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PROMOTING KERATINASE ACTIVITY FROM NEWLY IDENTIFIED STRAIN *Strenotrophomonas maltophilia* B6 THROUGH OPTIMIZATION AND CHARACTERIZATION

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ABSTRACT

Strenotrophomonas maltophilia B6 was a newly identified keratinolytic bacteria isolated from a landfill in North Sumatra. Keratinolytic activitiy of this strain was investigated through optimization and characterization of its enzymatic properties. Amino acid profiles were quantified using High Performance Liquid Chromatography (HPLC) after a 96-h of incubation resulted in 2.2569 mg mL^{-1} amino acids from a 40-mL fermentation supplemented with 10 g/L chicken feathers. The keratinolytic activities were enhanced through addition of 1% glucose yielded 67.41 ± 1.1 UmL^{-1} and 0.5% peptone yielded 90.24 ± 0.7 UmL^{-1}. Keratinolytic activity enhanced with the addition of Ca^{+} (116%), Na^{+} (111%), Mg^{+} (110%) at 10 mM concentration. Zymogram analysis showed a protein band in the 26 kDa of molecular mass, similar to serine protease. Physical and chemical characteristics of degradation products were analyzed using Scanning Electron Microscope (SEM) and Fourier Transform Infra Red (FTIR). Qualitatively, the optimum activity occurred at 96-h of incubation through SEM image analysis. The FTIR peaks detected were peak 3747 cm^{-1} (-OH); peak 2972 cm^{-1} (asymmetric -CH3); peak 1641 cm^{-1} (amide I); peak 1438 cm^{-1} (amide II); peak 1092 cm^{-1} (-C-C-); peak 523 cm^{-1} (-S-S-). Thus, *Strenotrophomonas maltophilia* B6 may be used as potential biodegradative bacterium to chicken feather wastes in nature.

Key words: Feather waste, keratinase, serine protease, *Strenotrophomonas maltophilia*

INTRODUCTION

Chicken feather waste is produced in large number as by-product of poultry processing industry which is also the source of pure keratin protein. The research conducted by Wang and Parsons (1997) reported that the protein meal content of poultry feather is 880.0 g kg^{-1}. It is estimated that almost 24 billion chickens are slaughtered every year resulting in the increase of feather waste production amounted to 4 billion kg (Revathi *et al*., 2013). The poultry feather waste is difficult to be degraded causing contamination to the environment. The wastes stored in the garbage dump, will be buried in the soil leading to a disruption of the balance of soil microbiota ecosystem. The unfinished degradation of feather waste is due to its natural structure, keratin dense and stable polypeptides (Brandelli *et al*., 2010). Keratin is a structural protein resistant to both chemical and physical environmental factors (Anitha & Palanivelu, 2012; Sivakumar *et al*., 2012). Protein is also insoluble in acidic and weak in alkaline water.

Keratin is hardly digested by enzyme such as trypsin, pepsin, or papain (Sivakumar *et al*., 2012; Gradisar *et al*., 2005). The difficulty to degrade the keratin is due to the high number of hydrogen bonds, hydrophobic interactions, and disulphide bonds (Brandelli *et al*., 2010). Keratin is mostly found in poultry's feather, wool, horn, nail, claw, beak and hair (Anitha & Palanivelu, 2012; Sivakumar *et al*., 2012). The presence of indigenous bacteria in the nature can help the process of keratin degradation of the feather by utilizing the specific protease enzyme (keratinase). In fact, bacterial-produced keratinase plays an important role for

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many applications such as bio fertilizer, leather manufacturing industry, high nutrition fodder, detergent, bio hydrogen production, textile industry and prion hydrolysis (Gupta & Ramnani, 2006; Brandelli, 2008). The selection of diversity of keratinolytic bacteria is carried out in order to obtain the potential bacteria. Based on the previous research results, it has been found that the bacteria producing the keratinase derived from Gram-positive bacteria (Daroit *et al*., 2009; Bach *et al*., 2011a) and Gram-negative bacteria (Bach *et al*., 2011b; Yamamura *et al*., 2002; Riffel & Brandelli, 2006).

Keratinolytic bacteria have been widely documented from Gram-positive strains, especially those capable of degrading β-keratin, substrate including the chicken feather (Daroit *et al*., 2009; Bach *et al*., 2011a; Tork *et al*., 2013). Extensive protease diversity is very contradictory with the previous application. This is due to the different physiological nature of the bacteria in every place. This interest has brought the researchers around the world to try to exploit the specific possessed characters and the application of the biotechnology (Rao *et al*., 1998; Gupta *et al*., 2002). Now, fewer application of keratinase becomes an important concern to isolate the novel of keratinase bacteria from various sources. The application of biotechnology products is supported by biodiversity of the keratinolytic bacteria. The different characters of each enzyme follow the diversity of keratinase bacteria. The cause of these character differences was influenced by the source of bacterial habitat, in other words, the living habitat of keratinolytic bacteria affects the keratinase variation.

The previous research has reported several strains of *Stenotrophomonas maltophilia,* which can produce keratinase (Fang *et al*., 2013; Jankiewicz *et al*., 2016; Jeong *et al*., 2010; Jankiewicz & Frąk, 2017; Cao *et al*., 2009). Different sources of isolation cause the variation of biochemistry. In addition to keratinase-related information, the isolation result of the flue liquid was selected to characterize its keratinase, of which 16S rRNA has been found as *Stenotrophomonas maltophilia* B6. Our study has characterized keratinase activity against the differences of temperature, pH and percentage of precipitation with ammonium sulfate (Suryanto *et al*., 2017). In order to evaluate the enzyme characteristics from strain B6, the aim of the present study is to investigate the optimization condition of different sources of carbon and nitrogen on the production media for the secretion of thermostable keratinase proteases and characterize the native enzymes.

MATERIALS AND METHODS

Bacterial strain and culture condition

Keratinolytic bacteria have been isolated previously from a landfill soil in North Sumatra. The proteolytic potential has been selected through the formation of halo zone in skim milk agar media and its ability to degrade the chicken feathers. Strain B6 was closely related to *S. maltophilia* strain BIW based on 16S rRNA genetic similarity. Strain B6 was the source of keratinases in the present study. The isolation sub-cultured on the feather meal agar (FMA) media, contains 15 g L^{-1} of feather meal broth (FMB), 0,5 g L^{-1} NaCl, 0,7 g L^{-1} K_2HPO_4, 0,4 g L^{-1} KH_2PO_4, 0,1 g L^{-1} $MgSO_4$ and 15 g L^{-1} agar (Cai *et al*., 2008). FMB was prepared in the same way as FMA without adding the agar. Skim milk powder (Oxoid) was also prepared with NB agar composition added by 1% of skim milk powder. All of cultivated media was autoclaved at 121°C, 1 atm for 15 minutes and cultivated at 37°C with rotary shaking at 180 rpm. The chicken feathers waste is obtained from the chicken slaughterhouses and washed using water until it was clean completely and ended by ablution using aquadest. Then, the feathers were cut into small fragments (± 2 cm). The pieces of feathers were washed with water and detergent and then soaked with acetone for 24 h. The feather pieces were then heated in oven at 40°C for 72 h.

Optimization of fermentation medium

The growth media was added by carbon sources such as sucrose, fructose, lactose, sorbitol, glucose, and starch (1%). pH and temperature were adjusted according to keratinase activity studied previously (Suryanto *et al*., 2017), where controls were production media without carbon source. Different sources of nitrogen include yeast extract, peptone, casein, tryptone, $NaNO_3$, KNO_3, and gelatin (0.5%) mixed in a production media with 1% glucose used as the best additive for keratinase activity. The provision of carbon and nitrogen sources to measure the keratinase activity at different incubation time intervals include 24, 48, 72, 96, 120, 144 and 168 h.

Production of crude keratinase

Erlenmeyer containing 50 ml FMB was inoculated in each isolation and incubated at 30°C in orbital shaker (150 rpm). After 24 h, 5% (v/v) inoculum (10^8 CFU ml^{-1}) was added into 150 ml FMB and incubated under the same condition (Bach *et al*., 2011b) for 4 days (bacterial logarithm phase). Keratinase was harvested by centrifugation

of bacterial culture at 8000 X *g* for 20 minutes at 4°C. Supernatant was precipitated using ammonium sulfate 70% (Suryanto *et al*., 2017) slowly at ±5°C evenly. The precipitated result was kept overnight (to maximize the precipitation). The precipitated supernatant was centrifuged on 8000 X *g* for 20 minutes at 4°C. The centrifugation product pellet was separated from the supernatant (10 mg) in order to be dialyzed by adding 5 ml (2:1) buffer A (25 mM Tris-HCl, pH 8) and dialyzed using 10 kDa cut-off dialysis membrane. Dialysis membrane was included in 600 ml of buffer B (50 mM Tris-HCl, pH 8) and rested for 24 h in hotplate stirrers (±5°C), in addition, the buffer was also replaced every 8 h (Gegeckas *et al*., 2014).

Purification of keratinase

The dialysis result was purified by Sephadex G-50 filtration gel chromatography, which ranges from 1.5 to 30 kDa (Sigma-Aldrich). The filtration gel was pre-equilibrated using Tris-HCl 25 mM buffer, pH 8. The samples were inserted into the gel slowly then eluted by Tris-HCl 25 mM buffer with pH 8 to separate the protein and stop it at the 13th fraction. Each fraction contains 1 ml of ready-made purified solution used for subsequent analysis.

Keratinase assay

The assay was performed as previously described (Sahoo *et al*., 2012) with some modifications. The keratinase activity was assayed with keratin (feather powder) as a substrate (Microbiology Laboratory, Universitas Sumatera Utara). Four milligram (0.004 g) of keratin powder was dissolves in 1 ml of 50 mM Tris–HCl, pH 8.0 used as substrate. Two hundred fifty microliters of substrate was added to 500 µl of enzyme solution with 250 µl 50mM Tris–HCl buffer pH 7.8 and was incubated for 30 min at 40°C in a waterbath. The reaction was terminated with 1 ml 10% trichloroacetic acid and incubated at 4°C for 10 min. Then, the reaction mixture was centrifuged at 10,000×g for 10 min, and the supernatant was used to measure the absorbance at 280 nm against a reagent blank using spectrophotometer. Tyrosine solution (50–500 µg ml^{-1}) was used for standard curve. One unit of keratinase activity was defined as the volume of enzyme which increases the absorbance of 0.01 at a wavelength of 280 nm.

Estimation of protein concentration

The protein concentration was essayed using a Bradford reagent. The composition of Bradford stock solution was 70 mg Coomassie blue G-250 (Sigma-Aldrich), 20 ml 95% ethanol, 40 ml 85% phosphoric acid homogenized with a magnetic stirrer, then filtered using Whatman qualitative filter. The Bradford reagent was prepared with a 425 ml distilled water, 20 ml 95% ethanol, 40 ml 85% phosphoric acid added by 30 ml Bradford stock solution homogenized with the magnetic stirrer, then filtered using Whatman qualitative filter. Protein content of keratinase was estimated using Bradford method (Bradford, 1976). Estimation of purified keratinase were based on SDS-PAGE visualization.

SDS PAGE and Zymography

Zymogram used a 10% separating gel with the addition of 0.2% keratin powder (chicken feathers). After the separation process, the gel was incubated in 2.5% Triton® X-100 at 37°C for 1 h. The gel was incubated in a 50 mM Tris-HCl buffer (pH 8) overnight. Gel was stained in 0.05% using Coomassie Brilliant Blue for 2 h. The last stage was to remove the remaining using coomassie® brilliant blue R-250 with the destaining solution until a white band appears.

Scanning electron microscopy (SEM) analysis of degradation products

Whole chicken feathers were degraded by keratinase strain B6 after the incubation of 48-96 h (37°C, 180 rpm). The controlled chicken feathers were cut into small pieces (±1 cm) and added with 2% coccodylate buffer. The fixation solution was removed, added by coccodylate and 1% tetratoxide and soaked for 1 h. The tetraoxide solution was removed and ethanol solution was given concentration levels of 70%, 80%, 90% and absolute ethanol. Solution was discarded and added by butanol. The sample piece of Au-coated metal was placed at the sample location in SEM (JEOL JSM-5310 LV, Japan) and tube was put into vacuum (0 Pa).

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) analysis was performed to find out the peak amino acid chicken feather degradation keratinase B6 after the incubation of 96 h. Fourier transform infrared spectroscopy (FTIR) analysis was based on the Wojciechowska method (Wojciechowska *et al*., 2004) with a slight modification. An IR spectroscopic analysis was performed using the FTIR 8201 PC, Shimadzu.

Profile of amino acids

Amino acid profilling was performed to observe the degraded product of keratin. The strain B6 was grown in the optimal feather medium for 5 days and the culture supernatant was obtained by centrifugation at 10.000 × g for 15 min. The quantity of amino acid was determined using a high-performance liquid chromatography (Waters Corporation USA), the column used was Pico tag 3.9 x 150 µm, UV wavelength detector set at 256 nm.

RESULTS AND DISCUSSION

Degradation of chicken feather waste

Chicken feather is a keratin substrate, which can activate keratinase produced by *Stenotrophomonas maltophilia* B6. Keratin is the only source of C and N for keratinase secretion. The formation of a clear zone around the colonies of strain B6 on NB agar and skim milk gave initial information on extracellular keratinase activity (Figure 1A). Figure 1B shows that biodegradation of chicken feathers occurred maximally after the treatment with B6 for 96 h of incubation at incubator shaker (180 rpm) at 40°C. Strain B6 was successfully isolated from landfill which effectively degrades chicken feather waste in moderate temperature. This is closely related to the temperature condition of the habitat of the isolation source. Native keratinase *Stenotrophomonas maltophilia* has various roles, such as reducing hexavalant chromium (Bhange *et al*., 2016), nematocidal activity (Jankiewicz *et al*., 2016), and plant growth-promoting activity (Jeong *et al*., 2010).

Optimization on carbon and nitrogen source of fermentation medium

The induction of carbon and nitrogen sources into the growth media greatly affects the production of keratinase by bacteria. Strain B6 utilizes chicken feathers as a major source of carbon and nitrogen for the continuity of cell growth. The addition of carbon and nitrogen sources was used as an additive to accelerate the continuity of cell division of strain B6. Great carbon supplementation affects the quantity of keratinase production (Figure 2A). Significantly, the keratinase activity increased up to 67.41 ± 1.1U mL^{-1} after the addition of sucrose to the media and culturing for 4 days. Glucose carbon sources were easily digestible by the bacteria because of their nature as monosaccharides. Meanwhile, the provision of other carbon sources can also increase the keratinase activity when it was compared to control. Sucrose has a positive influence on increased keratinase activity of *Thermoactinomyces* sp. YT06 (Wang *et al*., 2017).

The provision of nitrogen sources may also affect the effectiveness of keratinase as an indicator of keratinase activity shown in Figure 2B. Peptone was able to increase keratinase activity by 90.24 U mL^{-1} after 96 h of incubation. In addition, $NaNO_3$ and casein (88.50 U mL^{-1} ± 0.61 and 88.49 ± 0.51 U mL^{-1}) also contributed in increasing the keratinase activity in our experiments. This result was the first report for *Stenotrophomonas maltophilia* that increased the keratinase activity by peptone which was previously reported for keratinase activity in *Bacillus thuringiensis* TS2 (197.60 ± 3.15 U mL^{-1}) (Sivakumar *et al*., 2012). Incubation of 96 h is the best time to increase the keratinase activity of strain B6.

Growth of strain B6 and soluble protein

The observation of bacterial cell growth and total dissolved protein in the treatment of glucose and

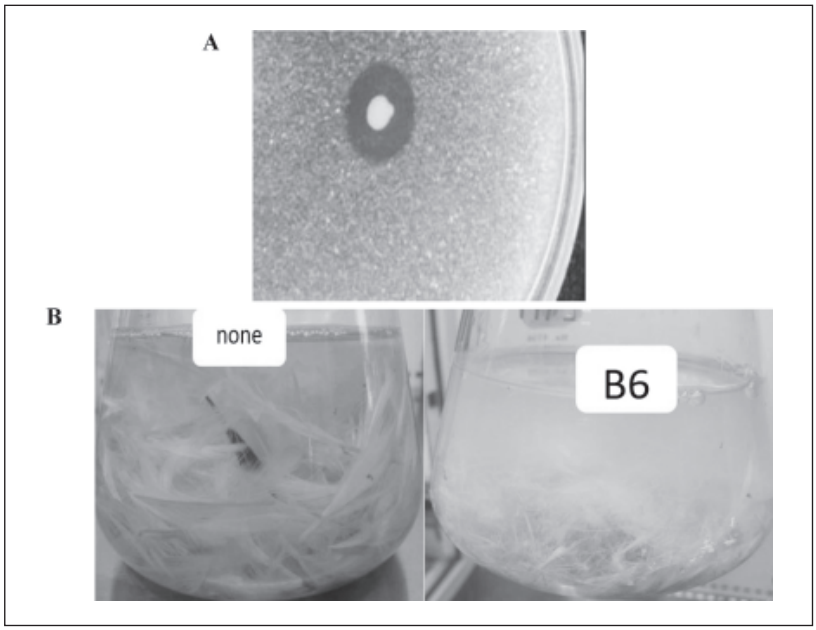

Fig. 1. The degradation process of chicken feathers by the strain B6 for 96 hours of incubation. (A) The formation of clear zone around the colonies of strain B6 which were grown on *skim milk agar* at 40°C for 24 hours. (B) The result of treatment comparison toward the chicken feathers with and without giving the B6 strain bacteria after 96 hr.

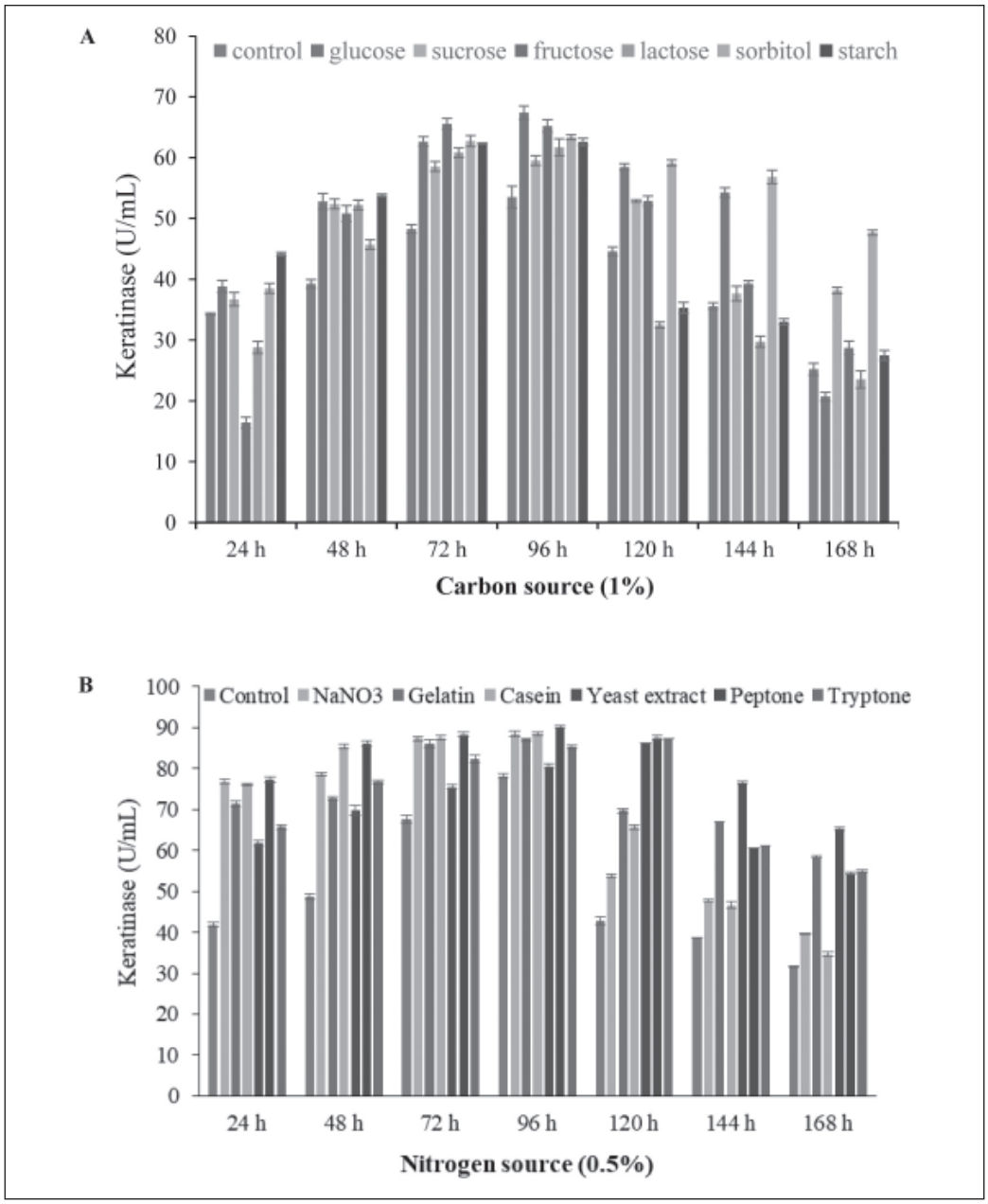

Fig. 2. Relative activity of strain B6 supplementation with different carbon and nitrogen sources. (A) Influence of carbon source. (B) Influence of nitrogen source. Values represent the mean ± SD of three replicates.

peptone after 96 h of incubation was also performed. The results shown that the addition of glucose and peptone treatment helps the growth of bacterial cells more rapidly which is sufficient for quorum as an initiation to increase keratinase production. The bacterial cell at 96 h reached 4.22×10^8 cells mL^{-1} (Figure 3A) and the soluble proteins reached 67.96 mg mL^{-1} (Figure 3B) on the treatment of glucose as a carbon source. Meanwhile, the number of bacterial cells of 5.49×10^8 (Figure 3C) and the soluble proteins of 93.36 mg mL^{-1} (Figure 3D) was treated after the addition of peptone. The relevance of increased keratinase activity to soluble proteins has been demonstrated in this study.

Table 1 shows that the total free amino acid concentration in the culture filtrate was 2.2569 mg mL^{-1} after the degradation of 96 h. Alanine, serine, threonine and tyrosine were the mostly found amino acids during B6 hydrolysis supernatant result. Biotechnological applications consider the use of keratin degrading microorganisms or keratinase enzymes in the production of amino acids and peptides. Moreover, amino acids resulting from the utilization of feather or keratin-containing materials are one important product that can promote several industries such as animal feed and bio-fertilizer. Amino acids released during the biodegradation of feather by the host.

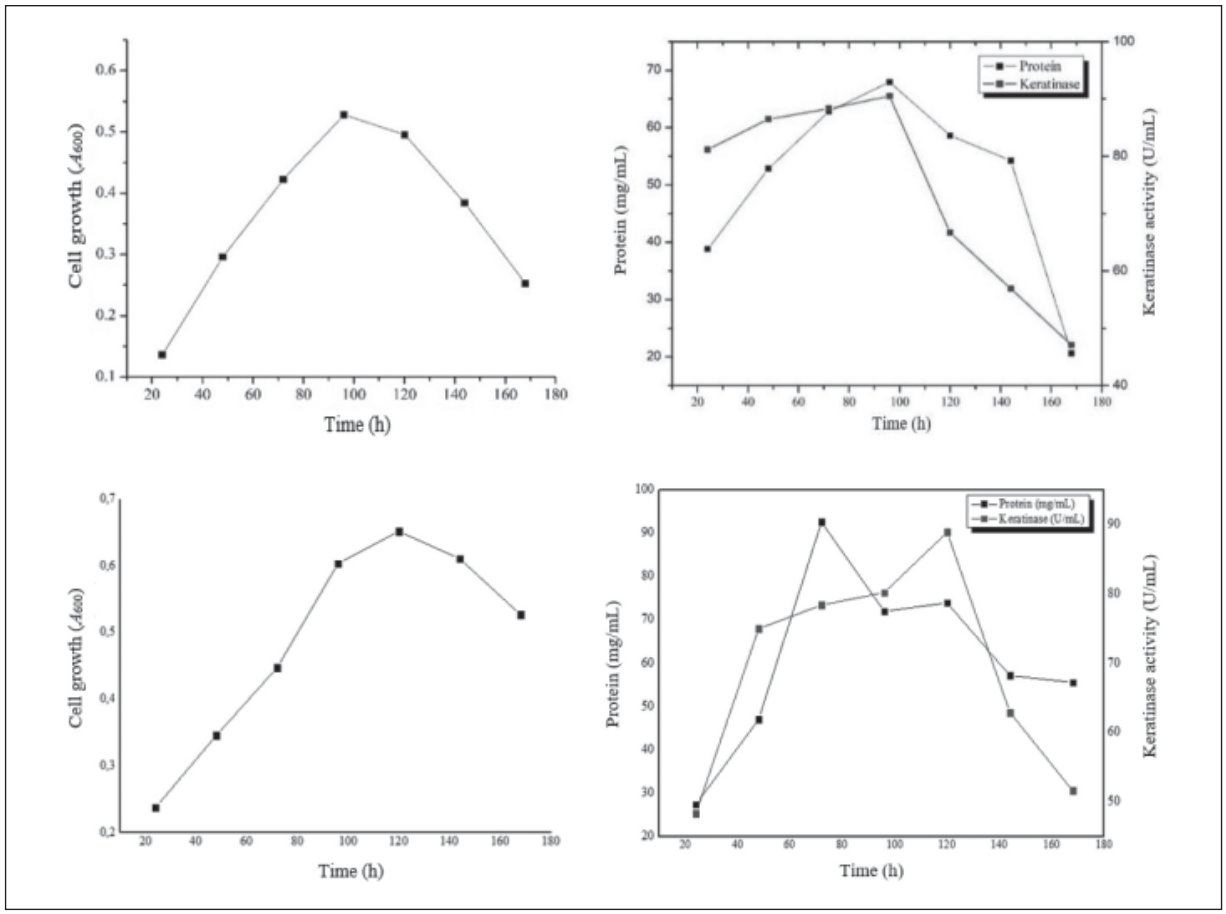

Fig. 3. The growth of bacterial cells and soluble proteins during the addition of the glucose and peptone. (A) The growth of bacteria which is enriched glucose 1% (w/v). (B) Soluble proteins and the activity of keratinase after the addition glucose 1% (w/v). (C) The growth of bacteria which is enriched peptone 0.5% (w/v). (D) Soluble proteins and the activity of keratinase after the addition peptone 0.5% (w/v).

Table 1. Monitoring the level of amino acids in the cell-free supernatant of strain B6 at day 4 of cultivation

Amino acids	Concentration (mg $^{mL-1}$)
Glycine	0.1207
Alanine	0.3387
Arginine	0.1664
Aspartic acid	0.0993
Glutamic acid	0.0941
Phenylalanine	0.0352
Histidine	0.0408
Isoleucine	0.0378
Leucine	0.0678
Lysine	0.1563
Methionine	0.0128
Proline	0.1716
Serine	0.2493
Cysteine	0.0484
Threonin	0.2393
Tyrosine	0.2228
Valine	0.1556
Total	2.2569

Effect of metal ions to keratinolytic activity

The keratinase activity of strain B6 is increased by ions Ca^{+}, Mg^{+}, Na^{+}, Co^{+} and K^{+}, respectively 116%, 110%, 111%, 111% and 109% (Table 2). The keratinase activity of strain B6 decreases due to the administration of 10 mM Mn^{+} and 10 mM Ba^{+}. The result of this test gives the consideration that Co^{+}, Mn^{+} and Ba^{+} do not increase the keratinase activity of both bacteria. The conformation of enzyme structure and complex substrate enzyme becomes stable and maintained by the salts formed by the metal ions. Moreover, cation plays an important role in maintaining the thermal stability of the enzyme**.** Brandelli *et al.* (2010) stated that the presence of divalent metal ions such as Ca^{2+}, Mg^{2+}, and Mn^{2+} always stimulate the activity of keratinase protein. Keratinase of strain B6 was positively stimulated by Ca^{2+} and Mg^{2+} (10 mM) (Table 2). Metal ions can be used for the process of hydrolysis optimization of keratin waste as the additive for keratinase stabilization. This result was confirmed by the previous study where the addition of Ca^{2+} and Mg^{2+} in the production of keratinase media

Table 2. The Effect of the Addition of several types of metal ions and inhibitor on the activity related to native keratinase strain B6

Metal ions and Inhibitors	Relative activity (%)	
	5 mM	10 mM
Control	100	100
Ca^{2+}	111 ± 0.7	116 ± 1.5
Mg^{2+}	108 ± 1.6	110 ± 1.5
Ba^{2+}	111 ± 1.3	76 ± 1.5
Mn^{2+}	83 ± 1.0	78 ± 1.0
Co^{2+}	107 ± 1.5	111 ± 2.0
Na^{+}	101 ± 1.0	111 ± 1.0
K^{+}	105 ± 1.5	109 ± 1.5
Pepstatin A	55 ± 2.0	16 ± 1.0
PMSF	56 ± 2.0	29 ± 1.5
DTT	55 ± 1.5	43 ± 1.6
EDTA	94 ± 1.0	114 ± 1.6
N-toyl-L-lysine	48 ± 1.0	31 ± 1.5
bromoacetic acid	42 ± 1.5	19 ± 1.5
chymostatin microbial	15 ± 1.0	11 ± 1.5
Iodoacetic acid	52 ± 1.0	32 ± 1.5
Benzamidine	31 ± 1.5	18 ± 1.6
Trypsin inhibitor	73 ± 2.0	40 ± 1.5
2-mercaptoethanol	66 ± 2.0	14 ± 1.0

increased their activity (Lateef *et al*., 2015; Tork *et al*., 2016).

Protease can be classified based on their sensitivity to various types of inhibitors (Rawlings *et al*., 2004). It is important to know the protease type of its keratinase of strain B6 and other enzymes involved in the keratin degradation. The treatment of inhibitor administration in both bacteria does not increase the keratinase activity, except the addition of 10 mM EDTA that can stimulate the keratinase activity of strain B6 (110%) (Table 2). From all of the tested inhibitors, PMSF actually inhibits the keratinase (Table 2). It shows that its keratinase was the serine protease. Bose *et al*. (2014), stated that the keratinase activity of *Bacillus amyloliquefaciens* was inhibited by 5 mM PMSF. The keratinase activity is only 1.25%, which indicates the production of serine protease. This research result shows that EDTA can give a positive effect on keratinase activity of both keratinase bacteria. Keratinase activity of *Bacillus subtilis* of 10 mM EDTA can stimulate the keratinase activity up to 144.8% (Cai *et al*., 2008).

EDTA as the chelator activates the active site of reductase and optimally hydrolyses the disulphide bond in the keratin structure resulting in 10 times of the increase of hydrolysis. The presence of reductase disulphide was beneficial not only to catalyse the reduction of the disulphide bond (S-S) but also the secretion of disulphide bond, which contains protein (Rahayu *et al*., 2012). The formation of disulphide bond is inhibited by metal complex ion EDTA (Trivedi *et al*., 2009). This result indicates that strain B6 produces other enzyme to degrade keratin namely disulphide reductase.

Purification of keratinase

The keratinase SmB6KER was purified by ammonium sulfate fractionation followed by dialysis membrane (10 kDa) and Sephadex G-50 (Table 3). The purity of protein was analyzed by SDS-PAGE and keratinase activity was determined by zymogram after SDS-PAGE using keratin from chicken feathers as the substrate. Dialysis membrane resulted in 6.24 fold purification with a 63.64% recovery rate. After Sephadex G-50 chromatography, the purification was 19.18 fold with a final recovery to 35.61% and 397.52 U/mg of specific activity. The molecular mass of this purified SmB6KER was determined to be 26 kDa by SDS-PAGE (Figure 4). An activity gel analysis confirmed that the purified SmB6KER was a single monomeric protein. Molecular mass of keratinase ranged from 18 to 240 kDa (Brandelli *et al*., 2010), 80 kDa (*Bacillus cereus*), 26 kDa (*Bacillus licheniformis*), 30 kDa (*Bacillus megaterium*), 36 kDa (*Stenotrophomonas maltophilia* N4) (Akhtar & Edwards, 1997), 36 kDa (*Stenotrophomonas maltophilia* DHHJ) (Gupta & Ramnani, 2006), 130 kDa (*Fervidobacterum pennavorans*) (Friedrich & Antranikian, 1996). We successfully reported that *Stenotrophomonas maltophilia* B6 has protein keratinase molecular mass of 26 kDa, which previously has never been reported.

SEM analysis of degradation product

SEM visualization shows that the degradation of chicken feathers by strain B6 occurred after 96 h of incubation (Figure 5). Feather barbicels were completely degraded by strain B6, also the length

Table 3. Purification profile of Keratinase from strain B6

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Concentrated Supernatant	12648	610.20	20.73	1	100
Ammonium Sulphate 70%	10380	303.94	34.15	1.65	82.07
Dialysis	8050	62.18	129.46	6.24	63.64
Sephadex G-50	4504	11.33	397.52	19.18	35.61

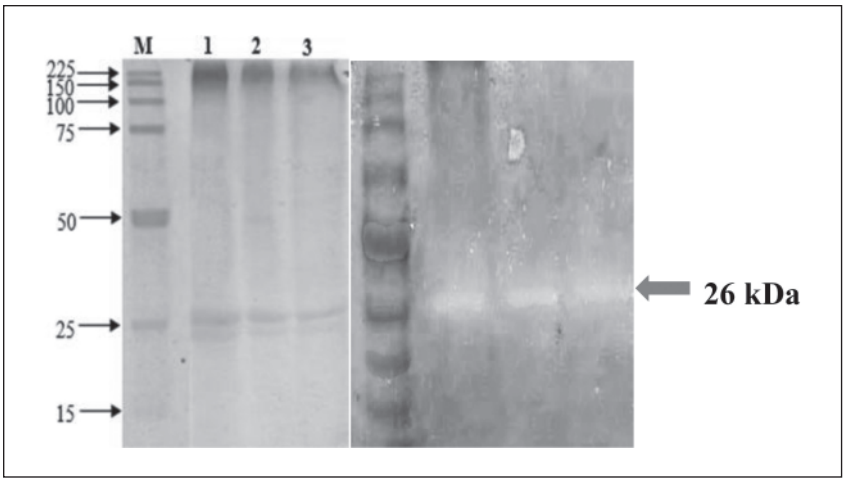

Fig. 4. SDS-PAGE and zymogram analysis of keratinase secreted by strain B6. (M) 10-225 kDa Protein Marker, (1) Precipitation of ammonium sulfate 70% (2) 10 kDa dialysis membrane (3) CM Sephadex G-50 chromatography. Arrows indicate the presence of protein bands.

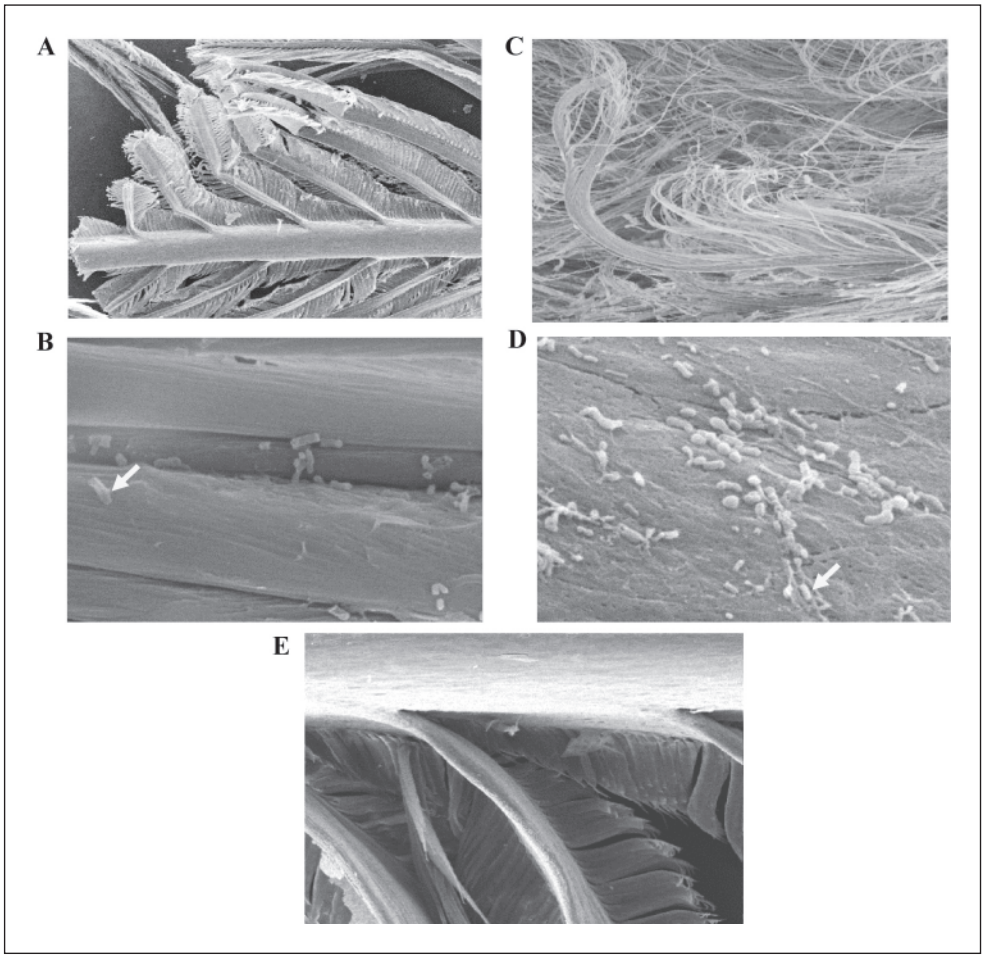

Fig. 5. Scanning electron micrographs of feather degradation by *S. maltophilia* B6. (A) The degradation of barb and barbules after 2 days; (B) The colonization of bacterial cells on feather surface after 2 days (yellow arrow); (C) The degradation of feather shaft after 4 days; (D) The colonization of bacterial cells on feather surface after 4 days (yellow arrow); (E) The uninoculated whole chicken feather and feather barbs.

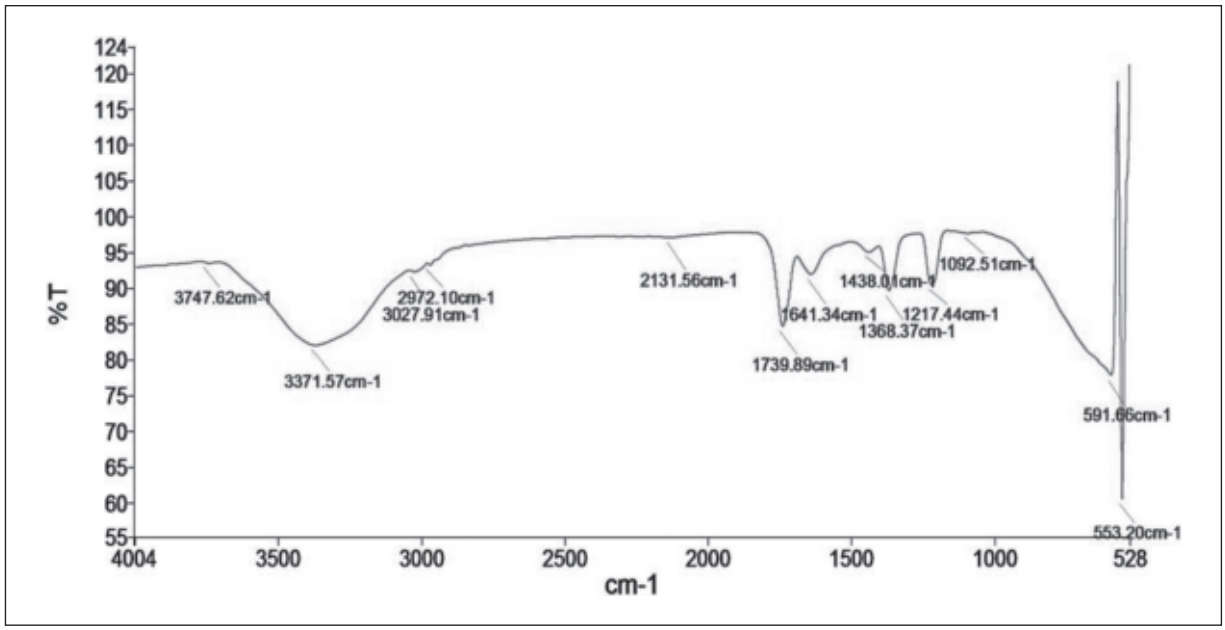

Fig. 6. IR spectra of degraded feathers. peak 3747 cm^{-1} (*–OH*); peak 2972 cm^{-1} (asymmetric -CH_3); peak 1641 cm^{-1} (amide I); peak 1438 cm^{-1} (amide II); peak 1092 cm^{-1} (–C–C–); peak 523-553 cm $^{-1}$ (–S–S–).

of the barbules and the width of the barbs were reduced (Figure 5C). The bacterial cells colonize on the surface of the chicken feather structure and cause the fiber structure to crack and disintegrate slowly (Figure 5D). This result was similar to the previous report that keratinase may damage the structure of the keratin substrate (Cedrola *et al*., 2012). Indirectly, chicken feathers were the only source of carbon and nitrogen for the growth of bacterial cells. This resulted was more convincing to use isolate B6 as a poultry feed additive. The abundance of chicken feather waste in the environment can be minimized through further research activities.

FTIR analysis of degradation product

The degradation of chicken feathers by strain B6 was confirmed using FTIR, keratin structure group was seen after the presence of peak spectrum FTIR is detected. FTIR spectra of degraded feather displayed that transmittance peaks nearby 523, 553, 1092, 1217, 1368, 1438, 1641, 1739, 2131, 2972, 3027, 3371 and 3747 cm^{-1}. The peak located in the range of 2700-3100 cm^{-1} indicating that the presence of CH groups, and the broad peak around 3747 cm^{-1} was usually caused by the vibration of hydrogen bonded –OH groups (Akhtar & Edwards, 1997). The transmittance peak for the amideI (1650 cm^{-1}) and amideII (1547 cm^{-1}) suggests that there was a presence of α-helix structure in the sample, moreover the amideI (1638 cm^{-1}) and amideII (1515 cm^{-1}) indicate the presence of a β-sheet type (Wojciechowska *et al*., 2004). The peak near 1100 cm cm^{-1} was observed, and this fact indicates that C–C groups presents in each of the two samples (Wojciechowska *et al*., 2004).

Figure 4 showed the chicken feathers incubated by adding culture strain B6 resulted in the peaks of disulphide bonds become weak. Disulphide bonds owing to the S–S stretching vibrations show a peak in the 500–550cm^{-1} (Wojciechowska *et al*., 2004; Wojciechowska *et al*., 2002). The peaks appear in the range of 523-553 cm^{-1} as shown in Figure 6, which represents disulphide bonds present in the sample. The cysteine composition (8.85%) on chicken feathers contributes to thiol structural element (SH) (Lee *et al*., 2015). Therefore, keratinase bacteria need to be utilized to hydrolyze the bond. Our experiment successfully reported that molecular mass of protein keratinase by strain B6. *Stenotrophomonas maltophilia* B6 has protein keratinase molecular mass of 26 kDa. This new isolated strain possesses high keratinase activity and is very effective in feather degradation, presenting potential use for biotechnological processes involving keratin hydrolysis.

CONCLUSION

In conclusion, optimization and characterization of a newly bacterial isolate, *Strenotrophomonas maltophilia* B6 (SmB6KER) has successfully enhanced the keratinolytic activity. The addition of glucose for 1% as the source of carbon is the best treatment in order to increase the keratinase's activity by 67.41 ± 1.1 U mL^{-1}, while the best source

of nitrogen is pepton for 0.5% with yielded 90.24 ± 0.7 U mL^{-1}. SmB6KER belongs to serine protease family since its relative activity is inhibited by 10 mM of PMSF (29 ± 1.5%), but its relative activity back to increase after the addition of 10 mM EDTA (114 ± 1.6), indicating that SmB6KER utilizes EDTA as chelating agent. SmB6KER has protein weight of 26 kDa of molecular mass. The analysis result using SEM indicates that SmB6KER is able to degrade the chicken feathers after 96 hours of incubation. It is encouraged by the result of FTIR, which indicates that the disulfide bond (-S-S-) is in the peak range of 523-553 cm^{-1}. The degradation of chicken feathers also produced 2.2569 mg mL^{-1} of amino acids from 40 mL fermentation medium. This result give significant idea that SmB6KER whose cell growth has been characterized and optimized can be used as excellent strain for the degradation process of chicken feathers substrate and synthesis product in the form of amino acid which further used as application aspect.

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