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Screening, identification and statistic optimization of a novel exopolysaccharide producing *Lactobacillus paracasei* HCT

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The exopolysaccharides (EPS) producing ability was evaluated by phenol-sulphuric acid method and the *Lactobacillus* HCT was the highest yield EPS-producing strain, which was isolated from feces of Bama centenarian in Guangxi of China. It was identified as *Lactobacillus paracasei* HCT with carbohydrate assimilation profiling and 16S rRNA gene sequencing. *L. paracasei* HCT was found to be a novel EPS producing strain. Preliminary one factor tests were employed to obtain the favorable conditions for EPS yield and microorganism growth in a chemically defined medium and found that carbon nitrogen ratio (C/N ratio), cultivation time and temperature had the most significant influences. Box–Behnken experimental design and response surface methodology (RSM) were adopted to further study the interactive effects of these three variables on EPS yield and cells growth. The optimal culture conditions for EPS production were: C/N ratio 9.090, cultivation time 60.67 h and temperature 29.2°C. In these conditions, the maximum EPS yield was 39.0736 mg/ml which was 4 times more than the original yield. But the optimal conditions for cell growth were: C/N ratio 8.643, 58.75 h and 31.9°C, logarithm value of viable cell count reached 7.921. These predicted values were also verified by validation experiments.

Key words: *Lactobacillus paracasei*, exopolysaccharides, chemically defined medium, Box-Behnken design.

INTRODUCTION

Several lactic acid bacteria (LAB) produce exopoly-saccharides (EPS) that are secreted into the growth media. Since 1990, several structural studies and functionality of EPS produced by different strains of LAB have been reported. And some of lactobacilli and bifidobacteria used as the probiotic bacteria produce EPS. In fact, it has been suggested that the health-promoting effect of EPS-producing strains are related to the biological activities of these biopolymers. EPS might contribute to human health

as prebiotics or due to anti-tumor, anti-ulcer, immuno-modulating or cholesterol-lowering activities (De Vuyst et al., 2001; Ruas-Madiedo et al., 2002). The food industry is interested in EPS producing food grade organisms such as lactic acid bacteria. EPS produced by lactic acid bacteria can not only be applied as natural additives but can also be produced *in situ*.

Most bacteria produce EPS under all conditions, but the quantities and the composition of EPS are strain dependent and affected by the nutritional and environmental conditions. So it is possible to increase the polymer production by manipulating the culture conditions (Looijesteijn 1999). In fact, mesophilic strains seem to produce maximum levels of EPS in suboptimal conditions for the bacterial growth, whereas EPS production appears to be growth associated in thermophilic strains (Degeest, 2001).

LAB is fastidious microorganisms requiring rich media

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like milk, whey ultrafiltrate or complex synthetic media such as MRS or M17 for growth. Among them, milk has been the most common culture medium used for EPS production, however, the isolation of poly-saccharides from fermented milk is tedious and includes the risk of degrading the polymer during manipulation (Cerning et al., 1992). When employing complex media, EPS are more easily isolated than in milk but they are heavily contaminated with interfering components (such as yeast and beef extracts, tryptone and peptone), which lead to an overestimation of the biopolymer produced and erroneous determinations of the EPS chemical composition (De Vuyst and Degeest 1999).

Based on the chemi-cally defined medium (CDM), the production of EPS was evaluated accurately and the metabolic flux changes of polysaccharide biosynthesis was clear to exploit. Moreover, EPS purified from fermentation broth will be more convenient and low-lost, and the residual media in broth were easy to be discarded by dialysis method. In this work, a CDM for cell growth and EPS production of *L. paracasei* was investigated for the first time.

Moreover, without the interaction of media components, EPS collected from the CDM was more proper to carry out in our further structural analysis. Response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models, searching optimum conditions of factors for desirable responses and evaluating the relative significance of several affecting factors even in the presence of complex interactions. The RSM has been recently used on modeling and optimization of bioprocesses such as fermentation media, cultivation and process conditions (Yao et al., 2009; Ruchi et al., 2008), enzyme production (Mohana et al., 2008; Burkert et al., 2004) and extracellular polysaccharide production of *Oudemansiella radicata* (Zou, 2005).

The aim of this study was to screen high yield EPS-producing strain and analyse the EPS production in a chemical defined medium to better understand the influence of different nutrients and cultivation conditions on the biopolymer yield and the cell growth. In this paper, a pure *Lactobacillus* strain HCT capable of producing a relative large amount of EPS was screened and identified. Single factor experiments were used to determine the optimum range of several parameters. And then the response surface methodology with Box-Behnken design was subsequently applied to determine the effects of significant parameters and their interactions and to identify the optimum values. Finally, the optimum conditions were experimentally validated.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The 60 strains of LAB used in this study to screen the EPS-producing strain were isolated from different sources, such as infant feces, centenarian feces, pig manure, freshwater fishes, microbial ecological agents, villi and shrimps and so on. The strains were analyzed for morphological, cultural and physiological characteristics and detection of EPS production yield in MRS medium with 5% sucrose to screen the strain which has relative higher EPS yield. The further identification like the sugar fermentation profiles and 16S rRNA gene sequencing were performed on the highest EPS yield strain.

For the optimization of the selected strain in the single factor experiments and RSM, it was grown at 37°C in a CDM since complex nutrients like beef extract, peptone and yeast extract containing some carbohydrate-polymer material, which interfere with EPS quantification (Vaningelgem et al., 2004). The CDM contained 50.0 g/L sucrose, 5.0 g/L Na_2HPO_4, 6.0 g/L KH_2PO_4, 2 g/L tri ammonium citrate, 1.0 g/L $MgSO_4 \cdot 7H_2O$ and 10 ml trace elements solution (consisting of 5 g/L $FeSO_4 \cdot 7H_2O$, 2.0 g/L $MnSO_4$, 1.0 g/L $CaCl_2$ and 1.0 g/L $ZnCl_2$ dissoved in 0.1 N HCl solution). The initial pH of the medium was 6.70 and it was sterilized at 115°C for 15 min.

Screening for EPS production

For EPS yield determination, all strains were grown in MRS broth medium containing 5% sucrose. After 24 h of incubation at 37°C, cultures were centrifuged at 8000 g for 10 min to remove cells using TGL-20M centrifuge (Changsha Pingan Instrument, China), 3 volumes of cold anhydrous ethanol were added to 1 volume of culture supernatants and the mixtures were stored overnight at 4°C. After ethanol precipitation and centrifugation, precipitates were resuspended in distilled water to the original volume (Montersino, 2008).

Total amount of EPS was determined by the total carbohydrate content of the precipitates by the phenol-sulfuric acid method using glucose as standard (Dubios et al., 1956). Briefly, 1 ml of EPS sample aliquots were mixed with 1 ml distilled water and 1 ml of 6% phenol aqueous solution and 5 ml of sulfuric acid 95% (v/v) were added quickly. After vigorously mixing, absrbance at 490 nm was measured using 722s spectrophotometer (Lengguang Tech., China). The concentration of EPS was determined in triplicate.

Identification of high-yield EPS-producing strain

The colony morphology of the selected strain was observed on MRS agar plates containing 5% sucrose after incubation at 37°C for 48 h. The cell morphology of it was examined after being cultured in MRS broth medium for 20 h by microscopy (magnification, ×1000). The physiological and biochemical characteristics of the strain were examined using standard procedures (Dong and Cai 2001). Gram staining, catalase-activities and other characteristics were investigated. The utilization of some carbon sources by the strain was performed based on Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986).

Sequence analysis of the 16S rRNA was performed by amplifying the 16S rRNA of the isolate with PCR using primers LPW57 and LPW205 (Woo et al., 2002). The obtained 16S rRNA sequence was manually corrected and aligned using Vector NTI 7.0 and subsequently aligned to sequences in GenBank Database using the BLAST program(http://www.ncbi.nlm.nih.gov/BLAST/). The aligned 16S rRNA sequences of related species were retrieved from the NCBI nucleotide database. The program Clustal X (version 1.8) with default parameters was run for multiple sequence alignment. Phylogenetic and distance analysis of the aligned sequences was performed by the program MEGA (version 3.1). The resulting unrooted tree topologies were evaluated by bootstrap analysis of un-weighted pair group method arithmetic averages clustering algorithm (UPGMA).

Table 1. Level and code of the Box-Behnken experimental design.

Variables	None code	Code	Code levels		
			-1	0	1
C/N ratio	A	x_1	7.0	9.0	11.0
Cultivation time (h)	B	x_2	50.0	60.0	70.0
Cultivation temperature (°C)	C	x_3	25.0	31.0	37.0

Single factor experiments for determining the optimal range of factors

To find the optimal media components and cultivation conditions, the following factors were investigated using CDM with the one-factor-at-a-time method, namely carbon source with replacement of sucrose by glucose, sucrose, lactose, fructose or galactose, glucose concentrations (5, 10, 15, 20 and 25% w/v), carbon to nitrogen ratio (1:1, 3:1, 6:1, 9:1 and 12:1), initial pH (4.30, 5.10, 5.90, 6.70 and 7.50), cultivation temperature (22, 27, 32, 37 and 42°C), and cultivation time (12, 24, 36, 48, 60, 72h). In each experiment, one factor was changed with the other factors remaining constant. The effects of these factors were evaluated by measuring the EPS yield and cell growth, where each experiment was triplicated.

Response surface methodology for optimizing the cultivation conditions and C/N ratio

Response surface methodology (RSM) has been in use for several decades. Therein, Box-Behnken design only has three levels and need fewer experiments, which make it more efficient and easier to arrange and to interpret in comparison with others (Box and Behnken 1960). Therefore, this statistical technique was used in the present study and the range and the levels of the variables investigated in this study were shown in Table 1. The quadratic polynomial model for predicting the optimal point was expressed according to Equation 1:

$$Y_i = c_0 + \sum_{i=1}^{n} a_i x_i + \sum_{j \leq i}^{n} b_{ij} x_i x_j \qquad (1)$$

where Y_i is the predicted response; the c_0 is the intercept term; the a_i values are linear coefficient; the b_{ij} values are quadratic coefficient; x_i and x_j are the level of the independent variables and the subscripts i and j takes values from 1 to the number of variables. In the present study, three variables are involved and hence n takes the value 3. The software Design Expert (Version 6.0.5) was used for experimental design, data analysis and quadratic model building. The optimal fermentation conditions for enhanced yield of EPS and cell growth were obtained by solving the regression equation and also by analyzing the response surface contour plots using the software Origin 8.0.

Analytical procedure and estimation of cell growth and EPS content

The strain was preliminarily inoculated in MRS broth medium for 24

h at 37°C. To avoid carryover of nutrients, cells in MRS broth medium were harvested by centrifugation (8000 g for 10 min at 4°C) using TGL-20M centrifuge (Changsha Pingan Instrument, China), washed twice with sterile 8.5% (w/v) saline solution, centrifugated at 8000 g for 10 min at 4°C, and suspended in the same solution to the original volume. This cell suspension was used to inoculate the different chemical defined media and cultivated in different conditions.

The cell growth was enumerated by the method of viable bacterial plate count. For EPS yield determination, cultures were centrifuged at 8000 g for 10 min at 4°C after incubation, 3 volumes of cold anhydrous ethanol were added to 1 volume of culture supernatants, and the mixtures were stored overnight 4°C. After ethanol precipitation and centrifugation (8000 g for 10 min at 4°C), precipitates were re-suspended in distilled water to the original volume.

To eliminate monosaccharide residues, EPS was membrane dialyzed (cutoff 8000 - 14000 Da) overnight at 4°C against distilled water and added to the initial supernatant volume (Montersino, 2008). And then the EPS content was measured by a phenol-sulfuric acid method using glucose as the standard as mentioned above. The main values and the standard deviation were calculated from the data obtained with triplicate trials. The value of the pH of the culture broth was determined using a digital pH meter (PB-10, Sartorius scientific Instruments, China).

RESULTS AND DISCUSSION

EPS production by different strains of lactic acid bacteria

Different EPS screening methods have been reported for LAB. The visual inspection of bacterial colonies on agar plates is most probably the easiest method, but it is insensitive and indicative. This method is unable to detect LAB strains that produce low amounts of EPS, unless they are very ropy (Smitinont et al., 1999; Vandenberg et al., 1993). In this study, none of these 60 isolates presented a ropy phenotype, but a more apparent mucoid phenotype than the other isolates analyzed. The partial purification of EPS through precipitation with ethanol or acetone and its spectrophotometrical quantification is another quick screening method (Welman et al., 2003). So, a total of 60 LAB strains isolated from different sources were screened for the production of EPS by this method in this study. EPS was produced in a range from 67.12 ± 3.45 to 238.23 ± 6.34 mg/l (Table 2). The highest EPS-producing strain HCT was selected for further analyses.

Table 2. Origin of EPS-producing strain and EPS yield in MRS broth medium containing 5% (w/v) sucrose after inculbation at 37°C for 24 h.

Code NO.	Origin	EPS (mg/L)	Code NO.	Origin	EPS (mg/L)	Code NO.	Origin	EPS (mg/L)
PCT	villi	67.12±3.45	205	centenarian feces	135.08±3.51	Y-38	infant feces	187.57±6.56
Y-33	infant feces	78.08±2.41	SC-2	Silver carp	139.20±4.37	KCT	villi	189.13±2.31
FCT	villi	79.83±7.01	B-4	centenarian feces	149.08±5.61	P-11	Micro-ecological agents	189.44±4.76
ACT	villi	90.56±3.01	W-9	pig manure	149.91±6.58	W-7	pig manure	191.09±5.98
A-9	centenarian feces	92.43±3.31	W-19	pig manure	152.38±7.01	S-2	shrimp	192.74±7.01
GCT	villi	99.52±8.56	Y-48	infant feces	155.67±6.32	Y-37	infant feces	198.50±7.61
J-1	Micro-ecological agents	101.22±2.56	RCT	villi	156.23±4.78	ICT	villi	201.11±3.21
BCT	villi	105.32±4.01	P-12	Micro-ecological agents	157.32±5.86	A-2	centenarian feces	202.62±8.12
Y-46	infant feces	107.83±3.56	P-2	Micro-ecological agents	165.56±5.96	J-21	Micro-ecological agents	203.44±5.98
OCT	villi	110.30±2.89	W-28	pig manure	165.56±6.03	MCT	villi	203.56±3.79
CCT	villi	112.73±4.58	C-16	carp	165.57±5.23	LCT	villi	205.78±7.95
C-18	carp	116.04±3.01	W-2	pig manure	168.03±8.01	P-8	Micro-ecological agents	210.86±4.81
GCT	villi	116.14±2.51	W-1	pig manure	170.25±6.34	QCT	villi	210.89±9.01
B-5	centenarian feces	119.44±3.65	NCT	villi	170.65±5.61	P-1	Micro-ecological agents	211.68±5.34
ECT	villi	191.23±5.61	P-7	Micro-ecological agents	172.15±4.37	P-3	Micro-ecological agents	215.79±6.21
A-22	centenarian feces	122.73±5.02	SCT	villi	172.97±7.32	W-11	pig manure	216.62±8.35
A-2	centenarian feces	124.20±3.58	W-3	pig manure	174.62±4.87	W-25	pig manure	217.56±9.42
DCT	Villi	125.44±3.67	Y-59	infant feces	175.44±5.49	Y-23	infant feces	218.49±3.28
W-6	pig manure	129.32±3.76	W-36	pig manure	176.26±6.03	Y-32	infant feces	219.86±6.62
W-16	pig manure	133.44±2.87	B-6	centenarian feces	182.03±5.96	HCT	centenarian feces	238.23±6.34

Identification of the highest-yield EPS-producing strain HCT

The highest-yield EPS-producing strain HCT was deter-mined to be pure after the visual observation of colonies on solid medium plates that had been transferred for several generations and the microscopic observation of cells (magnification, ×1000) were demonstrated in colonies and cells uniform in morphology. Strain HCT was found to be a gram-positive, rod-shaped, facultative anaerobic, asporogenic and catalase negative bacterium. Strain HCT formed smooth-looking white mucoid colonies after 48 h of incubation at 37°C on MRS agar medium containing 5% sucrose.

The isolate HCT fermented glucose, cellobiose, ribose, mannose, fructose, melezitose, maltose, salicin, amygda-lin, galactose, melibiose, lactose, trehalose and mannitol and not fermented arabinose, raffinose and rhamnose. So, based on carbohydrate fermentation reactions isolate HCT was closely related to *Lactobacillus casei* (95.2% certainty).

But this was confirmed further with 16S rRNA gene sequence analyses. An approximately 1.5 kb 16S rRNA fragment was amplified from the total DNA of strain HCT and partially sequenced. After alignment with other 16S rRNA sequences in GenBank, the similarity to other members of genus *Lactobacillus* was above >88%. The 16S rRNA sequence was further aligned with the corres-ponding sequences from additional strains of species of *Lactobacillus* in Bergey's Manual of Systematic Bacterio-logy, as well as representatives of other constituent taxa of EPS-producing bacteria were retrieved from GenBank. A phylogenetic tree based on all known related species was shown in Figure 1. Phylogenetic analysis revealed that strain HCT was clustered closely with *L. casei* and *L. paracasei*.

L. casei, *L. paracasei* and *Lactobacillus rhamnosus* form a closely related taxonomic group within lactobacilli and these three species are difficult to differentiate using traditional fermentation profiles. The taxonomic position of the *L. casei* group has been the subject of some debate. Bergey's Manual of Systematic Bacteriology lists four subspecies of *L. casei*, reclassified into three species on the basis of DNA homology data (Collins et al., 1989).
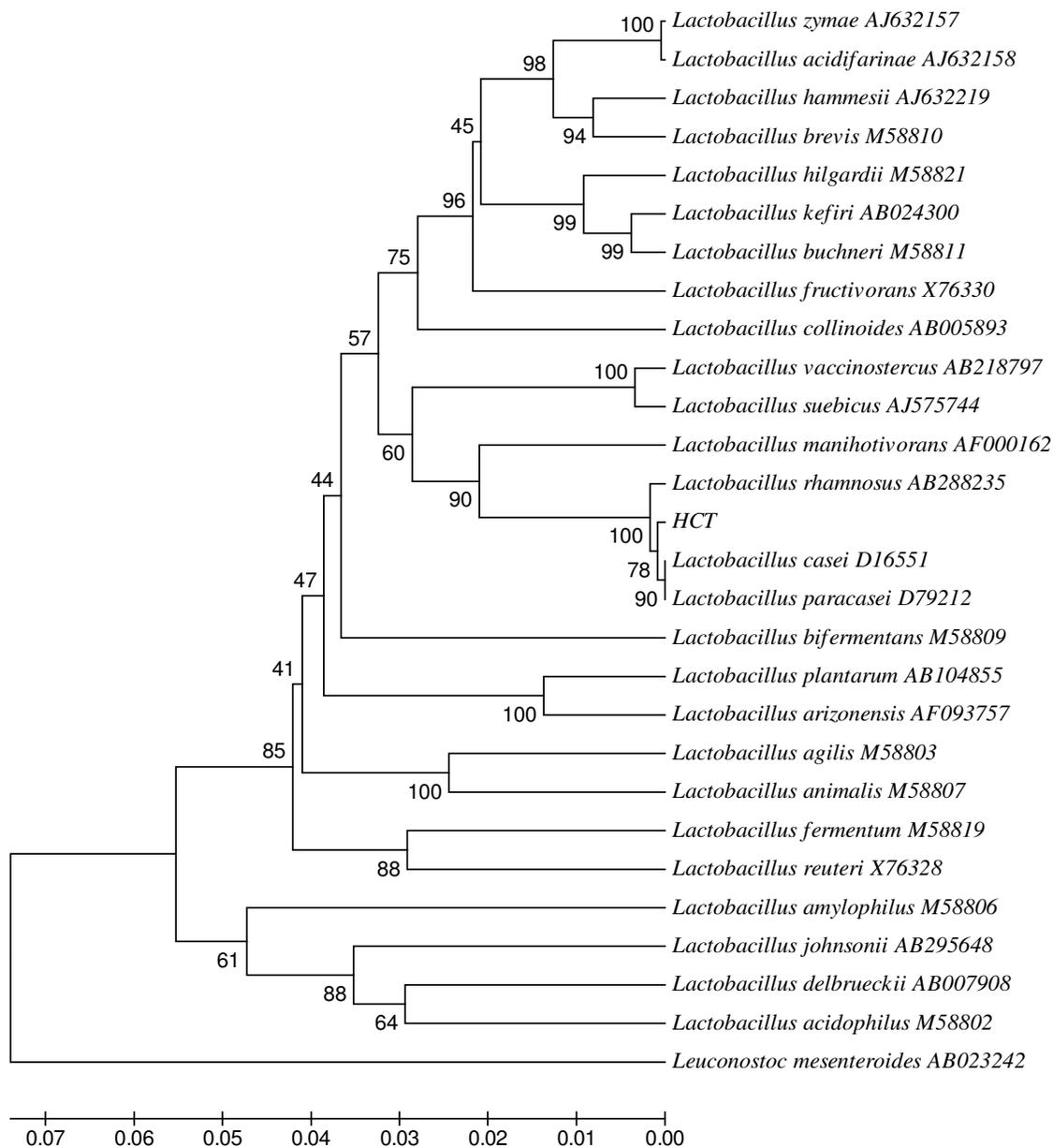
Figure 1. Unrooted tree based on the polygenetic analysis of 16S rRNA sequences showing the position of strain HCT. The tree was evaluated by bootstrap analysis of the un-weighted pair group method, arithmetic averages clustering algorithm UPGMA

Differences in the V1 region of the 16S rRNA were observed for the three *casei*-group species (Klijn et al., 1991). And it had been reported that these sequence signatures from the 16S rRNA allowed differentiation of these species (Mori et al., 1997; Ward et al., 1999). The 16S rRNA gene sequences of *L. casei* (GenBank D16551), *L. paracasei* (GenBank D79212) and the target strain HCT were aligned and the strain HCT had the same sequence of V1 region of 16S rRNA as *L. paracasei* D79212, so it was identified as *L. paracasei*, which was first reported to be an EPS-producing strain.

Single factor experiments to approach the optimum range of three factors

Effect of different carbon source and initial carbon source concentrations on EPS yield

To determine the most suitable carbon source for EPS production, *L. paracasei* HCT was grown in CDM with 5% (w/v) of carbohydrate at 37°C. The effect of carbon source (glucose, lactose, galactose, fructose and sucrose) on cell growth and EPS formation was shown in
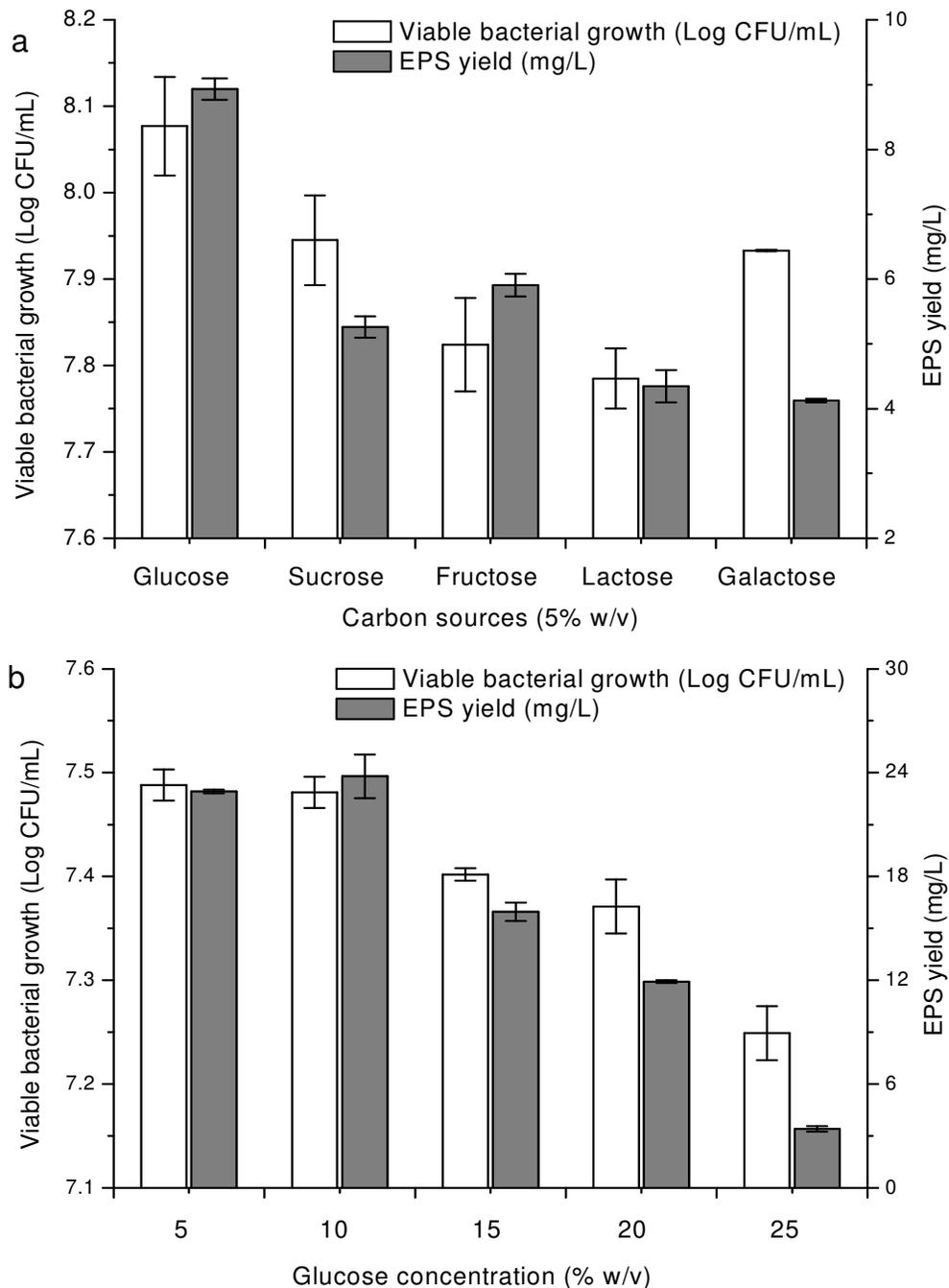
Figure 2. Effect of carbon sources and glucose concentration on EPS production and the cell growth by *L. paracasei* HCT. Data are average of three determinations and standard deviations. a) Different carbon sources at the concentration of 5% w/v. b) Different glucose concentration (% w/v).

Figure 2a. It showed that the carbon source had a marked influence on growth and EPS production by *L. paracasei* HCT and glucose was the best sugar source for cell growth and EPS synthesis. The difference of EPS yield between galactose and lactose was not significant. The EPS production in sucrose was significantly lower than glucose, so the EPS-producing strain should be screened in a medium with mixed carbon sources. Fortunately the strain HCT was the highest EPS-producing strain in sucrose in this study.

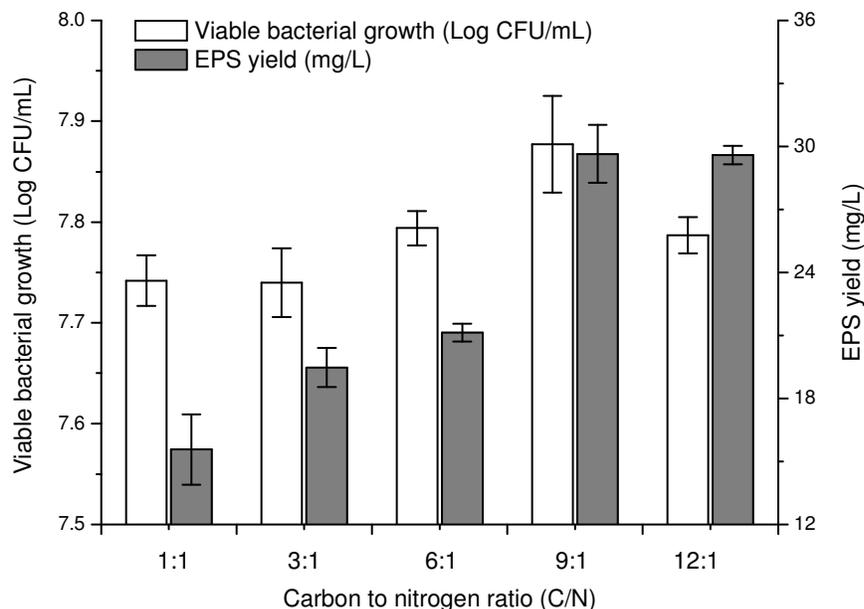The optimum glucose concentration of 10% (w/v) was observed, the corresponding maximum cell growth and

Figure 3. Effect of carbon to nitrogen ratio (C/N) on EPS production and the cell growth by *L. paracasei* HCT. Data are average of three determinations and standard deviations.

EPS yield were 7.481 (log CFU/ml) and 23.7860 mg/L (Figure 2b). A very high glucose concentration resulted in a relatively lower value of both cell growth and EPS yield. The concentration of EPS decreased from 15.9548 mg/L to 3.4024 mg/L when the concentration of glucose increased from 15 to 25% (w/v). The influence of glucose concentration on the cell growth was similar to its effect on EPS production.

Effect of the carbon to nitrogen ratio (C/N) on EPS yield

To study the effect of C/N ratio, the nitrogen concentration of CDM medium was kept constant and the concentration of glucose was varied from 1 to 12% (w/v) and the highest glucose concentration was controlled below the upper limit mentioned above. The C/N ratio is an important factor affecting the biosynthesis of many metabolites. The lower C/N ratio was beneficial for the cell growth, but the EPS yield increased obviously with the increasing C/N ratio, as shown as Figure 3. The results of EPS yield at the C/N ratio of 9.0 and 12.0 were not statistically significant ($p > 0.05$), so the EPS yield achieved a plateau of 29.6498 mg/L, with a relatively lower logarithm value of viable cell count 7.742.

Effect of cultivation conditions on EPS yield

A CDM supplemented with 9% (w/v) of glucose was used to find the optimal cultivation conditions for EPS pro-

duction and cell growth. The influence of cultivation time was studied at 37°C and pH 6.70, and results were shown in Figure 4a. The EPS yield reached a plateau of 30.1108 mg/L at 60 h and the highest cell growth (log CFU/ml = 7.941) was reached at the same time.

The effect of initial pH on growth and EPS formation was examined at 37°C for 60 h (Figure 4b). The highest EPS yield (35.241 mg/L) was found at pH 5.90 while the cell growth (log CFU/ml = 8.065) was the highest at pH 6.70. But the differences of EPS yield at varied pH value were not statistically significant, so it was not further optimized in RSM. To find out the optimal temperature for cell growth and EPS production, *L. paracasei* HCT was cultivated at various temperatures ranging from 22 to 42°C at pH 5.90 for 60 h. The highest concentration of EPS (39.2635 mg/L) was found at 27°C (Figure 4c). At the general growth temperature of 37°C, EPS biosynthesis was slightly lower. The increase of EPS production at low temperature seems to be a common feature for mesophilic EPS-producing LAB, in which suboptimal growth conditions result in improved EPS production (Degeest et al., 2001).

Response surface methodology (RSM)

The optimum ranges of three critical factors (C/N ratio, cultivation temperature and cultivation time) affecting the EPS yield and cell growth were identified by signal factor experiments andselected for further optimization by response surface methodology.
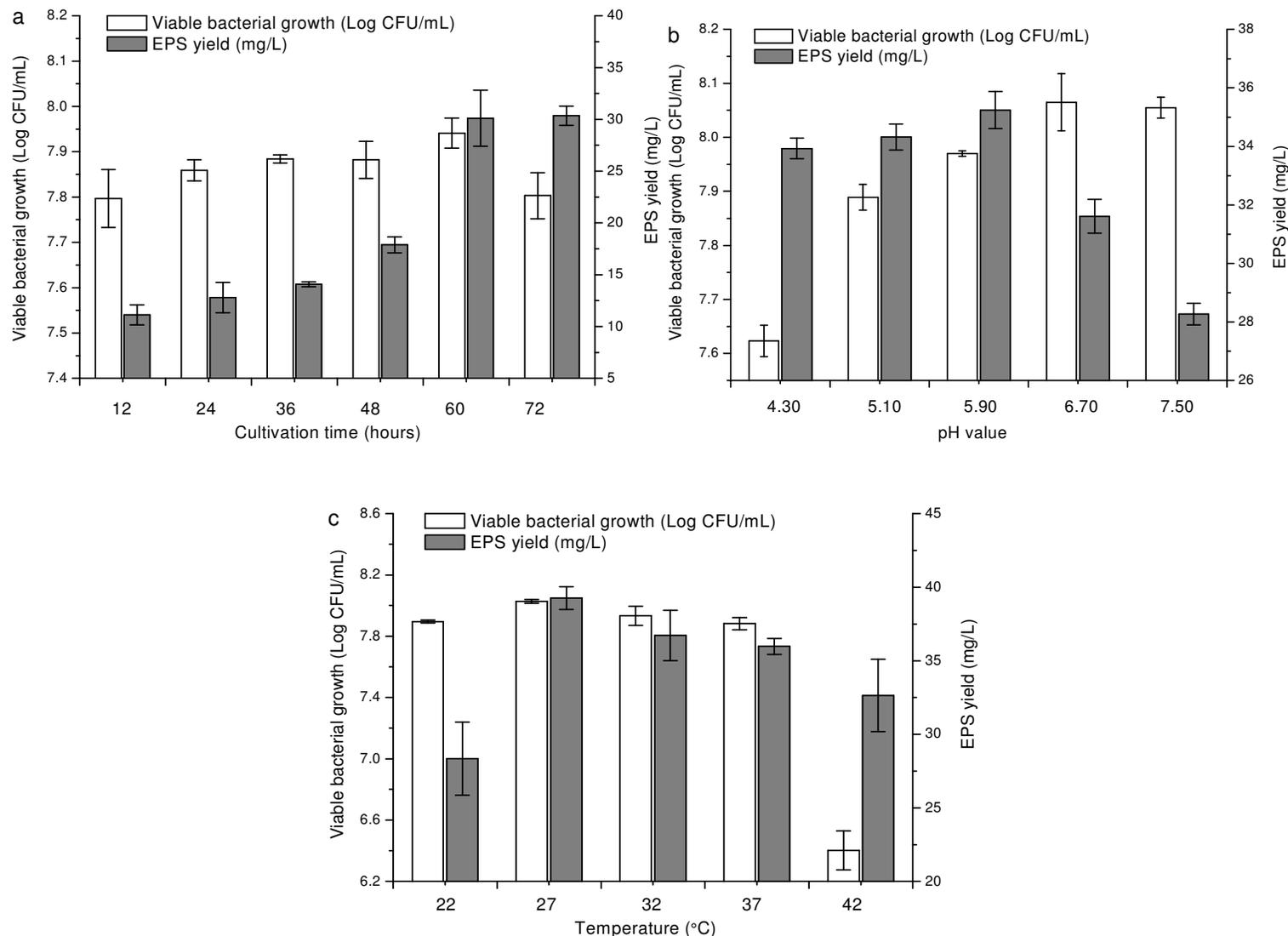
The design matrix of the variables in coded units was

Figure 4. Effect of cultivation conditions on EPS production and the cell growth by *L. paracasei* HCT. Data are average of three determinations and standard deviations. a) Cultivation time, b) Initial pH value of growth media, c) Cultivation temperature.

given in Table 3. Each run was performed in triplicate and thus the values of EPS yield and cell growth were given in Table 3. The predicted values of responses were obtained from quadratic model fitting techniques using the software Design Expert 6.0.5.

The experimental results were modeled with a second-order polynomial equation to explain the dependence of EPS yield and cell growth on the different factors. By applying multiple regression analysis methods, the data obtained were analyzed based on Equation 1, thepredicted response Y_i for EPS yield can be obtained and given as:

$$Y_{EPS}=38.52+0.65x_1-0.25x_2-3.53x_3-7.89x_1^2-4.73x_2^2-5.96x_3^2 \\ +2.69x_1x_2+0.40x_1x_3-2.50x_2x_3 \tag{2}$$

where Y_{EPS}, the yield of EPS (mg/L), are the predicted response variable; x_1 - x_3 are the coded values of the independent variables, viz., C/N ratio, cultivation time and cultivation temperature, respectively.

The statistical significance of Equation 2 was checked by F-test, and the analysis of variance (ANOVA) for response surface quadratic model was summarized in Table 4. The model of EPS yield was very significant, as is evident from the model F-value and a very low probability value ((P_{model}>F) = 0.0001). The value of adjusted determinant coefficient (adj-R^2 = 0.9372) suggested that the total variation of 94% for EPS was attributed to the independent variables and about 7% of the total variation cannot be explained by the model. Here, the value of R (0.9862) indicated good agreement between the experimental and predicted values of EPS.

Table 3. Box-Behnken design matrixes along with the experimental and predicted values of EPS yield and cell growth.

Std.	x_1 (C/N)	x_2 (time)	x_3 (temperature)	EPS yield (mg/L)		Cell growth (Log value)	
				Experimental	predicted	Experimental	predicted
1	-1	-1	0	28.868	28.190	7.903	7.901
2	1	-1	0	23.776	24.100	7.865	7.866
3	-1	1	0	22.628	22.304	7.872	7.872
4	1	1	0	28.309	28.987	7.884	7.887
5	-1	0	-1	26.692	27.950	7.882	7.881
6	1	0	-1	28.192	28.448	7.882	7.878
7	-1	0	1	20.341	20.086	7.892	7.896
8	1	0	1	23.439	22.181	7.878	7.879
9	0	-1	-1	29.695	29.115	7.875	7.878
10	0	1	-1	34.539	33.605	7.881	7.883
11	0	-1	1	26.107	27.040	7.895	7.894
12	0	1	1	20.971	21.550	7.885	7.882
13	0	0	0	37.247	38.322	7.919	7.920
14	0	0	0	37.833	38.322	7.919	7.920
15	0	0	0	40.420	38.322	7.913	7.920
16	0	0	0	40.530	38.322	7.934	7.920
17	0	0	0	36.577	38.322	7.915	7.920

Table 4. Analysis of variance (ANOVA) for the fitted quadratic polynomial model of EPS yield.

Source	Sum of squares	DF	Mean square	*F*-value	Probability (*P*)>*F*
Model	720.57	9	80.06	27.52	0.0001
Lack-of-fit	6.84	3	2.28	0.67	0.6116
Pure error	13.53	4	3.38		
Corrected total	740.93	16			

$R^2 = 0.9725$, adj-$R^2 = 0.9372$, $R = 0.9862$

The value of lack-of-fit for regression Equation 2 was not significant ($P = 0.6116$), indicating that the model equation was adequate for predicting the EPS under any combination of values of the variables. The three-dimensional response surfaces were generated by Origin 8.0 program to directly study the interactions among the three factors tested and to visualize the combined effects of factors on the EPS yield (Figure 5). The response surface plots showed EPS yield by *L. paracasei* HCT as function of two factors, while the other variables set at their zero level.

It was clear from the figures that in the investigated area, the yield of EPS by *L. paracasei* is sensitive to minor alterations of the test variables, that is, temperature, cultivation time and C/N ratio. Especially, the cultivation temperature played an important role in EPS yield ($p = 0.0006$). The shapes of the contour plots, circular or elliptical, also show if the mutual interactions between the variables were significant or not. So, the elliptical contour plots shown in Figure 5 indicated that the mutual interactions between the cultivation time and

temperature ($p = 0.0160$) and between C/N ratio and cultivation time ($p = 0.0222$) are significant. Figure 5 showed the interaction between C/N ratio and cultivation time, it was obvious that the EPS yield of *L. paracasei* HCT increased remarkably as C/N ratio value were increased and cultivation time lasted for longer time, but then it decreased.

The results were in agreement with the single factor tests data. A similar behavior was also observed in case of cultivation time and temperature. The interaction effect between C/N ratio and temperature was not significant ($p = 0.6538$), but the plot and contour showed that optimal conditions for the highest yield of EPS production was in the range. By solving the inverse matrix using Expert Design 6.0.5, the optimal values of the test variables in natural units were C/N ratio = 9.090, cultivation time = 60.67h and 29.2°C. Under these conditions, the maximum predicted yield of EPS produced by *L. paracasei* HCT was about 39.0736 mg/L. From the above analysis, the best value of EPS occurs at low temperature and an appropriate C/N ratio and cultivation time. The second-
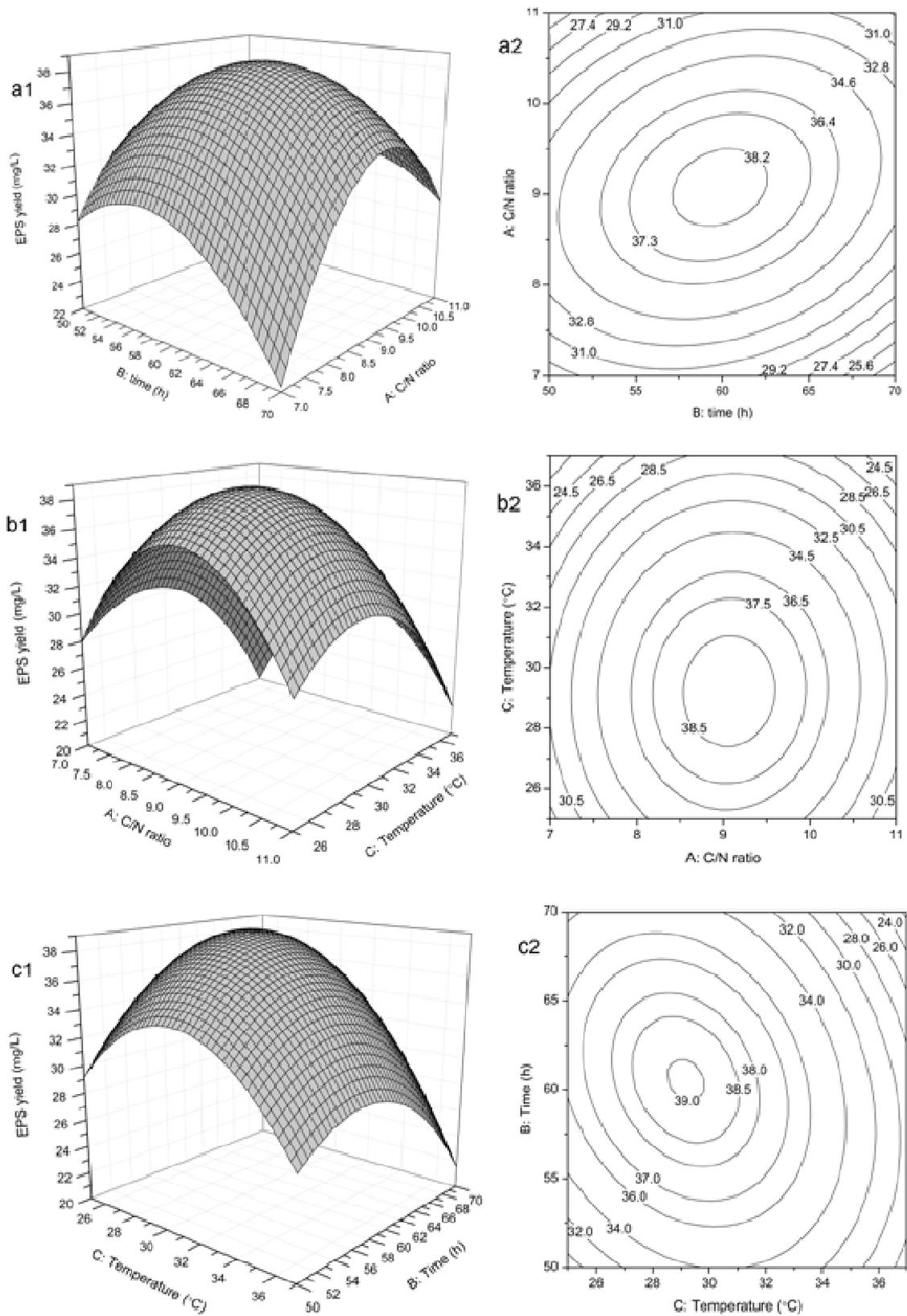
Figure 5. Three-dimensional plots and corresponding contour plots of the effect of three variables on EPS yield. When the effect of two variables was plotted, the other variable was set at central levels. a) C/N ratio and cultivation time. b) C/N ratio and temperature. c) Temperature and time.

Table 5. Analysis of variance (ANOVA) for the fitted quadratic polynomial model of cell growth.

Source	Sum of squares	DF	Mean square	F-value	Probability (P) > F
Model	5.979E-003	9	6.643E-004	13.74	0.0011
Lack-of-fit	6.650E-005	3	2.217E-005	0.33	0.8080
Pure error	2.720E-004	4	6.800E-005		
Corrected total	6.317E-003	16			

R^2 =0.9464, adj- R^2 =0.8775, R =0.9728

Table 6. Model validation experiments.

NO.	x_1 (C/N)	x_2 (time)	x_3 (temperature)	EPS yield (mg/L)		Cell growth (Log value)	
				predicted	Experimental	predicted	Experimental
1	9	60	27	38.227	28.597	7.910	7.996
2	9	60	37	29.028	39.264	7.907	7.917
3	9	70	32	32.369	33.670	7.898	7.854
4	10	70	37	21.447	23.450	7.879	7.589
5	9.1	60.7	29	39.070	40.029	7.917	7.773

order polynomial equation for the cell growth (logarithm value of viable cell count), Y_{cell}, is as follows:

$$Y_{cell} = 7.92 - (5.00E - 03)x_1 - (2.00E - 03)x_2 + (3.75E - 03)x_3 - 0.020x_1^2 - 0.019x_2^2 - 0.017x_3^2 + 0.012x_1x_2 - (3.50E - 03)x_1x_3 - (4.00E - 03)x_2x_3$$

(3)

where Y_{cell} are the predicted response variable; x_1 - x_3 are the coded values of the independent variables the same to Equation 2.

The statistical significance of the second-order model, Equation 3 was checked by F-test (ANOVA) and data shown in Table 5. The model F-value of 13.74 implied the model is significant. And there was only 0.11% chances that a "model F-value" this large, could occur due to noise. The fit-value, adj-R^2 of the second-order polynomial prediction (Equation 3) is 0.8775, indicating that the variability of approximately 94% for cell growth could be explained by the fitted model. The values of correlation coefficient R (0.9728) and determinant coefficient R^2 (0.9464) showed a close agreement between the experimental results and the theoretical values predicted by the polynomial model (Pujari, 2000). The value of lack-of-fit for regression Equation 3 is not significant (p = 0.8080), indicating that there is only 0.11% chance that a "model F-value" this large, could occur due to noise. The fitted response surface for cell growth by the above empirical model was generated using Origin 8.0 software and given in Figure 6. Figure 6a showed the effect of interaction of C/N ratio and cultivation time on the cell growth at fixed temperature 31°C (central point). It is evident that the cell growth of the strain HCT is sensitive to minor

alterations of C/N ratio. The cell growth significantly increased on increasing C/N ratio and cultivation time, but then decreased sharply probably the high osmotic stress coursed by high glucose concentration. However, the effect of cultivation temperature on the growth of this strain is insensitive within the tested range.

By solving the inverse matrix using Expert Design 6.0.5, the optimal values of the test variables in natural units were C/N ratio = 8.643, cultivation time = 58.75 h and 31.9°C. Under these conditions, the maximum predicted logarithm value of viable cell count was about 7.921. And the optimal conditions for both of EPS yield and cell growth were C/N ratio = 8.916, cultivation time = 59.60 h and 30.5°C. Under these conditions, the maximum predicted logarithm value of viable cell count was about 7.920 and EPS yield 38.7498 mg/L.

Validation of the models

In order to validate the adequacy of the model equations, a total of five verification experiments were carried out under various conditions (within the experimental range). Table 6 presented the design matrix of the independent variables in natural units along with the experimental results and theoretical values predicted by Equation 2 for EPS yield and Equation 3 for cell growth, respectively. The validation data of both were separately analyzed by using the SPSS software (Version 11.5). The correlation coefficients (R) between the experimental and predicted values are 0.9926 for EPS yield and 0.9930 for cell growth. The results of analysis indicated that the two groups of experimental values were in good agreement
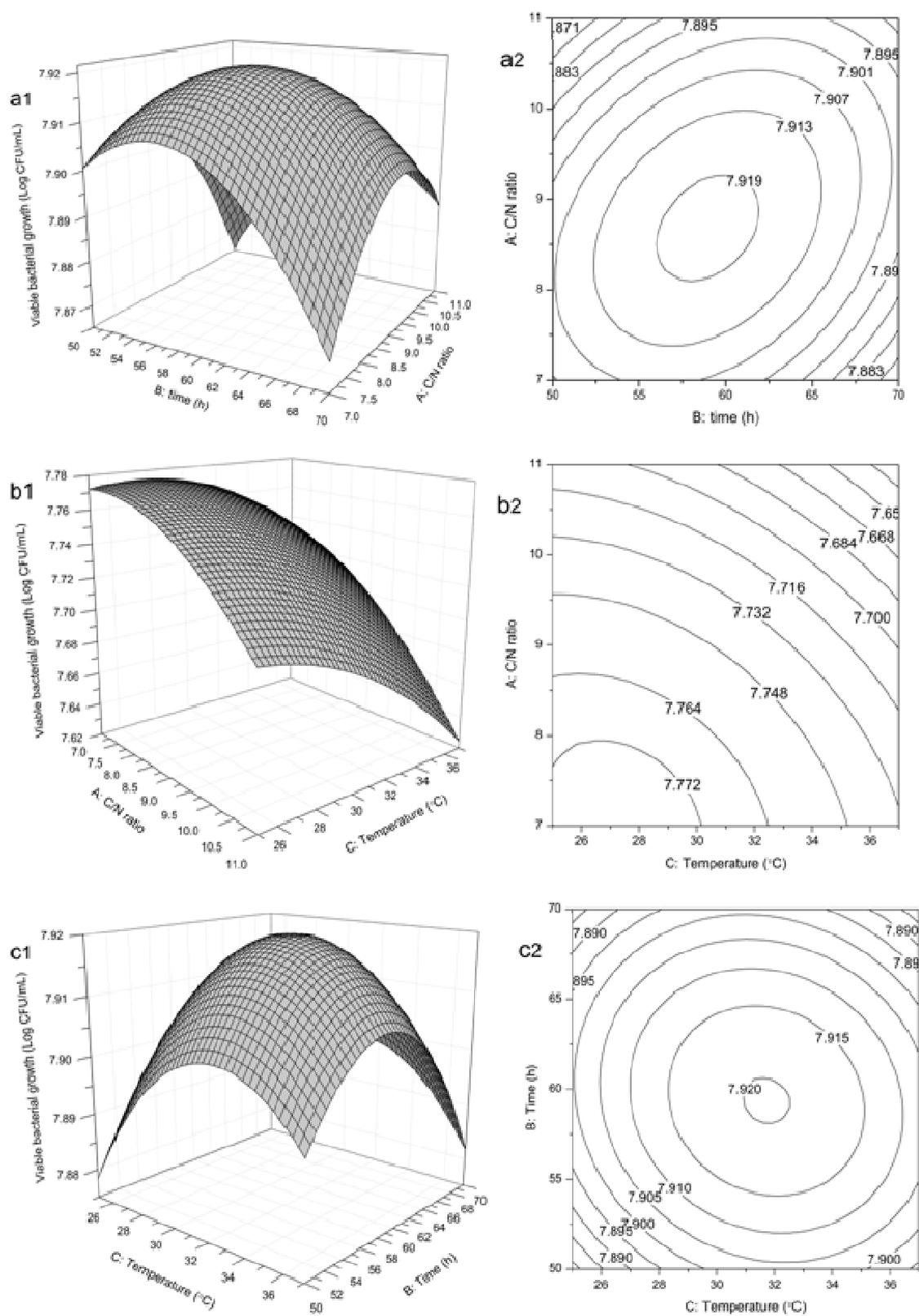
Figure 6. Three-dimensional plots and corresponding contour plots of the effect of three variables on cell growth. When the effect of two variables was plotted, the other variable was set at central levels. a) C/N ratio and cultivation time. b) C/N ratio and temperature. c) Temperature and time.

with the predicted ones, and also suggested that the models of Equations 2 and 3 are satisfactory and accurate.

Conclusion

A pure bacterial strain capable of producing EPS from Bama centenarian feces was isolated and identified as *L. paracasei* HCT by carbohydrate fermentation profile and sequence analysis of 16S rRNA. The three important parameters (C/N ratio, cultivation time and temperature) had significant positive effects on the EPS production and cell growth.

The optimum values of these three variables were identified by a three-factor-three-level Box-Behnken Design and response surface analysis. The results indicated a novel EPS-producing strain *L. paracasei* HCT and illustrated the effect of different nutrients and cultivation conditions on its EPS production and cell growth. Moreover, the work built up a proper model to optimize the EPS yield and cell growth of *L. paracasei* HCT by response surface methodology using Box- Behnken design.

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