ISSN: 0976-1675

www.rjas.org

Research Journal of Agricultural Sciences

Research Paper

DI: 5983-1203-114

Statistical Based Multi Response Optimization of Exopolysaccharide Production by *Cronobacte rmuytjensii ATCC 51329 (T)* and Characterization of EPS Produced

Gitanjali G Mane[1], **Pranav R Kshirsagar**[2] **and Venkat S Hamde***[1]

Department of Microbiology,

Yogeshwari Mahavidyalaya, Ambajogai, District Beed - 431 517, Maharashtra, India

Received: 12 March 2020; Revised accepted: 01 May 2020

A B S T R A C T

In present investigation statistical based optimization methods Plackett-Burman (PB) design and central composite design (CCD) were applied to optimize fermentation parameters for exopolysaccharide production from *Cronobacter muytjensii ATCC 51329 (T)*. Total eight parameters percent (%) glucose, percent (%) peptone, NaCl concentration, $MgSO_4 \cdot 7H_2O$ concentration, initial pH, temperature, % inoculum, incubation period were optimized to improve exopolysaccharide production. The significant factors were screened using set of 23 runs using Plackett-Burman design (PBD) and significant factors subsequently optimized with central composite design experiments. EPS yield 4.25 g L^{-1} was obtained under optimum conditions. The polynomial equations were developed for prediction of EPS yield and cell growth. The developed mathematical models were very well fitted (R^2>0.75) value) and suitable for prediction of crude EPS, g L^{-1}, EPS µg ml^{-1} and cell growth. Extracted EPS was characterized by SEM, TEM and FTIR analysis. The SEM analysis of EPS showed flakes like structural unit and highly compact structure with smooth glittering surface. These properties of EPS specify the potential use of the EPS as water holding, viscosifying, and thickening or as stabilizing agent. The FTIR spectrum showed that the obtained EPS was heteropolymeric in nature.

Key words: Exopolysaccharide, Plackett-Burman design, Central composite design, SEM, TEM, FTIR

Exopolysaccharides (EPS) are high molecular weight polymers biosynthesized by a wide range of microorganisms (Vijayabaskar *et al*. 2011). Physico-chemical and structural properties of microbial EPS are suitable for variety of application, hence widely used as emulsifiers, stabilizers, gelling agent, thickening agent, coating agent in different fields such as in food and pharmaceutical (Chawla *et al*. 2009, Mane and Hamde 2018). Microorganisms present in the soil plays very important role in the maintenance of quality and health of soil. Leguminous plants are major source of biological nitrogen fixation through root nodule formation. Iverson and Forsythe (2003) hypothesized that the *Cronobacter species* might be of plant origin due to its physiological features such as gum like extracellular polysaccharide production, and its desiccation resistance (Iversen and Forsythe 2003).

Many factors were reported that could be influenced to the production and characteristics of EPS. EPS production could be improved by manipulating the culture conditions (Sivakumar *et al*. 2012). Optimization of production conditions play very important role in reducing the production cost (Arun *et al*. 2014). Optimization of medium by one-factor-at-a-time method is very time consuming, extremely laborious, and non-reliable especially for a large number of variables. All these drawbacks of one-factor-at-a-time process can be eliminated by using combined statistical treatment of optimization factorial design experiments and RSM (Response Surface Methodology) (Li *et al*. 2013). Statistical experiment design and data analysis have been applied successfully for optimization of medium constituents and other variables responsible for exopolysaccharide production (Li *et al*. 2013). The aims of present study were, to optimize EPS production using one-

[1]*Venkat S. Hamde, (venkathamde@gmail.com), Department of Microbiology, Yogeshwari Mahavidyalaya, Ambajogai, Beed - 431 517, Maharashtra

[2]Pranav R. Kshirsagar (prkshirsagar@aripune.org), Bioenergy Group, MACS-Agharkar Research Institute, Pune - 411 004, Maharashtra

factor-at-a-time method followed by PBD and RSM, to develop mathematical equation for prediction of EPS yield and cell growth and characterization of obtained EPS using scanning electron microscopy, transmission electron microcopy and Fourier transform infrared radiation (FTIR).

MATERIALS AND METHODS

Isolation and identification of bacterial culture

For isolation of EPS producing bacterial culture, healthy, pink colored, unbroken root nodules were selected. The root nodules of different leguminous plants selected and surface sterilized with 0.1% $HgCl_2$ for 3-5 minutes and 70% ethanol for 30 seconds and washed multiple times with sterilized distilled water. Surface sterilized root nodules crushed and serially diluted, higher dilutions were spread on yeast extract mannitol agar plates (YEMA) supplemented with 0.025% Congo red dye and incubated at 30° C for 3 to 5 days. The bacterial culture purified by repeated plating and stored on YEMA slants at 4°C for further studies (Balamurugan and Prakash 2012, Gharzauli *et al*. 2012, Nirmala *et al*. 2011).

Determination of EPS production gravimetrically

Seed inoculum was developed by inoculating the culture in 50 ml YEM medium and incubated flask at 30°C for 24h. 2% (v/v) inoculum was transferred aseptically in 100 ml YEM medium and incubated at 30°C for 72 h. Further the broth was centrifuged at 10000 rpm and supernatant was recovered. Three volume of ice cold ethanol was added in supernatant and kept the solution for EPS precipitation at 4°C for 24 h. EPS precipitate was recovered by centrifugation at 2500 rpm for 15 min. The obtained EPS precipitate was further treated with equal volume of distilled water and ice cold ethanol. Again centrifugation carried out 2500 rpm for 15 min and EPS was recovered. The obtained EPS was dried at 60°C using oven and expressed EPS production in g L^{-1} (Balamurugan and Prakash 2012, Gharzauli *et al*. 2012, Nirmala *et al*. 2011).

Determination of EPS production spectro-photometrically

Phenol-sulphuric acid method (Dubois *et al*. 1956) was used for determination of total carbohydrate contents of EPS. 1 ml of EPS solution was treated with 1 ml of phenol (5% v/v) in test tube. 5 ml concentrated H_2SO_4 added in reaction mixture and vigorously shook for 10 min and allowed to stand for 20 min for development of color. The absorbance was read at 490 nm spectro-photometrically and expressed EPS in µg ml^{-1}. Glucose was used as a standard.

Medium optimization for EPS production by one-factor-at-a-time method

In one- factor- at –a- time experiments, one parameter was varied and other parameters were kept at fix level. Effect of six parameters incubation time, temperature, carbon source, nitrogen source, metal ion concentration and initial pH of the medium were investigated on EPS production. In case of the parameter incubation time EPS yield were tested at four different times 24, 48, 72 and 96 h. The parameter temperature was varied at 10, 25, 30, 35, 40 and 45°C, and incubated for 72 h. Effect of carbon source on EPS yield was tested by adding 2% (w/v) of carbon source in the medium. Different carbon sources viz. mannitol, D-glucose, sucrose, maltose, arabinose, xylose, fructose, and lactose were added in basel medium. Similarly effect of different nitrogen sources were investigated using 0.2% (w/v) of one of the following nitrogen source viz. yeast extract, beef extract, peptone, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, glycine and urea. Effect of different metal ion concentration studied using 300µg/ml of one of the following metal ion $FeSO_4$, $MnSO_4$, $ZnSO_4$, $MgSO_4$, $HgCl_2$ and $ZnCl_2$. Effect of different pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9.0 were tested on EPS production. In case of the parameters carbon source, nitrogen source, metal ion and initial pH of the medium flask were incubated at 37°C for 72 h. All the experiments executed in duplicate and using 2% (v/v) inoculum.

Screening of parameters for EPS production using Plackett - Burman design

The factors included in the Plackett-Burman design (PBD) are listed in (Table 1). The parameters % glucose (source of carbon), % peptone (source of nitrogen), NaCl concentration, $MgSO_4.7H_2O$ (source of metal ion concentration), initial pH of the medium, % inoculum, temperature and incubation time were considered in the design. In this design the parameters were set up at -1 and +1 level. Total 23 run were carried out (Table 2). The experiment no.6, 8 and 12 were performed at '0' level to confirm the reproducibility of the experiment. Regression analysis of the PBD was carried out by considering the linear terms in the analysis. The regression analysis provided the P-values, which were used to evaluate the significance of the parameters. The parameters resulted with P-value <0.05 were considered the significant parameter and P > 0.05 was considered non-significant parameter.

Table 1 Factors and their levels for P–B experimental design and RSM using CCD

Factor (Factor code)	Levels				
	-2	-1	0	1	2
X_1: % glucose (w v^{-1})	1	2	3	4	5
X_2: % Peptone (w v^{-1})	0.1	0.2	0.3	0.4	0.5
X_3: NaCl conc. (µg ml^{-1})	100	200	300	400	500
X_4: Metal ion Conc. (µg ml^{-1}) ($MgSO_4$)	100	200	300	400	500
X_5: Initial pH	5	6	7	8	9
X_6: % Inoculum (V V^{-1})	0.5	1	1.5	2	2.5
X_7: Temperature, °C	25	30	35	40	45
X_8: Incubation time, h	24	48	72	96	120

Table 2 Plackett- Burman design matrix for screening of significant parameters from 8 factors with coded value

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Crude EPS yield (g L^{-1})	EPS estimated (µg ml^{-1})	Growth (OD)
1	-1	-1	1	1	-1	1	1	-1	1.99	705	1.665
2	1	-1	1	-1	1	1	1	1	1.945	620	0.874
3	-1	-1	-1	-1	1	-1	1	-1	1.625	520	1.279
4	-1	1	1	-1	1	1	-1	-1	1.875	560	1.708
5	1	1	-1	1	1	-1	-1	-1	3.12	860	1.743
6	0	0	0	0	0	0	0	0	3.625	940	1.853
7	-1	-1	1	-1	1	-1	1	1	1.038	425	1.038
8	0	0	0	0	0	0	0	0	3.765	975	1.685
9	1	-1	1	1	-1	-1	-1	-1	3.19	845	1.377
10	1	-1	1	1	1	1	-1	-1	2.425	720	1.441
11	-1	-1	-1	1	-1	1	-1	1	2.515	715	1.51
12	0	0	0	0	0	0	0	0	4.075	970	1.79
13	-1	1	1	1	1	-1	-1	1	1.99	700	1.695
14	-1	1	1	-1	-1	-1	-1	1	1.82	540	1.78
15	1	1	-1	-1	-1	-1	1	-1	2.775	730	1.173
16	1	1	1	1	-1	-1	1	1	2.685	435	1.376
17	1	1	-1	-1	1	1	-1	1	2.935	785	1.158
18	-1	-1	-1	-1	-1	-1	-1	-1	2.29	355	1.117
19	-1	1	-1	1	-1	1	1	1	1.415	365	1.015
20	-1	1	-1	1	1	1	1	-1	1.425	335	1.112
21	1	1	1	-1	-1	1	1	-1	2.29	365	1.052
22	1	-1	-1	1	1	-1	1	1	1.36	370	0.625
23	1	-1	-1	-1	-1	1	-1	1	2.225	695	1.472

Optimization of EPS yield using central composite design

Regression analysis of PBD experiments was performed for three responses exopolysaccharide (EPS) measured gravimetrically; exopolysaccharide measured spectro-photometrically and cell growth. The responses EPS (g L^{-1}) measured gravimetrically, EPS µg ml^{-1}spectro-photometrically and cell growth were regressed simultaneously. EPS measured gravimetrically was crude EPS. Analysis revealed that the parameters glucose (X_1) and temperature (X_7) were found significant in case of response EPS (gL^{-1}) measured gravimetrically. In case of response EPS µg ml^{-1} measured spectro-photometrically and cell growth the parameters peptone (X_2) and temperature (X_7) were found significant. The main objective of the experiment was improvement of EPS yield therefore the CCD experiments were planned with the parameters glucose (X_1) and temperature (X_7). CCD matrix generated is shown in (Table 3). In CCD, total 14 run were carried out. The parameters were set at five different levels -2, -1, 0, 1, and 2. In experiment design 4 runs were set up using factorial point (-1 and +1), 4 runs were set up using axial points (-2 and +2) and 6 runs were set up using the center point (0). In CCD experiments non-significant parameters X_2, X_3, X_4, X_5, X_6 and X_8 were kept at '0' level.

Table 3 Central composite design (CCD) experiment

X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	EPS yield g/l	EPS µg/ml	Cell growth (OD)
0	0	0	0	0	0	2	0	1.095	350	0.3075
0	0	0	0	0	0	0	0	2.965	700	1.5515
0	0	0	0	0	0	0	0	3.58	950	1.6925
0	0	0	0	0	0	-2	0	1.625	520	0.549
0	0	0	0	0	0	0	0	3.16	800	1.354
2	0	0	0	0	0	0	0	2.985	770	1.1015
-2	0	0	0	0	0	0	0	0.925	300	1.585
-1	0	0	0	0	0	1	0	1.275	450	0.435
1	0	0	0	0	0	-1	0	1.955	560	1.736
0	0	0	0	0	0	0	0	3.375	880	1.435
0	0	0	0	0	0	0	0	3.125	910	1.431
0	0	0	0	0	0	0	0	3.245	850	1.3785
-1	0	0	0	0	0	-1	0	2.4	470	1.836

The factors level were coded and decoded according to Eq. (1) and (2).

$$x_i = \frac{X_i - \overline{X_i}}{\Delta X_i} \quad \text{--------- (1)}$$

$$X = x_i.\Delta X + \overline{X} \quad \text{--------- (2)}$$

The general form of second order polynomial equation is expressed by Eq. (3) as function of independent variable.

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n}\sum_{j=1}^{n} b_{ij} x_{ij} + \sum_{i=1}^{n} b_{ii} X_i^2 \quad \text{--------- (3)}$$

Where *Y*, was the predicted value of crude EPS gL^{-1} or EPS µg ml^{-1} or cell growth OD. Term $\Sigma b_i x_i$ is linear terms and their coefficients, $\sum_i \sum_j b_{ij} x_{ij}$ is the interactive terms and their coefficients and $\sum_i b_{ii} x_i^2$ is the quadratic terms and their coefficients. The regression analysis was carried out to estimate the coefficients in equation (3). The statistical software Minitab 15 was used for the design of experiment matrix; 3D surface plots, equation development and to find out parameter optimum conditions. In recent years, both RSM and CCD have been widely used to optimize the medium composition for production of different metabolites, which have also been proved to be efficient, practical and precise (Li *et al*. 2012).

SEM/TEM analysis of EPS

Scanning electron microscopy and transmission electron microscopy techniques were used for morphological characterization of bacteria and EPS. For SEM analysis of the bacteria, a colony from a plate of active culture of representative bacterial strain was picked using sterile loop and spread as a thin film over a freshly cleaved mica-sheet 224 N.E. and left to dry for 1 h at room temperature. Similarly for SEM analysis of EPS, the lyophilized sample of EPS (1mg/ml) were spread as a thin film over a freshly cleaved mica-sheet 224 N.E. and left to dry for 1 h at room temperature. Samples were sputter coated with gold and imaged using a Zeiss Supra 40VP field emission scanning electron microscope (Carl Zeiss NTS Ltd., Cambridge, UK) operating at an accelerating voltage of 20 kV. In case of TEM analysis lyophilized EPS sample was prefixed using 2.5% (v/v) glutaraldehyde in PBS at 4°C for overnight. After pre-fixation with 2.5% glutaraldehyde overnight, the EPS sample was washed 3 times with PBS and post-fixed with 2% osmium tetroxide in PBS for 70 min. The samples were washed two times with PBS, followed by dehydration for 9 min in a graded ethanol series (50%, 70%, 90% and 100%), and incubated for 10 min each in 100% ethanol, a mixture (1:1) of 100% ethanol and acetone, and absolute acetone. These samples were then incubated at 37°C overnight. Finally, the specimens were observed using a transmission electron microscope.

FTIR spectroscopic analysis of EPS: Fourier transform infrared (FTIR) of EPS sample was recorded to detect the functional groups of purified EPS. 2 mg of EPS was ground with 200 milligram of dry potassium bromide (KBr). The ground mixture was pressed in hydraulic press machine and compressed pellet was obtained. The FTIR spectra were recorded using % transmittance mode, 4 cm^{-1} resolution, wavenumber range of 4,000–400 cm^{-1} and FTIR Bruker Optics, GmBH, Germany.

RESULTS AND DISCUSSION

Isolation and identification of bacteria

EPS producing bacterial strain was isolated from root nodules of leguminous plant soybean (*Glycin max*) and identified as *Cronobacter muytjensii ATCC 51329(T)* by grams staining, some biochemical characters (Table 4) and by 16s rRNA sequencing (Fig 1). The sequence was deposited in GenBank with accession number KT936150.

Table 4 Morphological and biochemical characterization

Characteristics	Inference
Grams nature	Negative
Cell shape	Rod
Surface	Mucoid
Indol	+
Citrate	+
Catalase	+
Oxidase	-

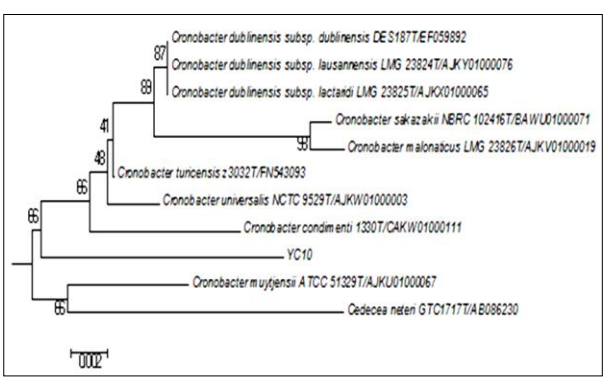

Fig 1 Phylogenetic tree of *Cronobactermuytjensii ATCC 51329(T)* identification

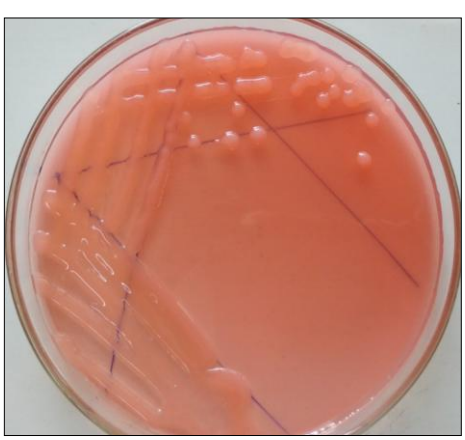

Fig 2 Growth of isolated bacteria *Cronobacter muytjensii ATCC 51329(T)* on YEMA+ CR plate

Large, circular, mucoid and white colonies were appeared on pink colored background (Fig 2). When colonies touched with sterile loop/tooth picks able to generate long viscous filament and that phenomenon indicate production of EPS (Gharzauli *et al*. 2012).

Effect of parameters on EPS production using one-factor-at-a-time method

One-factor-at-a-time was carried out to study the effect of various parameters on EPS production. One factor at a time, although time consuming and laborious method, this experiment aided in selecting the center points for the optimization study using P-B design and RSM. Influence of various process parameters on EPS production was investigated under batch mode. It is important to optimize the incubation time to ensure the length of fermentation. Maximum production of EPS 3.48 g $L^{-1}$was obtained after 72 h of incubation and upon further increase in incubation time, the EPS production was decreased (Fig 3a). A constant temperature is essential to inhibit the negative effect on EPS production. Hence temperature optimization study was carried out to reveal the optimum temperature condition, Maximum EPS production 3.01 g L^{-1}obtained at 30°C (Fig 3b). Deviation in temperature at lower side from optimum temperature resulted decrease in EPS production and reached to its minimum around 0 at 10°C. When temperature was increased above the optimum temperature, the EPS production decreases by 30 % in comparison with optimum EPS production at 30°C. Carbon source expected to have significant impact on the EPS production. Maximum EPS production 3.745 g L^{-1} was obtained using carbon source D-glucose (Fig 3c). In presence of carbon source mannitol and sucrose comparable EPS production around 3 g L^{-1} was observed. In case of other carbon sources maltose, lactose, xylose and arabinose EPS production was greatly influenced and decreased to 1 g L^{-1}. Carbon source is one of the most important factors affecting EPS production. The carbon source that leads to high cell weight does not always result in high EPS production (Kumar *et al*. 2007). Previous reports suggested that EPS production is favored by a high carbon to nitrogen ratio (Miqueleto *et al*. 2010). In present study, glucose was found to be the best carbon source for EPS production. Nahas *et al*. (2011) reported a maximum EPS production 9.01 g L^{-1} by a marine bacterium with glucose as best carbon source and at neutral pH 7 in seven days of incubation period. *Ganoderma* produced 1.7 g L^{-1}EPS with 70 g L^{-1} glucose concentration (Kim *et al*. 2006). *Lentinus edodes* produced 6.88 g L^{-1} EPS in presence of 15.88 g L^{-1}glucose concentration (Feng *et al*. 2010). Chowdhury *et al*. (2011) with *B. megaterium RB-05* reported that glucose is the better carbon source over others such as sucrose, fructose, maltose and lactose for maximum EPS yields. Previous research studies reported that the polysaccharide production was greatly influenced by higher amounts of carbon and limited nitrogen concentration (Sirajunnisa *et al*. 2016).

In the present investigation among the different nitrogen sources, maximum EPS production 4.075 g L^{-1} was obtained using peptone (Fig 3d). Other nitrogen sources resulted with significantly less EPS production except yeast extract and beef extract. Due to its proteins, amino acids and vitamin contents peptone stimulates the EPS production. Liu *et al*. (2011) reported a maximum EPS production (8.90 g L^{-1}) from *Zunonwangia profunda* bacteria isolated from a deep sea with peptone as best nitrogen source. *Cronobacter dublinensis subsp. dublinensis DES187(T)* showed maximum EPS production (770µg/ml) with 0.2% beef extract as nitrogen source (Mane and Hamde 2014). Srinivas and Padma (2014) reported that the organic nitrogen sources were much more suitable than inorganic nitrogen sources for the microbial EPS production.

Effect of six different mineral ions on EPS production were examined in which magnesium sulphate ($MgSO_4$) gave maximum EPS production (4.075 g L^{-1}**)** followed by Mg^{2+} ion $FeSO_4$ showed maximum production (Fig 3e). Researcher suggested that concentrations of magnesium can change polysaccharides composition rather than polysaccharide production (Hsieh *et al*. 2006). Effects of different mineral ions were studied at different concentrations and revealed that $CaCl_2$, at 0.05% concentration, gave the maximum EPS yield (1.432 g L^{-1}). Sayyed *et al*. (2015) isolated the heavy metal resistant bacteria from field soil and identified as *Enterobacter species RXS5*, and cultural conditions were optimized for EPS production. Threshold level of 50 µM for Fe^{2+} yield maximum EPS (3.22 g L^{-1}), the concentration of 60 µM of Ca^{2+} yielded maximum EPS (2.82g L^{-1}) and among the different concentrations of Mg^{2+} used 60 µM yielded optimum EPS (2.82g L^{-1}) (Sayyed *et al*. 2015).

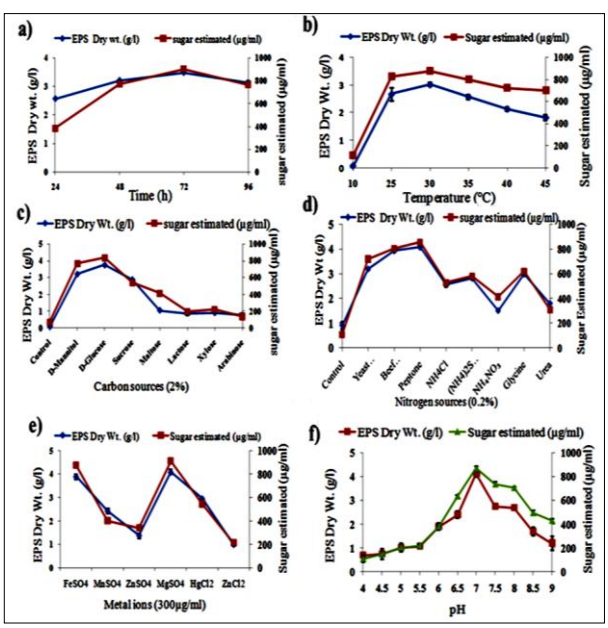

Fig 3 Influence of physical and chemical factors on EPS yield by *Cronobacter muytjensii ATCC 51329(T)*

a) Effect of incubation time. b) Effect of temperature. c) Effect of carbon sources. d) Effect of nitrogen sources. e) Effect of metal ions. f) Effect of pH

The EPS production was greatly influenced by initial pH of the medium (Fig 3f). In the present investigation it

was observed that the pH 7 was the optimum and resulted with EPS production 4 g L^{-1} (Fig 3f). At pH 4.0 EPS production was decreased by 85% and at pH 9.0 by 75% in comparison with optimum. Thus the incubation period 72 h, temperature 30°C, carbon sucrose –glucose, nitrogen source peptone, metal ion $MgSO_4$ and pH 7.0 were the optimum condition obtained using single parameter optimization approach. Using one-factor-at-a-time method interactions occurring between two or more variables were unable to detect. Hence the two stage process of optimizations Plackett Burman and CCD approach effectively were applied for improvement of EPS yield production and to understand interaction between the factors using *Cronobacter muytjensii ATCC 51329(T)*.

Identification of significant factors using Plackett-Burman design

Plackett-Burman design, EPS yield and cell growths are shown in (Table 5). The experiment 6, 8 and 12 showed that the response EPS yield and growth were reproducible. The regression analysis of Plackett-Burman experiments is shown in (Table 5)**.** The regression analysis showed that the parameter glucose (X_1) and temperature (X_7) were significant at 1% level in case of response EPS, g L^{-1}. Similarly in case of response EPS, µg ml^{-1}, parameter temperature (X_7) was significant at 5% level. The parameters peptone (X_2) and temperature (X_7) were significant at 1% level for response cell growth. In case of response EPS g L^{-1} and cell growth the parameters glucose (X_1) and peptone (X_2) showed positive effect value 0.646 and 0.239 respectively. This positive effect value of the parameter X_1 and X_2 indicates that it will influence on the positive side of '0' level of the parameter. The parameter temperature (X_7) showed negative effect. The X_7 will significantly influence on negative side of '0' level of the parameter. Placket-Burman results indicated that the parameter glucose and temperature had significant influence on EPS production. Jian-Dong Cui *et al.* (2010) demonstrated application of PBD for screening of significant independent factors for EPS production and the result indicated that the glucose and peptone were significant factors ($p<0.05$). The regression equation obtained from analysis of variance (ANOVA) indicated that the correlation coefficient of R^2 is 0.931 and the model can explain 93.1% variation in the response. Furthermore, the interactive effects of glucose and peptone was also highly significant ($p<0.01$) (Cui *et al*. 2010).

Table 5 Plackett-Burman design with EPS yield and cell growth

Response	Crude EPS, g/L		EPS, µg/ml		Cell growth, OD	
Term	Effect	p-value	Effects	p-value	Effects	p-value
Constant	-	0.000	-	0.000	-	0.000
X_1	0.6460	0.002**	121.00	0.105	-0.0565	0.483
X_2	0.1220	0.477	-29.00	0.683	0.2398	0.009**
X_3	0.0070	0.967	18.00	0.800	0.1601	0.061
X_4	0.0790	0.643	45.00	0.529	0.0709	0.381
X_5	-0.2950	0.100	15.00	0.833	-0.0731	0.367
X_6	-0.1360	0.429	8.00	0.910	-0.0315	0.693
X_7	-0.5330	0.007**	-191.00	0.017*	-0.4852	0.000**
X_8	-0.2570	0.147	-34.00	0.633	-0.0056	0.944

**Significant at 1% level; *Significant at 5% level

Development of mathematical model for EPS yield and cell growth

RSM is a collection of mathematical and statistical techniques used for designing experiments, building models, searching optimum conditions of factors for desirable responses and evaluating the relative significance of several affecting factors even in the presence of complex interactions (Ruchi *et al*. 2008, Mohana *et al*. 2008). Most researches have been indicated that incubation time, fermentation temperature, and inoculum volume are the most significant influencing factors in the optimization with RSM (Gao *et al*. 2007). In present study the most significant factors in fermentation of optimization conditions were glucose, incubation temperature and inoculum volume. The central composite design and the results obtained for EPS yield and cell growth are summarized in (Table 4). Experiment No. 2, 3, 5, 10, 11, and 12 showed that the response EPS yield and cell growth were reproducible. The EPS yield was varied in the range of 0.925-3.58 g L^{-1} in gravimetric measurement and 300-950 µg ml^{-1} in spectrophotometric measurement. The cell growth was varied in the range 0.307 to 1.83 OD. The regression analysis was carried out by subjecting these data to MINITAB software and regression analyses results are shown in (Table 6). The regression analysis for response EPS g L^{-1} revealed that the linear term X_1, quadratic terms X_1*X_1 and X_7*X_7 and interactive terms X_1*X_7 were significant. Similarly for response EPS µg/ml linear terms X_1 and quadratic terms X_1*X_1 and X_7*X_7were significant. In case of response cell growth only term X_7 was significant at 5% level. The coefficients obtained using regression analyses were used for development of the polynomial equation (1), (2) and (3). The equation (1) (2) and (3) included significant terms found in regression analyses.

$$Y_{EPS,g/L} = 3.198 + 0.425X_1 - 0.357X_1^2 - 0.5065X_7 + 0.4657X_1X_7 \text{ ------ (1)}$$

$$Y_{EPS,\mu g/ml} = 836.286 + 93.75X_1 - 95.542X_1^2 - 113.042X_7^2 \text{ ----------(2)}$$

$$Y_{Cell\ growth,OD} = 1.4817 - 0.248X_7 - 0.269X_7^2 \text{ ----------(3)}$$

Table 6 Estimated coefficient using regression analysis for EPS yield and cell growth

Crude EPS, g/L				
Term	Coefficient	SE Coefficient	t-value	P-value
Constant	3.1986	0.11816	27.069	0.000
X_1	0.4250	0.08605	4.939	0.002**
X_7	-0.1200	0.08605	-1.395	0.206
X_1*X_1	-0.3577	0.06309	-5.670	0.001**
X_7*X_7	-0.5065	0.06309	-8.028	0.000**
X_1*X_7	0.4675	0.14904	3.137	0.016*
EPS, µg/ml				
Term	Coefficient	SE Coefficient	t-value	P-value
Constant	836.286	45.49	18.384	0.000
X_1	93.750	33.13	2.830	0.025*
X_7	-33.750	33.13	-1.019	0.342
X_1*X_1	-95.542	24.29	-3.934	0.006**
X_7*X_7	-113.042	24.29	-4.654	0.002**
X_1*X_7	1.250	57.38	0.022	0.983
Cell growth OD				
Term	Coefficient	SE Coefficient	t-value	P-value
Constant	1.4817	0.147	10.03	0.000
X_1	-0.071	0.107	-0.668	0.526
X_7	-0.248	0.107	-2.308	0.05*
X_1*X_1	-0.040	0.078	-0.511	0.625
X_7*X_7	-0.269	0.078	-3.41	0.011*
X_1*X_7	0.076	0.186	0.408	0.695

**Significant at 1% level; *Significant at 5% level

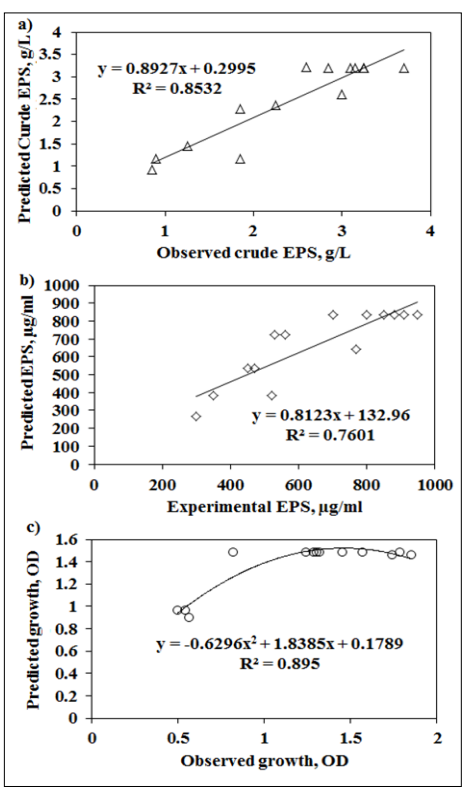

Fig 4 Predicted and observed value plots of EPS yield and cell growth

The suitability of the equation (1), (2) and (3) were further confirmed by simulating the equations using the coded value in CCD matrix and predicted EPS yield and cell growth were obtained. The predicted values of the EPS yield and cell growth plotted against experimental EPS yield and cell growth respectively (Fig 4).

Trend line was fitted and obtained the coefficient of determination R^2. The R^2 value for response EPS g L^{-1}, EPS µg ml^{-1} and growth OD were 0.85, 0.76 and 0.89 respectively (Fig 4a,b,c). The R^2>0.75 specify the suitability of the model.

The regression analyses revealed that the parameter glucose and temperature were most influencing factors. The 3D surface plots showed that appropriate levels of glucose in the medium and process temperature improved the EPS yield. Under optimized condition EPS increased to 4.25 g L^{-1} and EPS to 983.33µg ml^{-1}. The developed mathematically models very well fitted and suitable for prediction of EPS, g L^{-1}, EPS µg ml^{-1} and cell growth. Arun *et al* (2014) optimized EPS production in *Halobacillus trueperi AJSKRSM* using RSM and found maximum production (12.93 g/l) with glucose and peptone as best carbon and nitrogen sources, at 35°C in 72 h by RSM using CCD. Cui *et al*. (2010) under optimum cultural conditions, the EPS production was enhanced from 0.78 to 1.96 g L^{-1} (Liu *et al*. 2011).

Interactive effect of significant factors on EPS yield and cell growth

The interactive effect of glucose and temperature is shown in (Fig 5). The EPS concentration was decreased with increase in parentage of glucose at low level of temperature -2 and reached to the minimum EPS production at glucose and temperature level intersection point (2, -2), whereas at temperature EPS concentration increased with increase in

the glucose concentration in the medium and reached to the highest value around 2.5 g L^{-1} (Fig 5a) at the 0 level of the both factors.

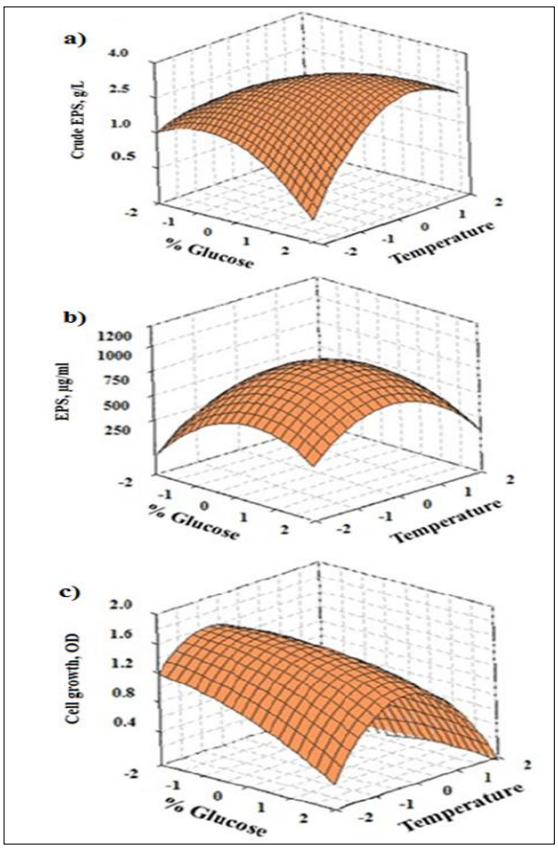

Fig 5 Interactive effect potential of glucose and temperature parameters on a) crude EPS, g/L b) EPS, µg/ml c) cell growth

A typical 3D surface of optimized conditions was observed in case of response EPS, µg ml^{-1}. Maximum EPS concentration of around 750 µg ml^{-1} was observed at '0' level of the both the parameters (Fig 5b). The cell growth increased with increasing in temperature till '0' level of temperature (Fig 5c). When temperature was increased further resulted with decrease in cell growth. Maximum cell growth 1.6 OD observed at lower level '-2' of glucose. This cell growth was steady till glucose level '0' in the medium and beyond that the cell growth was decreased. Interestingly at lower (-2) and higher level (+2) of temperature, the cell growth was minimum when glucose level in the medium at 2. These results proved that the optimum levels of glucose and temperature in the medium was essential for growth of cells as well as EPS production.

Demonstration of validation experiment

The optimized levels obtained using optimizer tool is shown in (Fig 6). The value of individual desirability of EPS g L^{-1}, EPS µg ml^{-1} and cell growth were 0.76, 0.81 and 0.73 respectively. The overall desirability was close to 1.0. If values of individual and overall desirability close to 1.0, then predicted value will be close to the experimental value. Raissi *et al*. (2009) have reported that when response approaches to it predicted value when desirability is close to 1.0. The results of EPS yield and cell growth obtained under optimized condition are shown in (Table 7). In preliminary experiments of screening of culture 3.0 g L^{-1} and µg ml^{-1} 590 EPS were obtained, when inoculated with 2% (v/v) inoculum. Under optimized condition EPS increased to 4.25 g L^{-1} and EPS yield increased to 983.33µg ml^{-1}, when inoculated with 1.5 % (v/v) inoculum. The CCD optimization revealed that appropriate levels of the medium components and process parameters improved EPS yield.

Table 7 EPS yield and cell growth under optimum condition

Parameter	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Crude EPS (gL^{-1})	EPS (μgml^{-1})	Cell growth (OD)
Coded	0.3838	0	0	0	0	0	-0.1414	0	4.25±0.08	983.33±5.77	1.4853±0.06
Real	3.38	0.3	450	300	7	1.5	34.29 ~ 35	72			

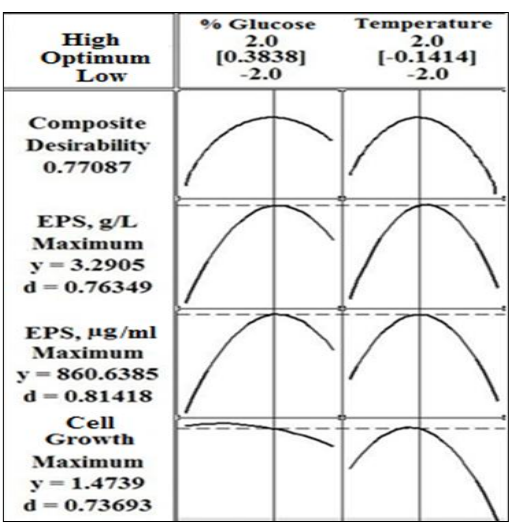

Fig 6 Optimized levels obtained using optimizer tool

SEM/TEM analysis of EPS

To study the surface morphology of the biopolymers, SEM/TEM has been a tool of choice reported by many scientists (Ahmed *et al*. 2013, Zhu *et al*. 2017). EPS obtained from *Cronobacter muytjensii ATCC 51329(T)* observed by SEM/TEM is shown in (Fig 7).

The EPS exhibits flakes like structural unit and highly compact, porous structure. This indicates the potential of EPS as water holding, viscosifying, and thickening or as stabilizing agent (Yadav *et al*. 2011). In SEM at 40000X and 45000X, the EPS is compact in structure composed of different layers with smooth surface. At higher magnification in SEM/TEM, smooth glittering surface with irregular lumps was even clearer; which is an indication of structural integrity of polymer. Small pores observed through SEM could give good water holding ability to EPS. Similar EPS structure was also reported with the galactomannan biopolymer isolated and purified from

fenugreek (Farhat *et al*. 2017). Singh *et al*. 2016 reported microstructures of the EPS from strains of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* by SEM analysis. The exopolysaccharide (EPS) showed a relatively stable three dimensional, porous, and smooth glittering structure. EPSs with smooth surface and good structural integrity are excellent candidates for film making and drug deliveries (Yadav *et al*. 2011).

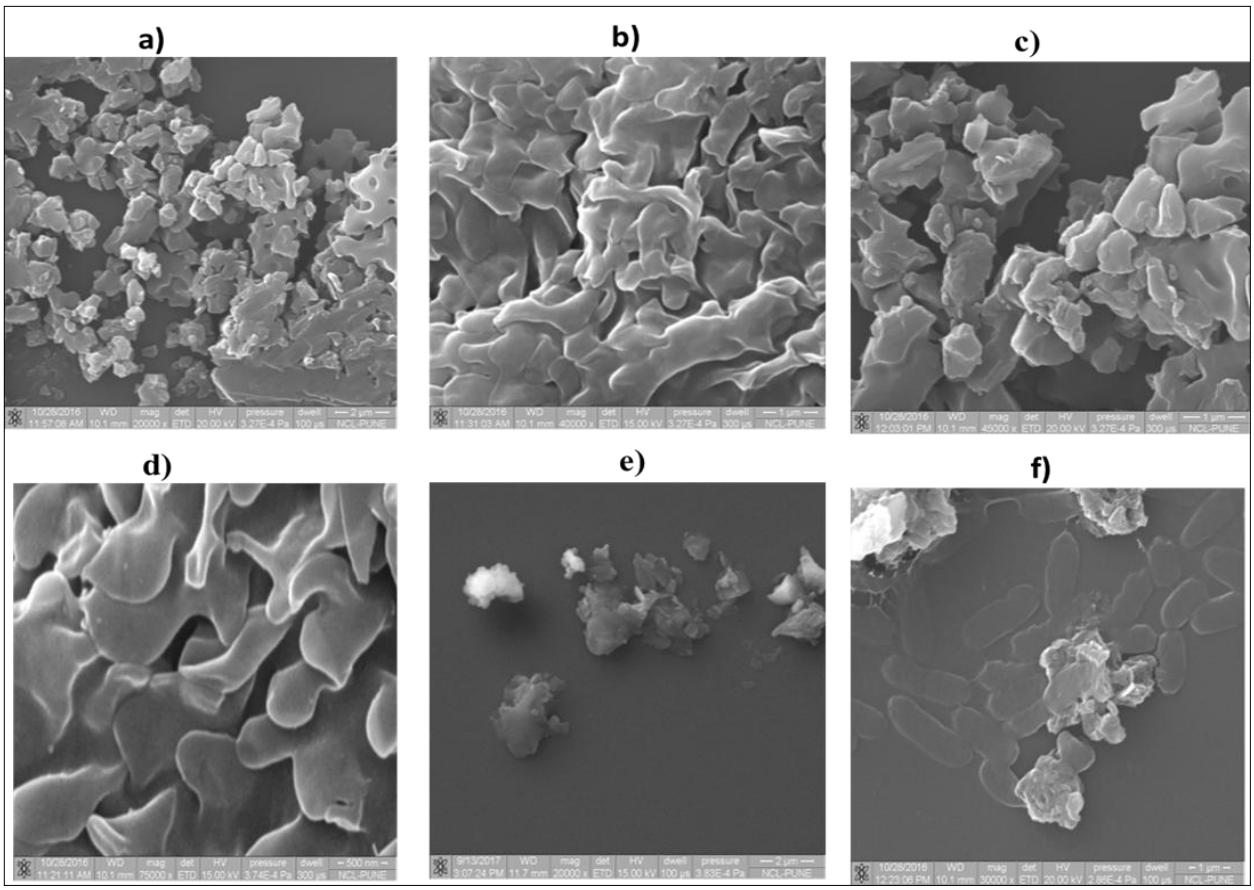

Fig 7(a, b, c, d) SEM images of EPS at magnification 20000x, 40000x, 45000x, 75000x
(e) TEM image of EPS at 20000x; (f) SEM image of bacteria at 30000x

FTIR spectroscopic analysis of EPS

FTIR spectrum of the EPS obtained from *Cronobacter muytjensii ATCC 51329(T)* is shown in (Fig 8). The characteristic hydroxyl stretching vibration (O-H) was observed by strong and broad observation of signal in the region 3600-3200 cm^{-1}. Castellane *et al*. (2015) demonstrated the EPS produced from different microbes isolated from rhizobia *Mesorhizobiumhuakuii* LMG14107, *M. loti* LMG6125, *M. plurifarium*LMG11892, *Rhizobium giardini* bv. *Giardini* H152T, *R. mongolense*LMG19141, and *Sinorhizobium* (= *Ensifer*) *kostiense* LMG19227 in a RDM medium with glycerol as a carbon source showed strong absorption of signal of O-H vibration at 3400 cm^{-1}. Similarly, Kanamarlapudi and Muddada (2017) reported the presence of O-H stretching vibration band at 3400 cm^{-1} for EPS obtained from *Streptococcus thermophiles* CC30. O-H stretching is responsible for solubility of EPS in water. Karbowiak *et al*. (2007) documented the similar results. Absorption of signal at 2950 cm^{-1} indicated by C-H stretching vibration of methyl or methylene group present in hexose like glucose. Absorption peak at 2156 .46 cm-1 represent presence of carboxylic group. The peak at 1732 cm-1, 1252 cm-1 and 1030cm^{-1} correspond to stretching vibration in C=O, C-O and C-O-C glycoside linkage respectively. The presence of α D-glucan is represented by absorption peak at 826.74 cm^{-1}.The shifting in absorption peak may be due to the type of glycosidic linkage, length of polysaccharide and secondary and tertiary structure of polysaccharide. Therefore the difference in chemical composition and quantity of each individual constituent were observed. However, FTIR spectrum showed the obtained EPS was heteropolymeric in nature. Kaur *et al*. (2013) showed the EPS obtained from *Alcaligenes Faecalis* B14 was heteropolymeric in nature.

The *Cronobacter muytjensii ATCC 51329(T)* was isolated from root nodules of leguminous plant soybean (*Glycin max*) and EPS production potential was estimated. The EPS production was optimized using statistical based design PBD and RSM and under optimized condition EPS production increased to 4.25 gL^{-1}. SEM and TEM analysis revealed that highly compact, multilayered, smooth glittering surface and structural integrity of EPS and can be explored in various industrial applications such as viscofying, thickening, stabilizing agent, and film making

agent. Small pores observed by SEM could give good water binding ability to exopolysaccharide (EPS). FTIR spectrum showed that the obtained exopolysaccharide (EPS) is heteropolymeric in nature.

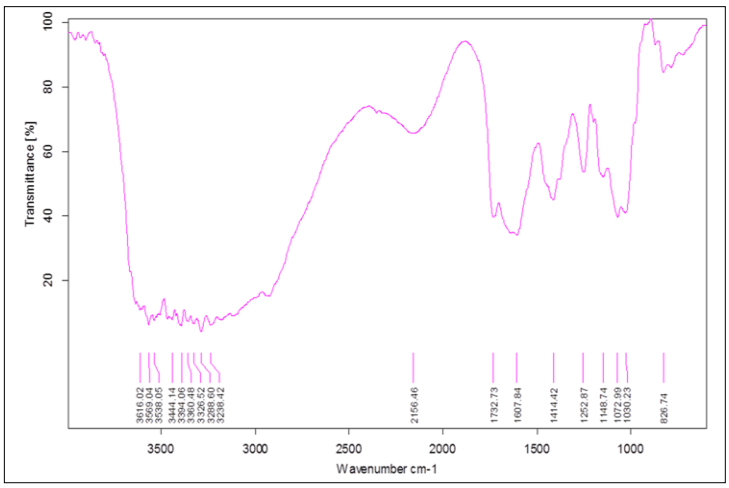

Fig 8 FTIR analysis of EPS obtained from *Cronobacter muytjensii ATCC 51329(T)*

Acknowledgements

The author is grateful to the INSPIRE DST (Department of Science and Technology) fellowship, New Delhi for providing financial support as INSPIRE fellowship. The author is also thankful to Yogeshwari Mahavidyalaya, Ambajogai, Maharashtra.

LITERATURE CITED

Ahmed Z, Wang Y, Anjum N, Ahmad A and Khan S T. 2013. Characterization of exopolysaccharide produced by *Lactobacillus kefiranofaciens ZW3* isolated from Tibet kefir e Part II. *Food Hydrocolloids* **30**(1): 343-350.

Arun J, Sathishkumar R and Muneeswaran T. 2014. Optimization of extracellular polysaccharide production in *Halobacillus trueperi* AJSK using response surface methodology. *African Journal of Biotechnology* **13**(48): 4449-4457.

Balamurugan G and Prakash S. 2012. Extraction, partial characterization and antibacterial efficacy of extra cellular polysaccharides from *Bacillus licheniformis* and *Klebsiella pneumonia* isolated from root nodules of *Tephrosia purpurea*. *International Journal of Pharma and Bio Sciences* **3**: 306-316.

Castellane T C L, Otoboni A M M B and Lemos E D M. 2015. Characterization of Exopolysaccharides Produced by *Rhizobia* Species, *Revista Brasileira de Cienciado Solo* **39**: 1566-1575.

Chawla P R, Bajaj I B, Survase S A and Singhal RS. 2009. Microbial Cellulose: Fermentative production and applications. *Food Technology and Biotechnology* **47**(2): 107-124.

Chowdhury R S, Basak R, Sen R and Adhikaria B. 2011. Optimization, dynamics, and enhanced production of a free radical scavenging extracellular polysaccharide (EPS) from hydrodynamic sediment attached *Bacillus megaterium* RB-05.*Carbohydrate Polymers* **86**: 1327-1335.

Cui J D and Jia S R. 2010. Optimization of Medium on exopolysaccharides production in submerged culture of *Cordyceps militaris. Food Science and Biotechnology* **19**(6): 1567-1571.

Dubois M, Gilles K A, Hamilton J K, Rebers P A and Smith F. 1956. Colorimetric method for determination of sugar and related substances. *Analytical Chemistry* **28**: 350-356.

Farhat R, Sarfraz H and Zaheer A. 2017. Extraction purification and characterization of galactomannan from fenugreek for industrial utilization. *Carbohydrate Polymers*. https://doi.org/10.1016/j.carbpol.2017.10.025.

Feng Y L, Li W Q, Wu X Q, Cheng J W and Ma S Y. 2010. Statistical optimization of media for mycelial growth and exo-polysaccharide production by *Lentinus edodes* and a kinetic model study of two growth morphologies. *Biochemical Engineering Journal* **49**(1): 104-112.

Gao H and Gu W Y. 2007. Optimization of polysaccharide and ergosterol production from *Agaricus brasiliensis* by fermentation process. *Biochemical Engineering Journal* **33**: 202-210.

Gharzauli R, Benahmed A, Benhizia Y and Benguedouar A. 2012. Influence of carbon sources on the production of exopolysaccharide by *Rhizobium sullae* and on the nodulation of *Hedysarumcoronarium* L. legume. *African Journal of Microbiology Research* **6**(30): 5940-5946.

Hsieh C, Tseng M H and Liu C J. 2006. Production of polysaccharides from *Ganoderma lucidum* (CCRC. 36041) under limitations of nutrients. *Enzyme and Microbial Technology* **38**: 109-117.

Iversen C and Forsythe S. 2003. Risk profile of *Enterobacter sakazakii*, an emergent 16 pathogen associated with infant milk formula. *Trends in Food Science and Technology* **14**: 443-454.

Kanamarlapudi S L R K and Muddada S. 2017. Characterization of Exopolysaccharide produced by *Streptococcus thermophilus* CC30. *BioMed Research International* Article ID 4201809. pp 11.

Karbowiak T, Ferret E, Debeaufort F, Voilley A and Cayot A. 2007. Investigation of water transfer across thin layer biopolymer films by infrared spectroscopy. *Journal of Membrane Science* **370**: 82-90.

Kaur V, Bera M B, Panesar P S and Chopra H K. 2013. Production and characterization of exopolysaccharide produced by *AlcaligenesFaecalis* B14 isolated from indigenous soil. *International Journal of Biotechnology and Bioengineering Research* **4**: 365-374.

Kim H M, Paik S Y, Ra K S, Koo K B, Yun J W and Choi J W. 2006. Enhanced production of exopolysaccharides by fed-batch culture of *Ganoderma resinaceum* DG-6556. *Journal of Microbiology* **44**(2): 233-242.

Kumar A S, Mody K and Jha B. 2007. Bacterial exopolysaccharides: A perception. *Journal of Basic Microbiology* **47**: 103-117.

Li H, Li J, Dou W, Shi J and Xu Z. 2013. Enhancing the production of a novel exopolysaccharide by *Bacillus mucilaginosus* CGMCC5766 using statistical experiment design. *Tropical Journal of Pharmaceutical Research* **12**(5): 711-718.

Li P, Xu L, Mou Y, Shan T, Mao Z, Lu S and et al. 2012. Medium optimization for Exopolysaccharide production in liquid culture of Endophytic fungus *Berkleasmium sp*. Dzf12. *International Journal of Molecular Sciences* **13**: 11411-11426.

Liu S B, Qiao L P, He H L, Zhang Q, Chen X L, Zhou W Z, Zhou B C and Zhang Y Z. 2011. Optimization of fermentation conditions and rheological properties of exopolysaccharide produced by deep-sea bacterium *Zunongwangia profunda* SM-A87. *PLoS One* **6**: e26825.

Mane G G and Hamde V S. 2015. Optimization of *Cronobacter dublinensis subsp. dublinensis* DES187 (T) isolated from root nodules of soybean for exopolysaccharide production. *International Journal of Global Science Research* **2**(4): 265-270.

Mane G G and Hamde V S. 2018. Isolation and identification of exopolysaccharide producing *Cronobacter* species from root nodules of leguminous plants. *Journal of Biochemical Technology* **9**(2): 1-6.

Miqueleto A P, Dolosic C C, Pozzi E, Foresti E and Zaiat M. 2010. Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for waste water treatment. *Bioresource Technology* **101**: 1324-1330.

Mohana S, Shah A, Divecha J and Madamwar D. 2008. Xylanase production by *Burkholderia sp*. DMAX strain under solid state fermentation using distillery spent wash. *Bioresource Technology* **99**: 7553-7564.

Nahas A M O, Darwish M M, Ali A E and Amin M A. 2011. Characterization of an exopolysaccharide-producing marine bacterium, isolate *Pseudoalteromonas sp*. AM. *African Journal of Microbiology Research* **22**: 3823-3831.

Nirmala P, Aysha O S, Valli S, Reena A and Kumar P V. 2011. Production of extracellular polysaccharides by a Rhizobium species from root nodules of *Vigna mungo* (Hepper). *International Journal of Pharmaceutical and Biological Archive* **2**(4): 1209-1214.

Raissi S and Farsani R E. 2009. Statistical process optimization through multi-response surface methodology. *International Journal of Mathematical, Computational, Physical, Electrical and Computer Engineering* **3**: 197.

Ruchi G, Anshu G and Khare S K. 2008. Lipase from solvent tolerant *Pseudomonas aeruginosa* strain: production optimization by response surface methodology and application. *Bioresource Technology* **99**: 4796-802.

Sayyed R Z, Patel P R and Shaikh S S. 2015. Plant growth promotion and root colonization by EPS producing *Enterobacter sp.* RZS5 under heavy metal contaminated soil. *Indian Journal of Microbiology* **51**: 294-300.

Singh P, Saini P, Puranik V, Gupta S and Dubey S. 2016. Optimization and characterization of exopolysaccharides produced by *lactobacillus strains* isolated from cabbage and cucumber. *Journal of Microbiology and Biotechnology Research* **6**(6): 27-35.

Sirajunnisa A R, Vijayagopal V, Sivaprakash B, Viruthagiri T and Surendhiran D. 2016. Optimization, kinetics and antioxidant activity of exopolysaccharide produced from rhizosphere isolate, *Pseudomonas fluorescens* CrN6. *Carbohydrate Polymers* **135**: 35-43.

Sivakumar T, Narayani S, Shankar T, and Vijayabaskar P. 2012. Optimization of cultural conditions for exopolysaccharides production by *Frateuria aurentia*. *International journal of applied biology and pharmaceutical technology* 3(3): 133-143.

Srinivas B and Padma P N. 2014. Screening of diverse organic, inorganic and natural nitrogen sources for dextran production by *Weissella Sps* Using Plackett-Burman Design. *International Journal of Scientific and Technology Research* **3**(4): 234-237.

Vijayabaskar P, Babinastarlin S, Shankar T, Sivakumar T and Anandapandian K T K. 2011. Quantification and characterization of exopolysaccharides from *Bacillus subtilis* (MTCC 121). *Advances in Biological Research* **5**(2): 71-76.

Yadav V, Siddalingaiya Gurudutt Prappulla S G, Jha A and Poonia A. 2011. A novel exopolysaccharide from probiotic *Lactobacillus fermentum* CFR 2195: Production, purification and characterization. *Biotechnology, Bioinformatics and Bioengineering* **1**(4): 415-421.

Zhu W, Xue X and Zhang Z. 2017. Structural, physicochemical, antioxidant and antitumor property of an acidic polysaccharide from *Polygonum multiflorum*. *International Journal of Biological Macromolecules* **96**: 494-500.