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Stability and Specific Substrate of Lipase from Microbes in Coconut Pulp Media

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A B S T R A C T

One source of lipase that is considered potential is from microbes because microbes can multiply rapidly. The advantage of microbes is being able to adapt quickly to the environment, including growing media. Coconut pulp still contains fat with lauric acid as the dominant fatty acid. The purpose of this study was to obtain temperature and pH stability and lipase specific substrate from microbes grown on coconut pulp media. To achieve this goal a partial purification of lipase was carried out with ammonium sulfate. The obtained lipases were tested for temperature and pH stability by hydrolysis activity. Specific substrates were determined by testing esterification activity using several types of fatty acids. The results showed that at the saturation level of 60% ammonium sulfate produced lipase with the highest level of activity. The resulting lipase has a stability of more than 90% at 40°C and pH 6 is the most stable activity. Substrates that show high activity are lauric acid and oleic acid.

Introduction

The need for lipase in the world continues to increase and the price is expensive, especially for lipases that have specific properties on the substrate or regiospecific (1) and (2). Lipases catalyze the breakdown of ester bonds in fat to produce fatty acids and glycerol (3). Lipase is also able to catalyze the esterification reaction at low water levels (4). Lipase is much needed for research and industrial needs such as food, pharmaceuticals, cosmetics, detergents and others so that it becomes an opportunity for commercial production of lipase. According to Ferraz *et al.*, (5) lipase has a different character so for its application it is necessary to know the character of the lipase. The most influential factor in its application is its stability against temperature and pH. One very potential source of lipase is microbes because microbes can be bred quickly. Bacteria and molds are widely used as a source of lipase,

such as *Aspergillus niger, Candida cylendracea, Mucor miehei, Penicillium camemertii and Pseudomonas cepacia*. Lipases from microbes usually have a high affinity for the dominant fatty acids in the growth media so that specific lipases will be produced. Lipases from *Aspergillus niger* MYA have high affinity for long chain fatty acids (C18) (6), whereas pancreatic lipases prefer PUFA. Lipases from Candida rugosa and Thermomyces lanuginose have higher activity than pancreatic lipases (7).

Coconut pulp has been considered as food processing waste after extracting coconut milk and is generally used as animal feed. In addition to utilizing coconut milk, coconuts are generally used as a source of vegetable fat. According to Kwon *et al.,* (8) mature coconut meat which has a water content of 40.9%, has a protein content of 3.8% and fat of 35.2%. Marina *et al.,* (9) conducted an analysis of commercial coconut oil sold in Malaysia and Indonesia. Lauric acid (C-12) is a fatty acid with the largest proportion ranging from 46.64 to 48.03%, followed by myristic acid (C14) 16.23-18.90%. If the coconut pulp is used as a medium for microbial growth, it is hoped that lipase can be isolated from microbes that have a specific substrate. The purpose of this study was to determine the temperature and pH stability and lipase specific substrate from microbes that grow on coconut pulp media.

Materials and Methods

Research Stages

The order of research to be carried out in outline is as follows

The obtained lipase is partially purified using ammonium sulfate.

The partially purified lipase was tested for its esterification activity using several types of fatty acids with glycerol to determine its specific substrate.

Stability of lipase temperature and pH is determined by testing the lipase hydrolysis activity at various temperatures and pH.

Time and Place

The study was conducted by the laboratory of the Faculty of Agricultural Technology, UPT Integrated Laboratory of Biotechnology and Bioscience and Analytical Chemistry Laboratory of Udayana University, Bali. The time for conducting research will be from June 2019 to October 2019

Raw Materials and Chemicals

Coconut pulp is made from coconuts taken from a traditional market in Denpasar, Bali Province. Chemicals such as isooctan, Na_2HPO_4, NaH_2PO_4, ammonium sulfate, glycerol, EDTA, Cu-acetate, pyridine from Merck Germany. Extremely high purity standard chemicals such as olive oil, fatty acid kits from Sigma Co., St. Louis, MO, USA.

Tools

Mortal, digital pH meter (TOA HM 605), magnetic stirrer, centrifuge (Herolap, Germany), water bath shaker (Shel-Lab), test tube, vortex, thin cotton cloth, Analytical Balance (Sartorius), Spectrophotometer UV-Vis (*Genesys* 10S UV-Vis),

Research procedure

Microbial growth on coconut pulp

Grated coconut flesh is then extracted to take coconut milk to obtain coconut pulp. Coconut pulp added tempe then covered with gauze and left at room temperature for 5 days. Coconut pulp that has been overgrown with microbes is extracted to obtain lipase

Preparation of modified enzyme extraction (10)

The enzyme was isolated at 4° C for all experiments carried out. The coconut palm pulp is homogenized for 10 minutes in 0.15 M phosphate buffer consisting of 0.6 M sucrose, 1 mM EDTA 10 mM KCl and 1 mM $MgCl_2$. Homogenate is centrifuged for 40 minutes at 10,000 rpm. The supernatant layer is taken for testing dissolved protein, hydrolysis activity, lipase esterification

Lipase Fractionation with Ammonium sulfate

Fractionation of lipases with ammonium sulfate was carried out by following the Doonan method (11).

Ammonium sulfate is added to the active extract with varying concentrations (20-40%, 40-60% and 60-80%). The addition of ammonium sulfate starts from the lowest concentration of 20%. Extract as much as 100 ml in a stirer at 4^0C and then centrifuged 5000 g for 30 minutes. The precipitate is separated and the supernatant is added to the ammonium sulfate to a saturation concentration of 40%. Centrifuged 5000 g for 30 minutes, the supernatant was saturated again with ammonium sulfate to 60%. Return centrifuged for 30 minutes at 5000 g. The supernatant was separated and saturated again at a concentration of 80%. Then centrifuged at 5000 g for 30 minutes. The pellet and the supernatant from the results of each concentration were tested for their activity by the method of Marseno (1998) (12) and dissolved protein by the Lowry-Folin method(13).

Lipase Hydrolysis Activity (12)

60% olive oil in isooktan added 10% crude lipase, divortex for 5 minutes. incubated at 37°C for 1 hour. After the incubation is immediately put into ice for a few moments. Samples were taken 5 ml and added 0.7 mL Cu pyridine acetate pH 6, gojok solution for 90 seconds by hand. After that the solution was centrifuged at 2000 rpm for 10 minutes, then absorbed at 715 nm wavelength

Lipase Specific Substrate

Crude lipase concentration of 5% plus 60 µmol / ml various fatty acids (myristic, lauric, oleic, palmitic, stearic) shaked for 10 minutes. Glycerol 20 µmol / ml is added to the mixture as soon as possible and incubated at 40°C for 24 hours. After the incubation is immediately put into ice for a few moments.

Furthermore the remaining fatty acids were analyzed using the method of Marseno, et.al (12) samples were taken 300 µL and added 2.7 mL isooktan and 0.6 mL Cu pyridine acetate pH 6, corner the solution for 90 seconds by hand.

After that the solution was centrifuged at a speed of 2000 rpm for 5 minutes, then absorbed at a wavelength of 715 nm. The highest amount of fatty acid that is etched shows the specific substrate.

Temperature Stability Test

Temperature stability is determined by heating the lipase solution at a temperature variation of 30, 40, 50 and 60°C for 1 hour then cooled. Residual activity is measured by the same method at its optimum temperature conditions. The reaction time for activity testing is done for 1 hour.

pH Stability Test

The pH stability test was carried out following the method of Mayordomo et.al (14). 0.15 ml lipase solution plus 0.15 M universal buffer with a pH of 4-10 was 1.35 ml and was incubated for 30 minutes. Furthermore lipase

hydrolysis activity was tested under optimum pH conditions.

Results and Discussion

Lipase fractionation with ammonium sulfate

The results of lipase precipitation from microbes on coconut pulp media using ammonium sulfate solution at varying concentrations resulted in different specific activities and recovery (Table 1).

The highest specific activity was obtained at a 60% concentration level. Likewise the highest recovery rate was obtained at 60% ammonium sulfate concentration. Based on the results of specific activities and recovery of lipase, the precipitation results were chosen at 40-60% ammonium sulfate concentration for further testing.

Temperature Stability

The results of microbial lipase stability test on coconut pulp in the temperature range of 30-55^{0}C with an interval of 5°C showed that up to 40°C was still stable (Table 2). Lipase activity at a temperature of 45°C has decreased and at a temperature of 55°C only 72.27%, meaning that microbial lipase on coconut pulp has stability at a temperature of 40°C. Illanes *et al.,* (2008) (15) state that high temperatures can cause the breakdown of non-peptide bonds to break so that the lipase structure changes resulting in decreased lipase activity. The stability of microbial lipase is similar to that of peanut in the temperature of 40°C, its activity is still stable, while at 50°C there is a drastic decrease in activity (Sanders and Pattee, 1975).(16)

Table.1 Specific Activities and Recovery of Lipase Resulting from Precipitation with Ammonium Sulfate

Ammonium Sulfate concentration	Activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg protein)	Total Activity (U)	Recovery (%)
0	0.542 ± 0.034	2.321 ± 0.527	0.241 ± 0.069	75.406 ± 5.625	100 ± 000
20	0.473 ± 0.006	0.559 ± 0.081	0.846 ± 0.016	1.419 ± 0.173	1.884 ± 0.028
40	0.482 ± 0.03	0.363 ± 0.025	1.333 ± 0.083	1.447 ± 0.010	1.939 ± 0.286
60	0.741 ± 0.108	0.384 ± 0.147	2.139 ± 0.102	4.03 ± 0.073	5.398 ± 0.664
80	0.314 ± 0.036	0.955 ± 0.486	0.388 ± 0.235	1.568 ± 0.179	2.117 ± 0.536

Table.2 Lipase Activity of Ammonium Sulfate Precipitation from 40-60% in Temperature Stability Test

Temperature (^{0}C)	Activity (U/ml)	Persen Activity
30	0.988 ± 0.001	99.62 ± 0.317
35	0.984 ± 0.007	99.23 ± 0.231
40	0.972 ± 0.009	97.99 ± 0.455
45	0.884 ± 0.055	89.09 ± 5.193
50	0.858 ± 0.062	86.46 ± 5.860
55	0.717 ± 0.036	72.27 ± 3.916
Control	0.992 ± 0.004	100 ± 0.003

Table.3 Lipase Activity of Ammonium Sulfate Precipitation from 40-60% in pH Stability test

pH	Aktivity (U/ml)	Persen Activity
3	1.666 ± 0.068	69.428 ± 0.549
4	2.065 ± 0.047	86.067 ± 0.908
5	2.277 ± 0.072	94.893 ± 0.147
6	2.387 ± 0.032	99.503 ± 1.957
7	2.231 ± 0.101	92.955 ± 1.121
8	1.809 ± 0.018	75.456 ± 3.265
Optimum	2.399 ± 0.079	100.003 ± 0.017

Table.4 Lipase Activity Resulting from Ammonium Sulfate Precipitation of 40-60% in the Substrate test

Fatty Acid	Esterification Activity
C 8 : 0	0.086 ± 0.011
C12 : 0	0.277 ± 0.011
C14 : 0	0.152 ± 0.028
C16 : 0	0.037 ± 0.005
C18 : 0	0.044 ± 0.006
C18 : 1	0.187 ± 0.090

pH Stability

The stability of microbial lipase on coconut pulp media to pH was carried out in the pH range 3-8.

The difference in pH is greater than the optimum pH causing lower stability (Table 3). At pH 6 the activity is the most stable, this corresponds to the optimum pH. The activity is relatively stable in the pH range 5-7, while at pH 4 and 8 there is a decrease in activity of about 14%.

At extreme pH it causes large structural changes making it difficult to return to the original structure after being returned to its optimum pH.

Specific Substrate

The results of esterification activity testing using various fatty acids obtained the highest activity in lauric acid and followed by oleic acid (Table 4). This means that microbial lipase is not specific to the length of the fatty acid chain but more specific to the type of fatty acid. This is in accordance with the types of fatty acids in coconut oil which are lauric acid and followed by oleic acid. According to Lotti and Alberghina (2007) (17), the selective nature of lipase to the substrate is strongly influenced by the shape and size of the pocket (Hole) on its active side.

The results of microbial lipase precipitation were obtained at a concentration of ammonium sulfate 60 percent, has a temperature stability of 40°C and a pH of 6. Lipase has specific properties for the types of fatty acids namely lauric and oleic acids.

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