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Full Length Research Paper

Production of β-galactosidase by *Aspergillus oryzae* in solid-state fermentation

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Fungal culture exhibiting β-galactosidase activity was isolated and identified as *Aspergillus oryzae* from soil polluted with milk dairy factory effluents. Optimization of β-galactosidase (E.C 3.2.1.23) was carried out in Solid State Fermentation (SSF). Wheat bran and rice husk supports the maximal growth and β-galactosidase production by *A.oryzae.* The fungal culture utilized several carbon sources for the β-galactosidase induction. Glucose serves as a best carbon source, followed by lactose, maltose and sucrose. Among the various nitrogen sources used in this study, sodium nitrate found to be the best. Certain fermentation parameters involving initial pH, moisture content and incubation temperature were studied separately. Maximal amount of β-galactosidase activity and protein content was obtained when SSF was carried out using wheat bran and rice husk in 1:1 ratio, having initial moisture of 90%, initial pH 5.0 and supplemented with (12.5%) w/w glucose and 1% w/w sodium nitrate at 30ºC for 7 days using 10 ml spore suspension (1 x 10^7 spores/ml) as inoculum's size.

Key words: *Aspergillus oryzae*, β-galactosidase, solid state fermentation.

INTRODUCTION

β-galactosidase (EC 3.2.1.23) has catalytic property to hydrolyze lactose into glucose and galactose. Due to its hydrolyzing property of lactose, it has been used for milk and fermented milk products. Potential beneficial effects on the assimilation of foods containing lactose, as well as the possible technological and environmental advantages of industrial application (Jurado et al., 2002; Gekas and López-Leiva, 1985) like elimination of lactose intolerance and formation of galacto-oligosaccharides during lactose hydrolysis. Therefore, the use of β-galactosidase is one of the most promising applications of enzymes to food industries (Pomeranz, 1964; Wendorff et al., 1971). Acid β-galactosidase produced from moulds is very stable and does not require metal ion cofactors for its action (Gonzalez and Monson, 1991). Recovery costs for this enzyme are primarily at the level of production and purification stages. The selection of an inexpensive and easily available substrate together with suitable producer microorganism, optimization of culture conditions, and effective downstream processing are essential to reduce the cost of enzyme preparation (Becerra and Siso, 1996).

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Solid State Fermentation (SSF) has several advantages (Holker and Lenz, 2005) over more conventional submerged fermentation, and many promising lab-scale SSF processes are periodically reported (John et al., 2006; Krasniewski et al., 2006; Lechner and Papinutti, 2006; Sabu et al., 2006).

A local isolate of *Aspergillus oryzae* with β-galactosidase activity was obtained from soil discharges with milk dairy factory effluents. *A. oryzya* has many advantages over many other microbial sources. It is widely accepted as source of enzyme used for food and feeds. *A. oryzae* has been accorded as GRAS (generally regarded as safe) status (Reichelt, 1983). Since this natural isolate produced very low concentration of β-galactosidase, attempts were made to increase the productivity by optimizing following parameters with emphasis on carbon, nitrogen source, moisture content, pH, and temperature by using wheat bran and rice husk as solid substrates constant.

MATERIALS AND METHODS

Organism

A local isolate of *A. oryzya* as used in this study was isolated from

soil contaminated with effluent of milk dairy factory. It was maintained on Potato dextrose agar (PDA) (Hi-media, Mumbai) medium. The slants were grown at 30°C for seven days and stored at 4°C for further studies.

Viable spore count

The viable spore number on a PDA slant was determined by colony count technique. The spores were suspended in 10 ml of distilled water with 0.01% Triton- X 100, using a sterile transfer needle and diluted serially. 1 ml of spore suspension was poured onto sterile Petri-plates, containing sterile potato dextrose agar (PDA) medium and spread uniformly. The inoculated Petri-plates were incubated at 30°C for 6 days. A plate that developed between 7 to 30 colonies was selected for counting. The spore density was calculated as the count multiplied by the dilution factor.

Inoculum preparation

10 ml of sterile distilled water containing 0.01% Triton–X 100 was transferred to a sporulated (7 days old) PDA slant culture. The spores were dislodged using the sterile inoculation needle under aseptic conditions and the suspension, with appropriate dilution was used as inoculum for experimental bottles.

Solid substrates and chemicals

Wheat bran and Rice husk are substrates used in this study. They are cheap and readily available in the local market, Nandyal, Andhra Pradesh, India. ONPG (o-nitrophenyl-β-D-galactopyranoside) was purchased from Sigma chemicals (USA).

Solid state fermentation

Mixture of wheat bran and rice husk (65 g each in 1:1 ratio) were taken in 3000 mL borosil bottle and moistened with distilled water containing lactose (12.5 and 1% w/w) sodium nitrate and pH was adjusted to 5.0 and sterilized. After sterilization 10 ml of fungal spore suspension (1 x 10^7 spores/ml) was inoculated and incubated at 30 °C for 6 days. All experiments were carried out in two sets.

Extraction of crude enzyme

The enzyme was extracted by mixing by 1 kg of fermented matter with distilled water (1:10 volume) at approximate room temperature and agitated for 1 h on a rotary shaker (180 rpm). The mixture was filtered through a double-layered muslin cloth and cotton wool and it was centrifuged at 10,000 rpm for 10 min. The clear supernatant obtained was used as the crude enzyme.

Enzyme assay

The enzyme was assayed with ONPG as substrate, previously described by Park et al. (1979). One unit of D-galactosidase activity was defined as the amount of the enzyme required to liberate 1 µmole of ONPG per minute under assay conditions.

Estimation of total soluble protein

Total protein content in the supernatant was determined by the method described by Lowry et al. (1951). Bovine serum albumin was used as standard protein.

Table 1. Effect of carbon source on protein content and β-galactosidase production by *A.oryzae.*

Carbon source	Protein content (mg/ml)	Enzyme activity*
Lactose	3.0	306.6
Glucose	3.3	386.6
Maltose	3.2	243.3
Sucrose	2.2	146.6

*Enzyme activity expressed in terms of µmoles of ONP/ml/min. Values represented in the table are mean of two separately conducted experiments.

Optimization of initial pH, incubation temperature and moisture content

Optimum initial pH for enzyme production was determined by adjusting the pH of media to varying ranges (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) with 0.1 M HCl and 0.1 M NaOH. The inoculated substrates were incubated at different temperatures to determine the optimum fermentation temperature for β-galactosidase production (20°C, 25°C, 30°C, 35°C, 40°C and 45°C). Optimal initial moisture content for β-galactosidase production was determined by adjusting the initial moisture content of the fermentation substrates to varying levels (60, 70, 80, 90 and 100%).

Carbon and nitrogen supplements

The ability of the strain to utilize different sugars (lactose, glucose, maltose, and sucrose) as a sole carbon sources (12.5%, w/w) was examined for β-galactosidase production. Various organic and inorganic nitrogen sources (1%, w/w) like peptone, urea, corn steep liquor, sodium nitrate, ammonium nitrate, are used in this study.

RESULTS AND DISCUSSION

Influence of supplementation of different carbon sources including glucose, lactose, sucrose and maltose to wheat bran-rice husk medium for β-galactosidase production by *A.oryzae* was examined and listed in Table 1. Among carbon sources tested in this study glucose served the best carbon source followed by lactose (control), maltose and sucrose for β-galactosidase production by *A.oryzae*. For instance the β-galactosidase activity of *A.oryzae* in the medium supplement with glucose showed 386.6 µmoles of ONP released per ml/min (1.26 fold) than lactose (306.6), and is also better than maltose (243.3) and sucrose (146.6). Basil (1981) observed that addition of certain agricultural by-products (molasses, whey) to growth medium, lactase activity was enhanced. Similarly, Eratt et al. (1984), Sumitra et al. (2004) reported lactose and maltose also improved the (α-amylase) activity on rice bran medium produced by *A. oryzae*. In contrast Srinivas et al. (1994) observed a negative effect up to 3 days of fermentation and then turned positive with presence of lactose as carbon source for α-galactosidase production by *Aspergillus niger.*

Table 2. Effect of nitrogen source on protein content and β-galactosidase production by *A. oryzae.*

Nitrogen source	Protein content (mg/ml)	Enzyme activity*
Control	1.2	80
Peptone	2.9	286.6
Urea	2.1	186.6
CSL	1.6	106.6
Sodium nitrate	3.3	386.6
Ammonium nitrate	1.9	173.3
Ammonium sulphate	2.5	250

*Enzyme activity expressed in terms of µmoles of ONP/ml/min.
Values represented in the table are mean of two separately conducted experiments.

Nitrogen source is an important amendment that effects enzyme production. In the present study six nitrogen sources were taken and assessed for β-galactosidase production and was compared with control (without any nitrogen source) (Table 2). The effectiveness of nitrogen source in supporting β-galactosidase production along with protein secretion and decreased in the following order sodium nitrate > peptone > ammonium sulphate > urea > ammonium nitrate > Corn steep liquor. β-galactosidase activity in culture filtrate of *A.oryzae* grown on medium with supplementation of sodium nitrate was 386.6 µmoles of ONP/ml/min (4.8 fold) than control. Among organic nitrogen sources used in this study, peptone improved the β-galactosidase activity with 286 µmoles of ONP/ml/min (Table 2). Maximum amount of protein was determined in filtrate extracted from media with sodium nitrate as a nitrogen source. Other authors found that ammonium sulfate (0.6% w/w) showed maximum protein induction using rice bran as substrate (Ravinder et al., 2006). Srinivas et al. (1994) reported, among nitrogen sources, urea contributed positively to α-galactosidase production by *A. niger*. Basil (1981) observed when nitrate, phosphate, ammonium, potassium, and sodium ions were added to the growth medium, enhanced α-galactosidase production. Falony et al. (2006) reported that the highest value of lipase activity was obtained in the presence of the combination of ammonium sulphate and urea, in contrast Sumitra et al. (2004), reported ammonium nitrate was best among all the nitrogen sources followed by peptone for production of α-amylase by *A.oryzae*,

The effect of initial pH of fermentation medium for enzyme production was studied in different ranges; 3, 4, 5, 6, 7, 8 and listed in Table 3. The highest enzyme activity was observed at pH 5 and β-galactosidase activity was gradually reduceded up to pH 8. Wang et al. (2004) reported that the β-galactosidase induced by a mutant *Penicillium* sp. in SSF is optimum at pH 5.5 - 6.5. Many kinds of fungi have more acidic pH optima during submerged fermentation. Rajoke et al. (2003) reported highest β-galactosidase by *Klumeromyces marxians* level occurred at pH 5.5. Basil, 1981, reported that production of extra cellular lactase from *Fusarium moniliforme* cultivated either in a whey liquid medium or on wheat bran solid medium, optimum pH was between 4 - 5.

Table 3. Effect of pH on protein and b β-galactosidase production *A.oryzae.*

pH	Protein content (mg/ml)	Enzyme activity*
3.0	1.4	96.6
4.0	2.8	288.8
5.0	3.3	386.6
6.0	3.1	322.2
7.0	2.6	273.3
8.0	1.6	126.6

*Enzyme activity expressed in terms of µmoles of ONP/ml/min.
Values represented in the table are mean of two separately conducted experiments.

The temperature control is the one of the factors that decides the good development of SSF and must be taken into account in an SSF process. The production of β-galactosidase from *A. oryzae* was studied on various incubation temperatures; 20°C, 25°C, 30°C, 35°C, 40°C, and 45°C. The present study reveals that the optimum temperature for β-galactosidase is 30°C (Table 4) for 6 days incubation period. The enzyme production was gradually increased with increasing temperature up to 30°C, and thereafter declined (Table 4). Basil (1981) and Wang et al. (2004) reported similar findings with *F. moniliforme* and *Penicillium*, respectively. Other reports made by Rajoke et al. (2003), Brady et al. (1994) and Furlan et al. (2000) indicate an optimum temperature of 35ºC for the maximum production of β-galactosidase by *K. marxianus.* Ravinder et al. (2006) reported 28ºC as optimum incubation temperature for maximum protein enrichment using rice bran as substrate by *A. oryzae.*

The initial moisture content is a crucial factor affecting the formation of products through solid state fermentation. The importance of moisture content for SSF process has been documented (Pandey, 1992). From experimental results, it was found that 90% moisture content was sufficient for β-galactosidase production for *A. oryzae* on

Table 4. Effect of temperature on protein content and β-galactosidase production by *A. oryzae.*

Temperature (ºC)	Protein content (mg/ml)	Enzyme activity*
20	2.5	266.6
25	3.1	333.3
30	3.3	386.6
35	2.9	288.8
40	2.2	153.3
45	1.6	86.6

*Enzyme activity expressed in terms of µmoles of ONP/ml/min.
Values represented in the table are mean of two separately conducted experiments.

Table 5. Effect of moisture content on protein content and β-galactosidase production by *A. oryzae.*

Moisture content (%)	Protein content (mg/ml)	Enzyme activity*
60	0.8	22.2
70	1.4	96.6
80	2.5	266.6
90	3.3	386.6
100	2.6	286.6

*Enzyme activity expressed in terms of µmoles of ONP/ml/min.
Values represented in the table are mean of two separately conducted experiments.

wheat bran and rice husk as solid substrates (Table 5). With increasing the moisture content, enzyme activity also improved up to 90% (386.6 µmoles of ONP released/ml/min). The β-galactosidase activity ceased at 100% moisture content. Similiar observation was made by Young et al. (1983). According to their observation, 80% moisture content was suitable for solid state fermentation. 70% moisture content was reported by Gowthaman et al., 2001. It is well established that lower moisture levels leads to particles agglomeration, gas transfer limitation and competition from bacteria. Szendefy et al. (2006) reported highest xylanase yields were attained by *A. oryzae* at 83 and 80% initial moisture content on eucalyptus and bagasse pulp, respectively. Ravinder et al. (2006) reported 60% as optimum for maximum protein enrichment using rice bran as substrate for *A. oryzae* The optimum moisture level for the cultivation of *A. niger* on rice was 40%, whereas on coffee pulp the level was 80%, which illustrates the unreliability of moisture level as a parameter for predicting microbial growth (Griffin, 1981). Wang et al. (2004) reported that 50% moisture content was favorable for galactosidase production by a mutant strain *Penicillium* sp. in SSF on wheat bran media.

Conclusion

From the above results, it can be concluded that *A. oryzae* isolated from soil polluted with dairy effluents exhibits β-galactosidase activity. Wheat bran and rice husk were superior solid substrates for supporting growth and β-galactosidase production from *A. oryzae* in solid state fermentation. Glucose and sodium nitrate served as best carbon and nitrogen sources, respectively. The optimum pH, temperature and moisture content were 5, 30ºC and 90% for β-galactosidase production by *A. oryzae,* respectively.

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