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Media Optimization for Exopolysaccharide Producing *Klebsiella oxytoca* KY498625 under Varying Cultural Conditions

Saad AM Moghannem, Mohamed M.S.Farag, A. M. Shehab*, Mohamed Salah Azab.

Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Nasr City, Cairo, Egypt. Post code 11884.
*Corresponding author: **Amr Mohamed Shehab.**
E-mail: *Amrshehab43@yahoo.com*

Abstract

The present work was designed for studying the efficiency of multi-factorial experimental designs to elucidate factors affecting exopolysaccharide production by *K.oxytoca* isolated from soil. Combinations of Plackett–Burman design (PBD) and Box – Behnken design (BBD) have been carried out for media optimization of various factors. Among 11 factors, yeast extract, molasses and incubation temperature were the most significant factors. These three factors were selected for further optimization using Box-Behnken design (BBD). The result obtained from data analysis showed that; the most optimum concentrations were yeast extract 6%, molasses12% and incubation temperature 37^0C. Bacterial isolate was identified as *Klebsiella oxytoca* using 16_SrRNAgene sequences. This isolate was submitted to the gene bank with accession number KY498625.Biopolymer was purified using DEAE-cellulose and identified using Fourier transform infrared (FTIR), gel permeation chromatography (GPC) and Gas mass chromatography spectroscopy (GC-MS).The result showed that; average molecular weight (Mw) of 4.05 x10^4 Da; major monosaccharide constituents were mannose, glucose, Arabinose and Galacturonic acid.

Keywords: Bacterial exopolysaccharide; Plackett–Burman; Box–Behnken; DEAE-cellulose.

Introduction

In the last decades, research has focused on the capabilities of microbes to secrete exopolysaccharides (EPS) that have novel, unique physical characteristics and application differ than commercial ones derived from plants or algae. Microbial exopolysaccharides (EPSs) composed mainly of carbohydrate polymers produced by many microorganisms including bacteria, fungi and yeasts (Freitas 2010). These biopolymers have been extensively applied in various biotechnological applications including; pharmaceutical, cosmetics, food, textile, oil recovery, metal mining and recovery (Satpute *et al*, 2010).These EPSs have many predominant advantages, such as easy production, less side effect, cost effectiveness and supply (Jaehrig *et al*, 2008).

Optimization of different fermentation conditions can be carried out either by means of conventional and/or statistical techniques. The conventional approach follows the 'one by one technique' in which one variable is modified even as all others are kept constant. This approach is time-consuming and

provides no information on interaction influences on overall productivity, in addition to incomplete understanding of the system's, ensuing in confusion and a loss of predictive capability (Hymavathi *et al*, 2010). Whereas, statistical methods are rapid and dependable, short lists significant nutrients, allows apprehend the interactions some of the nutrients at various concentrations .Plackett–Burman is well established and widely used in the statistical designs for selection of medium components, which can screen the important variables as well as their significance levels (Plackett and Burman 1946). Response surface methodology (RSM) has eased process development and has been of significant use at industrial level, among which Box–Behnken design methodology considers as best as it predicts the interaction effects among the variables (Li *et al*, 2008). RSM is a group of statistical techniques for designing experiments, building models, evaluating the effects of factors and attempting to find optimum conditions of factors for appropriate responses (Khuri and Mukhopadhyay, 2010). It gives vital information for designing and optimization in addition to useful analysis of a couple of responses at the same time.

In this study, statistical experimental design was used for optimization of different culture condition for exopolysaccharide production by *Klebsiella oxytoca* **KY498625** isolated from soil.

Materials and Methods

Isolation and Purification of Bacteria producing exopolysaccharide (EPS):

Seven soil samples were collected from Al-Bahariya Oasis, Egypt 28°24¢17″N 28°52¢25″E. Each sample was serially diluted from 10^{-1} to 10^{-7} in phosphate buffered saline, pH 7.2 ± 0.2. One mL of each dilution was inoculated into tubes containing 9 mL EPS culture medium [0.2g KH_2PO_4; 1.5g K_2HPO_4; 0.2g $MgSO_4.7H_2O$; 0.1g $CaSO_4.2H_2O$; 2.0mg $FeCl_3$; 0.5g Yeast Extract, 20g sucrose(per liter of medium)] the majority of organic and inorganic chemicals were products of Aldrich, (BDH, Fluka)and Sigma companies.

Phenotypic analysis of Exopolysaccharide production:

Exopolysaccharide (EPS) production was assessed by cultivating the strains on Congo Red Agar (CRA) using a method modified by (Freeman *et al*, 1989). The CRA allows the detection of exopolysaccharide production by variation in the color of colonies in the medium, Red or pink colonies on Congo red plates indicate the binding of Congo red to extracellular polysaccharide around colonies. The medium was prepared by adding 0.8 g of the Congo red dye (Merck®) in 1L of Brain Heart Infusion Agar (BHI, Himedia®) supplemented with 3% sucrose. The bacterial isolates were streaked onto Congo Red Agar plates and incubated at 37 °C for 24 hours.

Quantification of Exopolysaccharide

After selection and purification of EPS producing bacteria (characterized by colonies of bacteria that form Red or pink colonies on Congo red plates);EPS was obtained through precipitation using cold absolute ethanol 2:1 culture broth, then the precipitate was obtained by centrifugation at 5,000rpm for 10min and finally; EPS was quantified using phenol-sulphuric acid method (DuBois *et al*, 1956).

Preparation of cane molasses as the carbon source

Sugarcane molasses was obtained from a sugar factory at (Sugar cane factory in Kafr El Sheikh, Egypt) that has been used as carbon source. Molasses was diluted with distilled water containing sodium di-hydrogen orthophosphate(2.0g/l) with ratio 1:1. The solution was autoclaved at 121°C for 10min and left to settle for 24hr (Prasanna *et al*, 2014). The clarified molasses were used as sole carbon source at different concentrations 1, 2, 3, 4, 5, 6, 7 and 10%.

Experimental Designs and Optimization:

Classical one factor at a time method: this method depend on change in one factor at a time where other factors remaining constant (Prasanna *et al*, 2014). Single-factor experiments were carried out in 500 ml flasks containing 100ml medium at 200rpm/min and 30°C. Seven carbon source (Glucose, Sucrose, Lactose, Maltose, soluble Starch, Fructose and sugarcane Molasses) and six nitrogen sources (yeast extract, peptone, urea, $(NH4)_2SO_4$, NH_4Cl and KNO_3) were used. Statistical analysis was generated by Microsoft excel 2010 software and p levels at 0.05 were considered as significant.

Optimization of EPS using plakett-burmann (PB) design:

The PB experimental design was applied to screen the significant variables that influence EPS production (Chen *et al*, 2011) Eleven variables of medium composition and culture conditions were tested at low

(-1) and high (+1) levels as shown in Table 1. Based on PB matrix design, two-level factorial design, it allows the investigation of 12 variables (n+1) (Plackett and Burman 1946). This design consisted of three replicated center points to avoid error.

$$Y=ß_0+\Sigma ß_{ixi} \quad (1)$$

Where Y is the predicted response, $ß_0$ is the model intercept; $ß_i$ is the linear coefficient and X_i=is the level of the independent variable.

EPS production was measured in triplicate and the average value was taken as the response as shown in Table 2.The variables significant at 95% level ($P< 0.05$) were considered to have significant effect on EPS production and thus used for further optimization.

Response surface methodology

Three factors Box-Behnkendesign (BBD) was used as model for this study (Khani *et al*, 2015). The center points and parameters were selected according to Plakett-Burmann design. Fifteen trials for three different variables included medium consisted of sugarcane molasses, yeast extract and incubation temperature were designed. Three levels, ''high (+1)'', ''middle (0)'' and 'low (-1)'' as shown in Table 3. Regression analysis was performed on the data obtained using software package 'Design Expert' software (Version 7.0) and confirmed by Microsoft Excel 2010 (Rahman *et al*, 2008).The accuracy of polynomial model equation was expressed by coefficient of determination R^2. All experiments were performed in triplicates. The design is represented by a second-order polynomial regression model as follows:

$$Y=ß_0+\Sigma ß_{ixi}+ \Sigma ß_{iixi}^2+ \Sigma ß_{ijxixj}+ \Sigma \quad (2)$$

Where Y: is the predicted response, $ß_0$: the intercept, $ß_i$: linear coefficient, $ß_{ij}$: the interactive coefficients, $ß_{ii}$: the quadratic coefficients, and Σ: is the error, and x_i: and x_j: are the coded independent variables.

Table 1. Low and high levels of each variable used in Plackett-Burman design

Medium components	Units	Symbol	+values	-values
Yeast extract	g/L	X_1	4	2
Sugarcane molasses	%	X_2	9	3
$MgSO_4$	g/L	X_3	0.6	0.2
K_2HPO_4	g/L	X_4	3	1
KH_2PO_4	g/L	X_5	0.6	0.2
Peptone	g/L	X_6	4	2
Incubation time	hours	X_7	72	24
Inoculum size	%	X_8	9	3
Temperature	^{0}C	X_9	37	32
pH	-	X_{10}	8.5	6.5
Shaking	rpm	X_{11}	300	100

Table 2-Plackett –Burmann experimental design for screening of culture conditions affecting EPS production

RUN	*X1*	*X2*	*X3*	*X4*	*X5*	*X6*	*X7*	*X8*	*X9*	*X10*	*X11*	*EPS g/l observed*	*predicted*
1	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	3.98±0.11	5.8
2	1.00	-1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	6.88±0.28	5.55
3	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	6.52±0.22	6.61
4	-1.00	-1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	2.57±0.44	3.14
5	1.00	-1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	5.87±0.33	7.18
6	1.00	1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	5.25±0.19	6.76
7	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	2.34±0.35	3.05
8	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	1.84±0.54	2.15
9	-1.00	1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	3.24±0.39	3.69
10	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	2.1±0.54	2.57
11	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.56±0.16	1.96
12	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	4.32±0.21	4.65
13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.65±0.36	4.69
14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.9±0.14	4.69
15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.89±0.21	4.69

Table 3-Experimental design and results of Box–Behnken optimization experiment

Trials	X_1(Molasses)		X_2(Yeast extract)		X_3(incubation temp.)		EPS (g/l)	
	Coded level	Real level (%)	Coded level	Real level(g/l)	Coded level	Real level (^{0}C)	**observed**	**predicted**
1	0.00	**8**	1.00	**6**	-1.00	**30**	**5.2±0.36**	**5.55**
2	1.00	**12**	-1.00	**2**	0.00	**35**	**7.45±0.48**	**7.55**
3	0.00	**8**	1.00	**6**	1.00	**40**	**7.35±0.36**	**6.87**
4	1.00	**12**	0.00	**4**	-1.00	**30**	**6.79±0.18**	**6.21**
5	-1.00	**4**	0.00	**4**	1.00	**40**	**4.1±0.45**	**4.84**
6	1.00	**12**	0.00	**4**	1.00	**40**	**6.9±0.22**	**7.15**
7	1.00	**12**	1.00	**6**	0.00	**35**	**7.68±0.15**	**7.89**
8	0.00	**8**	-1.00	**2**	-1.00	**30**	**3.88±0.41**	**4.35**
9	-1.00	**4**	0.00	**4**	-1.00	**30**	**4.1±0.33**	**3.84**
10	-1.00	**4**	-1.00	**2**	0.00	**35**	**4.21±0.26**	**3.99**
11	0.00	**8**	-1.00	**2**	1.00	**40**	**5.32±0.10**	**4.96**
12	0.00	**8**	0.00	**4**	0.00	**35**	**4.65±0.28**	**4.83**
13	-1.00	**4**	1.00	**6**	0.00	**35**	**6.88±0.21**	**6.7**
14	-1.00	**4**	0.00	**4**	1.00	**40**	**5.01±0.31**	**4.8**
15	0.00	**8**	0.00	**4**	0.00	**35**	**5.02±0.44**	**4.8**

X1: molasses (%); X2: yeast extract (g/l) andX3: incubation temperature (hours)

Identification of bacterial Isolate:

Identification of EPS producing isolate was performed using 16S rRNA sequence analysis. This step was carried out in Sigma Company for Research, Cairo, Egypt. Forward and reverse primers used for PCR amplification were:
27f5'AGAGTTTGATCCTGGCTCAG3' and 1492r5'GTTACCTTGTTACGACTT3'.

PCR Sequence was carried out in GATC (GuaninAdeninThyminCytosine) German Company using ABI 3730 X1 DNA sequencer. Sequence analysis was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis was performed using a MEGA version 4.1 program by neighbor joining (NJ)/maximum parsimony (MP) (Kumar *et al*, 2004).

Purification of EPS by Di-ethylaminoethyl-cellulose (DEAE-cellulose):

DEAE-cellulose was used as an anion exchanger as recommended by Peterson and Sober,[22] it was equilibrated with 0.05 M Tris buffer (pH 7.5) to be used in chromatography. The lyophilized crude EPS sample was dissolved in small volume of 0.1 M NaCl. Then fractions was initially purified by DEAE–cellulose column chromatography and eluted with 25 Mm Tris–HCl (pH 8.5). Polysaccharide containing fractions were determined for the presence of the total sugar by phenol-sulfuric acid method (DuBois *et al*, 1956) the fractions containing sugar were pooled, concentrated by ultra-filtration, and precipitated with 2 volumes of ethanol, for further Characterization.

Preliminary Characterization and identification of purified fraction:

The purified fraction was characterized using the following spectroscopic analysis, Number and weight average molecular weights (Mn and Mw, respectively), as well as the polydispersity index (Mw/Mn) of the purified EPS were obtained by gel-permeation chromatography (GPC) (Wang *et al*, 2014). The EPS were detected using a refractive index detector (RI) at an internal temperature of 40°C. The column was eluted with 0.1 mol/L $NaNO_3$ solution at a flow rate of 0.5 mL/min, and the injection volume of sample was 200 µL. Data processing were performed with Wyatt Astra software (Version 5.3.4.14, Wyatt Technology, USA).

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 4000-400cm^{-1}. The exopolysaccharide was characterized using a Fourier Transfer Infrared Spectrophotometer (Bruker Optics GmBH, Germany).

The monosaccharide composition according to (Seviour *et al*, 2010) for purified polysaccharide from *K.oxytoca* was identified by GC-MS of their alditol acetate derivatives. After hydrolysis with 2 N (final concentration) trifluouoacetic acid at 121^{0}C for 1 h, the hydrolysate was reduced and acetylated with NaBD4and aceticanhydride/pyridine, respectively. After it was evaporated with toluene, it was extracted with dichloromethane. The alditol acetates were analyzed with a Shimadzu GC-MS-QP5050 Thermo Scientific Prop, equipped with capillary column (ø 0.25 mm×60 m). Its temperature program was 190^{0}C for 4 min and then1^{0}C min to 220 ^{0}C and holding for 20 min at 220^{0}C. As references, different concentrations of the following neutral sugar standard mixtures were also prepared, converted to their derivatives, and analyzed: rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose.

Results and Discussion

Phenotypic assay for detection of the exopolysaccharide production

All purified isolates were tested for Exopolysaccharide production on Congo Red Agar (CRA) with sucrose. Colonies that appears with black and opaque colonies were selected. Negative EPS producing Colonies appears with red color (Figure 1).The most potent bacterial isolates producing EPS were M7 (30), E1 (52) and 9I (55) that have been selected from sixty isolate using phenol–sulphuric acid assay. The isolate M7 (30) was the maximum productivity of about 3mg/ml that has been selected for further study of research.

Figure 1: Congo red agar media for testing Exopolysaccharide production.

Positive Exopolysaccharide producing isolate appears with black colonies (+ve), while negative isolate appears with red and smooth colonies (-ve).

The color of the colonies ranged from very dark to almost black, according to the classification described by (Arciola *et al*, 2002**),** and considered as positive for the production of EPS. The concentration of sucrose in medium was a limiting factor for EPS production.

Kinetics of Growth and EPS Production:

The synthesis of EPS as a function of the growth was investigated by monitoring the changes of cell density (OD_{600}) and the production of EPS (g/L) during the entire fermentation period. As shown in Figure 2, there was strong correlation between bacterial growth and exopolysaccharide.

Production in culture media that confirmed from our study; EPS productionby isolate (M7) was gradually increased after 16 h, and its production was maintained at a constant level after 96 h.

This was probably because of the action of glycohydrolases possibly produced in the culture that catalyzed the degradation of polysaccharides, resulting in decreased EPS yields after stationary phase. These results indicate that the synthesis of EPS occurred only in the post stationary growth phase of the strain (growth dissociated synthesis). Therefore, following optimized experiments only focused on EPS production. This result is agreed with that obtained by (Reshetnikov *et al*, 2001).

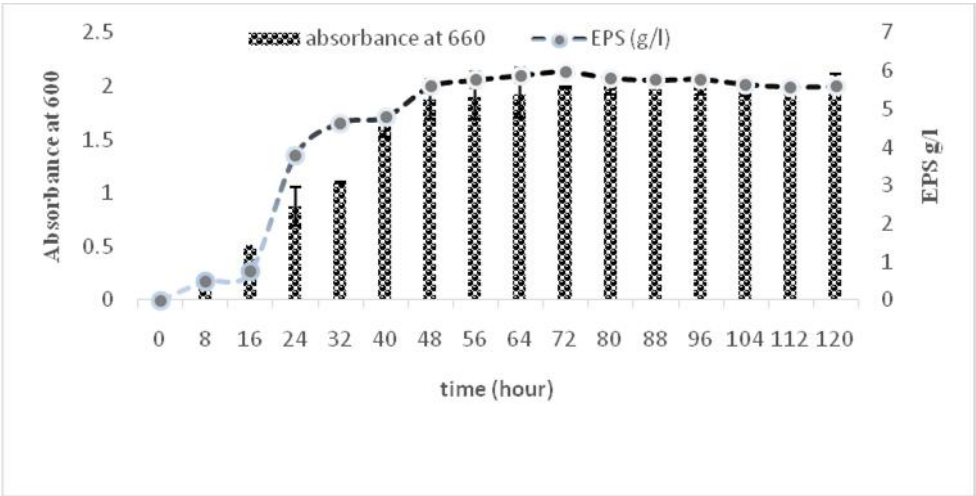

Figure 2- Correlation between Bacterial growth curve and EPS production of bacterial isolate (**M7).**

EPS production was significantly affected by the types of carbon sources and their concentrations in the medium (Rahman *et al*, 2008). EPS yields varied from 1.2 g/L to 4.6 g/L using different carbon sources. The highest yield (4.6 g/L) of EPS was obtained when sucrose was used as the carbon source, whereas the lowest yield (1.2 g/L) occurred when soluble starch was used as the sole carbon source. Glucose, soluble starch, maltose and lactose were also favorable carbon sources for EPS production. Nevertheless, sucrose was most effective and economical for large scale production of EPS among these carbon sources.

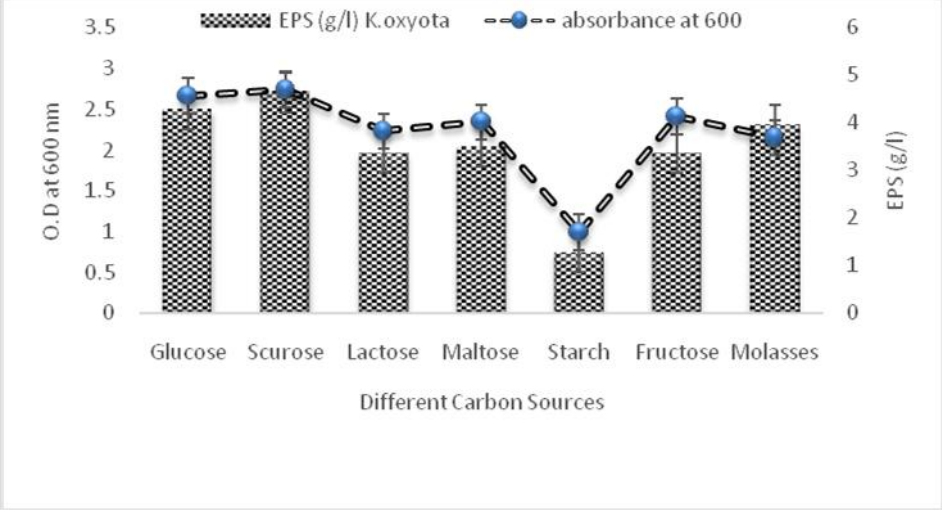

Figure 3- Effect of different carbon sources on EPS production.

Higher yields were achieved by the addition of inorganic nitrogen and organic nitrogen sources. Yeast extract was the most favorable source of all different nitrogen sources used for EPS production (4.1 g/L) at concentration of 3 g/L yeast extract (Figure 4). The experiment of single factor optimization was designed to identify the key variables which can affect EPS production keeping other factors constant (Ismail and Nampoothiri 2010).The results obtained from this experiment indicated that; EPS production was significantly affected by the type and concentration of carbon source in medium. Molasses was the most effective and economically for large scale production of EPS among the carbon sources. Carbon and Nitrogen sources are well known factors that affect both cellular metabolism and EPS production (Wang *et al*, 2015). The present study was carried out to study the influence of various parameters on elevation of EPS yield.

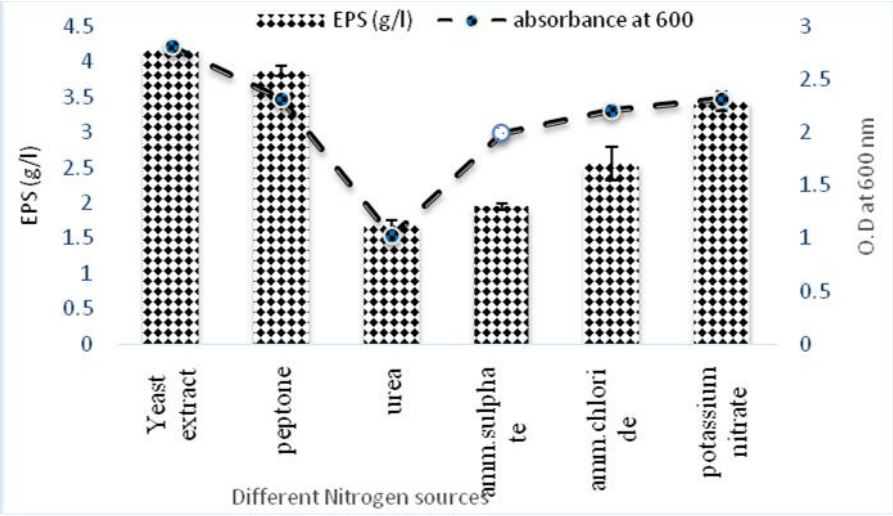

Fig.4- Effect of different nitrogen sources on EPS production.

Screening significant variables using Plackett–Burman design:

Eleven factors of media components were examined in the Plackett–Burman design experiments with fifty different trials, and maximum EPS production was obtained for trial number (**2**), while the lowest production was obtained for trial number (**11**). The regression coefficients (t value) and confidence level are given in Table 4. The media components showed both positive and negative effects on EPS production. Statistical analysis (t values) demonstrated that molasses and yeast extract and incubation temperature had significant positive influences on the EPS production with main effects of 3.47, 1.77 and 1.12 respectively (Fig.5). The ANOVA of PB design for EPS production is shown in Table 4 where the determinant coefficient R^2 of the first-order model was 0.95 for EPS production. The significant F-values of the models were 0.010 indicating that the models were significant. One factor at a time, although difficult and labor intensive method, this experiment supported 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.

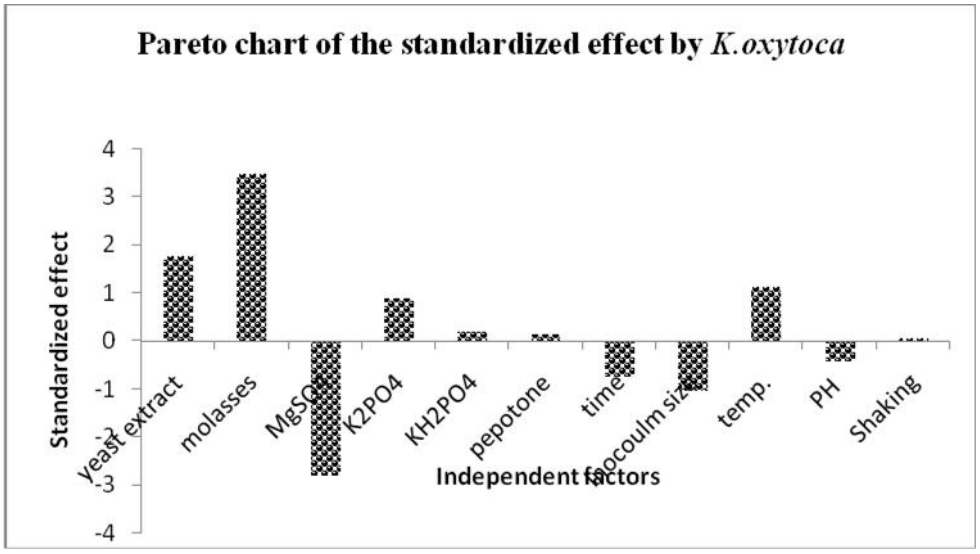

Figure 5- Effect of environmental and media composition on EPS production by PB design.

Table 4- Identification of significant variables for EPS production of strain (M7) using PB design.

EPS Production				
Variables	**Coefficients**	**Standard Error**	**t Stat**	**P-value**
Intercept	4.52	0.12	36.46	4.535
X_1 (yeast extract)	1.77	0.14	12.19	0.001
X_2 (sugarcane molasses)	3.47	0.58	5.910	0.009
X_3 ($MgSO_4$)	-2.84	0.50	-5.59	0.011
X_4 (K_2HP_4)	0.89	0.14	6.12	0.008
X_5 (KH_2PO_4)	0.19	0.14	1.32	0.27
X_6 (Peptone)	0.14	0.14	0.96	0.403
X_7 (Incubation time)	-0.78	0.14	-5.15	0.014
X_8 (Inoculum size)	-1.02	0.14	-7.06	0.005
X_9 (temperature)	1.12	0.14	7.72	0.004
X_{10} (pH)	-0.40	0.14	-2.73	0.067
X_{11} (Shaking)	0.068	0.14	0.47	0.66
Regression Statistics				
Multiple R	0.994	Adjusted R Square	0.953	
R Square	0.989	Standard Error	0.37	

Response surface methodology:

Based on above results, three key factors (Yeast extract, sugarcane molasses and incubation temperature) were significantly affected EPS production. These factors were selected for further analysis with Box-BehnkenDesign (BBD) (Table 3). The following second-order polynomial equation was:

$$Y= 4.83+1.17X_1+0.78X_2+0.48X_3-0.61X_1X_2 -0.01X_1X_3 +0.17X_2X_3+0.89X_1X_1+0.82X_2X_2-0.21X_3X_3 \quad (3)$$

where Y represents EPS production (g/L); 4.83 is the intercept; 1.17, 0.78 and 0.48 are the linear coefficients; -0.61, -0.01 and 0.17 are the interactive coefficients, 0.89, 0.82 and -0.21 are the quadratic coefficients; and X_1, X_2 and X_3 are the concentrations of sugarcane molasses, yeast extract and incubation temperature, respectively. The statistical significance of equation (3) was evaluated by the F-test and ANOVA analysis (Table5). The three-dimensional graphs that obtained from Equation 3 were very reliable, with R^2 value of 0.92, which indicated that 99% of the variability in the response explained by this model (Fig. 6**)**. The Model F-value of 7.18 implies the model is significant. There is only a 2.14% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob> F" less than 0.0500 indicate model terms are significant. In this case, the mutual interactions between every two of the three variables were significant. By solving the inverse matrix using Expert-Design software, the optimal concentrations of molasses, yeast extract and incubation time were 10 % (w/v), 5 g/L and 30 ^{0}C, respectively.

Table 5-Identification of significant variables for EPS production of strain (9I) using PB design

ANOVA	df	SS	MS	F	Significance F
Regression	9	24.83	2.75	7.18	0.021
Residual	5	1.91	0.38		
Total	14	26.75			
	Coefficients	**Standard Error**	**t Stat**		
Intercept	4.83	0.438	11.03		
X_1	1.17	0.21	5.54		
X_2	0.78	0.21	3.56		
X_3	0.48	0.21	2.29		
X_1X_2	-0.61	0.30	-1.96		
X_1X_3	-0.01	0.28	-0.05		
X_2X_3	0.17	0.30	0.572		
X_1X_1	0.89	0.34	2.63		
X_2X_2	0.827	0.34	2.40		
X_3X_3	-0.21	0.34	-0.64		

Multiple R = 0.96961 R^2 (coefficient of determination) = 0.928; Adj. R^2 (Adjusted coefficient of determination) = 0.799; Model are significant.

Data obtained from single factor optimization was followed by Plackett–Burman design for screening of significant variables that depend on multi-variables at a time. Data analysis obtained from this model indicted the use of yeast extract as nitrogen source and sugarcane molasses during production of polysaccharides by isolate code (M7). Moreover, yeast extract could provide growth factors such as vitamins and amino acids that support much bacterial growth (Luthra *et al*, 2015).

With the same consequence; RSM depend on data obtained from Plackett–Burman and the data analysis indicated that the maximum yield was estimated to be 7.68 g/L, and the actual yield obtained with optimal medium was 7.88 g/L (the average value of triple experiments), which in close accordance with the model prediction. In addition, the sole carbon source was used in the fermentation medium for maximum EPS production, which would be advantage to reduce production cost and operation process of EPS (Lee *et al*, 1997).

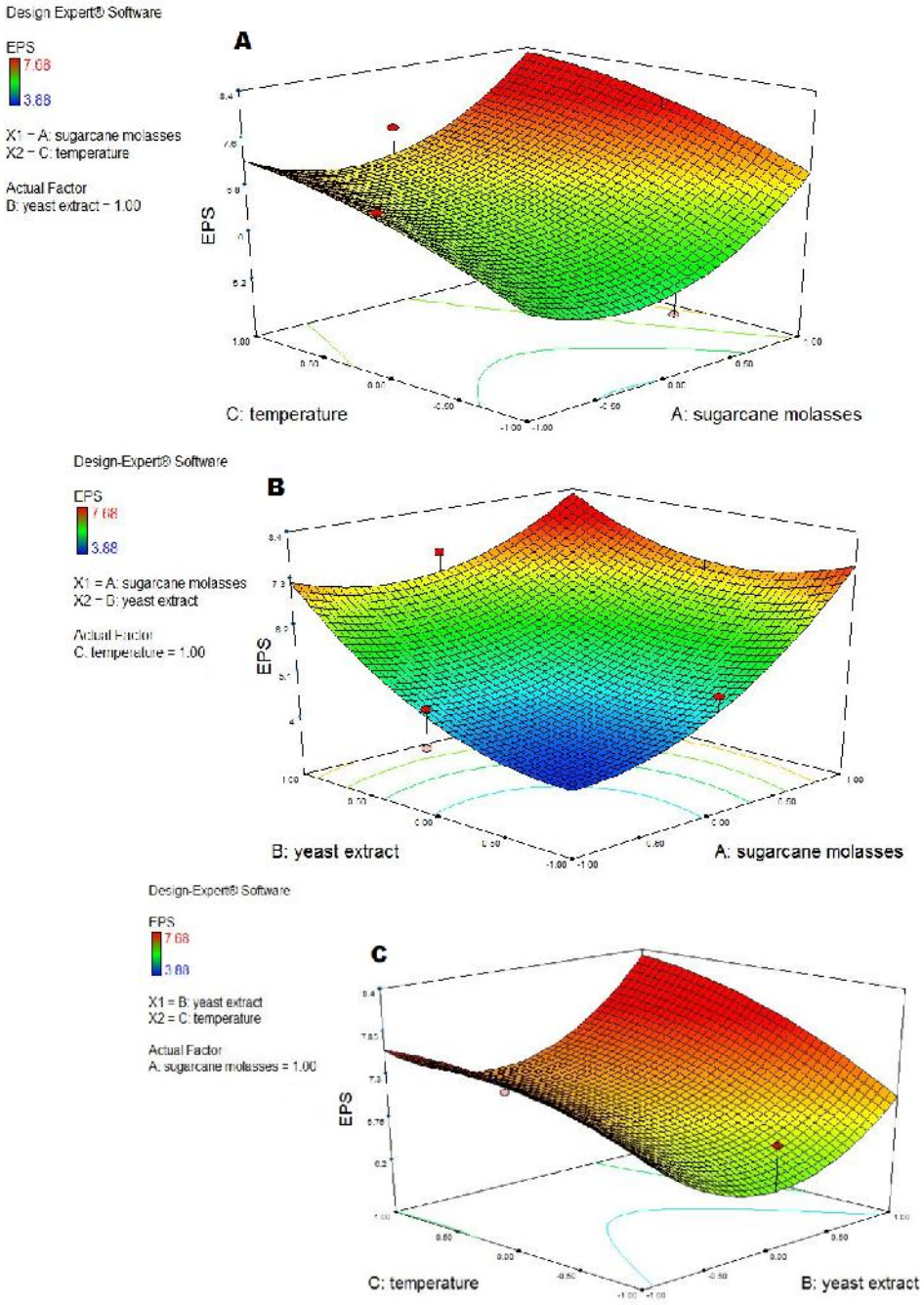

Figure 6- Response surface plots of three variables on EPS production in medium.
A, interaction of Molasses and incubation temperature;
B, interaction of molasses and yeast extract;
C, interaction of yeast extract and temperature.

Molecular Characterization

The amplification of 16S rRNA gene was sequenced and aligned using NCBI Based on the sequence alignment BLAST revealed that isolate belongs to *Klebsiella* species with maximum evolutionary relation to *K.oxytoca* 99% sequence similarities. The sequence was deposited in NCBI using Bankit program and assigned with the accession number KY498625. The phylogenetic tree was constructed using the neighbor-joining tree making algorithm of the MEGA 4 package (Fig. 7)

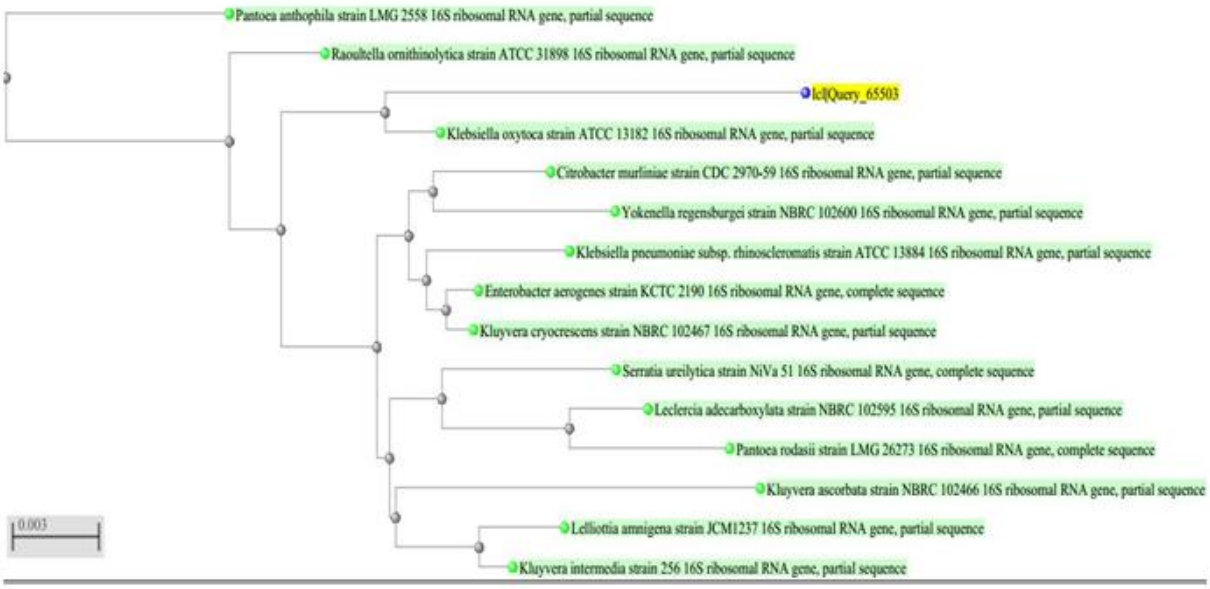

Figure 7- Phylogenetic tree of the partial sequence of 16S rDNA of the local isolate M7 with respect to closely related sequences available in GenBank databases

Purification of bacterial Exopolysaccharide using DEAE-Cellulose

Ion exchange chromatography was performed for purification of EPS using DEAE-Cellulose column after purification by tri-chloroacetic acid; EPS (eluted with 0.1M NaCl) was collected. Chromatography on DEAE cellulose column afforded 61 fractions, the first recovered polysaccharide in the nine eluting solution (fraction no. 4 to 17) that confirmed through phenol-sulphuric acid assay.

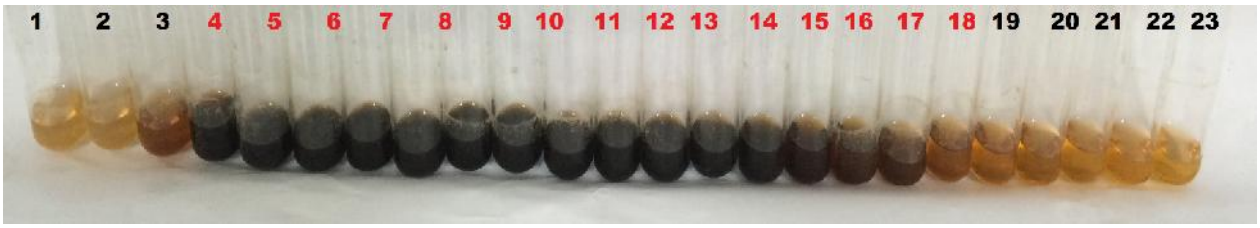

Figure 8-Detection of bacterial EPS in the collected fractions from DEAE-cellulose column chromatography using phenol-sulfuric acid method.

Figure 9: Different forms of Exopolysaccharide were;
(A) Crude Exopolysaccharide after ethanol precipitation
(B) EPS after first purification using DEAE-Cellulose and
(C) EPS after second purification using DEAE-Cellulose.

Determination of molecular weight by gel permeation chromatography (GPC):

The weight-average (Mw) and number-average (Mn) molecular weights and polydispersity (Mw/Mn) of purified EPS was analyzed by GPC and found to have a weight average molecular weight (Mw) of 4.05 x10^4 Da, number average molecular weight (Mn) of 4.28 x10^4 Da and a size average molecular weight (Mz) of 4.07 x10^4 Da (Fig. 10). The Molecular Weight and Polydispersity Index (PDI) (Mw/Mn) of the exopolysaccharide was obtained as 0.94, which is a measure of the distribution of molecular mass in the sample.

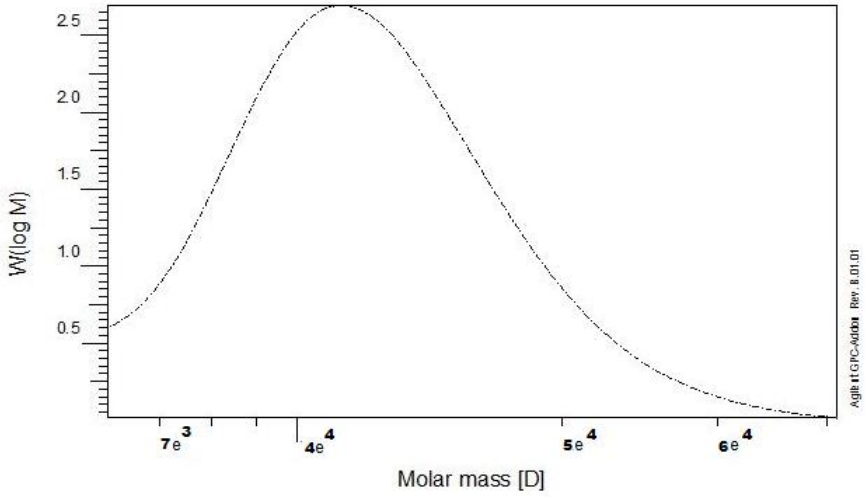

Figure 10- GPC chromatogram of partially purified exopolysaccharide from *K.oxytoca*

Fourier transform infrared spectroscopy (FTIR):

The results obtained from FTIR spectrum of purified EPS for detection of functional groups showed absorption band appeared at 1648cm^{-1} (Fig. 11) and assigned to the stretching vibration of carbonyl group (C=O). The absorption peak observed at 522 cm^{-1} was known to be typical characteristics of sugar derivatives, this also agree with (He *et al*, 2007) a broad intense peak at 2989cm^{-1} stretch of –CH_2 groups. Absorption peak at 1063.57cm^{-1} was assigned to carbohydrate C–O stretching vibrations and dominated by glycosidic linkage -(C–O-C)-stretching vibration. Polysaccharides possessing carbonyl group is reported previously. The IR spectrum of polymer proved the presence of carbonyl group which may serve as a binding site for divalent cations. This carboxyl group may also work as a functional moiety to generate an innovative new or modified polymer, by using different novel approaches like synthetic polymers. Carbohydrates showed high absorbencies in the region 1200–950cm^{-1}, that is within the so-called fingerprint region, where the position and intensity of the bands are specific for every polysaccharide, allowing it possible identification. These results are harmony with (Sun *et al*, 2007)

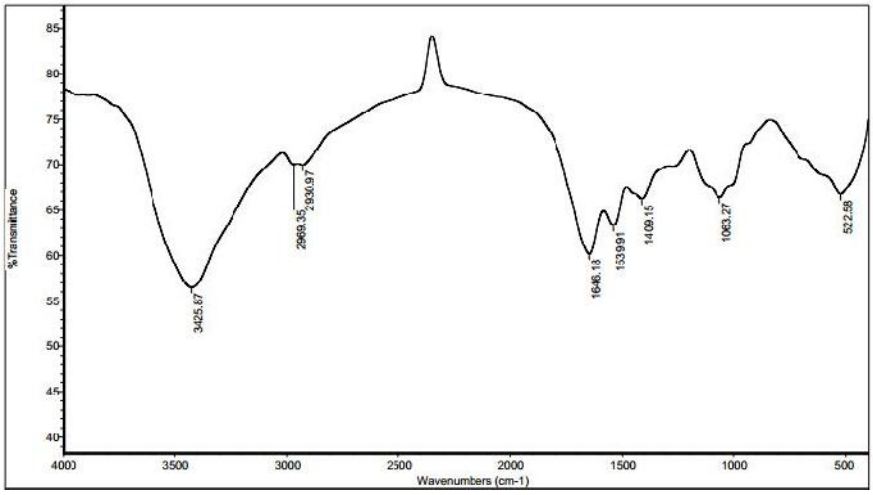

Figure11- FTIR spectroscopy analysis of *K. oxytoca* polysaccharide extracted showing the transmittance trough at different wave number.

Gas-mass chromatography (GC-MS):

In order to understand chemical structure, the purified EPS was hydrolyzed then methylated and applied to a GC-MS. Data analysis obtained from GC-MS library resulted in the detection of several components including mannose, glucose, Arabinose and Galacturonic acid (Fig. 12A-B).The peaks mannose, glucose, Arabinose and Galacturonic acid were confirmed by using the standard sugars treated with same reactions. The MS fragmentation patterns obtained from the GC generated by each peak, were subsequently analyses.It shows the characteristics of aldohexoses in sugar alditol acetate form. Arabinose appears most often, while mannose is seldom found as a component of the EPS. In particular, (KenneandLindberg 1983) reported that uronic acids, e.g. glucuronic and galacturonic acids, are common components of bacterial extracellular polysaccharides Therefore, the results reported here demonstrate that *K.oxytoca* likely produces similar common polysaccharides and anionic groups as other *Klebsiella* species.

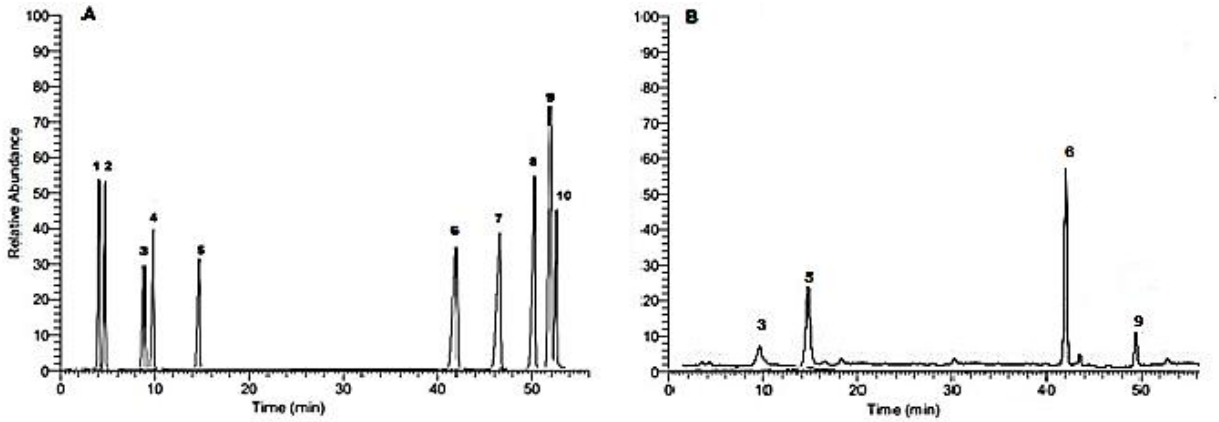

Figure (12A): Gas chromatographic of standard sugar are as follows:1.Galactose; 2.Fucose; 3.Mannose 4.Xylose; 5.Glucose; 6.Arabinose; 7.Mannuronic acid; 8.Glucuronic acid; 9.Galacturonic acid; 10.Mannuronic acid.
Figure (12B): Gas chromatograms of extracellular polysaccharides produced by *K.oxytoca*

Conclusion

Sixty different isolates from soil were screened for EPS producing activity. EPS production was optimized under different Nutritional and environmental conditions using *Klebsiella oxytoca* KY498625 isolated from soil. Two statistical experimental design Plackett–Burman and Box-Behnken were used to improve exopolysaccharide production. The production of EPS under optimal conditions obtained from data analysis succeeded to increase productivity from 4.1 to 7.6 g/L compared to the initial EPS production. Industrial production of EPS requires cost effective and optimal culture medium. The purified EPS has molecular weight 4.05 $x10^4$ Da with number average molecular weight (Mn) 4.28 $x10^4$Da that consists mainly of mannose, glucose, Arabinose and Galacturonic acid.

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