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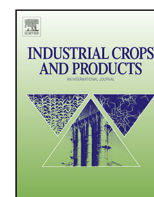

Integrated process for fungal citric acid fermentation using apple processing wastes and sequential extraction of chitosan from waste stream

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ABSTRACT

The present study was carried out to explore the possibility of extracting chitosan (CTS) from waste fungal mycelium as co-product during citric acid (CA) fermentation. CA biosynthesis was carried out through solid-state (SSF) and submerged fermentation (SmF) in lab scale fermenters using apple pomace (AP) and apple pomace ultrafiltration sludge (APS) by *Aspergillus niger* NRRL 567. CA production of 182.8 ± 8.2 and 294.2 ± 13.2 g/kg dried AP and 18.4 ± 1.24 and 40.3 ± 2.0 g/L APS was achieved through SSF and SmF after 120 h and 132 h fermentation period, respectively in control and in treatment with methanol as an inducer.The resulting waste fungal biomass during CA production was used for CTS extraction using ambient conditions. Extractable CTS was found to be higher in control (treatment with no inducer) with 6.40% and 5.13% of dried fungal mycelium resulting from the SSF and SmF, respectively as compared to treatments supplemented with inducers (ethanol and methanol). Degree of deacetylation of the CTS ranged from 78 to 86% for fungal biomass obtained from SSF and SmF. The viscosity of fungal CTS was in a range of 1.02–1.18 Pa s^{-1}, comparable to the commercial crab shell CTS. The study indicated the possibility of sequential extraction of superior quality CTS from waste fungal biomass resulting from various fungal-based biotechnological industries including CA.

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1. Introduction

There is an increasing demand of the molecules having non-toxic, biodegradable, biocompatible and environmentally safe nature (Dhillon et al., 2011a). The natural occurrence of citric acid (CA) as metabolic intermediate product in mostly all organisms and chitosan (CTS) in some fungal strains assures their non-toxic nature. CA has emerged as one of the important biochemical with great potential in the food, pharmaceuticals and personal care products, environmental and agricultural sectors (Soccol et al., 2006; Ashkan et al., 2010; Dhillon et al., 2010; Guillermo et al., 2010). However, CA market has been under tremendous pressure for the past few years as high cost of raw materials and energy has transformed the once lucrative CA production sector into an unprofitable market. The search for inexpensive substrate alternative is vital to reduce the production cost.

Another viable option to stabilize the CA markets is to extract CTS as co-product, from waste fungal mycelium resulting during CA production (Dhillon et al., 2012). CTS is a natural biopolymer having unique polycationic, chelating and film forming properties due to the presence of active amino and hydroxyl functional groups.Owing to these unique properties, CTS is widely used in diverse fields ranging from medicine, pharmaceutical, cosmetics, food and nutrition and agriculture (Dhillon et al., 2013; Kaur and Dhillon, 2013). CTS also exhibits an array of biological properties, such as antimicrobial activity, induced disease resistance in plants and diverse stimulating or inhibiting properties toward a number of human cell lines (Dhillon et al., 2012, 2013; Kaur and Dhillon, 2013).

Traditionally, CTS is derived from naturally occurring crustacean chitin, such as crab and shrimp shell by *n*-deacetylation with harsh chemical treatments. However, the CTS obtained by such treatments suffer from some inconsistencies, such as protein contamination and inconsistent levels of degree of deacetylation (DD) and molecular weight (MW) which results in variable

Abbreviations: AAIM, acid-and alkali insoluble material; AIM, alkali soluble material; AP, apple pomace; APS, apple pomace ultrafiltration sludge; CA, citric acid; CTS, Chitosan; DD, degree of deacetylation; EtOH, Ethanol; MeOH, Methanol; SmF, submerged fermentation; SSF, solid-state fermentation.

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physico-chemical characteristics of CTS. There are some additional issues due to use of toxic chemicals, limitation of shell supply, geographical limitation, seasonal variation and high cost (Dhillon et al., 2012). Therefore, a biological approach for the CTS synthesis seems to be a novel green route. Recent advances in fermentation technology suggest that the mycelia of various fungi including *Zygomycetes*, *Ascomycetes*, *Basidiomycetes* and *Deutermoycetes* are alternative potential sources of chitin and CTS (Pochanavanich and Suntornsuk, 2002; Dhillon et al., 2012). Fungal cells wall contains up to 50% chitin as compared to crustacean shells which contain 14–27% on dry biomass basis (Zamani et al., 2007). Production and purification of CTS from cell walls of fungi grown under controlled conditions offers greater potential for highly consistent product. Moreover, the fungal CTS can have unique properties compared with those derived from crustaceans, such as: (1) free of allergenic shrimp protein and; (2) the MW and DD of fungal CTS can be controlled by varying the fermentation conditions (Dhillon et al., 2012). Due to the abundant and inexpensive availability, the waste mycelia could be viewed as the possible source of industrial production of CTS.

Aspergillus strains are widely employed for the production of CA and various biotechnological and pharmaceutical products on industrial scale (Dhillon et al., 2010). Moreover, due to the increasing demand of CA, its annual worldwide production is estimated to be 1.7 million tons which will result in 0.34 million tons of *Aspergillus niger* mycelium waste per annum and furthermore increasing at an annual growth rate of 5% (Dhillon et al., 2012). The alkali-insoluble cell-wall residue of the *A. niger* biomass consists mainly of CTS, chitin and β-glucans, with a significant prevalence of (1,3)-β-D-glucan. Generally, the waste fungal mycelium resulting from fermentation is incinerated. This mandates an obvious need to develop some integrative technology to utilize the unlimited waste mycelium resulting from CA industries for co-product, CTS extraction. This will also support in the stabilization of CA prices. The waste fungal biomass from CA or other biotechnological industries can be utilized as free and rich alternative sources of CTS beside the traditional industrial source – marine shell wastes.

The present study was carried to explore the possibility for extraction of important co-product, CTS from *A. niger* NRRL 567 waste mycelium produced during CA production. In this context, the bidentate approach was applied as follow: (1) the economical production of CA from apple pomace (AP) and apple pomace sludge (APS) under solid-state fermentation (SSF) and submerged fermentation (SmF) conditions and; (2) extraction of CTS from resulting waste fungal mycelium. The characterization of the properties of the produced CTS was carried out. As per the published literature, so far to the best of our knowledge, there is no study reported on the utilization of fruit processing waste for the combined production of CA and sequential extraction of CTS from waste fungal mycelium.

2. Materials and methods

2.1. Microorganisms and inoculum preparation

A. niger NRRL 567 was procured from Agricultural Research Services (ARS) culture collection, IL-USA. The culture conditions, maintenance of fungus and inoculum production are described elsewhere (Dhillon et al., 2011b).

2.2. Substrate procurement and pretreatment

AP and APS (from Lassonde Inc., Rougemont, Montreal, Canada), was selected as substrate for CA production. AP was already supplemented with rice husk (1%, w/w), as a common practise of supplementing the apples with rice husk during the extraction of juice for a better hold on the apples in the industry during meshing and filtration. AP was completely dried at 50 ± 1 °C in a hot air oven till constant weight, grounded and was passed through sieves to get the desired particle size of 1.7–2.0 mm which was used in this study.

APS is the liquid sludge obtained after the ultrafiltration of crude juice. The apples are mashed in the initial step of processing and the solid waste is separated, and the crude juice so obtained is again filtered twice through ultrafiltration system which leaves APS accounting for 5–10% of total processed apples. APS containshigh macro- and micronutrient contents (total carbon 51.9 g/L, total nitrogen 2.94 g/L, carbohydrates 66.0 ± 1.7 g/L, lipids 5.9 ± 0.3 g/L and protein 33.75 ± 2.0 g/L, among others) (Dhillon et al., 2011b).

2.3. SSF

SSF was performed in a 12 L rotating drum type bioreactor, Terrafor (Infors HT, Switzerland). Approximately, 3 kg of rehydrated AP (moisture 75%, v/w) was sterilized in autoclave at 121 ± 1 °C for 30 min and was transferred into the sterilized bioreactor under aseptic condition. After transferring the substrate into the bioreactor, the sterilization was done again to ensure complete decontamination. The 3 different set of experiments were performed as follows: (1) control without any inducer and; (2) supplemented with 3% (v/w) of inducer, ethanol (EtOH) and; (3) supplemented with 3% (v/w) of methanol (MeOH) as an inducer. The initial pH (3.5 ± 0.1) was taken as such without any adjustment. The inoculation was carried out using the spore suspension having 1×10^7 spores/g AP. For final moisture content of 75% (v/w), the volume of inducers and spore suspension was also taken into account. The fermentation was carried out in a controlled environment at 30 ± 1 °C with intermittent agitation rate of 2 rpm (for 1 h after every 12 h) and aeration rate of 1 vvm. The fermentation was carried out till 144 h, and under aseptic conditions samples were harvested every 24 h for CA and total spore count analysis. CA was extracted from 1 g of a fermented sample macerated with 15 mL of distilled water and shaken at 200 rpm for 20 min in an incubator shaker at 25 ± 1 °C. Supernatant was filtered through glass wool and 100 μL sample was taken in Eppendorf tubes for viability (total spore count) assay using haemocytometer and the remaining sample was again centrifuged (Sorvall RC 5C plus by Equi-Lab Inc., Québec, Canada) at $9000 \times g$ for 20 min and the clear supernatant was analyzed for CA concentration.

2.4. SmF

All the experiments were performedin a 7.5 L capacity fermenter with 4.5 L working volume (Labfors, HT Bottmingen, Switzerland). APS having 25 g/L suspended solids (SS) concentration was used as a substrate for CA bioproduction. APS was sterilized at 121 ± 1 °C for 30 min. After sterilization, the substrate was supplemented with inducers [3% (v/v) EtOH and MeOH] in different treatment. The medium was inoculated with 10% (v/v) inoculum concentration. Temperature and pH of the fermentation medium was controlled at 30 ± 1 °C and 3.5, respectively. To maintain dissolved oxygen concentration above 20% saturation (critical oxygen concentration), the medium was agitated at a speed of 300 rpm, and the air flow rate was automatically controlled using a computer controlled system. Samples were withdrawn from the fermenter at 24 h intervals for the CA and biomass estimation. All the experiments were performed in triplicates and the values given were average along with standard deviation.

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