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Utilization of Extracted Protein from Fish Fin and Chicken Feather Waste for Alkaline Protease Production by Indigenous Bacteria

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Abstract

Microbial Alkaline Proteases (APs) are of considerable interest in view of their activity and stability at alkaline pH. The present study aims to utilize keratin and collagen extracted from Fish Fin (FF) and Chicken Feather (CF) waste, respectively, for the production of AP enzyme by indigenous bacteria. Both wastes can be sources of solid waste contamination; hence, they were investigated for AP production through microbial degradation. The proteins extracted were added into the production medium containing the bacterial suspension, and assayed for AP production. The process parameters were optimized by One Factor At a Time (OFAT) and the optimum conditions for CF and FF were pH 9.0, temperature of 28°C for CF and 40°C for FF, incubation period was 6 and 10 days for CF and FF, respectively. The optimum carbon source was galactose and glucose for CF and FF, respectively, and the optimum nitrogen source was ammonium chloride and beef-extract, respectively. The inoculum size of 1.5 mL and a protein volume of 0.5 and 2.0 mL for CF and FF, respectively, was recorded. The present study indicates that the protein was successfully extracted from the waste used and degraded by AP enzyme that produced and optimized using OFAT by indigenous bacterial isolates.

Keywords: Animal waste; Environmental pollution; Indigenous bacteria; Protein Extraction.

1. Introduction

Proteases are a group of enzymes that hydrolyze the peptide bond of proteins, breaking them into polypeptides or free amino acids. They constitute 59% of the global market for industrial enzymes (Deng *et al.*, 2010). They have a wide range of application in detergents, leather, food and pharmaceutical industries (Bhaskar *et al.*, 2007 and Jellouli *et al.*, 2009). The sources of proteases include all forms of life, including plants, animals, and microorganisms. Based on their acid-base behavior, proteases are classified into three groups which are acid, neutral and alkaline proteases. The acid proteases perform best at a pH range of 2.0 - 5.0 and are mostly produced by fungi. Proteases with pH optima of 7.0 are called neutral proteases, mainly of plant origin. Proteases that have optimum activity at a pH range of 8 and above are classified as APs, mostly produced by microorganisms. Proteases produced from microorganisms play an important role in several industries, such as detergent, tanning, photographic and pharmaceutical industries (Gupta *et al*., 2002). However, the high cost and lack of long-term stability under storage and process conditions often hampered their applications (Binod *et al.*, 2013; Cavaco-Paulo and Gubitz, 2003). Pollution can be described as the introduction of contaminants which may harm or discomfort living beings into the environment. Pollutants can be in the form of naturally occurring substances or energies; however, they are considered contaminants when in excess of the natural levels (Santos, 1990). The decomposition of nutrients is facilitated by gastrointestinal bacteria through the secretion of physiologically active enzymes, amino acids, and vitamins (Sugita *et al.*, 1997). Besides, few proteolytic bacteria have been previously reported to be related to fresh water and marine fish processing wastes (Sudeepa *et al.*, 2007; Triki-Ellouz *et al.*, 2003). The catalytic properties of AP proved that it is a suitable candidate for industrial applications as in tannery and detergent formulations (Fouzia *et al*., 2017). The present study aims to extract, utilize and optimize the keratin and collagen from CF and FF, respectively, for the production of AP enzyme by indigenous bacteria. The process was optimized to achieve zero solid waste from animal sources.

2. Materials and Methods

2.1. Samples Collection and Preparation

Two types of solid waste, namely Chicken Feather (CF) and Fish Fin (FF) were collected from the wet market, Kuantan area, Pahang, Malaysia, from Sept. 2015

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to April 2016. The wastes moved to the lab immediately, washed, dried at room temperature, and blended into small pieces to a size range of about 2-3 mm (Raid *et al*., 2017).

2.2. Extraction of Keratin from Chicken Feathers

The extraction of keratin from the CF was done as described by Gupta *et al*. (2012). About 25 g of ground CF was added into 1 L of 0.5M sodium metabisulfite (pH 5.0) and incubated at 30 °C with continuous stirring for 6 h. After the incubation period, the solution was filtered and centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and filtered again using filter paper (Whatman 125 mm Ø). Then, 100 mL of the CF filtrate was added into a beaker and placed on a magnetic stirrer before adding 100 mL of ammonium sulfate drop wise. The mixture was later kept in the chiller at 4 °C for further experiments.

2.3. Extraction of Collagen from Fish Fin Wastes

The extraction of collagen from the FF was done as described by Hashemi-jokar (2014). About 5 g of the ground FF was added in 100 mL of 1M NaOH and stirred continuously using magnetic stirrer for 6 h. To get rid of the stirred non-collagen proteins, the suspension was centrifuged at 7000 rpm for 5 minutes. The pellets obtained were washed with distilled water and mixed by vortex before centrifuging again. Then, 0.5M of acetic acid was added to the pellets and kept for 3 days before centrifuging at 2000 rpm for 1 h. The supernatant was removed and the pellets washed with distilled water. The insoluble materials were soaked in 0.5M EDTA at pH 8.0 for 5 days to remove calcium. After 5 days, the pellets were washed with distilled water by centrifuging at 10,000 rpm for 1 hour. The distilled water was discarded and 0.5M of acetic acid was added until it covered the insoluble pieces. Then, ammonium sulfate was added at a ratio of 1:1 and the mixture centrifuged again at 8000 rpm for 5 minutes. The protein content of the pellets was determined using Lowry *et al*. (1951) method.

2.4. Ammonium Sulfate Precipitation

The crude protein filtrate (100 mL) was poured into a beaker and stirred before slowly adding particles of 80% ammonium sulfate into the filtrate. The calculation of the solid ammonium sulphate to be added at any concentration was obtained by the chart of (Gomori, 1955) as mentioned by (Dixon and Webb, 1964). The solution was centrifuged at 10,000 rpm for 5 minutes; the formed solids were carefully gathered by rinsing with distilled water. More precipitates were formed by the addition of more ammonium sulfate into the supernatant. The protein content was determined by Lowry method of protein assay.

2.5. Inoculum and Production Media Preparation

The bacterial suspension was prepared by mixing 1 g of the waste with autoclaved distilled water. The solution was shaken for three to five minutes; 1 mL of the clear supernatant from the waste suspension was inoculated into 50 mL nutrient broth and incubated for 48 h at 37ºC. M9 minimal salts stock solution (5X) (Sigma-Aldrich) was used (g/L): $Na_2HPO_4.7H_2O$, 64; KH_2PO_4, 15; NaCl, 2.5; NH_4Cl, 5 (Stukus, 1997), after autoclaving, the media was mixed uniformly and cooled down. Then, 2 mL of 1M $MgSO_4$, 0.1mL of 1M $CaCl_2$, and 20 mL of 20% glucose was added to the autoclaved stock solutions. 200 mL of the M9 medium was mixed with 0.5 mL of the extracted protein from CF and FF and inoculated with 1 mL of the bacterial suspension in a conical flask before incubating at 37 °C for 48 h. After the incubation period, the solution was centrifuged to get the Cell-Free Filtrate (CFF) used for AP assay (Raid *et al*., 2017).

2.6. Alkaline Protease Assay

Alkaline protease was determined using the Folin-Lowry method as described by Nisha and Divakaran (2014). A 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 of CFF and incubated for 30 minutes at 30 °C. Next, 3 mL of 5% Trichloroacetic Acid (TCA) was added and incubated for 10 minutes at 4 °C before centrifuging at 5000 rpm for 15 minutes. The supernatant (0.5 mL) was added to 2.5 mL of 0.5M of sodium carbonate, mixed and incubated for 20 minutes. Thereafter, 0.5 mL of Folin reagent was added and analyzed under UV-Vis at 660 nm. The concentration of protease was measured using a tyrosine standard graph (Takami *et al*., 1989). One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per milliliter per minute under the stated experimental conditions.

2.7. Optimization of the Parameters

To optimize the culture conditions for maximum AP production, different process parameters, such as initial pH-values (5.0, 6.0, 7.0, 8.0 and 9.0), temperature (20, 30, 40, room temperature (RT) and 50°C), carbon sources (glucose, starch, maltose, galactose, xylose, lactose and fructose), nitrogen sources (yeast extract, beef extract, peptone, urea, ammonium chloride, sodium nitrate and ammonium sulfate), incubation periods (2, 4, 6, 8 and 10 days), extracted protein volume (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mL), and bacterial inoculum sizes (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mL), were studied using One Factor At a Time method (OFAT). The effect of these parameters on the production of AP was investigated. All statistics were performed are shown as means ± standard deviations (SD) with sample size (n) indicating the number of independent experiments, and analysis of variance (ANOVA) was used to analyze the observed differences ($p<0.05$).

3. Results and Discussions

3.1. Total Protein Determination

Different quantities of FF and CF samples were used for the extraction of protein content. Table 1 shows the protein contents of the extracted samples determined by the Lowry method of protein estimation. A high protein content was observed in the supernatants of all the samples. The waste pellets were completely dissolved during the extraction steps, leaving no waste from the FF and CF. However, the protein content of FF was lower (0.852 ± 0.050 mg/mL) compared to that of CF (1.183 ± 0.035 mg/mL). The protein content of the samples was higher compared to the control. This may be due to the higher surface area of waste pellets which made more room for the interaction of the chemicals with the small pieces of FF waste. The indigenous bacteria grew well in

the minimal salt medium containing CF as the only carbon source, and degraded 91% of the CF in a period of 7 days (Avinash *et al*., 2011). Similar results were reported for other bacterial isolates (Williams *et al*., 1991) and fungal strains (Kaul and Sumbali, 1999). The pH of the medium was continuously monitored during the course of CF degradation; a gradual increase was observed from an initial value of 7.0 to 8.5; which suggests the possible deamination of peptides and amino acids resulting in the production of ammonia. Such alkalization of media was also reported in the case of keratolytic fungi (Avinash *et al*., 2011).

3.2. Alkaline Protease Assay

The AP activity of the extracts was determined in the presence of the extracted protein by the indigenous bacteria. The concentration of AP was slightly higher in CF (0.254 ± 0.001 U/mL) compared to FF (0.246 ± 0.014 U/mL). This might be due to the amount and/or the type of protein present in each sample (Table 1). The higher the protein content t in the sample, the higher the production of AP enzyme. Casein served as the substrate; tyrosine was liberated during the enzymatic degradation of the Casein either as amino acids or peptide fragments. Folin's reagent was used to develop the color from the reaction with free tyrosine. Hence, the higher the amount of tyrosine from casein, the higher the produced chromophores, and the stronger the protease activity.

Table 1. Screening of AP production and extracted protein content by indigenous bacterial isolates in both FF and CF waste samples

Samples	AP activity (U/mL)	Protein content (mg/mL)
Control	0.0273 ± 0.002	0.058 ± 0.004
CF	0.254 ± 0.001	1.183 ± 0.035
FF	0.246 ± 0.014	0.852 ± 0.050
F-value	3099.706	33.619
P-value	0.00[a]	0.03[a]

Each value represents the AP activity and protein content extracted from CF and FF waste by indigenous bacterial isolates in column of means compared to control. P-value= [a]Significant at $p < 0.05$.

3.3. Optimization of Enzyme Production

The effect of temperature on AP enzyme activity was determined at different temperatures, as presented in Figure 1. The AP enzyme was active at the temperature range of 20–50°C, with an optimum at RT (27 ± 2°C) and 40°C for both CF (0.362 ± 0.016 U/mL) and FF (0.342 ± 0.030 U/mL), respectively. The activity decreased rapidly above this temperature range.

Earlier reports have shown that protease production is maximum at 30°C, and there is a reduction in the enzyme production above this range as the enzyme undergoes thermal inactivation. The enzyme production can be affected by temperature through changes in the physical properties of the cell membrane (Goma, 2013). Nisha and Divakaran (2014) also reported that protease production was highest at 40°C using *Bacillus subtilis*. The incubation temperature to be used generally depends on the microorganisms (Gomaa, 2013). However, in the present study, a mixed culture was used, which explained why the highest temperature for alkaline protease production from the extracted proteins was different. At high temperatures, the enzyme was inactivated, resulting in the low enzyme activity at 50°C for both CF (0.195 ± 0.000 U/mL) and FF (0.201 ± 0.002 U/mL). The effect of temperature on the enzyme production was studied by varying the temperature from 24 to 39°C, with an increment of 3°C while keeping the other parameters constant. It was found that protease production was maximum (397.19 U/g) at 33°C. Also, the enzyme production was favored at a temperature range of 30 to 39°C, showing the ability of the organism to reduce protease enzyme over a wide range of temperature (Renganath *et al*., 2017).

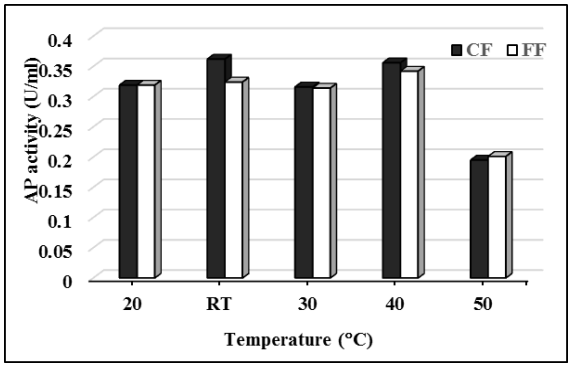

Figure 1: Effect of temperature on AP activity against extracted proteins of CF and FF samples.

Media pH strongly affects many enzymatic processes and transport of compounds across cell membranes. The maximum AP production was achieved at pH 9.0 in both CF (0.217 ± 0.013 U/mL) and FF (0.277 ± 0.034 U/mL) as substrates (Figure 2). The enzymes were inactivated in the acidic medium, resulting in low enzymatic activity. In addition, as the pH deviates from the optimal level, the enzymatic process can be altered. This indicates the low level of enzyme saturation due to pH effect on their stability (Dixon and Webb, 1979). The organism was efficient in protease production at alkaline pH conditions compared to neutral pH (Renganath *et al*., 2017). The maximum protease production was achieved at medium pH 9, while the least was recorded at medium pH 5. The production of protease increased as the pH of the medium increased towards pH 9. After pH 9, there was a decrease in the enzyme production, suggesting a stimulation of enzyme production at alkaline pH. This could be indicative of the alkalophilic nature of the microorganism (Sunita *et al*., 2016).

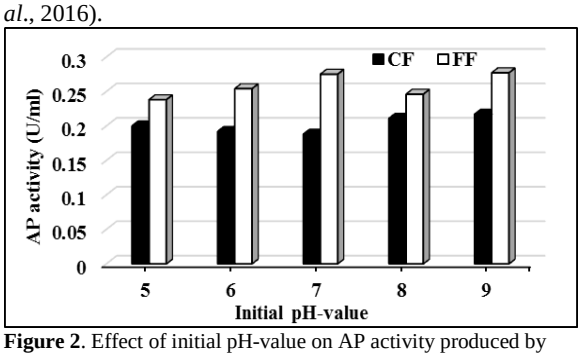

Figure 2. Effect of initial pH-value on AP activity produced by indigenous bacterial isolates from protein extracted based waste

To investigate the effect of different carbon sources on AP production by indigenous bacteria, the glucose of M9

medium was substituted with equal amounts of complex or simple carbon sources. It was observed that galactose showed the highest AP production (0.230 ± 0.011 U/mL), followed by glucose and starch (0.206 ± 0.008 U/mL) in the presence of CF as substrate. Meanwhile, glucose showed the highest AP production (0.233 ± 0.004 U/mL), followed by lactose (0.169 ± 0.010 U/mL) in the presence of FF as substrate (Figure 3). A similar effect of galactose on AP production was observed by Pant *et al*. (2015) who found that galactose gave the maximum amount of alkaline protease, while sucrose gave the lowest amount. The present study shows that the lowest enzyme activity was in the presence of maltose for CF (0.201 ± 0.004 U/mL) and starch for FF (0.148 ± 0.007 U/mL). Generally, the results obtained indicate that monosaccharide sources produced a higher amount of alkaline protease compared to disaccharide and polysaccharides. The difference in enzymatic activities on the carbon sources between CF and FF may be due to the different types of protein present in each sample. Since a mixed culture of microorganisms was used for both samples, different types of microbes utilized different carbon source to produce alkaline protease. The production of alkaline protease was dependent on the available carbon and nitrogen sources in the medium. The addition of carbon sources in the form of either monosaccharides or polysaccharides could influence the production of an enzyme (Sudharshan *et al*., 2007).

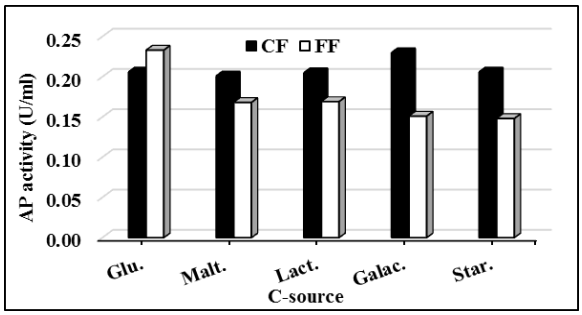

Figure 3. Effect of carbon source on AP production from extracted proteins of CF and FF waste by indigenous bacteria

Various types of nitrogen sources (including organic and inorganic) were evaluated in the M9 medium containing the extracted proteins (Figure 4). The nitrogen source in the M9 medium was substituted with a nitrogen equivalent. It was found that beef extract (0.350 ± 0.000 U/mL) and ammonium chloride (0.414 ± 0.050 U/mL) served as the best organic and inorganic sources for enhancing AP activity in presence of both CF and FF extracted proteins, respectively. Nisha and Divakaran (2014) reported that beef extracts gave the highest alkaline protease production compared to yeast extract, ammonium sulfate, ammonium chloride, urea, and peptone. The requirement for a specific nitrogen source for protease production differs from organism to organism, and also, the alkaline protease biosynthesis depends on the presence of both nitrogen and carbon sources in the production medium (Kole *et al*., 1988). The outcome of the present study is in the line with the findings of Shafee *et al*. (2005) who reported that beef extract, among the different organic nitrogen sources and ammonium chloride among the inorganic nitrogen sources, leads to a high proteolytic activity by *Bacillus sp.* after 48 h of incubation.

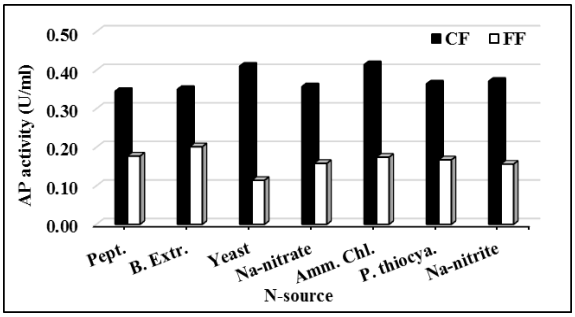

Figure 4: Effect of nitrogen source on AP production from extracted proteins of CF and FF wastes by indigenous bacteria

The optimum volume of the extracted protein required for maximum activity of AP was 0.5 mL (0.364 ± 0.016 U/mL) in CF and 2.0 mL (0.251 ± 0.060 U/mL) in FF (Figure 5). The production of alkaline protease was higher when 0.5 mL of the protein was inoculated. This may be due to the presence of more active sites for more substrate binding. Hence, more alkaline protease enzyme can be produced from lesser enzyme volumes compared to higher protein volume. Conclusively, the lowest enzymatic activity can be observed in the absence of proteins due to the reduced rate of substrate binding. Chandran *et al*. (2016) reported an increase in the protein content at 0.5% (v/v) protein volume. The proteins isolated from 0.1-0.5% (w/v) of the substrates were used as the substrate for the production of protease. The maximum protein content of 0.5% contrasted with our results at 5% protein content (the equivalent of 2.5 mL); though, 2 mL was found as the highest level for AP enzyme production.

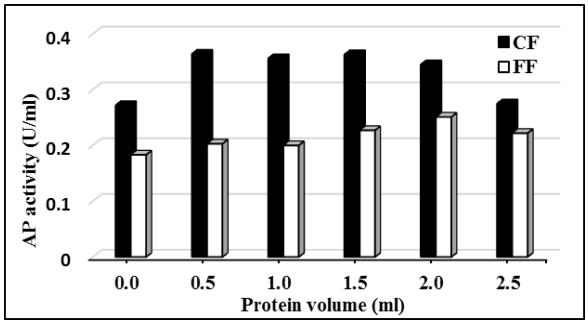

Figure 5. The effect of extracted protein volumes on AP activity by indigenous bacteria

To investigate the effect of incubation period on the production of AP enzyme, the M9 medium was inoculated and incubated at different periods ranging from 2-10 days. The maximum AP production was found after 6 and 10 days of incubation, with enzymatic activities of 0.290 ± 0.001 U/mL and 0.336 ± 0.019 U/mL in the presence of CF and FF, respectively (Figure 6). As for the CF, the production of protease declined at 10 days of incubation because, the enzyme production could have ended with auto proteolysis (Nisha and Divakaran, 2014). However, the production of AP was high after 10 days of incubation with FF likely due to the different proteins in FF compared to CF. The proteins in the FF can yield more alkaline protease when incubated for 10 days. In addition, Kaur *et al*. (1998) reported that the synthesis of enzymes can be associated with the growth of the cell and the incubation period.

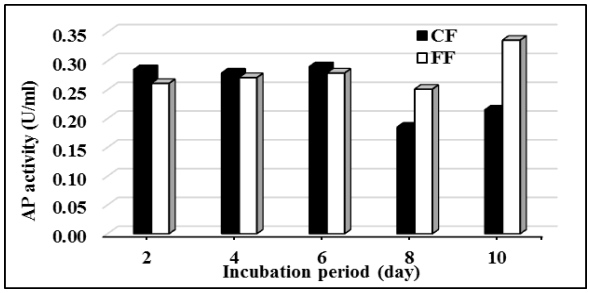

Figure 6. The effect of incubation period on AP activity by indigenous bacteria

The effect of various inoculum sizes (0.25-1.5 mL) was tested and the results are presented in Figure 7. The maximum AP activities of 0.308 ± 0.001 U/mL and 0.450 ± 0.013 U/mL were found with 1.5 mL of the indigenous bacterial inoculum in the presence of CF and FF extracted protein, respectively. Generally, it can be concluded in the present study that large sizes of bacterial inoculum produced the maximum amounts of protease in the presence of both CF and FF. This is because the increased bacterial concentration can increase AP production as more bacteria will be available to degrade the protein in the production medium. On the other hand, smaller inoculum sizes gave the lowest AP production because of the lack of enough bacteria to degrade the protein. Moreover, an upgraded distribution of dissolved oxygen and high nutrient uptake can increase AP production. However, 0.5 mL of the inoculum gave considerable results for FF as AP synthesis with small inoculum size had larger surface areas which contributed to more protease production (Shafee et al., 2005). Renganath *et al*. (2017) reported the highest the protease activity with an inoculum size of 15% after studying a concentration range of 5 to 25%. Furthermore, Divakar *et al*. (2006) reported a higher protease activity with inoculum a concentration of 20% using Wheat bran as the substrate.

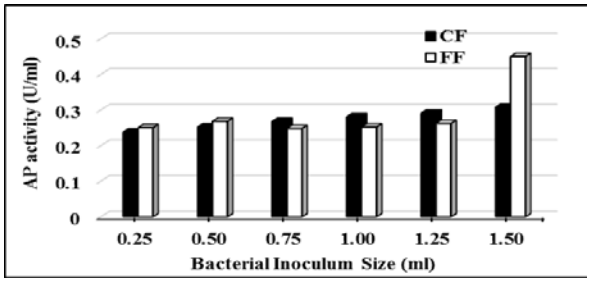

Figure 7. The effect of inoculum size on AP activity by indigenous bacteria

4. Conclusion

The ability to produce AP from waste chicken feathers and fish fins using indigenous bacteria was investigated. The M9 minimal media efficiently supported the production of AP from the extracted CF and FF proteins. The process parameters were optimized for optimum AP production and to reduce the cost of the AP production process industrially. Furthermore, the protein extraction process completely utilized the waste materials, leaving no solid waste afterward. Moreover, CF and FF are inexpensive protein sources for keratin and collagen needed for the cost-effective production of AP. It is revealed that CF and FF can be a potential source of alkaline proteases for use as bacterial additives in many industrial applications. These proteases have good activities at high alkaline pH levels and wide temperature ranges; thereby, permitting their wide biotechnological application potentials in many industries.

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Authors' Contributions

Study Design-Raid D. Thanoon and Essam A. Makky; Data Collection-Raid D. Thanoon and Rubaaini Subramaniam; Statistical Analysis-Essam A. Makky; Data Interpretation- Raid D. Thanoon, Rubaaini Subramaniam and Essam A. Makky; Manuscript Preparation and Literature Search-Raid D. Thanoon and Rubaaini Subramaniam; Funds Collection-Essam A. Makky; Manuscript Revision and Supervision-Essam A. Makky, Mashitah M. Yusoff.

Conflict of Interest Disclosure

The above-mentioned manuscript has not been published before and is not under consideration for publication anywhere else. The publication of this article was approved by all authors, as well as by the responsible authorities.

References

Avinash S, Anshul S and Vuppu S. 2011. Feather waste biodegradation as a source of amino acids. *Eur J of Exp Biol.* **1(2):** 56-63.

Bhaskar N, Sudeepa ES, Rashmi HN and Selvi AT. 2007. Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. *Bioresour Technol*. **98**: 2758-2764.

Binod P, Palkhiwala P, Gaikaiwari R, Nampoothiri KM, Duggal A, Dey K and Pandey A. 2013. Industrial enzymes present status and future perspectives for India. *J Sci Ind Res*. **72**:271-286.

Cavaco-Paulo A and Gubitz GM. 2003. **Textile Processing with Enzymes**. Woodhead Publishing Ltd & CRC Press LLC.

Chandran M, Balaji E, Vigneshwar J and Parthasarathy N. 2016. Application of response surface methodology (RSM) for protease production from *Enterococcus hirae* and using algae as substrate. *Biotechnol. BTAIJ*. **12(3)**:145-155.

Deng A, WU J, Zhang Y, Zhang G and Wen T. 2010. Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. *Bioresour Technol*. **101**: 7100-7116.

Divakar G, Sunitha M, Vasu P, Udaya Shanker P and Ellaiah P. 2006. Optimization of process parameters for alkaline protease production under solid-state fermentation by *Thermoactinomyces thalophilus* PEE 14. *Indian J Biotechnol*. **5**: 80–83.

Dixon M and Webb EC. 1964. **Enzymes**" 2nd Edit. Academic Press Inc. New York.

Dixon M and Webb EC. 1979. Enzyme Kinetics. In: Dixon M, Webb EC, (Eds). **Enzymes**. Vol. 3. Academic Press; New York. 47–206.

Fouzia H, Shagufta K, Saima R, Muhammad A, Ismat B, Tanvir A and Hafiz MN I. 2017. Alkaline protease production using response surface methodology, characterization and industrial exploitation of alkaline protease of *Bacillus subtilis* sp. *Catal Letters*. **147**:1204–1213. DOI 10.1007/s10562-017-2017-5.

Gomaa EZ. 2013. Optimization and characterization of alkaline protease and carboxymethyl-cellulase produced by *Bacillus pumillus* grown on *Ficus nitida* wastes, *Braz J Microbiol*. **44**: 529–537.

Gomori G. 1955. Preparation of buffers for use in enzyme active studies. *Method in Enzymol*. **I**, 138-146. Academic Press, London.

Gupta A, Kamarudin NB, Yeo C, Kee G, Bin R and Yunus M. 2012. Extraction of keratin protein from chicken feather. *J Chem Eng*. **6**: 732–737.

Gupta R., Beg QK, Khan S and Chauhan B. 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Appl Microbiol Biotechnol.* **60**: 381-395.

Hashemi-jokar S. 2014. Comparison of collagen extracted from the skin and fin of long tail tuna Thunnus Tonggol. *Int Res J Appl and Basic Sci*. **8(7):** 904–910.

Jellouli K, Bougatef A, Manni L, Agrebi R, Siala R., Younes I and Nasri M. 2009. Molecular and biochemical characterization of an extracellular serine-protease from *Vibrio etschnikovii*. *Microbiol Biotechnol*. **36**: 939-948.

Kaul S and Sumbali G. 1999. Production of extracellular keratinases by keratinophilic fungal species inhabiting feathers of living poultry birds (*Gallus domesticus*): A comparison *Mycopathol*., **146**: 19–24.

Kaur M, Dhillon S, Chaudhray K and Singh R. 1998. Production, purification and characterization of a thermostable alkaline protease from *Bacillus polymyxa*. *Indian J Microbiol*. **38**: 63-67.

Kole M, Draper I and Gerson DF. 1988. Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. *J Chem Technol Biotechnol*. **41**: 197-206.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951. Protein measurment with the Folin phenol reagent, *J Biol Chem*. **193**: 265-275.

Nisha SN and Divakaran JD. 2014. Optimization of alkaline protease production from *Bacillus subtilis* NS isolated from sea water. *Afr J Biotechnol.* **13(16)**: 1707-1713. http://doi.org/10.5897/AJB2014.13652

Pant G, Prakash A, Pavani JVP, Bera S, Deviram GVNS, Kumar A and Gyana R. 2015. Production, optimization and partial purification of protease from *Bacillus subtilis*. *Integr Med Res.* **9(1):** 50–55. http://doi.org/10.1016/j.jtusci.2014.04.010

Raid DT, Rubaaini S, Mahmood HM, Osama AM, Makky E A and Yusoff MM. 2017. FGIC 1st Conference on Governance & Integrity, 2017 "Innovation & Sustainability Through Governance" 3 – 4 April 2017, Yayasan Pahang, Kuantan, Malaysia. pp: 308-322. ISBN 978-967-2054-37-5

Renganath Rao R, Vimudha M, Kamini NR, Gowthaman MK, Chandrasekran B and Saravanan P. 2017. Alkaline Protease Production from Brevibacterium luteolum (MTCC 5982) Under Solid-State Fermentation and Its Application for Sulfide-Free Unhairing of cowhides, *Appl Biochem Biotechnol*. **182**: 511–528. DOI 10.1007/s12010-016-2341-z.

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

Shafee N, Aris SN, Noor R, Abd Z, Basri M and Salleh AB. 2005. Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus* Strain 146. *J Appl Sci Res*. **1(1):** 1–8.

Stukus PE. 1997. **Investigating Microbiology: A Laboratory Manual for General Microbiology**. Saunders College Publishing. Harcourt Brace, College Publishers.

Sudeepa ES, Rashmi HN, Tamil SA and Bhaskar N. 2007. Proteolytic bacteria associated with fish processing waste: Isolation and Characterization. *J Food Sci Technol*. **44(3):** 281-284.

Sudharshan RK, Dutt L and Nayyar R. 2007. A highly thermostable and alkaline amylase from a *Bacillus*. sp. PN5. *Bioresour Technol*. **21**: 25-29.

Sugita H, Kawasahi J and Deguchi Y. 1997. Production of amylase by the intestinal microflora in cultured fresh water fish. *Lett Appl Microbiol*. **24**: 105–108.

Sunita BP, Poonam BC and Mayur G. 2016. Assessment of process parameters for enhanced production of microbial alkaline protease. *Int J Adv Res Biol Sci*. **3(5):** 28-35 SOI: http://s-o-i.org/1.15/ijarbs-2016-3-5-5.

Takami H, Horikaoshi T and Akiba K. 1989. Production of extremely thermostable alkaline protease from *Bacillus* sp. No.AH-101. *Appl Microb Biotechnol.* **30**: 120-124. DOI: 10.4236/aer.2013.13005.

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.

Williams CM, Lee CG, Garlich JD and Shih JCH. 1991. Evaluation of a bacterial feather fermentation product, feather lysate, as a feed protein. *Poult Sci*. **70**: 85-94.