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Enzymatic Dehairing of Goat Skin Using Keratinase from *Bacillus sp*. MD24, A Newly Isolated Soil Bacterium

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ABSTRACT

A newly keratin-degrading bacterium was isolated from a residential soil at Wajak district, Malang, East Java, Indonesia. Sequence homology analysis showed the 16S rRNA had only 89% sequence identity to the available bacterial 16S rRNA which led to a discovery of a new species of *Bacillus*. The bacterium was named *Bacillus sp* MD24. The isolated bacterium degraded 71% mass of whole chicken feathers within 10 days. Keratinase fermentation using 1% of chicken feathers as sole source of carbon and nitrogen exhibited highest enzyme activity at third day under optimum condition (pH of 8 and temperature of 37^{o}C). At enzyme concentration of 0.3 U/mL, the crude extract keratinase started to exhibit dehairing activity on goat skin after an overnight incubation; the best incubation was achieved at 72 hours. Surface of enzymatically dehaired goat skin was compared to chemically dehaired skin goat. The result showed similar or improved surface of the skin which makes the crude keratinase from *Bacillus sp* MD24 a potential candidate for application in leather industry to avoid pollution problems due to the use of chemicals.

Keywords: *Bacillus*, dehairing, keratinase, dehaired goat skin, keratin-degrading bacterium

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INTRODUCTION

Leather industry contributes greatly to the Indonesian economy but there has been an increasing environmental concern regarding various pollutants as by-products of this industry (Kolomaznik, Adamek, Andel, & Uhlirova, 2008; Lofrano, Meriç, Zengin, & Orhon, 2013; Syed et al., 2010; Zhang, 2007). Biotechnological processes such as enzymatic process offer cleaner technologies

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which minimise production of hazardous waste. Dehairing is an important step during leather processing. Hair is composed primarily of strong fibrous proteins named as keratin (McKittrick et al., 2012) which contains of large amount of cysteine residues. The thiol groups form cysteine residues form strong covalent disulphide bonds that cross links the polypeptide chains together. Sodium sulphide was conventionally applied for hair removal during dehairing process to break the disulphide bonds. Although this process is very effective, it generates hydrogen sulphide gas, obnoxious odour and toxic gas. Single inhalation exposure to hydrogen sulphide in experimental animals can result respiratory, immunological/lymphoreticular, cardiovascular, neurological effects and even death (Selene & Chou, 2003). Short term inhalation studies of hydrogen sulphide showed ocular, neurological, cardiovascular, metabolic, reproductive, and developmental effects at lowest concentration tested at 28 mg/m^3.

Microbial keratinases have been reported to be promising enzymes for application in dehairing process. Various extracellular keratinase showed capability to degrade keratin, such as keratinase produced by *Bacillus subtillis* (Andre & Macedo, 2014), *Bacillus halodurans* strain PPKS-2 (Prakash & Jayalakshmi, 2010), *Brevibacillus brevis* US575 (Jaouadi et al., 2015) and *Bacillus licheniformis* ER-15 (Tiwary & Gupta, 2010). Keratinase application in dehairing step accomplishes many advantages especially to overcome environmental pollution and leather quality (Ismail, Housseiny, Abo-Elmagd, El-Sayed, & Habib, 2012) Biochemical and biophysical properties of keratinases vary greatly among microorganisms isolated from different environment. Hence, isolation of a new microbe might be directly correlated to finding a novel keratinase.

A new *Bacillus* sp. was isolated from decomposed chicken feather containing soil. The isolate produces keratinase and the enzyme showed activity in dehairing of goat skin. This finding adds to the library of keratinase producing microbial collection for sources of keratinase, a potential replacement agent of the harmful chemicals commonly applied in leather dehairing, as eco-friendly and economically viable alternatives.

MATERIALS AND METHODS

Isolation and Characterisation of Keratinase Producing Bacterial Strain

Chicken feather was decomposed by mixing it with moistened soil and incubated for approximately 1 month. The mixture was mosturized daily with ±10% (v/w) of water. Microbial strains were isolated from decomposed chicken feather containing soil. A soil suspension was prepared by dissolving around 1 g decomposed chicken feather in 100 mL of 0.85% NaCl and serial dilution of 1000-fold was created from the suspension. A series of 100 µL diluted suspension was plated on a skim-milk medium (0.5% NaCl; 0.1% MgSO4; skim milk 5.0%, and 1.5% agar with a pH of 7.5) and incubated at 37^0C overnight.

Colonies with clear zone were picked up and tested for their ability to degrade keratin by placing it on keratin medium (0, 5% keratin powder; 0.03% K_2HPO_4; 0.04% KH_2PO_4; 0,05% NaCl; 0,01 % $MgCl_2.6H_2O$; and 1,5% agar). A colony called MD24 with the highest proteolytic index was selected and maintained for further analysis. Strain identification was done by homology study of 16S rRNA gene sequence. Amplification and sequencing of 16S rRNA gene was performed based on Macrogen (Seoul, Korea) using internal primer 785F ($^{5'}$GGATTAGATACCCTGGTA$^{3'}$). Resulting sequence was used as query sequence to find similar 16S rRNA genes at data base using BLASTN program provided by National Centre for Biotechnology Information homepage. Ten 16S rRNA gene sequences with the highest identity were selected and aligned with the 16S rRNA from the isolate bacterium. Sequence alignment was performed using Clustal X2.1 and phylogenetic tree was constructed using MEGA 6 with the nearest neighbour-joining method.

Chicken Feather Degradation

Chicken feathers are potential carbon and nitrogen sources for keratinase production. The selected isolate was tested for its capability to degrade whole chicken feathers. The isolate was grown in a 100 mL medium containing 5.0 % NaCl; 1.0 % $MgSO_4$; 0.5 % K_2HPO_4; and ± 0,1 g chicken feathers for 10 days at 37^{o}C. Chicken feather degradation was followed by weight loss of feathers and increasing tyrosine concentration.

Optimisation of Keratinase Production

Optimisation of keratin production was done based on carbon source, pH medium, and temperature incubation. Keratin production was examined using 1% chicken feathers as a sole carbon and nitrogen source and combination of 1% chicken feathers with 1% of additional carbon source (glucose, sucrose or glycerol). The physical condition, pH (in the range of 6-9) and temperature (in the range of 35-41°C), were optimised for maximum yield of keratin production.

Keratinase Activity Measurement

Keratinase activity was measured spectroscopically by measuring tyrosine released through enzymatic hydrolysis. A reaction mixture containing 1 mL of 1% keratin and 1 mL of Tris-HCl buffer pH 8 and 1 mL of crude extract keratinase was incubated at 37°C for 30 minutes. The enzymatic reaction was stopped by adding 1 mL of 10% trichloroacetic acid (TCA). Subsequently, the reaction mixture was incubated on ice for 20 minutes. The mixture was then centrifuged at 5000 rpm for 5 minutes and the absorbance of supernatant was measured at 280 nm. Catalytic site of keratinase was tested through inhibition test using phenazine methosulphate (PMSF) at 0.5 and 1 mM and Ethylenediaminetetraacetic acid (EDTA) at 0.5 and 1 mM. Dependency of keratinase on metal ions were tested using 0.5 and 1 mM divalent cations (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, an Ni^{2+}). Optimum temperature and optimum pH were determined under the best divalent ion activity induction. In order to know the

increase of absorbance due to non-enzymatic activities, control reaction was performed using same chemical composition but the enzyme was added after the addition of TCA.

Dehairing of Goat Skin

Sundried goat skin was prepared without adding any chemicals. It was cut into 1 × 1 cm pieces and soaked in a 20 mL of 0.3 U/ml crude extract keratinase at optimum keratinase activity condition (37^{o}C, pH 8, in 1 mM $CaCl_2$) overnight (16 h), 48 h, and 72 h. Skin was then gently scraped to remove hairs. Chemical dehairing was done using a 20 mL solution containing 2% technical grade sodium sulphide (SN) and 2% technical grade lime (this method is used at local tannery industry at Mageta district, East Java, Indonesia). Sodium sulphide and lime were collected from local tanning industry. Chemical dehairing was done for 24 hours. Skin was also gently scraped to remove hairs and washed with demineralised water. Dehaired skin surfaces were examined using Scanned Electron Microscopy (SEM).

RESULTS AND DISCUSSION

Screening of Keratinase Producing Bacterial Strain

A total of 22 colonies exhibited production of extracellular proteases after 24 h incubation. The ratio of clear zone and colony diameters served as a proteolytic index for selection of a strain with highest protease production ability. A selected colony called MD24 with the ratio of 4.7 was subsequently transferred to keratin containing medium (Figure 1A). Figure 1B shows the MD24 isolate after 16 h incubation on keratin containing medium.

Morphological characterisation of MD24 isolate indicated that the isolate was a *rod*-shaped gram-positive *Bacillus* (Figure 2). Sequence alignment of 16S rRNA gene against available bacterial 16S rRNA gene

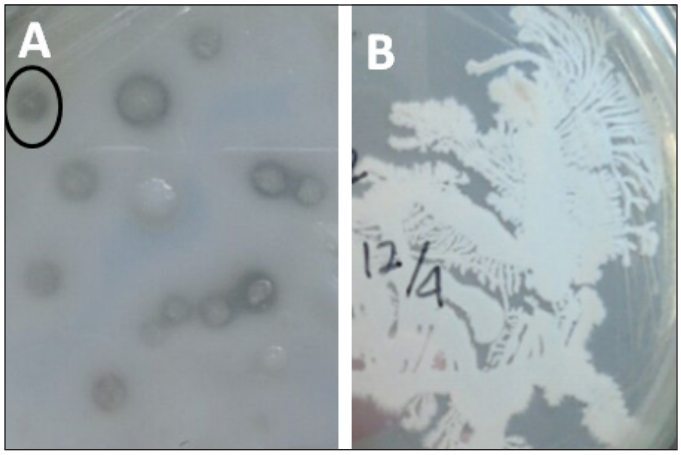

Figure 1. Screening keratinase bacterial strain. A. Selected strain from skim milk medium. B. Selected bacterial strain grown on keratin containing medium.

at the database exhibited 89% maximum of sequence identity. "A prokaryotic species is considered to be a group of strains that are characterized by a certain degree of phenotypic consistency, showing 70% of DNA–DNA binding and over 97% of 16S ribosomal RNA (rRNA) gene-sequence identity" (Gevers et al., 2005; Stackedbrandt & Goebel, 1994). Therefore, it can be concluded that MD24 isolate is a new isolated *Bacillus* strain and named as *Bacillus* sp MD24. Ten of 16S rRNA gene sequences with 89% identity were aligned using Clustal X2 and a phylogenetic tree was constructed using MEGA6. Figure 3 shows a phylogenetic tree of the 10 selected strain and *Bacillus* sp. MD24. Horizontal dimension represents the genetic change. The tree exhibits one common ancestor that broken into two branches. Ten strains fall into one branch and *Bacillus* sp falls into different branch. Therefore, *Bacillus sp* MD24 might develop a different metabolic pathway compared with another branch.

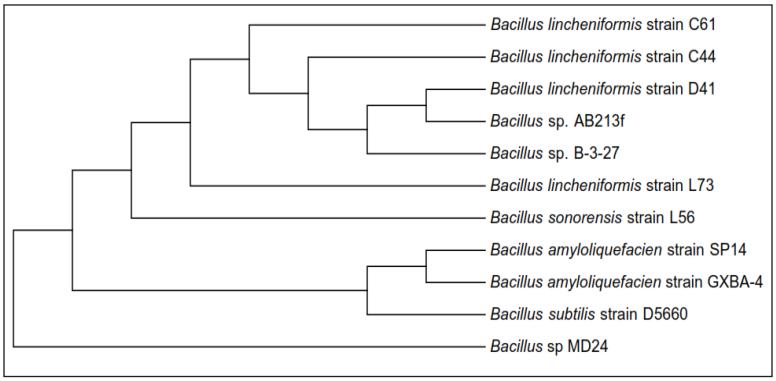

Figure 3. Neighbour-joining phylogenetic tree of *Bacillus* sp. MD24 with closest *Bacillus* strains based on 16S rRNA partial gene sequences

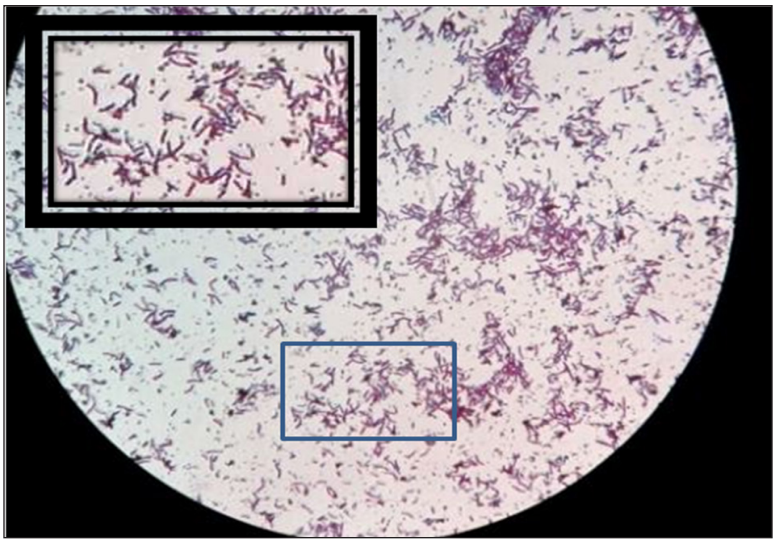

Figure 2. Morphology of *Bacillus* sp MD24. Insert is magnification of the figure signed by a rectangle

Chicken Feather Degradation by *Bacillus sp. MD24*

The poultry industry all over the world resulted in the generation of millions of tonnes of chicken feather waste (Prakash & Jayalakshmi, 2010). Chicken feathers contain 90% or more keratin (Tork, Shahein, El-Hakim, Abdel-Aty, & Aly, 2013) that act as keratinase inducer and cheaper carbon and nitrogen sources for keratinase production (Gurav & Jadhav, 2013). Chicken feathers act as keratinase inducer and it can be applied as medium for keratinase fermentation. Degradation of chicken feathers by *Bacillus* sp. MD24 was examined. Fermentation was done for 10 days using chicken feathers as sole carbon and nitrogen sources. Chicken feather degradation was followed by its weight loss and production of soluble tyrosine. Table 1 shows chicken feather degradation using 1% initial weight of chicken feathers. About 71% of chicken feathers were degraded within 10 days of incubation. Decreasing the weight of chicken feathers was consistent with increasing tyrosine concentration which indicated the enzymatic hydrolysis of keratin into smaller molecule e.g. polypeptides and amino acids. Keratinolytic proteases has been reported from many *Bacillus* strains (Abdel-Naby, El-Araby, & El-Refai, 2015; Macedo et al., 2005; Prakash & Jayalakshmi, 2010). However, *Bacillus* strains are unique and keratinase activity of each strain is unique.

Table 1
Chicken feather degradation by Bacillus sp. MD24

Incubation time (days)	*%Weight loss*	Tyrosine concentration (µmol/mL)	Left over chicken feather
2	37.87%	0.06	
4	45.04%	0.61	
6	48.73%	0.81	

Table 1 *(continue)*

Incubation time (days)	*%Weight loss*	Tyrosine concentration (µmol/mL)	Left over chicken feather
8	63.41%	0.89	
10	71.01%	1.41	

Finding a new keratinase from a new isolate might provide better enzyme for specific applications.

Enzyme Production

Optimum enzyme production was observed at pH 8, 37°C, and 72 h. Although chicken feathers can be degraded up to 71 % within 10 days, optimum enzyme activity was obtained on the third day and decreasing in the following days (Figure 4). In addition of chicken feathers, carbon sources (glucose, sucrose, and glycerol) were examined. Unlike previous finding that found the positive influence of additional carbon sources toward keratinase production (Cavello, Chesini, Hours, & Cavalitto, 2013; Ramnani & Gupta, 2004), *Bacillus* sp. MD24 faithfully using keratin for the best keratinase production when glucose, sucrose or glycerol are present. When cells were grown under keratin as a sole carbon and nitrogen sources, it will produce keratinase to degrade keratin into amino acids and use it for energy as well as building cell chemical. However, although simple carbohydrates might increase cell growth, when keratin is also available in the environment, the cells may prefer to use simple carbohydrates instead of keratin for energy. This will reduce the production of keratinase. In this study, a combination of chicken feathers and complex carbohydrate are used to improve keratinase production. While simple carbohydrates reduce keratinase, complex carbohydrate might increase keratinase production due to a slow-release of simple carbohydrate enzymatically. Cell would balance keratinase and carbohydrate degrading enzyme to provide amino acids and simple carbohydrate for growth.

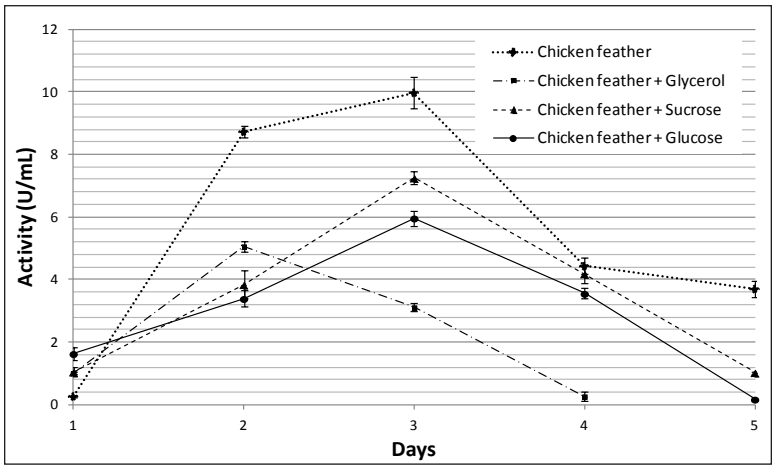

Figure 4. Keratinase production with chicken feathers as sole carbon and nitrogen source and the influences of addition carbon sources on enzyme production

Effect of Inhibitors on Enzyme Activity

The nature of protease was studied using specific class of protease inhibitors, PMSF and EDTA. As shown in Table 2, both PMSF and EDTA inhibit keratinase activity. The results indicated that keratinase from *Bacillus* sp. MD24 is a serine type protease which also depends on the presence of metal. Serine keratinase are reported in several bacteria (George, Chauhan, Kumar, Puri, & Gupta, 2014; Jaouadi et al., 2013; Tiwary & Gupta, 2010).

Table 2.
The effect of PMSF and EDTA on keratinase activity

	Percent Activity	
	0.5 mM	1 mM
Crude extract	100	100
Crude extract + PMSF	32.14	14.29
Crude extract +EDTA	25.00	10.71

The Effect of pH and Temperature on Keratinase Activity

The effect of pH was examined in the range of 7 to 9. The enzymes are active at the pH range of 7-9, but optimum activity was achieved at pH 8, and increasing pH at 9, the activity only remains about 50% of its activity at pH 8 (Figure 5A). Temperature optimum of the enzyme was obtained at 37^{o}C (Figure 5B). At temperature of 25^{o}C, almost no activity was observed for 30 min incubation time.

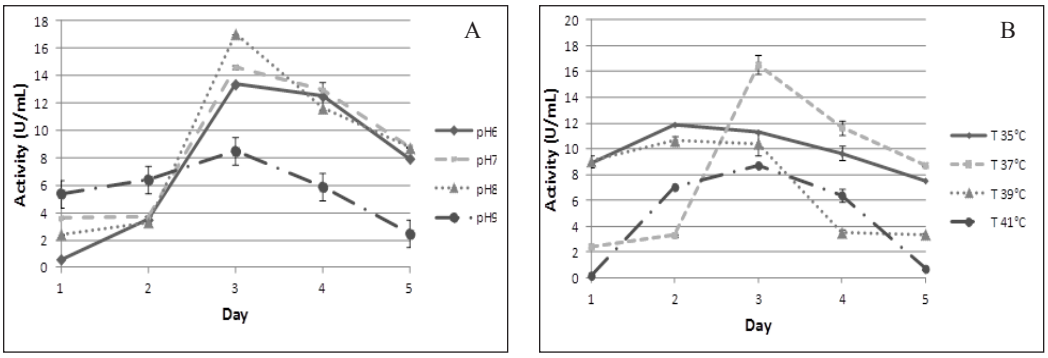

Figure 5. Effect of pH (A) and temperature (B) on keratinase activity

The Effect of Divalent Cations on Enzyme Activity

Table 3 shows the effect of several divalent cations. Addition 1 mM of Ca^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} cations increased enzyme activity by factor of 4.21, 4.21, 3.18, and 2.64 respectively, while Mg^{2+} reduced the activity by a factor of 0.5. Calcium ion has been reported to induce keratinase activity (Kim, 2005; Poopathi, Thirugnanasambantham, Mani, Lakshmi, & Ragul, 2014) Co^{2+}, Mn^{2+}, and Ni^{2+} inhibit keratinase from *Bacillus subtilis* (George et al., 2014; Tork et al., 2013).

Table 3
The effect of divalent cations on keratinase activity

Cation ions	Activity	
	0.5 mM	1 mM
Crude extract	1.00	1.00
Crude extract + Ca^{2+}	2.07	4.21
Crude extract + Co^{2+}	0.89	4.10
Crude extract + Mg^{2+}	0.64	0.50
Crude extract + Mn^{2+}	1.75	3.18
Crude extract + Ni^{2+}	2.00	2.64

Dehairing of Goat Skin

Goat skin hair was removed enzymatically and chemically. Figure 6 shows dehaired skins in 3 consecutive observation days. After an overnight of incubation, the hairs began to fall out. The hairs were easily removed after 72 hours of incubation. Keratinase removes hair skins as good as removal by Na_2S and lime. For 1 × $1cm^3$ of goat skins, hair removal using 20 ml of 2% of Na_2S and 2% lime for overnight can be replaced by enzymatic method using 20 ml of 0.3 U/ml keratinase for 72 h. The incubation time might be shorter by using higher enzyme activity. However, although the enzyme was produced using only carbon and nitrogen, collagenase activity must be measured in the keratinase crude extract.

Increasing keratinase concentration should be done thoroughly to find the best enzyme concentration without damaging collagen structure. Surface quality assessment of dehaired goat skins using SEM showed that hairs were removed effectively using both methods (Figure 7). However, although dehaired skins were washed several times with water, chemical precipitations were observed on chemically dehaired skin surfaces. Enzymatic dehaired skin showed cleaner surfaces. Effective dehairing of goat skin using keratinase has been reported by (George et al., 2014; Paul, Das, Mandal, Jana, & Maity, 2014). This study showed skin surface became more compact and fibre bundle are irregular after enzyme treatment. Keratinase from *Bacillus* sp. MD24 leaves smoother and regular fibre bundle traces.

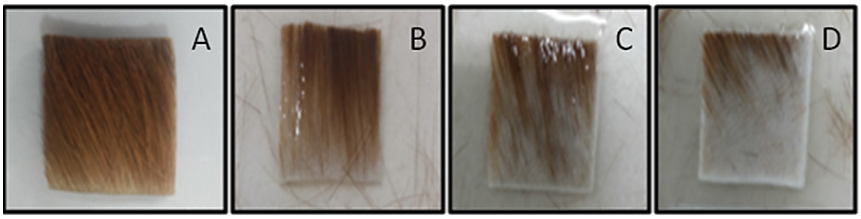

Figure 6. Enzymatic dehaired goat skins. The skin of about 1 cm^2 was soaked in a 20 mL of 0.3 U crude extract keratinase. A. Unhaired goat skin. B. Goat skin after overnight incubation. C. Goat skin after 48 h incubation. D. Goat skin after 48 h incubation.

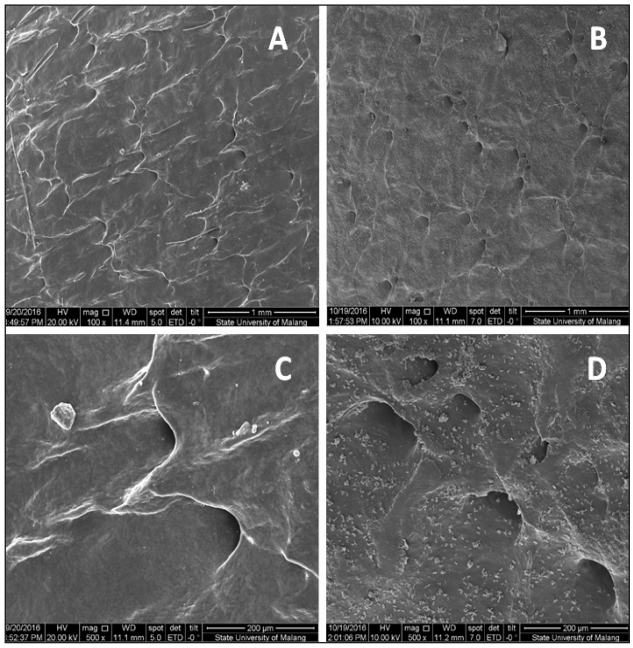

Figure 7. SEM images of surface dehaired skin. A. Enzymatically dehaired skin under 50× magnification. B. Chemically dehaired skin 50× magnification. C. Enzymatically dehaired skin 500× magnification. D. Chemically dehaired skin 500× magnification.

CONCLUSION

Keratinolytic bacterium was isolated from feather contaminated soil. The isolate was identified as a new strain bacterium named *Bacillus* sp. MD24. The keratinase can be produced under chicken feathers as sole nitrogen and carbon sources and the production is repressed by addition of glucose, sucrose, and glycerol. Under experimental condition, maximum enzyme production was obtained at pH 8, temperature of 37°C for incubation time of 3 days. The enzyme activity was also observed at the same pH and temperature conditions. The enzyme is a serine type protease induced by of Ca^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} cations and inhibits by Mg^{2+}. Finally, enzyme application on dehairing of goat skin showed a better quality surface compared with treatment using chemical method. However, further works need to be done such as optimising enzyme and ratio, increasing enzyme yield and scale up fermentation to obtain sufficient enzyme for industrial applications.

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