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Production of exopolysaccharide by bifidobacteria and its viscometric analysis

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Abstract

The exopolysaccharide production by three *Bifidobacterium* strains was evaluated by optimizing two parameters (temperature and time). In addition, the role of EPS on viscosity of solutions was observed. Bacterial cultures were grown in MRS broth supplemented with 0.5 % (w/v) cysteine HCl in anaerobic conditions. Among the different time (24 h, 48 h and 72 h) and temperature (30°C, 37°C and 42°C) conditions, high EPS production was observed at 42 °C after 72 h of incubation. At these conditions maximum amount of EPS was produced by *Bifidobacterium breve* 11815 with the yield of 94.64 ± 0.25 ug/ml, followed by *B. longum* 11818 and *B. animalis* ssp. *lactis* Bb12 with the yield of 90.53 ± 0.34 ug/ml and 58.8 ± 0.25 ug/ml respectively. Viscometric analysis of EPS performed by viscometer showed highest viscosity of milk (23 ± 1.41 cp) by using EPS produced by *B. animalis* ssp. *lactis* Bb12. This study suggests that the foods in which bifidobacteria are used as starter culture should be incubated at 42 °C to obtain maximum probiotic dose and EPS. Finally, EPS produced by *B. animalis* ssp. *lactis* Bb12 can be used for reducing syneresis and improving texture and viscosity of food products.

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Introduction

Nowadays food grade bacteria including lactic acid bacteria (LAB) and bifidobacteria are widely used for the production of exoplysaccharide (EPS) because these are GRAS (Generally regarded as safe) organisms. The term probiotic is used for microorganisms that include both bacteria and fungi which when used in sufficient amount, provide health benefits (Kerry *et al*., 2018).

There are different properties of microorganisms due to which they are considered as probiotis and these properties include, 1) they are safe, 2) they are resistant to stomach acids, 3) they are resistant to bile acids, 3) they colonize the intestine to compete the pathogenic organisms, 4) they kill the pathogens by producing bacteriocins, hydrogen peroxide, organic acids and short chain fatty acids, 5) they maintain the intestinal pH, 6) they boost the nonspecific immune system by stimulating mucin production (Alp *et al*., 2010; Fanning *et al*., 2012; Hughes *et al*., 2017; Reid *et al*., 2019).

EPSs produced by food grade bacteria are used as stabilizers, emulsifiers, gelling agents, thickeners and water binding agents and thus they contribute in maintaining rheology and texture of fermented milk products (Sutherland, 1998; Sengupta *et al*., 2018).

EPSs are either homopolysaccharide polymers (composed of same monosaccharide units) or heteropolysaccharides (composed of different monosaccharide units). Based on the composition of monosaccharides and the chemical bonds between them, there is great diversity in EPSs synthesized by different bacteria. EPSs are loosely attached to the cells and are mostly diffused in surroundings (Tallon *et al*., 2003). EPSs produced by bacteria can be ropy or non-ropy. Ropiness of EPSs increases the viscocity of food products in which they are used. (Mende, 2016).The production of EPSs by Bifidobacteria is one of the suggested mechanisms for their probiotic activities. EPSs produced by Bifidobacteria, have got role in maintaining commensalism between human host and bacteria by altering the physical properties of cell surfaces (Hughes *et al*., 2017).

There are different factors that may affect on the production of EPSs including, 1) Composition of the medium, 2) Bacterial strains and 3) Growth conditions. To achieve the maximum production of EPSs it is very important to optimize the conditions (Ismail and Nampoothiri, 2010).

As mentioned previously many lactic acid bacteria and bifidobacteria have been reported to produce EPSs but unfortunately very few of them have been used commercially. This is either due to their inability of producing constant amount of EPS or lacking in improved properties. Therefore, quality of fermented food product in which EPSs are used is affected. To avoid inconsistency in EPS production, this study was carried out to optimize the cultural conditions for production of constant and high quantity of EPS by three *Bifidobacterium* spp. In addition, role of EPS in enhancing the viscosity of milk was also assessed.

Material and methods

Microorganisms, media and culture conditions

Bifidobacterium animalis ssp. *lactis* Bb12 (Christian Hansen, UK), *Bifidobacterium breve* 11815 (NCTC) and *Bifidobacterium longum* 11818 (NCTC) were sub cultured twice on de Man, Rogosa and Sharpe agar (Oxoid, UK) supplemented with 0.5% L-cysteine HCl under anaerobic conditions at 37 ^{o}C for 48 h. Anaerobiasis was generated by using anaerogen sachet (Oxoid, UK) (Audy *et al*., 2010). Gram staining was performed to confirm their Gram reaction and absence of contaminant bacteria. For routine experiment, bacterial strains were subcultured and maintained on De Man, Rogosa and Sharpe (MRS) agar supplemented with 0.5% L-cysteine HCl and were stored at -18 ^{o}C in cryogenic vials as stock cultures (Novik *et al*., 2008).

Effect of culture parameters on growth and EPS production

Before experimental use bacterial strains were propagated twice by method described in previous section. For experiment 1% (v/v) of each bacterial

culture was inoculated in MRS broth supplemented with 0.5% cysteine HCl. To optimize the growth conditions that lead to highest EPS production, all three cultures were grown anaerobically under different variables: incubation time (24 h, 48 h and 72 h) and incubation temperature (30 °C, 37 °C and 42 °C). Unioculated control was also kept for each batch. Before incubation and after each 24 h, samples from each cultures were collected aseptically for viable counts to create growth curves, and EPS quantification.

Enumeration of bacteria

One mililitre of each bacterial culture was diluted with 9ml of maximum recovery medium (MRD) and mixed by using vortex mixer. Serial dilutions were prepared followed by inoculation and spreading with sterilized glass spreader on MRS agar to determine viable counts (cfu mL^{-1}) (Ayala-Hernandez *et al.*, 2009). Plates were incubated anerobically at 37°C for 48 h. Plates containing 30-300 colonies were enumerated and recorded. (Prasanna *et al.*, 2012)

Extraction of EPS

The extraction of EPS was performed by method described previously by Yang *et al.*, (1999) and reviewd by Zhang *et al.*, (2011). In brief, 10ml of each sample culture was heated at 100 °C for 15 min. Samples were then cooled and mixed with 4% (w/v) trichloroacetic acid and were centrifuged at 12500 × g for 30 min at 4 °C. Double voulme of cold ethanol was added in supernatant and then stored at 4°C for 24 h. Samples were centrifuged at 12500 × g for 30 min at 4 °C to collect the precipitated EPS. EPS was then dried at 60 °C in oven.

Quantification of EPS

EPS (Dried) was mixed with 2ml of distilled water and total EPS (expressed as mg L^{-1} glucose) produced by each bacterium was estimated by phenol sulphuric acid method (Dubois *et al.*, 1956) using glucose as a standard on spectrophotometer (Torino *et al.*, 2001).

Amount of EPS (carbohydrate equivalent) was calculated in the sample solution using the standard graph.

Viscometric analysis of EPS

EPS was mixed in whole milk supplemented with skimmed milk (6%). The viscosity of the solutions was measured with a viscometer (Brookfield, DV-E, USA) at 25°C by applying different shear rate (50, 60, 100 rpm) and LV 4 spindle for all the solutions (Bejar *et al.*, 1998).

Statistical analysis

All anaylses were run in triplicates (n=3). Results are expressed as mean ± standard deviation (S.D). Significant differences were considered at $P < 0.00001$. Data were analyzed with one way analysis of variance (ANOVA) using R (Version, 2.15.1) software.

The Tukey's significant different test was also performed to compare the means.

Results and discussion

Phenotypic identification of Bifidobacterium strains

All the cultures exhibited typical Bifidobacterial morphological characteristics and were observed as Gram positive, pleomorphic rods with uniform to branch or club shaped cells.

Table 1. Viscocity of EPS produced by Bifidobacterial species.

EPS sample	Viscocity (cp) (Mean ± SD) n=3
B. animalis ssp *lactis* Bb12	23±1.15
B. breve 11815	19±1
B. longum 11818	19±0

Effect of temperature and time on growth of Bifidobacterium strains

High cell growth 7.56 ±0.02 log was observed for *B. breve* 11815 at 37 °C temperature followed by 7.51 ± 0.13 at 42 °C after 72 h of incubation (Fig. **1.**). For *B. longum* 11818 high cell growth (7.5 ± 0.04 log) was observed at 37 °C followed by 7.37± 0.07 log at 42 °C (Fig. 1.). In contrast to above strains for *B. animalis*

ssp. *lactis* Bb12 high cell growth (7.4 ± 0.005 log) was observed at 42 ºC followed by 7.23 ± 0.005 log at 37 ºC (Fig. **1.**). ANOVA showed that there was significant difference in growth rate ($p < 0.0001$) among these three strains.

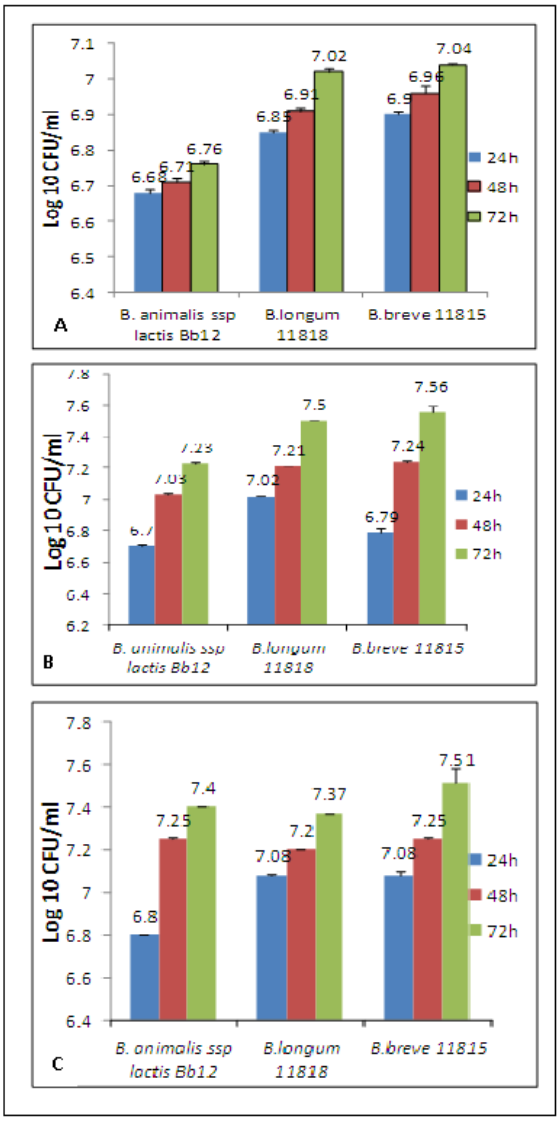

Fig. 1. Viable cell count of *Bifidobacterium strains* at various temperature, A) 30°C, B) 37°C, C) 42°C

In this study results show that with the increase of time (maxiumum 72 h), cell count also increases. In contrast with these results Ostile *et al.*, (2005) observed reduction in cell counts of *B. animalis* BB12. Generally high cell count in food products results in good quality. According to Roy *et al.*, (2001), number of bifidobacteria in fermented milks should be $>10^6$ bifidobacteria/g at the time when strain is mixed to the product. Therefore, rapid and reliable methods should be used in food industry to routinely determine the initial inoculum added in food product and also to evaluate the viability of bifidobacteria during storage time period.

Effect of temperature and time on EPS production by Bifidobacterium strains

The influence of temperature and time on the production of EPS by *Bifidobacterium* strains was also determined by incubating the organisms at different temperature (30 ºC, 37 ºC and 42 ºC) over a range of time (24 h, 48 h and 72 h). EPS production was quantified with spectrophotometer by using glucose as standard. Calibration curve was prepared (Fig. 2.).

By analysing the data through ANOVA, it was concluded that organisms were significantly different ($p < 0.0001$) in their EPS production. Genearlly all the *Bifidoacterium* strains produced high amount of EPS at 42 ºC after 72 h of incubation ranging from 58.8 ± 0.25 to 94.64 ± 0.25 ug/ml. At these conditions maximum amount of EPS was produced by *B. Breve* 11815 (Fig. 3B) with the yield of 94.64 ± 0.25 ug/ml, whereas, lower amount of EPS was produced by *B. animalis* ssp. *lactis* Bb12 (Fig.3B) with the yield of 58.8 ± 0.25. Compared to 42 ºC, low amount of EPS was produced at 37 ºC (Fig. 3A). EPS was not produced by either strain at 30 ºC.

With the increase in time of incubation, EPS production was also increased. This could be because of increase in total viable count of bacteria as mentioned earlier with the passage of time. This indicated the correlation of bacterial count and amount of EPS produced. However at 30 ºC no EPS was produced at all although results of total viable count showed the presence of viable bacteria in the samples; In contrast to this study which suggests that by increasing time of incubation EPS production also increases, some reports show that EPS concentration decreases during prolonged incubation for strains of *Lactobacillus pentosus* (6 ug/ml) (Sanchez *et al.*, 2006) and *Streptococcus thermophiles* (136 ug/ml) (Zisu and Shah, 2003). Decrease in EPS concentration could be because of presence of

degradative enzymes which hydrolyze the EPS as reported by Degeest *et al.*, (2001). However, there is no any report about EPS degradation of EPS by bifidobacteria, therefore this should be researched. High production of EPS at high temperature could be due to the fact that some bacteria produce EPS in stress conditions as reported by Prasanna *et al.*, (2012). In this study high production of EPS was observed at 42 °C, where as some researchers got the higher cell count and higher EPS production at 37 °C for *Streptococcus thermophilus* and *L. casei* strains (Gancel and Novel, 1994; Mozzi *et al.*, 1996). In our study no EPS was produced at 30 °C by bifidobacteria. In contrast in previous studies significant amount of EPS was produced by mesophilic EPS producing lactic acid bacteria (Petry *et al.*, 2000 and Sanchez *et al.*, 2006).

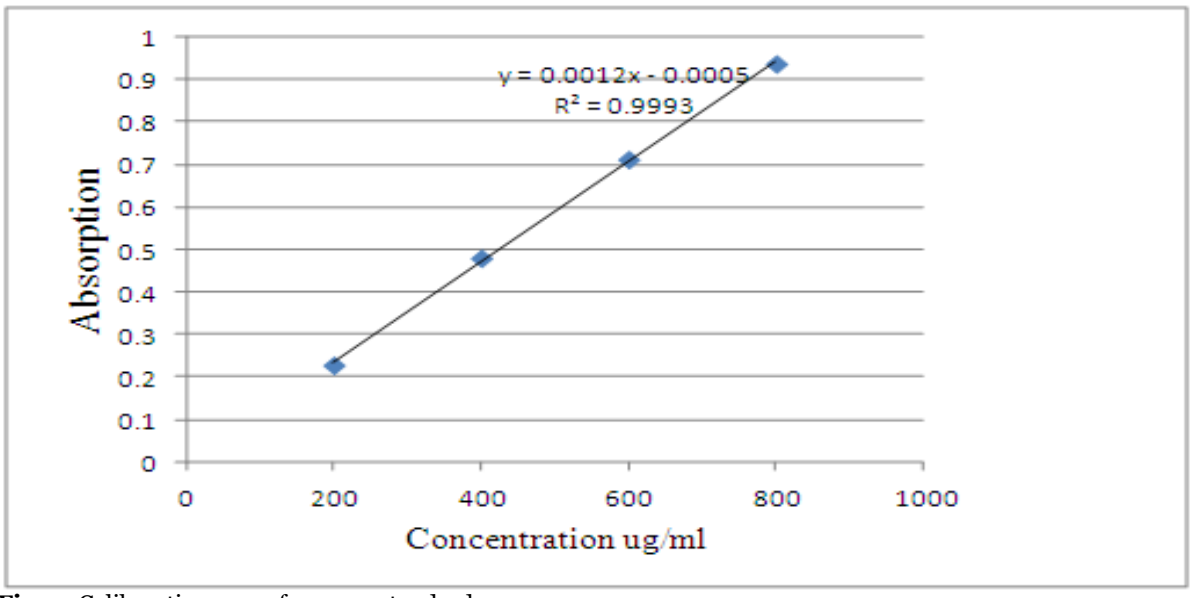

Fig. 2. Caliberation curve for sugar standards.

In this study time and temperature were optimized to produce the high amount of EPS by bifidobacteria. However, EPS production could be improved by manipulating medium composition by adding different sugar source (Audy *et al.*, 2010), providing different environmental conditions such as oxygen tension (Gamar-Nourani *et al.*, 1998) and different amount of carbon dioxide (Ninomiya *et al.*, 2009).

There are certain limitations of the method by which EPS was quantified in this study. Phenol sulphuric acid method was used to quantify the EPS by using spectrophotometer; this method is used to detect the carbohydrates in sample. However there is possibility that EPS produced by bifidobacteria may also contain other components such as proteins, peptides, and phosphate. Therefore research is needed about chemical analysis of EPS. Moreover change in pH of the medium could also effect on the production of EPS and this was confirmed by Grobben *et al.*, (1997) and reported that under controlled condition of pH, significantly higher amount of EPS is produced compared to batch fermentations with uncontrolled pH. In another study, De Vuyst and Degeest, (1999), reported that in some cases controlled pH conditions produced more exopolysaccharide than that of supplementation with nutrients and molecular structure and sugar composition of the EPS was also dependent upon fermentation conditions.

Viscometric analysis of EPS

Highest viscosity, 23 ± 1.41 cp was observed for EPS produced by *B. animalis* ssp. *lactis* Bb12, followed by equal viscocity 19 ± 0 cp by *B. breve* 11815 and *B. longum* 11818 (Table 1). Viscometric analysis of EPS solutions was recorded at 25 °C. Due to very low difference in values, ANOVA could not analyse the data. Although viscosity was measured at different

shear rate such as 50, 60 and 100 no change in viscosity was detected at these shear rates.

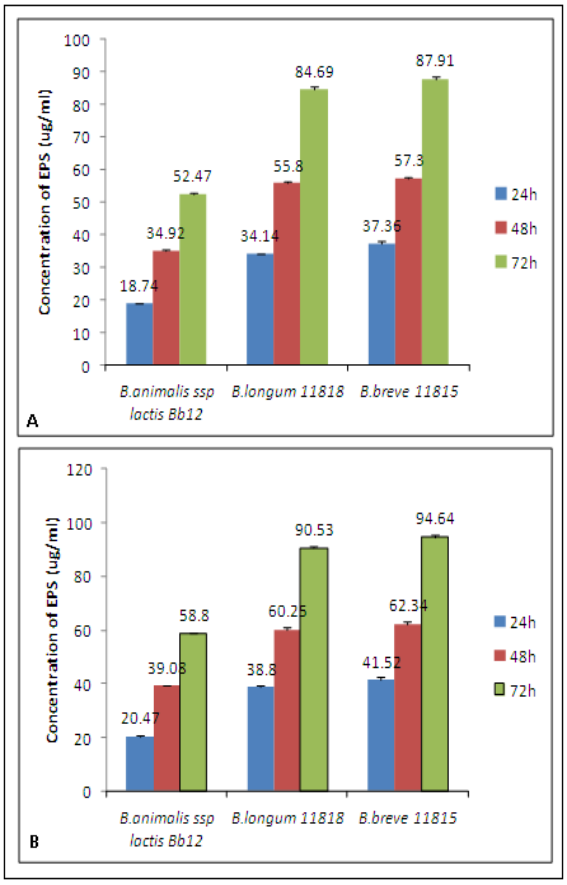

Fig. 3. Production of EPS by *Bifidobacterium* strains at various temperature, A) 37°C, B) 42°C.

EPS produced by *B. animalis* ssp. *lactis* Bb12 showed maximum viscosity. However, this organism produced a lowest amount of EPS. On the other hand *B. breve* 11815 produced high amount of EPS but its EPS showed lower viscosity. This suggests that that EPS produced by *B. animalis* ssp. *lactis* Bb12 could be used in industry to increase the viscosity of food products. However, further research is needed on the optimization of media and other cultural conditions to stimulate the growth of this organism and achieve high yield of EPS. Nevertheless, there is no correlation between production of EPS and viscosity of medium (Bouzar *et al.*, 1996). In this study, overall it was observed that the viscosity of the EPS solutions was very low and ranged from 19 to 23 cp. This could be because very low amount (2g/100 ml) of EPS was mixed with milk to detect viscosity. In addition, viscosity could be increased by lowering the pH of EPS solutions as observed by Bejar *et al.*, (1998). In their study viscosity of the EPS solutions ranged from 15 to 32.1 cP in complex medium and 26.1 to 100 in minimal medium at pH 7.0, but by decreasing pH of the solutions, maximum viscosity was recorded with 16600 cP in complex medium and 3000 cP. Other factors such as temperature, salt concentration of medium, protein content in the medium, time of fermentation, EPS conformation or interactions between EPS and growth media microstructure could also effect on the viscosity.

Conclusion

Initially, to observe the role of EPS produced by *Bifidobacterium* strains in enhancing the viscosity of medium, EPS was dissolved in distilled water and viscosity was measured with viscometer (Brookfield, DV-E, USA). Unfortunately, viscometer could not detect the viscosity of solution. This could be because the viscosity of solution was very less than the lowest detection limit of the viscometer used. To overcome this problem distilled water was replaced by whole milk (liquid) but yet there was same problem. Finally milk was supplemented with 6 % (w/v) skimmed milk and then viscometer could detect the viscosity of solutions and effect of EPSs on viscosity of milk was measured.

The current study investigated three *Bifidobacterium* strains for their potential of EPS production. Findings revealed that highest EPS was produced by *B. breve* 11815 followed by *B. longum* 11818 at 42 °C. Thus, these strains can be used as starter culture in food products including yogurt. In this study, *B. animalis* ssp. *lactis* Bb12 produced less EPS but EPS produced by this strain was found to be effective in increasing viscosity of milk samples and therefore can be used for reduction of syneresis and improvement of texture and viscosity of food products. Further research is needed to optimize the various conditions to increase the production of EPS by *B. animalis* ssp. *lactis* Bb12.

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