Malaysian Applied Biology (2023) 52(4): 27-34
https://doi.org/10.55230/mabjournal.v52i4.j171

Research

Effect of Temperature and Fermentation Time on Protease Production Using *Decapterus macarellus* Fish Waste

Noralia Mohd Alias, Chua Gek Kee, Chew Few Ne, Noraziah Abu Yazid, Rozaimi Abu Samah, and Siti Hatijah Mortan*

Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang Al-Sultan Abdullah, Lebuh Persiaran Tun Khalil Yaakob, 26300 Kuantan, Pahang, Malaysia
*Corresponding author: hatijah@umpsa.edu.my

ABSTRACT

The fish processing industries generate huge amounts of by-products which cause serious environmental and health problems. The environmental problems related to waste disposal can be reduced as the low cost of fish by-products contains nutrients that can enhance microbial growth and are useful for enzyme production. This research aims to investigate the effect of temperature and fermentation time on protease production from fish waste hydrolysate by using the *Bacillus* strain (*Bacillus cereus*). The pre-treatment and fish waste hydrolysate were carried out and continued with the production of protease. The effect of fermentation time was studied every 4 h for 72 h while the effect of temperature was investigated at temperatures ranging from 30 °C to 60 °C. The results showed the maximum protease production of 45.63 U/mL at 48 h of fermentation time and 44.908 ± 6.14 U/mL at a temperature of 50 °C. The current study provides insight into the effects of cultivation conditions on protease production from local fish waste industries for further optimization study to enhance protease production.

Key words: *Bacillus cereus*, *Decapterus macarellus*, fish waste hydrolysate, protease, submerged fermentation

Article History
Accepted: 24 July 2023
First version online: 31 October 2023

Cite This Article:
Mohd Alias, N., Kee, C.G., Ne, C.F., Yazid, N.A., Samah, R.A. & Mortan, S.H. 2023. Effect of temperature and fermentation time on protease production using *Decapterus macarellus* fish waste. Malaysian Applied Biology, 52(4): 27-34. https://doi.org/10.55230/mabjournal.v52i4.j171

Copyright
© 2023 Malaysian Society of Applied Biology

INTRODUCTION

Protease is a hydrolysis class of enzymes that perform the proteolytic action to hydrolyze the peptide bonds of a polypeptide chain (Rashmi & Dubey, 2017). Proteases are being commercialized for nearly 60% of the total enzyme industrial market. Many applications of proteolytic enzymes can be found in industries such as pharmaceuticals, detergents, and food. Proteases have been synthesized by different kinds of microbes such as fungi, bacteria, and yeasts, and by some plants and animal tissues. Microbes were selected as a preferred source of protease enzymes due to their rapid growth and less space requirement for cultivation (Shafee *et al*., 2005).

Many investigators have searched some methods of producing microbial proteases using low-cost media. Based on their findings, fish processing wastes such as fish meat wastes offer good potential for this purpose (Wang & Yeh, 2006; Vázquez *et al*., 2008; Haddar *et al*., 2010). Fish wastes which are rich in carbon and nitrogen contents can be a great alternative for the production of valuable added products such as enzymes, gelatin, and collagen. The elements in the fish waste consist of 58% protein, 19% fat, and minerals such as magnesium, potassium, iron, zinc, phosphorus, manganese, and copper act as cofactors which important for the growth of microbes for various metabolic activities (Ramakrishnan *et al*., 2013). Currently, protease has been produced by waste from several fish species such as Sardinella, carps, anchovy, mullet, red snapper, tuna, goby, and ray fish (Esakkiraj *et al*., 2013; Ramakrishnan *et al*., 2013; Ramkumar *et al*., 2018).

Production of protease was influenced by nitrogen sources as different bacterial strains may utilize different substrates as nitrogen sources for effective protease production (Mehta *et al*., 2006). A few types of nitrogen sources have been used by several industries for the growth

process of bacteria in producing proteases such as skim milk powder, peptone, beef extract, urea, fish hydrolysate, and tryptone (Gupta & Khare, 2007). Some studies were using fish waste hydrolysate as a substrate for protease production as fish was known to contain higher nitrogen content. This substrate is widely used in protease processing industries such as food, casein, soy, and gelatin (Al-Abdalall & Al-Khaldi, 2016).

The growth and protease production by various bacteria is greatly affected by the culture conditions such as temperature, pH, agitation time, and fermentation time (Cui *et al*., 2015; Asha & Palaniswamy, 2018). To obtain high activity of protease, the best condition of these parameters needs to be achieved. However, temperature and fermentation time are among the factors that highly contribute to protease production. The activity of the enzyme will be reduced by its thermal denaturation when at higher temperatures and can decrease the hydrolysis rate due to insufficient energy provided by the temperature for the alcalase (commercial protease) to react with the substrates (Salwanee *et al*., 2013). Enzymes will become inactivated at higher temperatures due to the unfolding of their structure (Soares da Silva *et al*., 2018). Aside from that, enzyme activity also will be decreased due to a prolonged fermentation period, though it does not significantly affect the growth of microorganisms (Ul-Haq *et al*., 2006). The study of the best fermentation conditions that can enhance the ability of the bacteria to withstand drastic changes will become advantageous for the protease production process (Cui *et al*., 2015).

The protease production economy is based on the type of substrate as they play an important role in minimizing the production cost. Thus, the low-cost, easily obtainable, and regularly available substrate is needed to cut down the cost of production (Barros *et al*., 2013). In this study, the low cost of raw material which is fish waste from the local fish food product business is used to produce protease by using the hydrolysation process and undergo submerged fermentation. The effect of the temperature and fermentation time on protease production is being compared experimentally.

MATERIALS AND METHODS

Materials

The fish waste used in this research was gathered from all parts of *Decapterus macarellus* locally known as ‘*Ikan Selayang*’ collected from CCM 115 Keropok Lekor Industry, Tanjung Lumpur, Kuantan. The *Bacillus cereus* (ATCC 10876) was obtained from Central Lab, Universiti Malaysia Pahang, Gambang, Kuantan.

Preparation of fish waste hydrolysate

Decapterus macarellus or ‘*Ikan Selayang*’ waste sample which mostly the head part of the fish was being processed into fine powders before the hydrolysation process. Fish wastes were washed with water until clean to remove the remaining blood and dirt before being steamed with water for about 20 min. The steamed fish wastes were pressed using a small cloth and dried in the oven at 80 °C for 24 h. The dried fish wastes were ground into fine powder and the acid hydrolysation process was carried out by mixing the powdered fish waste with distilled water at a 1:2 (w/v) ratio. 2N HCl was mixed in the mixture until pH 3.3 and was agitated in the shaking water bath at 85 °C. The acidic hydrolysis was terminated using 2N NaOH at pH 7. The neutralized mixture was centrifuged and the supernatant was collected (Mukhtar & Ul-Haq, 2012; Contesini *et al.*, 2018). To ensure the consistency of the hydrolysate for each fish waste batch, the supernatant was freeze-dried to obtain a solid form of hydrolysates by the method developed by Zainuddin *et al.* (2020).

Preparation of media and cultures

Bacillus cereus (ATCC 10876) was cultivated on nutrient agar plates at 30 °C for 24 h (Mushtaq *et al*., 2014). The culture was stored at 4 °C until further use.

Protease production

The selected proteolytic isolates and the isolates were grown separately in 20 mL production media for each flask containing 18 mL supernatant hydrolysate, 2 g/L ammonium sulphate (NH4)2SO4, 1 g/L dipotassium phosphate K_2HPO_4, 1 g/L monopotassium phosphate KH_2PO_4, 0.4 g/L magnesium sulfate heptahydrate $MgSO_4.7H_2O$, 0.01 g/L manganese sulfate $MnSO_4$, 0.01 g/L iron (II) sulfate heptahydrate $Fe_2SO_4.7H_2O$, 1 g/L yeast extract and 10% (v/v) inoculum, maintained at 35 °C for 48 to 72 h in a shaking incubator at 200 r.p.m (Ramkumar *et al*., 2016).

Effect of temperature on protease production

For the effect of temperature, the microbial broth was incubated at five different temperatures (30, 40, 50, 55 & 60 °C). The fermented broth was analyzed for proteolytic activity, total protein content, and cell biomass concentration after 48 h incubation time (Ovissipour *et al*., 2012). All activity test was performed in duplicate.

Effect of fermentation time on protease production

The microbial broth was incubated under submerged fermentation for 72 h and the protease activity, total protein content, and cell biomass concentration were analyzed every 4 h (Ovissipour *et al*., 2012).

Protease assay

1 mL of the supernatant from the fermentation was added to 5 mL of a 2% (w/v) casein solution and incubated for 1 h at 50 °C and 100 rpm. The sample was added with 5 mL of 15% (w/v) trichloroacetic acid, TCA to terminate the reaction. The samples were centrifuged for 10 min at 10000 r.p.m and 4 °C and 0.5 mL of an aliquot of the supernatant was added to the alkaline reagent before the addition of 0.5 mL of 25% (v/v) Folin-Ciocalteu phenol reagent. The solution was incubated in a dark place at room temperature for 1 h. The absorbance was measured at 700 nm with tyrosine as a standard (Ul-Haq *et al*., 2006). All assays were performed in triplicate. One unit (U) of protease activity is defined as the amount of enzyme that releases l µg tyrosine per minute under standard assay conditions. The protease activity was calculated using Equation 1:

$$\text{Protease activity } \left(\frac{U}{mL}\right) = \frac{C \times 15}{1 \times t \times 0.5}$$

Where C is the concentration of nitrogen source (µg/mL), 15 is the total volume of chemicals used during protease assay (mL), t is the time of incubation (min) and 0.5 is the volume of supernatant (mL).

Total protein content determination

The total protein content was measured by a Bradford method using 2% (w/v) bovine serum albumin (BSA) as a standard protein (Hamza, 2017). 0.05 mL of cell-free supernatant was mixed with 1.5 mL Bradford reagent and the intensity of color developed was read at 595 nm by using Thermo Scientific™ Evolution™ 350 UV-Vis Spectrophotometer (Tryte Technology, Hunan). All samples were tested in triplicate.

Determination of cell biomass concentration

The cell biomass concentration was determined using dry weight measurement with a slight modification (Asker *et al*., 2013). The absorbance of 1.5 mL fermented medium was measured at 600 nm by using the same UV-Vis Spectrophotometer.

RESULTS AND DISCUSSION

Effect of temperature on protease production

In the present study, the protease activity was observed at five different temperatures; 30°C, 40°C, 50 °C, 55 °C and 60 °C. Figure 1 shows the protease activity gradually increases at lower temperatures (30 °C to 50 °C) and then started to decline at a higher temperature of 55 °C and 60 °C. The highest recorded protease activity was 44.908 ± 6.14 U/mL at 50 °C. This temperature is relatively high in comparison with other proteases produced from fishery waste using *Bacillus* sp. where most of the optimum temperature is between 30 – 37 °C (Sellami-Kamoun *et al*., 2010; Souissi *et al*., 2010; Ghorbel-bellaaj *et al*., 2012). Protease from Sardinella, catfish, cuttlefish, rayfish species wastes using *Bacillus thuringiensis* produced optimum protease at 37 °C while other fish waste from anchovy (using *Bacillus pumilus*) and tuna (using *Bacillus licheniformis* NK) obtained the highest protease activity at 30 and 40 °C (Gupta *et al*., 2012; Esakkiraj *et al*., 2013; Ramkumar *et al*., 2016). The energy metabolism and enzyme synthesis in bacteria were controlled by the temperature. *Bacillus cereus* is a mesophilic bacteria that can grow in moderate temperatures, neither too cold nor too hot, with an optimum growth range. This is the reason why most of the proteases from Bacillus sp. have an optimum temperature between 30 – 37 °C.

However, similar to our results, some other research on protease from fishery waste and using *Bacillus* sp., also obtained the highest protease activity at thermophilic conditions. Ramkumar *et al*. (2018) observed maximum protease activity of 75.52 U/mL at 46 °C for protease using gut waste *Sardinella longiceps* by *Bacillus licheniformis* while Annamalai *et al*. (2013) discovered the highest activity of 3413 U/mL at 50 – 55 °C for protease from shrimp shell by *Bacillus holidurans* (Annamalai *et al*., 2013; Ramkumar *et al*., 2018).

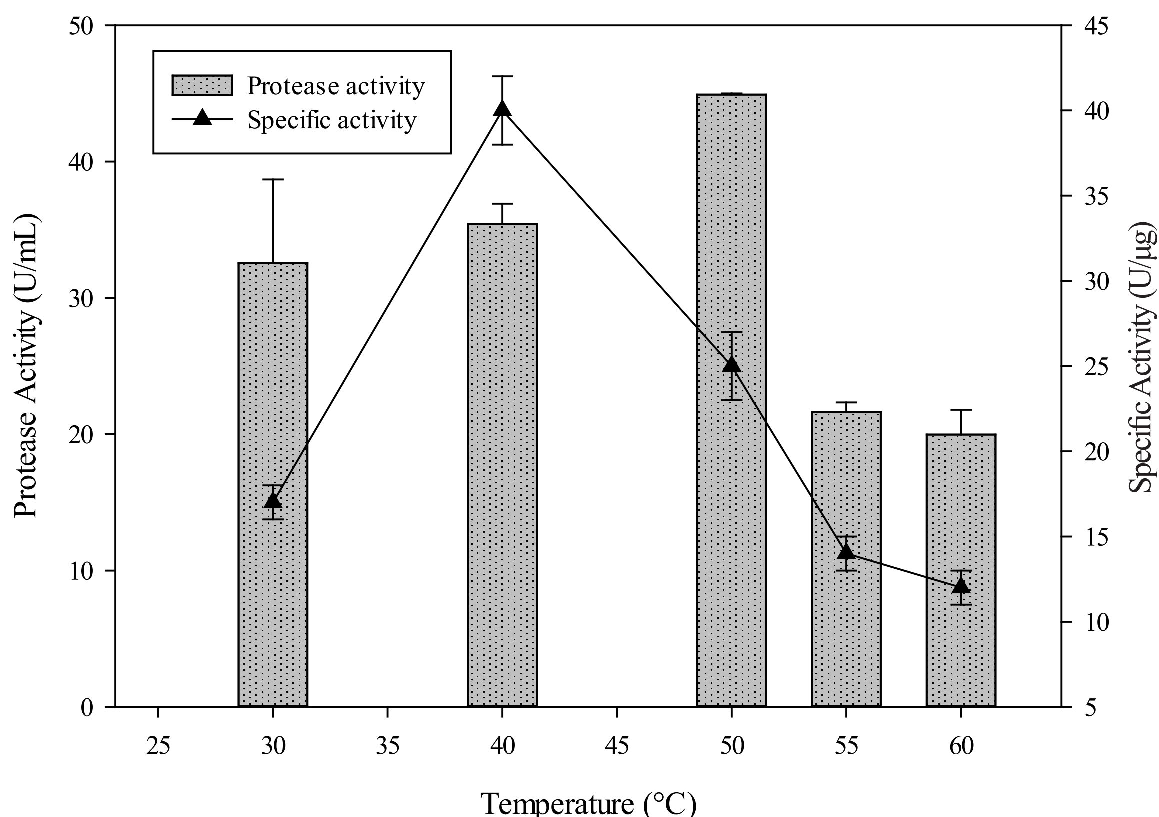

Fig. 1. Protease activity at different temperatures.

This could be further explained by the results we got for cell biomass concentration at different temperatures as shown in Figure 2. As can be seen from the figures, the biomass concentration was in inverse relationship with the protease activity depicted in Figure 1, where lower biomass concentration was observed at higher protease activity. This indicates that the protease activity in this study is not directly related to the biomass concentration. On the contrary, the highest protein content at 5.699 (µg/mL) was also obtained at 50 °C as the protease activity. Similar trends were observed for total protein in Figure 2 and protease activity in Figure 1, where higher total proteins were observed at thermophilic conditions. Regardless of that, as most of the previous studies used different types of microorganisms than us, we cannot certainly say that the species of fish waste used influenced the optimum temperature of the protease production.

Effect of fermentation time on protease production

In this study, the protease activity was observed every 4 h for a total of 72 h of fermentation time. Figure 3 shows that maximum protease activity of 45.63 U/mL was exhibited at 48 h of fermentation time. This is in agreement with other research on protease from fishery waste where the highest protease activity is observed at the late exponential phase or early stationary phase. Protease from head and viscera *Sardinella aurita* (8473 U/mL), the fish scale of *Labeo rohita* (28150 U/mL), shrimp (4000.6 U/mL) and crab shell (1398.2 U/mL) wastes also exhibited the highest protease activity at 48 hr of fermentation time (Sellami-Kamoun *et al*., 2010; Maruthiah *et al*., 2015; Harikrishna *et al*., 2017).

The protease enzyme synthesis in *Bacillus* species was controlled by mechanisms operative during the transition state between stationary and exponential growth phases (Martínez-Medina *et al*., 2019). Figure 4 shows that the protease activity was increased during the exponential phase while the culture was metabolically active from 4 h up to 48 h, thereafter the growth was unstable and leading to the death phase. The enzyme activity gradually decreased from 52 h to 72 h. Prolonged incubation time will decrease the enzyme activity, however, the growth of the microorganism was not significantly affected (Huang *et al*., 2010).

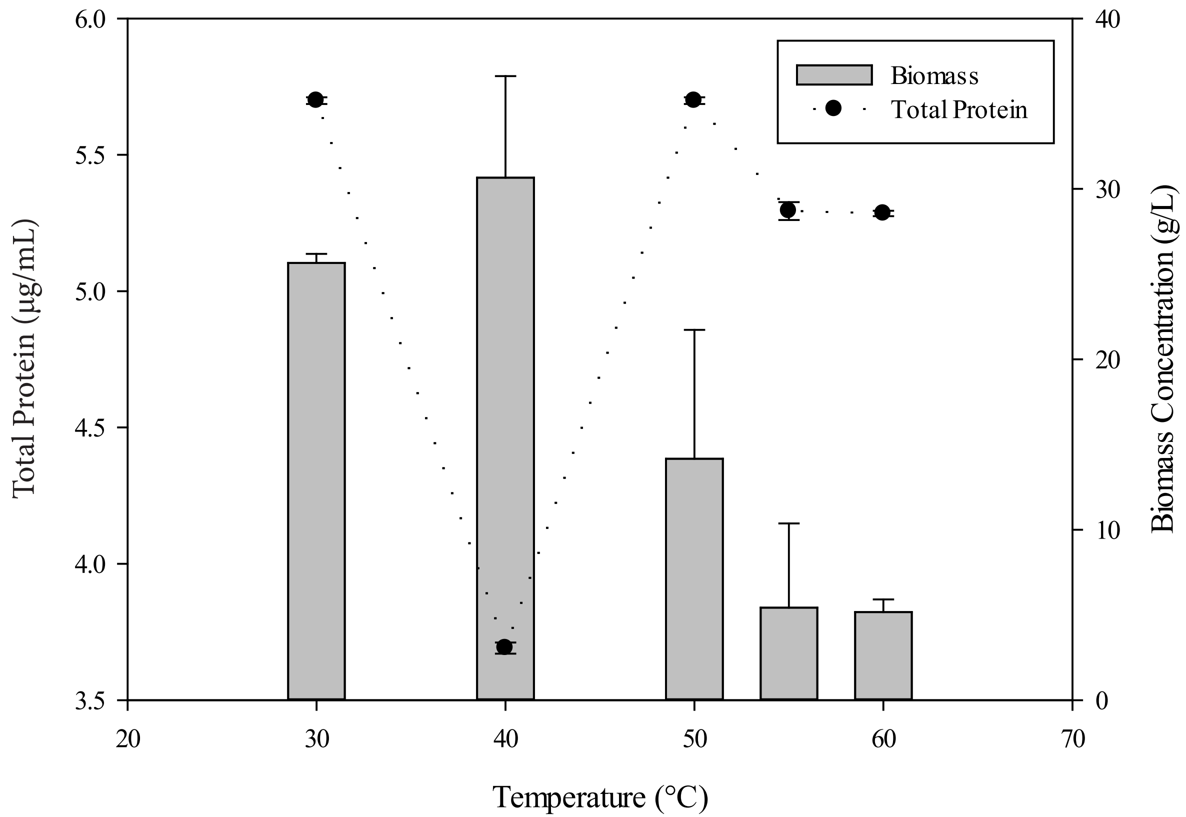

Fig. 2. Biomass concentration and protein content at different temperatures.

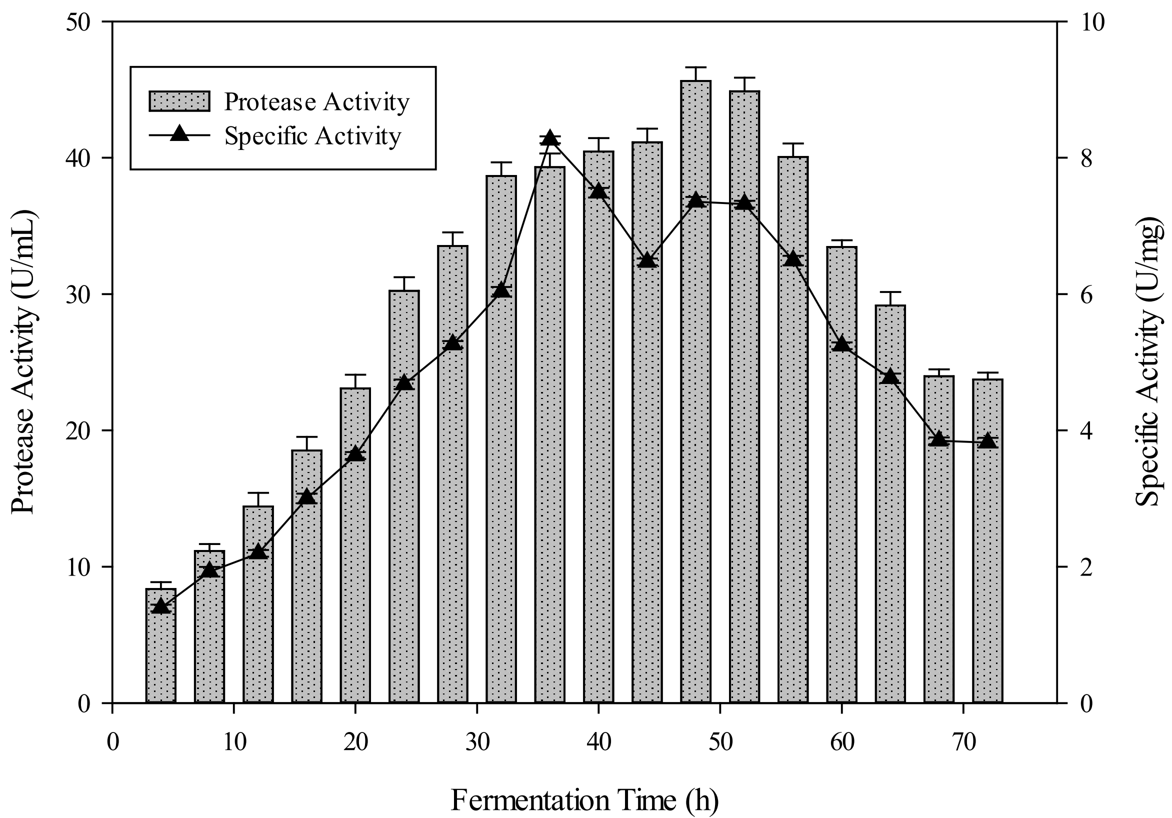

Fig. 3. Protease activity at different fermentation times.

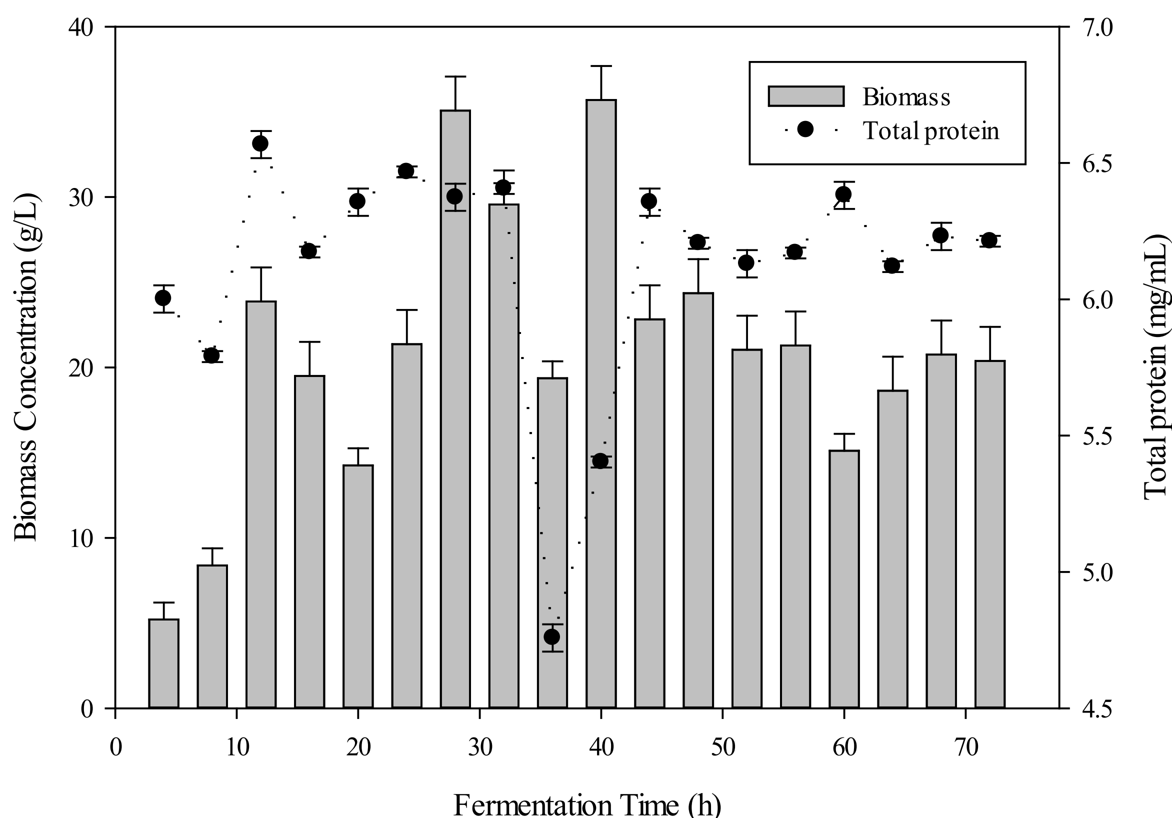

Fig. 4. Biomass concentration and protein content at different fermentation times.

Based on Figure 4, the protein content and cell biomass concentration at 48 h were quite low at 6.206 µg/mL and 24.353 g/L, respectively. Figures 3 and 4 show that the highest protease activity is not observed at the highest protein content and cell biomass concentration. At a fermentation time of 36 h, the protease activity was quite high at 39.32 U/mL while protein content and cell biomass concentration were lowest at 4.76 µg/mL and 19.36 g/L, respectively. However, looking into the overall trends of the protease activity and the biomass concentration in Figure 3 and 4, both figures show lower protease activities at lag and exponential phases, the highest activity at the end of the exponential phase, and constant activities at the stationary phase. Most of the protease produced by *Bacillus* species occurred during the late exponential phase and it was related to the high rate of protein turnover during endospores formation (Chu, 2007). Moreover, protease production can be improved due to high lipid content that is influenced by nutritional factors which are acting as inducers (Triki-Ellouz *et al*., 2003).

CONCLUSION

Fish waste hydrolysate is one of the nitrogen sources that can produce protease. The highest amount of protease activity obtained by 1% (w/v) fish waste hydrolysate was (44.908 ± 6.14 U/mL) at a temperature of 50 °C. The highest protease activity 45.63 U/mL by 1% (w/v) fish waste hydrolysate was recorded at 48 hr. Thus, this study shows that at best conditions of temperature and fermentation time, local fish waste as a nitrogen source has the potential to produce a high amount of protease that can be used in various applications such as in pharmaceuticals, detergents, and food.

ACKNOWLEDGEMENTS

The authors would like to acknowledge with much appreciation Universiti Malaysia Pahang for the financial assistance through Flagship Research Grant RDU182204-2.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Al-Abdalall, A.H. & Al-Khaldi, E.M. 2016. Production of alkaline proteases by alkalophilic *Bacillus subtilis* during recycling animal and plant wastes. African Journal of Biotechnology, 15(47): 2698-2702. https://doi.org/10.5897/AJB2016.15430

Asha, B. & Palaniswamy, M. 2018. Optimization of alkaline protease production by *Bacillus cereus* FT 1 isolated from soil. Journal of Applied Pharmaceutical Science, 8(2): 119-127.

Asker, M.M.S., Mahmoud, M.G., El Shebwy, K. & Abd el Aziz, M.S. 2013. Purification and characterization of two thermostable protease fractions from *Bacillus megaterium*. Journal of Genetic Engineering and Biotechnology, 11(2): 103-109. https://doi.org/10.1016/j.jgeb.2013.08.001

Barros, F.F.C., Simiqueli, A.P.R., de Andrade, C.J. & Pastore, G. M. 2013. Production of enzymes from agroindustrial wastes by biosurfactant-producing strains of *Bacillus subtilis*. Biotechnology Research International, 2013: 103960. https://doi.org/10.1155/2013/103960

Chu, W.H. 2007. Optimization of extracellular alkaline protease production from species of *Bacillus*. Journal of Industrial Microbiology and Biotechnology, 34(3): 241-245. https://doi.org/10.1007/s10295-006-0192-2

Contesini, F., Melo, R. & Sato, H. 2018. An overview of *Bacillus* proteases: from production to application. Critical Reviews in Biotechnology, 38(3): 321-334. https://doi.org/10.1080/07388551.2017.1354354

Cui, H., Yang, M., Wang, L. & Xian, C.J. 2015. Identification of a new marine bacterial strain SD8 and optimization of its culture conditions for producing alkaline protease. PLoS ONE, 10(12): e0146067. https://doi.org/10.1371/journal.pone.0146067

Esakkiraj, P., Maruthiah, T., Mohideen Askar Nawas, P., Immanuel, G. & Palavesam, A. 2013. Solid state protease production by *Bacillus thuringiensis* AP - CMST using trash fish meal as substrate. Journal of Pure and Applied Microbiology, 7(4): 1-7.

Gupta, A. & Khare, S.K. 2007. Enhanced production and characterization of a solvent stable protease from solvent tolerant *Pseudomonas aeruginosa* PseA. Enzyme and Microbial Technology, 42(1): 11-16. https://doi.org/10.1016/j.enzmictec.2007.07.019

Gupta, R.K., Prasad, D., Sathesh, J., Naidu, R.B., Kamini, N.R., Palanivel, S. & Gowthaman, M.K. 2012. Scale-up of an alkaline protease from *Bacillus pumilus* MTCC 7514 utilizing fish meal as a sole source of nutrients. Journal of Microbiology and Biotechnology, 22(9): 1230-1236. https://doi.org/10.4014/jmb.1203.03021

Haddar, A., Fakhfakh-Zouari, N., Hmidet, N., Frikha, F., Nasri, M. & Kamoun, A.S. 2010. Low-cost fermentation medium for alkaline protease production by *Bacillus mojavensis* A21 using hulled grain of wheat and sardinella peptone. Journal of Bioscience and Bioengineering, 110(3): 288-294. https://doi.org/10.1016/j.jbiosc.2010.03.015

Hamza, T.A. 2017. Bacterial protease enzyme: safe and good alternative for industrial and commercial use. International Journal of Chemical and Biomolecular Science, 3(1): 1-10.

Huang, G., Dai, D., Hu, W. & Jiang, J. 2010. Optimization of medium composition for thermostable protease production by Bacillus sp. HS08 with a statistical method. African Journal of Biotechnology, 7(8): 1115-1122.

Martínez-Medina, G.A., Barragán, A.P., Ruiz, H.A., Ilyina, A., Hernández, J.L.M., Rodríguez-Jasso, R.M., Hoyos-Concha, J.L. & Aguilar-González, C.N. 2019. Fungal proteases and production of bioactive peptides for the food industry. In: Enzymes in Food Biotechnology: Production, Applications, and Future Prospects. M. Kuddus (Ed.). Academic Press. pp. 221-246. https://doi.org/10.1016/B978-0-12-813280-7.00014-1

Mehta, V.J., Thumar, J.T. & Singh, S.P. 2006. Production of alkaline protease from an alkaliphilic actinomycete. Bioresource Technology, 97(14): 1650-1654. https://doi.org/10.1016/j.biortech.2005.07.023

Mukhtar, H. & Ul-Haq, I. 2012. purification and characterization of alkaline protease production by a mutant strain of Bacillus subtilis. Pakistan Journal of Botany, 44(5): 1697-1704.

Mushtaq, Z., Adnan, A., Mehmood, Z. & Syed, Q. 2014. Process optimization by response surface methodology for extracellular alkaline protease production from *Bacillus subtilis*. Pakistan Journal of Botany, 46(2): 699-704.

Ovissipour, M., Safari, R., Motamedzadegan, A. & Shabanpour, B. 2012. Chemical and biochemical hydrolysis of persian sturgeon (*Acipenser persicus*) visceral protein. Food and Bioprocess Technology, 5(2): 460-465. https://doi.org/10.1007/s11947-009-0284-x

Ramakrishnan, V., Ghaly, A., Brooks, M. & Budge, S. 2013. Enzymatic extraction of aminoacids from fish waste for possible use as a substrate for production of jadomycin. Enzyme Engineering, 2(2): 1-9.

Ramkumar, A., Sivakumar, N. & Victor, R. 2016. Fish waste-potential low cost substrate for bacterial protease production: a brief review. The Open Biotechnology Journal, 10(Suppl-2, M6): 335-341.

https://doi.org/10.2174/1874070701610010335
Ramkumar, A., Sivakumar, N., Gujarathi, A.M. & Victor, R. 2018. Production of thermotolerant, detergent stable alkaline protease using the gut waste of *Sardinella longiceps* as a substrate: optimization and characterization. Scientific Reports, 8(1): 12442 (2018). https://doi.org/10.1038/s41598-018-30155-9
Rashmi, P. & Dubey, S.K. 2017. Characterization of protease production efficiency of bacteria present in the poultry farm soil. International Journal of Science and Research (IJSR), 6(1): 1025-1028. https://doi.org/10.21275/ART20164246
Salwanee, S., Wan Aida, M., Mamot, S., Maskat, M.Y. & Ibrahim, S. 2013. Effects of enzyme concentration, temperature, pH and time on the degree of hydrolysis of protein extract from viscera of tuna (*Euthynnus affinis*) by using alcalase. Sains Malaysiana, 42(3): 279-287.
Shafee, N., Aris, S.N., Zaliha, N., Rahman, A., Basri, M. & Salleh, A.B. 2005. Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus* strain 146. Journal of Applied Sciences Research, 1(1): 1-8.
Soares da Silva, O., Lira de Oliveira, R., de Carvalho Silva, J., Converti, A. & Souza Porto, T. 2018. Thermodynamic investigation of an alkaline protease from *Aspergillus tamarii* URM4634: A comparative approach between crude extract and purified enzyme. International Journal of Biological Macromolecules, 109: 1039-1044. https://doi.org/10.1016/j.ijbiomac.2017.11.081
Triki-Ellouz, Y., Ghorbel, B., Souissi, N., Kammoun, S. & Nasri, M. 2003. Biosynthesis of protease by Pseudomonas aeruginosa MN7 grown on fish substrate. World Journal of Microbiology and Biotechnology, 19(1): 41-45. https://doi.org/10.1023/A:1022549517421
Ul-Haq, I., Mukhtar, H. & Umber, H. 2006. Production of protease by *Penicillium chrysogenum* through optimization of environmental conditions. Journal of Agriculture & Social Sciences, 2(2): 23-25.
Vázquez, J.A., Docasal, S.F., Prieto, M.A., González, M.A.P. & Murado, M.A. 2008. Growth and metabolic features of lactic acid bacteria in media with hydrolysed fish viscera. An approach to bio-silage of fishing by-products. Bioresource Technology, 99(14): 6246-6257. https://doi.org/10.1016/j.biortech.2007.12.006
Wang, S.L. & Yeh, P.Y. 2006. Production of a surfactant- and solvent-stable alkaliphilic protease by bioconversion of shrimp shell wastes fermented by Bacillus subtilis TKU007. Process Biochemistry, 41(7): 1545-1552. https://doi.org/10.1016/j.procbio.2006.02.018
Zainuddin, F.H., Chua, G. K., Chew, F N., Samah, R A., Mortan, S.H. & Yazid, N.A. 2020. Effect of type of nitrogen sources and concentration on protease production: Fish waste hydrolysate as a potential low cost source. IOP Conference Series: Materials Science and Engineering, 991(1): 012063. https://doi.org/10.1088/1757-899X/991/1/012063