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SCREENING OF ENDOPHYTIC FUNGUS *ASPERGILLUS* SP. FOR AMYLASE PRODUCTION

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Abstract

In the present study, five isolated endophytic fungal spp. were tested for amylase production. A representative isolate AS-05 of *Aspergillus spp.* yielded an amylase chosen for further characterization for amylase production. The enzyme was further evaluated for its biochemical properties for optimization of carbon, nitrogen sources and pH. The optimum production of enzyme was observed when the production media containing maltose as a source of carbon, ammonium sulphate as a source of nitrogen and the broad range of pH with optimum yield at 7. This strain was found to produce amylase with enzymatic activity of 4.17µmol min^{-1} mg^{-1} at pH 7 and the protein content was found to be 32.7µg/ml. This study concluded that the diverse endophytic fungi can be potential source of enzymes and various industrially important biomolecules.

Keywords: Endophytic fungus, *Aspergillus sp.*, Amylase production.

Introduction

Endophytic fungi 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 phyto-pathogenic microorganisms[1]. Their presence implied a symbiotic interaction, in all the plants investigated until now[2]. They were revealed for the first time by Bary in 19th century[1], gaining interest from 1970's. This is due to the possibility of these microorganisms producing pharmacologically active substances with biotechnological applications such as antitumor agents, antifungal agents (*Cryptosporiopsis spp., Aspergillus spp., Mucor spp., Alternari spp., etc.*,), besides producing factors of plant growth, toxins and enzymes, including some microorganisms being used as biological controllers of many diseases[3,4]. Amylase enzymes are essential enzymes employed in starch processing industries for hydrolysis of polysaccharides such as starch into simple sugar constituents [5, 6]. They have diverse application in industries such as food, fermentation, textile, paper,

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detergent, pharmaceutical and sugar industries[7]. Though 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[8]. Molds are capable of producing high amount of Amylase; *Aspergillus niger* was 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[9]. The present study was undertaken to screen an enzyme producing ability of endophytic *Aspergillus spp*.

Materials and methods

Selection of endophytic *Aspergillus spp*. isolates

Five isolates of endophytic *Aspergillus spp.* previously isolated from root segments of *Rhamnus prinioids*, were selected for screening for production of amylase[10].

Screening for Amylolytic activity

All the *Aspergillus spp*. isolates were tested for amylase production by starch hydrolysis. When starch agar medium (Peptone – 0.5g, Beef Extract – 0.15g, Yeast extract – 0.15g, NaCl – 0.5g, Starch – 1g, Agar – 2g, Distilled water – 100ml) was inoculated with the organism and subsequently flooded with iodine solution (Iodine – 0.2%, Potassium Iodide – 0.4%, Distilled water – 100ml), the zone of clearance around the fungal growth indicated the production of amylase. On the basis of the area of clearance, the strain AS-05 out of thefive fungal isolates was selected for further studies on amylase production[11].

Production of Amylase

Growth medium and inoculation

The growth media was selected which contained (g/L) starch 20, yeast extract 0.5, KH2PO4 1.5, MnSO4.7H2O 0.015, MgSO4.7H2O 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 such as glucose, fructose, maltose and galactose were taken in the media for checking the fungal growth. The flasks were incubated at 37°C for 5 days. Samples were taken at regular intervals and analyzed for amylase activity. The absorbance was taken at 600 nm. The most suitable carbon source was determined [12].

Effect of Nitrogen source

The nitrogen source was optimized by using urea, ammonium sulphate and ammonium sulphamate. The flasks were incubated at 37°C for 3 days. Samples were taken at regular intervals and analysed for amylase activity. The suitable nitrogen source was determined[11].

Effect of pH on enzyme activity

The pH's selected for optimizations were 6, 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 analysed for amylase activity. The optimum growth was seen at pH 7, and hence selected for the production media[13].

Production Medium 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 completion of incubation period the fungal growth was observed. Then media along with fungal mesh was filtered through Whatmann filter paper. The filtrate was then precipitated through ammonium sulphate by the process of ammonium sulphate fractionation[14] and kept overnight then centrifuged (8000rpm, 15 min, -2°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 buffers (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 [15].

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 25ºC for 20 min by the Dinitrosalicylic acid method[16]. 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 using bovine serum albumin as the standard[17].

Results

The carbon sources used in this study were glucose; fructose, maltose and galactose. These were added in the media for checking the fungal growth. The flasks were incubated at 37°C for 5 days. Samples were taken at regular intervals and analysed for amylase activity. The absorbance was taken at 600 nm. The optical density for glucose, fructose, maltose and galactose were 0.14, 0.15, 0.18 and 0.07 respectively. So when maltose as carbon source was used, the optimum production was obtained (Fig. 1).

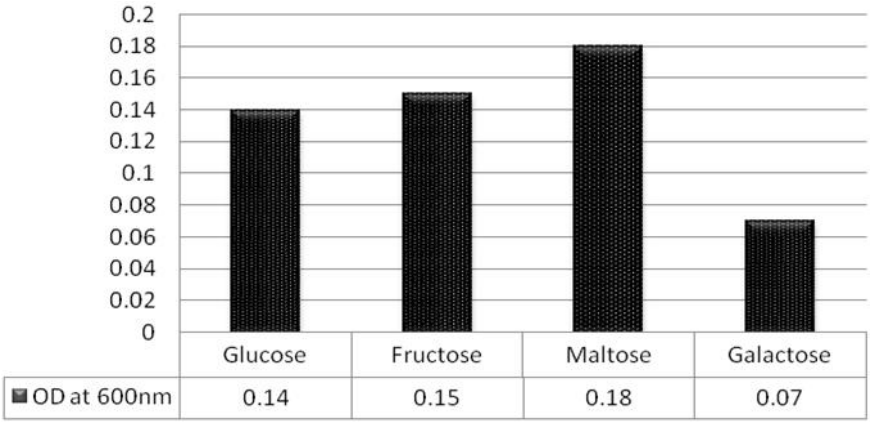

Fig. No. 01: Optimization of carbon source for the production of amylase from *Aspergillus spp.*

The nitrogen source was optimized by using urea, ammonium sulphate and ammonium sulphamate. The flasks were incubated at 37°C for 5 days. Samples were taken at regular intervals and analysed for amylase activity. The optical density for urea, ammonium sulphate and ammonium sulphamate were 0.05, 0.09, and 0.02 respectively. Ammonium sulphate as a nitrogen source the optimum production of amylase was obtained (Fig. 2).

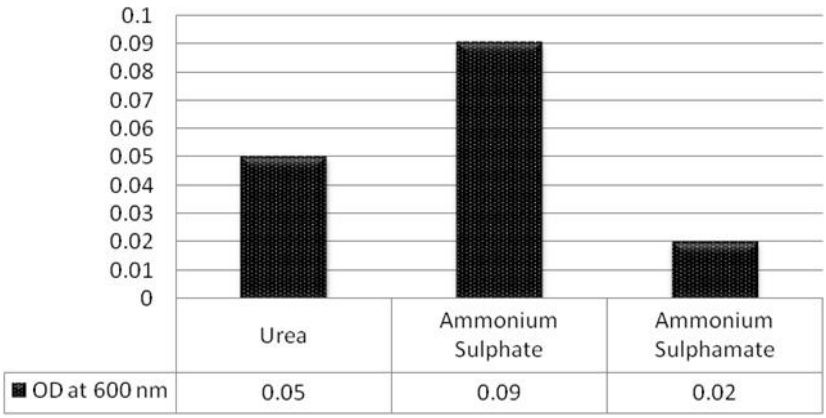

Fig. No. 02: Optimization of nitrogen source for the production of amylase from *Aspergillus spp.*

The various pH ranges selected for optimization of amylase production were 6, 7, 10, and 13. The optical density for pH 6, 7, 10, and 13 were 0.52, 0.71, 0.19 and 0.14 respectively. The pH 7 showed the optimum growth and production (Fig. 3).

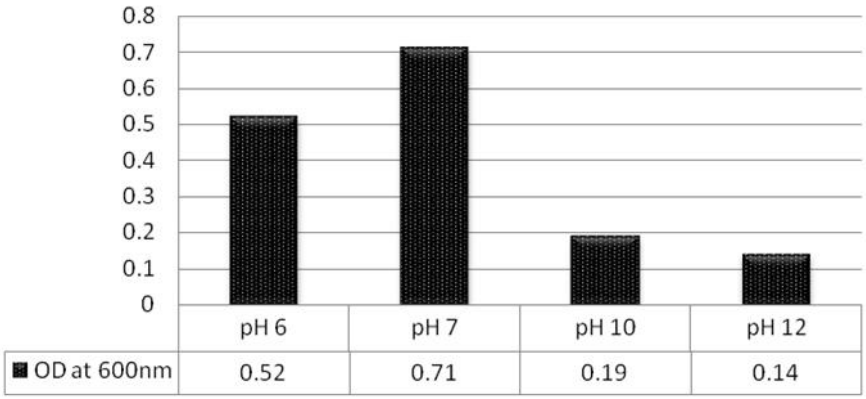

Fig. No. 03: Optimization of pH for the production of amylase from *Aspergillus spp.*

Partial Purification

The enzyme was precipitated using ammonium sulphate fractionation technique. The crude enzyme precipitation was subjected for dialysis using 0.02 M Tris-HCl buffers (pH 8.0) resulted in 14.7% recovery.

Assay of amylase Activity

The standard curve of maltose was plotted and the experiment was accomplished. 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 enzyme was assayed for determination of enzyme activity using DNS Assay reagent and the impressions were plotted on the standard maltose curve. The Optical density gained for 20% saturation was greatest and found to be 0.9. The Optical density when plotted on standard maltose curve, the enzyme activity was found to be 4.17µmol min^{-1}.

Assay of protein concentration

Similarly standard curve for BSA was plotted and the experiment was performed. The mean of the values were taken and plotted. The lower limit of quantification was found to be at Optical density of 0.06. The enzyme was assayed for determination of Protein content using Lowry's method and the readings were plotted on the standard BSA curve. The Optical density obtained for 20% saturation was optimum and found to be 0.19. The Optical density when plotted on standard BSA curve, the protein content was found to be 32.7µg/ml.

Discussion

Endophytic fungi are those that reside inside of a plant at least in a period of its vital cycle, and are establish in tissues such as roots, leaves and branches. Actually, they do not cause any impairment to the host, which differentiates them from the phyto-pathogens [1]. Amylase enzymes are important enzymes employed in starch processing for hydrolysis of polysaccharides into simple sugars[6]. In the present study, the screening was carried out on the endophytic *Aspergillus spp*. for the production of amylase enzyme. Previously isolated endophytic *Aspergillus spp*. were screened for this study, isolate SA-05 was selected based on its optimum ability to hydrolyse starch agar. Various 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[2].Maltose as carbon source was revealed the optimum production of enzyme in this study. On the contrary glucose and sucrose supplementation resulted in the repression of enzyme production[18]. Similar results of catabolite repression of enzyme production by glucose, for *A. niger* CFTRI 1105 and for *Aspergillus sp.* JGI 12 [19, 20].In the present study the nitrogen source was optimized by using urea, ammonium sulphate and ammonium sulphamate and ammonium sulphate in the media yielded optimum enzyme. Similar studies revealed that the supplementation with different nitrogen sources to amylase production by fungi is done with success increasing the yield of the enzyme in SSF [21]. Our results are in agreement

and found that the various nitrogen supplementations enhance the production of the organism and have increased in the biomass production [22]. Previous findings have shown that peptone, sodium nitrate and casein hydrolysate are good nitrogen supplements for amylase production in *A. fumigatus*[23], *A .niger* [24] and A. oryzae [25].The optimum growth of fungi and enzyme production was observed at the neutral pH 7. In contrary to our results reported the maximum enzyme activity of 75 U/mg of protein at pH 9.5[26]. Others have reported acidic pH optima for amylases from *A. niger*[27].This study revealed that the enzyme activity was found to be 0.14 µmol min^{-1} and the protein content was found to be 32.7µg/ml. 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[28].

Conclusion

This study concluded that the diverse endophytic fungi can be potential source of enzymes and other various industrially important substances.

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