EXOPOLYSACCHARIDE PRODUCING LACTIC ACID BACTERIUM FROM TRADITIONAL LACTIC FERMENTED PREPARATIONS: SCREENING, BIOSYNTHESIS DYNAMICS, COMPOSITIONAL ANALYSIS OF EXOPOLYSACCHARIDE AND EVALUATION OF ITS PROBIOTIC POTENTIAL

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Abstract
L.plantarum MCC2034, a Takrarista (Ayurvedic preparation) isolate, was selected for the present study on the basis of the screening trials, which indicated its capability for stable and enhanced production of EPS. The yield of the EPS produced by L.plantarum MCC2034 in mod-MRS medium was 1,059 mg/L. In addition, the kinetics of growth and biosynthesis of EPS in mod-MRS medium were evaluated. The results indicated that, L.plantarum MCC2034 has specific growth rate (μ) of 0.217 h^{-1}. The growth yield coefficient ($Y_{X/S}$) was 0.019 ± 0.006 g of biomass g^{-1} sucrose. The product yield coefficients, $Y_{P/S}$ and $Y_{P/X}$, were 0.026 ± 0.005 g of EPS g^{-1} sucrose and 0.026 ± 0.005 g of EPS g^{-1} biomass, respectively. The compositional analysis of EPS indicated that it is a heteropolysaccharide. The FTIR analysis revealed the presence of key functional groups viz. –OH group, and C-H group at 3301 and 2498 wave cm $^{-1}$ respectively, indicating the chemical nature of the EPS. The effects of low pH and bile salts on the viability of the selected strain were studied. The results indicated that L.plantarum MCC2034, is capable of withstanding the adverse conditions encountered during the passage through the gastrointestinal tract. Thus, L.plantarum MCC2034, a novel strain of LAB isolated from traditional Indian fermented preparation, is capable of producing EPS and also a potent probiotic bacterium. L.plantarum MCC2034, a promising EPS producing LAB has demonstrated good potential as probiotic and could open up new avenues in the management of gastrointestinal health, besides contributing to improved understanding of the active ingredients involved in the traditional Ayurvedic preparation.

Keywords: lactic acid bacteria, fermentation, exopolysaccharide, ayurvedic, biosynthesis

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1.INTRODUCTION

Exopolysaccharides (EPS) are widely produced by Lactic acid Bacteria (LAB). They are long chain polysaccharides made of branched repeating units of sugars (*e.g.*, glucose, galactose, rhamnose, etc.). EPS of microbial origin are long chain, high-molecular-mass polymers that have potential applications in food industries as texturizers, viscosifiers, emulsifiers and synerisis-lowering agents for their pseudoplastic, rheological behaviour and water-binding capacity (Galle et al. 2011). Many food grade bacteria that have been awarded generally regarded as safe (GRAS) status, *e.g.* LAB, propionibacteria and bifidobacteria, are capable of producing EPS. In some countries of European Union (EU) and the United States of America (USA), where addition of synthetic texture-promoting agents in food and dairy products is prohibited, EPS can be successfully used as food additives to enhance texture. This produces good impact on the development of novel food products with improved appearance, stability and rheological properties. EPS produced by LAB display a great variety of structures (De Vuyst and Degeest 1999). LAB produce a wide variety of EPS that can be broadly divided into two categories: Homopolysaccharides, composed of a single monomer (*e.g.* Glucan, fructan), and Heteropolysaccharides, composed of more than one monomer, usually an oligomer composed of 3 to 5 monosaccharides. EPS are involved in a wide variety of biological functions that benefit the host cell including prevention of

desiccation, protection from environmental stresses, adherence to surfaces, pathogenesis and symbiosis (Roberts 1996).
The physiological functions of these carbohydrate polymers have not yet been clearly understood. EPS have been known to act as prebiotics, when degraded by probiotic LAB (Salazar *et al.* 2008). It has been suggested that EPS produced by certain LAB could exert beneficial effects on the gastro-intestinal (GI) health in humans (Korakli *et al.* 2002). Additionally, EPS have also been reported to provide a wide variety of health benefits such as hypocholesteremic (Martensson *et al.* 2005), immunogenic (Chabot *et al.* 2001, anticancer (Russo *et al.* 2007), anti-mutagenic (Sreekumar and Hosono 1998) anti-hypertensive- (Ai *et al.* 2008), effect and inhibition of biofilm formation by pathogenic bacteria (Kim *et al.* 2009).
Ayurveda is an ancient system of medicine that has been practiced in India across millennia. In the last few decades, it has been steadily gaining popularity among the consumers. *Takrarista, Sandaki and Kanjika* are *Ayurvedic* lactic fermented preparations used for the treatment of various gastro-intestinal maladies. They represent indigenous lactic fermented preparations that possess health promoting properties. *Takrarista* is a butter milk based preparation containing added spices. *Kanjika* is prepared by adding chopped vegetables and spices to starchy water obtained by cooking rice with excess of water, followed by fermentation for seven days at ambient temperature. *Sandaki* is a lactic fermented preparation prepared using primarily radish and mustard. *Tushambu* is a lactic fermented preparation consisting of primarily the husk of black gram and barley. In this context, LAB isolates from the sources mentioned above were screened for the production of EPS and characterized.

2. MATERIAL AND METHODS

2.1. Chemicals

The chemicals used were of analytical grade, unless otherwise mentioned. Ruthenium Red, dextran standards, Oxgal and organic components of the media were purchased from Himedia Laboratories Pvt Ltd, Mumbai, India. The rest of the chemicals were procured from Rankem Pvt Ltd, New Delhi, India. Dialysis membrane (Molecular weight cutoff: 12 kDa) was from Sigma, St. Louis, USA.

2.2. Microorganisms, media and culture conditions

LAB strains (38 nos.) that were part of the culture collection of the research group were used for screening of EPS production. The strains were isolated from the *Ayurvedic* lactic preparations, *viz. Takrarista, Sandaki, Kanjika and Tushambu.* The cultures were maintained in glycerol stocks at 4^{o} C. *S.thermophilus* ATCC 19258, a known producer of EPS, was used as the positive control for the screening experiments. The isolates were cultured twice in MRS broth at 37^{o} C for 24 h prior to use.

2.3. Screening of LAB for the production of EPS

The screening of LAB for the production of EPS was carried out using modified MRS (mod-MRS) agar medium where sucrose (10 % w/v) served as the sole source of carbohydrate. The medium was sterilized at 121^{o} C for 15 min. Sucrose was sterilized separately and added to the medium under aseptic condition. Ruthenium Red [0.08% (w/v)] was added to the cooled sterile medium (45^{o}C) (Stingele *et al.* 1996). LAB isolates were streaked on the mod-MRS agar medium with Ruthenium Red and the plates were incubated at 37^{o}C for 48 h. The plates were observed for bacterial growth.

2.4. Extracellular biosynthesis, isolation and quantification of EPS

LAB isolates that tested positive for EPS were cultured in 200 mL of EPS production medium (EPM) taken in Erlenmeyer flasks (500mL) (Sanchez *et al.* 2006). EPM [modified MRS broth wherein the glucose component was substituted with sucrose (10% w/v)] is the same as that of the media used for screening trials devoid of agar and Ruthenium Red. The pH of the medium was adjusted to 6.5. The

EPM was inoculated with overnight grown LAB isolates (10% v/v; viable count: 1 x 10^7 cfu/mL) and incubated at 37°C for 48h. The EPS was precipitated from the cell-free, deproteinized (10% w/v Trichloroacetic acid) supernatant by the addition of distilled ethanol (1:2), and kept overnight at 4°C, and recovered by centrifugation. The EPS pellet was dissolved in distilled water and the low molecular weight contaminants were removed by dialysis (Mol wt cut-off: 12 kDa) against distilled water with five changes, for 48 h at ambient temperature. The dialyzed EPS was subjected to lyophilization and the lyophilized powder was stored at ambient temperature in an airtight vial. The quantification of the EPS was carried out by Phenol-Sulphuric acid assay (Dubois *et al.* 1956) for total carbohydrates using dextran as the standard.

2.5. Studies on the kinetics of Growth and biosynthesis of EPS by *L.plantarum* MCC2034

The time course and kinetics of the growth of *L.plantarum* MCC2034 were studied at 37°C, in EPM. The chief parameters *viz.* microbial growth, pH and residual sucrose were monitored every four hours. Microbial growth was evaluated by determining the dry biomass at the specified time intervals. The residual sucrose concentration was determined by HPLC (Shimadzu LC-20, Shimadzu Corp., Japan), using Zorbax Amino column (Agilent Pvt Ltd, USA) with the aid of Refractive Index Detector (RID). The mobile phase used was acetonitrile and water (70:30), in isocratic mode at a flow rate of 1 ml/min at ambient temperature. The Monod model as given below was fitted with the experimental data for the determination of specific growth rate (μ), and growth yield co-efficient ($Y_{X/S}$) and the production yield co-efficient with respect to unit substrate consumed ($Y_{P/S}$) as well as production of unit biomass ($Y_{P/X}$).

$\mu = \mu_{max} . S / K_S + S$ **(1)**

$Y_{X/S} = dX/dS$ **(2)**

Where, S – Substrate concentration and K_S – Half saturation constant.

2.6. Compositional analysis of EPS

The monosaccharide composition of the EPS produced by *L.plantarum* MCC2034 was determined subsequent to its hydrolysis. A known quantity of EPS dissolved in distilled water was refluxed with 2M Trifluoroacetic acid (TFA) at 121°C for 1h in an oil bath (Calsteren *et al.* 2002). The hydrolyzed EPS sample was neutralized and diluted appropriately prior to HPLC analysis. HPLC analysis was carried out using Zorbax Amino column (Agilent Pvt Ltd, USA), with the aid of Refractive Index Detector (RID) for detection of the constituent monosaccharides, with reference to the carbohydrate standards (Himedia Pvt Ltd, Mumbai). The mobile phase used was acetonitrile and water (75:25), in isocratic mode at a flow rate of 1 ml/min at ambient temperature.

2.7. Infrared spectroscopy

The infrared analysis of the EPS produced by *L.plantarum* MCC2034 was carried out using a fourier transform-infrared spectrophotometer (FTIR, Thermo Nicolet, America) for the detection of functional groups present in the EPS. The lyophilized EPS (5 mg) was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into a 1 mm pellet. The FTIR spectrum was recorded in the frequency range of 4000–400 cm^{-1}.

2.8. Evaluation of the potent probiotic properties of EPS producing LAB strains using *in vitro* methods

The EPS positive LAB strain, *L.plantarum* MCC2034, was evaluated for its potent probiotic properties using *in vitro* methods which primarily assess the ability of the strains to survive during their passage through the gastro-intestinal tract *viz.* acid tolerance and bile tolerance.

2.9. Acid tolerance

The ability of *L.plantarum* MCC2034 to survive in low pH was studied according to the method described by Yeong *et al.* (2002). *L.plantarum* MCC2034 (10% v/v) was inoculated into the modified MRS broth whose

pH was adjusted to 2.0, 2.5 and 3.0 with 5.0 M HCl; MRS broth whose pH was adjusted to 7.0 served as control. The tubes were incubated at 37^{o}C up to 180 min and samples were withdrawn every 30 min for evaluation of the viability using spread plate method. Serial dilutions of the samples were thoroughly mixed for 30 seconds individually before inoculation to MRS agar plates. Plates were incubated aerobically at 37^{o}C for 3 h. Acid tolerance was determined by comparing the final plate count (3 h) with the initial plate count (0 min).

2.10. Bile tolerance

Bile tolerance of *L.plantarum* MCC2034 was studied according to the method described by Yeong *et al.* (2002). The test medium consisted of MRS broth containing 0.3% (w/v) of Oxgal, adjusted to pH 5.8 using 5.0 N HCl. *L.plantarum* MCC2034 (10% v/v) was inoculated into the MRS broth with bile salt and incubated at 37^{o}C for up to 4h. MRS broth without added bile salt served as the control. Bile tolerance was evaluated by monitoring viable cell counts at 0, 30,120 and 240 min using plate count method

2.11. Statistical analysis

All the experiments on the screening, kinetics of growth and biosynthesis of EPS were carried out three times. Compositional analyses of EPS were done in three independent repetitions. All the data were expressed as means ± standard deviation.

3. RESULTS AND DISCUSSION

In the past three decades, the application of EPS producing LAB as functional starter cultures for the production of fermented foods (*e.g.* yoghurt, cheese, etc.) has attracted wide interest, because of the interesting technological properties of EPS (Ruas-Madiedo *et al.* 2002). Although genetic modifications have been shown to be successful in enhancing the production of EPS (Levander and Radstrom 2001), the public opinion and respective national legislations restrict the use of GMOs (Genetically Modified Organisms) for food applications (European Council Directive 2001). Therefore, exploration of the biodiversity of wild LAB strains, concerning their EPS production, seems to be the most suitable approach (Ruas-Madiedo and Gavilan 2005).

Takrarista, is a butter milk based *Ayurvedic* lactic fermented preparation that has a long history of consumption in India, for its favourable effects in the management of various gastro-intestinal maladies. In addition, it is also a rich source of native LAB that are actively involved in the fermentation process. In this context, the present investigation was carried out with the objective of improving the understanding of knowing the active ingredients produced by native LAB that contribute to the beneficial effects of *Takrarista*, with the ultimate aim of utilization of these beneficial LAB for varied food applications, more towards the current trend of 'Health Foods' and thus harnessing their beneficial attributes to their full potential. To the author's knowledge, this is the first report detailing with the screening, quantification and characterization of EPS producing LAB from an *Ayurvedic* lactic fermented preparation.

3.1. Screening of LAB for the production of EPS

A total of 38 LAB isolated from different *Ayurvedi*c preparations *viz. Takrarista, Kanjika* and *Sandaki,* were screened for their ability to produce EPS, out of which four of the strains were tested positive for EPS production. The production of EPS was detected by the presence of white, opaque, mucoid colonies having a characteristic sheen against the red background of the medium compared with that of EPS negative strains, indicated by the presence of transparent colonies taking up the colour of the media. The LAB isolates that tested positive for EPS were T 5-1, K1 and S5-2 isolated from ayurvedic lactic fermented preparations *viz. Takrarista, Kanjika* and *Sandaki*, respectively. Among these EPS positive LAB strains, T5-1, a *Takrarista* isolate, which has been identified as *L plantarum* (unpublished data) was chosen for

further studies, on the basis of its consistent and better EPS production.

3.2. Extracellular biosynthesis, isolation and quantification of EPS

The yield of EPS as function of fermentation time is depicted in **Figure 1**. As can be seen from the results, maximum yield of EPS was obtained at 40 h of fermentation. From the figure, it is evident that the growth of the isolate entered the log phase around 4 h, accompanied by a decrease in pH due to the production of lactic acid and concomitantly, EPS production also showed a visible increase. After 8-12 h, there was a transition from growth to stationary phase, and the pH also showed maximum decrease and stabilized thereby indicating the end of the growth phase. However, the production of EPS which commenced in tandem with the growth phase continued at a steady rate well into the stationary phase up to 40 h, where maximal yield of EPS was observed. The results revealed that the yield of EPS produced by *L.plantarum* MCC2034 was 1,059 mg/L.

3.3. Kinetics of growth and biosynthesis of EPS by *L.plantarum* MCC2034

The time course study of biomass and pH profile are shown in **Figure 1**. The biomass curve of *L.plantarum* MCC2034 indicates that in the EPM, lag phase lasts for the first four hours, following which the cells enter into the log phase of growth, indicated by the exponential increase in the biomass yield. The highest biomass yield of 605 mg/L was obtained after 16 h of incubation. The pH profile reiterates the transition of *L.plantarum* MCC2034 through different growth phases, since change in pH is directly related to the growth in LAB. The results show that in EPM, *L.plantarum* MCC2034 has specific growth rate (μ) of 0.217 h^{-1}. The growth yield coefficient ($Y_{X/S}$) was evaluated to be 0.019 ± 0.006 g of biomass g^{-1} sucrose. The product yield coefficients, $Y_{P/S}$ and $Y_{P/X}$, were 0.026 ± 0.005 g of EPS g^{-1} sucrose and 0.026 ± 0.005 g of EPS g^{-1} biomass, respectively. The studies on the growth curve *vis-à-vis* EPS production revealed that *L.plantarum* MCC2034 produced EPS in EPM throughout the growth phase, and continued well into the stationary phase, reaching the maxima at 40 h. The results are concurrent with earlier reports, where growth-associated production has been observed with most EPS from LAB (Ricciardi and Clementi 2000). The results indicate that *L.plantarum* MCC2034 could be utilized in the preparation of a variety of fermented food products that provide the added benefits attributable to the EPS producing LAB. This complies well with the original goal of screening and characterization of EPS producing LAB from *Ayurvedic* lactic fermented preparations and exploiting the same in the preparation of a variety of health promoting food products. The period taken by *L.plantarum* MCC2034 for the production of EPS is also reasonable, in terms of assessing its suitability in the production of the targeted food products at an industrial scale. The growth profile clearly indicates that the EPS production is directly related to the growth of the strain, wherein higher growth resulted in enhanced production of EPS. It was observed that the growth of *L.plantarum* MCC2034 decreased slightly at 12 h (Figure 1). The pH of the culture reached a minimum of 3.8 during the period between 12 and 16 h (Figure 1). The reasons for these two important findings can be attributed to the fact that the co-production of lactic acid might have temporarily thwarted the growth of the culture. In reaction to this situation, further production of lactic acid by the bacterial culture gets impeded. It is also imperative to notice that maximum biosynthesis of EPS was achieved when the growth was in stationary phase (Figure 1). It has been reported that bacterial EPS exert protective actions on the host bacterium (Knoshaug *et al.* 2000). EPS degradation upon prolonged incubation has been observed (De Vuyst *et al.* 1998) and has been attributed to the production of glycohydrolases (Gancel and Novel 1994). The yield of the EPS, being a heteropolysaccharide was also in accordance with the reported results (Vaningelem *et al.* 2004).

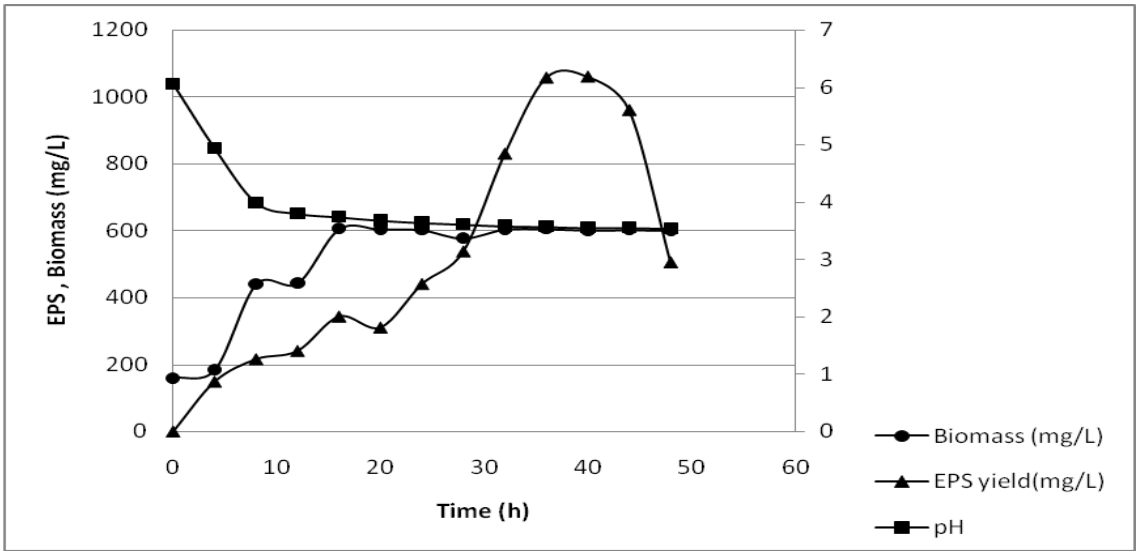

Figure 1 Time course study of the growth, biomass production , change in pH and EPS yield by *L.plantarum* MCC2034

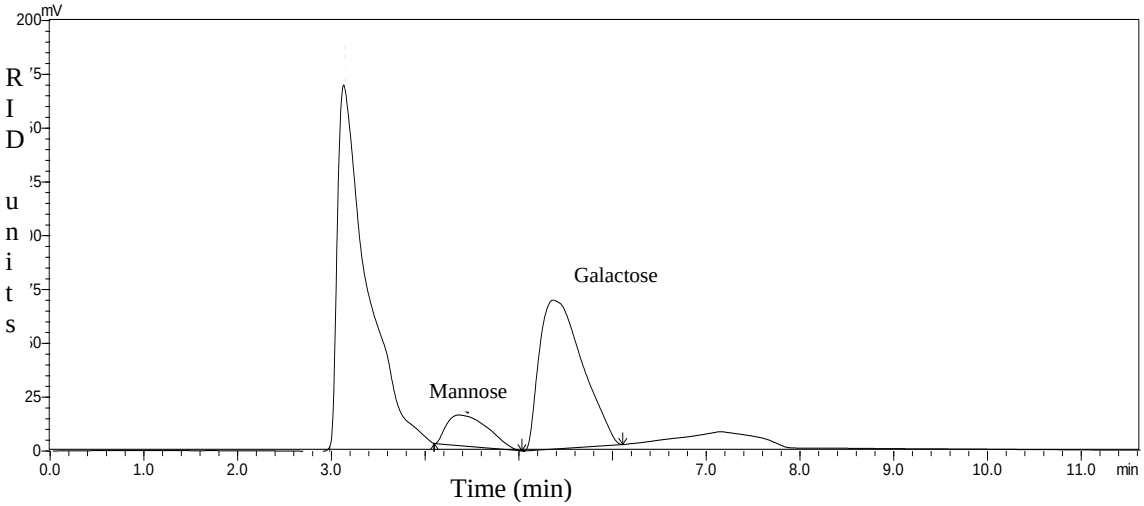

Figure 2 Compositional analysis of the EPS produced by *L.plantarum* MCC2034 in mod-MRS media. Peaks at 4.35 and 5.38 min represent mannose and galactose respectively

3.4. Compositional analysis of EPS

The HPLC chromatogram shows that the EPS produced by LAB isolate, *L.plantarum* MCC2034, is a heteropolysaccharide comprising of primarily galactose and mannose (**Figure 2**). The monosaccharides were identified by comparison with the respective standards.

3.5. Infrared analysis

The FTIR spectrum of the EPS indicating the major functional groups and the chemical bonds is presented in **Figure 3**. The broad peak at 3312 cm^{-1} was the -OH stretching peak and the peak at 2894 was indicative of the presence of C-H group.

The FTIR spectrum analysis of the EPS showed characteristic absorption peaks indicating the presence of –OH group and C-H group which have been reported to be major components present in the FTIR analysis of EPS. The results are consistent with the earlier reports of FTIR analysis of the EPS isolated from LAB and provide further indication about the chemical nature of the constituents of the EPS produced by *L.plantarum* MCC2034 (Vijayendra *et al.* 2008).

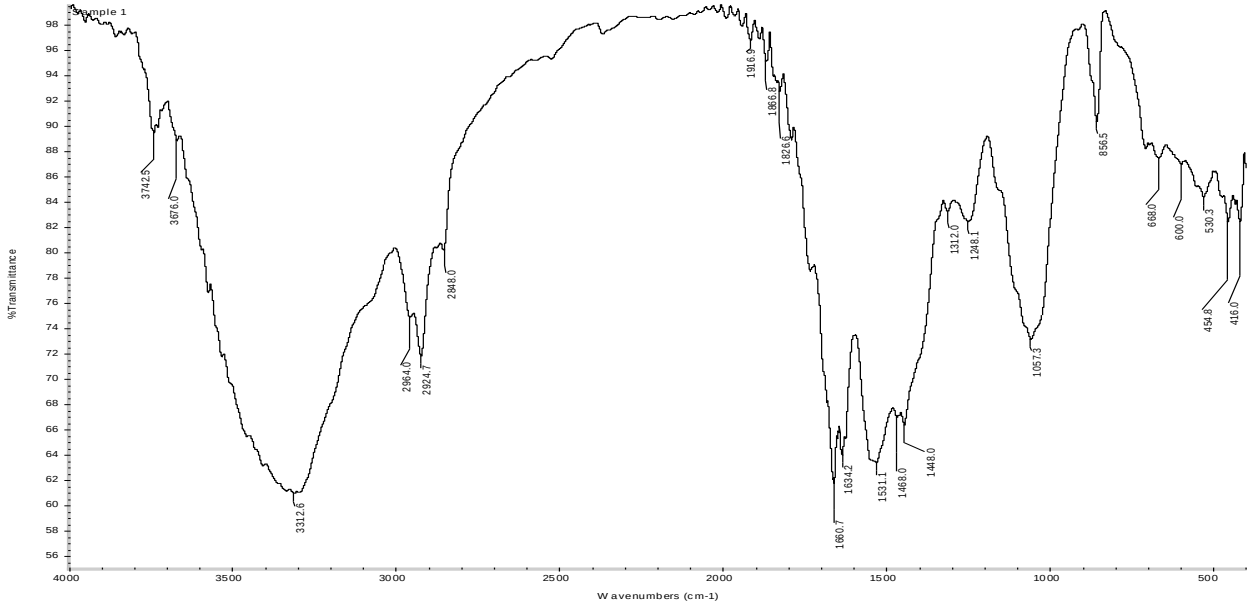

Figure 3 FTIR spectrum of the EPS produced by *L.plantarum* MCC2034

3.6. Acid tolerance

The viability of *L.plantarum* MCC2034 under acidic conditions is shown in Table 1. The initial viable count (0 min), at pH 2.0, 2.5, 3.0 and 7.0, was an average of 7.49, 8.60, 10.38 and 9.30 log CFU/mL, respectively. There was a steady decrease in the viability of the strain when exposed to acidic conditions, with the severity directly proportional to the acidity of the growth medium. Consequently, after one hour of incubation, the viability decreased to 6.95, 7.74 and 7.70 log CFU/mL at pH 2.0, 2.5 and 3.0, respectively.

Table 1 Evaluation of the acid tolerance of *L.plantarum* MCC2034

Viable count (log CFU/ml)

Time (min)	pH			
	2.0	2.5	3.0	7.0
0	7.76±0.	8.61±0.	10.20±0.	9.35±0.1
60	7.00±0.	7.68±0.	7.69±0.1	9.56±0.1
120	6.91±0.	7.67±0.	7.47±0.3	9.76±0.1
180	5.20±0.	7.61±0.	7.49±0.3	9.61±0.2

The experiments were conducted in triplicates and the values are expressed as mean±SD

This was in contrast to the viability of the control (pH 7.0), which showed a predictable increase of 9.41 log CFU/mL. After three hours of incubation, the final viable count of *L.plantarum* MCC2034 was 5.0, 7.39, 7.27 and 9.89 log CFU/mL at pH 2.0, 2.5, 3.0 and 7.0, respectively.

3.7. Bile tolerance

The effect of the bile salt (Oxgal) on the viability of the *L.plantarum* MCC2034 is presented in Table 2. The results indicate a positive effect on the viability of *L.plantarum* MCC2034 when exposed to Oxgal. The initial viable count of 9.24±0.22-log CFU/mL increased to 9.17±0.17-logCFU/mL after 30 min of exposure and the average viable count at the end of 240 min was 9.67±0.22 and 10.00±0.27 for *L.plantarum* MCC2034 with added bile salts and control, respectively.

The EPS producing LAB, *L.plantarum* MCC2034, was also evaluated for its probiotic potential, considering the fact that is a native LAB strain isolated from a preparation consumed for its beneficial properties. The EPS producing strain, *L.plantarum* MCC2034, was evaluated using primary probiotic tests that assess its ability to survive in low pH and also in the presence of added bile salts (0.3% w/v Oxgal). It is imperative that for any microorganism to be categorized as a probiotic, it should be capable of surviving the above mentioned tests since the bacteria face these

adverse conditions during their transit *via* the gastro-intestinal tract prior to reaching the more habitable colon region, the targeted area of colonization. Although the concentration of bile acid and pH varies largely between individuals, the levels used in the present study were within the physiological concentrations found in the human duodenum (Floch 2002). The results of these primary probiotic tests indicate that *L.plantarum* MCC2034 is capable of surviving at low pH for up to three hours and retain its viability (Table 1). The results of the experiments to determine the ability of *L.plantarum* MCC2034 to survive in the presence of bile salts indicate that the strain is capable of effectively countering the adverse effects of bile salts. Molecular characterization of the isolate, *L.plantarum* MCC2034, detailed chemical characterization and also the evaluation of bioactive properties of the EPS produced are under progress.

Table 2 Evaluation of the bile tolerance (0.3% w/v Oxgal) of *L.plantarum* MCC2034

	Viable count (log CFU/ml)			
Time (min)	**0**	**30**	**120**	**240**
Control	9.83±0.06	9.79±0.16	9.87±0.18	10.00±0.27
0.3% w/v Oxgal	9.24±0.22	9.17±0.17	8.08±0.23	9.67±0.22

The experiments were conducted in triplicates and the values are expressed as mean±SD

4. CONCLUSIONS

In the present study, *L.plantarum* MCC2034, a novel strain of LAB isolated from *Takrarista*, a traditional Indian fermented preparation, was evaluated for its suitability for the fermentative production of EPS. The results of the experiments discussed above, including the kinetics of EPS production and biomass yields, indicated that *L.plantarum* MCC2034 could be used effectively for the fermentative production of a heteropolysaccharide. Additionally, the strain also proved to be capable of surviving acid and bile conditions, thereby enabling its utilization as EPS producing LAB, with potent probiotic properties. This could open up new avenues in the management of gastrointestinal health, besides contributing to improved understanding of the active ingredients involved in the traditional *Ayurvedic* preparations.

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6. REFERENCES

[1] Galle,S., Schwab, C., Arendt, E.K., Ganzle, M. Structural and rheological characterisation of heteropolysachharides produced by lactic acid bacteria in wheat and sorghum sourdough. Food Microbiol., 2011, 28,547-553.

[2] De Vuyst, L., Degeest, B. Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol. Rev., 1999, 23,153–157.

[3] Roberts, I.S. The biochemistry and genetics of capsular polysaccharide production in bacteria. Ann. Rev. Microbiol., 1996, 50(1), 285–315.

[4] Salazar, N., Gueimonde, M., Hernandez-Barranco, A.M., Ruas-Madiedo, P., De los Reyes-Gavilan, C.G. Exopolysaccharides produced by intestinal *Bifidobacterium* strains act as fermentable substrates for human intestinal bacteria. Appl. Env. Microbiol., 2008, 74(15), 4737-4745.

[5] Korakli, M., Gänzle, M., Vogel, R. (2002) Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. J. Appl. Microbiol., 2002, 92(5), 958-965.

[6] Martensson, O., Biörklund, M., Lambo, A.M., Duenas-Chasco, M., Irastorza, A., Holst, O.E., Welling, G., Oste, R. and Onning, G. Fermented, ropy, oat-based products reduce cholesterol levels and stimulate the bifidobacteria flora in humans. Nutr. Res. 2005, 25(5), 429–442.

[7] Chabot, S., Yu, H.L., De Léséleuc, L., Cloutier, D., Van Calsteren, M.R., Lessard, M., Roy, D., Lacroix, M., Oth, D. Exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M stimulate TNF, IL-6 and IL-12 in human and mouse cultured immunocompetent cells and IFN-γ in mouse splenocytes. Lait, 2001, 81, 683–697.

[8] Russo, F., Orlando, A., Linsalata, M., Cavallini, A., Messa, C. Effects of *Lactobacillus rhamnosus* GG on the cell growth and polyamine metabolism in

HGC-27 human gastric cancer cells. Nutr. Cancer, 2007, 59, 106–114.
[9] Sreekumar, O., Hosono, A. The antimutagenic properties of a polysaccharide produced by *Bifidobacterium longum* and its cultured milk against some heterocyclic amines. Can. J. Microbiol., 1998, 44(11), 1029–1036.
[10] Ai, L., Zhang, H., Guo, B., Chen, W., Wu, Z., Wu, Y. Preparation, partial characterization and bioactivity of exopolysaccharides from *Lactobacillus casei* LC2W. Carb. Pol., 2008, 74(3), 353–357.
[11] Kim, Y., Oh, S., Kim, S.H. Released exopolysaccharide (r-EPS) produced from probiotic bacteria reduce biofilm formation of enterohemorrhagic *Escherichia coli* O157: H7. Biochem. Biophys. Res. Comm., 2009, 379(2), 324–329.
[12] Stingele, F., Jean-Richard, N., Beat, M. Identification and characterization of the *eps* (exopolysaccharide) gene cluster from S*treptococcus thermophilus* sfi6. J. Bacteriol., 1996, 178(6), 1680–1690.
[13] Sanchez, J.I., Martinez, B., Guillen, R., Jimenez-Diaz, R., Rodriguez, A. Culture conditions determine th*e balance between two diff*erent exopolysaccharides produced by *Lactobacillus pentosus* LPS26. Appl. Env. Microbiol., 2006, 72 (12): 7495–7502.
[14] Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith F. Anal. Chem. 1956, 28,350-356.
[15] Calsteren, V., Marie-Rose, Pau-Roblot, Andre, C.B., Denis, R. Structure determination of the exopolysaccharide produced by *Lactobacillus rhamnosus* strains RW-9595M and R. Biochem. J., 2002, 363, 7-17.
[16] Yeong, S., Ji-Young, L.P., Young-Sum, K., Dong-Hwa, S. Isolation and characterization of lactic acid bacteria from feces of new borne baby and from *Dongchimi*. *J Agric Food Chem 2*002, 50, 2531-2536.
[17] Ruas-Madiedo, P., Hugenholtz, J. and Zoon, P. An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. Int. Dairy J., 2002, 12, 163–171.
[18] Levander, F., Radstrom, P. Requirement for phosphoglucomutase in exopolysaccharide biosynthesis in glucose- and lactose-utilizing *Streptococcus thermophilus*. Appl. Environ. Microbiol., 2001, 67, 2734–2738.
[19] European Council Directive. Council Directive 2001/18/EC. The deliberate release into the environment of genetically modified organisms. Official Journal L., 2001, 106, 1–39.
[20] Ruas-Madiedo, P., De los Reyes-Gavilan, C.G. Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. J. Dairy Sci., 2005, 88, 843–856.
[21] Ricciardi, A., Clementi, F. Exopolysaccharides from lactic acid bacteria: structure, production and technological applications. Italian. J. Food Sci., 2000, 12, 23-45.
[22] Knoshaug, E.P., Ahlgrent, J.A., Trempy, J.E. Growth associated exopolysaccharide expression in *Lactococcus lactis* ssp. *cremoris* Ropy352. J. Dairy Sci., 2000, 83: 633–640.
[23] De Vuyst, L., Vanderveken, F., Van de Ven, S., Degeest, B. Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in milk medium and evidence for their growth associated biosynthesis. J. Appl. Microbiol., 1998, 84, 1059–1068.
[24] Gancel, F., Novel, G. Exopolysaccharide production by *Streptococcus salivarius* ssp. *thermophilus* cultures. Distinct modes of polymer production and degradation among clonal variants. J. Dairy. Sci., 1994, **77**, 689–695.
[25] Vaningelgem, F., Zamfir, M., Mozzi, F., Adriany, T., Vancanneyt, M., Swings, J., De Vuyst, L. (2004). Biodiversity of exopolysaccharides produced by *streptococcus thermophilus* strains is reflected in their production and their molecular and functional characteristics. *Appl Environ Microbiol*, 2004, 70 (2), 900–912.
[26] Vijayendra, S.V.N., Palanivel, G., Mahadevamma, S.,Tharanathan, R.N. Physico-chemical characterization of an exopolysaccharide produce by a non-ropy strain of *Leuconostoc* sp. CFR 2181 isolated from *dahi* ,an Indian traditional fermented product. Carb. Pol., 2008, 72, 300-307.
[27] Floch, M.H. Bile salts, intestinal microflora and enterohepatic circulation. Dig. Liver. Dis., 2002, 34, 54–7.