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Chitooligomers preparation by chitosanase produced under solid state fermentation using shrimp by-products as substrate

T. Nidheesh, Gaurav Kumar Pal, P.V. Suresh*

Academy of Scientific and Innovative Research, Meat and Marine Sciences Department, CSIR-Central Food Technological Research Institute, Mysore 570020, India

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ABSTRACT

Solid state fermentation (SSF) conditions were statistically optimized for the production of chitosanase by *Purpureocillium lilacinum* CFRNT12 using shrimp by-products as substrate. Central composite design and response surface methodology were applied to evaluate the effect of variables and their optimization. Incubation temperature, incubation time, concentration of inoculum and yeast extract were found to influence the chitosanase production significantly. The R^2 value of 0.94 indicates the aptness of the model. The level of variables for optimal production of chitosanase was 32 ± 1 °C temperature, 96 h incubation, 10.5% (w/v) inoculum, 1.05% (w/w) yeast extract and 65% (w/w) moisture content. The chitosanase production was found to increase from 2.34 ± 0.07 to 41.78 ± 0.73 units/g initial dry substrate after optimization. The crude chitosanase produced 4.43 mM of chitooligomers as exclusive end product from colloidal chitosan hydrolysis. These results indicate the potential of *P. lilacinum* CFRNT12 for the chitosanase production employing cost effective SSF using shrimp by-products.

1. Introduction

Seafood processing, one of the major agro-industry in tropical and subtropical countries based on aquaculture and marine fishing, has assumed great importance during the last couple of decades due to the ever increasing demand for processed shrimps in the international market (Kumar & Suresh, 2014; Nidheesh & Suresh, 2014). The industrial processing of shrimp involves the removal of cephalothorax and carapace as non-edible by-products, which accounts for 50–70% by total volume of raw materials, depending upon the species and processing methods (Kumar & Suresh, 2014; Nidheesh & Suresh, 2014; Olsen, Toppe, & Karunasagar, 2014). Annually, India produces more than 100,000 tons of shrimp by-products, of which only an insignificant quantity is used for the isolation of chitin. The rest of the produce is either discarded into the coastal and nearshore area, or under-utilized (Kumar & Suresh, 2014; Nidheesh & Suresh, 2014). Considering the potentials of shrimp by-products and society's current levels of awareness on negative impact of pollution, possible utilization of shrimp by-products as media substrate for microbial fermentation and production of economically useful products is proposed as a cost effective and ecofriendly waste management approach (Jayakumar, Prabaharan, Nair, & Tamura, 2010; Kumar & Suresh, 2014; Suresh, 2012; Wang, Hsu, & Liang, 2010; Wang, Liang, & Yen, 2011; Wang, Peng, Liang, & Liu, 2008). In spite of number of study reports available on the utilization of shrimp by-products as fermentation media, so far no commercial venture based on such by-products is known. Thus an ideal bioprocess, at commercial scale, for efficient utilization of shrimp by-products is warranted

Chitosan, the linear cationic (1-4)-2-amino-2-deoxy-β-D-glucan produced from chitin by partial deacetylation, is a prominent polysaccharide owing to its biocompatibility (Jayakumar et al., 2010; Muzzarelli et al., 2012). Chitooligomers (COS) are readily soluble in water and exert, better than chitosan, antimicrobial, antitumor and immunomodulatory activities (Kim & Rajapakse, 2005; Muzzarelli, 2009; Thadathil & Velappan, 2014; Wee, Reddy, Chung, & Ryu, 2009). Thusly, the production of chitooligomers has received increased attention in the food and biomedical industries (Gao, Ju, Jung, & Park, 2008; Nguyen et al., 2014; Pechsrichuang, Yoohat, & Yamabhai, 2013; Wang, Wu, & Liang, 2009; Wee et al., 2009). The enzymatic production of chitooligomers has numerous advantages over the chemical methods including a high yield, and less environmental impact (Kim & Rajapakse, 2005; Sun, Han, Liu, Zhang, & Gao, 2007; Wang et al., 2009; Wee et al., 2009).

* Corresponding author. Tel.: +91 821 2514840; fax: +91 821 2517233.
E-mail addresses: drsureshpv@cftri.res.in, drsureshpv@hotmail.com (P.V. Suresh).

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Chitosanases (EC 3.2.1.132, Chitosan *N*-acetyl glucosaminohydrolase) are hydrolytic enzymes acting on β-(1 → 4) glycosidic linkage of chitosan resulting in low molecular weight chitooligomers as exclusive end products (Thadathil & Velappan, 2014). Therefore, chitosanases, can be effectively used for the preparation of chitooligomers from chitosan substrates. However, the bulk production of chitosanases for commercial applications is still scanty (Lee, Xia, & Zhang, 2008; Da Silva, Honorato, Franco, & Rodrigues, 2012). Hence, screening of high yielding enzyme-productive microorganisms and development of low-cost cultivation media, as well as different bioprocessing approaches for more economic production of the chitosanases are being intensively pursued by scientific community.

Solid state (substrate) fermentation (SSF) is defined as a fermentation process in which microorganisms grow on solid materials without the need or, presence of free liquid and the moisture necessary for microbial growth exists in adsorbed state with solid matrix (Pandey, Selvakumar, Soccol, & Nigam, 1999). SSF has been reported to be economical and an effective alternative to submerged fermentation (SmF) due to its cost effective production of high titers of microbial extracellular enzymes. SSF can be carried out using cheaply available agro-industrial residues such as wheat bran, shrimp by-products, etc. (Suresh, Kumar, & Sachindra, 2011; Suresh, Sachindra, & Bhaskar, 2011; Thadathil, Kuttappan, Vallabaipatel, Kandasamy, & Velappan, 2014). Despite number of reports available on production of chitosanase by SmF, to the best of our knowledge only one report (Da Silva et al., 2012) is available on the chitosanase production from *Trichoderma koningii* under SSF using wheat bran supplemented with chitosan. Hence, the present study was aimed at developing an ideal bioprocess for the production of chitosanase by a soil isolate of *Purpureocillium lilacinum* CFRN12 through statistical optimization of process variables, employing the cost-effective SSF process using the shrimp by-products as the solid substrate. In addition the hydrolytic activity of the crude chitosanase obtained from the fungus for the production of bioactive chitooligomers from chitosan was investigated.

2. Materials and methods

2.1. Materials

Potato dextrose agar (PDA, Himedia, Mumbai, India), D-glucosamine (Sisco Research Laboratory, Mumbai, India); chitosan, glycol chitosan, ρ-nitrophenyl *N*-acetyl-β-D-glucosaminide (ρ-NPGlcNAc) (Sigma Chemical Co., St. Louis, USA), High Performance Liquid Chromatography (HPLC) standards for monomeric GlcN and oligomeric GlcN (Associates of Cape Cod Inc., East Falmouth, MA, USA), were procured. All other reagents and chemicals were of analytical grade.

2.2. Preparation of shrimp by-products substrate

Marine shrimp (*Penaeus* sp.) processing fresh by-products containing cephalothorax and carapace were collected from local seafood market and transported to the laboratory in chilled condition in an icebox. They were minced with a wet mill (Stephen Mill, UM 5 Universal, Germany) into about 1–2 cm particle size and washed twice with tap water (1:10, w/v) using a planetary mixer in order to remove the dirt, soil and sand adhering to the by-products. The washed shrimp by-product chips were dried at 55 ± 2 °C for 12 h in a hot air drying oven (Kilburn, Bombay, India), milled with an electric wearing blender, and sieved through 22 mesh sieve. The shrimp by-products powder, thus prepared, was used as the substrate for SSF studies without any further treatment.

2.2.1. Proximate and chemical analysis of shrimp by-products

The moisture, lipid and ash content were determined according to standard methods of AOAC (2000). Total nitrogen content of shrimp by-products was estimated using Kjeldahl method (AOAC, 2000). Chitin and pH of shrimp by-products were determined according to Suresh et al. (2011a).

2.3. Microorganisms and inoculum preparation

P. lilacinum CFRNT12 (GenBank accession No. KJ524453) and *Penicillium decumbens* CFRNT15 (GenBank accession No. KJ563941), used in the current investigation, were isolated from the native soil sample (unpublished data, Fig. 1) during the course of an earlier investigation. These microorganisms were maintained on PDA slants, under refrigeration, in the culture collection of the laboratory (MMS Department, CSIR-CFTRI, Mysore, India). The identity of the cultures was confirmed by the standard identification techniques for fungi and 18S DNA sequencing. Spore inocula were prepared by growing the cultures on PDA slants at 30 ± 2 °C for 5 days. Later, the spores were dispersed in distilled water containing 0.1% (w/v) Tween 80. The concentration of spore suspension was adjusted to ∼log 7.5 colony-forming units/ml, and then used as inoculum (unless otherwise mentioned).

2.4. Production of chitosanase under SSF

2.4.1. Preparation of solid substrate medium and fermentation

Five gram of solid shrimp by-product substrate taken in Erlenmeyer conical flask (100 ml) was moistened with 5.5 ml of tap water and autoclaved at 15 psi pressure for 30 min. pH of the solid shrimp by-products substrate medium was adjusted to 6.0 ± 0.2 using 0.5 M HCl solution before autoclaving. After cooling, the solid substrate medium was inoculated with 2 ml of prepared spore suspension and incubated at 32 ± 2 °C in an incubator without air moisture control. The solid substrate medium had 55% (w/w) initial moisture content after the addition of the inoculum. After incubation, the fermented solid substrate in each flask was added with 50 ml of chilled acetate buffer (pH 5.6, 0.1 M) and mixed well on an environmental shaker (150 rpm) for 20 min. The slurry was then allowed to settle for about 5 min, and the supernatant was collected by filtration through a dampened cheese cloth. Later the collected supernatant was clarified by centrifugation (12,000 rpm) at 4 °C for 20 min and used for various assays.

2.5. Assay of chitosanase

Chitosanase (EC 3.2.1.321) activity was assayed using glycol chitosan as the substrate (Da Silva et al., 2012). The enzyme assay mixture consisting of 400 μl of 0.1% (w/v) glycol chitosan, and 100 μl of enzyme solution was incubated at 32 ± 2 °C for 30 min. The reaction was terminated by heating the reaction mixture in a boiling water bath for 10 min. Heat inactivated enzyme with the substrate was used as a blank. The amount of reducing sugar produced in the supernatant was estimated using dinitrosalicylic acid reagent (Miller, 1959). D-glucosamine was used as the standard. One unit of the chitosanase activity was defined as the amount of enzyme which released one micromole of D-glucosamine per minute under the conditions mentioned. The enzyme production in SSF was expressed as units/g of initial dry substrate (U/g IDS) (Da Silva et al., 2012; Thadathil et al., 2014).

2.6. Non- specific chitosan degrading enzyme assays

Non-specific chitosan hydrolyzing enzymes like endo-chitinase (EC 3.2.1.14) using ethylene glycol chitin (Suresh, Sachindra, et al., 2011), β-*N*-acetylhexosaminidase (EC 3.2.1.52) using ρ-NPGlcNAc