Original Research

Statistical Optimization of Medium for Enhanced Production of Exopolysaccharide by Marine Bacteria *Enterobacter cloacae* VHP-34

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Abstract

Response surface methodology was used to optimize the composition of medium for exopolysaccharide production by Enterobacter cloacae *VHP-34, a gram-negative coliform bacteria isolated from the costal area of Umargam (20.1738° N-72.7640° E), Gujarat. Using Central Composite Design (CCD) and ANOVA analysis, the optimum values of the tested variables were found to be glucose 10.0%, yeast extract 2.0%, peptone 0.2%, and NaCl 5.0%. This combination yielded exopolysaccharide production of 39.4 g/L, a 1.21-fold increase, by statistical optimization compared with manual method.*

Keywords: *Enterobacter cloacae*, CCD, ANOVA, Response Surface Methodology, coliform

Introduction

Most extracellular polymers produced by bacteria are polysaccharides, although some bacteria produce capsules made of polypeptides of organic compound residues. The mass and even the composition of these extracellular polysaccharides can vary depending on culture conditions.[1] Distinct physical-chemical properties of polysaccharides, such as water retention capacity, rheology and film-forming capacity, allow their application in several industries.[2]

Carbohydrates are among the most commonly used substrates for microbial cultivation. In fact, in many industrial bioprocesses for the assembly of microbial exopolysaccharides, like xanthan, gellan, and pullulan, sugars like glucose or sucrose are used as carbon sources because they enable prime productivities and yields.[3] Although diverse microbial exopolysaccharide (EPS) have been studied in recent years, little information is available regarding potentials for hyper production. Some microbial EPS producers and related fermentation strategies and genetic tools have been adopted to reinforce EPS yields. Features of the complex EPS production process can also be varied to extend polymer yields. These include medium composition, temperature, pH and aeration. Several studies and methods are also being developed to intervene at the genome level to increase EPS productivity.[4]

Optimization of fermentation conditions can be administered by conventional and statistical techniques. The traditional approach follows the 'one by one technique' during which one variable is modified while all others are kept constant. This approach is time-consuming and provides no information on interaction influences on overall productivity. It also leads to incomplete understanding of the systems, creating confusion and loss of predictive capability.[5]

Response surface methodology (RSM), a set of statistical techniques for designing experiments, uses quantitative data from appropriate experiments to simultaneously solve multivariate equations. The process includes building models, evaluating results, and analyzing optimum conditions for desired responses.[6,7] A key objective to RSM is determining optimum settings of the control variables that result in a maximum (or a minimum) response over a particular variable of interest.[8]

Materials and Methods

MICROORGANISM AND MEDIUM

Bacterium used throughout this study was isolated from the Umargam beach of Gujarat, India. The medium used for the isolation and screening was GYEA broth (medium composition: 1% Peptone, 1% Yeast Extract, 3.5% NaCl, 5% Glucose, 3% agar; pH 7). Previous studies support the same medium for the growth of *Bacillus subtilis,* which was maintained on a GYE solid medium containing (%, w/v) NaCl (4.45), peptone (0.5), yeast extract (1.0), glucose (0.1) and granulated agar (2.0).[9] DNA was isolated from the culture. The quality of DNA was evaluated on 1.0% agarose gel, and a single band of high-molecular weight DNA was observed. A 16s fragment of rRNA gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on agarose. The PCR amplicon was purified by column purification to removecontaminants. DNA sequencing reaction of PCR amplicon was carried out with 357F & 1391R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl GeneticAnalyzer. The 16S rRNA sequence was used to carry out BLAST with the database of NCBI GenBank. Phylogenetic analyses of the aligned sequences were performed with the help of program MEGA7.[10]

QUANTIFICATION OF EPS BY SOLVENT PRECIPITATION

Fermented broth was centrifuged (Centrifuge REMI Model R-24) at 10,000 rpm for 20 min and supernatant was collected in

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centrifuge tube and mixed with double volume of chilled acetone (RANKEM, 99% pure). EPS can be precipitated with this method. The solvents reduce the dielectric constant of the medium, which reduces the solubility of EPS and results in precipitation.[11]

OPTIMIZATION OF PHYSICAL PARAMETERS

Isolation of VHP-34 *Enterobacter cloacae* was optimized according to the following physical paramaters:

Effect of incubation time on EPS production. At various incubation times, starting from 24 h to 120 h, fermented broth was suspended and centrifuged. Supernatant was collected mixed with chilled acetone to measure extracted EPS. Study proved that the highest EPS yield (0.22%) was obtained after 72 h for *Enterobacter cloacae* WD7 in a basal medium.[1]

Effect of inoculum size on EPS production. Bacterial cell number is an effective parameter in EPS production.[12] Different concentrations of inoculum of VHP-34 ranging from 2% to 20% v/v (2.6×10^6 cells/mL) was inoculated in GYE broth. At 72 h, fermented broth was suspended in centrifuge tubes. Supernatant was collected and mixed with chilled acetone to measure extracted EPS.

Effect of pH on EPS production. GYE broths at pH 5, 6, 7, 8 and 9 were inoculated with VHP-34 and withdrawn at 72 h and centrifuged. Supernatant was collected and mixed with chilled acetone to measure extracted EPS. Studies have suggested that maximum EPS production in many organisms is observed in the range of pH 5 to 7.[13,14]

Effect of temperature on EPS production. GYE broths were inoculated with VHP-34 and incubated at 25, 30, 35 and 45°C. Fermented broth was withdrawn at 72 h and centrifuged. Supernatant was collected and mixed with chilled acetone to measure extracted EPS. Previous work suggested that using higher or lower temperatures rather than the optimum growth temperature of the bacterium (30°C) significantly decreases EPS production yield.[12]

Effect of carbon sources on EPS production. Carbon source is one of the most important factors affecting EPS production.[15] Medium was supplemented with different carbon sources, including glucose, sucrose, lactose and manitol. Fermented broth was withdrawn at 72 h and centrifuged. Supernatant was collected and mixed with chilled acetone to measure extracted EPS.

Effect of nitrogen sources on EPS production. Medium was supplemented organic nitrogen sources like meat extract, beef extract, yeast extract, peptone as well as inorganic nitrogen sources such as KNO_2, and KNO_3. Fermented broth was withdrawn at 72 h and centrifuged. Supernatant was collected and mixed with chilled acetone to measure extracted EPS. It has been reported that organic nitrogen sources are absorbed by cells easier than inorganic sources.[16,17]

Relative viscosity of fermented broth. To support EPS study, culture media of *Lactobacillus fermentum* collected at 0, 8, 16, 24, 32, 48, 96, and 144 h of cultivation were measured using a VT-03F rotational viscometer. Furthermore, aqueous solutions of HePS48hr and HePS144hr were dissolved in MRS broth to give a polysaccharide concentration of 0.02% (w/v) for viscosity measurement.[18]

STATISTICAL ANALYSIS

The statistical experimental design was implemented to optimize the EPS by RSM. Central Composite Design (CCD) matrix was used for RSM. For this experiment, peptone (A), yeast extract (B), NaCl (C) and glucose (D) were set as continuous factors. The response, EPS production (R1), was optimized using response surface methodology in Design Expert software trial version 13.0.2.0. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) in case of model fitness.The quality of the polynomial model equation was judged statistically by the coefficient of determination, and its statistical significance was determined by theF-test.[19]

Results and Discussion

Enterobacter cloacae VHP-34 (GenBank, Accession No: MW492544) is a potent exopolysaccharide producer bacterium identified by 16s rRNA Sequencing method.[10]

OPTIMIZATION WITH PHYSICAL PARAMETERS

Effect of incubation time on EPS production. VHP-34 was inoculated in GYE fermented broth at different intervals. 72 h was the optimal incubation time (*Fig. 1*), as the isolate gave 32.5 g/L of dry EPS by extraction with chilled acetone. Mikhlid et al. found that an incubation period of 72 h was optimal for the selected isolate *Enterobacter* sp.to achieve the highest EPS yield.[20]

Effect of inoculum size on EPS production. 2.6×10^6 cells/mL was inoculated in GYE fermented broth with different concentrations of inoculum. 2% inoculum concentration isolate gave 32.3g/L of dry EPS (*Fig. 1*) by extraction with chilled acetone. Previous work showed that inoculam concentration for EPS production was higher in populations of 10^6 and 10^7 compared to10^8.[1]

Effect of pH on EPS production. VHP-34 was inoculated in GYE fermented broth at varied pH. An ideal pH for this isolate was 7 (*Fig. 1*), which yielded 32.1 g/L of dry EPS by extraction with chilled acetone. Previous work by Torres et al. Showed that EPS productivity of *Enterobacter* A47 increased in at pH range 6.0–8.0.[21]

Effect of temperature on EPS production. Inoculated GYE broth was set at different temperatures. An incubation tempertaure of 37°C (*Fig. 1*) was found to be optimal at inducing maximum dry EPS of 31.7g/L when extraction via chilled acetone. Torres et al. demonstrated that EPS productivity of *Enterobacter* A47 increased in the temperature range 25°C–37°C.[21]

Effect of carbon sources on EPS production. GYE fermented broth was employed with different carbon sources. Glucose

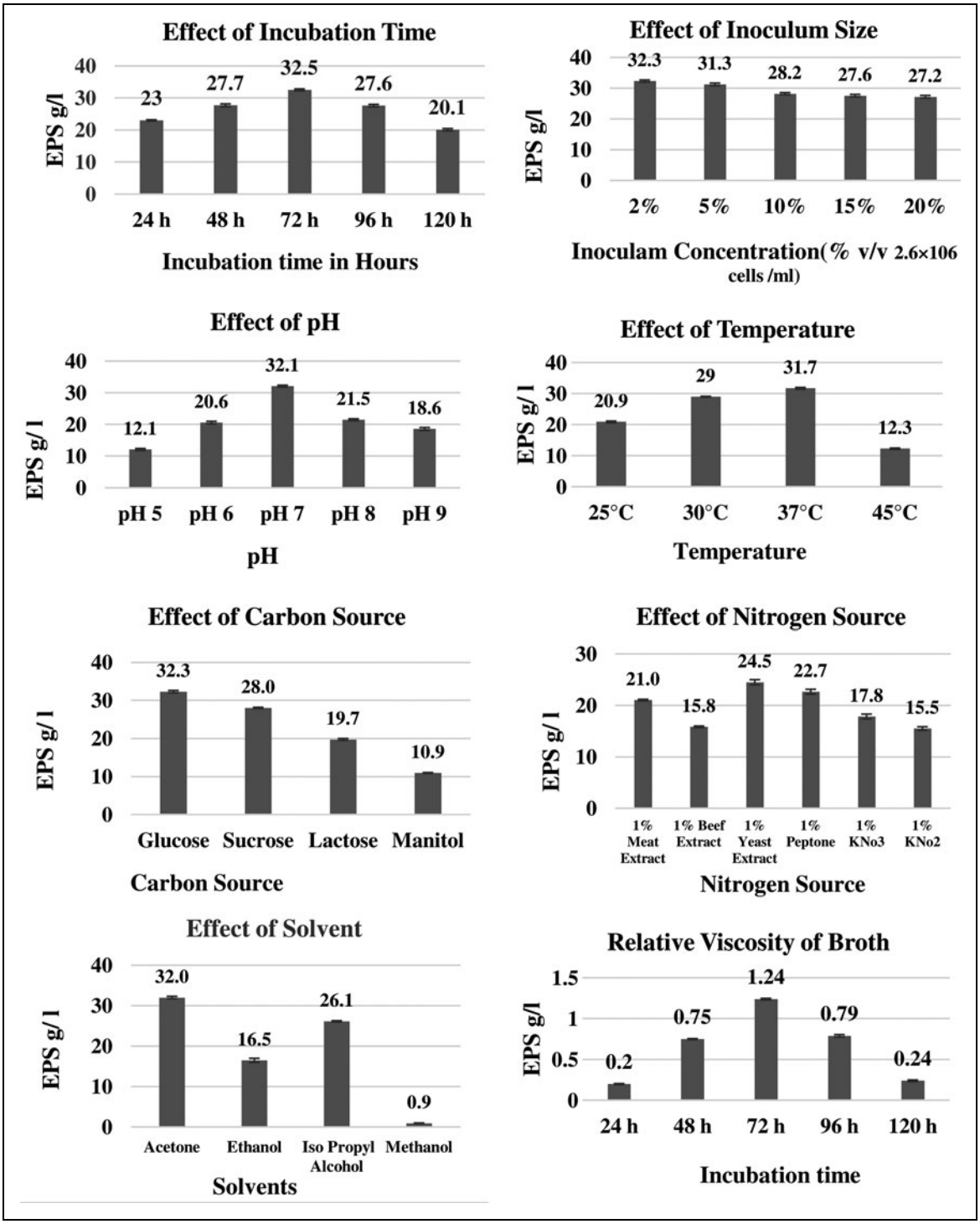

Fig. 1. Effect of incubation time, inoculum size, pH, temperature, carbon source, nitrogen source, different solvent and relative viscosity on EPS production by VHP-34.

Table 1. Experimental Design and Results of the Central Composite Design for EPS Production by VHP-34

RUN	FACTOR 1 A: PEPTONE (gm/L)	FACTOR 2 B: YEAST EXTRACT (gm/L)	FACTOR 3 C: NACL (gm/L)	FACTOR 4 D: GLUCOSE (gm/L)	ACTUAL VALUE RESPONSE 1 EPS (gm/L)	PREDICTED VALUE EPS (gm/L)
1	2	2	0.5	10	14.00	12.51
2	0.2	0.2	5	10	21.30	23.23
3	2	2	5	10	38.00	33.66
4	2	2	0.5	1	8.45	10.20
5	2	0.2	5	1	16.40	19.82
6	2	2	5	1	22.40	24.44
7	1.1	1.1	2.75	5.5	19.40	19.05
8	-0.7	1.1	2.75	5.5	18.50	15.83
9	0.2	2	5	1	20.20	20.11
10	1.1	1.1	7.25	5.5	29.00	29.36
11	1.1	1.1	2.75	5.5	20.00	19.05
12	0.2	2	5	10	39.40	39.77
13	1.1	1.1	-1.75	5.5	13.20	8.74
14	2	1.1	2.75	14.5	19.40	26.94
15	2	0.2	5	10	34.40	30.07
16	1.1	0.2	0.5	1	5.00	9.34
17	2	1.1	2.75	5.5	22.00	20.66
18	0.2	0.2	0.5	10	18.10	16.85
19	1.1	2	0.5	10	18.50	17.94
20	1.1	2.9	2.75	5.5	21.30	24.61
21	1.1	1.1	2.75	-3.5	12.80	7.55
22	0.2	1.1	2.75	5.5	17.20	17.44
23	0.2	0.2	0.5	1	1.0000	3.07
24	0.2	2	0.5	1	7.50	10.60
25	0.2	0.2	0.5	10	15.10	16.85
26	1.1	0.2	5	1	10.10	11.18
27	1.1	1.1	2.75	5.5	21.30	19.05
28	1.1	-0.7	2.75	5.5	14.30	13.49
29	1.1	1.1	2.75	5.5	22.00	19.05
30	2.9	1.1	2.75	5.5	22.50	22.27

showed the highest production (*Fig. 1*), yielding 32.3 g/L of dry EPS by extraction with chilled acetone. A study carried out by Mikhlid et al. using same bacterium achieved highest EPS production with 15% sucrose (EPS 7.8 g/L), followed by 3% glucose as a control (EPS 5.51 g/L) and 10% fructose (EPS 4.28 g/L).[20]

Effect of nitrogen sources on EPS production. GYE fermented broth was employed with different nitrogen sources. Peptone and yeast extract (*Fig. 1*) were the simplest nitrogen sources and yielded 24.5g/L and 22.7g/L, respectively, of dry EPS by extraction with chilled acetone. Work by Prasertsan et al. reported similar results where the addition of organic nitrogen

Table 2. Analysis of Variance (ANOVA) Fitted for 2FI Model for EPS Production by VHP-34

SOURCE	SUM OF SQUARES	DEGREE OF FREEDOM	MEAN SQUARE	F-VALUE	P-VALUE
Model	1920.10	10	192.01	15.12	< 0.0001
A-Peptone	49.75	1	49.75	3.92	0.0625
B-Yeast Extract	157.65	1	157.65	12.41	0.0023
C-NaCl	544.64	1	544.64	42.87	< 0.0001
D-Glucose	730.00	1	730.00	57.47	< 0.0001
AB	99.52	1	99.52	7.83	0.0114
AC	14.37	1	14.37	1.13	0.3009
AD	91.99	1	91.99	7.24	0.0145
BC	80.80	1	80.80	6.36	0.0208
BD	0.9716	1	0.9716	0.0765	0.7851
CD	45.32	1	45.32	3.57	0.0743
Residual	241.36	19	12.70		
Lack of Fit	232.63	15	15.51	7.11	0.0356
Pure Error	8.73	4	2.18		
Cor Total	2161.46	29			

(polypeptone) was found to influence growth of *Enterobacter cloacae* WD7, with the highest value in the presence of 0.3% polypeptone (OD_{660} of 2.67).[1]

Effect of various solvents for EPS extraction. After 72 h incubation, fermented broth was treated with different solvents for precipitation of EPS. Acetone was the optimal solvent (*Fig. 1*), yielding 32.0 g/L of dry EPS.

Relative viscosity of fermented broth. At different incubation time intervals, fermented broth was checked for relative viscosity and shear rate. 72 h gave the best viscosity of 1.24 cp (*Fig. 1*) in Brookfield Viscometer.

STATISTICAL ANALYSIS BY RESPONSE SURFACE METHODOLOGY

Central Composite Design of RSM was employed to optimize EPS with four significant factors: peptone, yeast extract, NaCl and glucose (*Table 1*). 30 runs showed different predicted and actual values of EPS g/L as a response. This design resulted in actual values from 1.0 g/L to 39.40 g/L of dry EPS. Previous work on EPS production by RSM analysis was used for the whole set of responses, and the second order model showed a good fit ($R^2 > 0.90$), except for the maximum EPS concentration (Y2), rhamnose content (Y8) and acetate content (Y12) responses.[22]

Data of Analysis of Variance (ANOVA) is shown in *Table 2*. The Model F-value of 15.12 implies the model is important. P-values 0.0500 indicate model terms are significant. Factors coded as B, C, D, AB, AD, and BC were significant in model terms, and the dearth of Fit F-value of 7.11 implies the dearth of Fit is important. There was only a 3.56% chance that a scarcity of Fit F-value could occur because of noise.

The Predicted R^2 of 0.6260 isn't as near the Adjusted R^2 of 0.8296 (*Table 3*) as might normally be expected; the difference is just 0.2. This could indicate an oversized block effect or a possible problem with model and/or data. Things to consider include model reduction, response transformation, and outliers. All empirical models should be tested by doing confirmation runs. Adeq Precision measures the signal to noise ratio, and a ratio greater than 4 is desirable. The ratio of 17.007 in this work indicates an adequate signal.

Our stastical data were compared with EPS production by probiotic yeast *Lipomyces starkeyi,* and showed Model F-value of 52.17, implying the model is significant. The total determination of the coefficient ($R^2 = 0.9766$) showed a realistic fit of the model to the experimental data. The adjusted coefficient value (adj $R^2 = 0.9579$) also proved that the model was highly significant with the coefficient of the variation (C.V) (7.59%).[19]

Table 3. Analyzed Values for Response Surface Model

Standard Deviation	3.56	R^2	0.8883
Mean	18.76	Adjusted R^2	0.8296
C.V. %	19	Predicted R^2	0.626
PRESS	808.49	Adeq Precision	17.0067

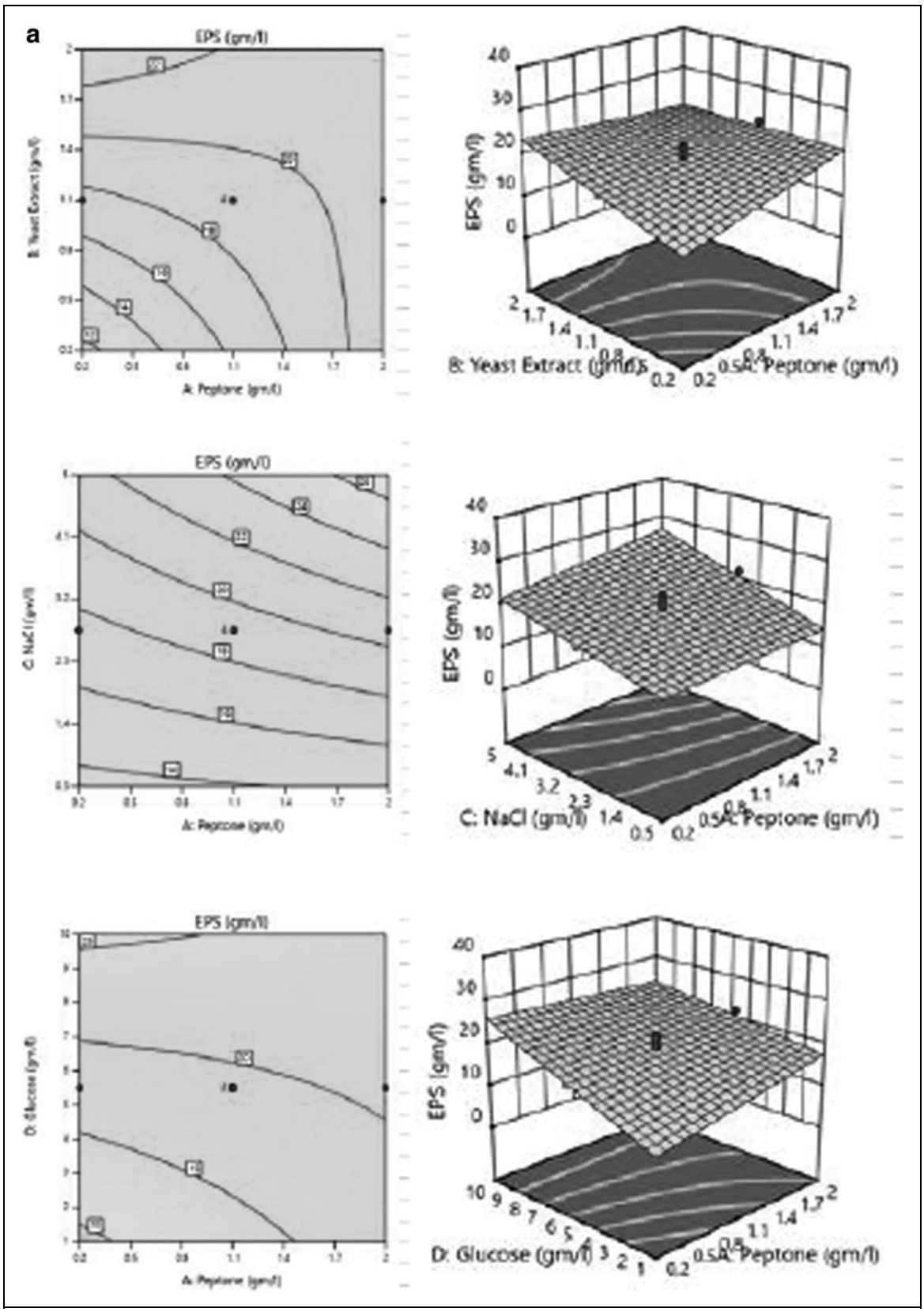

Fig. 2. (a) Contour and 3D graph for significant factors interaction for EPS production by VHP-34; **(b)** Contour and 3D graph for significant factors interaction for EPS production.

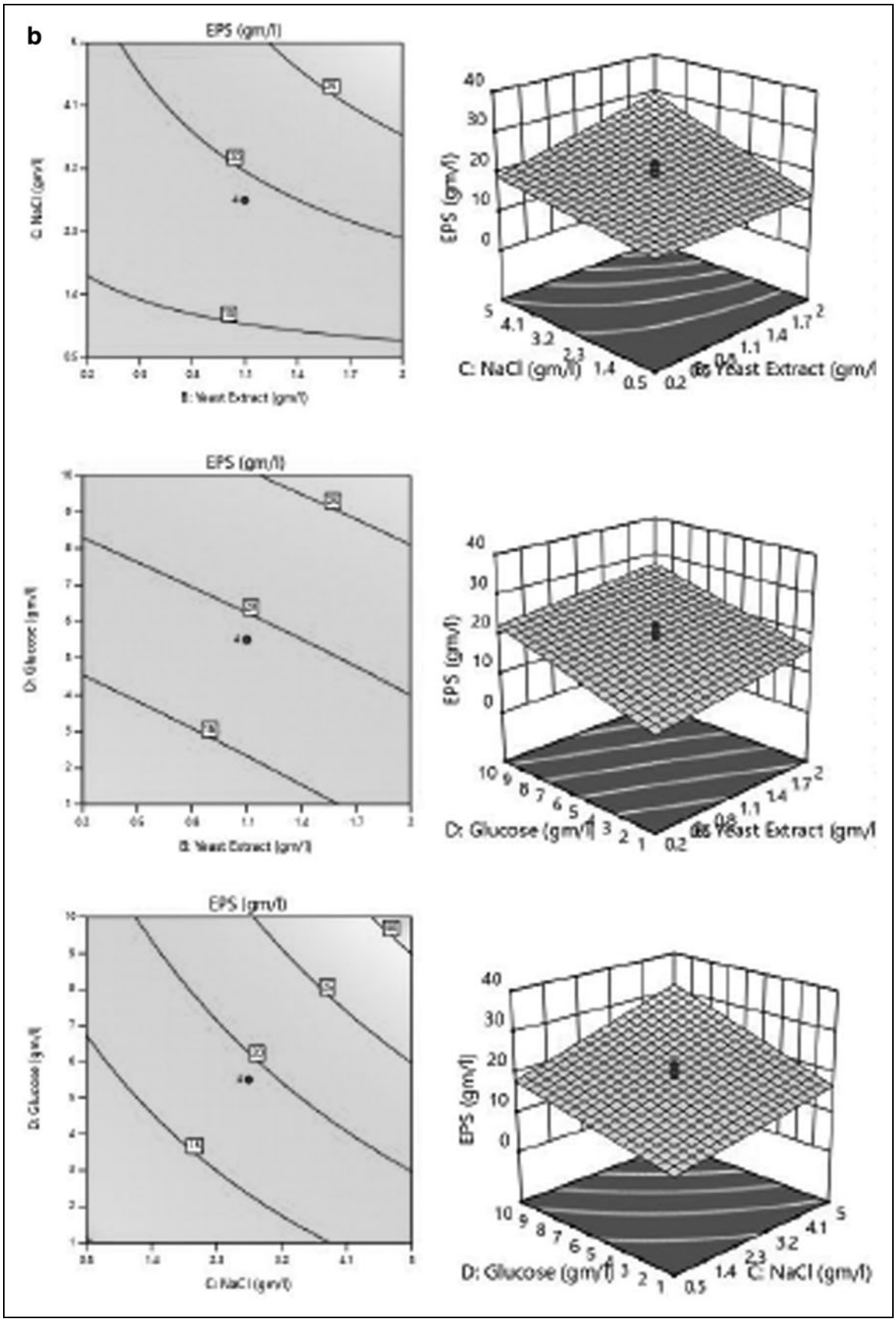

Fig. 2. (*Continued*).

The Final Equation for EPS in Terms of Coded Factors was:

$$EPS = +19.05 + 1.61\ A + 2.78\ B + 5.15\ C + 5.75\ D - 3.24\ AB + 1.18\ AC - 2.61\ AD + 2.51\ BC - 0.2563BD + 1.73\ CD$$

In terms of coded factors, this equation predicts the response for given levels of every factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is helpful for identifying the relative impact of the factors by comparing the factor coefficients.

The graphical representation of the factors in 3D response surface and 2D contour plots show the association between the response and the levels of every factor.[3] The interactive effects of the variables and optimal levels of every variable were determined by plotting the 2D contour and 3D response surface plots. These plots are the function of two factors at a time, and all other factors are maintained at fixed levels. This helps in understanding both the maximum effects and also the interaction effects of any two given factors. The 2D contour and 3D response surface plots were plotted to work out the interactive effects of the variables and the optimal levels of every variable.[22] The interaction between factors peptone, yeast extract, NaCl and glucose for the EPS production are shown in *Fig. 2*, which includes six contours and 3D surface plots for these 4 factors. From these, NaCl-yeast extract, glucose-yeast extract and glucose-NaCl gave the best results for optimum EPS production.

This study is focused on EPS production from *Enterobacter cloacae* VHP-34 isolated from the water and soil of Umargam, Gujarat, India. CCD was used to identify 30 runs with duplicate sets of experiments to provide predicted as well as actual values of EPS production from 1.0 g/L to 39.4 g/L. Optimum physical parameters for EPS production were 72 h incubation period, pH 7, 37°C temperature and optimized media for EPS production were glucose-10.0% (carbon source), yeast extract-2.0% (nitrogen source), peptone-0.2% (nitrogen source), NaCl- 5.0% (salt) and inoculum concentration 2%. EPS production from *Enterobacter cloacae* under the optimized conditions was higher than that under the fundamental substance and initial conditions.

Related studies on same bacterium showed that isolated marine *Enterobacter* sp. ACD2, under optimum medium conditions (sucrose 15%, peptone 0.5%) and all fermentation medium salts will give the best EPS yields (8.6 g/L) when grown at pH 7.5–8.0, incubation temperature 37°C with shaking speed 150 rpm for 72 h.[20] The EPS attained a maximum concentration of 13.28 ± 0.74 g/L after about 4 days of cultivation. There was no further improvement in EPS production during the last 3 days of the cultivation since its concentration within the culture broth remained almost constant. Considering the time of effective EPS production (3–8 days), the productivity was 3.64 g/L/d. In another experiment performed with *Enterobacter* sp. grown on glycerol under different operating conditions, both the ultimate EPS concentration and its productivity were improved. The cultivation for EPS production takes 4–7 days, with a final production of 12.0–18.0 g/L of EPS and maximum productivities of 3.6–4.8 g/L/d.[23]

Conclusion

Response surface method had been proven effective for optimizing exopolysaccharide production by *Enterobacter cloacae* VHP-34. The optimum parameters were glucose-10.0%, yeast extract-2.0%, peptone-0.2%, NaCl-5.0%, inoculum concentration 2%, 72 h incubation, pH 7, and 37°C temperature. This resulted in an overall 1.21-fold increase compared to using the original medium. *Enterobacter cloacae* VHP-34 was isolated from Umargam beach, Gujarat. The beach and surrounding costal area were found to be novel marine site for isolation of EPS-producing bacteria, although this site has not been reported for in other EPS-production studies.

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