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Regular Article

Optimization and cultural characterization of alkalophilic protease producing *Bacillus* sp. GPA4

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A new bacterial isolate belonging to Genus *Bacillus* coded as GPA4 was analyzed for protease activity. The strain GPA4 had the ability to produce extracellular protease. Nutrient media supplemented with 0.1% of casein showed maximum production of protease of 305 U/ml during the stationary phase. Maximum growth of the organism was observed at pH 7 but alkaline pH favored protease production. An increased level of 390 U/ml of enzyme was produced at pH 8. At 30°C under mild shaking conditions 100 rpm it produced 399.4 U/ml of enzyme compared to static and shaking incubation at 37° C. Among different metals, addition of Zn^{2+} induced more enzyme production. Addition of carbon source in the form of glucose and nitrogen source as soy bean meal increased protease production to 451.4 U/ ml and 465 U/ml respectively.

Keywords: Bacillus, alkalophilic, GPA4, alkaline protease.

Enzymes are organic catalysts that play a vital role in many aspects of life. They have been used by civilizations since centuries. Enzymes play a huge role in day to day life from simple fermentation to complicated gene expressions.

Protease are enzymes that have the ability to degrade protein by the breaking of the hydrogen bond that binds protein into peptides and protein. Microbial proteases belong to acid, neutral or alkaline based on their pH optimum for activity and the active sites viz., metallo (EC3.4.24), aspartic- (EC.3.4.23), cysteine- or sulphydryl- (EC.3.4.22), or serine-type (EC.3.4.21). Alkaline proteases are stable in alkaline pH and posses a serine residue at the active site. Microbial proteases have been extensively studied and are used in numerous fields ranging from detergent industry, leather processing, molecular biology, Genetic engineering, medicine to food industry. Proteases form 60% of the total global enzyme market estimated to be $6 billion by 2011(Gupta et al., 2002).

Bacillus is the major producer of protease. This paper presents the results of the characterization of an alkaline protease produced by *Bacillus* GPA4.

Materials and methods

Screening for protease production

The Bacterial isolate used in this present study was isolated by Dr. V. Thankamani, which is a *Bacillus* and coded as GPA4. The organism was screened for extra cellular protease production by inoculating in skim milk agar at 37°C for 24 to 48 hours.

Characterization of the Isolate

The strain was primarily characterized by microscopy and cultural characterization. Further characterization was done for biochemical, sugar fermentation and the effect with environmental parameters such as temperature, pH and sodium chloride were done.

Growth Curve

Nutrient broth containing 0.1% casein was used as growth media inoculated with 10% of overnight grown culture of *Bacillus* GPA4. The inoculated broth was incubated at 37° C under static condition. Samples were drawn at every 4 hr interval up to 48 hours and were centrifuged at 10,000 rpm for 15 minutes at 4° C. The parameters measured included pH, biomass, protein content and protease enzyme activity by caseinolytic activity (Kanekar et al., 2002).

Protein assay

Protein content of the sample was tested by Lowry's method (Lowry et al., 1951). To 1ml of sample, 5 ml of Lowry's reagent was added and incubated at 37° C for 10 min and 0.1 ml of Folin Ciocalteu reagent was added followed by incubation at room temperature for 40 min and the absorbance was measured at 660 nm. Concentration was calculated from the standard graph plotted with known BSA concentration.

Protease Assay

Protease production by *Bacillus* GPA4 was measured by caseinolytic activity. To 2ml of casein substrate (2%) 0.5 ml of sample containing enzyme was added and the substrate-enzyme complex was incubated at 37° C for 10 minutes. The reaction was stopped by adding 0.5 ml of trichlor acetic acid (100 mM) and incubated at 37° C for 30 min. The precipitate was centrifuged at 10,000 rpm for 10 min. To 1 ml of clear supernatant obtained 3 ml of 0.5M sodium carbonate and 0.2 ml of folin's reagent (1N) was added. Absorbance was measured at 660 nm. The values were compared with the standard graph plotted with varying concentration of tyrosine. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µmol of tyrosine per min under standard assay conditions.

Optimization of the growth curve

Effect of pH and temperature

Influence of pH (Naidu et al., 2005) on growth and protease production was determined in different pH values ranging from 7.0 to 9.0. Flasks were incubated at 37° C under static condition. Influence of temperature (Shaheen et al., 2008) was determined by incubating the flasks at different temperature ranges 20 to 40°C at a constant pH 8.0. Samples were drawn at regular intervals, centrifuged at 10,000 rpm for 15 min at 4°C and analyzed for pH, biomass, protein content and enzyme production.

Effect of static, ambient shaking and shaking incubation

The effect of external parameters such as static, shaking at ambient temperature (Venkatadri et al., 1990) and shaking incubation at higher temperature on growth and protease production was determined by incubation of inoculated flasks at ambient non shaking condition; shaking (100 rpm) at room temperature and in a shaker incubator 37° C (100 rpm). Samples were drawn at regular intervals and analyzed for the above mentioned parameters.

Effect of Metal Ions

To determine the effect of different metals (Darani et al., 2008) on the growth and protease production, the growth medium was supplemented with different metallic salts (0.05%) such as $CaCl_2$, $CuSo_4$, $MgSo_4$ and $ZnCl_2$ and inoculated with 10% overnight

culture and incubated in shaker (100 rpm). Samples were drawn out periodically and analyzed for biomass, pH, protein and enzyme content.

Effect of carbon, organic and inorganic nitrogen sources

Effect of carbon (Thys et al., 2006) source and nitrogen (Abidi et al., 2008) sources on the growth and protease production was determined by supplementing growth medium with 0.1% of different carbon (glucose, fructose, lactose, sucrose and starch), 0.05% of organic nitrogen (malt extract, peptone and soy bean meal) and inorganic nitrogen sources (ammonium sulphate, sodium nitrate). 10% of culture was inoculated to the media and incubated in shaker (100 rpm). Samples were drawn periodically. The parameters measured were biomass, pH, protein content and enzyme production.

Results and Discussion

Screening for proteolytic activity

The isolate incubated with skim milk agar showed clear zone of hydrolysis due to proteolytic digestion which indicated the production of protease by the isolate *Bacillus Sp.* GPA4 (Fig. 1).

Characterization of the Isolate

The strain was Gram positive, non motile, rod shaped bacilli. It showed positive reaction in methyl red, citrate, nitrate, catalase, urease, starch hydrolysis and gelatin liquefaction tests and could ferment glucose. The isolate was able to grow over a temperature range of 20^{o} C to 40^{o} C and at pH 7.0 to pH 9.0 and could tolerate salt up to 9% (g/l).

Growth curve

Growth of the organism and enzyme production by the culture was determined periodically by drawn sample at every 4 hours interval up to 48 hours. Lag phase was very short and the log phase was up to 4hr followed by the stationary phase till 16th hour, after that decline phase was observed. Protease production was observed during the stationary phase. Enzyme production was maximum in 24 to 36 hours (13.344 g/L, 305 U/ml) after which the production started to decline. A similar study on *Bacillus sp* (Patel R et al., 2005) showed maximum enzyme production in stationary phase. During growth the pH changed towards alkaline (pH 8.05) which indicated that alkaline condition was favourable and thus the *Bacillus GPA4* was alkalophilic (Fig. 2).

Effect of pH

The influence of pH on the growth and enzyme production was studied by inoculating the isolate in media with different pH, 7.0 to 9.0, as the growth was maximum in this pH range. The growth pattern changed when inoculated into higher pH medium, the log phase was extended (up to 24 hrs) and the biomass also increased with increase in pH (Fig 3 and 4). Regarding enzyme production, it followed the same trend as growth, showing the maximum values in pH 8.0 (biomass 9.80 g/L, enzyme activity 390 U/ml) and at pH 9.0 (9.45g/ L, 374.6 U/ml) (Fig 3). Similar study on *Bacillus subtilis* (Das et al., 2010) showed maximum production at pH 8.0 and 9.0. Growth of *Bacillus* GPA4 was high at pH 7.0 but enzyme production was higher in alkaline pH which coincided with the previous study by *Bacillus amovivorus* (Sharmin et al., 2005).

Effect of Temperature

Effect of temperature on growth and enzyme production was observed at different temperatures including 20, 30 and 40^{o}C. Above 40^{o} C, there was no growth, thus it was a mesophilic bacterium as it preferred moderate temperature for growth. There was no significant difference in the enzyme

production in all the 3 different ranges of temperature. Highest protease production was observed at 30° C (399.3 U/ml) at the stationary phase (Fig 5). A similar study on *Bacillus Sp*. I18 (Genckal and Tarib, 2006) showed maximum enzyme production at 30° C.

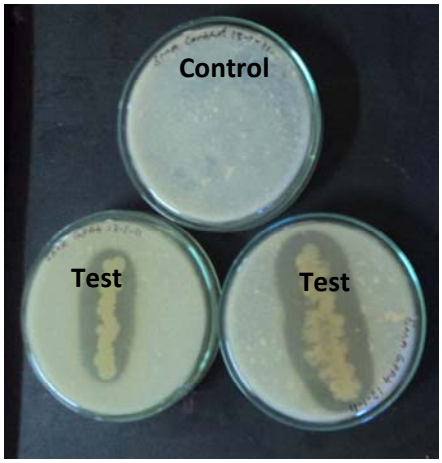

Fig 1: Skim Milk Agar incubated with *Bacillus* GPA4 showing clear zone of hydrolysis due to proteolysis after 24 hrs of incubation at 37˚C.

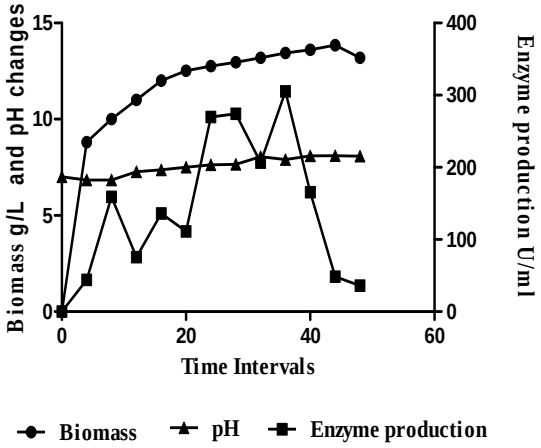

Fig 2: Growth curve of *Bacillus* GPA4 in nutrient broth containing casein at pH 7 incubated under static condition at 37° C.

Effect of Static, shaking at ambient temperature and shaking Incubation

Growth and enzyme production was observed in three different conditions viz., shaking at room temperature, shaking incubation at 37° C (100 rpm) and static condition. Shaking incubator yielded maximum biomass production (16.15 g/L) compared to shaking at room temperature and static incubation at 37° C (Fig 6). Enzyme production was found to be high in shaking at room temperature (399.4 U/ml) (Fig 6). A similar study on *Bacillus sp* I18 (Genckal and Tarib, 2006) showed highest enzyme production at 100 rpm In contrast, a study with *Bacillus cereus* (Shafee et al., 2005) showed lesser production at 100 rpm.

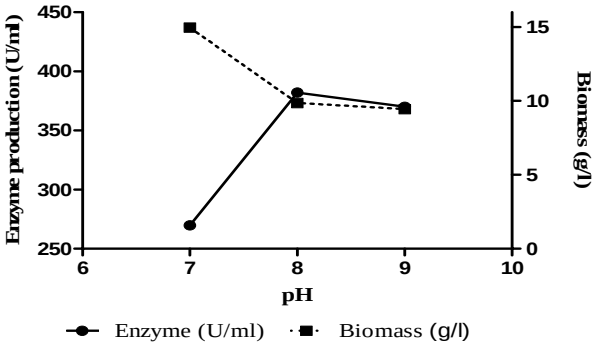

Fig 3: Effect of pH on growth and enzyme production by *Bacillus* GPA4 (32 hours)

Effect of Metal ions

Influence of metals on the growth and enzyme production was observed by the addition of metallic salts to the basal growth medium. Addition of metallic salts increased the enzyme production. Zinc had significant effect on growth and enzyme production (7.05 g/l and 376.6 U/ml) followed by copper (13.39 g/ L and 333.3 U/ ml) and calcium (11.10 g/ L and 351.3 U/ml) (Fig 7). In contrast, a study showed reduction in production with zinc by *Bacillus licheniformis* (Mabrouk et al., 1999).

Effect of Carbon source

Carbon sources (0.1%) such as glucose, fructose, lactose, sucrose and starch were supplemented in the growth media to study their effect on growth and enzyme production. The growth was not significantly

affected by the addition of sugars. Biomass level was high with starch (14.3 g/l) and minimum with lactose (9.6 g/l).There were slightly increased enzyme production in all additional sugar containing media including starch (450 U/ml), sucrose (449 U/ml) and fructose (448 U/ml) similar to glucose (Fig 8). A similar study with *Bacillus thuriengensis* (Chudasama, et al., 2010) showed increase in production by addition of glucose and fructose. In all the sugars maximum enzyme production was observed at 24 hours, which is similar to a study by *Bacillus cereus* (Bhatiya et al., 2010) *that* showed maximum production at 24 hours. On the contrary, a report showed that glucose addition repressed enzyme production by *Bacillus aquimaris* (Mahendran et al., 2010).

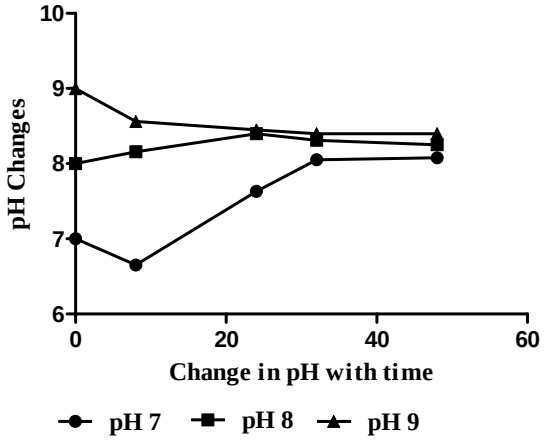

Fig 4: pH changes from the initial medium pH over time

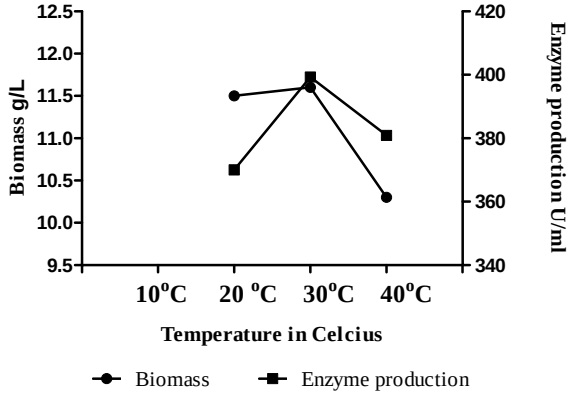

Fig 5: Effect of temperature on biomass and enzyme production (24 hours)

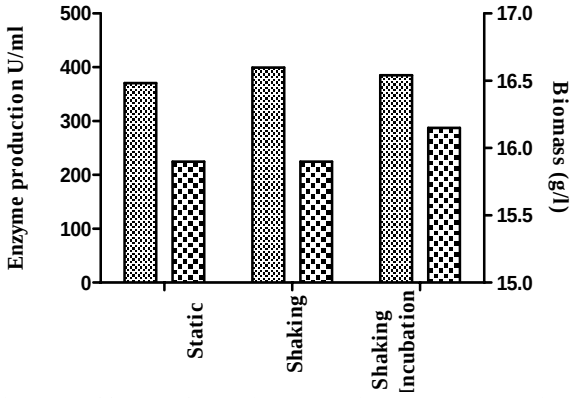

Fig 6: Effect of static, shaking and shaking incubation on growth and enzyme production (32 hours)

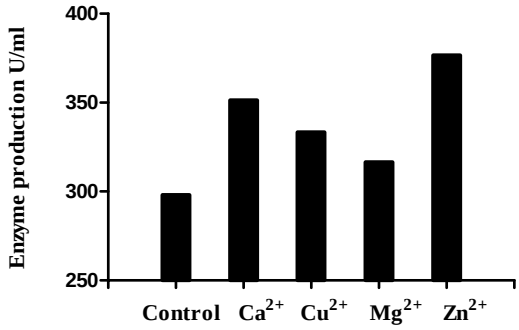

Fig 7: Effect of metallic salts on enzyme production under shaking at room temperature (8 hours)

Effect of inorganic and organic nitrogen source

The influence of organic and inorganic nitrogen source (0.05 %) on the growth and enzyme production was observed. Addition of different nitrogen sources had not much significant influence on the growth. Maximum growth was seen in peptone (15.25 g/L) and least with ammonium sulphate (11.35 g/l). Enzyme production was higher than the control flask. Regarding enzyme production soy bean meal increased the production (465 U/ml), followed by peptone, malt extract, sodium nitrate and ammonium sulphate with 463.2 U/ml, 460.2 U/ml, 458 U/ml and 456 U/ ml respectively (Fig 9). A previous study on *Bacillus* sp (Chauhan et al.,

2004) and *Conidiobolus coronatus* (Laxman R. S et al., 2005) also showed increase in enzyme activity on the addition of organic nitrogen source soy bean meal. *Aureobasidium pullulans* (Chi et al., 2007) also showed increased enzyme production on the addition of inorganic source like sodium nitrate.

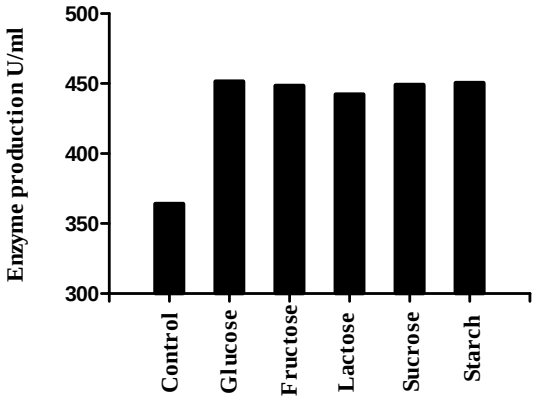

Fig 8: Effect of various carbon source (0.1%) on enzyme production under shaking at room temperature, initial pH 8 for 24 hours

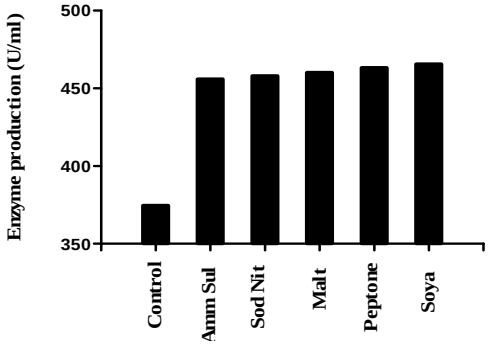

Fig 9: Effect of various organic and inorganic sources on growth and enzyme production under shaking at room temperature, initial pH 8 for 24 hours

Conclusion

The isolate *Bacillus* Sp. GPA4 was characterized in detail by biochemical methods. Growth curve showed that the lag phase was very short or almost absent and log phase lasted for 4 hours followed by stationary phase. After 44 hours decline in biomass level was observed. This might be the beginning of the death phase. The enzyme production was observed to be at maximum rate from 24 to 36 hours. The isolate had the highest enzyme activity at alkaline pH which indicated that the protease was alkalophilic. The temperature of 30º C had positive influence on the enzyme production which was found to be equivalent at this range. The isolate was not able to survive above 40˚ C. Mild shaking of 100 rpm enhanced enzyme production compared to static condition. Among the metal ions Zn^{2+} enhanced enzyme production at the log phase itself. The sugars dextrose, fructose, lactose, sucrose and starch had nearly equally significant influence on the enzyme production. Inorganic nitrogen sources such as ammonium sulphate, sodium nitrate and organic nitrogen sources such as malt, peptone and soy bean meal also had similar effects on the enzyme production.

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