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Optimization of *Penicillium Lagena* Medium Cultivication on Antifungal Pathogen of *Phellinus Lamaoensis* Using Surface Methode

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Abstract

Phellinus lamaoensis (Murr.) Hein is fungal pathogen that can cause brown root rot disease in cocoa, tea, rubber, and coffee plants. Endophytic fungi, Penicillium lagena, isolated from bandotan (Ageratum conyzoides Linn.), medicinal plant, is able to inhibit the growth of pathogenic, P. lamaoensis. The effect of carbon source, nitrogen source, and mineral solution was studied. Lactose, yeast extract, and mineral solution were media components which showed significant effect toward production of P. lagena active compound. Composition optimization of these three medium components was done by response surface methodology (RSM). The Optimal response region of the significant factor was predicted by using a second order polynomial model with statistical design, central composite design (CCD). Higest production of P. lagena active compound by quadratic model was predicted to be 69.233% with medium composition 44.77 g L^{-1} lactose, 13.02 g L^{-1} yeast extract, and 15.95 mL L^{-1} mineral solution. Verification value in laboratory is 58.365%, lower 15.7% than its prediction. Optimization increase P. lagena active compound 9 fold compared to unoptimize media.

Keywords: *active compound; optimization; Penicillium lagena; RSM*

Abstract

[Judul: Optimasi Media Kultivasi Penicillium Lagena pada patogen anti jamur Phellinus lamaoensis Menggunakan Metodologi Surface] *Phellinus lamaoensis (Murr.) Hein merupakan jamur patogen yang dapat menyebabkan penyakit akar cokelat pada tanaman kakao, teh, karet, dan kopi. Fungi endofit Penicillium lagena yang diisolasi dari tanaman obat bandotan (Ageratum conyzoides Linn.) diketahui mampu menghambat pertumbuhan patogen P. lamaoensis. Penelitian ini bertujuan untuk mencari sumber karbon, nitrogen, dan mineral terbaik yang menghasilkan senyawa aktif P. lagena sebagai antifungi patogen P. lamaoensis dengan kadar tertinggi. Laktosa, yeast extract, dan larutan mineral adalah komponen media yang menunjukkan pengaruh yang nyata terhadap produksi senyawa aktif P. lagena. Optimasi ketiga komponen media tersebut dilakukan dengan response surface methodology (RSM). Optimasi terhadap faktor yang signifikan diprediksi dengan model ordo dua melalui rancangan statistika central composite design (CCD). Produksi senyawa aktif P. lagena tertinggi yang diprediksi oleh model kuadratik mencapai 69.233% dengan komposisi media 44.77 g L^{-1} laktosa, 13.02 g L^{-1} yeast extract, dan 15.95 mL L^{-1} larutan mineral. Hasil verifiksasi di laboratorium sebesar 58.365%, lebih rendah 15,7% dibandingkan prediksinya. Optimasi meningkatkan senyawa aktif P. lagena 9 kali lipat dibandingkan sebelum optimasi*

Kata kunci: *optimasi; Penicillium lagena; RSM; senyawa aktif*

1. Introduction

Phellinus lamaoensis (Murr.) Hein, which the synonyms are *Fomes noxius* Corner or *Fomes lamaoensis* Murr., causes brown root rot disease in cocoa, tea, rubber, and coffee plants (Semangun, 2000). The attack of brown root rot disease is able to decrease 50 percent of the cacao plantation population (Pusat Penelitian Kopi dan Kakao Indonesia 2010). *P lamaoensis* attacks cacao in several countries, such as Indonesia, Ghana, Nigeria, Sri Lanka, Malaysia, Papua New Guinea, and Samoa. The spread of root disease is through direct contact between healthy roots with infected root. Trees that are infected will die after six months of infection (Wood, Lass, 1989).

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Prevention brown root rot disease was late because the spread of disease was slow and the infection of pathogens was unknown since beginning. Plants that have died which were caused by the disease were dismantled and burnt, then the remaining holes were given 100-600 grams of sulfur and this land can be used again one year later (Nazaruddin, Paimin, 1992; Eden, 1965). Gas of SO_2 or SO_3 in sulfur can react with water vapor in the air to form H_2SO_3 or H_2SO_4 which can cause acid rain (Triharso, 1994). Acid rain causes the damage of forests and eroded the fertile soil layer. Disease control using chemicals are less effective and could negatively affect the environment and human health (Mejía *et al*., 2008).

Controlling the disease can be done with biological control, endophytic fungi. Endophyte are microorganisms (normally fungi and bacteria) that exist in almost every tissue of a plant but do not cause harm or infect plants. Some endophyte produce active compounds in secondary metabolites as anticancer, antioxidant, antiviral (Selim *et al.*, 2012), antibacterial and antifungal (Tran *et al.*, 2010). Endophyte fungi, *P. lagena*, which was isolated from herbal, bandotan (*Ageratum conyzoides* Linn.), is able to inhibit the growth of pathogenic, *P. lamaoensis* (Kaswati, 2013). Bandotan is an annual herb plant in Asteraceae family (Dalimartha, 2006).

Herbal plants are shortages caused by the slow regeneration and the increasing human population resulting in environmental degradation and decline in biodiversity (Wilson, 1988). Scarcity of herbal plants becomes obstacles to isolation *P. lagena*. Therefore, It needs to optimize growth medium *P. lagena* to produce the active compound which is able to kill the pathogen *P. lamaoensis*. The conventional method for the optimization of cultivation media is done by changing one parameter while other parameters are fixed (Liu,Tzeng, 1998). This method may lead to misinterpretation of the results, especially when the effects of the interaction between different factors are ignored. Response Surface Methodology (RSM) is a combination of mathematical and statistical techniques to establish an empirical model. Optimization cultivation using statistical methods can minimize the number of experiments carried out so that it can save costs, time, and effort. RSM in optimization of cultivation can combine all the factors involved in cultivation (Lepsch, McMillin, 1998).

2. Materials and Methods

2.1 Microorganism

Penicillium lagena and *Phellinus lamaoensis* are cultural stock in BPPT-Biotek Serpong, Tangerang Selatan. It was grown on agar medium and was incubated at 28ºC for 5 days.

2.2 Inoculum preparation

6 mL NaCl physiologic is augmented to *Penicillium lagena* on agar medium and 1 mL was transferred to 50 mL sterile vegetative medium in 500 mL Erlenmeyer with glass bead. The composition of vegetative medium consisted of 10 g L^{-1} rice flour, 10 g L^{-1} glucose, 20 g L^{-1} soybean meal, 1 g L^{-1} KH_2PO_4, and 0.5 g L^{-1} $MgSO_4.7H_2O$, pH adjusted at 5.8. Vegetative culture is incubated at 25ºC, 220 rpm for 48 hours.

2.3 Production culture for determination of the best of carbon and nitrogen source

Cultivation medium for determination of the carbon source consisted of 10 g L^{-1} malt extract, 10 g L^{-1} yeast extract, 1 g L^{-1} tryptone, 1 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4NO_3, and carbon from four different sources: lactose, galactose, maltose, and glucose. Cultivation medium for determination of the nitrogen source consisted of 30 g L^{-1} glucose, 20 mL L^{-1} glycerol, 10 g L^{-1} dexstrin, 1 g L^{-1} KH_2PO_4, and nitrogen from four different sources, namely yeast extract, malt extract, tripton, and NH_4NO_3. Cultivation *P. lagena* was conducted with shake culture 25 mL volume pH adjusted at 5.9, 25ºC, 220 rpm for 5 days on Erlenmeyer 250 mL. Active compound from each medium was extracted and injected to HPLC. The wide area curved was considered as concentration of active compound of *P. lagena*.

2.4 Production culture for determination of the best of mineral concentration

According to Ghatora et al. (2006) mineral solution for cultivation medium of *P. lagena* consists of 0.02 g L^{-1} $(NH_4)_2S0_4$, 0.05 g L^{-1} KCl, 0.01 g L^{-1} $CaCl_2$, 0.05 g L^{-1} $MgSO_4$, 0.001 g L^{-1} $ZnSO_4$, dan 0.0005 g L^{-1} $CuSO_4$. Mineral solution was augmented to cultivation medium of *P. lagena* 0, 20, 40, 60, and 80 mL L^{-1}, respectively. Cultivation *P. lagena* was conducted with shake culture 25 mL volume pH adjusted at 5.9, 25ºC, 220 rpm for 5 days on Erlenmeyer 250 mL. Medium with highest concentration of active compound was used for optimization medium.

2.4 Extraction of active compound

According to Kaswati (2013), bioactive compound of *P. lagena* is extracellular. Cultivation medium was centrifuged at 8000 rpm for 15 second. Supernatant was extracted with ethyl acetate ratio 1:1 (v/v) and shaken for 30 second. Ethyl acetate phase was dried with centrifugal concentrator. Extracellular extract was weighed and was adjusted to 5000 ppm concentration and was injected to HPLC.

2.5 Experimental design and optimization by RSM

Designing of optimization medium uses central composite design (CCD). The CCD consisted of three designs, namely design of factorial 2^3, starting point, and center point. Factorial and starting point were done in two replicates whereas center point was done six replications, resulting in 34 experiments. Factors and levels that were

used in central composite design is shown on Table 1. Cultivation *P. lagena* was conducted with shake culture 25 mL volume pH adjusted at 5.9, 25ºC, 220 rpm for 5 days on erlenmeyer 250 mL.

Table 1. Factors and levels that were used in central composite design

Factors	Levels				
	-1.68	**-1**	**0**	**1**	**1.68**
Lactose (g L^{-1})	25.99	33.61	44.81	56.01	63.63
Yeast extract (g L^{-1})	3.76	7.16	12.16	17.16	20.56
Mineral solution (mL L^{-1})	0	4.05	10	15.95	20

The behavior of the system was explained by the following second order polynomial equation:

$$Y = b_0 + b_1X_{1i} + b_2X_{2i} + b_3X_{3i} + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3$$

Y = wide area (response)
X_1 = source of carbon concentration (g L^{-1})
X_2 = source of nitrogen concentration (g L^{-1})
X_3 = volume of mineral solution (mL L^{-1})

The model was verified with five replication in the laboratory for the testing the suitability of the model.

3. Results and Discussion

Microorganisms need water, carbon, nitrogen, minerals, and oxygen if the aerob is in the process of cultivation. Materials with the appropriate concentration in the cultivation medium can produce the desired product optimally. Relative percentage wide area of antifungal compound of lactose is the highest than galactose, maltose and glucose. The result of further test with Duncan test on a probability level 0.05 shows that lactose, maltose, and glucose were significantly different but lactose and galactose were not significantly different (Figure 1). Lactose was chosen as the best carbon source.

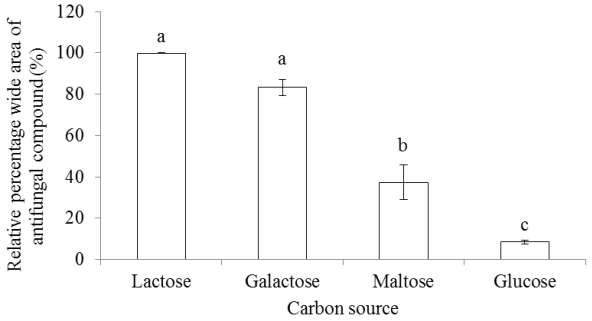

Figure 1. Relative percentage wide area of antifungal compound of some carbon sources. The vertical line at the top of each block of data indicates standard error and letters above blocks of data shows the comparison of the mean of carbon source based on Duncan multiple comparison test with probability level of 0.05

In the HPLC, a wide area is proportional to the concentration of the solute in the flow cell (tested compounds) (Lough, Wainer, 1996). Carbon is required by microorganisms for biosynthesis and as a source of energy (Stanbury *et al.*, 2003).

Relative percentage wide area of antifungal compound of yeast extract is the highest than malt extract, tripton and NH_4NO_3. The result of the further test with Duncan test on a probability level 0.05 shows that yeast extract was significantly different from tripton and NH_4NO_3 but it was not different from malt extract. Malt extract with tripton was not also different (Figure 2). Yeast extract was chosen as the best nitrogen source.

Nitrogen source was as the supply of amino acid, protein, or urea (Stanbury *et al.*, 2003). Yeast extracts are excellent substrates for many microorganism on fermentation process because it contains amino acid and peptides, water-soluble vitamins, and carbohydrates (Crueger, Crueger, 1984).

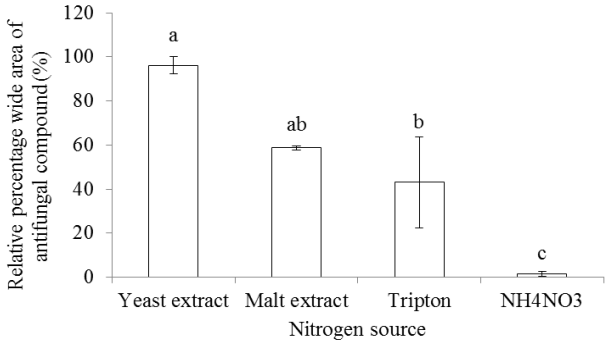

Figure 2. Relative percentage wide area of antifungal compound of some nitrogen sources. The vertical line at the top of each block of data indicates the standard error and letters above blocks of data shows the comparison of the mean of nirogen source based on Duncan multiple comparison test with probability level of 0.05

Relative percentage wide area of antifungal compound in cultivation medium without mineral solution is lower than cultivation medium with mineral solution. The results of further test with Duncan test on a real level 0.05 shows that relative percentage wide area of antifungal compound of cultivation medium without mineral solution was different with cultivation medium with mineral solution. However the concentration of mineral solution (20, 40, 60, and 80 mL L^{-1}) was not significantly different (Figure 3). Mineral with concentration 0 – 20 mL L^{-1} was chosen for the next step. Minerals are needed by all microorganisms for growth and metabolism (Stanbury *et al*., 2003).

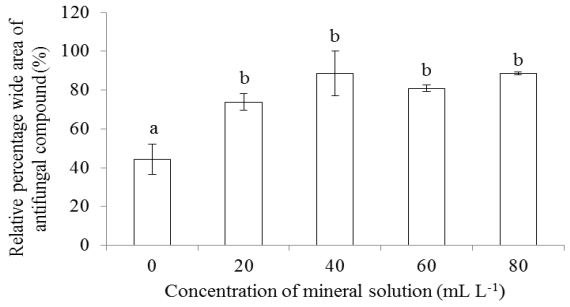

Figure 3. Relative percentage wide area of antifungal compound of several concentration of mineral solution. The vertical line at the top of each block of data indicates standard error and letters above blocks of data shows the comparison of the mean of concentration mineral solution based on Duncan multiple comparison test with probability level of 0.05

The design of optimization medium is using a central composite design (CCD) with three variables, namely lactose, yeast extract, and minerals. The experimental design is encoded for each factor, -1 lower limit, 0 middle limit, 1 upper limit, and 1.68 or -1.68 starting point with value for each code and factor based on Table 1. Data analysis of responses to the optimization of cultivation media (Table 2) were tested with Sequential Model Sum of Square (Table 3) and indicates that the quadratic vs. 2FI model is significant ($p = 0.0002$) and suggested. Model summary statistics (Table 4) shows that the quadratic model has the largest adjusted R -squared value among the others, i.e. 0.5025. Adjusted R-squared shows that three variables effect of diversity responses by 50.25 % while the rest is influenced by the other variables which were not examined. PRESS value (prediction error sum of squares) quadratic model is the lowest. This suggests that the quadratic model is the best fit model.

Results of analysis of variance for quadratic model (Table 5) shows that the quadratic model significantly ($p = 0.0011$) affect the response. The influence of linear (X_2X_3) and quadratic also showed significant results ($p < 0.05$). The result of Lack of fit was not significant ($p = 0.1846$) indicating that the quadratic model is the right model. The test of center point produces error pure value. Comparison of the mean square lack of fit with pure error results in the F test that if the results are not significant indicates that the model is the right model (Bradley, 2007). Quadratic equation obtained:

$$Y = 72.6459 - 2.1532\ X_1 + 1.4704\ X_2 + 4.1495\ X_3 + 2.5949\ X_1X_2 + 5.2990\ X_1X_3 + 6.9158\ X_2X_3 - 13.3402\ X_1^2 - 10.4027\ X_2^2 - 8.6880\ X_3^2$$

Y = relative percentage wide area of antifungal compound (response)

X_1 = source of carbon concentration (g L^{-1})

X_2 = source of nitrogen concentration (g L^{-1})

X_3 = volume of mineral solution (mL L^{-1})

The negative sign (-) in the quadratic coefficient (X_1^2, X_2^2, and X_3^2) indicates that the response graph obtained is a maximum or graph a parabola opens downward (Montgomery, 2001).

Table 2. Data analysis of responses to the optimization of cultivation media with central composite design

Std	Lactose (g L^{-1})	Yeast extract (g L^{-1})	Mineral (mL L^{-1})	Relative percentage wide area of antifungal compound (%)
1	-1	-1	-1	54.990
2	-1	-1	-1	60.164
3	1	-1	-1	29.272
4	1	-1	-1	44.425
5	-1	1	-1	65.682
6	-1	1	-1	28.023
7	1	1	-1	17.480
8	1	1	-1	29.121
9	-1	-1	1	44.413
10	-1	-1	1	45.183
11	1	-1	1	33.324
12	1	-1	1	30.799
13	-1	1	1	40.994
14	-1	1	1	56.071
15	1	1	1	59.987
16	1	1	1	58.774
17	-1.68	0	0	34.499
18	-1.68	0	0	15.591
19	1.68	0	0	22.471
20	1.68	0	0	47.555
21	0	-1.68	0	30.447
22	0	-1.68	0	38.320
23	0	1.68	0	40.237
24	0	1.68	0	44.347
25	0	0	-1.68	29.144
26	0	0	-1.68	35.543
27	0	0	1.68	53.441
28	0	0	1.68	54.623
29	0	0	0	59.093
30	0	0	0	77.269
31	0	0	0	55.090
32	0	0	0	70.436
33	0	0	0	77.340
34	0	0	0	100

Table 3. Sequential Model Sum of Square for response for relative percentage wide area of active compound *P. lagena*

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	Description
Mean vs. Total	73809.8353	1	73809.8353			
Linear vs. Mean	655.9748	3	218.6583	0.6082	0.6149	
2FI vs. Linear	1322.2684	3	440.7561	1.2576	0.3086	
Quadratic vs. 2FI	5323.1898	3	1774.3966	10.2874	0.0002	Suggested
Cubic vs. Quadratic	796.9907	4	199.2477	1.1922	0.3447	Aliased
Residual	3342.6059	20	167.1303			
Total	73809.8353	1	73809.8353			

Table 4. Model summary statistics for response for relative percentage wide area of active compound *P. lagena*

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	Description
Linear	18.9605	0.0573	-0.0369	-0.1289	12915.9111	
2FI	18.7209	0.1729	-0.0109	-0.0921	12494.7281	
Quadratic	13.1333	0.6382	0.5025	0.2955	8059.8775	Suggested
Cubic	12.9279	0.7078	0.5179	0.2100	9037.9833	Aliased

Table 5. Analysis of variance for response surface of quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Description
Model	7301.4330	9	811.2703	4.7035	0.0011	Significant
X_1-Laktosa	126.6323	1	126.6323	0.7342	0.4000	
X_2-Yeast Extract	59.0532	1	59.0532	0.3424	0.5639	
X_3-Mineral	470.2893	1	470.2893	2.7266	0.1117	
X_1X_2	107.7385	1	107.7385	0.6246	0.4371	
X_1X_3	449.2673	1	449.2673	2.6047	0.1196	
X_2X_3	765.2625	1	765.2625	4.4367	0.0458	Significant
X_1^2	4012.4878	1	4012.4878	23.2631	< 0.0001	Significant
X_2^2	2439.9457	1	2439.9457	14.1460	0.0010	Significant
X_3^2	1701.8485	1	1701.8485	9.8668	0.0044	Significant
Residual	4139.5966	24	172.4832			
Lack of Fit	1276.0813	5	255.2163	1.6934	0.1846	Not Significant
Pure Error	2863.5153	19	150.7113			
Cor Total	11441.0295	33				

Response surface plot obtained as function of lactose versus yeast extract concentration (Figure 4) shows that the increase of lactose and yeast extract concentration effect on the production of active compounds *P. lagena*. Lactose concentration above 44.81 g L^{-1} level (0) and yeast extract concentration above 12.16 g L^{-1} level (0) cause the decrease of production of active compounds *P. lagena*. Interaction between lactose with yeast extract no effect on the production of active compounds *P. lagena* (p = 0.4371). High substrate concentrations can inhibit cell growth of microorganisms (Edwards, 1970). Inhibited cell growth causes the decrease of secondary metabolites produced.

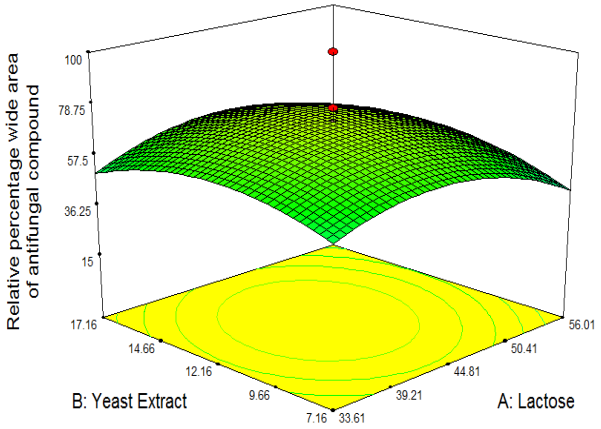

Figure 4. Response surface plot between lactose and yeast extract on the production active compound *P. lagena*

Response surface plot obtained as function of lactose versus mineral concentration (Figure 5) shows that the increasing of lactose and mineral concentration affect on the production of active compounds *P. lagena*. Lactose concentration above 44.81 g L^{-1} level (0) causes the decrease of the active compounds production and mineral concentration above 10 mL L^{-1} level (0) causes the decrease of active compounds *P. lagena* production. Ghatora et al (2006) used mineral solution for xylanase cultivation of thermophilic and thermotolerant fungi with concentration 1% or 10 mL L^{-1}. *P. lagena* is thermotolerant fungi. Interaction between lactose with mineral no effect on the production of active compounds *P. lagena* (p = 0.1196).

Response surface plot obtained as function of yeast extract versus mineral concentration (Figure 6) shows that the increase of yeast extract and mineral concentration affects the production of active compounds *P. lagena*. Yeast extract concentration above 12.16 g L^{-1} level (0) causes the decrease of production of active compounds and mineral concentration above 10 mL L^{-1} level (0) causes the decrease of production of active compounds *P. lagena*. Interaction between yeast extract with mineral effect on the production of active compounds *P. lagena* (p = 0.0458).

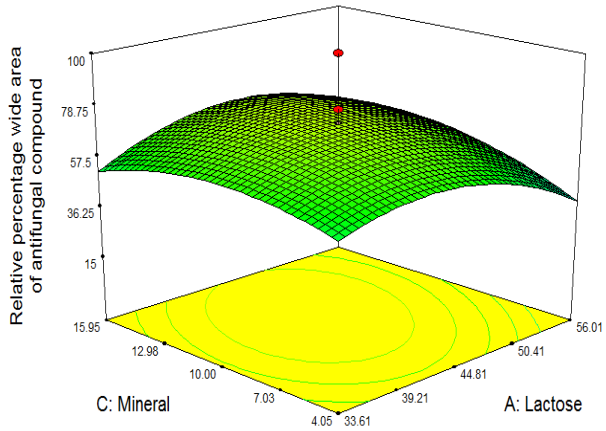

Figure 5. Response surface plot between lactose and mineral on the production of active compound *P. lagena*

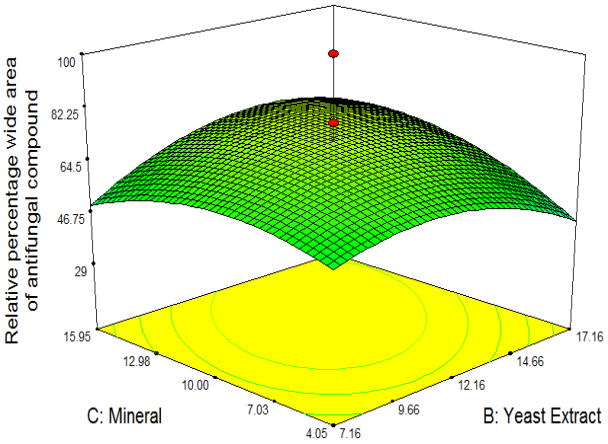

Figure 6. Response surface plot between yeast extract and mineral on the production of active compound *P. lagena*

The maximum percentage relative wide area response indicating the concentration of active compound *P. lagena* was to be predicted at 69.233% with media composition 44.77 g L^{-1} lactose, 13.02 g L^{-1} yeast extract, and 15.95 mL L^{-1} mineral. The result of the verification in the laboratory produces the active compound *P. lagena* at 58.365%. This value was found to be 15.7 % less than the predicted value. Results of optimization increase the production of active compounds *P. lagena* about 9 times compared with that of unoptimized medium (basal medium).

4. Conclusion

Components of cultivation media which are able to increase the production of active compounds *P. lagena* are lactose as a carbon source, yeast extract as a nitrogen source, and mineral additions. Production of the active compound *P. lagena* was the highest on media with the composition 44.77 g L^{-1} lactose, 13.02 g L^{-1} yeast extract, and 15.95 mL L^{-1} minerals. Optimization of the composition media is able to increase the production of the *P. lagena* active compound 9 times more compared to basal medium.

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