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Culture Condition for the Production of Thermostable Amylase by *Penicillium rugulosum*

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Abstract: Studies on α-amylase production is carried out with a fungal strain *Penicillium rugulosum* isolated from a soil sample, which is found to be thermophilic in nature. The conditions for production of α-amylase is optimized. Maximum production of amylase by *Penicillium rugulosum* is observed at 3rd day of incubation. Amylase production is improved in the presence of galactose as sole carbon source. Addition of peptone shortened the lag period and improved α-amylase synthesis. Initial pH of medium for amylase synthesis by organism is 7.0. The optimal pH and temperature for activity of amylase is 7.0 and 57°C. The enzyme activity is inhibited in the presence of EDTA and increases in presence of metal ion Mn^{2+} and Fe^{2+}.

Key words: α-amylase · thermophilic fungi · thermostable enzymes

INTRODUCTION

Recent discoveries of starch degrading enzymes have led to increase application of amylases in various industrial processes [1]. The α-amylase (1, 4-α-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyses α-1, 4 glucosidic linkages in starch and related substrates [6]. This enzyme has extensive commercial applications in starch liquefication, brewing and sizing in textile industries, paper and detergent manufacturing processes [5, 12]. Thermostability is a feature of most of the enzymes sold for bulk industrial usage and thermophilic organisms are therefore of special interest as a source of novel thermostable enzymes. Recent research with thermostable α-amylases has concentrated on the enzymes of thermophiles and extreme thermophiles [2, 3, 7, 9 and 10] and little is known about the properties of the enzymes produced by these organisms. The present study deals with the isolation and identification of thermophilic fungi and describes the effects of culture conditions on the activity of α-amylase.

MATERIALS AND METHODS

Culture medium-Agar Plate 'A' consisted of Czapexdox medium at pH 7.3, which is used for selection of thermophilic fungi.

Agar Plate 'B' contained 1% starch in Czapexdox medium, which is used for screening fungi capable of producing starch digesting enzymes.

Isolation and determination of thermophilic fungi producing α-amylase: Soil suspensions in sterilized water are poured and spread on agar plate 'A'. These plates are incubated at 55°C at 3 days for incubation period. The colonies that are found on the plates are transferred on agar plates 'B'. Again these plates are incubated at 55°C for 3 days. Several amylase-producing fungi are isolated. The plates are exposed to iodine vapors and examined for starch hydrolysis around the line of growth; a clear area around the growth line give a positive result for amylase production while a negative reaction indicated by dark coloration of the media which are not producing amylase. Among fungi, species that yield a high level of α-amylase are selected and identified to be used for further experiments.

Identification of fungi: The isolated fungi i.e. *Penicillium rugulosum* are further identified from available literature [4, 11.] and reference slides and finally by authentic authority.

Preparation of crude enzyme: It has been generally believed that starch serves as inducers for the amylase biosynthesis [8]. Mass culturing of organism are carried out for production of large amount of biomass. The selected starch hydrolyzing *Penicillium rugulosum* is inoculated into 50ml of Czapexdox broth containing 1% starch and incubated for 5 days for incubation period. The mycelial extract is used for intracellular enzyme source and culture broth is used as extracellular enzyme source. The mycelial mats are washed with cold normal saline and one

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gram of mycelial mats is weighed and thoroughly homogenized in 10ml cold normal saline with the help of pestle mortar. The mycelial extract are centrifuged at 3000 rpm for 15 minutes at room temperature. The resulting supernatant is taken directly as source of intracellular crude enzyme for assay of activity.

Preparation of extracellular enzyme: After harvesting the mycelial mat the culture broth is centrifuged at 3000 rpm for 15 minutes at room temperature to obtain cell free supernatant, which served as source of extracellular enzyme.

Enzyme assay: For both the samples amylase activity is measured according to the method of Yoo *et al.* [14].

Protein estimation: Quantity of Protein in both the samples is estimated by the method of Lowry *et al.* [13] using serum albumin as standard protein.

Culture amendments for optimum production of extracellular amylase: The objective of this study is to elaborate best conditions for the production of extracellular α-amylase from *Penicillium rugulosum.* Effects of different carbon sources, nitrogen sources, pH and incubation time on the production of extracellular amylase are studied using Czapexdox broth medium.

Effects of carbon sources: To study the effect of carbon sources on the production of extracellular amylase, lactose, galactose, arabinose, maltose and dextrose are selected as carbon source. Sucrose is replaced in the basal medium by equal amounts of other sugars to be tested, while all the other ingredients remained the same. *Penicillium rugulosum* is grown in duplicate flasks for 5 days with each carbon sources and amylase activity assayed in fungal filtrate.

Effect of nitrogen sources: Nitrogen sources selected for the present study are tryptone, yeast extract, peptone, ammonium nitrate and ammonium sulphate. Sodium nitrate is substituted by other nitrogen sources in culture medium of *Penicillium rugulosum.* The culture filtrate is taken and amylase activity is determined.

Effect of different pH: In order to investigate the effect of pH on extracellular amylase production pH of growth medium is adjusted to 1, 3, 5, 7 and 9 prior to sterilization.

Effect of incubation time: Effect of incubation time on production of amylase is studied by harvesting the culture filtrate after each 24 hrs and amount of starch is quantitated.

Characterization of extracellular amylase from *Penicillium rugulosum*

Effect of pH on the activity of amylase: The enzyme is incubated at different pH 1, 3, 5, 7, 9 for determination of optimum pH of amylase. The pH is maintained in the reaction mixture by preparing substrate in 20 mM phosphate buffer of respective pH and activity is determined.

Effect of temperature on the activity of amylase: The enzymatic reaction is carried out at different temperature 27, 37, 47, 57, 67 and 77°C. Reaction mixtures are incubated at above respective temperature and enzyme activity is determine in order to find out the optimum temperature for amylase.

Effect of metal ions on the activity of amylase: The effect of metal ions on the activity of amylase is determined by adding different metal ions in the reaction system. The metal ions selected for present study are $HgCl_2$, $CaCl_2$, $CoCl_2$, $FeSO_4$ and $MnSO_4$ at 1mM concentration and added to enzyme mixture and incubated for 10 minutes.

The effect of different inhibitors on the activity of amylase: The inhibitors used are SDS (sodium dodecyl sulphate), EDTA (ethylenediamine tetra acetic acid) Acryl amide and Urea. All the inhibitors are added in the incubation mixture at 1mM concentration and enzyme assay is carried out discussed as above.

RESULTS AND DISCUSSION

Screenings for amylase: Species isolated from soil sample are screened for amylase in Starch Agar Plate. *Penicillium rugulosum* is chosen for further studies.

Assay method: Enzyme assay is performed as per method of Yoo *et al.* Enzymatic activity is recorded as 3.9 mg ml^{-1} from intracellular part of *Penicillium rugulosum* and 1.28 mg ml^{-1} from extracellular culture filtrate. For further experiment only extracellular enzyme is taken.

Table 1: Effects of carbon sources on the production of extracellular amylase

S. No	Carbon sources	Enzyme activity
1	Galactose	2.23
2	Arabinose	2.16
3	Maltose	1.03
4	Dextrose	2.18
5	Lactose	1.72

Table 2: Effect of Nitrogen sources on the production of extracellular amylase

S. NO	Nitrogen Sources	Enzyme activity
1	Peptone	2.41
2	Tryptone	1.26
3	Yeast extract	1.95
4	Ammonium nitrate	2.00
5	Ammonium sulphate	1.98

Table 3: Effect of different pH on production of amylase

S. No	pH	Enzyme activity
1	1	1.72
2	3	1.67
3	5	1.94
4	7	2.16
5	9	0.00

Table 4: Effect of incubation time on production of amylase

S. No	Days	Enzyme activity
1	1st	2.13
2	2nd	2.58
3	3rd	2.68
4	4th	2.47
5	5th	2.00

Characterization of enzyme activity

Protein estimation: Protein concentration from *Penicillium rugulosum* is measured by Lowry's method. It is found that extracellular protein concentration is 3.07 mg ml^{-1}.

Culture amendments for optimum production of extracellular amylase

Effect of carbon source: The effect of different carbon sources is studied during present investigation on the production of amylase by *Penicillium rugulosum.* Galactose, Arabinose, Lactose, Maltose and Dextrose are used as different carbon source in the broth. It is observed that with Galactose highest production of extracellular amylase occurs. (2.23 mg ml^{-1}). (Table 1).

Table 5: Effect of pH on enzyme activity

S. No	pH	Enzyme activity
1	1	2.07
2	3	1.27
3	5	1.05
4	7	2.12
5	9	1.80

Table 6: Effect of temperature on enzyme activity

S. No	Temperature (°C)	Enzyme activity
1	27	2.17
2	37	2.28
3	47	2.23
4	57	2.50
5	67	2.24
6	77	2.06

Table 7: Effects of metal ions on enzyme activity

S. No	Metal salts	Enzyme activity
1	Mercuric chloride	1.18
2	Calcium chloride	1.38
3	Cobalt chloride	1.42
4	Ferric chloride	1.62
5	Manganese chloride	1.94

Table 8: Effects of enzyme inhibitors

S.No	Inhibitors	Enzyme activity
1	EDTA	0.98
2	SDS	1.00
3	Acrylamide	1.30
4	Urea	1.37

Effect of nitrogen source: The amylase synthesis by *Penicillium rugulosum* in the presence of complex nitrogen sources is measured. Peptone, yeast extract, tryptone, ammonium sulphate and ammonium nitrate are added in growth medium. Peptone in the broth shortened the lag period and increased enzyme synthesis (2.41 mg ml^{-1}). The result suggests that the peptone favors the growth of the fungi and synthesis of amylase by the organism studied. The results are similar to the findings of Carlos *et.al.* who found maximum production of amylase with peptone (Table 2).

Effect of pH: *Penicillium rugulosum* is inoculated in the broth of different pH [1, 3, 5, 7 and 9]. At pH 7 extracellular amylase production is found to be highest (2.16 mg ml^{-1}) (Table 3).

Effect of incubation time: Effect of optimum time for production of amylase is studied and after inoculation of *Penicillium rugulosum* in broth, filtrate is harvested after every twenty-four hour (24 h) up to fifth (5^{th}) day of incubation. It is observed that *Penicillium rugulosum* give maximum production of amylase on 3^{rd} day (2.68 mg ml^{-1}) and which decreased there after. (Table 4).

Characterization of enzyme activity

Effect of pH on enzyme activity: Effect of pH on enzyme activity is measured by incubating reaction mixture at pH 1, 3, 5, 7, 9. The maximum enzyme activity is observed at pH 7.0. Amylase activity is optimum at pH 7 (2.12 mg ml^{-1}) but the enzyme is acid tolerant and also shows slight activity at pH 1 (2.07 mg ml^{-1}). (Table 5).

Effect of temperature on enzyme activity: Effect of temperature on amylase activity is measured by incubating the reaction mixture at 27, 37, 47, 57, 67 and 77°C with 10°C increasement. The activity of enzyme increased from 27°C to 57°C and decreased thereafter. 57°C (2.50 mg ml^{-1}) is the optimum temperature for amylase activity and this indicates that the enzyme is thermotolerant (Table 6).

Effects of metal ions on enzyme activity: Different metal ions are used to study their effect on amylase activity. Hg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+} and Co^{2+} are used in 11 mmol. Concentration. Enzyme reaction mixture are incubated with each metal ion and it is found that with Mn^{2+} (1.94 mg ml^{-1}) and Fe^{2+} (1.62 mg ml^{-1}) amylase showed high activity. Hence the enzyme is Mn^{2+} and Fe^{2+} dependent metalloenzyme (Table 7).

Effects of enzyme inhibitors: Different chemicals are used to analyze the effect of inhibitors on enzyme activity. EDTA, SDS, acrylamide and urea are used to find out best inhibitor of amylase. EDTA (0.98 mg ml^{-1}) and SDS (1.0 mg ml^{-1}) are found to inhibit amylase activity. In the presence of acrylamide and urea very low activity of amylase is observed. (Table 8).

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