Optimization of an Exopolysaccharide Production from *Bacillus Aerogenes* by Response Surface Methodology

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ABSTRACT

There is an ever-increasing demand for inexpensive & environmentally acceptable viscosifiers, bioflocculants, bioemulsifiers and biodegradable polymers. In the presence study, the feasibility of production of such a novel exopolysaccharide is using Bacillus aerogenes (NCIM 2239) and optimization of the media for higher yield using Response Surface Methodology. During the course of investigation, production of exopolysaccharide the Bacillus aerogenes and its mutant were respectively. They were characterized, EPS S showed better flocculation and emulsification activity, but the yield was low. Hence the optimization of media for higher yield was performed. The three factors peptone, potassium dihydrogen phosphate and potassium sulphate identified by Plackett-Burmann Design were studied at five levels under central composite design. The exopolysaccharide (EPS OM) produced using optimized media was also characterized. The yield of EPS OM was 16 folds higher than EPS S; the total sugar content of EPS OM was increased when compared to EPS S and EPS M. The EPS OM was also found to be superior over EPS S and EPS M for bioflocculant & bio emulsification activities.

Keywords: Exopolysaccharide, Bioflocculant, Bioemulsification, Plackett-Burman design, Pareto chart, Central composite design.

INTRODUCTION

Exopolysaccharide[1,2,3,4] are high- molecular weight polymers that are composed of sugar residue and are secreted by a microorganism into the surrounding environment. Exopolysaccharide (EPS) generally constituted of monosaccharides and some non-carbohydrate substituent (such as acetate, pyurvate, succinate and phosphate). Many microbial EPS provide properties that are almost identical to the gum currently in use. Exopolysaccharide by bacteria are susceptible to biodegradation and contribute less to environmental pollution than synthetic polymers. Some of these applications include their use as emulsifier, stabiliser, binders, gelling agents, coagulants, lubricants, film formers, thickening agent and suspending agent[5, 6]**.** The rheological properties depend largely on intrinsic physico chemical characteristics. The composition of media plays a vital role in the production of exopolysaccharide. These exopolymers are often produced during stationary phase of bacterial growth.

Optimization[7, 8]

Optimizing refers to improving the performance of a system, a process, or a product in order to obtain the maximum benefit from it. Traditionally, optimization of media has been carried out by monitoring the influence of one factor at a time on an experimental response. As a consequence, this technique does not depict the complete effects of the parameter on the response. In order to overcome this problem, the optimization of analytical procedures has been carried out by using multivariate statistic techniques. Among the most relevant multivariate techniques used is response surface methodology (RSM).

Response surface methodology is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which must describe the behaviour of a data set with the objective of making statistical previsions. The objective is to simultaneously optimize the levels of these variables to attain the best system performance. Numerous variables may affect the response of the system studied, and it is practically impossible to identify and control the small contributions from each one. Therefore, it is necessary to select those variables with major effects. Full or fractional two-level factorial designs may be used for this objective principally because they are efficient and economical. After acquiring data related to each experimental point of a chosen design, it is necessary to fit a mathematical equation to describe the behaviour of the response according to the levels of values studied.

The more reliable way to evaluate the quality of the model fitted is by the application of analysis of variance (ANOVA). The selection of independent variables of major effects on the system through screening studies and the delimitation of the experimental region and the choice of the experimental design and carrying out the experiments according to the selected experimental matrix were the key process variables to getting maximum exopolysaccharide yield. The mathematical statistics treatment of the obtained experimental data through the fit of a polynomial function and the evaluation of the model's fitness and obtaining the optimum values for each studied variable. A statistical method, central composite designs (CCD), was adopted to optimize the three key process variables.In this present work we investigate the novel exopolysaccharide from **Bacillus *aerogenes* NCIM 2239** and its mutant**.** Also the optimization of the media for higher EPS yields using response surface methodology**.**

MATERIALS AND METHODS

The Bacillus *aerogenes* NCIM 2239 were screened[7,8] for exopolysaccharide were selected in production on modified MRS agar plates were visually inspected for production of slime (shiny colonies) and ropiness and then mutated by UV-mutagenesis method. Selected strain and mutant were cultivated in the nutrient broth medium. The media with composition shown in table 1 were sterilized and inoculated with 3 % v/v of the mutant of the *Bacillus aerogenes* NCIM 2239. The flasks were incubated at 32+2º C at 200 RPM in an orbital water bath shaker for 8 days. [18,19] After an incubation of 8 days the media subjected to centrifugation and the supernatant precipitated by adding three volumes of ice cold acetone[9,10]. The EPS precipitates were recovered by centrifugation at 8000 rpm for 20 minutes at 40^0C and purified by dialysis (12K Da membrane) against distilled water at 4^0C for minimum 12 hours. The purified strain and mutant exopolysaccharide (EPS S & EPS M) was freeze dried (at -55^0C, for 6 hours) to obtain in dry form. The purified EPS S & EPS M were analyzed for the following characteristics [11,12, 13]. The total carbohydrate and protein were measured by Phenol sulfuric acid method and Lowery's method respectively[14, 15,16,17]. The compositional analysis were performed on both strain and mutant lyophilized exopolysaccharide respectively. The monosaccharide composition was identified using HPLC. A reverse phase column C18 (Princeton SPHRE-100, 240*40 mm, 100 A, 5 u) was used with mobile phase tertiary butyl ammonium iodide - pH 4.0 (solvent A) and acetonitrile (solvent B) in the ratio of 80:20 at the flow rate of 1ml/min.[20,21]. The bioflocculant studies[22,23]

were carried according to the method of Kurane et.al., using a suspension of kaolin clay as a test material. Emulsification assays were carried out by method described by cooper and Goldenberg at a concentration of 5mg/ml of exopolysaccharide, the emulsifying activity of the EPS S & M were studied against hydrocarbon (xylene) and compared with standards Tween 20, Tween 80 and Triton X-100.

RESPONSE SURFACE METHODOLOGY [9, 10]

For the screening, various medium components and culture parameters were evaluated. Using a Plackett-Burman factorial design, each factor was examined on two levels: -1 for a low level and +1 for a high level. Table 2 illustrates the design matrix of the studied variables. The Plackett - Burman experimental design was based on a first-order model.

RSM enables evaluation of the effects of multiple parameters, alone or in combination, on response variables and also predicts their behavior under given sets of conditions. A statistical method, central composite designs (CCD), was adopted to optimize the three key process variables for getting maximum yield of exopolysaccharide optimized media (EPS OM). A Central Composite Design for three variables at two levels with 5 replicates at the center point leads to the total number of 20 experiments (known also as trails or runs). Details of upper limit and lower limit are shown in Table 3. The selection of low, middle and high levels for all these variables were based on prior screening studies. The behavior of the present system was described by the following equation (1), which includes all interaction terms regardless of their significance:

$$y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_2 AB + \beta_2\beta_3 AC + \beta_1\beta_3 AC \quad \text{---------- (1)}$$

Where y is predicted response, *ie* EPS yield; A, B and C are independent variables; β_0 is coefficient constant for offset term; β_1, $\beta_2$and β_3 are coefficient constant for linear effects and $\beta_1\beta_2$, $\beta_2\beta_3$ and $\beta 1\beta 3$ are coefficient constant for interactions effects. The model evaluates the effect of each independent variable to a response. The experimental designs are shown in the following table 4. The statistical software package Design-Expert 8 (Stat-Ease, Inc., Minneapolis, USA) was used for regression analysis of experimental data and to plot response surface.

RESULTS AND DISCUSSION

Screening of microorganisms

The formation of mucoid colonies in the MRS agar plate is an indication for the possible exopolysaccharide production by the microorganism shown in figure 1. The selected culture was genetically modified by mutation using ultraviolet (UV) radiation as mutagenic agents. The radiation was supplied for various time intervals as shown in table 5 and the results are tabulated in table 6. The exopolysaccharide was precipitated from supernatant by adding three volumes of ice cold acetone and the precipitate yield was purified then Lyophillization performed, thereby obtaining purified dried exopolysaccharide.

Characterization

The purified exopolysaccharide of *Bacillus aerogenes* mutant are Crystalline and whitish powder in appearance. The exopolysaccharides at concentration (1mg/ml) were analyzed for total sugar and total protein contents by Phenol-sulfuric acid method and Lowry's method respectively. The total ash value, pH, melting point of EPS S and EPS M were also estimated and shown in table 7. The Bacillus aerogenes mutant produced a yield of 490 mg/L whereas Strain produced 80 mg/L. The total sugar was found to be 40 % w/ v for mutant whereas for strain 80 % w/ v respectively. From the above all results it was observed the mutant produced higher yield comparing strain. The various concentrations of exopolysaccharide were investigated for bio-flocculation activity. The various concentration (1 to 0.001 mg/ml) of EPS S & M were studied and the results are shown in the table 8. The EPS S at concentration of 0.1 mg/ml shows better flocculation rate and activity of 83.09 % and 23.74 respectively. Whereas the EPS M showed maximum bioflocculant property at 0.01 mg/ml concetration showed 43.47 % flocculation rate and 6.58 flocculation activity respectively. Emulsification assays were carried out by method described by cooper and Goldenberg the EPS S & M showed an critical parameters were made and the results are given in the following table 9.

On comparing the EPS S and EPS M the following important findings were observed. The yield was higher with mutant. The total sugar, flocculant proeprty and emulsification activity were better in EPS produced by strain. As the yield was much lower with the *Bacillus aerogenes* strain, the statistical media optimization using Response surface methodology was performed to obtain a novel exopolysaccharide by using EPS OM.

RESPONSE SURFACE METHODOLOGY

Determination of active factors - Plackett - Burman Screening Design

From the Plackett - Burman design the major influence on the EPS production showed in pareto chart figure 2. The figure 3 results also shown that the two factors sodium chloride and temperature are having negative impact on the EPS production. pH being the factor which affects the production the most, it was decided to maintain the pH for the further studies at high value.From the Plackett - Burman study it was decided that to optimize three major active factors viz. Peptone, Potassium dihydrogen phosphate and Potassium sulphate using response surface methodology.

Central Composite Design and statistical analysis by ANNOVA

The media optimization was perforemd using Central composite design by selecting three variables viz Potassium dihydrogen phosphate, potassium sulphate and peptone. Table 10,11,12 & 13 shows the response (yield) obtained and also predicted for the central composite design. The design summary showed in table 14.

In statistical analysis of ANNOVA showed in table 15, The Model F-value of 10.42 implies the model is significant. There is a 13.97% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good --this model is fit. The equation was shown in table 16.

The **FDS (Fraction of Design Space) Graph** is a line graph showing the relationship between the "volume" of the design space (area of interest) and amount of prediction error. The curve indicates what fraction (percentage) of the design space has a given prediction error or lower. In general, a lower and flatter FDS curve is better. Lower is more important than flatter. A lower curve translates to a higher Fraction of Design space - more of the design has useful precision (Figure 4)

Diagnostic - Box plot provides a guideline for selecting the correct power law transformation. A recommended transformation is listed, based on the best lambda value, which is found at the minimum point of the curve generated by the natural log of the sum of squares of the residuals. If the 95% confidence interval around this lambda includes 1 then the software does not recommend a specific transformation. This plot is not displayed when either the logit or the arcsine square root transformation has been applied. The plot is shown in the figure 6. In following figure 10 shows the effects of PDP and PS on the production of Exopolysaccharide from *Bacillus aerogenes.* The graph figure 7 indicates that EPS production Potassium sulphate should be used at high level and potassium dihydrogen phosphate at low level. Figure 11 shows the effects of PDP and Peptone on the production of Exopolysaccharide from *Bacillus aerogenes.* The graph figure 8 indicates that for high EPS production Peptone should be used at high concentration while potassium dihydrogen phosphate at low level. Figure 12 shows the effects of Potassium sulphate and Peptone on the production of Exopolysaccharide from *Bacillus aerogenes.* The graph figure 9 indicates that for high EPS production Peptone and potassium sulphate should be used at high concentrations.

From the above statistical experimental design it was found out that the best EPS production was observed when the factors are used at the following levels Peptone : 0.2 or 0.6 % w/v, Potssium dihydrogen phosphate: 0.3 % w/v and Potassium sulphate: 0.6 % w/v . Hence from the optimization of the production media and other parameter using response surface methodology, it was found out that for getting higher yield of EPS the following media is recommended (table 17). The exopolysaccharide produced using the above said media (EPS OM) was characterized shown in table 18 and compared with the EPS S and the results are given in the following table 19.

Monosaccharide composition

The EPS OM after being hydrolysed with Tri Fluro-acetic acid (TFA) was anlaysed for its sugar composition by HPLC. The HPLC Chromatograms (figures 13, 14 & 15) shows that the EPS OM was composed mainly of glucose (aldohexose) and fructose (ketohexose).

Functional group analysis

The Infra red spectra (figure 16) of the EPS OM shows the presence of Carboxyl (1400cm^{-1}) group. The sample having amine group, due to presence of N-H band around 3140 cm^{-1} – 3500 cm^{-1} and chance for methylated polysaccharide containing uronic acid due to presence of O-H broad spectra (3400 cm^{-1} – 3650 cm^{-1}). The absorption peak at 2935.76cm^{-1} indicates the weak C-H stretching band. The peak around 1650 cm^{-1}– 1580 cm^{-1}

& 1654 cm^{-1} shows the presence of N-H bending and C=O stretching peak. The peak around at 1118.25 cm^{-1} indicate the presence of glucouronic acid, mannuronic acid and O-acetyl ester. The sharp peak at 619.17 cm^{-1} is due to presence of C-Halide group.

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Table 1: Composition of the media used for the EPS production

S. N	Ingredients	Qty (% w/v)
1.	Glucose	2
2.	Peptone	0.05
3.	Potassium dihydrogen phosphate	0.3
4.	Sodium chloride	0.1
5.	Potasssium sulphate	0.1
6.	Magnesium sulphate	0.02
7.	Ferrous sulphate	0.0001
8.	Calcium chloride	0.002

Table 2: Variables selected for determining the active factors

Variables	**High level (H)**	**Low Level (L)**
Glucose (C)	3 %	1 %
Peptone (N)	0.1 %	0.01 %
Potassium dihydrogen phosphate (PDP)	0.4 %	0.2 %
Sodium chloride (SC)	0.2 %	0.05 %
Potassium sulphate (PS)	0.2 %	0.05 %
pH	7.5	5.5
Temperature (Temp) ^{0} C	37	27

Table 3: Upper and Lower limit of selected variables of CCD

Variable	**Potassium sulphate (% w/v)**					**Potassium-dihydrogen phosphate (% w/v)**					**Peptone (% w/v)**				
Coded Value	- α	- 1	0	+ 1	+ α	- α	- 1	0	+ 1	+ α	- α	- 1	0	+ 1	+ α
Real Value	0.0636	0.2	0.4	0.6	0.7364	0.1646	0.3	0.5	0.7	0.8364	0.0636	0.2	0.4	0.6	0.7364

Table 4: Experimental matrices for Central Composite Design (Coded Values)

Factor	**Factor A: PS (%w/v)**	**Factor B: PDP (%w/v)**	**Factor C: Peptone (%w/v)**
Factorial design	-1	-1	-1
	-1	-1	+1
	-1	+1	-1
	-1	+1	+1
	+1	-1	-1
	+1	+1	+1
	+1	-1	1
	+1	+1	-1
Axial points	-α	0	0
	+α	0	0
	0	-α	0
	0	+α	0
	0	0	-α
	0	0	+α
Central points	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0

Table 5: UV radiation duration – isolation of mutant

TIME (seconds)	NUMBER OF COLONIES IN VARIOUS DILUTIONS		
	10^{-6}	10^{-7}	10^{-8}
10	19	4	9
30	11	15	12
70	5	4	3
130	2	**1**	**1**
210	0	**1**	**1**
310	0	**0**	**1**
430	0	0	0

Table 6: Replica Plating

TIME (seconds)	Replica Plating					
	10^{-7}	Plate A	Plate B	10^{-8}	Plate A	Plate B
130	1	+	+	1	+	+
210	1	-	-	1	-	-
310	0	-	-	1	-	-

+ Growth of the microorganism - No growth of the microorganisms

Table 7: Characteristics of EPS S and EPS M

CHARACTERISTICS	EPS S	EPS M
APPEARANCE	White, crystalline under microscope	White, crystalline under microscope
SOLUBILITY	Water: soluble Organic solvents: insoluble	Water: soluble Organic solvents: insoluble
YIELD	80 mg/L $\pm$ 0.12	490 mg/L $\pm$ 0.14
TOTAL SUGAR	80% w/v $\pm$ 0.31	40% w/v $\pm$ 0.11
TOTAL PROTEIN	0.12% w/v $\pm$ 0.17	0.12% w/v $\pm$ 0.18
TOTAL ASH VALUE	49% w/w $\pm$ 0.42	65% w/w $\pm$ 0.36
pH	4.6 ± 0.3	3.6 ± 0.4
MELTING POINT	185 – 190 ^{o}C	195 -205 ^{o}C
VISCOSITY	1.010 cps	1.0844 cps

Table 8: Flocculation property

CONC.	FLOCCULATION RATE		FLOCCULATION ACTIVITY	
	EPS S	EPS M	EPS S	EPS M
1 mg/ml	18.82 % $\pm$ 0.23	6.6% $\pm$ 0.14	2.73$\pm$ 0.21	0.79$\pm$ 0.20
0.1 mg/ml	**83.09 %** $\pm$ 0.11	44.18%$\pm$ 0.32	**23.74**$\pm$ 0.26	4.6$\pm$ 0.34
0.01 mg/ml	46.07% $\pm$ 0.22	**43.47%**$\pm$ 0.18	2.8$\pm$ 0.78	**6.58**$\pm$ 0.37
0.001 mg/ml	13.14% $\pm$ 0.31	34.48%$\pm$ 0.22	0.48$\pm$ 0.89	2.95$\pm$ 0.21

Table 9: Bioemulsification Activity

Hydrocarbon	Emulsant	Type	Emulsification Index E_{24}
Xylene	**EPS S**	**o/w**	**48.2%**± 0.23
Xylene	EPS M	o/w	43.3%± 0.87
Xylene	Triton X-100	o/w	48.27%± 0.63
Xylene	Tween 80	o/w	50.0%± 0.23
Xylene	Tween 20	o/w	50.0%± 0.12
Xylene	Water (control)	o/w	-

Table 10: The experimental design using the Plackett – Burman method for screening of nutrients

TRIAL / VARIABLES	N	C	pH	PDP	SC	PS	TEMP	Yield mg/L
A	H	H	H	H	L	H	L	**680**
B	L	L	H	H	H	H	L	**320**
C	L	H	H	L	L	L	H	56
D	H	H	L	H	L	H	H	108
E	H	L	H	L	H	L	H	**236**
F	L	H	L	H	H	H	H	116
G	H	L	L	L	H	L	L	108
H	L	L	L	L	L	L	L	56

Table 11: Influence of the various factors on the EPS production

S.N	Factor	Effect
1	Peptone	**18.25**
2	Glucose	7.5
3	pH	28.25
4	Potassium dihydrogen phosphate	**24**
5	Potassium Sulphate	**24**
6	Sodium Chloride	- 3.75
7	Temperature	- 20.25

Table 12: Experimental design with actual value

Std	Run	Factor A: PS (% w/v)	FactorB: PDP (% w/v)	Factor C: Peptone (% w/v)
1	5	0.20	0.30	0.20
2	8	0.60	0.30	0.20
3	2	0.20	0.70	0.20
4	7	0.60	0.70	0.20
5	10	0.20	0.30	0.60
6	9	0.60	0.30	0.60
7	4	0.20	0.70	0.60

8	3	0.60	0.70	0.60
9	6	0.40	0.50	0.40
10	11	0.40	0.50	0.40
11	12	0.40	0.50	0.40
12	1	0.40	0.50	0.40
13	14	0.06	0.50	0.40
14	20	0.74	0.50	0.40
15	17	0.40	0.16	0.40
16	19	0.40	0.84	0.40
17	15	0.40	0.50	0.06
18	16	0.40	0.50	0.74
19	18	0.40	0.50	0.40
20	13	0.40	0.50	0.40

Table 13: Experimental design result showing the obtained value for EPS production

Std	Run	Block	Factor A: Potassium sulphate (% w/v)	Factor B: Potassium dihydrogen phosphate (% w/v)	Factor C: Peptone (% w/v)	Response Yield (mg/100 ml)
1	5	Block 1	0.20	0.30	0.20	31
2	8	Block 1	0.60	0.30	0.20	126
3	2	Block 1	0.20	0.70	0.20	51
4	7	Block 1	0.60	0.70	0.20	61
5	10	Block 1	0.20	0.30	0.60	49
6	9	Block 1	0.60	0.30	0.60	102
7	4	Block 1	0.20	0.70	0.60	74
8	3	Block 1	0.60	0.70	0.60	101
9	6	Block 1	0.40	0.50	0.40	49
10	11	Block 1	0.40	0.50	0.40	30
11	12	Block 1	0.40	0.50	0.40	30
12	1	Block 1	0.40	0.50	0.40	30
13	14	Block 2	0.06	0.50	0.40	32
14	20	Block 2	0.74	0.50	0.40	85
15	17	Block 2	0.40	0.16	0.40	42
16	19	Block 2	0.40	0.84	0.40	32
17	15	Block 2	0.40	0.50	0.06	30
18	16	Block 2	0.40	0.50	0.74	78
19	18	Block 2	0.40	0.50	0.40	30
20	13	Block 2	0.40	0.50	0.40	30

Table 14: Design Summary

Study Type	Response Surface	**Runs**	20
Design Type	Central Composite	**Blocks**	2
Design Model	Quadratic	**Build Tim(ms)**	2.91

Factor	**Name**	**Units**	**Type**	**Subtype**
A	Potassium sulphate	% w/v	Numeric	Continuous
B	Potassium dihydrogen phosphate	% w/v	Numeric	Continuous
C	Peptone	% w/v	Numeric	Continuous

Factor	**Minimum**	**Maximum**	**-1 Actual**	**+1 Actual**	**Mean**	**Std. Dev.**
A	0.06	0.74	0.20	0.60	0.40	0.17
B	0.16	0.84	0.30	0.70	0.50	0.17
C	0.06	0.74	0.20	0.60	0.40	0.17

Response	**Name**	**Units**	**Obs**	**Analysis**	**Model**
Y1	Yield	mg/L	20	Polynomial	Quadratic

Response	**Minimum**	**Maximum**	**Mean**	**Std. Dev.**	**Ratio**	**Trans**
Y1	30	126	54.65	29.7627	4.2	None

Table 15: Statistical analysis by ANOVA

Response Yield

ANOVA for Response Surface Quadratic Model

Source	Sum of squares	df	Mean Square	F Value	p value Prob > F
Block	1274.01	1	1274.01		
Model	14194.04	9	1577.12	10.42 (Significant)	0.0009
A-PS	5502.73	1	5502.73	36.35	0.0002
B-PDP	104.72	1	104.72	0.69	0.4271
C-Peptone	1388.93	1	1388.93	9.17	0.0143
AB	1540.13	1	1540.13	10.17	0.0110
AC	78.13	1	78.13	0.52	0.4908
BC	595.13	1	595.13	3.93	0.0787
A^2	2906.51	1	2906.51	19.20	0.0018
B^2	628.34	1	628.34	4.15	0.0721
C^2	2291.98	1	2291.98	15.14	0.0037
Residual	1362.50	9	151.39		
Lack of Fit	1091.75	5	218.35	3.23	0.1397 (Nonsignificant)
Pure Error	270.75	4	67.69		
Cor Total	16830.55	19			

Std. Dev.	12.30	R-Squared	0.9124
Mean	54.65	Adj R-Squared	0.8248
C.V. %	22.51	Pred R-Squared	0.3329
PRESS	10377.25	Adeq Precision	9.803.

**"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here the ratio of 9.803 indicates an adequate signal. This model can be used to navigate the design space.

Table 16: Statistical analysis by ANOVA

Final Equation in Terms of Coded Factors:
Yield = 30.06 + 20.07 * A - 2.77 * B + 10.08 * C - 13.87 * A * B - 3.12 * A * C
+8.62 * B * C + 14.21 * A^2 + 6.61 * B^2 + 12.62 * C^2
Final Equation in Terms of Actual Factors:
Yield =+ 86.49424
+ 20.91155 * Potassium sulphate
- 126.48746 * Potassium dihydrogen phosphate
- 278.46022 * Peptone
- 346.87500 * Potassium sulphate * Potassium dihydrogen phosphate
- 78.12500 * Potassium sulphate * Peptone
+ 215.62500 * Potassium dihydrogen phosphate * Peptone
+ 355.17664 * Potassium $sulphate^2$
+ 165.14169 * Potassium dihydrogen $phosphate^2$
+ 315.40189 * $Peptone^2$

Table 17: Recommended media for EPS production from *Bacillus aerogenes*

S. N	Ingredients	Qty (% w/v)
1.	Glucose	2
2.	Peptone	0.2
3.	Potassium dihydrogen phosphate	0.3
4.	Sodium chloride	0.1
5.	Potasssium sulphate	0.6
6.	Magnesium sulphate	0.02
7.	Ferrous sulphate	0.0001
8.	Calcium chloride	0.002

Table 18: Characteristics of EPS OM

CHARACTERISTICS	EPS OM
Appearance	Slight brown, crystalline under microscope
Solubility	Water: soluble Organic solvents: insoluble
Yield	**1260 mg/L ± 0.02**

Total Sugar	92 % w/v ± 0.12
Total Protein	0.12% w/v ± 1.18
Total Ash Value	57 % w/w ± 2.14
Ph	4.8 ± 0.2
Melting Point	180 – 185 °C
Viscosity	1.0313 cps
FLOCCULANT PROPERTY a) 1mg/ml Rate : Activity : b) 0.1 mg/ml Rate : Activity :	 90 % ± 1.24 43 ± 2.14 **94.7 %** ± 0.84 **39.8** ± 1.12
EMULSIFICATION INDEX E $_{24}$ a) Xylene (o/w type) b) Sunflower oil (o/w type) c) Cotton seed oil (o/w type)	 **61.5%** ± 1.23 46.48% ± 2.14 44.11% ± 2.22

Table 19: EPS S and EPS OM Comparison

S.N	Property	EPS S	EPS OM
1	Appearance	White, crystalline under microscope	Slight brown, crystalline under microscope
2	Solubility	Water: soluble Organic solvents: insoluble	Water: soluble Organic solvents: insoluble
3	Yield	80 mg/L	**1260 mg/L**
4	Total Sugar	80 % w/v	**92 % w/v**
5	Total Protein	0.12 % w/v	0.12 % w/v
6	Total Ash Value	49 % w/w	57.79 % w/w
7	Ph	4.6	4.8
8	Melting Point	185 – 190 °C	180 -185 °C
9	Viscosity	1.010 cps	1.0313 cps
10	Flocculant Property Rate: Activity:	(0.1 mg/ml) 83.09 % 23.74	**(0.1 mg/ml)** **94.7 %** **39.8**
11	Emusification Index E $_{24}$	48.2 %	**61.5 %**

Fig 1: Mucoid colonies in MRS agar plate.

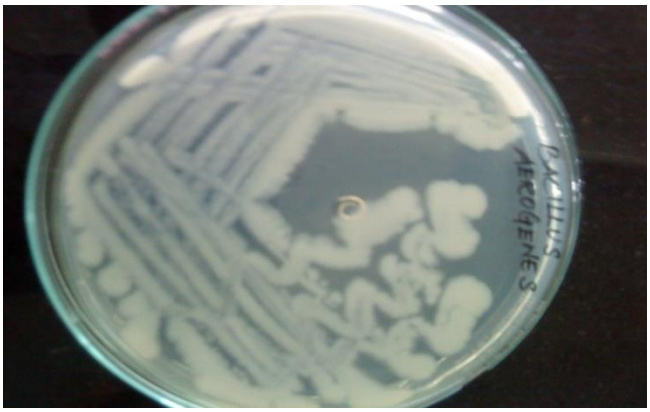


Figure 2: Pareto Chart

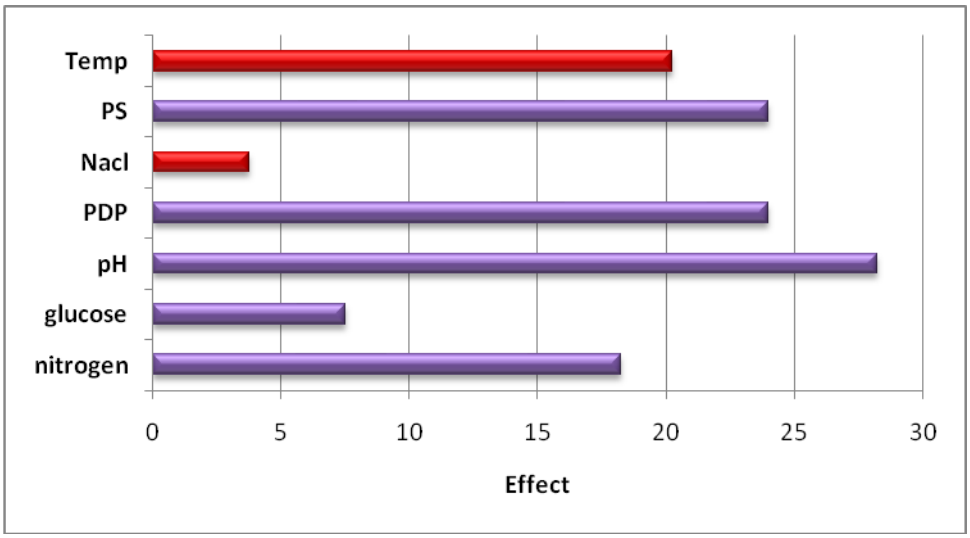


Figure 3: Effect of various factors on the EPS production

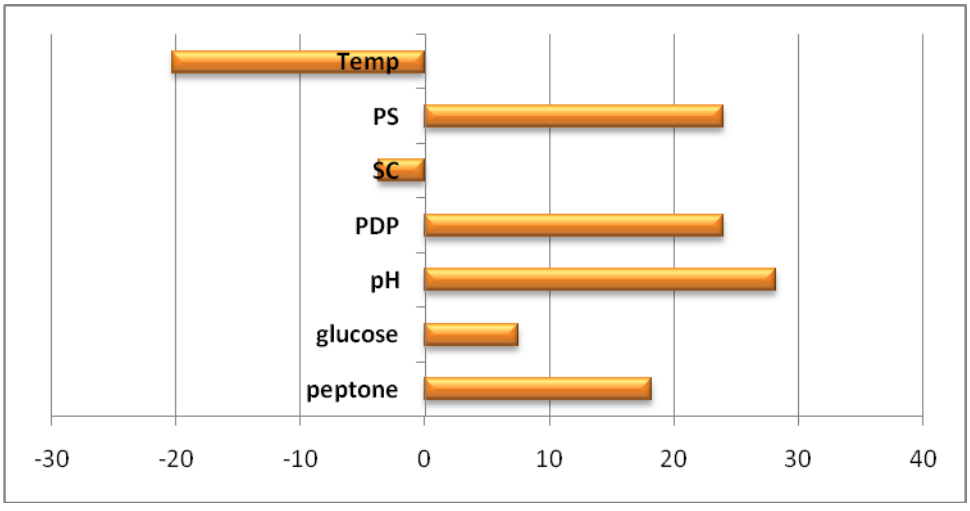
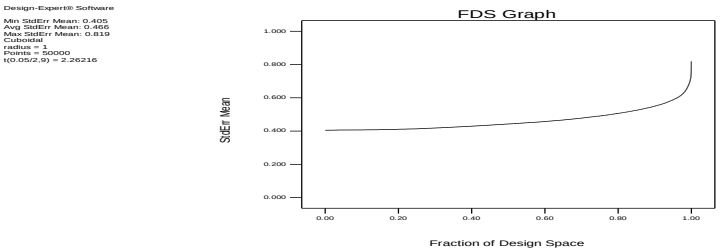
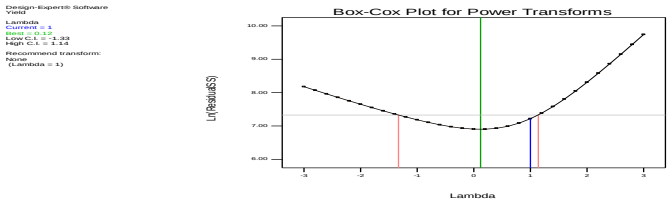


Figure 4: FDS graph



**The lower curve specifies that the design space has less prediction error.

Figure 6: Box-Cox plot



Graph columns

Fig 7: EPS yield plotted against Potassium sulphate

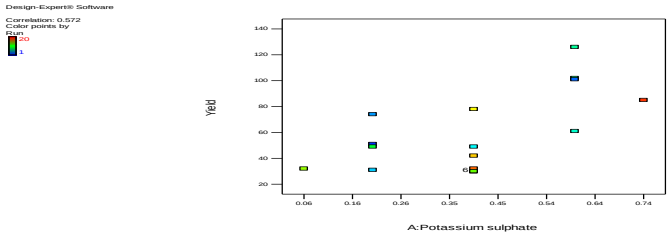


Fig 8: EPS yield plotted against Potassium dihydrogen phosphate

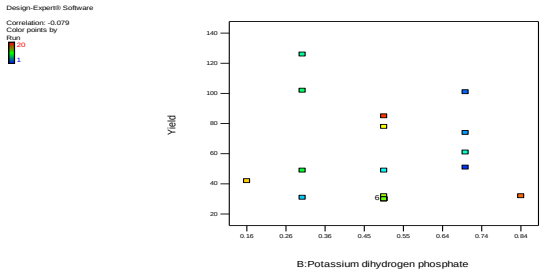
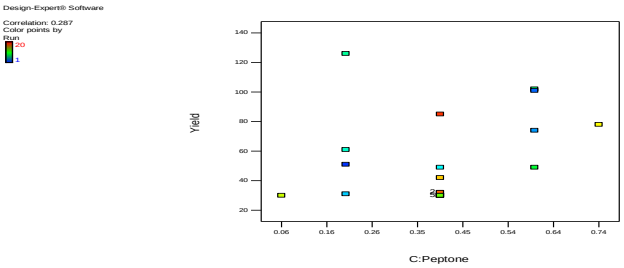


Fig 9: EPS yield plotted against Peptone



Response Surface Plot

Fig 10: The three-dimensional, response surface plot for PDP: PS on EPS Production.

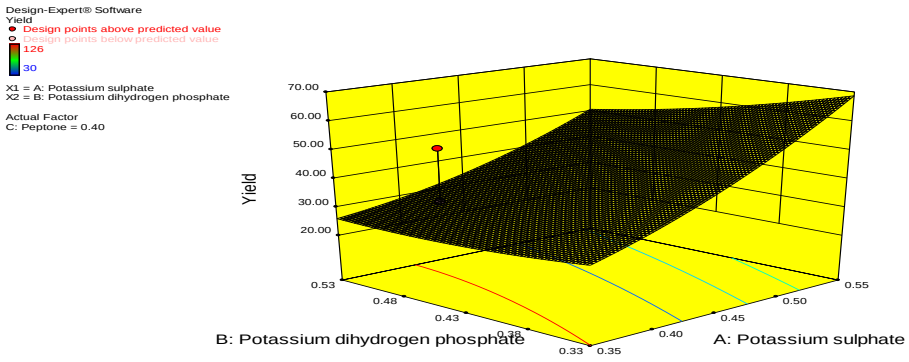


Fig 11: The three-dimensional, response surface plot for PDP: Peptone on EPS Production.

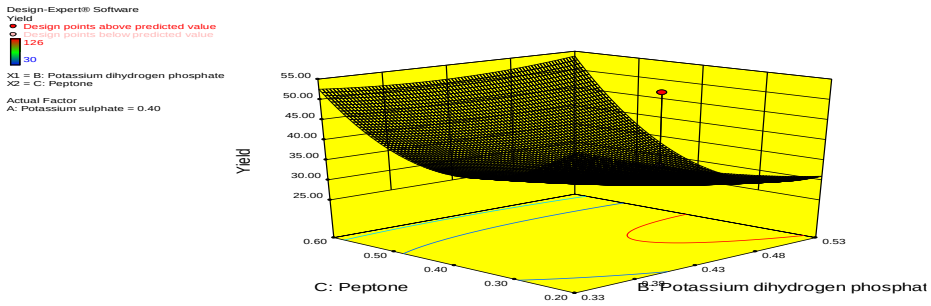


Fig 12: The three-dimensional, response surface plot for PS: Peptone on EPS Production

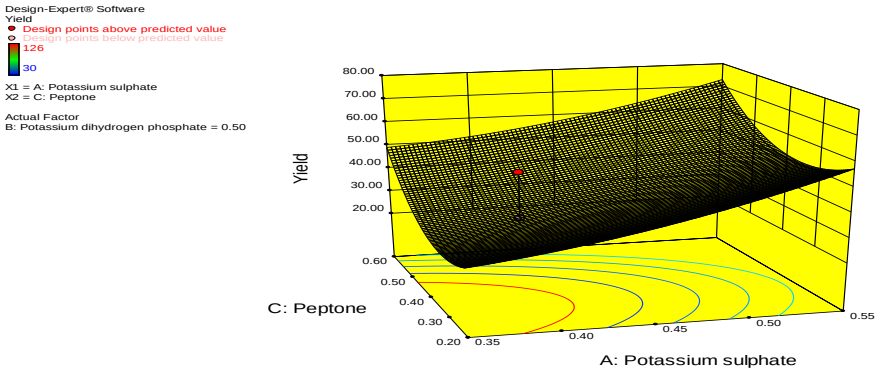


Fig 13: EPS OM - HPLC chromatogram

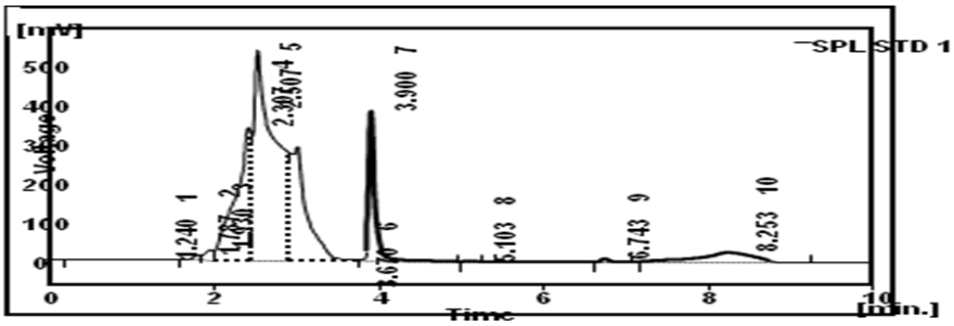


Fig15:Standard Glucose HPLC chromatogram

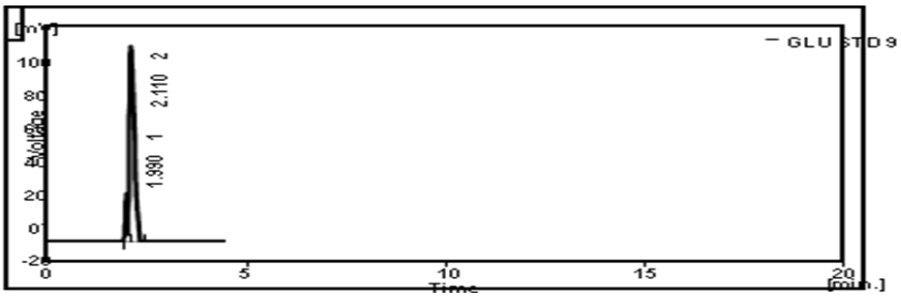


Fig 15: Standard Fructose HPLC chromatogram

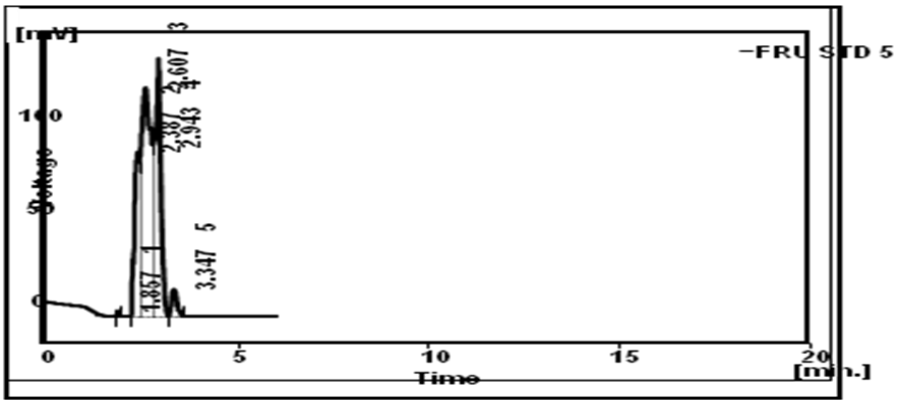
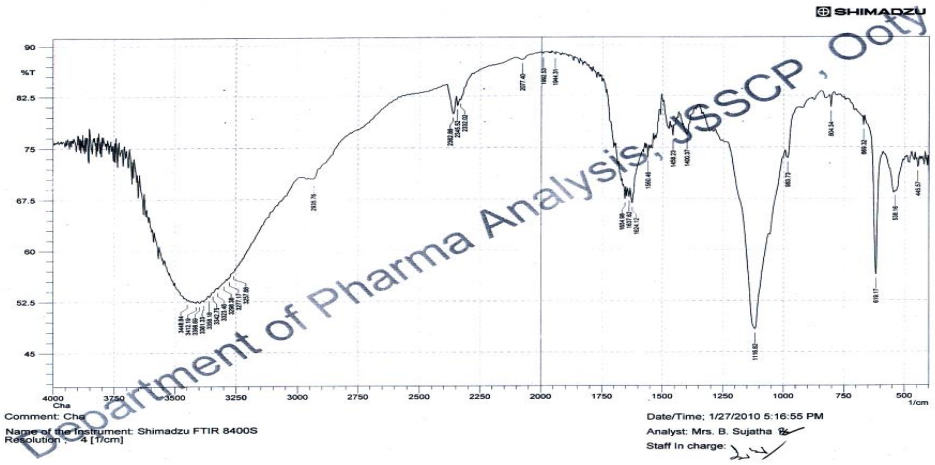


Fig. 16 IR Spectrum of EPS OM



ABBREVIATION

NCIM	National Collection of Industrial Microorganisms
EPS	Exopolysaccharide
EPS S	Exopolysaccharide Strain
EPS M	Exopolysaccharide Mutant
EPS OM	Exopolysaccharide Optimized Media
HPLC	High Performance Liquid Chromatography
PDP	Potassium Dihydrogen Phosphate
PS	Potassium Sulphate
FDS	Fraction of Design Space
RSM	Response Surface Methodology