FGIC 1st Conference on Governance & Integrity, 2017
"Innovation & Sustainability Through Governance"
3 – 4 April 2017, Yayasan Pahang, Kuantan, Malaysia
ISBN 978-967-2054-37-5

WASTE MANAGEMENT BIOTECHNOLOGY FOR ALKALINE PROTEASE PRODUCTION AND OPTIMIZATION

Raid D. Thanoon
Faculty of Industrial Sciences & Technology,
Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
raeid_dodo@yahoo.com*

Rubaaini Subramaniam
Faculty of Industrial Sciences & Technology,
Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
rubaaini_sk@yahoo.com

Mahmood H. Mahmood
Faculty of Industrial Sciences & Technology,
Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
anfalanfal87@yahoo.com

Osama A. Munem
Faculty of Industrial Sciences & Technology,
Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
golden5eagle@yahoo.com

Essam A. Makky
Faculty of Industrial Sciences & Technology,
Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
essammakky@ump.edu.my

Mashitah M. Yusoff
Faculty of Industrial Sciences & Technology,
Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
mashitah@ump.edu.my

ABSTRACT

Environmental pollution is one of the major issues in either well developed or in developing countries. Pollution can also come from animal wastes such as livestock, wastewater and feed that have been wasted. This type of circumstances occurs when there are abundant of waste generated in animal farms in certain area that is difficult to be disposed. For example, the fish residues such as fish fin, viscera, and scales can be one of the sources of contamination that can be the major problem. Moreover, chicken feather (CF) is widely disposed that results in environmental pollution as it cannot be solved with ease. Hence both

ISBN 978-967-2054-37-5

fish fin (FF) and CF can be utilized to produce alkaline protease (AP) enzyme by using microbial fermentation power. Protein from each sample was extracted and determined. Then, the extracted protein from both wastes was employed in the production media together with bacterial suspension which was incubated. Next, alkaline protease assay was carried out to test for presence of alkaline protease in the samples. Optimization parameters controlling alkaline protease productivity were screened and determined. The optimum pH of CF and FF was 9.0, the optimum temperatures recorded at 28 and 40°C for CF and FF respectively, the production incubation periods were 6 and 10 days for CF and FF respectively. While, galactose and glucose exhibited the optimum carbon sources for CF and FF respectively from one hand, and the optimum nitrogen sources recorded to be ammonium chloride and beef extract for CF and FF respectively from the other hand.

***Keywords*:** Alkaline Protease, Fish fin, Chicken feather, Waste management, Protein extraction, Optimization.

INTRODUCTION

Environmental pollution is one of the major issues in either well developed or in developing countries. In another word, pollution can be described as the introduction of contaminants into the environment which may result in harm or discomfort to living beings or that harm the environment. Pollutants can come in naturally occurring substances or energies, however, it is considered as contaminants when in excess of natural levels. Pollutants can be divided into two categories namely biodegradable and non-biodegradable. Biodegradable pollutants are considered as the one can be broken down and can be processed by living beings, these include organic waste products, phosphates, and inorganic salts. On the other hand, non – biodegradable pollutants are the ones that cannot undergo decomposition by living organisms which mean the pollutants will remain in ecosphere for extremely long periods of time (Miguel, 1990). Pollution can also come from animal wastes such as livestock and poultry manure, waste water and feed that have been wasted. This situation arises when there are abundant of waste produced by animal farms in certain area that cannot be disposed of or recycle. Unfortunately, this type of waste will affect the nature of air and water if it is not treated well. This is because, it will be big danger if the untreated animal waste wash into rivers or water streams and this is considered as non-point source (NPS) pollution .In United States this type of pollution is in critical level among water quality issues (Fafioye, 2012) as this can lower down the dissolved oxygen (DO) and affect aquatic life. Similarly, chicken feather waste and fish waste considered as pollutants that can contribute to environmental pollution (Gray, 2012). An industry that deals with fish processing generate considerable quantities of by-products as waste including viscera, shell (from crustacean and molluscan processing) scales, frame bones and fins. These kinds of wastes are largely produced in all over the countries. For instance, India alone produces <2mmt of by - products resulting from fish processing activities. Furthermore, unlike the seafood processing sector, fresh water fish or the inland fisheries sector carry out un-organized which lead to distinct level of waste disposal issues. Visceral waste alone can contribute as much as 300 000 ton (Mahendrakar,

ISBN 978-967-2054-37-5

2000). However, despite it is considered as waste viscera are good source of proteins including fats and enzyme in it. The fish's digestive tract contains micro flora which can generate antibacterial materials, these antibacterial materials able to prevent the pathogenic bacteria from getting into an organism (Sugita *et al*, 1996). The decomposition of nutrients is done by the gastrointestinal bacteria and it provides physiologically active materials such as enzymes ,amino acids and vitamins for the macro organism.(Sugita *et al*,1997).Besides, few proteolytic bacteria previously have been reported to be related with both fresh water and marine fish processing wastes (Sudeepa *et al.*,2007). In addition, it is also known that the aquatic microbes produce exo-enzymes identified as inducible catabolic enzymes. The waste that has been disposed from the fishery processing industry contributes to higher environmental and health problem. Hence, to lower the wastage of these by products, variety of method to dispose them have been developed which includes the process of ensilation , fermentation, hydrolysate and fish oil production (Faid et al.,1997)Another interesting information is that the by–products of fish able to provide excellent nutrient source for the growth of microbes that can be very useful in enzyme production process, which is widely governed by the cost associated with growth media (Coello *et al*., 2000). Fish wastes such as heads, fins, bones, viscera, chitinous material, waste water, and so on were prepared in order to test as growth substrates for production of microbial enzymes. Example of microbial enzymes are protease, lipase chitinolytic and ligninolytic enzymes (Triki-Ellouz*et al.*2003). Apart from fish processing waste, another type of waste that can contribute to pollution is from poultry industry where the waste from this industry eventually produces a lot of wastes. The large quantity of waste feathers generated by the poultry industry every year is around 1.8 million tons in US. This leads to disposal issues as it is environmentally difficult. Hence, if we consider this problem economically and environmentally, it will be easy to come up with effective and profitable techniques to utilize this source of waste (Gessesse, et al & Mattiasson, 2003). Feathers are generated in bulk amount at poultry processing plants and clearing away is big issue in processing plants (Ichida et al., 2001). Beta keratin is one of the components of feathers that repel chemical agent and enzymatic lysis. The keratin in feathers are linked by certain angle to the cysteine and two cysteine molecule linked by disulphide bond that contributes to huge durability of keratin (Esawy, 2007). Protease can be considered as one of significant groups of enzyme and revealed over animal kingdom such as plants and microbes (Imtiaz & Mukhtar, 2013).The amount of enzyme protease attained from source of plant and animal were inadequate to meet present requirements. Currently, there has been remarkable increase in the application of alkaline protease enzyme that functions as industrial catalyst. This particular enzyme approaches other benefits compared to the utilization of conventional chemical catalyst for number of reasons. This reason can be explained by, alkaline protease they demonstrate high catalytic activity and high rank of substrate specificity. It also can be generated in huge amounts and at the same time are economically viable (Alencar et al., 2003).

ISBN 978-967-2054-37-5

METHODOLOGY

Samples Collection

Two different types of wastes namely chicken feather, fish fin was collected from the wet market at Kuantan. The fish fin collected was washed and dried under room temperature by using fan and after drying it was blended using a blender to ensure it is in small pieces. As for the chicken feather (CF), it was washed and dried as same method as fish fin (FF) and grinded using grinder to make it small pieces.

Pre-treatment of Fish Fin

The extraction of protein from fish fin was done as described by Hashemi-jokar, 2014. Small pieces of fish fin and blended fish fin of 5 g was weighed and added in 100 mL of 1M sodium hydroxide. The solution was stirred continuously using magnetic stirrer for 6 hours. This is to get rid of stirred non-collagen proteins to be extracted.

Extraction of Collagen from Fish Fin

After 6 hours in the sodium hydroxide, the suspension was centrifuged at 7000 rpm for 5 minutes. The pellet obtained was more like colloid. Distilled water then added to the pellet and mixed well by vortexing then centrifuged again to obtain the pellet after washing it. Then, 0.5M of acetic acid was added until it covers the insoluble material and kept for 3 days. After 3 days, the solution was centrifuged at 2000 rpm for 1 hour. The supernatant was removed and the insoluble material collected was washed with distilled water. 0.5M of EDTA was prepared and the pH was adjusted at 8 as it will only dissolve at pH 8.The insoluble material was soaked in EDTA for 5 days in order to remove calcium. After 5 days of soaking, the insoluble material of fish fin was washed with distilled water twice. This was done by centrifuging the insoluble material of fish fin with distilled water 10,000 rpm for 1 hour. The distilled water then discarded and 0.5M of acetic acid was added until it covers the insoluble pieces of fish fin. The total volume after addition of acetic acid was 25 mL. Then ammonium sulfate was added at ratio of 1:1 which means 25 mL of ammonium sulfate was added into 25 mL of fish fin suspension. The mixture was centrifuged again at 8000 rpm for 5 minutes. Both the supernatant and pellet was tested for presence of protein determination using Lowry assay method.

Dissolving of Chicken Feathers

Grinded chicken feather of 25 g was weighed and added into 1L of 0.5M sodium metabisulfite. The solution was heated at 30°C, at pH 5 and stirred regularly for 6 hours. After that, the solution was filtered using a sieve and centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and filtered again using filter paper to make it particle free.

ISBN 978-967-2054-37-5

Ammonium Sulfate Solution Preparation

Ammonium sulfate of 525 g was dissolved in 1L distilled water and stirred continuously until all particles of ammonium sulfate were dissolved completely. Again the solution was filtered using filter pump to remove any particles.

Precipitation of Protein

The 100 mL filtrate collected after filtration was poured into beaker and stirred well using a glass rod. Next, 100mL ammonium sulfate was added drop wise slowly into the filtrate. The ratio of ammonium sulfate and feather filtrate used was 1:1.Then, the solution was centrifuged at 10,000 rpm for 5 minutes and the solids formed were gathered carefully by rinsing it with distilled water. The supernatant after centrifugation was used to repeat the addition of ammonium sulfate at 1:1 ratio and centrifuged again to collect the both supernatant and pellet. The whole procedure of keratin extraction was done as described by (Gupta et al., 2012) The pellet was suspended in distilled water until it covers the pellet. Both the supernatant and pellet was kept in chiller for protein determination by Lowry assay (1951).

Alkaline Protease Production

M9 minimal salts solution (5X) was prepared as follows: To 800 mL of distilled water, all chemicals were added (g/L); $Na_2HPO_4.7\ H_2O$ 64; KH_2PO_4 15; NaCl 2.5g; NH_4Cl 5, the volume was topped up to 1000 mL and then autoclaved. Next, for the preparation of 1L of media, 200 mL of 5 X M9 salt solutions was added to 800 mL of distilled water. After autoclaving, the media was mixed uniformly and was cooled down until it reaches room temperature. Then, 2 mL of 1M $MgSO_4$, 0.1mL of 1M $CaCl_2$, 20 mL of 20% glucose was added to the autoclaved M9 5 X stock solutions. The mixture was swirled evenly. 1g of waste (fish fin and chicken feather) was weighed and mixed with autoclaved distilled water until it covers the waste. Then, the solution was shaken for three to five minutes, 1mL of clear solution from the waste solution was taken and inoculated in 50 mL nutrient broth media. The inoculated media was incubated for 48 hours at 37ºC. In 200 mL M9 media was mixed with 0.5 mL of waste (protein containing waste) with 1 mL prepared bacterial suspension that was added into conical flask and be incubated at 37°C for 48 hours. After the incubation period, the solution was centrifuged in order to obtain the crude enzyme to proceed alkaline protease assay.

Protease Assay

Protease was determined by using the method of Folin Lowry method as described in (Nisha & J., 2014). 1.25 mL of Tris buffer (100 mM, pH 9) and 0.5 mL of 1% aqueous casein solution was added into 0.25 mL culture supernatant. It was incubated for period of 30 minutes at 30°C.Next, 3 mL of 5% tricholoroacetic acid (TCA) was added into this mixture and it will form precipitate .The mixture was incubated for 10 minutes at 4°C, and centrifuged

ISBN 978-967-2054-37-5

at 5000 rpm for period 15 min. Then, 0.5 mL of the supernatant was taken and 2.5 mL of 0.5M of sodium carbonate was added to the supernatant, mixed well and incubated for 20 minutes. Thereafter, 0.5 mL of folin reagent was added to the mixture and analyzed under UV-VIS at 660 nm. Then, the amount of protease was measured using a tyrosine standard graph (Takami et al., 1989).

Process Optimization for Maximum Protease Production

The method carried out to test different parameters to screen alkaline protease enzyme and determine the optimum conditions described by Nisha and J. Divakaran, 2014. The ideal initial pH was determined by altering the production medium to various pH values, where the pre-autoclaved medium was prepared individually at pH 5, 6, 7, 8 and 9 was inoculated with bacterial suspension and protein extracted from chicken feather and fish fin incubated at 37°C for 48hrs. The effect of different temperatures on protease production was studied by incubation at temperature of 20, 30, 40, 50, and room temperature for 48 hours at 37°C. The development of different carbon sources for instance glucose, starch, maltose, lactose, xylose and fructose was determined and the various nitrogen sources such as yeast extract, beef extract, peptone, urea, ammonium chloride, sodium nitrate and ammonium sulphate was investigated for their development on protease production. The effect of incubation period was studied for 2, 4, 6, 8 and 10 days. The effect of protein volume on the production of alkaline protease was studied using volumes (mL) such as 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 of protein extracted from waste under study was added and incubated with bacteria for two days. The bacterial inoculum sizes (mL) inoculated was varied viz; 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 into M9 minimal media together with the protein extracted and kept in incubator at 37°C for two days. After incubation period alkaline protease was determined.

RESULTS AND DISCUSSION

Data recorded in table 1 showed the protein content in chicken feather and fish fin measured using 96 well microplate at 700nm by conducting Lowry assay. According to the results above, we can conclude that the supernatants of all the samples contain high protein content compared to pellet. Hence, the supernatant and pellet was mixed together as the crude extracted protein from the chicken feather and fish fin. However, the protein content for fish fin blended was low compared to fish fin pieces, as a matter of fact; the fish fin blended was not preceded further in this experiment. Fish fin pieces was used throughout this study as it shows better and logical results. Fish fin pieces provided better results as the surface area of pieces was higher which makes efficient for the chemicals to react with small pieces of fish fin rather than blended one.

ISBN 978-967-2054-37-5

Table 1: Protein content in both the samples

Protein Sample	Protein at 660nm
Control	0.058 ± 0.004
FFPP	0.393 ± 0.063
FFSP	0.852 ± 0.050
FFPB	0.084 ± 0.010
FFSB	0.058 ± 0.001
CFP	1.183 ± 0.035
CFS	4.894 ± 0.518

FFPP: Fish fin pellet pieces; FFSP: Fish fin supernatant pieces; FFPB: Fish fin pellet blended; FFSB: Fish fin supernatant blended; CFP: Chicken feather pellet; CFS: Chicken feather supernatant.

Data recorded in table 2 showed the readings for alkaline protease assay which was conducted to test the presence of alkaline protease enzyme in chicken feather and fish fin and, it is proven that the extracted protein contains the presence of alkaline protease enzyme in both chicken feather and fish fin. The amount of alkaline protease enzyme is slightly higher in chicken feather compared to fish fin and this might be due to the amount of protein present in each sample. The higher the protein amount in the sample, the higher the production of alkaline protease enzyme. Moreover, casein was not added for the control in this particular step, instead distilled water was added as addition of casein in the control results in high reading as it can react with the chemicals added in protease assay. This is because the casein plays the role of substrate, as the enzyme protease we are looking for digests casein, tyrosine which is the amino acid was liberated including either amino acids or peptide fragments. Folin's reagent was used so that there is reaction between free tyrosine in order to generate blue colored chromophore that can be quantified by measuring the absorbance. Hence, the higher amount of tyrosine generated from casein, the higher the chromophores produced, stronger the protease activity.

Table 2: Protease assay readings for chicken feather and fish fin

Samples	Alkaline Protease at 660nm
Control (Casein Free)	0.0273 ± 0.002
Chicken Feather	0.254 ± 0.001
Fish Fin	0.246 ± 0.014

According to the Figure 1, it was found that alkaline protease activity is highest at room temperature which is at 28° C (0.362 /mL) and minimum production of enzyme is at 50°C (0.195 U/mL) for CF. (Goma, 2013), were also reported that the protease production was highest at 30°C and there was reduction in enzyme production as the enzyme undergo thermal inactivation. However, it can also be said that enzyme activity at 40°C is the second highest by few points only for CF which indicates the enzyme activity can be optimized at 40°C also. The highest enzyme activity for FF is at 40°C (0.342 U/mL) and lowest at 50°C (0.201 U/mL).The enzyme production is affected by temperature where the temperature change able

ISBN 978-967-2054-37-5

to alter the physical properties of cell membrane. (Nisha and Divakaran, 2014) also reported that the protease production was highest at 40°C where the microorganism that they utilized to produce protease was *Bacillus subtilis*. The incubation temperature generally concluded by considering the isolated microorganisms (Gomaa, 2013). However, in this paper there was no specific microorganism that was isolated instead mixed culture was used which explains why the highest temperature for alkaline protease production was different for chicken feather and fish fin. At high temperature the enzyme was inactivated which describes the low enzyme activity at 50°C for both CF and FF.

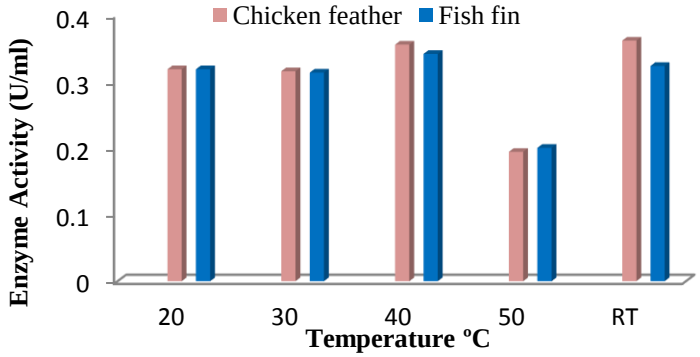

Figure 1: Enzyme activity of CF and FF against different temperatures (°C)

According to fig. 2 it has been found that for CF the highest enzyme activity was at pH 9 (0.217 U/mL) and the minimal enzyme activity was at pH 7 (0.188 U/mL).This clearly shows the alkaline protease from chicken feather can be produced optimally in alkaline condition by adjusting the production media to pH 9.The production of protease was less at pH 7 which means low amount of alkaline protease was produced neutral condition. As for the FF, the highest protease production was also at pH 9 (0.277 U/mL) and lowest production was at pH 5 (0.238 U/mL).As for FF, the production of alkaline protease was low at pH 5 which means the production of enzyme cannot be optimized in acidic condition, the acidic medium causes inactivation of enzyme resulting in low enzymatic activity. In addition, as the value of pH deviate from the optimal level, the productive operating of the enzyme can be altered. This indicates the low level of saturation of the enzyme to reduced affinity due pH effect on the stability of enzyme (Dixon and Webb,1979).The control for assay was carried out which gives the reading of, and it can be compared with the other values obtained to analyze the amount of protease produced.

ISBN 978-967-2054-37-5

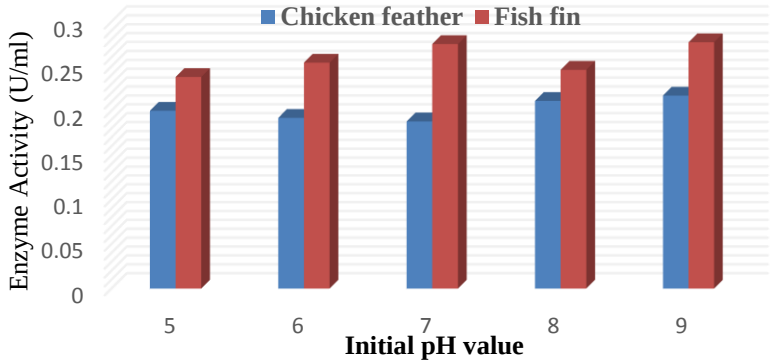

Figure 2: Graph of enzyme activity (U/mL) against pH

Figure 3 revealed that the various type of carbon sources employed in the production medium which includes all 3 classes of sugar, monosaccharide, disaccharide and polysaccharide. It has been found the best sugar for the production of alkaline protease in CF is galactose with enzymatic activity of 0.230 U/mL and the lowest production of enzyme is when maltose was employed with enzymatic activity of 0.201 U/ mL. Pant, (2015) was also reported that the galactose gave the maximum amount of alkaline protease and sucrose gave the lowest amount. On the other hand, carbon source that gave high amount of alkaline protease was glucose (0.233 U/mL) and lowest amount was starch with enzymatic activity of 0.148 U /mL. The control gave reading of 0.50 U/mL which can be compared with other readings for the production of alkaline protease. Generally, this overall reading indicates that monosaccharide sugar which was used as carbon source able to produce higher amount of alkaline protease compared to disaccharide and polysaccharide sugar. The different enzymatic activity on carbon source between CF and FF can be due to the different type of protein present in each sample. Since, mixed culture of microorganisms used for both samples, different type of microbes utilized different type of carbon source to degrade the protein and to produce alkaline protease.

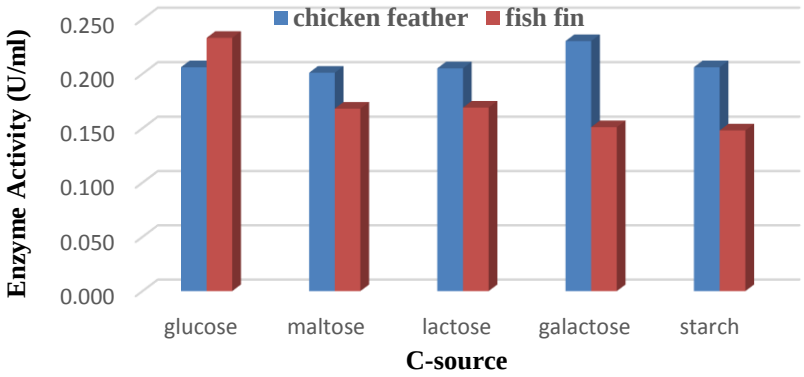

Figure 3: Graph of enzyme activity (U/mL) against carbon sources

ISBN 978-967-2054-37-5

Various type of nitrogen sources including organic and inorganic nitrogen were employed in the production medium. The results represented in Figure 4 indicated the highest enzymatic activity for CF was when nitrogen source ammonium chloride was used which gives reading of (0.414 U/mL) and the minimum amount of protease was for peptone (0.345 U/mL).The control used for CF gave production of 0.050 U/mL. This shows inorganic nitrogen source give higher enzyme compared to organic nitrogen for CF. However, nitrogen source of yeast and beef are also comparable as the production of alkaline protease enzyme in these both nitrogen source were high also. As for FF, beef extract gave high result with production of 0.201 U/mL and lowest reading of 0.114 U/mL for yeast. The control for FF produced 0.003 U/ mL. In this case, organic nitrogen gave higher reading compared to inorganic nitrogen. In a journal reported by N.S Nisha and J.Divakaran, (2014) beef extract gave the highest production of alkaline protease also. The optimal nitrogen source for CF and FF gave different result as the type of protein in each sample is different.

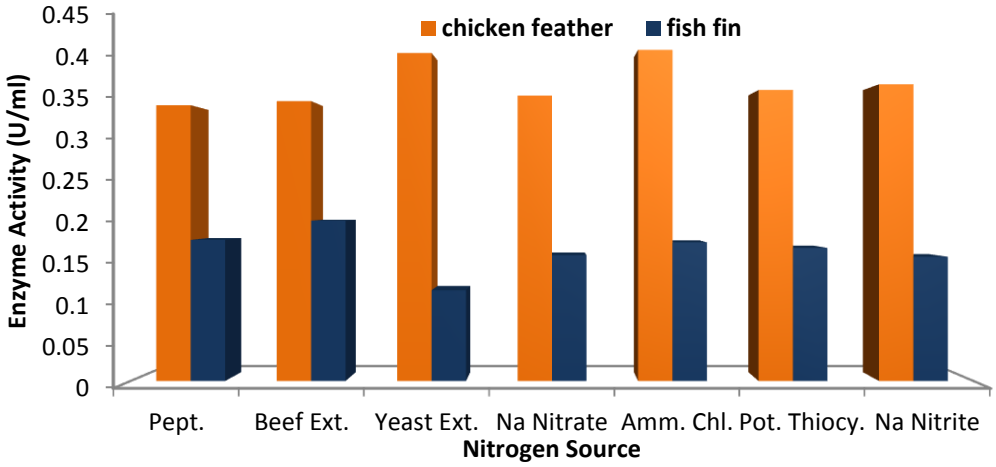

Figure 4: Graph of enzymatic activity (U/mL) against nitrogen sources

Data represented in Figure 5 was constructed based on varying the protein volume that was extracted from CF and FF to check on its enzymatic activity, (U/mL).It has been found that for CF the protease production was maximum when 0.5mL of protein volume inoculated as it gave enzymatic activity of 0.345 U/mL and minimum production of 0.272 U/mL at 0.0 mL protein volume. The control gave enzymatic activity of 0.027 U/mL for CF. The production of alkaline protease was higher when 0.5 mL of protein inoculated is because the protein in this amount of volume might have more active site so that more substrate able to bind to it. Hence, the more alkaline protease enzyme can be produced compared to higher volume protein, for instance, 2.5 mL of protein produce lesser amount than 0.5 mL, this is because at higher volume of protein, all the substrate available bonded at free active site and the bacteria present is constant for all the protein volume which means low amount of bacteria can only degrade certain amount of protein that will result in production of alkaline protease. As for the FF, the highest enzymatic activity was at 2 mL of protein volume with amount of 0.251 U/mL and the lowest animatic activity was at 0.0 mL (0.183 U/mL).The control for FF for the assay was 0.026 U/mL. In this case, the protein inoculated 2 mL shows higher reading

ISBN 978-967-2054-37-5

because the protein present have more active site so that the substrate can bind where this make an advantage when the volume of protein was increased. Conclusively, the lowest enzymatic activity was when there is absence of protein as there is less substrate binding occur.

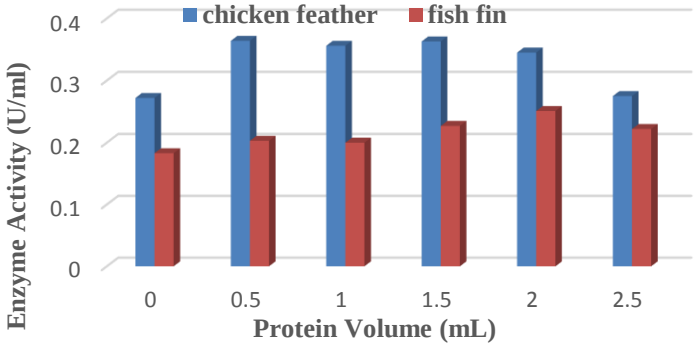

Figure 5: Graph of enzymatic activity (U/mL) against protein volume (mL)

One of the parameter is to vary the incubation period of production medium that is inoculated with bacteria and protein extracted from CF and FF (Figure 6). The incubation period was between 2 to 10 days maintained at 37°C. In the case of CF, the production of alkaline protease enzyme is maximum at 6 days of incubation with enzymatic activity of 0.290 U/mL and, minimum production of alkaline protease was found to be 0.185 U/mL at 8 days of incubation. As for the FF, the maximum production of alkaline protease were found to be at 10 days of incubation with enzyme activity of 0.336 U/mL and the lowest activity is 0.251 U/mL which is at 8 days of incubation. As for the CF, the production of protease decline at 10 days of incubation and this is because the production of enzyme could have terminated along with auto proteolysis (Nisha and Divakaran, 2014). However, the production of alkaline protease is high during 10 days of incubation and this might be due to the different protein in fish fin compared to chicken feather, where the protein in fish fin which is the collagen able to produce more alkaline protease after incubation of 10 days. In addition, the synthesis of enzyme is associated with growth of cell and incubation period and production of enzyme indeed related to each other (Kaur et al., 1998).

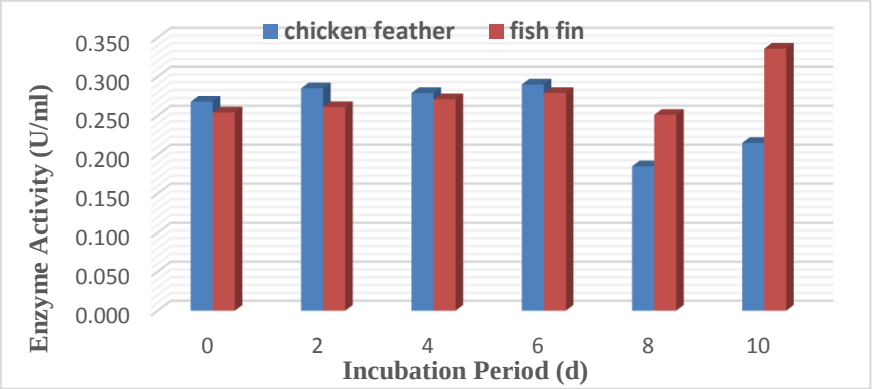

Figure 6: Graph of enzymatic activity against incubation period (days)

ISBN 978-967-2054-37-5

Another parameter tested was the size of bacterial inoculum where different volume of bacterial suspension was added in the production media together with the extracted protein from CF and FF. According to Figure 7, it can be concluded that maximum production of alkaline protease is at 1.5 mL of bacteria where the enzyme activity is 0.308 U/mL and the lowest enzymatic activity is 0.238 U/mL for 0.25 mL. The control for CF is 0.045 U/mL of enzymatic activity. As for FF, the maximum alkaline protease production is also at 1.5mL of bacterial inoculum with enzymatic activity of 0.450 U/mL and the minimum enzymatic activity obtained is 0.25 U/mL for bacterial size 0.25mL.The control gave enzymatic activity of 0.130 U/mL. Generally, from this paper, it can be concluded that large size of bacterial inoculum has the maximum amount of protease production for both CF and FF. This is because the presence of large number of bacteria will increase the alkaline protease production as more bacteria will be available to degrade the protein in the production medium. On the other hand, smaller inoculum size gave low amount of alkaline protease enzyme and this is because there is lack of enough bacteria to degrade the protein that results in low amount of protease production. Moreover, upgraded distribution of dissolved oxygen and also high nutrient uptake can increase the alkaline production. However, 0.5mL of size of inoculum also gave considerable result for FF as alkaline protease synthesis with small inoculum size has larger surface area which contributes to more protease production. (Shafee et al., 2005).

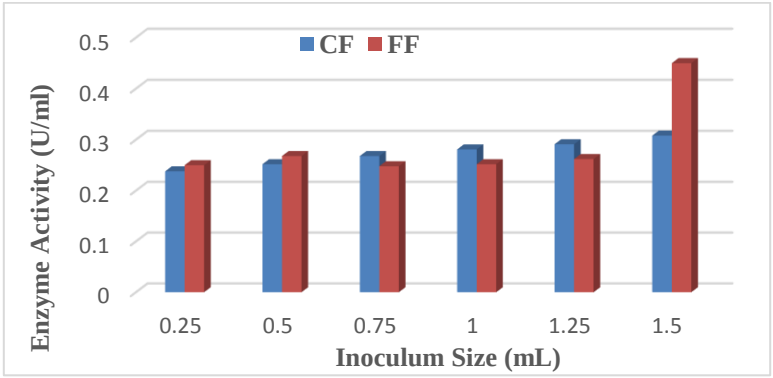

Figure 7: Graph of enzymatic activity (U/mL) against bacterial inoculum size

CONCLUSION

The study concludes that protein containing waste CF and FF able to produce alkaline protease enzyme using microbial fermentation. The protein extracted was further confirmed with Lowry method as it shows there is presence of protein in chicken feather and fish fin. The protein extracted was obtained in the form of supernatant and pellet. Next, the protein extracted was employed in the M9 minimal media together with bacterial suspension from CF and FF and tested for the presence of alkaline protease enzyme. The alkaline protease assay was carried out for both samples and the test gave positive results which confirm there is production of alkaline protease enzyme. Then, seven different parameters were tested to screen for the alkaline protease enzyme that gives the optimal result for each parameter. In

ISBN 978-967-2054-37-5

this study it has been found that the production of alkaline protease was highest at all these conditions.

ACKNOWLEDGMENTS

The author would like to thank Faculty of Industrial Sciences & Technology (FIST), Universiti Malaysia Pahang (UMP) for technical assistance during this research. Moreover, the financial support from RDU160333 is highly acknowledged and appreciated.

REFERENCES

Alencar R.B, Biondi M.M, Paiva P.M.G, Vieira V.L.A, Carvalho L.B Jr., R.S. Bezerra.(2003) Alkaline proteases from digestive tract of four tropical fishes *Brazilian Journal of Food Technology, 6* pp. 279–284.

Coello N, Brito L, Nonus M (2000) Biosynthesis of L-lysine by Corynebacterium glutamicum grown on fish silage. Bioresour Technol 73:221–225

Dixon M, Webb EC. Enzyme Kinetics. In: Dixon M, Webb EC, editors. Enzymes. Vol. 3. Academic Press; New York: 1979. pp. 47–206.

Esawy, M. A. (2007). Isolation and Partial Characterization of Extracellular Keratinase from a Novel Mesophilic Streptomyces albus AZA. *Journal of Agriculture and Biological Sciences*, *3*(6), 808–817.

Fafioye, O. O. (2012). Problem of animal wastes disposal on the environment ; a case study of s and d farms , odeda , nigeria . *Journal of Environmental Research and Management.*, *3*(4), 84–87.

Faid, M., Zouiten, A., & Elmarrakchi, A. (1997). Biotransformation of fish waste into a stable feed ingredient. *Food Chemistry*, *60*(I), 13–18.

Gessesse, A., Hatti-Kaul, R., Gashe, B. A., & Mattiasson, B. (2003). Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. *Enzyme and Microbial Technology*, *32*(5), 519–524. http://doi.org/10.1016/S0141-0229(02)00324-1

Gomaa, E. Z. (2013). Optimization and characterization of alkaline protease and carboxymethyl-cellulase produced by Bacillus pumillus grown on Ficus nitida wastes, *537*, 529–537.

Gray, I. (2012). Environmental Pollution, Its Sources and Effects. *Tropical-Rainforest-Animals.com*. Retrieved from http://www.tropical-rainforest-animals.com/Environmental-Pollution.htmL

ISBN 978-967-2054-37-5

Gupta, A., Kamarudin, N. B., Yeo, C., Kee, G., Bin, R., & Yunus, M. (2012). Extraction of Keratin Protein from Chicken Feather. *Journal Chemical Engineering*, *6*, 732–737.

Ichida, J. M., Krizova, L., Lefevre, C. A., Keener, H. M., Elwell, D. L., & Burtt, E. H. (2001). Bacterial inoculum enhances keratin degradation and biofilm formation in poultry compost. *Journal of Microbiological Methods*, *47*(2), 199–208.

Imtiaz, S., & Mukhtar, H. (2013). Production of alkaline protease by Bacillus subtilis using solid state fermentation. *African Journal of Microbiology Research*, *7*(16), 1558–1568. http://doi.org/10.5897/AJMR12.1845

Kaur, M., Dhillon, S., Chaudhray, K., and Singh, R.(1998)**.***Indian Journal of Microbiology, (38):* 63-67

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurment with the Folin phenol reagent, *Journal of Biology Chemistry.* 193: 265-275.

Mahendrakar, N.S., 2000. Aquafeeds and meat quality of cultured fish. In:John, G., Ninawe, A.S. (Eds.), Aquaculture – *Feed and Health.Biotechnology Consortium India Ltd.*, New Delhi, pp. 26–30.

Nisha S. N., & J., Divakaran (2014). Optimization of alkaline protease production from Bacillus subtilis NS isolated from sea water. *African Journal of Biotechnology*, *13*(16), 1707–1713. http://doi.org/10.5897/AJB2014.13652

Pant, G., Prakash, A., Pavani, J. V. P., Bera, S., Deviram, G. V. N. S., Kumar, A., Gyana, R. (2015). Production , optimization and partial purification of protease from Bacillus subtilis. *Integrative Medicine Research*, *9*(1), 50–55. http://doi.org/10.1016/j.jtusci.2014.04.010

Santos, M. A. (1990). *Managing Planet Earth: Perspectives on Population, Ecology, and the Law*. Westport, CT: Bergin & Garvey, **p. 44**. Retrieved December 23, 2011 from Questia.com

Shafee, N., Aris, S. N., Noor, R., Abd, Z., Basri, M., Salleh, A. B., & Sciences, 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.

Sudeepa, E.S., Rashmi, H.N., Tamilselvi, A., Bhaskar, N., 2007. Proteolytic bacteria associated with fish processing wastes: isolation and characterization. *Journal of Food Science and Technology 44*,in press

Sugita, H., Shibuya, K., Shimooka, H., Deguchi, Y., 1996. Antibacterial abilities of intestinal bacteria in fresh water cultured fish. Aquaculture 145, 195–203

Sugita, H., Kawasahi, J., Deguchi, Y., 1997. Production of amylase by the intestinal microflora in cultured fresh water fish. *Letters in Applied Microbiology 24*, 105–108.

ISBN 978-967-2054-37-5

Takami, H. (1989). Applied Microbiology Biotechnology Production of extremely thermostable alkaline protease from. *Applied Microbiology and Biotechnology*, *21*, 120–124.

Triki-Ellouz, Y., Ghorbel, B., Souissi, N., Kammoun, S., and Nasri, M.(2003). Biosynthesis of protease by Pseudomonas aeruginosa MN7 grown on fish substrate, World J. *Microbiol. Biotechnol.*, *19*, 41–45.