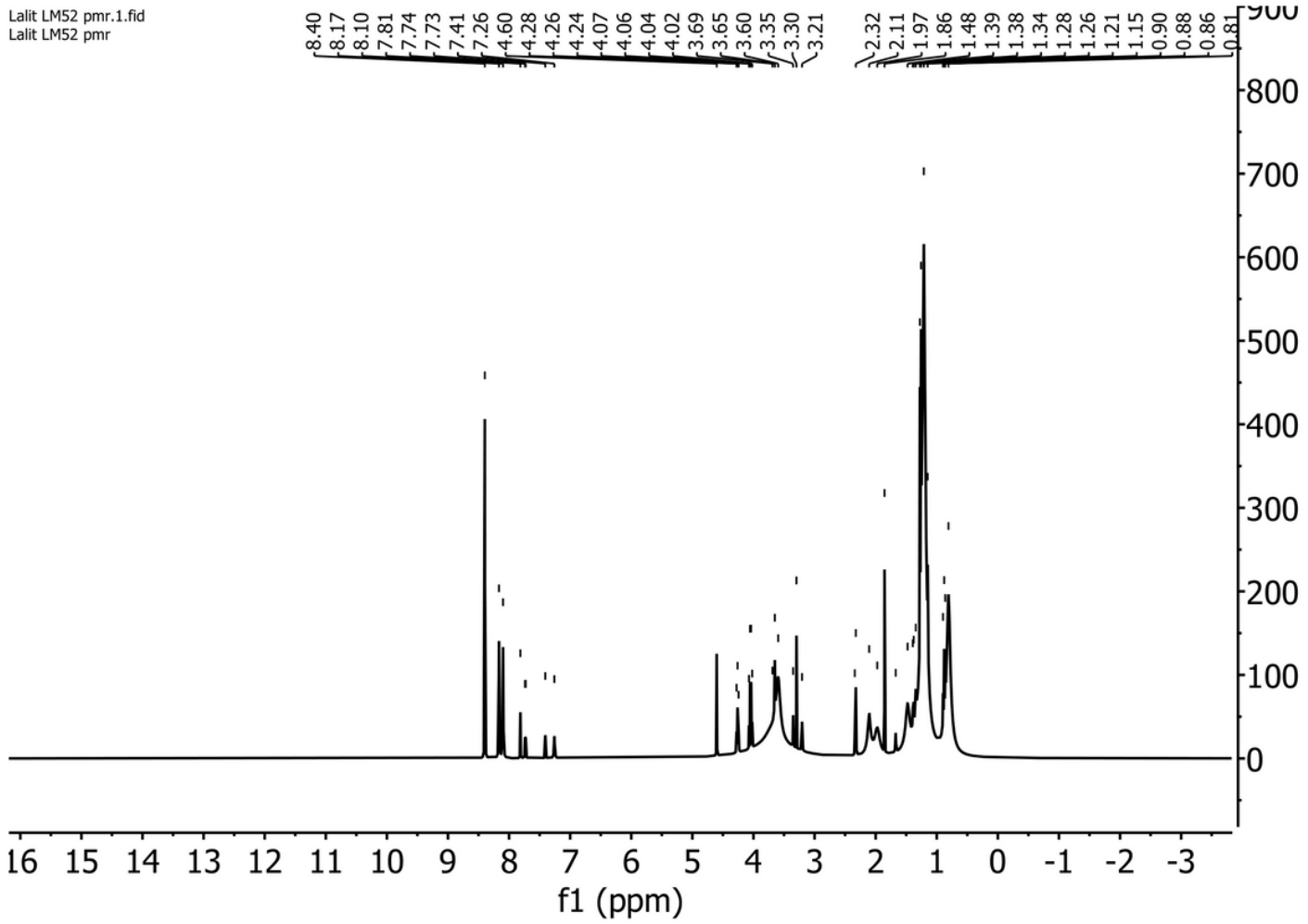

Figure 4

H1 NMR spectroscopic pattern of purified biosurfactant form Enterococcus faecium LM5.2 dissolved in deuterated methanol-d4 solvent. The peaks were developed in the range of 1.2 to 1.8 ppm indicates hydrocarbon chains of fatty acids; however from 2.1 to 8.3 showed the different bonds of peptidal chain.

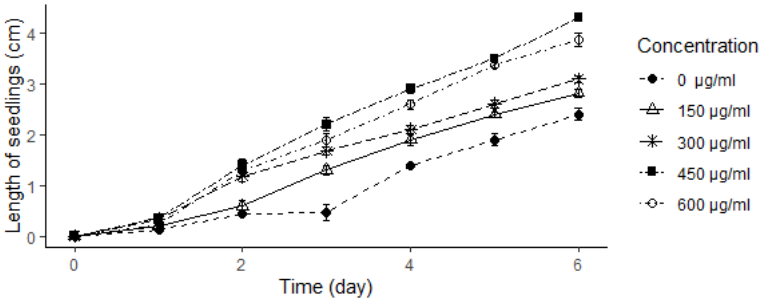

Figure 5

Effect of different concentrations of biosurfactant produced by Enterococcus faecium LM5.2 on the germination and growth of corn seeds under in-vitro condition.

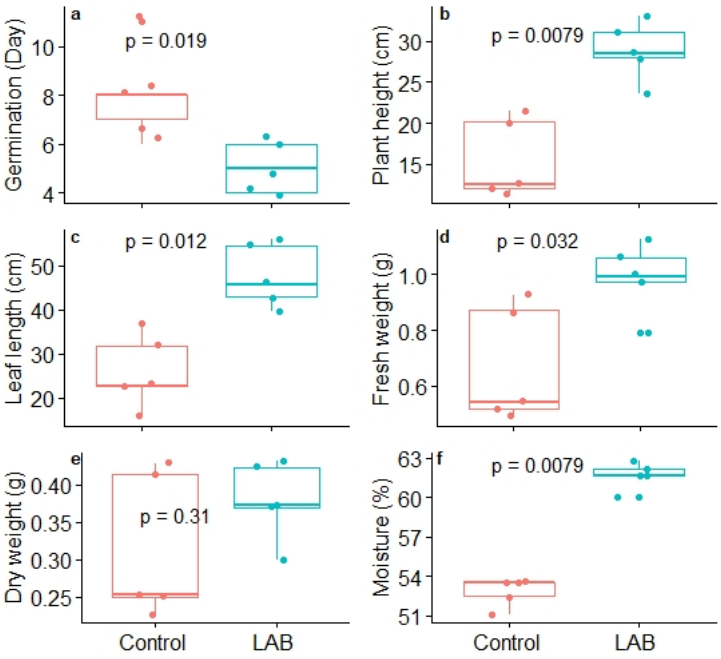

Figure 6

Boxplot showing effect of E. faecium LM5.2 on the plant growth with the respective p-values. (a) Effect on the germination time, (b) Effect on the height of the plant, (c) Effect on the leaf length, (d) Effect on the fresh weight of the plant, (e) Effect on the dry weight of the plant (f) Effect on the moisture content. Boxplot with red color represents the control set while the boxplot in peacock green represents the treatment group (LAB).

Supplementary Files

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- Supplementarytable1.docx