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Fungal biosynthesis of endochitinase and chitobiase in solid state fermentation and their application for the production of *N*-acetyl-D-glucosamine from colloidal chitin

Parameswaran Binod [a], Chandran Sandhya [a], Pradeep Suma [a], George Szakacs [b], Ashok Pandey [a,*]

[a] *Biotechnology Division, Regional Research Laboratory, CSIR, Trivandrum- 695 019, India*
[b] *Department of Agricultural Chemical Technology, Technical University of Budapest, Hungary*

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Abstract

The present study was directed to the production of *N*-acetyl-D-glucosamine using endochitinase and chitobiase from fungal cultures in solid culturing. Fifteen fungal strains were evaluated for endochitinase and chitobiase production under solid-state fermentation using agro-industrial residues, of which *Penicillium aculeatum* NRRL 2129 showed maximum endochitinase activity whereas *Trichoderma harzianum* TUBF 927 showed maximum chitobiase activity. Eleven substrates, alone and in combination with chitin, were evaluated for the enzyme production. Optimization of physico-chemical parameters such as incubation period and initial moisture content, and nutritional parameters such as chitin source, inorganic and organic nitrogen sources, were carried out. Optimization resulted in more than 3-fold increase in endochitinase production (from 3.5 to 12.53 U/g dry weight of substrate) and about 1.5-fold increase in chitobiase production (from 1.6 to 2.25 U/g dry weight of substrate). Studies on the degradation of colloidal chitin to *N*-acetyl-D-glucosamine showed improved efficiency when endochitinase and chitobiase were used in combination.
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1. Introduction

N-Acetyl-D-glcosamine (NAG), the monomer of chitin, is a valuable pharmacological agent in the treatment of a wide variety of ailments. It has also been used as a food supplement. It is more suitable than D-glucosamine for oral administration due to its sweet taste (Sashiwa et al., 2002). Since NAG is produced by the acid hydrolysis of chitin, it has some disadvantages, such as low yield (below 65%) and acidic waste by the usage of concentrated hydrochloric acid and the cost of production is high. In this context, chitinases can be good alternative, which hydrolyse chitin into NAG. Recently, the production of NAG from α-chitin or β-chitin by the usage of crude enzymes has been reported (Sashiwa et al., 2002). Chitin together with its derivatives can also be used as agrochemicals. However, there is only little effort to clarify its utilization in the food industry (Haki and Rakshit, 2003).

Chitinases (EC 3.2.1.14) are a group of complex hydrolytic enzymes that catalyze depolymerisation of chitin. Chitin degradation can be initiated by chitinases (endochitinase) to oligo *N*-acetyl glucosamine chains. The oligomers, tri NAG and di NAG are, subsequently degraded to NAG monomers by chitobiase also called β-*N*-acetylglucosaminidase (EC 3.2.1.52). Several reports reveal that fungi are excellent producers of extracellular chitinases, mainly species of genera *Aspergillus* and *Penicillium*

* Corresponding author. Tel.: +91 471 2495949/2515279; fax: +91 471 2491712.
E-mail addresses: pandey@csrrltrd.ren.nic.in, ashokpandey56@yahoo.co.in (A. Pandey).

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(Hodge et al., 1995; Hunkova et al., 1996; Binod et al., 2005). It is reported that some commercial crude enzyme preparation of fungal origin are rich in chitobiase and hence provided a convenient source for its isolation (Scigelova and Crout, 1999). Most of the microorganisms have the ability to produce extracellular chitobiase. However, this could also be produced intracellularly by *Candida albicans*, *Aspergillus niger*, *Dictyostelium discoideum* and *Phycomyces blakesleeanus*. (Berkeley et al., 1973; Sullivan et al., 1984). Membrane bound chitobiase has also been reported, e.g., from *Vibrio* (Soto-Gill and Zyskind, 1989) and *Alteromonas* (Tsujibo et al., 1995a) cultures.

Most of the studies on chitinase production have been carried out in submerged fermentation (SmF), although some attempts have been made in more recent times on solid-state fermentation (SSF). SSF offers several economic advantages over conventional SmF such as use of simple growth and production media comprising agro-industrial residues, simple processes, use of low volume equipment (lower cost), and are yet effective by providing high product titres (Pandey et al., 2000; Pandey, 1992, 2003; Binod et al., 2004). Among the various groups of microorganisms used in SSF, the filamentous fungi are the most widely exploited because of their ability to produce a wide range of extracellular enzymes. SSF offers a cost effective technique for the production of chitinase (Pandey et al., 1999; Sandhya et al., 2005).

The aim of the present study was to find out a cost effective, eco-friendly enzymatic method for the production of NAG. The first objective was to produce chitinase and chitobiase in solid-state fermentation (SSF) and then to check the feasibility of these enzymes for the production of NAG. SSF involved selection of suitable microbial cultures and substrate, and optimization of physico-chemical and nutritional parameters to enhance the enzymes production.

2. Methods

2.1. Microorganisms and maintenance

Beauveria bassiana ATCC 7153, *B. bassiana* ATCC 481023, *Trichoderma harzianum* TUBF-690, *T. harzianum* TUBF 699, *T. harzianum*TUBF 700, *T. harzianum* TUBF 763, *T. harzianum* TUBF 773, *T. harzianum* TUBF 789, *T. harzianum* TUBF 927, *T. harzianum* TUBF 947, *T. harzianum* TUBF 967, *Penicillium aculeatum* NRRL 2129, *Penicillium funiculosum* NRRL 1132, *P. funiculosum* NRRL 1768, and *Penicillium pinophilum* NRRL 1066 were used in this work. They were purchased from the American Type Culture Collection (ATCC), Manassas, Virginia, from the Northern Regional Research Center (NRRL), USDA, Peoria, Illinois and from the Technical University of Budapest (TUB), Budapest, Hungary collections. Spores suspension was obtained by growing the fungus in potato dextrose agar (PDA) at 30 °C for one week and harvested the spore with 0.1% Tween-80 in distilled water and spore count was adjusted to 5×10^7 spores ml^{-1}.

2.2. Screening of fungal strains

The fungal strains evaluation for endochitinase and chitobiase production was carried out in cotton-plugged 250 ml Erlenmeyer flasks. Five grams of wheat bran containing 1% (w/w) chitin flakes in 250 ml Erlenmeyer flasks were moistened with 10 ml of salt solution containing (%, w/v) 0.1 $NaNO_3$, 0.5 KNO_3, 0.5 $(NH_4)_2HPO_4$, 0.1 $MgSO_4 \times 7H_2O$, 0.1 NaCl and 0.1 (v/v) trace element solution, pH 5.0. The composition of the trace element solution (%, w/v) was the following: 0.08 $MnSO_4$, 0.17 $ZnSO_4 \times 7H_2O$, and 0.25 $FeSO_4 \times 7H_2O$. The medium was autoclaved at 121 °C for 15 min, cooled, inoculated with 1 ml of spore inoculum prepared as above and incubated at 30 °C for 72 h without shaking (still culture). The best endochitinase and chitobiase producing strains were selected for further process parameter optimization.

2.3. Screening of Agro-industrial residues

Eleven agro-industrial residues such as wheat bran (WB), rice husk (RH), prawn shell powder (PSP), coffee husk (CH), coconut oil cake (COC), ground nut oil cake (GOC), sesame oil cake (SOC), palm kernel cake (PKC), tamarind seed powder (TSP), jack fruit seed powder (JSP) and soybean meal (SM) alone, and in combination with 1% (w/w) chitin flakes were screened for endochitinase production by *P. aculeatum* NRRL 2129 and chitobiase by *T. harzianum* TUBF 927. The protocol followed was same as described above.

2.4. Process and nutritional parameter optimization

Different incubation periods (24–96 h) were employed to study the endochitinase and chitobiase production. Optimum initial moisture content was determined by varying the volume of distilled water in the medium so as to achieve 50–70% initial moisture. To optimize the chitin sources, SSF medium was supplemented with different chitin sources (1%, w/w) such as chitin flakes (CF), colloidal chitin (CC), prawn shell powder (PSP) and cell wall of *R. oryzae* (RO) *A. ficuum* (AF), and *C. gloeosporioides* (CG). For the isolation of chitin from fungal cell walls, the different fungal spores were inoculated separately in PDB (potato-dextrose-broth) and incubated for six days at 30 °C. It was then autoclaved at 121 °C for 15 min and filtered through muslin cloth. It was then dispersed in water, homogenized, filtered and washed three times with water, followed by washing with ethanol and acetone. The cell wall obtained was dried in hot air oven at 70 °C for 24 h and powdered. The effect of additional nitrogen sources were studied by adding 1% (w/w) organic and inorganic nitrogen sources such as yeast extract, casein, peptone, tryptone, beef extract, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $NaNO_3$ and urea to the medium.

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