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Screening of Factors Influencing Exopolymer Production by *Bacillus licheniformis* Strain T221a Using 2-Level Factorial Design

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1. Introduction

The term exopolymer can be defined as biopolymer that is secreted by microorganisms outside the cell. Exopolymer is also known as exopolysaccharides (EPS) (Verhoef, 2005; Beech *et al.,* 1999). The exopolymer consist of polysaccharides that are either found associated with the microbial cell wall in the form of capsules or completely dissociated from the microbial cell. Besides, nucleic acids, proteins and also amphiphilic compounds including (phospho-) lipids can also be present in exopolymer. The contents of carbohydrate, protein and nucleic acid in exopolymer have a substantial effect on the flocculation of bacteria (Verhoef, 2005; Beech *et al.,* 1999, Sheng *et al.,* 2005). Generally bacteria exopolysaccharides have unique rheological properties because of their high purity and regular structure. These bacteria produce wide variety of exopolymers that have been used to cope in various ways with the external environment (Kornmann *et al.,* 2003). The exopolymer has advantages to enhance the viscosity of the solution thus making it applicable as thickeners, emulsifiers, and suspending agents in food, dairy product, pharmaceutical and petroleum industries (Lungmann *et al.,* 2007; Gandhi *et al.,* 1997, Yakimov *et al.,* 1997; Lee *et al.,* 1999; Degeest *et al.,* 2001; Torino *et al.,* 2005).

In Microbial Enhanced Oil Recovery (MEOR) application, the exopolymer-producing bacteria are highly dependent on both physical and chemical environment (Yakimov *et al.,* 1997; Haghighat *et al.,* 2008; Duta *et al.,* 2006, Lin *et al.,* 2007). Physical environment include the fermentation conditions such as temperature, pH environment and incubation time. The chemical environments include the carbon and nitrogen source of the medium (Liu *et al.,* 2009; Degeest *et al.,* 2001; Cerning *et al.,* 1994). Temperature and pH are important factors that affect the performance of cells and exopolymer production (Lee *et al.,* 1997, Degeest *et al.,* 2001; Tharek *et al.,* 2006). The acidity and alkalinity could result in the inhibition of bacterial growth, thus retarding its metabolic production (Norell and Messley, 2003). Ghaly

et al., 2007 reported that bacteria *Bacillus licherniformis* produced exopolymer (levan) which has potential applications as a selective plugging agent in microbial enhanced oil recovery when grown in sucrose. The bacteria was able to grow in sucrose, glucose, and fructose, but produced exopolymer only in the presence of sucrose. Exopolymer production will be very low if one of these environmental factors is not controlled at the proper levels (Turimin, 2003). Therefore, intensive research on optimization of exopolymer production should be conducted at effective low cost medium and little manpower.

Recently, the results analyzed by a statistical planned experiment are better acknowledged than those carried out by the traditional one-variable-at-a-time method. Statistical experimental designs have been used for many decades and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response (Duta *et al.,* 2006; Cui *et al.,* 2006; Lungmann et al., 2007; Reddy *et al.,* 1999; Casas *et al.,* 1997). The 2-level factorial design can be considered to be a multivariable sequential search technique in which the effects of two or three factors are studied together and the responses are analyzed statistically to arrive at a decision (Tunga *et al.,* 1998, Duta et al., 2006; Anbu *et al.,* 2006). It is useful to identify the important nutrients and interactions between two or more nutrients in relatively few experiments as compared to the one-factor-at-time technique (Ooijkaas *et al.,* 1998, Luo et al., 2009).

In this study, five statistically significant parameters which are temperatures, pH, sucrose, NaCl and peptone concentrations were used for screening process using *DESIGN EXPERT 6.0.4 software*. Each independent variable was investigated at a high (+1) and a low (-1) level. The design consists of 37 experiments which include five replicates at center points.

2. Materials and methods

2.1 Microorganism

Bacillus licheniformis strain T221a was used in the experiments. The bacteria was locally isolated from Tiong A27 Petroleum Reservoir in Sarawak (Tharek *et al.,* 2006). The stock culture was maintained in cryopreservation beads and stored at -80^{o}C.

2.2 Culture media

This microorganism was grown and maintained on Modified Reinforced Clostridia (RGM1) agar medium. This medium contained NaCI 5.0 g/L, peptone 10.0 g/L, yeast extract 3.0 g/L, glucose 5.0 g/L, NH_4NO_3 2.0 g/L, meat extract 10.0 g/L, soluble starch 1.0 g/L, L-Cystein HCl 0.5 g/L and sodium acetate 3.0 g/L. The pH of the media was adjusted to approximately 8.5. *B. licheniformis* was grown on this media at 50^{o}C for 12 hours. After growth, the cultures were stored at 4^{o}C.

The stock culture was used for preparing the inoculum in 150 mL serum bottles containing 100 mL of RGM1 broth medium. The preparation of the RGM1 broth was conducted under anaerobic condition. 1 mL of trace mineral and 1 mL of vitamin was added into the medium. Resazurin solution (0.1% w/v) was added as indicator of anaerobiosis. The medium was sparged for 10 minutes to remove oxygen gas. The culture medium was incubated at 50^{o}C for 12 hours without agitation until the optical density at 660nm was above 0.6.

2.3 Exopolymer production medium

The enhancement of exopolymer production was performed in Modified Exopolymer Production (MM2) medium which consisted of the following composition (g/L): KH_2PO_4

(0.5), K_2HPO_4 (0.5), cystein HCI (0.5), NH_4NO_3 (2.0), and sodium bicarbonate (10.0). The concentration of sucrose, peptone and NaCl was added in the range according to the experimental design; sucrose (30.0 - 70.0 g/L), peptone (4.0-8.0 g/L) and NaCI (10.0 – 30.0 g/L). The initial pH of the medium was adjusted in the ranged accordance to the experimental design which was 7 - 10. Preparation of production medium was performed in 150 mL serum bottles under aerobic condition. All the culture media were sterilized for 15 minutes at 121°C.

2.4 Production of exopolymer in batch culture

Batch culture was initiated by inoculating approximately 10% (v/v) of culture from serum bottles that were prepared previously and transferred into 100 ml sterile MM2 medium. The experiments were done in duplicate. Culture broth was incubated at their respective temperatures for 16 hours without agitation. Withdrawn samples were centrifuged at 5,000 rpm for 30 minutes. The supernatant was used for the determination of exopolymer concentration and sucrose concentration. The pellet (cells) was used for the determination of cell concentration.

2.5 Exopolymer quantification

Exopolymer concentration was determined via product-dry weight method by centrifuging culture sample (5 mL) at 5000 rpm for 30 minutes. The supernatant was kept and two volumes of chilled (4°C) ethanol (99.8%, v/v) were added to one volume of supernatant in order to precipitate out the soluble exopolymers. After the exopolymers total precipitation, the suspended material was filtered through pre-weighed 0.2um nylon membrane filter (Whatman) and was washed twice with chilled (4°C) ethanol (99.8%, v/v). Finally, the filtered soluble exopolymers was dried in an oven at 70°C until a constant weight was produced (Duta *et al.,* 2006).

2.6 Determination of sucrose

The procedures described by Mokrasch (1954) were followed to determine the sucrose content. The Anthrone solution was prepared in a mixture of concentrated sulphuric acid and water at ratio of 5:2 and was chilled on ice until it becomes cold. The Anthrone solution was stored at 4°C in the dark. 1 mL of sample was spun down and 5 mL of chilled Anthrone solution was added and mixed. The mixture was kept on ice about 5 minutes to form blue-green colour before reading on spectrophotometer at 620nm.

2.7 Cell concentration determination

The cell concentration was determined by dry-cell weight method (Soni *et al.*, 1987). The culture sample 5 mL was centrifuged at 5,000 rpm for 30 minutes and the supernatant was decanted. The cells was washed twice with distilled water by filtering it through a pre-weighed 0.2 μm cellulose nitrate membrane filter (Whatman) prior to drying it in an oven at 95°C until a constant reading was produced.

2.8 Experimental design

The factors that influenced the exopolymer production were screened using 2-level factorial design created by *DESIGN EXPERT software* (State-Ease Inc., Statistic made easy, Minneapolis, MN, USA, Version 6.0.4). Five variables factors, which are temperature, pH,

NaCl, sucrose (carbon source) and peptone (nitrogen source) concentration were expected to have a significant effect on exopolymer production. The design contains a total of 37 experimental trials involving five replicates at centre points. Each independent variable was investigated duplicate at superior a high (+1) and a low (-1) level (Table 1). Runs of center points were included in the matrix and statistical analysis was used to identify the effect of each variable on exopolymer production. The runs were randomized for statistical reasons.

* Factor	Unit	Low Level (-1)	High Level (+)
Temperature (A)	^{o}C	40	60
pH (B)	-	7	10
NaCl concentration (C)	g/l	10	30
Sucrose concentration (D)	g/l	30	90

* The data of the factors and the low level (-1) and high level (+1) according to the conventional factors that give the significant influenced towards exopolymer production.

Table 1. Factors in real value, for screening by the 2-level fractional factorial design.

3. Results & discussions

3.1 Factors significantly affecting exopolymer production

The medium components and fermentation condition have played an important role for exopolymer production in batch culture. In order to find out the key ingredients significantly affecting the production of exopolymer, the relative significance of five variable factors (temperature, pH, sucrose, peptone and NaCl concentration) were investigated by the 2-level factorial design. The design consisted of 37 experiments in duplicate plus five center point (Table 2). All the experiments were conducted in static flask culture.

The results of the 2-level factorial design model in the form of analysis of variance (ANOVA) are shown in Table 3. ANOVA is a statistical technique that subdivides the total variation of a set of data into component associated to specific sources of variation for the purpose of testing hypotheses for the modeled parameters (Duta *et al.*, 2006). According to the ANOVA, the Fisher's F-test with a very low probability value [($P_{model} > F$) < 0.005] indicated the model was highly significant on exopolymer production. The larger the magnitude of t-test and smaller the P-value, the more significant is the corresponding coefficient. Among the variables screened, the concentration of sucrose (D), temperature (A) and pH (B) were determined as the most significant variables influencing exopolymer production. Concentration of peptone (E) and NaCl (C) in exopolymer production did not result in significant variation due to the P-value is greater than 0.100.

The goodness of fit of the model was examined by determination coefficient R^2 = 0.9360, which implied that the sample variation with more than 93.6% was attributed to the variables. However, only 6.40% of the total variance could not be explained by the model. The adjusted determination coefficient (Adj R^2 = 0.8879) was also satisfactory to confirm the significance of the model. Also, the model has an "adequate precision value" of 15.392, which suggests that the model can be used to navigate the design space.

The predicted optimum levels of tested variables (temperature (A), pH (B), NaCl (C), sucrose (D) and peptone (E)) were obtained from ANOVA. The optimal levels for the variables were as follows: 90.0 g/L sucrose, 4.00 g/L peptone, 29.98 g/L NaCl, 40^{o}C temperature and initial pH 10 with the corresponding Y = 4.22 g/L. To validate this model, these predicted parameters were tested in the laboratory and the samples were taken at

Sd	Run	Block 1	Temp (°C)	pH	[NaCl] (g/L)	[sucrose] (g/L)	[peptone] (g/L)	*Exopolymer Production (g/L)	
								Act. Value	Pred. Value
1	32	Block 1	40	7	10	30	8	2.48	2.38
2	12	Block 1	40	7	10	30	8	2.28	2.38
3	35	Block 1	60	7	10	30	4	0.22	0.24
4	9	Block 1	60	7	10	30	4	0.26	0.24
5	18	Block 1	40	10	10	30	4	2.36	2.75
6	27	Block 1	40	10	10	30	4	3.14	2.75
7	36	Block 1	60	10	10	30	8	1.40	1.58
8	4	Block 1	60	10	10	30	8	1.74	1.58
9	24	Block 1	40	7	30	30	4	2.40	2.36
10	1	Block 1	40	7	30	30	4	2.32	2.36
11	33	Block 1	60	7	30	30	8	0.34	0.32
12	17	Block 1	60	7	30	30	8	0.30	0.32
13	21	Block 1	40	10	30	30	8	1.72	1.60
14	14	Block 1	40	10	30	30	8	1.48	1.60
15	3	Block 1	60	10	30	30	4	1.26	1.03
16	7	Block 1	60	10	30	30	4	0.80	1.03
17	19	Block 1	40	7	10	90	4	1.54	1.58
18	10	Block 1	40	7	10	90	4	1.62	1.58
19	2	Block 1	60	7	10	90	8	0.24	0.61
20	25	Block 1	60	7	10	90	8	0.98	0.61
21	30	Block 1	40	10	10	90	8	3.50	3.67
22	26	Block 1	40	10	10	90	8	3.84	3.67
23	29	Block 1	60	10	10	90	4	1.98	1.16
24	13	Block 1	60	10	10	90	4	0.34	1.16
25	31	Block 1	40	7	30	90	8	0.28	0.32
26	15	Block 1	40	7	30	90	8	0.36	0.32
27	5	Block 1	60	7	30	90	4	1.88	1.66
28	11	Block 1	60	7	30	90	4	1.44	1.66
29	28	Block 1	40	10	30	90	4	3.84	4.24
30	8	Block 1	40	10	30	90	4	4.64	4.24
31	37	Block 1	60	10	30	90	8	2.50	2.85
32	6	Block 1	60	10	30	90	8	3.20	2.85
33	16	Block 1	50	8.5	20	70	6	1.38	1.36
34	22	Block 1	50	8.5	20	70	6	1.26	1.36
35	20	Block 1	50	8.5	20	70	6	1.28	1.36
36	34	Block 1	50	8.5	20	70	6	1.58	1.36
37	23	Block 1	50	8.5	20	70	6	1.32	1.36

* Exopolymers were determined after cultivation of the bacteria in MM2 medium. The actual values of exopolymer production were compared to the predicted values given by 2-level factorial design. The actual experimental results were in agreement with the prediction.

Table 2. Experimental designs for the screening of significant factors that influences exopolymer production using 2-level factorial design.

Source	Sum of Squares	Mean Square	F Value	Prob > F	
*** Model**	42.96	2.86	19.49	< 0.0001	*Significant*
A (Temperature)	11.16	11.16	75.94	< 0.0001	
B (pH)	11.07	11.07	75.30	< 0.0001	
C (NaCl)	0.021	0.021	0.14	0.7093	
D (Sucrose)	1.83	1.83	12.47	0.0021	
E (Peptone)	0.36	0.36	2.43	0.1348	
AB	0.42	0.42	2.85	0.1070	
AC	2.13	2.13	14.51	0.0011	
AD	0.71	0.71	4.86	0.0394	
AE	2.24	2.24	15.22	0.0009	
BC	0.063	0.0693	0.43	0.5201	
BD	4.64	4.64	31.54	< 0.0001	
BE	0.93	0.93	6.34	0.0205	
CD	1.70	1.70	11.58	0.0028	
CE	5.63	5.63	38.29	< 0.0001	
DE	0.060	0.060	0.40	0.5318	
Curvature	0.72	0.72	4.89	0.0387	*Significant*
Pure Error	2.94	0.15			
Cor Total	46.62				
Std. Deviation	0.38	**Adj R-squared**		0.8879	
Mean	1.72	**Pred R-quared**		0.7513	
R-squared	0.960	**Adeq Precision**		15.392	

* The data were shown that the model was highly significant towards exopolymer production by *Bacillus licheniformis.*

Table 3. Regression analysis (ANOVA) for the production of exopolymer

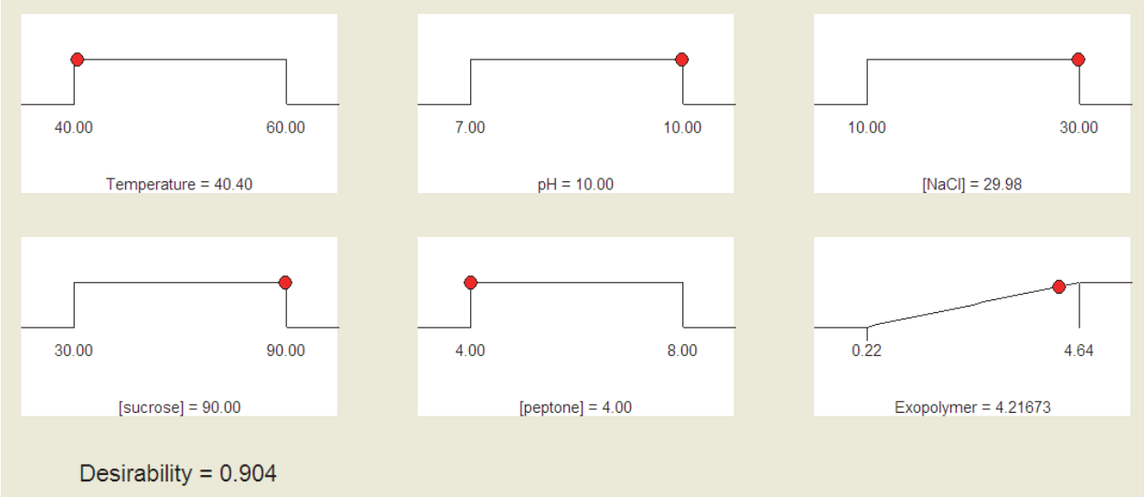

Fig. 1. Predicted optimum levels of five independent variables which indicate the highest exopolymer production.

certain interval during fermentation hour for exopolymer production and other analysis. The final exopolymer concentration obtained was 4.04 g/L, which is almost reaching the predicted value under same condition. This result corroborated the validity and the effectiveness of this model. Figure 1 shows the predicted optimum levels of five independent variables with desirability 90.04%.

3.2 Time course and kinetic evaluation of exopolymer production in batch culture

The suggested optimal values for each variable obtained from 2-level factorial design, were tested in the laboratory and the kinetic was studied. The pattern of exopolymer production by microorganisms may either be growth-associated, non-growth-associated or mixed (Gandhi et al., 1997; Desai and Banat, 1997). Time course of exopolymer production by *B. licheniformis* strain T221a in static culture is presented in Figure 2. The growth of *B. licheniformis* strain T221a increased rapidly during the first 16 hours of incubation. After 16 hours incubation, the bacteria entered death phase and the cell mass decreased. However, exopolymer production kept increasing after 16 hours of incubation even though the cell mass entered death phase. The profile of exopolymer production by *B. licheniformis* strain T221a assigned it as growth-associated. This result is similar to other bacteria such as *Ralstonia eutropha* ATCC 17699 (Wang and Yu, 2007), *Lactobacillus sakei 0-1* (Degeest *et al.*, 2001), *Lactobacillus salivarius* BCRC 14759 and *Bifidobacterium bifidum* BCRC 14615 (Liu *et al.*, 2009) which produced same trends of exopolymer production. Maximum exopolymer production (P_{max} = 7.12 g/L) produced by *B. licherniformis* was observed at 24 hours.

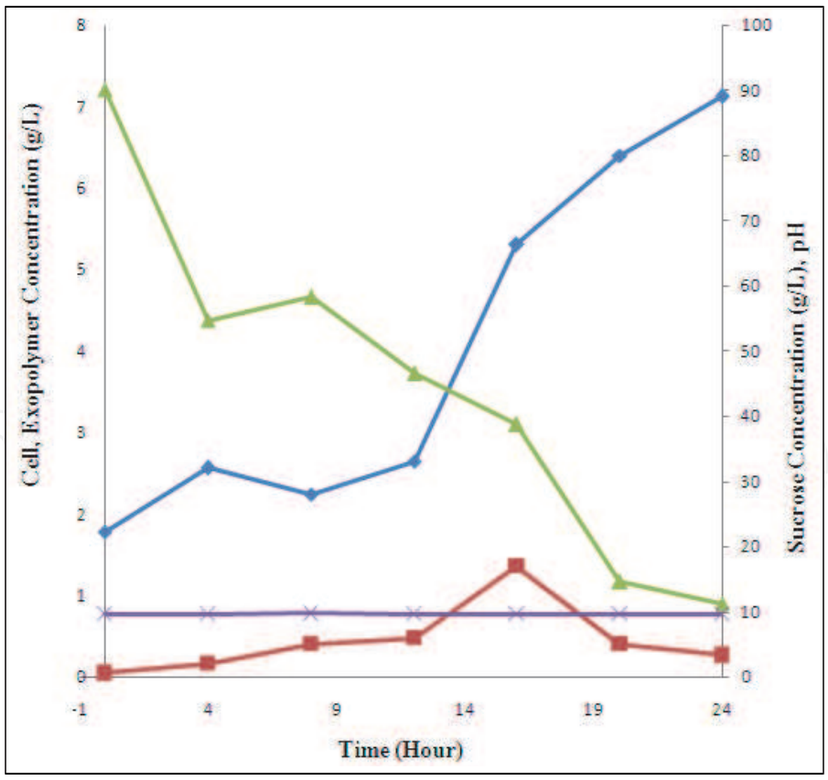

Fig. 2. The time course of exopolymer production in batch culture by *Bacillus licheniformis.* Symbols represent: (►) Sucrose concentration, (■) Cell mass concentration, (♦) Exopolymer Concentration, (x) pH

The maximum concentration of cells mass (X_{max} = 1.36 g/L) was obtained at 16 hours of incubation. During active cell growth and exopolymer production, sucrose was actively utilized. The concentration of sucrose decreased exponentially reaching about 11.26 g/L after 24 hours. Depletion of sucrose caused a decrease in bacterial cell mass but not in exopolymer production. According to Desai and Banat (1997), the exopolymer can also be produced under nitrogen-limiting conditions. A number of investigators have demonstrated that an overproduction of biopolymer by *Pseudomonas* spp. was obtained when the culture reaches the stationary phase of growth due to the limitation of nitrogen and iron.

The kinetic evaluation of exopolymer production by *B. licheniformis* strain T221a showed the yield of exopolymer formation per gram of substrate utilized (Yp/s) was two times higher than yield of cell formation per gram of substrate utilized (Yx/s), 0.025 g/g. The maximum and overall productivities of exopolymer were 0.64 g/L/h and 0.297 g/L/h, respectively. This result indicated that the predicted parameters in medium composition are more suitable for production of exopolymer rather than the cell mass concentration.

4. Conclusions

2-level factorial design is useful to screen the effects of five variables factors that influenced the exopolymer production. A fitted model obtained showed suitable prediction response that indicates improvement of a model. Based on the data obtained, it was proven that pH, temperature and sucrose concentration was highly significant towards exopolymer production. This design suggested that the optimal value for each variable are; 90.00 g/L sucrose, 4.00 g/L peptone, 29.98 g/L NaCl, 40^{o}C temperature and initial pH 10 with predicted exopolymer of 4.22 g/L. This predicted value was performed in laboratory and 4.04 g/L of exopolymer yield was obtained. The actual experimental results were in agreement with the prediction. This statistical design proved to locate the optimum levels of the most significant parameters for exopolymer production, with minimum effort and time.

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