Research Paper

Biosynthesis, partial purification and characterization of invertase through Carrot (Daucus carota L.) Peels

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ABSTRACT

The current work was investigated on the production of Invertase under solid-state fermentation (SSF) through Aspergillus species, by utilizing peels of Carrot (Daucus carota L.) as a substrate. The maximum production of Invertase (7.95±0.1 U/ml) was attained by using Aspergillus niger with 1x10^6 spores/ml at 90% moisture content after incubation period of 72 h. Through ammonium sulphate precipitation the enzyme was purified about 1.42-fold. Invertase showed maximum thermal stability ranges from 20-70°C at a pH range (5.5 to 6.5) with utmost activity at pH 5.5 and 50°C. However, the enzyme was highly active at two different concentrations of sucrose that is, 0.1 and 0.5 M respectively, but it indicates less activity at glycerol. While the enzyme was completely repressed by Hg2+ (1 mM) and slightly enthused by Co2+ and Na+ at the similar application.

Key words: Food wastes, carrot peels, invertase, production, Aspergillus niger, solid-state Fermentation.

INTRODUCTION

Agriculture wastes and by-products are sustainable form of resources generates all around the world. Sugar cane bagasse, wheat straw, rice bran, citrus and mango peel and corn cobs etc are some of vital wastes of food processing industries. These materials are accrued in massive amounts in Pakistan and become a source of environmental pollution (GOP, 2009). Transformation of carbohydrate wastes is getting high attention in sight of the fact that these wastes can be used as agents for the conversion of useful biomaterials and chemical intermediates with increase industrial development, today, there is a requirement of environmentally sustainable processes, and it is generally accepted that sustainable environmental protection can only be acquired through integration of general environmental awareness. In recent times, changing of renewable raw materials into chemicals has become a foremost subject of research and development around the globe (Jin et al., 2005).

Rising apprehensions about pollution generated from wastes of agriculture and the industry sector has motivated the conversion of waste resources into commercially functional products. Food industry generates huge quantity of wastes in both solid and liquid forms. Undoubtedly, food processing waste material causes pollution and having hazardous effects yet, they have potential for reprocessing raw materials and convert it into valuable product of superior value (Sangeetha et al., 2004; Mamma et al., 2008; Rashad and Nooman, 2008; Guimaraes et al., 2009).

Carrots (Daucus carota L.) are rather inexpensive and highly nutritious as it contains appreciable amount of vitamins and minerals. They are rich source of energy because it contains a lot of sucrose (Manjunatha et al., 2003). Carrot wastes, such as peels, pomace, are generally dumped or used as animal feed after processing. Though, carrot derivative still have high contents of useful substances, in particular bioactive compounds with antioxidant activities (Zhang and Hamazu, 2004).

Advent of biotechnology facilitates to open innovative features by utilizing newly derived enzymes. Microorganisms play a pivotal function in this development and presently the fermented products provide adequate contribution. Their activity in conversion process of wastes...
into significance products has been enhanced in recent times (Pandey et al., 2000; Ahuja et al., 2004). The employment of microbial enzymes has found extensive purpose in various industrial applications. Among a variety of commercial enzymes most of them are achieved through fermentation of fungal species (Piccoli-valle et al., 2001). The amplify use of enzymes substitute conventional chemical transformation processes is infatuated by an objective for better production economics, innovative product, better security, and an escalating wish to diminish the environmental pollution. To execute this rising demand for enzymes, many of the fresh enzymes are developed from fungal or bacterial kingdom in large-scale fermenters by consuming agro-industrial wastes by consuming agro-industrial wastes (Cherry et al., 2001).

Invertase (β-D-fructofuranosidases fructohydrolases, EC. 3.2.1.26) is utilized for the development of invert sugar and high fructose syrup (HPS). This enzyme is extensively use in food industry where fructose is favored particularly in the preparation of jams and candies where it is serve as a sweetener and could not crystallize easily D-glucose and D-fructose at lower concentrations, therefore it makes these enzymes appropriate for biotechnological processes (Uma et al., 2010). In nature, active enzymes can be extracted from any living organisms while microbes are favored as compared to plants and animals, because of less risky materials of plant and animal tissues. The mainstream of enzymes utilized in industrial / biotechnological applications is attained from fungal and bacterial origin (Hussain et al., 2009). Invertases have also been characterized from Aspergillus sp. and several other filamentous fungi (Ashokkumar et al., 2001; Nguyen et al., 2005; Guimaraes et al., 2007, 2009).

Most work have been performed on preparation of synthetic medium for Invertase production whereas, less attention has been made for its production from inexpensive and economical sources (Vitolo et al., 1995; Ashokkumar et al., 2001; Rashad et al., 2006; Guimaraes et al., 2007, 2009).

Present study was conducted to produce Invertase enzyme by utilizing agro waste i.e. carrot peels through Aspergillus niger under solid state fermentation when grown in shaken cultures. Further enzymes was purified and characterized to get pure enzyme.

**MATERIALS AND METHODS**

**Microorganism**

Five pure culture of the Aspergillus spp. i.e. A.niger L. FCBP-840, A.fumigatus L. FCBP-971, A.flavus L. FCBP-1002, A.terreus L. FCBP-1011 and A.candidus L. FCBP-844 were acquired from the First Fungal Culture Bank of Pakistan, Institute of Agricultural Sciences, University of the Punjab, Lahore. The cultures were propagated on malt extract agar (MEA) slants.

**Food processing waste**

Carrot peels (D. carota L.) were used as a substrate for Invertase production. The peels obtained from local juice shops and households wastes, were rinsed with distilled water, excess water was dried. After fine chopping, peels were stored in plastic bags at 4°C for subsequent use as substrate for fermentation medium (Chantaro et al., 2008).

**Chemical analysis of carrot peels**

Proximate analysis of carrot peels were analyzed for moisture content, ash, crude fiber, crude fat, crude protein, total carbohydrates and pH (AACC, 2000).

**Fermentation media and growth conditions**

For solid state cultivation and fermentation carrot peels were used as substrate were taken in 250 mL conical flasks containing 10 g in each were sterilized at 121±1°C for 20 min. For inoculatory development, 2 mL of sterilized water was added in each slant having pure culture in order to scratch fungal mass by using a glass rod. After that spore suspension was decant in sterilized flasks. All work was performed under aseptic conditions to evade contamination. The number of spores per mL was calibrated by utilizing heamatocytometer. Cotton plugged flasks were allowed to incubated at 30±2°C for 72 h statically (Rashad and Nooman, 2009).

**Enzyme extraction**

After fermentation period, distilled water of 50 mL was poured in each flask. At 250 rpm enzyme extraction was carry out on rotary shaker for 90 min at 30±2°C. Crude extracellular enzyme was filtered by using filter paper (Whatman No.1) for measuring activity (Sangeetha et al., 2004).

**Enzyme assay**

Sumner and Howell method was used to calibrate Invertase activity in culture filtrate through liberation of reducing sugar released by the hydrolysis of sucrose by using glucose as a standard (Sumner and Howell, 1935). One Invertase unit is characterizes as the quantity of enzyme which liberate 1 mg of inverted sugar in 5 min at 20°C, at
pH 4.5.

**Protein determination**

Lowry et al. (1951) method was demonstrated to calibrate protein by using bovine serum albumin (BSA) as standard.

**Dry cell mass evaluation**

After requisite fermentation period, weight of dry fungal mycelium was measured which was collected after filtration by using filter paper. Weight was estimated by placing mycelium for at least six hours in electric oven at 105°C (Myers et al., 1997). Final weight was taken to obtain dry cell mass.

**Optimization of cultural conditions for Invertase production**

**Moisture content**

In order to obtain maximum production of Invertase, fermentation was done by facilitating the growth of organism on their particular substrate with different percentage of moisture content i.e. 10, 30, 50, 70 and 90% at 30±2°C for 72 h.

**Incubation period**

Invertase production varies with the increase in incubation period. For this purpose, different incubation times at 36, 48, 60, 72, 84 and 96 h were optimized at 30±2°C. Enzyme activity was anticipated after 12 h under pre-optimized conditions.

**Inoculum’s size**

Five various Inoculum’s size (1×10³, 1×10⁴, 1×10⁵, 1×10⁶, spores/mL) were evaluated for the maximum enzyme production under optimized conditions at 30±2°C.

**Partial Purification of Invertase**

**Ammonium Sulphate Precipitation**

The method of Madhan et al. (2010) was followed for purification of Invertase enzyme. Different levels of ammonium sulphate (40, 50, 60, 70 and 80%) in acetate buffer (pH 4.5) were used for the enzyme precipitation.

Crude culture filtrate was used to purify Invertase which was primarily subjected to 40% precipitation for 20 min at 4°C with ammonium sulfate and pelleted at 10,000 rpm. The acquired pellet was then suspended in 1 mL of 0.2 M acetate buffer. At 80% concentration of ammonium sulfate supernatant was additionally saturated and pelleted as earlier. Then pellet was again dissolve in 1 mL of acetate buffer to estimate activity in fractions.

**Charaterization of Invertase**

**Activity and Stability of Invertase at different pH**

Four buffering agents i.e. citrate (pH 3.0-4.0), acetate (pH 4.5-5.5), phosphate (pH 6.0-7.0) and Tris-HCl (pH 7.5-9.0) at 0.1 M were assayed for small aliquots of enzyme and pH profile was measured at standard assay conditions.

In order to measure the effect of pH on stability of enzyme it was allow to pre-incubate at 30°C for 30 min with four buffering agents after it, enzymatic and relative activity was calibrate.

**Activity and stability of Invertase at different temperatures**

Enzyme activity and thermal stability was measured at various incubation temperatures ranges from 20-70°C for 1 h after that enzyme activity was assayed under standard assay conditions.

**Determination of substrate specificity**

**Influence of metal ions**

A volume of 1 mM solutions of various metals were prepared. A volume of 1 mL of these solutions was earlier incubated at 30±2°C for 30 min with 1 mL of enzyme solution. Then Invertase activity was measured and % reduction in Invertase activity was estimated. Blank was taken before adding the metals. The chemicals which were used were, Nickel chloride (NiCl), Zinc sulphate (ZnSO₄), Cobalt chloride (CoCl₂), Sodium chloride (NaCl), Ferrous sulphate (FeSO₄), Copper sulphate (CuSO₄), Calcium chloride (CaCl₂), Mercuric chloride (HgCl₂), Magnesium chloride (MgCl₂).

Invertase activity on various substrates such as sucrose, maltose, fructose, and glycerol were analyzed. Concentrations of 0.1 and 0.5 M of all substrates along with glucose as a standard were utilized to determined reducing sugars.
Statistical analysis

Software package Co-stat Version 3.03 was used to calculate data obtained from various parameters of treatment means, standard error and Duncan's multiple range test (Steel and Torrie, 1980).

RESULTS

Experiments were run in three replicates, and all the values of data have been given as means (standard errors (SE)) which have been shown as error bars in the figures evaluated by (DMR) Duncan's multiple range test. In this work, five strains of Aspergillus spp. were selected for Invertase production. The cultivation was carried out on carrot peels by using solid state fermentation (SSF) devoid of any chemical addition. A wide variation in the yield of enzyme has been depicted by data on carrot peels (4.49±0.08-7.95±0.1 U/ml) The maximum production of Invertase was observed by using A. niger in case of carrot peels as a substrate, followed by A. candidus, A. terreus and A. flavus (7.71±0.06, 6.86±0.1 and 6.64±0.07 U/ml) respectively, which showed considerable amount of Invertase while negligible amounts of the enzyme was detected in A. fumigatus (Figure 1). During higher production of Invertase by A. niger dry cell mass and sugar consumption were 8.96±0.15 and 20.6±0.51 mg/ml, respectively and 1.01±0.05 mg/ml protein activity was recorded.

It was noted that Invertase production was affected by various factors. It was noted that production of invertase increased by increasing the moisture content from 30 to 90% as depicted in Figure 2. A. niger on carrot peels showed maximum Invertase (7.95±0.13) production during 90% of moisture. While dry cell mass, sugar consumption and protein were 8.04±0.35, 19.3±0.83 and 1.02±0.11 mg/ml, respectively. Incubation period had an obvious effect on Invertase production by A. niger, it seems from the results that a lag phase was observed during the first 24 h of spore germination with practically less enzyme synthesis. The effect of incubation periods on the production of Invertase is demonstrated in Figure 3. Maximal Invertase productivity was observed at the end of 72 h in A. niger (7.95±0.05 U/ml) after which a decline in enzyme activity was observed. The dry cell mass, sugar consumption and protein were 8.04±0.35, 19.3±0.83 and 1.02±0.11 mg/ml, respectively.

Concerning the effect of inoculum’s size, the obtained results revealed that, the optimal inoculum’s size needed to produce the highest yield of enzyme. The effect of inoculums size on the production of Invertase is demonstrated in Figure 4. Maximum enzyme production (10.24±0.06 U/ml) from A. niger on carrot peels was achieved at Inoculum’s size 1×10^6(spores/ml). While dry cell mass, sugar consumption and protein were estimated to 8.94±0.3, 19.5±0.63 and 1.21±0.1 mg/ml, respectively.

The proximate composition of the fermented and non-fermented carrot peels in Table 1 reveals that fermentation of the peels increases moisture content of carrot peels (26.7±0.29 %) as compared to the non-fermented dry sample (7.46±0.35%). There is slight increase in protein of fermented carrot in contrast to non-fermented sample. There was significant decrease in the fiber, fat and ash content of the fermented sample. This affected the carbohydrate content (calculated by difference) which was high in non-fermented sample. However pH of the fermented carrot peel also show decline as compare to non-fermented one.

The crude enzyme preparation was obtained by conducting fermentation processes under the optimal culture conditions tested in the present study. The whole optimum culture media obtained from several batches were collected and the culture filtrate was concentrated to be used in the trials of getting the enzyme in a pure form. The crude culture filtrate containing Invertase was subjected to ammonium sulfate precipitation. After conducting some preliminary trials, 80% concentration was selected for the enzyme precipitation. The results indicate specific activity of 8.09 U/mg, respectively. However in partially purified form specific activity was 11.5 with 1.42 purification fold (Table 2).

The enzyme was characterized to find the conditions at which it showed best performance. The activity and stability of purified enzyme were investigated at different temperatures and pH in (Figure 5 and 6). The enzyme showed maximum activity at 50°C however, Invertase was found to be stable from pH 5.5 to 6.5, respectively. The purified extracellular Invertase was tested for substrate specificity. Invertase was highly active towards sucrose at both concentrations viz: 0.1 and 0.5 M, but it showed less activity towards glycerol (Figure 7).

The effects of metal ions on the activity of the Invertase are shown in Figure 8. Different metals were assessed in present work and results showed that Hg²⁺ inhibited the enzyme activity. While slight inhibition was noted by other metals however increase in Invertase activity was observed in case of Co²⁺.

DISCUSSION

In recent years, the bioconversion of food processing residues matters represents possible and utilizable resources for conversion to useful products. Food processing wastes are produced in huge amounts and since they are rich in carbohydrates and other nutrients, they can serve as a substrate for the production of bulk chemicals and enzymes using SSF technique (Couto and Sanroman, 2006).
In present study, carrot peels were used as substrate for invertase production after screening, by the *Aspergillus* spp. The results indicate that in case of carrot peels *A. niger* gave the higher units in comparison to other species. Santos et al. (2004) reported that fungi have been considered to be the organisms most adapted to SSF because their hyphae can grow on particle surfaces and penetrate into the inter particle spaces and thereby colonizing solid substrates. Viniegra-Gonzalez et al. (2003) found that *A. niger* grew more efficiently and produced more Invertase in SSF.
culture than in submerged culture when sucrose levels were high. His studies suggested that higher sugar levels prevented penetration of Invertase in SSF. Mamma et al. (2008) found that the highest Invertase activity produced by \textit{A. niger} cultivated on dry orange peel was 72.5 U/g dry substrate at initial pH 5 and 90% moisture content, while the highest activity which was produced by \textit{Neurospora crassa} was 74.0 U/ g dry substrate under the same conditions. These values are much higher than those reported in the present study, by the same microorganism grown on different carbon source. Madhan et al. (2010) investigated the efficiency of \textit{A. niger} to produce invertase by using powdered stem of Lemon grass as sole substrate and sole carbon source for the microorganism.

The economic feasibility of the microbial enzymes production application generally depends on the cost of its
production processes. In order to obtain high and commercially viable yields of Invertase enzymes, it was essential to optimize the fermentation medium used for fungal growth and enzymes production. In SSF, the existence of an optimum moisture content of the medium has been stressed as it has profound effects on growth kinetics, and on the physicochemical properties of solids, which in turn affects productivities (Lonsane et al., 1992).

So, in the present study the effect of different initial moisture contents on the production of the enzyme was studied. Krishna (2005) found that lower moisture tends to reduce nutrient diffusion, microbial growth, enzyme stability and substrate swelling. Similar results were reported by Mamma et al. (2008) who found that the optimum moisture content for the production of Invertase from different fungal strains by SSF using orange peels was 90%.

Incubation period had an obvious effect on invertase production by A. niger it seems from the results that a lag phase was observed during the first 24 h of spore germination with practically less enzyme synthesis. Maximal invertase productivity was observed at the end of 72 h in after which a decline in enzyme activity was observed. Similar results were concluded by Rashad et al.
(2005) from *Saccharomyces cerevisiae*. Sirisansaneeyakul et al. (2000), reported incubation period of 72 h for both intra and extracellular Invertase from *A. niger* which was similar to present results. It was very essential parameter that has to be controlled because it varies from organism to organism. Parks and Pastores (2006) observed that extracellular Invertase from *A. niger* reached at maximum activity after 4 days of incubation followed by a decline in the enzyme activity till the 6th day of incubation.

Concerning the effect of inoculum’s size, the obtained results revealed that, the optimal inoculum’s size needed to produce the highest yield of enzyme. Inoculum’s size above the previously optimum recorded value gave value gradually decreasing to that of the optimal one. However,
after certain limit the competition for the nutrients resulted in a decrease of the metabolic activity of the organism. With optimum inoculum’s size, there was a balance between biomass synthesis and availability of nutrients that supports production of enzyme (Nampoothiri et al., 2004).

Chemical analysis of substrate revealed different composition of nutrients which suggested that it can be utilized for microbial growth. Krishna (2005), reported that numerous nutrients can regulate sporulation through metabolic effects in SSF. These nutrients include carbon and nitrogen sources, minerals, and vitamins or cofactors. The present study depicted that moisture content of fermented peels was high as compare to non-fermented, so it can serve as substrate for the production of enzymes using SSF technique. There is slight increase in protein content. The increase in protein of the fermented peels samples may be due to the fact that the microorganisms identified which degrades the sample readily may have secreted extracellular enzymes in the peels which subsequently increases the protein content of the fermented sample as well as microbial biomass (Odetokun, 2000). There was no considerable difference in the fat, while there was a decrease in fiber content of the fermented sample. This affected the carbohydrate content (calculated by difference) in which there was no considerable difference. Aykroyd and Doughy (1982), Brough and Azam-Ali (1992), and Odetokun (2000) reported that increase in carbohydrate content during fermentation may be due to a reduction in the fiber content and increase in both reducing sugars and total soluble sugars. Suutarinen et al. (2003) reported proximate composition of carrot and to determine the enzymatic potential of various vegetable peels. Rashad and Nooman (2009) worked on chemical analysis of red Egyptian carrot for the production of extracellular Invertase from S. Cerevisiae. Various authors reported this analysis on the basis of their substrate conditions. Change in pH in fermenting carrot was also observed and it was noted that there was decrease in pH throughout the fermentation. Raimbault and Tewe (2001) indicated that the pH of a culture may change in response to metabolic activities. The most obvious reason being the secretion of organic acids as citric, acetic or lactic this causes pH to decrease.

The present study describes the purification of crude enzyme by different fractions of ammonium sulphate precipitation. The results presented here indicate specific activity of 8.09 in carrot peels. However in partially purified form specific activity was 11.5 with 1.7 purification fold. Hocine et al. (2000) reported purification of extracellular \( \beta \)-d-fructofuranosidase from A. ochraceus. The results of ammonium sulfate fractionation showed that 80% of total fructosytransferase and Invertase activities could be recovered in the fraction of 30–100% ammonium sulfate saturation with a purification of 5.3 and 5.1 fold, respectively. Bhatti et al. (2006) purified the Invertase from Fusarium solani with 3.1 purification fold. Akardere et al.
(2010) used three-phase partitioning (TPP) to purify Invertase from Baker's yeast (S. cerevisiae) in a single step. They concluded that under optimized conditions the Invertase was purified to 15-fold with 363% activity recovery. The efficiency of the salting out of proteins will depend on sulfate concentration as well as, on the net charge of the proteins. Therefore, ammonium sulfate saturation is of critical importance and must be optimized (Dennison and Lovrein, 1997; Sharma et al., 2000). Invertase yield obtained by this purification procedure was low; nevertheless, our goal was to purify and characterize the isolated enzyme.

The activity and stability of purified enzyme were investigated at different temperatures and pH in the present work. The enzyme showed maximum activity at 50°C in A. niger this result was similar to that, reported by Nguyen et al. (2005) who found that the optimum temperature for Invertase production by A.niger was 50°C. Akardere et al. (2010) found Invertase activity at 60°C. Higher values of optimum Invertase temperatures were reported by many authors (Rubio et al., 2002; Guimaraes et al., 2007; Hussain et al., 2009), while lower value (30°C) was reported by Rashad et al. (2006). The thermal stability of the Invertase was found from 20-40°C. The enzyme stability in A.niger was in the same range as illustrated by Quiroga et al. (1995), Rashad and Nooman (2009). However Madhan et al. (2010) found that Invertase from A. niger was found to be stable till a temperature of 55°C. The enzyme showed maximum activity at pH 5.5. The similar results were described by Nguyen et al. (2005) for A.niger. The optimum pH value was higher than those reported by many authors (Quiroga et al., 1995; Rubio et al., 2002; Rashad et al., 2006; Guimaraes et al., 2007, 2009), while it was lower than that reported by Vorster and Botha (1998) who found that the optimum pH of neutral Invertase was 7.2. While in present study Invertase was found to be stable from pH 5.5 to 6.5. Nguyen et al. (2005) found that A.niger Invertase was stable in the pH range from 5.0 to 6.5. Different pH-stability profiles of Invertases have been obtained depending on the enzyme source and also assay method applied (Bhatti et al., 2006; Nguyen et al., 2005; Belcarz et al., 2002).

Invertase was highly active towards sucrose (100%) and slightly active towards raffinose (14.7%) but it nearly did not show any activity towards maltose and glycerol (Rashad and Nooman, 2009). Similar behavior of extracellular Invertases towards sucrose and raffinose has been detected by many workers (Rubio et al., 2002; Rashad et al., 2006; Guimaraes et al., 2009) using different microorganisms (R. glutinis, S. cerevisiae and A. niger) respectively. Similar results were depicted in present work; invertase was highly active towards sucrose at both concentrations viz: 0.1 and 0.5 M, but it showed less activity towards glycerol. These results suggest that the enzyme is a β-D-fructofuranoside fructohydrolase, able to attack the β-D-fructofuranosides from the fructose end.

Different metals were assessed in present work and results showed that Hg²⁺ inhibited the enzyme activity. While slight inhibition was noted by other metals however increase in invertase activity was observed in case of Co²⁺. Rashad and Nooman (2009) reported similar results by detecting complete inhibition by Hg²⁺, as well as noticed slight increase in the enzyme by using Co²⁺ at low concentration (1 mM). The inhibition of Invertase by Hg²⁺ was reported by many authors (Ghosh et al., 2001; Rashad et al., 2006; Guimaraes et al., 2009) and they suggested that thiol groups at the catalytic site are important for the Invertase activity. Stimulation of Invertase activity by Co²⁺ was also reported by Rubio et al. (2002) and Rashad et al. (2006), while Nguyen et al. (2005) found that the enzyme was slightly inhibited by addition of 1 mM Co²⁺. Guimaraes et al. (2007) also reported that Cu²⁺ inhibited the extracellular β-fructofuranosidase activity of A. ochraceus. Salt addition may affect amino acid residues both at the active site and the exterior surface of enzymes, possibly inducing charge alterations of the catalytic amino acids and/or structure distortions (Salis et al., 2007). So, this study indicates the potential for the use of food processing wastes such as red carrot peels for the efficient production of Invertase by A.niger in SSF, thereby resulting in recycling waste materials for conversion into useful product of higher value as a by-product.

CONCLUSION

This study indicates the potential for the use of food processing wastes such as red carrot peels for the efficient production of Invertase by A.niger in SSF, thereby resulting in recycling waste materials for conversion into useful product of higher value as a by-product.

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