KINETIC AND THERMODYNAMIC CHARACTERIZATIONS OF THE PROTEASE FROM *Bacillus licheniformis* (ATCC 12759)

Vivi Mardina[*], Faridah Yusof[**], Md. Zahangir Alam[***]

[*]*Department of Biology, Faculty of Engineering, Samudra University, Langsa, Aceh, Indonesia, vmardina@unsam.ac.id*

[**]*Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, Kuala Lumpur, Malaysia, yfaridah@iium.edu.my*

[**]*Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, Kuala Lumpur, Malaysia, zahangir@iium.edu.my*

Email Correspondence : vmardina@unsam.ac.id

Received : July 21, 2020 Accepted : December 16, 2020 Published : December 31, 2020

Abstract: In this study, the kinetic of a thermo-stable extracellular protease produced by *Bacillus licheniformis* (ATCC 12759) cultured in skim latex serum fortified media was investigated. The enzyme was stable up to 65 ^{o}C after incubation for 60 min at pH 8. The Lineweaver-Burk exhibited v_{max} (maximum rate) of 37.037 U/mg min^{-1} and K_M (Michaelis-Menten constant) of 8.519 mg/mL. The activation energy (E_a) of casein hydrolysis and temperature quotient (Q_{10}) were found to be 4.098 kJ/mol and 1.038 - 1.034, respectively, at a temperature ranging from 35 ^{o}C to 65 ^{o}C. The results of the residual activity test allowed estimating activation energy for irreversible inactivation of the protease (denaturation) which was approximately $E_{a(d)}$ = 62.097 kJ/mol. The thermodynamic parameters for the enzyme irreversible denaturation were as follow enthalpy (59.286 $\leq \Delta H^*_d \geq$ 59.535 kJ/mol), Gibbs free energy (97.375 $\leq \Delta G^*_d \geq$ 93.774kJ/mol), and entropy (-122.797 $\leq \Delta S^*_d \geq$ -101.992 kJ/mol). These thermodynamic parameters inferred that the thermo-stable proteases could be potentially important for industrial application, for example, in the detergent industries.
Keywords: *Bacillus licheniformis*; kinetic; protease; skim latex serum; thermostability

Abstrak: Pada penelitian ini, kinetika protease ekstraseluler termo-stabil yang diproduksi oleh *Bacillus licheniformis* (ATCC 12759), yang dikultur dalam media yang diperkaya serum lateks skim diselidiki. Enzim stabil hingga 65 ^{o}C setelah diinkubasi selama 60 menit pada pH 8. Lineweaver-Burk menunjukkan v_{max} (laju maksimum) adalah 37.037 U/mg min^{-1} dan K_M (konstanta Michaelis-Menten) 8.519 mg/mL. Energi aktivasi (E_a) dari hidrolisis kasein dan suhu quotient (Q_{10}) ditemukan masing-masing sebesar 4.098 kJ/mol dan 1.038 - 1.034, pada suhu yang berkisar dari 35 ^{o}C hingga 65 ^{o}C. Hasil uji aktivitas residu memungkinkan estimasi energi aktivasi untuk inaktivasi ireversibel dari protease (denaturasi) yang kira-kira Ea (d) = 62.097 kJ/mol. Parameter termodinamika untuk denaturasi enzim ireversibel adalah sebagai berikut entalpi (59.286 $\leq \Delta H * d \geq$ 59.535 kJ / mol), energi bebas Gibbs (97.375 $\leq \Delta G * d \geq$ 93.774kJ / mol) dan entropi (-122.797 $\leq \Delta S * d \geq$ -101.992 kJ / mol). Parameter termodinamika pada penelitian ini menyimpulkan bahwa protease termo-stabil dapat berpotensi penting untuk aplikasi industry seperti dalam industri deterjen.
Kata kunci: *Bacillus licheniformis*; protease; serum lateks skim; termostabilitas

Recommended APA Citation :

Mardina, V., Yusof, F., & Alam. M. Z. (2020). Kinetic and Thermodynamic Characterization of the Protease From *Bacillus licheniformis* (ATCC 12759). *Elkawnie*, *6*(2), 178-188. https://doi.org/10.22373/ekw.v6i2.7530

Introduction

Proteases or proteolytic enzymes are natural catalysts that act in the degradation of protein by hydrolyzing peptides bonds (Rao *et al*., 1998; Aguilar *et al*., 2019; Jisha *et al*., 2013). They usually require metals ions and or cofactors for the maximal efficiency. The catalytic activity of protease is affected by temperature, pH, the concentration of substrate, and the presences of similarly sized and shaped molecules called inhibitors (Mienda *et al*., 2014; Bhunia *et al*., 2012). As a catalyst, protease has a pivotal role in application areas such as food, textile, leather, environmental pollution abatement, pharmaceutical, and healthcare product (Bhunia *et al*., 2012). In order to perform their application effectively, the kinetic and thermodynamic studies of protease need to be investigated. The kinetic analysis of enzymes constitutes the information about activation and deactivation of enzymes and describes the capability of the enzyme to catalyze and enhance in a process. In biotechnological processes, studies of deactivation enzyme are essential because it provides the data to illustrate the enzyme stability and control over the activation processes. Moreover, the deactivation studies support investigates the correlation between structure and function of particular enzymes in which will provide valuable physical insight into the structure and function of enzymes (Prajapati *et al*., 2014; Bhuni *et al*., 2013).

However, finding the protease with novel and excel properties such as having stability and activity at high pH and temperature are desirable due to providing an economic advantage such as minimized enzyme consumption (Souza *et al*., 2015), enhancing the mass-transfer and reducing the substrate viscosity during the progress of hydrolysis substrate. Furthermore, thermostability of the enzyme at 60^{o}C or above can avoid or reduce microbial contamination, particularly bioreactor containing immobilized enzyme (Nigam *et al*., 2013). Many researchers reported proteases that possess stability and activity at high pH and temperature using agri-industrial waste such as groundnut oil cake (Elumalai *et al*., 2020), soy fibre (Marin *et al*., 2018), and dried powder of wheat bran, rice bran, sugarcane bagasse, and sugarcane molasses (Rathod and Pathak, 2014). On the other hand, in the best of our knowledge, only a few studies about proteases production using skim latex serum as a basal medium for microbial growth (protease *Bacillus licheniformis*, ATCC 12759). Thus, in this study, we reported the heat-stable microbial protease from *Bacillus licheniformis*, ATCC 12759 that was the culture in much waste (skim latex serum) by different reporting method that is the deactivation kinetics of protease. As a consequent, the objective of this study was to examined kinetic parameters (Michaelis constant, K_M and maximum

velocity, v_{max}) and its relation toward thermodynamic parameters (activation and deactivation energy, enthalpy, entropy and Gibbs free energy) of the protease from *B. licheniformis* (ATCC 12759).

Materials and Methods

Microorganism and Cultured Condition

The *Bacillus licheniformis* (ATCC 12759) used in this study was ordered from American Type Culture Collection (ATCC) USA. They were cultured in the nutrient broth media and routinely re-cultured every 12 days on Nutrient Agar (NA) medium at 37 ^{o}C, then stored at 4 ^{o}C (Mardina *et al.,* 2018). As suggested in our previous optimization study (Mardina *et al.,* 2015), the production of the protease was conducted using the prepared media containing the skim latex serum (70 %, v/v), galactose (1 %, w/v) and Luria Bertani (LB) (1 %, w/v). One hundred millilitres of the modified media was then inoculated with 4 % (v/v) of the inoculums ($3x10^9$ cells (CFU/mL) that were previously prepared in LB broth). The prepared started was cultured at 35 ^{o}C with a specific of pH 7, 75 rpm of rotation and 24 h for agitation and incubation period. All the liquid state fermentations were performed in triplicates. The extraction of protease from the production medium was carried out by centrifugation (10,000xg) for 10 min at 4 ^{o}C. The obtained cell-free supernatant (called crude enzyme) was stored for protease activity determination.

Assay of Protease Activity

The produced protease activity was confirmed using a casein substrate which was previously described by in our previous work (Mardina *et al.,* 2015). An aliquot of 2.5 mL of the casein solution 1 % (w/v) was mixed to the protease and followed by 2.5 mL trichloroacetic acid (TCA, 0.11 M) and then incubated in the water bath for 10 min at 37 ^{o}C. Subsequently, the mixture was allowed at room temperature for 45 min. 1 mL supernatant was pick up from centrifugation process (10,000 xg for 10 min at 4 ^{o}C), then subjected to the mixture of 2.5 mL Na_2CO_3 (0.5 M) and 0.5 mL Folin reagent (0.1 N). The average of triplicate optical density at the wavelength of 660 nm was calculated for the determination of protease activity with the equation:

$$\text{Protease activity (U/mL)} = \frac{\mu\text{g of Tyrosine release x reaction volume}}{\text{sample vol x reaction time x vol assayed}} \quad \ldots\ldots\ldots\ldots(1)$$

The quantity of enzyme that liberates 1 µg tyrosine per millilitre in 1 min under the assay condition was defined as one unit of protease activity.

Kinetic Parameters Determination

K_M and v_{max} of the enzyme were calculated through a double reciprocal (*Lineweaver-Burk*) plot of the protease activity versus casein concentrations (2 – 30 mg/mL). The calculation was carried out at 37^{o}C and pH 8. The protease assay was performed using equation (1). In order to read the sample absorbance, 2.5 mL Na_2CO_3 and 0.5 mL Folin reagents were used to colour the substrate. After homogenically mixed, the substrate was tested in a specific absorbance reading of 660nm in every 1 minute and repeated for three times. The obtained data were then averaged to obtain the curve by plotting Δ660 nm versus time. The slope was calculated using the equation (2) and considered as the rate of reaction. The value of quality was converted using L-tyrosine standard curve and expressed in U/mg, and the concentration of casein [S] was reported in mg/mL.

$$m = \frac{(y1-y2)}{x1-x1} \text{.....(2)}$$

Thermodynamic Parameters Determination

Determining the energy activation and temperature coefficient (Q10)

The energy required for the activation was determined using methods developed by Souza *et al*. (2015), Castro and Sato (2014), and Aguilar *et al*. (2019). First, the protease was incubated on 1 % of casein solution at various temperatures (35 to 65 ^{o}C) for 10 min. The correlation of the rate constants with temperature was expected to follow the Arrhenius law where E_a was estimated from the slope of the plot of 1/T (in Kelvin) vs natural log (*ln*) of the enzyme activity, E_a = -slope x R, R is the gas constant with the value of 8.314 JK^{-1} $mol^{-1,}$ and T is the absolute temperature (K) (Castro and Sato, 2014; Pogaku *et al*. (2012). The effect of temperature on the rate of reaction was expressed as (Q_{10}). The temperature quotient was defined as a measure of the rate increases as a consequence of increasing the temperature by 10 ^{o}C and determined by the equation (Eq. 3) given by Castro and Sato (2014) and Aguilar *et al.* (2019).

$$Q_{10} = \text{antilog}_{\varepsilon} \left(E_a \text{ x} \frac{10}{RT^2}\right) \text{ .....(3)}$$

Kinetic parameters for thermal inactivation

The inactivation process of protease due to thermal effect (denaturation) was calculated by first-order kinetics as a function of the time (Eq. 4 and Eq. 5). The protease was incubated without substrate at various temperatures (range 35 ^{o}C – 75 ^{o}C) for 60 min. The residual activity was measured at 20, 40 and 60 min.

$$\frac{dA}{dt} = -K_d A \text{ .....(4)}$$

so that,

$$In\left[\frac{A_t}{A_o}\right] = -K_d\, t \quad \text{.....(5)}$$

Where t was time, A_0 was the initial protease activity, A was the protease activity at a particular time t, and K_d was deactivation rate constant which is determined from the slopes gained by plotting ln [A/A_0] or *ln* [residual activity] versus t (time) at an assay temperature (Castro and Sato, 2014; Pogaku *et al*. (2012). From this, the deactivation energy ($E_{a(d)}$ could be determined based on the Arrhenius equation as given by Prajapati *et al*. (2014) and Bhunia *et al*.(2013).

$$K_d = K_o\, exp\left[-\frac{E_{a(d)}}{RT}\right] \quad \text{.....(6)}$$

The half-life ($t_{1/2}$) of the enzyme was the time when its residual activity reaches 50 % of the initial activity and calculated, as shown in Eq. 7 (Castro and Sato, 2014).

$$t_{(1/2)} = \frac{\ln(0.5)}{K_d} \quad \text{.....(7)}$$

Furthermore, D-value of the enzyme that was related to K_d can be defined as the time required for a 90 % reduction in the initial enzyme activity at a specific temperature and was calculated as shown in Eq. 8 (Prajapati *et al.*, 2014).

$$D = \frac{2.303}{K_d} \quad \text{.....(8)}$$

The remaining thermodynamic parameters of the enzyme were determined according to the equation given by Souza *et al*. (2015) and Aguilar *et al.* (2019).

$$\Delta H^*_d = E^*_d - RT \quad \text{.....(9)}$$

$$\Delta G^*_d = -RT \ln\left[\frac{k_d h}{K_B T}\right] \quad \text{.....(10)}$$

$$\Delta S^*_d = \frac{\Delta H^*_d - \Delta G^*_d}{T} \quad \text{.....(11)}$$

where ΔH^*_d, ΔG^*_d, ΔS^*_d, are the changes of enthalpy, Gibbs free energy and entropy between the denatured and the active form of the enzyme respectively; K_B and *h* are the Boltzmann and Planck constants, respectively. The Boltzmann's constant (*R/N*) is 1.38 x 10^{-23} JK^{-1}, *T* is the absolute temperature (K), *h* is Planck's constant with the value of 6.626 x 10^{-34} Js, *N* is Avogadro's number (6.02 x 10^{-23} mol^{-1}) and R is gas constant at 8.314 JK^{-}

Results and Discussion

Kinetic Studies

The kinetic parameters K_M and v_{max} were estimated at pH 8 and 37 ^oC varying S_0 from 2 – 30 mg/mL. The result of *Lineweaver-Burk* of the enzyme showed Michaelis-menten-type kinetics with v_{max} = 37.037 U/mg min^{-1} and K_M = 8.519 mg/mL, with good correlation (R^2 = 0.993). According to Souza *et al*. (2015), the activity of an enzyme depends on the type of substrate used and the purity of the enzyme. Thus it isn't easy to compare. However, the K_M value of this result that is quite closed by other reports for protease from *Bacillus licheniformis* (5 mg/mL) (Nasdeem *et al*., 2013) and alkaline protease from *Lactobacillus brevis* (3.33 mg/mL) (Ola *et al*., 2012) demonstrated the good affinity for the substrate casein.

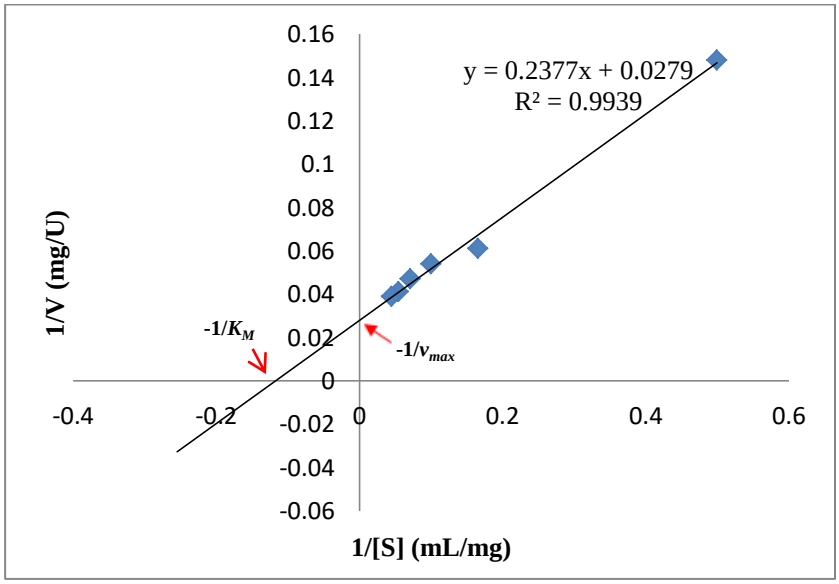

Figure 1. Lineweaver-Burk plot for determining of K_M and V_{max} (casein as substrate).

Thermodynamic Studies

Proteases are biocatalysts that minimize the activation energy (E_a), which was required to convert the substrates into the product. In this study, the activation energy barrier was carried out at pH 8.0, S_0 = 100 mg/mL and varying temperature from 35 ^oC to 65 ^oC. In order to reduce the effect of temperature on the loss of protease activity due to denaturation, only the initial of protease activity (v_0) was occupied into consideration as suggested by Souza *et al.* (2015). The apparent activation energy for the hydrolysis of casein value was calculated from the slope of the Arrhenius plot (Fig. 2) and found to be 4.098 kJ/mol with satisfactory correlation (R^2 = 0.852). The lower value of E_a thermodynamically demonstrated the feasibility of casein hydrolysis reaction by protease at a lower temperature. This result was lower than those of similarly reported proteases; for

instance, E_a value of 19.03 kJ/mol, 15.53 kcal/mol (64.98 kJ/mol), 10.59 kcal/mol, and 44.30 kJ/mol for protease from *Aspergillus foetidus*, *Yersinia ruckeri, and Bacillus clausis and Bacillus licheniformis* LBA 46 respectively. If the low E_a value in this study, it might indicate that only small amount energy was required to create the activated complex of casein hydrolysis and the reaction is also highly reactive (Souza *et al.*, 2015; Kezia *et al.*, 2011; Aguilar *et al.* (2019).

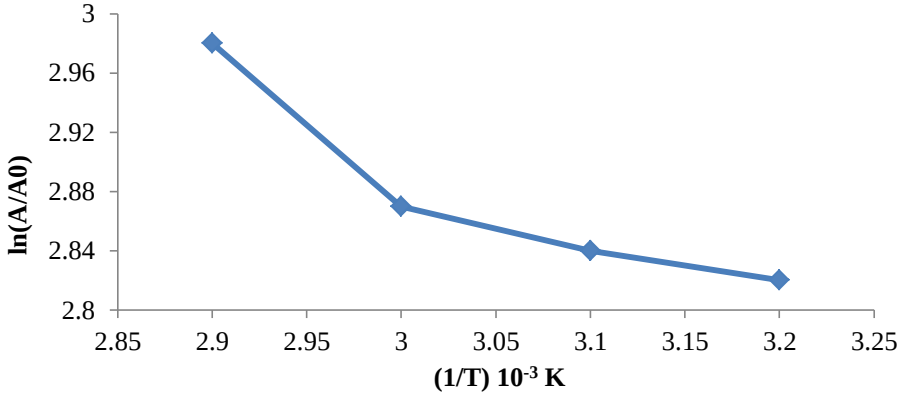

Figure 2. Arrhenius plots for determining the activation energy (E_a) of *B. licheniformis* protease in the temperature range from 35 – 65 ^{o}C.

The Q_{10} values were determined to infer the influence of reaction that being examined was controlled by temperature and or other factors. Samanta *et al*. (2014) and Aguilar *et al.* (2019) stated that enzymatic reaction generally had Q_{10} value between 1 and 2 and any deviations from these value indicate the involvement of some factors other than the temperature in regulating the reaction rate. A Q_{10} value of 2 recommended doubling of the rate of reaction with every 10 ^{o}C increase in temperature. The Q_{10} value of the *Bacillus licheniformis* protease in this study showed in 1.038 – 1.034 at a temperature ranging from 35 ^{o}C to 65 ^{o}C. This finding was supported by Castro and Sato (2014), who reported protease from *A. oryzae* had a Q_{10} value of 1.64 – 1.53 at the temperature range of 30 to 55 ^{o}C. Other study reported the Q10 in the range of 1.59-1.87 for protease from *Bacillus licheniformis* LBA 46 (Aguilar *et al.* (2019).

In term of the economic parameter in industries, $t_{1/2}$ and *D* values were significant, due to if higher their value, it means higher enzyme thermostability. The thermodynamic results of these assessments were summarized in Table 1 that exhibited the K_d value of 0.0015 min^{-1}, t $_{1/2}$ of 7.77 h and a *D* value of 25.59 h at 55 ^{o}C. These values were according to the trend of the enzymes reported in the literature, such as protease from *Aspergillus foetidus* (K_d = 0.018 h^{-1}); t $_{1/2}$ = 37.6 h; T = 55 ^{o}C) (Souza *et al*., 2015) and A*spergillus oryzae* ((K_d = 0.071 min^{-1}); t $_{1/2}$ = 97.63 h; T = 57.2 ^{o}C) (Castro RJS and Sato, 2014), thus this produced enzyme acted to be thermostable.

Table 1. Thermodynamic parameters for inactivation of the produced protease

T (^oK)	K_d (min^{-1})	$t_{1/2}$ (h)	D (h)	R^2	ΔH^*_d (kJ/mol)	ΔG^*_d (kJ/mol)	ΔS^*_d (J/mol K)
308.15	0.0002	57.75	191.9	0.8322	59.535	97.375	-122.797
318.15	0.0013	8.88	29.5	0.9616	59.452	95.663	-113.817
328.15	0.0015	7.77	25.59	0.9597	59.369	95.366	-109.696
338.15	0.0023	5.02	16.69	0.9968	59.286	93.774	-101.992

Semi-log plot of In k_d versus 1/T recommended for calculating the activation energy of protease denaturation or energy required for thermal inactivation (E^*_d) that was found to be 62.097 kJ/mol (R^2 = 0.7938). This value is similar to those reported for alkaline protease from *Bacillus licheniformis* (32.79 – 41.69 kJ/mol) (Bhunia *et al*., 2013) and *Aspergillus fumigatus* (69 kJ/mol) (Martinez *et al.* 2011) and *Bacillus licheniformis* LBA 46 (144.50 kJ/mol) Aguilar *et al.* (2019). E^*_d means enzymes require a similar amount of energy to be inactivated.

Besides, there was the relation between E^*_d in Eq. (7) and the activation enthalpy of denaturation (ΔH^*_d), which was another essential thermodynamic parameter expressing the total amount of energy necessary to denature the protease; thus, large and positive values of E^*_d and ΔH^*_d should be associated with high enzyme thermostability. The protease in this study had a ΔH^*_d at 65 ^{o}C (59.29 kJ/mol) (Table 1) which was much higher than alkaline protease from *B. licheniformis* (30.06 – 39.97 kJ/mol) (Bhunia *et al*., 2013).

The other reliable predicting parameters for evaluating the stability of enzyme due to thermal denaturation include the Gibb free energy (ΔG^*_d) and the activation entropy (ΔS^*_d). The ΔG^*_d and ΔS^*_d of irreversible thermal inactivation (a 55 ^{o}C) of *B. licheniformis* protease was equal to 93.774 kJ/mol and -101.992 J/mol K respectively (Table 1). An increase in temperature would decrease in free energy while there was a slight increase in entropy. However, a slight decrease in ΔG^*_d in this study exhibited resistance of the enzyme to denaturation (thermostable). The lower value of ΔG^*_d of protease from *B. licheniformis* indicates its higher affinity to catalyse soluble casein hydrolysis (Souza *et al.*, 2015). Furthermore, the low entropy values in this study also showed compaction of the enzyme molecule and such changes could arise from the formation of charged particles around the enzyme molecule and the ordering of solvent molecule (Prajapati *et al.*, 2014).

Conclusions

The protease obtained from *Bacillus licheniformis* (ATCC 12759) cultured in skim latex serum basal media potentially used in several industries such as detergent industry because it has high stability up to 65 ^{o}C after incubation for 60 min at pH 8.

Acknowledgements

The authors thank to the Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia for providing financial and laboratory supports.

References

Aguilar, J. G., de Castro, R. J. S., Sato, H.H. (2019). Alkaline protease production by *Bacillus licheniformis* LBA 46 in a bench reactor: effect of temperature and agitation. *Brazilian Journal of Chemical Engineering*, 36(2), 615 - 625. Doi.org/10.1590/0104-6632.20190362s20180014.

Bhunia, B., Basak, B., Dey, A. (2012). A review on production of serine alkaline protease by *Bacillus sp. Biochemical Technology*, **3**(4), 448-457.

Bhunia, B., Basak, B., Mandal, T., Bhattacharya, P., Dey, A. (2013). Effect of pH and temperature on stability and kinetics of novel extracellular serine alkaline protease (70 kDa). *International journal of biological macromolecules*, 54, 1 – 8. Doi.10.1016/j.ijbiomac.2012.11.024

Castro, R. J. S., & Sato, H. H. (2014**).** Protease from *Aspergillus oryzae*: Biochemical characterization and application as a potential biocatalyst for production of protein hydrolysates with antioxidant activities. *Journal of food processing*, 1 – 11. https://doi.org/10.1155/2014/372352

Elumalai, P., Lim, J. M., Park, Y.J., Cho, M., Shea, P.J., Oh, B. T. (2020). Agriculture waste materials enhance protease production by *Bacillus subtilis* B22 in submerged fermentations under blue light-emitting diodes. *Bioprocess and biosystem engineering*, 43(2020), 821 – 830.

Jisha, V. N., Smitha, R. B., Pradeep, S., Sreedevi, S., Unni, K. N., Sajith, S., Priji, P., Josh, M. S., Benjaminn, S. (2013). Versatility of microbial proteases *Advances in Enzyme Research*, 1 (3), 39 – 51. https://doi.org/10.4236/aer.2013.13005

Kezia, D., Chandrakala, G., Prasanthi, V., Naidu, S. V., Rao, M. N. (2011). Influence of different factors on production of purified protease by *Bacillus subtitis* DKMNR. *Biotechnology*, 2(3), 73 – 85. Doi.10.4236/aer.2013.13005

Mardina, V., & Yusof, F. Chapter 15, Skim Latex Serum as an Alternative

Nutrition for Microbial Growth. In: Amid, A., Sulaiman, S., Jimat, D., Azmin, N. (eds). (2018). Multifaceted Protocol in Biotechnology. *Springer Nature, Singapore*, 179-196. Doi.10.1007/978-981-13-2257-0_15

Mardina, V., Yusof, F., Alam, M. Z. (2015). Statistical optimization of physichochemical factors for protease production by *Bacillus licheniformis* on skim latex serum fortified media. *Journal of Engineering Science and Technology*, 6, 42 – 52.

Marin, M., Artola, A., Sanchez, A. (2018). Production of proteases from organic waste by solid state fermentation: downstream and zero waste strategies. *Biotechnology*, 8 (205), 1 – 16. https://doi.org/10.1007/s13205-018-1226-y.

Martinez, R. H., Sanchez, G. G., Bergmann, C. W., Corral, O. L., Dominguez, A. R., Ochoa, S. H., Gonzalez, C. R., Prado-Barragan, L. A. (2011). Purification and characterization of a thermodynamic sable serine protease from *Aspergillus fumigatus*. *Process biochemistry*, 46, 2001–2006. Doi.10.1016/j.procbio.2011.07.013.

Mienda, B. S., Yahya, A., Galadima, I. A., Shamsir, M. S. (2014). An overview of microbial proteases for industrial applications. *Pharmaceutical, Biological and Chemical Sciences*, 5(1), 388–386. https://doi.org/10.1371/journal.pone.0188724

Nasdeem, M, Qazi JI, Syed Q, Gulsher M. (2013). Purification and characterization of an alkaline protease from *Bacillus licheniformis* UV-9 for detergent formulations *Songklanakarin J. of Sci. and Tech.*, 35 (2), 187–195.

Nigam, P.S. (2013). Review Microbial enzymes with special characteristics for biotechnological applications. *Biomolecules*, 3, 597–611. Doi:10.3390/biom3030597

Ola, T. O. F. & Oladakun, D. O. (2012). Partial purification and characterization of a thermostable alkaline protease from isolate *Lactobacillus brevis Malaysian Journal of Microbiology*, 8 (1), 1 – 5. Doi.10.21161/mjm.28310

Pogaku, R., Raman, J. K., Ravikumar, G. (2012). Evaluation of activation energy and thermodynamic properties of enzyme-catalysed transesterification reactions. *Advances in Chemical Engineering and Science*, (2), 150 – 154. http://dx.doi.org/10.4236/aces.2012.21018

Prajapati, V. S., Trivedi, U. B., Patel, K. C. (2014). Kinetic and thermodynamic characterization of glucoamylase from *Colletotrichum sp.* KCPI: *Indian journal microbiology*, 54(1), 87 – 93. Doi: 10.1007/s12088-013-0413-0

Rao, M. B., Tanksale, A. M., Ghatge, M. S., Deshpande, V. V. (1998). Molecular and biotechnologycal aspects of microbial proteases. *Microbiology and*

Molecular Biology Reviews, **62** (2), 579 – 635. Doi.10.1128/MMBR.62.3.597-635.1998

Rathod, M. G. & Pathak, P. (2014). Wealth from waste: optimized alkaline protease production from agro-industrial residues by *Bacillus alcalophilus* LW8 and its biotechnological applications. *Journal of Taibah University for Science*, 8 (4), 307 – 314. https://doi.org/10.1016/j.jtusci.2014.04.002.

Samanta, S., Das, A., Halder, S. K., Jana, A., Kar, S., Mohapatra, P. K. D, Pati, B. R., Mondal, K. C. (2014). Thermoodynamic and kinetic characteristics of an α-amylase from *Bacillus licheniformis* SKB4. *Acta Biologica Szegediensis*, 58 (2), 147 – 156. http://www2.sci.u-szeged.hu/ABS.

Souza, P. M., Aliakbarian, B., Filho, E. X. F., Magalhaes, P. O., Junior, A. P., Converti, A., Perego, P. (2015). Kinetic and thermodynamic studies of a novel acid protease from *Aspergillus foetidus*. *International Journal of Biological macromolecules*, 81, 17–21. http://doi.org/10.1016/j.ijbiomac.2015.07.043.