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Isolation and screening of gut microflora of a freshwater crab *Paratelphusa jacquemontii (*Rathbun) for amylase production

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Abstract

The fresh water edible crab is abundantly found in the Orathanadu region, Pattukottai district, Tamilnadu and its gut microflora was studied. The density of the gut bacteria was ranged from $1.4x10^5$ to $2.1x10^3$ CFU/g. Among the isolates *Pseudomonas aeruginosa* (8 %) constituted the highest percentage of occurrence followed by *Bacillus amyloliquefaciens* (6 %), *Escherichia coli* (6 %), *Lactobacillus cellobiosus* (5 %), *Pseudomonas fluorescens* (4 %), *Pseudomonas putida* (2 %), *Lactobacillus casei* (2 %), *Lactobacillus delbrucckii* (2 %), *Bacillus subtilis* (1 %), and *Micrococcus luteus* (1 %). Potential enzyme producers were identified and this paper deals with an amylase producing *Bacillus amyloliquefaciens*. Optimization of various physicochemical parameters on growth and enzyme production revealed that 36 hrs of incubation period pH 8, 35°C, 0.5% NaCl concentration, starch as carbon and peptone as nitrogen source were found as ideal conditions for both growth as well as amylase production. Mass scale production with the optimum conditions produced growth of 2.24 OD and 682U/ml/min. of amylase.

Keywords: Gut microflora, freshwater crab, *Paratelphusa jacquemontii*, enzyme production, amylase

Introduction

The gut serves as the natural habitat for numerous bacteria, some are beneficial to the host and others are harmful. The primary function of the gut is to take up water and nutrients. The resident colonic gut microflora play a vital role in digestion by fermenting the nutrients in the food that cannot be digested by the host intestine, also in growth and disease resistant of the host (Olsson *et al.*, 1992). Some organisms have been shown to maintain a permanent and consistent microbiota in the gut, which is significantly different from that of the surroundings, for example in the giant prawn *Macrobrachium rosenbergi* (Colorni, 1985).

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 (Hungate, 1975 and Saha *et al.*, 2006). Amylase production by the intestinal bacteria in one mangrove crab and seven fish species has been reported (Sugita *et al.*, 1996). Though the gut microflora of fishes and prawns have been well studied, only few studies are available on edible crabs from Indian waters especially in freshwater crabs. Hence the present study was aimed to analyze the gut bacterial populations in a freshwater crab *Paratelphusa jacquemontii* found in paddy fields and also the bacterial isolates were screened for amylase production.

Materials and Methods

Collection of experimental animals

The fresh water edible crabs are abundantly found mainly in and around paddy fields of Orathanadu region, Pattukottai Dist, Tamilnadu, India. It is an important nominee and cultivable for aquaculture in India and are cultured in the ponds of delta districts of Tamilnadu. Animals with a body weight of 30±3 g (carapace width of 34±3 mm) were collected and they were identified as *Paratelphusa jacquemontii*. The collected fresh animals were immediately used for the isolation of gut bacteria.

Isolation of gut bacteria from *Paratelphusa jacquemontii*

Before separating the gut, the crab samples were clearly washed with 10% formalin solution for a minute, subsequently washed with tap water and then finally with sterile distilled water to remove the surface microflora. The carapace of the crabs was gently removed, by using a sterile scalpel the gut was aseptically removed and thoroughly washed 2 to 3 times with 0.9% saline to remove the non adherent bacteria (Talpur *et al.*, 2011). The fore gut, mid gut and hind guts were used. They were homogenized in saline, were three fold serial diluted and directly plated on sterile nutrient agar (NA) medium (Hi Media, Mumbai, India) using spread plate method. Plates were inoculated and incubated at room temperature for 24-48 hrs.

Microbial density in the sample were expressed as colony forming units (CFU) CFU/g of the sample and it was calculated using the formula: Total microbial load in the given sample ($CFUg^{-1}$) = Total number of colonies/ Total volume of the sample x Volume of sample plated (0.1 ml) × dilution factor. Morphologically different colonies were isolated and streaked on NA slants and were maintained at 4°C.

Screening of gut bacterial isolates for amylase production

In the present morphologically different isolates isolated from the gut of *Paratelphusa jacquemontii* were screened for the production of amylase. Initial screening for amylase production was qualitatively assessed by spot inoculation of morphologically different colonies on starch agar plates using sterile tooth pick. After incubation at 28 ± 2°C for 24-48 hrs, plates were flooded with an aqueous solution of iodine solution (1mg/ml) for 15 min. The iodine solution was poured off and the formation of zone of clearance was considered as the positive result. For the quantitative assessment of the most potent amylase producing strain (based on the highest zone of clearance) was used and it was further inoculated in to respective broth and incubated at 30°C for 24 to 48 hrs. After the incubation period the culture broth was centrifuged at 10000 rpm at 4°C for 10 min. and the clear supernatant was used as a source of crude enzyme for quantitative measurement.

Amylase activity assay

Amylase activity was quantitatively assessed as described by Palanivelu *et al.*, 2001. The reaction mixture consisting of 1.25 ml 1% (w/v) soluble starch (Merck) solution, 0.25 ml, 0.1 M Sodium acetate buffer (pH 5.0), 0.25 ml of distilled water, and 0.25 ml of properly diluted crude enzyme extract was taken and after 10 min. incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitro salicylic acid method (Miller, 1959). One unit (U) of α-amylase is defined as the amount of enzyme releasing 1µmol glucose equivalent per minute under the assay conditions.

Identification of gut bacteria

Bacterial strains were identified using Bergey's manual of Determinative Bacteriology (Buchanan *et al.*, 1974).

Optimization of growth and amylase production

The strain was further selected and optimized for various physiochemical parameters such as incubation period (0-48 hrs with 6 hrs interval), pH (5, 6, 7, 8, 9 and 10), temperature (25°C - 50°C with 5°C interval), NaCl concentration (0%, 0.5%, 1%, 1.5%, 2% and 2.5%), 1% carbon such as glucose, fructose, maltose, sucrose, starch and cellulose and 0.5% of different nitrogen sources such as beef extract, yeast extract, peptone, ammonium nitrate and sodium nitrate were tested using one parameter at a time. The optimum parameter achieved by each step was fixed for the subsequent steps. For growth absorbance was measured at 600nm using a UV spectrophotometer (Systronics, Double beam spectrophotometer - 2202). As growth and enzyme production was found to be highest at the 36th hr of incubation further parameters tested were tested at the same incubation period.

Mass scale culture in shake flask

The optimum conditions for growth and enzyme production such as 36 hrs of incubation period pH 8, 35°C, 0.5% NaCl concentration, starch as carbon and peptone as nitrogen source were used for mass scale production in a shake flask (1L conical flasks with 0.75L of the medium) at 150 rpm. Growth and enzyme production was assessed for every 6 hrs as previously mentioned.

Results and Discussion

The density of the gut bacteria was ranged from $1.4x10^5$ to $2.1x10^3$ CFU/g. Janczyk *et al.*, 2007 and Liu *et al.*, 2011 studied the intestine microbial diversity of terrestrial and aquatic animals isolated from different environments. Typical viable counts in a mud crab intestine (10^6 to 10^8) are significantly lower than those reported for humans and terrestrial animals (approx. 10^{11} CFU g^{-1}) (Mead, 1997).

The species composition (diversity) of isolates showed different species of organisms ranged from aerobic (*Pseudomonas* spp. and *M. luteus*) to facultative anaerobic forms such as *Lactobacillus* spp., and *Bacillus* spp. Among the isolates *Pseudomonas aeruginosa* (8 %) constituted the highest percentage of occurrence, followed by *Bacillus amyloliquefaciens* (6 %), *Escherichia coli* (6 %), *Lactobacillus cellobiosus* (5 %), *Pseudomonas fluorescens* (4 %), *Pseudomonas putida* (2 %), *Lactobacillus casei* (2 %), *Lactobacillus delbrucckii* (2 %), *Bacillus subtilis* (1 %), and *Micrococcus luteus* (1 %) (Fig. 1 and Table 1).

Fig. 1 – Isolation of gut bacteria from *Paratelphusa jacquemontii*

Table 1 – Diversity (species composition) of gut bacteria from *Paratelphusa jacquemontii*

Strain	% of occurrence
Pseudomonas aeruginosa	8 %
Pseudomonas putida	2 %
Pseudomonas fluorescens	4 %
Bacillus subtilis	1 %
Bacillus amyloliquefaciens	6 %
Lactobacillus cellobiosus	5 %
Lactobacillus casei	2 %
Lactobacillus delbrucckii	2 %
Escherichia coli	6%
Micrococcus luteus	1 %

In a mud crab *S. serrata;* Najiah *et al.,* 2010 isolated 91 bacterial strains belonged to 12 species, in the present study 10 different species belonged to 5 different genus were isolated from the freshwater crab *Paratelphusa jacquemontii*. Variations in the microbial species composition ranged from *Bacillus, Micrococcus, Cornyforms, Vibrio, Pseudomonas, Aeromonas, Achromobacter, Flavobacterium* and *Enterobacterium* in the gut samples of different crabs such as *S.serrata, C.helleri* and *P.pelagicus* were observed by Rameshkumar *et al.,* 2009. Presence of these microbes may have significant role in the growth, resistance to diseases and digestive process (Lapez *et al.,* 1977). Gut bacteria have shown to play an important role in cellulose digestion, nitrogen acquisition and conservation in ruminants and termites. Existence of these gut microflora as an endosymbiotic associations in some aquatic invertebrates had long been recognized.

Sugita *et al.,* 2002 observed *Bacillus, Coryneforms, Enterobacteriaceae, Flavobacterium, Micrococcus, Pseudomonas* and *Vibrio* sp. in the intestinal tracts of Japanese flounder. Whereas Harris (1993) observed *Vibrio, Pseudomonas, Flavobacterium, Aeromonas, Micrococcus* and *Staphlycoccus* as the most commonly isolated bacterial genera in the gut microbiota of crustaceans. Zhang *et al.,* 2016 studied the symbiotic bacteria in gills and guts of Chinese mitten crab (*Eriocheir sinensis*) differ from the free-living bacteria in water found 5 dominant intestinal bacterial phyla *Tenericutes* (46.62% to 98.94%), *Bacteroidetes* (0.08% to 36.11%), *Proteobacteria* (0.23% to 5.44%), *Firmicutes* (0.21% to 6.01%) and CKC4 (0.03% to 6.25%). Sankar *et al.,* 2013 isolated more frequent incidences of *Micrococcus* spp. in ghost crabs whereas in the present study the frequency *Micrococcus* spp. (1%) was found to be the least one.

The role of enzymes in many industries has a long history and more than 3000 different enzymes have been screened and characterized from mesophilic organisms for the commercial exploitation of the microbial world (Gupta *et al.,* 2002). The first amylase was isolated by Anselme Payen in 1833 and they are extensively used in various industries such as bread making, paper, textile and garment making and also used in liquefaction of starch, production of food, sugar and adhesives (Udani and Hardy, 2004). Apart from amylolytic activity these enzymes have great significance in several industries including lots of biotechnological applications (Lin *et al.,* 1997).

Bacteria living in the gut region have the ability to digest the carbohydrates (Galli and Giese, 1959). The midgut of crabs supports good growth of many enzyme producing bacteria such as proteolytic (Vankateswaran *et al.,* 1981), amylase-producing in gut bacteria of one mangrove crab and seven fish species (Sugita *et al.,* 1996). Presence of symbiotic organisms in the guts of some aquatic invertebrates has been known for a long time for example cellulose-degrading bacteria in the gut of *Teredo* (Hidaka, 1954) and *Cristispira* in *Saxidames gigantes* (Berkeley, 1959).

In the present study the bacterial isolates from the gut were qualitatively screened for industrially important enzyme production (i.e.) amylase production. The most potent enzyme producing strain was selected based on the highest zone of clearance and were further screened and quantitatively assessed for respective enzyme production. Based on the result the most potential amylase producing strain was identified as *Bacillus amyloliquefaciens* (Fig. 2 and Table 2).

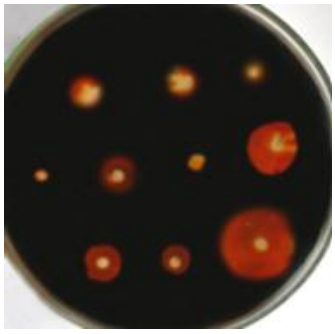

Fig. 2 - Screening for amylase production

Table 2: Screening of gut bacterial isolates for amylase production

Strain	Screening for amylase production Zone of clearance (mm)
Pseudomonas aeruginosa	14
Pseudomonas putida	15
Pseudomonas fluorescens	9
Bacillus subtilis	12
Bacillus amyloliquefaciens	28
Lactobacillus cellobiosus	16
Lactobacillus casei	8
Lactobacillus delbrucckii	11
Escherichia coli	9
Micrococcus luteus	-

The strain was further selected and optimized for various physiochemical parameters such as incubation period, pH, temperature, NaCl concentration, carbon and nitrogen sources for mass scale production. The results showed that 36 hrs of incubation period pH 8, 35°C, 0.5% NaCl concentration, starch as carbon and peptone as nitrogen source were found as ideal conditions for both growth as well as amylase production (Figs. 3-8). With the optimum conditions in the mass scale medium highest growth and amylase production of 2.24 OD and 682U/ml/min. was observed respectively (Fig. 9).

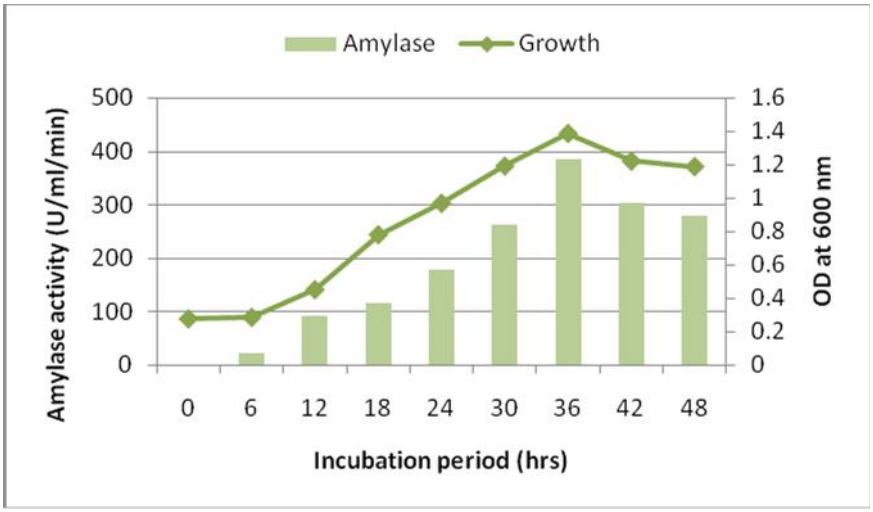

Fig. 3 - Effect of incubation period on growth and amylase production by *Bacillus amyloliquefaciens*

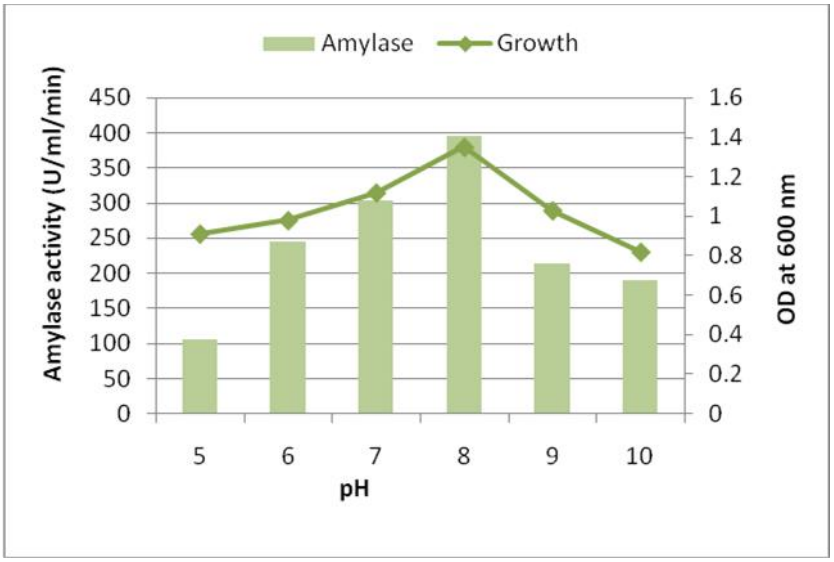

Fig. 4 - Effect of pH on growth and amylase production by *Bacillus amyloliquefaciens*

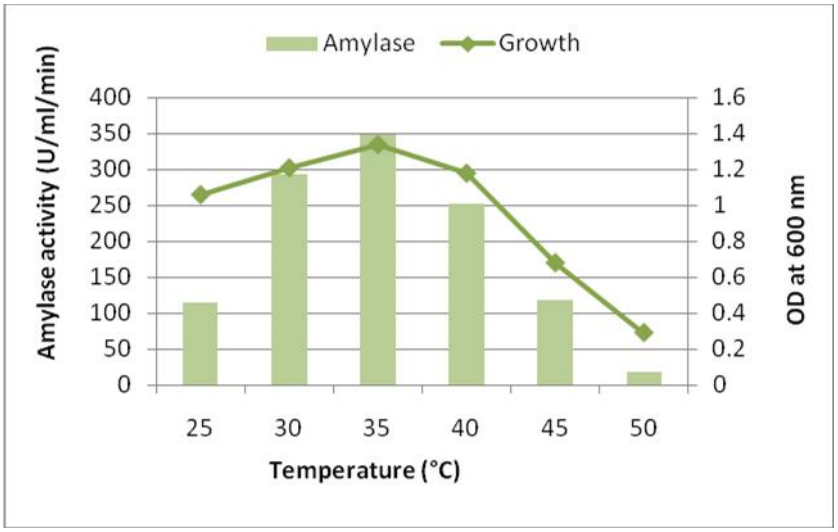

Fig. 5 - Effect of temperature on growth and amylase production by *Bacillus amyloliquefaciens*

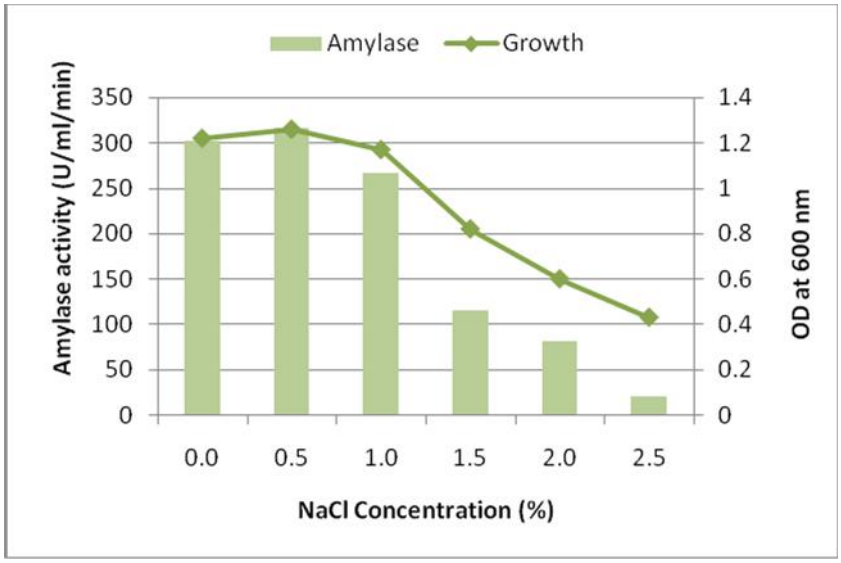

Fig. 6 - Effect of NaCl concentration on growth and amylase production by *Bacillus amyloliquefaciens*

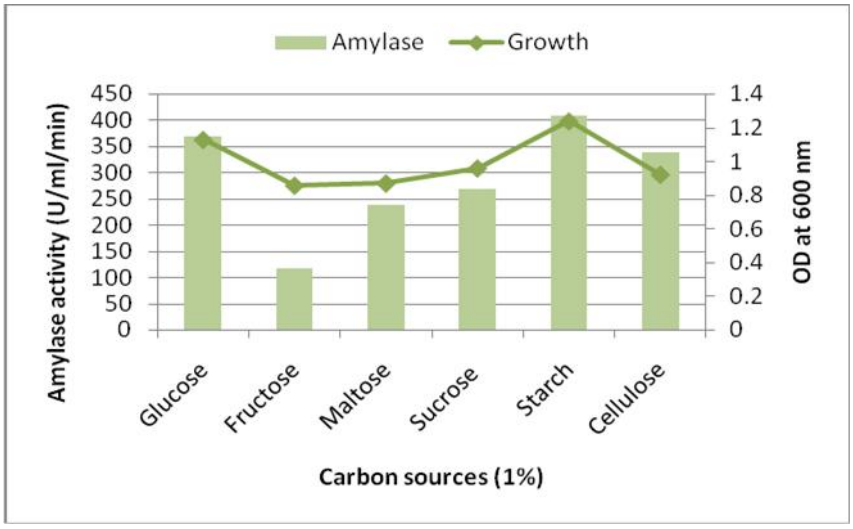

Fig. 7 - Effect of carbon source (1%) on growth and amylase production by *Bacillus amyloliquefaciens*

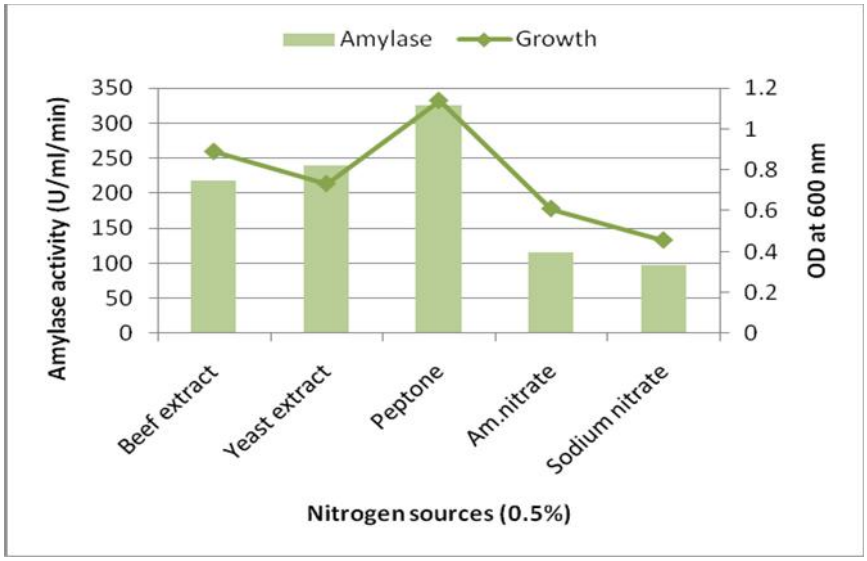

Fig. 8 - Effect of nitrogen source (0.5%) on growth and amylase production by *Bacillus amyloliquefaciens*

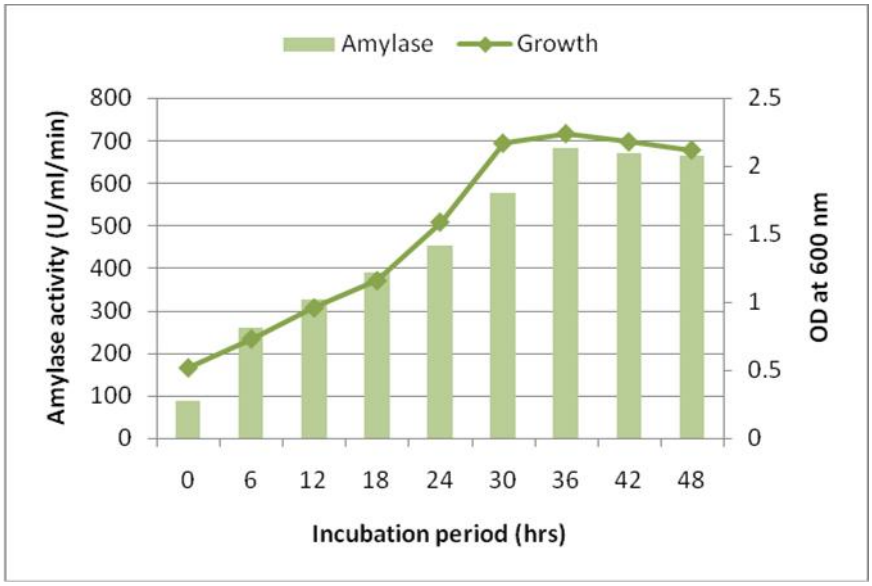

Fig. 9 - Mass culture and amylase production from *Bacillus amyloliquefaciens*

Like in the present study, Rukhaiyar and Srivastava (1995) observed 1% starch supported maximum amylase production. Similar to the present investigation peptone as the ideal organic nitrogen, Hayashi *et al.,* 1988 found organic nitrogen sources supported maximum amylase production, Soni *et al.,* 1996 observed maximum amylase production when using soyabean meal as the sole nitrogen by *Saccharomylopsis capsularis* and in *Botryodiplodia thebromae*. On the other hand Das *et al.,* 2004 found ammonium chloride as inorganic nitrogen supported highest amylase production in a *Bacillus subtilis* DM-O3 strain.

The amylase production by the gut microbe *P. jacquemontii* showed that it might feed on detritus also. Amylase and other enzymes of various gut microflora of this crab can be exploited for commercial purposes.

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