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Investigation on α-Galactosidase Production by *Streptomyces griseoloalbus* in a Forcefully Aerated Packed-Bed Bioreactor Operating in Solid-State Fermentation Condition

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Abstract Solid-state fermentation (SSF) was carried out for the production of extracellular α-galactosidase by *Streptomyces griseoloalbus*. Soybean flour was the best solid substrate for α-galactosidase production. Packed-bed bioreactor performed well in enhancing the enzyme yield and resulted in a highest yield of 197.2±0.02 U/gds with a forced aeration of 2 vvm, which was approximately twofold the yield obtained in flasks. The α-galactosidase production was growth-associated, and the highest enzyme yield was obtained after 96 h of incubation. It is significant that this is the first report on α-galactosidase production by a filamentous bacterium in SSF using packed-bed bioreactor.

Keywords α-Galactosidase · Filamentous bacteria · *Streptomyces griseoloalbus* · Solid-state fermentation · Packed-bed bioreactor

Introduction

Solid-state fermentation (SSF) is defined as any fermentation process occurring in the absence or near absence of free water, using an inert substrate or a natural substrate as solid support [1]. It continues to be an interesting alternative to submerged fermentation due to the possibility of using cheap and abundant agro-industrial products as substrates, and the metabolites so obtained are more concentrated, and purification procedures are less costly. The successful development of commercial-scale SSF processes is limited due to the lack of well-established knowledge regarding how to design and operate large-scale bioreactors for SSF. The simplest bioreactor design for SSF, next to trays, involves the packed-bed bioreactors, which typically have static substrate beds and are suited for SSF processes in which mixing is deleterious. Filamentous microorganisms are most exploited in SSF processes because of their ability to grow on complete solid substrate and produce a wide range of extracellular enzymes.

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α-Galactosidase or melibiase (α-D-galactoside galactohydrolase, EC 3.2.1.22) is an exoglycosidase that catalyzes the hydrolysis of terminal non-reducing α-1-6-linked galactosyl residues from a wide range of galacto-oligosaccharides and polysaccharides [2]. α-Galactosidases are of particular interest in view of their many potential biotechnological and medicinal applications. They play a crucial role in improving the nutritional value of legume-based food. They can be applied for the reduction or removal of antinutritive galacto-oligosaccharides such as raffinose family sugars that cause flatulence [3, 4]. Microbial α-galactosidases are useful enzymes in sugar-making industry where they eliminate raffinose and/or stachyose that negatively affect the crystallization of sucrose [5]. Transglycosidase activity was also demonstrated in some of the α-galactosidases [6]. α-Galactosidases have interesting applications in the pulp and paper industry [7]. Though there has been considerable interest in producing α-galactosidase by SSF [8–10], information about α-galactosidase production in SSF bioreactors is limited to the few studies dealing with tray fermentation [11, 12]. We have previously reported α-galactosidase production by the filamentous actinobacterium, *Streptomyces griseoloalbus* under SSF at flask level [13, 14], and the potential of this enzyme in the removal of flatulence-causing oligosaccharides present in legume seed flours [15] was also demonstrated. The goal of the present study was to get a clear picture about the process conditions conducive for enhanced production of α-galactosidase by *S. griseoloalbus* under SSF in a packed-bed bioreactor.

Materials and Methods

Microorganism and Inoculum Preparation

The actinomycete *S. griseoloalbus* producing large amounts of extracellular α-galactosidase was isolated in our laboratory from a soil sample collected from the mangrove regions along the West Coast of India, identified by Institute of Microbial Technology, Chandigarh, India and deposited in Microbial Type Culture Collection, with accession number 7447. The organism was maintained at 4°C on starch casein agar (SCA) slants and was subcultured fortnightly.

Inoculum was prepared by transferring a loopful of culture from fresh SCA slants into sterile medium (100 mL in 250 mL Erlenmeyer flask) composed of 10 g/L locust bean gum, 3 g/L yeast extract, 3 g/L $(NH_4)_2HPO_4$, 1 g/L KH_2PO_4, 0.4 g/L $MgSO_4 \cdot 7H_2O$, and 1 mL of trace element solution. The trace element solution was composed of 0.1 g/L $FeSO_4 \cdot 7H_2O$, 0.1 g/L $MnCl_2 \cdot 4H_2O$, and 0.1 g/L $ZnSO_4 \cdot 7H_2O$. The flasks were incubated at 30°C on a rotary shaker at 175 rpm. A 48-h-old culture containing 3×10^6 CFU/mL was used as the inoculum.

Preparation and Inoculation of Solid Substrate

Packed-bed bioreactor studies were conducted based on the results obtained from the optimization experiments at flask level [13, 14]. Soybean flour, which was found to be the best substrate for α-galactosidase production at flask level [13], was used for enzyme production in packed-bed bioreactor. Fifty grams of soybean flour, in the particle size range of 0.5–1 mm, was moistened with mineral salt solution (containing 1 g/L KH_2PO_4, 0.4 g/L $MgSO_4$; pH 7.0) and thoroughly mixed and autoclaved at 121°C for 30 min. The cooled medium was inoculated with 1.9×10^6 CFU/g of initial dry substrate. The moisture content of the medium after inoculation was 40%.

Solid-State Fermentation in Packed-Bed Bioreactor

The heat-sterilized vertical glass column reactor (length, 22 cm; inner diameter, 5 cm) was aseptically filled with pre-inoculated soybean flour leaving a head space of about 5 cm at the top of the column. Air from an aerator pump was filtered through a glass column filter filled with glass wool before entering the humidification flask. In the humidification flask, air was saturated, and the saturated moist air was then continuously supplied through the bottom of the column. The outlet air from the top of the column was directed to the air exit unit. The diagrammatic representation of the experimental set up is shown in Fig. 1. The aeration rates were varied from 1 to 3 vvm (vessel volume/min), and fermentation was carried out for 120 h at 30±1°C. For comparison, a similar glass column filled with the pre-inoculated substrate but without aeration and a 250-mL Erlenmeyer flask containing 10 g of inoculated substrate were incubated at 30±1°C for 120 h.

Enzyme Extraction

Enzyme extraction was carried out by mixing the fermented matter with distilled water (1:5, *w*/*v*) on a rotary shaker at 200 rpm for 1 h. The thoroughly agitated fermented matter was then filtered through muslin cloth, and the filtrate obtained was centrifuged at 10,000 rpm for 20 min. The resultant supernatant was used as the α-galactosidase enzyme.

Enzyme Assay

The activity of α-galactosidase was routinely determined according to the method of Dey and Pridham [16] using *p*-nitrophenyl α-D-galactopyranoside (*p*NPG), with minor modifications. The *p*NPG hydrolyzing activity was estimated by incubating 100 μL of suitably diluted enzyme with 50 μL of 2 mM *p*NPG and 850 μL of 0.1 M McIlvaine buffer (citrate–Na_2HPO_4, pH 7.0) at 55°C for 10 min. The reaction was terminated by the addition of 2 mL of 1 M sodium carbonate. The *p*-nitrophenol released was estimated spectrophotometrically by absorbance at 400 nm. One unit (U) of enzyme activity was defined as the amount of enzyme in 1 mL of the enzyme preparation that liberated 1 μmol of *p*-nitrophenol per minute under the assay conditions. α-Galactosidase yield under SSF was expressed as units per gram dry fermented substrate.

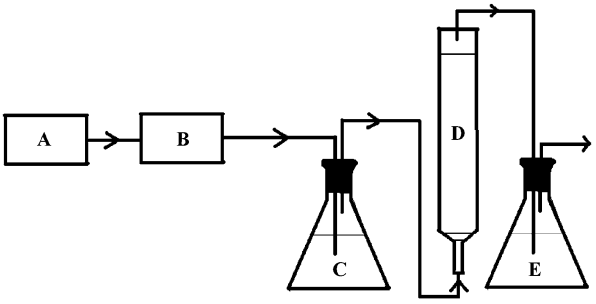

Fig. 1 Diagrammatic representation of packed-bed bioreactor. *A* Aerator pump, *B* air sterilization filter filled with glass wool, *C* air humidification unit, *D* packed-bed column, *E* air exit unit. The *arrow marks* show the direction of air flow

Biomass Estimation

The biomass was estimated by determining the *N*-acetyl glucosamine released from the cell wall of the actinomycete by acid hydrolysis [17] and was expressed as milligram of glucosamine per gram dry fermented substrate.

Statistical Analysis

All experiments were carried out in triplicate to check the reproducibility of results. The data presented here are the mean of triplicate determinations ± SD.

Scanning Electron Microscopy

Growth distribution of *S. griseoloalbus* on soybean flour was examined using a scanning electron microscope (JEOL JSM 5600LV, 115 Japan). The fermented sample (96 h) was adequately dried and mounted on a brass stud followed by a mild gold coating (0.01 μm) and was subjected to electron microscopy at an accelerating voltage of 10 kV.

Results and Discussion

Effect of Aeration on α-Galactosidase Production in Packed-Bed Bioreactor

Different air-flow rates were evaluated in order to identify the best condition for α-galactosidase production by *S. griseoloalbus* in a vertical glass column bioreactor. For enzyme extraction and assay, the entire substrate bed was divided into three equal zones, i.e., upper, middle, and lower, and growth of the culture and enzyme yield was monitored in each segment. The results (Table 1) showed that growth and α-galactosidase yield was highest in the upper zone and lowest in the lower zone, irrespective of the aeration rate provided. Forced aeration of 2 vvm resulted in highest α-galactosidase yield of 197.2± 0.02 U/gds which was approximately twice the yield obtained in flasks. In the control column without any aeration, slight growth and enzyme yield was noted (Table 1), which was restricted to the upper zone, and in the lower zones, visible growth was absent, but a negligible α-galactosidase activity was obtained, which could be due to the constitutive α-galactosidase [18, 19] present in the inoculum. The negligible amount of glucosamine recorded in the lower zones of the control could be due to the presence of culture added as inoculum. The α-galactosidase activity obtained in the upper zone of the control could be due to the oxygen trapped in the head-space at the top of the column, indicating the significance of aeration in enhancing the growth and metabolic activities of the strictly aerobic microorganism. Enhancement of enzyme productivity in traditional SSF by oxygen enrichment has been reported previously by many workers [20–22]. Lower enzyme yields with aeration lower than 2 vvm could be due to inadequate oxygen supply. At higher rates of aeration, though the oxygen supply was higher, the reduction in α-galactosidase yield could be due to reduction in water content of the fermented matter below the critical level, which adversely affected the growth and microbial activity. As a result of end-to-end aeration, axial gradients of oxygen distribution are impossible to prevail in packed-bed bioreactors, and temperature gradients have a greater potential to limit reactor performance than oxygen gradients [23–25]. These axial temperature gradients promote evaporation even if saturated air is used to aerate the column because the water carrying capacity of air

Table 1 Growth and α-galactosidase production by *S. griseoloalbus* in packed-bed bioreactor with different rates of aeration.

Aeration rate (vvm)[a]	α-Galactosidase yield (U/gds)			Glucosamine (mg/gds)		
	Upper zone	Middle zone	Lower zone	Upper zone	Middle zone	Lower zone
1	117.6±0.04	106.7±0.04	99.4±0.03	63.2±0.01	27.3±0.07	21.4±0.27
1.5	148.9±0.07	119.1±0.16	101.3±0.01	81±0.21	42.5±0.09	36.1±0.13
2	197.2±0.11	139.4±0.08	126.3±0.17	83±0.18	47.7±0.04	40.7±0.16
2.5	158.1±0.27	124.2±0.09	111.8±0.25	82.4±0.22	42.5±0.14	40.3±0.17
3	127.2±0.14	108.9±0.06	88.7±0.15	78.6±0.24	28.7±0.31	23±0.26
Control column without aeration	7.3±0.15	2.8±0.16	2.4±0.28	10.9±0.15	2.6±0.23	2.3±0.2
Control (flask)	117.8±0.11			56.7±0.09		

For enzyme extraction and assay, the entire substrate bed was divided into three equal zones, i.e., upper, middle, and lower, and growth of the culture and enzyme yield was monitored in each segment. The data are mean and standard errors of three independent samples with triplicate determinations.

[a] Vessel volume per minute

increases as it heats up. This evaporation can remove 65% of the waste metabolic heat but is undesirable since drying out of the substrate will inhibit growth, while replenishment of water is not practical within an unmixed bed [24]. In packed-bed bioreactor, most of the metabolic heat and CO_2 released from the fermented mash could be removed by forced aeration with humidified air, thus minimizing the rise in temperature of the fermenting substrate. The packed-bed bioreactor offers several advantages over the tray fermentation [26] previously reported by several workers for α-galactosidase production in SSF. It allows better control of fermentation parameters than is possible in trays [27].

Time Course of α-Galactosidase Production

The growth of *S. griseoloalbus* as determined by the glucosamine content of fermented matter (Fig. 2) showed a lag phase up to 24 h followed by the exponential growth phase

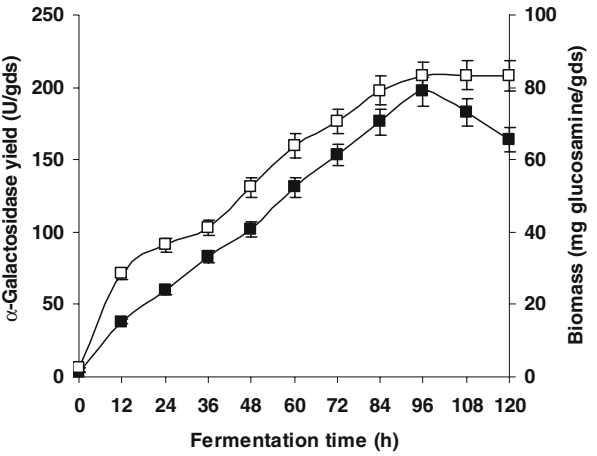

Fig. 2 Fermentation profile of *S. griseoloalbus* showing α-galactosidase yield (*closed squares*) and growth kinetics (*open squares*) at different hours of incubation in a packed-bed bioreactor with a forced aeration of 2 vvm

extending from 24 to 96 h with rapid increase in the biomass, and subsequent to this, there was the stationary phase. There was a very good correlation between the initial trend of α-galactosidase biosynthesis and the growth profile of *S. griseoloalbus*. The initial production of α-galactosidase is constitutive [18, 19], and the action of this constitutive α-galactosidase releases galactose from the substrate which is then utilized as the carbon source for growth of the organism which in turn synthesizes more enzyme. The organism colonized well on the solid substrate and exhibited a good growth on the surface after 36 h of incubation, but enzyme production reached the maximum at 96 h when the organism had established itself well in the deeper layers of the solid medium. After 96 h, the enzyme titer declined due to the depletion of nutrients that were then utilized mostly for cell multiplication and compensation of cell death in the stationary phase. Moreover, the already synthesized enzyme molecules might have undergone proteolytic degradation to complement for cell multiplication. Similar results of growth associated α-galactosidase production in SSF system have been reported by Srinivas et al. [28] from *Aspergillus niger* NCIM 839.

Scanning Electron Micrograph Studies

Figure 3 shows the scanning electron micrograph elucidating the extensive growth of *S. griseoloalbus* on soybean flour. The mycelial morphology of actinomycetes is well suited for the invasive growth on solid substrate. As observed from scanning electron micrograph, the average diameter of the filaments was between 1 and 2 μm. The filaments of *S. griseoloalbus* grown on soybean flour are slender compared to the fungal counterparts such as *A. niger*, which are having filaments of diameter around 3 μm. It is advantageous because slender cells have faster reaction rates without having diffusion problems [14]. It is very significant that this information is relevant for future studies of modeling the diffusion and consumption of oxygen across the network of filamentous microbial growth.

The results obtained during the course of this study indicate the scope for utilization of actinomycetes in SSF systems for harnessing their immense potential as source of exo-enzymes. Continued development of bioreactor models will contribute to the development of SSF technology, enabling it to be routinely evaluated as one of the possible production modes in the production of biotechnological products.

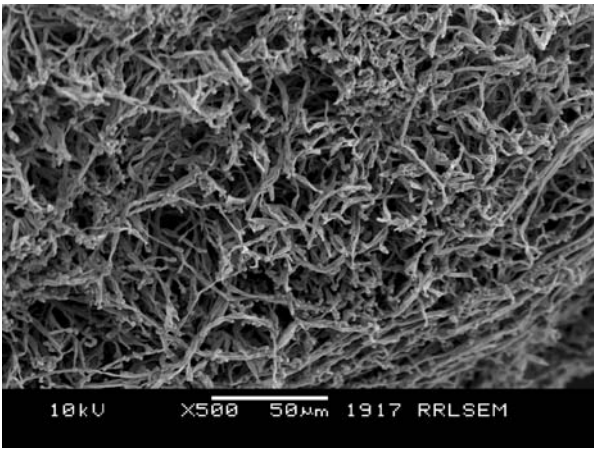

Fig. 3 Scanning electron micrograph showing extensive growth of *S. griseoloalbus* on soybean flour

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