

Screening, Gene Sequencing and Biosurfactant Production from *Pichia Fermentans* Isolated From Dairy Effluents

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Abstract : Microbial surfactants are surface metabolites that produced by bacteria, yeast, and fungi having varied chemical structure and properties. These molecules reduce surface tension between aqueous solutions and hydrocarbon mixtures. Recently biosurfactants are potential alternatives for chemically synthesized surfactants having varied industrial applications. In this study, we collected fermented dairy whey waste samples from a small dairy farm house located in Salem and Coimbatore and a total of fifty yeast strains were isolated, screened for biosurfactant activity, and cultured by enriching carbon and nitrogen sources. These were conducted using oil displacement technique, drop collapse method, microplate assay method, and emulsification activity determination (EA). Among fifty isolates, SS2 yeast strains exhibited positive for biosurfactant activity. The yeast strain SS2 exhibited an oil clearance diameter of 12 mm with an interpolated surfactant concentration of 2.77 micrograms and measured an emulsification index is 61.42 with a height of emulsion 17.2 mm after 120 hours. Biochemical analysis and molecular characterization with 18S rRNA gene sequence indicates that yeast strain SS2 belongs to *Pichia* species and closely related to *Pichia fermentans*. Studies of biosurfactant production on various carbon and nitrogen sources were performed. The communication looks at the future perspective of biosurfactant production and its application in the healthcare and pharmaceutical sector.

Keywords: Microbial surfactants, 18S rRNA-ITS gene sequencing, *Pichia fermentans*

I. Introduction

Microbial surfactants are important biotechnological products with a wide range of applications in many industries such as food, cosmetics, and pharmaceutical industries [1]. These surfactants are produced by a variety of microorganisms, secreted either extracellular or attached to parts of cells, predominantly during growth on water immiscible substrates. They are categorized mainly by their chemical composition and their microbial origins. The microbial surfactants are structurally diverse group of surface active molecule which can be categorized as glycolipids, lipopeptides, fatty acids, polysaccharide-protein complexes, phospholipids, peptides, and neutral lipids [2]. These amphiphilic compounds contain a hydrophobic and a hydrophilic moiety, and have the ability to reduce the interfacial tension between different fluid phases. It is less toxic, highly biodegradable, with better environmental compatibility, high foaming and selectivity, and specific at extreme temperature, pH, and salinity [3]. These molecules reduce surface and interfacial tensions in both aqueous solution and hydrocarbon mixtures [4]. Its biodegradability, ecological safety, production from renewable resources, and functionality under extreme conditions make these microbial surface active agents superior to chemical surfactants [5]. The surface active compounds commonly used in medical and industrial applications are chemically synthesized which can be replaced by biosurfactants produced from microorganisms [6,7]. The surfactant market exceeds US\$9.4 billion per annum with the demand expected to increase at the rate of 35% per year. According to the Technical Insight, a division of Frost & Sullivan, microbial surfactants have begun to enjoy a market [8]. According to the Transparency Market Research, the global biosurfactant market was worth USD 1,735.5 million in 2011 and is expected to reach USD 2, 210.5 million in 2018, growing at a CAGR of 3.5% from 2011 to 2018 [9]. Many studies point out to the increased demand for synthetic surfactants globally in spite of that being a reason for an environmental hazard. Minimal studies have been reported on microbial surfactants synthesized by yeasts [10], and most of them are related to bacteria [11]. Recently yeasts are known for producing microbial surface active agents in higher concentration than bacteria, especially due to their GRAS (generally regarded as safe) status [12]. The microorganisms use agro-industrial wastes as carbon sources and several other renewable substrates for the production of microbial surfactants. The choice of cheap raw materials is important to the overall economy of the process and also reduces the expenses with waste treatment [13]. The usages of low cost substrates as carbon sources make the production of microbial surfactants economically viable. Agricultural wastes, fermented whey waste, effluents from food industries, and residual waste containing high levels of carbohydrates and lipids with reduced production costs and minimal volume of waste dumped into the environment [14]. The effluents of vegetable oil industries, petrochemical oil residues or by the byproducts from the food industries have been extensively studied for microbial production at an experimental scale [15]. Recently there has been worldwide interest on molecular techniques [16].

Amplification and sequencing of target regions within the ribosomal DNA has emerged as a useful tool for the identification of yeast strains [17].

The present work was done to assess, isolate, and select yeast strains from fermented dairy whey waste (effluent), collected from in and around Coimbatore and Salem districts, which possesses a potential for the production of biosurfactant.

II. Materials and Methods

2.1 Sample collection and microbial culture maintenance

Fermented dairy whey waste was collected from a small dairy farm house located in Mettur, Salem district and Avinashi, Coimbatore and stored at 4°C until analysis. The collected sample were serially diluted in buffered peptone sodium chloride solution (pH 7) and pour plate technique was performed and inoculated into Yeast Extract Peptone Dextrose (YEPD) and Rose Bengal Chloramphenicol agar (RBCA) and incubated at 30°C for 48 hr. Transfers were done to fresh agar slants each month to maintain viability.

2.2 Isolation of biosurfactant producing yeast strains

Screening for a total of fifty colonies with yeast like colony morphology were stained and observed. Yeast like cells were subcultured and maintained in YEPD and transfers were done to fresh agar slants to maintain viability. All chemicals used for the work were of analytical grade and media components were purchased from Hi-media (Mumbai, India). To determine the biosurfactant production ability, a total of fifty yeast isolates were inoculated into mineral salt medium (2.5 g/l of NaNO₃, 0.1 g/l of KCL, 3.0 g/l of KH₂PO₄, 7.0 g/l of K₂HPO₄, 0.01 g/l of CaCl₂, 0.5 g/l of MgSO₄.7H₂O) supplemented with 5ml of trace element solution (0.116g/l of FeSO₄.7H₂O, 0.232 g/l of H₃BO₃, 0.41 g/l of CoCl₂.6H₂O, 0.008 g/l of CuSO₄.5H₂O, 0.008 g/l of MnSO₄.H₂O, 0.022 g/l of [NH₄]₆Mo₇O₂₄, 0.174 g/l of ZnSO₄) and final pH was adjusted to 7.0. Five percent of the inoculums were inoculated into MSM medium containing 2% of a mixture of fermented whey wastewater and glucose as a source of carbon and energy source, incubated at 200 rpm at 30° C for 7 days. The culture suspension was then subjected for screening the biosurfactant production capacity of yeast isolates.

2.3 Biochemical analysis of SS2 yeast isolate

Yeast strain SS2 was isolated, identified by physiological activities and biochemical characteristics and confirmed by the Internal Transcribed Spacer (ITS) rDNA sequence analysis. Yeast Nitrogen base broth (YNB) was used. YNB broth was prepared by adding following ingredients: (g/l) Ammonium sulfate 5g, KH₂PO₄ 1g, MgSO₄ 5g, NaCl 0.1g, CaCl₂ 0.1g, Inositol 0.002g. The medium was filter sterilized with 1% Carbohydrate used in the experiment. After sterilization the media was added into microtiter plates and inoculated with the respective isolates and incubated for 24 to 48 hr at 30°C.

2.4 Identification of SS2 yeast isolate using 18S –ITS sequence of rRNA based molecular technique

Samples of the isolates were sent to Aristogene Biotech at Bangalore for molecular characterization. Fungal DNA was isolated from the respective isolate that showed positive biochemical analysis. DNAzol method [18] was carried out for yeast DNA isolation. The yeast cell pellet was with 0.5 ml of PBS, centrifuged for five minutes at 6000 rpm. Pellets were dispersed and treated with 3ul of 10mg/ml Proteinase K for 30 minutes at 65°C. Then 0.5 ml of DNAzol was added and incubated at 45 °C for one hour with intermittent mixing. This was centrifuged at high speed for fifteen minutes and supernatant was transferred into fresh vial. Equal volumes of isopropanol was added and mixed well. It was then centrifuged at high speed for fifteen minutes and supernatant was discarded. To the pellet, 70% ethanol was added and centrifuged. Then pellet were air dried and suspended in 30 µl of TE. Silica spin columns and buffers were obtained from Qiagen. Following lysis, purification and quantification of eluted DNA samples were done by loading into the Agrose gel [19]. 18s rRNA gene primers were used to amplify ~1.1Kb gene from the genomic DNA isolated. Both forward and reverse primers were used for species identification according to the method suggested by Olmos *et al*, 2002 [20]. PCR product obtained was gel purified, quantified, and sent for sequencing. The analysis of nucleotide sequence was done in Blast-n site at NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>) and consensus sequence generated from forward and reverse sequences using Aligner software. This sequence was subjected to BLAST with NCBI Genbank database. A phylogenetic tree was constructed for SS2 yeast sequence.

2.5 Preliminary screening for biosurfactant activity

The culture supernatant was tested for biosurfactant activity by oil spreading technique, drop collapse technique, and Microplate assay method.

Oil displacement technique: For the oil spreading technique, 50 ml of distilled water was added to a large petri dish followed by addition of 20µl of crude oil to the surface of the water. Ten microliters of culture were then added to the surface of oil [21]. The diameter of the clear zone on the oil surface was measured. The

diameters of duplicate samples from the same culture of each strain were determined. The unknown biosurfactant concentration was determined by comparing with the concentration of the standard surfactant Tween-80 in various dilutions and a standard graph was plotted.

Drop collapse technique: For this technique, each well was coated with 7 μ l mineral oil and left for 24 hr at room temperature. After that, 20 μ l of supernatant and 20 μ l of each control solution were added to each well by the syringe at an angle of 45°. After 1 min the drops were examined visually. The syringe was rinsed three times with distilled water, three times with alcohol and three times with ether between each measurement. All experiments were repeated three times. Biosurfactant-producing cultures gave flat drops with scoring system ranging from '+' to '++++' corresponding to partial to complete spreading on the oil surface. Aliquots from a culture of each strain were analyzed on two separate microtiter plates [22].

Microplate assay technique: The surface activity of individual strains can be determined qualitatively with the microplate assay technique developed and patented by Vaux and Cottingham [23]. This assay is based on the change in optical distortion that is caused by surfactants in an aqueous solution. Pure water in a hydrophobic well has a flat surface. The presence of surfactants causes some wetting at the edge of the well and the fluid surface becomes concave and takes the shape of a diverging lens. For this assay, a 100 μ l sample of the supernatant of each strain is taken and put into a microwell of a 96-mircowell plate. The plate is viewed using a backing sheet of paper with a grid.

2.6 Emulsification activity and emulsification index calculation

The results of preliminary screening indicated highest biosurfactant producing activity in SS2 yeast isolate, among fifty isolates. Emulsification activity was done for qualitative analysis of its biosurfactant producing property. It was determined by the addition of 2 ml of n-hexadecane to the same volume of culture or purified biosurfactant or commercial chemical surfactant solutions in a test tube which was mixed vigorously with vortex for 2 min. The tubes were incubated at 25°C and the emulsification index (EI) was determined after a given time (t) according to Equation below:

$$EI_t = \left(\frac{H_e}{H_t} \right) \times 100$$

where H_e and H_t are the height of emulsion and total height of the liquid in the tube, respectively. All emulsification indexes were performed in triplicate. To study the emulsion stability the same protocol was used; the emulsification index [24, 25].

2.7 Production of Biosurfactant

2.7.1. Biosurfactant production by identified yeast strain SS2 grown on various carbon sources

The biosurfactant is produced by cultivation of SS2 yeast strain (*Pichia sp.*) in a 250ml production medium containing 0.2% NaNO₃, 0.02% KH₂PO₄, 0.02% MgSO₄.7H₂O, 0.1% Yeast extract, 4% and 8% of various carbon sources such as glucose, corn starch, glycerol, coconut oil, and soybean oil in a 500ml Erlenmeyer flask with shaking at 200 rpm for 7 days at 30°C. Cell culture (6% v/v) was used as inoculums which was an overnight culture, maintained at pH 4.5. Aliquots of the culture were removed periodically for growth and biosurfactant activity analysis. To determine the optimum concentration of the carbon source, concentrations of 1-8% were used to determine biosurfactant producing activity. Biosurfactant activity in the culture was determined by measuring the oil displacement activity as described by Morikawa *et al.*

2.7.2. Biosurfactant production by identified yeast strain SS2 grown in various nitrogen sources

The biosurfactant is produced by cultivation of SS2 yeast strain (*Pichia sp.*) in a 250ml medium containing 0.02% KH₂PO₄, 0.02% MgSO₄.7H₂O, 0.1% Yeast extract, 4% of soybean oil and various nitrogen sources such as NH₄NO₃, (NH₄)₂SO₄ and NaNO₃ at 0.2% and 0.4% in 500ml flask with 250ml medium with shaking at 200 rpm for 7 days at 30°C. Aliquots of the culture were removed periodically for growth and biosurfactant activity analysis. To determine the optimum concentration of the nitrogen source, concentrations of 0.1%-0.5% were used to determine biosurfactant producing activity. Cell culture (6% v/v) was used as inoculums which was overnight culture. Biosurfactant activity in the culture was determined by measuring the oil displacement activity as described by Morikawa *et al.*

2.7.3. Effect of pH on biosurfactant production

To determine the optimum pH of the production medium, cells were cultivated in media containing 4% soybean oil as a carbon source and 0.4% NaNO₃ as a nitrogen source at various pH levels, ranging from 3.5 to 6.5. It was incubated at 30°C with shaking at 200 rpm for 7 days. The supernatant were collected and analyzed for biosurfactant activity every day for 7 days [26].

2.7.4. Production and extraction of Biosurfactant

Biosurfactant production was analyzed in 1000 ml production medium with 4% Soybean oil as carbon source and 0.4% Na NO₃ as nitrogen source with pH 5.5 with shaking at 200 rpm for 7 days at 30°C. After 7 days cells were removed by centrifugation at 7,500 rpm for 15 min. The supernatants were kept at 4°C overnight, after which clear separation of the two phases was observed. The aqueous phase was harvested and extracted 3 times with ethyl acetate. The solvent layer was evaporated to dryness in a vacuum at 40°C. The crude extracts obtained were weighed and analyzed for biosurfactant activity by oil displacement technique.

III. Results

3.1 Microorganism and Identification

Among the 50 yeast isolates, only 6 isolates were active for biosurfactants, of which SS2 exhibited the highest activity (Table 1). The morphological and physiological characteristics of the yeast strain SS2 were identified. The results of assimilation test, growth characteristics, and gene sequence analysis suggest that it belongs to the genus *Pichia* and according to 18Sr RNA- ITS gene sequence analysis, it suggested similarity to the strain *Pichia fermentans* ATCC 10651 ([GQ458040.1](#)).

Table 1: Isolation of Biosurfactant producing yeast strains

	YEPD
Total isolates	50
Biosurfactant activity	6

3.2 Biochemical analysis of SS2 yeast isolate

The characteristics of yeast strain SS2 grown on differential medium were off-white, oval shaped and smooth colonies. On microscopy, it showed budding yeast like cells (Figure 1). The presence of growth of SS2 isolate in yeast nitrogen base indicated the utilization of carbohydrates including glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, mannose, salicin, sorbitol, sucrose, trehalose and xylose (Table 2).

Figure 1: Microscopic identification of SS2 yeast isolate

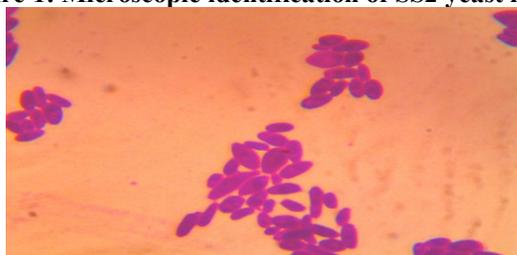


Table 2: Carbohydrate Utilization of SS2 yeast isolate

S.No.	Experiment	Isolate SS2
		Growth
1	Adonitol	-
2	L-Arabinose	-
3	Cellobiose	-
4	Glucose	++
5	Dulcitol	-
6	Galactose	-
7	Glycerol	++
8	Inositol	+
9	Inulin	+
10	Lactose	++
11	Maltose	++
12	Mannitol	++
13	Mannose	++
14	Melibiose	-
15	Raffinose	-
16	Rhamnose	-
17	Salicin	+

18	Sorbitol	++
19	Sucrose	++
20	Trehalose	++
21	Xylose	++
22	Blank	-

3.3 Identification of SS2 yeast isolate using 18S –ITS sequence of rRNA based molecular technique

Based on the maximum identity score in the Blast n results, homology and phylogenetic data, SS2 yeast strain belongs to *Pichia* species and has a maximum score with 92% identity towards *Pichia fermentans* strain ATCC 1065 (GQ458040.1) for a a query cover of 99%. The 18S rRNA forward query gene sequence of the SS2 yeast isolate was compared with the data present in NCBI. Phylogenetic tree was constructed and relationship was determined (Table 3).

Table 3: Query sequence, BLAST results, and Phylogenetic tree – SS2 yeast isolate

Query sequence: AAGTTAGAGACTTCTCTTGATCTTACGGGTGGTGGTGCATGGCCGTTTTAGTCCTTGGAGTGATTGTCTGCTTAATTG CGATAACGGACGAGACCTAACCTGCTAAATAGGGCTGCGAGCATCTGCTCGGGTGCTCTTCTTAGAGGGACTATGGGTA TCAAACCCATGGAAGTTGAGGCAACAACAGGTCTGTGATGCCCTTAGACGTTCTGGGCCGCACGCGCTACACTGACG GAGCCAGCAAGTCCAACCTTGGTCGAGAGGCCGGGTAATCTCGTGAACCTCCGTGCTGGGGATAGAGCATTGTAAT TTTTGTCTTCAACGAGGAATTCCTAGTAAGCGCAAGTCATCAGCTTGCCTGATTACGTCCTGCCCTTTGTACACACC GCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCTCAAGATTGGCGCCGCGGGAGGGGCAACTTCCCATGGGGCC GAGAATCTAGTCAAACCTGGTCATTTAGAGGTCGTAAGTTCGTAACAAGGTTCCGTAAGGTGAACCTGCGGAAGGATCA TTAGTGTGATTTAGTACTACACTGCGTGAGCGGAACGAAAACAACAACACCTAAAATGTGGAATATAGCATATAGTCGAC AAGAGAAAATCTACGAAAAACAACAAAACCTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAAATG CGATACCTAGTGTGAAGTGCAGCCATCGTGAATCATCGAGTCTTGAACGCACATTGCGCCCTCGGCATTCCGGGGGGC ATGCCTGTTTGGCGTCTGTTCCATCTTGGCGGTGC							
Description (SS1 yeast isolate)	Max score	Total score	Query cover	E value	Identity	Accession no	
Pichia fermentans strain ATCC 10651 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1122	1122	99%	0.0	92%	GQ458040.1	
Pichia sp. 11-485 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1088	1088	99%	0.0	91%	JX112045.1	
Phylogenetic tree 							

3.4 Preliminary screening for biosurfactant activity

As per the gene sequencing results, SS2 yeast isolate exhibited close and comparable similarity with *Pichia fermentans*. This isolated was then screened for biosurfactant activity which gave an oil clearance diameter of 12 mm with an interpolated surfactant concentration of 2.77 micrograms. The supernatant of SS2 yeast isolate collapsed on the oily surface and gave a flat drops with scoring system ranging from ‘+’ to ‘++++’ corresponding to partial to complete spreading on the oil surface. In microplate assay screening for yeast isolate SS2, the presence of surfactants caused some wetting at the edge of the well and the fluid surface becomes concave and took the shape of a diverging lens and the concave surface distorted the image of the grid. The optical distortion of the grid provides a qualitative assay for the presence of biosurfactants (Table 4 and Figure 2). The diameter of the clear zone on the oil surface was measured and related to the concentration of biosurfactant by using a standard curve prepared with a commercially available biosurfactant Tween-80 (Sigma, St. Louis, MO). The unknown biosurfactant concentration was determined by comparing with the concentration of the standard surfactant Tween-80 in various dilutions and a standard graph was plotted. Commercial surfactant Tween-80 exhibited an oil clearance diameter of 12 mm with a concentration of 2.62 microgram in 1:16 dilution (Figure 3) (Table 5 and 6). The SS2 isolate measured an emulsification index of 50.00 with a height of emulsion 14 mm after 24 hours. After 120 hours the emulsification index is 61.42 with a height of emulsion 17.2 mm (Table 7 and Figure 3). The present study showed that the surface activities of biosurfactant yeast species were found to be approximately similar to the commercial surfactant Tween-80. The results suggested that the biosurfactant from *Pichia* species or strain similar to *Pichia fermentans* provides excellent emulsification property.

Table 4: Screening of SS2 yeast isolate for Biosurfactant Activity

S.No.	Culture Isolate	Oil clearance diameter (mm)	Presence of Drop Collapse	Microplate assay
1	SS1	<5	-	-
2	SS2	12	+++	+++
3	SS3	5	+/-	+
4	SS4	5.5	+	+
5	SS5	8	++	+
6	SS6	<2	-	-

Figure 2 : Biosurfactant Activity by Oil Spreading Technique

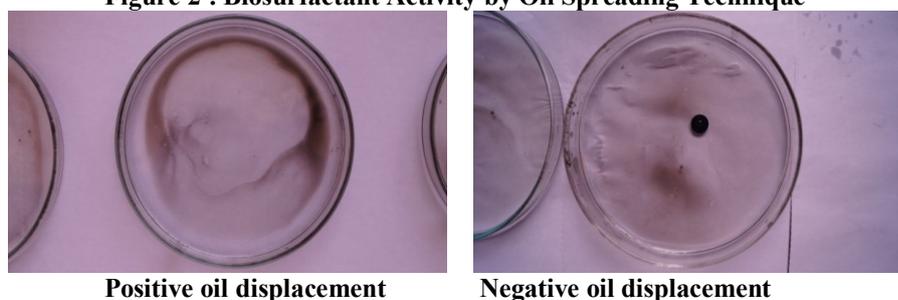


Table 5: Standard Surfactant activity

Dilution	Tween-80 Concentration	Oil clearance diameter (mm)
1:1	42.0000	>40
1:2	21.0000	35
1:4	10.5000	18
1:8	5.25000	17
1:16	2.62500	12
1:32	1.31250	10
1:64	0.65625	6
1:128	0.32812	<6

Table 6: Interpolated Approximate Concentration of Biosurfactant from Standard Plot

Sample Isolate	Concentration (µg)	Oil Clearance Diameter (mm)
SS2	2.774782	12.000
SS5	1.655955	8.000

Figure 3: Determination of Unknown biosurfactant concentration compared with standard

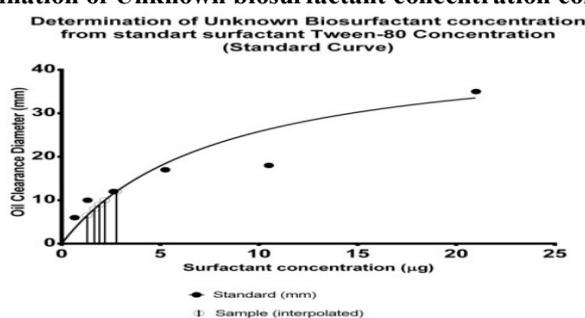
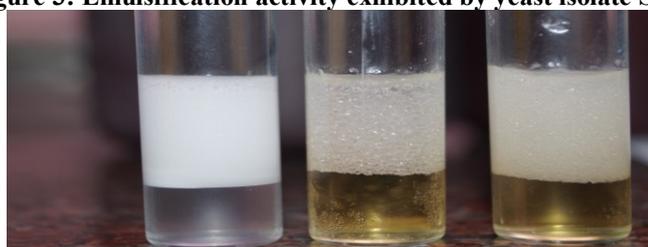


Table 7: Emulsification activity exhibited by SS2 yeast isolate

	Height of emulsion (He) in mm	Total height of the Liquid (Ht) in mm	Emulsification Index (EI)
0.5 % Tween Standard	19	27.5	69.09
Isolate SS2 (24 hr)	14	28	50.00
Isolate SS2 (120 hr)	17.2	28	61.42

Figure 3: Emulsification activity exhibited by yeast isolate SS2



1. 0.5 % Tween Standard 2. Isolate SS2 (24 hr) 3. Isolate SS2 (120 hr)

3.5 Production of Biosurfactant from SS2 yeast isolate (*Pichia fermentans*)

3.5.1. Biosurfactant production and extraction by identified yeast strain SS2 grown in various Carbon and nitrogen sources, and pH concentration

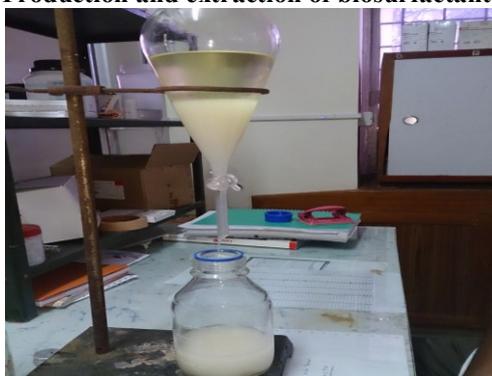
Six different carbon sources were used: glucose, corn starch, glycerol, crude oil, coconut oil, and soybean oil. These sources were chosen according to the previously reported results [27] and commercially available in areas around Coimbatore. In this study biosurfactant production was determined by biosurfactant activity in terms of oil displacement test. Compared to hydrophilic substrate glycerol, the hydrophobic substrate soybean oil exhibited a maximum oil displacement activity of 9mm in diameter. Based on the oil displacement activity, it was found that 8% soybean oil was the most effective carbon source for the biosurfactant production by the SS2 yeast isolate which has a 92% similarity to *Pichia fermentans*. The nitrogen source and its concentration also affected biosurfactant production. It was found that 0.4% nitrogen source NaNO₃ supported biosurfactant production by displacing the oil drop to a diameter of 9mm when compared to other nitrogen sources. The influence of the pH of the culture medium on biosurfactant production was studied in medium containing 8% soybean oil as carbon source and 0.4% NaNO₃ as nitrogen source. The results exhibited that oil was displaced at a diameter of 9mm at pH 5.5 (Table 8). Crude extracts of biosurfactants were prepared from the culture media as described in 'Materials and Methods'. Clear separations of two phases were observed when supernatant was kept undisturbed at 4°C overnight. The aqueous phase was harvested, extracted with ethyl acetate, and solvent layer was vacuum dried at 40°C. The dry crude extract containing biosurfactant obtained was weighed at 6.24 g/l which displaced an oil drop at a diameter of 9mm (Figure 4).

Table 8: Biosurfactant production by identified yeast strain SS2 grown in various Carbon and nitrogen sources, and pH concentration

S.No.	Carbon source	Oil Displacement activity (Diameter in mm)
1	Glucose 4%	< 4 mm
2	Glucose 8%	< 5 mm
3	Corn Starch 4%	< 5 mm
4	Corn Starch 8%	Nil
5	Glycerol 4%	7 mm
6	Glycerol 8%	8 mm
7	Crude oil 4%	Nil

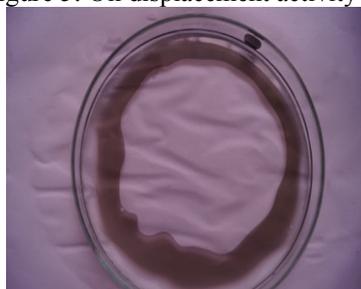
8	Crude oil 8%	Nil
9	Coconut oil 4%	< 3 mm
10	Coconut oil 8%	< 5 mm
11	Soybean oil 4%	9 mm
12	Soybean oil 8%	9 mm
S.No.	Nitrogen source (0.4%)	Oil Displacement activity (Diameter in mm)
1	0.2% NH ₄ NO ₃	< 3mm
2	0.4% NH ₄ NO ₃	< 5 mm
3	0.2% (NH ₄) ₂ SO ₄	< 4mm
4	0.4% (NH ₄) ₂ SO ₄	<5 mm
5	0.2% NaNO ₃	7 mm
6	0.4% NaNO ₃	9mm
S.No.	pH	Oil Displacement activity (Diameter in mm)
1	3.5	< 5mm
2	4.5	< 5mm
3	5.5	9 mm

Figure 4: Production and extraction of biosurfactant



Separation of two phases (gravity separation recovery)

Figure 5: Oil displacement activity of dry crude extract containing biosurfactant



Positive for biosurfactant activity



Negative for biosurfactant activity

IV. Discussion

Dairy industry is one of the major food industries in India [28]. The wastewaters generated from dairy industries contain large amount of fats and oils which gets accumulated in the environment. These wastewaters are considered high in biodegradable carbon and nitrogen sources, its utilization has a low cost fermentative media for the production of biosurfactants. For the screening of biosurfactant producers, enrichment cultures utilizing hydrophobic compounds are used as the sole carbon sources [29]. All results obtained help in reporting a study that examined biosurfactant production by *Pichia* species which showed 92% identity with *Pichia fermentans* isolated from fermented dairy whey waste. A phylogenetic analysis of SS2 yeast isolate was performed to determine how the 18S rRNA of SS2 yeast isolate and its related strain derived during evolution. The homology assay result indicated that the SS2 yeast isolates were in the phylogenetic branch of *Pichia* species. The identification of SS2 as *Pichia fermentans* was on the basis of the obtained 92% sequence similarities for 99% query [27]. The ITS regions located between highly conserved small 18S ribosomal subunit genes in the rRNA operon are known to have sufficient sequence variability to allow identification of the species level for many fungi and yeast. The evolutionary relationships among the sequences were depicted as a phylogenetic tree. The branching construction of the tree reflects the degree to which different sequences are related. Sequences that are alike were located as neighboring branches joined together to form a common branch

beneath them. When the query and reference sequence is 99% and above, then the unknown isolate would be assigned to reference species. If the percentage similarity is between 95-99%, the unknown isolate would be assigned to the corresponding genus, and when the percentage similarity is less than 95%, the unknown isolate would be assigned to a family. The extraction of the genomic DNA of the isolated yeast was done according to the method suggested by Hoffman & Winston [30]. For the amplification of the gene, 18S, ITS conserved sequences and a pair of forward and reverse primers was used by Aristogene Private Limited, Bangalore which was also suggested by WHITE et. al [31]. The sequences obtained were compared to the ones deposited in the "NCBI" (National Center for Biotechnology Information). Blast (Basic Local Alignment Search Tools) was used as a tool for the comparison of the sequence [32]. It shows that the phylogenetic relationship between the isolate and other related yeast species. The influences of the concentration of various carbon and nitrogen sources, along with the impact in the culture media at various pH levels were studied. The aim behind screening of new biosurfactants is to find new structures with strong interfacial activity, high emulsion capacity, and discovery of good production strains with high yields. Growth associated biosurfactant production has a parallel relationship between substrate consumption, growth and biosurfactant production[33]. The enrichment culture provides growth conditions that are very favorable for the SS2 yeast strains to grow and as unfavorable as possible for the competing organisms. Drop collapse assay[34] relies on the destabilization of liquid droplets by surfactants. The stability of those drops is dependent on surfactant concentration and its correlation with surface tension. Based on the results obtained after the study, 8% soybean oil and 0.4% NaNO₃ can be considered as the best carbon and nitrogen sources with an optimum pH of 5.5. The findings can be supported by some previous reports which state the soybean oil and NaNO₃ with pH 5.5 was the optimum carbon, nitrogen and pH for biosurfactant production of *Pichia anomala*. *Pichia* is a genus of yeasts in the family *Saccharomycetaceae* with spherical or oblong cells. It is a teleomorph and produce round ascospores during sexual reproduction. The anamorphs of *Pichia* species are *Candida* species. One of the *Pichia* species isolated from fermented whey waste, *Pichia fermentans* has proved to be attractive yeast for producing biosurfactants and its role in industrial applications.

V. Conclusion

Biosurfactants are ecologically accepted, low toxic, biodegradable, and effective than chemical surfactants. Most of the literatures throw light on the production of biosurfactants from bacterial species, but most of the byproducts are inappropriate to be used in industrial, food, or pharmaceutical industries; due to its pathogenic nature. Yeast, being a GRAS (generally regarded as safe) status microorganism, produces higher quantity of biosurfactants when compared to bacterial species. But the cost for its production makes it economically non feasible. Usage of renewable resources or accumulated waste effluents as substrates can be a possible strategy to reduce its production cost and help extend its application in all industries. Also it was recovered by simple and inexpensive techniques such as gravity separation and solvent extraction. In this work, one of the *Pichia* species was isolated from fermented whey waste which showed 92% similarity with *Pichia fermentans* that was screened to detect its ability to produce biosurfactants with soybean oil and NaNO₃ as carbon and nitrogen sources.

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