Current Journal of Applied Science and Technology

41(26): 12-23, 2022; Article no.CJAST.89657
ISSN: 2457-1024
(Past name: British Journal of Applied Science & Technology, Past ISSN: 2231-0843, NLM ID: 101664541)

Isolation of Biosurfactant Producing *Pediococcus pentosacaeus* from Laboratory Controlled (Simulated) Fermentation of Indian Wheat-based Seera

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Authors' contributions

The present study was carried out in the collaboration among all authors. Author TK conducted the study, managed literature, performed statistical analysis and wrote the first manuscript. Authors AS and NB designed the study and helped in the analysis of data. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CJAST/2022/v41i2631778

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/89657

Original Research Article

Received 14 May 2022
Accepted 19 July 2022
Published 22 July 2022

ABSTRACT

Biosurfactants are amphiphilic molecules produced by certain micro-organisms that have numerous applications in diverse sectors. Lactic acid bacteria (LAB) are preferred over other biosurfactant producing micro-organism due to their Generally Recognized As Safe (GRAS) status. The current study aims to isolate and screen biosurfactant producing LAB from laboratory controlled fermentation of Indian wheat-based Seera. Biosurfactant-producing isolates were screened by a battery of tests including drop collapse assay, surface activity, hemolytic activity, and emulsifying activity. The strain S-2 with better emulsification index i.e. 63.27±0.08, surface activity i.e 42.32±0.17, and biosurfactant yield i.e. 1.2±0.02g/L was chosen for further characterization. Morphological characterization of the strain S-2 was carried out and the strain was found to be gram-positive, coccus shaped, and lack endospore. Biochemical characterization and 16S rRNA sequencing confirmed the selected strain as *Pediococcus pentosacaeus.*

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Keywords: Biosurfactants; fermented food; Lactic acid bacteria (LAB); Pediococcus pentosaceus and Seera.

1. INTRODUCTION

Different ethnic groups of India consume a variety of fermented foods that are produced by different micro-organisms or enzymes, resulting in beneficial biochemical changes that produce significant modifications to the food, conferring several health advantages [1]. Numerous microorganisms are responsible for food fermentation including *Acetobacter, Bacillus,* yeasts, and molds. One of the major classes of micro-organism involved in fermentation is Lactic acid bacteria (LAB) which is responsible for the fermentation of an enormous variety of dairy products (fermented milk, cheese, yogurt), cereal, meat, vegetable products, etc. [2]. LAB including *Pediococcus, Enterococcus, Oenococcus, Lactococcus, Streptococcus, Lactobacillus, Leuconostoc, Weissella etc.* are prevalent in fermented foods. LAB are Generally Recognized as Safe (GRAS) according to the US Food and Drug Administration owing to their non-pathogenic nature [3].

Fermented foods are consumed in diverse forms, some are cereal-based while others are pulse-based, dairy-based, vegetables, and meat-based [4]. The majority of traditional fermented foods (wheat/barley/rice/buckwheat) are cereal-based. LAB further secretes a variety of metabolites such as organic acids, hydrogen peroxide, carbon dioxide, surface-active molecule, diacetyl, broad-spectrum antimicrobials such as reuterin, and bacteriocins [5,6]. Diverse LAB species produce surface-active compounds or biosurfactants in order to adapt and thrive on a different substrate along with other functions [7].

Biosurfactants are amphiphilic molecules having polar (hydrophilic) and nonpolar (hydrophobic) moieties that reduce the surface tension at the oil–water interface, improving the solubility of water-immiscible substances. They serve as an eco-friendly alternative to synthetic surfactants which have a detrimental environmental impact due to their non-degradable nature [8].

They are broadly classified as low molecular-weight microbial compounds (lipopeptides, glycolipids, etc.) and high molecular-weight microbial compounds (polysaccharides, lipopolysaccharides proteins, or lipoproteins), with the ability to reduce surface and interfacial tension and stabilize emulsions [9]. Many microorganisms such as *Acinetobacter sp., Bacillus sp.,Candida antartica*, and *Pseudomonas aeruginosa*, etc. are known to synthesize biosurfactants. Among all micro-organism, *Bacillus* and *Pseudomonas* are known to synthesize biosurfactants with high yield, but their applications are limited owing to their pathogenic nature, so there is a need to explore safer micro-organism i.e LABfor biosurfactant production. Several studies have shown that LAB strains produce biosurfactants, which are mostly a complex combination of proteinaceous substances, glycolipids, glycoproteins, or glycolipopeptides [10]. Probiotic biosurfactants impart effective antibacterial, anti-adhesive, anticancer, and antibiofilm properties. Furthermore, they have an advantage over traditional microbial surfactants since probiotics are an essential element of normal human microflora and their biosurfactants are non-toxic to humans, allowing them to be used safely and efficiently in the food and cosmetics industries. Moreover, biosurfactants due to their stability at extreme pH/ temperature, biodegradable nature, and low toxicity, are employed in various fields of bioremediation, food, pharmaceutical, agricultural, and cosmetics sectors [11].The present study aims to isolate biosurfactant producing strain from the fermentation of wheat-based Seera under laboratory conditions.

2. MATERIALS AND METHODS

2.1 Sample Preparation

10 grams of wheat (*Triticum aestivum*) grains were soaked in 150 ml of distilled water and allowed to ferment for 4 days in a sterile flask. Water was replaced at regular intervals and after fermentation grains were ground and incubated at room temperature for 1hour to allow the starch granules to settle. The surface water was discarded, and the starchy residue was collected aseptically [12].

2.2 Isolation of Lactic Acid Bacteria

A fermented Seera sample was homogenized and serially diluted with Phosphate Buffered Saline (PBS) solution (pH 7) aseptically.100µL of dilutions from 10-5 to 10-7 were spread on sterile De Man, Rogosa, Sharpe (MRS) agar plates and incubated for 24 hours at 37°C under anaerobic conditions. Different morphological colonies were

picked and streaked multiple times to obtain pure colonies [13].

2.3 Selection of Biosurfactant-producing Isolates

2.3.1 Hemolytic test

The screening of biosurfactant-producing Lactic acid bacteria (LAB) was carried out by streaking various isolates on a sheep blood agar plate (Himedia-MP1301) and incubating them at 37°C for 48 hours and observed for hemolytic activity [14].

2.3.2 Oil displacement method

In this test, distilled water (25 mL) was poured in an empty petri dish, followed by the addition of 25 µL of motor oil in the center of the dish to form an oil layer, and then 25 µL of cell-free supernatant of isolate was added to the oil layer. The presence of biosurfactants in cell free supernatant result in the displacement of oil by forming a clear zone [15].

2.3.3 Drop Collapse method

In drop collapse test, 15 µL of cell-free supernatant was pipetted on thin film and left undisturbed for 3 to 4 minutes. The flattening or spreading of droplet was observed. As a negative control, distilled water was used. The presence of biosurfactant in cell-free supernatant results in reducing force or interfacial tension between the liquid drop and the hydrophobic surface eventually resulting in the spreading and collapsing of droplets [16].

2.3.4 Emulsification activity

This activity was determined by adding 2 mL of cell free supernatant and 2 mL of vegetable oil to a test tube. This mixture was vortexed for 3 minutes at high speed and left undisturbed at room temperature foe 24hrs and Emulsification index (E24) was determined by following equation [17].

$$\text{Emulsification index} = \frac{\%\ \text{height of emulsified layer}}{\text{Total height of the column}}$$

Emulsification activity is proportional to the Emulsification index.

2.3.5 Surface tension

The surface tension was measured with the help of a Stalagmometer by drop weight method. Completly clean and dry stalagmometer was fixed with a clamp and distilled water was drawn into the capillary tube up to mark A and then allowed to fall naturally due to gravity in dry beaker. Upon collection of 30 drops in beaker, weight of the droplets was determined by using an electronic balance. The same process was carried out for cell free supernatant of different isolates. Modified MRS (without tween 80) without any inoculation used as control. Surface tension of sample was measured by using the following equation [18].

$$V_1 = \frac{W_1}{W_2} V_2$$

Where V1= surface tension of test liquid
V2 = surface tension of distilled water
W1=Weight of test liquid
W2= Weight of distilled water

2.3.6 Biosurfactant production

The production of biosurfactants was carried out for the strains that exhibited the presence of biosurfactant in their supernatant after a series of screening assays. 2 mL of overnight culture was inoculated in 150 mL of sterile modified MRS broth without tween 80 and incubated at 37°C for 48 hours after that culture was centrifuged at 9000 rpm for 15 minutes to obtain cell-free supernatant (CFS). The cell pellet was suspended in PBS and agitated gently for 2 hours to allow adhered biosurfactants to release from the pellet. It was again centrifuged at 9000 rpm for 15 minutes to recover the adhered biosurfactant [19].

2.3.7 Biomass determination

To determine biomass, 20 mL of culture broth was centrifuged at 8000 rpm for 15 minutes in pre-weighed centrifuge vials. The cell pellet was dried in an oven at 80°C for 24 hours after two washing with PBS, and the biomass weight was determined [20].

2.3.8 Biosurfactant extraction

The supernatant (CFS) was acidified to pH 2 with 2N HCl and stored at 4°C overnight. After three extractions with chloroform/methanol, the organic fraction was vacuum evaporated (2:1). Acetone was employed to recover the biosurfactant [21].

Table 1. Details of PCR primers

NO.	Primer	Sequence(5`à 3`)	Tm (°C)
1	16s Forward	GGATGAGCCCGCGGCCTA	73
2	16s Reverse	CGGTGTGTACAAGGCCCGGGAACG	78

Table 2. Cycling condition for PCR

Initial Denaturation	3 minutes at 94°C	
Denaturation	1 minute at 94°C	
Annealing	1 minute at 65°C	30 Cycles
Extension	2 minutes at 72°C	
Final Extension	7minutes at 72°C	

2.3.9 Determination of biosurfactants yield

A sterile glass petri dish was taken, and its weight was noted after that recovered biosurfactants were added to it. Then petri dish was kept in a hot air oven for a 30-minute at 100°C. Upon complete drying, the plate was weighed again.The following formula was used to determine the biosurfactant yield [22].

Yield of biosurfactants = (Weight of the plate after drying - weight of the empty plate).

2.3.10 Characterization and Identification of selected LAB

Among biosurfactant-producing isolates, the isolate with the highest biosurfactant activity and yield was chosen and identified morphologically, biochemically, and genetically. According to Bergey's Manual of Determinative Bacteriology, the selected strain was identified on the basis of morphological and biochemical characterization [23]. Gram staining, spore formation, and motility were utilized for morphological identification of LAB while for biochemical identification oxidase, catalase, indole production, nitrate reduction, methyl red, Voges-Proskauer citrate, H_2S production, and carbohydrate utilization assay were performed [24].

2.3.11 DNA isolation

DNA extraction was carried out as per the method reported by Leenhouts et al. [25]. 5 mL of culture was centrifuged after overnight growth. The pellet was rinsed with distilled water before being suspended in 1 mL of lysis solution containing 5 mg of lysozyme and of mutanolysin (30 U/ mL). The mixture was incubated at 37°C for 30 minutes before adding 20 µL of proteinase K (20 mg/mL) and 50 µL of 10% sodium dodecyl sulfate and then incubated at 60°C for 50 minutes. The lysate was extracted five times with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 2 volumes of 96 percent ethanol (-20°C) after 3 M sodium acetate (0.1 volume) was added. The DNA was dissolved in 150 µL of TE (10 mM Tris [pH 8],1 mM EDTA) with 20 µg of RNase. DNA concentration was determined using a spectrophotometer at an absorbance of 260nm.

2.3.12 Sequencing and phylogenetic analysis

DNA of selected strain was amplified according to the method described by Khedkar and Shanker [26] technique using 16s F243, 5′ GGATGAGCCCGCGGCCTA-3′ and reverse, R1378 5′CGGTGTGTACAAGGCCCGGGAACG-3′ primers.

The amplification product was purified and sent to BioKart Pvt. Ltd. in Bengaluru, India, for 16S rRNA sequencing and BLAST analysis was carried out to compare with the sequences available in the NCBI GenBank database. The sequences that showed the highest similarity were selected based on maximum identity score and phylogenetic tree was generated [27].

3. RESULTS AND DISCUSSION

3.1 Preliminary Screening of Biosurfactant Producing Strain

LAB isolated from freshly prepared Seera sample was screened for biosurfactant production. Only oxidase and catalase-negative strains were chosen as these are two important

characteristics of the biochemical traits ofLAB. Seven out of thirteen isolates tested negative for catalase and oxidase, and they were marked as S1, S2, S3, S4, S5, S6, and S7. These isolates were inoculated in a modified MRS medium (without Tween 80) for 24 hours at 37^{o} C and cell-free broth (supernatant) from various cultures were further tested for biosurfactant production using a battery of tests such as Hemolytic activity, Drop collapse assay, Oil displacement technique, surface tension, and Emulsification method, as shown in Table 3.

As illustrated in Table 3, out of seven isolates only three strains exhibited β- hemolytic activity, one strain exhibited alpha- hemolytic activity and three strains exhibited gamma hemolytic activity. The blood agar assay was used as the primary method for screening of biosurfactant producing strains by Carrillo et al. [28]. Many biosurfactant-producing strains exhibited hemolytic activity due to lysis of erythrocyte according to Puphan et al. [29]. However, there are certain strains that produce biosurfactants without showing any hemolytic activity [30]. This method is not selective since many lytic enzymes are known to produce clear zones by lysing RBC. Secondly, hydrophobic substrates cannot be employed as the sole carbon source in blood agar assay. Furthermore, various surfactants have varying diffusion properties, which might impede the production of a clean zone. As a result, this assay produces a large number of false negative and false positive outcomes, therefore hemolytic test alone cannot predict the biosurfactant producing nature of micro-organism. This assay should be employed with other surface activity assessment-based methods for preliminary screening of biosurfactant [31].

The presence of biosurfactant in the supernatant was indicated in the oil displacement test by isolate S-2 in Fig. 1.

Table 3. Preliminary screening for biosurfactant production by isolated strains

Isolate	Hemolytic activity	Drop collapse	Oil displacement method	Emulsification activity	Surface tension (mN/m) reduced To
S-1	β	+	11.2±0.05	36.56±0.03	69.35±0.02
S-2	γ	+++	40.3±0.06	63.27±0.08	42.32±0.17
S-3	γ	-	00.00	31.04±0.06	67.53±0.15
S-4	γ	-	00.00	13.64±0.05	70.36±0.13
S-5	β	-	05.5±0.03	19.33±0.12	68.57±0.08
S-6	β	++	23.4±0.50	54.24±0.05	59.24±0.03
S-7	α	++	27.2±0.44	49.36±0.07	52.74±0.05

(- = Negative, +++ = complete collapse after 1 min, ++ = collapse after 2 mins, + = collapse after 4 mins)
Values are mean ± standard error of means
Surface tension of distilled water was 72.21±0.03 mN/m.

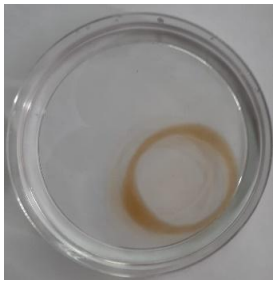

Fig. 1. Oil displacement test

Fig. 2. Drop collapse test of supernatant of different isolates

In the oil displacement test, cell-free supernatant of isolate S-2 showed maximum oil displacement i.e., 40.3±0.06 while S-6 and S-7 showed displacement of 23.4±0.50 and 27.2±0.44 respectively. Distilled water was used as negative control while SDS was used as positive control. The oil spreading approach is a reliable method for detecting biosurfactant synthesis by various microorganisms [32,33]. The presence of a biosurfactant decreases surface (liquid-air) and interfacial (liquid-liquid) tensions, hence oil was displaced at the interface between the two immiscible fluids (oil and water). The repulsive forces that exist between immiscible were diminished, enabling them to mix and interact more freely. As a result, the oil was pushed off the surface of the water, resulting in the formation of a clear zone [34]. The findings matched with the study of (Ghasemi *et al.* in terms of lactic acid screening [35].In this assay, the oil displacement area is proportional to the biosurfactant concentration present in the cell-free supernatant [36].

In the drop collapse assay, distilled water denoted by 0 act as negative control and Modified MRS broth (without tween 80) acts as a control which was denoted by C Fig. 2 depicts the differences in the diameter of droplets from different isolates on thin parafilm in a drop collapse test. Flattening of droplets of strain S-2 occurred within 60 seconds whereas strains S-6, and S-7 indicated a significant increase in drop diameter after two minutes. In drop collapse method, if no surfactants are present in the liquid, the polar water molecules are repelled off the hydrophobic surface, and the droplets stay stable, whereas presence of surfactants diminish the force or interfacial tension between the liquid drop and the hydrophobic surface, causing the droplets to spread or even collapse [37]. Drop stability is affected by surfactant concentration and is related to surface and interfacial tension. The results of the study were in accordance with the study reported by Cornea *et al*. for the drop collapse test for screening biosurfactant synthesizing micro-organisms [38].

According to the emulsification assay presence of biosurfactant in the supernatant significantly reduce the oil-water interfacial tension resulting in the emulsification of two immiscible liquids into a semi-stable mixture. As illustrated in Table 3, maximum emulsification activity was observed for isolate S-2 i.e., 63.27±0.08 among all isolates. The emulsification index correlates with the amount of biosurfactant. According to this study the emulsification activity of biosurfactant producing strains S-2, S-6, and S-7 also lie within the range reported by Akintokun *et al*. that was from 42.5 to 74.4% for biosurfactant producing strains [39].

Surface activity is the most reliable criteria for the selection of biosurfactant producing isolates. Biosurfactant potential is determined by its capacity to lower the surface tension of the productive medium [40].

During biosurfactant screening assay, the maximum reduction in surface tension was reported by strain S-2 from 72.21±0.03 to 42.32±0.17 followed by S-6 and S-7 isolates i.e., 59.24±0.03 and 52.74±0.05 respectively. According to the study reported by Rodrigues *et al.* LAB has tendency to reduce surface tension from 72 to 39 mN/m in case of *Streptococcus thermophilus* and 72 to 37 mN/m in case of *Lactobacillus fermentum* [41]. Based on the different screening assays strains S-2, S-6 and S-7 were selected further for biomass and biosurfactant production.

3.2 Biomass and Biosurfactant Determination

Among three isolates, the biomass and biosurfactant yield of strain S-2 was comparatively higher. Biomass yield of strain S-2 was found to be 3.7 ± 0.03 g/L whereas biosurfactant yield was 1.2±0.02 g/L, hence selected for further characterization.

According to the study reported by Ghasemi et al., biosurfactant yield from LAB*Pediococcus dextrinicus* was found to be 0.7g/L [42], whereas Souza *et al*. reported biosurfactant yield of *Lactobacillus lactis* to be 0.1–4.6 g/L [43]. Variation in biosurfactant yield was caused due to the distinct nature of the biosurfactant-producing strains and the different sources from which strains were isolated. Many additional factors impacted the yield, such as changes in growing conditions including temperature, pH, and medium composition. According to many studies, it was found that biosurfactants producing LAB were mainly isolated from fermented batters, fruits, vegetables, and milk products [44,45].

Table 4. Biomass and biosurfactant yield of isolates S-6, S-2, and S-7

Isolates	Biomass determination (g/L)	Biosurfactant determination (g/L)
S-6	2.2 ±0.04	0.4 ±0.01
S-2	3.7 ±0.03	1.2 ±0.02
S-7	2.7 ±0.01	0.7 ±0.02

Values are mean ± standard error of means

Table 5. Characterization of strain S-2

Morphological Characterization									
Colour					Off white				
Motility					-				
Cell shape (Negative staining)					Coccus				
Gram staining					+				
Endospore					-				
Biochemical characterization									
Catalase					-				
Oxidase					-				
Methyl Red					+				
VP reaction					+				
Indole					-				
Citrate					-				
Nitrate reduction					-				
H2S					-				
Carbohydrate utilization test									
Lactose	+	Trehalose	+	Glycerol	+	α- methyl-D – glucoside	+	Esculin hydrolysis	+
Xylose	+	Melibiose	+	Dulcitol	+	Rhamnose	+	D – Arabinose	+
Maltose	+	Sucrose	+	Inositol	+	Cellobiose	+	Citrate utilization	-
Fructose	+	L-arabinose	+	Sorbitol	+	Melezitose	+	Sorbose	+
Dextrose	+	Mannose	+	Mannitol	+	α- methyl-D – mannoside	+	Erythritol	+
Galactose	+	Insulin	+	Adonitol	+	Xylitol	+		
Raffinose	+	Sodium gluconate	+	Arabitol	+	ONPG	-		

3.3 Characterization and Identification of Selected LAB

Strain S-2 was selected and studied for morphological characteristics such as gram staining, morphology, spore formation, and motility using the LAB procedures outlined by Kozaki et al. [46]. Strain S-2 was found to be Gram-positive, cocci-shaped, non-motile, catalase-negative, and lack Endospore. The morphological findings were matched with the studies reported by Todorov and Dicks [47] & Cai et al [48].

Different biochemical tests were performed for the strain S-2 and the findings are reported in Table 5. Catalase, oxidase, citrate, nitrate reduction, and H_2S activities were absent while selected strain S-2 was found to be positive for MR, VP, and indole. The results of biochemical tests were also in accordance with the study reported by (Vidhyasagar et al. [49].

Carbohydrate utilization test was performed using Himedia carbokit, it was found that the S-2 strain has the ability to ferment all carbohydrates except ONPG and citrate as illustrated in Table 5. Fermentation of many sugars i.e. glucose, sucrose, galactose, mannose, xylose, fructose, and maltose was in accordance to study described by Albano et al. [50] but did not match with the fermentation pattern of arabinose, glycerol, sorbitol, and CMC (carboxy methyl cellulose). The present findings were also in accordance with the study reported by Cai et al. except for sorbitol and Ramnose [51]. The differences in the pattern of sugar fermentation were due to the isolation of strains from diverse origins with different growth conditions.

3.4 Agarose Gel Analysis

Fig. 3 depicts the result of gel electrophoresis. Lane 1 indicates Ladder that contains 10 DNA fragments of size 500 bp, 1000 bp, 1500 bp, 2000 bp, 2500 bp, 3000 bp, 3500 bp, 4000 bp, 5000 bp. Lane 2 indicates the size of PCR product that was approximately 1500 bp.

3.5 Phylogenetic Analysis

The 16S rRNA sequence of the selected strain S-2 was compared with the sequences which were already present in the GenBank database, it was found that the isolated strain was closely related with *Pediococcus genus*. The BLAST analysis revealed that the genus *Pediococcus* had 99.86 percent identity with *Pediococcus pentosaceus*. In Fig. 4, the length of a branch in a phylogenetic tree represents the degree of genetic change of 0.01. The selected strain was found to be the closest homologue to *Pediococcus pentosaceus* strain (KX886792.1) and the next the closest homologue was found to be Pediococcus pentosaceus strain (NR 04058.1). 16S rRNA sequence of strain S-2 *i.e. Pediococcus pentosaceus* was submitted to GenBank (National Center for Biotechnology Information, USA) and the accession number was obtained as OM843219.

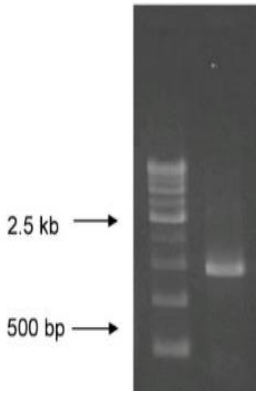

Fig. 3. Lane Description: Lane 1: Ladder; Lane 2: Sample

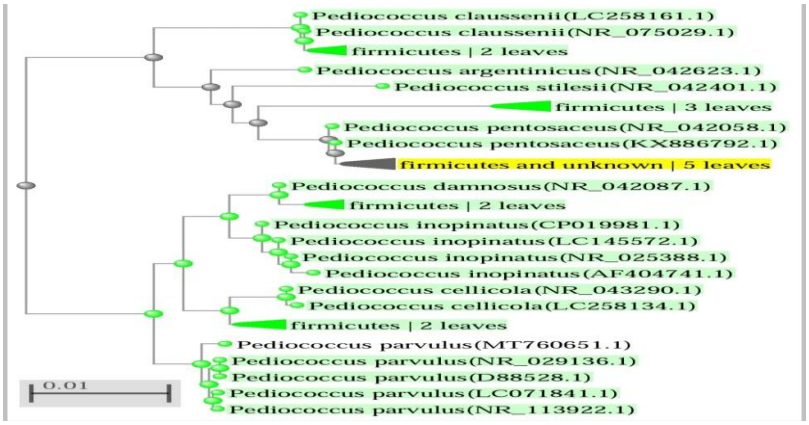

Fig. 4. Phylogenetic analysis of *Pediococcus pentosaceus* based on 16S rRNA genes

4. CONCLUSION

Fermented food products are a versatile source of LABthat produce a variety of metabolites. In the present study biosurfactant-producing LAB strains were isolated from fermented wheat product. Strain S-2 with the highest emulsification index of 63.27±0.08, surface activity of 42.32±0.17, and biosurfactant yield of 1.2g/L was selected among other isolates. Selected strain S-2 was found to be coccus-shaped, gram-positive, non-motile, and non-spore-forming micro-organism that had tendency to utilize different carbohydrates. The biochemical characterization and 16S rRNA sequencing confirmed the selected strain as *Pediococcus pentosaceus*. However, further research will be carried out, to determine the chemical nature of the biosurfactants, to optimize the culture conditions for enhancing biosurfactant yield and evaluate their anti-oxidative activity, antibacterial, antiadhesive activities, and anti-biofilm potential so it can be utilized in different sectors including pharmaceutical, cosmetics and food industries. Due to its unique properties, biosurfactant can act as a better candidate for replacement of synthetic surfactants.

ACKNOWLEDGMENTS

The authors greatly appreciate the Indian Council of Medical Research (ICMR), Delhi for the financial support to the first author for research in the present field.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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