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Original Article

Isolation and identification of keratinolytic bacteria from Jember, Indonesia as a biodegradation agent of chicken feather wastes

Sutoyo Sutoyo[1,2*], Subandi Subandi[3], Tri Ardyati[1], Suharjono Suharjono[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, 65145 Malang Jawa Timur, Indonesia

[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Jember, Jl. Kalimantan 37, Kampus Tegalboto, 68121 Jember, Jawa Timur, Indonesia

[3]Department of Biochemistry, Faculty of Mathematics and Natural Sciences, Malang State University, Jl. Semarang 5, 65145 Malang, Jawa Timur, Indonesia

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**Corresponding author email:*
sutoyo.fmipa@unej.ac.id

Abstract

Chicken feather waste contains compounds consisting of the protein called keratin and other protein. Keratin is derived from chicken feathers and the proteins from chicken organs and blood. The degradation of chicken feather wastes require microorganisms which actively degrade especially keratin compounds, as well as other proteins and blood. This study aimed to screen and identify keratinolytic bacteria hydrolizing other proteins and blood from chicken feather wastes. The degradation of chicken feather keratin and hydrolysis of other protein were assayed by measuring the hydrolysis index of chicken feather keratin and skim milk protein, respectively. Hydrolysis of blood was tested qualitatively using blood agar. Identifying selected isolates used phenotypic and phylogenetic analysis. The strains of GB22.2 and GB23.4 revealed the highest keratinolytic and proteolytic activities, and they could hydrolyze blood. The GB22.2 isolate was most closely related to *Bacillus velezensis* BCRC 17467^T (100% similarity). It is proposed as *Bacillus velezensis* GB22.2-a novel keratinolytic bacterial species. *Bacillus velezensis* GB22.2 is species *Bacillus velezensis* which is firstly reported to be able to degrade chicken feather keratin. The strain of GB23.4 was closely related to *Bacillus cereus* ATCC 14579^T (100% similarity) and it is considered as a new keratinolytic strain of *Bacillus cereus*. The isolated bacterial strains are new candidates for chicken feather degradation agent.

Keywords: Keratinolytic, Proteolysis, Chicken feather, *Bacillus velezensis*, *Bacillus cereus*

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Introduction

Chicken feather wastes are a vital source of protein and include the structural protein determined as keratin. Chicken feather keratin is difficultly degraded due to its structure, which provides β-keratin of low molecular mass with high sulfur contents and forms an amorphous matrix (Wang et al., 2016). The keratin

content of chicken feathers is high, and it consists of various amino acids (Fan et al., 2016). The quantity of chicken feather wastes has globally. Based on the characteristics and availability of keratin-containing chicken feathers continuously, chicken feather wastes can be utilized on an industrial scale (Kwiatkowski et al., 2013). Keratin in chicken feather wastes can be used in the form of polymers (no hydrolyzed product) and hydrolyzed products such as soluble proteins, pure peptides, and free amino acids. Polymers derived from chicken feather keratin are used in various fields, including biomedical applications (Wang et al., 2017), films (Xing et al., 2016), sponges (Posati et al., 2016) and nanofibers (Aluigi et al., 2013). Hydrolyzed keratin can be implemented for improving soil fertility (Jain et al., 2016), methane production (Forgács et al., 2014), amino acids production, and the soluble proteins used as animal feeds (Veselá and Friedrich, 2009) and for biological control in agriculture (Gousterova et al., 2011).

The use of keratin-derived polymer in various fields requires special treatment and advanced technology. Also, the amount of chicken feather wastes used in the industry employing such technology is lower than compared to the total available every year. Therefore, it does not have a significant impact on reducing the chicken feather waste quickly. In contrast, the hydrolysis process of chicken feather keratin requires only one step of transformation technology. Also, the amount of chicken feather waste is going to rapidly reduced due to the many applications and the use of keratin hydrolysate products annually. The most effective and environmentally friendly process to produce hydrolyzed keratin is the use of protease as a biocatalyst (Thyagarajan et al., 2013). Moreover, proteases from certain bacterial strains degrade keratin in chicken feather waste without leaving solid waste later (Thanoon et al., 2018).

Microorganism is a valuable source for the keratinolytic protease. Exploration of new keratinolytic microbes are still required to obtain the potential keratinolytic isolate. Keratinolytic microorganism which have been studied are fungi, bacteria as well as actinomycetes. *Bacillus* is one which is the most frequently reported as the producers of keratinolytic protease. Keratinolytic *Bacillus* species have been applied in various areas such as bioremediation (Zhang et al., 2015) and biological control agents (Krimi et al., 2016).

The purpose of this study was to obtain keratinolytic *Bacillus* isolates present in soil mixed with chicken feather waste around the environment of Jember chicken meat production house, Indonesia. The screening was attempted to obtain strains that were able to degrade keratin chicken feathers, pure proteins, and blood. In the future, the bacteria will be developed as the candidate of biodegradation agent of chicken feather wastes.

Material and Methods

Soil chicken feathers sampling and media preparation

Samples of soil mixed with chicken feathers were collected from the surrounded area of slaughtering house in Jember, Indonesia. The site of sampling is S08º10.082'; E113º41.369'. The soil sample was taken with a sterile scalpel and put in a sterile polypropylene bag.

Chicken feathers were collected from slaughtering house. It was required as an ingredient of isolation medium of keratinolytic bacteria. Firstly, they were washed with tap water and dried under sunlight and then continued in the oven at 45ºC for two days. The dried chicken feathers were grounded into small filament with a particle size less than 200 µm. The keratinolytic microbes contained in the sample have been cultured in the modified chicken feather meal broth basal medium consisting of 0.5% (w/v) glycerol, 0.05% (w/v) K_2HPO_4, 0.1% (w/v) yeast extract, 1% (w/v) chicken feather meal, and distilled water (Tatineni et al., 2008). The growing bacteria were then transferred to the chicken feather meal basal agar medium (without glycerol and yeast extract). Cycloheximide was also added as an antibiotic compound (50 µg/mL) to inhibit fungi (Chao et al., 2007). The bacterial strains were maintained on nutrient agar media consisting of 0.3% (w/v) beef extract, 0.5% (w/v) peptone and 1.5% (w/v) agar. Skim milk agar media was used to assay the protease activity (Chu, 2007). Blood agar base (Oxoid) was used for hemolytic tests. It consisted of 1% (w/v) Lamco meal, 1% (w/v) peptone, 0.5% (w/v) sodium chloride, 1.5% (w/v) agar, and 5% (w/v) sheep blood (Ghojavand et al., 2008).

Isolation and screening of keratinolytic bacteria

Soil-chicken feather waste samples of 25 g were suspended into 225 mL of physiological saline solution (0.85% w/v sodium chloride). The sample suspension (10% v/v) was cultured in a 100 mL volume of the chicken feather meal broth basal

medium. The culture had been incubated on an orbital incubator (Stuart) at 30°C and 180 rpm for seven days. The bacteria were isolated by spreading the serial diluted culture suspension on the agar medium of chicken feather meal without glycerol and yeast extract. The culture of bacteria had been incubated at 30°C for four days. Individual colonies grown with distinct morphological features were selected by growing on the same agar medium. The clear zone around the colony which picked up become more visible after staining with Coomassie Brilliant Blue (CBB) G250 reagent (Hassan et al., 2013).

The degradation ability to chicken feather keratin in the selected bacterial isolate was tested by semi-quantitative analysis. Stock cultures of bacterial strains were subcultured in chicken feather meal broth basal medium without glycerol and yeast extract at 30°C for three days. By using the drop method, 5 µL of each of these culture suspensions was inoculated over agar medium containing minimal minerals and chicken feather meal without glycerol and yeast extract. The culture plate was incubated at 30°C for four days. The activity of keratinolytic isolates in degrading the chicken feather meal was estimated through its activity index based on the ratio of clear zone diameters and the colony (Tatineni et al., 2008). Each experiment was carried out in triplicates. The index of keratinolytic activity of isolates was analyzed by analysis of variance (ANOVA), $p<0.05$, using SPSS version 15.0.

Phenotypic identification of keratinolytic bacteria

The phenotype of keratinolytic bacteria were characterized, including morphology of colonies, cell, and biochemical activities. Identification of the genus level for isolates was made by using the matching profile method among the phenotypic characteristics of the study results and the database in Bergey's Manual of Determinative Bacteriology.

Phenotypic characteristics associated with non-keratin protein degradation and red blood cell lyses ability were tested with bioassays. The semi-quantitative activity of casein hydrolysis was measured with a degradation index by measuring the ratio of clear zone diameters and the colony (Chu, 2007). The ability of isolates to lyse red blood cells was determined on agar plates at 37°C for 48 h. The presence of clear zones around the colony indicated that the isolate produced biosurfactants that lyse red blood cells (Ghojavand et al., 2008).

Phylogenetic identification of keratinolytic bacterial isolates

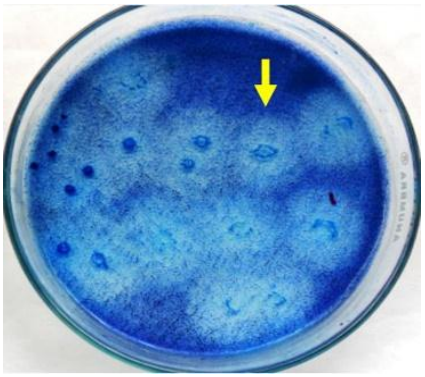
The bacterial strains were identified based on phylogenetic analysis of the nucleotide sequences of 16S rDNA. The chromosomal DNA of the bacteria was extracted by DNA extraction mini kit (Intron Biotechnology). The 16S rDNA sequence was amplified by PCR (Eppendorf, Mastercycler personal) using a universal primer consisting of 27F (5'-GAG AGT TTG ATG CTG GCT CAG-3') and 1495R (5'-CTA CCTG TGT TAC GA-3') (Weisburg et al., 1991). The composition of 50.0 µL PCR mix were 25.0 µL PCR mix master, 2.0 µL of each primer, 19.0 µL of ddH_2O, and 2.0µL of chromosomal DNA as a template. The program to amplify of 16S rDNA was 35 cycles consisting of predenaturation at 94°C for 5 min, denaturation at 94°C for 20 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 5 min and a final extension at 72°C for 5 min. The sequence of 16S rDNA (about 1500 base pairs) was confirmed by electrophoresis on a 1.5% (w/v) agarose gel in 1x TBE buffer and stained with ethidium bromide. The band of 16S rDNA was documented using Geldoc apparatus.

The 16S rDNA was purified and sequenced at the 1st BASE Company, Malaysia, using an Applied Biosystems sequencer with the Big Dye® Terminator v3.1 cycle sequencing kit chemistry. The sequence of 16S rDNA from bacterial isolates was combined with the BioEdit Program for Windows (Hall, 1999). The homology of the 16S rDNA sequence between the bacterial strain and reference isolates in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) was analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). The 16S rDNA sequences of keratinolytic bacteria were aligned with sequences of the type strains obtained from Gene Bank, Ribosomal Database Project (RDP), and Strain Info Projects to identify the similarity and construct a phylogenetic tree. The alignments were carried out by the Neighbour-Joining statistical method and Tamura-Nei algorithm method. Moreover, evaluation of the branching pattern robustness used the bootstrap 1000 replications (Fig.4). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 7 (Kumar et al., 2016).

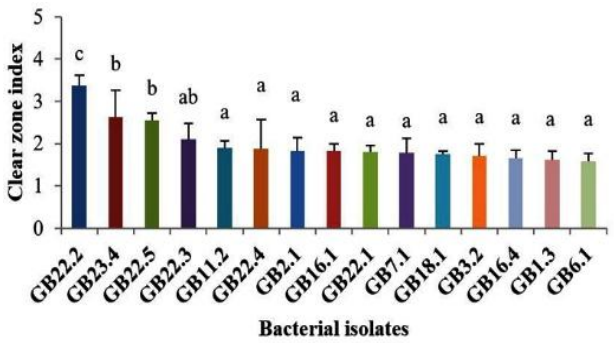
Results

Keratinolytic bacteria growth on chicken feather meal medium

A total of 15 bacterial isolates was able to degrade chicken feathers meal. The degradation activity of each strain was determined based on the presence of a clear zone around the bacterial colonies (Fig. 1.A). The isolates were selected and further evaluated for its potency as keratinolytic bacteria. According to the diameter of clear zone and then supported with the statistical analysis, they showed that bacterial isolates of GB22.2 and GB23.4 had the highest keratinolytic activity for keratin degradation (Fig. 1.B).



A



B

Figure-1: Degradation activity of indigenous bacterial isolates towards chicken feather meal (Note: the same notation among bacterial isolates indicates their keratinolytic activity is not significantly different)

Phenotypic characteristics and the potency of keratinolytic bacteria

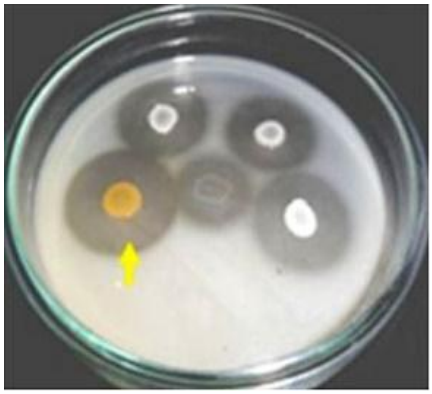
The two keratinolytic bacterial isolates with the highest activity (GB22.2 and GB23.4) showed relatively similar phenotypic characters. These two isolates had identical morphological features.

Table-1: Phenotypic characteristics of GB22.2 and GB23.4 isolates

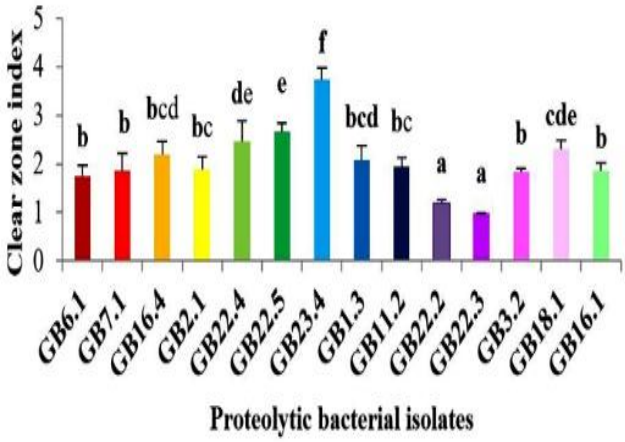
Characteristic	Isolates	
	GB22.2	GB23.4
Morphological Feature		
Cell characteristics		
Cell shape		
Rod	+	+
Endospore formation	+	+
Motility	+	-
Gram		
Positive	+	+
Colony characteristics		
Edge		
Uneven	+	-
Even	-	+
Colour		
White	+	-
Beige	-	+
Convex elevation	+	+
Consistency		
Mucoid	+	-
Dry	-	+
Diameter (mm)		
3.39	+	-
2.34	-	+
Biochemical characteristics		
Catalase reaction	+	+
Indole production	-	-
Citrate utilization	-	+
TSIA test (Sucrose fermentation test)		
Acid/Acid, Gas, H_2S	+	-
Acid/Acid	-	+
VP (Voges-Proskauer test)	+	+
Oxidase reduction	+	+
Nitrate reduction	+	-
Lysine-decarboxylation and Lysine deamination	-	-
Ornithine decarboxylase	-	-
Fermentation of carbohydrate		
Glucose	+	+
Lactose	-	-
Mannitol	+	+
Xylose	-	+
Malonate	-	-
Inositol	-	-
Rhamnose	-	-
Sucrose	-	-
Arabinose	+	+
Adonitol	-	-
Raffinose	-	-
Arginine utilization	-	-
Urease activity	-	-
Hydrolysis of Gelatine	+	+
ONPG (β-galactosidase activity)	+	+
TDA (tryptophan deaminase activity)	-	-

+ = positive reaction; - = negative reaction; A/- = acid reaction/no gas; -/- = no acid/no gas.

Besides, the isolates had equal in some common biochemical characteristics, such as producing catalase; fermenting glucose, fermenting mannitol and fermenting arabinose; producing acetoin (Voges-Proskauer test); oxidation-reduction; gelatin hydrolysis; and ONPG (β-galactosidase activity). However, their biochemical characters were different. Instead of using citrate, the GB22.2 isolate produced acid, gas, and H_2S during sucrose fermentation. Meanwhile, GB23.4 strain did not reduce nitrate, but fermentation of xylose occurred (Table 1).



A



B

Figure-2: Proteolysis activity of keratinolytic bacterial isolates grown in skim milk media (Note: the same notation among bacterial isolates indicates their proteolytic activity is not significantly different)

Isolates of keratinolytic bacteria grew and hydrolyzed casein as a source of nitrogen, as indicated by the clear zones index. This study showed that these isolates were able to hydrolyze casein and hemoglobin proteins, in addition to degrading the keratin in chicken feathers. The GB23.4 isolate showed the highest casein hydrolysis activity ($P<0.05$) among all the keratinolytic bacterial strains. Meanwhile, the proteolysis activity of GB22.2 isolate was higher than the others, but it was not significantly different from GB18.1 and GB22.4 isolate (Fig.2).

The isolates of keratinolytic bacteria cultured on blood agar medium showed that the GB22.2 and GB23.4 strains grew and produced a clear zone around the colony. Hemolytic bacteria produced and secreted an active compound in the blood agar media. The compound lysed the blood cells around the isolated colony. The compounds were thought to be surfactants (including glycolipids) (Kügler et al., 2015), a serine protease (Horowitz et al., 1990) and lipopeptide surfactin (Vallet-Gely et al., 2010). The hemolytic activity of the GB22.2 isolate (Fig. 3.A) was higher than that of the GB23.4 strain (Fig. 3.B).

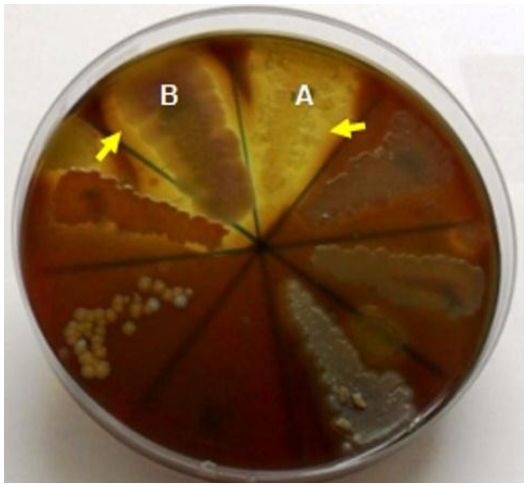


Figure-3: Hemolytic activity of keratinolytic bacterial isolates; (A) GB22.2 and (B) GB23.4 in the blood agar medium indicated as the clear zone around colony (arrow sign)

Taxonomy of keratinolytic bacterial isolate

Based on phenotypic characters analysis, the bacterial strains of the GB22.2 and GB23.4 were identified as members of the *Bacillus* genus. Amplification of the 16S rDNA from the GB22.2 and GB23.4 isolates produced approximately 1500 bp DNA fragments. The sequencing process of the amplicons and development with the BioEdit version 7.2.3 program (Hall, 1999) generated a 1413 bp DNA fragment for the GB22.2 isolate and a 1415 bp for GB23.4 isolate. The alignment results of the 16S rDNA sequences from GB22.2 and GB23.4 strains were homologous with several references for the *Bacillus* genus in the NCBI

database. The maximum homology identity for the data was 99%. The evolutionary analysis of the selected and reference nucleotide sequences produced genetic information about the similarity of 16S rDNA sequences. The16S rDNA sequence of the GB22.2 isolate and other isolates showed similar values of 74.78–100%. Genetic relationship between *Bacillus* sp. GB22.2 and *Bacillus velezensis* BCRC 17467^T were very close, based on the similarity of the 16S rDNA sequence (100%). The similarity value for the 16S rDNA sequence of the GB23.4 strain compared with other bacterial references was in the range of 75.25-100%. *Bacillus* sp. GB23.4 and *Bacillus cereus* ATCC 14579^T had the highest similarity (100%).

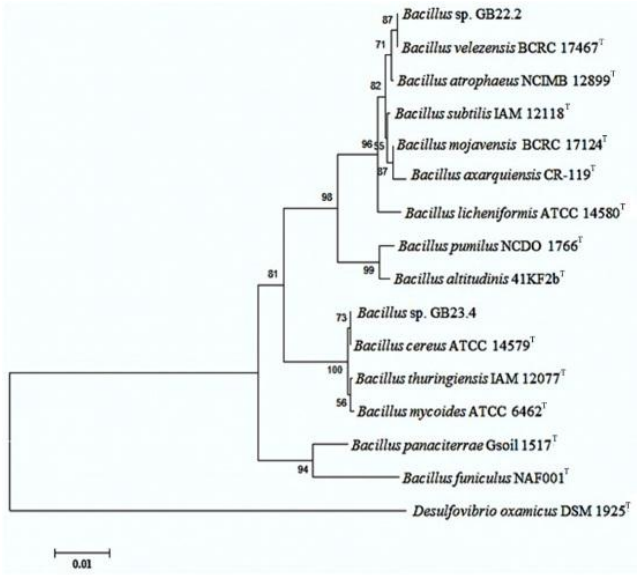


Figure-4: Phylogenetic tree of GB22.2 and GB23.4 keratinolytic bacteria with reference type strains of bacteria, based on similarity of 16S rDNA sequence

Likewise, the results of molecular identification based on a 16S rDNA analysis confirmed that GB22.2 and GB23.4 strains were different members of the *Bacillus* genus. In the phylogenetic tree (Fig. 4), *Bacillus* sp. GB22.2 was included in the *Bacillus subtilis* group while *Bacillus* sp. GB23.4 belonged to the *Bacillus cereus* group. Phylogenetic analysis demonstrated that *Bacillus* sp. GB22.2 was in one large clade with reference species. All species of bacteria in this clade were members of the *Bacillus subtilis* group. *Bacillus velezensis* BCRC 17467^T and *Bacillus* sp. GB22.2 were in the same sub-clade. *Bacillus* sp. GB23.4 in the phylogenetic tree was in one clade of the *Bacillus cereus* group. The *Bacillus* sp. GB23.4 and Bacillus cereus ATCC 14579^T was in the same sub-clade.

Discussion

The discovery of keratinolytic bacteria with high degradation activity for chicken feathers is a great demand recently. Keratinolytic bacteria have played an essential role in the biological process for converting chicken feather keratin into an amino acid and soluble protein (Rai et al., 2009). In this study, fifteen bacterial isolates can degrade chicken feather. All strains degraded keratin which was mainly a constituent of chicken feathers. The degradation activity of chicken feathers around colonies of keratinolytic strains caused the formation of clear zones. Solubilityzation of keratin occured because the isolate colonies secrete keratinase and actively hydrolyze keratin to become some soluble protein and free amino acid (Rahayu et al., 2007).

Two bacterial isolates, GB22.2 and GB23.4 were selected as candidates to be developed as chicken feather degradation agents. Both bacteria were able to grow, and had the highest degrading activity, in media containing chicken feathers as a sole carbon and nitrogen source. They formed a clear zone with the largest size around the colony of the bacterial isolates (Fig. 1). Clear zones were created because the bacterial keratin proteinase degraded the chicken feathers around the colony (Zaghloul et al., 2011).

The method of screening in this study was adequate for selecting isolates of keratinolytic bacteria directly and showed the most visible activity of keratin degradation of chicken feathers. In the previous study, the first screening used a casein protein substrate in the form of a simple protein structure, followed by a keratin substrate (Adigüzel et al., 2009). This study has successfully selected bacterial isolates of GB22.2 and GB23.4 as the potential keratin biodegradation agents of the chicken feathers.

The phenotypic characteristics of the GB22.2 and GB23.4 isolates showed that both bacterial strains had similarities in cell and colony morphology and biochemical activity (Table 1). These characteristics were identical to those for the *Bacillus* genus in a database of bacterial features in Bergey's Manual of Determinative Bacteriology. Genus identification of these bacterial was based on the phenotype analysis as *Bacillus* sp. GB22.2 and *Bacillus* sp. GB23.4.

A further test demonstrated that the selected keratinolytic bacteria, *Bacillus* sp. GB22.2 and GB23.4 strains had high potency as candidate agents for feather biodegradation. Several studies have reported that *Bacillus cereus* LAU08 (Lateef et al.,

2010), *Bacillus cereus* KB043 (Nagal and Jain, 2010) and *Bacillus cereus* Wu2 (Lo et al., 2012) have potency as biodegradation agents of feather keratin. In this study, both of the bacterial strains actively degraded chicken feather, red blood cells as well as hydrolyzed skim milk, as shown by the formation of a clear zone on the agar medium. These bacteria are espected that they can degrade the keratin, blood protein, and protein contained in the chicken feather waste.

Enzymes and surfactants produced by bacterial isolates are thought to support their degradation capacity. Keratin protease degrades the fiber proteins in chicken feathers soluble protein, oligopeptides and amino acids (Jeevana Lakshmi et al., 2013). At the same time, other proteases can degrade casein protein (Smid et al., 1991). The microbial metabolites including biosurfactants composed of lipid molecules which have surface properties that readily interacting with different lipid molecules. The interactions of these biosurfactants with lipid membranes form pores which result in cell leakage (Brotz and Sahl, 2000; Carrillo et al., 2003; Heerklotz and Seelig, 2007). Also, hemoglobin excreted from red blood cells can be lysed by lysine proteins produced by the bacteria (Pornsunthorntawee et al., 2008). The strains had a high potency not only to degrade chicken feathers keratin but simultaneously hydrolyze single proteins and red blood cells contained together in chicken feather waste.

Bacillus sp. GB22.2 isolate had a robusthemolytic activity, whereas *Bacillus* sp. GB23.4 isolate only partially lysing red blood cells (Fig. 3). The ability of strains to hydrolyze red blood cells strongly demonstrates the potency of both isolates as producers of organic surfactants for bioremediation (Bhange et al., 2016), detergent formulation (Barbosa et al., 2013), and as cleaning agents for medical impurities.

Phylogenetic analysis demonstrated that *Bacillus velezensis* BCRC 17467^T and *Bacillus* sp. GB22.2 were in the same sub-clade. Some researchers stated that among organisms sharing the base sequence of 16S rDNA nucleotides, more than 97% is considered as the same species. This study proposed that *Bacillus* sp. GB22.2 is *Bacillus velezensis* GB22.2. *Bacillus velezensis* GB22.2 is the first reported as a new type of *Bacillus velezensis* which degrades keratin chicken feathers and also hydrolyzes non-keratin proteins and hemolytic of red blood cells. *Bacillus velezensis* NRRL B-41580^T, which has been reported only as a surfactant-producing bacteria (Ruiz-Garcia et al., 2005). It has never been reported as keratinolytic bacteria.

According to Jung et al. (2011) and Jiménez et al. (2013) *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus anthracis*, *Bacillus mycoides*, and *Bacillus pseudomycoides* are one clade (Fig.4) and have sufficient similarities to form the group of *Bacillus cereus*. According to Rasko et al. (2005) *Bacillus thuringiensis*, *Bacillus cereus*, and *Bacillus anthracis* are difficult to distinguish as different species based on analysis of 16S rDNA sequences. In this study, *Bacillus* sp. GB23.4 showed proximity at the species-level with bacteria belonging to the *Bacillus cereus* group. *Bacillus* sp. GB23.4 was proposed as a new strain of *Bacillus cereus* ATCC 14579^T because it had the highest similarity (100%). Based on the phylogenetic analysis, the strain was identified as *Bacillus cereus* GB23.4. It has potential as a candidate agent for the biodegradation of chicken feather keratin. *Bacillus cereus* ATCC 14579^T is non-pathogenic *Bacillus cereus*.

Conclusion

This study found that the soil bacteria of *Bacillus velezensis* GB22.2 and *Bacillus cereus* GB23.4 have the highest capacity to degrade chicken feathers. *Bacillus velezensis* GB22.2 is proposed as–the first keratinolytic species of *Bacillus velezensis*. On the other hand, *Bacillus cereus* GB23.4 is another keratinolytic species of *Bacillus cereus*. Both of *Bacillus velezensis* GB22.2 and *Bacillus cereus* GB23.4 have high potential as a candidate agent for chicken feather wastes degradation.

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Contribution of Authors

Sutoyo S: Research design, Experiments, Processed experimental data and analyzed the results, Wrote the manuscript

Subandi S: Research design, discussed the results and Commented on the manuscript.

Ardyati T: Research design, Processed experimental data and Analyzed the results, Discussed the results and commented on the manuscript.

Suharjono S: Research design, Processed, experimental data and Analyzed the results, Discussed the results and Commented on the manuscript.