Extracellular chitin deacetylase production in solid state fermentation by native soil isolates of *Penicillium monoverticillium* and *Fusarium oxysporum*

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Abstract Extracellular chitin deacetylase production by native soil isolates of *Penicillium monoverticillium* CFR 2 and *Fusarium oxysporum* CFR 8 in solid state fermentation (SSF) using commercial wheat bran (CWB) and shrimp processing by-products (SPP) as solid substrate has been studied. *P. monoverticillium* produced maximum chitin deacetylase activity of 547.7 ± 45 and 390.2 ± 31 units/g initial dry substrate (U/g IDS) at 96 h of incubation in CWB and SPP media, respectively. While, *F. oxysporum* produced maximum chitin deacetylase activity of 306.4 ± 22 U/g IDS at 72 h of incubation in CWB medium and 220.1 ± 20 U/g IDS at 120 h of incubation in SPP medium. Along with chitin deacetylase, *P. monoverticillium* and *F. oxysporum* produced other chitin degrading enzymes such as endo-chitinase and β -N-acetylhexosaminidase. *P. monoverticillium* produced maximum activity (U/g IDS) of endo-chitinase 4.6 ± 0.20 at 120 h incubation and β -N-acetylhexosaminidase 82.6 ± 03 at 120 h incubation in CWB medium. While, *F. oxysporum* produced maximum activity (U/g IDS) of endo-chitinase 7.8 ± 0.20 at 144 h incubation and β -N-acetylhexosaminidase 38.3 ± 02 at 120 h incubation in CWB medium. Production of extracellular chitin deacetylase by *P. monoverticillium* CFR 2 and *F. oxysporum* CFR 8 in SSF is being reported for the first time.

Keywords *Penicillium monoverticillium* · *Fusarium oxysporum* · Chitin deacetylase · Solid state fermentation

Introduction

Chitosan is a linear copolymer of β -(1 $\rightarrow$ 4)-2-acetamido-D-glucose and β -(1 $\rightarrow$ 4)-2-amino-D-glucose units with the latter usually exceeding 80% (Jeon et al. 2000). It is polycationic polysaccharides; possess unique functional properties favorable for wide variety of applications in food, biomedicine, cosmetic, agriculture, biotechnological and pharmaceutical industry (Amorim et al. 2001; Synowiecki and Al-Khateeb 2003). However, its application has been restricted especially in food and bio-medicine because lack of commercial availability of high quality chitosan (Tsigos et al. 2000; Amorim et al. 2001; Suresh et al. 2011a). Chitosan is commercially prepared from chitin. Chitin is the most abundant natural biopolymers on the earth after cellulose, extracted commercially from an abundant source of marine crustacean processing by-products (Synowiecki and Al-Khateeb 2003; Kurita 2006; No et al. 2007). Efforts for the use of chitin and chitosan have intensified since efficient utilization of marine biomass resources has become an environmental priority (Jeon et al. 2000). Currently, chitosan is produced by harsh thermo-chemical N-deacetylation of chitin (Synowiecki and Al-Khateeb 2003). This chemical approach has some problems, such as environmental pollution, high energy consumption and uncontrolled reaction process, resulting poor quality chitosan with inconsistent property (different deacetylation grades and molecular weights) (Synowiecki and Al-Khateeb 2003; Chang et al. 2007; No et al. 2007; Zhou et al. 2010; Suresh et al. 2011a). Therefore, biological approach using microbial chitin deacetylase has been proposed as an attractive alternative to alkali

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deacetylation of chitin to chitosan (Martinou et al. 1997; Tsigos et al. 1999, 2000; Synowiecki and Al-Khateeb 2003; Beaney et al. 2007; Suresh et al. 2011a).

Chitin deacetylase (Chitin amidohydrolase, EC 3.5.1.41) is an enzyme that catalyzes the conversion of chitin to chitosan by the deacetylation of *N*-acetyl-D-glucosamine units (Tsigos et al. 2000; Suresh et al. 2011a). The presence of this enzyme activity has been reported in several fungal (Tsigos and Bouriots 1995; Alfonso et al. 1995; Gao et al. 1995; Tsigos et al. 1999, 2000; Nahar et al. 2004; Cai et al. 2006; Kim et al. 2008) and in some bacterial species (Kadokura et al. 2006; Zhou et al. 2010). However, most of these reports deal with purification and characterization of intracellular chitin deacetylase. Chitin deacetylase production is mainly intracellular, however, some microorganisms also synthesis this enzyme as extracellular for their nutritional purpose (Tsigos et al. 2000; Suresh et al. 2011a). Production of extracellular chitin deacetylase has attracted much interest in recent years, owing to its potential in chitosan preparation in industry (Kuk et al. 2005; Kim et al. 2008; Zhou et al. 2010; Suresh et al. 2011a). However, currently there is no commercial process available for the production of this enzyme, mainly due to the reasons such as lack of potential microbial strains and complicated fermentation requirements in chitin deacetylase production by most of the strains reported (Zhou et al. 2010; Suresh et al. 2011a). Solid state fermentation (SSF) a low cost fermentation processes especially for developing nations and offer several advantages over conventional submerged fermentation (SmF) (Suresh and Chandrasekaran 1999; Suresh et al. 2011a, b). SSF has been exploited successfully for the cost effective production of number of fungal enzymes. However, except one report (Suresh et al. 2011a) the production of commercially potent chitin deacetylase by SSF is remains unexploited. In this paper, we report the potential of two native soil fungal strains, for extracellular production of chitin deacetylase by SSF using different solid substrates.

Materials and methods

Preparation of solid substrates

Fresh marine shrimp (*Penaeus* sp.) processing by-products (SPP) containing head and carapace were collected from a local fish market and transported to the laboratory under chill condition. It was dried in a mechanical drier (Kilburn, Mumbai, India) at 55 ± 2 °C for 12 h to constant moisture content ($\sim 10\%$, w/w) and stored at room temperature (30 ± 2 °C) until use. The dried SPP were ground into about 1–2 cm size with an electric wearing blender, washed with tap water (1:10, w/v) for two times using a planetary mixer in order to remove dirt, soil and sand particles and dried at 55 ± 2 °C for 12 h in a mechanical drier. The dried SPP chips

were milled with an electric wearing blender and sieved through a 22 mesh sieve. The SPP powder with <22 mesh particle size were used for SSF without any further demineralization or deproteinization treatment. Commercial wheat bran (CWB) procured from a local market was washed with tap water (1:5, w/v) for two times using a planetary mixer in order to remove dirt and soluble sugars and dried at 55 ± 2 °C for 12 h in a mechanical drier. The dried CWB was stored at room temperature in air tight container and used as second substrate for SSF.

Microorganism and preparation of inoculum

Soil isolates of *Penicillium monoverticillium* CFR 2 and *Fusarium oxysporum* CFR 8 (Suresh et al. 2011b) were used in the present study. The fungal cultures were maintained in the Department culture collection on potato dextrose agar (PDA, Himedia, Mumbai, India) slants at 4 °C and subcultured in every 2 months. The inoculum was prepared by growing the cultures on PDA slants at 30 ± 2 °C for 5 days and the spores were dispersed in distilled water containing 0.1% (w/v) Tween 80 and used as inoculum.

Preparation of solid media and fermentation

Enzyme production by SSF using CWB and SPP as solid substrates was carried out essentially by the procedure reported earlier (Suresh et al. 2011a). Briefly, 5 g of solid substrate in Erlenmeyer conical flasks (100 ml) was moistened with 5.5 ml tap water and autoclaved at 15 psi pressure for 30 min. Chitosan (Sigma Chemical Co., St. Louis, USA) powder was supplemented with at 1% (w/w) as an inducer in CWB medium. After cooling, the solid medium was inoculated with 2 ml spore suspension (4.6×10^9 cfu/ml) and incubated at 32 ± 2 °C for the desired period in an incubator with out air moisture control. After incubation, enzyme from the moldy substrates were extracted with chilled (4 °C) phosphate buffer (pH 7, 0.1 M) and clarified by centrifugation (10,000 rpm, 4 °C).

Enzyme assays

Chitin deacetylase activity was assayed using ethylene glycol chitin prepared from ethylene glycol chitosan (Sigma Chemical Co., St. Louis, USA) as substrate essentially by the procedure reported earlier (Suresh et al. 2011a). One unit of chitin deacetylase activity was defined as the amount of enzyme which formed 1 μ g chitosan, measured using GlcN as reference standard under the reaction conditions and the enzyme production in SSF was expressed as units/g of initial dry substrate (U/g IDS, Suresh et al. 2011a).

Endo-chitinase (EC 3.2.1.14) activity was assayed using colloidal chitin as substrate essentially by the procedure

reported earlier (Suresh et al. 2011a). Pure α -chitin from fresh shrimp abdominal shells and colloidal chitin from pure α -chitin were prepared by the methods reported (Suresh et al. 2011a). One unit of endo-chitinase activity was defined as the amount of enzyme which released 1 μ mol of GlcNAc under the reaction conditions and enzyme production in SSF was expressed as U/g IDS (Suresh et al. 2011a).

β -*N*-acetylhexosaminidase (EC 3.2.1.52) activity was assayed using *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma Chemical Co., St. Louis, USA) as the substrate essentially the procedure reported earlier (Suresh et al. 2011a). One unit of β -*N*-acetylhexosaminidase activity was defined as the amount of enzyme which released 1 μ mol of *p*-nitrophenol under the reaction conditions and enzyme production in SSF was expressed as U/g IDS (Suresh et al. 2011a).

Scanning Electron Microscopy (SEM)

The growth and performance of *P. monovercillium* CFR 2 and *F. oxysporum* CFR 8 on moist CWB and SPP medium in SSF were observed under SEM as described below. Samples were collected from fermented solid substrates, fixed by immersion in 2.5% (v/v) glutaraldehyde for 12 h at 4 °C, washed with phosphate buffer (0.1 M, pH 7.0) for 1 h at room temperature and further dehydrated in a graded 20–100% (v/v) ethyl alcohol series and dried under vacuum at room temperature. The dried samples were coated on a double sided conducting adhesive tape pasted on to a metallic stub and subjected to gold covering (~100 Å). Finally the prepared samples were examined in a Scanning Electron Microscope (LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, UK) at 20 kV.

Analytical methods

The moisture, crude protein, fat and ash content of the SPP substrate (AOAC 2000), chitin content of the SPP substrate (Spinelli et al. 1974), pH of solid SPP and CWB substrates (Suresh and Chandrasekaran 1998) were determined.

Statistical analysis

Statistical analyses were carried out with the statistical software STATISTICA (STATSOFT 1999). Effect of two different substrates; two different cultures and the incubation time on the enzyme productions were analyzed using the Analysis of Variance techniques (ANOVA). All experiments were conducted in triplicate and the mean values $\pm$ SD are reported.

Results and discussion

Washed SPP substrate used in the study contained (w/w, dry weight basis) 25.1 $\pm$ 0.2% protein, 24.8 $\pm$ 0.30% chitin, 0.08 $\pm$

0.02% fat and 42.3 $\pm$ 0.60% ash. The washed and dried CWB and SPP substrate had a pH of 6.3 $\pm$ 0.20 and 8.8 $\pm$ 0.20, respectively. Proximate composition of SPP and pH of SPP, and CWB substrates used in the present study was found similar to an earlier report (Suresh et al. 2011a). The moist solid substrate (CWB and SPP) media after inoculation had 55% (w/w) initial moisture content. The autoclaved solid CWB and SPP media had initial pH of 6.4 $\pm$ 0.20 and 8.7 $\pm$ 0.20, respectively. Visual observation of moldy substrates showed that both *P. monovercillium* CFR 2 and *F. oxysporum* CFR 8 grew profusely and uniformly throughout on moist CWB and SPP medium as a white cottony mass. The fungal hyphae completely covered the entire CWB medium within 48 h of incubation. Both fungi were tolerated the adverse conditions of high protein and CaCO₃ presence of other components and high alkaline pH (8.8 $\pm$ 0.20) of SSP. However, the growth was slow and less profuse on SPP medium as compared to the growth on CWB medium. Similar observation was reported earlier (Suresh et al. 2011a). Figure 1 shows the SEM micrograph of moldy substrates revealing the invasion of *P. monovercillium* CFR 2 and *F. oxysporum* CFR 8 over the solid CWB and SPP substrates.

Enzyme production

As shown in Fig. 2a in CWB medium supplemented with chitosan, *P. monovercillium* CFR 2 produced maximum chitin deacetylase activity of 547.7 $\pm$ 45 U/g IDS at 96 h of incubation and by *F. oxysporum* CFR 8 was 306.4 $\pm$ 22 U/g IDS at 72 h of incubation. Similarly in SPP medium, *P. monovercillium* CFR 2 recorded higher chitin deacetylase activity of 390.2 $\pm$ 31 U/g IDS at 96 h of incubation as compared with the maximum activity of 220.1 $\pm$ 20 U/g IDS at 120 h of incubation by *F. oxysporum* CFR 8. CWB is a frequently used substrate for SSF production of exo-enzyme by fungi due to its favorable properties as a solid substrate for fungal growth (Suresh and Chandrasekaran 1999; Suresh et al. 2011a, b). From the results (Fig. 2a) it was found that no significant ($p \leq 0.05$) variation in chitin deacetylase activity between CWB and SPP medium. However, the use of inexpensive SPP a by-product from seafood processing industry as substrate in SSF for enhanced production of chitin deacetylase will have economic advantage. Similarly, chitin deacetylase production by *P. monovercillium* CFR 2 and *F. oxysporum* CFR 8 did not differ significantly ($p \leq 0.05$), indicating their potential for large scale application in industry. Incubation time had significant ($p \leq 0.001$) effect on maximal chitin deacetylase production by both *P. monovercillium* and *F. oxysporum*. The chitin deacetylase production by both *P. monovercillium* and *F. oxysporum* was slowly denatured beyond optimum periods of incubation in both media. The reduction in chitin deacetylase activity in both media after optimum fermentation

Fig. 1 Scanning electron micrograph of growth of *P. monovercillium* CFR 2 on solid commercial wheat bran (a) and shrimp processing by-products (b) and *F. oxysporum* CFR 8 on solid commercial wheat bran (c) and shrimp processing by-products (d) in solid state fermentation

period is probably due to denaturation of this enzyme by protease secreted by the organism and also due to the depletion of available nutrients in the solid medium. A

similar drop in enzyme activity has been reported in SSF production chitin deacetylase by *Colletotrichum lindemuthianum* ATCC 56676 (Suresh et al. 2011a).

Fig. 2 Time scale of chitin deacetylase (CDA) production (a), endo-chitinase (chitinase) production (b) and β -N-acetylhexosaminidase (NHase) production (c) by *P. monovercillium* CFR 2 and *F.*

oxysporum CFR 8 in solid state fermentation using commercial wheat bran (CWB) and shrimp processing by-products (SPP) media

Not much information regarding the fermentation processes and yield of chitin deacetylase is available except few reports (Kim et al. 2008; Zhou et al. 2010; Suresh et al. 2011a). Most of the works on chitin deacetylase were carried out from the viewpoint of their purification and characterization, mode of action on substrate and their substrate specificity (Kauss and Bauch 1988; Kafetzopoulos et al. 1993; Tsigos and Bouriotis 1995; Tokuyasu et al. 1996; Tsigos et al. 1999; Kadokura et al. 2006). Recently some researchers have studied the production of microbial extracellular chitin deacetylase by different fermentation methods due to its potential in industry for the preparation of chitosan with unique quality (Kim et al. 2008; Zhou et al. 2010; Suresh et al. 2011a). Kim et al. (2008) reported a maximal extracellular chitin deacetylase yield of 0.6 U/ml on 3 d of fermentation in a yeast peptone glucose medium employing SmF from *Mortierella* sp. DY-52. They assayed chitin deacetylase activity using a water soluble chitin (WSCT-50) as substrate and observed release of acetic acid. The production of an extracellular chitin deacetylase by a soil bacterium *Bacillus amyloliquefaciens* in SmF has been reported by Zhou et al. (2010). They reported a maximum of 17.84 U/ml chitin deacetylase activity in a mineral medium supplemented with chitin. They assayed chitin deacetylase activity using hexa-*N*-acetylchitohexose as substrate and monitored the release of acetate. Very recently, Suresh et al. (2011a) studied the production of extracellular chitin deacetylase from *C. lindemuthianum* ATCC 56676 by SSF using different solid substrates. They reported a maximum chitin deacetylase activity of 460.4 ± 15 U/g IDS at 96 h of incubation in a CWB medium supplemented with chitosan and 392.0 ± 06 U/g IDS at 192 h of incubation in a shrimp shell chitin waste medium. Suresh et al. (2011a) assayed chitin deacetylase activity based on the formation of ethylene glycol chitosan from ethylene glycol chitin. The production of high level of extracellular chitin deacetylase by *P. monovercillium* CFR 2 and *F. oxysprum* CFR 8 in SSF has the advantages for its industrial application as compared with earlier report (Suresh et al. 2011a).

It is well known that chitin deacetylase has multiple functions in fungi along with other chitin degrading enzymes such as endo-chitinase and β -*N*-acetylhexosaminidase (Suresh et al. 2011a). Chitin deacetylase was extracted from fungi in addition to chitinases (Nahar et al. 2004; Kim et al. 2008; Suresh et al. 2011a). *P. monovercillium* CFR 2 and *F. oxysprum* CFR 8 produced endo-chitinase and β -*N*-acetylhexosaminidase along with chitin deacetylase in SSF. As shown in Fig. 2b *F. oxysprum* CFR 8 produced significantly ($p \leq 0.05$) higher endo-chitinase activity (7.8 ± 0.20 U/g IDS at 144 h) as compared with the activity produced by *P. monovercillium* CFR 2 (4.6 ± 0.20 U/g IDS at 120 h) in CWB medium supplemented with chitosan. While, in SPP medium *P. monovercillium* CFR 2 recorded significantly ($p \leq 0.05$) higher endo-chitinase activity (0.44 ± 0.02 U/g IDS at 96 h) as compared with the enzyme activity

produced by *F. oxysprum* CFR 8 (0.12 ± 0.01 U/g IDS at 96 h). In the case of β -*N*-acetylhexosaminidase production, *P. monovercillium* CFR 2 recorded highest activity (82.6 ± 03 U/g IDS at 120 h) as compared with the activity produced by *F. oxysprum* CFR 8 (38.3 ± 02 U/g IDS at 120 h) in CWB medium (Fig. 2c). Similarly, in SPP medium, *P. monovercillium* CFR 2 produced higher β -*N*-acetylhexosaminidase activity of 1.8 ± 0.07 U/g IDS at 120 h as compared with 0.28 ± 0.01 U/g IDS at 96 h of incubation by *F. oxysprum* CFR 8. When comparing endo-chitinase and β -*N*-acetylhexosaminidase activity obtained in different media, CWB supported significantly ($p \leq 0.001$) higher production. Similar to chitin deacetylase activity the incubation period significantly ($p \leq 0.05$) affected the overall endo-chitinase and β -*N*-acetylhexosaminidase activity. The production of endo-chitinase activity was significantly ($p \leq 0.05$) affected by the organisms. However, there was no such difference ($p \leq 0.001$) in β -*N*-acetylhexosaminidase activity due to organisms. Elaborate discussion on the production of endo-chitinase and β -*N*-acetylhexosaminidase is not provided as the emphasis was on chitin deacetylase production in SSF process by these organisms.

Conclusions

Chitin deacetylase, an industrially important enzyme, can be produced by SSF using CWB and SPP as solid substrates. In SSF cultivation, *P. monovercillium* CFR 2 produced maximum chitin deacetylase activity (U/g IDS) of 547.7 ± 45 in CWB medium and 390.2 ± 31 in SPP medium at 96 h of incubation. Similarly, *F. oxysprum* CFR 8 produced maximum chitin deacetylase activity (U/g IDS) of 306.4 ± 22 at 72 h in CWB medium and 220.1 ± 20 at 120 h of incubation in SPP medium. The utilization of inexpensive SPP, a by-product of seafood processing industry as a substrate in SSF production of chitin deacetylase will have economic advantage and offers great relevance in environmental hazards due to seafood processing by-products disposal. The result of the study indicates the potential of *P. monovercillium* CFR 2 and *F. oxysprum* CFR 8 for the extracellular chitin deacetylase production by SSF. Investigation works are in progress to optimize various physico-chemical processes parameters for chitin deacetylase production in SSF by *P. monovercillium* CFR 2 and *F. oxysprum* CFR 8.

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