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Enzymatic degradation of chitosan and production of D-glucosamine by solid substrate fermentation of exo-β-D-glucosaminidase (exochitosanase) by *Penicillium decumbens* CFRNT15

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ABSTRACT

Exo-β-D-glucosaminidase is an enzyme acting on β-(1 → 4) linkage of chitosan from its non-reducing end and produces D-glucosamine as the exclusive end product. Solid substrate fermentation (SSF) production of exo-β-D-glucosaminidase by a novel isolate of *Penicillium decumbens* (CFRNT15) was optimized employing Box-Behnken design and response surface methodology. The optimized conditions for maximal production of exo-β-D-glucosaminidase were 22 ± 1 °C temperature, 75% (w/w) moisture, 120 h fermentation, 5.0% (w/w) chitosan, 0.05% (w/w) $CaCl_2$, 0.5% (w/w) $MgSO_4$, and 15.1% (v/v) inoculum. The close agreement of experiential results with the model prediction showed that the model was precise and dependable for predicting the exo-β-D-glucosaminidase production. Statistical optimization resulted in an overall increase of exo-β-D-glucosaminidase activity from 116.7 ± 4.6 to 2335.1 ± 12.4 unit/g initial dry substrate. Crude exo-β-D-glucosaminidase was found to have optimum activity at 42 ± 1 °C and pH 5.6. Crude exo-β-D-glucosaminidase produced the maximum D-glucosamine yield of 410–546 μM from different α-chitosan substrates hydrolysis. Thin layer chromatography analysis revealed that D-glucosamine was the sole end product of the chitosan hydrolysis. These results indicated the potential of *P. decumbens* for the production of the exo-β-D-glucosaminidase employing the cost-effective SSF process and the significance of the exo-β-D-glucosaminidase for the preparation of commercially important D-glucosamine and to cope seafood processing chitinous biomaterials.

Introduction

Chitosan is a linear co-polymer of β-(1 → 4) linked *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) units with latter usually exceeding >70% and is commercially obtained by *N*-deacetylation of chitin that has been isolated from an abundant source of seafood (shrimp, crab, lobster, etc.) processing by-products (Kurita, 2006; Muzzarelli et al., 2012; Thadathil and Velappan, 2014). Chemical structure, availability, methods of production and applications of chitin and chitosan are presented in our recent publications (Suresh and Kumar 2012; Thadathil and Velappan, 2014; Nidheesh and Suresh, 2014). Chitosan and its derivatives (hydrolytic or chemical) have high commercial value due to their versatile biofunctional properties and the broad spectrum of applications in many fields (Muzzarelli et al., 2012; Thadathil and Velappan, 2014). Even though, a number of reports published on the potential high-value applications of chitin and chitosan, no real commercial development has occurred thus far (Olsen et al., 2014).

D-glucosamine (2-amino-2-deoxy-D-glucose), an amino monosaccharide the basic structural unit of chitosan, is a useful medicament in various fields of medicine and dentistry (Sitanggang et al., 2012; Igawa et al., 2014). D-glucosamine is known to possess many biological roles and has received a special attention for the treatment of osteoarthritis (Krikham and Samarasinghe, 2009). Research on D-glucosamine in antipain effect in patients with osteoarthritis has highlighted its ability to suppress the pain associated with a disability of osteoarthritis. This amino sugar synthesized naturally in the body and found in mammalian articular cartilage and has been evaluated as a food supplement or nutraceutical (Hiroshi, 2011). D-Glucosamine also finds applications in wound healing, bone regeneration and antibacterial agent in

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dentistry (Muzzarelli et al., 2012). Because of its widespread applications, there are emerging commercial interests in the commercialized production of D-glucosamine. Commercially available D-glucosamine is mainly derived from marine crustacean chitin, by the hydrolysis with concentrated HCl (Krikham and Samarasinghe, 2009; Sitanggang et al., 2012). Besides to acid hydrolysis, direct hydrochlorination of chitin can be applied for D-glucosamine preparation (Chang et al., 2011). However, these chemical methods of D-glucosamine preparation has several drawbacks, including low yield, high cost, formation of acidic effluent, environmental pollution due to acidic effluent, etc. Thus, biological methods especially enzymatic hydrolysis of chitosan for the production of D-glucosamine has received considerable attention, in order to apply mild reaction conditions as well as product consistency (Sashiwa et al., 2003; Sujata et al., 2011). The enzymatic hydrolysis of chitosan to D-glucosamine is carried out by specific chitosan hydrolyzing enzyme 'exo-β-D-glucosaminidase'. However, no real commercial progress has occurred so far on enzymatic production of D-glucosamine, mainly due to the non-availability of exo-β-D-glucosaminidase commercially (Sujata et al., 2011).

Exo-β-D-glucosaminidase (EC 3.2.1.165) is a hydrolytic enzyme acting on β-(1 → 4) glycosidic linkage of chitosan or chitosan oligosaccharides from their non-reducing termini and successively removes D-glucosamine as the exclusive end product (Thadathil and Velappan, 2014). Exo-β-D-glucosaminidase is also known as exo-1,4-β-D-glucosaminidase, exochitosanase and GlcNase. Some of the exo-β-D-glucosaminidase have also shown transglycosylation activity beneficial for the synthesis of designer oligosaccharides. However, very few chitosan hydrolytic enzymes have been shown to have only exo-β-D-glucosaminidase activity (Sujata et al., 2011). Despite the availability of a number of reports on the purification and characterization of exo-β-D-glucosaminidase from fungi (Nogawa et al., 1998; Zhang et al., 2000; Ji et al., 2003; Chen et al., 2005; Cheng et al., 2006; Chao et al., 2013) and bacteria (Nanjo et al., 1990; Tanaka et al., 2003); to the best our knowledge no reports are available on the process optimization and production of exo-β-D-glucosaminidase by any fermentation methods.

Solid-state (substrate) fermentation (SSF) is one of the oldest cultivation techniques of microorganisms and has received a considerable attention in recent years by the biotechnological industry owing to the potential for the production of commercially important various metabolites (Singhania et al., 2009). The commercial advantages of the SSF over the conventional submerged fermentation (SmF) for the cost-effective production of secondary metabolites and extracellular enzymes especially from the filamentous fungi has been reported (Pandey et al., 2008; Singhania et al., 2009; Suresh et al., 2011a,b; Sharma and Aror, 2011; da Silva et al., 2012; Mazotto et al., 2013; Patil and Yadhav, 2014). Several inexpensive substrates, mainly agro industrial by-products/co-products in the solid media have reported for the SSF production of fungal extracellular enzymes. However, the wheat bran was reported as one of the best substrates for fungal enzymes production in the SSF owing to its favorable properties as a solid substrate for mold growth (Joseph et al., 2010; Suresh et al., 2011b,da Silva et al., 2012).

In view of advantages of statistical experimental approaches and response surface methodology (RSM) over traditional 'one variable at a time' methods (Joseph et al., 2010; Suresh, 2011a; Patil and Yadhav, 2014; Thadathil et al., 2014; Wu and Nian, 2014) the current study was aimed at the optimization of process variables by RSM to maximize the exo-β-D-glucosaminidase yield from a soil isolate of *Penicillium decumbens* CFRNT15 in the cost-effective SSF process. Further, the chitosan degrading activity of the crude enzyme to produce monomeric D-glucosamine was investigated.

Materials and methods

Materials

Standards for monomeric and oligomeric GlcN (Associates of Cape Cod Inc., Associates of Cape Cod, Inc. East Falmouth, MA, USA), low molecular weight chitosan and glycol chitosan (Sigma Chemical Co., St. Louis, USA), HiCarboTM Kit, HiPurATM DNA-XpressTM Kit, Polymerase chain reaction (PCR) purification kit, potato dextrose agar (PDA) and potato dextrose broth (PDB) (HiMedia, Mumbai, India) and D-glucosamine (Sisco Research Laboratory, Mumbai, India), Taq DNA polymerase and other molecular biology reagents (Genei, Bengaluru, India) and PCR primers (BioServe, Hyderabad, India) were procured. All other chemicals and reagents were of analytical grade.

Enrichment isolation and screening of chitosan hydrolysing microorganisms

A mineral medium (MM) (g/l, Na_2HPO_4, 1.3; KH_2PO_4, 3.0; NaCl, 5.0; NH_4Cl, 1.0; $MgSO_4$, 0.24; $CaCl_2$, 0.01; pH 6.5) containing chitosan powder (~0.5%, w/v) was used as a selective medium to enrich fastidious chitosan degrading microorganisms. Various samples including, water, soil, marine water, marine sediment, decomposing crustaceans, etc. were collected from different parts of South India and transported to the laboratory under chilled condition (~4 °C) in an ice box, and processed within 48 h. The prepared medium was inoculated with 0.5 ml of sediment/solid sample after 1:10 (w/v) dilution with sterile physiological saline (0.85% NaCl, w/v) or 0.5 ml of undiluted water sample to 10 ml to medium taken in 100 ml Erlenmeyer conical flasks for the enrichment of chitosan degrading microorganisms. The inoculated was incubated flasks at 32 ± 2 °C for two weeks. A full loop aliquots from the enriched samples were streaked on colloidal chitosan-mineral agar (CCMA, pH 6.0) plates and incubated at 32±2 °C for one week in order to obtain pure cultures. The CCMA was prepared by mixing 25 ml of 2% (w/v) colloidal chitosan (prepared in 1%, v/v acetic acid) with 75 ml of MM and 2 g agar. Colonies that showed a zone of clearance against the white-creamy background were regarded as chitosan hydrolysing microorganisms. They were selected and re-streaked on CCMA plates until pure cultures obtained. The purified cultures were maintained on PDA agar slants containing 0.01% (w/v) colloidal chitosan under refrigeration condition at the culture collection of the Meat and Marine Sciences Department, CSIR-CFTRI, Mysore, India and subcultured every two months.

All the isolates obtained from the enriched samples were initially screened for extracellular production (qualitative) of chitosan hydrolysing enzyme by growing them on CMMA plates (~5 mm thickness) for 5 days at 32 ± 2 °C. Then the diameters of colony (DC) and clearance zones (DZ) were measured and activity ratios (DZ/DC) calculated. Five fungal isolates, which showed comparatively high DZ/DC ratio were further subjected to SSF production (quantitative) of extracellular chitosan hydrolysing enzyme (exo-β-D-glucosaminidase) using a commercial wheat bran medium [commercial wheat bran 5.0 g, chitosan powder 50 mg, initial moisture content 70% (w/w), initial pH 7.0] at 32 ± 2 °C for 48 h as described below under Section 2.4. A fungal culture (CFRNT15) showing the highest production of chitosan hydrolysing enzyme was further characterised.

Identification of chitosan hydrolysing fungal isolate CFRNT15

The culture (CFRNT15) was characterised for morphological features after growing on a PDA plate for 14 days. The conidial head