Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 111-122

Extraction, Partial Purification and Characterization of Inulinase Produced from *Aspergillus niger* AN20 by Solid State Fermentation

Ghazi M. Aziz[1], Zainab W. Abdulameer[1], Ali J. R. AL-Sa'ady[1]

[1]Biotechnology Dept., College of Science, University of Baghdad, Iraq

Received: June 16, 2015 / **Accepted**: September 2, 2015

Abstract: Inulinase produced by *Aspergillus niger* AN20 was partially purified using two chromatographic techniques, ion exchange chromatography by DEAE-cellulose and gel filtration by Sephadex G-150. The two steps gave specific activity of 810 U/mg proteins, the purification fold was 8.1 and enzymatic yield was 33.5 %. Some biochemical characteristics of the partially purified inulinase were determined and the results revealed that the enzyme have a molecular weight of 42 KDa., optimum pH for inulinase activity was 4.5, and the pH of the enzyme stability was the range 4.0-8.0. The maximum activity of purified inulinase activity from *A. niger* AN20 was determined as 50 ^{O}C, while the thermal stability ranges from 20-50 C^{o}. The aim of this study was the extraction and purification of inulinase produced from *Aspergillus niger* AN20 by solid state fermentation.

Key words: *Aspergillus niger* ; Inulinase **;** Purification ; Characterization.

Corresponding author: should be addressed (Email: Ali.jabbar15@yahoo.com)

Introduction

Inulinases are enzymes that degrade the β-(2, 1) linkages of β-fructans, like inulin. Inulinases are classified into endo- and exo-inulinases, depending on their mode of action. They act by two mechanisms: exo-inulinases (EC 3.2.1.80) sequentially split-off the terminal β-(2, 1) fructofuranosidic bonds, while endo-inulinases (EC 3.2.1.7) hydrolyze the internal linkages in inulin and release inulooligosaccharides. Inulinases are produced from several fungal species (1). The genus *Aspergillus* is one of the most important filamentous fungal genera. *Aspergillus* species are used in the fermentation industry (2).

Aspergillus niger is a soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose. A variety of these enzymes from *A. niger* are important in the biotechnology industry (3). Production of inulinase by fermentation which is a method of generating enzymes for industrial purposes. Fermentation involves the use of microorganisms, like bacteria and yeast to produce the enzymes.

There are two methods of fermentation used to produce enzymes. These are submerged fermentation and solid-state fermentation (SSF) (4). The production of enzymes by SSF has gained much attention in biotechnology studies for production of lipases, inulinase, prote-

ases, etc. The use of low cost residues, higher productivities, low energy requirements, lower waste water production, extended stability of products and low production costs are some of the main advantages of SSF (5).

Materials and Methods

Chemicals and Media

Potato-dextrose agar (PDA) was obtained from Hi-media**s**. Sephadex G-150 and DEAE-cellulose column were purchased from Pharmacia Fine Chemicals. Coomassie brilliant blue and bovine serum albumin (BSA), were obtained from Sigma Co. other chemicals were supplied by BDH Chemicals.

Inulinase Production

Aspergillus niger AN20 isolate was cultured on potato dextrose agar. Then inulinase production from this isolate were determined by using a medium composed of sugar cane moisten with corn steep liquor 5:5 (v\w) at initial pH 5.0 and incubated for 96 hours at 30^{O}C.

Inulinase Extraction

Enzyme assay was performed according to the method described by mazutii *et al.* (6). After four days incubation, 50 ml of sodium acetate solution (0.1 M, pH 4.8) was added to each flask, and incubated in shaker incubator for 30 min. at 30 ^{o}C and 150 rpm for enzyme extraction, and then the extract was filtrated by Whatman filter No.1 paper. The filtrate was considered as crude enzyme, then the enzyme activity and protein concentration were estimated.

Estimation of Inulinase Activity and Concentration

Inulinase activity was estimated in solutions resulted after extraction of the enzyme, by the method described by Miller (7), which depends on estimation of reducing sugar as liberated from inuline after hydrolysis by inulinase (substrate concentration inuline 1% in sodium acetate pH 5). Unit of enzyme activity is defined as the amount of enzyme that liberate 1μM of fructose per minute in standard conditions. Protein concentration was estimated according to the method described by Bradford depending on BSA standard curve and using of Coomassie blue G-250 and measured at 595 nm (8).

Partial Purification of Inulinase

Inulinase Purification by Ion Ex-Change Chromatography

DEAE-cellulose was prepared as described by Whitaker (9). An amount of 10 ml of inulinase concentrated by sucrose was passed through DEAE-cellulose column (16×1.5 cm) carefully using pasture pipette, then washed with 0.005 M phosphate buffer pH 7.0, the fractions (proteins) were eluted with phosphate buffer pH 7.0 and NaCl gradient 0.1-1 M, at a flow rate of 45 ml/hour, 3 ml fractions of eluted were collected. Protein contents were traced in accordance to absorbency at 280 nm, the enzyme activity was estimated. The fractions which contained enzyme activity were collected and concentrated by sucrose.

Inulinase Purification by Gel Filtration Chromatography

Sephadex-G150 was prepared and packed following the instructions described by the manufacturing company (Pharmacia Sweden). Nine ml of the enzyme solution was passed carefully through the column (37×1.5 cm) using a pasture pipette. Protein sample was eluted by 0.2 M phosphate buffer pH 7.0, and 3 ml fractions were collected. Protein content of each fraction was determined spectrophotometrically at 280 nm. The peaks were estimated by plotting the absorbency versus fraction number, and then protein concentration and enzyme activity were estimated.

Inulinase Characterization

Inulinase Molecular Weight

Molecular weight of inulinase from *A. niger* AN20 was estimated according to methods described by Whitaker; Stellwagen (10, 11), using Sephadex G-150 column (37×1.5 cm) with standard proteins (Urease, Trypsin, Ovatransferrin and Bovine serum albumin).

Optimum pH for Inulinase Activity

The substrate solution (1 % inuline) was prepared of different pH (2-8). Enzyme activity for the prepared substrate was estimated for the different pH, the relation between enzyme activity and pH was drawn to determine the optimum pH of enzyme activity.

Optimum pH for Enzyme Stability

Two ml of the enzyme was mixed with 2ml of buffers previously prepared at pH range 2-8, in test tubes and incubated in a water bath at 50 ^{o}C for 30 min, then cooled directly in ice bath and the enzyme activity was estimated for each treatment, the relation between remaining activity % and pH was drawn to determine the pH of inulinase stability.

Optimum Temperature for Inulinase Activity

The substrate solution (0.9 ml) was added to 0.1ml of enzyme solution and incubated for 15 min. in water bath at different temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70) ^{O}C**,** then the activity was estimated for each treatment, and the relation between enzyme activity and temperature was drawn to determine the optimum temperature for inulinase activity.

Thermal Stability of Inulinase

One ml of partial purified inulinase was incubated in a water bath at different temperature degrees (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85) ^{O}C for 30 min. and then the test tubes containing the enzyme were transferred directly to ice bath, then the enzyme activity was estimated. The relation between remaining activity (%) and temperatures was drawn to determine the thermal stability of the enzyme.

Results and Discussion

Purification of Inulinase

Ion Exchange Chromatography

Ion exchange chromatography was done to the crude enzyme after concentration by sucrose, DEAE–cellulose used as ion exchanger in presence of phosphate buffer 0.02 M, pH 7.0. The results showed two peaks of protein in the wash step, and only one peak of enzyme

activity in fractions (19-34). Three peaks appeared in elution step with no enzyme activity. The purification fold of this step was 5.3 and yield 60 % and gave specific activity of 532.5 U\mg (table 2) (figure 1). Ion exchange chromatography was used in many studies of inulinase purification from fungi, Ertan *et al.* (12) used DEAE- cellulose in inulinase purification from *Rhizoctonia solani* and the purification fold of his experiment was 5.21 and the yield 17.93%. This enzyme was also purified from *Alternaria alternata* by DEAE–cellulose exchanger resulted in purification fold 65.89 and enzymatic yield 11.56% (13), while Souza- Motta *et al.*(14) used DEAE-52 to purify inulinase from *Aspergillus niveus* with purification fold 32.78 and yield 83.05%.

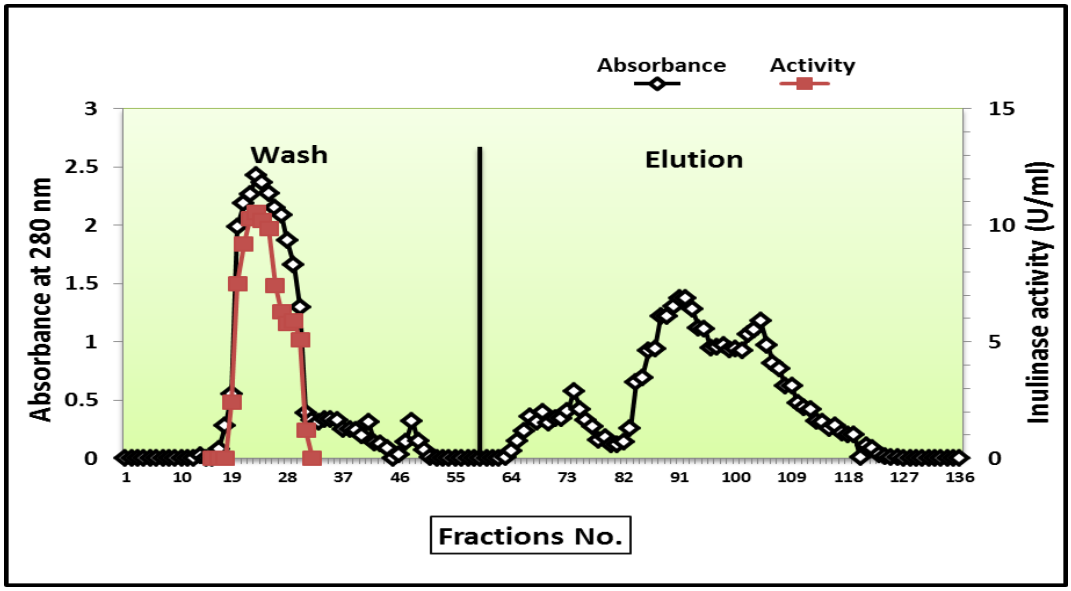

Figure (1): Ion exchange chromatography for inulinase purification from *A. niger* AN20 by DEAE-cellulose column (16-1.5 cm) equilibrated with phosphate buffer (0.02 M , pH 7.0), eluted with phosphate buffer with NaCl gradient 0-1 M in flow rate 45 ml/hr., 3 ml for each fraction.

Gel Filtration Chromatography

Gel filtration step followed ion exchange, in two steps. Figure (2) shows results of the first step of gel filtration. Three peaks of protein were separated. After estimating enzymatic activity of each peak, only one shows activity in fractions 19-28, the fractions which show activity are collected and mixed together and the solution was concentrated by sucrose to 9 ml. The first step of gel filtration gives specific activity of 630 U\mg, purification fold 6.3 and enzymatic yield 35.5 % (table 1).

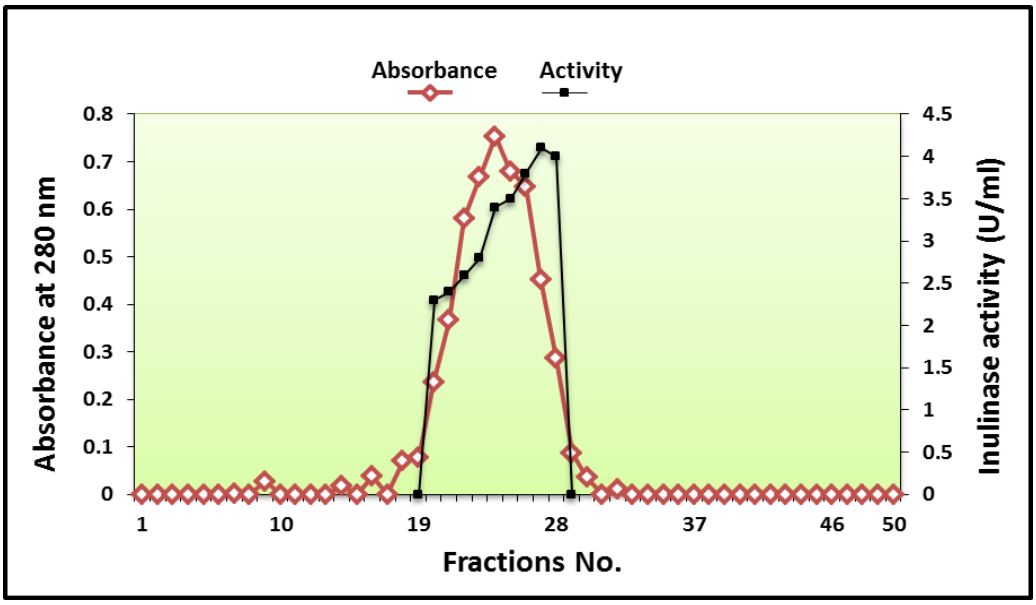

Figure (2): Gel filtration chromatography (First step) for partial purification of inulinase from *A. niger* AN20 by using Sephadex G-150 column (37×1.5 cm) equilibrated with 0.2 M phosphate buffer pH 7.0 with a flow rate of 36 ml/hour, 3 ml for each fraction.

The second step of gel filtration was done after the first step of gel filtration, where the fractions which showed activity were gathered and concentrated, then passed through the gel filtration column (figure 3). Only one peak appeared contain enzymatic activity identical to the protein peak. This proves the purity of the enzyme (9). After second step of gel filtration, the specific activity became 810 U/mg, purification fold 8.1 and enzymatic yield 33.5 % (table 1). Sphadex G-150 gel was chosen because it permits passage of proteins between 5-300 kilo Dalton, which is the range that cover the molecular weights of inulinases that are purified from different *A. niger* isolates. Singh & Gill (15) referred that the molecular weights of inulinases relies are between 28-300 kilo dalton. Sephadex G-150 was used in many studies of inulinase purification, Kochhar *et al.* (16) used sephadex G-150 for purification of inulinase extracted from *Aspergillus versicolor* and the purification fold of his study was 50. Chen *et al.* (17) used Sephadex G-150 and obtained purification fold 67 and enzymatic yield of 25.5%.

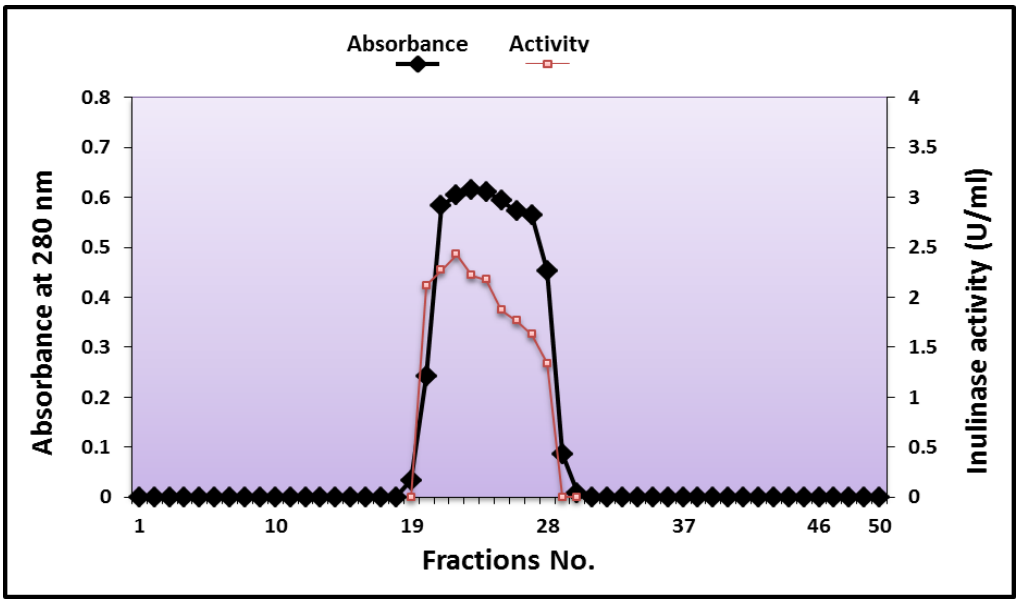

Figure (3): Gel filtration (second step) for partial purification of inulinase from *A.niger* AN20 by using Sephadex G-150 column (37×1.5 cm) equilibrated with 0.2 M phosphate buffer pH 7.0, with a flow rate of 36 ml/hour, 3 ml for each fraction.

Table (1): Purfication steps of inulinase from local isolate *A. niger* AN20:

Purfication steps	Volume (ml)	Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total Activity U	Purific-ation fold	Yield (%)
Crude extract of enzyme	80	2	0.02	100	160	1	100
Cocentration of enzyme solution by sucrsoe	10	14.8	0.11	134.5	148	1.34	92.5
Ion-exchange chromatocraphy DEAE-cellulose (wash) after concentration by sucrose	9	10.65	0.02	532.5	95.85	5.3	60
Gel filtration chroma-tography after concen-tration by sucrose (First step)	9	6.3	0.01	630	56.7	6.3	35.4
Gel filtration chroma-tography (Second step)	22	2.43	0.003	810	53.46	8.1	33.4

Characterization of Inulinase from Local Isolate *A. niger* AN20

Enzyme Molecular Weight

Gel filtration chromatography was used to estimate inulinase molecular weight, according to the standard curve which represent the relationship between the log of molecular weight of standard proteins versus (Ve\Vo) as illustrated in (figure 4) which shows that the molecular weight of inulinase purified from *A. niger* AN20 was 42 kilo Dalton. Singh & Gill (15) found that the molecular weights of inulinases lie between 28-300 kilo Dalton.

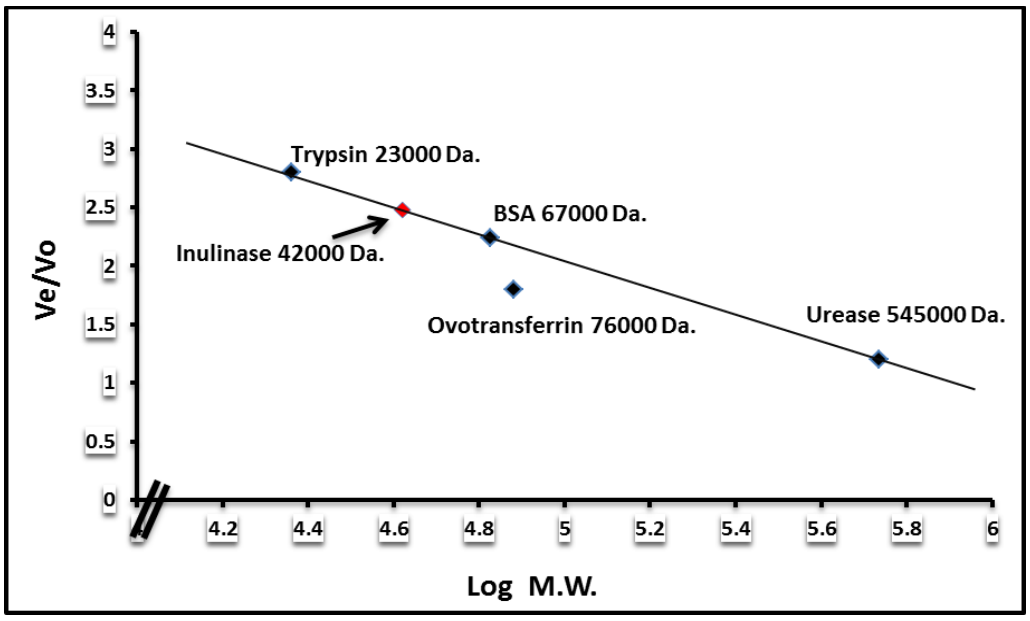

Figure (4): Estimation the molecular weight of inulinase from local isolate *A. niger* AN20 by gel filtration chromatography.

Another study by Nakamura *et al*. (18) showed that the molecular weights of endo-inulinases isozymes purified from *A. niger* strain 817 were 70 and 68 for (P-IA) and (P-IB) respectively. Ettalibi & Baratti (19) who found molecular weights of inulinase isozymes from *Aspergillus ficuam* were 74 and 64 KDa., while the other study showed that 66 KDa., was the molecular weight of inulinase purified from *Aspergillus ficuum* (Uhm *et al*.) (20).

Optimum pH for Enzyme Activity

The effect of pH on Inulinase activity was studied, figure (5) shows results that the pH 4.5 is the optimum pH for inulinase activity, while the activity decreased in pH below and above 4.5. The pH of a solution had several effects on the structure and activity of enzymes; pH could effected on the state of ionization of acidic or basic amino acids. Acidic amino acids had carboxyl

functional groups in their side chains. Basic amino acids have amine functional groups in their side chains. If the state of ionization of amino acids in a protein was altered then the ionic bonds that help to determine the 3-D shape of the protein can be altered. This can lead to altered protein recognition or an enzyme might become inactive. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or the properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis (Chesworth *et al*.) (21).

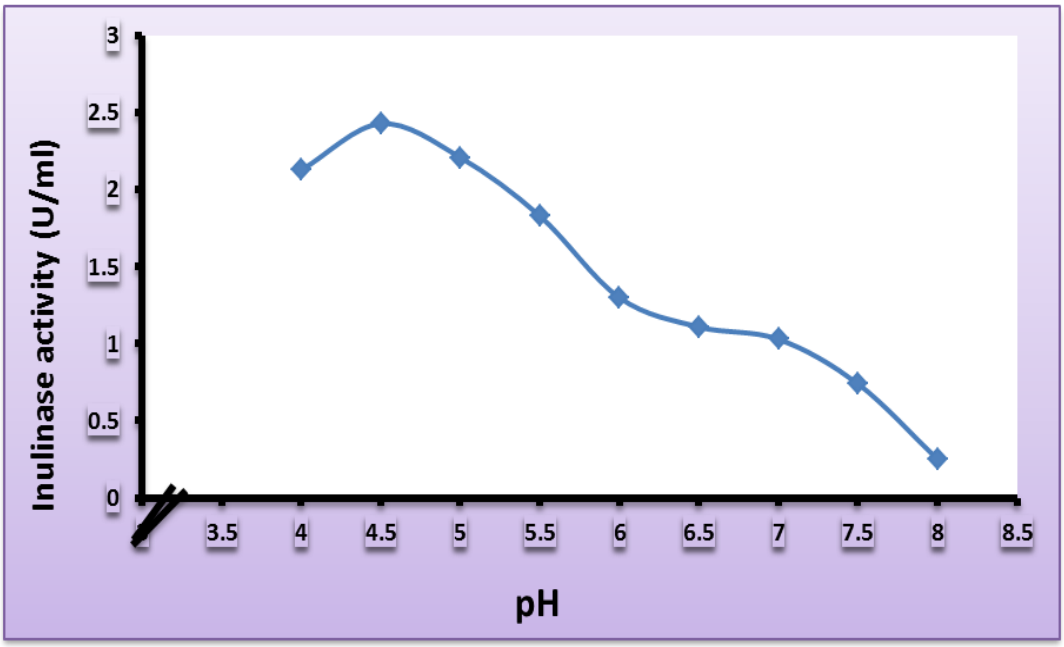

Figure (5): Optimum pH for purified inulinase activity from local isolate *A. niger* AN20

Arand *et al*. (22) found that the optimum pH for inulinase activity from *A. awamorii* was 4.5; while Derycke & Vandamme (23) found the optimum pH for inulinase from *A. niger* was 4.3.

Enzyme Stability pH

The pH of enzyme stability was studied because it is an important criterion in determining the optimum conditions in purification and storage of the enzyme. Figure (6) shows the results of the study, where the range of inulinase stability was from 4.0 - 8.0, while the activity was very low at acidic pH (below 4) and basic pH between 9 - 10. The lowering in enzymatic activity in extreme acidic and extreme basic conditions may be due to changes in tertiary structure of the enzymatic residues as well as changes in ionic state of the active site of the enzyme and substrate (24, 25).

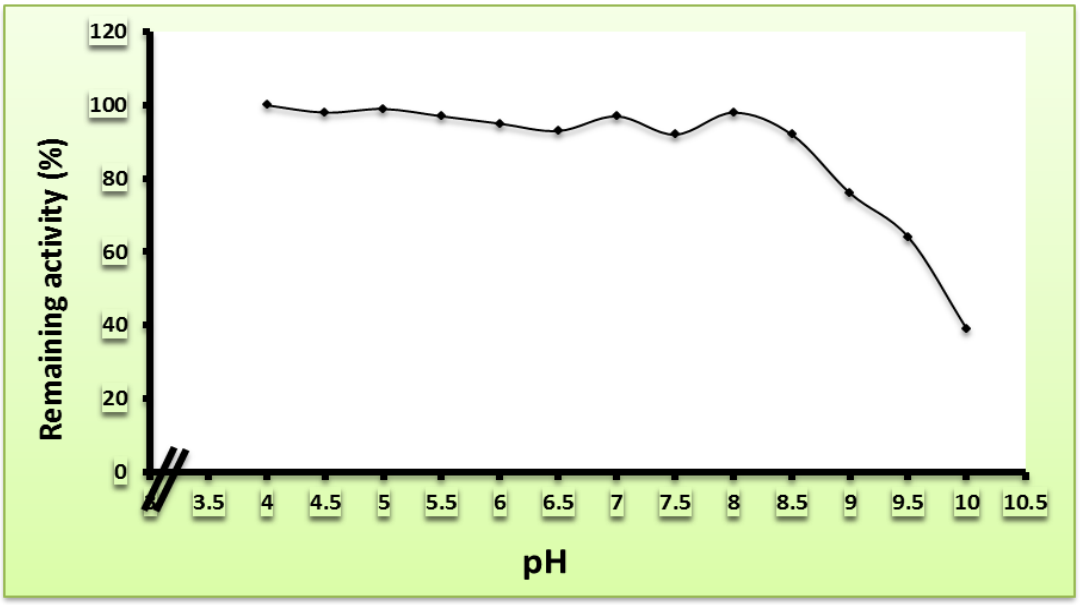

Figure (6): Stability pH for purified inulinase from local isolate *A. niger* AN20

Nakamura *et al*. (18) in a study to determine the pH for inulinase isozymes found that *A. niger* strain PII isozyme was stable in pH ranging from 3.2–7, while PIII is stable in pH ranges from 5–7. Skowronek & Fidurek (26) study showed that *A. niger* 20 OSM endoinulinase is stable in range 4 – 7 these results were close to the results of the present study.

Optimum Temperature of Inulinase Activity

Optimum temperature of inulinase activity was studied by incubating the enzyme with the substrate at different temperatures 20–70 ^{O}C for 15 min, the results showed that the optimum temperature for inulinase activity from *Aspergillus niger* AN20 was 50 ^{O}C, where it gave highest activity at this temperature 1.92 U/ml (figure 7), and the activity decreases above and below this temperature, and lost completely at 70 ^{O}C. The results showed an increase in reactions speed until it reached 50 C^{o} then began to decrease over 55C^{o}. This belongs to the increase of clash between the enzymatic molecules participating in the reaction with the substrate as a result of increasing the kinetic energy of the molecules, while the decrease in the enzymatic activity by temperature degrees over 55C^{o} is a result of the denaturation of protein structure and changes in the active sites which leads to loss of the activity (27). These results agreed with the results found by Rouwenhorst *et al*. (28), where they found that the maximum activity lied between 40-50 ^{o}C.

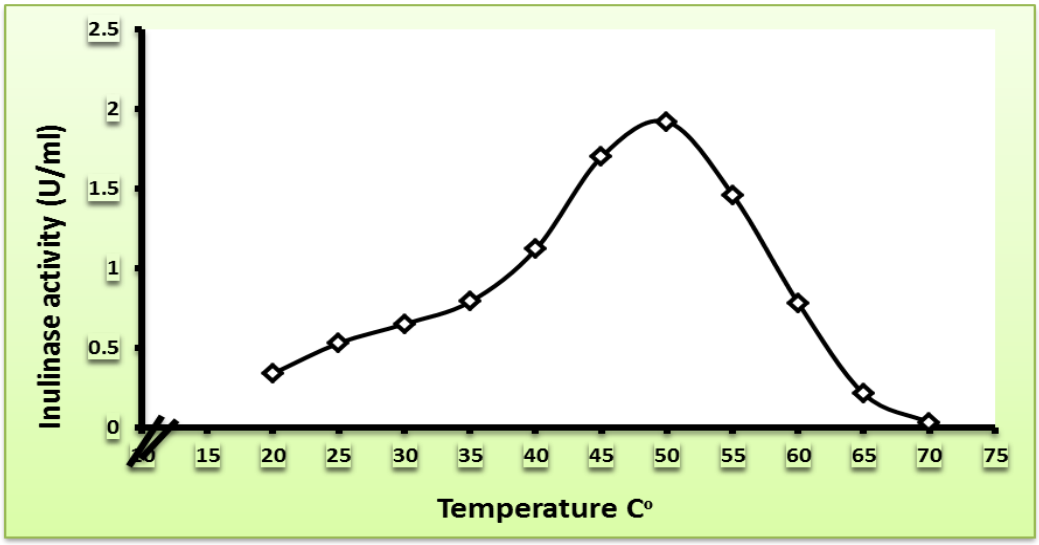

Figure (7): Optimum temperature for purified inulinase activity from local isolates *A. niger* AN20.

Thermal Stability of Inulinase

Incubation of inulinase from *A. niger* AN20 in different temperature degrees between 20 – 70 C° for 30 min, showed that the enzyme was stable in temperatures between 20 –50 C°, then the activity began to decrease and was completely lost in 75°C (figure 8). This decrease in enzymatic activity may be due to the thermal effect on the enzyme structure then it's denaturation (29).Where temperature may effect on the protein structure by breaking the bonds that stabilizes secondary and tertiary structure of proteinwhich results to denaturation (21).

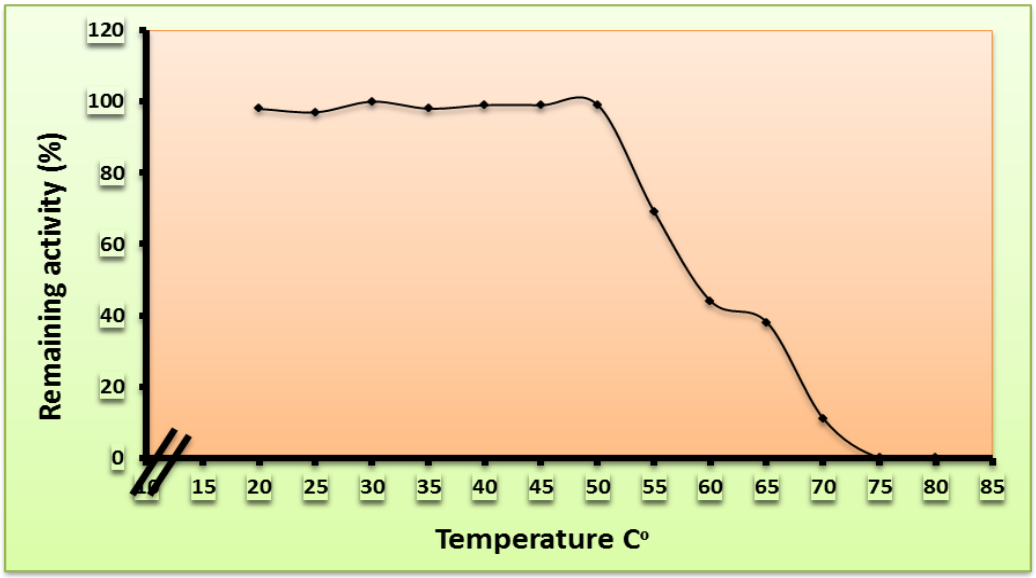

Figure (8): Thermal stability of inulinase purified from local isolate *A. niger* AN20.

Studies of thermal effect on enzymatic activity showed different results. Nakamura *et al.* (18) found that inulinase isozymes PII and PIII from *Aspergillus niger* Strain 12, keeps its activity when enzyme incubated for 30 min, at 50 and 60 ^{o}C, while they lose thier activity at 70 and 80 ^{o}C . The thermal stability of inulinase from *Chrysosporium pannorium* studies showed that the enzyme was thermally stable when incubated for 30 min at temperatures up to 45^{o}C, but loses 15% of its activity when incubating at 50 ^{o}C, and completely loses its activity at 60 ^{o}C (30).

References

1- Ertan, F. ; Ekinci, F. and Aktac, T. (2003). Production of inulinase from *Penicillium spinulosum*, *Kluyveromyces marxianus*, *Aspergillus parasiticus* NRRL2999 and *Trichoderma viride*. *Pakistan J. Biol. Scien.*, 6(15): 1332-1325.

2- Roze, L.V. ; Beaudry, R. M. ; Arthur, A. E. ; Calvo, A. M. and Linz, J. E. (2007). *Aspergillus* volatiles regulate aflatoxin synthesis and asexual sporulation in *Aspergillus parasiticus*. *Appl. Environ. Microbiol.*, 73(22):7268-7276.

3- Perfect, J. R. ; Cox, G. M. and Lee, J. Y. (2001). The impact of culture isolation of *Aspergillus* species: a hospital-based survey of aspergillosis. *Clin. Infect. Dis.*, 33: 1824-1833.

4- Weir, E. (2000). Production of Industrial Enzymes in Fermentation. *J. Biol.*, 5:213-224.

5- Mutanda, T. ; Wilhelmi, B. S. and Whiteley, C. G. (2008). Response surface methodology: Synthesis of inulo-oligosaccharides with an endoinulinase from *Aspergillus niger* .*Enzyme and Microbial.Tech.*, 43:362-368.

6- Mazutii, M. ; Bender, J. P. ; Treichel, H. and Di Luccio, M. (2006). Optemization of inulinase production by soild state fermentation using sugarcane bagasse as substrate. *J. Biotechnol.*, 12: 1123-1131.

7- Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.*, 31: 426-428.

8- Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.*, 72 : 248-254.

9- Whitaker, J. R. (1972). Principle of enzymology for the food science. *Marcel Dekker Inc.*New York., 5: 153-158.

10- Whitaker, J. R. (1963). Determination of molecular weight of protein by gel filtration on sephadex. *Anal. Chem.*, 35: 1950-1953.

11- Stellwagen, K. (1990). Gel filtration. In:"Methods in Enzymology" Deutscher, M.P.(edt.). 182: 317-328. *Academic Press*, New York.

12- Ertan, F. ; Sanal, F. E. ; Kaboglu, A. C. ; Actac, T. and Bakar, E. (2005). Some properties of Inulinase from *Rhizoctonia solani*. *J. Biol. Scien.*, 5(3): 330-334.

13- Sanal, F. E. ; Ertan, F. and Aktac, T. (2005). Production of exo- inulinase from *Alternaria alternata* grown on Jerusalem artichoke and some biochemical properties. *J. Biol. scien.*, 5(4): 497-505.

14- Souza - Motta, C. M. ; Cavalcanti, M. A. Q. ; Porto, A. L. F. ; Moreira, K. A. and Filho, J. L. L. (2005). *Aspergillus niveus* Blochwiz 4128URM: New Source for Inulinase Production. *Brazilian Archives of Biol, and Technol.*, 48(3): 343-350.

15- Singh, P. and Gill, P.K. (2006). Production of inulinases: recent advances, Food Technol. *J. Biotechnol.*, 44 (2): 151-162.

16- Kochhar, A. ; Karur, N. and Gupta, A. K. (1997). Inulinase from *Aspergillus versicolor*: A potent enzyme for production fructose from inulin. *J. Sci. Ind. Res.*, 56: 721-726.

17- Chen, G ; Sun, Z. ; Wang, Y. and Qian, X.(1997). Purification and properties of inulinase from *Aspergillus niger*. *Wieshengwu Xuebae*., 37: 362-376.

18- Nakamura, T. ; Nagatomo, Y. ;Hamada, S. ; Nishino, Y and Ohta, K. (1994). Occurnace of two forms of extracellular endoinulinases from *A.niger* mutant 817. *J. Ferment. Bioeng*., 78: 134-139.

19- Ettalibi, M. and Baratti, J. C. (1987). Purification, properties and comparison of invertase, exoinulinase and endoinulinase of *Aspergillus ficuam*. *Appl. Microbiol. Biotechnol*., 26: 13-20.

20- Uhm, T. B ; Chung, M. S. ; Lee, S. H. ; Gourronc, F. ; Housen, J. ; Kim, J. H. ; Beeumen and Haye, B.(2000). Purification and characterization of *Aspergillus ficuum*

endoinulinase. *Biosci. Biotechnol.*, 6: 146-151.

21- Chesworth, J. M.; Stuchbury, T. and Scaif, J. R. (2010). An Introduction to agricultural biochemistry. *Chapman & Hall, London*., 5(2): 215-223.

22- Arand, M. ; Golubev, A. M. ; Brnando neto, J. R.; Polikaprov, I. ; Wattiez, R. ; Korneeva, O. S. ; Eneyskaya, E. V. ; Kulminskaya, A. A. ; Shabalin, K. A. ; Shishliannikov, S. M. ; Chepurnaya O. V. and Neustroev, K. N.(2002). Purification, characterization, gene cloning and preliminary X-ray data of the exo-inulinase from *Aspergillus awemorii*. *Biochem. J*., 362: 131-135.

23- Deryck, D. G. and Vandamme, E. J. (2014). Production and properties of *Aspergillus* niger Inulinase. *J. Chem. Tech. Biotechnol*., 34:45-51.

24- Lehmacher, A. and Bisswanger, H. (1990). Isolation and characterization of an extremely thermostable D-glucose/ Xylose isomerase from *Thermus aquaticus* HB8. *J. Gen. Microbiol*., 136: 679-686.

25- Moat, A. G. ; Foster, J. W. and Spector, M. P. (2002). Microbial Physiology. 4th ed. Wiley-Liss, Inc., New York., 1: 1-28.

26- Skowronek, M. and Fidurek, J. (2006). Purification and properties of extracellular endoinulinase from *Aspergillus niger* 20 OSM. *Food technol. Biotechnol*., 44(1): 53-58.

27- Kochhar, A. ; Gupta A. K. and Kaur, N. (2001). Purification and immobilization of inulinase from *Aspergillus candidus* for producing fructose. *J. Sci. Food and Agriculture*, 79: 549-554.

28- Rouwenhorst, R. J. ; Visser, L. E. ; Scheffers, W. A. and Van Dijken, J. P. (1988). Production, Distribution, and Kinetic Properties of Inulinase in Continuous Cultures of *Kluyveromyces marxianus* CBS 6556. *Applied and Environmental Microbiology*., 54(5): 1131-1137.

29- Meng, M. ; Bagdasarian, M. and Zeikus, J. G. (2013). Thermal stabilization of xylose isomerase from *Thermoanaerobacterium* therm sulfurgenes. *J. Biotechnol*., 11: 1157-1161.

30- Xiao, R. ; Tanida, M. and Takao, S. (2009). Purification and characterization of two exoinulinases from *Chrysosporum pannorum. J. Ferment. Bioeng*., 67:331-334.