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Evaluation of the Potential of Fungal Acid Proteases of *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* sp. to Produce Shrimp-Waste Protein Hydrolysates with Degraded Antigenic Proteins

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Abstract

The waste produced by the shrimp industry can be a great source for the recovery of important bioactive compounds with potential applications in food industry. The present study aimed to characterize extracted proteases of *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* sp. to be utilized as potential sources to produce shrimp waste protein hydrolysates with degraded antigenic proteins. The extracellular fungal acid protease enzymes were extracted under solid substrate fermentation and partially characterized the enzyme activities,

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concerning the reaction temperature and pH of the reaction medium. Then, the capabilities of extracted fungal protease enzymes to reduce the potential allergens of shrimp waste protein hydrolysates were examined by SDS-PAGE. After incubation of all three fungal species at 37 °C and pH 3.0 for 5 days, the highest protease yield, 527.40 µg/mL was achieved from *A. flavus*. The optimum temperature for *A. flavus* for the highest protease activity was 45 °C, while for other two microorganisms the optimum temperature was 50 °C. The optimum pH values for all three proteases were pH 3. According to the SDS-PAGE protein hydrolyzing patterns, the crude acid protease extracted from *A. flavus* was the best protease to degrade the potential antigenic proteins over the other two acid proteases extracted from *A.niger* and *Penicillium* sp., while all three enzymes had shown the potential to reduce the allergenic capacity of shrimp waste proteins through enzymatic hydrolysis.

Keywords: *Aspergillus flavus*; *Aspergillus niger*; Fungal protease; *Penicillium* sp.; Shrimp waste; Solid-state fermentation

Introduction

Proteases account for nearly 60% of the global enzyme industry due to the diverse range of commercial applications in many industries (Rao *et al*. 1998). Fungi species are highly exploited since they produce varied extracellular enzymes and are able to grow on low-cost substrates (Gupta *et al*., 2002). The fungal extracellular protease enzymes are reported to be extracted as acid, alkaline and neutral proteases. The acid proteases are extensively utilized in the food industry (Mukhtar, 2009). Shrimp solid wastes, head, shell and tail portions, have generated many environmental problems. Traditional waste treatment methods are based on chemical techniques which cause environmental pollution, depletion of resources and upsurge the associated costs. Better economic use of shrimp waste would minimize the environmental problems and generate added profits for the industry.

As reported by FAO and WHO, crustaceans are one of eight main allergenic foods and, the crustacean allergic cases have been increased annually (Khanaruksombat *et al*., 2014). The shrimp muscle protein tropomyosin (34-38

kDa), is identified as the most important heat-stable allergen. Antigenic proteins are extensively studied on shrimp muscle though studies on shrimp waste portions were under-discussed. Consequently, it is important to produce shrimp waste with degraded potential antigenic proteins for future applications in the food industry. Therefore, the present study aimed to produce and characterize the fungal proteases extracted from *A. flavus*, *A. niger,* and *Penicillium* sp. as potential sources to produce shrimp waste protein hydrolysates with degraded antigenic proteins.

Materials and Methods

Materials

Three fungal species, *A. flavus*, *A. niger,* and *Penicillium* sp. which were identified under standard protocols during a preliminary study were applied in the present experiments. *Penaeus monodon* including the head, shell and tail were purchased from local market and stored at freezing temperature (-18 °C). The raw white *samba* rice of commercial-grade was purchased from the local market. The standard method described by Lakshman *et al.,* (2010) was followed to produce the fungal protease enzymes by solid-state fermentation. The raw white *samba* rice was used as the solid substrate based on the standard protocol followed by Lakshman *et al.,* (2010) using polished rice for the similar purpose.

Preparation of solid substrate for fungal culture

The solid-state fermentation was conducted to produce enzyme by soaking raw *samba* rice for 18 hours in 500 mL Erlenmeyer flasks. The 30 g substrates were put into the Erlenmeyer flasks, and then, they were plugged with cotton wool. Then they were sterilized at 121 °C for 20 min in an autoclave. The Erlenmeyer flasks were allowed to cool to room temperature.

Solid-state fermentation of fungal culture

The soaked *samba* rice was inoculated with the fungal spore suspensions. Firstly, sterilized 0.005% Dioctyl sodium sulphosuccinate (10 mL) was added to fungal culture. Then the fungal spores were scratched using an inoculating

needle. In order to prepare the homogenized spores suspensions, the test tubes were shaken thoroughly. Then 1 mL each from the homogenized spore suspensions of *A. lavus, A. niger* and *Penicillium* sp. were transferred to the pre-sterilized Erlenmeyer flasks which contained soaked *samba* rice. Then they were incubated at the optimum temperature and pH level in the fermentation media for 5 days. The flasks were shaken two times per day and the fermentation was triplicated.

Extraction of fungal acid protease enzymes

On the fifth day of incubation, the fermented rice was mixed with citrate/phosphate buffer (pH 3) in the ratio of 30 mL of buffer with 10 g of rice, and the resultant mixture was kept at 4 ℃ while shaking for 1 hour. Then it was filtered using a Whatman filter paper No. 44, and the filtrate was centrifuged at 3000 rpm for 15 minutes at 4 °C. The crude enzyme was collected from the supernatant.

Optimization of fermentation temperature and pH

The protease enzyme production conditions were optimized by incubating the fermentation flasks at different temperatures (20, 28, 37, 45, 50 and 60 °C) at neutral pH for 5 days. Once the optimum temperature was found at neutral pH, the optimum incubation pH was studied at different levels (2.0, 2.5, 3.0, 4.5 and 5.0) for 5 days of incubation. The used buffer systems included, Citrate-sodium phosphate buffer of pH 5.0-7.0, lactate buffer of pH 3.0-3.5 and Sodium acetate buffer of pH 2.0-2.5.

Enzyme activity of extracted proteases

The universal protease activity assay was conducted as explained by Lakshman *et al*., (2010) using casein as the substrate. Firstly 2% casein solution (0.25 mL) was added to vials, and then, they were allowed to equilibrate in a water bath at 50 °C for about 10 minutes. After that, 0.025 mL volume of isolated crude enzyme was added to two of the test sample vials, but not the blank.

After 10 minutes of incubation at 50 °C, the reaction was terminated by adding 0.4 $molL^{-1}$ trichloroacetic acids (0.5 mL). The supernatant was collected by centrifugation (at 3000 rpm for 5 minutes) of the terminated reaction mixture. The colorimetric method was used to determine the liberated amount of tyrosine by adding 5 × *Folin-Ciocalteu's* reagent (0.2 mL) and 0.5 mol L^{-1} Na_2CO_3 (1 mL) to the supernatant (0.2 mL). A spectrophotometer (UV mini 1240, Japan) was used to measure the optical density at 660 nm. The tyrosine standard curve was developed by conducting a tyrosine assay and then, it was used to calculate the amount of released tyrosine. One unit of the acid protease activity was defined as the amount of enzyme that yielded 1 µg of tyrosine per minute at the temperature and pH of the reaction mixture. The protease activity assay was done in duplicates, and the mean values were calculated.

Partial characterization of protease enzyme activity

The optimum temperature for the highest enzyme activity was studied at different temperatures (20, 28, 37, 45, 50 and 60 °C) at neutral pH. The optimal pH for the maximum protease activity was evaluated at a series of pH levels (2.0, 2.5, 3.0, 5.0 and 7.0) at their optimum temperatures.

Production of shrimp waste protein hydrolysates with protease treatment

Shrimp muscle and waste protein hydrolysates were separately prepared by following the methods explained by Limam *et al*., (2008) with slight modification. Shrimps were washed thoroughly under running water and Shrimp muscle and waste portions (head and shell) were separately minced using a blender, and 100 g of each sample was taken for each hydrolysis. Then, they were mixed with distilled water at the 1:1 (w/v) proportion, and a glass rod was used to shake and homogenize the solution. The incubation period was extended for 1 hour and during this period, it was occasionally stirred. The reaction mixture was kept at 95 °C for 5 minutes to inactivate the endogenous hydrolyzing enzyme. Shrimp proteins were hydrolyzed at the ideal temperature and adjusted the pH of the respective enzymes. The reaction mixture was heated at 90 °C for 5 minutes to terminate the proteolysis. Then, they were kept under the ambient temperature and filtered using a muslin cloth. Then, it was centrifuged at 3000 rpm and 4 °C for 15 minutes and the supernatant was

collected. For the explained experiment, 0.5 mL of shrimp protein samples were treated with 0.025 mL of extracted crude enzymes of three fungal species.

SDS PAGE for shrimp waste protein hydrolysate

The standard protocol explained by He, (2011) for SDS-PAGE was conducted to observe the separation of protein bands based on their molecular weights in shrimp waste protein hydrolysate. The polyacrylamide of separation gel (12%) and polyacrylamide of stacking gel (4%) were arranged by the following standard procedures. The protein band patterns of shrimp muscle and waste samples prior to and after treatment were observed through the SDS PAGE analysis. Shrimp protein samples were mixed with Laemmli sample buffer (ratio 2:1) and then, the sample was heated in a water bath (95 °C for 5 minutes). Then, the samples were cooled to ambient temperature. Then the mixtures were centrifuged (3000 rpm and 5 minutes) to settle any precipitated particles prior to being loaded into the wells. Then, 20 µL of each protein mixture was loaded onto wells and the gel was run at 300 V (2 h) until reaches the separating gel bottom. The Coomassie brilliant blue R-250 was used to stain the gels for the detection of the separated protein bands. Finally, the gel was de-stained overnight till the protein bands were visible as dark blue bands against a transparent background. The standard protein marker ranged from 14.4 to 97.4 kDa was used in SDS PAGE as a reference to estimate the molecular weight of separated protein bands.

Results and Discussion

Protease yield

Under solid – state fermentation of all three fungal species, the highest protease yield, 527.40 µg/mL was achieved from *A. flavus* over *A. niger* (234.52 µg/mL) and *Penicillium* sp. (147.60 µg/mL) after 5 days of incubation. However, the present results are not following the findings of Anand. K, (2016) who stated that *A. niger* grown at 37 °C showed the maximum enzyme production over *A. flavus* under submerged fermentation of rice bran. In contrast to the present results, Sethi and Gupta, (2015) had gained a higher alkaline protease activity by fermentation of *Penicillium chrysogenum* than that of *A. niger*.

Effect of temperature and pH on protease production

As presented in the Figure. 1, 37 °C was the optimum temperature since protease production was gradually increased over the elevated incubation temperatures 20, 28, and 37 °C, and thereafter, the enzyme yield of all three fungal species were started to decline with further increase of incubation temperature for all studied fungal species. The higher temperature might reduce the fungal growth, and thus, reduce the enzyme yield as enzyme is a secondary metabolite produced through the exponential growth phase of microorganisms. Although the rates of physiological processes are increased by rising the temperature up to a certain extent, and it starts to diminish the rates due to sensitivity of enzymes at elevated temperatures (Mukhtar, 2009). The present findings are following the results of Anand (2016) who had revealed the highest protease enzyme production at 37 °C under submerged fermentation of rice bran by *A. flavus* and *A. niger*. As evidenced by previous studies, *Aspergillus* species, have a capacity to secrete high levels of enzymes in their growth environment (de Souza 2015).

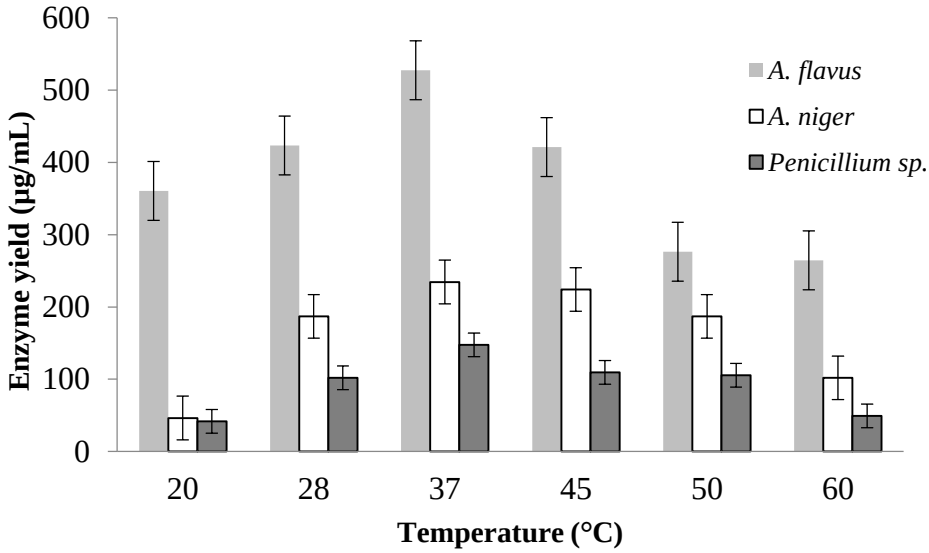
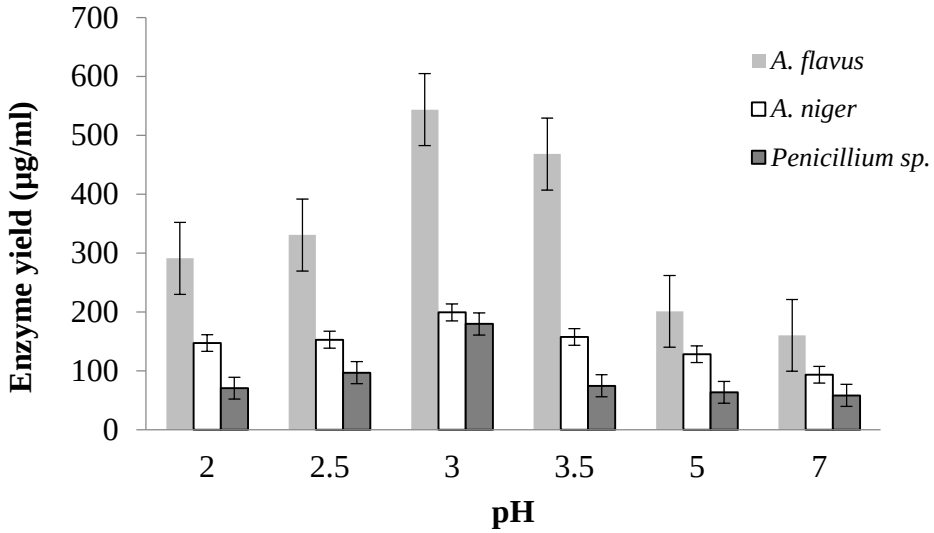


Figure 1: Protease production at different incubation temperatures and neutral pH

As per the basic facts, fungal protease enzyme is a secondary metabolite secreted during their exponential growth phase. The incubation at high temperature could lead to lessen the growth rates, and thus a reduction in enzyme yield could be observed.

Based on the results illustrated in Figure. 2, the highest enzyme yields were observed at pH 3.0 for three fungal species.



F**igure 2:** Protease production at different incubation pH levels and optimum temperatures

The pH significantly impacts on the production of enzymes, whereas the variation in pH may cause to denature the enzyme, resulting in loss of catalytic activity. In correspondence with present results, Radha *et al*. (2012) had stated the same figures with *Aspergillus* sp. under the solid – state fermentation of wheat bran. In the controversy with the present findings, Shivakumar (2012) stated that the optimum pH was 5.0 for acid protease production of *A. niger* by wheat bran fermentation, while that was 9.0 in the case of both *P. chrysogenum* and *A. niger* as reported by (Sethi and Gupta, 2015).

Partial characterization of protease enzyme activity

As illustrated in Figure. 3, the optimum temperature for highest protease activity was 45 °C for *A. flavus* enzymes, while that was 50 °C for other two fungal species.

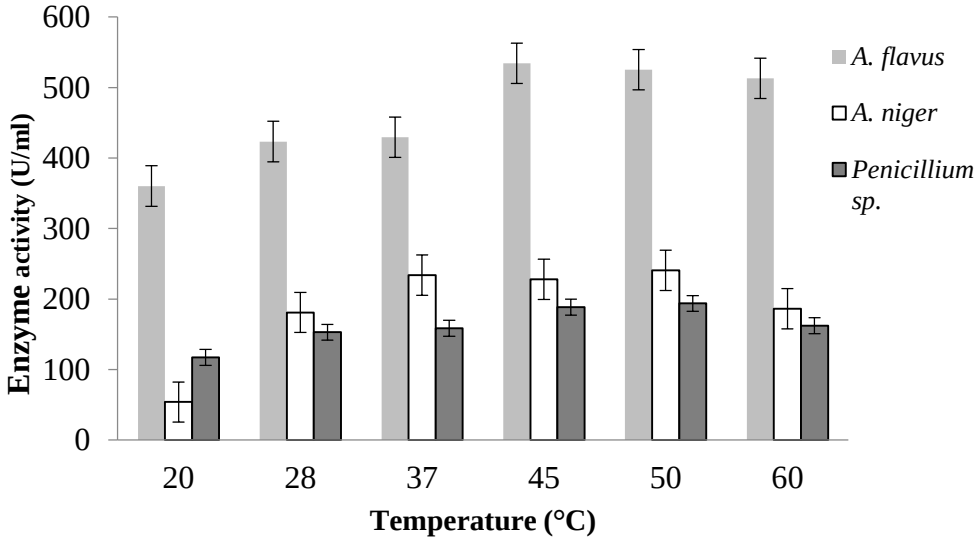


Figure 3: Optimum temperature for the highest enzymatic activity

A recent research article by Anand (2016) has reported that the enzymes produced by submerged fermentation of rice bran by *A. flavus* and *A. niger* were stable at a temperature, ranging from 50 to 60 °C with the gradual increase of enzyme activity with increasing temperature followed by a steep decrease above 60 °C. This result showed that the protease produce by *A. flavus* is a thermo tolerant enzyme which is stable with its structural integrity at above 60 ˚C temperature (Rukmi, 2020). In controversy, Chandrasekaran *et al*. (2015) had stated that, the optimal temperature for protease production by *A. flavus* extracted from paddy soils was 30 °C, while Radha *et al*. (2012) had published that the maximum protease production by *Aspergillus* sp. was observed at temperature 32±2 °C, and at 40 °C. The minimum protease production was noticed. In the argument with the present findings, Shivakumar (2012) has found that the highest protease productivity by *A. niger* was shown at 30 °C. As

reported by Sethi and Gupta, (2015), protease activity of *P.chrysogenum* was highest at 35 °C and completely inactivated at 60 °C.

The pH profile of the purified proteases is shown in Figure. 4, and the results revealed that the optimum pH values were similarly aligned at pH 3.0 for all tested proteases.

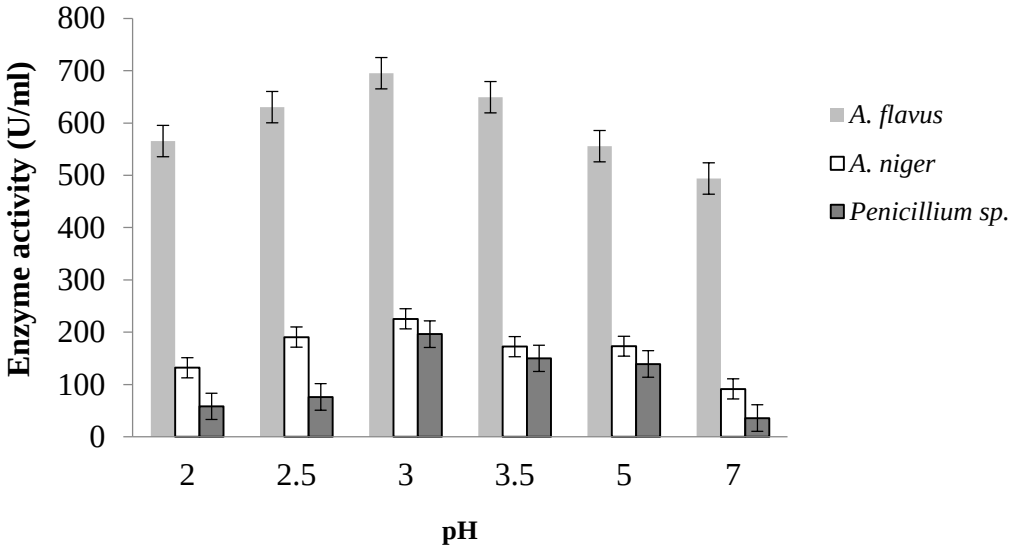


Figure 4: Optimum pH for the highest enzyme activity

These findings are in line with a similar study as mentioned that protease enzymes from *A*. *niger* were most active at pH 3.0 (Siala *et al*., 2009). However, some controversies were also found in the literature. Tremacoldi *et al*. (2004) indicated that, the optimum pH for *Aspergillus clavatus* protease activity was 2.0, and as reported by Oseni (2011), it was 5.5 for *Penicillium* sp.

SDS PAGE for shrimp proteins

The untreated protein extracts of shrimp muscle, shell and head were profiled by SDS PAGE and the characteristic patterns were obtained for all the extracts of the shrimp samples as illustrated in Figure. 5. There was no observable dissimilarity in patterns of protein bands in between shrimp muscle and shell or head. Four major bands with molecular weights of ~66.2 kDa, ~45 kDa, ~31

kDa and ~21.5 kDa were found in all shrimp protein samples. Literature supports well on the flesh of shrimp, but allergenicity of shrimp shell and head were under-provided.

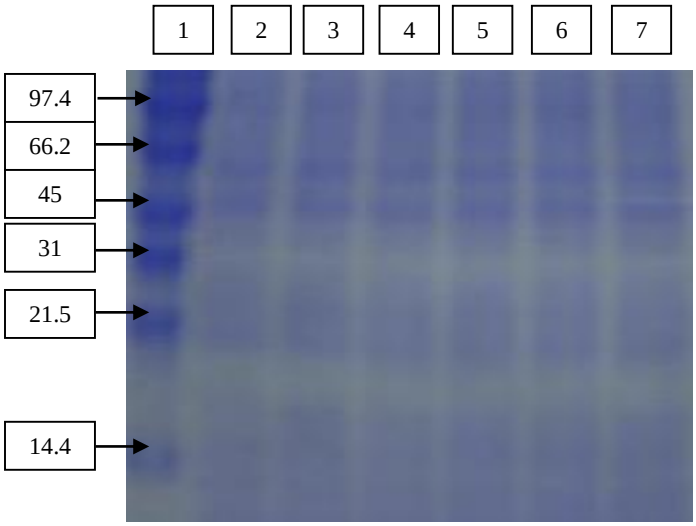


Figure 5: SDS-PAGE for untreated shrimp protein hydrolysate, Lane 1: Protein marker, Lane 2,3: Shrimp muscle, Lane 4,5: Shrimp shell, Lane 6,7: Shrimp head

The major allergens identified from black tiger shrimp were tropomyosin (33kDa), myosin light chain (20 kDa) and argine kinase (40 kDa) (Velickovic and Jankulovic, 2014). All of the samples of shrimp extracts had shown similar bands in the range of 45 – 31 kDa, and the protein bands at ~31 kDa would be the major allergen, tropomyosin ~45 kDa would be the arginine kinase, and ~21.5 kDa supposed to be sarcoplasmic calcium-binding proteins. Several research reviews and articles have mentioned that *tropomyosins* are the cytoskeletal proteins. *Tropomyosins* play a key role in the muscle contraction regulation with other contractile proteins actin and myosin. *Tropomyosins* form head-to-tail polymers along the actin filaments (Stafford *et al*., 2012). *Tropomyosin* regulates the stability of actin filaments in non-muscle cells (Stafford *et al*., 2012). These proteins may remain in the derived products from

the shrimp waste. Therefore, it is very important to degrade the potential antigenic protein in shrimp waste before further processing.

SDS PAGE for protease treated shrimp waste protein hydrolysate

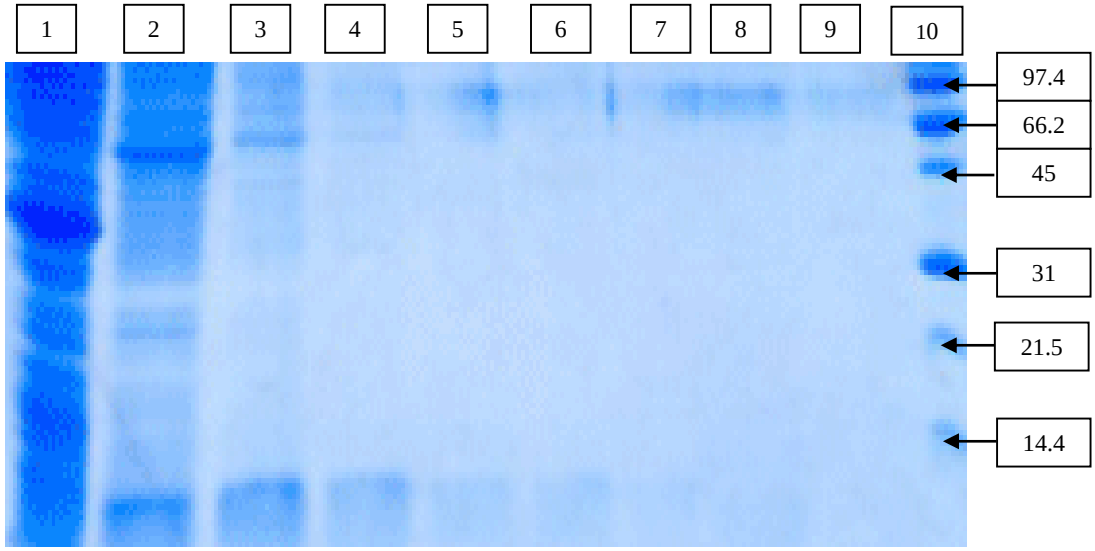


Figure 6: *SDS-PAGE for protease treated shrimp protein hydrolysates* 1-Untreated Shrimp muscle protein extract, 2- Untreated Shrimp waste (head and shell) protein extract, 3-shrimp waste protein hydrolysate treated with mixture of extracted fungal enzymes 4-Shrimp waste protein hydrolysate treated with enzyme extracted by *Aspergillus niger*, 5-Shrimp waste protein hydrolysate treated with enzyme extracted by *Penicillium* sp., 6-Shrimp waste protein hydrolysate treated with enzyme extracted by *Aspergillus flavus,* 7-Shrimp waste protein hydrolysate treated with enzyme extracted by *Aspergillus niger*, 8-Shrimp waste protein hydrolysate treated with enzyme extracted by *Penicillium* sp., 9-Shrimp waste protein hydrolysate treated with enzyme extracted by *Aspergillus flavus,* 10- Protein Marker

As per Figure. 6, the shrimp waste samples treated with three fungal proteases had a great reduction in protein bands compared to the untreated sample proving that the extracted crude acid protease enzymes affected on hydrolyzing the shrimp proteins. According to Figure. 6, lanes 3, 4, 5, 7, and 8 have much darker bands at the bottom (<14.4 kDa) than in lanes 6 and 9, indicating that

there is a much greater concentration of smaller peptides because hydrolysis of shrimp waste protein is a process to degrade the long protein chain in shrimp head and shell into small peptides and amino acids. This would suggest that crude acid protease extracted from *A. flavus* does a great degree of hydrolyzing protein than the other two enzymes.

The further increase of enzyme concentrations of none of the enzymes is further effective on hydrolyzing potential antigenic proteins. The literature survey had found many reports with evidence, when the concentration of enzyme added reaches a certain point, the increase of soluble protein in hydrolysate would not increase significantly or even does not increase at all. Similar findings were investigated by Benito *et al*. (2002) who has found that protease extracted from *P. chrysogenum* Pg 222 was effective against the myofibrillar proteins myosin, actin and tropomyosin. Another study Paul *et al*. (2015) has stated that, shell of the black tiger shrimp was a good protein source, while a different recent study has invented that crude alkaline proteases from *Bacillus invictae* could be utilized to degrade the shrimp shell proteins (Hammami *et al*.. 2017). The same kind of investigation has been found after treatment with pepsin (Mejrhit *et al*., 2017). The experiments of Shimakura *et al*. (2005) and Liu *et al*. (2011) have also found the effectiveness of protease to degrade *tropomyosin.*

Conclusion

A. flavus, *A. niger* and *Penicillium* sp. are effective protease producers under solid-state fermentation of white *samba* rice. Their extracellular crude acid proteases have higher proteolytic activity against potential antigenic proteins in shrimp waste. The crude acid protease extracted by *A. flavus* is found to be the best to degrade potential antigenic proteins of shrimp waste over the other two acid proteases extracted by *A. niger* and *Penicillium* sp. Those fungal acid proteases were found to be potential sources to produce shrimp – waste protein hydrolysates with degraded antigenic proteins.

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