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Effect of mixing events on the production of a thermo-tolerant and acid-stable phytase in a novel solid-state fermentation bioreactor

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ABSTRACT

Mixing constitutes a critical design parameter in solid-state fermentation (SSF) bioreactor and its effect on heat and water transport, and microbial growth in substrate bed can significantly influence overall productivity. Effect of mixing events on the production of a thermo-tolerant and acid stable phytase, by *Rhizopus oryzae*, was studied in a novel SSF bioreactor, using optimized growth medium containing wheat bran and linseed oil cake (1:1) as main substrate. A critical mixing phase was identified, in the absence of which fungal growth led to the onset of heat accumulation and subsequent bed drying. The tensile strength of hyphal bonds between two substrate particles, at this critical phase, was estimated and related to the mixing intensity in the bioreactor that resulted in an optimum working value of 15 rpm. Effect of mixing time on bioreactor performance was also investigated where a 3 min mixing duration, at every 6 h, increased biomass and phytase productivity to 2.2- and 4.5-fold, respectively, in comparison to packed bed bioreactor (PBR). The proposed bioreactor system with intermittent mixing gave superior performance than PBR and tray bioreactor, in terms of maximum bed temperature, axial bed temperature and biomass gradient, average bed moisture content, biomass and phytase productivity.

1. Introduction

Solid-state fermentation (SSF) may be defined as the growth of microorganisms on solid substrate-support in the absence or near absence of free water. In the last two decades, significant effort has been devoted towards heat and mass transfer studies in static SSF processes. Relatively, less information is available regarding the effect of mixing on process productivity and most of the relevant work has been restricted to a specific reactor type, in particular, the rotating drum bioreactor. Moreover, as the popularity of SSF bioreactor designs tends to shift from conventional tray to modular mixed bioreactor system, the optimization of mixing events, and study of their effect on heat and mass transport holds paramount importance. Static beds are generally preferred for SSF operation, since the microenvironment resembles the natural habitat of mycelial organisms [1], and are often accredited for high product yields [2]. However, at high substrate loading, static beds are prone to heat accumulation, heat and mass heterogeneity, bed compaction, shrinkage, high pressure drop across substrate bed and air channelling [3,4]. Addition of inert support such as sugarcane bagasse, perlite, vermiculite, polyurethane and glass fiber [5] constitutes an interesting strategy to address these issues, especially if the microorganism is shear sensitive, but this may result in low substrate loading rates. Mixing circumvents these problems by disruption of hyphal bonds and increasing surface area of fermenting solids exposed to conditioned inlet air [6]. However, aggressive or continuous mixing may be detrimental to microbial growth and result in low productivity [7], substrate agglomeration [8] and energy intensive process. Therefore, at certain stage during fermentation on a static bed involving a fast growing fungus, a trade off between development of mycelial network and transport processes is highly likely, and as a result, mixing events may have to be initiated and carefully designed to avoid process failure, provided the organism is able to tolerate mild agitation.

Most reported studies on mixing have been limited to flasks, columns and reagent bottles at laboratory scale with few exceptions of rotating drum bioreactors [9–12]. One major reason for this could be lack of suitable modular mixed SSF bioreactor system at lab and pilot scale. Moreover, conflicting results have often been reported concerning the effect on enzyme production with mixing action [13–16]. Profound variations in fungal morphology and nature of substrate make it impossible to select a general mixing strategy for a broad spectrum of bioprocesses. Intermittent mixing, however, is an interesting prospect where optimized gentle agitation may strike a balance between kinetic and transport processes to yield high productivity [17–19].

Phytase is an important enzyme in the food or feed industry because

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it catalyzes the hydrolysis of phytate, an antinutrient compound present in cereal, grains etc., thereby obliterating the need for additional supply of nutrients and rendering phosphorus and other vital supplements, present in feed, available to mono gastric animals [20]. In our previous work [21], a phytase from *Rhizopus oryzae* MTCC 1987, possessing novel physico-chemical properties was studied and reported. Growth media, containing wheat bran (WB) and linseed oil cake (LOC) as sole source of nutrients were optimized and utilized for the production of phytase under static condition in flasks and trays. The objective of this work was to evaluate the effect of intermittent mixing on phytase production by *R. oryzae*, in a novel SSF bioreactor. Mixing events were designed based on the tensile strength of *R. oryzae* hyphal bonds; their effect on temperature, moisture and respiratory profiles were investigated. The performance of the proposed SSF bioreactor system under optimized mixing regime was compared with tray bioreactor (TB) and PBR operating at same substrate loading rates.

2. Materials and methods

2.1. Microorganism and inoculum preparation

R. *oryzae* procured from Microbial Type Collection Centre (MTCC), Chandigarh, India, was routinely grown on Potato Dextrose Agar (PDA) for six days at 30 °C. Viable spore suspension (~1×10^6 CFU/mL) was harvested from PDA slants using 0.1% (v/v) Tween 80 solution, to be used as inoculum, in subsequent fermentations.

2.2. Substrate and medium

Wheat bran and linseed oil cake (~1 mm size) were used as solid substrate and were procured from local retail feedstuff outlet from Roorkee (Uttarakhand, India). The media composition was optimized in our laboratory in a previous work [22] and its composition is as follows: wheat bran and linseed oil cake (1:1) supplemented with 2.05% (w/w) mannitol, 2.84% (w/w) ammonium sulphate, 0.38% (w/w) di-potassium hydrogen phosphate/di-sodium hydrogen phosphate and 20% (v/w) mineral salt solution ((w/w); 0.3% NaCl; 0.3% $MgSO_4 \cdot 7H_2O$; pH 5.6).

2.3. SSF bioreactor

SSF was carried out in a single module of a modular bioreactor system with a capacity of 200 g. As shown in Fig. 1B, each module consisted of two chambers separated by a perforated plate. The central pipe facilitated moist air supply and also functioned as a shaft of the mixing apparatus (Fig. 1A and B). Moist air was received in the lower chamber, through an opening in central pipe, and then traversed through the perforated plate into the upper chamber. The upper chamber housed the substrate, and was the site of fermentation. The upper chamber also harboured mixing assembly and consisted of a revolving plate to which a number of mixing blades were fixed, to mix the substrate bed. The revolving plate was connected to central pipe through a gear system. In addition to a variable diameter exhaust vent, three spray nozzles were provided on the wall of upper chamber for addition of inoculum, water, extracting fluids etc. Multiple such modules could be stacked vertically. The proposed bioreactor operated as a PBR when no mixing events were initiated, and as TB when both mixing and forced aeration were absent.

2.4. Solid-state fermentation: set up and operation

Inlet air and ambient room temperature was maintained at 30 °C. Inlet air with 80% relative humidity (RH) was supplied to substrate bed through the central pipe. Desired RH was maintained by passing air through a temperature controlled water column and monitored through a thermo-hygrometer (ZEAL, United Kingdom). An optimized air flow rate of 4 L/min (unpublished data) was maintained with a flowmeter (KDH, England). Growth media were sterilized in situ at 121 °C for 30 min. After cooling the reactor contents to room temperature, medium was inoculated aseptically with spore suspension (~2×10^5 CFU/g dry solid) through spray nozzle in the upper chamber using a peristaltic pump (Applikon, Nederlands) and the fermentation was carried out for 72 h. For mixing experiments, bed was continuously mixed for 10 min immediately after inoculation, whereas no mixing events were initiated when the bioreactor was operated as PBR and TB. A working bed height of 3.5 cm was employed in module and temperature was recorded along the height of bioreactor at every 6 h using thermocouples (Pt 100 sensors (Nutronics, India)), through a sterile opening on the reactor wall. A sketch of experimental set up is depicted in Fig. 1A.

2.5. Analyses

2.5.1. Biomass estimation

Fungal biomass was estimated by determining *N*-acetyl glucosamine released by acid hydrolysis of chitin, a component specific for fungal cell wall [23]. Here, 0.5 g of dried fermented solid was mixed with 2 mL of concentrated sulphuric acid and kept at 30 °C for 24 h. The

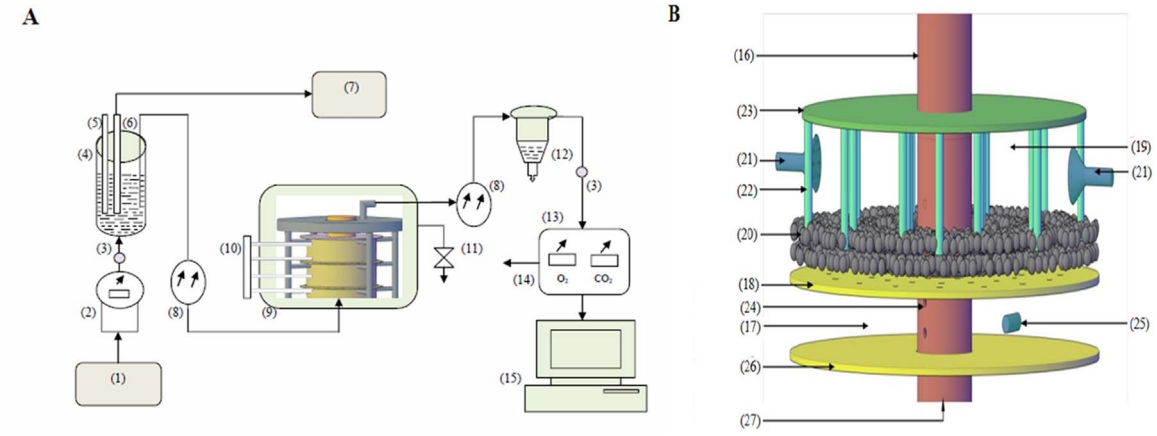

Fig. 1. A and B. Schematic representation of experimental set up and bioreactor module. A. Schematic representation of SSF experimental set up. (1) air compressor (2) air flowmeter (3) air filter (4) humidifier column (5) heating rod (6) cooling coil (7) temperature controller (8) thermo-hygrometer (9) SSF bioreactor(10) thermocouples (11) sampling port (12) moisture remover (13) exit gas analyzer (14) exhaust (15) PC (data logger). B. Schematic representation of a single module of SSF bioreactor. (16) central pipe (17) lower chamber (18) perforated disc (19) upper chamber (20) substrate bed (21) spray nozzles (22) mixing blades (23) revolving plates (24) opening for steam/moistened air (25) exit channel at lower chamber (26) bottom plate of module (27) air inlet to central pipe.