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Isolation, Characterization and Optimization of Amylase Producing Micro-Organism from Gastrointestinal Tract of *Catla Catla*

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ABSTRACT: Fresh water dwelling fishes have scanty information on gut microflora associated with them and there is a paucity of knowledge regarding microbial enzyme activity in fish gastrointestinal tracts. Although some of the enzymes like cellulase, proteases have been reported to be associated in the gut of fish.Amylase is one of the most important enzymes known and is of great significance and contributes of about 25% of the total enzyme market. It has a number of potential applications in food, pharmaceutical and fine chemical industries. This work describes the isolation, characterisation and optimization of amylase producing micro-organisms from gastrointestinal tract of*Catla catla*. Biological production of amylase by bacterial isolate PAC4 isolated from fish gut was identified to be *Bacilluscereus*. The maximum amylase production was reached to 92$\pm$ 3 U/ml in 48 hours of incubation.The various physico-chemical factors for optimum amylase fermentationwere found to be 3% sodium chloride concentration,37 °C temperature and pH 7.

KEYWORDS: *Catla catla*, *Bacillus cereus*, amylase, fermentation, media optimization

I. INTRODUCTION

In general, the bacterial flora of the gastrointestinal tract represent a very important and diversified enzymatic potential and it seems logical to think that the enzymatic mass lodged in the digestive tract might interfere in a considerable way with a major part of the metabolism of the host animal [1].Though considerable informationis available regardingtheintestinalmicrofloraofhomeothermsandtheirroleindigestion, reports On the bacterial population in the gastro intestinal tract of poikilotherms, including fish, and their role in digestion are scanty. Although few reports concerning microbial enzyme production in the gastro Intestinal tract of fish are available[2],[3],[4],[5],[6]information on the distribution of these enzyme-producing endosymbiont sin different regions of the gutarescarce[7],[8],[9],[10],[11].

Amylases are a group of hydrolyses that can specifically cleave the O-glycosidic bonds in starch. Two important groups of amylases are glucoamylase and α-amylase. Glucoamylase (exo- 1, 4-α-D-glucan glucanohydrolase) hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner[12]. The α- amylases (endo-1, 4-α-D-glucan glucohydrolase) are extracellular enzymes that randomly cleave the 1, 4-α-D-glucosidic linkages between adjacent glucose units inside the linear amylose chain[13],[12].The α-amylases are widely distributed in nature and can be derived from various sources such as plants, animals and microorganisms[13],[14].For the microbial α-amylase production, two types of fermentation methods are mainly used i.e. submerged and solid state[15]. Submerged fermentation (SmF) is comparatively advanced and commercially important enzymes are traditionally produced by this method[16],[17] whereas, solid state fermentation (SSF) is an old technology. However, in recent year SSF has emerged as a well developed biotechnological tool for the production of enzymes [18].

Amylases are among the important industrial enzymes & also have great significance in biotechnological studies. Microbial production of amylase is more effective than that of other sources as the technique is easy, cost effective, fast & can be modified to obtain enzymes of desired characteristics. The microbial amylase could be potentially useful in

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various pharmaceutical, fine-chemical industries etc., with the event of new frontiers in biotechnology, use of amylase has widened in clinical research, medical chemistry & starch analytical chemistry. Amylases are also used in baking, brewing, textile, detergent, paper &distilling industries[19]. These uses have placed greater stress on increasing indigenous amylase production & search for more efficient processes. Many microorganisms are known to produce this enzyme which includes bacteria and fungi[19]. The one most commonly used for their industrial production are *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Aspergillus niger*. The first enzyme produced industrially was an amylase from a fungal source in 1894, which was used for the treatment of digestive disorders.

The production amylase by fermentation has been thoroughly investigated and it is affected by variety of physicochemical factors. Most notable among these are composition of growth medium, inoculums age, pH, temperature, carbon and nitrogen source[13],[14].

Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of the digestive tract of fish confers a favorable culture environment for the microorganisms. Endogenous digestive enzymes in fish have been studied by several workers[3],[20]. In the present study, an attempt has been made to investigate the relative amount of amylase producing bacteria in the gastrointestinal (GI) tracts of two fresh water teleosts, namely the Indian major carp, *Catla Catla.*

II. MATERIALS AND METHODS

Sample-Collection:

In the current study the fish catla were sampled from the village pond located at Mogri, District of Anand, Gujarat (India). The average weight, total length and gut length of the fish studied are presented in Table 1.

Table 1 : Body Weight and Gut weight with their length of Catla fish.

Fish species	Body weight(gm)	Total length (cm)	Gut weight (gm)	Gut length (cm)
Catla catla	233	6.09	10.90	27

Homogenate Preparation:

The gut of the two healthy fish was dissected out in cold and aseptic condition, weighed, rinsed in sterile phosphate buffer (0.2M). Then it was mechanically homogenized in sterile phosphate buffer give 10^{-1} diluted sample. Homogenates were then centrifuged for 15 min at 7000 rpm. Supernatant was stored in a refrigerated at 4°C for further studies.

Isolation and Screening of microorganisms producing Amylase:

Isolation of bacteria was performed by serial dilution and spread plate method. The samples were serially 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 plate. The sample was spread inoculated uniformly using a sterile glass spreader. The plates were incubated at 37°C for 24 hrs. The bacterial isolates were further sub-cultured on a nutrient slant and maintained at 4°C in refrigerator for further studies.Bacterial isolates were screened for amylolytic properties by starch hydrolysis test on starch agar plate as described by Jacob and Gerstein (1960).

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 amylase production using Starch agar plates containing peptone (0.1% wt/vol), NaCl (0.5% wt/vol), agar (2.0% wt/vol), and Soluble starch (1% wt/vol) pH-7.0. The plates were incubated at 37°C for 24 hrs. A clear zone of hydrolysis after Lugol's Iodine solution addition gave an indication of amylolytic microorganisms. The organisms which were producing maximum zone size of hydrolysis surrounding the colony on starch agar plate selected for further shake flask testing.

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Amylase Production in shake flasks:
The culture medium used in this work for protease production contained (g/L) Starch, 10; peptone, 10; yeast extract, 20; KH_2PO_4, 0.05; $MnCl_2.4H_2O$, 0.015; $MgSO_4.7H_2O$, 0.25; $CaCl_2.2H_2O$, 0.05; and $FeSO_4.7H_2O$, 0.01 and pH maintained to 7.0.
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 amylase assay.

Determination of amylase activity:
Amylase activity was determined by sepctrophotometric method as described by Fisher and Stein. According to procedure 1.0 ml of culture filtrate was taken in test tube in duplicate and 1.0 ml of substrate (2% starch) was added. The test tubes were covered and incubate in water bath maintained at 37 °Cfor 15 minutes. The reaction was stopped by addition of 2.0 ml DNS reagent to each tube and kept in boiling water bath for 5 minutes. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer. A unit of amylase activity was defined as the amount of amylase required to catalyze the liberation of reducing sugar equivalent to one □mol of maltose per minute under the assay condition [21].

MEDIA OPTIMIZATION
Effect of different carbon sources as substrates on Amylase Production:
Starch in production medium was replaced by 1% each of sucrose, lactose, maltose, and Glucose. 50ml medium was inoculated with 5% inoculum of freshly grown culture and incubated at 37^{o}C on an orbital shaker at 100rpm speed for 72hrs. 2.0ml of broth was withdrawn aseptically after a period of 24, 48 & 72 hours and centrifuged at 10,000 rpm for 20 min at 4^{o}C and the resulted supernatant was used for amylase assay.

Effect of different Nitrogen Sources on Amylase Production:
Medium was supplemented with 0.5% each of meet extract, beef extract, and yeast extract. 50ml medium was inoculated with 5% inoculum of freshly grown culture and incubated at 37^{o}C on an orbital shaker at 100rpm speed for 72hrs. 2.0 ml of broth was withdrawn aseptically after a period of 24, 48 & 72 hours and centrifuged at 10,000 rpm for 20 min at 4^{o}C and the resulted supernatant was used for amylase assay.

Effect of varying NaCl Concentrations on Amylase Production:
The effect of different salt concentration was studied by supplementing the production medium with varied concentrations of NaCl such as 1%, 2%, 3%, 4%, and 5%. 50ml medium was inoculated with 5% inoculum of freshly grown culture and incubated at 37^{o}C on an orbital shaker at 100rpm speed for 72hrs. 2.0 ml of broth was withdrawn aseptically after a period of 24, 48 & 72 hours and centrifuged at 10,000 rpm for 20 min at 4^{o}C and the resulted supernatant was used for amylase assay.

Effect of varying pH on Amylase Production:
To study the effect of different pH on amylase production, the pH of production medium was set as 6, 7, 8, 9 and 10. 50ml medium was inoculated with 5% inoculum of freshly grown culture and incubated at 37^{o}C on an orbital shaker at 100rpm speed for 72hrs. 2.0 ml of broth was withdrawn aseptically after a period of 24, 48 & 72 hours and centrifuged at 10,000 rpm for 20 min at 4^{o}C and the resulted supernatant was used for amylase assay.

Effect of varying Temperature on Amylase Production:
To study the effect of different temperature on amylase production, 50ml medium was inoculated with 5% inoculum of freshly grown culture and incubated at 30^{o}C, 37^{o}C, 40^{o}C and 45^{o}C on an orbital shaker at 100rpm speed for 72hrs. 2.0 ml of broth was withdrawn aseptically after a period of 24, 48 & 72 hours and centrifuged at 10,000 rpm for 20 min at 4^{o}C and the resulted supernatant was used for amylase assay.

PCR amplification of the 16S rDNA and sequence determination of Isolate:
The purified amylase producing bacterial isolates were grown in nutrient agar slant and send for the PCR amplification of the 16S rDNA and sequence determination to Xcelris genomics, laboratory, Ahmedabad, Gujarat.

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II. RESULTS AND DISCUSSIONS

Isolation & screening of amylase producing bacteria:

Total five isolates were obtained on Nutrient agar plate with pH 7.0 from the catla.The isolates were further screened for starch hydrolysis testing on starch agar plate. The zone formation around the bacterial colony upon addition of Lugols Iodine solution indicated the amylase positive strain which is due to hydrolysis of starch. Hence these bacterial strains were identified as an amylase producer.One isolate wasidentified as amylase producers from catla showed maximum zone around colony and labelled asPAC4 (Figure No.1).

Morphological characteristics and cultural characteristics of PAC4 were given in table 2.The biochemical test results were shown in table 3 for PAC4 Isolate; and it was positive for Vogas Proskauer, Starch hydrolysis and Negative for Methyl red, Indole and Citrate (Table 3). The Biochemical test results of organism suggested PAC4 is found to be *Bacillus* spp. producing amylase enzyme. Das & Prasad also reported similar kind of result of biochemical test of organisms P5 and found the strain to be *Bacillus sp.*[22]. The production of α-amylase using a suitable organism and an economical medium has been a worth parsing achievement in the field of industrial biotechnology. Isolation and selection of suitable organisms is very essential for the production of extracellular α-amylase in abundance. Members of genus *Bacillus* were found to be better producer of amylase[23],[24],[25]. Some *Bacillus* produces the enzyme in the exponential phase, where as some others in the mid-stationary phase. Though the pattern of growth and enzyme profile of *Bacillus spp*. have similarities, the optimized conditions for the enzymes differ widely, depending upon the strain. The various physical and chemical composition of medium affects the production of enzyme like carbon sources, nitrogen sources, salt, pH and temperature[26]. Slight changes in temperature and pH have adverse effect on the growth of microorganisms as well as on the productivity of α-amylase[13],[26],[27].

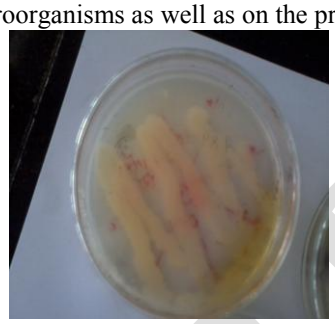

Figure 1 (a) : Colony growth on starch agar plate

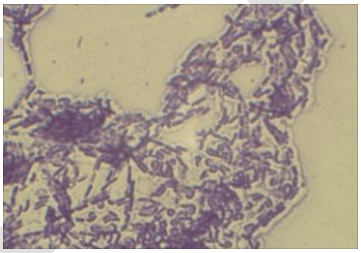

Figure 1 (b) : Gram Staining of PAC4

Table 2 :Morphological characteristics and cultural characteristics of PAC4

Size	Small
Shape	Round
Margin	Regular
Elevation	Convex
Texture	Smooth
Opacity	Opaque
Pigmentation	No pigment
Gram Nature	Gram positive rod shape

Table 3 : Biochemical tests of Amylase producing bacteria isolated fromCatla

Sr No.	Tests	Results PAC4
1	Methyl Red Test	+
2	Vogus Proskauer Test	-
3	Indol Test	-
4	Citrate Utilization	-

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5	Starch Hydrolysis	+
6	H_2S Production	-
7	Casein Hydrolysis	-
8	Acid production from carbohydrate	
	Glucose	+
	Lactose	+
	Maltose	+

MEDIA OPTIMIZATION

Effect of different carbon sources as a substrate on amylase production:

To study the influence of carbon source on the amylase activity the medium was supplemented with different sugars like Glucose, Sucrose, Maltose, and lactose at 1.0% concentration. The isolated bacteria showed efficient growth in all the substrates and amylase production but maximum amylase production was found in production media containing starch as carbon source with amylase production; 93U/ml, whereas minimum amylase production was found in media containing lactose as a carbon source with Amylase activity; 16U/ml as shown in the figure 2. Results are supported by earlier studies carried out for production of amylase with starch as carbon source for *Bacillus* sp.[28],[29]. Similar results were also been reported for the best growth and amylase activity in the presence of starch as carbon source at 37^{o}C for *Bacillus sp.*[20].It has been observed that lack of starch as carbon source in a media resulted in dramatic decrease in the enzyme production[28].*Bacillus megaterium* strain was found to be maximum growth with 3% starch as a substrate[30].

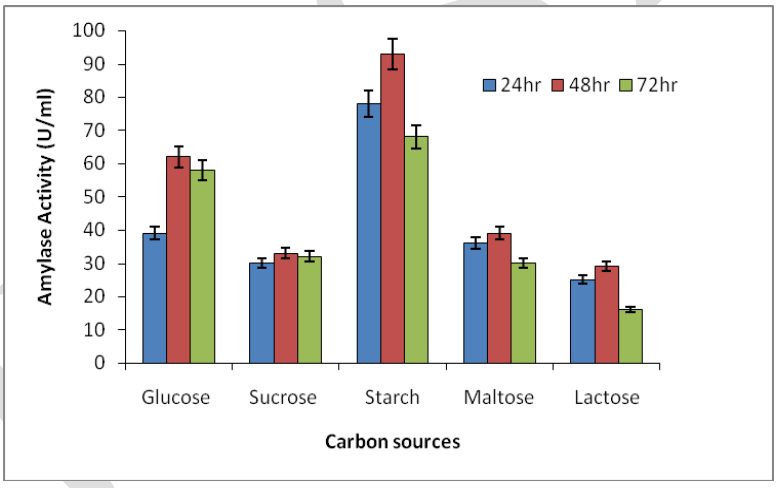

Figure 2 : Effect of different Carbon sources on amylase production

Effect of different nitrogen source on amylase activity:

To study the influence of nitrogen source on the amylase activity the medium was supplemented with different nitrogen source like meet extract, beef extract, yeast extract and nutrient broth. The bacteria for each co-substrate given efficient growth and amylase production but maximum amylase activity was found to be 95U/ml with medium containing yeast extract as nitrogen source, whereas minimum enzyme activity was found in medium containingmeet extractwith amylase activity; 29U/ml as seen in figure 3.Similar results are also been reported for the maximum production of enzyme using yeast extract as nitrogen source[20]. Strain of *Bacillus stearothermophillus* and *Bacillus amylolyticus* secreted maximum α-amylase in a medium supplemented with 0.5% yeast extract under various shaking conditions[31].

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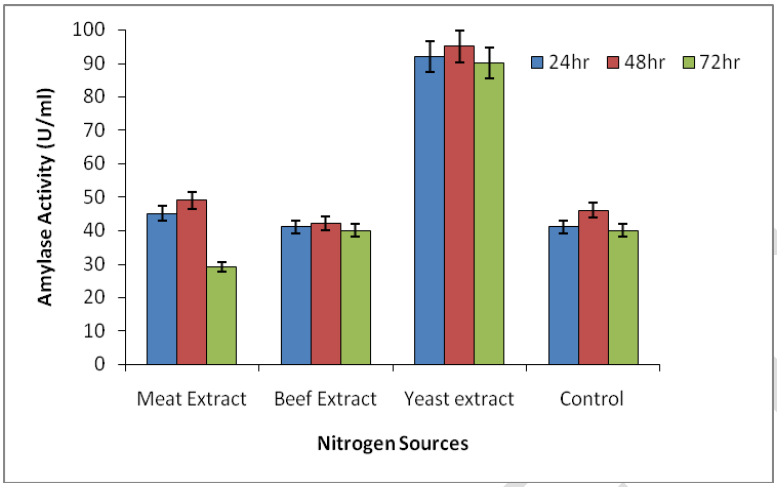

Figure 3 : Effect of different Nitrogen sources on amylase production by PAC4

Effect of different NaClconcentration on amylase activity:

It is necessary to check the behavior of bacteria in different NaCl concentration for optimum growth of organisms and to achieve maximum enzyme production. The experimental media were supplemented with different concentration of NaCl (1%, 2%, 3%, 4% and 5%) and incubated at 37°C.The maximum amylase production was found at 3% NaCl concentration showing amylase activity; 88U/ml, whereas below and above the 3% NaCl concentration decrease in amylase production was observed (figure 4). In one of the previously studied reports the production of extracellular α-amylase from *Bacillus marini*showed the maximum enzyme production at 5.5% NaCl concentration [28].

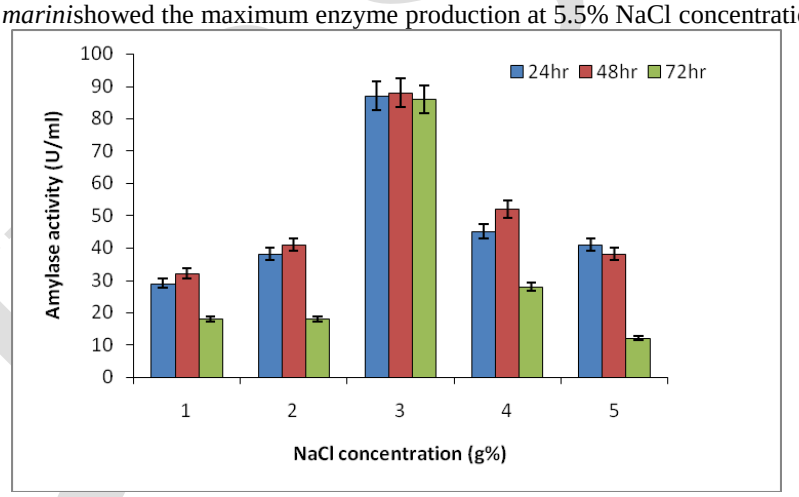

Figure 4 : Effect of different NaCl concentration on amylase production by PAC4

Effect of different pH on amylase production:

It was found that pH can significantly affect the growth of microorganisms because change in pH can influence fermentation by change in growth pattern of the culture as a result it will influence the metabolic activities. Thus, to check the effect of different pH on enzyme production, the experimental design with production medium adjusted to different pH (6, 7, 8, 9, and 10) inoculated with the culture and was incubated at 37°C. The maximum enzyme production was found at pH 7.0 (94U/ml) in 48 hours of incubation whereas further incubation did not show further increase in enzyme production (figure 5).Similar results have been reported by earlier studies carried out for production of amylase[32]. In *Bacillus megaterium* strain the maximum amylase production was found at pH 7.5[30]. Similarly,

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invarious *Bacillus spp.* maximum amylase production was observed in pH range of 6.5 to 7.5[33]. Earlier studies have revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth. The pH is known to affect the synthesis and secretion of α-amylase just like its stability.

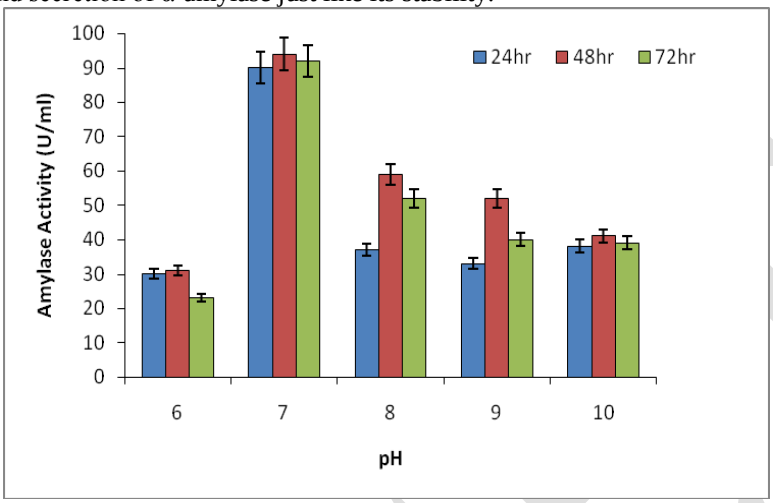

Figure 5 : Effect of different pH on the amylase production by PAC4

Effect of temperature on amylase production:

Temperature is an effective parameter for production of amylase enzyme by bacteria. To study the effect of temperature on amylase activity, the experimental flask containing starch medium were incubated at different temperature (30°C, 37°C, 40°C and 45°C) respectively. Production of amylase was increased from 30°C to 37°C i.e. 50 U/ml to 90 U/ml activity but further increase in the temperature showed decrease in amylase production (figure 6). Results are supported by earlier studies carried out for maximum production of amylase at temperature 37°C[34],[35],[30]. Bacterial amylase are produced widely range of temperature *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus lichniformis* are among the most commonly used *Bacillus sp.* reported to produced α-amylase at temperature ranging 37°C–60°C[34]. Ashwini et al also reported that *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 45°C and no enzyme production was observed at 50°C as the temperature increased or decreased, there was gradual decrease in the protein content and enzyme activity (Fig 6). At 50°C, the production of amylase and protein content was extremely low. These results may be due to virtual inactivity of enzyme at 20°C and denaturation of the enzyme above 50°C[36].

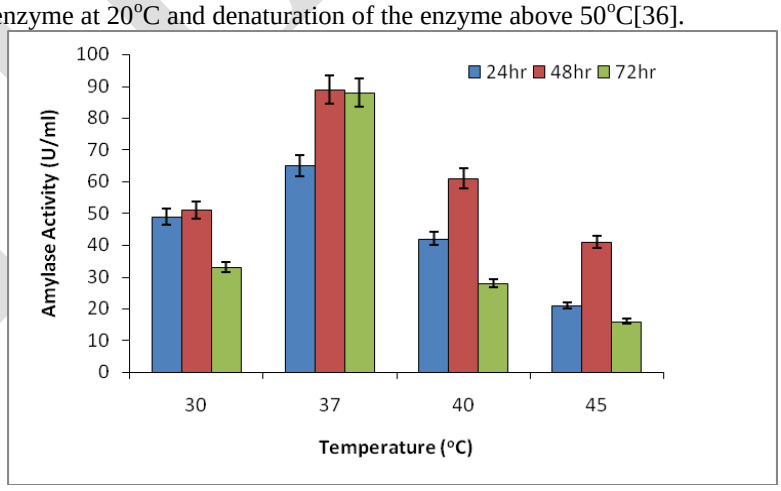

Figure 6 : Effect of different Temperature on the amylase production by PAC4

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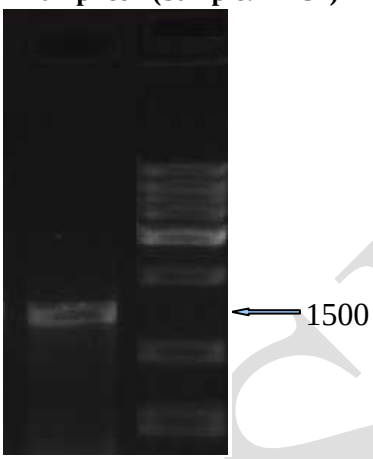
Colony PCR and 16S rDNA sequence analysis of PAC4:

Initial morphological and biochemical identification showed that the isolate wasGram-positive and rod-shaped (figure 1-b). The PCR amplification of 16S rDNA gene revealed a single band of amplified DNA product of ~1500-bp, indicating efficient amplification (figure 7). The DNA sequences were published in the NCBI databases under the specific accession numbers. The isolate **PAC4** was found to be ***Bacillus cereus* strain Pf-1** (GenBank Accession Number: **KC152883.1**) based on nucleotide homology andphylogenetic analysis. Information about other close homologs for the microbe can be found from the Alignment View table 4 and Phylogenetic tree (figure 8).

Figure 7 : Gel Image of 16S rRNA amplicon (Sample: PAC4)



[Lane 1:16S rDNA amplicon band; Lane 2: DNA marker]

Table 4 : Sequence Producing Significant Alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KC152883.1	*Bacillus cereus strain Pf-1*	2547	2547	100%	0.0	100%
JX134461.1	*Bacillus sp. A5-4*	2547	2547	100%	0.0	100%
KC484953.1	*Bacterium CulaenoE10E*	2547	2547	100%	0.0	100%
KC679054.1	*Bacillus cereus strain JY13*	2547	2547	100%	0.0	100%
KC439345.1	*Bacillus anthracis strain APT10*	2547	2547	100%	0.0	100%
KC153275.1	*Bacillus sp. G1-24*	2547	2547	100%	0.0	100%
KC153269.1	*Bacillus sp. G1-18*	2547	2547	100%	0.0	100%
FR821128.1	*Bacillus sp. AB358d*	2547	2547	100%	0.0	100%
FR821124.1	*Bacillus sp. AB289d*	2547	2547	100%	0.0	100%
JX544748.1	*Bacillus cereus strain CP1*	2547	2547	100%	0.0	100%

Phylogenetic Tree:

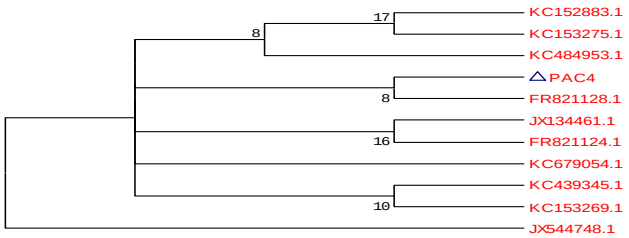


Figure 8 : Evolutionary relationships of 11 taxa

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The evolutionary history was inferred using the Neighbor-Joining method[37]. The bootstrap consensus tree inferred from 500 replicates[38]is taken to represent the evolutionary history of the taxa analyzed[38]. 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[38]. The evolutionary distances were computed using the Kimura 2-parameter method[39] 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 eliminatedfrom the dataset (Complete deletion option). There were a total of 1379 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4[40].

III. CONCLUSION

The present study demonstrates that the *Bacillus cereus* strains isolated from the fish gut shows amylase production which is non conventional source. The results obtained for media optimization for better amylase production needs to be done using statistical design analysis tool for better amylase production which will in turn shows its the industrial potentiality in amylolytic ability which would lead to the significant improvement of enzymatic hydrolysis of starchy materials. Thus the amylase producer strain obtained would be helpful for starch hydrolysis on large scale.

IV. ACKNOWLEDGEMENT

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