Research Article

Isolation, Identification and Optimization of Exopolysaccharide Producing Lactic Acid Bacteria from Raw Dairy Samples

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ABSTRACT

Most of the Gram-positive bacteria, including lactic acid bacteria, produce exopolysaccharides (EPS). EPS are either excreted into the growth medium (slime) or attached to the bacterial cell wall (capsules). They are long-chain, high molecular carbohydrate polymers. In dairy industry, EPS producing cultures provide the fermentation produced with viscosity, stability and water binding functions. The present study was isolation, identification and optimization of Exopolysaccharides producing lactic acid bacteria from raw dairy materials. In this study, EPS production of selected isolates was analyzed between 0 hour and 54 hours in MRS medium. It was determined that EPS production was gradually increased and the maximum production was observed during 24th hour. After 24 hours of incubation, EPS production decreased gradually. EPS production increased during the exponential growth phase and no further production was observed in the stationary growth phase. Based on the results of the present study, it is concluded that *Lactobacillus lactic ssp. lactis*isolated from dairy samples showed better characteristic EPS producing ability.

Keywords: Lactic acid Bacteria, Exopolysaccharide, MRS medium and Raw dairy materials.

1. INTRODUCTION

Exopolysaccharides (EPSs) are long-chain polysaccharides that are secreted mainly by bacteria and microalgae into their surroundings during growth and that are not permanently attached to the surface of the microbial cell. The physical characteristics of EPSs are responsible for the slime-forming or mucoid trait of many microorganisms. A second group of polysaccharides that are structurally similar but that are permanently attached to the cell surface are classified as capsular polysaccharides.

Nevertheless, now, for industrial purposes, many new polysaccharides are developed from bacteria; exocellular polysaccharides are produced in a large scale by the usual techniques of microbiology and fermentation – this procedure allows a good control of the characteristics of polymers and allows the purification of polysaccharides more easily than from other natural sources. Extension of such production also allows reducing the price and extending the range of applications(Andrew Laws *et al.,* 2001).

1.1. LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) constitute a group of gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. The general description of the bacteria included in the group is gram-positive, non sporing, non respiringcocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates.

The boundaries of the group have been subject to some controversy, but historically the genera *Lactobacillus, Leuconostoc, Pediococcus,* and Streptococcus form the core of the group. Taxonomic revisions of these genera and the description of new genera mean that LAB could, in their broad physiological definition, comprise around 20 genera.

In addition, the present taxonomy relies partly on true phylogenetic relationships, which have been revealed by extensive work on determining 16S rRNA sequences Most genera in the group form phylogenetically distinct groups, but for some, in particular *Lactobacillus* and *Pediococcus,* the phylogenetic clusters do not correlate with the current classification based on phenotypic characters (Savadogo*et al.,* 2004).
Nonetheless, most studies have been performed *in vitro* and there is a lack of clinical evidence demonstrating these putative probiotic characteristics after oral administration of EPSs in functional foods. In addition, the mechanism(s) and EPS-parameter(s) involved in these biological effects still remain poorly understood(Goh*et al*.,2005). Based on the comparative sequence analysis of 16S rRNA (16S ribosomal ribonucleic acid) genes, bacteria can be divided into 12 major phyla (Figure - 1).

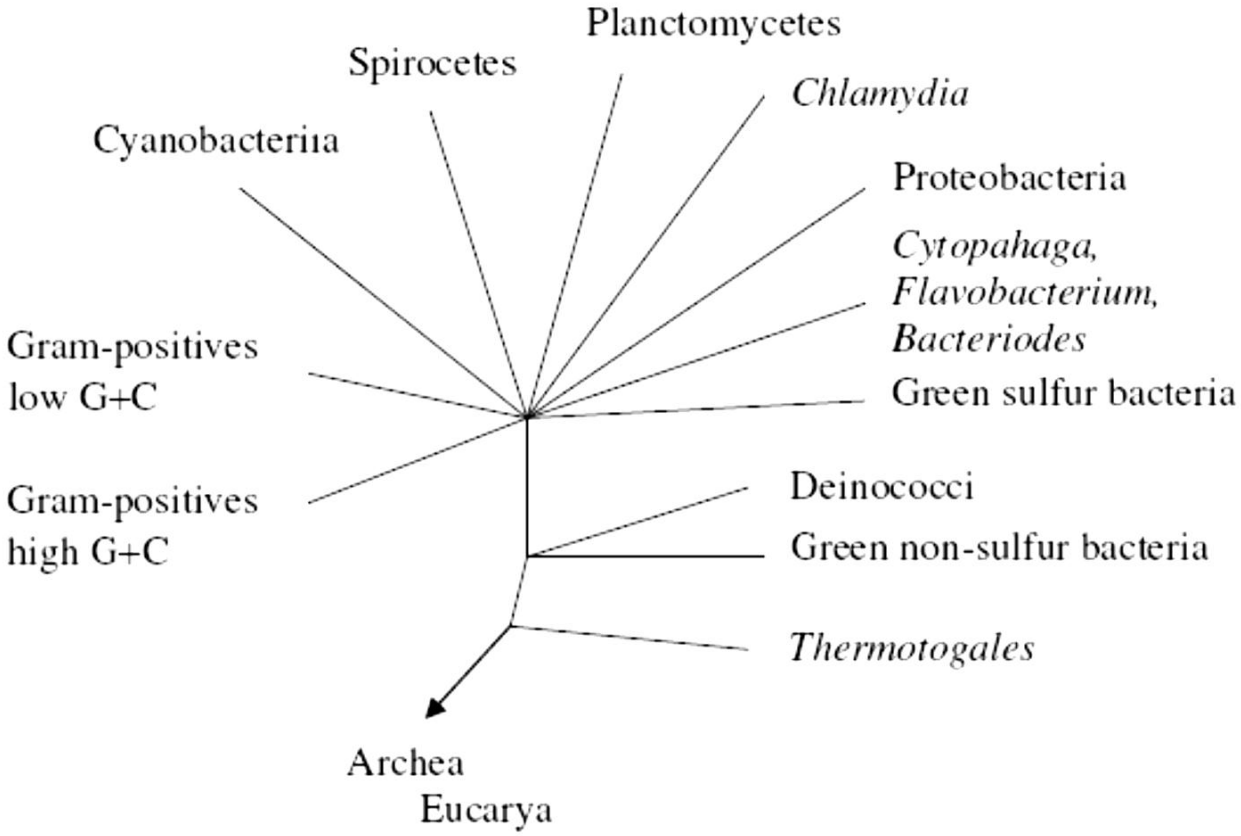

Fig. 1: Phylogenetic tree of bacteria based upon 16S rRNA sequence comparison

2. MATERIALS AND METHODS

The bacteria involved in this study were isolated from different raw dairy samples present in Chennai. Raw and treated (Branded) samples were collected using sterile container. A total of 5 different samples were collected and transported to laboratory for further processing.
Samples were serially diluted upto 10^9 dilutions using sterile saline as a blank and the diluted samples were plated into the sterile Nutrient agar medium using spread plate method.
The plates were incubated at 37°C for 24 to 48 hours. Plates were examined and different isolates were further purified by repeated single colony isolation and purity of cultures checked periodically by streaking liquid cultures onto Nutrient agar. The isolated cultures were used for further studies.
Screening for Lactic acid bacteria was followed by Annamaria*et al.,* 1997.The positive strain that produces maximum Exopolysaccharides was selected and given for identification in IBMS, University of Madras, Taramani.The spectrometric chemical assay for the determination of exopolysaccharide from the

sample was estimated using Phenol sulphuric acid method (Michel Dubois *etal.,* 1956).
Estimation of exopolysaccharides from Lactic acid bacteria was followed by Annamaria*et al.,* 1997. **T**he measurement of EPS concentration (as mg glucose^{-l}) with the phenol-sulphuric acid and Parameter optimisation studies for production of EPS was followed by Michel Dubois *et al.,* 1956.
The time course of production of Exopolysaccharide during fermentation, effect of temperature, influence of different pH levels, different carbon,nitrogen sources on their overall production and Production of EPS from Agro productswere investigated using MRS production medium. Isolation of Genomic DNA from lactic acid bacteria were carried out by standard procedure.

3. RESULTS

Exopolysaccharides are naturally produced by lactic acid bacteria during the life cycle of the organisms, and therefore are referred to as natural polymers. Exopolysaccharides (EPSs) play important roles in the attachment of bacterial cells to a surface and in building and maintaining the three-dimensional, complex structure of bacterial biofilms.

3.1. SAMPLE COLLECTION AND ISOLATION

In this study, a total of 41 bacterial strains were isolated from raw and treated dairy samples collected from Chennai outlets.

3.2. SCREENING FOR LACTIC ACID BACTERIA (LAB)

The isolates were screened for lactic acid production using MRS agar medium. Among the 41 strains, 13 produced cream coloured colonies in MRS agar plate indicating the presence of potential lactic acid production (Figure1).

3.3. ISOLATION OF BEST EXOPOLYSACCHARIDE PRODUCER

The selected 13 isolates were inoculated in MRS production medium for identifying the best exopolysaccharide producer. Among the 13 isolates the best exopolysaccharide producer (s6) was selected and subjected for further optimization studies (Figure 2).

3.4. IDENTIFICATION OF BACTERIA

The identification study showed that the isolated positive strain has been identified as *lactococcuslactisssp .lactis***(**Identification done by Dept. of Microbiology, IBMS, University of Madras, Taramani.) (Figure 3).

3.5. CHEMICAL ASSAY

The exopolysaccharides was estimated by using phenol – sulphuric acid method (Michel Dubois *et al.,* 1956) (Figure 4).

3.6. GROWTH STUDY

The growth study of the organism is essential for the production of exopolysaccharides. Growth study was performed for the selected isolate using MRS broth medium. In order to determine the optimum production time for maximum Exopolysaccharides production, the samples were collected at 6 hours intervals and analyzed for the estimation of exopolysaccharides. In the growth study we found that upto 12th hour the production of EPS was very low and then there is a gradual increase in the production. Maximum EPS production was observed from 18th hour to 54thhour, from there onwards gradual decrease in the EPS production was observed. Based on the results analyzed at different time intervals, it was determined that the maximum production of EPS was at 24th hour (Table 1 and figure 5).

3.7. Different Parameters

The environmental parameters show great influence on the growth of the organisms and the production of exopolysaccharides. The main parameters like Temperature, pH is considered as the essential parameters for the production of EPS.

3.8. Effect of Temperature

In order to determine the effect of the incubation temperature for the better exopolysaccharides production, different incubation temperatures were maintained for production process. Based on the readings it was observed that the selected strain have a temperature optima at 37°C (Table 2 and figure 6).

3.9. Effect of pH

The optimal pH for EPS was determined by analyzing the EPS production using phenol – sulphuric acid. Based on the readings it was observed that the selected strain shows maximum EPS production when it was maintained at pH 6.5 (Table 3 and figure 7).

3.10. Effect of Carbon Sources

Different carbon sources were screened for maximum production of exopolysaccharides for

the selected isolates (MRS medium). As it is seen from Table- 4, except for maltose, the rest of the carbon sources gave satisfactory production of EPS. However if maximum productivity was considered Glucose was taken as best carbon source (Table 4 and figure 8).

3.11. Effect of Nitrogen Sources

The Nitrogen sources are of secondary energy sources for the organisms which play an important role in the growth of the organism and the production. Different nitrogen sources were screened for maximum production of exopolysaccharides for the selected isolates. As it is seen from Table, the lowest exopolysaccharides production was obtained with casein and gelatin. Peptone and Potassium nitrate gave much more satisfactory result among the selected nitrogenous source. Since productivity is concerned, Potassium nitrate shows the maximum EPS production and considered as best sources in this study (Table 5 and figure 9).

3.12. Production of EPS from Agro products

The most economical and valuable bioproducts are produced from the natural sources and industrial wastes. One of the limiting factors in the commercial success of EPS production schemes is the cost of the sugar substrate used for EPS formation. In this study, several natural and agro products have been used as substrates. The results revealed that, maximum production was observed in sugar Rice bran (Table 6 and figure 10).

3.13. DNA ISOLATION

The genomic DNA was isolated from the selected isolate. The sample was run in 0.7% agarose gel containing ethidium bromide and the band was observed under UV-transilluminator confirming the presence of genomic DNA (Figure 11).

Fig. 2: *Lactococcus lactic ssp. Lactis* in MRSagar medium

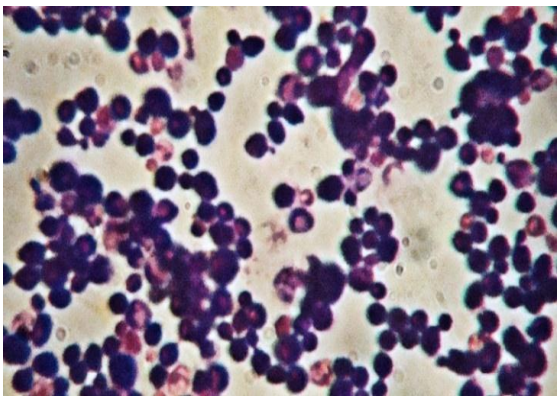

Fig. 3: Identification of *Lactococcus lactic ssp. lactis*

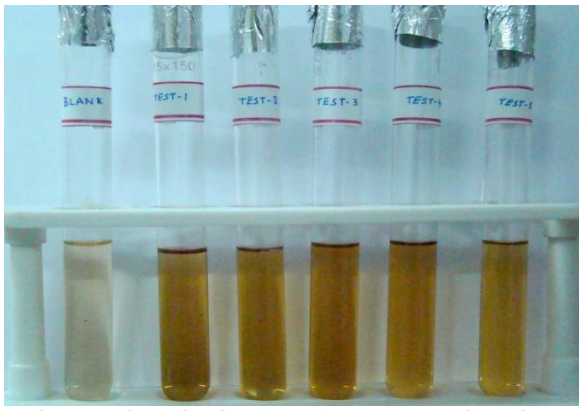

Fig. 4: Chemical Assay for EPS Estimation

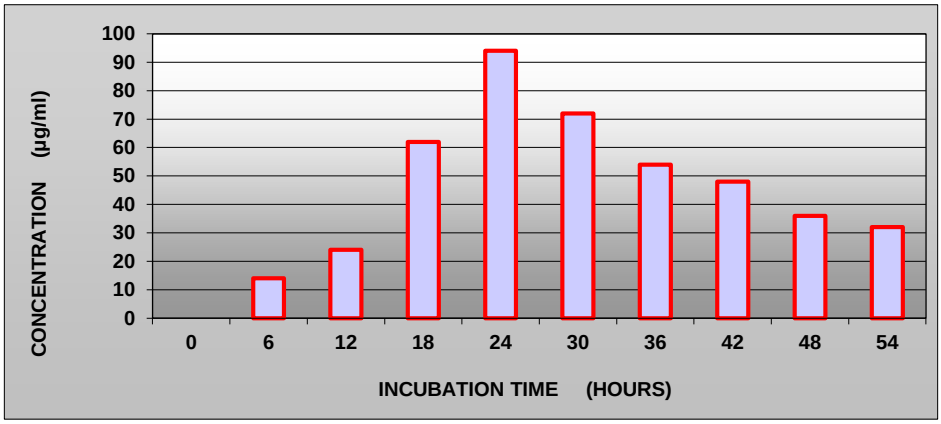

Fig. 5: EFFECT OF INCUBATION TIME ON EPS PRODUCTION

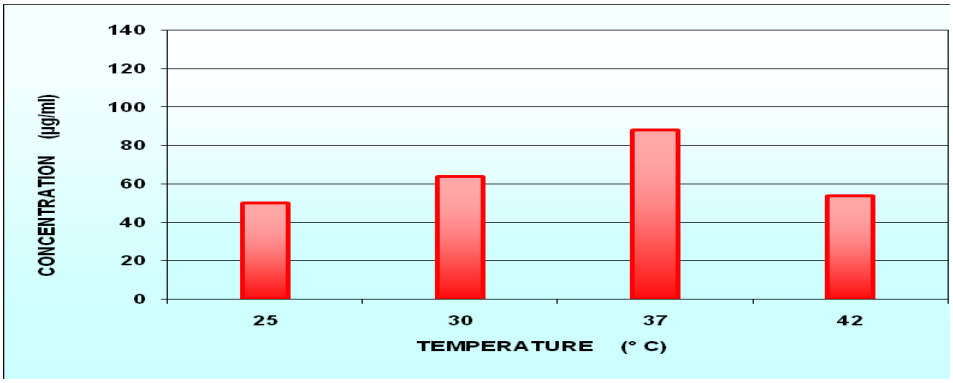

Fig. 6: EFFECT OF TEMPERATURE ON EPS PRODUCTION

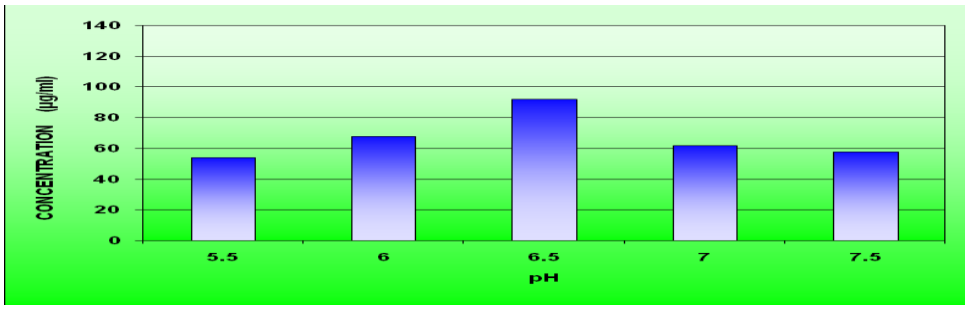

Fig. 7: EFFECT OF pH ON EPS PRODUCTION

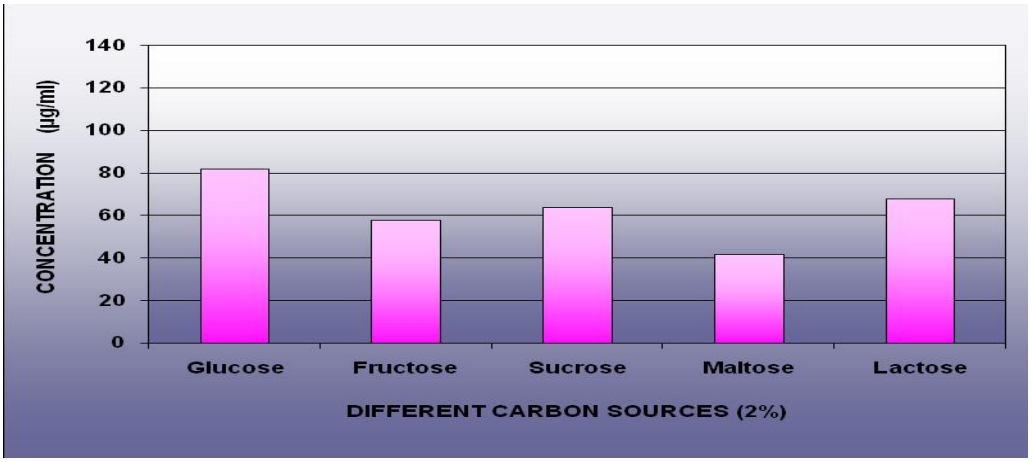

Figure: 8 EFFECT OF NITROGEN SOURCES ON EPS PRODUCTION

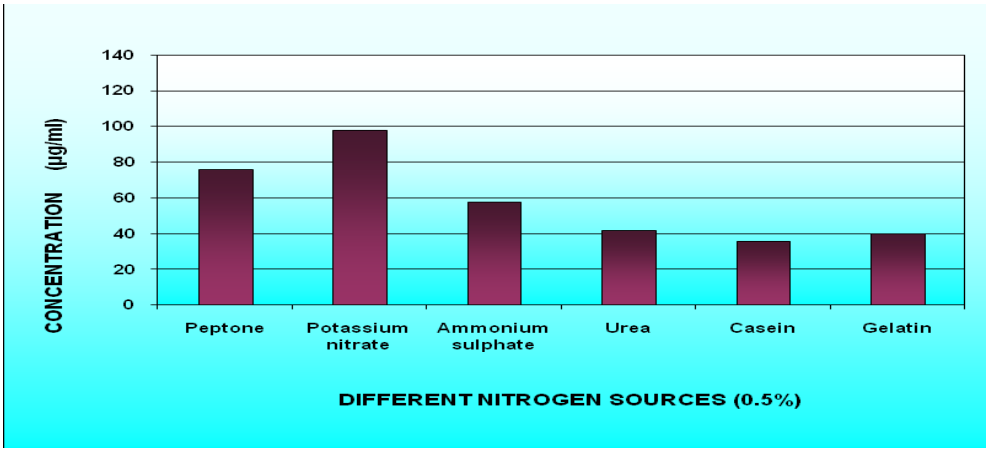

Fig. 9: EFFECT OF CARBON SOURCES ON EPS PRODUCTION

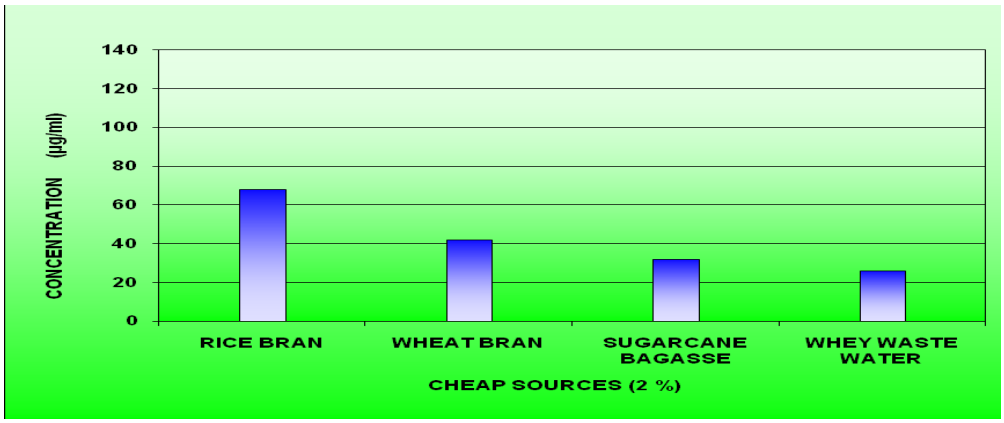

Fig. 10: PRODUCTION OF EPS USING DIFFERENT AGRO CHEAP SOURCES

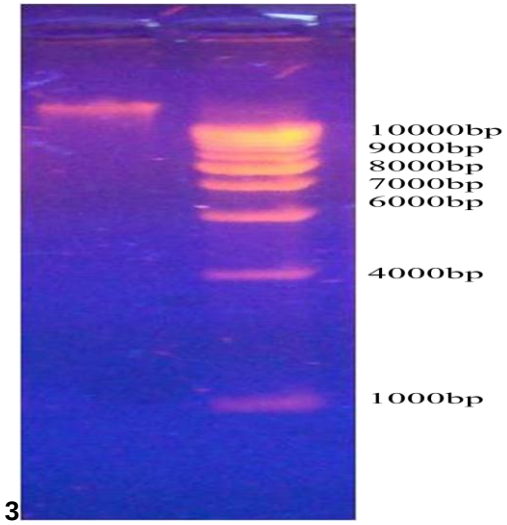

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Fig. 11: Genomic DNA

Table 1: Effect of Incubation Time on EPS Production

Time of culture withdrawal	6th hour	12th hour	18th hour	**24th hour**	30th hour	36th hour	42th hour	48th hour	54th hour
EPS (µg/ ml)	14	24	62	**94**	72	54	48	36	32

Table 2: Effect of Temperature on EPS production

Temperature (°C)	25	50	25	50
EPS (µg/ ml)	30	64	30	64

Table 3: Effect of pH on EPS Production

pH	5.5	6	6.5	7	7.5
EPS (µg/ ml)	54	68	92	62	58

Table 4: Effect of Different Carbon Sources on EPS Production

Carbon source	Glucose	Fructose	Sucrose	Maltose	Lactose
EPS (µg/ ml)	82	58	64	42	68

Table 5: Effect of Different Nitrogen Sources On EPS Production

Nitrogen sources	Peptone	Potassium nitrate	Ammonium sulphate	Urea	Casein	Gelatin
EPS (µg/ ml)	76	98	58	42	36	40

Table 6: Production of EPS from Agro products

Cheap sources	Rice bran	Wheat bran	Sugarcane Bagasse	Whey waste water
EPS (µg/ ml)	68	42	32	26

4. DISCUSSION

Bacterial polysaccharides that are secreted into the environment are termed exopolysaccharides (EPS). Lactic acid bacteria produce a wide variety of exopolysaccharides , which are mainly involved in cell adhesion and protection. Until recently, industrial interest has resulted from their physical–chemical properties, but these polysaccharides have now raised new interest due to their potential for nutritional and health applications.

In this study, EPS production of selected isolates was analyzed between 0 hour and 54 hours in MRS medium (Table 1). It was determined that EPS production was gradually increased and the maximum production was observed during 24th hour. After 24 hours of incubation, EPS production decreased gradually. EPS production increased during the exponential growth phase and no further production was observed in the stationary growth phase.

These results are in agreement with observations on other researchers (De Vuyst*et al.,* 1998; Grobben*et al.,* 1998; Tallon*et al.,* 2003). Marshall *et al.,* (1995) indicated that the onset of EPS production from a strain of *L. lactis subsp. cremoris* is observed towards the end of the exponential phase of growth. Other investigators observed continued EPS production beyond or only in the stationary phase of growth (Manca De Narda*et al.,* 1985; Gancel& Novel 1994; Bouzar*et al.,* 1996). Such conclusions were however often based on optical density measurements of microbial growth, which is not always a valid parameter when using complex media (De Vuyst&Degeest, 1999).

The effect of temperature is highly variable and is dependent on the strain used and the experimental conditions. The influence of temperature on bacterial growth and EPS production is presented in Table 2. Four temperatures were tested: 25, 30, 37 and 42°C. In all strains, there was a direct relationship between EPS production, growth and temperature. Maximum EPS production was attained at 37°C.

Contradictory effects of temperature on EPS production by LAB have been reported. Grobben*et al.,* (1995) and De Vuyst*et al.,* (1998) found that a greater amount of EPS was produced by *L. delbrueckii spp. bulgaricus* and *S. thermophilus*, respectively, at temperatures within the optimum growth temperature range of the test organism. In contrast, Van den Berg *et al.,* (1995) and Degeest*et al.,* (2001) reported that more EPS was produced by *L. sake* 0-1 and *L. sakei* 0-1, respectively, at a temperature lower than the optimum temperature of the test organism.

In order to improve EPS production of the selected isolate, the influence of pH was studied. Results for growth and EPS production are shown in Table 3. pH affects both growth and EPS production. There was a general increase in EPS production and growth of bacteria with increasing pH. Maximum EPS production was observed at a pH of 6.5 in the production medium.

Various investigators have involved their efforts in examining the effects of pH on EPS production by LAB. Mozzi*et al.,* (1995) measured maximum EPS synthesis (488 mg/l) and highest cell numbers at a constant pH of 6.0 for *L. casei* CRL 87. Ricciardi*et al.,* (2002) reported that the best pH for EPS production in *S. thermophilus SY* strain was 6.4. De Vuyst*et al.,* (1998) also found that optimal pH was 6.2 compared with the results obtained at pH 4.9,

5.5 and 6.9. The optimal pH for EPS production has been found to vary in different strains of LAB (De Vuyst&Degeest 1999; Ricciardi*et al.,* 2002). However, the optimal pH for EPS production is often close to 6.5 (De Vuyst*et al.,* 1998; Van den Berg *et al.,* 1995) which is in agreement with our findings.

Carbon source in the culture medium has been found to affect the yield and sugar composition of EPS produced by LAB. Different carbon sources (Table- 4) were screened as main industrial carbon sources (2 % conc.) in the formulation of the production medium. As it is seen from Table 3, glucose shows the maximum EPS production when compared with the sugars subjected for analysis.

Comparing the results to the literature the total yield of EPS produced by the lactic acid bacteria depends on the composition of the medium and conditions in which the organisms grow (i.e., medium, temperature and incubation time) (Cerning*et al.,* 1990; Cerning*et al.,* 1994). Similar observations have been made for gram negative bacteria, including *Klebsiella*sp., *Acinetobactercalcoaceticus*and *Aeromonassalmonicida*.Zehra and Belma reportedglucose was the most efficient carbon source in studies with *Lactobacillus delbrueckiisubsp.bulgaricus*(B3, G12) and *Streptococcus thermophilus*(W22) in the medium containing various carbon sources.Gamar*et al.,* (1997) reported that EPS production and yield were influenced by the carbon source and concentration.

Different nitrogen sources were screened for maximum production of exopolysaccharides for the selected isolate. As it is seen from Table - 5, among the Peptone, Potassium nitrate, Ammonium sulphate, Urea, Casein, Gelatin used for exopolysaccharides production studies, potassium nitrate shows a higher productivity rate. EPS characteristics and amounts can be influenced by several factors such as composition of the medium (carbon and nitrogen sources), as well as incubation conditions (temperature, pH, time, etc.) (Looijesteijn*et al.,* 1999; De Vuyst and Degeest, 1999; Tallon*et al.,* 2003).

The major restriction in the industrial production of EPS is their high production cost. The use of readily available cheap agro-industrial residues as the carbon sources may reduce the higher cost. Several studies have shown the utilization of various carbon sources for different bacterial strains. In our study different agro products like rice bran, wheat bran, sugarcane bagasse and whey waste water (Table – 6) from dairy industry utilized for EPS production. Among the cheap sources Rice bran shows the best cheap substrate for EPS production.

5. CONCLUSION

The *Lactococcuslactis ssp. lactis*was isolated from different raw dairy samples present in Chennai using sterile container. The isolated strain was screened for the lactic acid production and further screened for exopolysaccharides production. After growth study, the production was done by shake flask fermentation. The various factors affecting production of exopolysaccharides was assayed, which include pH, different agro substrate, temperature and additives. Besides, production was made using different carbon sources and nitrogen sources. Results showed that pH 6.5 and temperature 37ºC is an optimum environmental parameter for the growth of the isolate and for its better production. In addition to this, glucose was found to be better carbon source, potassium nitrate as better nitrogen source and rice bran as a better agro substrate for better production of exopolysaccharides. Based on the results of the present study, it is concluded that *Lactobacillus lactic ssp. lactis*isolated from dairy samples showed better characteristic EPS producing ability.

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