Characterization of Crude Amylase Activity from Bacteriocin-Producing *Lactobacilli*

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ABSTRACT

Bacteriocin-producing Lactobacilli have been successfully isolated from inasua, a fermented fish product from Teon, Tilapia, and Serua, Province of Maluku Indonesia. In addition to producing bacteriocin, Lactobacilli could produce amylase enzymes. This study aims to characterize crude amylase enzyme activity from bacteriocin-producing *Lactobacillus plantarum* ITN13 and *Lactobacillus rhamnosus* IN13 based on pH and temperature optimum enzyme activity. Both isolates were grown on media de Mann Rogosa Sharpe Agar (MRSA) + 1% starch and media Nurient Agar (NA) + 1% starch. Amylolytic index (AI) of *L. plantarum* ITN13 and *L. rhamnosus* IN13 were 1.33 and 1.00, respectively. Amylase production of *L. rhamnosus* IN13 maximum at 3 hours incubation was 0.123 (Unit / mg protein) while *L. plantarum* ITN13 at 6 hours was 0.092 (Unit / mg protein) in the exponential phase, respectively The optimum amylase activity from *L. rhamnosus* IN13 at 45°C and pH 5, while the optimum of amylase *L. plantarum* ITN13 at 35°C and pH 6.
Keywords: *amylolytic index, inasua, MRSA, pH optimum.*

1. INTRODUCTION

Inasua is a traditionally product of wet salt fish fermentation produced by Teon, Nila and Serua (TNS)
Communites in Central Maluku, Indonesia [1]. Coconut sap is also added to the inasua fermentation to extend the shelf life of this product until more than a year. Mahulette *et al*. reported having successfully isolated a number of lactic acid bacteria (LAB) from inasua, among others *Lactobacillus plantarum* ITN13, *L. rhamnosus* IN13, *L. paracasei* IN17, dan *Leuconostoc mesenteroides* ITN17. *Lactobacillus plantarum* is the dominant LAB found in inasua [2]. LAB is Gram positive bacteria that can produce lactic acid in carbohydrate metabolism. The bacteria play an important role in preserving food because they produce bacteriocin, a peptide compound used as a biopreservative agent. Monafathia *et al*. has selected *L. plantarum* ITN13 and *L. rhamnosus* IN13 for producing bacteriocins at optimum condition with the addition of 4 g/L glucose, 8.11 g/L yeast extract, pH of 5.3, and temperature of 30°C [3].
The use of amylases produced from *Lactobacillus* is considered as safe because they are non-pathogenic and the end product of fermentation is lactate which is used as flavoring agent in the food industry. Some of the lactic acid bacteria exhibiting amylolytic activity i.e. *L. amylovorus, L. plantarum, L. manihotivorans,* and *L. fermentum* [4]. The advantage of using microbial amylases in industrial application is that they are more stable than any other source and easy to manipulate to obtain enzyme of desirable characteristics in bulk production [5]. The studies that mention LAB isolates which produce bacteriocin also produce amylase so far have not reported. This study aims

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to characterize crude amylase enzyme activity from bacteriocin-producing *L. plantarum* ITN13 and *L. rhamnosus* IN13 based on pH and temperature optimum enzyme activity.

2. METHODS

2.1. Materials

Bacteriocin producing LAB i.e. Lactobacillus plantarum ITN13 and *Lactobacillus rhamnosus* IN13 has been isolated by Mahulette *et al.* (2016). The indicator bacteria used were *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644. All bacterial are collected at IPBB Culture Collection (IPBCC).

2.1.1. Preparation of bacterial isolates

The LAB isolates of *L. plantarum* ITN13 and *L. rhamnosus* IN13 were grown on Mann de Rogosa Sharpe Agar (MRSA) media added with 1% $CaCO_3$ and then incubated at 37ºC for 24 hours. Indicator isolates used were *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644 grown on agar nutrient media (NA) at 37ºC for 24 hours [3]. The catalase test was carried out using 3% H_2O_2 which was dropped one drop onto the glass preparation. Bacterial isolates are taken one loop and placed on glass preparations that have been dripped with 3% H_2O_2. Positive results are indicated by the formation of bubbles on H_2O_2. Gram staining is carried out following the standard microbiological Gram staining procedure.

2.1.2. Production and detection of bacteriocin

Lactobacillus isolate was inoculated into MRSB media and then incubated for 24 hours at 37ºC. One mL of bacterial culture was then inoculated into 50 mL of MRSB and incubated at 37ºC for 24 hours. A total of 10 mL of bacterial isolates were then centrifuged at 6000 g for 10 minutes. The supernatant was adjusted to pH to 6.5 and filtered using milipore 0.22 µm. The filter obtained was a neutral cell-free supernatant.

The bacteriocin activity test was carried out using the agar diffusion well method Vennigalla *et al.* [6]. A total of 50 µL bacteriocin was put into the well in the Mueller Hinton Agar medium which had been inoculated with tested (indicator) bacteria, i.e. *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogens* ATCC 7644 with a cell density of 10^6 CFU / mL. The cultures were then incubated at 37ºC for 24 hours. After 24 hours a clear zone was formed which formed around the well. The bacteriocin activity unit is expressed in the Activity Unit (AU). 1 AU is defined as the area of inhibition per unit volume of sample of the bacteriocin tested [7]. Measurement of bacteriocins activity is carried out by the following formula: Activity of bacteriocins = (AZ - AW) : V , where AZ means clear zone area (mm^2), AW means Area of wells (mm^2); V means volume of sample (mL). Bacteriocin sensitivity testing of protease enzymes was carried out by adding 10 µL proteinase K enzyme (2 mg / mL) to 100 µL cell-free supernatant then incubating for 2 hours at 37ºC. The inhibitory activity was tested against to bacteria. The control treatment was supernatant without the addition of enzymes [8]. If the results do not form a zone of inhibition, this indicates that the active compound tested is bacteriocin.

2.1.3. Measurement of amylolytic index

One ose/loop of LAB isolate inoculated on MRSA media added with 1% soluble starch and then incubated at 27ºC for 48 hours. Measurement of amylolytic index is carried out by the following formula: Amylolytic Index= (diameter of clear zone (mm) – diameter of colony diameter (mm): diameter of colony (mm).

2.1.4. Measurement of growth cell and crude amylase activity

As much as 1-2 ose of bacterial isolates were grown on MRSB 30 mL containing 1% starch as cultivation media. The culture which used as inoculum was incubated in a shaking incubator for 12 hours at 37 ° C. As much as 0.5 mL inoculum then put into 50 mL of other MRSB media containing 1% starch as production media. Cell turbidity, enzyme activity, and protein content were measured every 3 hours for 36 hours of incubation. The linear regression equation of the turbidity versus total cell is used to measure the total of cells. Amylase enzymes are extra cellular enzymes. Supernatant containing crude amylase is obtained by centrifugation at 4° C for 10 minutes using centrifuge Hermle Labortechnik GmbH Z 326 K) speed of 10000 rpm (rotation per minute)

2.1.5. Measurement of amylase activity and protein concentration

The amylase activity of crude enzyme was measured according to the Bernfeld method using 3,5-dinitrosalicylic acid (DNS) reagent to detect production of reducing sugars [9]. One unit of amylase activity is defined as the amount of an enzyme that can produce 1 µmol of maltose as a reducing sugar per minute under measurement conditions.

Protein content was measured by the method of Bradford and bovine serum albumin (BSA) is used

as a standard protein measured at a concentration of 0.1-1.0 mg / mL protein [10]. The specific activity of crude amylase was calculated by dividing unit activity with protein content.

2.1.6 Characterization of pH and temperature optimum of crude amylase activity

The optimum pH of crude amylase activity from LAB isolate was measured by using 0.05M buffer citrate phosphate for pH 4.5 and 6, 0.05M buffer Tris-HCl for pH 7 and 8, and 0.05M glycine-NaOH buffer for pH 9. Optimum temperature of crude amylase activity was measured in the temperature range: 25,30,35,40, 45, and 50 ºC.

3. RESULTS AND DISCUSSION

3.1. Characteristic of Lactobacilli isolates

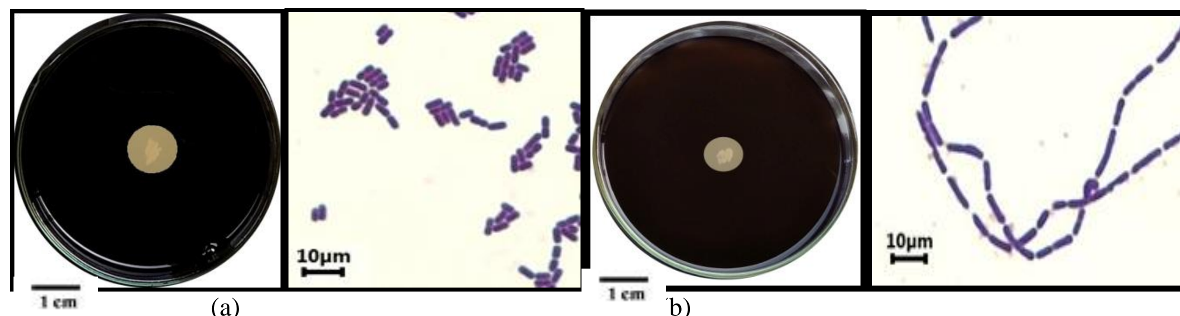
Isolates of *L. plantarum* ITN13 and *L. rhamnosus* IN13 which grew on MRSA media at 37ºC for 48 hours revealed a colony morphological form: white, slippery edges and circular in shape. The cell forms of the LAB are rod and Gram positive. The catalase test results are negative.

The growth of isolate on MRSA media containing 1% starch incubated at 37ºC for 48 hours was able to form a clear zone around the colony after dropping with iodine solution (Figure 1). The clear zone showed the cells produced extracellular amylase enzymes. The amylolytic index of *L. plantarum* ITN 13 was greater than *L. rhamnosus* IN13 (Table 1).

Both isolates produced bacteriocin which found in the supernatant. Test results with proteinase K showed that bacteriocin is hydrolyzed by proteases (sensitive). The results of bacteriocin testing of each isolate against two indicator isolates viz *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogens* ATCC 7644 produced inhibitory zones (Table 2).

Table 1 Characteristic of *Lactobacillus plantarum* ITN13 and *L. rhamnosus* IN13

Isolate	Colour of colony	Edge of colony	Colony shape	Cell shape	Gram	Catalase	Amylolytic Index
L. plantarum ITN13	White	Smooth	Circular	Rod	Positive	Negatif	1.3
L. rhamnosus IN13	White	Smooth	Circular	Rod	Positive	Negatif	1.0



(a) (b)

Figure 1 Amilolytic zone around the bacterial colony and morphology of bacterial cell: (a) *Lactobacillus plantarum* ITN13 and (b) *Lactobacillus rhamnosus* IN13

Table 2 Antagonistic activity of bacteriocins producing Lactobacilli toward indicator bacteria

Isolate	Inhibitory area (AU/mL)	
	Listeria monocytogens	*Salmonella typhimurium*
L. plantarum ITN 13	1383 ± 38.3	1579 ± 46.1
L. rhamnosus IN13	1417 ± 73.1	1210 ± 20.3

3.2. Growth of Lactobacilli isolates and amylase production

L. rhamnosus IN13 and *L. plantarum* ITN13 grown well on MRSB media incubated at 37 ^{0}C. The exponential phase *L. rhamnosus* IN13 and *L. plantarum* ITN13 ended at the 9th hour and the

12th hour, respectively. Amylase production of *L. rhamnosus* IN13 maximum at 3 hours incubation was 0.123 (Unit / mg protein) while *L. plantarum* ITN13 at 6 hours was 0.092 (Unit / mg protein) in the exponential phase, respectively (Figure 2).

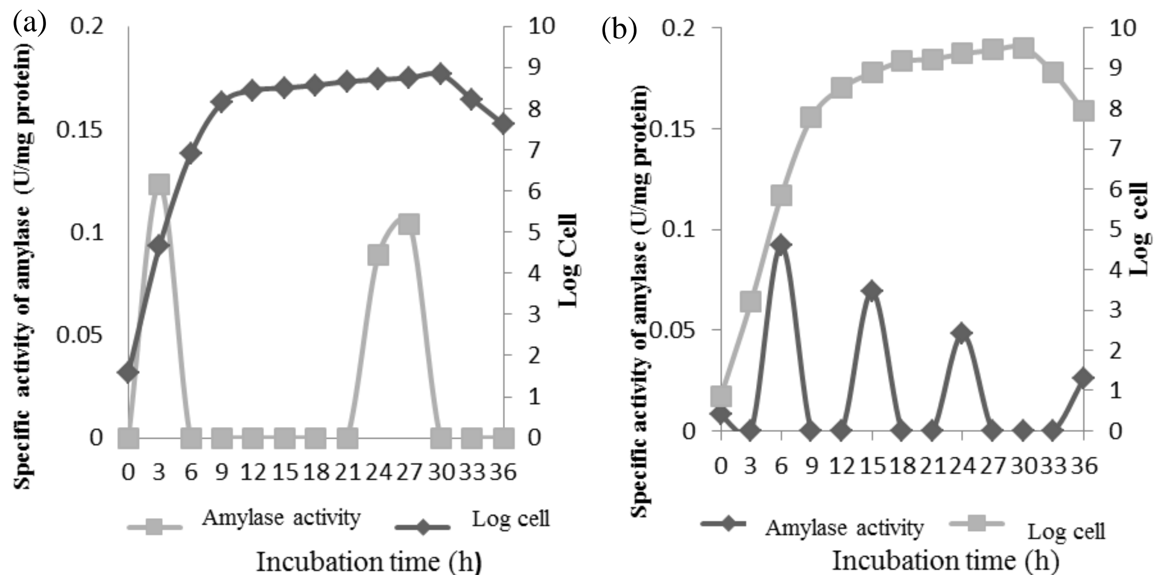


Figure 2 The growth curve and amylase production curve (a) *Lactobacillus rhamnosus* IN13 dan (b) *Lactobacillus plantarum* ITN13

Both of *L. rhamnosus* IN13 and *L. plantarum* ITN13 are showed bacteriocin-producing activities that inhibit pathogenic bacteria such as *S. typhimurium* ATCC 14028 and *L. monocytogens* ATCC 7644. Bacteriocin produced by *L. rhamnosus* IN13 begins to be produced at 12-hour incubation, the optimum of bacteriocin production at the 30th hour [11]. But amylase production of *L. rhamnosus* IN13 maximum at 3 hours incubation while *L. plantarum* ITN13 at 6 hours in the exponential phase, respectively. Thus both isolates produce amylase not at the same time as bacteriocin production.

More than one peak of amylase activity formed during 36 hours of incubation, it is thought there is more than one type of amylase produced.The optimum production of enzymes in the exponential phase indicates that the amylase enzymes production are used for growth and cell metabolism, while the optimum enzymes produced in the stationary phase are more functioned as a self-defense or antagonistic response.The production of enzymes by a bacterium is strongly influenced by media components, especially carbon and nitrogen. In addition, enzymes are also influenced by physical factors such as temperature, pH, length of incubation of the inoculum, and density of the inoculum [12].

3.3. Characterization of amylase activity

Isolate of *L. rhamnosus* IN13 had optimum conditions of amylase activity at pH 5, while *L. plantarum* ITN13 at pH 6. The optimum temperature of amylase activity of *L. rhamnosus* IN13 at 45 ° C and *L. plantarum ITN13* at 35 °C (Figure 3). The amylase activity of the Lactobacilli isolates were in the same range as previously published, viz *L. fermentum* Ogi E1 and *L. manihotivorans* LMG 18010T which had optimum pH of 5.0 and 5.5, respectively (Souza and Magalhães 2010). The optimum temperature of *L. fermentum* Ogi E1 and *L. manihotivorans* LMG 18010T amylase activity at 30^0C and 55 ^{0}C respectively were differed from the Lactobacilli tested.

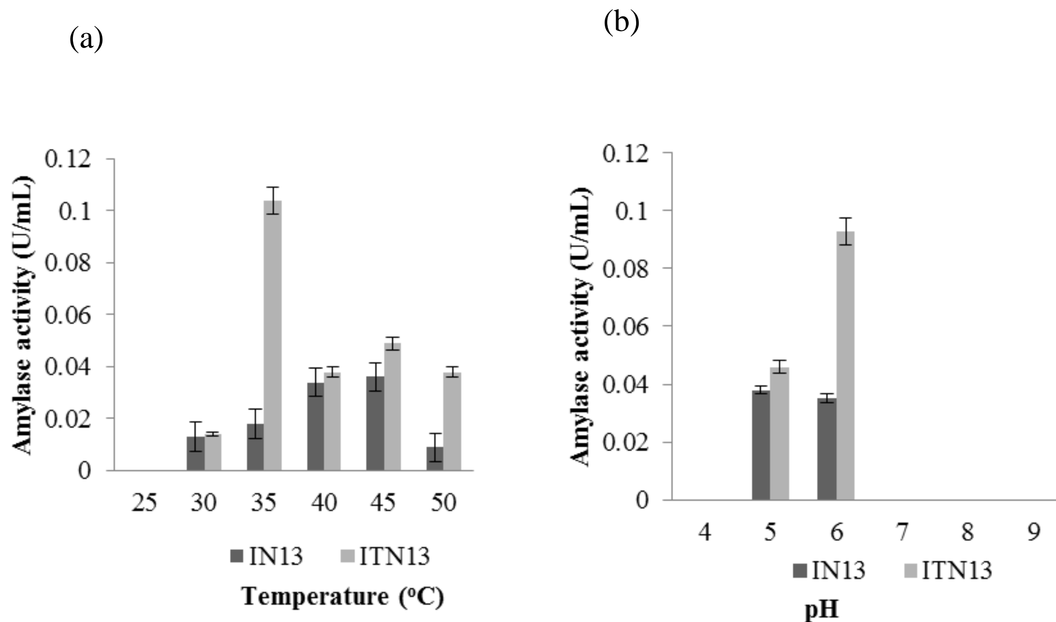


Figure 3 The optimum amylase activity of *L. rhamnosus* IN13 and *L. plantarum* ITN13 (a) temperature and (b) pH

4. CONCLUSION

Lactobacillus rhamnosus IN13 and *L. plantarum* ITN13 which previously reported as bacteriocin producing also produced amylase enzyme at exponential phase in MRSB medium containing 1% starch. The optimum amylase activity from *L. rhamnosus* IN13 at 45°C and pH 5, while the optimum of amylase *L. plantarum* ITN13 at 35°C and pH 6.

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