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AMYLASE PRODUCTION BY ENDOPHYTIC FUNGI *CYLINDROCEPHALUM* SP. ISOLATED FROM MEDICINAL PLANT *ALPINIA CALCARATA* (HAW.) ROSCOE

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ABSTRACT

Amylases are among the most important enzymes used in modern biotechnology particularly in the process involving starch hydrolysis. Fungal amylase has large applications in food and pharmaceutical industries. Considering these facts, endophytic fungi isolated from the plant *Alpinia calcarata* (Haw.) Roscoe were screened for amylolytic activity on glucose yeast extract peptone agar (GYP) medium. Among thirty isolates of endophytic fungi, isolate number seven identified as *Cylindrocephalum* sp. (Ac-7) showed highest amylolytic activity and was taken 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 amylase production was found to be at 30°C and at pH 7.0 of the growth medium. Among the various carbon and nitrogen sources tested, maltose at 1.5% and Sodium nitrate at 0.3% respectively gave optimum amylase production.

Key words: *Alpinia calcarata* Roscoe; Endophytic fungi; *Cylindrocephalum* sp.; Amylase.

INTRODUCTION

Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry. Fungal amylases have been widely 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 (6). These enzymes account for about 30% of the world's enzyme production (40). Amylases are starch degrading enzymes that catalyze the hydrolysis of internal glycosidic bonds in polysaccharides with the retention of anomeric configuration in the products. Most of the amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability (5). Amongst starch degrading enzymes are endo-amylases, exo-amylases, debranching enzymes and glycosyltransferases (21). The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps (36). Because of the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases, which are suitable for industrial applications and their cost effective

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production techniques.

Most of the amylases have been produced from soil fungi such as *Aspergillus, Penicillum and Rhizopus* (31). Very few reports are available on amylases from endophytic fungi, which are mainly explored for beneficial secondary metabolites with different bioactivity (25). Endophytic fungi usually live asymptomatically within tissues of their host plants protecting them from natural enemies (10). Caldwell *et al*., (7) reported the ability of dark septate root endophytic fungi, *Philaophora finlandia* and *P. fortinii* isolated from Alpine plant communities, which were able to breakdown the major polymeric forms of carbon, nitrogen and phosphorus found in plants. Marlida *et al*., (26) reported raw starch degrading enzyme from endophytic fungi *Gibberella pulicaris*, *Acremonium* sp. *Synnematous* sp. and *Nodilusporium* sp. Maria *et al*., (24) also reported amylase production by few endophytic isolates from mangrove angiosperm *Acanthus ilicifolius* L. and mangrove fern, *Acrostichum aureum* L. There are no reports of amylase production from endophytic fungi isolated from *Alpinia calcarata.* This led us to explore the amylolytic potential of the endophytic fungi isolated from this plant.

The aim of the present study was to screen the different endophytic fungi for amylase production isolated from *A. calcarata* and study the capability of hydrolyzing a wide range of cheap and easily available starch source as substrates.

MATERIALS AND METHODS

Sources of endophytic fungi

Endophytic fungi were isolated from fresh material of healthy wild medicinal plant *Alpinia calcarata* Roscoe, which belongs to the family *Zingiberaceae* collected from the Charaka Sushrutha Vana, situated in Jnanabharathi Campus, Bangalore University, Bangalore, India. The Charaka Sushrutha Vana consists of five hundred medicinal plants grouped into fifty, based on their treatment to ailments, each group comprising of ten plants. These plants are planted in three concentric circles, the inner circle consisting of ten plants, outer circle consisting of twenty plants and the outermost circle with twenty. The medicinal garden is maintained by Sri Adichunchangiri Shikshana trust, Sri Kalabhairaveshwara Swamy Medical College, Hospital and Research Centre, Vijayanagara, Bangalore, India.

Isolation & identification of the endophytic fungi

The endophytic fungal strains were isolated from the medicinal plant *A. calcarata* Roscoe. The plant samples along with flowers and leaves were deposited for herbarium (RRCBI-Mus/09) and authenticated by National Ayurveda Dietetics Research Institute (Central Council for Research in Ayurveda and Siddha), Department of AYUSH, Ministry of Health and Family Welfare, Govt. of India, (New Delhi) Jayanagar, Bangalore, India.

Different parts of the *A. calcarata* such as leaves, midrib, petiole and stem were cut with the help of knife disinfected with 70% ethanol and brought to the laboratory. The collected samples were washed with tap water, cut into 0.5 cm^2 segments and were surface sterilized with standard triple ethanol-sodium hypochlorite-ethanol (16). Fifteen segments from each individual plant parts were placed in Petri plate containing Potato dextrose agar (PDA; HiMedia), autoclaved at 121°C, 15lbs for 15minutes and amended with tetracycline (150 mgL^{-1}). The Petri plates were then sealed with cellophane tape and incubated in a light chamber at 28°C with 12 hrs light followed by 12 hrs darkness. Regular observation of the Petri plates was done from the second day onwards for a period of 3–4 weeks for the fungal colonies (4). The fungi growing from internal tissues were transferred to fresh PDA slants and stored at 4°C. The fungi were identified based on the cultural, morphological characteristics of the fruiting bodies and spores using standard manuals (3, 15, 38).

Taxonomic identification of the fungus

The colonies were effuse, grey or olivaceous measuring

about 6–7cm in 7 days on PDA. Mycelium hyaline, conidiophores were mononematous, short (20–30µm), cylindrical and pointed at the apex, arising from aerial hyphae. Conidia were one celled, hyaline, ellipsoid (11–14 x 1.2. –2.5 µm) produced successively at the apex of the conidiophore and held together in loose clusters. The fungus was identified as *Cylindrocephalum* sp. (3).

Screening of endophytic fungi for amylase production on solid media

Endophytic fungal culture grown on PDA was cut into 5 mm discs with the help of borer. Three discs were placed on Petri plate containing autoclaved 15 mL of glucose yeast extract peptone agar (GYP) medium (glucose 1 g, yeast extract 0.1 g, peptone 0.5 g, agar 16 g and distilled water 1000 mL) with 0.2% soluble starch at pH 6.0. (20). After incubation, the plates were flooded with 1% iodine solution in 2% Potassium iodide. Zone of clearance around the colony was measured (24).

Optimization of culture conditions for amylase production

The procedure adopted for optimization of various parameters influencing amylase production was to evaluate the effect of each parameter independently keeping others as constant. The optimized parameters were incorporated in subsequent experiments. All experiments were done in triplicate and the mean values are presented.

Out of thirty isolates, eleven of them showed positive for Iodine test on solid media. Among these, the isolate number 7 of *A. calcarata* identified as *Cylindrocephalum* sp. (Ac-7) which gave maximum activity was selected for the optimization of amylase activity in liquid media. The organism was grown in 25 mL of basal media g/L ($NaNO_3$ 3 g, $MgSO_4.7H_2O$ 0.5 g, KCl 5 g, KH_2PO_4 1 g, $FeSO_4.7H_2O$ 0.01 g, $CaCl_2$ 0.1 g) and supplemented with 1.5% starch in 150 mL Erlenmeyer flasks and autoclaved at 121ºC, 15 lbs for 15minutes. After sterilization, the flasks were cooled to room temperature and 0.1 mL spore suspension of the fungal strain was inoculated and incubated for 7 days at different parameters as described below by taking one parameter at a time. An uninoculated flask served as control.

Effect of temperature, pH, carbon and nitrogen sources on Amylase activity

Present study was carried out at different temperature (15, 25, 30, 37 and 45°C) and pH (3, 5, 7, 9 and 11). Different sources of carbon such as corn flour (COF), cassava flour (CAF), rice bran powder (RBP), wheat bran powder (WBP), maltose (MAL), starch (STR) at 1.5 % (w/v) and Nitrogen sources, peptone (PEP), tryptone (TRP), beef extract (BE), yeast extract (YE), ammonium nitrate (AN) and sodium nitrate (SN) at 0.3% (w/v) were used to determine their effect on the production of amylase activity.

Determination of fungal biomass

The biomass of fungal culture 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 2795 *g* for 10 minutes 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 1mol L^{-1} sodium acetate buffer (pH 6.0), 0.5 mL 1% starch (w/v) and 0.5 mL of the crude enzyme extract (20). After 20 minutes of incubation, the liberated maltose was estimated by dinitrosalicylic acid (DNS) method (27). One unit of amylase activity (U) is defined as the amount of enzyme releasing one µmol of reducing sugar mL^{-1} min^{-1}, with maltose as standard under the assay conditions mentioned above. The denatured culture filtrate served as control.

Statistical analysis

Analysis of Variance was conducted by ONE-WAY ANOVA test using SPSS 16.0 from MICROSOFT WINDOWS and the means of the triplicates were compared by Duncan's multiple range test (13) at the 0.05 level of significance.

RESULTS AND DISCUSSION

Screening of endophytic fungi for amylase production on solid media

Out of thirty endophytic isolates screened for the amylolytic activity on solid media, eleven of them showed positive results. Among these the isolate (Ac-7) *Cylindrocephalum* sp., which showed maximum zone of clearance, was selected for the optimization of amylase activity in liquid media. Mean results are represented in Fig. 1.

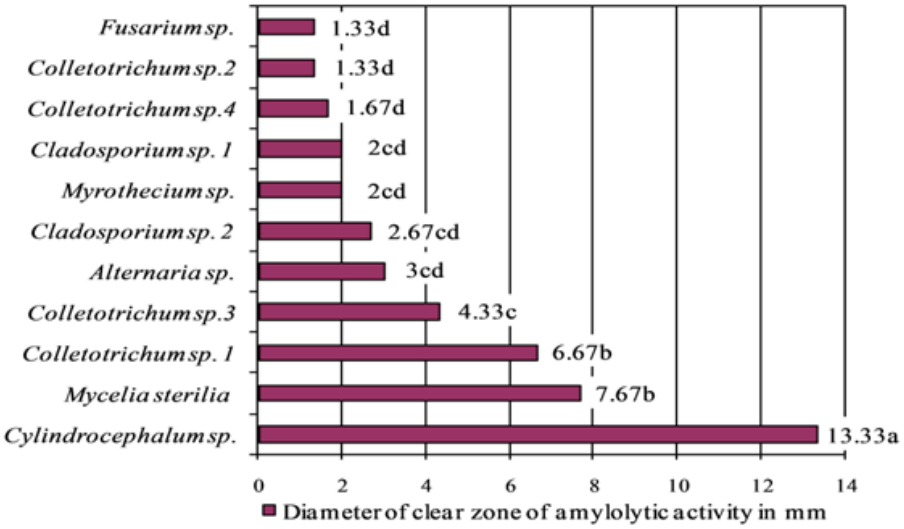

Figure 1. Amylolytic activity of endophytic fungi

Optimization studies

Optimization of various parameters and manipulation of media are one of the most important techniques used for the production of enzymes in large quantities to meet industrial demands (39). Production of amylase enzyme in fungi is known to depend on both morphological and metabolic state of the culture. Growth of mycelium is crucial for extracellular enzymes (8). Various physical and chemical factors have been known to affect the production of amylase such as temperature, pH, carbon sources acting as inducers, and nitrogen sources respectively. Interactions of these parameters are reported to have a significant influence on the production of the enzyme (37).

Effect of incubation temperature on amylase activity

The amylase activity and the biomass were nil at lower (15° C) and higher incubation (45° C) temperature (results not shown). The optimum incubation temperature for amylase production was found to be at 30° C (Fig. 2.) and biomass also correlated with the results. The influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature for enzyme production depends on whether the culture is mesophilic or thermophilic. Among fungi, most of the amylase production studies have been done with mesophiles within the temperature range of 25–37° C (17, 28). Kundu and Das (22) and Ray (35) have reported that amylase activity was optimum at 30° C in *Aspergillus oryzae*, *Botryodiplodia theobromae* and *Rhizopus oryzae*. A raw starch degrading α-amylase was also produced by *A. ficuum* at 30° C (19). Kathiresan and Manivannan (20) also reported 30° C as optimum temperature in case of *Penicillium fellutanum* isolated from mangrove rhizosphere soil.

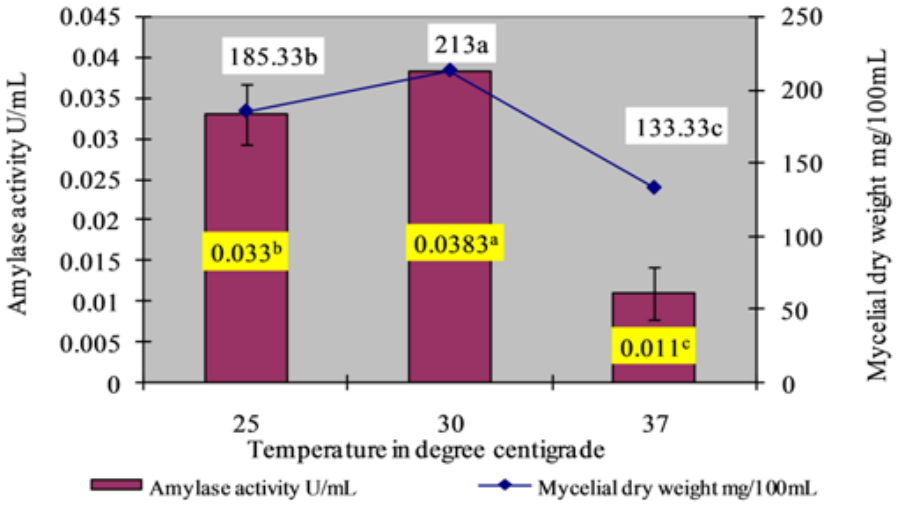

Figure 2. Effect of temperature on amylase production. The mean values followed by the same letter are not significantly different according to DMRT at p=0.05.

Effect of pH of the culture media on amylase activity

The pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. The pH is known to affect the secretion of α-amylase and its stability (18). The optimum pH for amylase production was 7.0 (Fig.3.), which is similar to the findings of Patel *et al.,* (32) who reported in *A. oryzae.* The biomass yield was found to be higher in case of pH 5.0 contradictory to the findings of Olama and Sabry (29) where the amylase activity and the biomass yield was maximum at pH 7.0 in *Aspergillus flavus* and *P. purpurescence*. *Aspergillus oryzae*, *A. ficuum* and *A. niger* were found to give significant yields of α-amylase between pH 5.0–6.0. (9, 12, 19).

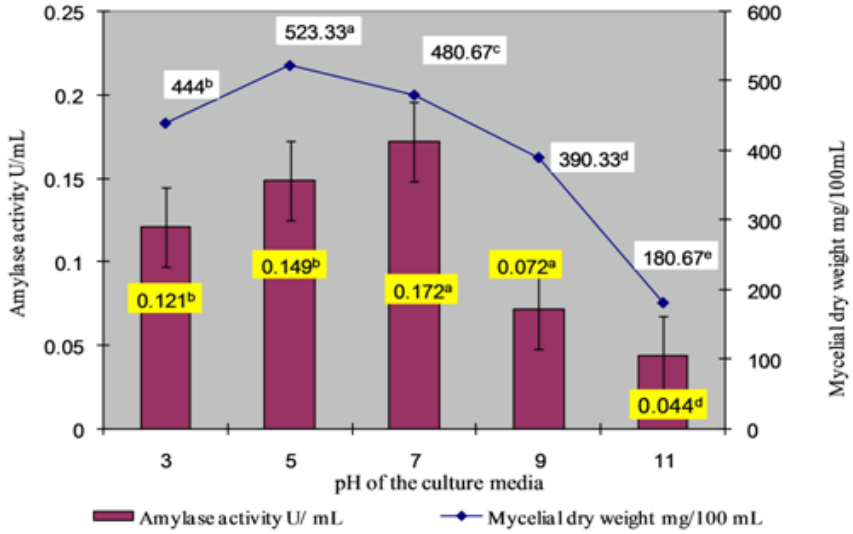

Figure 3. Effect of pH on amylase production. The mean values followed by the same letter are not significantly different according to DMRT at p=0.05.

Effect of different carbon sources on amylase production

The fungus was able to grow on all the tested carbon sources. There were significant differences in the yield of the biomass and amylase production. Among the various substrates screened for amylase production, maltose gave the highest enzyme activity followed by starch. The order of usability of substrate was maltose > starch > wheat bran powder > corn flour > cassava flour > rice bran powder (Fig.4.). There was high increase in biomass yield in case of wheat bran powder and rice bran powder, but the enzyme activity was low in contrast to the findings of Ellaiah *et al.,* (14) where amylase activity was maximum in *Aspergillus* spp. This indicates that the nature and amount of carbon source in culture media is important for the biomass growth and production of extracellular amylase.

Among chemical parameters, carbon source of the growth medium plays a very important role in inducing enzyme secretion. Amylase is generally induced in the presence of carbon sources such as starch and its hydrolytic products. Similarly, such findings were reported by Kathiresan and Manivannan (20) as maltose as best carbon source to enhance the amylase activity in *P. fellutanum.* Compared to defined carbon sources, the biomass yield was higher in undefined carbon sources similar to the findings of Oliveira *et al.,* (30) in case of Rhizobial strains. Kuo and Hartman (23) showed that *Thermoactinomyces vulgaris* produces best yields of α-amylase when starch or maltose is used as carbon source.

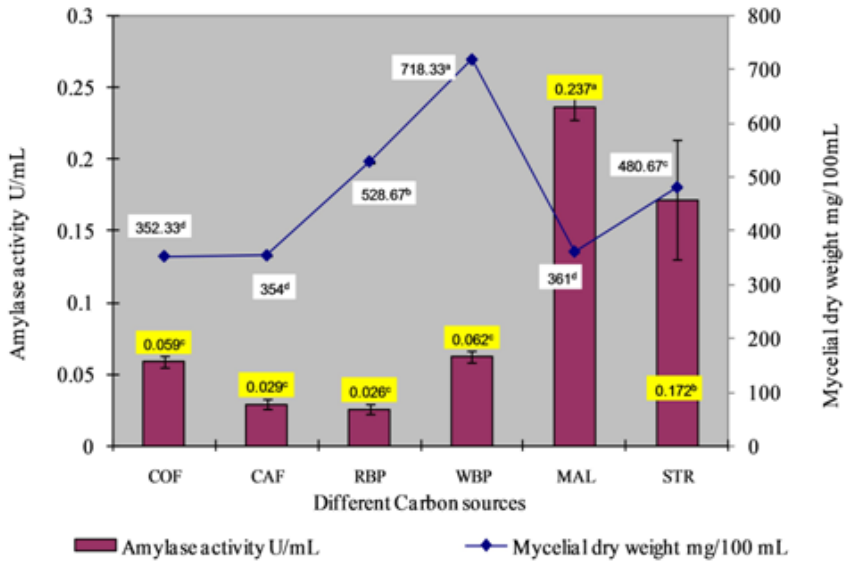

Figure 4. Effect of different carbon sources on amylase production. The mean values followed by the same letter are not significantly different according to DMRT at p=0.05.

Effect of various nitrogen sources on amylase production

Nitrogen sources have been reported to have an inducing effect on the production of various enzymes including α-amylase in SSF system (1, 33, 34). Earlier reports show that among various inorganic nitrogen sources tested, ammonium sulphate, ammonium chloride and ammonium hydrogen phosphate favored growth and enzyme secretion (28). Similar observations were recorded by Chandra *et al.,* (11) Babu and Satyanarayana (2). However, in our studies, there was no significant increase in enzyme yield in the case of the supplementation with either inorganic or organic nitrogen sources (Fig.5.). A marginal increase in amylase activity was noted with the addition of Sodium nitrate. A minimum activity was observed when Ammonium nitrate was used as nitrogen source. There was no significant difference in amylase activity with peptone, tryptone and beef extract indicating that any of these sources can be alternatively used.

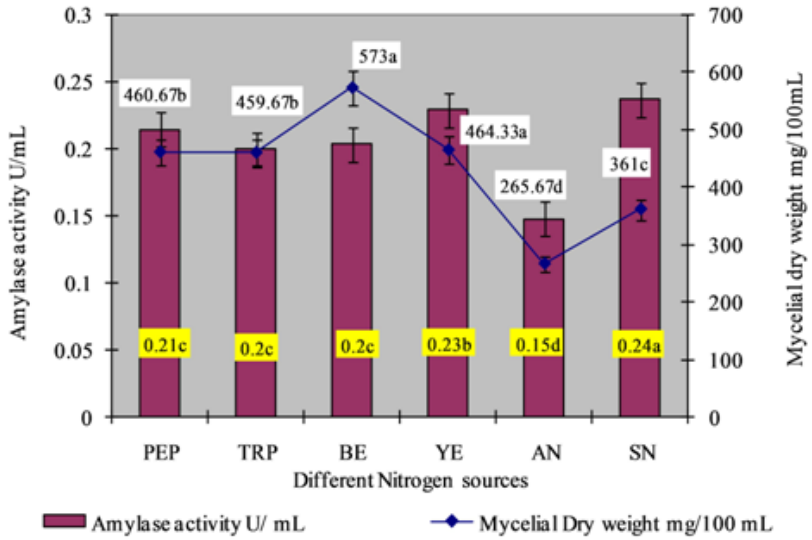

Figure 5. Effect of different nitrogen sources on amylase production. The mean values followed by the same letter are not significantly different according to DMRT at p=0.05.

CONCLUSION

In the present study, an attempt has been made to offer an endophytic fungus as source of enzymes for industrial requirements. The growth parameter of *Cylindrocepahalum* sp. for the maximum amylase production has been standardized, which can effectively be used in large scale production. Though, the cheaper agricultural byproducts are feasible for the production of amylase enzyme, but are not significant to the maltose when used as substrate. However, more detailed investigation is required to characterize this enzyme, which may be used in the large-scale production for commercial purpose in future.

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