

Keratinolytic Enzymes for Cleaning Edible Bird's Nest

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Edible bird's nest (EBN) as a kind of functional food has high economic value depending on the quality such as color and hygiene. The purpose of this research was to find optimum condition for application of keratinolytic enzymes *Bacillus* sp. MTS in cleaning EBN. Activating agents for both enzymes were cation divalents, EDTA, reducing agents, organic solutions, and antibacterial agents. Additives compound that able to increase keratinase activity were used to make cleaning solution and its tested on EBN and human hair. Alcoholic solutions (25% ethanol, 25% methanol, 25% glycerol), and some divalent metallic ions (Ca²⁺, Mg²⁺, Mn²⁺, Zn²⁺) were able to increase keratinase while disulfide reductase was solely activated by 0.05 mM EDTA. The activity of both enzymes was inhibited by NaCl and Na-azide. The activity of keratinase of *Bacillus* sp. MTS in cleaning solution formulated in this research was 2-3 fold as much as control (crude extract) in human hair substrates. Glycerol and cations divalent increasing 2-3 fold keratinase activity in cleaning solution. The solution was successfully applied to cleaning EBN with weight loss 2.3-2.5% approximately.

Keywords: keratinase, *Bacillus*, cleaning, edible bird's nest.

Edible bird's nest (EBN) is one of the most expensive animal products, its a saliva produced by two specific swiftlets, namely *Aerodramus fuciphagus* (white-nest) and *Aerodramus maximus* (black-nest)¹. EBN is a functional food containing high quality nutrients such as protein, carbohydrate, iron, inorganic salts and fiber^{2,3,4}. EBN also serves medical function as antiaging, anticancer, immunity enhancing agent, inhibiting influenza virus infection and improving respiratory and digestive problems^{5,6}. EBN is a good economic value namely 20 million and 10 million rupiahs per kg for white nest and red nest, respectively depending on the quality⁷. The primary factor of EBN quality is color and hygiene,

therefore the whiter and cleaner EBN, the higher is the price.

The steps in cleaning EBN is a tedious work for being meticulous and perseverant to obtain high quality product. Koon and Cranbrook⁷ informed that it takes eight hours to have someone clean 10 nests through soaking process in cleaning solution. The solution however cannot wash away the bird's feather that is stuck inside the nest; therefore, it takes a time-consuming manual process to singly discard the feathers using pincers.

The commonly used cleaning solution among the farmers/collectors is chemical-based solution containing hydrogen peroxide (H₂O₂) known as whitening/bleaching agent. Replacing the chemicals in cleaning process with natural body-safe bleaching namely protease enzyme is one of the solution. Several alkaline proteases have been purified and characterized from many *Bacillus*

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strains^{8,9,10,11}. We had isolated a feather degrading bacteria and its referred to *Bacillus* sp. MTS. This bacillus strain was aerobic mesophilic bacteria, its very effective in degradation of whole chicken feather and this appeared to be related to activity of the extracellular keratinase and disulfide reductase enzymes. The crude extract from the isolate has been showed capable of degrading whole chicken feathers, silk, cocoon, hair and fish scales¹². The purified enzymes of *Bacillus* sp. MTS worked optimally at alkaline pHs, for keratinase at pH8–12, and for disulfide reductase at pH8–10. Optimum temperature for the extracellular keratinase was within 40–70°C, while that for disulfide reductase was 35°C¹³. We attempted to apply this enzymes to solve the problem contaminant feather that is stuck in edible bird's nest.

In the present study, we report the effect of several compound in a cleaning formula containing keratinolytic enzymes and testing the formula to clean EBN. This cleaning solution is expect to be environment-friendly and the enzymatic hydrolysis can shorten the cleaning process of EBN.

MATERIALS AND METHODS

Growth conditions and Enzymes Production

The aerobic mesophilic *Bacillus* sp. MTS was used in these experiments. Its screened and isolated from Tangkuban Perahu crater West Java-Indonesia. The agar medium for culture maintenance pursuant to Macedo *et al.*¹⁴ with few modification viz. contained 0.6% crushed dried feather (powder). For enzyme production, 250 ml medium containing several inorganic salts with 1.0% chicken feather powder was used as substrate, pH was adjusted to 7.5. Incubation was carried out in a 1 l flask at 37°C 100 rpm for 48h. After incubation the culture was strained and centrifuged at 4000 g 4°C for 10 min to harvest the extracellular enzymes.

Protein and Enzymes Assay

Protein concentration was measured at 595 nm in accordance with Bradford, using bovine serum albumin as the standard protein¹⁶.

Keratinase activity was measured according to Walter¹⁷ using 1% feather powder in Tris/HCl (50 mM, pH8.0) as substrate and absorbance of the samples were read at 660 nm. A tyrosin standard curve was made for

quantification. One unit of enzyme activity was as signed as the amount of enzyme which liberate 1 mmol tyrosine in one min.

Disulfide reductase activity was assayed as described by Serrano *et al.*¹⁸ with a few modifications. Enzyme was mixed with 500 ml of Tris/HCl buffer (0.13 mM, pH9.0) containing 0.05 mM oxidized glutathione (GSSH) and 0.05 mM EDTA then incubated at room temperature for 10 min. After centrifuged at 1000 g 4°C for 10 min, the reaction product was detected by addition of DTNB (dithiois-nitrobenzoic acids). Absorbance was measured at 405 nm after 2 min of stable color development¹³.

Effect of Additives compound

Additives compound tested were divalent cations (Mg⁺⁺, Zn⁺⁺, Ca⁺⁺, Mg⁺⁺), EDTA (ethylenediaminetetraacetic acid), reducing agents viz. dithiothreitol (DTT) and 2-mercaptoethanol (BMT). Organic solvents tested were ethanol, methanol, glycerol and tween 20 and antibacterial agents namely NaCl and Na-azide. Various additives and concentration were tested and observed for the effect on keratinase and reductase activity. An concentration to increase enzyme activity was chosen to formulate with the crude extract of *Bacillus* sp. MTS as the cleaning solution formula.

Cleaning Formulation

The detergent formulations were prepared by mixing a 1 liter crude extract of *Bacillus* sp. MTS with 2% (v/v) glycerol, 2 mM CaCl₂, 2 mM MgCl₂, 2 mM ZnCl₂ and 5 mM MnCl₂, the experiments were done with 0.16 U/mg.

Cleaning Test

Cleaning formula was tested on human hair and its was performed at 50°C for 0, 20, 40, 60 and 90 minutes. Hydrolyzed keratin products were then measured using a spectrophotometer to obtained the exact time and temperature to react cleaning solution with substrate. The solution also tested for cleaning whole EBN. The first step, EBN was cleaned with aquadest and then 25% ethanol. Hereafter, it's dipped in cleaning solution, then left at room temperature for 10 minutes and incubated at 50°C for 20 minutes. After repeating the feather, EBN was dried on 40°C for 40 hours. EBN was weighed before and after cleaning processes.

RESULTS AND DISCUSSION

Effect of several compound to keratinase and disulfide reductase activity

Proteases have extensive applications in a range of industrial products and processes including detergent, food, pharmaceuticals, tannery, waste treatments, resolution of amino acid mixtures, silk and silver extraction from used X-ray films¹⁹. Keratinase is known as keratinolytic protease capable of binding and hydrolyzing solid substrate like feather or hair. This capability is important since detergent enzyme is required to react with protein substrate (keratin) sticking on fabric such as collar²⁰.

Activating keratinolytic enzymes in crude extract of *Bacillus sp.* MTS was increased by 25% (v/v) ethanol and methanol, the increased activity was 49% and 46% for ethanol and methanol, respectively, higher than that of control (Figure 1). Skrzydlewska *et al.*²¹ reported that ethanol increased protease cytosol (cathepsin) higher than ethanol. Methanol increased cathepsin activity C and E as much as 28% and 34%, respectively, while ethanol cathepsin C and B was as much as 45% and 42%, respectively. Ethanol and methanol are two additives extensively used as cleaning solution. Ethanol is also used as anti-microbial agent to kill or inhibit the growth of disease and odor-stimulating microbe. Economy and toxicity consideration has made ethanol chosen in cleaning solution of edible bird's nest.

Glycerol concentration of 25% can increase keratinase activity as much as 24% higher than that of control, while Tween 20 inhibited activity of keratinase *Bacillus sp.* MTS (Figure 2). Protein stability has important role in keeping biological function of the protein for example during protein design, refolding and storage. Glycerol has long been used to protect enzyme activity and native protein structure against denaturation. Glycerol as reported by Meng *et al.*²² is able to increase structure of native creatin kinase. Glycerol enhanced the keratinase activity at concentration of 25% whereas Tween 20 inhibited its activity. Tween 20 is a nonionic detergent, used extensively for solubilization of membrane proteins and their biochemical characterization. Fig. 1B showed that Tween-20 was ineffective for this

purpose, therefore it could not be used as additive for cleaning solution.

Reducing agents viz dithiothreitol (DTT) and 2-mercaptoethanol (BMT) significantly affect keratinase activity. When the enzyme reacted with reducing agent (E*), hydrolyzed product drastically decreased, indicating enzyme damage. However, when substrate (chicken feather) was pre-incubated with reducing agent before being reacted with enzyme (E+S*), hydrolyzed product increased (Figure 3). DTT and BMT significantly affect keratinase activity. When the enzyme reacted with reducing agent, hydrolyzed product drastically decreased, indicating enzyme damage. However, when substrate (chicken feather) was pre-incubated with reducing agent before being reacted with enzyme, hydrolyzed product increased (Figure 3). Several researches reported factors of disulfide bond reduction in the activity of keratin-user microorganism. *Bacillus sp.* MTS produces keratinase and disulfide reductase, and the reaction of both enzymes results in drastic increase of keratinolytic activity (more than 20 fold) compared to sole keratinase¹³. It showed that keratinase affinity is higher when keratine substrate has priorly been reduced by either reducing agent or reductase disulfide enzyme.

For maximum activity, protease alkali needs cation divalent such as Ca²⁺, Mg²⁺ and Mn²⁺ or the combined cations. Cation is also needed to increase thermal stability of the alkaline protease of *Bacillus*. Cation protects enzyme from thermal denaturation effect and importantly maintain active enzyme conformation at high temperature¹⁹. Some cations increase the keratinase activity of *Bacillus sp.* MTS at different concentration. At 2 mM concentration Ca²⁺, Mg²⁺ and Zn²⁺ cation increases keratinase activity as much as 266%, 266% and 166%, respectively. While Mn²⁺ at 5 mM concentration increases keratinase activity to 360%, higher than that of control (Figure 4). Rahayu *et al.*¹² informed that *Bacillus sp.* MTS produced six proteases, their molecular weights are 17, 25, 32, 53, 96 and > 97 kD. Keratinase *Bacillus sp.* MTS activated by Ca²⁺, Mg²⁺ and Zn²⁺ (Fig. 4), this result is in line with Bernard²³ that >97 kDa and 96 kDa *Bacillus sp.* MTS protease were activated by Mg²⁺ and Mn²⁺ and inhibited by 2 mM EDTA. It indicated that both protease belong to metal protease.

The effect of additives to *Bacillus* sp. MTS reductase activity showed that reductase enzyme was obstructed by various tested additives. Activity increase was observed when reductase was reacted with 0.05 mM EDTA (Table 1). Reductase enzyme (E.C. 1.6.4) is active enzyme that catalyzes reduction of disulfide bonds and both are included in oxidoreductase. The active site of thiol-disulfide oxidoreductase bears Cys-Xxx-Yyy-Cyst motifs and both residual cysteine contribute to oxidized disulfide cycle and reduced thiol (redox reactions)²⁴. EDTA as chelate agent made reductase active site work optimally in hydrolyzing disulfide bonds in keratin structure, resulting in 10 fold increase of hydrolysis product. Inorganic ions could relate to

protein side chain or interact with the active site in which these interactions might not affect the structure but facilitate or complicate substrate molecule to be or relate with enzyme active site²⁵. The interrupted interaction between substrate and enzyme active site caused the catalytic activity of enzyme to decrease. The presence of salt usually affects the conformation, folding, stability and activities of enzymes. Some enzymes are affected by monovalent cations, other and in most cases are affected by divalent cations. Monovalent cation and monovalent anions may neutralize protein charges, and that may change protein structure with no effect or decreasing or increasing enzyme activity. In this case the salt (NaCl) might be unfavorable

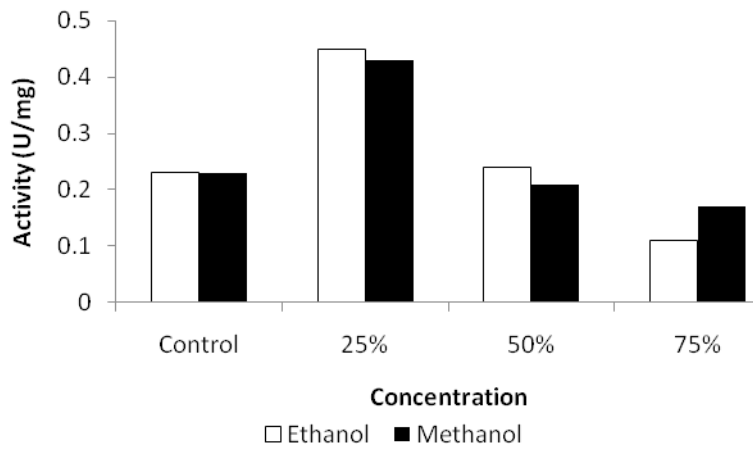


Fig. 1. Effect of ethanol and methanol on keratinase of *Bacillus* sp. MTS

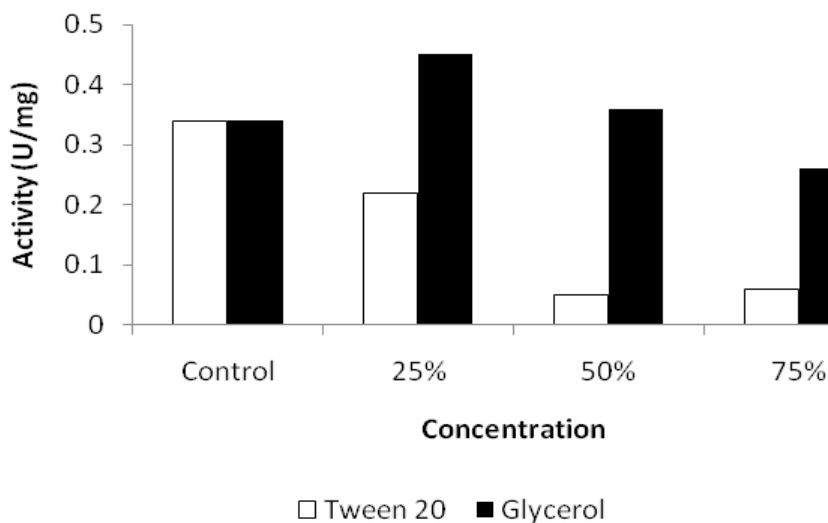


Fig. 2. Effect of Tween 20 and glycerol on keratinase of *Bacillus* sp. MTS

to the enzyme conformation and enzyme substrate interaction¹³.

Based on the test on various additives towards keratinase activity and reductase, one formula of cleaning solution for edible bird's nest was composed. The formula was then tested on human hair at 50°C at various incubation periods. Keratinase in the formula was generally 2-3 times higher than that of control (Figure 6).

Effect of several compound to activity of keratinase in cleaning solution

Bacillus sp.MTS produces six protease molecules in its cell-free filtrates, two of which

are kreatinase. Various types of keratinase in *Bacillus* sp.MTS enable the bacteria to degrade keratin substrate such as chicken feather, human hair, cocoon, silk, fish scale and horn¹². Edible bird's nest is mainly composed of glycoprotein with carbohydrate components of 9% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose and 0.7% fructose. Protein of edible bird's nest is mainly composed of serine amino acid, threonine, aspartic acid glutamate acid, proline and valine¹. Marcone² reported that edible bird's nest contained fat (0.14–1.28%), ash (2.1%), carbohydrate (25.62–27.26%) and protein

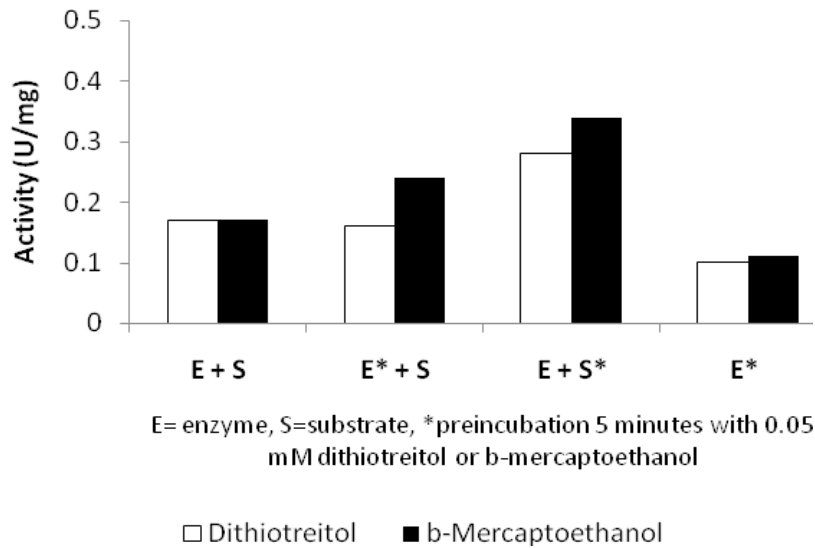


Fig. 3. Effect of reducing agents on keratinase of *Bacillus* sp. MTS

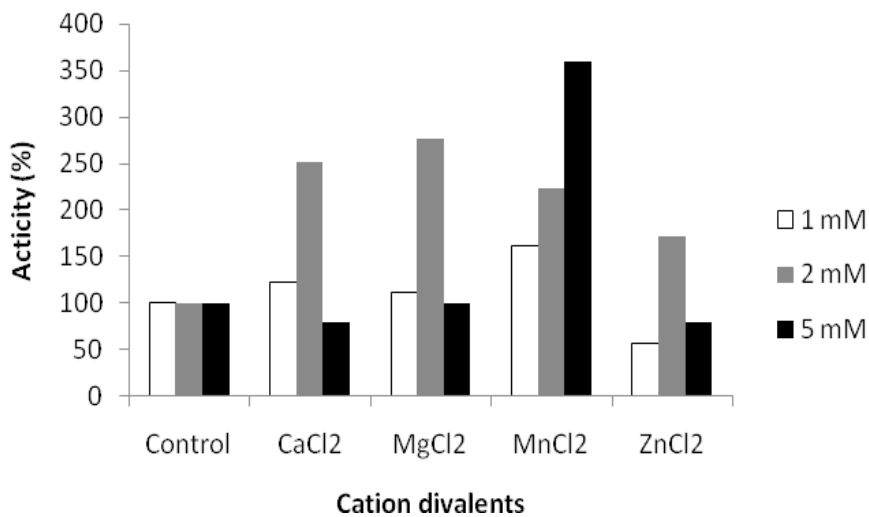


Fig. 4. Effect of divalent cation on keratinase of *Bacillus* sp.MTS

(62–63%). Furthermore, 10% feather was found stuck in the nest. Protein substrate and keratin in edible bird’s nest enabled enzyme in cleaning solution to function well and produce hydrolysis.

Keratinase activity of *Bacillus sp.* MTS in human hair was higher (Figure 6). Cysteine content in keratin was approximately 8% and absent in other proteins, while cysteine content in human hair was double of that in chicken feather (15.6-21.2% vs 7.05-12.2%)²⁶. Keratin structure became very solid due to disulfide bridge between two amino acids (cysteine). Keratinase and reductase disulfide of *Bacillus sp.* MTS was observed to perform specific and synergic hydrolysis in peptide and disulfide bonds of human hair. Specificity of the two enzymes resulted in higher substrate hydrolysis on human hair than on edible bird’s nest (data not

showed). However, it resulted in beneficial effect as cleaning solution because low keratinase activity would prevent edible bird’s nest from protease enzyme breakdown.

Cleaning edible bird’s nest (EBN) need several steps before its cleaned by enzymes viz. in dip several times using aquadest, 25% ethanol and enzyme solution. Aquadest and 25% ethanol removing the dust and faeces on the EBN, it’s also preparing EBN for enzyme activity. Then it’s incubated at room temperature for 10 minutes and 50°C for 20 minutes after immersion in enzyme solution. These incubation processes will provide opportunities the enzymes for loosening the bond between feather and EBN. The next stage was taken away the bird’s feather that is stuck inside the nest using feather Plucker. All steps effectively cleaning

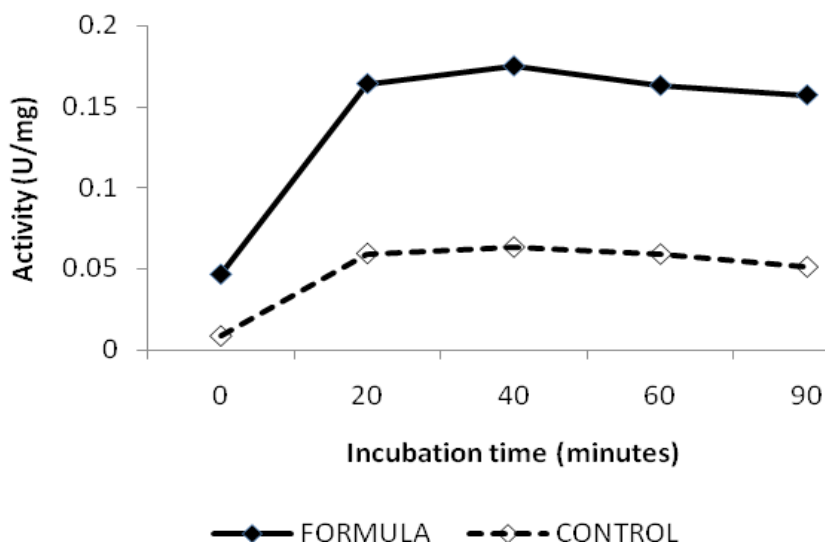


Fig. 5. Keratinase activity in the cleaning solution on human hair



Fig. 6. Edible bird’s nest unprocessed (left) and processed (right)

Table 1. Effect of several compound on reductase activity of *Bacillus sp.* MTS

Compound	Concentration	Activity (%)
EDTA	0 mM	100
	0.05 mM	1025
	0.25 mM	0
	0.5 mM	0
Alcohol		
Ethanol	25%	25
Methanol	25%	0
Glycerol	25%	95
Cations		
NaCl	0%	100
	5%	30
	10%	0
	20%	0
ZnCl ₂	0 mM	100
	1 mM	58
	2 mM	20
	3 mM	8
MgCl ₂	0 mM	100
	1 mM	38
	2 mM	0
	3 mM	0
CaCl ₂	0 mM	100
	1 mM	42
	2 mM	16
	3 mM	0

EBN, it's appeared white and neat (figure 6). This cleaning process demonstrated that keratinolytic enzymes in solution capabilities to clean EBN, the weight loss of EBN was 2.3-2.5% approximately (data not showed).

CONCLUSIONS

Keratinase in crude extract of *Bacillus sp.* MTS was activated by 25% ethanol, 25% methanol, 25% glycerol, and metal Ca²⁺, Mg²⁺, Mn²⁺, Zn²⁺ and it was inhibited by NaCl and Nazide. While reductase disulfide enzyme was solely activated by 0.05 mM EDTA.

Keratinase activity of *Bacillus sp.* MTS in cleaning solution formula increased 2-3 fold compared to that of control (crude extract) in human hair substrates. The solution was successfully to clean EBN with weight loss 2.3-2.5% approximately.

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