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# Influence Of Various Parameters On Exopolysaccharide Production From Bacillus subtilis

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**Abstract:** Optimization of different parameters namely temperature, pH, incubation time and inoculum concentration was carried out in the present investigation, to enhance the production of exopolysaccharide by *Bacillus subtilis*. A one factor at a time method was performed to obtain the central values for the further optimization by Response Surface Methodology (RSM). A Central Composite Design was developed to study the interactive effects of the four significant variables on EPS production. The maximum production was found to be 5.56 g $L^{-1}$. The optimal parameter values were temperature $35^0$C, pH 7, incubation time 72h and inoculum concentration 2 ml. This set of experiment gave 5.59 g $L^{-1}$ which was in good agreement with the predicted value of 5.62 g $L^{-1}$.The structure of extracted EPS was investigated by FTIR spectroscopy showing the presence of sucrose units. The extracted polysaccharide could thus be an ecofriendly product which can be subjected to various industrial and pharmaceutical applications.
**Keywords:** *Bacillus subtilis,* Exopolysaccharide, Central Composite design, FTIR Spectroscopy.

## 1. Introduction

Microorganisms synthesise a wide spectrum of multifunctional polysaccharides including polysaccharides, structural polysaccharides and extracellular polysaccharides (EPS) [1]. EPSs of microorganisms has generated greater attention of researchers due to then diversified applications and advantages of their complexity. Bacterial exopolysaccharides are primarily the constituents of glycocalyx, with which the cells adhered to surfaces [3]. These form the major components of the biofilms [4]. Microbial EPS may be non-ionic or may process cationic, anionic or neutral charges. Various applications make EPS advantageous. EPS are found to the involved in pharmaceutical industrial as they are immunomodulatory, antiviral, antiulcer and antioxidants and used as matrix for sustained release of drugs, also involve in pathogenesis, symbiosis, protecting against osmotic shock, toxic stress and antibacterial compounds [5,6]. In food industry, EPSs are used as stabilizers, emulsifiers, gelling agents [7]. Contain 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 constitute less to environmental pollution than synthetic polymers [10].

EPS exhibit remarkable thickening and shear thinning properties and display high intrinsic viscosity. EPS synthesized by *Bacillus* spp. has comparatively elevated viscosity and superior pseudo plastic properties [11].

Bacillus sp. produces complex EPSs, which could be homopolysaccharide with repeated similar units of sugar or heteropolysaccharide containing different sugar moieties. Widely studied species for EPS production are *B.licheniformis*, producing levan [11], *B. coagulans*, producing a heteropolysaccharide, *B. polymyxa* [12], *B. megaterium* [13] and *B. mucilaginosus* [14].

*Bacillus subtilis* is also one of the major producers of EPS among Bacillus sp. This organism essentially constitute for studies involved in biofilms formation. In major investigations, *B.subtilis* is mostly found to produce a biopolymer poly-γ-Glutamate (PGA) [15,16], levan fructan is produced by a strain *B.subtilis* natto [17].

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 favoured 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].

Optimization of the parameters for fermentation process could be carried out 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 variable constant. RSM tool is reliable, a fast experimentation technique and provides with individual effects of nutrients and their interactive effects. For the production of EPS from *B.subtilis*, a Central Composite Design was used to optimize the definite level of the significant parameters.

## 2. Materials and Methods

### 2.1 Bacterial culture isolation

Soil sample, 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 sterile nutrient agar plates composed of peptone (5g/l), yeast extract (2g/l) NaCl (5g/l) and Agar (20g/l). The fixed pH was adjusted to 7 using 0.1N NaOH and diluted HCl. The inoculated plates were incubated at $37^0$C for 24 hr. After incubation, colonies that were cream colored, highly mucoid with undulated edges 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*. The organism was stored on nutrient agar slant and maintained at $4^0$C for further studies. One loopful of culture was taken and inoculated in an Erlenmeyer flask with 50ml broth, which was incubated at $37^0$ C for 24 h. This overnight culture was being used for the further optimization studies.

### 2.2 One factor at a time experiments

The medium comprised the following components: cane molasses, 2%; yeast extract, 0.5%; NaCl, 0.7% and $CaCl_2$, 0.05%. Commercial sucrose was replaced by cane molasses which has higher sucrose content, is an agro waste and cost effective.

One factor at a time method was carried out to examine the parameters influencing the growth of the culture and fermentation of the product. Overnight culture of *B. subtilis* was used to inoculate the sterilized culture medium. To study the effect of temperature for EPS production, the inoculated culture broths were incubated at 10, 15, 20, 25, 30, 35, 40, 45 and 50ºC. The initial pH for higher product yield was determined by adjusting pH with 1M HCl and 1M NaOH before sterilization at different pH as 1, 4, 7, 10 and 13. Different concentrations of inoculum were added to the medium to study its influence at varying concentrations like 1, 2, 3, 4 and 5 %. The effect of incubation time was investigated by varying time periods as 24, 48, 72, 96 and 120 h. For the study of effects of temperature, pH and time, 1% culture was used as constant inoculum size.

### 2.3 Response Surface Methodology (RSM)

Response surface methodology was used to determine the optimum concentration of these variables for the enhancement of the production of EPS. The significant variables (temperature, pH, incubation time and inoculum concentration) were optimized by Central Composite Design (CCD). The four independent variables were evaluated at five different levels (-2, -1, 0, +1 and +2) and 30 experiments, containing 6 replications at the center point.

The behavior of the system was explained by the following second order polynomial equation,

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

where, Y is the predicted response, $\beta_0$ is the scaling constant, $X_1$- $X_4$ are the coded levels of the factors, $\beta_1$ - $\beta_4$ are the linear coefficients, $\beta_{12}$- $\beta_{34}$ are the interactive coefficients and $\beta_{11}$- $\beta_{44}$ are the quadratic coefficients.

The responses were analysed using the software-Design Expert, version 8.0.7.1 trial (Stat – Ease, Inc., Minneapolis, USA). ANOVA and regression analyses were also carried out used the above software. The quality of the polynomial equation was confirmed by the determination of coefficient $R^2$ and its statistical significance was determined by Fisher's test, F value.

### 2.4 Production and Isolation of exopolysaccharides

EPS production was carried out in inoculated, unagitated 50ml medium as per the framed experimental design and incubated according to the runs and the EPS content was calculated. EPS was extracted by precipitation method using ethanol. The culture was centrifuged at 11,000 rpm for 10 min at $4^0$C. The supernatant obtained was mixed with two volumes of ice cold ethanol and kept at $4^0$ C for 24 hr. The mixture was then centrifuged at 2500 rpm for 20 min at $4^0$C. The obtained pellet was suspended in distilled water, which was centrifuged at 2500 rpm for 30 min at $4^0$ C with two volumes of ice cold ethanol [21]. 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 [22] using glucose as standard. To check the presence of proteins, concentration of protein was estimated using Lowry et al method with BSA as standard [23].

### 2.5 FTIR Spectroscopy analysis

A quantity of 50mg of lyophilized EPS was taken, mixed with 150mg of KBR powder and ground well to fine mixture. The mixture was pressed to a disc using a hydraulic press. The disc was subjected to FTIR spectral measurement in the frequency range of 4000-400$cm^{-1}$. The exopolysaccharide was characterized using a Fourier Transfer Infrared Spectrophotometer (Bruker Optics GmBH, Germany).

## 3. Results and discussion

### 3.1 One factor at a time

The present study was carried out to study the influence of various parameters on elevation of EPS yield by *B. subtilis* in agro waste containing medium. One factor at a time, although difficult and labor intensive method, this experiment aided in selecting the center points for the optimization study using RSM. This method of using single variables is disadvantageous as the interactive effects between two factors cannot be studied.

### 3.2 Central Composite Design – Response Analysis

Exopolysaccharide was produced by conventional batch fermentation involving cultures growth and EPS synthesis, which was extracted by precipitating with ethanol. The responses obtained under varying temperature, pH, incubation time and inoculum concentration are represented in Table 1.

**Table 1: Central Composite Design matrix of four variables with experimental response data showing coded, actual and response values**

| Run Order | $X_1$ Temperature ($^0$C) | $X_2$ Time (h) | $X_3$ pH | $X_4$ Inoculum concentration (ml) | Y EPS ( g $L^{-1}$) |
|---|---|---|---|---|---|
| 1 | 1.00 (38) | -1.00 (60) | -1.00 (6) | 1.00 (2.5) | 4.78 |
| 2 | 0.00 (35) | 0.00 (72) | 0.00 (7) | 0.00 (2) | 5.56 |
| 3 | 0.00 (35) | 2.00 (96) | 0.00 (7) | 0.00 (2) | 4.61 |
| 4 | 0.00 (35) | 0.00 (72) | 0.00 (7) | 0.00 (2) | 5.56 |
| 5 | 1.00 (38) | 1.00 (84) | 1.00 (8) | -1.00 (1.5) | 4.86 |
| 6 | -1.00 (32) | -1.00 (60) | 1.00 (8) | 1.00 (2.5) | 4.54 |
| 7 | 0.00 (35) | 0.00 (72) | 0.00 (7) | -2.00 (1) | 3.69 |
| 8 | 0.00 (35) | 0.00 (72) | 0.00 (7) | 2.00 (3) | 4.06 |
| 9 | 0.00 (35) | 0.00 (72) | 0.00 (7) | 0.00 (2) | 5.56 |
| 10 | 1.00 (38) | 1.00 (84) | -1.00 (6) | 1.00 (2.5) | 4.94 |
| 11 | -1.00 (32) | -1.00 (60) | -1.00 (6) | -1.00 (1.5) | 3.76 |
| 12 | -1.00 (32) | 1.00 (84) | 1.00 (8) | 1.00 (2.5) | 4.63 |
| 13 | 1.00 (38) | -1.00 (60) | 1.00 (8) | -1.00 (1.5) | 4.81 |
| 14 | 0.00 (35) | 0.00 (72) | 2.00 (9) | 0.00 (2) | 4.24 |
| 15 | -2.00 (29) | 0.00 (72) | 0.00 (7) | 0.00 (2) | 3.51 |
| 16 | 0.00 (35) | 0.00 (72) | 0.00 (7) | 0.00 (2) | 5.56 |
| 17 | -1.00 (32) | 1.00 (84) | -1.00 (6) | 1.00 (2.5) | 4.9 |
| 18 | 1.00 (38) | 1.00 (84) | -1.00 (6) | -1.00 (1.5) | 4.78 |
| 19 | 0.00 (35) | 0.00 (72) | 0.00 (7) | 0.00 (2) | 5.56 |
| 20 | 0.00 (35) | 0.00 (72) | 0.00 (7) | 0.00 (2) | 5.56 |
| 21 | 2.00 (41) | 0.00 (72) | 0.00 (7) | 0.00 (2) | 3.46 |
| 22 | 0.00 (35) | -2.00 (48) | 0.00 (7) | 0.00 (2) | 3.81 |
| 23 | -1.00 (32) | 1.00 (84) | -1.00 (6) | -1.00 (1.5) | 4.62 |
| 24 | -1.00 (32) | -1.00 (60) | 1.00 (8) | -1.00 (1.5) | 4.69 |
| 25 | 1.00 (38) | -1.00 (60) | 1.00 (8) | 1.00 (2.5) | 5.02 |
| 26 | -1.00 (32) | -1.00 (60) | -1.00 (6) | 1.00 (2.5) | 4.42 |
| 27 | 0.00 (35) | 0.00 (72) | -2.00 (5) | 0.00 (2) | 3.98 |
| 28 | -1.00 (32) | 1.00 (84) | 1.00 (8) | -1.00 (1.5) | 4.61 |
| 29 | 1.00 (38) | -1.00 (60) | -1.00 (6) | -1.00 (1.5) | 4.32 |
| 30 | 1.00 (38) | 1.00 (84) | 1.00 (8) | 1.00 (2.5) | 5.15 |

Based on the results of the experimental designs, a second order polynomial equation was developed, describing the correlation between the variables used for study. The EPS yield, Y (g/l) could be represented as

$$Y = 5.56 + 0.21\ x_1 + 0.2\ x_2 + 0.14\ x_3 + 0.19\ x_4 + 0.028\ x_1x_2 - 0.019\ x_1x_3 + 0.019\ x_1x_4 + 0.043x_2x_3 + 0.012\ x_2x_4 + 0.004\ x_3x_4 - 0.38\ x_1^2 - 0.28\ x_2^2 - 0.35\ x_3^2 - 0.3\ x_4^2$$

ANOVA and regression analysis done for the experimental designs are tabulated (Table 2). Correlation measure for testing the goodness of fit of regression equation is the adjusted determination coefficient, $R^2$. For the experimental designs performed, the second order model showed a good fit with $R^2 = 0.9551$ and adjusted $R^2 = 0.9132$, indication a high correlation between the experimental and observed values for EPS production. The coefficient $R^2$ indicated that only 4.49% of the model was affected by the variable. The larger t-value and smaller P value suggest higher significance of the corresponding coefficient. The regression model showed that the model was highly significant which was confirmed from the evaluated F value-22.79 and probability values ($p < 0.0001$). The model also showed lack of fit ($p < 0.05$), which demonstrated that predicted errors were more than errors of replicas involved in the model. A lower value of coefficient of variation (CV = 4.27) also indicated better precision and reliability of experiments performed.

**Table 2 : ANOVA and regression analysis of the experiment**

| Source | Sum of Squares | DF | Mean Square | F value | Prob > F |
|---|---|---|---|---|---|
| Model | 11.85 | 14 | 0.85 | 22.79 | < 0.0001 |
| $X_1$ | 1.11 | 1 | 1.11 | 29.76 | < 0.0001 |
| $X_2$ | 0.92 | 1 | 0.92 | 24.68 | 0.0002 |
| $X_3$ | 0.46 | 1 | 0.46 | 12.29 | 0.0032 |
| $X_4$ | 0.83 | 1 | 0.83 | 22.42 | 0.0003 |
| $X_1X_2$ | 0.013 | 1 | 0.013 | 0.34 | 0.5680 |
| $X_1X_3$ | 0.005 | 1 | 0.005 | 0.001 | 0.9695 |
| $X_1X_4$ | 0.005 | 1 | 0.005 | 0.001 | 0.9695 |
| $X_2X_3$ | 0.030 | 1 | 0.030 | 0.80 | 0.3848 |
| $X_2X_4$ | 0.002 | 1 | 0.002 | 0.061 | 0.8086 |
| $X_3X_4$ | 0.003 | 1 | 0.003 | 0.008 | 0.9288 |
| $X_1^2$ | 3.93 | 1 | 3.93 | 105.91 | < 0.0001 |
| $X_2^2$ | 2.15 | 1 | 2.15 | 57.87 | < 0.0001 |
| $X_3^2$ | 3.43 | 1 | 3.43 | 92.39 | < 0.0001 |
| $X_4^2$ | 2.51 | 1 | 2.51 | 67.55 | < 0.0001 |
| Residual | 0.56 | 15 | 0.037 | | |
| Lack of Fit | 0.56 | 10 | 0.056 | | |
| Pure Error | 0.000 | 5 | 0.000 | | |
| Cor Total | 12.41 | 29 | | | |

**$X_1$ :** temperature , **$X_2$** : pH , **$X_3$ :** Time **, $X_4$:** Inoculum concnentration

| | | | |
|---|---|---|---|
| Std. Dev. | 0.19 | R-Squared | 0.9551 |
| Mean | 4.51 | Adj R-Squared | 0.9132 |
| C.V. % | 4.27 | Pred R-Squared | 0.7414 |
| PRESS | 3.21 | | |

Three dimensional response surface plots represent regression equations and illustrate interaction between the response and experimental levels of each variable. Two dimensional contour plots are graphical representations of regression equation. A circular plot indicates that the interaction is negligible and an elliptical plot represents the significance of the interactions between corresponding factors. Figure 1 illustrates the response surface and contour plots exhibiting interaction of the four variables.

The graphical plots showed that interactions between the variables were elliptical indicating that the significance of the factors on the EPS production. The results showed that the EPS yield varied between 3.51 – 5.56 g $L^{-1}$. Maximum productivity of EPS was achieved at central point conditions of 35$^{\circ}$C and pH 7 at 72h with 2 % inoculums (5.56 g $L^{-1}$). The production decreased under other conditions, irrespective of higher or lower than the central points.

Environmental parameters - temperature and pH play a vital role in the synthesis of exopolysaccharide. pH is a significant factor influencing the physiology of a microorganism by affecting nutrient solubility and uptake, enzyme activity, cell membrane morphology, by product formation and oxidative- reductive reactions [24]. Higher temperature could not help the growth of the organism, since the stability of the cell structure might be affected. Since *Bacillus subtilis* is mesophilic, decrease in temperature did not enhance the multiplication of cells and since the biosynthetic pathway of exopolysaccharides would be inhibited below optimal temperature [25], lesser production of EPS was observed. Incubation time is an essential factor determining the enhancement of EPS synthesis in the culture. As EPS is highly synthesized during late exponential growth phase or in the stationary phase, decrease in incubation time may lower the production. Higher incubation time might affect the yield due to the production of certain enzymes, saccharases, along with EPS, might act upon polysaccharides, thus deteriorating the product formation.

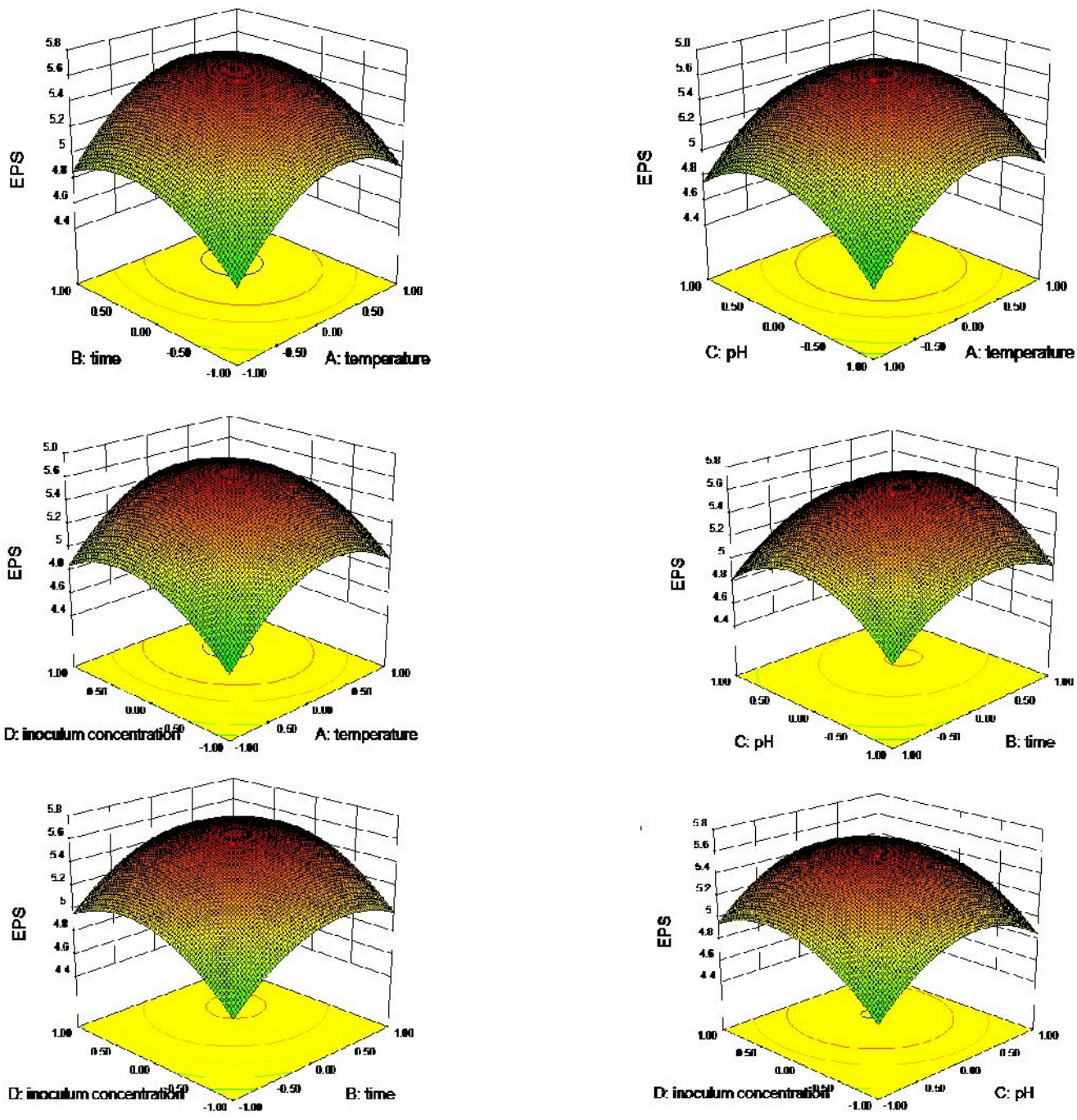


**Figure 1: Response and Contour plots of the interactive effects of four significant variables**

The obtained results from the present work are consistent with the similar studies carried out earlier. *B.subtilis* produced polysaccharide (1.58±0.13mg/100ml) at 72h when temperature was $37^0$C when cultured in basal medium [26]. Optimal pH was 7-8 for the batch fermentation of EPS, for 31 h, produced by *B. polymyxa* (33 g $L^{-1}$) in sucrose containing medium. Relatively low concentration of EPS was obtained, during this study, when pH lowered from 7 to 4.5, since the medium turned acidic [12]. A temperature range of 30-$35^0$C and pH 7 were found to be optimum for the production of exopolymer by Bacillus sp. [13]. *Enterobacter* sp. synthesized fucose containing polysaccharides at a temperature range of 30-$35^0$C and pH ranging between 6 and 8 [27].

The optimized values of the parameters were validated by batch process in duplicate sets of experiments and the maximum EPS production was 5.59 g $L^{-1}$, which was in good agreement with the predicted value, 5.62 g $L^{-1}$.

### 3.3 Characterisation of EPS

The sugar content was estimated to be 81.81% of total carbohydrates in lyophilized EPS. Proteins were analyzed and accounted to only 9.02% of total EPS content, confirming that the extracted was majorly a polysaccharide. The vibrational spectral analysis plays a major role in identifying the sugars. Analysis of composition of EPS, isolated in the present study, by FTIR spectroscopy revealed the presence of the functional groups (Figure 2). Absorption peak at 3494.91 indicated the presence of OH groups. Ester group is assigned to peak 1665.00. Vibrational stretch of C-O-C group is found at absorption peak 1058.48. An absence of β configuration was indicated as there was no absorption peak at 890 $cm^{-1}$.

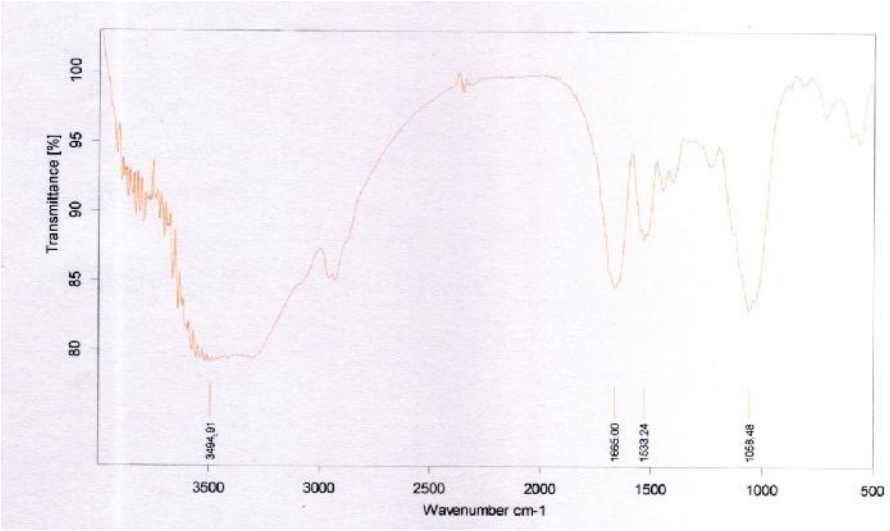


**Figure 2 : FTIR Spectrum of lyophilized EPS**

## 4. Conclusion

The present work dealt with the production of exopolysaccharide from *B.subtilis* and optimization of environmental parameters for its production. The central values were obtained from one factor at a time method. Using Central composite design, the combined effects of the four independent variables were studied. The results showed that temperature $35^0$C, pH 7, time 72h and 2% inoculum gave the maximum yield of EPS – 5.56g $L^{-1}$. The structural characterization was done by FTIR spectroscopy, which exhibited the presence of sucrose units. Exopolysaccharides find greater applications in pharmaceuticals as therapeutic agents, cosmetics and are ecofriendly as they are biodegradable in nature.

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