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Medium optimization and in vitro antioxidant activity of exopolysaccharide produced by *Bacillus subtilis*

Sirajunnisa Abdul Razack†, Vijayagopal Velayutham, and Viruthagiri Thangavelu

Bioprocess Laboratory, Department of Chemical Engineering, Faculty of Engineering and Technology, Annamalai University, Annamalainagar, Tamilnadu-608002, India
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Abstract−The present study involves medium formulation using an agro waste, cane molasses, as the carbon substrate that was used instead of sucrose, to produce exopolysaccharide from *Bacillus subtilis*. Plackett Burman design was applied to evaluate twenty selected components, from which cane molasses, yeast extract, $CaCl_2$, NaCl were found to be significant for fermentation. To study the concentration of each component, response surface methodology experimental design was performed using central composite design. The response plots resulted in optimized conditions--Cane molasses- 2.36%, Yeast extract- 0.56%, NaCl - 0.71%, $CaCl_2$ - 0.05%--which yielded 4.92 g/L at 48 h, at a temperature of 37 °C, initial pH 7 under still conditions. Antioxidant activity of EPS on DPPH resulted in a reducing capacity of 61.19%, at a concentration of 0.8mg/ml, greater than standard, Vitamin C. The biopolymer could thus be an ecofriendly product which can be subjected to various industrial and pharmaceutical applications.

Keywords: *Bacillus subtilis*, Exopolysaccharide, Optimization, Response Surface Method, FTIR Spectrophotometry, Antioxidant Activity

INTRODUCTION

Microorganisms synthesize a wide spectrum of multifunctional polysaccharides including polysaccharides, structural polysaccharides and extracellular polysaccharides (EPS) [1]. Bacterial exopolysaccharides are primarily the constituents of glycocalyx, with which the cells adhere to surfaces [2]. These form the major components of the biofilms [3]. Various applications make EPS advantageous. EPSs are found to be involved in pathogenesis, symbiosis, protecting against osmotic shock, toxic stress [4,5]. EPSs also possess immunomodulatory, antibacterial, antiviral, antiulcer and antioxidant effects [6]. In the food industry, EPSs are used as stabilizers, emulsifiers, and gelling agents [7]. EPSs help in capturing nutrients, act as anti-corrosive agents [8] and bioflocculants [9]. EPS synthesized by bacteria are susceptible to biodegradation in nature, thus contribute less to environmental pollution than synthetic polymers [10]. EPS exhibits remarkable thickening and shear thinning properties and display high intrinsic viscosity.

Bacillus sp. produces complex EPSs, which could be homopolysaccharide with repeated similar units of sugar or heteropolysaccharide containing different sugar moieties. EPS synthesized by *Bacillus* sp. have comparatively elevated viscosity and superior pseudoplastic properties [11]. Widely studied species for EPS production are *B. licheniformis*, producing levan [11], *B. polymyxa* [12], *B. megaterium* [13] and *B. mucilaginosus* [14]. *B. subtilis* essentially constituted for studies involved in biofilms formation. In major investigations, *B. subtilis* is mostly found to produce a biopolymer poly-γ-Glutamate [15,16]; levan (fructan) is produced by a strain *B. subtilis* natto [17].

Bacillus subtilis has been selected for study due to its structural and genetic complexity. Wild strains of B.subtilis are capable of forming architecturally complex communities of cells known as biofilms, which are predominantly composed of exopolysaccharides (EPS). This organism is highly utilized for biofilm and endospore formation studies. *B. subtilis* has the capacity to transform from the motile state to the nonmotile state. In nonagitated liquid broth, the highly motile cells, swimming singly, accumulate as bundled chains forming pellicles and ultimately form a thick biofilm on the air liquid interface. Larger pellicle formation would eventually aid in higher yield of EPS. A 15-gene eps operon designated as eps A-O, facilitates EPS production and biofilms formation in *Bacillus subtilis* [18].

Growth medium and environmental parameters play important roles in fermentation of EPS. Studies showed that the medium composition can affect the specific rate of EPS synthesis, molecular size of EPS, its degree of branching and composition [19]. Production is generally favored by high calcium and low nitrogen ratio in the medium [20]. Carbohydrate components of the medium affect the yield of EPS but do not influence their chemical structure. They also affect viscosity of EPS, possibly owing to the heterogeneity in the molecular weight [10].

The medium composition for fermentation process could be optimized by using a statistical tool, response surface methodology (RSM). This method is advantageous over the conventional method, which involves numerous experiments by changing one variable at a time, keeping other independent variables constant. RSM is reliable, fast and provides interactive effects of nutrients and their individual effects. For the production of EPS from *B. subtilis*, a Plackett-Burmann Design was performed to screen the significant nutrients to enhance the yield of EPS, and then a central composite design (CCD) was carried out to optimize the concentration of essential medium com-

†To whom correspondence should be addressed.
E-mail: siraj.razack@gmail.com

ponents that were screened Plackett-Burman design. The efficiency of the isolated EPS as a potent antioxidant was also studied by its scavenging or reducing capacity of DPPH free radicals.

MATERIALS AND METHODS

1. Bacterial Culture Isolation

Soil sample was collected from the campus of Annamalai University (Tamilnadu, India), was suspended in sterile distilled water and subjected to serial dilution (10^{-1}-10^{-7}). An aliquot of 0.1 ml of each dilution mixture was spread on nutrient agar medium containing peptone (5 g/*l*), yeast extract (2 g/*l*), NaCl (5 g/*l*) and Agar (20 g/*l*). The final pH was adjusted to 7 using 0.1 N NaOH and diluted HCl. After sterilization the plates were incubated at 37 °C for 24 hr. After incubation, cream colored, highly mucoid with undulated edges colonies were selected and purified by repeated plating. The isolated, pure colonies were then subjected to routine microbiological and biochemical characteristic techniques, with which the culture isolated was identified as *B. subtilis* [21]. The organism was stored on nutrient agar slant and maintained at 4 °C for further studies. One loopful of culture was taken and incubated in an Erlenmeyer flask with 50 ml broth, which was incubated at 37 °C for 24 hr. 2% inoculum (v/v) was used from this culture in 100 ml medium for EPS production.

2. Production and Isolation of Exopolysaccharides

EPS production was performed in 50 ml medium as per the framed experimental design. The unagitated, inoculated flasks were incubated at 37 °C for 48 hr. All the runs were carried out and the EPS content was estimated. EPS was extracted by precipitation method using ethanol. The culture was centrifuged at 11,000 rpm for 10 min at 4 °C. The supernatant obtained was mixed with two volumes of ice cold ethanol and kept at 4 °C for 24 hr. The mixture was then centrifuged at 2,500 rpm for 20 min at 4 °C. The obtained pellet was suspended in distilled water, which was centrifuged at 2,500 rpm for 30 min at 4 °C with two volumes of ice cold ethanol [22]. The process was repeated twice and the EPS obtained was dried, weighed and lyophilized. The total carbohydrate content of the biopolymer was studied by phenol sulfuric acid method [23] using glucose as standard.

3. Media Optimization

3-1. Plackett Burman (PB) Design

The screening of significant nutrients was carried out using Plackett Burman Design [24]. Based on one-factor at a time experiments, carbon, nitrogen, vitamin, amino acids, trace metal ions and minerals were screened by one factor at a time and the significant nutrients were used for study. Based on this, 20 independent variables were selected for the study, evaluated in 24 experiments trials. Each nutrient was used at two concentrations (high and low), designated as +/– levels. The concentration levels were also selected by one factorial experiment. Plackett Burman Design is showed on the first order polynomial model,

$$Y = \beta_0 + \sum \beta_i X_i$$

where Y is the response (EPS yield), β_o is the model intercept and β_i is the linear coefficient, and X_i is the level of the independent variable. This model does not derive the interactive effects but is used to screen the essential nutrients implementing the yield of EPS (Y) [25]. The experimental design and statistical analysis of the data were done by Minitab statistical software package (v 16.0). In the present study the trials were run in duplicates and the analyzed EPS was taken as the response. Regression analysis determined the components, based on the significance level of 95% ($p<0.05$).

3-2. Central Composite Design (CCD)

A central composite design was experimented to optimize the four variables screened by Plackett Burman design that significantly influenced EPS production. Design Expert software (Version 8.0.7.1 Trial, Stat-Ease Inc., Minneapolis, USA) was used to frame the experimental designs and statistical analyses. The four independent variables were evaluated at five levels (−1, −2, 0, +1, +2) with 30 experimental runs and six repetitive central points. The experiments were conducted in 250 ml Erlenmeyer flasks with 100 ml of media, under nonagitating condition 37 °C for 48 h, prepared according to the design. The response obtained could be represented by a second-degree polynomial equation as:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4 +\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{34}X_3X_4+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{44}X_4^2$$

where Y is the predicted response, β_0 was the constant, X_1, X_2, X_3 and X_4 were the input variables, β_1, β_2, β_3 and β_4 were the linear coefficients, β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} were the second-order interactive coefficients and β_{11}, β_{22}, β_{33} and β_{44} were the quadratic coefficients. The experiments were carried out in triplicate. The response (yield of EPS gL^{-1}) was the dependent variable. The 3D graphical plots obtained would illustrate the mutual interactions between each significant factor, thus evaluating the optimized medium components.

4. Fourier Transform Infra Red (FTIR) Spectrophotometry

A quantity of 50 mg of lyophilized EPS was taken, mixed with 150 mg of KBr powder and ground well to fine mixture. The mixture was pressed to a disc by using a hydraulic press. The disc was subjected to FTIR spectral measurement in the frequency range of 4,000-400 cm^{-1}. The exopolysaccharide was characterized using a Fourier transform infrared spectrophotometer (Bruker Optics, GmBH, Germany).

5. Assay of Antioxidant Activity

The antioxidant activity of the isolated EPS was evaluated on the basis of the free radical scavenging effect of 1, 1-diphenyl - 2 picrylhydrazyl (DPPH), by the method of Shimada et al. (1992) and Liu et al. (2010) [26,27] with slight modification. In brief, sample solutions at various concentrations of 0.2, 0.4, 0.6, 0.8 mg/ml were made up to to 1 ml with distilled water. 1 ml of DPPH solution (0.004% in methanol) was added to sample and standard solutions. After the solutions were incubated for 30 min in dark, the absorbance was read at 517 nm. Vitamin C and distilled water with DPPH were used as the reference and blank, respectively. The percent scavenging ability was calculated using the formula:

$$\text{Percent (\%) scavenging activity} = 1-(A/B)\times 100$$

RESULTS AND DISCUSSION

1. Variables Affecting EPS Production

Exopolysaccharide was produced by conventional batch fermentation involving culture growth and EPS synthesis. The variables influencing the synthesis of EPS were investigated. Plackett Bur-

Table 1. Plackett-Burman Design for two levels of 20 variables along with the observed EPS yield

Run	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	Y
1	1	3	3	1	0.85	0.25	0.85	0.85	0.9	0.09	0.09	0.01	0.01	0.0025	0.0025	0.0075	0.0025	0.0035	0.0025	0.0025	4.349
2	3	3	3	3	0.85	0.25	0.25	0.25	0.5	0.09	0.01	0.09	0.01	0.0025	0.0075	0.0075	0.0025	0.0025	0.0035	0.0035	4.486
3	1	1	1	3	0.25	0.85	0.85	0.85	0.9	0.01	0.09	0.09	0.09	0.0075	0.0075	0.0025	0.0025	0.0025	0.0025	0.0035	4.492
4	3	1	3	3	0.85	0.85	0.85	0.25	0.5	0.01	0.01	0.09	0.01	0.0075	0.0025	0.0025	0.0075	0.0035	0.0025	0.0025	4.246
5	1	1	1	1	0.25	0.25	0.25	0.25	0.5	0.01	0.01	0.01	0.01	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	3.894
6	1	1	3	3	0.25	0.25	0.85	0.85	0.5	0.09	0.01	0.09	0.09	0.0075	0.0075	0.0075	0.0025	0.0025	0.0025	0.0025	4.168
7	3	3	1	1	0.25	0.25	0.85	0.25	0.9	0.01	0.01	0.09	0.09	0.0025	0.0025	0.0075	0.0075	0.0025	0.0035	0.0025	4.532
8	3	1	1	3	0.85	0.25	0.85	0.25	0.9	0.09	0.09	0.09	0.09	0.0025	0.0025	0.0025	0.0025	0.0035	0.0025	0.0035	4.681
9	1	1	3	3	0.25	0.85	0.25	0.85	0.9	0.09	0.09	0.09	0.01	0.0025	0.0025	0.0025	0.0075	0.0025	0.0035	0.0025	4.213
10	3	3	3	1	0.25	0.25	0.25	0.85	0.5	0.09	0.01	0.01	0.09	0.0075	0.0025	0.0025	0.0075	0.0035	0.0025	0.0035	4.478
11	1	3	3	3	0.85	0.85	0.25	0.25	0.5	0.01	0.09	0.01	0.09	0.0025	0.0025	0.0075	0.0075	0.0025	0.0025	0.0035	4.164
12	3	3	1	3	0.25	0.85	0.85	0.85	0.9	0.09	0.01	0.01	0.01	0.0025	0.0075	0.0025	0.0075	0.0025	0.0025	0.0035	4.472
13	3	1	1	1	0.25	0.85	0.25	0.85	0.5	0.09	0.09	0.09	0.09	0.0025	0.0025	0.0025	0.0025	0.0035	0.0025	0.0035	4.681
14	1	1	3	1	0.85	0.25	0.25	0.85	0.9	0.01	0.01	0.09	0.09	0.0025	0.0075	0.0025	0.0075	0.0035	0.0035	0.0035	4.246
15	3	1	3	1	0.85	0.85	0.85	0.85	0.9	0.01	0.01	0.01	0.01	0.0075	0.0025	0.0075	0.0025	0.0025	0.0035	0.0035	4.536
16	1	3	1	3	0.85	0.85	0.85	0.85	0.5	0.01	0.01	0.01	0.09	0.0025	0.0075	0.0025	0.0025	0.0035	0.0035	0.0025	4.316
17	3	3	1	1	0.85	0.85	0.25	0.85	0.5	0.09	0.09	0.09	0.09	0.0075	0.0025	0.0025	0.0025	0.0025	0.0035	0.0025	4.693
18	3	3	3	3	0.25	0.25	0.25	0.25	0.9	0.09	0.01	0.09	0.01	0.0075	0.0075	0.0075	0.0075	0.0035	0.0025	0.0025	4.311
19	1	3	1	1	0.85	0.85	0.25	0.25	0.9	0.09	0.01	0.09	0.01	0.0075	0.0075	0.0075	0.0075	0.0035	0.0025	0.0025	4.311
20	1	1	1	3	0.25	0.85	0.25	0.25	0.9	0.09	0.01	0.01	0.09	0.0075	0.0025	0.0075	0.0025	0.0035	0.0035	0.0035	4.243
21	1	1	1	1	0.85	0.25	0.85	0.25	0.5	0.09	0.09	0.01	0.01	0.0075	0.0075	0.0025	0.0075	0.0025	0.0035	0.0035	4.321
22	1	3	1	3	0.25	0.25	0.85	0.85	0.5	0.01	0.09	0.09	0.01	0.0075	0.0025	0.0075	0.0075	0.0035	0.0035	0.0035	4.272
23	3	1	1	3	0.85	0.25	0.25	0.85	0.9	0.01	0.09	0.01	0.09	0.0075	0.0075	0.0075	0.0075	0.0025	0.0025	0.0025	4.534
24	3	1	3	1	0.25	0.85	0.85	0.25	0.5	0.09	0.09	0.01	0.09	0.0025	0.0075	0.0075	0.0075	0.0035	0.0035	0.0025	4.321

A: cane molasses, B: glucose, C: fructose, D: lactose, E: yeast extract, F: peptone, G: NH_4Cl, H: $NaNO_3$, I: NaCl, J: KH_2PO_4, K: $MgSO_4$, L: $FeCl_3$, M: $CaCl_2$, N: L-Asn, O: L-Gly, P: L-Cys, Q: L-Gln, R: Vit B1, S: Ascorbic acid, T: Vit. B12, Y: EPS (gL^{-1})

man design was employed for preliminary screening of nutrients obtained through one-factor-at a time design. The averages of EPS yield (g/*l*) for 24 different trials of 20 medium components are represented in Table (1).

The variables which had a significant effect ($p<0.05$) were observed to be involved in EPS synthesis. The most significant four variables, namely cane molasses (sucrose substitute), yeast extract, sodium chloride and calcium chloride, were used for the further optimization as they were found to be highly effective in biopolymer production. Though Vitamin B_{12} and ascorbic acid were significant, they did not aid in enhancing the yield of EPS, hence were neglected from the further optimization process (Table 2), and thus were not included in this medium formulation as nutrients.

Based on the results of design, a polynomial, first-order equation was developed, excluding the insignificant variables, describing the correlation between the variables used for study. The EPS yield, Y (g/*l*) could be represented as

$$Y\ (\text{EPS}\ gL^{-1}) = 3.267 + 0.114\,A + 0.148\,E + 0.345\,I + 1.077\,M + 1.713\,N + 6.65\,S$$

EPS depends on C/N ratio of the growth medium; more carbon and limiting nitrogen sources elevate the production of the biopolymer. Cane molasses and yeast extract helped the microorganism in its growth. Cane molasses acts as the main carbon substrate, and EPS production by *Bacillus subtilis* is ascertained to be growth associated, as identified through one-factor-at-a-time process for parameter estimation through our previous study [21], substantially enhanced

Table 2. Statistical analysis of Plackett-Burman Design showing coefficient, T and P values for each variable

Term	Coefficient	T	P
Intercept	4.363	429.44	0.000
Cane molasses (A)	0.113	11.17	0.002
Glucose (B)	0.065	6.44	0.910
Fructose (C)	−0.007	−0.66	0.554
Lactose (D)	0.001	0.12	0.078
Yeast Extract (E)	0.044	4.36	0.022
Peptone (F)	−0.015	−1.48	0.234
NH_4Cl (G)	0.029	2.91	0.062
$NaNO_3$ (H)	0.007	0.73	0.518
NaCl (I)	0.069	6.80	0.007
KH_2PO_4 (J)	0.032	3.16	0.059
$MgSO_4$ (K)	0.035	3.47	0.069
$FeCl_3$ (L)	0.013	1.25	0.301
$CaCl_2$ (M)	0.043	4.24	0.024
L-Asn (N)	0.043	4.22	0.709
L-Gly (O)	0.004	0.41	0.051
L-Cys (P)	−0.023	−2.23	0.112
L-Gln (Q)	−0.020	−1.98	0.142
Vit. B_1 (R)	−0.013	−1.26	0.296
Ascorbic acid (S)	0.033	3.27	0.047
Vit. B_{12} (T)	0.017	1.67	0.194

S=0.0498 **PRESS**=0.475 $\mathbf{R^2}$=99.09% **Adj.** $\mathbf{R^2}$=93.02%

EPS production. During the synthesis enzymes involved in breaking down sucrose could have been induced, due to which EPS yield had enhanced [28]. Commercially available sucrose had been used as the main carbon source in various reports. *B. licheniformis*, a type of *B. subtilis*, produced levan (0.36 gL^{-1}) using sucrose in medium, cleaving the carbon source into levan and glucose with the aid of levan sucrase [11]. Sucrose would be used by bacterial cells for maintenance and growth [11]. Many kinds of agrowaste, rich in sugars, have been employed to learn their effects on fermentation of various products. Cane molasses, an agro residue of sugar factories, was used as the low cost supplementary for the production of EPS. Molasses is effective as it consists of certain amount of vitamins and minerals, thus possessing significant growth stimulatory effect [29]. Homopolysaccharide was produced by *Weisella* strains, taking up sucrose from sugarcane molasses and white sugar, giving comparatively higher yield of 8.65 gL^{-1} than synthetic sucrose [30]. *Zymomonas mobilis* was able to synthesize levan of 40.14 gL^{-1}, using sugarcane molasses catalyzed by levansucrase [31]. Due to its many advantages like high sucrose and other nutrient contents, low cost, ready availability, and ease of storage, molasses has been used as a substrate for fermentation production of commercial polysaccharides like curdlan, xanthan, dextran, scleroglucan, and gellan [32]. Previous experiments reported on the use of molasses for EPS production. B. cereus B-11 was able to produce biopolymers in a medium containing molasses waste-water, replacing glucose and yielding 500 mg/L [33]. A fungus, Mucor rouxii, produced 87% EPS in medium with 3% beet molasses [34]. A. pullulans produced 16.9% pullulan in molasses medium with initial sugar concentration of 50 g/L at pH 7.5 [35]. Azotobacter was able to produce 7.5 mg EPS/mL of medium with 2% beet molasses [36]. Nitrogen sources, in a lesser quantity, were required for the biopolymer generation. Yeast extract was found to be the best nitrogen source for this fermentation. Organic nitrogen components yielded larger amount of EPS than that of inorganic sources. This might be due to the reason that cells absorb organic nitrogen sources easier than the inorganic ones [37]. It was also suggested that the essential amino acids cannot be synthesized from inorganic nitrogen [37]; thus EPS production might have been retarded. Yeast extract has been often used to provide

Table 3. Central Composite Design with experimental and predicted responses

Runs	Coded levels (actual levels)				EPS (gL^{-1})	
	X_1 (Sucrose) gL^{-1}	X_2 (Yeast extract) gL^{-1}	X_3 (NaCl) gL^{-1}	X_4 ($CaCl_2$) gL^{-1}	Observed value	Predicted value
1	0(20)	0 (5)	−2(3)	0(0.5)	4.43	4.21
2	−1(15)	1(7)	1(9)	1(0.7)	3.76	3.86
3	1(25)	1(7)	1(9)	1(0.7)	4.52	4.38
4	0(20)	0(5)	0(7)	−2(0.1)	4.17	3.92
5	1(25)	1(7)	1(9)	−1(0.3)	4.16	4.19
6	1(25)	−1(3)	1(9)	−1(0.3)	3.62	3.85
7	2(30)	0(5)	0(7)	0(0.5)	3.51	3.48
8	1(25)	1(7)	−1(5)	1(0.7)	3.92	4.03
9	1(25)	−1(3)	−1(5)	1(0.7)	3.89	3.88
10	−1(15)	−1(3)	−1(5)	−1(0.3)	3.49	3.73
11	−2(10)	0(5)	0(7)	0(0.5)	2.96	2.81
12	0(20)	0(5)	2(11)	0(0.5)	4.32	4.36
13	1(25)	−1(3)	−1(5)	−1(0.3)	3.89	3.88
14	−1(15)	−1(3)	1(9)	−1(0.3)	3.54	3.52
15	−1(15)	1(7)	1(9)	−1(0.3)	3.79	3.76
16	0(20)	−2(1)	0(7)	0(0.5)	4.09	4.16
17	1(25)	−1(3)	1(9)	1(0.7)	4.21	4.08
18	−1(15)	1(7)	−1(5)	1(0.7)	3.82	3.68
19	1(25)	1(7)	−1(5)	−1(0.3)	3.91	3.94
20	0(20)	2(9)	0(7)	0(0.5)	4.15	4.16
21	−1(15)	1(7)	−1(5)	−1(0.3)	3.47	3.68
22	−1(15)	−1(3)	1(9)	1(0.7)	3.60	3.67
23	−1(15)	−1(3)	−1(5)	1(0.7)	3.72	3.77
24	0(20)	0(5)	0(7)	2(0.9)	4.09	3.91
25	0(20)	0(5)	0(7)	0(0.5)	4.92	4.92
26	0(20)	0(5)	0(7)	0(0.5)	4.92	4.92
27	0(20)	0(5)	0(7)	0(0.5)	4.92	4.92
28	0(20)	0(5)	0(7)	0(0.5)	4.92	4.92
29	0(20)	0(5)	0(7)	0(0.5)	4.92	4.92
30	0(20)	0(5)	0(7)	0(0.5)	4.92	4.92

necessary growth factors, but higher concentration of this could deteriorate the use of carbon sources, causing reduction of metabolites [38]. Impact of salinity on EPS synthesis was also studied. NaCl had a strong influence on the production, increasing the growth and metabolite production, when compared to the NaCl-free medium. This might be due to its importance in maintaining the bacterial cells' osmotic stability. The study of effect of minerals on EPS yield revealed that $CaCl_2$ was found to be the most influential mineral. Cations generally influence the production qualitatively and quantitatively [39]. Ca^{2+} had the ability to enhance the biofilm formation, aiding as an inducer during metabolism, hence providing with a maximum yield of exopolymeric substance.

2. Optimization of Components for EPS Production Using RSM

Sucrose, yeast extract, NaCl and $CaCl_2$ were eventually screened to be the most significant factors based on the results of Plackett Burman design, identified from 20 variables, which were screened using the one-factor-at-a-time method. The CCD for four variables with six central points and the relevant data are represented (Table 3). An overall second-order polynomial equation was developed for the EPS production as represented below:

$$Y\,(EPS)=4.92+0.17X_1+0.063X_2+0.036X_3+0.059X_4+0.026X_1X_2 +0.044X_1X_3+0.022X_1X_4+0.071X_2X_3-0.012X_2X_4 +0.024X_3X_4-0.44\,X_1^2-0.22\,X_2^2-0.16X_3^2-0.22X_4^2$$

where, Y is the EPS yield, X_1 is sucrose, X_2 is yeast extract, X_3 is NaCl, X_4 is $CaCl_2$ respectively.

The goodness of fit of regression equation developed could be measured by adjusted determination coefficient. The R^2 value of 0.9480 and adjusted R^2 of 0.8995 show that the model could be significant predicting the response and explaining 95% of the variability in the EPS synthesis. The predicted R^2 of 0.7006 is in reasonable agreement with the Adjusted R^2 of 0.8995. Adequate precision measures the signal, i.e., response to noise (deviation) ratio. A ratio greater than 4 is desirable. The ratio of 17.713 indicates an adequate signal for this model. The statistical significance of the equation was evaluated by F-test and ANOVA (analysis of variance) which showed that the model was statistically significant at 95% confidence level ($p<0.05$). ANOVA reported the model F-value of 19.54, implying that the modelis significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise (Table 4).

P-value denotes the importance of each coefficient, helping in understanding the interactions among the variables. The most significant factors of this model are X_1, X_1^2, X_2^2, X_3^2 and X_4^2. Values of p greater than F and less than 0.0500 indicate model terms are significant. P-values greater than 0.1000 indicate the model terms are not significant. The model also depicted the statistically nonsignificant lack of fit ($p>0.05$), indicating that the responses are adequate for employing in this model.

Three-dimensional response surface plots represent regression equations and illustrate the interactions between the response and experimental levels of each variable. These plots let us locate the optimum levels of each variable for the highest EPS yield. Fig. 1 illustrates the response surface plots and represents the pairwise interaction of the four variables. Higher interaction between sucrose, yeast extract resulted in larger significance of EPS production. From this optimization study, the optimal concentration of sucrose, yeast extract, sodium chloride and $CaCl_2$ was 2.36, 0.56, 0.71 and 0.05 g/*l*, respectively. The maximum production was estimated to be 4.92 gL^{-1} and the actual production obtained with the optimal medium was also 4.92 gL^{-1}, which is in complete agreement with the prediction of the model.

Table 4. Analysis of variance of optimisation of EPS production by *B. subtilis*

Source	Sum of squares	df	Mean square	F value	P-value
Model	7.76	14	0.55	19.54	0.0001
X_1	0.68	1	0.68	23.85	0.0002
X_2	0.095	1	0.095	3.35	0.0872
X_3	0.032	1	0.032	1.11	0.3084
X_4	0.083	1	0.083	2.92	0.1081
X_1X_2	0.011	1	0.011	0.37	0.5519
X_1X_3	0.032	1	0.032	1.11	0.3086
X_1X_4	0.007	1	0.007	0.27	0.6110
X_2X_3	0.080	1	0.080	2.81	0.1142
X_2X_4	0.002	1	0.002	0.08	0.7818
X_3X_4	0.01	1	0.01	0.34	0.5713
X_1^2	5.39	1	5.39	190.12	0.0001
X_2^2	1.35	1	1.35	47.73	0.0001
X_3^2	0.69	1	0.69	24.27	0.0002
X_4^2	1.32	1	1.32	46.66	0.0001
Residual	0.43	15	0.028		
Lack of fit	0.63	10	0.063		
Pure error	0.000	5	0.000		
Cor total	8.19	29			

Std. Dev.	0.17	R^2	0.9480
Mean	4.09	Adjusted R^2	0.8995
C.V. %	4.12	Predicted R^2	0.7006
PRESS	2.45	Adequate Precision	17.713

The model was validated by carrying out three experiments in non-agitated, optimized medium formulation for EPS production. The mean value obtained was 4.89 gL^{-1}, which was in good agreement with the predicted response.

3. Characterization of EPS

Analysis by phenol sulfuric acid method showed that the EPS constituted 81.83 (w/w) of total sugars. This indicated that the biopolymer extracted was majorly a polysaccharide. The isolated and lyophilized EPS sample was subjected to FTIR spectrometric analysis, revealing the presence of functional groups in it (Fig. 2). An intense peak at 3,307 cm^{-1} denoted the presence of hydroxyl groups. A vibrational stretching band was observed at 2,975 cm^{-1} indicating -C-H group. -C=O- vibrational stretching of carboxyl group was identified in the band at 1,651-1,400 cm^{-1}. An absorption peak at 1,261 cm^{-1} indicated -C=O- stretching in alcohol. Band at 1,058 cm^{-1} was due to methoxyl group. The absorption peaks between 1,100-1,000 cm^{-1} were characteristic for most of the sugar moieties [39-41].

4. DPPH Scavenging Activity

This activity results in the reduction of stable DPPH radical (purple) to non-radical DPPH-H (yellow) form [26,27]. The isolated EPS along with the reference antioxidant was checked for their DPPH

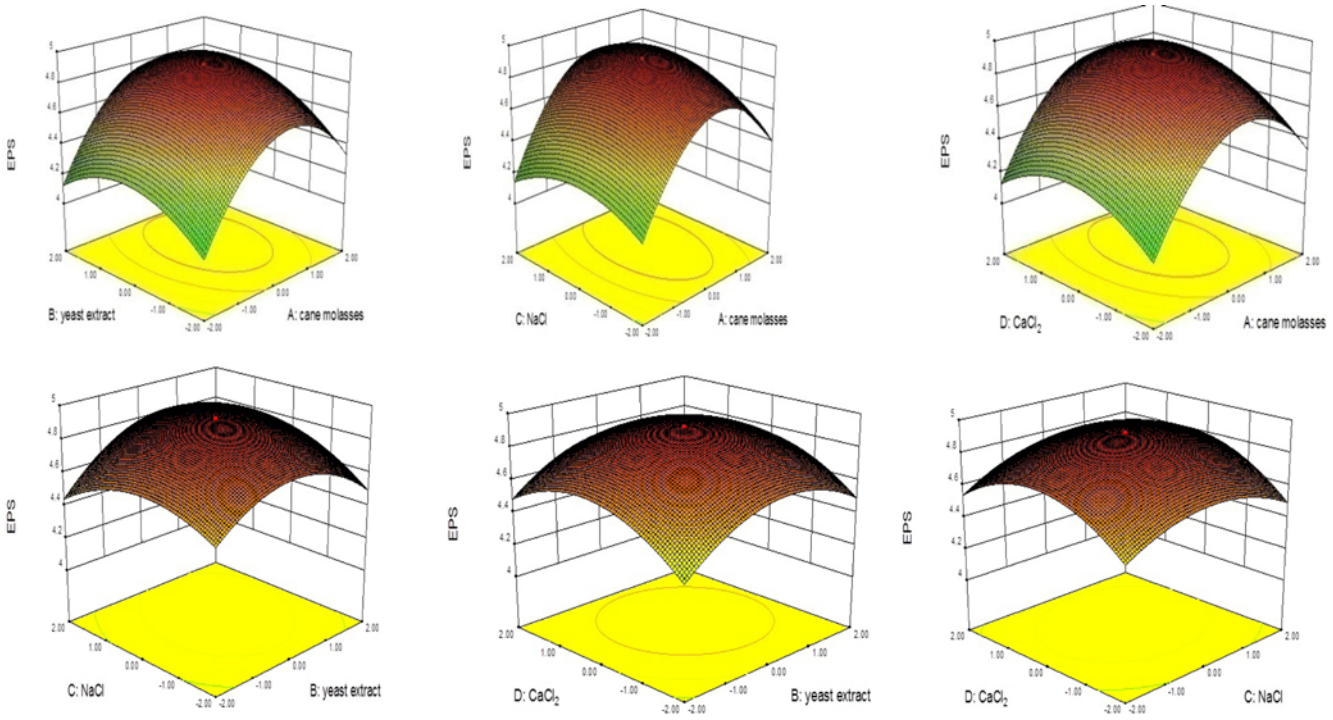

Fig. 1. Response surface three-dimensional plots of the interactive effects of variables, Cane molasses (X_1), Yeast extract (X_2), NaCl (X_3) and $CaCl_2$ (X_4), on EPS production.

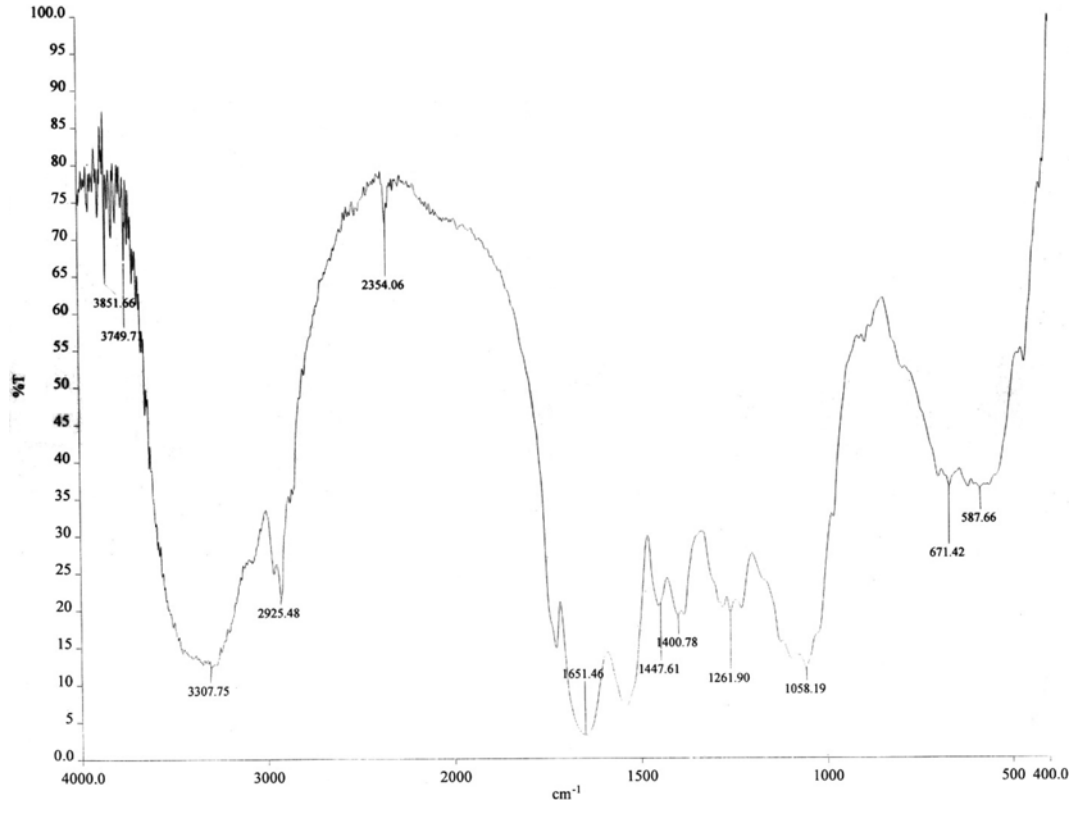

Fig. 2. FTIR spectrum of lyophilized EPS.

reducing capability. The crude EPS was found to be a stronger antioxidant than the standard Vitamin C (Vc). In the present study, the maximum antioxidant activity of EPS, as shown in Fig. 3, was at the concentration of 0.8 mg/ml with a maximum percentage inhibition of 61.19%, which was comparable with that of reference (32.81%). As the concentration increased, the reducing capacity also elevated,

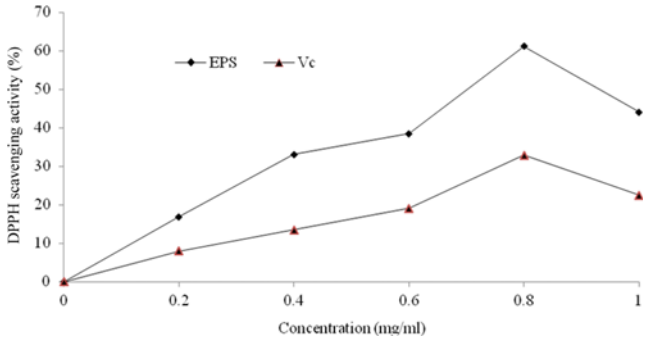

Fig. 3. Representing DPPH scavenging activity of EPS.

but after the concentration of 0.8 mg/ml, the ability deteriorated. Though similar works showed high reducing effects, our investigation showed greater activity at very low concentrations, in contrast to various reports. *Paenibacillus* sp. TKU023 showed a maximum effect at 9 gL^{-1} [42]. *Paenibacillus polymyxa* EJS 3 also had a strong antioxidant activity at a concentration of 4 mg/ml [24]. An endophytic fungus, *Aspergillus* sp. Y16 potentially reduced DPPH, but highest effect of 42.79% was observed only at 10 mg/ml [43]. Reducing ability of a compound is the most essential factor of antioxidant activity. The activity is apparently due to the presence of reducing sugars or monosaccharides in the biopolymer [37]. Along with the sugars, presence of trace amounts of proteins, amino acids and other organic components might effectively play a role in the scavenging activity [44,45]. The scavenging activity is also due to hydrogen donating ability [24]. The results clearly indicated that the extracted EPS was a potent antioxidant.

CONCLUSION

The present investigation dealt with the production of exopolysaccharide from an isolated soil bacterium, *Bacillus subtilis*. The present process was focused on cost effective production of EPS using agricultural waste, cane molasses as carbon substrate. RSM was carried out using CCD to optimize the medium components which were screened by Plackett Burman design. The experiments found the most significant factors that highly affected the production as Cane molasses, Yeast extract, NaCl and $CaCl_2$, yielding 4.92 g of EPS/L. Characterization of EPS by FTIR Spectrophotometer revealed the presence of functional groups indicating sugar moieties. The isolated EPS could be a potential source of various biological activities, as the study clearly disclosed the role of EPS as an antioxidant and also it would be an efficient alternative for synthetic polymers.

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