DETERMINATION OF AMYLASE ACTIVITY OF *RHIZOCTONIA SOLANI* ISOLATED FROM SOYBEAN (GLYCINE MAX L.) SEEDS

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ABSTRACT

The present study was aimed to determine the production and activity of amylase enzyme of *Rhizoctonia solani*isolated from the soybean seed. The *R. solani* was isolated from 0.1% $HgCl_2$ treated soybean seeds.The effects of pH, temperature, incubation time, sources of carbon and nitrogen were tested in submerged fermentation process in production of amylase by *R. solani*.The production medium without addition of starch and with provision of maltose as carbon source, peptone as nitrogen source, incubated for 120 h, maintained with pH of 6.5 at 30^0C, was found optimal for production of amylase by *R. solani.*

Key words: Amylase, Carbon, Nitrogen sources, *Rhizoctonia solani,* Soybean seeds

INTRODUCTION

Amylase is an important metabolic enzyme which hydrolyzes starch molecules to give diverse products including dextrin. Amylases are group of enzymes that have been found in several microorganisms including fungi (Fadel 2000). It is commercially important enzyme in the starch bioprocessing and brewing industries responsible for breakdown of starch or glycogen into simple sugar constituents. Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries (Lin et al., 1997; Pandey et al., 2000). Mass production of extracellular alpha amylase was reported by fungi like *Aspergillus awamori* (Castro et al 2010). Extracellular amylase produced by several filamentous fungi has been used in baking, detergent, paper, textile & food industry (Emmanuel & Saleh 2004; Mishra & Dadhich 2010).

With the help of new frontiers in biotechnology, the amylase application has used in many other fields. Many new areas have opened for their utilization as raw materials for the production of value added fine products (Pandey et. al., 2000, 2000 a). The increasing demand from modern biotechnological industries for enzymes can fulfilled with increased survey of microorganisms producing enzymes. Different types of fungi were isolated from soybean seeds on potato dextrose agar. Further *R. solani* selected and studied for amylase production and optimization of cultural conditions also done.

MATERIALS AND METHOD

Determination of seed mycoflora (Boukhout & Robert 2003)

Different types of fungi were isolated from soybean seeds obtained from various locations by Sabouraud Glucose Agar with an antibiotic (Chloramphenicol 0.1 g l-1) after surface sterilization by $HgCl_2$. The fungus, *Rhizoctonia solani* was selected for further study.

Chemicals

All analytical reagents and media components were purchased from Hi-Media (Pune, India) and Sigma chemicals (Pune, India).

Growth media

For growth of *R. solani* Sabouraud Glucose Agar medium containing glucose 20 g, peptone 10 g, agar 20 g, and distilled water 1000 ml was used.

Production medium

Production of amylase was studied by growing the *R. solani*in liquid medium containing starch- 1%, KNO3-0.25%, KH2PO4- 0.1% and MgSO4.7H2O-0.05%, 100 ml distilled water. The pH of the medium was adjusted to 6.5. Twenty five ml the medium was poured in 100 ml conical flasks, autoclaved for 15 min at 121°C. The media were inoculated separately with 1 ml spore suspension (20 OD and 350 wavelengths) of the *R. solani* which was maintained on PDA slants for 7 days. The flasks were incubated for 6 days at 30 ±2°C with diurnal periodicity of light in an orbital shaker set at 100 rpm for 72 h. On 7th day the flasks were harvested by filtering the contents through Whatman filter No.1. The filtrates (media) were collected in pre-sterilised bottles and were centrifuged at 5,000 rpm for 15 min to obtain crude enzyme solution

Confirmation of amylase production

Amylase production by *R. solani*was confirmed (Hols et al., 1994; Capuccino & Sherman 2001) on starch agar plates containing peptone 5 g, yeast extract 1.5 g, soluble starch 2.0 g, NaCl 5.0 g, agar 15 g, and distilled water 500 ml 15ml of the medium were poured in each Petriplates. On solidifying the medium, a cavity (8 mm diameter) was made in the centre with the help of Cork borer (No.4) and was filled with 1ml culture filtrate (crude enzyme preparation). The plates were incubated at 28^{0}C for 24 hours Amylase production was detected after flooding the plates with Lugol´s iodine solution as an indicator. A clear, non-blue, circular zone was obtained surrounding the central cavity. The diameter was measured (mm) as the amylase activity zone. Similar procedure was followed for the control except pouring of autoclaved culture filtrate in the central cavity instead of the active enzyme.

Enzyme assay (Miller, 1959)

Amylase assay was made by using a reaction mixture (4 ml) consisted of 1 ml of enzyme solution and 2 ml of soluble starch in phosphate buffer, pH 6.5 (Wood & Bhat 1988). The mixture was incubated for 10 min at 30°C. Level of reducing sugars was determined by dinitrosalicylate method and is expressed in units (one unit is the amount of enzyme which releases 1 μ mole glucose).

Optimization of culture conditions

The factors such as temperature, pH, sources of carbon and nitrogen affecting production of amylase were optimized by varying parameters one at a time. The experiments were conducted in 100 ml Erlenmeyer flask containing production medium. After sterilization by autoclaving, the flasks were cooled and inoculated with culture and maintained under various operational conditions separately such as pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5), temperature (25, 35, 45°C), carbon source (glucose, galactose, maltose, lactose, sucrose and xylose each at 1%), nitrogen source (peptone, beef extract, yeast extract, meat extract and casein each at 0.5%) After 72 h (except for incubation period effect), the culture filtrate was assayed in triplicate for amylase activity.

RESULTS

The culture filtrate of *R. solani* after 72 h exhibited amylase activity of 94 U/ml, at pH 6.5 and 30°C. The activity was about 31% higher at pH 6.5 than pH 5.0; and 30% higher at 30°C than 40°C (Tables 1 and 2). When the culture was incubated at 96 h, the maximum activity detected was 136 U/ ml. There was a 2 fold increase in activity at 96 h incubation as compared to 24 h (Table 3). Among carbon sources, maltose was the best to enhance the enzyme activity of 146 U/ml which was 7% higher than sucrose (Table 4).

Among nitrogen sources, peptone was ideal to increase the enzyme activity of 150 U /ml, which was about 9% higher than yeast, meat and casein

However, there are only a few reports concerning the optimization of media composition especially for fungal strains in amylase production (Quang et al., 2000). The growth requirements for many fungi may vary from strain to strain although cultures of the same species and genera tend to grow best on similar media (Smith & Onions 1994). Temperature optimum for amylase was found to be in a range between 25 and 37°C for the mesophilic fungi (Gupta et al., 2003) and the present study recorded 30°C as optimal, which agrees with earlier findings. Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium (Gupta et al., 2003). Most of the earlier studies revealed the optimum pH range between 6.0 and 7.0 for the growth of bacterial strains and enzyme production (Gupta et al., 2003). This is also true of strain of *R. solani used* in the present study. However, *Aspergillus oryzae* released amylase only in alkaline pH above 7.2. In the present study the amylase activity increased steadily and reached maximum at 96 h of incubation (Table 3), as against a short duration of 24 h in the case of bacteria (Dharani, 2004). Contrary to this, Lactose induced amylase production (148 U/ml) by *R. solani* (Table 4). Most reports available on the induction of amylase in different strains of fungal species suggest that the general inducer molecule is maltose which increases many fold enzyme activity (Eratt et al., 1984). However, there is statistically significant variation among the carbon sources used in our study (Table 4).

Table 1 Effect of pH on Amylase Production by *R. solani*

Sr.No.	pH	Amylase Activity* (U/ml)
1	5.0	47
2	5.5	90
3	6.0	39
4	6.5	32
5	7.0	10
6	7.5	17

*One unit of amylase activity was defined as the amount of enzyme which released 1µmole glucose under the assay conditions. Values are mean ± S.E

Graph 1Effect of pH on Amylase Production by *R. solani*

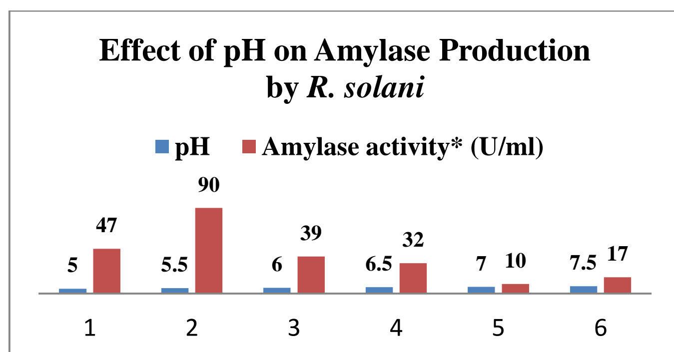


Table2 Effect of Temperature on Amylase Production by *R. solani*

Sr. No.	Temperature (°C)	Amylase Activity* (U/ml)
1	25	30
2	35	43
3	45	10

*One unit of amylase activity was defined as the amount of enzyme which released 1µmole glucose under the assay conditions. Values are mean ± S.E

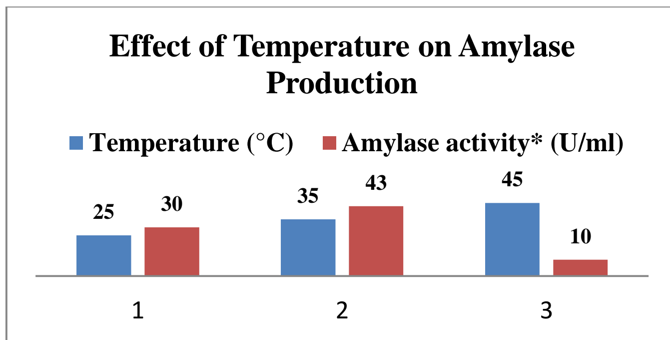


Table 3 Effect of Nitrogen Sources on Amylase Production by*R. solani*

Sr. No.	Nitrogen Sources (0.5%)	Enzyme Activity (U/ml)	O.D. (350)
1	Beef Extract	72	0.72
2	Meat Extract	63	0.63
3	Yeast Extract	25	0.25
4	Casein	51	0.51
5	Peptone	84	0.84

*One unit of amylase activity was defined as the amount of enzyme which released 1µmole glucose under the assay conditions. Values are mean ± S.E.

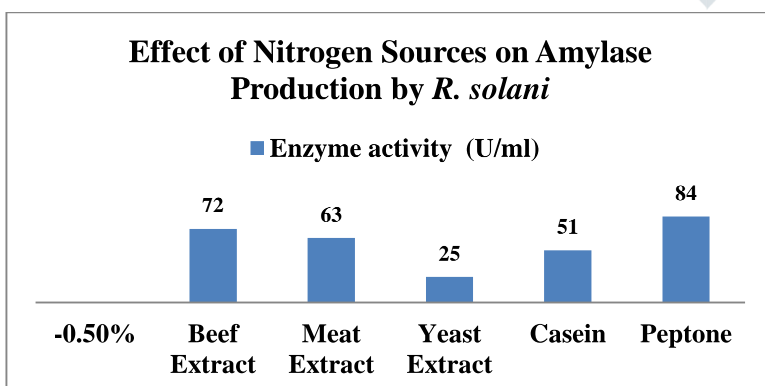
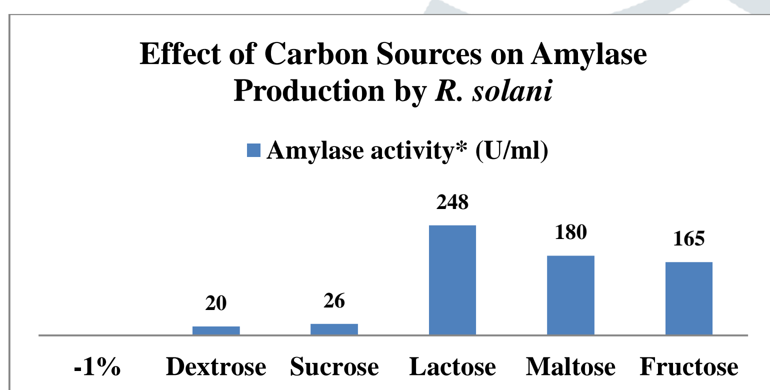
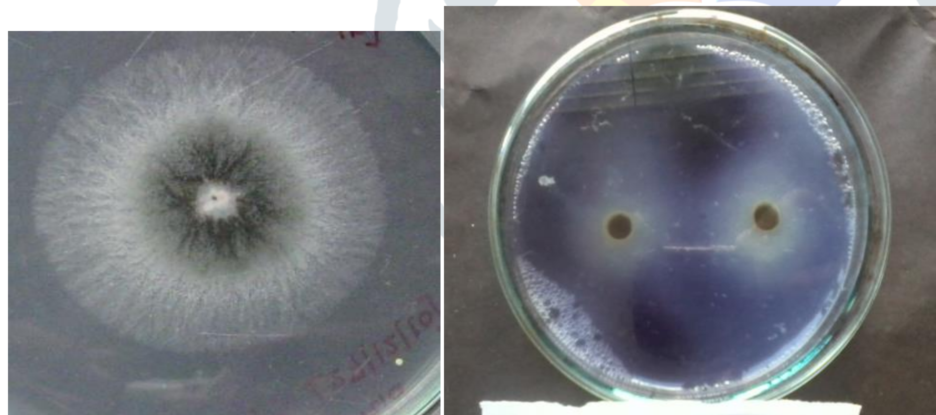


Table 4 Effect of Carbon Sources on Amylase Productionby *R. solani*

Sr. So.	Carbon Sources (1%)	Amylase Activity* (U/ml)
1	Dextrose	20
2	Sucrose	26
3	Lactose	248
4	Maltose	180
5	Fructose	165

One unit of amylase activity was defined as the amount of enzyme which released 1µmole glucose under the assay conditions. Values are mean ± S.E.





***R. solani* growth on PDA** **Amylase Inhibition Zone**

DISCUSSION

The media optimization is an important aspect to be considered in the development of fermentation technology. However, there are only a few reports concerning the optimization of media composition especially for fungal strains in amylase production (Quang et al., 2000). Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium (Gupta Rani et al., 2003). Most of the earlier studies revealed the optimum pH range between 6.0 and 7.0 for the growth of bacterial strains and enzyme production (Gupta Rani et al., 2003; Kundu et al., 1973; Castro et al., 1992). The similar results are observed in case of strain of *Rhizoctonia solani used* in the present study. However, *Aspergillus oryzae* released amylase only in alkaline pH above 7.2 (Yabuki et al., 1977). Temperature optimum for amylase was found to be in a range between 25 and 37°C for the

mesophilic fungi (Kundu et al., 1973; Ueno et al., 1987; Gupta Rani et al., 2003) and the present study recorded 30°C as optimal, which agrees with earlier findings. The incubation period varies with enzyme productions (Smitt et al., 1996). Short incubation period offers potential for inexpensive production of enzymes (Sonjoy et al., 1995). In the present study the amylase activity increased steadily and reached maximum at 96 h of incubation (Table 3), as against a short duration of 24 h in the case of bacteria (Dharani Aiyer, 2004). Amylase is an inducible enzyme and is generally induced in the presence of carbon sources such as starch, its hydrolytic product, or maltose (Yabuki et al., 1977; Tonomura et al., 1961; Lachmund et al., 1993; Morkeberg et al., 1995). (Arst and Baile, l977). Still the role of glucose in production of amylase is controversial. Xylose has been reported to strongly repress amylase production, although the carbon source supports good growth in *A. nidulans* (Arst and Baile, 1977). Contrary to this, Lactose induced amylase production (148 U/ml) by *Rhizoctonia solani* (Table 4). Most reports available on the induction of amylase in different strains of fungal species suggest that the general inducer molecule is maltose which increases many fold enzyme activity (Eratt et al., 1984). However, there is statistically significant variation among the carbon sources used in our study (Table 4). Organic nitrogen sources are preferred for the production of amylase. A maximum amylase production was supported by yeast extract; peptone or beef extract (Hamilton et al., 1999; Emanuilova and Toda, 1984; Krishnan and Chandra, 1982; Hayashida et al., 1988). Although, peptone appears to be ideal source, there is no statistically significant variation among the nitrogen sources used in our study (Table 3 The nature of culture conditions and composition of media for optimal production of amylase by has *R. solani* been developed in this study.

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This paper reports the results of a study on identification and isolation of fungal species, having extra-cellular amylase activity

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