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Optimization of pH for the Production of Amylase by Soil Mycotic Flora of Jabalpur Region.

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ABSTRACT

Amylases have potential application in the food fermentation, textile, paper and pharmaceutical industries. There are several processes in the medical and clinical areas that involve the application of amylase. The nutritional and cultural conditions are required for the optimum growth and production of amylase from the mycotic flora. The enzymes are very sensitive to pH and development of an optimal pH control strategy is helpful in obtaining higher protein productivity. The present work deal with the study of effect of pH on fungal amylase production. This comprises isolation and characterization of amylase producing fungi from different soil sources. These fungi were isolated using spread plate technique. The identification of the fungal isolates was done on the basis of morphology and biochemical tests. The probable genera identified were *Aspergillus species, Rhizopus species* and *Fusarium species* .The enzyme activity was observed for the isolates at pH range of 6.5, 5 and 9 and it was inferred that selection of optimum pH is essential for the maximum enzyme production. It was observed that *Aspergillus flavus, Aspergillus niger* and *Rhizopus species* were best amylase producers at different pH range.

INTRODUCTION

The demand for novel amylases worldwide is increasing day by day, as the application spectra of these enzymes are spreading in various industrial sectors. The classical approach is the isolation of microbial species, which produce novel enzyme from exotic environments and would offer a competitive advantage over the existing products. Subsequent characterization of these microbes under fermentation conditions to optimize the enzyme production properties plays vital role in evaluation of economic significance. Starch degrading amylolytic enzymes is of great importance in biotechnological application ranging from food, fermentation and textile to paper industries etc. Amylases are widely distributed and are one of the most studied enzymes [1]. Alpha-amylase is a key enzyme in metabolism of spacious diversity of living organism which utilizes starch as carbon and energy sources. It can hydrolyse starch, glycogen and related polysaccharide by randomly cleaving internal alpha-1, 4 glycosidic linkages to produce different sizes of oligosaccharides. Amylase is a very significant enzyme, having biological, clinical, biochemical and even industrial importance [2]. It could be obtained from different types of bacteria and fungi. Isolating amylolytic organisms, particularly fungi, from soil is very simple and yields accurate results [3].

Molds are capable of producing high amounts of amylase; *Aspergillus niger* is used for commercial production of α-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 [4]. It is possible to enlist the use of amylases under extreme condition of pH and temperature using thermo-acidophilic and alkaline amylases. Since the most effective preparation of some

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applications contain other enzymes, especially amyloglucosidases and submerged methods give a narrow spectrum of additional enzymes and it is worthwhile to isolate suitable strains of *Aspergillus niger* for efficient mechanism [5]. The production of α -amylase by molds has been greatly affected by cultural and nutritional requirement.

Solid State Fermentation holds tremendous potentials for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source [6].

Among the physical parameters, the pH of growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. The pH change observed during the growth of the organism also affects product stability in the medium. In fungal processes, the buffering capacity of some media constituents sometimes eliminates the need for pH control [7].

The pH value also serves as a valuable indicator of the initiation and end of enzyme synthesis. It is reported that *Aspergillus oryzae* accumulated α amylase in the mycelia when grown in phosphate or sulphate deficient media and was released when the mycelia were replaced with alkaline medium [3,8].

MATERIALS AND METHODS

Isolation of Fungal Isolates

Soil samples were collected from the Jabalpur region. The samples were collected in a sterile container and brought to the laboratory for further processing. This soil sample was serially diluted up 10^{-6} dilution by the serial dilution method and spreaded on potato dextrose agar media plate containing antibiotic chloramphenicol to avoid bacterial contamination.

Identification of Amylolytic Fungal Isolates

After 4-5 days, the Petri plates were observed for the growth of various fungi. These fungi were then identified of the basis of morphological (microscopic and macroscopic) characteristics and starch hydrolysis test. All the plates were observed for macroscopic characters of fungi that is for color, hyphae and texture. The fungi were observed microscopically by lacto phenol cotton blue staining method.

All the fungal colonies were examined for amylase production by starch hydrolysis test. The test is performed to check the ability of the organism to utilize starch by producing amylase.

Study of Growth Parameters of Isolates

Growth parameters of fungal isolates showing maximum hydrolysis were studied in term of growth kinetics [9]. The Growth curve of the fungal isolates was studied in order to get an idea about phases of growth. The PDB media was inoculated with loop full of fungal isolates (showing maximum hydrolysis) and incubated at 28°C. The growth of isolates was tracked for 7 days by reading the 600nm against blank. After that a curve was plotted between days on X axis and OD at600nm.

Preservation of Culture

The fungal isolates were preserved in starch agar slants covered with parafilm at low temperature for future studies.

Fermentative Production of Amylase

Inoculum Preparation

10 ml of sterilized distilled water was added on slants containing fungal spores and then scraped to loosen the spores. A standard amount of inoculum (10ml) was transferred into a flask containing growth medium.

Submerged Fermentation

Submerged fermentation was carried out in Erlenmeyer flask by taking 100 ml of amylase production medium ,containing KH_2PO_4 (1.4 g L^{-1}), NH_4NO_3 (10 g L^{-1}) ,KCl (0.5gL^{-1}) ,$MgSO_4.7H_2O$ (0.1g L^{-1}),$FeSO_4.7H_2O$ (0.01g L^{-1}),Soluble starch (20 g L^{-1}) ,and pH adjusted 6.5.

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The flask was autoclaved, cooled at room temperature and inoculum (showing maximum hydrolysis during screening) was incubated for 72 hrs. at 28°C±2°C on a shaker at 150 rpm.

Extraction of Crude Enzyme

Crude enzyme was extracted from fermented media by adding Tris buffer pH 6.5, agitating the flask in shaker at 180rpm for 1 hour, the mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4°C for 5 minutes. The supernatant was collected and treated as crude enzyme.

Protein Estimation

The concentration of protein in crude enzyme (extracted from flask) was determined by Lowry's methods of protein estimation in which enzyme was reacted with the Lowry's reagents and the absorbance obtained was compared with a standard graph plotted by reacting a standard protein with known concentration with the Lowry's reagents [11]. Then a graph was plotted between concentration of standard protein on X axis and absorbance at 650nm on Y axis.

Enzyme Assay in Crude Enzyme

0.5ml of crude enzyme extract was taken into a test tube to which 0.5 ml of 1% soluble starch was added. The test tube was then incubated in a water bath at 100°C for 15 minutes For blank, 0.5 ml of enzyme extract (that has been boiled for 15 minutes in order to inactivate the enzyme) was added to starch solution and treated with same reagent as the experimental tubes. The reaction was ceased by adding 1 ml of DNS Reagent. The test tube was boiled for 15 minutes and cooled immediately. 10 ml of distilled water was added and color intensity at 540 nm was determined .Amount of maltose released was determined by comparing the absorbance reading of the test enzyme at 540nm with the standard graph plotted by reacting the known concentration of maltose ranging from 0.05 to 0.5 mg/ml [10,12,13].

Culture Amendments for Optimum Production of Extracellular Amylase at Different pH

The aim of the study comprises of pH optimization for the production of extra cellular alpha-amylase from different fungal isolates .In this study the effect of different pH was seen for maximum amylase production to investigate that the culture conditions affects the enzyme production from microorganisms.

Enzyme Activity at Different pH

The enzyme activity was investigated by the study of effect of pH on extracellular amylase production. For this, the pH of growth medium is adjusted to 6.5,5 and 9 prior to sterilization.

RESULTS AND DISCUSSION

The main object of the work was to focus on the study of effect of pH on amylase production and isolation of the amylase producing fungi from soil, their characterization and crude enzyme assay. In the present study it was observed that the soil harbors a dominant amylolytic fungal population. *Aspergillus species, Rhizopus species* And *Fusarium species* was the most common and abundant amongst amylolytic soil mycotic flora. This finding is in agreement with the studies reported earlier [14]. Thus, soil is known to be a repository of amylase producers. The amylases from different isolates differ in their in their pH optimum, temperature optimum and other physicochemical properties depending on the species origin. The enzymes are very sensitive to pH and the selection of pH is very essential for the production of amylase. A pH regulatory system may be essential and cultivation pH can also affect fungal morphology.

Isolation of Fungal Solates

Several fungal species were isolated from the soil sample. These were identified on the basis of colony morphology. The fungal isolates from soil sample were identified on the basis of the morphological (microscopic and macroscopic) characteristics. These isolates were identified with the help of www.docterfungus.com.

The probable fungal isolates in the soil sample were *Aspergillus flavus, Aspergillus niger, Fusarium species* and *Rhizopus species.* Apart from these isolates *Unidentified species, Absidia species, Alternaria alternata Pythium species and Curvularia lunata* were also reported in the samples.

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Study of Amylase Production

Primary screening of the fungal isolates was carried out for amylase production by starch hydrolysis test. The four fungal isolates i.e. *Aspergillus flavus, Aspergillus niger, Fusarium species* and *Rhizopus species* were found to give positive results. Among these fungal isolates, *Aspergillus flavus, Aspergillus niger* and *Rhizopus species* were capable of producing excessive amylase. Though the amount of amylase produced was different in each case. But *Unidentified species* was found to be less amylase producer. These fungal isolates were then used for the crude enzyme production using fermentation method .

Study of Growth Kinetics of the Isolates

Study of the Growth parameter gives proper idea of the stationary phase so that suitable environment could be provided during fermentation procedure. The fungal isolates were grown at 28ºC temperature and pH 6.5. Growth kinetics statistics of the isolates was studied and it was observed that stationary phase reached between days 4 to 5.

Enzyme assay

A variable pattern of enzyme activity was observed from the enzymes of amylolytic soil mycotic flora. The different fungal isolates from soil sample showed different enzyme activity at different pH conditions. The amylase production by the isolates was observed in a range of pH (5, 6.5 & 9).

Table No.1: Identification of fungal isolates

S.No	Strain code	Macroscopic characteristics	Microscopic characteristics	Probable genera
1.	A	Colonies were powdery in texture and black in colour with conidial production. Reversed plate showed pale yellow coloured due to pigmentation	Hyphae septate and hyaline ichotomously branched vesicle, round, radiate head, conidia were black in colour (long and smooth). Conidiophores erect, simple and thick walled.	*Aspergillus niger*
2	B	Colonies were lime green to cream colour texture woolly on maturity and were dark brown.	Hyphae septate, vesicles globose. Conidiophores colourless and rough.	*Aspergillus flavus*
3	C	Smooth texture with radial ridge. Surface colour was found to be whitish.	Conidiophores were long and arise from aerial hyphae.	*Rhizopus species*
4	D	Grayish white at the beginning which later darkens and becomes greenish black or olive brown with a light border.	Septate brown hyphae. Conidiophores are also septate and brown in colour, occasionally producing a zigzag appearance. They bear simple or branched large conidia which have both transverse and longitudinal septation.	*Alternaria alternata*
5	E	Brown with white surrounding circular colony with cottony appearance reversed plate showed dark yellow coloured due to pigentation.	Septate hyphae	*Unidentified species*
6	F	White, ovate velvet like smooth yellow colony.	Hyphae are non septate	*Pythium species*
7	H	Mass green with white margin, cottony, smooth and flat colony	Hyphae wide, non septate sporangiophore,branched present on stolon columella	*Absidia species*
8	I	White pink colour smooth swarming and raised colony	Multicelled spores(conidia) are oval shaped and attached to conidiophores arising from a septate mycelium	*Fusarium species*

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The amylolytic activity in pH 6.5, pH 5 and pH 9 of *various fungal isolates can be seen from the table*. From this data it was inferred that *Aspergillus flavus, Aspergillus niger* and *Rhizopus species* were best amylase producers.

Table No 2: Starch Hydrolysis test

S.No.	Fungi	Inference
1	*Aspergillus flavus*	Abundant (+++)
2	*Aspergillus niger*	Abundant (+++)
3	*Fusarium species*	Average (++)
4	*Rhizopus species*	Abundant (+++)
5	*Unidentified species*	Below average (+)

Table No 3: Growth kinetics Study of different fungal isolates.

Sl. No.	Name	O.D. at 600 nm						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	*Aspergillus flavus*	0.01	0.2	0.3	0.42	0.83	0.89	0.92
2	*Aspergillus niger*	0.01	0.4	0.41	0.45	0.78	0.97	1
3	*Fusarium species*	0.04	0.47	0.53	0.6	0.72	0.8	0.92
4	*Rhizopus species*	0.01	0.05	0.07	0.14	0.2	0.38	0.45
5	*Unidentified species*	0.01	0.03	0.05	0.7	0.18	0.23	0.55

Table No 4: Enzyme assay at different pH

S.No.	Name of organism	Enzyme activity (U/ml)		
		pH 6.5	pH 5	pH 9
1	*Aspergillus flavus*	8.4	6.33	10.3
2	*Aspergillus niger*	8.3	5	18
3	*Rhizopus species*	9	6	15.66
4	*Fusarium species*	6.66	5.6	6.66
5	*Unidentified species*	7.3	9.6	6.66

CONCLUSION

The enzymes are very sensitive to pH and development of an optimal pH control strategy is helpful in obtaining higher protein productivity. The enzyme activity was observed for the isolates and it was inferred that selection of optimum pH is essential for the maximum enzyme production. These isolates can be used further to study the effect of other cultural parameters like temperature, substrate concentration, nitrogen source, carbon source, surfactants etc. for the maximum amylase production in fermentation broth. The crude enzyme extracts can be used further for different industrial application after purification.

Also mutagenesis based strain improvement methods can be applied for enzyme production. The application of recombinant DNA technology can reveal important information on the molecular basis of fungal enzyme production.

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