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Comparison of exopolysaccharide production by indigenous *Leuconostoc mesenteroides* strains in whey medium

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ABSTRACT

Two *Leuconostoc mesenteroides* strains NCDC 744 and NCDC 745 were evaluated for exopolysaccharides (EPS) production in whey and MRS medium. Paneer whey medium supplemented with 10% sucrose, 0.1 % yeast extract and 0.1 % K_2HPO_4 was found to be proferred medium for EPS production. Among two strains *L. mesenteroides* NCDC 744 produced significantly higher EPS (12.7 ± 0.24 gm/L) as compared to NCDC 745 (10.51 ± 0.18 gm/L). The EPS powder obtained from NCDC 744 after oven drying was white, crystalline, water soluble and contained 89.56 ± 0.37 % of total carbohydrate and 3.49 ± 0.14 % protein. Scanning Electron Micrograph (SEM) study revealed that the EPS from NCDC 744 was porous, compact and crystalline in appearance.

Key words: Exopolysaccharides, *Leuconostoc mesenteroides*, MRS, Paneer whey, SEM.

INTRODUCTION

Leuconostocs, one of the predominant members of lactic acid bacteria (LAB) group, is a gram-positive, non-motile and non-spore forming cocci, usually present as pairs or short chains. It plays an important role in fermentation of foodstuffs (sauerkraut, pickles, fermented milks and meat products) as a secondary or adjunct starter for the production of gas (CO_2) in cheeses resulting in open texture and production of flavour compounds in multiple dairy products. Certain strains of *Leuconostocs* are commercially exploited for production of exopolysaccharides (EPS) such as homopolysaccharides dextran, alteran and mutan from sucrose metabolism (Korakli *et al*., 2003; Han *et al.*, 2014). EPS producing lactic acid bacteria are of great importance in food industry as they impart better physical, rheological and sensory properties in low-fat dahi, lassi and yoghurt (Behare *et al*., 2013). These polymers are used as viscosifying, stabilizing, emulsifying, sweetening, gelling or water-binding agents, in the food as well as in the non-food industries (Korakli *et al*., 2003). Among various biopolymers, dextran produced by *Leuconostocs* species has many industrial applications. Dextran is an extracellular bacterial polymer of D-glucopyranose with predominantly α-(1→6) linkage in the main chain and a variable amount of α-(1→2), α-(1→3) and α-(1→4) branched linkages (Monsan *et al*., 2001). When, *Leuconostocs* are grown in a sucrose-rich media they release dextransucrase, enzyme, which converts excess sucrose to dextran and fructose (Tsuchiya *et al*., 1952; Torres-Rodriguez *et al*., 2014).

Current challenges in the utilization of EPS from LAB include not only strain improvement and enhancement of EPS production, but also selection of cost effective medium that facilitates easy extraction of these polymers (Rimada and Abraham, 2003). Several media including milk based, whey based and modified exopolysaccharides selection media (ESM) have been used to enhance EPS production and its subsequent extraction (Behare *et al*., 2009). However, most EPS production and isolation procedures are tedious, time consuming and include the risk of loss of polymer (Rimada and Abraham, 2003). Further, presence of interfering molecules like protein and media component greatly affect EPS production and extraction. Recently, synthesis of dextran in tomato-juice supplemented with sucrose as an alternative medium to manufacture dextran on a large scale has been proposed (Han *et al*., 2014).

Whey is one of the major by-products of dairy industry containing in valuable nutrients. In India, there has been a substantial increase in the production of paneer and cheese at commercial level, resulting in an increased availability of whey. India's annual paneer production is estimated at 1, 50,000 tonnes resulting in 2 million tonnes of whey, containing about 1, 30,000 tonnes of valuable milk nutrients (Goyal and Gandhi, 2009). Adequate utilization of

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whey is the biggest challenge in most dairy plants due to lack of infrastructure and unavailability of sophisticated equipment required for recovery of whey components especially lactose and minerals. Disposal of whey leads to economic loss and environmental problems. In this context, utilization of whey for production of EPS may be an economical and useful alternative. As there were few reports regarding utilization of whey for cost effective production of EPS, the present study aimed to utilize whey media for maximum EPS production by an Indigenous strain of *L. mesenteroides*.

MATERIALS AND METHODS

Leuconostoc mesenteroides strains NCDC-744 and NCDC 745 were obtained from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute (NDRI), Karnal, India. The cultures were maintained in modified MRS agar (Glucose replaced with 1% sucrose) as stab at 4°C and sub-cultured every 2 weeks.

Screening for EPS production on sucrose agar: The actively grown cultures in MRS broth (30°C / 24 h) were streaked on sucrose agar and incubated at 30°C for 24 h. The formation of mucoid or viscous colonies on sucrose agar was considered as positive sign for EPS production (Garvie, 1984).

Preparation of whey medium and production of EPS: Cheese and paneer whey was procured from Experimental Dairy Plant, NDRI, Karnal, and heated at 100°C for 30 min. It was filtered to remove visible milk solid residues, and centrifuged at 10,000g for 10 min to obtain clear solution with light greenish colour (Abiodun *et al*., 2002).

Whey medium (cheese and paneer whey) was prepared by supplementation of 10% sucrose (w/v), 0.1% yeast extract (w/v) and 0.1% K_2HPo_4 (w/v). Similarly, MRS media was prepared containing sucrose at different concentration (5, 7.5 and 10% w/v) in place of glucose. Initial pH of the media was adjusted to 7.0, before autoclaving. Production of EPS in whey medium and MRS media was carried out by inoculating the active grown cultures (O.D 1 at 600 nm) with 5% level of inoculum and fermentation was carried out at 30°C for 18 h.

Production, extraction and quantification of EPS: The production of EPS was carried out in 250 ml Erlenmeyer flasks containing 100 ml medium inoculated with 5% culture inoculum. The EPS from fermented whey and MRS medium was extracted as per the method given by Majumder *et al*., (2009). After fermentation the culture supernatant was obtained by centrifugation of the media at 10000 x g at 4°C for 10 min. The crude EPS was precipitated by the addition of 3 volumes of 95% (v/v) pre-chilled ethanol at 4°C and centrifuged at 13000 x g. The process of precipitation was repeated to remove any trace impurities or free reducing sugars. EPS was quantified by phenol-sulfuric acid method and further analyzed for moisture and protein content. For moisture content, the dried EPS was taken in a dish, which was previously dried and weighed. EPS containing dish was placed along with its lid in an oven maintained at 105°C for 5 h and cooled in a desiccator. Drying was repeated till a constant weight was obtained and the moisture content calculated. Protein content in EPS was determined by Lowry method (Lowry *et al*., 1951) and carbohydrate content by phenol-sulfuric acid method (Dubois *et al*., 1956).

Scanning electron microscopic analysis (SEM): Surface morphology of EPS producing cells and dried EPS harvested from paneer whey were carried out using a Scanning Electron Microscope (EVO® 18, Carl ZEISS Special Edition-UK). Cells were harvested from the fermented media by centrifugation (10000 x g, 10 min) and were resuspended in 1 volume of 0.1 mol L^{-1} phosphate buffer (pH 7.4). Aliquots of samples (5-10µl) taken on glass cover slips and air dried and were fixed in a solution of 2.5% glutaraldehyde in 75 mM Phosphate buffer, (pH 7.4) for 45-60 min. Samples were rinsed three times for 15 min at a time in 50% 75 mM phosphate buffer. After rinsing, samples were serially dehydrated in ethanol concentrations each of 30, 50, 70, 80, 90 and 100% for 15 min. Samples were then mounted on stub followed by coating with gold. In case of dried EPS (extracted from paneer whey), sample was mounted on SEM stub with the assistance of a double-sided tape (Siddiqui *et al*., 2014) and encrusted with gold at approximately 100-200 Å thickness on Hitachi IB-3 ion coater. The ion current was kept at 6-8mA at fine vacuum of 0.05-0.07 torr for 2-4 min furthermore, the coated sample was finally visualized.

Statistical analysis: The results were mean of three independent experiments. The data were analyzed by one-way analysis of variance (ANOVA) with Statgraphics Centurion v16.1.15 software. After ANOVA, Duncan's multiple range tests were applied to compare sets of means with a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Both *Leuconostocs* strains were able to produce shiny, mucoid or viscous colonies on sucrose agar (Figure 1A) and when touched with sterile spreader, thick viscous strand was formed (Figure 1B), which may be used as a visual indicator for screening of higher EPS producing strains. In the past also several EPS producing lactic strains have been isolated using various sugars as carbon source that improved screening of potential EPS strains (Garvie, 1984). Comparison of EPS production in different whey medium

FIG 1: (A) Shiny mucous/viscous colonies upon streaking on sucrose agar, (B) Highly mucous colonies when touched with sterile spreader

FIG 2: Dried EPS from NCDC 744

comprising carbon and nitrogenous sources indicated that the polymer production was significantly higher in paneer whey supplemented medium followed by cheese whey and MRS in decreasing order Paneer whey medium fermented by *L. mesenteroides* NCDC-744 was observed to be extremely viscous. Biosynthesis and secretion of EPS from *Leuconostocs* occur during different growth phases, and both the amount and type of polymer influenced by composition of medium and growth conditions (Sutherland 1972). Moosavi-Nasab *et al*., (2010) reported fermentative production of dextran using food industry wastes (whey + molasses) by *L. mesenteroides* NRRL B-512F yielded 9.51±0.36 gm/L of dextran. In our study, *L. mesenteroides* NCDC-744 produced higher EPS i.e. 12.7 gm/L in paneer whey medium containing sucrose, yeast extract and K_2HPO_4 (Table 1). Irrespective of the strain, negligible amount of EPS was produced in paneer and cheese whey without supplementation of sugar and yeast extract. This may be due to lack of carbon and nitrogenous sources required for the growth and production of EPS by *Leuconostoc* spp. Fermentation of MRS media containing different concentration of sucrose yielded lower quantity of EPS by both the cultures although increase in sucrose concentration slightly increased EPS production. This means MRS medium even if supplemented with the sugars is not the ideal medium for EPS production by *Leuconostocs* strains. Media containing complex nutrients are not suitable because of interference of these compounds with the monomer synthesis, thereby total yield and structure of EPS (Kimmel and Roberts, 1998). The EPS yield from *L. mesenteroides* NCDC-744 in whey based medium was in tandem or higher as compared to previous reports using other strains of *L. mesenteroides* CMG713 and *L. mesenteroides* NRRL B-512F, which is commercial dextran producing strain (Sarwat *et al*., 2008; Moosavi-Nasab *et al*., 2010). Figure 2 shows the physical appearance of EPS powder obtained from NCDC-744. Comparison of physical and chemical properties of EPS produced by *L. mesenteroides* NCDC-744 with that of *L. mesenteroides* NRRL B512F and CMG713 are presented in Table 2. EPS produced by NCDC-744 was white crystalline as compared to other variants of *L. mesenteroides*. Physical variations may be due to differences in molecular aggregation

TABLE 1: EPS production in different media by *L. mesenteroides* NCDC 744 and NCDC 745

Fermentation media	EPS concentration gm/Litre	
	Strain NCDC 744	Strain NCDC 745
Paneer whey	0.047±0.013^{g}	0.030±0.02^{g}
Paneer whey Supplemented (10% sucrose, 0.1% YE & K_2HPO_4)	12.7±0.24^{a}	10.51±0.18^{a}
Cheese whey	0.50±0.03^{f}	0.36±0.01^{f}
Cheese whey Supplemented (10% sucrose, 0.1% YE & K_2HPO_4)	11.6± 0.27^{b}	9.40±0.19^{b}
MRS medium Supplemented with 5% Sucrose	1.34±0.13^{e}	1.33±0.11^{e}
MRS medium Supplemented with 7.5% Sucrose	2.21±0.17^{d}	1.89 ±0.14^{d}
MRS medium Supplemented with 10%Sucrose	2.74±0.21^{c}	2.36 ±0.24^{c}

Mean±Standard deviations, n=3

*Means without a common superscript in columns are signiûcantly different ($P < 0.05$)

TABLE 2: Physico-chemical characteristics of crude EPS of NCDC 744 and reported *L. mesenteroides* strains

Characteristics	*Leuconostocs mesenteroides*BA08	*Leuconostoc mesenteroides* CMG713[a]	*Leuconostoc mesenteroides* NRRL B512F[b]
Color& Texture	White, Crystalline	White, amorphouspowder	Colourless-light
Carbohydrate (%)	89.56 ± 0.37	79	25 (% reducing sugar only)
Total Protein (%)	3.49 ± 0.14	1.9	1.0
Moisture (%)	6.5 ± 0.27	10.2	-
Solubility (In water)	Soluble	Soluble	Soluble

[a] Sarwat *et al*., 2008
[b] Moosavi-Nasab *et al*., 2010

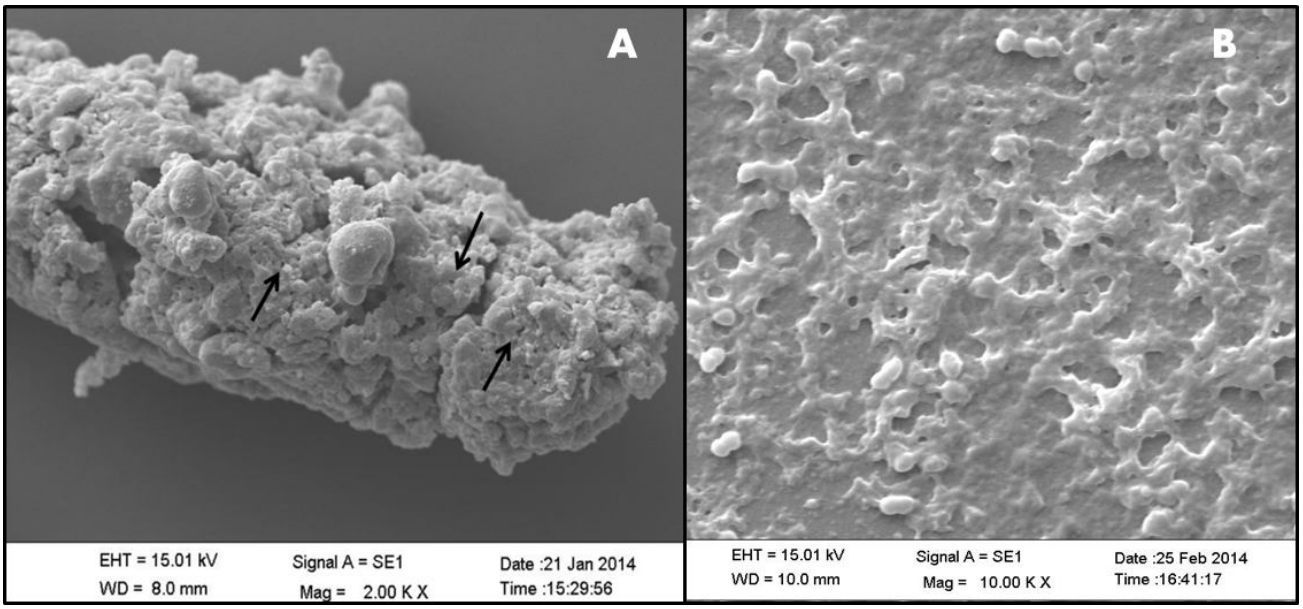

FIG 3: Scanning electron micrograph of dried EPS (A) and EPS producing cells of NCDC 744 (B);
FIG. 3A: Arrow indicates porous crystalline structure of dried EPS
FIG. 3B: Cells harvested from fermented paneer whey medium showing liberated EPS and slime around the cells.

(Sarwat *et al*., 2008). However, the protein content of EPS from NCDC-744 was higher as compared to previous reports, which may be due to cells, which remain intact in the polymer during extraction process or due to contamination of media component. Scanning electron microscopic study of oven dried EPS (Fig. 3A) from higher producing strain NCDC-744 reveals porous, compact and crystalline structure that might facilitate its solubilization in water based solutions (Siddiqui *et al*., 2014) and could be useful as an alternative source for commercial plant based texturing, stabilizing and water binding agents in food and pharmaceutical industry. Moreover, cells harvested from fermented paneer whey media showed liberated EPS or slime around the cells (Fig. 3B).

CONCLUSION

Paneer whey supplemented with sucrose, yeast extract and K_2HPo_4 is best suited for the production of EPS from the *Leuconostoc* species. Further, the fermented viscous whey produced after fermentation could be dried by lyophilisation or spray drying and may find its use as a food ingredient where milk solids, whey, thickeners or stabilizers are used in foods. Besides economical production of bio-thickener, the developed process offers efficient and environment friendly means of utilization of whey and its valuable nutrients. The structural identification and optimization of nutrient conditions to scale up the production of this multifunctional biomolecule need to be studied in detail for its commercial application.

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