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Determination of Activity Optimum Inulinase from Bacteria Screened from Dadih (Curd)

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ABSTRACT

Dadih (curd) contains Lactic Acid Bacteria (LAB), most of them are probiotics. Probiotics favour nutritional supplements called prebiotics. One compound that belongs to the prebiotic group is inulin. Inulinase hydrolyses inulin into fructose and FOS. The aim of the study was to determine the optimum activity of inulinase from bacteria screened from dadih (curd) at variation of pH (4.5; 5; 5.5; 6; 7; 8), temperature (20°C, 30°C, 37°C, 45°C) and variation of substrate concentration (0.5%; 1%; 1.5%; 2%; 2.5%). Determination of inulinase activity was determined using Dinitrosalicylic Acid (DNS) reagent method. Absorbance was measured using spectrophotometer at 488 wavelengths. The results obtained from this study were the optimum activity of inulinase (bacterial inulinase degrading inulin) at pH 5.5; temperature 37°C and substrate concentration 2.5%.

Key Words: Activity of Inulinase, Dadih (Curd), Inulinase.

1. INTRODUCTION

One example of a traditional food that is safe to consume and has many benefits for the body is fermented buffalo milk, known as dadih. In the fermentation process of buffalo milk, milk lactose is fermented into lactic acid, giving the milk a sour flavour. Dadih is a fermented buffalo milk product native to West Sumatra that has a thick texture like tofu. Dadih is made from buffalo milk that is put into bamboo and covered with banana leaves that have been weathered, then left for 48 hours at room temperature. Good quality curd is white in colour and has a distinctive sour milk aroma. The nutritional composition of dadih were 82.10% water, 6.99% protein, 8.08% fat, and pH 4.99 [1].

Dadih (curd) contains Lactic Acid Bacteria (LAB), most of them are good bacteria or probiotics. The growth of good bacteria or probiotics requires prebiotics. One of the compounds included in the prebiotic group is inulin [2]. Inulin is a carbon source for bacterial growth. Lactic acid bacteria in curd prefer inulin as a prebiotic. Inulin is the best prebiotic [2,3]. Inulin is metabolised by probiotics to produce *Short Chain Fatty Acids* (SCFA) [4,5].

Lactic Acid Bacteria found in dadih (curd) include *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus acidophilus, Lactobacillus paracasei, Streptococcus faecalis subsp. liquefaciens, Leuconostoc mesenteroides, Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Lactococcus casei subsp. diacetylactis, and Bifidobacteria* [5,6,7,8]. The first-order reaction in inulin hydrolysis involves the enzyme catalyst inulinase. The ability of the inulinase enzyme as a catalyst in hydrolysing inulin is called inulinase activity [9]

Inulinase activity is the ability of the inulinase enzyme to catalyse the hydrolysis reaction of inulin which is proportional to the fructose formed under certain conditions. Some factors that affect inulinase activity include pH, temperature and substrate concentration. Enzymes have an optimum pH and temperature so that enzymes can work effectively. Inulinase enzyme activity can be determined using *Dinitrosalicylic Acid* (DNS) reagent method[10]. Enzyme activity with *Dinitrosalicylic Acid* (DNS) method has the principle that the reducing sugar will react with DNS reagent to form a compound of 3-amino-5-nitrosalisilic acid which is brownish yellow [11][12]. This study aims to determine the optimum activity of inulinase that is screened from dadih (Curd)

2. MATERIALS AND METHODS

2.1 Materials :

The materials used were bacteria, inulin, DNS reagent, lugol iodine solution. Degrading bacteria were explored from dadih using media with the following composition (g/L): 2g $(NH_4)2SO_4$, 14g KH_2PO_4, 6g $K_2HPO_4.3H_2O$, 0.2g $MgSO_4.7H_2O$, 1g trisodium citrate, 20g bacto agar, 10g inulin [13].

a. Screening of Inulin-Degrading Bacteria from dadih (curd)

Screening was performed aseptically, one gram of curd was weighed using a watch glass and placing it to an Erlenmeyer containing 9 mL of sterile liquid media (10^{-1} dilution). Incubate the Erlenmeyer in a shacker incubator at 37°C for 24 hours. Next, pipette 1 mL of bacterial culture from dilution 10^{-1} and transfer it into another Erlenmeyer containing 9 mL of sterile liquid medium (dilution 10^{-2}). Repeat this process sequentially until reaching dilution 10^{-5}. One mL of bacterial culture from each dilution was spread onto a petri dish containing sterile solid media using the spread plate method. Incubate the petri dish at 37°C for 24 hours. Single colonies were selected and streaked on fresh solid media for colony purification, then incubated at 37°C for 24 hours. Pure isolates were then inoculated onto agar media and transferred into 9 mL of sterile liquid media three times (twice for working culture, once for stock culture), and incubated in a shaker at 37°C for 48 hours. For stock culture preparation, bacterial isolates were mixed with sterile glycerol in the ratio of 0.85 mL isolate and 0.15 mL glycerol. The mixture was put into a micro tube and homogenised. The stock culture is stored in the freezer [14][15].

b. Bacterial Screening using Lugol Iodine Solution

The inulinase activity of the isolates was screened with solid media containing 2% agar and 1% inulin powder, then the media was spotted with 100µL of bacterial culture from the liquid media and the solid media was incubated at 37°C for 24-48 hours. To see the inulinase activity by the isolates, lugol iodine was poured on the petri for 3-5 minutes then discarded, then poured distilled water to remove the remaining lugol iodine. The clear zone indicates that the bacteria express inulin [16][17].

c. Crude inulinase extraction

A single bacterial colony was taken using an ose needle and placed into 15 mL liquid medium in a 50 mL Erlenmeyer and then suspended for 18 hours at 37°C at 120 rpm. The bacterial culture was transferred into a microtube for centrifugation at 8000 rpm at 4°C for 30 minutes. The clear extract that had been separated from the bacterial cell sediment was pipetted into a clean and sterile microtube. The extract is the crude enzyme that will be used for the determination of inulinase activity

d. Measurement of Absorbance of Fructose Standard Solution

Absorbance measurements were taken for 100-500 µg/mL standard solutions, which were diluted from a 1000 µg/mL master solution. Each solution was pipetted (75 µL) into a microtube, followed by the addition of 75 µL of DNS reagent. The microtubes containing the solutions were heated in boiling water for 10 minutes, then cooled to room temperature before adding 850 µL of distilled water while homogenizing. Absorbance was measured at λ488 nm. For the blank, 75 µL of distilled water was used as a substitute for the fructose standard solution, following the same procedure as described above [9].

e. Measurement of inulinase enzyme activity

The inulinase activity of inulin-degrading bacteria was determined at pH variations of 4.5, 5, 5.5, 6, 7, and 8; temperature variations of 20°C, 30°C, 37°C, and 45°C; and substrate concentrations variations of 0.5%, 1%, 1.5%, 2%, and 2.5%. Two microtubes were prepared with different mixtures. The first microtube, labelled Sample Enzyme (ES), was filled with 50 µL of 2% inulin solution, 50 µL of acetate buffer or 0.2 M phosphate buffer (for temperature variation), and 50 µL of crude inulinase enzyme. The second microtube, labelled Enzyme Control (EK), was filled with 50 µL of 2% inulin solution, 50 µL of acetate buffer or 0.2 M phosphate buffer (for temperature variation), and 50 µL of inactivated crude enzyme as control.

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Microtube were incubated at room temperature for 30 minutes and then heated in boiling water for 10 minutes. Microtube were then cooled to room temperature, and 150 µL of DNS reagent was added. Microtube were heated in boiling water for 10 minutes, cooled to room temperature, and 700 µL of distilled water was added. The solution was homogenized, and the absorbance was measured at λ 488 nm. microtube, filled with distilled water instead of sample, received the same treatment (note: Microtube as blank containing no enzyme and substrate). This procedure was repeated for the specified variations of pH, temperature, and substrate concentration [18].

f. The data analysis technique

This study used 2 data, namely qualitatively and quantitatively. Qualitative analysis obtained clear zone data. Quantitative analysis of the absorbance data of fructose standard solution and inulinase activity data on variations in pH, temperature and substrate.

3. Result and Discussion

a. Screening of Inulin-degrading Bacteria from Dadih

Screening and isolation were carried out by 2 methods, namely *undirect isolation method* and using lugol iodine solution. undirect isolation method can be done in 2 ways, namely spread plate technique and streak plate technique. The results of *undirect isolation method* can be seen in Figures 1 and 2

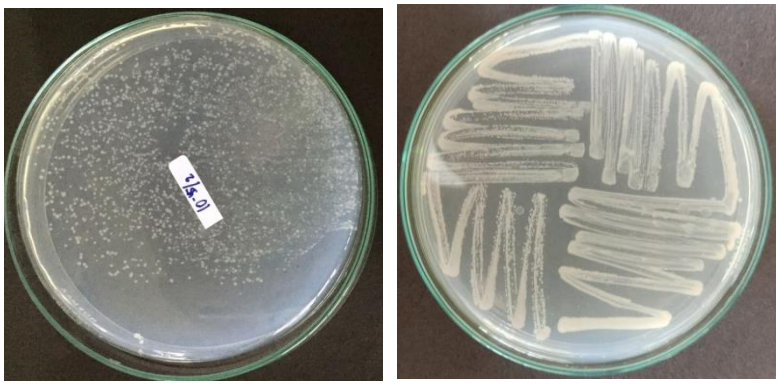

Figure 1. Screening with the spread plate method 2. Screening with the streak plate method

The results of the screening the bacteria using Lugol's iodine solution can be seen in Figure 2, undicated by the clear zone that formed

Figure 3. skrinning using lugol iodine solution

b. Standard Curve Absorbance of Fructose Solution

The absorbance of the standard curve was measured at concentrations of 100-500 µg/mL using a Genesys 30 spectrophotometer at a wavelength of 488 nm with the addition of DNS (*Dinitrosalicylic Acid*) reagent. Based on the absorbance results of the fructose standard solution, a graph was obtained showing the relationship between the concentration of the solution and the absorbance. The graph illustrating this relationship can be seen in Figure 4

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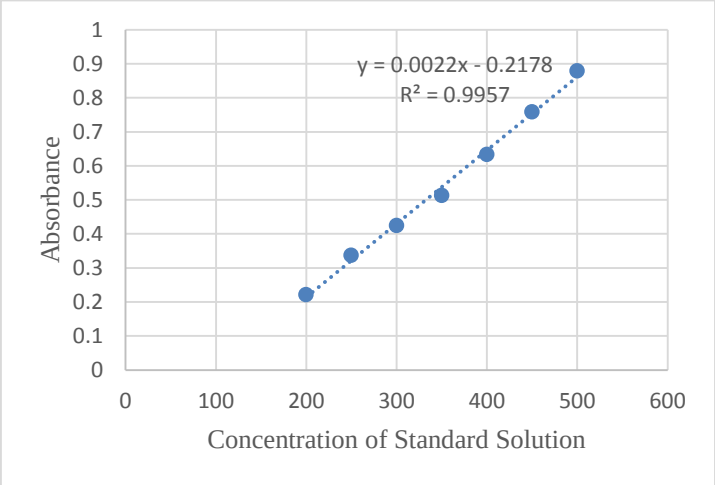

Figure 4. Standard Curve of Fructose Solution

c. Determination of Inulinase Activity at Various pH

Determination of activity based on pH variation can be seen in Figure 5. Based on the research that has been done, the optimum pH of inulinase is 5.5.

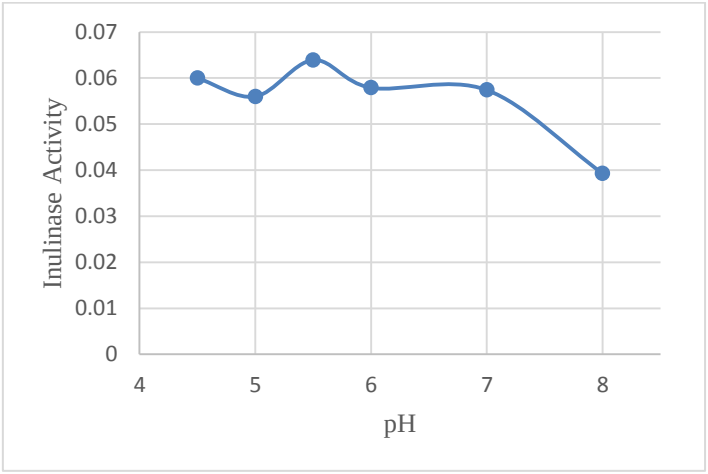

Figure 5. Inulinase activity in pH variation

d. Determination of Inulinase Activity at Variation of Temperature (optimum pH)

The determination of inulinase activity based on temperature variation can be seen in Figure 6. Based on the research that has been done, the optimum temperature of inulinase is 37°C.

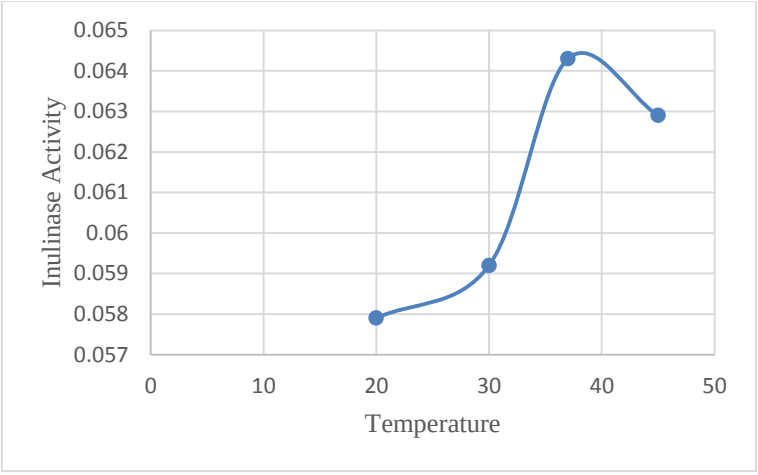

Figure 6. Inulinase Activity on Temperature Variation

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e. Determination of Inulinase Activity at Varying Substrate Concentrations

The determination of inulinase activity based on variations in substrate concentration can be seen in Figure 7. Based on the research that has been done, the optimum temperature of inulinase is 2.5%.

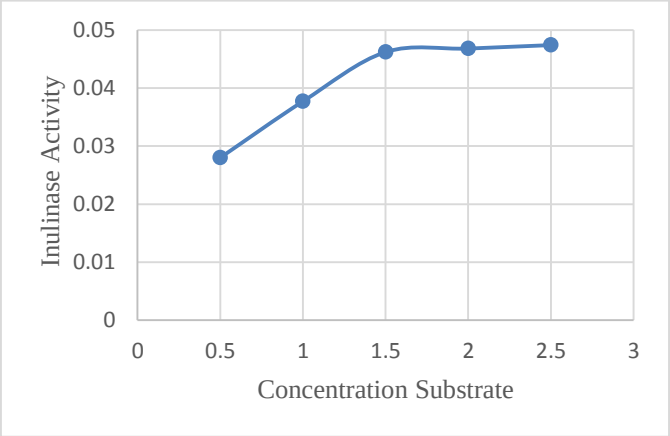

Figure 7. Inulinase Activity with respect to Substrate Concentration

3. DISCUSSION

a. Screening of Inulin-Degrading Bacteria from Dadih (Curd)

The dadih (Curd) samples used in this study came from the Tilatang Kamang area of Agam Regency, West Sumatra, as shown in Figure 8. Screening of inulin-degrading bacteria was carried out using 2 methods, namely undirect isolation method and using lugol iodine solution. In the undirect isolation method, it is done in 2 ways, namely spread plate and streak plate. spread plate is a technique that serves to count the number of bacterial colonies in a certain volume. Streak plate technique serves to obtain pure cultures of bacteria without being contaminated with other microbes.

Figure 8. Dadih (Curd)

The growth of inulin degrading bacteria at the dilution level of 10^{-1} to 10^{-4} produces a tight group, the morphology is still difficult to observe and difficult to take a single colony. At the dilution level of 10^{-5} contained in Figure 1, the resulting bacteria have formed single colonies that are not tight and their morphology can be observed such as convex round shape with flat edges, white in colour and have different sizes (medium and small).

Colonies selected for isolation of inulin degrading bacteria from curd are specific single colonies that are small in size. These single colonies were inoculated onto agar media using streak plate method and incubated at 37°C for 24 hours to obtain pure bacterial isolates. Pure bacterial isolates produced on solid media can be seen in Figure 2. Pure bacterial isolates were inoculated into liquid media and shaken at 37°C for 48 hours. Pure isolates that have been dishacker then stored in glycerol stock and tested.

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Previous researchers have successfully isolated inulin degrading bacteria using the undirect isolation method derived from soil bacteria [19]. [15,20]have successfully isolated inulin degrading bacteria from hot springs in Solok, West Sumatra. [14] has successfully screened inulin degrading bacteria from dahlia tuber rhizosphere using dahlia tuber inulin. The success of the undirect isolation method in isolating inulin degrading bacteria suggests that, if inulin is present inulin degrading enzymes will be expressed [21].

b. Determination of Fructose Solution Standard Curve

Based on the results of measuring the absorbance of fructose standard solution, a graphical relationship between the concentration of the solution and the absorbance of the solution was obtained. Linear regression equation was obtained y=0.0022x - 0.2178 with a regression of 0.9957 (Figure 4). This equation serves to determine the fructose content of the sample which is the product of inulin hydrolysis using inulinase.

c. Determination of Inulinase Activity.

Inulinase activity is the amount of inulinase required to produce 1 µmol of fructose or fructooligosaccharide per minute under certain reaction conditions. Inulinase activity is expressed in units of U/mL which is the amount (µmol) of fructose or fructooligosaccharide per minute, the more fructose produced, the better the inulinase activity. The absorbance measurement results of reducing sugars were substituted into the linear regression equation of the fructose standard curve to determine the fructose content of inulin hydrolysis due to inulinase. . One unit of enzyme activity is the amount of enzyme that can liberate one µmol of inulin fructose per minute. Inulinase activity can be determined using the formula. enzyme activity :

$$\frac{[X]}{Mr\ fruktosa\ .t} Unit/mL$$

Inulinase activity is influenced using several factors such as pH, temperature and substrate concentration. Another factor that affects the amount of product produced is the incubation time, the longer the incubation time, the more product will be produced so that all substrates or enzymes will finish reacting. Each enzyme has an optimum pH and temperature for work. If the enzyme works above or below the optimum pH and temperature, the enzyme does not work optimally.

1). Variation of pH

pH is one of the most important factors in enzyme activity. Enzymes are able to work at a certain pH, enzymes will work optimally at the optimum pH. From the research that has been done, the optimum pH of Lactic Acid Bacteria is 5.5 which can be seen in Figure 8 with pH variations of 4.5-8 at room temperature.

Changes in pH will change the conformation of the enzyme, the group in the active position and the binding of the enzyme substart. This is because the enzyme has a group that is easily ionised called the prototropic group which is in the chain up to the amino acid. The pH conditions are too far from the optimum pH causing the enzyme to denature due to the increasing number of prototropic groups that are charged similarly causes the repulsive force of large protein molecules and the opening of folds of protein molecules causes the catalytic lost [18].

This happens because the change in pH value is in line with the change in the charge of amino acid residues in the enzyme. The higher the pH value, the less [H^+] in the solution. As a result, the active group loses its positive charge which inhibits enzyme activity because the bond between the enzyme and the substrate becomes inappropriate [22]. Based on previous research, inulinase derived from Aspergillus sp fungus has a pH in the range of 4.5-5.0 [23].

Inulinase isolated from mesophilic bacteria rhizosphere dahlia tubers has optimum activity at pH 4 [18]. The optimum activity of inulinase derived from Bacillus Cereus bacteria has an optimum pH of 7 [24]. According to Saryono, inulinase derived from Aspergillus niger has an optimum pH of 4.6 [25].

2). Temperature variations

Besides pH, enzyme activity is also affected using temperature. Inulinase activity increases with an increase in temperature. If the temperature is high, the enzyme will work faster to reach the optimum. Enzymes produced from different sources have different optimum temperatures.

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Based on the research that has been done, the activity of inulinase derived from Lactic Acid Bacteria has an optimum temperature of 37°C with a large activity of 0.0643 U/mL, the activity decreases after passing the temperature of 37°C, this shows that the enzyme is denatured after passing the optimum temperature. Temperature can accelerate catalytic reactions because there is an increase in catalytic energy so as to increase the chances of enzyme binding to the substrate, but in too high temperature conditions the enzyme catalysis reaction will decrease [18].

3). Substrate Concentration Variations

Substrate concentration is a factor that can affect inulinase activity. Based on the research conducted, inulinase activity increases with increasing substrate concentration up to 2.5% with an optimum activity of 0.0677 U/mL, the higher the substrate concentration, the lower the enzyme production. Inulin is a carbon source that acts as an enzyme growth. The enzyme will work optimally at the optimum substrate concentration, but will be constant if the substrate concentration continues to be added under fixed enzyme conditions. The addition of substrate to the enzyme causes more substrate to bind to the active side of the enzyme and will increase enzyme activity [18].

4. CONCLUSIONS

The optimum inulinase activity of inulin-degrading bacteria screened from curd had an optimum pH of 5.5; temperature of 37°C and substart concentration of 2.5%.

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