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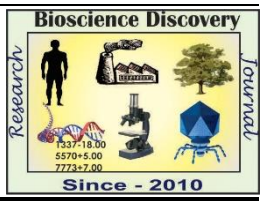
Research Article



Screening of inulinase producing bacteria from diverse sources

Sai Tharun Kumar Guttikonda, S. Vanitha and K. Anuradha

Department of Microbiology, Bhavan's Vivekananda College, Sainikpuri, Secunderabad – 500094.
Email: kanuradha_7@yahoo.co.in

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Abstract

Inulinases are β-fructosidases that release fructose molecules from inulin. Different inulinase producing bacterial isolates were screened from soil samples of rhizospheres of Onion, Garlic, Dahlia, Taro, etc. Enrichment culture technique was used for isolation of inulinases that have major applications in the production of high fructose sugar from inulin and inulin rich materials. Modified Czapek-Dox agar with 1% inulin was used for screening inulinase producing bacteria. Inulinolytic activity was indicated by a zone of inulin hydrolysis using the Lugol's iodine. Colonies positive for inulinolytic activity from different rhizosphere samples were further assayed for inulinase production in broth at 37 ° C. Different inulinolytic bacterial isolates were obtained and were identified microscopically belonging to the genera *Bacillus, Acetobacter, Pseudomonas, E. coli, Staphylococcus.* Among all the isolates one isolate was found to be a good inulinase producer and so was tested for their inulinolytic activity on different inulin-containing plant extracts derived from Onion, Garlic and Dhalia. One efficient bacterial isolate identified as *Bacillus sp* showed good enzyme production at 37 ° C and pH 6 using Dhalia as raw inulin source. The selected inulinolytic *Bacillus sp* could have ample application not only for enzyme production but also in the food industry for the production of fructose and fructose syrups.

INTRODUCTION

A poly fructan, inulin is a storage carbohydrate containing repeating units of (2, 1) linked fructose residues with sucrose as its terminal residue. As it has fructose as its major repeating unit it has become an alternative substrate for production of fructose syrup and fructooligosaccharides (FOS) (Maarten *et al*., 2015). In nature many mono and dicotyledonous plant species namely *Liliaceae, Amaryllidaceae, Gramineae, Compositae* and *Asteraceae* store inulin in their bulbs, tubers and roots except *Gramineae.* Few plants like Jerusalem artichoke (*Helianthus tuberosus*), Chicory (*Cichorium intibus*), Dahlia (*Dahlia pintana*), Onion (*Allium cepa)*, garlic *(Allium sativum)* and Taro *(Colocasia esculenta)* contain inulin as a storage compound in various amounts (Nagen *et al.*, 2004; Molina, 2005; Singh and Gill, 2006; Chi *et al.*, 2011).

Inulinases catalyse inulin to produce inulo-oligosaccharides, fructose and glucose as their end products. It is categorized into two types mainly exo-inulinases which act from the non-reducing ends to produce fructose as its main product, whereas endo-inulinases acts on internal glycosidic linkages to yield fructose oligosaccharides as its products (Ertan *et al.*, 2003; Chi *et al.*, 2009; Chi *et al.*, 2011).

A large number of microorganisms like bacteria, fungi and yeasts are used to produce inulinases. Bacterial strains are used for inulinase production, mainly because of their thermostability, many researchers are looking forward to screen and isolate a unique strain to produce inulinases that can

be used for various applications. *Bacillus* strains were isolated that produce inulinases on sucrose substrate only (Zherebtsov N.A, 2002) and strains acting on inulin as a substrate is our interest of this study. Researchers are focused on the production of inulinase producing microorganism as their products have varied applications in industries like food industry for the production of high fructose syrup, single cell oil, single cell protein and bioethanol whereas, in chemical industry it is used for the production citric acid, butylene glycol, alcohols and lactic acid and also in pharmaceutical industry (Pandey A *et al.*, 1999; Chi Z *et al.*, 2009; Liu X.Y *et al.*, 2010; Chi Z. M *et al.*, 2011). The aim of this study is to isolate an efficient bacterial strain that produces inulinase using inulin as its substrate.

MATERIALS AND METHODS

Screening and selective isolation of inulinolytic organisms: Sample from diverse inulin rich plant sources and soil from rhizospheres of Taro, Dahlia, Garlic and Onion were screened for inulinolytic isolates. These samples were collected aseptically and inoculated on Czapek-Dox agar plates enriched with inulin (1% w/v) (make) and incubated at 37° C for 24 hours and duplicates of the plates were kept to facilitate the test for inulin hydrolysis by lugol's iodine (Ai-Xia Li *et al.*, 2011). The plates were treated with lugol's iodine solution constituting potassium iodide (1.5% w/v) and iodine (1% w/v) (Ai-Xia Li *et al.*, 2011), for 3 to 5 minutes and washed with distilled H_2O to remove unbound iodine and then incubated for 1 hr at room temperature. Isolates with clear colourless zones around the colonies indicating inulin hydrolysis were observed. Six bacterial colonies with larger zones of inulin hydrolysis were subcultured, the obtained strains were identified morphologically and tested further for inulinase production in a liquid medium using commercially available Inulin as its substrate.

Enzyme Production: The enzyme production was performed by submerged fermentation in 250ml Erlenmeyer flasks containing 100ml of Czapek-Dox broth enriched with Inulin (1% w/v). The flasks were incubated at 37 ° C for 24-48 hours. Broth samples were collected and used for the assay of inulinase activity.

Fermentation Condition for Inulinase Production: Submerged fermentation studies were carried out with selected isolate *Bacillus sp* 2 for the production of inulinase using inulin enriched Czapex-Dox broth at different fermentation condition's like pH (a range of 4-7), temperature (ranging from 25-40 ° C).

Assay of Inulinase: Fermented broth was centrifuged at 3500 rpm for 10 minutes at room temperature the supernatant obtained was used to determine inulinase activity using 3, 5 dinitro salicylic acid (DNS) method. Fructose (1 mg/ml) was used for the standard curve. To 0.2ml of supernatant taken in a test tube 1.8ml of 1% inulin prepared in 0.2M sodium acetate buffer (pH 5) was added and incubated at 50 ° C for 30 minutes. After incubation, liberated reducing sugars were estimated by DNS reagent (3ml) and the tubes were placed in a boiling water bath for 5 minutes (Miller GL, 1959). Tubes were allowed to cool to room temperature and reddish brown colour obtained and their absorbance was measured at 540 nm against a reagent blank.

Thin Layer Chromatography: Thin layer chromatography was performed on precoated silica gel plates (Merck, Germany). The broth samples were spotted on a TLC plate using microcapillary tubes and the plate was developed with the solvent system containing 2 parts of isopropyl alcohol and 2 parts of ethyl acetate and 1 part of water. The spots were visualised by spraying with α-naphthol (0.5% w/v), concentrated sulfuric acid (5% v/v) in absolute ethanol and purple coloured spots were observed after heating the plate at 100 ° C for 10 minutes and compared using commercially available fructose as standard (Naveen Kango, 2007).

RESULTS AND DISCUSION

Inulin rich various plant source samples gave fifty primary inulinolytic isolates by enrichment culture technique using Inulin (1% w/v). Lugols iodine plate assay was employed for rapid screening to determine primary isolates that depolymerised inulin which was indicated by the development colourless hydrolytic zones (Fig. 1). The isolates having 0.1 - 0.5 cm zones of hydrolysis were considered as potential inulinase producers, as indicated in (Fig. 2). A morphological study of the primary isolates indicated the presence of species like *Bacillus, Acetobacter, Pseudomonas, E. coli, Staphylococcus* and was tested for inulinase production by submerged fermentation. While the inulinase production was confirmed by the release of fructose into the medium which was detected qualitatively by thin layer chromatography (Fig. 3). Among the inulinase producers, one bacterial isolate namely

Bacillus sp 2 from soil rhizosphere of Onion showed high inulinase activity (41.16 U/ml) represented in (Fig. 4). This isolate *Bacillus sp* 2 was selected for further study and its optimal conditions were determined by submerged fermentation studies using inulin enriched cazpek-dox broth at different fermentation conditions like pH (a range of 4-7) (Fig. 5), temperature (25-40 ° C) (Fig. 6). Highest enzyme production was observed after 24 hours at pH 6 and at a temperature of 37 ° C. The selected isolate *Bacillus sp* 2 was further studied for inulinase production using different raw sources like onion, garlic, dahlia as a carbon source in the fermentation medium. The inulinase production was found to be high in broth containing onion as carbon source (25.9 U/ml) when compared to dahlia (12.02 U/ml) and garlic (4.61 U/ml) represented in (Fig. 7).

Inulin is one of the important raw material for the production of high fructose syrup (Ricca E *et al.*, 2007) which is used as a safe sweetener in the food industry and fructooligosaccharides (P. T. Sangeetha *et al.*, 2005) which is used as a potent prebiotic and dietary fibre.

Inulin being predominant storage component in raw sources like Onion, Taro, Dahlia and Garlic have enhanced the growth of inulinolytic isolates and hence efficient Inulinolytic organisms were obtained from rhizosphere samples of Onion.

Lugol's iodine method was employed as it specifically binds to inulin giving brown colour and a colourless halo zone in absence of inulin hence lugol's iodine plate assay was an efficient and significant screening strategy (Ai-Xia Li *et al.*, 2011). Many bacterial isolates like *Bacillus sp*, *Xanthomonas sp* and *Clostridium sp* are known to produce inulinases at different temperatures and pH (Allais JJ *et al.*, 1986; D. L. Vullo *et al.*, 1991; Nakamura *et al.*, 1997). When compared to other microorganisms the bacterial isolates are able to thrive in adverse environmental conditions so they can be the choice for commercial production of inulinase on inulin. As this study shows the high efficiency of *Bacillus sp* 2 in inulinase production with the three raw plant sources which are available locally of which onion was found to be the best source when compared to others. Commercial production of inulinase from such locally available sources can be economically beneficial. So this isolate could be further studied for the production of fructose and fructose oligosaccharides as they are of great demand in the food industry.

An efficient inulinolytic bacterial isolate identified as *Bacillus sp* 2 was screened from rhizosphere soil samples of onion. The isolate showed maximum enzyme production at pH 6 and at a temperature of 37 ° C. Its activity was found to be 41.16 U/ml when commercial inulin was used as a substrate and with raw onion source it was found to be 25.9 U/ml. The enzyme products have wide application in food and pharmaceutical industry.

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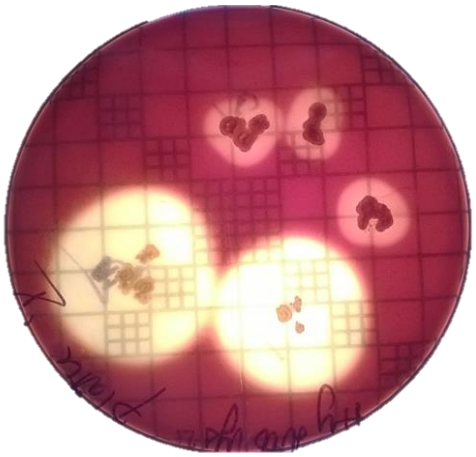

Figure-1: Zone of inulin hydrolysis.

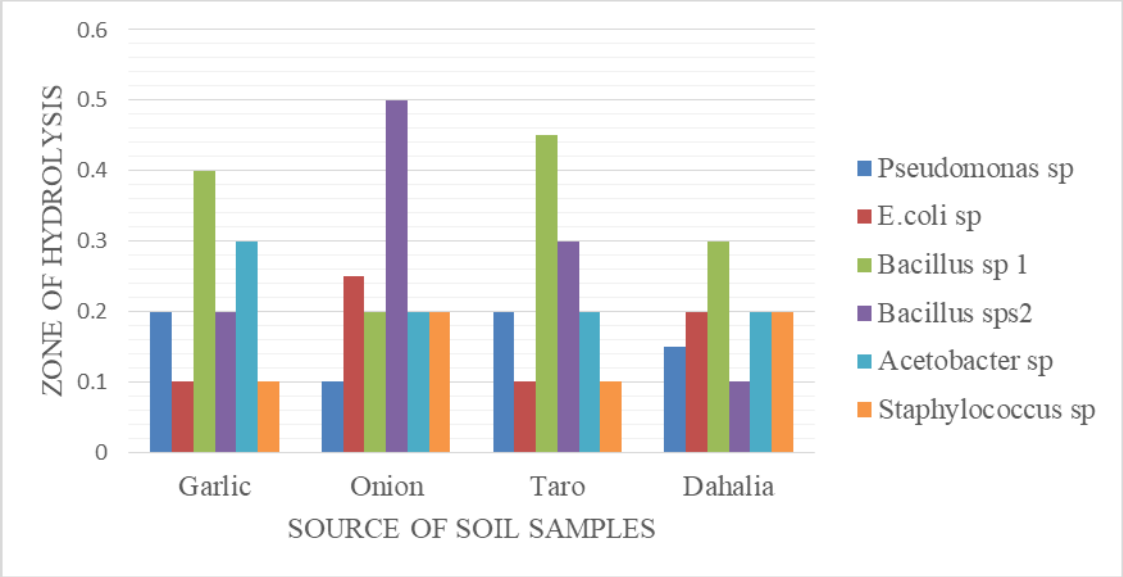

Figure-2: Inulinolytic bacterial isolates from various rhizospheres of Garlic, Onion Taro, and Dahlia.

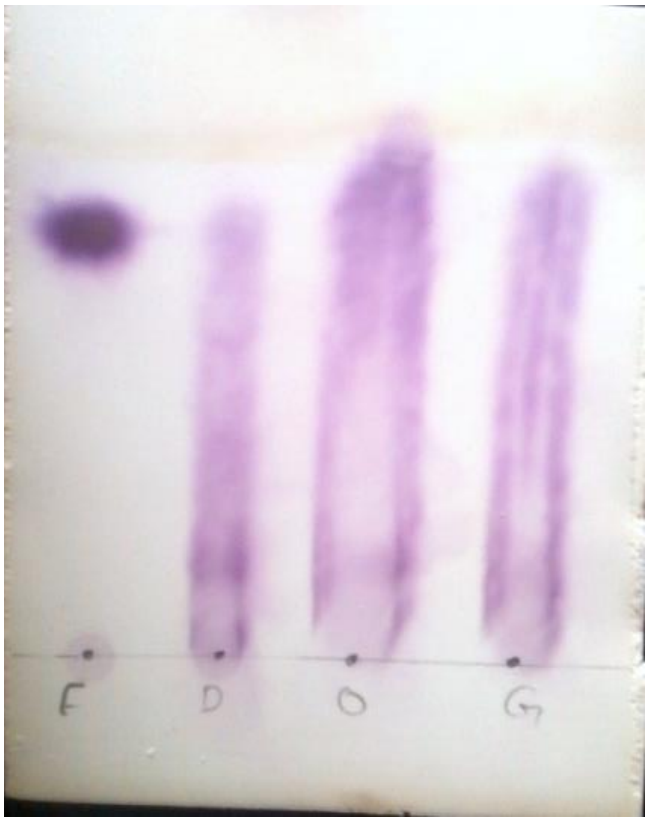

Figure-3: TLC Sheet after development.

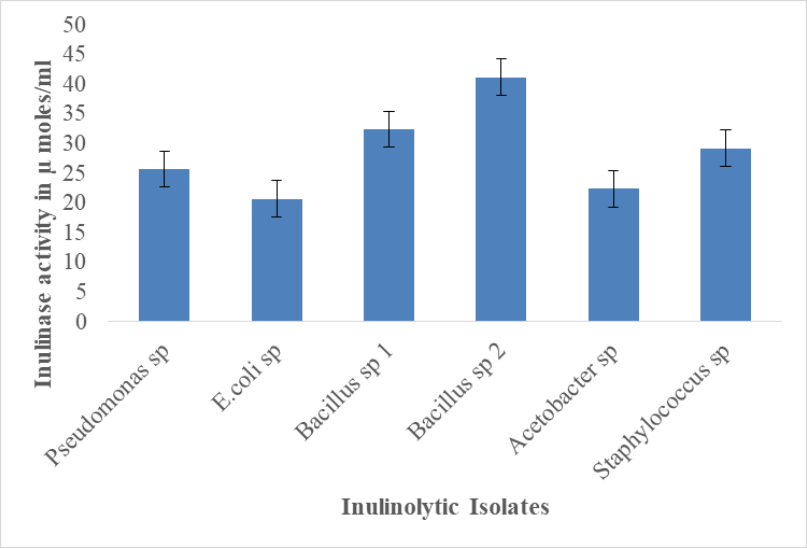

Figure-4: Inulinase activity of selected inulnolityic bacterial isolates in Czapex-Dox broth.

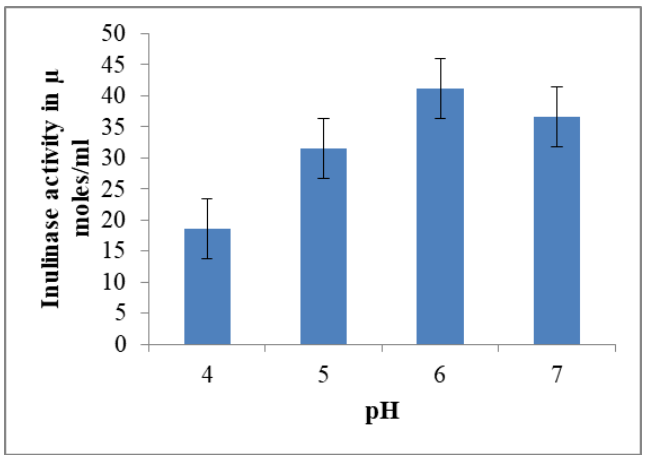

Figure-5: Inulinase production by *Bacillus sp* 2 at different pH.

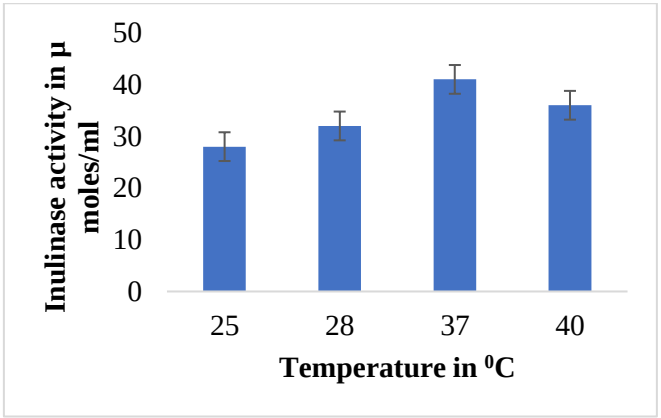

Figure-6: Inulinase production by *Bacillus sp 2* at different Temperatures

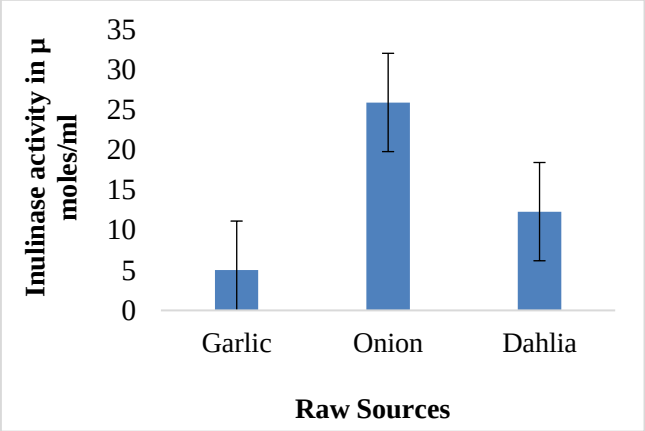

Figure-7: Inulinase production from raw sources

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