

## Use of Agro Industrial Residues for the Production of Amylase by *Penicillium* sp. for Applications in Food Industry

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### Abstract

Glucoamylase production has been investigated by solid state fermentation using agro-industrial residues, rice bran, wheat bran and banana peel by *Penicillium* sp. Out of the different agricultural residues tested for enzyme production, rice bran yielded the maximum glucoamylase activity ( $10.29 \pm 0.07$  Units/ml), protein content ( $1.22 \pm 0.0014$  mg/ml) and specific activity (8.4 Units/mg protein) with *Penicillium* sp. after 7th day of fermentation as compared to other agro residues. Media supplementation with carbon and nitrogen sources enhances the enzyme activity. For maximum production of enzyme, sucrose ( $13.70 \pm 0.77$  Units/ml) (1% mass level) and yeast extract ( $14.41 \pm 0.07$  Units/ml) (1% mass level) as carbon and nitrogen source respectively were found optimum on rice bran with *Penicillium* sp. Optimum enzyme activity was observed at 90°C, pH 9. The partial purification of enzyme from rice bran by *Penicillium* sp. was done with 60% ammonium sulphate precipitation showed maximum enzyme activity. Enzyme hydrolysis showed maximum activity (3.69 Units/ml) with potato starch from rice bran by *Penicillium* sp.

**Keywords:** Glucoamylase; *Penicillium* sp.; Solid state fermentation; Agro residues

### Introduction

Recent discoveries on the use of microorganisms as sources of industrially relevant enzymes have led to an increased in the application of microbial enzymes in various industrial processes. Nowadays, the use of microorganism as biotechnological source of industrially relevant enzymes has interest in exploration of extracellular enzymatic activities in several microorganisms. Amylases are used in dextrose production, in the baking industry, in the brewing of low-calorie beer and in whole gain hydrolysis for the alcohol industry [1]. The amylase family has two major classes, namely amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3). Alpha-amylase are extracellular enzymes which hydrolyze starch into maltose, glucose and maltotriose by cleaving the 1,4-D-glucosidic linkages between adjacent glucose units in the linear amylose chain [2].

Glucoamylase (GA) hydrolyses single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner and produces glucose as the sole end-product from and related polymers. Unlike alpha amylase, most glucoamylases are also able to hydrolyse the 1,6-linkages at the branching points of amylopectin, at a lower rate than 1,4-linkages [3]. Alpha amylase is secreted as a primary metabolite from many microorganisms like several bacteria, fungi and its secretion is growth associated [4]. It is an important group of enzymes in starch processing. They are second to the proteases in worldwide distribution and sales among industrial enzymes.

Production of glucoamylase (GA) is performed mostly by two methods, Solid State or Solid-Substrate Fermentation (SSF) and Submerged Fermentation (SMF) [5]. SSF has many advantages over SMF, such as simple technique, superior productivity, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up and reported to be the most appropriate process for developing countries [6]. Selection of a suitable microorganism is one of the most important parameters in SSF. Several groups of microorganism such as filamentous fungi group can grow on solid substrates.

Agro-industrial residues are considered as the best substrates for the SSF processes [7]. The use of agricultural by products is suitable with low cost fermentation medium for production of  $\alpha$ -amylase [8].

Fungal species such as *Aspergillus*, *Rhizopus* and *Penicillium* are considered to be the chief producers of amylase [9,10]. Among large number of agro-industrial wastes (wheat bran, wheat straw, rice bran, rice husk, rice straw, sago hampas, grapevine trimmings dust, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch and bagasse) [11] are available as substrate for the production of enzyme.

In the present study glucoamylase production has been studied with a natural isolate of the fungus *Penicillium* sp. using the rice bran, wheat bran and banana peel as growth substrates.

### Methods

#### Agro residues preparation

Rice bran and wheat bran were purchased from local market of Haryana. The ripen banana purchased from local market was peeled off and pulp (fruit) was separated. The peels were cleaned by washing in tap water and were cut into small pieces followed by homogenization in a blender. Little amount of water was added to facilitate the homogenization. The paste of banana peel was autoclaved to remove microbial contamination.

#### Microorganism and its maintenance

*Penicillium* sp. was propagated on Potato Dextrose Agar (PDA) medium. Slants were grown in test tubes at 30°C for 5 days and stored at 4°C. Sub culturing was done after every 15 days.

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## Preparation of basal media

Agro residues wheat bran (WB), Rice Bran (RB) and Banana Peel (BP) moistened with mineral salt solution having composition (mg/gds)  $(\text{NH}_4)_2\text{SO}_4$  4,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.02,  $\text{K}_2\text{HPO}_4$  1.4 and  $\text{KH}_2\text{PO}_4$  0.6. The pH of the media was adjusted to 7.0 [5].

## Enzyme production and extraction

The production media contained 10 g of solid substrate and 20 ml mineral solution in 250 ml Erlenmeyer flasks initially maintained at pH 7. The media were inoculated with 2 ml of inoculum having  $10^6$  spores/ml collected from 72 h grown culture of *Penicillium* sp. at  $28 \pm 2^\circ\text{C}$ . Inoculated production media were incubated under static conditions at  $28 \pm 2^\circ\text{C}$  and enzyme production was checked after every 24 h for 5 d. Enzyme was extracted in 100 ml, 0.1 M sodium acetate buffer on a rotary shaker at 100 rpm for 60 min. The content was filtered through muslin cloth and the filtrate was used as the enzyme source.

## Measurement of protein content and enzyme activity of crude enzyme extract

**Protein estimation:** Protein content was determined by using Bradford method. Bradford reagent was made by dissolving 100 mg Coomassie Blue G-250 in 50 ml of 85% ethanol and adding 100 ml of 85% (w/v) phosphoric acid and make volume to 1 l. 1 ml of Bradford reagent was added to protein sample and incubated at room temperature for 10 min. Standard curve was prepared by using BSA, stock solution having concentration of 100 mg/ml. The absorbance of each sample was measured at 595 nm using a UV-visible spectrophotometer [12].

**Enzyme activity:** The enzyme activity was determined by incubating a reaction mixture containing 0.95 ml of 0.1 M sodium acetate buffer (pH 5), 1.0 ml starch solution (1%, w/v) and 0.05 ml of crude enzyme at  $55^\circ\text{C}$  for 15 min [13]. The reducing sugar released after 10 min were measured with 3,5-dinitrosalicylic acid (DNSA) reagent [14] using glucose as a standard. Glucoamylase activity unit (U) was expressed as the amount of enzyme releasing 1  $\mu\text{mol}$  of glucose equivalent per minute under assay condition and enzyme activity is expressed in terms of units per gram dry fermented substrate (U/gdfs).

## Growth kinetics

Wheat bran, rice bran and banana peel (10 g) were kept separately in a 250 ml flask and then moistened with 20 ml of mineral solution containing (mg/gds)  $(\text{NH}_4)_2\text{SO}_4$  4,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.02,  $\text{K}_2\text{HPO}_4$  1.4 and  $\text{KH}_2\text{PO}_4$  0.6, initially maintained at pH 7 [5] and sterilized at  $121^\circ\text{C}$  for 30 min. The fermentation process was started by adding 2 ml of spore suspension as prepared above. The whole content was mixed thoroughly and then incubated at  $30^\circ\text{C}$  for 3 days, 5 days, 7 days and 10 days in a stationary condition.

## Separation and identification of sugars by thin layer chromatograph

Sugars get separated on the basis of differential adsorption onto silica gel. The reaction mixture contains 1 ml of enzyme and 1 ml of standard solution which was prepared as 1% solution of standard sugars such as glucose, maltose in 10% iso-propanol (v/v). Carefully the solution of individual standard sugars and the mixture of the samples applied on the separate marked spots. The chromatogram was developed with solvent system of ethyl acetate: iso-propanol:water:pyridine (26:14:7:2, v/v). Due to capillary action solvent reaches the marked line on the upper side of the silica plate. Plate was removed from chromatographic chamber and let it dry at room temperature. The spots were visualised

by spraying aniline diphenylamine reagent (mix 5 volumes of 1% aniline and 5 volumes of 1% diphenylamine in acetone with 1 volume of 85% phosphoric acid) and heating at  $100^\circ\text{C}$ . The sugar spots appeared as dark brown spots.

Identification of the sugars was done by comparing the relative fraction (Rf) values of the samples with that of the standards.

$R_f = \text{Distance travelled by unknown amino acid} / \text{Distance travelled by solvent system}$

## Characterization of glucoamylase

Soluble starch used in assay system for enzyme-substrate reaction was supplemented as 1%. Enzyme activity was checked individually at four different pH as 3, 5, 7 and 9 of the 0.1 M Sodium acetate buffer used and at four different temperatures  $35^\circ\text{C}$ ,  $55^\circ\text{C}$ ,  $75^\circ\text{C}$  and  $90^\circ\text{C}$ .

**Effect of temperature:** To check the optimum temperature for glucoamylase activity, crude enzyme of 7<sup>th</sup> day extract was incubated with 1% soluble starch in 0.1 M acetate buffer, pH 5 at different temperatures as  $35^\circ\text{C}$ ,  $55^\circ\text{C}$ ,  $75^\circ\text{C}$  and  $90^\circ\text{C}$ . Enzyme activity was checked individually.

**Effect of pH:** To check the optimum pH for glucoamylase activity, the pH of 0.1 M sodium acetate buffer was varied as 3, 5, 7 and 9 and the enzyme activity was checked individually.

**Effect of carbon and nitrogen additives:** The optimum SSF medium of wheat bran and rice bran was supplemented with different carbon (glucose, fructose, sucrose and starch) and nitrogen sources (urea, yeast extract, soybean meal and peptone) at 1% (by mass) level to study their effect on GA production by *Penicillium* sp. The fermentation was carried out at pH 5 and  $30^\circ\text{C}$  for 7 days [15].

## Partial purification of crude enzyme

**Ammonium sulfate precipitation:** 100 ml of the crude enzyme were first brought to 40% saturation with solid ammonium sulfate (enzyme grade) and the process was carried out at  $4^\circ\text{C}$  [16]. The whole apparatus was placed on a magnetic stirrer and ammonium sulphate was allowed to dissolve in the enzyme solution. The precipitated proteins were regimented by centrifugation for 15 min at 1000 rpm. The resulted pellet was dissolved in 3 ml of 0.2 M phosphate buffer at pH 6.2. The supernatant was applied again with ammonium sulfate to achieve 60% saturation. Both enzyme activity and protein content were determined for each separate fraction [17].

**Dialysis:** Pellet was dissolved in sodium acetate buffer (0.2 M) and dialysed against the same buffer (pH 5) with 3 equal changes of buffer after every hour to ensure the complete removal of the ammonium salt, enzyme was dialysed for 3 h at  $4^\circ\text{C}$ . The dialysed fraction referred to as partially purified glucoamylase [18].

## Application of glucoamylase

**Enzyme hydrolysis of various starches** The ability of the crude amylase to hydrolyze different native starches was studied using corn and potato starches. Commercially available soluble starch was used as standard. The assay mixture consisted of 1% of various starch in 0.2 M citrate-phosphate buffer (pH 6) and 0.05 of the enzyme. Enzyme assay was carried out after incubation at  $40^\circ\text{C}$  for 10 min [19].

## Results and Discussion

### Glucoamylase production using solid substrates

Rice bran, wheat bran and banana peel was used as substrate for the

production of glucoamylase by *Penicillium* sp. for 5 days in solid state fermentation. The results showed that maximum activity was shown by *Penicillium* sp. on rice bran (8.029 ± 0.014 Units/ml) followed by wheat bran and banana peel.

### Growth kinetics

Wheat Bran, Rice Bran and Banana Peel (10 g) (Figure 1) which were incubated for 3 days, 5 days, 7 days and 10 days in stationary condition showed enzyme activity. *Penicillium* sp. on rice bran (10.29 ± 0.07 Units/ml) at 7<sup>th</sup> day showed maximum enzyme activity followed by wheat bran and banana peel. After 7 days enzyme activity decreased.

### Thin layer chromatography of hydrolysis products

Glucoamylase is an exo-acting enzyme that catalyses the production of β-glucose from the non-reducing ends of amylose, amylopectin and glycogen. It consecutively hydrolyses α-1,4; α-1,6 and rare α-1,3 linkages [20]. TLC (Figure 2) revealed only glucose as the hydrolysis product of starch which indicates that the crude enzyme was a glucoamylase [21]. R<sub>f</sub> values of WB(A),WB(P),RB(A) and RB(P) are 0.85, 0.78, 0.75 and 0.89 respectively and R<sub>f</sub> values of glucose and maltose of WB(A),WB(P), RB(A) and RB(P) are 0.85, 0.89; 0.78, 0.74; 0.75, 0.7; 0.89, 0.85, respectively.

### Characterization of enzyme

**Effect of temperature:** Glucoamylase activity was tested at different temperatures. *Penicillium* sp. showed the maximum activity in case of WB (9.23 ± 0.10 Units/ml) at 55°C and for RB (10.66 ± 0.0007 Units/ml)

at 90°C. Banana peel showed very less activity as comparative to other agro-residues (Figures 3 and 4).

**Effect of pH:** Enzyme activity was maximum at pH 9, for *Penicillium* sp. on RB and WB using 1% soluble starch for enzyme assay (Figures 5 and 6).

**Effect of carbon sources and nitrogen additives on enzyme activity:** Addition of different sugars as additives to wheat bran and rice bran medium enhanced glucoamylase production by *Penicillium* sp. The

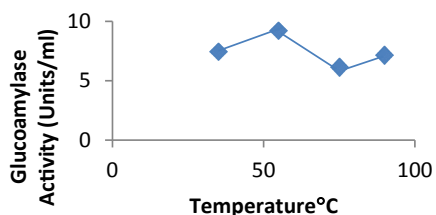


Figure 3: Effect of temperature (°C) on enzyme activity (Units/ml) [WB (P)].

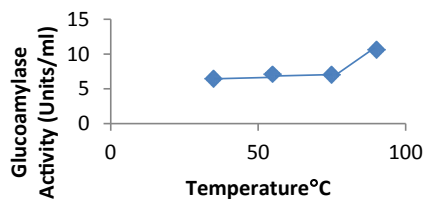


Figure 4: Effect of temperature (°C) on enzyme activity (Units/ml) [RB (P)].

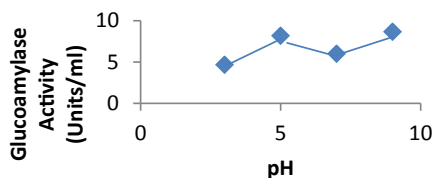


Figure 5: Effect of pH on enzyme activity (Units/ml) [WB (P)].

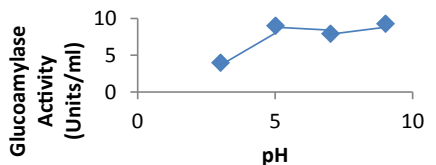


Figure 6: Effect of pH on enzyme activity (Units/ml) [RB (P)].

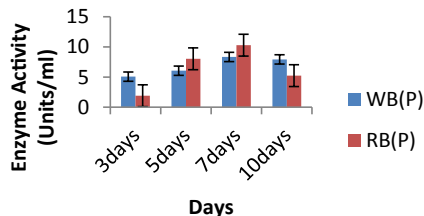


Figure 1: Effect of days on enzyme activity (U/ml) by *Penicillium* sp. on different substrates.

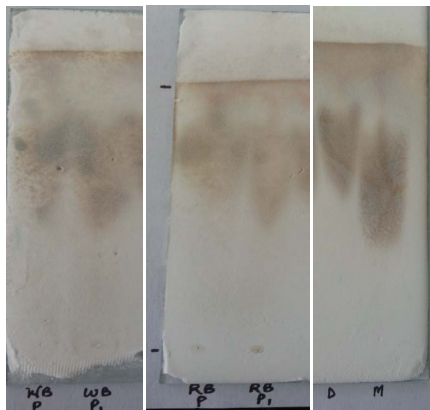


Figure 2: Thin layer chromatography of the reaction product hydrolysed by glucoamylase from *Penicillium* sp. glucose and maltose was used as standard.

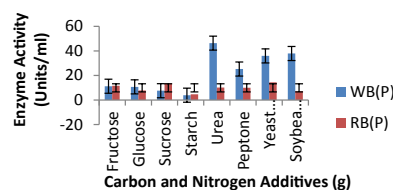
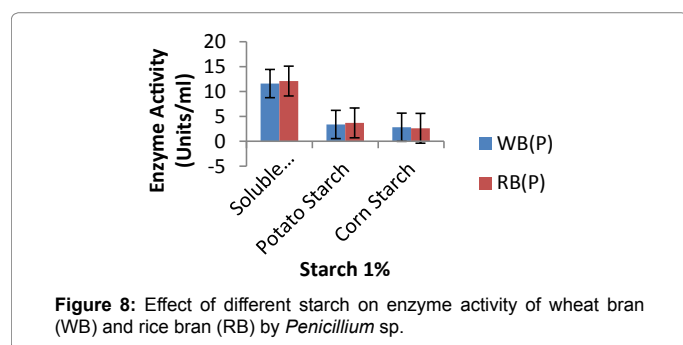


Figure 7: Effect of different carbon and nitrogen additives on enzyme activity (Units/ml) of wheat bran (WB) and rice bran (RB) by *Penicillium* sp.

Substrates	Enzyme Activity (Units/ml)	
	40%	60%
WB (A)	9.641 ± 0.037	9.84 ± 0.044
WB (P)	14.52 ± 0.014	8.62 ± 0.035
RB (A)	14.24 ± 0.014	6.15 ± 0.037
RB (P)	13.86 ± 0.022	14.32 ± 0.014

**Table 1:** Enzyme activity (Units/ml) of partial purified enzyme.



**Figure 8:** Effect of different starch on enzyme activity of wheat bran (WB) and rice bran (RB) by *Penicillium* sp.

maximum activity was shown by *Penicillium* sp. on RB with sucrose (13.70 Units/ml) as carbon source and yeast extract (14.41 Units/ml) as nitrogen source. Banana peel activity was less as compared to other agro-residues (Figure 7).

### Partial purification of glucoamylase

Glucoamylase was purified by ammonium sulfate precipitation and dialysis process. *Penicillium* sp. in rice bran and wheat bran showed an increase in enzyme activity as comparative to crude enzyme (Table 1).

### Application of glucoamylase

**Enzyme hydrolysis of various starches:** The rate of the amylase hydrolysis of different native starches was evaluated. Figure 8 showed that the amylase from the *Penicillium* sp. and *A. terreus* was able to hydrolyse different starches to varying degrees. Highest rate of hydrolysis was observed for potato starch, followed by corn starch (Figure 8).

### Conclusion

On the basis of the finding of the present study it can be concluded that out of the all different agricultural residues which is used to test for enzyme production, rice bran has the maximum glucoamylase activity (10.29 ± 0.07 Units/ml), protein content (1.22 ± 0.0014 mg/ml) and specific activity (8.4 Units/mg protein) with *Penicillium* sp. at 7<sup>th</sup> day as compared to other agro residues wheat bran and banana peel. By usage of Media supplements with carbon and nitrogen sources increases the enzyme activity. Sucrose (13.70 ± 0.77 Units/ml) (1% mass level) and yeast extract (14.41 ± 0.07Units/ml) (1% mass level) as carbon and nitrogen source respectively were found optimum on rice bran with *Penicillium* sp. Optimum enzyme activity was observed at 90°C, pH 9. The enzyme was partially purified using 60% ammonium sulphate precipitation. Enzyme

hydrolysis showed maximum activity (3.69 Units/ml) with potato starch from rice bran by *Penicillium* sp.

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