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Different Agroresidues Used in Solid Substrate Fermentation for α-Amylase Production by *Bacillus subtilis*-239

M. Yousaf *, M.B. Bhatty, S. Baig, M. Nadeem and Z. Nasreen

Food and Biotechnology Research Centre, PCSIR Laboratories Complex, Lahore - 54600, Pakistan

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Abstract. The best mass ratio for agroresidue fermentation for α-amylase production by locally isolated *Bacillus subtilis*-239 was found to be wheat bran to rice bran 2:1 with 70% initial moisture content for 60 h incubation time. Among different inorganic nitrogen sources supplemented, sodium nitrate and ammonium chloride (0.5% w/w) increased the enzyme yield upto 178 U/ml and 176 U/ml, respectively, whereas all the organic nitrogen sources decreased the enzyme production. Addition of glucose (1% w/w) as a carbon source enhanced α-amylase synthesis to 185 U/ml as compared to the control (134 U/ml).

Keywords: α-amylase production, fermentation, *Bacillus subtilis*, agroresidues

Introduction

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the area of industrial environment and food technology utilize enzymes, at some stage or the other. Solid substrate fermentation holds tremendous potential for the production of various enzymes such as proteases, lipases, pectinases and amylases. It can be of special interest in those processes where crude fermented products may be used directly as enzyme source (Pandey *et al*., 2000; Benjamin and Pandey, 1998). Amylases are among the most important and widely used enzymes whose spectrum of applications has widened in many sectors such as baking, brewing, detergent, textile, paper and distillery industries (Ramachandran *et al*., 2004).

Amylases are reported to occur in microorganisms, although they are also found in plants and animals. Two major classes of amylases have been identified in microorganisms, namely α-amylase and glucoamylase. α-amylase (endo-1,4-α-D-glucan glucohydrolase E.C. 3.2.1.1) is extracellular enzyme that randomly cleaves the 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Glucoamylase (exo-1,4-α-D-glucan glucanohydrolase E.C. 3.2.1.3) hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin in stepwise manner (Pandey *et al*., 2000). The cost of enzyme production in submerged fermentation is high which can be reduced by adopting alternate method i.e. solid substrate fermentation. The contents of synthetic media are very expensive and might be replaced with more economically available agricultural by-products to reduce the cost of production medium. The use of agricultural wastes makes solid substrate fermentation an attractive alternate method for enzyme production (Ellaiah *et al*., 2002).

In the present study, α-amylase production through fermentation by locally isolatedby *Bacillus subtilis*-239 has been investigated, using various agricultural wastes like wheat bran, rice bran, soybean meal, cassava bagasse, rice husk and their combinations in different mass ratios.

Materials and Methods

Organism and cultivation conditions. The organism used in this study was *B. subtilis*-239, earlier isolated in our laboratory (Yousaf *et al.,* 2007). The culture was maintained on nutrient agar medium, containing (g/l) peptone 6.0, casein hydrolyzate 4.0, yeast extract 3.0, glucose 2.0, beef extract 1.5 and agar 15.0. After inoculation the culture was incubated at 37 °C for 48 h.

Inoculum preparation. A 24 h old vegetative inoculum was employed in the present work. Nutrient broth, (50 ml) was sterilized in 250 ml conical flask at 121 °C for 15 min. After cooling, the medium was inoculated with culture of *B. subtilis*-239 and incubated at 37 °C for 24 h on a rotary shaker with 150 rpm.

Fermentation procedure. In order to choose the best substrate for fermentation, 40 g of each of various locally purchased defatted agricultural wastes like rice bran (RB), soybean meal (SBM), cassava bagasse (CB), wheat bran (WB), rice husk (RH), cotton seed oil cake (CSOC) and mustard oil cake (MOC) were taken separately, in 1:1 and in different mass ratios in 1 litre cotton plugged conical flask and moistened with 20 ml of phosphate buffer (pH 7.0). The flasks were autoclaved at

*Author for correspondence; E-mail: usaf_374@hotmail.com

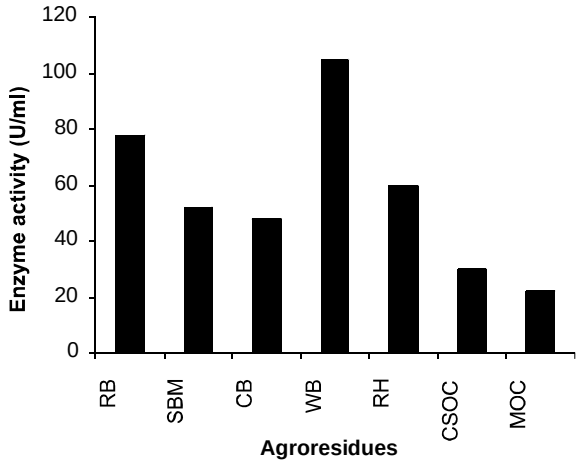
121 °C for 15 min and the sterilized solid substrates were inoculated with 8 ml of 24 h old inoculum and incubated at 37 °C for 48 h.

Preparation of enzyme extract. 400 ml of phosphate buffer (pH 7.0) was added to the fermented substrate for the extraction of crude enzyme. The flask was placed on a rotary shaker for 10 min (150 rpm). The substrate suspension was filtered using ordinary filter paper and the filtrate was used for the enzyme assay.

Analytical method. Enzyme activity was assayed by adding 1 ml of diluted enzyme solution to 1 ml (1% w/v) starch solution and incubated at 25 °C for 3 min. The reaction was terminated by adding 2 ml of 1% DNS (3,5-dinitro salicylic acid) reagent. Colour due to the liberated reducing sugars was developed by placing the reactants in boiling water for 5 min, then rapidly cooling them to room temperature. The extinction values were determined at 550 nm on spectrophotometer (Stein and Fisher, 1961). One amylase unit liberates reducing sugar equivalent to 1 mg maltose hydrate under the assay conditions. Triplicate samples were assayed for the enzyme activity and the confirmation of the result, whereas average of the values are shown in tables and figures.

Results and Discussion

Screening of agroresidues as substrate. Various defatted agroresidues were used for the production of α-amylase by *B. subtilis*-239 (Fig. 1). Among these, wheat bran gave the highest enzyme activity (108 U/ml). Wheat bran has also been reported to be the best substrate in solid substrate fermentation by *Bacillus* species because the produced metabolites are concentrated and purification procedure is less costly (Malimani and Ramalingam, 2000). Most of the mixtures of agroresidues containing wheat bran caused sufficient increase in enzyme production but the highest activity of the enzyme was at 156 U/ml, when the mixture of wheat bran and rice bran was used as substrate in mass ratio 1:1 (Table 1). The maximum activity of enzyme in different ratios of wheat bran and rice bran achieved was (178 U/ml) in a mass ratio 2:1 (Table 2). Agroindustrial wastes are rich sources of carbon, protein, minerals and other growth factors for microorganisms to produce amylases and in the present study a mixture of wheat bran and rice bran in mass ratio 2:1 has proved to be the best substrate for α-amylase production, whereas in the past, a mixture of wheat bran and coconut oil cake was found to be the best substrate for α-amylase production by fungal culture with optimum yield of 160 U/ml (Ramachandran *et al*., 2004).



RB = rice bran; SBM = soybean meal; CB = cassava bagasse; WB = wheat bran; RH = rice husk; CSOC = cotton seed oil cake; MOC = mustard oil cake

Fig. 1. Screening of agroresidues for the production of α-amylase by *B. subtilis*-239

Effect of initial moisture content of the medium on production of α-amylase. Solid substrate fermentation is the microbial growth on solid particles without presence of free water. The

Table 1. Screening of substrate mixture (1:1) for the production of α-amylase by *B. subtilis*-239

Substrate mixture (1:1)	Enzyme activity (U/ml)
RB + SBM	84
SBM + CB	50
CB + WB	122
WB + RB	156
WB + RH	118
WB + CSOC	63
WB + MOC	52
RH + CSOC	42
CSOC + MOC	26

RB = rice bran; SBM = soybean meal; CB = cassava bagasse; WB = wheat bran; RH = rice husk; CSOC = cotton seed oil cake; MOC = mustard oil cake

Table 2. Production of α-amylase by *B. subtilis*-239 in different ratios of wheat bran (WB) and rice bran (RB)

WB:RB	Enzyme activity (U/ml)
1:1	151
2:1	178
3:1	122
1:2	135
1:3	89

water remains in complex form within the solid matrix or as a thin layer either absorbed to the surface of the particles or less tightly bound within the capillary region of the solid substrate. Moisture level is a critical factor for the production of enzyme in solid substrate fermentation and generally bacterial growth requires higher water activity. The necessary moisture in solid substrate fermentation is likely to be more advantageous for growth because of the possible efficient oxygen transfer (Raghavarao *et al*., 2003). Enzyme synthesis gradually increased with increase in moisture content and maximum activity (182 U/ml) was achieved when substrate moisture was 70% (v/w) (Fig. 2). At insufficient moisture content, enzyme yield is found to be less as it does not allow good diffusion of solutes and gases due to which cell metabolism slows down or stops completely (Gervais and Molin, 2003). Higher moisture level also decreases the enzyme production because it decreases porosity, changes particle structure, increases stickiness and lowers the oxygen transfer (Ramesh and Lonsane, 1991). Optimal moisture level of 60-75% has been recommended by Lonsane *et al*. (1985) for α-amylase production by *B. licheniformis* in solid state fermentation, whereas in our study, the moisture level is within this range.

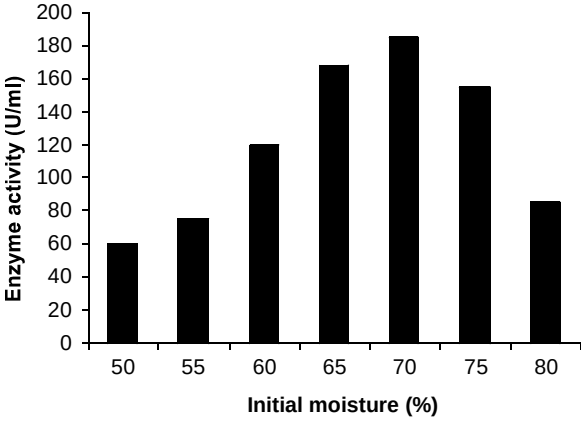


Fig. 2. Effect of initial moisture content of medium on the production of α-amylase by *B. subtilis*-239.

Effect of incubation period on α-amylase production. In solid substrate fermentation process, incubation period is very critical which depends upon the characteristics of the culture, growth rate and production of enzyme. There is a regular increase in enzyme formation and it becomes maximum i.e. 184 U/ml after 60 h incubation, after which a gradual decrease in enzyme production is observed with further increase in fermentation time (Fig. 3); this is caused by denaturization or decomposition of α-amylase due to interaction with other components of the medium (Ramesh and Lonsane, 1987).

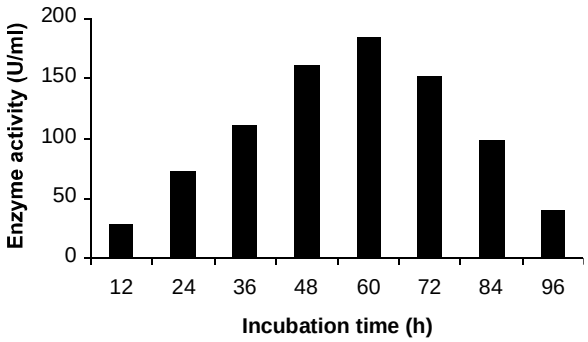


Fig. 3. Effect of incubation period on α-amylase production.

Effect of inorganic nitrogen sources on α-amylase production. Studies on the supplementation of different inorganic nitrogen sources at 0.5% (w/w) concentration to the fermentation medium showed a mixed trend on enzyme production (Table 3). Among the added inorganic nitrogen sources, sodium nitrate (178 U/ml) and ammonium chloride (176 U/ml) were found to increase enzyme yield as compared to the control (135 U/ml) whereas other sources inhibited the enzyme synthesis by *B. subtilis*-239. The increase in the enzyme activity on addition of ammonium chloride and sodium nitrate shows that any one of these two can be used alternatively as nitrogen source to enhance the enzyme yield; this is in accordance with the results reported earlier by Ikram *et al*. (2002). The inhibitory effects of some inorganic nitrogen sources on α-amylase production by *Aspergillus niger* using wheat bran in submerged and solid-state fermentation were also observed by Kocher *et al*. (2003).

Effect of complex nitrogen source on α-amylase production. The nutritional requirements of *Bacillus* sp. are reported to be complex for the growth and the enzyme synthesis as the structural macromolecules of agroresidues provide an inert matrix within which various organic acids are already present to provide complex nitrogen in solid substrate fermentation.

Table 3. Effect of inorganic nitrogen sources on α-amylase production

Nitrogen source	Enzyme activity (U/ml)
Sodium nitrate	178
Potassium nitrate	85
Ammonium sulphate	92
Ammonium chloride	176
Ammonium bicarbonate	72
Ammonium oxalate	60
Ammonium acetate	53
Control	135

Different organic nitrogen sources in complex form such as casein, yeast extract, urea, peptone, tryptone and beef extract were supplemented at the rate of 1% w/w to the medium for biosynthesis of α-amylase (Fig. 4). Addition of all the organic nitrogen sources resulted in considerable decrease in α-amylase production by *B. subtilis*-239. This result is in agreement with the findings in which repression in production of α-amylase was observed during the solid-substrate fermentation when organic nitrogen sources like casein, gelatin and soy meal were added (Malimani and Ramalingam, 2000). However these results differ with the investigations in which organic nitrogen sources along with wheat bran in solid substrate fermentation by *Aspergillus niger* enhanced α-amylase production (Kocher *et al*., 2003). On addition of complex nitrogen sources, the reduction in α-amylase yield may be due to the production of protease or other unfavourable catabolites.

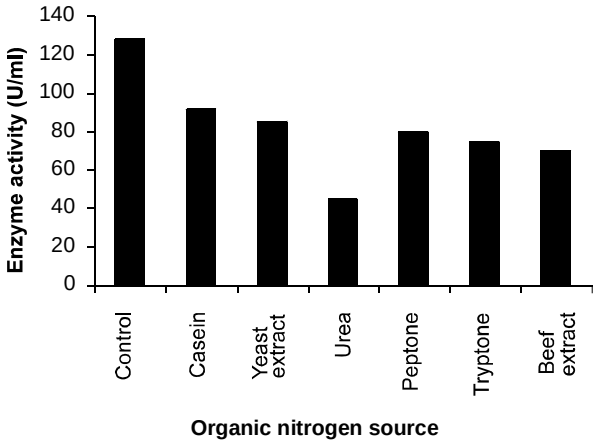


Fig. 4. Effect of complex nitrogen sources on α-amylase production.

Effect of carbon sources on α-amylase production. To investigate the effects of various carbon sources on α-amylase production, *B. subtilis*-239 was grown in the medium containing different monosaccharides, disaccharides and polysaccharides such as glucose, galactose, maltose, lactose, sucrose and starch at the rate of 1% (w/w). Maximum amylase yield (185 U/ml) was obtained in the medium containing glucose (Fig. 5). The glucose has been reported to be the best carbon source for the production of α-amylase by *B. theroolevorans* (Narang and Satyanarayana, 2001). It was also observed that lactose and starch enhanced the enzyme production to marginal limits whereas galactose, maltose and sucrose inhibited α-amylase synthesis. It has also been reported that formation of carbohydrate degrading enzymes in most

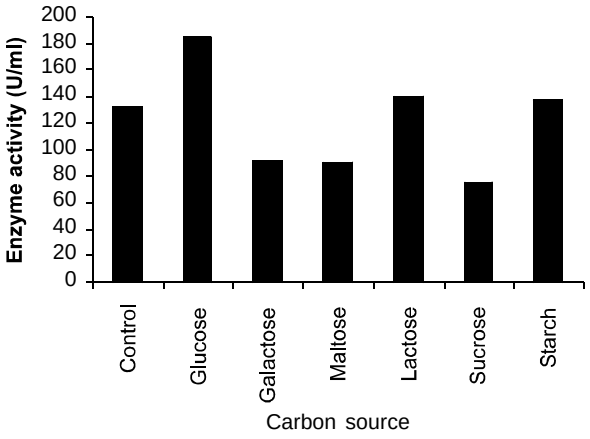


Fig. 5. Effect of carbon sources on α-amylase production.

species of the genus *Bacillus* were subjected to catabolic repression by some of the readily used carbon sources (Lin *et al*., 1998).

Conclusion

The production of α-amylase by *Bacillus subtilis*-239 using a combination of wheat bran and rice bran in the ratio 2:1, under the optimized conditions is an economical method for the production of enzyme for industrial use as both of the agroresidues are cheap and easily available.

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