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Chitobiose production by using a novel thermostable chitinase from *Bacillus licheniformis* strain JS isolated from a mushroom bed

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ABSTRACT

The thermophilic *Bacillus licheniformis* strain JS was isolated from a bed of mushrooms, *Pleurotus sajor-caju*. The organism could produce a novel, single-component, thermostable chitinase that was purified by ion-exchange chromatography using DEAE-cellulose in 7.64% yield and in an 8.1-fold enhancement in purity. Its molecular weight is 22 kDa. The enzyme is a chitobiosidase, since the chitin hydrolysate is N^{I},N^{II}-diacetylchitobiose. The optimum temperature for enzyme activity is 55 °C, and the optimum pH is 8.0. It was completely inhibited by Hg^{2+} ions whereas Co^{2+} ions served as an activator. The thermostability of this enzyme is important in the bioconversion of chitinous waste and for the production of chitooligosaccharides.

1. Introduction

Chitin, a β-1→4 linked polymer of *N*-acetylglucosamine, is an abundant biopolymer[1] being found in the outer shell of crustaceans, in the exoskeleton of insects, and in the cell wall of most fungi and nematodes, etc.[2] Chitinous wastes are also produced in large amounts by industries such as the seafood processing industry. These wastes sometimes become an environmental threat due to their accumulation and slow degradation. Therefore, organisms that produce thermostable chitinases can bring about bioremediation of these wastes and at the same time help maintain the carbon and nitrogen biogeochemical cycles in the environment.[3] During the microbial degradation of chitin using chitinase (E.C. 3.2.1.14), chitooligosaccharides are produced, which are further degraded to *N*-acetylglucosamine by chitobiase (E.C. 3.2.1.30).[4] Chitooligosaccharides possess a variety of therapeutic uses such as antibacterials, antitumor agents, and immuno-enhancing drugs.[5]

Chitin can be hydrolyzed into oligomers and monomers by acid hydrolysis. However, the enzymatic process is preferable in order to control the extent of hydrolysis and the consistency of the product. *Bacillus* sp. WY22 produces an extracellular chitinase that hydrolyses chitin to chitotriose as the major product from colloidal chitin and glycol chitin.[6] Tanaka et al. reported an extracellular chitinase from the hyperthermophilic archaeon *Pyrococcus kodakarensis* KOD1, producing chitobiose as the major end product.[7] Several reports exist on multiple forms of chitinases of the *Bacillus* genus that produce *N*-acetylglucosamine as an end product of chitin hydrolysis.[8–12]

This investigation documents the purification and characterization of a novel thermostable chitinase from a *Bacillus licheniformis* strain JS, which was isolated from a mushroom cultivated bed, for the production of chitobiose.

2. Materials and methods

2.1. Isolation and identification of the thermophilic microorganism

The isolation of the organisms was from a mushroom cultivation bed left over after the production of oyster mushroom *Pleurotus sajor-caju*. This mushroom was grown on a paddy straw bed at room temperature, which is unlike the *Agaricus* mushroom (also known as the button mushroom), which grows at a much lower temperature on a compost bed. The sample from the bed was enriched in a mineral base medium ($NaNO_3$, 0.3%; K_2HPO_4, 0.1%; KCl, 0.05%; $MgSO_4{\cdot}7H_2O$, 0.05%; $FeSO_4$, 0.001%) containing 3% colloidal chitin as the carbon source (pH 7.0). The colloidal chitin was prepared by the method of Hsu and Lockwood.[13] The flask was incubated at 55 °C on a rotary shaking incubator. After every 24-h interval 0.1-mL sample was inoculated on a solid medium containing colloidal chitin. These samples were incubated at 50 °C and observed for zone of hydrolysis around the colonies. The colony showing maximum zone of hydrolysis was isolated and was later identified on the basis of morphological and biochemical tests according to the methods described in *Bergey's*

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Manual of Systematic Bacteriology.[14] Sequencing of the 16S r-DNA was also carried out (by GeneOmbio Technology, Pune, India), and the results were submitted to Genebank (Accession No. GU172369) and analyzed on the NCBI server using BLAST tool. The phylogenetic tree was constructed by MEGA4 software.[15]

2.2. Thermophilic chitinase production

The chitinase production was done in the same mineral-based medium containing colloidal chitin that was used for isolation. The effect of different concentrations of colloidal chitin on chitinase production by *B. licheniformis* strain JS was studied when the culture was inoculated into media containing 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% colloidal chitin. The incubation was carried out at 50 °C for 72 h, and the chitinase activity was quantitated by methods described in the enzyme assay section. The effect of other sugars such as *N*-acetylglucosamine, lactose, sucrose, galactose, glucose, mellibiose, raffinose, cellulose, and cellobiose at concentrations of 1% was monitored for chitinase production. The protein content was estimated by the method of Lowry et al.[16] using bovine serum albumin (BSA) as the standard.

2.3. Purification of thermophilic chitinase

The organism was inoculated into the medium containing 2% colloidal chitin. After 72 h of incubation the liquid medium was centrifuged at 2795*g* for 20 min to obtain a cell-free medium that was precipitated overnight by ammonium sulfate at 4 °C. The precipitate was collected after centrifugation at 11180*g* for 30 min at 4 °C. It was dissolved in 25 mM sodium phosphate buffer (pH 7.4) and dialyzed overnight against the same buffer at 4 °C. The enzyme in the dialysate was separated by ion-exchange chromatography on a DEAE-cellulose column and eluted with a NaCl gradient (0–0.5 M) at a flow rate of 0.5 mL min^{-1}. The protein content was measured by the method of Lowry et al.[16] using BSA as the standard. The purity of fractions showing chitinase activity was analyzed by SDS–PAGE.

2.4. Electrophoresis

SDS–PAGE of the protein fractions was carried out by the method of Laemmli.[17] The molecular mass of the chitinase was determined on a scale calibrated with standard molecular marker proteins (phosphorylase b, 98 kDa; BSA, 66 kDa; ovalalbumin, 43 kDa; carbonic anhydrase, 29 kDa; and soyabean trypsin inhibitor, 20 kDa).

2.5. Enzyme assay

The chitinase assay was carried out using the assay mixture containing 1 mL of 0.5% colloidal chitin, 0.5 mL of 25 mM sodium phosphate buffer (pH 7.4), and 0.5 mL of enzyme solution. This was incubated for 1 h at 60 °C. The released reducing sugar was quantitated by the dinitro salicylic acid method[18] relative to *N*-acetyl-β-D-glucosamine standards from 100 to 1000 μg mL^{-1} concentration. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per min from colloidal chitin under standard assay conditions.

The value of K_m was obtained graphically using the software 'Sigma Plot v.10' (Systat, Inc., USA). The substrate specificity of the enzyme was studied with the various substrates including glycol chitin, glycol chitosan, cellulose, and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside (*p*NP GlcNAc). Chitinase activity was expressed as reducing sugar for all the substrates, except for *p*NP GlcNAc, where it was expressed as release of *p*-nitrophenol per min, which was measured as absorbance at 410 nm.

2.6. Characterization of thermophilic chitinase

The effect of pH on enzyme activity was studied over a range of pH 3.0–10.0 using citrate–phosphate buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–8.0), and glycine–NaOH buffer (pH 9.0–10.0). The effect of temperature on enzyme activity was studied between temperatures of 10 and 90 °C. The effect of temperature on stability of chitinase was determined by exposure of the enzyme solutions to different temperatures for 3 h in 25 mM sodium phosphate buffer, pH 8.0. The effect of different metal ions was studied by pre-incubating the enzyme for 10 min in 25 mM sodium phosphate buffer of pH 8.0 containing metal ions Ca^{2+}, Hg^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, and Co^{2+} by using their respective water-soluble salts at 10 mM concentration. Residual enzyme activity was then measured under standard assay conditions using colloidal chitin as substrate.

2.7. Analysis of hydrolyzed product

The purified enzyme was incubated with colloidal chitin at 60 °C. At every 1-h interval, aliquots of the reaction mixture were analyzed by TLC and HPLC. The TLC analysis was carried out using 26:14:7:2 EtOAc–2-PrOH–H_2O–pyridine as the mobile phase, and the products were detected by spraying with aniline–diphenylamine reagent. In case of HPLC studies, a Waters 2690 system was used having a C_{18} column (4.6 × 250 mm). Elution was done with 70% acetonitrile at a flow rate of 1.0 mL min^{-1}. The elution was monitored by measuring UV A_{215} with a Waters Lambda-Max model LC Spectrophotometer.

2.8. Statistical analysis

All the experiments were performed independently in triplicate, and the values given are the means of the values thus obtained. Standard deviation was within 10% (GraphPad InStat version 3.00, GraphPad Software, San Diego California USA).

3. Results

3.1. Isolation and identification of the thermophilic microorganism

The thermophilic bacterium, *B. licheniformis* strain JS, which was isolated in our laboratory, produced a thermostable chitinase when grown on colloidal chitin as the limiting substrate. The microorganism was identified by standard morphological, physiological, and biochemical tests according to *Bergey's Manual of Systematic Bacteriology*. The organism was a Gram-positive rod, and the colonies showed extensive ramifications with hairlike projections around at the edge of colony, when observed under a light microscope. The organism was capable of growing in a wide range of temperatures between 30 and 65 °C, with the optimum temperature of growth being 50 °C.

The identification of the microorganism was further confirmed with partial 16S rDNA sequencing having a length of 1344 bp nucleotide. The partial sequence of 16S rDNA was deposited in the Gene Bank (Accession No. GU172369). The phylogenetic relation of *B. licheniformis* strain JS is shown in Figure 1. The strain was deposited with National Collection of Industrial Microorganisms (NCIM) (National Chemical Laboratory, Pune, India) as *B. licheniformis* NCIM 5343.

3.2. Production of thermophilic chitinase

It was observed that in the mineral-based medium containing colloidal chitin, *B. licheniformis* strain JS produced maximum