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Design of experiments for enhanced production of bioactive exopolysaccharides from indigenous probiotic lactic acid bacteria

Bilqeesa Bhat, Surbhi Vaid, Bisma Habib & Bijender Kumar Bajaj*

School of Biotechnology, University of Jammu, Jammu-180 006, Jammu and Kashmir, India

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Exopolysaccharides (EPS) produced by several bacteria including the probiotic lactic acid bacteria (LAB) not only help them to execute certain vital life functions, but offers huge potential for applications in sectors like medical/pharmaceutical, food, agriculture, and environmental health. However, low yield of EPS from probiotic LAB has always been a challenge. Previously we have reported that EPS from two LAB probiotic strains *i.e. Enterococcus faecium* K1 (isolate from *kalarei*), and *Lactobacillus paracasei* M7 (isolate from human breast milk) possessed several bioactive functional attributes like hypocholesterolemic activity, antioxidant potential, antibiofilm activity, antimicrobial activity, emulsification ability, and desirable physiochemical properties. However, the EPS yield was low. Current study reports optimization of process variables by Design of Experiments (DoE) to enhance EPS yield from these bacteria. The most effective process variables for EPS production were earmarked for *E. faecium* K1 (lactose, ammonium citrate, incubation time and pH), and for *L. paracasei* M7 (glucose, incubation time and pH), by Plackett–Burman design, and the same were optimized using central composite design (CCD) of response surface methodology (RSM). The EPS yield from *E. faecium* K1 was enhanced by 101.40% at optimal level of variables (lactose 10.07 g/L, ammonium citrate 2.49 g/L, incubation time 94.05 h and pH 5.4). Similarly, EPS yield was enhanced by 79.6% from *L. paracasei* M7 using optimal level of variables (glucose 10 g/L, incubation time 48 h and pH 7.6). Thus, DoE represents a powerful approach for optimization of process variables.

Keywords: *Enterococcus faecium* K1, *Lactobacillus paracasei* M7, Optimization, Probiotics, Response surface methodology

The exopolysaccharides (EPS) are usually homo- or heteropolysaccharides that are composed of different sugars and sugar derivatives. A wide range of microorganisms has been reported to produce EPS that is either secreted out of the cell or attached to cell wall surface[1]. EPS producing ability has been reported widely among microorganisms, and it assists the producer bacteria against desiccation, phage attack, environmental stress such as osmotic stress, and help them for symbiosis, and adhesion[2-4]. The microbial EPS offers huge application potential for a variety of industries. For instance due to their bioactive properties such as immunomodulatory, hypocholesterolemic, anti-viral and anti-tumor, apt-adjutants, and others, EPS may have applications for medical/ pharmaceutical industries[5]. In food processing industries, EPS may be used as emulsifiers, thickeners, stabilizers, bodying or gelling agents, and/or as appropriate fat replacers[6]. Besides, microbial EPS may be utilized for bioremediation or detoxification of polluted soil/water habitats, and due to their water-holding capacity, EPS may be important for agriculture. Industrial application of EPS mostly depends on their monosaccharide constituents, their size and extent of branching[4,7].

Various bacteria including the probiotic lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Enterococcus* spp., and *Weissella* spp., among several others have been reported to produce EPS[1,8]. EPS produced by bacteria offers discreet advantages to the producers such as it provides protection under hostile environments like desiccation, osmotic stress, extremes of pH, and metal toxicity, plays a significant role in cellular recognition, quorum sensing, and colonization[12]. EPS from probiotic LAB enhances the gut colonization by contributing to autoaggregation, and cell surface adhesion[9]. Furthermore, the bioactivity potential of EPS augments the health benefitting prospective of probiotics[8,10]. EPS from probiotics are 'generally recognized as safe' (GRAS), and may be used as functional food ingredients, thus, conferring both health and economic benefits to the consumers[1]. EPS are widely used in fermented foods, especially in the dairy industry, for improving the organoleptic, gustatory and rheological properties of the finished food products[11]. EPS from probiotic bacterial species

*Correspondence:
E-mail: bajajbijenderk@gmail.com

such as *Lactobacillus*, *Enterococcus*, and *Weissella* has been used in functional food preparations[1,8].

Considering the safe status, and diverse application potential of probiotic based EPS, there is a huge scope for developing nutritive, healthy and/or functional foods that are augmented with EPS or EPS producing probiotics. This has triggered an intensive research on probiotics-EPS[4,12]. Probiotic LAB isolated from diverse ecological niches have been reported to produce EPS[12-16]. But low yield of EPS from most of the probiotic bacteria remains a major limiting factor that hampers the application of EPS in different food/pharmaceutical industries[2,3]. Therefore, enhancing EPS yield from probiotics may be an imperative and valuable approach for realizing their industrial application potential.

Probiotics exhibit inherent variations with respect to their health benefitting functional attributes including the EPS producing potential, therefore, isolation and characterization of new probiotic strains from unexplored econiches has been a continuous practice[17-19]. Previously in our laboratory, some potential probiotic LAB have been isolated and characterized such as *Enterococcus faecium* K1 from an indigenously fermented milk product *kalarei*[13], and *Lactobacillus paracasei* M7, from human breast milk[12], which possessed several functional attributes[20] including EPS producing ability. *E. faecium* K1 produced 355 mg/L of EPS while *L. paracasei* generated 376 mg/L of EPS, and interestingly EPS from both probiotic strains exhibited desirable bioactive functional attributes such as hypocholesterolemic activity, antioxidant potential, antibiofilm activity, and emulsification ability[12]. EPS from both LAB probiotics were characterized for physiochemical/structural properties by scanning electron microscopy (SEM), transmission electron microscopy (TEM), fourier-transform infrared spectroscopy (FT-IR), X-ray analysis, and thermal analysis by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC), to demonstrate their implications for food/pharmaceutical applications[12,13].

Process optimization has always been an instrumental approach for enhancing product yield[21-23]. Design of Experiments (DoE) based optimization has gained momentum due to severe limitations of the conventional 'one-variable-at-a-time' (OVAT) approach[24]. Plackett–Burman design (PB) is used for earmarking the most imperative process parameters which influence the product yield, and subsequently response surface methodology (RSM) is used for optimization of such selected parameters[24]. Considering that EPS from *E. faecium* K1[13], and *L. paracasei* M7[12] possessed very imperative functional and desirable physicochemical and structural attributes, but EPS yield was low, the current study aimed to enhance the EPS yield by optimization of the process variables. Most effective variables were selected based on Plackett–Burman (PB) designed experiments, and the same were optimized by response surface methodology.

Materials and Methods

Chemicals, media and media components

The chemicals, media and media components used in the current study were purchased from various suppliers like HiMedia Laboratories Ltd. (Mumbai, India); Sigma-Aldrich Chemicals Ltd. (St. Louis MO, USA); Merck and Co. Inc. (White House Station, NJ, USA); and Qualigens Fine Chemicals Ltd. (Mumbai, India). All the reagents, chemicals, media and media components were of standard analytical grade.

Exopolysaccharide producing probiotic bacteria

The probiotic bacteria used in the current investigation were isolated, and characterized in previous studies[12, 13]. *Enterococcus faecium* K1 was an isolate from indigenously fermented milk product *kalarei*[13], and *Lactobacillus paracasei* M7 was isolated from human breast milk[12]. Both the potential probiotic bacteria were characterized for desired probiotic functional attributes, and EPS producing ability. The EPS from both LAB probiotics exhibited numerous bioactivities such as hypocholesterolemic activity, antioxidant potential, antibiofilm activity, and emulsification ability. EPS were characterized by physiochemical/structural properties by SEM, TEM, FT-IR, X-ray studies, and thermal analysis (TGA, DSC).

Submerged fermentation for EPS production

Submerged fermentation was executed for EPS production from *E. faecium* K1 and *L. paracasei* M7, and resultant EPS was purified from the cultural broth[8]. Probiotic LAB *E. faecium* K1 or *L. paracasei* M7 were activated by growing them on MRS agar slants at 37°C for 24 h. LAB culture from slant was transferred to MRS broth and cultivated at 37°C under shaking (180 rpm) for 18 h. Cultural broth was centrifuged at 10000 × g for 10 min at 4°C (Eppendorf centrifuge 5804 R), and cell pellet was recovered and washed twice with phosphate buffered saline (PBS, 50 mM, pH 6.8). Cells of freshly grown *E. faecium* K1 or *L. paracasei* M7 were inoculated (10^9 cfu/mL) in EPS

production medium (MRS, pH 6.5±0.2), and fermentation was allowed to proceed for EPS production at 37°C under shaking (180 rpm) for 72 h.

Extraction and purification of EPS from cultural broth of LAB

The cultural broth was heated to 100°C for 10 min, cooled to ambient temperature, and centrifuged at 10000 × g 15 min at 4°C (Eppendorf centrifuge 5804 R). The cell free supernatant (CSF) was added with three volumes of chilled ethanol (99%), and incubated at 4°C for 48 h. The contents were centrifuged at 10000 × g for 10 min at 4°C (Eppendorf centrifuge 5804R), and the crude EPS pellet was collected. The EPS pellet was suspended in phosphate buffered saline (PBS, 50 mM, pH 7.0), and dialyzed against PBS for 3 days with change of buffer every 6-8 h. The dialyzed retentate was lyophilized (CRIST, ALPHA 2-4 LD Plus, Germany) into dried powder. Then, the EPS was dissolved at 5 mg/mL in Tris-HCl (50 mM, pH 7.5) containing $MgSO_4 \cdot 7H_2O$ (10 mM). To further reduce the content of DNA and protein, crude EPS solution was first treated with DNAse type I (2.5 µg/mL) for 6 h at 37°C, and then with protease (50 µg/mL) for 18 h at 37°C. Trichloroacetic acid (12%) was added in the reaction mixture, and contents were stirred for 30 min, and then centrifuged. The supernatant was dialyzed against milli Q water for 48 h at 4°C, and finally lyophilized to obtain pure EPS. Total sugar content in EPS preparation was examined[13].

Screening of process variables by PB design for EPS production

Eight process variables which included either medium components or environmental variables such as glucose (g/ L), lactose (g/ L), yeast extract (g/ L), beef extract (g/ L), ammonium sulphate (g/ L), ammonium citrate (g/ L), incubation time (h) and pH, were examined based upon Plackett–Burman design (PB, Design-Expert 6.0: Stat Ease, Minneapolis, Minnesota, USA) to earmark the most effective ones which influence the EPS production from *E. faecium* K1. Similarly, the same set of variables (except that the beef extract was replaced with tryptone), were screened by PB design for identification of the most effective variables for EPS production from *L. paracasei* M7.

For screening of the process variables by PB design, the low and high values of various variables used to design the model were set based upon previously published reports which are as follows: glucose 10 and 15 g/L, lactose 10 and 15 g/L, yeast extract 10 and 20 g/L, beef extract for *E. faecium* K1 while tryptone for *L. paracasei* M7 10 and 20 g/L, ammonium sulphate 1.5 and 2.5 g/L, tri-ammonium citrate 1.5 and 2.5 g/L, incubation time 24 and 96 h and pH 5 and 7[25]. A full experimental design with 12 experiments for both *E. faecium* K1 and *L. paracasei* M7 was developed using such process variables. The experiments were conducted by running submerged fermentation for EPS production from E. *faecium* K1, and from *L. paracasei* M7. EPS produced was quantified. The significance level (*P* value) of each variable was determined using Student's *t*-test. A polynomial equation was generated by the design after analysis of variance (ANOVA) using the data (EPS production) of each experimental run. All the experiments were carried out in triplicates.

Optimization of process variables for EPS production by RSM

The process variables identified based on PB design were optimized using DoE by central composite design (CCD) of response surface methodology (RSM) to determine the best operating conditions for EPS production. Four most significant variables were earmarked for EPS production from *E. faecium* K1 which included lactose, ammonium citrate, incubation time and pH. However, three significant variables *i.e*. glucose, incubation time and pH, influenced the EPS production from *L. paracasei* M7. The high and low level of these variables used in the experimental design for *E. faecium* K1 were: lactose 10 and 15 g/L, ammonium citrate 1.5 and 2.5 g/L, incubation time 24 and 96 h, and pH 5 and 7, respectively. For *L. paracasei* M7 level was: glucose 6 and 14 g/L, incubation time 24 and 72 h, and pH 5 and 7, respectively. The full experimental design was developed for *E. faecium* K1 and *L. paracasei* M7 with 30 and 20 experimental sets, respectively. Experiments were conducted in triplicates, at specified conditions by running submerged fermentation, and the EPS yield (the response) was quantified. The response generated was fed into the respective software designs for both the organisms, *E. faecium* K1 and *L. paracasei* M7.

The data was analyzed using multiple regression analysis and a polynomial equation was derived. Analysis of variance (ANOVA) for the designed model was done by the software. Response surface plots (3D) were generated by the design, and used to predict the correlation between the response *vis-a-vis* experimental level of each variable and the interaction between the variables.

Validation of process model

The point prediction tool of the software was used to predict the optimal level of various variables, and experiments were executed using the predicted values of variables. Validation of the model was done based on the comparison of the actual experimental results (responses) with the predicted results.

Statistical analysis

All the analytical experiments were executed in triplicates as three independent runs, and the results were expressed as mean ± SD. Statistical analysis was performed using IBM SPSS software version 25. The level of statistical significance was estimated using either the Student's *t*-test or analysis of variance where ever appropriate. The *P* value ($P < 0.05$) was used to statistically validate the data. Differences were considered statistically significant when the *P* value was $P < 0.05$.

Results and Discussion

EPS production from probiotic bacteria

The potential probiotic LAB *Enterococcus faecium* K1, an isolate from indigenously fermented milk product *kalarei*[13], and *L. paracasei* M7 from human breast milk[12] possessed several functional probiotic attributes including the EPS producing ability[20]. The EPS from both the organisms possessed functional characteristics like hypocholesterolemic activity, antioxidant activity, antibiofilm potential against several human pathogens, and emulsifying ability. The EPS was characterized for several physicochemical/structural properties by SEM, TEM, FT-IR, XRD, TGA and DSC, to elucidate their commercial application spectrum[12,13].

Despite excellent bioactivity potential, and desirable physicochemical/structural properties of EPS from *E. faecium* K1, and *L. paracasei* M7, their commercial application prospective remains limited due to low yield of EPS. Optimization of process variables represents an imperative and significant tool for enhancing product yield in a commercial production process. Even minor yield improvement may be reflected in terms of substantial economic gains. Optimization by conventional 'one-variable-at-a-time' (OVAT) approach though has been used extensively but poses serious limitations like it is highly laborious, time consuming, expensive, and ignores the combined interaction(s) among various physical and/or nutritional process variables. However, statistical tools based design of experiment (DoE) approach not only obviates the drawbacks of OVAT but offers economic, more practical and inclusive solutions for process optimization. Therefore, DoE based optimization of process variables for EPS production from *E. faecium* K1, and *L. paracasei* M7 was executed in the current study to enhance yield of EPS. PB design was used to identify the most imperative variables that influence EPS production (yield), and the same were optimized by response surface methodology (RSM).

Plackett-Burman design for identification of most effective process variables

The microbial bioprocess may be influenced by several variables like components of growth/production medium (carbon/nitrogen sources, salts), physiological factors (pH, incubation temperature, and incubation time), and biological factors (genetic nature, metabolic/biochemical behaviour, metabolite production pattern of the organism). Therefore, the first aim of developing an optimized bioprocess is to identify the most important variables which influence the product yield. Plackett-Burman (PB) design is a superior and quick screening procedure which mathematically computes the importance of large number of variables in fewer experiments. Thus, in the current study, eight variables were screened (glucose, lactose, yeast extract, beef extract or tryptone, ammonium sulphate, ammonium citrate, incubation time and pH) to earmark the ones which have the most significant influence on EPS production from probiotic *E. faecium* K1 and *L. paracasei* M7, by using PB design.

Each of the selected variables is imperative for growth and EPS production by lactic acid bacteria *E. faecium* K1 and *L. paracasei* M7. The individual carbon and nitrogen sources, and their concentrations employed in medium, may substantially influence the growth of probiotic bacterium, and yield, and even the composition of EPS[15]. Glucose and lactose are considered as good carbon sources for bacterial growth and EPS production. Glucose is readily utilizable as all of the enzymes required for its catabolic breakdown are constitutive in nature, and are always available. However, for catabolic metabolism of lactose certain inducible enzymes are required, therefore, lactose is not utilized as readily as glucose. The process organisms' may have individual preferences for various carbon sources for efficient growth/EPS production depending upon their biochemical or metabolic potential and genetic make-up. LAB employ several housekeeping enzymes such as glycosyl transferases, translocases, polymerases, others, for producing polysaccharides by utilizing sugars and other medium components[15].

Yeast extract acts as a very important organic nitrogen source, and composed of various amino acids, peptides, water soluble vitamins and some carbohydrates. Addition of yeast extract in the medium also ensures supply of trace elements, salts and vitamin B-complex to the process organism. Similarly, beef extract has many nutritive properties, and is constituted of mixture of peptides, amino acids, nucleotides, organic acids, minerals, phosphates, energy sources, and some vitamins. Inclusion of beef extract in the medium adequately supports the growth and metabolism for better functionality of the process organism. Ammonium sulphate has been reported as an effective nitrogen source for EPS production from various LAB[26].

Medium pH may affect the growth rate and metabolic activity of LAB, and hence EPS production. Various metabolic or enzymatic activities required for EPS production may need an optimum pH, therefore, medium pH is an important process parameter. During growth LAB generally produce lactic acid in the production medium which may lower down the pH, thereby affecting their growth. Thus, it is obligatory that the medium must contain appropriate buffering agent(s). Ammonium citrate may serve as a suitable buffering agent in the medium so as to maintain appropriate pH for proper growth of LAB[27]. An optimum incubation time (time period of submerged fermentation) is another important factor that may influence the EPS yield from a particular process organism. A lesser incubation time period than optimum may lead to low yield of EPS while the prolonged incubation time may cause degradation of EPS due to glycohydrolase activity, other side reactions, and reduction in the overall productivity of the process[21].

A varied EPS yield in the 12 PB designed experimental runs showed that level of variables was significant for EPS production by *E. faecium* K1. The maximum EPS production was observed in the run order 3 (492.93 mg/L) at following level of process variables *i.e*. glucose 10 g/L, lactose 15 g/L, yeast extract 10 g/L, beef extract 10 g/L, ammonium sulphate 2.5 g/L, ammonium citrate 1.5 g/L, incubation time 96 h and pH 7. However, the experiment run 8 yielded the least EPS yield (172.01 mg/L) at different level of variables *i.e.* glucose 15 g/L, lactose 10 g/L, yeast extract 20 g/L, beef extract 20 g/L, ammonium sulphate 1.5 g/L, ammonium citrate 2.5 g/L, incubation time 24 h and pH 5 (Table 1).

Analysis of variance (ANOVA) was performed (Table 2). Probability values (p-value) less than 0.05 indicates the significance of model as well of each variable studied for EPS production. The model was significant with p-value 0.0074. Of the eight variables screened, lactose (B), ammonium citrate (F), incubation time (G) and pH (H) were earmarked as the significant ones. Incubation time with *P* value of 0.0016 was observed to be the most significant variable followed by pH (*P* value 0.0041), lactose (*P* value 0.0080), and ammonium citrate (*P* value 0.0187) (Table 2).

The determination coefficient R-squared (R^2 0.9890) showing the aptness of fit of the model implied that the 98.90% variability in the response can be explained by the model, while 1.1 % of the total variance could not be explained by the model. R-squared value closer to 1 represented a better correlation between the experimental and the predicted values. A lower value of coefficient of variation (CV=4.76) substantiated the

Table 1 − Experimental and predicted yield of EPS from *E. faecium* K1 based on Plackett–Burman design

S. No.	Experimental variables*								EPS production (mg/L)	
	A	B	C	D	E	F	G	H	Actual value	Predicted value
1	10	10	20	20	2.5	1.5	96	7	460.48	474.73
2	15	15	10	20	1.5	1.5	24	7	399.06	400.05
3	10	15	10	10	1.5	2.5	96	7	492.93	496.15
4	15	10	20	10	1.5	1.5	96	7	433.13	418.88
5	15	15	20	10	2.5	2.5	24	7	340.76	350.81
6	15	10	10	10	2.5	2.5	96	5	345.79	346.78
7	10	15	20	10	2.5	1.5	24	5	356.61	346.56
8	15	10	20	20	1.5	2.5	24	5	172.01	175.23
9	10	10	10	20	2.5	2.5	24	7	358.88	344.63
10	15	15	10	20	2.5	1.5	96	5	459.04	458.05
11	10	15	20	20	1.5	2.5	96	5	385.82	382.6
12	10	10	10	10	1.5	1.5	24	5	274.48	284.53

*A: Glucose (g/L), B: Lactose (g/L), C: Yeast extract (g/L), D: Beef extract (g/L), E: Ammonium sulphate (g/L), F: Ammonium citrate (g/L), G: Incubation time (h) and H: pH

Table 2 — Results of ANOVA for EPS production from *E. faecium* K1 based on PB designed experiments using different variables*

Source	Sum of Squares	DF	Mean Square	F Value	Prob> F	
Model	85301.92	8	10662.74	33.8056	0.0074	Significant
A	2682.329	1	2682.329	8.504167	0.0617	
B	12639.28	1	12639.28	40.07208	0.0080	Significant
C	2741.256	1	2741.256	8.690993	0.0601	
D	5.894008	1	5.894008	0.018687	0.8999	
E	2244.888	1	2244.888	7.117287	0.0758	
F	6845.441	1	6845.441	21.70307	0.0187	Significant
G	38012.64	1	38012.64	120.5168	0.0016	Significant
H	20130.2	1	20130.2	63.82163	0.0041	Significant
Residual	946.2404	3	315.4135			
Cor Total	86248.16	11				

*A: Glucose (g/L), B: Lactose (g/L), C: Yeast extract (g/L), D: Beef extract (g/L), E: Ammonium sulphate (g/L), F: Ammonium citrate(g/L), G: Incubation time (h) and H: pH

precision with which the experiments were executed. The predicted R-squared value (0.8245) was in reasonable agreement with the adjusted R-squared value of 0.9598. Adequate precision (signal to noise ratio) of 20.865 indicated an adequate signal. The regression equation 1 generated based upon the PB designed experimental results after ANOVA showed the individual effects of various variables on EPS production:

$$\text{EPS (mg/L)}=+373.25-14.95A+32.45B-15.11C-0.70D+13.68E-23.88F+56.28G+40.96\,H \quad (1)$$

where A: Glucose, B: Lactose, C: Yeast extract, D: Beef extract, E: Ammonium sulphate, F: Ammonium citrate, G: Incubation time and H: pH

From the equation 1 it was inferred that of all the four significant variables ammonium citrate showed negative significance on EPS yield while lactose, incubation time and pH showed positive significance on the response.

Similarly, PB design was applied to select the most significant variables for EPS production from *L. paracasei* M7. The same set of eight variables was examined except that beef extract was replaced with tryptone. As indicated in the designed matrix, all the 12 experimental runs were executed and a varied combination of selected variables resulted in EPS yield from 300.00 - 584.28 mg/L, thus, substantiating that level of variables was quite imperative for EPS production from *L. paracasei* M7.

ANOVA results showed that the model was significant with *P* value 0.0357, and of all the eight variables, pH showed the most significant effect on EPS production (*P* value 0.0094), and was followed by glucose (*P* value 0.0172) and incubation time (*P* value 0.0238). R-squared value of 0.9678 showed that 96.78% of the variability in the response could be explained by the model except for 3.22% variability. A low CV value of 8.10 suggested the precision and reliability of the experiments conducted. The regression equation 2 for EPS production from *L. paracasei* M7 in terms of coded factors is as follows:

$$\text{EPS production (mg/L)}=+465.33-52.29A+15.66B-16.01C-31.09D+9.57E-6.29F+46.29G-4.99H \quad (2)$$

where A: Glucose (g/L), B: Lactose (g/L), C: Yeast extract (g/L), D: Tryptone (g/L), E: Ammonium sulphate (g/L), F: Ammonium citrate (g/L), G: Incubation time (h) and H: pH

From the equation 2, it was observed that incubation time had the positive effect, while glucose and pH had the negative effect on EPS production from *L. paracasei* M7.

Thus, based upon PB designed experimental results, lactose (B), ammonium citrate (F), incubation time (G) and pH (H) were identified to have the significant effect on EPS production from *E. faecium* K1, while pH (H), glucose (A) and incubation time (G) were earmarked as the most effective variables for EPS production from *L. paracasei* M7. These significant variables were optimized by RSM for EPS production from *E. faecium* K1 and *L. paracasei* M7.

Optimization of selected variables for EPS production

Four significant variables (lactose, ammonium citrate, incubation time and pH) were earmarked for EPS production from *E. faecium* K1 by PB design, while three variables (pH, glucose and incubation

time) were found significant for EPS production from *L. paracasei* M7. Optimization of these significant variables was executed by using central composite design (CCD) of RSM, keeping the other variables at a fixed level.

A total of 30 experiments designed by the software were executed for EPS production from *E. faecium* K1. The experimental results obtained for EPS production (response) were fed into the response column of the design. The close proximity between observed and predicted values inferred the accuracy of the model, and precision of experimentation. The maximum EPS production from *E. faecium* K1 was observed in the run order 10 (715.75 mg/L) while the least was observed in the run order 18 (230.47 mg/L) obviously at differentially specified level of process variables (Table 3).

ANOVA results (Table 4) revealed that the model was significant with *P* value of 0.0406. The model F-value of 2.56 implied that there was only 4.06% chance that this large F-value of model could be due to noise. The two interactive terms *i.e*. BD (ammonium citrate and pH), and CD (incubation time and pH), were significant with *P* value 0.0069 and 0.0064, respectively. The other model terms *i.e*. linear or squared terms of variables were statistically not significant. The lack of fit was not significant with F-value of 1.21 which implied the strength of the model. The R-Squared value of 0.7051 indicated that 70.51% of variability could be explained by the model. The adequate precision (signal/noise ratio) of 7.45 indicated the satisfactory signal. The regression equation 3 showing the individual effects

Table 3 — Experimental and predicted yield of EPSfrom *E. faecium* K1 based on response surface methodology

Run order	Experimental variables*				EPS production (mg/L)	
	A	B	C	D	Actual value	Predicted value
1	12.5	1	60	6	434.98	464.98
2	12.5	2	60	8	512.3	549.85
3	10	2.5	24	7	550.2	467.58
4	15	2.5	96	5	414.9	428.62
5	10	1.5	24	7	489.1	539.94
6	7.5	2	60	6	550.8	491.33
7	10	2.5	96	7	348.8	424.18
8	15	2.5	24	7	459.93	447.21
9	15	1.5	96	5	442.98	445.8
10	15	1.5	24	7	715.75	665.71
11	10	1.5	24	5	320.64	243.68
12	15	2.5	24	5	443.39	385.28
13	15	1.5	96	7	551.47	504.81
14	12.5	2	132	6	514.98	484.63
15	10	1.5	96	5	444.6	521.87
16	10	2.5	96	5	680.6	650.84
17	10	2.5	24	5	320.11	431.33
18	15	2.5	96	7	230.47	227.63
19	12.5	2	12	6	320.6	423.17
20	12.5	2	60	6	375.22	432.61
21	12.5	2	60	4	476.89	454.58
22	15	1.5	24	5	354.6	343.78
23	12.5	2	60	6	400.1	432.61
24	10	1.5	96	7	576.89	555.21
25	12.5	3	60	6	390.2	375.44
26	12.5	2	60	6	462.52	432.61
27	12.5	2	60	6	589.8	432.61
28	12.5	2	60	6	415.93	432.61
29	17.5	2	60	6	320.18	394.89
30	12.5	2	60	6	409.1	432.61

*A- Lactose (g/L), B- Ammonium citrate (g/L), C- Incubation time (h), D-pH

Table 4 — Results of ANOVA for EPS production from *E. faecium* K1 based on response surface methodology using different variables*

Source	Sum of Squares	DF	Mean Square	F Value	Prob> F	
Model	2.48E+05	14	17695.14	2.56	0.0406	Significant
A	13953.42	1	13953.42	2.02	0.1757	
B	12023.88	1	12023.88	1.74	0.2069	
C	4378.14	1	4378.14	0.63	0.4384	
D	13614.08	1	13614.08	1.97	0.1807	
A^2	191.67	1	191.67	0.028	0.8699	
B^2	267.63	1	267.63	0.039	0.8466	
C^2	577.5	1	577.5	0.084	0.7764	
D^2	8427.25	1	8427.25	1.22	0.2868	
AB	21359.09	1	21359.09	3.09	0.0991	
AC	31036.75	1	31036.75	4.49	0.0511	
AD	659.33	1	659.33	0.095	0.7616	
BC	3443.05	1	3443.05	0.5	0.491	
BD	67598.7	1	67598.7	9.79	0.0069	Significant
CD	69128.24	1	69128.24	10.01	0.0064	Significant
Residual	1.04E+05	15	6908			
Lack of Fit	73376.95	10	7337.69	1.21	0.4401	Not significant
Pure Error	30243.07	5	6048.61			
Cor Total	3.51E+05	29				

*A- Lactose (g/L), B- Ammonium citrate (g/L), C- Incubation time (h), D-pH

of various variables on EPS production from *E. faecium* K1 is as follows:

$$\text{EPS production (mg/L)} = +432.61-24.11A-22.38B+14.65C+23.82D+2.62A^2-3.10B^2+5.68C^2+17.40D^2-36.54AB-44.04AC+6.42AD-14.67BC-65.00BD-65.73CD \quad (3)$$

where, A: Lactose (g/L), B: Ammonium citrate (g/L), C: Incubation time (h), D: pH

The 3-D response surface plots showed the interactive effects of variables (lactose, ammonium citrate, incubation time, pH) on EPS production from *E. faecium* K1 (Fig. 1). The interaction between lactose and pH (AD) was though positive but statistically insignificant (Fig. 1A). The interactive effect of ammonium citrate and pH (BD) on EPS production (Fig. 1B) was statistically significant as indicated by ANOVA. At low ammonium citrate concentration, and high pH EPS production was the maximum. The interaction between incubation time and pH (CD) (Fig. 1C) was also statistically significant. Increasing the level of one variable and decreasing that of other resulted in an increase in EPS production. Variation in response for EPS production from *E. faecium* K1 when the level of individual variable changed from the reference point keeping the other factors constant is shown in (Fig. 1D). Lactose (A) produced maximum while the incubation time (C) shows minimum effect on the response.

Similarly, RSM based optimization of process variables for EPS production from *L. paracasei* M7 was done. A total of 20 experimental runs was executed as designed by the software. The maximum and minimum EPS production was obtained in run 3 (676.00 mg/L) and 1 (426.00 mg/L), respectively. The experimental design was tested statistically for ANOVA. It was inferred from the results that the model was significant with *P* value of 0.0001. It was further observed that all the three variables *i.e.* glucose (A), incubation time (B) and pH (C) were significant in squared terms (A^2, B^2, C^2) but none was significant in linear terms (A, B, C). Furthermore, C^2 was the most significant model term (*P* value < 0.0001), and was followed by B^2 (*P* value 0.0013), and A^2 (0.0024). However, in interactive terms the interaction between glucose (A) and incubation time (B) was the most effective one (*P* value 0.0009), and was followed by that between glucose (A) and pH (C), (*P* value 0.0010), and that between incubation time (B) and pH (C) (*P* value 0.0017). The high R-squared value of 0.9433 indicated that 94.33% of variability could be explained by the model. Adequate

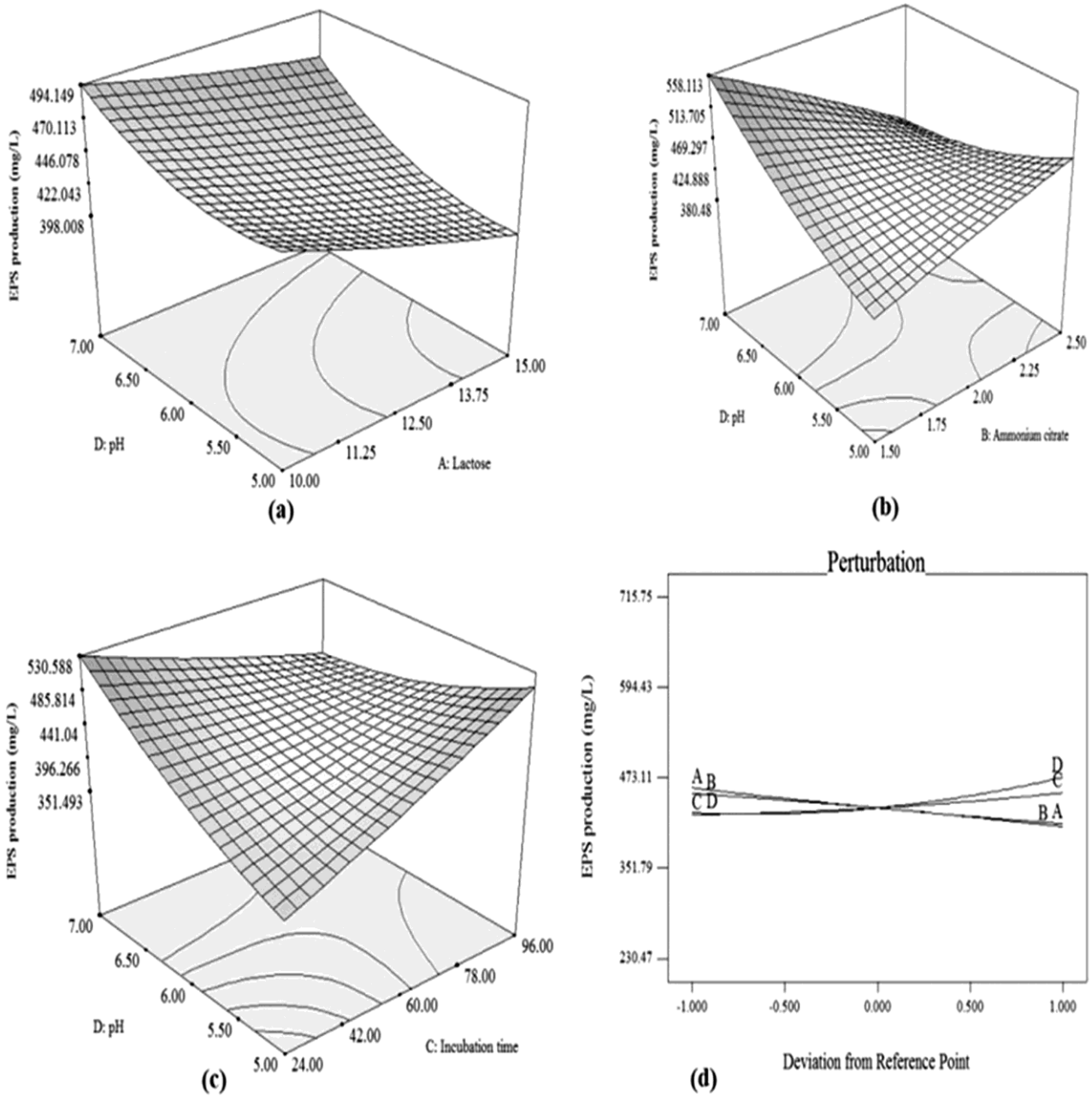

Fig. 1 — Response surface plots showing the interactions between variables for EPS production from *Enterococcus faecium* K1 *i.e.* between (A) lactose and pH; (B) ammonium citrate and pH; (C) incubation time and pH; and (D) the perturbation plot showing the response changes as each variable moves from the chosen reference point, keeping the value of all other variables constant

precision (signal to noise ratio) of 13.661 indicated the adequacy and strength of the designed model. The regression equation 4 for EPS production from *L. paracasei* M7 in terms of coded factors is shown as follows:

EPS Production (mg/L)
=+522.38−9.80A−2.80B+4.70C−25.30A2−27.77B2+ 47.54C2+39.00AB+ 38.50 AC +35.75BC (4)

where A: Glucose (g/L), B: Incubation time (h) and C: pH

The response surface plots for EPS production from *L. paracasei* M7 showing the interactive effect of different process variables (glucose, incubation time and pH) are presented in (Fig. 2). Since, AB (interaction between glucose and incubation time), AC (interaction between glucose and pH), and BC (interaction between

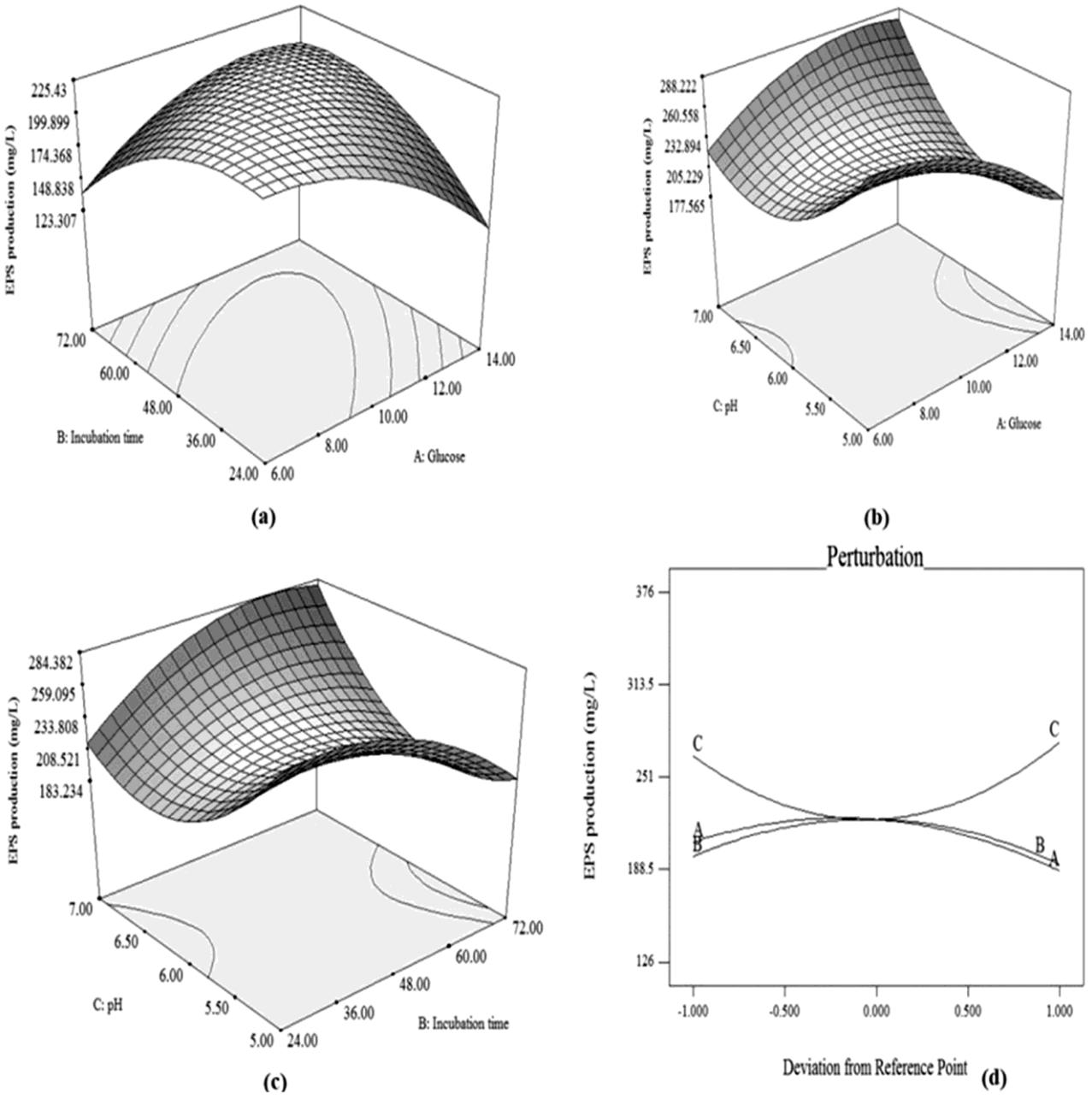

Fig. 2 − Response surface plots showing the interactions between variables for EPS production from *Lactobacillus paracasei* M7 *i.e.* (A) glucose and incubation time; (B) glucose and pH; (C) incubation time and pH; (D) and the perturbation plot showing the response changes as each variable moves from the chosen reference point, keeping the value of all other variables constant

incubation time and pH) were the statistically significant interactive terms for EPS production from *L. paracasei* M7, it implied that the combined effect of all the interactions was very critical for EPS production from *L. paracasei* M7. However, individually all the three variables did not have much effect on the yield.

The interaction between glucose and incubation time shows that increasing level of both these variables resulted in a decrease in EPS production. It was inferred from the glucose-incubation time (AB) interaction that high concentration of glucose inhibited the EPS yield over a longer duration, while low glucose concentration over a short duration (in the beginning) supported higher EPS yield (Fig. 2A). The interaction between glucose and pH (AC) showed an increased response (EPS yield) at low concentration of glucose and at low pH (Fig. 2B). Similarly, the

interaction between incubation time and pH (BC) (Fig. 2C) indicated that increase in EPS production occurred at low pH and short incubation time. The variation in response when the value of one variable changes with respect to the reference point while keeping the other variables constant is shown in perturbation plot (Fig. 2D). The variable A *i.e*. glucose diverged maximally, while the variable C (pH) deviated the least with respect to the reference point.

Validation of the model

The statistical model was validated for EPS production from both the probiotic bacteria *i.e*. *E. faecium* K1 and *L. paracasei* M7. The optimal level of the process variables was predicted by point prediction tool of the design software. The optimal values of variables determined for EPS production from *E. faecium* K1 were: lactose 10.07g/L, ammonium citrate 2.49 g/L, incubation time 94.05 h and pH 5.4. The experiments were performed using the optimum values of the variables. The predicted response (665.71 mg/L of EPS yield) and the experimental response (715.05 mg/L of EPS yield) were in close proximity which showed that the model was valid. A substantial EPS yield enhancement of 101.40% was obtained due to optimization of the process variables from *E. faecium* K1 as compared to that under unoptimized conditions (355±0.019 mg/L).

Similarly, EPS production from *L. paracasei* M7 was validated. The optimum value of variables *i.e*. glucose 10 g/L, incubation time 48 h and pH 7.6, deduced on the basis of point prediction tool of design software, were used for EPS production from *L. paracasei* M7 under submerged fermentation. The close agreement between experimental (676.00 mg/L), and the predicted (664 mg/L) EPS yield substantiates the validity of the model. Thus, optimization of process variables resulted in 79.6% enhanced EPS yield from *L. paracasei* M7 compared to that under unoptimized conditions (376.4 mg/L).

The EPS production from an organism may be influenced by a variety of parameters. The environmental factors like time period and temperature of fermentation, culture medium pH, and medium components like carbon and nitrogen source, salts, and buffering agents, may have considerable influence on EPS production[28]. The EPS production is also determined by the type of microorganism, its biochemical/metabolic and genetic potential, and cultural and environmental conditions used for EPS production[29,30]. Generally the optimum pH for EPS production is around 6-7, however, it may vary depending on the type of bacterial strain, and the experimental conditions. In current study, optimum pH for EPS production from *E. faecium* K1 was 5.4, while *L. paracasei* M7 produced maximum EPS at pH 7.6. Khanh[25] reported an optimum pH of 5.5 for EPS production (397.72 mg/L) from *L. plantarum* T10. However, *L. fermentum* Lf2 showed maximum EPS production at pH 6.5, and yielded low EPS at other pH used in the study[31].

Incubation time *i.e*. time period of fermentation process has got an impact on EPS yield. Generally bacteria yield maximal EPS yield after 48-96 h of fermentation. The current study reports that *E. faecium* K1 produced maximum EPS after 94.05 h of fermentation, while *L. paracasei* M7 showed highest EPS production after 48 h. The prolonged fermentation time periods (extended incubation time) may cause EPS degradation probably due to extracellular glycohydrolase activity of bacteria, and/or some other side reactions[28]. Various carbon/nitrogen sources may stimulate or inhibit the EPS production[32]. It was observed that different LAB might have different individual preferences for carbon and nitrogen sources for growth and metabolism, and for EPS production, and it may be a strain dependent phenomenon[28]. Therefore, the results demonstrate that optimization of process variables for enhancing EPS production cannot be generalized but optimum level of variables is specifically required to be optimized for each LAB strain.

EPS production from probiotic yeast *Lipomyces starkeyi* VIT-MN03 was optimized using Box-Behnken design (BBD) of RSM[32]. Optimization of the cultural conditions *viz*. sucrose concentration (1–3, *w/v*), NaCl concentration (2–4 *w/v*), pH (3–5), temperature (20–30°C), and incubation period (20–40 days) resulted in a 6-fold increase in EPS production (4.87 g/L^{-1}) from *L. starkeyi* VIT-MN03 as compared to that in unoptimized conditions[32]. EPS production of 15.61 g/L was reported for *Bacillus thuringiensis* under optimized conditions as compared to that in unoptimized condition (4.43 g/L)[33]. In another study, PB design and CCD of RSM was used to optimize production of phytase using 'one-variable-at-a-time' (OVAT) approach, and it was found that probiotic strain, *Lactobacillus paracasei* SMVDUDB1 showed maximum phytase activity (5.92 U/mL)[34]. Midik *et al*.[28] studied the effect of different cultural conditions such as temperature, incubation time, pH, NaCl concentration, carbon, nitrogen, and minerals on the EPS production by indigenous LAB strains (*L. plantarum*, *L. namurensis*,

and *Pediococcus ethanolidurans* species) isolated from pickles. Of all the strains *L. plantarum* MF460 produced the highest amount of EPS (515.48 mg/L) at 30°C after 48 h of incubation. Nitrogen sources such as glycine, $NaNO_3$ and KNO_3, and biotin were reported to be effective for EPS production from different *Rhizobium* spp. during the optimization of process[35]. Lakra *et al*.[30] reported that *Weissella confusa* MD1 produced maximum EPS yield (10.07 g/L) when cultivated in MRS broth (pH 6.5) supplemented with galactose (4%, *w/v*) and ammonium nitrate (1%, *w/v*) at 35°C for 36 h. Thus, different bacterial strains may produce maximum EPS under different set of conditions.

Considering that bacterial EPS plays a vital role in several life governing functions of producers like cellular recognition, quorum sensing, colonization, and protection against metal toxicity, and other hostile environments like desiccation, osmotic stress, and extreme pH[12]. It is very important to investigate EPS producing ability, and the mechanisms by which it imparts benfits to the producer. Furthermore, EPS may have applications in various industrial/environmental for processes like bioemulsification, bioflocculation, heavy metal sequestration, and others. Additionally the EPS from probiotic bacteria offers a wide range of health benefits such as hypocholesterolemic activity, antihypertensive, immunomodulatory and antioxidant activities, and others, and may have very important implications in food/pharmaceutical industries[13].

Conclusion

The current study concludes that EPS production from probiotic LAB isolates *E. faecium* K1 and *L. paracasei* M7 could be enhanced substantially by application of DoE based optimization. Furthermore, the optimal process conditions like medium components and environmental variables may be strain specific, and need to be specified for each such process organism. Considering multifaceted bioactivity potential of EPS from these probiotic bacteria, yield enhancement is an essential and vital prerequisite for future commercial connotations. The optimized process parameters may be investigated further in a scaled-up process, and the genetic determinants of EPS yield must be deciphered.

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Conflict of Interest

All authors declare no conflict of interest.

References

1 Oleksy M & Klewicka E, Exopolysaccharides produced by *Lactobacillus* sp: Biosynthesis and applications. *Crit Rev Food Sci*, 58 (2018) 450.

2 Cirrincione S, Breuer Y, Mangiapane E, Mazzoli R & Pessione E, 'Ropy' phenotype exopolysaccharides and metabolism: Study on food isolated potential probiotics LAB. *Microbiol Res*, 214 (2018) 137.

3 Xu Y, Coda R, Holopainen-Mantila U, Laitila A, Katina K & Tenkanen M, Impact of in situ produced exopolysaccharides on rheology and texture of fava bean protein concentrate. *Food Res Int*, 115 (2019) 191.

4 Liu Q, Huang X, Yang D, Si T, Pan S & Yang F, Yield improvement of exopolysaccharides by screening of the *Lactobacillus acidophilus* ATCC and optimization of the fermentation and extraction conditions. *Excli J*, 15 (2016) 119.

5 Moscovici M, Present and future medical applications of microbial exopolysaccharides. *Front Microbiol*, 6 (2015) 1012.

6 Saravanan C, Kavitake D, Kandasamy S, Devi PB & Shetty PH, Production partial characterization and antioxidant properties of exopolysaccharide α-d-glucan produced by *Leuconostoc lactis* KC117496 isolated from an idli batter. *Int J Food Sci Tech*, 56 (2019) 159.

7 Lin T, Chen C, Chen B, Shaw J & Chen Y, Optimal economic productivity of exopolysaccharides from lactic acid bacteria with production possibility curves. *Food Sci Nutr*, 7 (2019) 2336.

8 Zhang Z, Liu Z, Tao X & Wei H, Characterization and sulfated modification of an exopolysaccharide from *Lactobacillus plantarum* ZDY2013 and its biological activities. *Carbohydr Polym*, 153 (2016) 25.

9 Jia K, Tao X, Liu Z, Zhan H, He W, Zhang Z & Wei H, Characterization of novel exopolysaccharide of *Enterococcus faecium* WEFA23 from infant and demonstration of its *in vitro* biological properties. *Int J Biol Macromol*, 128 (2019) 710.

10 Dilna SV, Surya H, Aswathy RG, Varsha KK, Sakthikumar DN, Pandey A & Nampoothiri KM, Characterization of an exopolysaccharide with potential health-benefit properties from a probiotic *Lactobacillus plantarum* RJF 4 LWT. *Food Sci Technol*, 64 (2015) 1179.

11 Zhang H, Ren W, Guo Q, Xiong Z, Wang G, Xia Y & Ai L, Characterization of a yogurt-quality improving exopolysaccharide from *Streptococcus thermophilus* AR333. *Food Hydrocoll*, 81 (2018) 220.

12 Bhat B & Bajaj BK, Hypocholesterolemic potential and bioactivity spectrum of an exopolysaccharide from a probiotic isolate *Lactobacillus paracasei* M7. *Bioact Carbohydr Diet Fibre*, 19 (2019) 100191.

13 Bhat B & Bajaj BK, Hypocholesterolemic and bioactive potential of exopolysaccharide from a probiotic *Enterococcus faecium* K1 isolated from kalarei. *Bioresour Technol*, 254 (2018) 264.

14 Deepak V, SRK, Sivasubramaniam SD, Nellaiah H & Sundar K, Optimization of anticancer exopolysaccharide production from probiotic *Lactobacillus acidophilus* by response surface methodology. *Prep Biochem Biotechnol*, 46 (2016) 288.

15 Imran MYM, Reehana N, Jayaraj KA, Ahamed AAP, Dhanasekaran D, Thajuddin N & Muralitharan G, Statistical optimization of exopolysaccharide production by *Lactobacillus plantarum* NTMI05 and NTMI20. *Int J Biol Macromol*, 93 (2016) 731.

16 Wang J, Zhao X, Yang Y, Zhao A & Yang Z, Characterization and bioactivities of an exopolysaccharide produced by *Lactobacillus plantarum* YW32. *Int J Biol Macromol*, 74 (2015) 119.

17 Khalil ES, Abd Manap MY, Mustafa S, Alhelli AM & Shokryazdan P, Probiotic Properties of Exopolysaccharide-Producing *Lactobacillus* Strains Isolated from Tempoyak. *Molecule*, 23 (2018) 398.

18 Gupta M & Bajaj BK, Functional characterization of potential probiotic lactic acid bacteria isolated from kalarei and development of probiotic fermented oat flour. *Probiotic Antimicrob Protein*, 10 (2018) 654.

19 Andrabi ST, Bhat B, Gupta M & Bajaj BK, Phytase-producing potential and other functional attributes of lactic acid bacteria isolates for prospective probiotic applications. *Probiotic Antimicrob Protein*, 8 (2016) 121.

20 Bhat B & Bajaj BK, Multifarious cholesterol lowering potential of lactic acid bacteria equipped with desired probiotic functional attributes. *3 Biotech*, (2020) DOI: https://doi.org/10.1007/s13205-020-02183-8.

21 Sharma S, Sharma V, Nargotra P & Bajaj, BK, Bioprocess development for production of a process-apt xylanase with multifaceted application potential for a range of industrial processes. *SN Appl Sci*, (2020) DOI: https://doi.org/10.1007/s42452-020-2541-6

22 Gupta M & Bajaj BK, Development of fermented oat flour beverage as a potential probiotic vehicle. *Food Biosci*, 20 (2017) 104.

23 Singh S & Bajaj BK, Medium optimization for enhanced production of protease with industrially desirable attributes from *Bacillus subtilis* K-1. *Chem Eng Commun*, 202 (2015) 1051.

24 Sharma K, Sharma N & Sharma R, Multiple parameter optimization for maximization of exopolysaccharide production from *Lactobacillus paraplantarum* KM1 by response surface methodology. *Curr Trends Biotechnol Pharm*, 11 (2017) 103.

25 Khanh TB, Optimal conditions for exopolysaccharide production by *Lactobacillus plantarum* T10. *Vietnam J Sci Technol*, 54 (2016) 40.

26 Shi T, Aryantini NPD, Uchida K, Urashima T & Fukuda K, Enhancement of exopolysaccharide production of *Lactobacillus fermentum* TDS030603 by modifying culture conditions. *Biosci Microb Food H*, 33 (2014) 85.

27 Saeed AH & Salam AI, Current limitations and challenges with lactic acid bacteria: a review. *Food Nutr Sci*, 11 (2013) 73.

28 Midik F, Tokatli M, Elmaci SB & Ozcelik F, Influence of different culture conditions on exopolysaccharide production by indigenous lactic acid bacteria isolated from pickles. *Arch Microbiol*, 1 (2020) 11.

29 Kumar BB, Razdan K, JJ Claes I & Lebeer S, Physico-chemical characterization of exopolysaccharides of potential probiotic *Enterococcus faecium* isolates from infants' gut. *Curr Biochem Eng*, 2 (2015) 90.

30 Lakra AK, Domdi L, Tilwani YM & Arul V, Physicochemical and functional characterization of mannan exopolysaccharide from *Weissella confusa* MD1 with bioactivities. *Int J Biol Macromol*, 143 (2020) 797.

31 Ale EC, Batistela VA, Correa OG, Ferrado JB, Sadiq S, Ahmed HI, Reinheimer JA, VeraCandioti L, Laws AP & Binetti AG, Statistical optimisation of the exopolysaccharide production by *Lactobacillus fermentum* Lf2 and analysis of its chemical composition. *Int J Dairy Technol*, 73 (2020) 76.

32 Ragavan ML & Das N, Optimization of exopolysaccharide production by probiotic yeast *Lipomyces starkeyi* VIT-MN03 using response surface methodology and its applications. *Ann Microbiol*, 69 (2019) 515.

33 Ramamoorthy S, Gnanakan A, Lakshmana SS, Meivelu M & Jeganathan A, Structural characterization and anticancer activity of extracellular polysaccharides from ascidian symbiotic bacterium *Bacillus thuringiensis*. *Carbohydr Polym*, 190 (2018) 113.

34 Bhagat D, Slathia PS, Raina N & Sharma P, Production of phytase from *Lactobacillus paracasei* strain and its probiotic profile. *Indian J Biochem Biophys*, 57 (2019) 839.

35 Sethi D, Mohanty S & Pattanayak SK, Effect of different carbon, nitrogen and vitamin sources on exopolysaccharide production of *Rhizobium* species isolated from root nodule of redgram. *Indian J Biochem Biophys*, 56 (2019) 86.