Characterization of -Amylase and Protease from Indigenous *Lactobacillus fermentum* EN17-2 and Its Use in Tuber Paste Flour

Characterization of α-Amylase and Protease from Indigenous *Lactobacillus fermentum* EN17-2 and Its Use in Tuber Paste Flour

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Abstract. α-Amylase and protease are biocatalyst which can be used in paste flour to produce natural sweet paste flour. Production of these enzyme in Indonesia are limited, so, to increase those enzyme production, α-amylase and protease from indigenous bacteria are needed. This research focused in characterization of α-amylase and protease from indigenous *Lactobacillus fermentum* EN17-2 and its use in tuber paste flour. Crude enzymes of α-amylase and protease were characterized. Tuber sources were cassava (*Manihot esculenta*) and sweet potato (*Ipomoea batatas*) with wheat (*Triticum*) as comparison. α-Amylase activity and reduction sugar were detected by DNS methods, while protease was detected by tyrosin method and protein degradation was detected by titration. The results show that optimum activity of α-amylase from *L. fermentum* EN17-2 was reached at pH 5.5, 45^{o}C, while that optimum activity of protease was at pH 6.5, 35^{o}C. Stability of α-amylase from *L.fermentum* EN17-2 was at pH 5.0-6.5, 30-60^{o}C; while that protease was at pH 4.5-8.0, 20-50^{o}C. The reduction sugar content of cassava and sweet potato paste flour were 13.50% and 23.70%, respectively, while that the reduction sugar content of those flour added α-amylase from *L. fermentum* EN17-2 were 32.54% and 24.30%. The protein degradation of those paste flour were 0.49% and 1.17%, respectively, while that the protein degradation of those flour added protease were 1.90 % and 1.68%. The increase of reduction sugar in those paste flour were 58.51% and 2.47%, while that protein degradation were 1.40% and 0.50%, respectively. Based on the increase of reduction sugar and protein degradation in those treated paste flour, it concluded that the characterized α-amylase and protease from *L. fermentum* EN17-2 was better to be used as biocatalyst in cassava pasta flour to produce natural sweet paste flour than that sweet potato.

1. Introduction

α-Amylase is enzyme which degrade carbohydrate containing amylose to glucose and maltose, while protease which degrade protein to peptides. Production of these enzymes in Indonesia were limited, so, to increase the production of α-amylase and protease from indigenous bacteria are needed. Some species of lactic acid bacteria (LAB) producing α-amylase were *L. manihotivorans* LMG 18010T, *L. plantarum*, and *L. fermentum* [1,2], while that of protease were *Lactococcus lactis* [3], *Lactobacillus plantarum* S3 [4], *Lactobacillus delbrueckii* subsp. *lactis* CRL 581[5].

α-Amylase and protease from lactic acid bacteria (LAB) can be applied in Indonesia local tuber flour. This tuber flour can be used as wheat flour alternative although its homogeneous not as good as wheat flour. The form of the α-amylase and protease substrates are both in powder or paste flour. Local tuber flour as substrate for α-amylase and protease varied, such as cassava (*Manihot esculenta*) and sweet potato (*Ipomoea batatas*) in which those flour can be made pasta flour.

α-Amylase activity which hidrolyze amylose in tuber flour carbohydrate to glucose and maltose [6,7,8], and protease activity which hidrolyze protein in tuber flour to peptides [9,10] were affected by the type of the tuber flour. The α-amylase with high activity produced more glucose and maltose than that low activity [7,8,11], while the protease with high activity produced more peptides than that low activity [10,12]. So, various tuber flour were screened to get the tuber flour which good as substrates for producing high activity of α-amylase [6,8,11] and protease [13,14],

The α-amylase concentration and the flour type as substrate affected the glucose and maltose contents produced [6,7], while the protease concentration and the flour type as substrate affected the peptide contents produced [15,16]. The different amylose and protein contents between those flour as substrates depend on the different type of tuber flour [7,8, 10,16]. The diferent amylose hidrolysis in flour carbohydrate by α-amylase to glucose and maltose was caused by the different concentration of α-amylase [6,8,11], while the diferent protein hidrolysis in flour protein by protease to peptides was caused by the different concentration of protease [14, 17].

α-Amylase in flour hidrolyzed amylose to glucose as one of reduction sugar and maltose in which the concentration of glucose produced was generally higher than maltose [6,7,11], and protease in flour degradated protein to peptides in which the protein degradation varied depend on the flour type used [18,19]. Characterization of microbial α-amylase and protease and its application in flour have been reported (8,11,14,17). However, characterization of α-amylase and protease from indigenous lactic acid bacteria species of *Lactobacillus fermentum* EN17-2 as biocatalyst and its application in local tuber paste flour haven't been known yet. This research focused in characterization and stabilization of crude α-amylase and protease from indigenous *Lactobacillus fermentum* EN17-2 and its aplication in tuber paste flour.

2. Methods

2.1.Sub-culture L. fermentum EN17-2

*L fermentum E*N17-2 as indigenous lactic acid bacteria (LAB) found from traditional fermented nira, Enggano Island, collected as working culture at Microbiology Division, Research Center for Biology identified molecularly were sub-cultured in MRS (de Mann Rogosa Sharpe) media which consist of 0.8% beef extract (Himedia RM002-500G), 1% peptone (Bacto TM211677), 0.4% yeast extract (Bacto TM 212750), 1% glucose (Merck 1.08337.1000**)**, 0.5% natrium aceetate (Merck 1.06268.0250), 0.2% triamonium citrate (Sigma A1332-100G), 0.02% magnesium sulphate monohidrate (Merck 1.05886.0500), 0.005% mangan sulphate tetrahidrate (Merck 1.02786.1000), 0.2% dinatrium hydrogen phosphate dihydrate (Merck 1.06580.0500) 0.1%, and tween 80 (Merck 8.22187.0500). The sub-cultured *L. fermentum* EN17-2 was then incubated at temperature 37^{o}C in incubator (Isuzu incubator Himawari).

2.2. Tube Paste Flour

Tube paste flour was made from tube flour of cassava (*Manihot esculenta*), sweet potato (*Ipomoea batatas*) with wheat (Triticum) as comparison. The tube flour was heated at 70^{o}C up to form paste flour

2.3. Carbohydrate Degradation of Wheat and Local Tube Paste Flour Additional α-Amylase and Protease

The 5 gr of each tube flour from cassava, sweet potato and wheat was soluted in 50 mL aquadest, heated, homogenized by thermomagnetic stirrer (Sibata MGH-320) up to temperature 70^{o}C up to form paste flour, added 1U/mL crude α-amylase and 1 U/mL protease from *L. fermentum* EN17-2**,** respectively and incubated by rotary shaker (V-Tech VTRS-1) at temperature 37^{o}C for 24 hours.

2.4. α-Amylase Production [1]

Each suspension of *L. fermentum* EN17-2 was inoculated into 50 mL MRSB media and incubated at temperature 37^{o}C for 24 hours in incubator (Isuzu incubator Himawari). Each of crude α-amylase and protease from *L. fermentum* EN17-2 was found by growing 2% that bacteria into 25 mL sterilized MRSB media. For α-amylase production, glucose (Merck 1.08337.1000) was changed by 2% soluble starch (Merck 1.01252.0100) with pH medium: 6, incubated for 24 hours at temperature 37^{o}C by using incubator (Isuzu incubator Himawari), centrifuged at 9000 rpm for 10 minutes at temperature 4^{o}C by centrifuge (Kubota 5910). Each crude α-amylase from that bacteria was then tested its α-amylase activity**.**

2.5. Production of protease [18]

The 2 % inoculum culture was poured into 50 mL media of nutrient broth with addition of 1% casein and it was incubated at temperature 37°C for 48 hours. The production media was then centrifuged at 3500 rpm for 15 minutes. Supernatant produced was protease crude extract.

2.6. α-Amylase Activit [19,20]

α-Amylase activities were measured by DNS methods. The 50 µl crude α-amylase from that bacteria was added into 50 µl 1% soluble starch (Merck 1.01252.0100) in pH 5.0-8.0, homogenized by vortex (Sibata MGH-320), incubated in waterbath (Memmert) at temperature 35^{o}C-65^{o}C for 10 minutes, added 100 µl DNS reagen (Sigma D0550-100G), vortexed, heated at temperature 100^{o}C for 5 minutes, added 800 µl aquades, and revortexed. The absorbance was read at λ540 by using spectrophotometer UV-Vis (Shimadzu UV-1700 Pharmaspec), after cooling solution. One unit activity of amylase from that bacteria was defined as the amount of enzyme in which its reaction resulted product which equal 1µmol glucose per minute at measured condition.

2.7. Protease activity [21]

Protease activity test was conducted by Horikoshi method (1971) modified. The 0.2 mL protease was poured into reaction tube, added 0.4 mL 2% casein and 0.4 mL buffer phosphate 0.05 M pH 8. The mix was incubated at temperature 37 ^{o}C for 10 minutes, added 1 mL 20% TCA and homogenized. Incubation was continued at temperature 37^{o}C for 10 minutes, and solution was centrifuged by rotation 3500 rpm for 5 minutes. Controle was made. One unit protease was defined as the amount of mL protease needed to produce 1 µmol tirosin every minute with casein as substrate.

2.8. Optimation of α-Amylase Activity in Various pH and Temperatures [22]

Optimazion of crude α-amylase from *L. fermentum* EN17-2 in various pH detected by pH meter (Horiba pH 1100 Scientific), at incubation time for 10 minutes were conducted at pH in the range of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5. The highest α-amylase activity at certain pH indicated optimum activity of α-amylase. Optimization of α-amylase from that bacteria in various temperatures at incubation time for 10 minutes were conducted at temperature in the range of 30, 35, 40, 45, 50, 55, 60, 65, 70^{o}C. The highest α-amylase activity of that bacteria at certain temperature indicated α-amylase optimum activity.

2.9. Optimization of protease activity in various pH and Temperatures [18]

Optimization of protease activity was conducted in pH 4.5. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The activity test was conducted at temperature: 37^{o}C and incubation time for 10 minutes. The highest protease activity in certain pH was stated as optimum protease activity. Optimization of protease activity was conducted in various temperatures: 20, 25, 30, 35, 40, 45, and 50^{o}C. The activity test was conducted in optimum pH, with incubation time for 10 minutes. The highest protease activity in certain temperature was stated as optimum protease activity.

2.10. α-Amylase Stability in Various pH and Temperature [23]
Stability of crude α-amylase from *L. fermentum* EN17-2 were conducted by measuring α-amylase relative activities at pH in the range of 3,5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5) with incubation time for 60 minutes. The ≥ 50% α-amylase relative activities were defined as stabilities of the α-amylase at certain pH range. Stabilities of α-amylase was also conducted by measuring α-amylase relative activities at temperature in the range of 30, 35, 40, 45, 50, 55, 60, 65, 70^{o}C. The ≥ 50% α-amylase relative activity was defined as stability of α-amylase at certain temperature range.

2.11. Stability of protease in various pH and Temperatures [17]
Stability of protease in various pH were conducted by measuring protease relative activities in pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The activity test was conducted in optimum temperature with incubation time for 60 minutes. Protease relative activities with values: ≥ 50% showed that those activities were in stable condition. Stability of protease in various temperatures was conducted by measuring protease relative activities in temperatures: 20, 25, 30, 35, 40, 45 and 50^{o}C. The activity test was conducted in optimum pH with incubation time for 60 minutes. Protease relative activities with value: ≥ 50% showed that those activities were in stable condition.

2.12. Reduction Sugar [24,25]
Reduction sugar was measured by DNS method. Reduction sugar (%) was measured by using standard curve equation of glucose solution. Carbohydrate degradation in tuber flour of cassava, sweet potato and wheat (with and without addition of *L. fermentum* EN17-2 crude α-amylase) was centrifuged at 9000 rpm for 10 minutes at temperature 4^{o}C. The 100 µl the treated tuber flour was then added 100 µl DNS reagen, vortexed, heated at 100^{o}C for 5 minutes, added 800 µl aquadest and vortexed. The solution was then leaved at a minute, and absorbance was read at λ540 by using spectrophotometer UV-Vis (Shimadzu UV-1700 Pharmaspec)..
Reduction Sugar Concentration (%) = [glucose concentration (mg/mL)/sample weight (mg)]xVolume of reaction total (mL) x 100%.....(1)

2.13. Protein degradation [26].
Protein degradation was tested by formol titration. Sample of 10 mL treated paste flour was added phenolphthalein, and neutralized by NaOH 0,1 N solution. The 10 mL of 37 % Formaldehyde was added into the solution, titrated by standard solution of NaOH 0,1 N up to the colur change to pink. The protein degradation was then calculated as % Nitrogen x Fc (Conversion Factor).

3. Result and Discussion
The activities of *L. fermentum* EN17-2 α-amylase in pH: 3.5-7.5 were in the range 1.160-2.340 U/mL with the optimum activity was reached at pH: 5.5 (2.340 U/mL), and in temperature: 30-70^{o}C were 2.040-3.210 U/mL with the optimum activity was at 45^{o}C (3.210 U/mL) (Figure 1-2)

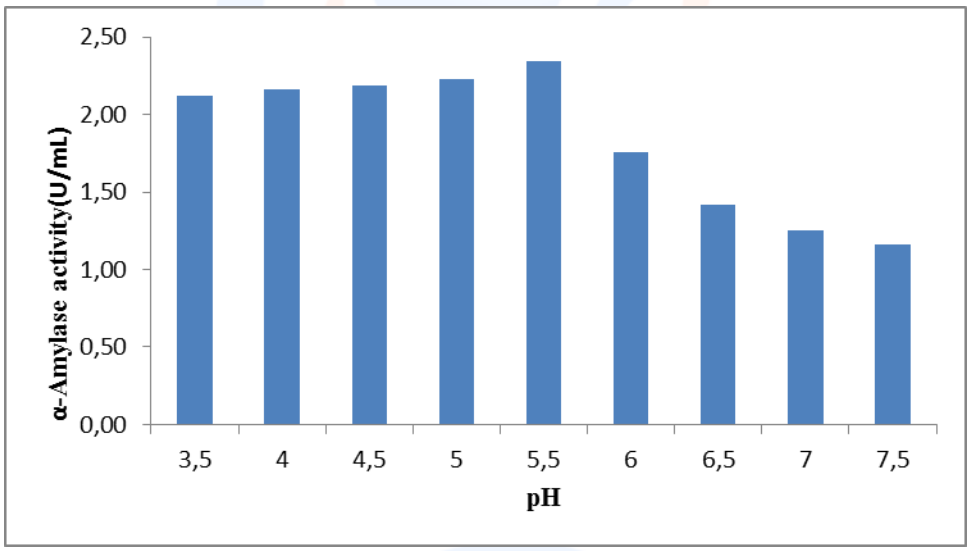

Figure 1 Activities of *L. fermentum* EN17-2 α-Amylase in Various pH

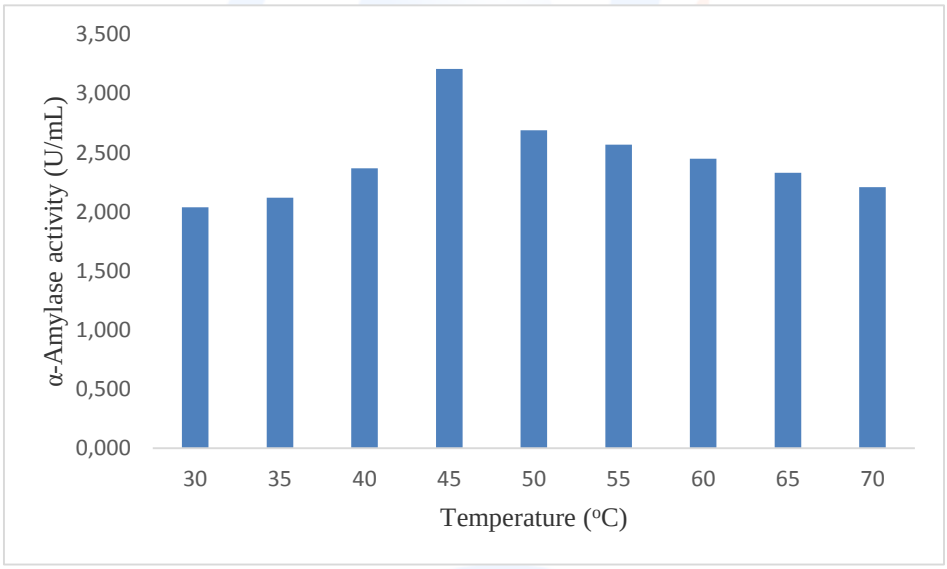

Figure 2. Activities of *L. fermentum* EN17-2 α-Amylase in Various Temperature

The optimum α-amylase activity from *L. fermentum* EN17-2 at a certain pH and temperature was affected by the type of bacteria species producing α-amylase. It has been reported that the optimum activity of α-amylase from lactic acid bacteria was affected by the type of species from lactic acid bacteria producing α-amylase [1,2,27].

The stability of *L. fermentum* EN17-2 α-amylase with ≥ 50% relative activities in incubation time for 60 minutes were reached at pH in the range of 5.0-6.5 (2.2889-2.9800 U/mL) with the relative activities were 76.81-100% (Figure 3a,b), while that at temperature in 30-60°C (2.150-4.120 U/mL) with the relative activities were 52.18-100% (Figure 4a,b). The highest stability of *L. fermentum* EN17-2 α-amylase was occurred at pH: 5.5 and temperature: 45°C (Figure 3b,4b)

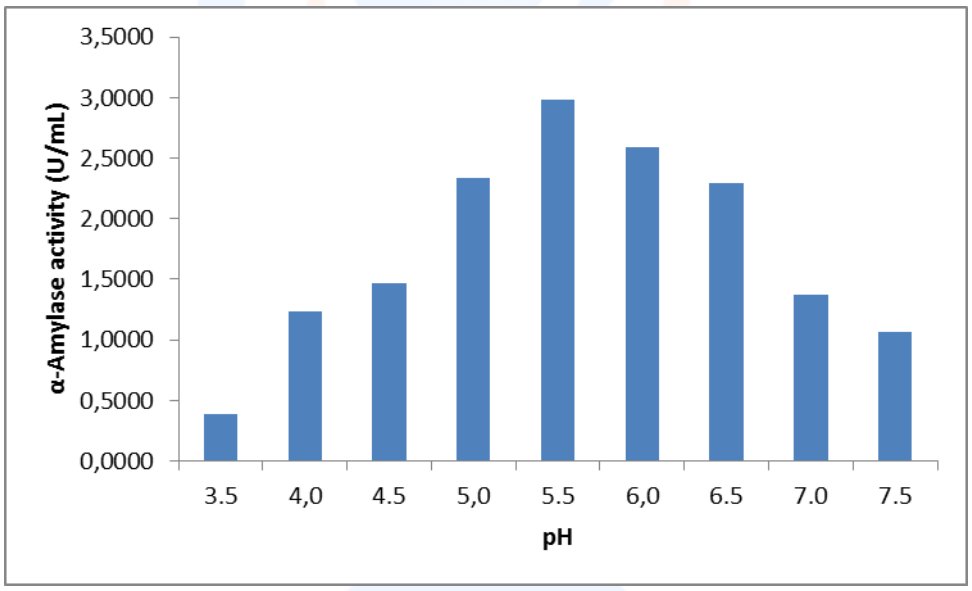

Figure 3a. Unit Activities of *L. fermentum* EN17-2 α-Amylase at Various pH in 60 Minutes Incubation

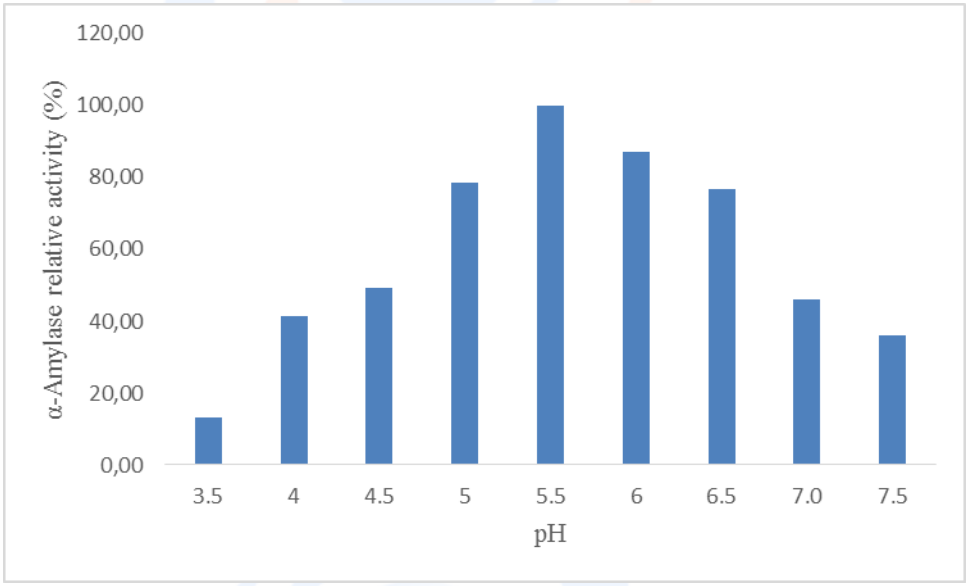

Figure 3b. Relative Activities of *L. fermentum* EN17-2 α-Amylase at Various pH in 60 Minutes Incubation

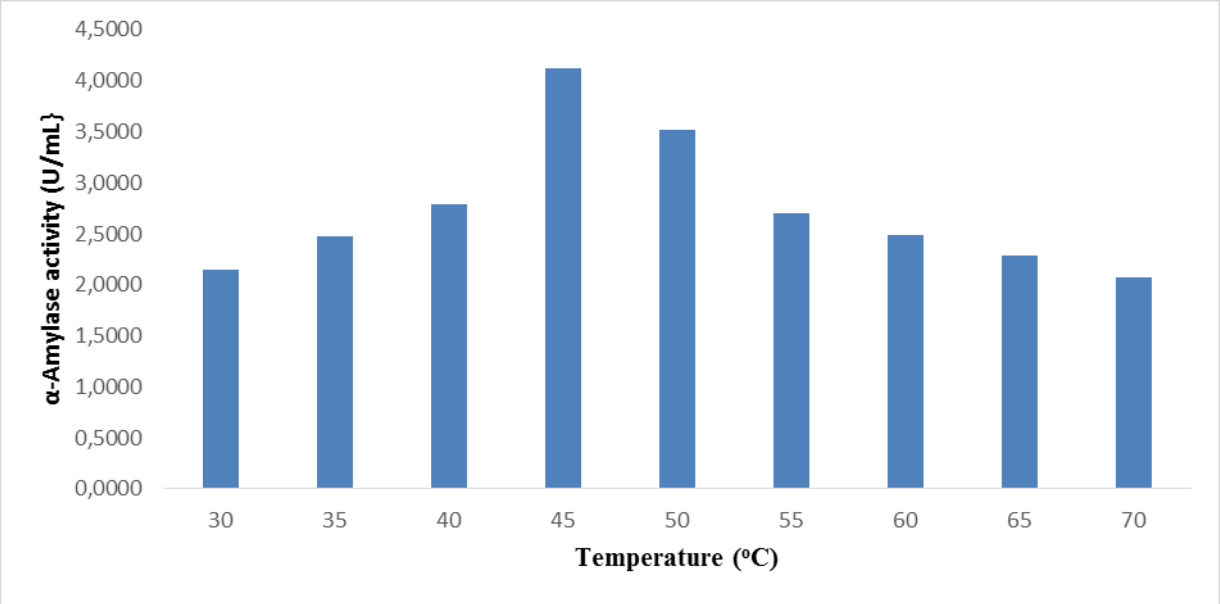

Figure 4a. Unit Activities of *L. fermentum* EN17-2 α-Amylase at Various Temperature in 60 Minutes Incubation

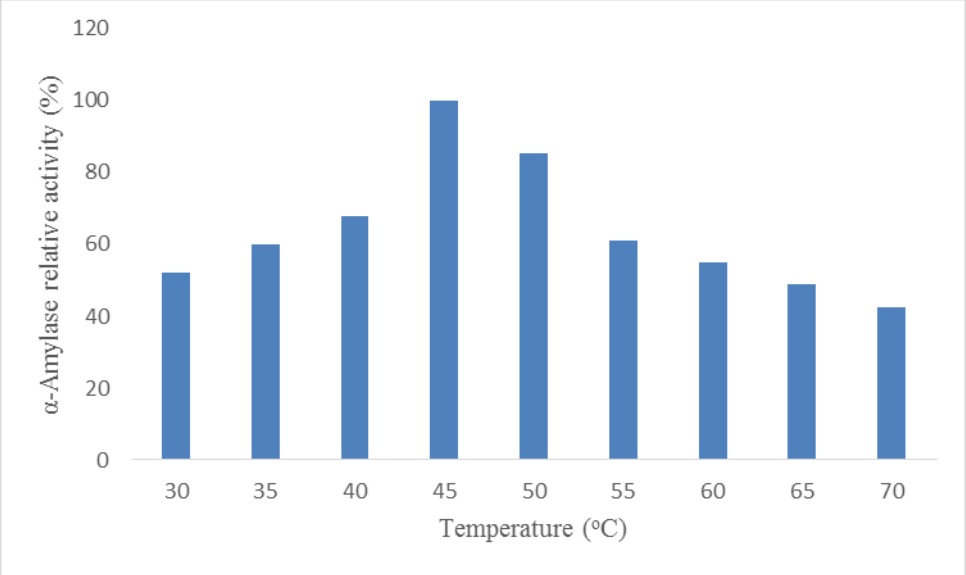

Figure 4b. Relative Activities of *L. fermentum* EN17-2 α-Amylase at Various Temperature in 60 Minutes Incubation

The α-amylase stabilities from *L. fermentum* EN17-2 in the certain pH and temperature were caused the type of spesies from that lactic acid bacteria. It has been reported that the optimum activity of α-amylase from species of lactic acid bacteri was affected by the type of species from lactic acic bacteria producing α-amylase [2, 11,20]

The increase of reduction sugar contents of cassava and sweet potato paste flour additional *L. fermentum* EN17-2 α-amylase were sequently 58.51% and 2.47% (Table 5). The reduction sugar content increase of cassava pasta flour (58.51%) additional *L. fermentum* EN17-2 α-amylase was

higher than that wheat paste flour (20.88%) (Table 9), while the reduction sugar content increase of the sweet potato pasta flour (2.47%) additional *L. fermentum* EN17-2 α-amylase were lower than that wheat paste flour (20.88%) (Table 1)

Table 1. The Contents and Increase Reduction Sugar in Local Pasta Tuber Flour Additional *L. fermentum* EN17-2 α-Amylase

Tuber paste flour types	Reduction sugar content (%)	Reduction sugar increase (%)
Cassava	32.540	58.51
Controle (without *L. sustensis* EN38-32)	13.500	
Sweet Potato	24.300	2.47
Controle	23.700	
Wheat	48.080	20.88
Controle	28.040	

The higher increase of reduction sugar contents from the cassava pasta flour additional *L. fermentum* EN17-2 α-amylase than that the wheat paste flour was because the carbohydrate contents of the cassava pasta flour additional *L. fermentum* EN17-2 α-amylase were higher than that wheat flour. On the contrary, the lowerr increase of reduction sugar contents from the sweet potato pasta flour additional *L. fermentum* EN17-2 α-amylase than that the wheat paste flour was because the carbohydrate contents of the sweet potato pasta flour additional *L. fermentum* EN17-2 α-amylase were lower than that wheat flour [22,28]

It has been reported that reduction sugar content of the tuber flour resulted from α-amylase activity in hidrolyzing flour carbohydrate.was affected by the types of flour and lactic acid bacteria producing α-amylase [6, 7,11.].

The activities of *L..fermentum* EN17- α-amylase in incubation time for 60 minutes in pH: 3.5-7.5 were in the range 0.3925-2.9800 U/mL and the α-amylase relative activities were in the range 13.18-100% (Figure 5); while that temperature: 30-70^{o}C were 2.0752-4.1203 U/mL and the relative activities were in 42.62-100% (Figure 6).

The activities of *L. fermentum* EN17-2 protease in pH: 4.5-8.0 were in the range 0.4451-0.7403 U/mL with the optimum activity was reached at pH: 6.5 (0.7403 U/mL) and in temperature: 20-50^{o}C were 0.4414-0.8146 U/mL with the optimum activity was at 35^{o}C (0.8146 U/mL) (Figure 5-6)

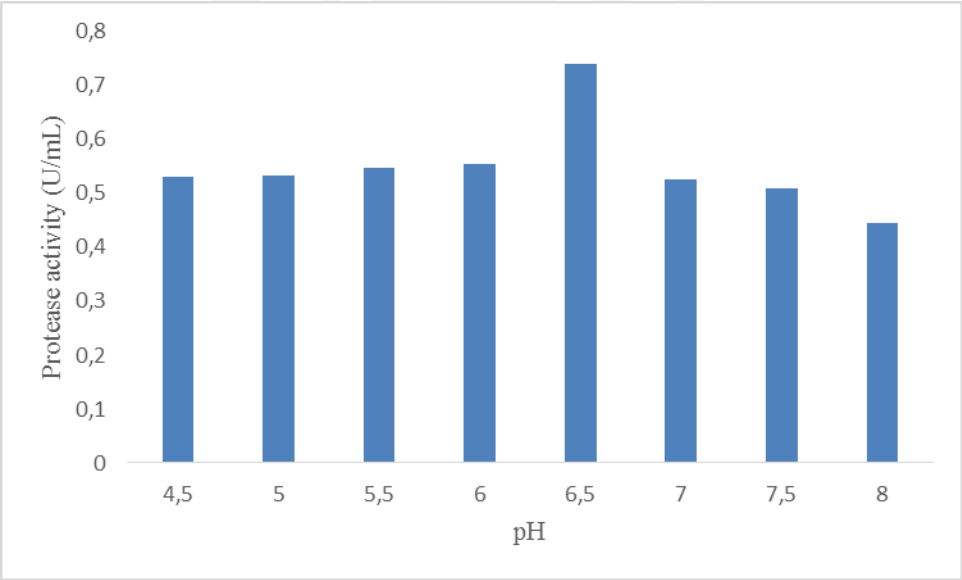

Figure 5. Activity of *L. fermentum* EN 17-2 Protease in Various pH

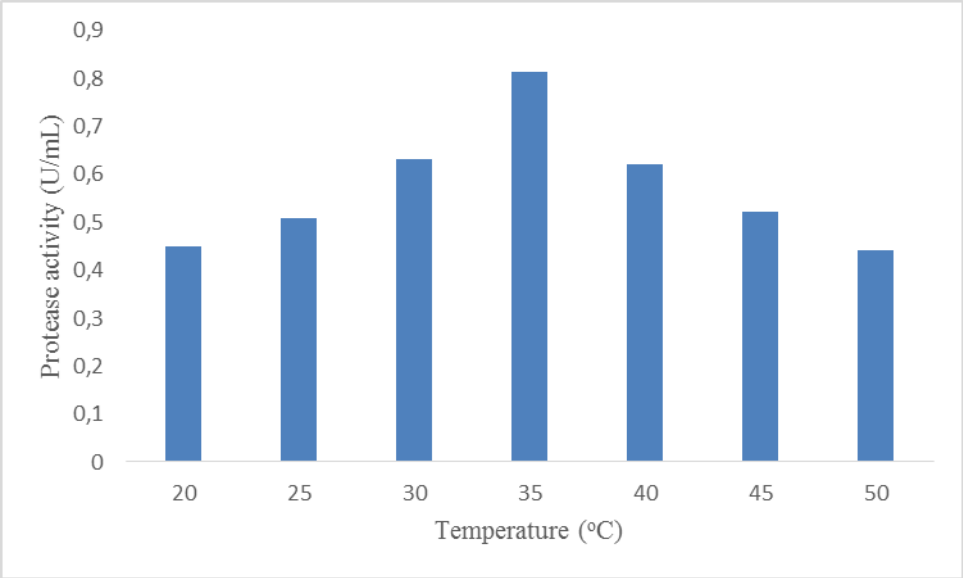

Figure 6 Activity of *L. fermentum* EN 17-2 Protease in Various Temperature

The optimum protease activity from *L. fermentum* EN17-2 at a certain pH and temperature was affected by the type of bacteria species producing protease. It has been reported that the optimum activity of protease from lactic acid bacteria was affected by the type of species from lactic acid bacteria producing protease [3,5,29]

The activities of *L. fermentum* EN17- protease in incubation time for 60 minutes in pH: 4.5-8.0 were in the range 0.4672-0.7346 U/mL and the protease relative activities were in the range 63.50-100% (Figure 7a,b), while that temperature:: 20-50^{o}C were 0.4598-0.8628 U/mL and the relative activities were in 53.29-100% (Figure 8a,b).

The stability of *L. fermentum* EN17-2 protease with ≥ 50% relative activities in incubation time for 60 minutes were reached at pH in the range of 4.5-8.0 (0.4672-0.7346 U/mL) with the relative activities were 63.50-100% (Figure 7a,b), while that at temperature in 20-50°C (0.4598-0.8628 U/mL) with the relative activities were 53.29-100% (Figure 8a,b). The highest stability of *L. fermentum* EN17-2 protease was occurred at pH: 6.5 and temperature: 35°C (Figure 7b, 8b)

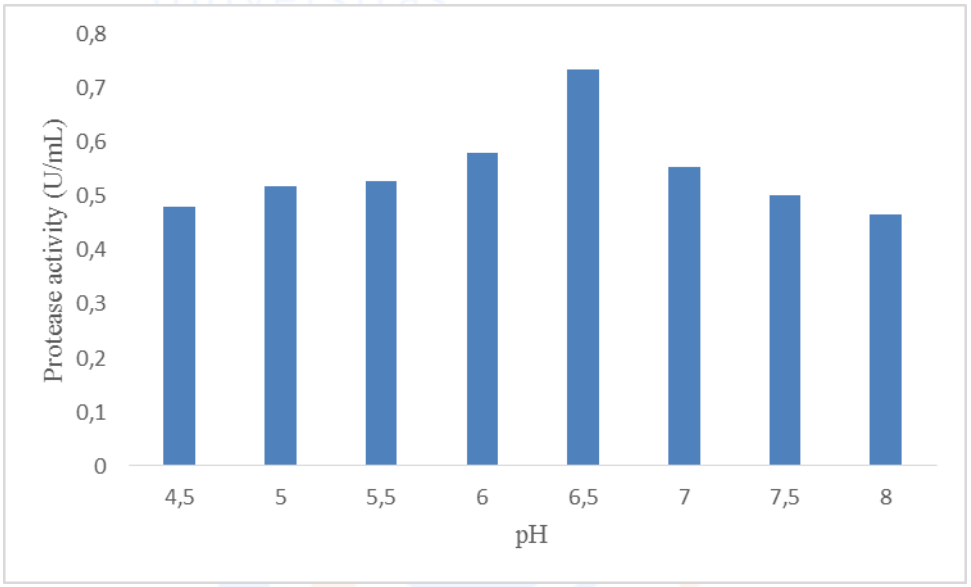

Figure 7a. Unit Activity of *L. fermentum* EN 17-2 Protease in Various pH

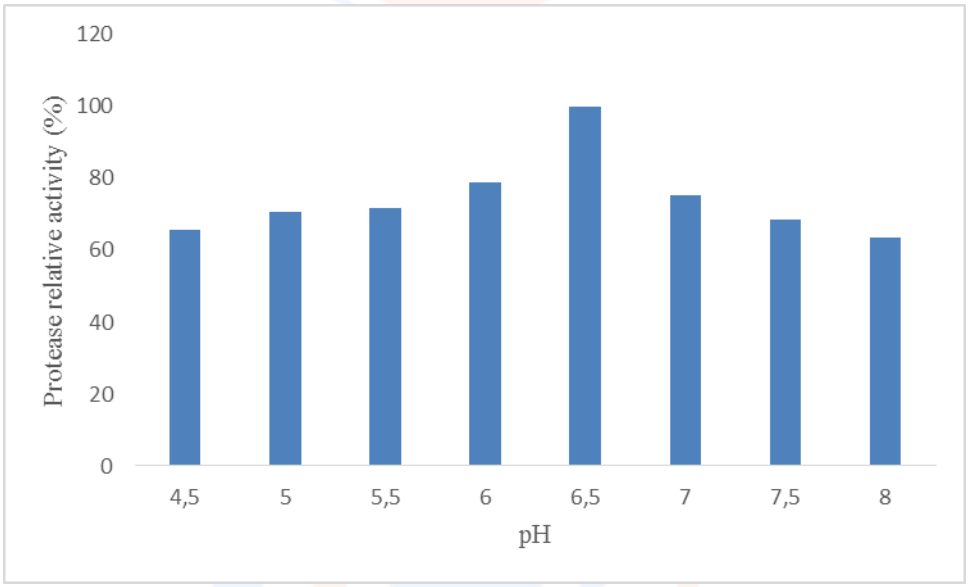

Figure 7b. Relative Activity of *L. fermentum* EN 17-2 Protease in Various pH

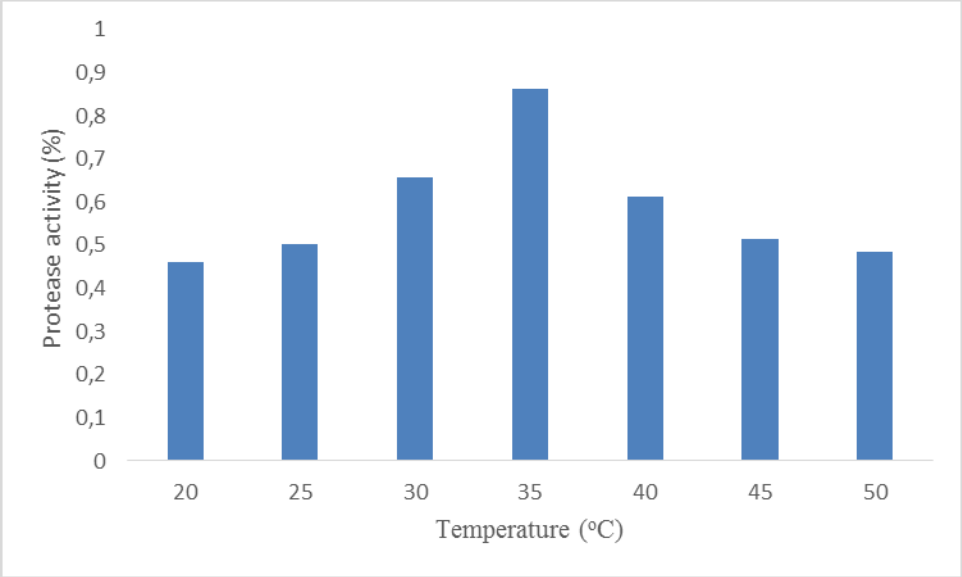

Figure 8a. Unit Activity of *L. fermentum* EN 17-2 Protease in Various Temperatures

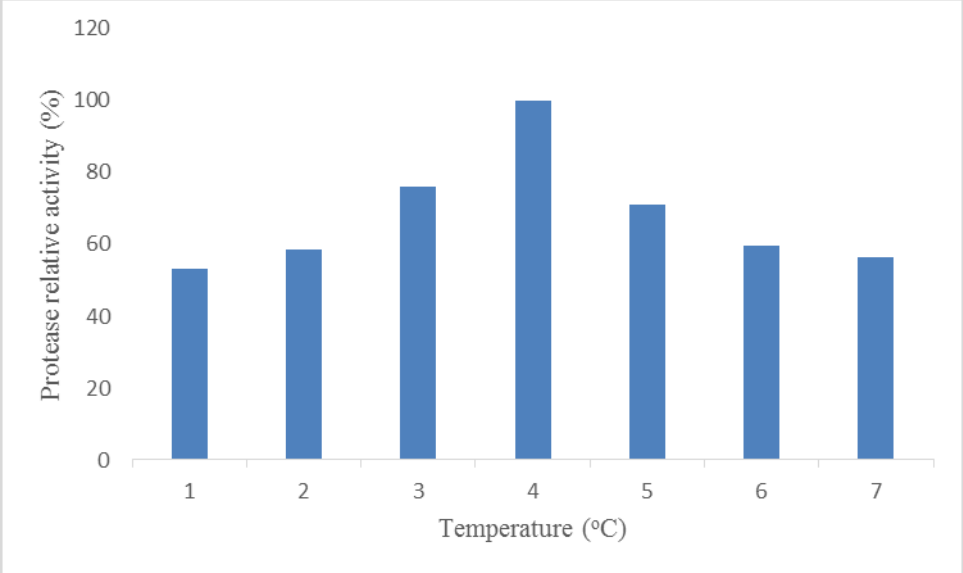

Figure 8b. Relative Activity of *L. fermentum* EN 17-2 Protease in Various Temperatures

The protease stabilities from *L. fermentum* EN17-2 in the certain pH and temperature were caused the type of spesies from that lactic acid bacteria. It has been reported that the optimum activity of protease from species of lactic acid bacteri was affected by the type of species from lactic acic bacteria producing α-amylase [3,4,5],

The increase of degraded protein contents of cassava and sweet potato paste flour additional *L. fermentum* EN17-2 protease were sequently 1.40% and 0.50% (Table 2). The degraded protein content increases of cassava (1.40%) and sweet potato (0.50%) additional *L. fermentum* EN17-2 protease were lowerr than that wheat paste flour (2.24%) (Table 2)

Table 2. Degraded Protein Content of Tuber Paste Flour Additional *L. fermentum* EN 17-2 Protease

Tuber paste flour types	Degraded protein content (%)	Degraded protein increase (%)
Cassava	1.900	1.40
Controle (without *L. sustensis* EN38-32)	0.490	
Sweet Potato	1.680	0.50
Controle	1.170	
Wheat	4.710	2.24
Controle	2.470	

The lower increase of degraded protein contents from the cassava and sweet potato pasta flour additional *L. fermentum* EN17-2 protease than that the wheat paste flour was because the protein contents of the cassava and sweet potato pasta flour additional *L. fermentum* EN17-2 protease were lower than that wheat flour [22,28].

It has been reported that degraded protein content of the tuber flour resulted from protease activity in hidrolyzing flour protein.was affected by the type of flour and lactic acid bacteria producing protease [9,19,13,14,15].

4. Conclusion

The activities of α-amylase and protease from *L. fermentum* EN17-2 reached optimum at pH 5.5, 45^{o}C and pH 6.5, 35^{o}C, respectively, while the stabilities of α-amylase and protease from *L. fermentum* EN17-2 reached in stable condition at pH 5.0-6.5, 30-60^{o}C and pH 4.5-8.0, 20-50^{o}C. The reduction sugar content of cassava and sweet potato paste flour additional α-amylase were higher than that without addition, and. the protein degradation content of those paste flour additional protease were also higher than that without addition. The increase of reduction sugar in those paste flour were 58.51% and 2.47%, while that protein degradation were 1.40% and 0.50%, respectively. It concluded that the characterized α-amylase and protease from *L. fermentum* EN17-2 was better to be used as biocatalyst in cassava pasta flour to produce natural sweet paste flour than that sweet potato, based on the increase of reduction sugar and protein degradation in those treated paste flour.

5. Acknowledgement

The Authors wish to acknowledge to DIPA Priority Project of Aplied Bioresources, Research Center for Biology, Deputy of Life Sciences, Indonesian Institute of Sciences for funding this research, and to Desy Septiani for her assistance in Laboratory during this researvh.

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