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OPTIMIZATION OF ALPHA AMYLASE FOR BETTER DOUGH PREPARATION

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ABSTRACT

Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. The present study mainly focused on screening of amylase producing *Bacillus subtilis*, production by solid-state fermentation using rice straw and banana pseudo stem and its optimization for amylase activity in dough preparation, effect on bread making and analysis of bread quality. Maximum production of amylase was obtained after 24hrs of incubation. The optimum pH for enzyme activity was found to be at pH 7 and the optimum temperature for the activity was found to be at the range of 30 – 70°C. The combination of 0.8g yeast and 300 U of amylase gave better results than enzyme alone for the better dough preparation.

Keywords: Amylase, *Bacillus subtilis*, solid state fermentation, optimization

INTRODUCTION

The industrial enzyme producers sell enzymes for a wide variety of applications and approximately more than 25% amylases represent as a class of industrial enzymes of the market **(Sidhu *et al*., 1997; Rao *et al*., 1998)**. Starch digesting amylase has found important application in bioconversion of starches and starch-based substrates **(Fogarty *et al*., 1983)**. Amylolytic enzymes are of great significance in biotechnological applications in food industry, amylases can be synthesized from numerous sources, like plants, animals and microorganisms. The enzymes from microbial sources commonly meet industrial demands and had made important contribution to the production of foods and beverages since, microbial amylases have almost completely replaced chemical hydrolysis of starch in starch processing industry **(Pandey *et al*.,2000a; Gupta *et al*., 2003; Kandra, 2003; Rajagopalan and Krishnan, 2008)**. Two major classes of amylases have been identified in microorganisms, namely, α-amylase and glucoamylase. α-Amylases (endo-1,4-a-D-glucan glucohydrolase) are extracellular enzymes that randomly cleave the 1,4-a-D-glucosidic linkages between adjacent glucose units in the linear amylase chain. Glucoamylase (exo-1,4-a-D-glucanm glucanohydrolase) hydrolyzes single glucose units from the nonreducing ends of amylose and amylopectin in a stepwise manner **(Rameshkumar and Sivasudha, 2011; Nigam and Singh, 1995)**. Alpha-amylase can produce by a wide spectrum of organisms, even though every source produces depend upon biochemical phenotypes that considerably differ in parameters such as pH and temperature optima as well as metal ion requirements. Submerged fermentation (Smf) has been a traditional way for production of industrially significant enzymes since long past due to multiple facilities like better control over environmental factors namely, pH, temperature, aeration, and moisture level. Cultures reported to be utilized for amylase production using SmF belong to a variety of *Bacillus* species like *Bacillus* sp.PN5, *Bacillus subtilis* JS-2004, *Bacillus* sp. IMD 435, *Bacillus* sp. I-3, *Bacillus caldolyticus* DSM405, *Bacillus licheniformis* GCBU-8 **(Saxena *et al*., 2007; Asgher *et al*., 2007; Hamilton *et al*., 1999; Goyal *et al*., 2005; Schwab *et al*., 2009; Haq *et al*., 2003)** since, Solid State Fermentation (SSF) replaces SmF as it mimics the natural habitat of microorganisms. SSF is alternative over SmF due to its low cost, lower energy requirement, simplicity, less water output, and lack of foam built up **(Couto and Sanroman, 2006; Pandey, 2003; Tanyildizi *et al*., 2007)**. Biotechnological innovations particularly in the area of fermentation technology and enzyme technology, SSF with agro wastes such as WB, RB, COC, and GOC has replaced the high cost media generally used in submerged fermentation for alpha amylase preparation and *Bacillus* species are frequently used for α-amylase production **(Mulimani and Ramalingam, 1999; Shukla and Kar, 2006; Vijayabaskar *et al*., 2012; Baysal *et al*., 2003; Mukherjee *et al*., 2009; Sodhi *et al*., 2005; Soni *et al*., 2003)**. The present study mainly focused on screening of amylase producing *Bacillus subtilis*, production by solid-state fermentation using rice straw and banana pseudo stem and its optimization for amylase activity in dough preparation, effect on bread making and analysis of bread quality.

MATERIAL AND METHODS

Isolation Of *Bacillus subtilis* from the environment

A cut piece of cut potato was buried about four inches deep, and covered with soil. After about 6-8 days, the potato was digged out; the soil was scarped off and was taken it to the lab in the ziploc bag or a plastic bag. The amylase producer was isolated from that soil sample. 10g of soil was suspended in 90ml of sterile distilled water, properly mixed. From the above, 10ml was transferred to 90ml of sterile distilled water. Two further dilutions were done in 90ml of sterile distilled water. 0.1ml of diluted sample was delivered in nutrient agar containing 1% (w/v) starch (corn starch) by means of pour plate techniques. The plate was incubated for 24 h at room temperature. Amylase producing colonies will have a clear area around them. To confirm, the plate was flood with Gram's iodine.

Optimization of pH for amylase activity

Nutrient agar containing1% (w/v) starch medium was prepared at different pH (3 - 10). The *Bacillus sp*. was streaked in the middle of the agar plates and incubated at room temperature for 24h. Amylase production was detected by the disappearance of blue colour around the colony when treated with iodine solution. Evaluation of the clear zones of each colony was estimated as radius (mm) of the clear zone minus the radius of the colony.

Bacterial amylase inoculation

Bacillus subtilis was inoculated into the bacteriological amylase production media (Bacteriological peptone –6gm, MgSO4.7H2O,-0.5gm, KCl-0.5gm, Starch-1gm Water -1000ml) and incubated at room temperature for 24 h.

Amylase production by solid state fermentation

Two sets of fermentation were carried out in 250ml Erlenmeyer flasks containing production media (Rice bran/ Banana pseudo stem -10g, Urea-0.05%, MgCl2 - 0.25%, KCl-0.25%,Sucrose-1%,Distilled water-30ml), which were autoclaved for 20min at 121℃. A cell suspension of 1% (v/w Rice bran/ Banana pseudo stem) containing viable cells/ml was used as inoculum. Incubation was carried out for 60 h at room temperature. Extraction was conducted using 10g fermented mass in 250ml conical flask. Soaking the fermented solid with a suitable solvent like glycerol, acetone, isopropyl alcohol and water for desired period. The crude extract was then squeezed out through cheese cloth. The clear extract obtained after centrifugation to remove insoluble, assayed for amylolytic activity.

Preparation of crude enzyme

Dialysis will remove residual sugars from the enzyme mixture. One end of a dialysis tube was tied and enzyme mixture was poured into dialysis tube. The other end was tied securely. Dialysis tube was put in distilled water in a beaker. The water was changed several times for 24h. Crude enzyme was poured into clean Universal bottles or screw cap tubes and stored in a freezer at about 0°C until needed. The dialysis sample that was collected in a sterile bottom flask was rotated in ice at 45°angles for the formation uniform thin layer of sample inside the flask and lyophilized. The enzyme powder collected from the flask was transferred into sterile vial and stored at 4°C.

Characterization of the crude enzyme

The effect of temperature on the crude amylase activity was assayed at temperature values ranging from 20°C to 90°C. The reaction mixture contained 0.1 ml of the crude enzyme in 1 ml of corn-starch (1% w/v). This was incubated for 30 mins at each chosen temperature. At room temperature, to 5ml of crude enzyme 0.5 ml of the extract was added and the enzyme activity was found by DNSA method and Bernfeld method **(Bernfeld, 1955)**.

Optimization of enzyme substrate concentration

One unit of enzyme activity was defined as the amount of enzyme that release 1µmole of reducing sugar as glucose per minute under the assay condition specified **(Soumen and Rintu, 2001)**. 1 to 10ml of 1% starch solutions were taken in different test tube and made up to 1ml with distilled water. 1ml of crude enzyme was added to the above starch solution. Incubated in water bath 60°C for 3min and the reaction was stopped by adding 1ml of DNSA reagent. The mixture was boiled for 5min and brought to room temperature then 10ml of distilled water was added and absorbance was measured at 540nm.

Optimization of enzyme concentration on dough preparation

The flour used was commercial standard white wheat flour, containing 30 ppm ascorbic acid, carbohydrate 73%, Protein 9.5%, fat 0.8%, crude fibers traces, minerals 0.3% and moisture contents 11.7%. Five different kinds of dough formulation were prepared for optimize the enzyme concentration. The above formulations prepared with 100g of wheat flour, 25ml of water containing 150 U, 300 U, 450 U, 0.8g yeast and 0.8g yeast with 300 U of amylase enzyme respectively. Then were kept for incubation at different period of time such as 0, 15, 30 minutes respectively. Six different dough formulations were given in the below table I.

Table 1 Six different dough formulations

Ingredients (in grams)	Types of Formulation					
	F1	F2	F3	F4	F5	F6
Flour	150	150	150	150	150	150
Dry yeast	-	1.2	-	-	1.2	1.2
Gluten	1	1	1	1	1	1
Amylase (Units)	-	-	200	400	200	-
Salt	3	3	3	3	3	3
Sugar	-	-	-	-	-	3
Water (ml)	75	75	75	75	75	75
Shortening	3	3	3	3	3	3

Analysis of bread

After baking the loaves volume were measured. Crumb stickiness, softness, and taste ware subjectively evaluated by well-experienced barking staff. A piece of crumb was squeezed between the thumb and two fingers for determine the stickiness of bread. Reducing sugar contents were analyzed by DNSA method. Softness was examined up to 7days. Reducing sugars were extracted from one-day bread crumb by mechanically stirring 2g crumb with 20ml distilled water at 25°C for 25 min and centrifuged at 12000 rpm for 15 min. The extracts were stored frozen if not used immediately. The resulted dates were calculated and tabulated.

RESULTS AND DISCUSSION

Screening of amylase producers

Clear zone around the colony were seen (figure not shown) when the plates were treated with iodine. This indicated the colonies are amylase producers. **Suman and Ramesh, (2010)** reported that the screening of purified cultures was done on MAM (Minimal agar media) supplemented with 1% starch the cultures growing in MAM were flooded with iodine solution and the zone of hydrolysis were obtained in the plates showing starch hydrolysis similar method in order to screen the microorganisms for amylase production.

Optimization of pH

pH and temperature have very important roles to play in amylase activity and stability. In this study the optimum pH range was found to be 7 but it works also in various pH ranges between 5 to 8. The colonies, which were grown in different pH media was treated with iodine solution. The zone of clearance was absorbed maximum in the pH 7 this indicate that the optimum pH for amylase production was 7 (Fig-1). Amylases are generally stable over a wide range of pH from 4 to 9 **(Vihinen and Mantsala, 1989)**; however, according to **Robyt *et al*. (**1971) amylases with stability showed in a narrow range of pH.

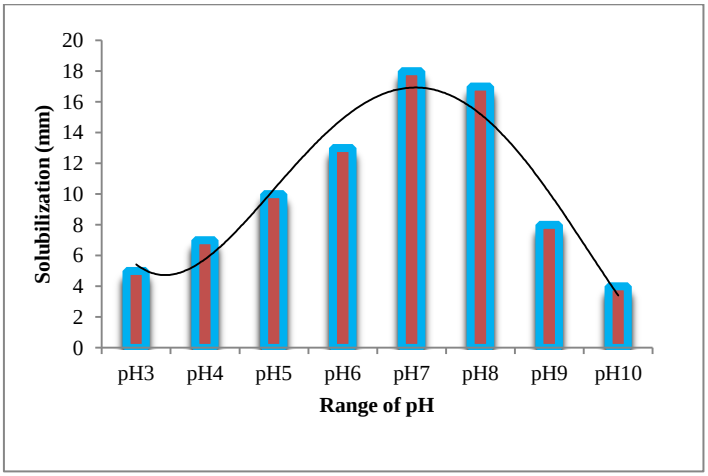

Figure 1 pH optimization by agar plate method

Solvent extraction

The extraction efficiency is critical to the recovery of the enzyme from the fermented biomass hence, selection of a suitable solvent is necessary. Different solvents selected were water, glycerol, and acetone. Among the solvent used glycerol gave the best result. Glycerol was found to be a suitable solvent to extract the extracellular amylase from the solid substrate fermentation medium. It showed the maximum release of 0.232 mg and 0.216 mg free glucose on banana pseudo stem and rice brawn substrate respectively followed by found to be isopropyl alcohol extraction (Table 2).

Table 2 Extraction of extra cellular enzyme from solid substrate by differed solvent systems

Type of solvent	Glycerol	Acetone	Isopropyl alcohol	Water
Free glucose (mg)/ 5 ml of Rice brawn as substrate (1%)	0.216	0.091	0.201	0.121
Free glucose (mg)/ 5 ml of Banana pseudo stem as substrate (1%)	0.232	0.086	0.226	0.176

Optimization of amylase enzyme activity

Effect of pH on amylase activity

The optimum pH was observed between the pH ranges of 5.0 - 8.0. After pH 8, a continuous decrease in enzyme activity was observed (Fig-2). Previous report

indicated the maximum production of amylase activity was obtained at pH of 7.0 **(Mukesh *et al*.,2012)**.

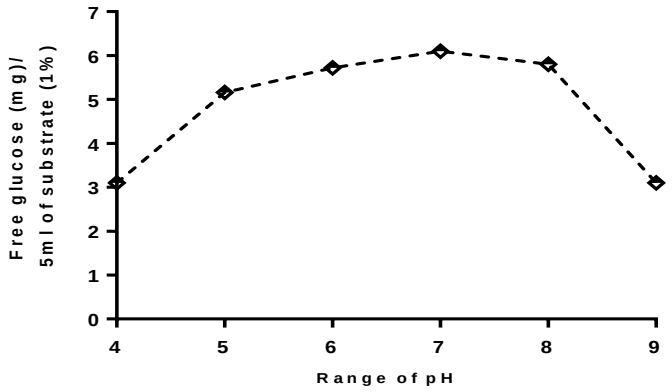

Figure 2 Effect of ph on amylase activity

Effect of temperature on amylase activity

The influence of temperature on amylase activity of the crude enzyme showed that enzyme activity increased progressively with increase in temperature from 20°C reaching a maximum at the range of 30 – 70°C. Above 70°C, there was a reduction in the amylase activity (Figure 3). Earlier studies indicated maximum amylase production occurred at the optimum growth temperature. The optimum temperature for enzyme activity was between 45°C and 55°C **(Mukesh *et al*., 2012)**. A reduction in enzyme activity was observed at values above 60°C. **Mukesh kumar *et al*. (2012)** reported the amylase activity attained its maximum at 50°C beyond which the enzyme activity was reduced gradually.

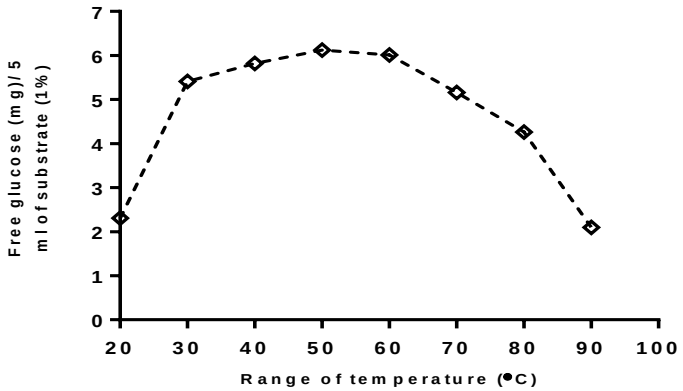

Figure 3 Effective on temperature on amylase activity

Effect of starch concentration on amylase activity

In the present study, amylase activity increased with increase in the starch concentration from 1ml to 10 ml of 1% starch concentration (Fig-4). The production of enzyme is greatly dependent on the condition of growth of the culture and composition of nutrient medium. The medium constituents have been predicted to play an important role in enhancing the production of enzyme from microbial sources **(Gupta *et al*., 2002).**

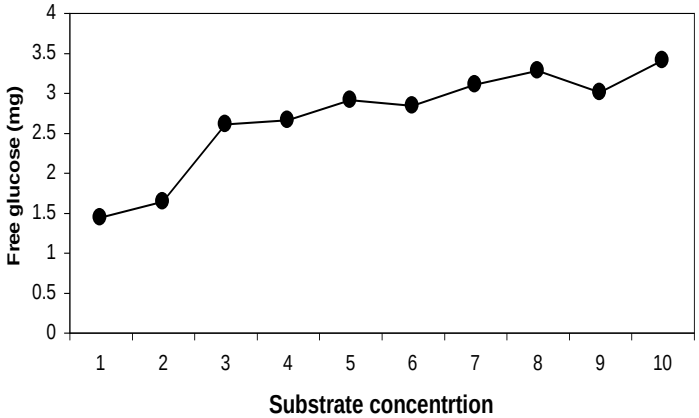

Figure 4 **Subtract concentration on amylase activity**

Optimization of enzyme concentration on dough preparation

The enzyme concentration was optimized based on the reducing sugar and incubation period. It was found to be 300 U for 30 min at 27°C in 100g of wheat flour. The combination of 0.8g yeast and 300 U of amylase gave better results than enzyme alone by means of dough volume. The detailed results were tabulated (Table- 3). Extra enzymes added to the dough improve control of the baking process, allowing the use of different baking processes, reducing process time, slowing-down staling, compensating for flour variability and substituting chemical additives **(Tramper *et al*., 2005).**

Table 3 Optimization of enzyme concentration on dough preparation

Enzyme/yeast	Flour gm	Incubation period	Reducing sugar (mg/gm of dough)
Amylase 150U	100	0 min	1.1
		15min	4.2
		30 min	6.7
Amylase 300U	100	0 min	1.82
		15 min	6.12
		30 min	9.62
Amylase 450U	100	0 min	2.27
		15 min	9.16
		30 min	13.72
Yeast 0.8g	100	0 min	0.42
		15 min	1.82
		30 min	3.62
Yeast 0.8g+300U amylase	100	0 min	2.16
		15 min	7.15
		30 min	10.82

Addition of α-amylase gave moderate crumb firmness after 7 days of storage than without enzyme product. The moisture content in breadcrumb has been shown to decrease during storage **(Herz, 1965)**. Addition of enzyme preparations caused significantly darker crusts (400U/150g of flour) compared to breads with 200 U and addition of yeast products. This can be explained by increased formation of reducing sugars when α-amylase is added and the estimated reducing sugar results were tabulated (Table-4) No differences were found between the other formulations like without enzyme, and without yeast products. The higher values for the intermediate resting time may be explained by assuming that, during the initial fermentation, the production of low-molecular weight sugars exceeded that metabolized by the yeast.

Table 4 estimation of reducing sugars of bread

Types of formulation	Reducing sugars (mg/gm of bread)
F1	3.1
F2	7.7
F3	14.23
F4	25.5
F5	20.6
F6	8.1

CONCLUSION

Amylase producing *Bacillus subtilis* was isolated from soil (potato tuber). The amylase enzyme activity was optimized at various parameters like pH, temperature and enzyme substrate concentration. It was found that the enzyme works wide ranges of pH (pH 5-8) and the temperature ranges from 30°C to 60°C. On SSF Banana pseudo stem reaches higher enzyme activity and glycerol was found to be a suitable solvent system for enzyme extraction. Crust darkness was significantly affected by addition of α-amylses. Independent of added α-amylase, resting time influences crust darkness, with maximum crust darkness obtained after 30 min. Addition of α- amylase reduced the dependence of loaf volume on mixing time, but gave slightly significant increase in bread volume. The present study concluded that the combination of α-amylase and yeast formulation showed better results compared with other formulations. So this enzyme system may be used under optimized concentration. Higher dosage of amylase gave high stickiness due to the formation of high levels of reducing sugars.

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