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*BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA*, September 2017. Vol. **14**(3), p. 989-996

# Keratinolytic Enzymes for Cleaning Edible Bird's Nest

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http://dx.doi.org/10.13005/bbra/2533

(Received: 28 July 2017; accepted: 08 August 2017)

**Edible bird's nest (EBN) as a kind of functional foodhashigheconomic value depending on the qualitysuch 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 cationdivalents, 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($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$)were able to increase keratinasewhile disulfide reductase was solely activated by 0.05 mM EDTA. The activity of both enzymes wasinhibited by NaCland 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. Gliserol and cations divalent increasing 2-3 fold keratinase activity in cleaning solution. The solution was successfully apllied 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 mostexpensive animal products, its a saliva produced bytwo specific swiftlets, namely *Aerodramusfuciphagus*(white-nest) and*Aerodramusmaximus*(black-nest)[1]. EBN is a functional foodcontaining high quality nutrients such as proteinx, carbohydrate, iron, inorganic salts and fiber[2,3,4].EBN also serves medical function as antiaging, anticancer, immunity enhancing agent, inhibiting influenza virusinfection 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 quality[7]. The primary factor ofEBN quality is color and hygiene, therefore the whiter and cleaner EBN, the higher isthe price.

The steps in cleaning EBN is a tedious work for being meticulous and perseverant to obtain high quality product. Koon and Cranbrook[7]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 ($H^2O^2$) 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 refered to*Bacillus* sp. MTS .This bacillusstrain was aerobic mesophillic 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 scale. The purified enzymes of *Bacillus* sp. MTS worked optimally at alkaline pHs, for keratinase at pH8–12, and for disulfide reductase at pH8–10.Optimumtemperaturefortheextracellularkeratinasewaswithin40–70$^0$C, while thatfordisulfidereductasewas35$^0$C[13]. We attempted to apply this enzymes to solve the problem contaminant feather that is stuck inedible bird's nest.

In the present study, we report the effect of several compound in acleaning formulacontaining keratinolytic enzymes and testing the formula tocleanEBN. 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 aerobicmesophilic*Bacillus* sp. MTSwasusedin these experiments .Its screened and isolated from Tangkuban Perahucrater West Java-Indonesia. Theagarmedium for culture maintenance pursuant to Macedoet *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 substrate15, pH was adjusted to 7.5. Incubation was carried out in a1 l flask at 37$^0$C 100 rpm for 48h. After incubation the culture was strained and centrifugedat 4000 g 40C 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 protein[16].

Keratinase activity was measured according to Walter 17 using 1% feather powder in Tris/HCl (50 mM, pH8.0) as substrate and absorbance of the samples were read at 660 nm. Atyrosin standard curve was made for quantification. One unit of enzyme activity was as signed as the amount of enzyme which liberate 1 mmoltyrosinein one min.

Disulfide reductaseactivitywasassayed asdescribedby Serrano *etal.*[18] with afewmodifications.Enzymewasmixed with500 ml ofTris/HCl buffer (0.13mM,pH9.0)containing0.05mMoxidizedglutathione (GSSH)and0.05mMEDTAthen incubated atroomtemperaturefor 10 min. Aftercentrifugedat1000g4$^0$C for 10 min, thereactionproductwasdetectedbyadditionof DTNB(dithiobis-nitrobenzoicacids). Absorbance wasmeasuredat405nmafter2minofstablecolor development[13].

### Effect of Additivescompound

Additives compound tested were divalent cations ($Mg^{++}$, $Zn^{++}$, $Ca^{++}$, $Mg^{++}$), EDTA (ethylenediaminetetraacetic acid), reducing agentsviz. 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 $CaCl_2$, 2 mM $MgCl_2$, 2 mM $ZnCl_2$ and 5 mM $MnCl_2$, the experiments were donewith 0.16 U/mg.

### Cleaning Test

Cleaning formula was testedon human hair and its was performed at 50$^o$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 withaquadest and then 25% ethanol. Hereafter, it's dipped in cleaning solution, then left at room temperature for 10 minutes and incubated at50°C for 20minutes. After repealing the feather, EBN wasdried on 40$^o$C for 40hours.EBN was weighed before and after cleaning processes.

## RESULTSAND DISCUSSION

### Effect of severalcompound 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 ofamino acid mixtures, silk and silver extractionfrom used X-ray films[19]. 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 collar[20].

Activitingkeratinolyticenzymes 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 ofkeratinase*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 vizdithiotreitol (DTT) and 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 keratinase[13]. 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 $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ 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 temperature[19]. Some cationsincrease the keratinase activity of *Bacillus* sp. MTS at different concentration. At 2 mMconcentration $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$cation increaseskeratinase activity as much as 266%, 266% and 166%, respectively. While $Mn^{2+}$ at 5 mM concentration increaseskeratinase activity to 360%, higher than that of control (Figure 4).Rahayu*et al.*[12]informed that *Bacillus* sp. MTS produced sixth proteases, their molecular weights are 17, 25, 32, 53, 96 and > 97kD.Keratinase*Bacillus* sp.MTS activated by $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$(Fig.4), this result is in line with Bernard[23]that >97 kDa and 96 kDa*Bacillus* sp.MTSprotease were activated by $Mg^{2+}$ and $Mn^{2+}$ and inhibited by 2 mM EDTA. Its indicated that both protease belong to metal protease.

The effect of additives to *Bacillus* sp. MTSreductase activity showed that reductase enzyme was obstructed by various tested additives. Activity increase was observed when reductase was reacted with 0.05 mMEDTA(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-Cystmotifs 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 site[25]. The interrupted interaction between substrate and enzyme active site caused the catalytic activity of enzymedecrease. The presence of salt usually affect 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 neutrilize 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

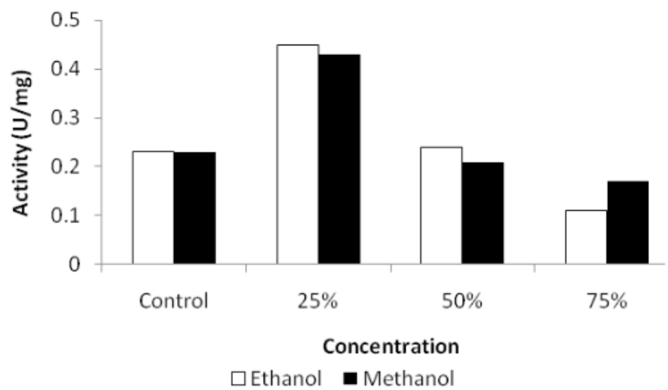


**Fig. 1.** Effect of ethanol and methanol onkeratinase of *Bacillus* sp. MTS

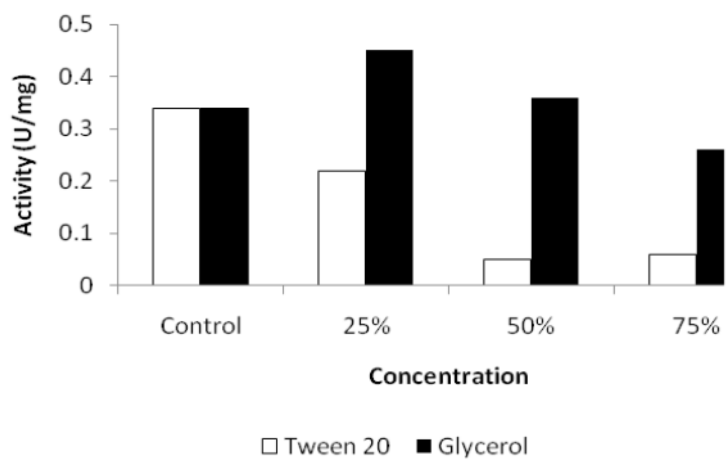


**Fig. 2.** Effect of Tween 20 and glycerol on keratinase of *Bacillus* sp. MTS

to the enzyme conformation and enzyme substrate interaction[13].

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 severalcompoundto activity of keratinasein cleaning solution**

*Bacillus* sp.MTS produces six protease molecules in its cell-free filtrates, two of which arekreatinase. 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 horn[12].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 valine[1]. Marcone[2]reported that edible bird's nest contained fat (0.14–1.28%), ash (2.1%), carbohydrate (25.62–27.26%) and protein

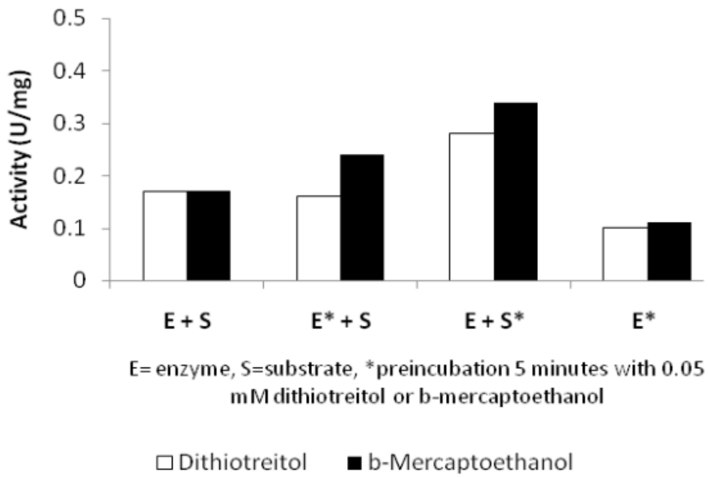


**Fig. 3.** Effect of reducing agents on keratinase of *Bacillus* sp. MTS

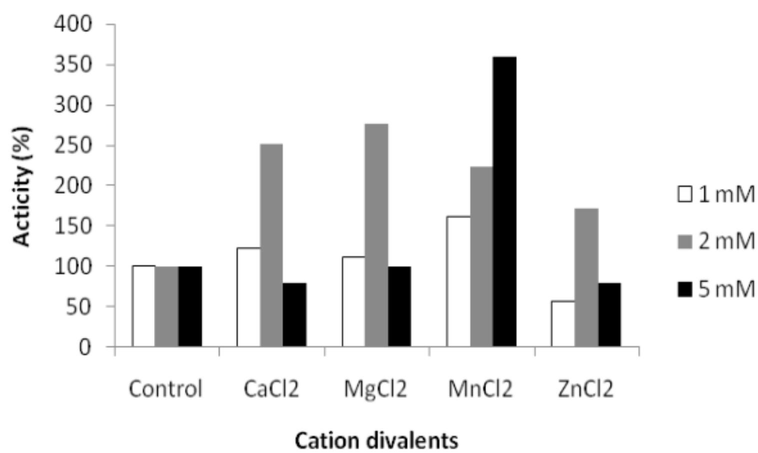


**Fig. 4.** Effect of divalent cation on keratinase of *Bacillus* sp.MTS

(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 enzymesviz. in dip several times using aquadest, 25% ethanol and enzymesolution. Aquadest and 25% ethanol removing the dust and faeces on the EBN, it's to preparing EBN for enzyme activity. Then it's incubated at room temperature for 10 minutes and 50°C for 20 minutes after immertion in enzyme solution. These incubation processes will provide opportunities the enzymes for loosening the bond between feather and EBN. The next stage wastaken away the bird's feather that is stuck inside the nest using feather Plucker. All steps effectively cleaning

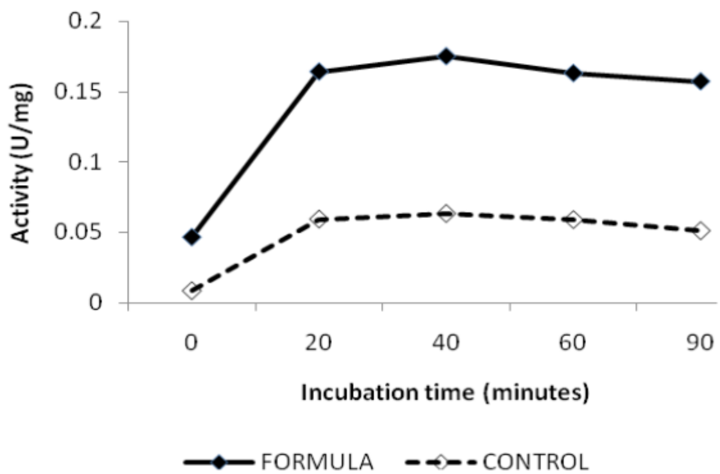


**Fig. 5.** Keratinase activity in the cleaning solution on human hair

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

**Tabel 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 |
| ZnCl2 | 0 mM | 100 |
| | 1 mM | 58 |
| | 2 mM | 20 |
| | 3 mM | 8 |
| MgCl2 | 0 mM | 100 |
| | 1 mM | 38 |
| | 2 mM | 0 |
| | 3 mM | 0 |
| CaCl2 | 0 mM | 100 |
| | 1 mM | 42 |
| | 2 mM | 16 |
| | 3 mM | 0 |

EBN, it's appearedwhite 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 $Ca^{2+}$,· $Mg^{2+}$,· $Mn^{2+}$,· $Zn^{2+}$ and itwas inhibited by NaCl and Na-azide. 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 cleanEBN with weight loss 2.3-2.5% approximately.


## ACKNOWLEDGEMENT

This research was financially supported by Ministry of Research and Technology Indonesia pass through Incentive Research SiNas programme.Thanks to IkaMalikah, Anastasia andMegahwatiEffendy for their practical support.


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