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PRODUCTION OF AMYLASE ENZYME BY ISOLATED BACTERIAL STRAIN *PANTOEA GAVINIAE* FROM *BOMBYX MORI*

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ABSTRACT

Amylases are classes of enzymes capable of digesting starch and has wide range of industrial applications from food to effluent treatment. The aim of study is to isolate starch degrading bacteria from *Bombyx mori* and to produce amylase using isolated bacterial strain. Materials and methods: 50 isolates were isolated from *Bombyx mori*, SACT2 strain exhibited higher amylase activity and identified as *Pantoea gaviniae.* The isolated strain was used for amylase production and optimization of media was done using various parameters.Starch degrading bacteria as *Pantoea gaviniae* was isolated and used for amylase production. The amylase production by the isolated strain was higher of 2.970(U/ml).

KEYWORDS: ***Starch, Amylase, Bombyx mori, Pantoea gaviniae***

INTRODUCTION

Starch is an abundant carbon source in nature, and amylases are starch degrading enzymes. They are widely distributed in microbial, plants and animals kingdoms. They degrade starch and related polymers to yield products are the characteristics of individual amylolytic enzymes. The starch degrading enzyme (amylase) is among the most important enzymes widely used in industries and commercial sectors. Microbial amylases are more stable, economical and easily available (Gupta *et al*., 2003). Isolation of amylases can be done from a number of sources, such as plants, animals and microbes, though microbial amylases are most preferred and used in industry (Dey and Banerjee, 2012).The major advantage of using microorganisms for bulk economical amylases production and also easy to manipulate to obtain the enzymes of desired characteristics (Aiyer, 2005). The microbial amylases meet industrial demands. A large number of them are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Bernfeld, 1955). Amylase production has increased dramatically due to its uses in food, textile, baking and detergent industries. Besides its use in the saccharification or liquification of starch, the enzyme is also used for the warp sizing of textile fibers, the clarification of haze formed in beer or fruits juices and for the pretreatment of animal feed to improve the digestibility (Kokab *et al*., 2003). Agronomic wastes have attracted worldwide attention as these can act as potential raw materials which may be utilized microbiologically for conversion into bio-based products and bioenergy. The use of agroindustrial wastes for enzymes production can become economically viable for the application of these bio-catalysts in large scale (Garica Martinez *et al*., 2010).

OBJECTIVES

The aim of the present study is to isolate and characterize a starch degrading bacteria from *Bombxy mori*. Present study is focussed on the standardization, production and assay of amylase with respect to temperature, pH and incubation period of growth.

MATERIALS AND METHODS

Collection of substrate

Garden leaves sample was collected from Srimad

Andavan Arts and Science College Gardern in Trichy district. The substrate was dried in sun shade and made into powder by means of mechanical blenders.

Isolation of bacterial strain

The first instar *Bombyx mori* larvae were purchased from Trichy district the larvae were reared from first to fifth instar in sterile cages at room temperature. The entire digestive track of Fifth instar larvae of *Bombyx mori* (approximately of 10 gm) was aseptically isolated in a UV- laminar flow hood. The isolated digestive tract was washed with sterile ice-cold NaCl (0.85%) solution, chopped with a sterile blade, homogenized and incubated for 30 minutes at 37 ^{0} C. The supernatant was taken and serially diluted then pour plate method was used to estimate total bacterial count on lysogenic broth agar plates containing 1% starch as respective substrate (Anand *et al*.,2009) .

Screening for Amylase Activity (Starch Iodine Test)

Isolated colonies were screened for amylolytic activity by inoculating them on starch agar plates and incubated at 37ºC for 24-48 hrs. Individual plates were flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5gm potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue colour forms, which confirms the amylolytic activity. The colonies which were showing zone of clearance in starch agar plates were maintained on to nutrient agar slants.

Identification of Microbial culture using rDNA based Molecular Technique Method

DNA was isolated from the provided bacterial culture using Qiagen DNA Extraction Kit. The quantity was measured using NanoDrop Spectrophotometer and the quality was determined using 2% agarose gel. A single band of high-molecular weight DNA has been observed.The microbial 16S ribosomal RNA (16SrRNA gene) region was amplified using universal primers 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 product was purified to remove contaminants.The purified PCR amplicon was sequenced using the Universal forward and reverse primers. Sequencing was done using BDT v3.1 Cycle sequencing kit on ABI 3500 Genetic Analyzer.

3Media optimization for amylase production

Effect of pH

The effect of pH of the medium (2, 4, 6, 8 and 10) on the production of amylase was studied.

Effect of Temperature

Incubation temperature from 20,30,40,50 and 60ºC was used to observe its effect on the amylase production.

Effect of various Incubation Time on Amylase Production

The effect of incubation time 24 to 120 hrs was used to observe its effect on the production of amylase.

Effect of Various Inoculum Concentrations on Amylase Production

Inoculum concentration from 0.5, 1, 1.5 and 2ml/100 ml was used to observe the effect of inoculum concentration on the production of amylase.

Effect of Carbon sources

The effect of different carbon sources namely maltose, sucrose and lactose on the production of amylase was studied.

Effect of Nitrogen sources

The effect of different nitrogen sources namely beef extract, casein and ammonium sulphate on the amylase production was studied.

Amylase enzyme production

Production medium contained (g/l) peptone- 20g, $MgSO_4.7H_2O$- 1g, K_2HPO_4 - 3 g, Starch- 5g, substrate -20g ,pH 8 was prepared in a conical flask. The flasks were sterilized in autoclave at 121°C for 15 min and after cooling the flasks were inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 40°C in shaker incubator for 24 hrs. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

3Total amylase activity

Glucose (100mg/100ml) was used as standard and different concentration of glucose between 0.2 to 1ml was taken and made up to 10 ml using distilled water used as stock solutions, 0.5 ml of working standard from the stock solutions were taken in test tubes. 5 ml of starch solution was added & incubated at 90 °C for 10 mins. The reaction was stopped by addition of 5 ml of 0.1N

Hcl, one ml 0f iodine solution was added to all tubes. Read absorbency at 640 nm (Palanivelu, 2004).

RESULTS

Amylase producing bacteria was isolated from *Bombxy mori* and screened for amylase production. Of 50 isolates from *Bombxy mori*, strain SACT2 showed higher amylolytic activity. The DNA sample was run on an agarose gel. Single band was visualized when observed under the Gel doc, which confirmed the purity of sample, as the bands of DNA were single, distinct and no traces of contaminants were observed. Results were finally photographed.Then, sequencing of the 16S rRNA gene of bacterium was done and the same was amplified by Taq DNA polymerase along with the DNA marker. This was then subjected to agarose gel electrophoresis. The sequence obtained was then blasted in NCBI database. Based on the 16s rRNA sequences, the above bacterium was confirmed as *Pantoea gaviniae.* The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the p distance method and are in the units of the number of base differences per site (Fig.1).On the optimization of production media using various parameters, the maximum amylase production was observed at pH 8.0 (0.498 U/ml) (Fig.2), where as optimum temperature was found to be 40°C (0.497 U/ml) (Fig.3). In the present study, the initial inoculum level has played an important role in amylase production by *Pantoea gaviniae*. The maximum amylase specific activity was registered at the1.5ml (0.296 U/ml) of inoculum level and 2g of substrate (0.471 U/ml) (Fig. 4 and Fig.5). Sucrose and casein exhibited higher amylase production. After the fermentation period, the total amylase activity was found to be 2.790(U/ml).

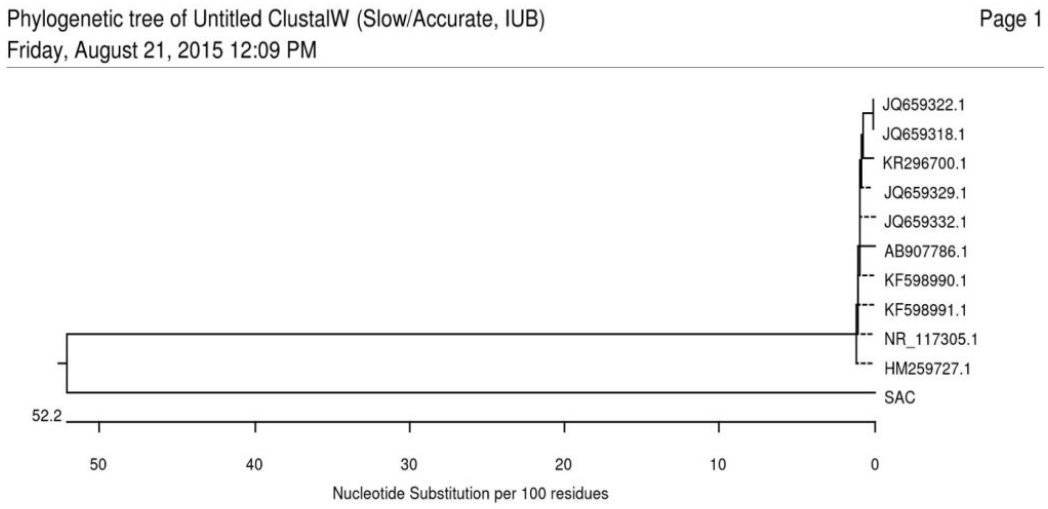

Figure 1
Phylogenetic analysis of identified bacterial sequence

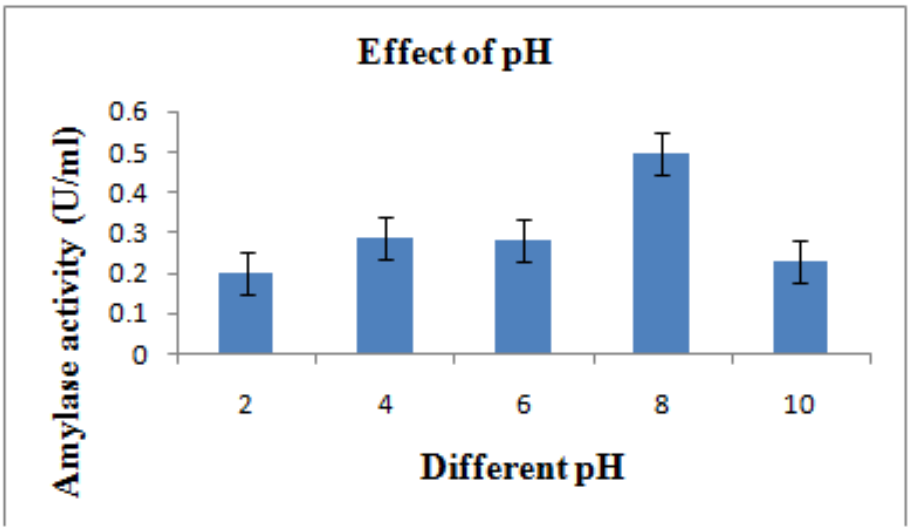

Figure 2
Effect of pH on amylase production

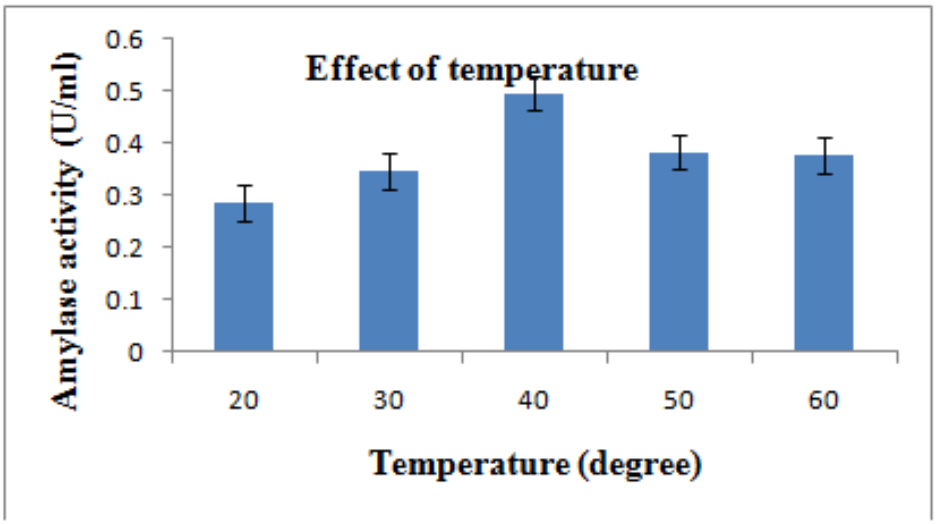

Figure 3
Effect of Temperature on amylase production

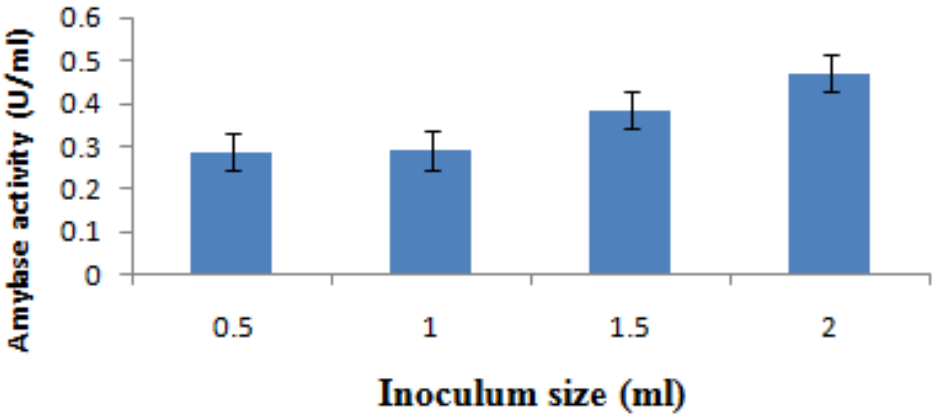

Figure 4
Effect of Inoculum size on amylase production

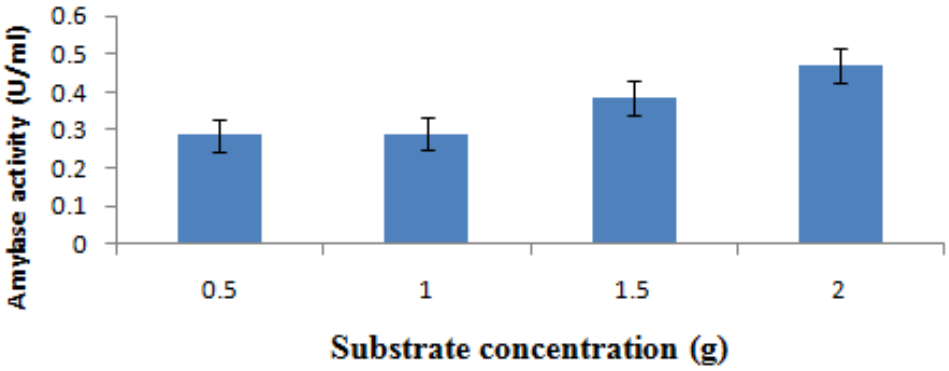

Figure 5
Effect of Substrate concentration on amylase production

DISCUSSION

Amylases are widely distributed and are one of the most studied enzymes .Nowadays, the renewed interest in the exploration of extracellular amylase production in bacteria and fungi is due to various industrial applications. Few attempts have been made to elucidate the control mechanism involved in the formation and secretion of the extracellular enzymes. In the present study, we observed that the optimum temperature for maximum growth of the bacterium and amylase production was 40°C, suggesting its thermophilic nature. The higher temperature (50°C and above) inhibited its growth and amylase activity (Romero *et al*., 2007). The high temperature may inactivate expression of gene responsible for amylase synthesis. pH of the growth medium is also among the important physical parameter that has to be optimized for the enzyme secretion. The pH range observed during the growth of microbes also affects enzyme production in the medium (Aiba *et al*., 1983). Most of the amylase secreting bacterial strains revealed pH range between 6 and 8 for the best growth of the organism and enzyme specific activity (Banerjee and Bhattacharyya, 1993, Bose and Das 1996). Similarly, under this category the optimum pH for maximal growth of *Pantoea gaviniae* was 8, and optimum pH for its maximum amylase activity was 0.498 U/ml. In another study, the activity of enzyme was also observed at slighly alkaline pH (at around pH 9) (Mishra and Benera, 2008). It has been reported that the enzyme activity is directly dependent on the period of incubation of bacterial strain in the culture medium (Deb *et al*., 2013). Some reports signify that with the increase in incubation time, enzyme activity decreased (Smits *et al*., 1996). In the present investigation, both growth as well as amylase activity increased with increasing period of incubation up to 24 hrs, followed by their decrease at further increase in the period of incubation. This suggested that the enzyme production in the isolated bacterial strain *Pantoea gaviniae* is a growth associated phenomenon. In some studies, maximum activity of amylase was reported at 12 hrs in *Bacillus* species (Aiyer, 2005), nevertheless more often amylase cannot be detected in the culture broth of *Bacillus* sp. before 12 hrs of incubation (Bozic *et al*., 2011).The maximum activity of *Pantoea gaviniae* occurred at 24 hrs of cultivation. The results were similar to those reported by (Devi *et al.,* 2012) for bacterial strain *Pantoea gaviniae* isolated from *Bombxy mori*. The isolated bacterium was non-pathogenic in nature, as tested on Himedia sheep blood agar plates. The biochemical, microscopic and morphological features of the isolated strain indicated it. Thus the non-pathogenicity of the starch degrading *Pantoea gaviniae* and its sensitivity to most of the antibiotics suggested the possibility of exploiting this bacterium for commercial production of amylase for diverse industrial applications. Further applicaton of enzyme alone or in combinaton with other enzymes like cellulase, hemicellulase and amylase may increase its applicability in many other applications.

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