International Multidisciplinary Research Journal 2012, 2(6):56-59
ISSN: 2231-6302
Available Online: http://irjs.info/

INTERNATIONAL MULTIDISCIPLINARY RESEARCH JOURNAL

Isolation and screening of proteolytic bacteria from freshwater Fish *Cyprinus carpio*

Balaji N [1], Rajasekaran K M [1], Kanipandian N [2], Vignesh V [2] and Thirumurugan R [2*]

[1]Department of Microbiology, The Madura College (Autonomous), Madurai – 625 011, Tamilnadu, India.
[2*]Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli-620 024, Tamilnadu, India.

Abstract
The present study was undertaken to isolate and characterize the protease producing potent bacteria from gastro intestinal tract of freshwater fish *Cyprinus carpio*. The protease producer was characterized as *Bacillus* sp. morphologically and biochemically. The optimization studies for growth and protease production of the isolate *Bacillus* sp. was carried out. The optimum temperature for the growth of isolated *Bacillus* sp. was 35^{0}C but the optimum temperature for the protease production was 45^{0}C, the optimum pH was 7.0 in which the isolate produced 18 mMol/ml and showed a remarkable number of Colony Forming Unit (CFU). Hence, it was suggested that protease enzyme production using bacteria could be a cheap and cost effective approach.

Keywords: Protease, *Bacillus* sp., *Cyprinus carpio,* pH and temperature

INTRODUCTION

The gastro intestinal tract of fish is filled with a number of beneficial bacteria called probiotics. The term probiotic means for life, as opposed to the term antibiotic which means against life [1]. Fish bodies have a symbiotic relationship with probiotics. They help us to digest food, kill harmful microorganisms and keep us functioning properly [2].

Fish get bacteria in the digestive tract from aquatic environment through water and food which are populated with bacteria. Being rich in nutrient, the environment of the digestive tract of fish confers a favorable culture environment for the microorganisms. There are only a few probiotics in the gut as it is highly acidic. The number of these beneficial bacteria increases dramatically in the intestine [3].

Proteolytic enzymes are produced by a variety of microorganisms and played an important role during fish sauce fermentation. Several protease-producing bacteria found in fish sauce fermentation, including halophilics, halotolerants and lactic acid bacteria. These bacteria hydrolyze fish protein to peptides and amino acids [4]. Protease-producing bacteria found in fish sauce are *Pseudomonas* sp. [5], *Bacillus* sp. *Micrococcus* sp. *Staphylococcus* sp. *Streptococcus* sp. *Pediococcus* sp. Coryneforms [6,7], *Halobacillus thailandensis* sp. nov.,[8], *Tetragenococcus halophililus* and *T.muriaticus* [9].

In the present study, an attempt has been made to isolate the potent protease producer and investigate the effect of physical factors like temperature and pH on protease producing bacteria *Bacillus* sp. isolated from gastrointestinal (GI) tracts of freshwater fish *Cyprinus carpio*.

Received: April 12, 2012; Revised: May 15, 2012; Accepted: June 02, 2012.

*Corresponding Author
Dr. R.Thirumurugan
Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli-24, Tamilnadu, India.

Tel: +91-94430 94199; Fax: +91-431-2407045
Email: ramthiru72@gmail.com, thiru72@rediffmail.com

MATERIALS AND METHODS

Collection of fish

The freshwater fish *Cyprinus carpio* was collected from the A.M. Aqua farm in Madurai. They were let to acclimatize for 10 days in laboratory. The number of incidental organism was reduced by washing fish skin with 70% ethanol. Then, the ventral surface was opened with sterile scissors. After dissecting the fish, 1*g* of the intestinal tract content of each fish was removed and homogenized with 0.1 mM of phosphate buffer solution under aseptic condition.

Isolation of Bacteria from GI tract

Serial dilution of Fish gut sample was performed using sterile saline. The dilutions were plated on Nutrient agar medium using spread plate method and incubated at 37°C for 24 hours.

Screening of Protease producer

The random individual colonies were picked and cultured separately on nutrient agar medium. The purified isolates were subcultured on the same medium. Proteolytic activity was tested using skim milk salt agar. A single colony of each strain was streaked on skim milk salt agar and incubated at 35ºC under aerobic condition for 5-7 days or until growth was observed. A positive reaction for the proteolytic test was indicated by clear zone around the colony and was collected for proteolytic activity measurement.

Characterization of bacterial isolate

The isolated bacteria from fish gut was identified using the guidelines described in the Bergey's Manual of Systematic Bacteriology [10]. It was maintained in nutrient agar slants using streak plate technique. The isolate was stored at 4°C in refrigerator

and was used for further process in the production of Protease.

Impact of pH and Temperature

The effect of chemical and physical factors on bacterial growth and protease were investigated. Selective isolate was tested for growth at different pH of 5.0 - 10.0 and temperatures 25^0- 50° C was tested using Nutrient Broth. Each condition was analyzed in duplicate and incubated under aerobic condition at 35^oC for 1-3 days. Then the inoculated cultures were spread on Nutrient agar medium and bacterial counts were made after 24-48 hours incubation. Petri plates with 30-300 colonies were selected and the total viable bacterial counts were made. The bacterial population was expressed as number of Colony Forming Units (CFU) per ml of sample.

RESULTS

The larger yellow colonies (Figure 1a & b) were selected and they were found to be gram positive, large motile, straight rods and spore forming bacteria. The Isolate was identified as *Bacillus* sp. based on the results of biochemical characteristics. The isolated colonies of *Bacillus* sp. was undergone to study the production of protease enzyme production using Skim Milk Agar plate method. After incubation, the plates had the clear zone around the colonies (Figure 2). The clear zone represented that the enzyme protease was synthesized for utilization of protein from the skim milk agar medium.

In this study the isolated *Bacillus* sp. from the fish gut was optimized using two parameters the first method was a physical method. In this method, the production of exocellular enzyme protease and colony forming units (CFU/ml) were estimated by physical parameter like temperature and pH. The isolated *Bacillus* sp. was grown well at various temperature ranging from 25-50^0 C. From this study at 35^0 C and 40^0 C, the isolate exhibited more number of colonies when compared with others. 72 x 10^4 CFU/ml at 35^0 C was effective for *Bacillus* sp. but at 40^0C, culture produced a low amount of Protease enzyme 20 mMol/ml (Figure 3a & b).

The second method was a chemical method, the production of extra cellular enzyme protease and colony forming units (CFU/ml) were estimated under various pH like 5.0-10.0. Microbial growth and metabolism inevitably lead to a change in the hydrogen ion balance and the pH of the culture medium. The effect of pH on growth and protease production was shown (Figure 4a & b). The isolate was capable of growing in the pH range of 6.0-10.0 with maximum growth at 7.0. The production of protease substantially decreased above and below the optimum pH 7.0. At pH 7.0 the protease production increased and leveled off around 18 mMol/ml.

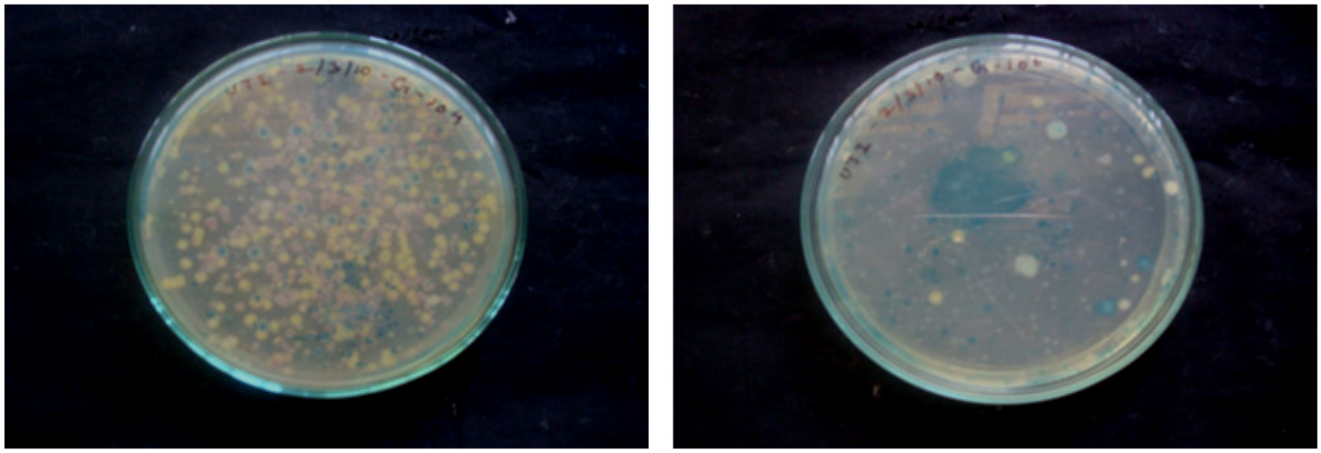


Fig 1a. Fig 1b.

Fig 1(a & b).Isolated Colonies of *Bacillus* sp. from gastrointestinal tract of freshwater fish *Cyprinus carpio*

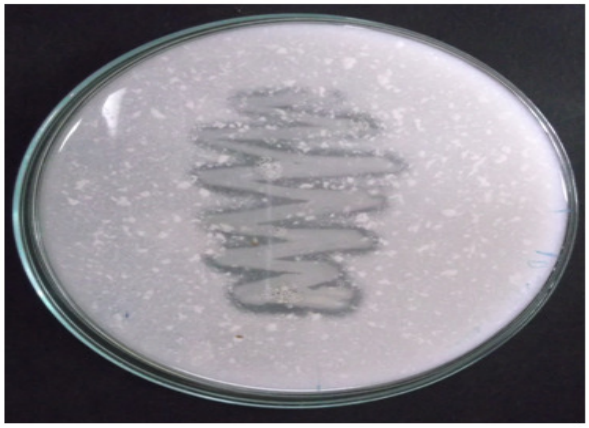


Fig 2. Qualitative analysis of protease enzyme production using Skim Milk Agar Plate. Plate showed Zone of Inhibition around the isolated colonies in Skim Milk Agar

Medium.

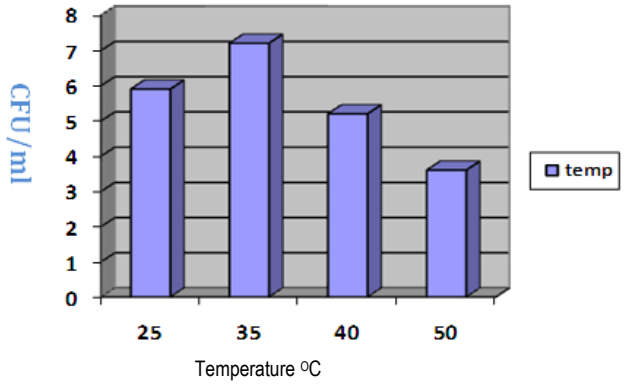


Fig 3a

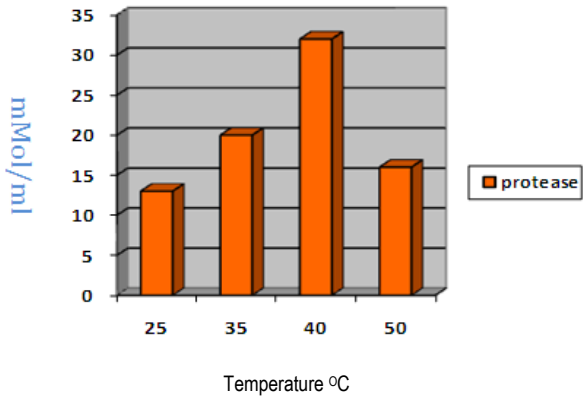
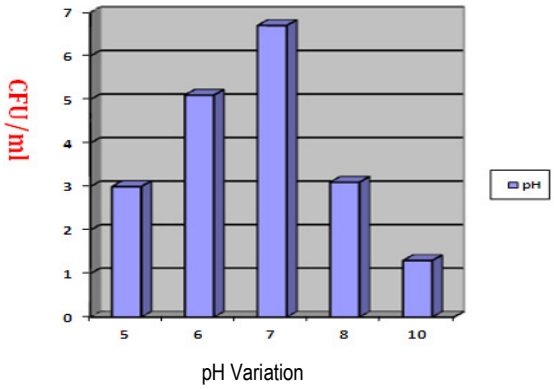


Fig 3b.

Fig 3(a & b). Effect of Temperature on growth and protease production of *Bacillus* sp.



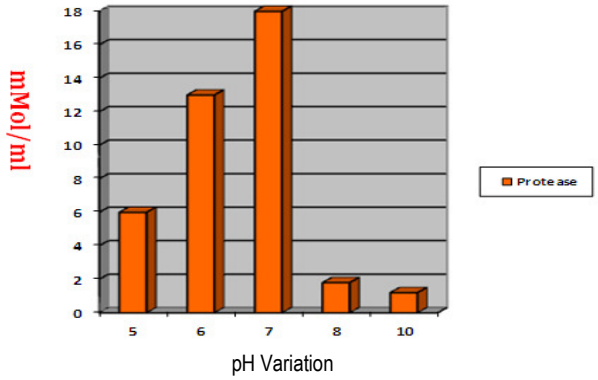


Fig 4(a & b).Effect of pH on growth and protease production of *Bacillus* sp.

DISCUSSION

The microenvironment of bacteria associated with the gastrointestinal tract of an animal influences the host in many ways, including the metabolism of nutrients. Given the importance in digestion and health, the composition, diversity and morphological characteristics of the gut microflora in many species of marine and freshwater fishes and invertebrates have been extensively researched [10-14]. The relationship between intestinal microflora and diet as well as their possible role in the digestion in both wild and cultured fish species have been studied [15]. In contrast to several studies describing bacteria associated with marine fishes [16-17].

The optimization for growth and protease production increased proteolytic activity and was useful in development of starter culture for acceleration the fermentation process [18, 19]. In this present study, it was concluded that the optimum pH was 7.0 for the production of high amount of protease enzyme. At this pH condition, the isolate produced 18 mMol/ml and showed high CFU when compared with other pH ranges. Similar results have been reported by Sugita *et al.,* [20]. Alkaline proteases mostly have their isoelectric points near to their pH optimum in the range of 8 to 11was studied by Gupta *et al.,* [21]

Rahman *et al.,* [18] found that enzyme synthesis and energy metabolism of bacteria was controlled by temperature. Moreover, temperature significantly regulated the synthesis and secretion of bacterial extracellular protease enzyme. Hence, our study was focused to optimize temperature for the efficient production of protease enzyme. It was found that optimum temperature of 35^0 C was effective condition to produce high amount of protease enzyme when compared with others. The temperature was found to influence extracellular enzyme secretion; possibly by changing the physical properties of cell membrane. Elsafey *et al.* [22] reported the same findings in production, purification and characterization of proteases enzyme from *Bacillus subtilis*. Related studies also reported that protease production by *Bacillus sp* was best at 35^0 – 40^0 C [23].

From an application point of view, it should be taken into consideration whether proteases activity or microbial cell is needed for development and acceleration of fish sauce fermentation process. To conclude, the aim of this present study was to isolate and identify potent protease producer from the gastrointestinal (GI) tracts of freshwater fish *Cyprinus carpio*. The optimum temperature and pH were determined for the isolate *Bacillus* sp. which yielded maximum amount of protease. Further studies have to be carried out in order to apply in different commercial fields.

ACKNOWLEDGEMENT

The authors are thankful to the UGC for providing financial support through Major research project (F.No: 38-239/2009 (SR)) and thankful to the Department of Zoology, The Madura College (Autonomous), Madurai and Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu.

REFERENCES

[1] Tanasupawat, S.,Thongsanit, J., Keeratipibul, S., and Jatikavanich, S. 2002. Characterization and Identification of *Tetragenococcus halophilus* and *Tetragenococcus muriaticus* strains from fish Sauce (Nam-pla). Jpn. *J. Lactic Acid Bact*. 13(1): 46-52.

[2] Sugita, H., Okano, R., Suzuki, Y., Iwai, D., Mizukami, M., Akiyama, N., Matsuura, S. 2002. Antibacterial abilities of intestinal bacteria from larval and juvenile Japanese flounder against fish pathogens. *Fish Sci.* 68: 1004-1011.

[3] Fukami, K., Ishiyama, S., Yaguramaki, H., Masuzawa, T., Nabeta, Y., Endo, K., Shimoda, M. 2002. Identification of distinctive volatile compounds in fish sauce. *J. Agric. Food Chem*. 50(19): 5412-5416.

[4] Lopetcharat, K., Choi, Y.J., Park, J. W., Daeschel, M. A. 2001. Fish sauce products and manufacturing: review. *Food. Rev. Int*. 17(1): 65-88.

[5] Vermelho, A.B., Meirelles, M.N.L., Lopes, A., Petinate, S.D.G., Chaia, A.A., and Branquinha. 1996. Detection of extracellular proteases from microorganisms on agar plates. *Mem. Inst. Oswaldo. Cruz*. 91(6): 755-760.

[6] Thongthai, C., and Suntinanalert, P. 1991. Halophiles in thai fish sauce (Nam Pla). Rodriguez-Valera, F. (ed.). General and applied aspects of halophilic microorganisms. pp.381-388. New York : Plenum Press.

[7] Noguchi, H., Uchino, M., Shida, O., Takano,K., Nakamura, L. K. and Komagata, K. 2004. *Bacillus vietnamensis* sp. nov., a moderately halotolerant, aerobic, endospore-forming bacterium isolated from Vietnamese fish sauce. *Int. J. Syst. Evol. Microbiol*. 54: 2117–2120.

[8] Chaiyanan, S., Chaiyanan, S., Maugel, T., Huq, A., Robb, F.T., Colwell, R.R. 1999. Polyphasic taxonomy of a novel *Halobacillus, Halobacillus Thailandensis* sp. nov. isolated from fish Sauce. *J. Appl. Microbiol*. 22: 360- 365.

[9] Trust, T.J., Sparrow, R.A.H. 1974. The bacterial flora in the alimentary tract of fresh water salmonid fishes. *Canadian Journal of Microbiology*, 20: 1219-1228.

[10] Spanggaard B., Huber I., Nielsen J., Nielsen T., Appel K.F. & Gram L. (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification, *Aquaculture*, 182, 1−15.

[11] Hoyoux C., Zbinden M., Samadi S., Gaill F. & Compère P. 2009 Wood-based diet and gut microflora of a galatheid crab associated with Pacific deep-sea wood falls. *Marine Biology,* 156, 2421−2439.

[12] Kuz'mina V.V. & Skvortsova E.G. 2002 Gastrointestinal bacteria and their role in digestion process in fish. *Uspekhi Sovremennoi Biologii* 122, 569–579.

[13] Cahill M.M. 1990 Bacterial Flora of Fishes: A Review, *Microbial Ecology,* 19, 21−41.

[14] Hansen G.H., Olafsen J.A. 1999 Bacterial interactions in early stages of marine cold-water fish. *Microbial Ecology*, 38, 1−26.

[15] Sun Y., Yang H., Ling Z., Chang J., Ye J. 2009 Gut microbiota of fast and slow growing grouper *Epinephelus coioides*. *African Journal of Microbiology Research,* 3, 713−720.

[16] Bairagi, A., Ghosh, K.S., Sen, S.K., Ray, A.K. 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquacul. Int*. 10:109- 121.

[17] Sugita, H., Hirose, Y., Matsuo, N., Deguchi, Y., 1998. Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. *Aquac.* 165: 269-280.

[18] Rahman, R. N. Z. A., Basri, L. P. G. M., Salleh, A. B. 2005. Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. *Bioresour. Technol*. 96: 429-436.

[19] Kawai, S., Ikeda, S. 1972. Studies on digestive enzymes of fishes. Effect of dietary change on the activities of digestive enzymes in carp intestine. *Bull. Jpn. Soc. Sci. Fish.,* 38: 265-270.

[20] Sugita, H., Takahashi, J., Miyajima, C., Deguchi, Y., 1991. Vitamin B12- producing ability of the intestinal microflora of Rainbow trout (*Oncorhynchus mykiss*). *Agric. Bio. Chem*. 92: 267-276.

[21] Gupta, R., Q.K. Beg and Lorenz, 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbial Biotechnolo*., 59: 15-20.

[22] El-Safey, E.M and Abdul-Raouf, M. 2004. Production, purification and characterization of protease enzyme from *Bacillus subtilis*. International conference for development and the environment in the Arab. World, pp: 14.

[23] Usharani, B. and M. Muthuraj, 2010. Production and characterization of protease enzyme from *Bacillus laterosporus*. *African J. Microbiology Res*., 4: 1057.