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Characterization of Biosurfactant Produced by Probiotic Bacteria Isolated from Human Breast Milk

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Abstract

Introduction: Breast milk is an important nutrient source for rapidly growing neonates since breastfeeding protects newborns against a variety of diseases. This effect may be due to the useful and natural microflora of breast milk. Biosurfactants are unique amphipathic compounds produced by some microorganisms. The present study demonstrates the isolation and characterization of biosurfactant generating bacteria from human breast milk samples.

Methods: The human breast milk samples were collected aseptically and then cultured in MRS agar media. The biosurfactant producing ability of the isolated strains was investigated by hemolytic assay, oil spreading method, drop collapse test and emulsification index assay. The screened isolates were identified by 16S rRNA gene sequencing. In vitro antibacterial activities of biosurfactants against a number of common bacterial strains were investigated by the agar disc diffusion method. This biosurfactant was characterized by Fourier-transform infrared spectroscopy (FTIR).

Results: In this study, 337 different colonies were isolated from 42 breast milk samples. The best isolates were identified as *Pediococcus pentosaceus* HM-1, *P. pentosaceus* HM-2 and *P. pentosaceus* HM-3 based on microscopic and 16S rRNA gene sequencing analysis. The biosurfactant extracted from screened strains exhibited a broad spectrum of antagonistic activity against some pathogenic bacteria.

Conclusion: Bacterial strains isolated here can be valuable sources for novel biosurfactants. The Human breast milk is a safe source for obtaining biosurfactant producing probiotic bacteria and for improving gut microflora of infants.

Keywords: Human breast milk, Biosurfactant, Probiotic, *Pediococcus*, FTIR

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Introduction

Human breast milk is the best food for infants because it fulfills all the nutritional requirements for them, additionally; it educates the infant immunity functions and confers a certain degree of protection against infectious diseases.[1] These effects seem a result of the action of many bioactive molecules, present in breast milk, including different antimicrobial compounds, immunoglobulins, and immune cells.[2,3] The breast milk contains prebiotic substances that preserve the beneficiary bacteria in the infant gut.[3,4] Human breast milk is constituted from several bacterial species, such as Staphylococci, Streptococci, Micrococci, Lactobacilli, Enterococci, Lactococci and Bifidobacteria.[5,6]

Biosurfactants are heterogeneous amphipathic surface active molecules that belong to a wide range of chemical classes. These are mostly excreted by microorganisms outside the cells, and in some cases attached to the cells.[7] Microbial biosurfactants produced by a wide variety of microorganisms, have a lower toxicity and a higher biodegradability than chemical surfactants. They are also effective at extreme conditions regarding temperatures, pH and saline concentration. Microbial surfactants play an important role in a wide variety of industrial and biotechnological applications such as agriculture, food-processing, cosmetics and medical fields as antibacterial, antifungal and antiviral

agents.[7-10] This paper describes the separation and characterization of biosurfactant generating probiotics bacteria from human breast milk.

Methods

Sample Collection

Human breast milk samples were obtained from 42 healthy mothers in Kerman province of Iran. The samples were collected in an aseptic condition and stored on ice until delivery to the laboratory.

Isolation of Probiotics Bacteria

Direct isolation of the microorganisms was carried out using serial dilution (up to 10^{-7}) of breast milk samples in 0.85% sterile saline. Serial dilutions were plated onto Man Rogosa Sharp (MRS; Biolife. Italia)) agar using the spreading method**.** The cultures were incubated anaerobically at 35°C for 5-7 days. Morphologically distinct colonies were isolated and purified by replicating on the MRS agar medium to obtain pure cultures.

Preliminary Characterization of Biosurfactant Producing Bacteria

Hemolytic Activity

The bacteria were screened on blood agar (Merck, Germany) plates containing 5% (v/v) sheep blood and incubated at 35°C for 72 h. Hemolytic activity was detected as the presence of a clear zone surrounding the bacterial colonies.[9,11-13]

Oil Spreading Method

Pure isolates were cultured in MRS broth at 35°C and 200 rpm for 7 days. The broth cultures were centrifuged at 20000 rpm for 45 minutes.[11,14] The supernatant was subsequently subjected to the screening methods using oil spreading and oil collapse methods as below. Distilled water (25 mL) was added followed by addition of crude oil (100 µL). Then, the cell-free culture broth (20 µL) was dropped on to the crude oil surface. The diameter of the clear zone on the oil surface calculated and compared with those of negative control (only 20 µL of distilled water).[9,11-13]

Oil Collapse Method

Ten microliters of the crude oil were poured into the Petri dishes. After that, 10 µL of cell-free culture broth was added and the picture of the drop on the oil surface was inspected after 1 minute. Biosurfactant production was considered positive when the cultures giving flat drops. Those isolates that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production.[11]

Complementary Screening of Biosurfactant Producing Bacteria

Bacterial isolates positive for at least one primary screening methods were subjected to the complementary assays to verify their ability to produce biosurfactant.

Emulsification Capacity (E24)

The emulsifying capacity of isolated strains was evaluated by an emulsification index (E24). Six milliliters of crude oil and 4 mL of culture supernatant were combined. This mixture was vortexed at high speed for 2 minutes and allowed to stand for 24 hours. The E24 index was calculated as the ratio of the height of emulsified layer (cm) divided respectively to the total height of the liquid column (cm). The results were compared with distilled water as negative control.[15,16]

Identification of Bacterial Isolates

Phenotypic Characterization

Different phenotypic characteristics were evaluated, as outlined in Bergey's manual of determinative bacteriology, such as motility, Gram reaction, oxidase and catalase.[17]

PCR Amplification of Bacterial 16S rRNA Genes

Genomic DNA was extracted from a pure culture of three isolated bacteria using DNA extraction kit (Cinnagen Cat No: DN8115C), according to the manufacturer's instructions. These isolates showed the highest biosurfactant production and were isolated from three independent samples obtained from the separate individual. The purity of the extracted DNA was checked by recording the absorbance in 260 nm and 280 nm. The 2 universal oligonucleotide primers used to amplify the 16S rRNA samples were as follows: forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-CGGTTACCTTGTTACGACTT-3′). Polymerase chain reaction (PCR) amplification was performed in a total volume of 50 µL mixture, containing 4 µL templates DNA (50 ng), 5 µL of F primer (10 pmol), 5 µL of R primer (10 pmol), 1 µL of 10 mM 4 dNTP mix, 1.5 µL of 50 mM $MgCl_2$, 5 µL of 10X PCR buffer, 0.5 µL of Taq DNA polymerase (5 U μL^{-1}, Fermentas, Germany), 28 µL of sterile distilled water and microcentrifuged briefly. An initial denaturing step of 94°C for 3 minutes was followed by 25 cycles of amplification (1 minute 94°C, 1 minute 56°C, 1.5 minutes 72°C) and a final extension step at 72°C for 5 minutes. PCR products were separated by electrophoresis of 5 µL of PCR product in a 1% agarose gel for 2 hours and by staining with ethidium bromide. Amplification products were stored at -20°C. The clean PCR product was subjected to cycle sequencing in both directions.

Phylogenetic Analysis

Phylogenetic analysis was carried out as follows: the sequences were checked using Bioedit V.5.0.9.[18] A BLAST was carried out at http://blast.ncbi.nlm.nih.gov/Blast.cgi to identify the nearest neighbors.[19] Alignment, phylogenetic and molecular evolutionary analyses were

conducted using MEGA version 5.[20] A bootstrap test and reconstruction were done based on 1000 replications to confirm the validity of the phylogenetic tree.[21] The nucleotide sequences of 16S rRNA gene of bacterial strains (*Pediococcus pentosaceus* HM-1, *P. pentosaceus* HM-2, and *P. pentosaceus* HM-3) reported in this study have been deposited in GenBank under Accession No: KU527555, KU527556 and KU527557, respectively.

Characterization of Obtained Biosurfactant

Biosurfactant Recovery

Ten milliliters of an overnight culture of bacterial strains, in MRS broth, was added to 500 mL of MRS broth and incubated for 7 days at 35°C. The bacteria were removed (10 000 rpm for 15 minutes) to recover the biosurfactant. The pH of the supernatant was adjusted to 2 with 6 N HCl and then the solution was stored at 4°C for 24 hours. The precipitated biosurfactants recovered using a combination of chloroform and methanol (2:1 v/v) and mixed vigorously to obtain the biosurfactant within the organic layer. The biosurfactants move from the hydrophilic phase (MRS broth) into the organic, hydrophobic phase. This layer was separated using a separating funnel and dried at 50°C for 4-5 hours to obtain dry mass and analyzed by FTIR spectroscopy.[22]

Fourier-Transform Infrared Spectroscopy

Four milligrams of partially purified biosurfactant was dried (applying a freeze dryer) and then grounded with 100 mg of potassium bromide (KBr) and pressed to obtain translucent pellets. Then analyzed in an FTIR (Bruker: Tensor 27, Germany), device at the range of 400–4000 wavenumbers (cm^{-1}).

Antibacterial Activity of Biosurfactant

In vitro antibacterial activities of biosurfactants against Gram-positive (*Staphylococcus aureus* PTCC 1431 and *Bacillus cereus* PTCC 1015) and gram-negative bacteria (*Salmonella entritidis* PTCC 1709, *Escherichia coli* PTCC 1270 and *Pseudomonas aeruginosa* CZO Accession number: JX441328) were investigated by the agar disc diffusion method. The biosurfactants were dissolved in double-distilled water, sterilized by filtration and stored at 4°C. The sets of four dilutions (1000, 500, 250 and 125 mg/mL) of biosurfactants were prepared in double-distilled water. Mueller-Hinton sterile agar (Himedia, India) plates were seeded with indicator bacterial strains (10^8 CFU/mL) and allowed to stay at 37°C for 3 hours. Control experiments were carried out under similar condition by using Ampicillin (10 µg/ml), Gentamicin (10 µg/mL), Amikacin (30 µg/mL), Amoxicillin (25 µg/mL) and ciprofloxacin (5 µg/mL; Padtan Teb, Tehran, Iran) for antibacterial activity as standard drugs. The zones of growth inhibition around the discs were measured after 18 to 24 at 37°C.

Results

Separation and Characterization of Biosurfactant Generating Bacteria

The initial isolation yielded a total of 337 morphologically distinct microbial colonies. Among them, 63 isolates (54 gram-positive, catalase-negative cocci and 9 gram-positive, catalase-negative bacilli) were isolated. Among the isolated bacteria, 25 isolates gave a positive response to hemolytic activity; of these 10 isolates showed positive oil spreading test (Figure 1) and 9 positive responses were obtained by the oil collapse method. Data presented in Table 1 exhibits the screening methods of biosurfactant producing bacteria. The positive isolates were further evaluated by the secondary assay; i.e. Emulsification activity test (Figure 2). Following complementary screening, three potential biosurfactant producing strains (HM-1, HM-2 and HM-3) were selected.

Phylogenetic Analysis

Phylogenetic affiliation of the screened strains was ascertained by 16S rRNA gene sequence analysis. In order to find the most similar available sequences, a BLAST search was done in NCBI database. 16S rRNA gene sequence data of most closely related species were retrieved and used in tree construction to demonstrate the taxonomy of these isolates. Figure 3 shows the inferred phylogenetic relationships derived from a

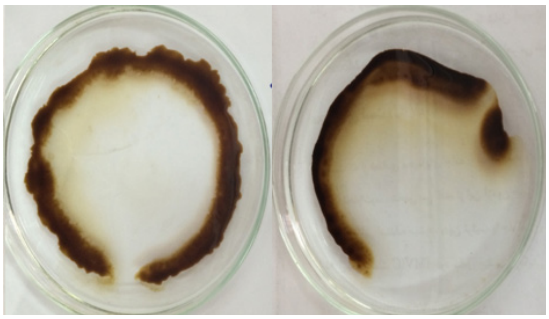

Figure 1. Oil Spreading Zone Exhibited by Isolated Strains.

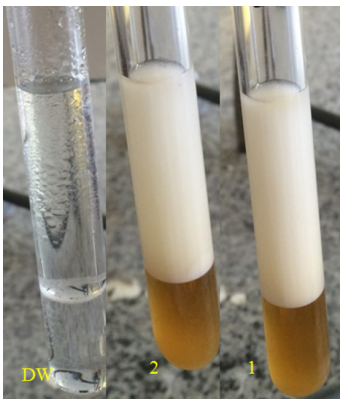

Figure 2. Emulsification Ability of Biosurfactant. 1: HM-1 strain, 2: HM-2 strain, 3: distilled water.

Table 1. Detection of Biosurfactant Producing Isolates by Preliminary and Complementary Screening Methods

Isolate	Preliminary Methods		Complementary Methods	
	Hemolytic Activity	Oil Spreading	Oil Collapse	E24
HM-1	+++	65*	+ + +	63.75 ± 0.56
HM-2	+++	77	+ +	62.33 ± 0.23
HM-3	+++	75	+ + +	21.11 ±0.25
HM-4	+++	85	+ + +	18.88 ± 0.33
HM-5	+++	64	+ + +	18.05 ± 0.23
HM-6	+++	64	+ + +	13.75 ± 0.3
HM-7	+++	67	+ + +	7.69 ± 0.46
HM-8	+++	47	+ + +	5.12 ± 0.91
HM-9	+++	50	+ + +	5.12 ± .25
HM-10	+++	58.3	+ + +	5 ± 0.23
HM-12	+++	0	-	0±0
HM-13	+++	0	-	0±0
HM-19	+++	0	-	0±0
HM-20	+++	0	-	0±0
HM-21	+++	0	-	0±0
HM-25	+++	0	-	0±0
HM-28	+++	0	-	0±0
HM-30	+++	0	-	0±0
HM-31	+++	0	-	0±0
HM-35	+++	0	-	0±0
HM-39	+++	0	-	0±0
HM-43	+++	0	-	0±0
HM-59	+++	0	-	0±0
HM-62	+++	0	-	0±0
HM-63	+++	0	-	0±0
Distilled water	-	0	-	1.25 ± 0.25

*Diameter of the clear zone in mm.

neighbor-joining analysis of 16S rRNA gene sequence of the *P. pentosaceus* HM-1, *P. pentosaceus* HM-2 and *P. pentosaceus* HM-3 with most validly described species of the genus *Pediococcus*.

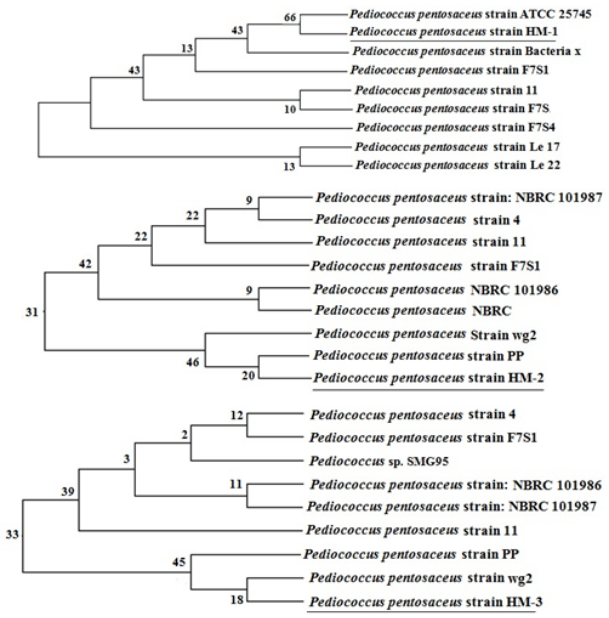

Figure 3. Neighbor-Joining Tree Based on 16S rRNA Gene Sequences, Showing Relationships of Screened Strains With Closely Related Members of the Genus *Pediococcus*.

Antibacterial Activity of Biosurfactant

The results regarding the antibacterial activity of the different concentrations of biosurfactants are indicated in Table 2. Biosurfactants isolated from screened strains showed a wide activity against the indicator strains (Table 2 and Figure 4). The antibiotic sensitivity pattern analysis of indicator strains was tested against 5 different commonly used antibiotics and the results were presented in Table 3.

Fourier-Transform Infrared Spectroscopy

The Fourier-transform infrared spectroscopy (FTIR) spectrum is illustrated in Figure 5, which shows broad stretching peaks at 3442 cm^{-1} (as the indicator of N–H stretching vibrations and strong hydrogen bonding). Absorption at 2997 and 2913 cm^{-1} are assigned to the symmetric stretch of CH_2 and CH_3 groups of aliphatic chains. The appearance of a weak absorbance signal at 2093 cm^{-1} may be due to C-N stretch. The bands observed at 1659 cm^{-1} is a definite indicator of linkages between the amides I and II. The absorbance in this region is significant in the presence of the peptide group in the molecule. The band peaks at 1437, 1408 and 1312 cm^{-1}, are characteristic of aliphatic chains' ($-CH_3$, $-CH_2$) bending vibrations. High-intensity peak in the region of 1047 cm^{-1} is assigned to O–C–O extend vibrations. The following vibrations observed at 500-1000 cm^{-1} are due to

Table 2. Antibacterial Activity of the Different Concentrations of Biosurfactants Against Indicator Strains

Strain	Biosurfactant	Inhibition Zone (mm)			
		1000 mg/mL	500 mg/mL	250 mg/mL	125 mg/mL
E. coli	BS-1	29 ± 0.577	16 ± 0.288	18 ± 0.763	11 ±0.5
	BS-2	27 ± 0.5	18 ± 0.288	14 ± 0.577	8 ± 0.763
	BS-3	26 ± 0.288	19 ± 0.5	13 ± 0.288	7 ± 0.5
S. aureus	BS -1	26 ± 0.5	20 ± 0.288	17 ± 0.763	12 ± 0.288
	BS-2	24 ± 0.763	23 ± 0.288	15 ± 0.763	10 ± 0.5
	BS-3	23 ± 0.577	19 ± 0.288	14 ± 0.5	11 ± 0.288
B. cereus	BS-1	23 ± 0.5	10 ± 0.763	0 ± 0	0 ± 0
	BS-2	22 ± 0.763	14 ± 0.763	0 ± 0	0 ± 0
	BS-3	22 ± 0.577	16 ± 0.5	2 ± 0.288	0 ± 0
P. aeruginosa	BS-1	24 ± 0.288	15 ± 0.763	14 ± 0.288	12 ± 0.577
	BS-2	23 ± 0.288	15 ± 0.577	14 ± 0.5	11 ± 0.5
	BS-3	22 ± 0.5	15 ± 0.288	13 ± 0.288	11 ± 0.288
S. entritidis	BS-1	45 ± 0.288	42 ± 0.288	44 ± 0.763	40 ± 0.288
	BS-2	43 ± 0.5	43 ± 0.5	40 ± 0.763	38 ± 0.288
	BS-3	49 ± 0.288	45 ± 0.288	0.5± 40	35 ± 0.5

BS1, BS-2 , and BS-3: The isolated biosurfactants from HM-1, HM-2 and HM-3 strains.

Table 3. Antibacterial Activity of the Standard Drugs Against Indicator Bacterial Strains

Strain	Inhibition Zone (mm)				
	Gentamicin	Ampicillin	Amoxicillin	Amikacin	Ciprofloxacin
E. coli	18 ± 0.288 S	9 ± 0.763 R	10 ± 0.288 R	15 ± 0.577 S	28 ± 0.288 S
S. aureus	17 ± 0.577 S	14 ± 0.288 S	20 ± 0.577 S	20 ± 0.763 S	29 ± 0.5 S
B. cereus	17 ± 0.763 S	43 ± 0.288 S	40 ± 0.288 S	14 ± 0.288 R	28 ± 0.5 S
P. aeruginosa	21 ± 0.763 S	0±0 R	0 ± 0 R	23 ± 0.288 S	33 ± 0.763S
S. entritidis	18 ±0.577 S	14 ± 0.5 I	18 ± 0.5 S	17 ± 0.5 S	27 ± 0.288 S

S: Susceptible, I: Intermediate, R: Resista.

out of plane C-H bending.

Discussion

Human milk is a complex biological fluid adapted to fulfill the nutritional requirements of the rapidly growing infant. The characterization of the breast milk microorganisms contributes to addressing the biological role of milk microbiota in the maintenance of health of the newborn and lactating mother.[2,3] The studies microbial features of human breast milk are restricted to those bacteria responsible for infancy infections. However, it is clear that the prevention of infant against pathogens is related to the natural flora of human milk.[3] Although there is limited knowledge about the commensal or probiotic bacteria in breast milk, Staphylococci, Streptococci, Micrococci, Lactobacilli, and Enterococci constitute the majority.[2,3,23] Biosurfactants are diverse groups of amphiphilic compounds with great diversity, environmental acceptability and a broad spectrum of functions and industrial applications.

The biosurfactant producing bacteria were checked using hemolytic activity, oil collapse and oil spreading tests. Selection of these methods was due to their strong advantages including simplicity, low cost, quick implementation and use of relatively common equipment that is accessible in almost every microbiological laboratory.[11] The results of our experiments indicated, 39.6% of total isolates were positive for hemolytic activity, 36% were positive for oil collapse and 40% were considered positive based on oil spreading and since these methods have shown differences, the isolates with more than one positive response were exposed to complementary screening including emulsion activity measurements. In the present study, three bacterial isolates (*P. pentosaceus*

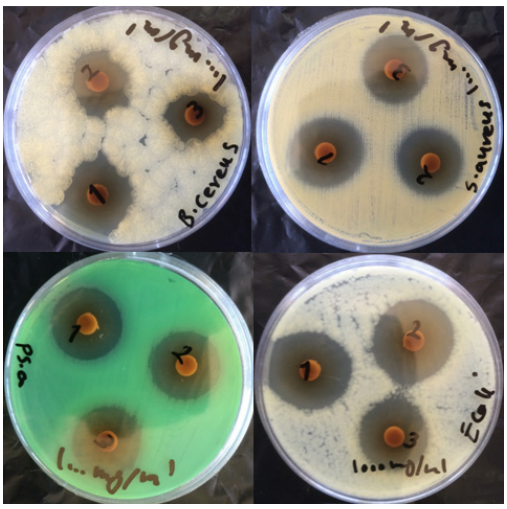

Figure 4. Antibacterial Activity of the Biosurfactants on the Growth of the Indicator Bacteria by Disc-Diffusion Method.

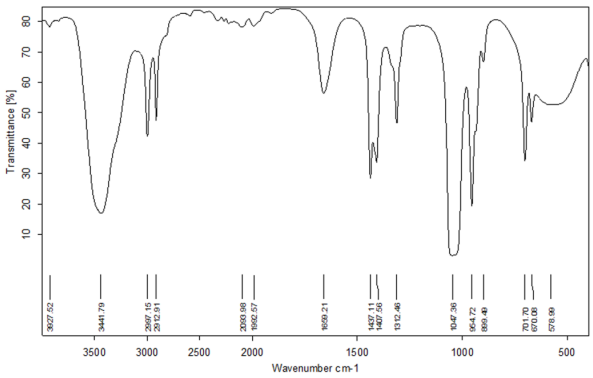

Figure 5. FTIR Spectra of the Biosurfactant Produced by HM-3 Strain

HM-1, *P. pentosaceus* HM-2 and *P. pentosaceus* HM-3) with biosurfactant-producing ability were isolated and screened from human milk samples. The biosurfactants derived from isolated strains showed significant antimicrobial activities against indicator bacteria at different concentrations. Baraka and Al-Rubayyi reported that the breast milk exerted bactericidal activity against *E. coli, P. aeruginosa S. aureus* and *Salmonella* sp.[24] The antimicrobial activity results generated from our studies are in agreement with that reported by Ibhanesebhor and Otobo for inhibitory activities of human colostrum against *S. aureus* and coliform organisms.[25]

Our findings demonstrated that biosurfactants produced by screened strains, compared with synthetic antibiotics, had acceptable antimicrobial activity. Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial effects of prebiotic bacteria are formed by producing a variety of active biological compounds.[26,27] For identifying types of functional groups in the unknown biosurfactants, FTIR analysis was used. The FTIR profile of the biosurfactant showed similarity to surfactin, a lipopeptide biosurfactant, and other lipopeptide biosurfactants like arthrofactin[28] and lichenysin[29] confirming the lipopeptide nature of the produced biosurfactant. According to Khopade et al, lipopeptide surfactants are potent antibiotics and had a wide antimicrobial activity.[30]

Conclusion

Biosurfactant producing probiotic bacteria could be safely isolated from human milk. These bacteria are necessary for improving intestinal microflora of infants. The antibacterial properties of the produced biosurfactant against bacterial strains suggest its potential use in the development of new pharmaceutical preparations.

Ethical Approval

Not applicable.

Competing Interests

None.

Acknowledgments

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