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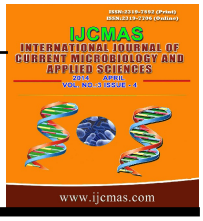

Original Research Article

Improved exopolysaccharide production by *Bacillus licheniformis* strain-QS5 and application of statistical experimental design

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K e y w o r d s

Exo-polysaccharide, *Bacillus licheniformis*, statistical experimental design, optimization

A B S T R A C T

Among a group of bacilli, *Bacillus* species strain-QS5 producing exopolysaccharide, locally isolated from Eastern Province in Saudi Arabia, was characterized and identified based on 16S rRNA sequencing. Phylogenetic analysis revealed its closeness to *Bacillus licheniformis*. The bacterium showed 1.9- and 2.8-folds increase in EPS production on basal salts medium containing glucose (M2) or sucrose (M3), respectively. Maximum yield of EPS by *B. licheniformis* strain-QS5 (3.78 g/g) obtained during growth on M3 medium supplemented with sucrose. While, slight decrease in biopolymer yield 3.64 and 3.5 (g/g) was recorded by the two other candidates *Bacillus* sp QC1 and *Bacillus* sp KSW21, respectively.Statistically based experiments were applied to detect the optimal medium composition for production of exopolysaccharide (EPS). The effect of four variables namely; sucrose, phosphate buffer and ammonium sulfate were examined for their significance on exopolysaccharide production using Box-Behnken design. Based on statistical analysis, maximal EPS production was reached under optimal conditions with approximately 4-folds increase in comparison with the amount produced on complex production medium M1 and the use of sucrose as carbon source was crucial (*p*-value 0.051). Verification experiment was carried out to examine model validation and revealed more than 78% validity.

Introduction

Exopolysaccharides are naturally formed macromolecules during growth of many organisms. Recently, vast number of bacterial exopolysaccharides (EPS) attracted attention of many scientists, their composition, structure, biosynthesis and functional properties have been extensively studied (Isobe *et al.*, 1992; Kumar *et al.*, 2007; Donot *et al.*, 2012). EPS have found diverse applications in food, pharmaceutical, medical and other biotechnological applications (Stewart *et*

al., 2001; Laue *et al.,* 2006; Rehm, 2010; Freitas*et al.,* 2011).

Production of EPS by several microbial groups; including strains belong to genus *Bacillus*, especially *Bacillus licheniformis*, have been investigated (Manca *et al.,* 1996; Larpin *et al.,* 2002; Arena *et al.,* 2006; Borgio *et al.,* 2009; Li *et al.,* 2009; Xiong*et al.,* 2010, Orsod *et al.,* 2012). Generally, synthesis of polysaccharide involves enzymatic catalysis (Flávia *et al.,* 2006, Rehm, 2010; Freitas *et al.,* 2011). Recently, the role of exopolysaccharides in biofilm formation has been extensively investigated (Mayer *et al.,* 1999; Sutherland, 2001; Vu *et al.,* 2009). For commercial production of exopolysaccharides, it is crucial to lower the production costs. Approaches might involve using cheaper substrates, improving product yield by optimizing fermentation conditions, or developing higher yielding strains and optimizing downstream processes (Rehm, 2010).

Application of experimental design techniques, as an effective alternative to one-variable-at-a-time (OVAT) approach has gained a lot of impetus for medium optimization and understanding interactions among various physiochemical parameters involved in EPS production from bacteria and fungi (Hsieh *et al.,* 2005; Bueno and Garcia-Cruz, 2006; Baskar *et al.,* 2011; Yeruva and Mantha, 2011; Abdul Razack *et al.,* 2013).

The main objective of the study was to isolate, characterize and identify an EPS producing Bacilli from soil and sewage samples, Eastern province, Saudi Arabia. The potent strain was identified by 16S rDNA analysis. Also, the use of different production media for optimized EPS production from *B. licheniformis* strain-QS5. Optimization of EPS production from *B. licheniformis* strain-QS5 was closely investigated using response surface methodology (RSM). Special emphasis was given to the impact of significant medium variables interactions on EPS biopolymer production using Box-Behnken design.

Materials and Methods

Microorganism; isolation and identification

Screening was carried out by enrichment and isolation of spore-forming bacilli by heat treatment of 1% of the soil or sewage sample obtained from cultivation field in Qateef area, Gobeil and sewage treatment plant in Al-Khobar, Eastern Province of Dammam, saudi Arabia, for 20 min at 90 ^{o}C. Subsequently, the strains were purified by cultivation on NA medium and screened for exopolymer production by cultivation on 3 different mineral agar media supplemented with sucrose or glucose as carbon source. Visible viscous colonies were selected for further experiments. Among the isolated bacterial strains, the most potent biopolymer producing bacterium (*Bacillus* sp strain-QS5) was isolated. Isolates maintained on nutrient agar slant composed of (g/L): peptone; 5, beef extract; 3, NaCl; 2 and agar; 20. Stock culture was subcultured at regular intervals of one month and stored under refrigeration.

The bacterium was characterized and identified by 16S rRNA gene sequencing using universal primers as descirbed by (Soliman *et al.,* 2005). The forward and reverse primers were of the following sequences, respectively: AGAGTTTGATCMTGGCTCAG and TACGGYACCTTGTTACGACTT. 16S

rDNA sequence was aligned with published sequences through BLAST sequence tool from the NCBI database. Subsequently, the sequence deposited in the GenBank under the accession number KC223618.

Growth and production conditions

The bacterium grew in 50ml aliquot of nutrient broth dispensed in 250ml Erlenmeyer flask and incubated at 37°C for 24h at (125 rpm). 1.5% inoculums of the overnight culture was used to inoculate a complex medium M1 of the following composition (g/L): Sucrose; 20, beef; 3, peptone, 5; K_2HPO_4; 3, NaCl; 5. Other modified basal salt production media of the following composition (g/L): K_2HPO_4; 8, KH_2PO_4; 2, $MgSO_4.7H_2O$; 0.5; $(NH_4)_2SO_4$; 10, yeast extract; 1, with glucose and sucrose (20 g/L) for medium M2 and M3, respectively, were tested. The cultures were incubated at 37°C for 96 h under shake condition.

For recovery of exopolysaccharide biopolymer EPS, modified method of Kumar *et al.*(2004) was applied. The cultivation medium was first centrifuged to remove cells (30min, 5000rpm at 4°C) and the EPS was precipitated using ice-cold ethanol (1:2 volume ratio) and kept at 4°C overnight. The crude precipitated EPS was separated by centrifugation. For further purification, the last step was repeated. Finally, the precipitate was dried overnight at 60°C and weighed. For estimation of cell dry weight, the harvested cells washed once with dist. Water, dried and weighed till constant weight.

Fractional factorial design

Box-Behnken design

For optimization of EPS production, Box-Behnken design (BBD) (Box and Behnken, 1960)was applied. As presented in Table 1, the four critical factors were prescribed into 3 levels, coded -1, 0, +1. Table 2 represents the design matrix of a 28 trials experiment. For predicting the optimal point, a second-order polynomial function was fitted to correlate relationship between independent variables and response represented by the amount of EPS produced. For the four factors the equation is:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{12}X_{12}+ \beta_{13}X_{13}+ \beta_{14}X_{14}+ \beta_{23}X_{23}+ \beta_{24}X_{24}+ \beta_{34}X_{34}+ \beta_{11}X_{11}+ \beta_{22}X_{22}+ \beta_{33}X_{33}+ \beta_{44}X_{44} \quad (1)$$

where Y is the predicted response, β_0 the model constant; X_1, X_2, X_3 and X_4 independent variables; β_1, β_2, β_3 and β_4 are linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are cross product coefficients and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients. Microsoft Excel 97 was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2.

Statistical analysis of the data

The data on the EPS production were subjected to multiple linear regressions using Microsoft Excel 97 to estimate *t*-value, *P*-value and confidence level. The significance level (*P*-value) was determined using the Student's t-test. The *t*-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. Factors having highest *t*-value and confidence level over 95% were considered to be highly significant on EPS production. Optimal value of activity was estimated using the solver function of MICROSOFT

EXCEL tools. The Statistica program was used for presentation of data.

Results and Discussion

Isolation, taxonomic classification and EPS production

Six bacterial strains isolated during a program for exploring the production of EPS from soil and sewage sample obtained from Quatief, Gobeil and Al-Khobar, Eastern Province, Saudi Arabia. The strains showed highly viscous growth on basal medium supplemented with sucrose or glucose as a sole carbon source. Indeed, the results indicated that the solid production medium M3 supported biomass as well as EPS production for most of the strains including *Bacillus* sp. strain-QS5 except *Bacillus* sp. QC22, where M2 was the preferred medium (Table 3).

Morphological and physiological characteristics of the *Bacillus* sp. Strain-QS5 showed that it is rod shaped, spore forming Gram positive bacterium, capable of growing on different sugars such as: glucose, fructose, mannose, xylose and arabinose. In addition, the isolate showed catalase, amylase and protease activities. To investigate phylogenetic affiliation of this strain, the complete 16S rRNA gene was amplified, sequenced and deposited in the GenBank and given the accession number KC223618. Comparison of the obtained sequence with other sequences available at NCBI database revealed the greatest similarity to the corresponding sequences of many *Bacillus lichniformis* strains. The sequence recorded very close similarity to *B. licheniformis* ATCC 14580 (accession No. CP000002) as well as *B. licheniformis* DSM 13 ATCC 14580 (accession no. AE017333) and a group of *B. licheniformis* strains involved in coal mining among of them *B. licheniformis* strain AIS68 (accession no. GU967450). Interestingly, the strain showed close relation to other biopolymer producing bacilli such as *B. licheniformis* S2 (accession no. AY052767) and *B. amyloliquefaciens* LL3 (accession no. CP002634).

Exopolysaccharide (EPS) production on different media

One of the most critical variables affecting EPS biopolymer production is the carbon source. In this concern, the effect of two most commonly used carbon sources, glucose and sucrose, on EPS production by *B. licheniformis* strain-QS5 was investigated. Results presented in Figure 1 revealed that maximum EPS production (13.75 g/L) was recorded during growth on M3 medium supplemented with sucrose. While, EPS production was remarkably decreased when sucrose was replaced by glucose in M2 production medium (8.5 g/L). Furthermore, dramatic decrease in EPS production was recorded during growth of *B. licheniformis* strain-QS5 on complex medium M1 (approxi. 70%) even in presence of sucrose. In comparison with other EPS producing bacilli, results in Table 3 revealed that maximum yield of EPS by *B. licheniformis* strain-QS5 (3.78 g/g) obtained during growth on M3 medium supplemented with sucrose. However, the use of glucose as carbon source in M2 production medium yield 2.7 g/g EPS. On the other hand, a slight decrease in biopolymer yield 3.64 and 3.5 (g/g) was recorded by the two other strains *Bacillus* sp QC1 and *Bacillus* sp KSW21, respectively.

Results collectively indicated that the EPS production by *B. licheniformis* strain-QS5 is enhanced by sucrose or glucose in

Table 1. Variables and their levels employed in Box-Behnken design for screening of culture conditions affecting on EPS production by *Bacillus* sp. QS5.

Variable code	Variable	Value		
		-1	0	+1
X_1	Sucrose (g/l)	20	25	30
X_2	K_2HPO_4 (g/l)	6	8	10
X_3	KH_2PO_4 (g/l)	0.5	1	1.5
X_4	Ammonium sulfate (g/l)	8	12	15

Table.2 Box-Behenken matrix representing the effect of significant variables affecting exopolysaccharide (EPS) production by *Bacillus* sp.SQ5.

Trail	Sucrose	K_2HPO_4	KH_2PO_4	$(NH_4)_2SO_4$	EPS (g/L)
1	-1	1	0	0	19.25
2	0	0	0	0	18.75
3	0	0	-1	-1	19.75
4	1	0	0	1	17
5	1	0	-1	0	19.75
6	-1	0	0	-1	15.25
7	0	1	0	1	22.5
8	0	0	1	-1	13
9	-1	0	1	0	14.75
10	0	1	1	0	16.25
11	0	0	-1	1	15.5
12	0	-1	0	-1	13.75
13	0	1	-1	0	22.75
14	0	-1	-1	0	13.5
15	1	0	0	-1	13.25
16	0	-1	1	0	14
17	0	0	0	0	16.25
18	1	0	1	0	14.5
19	0	-1	0	1	9.74
20	-1	0	-1	0	9.5
21	1	1	0	0	14.75
22	0	0	0	0	16.75
23	0	0	1	1	11.5
24	-1	0	0	1	11.25
25	-1	-1	0	0	9
26	0	0	0	0	17.5
27	0	1	0	-1	14.5
28	1	-1	0	0	11

Table.3 Production of exopolysaccharide (EPS) by bacteria belong to genus *Bacillus* sp. during growth on different cultivation media

Bacillus strain	Cultivation media					
	M1		M2		M3	
	CDW (g/L)	EPS Yield (g/g)	CDW (g/L)	EPS Yield (g/g)	CDW (g/L)	EPS Yield (g/g)
Bacillus sp. QC1	2.3	2	2.1	2.33	3.4	3.64
Bacillus sp.Gob4	2.2	1.3	3.2	3.4	4.3	3
Bacillus sp. QC22	0.2	ND	2.5	3.5	0.7	2.95
Bacillus sp. QS5	3.3	1.36	3.2	2.7	5.2	3.78
Bacillus sp. KSW21	2.4	1.6	3.8	2.7	4.0	3.5
Bacillus sp. V12	0.8	2.2	2.0	3.4	2.4	3.4

CDW: cell dry weight (g/l)
EPS: Exopolysaccharide (g/g)
M1: complex medium
M2, M3: Basal production media
ND: not detected

Table.4 Statistical analysis of Box-Behnken design showing coefficient values, *t*- and *P*-values for each variable

Variables	**Coefficient**	***t-Stat***	***P*-value**
Sucrose	1.0	2.16	0.05171
K_2HPO_4	2.8	5. 6	0.00012
KH_2PO_4	-1.1	-2.4	0.03352
$(NH_4)_2SO_4$	0.07	0.14	0.89221
X1X2	-1.0	-1.1	0.29787
X1X3	-1.6	-1.9	0.08793
X1X4	0.7	0.8	0.43002
X2X3	-2.0	-2.4	0.03430
X2X4	1.9	2.1	0.05683
X3X4	1.0	1.2	0.25809
X1X1	-2.4	-3.6	0.00351
X2X2	-0.6	-0.9	0.39187
X3X3	-1.0	-1.5	0.17186
X4X4	-1.8	-2.6	0.02171

Figure.1 Production of exopolysaccharide (EPS) by *Bacillus licheniformis* strain-QS5 during cultivation on different production media

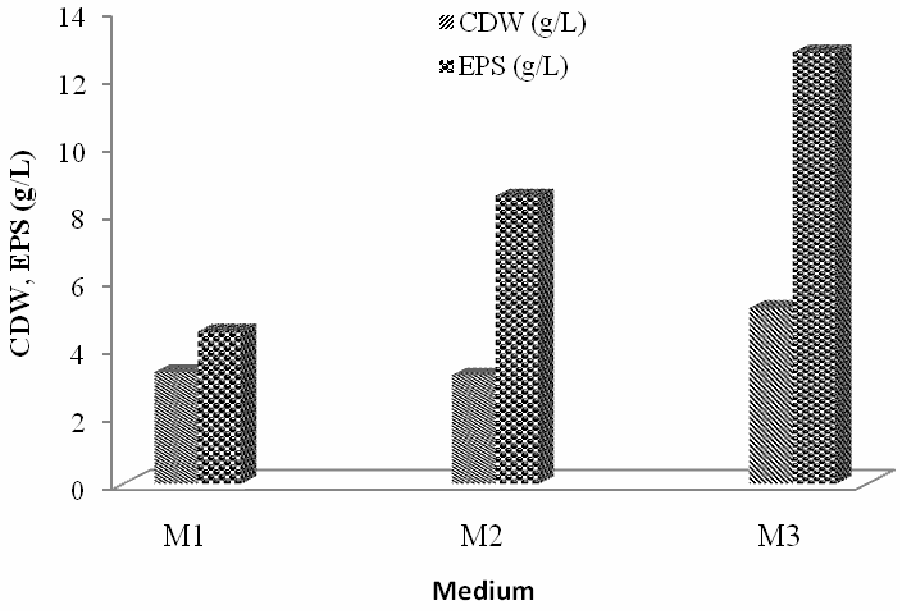
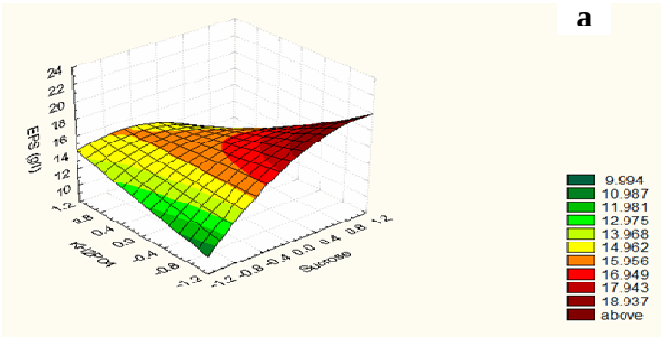
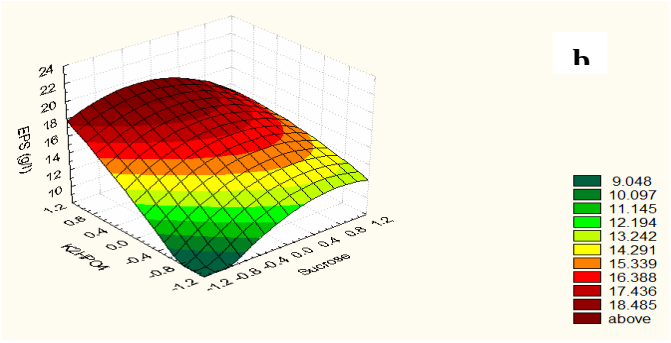
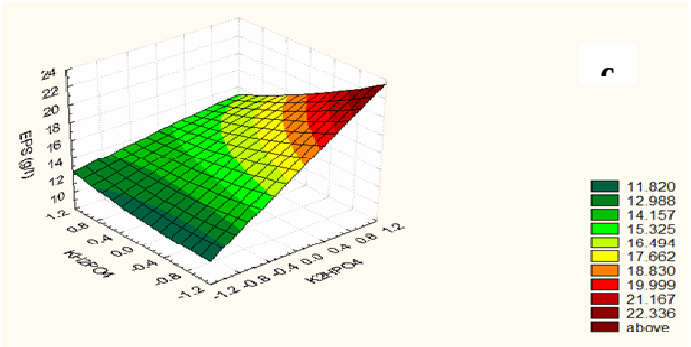
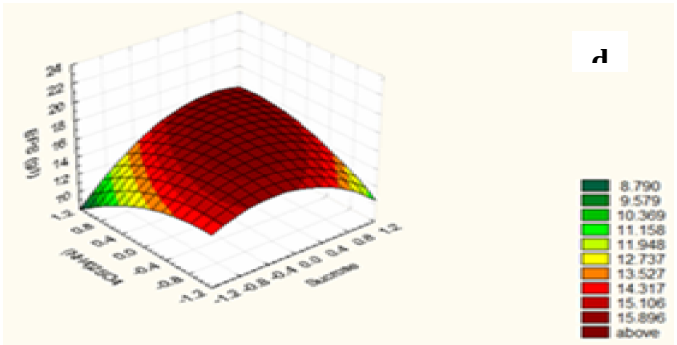


Figure.2a-d Three dimensional response surface graphics showing the behavior of EPS production by *B. licheniformis* strain-QS5 as affected by different cultivation parameters









presence of trace amount of yeast extract. Interestingly, production of extracellular polysaccharide in presence of yeast extract was previously described (Ebube *et al.,* 1992; Tallgren *et al.,* 1999; Mukherjee *et al*., 2011). Furthermore, the use of yeast extract as nitrogen source during production of three polysaccharides by *Bacillus licheniformis* SVD1 was reported by van Dyk *et al.* (2012). Moreover, yeast extract could provide growth factors such as vitamins and amino acids that support many bacterial growth (Ebube *et al.,* 1992). Interestingly, EPS production by *B. licheniformis* strain-QS5 was increased in stationary phase, as recognized in most bacteria where EPS formation is increased after cessation of microbial growth (Petry *et al.,* 2000).

Application of Box-Behnken design and data analysis

Due to simplicity of production medium, the effect of the four main medium components namely; Sucrose, K_2HPO_4, KH_2PO_4, and $(NH_4)_2SO_4$ were evaluated. The quadratic model consisting of 28 trials presented in Box-Behnken design. As shown in Table 1, the chosen variables were prescribed into 3 levels, coded -1, 0, +1. The design of this experiment is given in Table 2 together with the experimental results. Regression analysis was performed to fit the response function (EPS production) with the experimental data. Analysis of variance for the four variables indicated that EPS production can be well described by a polynomial model with a relatively high coefficient of determination (R^2 = 0.85). The statistical analysis of the full model showed that each sucrose, ammonium sulfate and K_2HPO_4 had a significant effect on EPS production. Interestingly, growth of *B. licheniformis* QS5 cells was significantly affected by the same parameters (data not shown). When presenting experimental results in the form of surface plot (Figure 2a-d) it can be seen that increased levels of sucrose, ammonium sulfate, and K_2HPO_4 supported high EPS production. For predicting the optimal point, within experimental constrains, a second-order polynomial function was fitted to the results of EPS production:

$$Y_{EPS} = 20.1+1.0X_1+2.8X_2- 1.1X_3+0.07X_4-1.0X_{12}-1.6X_{13}+0.7X_{14}-2.0X_{23} +1.9X_{24}+1.0X_{34}-2.4\ X_{11}- 0.6X_{22}- 1.0X_{33}-1.8X_{44} \quad (2)$$

where X_1, X_2, X_3 and X_4 represent codified values for sucrose, K_2HPO_4, KH_2PO_4 and $(NH_4)_2SO_4$, respectively. The closer the value of R is to 1, the better is the correlation between the observed and the predicted values. In this experiment, the value of R was 0.954 for EPS production. This value indicates a high degree of correlation between the experimental and the predicted values. The value of determination coefficient R^2 = 0.91 being a measure of fit of the model, indicates that about 10% of the total variations are not explained by the EPS production model.

Interestingly, results presented in Figure 2a-d and ststistically analyzed in Table 4 clearly indicated that EPS production was enhanced due to interaction between sucrose with ammonium sulfate and K_2HPO_4. Generally, EPS production by many bacilli belongs to genus B. *licheniformis* require sucrose in the production medium, others do not. In this work, enhanced EPS production by *B. licheniformis* strain-QS5 in presence of sucrose supported by the results obtained by many scientists (*p*-value 0.051). Where, sucrose was recorded as the prefered

carbon sources for production of EPS by many *B. licheniformis* candidates (Ebube *et al.,* 1992; Isobe *et al.,* 1992; Ghaly *et al.,* 2007; Liu *et al.,* 2010; van Dyk *et al.,* 2012). Spanò *et al.* (2013) reported that the haloalkaliphilic, thermophilic *Bacilluslicheniformis* T14 requires sucrose for growth and EPS production. On the other hand, results presented in this work indicated higher phosphate concentration namely; K_2HPO_4 (*p*-value 0.00012 and 0.033 for K_2HPO_4 and KH_2PO_4, respectively), plays a key role for optimal EPS production (Table 4). In concordance, Ebube *et al.* (1992) reported that *B. licheniformis* requires phosphate ions for EPS production. It is presumed that phosphate ions may provide energy that is required during polymer synthesis.

Verification experiment was performed and the maximum yield of EPS by *B. licheniformis* strain-QS5 was about 4-folds increased when the strain was cultivated in the optimal medium developed by experimental design. Therefore, experimental design may prove to be a powerful and useful tool for enhancing EPS production and urge the need for optimization process.

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