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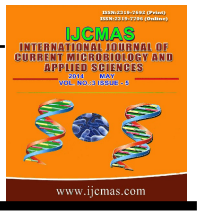

Original Research Article

Optimization of Exopolysaccharide production by *Lactococccus lactis* NCDC 191 by Response Surface Methodology

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Keywords

Exopoly-saccharide, Lactococcus, Whey, RSM

A B S T R A C T

An experimental design comprising of incubation temperature, pH, level of glucose, level of casein hydrolysate as factors was designed for production of exopolysaccharide (EPS) by *Lactococcus lactis* NCDC 191 in a whey based medium and responses were measured in terms of EPS production, absorbance, viscosity and plate count using response surface method. A great variation in EPS production was observed that ranged from 0 to 153.39 mg/l. The highest EPS production was achieved when the cultures were incubated at 25°C in deproteinized whey medium maintained at pH 6.8 supplemented with 1.0% casein hydrolyzate and 10 mg of glucose. There was no significant difference between the predicted values and actual values of response were found and findings reflect the adequacy of RSM for optimization of EPS production by NCDC 191. The adequacy of this model for further analysis was possible since R^2 was greater than 0.70. The outcomes of study can be used to scale up the EPS production commercially at fermenter level using dairy industry byproduct (whey) and giving possibility to study the functional attributes of produced biomolecule in food system by a potential strain.

Introduction

The term exopolysaccharide (EPS) refers to all forms of bacterial polysaccharide, both slime and capsule, found outside the cell wall (Sutherland 1972). Several lactic acid bacteria (LAB) are capable of forming EPS with potential application in the food industry as texturizers, viscosifiers, and syneresis-lowering agents because of their pseudoplastic rheological behavior and water-binding capacity (van den Berg, D. J. C. 1995). Besides, they have a couple of significant advantages for their industrial use, they have a GRAS (generally regarded as safe) status and can be produced either *in-vitro* or *in-situ* (De Vuyst Degeest 1999). These days, there is a considerable interest in finding new EPSs that are suitable for special applications or have a potential industrial relevance, either by applying different culture conditions or by using novel bacterial strains (Crescenzi 1995). Hence, several slime-producing lactic acid bacterium strains and their biopolymers

have been found to have interesting functional and technological properties. This wealth of biological information can be harnessed to develop applications in carbohydrate engineering for improving technological attributes of food systems e.g. viscosity, suspension of particulates, inhibition of syneresis, stabilization, emulsification that may contribute positively to the mouth-feel, texture, and taste perception of fermented dairy products low-milk-solid yogurt/dahi, low-fat yogurt/dahi, sour cream, lassi, low moisture cheeses etc. EPS provide functions that benefit reduced-fat dairy products. They bind water and increase the moisture in the nonfat portion, interfere with protein-protein interactions and reduce the rigidity of the protein network, and increase viscosity of the serum phase (Hassan 2008).

Whey protein aggregates are present in yogurt milk during acidification, and it could be hypothesized that these aggregates form a complex with the EPS produced by LAB. Such complexes significantly contribute to the structure development in milk fermented with EPS-producing strains (Ayala-Hernandez 2009; Kristo et al., 2011).

Some of these EPSs have also been observed to be endowed with advantageous biological properties, such as antitumour, antiulcer, antioxidative (↑ the CAT and SOD activity) and enhances the immune response via enhancing the macrophage and spleen lymphocyte stimulation (Oda et al., 1983; Nagakoya et al., 1994; Pan and Mei, 2010; Pan et al., 2014).

Biosynthesis and secretion of EPSs from LAB occur during different growth phases, and both the amount and type of polymer is influenced by growth conditions. The total yield of EPS produced by the LAB depends on the composition of the medium (carbon and nitrogen sources, growth factors, etc.) and the conditions in which the strains grow, i.e. temperature, pH, oxygen tension, and incubation time. Media containing complex nutrients like beef extract, peptone and yeast extract are not suitable because of interference of these compounds with the monomer and structure analysis of HePS (Kimmel and Roberts 1998). Biosynthesis of biomass and EPS biosynthesis follows roughly the same metabolic pathways which results in the same metabolic control for EPS production and growth. The uncoupling of growth and acid production explains the reduction in efficiency of EPS production in the cultures not glucose-limited.

Production of EPS and synthesis of cell surface polysaccharides both require isoprenoid lipid carriers, sugar nucleotides and energy, and competition between the two processes is possible for any of these factors (Sutherland 1972). For *Lc. lactis* ssp *lactis*, a higher HePS production and a better cell growth is observed for growth on glucose compared to fructose, although the transcription level of the EPS gene clusters is independent of the carbohydrate source (Looijesteijn et al., 1999). Cerning et al (1990) found that casein stimulates the HePS production, but not growth of *Lb. delbrueckii* subsp *bulgaricus*.

Enhanced HePS production and growth were initially obtained when (hydrolysed) casein was added to skim milk cultures of *Lb. delbrueckii* subsp *bulgaricus* (Garicia-Garibay and Marshall 1991). For some HePS producing bacteria such as *Xanthomonas, Pseudomonas* and *Rhizobium* spp, nitrogen limitation results

in increased HePS production (Sutherland 1990). This seems not to be the case for the LAB strains (De Vuyst et.al., 1998). Moreover it has been shown that an optimal balance between the carbon and nitrogen source is absolutely necessary to achieve high HePS yields (Degeest and De Vuyst 2000). This may be explained by the fact that the LAB are dependent on the nitrogen source for cell synthesis, whereas the carbon source is mainly utilized for energy generation (Degeest et al., 2001). Gancel and Novel (1993) observed EPS production by an Eps^+ *S. thermophilus* strain in stationery phase of growth in a synthetic medium and also found that temperatures or sugars that decrease growth rate increased polymer synthesis.

Conditions like temperature, pH, growth or HePS production might allow the uncoupling of growth and EPS production in mesophiles (Looijesteijn and Hugenholtz 1999). Also with mesophilic LAB, the HePS production may be further enhanced on varying the environmental factors once enough cells have been formed in the trophophase. Reports pertaining to the work examining the effect of temperature on EPS production by LAB in literature have been contradictory. Some workers have found greater amount of EPS produced at temperature within the optimum growth range, while others have suggested that more EPS is produced at suboptimum temperatures. A number of researchers have observed increase in EPS production at lower temperatures of incubation (Cerning et al., 1992; Looijesteiin and hugenholtz 1999; Marshall et al., 1995). This effect has been explained, based on information for EPS production from Gram negative bacteria, by the fact that slowly growing cells exhibit much slower cell wall polymers biosynthesis making more isoprenoid lipid carrier molecules available for biosynthesis. Although a higher EPS production has also been associated with optimal growth conditions (De Vuyst et al., 1998; Knoshaug et al., 2000).

The optimal pH for growth and product formation has been established to be around 6 for lactococci (Bibal et al., 1989). The proteolytic activity of *Lc. lactis* was found to be highest at temperature above 45°C and at around pH 5.5. Since *Lc. lactis* subsp *lactis* and *Lc. lactis* subsp *cremoris* regulate their internal pH between 7.0 and 7.5 when the external pH ranges from 5.5 to 7.5, the failure to grow at alkaline and acidic pH is unlikely to be caused by a limitation of cytoplasmic processes. The growth rates of *Lc. cremoris* and *Lc. lactis* were limited at alkaline pH by their capacity to accumulate glutamate. Glutamic acid uptake rate and μ_{max} decreased logarithmically when pH was above 6.5 and because glutamic acid an essential amino acid this could be one of the reasons why LAB do not grow at higher pH (Van Niel et al., 1999). Gassem et al (1997) suggested that maintenance of higher pH will result in increased EPS production by increasing the time the culture is in the exponential growth phase.

Higher pH also results in a longer stationary phase, which would decrease peptidoglycan and teichoic acid synthesis and could result in increased EPS production. It is further shown that EPS production under growth conditions with continuously controlled pH is significant higher than in acidified batch cultures.
Several investigators find higher EPS production by LAB strains at higher cultivation temperature and under conditions optimal for growth for instance

with respect to pH and oxygen tension. Aeration is not required, since higher EPS yields are obtained with a lower O_2 tension as well as anaerobically. Van den Berg et al (1995) postulated that conversion of sugar to EPS is more efficient at pH 5.8 but sugar is more efficiently converted to biomass at pH 6.2 (De Vyust et al., 1999). Whereas mesophilic strains seem to produce maximal amounts of HePS under conditions not optimal for growth, for instance low temperatures, the HePS production from thermophilic LAB strains appears to be growth-associated, i.e. maximal production during growth and under conditions optimal for growth (De Vuyst et al., 1998). Marshall et al (1995) indicated that the onset of the HePS biosynthesis from a strain of *Lc. lactis* subsp. *cremoris* and *Lb. rhamnosus*, respectively, is observed towards the end of the exponential growth phase. Gassem et al (1995) observed absence of association between growth rate or acid production and the HePS production in different media by the LAB strains. Most of the HePS are produced during the exponential growth phase when the cells are grown on the glucose, while during growth on fructose, about 60% of the HePS are produced in the stationary phase (Looijesteijn et al., 1999).

In *Lc. lactis* subsp. *cremoris* Ropy352, expressing two phenotypically distinct HePS, optimal growth conditions parallel optimal production of the ropy HePS, whereas poor growth conditions parallel optimal production of the mucoid HePS (Knoshaug et al., 2000). Other investigators observed continued HePS production beyond or only in the stationary phase, and hence consider the HePS as secondary metabolites (Gancel and Novel 1994; Looijesteijn and Hugenholtz 1999; Petry et al., 2000). A possible interpretation is that isoprenoid phosphate carriers are primarily needed for cell wall synthesis during growth. Upon cessation of growth, there is a greater availability of this molecule for the HePS biosynthesis (Petry et al., 2000; Degeest et al., 2001).

The traditional optimization of fermentation processes with respect to physical and chemical factors employing rationally selected strains is a promising strategy. Therefore, the influence of both nutritional as well as environmental factors on bacteria growth and EPS production needs to be understood. The general practice of determining the optimal operating conditions is by varying one parameter while keeping the others at a constant level. The major disadvantages of single variable optimization is that it does not include interactive effects among the variables and therefore, it does not depict the net effect of various parameters on the reaction rate. In order to overcome this problem, optimization studies have been carried out using response surface methodology (RSM). RSM is an ideal method to study and quantify the individual and combined effect of different of different parameters. It is a statistical method that uses quantitative data from appropriate experiments to determine and simultaneously solve multivariate equations. The RSM is based on comprehensive theory and may find its application in optimization processes because of its higher efficiency hence simplicity (Arteaga et al., 1994).

The present study was carried out with the objectives to determine the optimum temperature, pH, casein hydrolysate (nitrogen source) and glucose (carbon source) concentrations for maximum production of EPS by *Lactococcus lactis*

subsp. *lactis* strain (NCDC 191), a strain of indigenous origin in a whey based medium using RSM.

Materials and Methods

Culture Propagation

EPS^+ strain of *Lc. lactis* subsp. *lactis* NCDC191 was obtained from National Collection for Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, India. Working cultures were prepared by propagating culture in M-17 broth at 30°C for 14-16h and were maintained at 4°C. The culture was sub cultured twice in M-17 broth before inoculation.

Culture Medium

The deproteinised (DP) whey was prepared from paneer whey obtained from Experimental dairy, NDRI. The obtained whey was centrifuged to remove fat (<.05%) and then adjusting the pH to 4.3. It was then heated for 30 min at 100°C and then filtered (Whatman No 1 paper). The resulting supernatant was adjusted to pH 6.8 with 1M NaOH, steamed for 30 min and filtered (Whatman No 1) to obtain DP whey. The organism was grown (3% inoculum) in sterile DP whey medium and propagated in same medium. Protein content was determined by Kjeldhal method (Conversion factor 6.8) and lactose by Anthrone method of DP whey obtained by above discussed procedure (data not shown).

Experimental Design

Experimental design selected for optimization purpose was Central Composite Rotatable Design (CCRD), for 4 independent variables at five levels. The experiments conducted in the first phase of investigation revealed that temperature of incubation, pH of growth medium, concentration of carbon and nitrogen sources were the most critical factors for the production of EPS using *Lc. lactis* subsp. *lactis* (NCDC191). Levels of these 4 independent variables were selected on the basis of laboratory trials. The variable factors considered for RSM studies were Temperature of incubation (25 to 40°C), pH (5.6 to 6.8), casein hydrolyzate concentration (0.5% to 2.0%) and glucose concentration (2.5% to 12.5%) as given in Table 1 The full factorial CCRD matrix in the coded and the actual levels of variables is presented in Table 2 and all experiments were conducted in random order, 3 each at a time. The responses were EPS (mg/L), Optical Density (600 nm), viscosity (cP) and plate count (cfu/ml). The data were analyzed using Design-Expert software (6.0.10 version) and generalized second degree polynomial (Equation 1) using the method of least squares (Snedecor and Cochran 1968).

$$Y= b_0 + b_1 x_1 + b_2x_2+ b_3x_3 + b_{11}x_1^2 +b_{22}x_2^2 +b_{33}x_3^2 + b_{12} x_1x_2 + b_{13} x_1x_3 + b_{23} x_2x_3 + Є \quad \ldots(\text{Eq. 1})$$

The coefficients of the polynomial models were represented by b_0 (constant term), b_1, b_2, b_3, b_4 (linear terms), b_{11}, b_{22}, b_{33}, b_{44} (quadratic terms); and b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{34} (interactive terms). Adequacy of model was evaluated using F ratio and co-efficient of determination (R^2). The lack of fit was also calculated. Model was considered adequate when F-calculated was more than table F-value and R^2 was more than 70 per cent (Henika 1982). The effect of variables at linear, quadratic and interactive level on individual response was described using significance at 1, 5 and 10 per cent level of confidence. The magnitude and sign of coefficients described the extent of dependency of variation on increasing or decreasing the

response depending on positive or negative sign of coefficient terms.

Response surface plots were also developed using second order polynomial models for all responses keeping the two processing variables at centre point. Response surface was used to determine the interaction between two variables on responses.

Fermentation

All optimization fermentations were conducted in 100 ml working volume. Glucose and casein hydrolysate were added to experimental design and pH was adjusted accordingly. The media was sterilized at 121°C/15min. the culture was inoculated at the rate of 1% and incubated at the designated temperature for 48hrs. Agitation was maintained at 100rpm throughout the fermentation. After incubation hours, samples were analyzed for EPS production, growth, viscosity and plate count.

Growth Measurement

The growth of the culture in DP-whey medium was measured as optical density (O.D) at 600nm using Jenway Genova (UK) spectrophotometer and by plating appropriate dilutions of fermented DP whey on M-17 indicator agar.

EPS Isolation

EPS isolation was done (Van Geel-Schutten et al., 1998) from fermented whey by heating in a boiling water bath for 15 min in order to dissolve the polysaccharide attached to cell and to inactivate the enzyme that could hydrolyze the polymer and cells were removed by centrifugation at 20°C (Sigma 3-18K centrifuge, Germany). Cell pellet was discarded and supernatant was treated with two volumes of ice-cold ethanol and maintained at –20°C for 24 h in order to precipitate polysaccharide. Then samples were centrifuged at 10 000 g for 15 min at 4°C. Pellets were resuspended in hot water and precipitated again by the addition of two volumes of cold ethanol. After storage at –20°C for 24 h and centrifugation (10000 g/15 min at 4°C), EPS pellets were resuspended in hot distilled water.

EPS Quantification

Total sugars from final solutions were determined by Anthrone method (Morris 1948), measuring absorbance at 630nm using glucose solutions as standards.

A standard solution of Glucose was prepared by dissolving 5 mg in a minimal quantity of distilled water and made up the volume to 100 ml in a volumetric flask to give a final concentration of 50mg/L. A series of dilutions were prepared from this standard solution to have glucose concentration s ranging from 5–25 μg/0.5 ml. 2.5 ml of the Anthrone solution was added for developing the colour. The standard curve was drawn by plotting the optical density against glucose concentration at 630nm. Five hundred micro liters of the diluted samples was treated with 2.5 ml of Anthrone solution in a capped tube. It was heated at 100°C for 15 minutes followed by cooling and color was read at 630nm.

The obtained O.D value (Y) was substituted in the standard curve equation to get the value for X which gives concentration of EPS in 0.5 ml of the sample. Conversion factor is used to calculate the EPS produced per litre of fermented DP Whey medium.

EPS (mg/L) = {(X / 0.5 * 10) * 1.1} * 100

The values for EPS were calculated by subtracting the amount of back ground interference in uninoculated media (control) from the amount detected in fermented broth

EPS= TS (fermented broth) – TS (control)

Results and Discussion

There was a great variation in amount of EPS (mg/L) which ranged from 0.003 to153.39 mg/L (Table 1). The highest EPS production was achieved when the cultures were incubated at 25°C in DP whey medium maintained at 6.8 supplemented with 1.0% Casein hydrolysate and 10% of glucose. However, no production was observed at incubation temperature of 40°C, pH 6.2, casein hydrolysate and glucose concentrations of 1.5 and 7.5% respectively.

The coefficient of determination, R^2 was 0.73 for EPS production, which showed that the model explained 73% variability in the data. There was no lack of fit and the calculated F value was 2.67 ($P< 0.05$). Therefore, the model was adjudged to be adequate for further analysis. Table 3 shows that EPS production depends upon on the temperature as its linear effect ($P<0.01$) as well as quadratic effect ($P<0.05$) are negative. The other variables were found to have no effect on EPS production. The increase in temperature linearly resulted in decreased EPS production. The response surface curve (Fig 1a-c) showed that increase in temperature by varying other variables caused lowered production of EPS. This signifies that linear effect of temperature was dominant over the quadratic effect.

Absorbance

The polynomial equation of the quadratic response surface model for optical density showed that the model F value is 4 times more than calculated F value and the co-efficient of regression was 0.91. This confirmed the adequacy of model to describe the effect of processing variables on optical density of the medium. From Table 3, it may be observed that incubation temperature affected the O.D of the medium at linear ($P<0.01$) and quadratic ($P<0.01$) level. The effect of incubation temperature was positive at linear level and any increase in temperature increased the O.D value.

However the negative quadratic effect of temperature on OD value was more significant than linear one and it can be seen from the (Fig. 2a) that any increase or decrease in temperature resulted in a decline in O.D value. The casein hydrolyzate concentration also had significant effect on OD value, as its linear effect is negative ($P<0.05$) and interactive effect is negative ($P<0.05$). The O.D value of the medium was affected by the glucose concentration at positive linear ($P<0.01$) and negative interactive ($P<0.01$) level. It is evident from the interaction plot (Fig. 2b) that maximum OD value was at higher incubation temperature and lower glucose concentration. The pH had negative effect on O.D value at quadratic level and maximum OD was at central value (Fig. 2c).

Viscosity

The model F- value of 2.96 was more than the calculated F-value and it confirmed the significance of polynomial quadratic model. The co-efficient of determination value was 0.75 and according to Henika (1982) R^2 value above 0.7 indicates the adequacy of model. It remains difficult to correlate rheology of growth medium with

the quantities of EPS produced. The viscosity of the medium was in the range of 5.33- 47.23 cp. The maximum viscosity was exhibited by the sample which was incubated at 30°C, pH of 6.2 and concentration of glucose and casein hydrolysate were 12.5% and 1.5% respectively. However the sample incubated at 35°C, pH of 5.2 and glucose and casein hydrolysate concentration of 10% and 1%respectively, resulted in lowest viscosity of growth medium. Among the processing variables temperature ($P<0.01$), pH ($P<0.05$) and glucose ($P<0.05$) have significant effect on viscosity of the medium at quadratic level. The effect was negative and it implies that any increase or decrease in these variables from central point lowered the viscosity of the medium. Fig. 3a-c showed that effect of temperature was more predominant on viscosity as compared to other variables.

Plate count

Lowest microbial count was noticed in growth medium that was incubated at 35°C, pH of 6.8 and casein hydrolysate and glucose concentration of 2 and 10 mg/L respectively. Maximum microbial growth was observed, where growth medium was supplemented with 1.5 mg casein hydrolysate and 2.5 mg of glucose, incubated at 30°C and pH was maintained at 6.2. The model F value of polynomial quadratic model was 2.95 and it was significant at 5% level of significance. The coefficient of determination was 0.75 and it means that the model was adequate for further analysis.

Among the processing variables the glucose had most significant effect at linear level ($P< 0.01$) and its effect was negative. Increasing the level of glucose in the medium suppressed the growth of microbes i.e. Lc lactis subsp. lactis. Temperature had a negative quadratic effect ($P< 0.05$) and the lower microbial count was obtained at lower or higher incubation temperatures (Fig. 4a-b). Among the other variables only casein hydrolysate exhibited a negative linear and pH exerted a negative quadratic effect at higher level of significance (Fig. 4b, c, d). It means supplementation of nutrients at higher level adversely affected the cell biomass.

Estimated Optima Response

Optimization was done with the objective of determining the best combination of temperature, pH, glucose (as a source of carbon) and casein hydrolysate (as a source of nitrogen) concentrations. The goals that were set for obtaining the best combinations are illustrated in table 4.

Optimization of EPS Production by Response Surface Methodology (RSM)

There was a great variation in amount of EPS (mg/L) which ranged from 0.003 to153.39 mg/L. In the present investigation mesophilic bacteria *Lc. lactis* subsp. *lactis* was used as inoculum and its optimum growth temperature lies between 30-35°C. The maximum EPS production in this study was observed at sub-optimal temperature. Published work examining the effect of temperature on EPS production by LAB has been contradictory. Some reports found greater amount of EPS produced at temperature within the optimum growth range, while others have suggested that more EPS is produced at temperatures that are less than the optimum.

Table 1: Experiment design matrix and Responses

Run	Temperature(°C)	pH	Casein Hydrolysate (%)	Glucose (%)	EPS(mg/l)	Absorbance (OD_{600nm})	Viscosity (cP)	Plate count(1X 10^6cfu/ml)
1	30	6.2	1.5	7.5	113.733	0.958	43.20	40.0000
2	30	6.2	1.5	7.5	113.646	0.936	43.20	40.0000
3	35	5.6	2.0	10.0	94.670	0.242	8.37	0.0047
4	35	5.6	1.0	10.0	122.498	0.728	5.33	1.9000
5	35	6.8	2.0	5.0	99.271	1.330	17.48	90.0000
6	35	6.8	2.0	10.0	100.016	0.180	18.73	0.0004
7	25	6.8	1.0	10.0	153.395	0.358	6.98	0.0860
8	35	5.6	2.0	5.0	96.247	1.109	16.80	4.6000
9	25	5.6	2.0	5.0	111.849	0.471	8.84	0.2500
10	30	6.2	1.5	7.5	113.427	0.918	40.50	35.0000
11	35	5.6	1.0	5.0	96.992	1.409	15.00	68.0000
12	35	6.8	1.0	5.0	97.113	1.559	21.90	84.0000
13	35	6.8	1.0	10.0	97.124	0.872	27.43	14.0000
14	30	6.2	1.5	7.5	114.084	0.966	44.20	37.0000
15	25	6.8	2.0	5.0	111.235	0.588	38.55	6.5000
16	25	5.6	2.0	10.0	110.622	0.279	23.73	0.0380
17	25	6.8	1.0	5.0	109.044	0.567	14.74	6.3300
18	25	5.6	1.0	5.0	122.809	0.440	13.33	0.2180
19	25	6.8	2.0	10.0	110.841	0.381	16.30	0.1140
20	25	5.6	1.0	10.0	131.526	0.275	7.63	0.0330
21	30	6.2	1.5	2.5	108.737	1.745	23.83	200.0000
22	30	5	1.5	7.5	103.259	0.920	36.42	27.0000
23	30	6.2	2.5	7.5	96.160	1.083	41.63	20.0000
24	30	6.2	0.5	7.5	96.291	1.408	42.14	140.0000
25	30	7.4	1.5	7.5	49.092	0.456	40.00	0.1500
26	30	6.2	1.5	12.5	98.219	0.598	47.23	0.6700
27	40	6.2	1.5	7.5	-1.912	0.321	7.30	0.0900
28	30	6.2	1.5	7.5	113.733	0.954	43.40	37.0000
29	30	6.2	1.5	7.5	114.391	0.975	44.20	37.0000
30	20	6.2	1.5	7.5	103.040	0.206	20.56	0.0028

Table 2 Full experimental design for Response Surface Methodology

Std	A:Temperature ºC	B:pH	C:Casein hydrolysate	D:Glucose
1	-1	-1	-1	-1
2	1	-1	-1	-1
3	-1	1	-1	-1
4	1	1	-1	-1
5	-1	-1	1	-1
6	1	-1	1	-1
7	-1	1	1	-1
8	1	1	1	-1
9	-1	-1	-1	1
10	1	-1	-1	1
11	-1	1	-1	1
12	1	1	-1	1
13	-1	-1	1	1
14	1	-1	1	1
15	-1	1	1	1
16	1	1	1	1
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0
21	-2	0	0	0
22	2	0	0	0
23	0	-2	0	0
24	0	2	0	0
25	0	0	-2	0
26	0	0	2	0
27	0	0	0	-2
28	0	0	0	2
29	0	0	0	0
30	0	0	0	0

Table.3 Partial co-efficient of regression equation of quadratic models for optimization conditions for EPS production

Source	EPS (mg/L)	Absorbance ($O.D_{600nm}$)	Viscosity (cp)	Plate count(cfu/ml)
	Partial coefficient	Partial coefficient	Partial coefficient	Partial coefficient
b_o(constant)	110.018	0.974	45.293	41.789
b_1(Temperature)	-15.304*	0.179*	-1.063	10.380
b_2(pH)	-4.896	-0.002	2.927	3.012
b_3 (Casein Hydrolyzate)	-4.001	-0.095**	1.476	-13.044
b_4 (Glucose)	2.296	-0.026*	0.610	-26.766
b_{11}	-11.032*	-0.199**	-9.974*	-14.611*
b_{22}	-4.629	-0.094	-3.905**	-11.228**
b_{33}	0.38	0.045	-2.986	5.378
b_{44}	2.197	0.027	-4.575**	10.461
b_{12}	-1.537	0.002	1.062	6.313
b_{13}	3.044	-0.112**	-3.314	-4.596
b_{14}	-1.673	-0.163*	0.594	-13.604
b_{23}	1.570	-0.008	0.223	3.591
b_{24}	0.831	-0.022	-0.894	-6.346
b_{34}	-5.065	-0.042	0.191	2.584
R^2	**0.73**	**0.91**	**0.75**	**0.75**

* Significant level at 1% level ($p<0.01$)

** Significant level at 5% level ($p< 0.05$)

Table.4 Solutions suggested by the Design Expert package for optimization of EPS production

Number	Temperature	pH	Casein hydrolysate	Glucose	EPS	O.D.	Viscosity	Plate Count	Desirability
1	25.00	5.60	1.43	9.96	120.287	0.452085	26.2948	13.8561	0.919547
*2	25.00	5.60	1.26	9.10	121.789	0.474121	26.3339	15.1227	0.917454
3	25.95	5.60	1.04	9.08	127.417	0.604796	26.28	27.6793	0.882708
4	25.00	5.60	2.00	6.71	105.625	0.617725	31.4049	4.61908	0.84648
5	29.61	5.60	1.98	10.00	105.093	0.587113	34.393	10.1474	0.73638

* Selected combination

Table.5 Comparison of predicted v/s actual of responses

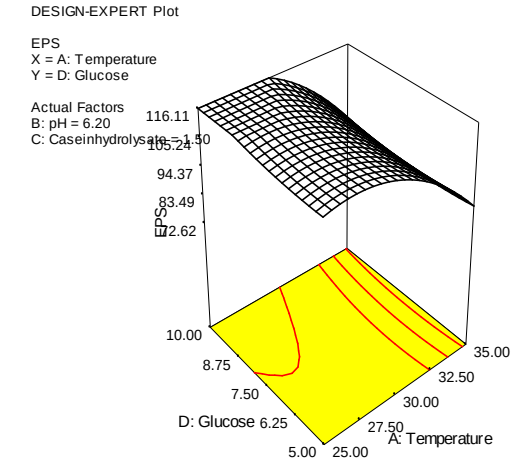
Attributes	Predicted results*	Actual results@	t-value#
EPS (mg/l)	127.417	131.193	0.629
Absorbance (OD_{600nm})	0.604	0.576	0.738
Viscosity (cp)	26.28	26.886	0.086
Plate count (cfu/ml)	27.68	24.333	0.744

* Predicted values of Design Expert 6.0.10 package

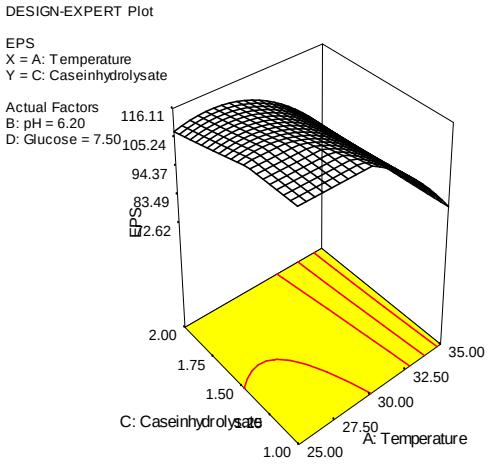
@ Actual values (average of 3 trials) of the optimized product

t-values found non significant at 5% level of significance (Tabulated value of t=4.303)

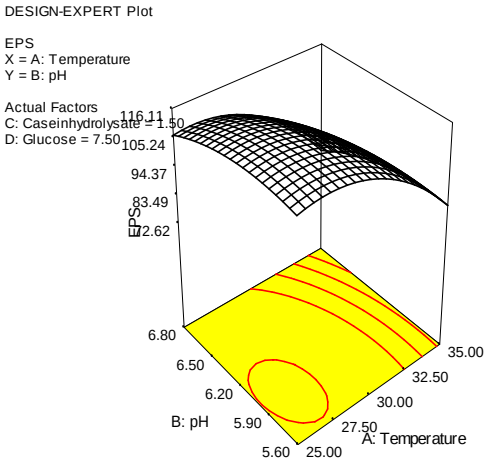
Figure.1 Surface plots for EPS Production



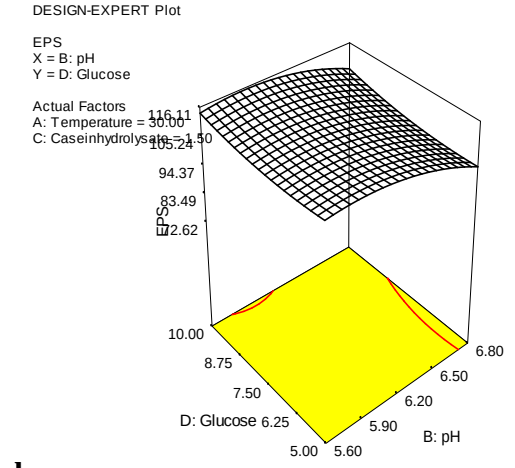
a



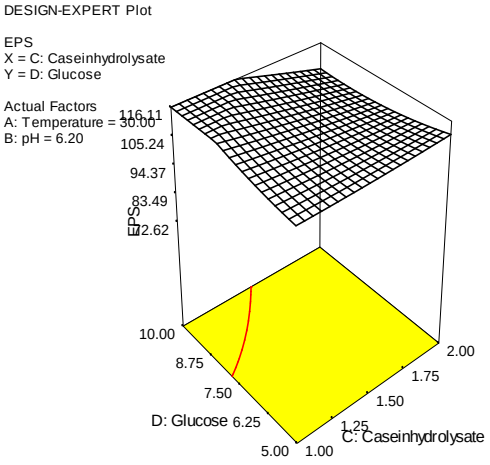
b



c

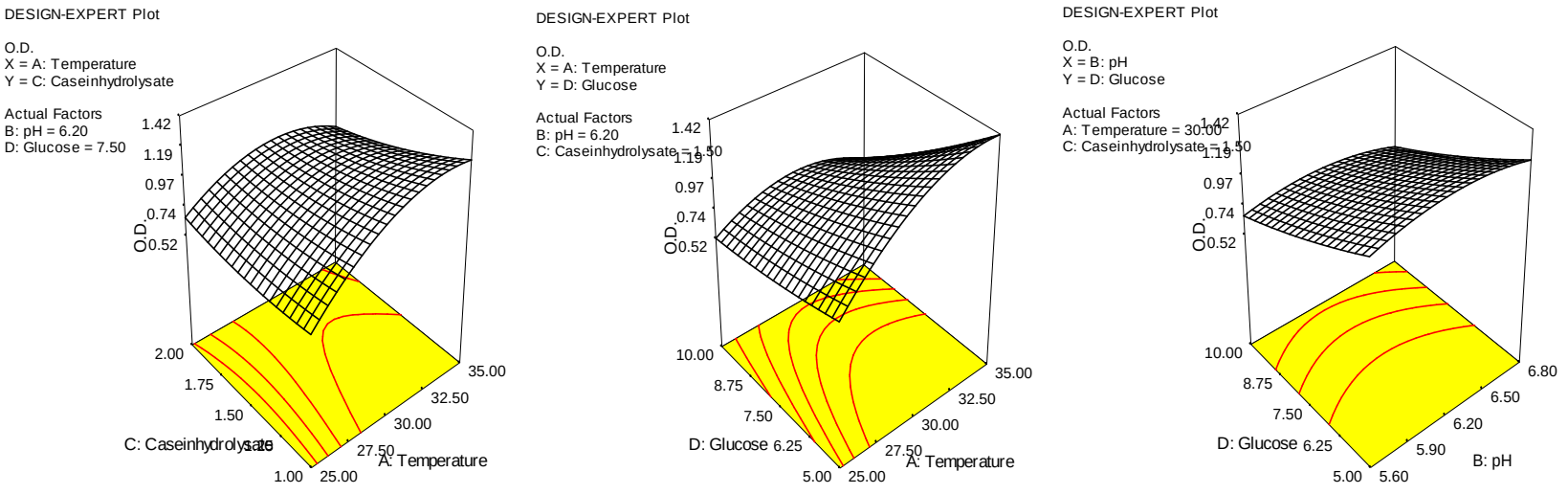


d



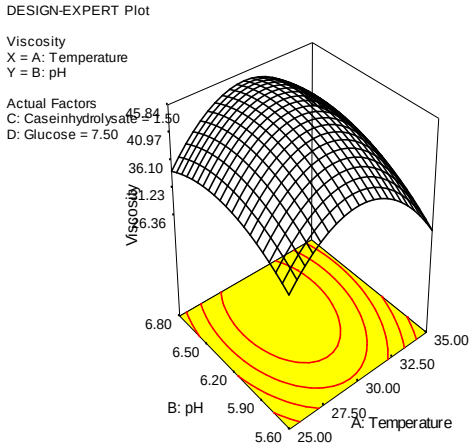
e

Figure.2 Surface plots for Absorbance



a **b** **c**

Figure.3 Surface plots for Viscosity



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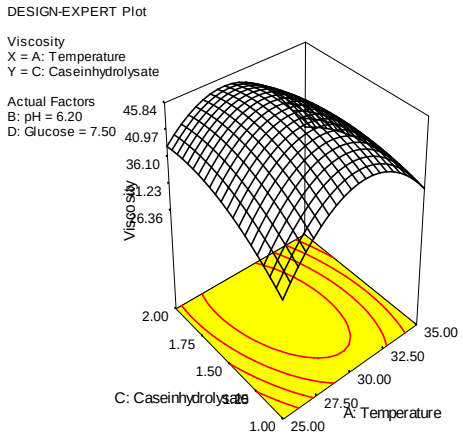
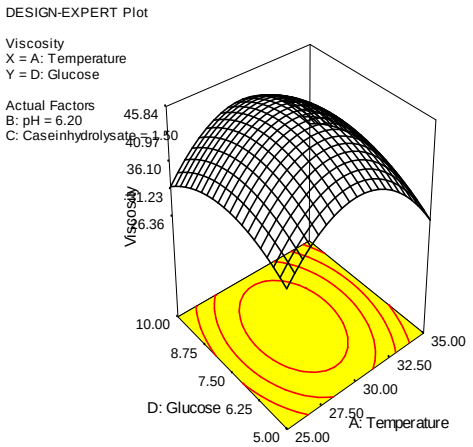
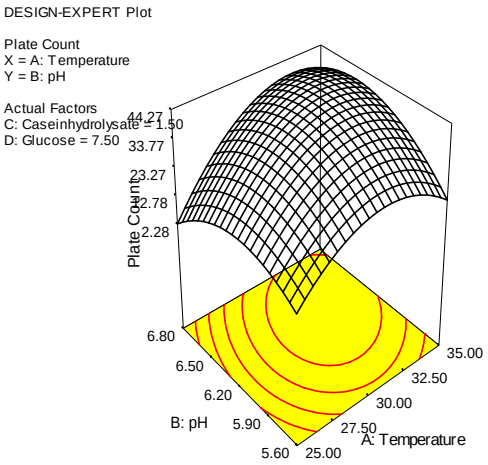


b

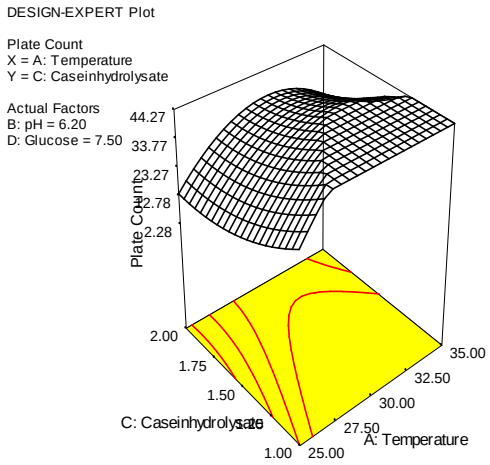


c

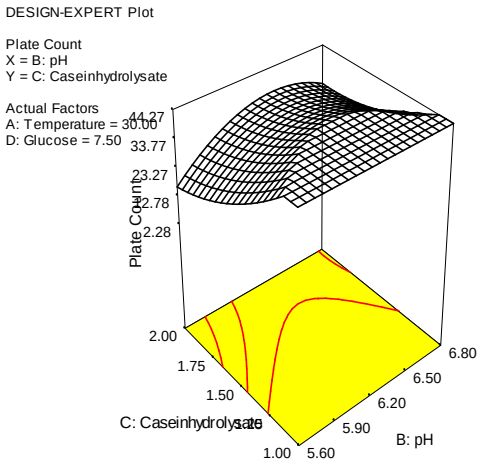
Figure.4 Surface plots for Plate Count



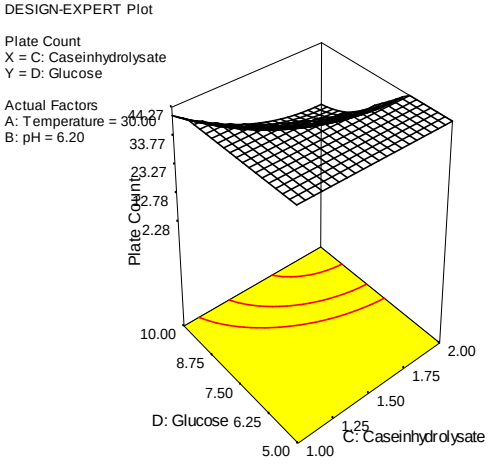
a



b



c



d

Discrepancies in the literature exists for a variety of reasons, including different ways of measuring EPS, including different ways of measuring EPS, different growth media, conditions and times of measurement, lack of pH control, and various means of expressing EPS production (Kimmel et al., 1998). There are a good number of reports in literature which suggests that lower temperatures enhance the production of EPS (Cerning et al., 1992; Looijesteiin and Hugenholtz 1999; Marshal et al., 1995). Although a higher EPS production has also been associated with optimal growth conditions (De Vuyst et al., 1998; Knoshaug et al., 2000).

Cerning et al (1992) showed that ropy strains of *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lactobacillus casei* when grown in milk and ultrafiltrate, produced 30-600 mg/L of EPS which was 50-60% higher when strains were grown at 25°C instead of 30°C. The yield of intracellularly synthesized Heteropolysaccharide (HePS) by different LAB strains varies roughly from 0.045 to 350 mg/L when the bacteria are grown under non-optimized culture conditions. Optimal culture conditions result in HePS yields from 150 to 600 mg/L, depending on the strain (Cerning 1990; Cerning and Marshall 1999).

Nakajima et al (1990) reported that the slime forming *Lc. lactis* subsp. *cremoris* SBT0495 isolated from starter culture of Finnish fermented "villi" yielded slime 500mg/L in whey permeate medium. One of the highest HePS concentrations (2775 mg/L) has been obtained when *Lactobacillus rhamnosus* RW was grown in whey permeate medium (Macedo et al., 2002). When a ropy strain of *S. thermophilus* is grown in association with a non-ropy strain of *Lb. delbrueckii* subsp. *bulgaricus* in milk, the HePS production can reach upto almost 800 mg/L (Cerning et al., 1990). An optimal carbon/nitrogen ratio in both milk and MRS media gives yields of 1500 mg/L of HePS with *S. thermophilus* LY03 (De Vuyst et al., 1998; Degeest and De Vuyst 1999, Degeest and De Vuyst 2000). Higher EPS production was observed under nitrogen-limited conditions than under carbon limited conditions (Mengistu et al., 1994; Marshall et al., 1995). With *Lb. sakei* 01 and *Lb. rhamnosus* 9595M, HePS yields of approximately 1400 mg/L and 1300 mg/L, respectively, are achieved (Van den Berg et al., 1995; Degeest et al., 2001). In general homopolysaccharides (HoPS) are produced in larger quantities than HePS.

The EPS quantity reported ranges from 50-350 mg/L for *S. thermophilus*, from 60-150 mg/L for *Lb. delbrueckii* subsp *bulgaricus*, from 50-60 mg/L for *Lb. casei* (Ruas Madiedo et al., 2002). However, the amounts of EPS produced by LAB in situ are low and their production is unstable, particularly in milk. Consequently, improvement of the EPS concentration in situ should result in an increased functionality of EPS producing LAB. Kimmel et al (1998) conducted a study to evaluate the effects of temperature, pH and Bacto-casitone concentration for production of EPS by *Lactobacillus delbrueckeii* subsp. *bulgaricus* RR in a semidefined medium using RSM. They observed 300mg/L of EPS production occurred at 38ºC and pH 5.0 respectively with a predicted yield of 295mg/L of EPS.

Our findings are in agreement with aforementioned reports and other workers who reported that slime producing organism looses this property more quickly at high temperature which is probably due to the loss or curing of plasmid at high temperatures (Bottazzi 1986; Ciric et al.,

1990). It can be observed from the Fig. 1d-e that increased in glucose concentration improved the EPS recovery. Kimmel et al. (1998) reported that higher glucose concentration (Carbon source) provides the higher yield of EPS. For *Lactococcus lactis* ssp lactis, a higher HePS production and a better cell growth is observed for growth on glucose compared to fructose, although the transcription level of the EPS gene clusters is independent of the carbohydrate source (Looijesteijn and Hugenholtz 1999).The sugar source is essential for the growth as well as EPS production by mucoid lactococci as it provides the energy necessary for both processes. Furthermore, a fraction of sugar source is used for the biosynthesis of biomass and EPS precursors. Amino acids are not directly involved in EPS biosynthesis but serve as carbon and nitrogen sources, which are essential for growth. Hence, one can expect that the amount of EPS produced per cell by Lc. lactis is lower under glucose limitation than under leucin limitation. However, there are reports in which higher EPS production was observed under nitrogen-limited conditions than under carbon limited conditions (Mengistu et al., 1994; Marshall et al., 1995). Gancel and Novel (1993) observed EPS production by an EPS^+ S. thermophilus strain in stationary phase of growth in a synthetic medium and also found that temperatures or sugars that decrease growth rate increased polymer synthesis.

Looijesteijn et al., (2000) studied the influence of different substrate limitations on EPS production by closely related organism namely *Lactococccus lactis* subsp. *cremoris*. They observed that reduction of the growth rate from 0.5 to 0.1 h^{-1} resulted in an increase of the specific EPS yield, but a further reduction of dilution rate to 0.05h^{-1} resulted in a decrease of the polymer yield. At all growth rates tested, the efficiency of EPS production (mg EPS/g glucose consumption) was slightly higher under glucose limitation than in complete chemically defined medium. Biosynthesis of biomass and EPS biosynthesis follow roughly the same metabolic pathways. This results in the same metabolic control for EPS production and for growth. The uncoupling of growth and acid production explains the reduction in efficiency of EPS production in the cultures not glucose-limited. Production of EPS and synthesis of cell surface polysaccharides both require isoprenoid lipid carriers, sugar nucleotides and energy, and competition between the two processes is possible for any of these factors (Sutherland 1994). At higher growth rates, more intermediates per time unit are needed for the biosynthesis of cell surface polysaccharides, and the intermediates are apparently used in favor of the synthesis of these polysaccharides; this may explain the reduction of the EPS yields at higher growth rates.

A steep inverted dome shaped curve was formed towards temperature axis as compared to other variables. This indicates that non-linear effect was more significant. Madiedo et al., (2002) also observed no clear relation between the EPS concentration and the viscosity of fermented milks. Not only the concentration but also the structure of the EPS is important for its thickening effect (Van Casteren et al., 1998). Since the polysaccharides of different LAB vary greatly in composition, charge, spatial arrangement, rigidity and ability to interact with proteins, it is not surprising that no clear correlation between observed EPS concentrations and apparent viscosity of the product could be established. Due to the complex physical, chemical process involved in texture generation, the mere ropiness trait of a culture strain does not guarantee an optimal, smooth and creamy

quality of the end product (Duboc and Mollet 2001). However, the reduced viscosifying properties of the EPS produced by such strains (Looijesteijn et al., 2000) can be harnessed to use this biomolecule as a bodying agent. It has a low biodegradability. (Ruijssenaars et al., 2000) and could therefore be used as a non-digestible food fraction in certain functional food products where an increase in viscosity is undesirable. It is obvious that the quantities of EPS are necessary to increase the viscosity, but the magnitude of increase varies because of differences in culture strains, incubation conditions, total solids of the medium, and viscosity measurements. Moreover viscosity may not only be affected by the amount of EPS released but also by an EPS with slightly different structure, resulting in different rheological characteristics of the medium.

The plate count varied from 4.7x 10^2 to 2 x 10^8 cfu/ml. Cerning et al (1992) reported that EPS production was stimulated in the presence of glucose at 25°C, the cell number was lower than the parent cultures. This is in agreement with the mechanism proposed by Sutherland (1972), who postulated that if the cells are growing slowly, then wall polymer synthesis will also be slow, thereby making more isoprenoid phosphate available for exopolymer synthesis. The design expert software recommended the first solution and was based on maximum desirability. But the third solution which predicted higher EPS yield though the lower desirability was selected for actual performance to find the adequacy of design of experiment. The final trial comprising of all the parameter suggested by the expert (incubation temperature 25°C pH 5.6, casein hydrolysate and glucose concentration of 1.04 and 9.08 respectively) was carried in triplicate in 1 liter working volume of DP whey. In conclusion, an increase in glucose concentration found to improve the EPS recovery and casein hydrolyzate concentration also had significant effect on OD value, as its linear effect is negative ($P<0.05$) and interactive effect is negative ($P<0.05$). Among the processing variables temperature ($P<0.01$), pH ($P<0.05$) and glucose ($P<0.05$) have significant effect on viscosity of the medium at quadratic level. Maximum microbial growth was observed, where growth medium was supplemented with 1.5 mg casein hydrolysate and 2.5 mg of glucose, incubated at 30^{o}C and pH was maintained at 6.2. The experimental values (actual values) were compared with that of predicted values (Table 5). There was no significant difference between the predicted values and actual values of responses as the calculated 't' values for all the parameters were found to be less than the table values. On the basis of the findings of the present study it is concluded that the selected combination is the best one in terms of optimization of responses delineated at the beginning of the study. The model can be adequately used for optimizing the parameters for higher EPS production.

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