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Research article

ISOLATION OF INULINASE PRODUCING BACTERIA FROM SUGARCANE SOIL

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ABSTRACT: Microbial inulinases have a great potential for industrial use in the production of fructose from inulin. Optimization of the growth parameters of the microbes is essential to obtain inulinase in sufficient quantity. The present work aimed to obtain inulinase from natural strains isolated from sugarcane fields and to optimize its growth parameters. In a total of ten isolates, four organisms *Bacillus subtilis, Lactobacillus casei, Pseudomonas aeruginosa,* and *Achromobacter* sp. were identified as efficient inulinase producers. The optimum temperature and pH of these organisms were found to be 40˚C and pH 5 respectively. Inulin was observed to be the suitable carbon source for these organisms. The molecular weight of this enzyme was estimated at 45 KDa using SDS-PAGE. On thin layer chromatography inulin hydrolysis showed mono and disaccharides as the main end products. The highest enzyme activity was obtained from *Achromobacter* spp. of 333 U/L at 22nd hour which showed exo-inulinase type.

Keywords: Inulinase, bacteria, inulin, temperature, pH, purification of inulinase.

INTRODUCTION:

Inulin is the reserve carbohydrate found in many plant families such as Jerusalem artichoke (*Helianthus tuberosus)*, Dahlia tubers or Chicory roots (*Cichorium endivia)*. It is a good source of high fructose and consists of linear -2, 1-linked polyfructose chains with terminal glucose unit (Susana kalil *et al*., 2001). This enzyme can be extracted from plants and vegetables, but it is difficult to obtain it in sufficient quantity and its production cost is also high. Therefore microbial inulinases EC.3.2.1.7) represents a good alternative and have become an important class of industrial enzymes (Vandamme and Derycke, 1983). These are inducible enzymes exhibiting exo/endo acting and fructotransferase activity. Generally exo acting enzymes are exhibited by many microorganisms, which catalyze the hydrolysis of inulin by splitting off the terminal fructosyl units (D-fructose) (Regina *et al*., 2001; Basso *et al*., 2010).

Hydrolysis of inulin leads to the formation of D-fructose, which is considered as a safe alternative sweetener to sucrose. Fructose in addition to higher sweetening capacity can also increase the iron absorption therefore more beneficial for the diabetic patients. Fructo-oligosaccharides also have good functional and nutritional properties such as low caloric diet, bifidus stimulating factor, growth inhibitory effect on tumors and hence used as a source of dietary fiber in food preparation (Andrelina Santos and Mangeri, 2006). These oligosaccharides therefore are now widely used to replace sugar in many food applications such as in confectionary, chocolate and dairy products. Inulinase are produced by different types of bacteria such as *Bacillus* sp, *Clostridium* sp, *Lactobacillus, Pseudomonas, Arthobacter & Staphylococcus* sp (Prabhjeet singh and Prabhjot Kaur Gill, 2006). Some fungi and yeast such as *Kluyveromyces fragilis, Kluyveromyces marxianus, Candida kefyr and Debraryonuyces* sp, *Aspergillus* sp, *Fusarium* sp, *Pencillium* sp (Allias *et al*., 2004; Figen Ertan Aktac *et al*., 2003) and actinomycete (Gill *et al*., 2003) are also used for inulinase production. Although much work on isolation of different inulinase producing microorganism were investigated still there is a need for isolating efficient inulinase producing microorganism from natural sources. The objective of this work is to screen the inulinase producing bacteria from sugar cane field soil and purification of the enzyme using partial characterization techniques.

MATERIALS AND METHODS

All chemicals were purchased from Hi-media and inulin was purchased from Sigma-Aldrich and sugars were filter sterilized using a 0.45µ pore size Millipore filter.

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(i) Screening for inulinase producing bacteria:

Soil samples were collected in sterile containers from two sugarcane fields at a depth of 3 cm located at Thumbal of Salem district and aseptically transferred to the laboratory. One gram of soil sample was aseptically suspended in 100 ml of distilled water. After appropriate dilution, aliquots of soil samples were spread on the inulin agar plates (g/L- Potassium dihydrogen phosphate 0.10; Magnesium sulphate 0.05; Potassium chloride 0.05; Ammonium dihydrogen ortho phosphate 1.00; Inulin 2; Agar 2; Final pH 4.5) and incubated at 37°C for 24 to 48 hours. Colonies grown on the plates were purified to homogeneity and used for further studies.

(ii) Submerged fermentation :

Fermentation was started with 5 % (v/v) inoculums that was inoculated in 100ml of production media (g/L- Potassium dihydrogen phosphate 0.10; Magnesium sulphate $7H_2O$ 0.05; Potassium chloride 0.05; Ammonium dihydrogen ortho phosphtae 1.00; Inulin 2; pH 5.5) (Marico mazutti *et al*., 2010); incubated on the rotary shaker at 100 rpm at 37°C for 72 hours.

(iii) Inulinase assay :

Fermented broth was centrifuged at 10,000 rpm for 10min at room temperature. The supernatant obtained was used for extracellular inulinase estimation using the DNS method (Joae Paulo *et al*., 2006). To 0.1ml of supernatant 9ml suspension containing 2% inulin prepared in Sodium acetate buffer (0.1M) at pH 4.5 was mixed. The suspension was incubated at 50˚C for 20 min and kept in boiling water bath (100°C) for 10 min to inactivate the enzyme and cooled to room temperature. The reaction was measured in a spectrophotometer at 575nm every hour and calibrated against a standard fructose. One unit of enzyme was defined as that required to liberate one micromole of fructose per minute from inulin under assay condition. For intracellular enzyme assay, sample was centrifuged at 10,000 rpm for 10min at room temperature. To the pellets 0.1ml of sodium acetate buffer at pH 4.5 was added for cell disruption followed by filtration with Whatmann filter paper. The filtrate was assayed for intracellular enzyme as described above.

(iv) Study of growth parameters of the isolates:

The growth rate of the isolates was studied by observing the growth pattern at stipulated intervals by measuring the optical density at 600 nm. In the same way the effect of initial pH (3, 4, 5, 6, 7, 8 and 9), temperature (30, 35, 40, 45 and 50˚C) and the effect of various carbon sources like inulin, sucrose, xylose, fructose and glucose were determined.

(v) Purification of inulinase:

The sample was centrifuged at 6000 rpm for 20 minutes and the obtained supernatant was treated with 80% Ammonium sulphate. To 20 ml of supernatant 50 ml of 80% ammonium sulphate was added, mixed and refrigerated overnight. The next day, centrifugation was done at 6000 rpm for 10 minutes and the supernatant was discarded. The precipitate was resuspended in 0.02 M phosphate buffer saline (pH 8.0); desalted on a dialysis bag against the same buffer. The partially purified sample was subjected to electrophoresis on 10% SDS-PAGE. The samples were run along with marker and molecular weight was estimated as described by Eman Tohamy, 2010.

(vi) Thin layer chromatography:

Thin layer chromatography was performed on pre-coated silica gel plates from Merk, Germany. The sample was developed using n-butanol, isopropanal, acetic acid and water (7:5:2:4, volume ratio) as solvent system. The sugars are visualized by heating the plates at 105˚C for 30 minutes after spraying with 3% urea in butanol, ethanol, water and orthophosphoric acid (80:8:5:7, volume ratio). Thin layer chromatography was followed as described by Wei *et al*., 2009.

RESULTS AND DISCUSSION

Isolation of efficient inulin hydrolyzing bacteria can be obtained from sugarcane fields (Drent *et al*., 1991; Zherelitsov *et al*., 2002). Therefore in the present study, ten soil samples were collected from two different sugarcane fields and screened for inulin hydrolyzing bacteria. Fifteen different colonies were selected based on the formation of clear zone of lysis around the colonies. These isolates were further purified and the enzyme activity was studied.

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Commercially high yield of microbial inulinase was obtained by submerged fermentation as presented in many literatures (Kalil *et al*., 2001; Silva-Santisteban boy and Maugeri, 2005). In order to obtain high yield, submerged fermentation was carried out for 72 hours and enzyme assay was performed using DNS method (Miller method). Results from enzyme activity revealed maximum enzyme activity in the supernatant for all the organisms rather than the pellet (data not shown). This indicated the extracellular activity of the enzyme and out of fifteen isolates four were selected for further optimization studies. These four isolates were identified as *Bacillus subtilis, Lactobacillus casei, Pseudomonas aeruginosa, and Achromobacter* sp. Figure1 to 4 shows the results of growth and enzyme profile of the four isolates. It is evident from the results that inulinase production was started from 8th hour and the maximum enzyme activity was seen during the late logarithmic phase. The maximum inulinase accumulation (333 U/L) was seen for the isolate *Achromobacter* sp. at 22nd hour of fermentation. Three other isolates *Bacillus subtilis, Lactobacillus casei, Pseudomonas aeroginosa* also produced maximum inulinase activities ranging from 221 to 333 U/L in the supernatant. Similar results were reported in *Xanthomonas oryzae No. 5* by Pessoa and Vital *et al*., 1999. The medium composition and growth parameters have profound effect on cell growth and enzyme activity. Media pH influences the optimal activity of a microbial strain, and hence optimization of pH is very essential. Figure 5 represents the effect of various initial pHs [3, 4, 5, 6, 7, 8, and 9] on bacterial growth at 35°C. The results showed that optimum pH for all the four bacterial isolates was slightly acidic ranging from pH 4.0 to pH 6.0. In terms of fermentation process, lower pH usually offer an advantage in fructose syrup production as it prevents microbial contamination (Pandey *et al*., 1999). Similarly the microbial activity varied with different temperature such as 30, 35, 40, 45 and 50˚C. It is evident from Figure 6 that all the four isolates grew well at mesophilic conditions. Most of the optimum temperature was found between 30˚C-55˚C for inulinase producers (Singh *et al*., 2007). The effects of process parameters on inulinase production by *Aspergillus niger* were studied in which the inulinase production was probably inducible and subjected to catabolite repression (Poorna and Kulkarni, 1995). In the absence of inducer, reasonable amount of enzyme could be produced. Inulin is the best carbon sources for inulinase production compared with other sugars (Jun sheng chi *et al*., 2003). Five different sugars were tested as carbon source (Sucrose, fructose, glucose, xylose and inulin) at 2% (w/v) concentration and results were illustrated in graph [Figure 7]. All the five sugars were utilized by bacteria but a slight increase in inulin and fructose utilization was observed.

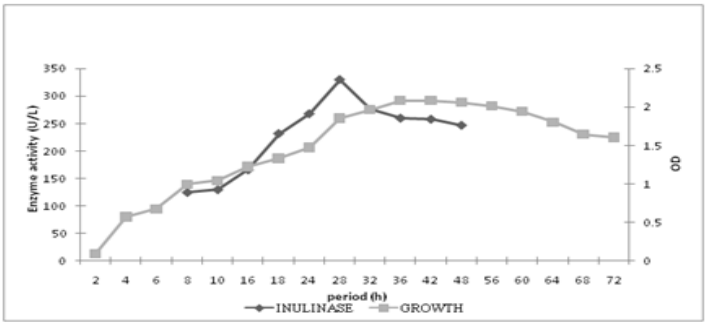

Figure 1: Growth and enzyme profile of *Achromobacter sp.*

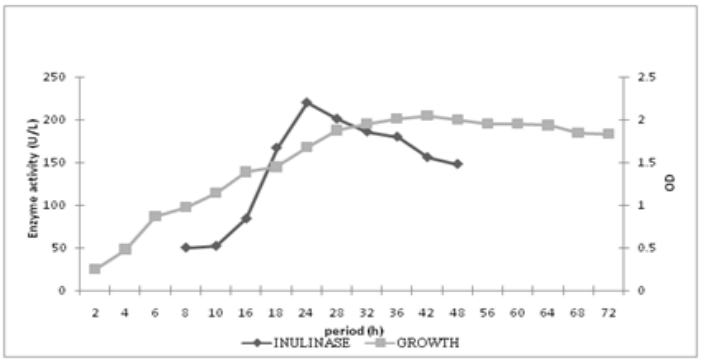

Figure 2: Growth and enzyme profile of *Bacillus subtilis*

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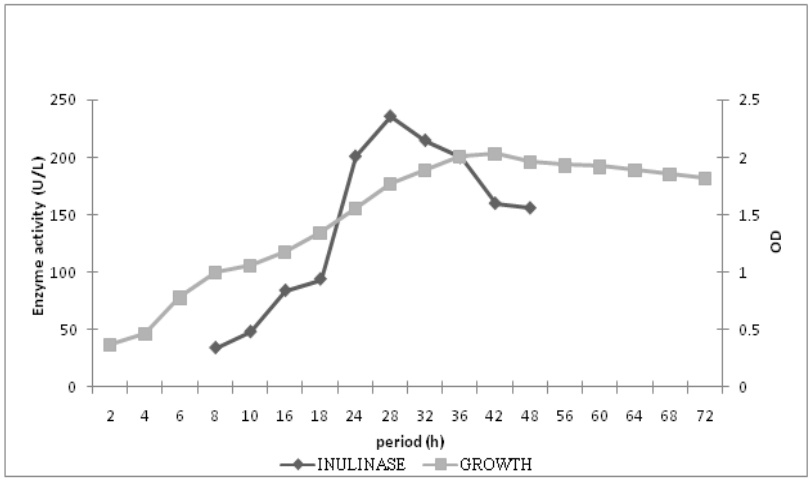

Figure 3: Growth and enzyme profile of *Pseudomonas aeruginosa*

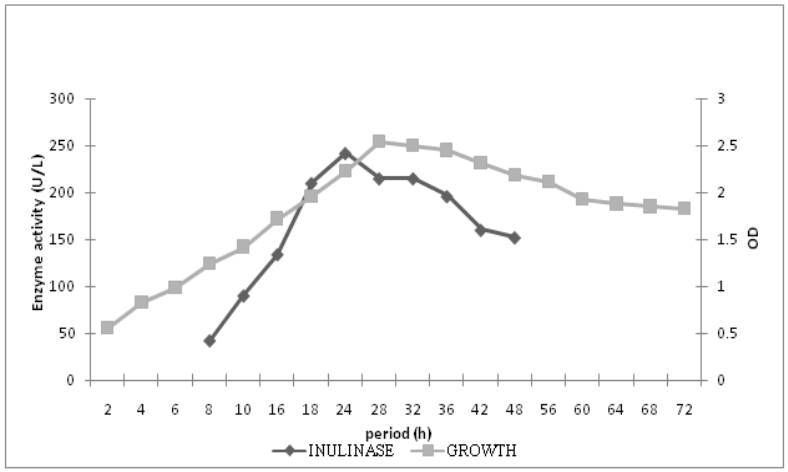

Figure 4: Growth and enzyme profile of *Lactobacillus casei*

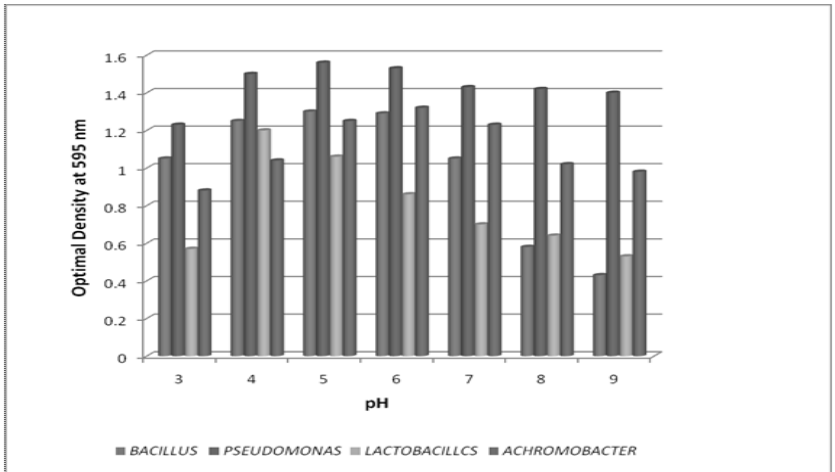

Figure 5: Effect of pH on the growth of bacteria

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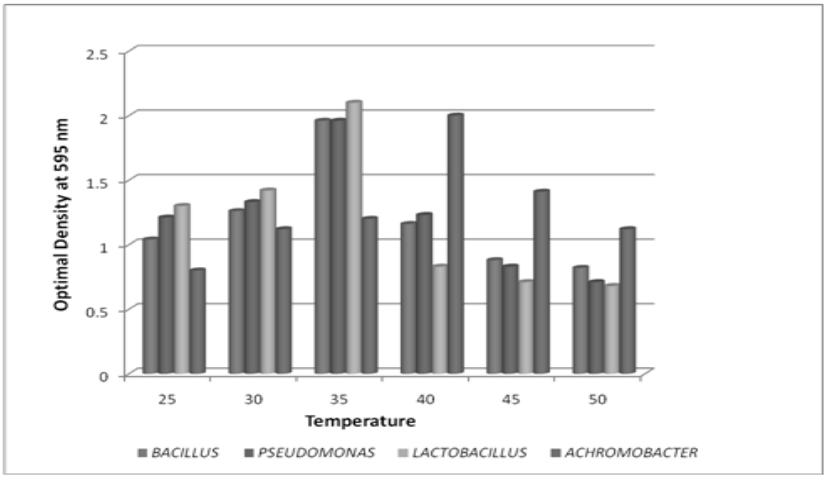


Figure 6: Effect of temperature on the growth of bacteria

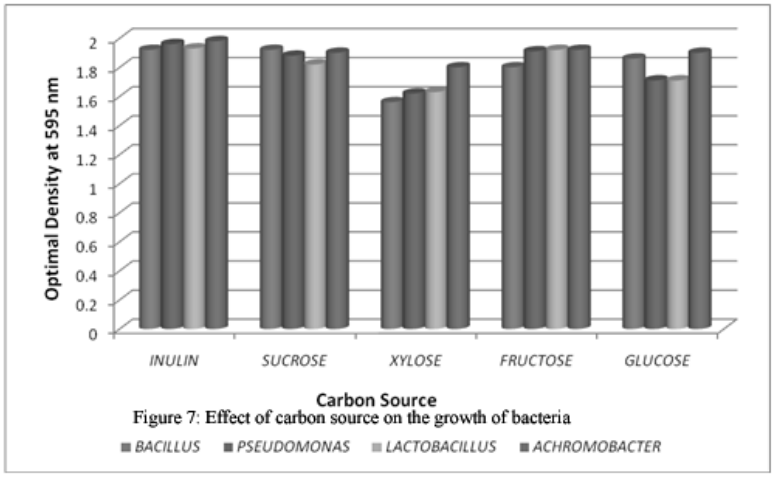
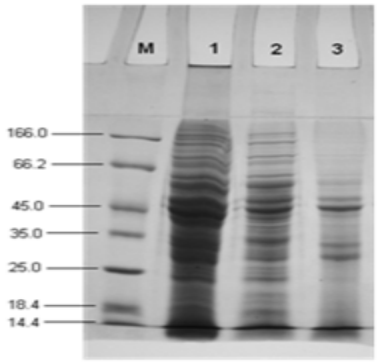


Figure 7: Effect of carbon source on the growth of bacteria



M- Marker;
Isolate 1: Bacillus subtilis;
Isolate 2: Pseudomonas aereginosa;
Isolate 3: Lactobacillus casei

Figure 8: Estimation of molecular weight of inulinase by SDS-PAGE

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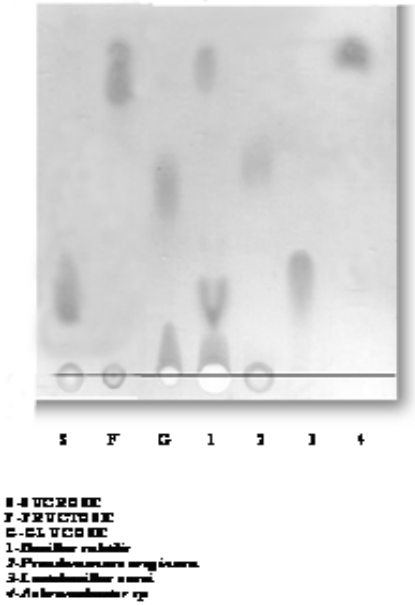

Figure 9: Separation of sugars by thin layer chromatography

Partial purification was carried out using 80% saturated ammonium sulfate followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The inulinase enzyme with the molecular weight of 45KDa was observed (Figure 8). Single band was observed in SDS-PAGE which indicates the presence of inulinase enzyme. Similar results were observed by Kushi *et al*., 2000 in *Bacillus smithii* and so far the highest molecular weight of inulinase was found to be 175 KDa in *Xanthamonas* sp (Youn Jeung Cho and Jong Won Yun, 2002). Thin layer chromatography was carried out with the crude extract. This technique was usually employed for the characterization of the mode of action of the enzyme, whether it is of an endo or exo inulinase type. Mono- and di-saccharides were detected in the reaction products, which suggested that the isolates are exo-acting enzymes (Figure 9). The exo-inulinase and endo-inulinase are used for the production of ultra high fructose syrup and oligosaccharides respectively (Dong Hyun *et al*., 1997). After the inulin hydrolysis to fructose and inulo-oligosaccharides by exo- and endoinulinases, these raw materials can be used for many practical applications as bioethanol production, in food industry, citric acid and other chemical production.

CONCLUSIONS

Inulinase is considered as an important class of industrial enzyme, requiring the need to identify inulinase with novel and specific properties. In this study, we have successfully isolated four efficient inulinase producing bacteria from sugarcane field soil. The temperature and the pH were optimized as 35 C and pH 5 respectively and Inulin was found to be the most suitable carbon source with exo-inulinase activity. *Achromobacter* sp. that showed exo-inulinase activity produced the highest inulinase activity (333 U/L) which is significant for industrial production.

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