OPTIMIZATION OF EXOPOLYSACCHARIDE PRODUCTION BY Lactobacillus casei AL.15

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Abstract: Exopolysaccharide (EPS) is one of the polysaccharides produced from microorganisms. This polysaccharide is usually produced by lactic acid bacteria and it is widely used for food products and pharmaceutical products. EPS are also very useful for stabilizers, emulsifiers, gelling agents, and have a good ability to bind liquids (water). This study aims to determine the optimization point of EPS growth, based on temperature, time, and the amount of glucose used in selective growth media of *Lactobacillus casei* (*L. casei*) AL.15, namely de Mann Rogosa Shape Broth (MRSB) media, using Response Surface Method (RSM) design to obtain optimum conditions that are more appropriate to produce EPS. Optimization of EPS production occurred at level -1; -1; 0, temperature 42 °C, incubation time 48 hours, and the addition of 10% glucose. *L. casei* AL.15 has a good ability to produce EPS at the temperature, time, and amount of glucose obtained. The result obtained under these conditions is 606.03 mg EPS/litre. This method can be used to produce large amounts of EPS and then these characteristics were analyzed.

Keywords: *optimization, exopolysaccharide, Lactobacillus casei AL.15*

INTRODUCTION

Lactic acid bacteria are EPS bacteria that have attracted much research recently. The production of EPS produced by lactic acid bacteria has considerable benefits in the fields of food production, agricultural land processing, marine industry, and pharmaceuticals. In food production polysaccharides, it has used as a thickening agent, stabilizer, emulsifier, gelling agent, or as a water-binding agent (Frietas et al., 2011). In the agricultural industry, EPS from bacteria can interact with soil particles through the formation of polymer bridges, thus having a role in the formation of microaggregates, and more importantly have the ability to stabilize soil aggregates (Lynch and Elliott, 1983). EPS in the marine industry has a great ability to bind heavy metals (Buffle et al., 1998). In the pharmaceumozzitical industry, EPS from LAB has health benefits such as antioxidant properties, anti-ulcer, anti-tumor, and immunomodulatory activities that play a role in (Liu et al., 2011).

The desire to produce EPS is widely researched and studied to apply the role of EPS in applied fields as described above. EPS production is influenced by various factors such as growth media, growth time, analysis method, temperature, growth pH, minerals, carbohydrates used, and growth techniques used. Optimization of the growth environment is important to achieve maximum EPS production by organisms (Kimmel et al., 1998).

Temperature, time, pH, minerals, and even the type of carbohydrate that used to produce the large amounts of EPS, have been carried out under several conditions as in previous studies. Growth temperature is one factor that has been studied by many previous researchers. The optimum temperature used as the optimum growth temperature is 37-42 °C, but the EPS produced is greater at 45 °C, even reaching 48 °C (Garcia-Garibay and

Marshall, 1991; Grobben et al., 1998). Mozzi et al. (2003) were able to achieve optimal production at 20 °C and also found optimization of EPS polymer synthesis at 30 °C in *L*. *casei* CRL 87.

Besides the temperature, media with bacto-casitone can also affects the amount of EPS produced. Kimmel et al. (1998) found that optimal conditions occur at a temperature of 39 °C with the addition of bacto-casitone as much as 30 g/L. The addition of bacto-casitone, which is quite a lot, requires approximately the same cost as the costs incurred, so it is necessary to add other alternative media to vary the growth of EPS production. This study aims to determine the optimization point of EPS growth, based on temperature, time, and the amount of glucose used in selective growth media of lactic acid bacteria, namely MRSB media, using RSM design to obtain optimum conditions that are more appropriate to produce EPS.

METHOD

Bacterial Strain and Culture Preparation

L. *casei* AL.15 is derived from lactic acid bacteria which is isolated from palm sap that produce EPS and maintained in MRSB (Himedia). Stock culture was prepared by mixing 1 mL of pure culture with 5 mL MRSB.

Culture Medium

The following is the composition of MRSB media (Himedia) in grams per liter: peptone protease 10 g, beef extract 10 g, yeast extract 5 g, dextrose 20 g, polysorbate 80 1 g, 2 g amonium citrate, sodium acetate 5 g, magnesium sulfate 0.1 g, manganese sulfate 0.05 g, potassium phosphate 2 g, and final pH (at 25 °C) 6.5 ± 0.2.

Screening of EPS production

For the determination of EPS yield, all strains were grown in MRSB media containing different glucose, temperatures, and incubation times. After the incubation, cultures were centrifuged at 5000 rpm for 30 min to remove cells using a Clements centrifuge (GS 150 centrifuge). Three volumes of cold anhydrous ethanol were added to 1 volume of culture supernatant and the mixture was kept overnight at 4 °C. After ethanol precipitation and centrifugation, the precipitate was suspended in distilled water to the original volume (Montersino et al., 2008). The total amount of EPS was determined by the total carbohydrate content of precipitate by phenol sulfuric acid method using glucose as a reference (Dubois et al., 1965). 1 mL aliquot of EPS sample was mixed with 1 mL of distilled water, 1 mL of 5% phenol solution, and 2.5 mL of 95% (v/v) H_2SO_4 were added rapidly. After shaking vigorously, the absorbance at 490 nm was measured using a thermo scientific spectometer (Evolution 201). EPS concentration was determined in triplicate.

Single Factor Experiments for Determining the Optimal Range of Factor

This study consists of three treatments, each of which consists of three levels. The first treatment (X1) was growth temperature of 30 °C, 37 °C, and 44 °C. The second treatment (X2) was incubation time of 16 hours, 24 hours, and 32 hours, and the third treatment (X3) was glucose concentration of 5%, 10%, and 15%. *L. casei* AL.15 which was grown on MRSB media. This study was designed using RSM with Central Composite Design (CCD) rules in MINITAB 14 program.

Experimental Design

The experimental design used RSM, following the rules of CCD with 15 designs and 5 replications in each experimental design. Each tube was designed following CCD in Table 1. A total of 20 combinations are shown in Table 2.

Table 1. Level and Experiment Code of CCD

Treatment	Code	-1.682	-1	0	1	1.682
Temperature °C (X_1)	A	25.246	30.0	37.0	44.0	48.774

Treatment	Code	-1.682	-1	0	1	1.682
Time (X_2)	B	10.544	16.0	24.0	32.0	37.456
% Glucose (X_3)	C	1.249	5.5	10.5	15.5	19.751

Table 2. Sample Design for CCD

Table Design					Sample Design		
Run	Block	A	B	C	Temperature (^{0}C)	Time (Hour)	Glucose (%)
1	1	-1	-1	-1	30	16	5
2	1	1	-1	-1	44	16	5
3	1	-1	1	-1	30	32	5
4	1	1	1	-1	44	32	5
5	1	-1	-1	1	30	16	15.500
6	1	1	-1	1	44	16	15.500
7	1	-1	1	1	30	32	15.500
8	1	1	1	1	44	32	15.500
9	1	-1.682	0	0	25.246	24	10
10	1	1.682	0	0	48.774	24	10
11	1	0	-1.682	0	37	2.544	10
12	1	0	1.682	0	37	45.456	10
13	1	0	0	-1.682	37	24	1.249
14	1	0	0	1.682	37	24	19.751
15	1	0	0	0	37	24	10
16	1	0	0	0	37	24	10
17	1	0	0	0	37	24	10
18	1	0	0	0	37	24	10
19	1	0	0	0	37	24	10
20	1	0	0	0	37	24	10

RESULTS AND DISCUSSION

EPS Production By RSM

The optimum ranges of three critical factors (temperature, time, and glucose concentration) affecting EPS yield and cell growth were identified by signal factor experiments and selected for further optimization by RSM. The variable design matrix in coded units is given in Table 3. Each experiment was performed in triplicate and thus the crude EPS values can be seen in Table 3. Velocity prediction values RSM fitting technique using Minitab 17 software.

The experimental results were modelled with a second order polymonial equation to explain the dependence of EPS crude oil production on three critical factors. By applying CCD for EPS crude oil, it can be obtained and given as:

$$YEPS = 507{,}02 + 113{,}31\ x1 - 98{,}21\ x2 + 32{,}60x3 - 75{,}93\ x1*x1 - 77{,}23\ x2*x2 - 85{,}65\ x3*x3 - 54{,}09\ x1*x2 + 33{,}47\ x1*x3 + 51{,}51\ x2*x3$$

Where YEPS, crude EPS (mg/L), is the predicted response variable; x_1-x_3 are the coded values of the independent variables, i.e. temperature, time, and glucose concentration.

Table 3. RSM Analysis at Minitab 17

Run	Temp.	Time	Glucose	EPS (mg/L)	Prediction (mg/L)
1	30.0	16.0	5.5	240.7	193.1
2	30.0	16.0	15.5	519.4	573.2

Run	Temp.	Time	Glucose	EPS (mg/L)	Prediction (mg/L)
3	30.0	32.0	5.5	45.1	85.2
4	30.0	32.0	15.5	468.7	449.8
5	44.0	16.0	5.5	228.7	252.8
6	44.0	16.0	15.5	300.4	265.6
7	44.0	32.0	5.5	263.5	215.0
8	44.0	32.0	15.5	159.4	212.3
9	25.2	24.0	10.5	370.3	356.5
10	48.7	24.0	10.5	200.7	207.0
11	37.0	10.5	10.5	370.3	375.6
12	37.0	37.4	10.5	252.7	240.0
13	37.0	24.0	2.0	116.3	137.8
14	37.0	24.0	18.9	484.2	455.2
15 (C)	37.0	24.0	10.5	511.4	533.8
16 (C)	37.0	24.0	10.5	502.5	533.8
17 (C)	37.0	24.0	10.5	582.4	533.8
18 (C)	37.0	24.0	10.5	564.3	533.8
19 (C)	37.0	24.0	10.5	519.4	533.8
20 (C)	37.0	24.0	10.5	521.7	533.8

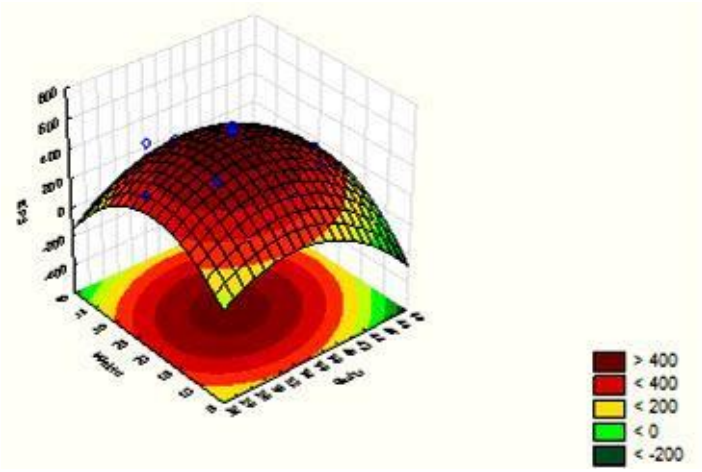

Figure 1. Respons surface and counter plot for the relationship of EPS, time, and temperature

The response graph and counter plot of EPS productivity are shown in Figure 1. The color of the graph shows the difference in EPS productivity with time and temperature combination.

The dark color on the graph and counter plot shows the EPS productivity of *L. casei* AL.15 that occurs at incubation temperature of 37 °C and incubation time of 24 hours, while the dark green color shows EPS production that decreases at temperature of 30 °C and incubation time of 32 hours.

The response graph on the counter plot of EPS productivity in Figure 2 shows the difference in EPS productivity with the combination of glucose and temperature. The dark red color on the counter plot graph shows the EPS productivity of *L. casei* AL15 occurs at 10.5% glucose and 37 °C incubation temperature, while the dark green color shows the EPS production decreases at 30 °C and 5.5% glucose.

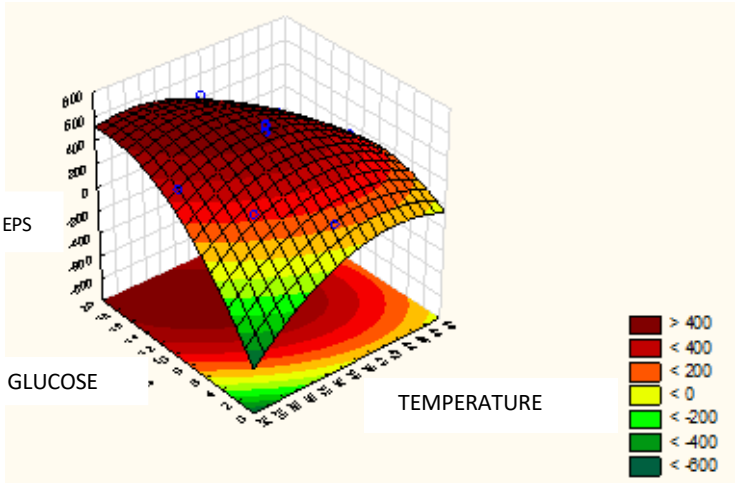

Figure 2. Respons surface and counter plot for the relationship of EPS, glucose, and temperature

The response graph and counter plot of the EPS productivity in Figure 3 shows the difference in EPS productivity with the combination of time and glucose. The dark red color on the graph and counter plot shows the EPS productivity of *L. casei* AL15 occurs at 24 hours incubation and 10.5% glucose level. While the dark green color shows the EPS production decreased at 32 hours incubation and 5% glucose level. The coefficient of determination (R) that obtained in this research is 0.9597. It means that the effect of three factor namely temperature, time, and glucose, affects EPS productivity by 95.9%, while the remaining (0.41%) is influenced by other factors such as pH, minerals, and other extrinsic factors.

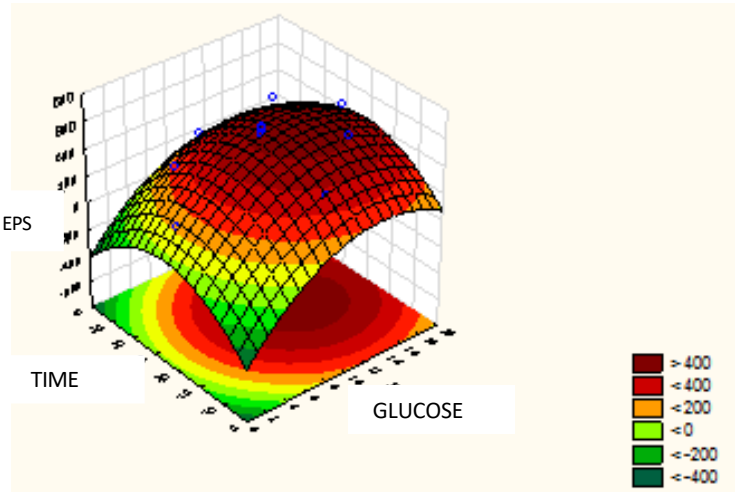

Figure 3. Respons surface and counter plot for the relationship of EPS, time, and glucose

According to de Vuyst and Degeest (1999), the amount of EPS produced by LAB is less stable due to incubation time extention that affects the plasmid or genome, so EPS selection needs to be done periodically to maintain EPS production properties. This study has not included the type of EPS produced by LAB. Therefore, this research needs to be developed again regarding the character of EPS, EPS production, and the application of EPS produced by LAB for food production development, pharmaceuticals, and other industries.

Optimization of EPS production in this study was designed using three factors, namely temperature, growth time, and glucose addition. The three factors

are thought to affect the amount of EPS production, so that it can be an optimization point. In previous studies, EPS production at optimal temperature of 30 °C for 24 h can produce a large amount of EPS. This happens due to influenced by total EPS measurement method which varies such as (milligrams of EPS, milligrams of EPS/liter or milligrams of EPS/CFR). The total EPS produced at the maximum point was 488 mg/L produced by *L*. *casei* CRL 87 (Mozzi et al., 1996).

Statistical analysis of the RSM provided varying information on each of the designed variables. In Figure 1 and Figure 2, the relationship between glucose and growth was limited to an ellipse bounded by the temperature of 30-37 °C, with the amount of glucose in the range of 1-5%. In Figures 3 and 4, the relationship between temperature and growth time is in the temperature range of 30-37 °C, and the growth time is 16-24 hours. The last graph is the relationship between glucose and growth time in Figure 3, shows that the growth is limited to the growth time of 16-24 hours and the glucose level is in the range of 1-5 g/L. Some of these growth conditions have a very significant relationship, related to the amount of EPS produced at the end point of growth. The three factors affecting the growth of exopolysaccharides carried out in this study, namely temperature, time, and glucose levels, greatly affect the productivity of LAB isolate from *L*. *casei* AL.15.

The total EPS produced from all bacterial isolates was influenced by all factors applied in this study. Temperature is one of the determining factors in EPS formation. The optimum growth temperature was achieved by *L*. *casei* AL.15 isolate at 30-37 °C with the range of EPS produced of 71.84-891.26 mg/L. In the type of *Lactobacillus delbrueckii* subsp. bulgaricus RR, the optimum temperature of EPS production occurs at 38 °C and is able to produce EPS of 217-374 mg/L at temperature of 30 °C (Kimmel et al., 1998). This growth is very different from other studies that the average optimum temperature is at 37-45 °C (Kandler and Weiss, 1986).

Besides the growth temperature, growth time is also a determining factor in achieving the optimization point of EPS growth. *Azotobacter* sp. LKM6 showed that EPS production reached the acceleration phase between 40 and 48 hours. The other studies also showed that the optimum growth time was at 48 hours (Hindersah and Sudirja, 2010). According to Kojic et al. (1992), *L*. *casei* CG11 was able to produce the large amounts of EPS at 72 hours of growth, or greater than 50% at 48 hours of growth, on BMM media. The total EPS of *L*. *casei* CG11 media was calculated based on the phenol-sulfate method.

Xu et al. (2010) added 5 grams of glucose in to MRS media to analyze the content of EPS that formed. The obtained total EPS test using phenol-sulfate ranged from 67.12 to 238 mg/L. In this study, the addition of 5% glucose without looking at the same treatment as the previous researcher, was able to produce EPS ranged from 3.88 to 650.49 mg/L. Further research was carried out on the same media, namely MRSi. At the highest EPS growth point, the amount of glucose added was 10%. These results indicate that EPS production is strongly influenced by the amount of glucose added to the growth medium.

Optimization can be defined as the production of goods or the highest of a process from several aspects that have been determined or available. In this study, EPS production optimization has been determined in such a way that at the end of the study, the optimal point or the best production points can be obtained as a reference for further production. The large differences in the results of this research were caused by several different EPS measurement factors, including growth media, conditions and measurements, the absence of pH control and total EPS measurements such as (milligrams of EPS, milligrams of EPS/liter, milligrams of EPS/CFU) (Mozzi et al., 1996). Different optimal growth from various research sources resulted in the opinion that EPS growth should be produced at temperatures smaller than

the optimal temperature (Scheellhaass, 1983).

CONCLUSION

A purified strain of *L*. *casei* AL.15 was able to produce very large EPS under modified MRS media growth conditions with a temperature of 37 °C, incubation time of 24 hours, and the addition of 10.5% glucose. These three factors of growth were very significant with a very large amount of EPS, compared to the research on the same type of MRS growth media. The analysis of CCD was able to show the optimum point use three EPS growth factors, so that it was expected to be a reference for greater EPS production for the development of *L*. *casei* AL.15 isolates in the future.

This research was designed using RSM with CCD rules in MINITAB 14 programme with 15 designs and 5 replicates in each experimental design. Each test tube was designed following the CCD. The optimum ranges of three critical factors (temperature, time, and glucose concentration) affecting EPS yield and cell growth were identified by signal factor experiments and selected for further optimisation by RSM. The modification of MRS media based on RSM using CCD showed that the addition of 10.5% glucose with incubation temperature of 37 °C and incubation time of 24 hours was able to produce EPS of 582.4 mg/L. This result indicates that modifying the bacterial growth medium, especially its MRS medium, can reach a sweet spot in EPS production that can be used for further studies later.

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