Biota
Biologi dan Pendidikan Biologi
http://biota.ac.id/index.php/jb
DOI: https://doi.org/10.20414/jb.v13i2.245

Research Article

The Ability of Soil *Candida albicans* Secreted Potential Protease and Lipase

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Abstract

Candida spp secreted a different kind of extracellular enzymes. Protease and lipase are the enzymes that are commonly used in the food and pharmaceutical industries. This study aimed to examine protease and lipase activity of *Candida tropicalis* isolated from the soil of the Medicinal Plant Garden of the Faculty of Pharmacy, Universitas Jenderal Achmad Yani, Indonesia. Candida isolate was subjected to the fermentation process to obtain bioactive metabolites. The result was analyzed using ANOVA within a 5% interval of confidence, continuing with PostHoc. The result showed the *Candida tropicalis* metabolite giving the best proteolytic index value (0,6556 ± 0,0090) U/mL. The metabolite isolate of *Candida tropicalis* had the highest activity, amounting to 5,776 ± 0,495 U/mL. The best results of lipolytic index value (0,394 ± 0,053) U/mL. The *Candida tropicalis* metabolites produced the highest lipase enzyme after nine days of fermentation in 5.2917±0,0167 U/mL.

Keywords: *Candida tropicalis*; fermentation; protease; lipase.

1. Introduction

Microorganisms are the most widely used source of enzymes compared to plants and animals. As a source of enzymes, microbes are more beneficial because of their rapid growth and can grow on inexpensive substrates more easily. The results are improved by regulating growth conditions and genetic engineering (Oktavia et al., 2018). Enzymes are macromolecules that can increase the rate of a chemical reaction. Enzymes can act as regulators that cause various metabolic functions to be coordinated harmoniously. Besides serving as a metabolic system for living things, enzymes can also be widely used in various research and industry fields. They require low energy, are selective to specific substrates, are biocompatible, biodegradable, and are obtained from renewable sources (Y. Liu & Chen, 2016). Extracellular enzymes produced by microbes are used to break down complex organic nutrient material to be transported into cells as a source of nutrition (Subagiyo et al., 2017).

Candida spp can produce various extracellular enzymes that are hydrolytic. The enzymes that are released can be virulent, giving rise to the pathogenesis of Candida. (Pandey et al., 2018; Zuza-Alves et al., 2017). Yeast can degrade polymers that cannot be absorbed by producing extracellular enzymes (Ramnath et al., 2017). *Candida tropicalis* is a type of yeast that can carry out two main processes in converting cellulose material into ethanol. The hydrolysis of cellulose degradation to glucose and fermentation of converting glucose into bioethanol (Hermansyah et al., 2016). Besides, *Candida tropicalis*

is also widely analyzed in the production of xylitol. *Candida tropicalis* can convert xylose to xylitol, which is used as an antibacterial *Streptococcus mutan* (Mahyati, 2017).

Protease is an enzyme that can hydrolyze proteins into simpler compounds such as peptides and amino acids. Microbial proteinase has potential for detergents, leather, silver recovery, milk, baking, beverages, and the pharmaceutical industry. This hydrolytic enzyme is efficiently involved in the food industry. That increases the nutritional value, digestibility, palatability, and taste, reduces allergies and manages domestic and industrial waste (Soeka & Sulistiani, 2017).

Lipase catalyzes the hydrolysis of fat in water and lipids' interface and carries various biotransformation reactions under low water conditions. Sources of lipases include microbial lipases, bacteria, and fungi (Gupta et al., 2015). Lipase is a hydrolytic enzyme widely used in industrial fields and potentially in the medical field (Bharathi et al., 2019). Microbial activity in lipid degradation is related to these microbes' ability to produce a lipase enzyme called lipolytic. Lipase is a soluble enzyme hydrolyzing triacylglycerol to free fatty acids and glycerol (Bestari & Suharjono, 2015). Research related to Candida tropicalis activity of the enzyme is a phospholipase isolated from the blood of patients in the intensive care unit Research on Candida tropicalis isolated from the soil to produce the extracellular enzyme with the addition of nutrients to the fermentation substrate has not been available

Based on the description above, research has been carried out to determine the extracellular enzyme activity of *Candida tropicalis* isolates by optimizing the fermentation substrate to obtain metabolites.

2. Material and Method

In this study, we were using a sample of *Candida tropicalis*, which was successfully isolated from the soil of the Medicinal Plant Garden of the Faculty of Pharmacy, Universitas Jenderal Achmad Yani, Cimahi. A total of 5 grams of soil was immersed in 50 mL PDB media, then homogenized and incubated for three days. Microbes that grow on PDB media are separated by repeating cultures on PDA media by incubating for three days at 25^{o}C. The isolation results obtained several isolates. The cultivation results got one isolate with the same macroscopic characteristics every time it was cultivated on PDA media. The process was repeated until a single isolate was obtained. These single isolates are genotypically identified. Identification was carried out on a single isolate at LIPI Bogor. Identification of isolates was carried out molecularly based on genetic analysis of 26S ribosomal DNA.

The fermentation is then performed using PDB media with an inoculum of 10% v/v of the fermentation media. Growth kinetics of isolates were analyzed by growing isolates in fermentation media with an incubation period of 14 days in an incubator at 30^{o}C and shaking at 125 rpm for 1-2 hours per day before measurement. Observations were made every 24 hours for 14 days by measuring the absorption at a wavelength of 640 nm (Weerasekera et al., 2016; A et al., 2017)

The cultivation results are dissolved in a NaCl solution, and the transmittance is measured at 25% with a visible spectrophotometer at a wavelength of 640 nm. A total of 10% v/v culture was inoculated on substrates containing different fermented nutrient compositions and subsequently incubated in an incubator at 30°C with a shaking speed of 125 rpm for 1-2 hours on the 3rd, 5th, and 13th days of fermentation. The results were centrifuged at 4000 rpm for 30 minutes. A supernatant is taken then tested the activity of the microbial test and enzyme potential test. The nutrients added to see its ability to help the growth of *Candida tropicalis*. Nutrient composition added to the fermentation substrate used as follows in Table 1:

Table 1. Nutritional composition of the fermented substrate

No	Composition of substrate	C (g/100mL)	T1 (g/100mL)	T2 (g/100mL)	T3 (g/100mL)
1	*Potato Dextrose Broth* (PDB)	2,4	2,4	2,4	2,4
2	KH_2PO_4	-	1	1,5	2
3	$MgSO_4$	-	0,5	0,5	0,5

The protease test using the Biuret test using 1 mL supernatant metabolite *Candida tropicalis* isolates were taken and added 1 mL of 10% sodium hydroxide (NaOH) solution to the test tube, stirring vigorously. Two drops of 0.1% Copper Sulfate (CuSO4) solution was added and mixed well. If no color arises, add a few drops of a solution of Copper Sulfate (CuSO4) to form a purple tint. The skim milk solution 1%(containing protein) is used as a positive comparison biuret test (Liu & Pan, 2017).

Proteolytic tests were performed using Skim Milk Agar (SMA) media, namely PDA media plus 2% skim milk. Media that has been perforated using a perforator. Supernatant metabolites of *Candida tropicalis* isolates were inoculated in the perforated media, then incubated for three days at 25°C. The proteolytic activity of microorganisms grown on Skim Milk Agar (SMA) media is indicated by the visible area of the transparent area around the colony formed. Furthermore, the clear zones that appear are measured in diameter and calculated semi-quantitative to obtain a proteolytic (Duanis-Assaf et al., 2020). The test was carried out with four repetitions. The result was analyzed using analysis of variance (ANOVA) within a 5% interval of confidence, continue with PostHoc.

The proteolytic index can be calculated as follow.

$$\text{Proteolytic Index} = \frac{\text{DZ} - \text{DK}}{\text{DK}}$$

DK: Diameter of the microorganism colony
DZ: Diameter of the clear zone

The protease test was carried out by adding 1 mL of the supernatant metabolite of *Candida tropicalis* isolate into 3 mL of 0.5% casein solution, then incubated at 37°C for 30 minutes. Enzyme activity was stopped by adding 5 mL of 5% solution of Trichloroacetic

Acid (TCA), then incubated at room temperature for 30 minutes, then centrifuged for 30 minutes to obtain a supernatant. Protease activity was determined by measuring the fermented supernatant's absorption value using a UV-Visible spectrophotometer at a wavelength of 280 nm (Ozturkoglu-Budak et al., 2016).

Lipolytic tests were performed using Potato Dextrose Agar (PDA) containing 2.5% Tween-80, 5% pure olive oil, and 0.01% methyl red. Media that has been perforated using a perforator. Supernatant metabolites of *Candida tropicalis* isolates were inoculated in solid media, then incubated for three days at 25^{o}C. The lipolytic activity of microorganisms is shown by a yellowish-white area considered a clear zone that appears around the formed colonies. Furthermore, the clear zone is measured in diameter and calculated quantitatively to obtain the lipolytic index (Bestari & Suharjono, 2015). The test was carried out with four repetitions. The result was analyzed using analysis of variance (ANOVA) within a 5% interval of confidence, continue with PostHoc.

The lipolytic index can be calculated as follow.

$$Lipolytic\ index = \frac{DZ - DK}{DK}$$

DK: Diameter of the microorganism colony
DZ: Diameter of the clear zone

Lipase activity was measured quantitatively by 1 mL supernatant metabolite of *Candida tropicalis* isolates plus 1 ml of sterile olive oil and 2 mL of phosphate buffer pH seven, then incubated at 40oC for 30 minutes. After incubation, 5 mL of acetone-ethanol (1: 1) solution is added and given two drops of phenolphthalein (PP) indicator then titrated with 0.05 N sodium hydroxide (NaOH) until it starts to turn pale pink (Bestari & Suharjono, 2015). The test was carried out with three repetitions.

Lipase activity can be calculated as follows.

$$\text{Lipase activity (U/mL)} = \frac{(\text{A} - \text{B})\ \text{x N NaOH x } 1000}{\text{VE x } 30}$$

A: Volume (mL) NaOH for sample titration
B: Volume (mL) NaOH for blank titration
1000: Convert mmol to µmol
30: incubation time
VE: Total supernatant volume

3. Results and Discussion

3.1. Results

Based on the research process carried out, protease and lipase test data can be seen in the following table.

Table 1. The results of qualitatively from the protease activity

No	Sample	Time of Fermentation (days)	Biuret reagent
	skim milk 1%		Purple
1	Sample 1(Metabolite + PDB)	3	Faded purple
2		5	Faded purple
3		9	Faded purple
4		13	Faded purple
5	Sample 2 (Metabolite + PDB + KH_2PO_4 1% + $MgSO_4$ 0,5%)	3	Faded purple
6		5	Faded purple
7		9	Faded purple
8		13	Faded purple
9	Sample 3 (Metabolite + PDB + KH_2PO_4 1,5% + $MgSO_4$ 0,5%)	3	Faded purple
10		5	Faded purple
11		9	Faded purple
12		13	Faded purple
13	Sample 4 (Metabolite + PDB + KH_2PO_4 2% + $MgSO_4$ 0,5%)	3	Faded purple
14		5	Faded purple
15		9	Faded purple
16		13	Faded purple

Semi-quantitative testing of the protease index uses a test substrate in the form of Potato Dextrose Agar (PDA), which has been added with 1% skim milk. The results of the index of protease showed in Figure 1. The protease activity's quantitative testing carried out using the Kunitz chemical method with a UV-vis spectrophotometer and casein as the substrate shown in Figure 2. The semi-quantitative testing of lipase use PDA media that has been added methyl red, tween, and olive oil showed in Figure 3. The results quantitative of the lipase activity showed in Figure 4.

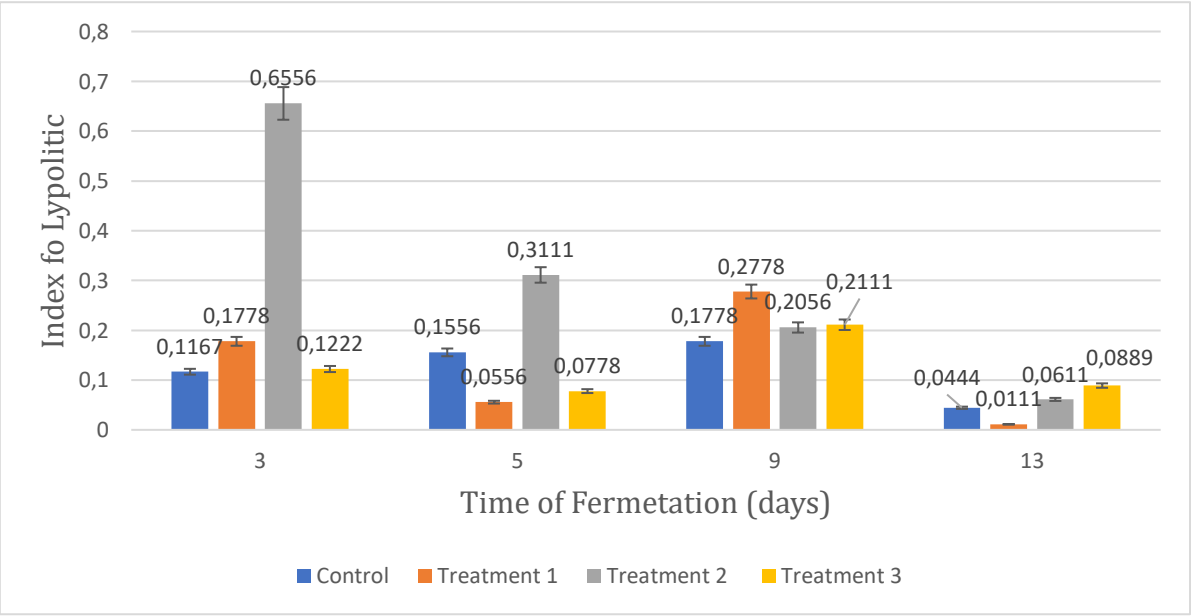

Figure 1. The results of the index of protease

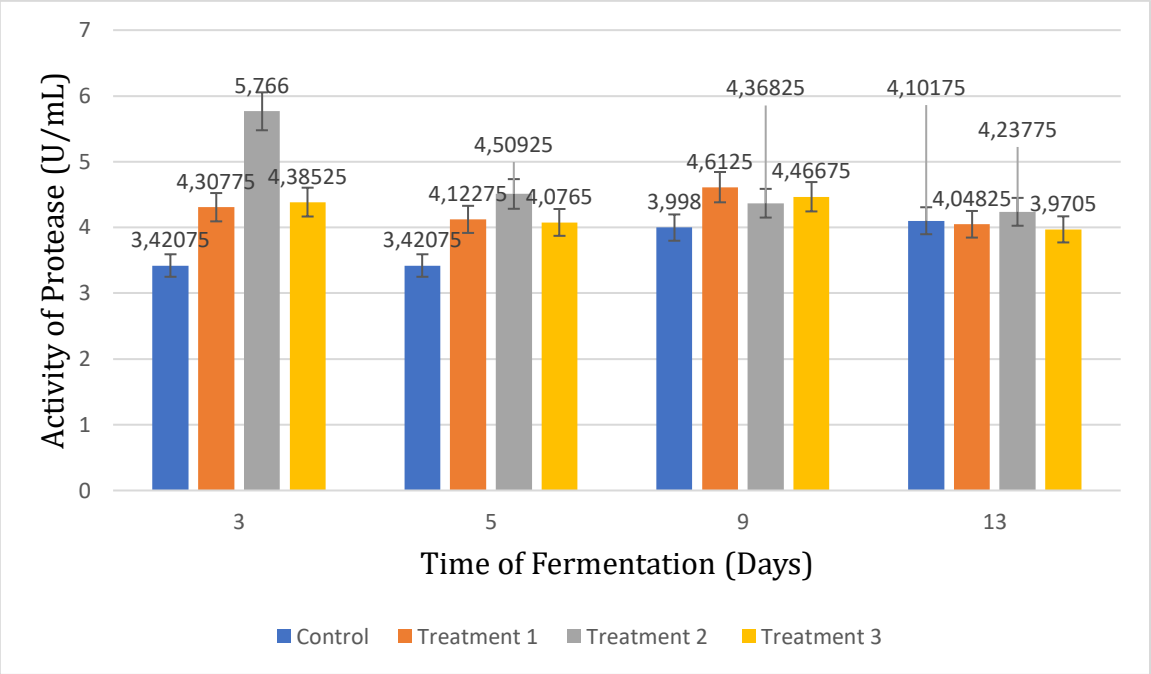

Figure 2. The results of the protease activity

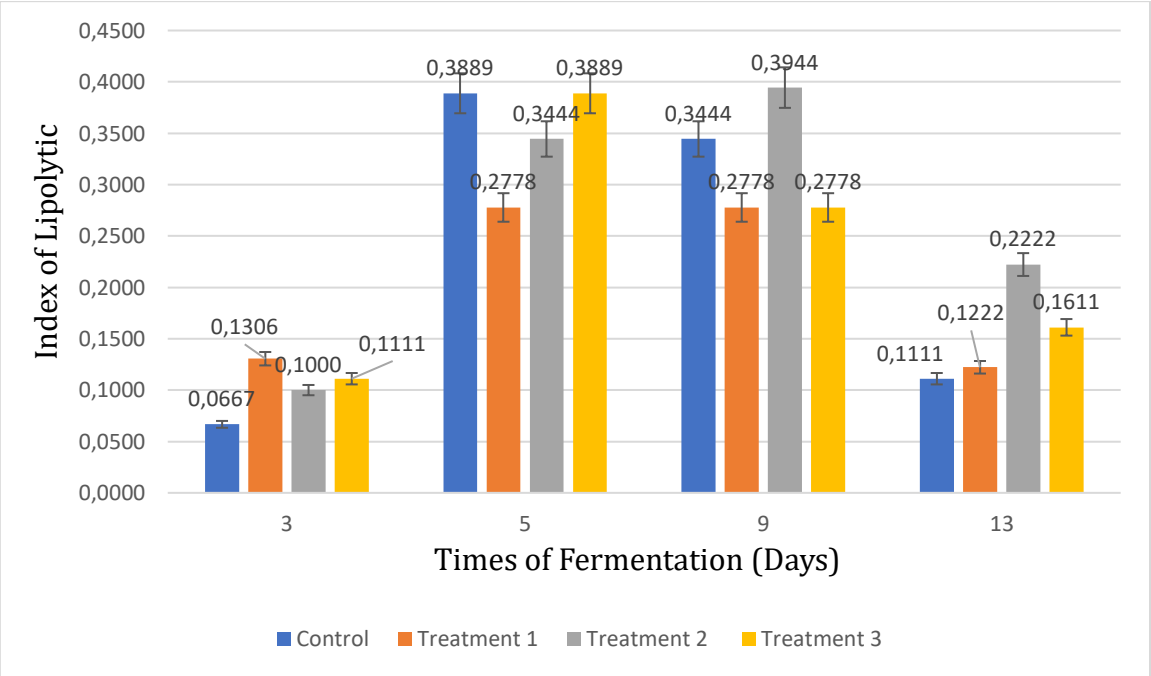

Figure 3. The results of index lipolytic

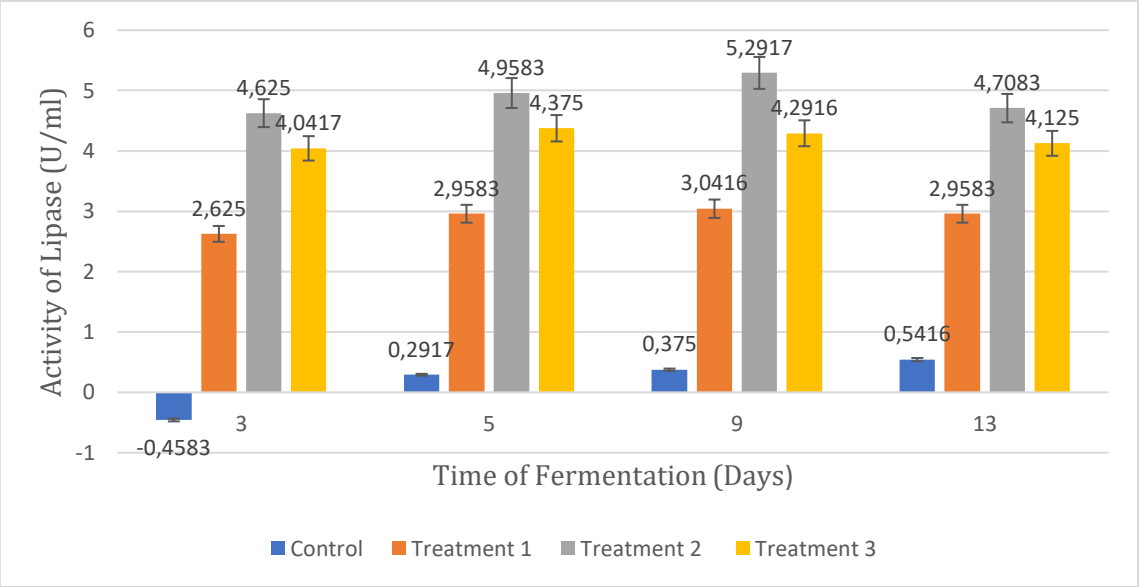

Figure 4. The results of lipase activity

3.2. Discussion

The fermentation process is influenced by internal factors (enzymes) and external cells (environment, temperature, pH, agitation, aeration). Then the composition of the fermentation media will significantly affect the cell. The piece of the fermentation substrate used includes mineral salts and glucose. Potato Dextrose Broth (PDB) is glucose used for carbon sources, increasing bioactive metabolites' production. The mineral salts used are KH_2PO_4 and $MgSO_4.7H_2O$. Some metals and minerals can affect the process of cell growth and fermentation. One is magnesium, and magnesium ions can affect yeast cells' development and growth and fermentative respiration metabolism. The addition of nutrients with different concentrations in this study was KH_2PO_4. The best enzyme testing results were obtained in sample 3, which contained PDB+ 1.5% KH_2PO_4 + 0.5% MgSO4. It is because the KH_2PO_4 mineral salt is used as a phosphate source. In fermentation, phosphate has an essential function because it plays a role in enzyme production and cell growth. The use of KH_2PO_4 above the optimum limit or high concentrations can inhibit cell growth and enzyme stability by increasing the medium's alkalinity (Carrazco-Palafox et al., 2018; Kalayu, 2019; Sun et al., 2015).

Previous research conducted by Soeka and Suliastini was isolating microbes from nature to produce proteases by fermentation with growth media without inducers. The protease activity was 315.88 U / mL, with a two-day fermentation time (Poernomo et al., 2017; Soeka & Sulistiani, 2017). The enzyme activities were tested using the supernatant metabolites of *Candida tropicalis* isolates, namely protease and lipase enzymes. *Candida tropicalis* isolate metabolites were observed in the presence of protease enzymes qualitatively, semiquantitatively, and quantitatively. Qualitative testing is done by t the

biuret test. The test results showed in table 1. The test's principle is to detect the presence of peptide bonds obtained by the reaction in the form of purple color in the solution that indicates protein presence. It happens because the Cu^{2+} ion (from the Biuret reagent) in an alkaline atmosphere reacts with polypeptides or peptide bonds that make up the protein to form a purple complex (violet) (Putri et al., 2016). The material used as an opposing substrate and comparison is 1% skim milk. The existence of a fading purple or blue color can be said to be a negative biuret test, which shows the presence of peptide bonds by the protease enzyme. The results of the biuret test for *Candida tropicalis* isolates are in Table 1. The results show a paler or fading purple color. It means that the metabolite isolate of *Candida tropicalis* produces a prosthetic enzyme.

Semi-quantitative testing uses a test substrate in Potato Dextrose Agar (PDA), added with 1% skim milk. Observations were made by looking at the clear zone produced. The results of this test showed in Figure 1. The results showed that a clear area formed on the test substrate. *Candida tropicalis* isolate metabolites containing the highest protease enzyme were obtained three days after fermentation of T2 (PDB + KH_2PO_4 1.5% + $MgSO_4$ 0.5%). The test results based on the same fermentation time showed that on day 3, the proteolytic index value of treatments 1 and 3 was more significant than the control ($p < 0.05$). The proteolytic index on day 5 showed that the group given treatment three was more significant than the control ($p < 0.05$) while the group's assigned treatment 1 and 3 had a smaller proteolytic index than the control ($p < 0.05$). At nine days of fermentation, the groups were given treatment 1, 2, and 3 had a higher proteolytic index than the control ($p < 0.05$). At 13 days of fermentation, the groups given treatment 1, 2, and 3 had lower proteolytic indexes than the control ($p < 0.05$). In this study, the highest proteolytic index of the control group was at the fermentation time of 9 days, in the treatment group 1 at nine days of incubation, the administration of treatment 2 with a fermentation time of 3 days, and the provision of treatment 3 with a fermentation time of 9 days. Based on these results, giving treatment 2 with a fermentation time of 3 days was the group that gave the best proteolytic index value (0,6556 ± 0,0090) U/mL among all treatment groups. The clear zone is caused by the activity of extracellular proteolytic enzymes produced by *Candida tropicalis* in hydrolyzing casein that is in skim milk on the media. The clear area is an indicator that isolates can utilize protein in the media as a nutritional source. The protease index produced by *Candida tropicalis* is smaller compared to the protease index produced by isolates from tofu wastewater (Asril & Leksikowati, 2019).

Quantitative testing carried out using the Kunitz chemical method with a UV-vis spectrophotometer and casein as the substrate. The results of this test showed in Figure 2. This method begins with the incubation of isolate metabolites in the substrate. Enzyme activity after incubation stopped with trichloroacetic acid (TCA) solution. The enzyme activity then measured the upper at 280 nm wavelength to determine the tyrosine amount formed from the hydrolysis of casein dissolved in TCA. One unit of enzyme is defined as an increase in 0.1 uptakes of TCA containing casein (Sumardi et al., 2019). Based on protease activity testing results, giving treatment two compared to the control ($p < 0.05$)

with a fermentation time of 3 days was the group that gave the best protease activity value among all treatment groups. The results showed that the metabolite isolate of *Candida tropicalis* after three days fermentation in T2 (PDB + KH_2PO_4 1.5% + $MgSO_4$ 0.5%) had the highest activity, amounting to 5,776 ± 0,495 U/mL. It is the need for added nutrients that have been fulfilled (Yuniarti et al., 2015). Based on the results of research that *Candida tropicalis* isolated from soil has protease activity. As in research conducted by Nizar microbial isolates from the mangrove, forest soils have a 1.9 x 10-4 U / mL (Nizar et al., 2015). The results of the protease enzyme activity test of *Candida tropicalis* isolates semiquantitatively and quantitatively showed the presence of protease enzyme activity on fermentation for three days. It is because three days of fermentation is an exponential phase where the metabolites produced are very high.

Microbes that can produce lipases are considered very important for several applications because they can be faster in finding natural sources of new lipases (Javed et al., 2018). Testing the existence of lipase enzymes is done semiquantitatively and quantitatively. Semi-quantitative testing uses PDA media that has been added methyl red, tween, and olive oil. The principle is to identify the presence of clear zones when isolate metabolites are incubated in the substrate, then the lipolytic index is calculated. Metabolite test results showed that the highest lipase enzyme activity produced by sample 3. The metabolite was obtained after 9 days fermentation in T2 (PDB + KH_2PO_4 1.5% + $MgSO_4$ 0.5%). The results showed in Figure 3. Based on these results, giving treatment 2 with a fermentation time of 9 days was the group that gave the best lipolytic index value (0,394 ± 0,053) U/mL among all treatment groups. The result is a clear zone around the bacterial colony after incubation and a change in the media's color to bright yellow. It means that *Candida tropicalis* isolate metabolites can hydrolyze fats into glycerol and fatty acids soluble in the media.

Quantitative lipase activity testing carried out using the acidimetry titration method with sodium hydroxide (NaOH) as a titrant, olive oil as a substrate, and phenolphthalein (PP) indicator as an indicator. The results showed in Figure 4. The appearance of changes indicates the existence of lipase enzyme activity in the solution from the original colorless to pink due to pH changes. The pink color appears when NaOH can no longer bind to fatty acids, thereby giving alkaline properties in the solution and giving rise to a pink color (Stoytcheva et al., 2012). The volume of NaOH obtained was then calculated by the activity value of the lipase enzyme. The isolate metabolites produced the highest lipase enzyme after nine days of fermentation of T2 (PDB + KH_2PO_4 1.5% + MgSO4 0.5%) in the amount of 5.2917±0,0167 U/mL. The high volume of NaOH needed indicates the more significant fatty acids produced from the lipid hydrolysis process. The results showed that *Candida tropicalis* could produce lipase. It is also found that another type of Candida, *Candida Antarctica,* and *Candida parapsilosis,* also has lipase (Ribas et al., 2019; Monteiro et al., 2020).

Conclusion

Based on these results, the *Candida tropicalis* metabolite giving the best proteolytic index value (0,6556 ± 0,0090) U/mL. The results showed that the metabolite isolate of *Candida tropicalis* had the highest activity, amounting to 5,776 ± 0,495 U/mL. The best results of lipolytic index value (0,394 ± 0,053) U/mL. The *Candida tropicalis* metabolites produced the highest lipase enzyme after nine days of fermentation in 5.2917±0,0167 U/mL.

Acknowledgments

Thank you to LPPM UNJANI for providing funding for this research.

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