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International Journal of Current Research
Vol. 4, Issue, 12, pp.103-107, December, 2012

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

PRODUCTION AND CHARACTERIZATION OF EXOPOLYSACCHARIDES USING *LACTOBACILLUS* SP. ISOLATED FROM MILK AND MILK PRODUCTS

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ARTICLE INFO

Article History:
Received 25th September, 2012
Received in revised form
29th October, 2012
Accepted 24th November, 2012
Published online 18th December, 2012

Key words:
Lactobacillus Sp,
Exopolysaccharides,
Plasmid DNA and
Antimicrobial activity.

ABSTRACT

Lactobacillus Sp isolated from various milk products (Milk, curd, cheese, butter, paneer, sourdough, ripened lemon, ripened banana) were analyzed and characterized by Cell morphology, biochemical tests, and carbohydrate fermentation test for the production of exopolysaccharides. *Lactobacillus* Sp were identified and screened on the basis of the molecular weight of its plasmid DNA (> 20 kb). The potentiality of *Lactobacillus* Sp. in EPS production was assessed. EPS production by *Lactobacillus* Sp is partially growth associated and about 1.2 gm of EPS/L was synthesized. The EPS production by *Lactobacillus* Sp was confirmed by thin layer chromatography (TLC) analysis. Antimicrobial activity of the produced EPS was tested against 6 pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa, Aeromonas hydrophila, Micrococcus* Sp., *Vibrio cholerae, Salmonella typhi*). Optimization of culture condition for mass EPS yield was studied. The maximum growth of *Lactobacillus* Sp and EPS production was achieved when optimizing the media at temperature 30°C, pH-3 and the concentration of sugar is 20 G/L. This ideal media providing maximum mass growth (1.5 G/L) and EPS production (1.3 G/L).

INTRODUCTION

Lactic acid bacteria are normally present in milk after milking and as they are able to ferment lactose, the major milk sugar, they are used as a starter cultures in dairy industries in the production of cultured dairy products such as yoghurt (Zehra Nur *et al.*, 2008). Exopolysaccharides produced by *Lactobacillus* Sp have gained increasing attention over the last few years because of their contribution to the rheology and texture of food products (Cerning and Marshall, 1999).*Lactobacilli* are rod shaped, gram positive, fermentative and organotrophs. They are usually straight, also can form spiral under certain conditions. *Lactobacilli* are naturally present or artificially introduced into the milk products in order to generate quality and health benefit for the consumer (Valeric Coeuret *et al.,* 2003).Under certain conditions they can produce exocellular polysaccharides (EPS). These polymers increase the viscosity of milk and decrease the susceptibility of syneresis (Cerning *et al.,* 1992). Interest on EPS producing lactic acid bacteria has increased recently because these organisms produce polymers which play an important role in determining the rheological properties of dairy products and also have applications in non dairy foods (Stacy *et al.,* 1997). When EPS added to food products, it acts as thickeners, stabilizers, emulsifiers, gelling agents, and water binding agents (Sutherland, 1990). With innovative approaches, efforts are under way supenrsedethe traditionally used plant and algal gums by their microbial counter parts more over, considerable progress has been made in discovering and developing new microbial EPS that posses novel industrial significance. Exopolysaccharides produced by lactic acid bacteria (LAB) have generated increasing attention among researchers for the last few years since LAB are food grade organisms and the EPS that they produce contribute to the specific rheology and texture of fermented milk products. In the present study the potentiality of EPS production by *Lactobacillus* Sp. isolated from milk sources were investigated and the antimicrobial activity of EPS also studied.

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MATERIALS AND METHOD

Sample collection: A total of eight different milk and milk products were collected from retail shops at different areas in Chennai.

Analysis: One gm of each sample was placed into 9ml of a sterile physiological saline (0.9% (w/v) NaCl) solution contained in a conical flask and for enrichment it was kept in an orbital shaker at room temperature for 4-8 hours. The processed samples were used for *Lactobacillus* isolation. Spread plate method was performed to isolate pure culture of *Lactobacillus Sp.,* and for viable cell counting. For each sample, successive decimal dilutions were made in sterile physiological saline (0.9% (w/v) NaCl) solution and 0.1ml of the appropriate dilution was spread plated on MRS agar plated by using a sterile L-rod. Plates were incubated at 37° C for 24 hours. After the incubation period the number of colonies formed on the surface of MRS agar was counted from that the number of viable cells (in colony forming units: CFU / ml) present in 0.1 ml of each sample were determined. For further

studies the working cultures were stored on nutrient agar slant at 4°C and sub culture prepared every 2 weeks. Identification was carried out based on the following main characteristic features: Microscopic examination of morphology and mobility, Gram staining, Endoscope staining, Catalase test, Oxidase test, Fermentation ability on different carbon sources. The isolated colonies were subjected to the above tests along with standard *Lactobacillus acidophilus* (MTCC strain no.447) for confirmation.

Screening of exopolysacharides production: MRS broth contained in a conical flask (250ml) was autoclaved (121°C for 20 minutes) and cooled to hand bearable warmth. The investigated strains were grown in 50 ml of MRS broth for 48 hours at 37°C.

Determination of biomass dry weight: After incubation the cells were removed by centrifugation (800 rpm for 15 minutes). The cell pellet was washed twice with 500ml of 1 % PBS buffer and dried at 37°C for overnight. The dry weight of the biomass was obtained by calculating the difference between the dry weight of the empty vial and the dry weight of the vial with biomass.

EPS Isolation and Purification: EPS were isolated and purified according to the method of Cerning *et al* (1992). The cultures were heated at 100°C for 15 min to inactive enzymes. Afterward, samples were treated with trichloroacetic acid (10% (w/v) with agitation for 30 min and centrifuged at 8000 rpm for 15 min for cell and protein removal. The EPS in the supernatant were concentrated by adding three fold volume of cold ethanol and incubated at 4°C for overnight. The precipitate was collected by centrifugation at 5000 rpm for 15 min and dissolved in 500 µl of 1% PBS buffer.

Estimation of Total Sugar Concentration: Total sugar concentration was determined by the phenol sulphuric acid method using glucose as a standard.

Thin Layer Chromatography (TLC): The hydrolyzed samples were subjected to thin layer chromatography along with the standard sugars (glucose, fructose, and mannitol) to confirm the presence of EPS.

Characterization of EPS

Screening for Antimicrobial Activity of EPS: The antimicrobial activity test was performed by agar well diffusion method.

Isolation of Plasmid DNA from Lactobacillus: The EPS producing *Lactobacillus* possess the plasmid DNA greater than 20 kb in size (Tallon *et al.,* 2003). Plasmid DNA was isolated from *Lactobacillus sp.* run on agarose gel under electrophoresis to determine the molecular weight.

Optimization of EPS Production: Influence of different carbon sources, varies pH, temperature in the EPS production was analysed by standard methods.

RESULTS AND DISCUSSION

In total of eight samples collected from retail shops at different areas in Chennai (Table 1), only two samples (Curd and Cheese) were found to contain *Lactobacillus* sp., while the remaining samples were containing Yeast and Micrococcus Sp. *Lactobacillus* sp., was used for the production of exopolysacccharides in vitro, since it is a non pathogenic, food grade organism, possess the status of generally recognized as safe (Cerning and Marshall., 1999 and Belma Aslim., 2008). The exopolysaccharides (EPS) produced by *Lactobacillus* sp., have received increased interest, mainly because of their rheological properties in food, and their potential health-beneficial properties. *Lactobacillus* sp., is identified on the basis of morphological and biochemical tests and the results are illustrated in table 2 and 3. The isolates from curd and cheese sample displayed as white, opaque, mucoid shape with glittering and slimmy appearance on MRS agar medium. Both the isolates were found to be gram positive, rod, non spore forming, catalase and oxidase negative and able to ferment different sugars such as glucose, sucrose, fructose, maltose and mannitol. The colony forming units (CFU/ml) of *Lactobacillus* Sp., on MRS agar were determined after incubation at 37°C for 24 hours and the results are shown in (Table 2). Colonies showing white, opaque appearance with waxy growth were considered as *Lactobacillus* Sp., and used for further investigation.

Lactobacillus sp., having the plasmid DNA of molecular size of above 20 kb are able to produce exopolysaccharides (Lamothe, 2000). The plasmid DNA was isolated from the *Lactobacillus* of curd and cheese origin and also from standard *Lactobacillus* strain and it's molecular weight was found out by running in agarose gel electrophoresis along with the λ/Hind III ladder. When comparing all the separated plasmid DNA with ladder DNA indicating that the plasmid DNA from all the three isolates are above 20 kb in size (Figure 1).The identified isolates were subjected to EPS production at 37°C for 48 hours along with the standard *Lactobacillus* strain (Figure 2). After incubation, the EPS production was determined by measuring the total carbohydrate content by Phenol sulphuric acid method (Dubois *et al.,* 1956). The quality of EPS product was studied in thin layer chromatography (TLC) with the presence of standard sugars such as glucose, sucrose, fructose and maltose. Brown colored spots were appeared on TLC plate when spraying with α-Napthol reagent (Figure 3).

The antimicrobial activity of EPS against six pathogenic organisms was studied and the results are illustrated in table 5. The EPS produced by the standard *Lactobacillus* strain was found to have better activity followed by the EPS produced by the *Lactobacillus* from cheese sample and from curd sample. EPS produced from all the three cultures show higher activity against *Staphylococcus aureus* followed by *Pseudomonas aeruginosa*. No significant difference found in the activity of EPS from standard *Lactobacillus* strain against *Vibrio cholerae, Salmonella typhi* and *Micrococcus* Sp.,. The EPS produced by the curd *Lactobacillus* had no activity against *Aeromonas hydrophila* and *Micrococcus* Sp. The activity of EPS from cheese *Lactobacillus* was found to be similar against *Aeromonas hydrophila* and *Salmonella typhi* (Figure 6). Several parameters, such as carbon source, temperature, pH and concentration were tested to determine the optimal conditions for the production of EPS. The influence of carbon sources on growth and EPS production were tested and the results are summarized as follows. Glucose provided the

highest EPS yield followed by sucrose whereas mannitol supported the lower EPS synthesis. No significant difference is observed between fructose and maltose (Figure 8). The biomass concentration was maximum in both sucrose and fructose MRS (Figure 9) and it is more or less similar in remaining sugars. When comparing the growth and EPS yield from different sugars, revealed that no correlation between the biomass and EPS yield. The effect of various concentration of glucose on EPS yield was tested and results were shown in (figure 10). The maximum EPS yield was attained with glucose at 50gm/L but no significant differences were obtained between glucose at 30 and 40gm/L. The sugar concentration of 20gm/L is the optimal value for the high growth and EPS yield. To determine the optimal temperature for maximum yield of EPS, batch cultures were performed with glucose (20gm/L) as the carbon source at five different incubation temperatures (Figure 8) for 48hours. The growth temperature of 30˚C was also optimal for EPS yield.

The influence of pH on EPS synthesis was determined in batch cultures performed with glucose (20g/L) as the carbon source at 37˚C for 48hours. The maximum EPS yield and biomass was attained at pH 3 (Figure 9). The optimal conditions for the maximum growth and PS yield were found to be glucose 20g/L, 30˚C, pH 3. There was a correlation between biomass and EPS yield at optimal conditions. However, it changes significantly at remaining conditions. The maximum viable count did not correspond with the maximum EPS production present results are also in accordance with Anderson *et al.,* (1998) who reported that the brown colored spots were obtained when spraying the TLC by α-Napthol reagent which indicates the presence of carbohydrates in the isolated EPS. Present results are correlated with the report of Anderson *et al.,* (1998). They reported that the EPS producing *Lactobacillus* possess a plasmid DNA of molecular weight greater than 20kb. The present study revealed that culture conditions have a clear impact on the growth and EPS production by *Lactobacillus* Sp. The carbon source has a remarkable influence on EPS production. Among the different sugar sources tested, glucose provides highest yield.

The biomass produced from the medium containing various carbon sources were analyzed for EPS production. Although many studies revealed the EPS production of *Lactobacillus* Sp is strongly linked to biomass levels (De Vuyst *et al.* 1998 and Torino *et al.* 2000*)* the present study is find out there is no correlation between the biomass levels and EPS yield. The quantity of carbohydrates in the medium affects EPS yield (Prasher *et al.* 1997) and high initial carbohydrate level tend to enhance the final EPS levels (De Vuyst *et al.* 1998). Our results are consistent with the previous reports (Cheirsilr *et al*. 2003) showing that the maximum EPS yield was obtained at the sugar concentration 20g/L. The effect of temperature on EPS production was studied. Incubation at 50˚C clearly affected the biomass and yield. The biomass produced the temperatures 35 - 45˚C have a correlation with the EPS yield. The optimal growth temperature of 30˚C was also optimal for EPS production. Although the biomass is similar at the pH range from 4 to 7, it does not having any effect on EPS yield. However the biomass and EPS yield was high at pH.

Table – 1: Sample Collection at Various Places in Chennai

SI.No	Sampling station	Samples
1.	Vadapalani	Milk
2.	CMBT	Curd
3.	Guindy	Cheese
4.	Vadapalani	Butter
5.	T.Nagar	Paneer
6.	Vadapalani	Sourdough
7.	Kodambakkam	Ripened lemon
8.	Nungambakkam	Ripened banana

Table – 2: Illustrates The Colony Forming Units (CFU) Of *Lactobacillus* Sp. in MRS Agar Plates

S. No	Samples	Dilution Range	No. Of Colonies	CFU/ml
1.	Milk	10^{-2}	42	$4.2X10^4$
2.	Curd	10^{-2}	12	$1.2X10^4$
3.	Cheese	10^{-2}	14	$1.4X10^4$
4.	Butter	10^{-2}	8	$0.8X10^4$
5.	Paneer	10^{-2}	10	$1.0X10^4$
6.	Sourdough	10^{-2}	4	$0.4X10^4$
7.	Ripened lemon	10^{-2}	6	$0.6X10^4$
8.	Ripened banana	10^{-2}	2	$0.2X10^4$

Table – 3: Illustrates The Morphological Tests For *Lactobacillus* Sp. Isolated From Various Samples

S. No	Samples	Morphology	Gram staining	
1.	Milk	White,opaque, waxy growth	+	Cocci
2.	Curd	White,opaque, waxy growth	+	Rod
3.	Cheese	White,opaque, waxy growth	+	Rod
4.	Butter	White,opaque, waxy growth	-	Cocci
5.	Paneer	White,opaque, waxy growth	+	Cocci
6.	Sourdough	White,abundant, rough growth	-	Cocci
7.	Ripened lemon	White,opaque, waxy growth	-	Cocci
8.	Ripened banana	White,opaque, waxy growth	-	Cocci

Table – 4: Illustrates The Biochemical Tests For *Lactobacillus* Sp.

Isolates	Catalase	Oxidase	Carbon source				
			Glucose	Fructose	Sucrose	Maltose	Mannitol
Cu 2	-	-	+	+	+	+	+
Cu 2	-	-	+	+	+	+	-
Ch 1	-	-	+	-	+	+	+
Ch 2	-	-	+	+	+	+	+
P 1	+	+	+	-	+	+	-
P 2	+	-	+	-	+	-	-

\+ - Positive
\- - Negative

Table – 5: Illustrates The Antimicrobial Activity Of Exopolysaccharides Against Various Pathogens

S. No	Test Organisms	Zone Diameter in mm		
		Standard	Curd	Cheese
1.	*Staphylococcus aureus*	32	26	29
2.	*Pseudomonas aeruginosa.*	24	16	24
3.	*Aeromonas hydrophila*	12	--	9
4.	*Micrococcus* Sp.,	10	--	8
5.	*Vibrio cholerae*	10	18	8
6.	*Salmonella typhi*	10	12	9

Table – 6: Illustrates The Estimation Of EPS Produced

Samples	Curd	Cheese	Standard
Amount of EPS	1.2	0.82	0.68
TLC	+	+	+
Plasmid DNA Isolation	+	+	+

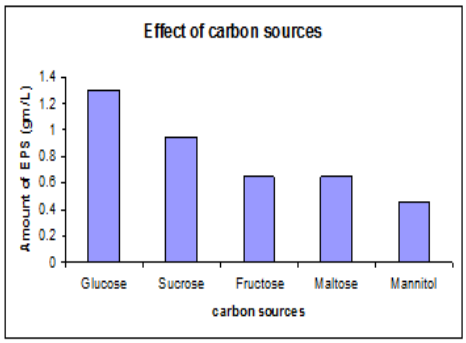

Figure – 1: Illustrates The Effect Of Various Carbon Sources On Exopolysaccharides Production

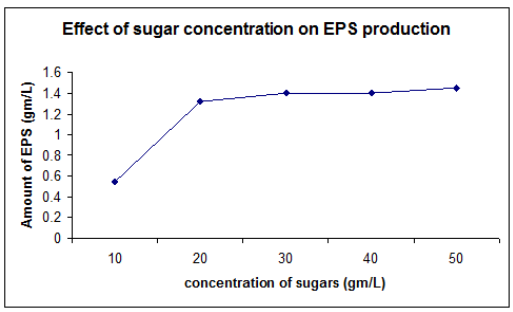

Figure – 2: Illustrates The Effect of Sugar Concentration On Exopolysaccharides Production

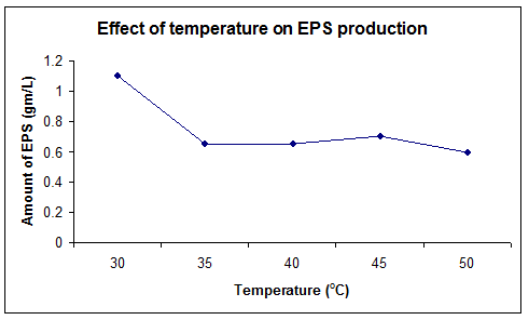

Figure – 3. Illustrates The Effect Of Temperature On Exopolysaccharides production

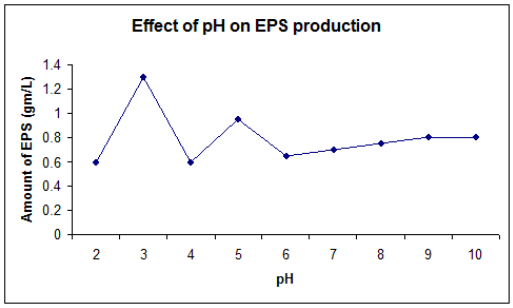

Figure – 4. Illustrates The Effect Of pH On Exopolysaccharides Production

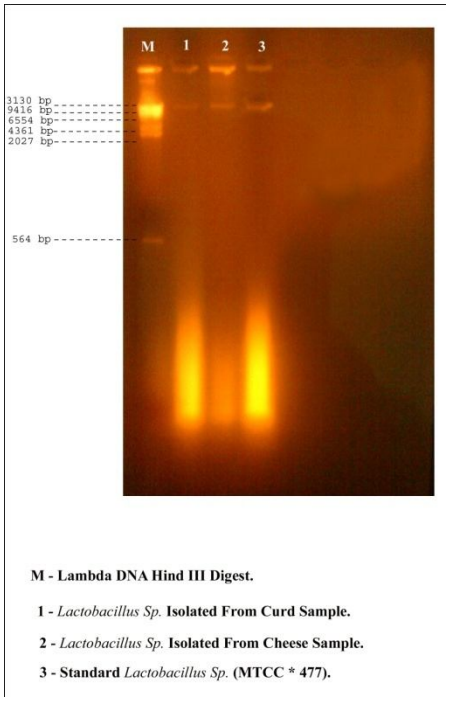

Plate 1: Plasmid isolation of lactobacillus from various milk products

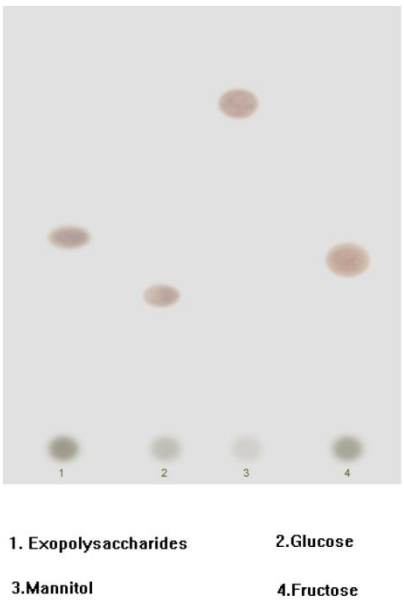

Plate 2:TLC Analysis of EPS with various sugars

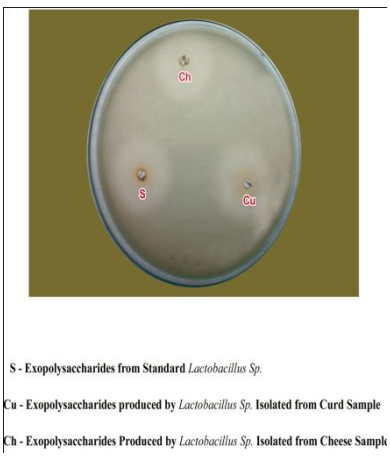

Plate 3:Antimicrobial activity of EPS produced from lactobacillus of various milk products

Conclusion

The present study concluded that the *Lactobacillus* isolated from various milk products were belongs to hetero fermentative and able to produce EPS in MRS medium. The production of EPS is partially growth associated and there is no correlation between the EPS yield and its antimicrobial activity. The EPS showed maximum antibacterial activity against *Salmonella aureus* and *Pseudomonas aeruginosa.*

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