© February 2022| IJIRT | Volume 8 Issue 9 | ISSN: 2349-6002

Extraction and Screening of Exopolysaccharide Producing Bacteria from Curd

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Abstract **- Exopolysaccharide (EPS) are environment friendly natural polymers consist of sugar moieties and secreted by microorganisms extracellularly having high molecular weight polymers. EPS has diverse applications in the food industry. In the present work, the two bacteria producing higher EPS was screened among eight other bacterial isolates from curd. The samples were plated on MRS agar plates and colonies showing ropiness, sticky and mucoid consistency were selected. The isolates were further used for EPS production and the only bacterium producing higher amount EPS used for further work. The identification and characterization of this superior EPS producing bacterium were done based on biochemical and morphological analysis. The EPS production carried out in a modified EPS selection medium (mESM) for about 48 hrs in shaking conditions. The extraction was done with the two-volume of prechilled isopropanol. The extracted EPS was analyzed by using the FTIR technique for functional group detection. We also analyzed for its emulsification activity. The emulsification was carried out with different oil as well as chemical hydrocarbons including benzene. The results were compared with standard surfactant i.e. Triton X-100. The EPS showed good emulsification activity against different oils. Thus, indicated its possible use as an additive in the food industry.**

Index Terms **- Exopolysaccharide (EPS), emulsification, FTIR, Biochemical and Morphological analysis.**

I.INTRODUCTION

Polysaccharides consist of multiple monosaccharides joined together often forming branched structures. It has a characteristic property that allows them to produce a material that makes them stick to each other on the surface. From several years, plants and sea weeds were used for the Polysaccharides production. However, over the past 20 years, a new class of microbial products: the microbial polysaccharides have gained industrial importance and led to increased attention in exopolysaccharide (EPS) production [1],[2],[3]. EPSs are eco-friendly, polymers and are secreted extracellularly and referred to as an extracellular polysaccharide or EPS material [4]. EPSs are branched, long-chains of various sugars. Particularly, sugars like Rhamnose, Glucose, Galactose in variable amount. In regards to characteristics, rheological properties are largely varied due to its charge, molecular weight [5]. EPSs from Lactic acid bacteria (LAB) are classified based on different criteria. Classification based on monomer composition is most preferred one. Two types of EPSs are homopolysaccharides and heteropolysaccharides based on repenting sugar units [6]. HePS has molecular weight ranges from 104 to 106 Da. Lactobacilli have been significantly used in the production of various meat and milk products due to its nutritional and preservation characteristics [7]. Also, EPSs from LAB have technical significance. These technological advantages are very important in improving the characteristics of fermented food formulation. EPSs can also be used as a source of oligosaccharides and sugar monomers. A variety of functional oligosaccharides can be produced by the LAB [8]. Oligosaccharides have numerous applications such as in prebiotics, sweeteners, humectants, colon cancer, and immune stimulators [9]. The EPSs has biological defenses against various stresses. Some EPSs produced by LABs have shown ability being used as a viscofier thickener, emulsifier or stabilizer in the food industry [10],[11]. The present work deals with isolation and screening of EPSs producing bacteria from curd.

II. MATERIALS AND METHODS

A. Collection of Samples

A homemade curd sample was collected in sterile screw-cap tubes and brought to the laboratory for microbiological analysis.

B. Isolation of EPSs Producing Bacteria

The sample was homogenized and then the liquid portion of it was serially diluted with saline, up to 10-1 to 10-8 dilutions. From each dilution tube, 0.1 ml of diluted sample was spread on MRS (Man, Rogosa and Sharpe) (Table.1) agar plates. All plates were incubated at 37°C for 48 hrs. After incubation, the colonies showing mucoid, as well as sticky consistency were selected and purified on new MRS agar plates and stored in the refrigerator at 40°C (Figure.1) [12],[13].

Figure.1 Bacterial isolates producing EPS on MRS medium

Table.1 Composition of MRS agar medium

Constituents	Gm/lit
Peptone	10
Beef extract	10
Yeast extract	5
Glucose	20
Tween-80	1ml
Ammonium citrate	2
Sodium acetate	5
Magnesium sulphate	0.1
Magnesesulphate	0.05
Dipotassium phosphate	2
Agar	5
PH	6.5 ± 0.2

C. Screening and Identification of Isolated Bacteria

The isolates were screened for EPS producing activity by propagating them in 20 ml modified EPS selection medium (mESM), (Table.2) [14]. After inoculation, the conical flasks were kept at shaking conditions at 100 rpm at 37°C for 48 hrs. The growth in medium, confirms that the isolates having the ability to synthesize EPSs [15] (Supplementary figure.2). By using chemical assay (Phenol sulfuric acid method), superior EPSs producing isolates were identified and screened. Also, various biochemical tests were performed to identify and characterize the superior EPS producing organisms. In 50 ml of D/W, 5% skimmed milk was dissolved in a conical flask and remaining ingredients were dissolved 50 ml D/W in another conical flask, autoclaved at 10 lbs/inch2 at 40 min.

Table.2 Chemical Composition of mESM media

Content	Quantity
Skim milk	5 %
Yeast Extract	0.35 % w/v
Peptone	0.35 % w/v
Glucose	5 % w/v

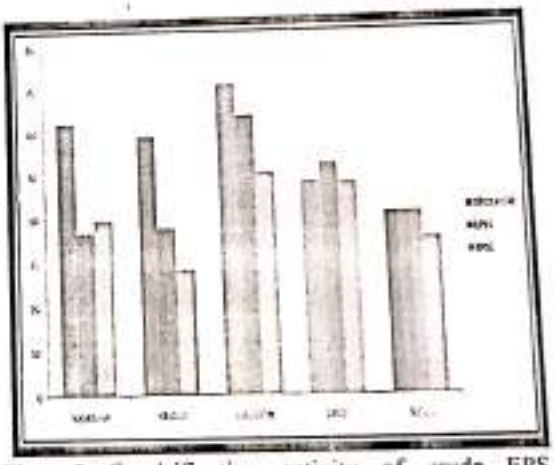

Figure.2 Emulsification activity of crude EPS extracted from isolate microorganism

D Morphological Characterization

1 Grams Character

Gram staining of superior EPS producing isolates were performed as described in microbiological Application: A laboratory Manual in General Microbiology by (Table.3), [16].

Table.3 Colony characteristics of efficient EPS producers

Sr. No.	Colony characteristics	Isolate1	Isolate2
1	Color	Off white	Off white
2	Size	1mm	1mm
3	Shape	Irregular	round
4	Surface	Rough	smooth
5	Consistency	Mucoid	Mucoid
6	Margin	Irregular	Regular

2 Biochemical Characterization

The various biochemical tests performed such as the Sugar fermentation test, IMViC TEST, (Indole, Methyl red, Voges proskauer and citrate utilization) Starch utilization test, Gelatin utilization test. The isolates were also checked for the production of enzymes like Oxidase test, Catalase test, Nitratase test, Urease test, Lysine decarboxylase test, and Phenylalanine deaminase test. Along with these tests, isolates were screened for other characters such as gas production, in regard to this, we performed Triple sugar iron (TSI) test (Table.4).

3 Study of growth at different NaCl Concentrations

To study the effect of different concentrations of NaCl, Nutrient broth with a range of NaCl concentration (1%, 2%, 3%, 4% and 5%) was prepared [17] (Table.4). Test organisms were inoculated and incubated at 37oC. The tubes were examined for growth for 48 hrs.

E Production of EPS

For the production of EPSs from isolated superior EPS producing bacteria, inoculum was prepared in MRS broth; a loopful of isolated bacterial culture from slant was inoculated in sterile 10 ml MRS broth and incubated at 37°C for 48 hrs. The activated culture was used as inoculum for the production on of EPS production. The medium was sterilized by autoclaving at 10 lbs/inch2 at 40 min the sterilized 50 ml of mESM medium was then inoculated with 1% inoculum the flask was inoculated on a rotary shaker at 100 rpm for 2 days at 37°C.

F Extraction of EPS

After incubation, the production media are treated with 80% TCA (17% v/v) and allow incubating at 370C on a rotary shaker for 30-40 min [18]. Then production media was centrifuged at 10,000 rpm for 20 min at 40°C. After centrifugation supernatant was collected in a conical flask and 2 volumes of prechilled isopropanol were added and stored at 40°C for 48 hrs. The precipitated material was collected by centrifugation at 10,000 rpm for 20 min at 40°C and the pellet was dried overnight at 55°C. The extracted EPS was estimated as a total carbohydrate by the Phenol-Sulphuric acid method (Table.5). For Phenol Sulphuric acid method 10 µl of EPS sample was added in a 190 µl distilled water in which 200µl Phenol reagent was added then 1 ml of 96% Sulphuric acid was added through the side of tube without disturbing the tubes and mix well after 10 min the contents in the test tube were kept in boiling water bath for 10 min and the O.D. values were noted at 490 nm. Glucose was used as a standard in the range of 10 to 100 µg concentration the standard graph was plotted with absorbance against concentration of glucose.

G Emulsification Index

The emulsification index (EI24) of culture samples was determined by adding 2 ml of oil to the same amount of extracted EPS solution (0.1%) [19],[20]. The contents were vortex for 24 hrs. The EI24 is given as a percentage of the height of the emulsified layer (mm) divide by total height of the liquid column (mm) (Figure.2).

Table.6 Emulsification Activity Index EI24

Oil	Isolate1	Isolate2	Triton X100
Soybean Oil	36.66	39.39	62.06
Olive Oil	37.50	28.12	58.82
Coconut Oil	63.33	50	70.96
Petrol	52	47.61	48
Benzene	40.99	35	40.90

H FTIR Analysis

The extracted EPS was dissolved in Sterile D/W or 1 % DMSO solution and used for FTIR analysis.

III. RESULT

I Isolation of EPSs Producing Bacteria

A total of 8 different bacterial colonies were isolated and purified from the Curd sample on the MRS medium.

J Screening and Identification of EPS producing bacteria

Screening of EPS producing colonies was carried out by the extent of stickiness and by estimating the EPS content by Phenol Sulphuric acid method (Dubois et al., 1956). Among 8 isolates, Isolate numbers 1 & 2 were superior EPS producing colonies (Table 3).

K Identification of EPS producing bacteria

The Efficient EPS producing bacterial isolates were identified based on cultural, morphological and biochemical Characteristics according to the Bergey's Manual of Systematic Bacteriology [21].

L Estimation of EPS

The concentration of EPS produced by superior EPS producing bacteria was estimated by phenol sulfuric acid test. The estimated production of EPS by isolate 1 and isolate 2 was found to be 195 µg/ml and 141µg/ml respectively (Figure.3).

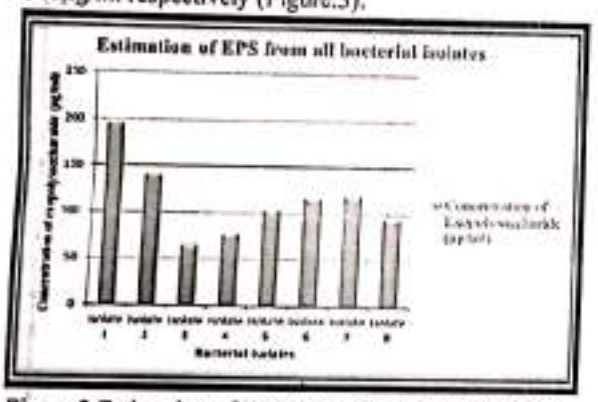

Figure.3 Estimation of EPS from all bacterial isolates

M FTIR Analysis

FTIR is an effective analytical method for detecting functional groups and characterizing covalent bonding information. The technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. The FTIR spectrum of purified EPS exhibited many peaks from 3910 to 526 cm-1. The EPS contains a large number of hydroxyl groups (O-H) stretching frequency, which showed a broad absorption peak around 3250–3440 cm-1 [22] .The peak in the range 2140-2100 cm-1 vibration may be of the acetylene group. The peak in the range between 1670-1640 cm-1 indicates the amide linkage. In the fingerprint region 600–850 cm-1 indicates these are halo derivatives. Therefore, it's confirmed that EPS contains hydroxyl, acetylene and amide linkage (Figure.4).

IV. DISCUSSION

EPS is well recognized for its physicochemical characteristics over the time but nowadays some other nutritional as well as health-related uses make them more valuable. EPSs perform a variety of different functions such as cellular defense and cellular binding (cell adhesion). Many lactic acid bacteria show EPS secretions in their surrounding environment. In the present study,

EPS production was initially low but gradually it increases with an increase in incubation timing (initial 24 hrs) and later on it follows the plateau stage and finally decreases to a considerable extent. It was observed that during stationary phase there is no significant growth as well as no EPS production [23], reported that EPS production may be at the peak towards completion of exponential phase in some strains of L. lactis subsp cremoris whereas other investigators reported at stationary phase only De [24]. Such conclusions were however often based on optical density measurements of microbial growth, which is not always a valid parameter when using complex media. Various other studies also reported EPS production other than LABs. Most of our results are in agreement with observations studied by other scientists [25]. The effect of temperature is variable and dependent on the strain used and the experimental conditions. Contradictory effects of temperature on EPS production by LAB have been reported. The influence of salt on bacterial growth is presented in Table 4. Five salt concentrations were tested: 1%, 2%, 3%, 4% and 5%. All isolates are salt-tolerant, having the capacity to survive at most of higher salt concentrations. Also, we have observed, in all isolates, there was a direct relationship between EPS production and growth. Maximum EPS production was attained at 37°C. The carbon source in the culture medium has been found to affect the yield and sugar composition of EPS produced by LAB. Glucose shows the maximum EPS production when compared with the sugars subjected to analysis.

In present study, superior EPS producing bacteria are gram-positive having cellular morphology described as Table 5. We observed that except Mannitol, both

the isolates utilize all other sugars such as glucose, maltose, sucrose, lactose, etc. Different nitrogen sources were screened for the maximum production of exopolysaccharides for the selected isolate. Also, on the other hand, both the isolates are unable to produce gas. It shows that these bacteria are simply aerobes or facultative anaerobes. Various factors such as media composition, temperature, pH and time etc., also influence the production as well as characteristics of EPS [26]. We observe that various biochemical tests such as Catalase, Methyl red, Starch hydrolysis, Lysine decarboxylase of superior EPS producing bacterial isolates show positive results. While that of all other tests shows negative results (Oxidase, Indole, VP, Citrate utilization, Urease, Phenylalanine deaminase, H2S production, Gelatin hydrolysis). The major restriction in the industrial production of EPS is its high production cost. By using cheaper dairy products or spoiled milk products may help in reduction of EPS cost. Earlier studies have already been reported about use of different carbon sources by different bacterial strains. In our study different household and dairy products like curd, spoiled milk, milk and whey wastewater from the dairy industry utilized for EPS production. Among the cheap sources, curd shows the best cheap substrate for EPS production.

IV. CONCLUSION

The two isolates were isolated from different dairy samples. The isolated strain was screened for EPS superior production. The various factors affecting the production of EPSs were assayed, which include different substrates and salt concentrations. Results showed that temperature ,37°C is an optimum environmental parameter for the growth of the isolates and its better EPS production. In addition to this, the study suggests glucose as better carbon source and curd as a better substrate for the production of EPSs. The present study reports that isolated bacteria's from curd has enhanced EPS producing ability and emulsification activity. Morphological and biochemical characteristics show that the present isolates from the curd sample may be identified as Streptococcus species (Isolate 1) and Lactobacillus sp. (Isolate 2) with superior EPS producing capacity.

ACKNOWLEDGMENT

Author is graceful to Shikshan Maharshi Dnyandeo Mohekar Mahavidylaya Kalamb, Dist.Osmanabad, and Maharashtra, India and also for providing necessary lab facility to this work.

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Table.4 Results of biochemical characterization

Sr No	Biochemical Test Performed	Isolate1	Isolate2
1	Gram staining	Gram positive	Gram positive
2	Oxidase test	Negative	Negative
	Sugar fermentation		
	a)Glucose	Acid production	Acid production
	b)Sucrose	Acid	Acid
	c) Mannitol	No acid	No acid
	d) Maltose	Acid	Acid
	e) Lactose	Acid	Acid

4	Catalase	Positive	Positive
5	Indol test	Negative	Negative
6	Methyl red	Positive	Positive
7	VP test	Negative	Negative
8	Motility test	Motile	Motile
9	Citrate Utilization	Negative	Negative
10	Starch Hydrolysis	Positive	Positive
11	Lysine Decarboxylase test	Positive	Positive
12	Urease test	Negative	Negative
13	Phenylalanine test	Negative	Negative
14	H2 S production	Negative	Negative
15	Gelatin hydrolysis test	Negative	Negative
	NaCl tolerance test a) 1 % b) 2 % c) 3 % d) 4 % e) 5 %	Tolerate all	Tolerate all

Table.5 Phenol Sulphuric Acid Method

Sr. No	Isolate	EPS Concentration	O.D Value	Phenol(µl)	[illegible]	Incubation	Optical density [illegible]
1	Isolate 1	10 µl					0.384
2	Isolate 2	10 µl					0.374
3	Isolate 3	10 µl				After 10	0.267
4	Isolate 4	10 µl				Min kept	0.317
5	Isolate 5	10 µl	190 µl	200 µl	1 ml	In Boling	360
6	Isolate 6	10 µl				Water	372
7	Isolate 7	10 µl				Bath	81
8	Isolate 8	10 µl					24

Isolate 1 Isolate 2

Figure.4 FTIR Analysis of isolate 1 & isolate 2