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Isolation and Screening of Keratinolytic Protease-Producing Bacteria from Soil in Abattoir Waste Disposal Area, Dessie, Ethiopia

Muhammed Seid Anbesaw*

Department of Biotechnology, School of Bio-Science and Technology, Wollo University, Dessie, Ethiopia

ABSTRACT

Management of keratin wastes with the help of microorganisms is a biotechnological alternative for recycling and valorization of these biomaterials. Keratinase belongs to a class of proteases that can serve as an important tool to convert keratin-rich wastes into diverse value-added products that are used for many applications. Therefore, the aim of this study was to isolate and screen keratinolytic bacteria from soil in abattoir waste disposal area. Screening of protease-producing isolates was performed on the medium containing 1% skim milk powder and 1.5% agar at 37ºC. A total of 34 isolates were selected after growth on nutrient agar media. Among 34 isolates, 17 were showing proteolytic activity on the skim milk agar medium and 3 isolates (MD1, MD4 and MD10) showed a clear zone greater than 25 mm. These isolates were again subjected to secondary screening for sheep hair degradation, and all showed complete degradation of sheep hair between 204 h and 360 h. Isolate MD4 was selected as the potent bacterium because it can degrade sheep hair within 204 h and produced maximum level of keratinase (899.30U/ml). Based on its morphological and biochemical characteristics, MD4 was tentatively identified as *Bacillus* sp. The results of this study revealed that isolate MD4 might have potential biotechnological applications that involve hydrolysis of keratin. It can be also used to manage keratin wastes and produce value-added products from these wastes. If further research such as enzyme characterization and genetic improvement of the organism is conducted, the isolate would be a good source of keratinolytic protease.

Keywords: Abattoir waste; Bacteria; *Bacillus* sp.; Keratinase; Sheep hair.

INTRODUCTION

Keratin is a rich resource in nature, and the amount of keratin-rich waste is increasing annually. Keratin wastes are considered environmental pollutants and are produced mostly by poultry farms, slaughterhouses, leather industries, and barber shops. About 75 %-85 % of the constituents of the leather and poultry industries are keratinous wastes that can be utilized as raw materials for the production of various value-added products (Tesfaye et al., 2017). Keratinous substrates such as feathers and wool can be degraded by microorganisms having the capability to utilize keratin as their sole carbon and nitrogen source (Ignatova et al., 1999; Lo et al., 2012). Currently, waste from the leather and poultry industries in Ethiopia are either collected and dumped in the lowlands or disposed of in an irregular manner, resulting in environmental pollution and public health hazards. Therefore, the keratinous biomass of the leather and poultry industries needs to be bio-converted into bio-resources through the production of value-added products such as enzymes, particularly keratinases. These enzymes are a group of proteolytic enzymes that have the capability of degrading keratinous materials (Lange et al., 2016).

*Corresponding author: muhaee@gmail.com

Keratinolytic proteases have gained much attention globally since they have the capability to convert keratinous wastes into various value-added products and are used in different biotechnological applications. As Lange et al. (2016) reported there are six bacterial keratin-degrading proteases that have been utilized by various industries on a commercial scale around the world. All these bacterial keratinases are from Bacillus. Keratinase can be produced by microorganisms using keratinous wastes as the raw material for their growth. (Latshaw et al., 1994).

A vast variety of bacteria, actinomycetes, and fungi are able to degrade keratin. Among the microorganisms, the genus *Bacillus* has the highest potential to completely degrade keratin wastes (Latshaw et al., 1994). Including this genus and others uses keratin wastes as the sole source of carbon, nitrogen and energy to perform fermentation. Therefore, the use of keratin waste as a nutrition substrate for the cultivation of microorganisms (Lo et al., 2012; Tesfaye et al.,

2017) and the production of their enzymes is considered as a green and sustainable approach to the production of keratinases for their further application such as in biomedicine (Gupta et al., 2013; Tesfaye et al., 2017), cosmetics (Gupta et al., 2013), bioenergy (Tesfaye et al., 2017), animal feed preparation (Gupta & Ramnani, 2006), biofertilizer preparation (Paul et al, 2018), dehairing application (Chaturvedi et al., 2014), detergent formulation (Haddar et al., 2010), production of inexpensive and biodegradable thermoplastics (Tesfaye et al., 2017), bio-control (Gousterova et al., 2011), textile (Gupta et al., 2013) and silver recovery from used x-ray films. This confirmed that the application of keratinases in various industries is already well established and various commercial products are available in the market such as Zymox, Versazyme, Prionzyme, PURE100 Keratinase and Keratoclean (Kumar & Takagi, 1999).

Although the importance of keratinases is well known and they are obtained from various microorganisms, there is still a demand for new ones with improved industrial properties such as higher catalytic efficiency on keratinous substrates and other properties. The industrial demand for new keratinases continues to stimulate the isolation and screening of new keratinolytic microorganisms. In recent years, demand for keratinolytic proteases has been increasing due to their various industrial applications. To meet the upward trend in demand, studies on the cost-effective production of industrially important keratinolytic proteases have become necessary today. With this regard, isolation and screening of microorganisms having the capability of degrading keratin wastes is becoming a priority because of their recalcitrant nature or resistance to biodegradation (Gupta & Ramnani, 2006). Therefore, the present study aimed to isolate and screen keratinolytic bacteria having the highest potential for the degradation of keratinacious wastes, particularly sheep hair, from Dessie abattoir waste disposal area.

MATERIALS AND METHODS

Materials and Sample collection:

Sheep hair was collected from animal hair waste disposal area of Kombolcha leather industry. Hair that was used for the entire experiment was washed gently with tap water until clear effluent was obtained and finally it was sun dried. All the chemicals and culture media used in this study were of analytical grade.

A soil sample was collected from abattoir waste-disposal area and kept in a sterile plastic bag. The sample was aseptically transported to the microbial biotechnology laboratory and kept at 4ºC until use (Masi et al., 2021).

Isolation and screening of keratinase producing bacteria:

One gram of soil was dissolved in sterilized distilled water and the suspension was shaken vigorously using a vortex. The mixture was serially diluted and 0.1 ml of the serially diluted sample was transferred to nutrient agar plates using the spread plate technique (Masi et al., 2021). The inoculated plates were incubated at 37ºC for 48 h. The colonies obtained through the spread plate method were sub-cultured to obtain pure colonies. The pure colonies were transferred to skim milk agar plates containing 1% (w/v) skim milk powder and 1.5% (w/v) agar. Finally, the plates were incubated at 37ºC for 60 h for screening of protease-producing bacteria (Marathe et al., 2018; Masi et al., 2021). The bacterial isolates producing a clear zone around their colonies were considered as protease producers. Isolates forming a clear zone greater than 25 mm were selected and subjected to further keratinase production test. The selected isolates were cultured on keratinase production media (see the keratinolytic protease production section) and an isolate having the capability to degrade the sheep hair in a short period of time as compared to other isolates was selected for further investigation in this study.

Time course of cell growth and keratinolytic protease production:

To determine the optimum cultivation period for maximum cell growth and keratinase production, 1% of fresh target isolate (MD4) was inoculated into enzyme production media and incubated at 37ºC on an orbital shaker at 120 rpm for 252 h. Determination of cell biomass assay was carried out by measuring the turbidity (OD) of the broth medium at 600 nm using a UV-7804C spectrophotometer at 12 h intervals. The time at which the isolate reached its maximum enzyme activity was also assessed using the same instrument by measuring the absorbance (OD) of the solution at 660nm.

Keratinolytic protease production and culture conditions:

The selected isolate (MD4), which was initially screened for keratinolytic activity, was cultivated using sheep hair as a carbon and nitrogen source under submerged fermentation in 250 ml Erlenmeyer flasks with a working volume of 100 ml of medium containing; 1% hair (w/v), 0.5% (w/v) glucose, 0.05% (w/v) yeast extract, 0.013% (w/v) $CaCl_2.2H_2O$, 0.02% (w/v) $MgSO_4.7H_2O$ (w/v), 0.1% (w/v) $K_2HPO_4.xH_2O$ and 1% Na_2CO_3 as described by Gessesse et al. (2003). Cultivation media was adjusted to pH 10 after it was autoclaved with 1% Na_2CO_3. Then, culture media was inoculated with 1% of 24 h pre-cultured target cells. Finally, cultivation was conducted at 37ºC

for 252 h on an orbital shaker at a speed of 120 rpm. At the end of the incubation period, the culture was filtered through a sieve (0.5 mm mesh) to separate the undigested sheep hair. The filtrate was washed twice with distilled water to remove bound cell biomass from the hair. The residue was transferred to a pre-weighed filter paper and kept in the dry oven at 70ºC for 6 h to remove any moisture content. The degradation percentage was calculated according to the following equation (Emran et al., 2020).

Degradation efficiency (%) = [(A – B) / A] x 100

Where A is the dry weight of sheep hair before fermentation process and B is the dry weight of the undigested sheep hair after keratinolysis /biodegradation.

Crude enzyme preparation:

After the target isolate (*Bacillus* sp. MD4 isolate) was cultivated in enzyme production media for 252 h, centrifugation was performed at 10,000 rpm for 5 min at 4°C. The clear supernatant (cell-free medium) was considered as the crude enzyme and used for further characterization of the enzyme as described by Gessesse et al. (2003).

Preparation of tyrosine standard curve:

For the preparation of the curve, 0.5 M of Na_2CO_3, 2 N Folin reagent (1:10 diluted), distilled water, and 1 mg/ml of tyrosine stock solution were used (Gessesse et al., 2003). A required amount of stock solution was added to each test tube except the blank to prepare working concentration of tyrosine. Distilled water was then added to each tube including the blank to make the volume 1000 µl. Then 2.5 ml of 0.5 M Na_2CO_3 was added to each test tube and the mixtures were held at room temperature for 10 min. After adding 500 µl of 2N Folin reagent to each test tube the solution was mixed immediately and left at room temperature for 30 min. Finally, the optical density (OD) was measured using spectrophotometer (UV-7804C) at 660 nm and the standard curve was plotted. To determine the crude keratinolytic protease activity a calibration curve equation ($Y = 0.0074X + 0.0115$) was generated from the standard curve with a regression coefficient of $R^2 = 0.999$.

Where Y is the value of optical density (OD) and X is the concentration of tyrosine (µg/ml) released from casein during reaction by enzyme.

[CT](µg/ml) = (OD-0.0115)/0.0074

µ mole of Tyrosine/ml = [CT]/MWT

Where [CT] is the concentration of Tyrosine released during reaction in µg/ml and MWT is the molecular weight of the Tyrosine.

Enzyme assay:

The enzymatic activity was measured by using casein as a substrate (Gessesse et al., 2003). Briefly, 50 µl of crude enzyme was mixed with 450 µl of glycine/NaOH buffer (0.1M, pH 10.0) containing 1% (w/v) casein and incubated in a digital water bath for 30 min at 40ºC. After 30 min of incubation, 500 µl of 10% trichloroacetic acid (TCA) was added to stop the reaction and all the reaction tubes were left at room temperature for 20 min to allow unreacted casein to precipitate. Centrifugation at 10,000 rpm for 5 minutes was performed to remove the precipitated casein. 500 µl supernatant was transferred to a clean test tube. Then after 2.5 ml of 0.5 M Na_2CO_3 and 500 µl 2 N Folin-Ciocalteau's phenol reagent (1:10 diluted) were added. The solution was then mixed with a vortex and kept at room temperature for 20 min. Finally, optical density (OD) of the solution was measured at 660 nm using Ultraviolet Visible Spectrophotometer (UV-7804C). The enzyme activity was measured using the tyrosine standard calibration curve and represented in units (U). One unit of keratinolytic protease enzyme is defined as the quantity of enzyme that releases 1µ mole of tyrosine per milliliter of crude extract per minute.

Enzyme unit (U/) = [CT]/T

Where [CT] is the amount of the product (Tyrosine) released in µ mole and T is the time of incubation in minutes.

Enzyme Activity (U/ml) = ([CT] X TV)/ (T X TE)

Where [CT] is the concentration of Tyrosine in µ mole/ml, TV is the total volume of the reaction in milliliter, T is the time of incubation in minutes, and TE is the total amount of enzyme used in the reaction in milliliter.

The total volume used in the enzyme assay reaction is 1ml (50 µl of enzyme, 450 µl of substrate and 500 µl of TCA); total amount of crude extract used in the reaction is 50 µl and time of incubation is 30 minutes.

Morphological and biochemical characterization of the bacteria:

A potent isolate (MD4) showing complete degradation of sheep hair within 204 h was characterized based on the methods and procedures described in Prescott (2002). Depending on the results of morphological and biochemical characterization, identification of the organism was performed using particular bacterial identification keys specified in Bergey's manual of determinative bacteriology (Breed et al., 1957).

Statistical analysis:

All results were reported by means of at least three measurements and Microsoft office Excel

worksheet 2010 was used for data analysis and presentation.

RESULTS

Isolation and screening of keratinolytic protease producing bacteria:

A total of 34 different bacterial colonies were isolated on the basis of their morphological characteristics after growing on nutrient agar medium and they were designated MD1 to MD34. All the 34 isolates were subjected to primary screening on skim milk agar plates and 17 showed proteolytic activity in terms of making a clear halo zone around their colony. Among those, 3 isolates (MD1, MD4 and MD10) showed a clear zone greater than 25 mm. Of these, isolate MD4 exhibited the maximum zone of proteolysis (31 mm) (Fig.1) and the other two isolates designated as MD1 and MD10 showed a 28 mm and 29 mm clear zone, respectively. The secondary screening of the three potent isolates on keratinolysis detection on sheep hair broth medium confirmed that all the isolates have the capability to degrade sheep hair completely during their growth. An isolate designated as MD4 showed the capability to degrade the sheep hair completely within 204 h and the other isolates designated as MD1 and MD10 degrade this keratin material at the end of 240 h and 360 h of incubation, respectively.

Identification of the isolates:

The isolate MD4, which showed complete degradation of sheep hair, was further characterized and the results showed that the isolate was gram positive, catalase positive, rod shaped, endospore former, and aerobic (Table 1). The colony appeared a creamy color. The isolate also showed good growth at a temperature range of 15 ºC -50ºC and showed optimum growth at 37ºC. Based on this, the isolate was grouped under the mesophile group of bacteria.

Sheep hair degradation efficiency of the organism:

Sheep hair degradation by isolate MD4 was started after 96 h of incubation. This particular fermentation was recognized as late exponential phases. The isolate showed complete degradation of the hair (99.9%) after 204 h of incubation, which was considered as late stationary phase (Fig. 2). The result showed that no intact hair remained in the culture medium after 204 h fermentation process.

Time course of cell growth and keratinolytic protease production/activity:

As shown in Fig. 3, the turbidity value (OD) or total cell biomass in the culture was increased until the organism entered its stationary phase. After 168 h of incubation the organism (MD4) showed a decrement in cell growth or the OD reading values. Cell growth and enzyme production show a nearly linear relationship until the early stationary phase. After that, cell biomass became constant and finally decreased at the end of the stationary phase. The finding of this study also agreed with Gupta

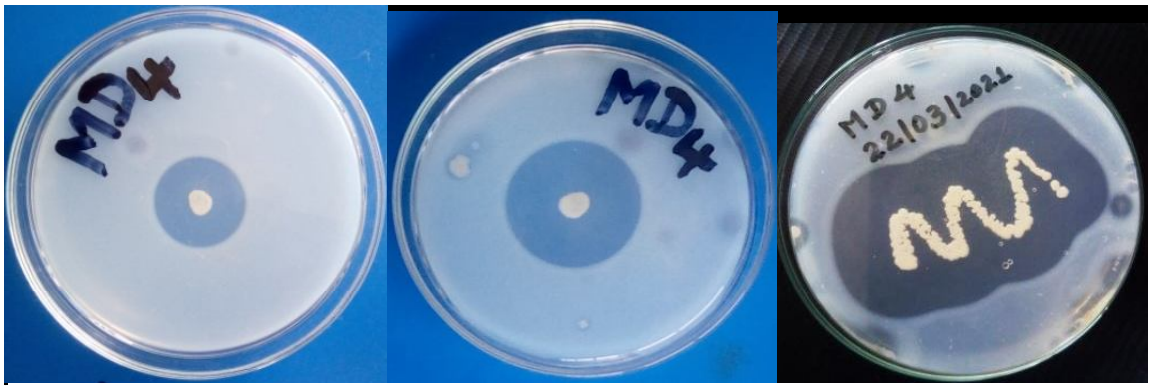

Fig. 1: Zone of hydrolysis by *Bacillus* sp. isolate MD4 on milk agar plate after 60 h incubation at 37ºC

Table 1: Morphological and biochemical characteristics of isolate MD4

Morphological tests	Observation	Biochemical tests	Observation
Gram stain	Gram +ve	Catalase	+ve
Shape	Rods	Acid from Glucose	+ve
Endospore formation	+ve	Hydrolysis of Casein	+ve
Growth		Hydrolysis of Gelatin	+ve
Aerobic /Anaerobic	Aerobic	Acid from Glucose	+ve
Temperature	15 ºC -50ºC	Gas from Glucose	-ve
Colony on Nutrient agar		Acid from Mannitol	+ve
Color	Creamy white		
Pigment	No		

& Ramnani (2006) and Oberoi *et al.* (2001) studies. Determination of cell growth associated with maximum enzyme production period is essential for identifying the fermentation period for maximal enzyme productivity and also makes it profitable to use the enzyme for several biotechnological applications.

Despite the fact that MD4 started degrading sheep hair after 96 h of incubation, its exponential phase, production of sufficient keratinolytic protease began in the late exponential phase. MD4 produced the highest level of keratinase (899.30 U/ml) after 8 and a half days (204 h) of cultivation, which is considered the stationary phase, under shaking conditions (120 rpm) at 37°C. Enzyme activity was relatively constant (> 97.5%–98.8%) within the 168-228h incubation time interval, after which it began to decline as the incubation duration increased. At the late stationary phase, MD4 showed a decrease in enzyme production.

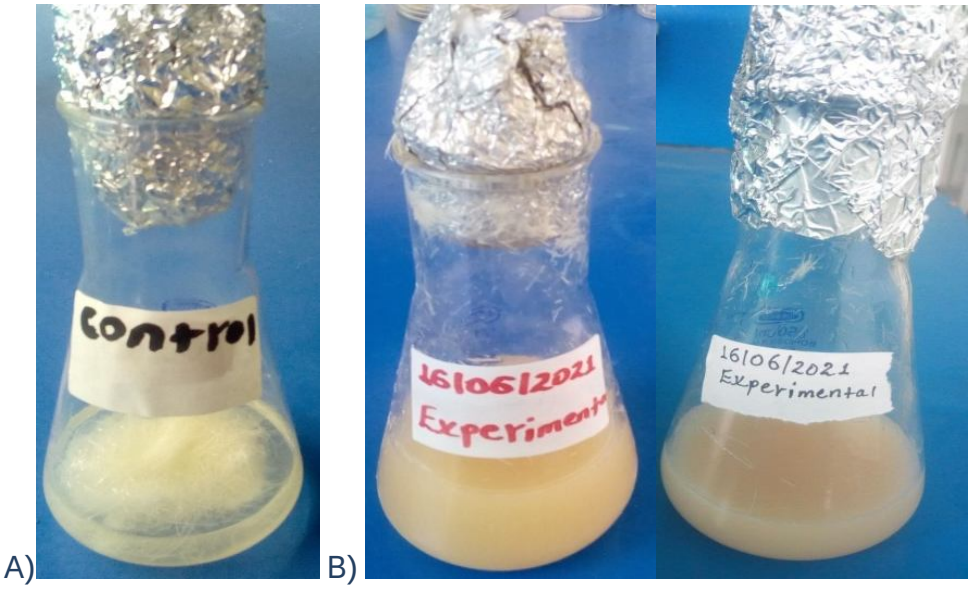

Fig. 2: A) Submerged fermentation with sheep hair as a carbon and nitrogen source but without MD4 (control); and B) similar fermentation condition as in case of (A), but with MD4 (experimental). The experimental flask shows complete degradation of the hair at the end of fermentation period (252h).

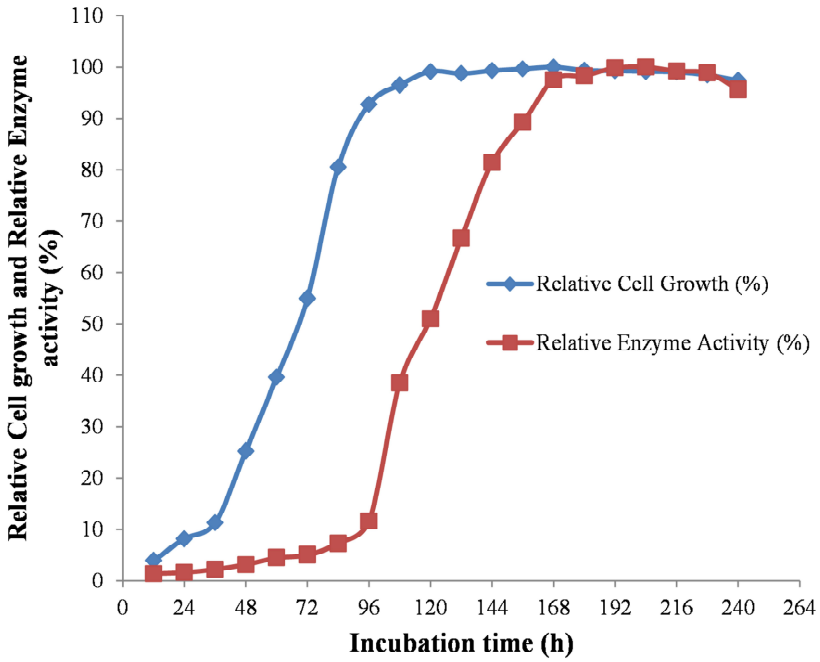

Fig. 3: Time course of cell biomass and enzyme activity profile at 24 h intervals

DISCUSSION

Keratinolytic microorganisms have tremendous potential applications in different biotechnology sectors because of their capability to degrade keratin wastes and convert into value added products. Therefore, isolation, screening and characterization of such organisms from different environmental samples have now become common practices for researchers. In this regard, the present study examined soil from an abattoir waste disposal area and the finding implies that keratinolytic microorganisms can be isolated from this particular place. The organisms isolated in this study showed an excellent zone of hydrolysis on skim milk agar plates. As compared to other proteolytic organisms including *Bacillus subtilis* MTCC (9102), which showed a maximum zone of hydrolysis of 18 mm (Balaji et al., 2008), isolate MD1, MD4 and MD10 were found to be the potent organisms that can form extremely large clear halo zone surrounding their colony on skim milk agar plates. It is imperative that investigation be made of an organism having the capability to make such a maximum clear halo zone around its colony because this may indicate its potential for extracellular enzyme production in large volume as well as high enzyme activity (Gomri et al., 2018).

Most of the literatures showed that keratinase-producing bacteria are frequently isolated from poultry wastes and a few are from hot springs and soil (Pandey et al., 2019). However, few studies have shown the keratinolytic microorganisms that are isolated from abattoir waste dumped soil except for the representative studies (Tatineni et al., 2008; Benkiar et al., 2013). As a result, the current study provides the baseline information from where keratinolytic microorganisms can be isolated because such novel habitats are promising for the discovery of new microbial strains. This means that abattoir waste disposal area can be targeted place for isolation of keratinolytic microorganisms in the future.

The morphological and biochemical characteristics of the isolated bacterium revealed that the organism belongs to the genus Bacillus because of its resemblance to this genus (Breed et al., 1957), and the isolate was tentatively identified as *Bacillus* sp. isolate MD4. The groups of bacteria that belong to this genus are very well studied and have an important role in industrial biotechnology for several reasons. Beside this, species belonging to this genus are also characterized by being non-pathogenic and generally recognized as safe. Furthermore, bacteria grouped under this genus are well known for their capability to secrete many industrially important extracellular enzymes directly into the culture medium (Jaouadi et al., 2010). Therefore, the newly isolated bacteria (MD4) may have the probability of being similar to *Bacillus* sp. which has already been extensively studied for biotechnological applications so far.

As expected for a micro-organism of environmental origin, MD4 showed optimum growth at a mesophilic temperature. Prescott (2002) also proved that the optimum temperature for the growth of a given microorganism is correlated with the temperature of the normal habitat of the microorganism. The organism isolated and screened in the present study, which is mesophilic, does not require high energy inputs for its growth unlike thermophilic keratin-degrading microorganisms and it may have important implications for industrial use.

The capability of the three isolates (MD1, MD4 and MD10) for complete degradation of the intact hair without any pretreatment implies that the organisms are keratinolytic protease producers and they are capable of using sheep hair for metabolic processes. The fact that MD4 grew significantly in a medium containing intact hard keratin (hair) suggests that the isolate has the ability to use hair as a carbon and nitrogen source. *Bacillus licheniformis* (Vigneshwaran et al., 2010) and *Thermoactinomyces candidus* (Ignatova et al., 1999) have also been shown to degrade hair and use it as a sole carbon and nitrogen source. MD4 has developed a viable microbial approach for getting keratinolytic enzymes by growing and producing large amounts of keratinolytic protease using hair as a cheap fermentation substrate.

The findings of Lin et al. (1999) and Emran et al. (2020) demonstrated that keratinolytic enzymes are mostly produced in mineral salts-based media with the commercially processed keratinous substrate. But the finding of the current study and related works (Gessesse et al., 2003; Kansoh et al., 2009) showed that the keratinolytic enzyme can be produced by using intact hair without any pretreatment. This implies that the inclusion of processed keratinous substrate is not always necessary for the production of keratinolytic enzymes.

The result indicated that isolate MD4 can utilize sheep hair, a material that is removed from the leather industry and considered as a waste as a substrate. Hence, utilization of sheep hair by MD4 as a growth substrate can be used for the production of keratinolytic proteases at the industrial level. This could result in a substantial reduction in the cost of enzyme production. Kalisz (1998) also reported that production of enzymes with locally available and cheap resources is one of the priority criteria for microbes to be used at industrial level because in the production of industrial enzymes, up to 30%-40% of the production cost is accounted for by the growth substrate. Moreover, the bacterial isolate used in

this study does not require any pre-treatment process like milling or other prior mechanical treatment of the sheep hair for biodegradation. This indicated that the organism (MD4) can degrade intact keratin waste directly without the cost of processing the substrate. Similar studies also suggested that the use of keratin waste as a nutrition substrate for the cultivation of microorganisms and the production of their keratinase is considered as cost effective, green and sustainable approach (Lo et al., 2012; Tesfaye et al., 2017).

Different carbon and nitrogen sources are essential for cultivation of industrially important microorganisms and they are considered among the most expensive nutritional components used in the culture of microorganisms. Therefore, the discovery of new low-cost media components including sheep hair as a substrate is therefore accepted as an important approach for industrial and biotechnological studies. Similarly, Tuysuz (et al., 2021) also demonstrated that biodegraded waste sheep hair to keratin hydrolysate could be used as an alternative peptone source for the cultivation of microorganisms.

The finding of the ability of MD4 to degrade intact sheep hair completely within a short period of time (8.5 days) is particularly notable. Ignatova et al. (1999) found that complete degradation of sheep hair was observed after 9 days of cultivation at 65°C by *Thermoactinomyces candidus*. Plentiful studies have been conducted and the information is available in Pandey et al. (2019) and Bhari et al. (2021a) with regard to the degradation of keratin wastes, mainly feathers. The results indicated that the ability of a microorganism to degrade keratin and the resulting levels of keratinase produced vary according to the species, chemical composition, the molecular structure of keratin substrates, and the culture conditions. With this regard, the time required for the degradation of feathers can vary in the range of 1.5-30 days. Numerous studies, including (Fakhfakh et al., 2011; Liu et al., 2014), have shown that biodegradation of feathers occurs quickly, whereas biodegradation of animal hair takes a long time (Ignatova et al., 1999). Most probably, the existence of a high number of disulfide bonds found in animal hair could make it more resistant to biodegradation by keratinolytic microorganisms than that of feathers. Therefore, isolation and screening of microorganisms having the capability of degrading keratin waste containing a high content of cysteine (high disulfide bonds) is becoming a priority because of their recalcitrant nature or resistance to biodegradation. Hence, the capability of the organism (isolate MD4) to degrade hard keratin (sheep hair), which is recalcitrant to biodegradation, makes it the best candidate for degradation of complex keratin materials. Furthermore, the organism could also be a source of keratinase genes that can be expressed in another host organism that is already established for enzyme production because MD4 has the ability to produce a sufficient amount of highly active keratinolytic protease.

The time course of MD4 cell growth and its keratinolytic protease production implies there is a nearly linear relationship between cell biomass/growth and enzyme production until the early stationary phase. However, at onset of the decline phase cell growth becomes impaired. This could be due to an increase in cell death (microbial growth reduction) associated with the depletion of available nutrients required for MD4 cell growth. Similar studies have also shown that extracellular keratinolytic protease enzyme production was detected maximally during the stationary or late exponential phase of microbial development (Gupta and Ramnani, 2006; Kansoh et al., 2009). As Kansoh et al. (2009) reported, the time course for maximum keratinolytic protease production differs according to the organism and its cultivation conditions. For example, the maximum keratinase production was observed after 60 h of incubation of *Bacillus cereus* (Kim et al., 2001), 72 h of *Bacillus pumilus* (Kim et al., 2001) and it may take 5-14 days to have the maximum keratinase activity of *Bacillus* sp. Fk46 (Suntornsuk and Suntornsuk, 2003). Therefore, time course of maximum keratinolytic activity of MD4 is bounded in the time frame that many organisms produce their maximum keratinase.

At the late stationary phase, MD4 showed a decrease in enzyme production which is consistent with earlier observations (Gupta & Ramnani, 2006; Kansoh et al., 2009). At the end of the stationary phase, no more enzyme was produced because the enzymes that had been produced previously may have undergone enzymatic autolysis and been inhibited by other end-products in the medium. This speculation is also supported by Syed et al. (2009). When incubation was prolonged after the cell population reached its late stationary phase, the viable cell population of MD4 decreased, which could be another cause of the drop in enzyme production during this growth phase. Conclusively, the time course of MD4 cell growth and its keratinolytic protease production implies enzyme production was typically most intensive at the end of the exponential and/or in the stationary phase, which is associated with the adaptation to the lack of nutrients. This suggests that the production or secretion of keratinolytic proteases may be regulated by nutritional stress, such as the lack of carbon and nitrogen sources. This is also supported by Daroit & Brandelli (2014).

The genus Bacillus MD4 produced 899.30 U/ml which is similar to the findings of Govarthanan et al., (2015) who reported keratinolytic activity of 1075 U/ml from *Bacillus* sp. MG-MASC-BT. On the other hand, MD4 is more efficient than other keratinase producing microorganisms (Kansoh et al., 2009; Liu et al., 2014; Chaturvedi et al., 2014; Mazotto et al., 2017; Abdel-Fattah et al., 2018; Marathe et al., 2018; Emran et al., 2020; Bhari et al. 2021b, Masi et al., 2021).

The result found in the present study and other similar investigations showed that there is no exact correlation between keratin degradation efficiency and keratinase activity (U/ml) of the keratinolytic microorganisms. For instance, MD4 degraded sheep hair after 8.5 days of incubation and produced 899.30 U/ml. Similarly, *Actinomadura keratinilytica* Cpt29 (Habbeche et al., 2014), *Bacillus subtilis* AMR (Mazotto et al., 2017) and *Bacillus subtilis* (Liu et al., 2014) degraded feathers completely within 3, 2, and 1.5 days of incubation, respectively. However, their keratinolytic activity was 24,000 U/ml, 360.6 U/ml, and 3.8 U/ml, respectively. This implied that MD4 takes more time to degrade the hair but it has the potential to produce a sufficient amount of keratinase enzyme. This is in contrast to the above-mentioned organisms had the potential to degrade feather within short period of time but their keratinolytic activity was limited. Therefore, it is possible to deduce that there is no a relationship between keratin degradation efficiency of microorganisms and the amount of keratinase they produce. This concern is also observed in bacterial species that belong to the same genus. The most probable reasons for the variation of the amount of keratinase produced by keratinolytic microorganisms and their keratinolysis activity might be the culture conditions (temperature, pH, etc.), nutritional factors (carbon and nitrogen sources, vitamins, etc.), the presence or absence of enzyme inhibitors and activators, and enzyme assay conditions (method of assay, incubation time, substrate used, etc.). Similarly, Kshetri & Ningthoujam (2016) also reported that physiological and nutritional factors greatly affect keratin degradation and keratinase production by keratinolytic microorganisms.

In conclusion, the newly isolated Bacillus sp. isolate MD4 produced a significant amount of keratinase. The enzyme production system is straightforward and easy to scale-up because the organism can grow on cheap media with sheep hair as its sole carbon, nitrogen, and energy source. Thus, utilization of animal hair as a fermentation substrate in conjunction with keratin-degrading microorganisms or their enzyme may be a better alternative to improve the nutritional value of animal hair and reduce environmental waste. The newly isolated bacterium (MD4) possesses high keratinolytic activity and is very effective in sheep hair degradation, presenting potential use for keratin hydrolysis. The use of this organism and its enzyme in various biotechnological applications should be investigated in future studies.

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