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Full Length Research Paper

Studies on the intestinal bacterial flora of tilapia *Oreochromis mossambicus* (Peters, 1852) and optimization of alkaline protease by *Virgibacillus pantothenticus*

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The intestinal bacterial flora of the fish *Oreochromis mossambicus* and optimization of alkaline protease production was carried out in this study. The fish, tilapia were collected from the Buckingham canal and were segregated into four size groups namely, 5 to 8, 9 to 12, 13 to 16 and 17 to 20 cm. The maximum number of gut bacteria (2.5×10^6 Cfu/g ml^{-1}) was enumerated in 5-8 cm group of fish and minimum (4.9×10^5 Cfu/g ml^{-1}) in 17 to 20 cm. The isolated strains were identified as *Virgibacillus pantothenticus, Bacillus cereus, Bacillus licheniformis, Enterococcus faecalis* and *Virgibacillus alginolyticus.* Among the isolated bacteria, *V. pantothenticus* gave the maximum yield of alkaline protease at pH of 9.5 and 40°C temperature. It is also inferred from the study that the seaweed, *Sargassum tenerrimum* and oil sardine, *Sardinella longiceps* fish waste were found as good carbon and nitrogen sources respectively for the production of alkaline protease. The studies on fish gut microflora are very much needed for the management both in aquaculture and public health. The result of alkaline protease production by *V. pantothenticus* is encouraging and it can be applied for bioconversion of fish waste and sea weed from *aquafeed* formulation and subsequent reduction in feed related waste outputs.

Key words: Intestinal bacterial flora, *Oreochromis mossambicus,* alkaline protease, *Virgibacillus pantothenticus.*

INTRODUCTION

Tilapia (*Oreochromis mossambicus*) is the third most important fish in aquaculture after carps and salmonids, with production reaching 1,505,804 metric tons in 2002 according to Fessehaye (2006). Globefish, a report from the U.S. total imports in 2007, was estimated at 170 000 tonnes. Taking a conversion factor of 2.85 for tilapia fillets, this gives about 400 000 tonnes of tilapia. Worldwide tilapia production should reach three million metric tons in future years. Intestinal microflora of animals can be changed by age, nutritional condition, developmental stages and stress. These changes can tempt disease and vice versa (Campbell and Buswell, 1983), so it may be possible to assay the susceptibility of

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fish to disease by observing the composition of gut microflora. If the onset of disease could be detected as early as possible, even before the symptoms appear, it will save a lot of time and labor. One of the possible detection methods would be to observe the intestinal microflora in fish. There are many reports concerning the normal microflora in marine (Aiso, 1968; Liston, 1957) and fresh water fish have been published. There is evidence that dense microbial population occurs within the intestinal contents, with numbers of bacteria much higher than those in the surrounding water, indicating that the intestine provides favourable ecological niches for these organisms (Austin and Austin, 1987).

In the aquaculture industry, microbial enzymes are very much essential for the preparation of high quality functional feeds through bioconversion of cost-effective feed materials. This is because the world wide sustainability of the aquaculture industry depends only on the inexpensive high quality feeds. However, very limited information is available in the area of gut bacterial enzymes especially proteases and its role in the intestine. In recent years, many results have also been revealed that alkaline protease in the intestine of fish can help in digesting the protein contained in the feed and also regulate the compound diets in the components. Therefore, alkaline proteases in the gut of fish have received much attention in recent years (Chong et al., 2002; Fu et al., 2005).

Further, the knowledge of the intestinal bacterial flora of commercially important fishes was found to be indispensable for two reasons, 1) Control of fish diseases, and 2) Preparation and handling of the proper fish feed. Tilapia is considered as a good candidate species for studying intestinal flora because of its ability to adapt to both freshwater and seawater. Its omnivorous diet includes phytoplankton, algae, and sometimes water plants (Sakata et al., 1980). The digestive tract of Tilapia is 5-7 times as long as its body length and abundant in commensal microbes. Due to this reason, the present research was carried out for studying the intestinal microflora of tilapia and was also focused on enzyme producing ability of isolated strains.

MATERIALS AND METHODS

Fish sample collection

The fish sample collection for the quantitative and qualitative analysis of gut microflora was carried out during the period of February to July 2009. The live and freshly dead fish samples of *O. mossambicus* were collected in morning times uniformly in the last week of every month from Buckingham canal near the Vellar Estuary. The collected samples were kept in sterile bags and ice boxes to avoid the multiplication of microorganism. The samples were immediately transferred to the laboratory and the fish were identified based on the morphometric and meristic key characters by Trewevas (1983).

Isolation of gut microflora

For every sampling, 25 to 30 fish with different size groups were collected for qualitative and quantitative analysis of gut microflora. Based on the length, fish were categorized into four groups (such as 5 to 8 cm < 9 to 12 cm < 13 to 16 cm and < 17 to 20 cm) and on the different dilution the qualitative analysis was carried out. The live fish were killed by physical destruction of the brain. The numbers of incidental organisms were reduced by washing the fish skin with 70% ethanol before opening.

The ventral surface of the fish was cut open with sterile scissors and then 1 g of gut content was taken aseptically. The content was homogenized with glass mortal adding 1 ml of 50% sterile sea water and transferred to 9 ml sterile blank solution. The homogenate was serially diluted upto 10^{-6} dilution and 0.1 ml of each dilution was spread onto Petri plates containing Zobell Marine Agar for enumeration of total heterotrophic bacteria. The plates were incubated at 37°C for 24 h, after which the total CFU (colony forming units) was calculated. The morphological observations for each colony were recorded and then isolated colonies were picked up for pure culture. The colonies were subjected to microscopic observation and biochemical tests for their identification using Bergey's manual of determinative bacteriology (Holt, 1986).

Screening for alkaline protease production by the isolated strains

The isolated strains were screened for alkaline protease production on 2% Gelatin agar (GA) medium. The GA media were prepared, sterilized and poured in Petri plates. After solidification, the isolated strains were streaked on the plates separately and incubated at 28°C for 48 h. After incubation, the incubated plates were flooded with $HgCl_2$ and the zone of clearance was observed. The formation of clear zone around the colony was considered as positive result. Further, the maximum activity shown strain (*Virgibacillus pantothenticus*) was inoculated into the 100 ml basal broth medium (glucose - 5 g/l; peptone- 5 g/l; skimmed milk - 2.5 g/l; yeast extract - 2.5 g/l; Na_2HPO_4 -1 g/l; $NaNO_3$ .10 g/l; pH -9.0) as described by Anandan et al. (2007) for further production and the flask was incubated at 28°C for 16 to 18 h. After incubation the culture medium was centrifuged at 3000 rpm for 45 min. The cell free supernatant was used for determining the enzyme activity and protein concentration. All the experiments were carried out in triplicate and average values were reported.

Protease assay

General proteolytic activity of culture supernatant was determined by the modified method of Keay and Wildi (1970). The reaction mixture containing 1 ml of 2% casein solution and 1 ml of diluted enzyme were incubated at 37°C for 10 min. Then the reaction was terminated by the addition of 2 ml of 0.4 M TCA and the mixture was incubated at 37°C for 20 min. The incubated solutions were filtered through Whatman No.1 filter paper. 1 ml of filtrate with 5 ml of 0.4 M Na_2CO_3 and 1ml of 0.5 M Folin phenol reagent were added and mixed well. Again the mixtures were incubated at 37°C

for 20 min and the final solution was measured at 660 nm by a spectrophotometer. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine in 20 min at 37°C. The amount of protein was spectroscopically estimated by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as the standard.

Partial purification

The cell-free supernatants were precipitated with 85% ammonium sulfate for 3 h at 4°C and the pellets were dissolved in small quantities of 20 mM Tris-HCl buffer, pH 7.4 and then dialyzed for 6 h against the same buffer. The partially purified enzymes were used for further studies.

Sephadex column chromatography

The dialyzed sample was loaded onto a Sephadex G-100 column, which was pre equilibrated with 20 mM Tris-HCL buffer containing 0.5 M NaCl at a flow rate of 6 ml per h and the active fractions were pooled together. Enzyme assay was carried out after each step to access the purification fold of the enzyme and characterized by lyophilization and used for further studies.

Optimization for alkaline protease production

The culture condition such as pH, temperature, various carbon and nitrogen sources that influence the production of proteases were optimized by varying the condition one at a time. The experiments were conducted in 250 ml Erlenmeyer flask containing production medium (Basal broth medium devoid of carbon and nitrogen sources). Effect of different pH (7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0) and temperature (28, 30, 35, 40 and 45°C) on alkaline protease production in basal broth medium inoculated with *V. pantothenticus* was performed.

To test the effect of carbon and nitrogen sources, the medium was supplemented with different carbon sources (Seaweed; *Sargassum tenerrimum,* sucrose, maltose, glucose and starch, each at 0.5 to 3%) and nitrogen sources (casein, fish waste (Oil sardine, *Sardinella longiceps*), skim milk, yeast extract, and sodium nitrate, each at 0.5 to 3%. The optimum pH and temperature are adjusted for alkaline protease production. After sterilization by autoclave, the flasks were cooled and inoculated with culture of *V. pantothenticus* and maintained at various operational conditions separately. After 48 h incubation period, the culture filtrate was assayed at triplicate samples for protease assay as well as for protein determination.

RESULTS AND DISCUSSION

Qualitative studies on gut microflora of *O. mossambicus*

Results on the qualitative analysis of gut microflora of *O. mossambicus* based on the length and on the different dilution are given in Figure 1. Length between 5 to 8 cm fishes possessing the bacterial loads with different dilutions ranged from 3.7×10^4 to 2.5×10^6 CFU/ml. The results of analysis of variance were significant ($P<0.05$) both in different lengths and various dilutions. The fishes ranged between 9 and 12 cm, the bacterial load ranged from 4.1×10^4 to 1.0×10^6 CFU/ml, whereas in the case of ANOVA both length and different dilutions results showed significant ($P<0.05$) values. Fishes had between 13 to16 cm lengths, and the bacterial load ranged from 3.9×10^4 to 8.3×10^5 CFU/ml. The ANOVA between lengths observed was significant ($P<0.05$) but in different dilutions showed non-significant values. In fish length between 17 to 20 cm, the bacterial load ranged from 4.1×10^4 to 4.9×10^5 CFU/ml. ANOVA showed significant (0.05) results in lengths and non-significant values in the different dilutions. In an ecosystem, the presence of microorganisms is regulated by local, ecological and physiological conditions in each of the locations, which differ among geographical and tropical niche.

Results of the present study revealed the high bacterial load only in a limited number of fishes. Tilapias have a high level of immunity compared to other fresh water fishes that guard against the infection which wipes out the whole population. In addition, the gastric fluids of tilapia are quite acidic. It is inferred from the study that high bacterial load in certain fish is mainly due to poor water quality and also cleaning of cattle along the canal which ultimately leads to the formation of hydrogen sulphide in the study area. According to Rheinheimer (1985), the bacterial load in the gut of tilapia was high and the reasons may be that the high ambient temperature in pond water was close to optimum for many mesophilic bacteria in natural systems. Al-Harbi and Uddin (2005) reported that the presence of a high bacterial load in gill and intestine of fish might be due to high metabolic activity of fish associated with increased feeding rates at higher temperature. They also reported that pond water and sediment bacteria influenced the bacterial composition of gills and intestine of tilapia.

According to Seki (1969), the bacterial load in a given time in the alimentary canal of a fish depends on the quantum and type of food recently ingested. Sakata et al. (1978) reported that the lower counts obtained are more likely to represent a realistic estimate of the size of aerobic bacterial population attached to, or in intimate association with, the gut epithelium. Further, throughout the present period of survey, there is no marked difference in the bacterial load in different size groups of tilapia. Yoshimizu et al. (1980) and Sugita et al. (1982) reported that the intestinal microflora of Salmonids fishes and tilapia changes with the development of fish, the possibility that the adult grass carp differs from the

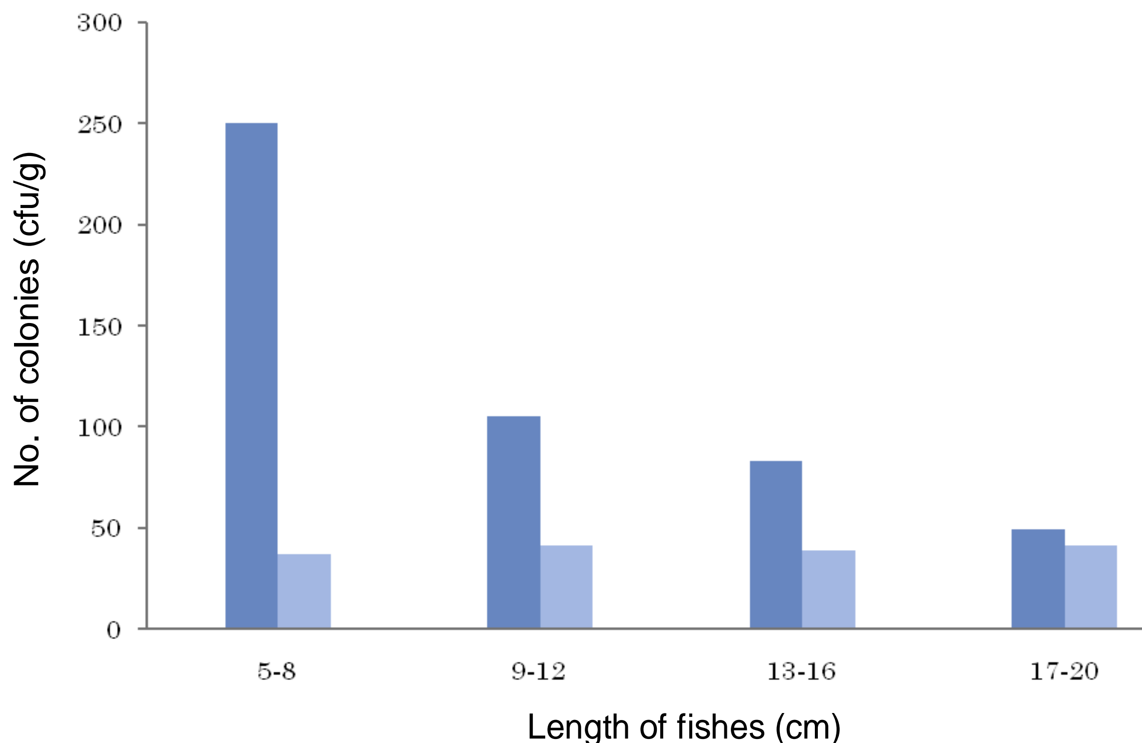

Figure 1. Gut bacterial load in *Oreochromis mossambicus*.

juvenile remains. MacDonald et al. (1986) suggested that the decline in numbers between the juvenile and adult fish is up till now an undescribed phenomenon, possibly reflecting subtle differences in the age of the surface with increasing age of the animal.

Quantitative studies on gut microflora of *O. mossambicus*

Totally, five different bacterial strains were isolated from the gut of *O. mossambicus* and were identified as *V. pantothenticus, Bacillus cereus, Bacillus licheniformis, Enterococcus faecalis and Vibrio alginolyticus.* In this study, 90% of the isolated bacteria were observed as Gram positive in the intestinal tract of tilapia (*O. mossambicus*). The bacterial strains isolated from the gut were identified as *B. cereus, B. licheniformis*, *V. pantothenticus, E. faecalis* and Gram negative *V. alginolyticus*. *Vibrio* sp. was present in small size group fish (5 to 8 cm) which has poor immune system. Sakata et al. (1984) observed that *Vibrio, Aeromonas* and *Pseudomonas* were predominant bacterial genera in tilapia intestine. However, it was different with Chowdhury et al. (1989), where *Micrococcus* sp. was the dominant bacterium in the intestine of tilapia which was not isolated from the tilapia fish in our finding. Mary et al. (1975) observed that *Bacillus* and *Corynebacterium* were predominant groups to tolerate the adverse effects of digestive enzymes, hence showed an increase in percentage.

Screening of isolated strains on protease production

Five bacterial strains were isolated from fish gut of *O. mossambicus*. All these strains were screened for the protease production in gelatin agar plate. Among these strains, *V. pantothenticus* showed the highest activity compared to others and hence used for alkaline protease production.

Effect of different pH on the production of alkaline protease

Results on the effect of different pH on alkaline protease production in basal broth medium inoculated with

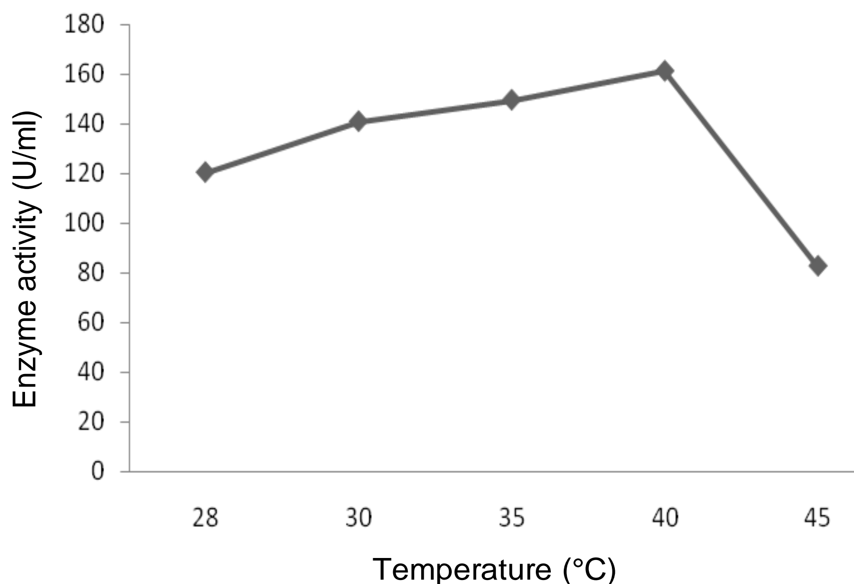

Figure 2. Alkaline protease production at different temperature.

V. pantothenticus are shown in Figure 2. The *V. pantothenticus* was found to grow well at pH ranges between 7 and 9.5. Alkaline protease production was found to be maximum at pH 9.5 and from the levels of pH 10 showed reduction in enzyme activity. In the present study, the five bacterial strains were separately screened for their proteolytic activity at various extents. The maximum zone of hydrolysis was observed in *V. pantothenticus* than the other isolates and selected for further optimization studies. In the present study, *V. pantothenticus* was found to grow well at pH ranges between 7 and 9.5. Alkaline protease production was found to be maximum at pH 9.5 and from the levels of pH 10 showed reduction in enzyme activity. Earlier studies also strongly supported that the protease production in *V. pantothenticus* was particularly sensitive to acidic pH as reported by Gupta et al. (2008).

Maximum protease production was obtained at pH 9.0. The protease production was to be thermostable alkaline by retaining its 100 and 85% stability at pH 10. Kumar et al. (2002) also reported that protease production was maximum at pH 7 and 9 for *Bacillus* sp. In the present study, results were in line with Kaliarasi and Sunitha (2009). They reported that the optimum pH for alkaline protease activity was at pH 9 and the reducing activity was observed at pH 10. Gupta et al. (2002) mentioned that alkaline protease was useful for detergent applications and was mostly active at 27% at pH 6.0 and lost 20% of its activity at pH 12.0. Gessesse (1997) and Mabrouck et al. (1999) studied that 100% of enzyme activity was retained in the pH range of 6 to 11 and the optimum pH value was 10.

Effect of different temperatures on the production of alkaline protease

A result on the effect of different temperature on alkaline protease production in basal broth medium inoculated with *V. pantothenticus* is shown in Figure 3. The optimum temperature for maximum alkaline protease production in *V. pantothenticus* was found at 40°C; however a reduction of enzyme production was observed at 45°C. In the present study, *V. pantothenticus* was capable of producing protease in the range of 28 to 40°C. The present investigation was in line with the study of Gupta et al. (2008), while the experiment of Beg and Gupta (2003) showed maximum protease activity at 60°C. This could be attributed to the addition of additives such as $CaCl_2$, which was not followed in the present study.

Similar results were found in the experiments of Johnvesly and Naik (2001) where the addition of Ca^{2+} ion gave the maximum yield at an optimum temperature of 77 to 80°C. Frankena et al. (1986) studied that a link existed between enzyme synthesis and energy metabolism in *Bacilli,* which was controlled by temperature and oxygen uptake. Shanthakumari and Ramesh (2009) stated that the protease showed maximum activity at 50°C, and the thermostable alkaline protease was characterized and exhibited maximum

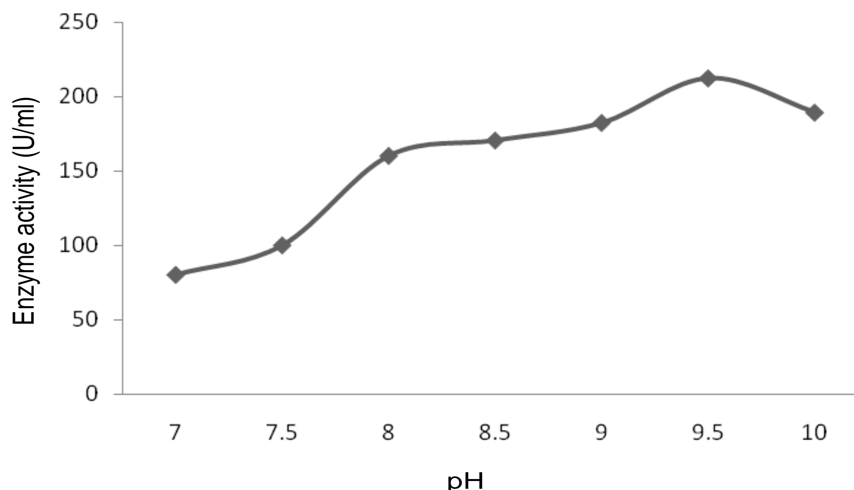

Figure 3. Alkaline protease production at different pH.

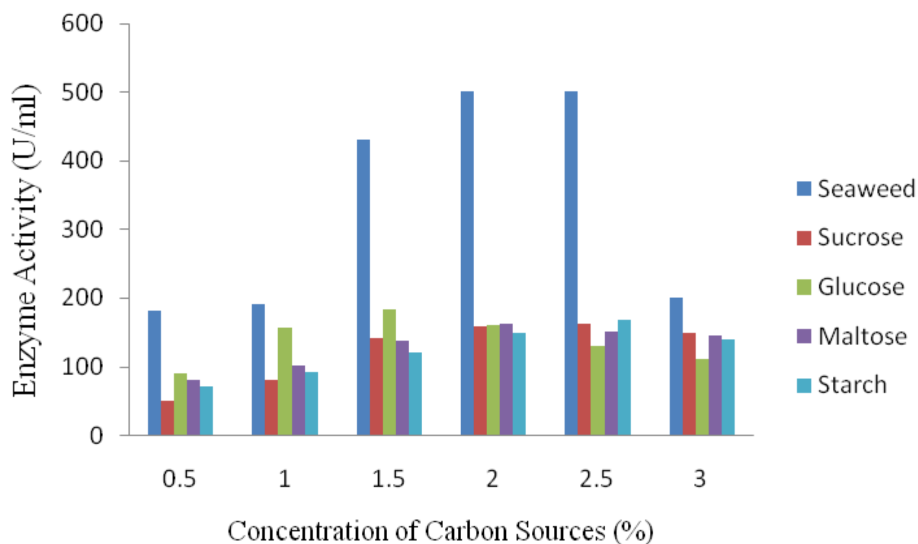

Figure 4. Alkaline protease production at different carbon sources.

activity at 36°C. Based on the observations by the present study, at 40°C, protease production was observed to be steady and in 45°C the protease production declined. It may be due to the stability and temperature tolerance of the strain.

Effect of different carbon sources on the alkaline protease production

Results on the different carbon sources (sucrose, glucose, maltose, seaweed and starch) were used to optimize the alkaline protease production in basal broth medium inoculated with *V. pantothenticus* as shown in Figure 4. The present study of the seaweed (*S. tenerrimum*) was considered as a good carbon source among these carbon sources, fructose and sucrose also showed high protease expression at 24 h, but drastically reduced by 48 h of incubation.

The addition of carbon sources in the form of either monosaccharide or polysaccharide could influence the production of enzyme (Sudharshan et al., 2007). It is inferred from the present study that the seaweed (*S. tenerrimum*) was considered as a good carbon source

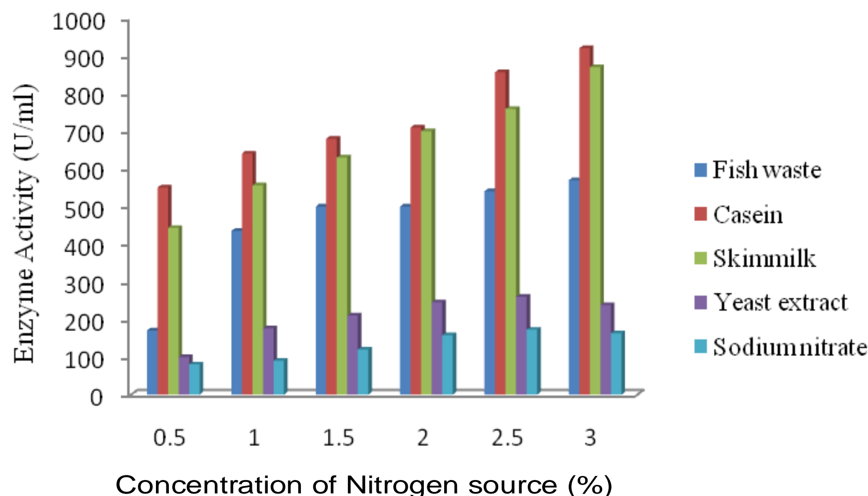

Figure 5. Alkaline protease production at different nitrogen sources.

because it provides maximum alkaline protease production when compared with other carbon sources. This may be due to the presence of high percentage of carbon (80%) and also the presence of cellulose in sea weed (Yoshiaki and Junji, 2004). Shafee et al. (2005) tested various carbon sources such as glucose, fructose, sucrose, maltose, starch and cellobiose were used to replace lactose which was the original carbon source in the growth media. Tari et al. (2006) screened different carbon sources, such as glucose, glucose syrup, fructose corn syrup, maltose (50%), glucose-fructose (30%), natural corn starch, potato starch, molasses and whey were used for alkaline protease production. Except for whey, molasses and starch, the rest of the carbon sources gave the satisfactory specific protease activity; the lowest specific activity was obtained with whey and the highest with maltose. Glucose-fructose of 30% and maltose of 50% can be considered as the best carbon sources. This observation agrees with previous report which suggested that the source of carbon affected the enzyme production by bacteria (Juhasz, 2003). Sonnleitner (1983) studied that starch, sucrose, and lactose proved appreciably good for the protease production. Lactose, starch, soy meal and sucrose are considered good for industrial protease production.

Effect of different nitrogen sources on the alkaline protease production

Five nitrogen sources were tested for the production of alkaline protease enzyme. These include casein, skim milk, fish waste (oil sardine), yeast extract, and sodium nitrate. Results on the effect of different nitrogen sources on alkaline protease production in basal broth medium inoculated with *V. pantothenticus* are shown in Figure 5. The alkaline protease production was influenced to a great extent by the dominance of casein along with the other nitrogen sources. The protease exhibited maximum activity toward casein. The present study reported that the casein, skim milk and fish waste (oil sardine) are incorporated in the production medium and enhanced the alkaline protease production and this is in accordance with the observations made by earlier workers from different parts of the world. Esakkiraj et al. (2007) suggested the defatted fish meat was found to be a better nitrogen source for maximizing protease production than the other tested preparations including the control medium with commercial peptone, alkali hydrolysates of tuna waste, and raw fish meat added medium.

The development of protease production in the defatted fish meat added medium may be attributed to the lipid free nature of the product, which could support the protease synthesis by the candidate species than other nitrogen source preparations. The protease production was high in defatted tuna waste meat supplemented media; its different concentrations were tested for protease production. 3% was found to be optimum to produce maximum protease and above this concentration the enzyme production declined. Despite the luxurious bacterial growth, the presence of yeast extract, peptone and tryptone resulted in low protease production. Oskouie et al. (2007) noted that the highest protease activity in *Bacillus clausi* was achieved in the presence of

yeast extract and potassium nitrate as organic and inorganic nitrogen sources. Gupta et al. (2008) suggested that casein was found to be the most suitable substrate followed by skim milk for maximum production of protease.

Purification of alkaline protease

The protein pellet obtained after acetone precipitation was dissolved in Tris HCl buffer and loaded onto sephadex G-100 column equilibrated with same buffer. From the elution profile, it was observed that the protease was eluted as well, resolved peak of casinase coinciding with a single protein peak at NaCl concentration of 0.5 mM. The fraction 10 to 21 exhibited higher protease activity among which fraction 1.9 was found to be maximum. The fractions were pooled, dialyzed and concentrated by lyophilization (IIshin, TDF 550) and used for further studies. Each purification step increased the specific activity and at the end of gel filtration chromatography purification fold of 12.1 was achieved with a specific activity of 5350 IV/mg.

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REFERENCES

Aiso K, Simidu U, Hasuo K (1968). Microflora in the digestive tract of inshore fish in Japan. J. Gen. Microbiol., 52: 361–364.

Al-Harbi AH, Uddin N (2005). Bacterial biodiversity of tilapia (*Oreochromis niloticus*) cultured in brackish water in Saudi Arabia. Aquaculture, 250: 566-572.

Anandan D, Marmer WN, Dudley RL (2007). Isolation, characterization and optimization of culture parameters for production of an alkaline protease isolated from *Aspergillus tamarii*. J. Ind. Microbiol. Biotechnol., 34: 339-347.

Austin B, Austin DA (1987). Bacterial fish pathogens: diseases in farmed and wild fish. Ellis Publishers, Chichester, P. 364.

Beg QK, Gupta R (2003). Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. Enzyme Microb. Technol., 32: 294-304.

Campbell AC, Buswell JA (1983). The intestinal microflora of farmed Dover sole (*Solea solea*) at different stages of fish development. J. Appl. Bacteriol., 55: 215-223.

Chong ASC, Hashim RH, Yang LC, Ali AB (2002). Partial characterization and activities of protease from the digestive tract of discus fish (*Symphysodon acquifasciata*). Aquaculture, 203: 321-333.

Chowdhury MBR, Muniruzzaman M, Uddin MN (1989). Study on the intestinal bacterial flora of tilapia *Oreochromis niloticus*. Bangladesh J. Aquac., 11: 65-70.

Esakkiraj P, Immanuel G, Sowmya SM, Iyapparaj P, Palavesam A (2008). Evaluation of protease producing ability of fish gut isolate Bacillus cereus for aqua feed. Food Bioprocess Technol., 2(3): 383-390.

Fessehaye Y (2006) Natural mating in Nile tilapia (*Oreochromis niloticus* L.) Implications for reproductive success, inbreeding, and cannibalism. Wageningen: Wageningen UR, P. 150.

Frankena J, Vanverseveld HW, Stouthamer AH (1986). A continuous culture study of the bioenergetics aspects of growth and production of exocellular protease in *Bacillus licheniformis*. Appl. Microbiol. Biotechnol., 22: 169-176.

Fu XY, Xue CH, Miao BC, Li ZT, Gao X, Yang WG (2005). Characterization of protease from the digestive tract of sea cucumber (*Stichopus japonicus*): high alkaline protease activity. Aquaculture, 246: 321-329.

Gessesse A (1997). The use of nug meal as low cost substrate for the alkaline proteases by the alkalophilic *Bacillus* sp. AR-009 and some properties of the enzyme. Bioresour. Technol., 62: 59-61.

Gupta A, Joseph B, Mani A, Thomas G (2008). Biosynthesis and properties of an extracellular thermostable serine alkaline protease from *Virgibacillus pantothenticus*. World J. Microbiol. Biotechnol., 24: 237-243.

Gupta R, Beg Q, Khan S, Chauhan B (2002). An overview on fermentation, downstream processing and properties of microbial alkaline protease. Appl. Microbiol. Biotechnol., 60: 381–395.

Holt JG (1986). Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, p. 1.

Johnvesly B, Naik GR (2001). Studies on production of thermostable alkaline protease from thermophilic and alkalophilic *Bacillus* sp. JB-99 in a chemically defined medium. Process Biochem., 37: 139-144.

Juhasz T, Kozma K, Szengyel Z, Reczey K (2003). Production of beta Glucosidase in mixed culture of *Aspergillus niger* BKMF 1305 and *Trichoderma reesei* RUT C30. Food Technol. Biotechnol., 41: 49-53.

Kaliarasi K, Sunitha U (2009). Optimization of alkaline protease production from Pseudomonas fluorescens isolated from meat waste contaminated soil. Afr. J. Biotechnol., 8(24): 7035-7041.

Keay L, Wildi BS (1970). Proteases of genus *Bacillus*. I. Neutral proteases. Biotechnol. Bioeng., 12: 179–212.

Kumar D, Gajju H, Bhalla TC (2002). Production of a thermostable protease by *Bacillus* sp. APR-4. *A.* J. Microbiol. Biotechnol. Environ. Sci., 4: 533-540.

Liston J (1957). Occurrence and distribution of bacterial types on flat fish. J. Gen. Microb., 16: 205-216.

Lowry OH, Rosebrough N, Farr A, Randall R (1951). Protein measurement with Folin phenol reagent. J. Biol. Chem., 193: 265–275.

Mabrouck SS, Hashem AM, El-Shayeb NMA, Ismail MS, Abdel-Fattah AF (1999). Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415. Bioresour. Technol., 69: 155–159.

MacDonald NL, Stark JR, Austin B (1986). Bacterial microflora in the gastrointestinal tract of Dover sole (*Solea solea* L.), with emphasis on the possible role of bacteria in the nutrition of the host. FEMS Microbiol. Lett., 35: 107–111.

Mary PP, Chandramohan D, Natarajan R (1975). The gut microflora of some commercially important fishes from Portonovo waters. Bull. Dept. Mar. Sci., 7(1): 185-199.

Oskouie SFG, Tabandeh F, Yakhchali B, Eftekhar F (2007). Enhancement of alkaline protease production by *Bacillus clausii* using Taguchi experimental design. Afr. J. Biotechnol., 6(22): 2559-2564.

Rheinheimer G (1985). Aquatic microbiology, 3rd ed. University of Kiel, West Germany. John Wiley and Sons, Chichester, New York, Brisbane, Tornoto, p. 257.

Sakata T, Nakaji M, Kakimoto D (1978). Microflora in the digestive tract of marine fish. I. General characterization of the isolates from yellow tail. Mem. Fac. Fish., 27: 65-71.

Sakata T, Okabayashi J, Kakimoto D (1980). Variation in the intestinal microflora of Tilapia reared in fresh water and seawater. Bull. Jpn. Soc. Sci. Fish., 46(3): 313-317.

Sakata T, Uno K, Kakimoto D (1984). Dominant bacteria of the aerobic

microflora in tilapia intestine. Bull. Jpn. Soc. Sci. Fish., 50: 489– 493.
Seki H (1969). Marine microorganisms associated with the food of young salmon. Appl. Microb., 17, 252-255.
Shafee N, Aris SN, Rahman RNZA, Basri M, Salleh AB (2005). Optimization of environmental and nutritional conditions for the alkaline protease by a newly isolated bacterium *Bacillus cereus* strain. J. Appl. Sci. Res., 1(1): 1-8, 146
Shanthakumari AR, Ramesh S (2009). Production and optimization of thermostable alkaline protease by Vibrio alginolyticus. Rec. Res. Sci. Technol., 1(5): 255-258.
Sonnleitner B (1983). In: Advances in biochemical engineering (Fiechter A ed.,) Springer-Verlag, p. 66.
Sudharshan RK, Dutt L, Nayyar R (2007). A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. Bioresour. Technol., 21: 25-29.
Sugita H, Ishida Y, Deguchi Y, Kadota H (1982). Aerobic microflora attached to the wall surface in the gastro-intestine of *Tilapia nilotica*. Bull. Coll. Agric. Vet. Med. Nihon Univ., 39: 302–306.
Tari C, Genckal H, Tokatali F (2006). Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. Process Biochem., 41: 659–665.
Trewevas E (1983). Tilapiine fishes of the Genera *Sarotherodon, Oreochromis* and *Danakilia*. British museum of Natural History, Publication Number 878.*Comstock Publishing Associates*. Ithaca, New York, p. 583.
Yoshiaki Y, Junji K (2004). The carbon, nitrogen, and phosphorus content of kajime (Seaweed; Ecklonia cava) which are grown on the artificial leaf. *Kanagawaken Kankyo Kagaku Senta Gyomu,* pp. 89-92.
Yoshimizu M, Kimura T, Sakai M (1980). Microflora of the embryo and fry of salmonids. Bull. Jpn. Soc Sci. Fish., 46: 967-975.