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Activity Test, Selectivity, Stability of Chitinase on Amobil Chitosan Membranes

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Abstract

The use of enzymes for industrial functions needs enzymes that are stable, selective and might be used repeatedly. The aim of the study was to determine the chitinase enzyme's function, selectivity, and stability in amobil chitosan membranes. The research method consisted of stages: production of the chitinase enzyme which included the manufacture of chitin colloidal substrate, rejuvenation of thermophilic bacteria, preparation of the inoculum and determining the optimum time of production, fractionation of ammonium sulfate, chitinase enzyme immobilization technique and activity, stability and selectivity test of amobil enzyme. The results demonstrated that chitinase activity, which incorporates the optimum temperature and thus the optimum concentration of production within the immobilization technique, had an optimum temperature of 65°C on day 4 of production time with an OD value of 0.9876. The selectivity of amobil chitinase with metal ions Cd (II), Pb (II), Zn (II), and Hg (II) demonstrated that amobil chitinase was selective for these ions. Eamobil chitinase was heat stable at 55-75°C and resistant to organic solvents, suggesting that it could be used repeatedly.

Keywords: *Chitinase Enzyme; Enzyme Immobilization; Chitosan Membrane*

1. Introduction

Chitinase is a direct chitin degradation enzyme that can transform chitin to chitosan [1]. This enzyme is commonly used to transform N-acetamide (-NH-CO-CH3) gums in 1,4 N acetyl glucosamine (chitin) to an amine group (-NH2) in 1,4 N glucosamine (Chitosan).

Chitinase enzymes from a variety of microorganisms (bacteria and fungi) are extracellular enzymes secreted into culture media via an external membrane [3]. The properties of the enzyme produced are affected by optimizing the fermentation conditions for the development of microbial chitinase [4]. The production of the chitinase enzyme depends on several factors such as temperature [5] growth, pH, nitrogen composition [6], carbon and lipid sources [7], inorganic salt concentration [8] as well as oxygen availability [9]. Chitinase produced from Bacillus Themal Lw – 411 is isolated from North Sulawesi waters has specific activity in the optimum temperature of 80°C [10]. Meanwhile, the optimum temperature of chitinase from thermophilic bacteria baciluus sp is 50°C [11]. Chitinase from

Colletotrichum capsici and Fusarium oxysporum has been purified and The optimum pH of chitinase is in the range of pH 11.5 -12 [12]. Chitinase from Actinomycetes isolateshas the optimum pH of 8, where the Optimum pH test is carried out using three kinds of buffers, namely phosphate citrate buffer (pH 5 - 7) phosphate buffer (pH 8-9) and NaOH buffer [13]. The use of isolated chitinase enzymes has several difficulties, namely the enzyme is not stable enough under operating conditions [14, 15]. As a water soluble free molecule, the enzyme is difficult to separate from the substrate and product, besides this enzyme is difficult to use repeatedly, thus increasing production costs [15, 16].

Nowdays, various attempts have been made to overcome this obstacle, that is by diffusing the enzyme amobil process into the reaction mixture and make it easier to recover the enzyme with simple separation techniques [17, 18]. Enzyme immobilization has several advantages, both in terms of economics and in terms of processes [19, 20]. Immobilization can help to solve some of the problems of handling enzymes as biocatalysts in industry, namely more stable, reusable after the reaction is complete, products are not mixed or contaminated with enzymes and product purification becomes easier, so that production costs can be reduced [21, 22].

Enzyme immobilization techniques can be carried out in various ways, such as: covalent binding of the enzyme to the support material [23], entrapment of the enzyme in the gel [24], enzyme encapsulation [25], adsorption of the enzyme on the surface of the solid [26], crosslinking of a double clustered material [27,]. Enzyme immobilization technique that has been developed recently is by means of adsorption on the surface of solids or membranes [28]. Enzymes can be more durable or less easily damaged when immobilized in a membrane, where the enzyme is separated in the pores of the membrane [29].

The advantage of the enzyme adsorption technique on the membrane compared to other immobiliation techniques is the separation of the products produced from the reactants, reagents and enzymes used [30]. Several factors influence enzyme immobiliation in the supporting membrane, namely enzyme activity, adsorption rate, enzyme stability, and membrane surface area [31].

The focus of the study was to determine the amobil chitinase enzyme's activity, adsorption rate, selectivity, stability, and the morphology of the amobil chitosan membrane. The study's findings result in chitinase enzymes with high activity, selectivity, and stability, which can be used in the food processing industry.

2. Materials and Methods

2.1 Material

The materials used were: Bacillus stearothermophilus thermophilic bacteria, chitin powder (shrimp waste), glucosamine (sigma), chitin glycol, concentrated HCl, concentrated NaOH, bakto agar, yeast extract, NaCl, (NH4) 2SO4, KH2PO4, MgSO4.7H2O, CaCl2, Indole, ammonium sulphate, glacial acetic acid.

2.2 Production of the Enzyme Chitinase

A total of 20 grams of chitin (shrimp waste) was added with 200 ml of concentrated HCL then closed tightly and left for 24 hours at 4 oC. The next stage, treatment in cold temperature, then filtered using a glasswoll, and the filtrate obtained was added with 200 ml of digin water then the pH of the solution was adjusted to 7.0 with the addition of 10 N NaOH and centrifuged at 3500 rpm, temperature 4

oC for 30 minutes. The filtrate is removed and the pellets are washed with cold water then centrifuged again. The pellets (colloidal chitin) are stored at cold temperature (ready to use).

Thermophilic Bacteria Rejuvenation

The rejuvenation of chitinolytic enzyme-producing thermophilic bacteria was selected in a solid medium of composition: bacto agar 1.5%, yeast extract 0.05%, 0.5% NaCl (NH4) 2SO4 0.7%, KH2PO4 0.1%, MgSO4 7 H2O 0.01%, CaCl2 0.025% and 2.0% colloidal chitin. In solid medium the incubation was carried out at pH 7.0 with an incubation temperature of 45oC. The colonies that form the fastest and widest clear zone (halo) are selected, and then stored in a refrigerator for selection in a liquid medium (fermentation medium).

2.3 Preparation of the Inoculum and Determining the Optimum Production Time

In the fermentation medium, the inoculum is the source cell for enzyme production. Selected bacteria inoculated as much as 4-5 ose in a total of 10 ml (medium inoculum) for 100 ml of fermentation medium. The medium contained 0.05 percent yeast extract, 0.5 percent NaCl, 0.7 percent (NH4) 2SO4, 0.1 percent KH2PO4, 0.001 percent MgSO47H2O, 0.025 percent CaCl2, and 0.5 percent chitin glycol. [32.] The inoculum that has been prepared above is poured into the fermentation medium (production medium) which has the same composition as the inoculum medium, then incubated under the same conditions for 4 days. Every 24 hours a sample was taken for measurement of OD (660 nm), protein content using the biuret test adhesion [33]. Culture filtrate (crude extract enzyme obtained by centrifuging the sample at 3000 rpm, temperature 4 oC for 30 minutes.

2.4 Ammonium Sulfate Fractionation

The crude extract enzyme was purified using ammonium sulfate fractionation with saturation levels of 40%, 50%, 60%, and 70%, which were progressively applied while stirring with a magnetic stirrer until completely dissolved and left overnight at 5°C. Centrifugation at 6000 rpm for 30 minutes at 4 oC isolated the enzyme filtrate, which was then dissolved in 0.1 M citrate phosphate buffer pH 7.0.

2.5 Chitinase Enzyme Immobilization Technique

A number of ingredients are mixed in various ratios consisting of acetic acid; chitosan: chitinase with a ratio of 1: 1: 1; 1: 2: 2; 1: 2: 3 and 1: 3: 3, stirring evenly. Then the membrane solution is poured on a square plate with the desired thickness of the membrane, then left in the open air for 48 hours so that the solvent evaporates so that a thin layer of chitinase immobilization membrane is formed.

2.6 Immobilized Chitinase Enzyme Activity Test

Chitinase activity was tested using the Ueda and Arai methods. The reaction mixture contained 0.3% colloidal chitin 0.1 M phosphate buffer and enzyme solution incubated at 55°C for 60 minutes. The remaining chitin in the mixture was measured for its turbidity at 660 nm. One unit Enzyme activity was defined as the amount of the enzyme that caused a decrease in absorption by 0.001 at 660 nm per minute.

2.7 Characterization of Immobilized Chitinase Enzymes

Biochemical characterization of chitinase enzyme was observed were optimal temperature and pH, heat stability, properties of divalent cations and molecular weight of the enzyme. Properties of temperature and pH using several types of buffers (pH: 3.0 - 11). Heat stability properties are measured by testing the enzyme at the optimal temperature for several hours / day, and each time interval a sample of the enzyme solution is taken lateractivity measured [34].

Selectivity

The coefficient of selectivity was determined by observing the percentage of inhibition against various standard solutions of the main Cd (II) ions and standard solutions of interfering ions (M n +). Each observation result was plotted in the inhibition percentage relationship curve with log [M n +] to obtain the measurement concentrations, sensitivity, and selectivity coefficients.

3. Result and Discussion

3.1 Bacillus Steraothermophilus Isolate

The culture of Bacillus steraothermophilus is an M-1 isolate which has chitinolytic activity and can grow optimally at temperature conditions of 50 °C with a pH of 7.0. Isolate M-1 was grown on a solid medium to rejuvenate the bacteria using colloidal chitin as the substrate. Colonies of M-1 isolates in solid medium at 55 °C and pH 7.0 can be seen in Figure 2.

3.2 Growth Curve Measurement

Isolate M-1 is grown further on the fermentation medium to determine the optimum production conditions, because the bacterial growth produced in the fermentation medium can be properly monitored under optimal conditions [35]. The growth of isolate M-1 in fermentation medium increased steadily until day -4 and began to decline on day -5. The relationship between optical density (OD) and incubation time can be seen in Figure 2.

Fig. 2 Opticel density against incubation time

Figure 2 indicates that bacterial growth is at its peak on day 4 with an OD value of 0.9956 and starts to decline on day -5. This is due to an accumulation of toxic products or nutrient depletion; as a result, some cells die while others grow and divide. Enzyme development is usually at its peak in the stationary period. [36]. In this phase, the growth rate is accompanied by the speed of deathcells [37]. Immobilization of the ctinase enzyme on the ktiosan membrane preserves the optimum conditions for the amobil chitosan membrane, namely the concentration ratio of 1: 3: 3 (acetic acid: chitosan: chitinsase) The morphology of the amobil chitinase enzyme on the chitosan.

3.3 Determination of Immobilized Enzyme Activity

In accordance with the growth curve calculation, the optimum activity of the amobil enzyme was also determined.

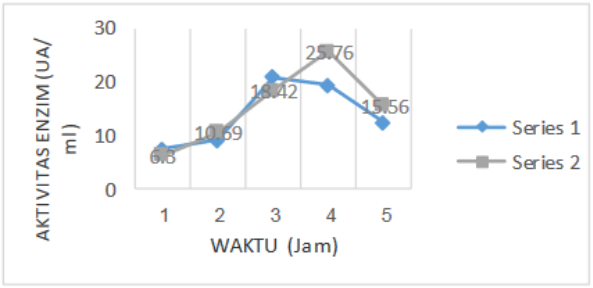


Fig. 3 Immobilized enzyme activity

The Amobil enzyme activity was obtained at the optimum optimization period on day 3 with an enzyme activity of 25.76 UA / ml, while for amobil enzyme, the enzyme activity was obtained at the optimum incubation time on day 4, namely sebesaer. The ability of amobil enzymes to break down the substrate into products is optimal in this situation [38].

3.4 Determination of the Optimal Temperature of Immobilized Chitinase

The activity of amobil chitinase is affected by temperature. Amobil chitinase activity can increase to optimal levels with increasing temperature, then decrease. This is due to the enzyme being denatured, which causes it to lose some of its function. Figure 4 depicts the effect of temperature on chitinase activity:

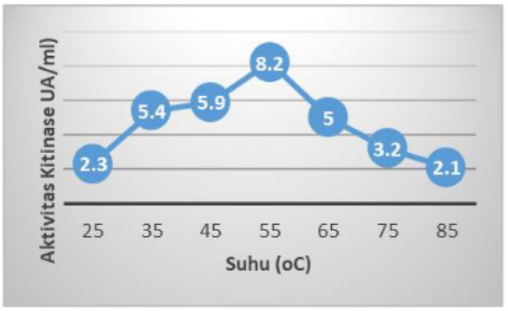


Fig. 4 Effect of temperature on immobilized chitinase activity

Increasing the temperature causes the enzyme kinetic energy to be higher. As a result of vibrational motion, transition, and rotation of the enzyme with the substrate will increase, so that the chances of both reacting are greater [39]. The maximum growth rate of some thermophilic bacteria ranges from an optimal temperature of 55 - 70 °C. In this range, the secreted enzyme production is optimal [40].

The activity of the amobil chitinase enzyme in chitosan support materials at different chitosan concentrations revealed that the greater the chitosan concentration, the greater the amobil enzyme activity.

Table 1 Specific activation of immobilized chitinase on chitosan membranes

No.	Chitosan concentration (mg)	Percentage of Immobilized (%)	Specific Activities (UA / mg)
1	2	46.29	0.63
2	4	44.80	0.71
3	6	36.79	1.89
4	8	21.47	1.74

Amobil chitinase activity increased with rising chitosan concentration, namely at a concentration of 2 mg the initial activity was 0.63 UA / mg and after the addition of 4 mg concentration the activity increased to 0.71 and amobil chitinase activity increased at its optimum condition at the concentration of chitosan reaching 6 mg with a particular activity of 1.89 and began to decline in increments of up to 8 mg [41] [42].

3.5 Selectivity of the Immobilized Chitinase Enzyme

Metal ions are needed as activators in order to increase the activity of specific enzymes [43]. Metal ions, on the other hand, can act as inhibitors at various concentrations [44]. The enzyme requires metal ion as a part on the active site [45]. The effect of metal ions on amobil kiinase action is depicted in the figure below.

Table 2 Addition of metal ions to immobilized chitinase

No.	Chitosan concentration (mg)	Percentage of Immobilized (%)	Specific Activities (UA / mg)
1	2	46.29	0.63
2	4	44.80	0.71
3	6	36.79	1.89
4	8	21.47	1.74

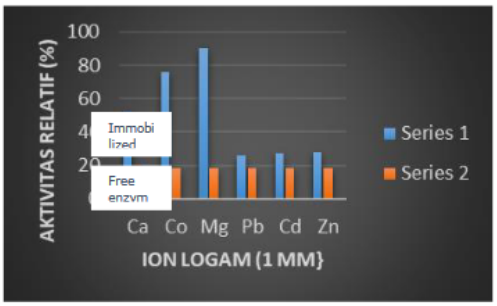


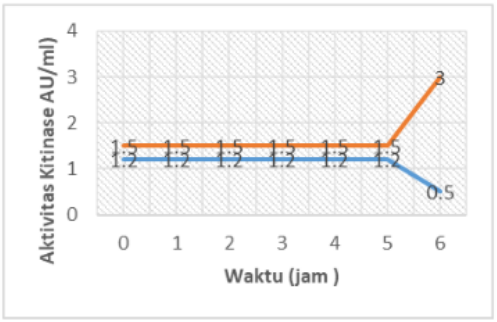
Figure 5 Addition of immobilized chitinase metal ion

Figure 5 shows that the addition of Mg ions increases amobil chitinase activity, while Ca, Co, Pb, Cd, and Zn ions decrease chitinase activity [46]. These ions function as chitinase inhibitors (inhibitors) [47]. The majority of thermophilic chitinases isolated belong to the cysteine chitinase group, including Bacillus Thermal LW-411 chitinase [48]. Lindetherium Collterium [49]. According to previous research, the addition of metal ions can increase amobil chitinase activity and decrease cysteine chitinase. In this study, the addition of Cd2 + metal ions to amobil chitinase tends to cause the formation of ES and EI complexes, which occur in the serine side chain [50]. The mechanism for the formation of the ES complex occurs because of the interaction between imidazole His 57 and H on serine [51]. So, it will cause the nucleophilic O serine attack on the C carbonyl substrate [52]. Furthermore, EI occurs because the Cd2 + metal ion is covalently bonded in coordination with 1 S atom from the 1 ar cysteine group [on N from the jhistidine specialty and 1 aotom O from the cymbal gus [48]. The active residue that does not bind to the subrarta because it is already bound to the metal ion [64]. So that the EI complex is formed [49]. The metal ion cd2 + inhibits the development of the ES complex, resulting in a small product that weaves the chitinase activity [50]. The bond between the metal ion and chitianse is tight, and the process is frequently defined as irreversible or irreversible [51]. The addition of the metal ion cd2 + inhibits the formation of the ES complex, so that the product is small and causes it to weave the chitinase activity [50]. The bond between the metal ion and chitianse is strong and is often described as an irreversible or irreversible process [51]. The addition of the metal ion cd2 + inhibits the formation of the ES complex, so that the product is small and causes it to weave the chitinase activity [50]. The bond between the metal ion and chitianse is strong and is often described as an irreversible or irreversible process [51].

3.6 The Adsorption Rate of Immobilized Chitosan Membranes

3.7 Termaostabitas Properties

Testing of chitinase termsotabity was carried out at two temperature levels, namely 55 °C and 75 °C. Incubation was carried out for 48 hours, during the first five hours sampling was carried out every hour, then sampling was carried out at 24 and 48 hours.



After 5 hours of incubation at 55°C, chitinase activity was reduced by approximately 34%. This is due to an increase in chitinase denaturation, which increases with increasing incubation time. According to [42], the chitinase activity of Colletgericum libdemutianum is stable up to 45°C and then decreases at 50°C with a residual activity of 20%.

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