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Research Article

SCREENING AND PRODUCTION OF α-AMYLASE FROM *ASPERGILLUS NIGER* USING ZERO VALUE MATERIAL FOR SOLID STATE FERMENTATION

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ABSTRACT

The aim of the study was to screen the *Aspergillus niger* for producing α-amylase, Fungal α Amylase, an enzyme which holds tremendous industrial and pharmaceutical applications is screened in the present investigation using the zero value material for solid state fermentative study using the modified *Czapek Dox* Media. The 1, 4-a-D-Glucan gluconohydrolase production is studied on two different substrates, wheat bran and potato peel + banana peel to identify the best source for the enzyme production using different parameters like incubation time, pH, temperature, Nitrogen source and the Carbon source.

Keywords: α Amylase, *Aspergillus niger,* Zero value material, Solid state fermentation.

INTRODUCTION

The starch degrading enzyme has gained importance in starch processing industry to form simpler sugar constitutes[1,2] by hydrolysis of polysaccharides by cleaving (1→ 4) linkage of starch. The enzyme α-amylase has wide applications in textile, paper food, and fermentation[3]. A wide range of application have also been found in the pharmaceutical industry[4]. It is used to cure digestive disturbance[5]. α-Amylase also called as 1,4-α-glucan gluconohydrolase (EC 3.2.1.1) is a glycoprotein having a single polypeptide chain about 475 residues[6]. It has 2 free 3H groups and four disulphide bridges and contains tightly bound Ca^{2+}[7], hence called as metalloenzymes and in whose absence the enzyme is unable to function[8].

Amylase is produced by a variety of living organisms, ranging from bacteria to plants & humans. Bacteria and fungi secrete amylases to the outside of their cell to carry out extracellular digestion. When they have broken down the insoluble starch, the soluble end products such as (glucose or maltose) are absorbed into their cells. We have taken fungal isolates for screening and measuring the production of amylases because it is very easy to remove the fungal mycelium from the enzyme production medium and soil fungi are a very good source of amylase production.

Amylase production comprises of about 30% of the world enzyme production[9] . The main microbial sources for Amylase production are Bacillus spp[10] and Asperigillus spp[11,12]. Molds especially *Aspergillus niger* produces high amount of commercially used α amylase. Amylase of fungal origin was found to be more stable than the bacterial enzyme on a commercial scale[13]. Because of the non-fastidious nutritional requirement and ubiquitous nature of the fungal amylase, developing countries have are depending on *Aspergillus niger* for the enzyme production[13]. The present investigation aims at the screening and production of the fungal amylases using the modified method, czapex dox media plate method and enhancing the amylase production by standardizing the parameters by using two different zero value substrates, wheat bran and potato peel + Banana peel.

MATERIALS AND METHOD

Screening and Isolation of α Amylase Producers

The fungi used in this study was collected from contaminated field soil in outer region of Hyderabad, for screening of best enzyme producing fungi from soil sample the below mention media is used as nutrient source.

Composition of media

After taking care of all the above said measures, the fungal culture from 10^{-5}dilutions were now transferred into the Petri dishes containing the modified Czapex dox media. One control is maintained without supplementing the Petri dish with starch, with the following media composition; Glycerol-4gl^{-},Na_2HPO_4-2gl^{-}, NaCl-2gl^{-}, $FeSo_4$-1gl^{-},Agar-20gl^{-},starch-8gl^{-},added in Distilled H_2O of 1000ml and the pH is maintained at 7.2

S. No.	Chemical name	g/l
1	Glycerol	4
2	Na2HPO4	2
3	NaCl	2
4	FeSO4	1
5	Starch	8
6	Agar	20

After incubating the plates for 16-20hrs, the plates are added with 50ml of iodine (I_2) solution to observe the zone of hydrolysis. The I_2 solution is prepared by adding Iodine- 0.5%, KI-0.8%, Distilled H_2O-50ml. After 5 hrs, a clear blue-black zone is observed around the α amylase producers.

Identification of the fungus

Depending on the maximum zone of clearance, whose diameter implies the amylolytic activity, the organisms were subjected to the staining technique for the microscopic morphological identification of fungi. The typical stain used is the Lactophenol Cotton Blue which typically stains the mycelium and is viewed under microscope at 45X.

Composition of lactophenol cotton blue

S. No.	Name of the Chemical	Weight in g or ml
1	Cotton blue	0.5
2	Phenol crystals	200
3	Glycerol	400
4	Lactic acid	200
5	Dist.water	200

Enhancing the enzyme production by SSF using zero value material

In order to obtain high quantity enzyme, the culture showing the high zone of clearance indicating the maximum hydrolysis was inoculated into different substrates for the solid state fermentation. The two different substrates used are the Wheat Bran and the Potato Peel+ Banana Peel hence forth referred to as PBP. The substrate PBP should be washed several times with distilled water and then dried thoroughly. It should then be washed with 95% ethanol thrice and should be cut into fine tiny pieces. After ensuring proper drying, both the potato peel banana peel should be mixed thoroughly.

The medium for the enzyme production by SSF should be prepared as follows: (NH_2) SO4-5g, NaCl-8g, KH_2PO_4-2g, $NH4NO_3$-2g, NH_4NO_3-8g added to 1000ml Distilled H_2O at pH-6.8. The moisture content is to be adjusted at 43-81%. It is sterilized in an autoclave at 121°C for 15lbs for 15min.

100ml each of the above medium is transferred into two sterilized and autoclaved 250ml Erlenmeyer flask and add 15g of wheat bran and 15g of PBP respectively. To each of these flasks 1% of inoculum which showed the maximum starch hydrolysis zone was added and is incubated for 4-6 days.

Extraction of the enzyme

The extraction of the crude enzyme is done by adding 20ml of 0.1M phosphate buffer, pH-7 to each of the flasks and is spinned thoroughly on a rotary shaker at 180rpm (rotations per minute) for 10 min. This mixture is filtered through cheese cloth and centrifuged at 8000 rpm at 4°C for 20 min. The filtrate is used as crude enzyme for further investigation. The enzyme is assayed by DNS method [14,15] to know its activity.

Specific activity

One unit of amylase activity is defined as the amount of enzyme, which released 1µM of glucose per minute per milligram protein (U/mg).

In the next few steps of investigation we tried to estimate which substrate yields the maximum enzyme at different parameters like Incubation Time, pH, temperature, Nitrogen source, Carbon source.

Effect of incubation time

The effect of incubation time on the enzyme production is studied by allowing the organism to incubate in two different substrates for 1, 2, 3, 4, 5, 6, &, 7days at pH 7.4 at 30°C temperature.

Effect of pH

The effect of pH on the solid state fermentative production was carried out with the following pH 2, 3, 4, 5, 6, 6.5, 7and 7.5 incubated for 6 days at 30°C temperature.

Effect of temperature

The temperature effect was studied with the following temperatures 25,30,35,40,45,50,55°C at pH-7.4 for 6 days.

Effect of nitrogen source

The Nitrogen source taken for the present investigation is Beef extract, peptone, Sodium Nitrate and Ammonium chloride respectively to study the effect of enzyme production on 2 different substrates used at pH 7.4 incubated for 6 days at 30°C temperature.

Effect of carbon source

The effect of enzyme production by different carbon sources was studied by taking the following: Starch, Maltose, Xylose & glucose at pH 7.4 incubated for 6 days at 30°C temperature.

Determination of the molecular weight

The molecular weight of the enzyme was detected by subjecting the enzyme α amylase to SDS-PAGE after treating the enzyme with Ammonium Sulphate PPT.

RESULTS AND DISCUSSIONS

Screening of the microorganism

The chemicals are to be dissolved in 1000ml of distilled water. Incubate for 16 -20 hrs of incubation, 50ml of I_2 solution was added and the following numbers of colonies were observed.

S. No.	Hours of incubation	No of blue colonies formed
1	16	3
2	18	4
3	20	6

The results are a clear indicative that at 20hrs of incubation and with the above used media the maximum no of colonies (starch hydrolysis) were observed. This proves that the media used is more effective in producing the Amylase producers at a very low incubation time of 20hrs

Identification by lactophenol cotton blue

All the six colonies when subjected to Lactophenol cotton blue staining technique and it is observed that the colony which showed the high zone of clearance was found to be *Aspergillus niger.*

Solid state fermentation

To the below given media composition 15g of substrate materials are to be added and incubated for 4-6 days. Thereafter the enzyme is extracted by adding 20ml of 0.1M phosphate buffer, its activity is determined by DNS method.

Media composition for SSF

S. No.	Chemicals	g/l
1	NH_4SO_4	5
2	NaCl	8
3	KH_2PO_4	2
4	NH_4NO_3	8

Enzyme assay by DNS method

S. No.	Substrate	Specific activity U/mg
1	Wheat Bran	80
2	PBP	92

After the enzyme is assayed, the production of the enzyme and its activity is determined by subjecting the enzyme to different parameters like incubation time, temperature, pH, Nitrogen source and Carbon source.

Effect of incubation time

The culture *Aspergillus niger* is inoculated into two substrates and is incubated for 2, 3, 4, 5, 6, 7 &, 8 days at pH 7.4 at 30°C temperature.

The enzyme is extracted and the specific activity is determined in different substrates at different incubation days.

S. No.	Incubation days	Specific activity U/mg	
		Wheat Bran	PBP
1	2	2	-
2	3	6	5
3	4	15	20
4	5	12	26
5	6	10	18
6	7	7	16
7	8	4	12

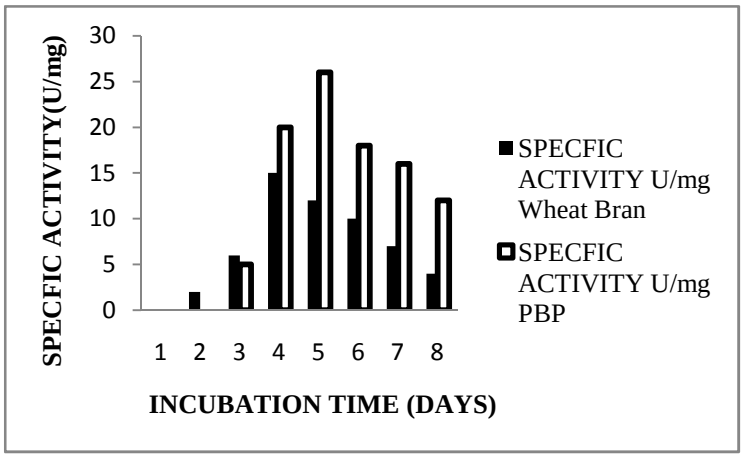
Maximum specific activity was observed at four days with wheat bran as 15 U/mg and maximum specific activity was observed at five days with PBP as 26 U/mg.

PBP at 2 Days of incubation

Wheat bran at 2 Days of incubation

PBP at 5 Days of incubation

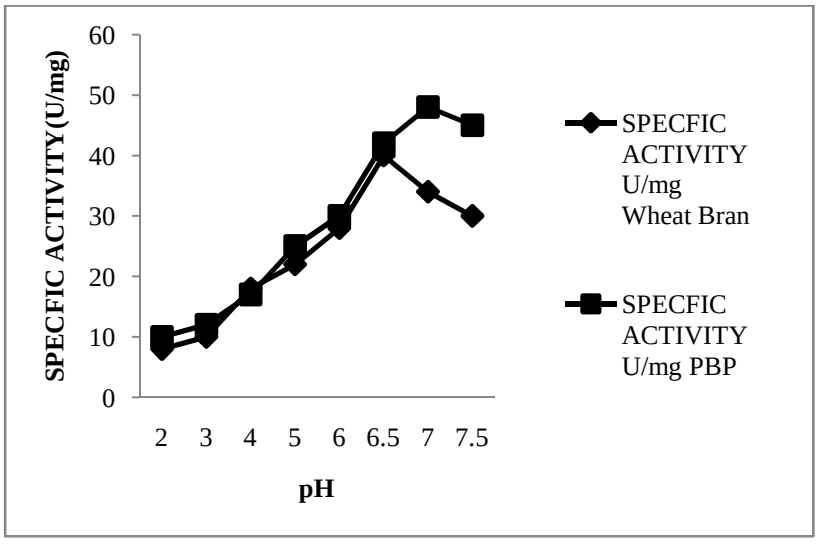
Wheat bran at 4 Days of incubation



Effect of pH

The pH effect on the enzyme production on two different substrates was studied by taking different pH gradient like 2, 3, 4, 5, 6, 6.5, 7, and 7.5 incubated for 6 days at30^{o}C.

S. No.	PH	Specific activity U/mg	
		Wheat Bran	PBP
1	2	8	10
2	3	10	12
3	4	18	17
4	5	22	25
5	6	28	30
6	6.5	40	42
7	7	34	48
8	7.5	30	45

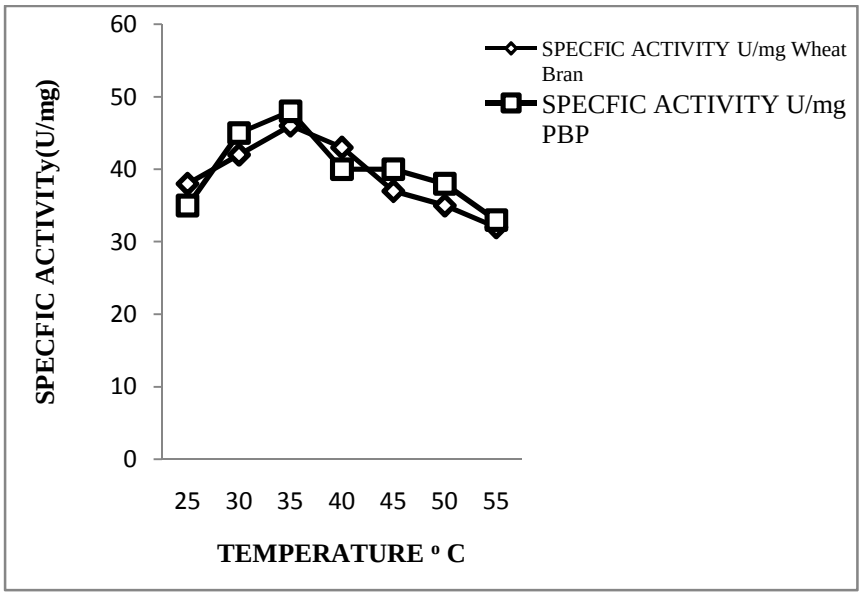


At the pH 6.5 the amylase specific activity was high for wheat bran and is found to be 40 U/mg and for PBP it is found to be 48 U/mg at pH7.

Effect of temperature

The temperature effect on the enzyme production is studied by inoculating the *Aspergillus niger* on two different substrates and studying the specific activity of the enzyme at 25,30,35,40,45, 50,55 ^{o}C respectively incubated for 6 days.

S. No.	P^{H}	Specific activity U/mg	
		Wheat Bran	PBP
1	25	38	35
2	30	45	42
3	35	46	48
4	40	43	40
5	45	37	40
6	50	35	38
7	55	32	33

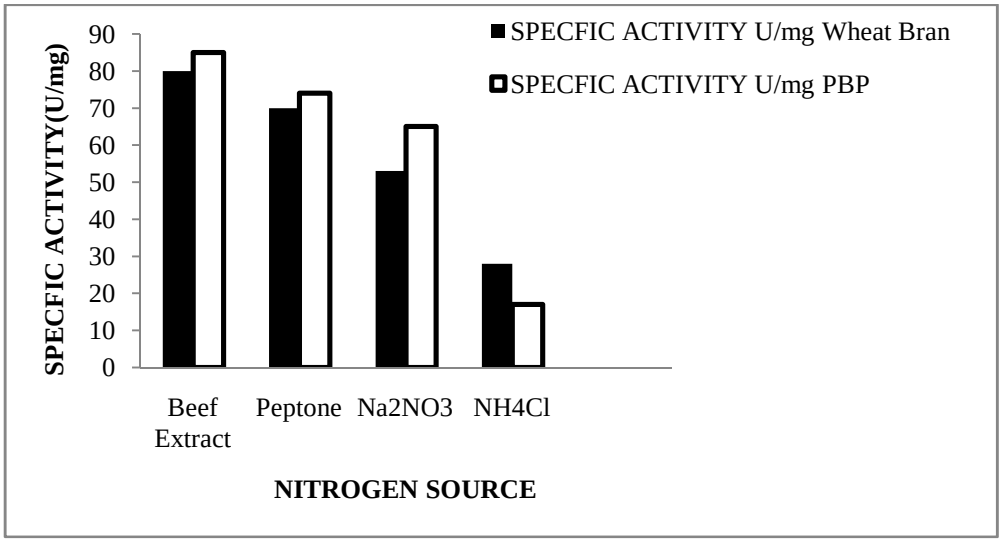


The specific activity was found to be maximum at 46U/mg at 35^{o}C for wheat bran and 48U/mg at 35^{o}C for PBP.

Effect of nitrogen source

The "N" effect on the enzyme production was studied by adding the following Nitrogen Sources.

S. No.	Nitrogen source	Specific activity U/mg	
		Wheat Bran	PBP
1	Beef extract	80	85
2	Peptone	70	74
3	Na_2NO_3	53	65
4	NH_4Cl	28	17

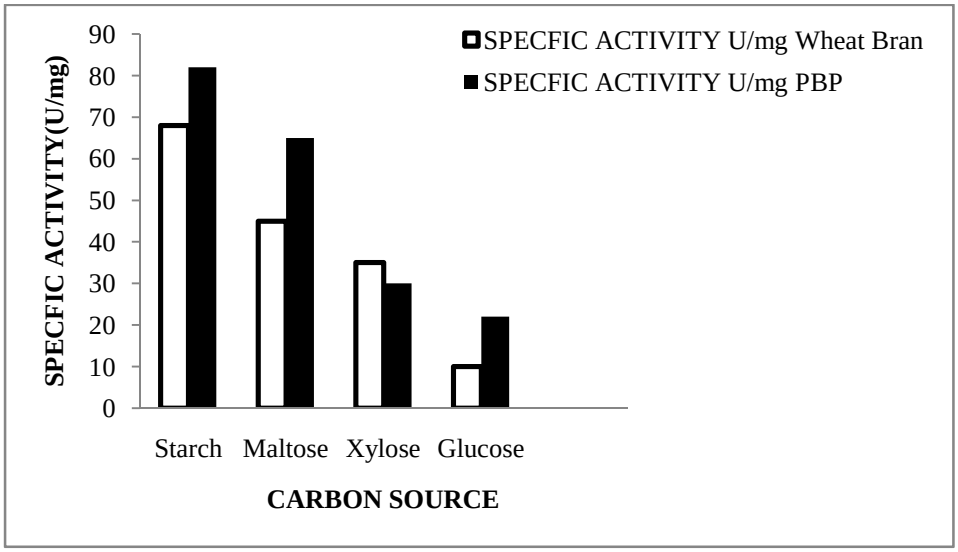


The best Nitrogen source for Wheat bran and PBP is found to be Beef extract with the specific activity of 85 U/mg and 80 U/mg respectively.

Effect of carbon source

The effect of enzyme production with the "C" source was studied taking the following:

S. No.	Carbon Source	Specific activity U/mg	
		Wheat Bran	PBP
1	Starch	68	82
2	Maltose	45	65
3	Xylose	35	30
4	Glucose	10	22



Starch has found to be the best Carbon source with the specific activity of 68 and 82 U/mg for wheat bran and PBP respectively.

Determination of the molecular weight

The molecular weight of the enzyme is found to be ≈ 43KD.

Lane I: Test Enzyme.

Lane II: Molecular Mass Markers.

From the above results it can be stated that the modified media used can produce the organism in much lesser time as reported and the results are also an indicative of PBP being a better substrate for the solid state fermentation than the wheat bran

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