Editor and Reviewer Decision

Kotak Masuk ×

Majalah ATK <majalah@atk.ac.id>
kepada saya

We have reached a decision regarding your submission to Berkala Penelitian Teknologi Kulit, Sepatu, dan Produk Kulit, " KERATINAS TANNING INDUSTRY ".

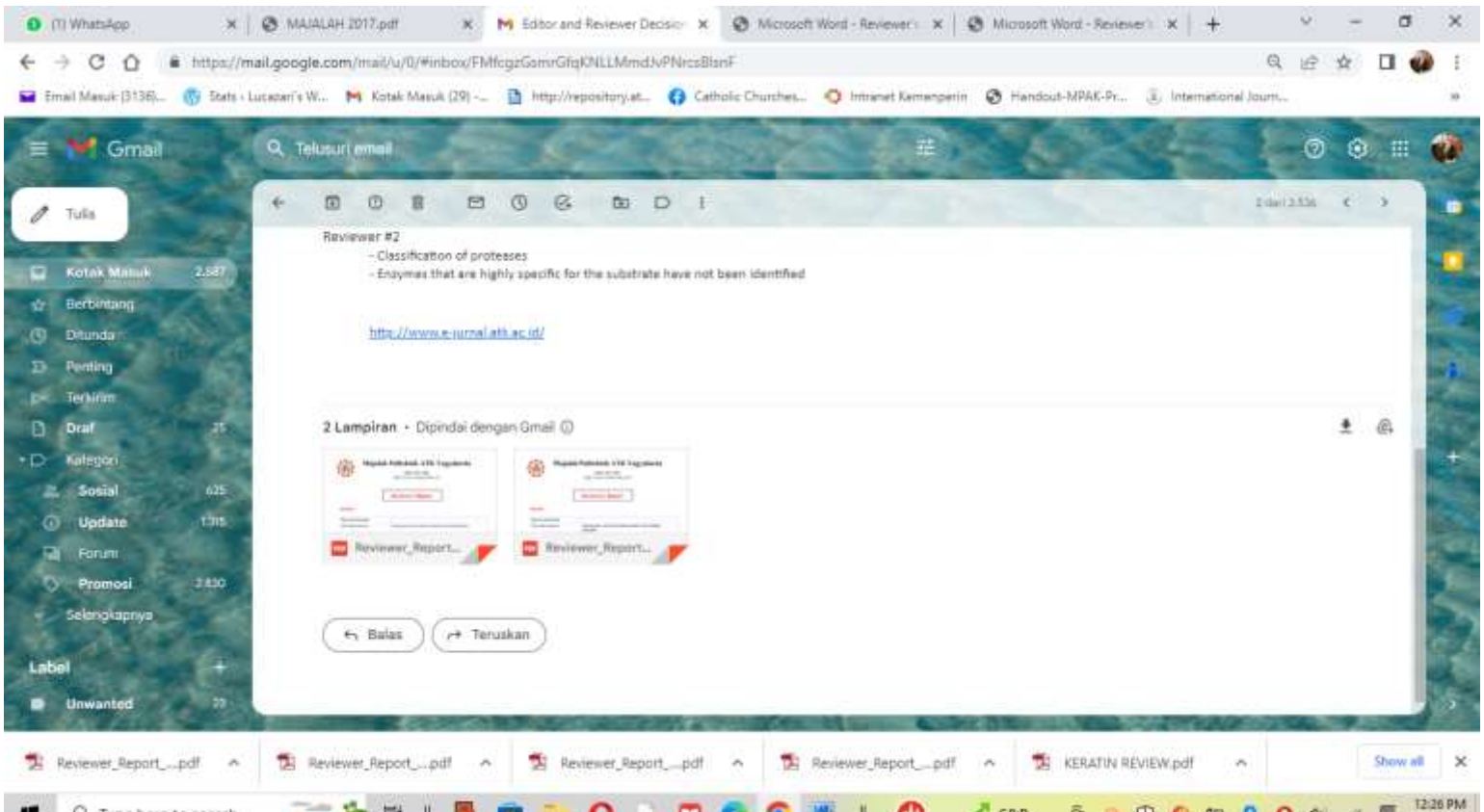
Our decision is: **Revisions Required**

Editor and Reviewer Comments

Reviewer #1

- So, at first, we organize the chapter of your paper. Following is many opinion,

1. Introduction of proteases
2. classification of proteases
3. function of keratinases
4. Industrial use of keratinases



Gmail **Alexius Lucas Ari Wibowo <alexius.lucaswibowo@gmail.com>**

Editor and Reviewer Decision

Majalah ATK <majalah@atk.ac.id> 15 Juli 2017 pukul 11.45
Kepada: alexius.lucaswibowo@gmail.com

We have reached a decision regarding your submission to Berkala Penelitian Teknologi Kulit, Sepatu, dan Produk Kulit, " KERATINASE PART OF PROTEASE USEFUL FOR TANNING INDUSTRY ".

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- Please discuss difference of the functions between exo- and endo-proteases
- How does keratinase react?

Reviewer #2

- Classification of proteases
- Enzymes that are highly specific for the substrate have not been identified

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Reviewer's Report

SECTION-I :

Manuscript Number:	
Title (with Aothors):	Keratinase Part of Protease Useful For Tanning Industry
Corresponding author	alexius.lucaswibowo@gmail.com
Date of receiving by Reviewer:	4 Juli 2017
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SECTION-II : Comments per Section of Manuscript

General comment:	**3 (Fair)**
Introduction and Literature Review:	**3 (Fair)**
Research Methodology:	**3 (Fair)**
Results and Discussion:	**3 (Fair)**
Bibliography/References:	**3 (Fair)**
Others:	**3(Fair)**

SECTION-III : Please rate the following
(1 = Excellent) (2 = Good) (3 = Fair) (4 = Poor)

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Originality:	**(Fair)**
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SECTION-IV : Recommandation
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- So, at first, we organize the chapter of your paper. Following is many opinion,
    1. Introduction of proteases
    2. classification of proteases
    3. function of keratinases
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Signature of the Reviewer
Date: 13 Juli 2017

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SECTION-I :

Manuscript Number:	
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Corresponding author	alexius.lucaswibowo@gmail.com
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SECTION-II : Comments per Section of Manuscript

General comment:	**3 (Fair)**
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Research Methodology:	**3 (Fair)**
Results and Discussion:	**3 (Fair)**
Bibliography/References:	**3 (Fair)**
Others:	**3(Fair)**

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Depth of Research:	**(Fair)**

SECTION-IV : Recommandation
(Kindly mark with an X)

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Requires Minor Revision:	**X**
Requires Moderate Revision:	
Requires Major Revision:	
Rejected for publication in RJC: (Please give reason)	

SECTION-V : Additional Comments (You may use additional sheet):

- Classification of proteases
- Enzymes that are highly specific for the substrate have not been identified

Signature of the Reviewer
Date: 14 Juli 2017

REVIEW
KERATINASE—PART OF PROTEASE USEFUL FOR TANNING INDUSTRY

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ABSTRACT

Proteolytic enzyme, which is able to hydrolyze keratin efficiently, is called keratinase, produced by microorganisms including fungi, bacteria and actinomycetes, and considered useful for various biotechnological approaches to waste management. Keratin is a fibrous, structural and insoluble protein, and it has high stability. Keratin is one of the main components for cell cytoskeleton, including hair and wool. Hair is difficult to degrade because it contains a lot of keratin with dense polypeptide structure, which is tightly bound by several hydrogen bonds, hydrophobic interactions, and cross-linkage of protein chain with cystine bridge. This condition makes hair have mechanical stability and resistant against degradation of proteolytic enzymes, such as pepsin, trypsin, and papain. Keratin has great strength, but it is not accumulated in nature because it can be hydrolyzed by several keratinolytic microbes.

Keratinase enzyme is a part of alkaline protease group, which is active in alkaline condition. Several keratinolytic bacteria produce keratinase enzyme which remains active at pH 8 to 13. The activity of keratinase enzyme also greatly varies by temperature. Keratinase enzyme has stable activity at 20 to 70^{o}C. Enzyme is protein which has catalytic activity and has certain molecular weight. Keratinase enzyme mostly has molecular weight which is less than 85 kDa.

Enzymatic hair removal process can be used as an alternative to avoide problems caused by using sodium sulfide in tannery. The benefit of enzymatic hair removal process is perfect hair removal, so that the skin is clean and smooth; removed hair is intact; and sodium sulfide usage is minimized. Keratinase enzyme specifically degrades keratin without damaging other structural proteins, such as collagen, so it has high potential for leather industry.

Keywords: *Proteolytic enzyme, keratinase, leather industry*

INTISARI

Enzim proteolitik yang mampu untuk menghidrolisis keratin secara efisien, disebut keratinase, diproduksi oleh mikroorganisme termasuk jamur, bakteri dan aktinomisetes dan dianggap penting untuk berbagai pendekatan bioteknologi untuk penanganan limbah Keratin merupakan protein serat, struktural, tidak larut, serta memiliki stabilitas yang tinggi, diantaranya berasal dari rambut dan wol. Rambut sulit didegradasi karena banyak mengandung keratin yang susunan polipeptidanya rapat, diikat kuat oleh beberapa ikatan hidrogen, interaksi hidrofobik, dan adanya ikatan silang rantai protein oleh jembatan sistin. Kondisi ini menyebabkan rambut memiliki kestabilan secara mekanis dan tahan dari degradasi enzim proteolitik pada umumnya seperti pepsin, tripsin, dan papain. Keratin memiliki kekuatan tinggi, tetapi tidak terakumulasi di alam karena dapat dihidrolisis oleh beberapa mikroba keratinolitik.

Enzim keratinase termasuk ke dalam kelompok alkalin protease yang aktif pada kondisi basa. Beberapa bakteri keratinolitik menghasilkan enzim keratinase yang tetap aktif pada pH 8 sampai dengan 13. Aktivitas enzim keratinase juga memiliki variasi yang besar terhadap suhu. Enzim keratinase memiliki aktivitas yang stabil pada suhu 20 sampai dengan 70^{o}C. Enzim merupakan protein yang memiliki aktivitas katalitik dan memilki berat molekul tertentu. Enzim keratinase sebagian besar memiliki berat molekul kurang dari 85 kDa.

Proses buang rambut enzimatis dapat digunakan sebagai alternatif untuk menghindari masalah yang disebabkan oleh penggunaan natrium sulfida dalam penyamakan kulit. Keuntungan proses buang rambut enzimatis adalah pelepasan rambut yang sempurna sehingga kulit menjadi bersih dan halus, rambut yang lepas kondisinya masih utuh, selain itu dapat meminimalisasi penggunaan natrium sulfida.

Enzim keratinase secara spesifik mendegradasi keratin tanpa merusak protein struktural lainnya seperti kolagen sehingga sangat potensial digunakan pada industri kulit

Kata Kunci: Enzim Proteolitik, Keratinase, Industri Kulit

INTRODUCTION

Protease is one of the major enzyme groups, which catalyze proteolysis of proteins or peptides by hydrolysis of peptide bonds. All living things belonging to animalia, plantae, fungi, bacteria, archaea and viruses possess several proteases, and proteases play a major role in various biological functions, from cell, organ to organism levels, by running metabolism reaction, regulation function (Rao et al., 1998).

Enzyme was used in industrial processing. Proteases used for industries are generally produced by microorganisms. The usage of microorganism for protease enzyme production has several advantages, including easy to produce in large scale, relatively short production time, and able to be produced sustainably at relatively low cost (Thomas, 1989). Proteases are also one of the most important industrial enzyme groups, because proteases have high economic value due to its broad applications, for example, detergent, leather, textile, food, hydrolyzed protein, milk processing, pharmacy, beer, waste and tannery industry (Moon et al., 1993).

Recently, tannery industry has utilized some kinds of enzymes as an alternative for environmentally friendly tanning. Enzymes can react with specific substrate depending on the purpose of the process, and this changes the paradigm from chemical-based production process into the using of biological agents in the process. Therefore, several studies are conducted to replace toxic chemicals (such as sodium sulfide in the hair removal process) with enzymes. The benefits of using enzymes in hair removal process are: better resulting hide because the hair can be removed perfectly, minimum usage of sodium sulfide which reduces dangerous hydrogen sulfide content in waste, good hair quality, and improved production efficiency.

Classification of proteases

The proteases have been classified to several classes by catalytic mechanisms, substrate specificities, enzymatic characters and physiological functions. For example, based on location of breaking peptide bond in protein

molecule, protease is classified into two groups, such as endopeptidase and exopeptidase (Bergman, 1942).

1. Exopeptidase: catalyzing the cleavage of the terminal peptide bond
    a. Carboxy (exo) peptidase, cutting peptide bond from carboxyl terminal
    b. Amino (exo) peptidase, cutting peptide bond from amino terminal
2. Endopeptidase: catalyzing the cleavage of the nonterminal peptide bond

Proteases, which catalyzes the breaking of peptide bond at the ends of polypeptide chain, is called exopeptidase, then endopeptidase is the protease, which catalyzes the cleavage of peptide bond inside polypeptide chain (Murray et al., 2003).

The International Union of Biochemistry and Molecular Biology in 1984 recommended using the term *peptidase* to refer to an enzyme that undertakes the hydrolysis of peptide chains. Peptidase is also known as protease. In general, a protease falls into the category of either an exopeptidase or an endopeptidase depending on the location where the enzyme functions. If an enzyme breaks down peptide bonds from the direction of amino acids or the carboxyl end of a substrate, it is an exopeptidase. On the other hand, if an enzyme breaks down peptide bonds away from the end of amino acids or the carboxyl end, it is an endopeptidase. Based on the way it works to deal with the N or C end, exopeptidases are divided into aminopeptidases and carboxypeptidases. Endopeptidases are divided into four groups based on the functional amino acid group of their active part, namely serine proteases, sulphurhydril proteases, acid proteases, and metal proteases. Serine proteases are an enzyme containing amino acids known as serine in the active part. This enzyme is inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP), not by the ethylenediaminetetraacetic acid (EDTA). This protease encompasses a number of types, namely trypsin, chymotrypsin, elastase, and subtilysin. Sulfhydryl or thiol proteases are a type of protease containing amino acids known as cysteine on the active part. These enzymes are sensitive to a number of oxidizing bases and some metals which can bind thiol groups in the active part. This enzyme group is comprised of papain,

fisin, and bromelain. Acid proteases are an enzyme that is active at an acidic pH, insensitive to both EDTA and serine protease inhibitors. Examples of these enzymes are pepsin, renin, and some fungal enzymes that are active at a low pH, i.e. ranging from 2 to 4. Neutral or metal proteases are an enzyme that shows maximum activities at a neutral pH and is sensitive to EDTA.

Protease is a group of very complex enzymes which occupies a central position in its application in physiology and commercial products. Extracellular protease mostly plays a role in hydrolysis of large polypeptide substrate. Intracellular protease enzyme has important role in metabolism and regulation process in animal, plant and microorganism cells, such as replacing protein, maintaining balance between degradation and protein. Intracellular protease plays a role in other physiological functions, such as digestion, hormone maturation, virus assembly, immune response, implantation, fertilization, blood coagulation, fibrinolysis, blood pressure control, sporulation, germination, and pathogenesis (Rao et al., 1998).

Proteases are now classified into seven families based on the nature of the catalytic residues.

1. aspartic peptidase:
    a. are a catalytic type of protease enzymes
    b. a highly specific family of proteases - they tend to cleave dipeptide bonds that have hydrophobic residues as well as a beta-methylene group
    c. Eukaryotic aspartic proteases include pepsins, cathepsins, and renins
2. cysteine peptidase:
    a. are commonly encountered in fruits including the papaya, pineapple, fig and kiwifruit
    b. also known as **thiol proteases**, are enzymes that degrade proteins
    c. catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic triad or dyad.

3. Serine peptidase:
    a. Has serine residual in its active location.
    b. Is endopeptidase
    c. Includes: trypsin, chymotrypsin, elastase and subtiline
4. metallo peptidase:
    a. the activeness depends on metal with 1 mol metal/1 mol enzyme stoichiometric relation
    b. can be inhibited by EDTA (Ethlene Diamine Tetra Acetic Acid) which can chelate metal to remove/reduce enzyme activeness.
    c. includes: carboxypeptidase for several aminopeptidase
5. threonine peptidase:
    a. are a family of proteolytic enzymes harbouring a threonine (Thr) residue within the active site
    b. use the secondary alcohol of their N-terminal threonine as a nucleophile to perform catalysis
    c. The threonine must be N-terminal since the terminal amine
6. glutamic peptidase:
    a. donates a proton to the amide nitrogen
    b. resulting in breakage of the peptide bond
    c. The active site of eqolosin contains a distinctive glutamic acid
7. asparagine peptidase:
    a. The catalytic mechanism of the asparagine peptide lyases involves an asparagine residue
    b. acting as nucleophile to perform a nucleophilic elimination reaction,
    c. These enzymes are synthesized as precursors or propeptides, which cleave themselves by an autoproteolytic reaction

Keratinase

Many keratinase preparations show good activity to collagen (Lin *et al*., 1992; Böckle *et al*., 1995; Nam *et al*., 2002; Gousterova *et al*., 2005), such as keratin. Keratin is fibrous protein but from a different structure which contains three helix conformation. It is stabilized by intermolecular covalent cross-linking

involving lysine and lysine derivative. Macedo *et al* (2005) state that keratinophylic bacteria is bacteria which produces keratinase enzyme, so it can degrade keratinized substrate into simpler building block. Keratin is insoluble fibrous protein found in hair, wool, feather, nail, horn, horse nail and other epithelial covers (Feughelman 1985). The most unique feature of keratin is high concentration of half-cystine residue which interlinks and gathers as polypeptide supercoil, providing high mechanical stability and making protein difficult to be degraded by common proteolytic enzymes, such as trypsin, pepsin, and papain (Dowling *et al*. 1986). Keratin waste can be degraded efficiently by various bacteria, actinomycetes, and fungi through keratinolytic protease synthesis (Onifade *et al*. 1998). Extacellular keratinolytic protease is purified and characterized from culture medium after growing using hornmeal (slaughterhouse side product) as the only source of carbon, nitrogen, and energy (Balaji *et al*, 2008).

Keratinase is a certain proteolytic enzyme group which shows ability to degrade insoluble keratin substrate. The enzyme has several potential applications to hydrolyze keratin substrates, such as a number of fibrous proteins which are insoluble as feather, hair, nail, horn and other byproducts in agroindustry processing (Brandelli, 2008)

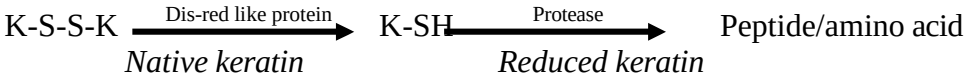
Ogawa *et al.*, (2008) state that hair degradation by keratinase is thought start with breaking disulfide bond, probably by sulfitolysis process. Reduced disulfide bond (SS) is changed into thiol (SH) and dithio group compounds, such as *dithiodiglycolic* acid and cystin. Meanwhile, Brandelli *et al*., (2010) state that the hydrolysis of keratin substrate by keratinase enzyme from microbe generally happens due to reduction of disulfide bond in keratin.

Keratinase enzyme (EC 3.4.99.11) of that number shows that the enzyme is a part of hydrolase class, peptide hydrolase or peptidase sub-class, and endopeptidase group which reacts to the inner part of the composing peptide chain. Most keratinase enzymes are included in extracellular serine protease group which can degrade keratin (Jouadi *et al.*, 2010).

Kunert (2000) state that the main factor of keratin degradation by fungi is caused by protease, alkalinization medium and sulfytolysis activities. Fungus excretes sulfite and ammonia. In alkaline condition, sulfite will cut disulfide bridge in substrat. Then slowly substrate will be denaturized and ease the work of protease. The main product of sulphitolysis in disulfide bridge is S-sulphocysteine in free form and combined by peptide.

A study by Kumar *et al.* (2008) explain that the growth of *B. pumilus* significantly increases pH in media during cow hair degradation. 605 keratin protein is degraded. It indicates high keratinolytic activity. The morphological change of cow hair shows that degradation happens due to enzymatic process. Microscopic observation shows that many parts of the substrate is degraded.

Complete degradation of hard keratin is only possible after denaturation by cutting disulfide bond. Thiol formation shows there is disulfide reductase activity along with keratinase activity. The research result shows that keratin degradation happens due to two mechanisms, sulphitolysis and proteolisis. Yamamura *et al.* (2002) report joint action of protein similar to disulfide reductase and protease produced by *Stenotrophomonas* sp. In degrading deer hair keratin, the keratin degradation mechanism is as follows:



Enzyme can only react with certain substrates consistent with the active side. A compound is designed to be a certain substrate to be changed into product with the help of enzyme. Change from substrate to product is the main detection, with the assumption that the amount of converted substrate is the same as the amount of product. The amount of product created or substrate converted by an enzyme in certain period (second, minute, hour) is called enzyme activity (Bisswanger, 2011).

Mazotto et al., (2011) state that the activity of keratinase enzyme is measured by several types of keratin substrate, such as feather meal and keratin azure (Jouadi *et al.*, 2010. Keratin azure is pure keratin made of wool dyed by azo dye. Keratin azure is mixed with water and reacted with certain enzyme to

produce blue reaction product (Sigma Aldrich, 1998). The amount of keratinase enzyme activity is stated as unit, where one unit (U) of keratinase activity is defined as the amount of enzyme required to increase absorbance by 0,01 between sample and control in 595 nm wavelength consistent with test condition (Wang *et al.*, 2008).

Enzyme is strongly affected by medium of pH because enzyme is a charged compound group which reacts to catalytic mechanism. Its protonation level determines its catalytic efficiency. Enzyme is also atmospheric substance with positive and negative charges on its surface and in its core to stabilize its original. Enzyme has the best catalytic activity at optimum pH and is usually in neutral condition between 6,5 and 8,5, but certain enzymes have alkaline pH or extreme alkali (Bisswanger, 2011).

Keratinase enzyme is included in alkaline protease group which has alkaline pH. *Streptomyces* sp. strain AB1 produces keratinase enzyme with high activity at pH 8 to 12, and optimum pH at 11,5 (Jouadi *et al.*, 2010). Tatineni *et al.* (2008) examines pH condition for keratinase enzyme produced by *Streptomyces* sp. with maximum activity at pH 9 to 12. The keratinase enzyme produced by *Bacillus halodurans* PPKS-2 is active at pH 9–13 (Prakash *et al.*, 2010).

Enzyme activity basically depends on the temperature. Based on Van't Hoff's postulate reaction speed is accelerated 2 – 3 times in every 10^{o}C increase in temperature. Increased temperature increases kinetic energy, so that enzyme activity will increase along with increased temperature, but because enzyme is protein, it has temperature limitation. At high temperature, enzyme is denaturized and deposited, losing its catalytic ability. Enzymes have varying denaturation temperatures, depending on various factors maintaining their stability. Several enzymes are very sensitive to temperature, but enzyme from thermophiliac organism has high temperature stability. Therefore, enzymes have varying optimum temperatures (Bisswanger, 2011). Keratinase enzyme has optimum activity at 25 to 70^{o}C, depending on each microorganism species producing it

(Farag dan Hasan, 2004). Similarly, the keratinase enzyme produced by *Bacillus* sp. 50-3 is stable at 20 –70^{o}C (Zhang *et al*., 2009)

Substrate concentration influences the rate of catalyzed reaction by enzyme. In low substrate concentration, initial reaction rate (V_o) equals to substrate concentration. Reaction rate will increase along with increased substrate concentration. Rate increase will decline as substrate concentration increases and will reach maximum point, so that the rate won't increase although substrate concentration increases. This rate limi t is called "maximum rate" (V_{max}). In this condition, enzyme is saturated by substrate and increased concentration won't increase rate anymore (Nelson and Cox, 2008).

The relation between substrate concentration and reaction rate forms hyperbole curve which applies for all enzymes. Leonor Michaelis and Maude Menten define the correct relation between substrate concentration and enzymatic reaction rate in a constant expressed as K_m or Michaelis-Menten constant. In other words, K_m is defined as substrate concentration when enzyme reaches half maximum rate. K_m value is unique for every enzyme with specific substrate at certain pH and temperature. Calculation of maximum reaction rate in hyperbole equation is difficult, so Lineweaver-Burk develops a straight line equation derived from Michaelis-Menten equation by changing the graph into 1/V and 1/[S]. The line has gradient K_m /V_{max} and the resulted line will cut Y axis with point of intersection 1/V_{max} and cut X axis with point of intersection -1/K_m, so K_m and V_{max} are found (Voet *et al.*, 2008).

Enzyme is protein which has catalytic activity. Protein is macromolecule which consists of monomers with molecular weight of 500 kDa or less. Molecular weight can be determined using sodium dodecyl sulfate (SDS). The molecular weight of keratinase enzyme from bacteria is around 18 kDa to 240 kDa (Brandelli *et al.,* 2010). The molecular weight of keratinase enzyme produced by *Thermoanaerobacter keratinophilus* is 85 kDa (Riessen and Antranikian, 2001), while *Streptomyces albidoflavus* produces keratinase enzyme with molecular weights of 40 and 70 kDa (Bressollier *et al*., 1999)

FACTORS INFLUENCING ENZYME ACTIVITY

Temperature

Reaction rate increases along with temperature rise, but as enzymatic reaction takes place, maximum point will be reached and reaction rate will decline as temperature rises. High temperature causes protein part of enzyme to break down or denaturized, inhibiting reaction. Enzyme only works effectively at optimum temperature (Saryono, 2011).

The nature of enzyme is temperature treatment can open protein structure and remove enzyme activity. The determining factor of enzyme stability in hot environment is non-covalent group in protein molecule which maintains secondary and tertiary structures (Sebayang, 2006). Enzyme reaction rate increases to optimum temperature and lowers to above optimum, usually due to enzyme damage. Most enzymes have optimum activity at 30 to 40°C (Volk and Wheeler, 1988).

Substrate Concentration

Enzyme activity is also influenced by substrate concentration. In low substrate concentration, enzyme doesn't reach maximum conversion because it's difficult to find substrate to react. As substrate concentration increases, reaction rate also increases because substrate is bound to enzyme faster. Substrate concentration increase at saturation point doesn't increase reaction rate anymore (Pertiwi, 2008). Enzyme activity rate will increase along with substrate to a point. Once enzyme is saturated with substrate, substrate increase won't influence reaction rate. The lower the substrate level which will produce maximum activity, the greater the relation between enzyme and substrate (Volk and Wheeler, 1988).

pH value

pH value in environment also influences enzyme activity rate in catalyzing a reaction. It's because hydrogen ion concentration influences three dimensional structure of enzyme and its activity. Every enzyme has optimum pH where its three dimensional structure is most conducive to increase substrate. If hydrogen ion changes from optimum concentration, enzyme activity progressively disappears until enzyme becomes non-functional (Lehninger, 1997). Change of

enzyme activeness due to change of pH in environment is caused by change of enzyme ionization, substrate or enzyme substrate complex. Enzyme has maximum activity at pH range called optimum pH. Around optimum pH, enzyme has high stability. Some enzymes have very extreme optimum pH, e.g. pepsin at pH 1,8 and arginase at pH 10,0 (Winarno, 1986).

Inhibitor

Enzyme activity is also influenced by inhibitor. If a compound reduces reaction rate, the compound is called inhibitor. Inhibitor can compete with substrate in bonding with enzyme, preventing substrate to be bound to enzyme active site (Poedjiadi et al., 1994).

LEATHER INDUSTRY

Leather and leather product industry is one of national leading industries. The materials of this industry are based on national natural resources, providing high value added. Leather and leather products from Indonesia are demanded by foreign markets. The favorite products of foreign customers include glove, footwear, apparel, jacket, and other leather garments. Indonesian glove product, especially golf glove, is famous among international customers, especially customers in America, Europe, and Japan. Indonesia dominates 36,3% world's market share for leather glove, 15% for sport shoe, 1 – 2% for non-sport shoe, 4,3% for leather apparel, jacket and garment, and 5% for bag, wallet, and belt. (Untari *et al*., 2009).

Indonesian leather products are accepted in international market because leather from Indonesia has advantages, such as soft, event and compact nerf surface, and strong and solid leather tissue structure. Since ancient times, cow hide from Indonesia, especially cow hide from Java, is known as *Java Boks. Java Boks* is famous across Europe. Sheepskin from Indonesia also has advantages compared with sheepskin from other countries because its surface nerf structure is smooth, even, and compact, so that if it's processed well, it will produce leather with good quality. Good leather quality makes Indonesia one of the leading

leather and leather product exporting countries in the world along with China, India, and Thailand (Thanikaivelan *et al*., 2005).

Leather industry is considered an important industry, but there are many problems to improve in it. One of the problems in the industry is production technology, such as tanning technology. Various chemicals are used in tanning, so the waste can cause pollution. Clean product issue and environmental issue are the major focus of many people. Foreign customers, especially European and Japanese customers require leather products to not contain dangerous substances and not pollute the environment.

Various efforts are developed by researchers across the world to make leather processing not dangerous for customer and environment. One of the approaches is by Thanikaivelan *et al*. (2004) by avoiding pollution source to minimize waste by subtitute eznyme with toxic chemicals. Meanwhile Kumar *et al.* (2011) suggest green chemistry concept by increasing efficiency of material usage, avoiding usage of dangerous and toxic chemical during production, and reducing waste production. One of the initial processes of tannery which produces polluting waste is unhairing process. Unhairing process uses dangerous chemicals such as Na_2S. Na_2S waste in tanning process can become dangerous because it may create toxic H_2S (Sunaryo, 2005).

Changing tanning process from chemical tanning process into using process using biological agent (bioprocess) as environmentally friendly tanning alternative. The biological agent which can be used is enzyme. Enzyme can react with specific substrate depending on purpose of process. It changes the thinking paradigm of chemical based production process into production process using biological agent. Therefore, several studies are performed to replace chemicals with enzyme such as Na_2S in unhairing process. The benefits of enzyme usage in unhairing process are: better leather product because hair can be removed perfectly, minimized usage of Na_2S to reduce H_2S content in waste, good quality of resulting hair, and increased production efficiency (Brandelli, 2008).

An enzyme which has been studied extensively as a bioprocess agent in tannery in protease enzyme. The enzyme is used in submersion, unhairing, and bating processes. The usage of protease enzyme in tanning process gives very good value added for the industry. The usage of protease enzyme in several tanning stages gives positive impact to the environment because pollution load is lower and the aether quality is better than the result of chemical tanning process.

The application of biotechnology in leather factory is by using enzyme to replace chemical. This can be an alternative to reduce pollution. Enzyme usage can be applied to different tanning stages, such as submersion, liming, unhairing, bating, dyeing, degreasing, and waste management, whether liquid or solid waste. Some benefits of using enzyme in leather processing is leather can be moistened again in short time and there is good leather fiber opening and quick removal of protein, fat and carbohydrate. Enzyme lso can be used in unhairing process, where epidermis and hair are removed and adipose residual component is removed, as well as in reducing pollution load of waste it produces. Unhairing enzyme in tanning process has been used as an alternative for sulfide. Continuous usage of sodium sulfide in unhairing process in tannery can cause dangerous smell and pollution because it's toxic. There have even been reports that it causes death. Alexander *et al* (2005) discover *Bacilus Subtilis* produced keratinase which potentially replaces sodium sulfide in unhairing process.

The effect on enzyme treatment on increasing leather quality is also reported by Kamini, *et al* (1999), who proves that enzyme usage in tannery improves leather quality, i.e.: leather is smoother/softer and easier to fold. Banerjee and Bhattacharyya (2001) show that tensile strength and shear force (elongation and tear strength) of leather are also better, increased by around 3-5%.

More specific enzyme usage for hair degradation has started to grow. Keratinase enzyme is developed because it can degrade substance made of keratin. Commercial use of keratinase is still very little, only to degrade feathers for feed material (Gupta, 2006). The usage of keratinase enzyme for unhairing in tanning process is only at laboratory research stage, such as using keratinase enzyme to remove sheepskin hair (Prakash *et al*., 2010). The usage of

keratinolytic protease enzyme is interesting in tanning process because it has very low collagenase activity, so the enzyme can remove hair well without affecting tensile strength of leather (Macedo *et al.*, 2005).

Several keratinolytic microbes from eukaryotic, bacteria, and archea groups can produce keratinase enzyme. Most keratinase enzyme is isolated from keratinolytic bacteria in various locations, from soil in the Antartics to hot water in mountains, including aerobic and anaerobic environments, poultry husbandry waste, alkaline lake, decaying feather, chicken slaughterhouse, and deer hair (Brandelli *et al.*, 2010).

Enzyme has very specific reaction to substrate. Selection of enzyme producing bacteria in nature can be adjusted with the substrate to be degraded. For example, to degrade chicken feather, the keratinolytic bacteria is isolated from poultry industry waste (Mazotto *et al.*, 2011) and to degrade wool, the enzyme is produced by keratinolytic bacteria is isolated Merino sheep wool (Infante *et al.*, 2010).

Conventional tanning method works in a great range of pH. Extreme pH change requires acid and alkaline chemicals to produce salt which increases total solid (TS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), chloride (Cl^-) and sulfate (SO_4^{2-}) in waste. It also produces toxic gas, such as hydrogen sulfide, which is produces in unhairing process. The toxicity of waste from unhairing and liming processes is up to 76% (Anonim, 1996).

Sharphouse (1983) states that various types of pre-tanning waste can affect environment, e.g.: chloride affects freshwater life, sulfide produces smell and reacts with acid to form toxic hydrogen sulfide, sulfate is generally not desired in aquatic life. Alkaline condition with high pH can inhibit plant, animal, and microbe lives.

Various studies are performed to replace both chemicals, but it's impossible to completely replace those (Thanikaivelan *et al.*, 2007). Keratinase enzyme is one of the alternatives which can be used to solve waste problem

caused by the usage of sodium sulfide. This enzyme is very effective in lysing hair, so it reduces sulfide content in waste and produces hair with good quality (Giongo *et al*., 2007).

The usage of keratinase enzyme in hair saving dehairing process has several benefits because it enables separating air and avoiding semi gelatinous hair meal material, so that there's little organic substance in the waste. Meanwhile, conventional hair saving dehairing process uses sulfide (hair destroying) destroying hair, increasing organic substance in the waste (Gupta and Ramnani, 2006). Enzymatic hair saving dehairing produces relatively whole hair so that the liquid waste has suspended solid content and low pH, and reducing BOD and COD contents (Triatmojo and Abidin, 2014).

Enzymatic hair saving dehairing process is currently used to reduce Na_2S usage (Thanikaivelan *et al.,* 2005). Enzymatic hair saving dehairing generally uses proteolytic enzyme and a small amount of sulfide and lime. The usage of enzyme in cowhide is 1% and 0,5% of sodium sulfide can remove hair perfectly. Complete unhairing of goat skin is achieved using 1% commercial enzyme without sodium sulfide, while for sheepskin, a combination of 1% enzyme and 0,25% sodium sulfide is required to remove hair perfectly. It's because natural fat content in sheep content can inhibit enzyme penetration to skin matrix and hair root (Thanikaivelan *et al.,* 2004).

Hair saving dehairing process using commercial enzyme also has been performed by several researchers, such as Saravanabhavan *et al*. (2007) who uses Biodart, and alkaline protease enzyme produced by local bacteria in India, with 100 U/g enzyme activity, optimum at pH 7,5 – 11 and $25^oC - 40^oC$, which is used in cowhide tanning process. Suyanto (2008) uses exolite enzyme as a substitute for Na_2S for hair saving dehairing process. Thanikaivelan *et al*. (2007) uses a combination of commercial enzyme Biodart and α-amylase enzyme (enzyme activity 1.000 U/g) to replace lime and Na_2S in hair saving dehairing process. The researches produce good unhairing with better pollution load than conventional method using chemical.

Laboratory scale enzymatic hair saving dehairing process uses keratinase enzyme from several bacteria has shown good unhairing activity. One of the studies is performed by Prakash *et al*. (2010) by submerging sheepskin piece in 10 mL rough keratinase enzyme produced by *Bacillus halodurans* Strain PPKS-2. Zambare *et al.* (2007) uses 1% (v/b) rough keratinase enzyme from *Bacillus cereus* MCM B-326 to submerge buffalo skin piece. Macedo *et al.* (2005) submerge cowhide piece in spinning drum by adding 1,0 mL rough keratinase enzyme from *Bacillus subtilis* S14 per gram skin. Giongo *et al*. (2007) submerge cowhide piece in keratinase enzyme (200 U mL^{-1}) from *Bacillus* sp. overnight. Sundararajan *et al*. (2011) cover the flesh part of goat skin with 2% (v/b) keratinase enzyme from *Bacillus cereus* VITSN04 and 10% (v/b) water rather than paste made of 10% (v/b) lime and 3% (v/b) sodium sulfide. The research result shows that enzymatic process produces better leather than chemical process. In fact the quality of the waste produced by the enzymatic process is better than the waste produced by the chemical process.

Keratinase enzyme specifically degrades keratin without damaging other structural proteins, such as collagen, so it has high potential for leather industry. Microscopic test on leather shows that keratinase enzyme hydrolize the outer part of hair root, so that hair is removed (Riffel *et al.*, 2003). Keratinolytic protease has very small collagenase and elastolase activities. Keratinolytic protease breaks keratin tissue effectively in follicle so that hair can be pulled wholly without affecting tensile strength of leather (Macedo *et al*., 2005).

Keratinase or alkaline protease enzyme is used to help digest epidermis, remove hair, and digest globular protein. The enzyme digests soft keratin in malphigian layer cells and hair root, so that hair can be removed to the root (Triatmojo and Abidin, 2014).

CONCLUSION

Keratinase is a group of protease enzymes capable to degrade substrates containing keratin. Hydrolysis of keratin substrate by keratinase enzyme from

microbes generally occurs because of the reduction of disulfide bond in keratin. Reduced disulfide bond (SS) is converted into thiol and dithio group (SH) compounds, such as dithiodiglycolic acid and cystine. Complete degradation of hard keratin is only possible after denaturation by cutting disulfide bond. The formation of thiol shows disulfide reductase activity in conjunction with keratinase activity. Keratin degradation occurs as a result of the action of two mechanisms: sulphitolysis and proteolysis.

Enzyme reacts very specific with its substrate. Keratinase enzyme can degrade keratin and avoid damage to other structural proteins such as collagen. It makes keratinase enzyme can be used in leather industry. Keratinase enzyme from bacteria is generally active and stable at pH 8 to 13, with a temperature ranging from 20 °C to 70 °C. Such conditions also allow keratinase from bacteria can be used to the process of hair removal in leather tanning because the hair removal process is generally performed at pH 10 to 12 with a temperature of 25 to 27 °C.

Keratinase does not hydrolyze collagen, so its use for hair removal in the process of leather tanning does not cause damage to collagen. Collagen damages such as denaturation of collagen will lower the physical quality of the skin. Collagen fibers contribute to the strength of the skin. Stability of the skin is generally determined by collagen bond with tanning material. The more collagen bind to tanning substances, the better the physical quality of the skin, such as tensile strength, elongation, and suture strength.

Leather tanning process is known as production process that produces quite a lot of waste. Pre-tanning process produces waste almost 70% of the total

waste of leather tanning. Approximately 80% of pre-tanning waste and all sulfide waste is derived from hair removal process. The process of conventional hair removal is using lime and sodium sulphide, changing hair into pulp, so that the load of contamination is getting high. Sulfide waste produces poison gas, while the solid waste can affect aquatic biota.

The process of enzymatic hair removal can be used as an alternative to avoid the problems generated by the sulfide waste in leather tanning. The advantages of enzymatic hair removal process: perfect hair removal, so that the skin becomes clean and smooth; the hair condition removed is still intact; in addition, it can minimize the use of sodium sulfide. Reduced use of sodium sulfide can reduce the content of sulphide and hair pulp in waste, thus reducing the value of BOD, COD, total solids and solids suspended in wastewater.

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