Purification and characterization of exoinulinase from *Pseudomonas putida* isolated from agricultural waste materials

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Abstract

Inulinase(2,1-b-D-fructano -hydrolases EC 3.2.1.7) is an enzyme catalyzing the hydrolysis of inulin into fructose and oligosaccharides, which are widely used as food additives. In this study we report inulinase from *Pseudomonas putida,* as in the past decade there isn't any report on inulinase from this bacteria, especially purification and characterization of this enzyme. *Pseudomonas putida* wh_2 gave the highest production level of inulinase, which purified to homogeneity by ammonium sulphate percipitation, gel filtration and ion-exchange chromatography with 109.1 fold of purification. The purified enzyme is a single peptide with approximate molecular mass of 72 kDa as assessed by SDS-PAGE. The enzyme is optimally active at 55℃ and pH 5, however it still possesses more than 70% of the maximal activity at pH ranging from 4.5 to 7.0, and it is stable at temperature up to 50℃. TLC analysis of end product (enzyme) revealed that inulinase hydrolyzed inulin with a large amount of monosaccharides (fructose) and a trace amount of oligosaccharides, indicating that the purified inulinase had a high exoinulinase activity.

Keywords: Purification, Inulinase, *Pseudomonas putida,* agricultural waste.

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تنقية وتوصيف الانيولينيز الخارجي من بكتريا *Pseudomonas putida* المعزولة من بقايا المواد الزراعية

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الخلاصة

الانيولينيز (2,1-b-D-fructano -hydrolases EC 3.2.1.7) انزيم يحفز تحلل الانيولين الى فركتوز وسكريات قليلة الوحدات التي تستخدم بشكل واسع كمضافات غذائية. في هذه الدراسة تم الحصول على الانيولينيز من *Pseudomonas putida* حيث لم يكن هناك في العقد الماضي اي دراسة عن هذا الانزيم من هذه البكتريا، خاصة تنقية وتوصيف هذا الانزيم. اعطت *Pseudomonas putida* wh_2 اعلى مستوى انتاج للانيولينيز، الذي تمت تنقيته بواسطة الترسيب بكبريتات الامونيوم، كروماتوغرافي الترشيح الهلامي والتبادل الايوني بعدد مرات تنقية تعادل 109.1.الانزيم المنقى هو ببتيد مفرد بوزن جزيئي يقارب 72 كيلو دالتون وفقا لما ظهر في تقنية SDS-PAGE . كانت الفعالية المثلى للانزيم بدرجة 55 ْم ورقم هيدروجيني 5 ، فضلا عن ذلك امتلك الانزيم اكثر من 70% من فعاليته القصوى عند مدى من الرقم الهيدروجيني يتراوح بين 4.5-7.0، كما انه كان مستقرا بدرجة حرارة تصل الى50 ْم. اظهر تحليل كروماتوغرافيا الورقة TLC للمنتج النهائي(الانزيم) انه يحلل الانيولين منتجا كميات كبيرة من السكريات الاحادية (الفركتوز) وكميات ضئيلة من السكريات قليلة الوحدات، مشيرا بذلك ان الانيولينيز المنقى يمتلك فعالية تحليلية خارجية عالية.

Introduction

The genus *Pseudomonas* is one of the most diverse gram-negative, heterogeneous and ecologically significant group of bacterial genera (1) isolated from sources ranging from plants to soils and water (2). *Pseudomonas* is characterized by their ability to grow in simple media at the expense of a great variety of simple organic compounds, without needing organic growth factors (2,3).

Pseudomonas putida is a type of gram-negative bacterium that is commonly found in water and soil (4), particularly around the roots of plants, it can protect from disease from

other microorganisms (5). *Pseudomonas putida* are significant to environtment due to its complex metabolism and ability to control pollution (3,6).

Inulin is present as a reserve carbohydrate in the roots and tubers of plants such as chicory, dahlia, onion and yacon. The yields of the roots and tubers are very high (7,8). Inulin consists of linear chains of β-2,1-linked D- fructofuranose molecules terminated by a glucose residue through a sucrose-type linkage at the reducing end (8,9).

Many inulin sources have recently received attention as a renewable raw material in the production of innulinase i.e. ethanol, acetone, butanol, pullulan, gluconic acid, sorbitol, inulooligosaccharides and ultra-high-fructose syrup in pharmaceutical industries (10,11).

Inulin is degraded by inulinase, which cleaves glycoside bonds to form largely (95%) D-fructose by a single-step process (12,13). Inulinase (2,1-b-D-fructano -hydrolases EC 3.2.1.7) hydrolyses the inulin in to pure fructose, being an excellent alternative for the production of fructose syrups (14,15). This sugar is used by the food and beverage industries, besides sweeteners and shows several adventages in comparison to sucrose, being less cariogenic, highly soluble and hygroscopic and therefore, less prone to form crystals, has low calory content and dose not cause arterioscelerosis (16).

Furthermore, fructose may be used by diabetic patients and mask the bitter taste of saccharm (16). The inulooligosacchrides acting as growth factors for improvments of the intestinal microbial flora, thus they are considered a prebiotic agents (12), relief of constipation, decrease of total cholesterol and lipid in the serum and promotion of animal growth (17).

Microorganisms are the best sourse of inulinases for commercial production. Microbial inulinases are an important class of industrial enzymes, which are usually inducible and extracellular (11). Earlier studies focused on inulinase production using various microorganisms such as yeasts, fungi and bacteria (12,14). Among the bacteria, *Bacillus* spp., *Pseudomonas spp.,* and *Streptomyces* sp. have been reported as high-yielding inulinase strains (18). The bacteria *Pseudomonas putida* has been rarely used for this purpose. To our

knowledge there is not any report about inulinase production and purification by *Pseudomonas putida.* Therefore, the aim of this study was to investigate inulinase production by *Pseudomonas putida* besides to purify and characterize this enzyme.

Materials and Methods

Collection of samples: Fourty samples of agricultural wastes were collected from different fields in Maysan governorate, these samples were included 10 samples each of wheat husk, rice husk, and rice straw. One gram of each of these samples was suspended in 10 ml of sterile distilled water and shake vigorously for 10 min. Later, 1 ml of the resulting liquid was spread on the surface of heart infusion agar plates. The plates were incubated at 30℃ for 24 hrs (5,19).

Isolation of *Pseudomonas putida*: The bacterial isolates were sub cultured to purity. For isolation of *Pseudomonas* cells were grown on *Pseudomonas* isolation agar, then several biochemical tests were done for differentiation of *Pseudomonas putida* from the other species such as a positive oxidase reduction, it's fruity oder, it's inability to grow at 4℃ and 42℃, it's inability to hydrolyze gelatin and production of flourescent pigment (20,21,22). In addition, Api 20NE identification to differential it from other types.

Primary screening for inulinase activity: All the bacterial isolates were inoculated in to the inuline agar plates containing 2g/L of inuline, 10g/L of yeast extract , 20 g/L of $MgSo_4.7H_2O$, 2g/L of KCl, 10% of NaCl, 20g/L of agar. Inulin was used as the sole source of carbon in this medium, thus, bacteria growth after 24 hrs of incubation at 30℃ shows this presence of inulinase activity (23).

Secondary screening for inulinase activity: The selected bacteria isolates were transferred into 10 ml of a medium contained (g/L): inulin 20, yeast extract 20, $(NH_4)_2H_2PO_4$ 5, $NH_4.H_2PO_4$ 2, $MnCl.4H_2O$ 0.5, KCl 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.01, and pH was adjusted to 7.0. The bacteria isolates were incubated on the rotary shaker at 100 rpm at 30℃

for 24 hrs. After removal of cells by centrifugation at 10000xg for 20 mins. the enzyme activity, protein content and specific activity were assayed (23,24).

Assay of inulinase: Endoinulinase activity was assayed by incubating 2 ml enzyme solution with 2% (w/v) inulin prepared in 0.1M sodium acetate buffer (pH 5) at 55 ̊c for 60 min. After incubation, the reaction tubes were kept in a boiling water bath for 10 min. to stop the enzyme reaction and then cooled to room temperature. The reaction mixture was assayed for reducing sugar as fructose by DNS method as described by (23,24) by reading the absorbance at 575 nm. The calibration curve was prepared with fructose solutions of known concentration and blanks were run simultaneously with enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme, which produced 1µ mole of fructose under the assay conditions.

Protein determination estimation: Protein content was determined by the method of (25), using bovine serum albumin as a standard.

Purification of inulinase: Inulinase was purified by modification the method that described by (26). At the end of incubation period, the cells were separated by centrifugation at 10000 rpm for 20 min (under cooling) and the clear supernatant (crude extract) was partially purified by salting out with ammonium sulphate at concentration (40-80%) saturations. All samples were left overnight at 4 ̊c. The precipitates were collected by centrifugation at 1000 rpm for 15 min, dissolved in 5ml of acetate buffer (0.2M, pH 6.0) and dialyzed overnight against the same buffer. Protein precipitate containing higher enzyme activity was then fractionated on sephadex G-100 column (1.5x80 cm) that was pre-equilibrated with acetate buffer. The column was eluted with same buffer at flow rate 0.5 ml/min. Protein concentration at 280 nm and inulinase were estimated and the active fractions (5 ml each) were pooled and subjected for further purification on DEAE- Sepharose column (2x20) was eluted with gradient of 0.1-0.6 M NaCl prepared in 0.2 M acetate buffer (pH 6.0) at a flow rate of 1ml/min and 5ml fractions were collected and assayed for inulinase activity and absorbance at 280 nm. The fractions showing the highest inulinase activity were pooled and assayed for protein content. The specific activity of purified enzyme fractions and fold purification were calculated.

Enzyme Characterization

1- Gel electrophoresis: SDS-PAGE was preformed at a concentration of 12% polyacrylamide according to the method of (27). The gel was stained with coomassie brilliant blue R-250. The standard protein marker used were: β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 67 kDa; and egg albumin, 45 kDa; carbonic anhydrase, 36 kD**a.**

2- Effect of temperature and thermal stability: The purified inulinase was incubated with inulin at various temperatures ranging from 30 to 70˚c. Enzyme activity was measured as previously described. However, for determination of thermal stability, the enzyme was pre-incubated in the absence of substrate at different temperatures, ranging from 30 to 70˚c and the remaining activity was determined under the standard conditions and reported as relative activity (%).

3- Effect of pH and pH stability: The optimum pH for the enzyme activity was determined by carrying out the reaction at different pH values using different buffers (0.1M phosphate, pH 4.0-7.0, 0.2M Tris-HCl, pH 7.5-9.0). To determine pH stability, the enzyme was incubated in the presence of pH values within the above cited for 30 min at 55˚c and the residual activity was estimated under standard assay conditions. The results were expressed as relative activity (%) referred to the activity observed before incubation.

4- Inulin hydrolysis: Thin layer chromatography (TLC) was performed on pre-coated silica gel plates. The plates were spotted with samples, developed by using n-butanol, isopropanol, 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 (23).

Results and Discussion

Isolation of *Pseudomonas putida* from agricultural wastes

The results showed that 16 samples of isolates of *Pseudomonas putida* were obtained out 40 samples of agricultural wastes (Table-1).

Eight (8) isolates of *Pseudomonas putida were* isolated from wheat husk samples, 5 from rice husk samples and 3 from rice straw samples. Thus wheat husk was showed the best medium for isolation of *Pseudomonas putida.* The nature and relative concentration of carbon, nitrogen and other nutrient sources in culture media are important for the growth and production of microbial growth (2). (28) revealed that three bacterial strains were isolated from decomposing rice straw, two of these bacteria, *Listeria* sp. and *Enterobacter* sp., were appeared during initial stages of decomposition whereas the other bacteria *Pseudomonas sp.,* became dominant towards the late stages of the process. Also (2) found that increased growth of *Pseudomonas spp.* were found at a combination of rice straw, rice husk, wheat husk and coconut water.

Screening of inulinase producing from *Pseudomonas putida*

From sixteen *Pseudomonas putida* isolates initiary subjected to rapid screening for extracellular inulinase production using inulin agar plates, Twelve isolates were found to be positive for inulinase activity by growth on this medium. To select the best inulinase producer, all 12 of these positive isolates were evaluated by secondary screening for inulinase in liquid culture and it was found that *Pseudomonas putida* Pwh_2 produced the highest level of inulinase with specific activity (1.47) (Figure -1). In this study, this isolate was selected for further studies.

Inulinase production by *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Lactobacillus casei* and *Achromobacter* sp. was started from 8th hour and the maximum enzyme activity was seen during the late logarithmic phase at 22nd hour of fermentation (22), in addition, *Flavobacterium multivorum* produced cell bound inulinase at the end of the growth phase

(29). Bacteria of genus *Bacillus* are also active producers of extracellular inulinase, 30-42 U/ml inulinase activity were obtained on sucrose as substrate (30). The maximum activity of endo-inulinase from *Pseudomonas sp.* was reached when 50g inulin/L as substrate was used (24). Inulin was the best carbon source for inulinase production by *Streptomyces* sp., the maximum enzyme activity of about 0.55 U/ml with pure inulin and about 0.85 U/ml with the inulin extract at 1% (w/v) (31).

Purification of inulinase

Endoinulinase was obtained as an extracellular enzyme in the culture broth of *Pseudomonas putida* Aw_2. Table (2) summarizes results of the three steps of purification of endoinulinase.

The first step of purification was carried out by precipitation of protein from the cell-free supernatant by using ammonium sulphate at 70% saturation. The supernatant obtained from ammonium sulphate precipitate was first loaded on gel filtration column sephadex G-100. In this step, inulinase was purified 39.5 fold with yeild of about 23.8%. The activity was located in peak 3 (Figure-2). The purification procedure was completed by ion exchange chromatography on DEAE-sephadex using sodium chloride gradient. The results showed that inulinase was purified 109.1 fold and yeild of 13% obtaining a final specific activity of 160.4 U/mg protein, the highest activity was detected in peak 2 (Figure-3).

The crude endoinulinase preparation is directly useful for large- scale production of inulooligosaccharides from inulin-containing agricultural crops (32). (15) reached yield of 53.6% of inulinase produced by *Aspergillus niveus*, using ammonium sulphate, DE-52 cellulose and sephadex G.15. In contrast, (31) reached yield of 1.8% of inulinase by *Streptomyces* sp., using ammonium sulphate followed by four steps of column chromatography.

Cho andYun (32) by using precipitation with ammonium and column chromatography on phenyl-sepharose and sulphate DEAE-sephacel obtained a purification of 29 fold with a yield of 5.5% for inulinase of *Xanthomonas oryzae*. Values higher than our results were mentioned

by (33) for the purification of the extracellular inulinase of *Bacillus smithii*, using ammonium sulphate precipitation and DEAE sepharose CL-6B and superdex75 , resulting a purification of 31.4 fold with a yield of 27.3%.

Characterization of inulinase

1- Molecular weight of inulinase: The purified enzyme was estimated as a single protein band with a molecular weight approximately 72 kDa by SDS-polyacrylamide gel electrophoresis (SDS- PAGE) as shown in (Figure-4). (23,33) reported that the molecular mass of endoinulinase from *Pseudomonas putida* and *Bacillus smithii* were 45 and 47 kDa, respectively. While (34) characterized the endoinulinase produced by *Arthrobacter* spp. and estimated its molecular weight at 75 kDa. So far results was observed by (32) who revealed that the highest molecular weight of inulinase was 139 kDa in *Xanthomonas oryzae* No.5. The molecular weights of many other microbial endoinulinase were around 64, 70, and 78 kDa from *Kluyveromyces marxianus* CBS, *Streptomyces* sp., and *Aspergillus niveus*, respectively (15, 31, 35).

2- Effect of temperature and thermal stability: The optimum temperature of purified inulinase was 55℃ (Figure-5) at pH 5, then the activity was declined retaining 32% of the residual at activity at 70℃. Compared to inulinases from *Streptomyces* sp. and *Aspergillus niveus*, which have optimal temperatures 5.5 and 45℃, respectively (31,15).

Thermal stability study of the purified enzyme revealed that it was stable up to 50℃. However it retained 12% of its activity after preincubation at 70% (Figure-6). Purified inulinase exhibiting optimum temperature at 50℃ and Stable up to 45℃ , has been reported from *Xanthomonas oryzae* (32). However, (33) revealed that the higher temperature optimum and greater thermal stability of inulinase purified from *Bacillus smithii* T7.

3- Effect of pH and pH stability: The effect of pH on endoinulinase activity was investigated by measuring the activity within the pH ranges from 4-9 by incubating the enzyme for 1hr. As shown in figure-7, the maximal activity of the enzyme was observed at pH 5. To determine the effect of pH on stability, the enzyme extract was incubated for 30 min

at 55℃ in different pH buffers. The enzyme retained almost 70% activity between 4.5-7.0, but at pH 7.5 and above, the activity registered a sharp decline (Figure-8). As pH value diverged from the optimum level, the efficient functioning of the enzyme was affected, most probably, due to the change in active site conformation which is determined, in part, by ionic and hydrogen bonding that can be affected by pH (26).

The inulinase that purified from *Bacillus smithii* has the highest activity at pH 4.5 and stable at pH range of 4.0-8.0 (33). In contrast, the inulinases produced by some bacterial strains hydrolyze inulin optimally at pHs 7 and 7.5, like *Arthrobacter* spp. (34) and *Xanthomonas oryzae* (32).

4- Inulin hydrolysis: The hydrolysis products of inulin by the purified inulinase were analysed by thin layer chromatography (TLC). A large amount of monosaccharides and a trace amount of oligosaccharides were detected after the hydrolysis (Figure-9). This means that the purified inulinase had a high exoinulinase activity. This characteristic may find potential applications in ultra-high fructose syrup production, food & acid industry, and ethanol production. The monosaccharides and oligosaccharides were also detected after inulin hydrolysis for more than 2 h by the purified exoinulinase produced by *Pseudomonas aeruginosa* (23). Also inulinases obtained from *Kluyveromyces marxianus* showed liberation of fructose from chicory inulin indicating only exoinulinase activity (36).

References

1. Franzetti, L.; Scarpelleni, M. (2007). Characterisation of *Pseudomonas* spp. isolated from foods. Annuals of Microbiology, 57: (1) pp.39-47.
2. Poorni, K. E.; Manikandan, A.; Geethanjali, S. and Petcy. K. (2011). Production of *Pseudomonas fluorescence* from agricultural wastes and its application in the preservation of selected vegetables. Advances in Applied Science Research. 2: (2), 156-160.
3. Choi, E. N., Cho, M. C.; Kim, K. C. K., and K. Lee. (2003). Expansion of growth substrate range in *Pseudomonas putida* F1 by mutations in both *cymR* and *todS* , which

recruit a ring-fission hydrolase CmtE and induce the *tod* catabolic pathway. Microbiology 149: 795-805.

4. Fonseca, P.; Moreno, R. and Rojo, F. (2011). Growth of *Pseudomonas putida* at low temperature: global transcriptomic and proteomic analyses. Environmental Microbiology Reports. Society for Applied Microbiology and Blackwell Publishing Ltd, pp.1-11.
5. Helga, G.; Arevalo, M. (2012). What Is *Pseudomonas Putida*? Printed by: 2003-2013, Conjecture Corporation.
6. Ruiz-Manzano, A.; Yuste, L.; and Rojo, F. (2005). Levels and Activity of the *Pseudomonas putida* Global Regulatory Protein Crc Vary According to Growth Conditions. Journal of Bacteriology. 187: p.3678-3686.
7. Yun, J.W.; Kim,D.H.; and Song, S.K. (1997). Production of inulo-oligosaccharides from inulin by immobilized endoinulinase from *Pseudomonas* sp. J.of fermentation & bioengineering. Vol.84(4): 369-371.
8. Chi,Z; Zang,T.; Yue,L. (2009). Inulinase expressing microorganisms and applications of inulinases. Applied microbial biotechnology. 82: pp. 211-228.
9. Basran,N.F.; Mustafa, S.; Shamsuddin,R.A.; Ali, A.M.; Noormi, R.; and Subramaniam,S. (2010). Isolation and Cloning of an Inulinase Gene from an Endophytic Bacteria. Advances in Environmental Biology, 4(3): 392-396.
10. Erdal,S.; Canli,O. and Algur, O.F. (2011). Inulinase production by *Geotrichum candidum* using Jerusalem artichoke as sole carbon source. Romanian Biotechnological Letters. Vol. 16(4): pp.6378-6383.
11. Lafuente-Castaneda, C.; Martinez, G.; Contreras-Esquivel, G.C.; Rodriguez-Herrera, R. and Aguilar,N. (2012). Inulinase Production by *Penicillium Citrinum* ESS in Submerged and Solid-State Cultures. American Journal of Agricultural and Biological Sciences. Vol. 7(2): pp. 129-134.
12. Nascimento, D.; Jiunio,V.; Fernades,P.; Ribero, G.; Danyo, M.; and Sandra, A. (2012) . Production, characterization and application of inulinase from *fungal endophyte* CCMB 328. Annals of the Brazilian Academy of Sciences. Vol. 84(2): 443-453.

13. Singh, P. and Gill, P.K. (2006). Production of Inulinases, *Food Technol. Biotechnol. Vol.44* (2): 151–162 .
14. Dilipkumar, D.; Rajasimman, D.; and Rajamohan, N. (2011). Responce surface methodology for the optimization of inulinase production by K. *Marxianus var. marixianus*. Journal of Applied Sciences in Environmental Sanitation, 6 (1): 85-95.
15. Souza-Mottal,S.; Cavalcanti,Q.; and Filho,L. (2005). Aspergillus niveus Blochwitz 4128 URM: new source for inulinase production. Brazilian Archives of Biology and Technology, 48(3): pp.343-350.
16. Nandagopal, S. and Kumari, B.D. (2006). Enhancment of inulinase production from Chicory and Fenugreek rhizosphere soil. american-Eurasian J. Agrric. & Environ.Sci., 1(3): 225-228.
17. Bharathi, S.; Saravanan, D.; Radhakrishnan, M.; Balagurunathan, R. (2011). Bioprospecting of Marine Yeast with special reference to Inulinase production. International Journal of Chem.Tech. Research., 3(3): pp 1514-1519.
18. SINGH, R.S. and SINGH, R.P. (2010). Production of Fructo-oligosaccharides from Inulin by Endoinulinases and Their Prebiotic Potential. *Food Technol. Biotechnol., 48* (4): p.435–450.
19. Kheng, P.P. and Songklanakarin, O.I. (2005). Xylanase production by a local fungal isolate, *Aspergillus niger* USM AI 1 via solid state fermentation using palm kernel cake (PKC) as substrate. J. Sci. Technol., 27(2): p.325-336.
20. Yang, G.; He, Y.; Cai, Z.; Zhao, X.; and Li Wang. (2011). Isolation and characterization of *Pseudomonas putida* WLY for reactive brilliant red x-3b decolorization. African Journal of Biotechnology, 10(51): pp. 10456-10464.
21. Blazevic, J.; Koepcke, H. and Matsen, M. (1973). Incidence and Identification of *Pseudomonas fluorescens* and *Pseudomonas putida* in the Clinical Laboratory. Applied Microbiology, Vol. 25(1): p. 107-110.
22. Holt, J.G. (1994). Bergey's manual of determinative bacteriology. 2nd ed. Vol.2, Williams and Wilkins. Baltimore.

23. S. Jenny Angel1, C. Kavitha1, G. Vidyadharani1, Priya Roy1 and R. Dhandapani1. (2012). Isolation of inulinase producing bacteria from sugarcane soil. International Journal of applied biology and pharmaceutical technology. Vol.3(4): p.320-326.
24. Kim, D.; Choi, Y.; Song, K. and Yun, W. (1997). Production of inulo-oligosaccharides using endo-inulinase from a *pseudomonas* sp. Biotechnology Letters. Vol.19(4): pp. 369–371.
25. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J.. (1951). "Protein Measurement with the Folin Phenol Reagent," *Journal of Biological. Chemistry*, Vol. 193(1): pp. 267-275.
26. Fawzi, E.M. (2011) . Comparative study of two purified inulinases from thermophile *Thielavia terresteris* NRRL 8126 and mesophile *Aspergillus foetidus* NRRL 337 grown on *Cichorium intybus* L. Brazilian Journal of Microbiology . 42: 633-649.
27. Laemmli, U.K. (1970). "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4". *Nature*, 227(5259): pp. 680-685.
28. Sirisena, D. M. and Manamendra, T. P. (1995). Isolation and characterization of cellulolytic bacteria from decomposing rice straw. J.Natn.Sci.Coun. SriLanka, 23(1): 25-30.
29. Nirobol, L.: Chantanaphan, R.; and Pinphanichakarn, P. (2012). Production, Purification and Characterization of Inulinase from a Newly Isolated *Streptomyces* sp. CP01 . *Natural Resources*, , 3, 137-144.
30. Camelia, N.; and Gabriela, B. (2011). Inulinase- a veasatile tool for biotechnology. Innovative Romanian Food Biotechnology Vol. 9(2): 1-11.
31. Laowklom, N.; Chantanaphan, R.; and Pinphanichakarn,P. (2012). Production, Purification and Characterization of Inulinase from a Newly Isolated *Streptomyces* sp. CP01. *Natural Resources*, 3: p. 137-144.
32. Cho, Y.; andYun, J.W. (2002). Purification and characterization of an endoinulinase from *Xanthomonas oryzae* No.5. Process Biochemistry 37: pp.1325–1331.

33. Gao, W.; Bao, Y.; Liu, Y.; Zhang, X.; Wang, J. and An, W. (2009). Characterization of Thermo-stable Endoinulinase from a New Strain *Bacillus Smithii* T7. Appl Biochem Biotechnol. 157:498–506.
34. Kang S.I., Chang Y.J., Oh S.J., Kim S.I. (1998). Purification and properties of an endo-inulinase from an *Arthrobacter* sp. Biotechnology Letters, 20 (10): pp. 983-986.
35. Rouwenhorst, R.J.; Hensing,M.; Verbakel, J.; Scheffers,W. and van Duken, J. (1990). Structure and properties of the extracellular inulinase of *Kluyveromyces marxianus* CBS. *Appl. Environ. Microbiol.*, 56(11):3337.
36. Jain,S.; Jain,P. and Kango, N. (2012). Production of inulinase from *Kluyvermyces marxianus* using dahlia tubers extract. Brazilian Journal of Microbiology: 62-69

Table (1) : Sources and numbers of *Pseudomonas putida* isolates.

Source of isolate	No. of isolates
Wheat husk	8
Rice husk	5
Rice straw	3
Total	16

Table (2) : Summary of treatments used for the purification of inulinase from *Pseudomonas putida* wh$_2$.

Purification step	Size (ml)	Protein conc. (mg/ml)	Inulinase activity (U/ml)	Specific activity (U/mg)	Total activity (U)	Purification (Fold)	Yield (%)
Crud extract	150	112.6	165.8	1.47	24870	1	100
(NH4)SO4 70%	70	34.3	240.7	7.01	16849	4.77	67.75
Sephadex G-100	20	5.1	296.8	58.19	5936	39.59	23.87
DEAE-Sepharose	10	2.02	324.2	160.4	3242	109.12	13.03

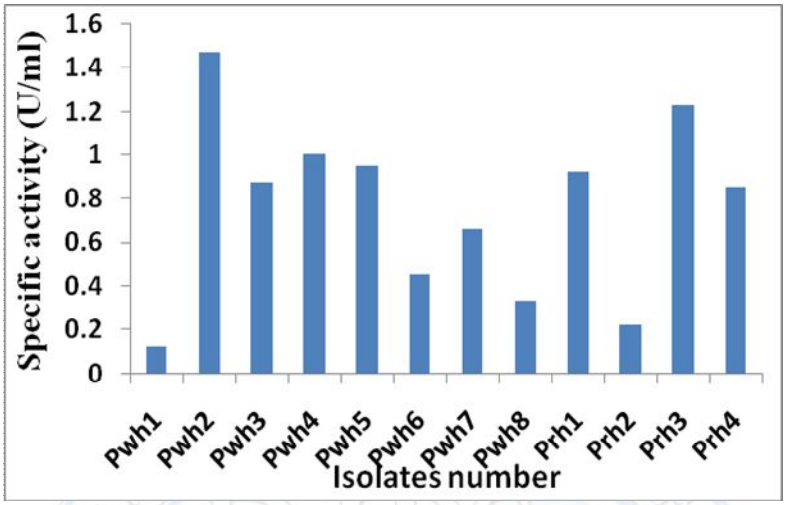

Figure (1): Inulinase production levels by *Pseudomonas putida* isolates in secondary screening.

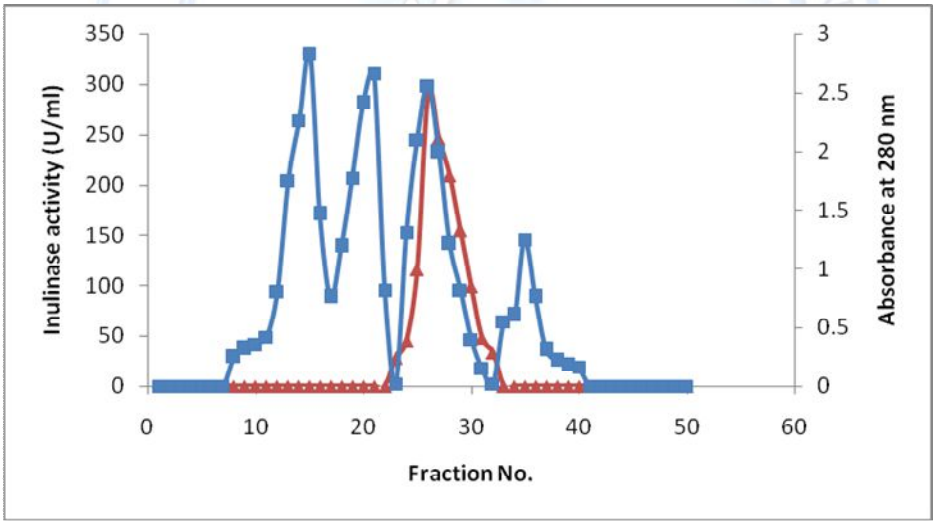

Figure (2): Purification of inulinase from *Pseudomonas putida* using gel filtration on sephadex G-100. (▲) refer to inulinase activity, (■) refer to protein concentration.

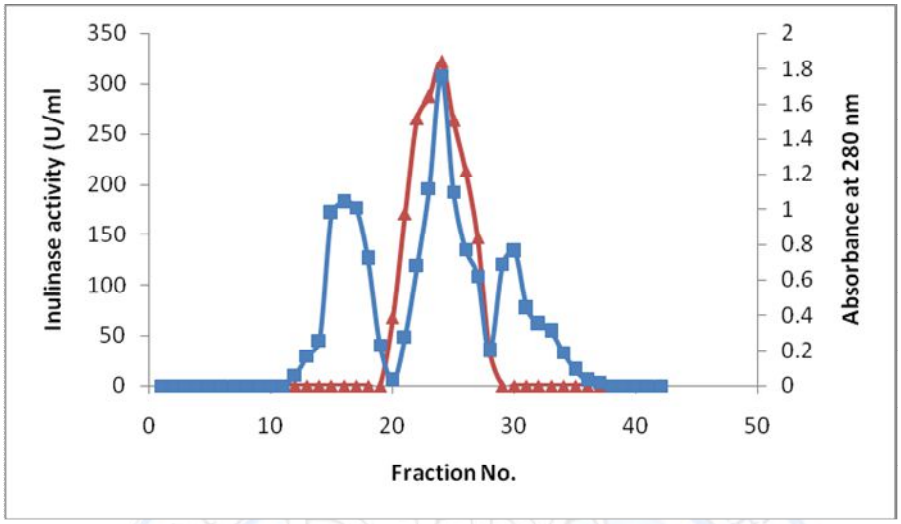

Figure (3): Purification of inulinase from *Pseudomonas putida* using ion exchange on DEAE- Sepharose. (▲) refer to inulinase activity, (■) refer to protein concentration.

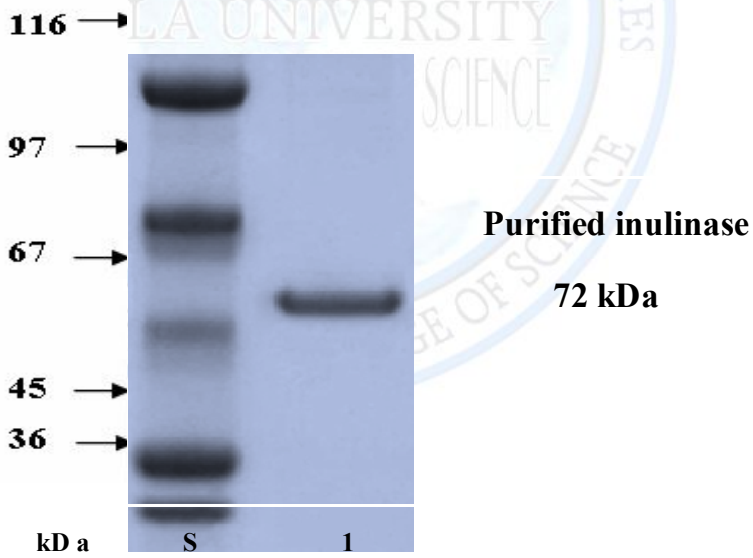

Figure (4): SDS-polyacrylamide gel electrophoresis of purified inulinase from *Pseudomonas putida* wh$_2$

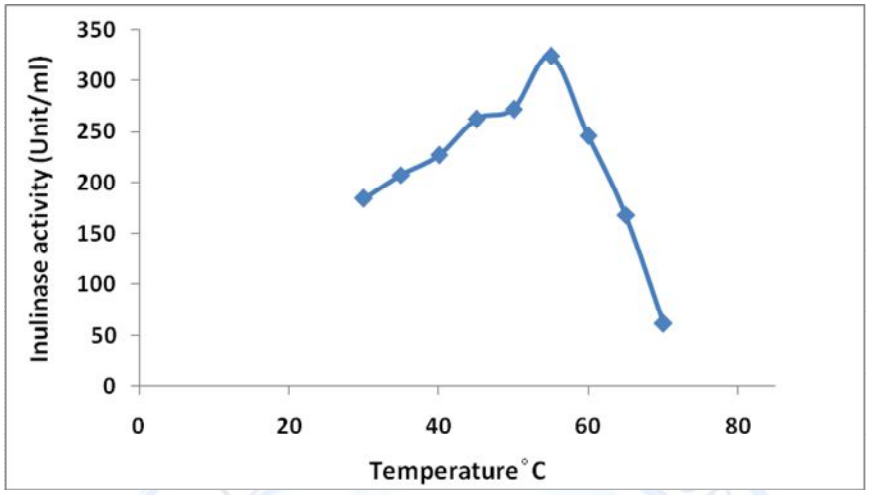

Figure (5): Effect of temperature on activity of the inulinase from *Pseudomonas putida* wb2

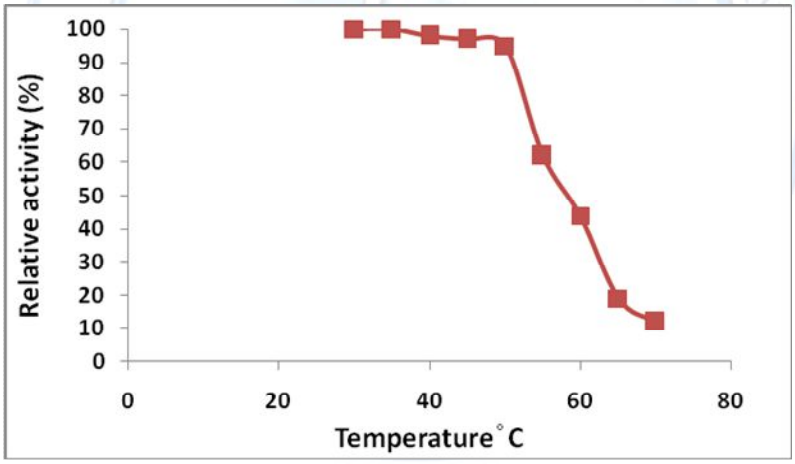

Figure (6): Effect of temperature on stability of the inulinase from *Pseudomonas putida* wb2

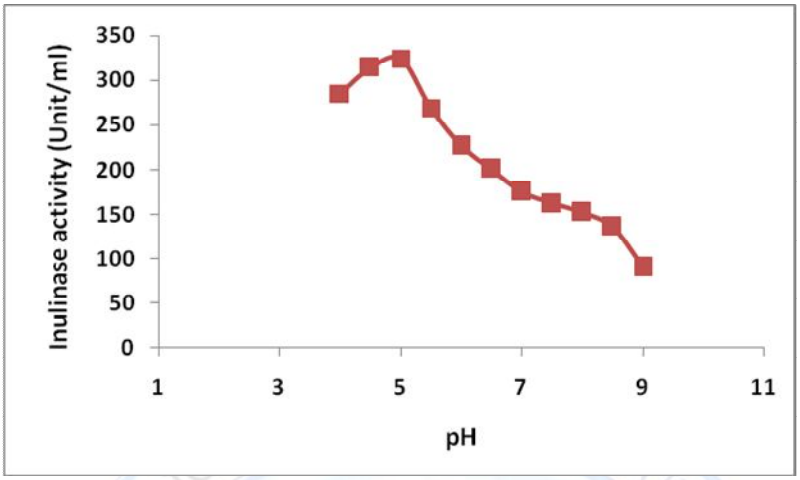

Figure (7): Effect of pH on activity of the inulinase from *Pseudomonas putida* wb$_2$

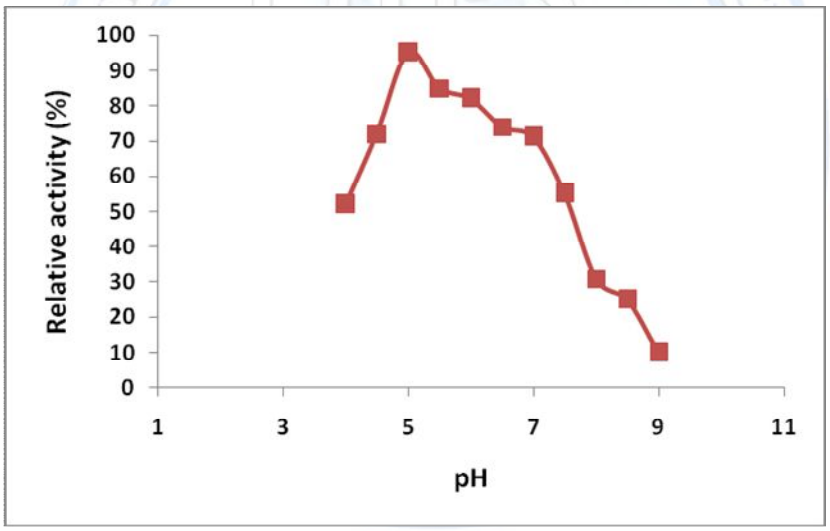

Figure (8): Effect of pH on stability of the inulinase from *Pseudomonas putida* wb$_2$

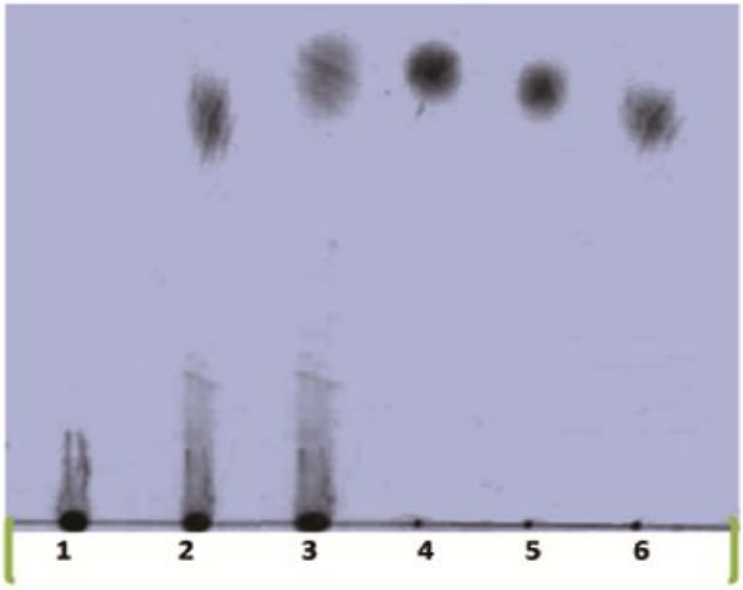

Figure (9): Thin layer chromatography of hydrolysis products of inulin with purified inulinase produced by *Pseudomonas putida* wh2. Lane 1 control (inulin+ inactivated inulinase at 100°C for 10 min); 2,3 hydrolysis products; 4 fructose; 5 sucrose; 6 kestose (trisaccharides)