Golden Research Thoughts
Impact Factor : 2.2052(UIF)
Volume-3 | Issue-11 | May-2014

STUDY OF AMYLASE PRODUCTION BY USING *ASPERGILLUS SPECIES* FROM SPOILED FRUITS.

C. B. Dalbhide.

P. G. Department of Microbiology, Yashwantrao Chavan College of Science, Karad, Dist. Satara. M. S. India

ABSTRACT

The study presents production of amylase by Aspergillus species in solid state fermentation using agro- industrial waste; wheat bran as a substrate. Attempt has been made to isolate Aspergillus species from native source i.e. spoiled fruits. Enzyme production was growth associated and maximum and minimum titres were obtained after 7 days of incubation at 27^{o}C. Amylase production was also carried out by using liquid state fermentation. The maximum yield obtained by submerged fermentation and surface culture fermentation. Solid state fermentation was found to produce the highest amount of enzyme amylase as compared with liquid state fermentation.

INTRODUCTION

Enzymes are biological catalysts. They are protein molecules produced in living cells having the very important function of catalyzing biochemical reactions that are necessary for the metabolic activity and thereby the living status of the cell. Enzymes are of plant, animal and microbial origin. Enzymes are among the most important products obtained for human needs through microbial sources (1). Enzymes can be produced commercially by using microorganisms for various purposes. Many types of industries, to aid in the generation of their products, utilize enzymes as in production of cheese, alcohol, bread etc. (2).

Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents Amylases are produced by a number of living organisms that include bacteria, fungi, plants etc. When insoluble starch is broken by amylase, soluble end products like glucose or maltose are formed

(3). Amylases are used in food, feed, textile and the pharmaceutical industries. In the food sector, they are mainly used for the liquefaction of starch where α-amylase disrupts the granular structure of starch and also causes thinning by breaking down amylase and amylopectin. The glucoamylases are acted upon where they saccharify starch (4). Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. This enzyme is also used for commercial production of glucose.

As amylase is extracellular in nature, recovery becomes easy. Morphology and physiology of molds enable them to penetrate and colonize various solid substrates (5). Amylase of fungal origin is found to be more stable than bacterial amylase as well as the yield potential is also higher. Fungi that are mainly used for commercial production of amylase are belonging to genus *Aspergillus* and *Rhizopus*. Among the mold species producing higher levels of amylase *Aspergillus oryzae* is used for commercial purpose. It can be isolated from various sources like soil, compost, spoiled fruits, contaminated food, indoor environments etc. *Aspergillus* causes a disease called black mold on various vegetables and fruits like onion, guava, peanuts, pomegranate etc. So, spoiled fruits having acidic pH are used for amylase production by solid state fermentation.

This study involves production of amylase by solid state fermentation using *Aspergillus spp.* because of its ubiquitous nature and non-fastidious nutritional requirement. Studies on solid state fermentation using local isolates belonging to fungi is carried out, for economic commercial production of amylase using cheapest raw materials like wheat bran and rice bran to search for economical process.

To prepare this extracellular amylase on a commercial scale, many attempts have been made to produce possible large amount of amylase by solid state fermentation with different types of raw materials.

MATERIAL AND METHODS

Collection of Samples: Three different types of spoiled fruits namely Guava (*Psidum guajava*), Pomegranate (*Punica granatum*), Apple (*Pyrus malus*) showing black moldy spots on the surface were collected from fruit market of Karad City. All fruits were separately kept into polythene bags. Bags were sealed tightly and were brought to laboratory and stored in refrigerator at 4^0C till further use.

Isolation and purification of *Aspergillus spp:* Isolation of *Aspergillus* cultures from each fruit samples was done by suspending small amount of moldy spot material with the help of sterile forceps in sterile saline aseptically. A loopful of each sample was streak inoculated on sterile Martin Rose Bengal agar medium and plates were incubated at 27^0C for 5 days. After incubation the suspected colonies of *Aspergillus* were further purified by re-streaking on Sabouraud's agar and maintained on Potato Dextrose Agar slants at refrigeration temperature. The fresh transfers were given after every 2 months.

Production of Amylase by Solid State Fermentation

a. Inoculum Preparation: The plates of Sabouraud's agar were spot inoculated with different isolates of *Aspergillus* from stock culture and incubated at 27^0 C for 3-4 days till the sporulation occurs. After heavy sporulation sterile 0.01 % Sodium Lauryl Sulfonate was poured on the

surface of plate and spore suspension was prepared by loose scrapping of spores with sterile nichrome wire loop. It was standardized using Neubauer's chamber to attain the number of 5 X 10^6 spores/ml.

b. Amylase Production: Amylase Production was done by using two kinds of substrates namely wheat bran and rice bran obtained from small scale flourmills and rice flake manufacturing industries in Satara City. The samples were in the form of finely ground powder and were collected separately in plastic bags. 50 gm of each of the brans was sterilized in separate vessels in an autoclave at 121^0C for 15 minutes and cooled to room temperature. Fine sand and gravels were also sterilized under same condition separately. Prepared inoculum was added into previously sterilized wheat bran and rice bran along with sand and gravels by vigorous mixing for aeration purpose. Appropriate amount of sterile distilled water was added to adjust the required moisture level. The mixture was then transferred to the specially designed containers with false perforated bottom. All the containers were incubated at 27^0C for 7 days.

c. Enzyme extraction: After the incubation, fermented cake (koji) was broken down into pieces and loaded on the funnel to obtain the extract of amylase produced, by giving 2 water washes. Each water washing gave a separate extract. Each extract was used as crude amylase and assayed for enzyme activity.

d. Enzyme Assay:

Enzyme activity was determined by DNSA method described by Mandels et al. (1976) using starch as the substrate. Quantitative determination of produced amylase enzyme was done by this method in which amount of reducing sugars was determined by reduction of dinitrosalicylic acid.

Standard Tables for Amylase Assay

Table No.1.1: Protocol A- Amylase Assay

Tube Type	Buffer (ml)	Substrate (ml)	Cofactor (ml)	D/w (ml)	Equilibrate at 37^0C for 10^{-1}	Enzyme (ml)	D/w (ml)	Incubate at 37^0C for 15^{-1}.	Inactivator (ml)	DNSA (Ml)	Boiling water bath for 10^{-1}	Abs at 540 nm
RC	2.5	2.5	1.0	-		-	0.5		0.5	0.5		
SC	2.5	2.5	1.0	-		-	0.5		0.5	0.5		540 nm
EC	2.5	-	1.0	2.5		0.5	-		0.5	0.5		
TC	2.5	2.5	1.0	-		0.5 (inactivator)	-		0.5	0.5		
Test	2.5	2.5	1.0	-		0.5	-		0.5	0.5		

Table No.1.2: Protocol B - Maltose Calibration Curve

Tube No	Buffer (ml)	Std. maltose solutio	D/w (ml)	Cofactor (ml)	D/w (ml)	Inactivator (ml)	DNSA (ml)		Abs. at 540 nm

		n (ml)							
0	2.5	0	2.5	1.0	0.5	0.5	0.5	Boiling water bath for 10^1	
1	2.5	0.1	2.4	1.0	0.5	0.5	0.5		
2	2.5	0.2	2.3	1.0	0.5	0.5	0.5		540 nm
3	2.5	0.3	2.2	1.0	0.5	0.5	0.5		
4	2.5	0.4	2.1	1.0	0.5	0.5	0.5		
5	2.5	0.5	2.0	1.0	0.5	0.5	0.5		
25	2.5	2.5	0.0	1.0	0.5	0.5	0.5		

The additions were done as per the protocol A. The reaction was terminated by the addition of 0.5 ml. DNSA solution. After stopping the reaction, the tubes were kept in boiling water bath for 10 minutes and then cooled. Absorbance was determined at 540 nm. The amount of maltose produced as a product was calculated by referring to standard maltose curve. The enzyme activity was expressed in terms of units. One unit of enzyme was defined as quantity releasing 1 μ mole of maltose from 1% starch in one minute at pH 4.6 obtained by using 0.16 M acetate buffer and at temperature 25^0C.

RESULTS AND DISCUSSION

In all 3 different fungal cultures from 3 different spoiled fruit samples were isolated and appropriately coded. The samples constitute one isolate each from spoiled Guava, Pomegranate and Apple. Table 2.1 represents the codes for fungal isolates obtained.

Table 2.1: Codes for fungal isolates obtained

Samples (Fruits)	Botanical Name	Isolate Code.
Guava	*Psidum guajava*	'Pgu'
Pomegranate	*Punica granatum*	'Pgr'
Apple	*Pyrus malus*	'Pma'

The size of colonies of all isolates ranged between 1.5-3.0 cm. in diameter viz. 'Pgu' to be 2.4 cm., 'Pgr' with 2.0 cm., 'Pma' with 3.0 cm. Colonies of all isolates were circular in shape, black coloured, with irregular margin .

Tentative identification was done by observing morphological characters that include septate hyphae, colour of conidiophores also colour and shape of conidia. The colour of conidiophore was colourless and brown for the isolates namely 'Pgr', 'Pma' And 'Pgu' respectively.

All the isolates showed circular shaped conidia. Colour of conidia was black and brown for isolates namely 'Pgr' and 'Pgu' respectively.

Microscopic observations of all 3 isolates showed the presence of foot cells, conidiophores, vesicle and sterigmata with blackish spores resembling *Aspergillus.*

On the basis of cultural and morphological characters, all 3 isolates appear to be belonging to genus *Aspergillus.* The amount of maltose released after breaking down of starch by amylase was determined from maltose calibration curve.

The results of amylase yield by solid state fermentation using seven different *Aspergillus* isolates and using two kinds of substrates namely wheat bran and rice bran are represented in table no.3 and 3.1 respectively.

Table No. 3- Results of solid state fermentation using wheat bran.

Sr. No.	Isolate Code	Enzyme Activity (U/50 gm.)		Total Enzyme Activity (U/50gm.)
		Fraction 1	Fraction 2	
1.	'Pgu'	161.88	149.96	311.34
2.	'Pgr'	99.31	92.36	191.67
3.	'Pma'	106.26	79.45	185.71

Table No.3.1- Results of solid state fermentation using rice bran.

Sr. No.	Isolate Code	EnEnzyme Activity (U/50gm)		Total Enzyme Activity (U/50 gm.)
		Fraction 1	Fraction 2	
1.	'Pgu'	82.43	70.51	152.94
2.	'Pgr'	91.37	70.51	161.88
3.	'Pma'	72.50	69.52	142.02

While the results of amylase yield by submerged fermentation and surface culture fermentation using seven different *Aspergillus* isolates are presented in table no.4

Table No.4- Results of Submerged Fermentation and Surface Culture Fermentation

Sr. No.	Isolate Code	Total Enzyme Activity (U/50 gm.)	
		Submerged Fermentation	Surface Culture Fermentation
1.	'Pgu'	72.50	62.56
2.	'Pgr'	70.51	50.65
3.	'Pma'	102.29	83.42

It can be seen from the tables that the amount of amylase produced by four different *Aspergillus* isolates on wheat bran and rice bran was variable.

On wheat bran isolate 'Pma' showed comparatively lower yield (185.71U / 50gm.). Isolate 'Pgu' gave comparatively maximum yield (311.34 U / 50gm.). The yield given by remaining different isolates were in between this range.

On rice bran isolate 'Pma' gave comparatively lower yield (142.02U / 50gm.) and isolate 'Pgr' gave comparatively higher yield (161.88U/50gm.) Remaining isolates produce amylase in between this range.

It can be seen from table no.4 that the quantity of amylase produced by three different *Aspergillus* isolates by submerged fermentation was variable.

Isolates 'Pgr' gave lowest yield of amylase (70.51U / 50gm.) while the highest yield of amylase was produced by isolates 'Pma' (102.29 U / 50gm.) Remaining isolates produce amylase in between this range.

It can be seen from table no.4 that the quantity of amylase produced by four different *Aspergillus* isolates by surface culture fermentation was variable.

Isolate 'Pgr' gave lowest yield of amylase (50.65 U / 50gm.) while the highest yield of amylase was produced by isolate 'Pma' (83.42 U / 50gm.) Remaining isolates produce amylase in between this range.

It can also be seen from tables that solid state fermentation gave superior yields as compared to submerged fermentation and surface culture for all 3 isolates of *Aspergillus.*

Among the two substrates, wheat bran and rice bran used for solid state fermentation, wheat bran was found to be the best substrate for amylase production for all 3 isolates of *Aspergillus* than the rice bran.

All the three isolates of Aspergillus viz 'Pgu', 'Pgr', 'Pma' produced amylase by solid state fermentation and submerged fermentation and surface culture method. All isolates were variable with respect to amount of amylase by different fermentation methods.

Among the 3 fermentation process, solid state fermentation was found to produce the highest amount of enzyme amylase as compared with submerged fermentation and surface culture fermentation.

Among the 3 isolates of *Aspergillus spp.*, isolate 'Pgu' produced the maximum yield of amylase through solid state fermentation using wheat bran as substrate (311.34U/50 gm). While isolate 'Pgr' gave the maximum yield of amylase through solid state fermentation using rice bran (161.88U/50 gm.). Thus wheat bran was found to produce the maximum yield of amylase than rice bran.

Among the two substrates, wheat bran and rice bran used for solid state fermentation, wheat bran was found to be the best substrate for amylase production for all 3 isolates of *Aspergillus* than the rice bran.

CONCLUSION

a. All the three isolates of *Aspergillus* viz 'Pgu', 'Pgr' And 'Pma' produced amylase by solid state fermentation and submerged fermentation and surface culture method.
b. All isolates were variable with respect to amount of amylase by different fermentation methods Among the 3 fermentation process, solid state fermentation was found to produce the highest amount of enzyme amylase as compared with submerged fermentation and surface culture fermentation.

c. Among the 3 isolates of *Aspergillus spp.*, isolate 'Pgu' produced the maximum yield of amylase through solid state fermentation using wheat bran as substrate (311.34U/50 gm). While isolate 'Pgr' gave the maximum yield of amylase through solid state fermentation using rice bran (161.88U/50 gm.). Thus wheat bran was found to produce the maximum yield of amylase than rice bran.
d. Among the two substrates, wheat bran and rice bran used for solid state fermentation, wheat bran was found to be the best substrate for amylase production for all 3 isolates of *Aspergillus* than the rice bran. Among the two substrates,
e. Wheat bran and Rice bran used for solid state fermentation, wheat bran was found to be the best substrate for amylase production for all 3 isolates of *Aspergillus* than the rice bran.

REFERENCES

1. Prescott S.C. and Dunn C.G. 1949 ^{4th} edition 'Mold enzyme preparation, uses and products' In' Industrial Microbiology' CBS publication and distributors, New Delhi 110 032.
2. Prescott S.C. and Dunn C.G. 1949 ^{4th} edition 'Mold enzyme preparation, uses and products' In' Industrial Microbiology' CBS publication and distributors, New Delhi 110 032.
3. Mitchell D. A.and Lonsane B. K., 1990. General principles of solid state fermentation. Publication of oxford London.
4. Prescott S.C. and Dunn C.G. 1949 ^{4th} edition 'Mold enzyme preparation , uses and products' In' Industrial Microbiology' CBS publication and distributors , New Delhi 110 032.
5. Jarun Chutmanop and et.al.2008. "Protease production by *Aspergillus oryzae* in solid state fermentation using agroindustrial substrates." Chem Technol Biotechnol, 83:1012-1018.