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Research Article

PRODUCTION OF AMYLASE BY SOLID STATE FERMENTATION FROM AGRO-INDUSTRIAL WASTES USING *Bacillus subtilis*

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ABSTRACT

In the present investigation, six isolates of *Bacillus subtilis* were isolated from soil of different areas in Nagpur district for production of amylase. All isolates were aerobic with straight rods, motile, endospore forming, gram positive, strongly catalase positive and indicative of *Bacillus subtilis*. The maximum clear zone surrounding the bacterial growth was observed in Isolate No. B.S-1 and B.S-3 indicating starch hydrolysis. The agro wastes used as substrates were Wheat Bran, Mustard Oilseed Cake and Gram Husk respectively. Crude enzyme obtained by using Wheat Bran as a substrate, showed maximum results for enzyme activity as compared to Mustard Oilseed Cake and Gram Husk. The results of the assay showed that bacterial isolate B.S-3 showed the highest enzymatic activity of 177mg/ml/min with wheat bran as substrate. *Bacillus* isolate B.S-3 and B.S-1 showed highest enzyme activity in presence of Wheat bran (177mg/ml/min, 151mg/ml/min) followed by Gram husk (126mg/ml/min, 71mg/ml/min) and low yield with Mustard oilseed cake by both isolates (91mg/ml/min).

INTRODUCTION

Enzymes are biological catalysts which are an indispensible component of biological reactions. The use of chemical catalysts has been followed for a very long time. Chemical catalysis though widely used was very cumbersome. The disadvantages that this method poses include need for high temperature and pressure for catalysis and the moderate specificity. These limitations were overcome by the use of enzymes. Enzymes work at milder conditions when compared to that required by chemical catalysts for operation. Also enzymes are highly specific and catalyze reactions faster than chemical catalysts [1]. Enzymes are now being used in various sectors of industry. They are used in detergents, paper industry, textile industry, food industry and many others industrial applications. Enzymes have been in use since ancient times [2] and they have been used in saccharification of starch, production of beverages like beer, treatment of digestive disorders and production of cheese from milk[3]. Among the many enzymes that are widely used α-Amylase has been in increasing demand due to its crucial role of starch hydrolysis and the applications of this hydrolytic action. The following sections elaborate on the types of amylases and their roles in enzymatic reactions.

Amylase is an enzyme that catalyses the hydrolysis of starch into sugars. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Foods that contain large amounts of starch but little sugar, such as rice and potatoes, may acquire a slightly sweet taste as they are chewed because amylase degrades some of their starch into sugar. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolysing α-1, 4-glycosidic bonds of amylose, amylopectin, glycogen and their degradation products. They act by hydrolysing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved. [4]

The pancreas and salivary gland make amylase (alpha amylase) to hydrolyse dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose to supply the body with energy. Plants and some bacteria also produce amylase. As *diastase*, amylase was the first enzyme to be discovered and isolated. Specific amylase proteins are designated by different Greek letters. All amylases are glycoside hydrolases and act on α-1, 4-glycosidic bonds. [4]

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α-Amylase can be isolated from plants, animals or microorganisms. The enzyme has been isolated from barley and rice plants [5]. It has been found that cassava mash waste water is a source of α-Amylase which is active in wide range of pH and temperature [6]. In the recent past, there has been extensive research on microbial production of α-Amylase. There are 2 major reasons for the increasing interest in microbial sources: 1) The growth of microorganisms is rapid and this will in turn speed up the production of enzyme. Microorganisms are easy to handle when compared to animals and plants. They require lesser space and serve as more cost effective sources. 2) Microorganisms can be easily manipulated using genetic engineering or other means. They can be subjected to strain improvement, mutations and other such changes by which the production of α-Amylase can be optimized.[7]

The microorganisms can be tailored to cater to the needs of growing industries and to obtain enzymes with desired characteristics like thermostability for example. Thermostable α-Amylases are desired as they minimize contamination risk and reduce reaction time, thus saving considerable amount of energy. Also when hydrolysis is carried out at higher temperatures, the polymerization of D-glucose to iso-maltose is minimized [8].

α-Amylase is produced by several bacteria, fungi and genetically modified species of microbes. The most widely used source among the bacterial species is the *Bacillus spp*. *B. amyloliquefaciens* and *B. licheniformis* are widely used for commercial production of the enzyme. Other species which have been explored for production of the enzyme include *B.cereus* and *B. subtilis* to name a few. α-Amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* show promising potential in a number of industrial applications in processes such as food, fermentation, textiles and paper industries [8,9]. *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-Amylase.

Fungal sources of α-Amylase are confined to terrestrial isolates, mostly to *Aspergillus* species and to only few species of *Penicillium*, *P. brunneum* being one of them[10]. *Penicillium fellutanum* has been used in the recent past to produce α-Amylase by submerged fermentation [10]. *Penicillium expansum* MT-1 has been used to produce the enzyme by solid state fermentation. Here, Loquat kernels were used as the substrate for growth of the fungi [11]. *Penicillium chrysogenum* was used as the microbial source for producing amylase by solid state fermentation using various substrates such as, corncob leaf, rye straw, wheat straw and wheat bran [12]. The fungal source used predominantly for commercial production of α-Amylase are the strains of *Aspergillus spp*. *Aspergillus oryzae, A. niger and A. awamori* are most commonly used species for commercial production among several others. *Aspergillus fumigatus* has been employed for production of the enzyme by submerged fermentation technique [13].

MATERIALS AND METHODS

Collection of Samples

The different soil samples for the isolation of amylase producing microorganisms were collected from the local area of Manish Nagar and Maharajbagh area of Nagpur district.

Isolation of bacteria from soil samples

The different soil samples collected were allowed to air dry. This was followed by serial dilution were 1 gram of each soil sample was added in 10 ml of sterilized distilled water. Further 1 mL of the suspension was removed with a sterile pipette and transferred to a 9 mL distilled water blank. The dilution was repeated seven times, each time with 1 mL of the previous suspension and a 9 mL distilled water blank. Labelling of the tubes were done sequentially starting from 10^{1}-10^{-9}.This resulted in serial dilutions of 10^{-1} through 10^{-9} grams of soil per mL. 100 µl from the dilution was spreaded on Hichrome Bacillus Agar plates. Plates were incubated in inverted position at 37 °C for 24 hours. After 24 hours, the plates were observed for colonies. [14]

Identification of the Bacillus species

The isolated colonies were identified on the basis of morphology by performing Gram staining and motility, biochemical by testing sugar fermentation using Glucose, Lactose, Mannitol, Maltose, Sucrose, IMViC Test, Catalase test, Oxidase test, Triple Sugar Iron (TSI) test, Urease test and cultural characteristics by inoculating bacteria on Hichrome Bacillus Agar.

Production of Enzyme Using Solid State Fermentation

Screening of Bacterial Isolates

Primary screening of bacterial isolates for production of alpha amylase was performed by the starch agar plate method. A fresh (16 to 18 hours) pure culture of test bacteria as an inoculation source was used. A single isolated colony was picked and single central streaked on the surface of the starch agar medium. A single starch agar plate was divided into four quadrants for four different inoculations followed by incubation of the plates for 24 to 48 hours at 37^{o}C in an incubator.[15]

Starch Hydrolysis Test

After proper inoculation and incubation, the surface of the starch agar plate was flooded with Gram's iodine solution and observed for the clear zone. The strains that showed the biggest zone of clearance in starch hydrolysis were selected for the production in Solid State Fermentation.[16]

Inoculum preparation

The selected bacterial strains that gave positive results during primary screening were selected for the enzyme production. For the preparation of the inoculum, these organisms were inoculated in sterile nutrient broth [consisting of (g L^{-1}): peptone-5; Beef extract-3; NaCl-5] followed by incubation at 37^{o}C for 24 hours to get a standardized inoculum (0.5 OD at 600nm with 3.5 x 10^{5} cfu/ml). [17]

Substrate

Three different types of agro-industrial wastes were used as substrate viz., Wheat bran, Mustard Oilseed cake and Gram husk. These substrates were procured from the local market and agricultural areas of Nagpur, air dried and powdered to obtain a particle size of 1.0 to 2.0 mm. Solid State Fermentation was performed with all the three substrates and their enzyme production was checked by assay.

Solid State Fermentation

The experiments were conducted in 250 ml Erlenmeyer flasks containing 25 g of the substrate impregnated with 100 ml of sterile Mineral Salt Medium (MSM) containing (%): [KH_2PO_4 - 0.1, NaCl- 0.25, $MgSO_4.7H_2O$- 0.01, $CaCl_2$- 0.01]. The moisture level of the content in the flasks was adjusted by addition of autoclaved distilled water followed by tightly sealing the mouth of the flasks with cotton plugs. The flasks were autoclaved and inoculated with 1ml of the prepared inoculum, thoroughly mixed and incubated at 37^oC for 5 days.[17]

Extraction of the Enzyme Produced

After incubation, the enzyme from the fermented bacterial bran was extracted. The slurry was squeezed with the help of muslin cloth. The extracts obtained were pooled and centrifuged at 4°C for 15 min at 10,000 rpm to separate the small particles of different substrates, cells and spores. The brown, clear supernatant was used in enzyme assay.

Determination of Enzyme Assay

The estimation of amylase activity was carried out according to the DNSA (3, 5 di-nitro salicylic acid) method. 1 ml of 1% starch was incubated with different crude enzyme extracts and 1ml of phosphate buffer (pH 7.0). The reaction mixture was incubated at 50^oC for 30 min. The reaction was stopped by adding 0.5 ml of DNS followed by keeping in boiling water bath for 5 minutes. The absorbance was read at 540nm using a Spectrophotometer, against maltose as the standard. One unit of enzyme activity is defined as the amount of enzyme, which releases 1µmole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min).[17]

RESULTS AND DISCUSSION

Isolation of Bacillus subtilis

On HiChrome Bacillus Agar plates, after incubation of 24 hours, species of *Bacillus subtilis* were isolated. These species were isolated and identified on the basis of their colour and colonies. *Bacillus subtilis* showed green coloured colonies on HiChrome Bacillus Agar plates.

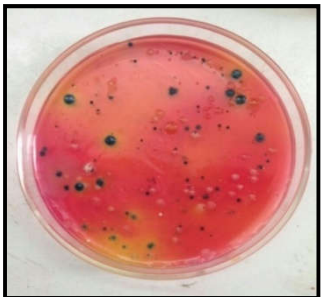

Figure No 1 Isolation of *Bacillus species* on HiChrome Bacillus Agar

Figure No 2 Isolated colonies of *Bacillus subtilis on* HiChrome Bacillus Agar slants

Isolation of Bacillus subtilis on Bacillus Differentiation Agar plates

On Bacillus Differentiation Agar plates, after incubation for 24 hours, *Bacillus subtilis* was isolated and identified on the basis of colour and colony morphology. *Bacillus subtilis* gave slightly transparent, yellow coloured colonies on Bacillus Differentiation Agar plates. For the identification of *Bacillus subtilis*, Gram staining was performed which showed that the organism is Gram positive and rod shaped. These isolates were further tested for their biochemical characteristics.

Biochemical Tests

The results of biochemical characteristics of the isolates of *Bacillus subtilis* are given in the following table:

Table No. 1 Observation of biochemical tests of *Bacillus* isolates

Samples	Indole	MR	VP	Citrate	Urease	TSI		
						Acid	Gas	H_2S
B.S-1	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve
B.S-2	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve
B.S-3	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve
B.S-4	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve
B.S-5	-ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve
B.S-6	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve

B.S.S- *Bacillus subtilis* (Isolate)

Table No. 2 Observation of Sugar fermentation of *Bacillus subtilis*

Samples	Glucose		Sucrose		Mannitol		Lactose	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
B.S-1	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
B.S-2	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
B.S-3	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
B.S-4	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve
B.S-5	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve
B.S-6	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve

B.S - *Bacillus subtilis* (Isolate)

In the present investigation, six isolates of *Bacillus* sp. were isolated from soil of different areas in Nagpur district. All isolates were aerobic with straight rods, motile, endospore forming, gram positive, strongly catalase positive and indicative of *Bacillus species* (*Bacillus subtilis*) on the basis of morphology and biochemical characterization which were designated as B.S-1 to B.S-6. [Table No. 1 & 2]

Screening of Bacterial isolates

After incubation of the streaked starch agar plates for 24 hrs, the surface of the plates were flooded with Gram's iodine solution and observed for clear zone. The results were

immediately recorded. The appearance of maximum clear zone surrounding the bacterial growth was observed in Isolate No. B.S-1 and B.S-3 indicating starch hydrolysis by the organism due to its production of the extracellular enzyme. The clear zone appeared yellow initially (from the iodine) and became progressively lighter yellow and then clear indicating that starch has been hydrolyzed. These isolates were selected for further use in the production of the enzyme amylase by Solid State Fermentation. On the other hand, the sample B.S-2, B.S-4, B.S-5, B.S-6 failed to showed the clear zone of hydrolysis, these isolates were unable to produce the extracellular enzyme. Out of the six bacterial isolates, two bacterial isolates i.e; B.S-1 and B.S-3 showed positive results and maximum clear zone of hydrolysis during screening. These isolates were further used for the production of amylase by Solid State Fermentation using agro wastes as substrates.

Determination of activity of enzyme

Table No. 3 Study of Alpha Amylase Activity of all the Substrates

Sr.No	Enzyme (ml)		1% Starch	DNS (ml)	O.D at 540 nm	Enzyme Activity mg/ml/min
Blank	**0**		**2.5**	**0.5**	**00**	**00**
Crude Enzyme (Wheat Bran)	Bacterial Isolate B.S -1	0.5	2.5	0.5	0.68	151
	B.S -3	0.5	2.5	0.5	0.80	177
Crude Enzyme (Mustard Oilseed Cake)	Bacterial Isolate B.S -1	0.5	2.5	0.5	0.41	91
	B.S -3	0.5	2.5	0.5	0.50	111
Crude Enzyme (Gram Husk)	Bacterial Isolate B.S -1	0.5	2.5	0.5	0.57	126
	B.S -3	0.5	2.5	0.5	0.32	71

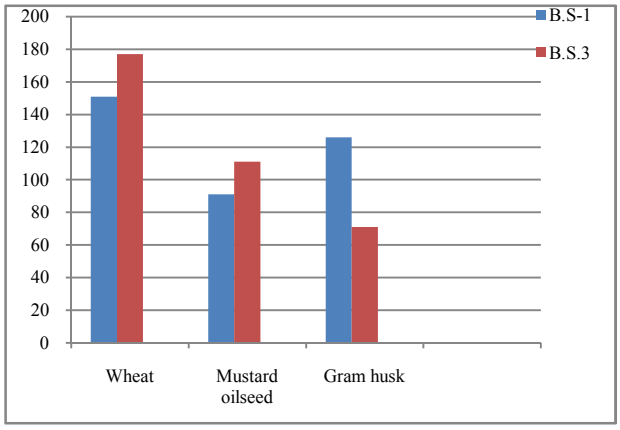

Figure No. 3 Comparative Study of Alpha Amylase Activity of all the Substrates

By determining the activity of amylase, the above graph has been plotted indicating the total enzymatic assay of the crude enzyme amylase obtained by Solid State Fermentation. Here, the agro wastes used as substrates were Wheat Bran, Mustard Oilseed Cake and Gram Husk respectively. Crude enzyme obtained by using Wheat Bran as a substrate, showed maximum results for enzyme activity as compared to Mustard Oilseed Cake and Gram Husk. The results of the assay showed that bacterial isolate B.S-3 showed the highest enzymatic activity of 177mg/ml/min with wheat bran as substrate. In the present study, the highest enzyme activity (177mg of maltose per ml of enzyme per minute) was observed from the extract obtained by using Wheat bran by B.S -3 isolate. Both the isolates showed appreciable amount of enzyme production such as 177mg of maltose per ml of enzyme per min, 151mg of maltose per ml of enzyme per min by using Wheat bran as a substrate using isolates B.S-3 and B.S-1 respectively. The enzyme activity of the crude enzyme obtained with Gram Husk as a substrate using isolate B.S-1 showed highest activity i.e. 126 mg/ml/min. However, overall, lower activity of enzyme amylase was observed when Mustard Oilseed Cake was used as a residue with both isolates of *Bacillus subtilis.* [Table & Figure No. 3]

Bacillus isolate B.S-3 and B.S-1 showed highest enzyme activity in presence of Wheat bran (177mg/ml/min, 151mg/ml/min) followed by Gram husk (126mg/ml/min, 71mg/ml/min) and low yield with Mustard oilseed cake by both isolates (91mg/ml/min).

B.S.-3 showed highest production of amylase in Mustard oilseed cake (111mg/ml/min).It can be concluded that Wheat bran can be a very good source of residue for the production of alpha amylase and can be helpful in reducing the production cost. [Table & Figure No. 3]

The isolates showed good growth on Mustard Oilseed Cake and so it was selected to be used as a substrate for amylase production. But an increase in the substrate did not increase the enzyme yield and the turbidity of substrate interfered with the optical density. On the other hand, enzyme activity (126 mg of maltose per ml of enzyme per minute) showed by *Bacillus subtilis* isolate B.S -1 using Gram husk as a substrate was also reported, indicating it to be a good source of substrate for the production of amylase. [Table & Figure No. 3]

Kindu Nibret Tsegaye and Amare Gessesse produced amylase under solid state fermentation using isolate W74 which was a potential strain for amylase production with wheat bran as a substrate. The extracted enzyme showed optimum enzyme activity at 70^0C, pH 6.5 and was found to have a better activity between moderately acidic and neutral pH values. This, to an extent proves that the utilization of Wheat bran as a solid substrate has a greater advantage and its low cost could lead to large scale production of amylase enzyme.[18]

However, studies done by Rajshree Saxena and Rajni Singhshowed that the best productivity was observed with Mustard Oilseed Cake with which about 5166 U/g of enzyme was produced which was much high compared to 1233 U/g of

enzyme produced by using Wheat Bran. This is slightly in contrast to the results obtained in the present study as according to various authors, the production of amylase by Solid State Fermentation using Wheat bran, Rice husk, Gram husk, Mustard Oilseed cake etc were affected by culture conditions such as moisture, pH and temperature. [17]

Investigations done on production and optimization of amylase using *Bacillus* by Vasantha Raj and N. Hemashenpagams howed maximum activity of amylase (41.21mg/ml) within 48 hrs of incubation with Wheat Bran as a substrate. In the present study, a significant production of amylase (activity being 177mg/ml/min) was observed using the same substrate when incubated for 120 hrs at 37^0C. [13]

The study done by Emaan Zakaria Gomaaconfirmed that Wheat bran is an ideal substrate for amylase production giving promising results due to the presence of sufficient nutrients and its ability to remain loose even under moist conditions, thus, providing a large surface area. Among five substrates screened, wheat bran (WB) gave the highest enzyme production reaching 74.60 and 56.66 U/g for *B. subtilis* and *B. cereus*, respectively. [19]

CONCLUSION

The present study showed that both the isolated amylolytic bacterial strains B.S-3 and B.S-1 produced appreciable amount of amylase enzyme by employing Solid State Fermentation using Wheat bran. Isolate B.S-1 produced variable amount of the enzyme with another substrate Gram husk. These isolates can thus be exploited for the synthesis of α- amylase which can have several industrial applications. In addition, wheat bran turned out to be a better substrate followed by Gram husk and Mustard oilseed cake, thus allowing itself to be a cheaper alternative to obtain amylolytic enzymes.

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