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Inulinase production from a new inulinase producer, *Penicillium oxalicum* BGPUP-4

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ABSTRACT

The present study was carried out for the isolation and characterization of an efficient inulinase producing fungal strain. Out of 31 fungal isolates, only 16 strains showed the hydrolytic zone surrounding the fungal growth on an inulin-rich medium. Screening of 16 inulolytic fungal strains was carried out based on activity of enzyme on inulin and sucrose and their I/S ratio. Fungal isolate BGPUP-4 shown maximum inulinase activity (11.06 IU/mL) after 5 days of cultivation and the corresponding I/S ratio was 2.64. This I/S ratio confirmed the inulinase nature of the enzyme. Thin layer chromatography and densitometer scanning of the hydrolysate were obtained after hydrolysis of inulin by inulinases from 5 fungal strains having high enzyme activity. Micromorphological and macromorphological characteristics of the fungal isolate BGPUP-4 exhibiting maximum enzyme activity, revealed it to be *Penicillium oxalicum*. Furthermore, 18S rRNA gene sequencing and phylogenetic analysis confirmed the identification of the fungal strain. Out of various carbon sources tested, lactose (2%) in combination with inulin (1.5%, as an inducer) showed a stimulatory effect on inulinase production (19.14 IU/mL). Agitation mode of cultivation in shake-flask fermentations, supported maximum inulinase production (38.52 IU/mL) after 5 days of incubation. This is the first report on inulinase production from *Penicillium oxalicum*.

1. Introduction

Inulinases are industrially important enzymes which act on β-2, 1 linkages of inulin. They are included in both transferase and hydrolases groups, depending upon their catalytic action on the substrate. Enzymes converting inulin into difructose anhydrides (DFA-I and DFA-III) and small amount of oligosaccharides through intramolecular transfructosylation reaction are termed as inulin fructotransferases (EC 4.2.2.17 and 4.2.2.18). Since the transfer is intramolecular and the reaction is elimination, the enzymes are considered lyase rather than transferase and hence termed as inulin lyases. The enzymes splitting β-2, 1 fructofuranosidic bonds of inulin by either exo- or endo action are called inulinases. Exoinulinase (EC 3.2.1.80; β-2-1-D-fructan fructohydrolase) acts sequentially on β-2, 1 bonds of inulin to produce fructose, while endoinulinase (EC 3.2.1.7; β-2-1-D-fructan fructanohydrolase) acts randomly to release fructooligosaccharides. The production of high fructose syrup (Singh et al., 2007a, 2007b, 2008; Singh and Chauhan, 2016) and fructooligosaccharides (Singh and Singh, 2010; Singh et al., 2016) are the two major applications of inulinases. Various other applications of inulinases are production of bioethanol, single cell oil, lactic acid, citric acid, pullulan, tequila etc. (Singh and Chauhan, 2016). Fructose is a low calorie sweetener which has a number of technical superiorities over conventional sweetener sucrose, whereas fructooligosaccharides are potent prebiotics. Conventionally, fructose is produced from starch by a multienzymatic process involving α-amylase, amyloglucosidase and glucose isomerase. The yield of fructose by this method is approximately 45%. Various ion-exchange techniques have also been developed to give syrups with D-fructose content over 90%, however these techniques add to the cost of production (Fleming and Groot-wassink, 1979). Single step enzymatic hydrolysis of inulin using inulinases is an attractive alternative, which yields as high as 95% of fructose.

Type of microorganism and media composition are the two key factors which affect inulinase production and its properties. Inulinases have been characterized from various microbial sources like yeast, bacteria and fungi which show a considerable variability in their biophysical and biochemical characteristics (Singh and Gill, 2006). Fungal strains are emerging as potent candidates for inulinase production, because they possess numerous desirable features like their cultivation occurs on low cost substrates and fungal enzymes are

Abbreviations: CYA, Czapek's yeast autolysate agar; DFA-I and III, Difructose anhydrides I and III; G25N, Glycerol nitrate agar; HPTLC, High performance thin layer chromatography; I/S ratio, Inulinase activity/Invertase activity; MEA, Malt extract peptone dextrose agar; and PDA, Potato dextrose agar

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more stable at high temperature and low pH. *Aspergillus* sp. and *Penicillium* sp. are the most common fungi used for inulinase production (Singh and Chauhan, 2016). Inulin is rendered as the prime inducer as well as a carbon source for the production of inulinases. Other pure substrates like glucose, fructose, lactose etc. have also been reported for inulinase production, but inulin (raw or pure) is considered as the best energy source. Inulin is a rich reserve polyfructan present in dicotyledonous and monocotyledonous plants representing families *Compositae, Campanulaceae, Poaceae, Liliaceae* etc. (Singh and Singh, 2010). It entails a linear chain of β-2, 1-linked D-fructosyl-fructose molecules terminated at the reducing end by a glucose moiety attached through a sucrose type linkage by α-(1, 2)-fructofuranosidic bonds. The current study was carried out to isolate a potent inulinase producing fungal strain. A fungal isolate BGPUP-4, exhibiting maximum inulinase activity has been characterized morphologically and by using molecular tools. Screening of various carbon sources was carried out for the production of inulinase and concentration of the best carbon source was optimized. Inulinase production from *Penicillium oxalicum* BGPUP-4 has also been investigated under stationary and agitation mode of cultivation. The maximum enzyme activity was expressed after 5 days of cultivation of the fungal isolate *Penicillium oxalicum* BGPUP-4. The literature survey reveals, this is the first report on inulinase production by *Penicillium oxalicum*.

2. Materials and methods

2.1. Isolation and maintenance of inulinase producing fungal strains

Isolation of inulinase producing fungal strains was carried out by collecting the soil samples from the vicinity of rhizosphere/root tubers/bulbs of inulin-rich plants growing in the Botanic Gardens, Punjabi University, Patiala, India. For the isolation, the soil samples (5%) were inoculated individually into sterilized enrichment medium (50 mL in 250 mL Erlenmeyer's flasks) consisting of (%): inulin 2, peptone 1, $NH_4H_2PO_4$ 1, NaCl 0.5, KH_2PO_4 0.1, $MgSO_4{\cdot}7H_2O$ 0.05, Chloramphenicol 0.01 and pH of the medium was adjusted to 5.5. Inulin was autoclaved separately at 10 psi for 15 min and added into the medium. The inoculated flasks were incubated at 30 °C for 5 days on a rotary incubator shaker (CIS-24 BL, REMI, Mumbai, India), under agitation at 150 rpm. Thereafter, spreading of the serially diluted culture broth was carried out on agar plates made from the same medium as described above. Agar plates were incubated at 30 °C for 5 days. Then, healthy and discrete colonies were picked up and maintained on potato dextrose agar (PDA) slants containing (%): potato extract 20, dextrose 2, agar 2 and adjusted to pH 5.5. All the cultures were subcultured at fortnight intervals and maintained on PDA slants at 4 °C, until further use.

2.2. Screening of inulinase producing fungal strains

Inulinase producing fungal strains were evaluated on the basis of their potency to form an inulolytic zone on inulin-rich medium, inulinase production and action pattern of the enzyme.

2.2.1. Primary screening

Primary screening was carried out on the basis of inulolytic zone formation by the isolated fungal strains on inulin-enriched medium using a rapid plate screening assay (Li et al., 2011). All the isolated fungal strains were grown on agar plates of inulin-enriched medium having the same composition as described above. Agar plates were incubated at 30 °C for 3–5 days. Thereafter, plates were flooded with Lugol's iodine solution (1.5% potassium iodide and 1% iodine) and kept undisturbed for 3–5 min. Then, plates were washed 2–3 times with distilled water and kept open for 15–20 min for the visualisation of the clear hydrolytic zone. Formation of the clear hydrolytic zone around the colonies confirmed the existence of extracellular inulinase. All the isolates exhibiting the hydrolytic zone on inulin-enriched medium were selected for secondary screening.

2.2.2. Secondary screening

Secondary screening was performed for the determination of enzyme activity of isolated fungal strains on inulin and sucrose as substrates. Inoculum was prepared by growing all inulolytic strains on PDA plates at 30 °C for 5 days. Erlenmeyer's flasks (250 mL) containing 50 mL production medium containing (%): inulin 2, $NaNO_3$ 0.2, $NH_4H_2PO_4$ 0.2, KH_2PO_4 0.2, KCl 0.1, $MgSO_4{\cdot}7H_2O$ 0.05, $FeSO_4{\cdot}7H_2O$ 0.001 and adjusted to pH 6.0, were inoculated with one agar disc (10 mm) covered with mycelia to ensure uniformity of the inoculum.

2.2.3. Tertiary screening

Tertiary screening was carried out to determine the action pattern of enzyme on inulin using thin layer chromatography (Coitinho et al., 2010). Only 5 fungal strains exhibiting maximum inulinase activity were selected for tertiary screening. Mycelium free extract was collected after centrifugation (5000*g*, 15 min at 4 °C) of fermented broth. For hydrolysis, reaction mixture was prepared by mixing 1% inulin in mycelium free crude extract (2.0 mL) and incubated at 55 °C for 5 h. After incubation, the reaction was terminated by heating the mixture in a boiling water bath for 10 min. Precoated silica gel TLC plates (SD Fine Chemicals Ltd., India) were activated at 110 °C for 10 min before their use. Thereafter, the TLC plates were spotted with the reaction mixture along with glucose, fructose and sucrose as standards, to analyse the end products. After spotting, the plates were air dried and kept in a developing solvent containing chloroform: acetic acid: water (30:30:5, v/v). Then the developed plates were again air dried and sprayed with aniline diphenylamine reagent (aniline 1%, diphenylamine 1%, phosphoric acid 10%) which on reacting with sugars emanates specific colours. Different coloured spots were visually examined after incubating TLC plates at 90 °C in a hot air oven for 15 min. The qualitative evaluation of the derivatized plates was also verified by high performance thin layer chromatography (HPTLC) for further assurance. Camag (Switzerland) TLC scanner 4 was implemented for the densitometer scanning of the plate at 500 nm. The scanner was operated by winCATS software version 1.4.9. Deutrium lamp was a continuous source of radiation emitting a spectrum between 100–800 nm. The slit dimensions were 5×0.45 mm with 20 mm/s scanning speed.

2.3. Phenotypic analysis of fungal isolate BGPUP-4

2.3.1. Macromorphology

Colonial characteristics of fungal isolate BGPUP-4 were studied on three different microbiological media, namely malt extract peptone dextrose agar (MEA), czapek's yeast autolysate agar (CYA) and glycerol nitrate agar (G25N) at 25 °C for 7 days. The compositions of all these media were same as described by Nagamani et al. (2005). Colony particulars like obverse and reverse colony colour, diameter, sporulation, exudate formation etc. were recorded. Emphasis was laid on the growth data obtained from these media for the identification of fungal isolate BGPUP-4.

2.3.2. Micromorphology

Microscopic characteristics like conidial colour, shape and size, rami size, phialides shape and size, verticillate, stipe length etc. of fungal isolate BGPUPU-4 were investigated. Microscopic slides were prepared from actively growing colonies of fungal isolate BGPUP-4. Determination of micromorphological structures of isolate was conceded by using lactophenol cotton blue dye. Light photomicrographs of fungal isolate were obtained by Leica DM 4000 B LED microscope fitted with digital camera DFC450C (Germany) using Leica application suite version 4.2. Fungal isolate BGPUP-4 was identified up to species specific level according to Nagamani et al. (2005).