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Research Article

OPTIMIZATION OF *ASPERGILLUS ORYZAE* FERMENTATION CONDITIONS FOR KOJIC ACID PRODUCTION

ADNINA FITRA AZZAHRA, DYAH KARINA PUSPITA SUKARNA, HERMAN SURYADI*

Laboratory of Microbiology and Biotechnology, Faculty of Pharmacy, Universitas Indonesia, Depok 16424, Indonesia.
Email: hsuryadi@farmasi.ui.ac.id

ABSTRACT

Objective: The objective of this study is to obtain the optimal fermentation conditions for *Aspergillus oryzae* by gradually optimizing the medium and fermentation conditions.

Methods: Fermentation condition were optimized by varying carbon and nitrogen sources, pH medium, temperature, and aeration condition. Levels of kojic acid were determined using thin-layer chromatography-densitometry with an ultraviolet detector at a wavelength of 318 nm.

Results: A combination of sucrose and yeast extract was chosen as the best source of carbon and nitrogen, respectively, from nine medium variations, producing 1.5425 g/L kojic acid. The optimum acidity of the medium was at pH 4.5, producing 1.7127 g/L of kojic acid, compared with pH 3.5 and 5.5. Compared with fermentation at room temperature, fermentation at 35°C yielded greater kojic acid production. The best aeration condition was 100 mL medium in 250 mL flask that produced the most kojic acid (1.6472 g/L).

Conclusion: The optimal medium for fermentation of kojic acid using *A. oryzae* uses sucrose and yeast extract as carbon and nitrogen sources, respectively, at pH 4.5. The optimal aeration and incubation conditions were used, a volume of 100 mL in a 250 mL conical flask, incubated at 35°C, obtaining the highest yield value of 0.0370 g/g.

Keywords: *Aspergillus oryzae*, Fermentation, Kojic acid, Optimization.

INTRODUCTION

Kojic acid is an organic acid with many uses, such as antibacterial, antifungal, anti-melanosis, and a chelating agent. It can be applied in areas such as chemistry, health, food, and cosmetics; however, it, currently, has limited commercial use despite the market being developed 40 years ago. The recent development of industries related to the application of these compounds, especially in the field of cosmetics, has fueled an increased interest in kojic acid [1].

Kojic acid is produced biologically by various microorganisms, including species of *Aspergillus* and *Penicillium*, and involves aerobic fermentation using various substrates. Kojic acid-producing microorganisms, such as *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus tamarii*, and *Aspergillus parasiticus* are reported to produce high levels of kojic acid [1-5].

A. oryzae is a filamentous fungus, or mold, well known in Indonesia. The use of this microorganism is not novel, and reports of soy sauce, one of its fermentation products, date back to 1680 [6]. Other common uses of *A. oryzae* in Indonesia include the manufacture of tauco [7].

The development of kojic acid fermentation has generally focused on genetic mutations to produce new strains or optimization of factors affecting fermentation [1]. Kojic acid fermentation itself is influenced by several factors, such as carbon and nitrogen sources as substrates, pH, temperature, aeration, and agitation [2].

The present study optimized each factor individually. First, the fermentation medium was optimized, focusing on carbon and nitrogen sources, and pH. Next, aeration and incubation temperature were optimized. Then, the fermentation results were analyzed by spectrophotometry to determine the remaining sugar content. The optimum conditions were determined through culture analysis using thin-layer chromatography (TLC)-densitometry to measure the levels of kojic acid produced by each variable. To strengthen our results, previously reported methods of analysis were used [8-10].

METHODS

Medium Rejuvenation, Medium Preculture/Inoculum Development containing 1% yeast extract and 5% technical sucrose, and medium fermentation phase optimization, containing a 5% carbon source, 0.25% variation of nitrogen source, 0.05% $MgSO_4$, and 0.1% KH_2PO_4 were used to prepare the media. Table 1 shows the variations of carbon and nitrogen sources. Verification of the kojic acid analysis method using TLC-densitometry was performed by generating an absorption curve, kojic acid calibration curve, and an accuracy and precision test. The optimization of the fermentation medium focused on optimizing the carbon and nitrogen sources, as well as the pH. The results obtained were based on the growth of cell biomass and the resulting kojic acid levels. Optimization of the fermentation conditions was performed by optimizing the aeration and incubation temperature. The results obtained were based on the growth of cell biomass and the resulting kojic acid levels. Quantitative analysis of the fermentation culture was performed. The dry cell weight method was used to analyze the cell biomass. Finally, the sugar content in the fermentation filtrate was determined.

RESULTS AND DISCUSSION

Results

Rejuvenation and inoculum preparation

After rejuvenation using potato dextrose agar (PDA) medium in tilted, screw-capped tubes for 7 days, a culture of dark green mold on the jelly surface was obtained. The newly rejuvenated mold was white for the first few day (Fig. 1). To multiply the cells and prepare the formed biomass for the fermentation stage, preculture was performed.

After incubation for 24 h at 180 rpm, it was produced mold cell culture in the form of pellets that are yellowish white in color (Fig. 2). Throughout the study, several schemes were performed for each fermentation condition.

Verification of kojic acid analysis method using TLC-densitometry

Absorption curve

TLC-densitometry requires that the maximum wavelength of the compound to be analyzed is known. This was determined by analyzing a solution of 100 μg/mL kojic acid at a wavelength of 200–400 nm. The maximum wavelength was found to be 318 nm and was used for subsequent analyzes of kojic acid using TLC-densitometry.

Calibration curve and linearity

After the maximum wavelength was determined, a calibration curve was generated using various concentrations of kojic acid to obtain the

Fig. 1: *Aspergillus oryzae* **culture colony in tilted potato dextrose agar medium after 7 days**

linear regression equation required to calculate the kojic acid content in each sample, and to find the value of r, seven different concentrations of kojic acid solution: 20.36, 40.72, 50.9, 61.08, 71.26, and 81.44 μg/mL were used to make the calibration curve. The seven kojic acid solutions were analyzed using TLC-densitometry at a wavelength of 318 nm. The results were plotted against concentration to generate a calibration curve for kojic acid. Linear correlation uses the correlation coefficient (r) in linear regression analysis, with an acceptance criterion of r≥0.99908. The calibration curve (Fig. 2) had the linear regression equation y=89.352x–435.55, with r correlation coefficient of 0.9993. Therefore, the TLC-densitometry method of quantitative analysis of the kojic acid showed linearity.

Accuracy and precision

Accuracy and precision tests were performed by adding standards of known kojic acid concentration to samples of fermented filtrate. The kojic acid standards added were 80%, 100%, and 120% of the amount of kojic acid in the sample. The result of the accuracy test was expressed as a percentage of recovery from the added analytes, while the precision test was expressed as the coefficient of variation.

The fermentation filtrate used in the accuracy and precision test used *A. oryzae* fermentation filtrate at a temperature of 35°C. Since 1 mL of filtrate contained 0.7 mg of kojic acid, 80%, 100%, and 120% of kojic acid standard were calculated as the addition of 0.63 mg, 0.73 mg, and 0.83 mg, respectively, of standard kojic acid.

The accuracy test gave an average range of the recovery rate from 98.68% to 100.88%. For the precision test, the variation coefficient was obtained in the range of 1.16–5.84%. Table 2 shows the data of the accuracy test result and complete precision.

The accuracy test met the criteria if the percentage recovery was within the range of 98–10%, while the precision test met the criteria if the relative standard deviation value or coefficient of variation (CV) was ≤2% [8]. Based on this, the accuracy requirement for the percentage recovery of all concentration is in the range 98–102%, while for precision, 80% concentration does not meet the requirements because the coefficient of variation is outside the range of 5.84%.

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Table 1: Variations of carbon sources and nitrogen source used in the optimization stage

Carbon source	Nitrogen source
Glucose	Yeast extract
Glucose	$CO\ (NH_2)_2$
Glucose	$(NH_4)_2SO_4$
Sucrose	Yeast extract
Sucrose	$CO\ (NH_2)_2$
Sucrose	$(NH_4)_2SO_4$
Fructose	Yeast extract
Fructose	$CO\ (NH_2)_2$
Fructose	$(NH_4)_2SO_4$

Table 2: Accuracy and precision test data for filtrate fermentation sample with standard addition of kojic acid

Addition (%)	Addition of standard (mg)	Theoretical mass (mg)	Experimental results area (AU)	Experimental results mass (mg)	Recovery (%)	Average (%)	Coefficient of variation (%)
80	0.63	1.37	10,985.9	1.27825	93.30	98.68	5.84
			12,288.3	1.42401	103.94		
			11,363.1	1.32047	96.38		
			12,015.7	1.39351	101.72		
			12,449.5	1.44206	105.26		
			10,764.3	1.25345	91.49		
100	0.73	1.47	12,852.6	1.48717	101.17	100.88	1.16
			12,702.4	1.47036	100.02		
			12,985.6	1.50205	102.18		
			12,922.0	1.49494	101.70		
			12,861.4	1.48815	101.23		
			12,565.5	1.45504	98.98		
120	0.83	1.57	13,484.5	1.55789	99.23	100.16	1.30
			13,587.5	1.56942	99.96		
			13,490.2	1.55853	99.27		
			13,455.0	1.55459	99.02		
			13,903.6	1.60479	102.22		
			13,768.2	1.58964	101.25		

Table 3: Determination of kojic acid content and dry cell weight for fermentation filtrate optimization of carbon and nitrogen source

Carbon source (50 g/L)	Nitrogen source (2.5 g/L)	Acquired area (AU)	Kojic acid (g/L)	Dry cell weight (g/L)
Glucose	Yeast extract	5163.8	0.6267	13.824
Glucose	$CO(NH_2)_2$	undetected	–	8.776
Glucose	$(NH_4)_2SO_4$	3859.4	0.4807	10.638
Sucrose	Yeast extract	13,168.4	1.5245	14.147
Sucrose	$CO(NH_2)_2$	2379.0	0.3150	11.082
Sucrose	$(NH_4)_2SO_4$	5349.3	0.6474	14.253
Fructose	Yeast extract	786.7	0.1368	12.395
Fructose	$CO(NH_2)_2$	2630.5	0.3431	11.154
Fructose	$(NH_4)_2SO_4$	cted undet	–	8.192

Quantitative analysis of fermentation culture

In the present study, the fermentation medium was optimized, focusing on the carbon and nitrogen sources as well as pH followed by optimization of aeration and incubation temperature. Quantitative analysis of the biomass and kojic acid was produced for the results of each culture condition. The dry cell weight method was used to perform the biomass analysis, while TLC-densitometry was used to analyze the kojic acid.

Optimization of the carbon and nitrogen sources

To determine the type of carbon and nitrogen to be used in the next stage of fermentation optimization, the carbon and nitrogen sources were optimized first. Glucose, sucrose, and fructose were selected as carbon sources while yeast extract, urea, and ammonium sulfate were used as nitrogen sources.

The dry cell weight yield indicated that, in general, fermentation culture using sucrose as a carbon source gave the highest average dry cell weight compared with other carbon sources. The mean dry cell weight for fermentation culture with sucrose as a carbon source was 12.669 g/L, but this only reached 10.807 and 10.349 g/L for glucose and fructose, respectively. Optimization of the nitrogen source in the medium showed that fermentation culture using yeast extract gave the highest average dry cell weight of 12.919 g/L compared with urea and ammonium sulfate that gave 10.138 g/L and 10.767 g/L, respectively. Table 3 shows that the largest dry cell weight (14.417 g/L) was seen with fermentation culture using sucrose as the carbon source and yeast extract as the nitrogen source (condition no. 4), while the smallest yield was 8.192 g/L, using fructose as a carbon source and ammonium sulfate as a source of nitrogen (condition no. 9).

Optimization of pH

Next, we optimized the pH used for fermentation. The acidity level was adjusted by adding a few drops of 1 N HCl into the fermentation medium until the desired pH was obtained. The optimization used pH 3.5, 4.5, and 5.5. Table 4 shows the results for the dry cell weight and total kojic acid content.

Aeration optimization

Aeration optimization was performed using four different volumes, 50, 100, 125, and 150 mL, in a 250 mL conical flask. Table 5 shows the data for the dry cell weight and total kojic acid.

Temperature optimization

Finally, the temperature was optimized by applying all the fermentation conditions obtained in the previous stages. Two temperature variations were performed: The first fermentation was carried out at room temperature in the fungus incubation room located on the third floor of the Faculty of Pharmacy University of Indonesia, with a mean temperature of approximately 30°C (range, 28–32°C). The second fermentation was carried out in a temperature-regulated incubator set at 35°C.

The results showed that fermentation at room temperature yielded a higher dry cell weight than fermentation at 35°C; however, fermentation at 35°C yielded higher kojic acid levels (Table 6).

Table 4: Kojic acid and dry cell weight of medium pH for optimization of fermentation culture

pH	Acquired area (AU)	Kojic acid (g/L)	Dry cell weight (g/L)
3.5	12,961.5	1.4994	13.603
4.5	14,868.1	1.7127	14.772
5.5	8117.3	0.9572	14.015

Table 5: Kojic acid concentration and dry cell weights for aeration optimization

Volume (mL)	Acquired area (AU)	Kojic acid (g/L)	Dry cell weight (g/L)
50	14,011.6	1.6169	13.542
100	14,282.7	1.6472	12.077
125	9767	1.1418	11.805
150	6022.1	0.7227	11.594

Table 6: Kojic acid concentration and dry cell weight of fermentation optimization temperature culture

Incubation Temperature (°C)	Acquired area (AU)	Kojic acid (g/L)	Dry cell weight (g/L)
30	5338.5	0.6462	8.552
35	6157	0.7378	8.057

Determination of sugar content in fermentation filtrate

The remaining sugar content in the filtrate was determined using sucrose as the selected carbon source. The analysis was performed using phenol sulfate and ultraviolet–visible spectrophotometry at wavelength of 490 nm [11].

After generating the calibration curve, the remaining sucrose content still presents in each sample was calculated. The sucrose concentration was measured using the fermentation filtrate from the optimization result that showed the highest kojic acid content, the optimum medium pH 4.5, and an incubation temperature of 35°C. The samples predicted to have a high absorption were diluted 1 in 1000.

Fermentation efficiency

The amount of product produced compared with the amount of the initial substrate used is known as the yield value (Yp/s). Tables 7 and 8 show the yield data for fermentation.

Discussion

Rejuvenation and inoculum preparation

The *A. oryzae* cultures obtained from Institut Pertanian Bogor Culture Collection were stored in the refrigerator to keep the culture dormant. To reactivate the culture for use in the preparation of the inoculum, rejuvenation of the cultures was performed in tilted tubes containing

PDA every 2 months for stock cultures, and 2 weeks for working cultures.

After incubation for 24 h at 180 rpm, the precultured form of pelletized *A. oryzae* cell biomass was yellowish-white. Throughout the study, precultures were used for each fermentation experiment. Fig. 2 shows there was a difference in the amount of biomass formed at the first preculture, conducted on March 27, 2017 (Fig. 2a), compared with the last preculture, conducted on May 27, 2017 (Fig. 2b), although both used the same medium with the same incubation conditions. The first preculture formed a greater amount of cell biomass compared with the last preculture. This may have been due to the age of the *A. oryzae* culture used in the inoculum preparation process. This is in accordance with previous studies that suggested that the age of the culture used to make a spore suspension in the preparation of inoculum has an effect on the preculture and fermentation [2].

Verification of kojic acid analysis method using TLC-densitometry

For the precision test, 80% concentration was not fulfilled because the coefficient of variation was outside the range of 5.84%. This may have been due to technical errors of implementation, such as at the time of bottling and elution that may have affected the repetition of levels obtained.

Quantitative analysis of the fermentation culture

Optimization of the carbon and nitrogen source

The determination of kojic acid levels using nine fermentation filtrates showed that the most significant difference between the highest concentration of kojic acid (1.5425 g/L) was obtained from filtrate no. 4 compared with the other filtrates. The results of dry cell weight and kojic acid content showed that medium containing 5% sucrose as a carbon source and 2.5% yeast extract as a nitrogen source produced the greatest biomass and kojic acid content. Unlike dry cell weights, the resulting kojic acid content was not only seen based on the carbon or nitrogen source alone but also on the physical and chemical properties of the combined carbon and nitrogen source used.

Optimum pH

Our results showed that both dry cell weight and the highest kojic acid content were produced by fermentation using the medium at pH 4.5, yielding 14.772 g/L dry cell weight, and 1.7127 g/L kojic acid. The
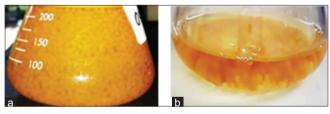

Fig. 2: (a and b) Preculture of *Aspergillus oryzae* after 24 h

Table 7: Determination of sucrose content in filtrate fermentation

Culture condition	Substrate levels (g/L)	Dilution	Absorbance	Sucrose levels (g/L)
pH 4.5	50	1000×	0.218	3.5510
35°C	50	1000×	0.404	6.5127

acidity level affects the function as well as the structure of the enzymes that play a role in the fermentation process. Production of kojic acid by fermentation increases with acidity, but acidity that is too strong can inhibit mold growth. However, this does not affect kojic acid fermentation using *A. oryzae*, since the optimal pH is highly dependent on the carbon and nitrogen source used [1].

Aeration optimization

The results showed the highest dry cell weight (13.542 g/L) using a volume of 50 mL, while the highest kojic acid content (1.6472 g/L) was produced by fermentation with a volume 100 mL. Kojic acid fermentation is aerobic; therefore, aeration is an important factor. Our results showed a decrease in dry cell weight formed when a smaller cavity remained in the conical flask. Higher amounts of oxygen in the 50 mL medium caused more mold to grow. While using a volume of 100 mL, cell growth was not as great as with 50 mL; however, the resulting kojic acid content was higher. This is because, with a volume of 50 mL, the substrate is used for cell growth rather than being converted into kojic acid. Fermentation using more than 100 mL of medium shows a decrease in dry cell weight, as well as the kojic acid level because the amount of available oxygen is not able to meet the demands of mold.

Temperature optimization

Our results showed that molds grow better grown at room temperature; however, the production of kojic acid was higher in the cultures incubated at 35°C. It was also noted that the average dry cell weight and kojic acid levels produced during temperature optimization were much lower than with the aeration optimization. This could have been caused by the quality of the inoculum or the preculture used for fermentation because the production of kojic acid and dry weight is influenced by the age of the culture used during the preparation of the inoculum, as well as the size of the inoculum [2].

Sugar levels in the fermentation filtrate

During fermentation, microbes convert the carbon source into cell biomass, and production of the product decreases the concentration of the carbon source in the medium. The total sugar remaining during fermentation was calculated to determine the total sugar left in the fermentation medium to measure mold productivity by comparing the amount of sugar consumed with the amount of product. The resulting glucose level in the fermentation filtrate was optimum at pH 4.5 and contained less glucose than the fermentation filtrate incubated at 35°C. This shows that the pH of the medium has a greater influence on glucose consumption by *A. oryzae* than the incubation temperature.

Fermentation efficiency

Based on the results shown in Table 8, fermentation of kojic acid using *A. oryzae* during the pH medium optimization stage showed a greater yield (0.0370 g/g) compared with fermentation performed at the temperature optimization stage (0.0170 g/g). These results show that the pH of the medium influences the fermentation efficiency to a greater extent than temperature. The efficiency of fermentation at each stage of fermentation increased due to previous conditions that were more optimal. This may be due to a decline in the quality or condition of mold culture shown by decreased mold growth in the rejuvenation and preparation of the previous inoculum performed.

CONCLUSION

The optimal medium for fermentation of kojic acid using *A. oryzae* uses sucrose and yeast extract as carbon and nitrogen sources, respectively, at pH 4.5. The optimal aeration and incubation conditions were used a

Table 8: Yield from the fermentation optimization results

Culture condition	Initial substrate level (g/L)	Sucrose (g/L)	Sucrose consumed (%)	Sucrose consumed (g/L)	Kojic acid (g/L)	Yield (g/g)
pH 4.5	50	3.5510	92.9	46.449	1.7127	0.0370
35°C	50	6.5127	86.97	43.4873	0.7378	0.0170

volume of 100 mL in a 250 mL conical flask, incubated at 35°C, obtaining the highest yield value of 0.0370 g/g.

CONFLICTS OF INTEREST

All authors have none to declare.

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