
Screening of endophytic fungus *Acremonium* sp. for amylase production

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Pranay Jain, Vibhor Aggarwal, Archit Sharma and Ram Kumar Pundir (2012) Screening of endophytic fungus *Acremonium* sp. for amylase production. Journal of Agricultural Technology 8(4): 1353-1364.

In the present study, the endophytic fungal isolates producing amylase were obtained during the primary screening. Secondary screening of these isolated fungi yielded an amylase producing isolate identified as *Acremonium* sp. The enzyme was further evaluated for its biochemical properties for optimization of pH, carbon and nitrogen sources. Results showed that the enzyme was produced in the broad range of pH with optimum yield at 7. When fructose as carbon source and ammonium sulphate as nitrogen sources were used in the production medium, the optimum enzyme yield was produced. This strain was found to produce amylase with enzymatic activity of 4.00 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ at pH 7. It concluded that this enzyme might be used for the various applications such as starch processing, bread making, production of high fructose corn syrups, detergent formulations and many other related industries.

Key words: Endophytic fungus, *Acremonium* sp., Amylase Production

Introduction

Endophytic microorganisms (fungi and bacteria) are those microorganisms that inhabit inside of a plant at least in a period of its vital cycle, and are found in tissues such as leaves, branches and roots. Apparently, they do not cause any damage to the host, which distinguishes them from the phytopathogenic microorganisms (Azevedo, 1998). Their presence implied a symbiotic interaction, in all the plants investigated until now (Azevedo, 2000). They were mentioned for the first time by Bary in 19th century (Azevedo, 1998), gaining interest from 1970's. This interest is due to the possibility of these microorganisms producing pharmacologically active substances with biotechnological potential such as antitumor agents (*Pestalotiopsis microspora*,

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taxol), antifungal agents (*Cryptosporiopsis criptocandina* quercine), besides producing factors of plant growth, toxins and enzymes, including some microorganisms being used as biological controllers of many diseases and plagues (Stierle, 1993; Azevedo, 1998; Araújo, 2002; Strobel, 2002). Amylase enzymes are important enzymes employed in starch processing industries for hydrolysis of polysaccharides such as starch into simple sugar constituents (Fogarty, 1980, Nigam, 1995 Akpan, 1999). They have diverse application in wide variety of industries such as food, fermentation, textile, paper, detergent, pharmaceutical and sugar industries (Gupta, 2003). Although amylases can be derived from several sources such as plants, animals and micro-organisms, enzymes from microbial sources generally meet industrial demands. Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable (Burhan, 2003). Molds are capable of producing high amount of Amylase; *Aspergillus niger* is used for commercial production of amylase. Studies on fungal amylases especially in developing countries have concentrated mainly on *Aspergillus niger*, probably because of their ubiquitous nature and non-fastidious nutritional requirements of these organisms (Abu, 2005). Considering the industrial importance of amylases the objective of this study was to isolate and identify amyolytic endophytic fungus from *Saraca asoca*, and to perform partial characterization of the enzyme production and its properties with regard to the effect of pH, carbon and nitrogen sources. *Saraca asoca* (Asoka) is a plant belonging to the *Caesalpinaceae* subfamily of the legume family. It is an important tree in the cultural traditions of the Indian Subcontinent and adjacent areas. The bark of the herb is strongly astringent and uterine sedative. It acts directly on the muscular fibers of the uterus. It has a stimulating effect on the endometrium and the ovarian tissue. It is also used for the uterine/ovarian fibroids, menorrhagia, bleeding hemorrhoids, bleeding dysentery. The aim of the present study was to isolate endophytic fungi from Asoka plant and to screen them for enzymatic activity. For determination of specific enzyme activity the enzyme activity and the protein concentration were evaluated.

Materials and methods

Screening Collection of Sample

Leaves from *Saraca asoca* (Asoka) were treated with surface disinfectant for getting the growth of endophytic fungi & avoiding epiphytic fungi. Firstly the leaves were washed with tap water and then washed with distilled water. After that leaves were optimally cut with and were dipped in ethanol (70%) for

30 seconds, Leaves were then kept in mercuric chloride for 5 minutes. Then these pieces of leaves were washed 3-4 times with autoclaved water. These pieces were then placed on Potato Dextrose Agar plates containing Streptopenicillin (Aneja, 2003).

Screening for Amylolytic activity

The fungal discs from various isolates were cut and kept on the starch agar plates. The clear zone of hydrolysis around the disc showed the amylolytic activity of fungus. This zone of hydrolysis becomes visible when 1% of iodine solution was layered on the agar plates (Pandey *et al.*, 1999, Pandey *et al.*, 2000).

Identification of endophytic fungus

The isolated endophytic fungus was further stained with lactophenol cotton blue for its identification. The prepared slides with observed under trinocular microscope. Detailed morphological study was carried out.

Production of Amylase

Growth media development and inoculation

The growth media was selected which contained (g/L) starch 20, yeast extract 0.5, KH_2PO_4 1.5, $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.015, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0025. Then a fungal plug was cut from the master plate and put in the growth media for growth of amylolytic fungi. This growth media was kept undisturbed for 5 days of incubation.

Optimization of fermentation conditions

Effect of Carbon source

The carbon source was optimized for the fungal growth and enzyme activity. The carbon sources viz. glucose, fructose and galactose were taken in the media for checking the fungal growth. The flasks were incubated at 37°C for 3 days. Samples were taken at regular intervals and analyzed for amylase activity. The absorbance was taken at 660 nm. The most suitable carbon source was determined (Mukerjee, 1993).

Effect of Nitrogen source

The nitrogen source was optimized by using ammonium sulphate, urea, and ammonium sulphamate. The flasks were incubated at 37°C for 3 days. Samples were taken at regular intervals and analyzed for amylase activity. The suitable nitrogen source was determined (Pandey *et al.*, 1999; 2000; 2001)

Effect of pH on enzyme activity

The pH's selected for optimizations were 7, 10, and 13. Adjustments of the pH were done by addition of hydrochloric acid (0.1N) and 0.1N sodium hydroxide to achieve acidity and alkalinity respectively. The flasks were incubated at 37°C for 48 h. Samples were taken at regular intervals and analyzed for amylase activity. The optimum growth was seen at pH 7, and hence selected for the production media (Yamasaki, 1977).

Production Media development and inoculation

The production media contained starch 20, yeast extract 0.5, KH₂PO₄ 1.5, MnSO₄·7H₂O 0.015, MgSO₄·7H₂O 0.0025, and the supplements for optimization of carbon and nitrogen source were added. The pH 7 was adjusted with NaOH. The growth media was then transferred to production media and kept for 5-6 days of incubation.

Partial Purification

After incubation the fungi growth occurred. Then it was filtered through Whatman filter paper. The filtrate was then precipitated through ammonium sulphate by the process of ammonium sulphate fractionation (20-100%) (Gomori, 1955) and allowed to stand overnight and then centrifuged (7500 g, 15 min, -1°C). The precipitate was then stored in a minimal volume of 3 M ammonium sulphate dissolved in 0.05 M K₂HPO₄. The 20% precipitated crude enzyme was then dialyzed through dialysis tubing 0.02 M Tris-HCl buffer (pH 8.0). The buffer was intermittently changed. Buffer was changed after 2 hours and the buffer was overall changed for 4 times. After that the enzyme was eluted through it and was stored at 4°C (Joshi, 2011).

Assay of enzyme activity

Amylase activity was estimated by analysis of reducing sugar released during hydrolysis of 1% (w/v) starch in 0.1 M phosphate buffer, pH 6.5, at

25°C for 20 min by the Dinitrosalicylic acid method (Miller, 1959). One unit of amylase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar as maltose per min under the assay conditions. Enzyme activity is expressed as specific activity, which is represented as U/mg of protein.

Assay of protein concentration

The protein concentration was determined by the Lowry's method (Lowry, 1951) using bovine serum albumin as the standard.

Results and discussions

Endophytic microorganisms (fungi and bacteria) are those that inhabit inside of a plant at least in a period of its vital cycle, and are found in tissues such as leaves, branches and roots. Apparently, they do not cause any damage to the host, which distinguishes them from the phytopatogens microorganisms (Azevedo, 1998). Amylase enzymes are important enzymes employed in starch processing industries for hydrolysis of polysaccharides such as starch into simple sugar constituents (Fogarty, 1980, Nigam, 1995, Akpan, 1999). Studies on fungal amylases especially in developing countries have concentrated mainly on *Aspergillus*, probably because of their ubiquitous nature and non-fastidious nutritional requirements of these organisms (Azevedo, 2000). Suganthi *et al.* (2011) selected one *Aspergillus niger* (BAN 3E) out of five fungal isolates which was selected for further studies on amylase production. Sidkey *et al.* (2011) isolated *A. flavus*, F2Mbb which was proved to be the most potent α - amylase producer strain, and it was selected for the purpose of production, purification and investigating properties of the enzyme (Suganthi, 2011). In the present study, the work was carried out on the enzymatic activity of endophytic fungi.

Strain selection

In the present study, two fungal isolates were obtained from different plants used that had cottony colonies and hydrolyzed starch (blue blackish color) and utilized glucose. One with larger zone of clearance was found to be *Acremonium sp.* when colony morphology and sporulation characteristics were studied (Fig. 1). *Acremonium* is a genus of Fungi in the *Hypocreaceae* family; it was previously known as "*Cephalosporium*". *Acremonium* species are usually slow growing and are initially compact and moist. *Acremonium* hyphae are fine and hyaline and produce mostly simple phialides. Their conidia are usually one-celled (i.e. ameroconidia), hyaline or pigmented, globose to cylindrical, and

mostly aggregated in slimy heads at the apex of each phialide. Its amylolytic activity was confirmed by the flooding of plates with Iodine, as the starch in the plates retained the color of iodine while the degraded starch didn't retain the color (Fig. 2).



Fig. 1. Growth of *Acremonium* after incubation



Fig. 2. Zone of Hydrolysis of starch shown by the *Acremonium*

Optimization of conditions

The carbon sources viz. glucose, fructose and galactose were taken in the media for checking the fungal growth. The flasks were incubated at 37°C for 3 days. Samples were taken at regular intervals and analyzed for amylase activity. The absorbance was taken at 660 nm. The optical density for glucose, fructose and galactose were 0.13, 0.17, and 0.08 respectively. So when fructose as carbon source was used, the optimum production was obtained (Fig. 3).

The nitrogen source was optimized by using ammonium sulphate, urea, and ammonium sulphamate. The flasks were incubated at 37°C for 3 days. Samples were taken at regular intervals and analyzed for amylase activity. The optical density for ammonium sulphate, urea, and ammonium sulphamate were 0.08, 0.01, and 0.03 respectively. With ammonium sulphate as nitrogen source the optimum production was obtained (Fig. 4).

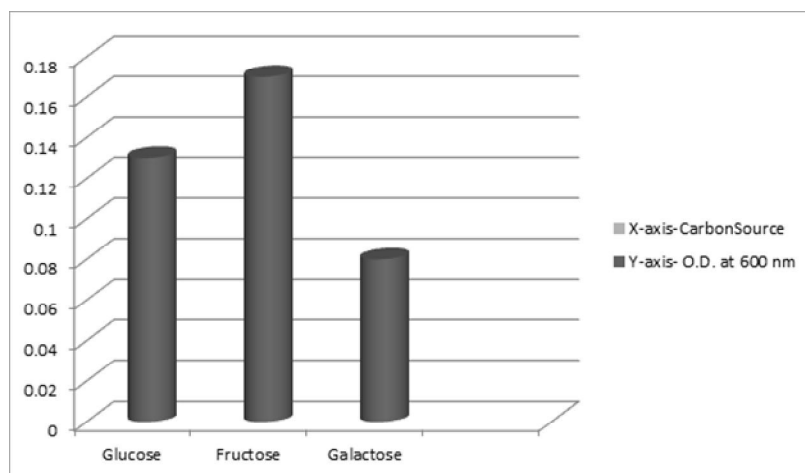


Fig. 3. Optimization of Carbon source done for the production of amylase

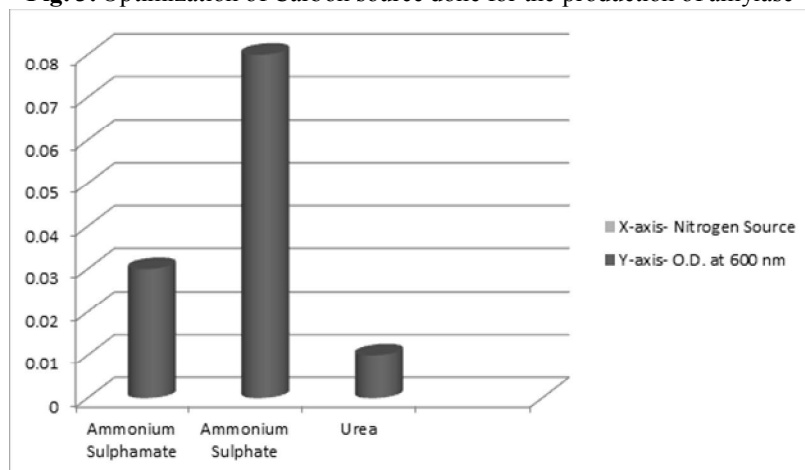


Fig. 4. Optimization of Nitrogen Source done for the production of amylase

The various pH values selected for optimization were 7, 10, and 13 respectively. The pH 7 showed the optimum growth and production (Fig. 5). Suganthi *et al.* (2011) found that there was marked increase in the yield of enzyme till pH 8, and then there was very minimum activity at pH 9 in all the substrates used in SSF. Varalakshmi *et al.* (2009) reported the maximum enzyme activity of 75 U/mg of protein at pH 9.5. Addition of different carbon source like glucose, maltose, lactose, and starch did not show remarkable change in the yield. Sucrose showed a better yield (specific activity was 50 U/mg). Sodium nitrate, ammonium sulphate, ammonium chloride, ammonium nitrate, and potassium nitrate were used as nitrogen source along with the solid substrates. Nitrogen source greatly increased the yield of the enzyme produced

(specific activity was 320 U/mg) in groundnut oil cake medium. There was remarkable increase in the production of amylase in ammonium nitrate supplemented black gram medium and in ammonium chloride supplemented Coconut oil cake medium. Hernandez *et al.* (2006) and Mitieri *et al.* (2006) have reported acidic pH optima for amylases from *A. niger* (Hernandez, 2006).

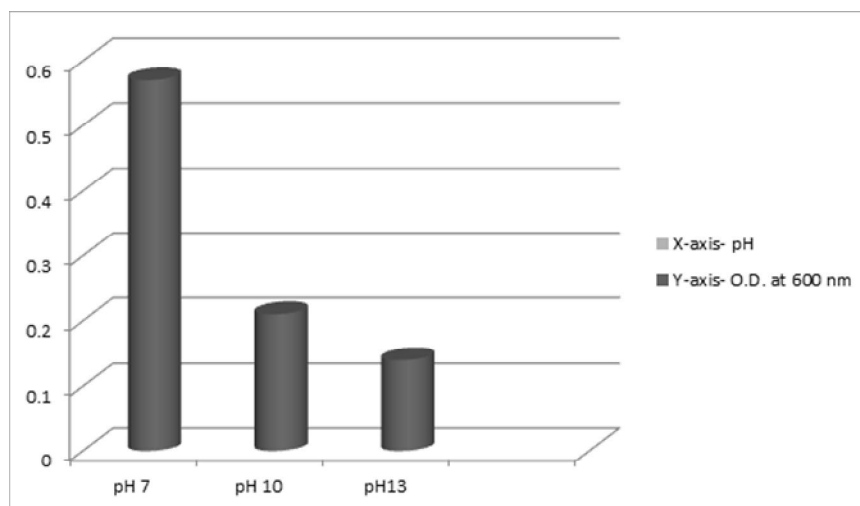


Fig. 5. Optimization of pH done for the production of amylase

Partial Purification

The enzyme was precipitated using ammonium sulphate fractionation technique. The precipitated crude enzyme was dialyzed through dialysis tubing 0.02 M Tris-HCl buffers (pH 8.0) (Fig 6). Sidkey *et al.* (2011) found that enzyme purification using 60% ammonium sulfate for precipitation and subsequent Sephadex G-200 gel filtration resulted in 15.74% recovery, specific activity of 4348 U/mg protein/ml (Sidkey *et al.*, 2011).



Fig. 6. Dialysis of Amylase for partial purification

Specific Activity of enzyme

The standard curve of maltose was plotted and the experiment was performed 5 times within run precision. The mean of the values were taken and plotted. The lower limit of quantification was found to be at Optical density of 0.07. The plot is shown in Fig.7. The enzyme was assayed for determination of enzyme activity using DNSA reagent and the readings were plotted on the standard maltose curve. The Optical density obtained for 20% saturation was greatest and found to be 0.8. The Optical density when plotted on standard maltose curve, the enzyme activity was found to be $0.12 \mu\text{mol min}^{-1}$.

Similarly standard curve for BSA was plotted and the experiment was performed 5 times within run precision. The mean of the values were taken and plotted. The lower limit of quantification was found to be at Optical density of 0.05. The plot is shown in Fig. 8. The enzyme was assayed for determination of Protein content using Lowry's method (Lowry, 1951) and the readings were plotted on the standard BSA curve. The Optical density obtained for 20% saturation was greatest and found to be 0.16. The Optical density when plotted on standard BSA curve, the protein content was found to be $30 \mu\text{g/ml}$. Specific activity was calculated and found to be $4.00 \mu\text{mol min}^{-1} \text{mg}^{-1}$. Suganthi *et al.* (2011) found *Aspergillus niger* BAN 3E to be the best amylase producer and specific activity of amylase produced by this strain was 52 U/mg in Submerged fermentation (Suganthi, 2011). Sidkey *et al.* (2011) performed different concentrations of ammonium sulphate and 60% concentration gave the highest enzyme activity of 202.05 (U/mg protein/ml) (Sidkey *et al.*, 2011).

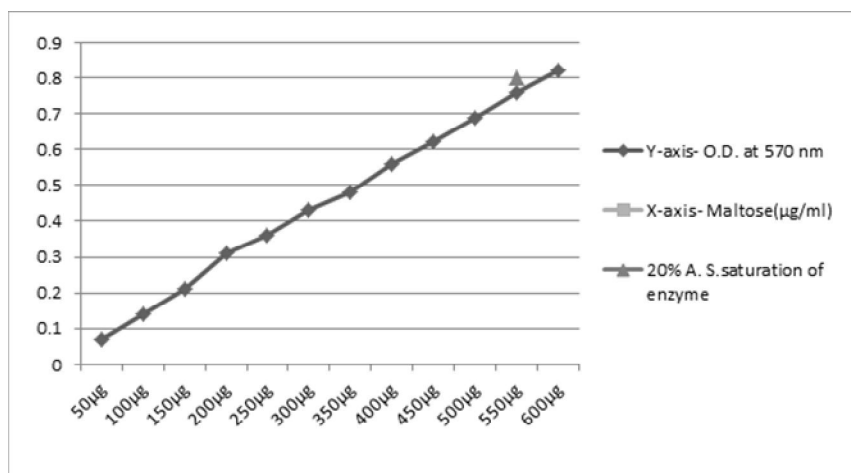


Fig. 7. Maltose standard curve prepared for the production of amylase

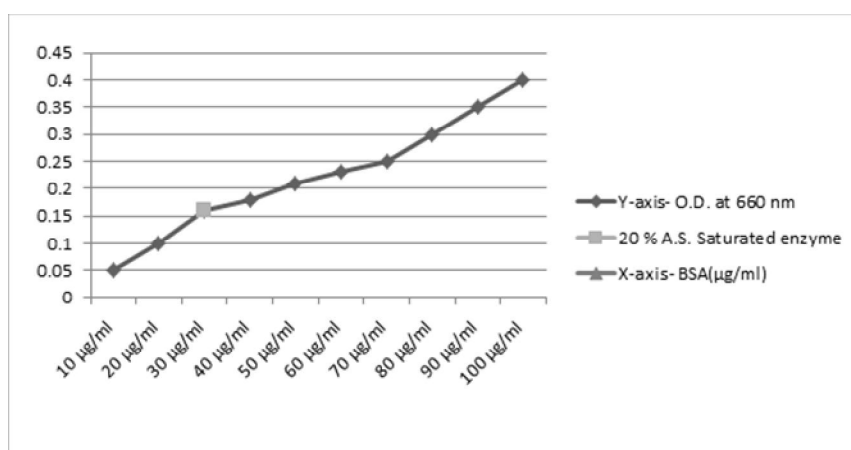


Fig. 8. BSA Standard Curve done for the production of amylase

Conclusion

It can be concluded that the enzyme isolated from endophytic fungus is very sensitive to pH. Therefore, the selection of optimum pH is essential for the production of amylases. In this study, the effect of pH on substrate was studied by maintaining the pH in the range of 7 - 13. Production of amylase was the best at pH 7. Further the effect of carbon and nitrogen sources was observed. Fructose and ammonium sulphate were selected as carbon and nitrogen sources respectively, as the optimum growth was observed when they were used. Amylase finds use in the industrial production of paper and detergents. Amylase also finds use in bread making and to break down complex sugars

such as starch (found in flour) into simple sugars. In molecular biology, the presence of amylase can serve as an additional method of selecting for successful integration of a reporter construct in addition to antibiotic resistance. As reporter genes are flanked by homologous regions of the structural gene for amylase, successful integration will disrupt the amylase gene and prevent starch degradation, which is easily detectable through iodine staining. As amylase is found to be an important enzyme so *Acremonium sp.*, can be industrially exploited for the synthesis of amylase and strain improvement studies can be carried out to enhance enzyme production.

Acknowledgement

The authors are grateful to the Hon'ble Vice-Chancellor, Kurukshetra University, Kurukshetra and the Director, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra for providing infrastructure facilities to carry out research work.

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(Published in July 2012)