Available online at www.ijpab.com

International Journal of Pure & Applied Bioscience

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **2 (3):** 124-134 (2014)

Research Article

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

Isolation, Characterization and Optimization of Protease Enzyme Producing Micro-Organism from Gastrointestinal Tract of *Labeo rohita*

Sandeep Chovatiya*, Keshal Dhola, Prerak Patel and Snehal Ingale
Ashok & Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences, New Vallabh Vidyanagar, Anand, Gujarat (India) 388121
*Corresponding Author E-mail: sandeepchovatiya@aribas.edu.in

ABSTRACT

Proteases which are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors especially in especially in food industry. The present study describes the screening of protease producer from gastrointestinal tract of Labeo rohita. Among the 7 isolates, PPR7 strain was found to be more suitable for protease enzyme production. The morphological, cultural, biochemical and molecular characteristics of isolate PPR7 revealed as Bacillus thuringiensis. The temperature and pH was optimized for the maximum production of this enzyme. The optimum temperature for the protease production (120 U/ml) was found to be 37 °C while the pH was found to be optimum at 7.00 for the protease production (100.0 U/ml). Medium optimization suggested that the most appropriate carbon source was glucose (122.0 U/ml), nitrogen source was glycine (130.4 U/ml) and NaCl concentration was 2% (85.2 U/ml) for the better protease production.
The present research will be helpful for fish nutritionists to utilize this enzyme producing bacterial isolates as a probiotic formulations, which is not only a cost-effective aqua feeds but is also especially for the larval stages when the enzyme system is not efficient.

Keywords: *Labeo rohita, Bacillus thuringiensis, Protease production, Media optimization, Isolation and Characterization.*

INTRODUCTION

An enzyme is a biocatalyst which accelerates the rate of biological reactions generally forming parts of the metabolic processes of the cells. They are essential to sustain life because most chemical reactions in biological cells would occur too slowly, or would lead to different products without enzymes. Enzymes are commercially exploited in the food, pharmaceutical, diagnostics, detergent and chemical industries[1]. More than 300 different enzymes have been isolated from various living sources such as identified plants, animals, bacteria and fungi and used in industrial and biotechnological applications[2].

Proteases (EC 3.4) are enzymes that hydrolyze proteins to small peptides or free amino acids. The very importance of enzyme as sources for industrial applications has been well recognized and it was reported that proteases count for nearly 65% of the world enzyme market[3]. Microbial sources of Proteases are alternatives over the enzymes from animal or plant sources since they possess all most all the characteristics desired for their biotechnological applications, particularly in food, detergent, weave, leather, pharmaceutical, chemical industries and waste processing industries[3,4]. Proteases are commonly classified according to their optimum pH: acidic protease, neutral protease and alkaline protease.

The micro flora present in gastro intestinal tract of fish can influence nutrition, growth, and disease susceptibility which may be essential in fish that feed on recalcitrant material or on material lacking vitamins. Presence of symbiotic bacteria in an animal's digestive tract often produce complement enzymes for digestion of plant foods as well as synthesize compounds that are assimilated by the host[5]. The biological diversity of fresh water bodies provides a wide array of enzymes with unique properties.

Endogenous digestive enzymes in fish have been studied by several workers[6,7]. However, information regarding the enzymes produced by intestinal dwelling bacteria, their source and significance in fish is scarce. With this in view, we have isolated *Bacillus thuringiensis* from the intestinal tract of *Labeo rohita* and characterized its protease production under various chemical and physical conditions.

MATERIALS AND METHODS

Sample-Collection

Herbivore, column feeder Rohu were sampled from the Bio-sciences department pond, Sardar Patel University for the present study. The average weight, total length (LT) and gut length (LG) of the fish studied are presented in Table 1.

Table 1 : Morphological characteristics of Rohu fish

Fish species	Body weight (gm)	Total length (cm)	Gut weight (gm)	Gut length (cm)
Rohu	6.96	5.15	0.487	32.27

Homogenate Preparation

The gut of the healthy fish was dissected out in cold and aseptic condition, weighed, rinsed in sterile phosphate buffer. Then it was mechanically homogenized in sterile phosphate buffer (0.2M) to give 10^{-1} diluted sample. Homogenates were then centrifuged for 15 min at 6000 rpm. Supernatant was stored in a refrigerated at 4°C up to further processing.

Isolation and Screening of microorganisms producing Protease

Isolation of bacteria was performed by serial dilution & spread plate method on nutrient agar plate. The samples were serially tenfold diluted in sterilized phosphate buffer to get concentrate range from 10^{-1}-10^{5}. A volume of 0.1 ml of each dilution was transferred aseptically to nutrient agar plates containing peptone (1% wt/vol), NaCl (0.5% wt/vol), agar (2.5% wt/vol), and meat extract (0.3% wt/vol) pH-7.4. The sample was spreaded uniformly using a glass spreader. The plates were incubated at 37°*C* for 24 hrs. The bacterial isolates were further subculture on a nutrient slant. Pure isolates were maintained at 4°*C* in refrigerator for further studies.

The isolates obtained were screened for protease production using skim milk agar plates containing peptone (0.1% wt/vol), NaCl (0.5% wt/vol), agar (2.0% wt/vol), and skim milk (10% vol/vol) pH-7.0. The plates were incubated at 37°C for 24 hrs. A clear zone of hydrolysis gave an indication of proteolytic microorganisms. The organisms which were producing maximum zone size of hydrolysis surrounding the colony on milk agar plate selected for further shake flask testing.

Protease production

The culture medium used in this work for protease production contained (g/L) Glucose, 10; Casein, 5.0; Yeast extract, 5.0; K_2HPO_4, 1.0; $MgSO_4$, 0.2 and pH maintained to 7.2.

50 ml production medium in 250ml Erlenmeyer flask was inoculated with 5% inoculum of overnight grown culture of Bacillus and incubated at 37°C on rotary shaker at 100rpm. 2ml of broth was harvested aseptically and centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant thus obtained was used for protease assay.

Protease Assay

Activity of protease was determined by Anson method with some modifications[8]. Enzyme solution (0.5ml) was mixed with 2.0 ml substrate (0.65% casein in 25 mM Tris-HCl buffer) at 37 ^{0}C for 30 minutes and after incubation TCA was added to attenuate the reaction. This mixture was centrifuged at 10,000 rpm for 5 minutes and the released animo acids were measured as tyrosine by the method of Folin and Ciocalteu[9].

Protein Determination

Protein concentration was estimated by Lowry's method[10] using Bovine Serum Albumin as standard.

MEDIA OPTIMIZATION

Effect of Different Carbon Sources on Protease Production

Glucose in production medium was replaced by 1% each of sucrose, lactose, maltose, starch. 50ml medium was inoculated on 250ml Erlenmeyer flask at 37°C in an incubator shaker at 100rpm. 2ml of broth was harvested aseptically and centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant thus obtained was used for protease assay.

Effect of Different Nitrogen Sources on Protease Production

Yeast extract in medium was replaced by 0.5% each of peptone, glycine and NH_4Cl. 50ml medium was inoculated on 250ml Erlenmeyer flask at 37°C in an incubator shaker at 100rpm. 2ml of broth was harvested aseptically and centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant thus obtained was used for protease assay.

Effect of varying Sodium chloride (NaCl) Concentrations on Protease Production

The effect of varying Sodium chloride (NaCl) concentration was studied by supplementing the production medium with varied concentrations of NaCl such as 2%, 4%, 6%, 8%, and 10%. 50ml medium was inoculated on 250ml Erlenmeyer flask at 37°C in an incubator shaker at 100rpm. 2ml of broth was harvested aseptically and centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant thus obtained was used for protease assay.

Effect of pH on Protease Production

To study the effect of different pH on protease production, the pH of production medium was set as 4, 6, 7, 8, 9, 10 and 11. 50ml medium was inoculated on 250ml Erlenmeyer flask at 37°C in an incubator shaker at 100rpm. 2ml of broth was harvested aseptically and centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant thus obtained was used for protease assay.

Effect of Temperature on Protease Production

To study the effect of different temperature on protease production. 50ml medium was inoculated on 250ml Erlenmeyer flask incubate at different temperature 30°C, 37°C, 40°C and 45°C in an incubator shaker at 100rpm. 2ml of broth was harvested aseptically and centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant thus obtained was used for protease assay.

Identification of the bacterial strain

The protease producing strain was identified by various biochemical test and 16S rRNA gene sequencing. Genomic DNA was extracted as per the standard protocol[11] and its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed.

Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants.

Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Consensus sequence of 1393bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

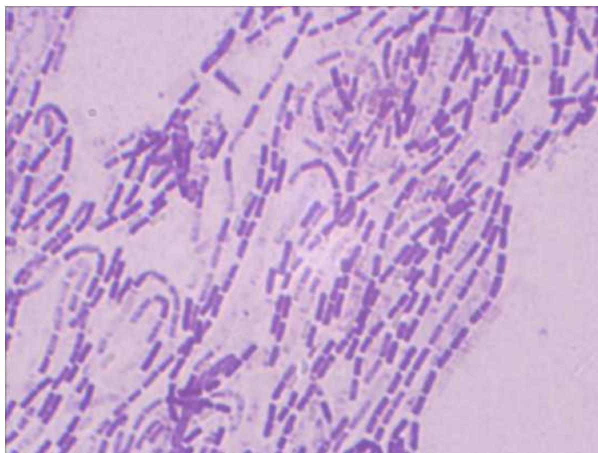
The PCR product was amplified and sequenced by Xcelris Labs Ltd. (Ahmedabad, India). The 16S rRNA gene sequence was submitted to GenBank under the accession number KC911634.

RESULT AND DISCUSSION

Isolation of protease producing bacteria

Total seven isolates were obtained on Nutrient agar plate with pH 7.2. The isolates were further screened for proteolytic activity on 5% skim milk agar plate. The zone casein hydrolysis around the bacterial colony indicates the protease producer (Figure–2). Three out of seven isolates were identified as protease producers. Isolate No.7 showed maximum zone around colony and stated as PPR7. Shake flask production of protease was done for isolates showing casein hydrolysis test on milk agar plate. The isolate PPR7 which showed maximum protease production was identified on the basis of morphological, cultural and molecular characteristics was found to be Bacillus thuringiensis as shown in Figure 1 and table no 2.

Fig.1 : Microscopic observation of PPR7 by Gram staining



Gram positive rods arranged in chains

Fig.2 : Zone of casein hydrolysis on skim milk agar plate

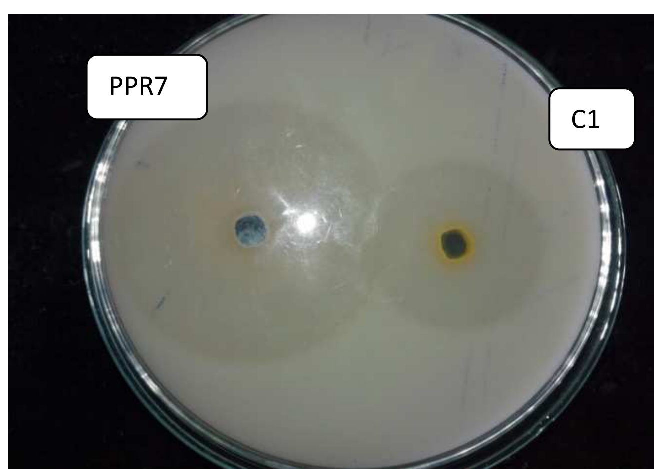


Table 2 : Morphological characteristics of well isolated colony of PPR7 on Nutrient agar medium

Shape	Round
Margin	Regular
Elevation	Convex
Texture	Mucoid
Opacity	Opaque
Pigmentation	No pigment
Gram Nature	Gram positive

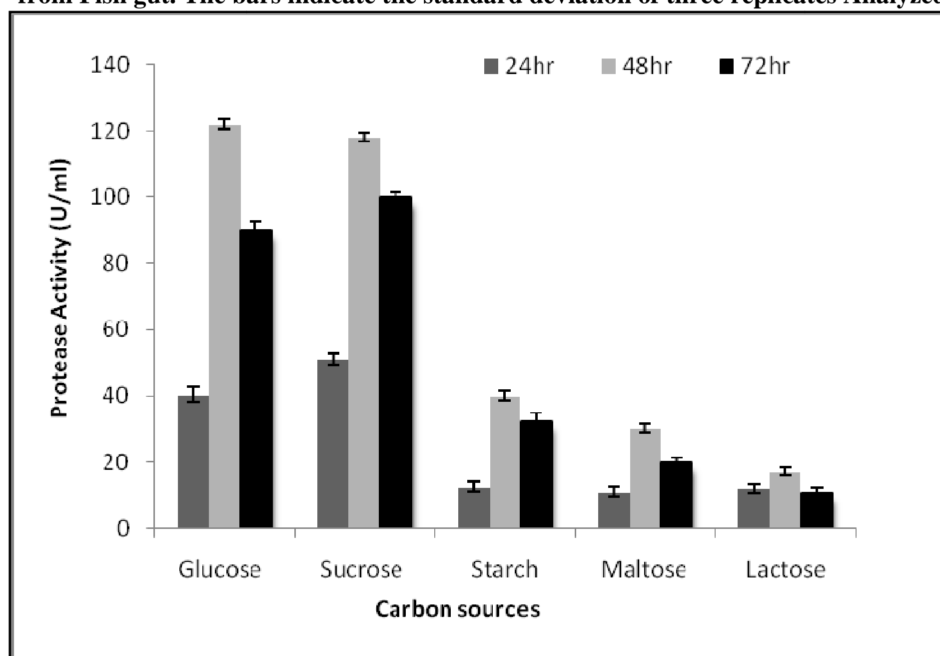
MEDIA OPTIMIZATION

Effect of carbon source of protease production

Among various carbon sources used, protease production was highest in the medium containing glucose (122.0 U/ml; 48 hrs.) and sucrose (118.2 U/ml; 48 hrs.) was found to be the best to support protease production (figure–3). While Lactose, maltose and starch showed the least protease production (17.0 U/ml; 30.1 U/ml & 40.0 U/ml respectively). Glucose and Sucrose can be easily utilized by our strain PPR7 compared to other saccharides. An increased yield of enzyme production from various carbon sources such as lactose[12], maltose[13] and sucrose[14] have been reported by other researchers.

Kumara *et al.,* 2012 and Ravishankar *et al.,* 2012 have evaluated various carbohydrates such as glucose, maltose, lactose, sucrose, mannitol, sorbitol, raffinose, xylose, fructose and starch for their effect on protease production. Carbon sources greatly influenced the enzymes production and the most commonly used substrate was reported to be glucose. It has been noticed that various sugars in the production and protease production depends upon the type of strain used under study[15,12].

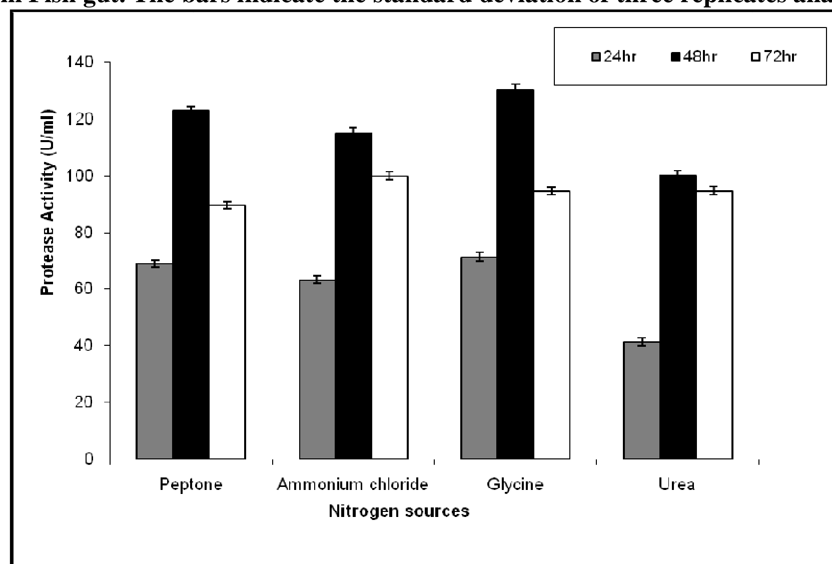
Fig.3 : Effect of Different Carbon sources on protease production in *Bacillus thuringiensis* (PPR7) isolated from Fish gut. The bars indicate the standard deviation of three replicates Analyzed



Effect of Nitrogen source of protease production

The various nitrogen sources on protease production were studied by replacing yeast extract in production media by various organic and inorganic nitrogen sources. Among the organic sources glycine and peptone showed high yield of protease production 130.4 U/ml and 123.0 U/ml respectively while inorganic nitrogen source like NH_4Cl and Urea showed 115.0 U/ml and 100.0 U/ml respectively (figure–4). In earlier reports, it was found that different nitrogen sources such as soybean meal, glucose, casamino acid, and peptone were effective medium ingredients for the protease production by Bacillus species[16,17,18]. Many researchers have been reported that organic nitrogen sources were better suited to *Bacillus* sp. for growth and enzyme production than inorganic sources. Both organic and inorganic nitrogen compounds were utilized by *Bacillus thuringiensis PPR7* strain giving almost the same protease production with less percentage of deviation. Its shows the versatility of the isolated bacteria utilizing a range of compounds as nitrogen source for protease production.

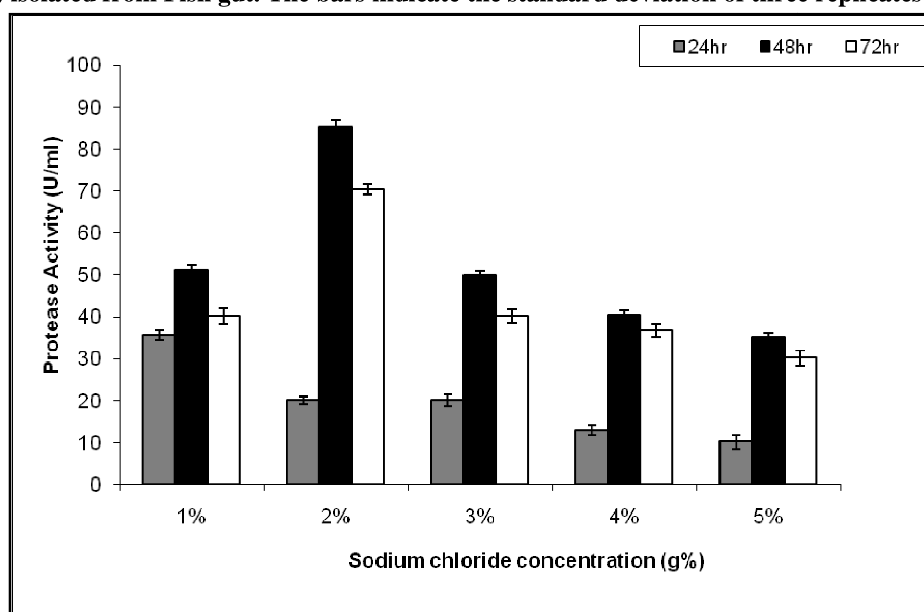
Fig.4 : Effect of Different Nitrogen sources on protease production in *Bacillus thuringiensis* (PPR7) isolated from Fish gut. The bars indicate the standard deviation of three replicates analyzed



Effect of varying NaCl Concentration on protease production

The effect of varying NaCl concentration was studied by supplementing the production medium with 1%, 2%, 3%, 4% and 5%, NaCl. Maximum protease production was observed at 2% NaCl (85.2 U/ml). Protease production was decreased remarkably in 4% (40.3 U/ml) and 5% (35.0 U/ml) NaCl concentration (figure–5). Mahendra *et al.* (2010) reported that *Bacillus aquimaris* showed growth between 1-3% NaCl and maximum protease activity at 2% NaCl concentration. An increased salt concentration creates change in the lipid composition of cell membrane. So, the growth rate decreases along with enzyme production. Mostly, gram positive moderate halophiles are often reported in the reduction of enzyme production at high salt concentration[19].

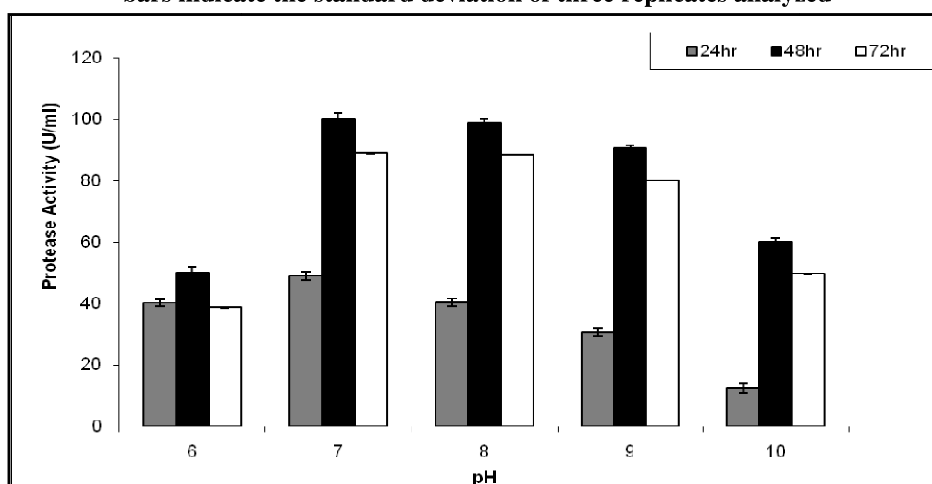
Fig.5 : Effect of varying Sodium chloride concentration on protease production in *Bacillus thuringiensis* (PPR7) isolated from Fish gut. The bars indicate the standard deviation of three replicates analyzed



Effect of pH on protease production

Bacillus thuringiensis PPR7 could grow and produce protease over a wide range of pH (2.0–10.0). Maximum protease production was observed at pH 7.0 (100.2 U/ml) (Figure-6). The production at pH 7 and 8 was relatively comparable. Protease production in *Bacillus spp.* has been reported to be optimum in the pH range 7.0 to 11.0 [20,12].

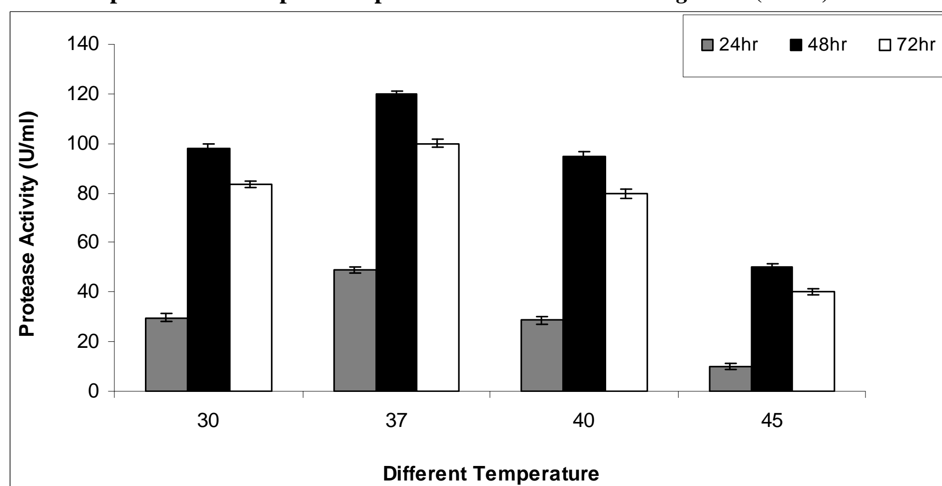
Fig.6 : Effect of pH on the protease production in *Bacillus thuringiensis* (PPR7) isolated from Fish gut. The bars indicate the standard deviation of three replicates analyzed



Effect of Temperature on protease production by PPR7

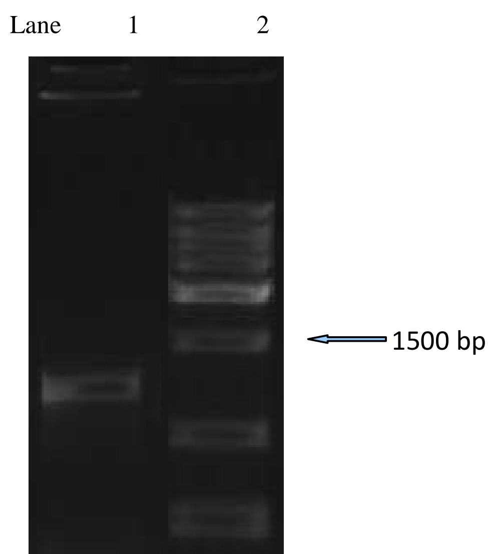
The effect of temperature on protease production was studied in production media incubating at different temperature 30°C, 37°C, 40°C and 45°C. The maximum protease production was obtained at 37°C (120 U/ml) and protease activity decreased remarkably in 40°C (95.0 U/ml) and 45°C (50.0 U/ml) (figure-7). Protease optimum activity of numerous protease from bacterial sources was between 30°C and 40°C[21,22]. The present study showed maximum protease production at 37°C in medium. However, *Bacillus* sp. was not capable of producing the enzyme at temperature below 25°C on other hand, a progressive decline in enzyme production was observed at 40°C and no enzyme production was observed at 50°C[13,6].

Fig. 7: Effect of temperature on the protease production in *Bacillus thuringiensis* (PPR7) isolated from Fish gut



The bars indicate the standard deviation of three replicates analyzed

Fig.8: Gel Image of 16SrDNA amplicon (Sample PPR7)



Lane 1:16S rDNA amplicon band
Lane 2: DNA marker

Phylogenetic tree of PPR7

The evolutionary history was inferred using the Neighbor-Joining method[23]. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed[24]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches[24]. The evolutionary distances were computed using the Kimura 2-parameter

method[11] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1387 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [25].

Fig.9: Chromatogram data file detail

(a) PPR-7_8F_S6952_D09_073.ab1: Data obtained with Forward primer

PPR-7_8F_S6952_D09_073 (985 bp)

GCGAATGAATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG
CCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTC
GAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC
AACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGT
TGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAG
TCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAG
AGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGG
CGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAAC
GCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTC
TGAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAG

(b) PPR-7_1492R_S6952_E09_071.ab1: Data obtained with Reverse primer

PPR-7_1492R_S6952_E09_071 (971 bp)

GCTGGCTCCAAAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTG
TACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGT
AGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTG
CAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTC
ATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTAAATGATGGCAACTAAG
ATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACACTGTCACT
CTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGC
GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCC
TTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCT
AACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTT
CGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCA
TTTCACCGCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCC
ACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCCAATAATT
CCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTA
GGTACCGTCAA

(c) Consensus Sequence PPR7 (1393 bp)

GCGAATGAATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG
CCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTC
GAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC
AACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGT
TGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG

GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAG
TCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAG
AGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGG
CGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAAC
GCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTC
TGAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAA
GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG
GGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATA
AAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCG
GATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTG
TAACACCCGAAGTCGGTGGGGTAACCTTTTGGAGCCAGC

Fig10 : Phylogenetic Tree: Evolutionary relationships of 11 taxa

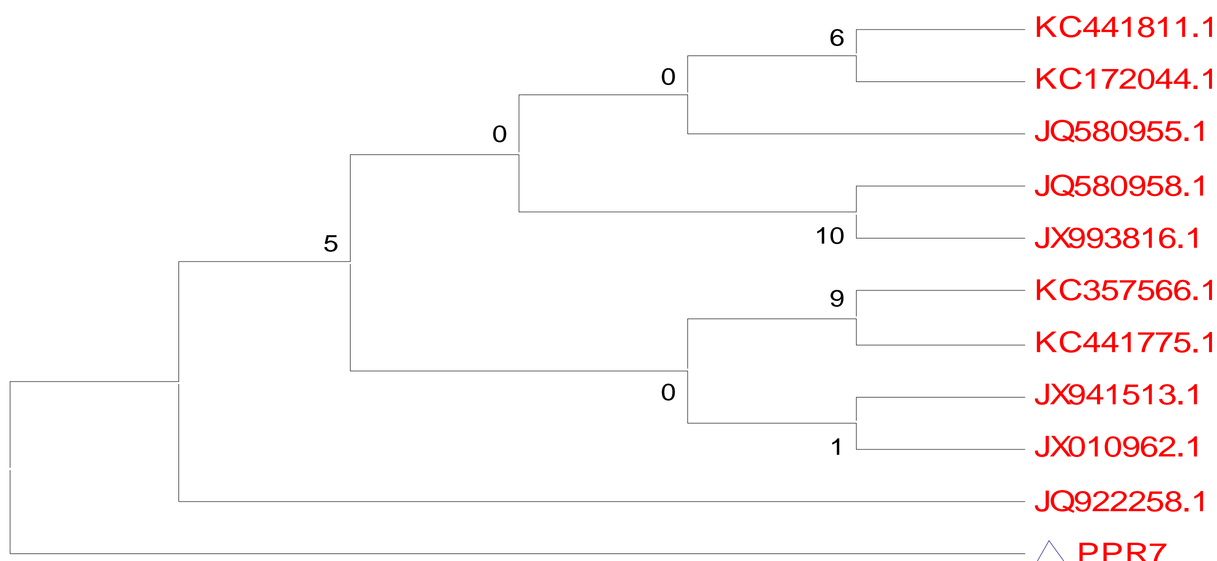


Table 3 : Sequence Producing Significant Alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JQ580958.1	Bacillus cereus strain KD125	2567	2567	100%	0.0	99%
JQ922258.1	Bacillus thuringiensis strain KUNi1	2514	2514	99%	0.0	99%
KPPR757566.1	Bacillus sp. P014	2507	2507	100%	0.0	99%
KC441811.1	Bacillus anthracis strain N34	2507	2507	99%	0.0	99%
KC441775.1	Bacillus anthracis strain D40	2507	2507	100%	0.0	99%
JX941513.1	Bacillus sp. B31(2012)	2507	2507	100%	0.0	99%
JX993816.1	Bacillus cereus strain XX2010	2507	2507	100%	0.0	99%
KC172044.1	Bacillus anthracis strain SH123	2507	2507	100%	0.0	99%
JX010962.1	Bacillus sp. A57	2507	2507	100%	0.0	99%
JQ580955.1	Bacillus cereus strain KD33	2507	2507	100%	0.0	99%

Table 4 : Distance Matrix

PPR7	1		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
JQ580958.1	2	0.001		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
JQ922258.1	3	0.001	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KPPR757566.1	4	0.001	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
KC441811.1	5	0.001	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000
KC441775.1	6	0.001	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
JX941513.1	7	0.001	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
JX993816.1	8	0.001	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
KC172044.1	9	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000
JX010962.1	10	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
JQ580955.1	11	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

CONCLUSION

The present study demonstrates that the many strains are on the market for commercial productions but the alternative source for isolation of better protease producer rather than conventional source i.e. from the gut of fish and eventually to better protease production by media optimization. The results obtained with the carbon and nitrogen sources were better with respect to the protease production which shows the potentiality and versatility of our culture *Bacillus thuringiensis* PPR7 which possesses a good proteolytic ability which lead to the significant improvement of enzymatic hydrolysis of proteins. Thus the protease producer strain obtained would be helpful for fish nutritionists for developing cost effective aqua feed on large scale.

Acknowledgement

Authors are very grateful to the laboratory facilities and financial support provided by the management of Ashok & Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences, New Vallabh Vidyanagar, Anand, Gujarat (India) and also wants to acknowledge Sophisticated Instrumentation Centre for Applied Research and Testing (SICART), Vallabh Vidyanagar, Gujarat (India) for providing instrumentation facilities. Authors also wish to acknowledge Xcelris Labs Ltd., Ahmedabad, Gujarat (India) for sequencing strain.

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