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Characterization of *Alcaligenes faecalis* GPA-1 producing thermostable extracellular α-amylase

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The bacterium coded GPA-1(isolated by Dr V Thankamani in 1990) was characterized by standard methods including microscopy, special stains, biochemical tests and growth on various types of media for systematic identification up to genus level. With 16S rRNA gene sequencing, the isolate was identified as *Alcaligenes faecalis* and deposited in NCBI with GenBank Accession number HQ 848384. The isolate was screened for the production of enzymes like amylase, protease and carboxy methyl cellulase (CMC). This isolate showed a clear zone of lysis on starch agar, yellow zone on CMC agar when stained with Congo red and a clear zone of casein hydrolysis in skim milk agar indicating amylase, cellulose and proteolytic activity respectively. Preliminary characterization of extra cellular amylase was done. The strain was found to be alkalophilic as it grew well in pH 9.0 and 10.0. The optimum temperature and salinity were found to be 37°C and 3% respectively. Growth curve experiments of the organism in nutrient broth containing 1% starch at varying physical and nutritional parameters were done up to 72 hrs, and the samples were also tested for pH changes, biomass, total protein, reducing sugars and α-amylase activity. Soluble starch (1%) in standard nutrient broth and pH 8.0, 37°C, shaking at100 rpm and 40-44 hours incubation were found to be the optimum conditions for maximal enzyme production.

Key words: *Alcaligenes faecalis* GPA-1, Alkalophilic, amylase.

From the beginning of 19th century the potential of using microorganisms as source of industrially relevant enzymes has stimulated interest in exploring microbes with extracellular enzymatic activity with various significant characteristic features like thermostability and alkalophilic nature (Akpan *et al*., 1999; Pandey *et al*., 2000; Abu *et al*., 2005; Thankamani and Lipin, 2011). Amylases are important enzymes employed in the starch processing industries for hydrolysis of starch into simple sugar constituents (Akpan *et al*., 1999; Mitchell and Lonsane, 1990). Some other industrial applications include baking, brewing, textile, paper industries and branches of chemistry including analytical and medical (Pandey *et al*., 2000). *Bacillus sp*are the most important and potent sources of α-amylase (Hema *et al*., 2006). As starch addition gives more amylase yield than un-supplemented substrate, this highlights the necessity of supplementation for higher and excess enzyme production (Alva *et al*., 2007).

The objective of this study was to characterize GPA-1 strain, screening for amylase production and optimization of growth conditions with various environmental parameters including pH, temperature and shaking.

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Materials and Methods:

Isolation of the microorganism

Garden soil sample collected from Angamali, Kerala, India (10°12′0″N, 76°24′0″E) was suspended in sterile saline. Aliquots were then inoculated on nutrient agar plates. The plates were incubated at 37°C for 24 hours. The colonies showing clear difference in cultural characteristics were further purified by sub culturing on nutrient agar and coded as GPA-1 and preserved on nutrient agar slopes in the refrigerator (Thankamani and Lipin, 2011).

Characterization of the isolate

The isolate was primarily characterized by Gram staining, biochemical tests including IMViC, catalase, nitrate reduction, TSI, urease, gelatin hydrolysis and carbohydrate fermentation to identify the isolate up to genus level. Further, it was characterized by 16S rRNA sequencing to find the species of the isolate. Results were analyzed as per Bergey's Manual of Systematic Bacteriology (Holding and Shewan, 1974).

Screening for multi enzyme production

Amylase

Amylase test was performed to determine the ability of organism to produce extracellular enzyme such as amylase to utilize the starch as carbohydrate source. The strain was inoculated in the media containing nutrient agar supplemented with 1% starch and incubated for 24 hours at 37°C. Iodine-Potassium iodide reagent was added to the plate and observed for presence of starch in the media which turned purple and clear zone formation around and within the colony which indicated starch utilization (Holding and Shewan, 1974).

Protease

The isolate was inoculated in skim milk agar (nutrient agar containing 1% skim milk), incubated at 37˚C for 24 - 48 hrs and monitored for zone of hydrolysis due to protein digestion (Thangam and Rajkumar, 2000).

Cellulase

Cellulase activity was determined by inoculating the isolate on nutrient agar containing 1% CMC and incubated for 24 hrs. After incubation, Congo red solution was poured on the plate followed by flooding with saturated sodium chloride solution (1M NaCl) and observed for the appearance of yellow zone due to the production of Cellulase (Huang and Monk, 2004).

Effect of physical parameters on growth:

The effect of physiological parameters such as temperature, pH and salinity on the growth of the organism was determined.

Effect of pH and temperature

The pH of culture medium plays a major role in the growth of an organism. Effect of pH on the growth of the organism was determined by subjecting it to different pH. The strain was streaked on nutrient agar of varying pH (7.0 to 12.0) and incubated at 37°C for 24 hours. After incubation, changes in growth were monitored.

Incubating an organism at its optimum temperature is essential for the growth of the organism. Effect of temperature on growth was determined by incubating the organism at different temperatures (Ambient (28-32˚C), 37, 50, 60, 70, 80 and 90˚C) and the growth parameters were monitored.

Effect of Salt

The salt tolerance level of the organism was determined by subjecting the organism to increasing salt concentrations. A loop full of culture was inoculated in to nutrient broth containing various salt concentrations from 1% to 10% (viz., 1%, 3%, 5%, 7% and 10%). Culture was incubated at 37° C for 24 hours and observed for growth.

Growth Curve of GPA-1

Two 500 ml Erlenmeyer flasks containing 200 ml of nutrient broth with 1% starch was inoculated with 5ml of the overnight grown culture and incubated in both ambient and incubator shaker for up to 72 hrs. At regular intervals, samples were withdrawn, centrifuged at 10000 rpm for 15 minutes and tested for the pH change of the medium, biomass, total protein (Lowry *et al*., 1951), reducing sugars and amylase enzyme activity as per standard protocol using soluble starch as the substrate in 0.1M phosphate buffer pH 7.0 (Ajayi, 2007).

Determination of reducing sugars

Bertrand *et al*., (2003) method was used to determine the reducing sugar content. 3, 5-Dinitrosalicylic acid (DNS) reagent was prepared by adding 1g of DNS in 20 ml NaOH (2N) and mixed with 50 ml of 60% potassium sodium tartarate and made up to 100 ml. The reducing sugar content from hydrolyzed starch was assayed by adding 2 ml of DNS reagent to 1 ml of the culture supernatant. The mixture was kept in a boiling water bath for 5 min, cooled and made up to 10 ml with distilled water. The absorbance was measured at 540 nm against reagent blank and enzyme control. Glucose was used as the standard.

Amylase assay

Amylase activity was determined by using soluble starch (dissolved in phosphate buffer, pH 7.0) as a substrate. Enzyme substrate complex (1+2ml) was incubated at 50^{o}C for 1hr, 0.5ml of this mixture was taken and added to 3ml of DNS reagent (Miller, 1959) and kept in a boiling water bath for 10 min, cooled, the volume of the sample was made to 10 ml by adding distilled water and then the absorbance was measured at 540 nm. Enzyme activity was expressed as micro grams of reducing sugars produced by one ml of the culture filtrate.

Comparison of Ambient shaking and Incubator (37^{o}C) shaking on Enzyme Production

Nutrient broth containing 1% starch was inoculated with the isolate and incubated at ambient shaking and incubator (37^{o}C) shaking and tested for α-amylase activity. Enzyme assay was estimated as described earlier.

Optimization of culture medium and incubation for maximum amylase production

The strain GPA-1 was inoculated in two sets of Nutrient broth maintained at three different pH (7.0, 8.0 and 9.0) and was incubated at both room temperature and 37˚C. After incubation, the culture broth was centrifuged at 10000 rpm for 10 min at 4˚C and analyzed for pH, biomass, total protein, glucose and enzyme.

Enzyme activity at various temperatures

Enzyme – substrate (1+2ml) mixture was incubated at temperature ranging from 40-90^{o}C for 1hr. Samples were tested with DNS reagent for amylase activity.

Enzyme activity at different substrate concentration

Effect of substrate concentration on enzyme activity was measured at different starch concentrations (1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%) (Alva *et al*., 2007) for different incubation periods viz., 1hr, 2hr and 4hr.

Thermo stability of Amylase

The thermal stability of the enzyme was determined by incubating the enzyme as such at temperatures ranging from 40-90˚C for 15mins, 30mins and 1hr and then carrying out enzyme assay as described above.

Results and Discussion

In the preliminary characterization, the organism was found to be a Gram

negative, motile bacillus based on microscopy and tentatively identified as genus *Alcaligenes* by biochemical tests (Table.1). Molecular characterization of the isolate was done and found to be *Alcaligenes faecalis by 16s rRNA* sequencing (NCBI GenBank Accession number HQ 848384).

Table: 1 Results of Gram Staining and Biochemical Tests.

Tests Performed	**Results**
Gram Staining	Gram Negative Bacilli
Indole Test	-
Methyl Red	-
Voges Proskauer	+
Citrate Utilization	+
Catalase Test	+
Nitrate Reduction	+
Triple Sugar Iron Agar	Alkaline slant and Butt; H_2S production
Urease test	-
Gelatin hydrolysis	Partial hydrolysis
Starch Hydrolysis	+
Carbohydrate fermentation	
Arabinose	-
Glucose	A
Lactose	-
Maltose	A
Manitol	A
Sucrose	-
Xylose	-
Growth on Nutrient Agar	+++
Growth on Mac Conkey	+++

A: Acid, (-) Negative and (+) positive

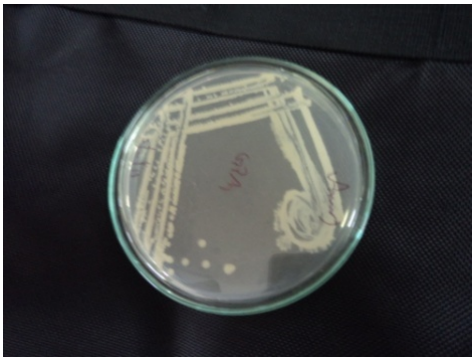

Fig 1: Growth of GPA1 On Nutrient Agar Plate

Fig 2: Growth of *GPA1* on Mc Conkey Agar Plate

Table 2: Response to Varying Physical Parameters

pH	7	+
	8	++
	9	+++
	10	++
	11	+
	12	-
Temperature	Ambient Temperature	++
	37^{o} C	+++
	50^{o} C	-
	60^{o} C	-
	70^{o} C	-
	80^{o} C	-
Salinity	1%	++
	3%	++++
	5%	+++
	7%	+
	10%	-

Screening for Extracellular enzymes of GPA1

Fig 3: NA+CMC – hydrolysis activity

Fig 4: Caesin agar -protease activity

Fig 5: Starch agar–amylase activity

Effect of pH, temperature and salinity

pH and temperature of the media were found to influence the growth of the isolate. The organism showed good growth on nutrient agar and could adjust to a wide range of pH (7.0 to 12.0) and in pH 9.0 the growth was maximum (Fig 6). The optimum temperature for growth of GPA1 was observed to be 37°C. In contrast, it was reported that 50 - 65˚C as optimum for *B. coagulans producing amylase* (Medda and Chandra, 1980). Regarding salinity, it showed maximum growth at 3% and slightly less in 5% (Fig 7). The isolate VTGP.A30808 identified as *Alcaligenes* species was also alkalophilic growing at pH 7-12 and in a wide range of NaCl concentrations, 1%, 2%, 5%, 7% and 10% (Thankamani and Lipin, 2011). Results are furnished in Table 2.

Fig 6: Growth of GPA-1 on varying pH from pH 7-12

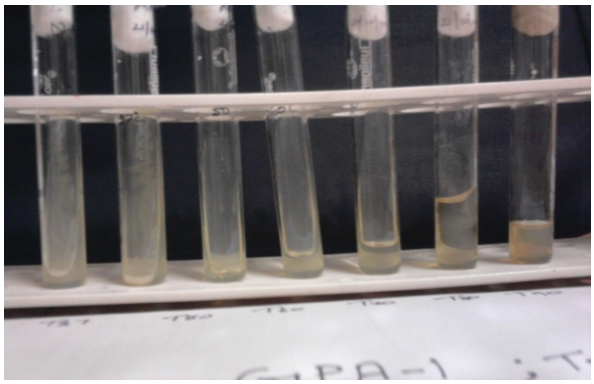

Fig 7: Growth of GPA-1 at different temperature from ambient to 90°C

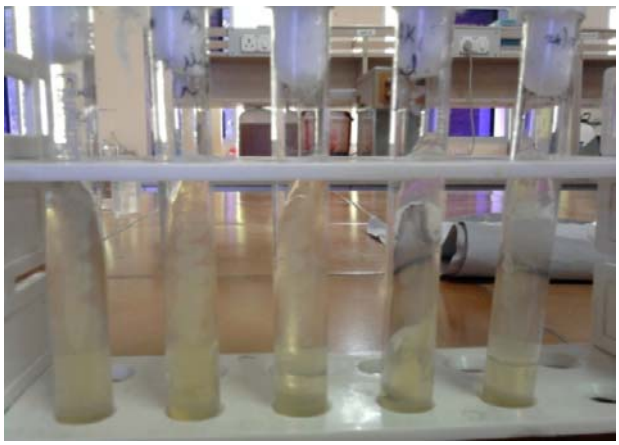

Fig 8:Growth of GPA-1 at different salt concentration ranging from1-10%.

Growth curve and Optimization

Influence of temperature and pH on amylase production was studied (Ramanathan *et al*., 2011). Maximum production of enzyme was observed between 36th and 48th hour which was 470.33 U/ml which was in ambient shaking condition. Optimization for suitable pH showed that maximum enzyme production was at pH 9 and with a slight difference at pH 8, which was calculated to be 700.33 units/ml and 650.33 units/ml respectively. The optimum pH for enzyme activity of other amylase producing bacteria *Bacillus subtilis* was 6.5 and 7.5 (Oduwole *et al*., 2009) and a lower optimum pH range of 5.5-6 was reported in *Bacillus licheniformis* (Amund and Ogunsina, 1987; Anthrin *et al*., 1990). Saliu and Bolanle Kudirat in 2009 reported an amylase producing gram-negative bacteria but the optimal pH was reported to be between 6.0 and 7.5.

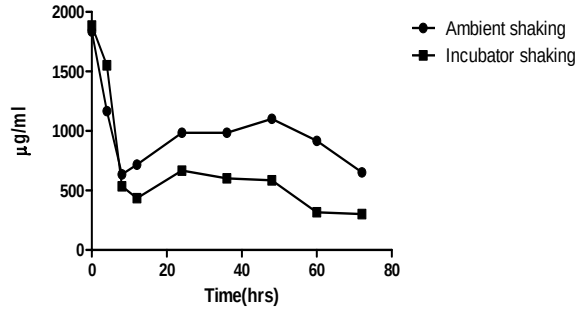

Fig 9: Total protein produced by GPA-1 at Ambient (28-32˚C) and Incubator (37˚C) shaking

At zero hour the protein concentration in the broths were 1835 and 1885µg/ml in the two flasks. After high rate of growth upto eight hours, the total protein content dropped. From eighth hour onwards till 48 hours, the concentration started increasing in both the flasks but the rates were significantly higher (1101.67µg/ml) in the medium incubated at ambient temperature compared to 37 ˚C (585µg/ml) (Fig 9).

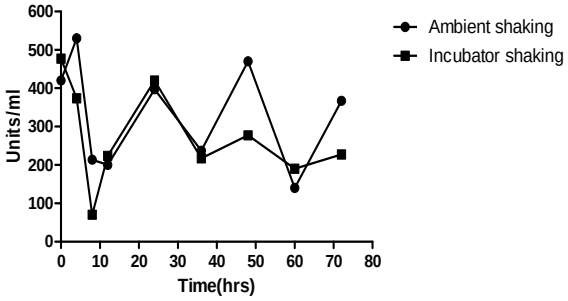

Fig 10: Amylase activity of GPA-1 at ambient (28-32˚C) and incubator (37˚C) shaking

The amylase production also was markedly different with wide variation in ambient temperature and 37˚C with 470.33 units/ml and 277 units/ml respectively. This clearly indicated that ambient temperature with shaking was better than 37˚C shaking. Previous works have reported maximum enzyme production at 96thhour in *Bacillus megaterium* which was 156 U/ml (Gurudeeban *et al*., 2011). It is also seen that production of α-amylase from GA2 (*Microbacterium foliorum*) was gradually increasing with incubation time and showed maximum yield (4090 units) at 120 hr (Roohi *et al*., 2011).

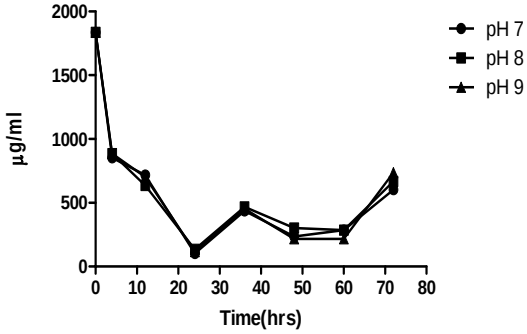

Fig 11: Influence of medium at pH 7, pH 8 and pH 9 on total proteins-GPA1

Similar to the observations in previous experiments, on the influence of incubation temperature on concentration of total proteins, the various initial medium pH showed a sudden fall in protein level from 0-4 hours and then from 12-24 hours. However, the pH did not have any apparent effect on the concentration of the soluble proteins at pH 7 (435 µg/ml), 8 (468.33 µg/ml) and 9 (451.67 µg/ml) at 36 hours (Fig 10).

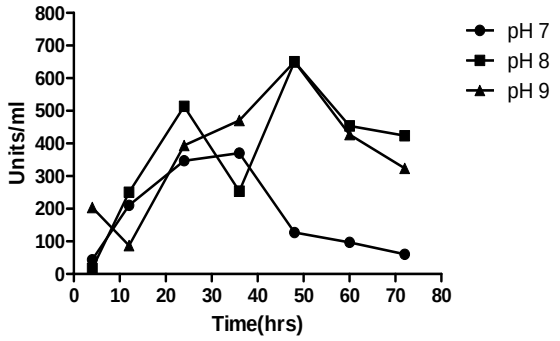

Fig 12: Amylase activity of GPA-1 at pH 7, pH 8 and pH 9

As seen in Fig.11, the maximum activity of amylase enzyme was observed at 48th hour with (663.67 units/ml) in pH 8, 127 units in pH 7 and 650 unts in pH 9. Earlier reports have stated that neutral pH was found to be optimal for the production of amylase by *B. thermooleovorans* NP54 and also reported in *B.coagulans* (Medda and Chandra, 1980), *B.licheniformis* (Krishna and Chandra, 1983) and *B.brevis* (Tsvetkov and Emanuilova, 1989).

Activity of amylase goes on increasing with respect to increase in substrate concentration but at 10% concentration of substrate, enzyme activity decreases. This decrease may be due to the unavailability of active sites due to the excess of substrate.

Crude amylase enzyme was found to be quite stable at different temperatures for various incubation periods. But the maximum activity was observed at 30 minute incubation at 50˚C which was 617 Units/ml. The enzyme remained stable even at very high temperature such as 80 and 90 ˚C with 423.6 and 363.3 Units/ml respectively. This observation also is possibly one of the first very few reports on *Alcaligenes fecalis* producing extracellular thermostable α-amylase enzyme.

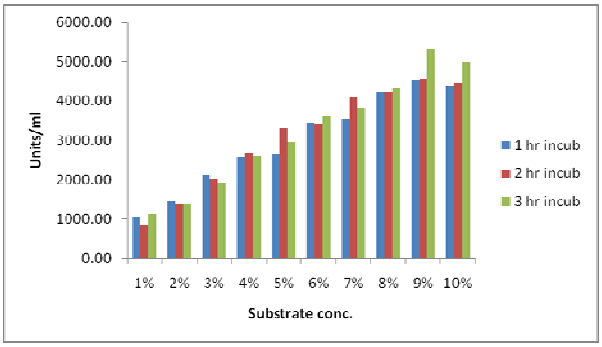

Fig 13: Influence of substrate and time on Amylase activity in culture filtrate

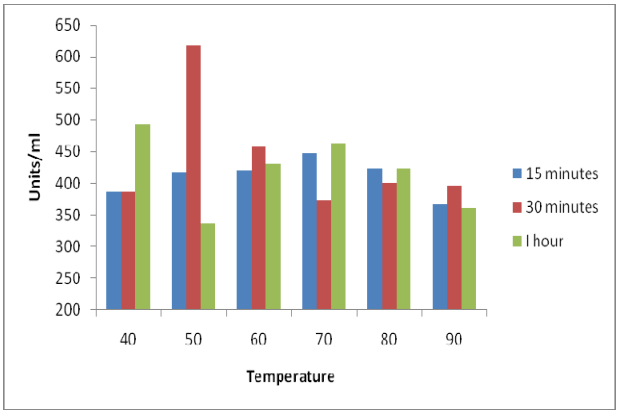

Fig 14: Stability of Amylase produced by GPA-1 at various temperatures

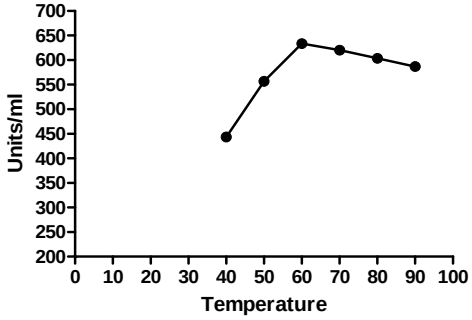

Fig 15: Amylase activity at different temperature from 40-90˚C

To determine the influence of temperature on activity of the α-amylase enzyme, the assay was carried out at 40, 50, 60, 70, 80 and 90˚C. Maximum amylase activity was observed at 60˚C (633.66 units/ml taken as 100%) and at 70˚C, the enzyme retained 90% of its activity. The activity decreased as the temperature was further increased. Similar findings were reported in *Bacillus cereus,* stating that, optimum temperature for amylase activity was 55˚C and 90% activity was retained at 70˚C (Hema *et al*., 2006).

Conclusion:

This work reports possibly for the first time that the gram-negative alkalophilic bacterium, an organism isolated from compost soil samples, identified as *Alcaligenes faecalis* by 16S rDNA sequencing and NCBI Acc. No: HQ 848384 was found to produce α-amylase enzyme which was highly thermostable, retaining enzyme activity up to even 90˚C.

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