

Production of antioxidant compounds of grape seed skin by fermentation and its optimization using response surface method

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Abstract. Skins and seeds of grape are waste generated from food industry. These wastes contain nutrients of which able to be utilized as an important source for antioxidant metabolite production. Through an environmentally friendly process, natural antioxidant material was produced. This study aimed to generate antioxidant compounds by liquid fermentation. Optimization was carried out by using *Schizosaccharomyces cerevisiae* in Katu leaf substrate. Optimization variables through response surface methodology (RSM) were of sucrose concentration, skins and seeds of grape concentration, and pH. Results showed that the optimum conditions for antioxidant production were of 5 g/L sucrose, 5 g/L skins and seed at pH 5, respectively. The resulted antioxidant activity was of 1.62 mg/mL. Mathematical model of variance analysis using a second order polynomial corresponding to the resulted data for the antioxidant was of $20.70124 - 3.86997 A - 0.65996 B - 1.88367 C + 0.19634 A^2 - 0.016638 B^2 + 0.28848 C^2 + 0.26980 AB - 0.068333 AC - 0.12367 BC$. From the gained equation, the optimum yield from all variables was significant. Chemical analysis of the antioxidant was carried out using 2,2-Diphenyl-1-picrylhydrazyl (DPPH).

1. Introduction

Skins and seeds of grape are by-products of food industry. High content of nutrients in skins and seeds of grapes can be utilized as a source of nitrogen, vitamins, and minerals. Utilization of skins and seeds of grapes as a medium for antioxidant flavonoid production is an attempt to enhance its add value and improving the quality of living things [1, 2]. However, these natural materials are oxidized easily. Fermentation technology is able to maintain natural materials stability as well as increasing its antioxidant activity. Microbial fermentation is considered as a potential technology for producing new bioactive compounds. Several fermentation processes are applied in the production of secondary metabolite. However, its bioconversion yield varies, depending on the application of fermentation parameters for instance the kind of microorganisms, medium used, temperature, and pH [3].

Engineering of antioxidant biosynthesis in microorganisms by fermentation has significant scientific and economically importance. Response Surface Methodology (RSM) is one of the strategies for increase antioxidant production yields [1, 4]. The skins and seeds of grape contain fiber and resveratrol which is one source of antioxidants. Antioxidant consists of quercetin, procyanidins, catechins, and anthocyanins. The chemical compounds, namely: terpenes (geraniol, linalool, terpineol, nerolidol), norisoprenoids (damascenone β , β ionone), thiols (hexan-1-ol-L-cysteine) [5].

In this study, RSM was used to determine the optimum conditions for antioxidant production with various pH, sucrose concentration and concentration of grape skins and seeds. With choose antioxidant activity in response, optimum conditions antioxidant production process using yeast *S. cereviceae* as biocatalysts can be determined [6-9].



2. Experimental

2.1. Materials

Skins and seeds of grape were obtained from food industry whereas Katu leaves from traditional market. Commercial sucrose was purchased from chemicals agent whilst *S. cerevisiae* was taken from collection of Research Unit for Clean Technology, Indonesian Institute of Sciences.

2.2. Method

2.2.1. Cultivation of microorganisms

S. cerevisiae was cultivated in potato dextrose agar (PDA) at 30°C for 48 hr.

2.2.2. Inoculum preparation

Pure culture of *S. cerevisiae* was activated by putting it to PDA slant medium for 48 hr and inoculated to PDB medium for 4 days. The phase of inoculum preparation was began with the stage of microorganism breeding.

2.2.3. Pulverizing of skins and seeds of grapes

Skins and seeds of grape were oven-dried for a week at 50°C then crushed with a grinder to form a powder.

2.2.4. Blanching of Katu leaves

Katu leaves were boiled for 15 min at 80 °C then blended in distilled water (1: 4) w /v [2]. The resulted liquor was separated from the mixture.

2.2.5. Fermentation process

The liquor of Katu leafs was inoculated with 10% w/v of *S. cerevisiae* in fermentation media without the skins and seeds of grape, then incubated in the dark room for seven days at 30 °C, pH 7.

2.2.6. Fermentation process optimization

Optimum condition for antioxidant production was carried out with experimental design of Response Surface Methodology with Design Expert 6.0.6. software supported 20 experiments including six-center point designed in Central Composite Design. Each experiment was done in duplicate. Variables were varied at pH (5-8), concentration of skins and seeds of grape (5-10) g/L, and concentration of sucrose (5-10) %.

2.2.7. Antioxidant analysis

Inhibition of DPPH free radicals were measured in fermentation broth without grape skins and seeds in Katu leafs substrate and compared with the broth containing skins and seeds of grape. Inhibition of the fermentation was carried out in 1-7 days. Antioxidant activity test was conducted to the highest inhibition. A total of 2 mL of the fermentation broth at a concentration of 25, 125, 250 and 500 µg /mL in 500 µL of methanol were mixed with 0.1 mM DPPH in methanol. Homogeny solution was incubated in dark room at 30°C for 30 min. A blank solution was made for each sample solution by mixing 500 µL of sample and 1.5 mL of methanol. As a negative control 500 µL of 0.1 mM DPPH solution was added with 1.5 mL of methanol. Absorbance was measured at of 515 nm using a spectrophotometer Hitachi U-2800.

$$\text{Inhibition} = (\text{control absorbance} - \text{sample absorbance}) / \text{control absorbance} \times 100\%$$

IC₅₀ values were determined on curve of inhibitions versus concentration obtain the regression equation. From the regression equation can be determined the amount of concentration of the fermentation broth that has the ability inhibition of DPPH activity by 50% [10].

3. Result and Discussion

3.1. Optimization of fermentation process by RSM

Observation of five points for optimization using CCD namely $(-\alpha)$, $(-)$, (0) , $(+)$ and $(+\alpha)$ is a factor of the variable pH, glucose concentration, and the concentration of yeast extract (Table 1). The optimum results for fermentation process by RSM that of activation of antioxidants as presented in the response and the rightmost column shows the average value of experiments in duplicate (Table 2). Variant Analysis (ANOVA) for the experimental results is presented in Table 3. From the analysis of the model fit summary of the results suggested that data is a quadratic model.

Table 1. Central Composite Design.

Parameter of optimization process	(-)	(+)	$(-\alpha)$	$(+\alpha)$	(0)
pH	5	8	3.98	9.02	6.5
Sucrose (%)	5	10	3.3	11.7	7.5
Grape seed skin (g/L)	5	10	3.3	11.7	7.5

The F value for the model 11.63 that shows the model is significant at 95% confidence level was showed in Table 3 whereas value of p (Prob> F) models was less than 0.05 (<0.0001) indicating that the overall variables was significant. In other words, variables in the model show significant effect on the response. In the above analysis, variables A, B, C, A2, B2, C2, AB, AC, BC were significant variables with p (Prob> F) values of less than 0.05. Meanwhile, AC and BC did not significantly affect towards antioxidant activity response, but still included in the model to get the model hierarchy. Mathematical equation model was presented in the equation I.1.

Table 2. Optimization of fermentation process by RSM.

Experiment	A:Sucrose (%)	B: grape seed skin (g/L)	C: pH	antioxidant (mg/mL)
1	10	10	8	9.44
2	7.5	7.5	6.5	2.65
3	7.5	7.5	6.5	2.52
4	7.5	3.3	6.5	2.18
5	7.5	7.5	3.98	3.76
6	7.5	7.5	6.5	2.33
7	5	5	8	4.82
8	7.5	7.5	6.5	2.77
9	5	5	5	1.62
10	7.5	7.5	6.5	2.72
11	11.7	7.5	6.5	8.53
12	7.5	7.5	6.5	2.68
13	5	10	8	3.39
14	3.3	7.5	6.5	3.05
15	7.5	11.7	6.5	1.87
16	7.5	7.5	9.02	4.55
17	10	10	5	9.12
18	5	10	5	1.86
19	10	5	5	1.95
20	10	5	8	4.31

The resulted models need to be examined its adequacy as well as the normal probability plots and residual plots. The normal probability plots of residuals and residuals show that in general are in a straight line, which means errors are normally distributed (Figure 1.a.). On the other hand, Figure 1.b. shows that the residuals do not show a specific pattern (random). It means the model becomes accurate for predicting the production of antioxidants to review pH, concentration of sucrose and concentration of the seeds and skins of grape.

Table 3. Varian analysis (ANOVA) for quadratic model by response of flavonoid antioxidant activities.

Variable	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	102.20	9	11.36	11.63	0.0003
A	36.56	1	36.56	37.44	0.0001
B	8.21	1	8.21	8.41	0.0159
C	5.59	1	5.59	5.73	0.0376
A ²	21.70	1	21.70	22.22	0.0008
B ²	0.16	1	0.16	0.16	0.6980
C ²	6.07	1	6.07	6.22	0.0318
AB	22.75	1	22.75	23.29	0.0007
AC	0.53	1	0.53	0.54	0.4802
BC	1.72	1	1.72	1.76	0.2139
Residual	9.77	10	0.98		

Antioxidant activity by analysis of variance for Response Surface Quadratic Model is

$$\text{Antioxidant} = 20.70124 - 3.86997 A - 0.65996 B - 1.88367 C + 0.19634 A^2 - 0.016638 B^2 + 0.28848 C^2 + 0.26980 AB - 0.068333 AC - 0.12367 BC \dots \dots \dots (I.1)$$

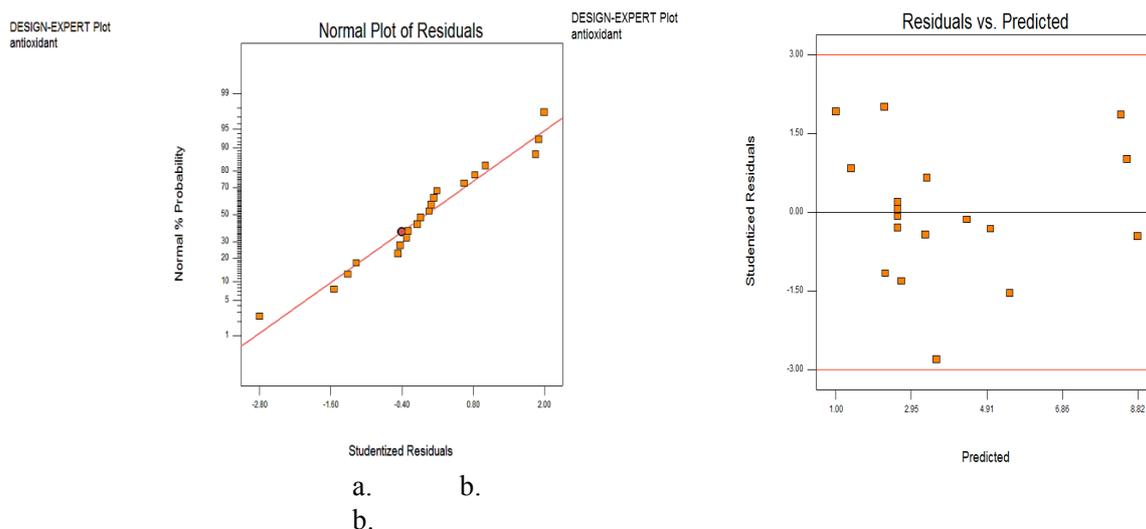


Figure 1. a. The normal probability for antioxidant activities.
b. Response for residuals and predicted for antioxidant activities.

The effect of pH and the concentration of sucrose against the production of antioxidants at concentrations of grape seed skin is presented in Figure 2.b. Antioxidant activity increased as rising

pH to reach maximum activity. Optimum pH production for antioxidants was of 5. Based on ANOVA analysis, pH was a variable that influences significantly for antioxidants production. These results indicated that *S. cerevisiae* produces antioxidants in accordance with pH condition.

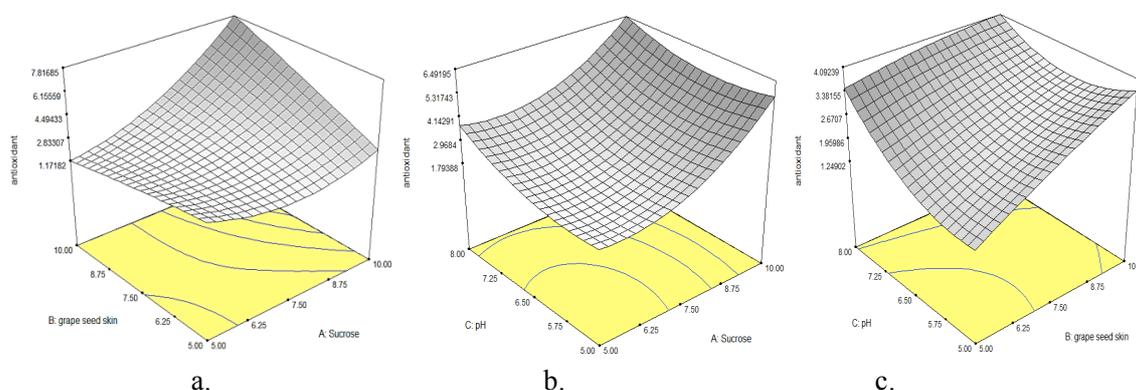


Figure 2. Three dimension graphic of the relation among sucrose 5%, grape seeds and skin, and pH 5.
 a. Three dimensions relation between sucrose and grape seed skin at pH 5
 b. Three dimensions relation between sucrose and pH at 5 g/L grape seed skin
 c. Three dimensions relation between grape seed skin and pH at 5% sucrose

The range of pH for *S. cerevisiae* to grows well is quite wide, of which its optimum around pH 5 [1], whereas others reported that the growth range *S. cerevisiae* was in the range 25-30°C and at pH of 5 [1,8]. Moreover, effect of sucrose and grape seeds and skins is shown in Figure 3.a. Optimum condition for antioxidant was achieved 5% of sucrose and of 5 g/L grape seed skin. Sucrose and grape seeds and skins are considered to represent carbon, and nitrogen of which provide a positive influence on the production of antioxidants [1,8]. Checking the accuracy for the optimum conditions generated by the software Design Expert was carried out through validation. Model validation was based on optimization as presented in Table 4. The tests showed that the predicted results and the experimental results differ greatly so the model is validated.

Table 4. RSM model validation between prediction and experiment.

Sucrose (%)	pH	Grape seed skin (g/L)	Antioxidant - prediction	Antioxidant - validation
5.07	5	5	1.70	1.62

3.2. Antioxidant analysis

The antioxidant activity of fermented liquid determined by DPPH. DPPH is purple free radical molecule. Antioxidant molecules will donate one hydrogen atom to be stable DPPH that marked with purple color changes to yellow. The antioxidant activity usually expressed as the concentration, which caused a loss of 50% DPPH activity.

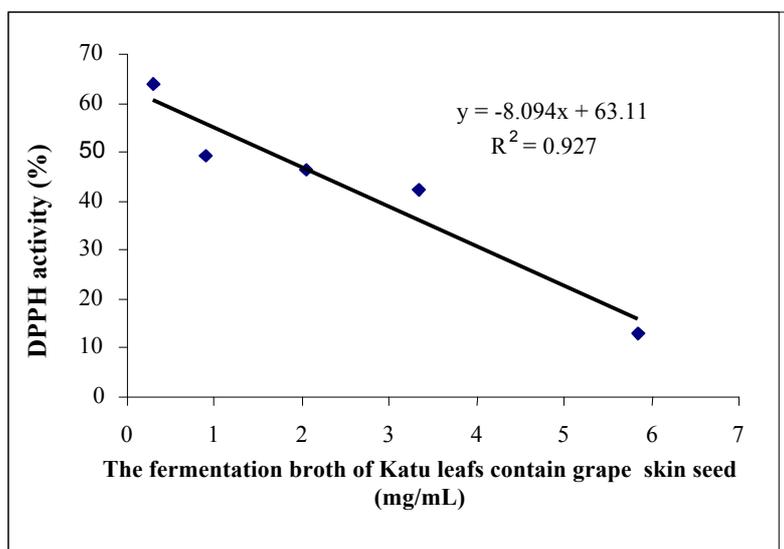


Figure 3. A regression equation to calculate the IC_{50} values by the broth fermentation in variation of 5g/L grape seed skin, 5% sucrose and pH 5.

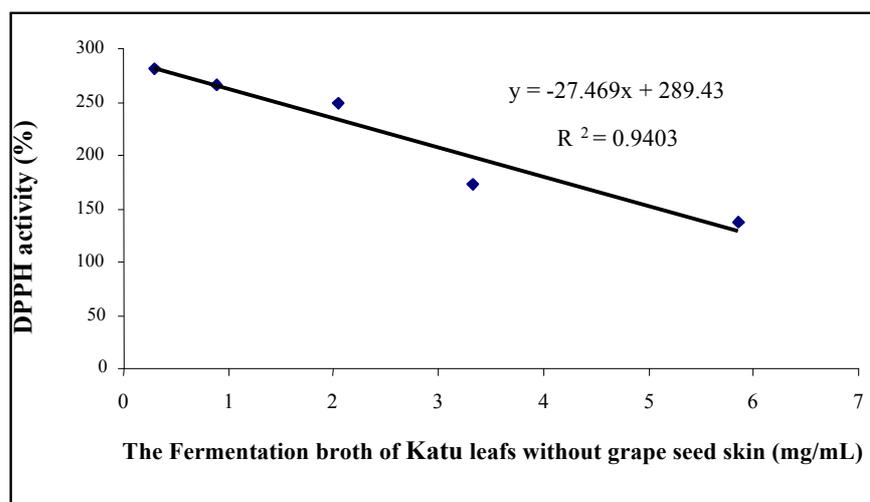


Figure 4. A regression equation to calculate the IC_{50} values by the broth fermentation in variation of 5% sucrose and pH 5.

Figure 3 and 4 show the DPPH radical activity decreases with increasing concentration of active ingredient. The optimum antioxidant activity containing 5 g/L grape seed skin, 5% sucrose and pH 5 with IC_{50} value was of 1.62 mg/mL whereas the fermentation broth that not contains grape seed skin was 8.72 mg/mL. The smaller the IC_{50} value, the greater the antioxidant activity in a test sample in reducing free radicals.

4. Conclusion

Antioxidant has been successfully produced from skins and seeds of grape. Antioxidant activity with IC_{50} value was of 1.62 $\mu\text{g/mL}$, increased 5 times higher than that of without skins and seed of grape.

Acknowledgement

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5. References

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