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The Chemical Modification of Cellulase from Locale Bacteria Isolate *Bacillus subtilis* ITBCCB148 with Glyoxylic Acid

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This research aims to increase the stability of cellulase obtained from *Bacillus subtilis* ITBCCB148 using glyoxylic acid as modifier. To achieve this aim, the research phases performed were production, isolation, purification and chemical modification on cellulase and were followed by characterization of the purified (native) and modified enzyme to know the stability increase of the enzyme. The result showed that the purified enzyme has optimum pH 6, temperature optimum 60ºC; k_i = 0,066 min.$^{-1}$; $t_{1/2}$ = 10.50 min.; and ΔG_i = 100.7330 kJ/mol, while the modified enzymes with glyoxylic acid with modification degree of 70.54; 78.68; 86.43% have similar optimum pH and temperature with the native enzyme, but the thermal stability of the three modified enzymes were k_i = 0.031; 0.033; and 0.037 min.$^{-1}$, $t_{1/2}$ = 22.35; 21.00; and 18.72 minutes, and ΔG_i = 102.8253; 102.6522; and 102.3354 kJ mol^{-1}, respectively. The chemical modification has been able to increase the thermal stability of the modified enzymes 1.8 – 2.1 times more than the native one.

Key words: *B. subtilis* ITBCCB148, cellulase, chemical modification, glyoxylic acid.

The use of enzyme in industrial sectors increases rapidly due to the rapid growth of industries especially in food, drink, textile and paper industries. Besides used in industrial sectors, enzymes might be used in the industrial waste processes[1]. Cellulase is a hydrolase enzyme that hydrolyze the cellulose at β-1,4-glycoside bond on cellulose molecule to produce glucose[2]. Cellulase can be obtained from variety of sources such as plants, animals and some microorganisms of fungi, bacteria and protozoa, where bacteria is the microorganism oftenly and commercially used to produce cellulase[3,4].

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The use of enzyme in industrial processes must fulfill some specific criteria for instance the enzyme is stable at high temperature and extreme pH[5].

In order to get enzyme having high stability and activity at extreme condition, it can be directly isolated from a natural organism living at the extremophilic condition or by doing chemical modification to enzyme isolated from organism not living at extreme condition mesophilic[6].

According to Mozhaev and Martinek[7], the stabilization of enzyme from mesophilic microbia is a preferred way to obtain a stable enzyme, while Mozhaev *et al*.[8] proposed the use of chemical modification to increase the stability of enzyme. Some researches have been performed towards some enzymes produced by locale bacteria isolate *B. subtilis* ITBCCB148, i.e. protease and a-amylase. The isolation and purification process has been carried out as to obtain pure protease which certain

grade purity. Attempts to increase the stability of purified protease have been performed using high molecular weight modifier i.e. NPC-PEG[9] and CC-PEG[10] and also low molecular weight such as dimethyladipimidate[11]. The result showed that the stability of modified enzymes have increased their stability compared to the native enzyme.

Chemical modification on α-amylase using low molecular weight modifiers such as dimethyladipimidate, glyoxylic acid and cytraconate anhydride[12-14], and immobilization with DEAE-cellulose, CM-cellulose and calcium alginate have also been performed[15-17], the result showed that all modified enzymes increased their stability compared to that of the native enzyme.

In this research, cellulase has successfully been isolated and purified from locale bacteria isolate *B. subtilis* ITBCCB148. To increase the stability of the cellulase towards temperature and pH, the chemical modification was applied using the glyoxylic acid as the modifier.

EXPERIMENTAL

Materials

All materials used in this research was pro analysis reagent and used without purification. Local bacteria isolate *B. subtilis* ITBCCB148 was obtained from Microbiology Laboratory, Chemical Engineering Department, Bandung Institute of Technology, Bandung, Indonesia.

Research phases performed were production, isolation, purification and characterization of the purified (native) enzyme similar to the procedures previously reported[18].

Cellulase activity test and protein content determination

The cellulase activity test was performed based on the Mandels method using dinitrosalycilic acid[19] and the protein content determination was done based on the method by Lowry *et al.* [20].

The modification of purified enzyme with glyoxylic acid[21]

10 mL of native cellulase (containing 0.4 µmol/mL) with cellulose 0.5% in phosphate-borate buffer (100 mM K_2HPO_4 and 500 mM H_3BO_3) pH 8.4 was added with 10 µmol glyoxylic acid and 8 µmol $NaBH_4$, the reaction was performed for 30 minutes at 4°C.

Characterization of native and modified enzymes

The characterizations performed were determination of modification degree, optimum pH, optimum temperature, thermal stability test, half live ($t_{1/2}$), inactivation rate constant (k_i), and change denaturation energy (ΔG_i)

Determination of modification degree

Determination of modification degree was done based on the method used by Synder and Sobocinski[22] and as follows: 0.1 mL of modified enzyme was dissolved into 0.9 mL borate buffer (pH 9.0) and then was added with 25 µl 0.3 M TNBS. The mixture was then shaken and left it at room temperature for 30 min. The standard solution was made with the same composition but using the native enzyme, while the blank solution contained 1 mL borate buffer 0.1 M pH 9 and 25 µl 0.3 M TNBS. The absorbance was measured at the λ_{max}. nm.

Thermal stability test of the enzyme before and after the modification

The determination of enzyme thermal stability and pH was done by measuring the residual activity of the enzyme after being incubated for a period of 60 minutes at optimum temperature and pH. The procedure applied was by measuring the enzyme activity after heating process every 10 minutes interval. The initial enzyme activity was given value of 100%[23].

Determination of half-life (t½), k_i and ΔG_i

Determination of k_i value (rate constant of thermal inactivation) of native enzyme and the modified enzyme was done using the first order of inactivation kinetics equation (Equation 1)[24]:

$$\ln(Ei / E0) = -k_i t \quad ...(1)$$

The denaturation energy change (ΔG_i) of the native and modified enzymes was done using the Equation (2)[24]:

$$\Delta G_i = -RT \ln (k_i h/k_B T) \quad ...(2)$$

RESULTS AND DISCUSSION

Determination of modification degree of native and modified enzyme

Determination of modification degree was based on the ratio of lysine residues of the enzyme before and after modification. The ammine group on lysine residue which has been modified i.e. bound to glyoxylic acid, will not react with TNBS and will produce a yellow colour complex that faded

with time. The ammine group that not react with TNBS will give yellow colour complex which getting intense with time. The careful observation indicated that the more faded the yellow colour complex, the higher the modification degree. The modified enzymes with glyoxylic acid produced modification degree of 70.54; 78.68; 86.43%, respectively. The result of modification degree determination is shown in Table 1.

Table 1. Determination of modification degree using 2,4,6-trinitrobenzene sulphonate acid[22]

Sample	ΔA_{420} nm	Modification (%)
Native Enzyme	0.884	0
Glyoxilic acid 5 mg	0.702	70.54
Glyoxilic acid 10 mg	0.681	78.68
Glyoxilic acid 15 mg	0.661	86.43

Table 2. The values of rate of thermal inactivation (ki), half-life ($t_{½}$) and The Energy Change Due To Denaturation (ΔGi) of native and modified enzymes with modification degree of 70.54; 78.68; 86.43%

Enzyme	k_i (min.$^{-1}$)	$t_{1/2}$ (min.)	ΔGi (kJ mol^{-1})
Native	0.066	10.50	100.7330
Glyoxylic acid 70.54%	0.031	22.35	102.8253
Glyoxylic acid 78.68%	0.033	21.00	102.6522
Glyoxylic acid 86.43%	0.037	18.72	102.3354

Determination of optimum pH of native and modified enzymes

Fig. 1 shows that the native and modified enzymes have the same optimum pH of 6.0. Although there is no change on optimum pH, the modified enzyme with modification degree of 78.68% showed a better stability compared to that of the native enzyme and the other modified enzymes (70.54% and 86.43%) at alkaline condition. The modified enzyme with modification degree of 70.54% was more stable at acidic condition. This result is because the modification process alter the charge on the modified enzyme, so the interaction occurred between charge increased the stability of modified enzyme.

Determination of optimum temperature of native and modified enzymes

The result of determination of optimum temperature for both native and modified enzyme showed that all enzymes have the same optimum temperature of 60°C as shown in Fig. 2. No change on the optimum temperature for native and modified enzymes indicated that the three dimension structure of the modified enzyme did not much change. This is because the enzyme modification occurred on lysine residues which were only at the surface of the enzyme, so the glyoxylic acid did not react with active site of the enzyme. As the three dimension structure did not much change, the activation energy did not change either, as a result the optimum temperature of the modified enzyme. Similar results were also reported by others[24,25] that the chemical modification did not always change the optimum temperature of the modified enzyme.

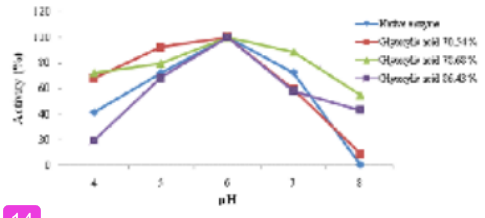

Fig. 1. Optimum pH of the native enzyme and modified enzymes with glyoxylic acid

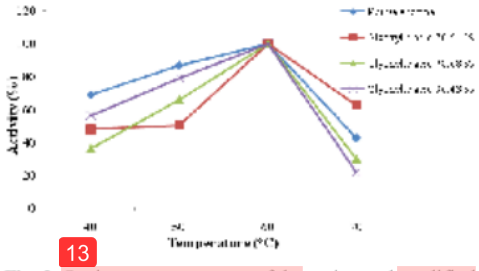

Fig. 2. Optimum temperature of the native and modified enzyme with glyoxylic acid

Determination of thermal stability of native and modified enzymes

Fig. 3. shows the % residual activity of the native and modified enzyme at 60°C for 60 minutes. The native enzyme has % residual activity of only 3%, while the modified enzymes with glyoxylic acid with modification degree of 70.54; 78.68; and 86.43 % have % residual activity of 13, 11 and 9%, respectively. The result indicated that the modified enzymes in general were much more stable than the native enzyme.

The values of rate of thermal inactivation (ki), half-life (t½) and the energy change due to denaturation (ΔGi) of native and modified enzymes are shown in Table 2. The determination of k_i of the native and modified enzymes can be seen in Fig. 4.

Half-life ($t_{1/2}$) and rate constant of thermal activation (k_i)

All modified enzymes have increased their half-life compared to the native enzyme. Stahl[26] stated that half-life of the enzyme will determine the stability of the enzyme. The decrease of k_i values for all modified enzymes indicated the decrease of denaturation rate of the modified enzymes. The increase of enzyme stability based on the decrease of k_i compared to the native enzyme was 1.8-2.1 times. The bond formation between glyoxylic acid and NH_2 group on the side change of lysine residue on the surface of the enzyme caused the k_i values getting lower, so the modified enzymes were not flexible in water and the structure of the modified enzymes became rigid, as a result the modified enzymes were more stable[27].

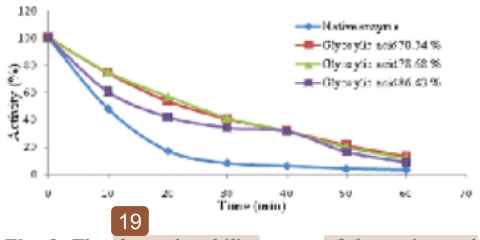

Fig. 3. The thermal stability curve of the native and modified enzyme with glyoxylic acid

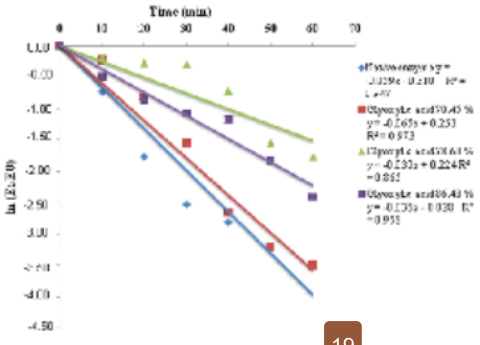

Fig. 4. The correlation of $\ln(E_i/E_0)$ of the native and modified enzyme for the determination of k_i, $t_{1/2}$ and ΔG_i

The energy change due to denaturation (ΔG_i)

The energy change due to denaturation (ΔG_i) in Table 2 illustrated that there was increase of ΔG_i values of all modified enzymes compared to that of the native enzyme. The increase of ΔG_i values indicated that the modified enzymes are more rigid and have the stronger bond, so the enzyme conformation is not easily opened and requires more energy to denaturate them. As can be seen in Table 2, although the increase of ΔG_i was not that high but there was significant increase.

CONCLUSIONS

The chemical modification of native cellulase with glyoxylic acid has successfully increased the thermal stability of the cellulase where the stability was increased 1.8-2.1 times. The decrease of k_i value, increase of half-life and ΔG_i indicated that the modified enzymes were more stable than the native cellulase.

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