Reaktor

p-ISSN 0852 – 0798
e-ISSN 2407 – 5973

Acredited: SK No.: 60/E/KPT/2016
Website : http://ejournal.undip.ac.id/index.php/reaktor/

Reaktor, Vol. 18 No. 1, March Year 2018, pp. 51-56

Effect of Alkaloids on Lactic Acid Fermentation from Cocoa Pod Husk using Lactobacillus Plantarum

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(Received: March 20, 2018 ; Accepted: April 16, 2018)

Abstract

Cocoa Pod Husk (CPH), about 70-75% of the fresh cocoa fruit, is the biomass waste of cocoa industry. It is gained after the digestion process of cocoa.. CPH contains active compounds of alkaloids which are thought to be the inhibitors of the lactic acid fermentation process using microorganisms. This study aimed to produce lactic acid from CPH by studying the influence of alkaloids on the fermentation using Lactobacillus plantarum bacteria. The fermentation was carried out at 50°C in a 100 rpm orbital shaker for 48 h. The consumption of substrate (glucose), dry weight of the cell, and the production of lactic acid were evaluated. Microorganisms at various treatments was performed based on the parameters values of the kinetic models. These models showed that the presence of alkaloids altered the growth patterns of products from growth-associated products into mixed patterns because the products were formed during the slow growth and stationary phases. The value of maximum growth rate (μm) and substrate inhibition constant (K_s) of inhibitor addition were likely to remain constant at the values of 0.69 h^{-1} and 3.89 g/L respectively, as these parameters were unaffected by the addition of inhibitor.

Keywords: *alkaloids; cocoa pod husk; fermentation; lactobacillus plantarum*

How to Cite This Article: Irwanto, D., Wiratni, and Rochmadi, (2018), Effect of Alkaloids on Lactic Acid Fermentation from Cocoa Pod Husk using Lactobacillus Plantarum, Reaktor, 18(1), 51-56, http://dx.doi.org/10.14710/reaktor.18.1.51-56

INTRODUCTION

Cocoa (Theobroma cacao L.) is one of the plantation crops grown widely in Indonesia. Cocoa plantation area reaches 1.6 million hectares, which is widespread in Sumatra, Java, Kalimantan, Sulawesi, Bali, West and East Nusa Tenggara, Papua, Maluku and North Maluku. With the cacao production of 539.000 tons per year, Indonesia has become the number three biggest producer of cocoa in the world after Ivory Coast and Ghana, each of which has a production capacity of 1.3 million and 900 thousand tons per year Regional (Directorate of Plantations, 2015).

Cocoa pod husk (CPH) is a byproduct of cocoa beans processing. CPH has not been used optimally. Currently, CPH is only used as animal feed after being properly fermented. CPH weight is ranged between 70 to 75% by weight of the whole fruit so that every ton of cocoa will produce 700-750 kg CPH (Cruz *et al*., 2012).

CPH is potential lignocellulosic biomass. Lignocellulosic or coarse fibers are composed of

cellulose, hemicellulose, and lignin, which are tightly bound to each other to form a unity. CPH contains of 35.4% cellulose, 37.0% hemicellulose, 14.7% lignin, and 12,3% ash (Daud *et al.*, 2013). CPH has a complex chemical composition. One of the chemical compounds having antimicrobial property is polyphenol, with total polyphenol content of CPH is 12.6% (Sartini, 2013). Active polyphenolic compounds contained in the CPH has a role as antimicrobial, antiviral and antioxidant (Ariestanto *et al.*, 2012). CPH contains alkaloid active compounds that has antimicrobial properties, namely theobromine (3,7-dimethylxantine) of 0.4% (Sartini, 2013).

The conversion of CPH to lactic acid is carried out through a series of processes that include delignification to remove lignin, hydrolysis to break cellulose polymers into glucose, and fermentation of glucose into lactic acid using microorganisms. Challenges with complex raw materials such as CPH are the emergence of inhibitor compounds that can disrupt the process of fermentation of glucose into lactic acid.

CPH contains active compounds of alkaloids that are thought to be the inhibitors of the lactic acid fermentation process using microorganisms. This study aims to produce lactic acid from CPH by studying further the influence of alkaloids on fermentation process using Lactobacillus plantarum bacteria. Lactobacillus plantarum is a gram-positive, rod-shaped bacteria. Research on lactic acid production was performed using four different bacteria: Lactobacillus plantarum, Pediococus acidilactici, L.brevis, and Ln. mesenteroides. Lactobacillus plantarum produces the highest lactic acid (Gardner *et al.*, 2001). Lactobacillus plantarum is one of the lactic acid-producing bacteria with an anaerobic life tendency, the final result of fermentation is only lactic acid. Lactobacillus plantarum in the fermentation process can produce the highest lactic acid (Filya and Sucu, 2007). Fermented corn flour by Lactobacillus plantarum bacteria at 30°C with a pH of 6-6.5 yields L (+) type lactic acid with lactic acid production 2.02 mg/mL with 72 hours incubation time (Indrarti *et al.*, 2005).

The study included evaluation of the effects of alkaloids to determine the maximum concentration of alkaloids that can still produce lactic acid without significant inhibitory effects.

The kinetic evaluation of the reactions was performed using a mathematical model to find the reaction constants that gave results most similar to the research data. The reaction kinetics model used to calculate the constants is the Monod Model, with modification of specific microbial mortality components (Busairi, 2002) in Equations (1).

$$\mu = \frac{\mu_m . S}{K_s + S} X - k_d X \quad (1)$$

The rate of production of lactic acid using the Luedeking-Piret mixed association (Shuler and Kargi, 1992) in Equation (2).

$$\frac{dP}{dt} = k_1 \frac{dX}{dt} + k_2 . X \quad (2)$$

The glucose conversion rate (S) as the substrate (Khanna and Srivastava, 2005) in equations (3).

$$-\frac{dS}{dt} = k_3 \frac{dX}{dt} + k_4 . X \quad (3)$$

This study aims to produce lactic acid from CPH by studying further the influence of alkaloids on fermentation process using Lactobacillus plantarum bacteria. This process was conducted to determine the effect of the addition of alkaloids by analyzing through the comparison between the consumption of substrate (glucose), dry weight of the cell, and the production of lactic acid. Evaluation of the differences in the performance of microorganisms at various treatments was performed based on the parameters values of the kinetic models prepared for the case studied.

RESEARCH METHOD

Materials

The CPH was obtained from the cacao of the genus Trinitario, which was harvested in Januari 2015, in Yogyakarta province, Indonesia. The CPH in this study contains 23.36% hemicellulose, 24.51% cellulose, 30.46% lignin, and 0.34% alkaloids. All chemicals used in the study were analytical grade. Lactobacillus plantarum FNCC 0020 has been purchased from Microbiology Laboratory, Inter-university Center, Universitas Gadjah Mada, Indonesia.

Inoculum Preparation

One mL of Lactobacillus plantarum isolate in activated MRS media was inoculated in 10 mL MRS medium and incubated at 37°C for 24 hours. This isolate is ready to be grown on fermentation media.

Fermentation Media

Fermentation media was prepared from MRS media. After dissolved completely, the media was sterilised using an autoclave at 121°C for 15 minutes.

Fermentation Process

Fifty mL of fermented media sterilized was added 0.5 ml of starter Lactobacillus plantarum. Fermentation was carried out in a incubator shaker with a stirring rate of 100 rpm at 37°C pH 5 for 48 hours. Sampling at 0; 2; 4; 6; 8; 10; 12; 24; 36; and 48 hours.

This process was conducted to determine the effect of the addition of alkaloids by analyzing through the comparison between the consumption of substrate (glucose), dry weight of the cell, and the production of lactic acid. The batch fermentation was run with variation of alkaloid concentrations 1, 2, 2.5, 3, and 3.5 g/L and as a controller the reactor was run without the addition of any inhibitor. Evaluation of the differences in the performance of microorganisms at various treatments was performed based on the

parameters values of the kinetic models prepared for the case studied.

Data Analysis

Glucose analysis was performed by Nelson Somogyi method. This method uses a UV-Vis Spectrophotometer at a wavelength of 546 nm.

Lactic acid analysis was performed on filtrate of fermentation. The fermented filtrate has been added 1-3 drops of pp indicator titrated with 0.1 N NaOH solution which has been standardized with oxalic acid solution. Total lactic acid was calculated using equation (4).

$$Lactic\ acid = \frac{V_1 x N x BM}{V_2 x 1000} x100 \quad (4)$$

The cell dry weight (X) was calculated by dividing the dry weight obtained from the biomass analysis by the total volume of the solution used in the experimental sample using equation (5).

$$X = \frac{m_{L.Plantarum}}{Total\ volume\ of\ solution} \quad (5)$$

$m_{L.\ Plantarum}$ obtained from the reduction of paper weight after and before filtration.

Based on the above equation obtained experimental data glucose concentration (S), lactic acid concentration (P), and microbial concentrations (X). Parameters to be calculated using the mathematical equations that have been compiled are μ_{max}, K_s, k_d, k_1, k_2, k_3, and k_4.

RESULTS AND DISCUSSION

Effect of Inhibitor on Fermentation Process

The effect of alkaloids on lactic acid fermentation using Lactobacillus plantarum bacteria at 50°C, agitation of 100 rpm in incubator shaker then analyzed by comparison between substrate consumption (glucose), dry cell weight, and lactic acid production. Evaluation of kinetics parameters for fermentation without inhibitor, fermentation with alkaloid inhibitor, shown in Figures 1 to 6.

In Figure 1, it can be seen that the production of lactic acid follows the growth of microbes. At 12 to 24 hours, the microbial growth slope is considerable, and a substantial increase in lactic acid production. This suggests that the production of lactic acid without inhibitors follows a growth associated product.

In Figure 2 to 4, the pattern of lactic acid formation follows a microbial growth pattern at the beginning of the experiment. At 0-10 hours, when microbial growth is slow, the production of lactic acid is small. after which microbial growth has no effect on product formation. At 12 to 48 hours of rapid microbial growth, the production rate of lactic acid is relatively unchanged. This suggests that the presence of inhibitors alters the trend of product growth patterns from associated patterns in fermentation without inhibitors, to a mixed pattern. The glucose consumption at the beginning of the experiment is mostly allocated for the microbial adaptation process in fermentation.

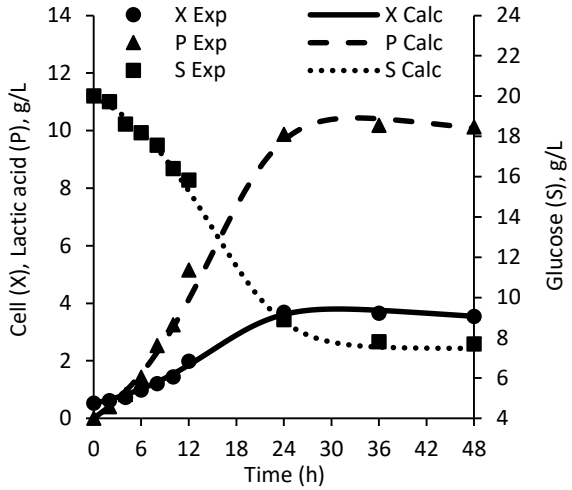

Figure 1. Glucose, dry cell weight, and lactic acid concentration in fermentation without inhibitor

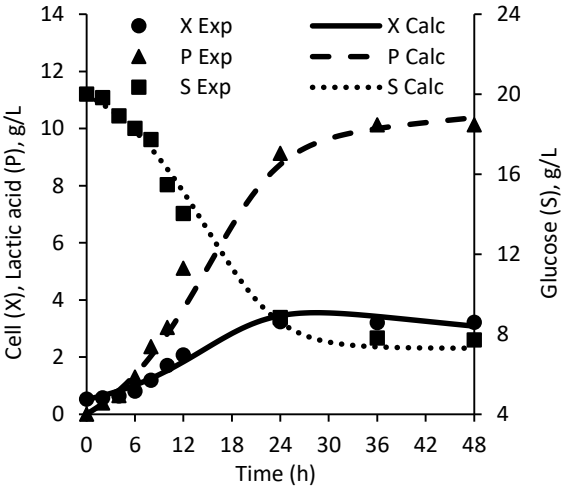

Figure 2. Glucose, dry cell weight, and lactic acid concentration in fermentation (1 g/L alkaloids)

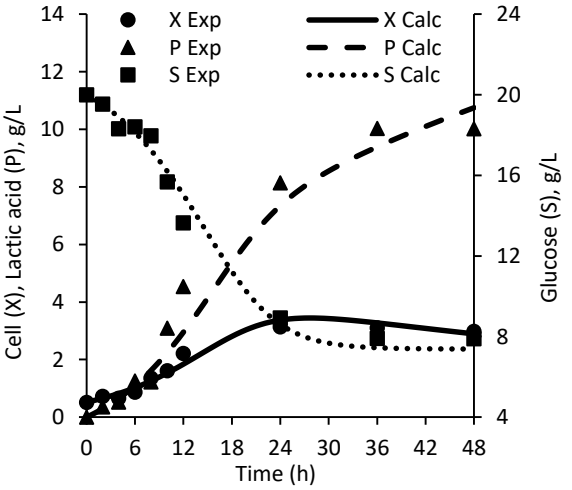

Figure 3. Glucose, dry cell weight, and lactic acid concentration in fermentation (2 g/L alkaloids)

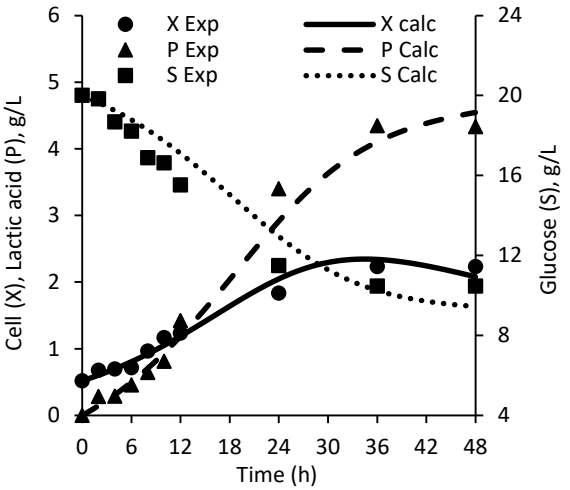

Figure 4. Glucose, dry cell weight, and lactic acid concentration in fermentation (2.5 g/L alkaloids)

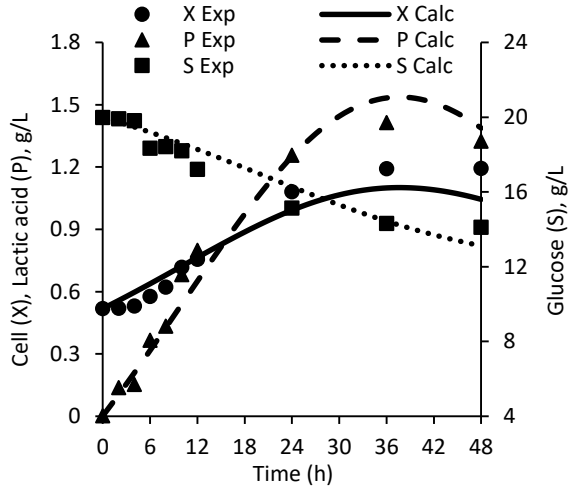

Figure 5. Glucose, dry cell weight, and lactic acid concentration in fermentation (3 g/L alkaloids)

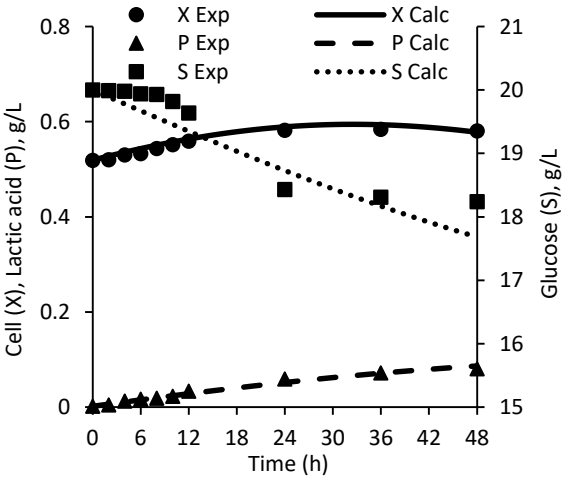

Figure 6. Glucose, dry cell weight, and lactic acid concentration in fermentation (3.5 g/L alkaloids)

In Figure 5 to 6, on the addition of 3 g/L inhibitor shows the difference with the previous condition of inhibitor condition ≤2.5 g/L where the glucose consumption is relatively low, microbial growth is low and the resulting product is relatively small. This is likely due to the adjustment of microbes in new environments where adaptations required by microbes in new environments with inhibitors require greater energy because the presence of inhibitors is enough to inhibit microbial growth.

Based on the calculation results obtained data of lactic acid fermentation reaction constants using Lactobacillus plantarum with the addition of alkaloids in Table 1.

Table 1. The kinetics constant(μ_{max}, K_s, k_d)of the lactic acid fermentation reaction with Lactobacillus plantarum and the addition of alkaloids using the Monod model

Alkaloids ($g\ L^{-1}$)	μ_{max} (h^{-1})	K_s ($g\ L^{-1}$)	k_d ($g\ h^{-1}$)
0	0.68	3.87	0.45
1	0.68	3.87	0.45
2	0.70	3.90	0.47
2.5	0.70	3.90	0.51
3	0.70	3.90	0.55
3.5	0.69	3.88	0.57

In Table 1, the values of maximum growth rate (μ_m) and substrate inhibition constant (K_s) obtained in each variation of inhibitor addition are likely to remain unaffected by the addition of inhibitor concentration. The values of μ_m and K_s are only influenced by the type and initial concentration of substrate, microbial type, and operating conditions (Charalampopoulos *et al*., 2009).

Inhibitors added to the fermentation process do not affect the two constants, so they can be considered the same for all variations. Comparison of μ_m and K_s values is presented in Table 2, lactic acid fermentation using Lactobacillus plantarum yields constants in nearly equal range. μ_m has a value similar to other studies in the range of 0.6 and K_s has various values in the range 2.4-3.8.

Specific death rate constant (k_d) values tend to remain up to 2 g/L alkaloid concentrations and begin to rise from the addition of 2.5 g/L alkaloids to 3.5 g/L. The value of k_d at various concentrations of alkaloids indicates that the higher the alkaloid concentration, the faster the rate of bacterial mortality.

Based on Table 3, the value of k_1 which is the coefficient of product formation associated with growth tends to change along with the addition of inhibitor. The coefficient value of product formation not associated with growth (k_2), the constant on the glucose consumption equation (k_3 and k_4) tends to remain unaffected by the addition of inhibitor concentration. These results indicate that the consumption of glucose by bacteria remains constant despite the addition of inhibitors.

Table 2. Comparison of kinetics constant of lactic acid fermentation reaction using Lactobacillus plantarum

Parameter	This research	Charalam-popoulos *et al.* (2009)	Sharma and Mishra (2014)
μ_m	0.69	0.703	0.62
K_s	3.89	2.445	-

Table 3. The kinetics constant (k_1, k_2, k_3, k_4)of the lactic acid fermentation reaction with Lactobacillus plantarum and the addition of alkaloids using the Monod model

Alkaloids (g L^{-1})	k_1 (g g^{-1})	k_2 (g g^{-1} h^{-1})	k_3 (g g^{-1})	k_4 (g g^{-1} h^{-1})
0	3.02	0.01	3.35	0.02
1	2.49	0.03	3.40	0.03
2	1.70	0.05	3.40	0.04
2.5	1.34	0.03	3.40	0.06
3	0.64	0.00	3.40	0.12
3.5	0.21	0.00	3.37	0.08

CONCLUSION

The maximum growth rate (μ_m) and substrate inhibition constant (K_s) obtained on each variation of inhibitor addition were likely to remain constant at the values of 0.69 h^{-1} and 3.89 g/L respectively, as these parameters were unaffected by the addition of inhibitor. In the addition of alkaloid the value of k_d remains relatively constant up to a concentration of 2 g/L and increases from the addition of 2.5 g/L to 3.5 g/L. k_d is the specific death rate constant. The results of k_d values at various concentrations of alkaloids indicate that the higher the concentration of alkaloids the faster the rate of bacterial death. The value of k_1 which is the coefficient of product formation associated with growth tends to change with the addition of inhibitor. The values of k_2, k_3, k_4 are fixed and unaffected by the addition of inhibitor concentration.

NOTATION

K_s = inhibitory constants by substrate, g/L
k_1 = coefficient of product formation associated with growth, $g.g^{-1}$
k_2 = coefficient of product formation not associated with growth, $g.g^{-1}.h^{-1}$
k_3 = constants on the glucose consumption equation, $g.g^{-1}$
k_4 = constants on the glucose consumption equation, $g.g^{-1}$ h^{-1}
k_d = specific mortality rate constants, g/h
μ = microbial growth rate, g/h
μ_m = maximum growth rate, h^{-1}
P = lactic acid concentration, g/L

S = glucose concentration, g/L
t = time, h
X = microbial concentrations, g/L
V_1 = volume of NaOH for titration, mL
V_2 = volume of titrated filtrate, mL
N = normality of NaOH solution

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