ISSN Online: 2349-7785

Detection of Exogenous and Endogenous (Cell Culture Based Analysis) Production of Cellulase in Grass Carp (*Ctenopharyngodon idella*)

Pushpita Mishra [1], Sriprakash Mohanty [1], Mrinal Samanta [1], Nikhil Kumar Maiti [1], Chandi C. Rath [2]*

[1] Microbiology Division, Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar-751002, India
[2] Department of Botany, College of Basic Science andHumanities, Orissa University of Agriculture & Technology (OUAT), Bhubaneswar-751003, India

Received 09 November 2015; accepted in revised form 28 November 2015

Abstract: Cellulase activity was found in forty eight bacteria isoalted from the grass carp intestine and intestinal content samples. Twenty six bacteria were positive towards congo red reaction. β-glucosidase and exoglucanase activity was measured in bacterial cultures but endoglucanase activity could not be detected. This paper reports the first attempt of using intestinal and hepatopancreatic cell culture for the establishment of the endogenous production of cellulase in *Ctenopharyngodon idella* (grass carp). EMEM media was used for cell culture. Initial experiment for primary cell culture by trypsinization showed no enzyme (β-glucosidase) production but enzyme was detected in explant culture samples. An increase (80 % of intestinal and 66 % of hepatopancreatic samples) in enzyme activity was seen when CMC was used as inducer.

Key words: *Ctenopharyngodon idella*, β-glucosidase, explants culture, carboxymethylcellulose, EMEM media.

Introduction

Freshwater aquaculture plays a very significant role in global aquaculture production. In 2011, 56.8 % of the global aquaculture production was freshwater fishes, and output amounted to 35.6 million tons [7]. Grass carp (*Ctenopharyngodon idellus*) is one of the most important freshwater fish that is native to China, and it plays an important role in aquaculture with 4.57 million tons produced in 2011, the highest in fish production worldwide [25]. Aquatic plants are an essential component of a well functioning aquatic ecosystem, but often reach levels of abundance that some pond owners consider excessive. In these situations, they look for methods to control the problem plant species. One commonly used technique is to stock grass carp (also known as white amur), a non-native, plant-eating fish that will reduce the abundances of some aquatic plants [25]. Hence due to its importance, now it has been introduced to more than 100 countries [23]. The fish also has a high growth rate [11] which increases its economic significance, and consequently the grass carp has been the subject of many investigations. Under natural condition, the grass carp is basically herbivorous feeding on certain aquatic plants [4]. Cellulose, the major component plant cell wall and the most common polysaccharide on earth, represents an important forage resource for such herbivores [27]. Thus cellulose degradation has a major impact on animal productivity. Despite a large body of indirect evidence, cellulose digestion

*Corresponding author (Chandi C. Rath)
E-mail: < chandicharanrath@yahoo.com >

in invertebrates has been ascribed until recently to the activity of microorganisms in the gut rather than the endogenous production of cellulase enzyme [15, 5, 24]. Apart from cellulytic bacterial isolation the objective of the present investigation was to define more clearly the endogenous production of cellulase in grass carp. Earlier reports [18] suggested cellulase activity in the guts of catfish in the absence of bacteria. Recently in a research [8] on catfishes suggested that the whole digestive strategy ranging from intake, to passage rate, digestive enzyme activities, gastrointestinal fermentation, and decreasing surface area in the distal intestine of these fishes are geared for the digestion and assimilation of soluble components of their detritus diet. Goldfish also showed endogenous cellulase activity in the absence of cellulolytic bacterial population [14, 2]. [13] indicated the indigenous origin of cellulase in mahseer *Tor khudree* [22] found the endogenous origin of cellulase in *Labeo rohita*.

Cell culture has become one of the major tools used in life sciences today. Cell culture provides a good model system for studying 1) basic cell biology and biochemistry 2) the interaction between disease causing agents and cells 3) the effect of drugs on cells 4) the process and triggers for aging and 5) nutritional studies. It is also used in toxicity testing, cancer research, virology, cell based manufacturing, genetic counseling, genetic engineering, gene therapy, drug screening and development etc.[20].

In order to establish the presence of endogenous source of cellulase in grass carp, cell culture was carried out. As per our knowledge this approach was perhaps first of its kind in the field of cellulase research.

Materials and methods

Sample collection for bacterial isolation and cell culture

Grass carp of different weight were collected from CIFA farm using a cast net and from local retail fish market in Bhubaneswar and ventrally dissected to extract the hepatopancreas and intestine separately. Grass carp weighing (70-100 gm) were maintained in laboratory tanks for further cell culture experimentation.

Isolation and Identification of cellulolytic bacteria

Isolation was carried out by enrichment culture technique. Intestinal tract weighing 1gm was triturated with 9 ml of sterilized 0.85 % NSS. 1 ml of triturated sample was inoculated into 9 ml of cellulase enrichment broth [(Peptone (0.5 %); $MgSO_4.7H_20$ (0.05%); KH_2SO_4 (0.1%); carboxymethylcellulose (1 %)] and incubated at 37°C for 24 hrs. Then enriched bacterial samples (100 µl) were spreaded on CMC agar plates (0.5 % CMC) and incubated at 37°C for 48 hrs. The colonies which appeared on CMC agar plates were pure cultured and the cellulose hydrolysis by these bacteria was detected by congo red binding assay. Identification of congo red positive bacteria were carried out by gram staining, colony characteristics and a panel of biochemical tests. Preservation of the bacterial cultures was carried out in 40 % glycerol.

Growth and enzyme activity

Assay of cellulose complex from exogenous (bacterial) source

The cells from bacterial culture enriched in cellulase enrichment broth (pH-8.0) were harvested by centrifuging at 10,000 rpm for 20 min at 4°C. The supernatant was used as crude enzyme solution and was used for estimation of cellulase (β-glucosidase, endoglucanase and exoglucanase).

β-glucosidase activity was measured following the method described [26] with some modifications. The reaction mixture containing 0.5 ml of enzyme solution was added to 0.5 ml of 0.1 % PNPG (p-nitrophenol β-D-glucoside) solution prepared in Tris HCl (pH-6.5) was incubated at 37°C in a shaker waterbath. After two hours the reaction was stopped by adding 1ml of 2 % Sodium carbonate. The yellow colour p-nitrophenol liberated was determined spectrophotometrically at 400 nm against a standard curve.

Endoglucanase activity was determined by following the methods [1,17] with some modifications. 0.5 ml of enzyme solution was added to a reaction mixture containing 1ml of 0.1M PBS buffer (pH-6.8) and 0.5 ml CMC (1 %) solution and incubated at 37°C for 1 hr. The reaction was stopped by adding 3 ml of DNS solution and

reducing sugar was determined spectophotometrically at 575 nm.

Exoglucanase was assayed in terms of filter paper activity modifying the method described elswhere [10]. Whatman filter paper weighing 1gm was used as substrate and rest of the protocol followed was same as in case of endoglucanase. The reducing sugar was determined spectophotometrically at 575nmt.

Determination of enzyme activity

The enzyme (endoglucanase and exoglucanase) activity was calculated by following formula.

Enzyme activity (units) = mg amout of glucose released / T X S X Mol. Wt. of glucose

Where, T is the time of incubation (1 hr); S is the volume of sample (0.5 ml); 180 is the molecular weight of glucose

The enzyme (β-glucosidase) activity was calculated by following formula.

Enzyme activity (units) = µg amout of p-nitrophnol released / T X S X Mol. Wt. of p-nitrophnol

Where, Time (T) = 2 hours; Sample volume (S) = 0.5 ml; 139.11= molecular weight of p-nitrophenol and the enzyme activity was expressed in units/ml/hr.

Primary cell culture

Grass carp was anesthetized and washed thoroughly with rectified spirit. Hepatopancreas and intestinal cells were aseptically collected (after removing scales from abdominal region) and placed in the maintenance media. [Eagles Minimum Essential Media (Sigma Chemicals Co. Louis, USA)]. Intestinal contents were flushed thoroughly and cells (intestinal and hepatopancreatic) were allowed to settle at the bottom of the beaker, supernatant was decanted and fresh maintenance media was added again. The cells were mined properly. Trypsin solution (0.1 % and 0.25 %) was prepared by adding Trypsin (Sigma Chemicals Co. Louis, USA) to HBSS solution (Sigma Chemical Co. Louis, USA) and pH adjusted to 7.6 and filler sterilized using Seitz Filter. The minced contents were transferred to two sterile trypsinization flasks containing 0.1 % and 0.25 % trypsin solution for hepatopancreas and intestinal samples respectively. After 15 minutes of trypsinization the contents were passed through muslin cloth and then centrifugation was carried out for 2000 rpm for 5 minutes. After decanting the supernatant the cells were placed in another flask containing 5 ml of maintenance media and Newborn calf Serum (Hyclone, Laboratories Inc., Logan, Utah) was added at the rate of 10 %. 1 ml of Antibiotic Antimycotic Solution (100 X). Sigma Chemicals, USA) was added to the culture vessel and then the flask was incubated in CO_2 incubator (Sanyo, Gallenkamp, UK) at 25°C with 5 % CO_2 tension for 48 hours. The cell growth was observed under inverted microscope (Leica, Germany).

Explant culture

Sample collection was carried out in the same way as in the case of primary cell culture. The hapatopancreas and intestine was collected aseptically and washed thoroughly in the maintenance media. Standardization of explant (hepatopanereas and intestine) culture was carried out. Hepatopancreas and intestine weighing 25 mg, 50 mg and 100 mg were tested with 0.5 %, 1 % and 1.5 % of CMC for enzyme production as shown in the checker board presented below (Table 1).

Table 1. Experimental set up for explants culture

25 mg	**25 mg**	**50 mg**	**50 mg**	**100 mg**	**100 mg**
25 mg 0.5 % CMC	25 mg 0.5 % CMC	50 mg 0.5 % CMC	50 mg 0.5 % CMC	100 mg 0.5 % CMC	100 mg 0.5 % CMC
25 mg 1 % CMC	25 mg 1 % CMC	50 mg 1 % CMC	50 mg 1 % CMC	100 mg 1 % CMC	100 mg 1 % CMC
25 mg 1.5 % CMC	25 mg 1.5 % CMC	50 mg 1.5 % CMC	50 mg 1.5 % CMC	100 mg 1.5 % CMC	100 mg 1.5 % CMC

After adding the samples (Intestine & Hepatopancreas) in two separate 24 well cell culture plates, serum was added to each cell at the rate of 10 %. Incubation was carried out in the CO_2 incubator at 25°C with 5 % CO_2 tension for 24 hours. Figure 1 shows the experimental set up of the carried out using intestinal and hepatopancretic explants with different concentration of the substrate (CMC). Enzyme (β-glucosidase) activity was measured after 24 hours following the method described earlier from explant culture.

Results

Screening and identification of cellulolytic bacteria

In total 48 bacteria were isolated based on colony morphology of which 26 (54.17 %) were reported to be cellulase positive on the basis of growth on CMC agar and congo red reaction. The zone size of the isolates is represented in (Figure 2). On the basis of Gram's reaction identification of 26 isolates revealed 19 gram-positive and 7 gram-negative strains. Through an array of biochemical characters the gram-negative species were tentatively identified as *Pseudomonas* (15.38 %), *Flavobacterium* (0.04 %), *Actinobacillus* (0.04 %) and *Erwinia* (0.04 %) whereas, the gram-positive species were identified as *Bacillus* (61.54 %), *Micrococcus* (0.04 %) and *Lactobacillus* (0.08 %). A total of 16 *Bacillus* species were isolated. All the *Bacillus* strains were further characterized on Hicrome Bacillus Agar (Himedia, India) on the basis of chromogenic reaction. *B. cereus* (25 %), *B. coagulans* (6.25 %), *B. megaterium* (6.25 %) and *B. subtilis* (25 %) appeared dark blue, pink, yellow, and green respectively on Hicrome Bacillus agar. The other *Bacillus* species isolated could not be identified by chromogenic reaction.

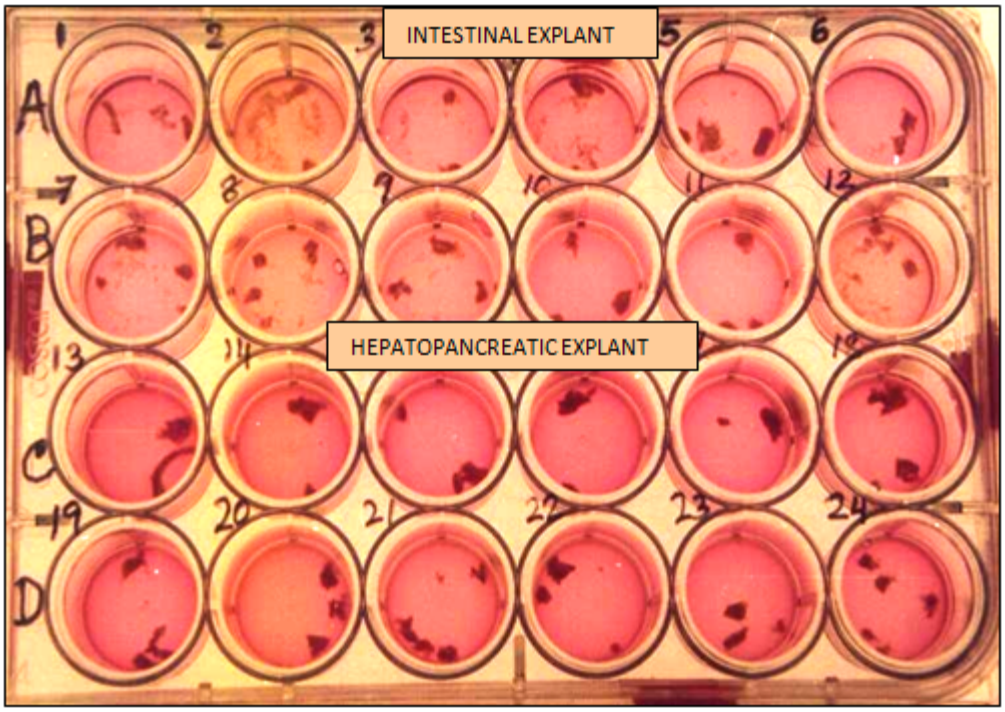

Fig. 1. Explant Culture of intestine and hepatopancreas in EMEM media

$A^{1,2}$ – 25 mg tissue
$A^{3,4}$ – 50 mg tissue
$A^{5,6}$ – 100 mg tissue
$C^{13,14}$ – 25 mg tissue+1% CMC
$C^{15,16}$ – 50 mg tissue+1% CMC
$C^{17,18}$ – 100 mg tissue+1% CMC
$B^{7,8}$ – 25 mg tissue+0.5% CMC
$B^{9,10}$ – 50 mg tissue+0.5% CMC
$B^{11,12}$– 100 mg tissue+0.5% CMC
$D^{19,20}$ – 25 mg tissue+1.5% CMC
$D^{21,22}$ – 50 mg tissue+1.5% CMC
$D^{23,24}$–100 mg tissue+1.5% CMC

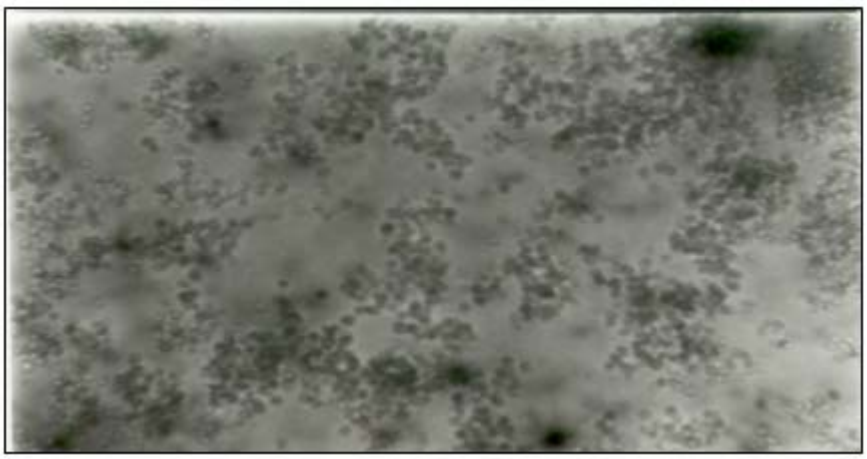

Fig. 2. Cell culture of grass carp intestine in EMEM media after 72 hours

Estimation of bacterial cellulase

β-Glucosidase estimation was carried out using standard protocol and standard curve was prepared. From the standard curve, y = 0.0632x and the R^2 value was 0.9997. Out of 48 isolates 10 (20.83 %) were found negative for β-glucosidase activity whereas 9 (18.75 %) isolates showed negligible (0.001 U/ml/hr) β-glucosidase activity. 13 (27.08 %) isolates were reported as good producers with ≥ 0.1U/ml/hr enzyme activity. The rest 17 (35.42 %) isolates produced ≥ 0.01U/ml/hr enzyme activity. According to our data good β-glucosidase producers were identified as *Actinobacillus*, *Erwinia* and *Bacillus* species.

Using standard protocol, for endoglucanase a standard curve was prepared. From the standard curve, y = 0.344x and the R^2 value was 0. Endoglucanase activity could not be detected from the bacterial samples. Out of 48 isolates 10 (20.83 %) were negative for exoglucanase whereas only one (2.08 %) isolate showed negligible (0.001 U/ml/hr) activity, 3 (6.25 %) were good producers with ≥ 0.1 U/ml/hr exo-glucanase activity and the rest 34 (70.83 %) showed exoglucanase activity ≥ 0.01 U/ml/hr. *Micrococcus* was identified as the best exoglu-canase producer according to our data. High exo-glucanase activity was also seen among *Bacillus* species.

Primary cell culture

The monolayer formation of the intestinal and hepatopancreatic cells was seen after 72 hours and the results are shown in figure 2 and 3.

Enzyme estimation from primary and explants culture

Primary cell culture of intestine and hepatopancreas did not show β-glucosidase activity in both control and test samples. Enzyme estimation from expalnt culture revealed that 100mg tissue and 10 % CMC produced maximum β-glucosidase activity. In the presence of CMC as induced and increase in the enzyme activity was found in 81.25 % of intestinal and 66.6 % of hepatopancreatic explants (Figure 4, 5).

Discussion

Bioconversion of cellulose, nature's most abundant polysaccharide is accomplished by the enzyme cellulase [12]. In general, complete cellulase activity, the activity by which cellulose is hydrolyzed to its final constituent unit (i.e. glucose) is considered to involve three synergistically acting enzymes (endoglucanase, exoglucanase & β-glucosidase) [19]. The literature pertaining to the cellulase digestion in fish is conflicting, cellulase enzyme activity of *Ctenopharymgodon idella* was studied for the first time by Das and Tripathy [5], and their report provided an indication for the presence of cellulase producing bacteria in fish intestine. Similar findings were also evidenced by Wu *et al.*,[27]. Analysing the enzyme activity pattern from both the endogenous and exogenous source in our study, it can be suggested that cellulose digestion in grass carp might be carried out in an interesting way where the endoglucanase produced by the fish, first breaks down the complex structure, generating

Fig. 3. Cell culture of grass carp hepatopancreas in EMEM media after 72 hours

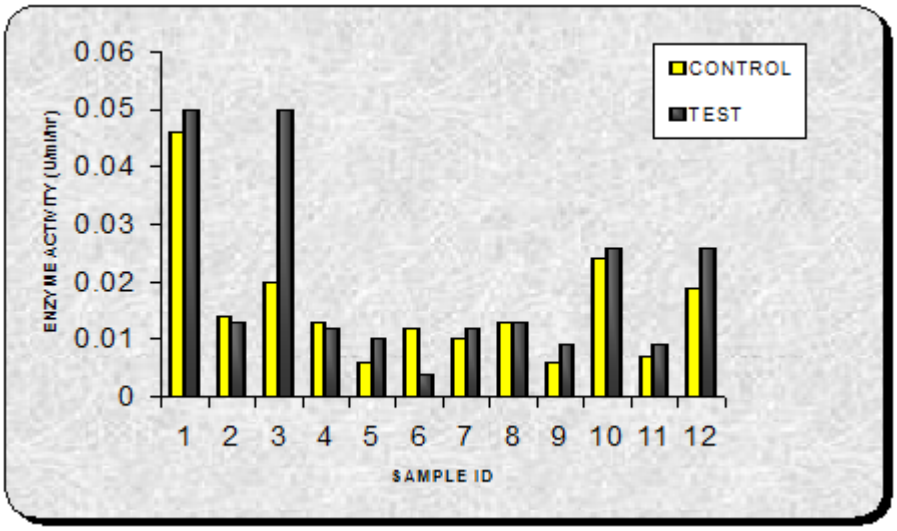

Fig. 4. Enzyme activity of intestinal explants

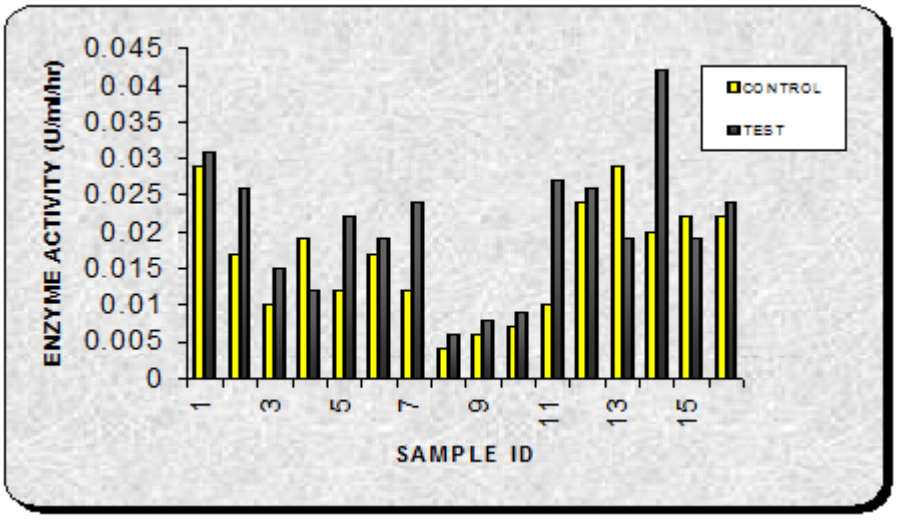

Fig. 5. Enzyme activity of hepatopancreatic explant

oligosaccharides of various lengths and consequently new chain ends which are later acted upon by the β-glucosidase produced from both bacteria and fish source and exoglucanase produced only from bacterial source. According to Masser [16] the large consumption of these carbohydrates present in water bodies by grass carp is due to a very short gut compared to other herbivores. This decreases the retention time (less than 8 h) in the gut and reduces the digestive efficiency to only 60 to 70 %. In our preliminary investigation high blood glucose level of grass carp (~32 mg/dl) was detected as compared to other fish species like *Catla catla* (catla) (~12 mg/dl), *Cirrhinus mrigala* (mrigal) (~13 mg/dl) and *Labeo rohita* (rohu) (~ 25 mg/dl). The high blood glucose level in grass carp may be due to the accumulation of more end product (glucose) after cellulose digestion. More than 60% of the isolates identified in this study, were *Bacillus* species (*B. subtilis*, *B. circulans*, *B. marinus*, *B. coagulans*, *B. cereus* and *B. megaterium*) based on their growth and pigmentation on Hicrome *Bacillus* agar [6,9,21] believed *Bacillus* species to be the potent producer of cellulase in fish gut.

Hence after detecting the exogenous source of cellulase in grass carp, in a new approach we tried to confirm the endogenous production of cellulase by grass carp using cell culture. Primary cell culture (where cells surgically removed from an organism and placed into a suitable culture environment, will attach divide and grow) was carried out. Initially primary cell culture using enzymatic dissociation method was used where trypsin was added to dissolve the cement holding the cells together. This created a suspension of single cells which were cultured and the enzyme activity was detected. Initially experiments to find out the enzyme activity from trypsinised culture failed to respond the inducement. The reason may be that a single cell is unable to produce enzyme instead cell to cell interaction is required for the enzyme secretory activity. Hence explant culture (small pieces of tissues are attached to a culture vessel) was carried out. Enzyme estimated from the explant culture of intestine and hepatopancreas suggested the endogenous source of cellulase in grass carp. The antibiotics used for cell culture were earlier tested to be sensitive to the cellulolytic microflora of grass carp with low degree of multiple antibiotic resistances. The antibiotic treatment in the growth media ruled out the possibility of the bacterial source of enzyme. CMC was used as inducer in our investigation based on the studies of Harchand & Singh [10] who also suggested that CMC is a very good inducer for β-glucosidase. β-glucosidase was detected as it is the most important enzyme, which cleaves oligosaccharides to glucose, which is an important step as cellobiose inhibits the action of many cellulose components [3]. The β-glucosidase activity showed an increase in the presence of inducer in more than 80% of the intestinal and 66 % of the hepatopancreatic samples.

Conclusion

The historical debate concerning the presence of endogenous cellulase in higher animals has been settled by cell culture experiments. There is no doubt that cellulase is a protein that can be potentially produced in any life form and a gene certainly encodes it. So, in the present investigation it is established that the cellulase production in grass carp is associated with the exogenous source of cellulase degrading bacteria inside the intestine in addition to the endogenous source and there is further scope for researchers to find out the cellulase gene in grass carp.

Acknowledgement

Authors are thankful to the Director, Central Institute of Freshwater Aquaculture for providing the necessary facilities to carry out this work.

References

1. **Agarwal, N. (2000).** Estimation of fibre degrading enzymes. Feed Microbiology. A short course Nov. 23 to Dec. 22, 2000. 283-291.
2. **Bairagi, A., Ghosh, K.S., Sen, S.K., Ray A.K. (2004).** Evaluation of the nutritive value of *Leucaena leucocephala* leaf meal, inoculated with fish intestinal bacteria *Bacillus subtilis* and

Bacillus circulans in formulated diets for rohu, *Labeo rohita* (Hamilton) fingerlings. Aquaculture. Research 35(5): 436-446.
3. **Beguin, P. (1990).** Molecular biology of cellulose degradation. Annual Review of Microbiology. 44: 219-248.
4. **Cui, Y.B., Wang, S.M., Liu, X.F., Chen, S.L. (1991).** Nitrogen budgets of young grass carp fed on plant or animal diets. Progress in Natural Science. 1: 449-451.
5. **Das, K.M., Tripathi, S.D. (1991).** Studies on the digestive enzymes of grass carp, *Ctenopharyngodon idella* (Val.). Aquaculture. 92(1): 21-32.
6. **Dey, R., Pal, K.K., Chauhan, S.M., Bhatt, D.M., Misra J.B. (2002).** Groundnut shell decomposition potential of some cellulolytic microorganisms. Indian Journal of Microbiology. 42: 165-167.
7. **Food and Agriculture Organisation. (2013).** FAO Fisheries and Aquaculture, Department Food and Agriculture Organization of the United Nations, Rome, Italy.
8. **German, D.P. (2009).** Inside the gut of wood eating catfishes: can they digest wood? Journal of Comparative Physiology and Biochemistry. 179(8): 1011-1023.
9. **Ghosh, K., Sen, S.K., Ray, A.K. (2002).** Characterization of *Bacilli* isolated from the gut of Rohu, *Labeo rohita* fingerlings and its significance in digestion. Journal of Applied Aquaculture. 12(3): 33-42.
10. **Harchand, R.K., Singh, S. (2001).** Induction of cellulases in *Streptomyces albaduncus* by different substrates. Indian Journal of Microbiology. 41: 45-49.
11. **Hickling, C.F. (1967).** On the biology of a herbivorous fish the white amur or grass carp, *Ctenopharyngodon idella* Val. Proceedings of the Royal Society. 70(1): 62-81.
12. **Karmakar, M., Ray, R.R. (2011).** Current trends in research and application of microbial cellulases. Research Journal of Microbiology. 6(1): 41-53.
13. **Kumar, M.B. (1995).** Effect of two commercially available feed additives on the growth and survival of cultivable carps. PhD Dissertation, University of Agricultural Sciences, Bangalore. 158 p.
14. **Lesel, R., Fromageot, C., Lesel, M. (1986).** Cellulose digestibility in grass carp, *Ctenopharyngodon idella* and in goldfish, *Carassius auratus*. Aquaculture. 54: 11-17.
15. **Linton, S.M., Greenaway, P., Towle., D.W. (2006).** Endogenous production of endo-β-1: 4-glucanase by decapod crustaceans. Journal of Comparative Physiology and Biochemistry. 176: 339-348.
16. **Masser, M.P. (2002).** Using grass carp in aquaculture and private impoundments. S.R.A.C. Publication. No. 3600.
17. **Miller, G.L. (1959).** Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 31: 426-428.
18. **Prejs, A., Blaszezyk, M. (1977).** Relationships between food and cellulose activity in freshwater fishes. Journal of Fish Biology. 11: 447-452.
19. **Ray, A.K., Bairagi, A., Ghosh, K.S., Sen, S.K. (2007).** Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. Acta Ichthyologica Et Piscatoria. 37(1): 47-53.
20. **Ryan, J.A. (2008).** Introduction to animal cell culture. Technical bulletin http://www.corning.com/lifesciences, Corning Inc, Corning, New York.
21. **Saha, S., Roy, R.N., Sen, S.K., Ray A.K. (2006).** Characterization of cellulose producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngodon idella* (Valenciennes). Aquaculture Research. 37(4): 380-388.
22. **Sethuramalingam, S., Haniffa, M.A. (2002).** Effect of formulated diet on digestive enzymes of *Labeo rohita* (Ham). Indian Journal of Experimental Biology. 40: 83-88.

23. **Song, X., Li, S.F., Wang, C.H., Xu, J.W., Yang, Q.L. (2009).** Grass carp (*Ctenopharyngodon idellus*) genetic structure analysis among native populations in china and introduced populations in USA, Europe and Japan based on mitochondrial sequence. Acta Hydrobiologica Sinica. 33: 709-716.
24. **Stickney, R.R., Shumway, S.E. (1974).** Occurrence of cellulose activity in the stomach of fish. Journal of Fish Biology. 6: 779-790.
25. **William, E.L. (2009)** Using Grass Carp to Control Aquatic Plants. FACT SHEET Agriculture and Natural Resources.
26. **Wood, T.M., Bhat, K.M. (1998).** Methods for measuring cellulase activities. Methods of Enzymology. 160: 87-112.
27. **Wu, S., Wang, G., Angert, E.R., Wang, W., Li, W., Zou, H. (2012).** Composition, Diversity, and Origin of the Bacterial Community in Grass Carp Intestine. http://www.plosone.org. 7(2)-e30440.Cited 20 Feb 2012.