CHARACTERIZATION AND PRODUCTION OPTIMIZATION OF KERATINASE FROM THREE *Bacillus* STRAINS

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ABSTRACT. Indonesia has large coastal areas. The fisheries are good for exploitation. In the previous studies, bacteria producing keratinase were isolated from fish market waste. Keratinase enzyme is able to degrade keratin on the skin. Enzyme activity is influenced by external conditions, such as pH, temperature, and incubation time. The study aimed to investigate the characteristics and the optimal conditions of the keratinase production. The materials used were keratinase from three *Bacillus* strains: *Bacillus thuringensis* BRAW_PT, *Bacillus aerius* BRAW_PB, and *Bacillus subtilis* BRAW_PI. The keratinase was investigated by sodium dodecyl sulfate (SDS PAGE) and nondenaturing polyacrylamide gel electrophoresis (Native PAGE). Conditions of the production were optimized by pH, temperature and incubation time on enzyme activity. The molecular weights of all keratinases from *Bacillus* species were 94.803 kDa and 70.115 kDa. The optimum activity of keratinase from *B. thuringensis* BRAW_PT and *B. firmus* BRAW_PI was obtained at pH 8, while keratinase from *B. aerius* BRAW_PB was optimal at pH 6-8. Keratinase from *B. thuringensis* BRAW_PT has maximum activity at 25°C, whereas keratinase from *B. aerius* BRAW_PB and *B. firmus* BRAW_PI at 29°C. All keratinases from *Bacillus* species are optimal at 90 minutes incubation. Based on the principal component analysis (PCA), *B. thuringensis* BRAW_PT was discriminated from the other enzymes.

KEY WORDS: production optimization, keratinase enzyme, unhairing, fish market waste

CARACTERIZAREA ȘI OPTIMIZAREA PRODUCȚIEI DE KERATINAZĂ DIN TREI TULPINI DE *Bacillus*

REZUMAT. Indonezia are mari zone de coastă, iar pescăriile pot fi exploatate. În studiile anterioare, s-au izolat bacterii producătoare de keratinază din deșeurile de pe piața de pește. Enzima keratinază este capabilă să degradeze keratina din piele. Activitatea enzimatică este influențată de condiții externe, cum ar fi pH-ul, temperatura și timpul de incubație. Studiul a urmărit să investigheze caracteristicile și condițiile optime de producție a keratinazei. Materialele utilizate au fost keratinaza din trei tulpini *Bacillus*: *Bacillus thuringensis* BRAW_PT, *Bacillus aerius* BRAW_PB și *Bacillus subtilis* BRAW_PI. Keratinaza a fost investigată utilizând electroforeza în gel de dodecil sulfat de sodiu poliacrilamidă (SDS PAGE) și cea fără denaturare (Native PAGE). Condițiile de producție au fost optimizate în ceea ce privește pH-ul, temperatura și timpul de incubație pentru activitatea enzimatică. Greutatea moleculară a keratinazelor din speciile *Bacillus* a fost de 94,803 kDa și 70,155 kDa. Activitatea optimă a keratinazei din *B. thuringensis* BRAW_PT și *B. firmus* BRAW_PI a fost obținută la pH 8, în timp ce keratinaza din *B. aerius* BRAW_PB a fost optimă la pH 6-8. Keratinaza din *B. thuringensis* BRAW_PT are activitate maximă la 25°C, iar keratinaza din *B. aerius* BRAW_PB și *B. firmus* BRAW_PI la 29°C. Toate keratinazele din speciile *Bacillus* sunt optime la 90 de minute de incubație. Pe baza analizei componentei principale (PCA), *B. thuringensis* BRAW_PT s-a evidențiat din rândul celorlalte enzime.

CUVINTE CHEIE: optimizare producție, enzimă keratinază, îndepărtarea părului, deșeuri de pe piața de pește

CARACTÉRISATION ET OPTIMISATION DE LA PRODUCTION DE KÉRATINASE À PARTIR DE TROIS SOUCHES de Bacillus

RÉSUMÉ. L'Indonésie a de vastes zones côtières et les pêcheries sont bonnes pour l'exploitation. Dans l'étude précédente, des bactéries produisant de la kératinase ont été isolées des déchets du marché aux poissons. L'enzyme kératinase est capable de dégrader la kératine sur la peau. L'activité enzymatique est influencée par des conditions externes, telles que le pH, la température et la durée d'incubation. L'étude visait à étudier les caractéristiques et les conditions optimales de la production de kératinase. Les matériaux utilisés étaient la kératinase de trois souches de *Bacillus* : *Bacillus thuringensis* BRAW_PT, *Bacillus aerius* BRAW_PB et *Bacillus subtilis* BRAW_PI. La caractérisation de la kératinase a été étudiée par électrophorèse sur gel de polyacrylamide en présence de dodécylsulfate de sodium (SDS PAGE) et par électrophorèse non dénaturante (Native PAGE). Les conditions de production ont été optimisées par le pH, la température et le temps d'incubation sur l'activité enzymatique. Les poids moléculaires de toutes les kératinases de l'espèce *Bacillus* étaient de 94.803 kDa et 70.115 kDa. L'activité optimale de la kératinase de *B. thuringensis* BRAW_PT et *B. firmus* BRAW_PI a été obtenue à pH 8, tandis que la kératinase de *B. aerius* BRAW_PB a été optimale à pH 6-8. La kératinase de *B. thuringensis* BRAW_PT a une activité maximale à 25°C, tandis que la kératinase de *B. aerius* BRAW_PB et *B. firmus* BRAW_PI à 29°C. Toute la kératinase des espèces de *Bacillus* est optimale à 90 minutes d'incubation. Sur la base de l'analyse des composants principaux (PCA), *B. thuringensis* BRAW_PT se démarque des autres enzymes.

MOTS CLÉS : optimisation de la production, enzyme kératinase, épilage, déchets du marché du poisson

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INTRODUCTION

Indonesia is the world's largest archipelagic state with 54,716 km of coastline, and 17,508 islands (of which 6,000 are inhabited), and the world's fourth most populous nation (247.5 million) [1]. After China, Indonesia is the second largest fish producer in the world, with capture fisheries and aquaculture production. Fish supply in Indonesia has been increasing over the past 50 years, from 0.8 million tons in 1,960 to 10.7 million tons in 2014 [2]. The proportion of the catch is consumed in dried, salted, smoked, boiled or fermented form, while 46 percent is consumed fresh from fish market [1].

In the previous study, Wibowo *et al*. [3] have isolated the bacteria producing keratinase enzymes from fish market waste. The enzyme is used in the unhairing process for environmentally friendly fish skin tanning. According to Tamersit and Bouhidel [4], unhairing generates heavily polluted solutions. Dettmer *et al*. [5] also stated that the conventional lime-sulfide in unhairing process leads to the destruction of the hair, causing emissions with high chemical oxygen demand (COD), biological oxygen demand (BOD), and total suspended solid (TSS) loads in the effluent of these industries. Moreover, during this step in leather industry, strong chemicals are used, such as sodium sulphide and lime, which represent approximately 80-90% of total pollution of leather manufacturing [6]. Keratinase enzyme is important for the environmentally-friendly tanning process by reducing the usage of sodium sulfide as chemical [7].

Utilization of enzyme in leather making is a promising application. Researchers discovered the keratinolytic enzymes, most of them produced from *Bacillus* strain, such as *Bacillus aerius* NSMk2 [8], *Bacillus* BPKer and BAKer [9], *Bacillus cereus* and *Bacillus polymyxa* [10], and *Bacillus suptillis* [11]. Enzyme production can be influenced by several factors, such as temperature, pH, and incubation time. Optimizing conditions is a crucial aspect of enzyme production [12]. In the current study, we report the characterization and optimization of keratinase from three different *Bacillus* strains as an innovative solution in supporting the cleaner production of the tannery.

EXPERIMENTAL

Production of Keratinase Enzyme

Preparation of Inoculum for Enzyme Production

The materials used in this study were: *B. thuringensis* BRAW_PT, *B. aerius* BRAW_PB, and *B. subtilis* BRAW_PI collected from isolation results of a previous study [3]. The fermentation media was: 0.5 g/l sodium chloride, 0.3 g/l potassium hydrogen phosphate, 0.4 g/l potassium dihydrogen phosphate. Stock solution (1 g/100 ml Yeast extract; 1 g/100 ml Biological peptone; 0,5 g/100 ml NaCl and 100 ml distilled water), ammonium sulfate, 20 mM Tris HCl pH 8, 12 kDa dialysis bag, 1 mM EDTA, $NaHCO_3$, distilled water. One dose of pure culture result isolate was inoculated from agar media into 5 ml preculture medium, then incubated in a shaker at 120 rpm overnight.

Enzyme Production

The enzyme production was based on the method of Hoq *et al*. [13]. One and a half milliliters isolate were inoculated into 50 ml liquid medium, then incubated in a shaker at 120 rpm overnight. Enzyme production is characterized by yellowing. Separation of the isolates from extracellular enzyme was performed by centrifuge at 4°C and 3500 rpm for 15 minutes. The resulting supernatant was raw enzyme source whose enzyme activity can be measured. The collected enzyme was measured for enzyme activity.

Enzyme Purification

One liter of fermentation product culture was centrifuged at 10.000g for 15 minutes at 4°C. The formed supernatant was separated from the pellet. The supernatant was an enzyme extract ready to be concentrated. Purification of enzyme by precipitated by 60% saturation of ammonium sulfate. Saturation was performed using ammonium sulfate [14]. Ammonium sulfate crystals were added slowly while stirring until dissolved. The solution was left for 24 hours at 4°C, then centrifuged at 10.000g for 15 minutes at 4°C. This step in order to purifying protein by removing other nutrient such as saccharides and minerals [15].

Characterization of Eznyme

Determination of Molecular Weight of Protein by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Materials for SDS-PAGE include 70% alcohol, sterilized water, acrylamide solution, Tris HCl, SDS, d H2O, TEMED, ammonium persulfate, glacial acetic acid, Coomassie blue, methanol. The buffers were sodium phosphate, glycine-NaOH, Tris acetate. The materials used to test keratinase activity were keratin azure, Tris HCl pH 7.5 and 10% TCA solution. Protein separation by SDS-PAGE method aims to separate protein in a sample based on molecular weight. The basic principle of SDS-PAGE is protein denaturation by sodium dodecyl sulfate, followed by separating molecules based on molecular weight by electrophoresis method using gel, in this case, polyacrylamide. Identification and analysis of SDS PAGE compared protein band which was previously separated with standard protein [16].

Protease Activity by Native or Nondenaturing PAGE Polyacrylamide Gel (Native-PAGE)

Native-PAGE was performed by Hiol *et al*. [17]. Materials for 10% concentrated Native-PAGE were 30% acrylamide solution; 0.8 % methylenebisacrylamide (Bis); 1.5 M tris HCl pH 8.8; 1.0 M. Tris HCl pH 6.8; N, N, N',N',tetramethyl-ethylenediamine (TEMED); 10% APS (amonium peroxidisulfate); 0.1% casein; 50% glycerol; A and B staining solutions; Tefco clear dry; Running buffer (1.5 g tris aminomethane; 7.2 g glycine, and 500 ml distilled water); 70 mm Advantec filter paper.

Determination of Vmax and Km

Kinetic parameters of the enzyme were determined by measuring the keratinase activity at different substrate concentrations. Lineweaver – Burk plot was drawn to determine the values of Km and Vmax of the enzyme [18].

Optimization of Enzyme Production

Keratinase Activity

Keratinase activity test was performed using keratin azure (Sigma-Aldrich, St. Louis, USA) as keratin substrate from wool and given azo stain. Keratinase activity test was performed based on Wang *et al*. [19]. Five hundred micro litres enzyme sample was incubated in 5 mg keratin azure solution in 500 µL 50 mM sodium phosphate buffer for 30 minutes at 30°C with constant agitation 180 rpm using shaker incubator. Enzyme reaction was stopped by adding 1 ml 10% TCA solution. The solution was put in ice for 30 minutes, then centrifuged at 13.000g for 5 minutes. The absorption of azo stain which was removed in supernatant was measured with 595 nm wavelength and compared with control tube. The control tube was given the same treatment except enzyme sample was replaced with sodium phosphate buffer.

The Effects of pH, Temperature, and Incubation Time on Keratinase Activity

Keratinase activity in purified enzyme was measured at pH 6; 7; 8; 9; and 10 using the following buffers: sodium acetate (pH 4,0-6,0), sodium phosphate (pH 7,0-8,0), and TrisNaOH buffer (pH 9.0-10.0). The optimum temperature was determined by incubation reaction combination at different temperatures, i.e. 25, 27, 29, 31 and 33°C. Beside temperature and pH, incubation time for keratinase characteristic was optimized for 30; 60; 90; 120 and 150 minutes [20].

Data Analysis

The results obtained from the production optimization were analyzed by analysis of variance (ANOVA) using IBM SPSS Statistics 25. Correlations among the variables were analyzed by Principal Component Analysis (PCA) using Minitab 18 Statistical Software.

RESULTS AND DISCUSSIONS

Characterization of Keratinase

SDS PAGE

B. thuringensis strain BRAW_PT, *B. aerius* BRAW_PB, and *B. firmus* strain BRAW_PI had similar molecular weights of enzyme between 72 kDa and 95 kDa. Electrophoresis method was used broadly in protein characterization, including determining molecular weight of

protein. The molecular weight of enzyme can be measured by calculating the molecular weight of protein which is electrophoresed by polyacrylamide sodium dodecyl sulfate (SDS-PAGE) gel and comparing it with molecular weight of standard protein. Bacteria from the genus *Bacillus*, generally secrete two types of extracellular peptidase, an alkaline peptidase and a neutral peptidase [19, 20]. This is in accordance with the results obtained by Mazotto *et al*. [21] Generally, all *Bacillus* spp. genus had keratinases with molecular weights between 13.8 and 140 kDa. The molecular weights of keratinase of the three isolates are presented in Figure 1. The keratinases were detected on various species of *Bacillus* spp. With molecular weight of 45 to 80 kDa on *B. subtilis* 1270, 15 to 100 kDa on *B. subtilis* 1273, and 63 to 140 kda on *B. licheniformis* 1274 [22]. Other extracellular keratinases such as on *B. subtilis* ks-1, *B. pumilus*, and *B. cereus* had molecules with masses of 25.4, 65, and 45 kDa [21, 23].

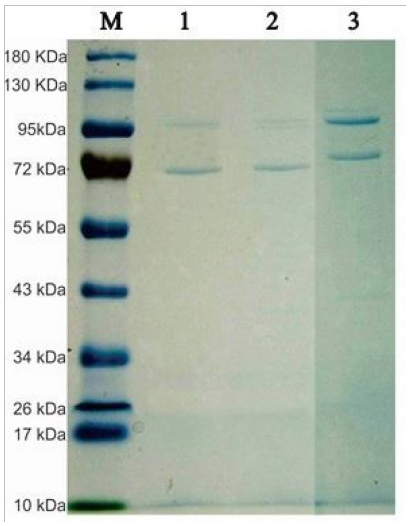

Figure 1. Molecular weight of keratinase from *B. thuringensis* BRAW_PT (1), *B. aerius* BRAW_PB (2), and *B. subtilis* BRAW_PI (3)

Native-PAGE

Native-PAGE analysis using casein substrate (Figure 2) demonstrated that the enzymatic extract obtained from three *Bacillus* strains was able to hydrolyze protein. Protein band of *B. thuringensis* BRAW_PT was very clear, followed by *B. aerius* BRAW_PB, while *B. firmus* BRAW_PI showed thin band. Native-PAGE method was used to determine protein bands, in this case protease activity of certain bacteria. Sattayasai [24] state that many proteins can be stained in gel by using their enzyme activity, native-PAGE are compatible with activity stains. Wilson and Walker [25] also stated that the sample in Native-PAGE process is not denaturized because it can make bonds in the secondary structure of protein to be destructed.

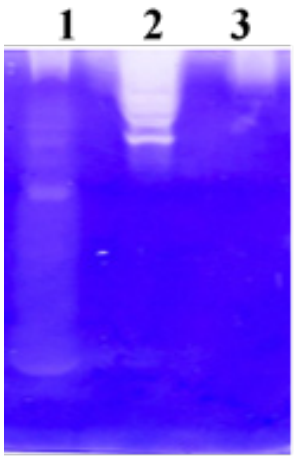

Figure 2. Native-PAGE results of enzyme keratinase from *B. thuringensis* BRAW_PT (1), *B. aerius* BRAW_PB (2), and *B. firmus* BRAW_PI (3)

Kinetics of Keratinase

The Lineweaver–Burk plot was represented against different concentrations of the substrate with the Michaelis–Menten plot (Fig. 3). The Michaelis constant (Km) of keratinase from *B. thuringensis* BRAW_PT, *B. aerius* BRAW_PB, and *B. firmus* BRAW_PI were found to be 1.09, 0.46, and 0.10 mg/ml, respectively and the maximum velocity of the reaction (Vmax) were 0.83, 7.29, and 37.4 mg/ml/min. Kinetic of enzyme was investigated to determine the enzyme reaction rate on different concentrations of the substrate through the Michaelis–Menten equation (Km). Keratinase produced by *Pseudomonas aeruginosa* KS-1 found higher Km which 1.66 mg/ml and Vmax which 3.1 mg/ml/min [26]. Purified keratinase from *B. thuringiensis* presented higher Km (5.97 mg/ml) [27]. The estimated Km and Vmax values for feather keratin were 6.6 mg/ml and 5.0 mg/ml/min, respectively [28].

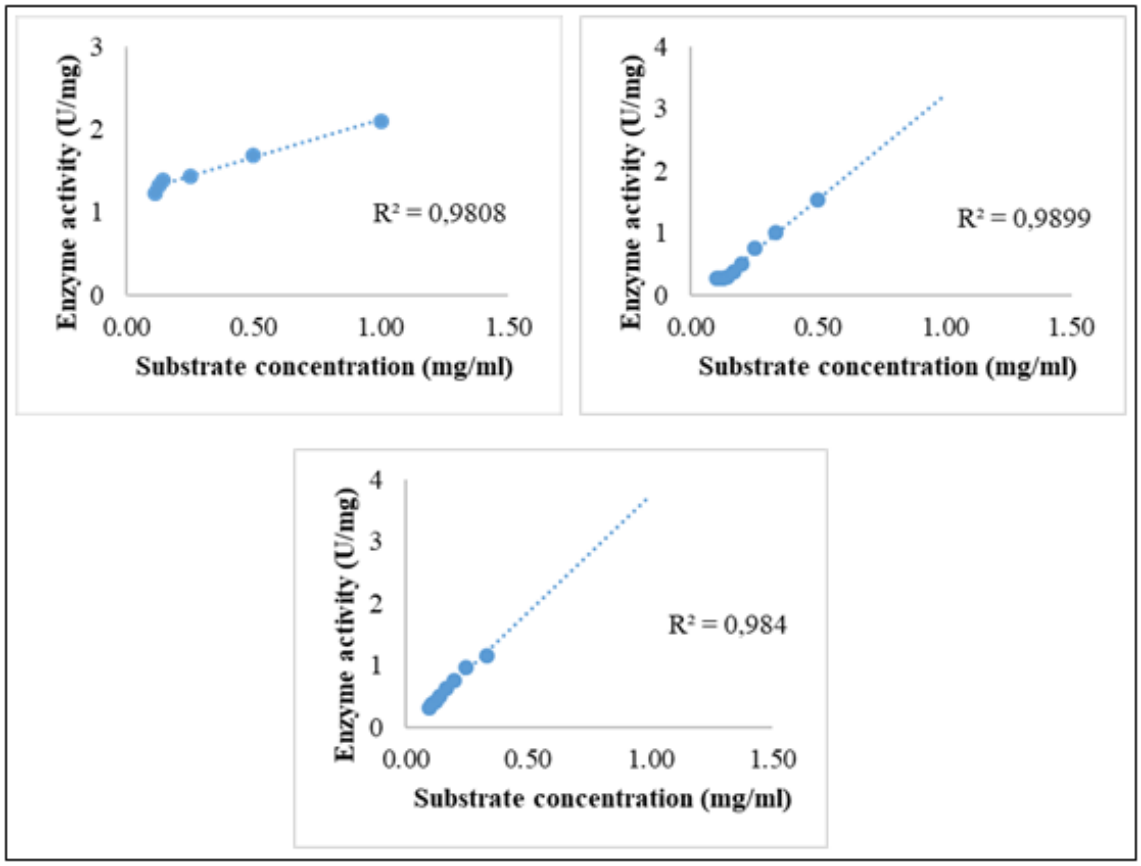

Figure 3. Graph of Line weaver - Burk plot of keratinase

Optimization of Keratinase Production

The effect of temperature on enzyme activity is depicted in Fig 4. The determination of optimum temperature of keratinase activity was performed by incubating at 25°C – 33°C. Enzyme from *B. aerius* BRAW_PB shows the highest enzyme activity at 29°C (9.39±0.05 U/mg). Enzyme from *B. firmus* strain BRAW_PI also had optimum activity at 29°C (8.25±0.10 U/mg). The optimum temperature of enzyme activity from these *Bacillus* sp stains were almost the same with the results of Balakumar *et al.* [29]. They state that *Bacillus subtilis* inoculated in medium and optimized the production at different temperature, and the increased production was identified at 30°C. However, enzyme from *B. thuringensis* BRAW_PT was optimum at 25°C, then its activity decreased when the temperature increased.

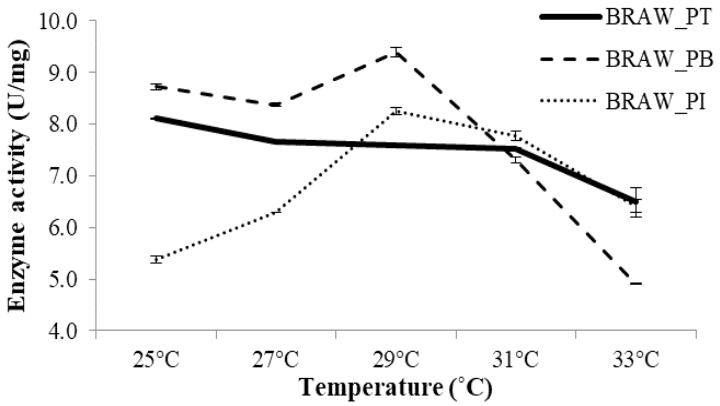

Figure 4. The effect of temperature on keratinase activity

Keratinase from *B. thuringensis* BRAW_PT and *B. firmus* BRAW_PI activities were optimal at pH 8 (Fig 5). However, keratinase activity of *B. aerius* BRAW_PB was optimal at pH in the range of 6-8. Selvam *et al*. [30] indicating that keratinase were produced by *Bacillus* sp at pH between 7 and 8. Keratinase from these bacteria strains can be categorized as alkaline protease because the optimum activity of the enzyme at alkaline pH. The result was almost similar to alkaline protease from APR-4 *Bacillus* sp which has optimum activity at pH 9 [23]. This result of keratinase activity of *B. aerius* BRAW_PB is in accordance with the optimum at pH 7.5 reported previously for azo keratin hydrolysis to hydrolyze fur with keratinase [31, 32]. Keratinases from *Meiothermus taiwanensis* WR-220 were also active in a broad range, which is between pH 4-11 [33].

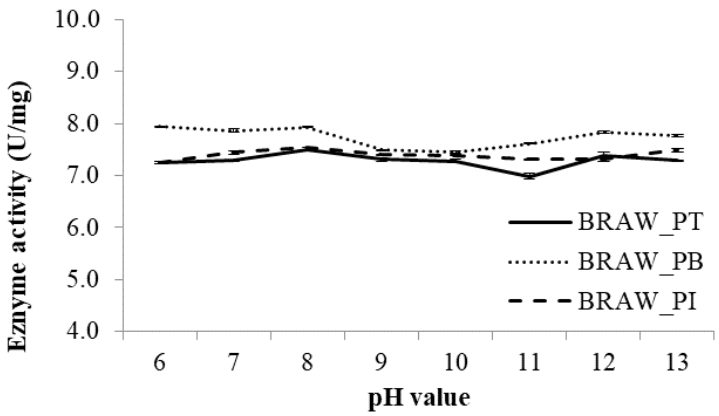

Figure 5. The effect of pH value on keratinase activity

Activity of all types of enzymes increased for 90 minutes, then it decreased by 9% after 90 minutes (Fig. 6). The highest activity is that of enzyme from *B. thuringensis* BRAW_PT (7.72±0.01 U/mg). Gupta *et al*. [28] have the same results with the optimum time incubation of keratinase from these bacteria strains, keratinase from *B. subtilis* stabilized up to 90 minutes, and decreased by 11% after 120 minutes. A different result was found in proteolytic enzyme of *B. subtilis* BLBc 11 [5] and commercial keratinase [34] that had stable activity for 120 minutes. Gessesse *et al*. [35] reported that enzyme from *B. pseudoformis* sp. was inactive after 20 minutes, moreover Ogino *et al*. [36] discovered proteolytic enzyme from *Bacillus* species that was inactive after only 10 minutes of incubation.

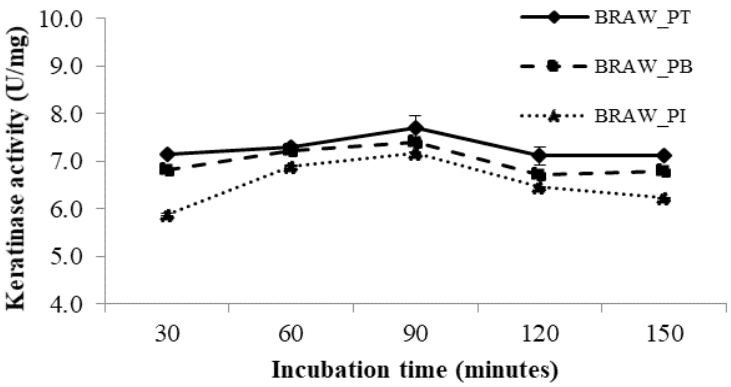

Figure 6. The effect of pH value on keratinase activity

Correlation among Variables Using Principle Component Analysis

Principal component analysis (PCA) is a statistical analysis method of combining several indexes into a few comprehensive ones. PCA is a calculation method allowing the reduction of variables, correlation among variables, and visualization data [37]. Reducing the amount of variables involves changing the initial set of variables into the new, reduced in number set of the so-called principal components [38]. In addition, the PCA makes the new variables be independent from each other, to achieve the purpose of simplification [39]. The variables that effected the principle component of enzyme activity are shown in Table 1. Temperature is the most effected to the first principle component (PC1), while the incubation time is the most effected second principle component (PC2). Table 1 provides the information about that there is a reducing variable, from 3 variables (temperature, pH value, and incubation time) to 2 variables (PC1 and PC2). The temperature is the most effected to the first principle component (PC1), while the incubation time is the most effected second principle component (PC2).

Table 1: Correlation between principle components

Variable	PC1	PC2
pH	0,555	-0,660
Temperature	0,643	-0,045
Time Incubation	0,527	0,750

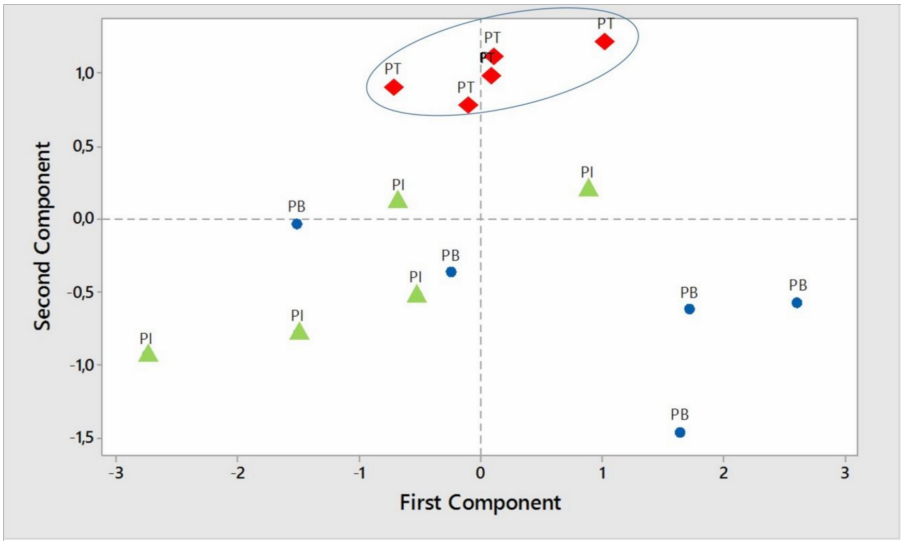

Figure 7. Score plot of type of bacterial producing keratinase strain. PT: Keratinase from *B. thuringensis* BRAW_PT; PB: Keratinase from *B. aerius* BRAW_PB; PI: Keratinase from *B. firmus* BRAW_PI

Moriya *et al*. [40] used PCA to discriminate the different enzymes. In this study, PCA showed a clear discrimination between keratinase from *B. thuringensis* BRAW_PT and other enzymes (Fig 6). Figure 7 showed the difference discriminant component of the variables of the bacterial strain. Keratinase from *B. thuringensis* BRAW_PT have a discriminant with other kinds of keratinase (from *B. aerius* BRAW_PB and *B. firmus* BRAW_PI).

CONCLUSIONS

B. thuringensis strain BRAW_PT, *B. aerius* BRAW_PB, and *B. firmus* strain BRAW_PI had similar molecular weights of enzyme between 72 kDa and 95 kDa. Protein band on native-PAGE of *B. thuringensis* BRAW_PT was very clear, followed by *B. aerius* BRAW_PB, while *B. firmus* BRAW_PI showed thin band. The Michaelis constant (Km) of keratinase from *B. thuringensis* BRAW_PT, *B. aerius* BRAW_PB, and *B. firmus* BRAW_PI were found to be 1.09, 0.46, and 0.10 mg/ml, respectively and the maximum velocity of the reaction (Vmax) were 0.83, 7.29, and 37.4 mg/ml/min. The optimum activity of keratinase from *B. thuringensis* BRAW_PT and *B. firmus* BRAW_PI was obtained at pH 8, while keratinase from *B. aerius* BRAW_PB at pH 6-8. Keratinase from *B. thuringensis* BRAW_PT has maximum activity at 25°C, whereas keratinase from *B. aerius* BRAW_PB and *B. firmus* BRAW_PI at 29°C. All keratinases are optimal at 90 minutes incubation. Keratinase has optimum activity at 29°C, pH 8 to 12, and 90 minutes of incubation time. The Principal Component Analysis (PCA) resulted some correlations among variables, and discriminated *B. thuringensis* BRAW_PT among other enzymes.

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