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Optimization of extracellular polysaccharide production in *Halobacillus trueperi* AJSK using response surface methodology

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The present study was conducted to optimize the media composition through response surface methodology (RSM) for extracellular polysaccharide (EPS) production in *Halobacillus trueperi* AJSK strain isolated from the salt pan. *Halobacillus trueperi* was identified with morphological, biochemical characteristics as well as 16S rRNA gene sequencing method. Production medium was optimized through central composite design. In the present study, the maximum EPS production was achieved under the optimal culture conditions of peptone, glucose, NaCl and $MgSO_4$ and they were 15.50, 22.24, 61.56 and 2.33 g/L, respectively at pH 9.0, 35°C in 72 h. An EPS production of 12.93 (g/L) which was well in agreement with the predicted value was achieved by this optimized procedure. Results of the present study proved that statistical media composition analysis with RSM enhanced the EPS production in *Halobacillus trueperi.*

Key words: Extracellular polysaccharide, *Halobacillus trueperi,* response surface methodology, salt pan bacteria.

INTRODUCTION

Marine bacteria are known to produce extracellular polysaccharides for their thriving fitness such as adhering purpose and surviving in adverse conditions. Microbial exopolysaccharides (EPS) are a heterogenous matrix of polymers comprised of different biological molecules such as polysaccharides, proteins, nucleic acids, phospholipids and other polymeric compounds thereby carrying different organic functional groups such as acetyl, succinyl or pyruvyl and some inorganic constituent like sulfate (Mishra and Jha, 2013; Nielsen et al., 1999).

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Microbial EPS are commonly present in two forms either in capsular or slime. In capsular form the polysaccharides are closely bound to the cell surface and in slime the polysaccharides are freely associated with the cell surface (Shauna and Reckseidler-Zenteno, 2012; Costerton, 1999). These EPS either remain attached to the cell surfaces or get released into the extracellular medium. Microbial EPS owing to their interesting physico-chemical and rheological properties has a wide range of industrial applications such as the production of textiles, detergents, adhesives, cosmetics, pharmaceuticals, food additives as well as applications in brewing, microbial enhanced oil recovery, wastewater treatment, dredging and various downstream processing processes, cosmetology, pharmacology and as food additives. The EPS also contribute to various physiological activities in human beings as anti-tumor, anti-viral and anti-inflammatory agents and can act as inducers for interferon, platelet aggregation inhibition and colony stimulating factor synthesis (Manivasagam et al., 2013; Lin and Zhang, 2004; Sutherland, 1999).

Optimization of bioprocess plays crucial role in reducing the production cost of all biotechnological commercial products. The optimization process using the 'one variable at a time' approach gives non reliable results and inter-active effects of different variables for the production also cannot be resolved by this approach. Statistical experimental strategies including factorial design and response surface methodology (RSM) are more reliable than classical experiments. Central composite design (CCD) is one of the most conventional experimental designs among different classes of RSM and this strategy particularly helps us to predict the better concentrations of substrates with less accidental errors (Sathiyanarayanan et al., 2013). Statistical optimization methods have been successfully employed for the optimization of EPS production through fermentation process (Fang et al., 2013).

The EPS production depends on several factors such as species employed, cultivation conditions and age of the cultures. The design of fermentation conditions is very vital (Allard and Tazi, 1993). Statistical design of experiments provides an economic and efficient method of optimizing several conditions at a time. Furthermore, the production of EPS is not species specific and each strain of same species may produce different kinds of EPS with different biotechnology properties. In the past decade more prominent research has been done in search for novel microbial EPS and EPS producing strains (Manivasagam et al., 2013). Still the search for EPS among halophilic bacteria and bacteria from saline soils holds to be pristine. In view of that, in this study, we report a strain of *Halobacillus trueperi* isolated from the Tamilnadu Salt and Marine Chemicals (TSMC) salt pan, Tuticorin, India. The production of EPS by *Halobacillus trueperi* AJSK was optimized by classical method followed by statistical experimental design.

MATERIALS AND METHODS

Isolation and identification of EPS producing halophilic bacteria

The soil samples collected from the TSMC salt pan, Tuticorin were brought to the laboratory within 6 h. Serially diluted samples were plated on Zobell marine agar plates (10% NaCl). Potential EPS producing strain was selected by observing for better mucoid colony morphology (Fusconi and Godinho, 2002) and the selected potential EPS producing strain was identified based on morphological and biochemical characteristics according to the Bergey's manual of determinative bacteriology (Garrity et al., 2001) and also confirmed through molecular characterization. Briefly, the bacterial genomic DNA was extracted by phenol chloroform method (Marmur, 1961) and the 16S rRNA gene was amplified by using forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'- GGGCGGTGTGTACAAGGC -3'). PCR was performed under the following conditions; initial denaturation at 95°C for 5 min followed by 35 cycles consisting of, denaturation at 95°C for 30 S, annealing at 55°C for 30 S and followed by final extension of 5 min at 72°C. The 16S rRNA forward and reverse sequences was obtained by an automated DNA sequencer (Megabace, GE) and homology was analyzed with sequences in the Gene Bank by using CLUSTAL X software. The phylogenetic tree was constructed by the neighbor-joining method (Saitou, 1987).

Microbial exopolysaccharides (EPS) analysis

EPS production was carried out with the production media consisting of peptone, 10 g/L; glucose, 15, g/L and NaCl, 75 g/L and the culture was incubated at 28°C, pH 10.0 for 72 h. The EPS were precipitated from the cell free liquid culture by adding two volumes of cold ethanol. Then the precipitates were collected by centrifugation, dissolved in distilled water and the EPS concentrations were determined by phenol-sulfuric acid method using glucose as standard (Dubois et al., 1956).

One-factor-at-a-time experiments

Classical method was used to investigate the EPS production by the strain *H. trueperi* AJSK. EPS production was carried out with the production media consisting of peptone, 10 g/L; glucose, 15, g/L, NaCl 75 g/L and $MgSO_4$ 1.5 (g/L). The time course of experiment was carried out for 72 h in 1 L flask containing 250 ml of

Table 1. Experimental design and EPS results of central composite design optimization experiment.

Run order	Peptone (g/L)	Glucose (g/L)	$MgSO_4$ (g/L)	NaCl (g/L)	EPS producion (g/L)	
					Observed	Predicted
1	10	10	1.0	40	5.1920	5.1859
2	20	10	1.0	40	6.7521	6.8232
3	10	30	1.0	40	6.5422	6.6587
4	20	30	1.0	40	7.8761	7.9104
5	10	10	4.0	40	5.4691	5.7321
6	20	10	4.0	40	6.5732	6.7648
7	10	30	4.0	40	7.1462	6.9673
8	20	30	4.0	40	7.7872	7.6145
9	10	10	1.0	90	5.3210	5.7463
10	20	10	1.0	90	6.3822	6.5946
11	10	30	1.0	90	8.0312	7.8731
12	20	30	1.0	90	8.3462	8.3358
13	10	10	4.0	90	4.0621	4.0614
14	20	10	4.0	90	4.1691	4.3052
15	10	30	4.0	90	5.7691	5.9506
16	20	30	4.0	90	5.7691	5.8088
17	5	20	2.5	65	4.3018	4.1236
18	25	20	2.5	65	5.7271	5.6191
19	15	0	2.5	65	4.3271	3.8239
20	15	40	2.5	65	6.5832	6.8003
21	15	20	0.5	65	6.1654	5.9660
22	15	20	5.5	65	4.0719	3.9852
23	15	20	2.5	15	11.0630	11.0467
24	15	20	2.5	115	10.0713	9.8015
25	15	20	2.5	65	12.1732	12.2035
26	15	20	2.5	65	12.1790	12.2035
27	15	20	2.5	65	12.1807	12.2035
28	15	20	2.5	65	11.9507	12.2035
29	15	20	2.5	65	12.5794	12.2035
30	15	20	2.5	65	12.3811	12.2035
31	15	20	2.5	65	11.9807	12.2035

culture medium. The optimum initial pH for EPS production was determined by adjusting various pH (4 to 11) with 1 M HCl and 1 M NaOH before sterilization and the optimum temperature for EPS production was evaluated by incubating the liquid cultures at various temperatures (20 to 45°C).

Central composite design (CCD)

Physical factor such as pH and temperature was selected by one-factor-at-a-time experiments and chemical factors were selected based upon the available literature, the media ingredients namely peptone, glucose, NaCl and $MgSO_4$ are the significant variables for statistical optimization (Mata et al., 2007; Liu et al., 2011; Nahas et al., 2011; Lu et al., 2011; Cerning et al., 1994). All the above said independent variables were evaluated at five different levels (-2, -1, 0, +1, +2) conducting 31 experiments. The central values of all variable were coded as zero. The minimum and maximum ranges of the variables and the full experimental plan with regard to their values in actual and coded form are presented in Table 1.

The data derived via RSM on production of EPS were analyzed with the analysis of variance (ANOVA). The results of the experiments were subjected to the response surface regression procedure with the given second order polynomial equation:

$$Y = \beta 0 + \sum \beta_i X_i + \sum \beta_{ii} X^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the predicted response, xi and xj are inde-

Table 2. Biochemical characteristics of the strain *H. trueperi* AJSK.

Characteristic	Activity
Indole	-
Methyl red	-
Voges Proskaevuer	-
Citrate	-
Catalase	+
Oxidase	+
Gelatin	+
Casein	-
H_2S	-
Fructose	+
Glucose	+
Maltose	+
Sucrose	+

pendent factors, $\beta 0$ is the intercept, βi is the linear coefficient, βii is the quadratic coefficient and βij is the interaction coefficient. The response values (Y) in each trial were presented as average of the triplicates. The statistical software package 'Minitab' (Version 16.0) was used to analyze the experimental design.

RESULTS AND DISCUSSION

Isolation and identification of *H. trueperi* AJSK

In the present study, a total of eight morphologically different isolates were investigated for potential EPS production. Among these, strain T7 produced significant mucoid colony in the preliminary screening. Furthermore, the strain was identified as *H. trueperi* Gram positive, rod shaped through biochemical characteristics (Table 2) and 16S rRNA analysis. Phylogenetic analysis revealed that the strain T7 belongs to the Firmicutes, Bacillaceae, *Halobacillus*. Evolutionary relation with other *Halobacillus* sp. is explained with the phylogenetic tree created by neighbor joining method (Figure 1). BLAST analysis with NCBI database retrieved a 96% similarity for strain T7 with *H. trueperi*. The 16S rRNA gene sequence of strain T7 was submitted to GenBank as *H. trueperi* AJSK with the accession number KC699491.

Effect of pH and temperature on EPS production

A series of experiments were carried out to study the effects of physical factors such as pH and temperature on EPS production in *H. trueperi* AJSK. Experiments were conducted using basal medium containing peptone 10 (g/L), glucose 15 (g/L) and NaCl 75 (g/L) and $MgSO_4$ 1.5 (g/L) for 72 h. The optimization of pH and temperature for EPS production revealed that pH 9.0 (3.73 g/L) and temperature 35°C (2.98 g/L) were found as optimum culture condition for maximum EPS production (Figures 2 and 3), respectively. The pH is an essential physical factor in EPS biosynthesis that may affect the uptake of various nutrients and EPS biosynthesis (Kim, 2005). Kanekar et al. (2008) has reported that 1.2 g/L of EPS production at an alkaline pH of 10 in *Vagococcus carniphilus* an alkalophilic bacterium isolated from alkaline Lonar Lake, India. *Pseudomonas polymyxa* EJS-3 is also reported to produce EPS at a slightly alkaline pH 8 (Liu, 2009). The present study reveals a good EPS production in an alkaline pH which is an industrially desired property. These results did not comply with the report of maximum EPS production at pH 7 by *P. fluorescens* (Raza et al., 2012). *Bacillus megaterium* RB-05 from the river sediment is reported to produce 0.895 g/L EPS at a neutral pH of 7.0 (Chowdhury et al., 2011). Mata et al. (2011) have reported a maximum EPS production at 32°C in *Alteromonadaceae* sp. a halophilic bacterium which is likely in agreement with the present study. In contrary, Liu et al. (2011) reported maximum production of EPS at 9.8°C from *Zunonwangia profunda*, a deep sea bacterium. Also reports of maximum EPS production at temperatures ranging from 28 to 37°C were reported (Raza et al., 2012; Chowdhury et al., 2011; Kaur et al., 2013).

Optimization of variables using central composite design (CCD)

Four variables such as peptone, glucose, NaCl and $MgSO_4$ were selected based on the results of previous literature reports for the CCD experiments. The values of the response (EPS) obtained under different experimental conditions are given in Table 1. Experiments were done as per the CCD experimental plan. The F value is a measure of variation of the data about the mean. High F value and a very low probability ($p>F=0.00$) indicates that the present model is in a good prediction of the experimental results.

The corresponding analysis of variance (ANOVA) is presented in Table 3. The regression equation is represented in the three-dimensional graphical response surface plots (Figure 4). The interest of using response surface methodology is to efficiently find out the accurate optimum values of the variables, with the maximized response.

The surface plots confirmed that the objective function is unimodal in nature, which shows an optimum in the centre. Also significant P-values (0.000) suggested that the obtained experimental data was a good fit with the model and it is also checked by the determination of coefficient (R^2) with R^2 (multiple correlation coefficient) of 99.52%. The predicted R^2 and the adjusted R^2 was

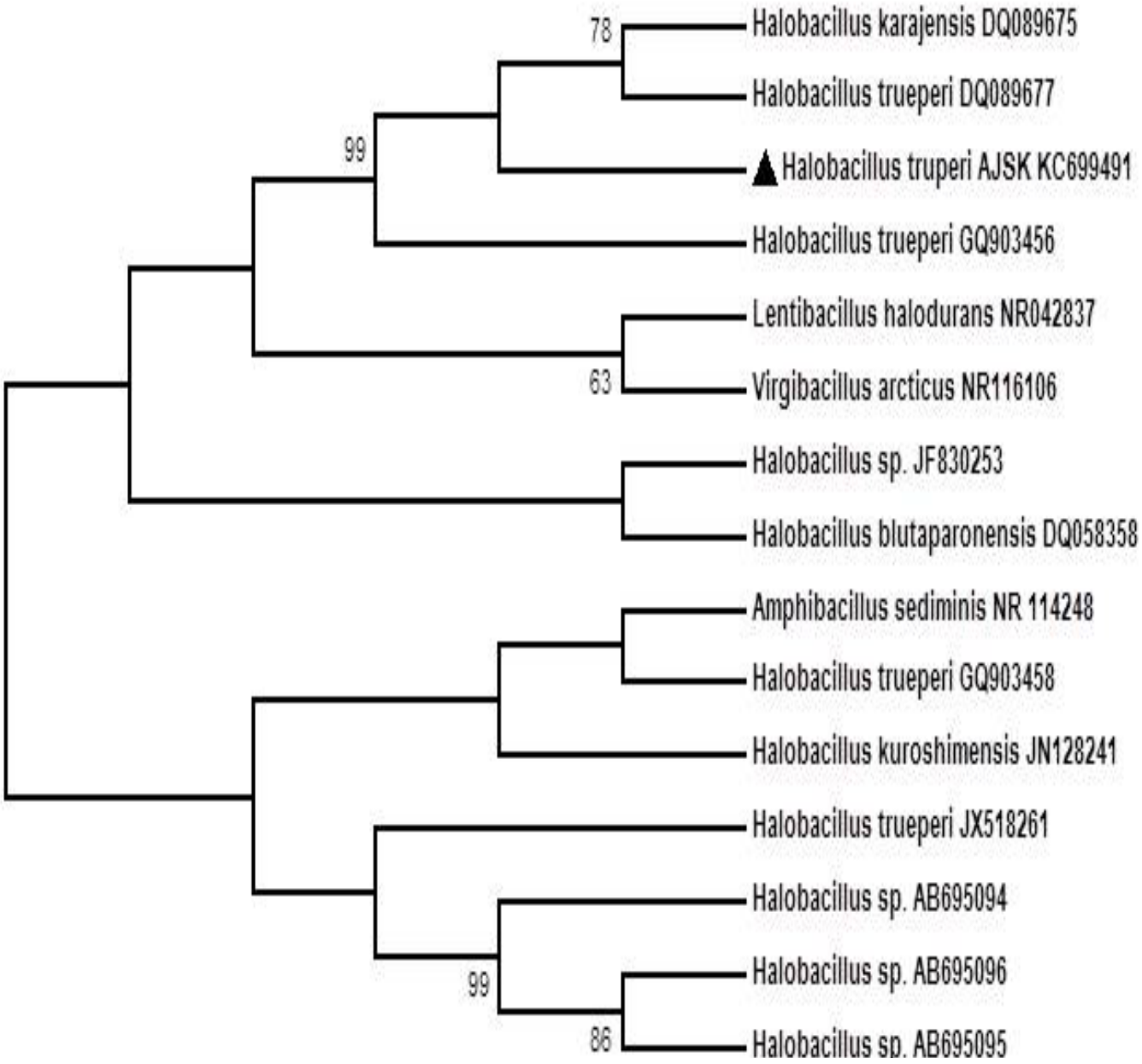

Figure 1. Phylogenetic tree of *Halobacillus trueperi* AJSK 16S rRNA gene sequence with other *Halobacillus* species/strains

Table 3. Analysis of variance for the fitted quadratic polynomial model for optimization of EPS production.

Source	DF	Seq SS	Adj SS	Adj MS	*F*- value	*P*- Value
Linear	4	24.855	101.778	25.4444	327.19	0.000
Square	4	226.280	226.280	56.5699	727.44	0.000
Interaction	6	6.599	6.599	1.0998	14.14	0.000
Residual error	16	1.244	1.244	0.0778		
Lack-of-Fit	10	0.956	0.956	0.0956	1.99	0.207
Pure error	6	0.288	0.288	0.0481		
Total	30	258.978				

DF, Degree of freedom; Seq SS, sequential sums of squares; Adj SS, adjusted sums of squares; Adj MS, adjusted mean square.

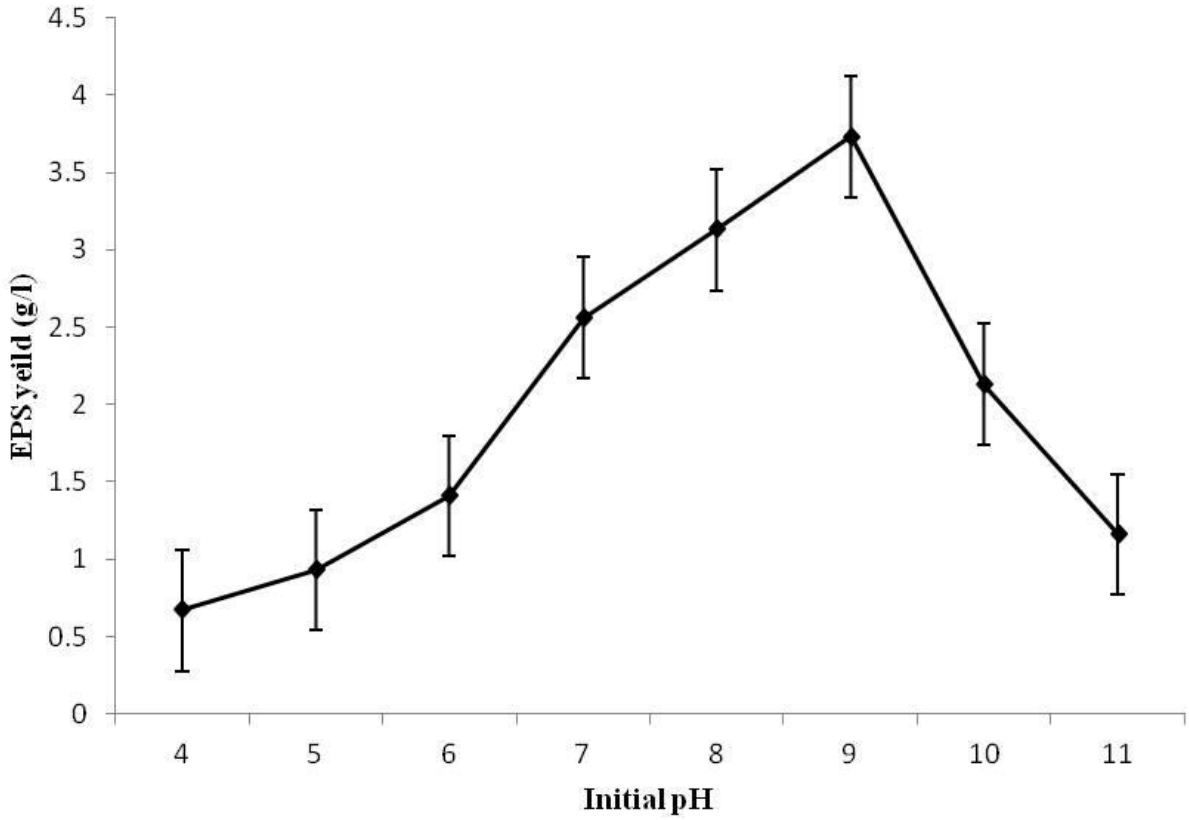

Figure 2. Effect of various pH on extracellular polysaccharide production.

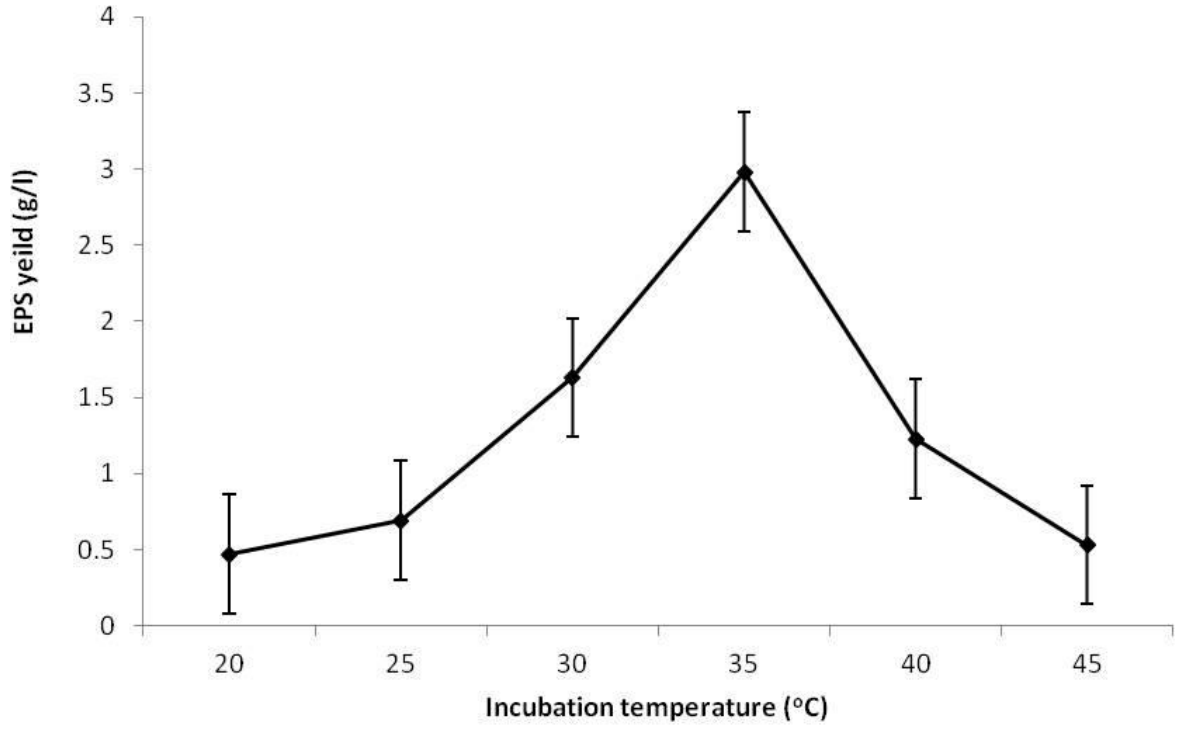

Figure 3. Effect of various temperatures on extracellular polysaccharide production.

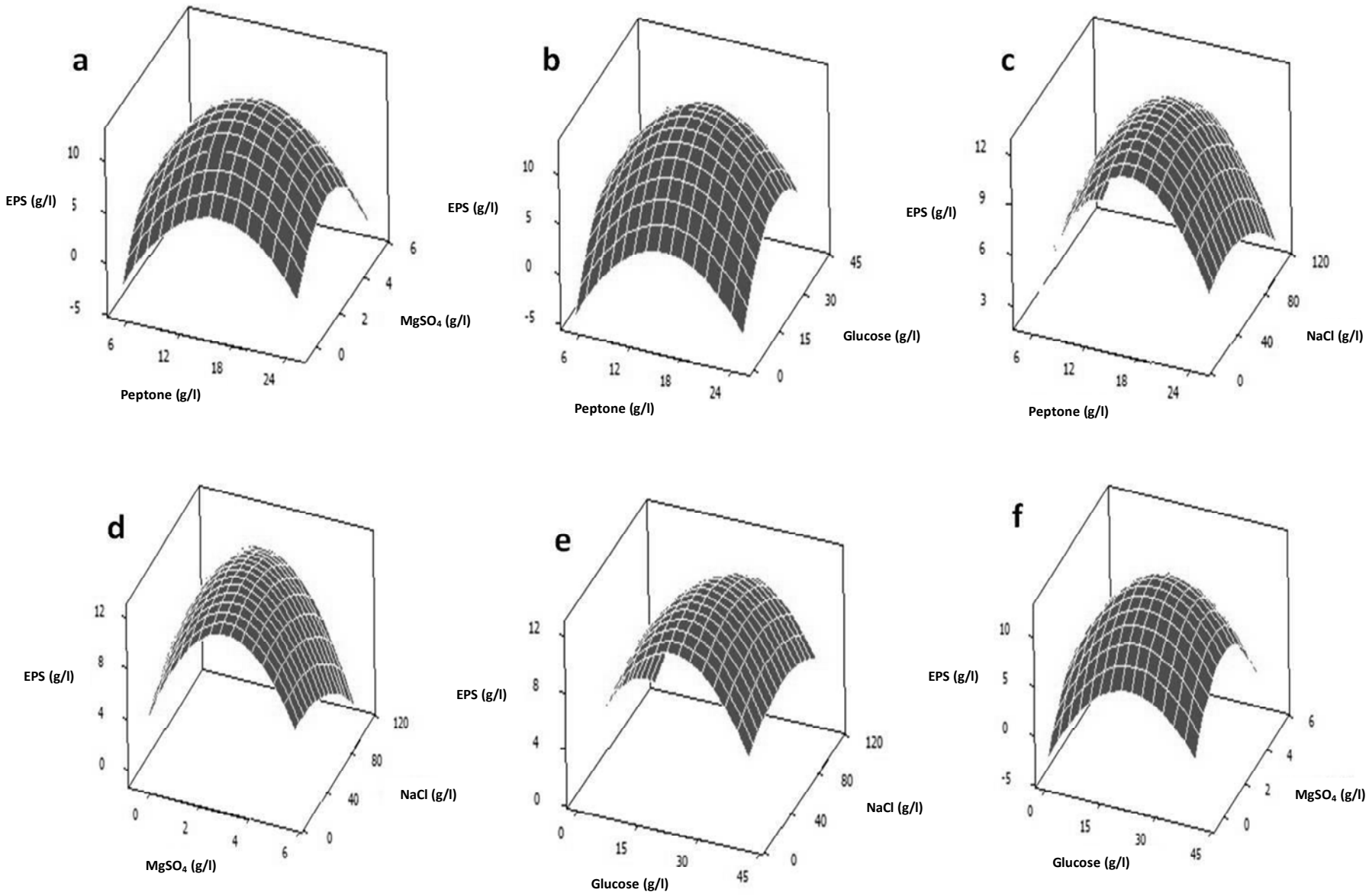

Figure 4. Three dimensional response surface plot for extracellular polysaccharide production showing the interactive effects of (a), peptone and $MgSO_4$ (b), Peptone and glucose (c), peptone and NaCl (d), $MgSO_4$ and NaCl (e), glucose and NaCl (f), glucose and $MgSO_4$.

Table 4. Results of regression analysis of the second-order polynomial model for optimization of EPS production.

Term	Coefficient	Estimate coefficient	*t*-Value	*p*-Value
Constant	-24.8245	1.15772	-21.443	0.000
X_1	2.4659	0.08172	30.177	0.000
X_2	0.7599	0.03699	20.541	0.000
X_3	5.0337	0.23990	20.982	0.000
X_4	0.1279	0.01567	8.159	0.000
$X1^2$	-0.0733	0.00209	-35.150	0.000
X_2^2	-0.0172	0.00052	-33.038	0.000
X_3^2	-0.8031	0.02318	34.651	0.000
$X4^2$	-0.0007	0.00008	-8.531	0.000
X_1x_2	-0.0019	0.00139	-1.383	0.186
X_1x_3	-0.0202	0.00930	-2.168	0.046
X_1X_4	-0.0016	0.00056	-2.829	0.012
X_2X_3	-0.0040	0.00465	-0.852	0.407
X_2X_4	0.0007	0.00028	2.345	0.032

about 97.72 and 99.10%, respectively (Table 4). The central optimum poin was evaluated by using gradient method in the direction of steepest ascend of media for the EPS production evaluated from the surface plots.

The response surface plots provide a visual interpretation of the interaction between variables and assist in determining optimal conditions by revealing the significance of the interaction among the variables. In this study the interaction between the variables glucose and NaCl is significant. Similarly Manivasagam et al. (2013) demonstrated a significant interaction between the variables glucose and NaCl using RSM in EPS production by *Streptomyces violaceus*. The optimal values of peptone, glucose, NaCl and $MgSO_4$ were estimated in actual units and they were 15.50, 22.24, 61.56 and 2.33 (g/L), respectively, with a predicted exopolysaccharide production of 12.35 (g/L). Conformation experiment was conducted for these predicted optimum conditions and extracellular polysaccharide production from the experiment was 12.93 g/L. This was little higher than the predicted value which reveals the higher accuracy of the model. A maximum EPS production of 9.01 g/L by a marine bacterium with glucose as best carbon source at pH 7 in seven days was reported by Nahas et al. (2011). In the present study we used glucose as the sole carbon source. Chowdhury et al. (2011) in *B. megaterium* RB-05 reported that glucose is the better substrate over fructose, sucrose, maltose and lactose for high EPS yields. Liu et al. (2011) reported a maximum of 8.90 g/L EPS production in *Zunonwangia profunda*, a deep sea bacterium with peptone as more influential than yeast extract. As reported by Srinivas and Padma (2014) the organic nitrogen sources were much more suitable than inorganic nitrogen sources for the microbial EPS production. Peptone with its peptide and amino acid composition serves an excellent nitrogen source for EPS production. Similarly, Wang et al. (2011) reported that beef extract, maltose, peptone and NaCl gave a maximum of 20.19 g/L EPS production in *B. thuringiensis* isolated from desert sand biological soil crusts using optimization by orthogonal matrix method. A maximum of 3.34 g/L EPS production in *Paenibacillus polymyxa* with galactose was reported by (Raza et al., 2011).

Conclusion

The present study lead to the optimization of key culture conditions with CCD designs for increased EPS production in halophilic bacterium, *H. trueperi* AJSK with promising properties for industrial exploitation. The RSM yielded a maximum of 12.93 (g/L) EPS production. Further investigation will identify the most befitting field of application. Numerous halophilic bacteria should be explored to reveal their potential for novel exopolysaccharides with biotechnolgically important properties to efficiently replace the synthetic polymers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article titled “Optimization of extracellular polysaccharide production in *Halobacillus trueperi* AJSK using response surface methodology”.

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