JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2017. Vol. **11**(1), p. 173-180

Partial Characterization of Keratinolytic Activity of Local Novel Bacteria Isolated from Feather Waste

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http://dx.doi.org/10.22207/JPAM.11.1.22

(Received: 25 November 2016; accepted: 17 January 2017)

Three keratinolytic bacterial isolates were characterized partially for their keratinase activity. Bacterial isolates were grown in feather meal agar. Ammonium sulfate precipitation followed by dialysis was performed to know the bacterial isolate keratinase activity in differet pH and temperature. Identification of the bacteria was done by using their 16S rRNA gene sequences. The result showed that bacterial growth was coinciding with keratinase activity. Precipitation with ammonium sulfate showed that keratinae activity of isolate A4 was optimum at 20% of ammonium sulphate, while B4 and B6 were more active at 70%. Keratinase activity increased after dialysis. Keratinase of A4 showed to have optimum activity at temperature of 45ºC and pH=8, B4 was optimum at temperature of 35ºC and pH=7, while B6 was optimum at temperature of 40ºC and pH=7, respectively. Identification of the bacterial isolates using 16S rRNA gen showed that A4, B4, and B6 were closed to *Leclercia adecarboxylata strain* M-X17B, *Azotobacter chroococcum strain* ABA-1, and *Stenotrophomonas maltophilia strain* BIW by 97%, 99%, and 98%, respectively. Two bacteria *L. adecarboxylata* and *A. chroococcum* were firstly reported to produce keratinase.

Keywords: Keratinase, Chikhen feather, Goat fur, *Azotobacter chroococcum*, *Leclercia adecarboxylata*, *Stenotrophomonas maltophilia*.

Poultry processing plants are producing millions of tons of feathers, which mainly consisted of keratin (Brandelli, 2008; Agrahari & Wadhwa, 2010; Mazotto *et al*., 2010; El-Ayouty *et al*., 2012). Keratin filament structures are stabilized by their high degree of cross-linking of disulfide bonds, hydrophobic interactions, and hydrogen (Brandelli, 2008; Xu *et al*., 2009), which make them unsoluble and resistant to most of the known proteases like trypsin, pepsin, papain and results in polluting the environment (Brandelli, 2008; Xu *et al*., 2009; Kansoh *et al*., 2009; Duarte *et al*., 2011; Sivakumar *et al*., 2012). However, keratin does not accumulate in nature (Rahayu *et al*., 2010).

Keratin sources such as feather, horn, nails and hair are abundantly available in nature as wastes. High protein content of keratin waste can be used as a good source of protein and amino acids by systemic recycling, which may provide a cheap and alternative protein feed stuff (Jeong *et al*., 2010; Duarte *et al*., 2011; Mazotto *et al*., 2011; Tiwary & Gupta, 2012). Feathers are currently used to produce feather meal using physical and chemical treatments, destroying certain amino acids and resulting in a low nutritional value product (Jeong *et al*., 2010; Duarte *et al*., 2011; Mazotto *et al*., 2011; Tiwary & Gupta, 2012).

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Certain microorganisms produce keratinases to degrade keratins and may be used to enhance the digestibility of feather keratin (Riffel & Brandelli, 2008). The nutritional upgrading of feather meal through microbial or enzymatic treatment has been described (Xu *et al*., 2009; Agrahari & Wadhwa, 2010; El-Ayouty *et al*., 2012). Bacterial genera such as *Burkholderia*, *Chryseobacterium*, *Pseudomonas*, *Microbacterium* sp. (Riffel & Brandelli, 2008), *Streptomyces* (Brandelli, 2008), and *Bacillus* (Mazotto *et al*., 2011; Brandelli, 2008; Lucas *et al*., 2003) have been reported to synthesize keratinase. The bacteria were grown on substrats such as feather meal, raw feathers, chicken nails, hair, and wool as sole carbon and nitrogen sources for the bacterial growth (Riffel & Brandelli, 2008).

So far, little attention has been given in study of utilization and degradation of feather waste by keratinolytic microorganisms in Indonesia. Rahayu *et al*. (2010) reported a study on keratinase of two Indonesian bacterial isolates. In this study, partial characterization of keratinolytic activity of three bacterial isolates from a feather decomposting waste was conducted. Their ability to degrade chikhen feather and goat hair has also been studied.

MATERIALS AND METHODS

Cell growth and keratinase activity

Bacterial culture was spreaded on feather meal agar (0,5 g/l NaCl; 0,7 g/l K_2HPO_4; 1,4 g/l KH_2PO_4; 0,1 g/l MgSO4 added with 10 g/l feather meal, and 15 g/l agar) and incubated at ambient temperature (Cai *et al*., 2008). Cell growth was measured everyday as colony forming unit/ml.

To measured keratinase activity, 500 µl crude enzyme was added with of phosphate buffer saline (PBS) of pH 7, and mixed with 500 µl of 0.5% keratin solution. Enzymatic reaction was stopped by adding 1 ml of 10% tricloroacetic acid, and spinned at 10.000 rpm for 30 minutes. Free amino acid in supernatant was measured using spectrophotometer at ë=280 nm. One unit of enzyme activity is defined as the increasing of absorbance of 0.01.

Ammonium sulphate precipitation and dialysis

Broth culture of bacterial isolates was centrifugated at 10.000 g for 10 minutes. Supernatant was precipitated using 20 to 70% of ammonium sulphate for 2 hours, and was spinned at 10.000 rpm for 10 minutes at 4°C. Precipitate and supernatant was subjected to keratinase activity assay. Precipate was solubilized with PBS of pH 7 prior enzyme activity assay. Precipitate showed higher activity was choosen for dialysis. Dialysis was conducted using PBS for 6 hours by changing the buffer twice. Keratinase activity of dialysis was measured as previously described.

Characterization of keratinase in different pH and temperature

Dialysed enzyme was subjected to be assayed in different temperature and pH. To characterize enzyme temperature, enzyme and substrate mixture was incubated at 31°C of optimum pH for 15 minutes. Effect of pH on keratinase activity was measured at varying pH of 4.0, 5.0, with Na-acetate buffer, 6.0, 7.0 with K-phosphate buffer, and 8.0, 9.0 with Tris-HCl buffer. The reaction was conducted at 31°C for 15 minutes. Keratinase activity was measured as previously described.

Identification of keratinolytic bacterial isolates based on its 16S rRNA genes

Keratinolitik bacterial DNA isolation WAS done by freeze and thaw method. One loop of bacterial culture1 of 24 hours put into microtube of 100 mL aquabidest. Suspension was frozen at -10°C and thawed with 90°C for 10 minutes for 5 times. Suspension was spinned and supernatan was taken out. The supernatan was used for 16S rRNA gene amplication using Ready To Go PCR Beads (*Pharmacia-Biothec*) with primer 63f (5'-C A G G C C T A A C A C A T G C A A G T C -3') dan 1387r (5'- GGCGGWGTGTACAAGGC-3') (Marchesi *et al.*, 1998).

Total volume of the PCR reaction (25 ml) consisted of 1.5 U *Taq* DNA polymerase, 10mM Tris-HCl (pH 9 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTPs and stabilizer including BSA. Reaction was conducted for pradenaturation at 94°C for 2 minutes, denaturation at 92°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C fro 1 minute, and post-PCR 72°C for 5 minutes in a thermocycler (Verity® 96-Well Thermal Cycler 437586, Applied Biosystems, Singapore). PCR was done for 30 reaction. PCR result was visualised with mini gel electrophoresis.

Construction of phylogenetic tree

Amplified 16S rRNA gene was purified and commercially sequenced. DNA sequence was compared to GenBank database of *The National Center for Biotechnology Information* (NCBI) (http://www.ncbi.nlm.nih.gov) using *Basic Local Alignment Search Tool* (BLAST). Cluster analysis was conducted using MEGA7.

RESULTS

Cell growth and keratinase actvity

Keratinase production profiles of each bacterial isolates were not similar. B4 showed to have higher cell number and keratinase activity at 5 days of incubation, while the other two A4 and B6 were higher at 4 days of incubation. However, it seemed that keratinase activity was in line with cell growth (Figure 1.). Maximum activity of keratinase of three isolates was shown at the end of exponential growth phase and at stasioner phase. Werlang & Brandelli (2005) reported that maximum keratinase activity of *Bacillus* sp. kr 16 incubated at 30ºC occurred at the end of exponential growth phase, as also showed by Zhang *et al*. (2009) of *Bacillus* sp. 50-3 incubated at 37ºC. Cell growth and keratinase production could be increased by using C-source such as starch (Gioppo *et al*., 2009).

Ammonium sulphate precipitation

Keratinase activity of precipitate and supernatant were measured after ammonium sulphate precipitation. It was observed that keratinase activity of fraction of 40 to 60% of ammonium sulphate were relative similar between pellet and supernatant of all isolates. Keratinase activity of pellet of B4 was higher at 70%. Interestingly, keratinase of the isolotes showed to have two peaks. Keratinase of A4 and B6 was optimum at 20 and 70%, while that of B4 was optimum at 30% and 70%, respectively (Figure 2.). This result indicated that the isolates might produce two different proteins. Rahayu *et al*., (2010) reported that *Bacillus* sp. MTS showed to produce 6 different proteins of 17, 25, 32, 53, 96 and 122 kDa showing keratinolityc activity.

Fraction of 20% of A4 protein, and of 70% of B4 and B6 were subjected to dialysis. Specific activity of keratinase increased after ammonium sulphate precipitation and dialysis, while total protein decreased, as shown in Table 1. *Bacillus pseudofirmus* keratinase activity precipited with ammonium sulphate increased its keratinase activity to 55.68 U/ml (Kojima *et al*., 2006). Keratinase activity of isolat L1 precipited with ammonium sulphate followed with dialysis increased 3 fold with yield of 42% (Cao *et al*., 2009).

Effect of pH on keratinase activity

Optimum pH of A4, B4, and B6 keratinase activity varied. B4 and B6 showed to have optimum activity at pH 7, while A4 was optimum at pH 8. Keratinase activity declined at pH above or below 7 and 8 (Figure 3.). Keratinase *Bacillus subtilis* MTCC (9102) (Kumar *et al*., 2010), *Stenotrophomonas maltophilia* DHHJ (Cao *et al*., 2009), and *B. subtilis* (Cai & Zheng, 2009), was optimum at pH 7, 7.8, and 8.5, respectively. Werlang & Brandelli (2005) reported that their bacterial isolate keratinase were active at pH 8 to 11, while *Bacillus* sp. 50-3 (Zhang *et al*., 2009) and *B. halodurans* PPKS-2 (Prakash *et al*., 2010) showed to have optimum pH of 10 and 11, respectively.

Effect of temperature on keratinase activity

Keratinase activity of all isolates showed to have different activity at different temperature (Figure 4.). The optimum temperature for A4 was at 45°C, B4 was at 35°C, while B6 was at 40°C. Keratinase of *Bacillus* kr 16 (Werlang & Brandelli, 2005) and *B. halodurans* PPKS-2 (Prakash *et al*., 2010) of chikhen feather waste were optimum at 45-65ºC and 60-70ºC, respectively. Zhang *et al*., (2009) reported keratinase of *Bacillus* sp 50-3 isolated from animal feces of Beijing Zoo was optimum at 60ºC. Keratinase of *Paracoccus* sp. isolated from poultry soil was optimum at 50ºC (Lee *et al*., 2004). *Stenotrophomonas maltophilia* DHHJ (Cao *et al*., 2009) and *B. subtilis* MTCC (9102) (Balaji *et al*., 2008) showed their optimum keratinase activity at 40ºC, while *B. subtilis* (Cai & Zheng, 2009) and isolate kr 6 (Riffel *et al*., 2003) were more active at 55ºC.

Identification of keratinolytic bacterial isolate based on its 16S rRNA gene

Isolates A4, B4, and B6 were identified by using their 16S rRNA gene sequences. A4, B4, and B6 was closely related to *Leclercia adecarboxylata* strain M-X17B, *Azotobacter chroococcum* strain ABA-1, and *Stenotrophomonas maltophilia* strain BIW by 97%, 99%, and 98%, respectively (Figure 5.). *Leclercia adecarboxylata* and *Azotobacter*

chroococcum has not been reported previously as keratinolytic bacteria. So far, this is the first report that these two bacteria produced keratinase. *Leclercia adecarboxylata* was previoulsy known as opportunistic pathogenic bacteria (Keren *et al*., 2014), and was utilised as biocontrol agents of *Leptinotarsa decemlineata* (Muratoglu *et al*., 2009). It was also reported to degrade polycyclic aromatic hydrocarbons of oil spills contaminated soil (Sarma *et al*., 2004). *Azotobacter chroococcum* was reported as nirogen fixing bacteria (Mahato *et al*., 2009; Damir *et al*., 2011) and in producing polyester polyhydroxialkanoic (Lopez *et al*., 1996).

One of the isolate B6 was identified as *Stenotrophomonas maltophilia*. This bacteria was isolated from chikhen feather composting soil in China. Growth and degradation activity of *S. maltophilia* DHHJ were optimum at at 40ºC in pH of 7,5-8 (Cao *et al*., 2009).

DISCUSSION

Keratin is a fibrous and insoluble structural protein found in feathers, hair, nails, horns and other epidermal tissue. Extensively cross-linked by disulfide, hydrogen and hydrophobic bonds makes it very resistant to digest by common proteases like pepsin, papain, dan tripsin (Werlang & Brandelli, 2005). Keratin can be degraded by breaking or break the disulfide bonds in keratin. This can be done with high heat (hydrothermal), chemical treatment and biological treatment. Hydrothermal treatment is done by setting a high temperature and pressure, as well as chemical treatments carried out by the addition of acid (HCl) and base (NaOH) at a high concentration (Cai & Zheng, 2009).

Many bacteria have been reported to produce keratinase. Keratinase production profile

Table 1. Purification of keratinase produced by the keratinolytic bacterial isolalates

Isolates	Purification step	Total protein (mg/ml)	Enzyme activity (U/ml)	Spesific activity (U/mg)	Yield (%)	Purification (fold)
A4	Supernatant	55.41	108.70	1.96	100	1
	Ammonium sulphate (20%)	38.88	95.90	2.47	88.22	1.26
	Dialysis	21.64	99.70	4.61	91.72	2.35
B4	Supernatant	52.18	65.80	1.26	100	1
	Ammonium sulphate (70%)	42.81	57.50	1.34	87.39	1.07
	Dialysis	20.20	63.20	3.13	96.05	2.48
B6	Supernatant	66.79	86.80	1.30	100	1
	Ammonium sulphate (70%)	49.22	71.20	1.45	82.03	1.11
	Dialysis	20.54	83.90	4.08	96.66	3.14

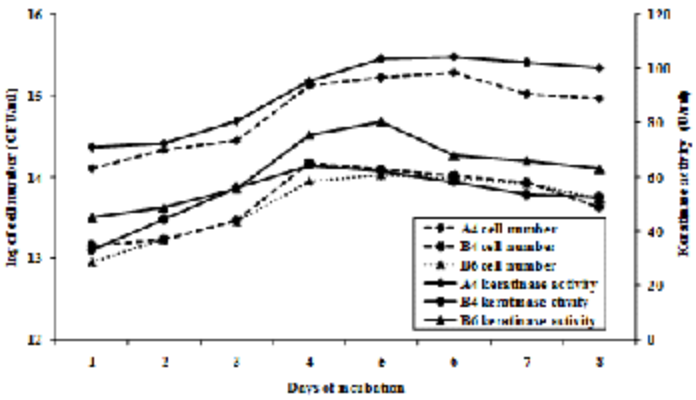

Fig. 1. Keratinase activity during bacterial cell growth in feather meal broth at 31°C and initial pH 6.5

of each microorganism can vary. It depends on the gene encoding the keratinase, the composition of culture medium as a substrate, concentration of carbon and nitrogen, temperature, pH, and aeration (Brandelli *et al*., 2010). Optimum temperature of bacterial enzyme activity is usually correlated with temperature in where the bacteria live. All of our isolates were isolated from chikhen feather waste which was progressively decomposed. The bacteria live in should be appropriate ones, having optimum enzyme acitivity of higher temperature, as also shown, for examples in Werlang & Brandelli (2005), Prakash *et al*. (2010), Zhang *et al*., (2009), Lee *et al*., (2004), and Cao *et al*. (2009) who isolated the bacteria from decomposing chikhen feather, animal feces, and poultry soil. Our keratinase might be induced by keratin substrat such as feather and fur, since the isolates were able to grow and produced keratinase in medium in which feather and fur served as a sole carbon and nitrogen source, as also shown by Suntornsuk *et al*. (2005).

Most keratinases are alkaline or netral protease with optimum pH of 7.5 to 9. However, some were extremely acidic or alkaline (Brandelli *et al*., 2010). Kumar *et al*., (2010), Prakash *et al*., (2010), Cao *et al*., (2009), Cai & Zheng (2009), Zhang *et al*., (2009), and Werlang & Brandelli (2005) showed that their bacterial keratinase activities were optimum at pH between 7 to 11. Our isolates showed to be more active in slightly alkaline or netral with optimum pH of 7 to 8, accordingly.

The keratinase of A4 and B6 reached maximum production in 5 days, while B4 reached it in 4 days, in which maximum growth was observed. This was similar to that of Suntornsuk *et al*. (2005) in which the maximum production of keratinase reached a maximum in the late logarithmic growth phase. Based on the maximum growth that were coinciding with maximum biomass and maximum specific production rates observed at the exponential growth phase, our keratinases were produced as a primary metabolite (Brandelli & Riffel, 2005). After reach maximum cell growth and enzyme activity, a loss of enzyme activity was observed probably because of enzymatic autolysis and end product inhibition (Suntornsuk *et al*., 2005).

The isolates showed only partial digestion of both chikhen feather and goat fur, with more chikhen feather degraded. Kansoh *et al*. (2009) showed that feather was more easy to be degraded compared tho that of hair, nail, and wool.

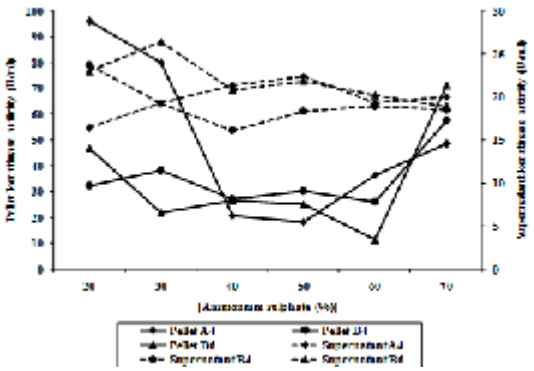

Fig. 2. Keratinase activity of pellet and supernatant of bacterial culture precipitated using ammonium sulphate

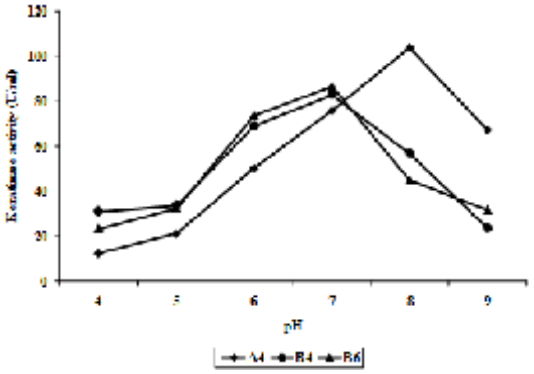

Fig. 3. Effect of pH on keratinase activity of bacterial isolates

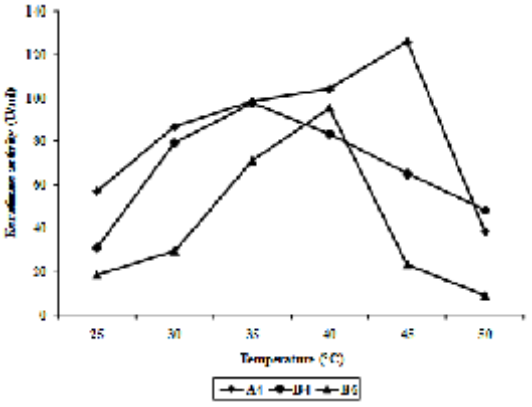

Fig. 4. Effect of temperature (°C) on keratinase activity of bacterial isolates

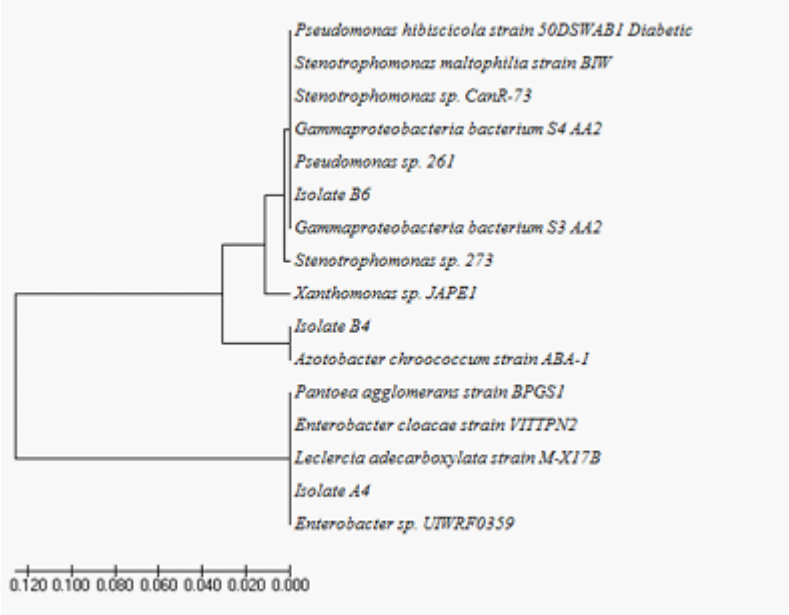

Fig. 5. Phylogenic tree of isolat A4, B4, and B6 based on their 16S rRNA genes

This result indicated that this enzyme could not completely cleave the disulphide bonds. Pre-treatment of these substrates such as chicken feather, nail and human hair by physical method or reducing agents, or detergents, or activation of the enzyme by adding metal salts are required for the improvement of their degradation (Suntornsuk *et al*., 2005).

ACKNOWLEGMENTS

We would like to thank to DRPM, Ministry of Research, Technology, and Higher Education, Republic of Indonesia to fully supporting this research.

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