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Optimization of amylase production from an endophytic fungi *Discosia* sp. isolated from *Calophyllum inophyllum.*

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Endophytic fungi isolated from the plant *Calophyllum inophyllum* were screened for amylolytic activity on solid media. Among the twelve isolates of fungi, Ci- 5 identified as *Discosia* sp. showed highest amylolytic activity and was selected for further study. Influence of various physical and chemical factors such as pH, temperature, carbon and nitrogen sources on amylase production in liquid media were studied. The maximal productivity was achieved at 30°C of incubation and at pH 7.0 of the cultural media. Among the various carbon sources, maltose at 1.5% gave the highest amylase production. Among different nitrogen sources 0.3% sodium nitrate was found to be optimum.

Key words: Endophytic fungi, *Discosia* sp., amylase production, optimization

Introduction

Enzymes are currently used in several different industrial products and processes and new areas of application are constantly being added. In a world with a rapidly increasing population and approaching exhaustion of many natural resources, enzyme technology offers a great potential to help many industries to meet the challenges they face in years to come (Kirk *et al*., 2002). Amylases are among the most important enzymes and are of great significance in the present day biotechnology. The most widely used enzyme in the industry for starch hydrolysis is α-amylase (EC 3.2.1.1, α-1, 4-glucan-4-glucanohydrolase), which catalyses the endocleavage of the α-1, 4-glycoside linkages and the release of short oligosaccharides and α-limit dextrin. This enzyme is used commercially for the production of sugar syrups from starch which consist of glucose, maltose, and higher oligosaccharides (Hagihara *et a1.,* 2001). It is also extensively used in starch liquefaction and paper, food, pharmaceutical and sugar industries. The

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amylases can be derived from several sources such as plants, animals and microorganisms. In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization (Burhan *et al*., 2003). Because of their short growth period, the enzymes from microbial sources generally meet industrial demands (Odee, 1997; Reddy, 1999). Microbial amylases are produced mainly from cultures of *Aspergillus*, *Bacillus*, *Clostridium*, *Pseudomonas*, *Rhizopus* and *Streptomyces* species (Pandey *et al.,* 2000). Hence, there is an increasing worldwide interest in the screening of new microorganisms producing amylases suitable for industrial applications. (Burhan *et al*., 2003; Gupta *et al*., 2003)

Materials and methods

Isolation & culture of the endophytic fungi

Different plant parts such as leaves, midrib, petiole and stem were cut from the plant *C. Inophyllum* (Fig 1) with the help of knife disinfected with 70% ethanol. The collected samples were washed with running tap water and cut into $0.5cm^2$ segments and were surface disinfected with standard triple ethanol-sodium hypochlorite-ethanol surface sterilization techniques (Fisher *et al.,* 1993). Fifteen leaf segments from each individual plant parts were placed in Petri dishes containing Potato dextrose agar (PDA) with tetracycline 150mg/lt to inhibit bacterial growth. The Petri dishes were then sealed with cellophane tape and incubated at 25°C with 12hrs photoperiod and regular observations was done from the second day onwards for a period of 3-4 weeks for the fungal colonies (Bills and Pollishook, 1992). The fungi growing from internal tissues were transferred to fresh PDA slants. The fungi were identified based on the cultural characteristics and the morphology of the fruiting bodies and spores using standard manuals (Barnett and Hunter, 1972). All the isolates were maintained on PDA slant.

Screening for amylase on solid media

Amylase activity was assessed by growing the fungi on glucose yeast extract peptone agar (GYP) medium (glucose-1g, yeast extract -0.1g, peptone- 0-5g agar–16g) with 0.2% soluble starch pH 6.0. After incubation, the plates were flood with 1% iodine solution in 2% Potassium iodide. A yellow zone around the colony in an otherwise blue medium indicated starch-degrading activity (Hankin and Anagnostakis, 1975; Maria *et al*., 2005).

Optimization studies

Effect of temperature, pH, Carbon and Nitrogen sources on α-Amylase Activity of the endophytic fungi**:** Out of twelve taxa of endophytic isolates screened for the amylase production on solid media nine of them showed positive. Among these the isolate Ci-5 *Discosia* sp. was selected for the optimization of amylase activity in liquid media. The fungus was grown in 25 ml of basal media g/l ($NaNO_3$, 3g, $MgSO_4.7H_2O$, 0.5g, KCl, 5g, KH_2PO_4, 1g; $FeSO_4.7H_2O$, 0.01g, $CaCl_2$, 0.1g and starch, 15g) in 150 ml Erlenmeyer flasks. After sterilization, the flask was cooled to room temperature and 0.5ml of spore suspension (10^6 spores/ml) of the fungal strain was inoculated and incubated for 7 days at different parameters as described in the following paragraphs by taking one parameter at a time. Uninoculated flask served as control. The procedure adopted for optimization of various parameters influencing amylase production was to evaluate the effect of independent parameters keeping others as constant and to incorporate it at the optimized level in the next experiment after optimizing other parameters.

Effect of incubation temperature: The effect of temperature was studied by incubating the isolate at 15, 25, 30, 37 and 45°C for 7 days. The optimum incubation temperature achieved by this step was fixed for subsequent experiments.

Effect of pH: The effect of pH was studied by varying the pH of the medium at 3.0, 5.0, 7.0, 9.0 and 11.0. After the inoculation of the fungus, the medium was incubated at 30°C for 7 days. The optimum initial pH of the solid substrate achieved by this step was fixed for subsequent experiments.

Effect of various carbon sources: The basal media was suspended with various carbon sources such as corn flour, cassava flour, rice bran powder, wheat bran powder, maltose and starch at 1.5% (w/v). The pH of the media was maintained at 7 and incubated at 30°C for 7 days.

Effect of various Nitrogen sources: The basal media was suspended with 0.3 % of various Nitrogen sources viz. peptone, tryptone, beef extract, yeast extract, ammonium nitrate and sodium nitrate. The broth was maintained at pH 7.0. After inoculation the flasks were incubated at 30°C for 7 days.

Determination of fungal biomass*:*

The biomass of fungal cell was expressed as dry weight by drying the mycelium in hot air oven at 80°C for 16h.

Enzyme assay

The culture broth was filtered using Whatman filter paper No.1, the filtrate was centrifuged at 5000 rpm for 10 min at 4°C and the supernatant was used for enzyme assay. Amylase activity was determined at room temperature in a reaction mixture containing 1ml of 1M sodium acetate buffer (pH 6), 0.5ml 1% starch (w/v) and 0.5ml of the crude enzyme extract. After 20mins of incubation, the liberated maltose was estimated by Dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of amylase activity is defined as the amount of enzyme releasing one µmol of reducing sugars per minute per ml, with maltose as standard under the assay conditions mentioned above. The denatured culture filtrate served as control. All the experiments were performed in triplicates and mean values are analyzed according to DMR Test comparisons (P=0.05).

Results and discussion

Enzyme activity of endophytic fungi on agar plate

The isolated fungus after identification was tested for their ability to produce extracellular amylase on solid media. Among the twelve taxa of isolates (Table1) nine of them showed positive for amylase. *Cladosporium* sp., *Fusarium* sp. *Penicillium* sp.1, 2 and *Pestalotiopsis* sp. were weakly positive (0-10mm), *Fusarium chylmadosporum*, *Xylaria* sp., *Isaria* sp. were moderately positive (10-20mm) and *Discosia* sp. (20-30mm) showed strong reaction on solid media. Since the *Discosia* sp. showed maximum activity, it was selected for the optimization of amylase activity in liquid media. On solid media, they were mixed with low nutrient content agar that had just nutrient for the mycelial growth to spread across the agar plates. In the liquid media used in this study, starch was used as substrate to induce enzyme production.Unlike the previous study by Choi *et al.* (2005) all the endophytes were not able to degrade soluble starch. The production of fungal enzymes in nature has a role in their pathogenicity or degradative capacity (Archer and Wood, 1995). Priest (1984) showed that there are several possible regulatory mechanisms in enzyme production, including enzyme induction.

Effect of incubation temperature on Amylase activity

The amylase activity and biomass was nil in lower (15ºC) and higher incubation (45ºC) temperature (Results not shown in the Table). The optimum incubation temperature for amylase production was found to be at 30ºC (Table 2). The biomass also correlated with the results. This is similar to the work of Kundu and Das (1970) and Ray (2004) also reported that amylase activity was optimum at

30ºC in *Aspergillus oryzae* and *Botryodiplodia theobromae* and *Rhizopus oryzae* respectively.

There is no significant correlation on amylase production when the culture was incubated at 25° C & 30°C. This indicates the influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic

Table 1. Enzyme activity of endophytic fungi on agar plate.

SL. No	Endophytic fungus	Amylolytic activity (mm)
Ci 1	*Penicillium* sp.1	8.0
Ci 3	*Fusarium* sp.	5.0
Ci 4	*Pestalotiopsis* sp.1	4.0
Ci 5	*Discosia sp.*	28.0
Ci 8	*Phyllosticta* sp.	---
Ci 12	*Isaria* sp.	20.5
Ci 13	*Xylaria* sp.	13.0
Ci 14	*Penicillium* sp.2	5.0
Ci 17	*Colletotrichum* sp.	---
Ci 19	*Fusarium Chylmadosporum*	11.0
Ci 20	*Cladosporium* sp.	6.0
Ci 31	*Pestalotiopsis* sp.2	---

Fig. 1. *Calophyllum inophyllum*.

Table 2. Effect of growth and Amylase activity at different incubation temperature.

Temperature	Amylase activity unit*/ml	Dry weight mg/100 ml
25°C	0.029±0.006[a]	344±5.6[b]
30°C	0.033±0.011[a]	384±5.3[a]
37°C	0.016±0[b]	200±5[c]

*Amount of maltose liberated in µmol per ml per minute

Effect of pH of the culture media ona activity

The pH of the medium played an important role in amylase production. The amylase activity was optimum at pH 7.0 (Table 3), which is similar to the findings of Patel *et al.* (2005) who also reported maximum amylase activity at pH 7.0. The biomass yield was found to be higher in case of pH 5.0 contradictory to the findings of Olama and Sabry (1989) where the amylase activity and the biomass yield was maximum at pH 7.0 in *A. flavus* and *P. purpurescence.*

Table 3. Effect of pH of the culture media on Amylase activity.

Initial pH	Amylase activity Unit*/ml	Mycelial Dry weight mg/100 ml
3	0.049±0.005[c]	293±3[b]
5	0.0607±0.005[b]	440±5[a]
7	0.077±0[a]	280±7[c]
9	0.049±0.005[c]	226±9 [d]
11	0.039±0.014[d]	133±1.2[e]

*Amount of maltose liberated in µmol per ml per minute

Effect of different carbon sources on amylase activity

Though the fungus was able to grow on all the carbon sources tested, there were significant differences in the yield of the biomass and the amylase. This indicates that the nature and amount of carbon source in culture media is important for the biomass growth and production of extracellular amylase. Significant growth and relatively high yields of activity was found in case of maltose, corn flour and cassava flour. The highest amylase activity was found incase of maltose (Table 4) indicating maltose as the best substrate among the tested carbon sources. This indicates that alternatively corn and cassava flour can be used in the medium as economically available products. Though there was high increase in biomass yield incase of wheat bran powder the enzyme activity was low. Compared to defined carbon sources the biomass yield was higher in undefined carbon sources similar to the findings of Oliveira *et al.* (2007) incase of Rhizobial strains. Kuo and

Hartman (1966) showed that *Thermoactinomyces vulgaris* produces best yields of α– amylase when starch or maltose is used as a carbon source.

Table 4. Effect of different Carbon sources on Amylase activity.

Carbon sources	Amylase activity Unit*/ml	Dry weight mg/100 ml
Corn flour	0.125±0.013[b]	376±2[b]
Cassava flour	0.125±0.016[b]	350±3[c]
Rice bran powder	0.018±0.006[d]	348±3[c]
Wheat bran powder	0.044±0.011[d]	536±2.6[a]
Maltose	0.425±0.034[a]	333±2.6[d]
Starch	0.077±0[c]	280±4.4[e]

*Amount of maltose liberated in µmol per ml per minute.

Effect of different nitrogen sources on amylase activity and production

Different organic and inorganic sources at 0.3% were used as nitrogen sources for amylase production. The biomass and amylase activity was maximum in medium supplemented with sodium nitrate. This result was similar to production of amylase in liquid media by a culture strain of *Aspergillus oryzae* by Kundu *et al.* (1970) at static condition in medium containing 0.9% sodium nitrate and 1% malt. The amylase yield was significantly low in the media supplemented with yeast extract and Tryptone. The lowest yield of amylase was found in the medium supplemented with beef extract, peptone and ammonium nitrate.

Table 5. Effect of different Nitrogen sources on Amylase activity and production.

Nitrogen sources	Amylase activityU*/ml	Dry weight mg/100 ml
Peptone	0.107±0.006[c]	213±3.6[e]
Tryptone	0.138±0.005[b]	180±2.6[f]
Beef extract	0.088±0.011[c]	260±2.6[d]
Yeast extract	0.159±0.016[b]	320±2[b]
Ammonium nitrate	0.094±0.006[c]	265.33±1[c]
Sodium nitrate	0.425±0.034[a]	333±2.6[a]

*Amount of maltose liberated in µmol per ml per minute.
In each column, mean values followed by the same letter are not significantly different a according to DMRT at p=0.05

This study reveals that the production of amylase is feasible by isolated endophytic *Discosia* sp. This is the first time that amylase enzyme is optimized in this genus, and there is no record in this matter before as our knowledge. Hence these results offer an alternative source of enzymes for industrial supply.

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