The Use of Response Surface Method in Optimization of Levan Production by Heterologous Expressed Levansucrase from Halophilic Bacteria *Bacillus licheniformis* BK2

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Abstract. Response surface method (RSM) is a recent common method used to identify culture condition for optimal production of particular metabolite. In the present study, RSM is used to optimize a catalytic reaction in levan production by heterologous expression of *lsbl-bk2* gene isolated from halophilic bacteria *Bacillus licheniformis*. Levan is a polyfructose polymer produced from sucrose by the action of extracellular levansucrase secreted by the microorganism. Three factors for levan production, namely sucrose concentration, pH, and temperature of reaction were optimized by full factorial and central composite designs in RSM. The result indicated that the optimum *in vitro* condition for levan production was achieved when the levansucrase catalytic reaction was performed at 32^{o}C, pH 8, in 12% (w/v) sucrose solution. Levan produced by this procedure was verified by FTIR and NMR spectroscopies.

1. Introduction

Levan is a fructose polymer produced by many bacteria and plants. The main chain of fructose polymer in levan consists of reiterating fructofuranosyl rings connected by β-(2,6)-glycosidic bonds, with β-(2,1)-glycosidic linkage in its branches [1,2]. Levan in different organisms are commonly produced as a storing energy [3]. In plants, as well as bacteria and fungi, levan is a very important compound for survival in cold and dry climates [4]. In some bacteria, levan is also used as a protector layer outside the cell for blocking host-pathogen recognition or toxic compounds [5,6].

Levan are white powder or yellow crystalline, insoluble in 75% (v/v) alcohol, soluble in water forming a nontransparent low viscosity solution, heat stable, non-irritant, and non-toxic [7,8,9]. These properties made levan utilization is superior in many commercial industrial sectors, including in food industries, pharmaceuticals, cosmetics, and chemical industries [3,9,10].

Levan is a bioactive polymer synthesized by microorganisms in a transfructosylation reaction catalyzed by levansucrase using a sucrose based-substrate [11]. Levansucrase (EC 2.4.1.10) are extracellularly produced by some genus of bacteria such as *Bacillus*, *Geobacillus*, *Lactobacillus*, and *Zymomonas* [3,9]. Sequence similarity classifications (CAZy) classified levansucrase as a member of 68 glycoside hydrolase family [12]. Bacterial GH68 family hydrolyses glycosidic bond of sucrose and take the energy released to transfer fructose into an acceptor molecule to synthesize polyfructan [13,14,15].

Many industries commonly use bacterial native extracellular levansucrase [3,16]. Study on molecular structure of this enzyme has been facilitated by gene cloning and expanded to using

heterologous system for better levan production [17]. In this study, we report the use of RSM to optimize *in vitro* levan production using levansucrase from *B. licheniformis* BK2 in which the levansucrase gene has been cloned and expressed in *E. coli*.

2. Material and Methods

2.1 Bacterial strains, plasmids, culture medium and chemicals

Bacterial strain used in this study is *E. coli* BL21(DE3)plysS carrying pET-*lsbl-bk2*. This recombinant clone contain levansucrase gene from *B. licheniformis* BK2 (accession number MF774878.1). *B. licheniformis* BK2 was initially isolated from Bledug Kuwu mud crater, Central Java, Indonesia.The *E. coli* BL21(DE3)plysS was obtained from Promega. pET-30a(+) was obtained from Novagen. The growth medium used is Luria Bertani (LB) medium [18] containing 1% (w/v) tryptone (Liofilchem), 0.5% (w/v) yeast extract (Liofilchem) and 1% (w/v) NaCl (Merck). Kanamycin was obtained from Biobasic, isopropyl-β-D-thiogalactopyranoside from Thermo Fisher Scientific**,** 3,5-dinitrosalicyclic acid from Sigma Aldrich, and sucrose, Na_2HPO_4, $NaH_2PO_4.H_2O$, ethanol, phenol, and H_2SO_4 97% were from Merck.

2.2 Expression of pET-lsbl-bk2 in E. coli BL21 (DE3)

The recombinant pET-*lsbl-bk2* in *E. coli* BL21 (DE3)plysS was employed for levansucrase production. A single colony of this clone was inoculated into 5 mL LB liquid medium containing 50 µg/mL kanamycin, was shaking overnight at 37^{o}C. A 2 mL of this fresh culture was transferred into 100 mL of the same medium and incubated for 2 hours at 37^{o}C to reach an OD_{600} 0.6-1, which then a final concentration of 1 mM IPTG was added. The medium containing levansucrase was collected after 4 hours further incubation by discarding the cell through centrifugation at 8000 rpm for 15 min. Levansucrase activity was measured by dinitrosalicylic acid (DNS) method to determine the release of reducing glucose at 510 nm. The crude extract of this levansucrase recombinant Lsbl-bk2 was then used to find an optimum condition of catalysis to produce levan by the RSM method.

2.3 Experimental design

The response surface method was applied to search for optimum condition in levan production catalysed by levansucrase. A set of experimental condition was determined by central composite designed (CCD). Three factors influenced catalytic levan production, namely sucrose concentration, pH, and temperature were varied based on preliminary experimental result. The predicted responses were analysed using RSM with the Minitab Statistical Software (version 18). The factorial was designed as 20 runs with one replicates at the central point (listed in **Table 1**).

2.4 In vitro levan production

In vitro levan production using 8 mL of crude recombinant levansucrase Lsbl-bk2 was performed in 8 mL of phosphate buffers with the condition according to **Table 1**, in which the temperature were varied from 20^{o}C to 54^{o}C, pH from 4 to 10, and sucrose concentration from 5% to 20% (w/v). The reaction mixtures were incubated for 24h, stopped by heating at 100^{o}C for 5 min, and cooled down to room temperature. The precipitated impurities were discarded by centrifugation at 8,000 rpm for 10 min [19]. The levan concentration in the supernatant was determined by phenol-sulfuric method at 490 nm [20,21].

2.5 Determination of levan concentration

Levan concentration was determined by phenol-sulfuric acid method to identify the total sugar concentration produce from sucrose hydrolysis catalysed by levansucrase [20,21,22]. A 0.45 mL of levan solution was mixed with 0.05 mL of 5% (w/v) phenol solution, and homogenized by vortexing for 30 s. The mixture was allowed to stand in ice bath for about 1 min and then 0.5 mL of concentrated sulfuric acid was carefully added. The reaction was stopped by heating at 70^{o}C for 5 min and cooled

down to room temperature water bath for 2 min. The absorbance of the yellow-orange formed solution was read at 490 nm. The fructose concentration was determined by aligning the observed absorbance to fructose standard curve.

2.6 Analysis of Levan

Levan produced in 50 mL of catalytic mixture in its optimum condition was precipitated out by the addition of cold ethanol 3:1 (v/v) (ethanol:supernatant). Levan was separated by means of centrifugation at 8,000 rpm for 20 min. The obtained levan pellet was washed in ethanol and ddH_2O, separated by re-centrifugation, and freeze dried for about 2 to 4 hours. The levan structure was analysed by Fourier Transform Infrared spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR) spectroscopy.

3. Result and discussion

3.1 Optimization of levan synthesis

The result of RSM approach in determining optimum condition of levan synthesis by varying temperature, pH, and sucrose concentration is presented in **Table 1**. The relationship between obtained levan concentration as dependent variable in each experimental condition was established by a second order polynomial equation (**Eq. 1**) that includes linear, quadratic, and interaction term correspond to CCD.

$$Y = -430.4 + 13.82(X_1) + 50.5(X_2) + 27.50(X_3) - 0.1430(X_1)^2 - 3.123(X_2)^2 - 1.065(X_3)^2 - 0.308(X_1X_2) - 0.1217(X_1X_3) + 0.645(X_2X_3) \quad (1)$$

Y = levan concentration (mg/mL) as dependent variable. The independent variables were X_1 = temperature (^{o} C), X_2 = pH, X_3 = sucrose concentration (% w/v). The regression coefficient: -430.4 is a constant coefficient; 13.82, 50.5, 27.50 are the linear coefficients; 0.1430, 3.123, 1.065 are the quadratic coefficients, and 0.308, 0.1217, 0645 are the interaction coefficient between variable X_1, X_2, X_3.

Table 1. Observed levan concentration from various experimental conditions determine by RSM using CCD approach

Run order	Temperature (^{o}C)	pH	Sucrose concentration (%)	Concentration of levan (mg/mL)
1	54	7	13	140
2	37	7	13	180
3	37	4	13	143
4	47	9	17	129
5	37	7	13	180
6	37	7	5	155
7	27	5	17	132
8	37	7	13	189
9	47	5	8	157
10	27	5	8	158
11	27	9	8	154
12	37	10	13	180
13	47	9	8	156
14	37	7	13	184
15	20	7	13	171
16	47	5	17	135
17	37	7	20	127
18	37	7	13	185
19	27	9	17	174
20	37	7	13	185

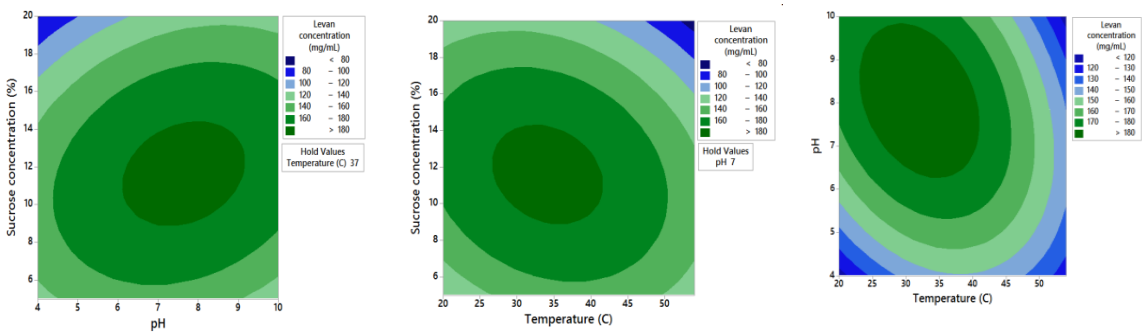
Analysis of variance (ANOVA) was performed to validate the quadratic model. The result provided correlation coefficient (R^2) and P-values of 92.57 % and 0.000 (< 0.05) respectively, indicated that the model is significantly fit to the variation observed. Therefore, the chosen regression model could be accepted and satisfactorily useful to explain levan production. This model gave highest levan concentration on 32.3^{o}C, pH 8, and 12% (w/v) of sucrose, in which the predicted levan concentration was 187.517 mg/mL. To validate this optimum condition, five replicates experiments were performed, which resulting in levan concentration of 188.269 mg/mL in average, highly agreed with the model predicted value.

In order to examine the correlation significance of each independent factor, the Minitab program was adopted and the result is presented in **Table 2**. Probability (p) values were used to check the significance correlation between temperature, pH, and sucrose concentration. Among the three factors tested, sucrose concentration appeared to have the highest impact on levan production as it had highest linear coded coefficient and its p-value is less than 0.05. The negative square coefficient found for temperature, sucrose concentration, and pH indicated that these independent variables were already in its optimum range. In term of interaction between the three tested independent variables, the obtained p-values are all > 0.05 indicating that there were no significant interaction between temperature, pH, and sucrose concentration on levan production.

Table 2. Coded coefficient and significance level (p) of model determined levan production

Source	Coded Coefficient	p-Value
Linear		
Temperature (^{o} C)	-6.89	0.009
pH	6.87	0.009
Sucrose concentration (%)	-7.69	0.005
Square		
Temperature (^{o} C) x Temperature (^{o} C)	-10.32	0.001
pH x pH	-8.13	0.003
Sucrose concentration (%) x Sucrose concentration (%)	-15.39	0.000
Interaction		
Temperature (^{o} C) x pH	-5.55	0.076
Temperature (^{o} C) x Sucrose concentration (%)	-5.48	0.076
pH x Sucrose concentration (%)	5.13	0.097

Further validation on independent variables interaction was performed by visualizing two-dimensional (2D) contour on its response to levan production. Contour showing effects of temperature, pH, and sucrose concentration was presented in Figure 1. Darker region on the contour indicates higher levan concentration. It could be seen in 1a that increase of levan concentration were obtained from pH 6 to 9, and 9% (w/v) to 13% (w/v) sucrose concentration. Figure 1b indicates that levan concentration was increased up to 40^{o}C within sucrose concentration of 9% (w/v) to 13% (w/v), but further increase of both variables appeared to decrease levan concentration. On the other hand, Figure 1c explained that highest levan concentration could be obtained in the range of 25^{o}C to 40^{o}C with pH of 6 to 9. However, levan concentration was drastically decreasing if the temperature was above 40^{o}C and pH above 9. This data suggested that interaction between temperature and pH was occurred.



(a) (b) (c)

Figure 1. Contour plots of levan concentration as function of pH and sucrose concentration (a), temperature and sucrose concentration (b), and temperature and pH (c).

3.2 Analysis of levan structure

The obtain levan sample was verified its structure by FTIR and NMR spectroscopic methods (Figure 2). FTIR characteristic absorption of the levan sample (Figure 2a) primarily showed hydroxyl (O-H) stretching vibration at around 3427.51-3414 cm^{-1}, C-H stretching vibration at around 2931.80 cm^{-1}, carbonyl (C=O) stretching vibration at 1658.78 cm^{-1}. The spectral feature appeared in a region within 925.83-1271.09 cm^{-1} was a fingerprint for most carbohydrates molecules. This region is also related to overlapping signals by glycosidic linkage (C-O-C) stretching of the polysaccharide. The overall FTIR spectral features were similar to those of levan from *Erwinia Herbicola*.

Further structural verification of the levan sample was carried out by ^{1}H-NMR and ^{13}C-NMR spectroscopies (Figure 2b and 2c). The ^{1}H-NMR spectral features of the levan sample were similar to those of levan from *Acetobacter xylinum* NCIM2526 [9]. ^{1}H NMR spectrum of the levan sample showed characteristic signals within the chemical shift between 3.4 and 4.3 ppm, which were correlated to sugar protons (Figure 2B). While the obtained ^{13}C-NMR spectrum exhibited six signals at 104.1 (C2), 80.2 (C5), 76.2 (C3), 75.1 (C4), 63.3 (C6), 59.8 (C1) ppm corresponding to the carbon position of β-fructofuranose. These chemical shifts were similar to those of carbon signals of levan from *Bacillus methylotrophicus* SK 21.002 and *Zymomonas subtilis* [23,24]. Both FTIR and NMR spectroscopic results accordingly verified that the carbohydrate sample is levan.

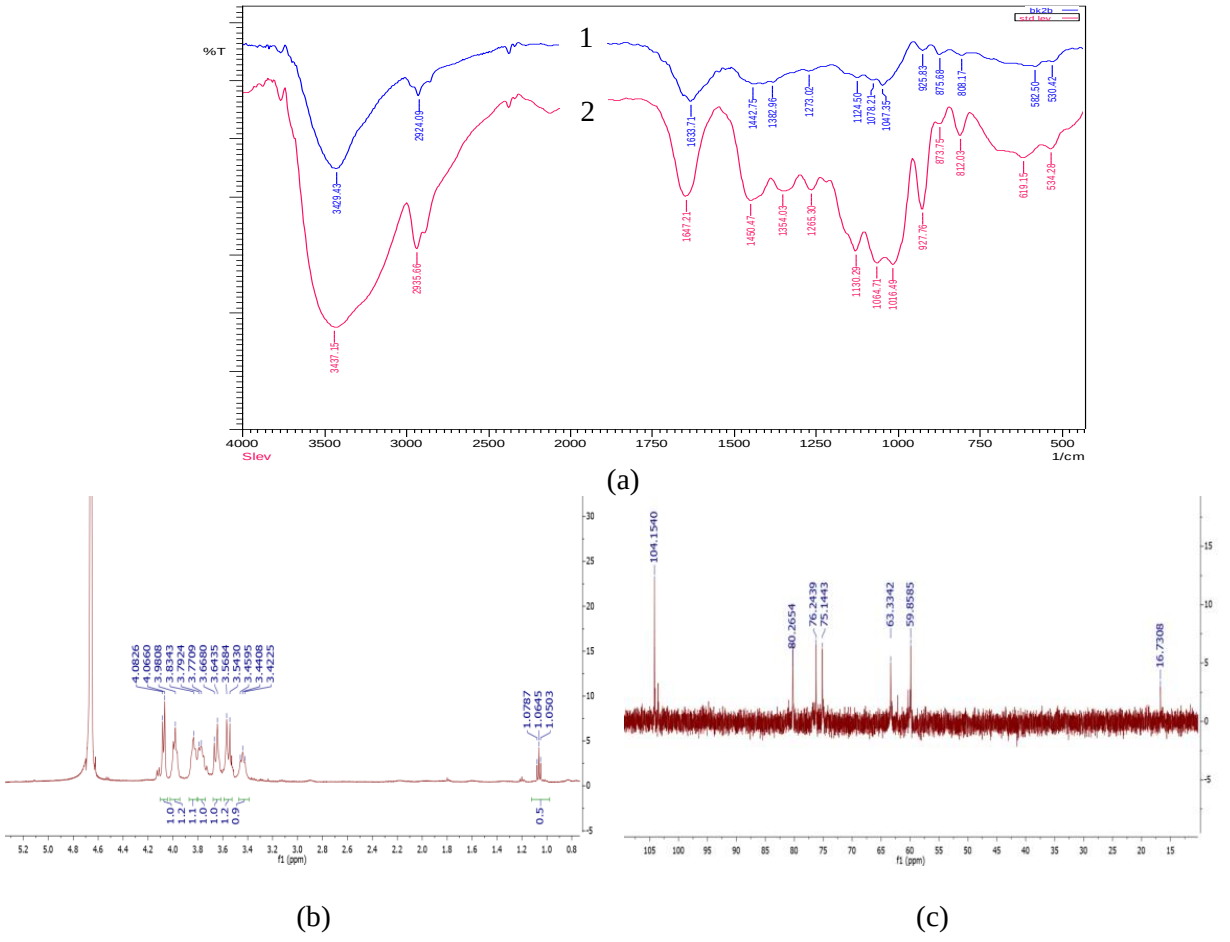


Figure 2. FTIR and NMR spectroscopics analysis of the levan sample synthesized by recombinant levansucrase from *B. licheniformis* BK2. (a) FTIR spectrum of the levan sample (spectrum 1) and the standard one from *E. herbicola* (spectrum 2). (b) ^{1}H-NMR and (c) ^{13}C-NMR spectra of the levan sample.

4. Conclusion

This study has shown that the response surface methodology (RSM) is a useful and effective methods to find the optimum production of levan through *in vitro* catalytic reaction by recombinant levan sucrase from *B. licheniformis* BK2. RSM revealed that 12 % (w/v) sucrose, pH 8, 32.3^{o}C were the optimum conditions for levan synthesis. FTIR and NMR spectroscopies verified the successful of levan synthesis.

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6. References

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