Isolation of exopolysaccharides producing lactic acid bacteria from dairy products

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ABSTRACT
Currently, much attention is being paid for improving the texture of food by screening the new exopolysaccharides (EPS) producing strains. The aim of the present work was to isolate EPS producing Lactic acid bacteria (LAB) strains from raw milk and milk products samples. Total of thirty eight dahi, lassi and raw milk samples were collected from different villages and towns of Karnal and Delhi District. The samples were plated on milk agar and colonies showing ropy polysaccharides production were subjected to biochemical test. After molecular identification 2 were found as S. thermophilus, 2 were Lb. rhamnosus and 2 were confirmed as Lb. fermentum. Two S. thermophilus strains (PD7 and PD11) and Lb. fermentum strains (AL6 and AD3) showed better curdling pattern, acidity, exopolysaccharides production, and sensory properties. These cultures can be used for manufacture of indigenous fermented milk products.

Key words: Dahi, Exopolysaccharides, Lb. fermentum, Lb. rhamnosus, S. thermophilus.

INTRODUCTION
Lactic acid bacteria (LAB) are gram-positive microorganisms that play an essential role in the industrial production of fermented dairy products. The metabolic products they generate during fermentation and ripening confer the rheological and organoleptic qualities desired by these products (Bennama et al., 2012). Some strains are known to produce exopolysaccharide (EPS), which play an important role in the development of the texture of yoghurt and other fermented milks, cheeses and low fat dairy desserts (Hassan, 2008). EPS from LAB are divided into two groups, homo- and hetero-EPS. Homo-EPS are composed of one type monosaccharide, whereas hetero-EPS consist of regular repeating units of 3-8 different carbohydrate molecules. EPS imparts highly desirable rheological changes in the food matrix such as increased viscosity, improved texture and reduced syneresis (Badel et al., 2011). Incorporation of EPS or EPS-producing starters in dairy foods can provide visciosifying, stabilizing, and water-binding functions. In situ production of EPS is very important in the manufacture of fermented dairy products, such as yogurt, drinking yogurt, cheese, cultured cream and milk-based desserts. EPS-producing LAB has a greater ability to withstand technological stresses (Stack et al., 2010) and survive the passage through the gastrointestinal tract compared to their non-producing bacteria.

In recent times, EPS produced by LAB have received mounting attention; mainly because of their health benefits. EPSs have been proved to show important health benefits like antioxidant, cholesterol lowering, antitumor, antiviral, and immunomodulatory activities (Madhuri and Prabhakar, 2014). Also, they reduce formation of pathogenic biofilms, help in modulation of adhesion to epithelial cells and increase levels of bifidobacteria showing a prebiotic potential (Hongpattarakere et al., 2012). Hence, the choice of EPS-producing starter cultures seems to give several advantages over non-producing ones. Also, LAB possess generally regarded as safe (GRAS) status which allows them to be incorporated in food without labeling. Most of the EPS-producing LAB can be isolated from different fermented foods such as dahi, lassi, yoghurt, cultured buttermilk, cheeses, yoghurt, kefir, and other fermented dairy products (Bunkoed and Thaniyavarn, 2014). Furthermore, EPS-producing strains can be also found in other environments such as gut of different animals and humans. Selection for LAB strains with newer properties can be used as new, functional starter cultures may lead to improved fermentation process and an enhanced quality of the end product. As information regarding isolation of EPS producing cultures from dairy products in Indian condition is scanty. The present study was undertaken to isolate EPS producing LAB from fermented dairy food for application in fermented milk products.

MATERIALS AND METHODS
Collection of samples: In an attempt to isolate some potential strains of EPS producing LAB, total 38 different

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samples of milk and milk products comprising of raw milk (12), dahi (16), and lassi (10) were collected in sterile plastic bottles from local market and rural and urban areas near Karnal and Delhi.

Reference strains and media: The standard cultures of *Lactobacillus fermentum* NCDC-141, *Lactobacillus rhamnosus* NCDC-24 and *Streptococcus thermophilus* NCDC-144 were procured from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute (NDRI), Karnal. *Lactobacillus* strains were isolated on Man, Rogosa and Sharpe (MRS) agar and modified MRS agar (Messens et al., 2002). The addition of a reducing agent such as cysteine 0.05% to MRS improves the specificity of this medium for *Lactobacillus* isolation (Shah, 2000). M17 and skim milk agar were used for *S. thermophilus* strains (Birollo et al., 2000). Skim milk agar medium was used for EPS producing strains. All strains were stored at -20 °C in their corresponding isolation medium, containing 30% (vol/vol) glycerol as a cryoprotectant.

Isolation of LAB: Appropriate dilutions of the samples were plated on the MRS and M17 agar. Viable counts were enumerated after incubation at 37 °C and 42 °C for 48-72 h. The individual colonies with different morphology were picked using tooth pick and grown in MRS and M17 broth. Further it was plated to check for purity. A total 80 presumptive LAB were isolated.

Screening for EPS production

Capsule formation: The ability of cultures to form a capsule was evaluated by capsule staining method (Anthony, 1931). A thin smear was prepared from actively grown culture in skim milk followed by air drying without hit fixing. Few drop of crystal violet were added for 2 min and smear rinsed with 20% (w/v) copper sulphate solution. The slides were air dried and examined under oil immersion lens, the capsules could be observed layer around the cell surface.

Ropy polysaccharide production: Production of roppy polysaccharide was observed visually. The culture were grown in skim milk tubes and after the curdling, tubes were vigorously shaken to break the curd. The broken curd was picked up with sterile loop or glass rod and observed for formation of long, roppy and viscous strand. The symbols for roppy polysaccharide production as observed visually was given as less roppy (+), medium roppy (+++) and highly roppy (+++).

Identification of LAB: The isolates were streaked on MRS and M17 with 4% sucrose medium for examination of morphological characteristics. The mucoid and roppy colonies on agar plate were observed by touching colonies with a sterile loop. The roppy colonies, formed a long filament when extended with a loop whereas the mucoid colonies have a slimy appearance on agar plates and were not able to produce strands by this method (Ruas-Madiedo and De Los Reyes-Gavilán, 2005). All isolates were tested for catalase and oxidase activity, Gram staining and spore formation. All Gram positive and catalase negative rods and cocci were tested for the growth in MRS and M17 broth respectively at 10, 37 and 45 °C (Abbas and Mahasneh, 2014). The suspected colonies were subjected to biochemical tests by using API 50 CHkit and CHL media (Biomérieux, France). Further, confirmation of *Lactobacillus* spp. (*Lb. fermentum* and *Lb. rhamnosus*) and *S. thermophilus* were done by molecular methods with some modification in PCR steps using reported primers by Song et al. (2000); Lick et al. (1996). The sequence of primers and the corresponding PCR cycle is given in Table 1. PCR was performed in a Biorad PCR System (S1000 Thermal cycler,Singapore). The amplified PCR products were then separated by agarose gel electrophoresis, stained with ethidium bromide and were subsequently visualized by UV transilluminator.

Technological screening: The milk (12% reconstituted skim milk) was fermented with identified EPS producing LAB strains at 37°C till curd is settled down. Technological parameters such as titratable acidity, curdling time, flavour, body and texture were evaluated. Titratable acidity of fermented milk was evaluated by titration method, while curdling time, flavour, body and texture simply by sensory method.

RESULTS AND DISCUSSION

Isolation of EPS producing LAB: Milk is the favourable environment for secretion of extracellular polysaccharide from several strains of LAB. In this study, total 80 isolates

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<th>TABLE 1: Species-specific primers and PCR cycle of LAB isolates</th>
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<td><em>Lactobacillus rhamnosus</em></td>
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<td><em>Lactobacillus fermentum</em></td>
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were obtained from milk and milk products collected from different sources. Based on morphological evaluation, some cocci and rod shaped isolates were selected (Fig. 1). Total 34 isolates were showed mucoid colonies on milk agar medium. All these EPS producing bacteria were of gram positive and catalase negative, which is a typical characteristic of LAB. These colonies showed clean lactic fermentation without any defects. The isolates with EPS were able to grow in milk and gave positive litmus milk test in 24 h as illustrated in Fig. 2. Repetitive streaking method was followed to purify all the suspected cultures. Previously, the numbers of EPS producing LAB were isolated from milk and milk products (Lavanya et al., 2011; Bennama et al., 2012).

**Screening for EPS production:** There are 2 types of EPS i.e. capsular polysaccharides (CPS) and ropy polysaccharides (RPS). CPS formation was evaluated by the Indian ink negative staining technique on the cells of LAB. Capsule staining revealed that around 14 isolates shown large capsule surrounding the cell surface while rest exhibited small or no capsule. Among the 14 isolates, PD11, AR2, AL6 produced larger and thick capsule (Fig.3). Isolate AD14 and PR5 did not show capsular polysaccharides production. Generally, capsular and ropy polysaccharides production greatly differs from culture to culture and certain strains were able to produce both types. Both CPS and RPS strains have been found in lactic acid bacteria (Mozzi et al., 2009). However, eighteen cultures showed the ropy polysaccharides production although long strand formation varied from strain to strain. Isolates AL6, AD3, AL18, AR2, PD11, PL7, PR3 shown highly ropy strand formation in curd, while isolates AD14, PD7, PD16, PD19, AL2, PD1 shown medium ropy strand in curd, and isolate PL5, PL6, AR5 shown less ropy strand in curd (Table 2). Figure 4 showed the ropy polysaccharide production by PD11 strain. As dairy industry point of view, the RPS producing strains were found to be more relevant as they are considered to be providing natural

![FIG 1: Morphology of LAB isolates [(a) & (b) – rods; (c)- cocci]

![FIG 2: Litmus milk test: left- control, right- positive test by strain DP 11]

![FIG 3: Capsule formation by isolates (a) PD11, (b) AR2, (c) AL6.]

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<th>TABLE 2: Technological Screening of EPS producing LAB strains</th>
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biothickness to the dairy product which enhances its technological properties. The use of yoghurt starter cultures that contain strains that produce EPS is a promising alternative. These polymers have been reported to improve the rheological behavior and texture of various fermented milk products (Badel et al., 2011). Even though both RPS and CPS increases the viscosity of the fermented product but only RPS can beneath a stretchable character provided by slime polysaccharides. The same was noticed in our isolates as well because the RPS producing cultures were found to be giving ropiness in the fermented milk therefore shown better technological attributes.

**Identification of EPS producing LAB:** The tentative isolates (n= 18) of LAB selected on the basis of microscopic and EPS production were further subjected to biochemical characterization. All isolates were tested for catalase and oxidase activity, Gram staining, spore formation and growth at 10, 37 and 45 °C (Abbas and Mahasneh, 2014). After biochemical test by using API 50 CH kit and CHL media (Biomérieux, France), 5 cultures were identified as *S. thermophillus* and 8 were *Lactobacillus* spp. Further, confirmation of *Lactobacillus* spp. (*Lb. fermentum* and *Lb. rhamnosus*) and *S. thermophillus* were done by molecular methods. Thirteen isolates were examined for their species specificity by using PCR reaction with reported primers (Table 1). A quite appreciable quality and amount of amplicons were observed in all isolates (Figure 5). The amplicons of all the 8 isolates showed the reported PCR product size along with the positive controls. Out of 13, 8 biochemically identified isolates were confirmed as LAB strains. Out of 8 isolates, 4 isolates (AR2, PR5, PD19 AND PD11) were confirmed as *S. thermophillus*, 2 isolates (AL6 and AD3) were confirmed as *Lb. fermentum* and 2 isolates (AL18 and PD7) were confirmed as *Lb. rhamnosus*.

**Technological screening of EPS producing LAB:** For the evaluation of technological properties of selected EPS producing LAB strains, acidity, curdling time and body and texture were used as indicator to select best strains. Table 2 shows result of technological properties and it was observed that four cultures AL6, AD3, PD7, and PD11 gave better results as compared to other four cultures. EPS production did not have effect on titratable acidity and curdling time hence clear cut relationship could not be established. The technological parameters which are greatly affected by EPS were flavour, body and texture and production of EPS improved these parameters. The effect of EPS cultures in improvement of properties of yoghurt, cheeses, dahi, lassi etc. are well demonstrated by rheological, sensory, and electron microscopic studies (Awad et al., 2005; Duboc and Mollet, 2001).

**CONCLUSIONS**

EPS producing LAB strains can be isolated from milk and traditionally made fermented milk products like dahi and lassi. These EPS producing cultures play important role in improvement of flavour and textural attributes. EPS also contributes to the mouth-feel, texture, and taste perception of fermented dairy products. However, EPS producing cultures should be initially screened for technological properties. The increasing demand for fat-free or reduced fat products is also raising interest in the use of EPS-producing LAB as natural bio-thickeners. There is need to establish specific EPS producing cultures for particular type of indigenous fermented milk products.

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