January 2022

THE SECOND INTERNATIONAL CONFERENCE ON GENETIC RESOURCES AND BIOTECHNOLOGY: Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture

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Preface: The Second International Conference on Genetic Resources and Biotechnology

The Second International Conference on Genetic Resources and Biotechnology, which is the continuation of the first event held in 2018, focuses on topics related to advances in biotechnology to create more opportunities for effective conservation and sustainable utilization of genetic resources for food and agriculture. This year conference's theme is Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture. The conference was organized by Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture, Indonesia, in collaboration with Indonesian Biotechnology Consortium and held on 24th-25th of May 2021virtually due to the pandemic of COVID-19.

The conference aims to share and exchange current scientific information and technological developments on biotechnology and their applications for conservation and sustainable use of genetic, to encourage and promote quality, efficiency, and modernization of management and utilization of genetic resources, and to facilitate national and international collaboration among participants. There are five scopes discussed in this conference. They are effective management of conservation and sustainable use of genetic resources for food and agriculture, application of genomics and molecular markers for genetic resource conservation and crop adaptation to climate change, application of innovative crop improvement techniques for conservation and sustainable use of plant genetic resources for food and agriculture, plant cell and tissue culture for conservation and effective utilization of genetic resources, and the use of microbial genetic resources as biological control agents of agricultural pests and diseases, and for soil bioremediation.

Five speakers from the United States of America, Japan, India and Indonesia were invited to discuss about their expertise and knowledge on relevant subjects in the plenary sessions. This conference was attended by more than 100 participants including 75 presenters and 44 listeners worldwide. They came from diverse governmental, private, or academic institutions and also scientific communities. The presented materials have undergone peer review processes and only qualified papers were selected. Furthermore, all papers were subjected to double blind peer-review and expected to meet the scientific criteria of significance and academic excellence to be published in a conference proceedings indexed in a well-known, reputable service.

We would like to express our sincere gratitude to our speakers, presenters and all participants for their contributions in this conference. We would also like to express our appreciation for the generosity of our sponsors that support this conference: PT CropLife, PT ITS Science Indonesia, PT Fajar Mas Murni and PT Prima Instrument Analitika. Lastly, special thanks to all committee members for their exceptional work and contributions in the conference and publication.

Chair of Organizing Committee

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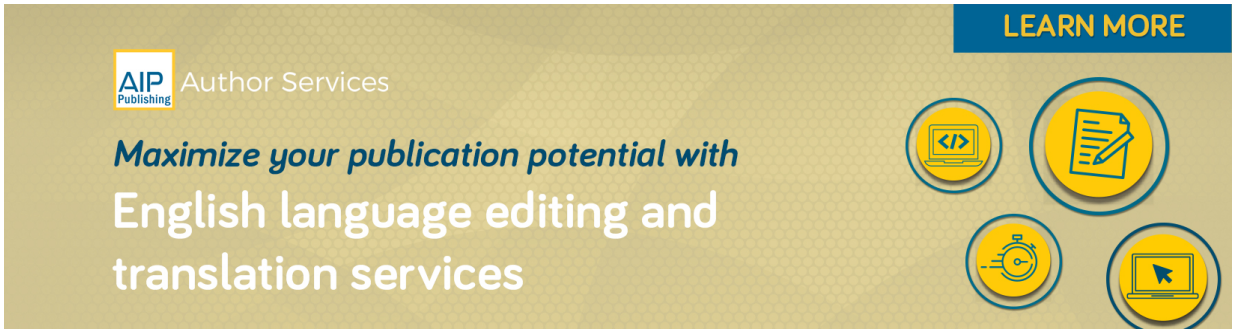

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Characteristic of Indigenous *Leuconostoc mesenteroides* EN 17-11 Protease and Its Stability during Storage at Cold and Freezing Temperatures

Tatik Khusniati[1, a)], Ika[2], Harry Noviardi[2] and Sulistiani[1]

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Abstract. Protease as protein hydrolyzer is an imported enzyme used in the food and pharmaceutical industries in Indonesia. To reduce imports, indigenous lactic acid bacteria can be used as a source of protease. This study aimed to determine the characteristics of indigenous *Leuconostoc mesenteroides* EN 17-11 protease and its stability during storage at cold and freezing temperatures. Protease activity was detected using the modified Horikoshi method. The protease stability after storage was tested at 0, 7, 14, 21, and 28 days. Relative protease activity with a value ≥50% indicates protease activity in stable conditions. All treatments were statistically analyzed using ANOVA. The results showed that the optimum protease activity of *L. mesenteroides* EN 17-11 was reached at pH 6.0 with activity of 0.9970 U/ml and temperature of 40°C with activity of 1.1166 U/ml ($p<0.05$). The protease stability at incubation time of 60 min was reached at pH 5.0–7.5 with a relative activity of 55.29–100.00%, and a temperature of 20–50°C with a relative activity of 51.92–100.00% ($p<0.05$). The protease stability at storage for 28 days with a relative activity of ≥50% at freezing temperature was higher than at cold temperature. The highest protease stability was reached at freezing temperature for 7 days with a relative activity of 77.31% ($p<0.05$). It was concluded that the protease stability of indigenous *L. mesenteroides* EN 17-11 was better at freezing than at cold temperature.

INTRODUCTION

The development of enzymes in the world is growing rapidly and has an important role in the fields of technology, industry, food processing, and cosmetics. Enzymes are natural catalysts produced by living organisms in various chemical processes needed by all living things, which are expected to reduce energy waste because the reaction does not require high energy, is nontoxic and catalyzes reactions without by-products [1–3].

One type of enzyme that has a very broad application is protease because it has high economic value in the industrial sector [4–6]. Industrial users of protease include the food industry as a meat tenderizer, cheese making, protein hydrolyzate and cracker manufacturing, and the nonfood industry, such as detergents, skins, and biomedicine [5, 7, 8].

In Indonesia, the need for protease is increasing, but this need still depends on imported production. One way to anticipate dependence on imported proteases is by making efforts to produce proteases by optimizing the utilization of microbial resources in Indonesia, including lactic acid bacteria. Lactic acid bacteria include GRAS (Generally Recognized as Safe) microorganisms, which are a group of microorganisms that are safe when added to food because of their nontoxic nature and are not a risk to health [5, 9, 10]. Several types of lactic acid bacteria that have proteolytic activity include *Lactococus lactic* [11], *Lactobacillus plantarum* S31 [7], *Leuconostoc mesenteroides* [12], and *L. delbrueckii* subsp. *lactis* CRL 58 [13].

The ability of proteases to accelerate reactions is influenced by several factors including pH, temperature, and metal as an activator or inhibitor [14] which causes enzymes to have certain characteristics. The protease produced

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by *L. satsumensis* EN 38-32 has optimum activity at pH 7 and an optimum temperature of 40°C with an activity value of 0.7214 U/ml [15]. However, the protease from *L. mesenteroides* EN 17-11 has not been characterized quantitatively. The stability of the protease *L. mesenteroides* EN 17-11 during storage is not known. Therefore, it is necessary to conduct research on the protease characteristics of *L. mesenteroides* EN 17-11 and their stability at various storage temperatures and times.

MATERIALS AND METHODS

Growth Media

Media used for the growth of *L. mesenteroides* EN 17-11 bacteria was de Man Rogosa Sharpe (MRS) and de Man Rogosa Sharpe Broth (MRSB). MRS consisted of 5 g peptone, 4 g beef extract, 2 g yeast extract, 10 g glucose, 2.5 g sodium acetate, 1 g tri ammonium citrate, 0.1 g $MgSO_4$, 0.025 g $MnSO_4$, 1 g Na_2HPO_4, and 0.5 g Tween 80, in 500 ml distilled water. The MRS solution was homogenized with a magnetic stirrer (SIBATA MGH-320), after which it was divided into two. Half of the media was added into an Enlenmeyer flask and left in liquid state, whereas the remaining was made solid by addition of 4.5 g of agar, and 1.25 g of $CaCO_3$ were also added, homogenized with a magnetic stirrer (SIBATA MGH-320), heated until dissolved and then sterilized in an autoclave (HIRAYAMA HICLAV), at a temperature of 121°C for 15 min. The MRS agar media was poured into sterile Petri dishes.

MRSB media, production media consisting of casein, peptone, and beef extract, tri chloro acetic acid (TCA) solution, phosphate buffer solution with the composition of Na_2HPO_4 and NaH_2PO_4, distilled water, casein, and L-tyrosine.

Subculture

Two loops of *L. mesenteroides* EN 17-11 isolated from local *nira* (sap obtained by tapping inflorescences of various palms to make sugar) of Enggano Island were inoculated to MRS medium and incubated at 37°C for 24 h.

Protease Production

As much as 2% of *L. mesenteroides* EN 17-11 inocula were transferred to 50 ml of production media (Nutrient Broth media with 1% casein added), then incubated in an incubator (Isuzu) at 37°C for 24 hours. After incubation, the media was centrifuged at 9,000 rpm for 15 min at 4°C to separate the supernatant from the cell biomass. The resulting supernatant was a crude protease and then tested for its protease activity.

Tyrosine Standard Curve Creation

Tyrosine standard curves were made at various concentrations, namely 0, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mg/ml, and compared with diluted tyrosine standard solution 3 mg/ml with 0.1 N HCl solvent. The standard solution was then measured for absorbance at $\lambda = 275$ nm with a UV-Vis spectrophotometer (Shimadzu).

Protease Activity Test

The protease activity test was carried out using the quantitative Horikoshi method [16]. Protease control with tyrosine standard was at various concentrations of 0, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mg/ml. This standard solution was obtained by diluting the standard solution of tyrosine 3 mg/ml with 0.1 N HCl solvent. The steps taken were 0.2 ml of protease solution was inserted into a test tube then added with 0.4 ml of 2% casein (w/v) and 0.4 ml of 0.05 M phosphate buffer (pH 8). The mixture was incubated at 37°C for 10 min. After that, 1 ml of the trichloro acetic acid (TCA) mixture was added to it and homogenized using the vortex (SIBATA). The incubation was continued at 37°C for 10 min, then the solution was centrifuged at 10,000 rpm for 5 min. The supernatant obtained was measured for its absorbance at $\lambda = 275$ nm.

Control was carried out by inserting 0.2 ml of protease solution into a test tube and adding 0.4 ml of 0.05 M phosphate buffer (pH 8). After that, 1 ml of TCA mixed solution was added, then incubated at 37°C for 10 min. To

this, 0.2 ml of 2% (w/v) casein solution was added. The solution was then vortexed (SIBATA) and incubated at 37°C for 10 min, and then centrifuged at 10,000 rpm. The supernatant obtained was measured for its absorbance at λ = 275 nm. One protease unit (U) was defined as the number of ml of enzyme required to produce 1 µmol of tyrosine per min with casein as substrate.

Measurement of protease activity was carried out by converting the absorption value into tyrosine concentration (µg/ml) with a standard tyrosine curve. The proteolytic activity of the enzyme was calculated by the formula:

$$\textit{Enzyme activity} = \frac{\textit{([tyrosine]} \times \textit{1,000)}}{\textit{Mr of tyrosine}} \times \frac{V}{p \times q} \times df$$

where [tyrosine] = tyrosine concentration (µg/ml), Mr of tyrosine = molecular weight of tyrosine (181.19 g/mol), v = total volume of sample (ml), p = enzyme volume (ml), q = incubation time (minute), df = dilution factor, 1,000= conversion of mmol to µmol.

Optimization of pH and Temperature

Determination of the optimum pH was carried out using a phosphate buffer and 2% casein substrate which was adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. Activity test was carried out at 37°C and incubation time for 10 min. The highest activity based on the protease activity test was the protease optimum pH. Determination of the optimum temperature was carried out by testing the protease activity at various temperatures, namely 20, 25, 30, 35, 40, 45, and 50°C. Activity test was carried out at 2% casein substrate concentration at optimum pH, with an incubation time of 10 min. The highest activity based on the protease activity test was the protease optimum temperature.

pH Stability at 60 Minutes Incubation Time

The pH stability was carried out by determining the relative activity of proteases at various pH, namely at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. To adjust the pH, a phosphate buffer was made with the composition of Na_2HPO_4 and NaH_2PO_4 dissolved with distilled water. Furthermore, the pH meter was calibrated at pH 4.0 and 7.0. A phosphate buffer solution was prepared by adding NaH_2PO_4 solution to Na_2HPO_4 until it reached the specified pH. The substrate used was a 2% casein solution in a 0.05 M phosphate buffer at the specified pH. The activity test was carried out at the optimum temperature from the optimization results with an incubation time of 60 min. The relative protease activity with a value of 50% indicated that the protease activity was in a stable condition.

Temperature Stability at 60 Minutes Incubation Time

Determination of temperature stability was carried out by determining the relative activity of proteases at various temperatures, namely 20, 25, 30, 35, 40, 45, and 50°C. For a temperature setting of 20°C, water was added with ice cubes little by little and a thermometer was used to find out that the temperature has reached 20°C. For setting the temperature of 25°C, tap water was used and a thermometer was used to determine that the temperature has reached 25°C. Temperature setting of 30–50°C was carried out on water bath. The optimum pH was tested using a 2% casein substrate and in an incubation time of 60 min. The relative protease activity with a value of 50% indicated that the protease activity was in a stable condition.

Protease Stability Test

The protease stability determination of *L. mesenteroides* EN 17-11 was carried out with proteases stored at cold temperature (4°C) and freezing temperature (-20°C), at various storage times of 0, 7, 14, 21, and 28 days. Each sample was tested for protease activity using the Horikoshi method [16]. Then, the relative protease activity was calculated at each storage temperature based on the following equation [15].

$$\textit{Protease relative activity (\%)} = \frac{\textit{Treatment activity (U/ml)}}{\textit{Highest activity}} \times 100\%$$

Experimental Design

The research design used in this study was a completely randomized design (CRD). The variables measured were protease activity at various pHs and temperatures. Data collection for each measurement was carried out three times. The data were analyzed using the analysis of variance (ANOVA). If the test results show a significant difference, then the Duncan's Multiple Range Test (DMRT) at the 5% significance level was applied. Data analysis was performed using SPSS 17.0 program.

RESULTS AND DISCUSSIONS

The protease activity of *L. mesenteroides* EN 17-11 after optimization was higher than before optimization (Table 1). The higher activity of protease after optimization was due to suitable pH and temperature during optimization, which was also reported on lactic acid bacteria [7, 11, 17].

TABLE 1. Activity of protease from *Leuconostoc mesenteroides* EN 17-11 before and after optimization.

Treatment	Protease activity (U/ml)
Before optimization*	0.4120
After optimization**	0.9970

*pH 8, incubation temperature: 37°C. **pH 6, incubation temperature: 40°C.

The range of protease activity at various pH (5.0–8.0) was between 0.6549U/mL±0.0193 to 0.9970U/mL±0.1251 (Table 2) with the optimum activity (0.9970U/mL±0.1251) of protease at pH 6.0. The protease activity at pH 6.0 was not significantly different to that at pHs 6.5, 7.0, and 7.5, but was significantly different to the protease activities at pH 5.0, 5.5, and 8.0 ($p<0.05$). The different protease activity at different pH was because the optimum activity of protease occurred at certain pH. The optimum protease activity occurred at a certain pH where the protease activity reached a maximum [18, 19, 20].

TABLE 2. Activity of protease from *Leuconostoc mesenteroides* EN 17-11 at various pH.

Treatment (pH)	Protease activity (U/ml)*
5.0	0.6549±0.0193 a
5.5	0.6825±0.0639 a
6.0	0.9970±0.1251 c
6.5	0.9363±0.0521 c
7.0	0.8977±0.0781 bc
7.5	0.8186±0.1254 abc
8.0	0.7413±0.1701 ab

*Mean value followed by the same letter was not significantly different according to DMRT at the significance level of 5%.

The range of protease activity of at various temperatures (20–50°C) was between 0.5371U/mL±0.0446 and 1.1166U/mL±0.0658 (Table 3). The optimum activity (1.1166U/mL ± 0, 0658) was reached at 40°C. The protease activity at 40°C was significantly different to that at temperatures of 20, 25, 30, 35, 45, and 50°C ($p<0.05$). Different protease activity at different temperature was because the optimum activity of protease occurred at certain temperature. The optimum protease activity occurred at a certain temperature where the protease activity reached a maximum [13, 21, 22].

TABLE 3. Activity of protease from *Leuconostoc mesenteroides* EN 17-11 at various temperatures.

Temperature (°C)	Protease activity (U/ml)*
20	0.7229±0.0165 b
25	0.7799±0.1376 bc
30	0.8719±0.1006 cd
35	0.9363±0.0627 d
40	1.1166±0.0658 e
45	0.5721±0.0291 a
50	0.5371±0,0446 a

*Mean value followed by the same letter was not significantly different according to DMRT at the 5% significance level.

The range of protease activity between pH 5.0–8.0 and 60 min incubation was between 0.0084U/mL±0.0068 and 0.0953U/mL±0.0110 (Table 4). The optimum protease activity (0.0953U/mL±0.0110) was reached at pH 6.0. The protease activity at pH 6.0 was not significantly different to that at pHs 5.5, 6.5, and 7.0, but was significantly different to the activity at pHs 5.0, 7.5, and 8.0 ($p<0.05$). The different protease activity at incubation time of 60 min and at different pH was because the optimum activity of protease occurred at certain pH. The optimum protease activity in a certain incubation time period occurred at a pH where the protease activity reach maximum [18, 20, 23].

TABLE 4. Unit activity and relative activity of protease from *Leuconostoc mesenteroides* EN 17-11 at various pH and incubation time of 60 min.

Treatment (pH)	Protease activity (U/ml)*	Protease relative activity (%)
5.0	0.0527±0.0005 b	55.29
5.5	0.0756±0.0210 cd	79.32
6.0	0.0953±0.0110 d	100.00
6.5	0.0947±0.0129 d	99.37
7.0	0.0928±0.0063 d	97.37
7.5	0.0591±0.0161 bc	62.01
8.0	0.0084±0.0068 a	8.81

*Mean value followed by the same letter was not significantly different according to DMRT at the 5% significance level.

The range of protease activity at various temperatures (20–50°C) and incubation time of 60 min was between 0.0848U/mL±0.0427 and 0.1633U/mL±0.0144 (Table 5) with the optimum activity (0.1633U/mL±0.0144) at 40°C. The protease activity at 40°C was not significantly different to the protease activities at temperatures of 35, 40, and 45°C, but was significantly different to that at 20, 25, and 50°C ($p<0.05$). The different protease activity at incubation time of 60 min and at different temperature was because the optimum activity of protease occurred at certain temperature. The optimum protease activity in a certain incubation time period occurred at the temperature where the protease activity was reach maximum [13, 22, 24].

TABLE 5. Unit activity and relative activity of protease from *Leuconostoc mesenteroides* EN 17-11 at various temperatures and incubation time of 60 min.

Temperature (°C)	Protease activity (U/ml)*	Protease relative activity (%)
20	0.0916±0.0170 a	56.09
25	0.1189±0.0069 ab	72.81
30	0.1544±0.0171 bc	94.54
35	0.1563±0.0037 c	95.71
40	0.1633±0.0144 c	100.00
45	0.1449±0.0032 bc	88.73
50	0.0848±0.0427 a	51.92

*Mean value followed by the same letter was not significantly different according to DMRT at the 5% significance level.

The range of protease activity at cold temperature at various storage times (7–28 days) was between 0.5003U/mL±0.0304 and 0.6015U/mL±0.0908 (Table 6). The highest relative activity of protease was reached at storage time of 7 days with a value of 0.6015U/mL±0.0908. The protease activity at storage time of 7 days was not significantly different to that at storage times for 14, 21, and 28 days, but was significantly different to that at storage times of 0 day ($p<0.05$). The relative activity of protease at cold temperature with a value of 50% indicated that protease stability occurred in 7, 14, 21, and 28 days. The different protease stability at different storage times was because the stability of protease occurred at certain storage time. The protease activity stored at cold temperature was relatively stable at the time of storage for a certain period [8, 25, 26].

TABLE 6. Unit activity and relative activity of protease from *Leuconostoc mesenteroides* EN 17-11 at cold temperature, at various storage times.

Storage time (day)	Protease activity (U/mL)*	Protease relative activity (%)
0	0.9970±0.3381 b	100.00
7	0.6015±0.0908 a	60.33
14	0.5048±0.0159 a	50.63
21	0.5003±0.0728 a	50.18
28	0.5003±0.0304 a	50.18

*Mean value followed by the same letter is not significantly different according to DMRT at the 5% significance level.

The range of protease activity at freezing temperature, at various storage times (7–28 days) was between 0.4120U/mL±0.0555 and 0.6144U/mL±0.0552 (Table 7). The highest relative activity of protease was reached at storage time of 7 days with a value of 0.6144U/mL±0.0552. The protease activity at storage time of 7 days was not significantly different to that at storage times of 14 days, but was significantly different to that at storage times of 0, 21, and 28 days ($p<0.05$). The relative activity of protease at freezing temperature with a value of 50% indicated that protease stability occurred in protease storage of 7, 14, 21, and 28 days. The different protease activity at different storage times was because the relative activity of protease occurred at certain storage time. The protease activity stored at freezing temperature was relatively stable at the time of storage for a certain period [2, 3, 5].

TABLE 7. Unit activity and protease activity of protease from *Leuconostoc mesenteroides* EN 17-11 at freezing temperature, at various storage times.

Storage time (day)	Protease activity (U/ml)*	Protease relative activity (%)
0	0.7947±0.1253 c	100.00
7	0.6144±0.0552 b	77.31
14	0.5242±0.0191 ab	65.96
21	0.4506±0.0303 a	56.70
28	0.4120±0.0555 a	51.84

*Mean value followed by the same letter was not significantly different according to DMRT at the 5% significance level.

The highest protease activity at cold and freezing temperatures, at various storage times of 7 days was 0.6015U/mL±0.0908 and 0.6144U/mL ± 0.0552, respectively (Table 8). The relative activity of protease at cold storage, at storage time of 7 days was lower than that at freezing temperature, at the same storage time ($p<0.05$).

TABLE 8. Unit activity and relative activity of protease from *Leuconostoc mesenteroides* EN 17-11 at cold and freezing temperatures for 7 days.

Temperature (°C)	Protease activity (U/ml)	Protease relative activity (%)
4	0.6015±0.0908 a	60.33
-20	0.6144±0.0552 b	77.31

*Mean value followed by the same letter was not significantly different according to DMRT at the 5% significance level.

The different protease relative activity at different storage temperature was because the relative activity of protease occurred at certain storage temperature. The protease activities stored both at cold and freezing temperatures were relatively stable at the time of storage for a certain period [6, 12, 26].

CONCLUSION

The optimum protease activity of *L. mesenteroides* EN 17-11 was reached at pH 6.0 and temperature of 40°C ($p<0.05$). The protease stability at incubation time of 60 min was reached at pH 5.0–7.5 with a relative activity of 55.29–100%, and temperature of 20–50°C with a relative activity of 51.92–100% ($p<0.05$). The protease stability stored for 28 days with a relative activity of ≥50% at freezing temperature was higher than that at cold temperature. The highest protease stability was reached at freezing temperature for 7 days with a unit activity of 0.6144 U/ml and a relative activity of 77.31% ($p<0.05$). It was concluded that the protease stability of indigenous *L. mesenteroides* EN 17-11 was better at freezing than at cold temperature.

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