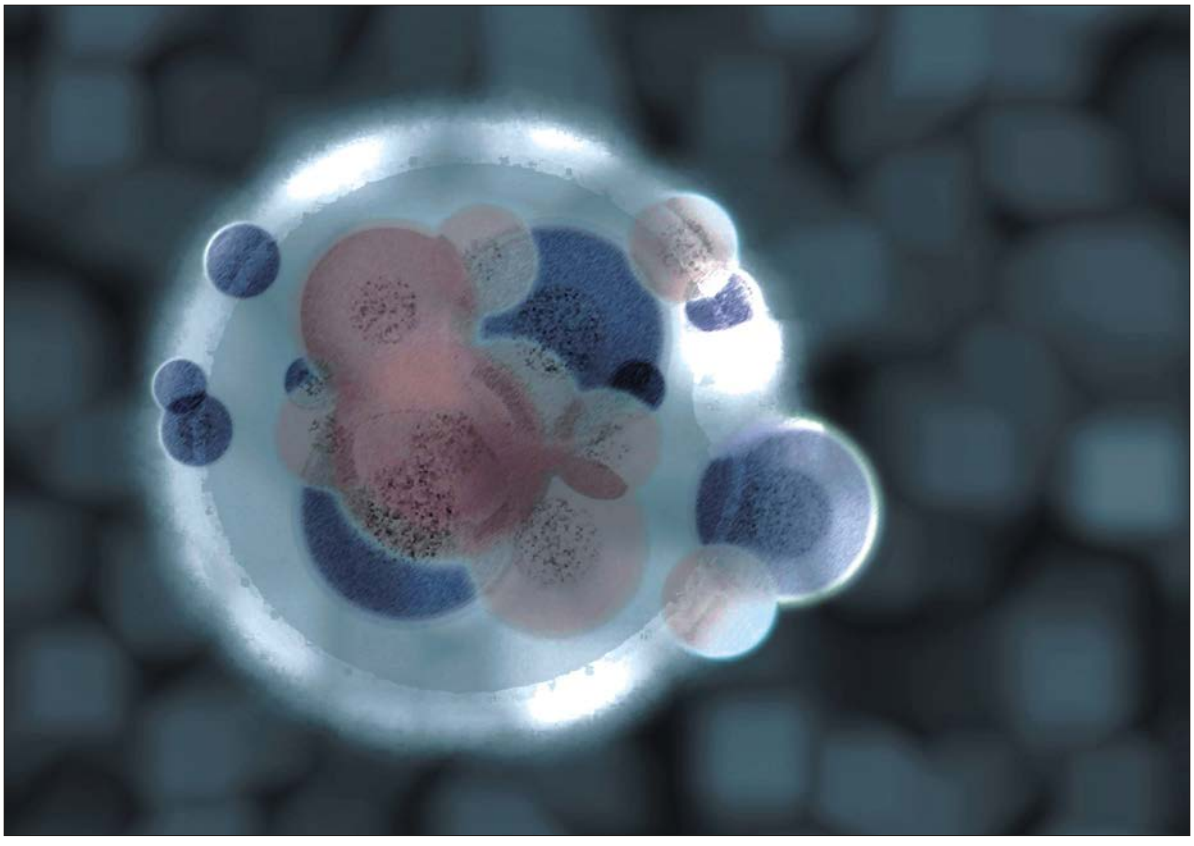

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Production of Exo-inulinase from *Alternaria alternata* Growth on Jerusalem Artichoke and Some Biochemical Properties

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Abstract.: The effect of different carbon and nitrogen sources in the medium on inulinase production were investigated in this research. The enzyme activity was maximum in media containing 3% Jerusalem artichoke powder. $NH_4H_2PO_4$ was found to be the better nitrogen source. $NH_4H_2PO_4 + NH_4NO_3$ combination had higher inulinase yields than other combined nitrogen sources. The enzyme was purified 65.89 fold by using dialysis, concentrating, gel filtration (Sephadex G-150) and ion exchange chromatography (DEAE-cellulose) techniques. The K_m and V_{max} values of inulinase for inulin were 0.52 mM and 8.69 mmol mL^{-1} min^{-1}, respectively. Optimum temperature and pH for the activity of the purified enzyme were found to be 30°C and 5.0 and the enzyme was found to be stable up to 40°C and in a pH range of 4.0 to 6.0. The enzyme was inhibited strongly by Mn^{2+} , KCN and DTNB. The molecular weight of the enzyme was estimated to be 66.000 and 68.000 Da by SDS polyacrylamide gel electrophoresis and gel filtration on sephadex G-200, respectively. The inulinase of *A. alternata* was hydrolised pure inulin and raw jerusalem artichoke powder in similar amounts. This results have industrial importance.

Key words: Inulinase, production, properties, Jerusalem artichoke, *Alternaria alternata*

INTRODUCTION

Fructose is the sweetest of the natural sugars and emerges as a safe alternative sweetener to sucrose which causes problems related to corpulence, cariogenecity, arteriosclerosis and diabetes. It increases the absorption of iron as a results of formation of an iron-fructose chelate complex which is better absorbed than inorganic iron. Fructose has beneficial effects in diabetic patients, increases the iron absorption in children and has a higher sweating capacity so it can be used in the diets of obese persons[1-3].

Conventional fructose production method is based on amylolysis of starch with amylase and amyloglucosidase followed by glucose isomerase catalysed conversion of glucose to fructose. The fructose yield by this method is about 45%, rest being glucose (50%) and different oligosaccharides (8%). Although ion exchange chromatography techniques have been developed for enrichment of fructose, these techniques increases the cost of production[1,2-4]. Direct production of fructose from fructose polymer inulin by inulinase can give 90% yield of fructose by a single step reaction. This fructose polymer is usually found as a reserve carbohydrate in roots and tubers of various plants such as Jerusalem artichoke, dandelion, chicory, dahlia and several other members of the family Compositae[5,6]. It 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. Such inulin sources have recently received attention as a renewable raw material for fructose syrup production and ethanol fermentation[7].

Inulinases (2,1-β-D fructan-fructanohydrolase E.C.3.2.1.7) have been characterised from inulin storing tissues of plants but their quantity is not enough to be used for commercial production of fructose from inulin. Therefore, microbial inulinases which can be induced by growing microorganisms on an inulin-based medium have received attention in various laboratories[7]. Enzymes of interest for fructose syrup preparation are inulinases elaborated by yeasts and filamentous fungi. However industrial application of these enzymes will only be feasible if they are available in large quantities at a competitive price.

Enzymatic production of fructose syrup has advantages over chemical processes: reaction conditions are mild, by-product formation is small because of high specificity for substrate and pollution problem is not serious[8]. Therefore, many researchers have focused their attention on the production of microbial inulinase. In our previous study we reported that *Alternaria alternata*

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produced active exo-inulinase. In this study, we described purification and some properties of inulinase from *A. alternata*.

MATERIALS AND METHODS

Organisms and enzyme production: *A. alternata* was isolated from Tekirdağ soil in Turkey and was identified in Trakya University, Faculty of Agriculture, Department of Plant Protection. The fungus was maintained on potato dextrose agar slants. *A. alternata* was aerobically cultivated with shaking at 30°C for two days in 250 mL flask containing 50 mL of cultivation medium. The cultivation medium used contained 3% Jerusalem artichoke powder, 0.05% $MgSO_4 7H_2O$, 0.05% $NH_4H_2PO_4$, 0.15% yeast extract, 0.1% KH_2PO_4 and 0.023% NH_4NO_3. The initial pH was 6.0. The medium was autoclaved at 115°C for 30 min[9]. The mycelia were removed by filtration using Whatman No:1 filter paper. The culture filtrate was centrifuged at 5000 rpm for 10 min. The supernatant was used as crude enzyme[10,11].

Preparation of Jerusalem artichoke powder: Jerusalem artichoke was washed with cold water, sliced and then dried in pasteur oven at 80°C. After milling the resultant fine powder was used directly as carbon source[9].

Enzyme assay: The enzyme reaction mixture contained 0.9 mL of 0.1% inulin in 0.1 M sodium acetate buffer (pH: 5.0). 0.1 mL enzyme solution was incubated at 30°C for 10 min and the liberated reducing sugar was measured by 3,5-dinitrosalicylic acid method[12]. The amount of the reducing sugar was estimated by comparison with a calibration curve that was made with fructose. One unit of inulinase activity was defined as micromole of fructose produced per minute by the enzyme solution.

Effects of carbon sources: Jerusalem artichoke powder in the medium was replaced by one of the following carbon sources at a concentration of 1%: fructose, glucose, maltose, sucrose, cellulose, pectin and inulin. As mentioned above, the enzyme activities were measured after cultivation and they were compared.

Effects of nitrogen sources: The nitrogen sources $NH_4H_2PO_4$ (4 mM) and NH_4NO_3 (2 mM) in the original cultivation medium were replaced by each of $NH_4H_2PO_4$, NH_4NO_3, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ and NH_4Cl at two different concentrations (5 mM and 10 mM). Moreover, the combined nitrogen sources (NH_4NO_3+ NH_4Cl, $NH_4H_2PO_4$+ NH_4Cl and $NH_4H_2PO_4$+ NH_4NO_3 at 10 mM) were replaced by each of nitrogen sources in the cultivation medium.

Preparation of inulinase: Purification of inulinase was performed at +4°C. The crude enzyme solution was dialysed against 0.1 M Na-acetate buffer (pH 5.0) and then the enzyme in dialysis tube was concentrated overnight at +4°C by embedding in silica gel-60 powder, which is a very strong hygroscopic substance. The concentrated enzyme solution was applied to a column of Sephadex G-150 (2.5x17 cm) equilibrated with 0.1 M Na-acetate buffer (pH 5.0). The fractions showing the enzyme activity were pooled. The pooled active fractions were loaded on to DEAE-Cellulose column (2.5x20 cm) previously equilibrated with 50 mM phosphate buffer (pH 7.0). The adsorbed enzyme was eluated with linear gradient of 0 to 0.4 NaCl.

Determination of protein: The amount of protein was determined by the method of Lowry[13] using bovine serum albumine as standard or by measuring absorbance at 280 nm[13].

Effects of pH , temperature and substrate concentration on inulinase activity: The enzyme reaction was performed at various pH values at 30°C. [Na-acetate (pH 3.0-5.0), phosphate (pH 6.0-7.0) and borate (pH:8.0) buffers]. To investigate the effect of temperature, the enzyme reaction was performed in the range of 25-60°C in 0.1 M Na-acetate buffer, pH 5.0. The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were determined from simple Line weaver-Burk plots using inulin (0.2-1.2 mM).

pH and thermal stability: The enzyme solution was kept at various pHs at 4°C for 30 min. Residual enzyme activity was measured. To investigate thermal stability, the enzyme solution was incubated at various temperature for 20 min at pH 5.0.

Molecular weight estimation: The molecular weight of inulinase was estimated by SDS-polyacrylamide gel electrophoresis[14] and by gel filtration on a Sephadex G-200 column. The electrophoresis was performed in 10% gel. In SDS-polyacrylamide gel electrophoresis, Sigma kit (SDS-6H) was used as standards. To visualise proteins, gel was stained with Comassie Brillant Blue R-250. In gel filtration (Sephadex G-200) phosphorylase (97.4 kDa), albumin bovine (66 kDa) and albumin egg (45 kDa) were used as molecular weight markers. The void volume of column (V_0) was determined by eluating blue dextran.

Effect of metal ions and other chemicals on inulinase activity: Enzyme assays were performed in the presence of various metals (Hg^{2+}, Cu^{2+}, Fe^{2+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Ag^{2+}) and other chemicals (EDTA, KCN, iodosobenzoic acid, β-mercaptoethanol, pCMB

(paracholoromercuribenzoate), DTNB [(5,5'-dithio-bis(2-nitrobenzoic acid)] (1 and 5 mM). The chloride salts of metals were used. The residual activity was measured after preincubation of enzyme in the reaction buffer containing different metals and other compounds at 30°C. The relative activity of the enzyme was compared with that buffer containing substrate only.

The hydrolysis of inulin and Jerusalem artichoke powder: Hydrolysis mixtures containing 0.5% inulin and 0.4 mL enzyme or 0.5% jerusalem artichoke 0.4 mL enzyme were prepared in sodium acetate buffer pH 5.0, 0.1 M and incubated for hydrolysis in different periods (10, 20 and 30 min) at 30°C. The amounts of the released fructose of each hydrolysis period were measured by DNS method.

RESULTS

Effect of carbon sources: The best inulinase production was observed in the presence of Jerusalem artichoke (3 and 1%) and inulin (1%) . The lowest activities were observed in media containing maltose and cellulose (Table 1).

Effect of nitrogen sources: The influence of inorganic nitrogen sources on inulinase production were investigated. The inorganic nitrogen sources in the original cultivation medium, $NH_4H_2PO_4$ (0.004 M) and NH_4NO_3 (0.002 M), were replaced by each of $NH_4H_2PO_4$, NH_4NO_3, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ and NH_4Cl at concentrations of 5 mM and 10 mM. Of these, a very high activity was observed in media containing $NH_4H_2PO_4$ 10 mM (Table 2).

When the effects of inorganic nitrogen sources on inulinase production were investigated it was observed that the combined nitrogen sources had higher inulinase yields than those obtained when they were added to the medium individually (Table 2). In other studies, it was reported that each fungal species has a preference for a specific nitrogen source for optimum production of inulinase[3].

Preparation of inulinase: Since preliminary experiments indicated that inulinase activity was lost when it was precipitated by ammonium sulphate[4], the ammonium sulphate precipitation was not used in our purification steps. Inulinase of *A. alternata* was purified by dialysis, concentrated with silica gel 60 and the sephadex G-150 and DEAE cellulose chromatographies. The purification procedure was summarised in Table 3. Inulinase from *A. alternata* was purified 65.89 folds from the culture filtrate with a yield of 11.6%. The purified inulinase had a specific activity of 29.52 U mg^{-1} towards inulin. The results of DEAE-Cellulose chromatography were given in Fig. 1.

Table 1: The effect of carbon sources on inulinase activity

Carbon source		Mycelial weight (g/50 mL)	Activity (U mL^{-1})
Fmctose	(1%)	0.50	0.47
Glucose	(1%)	0.22	1.10
Maltose	(1%)	0.30	0.23
Sucrose	(1%)	0.10	0.86
Starch	1(%)	0.40	0.82
Cellulose	(1%)	0.42	0.27
Pectin	(1%)	0.10	0.58
Inulin	(1%)	0.30	1.72
Jemsalem artichoke (1%)		0.43	1.50
Jemsalem artichoke (3%)		0.75	2.70

Table 2: The effect of nitrogen sources on inulinase activity

N source (mM)	Mycelial weight (g/50 mL)	Inulinase activity (U mL^{-1})
Control *	0.60	2.30
5 $(NH_4)_2HPO_4$	0.70	1.84
10 $(NH_4)_2HPO_4$	0.84	2.64
5 NH_4SO_4	0.58	1.72
10 NH_4SO_4	0.77	1.95
5 NH_4NO_3	0.54	2.64
10 NH_4NO_3	0.62	2.76
5 $NH_4H_2PO_4$	0.63	2.41
10 $NH_4H_2PO_4$	0.80	2.87
5 NH_4Cl	0.52	2.53
10 NH_4Cl	0.56	2.64
10 NH_4NO_3 + 10 NH_4Cl	0.58	3.22
10 $NH_4H_2PO_4$ + 10 NH_4Cl	0.83	3.10
10 $NH_4H_2PO_4$ + 10 NH_4NO_3	0.70	3.33

*$NH_4H_2PO_4$ (4 mM) and NH_4NO_3 (2 mM)

Table 3: Summary of purification results of inulinase by *A. alternata*

Step	Total protein (mg)	Total activity (Units)	Specific activity (U mg^{-1})	Purification (Fold)	Yield (%)
Crude enzyme	487.70	217.0	0.448	1.00	100.00
Dialysis+concentrated by silica gel	201.90	80.5	1.130	2.52	37.00
Sephadex G-150	3.75	45.3	12.080	26.96	20.87
DEAE-Cellulose	0.85	25.1	29.520	65.89	11.56

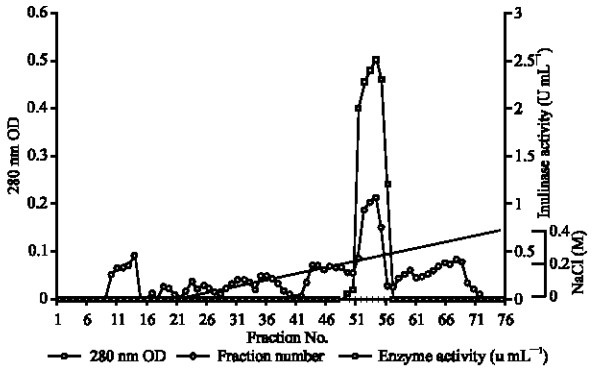

Fig. 1: Chromatography of inulinase from *A. alternata* a DEAE cellulose column

Molecular weight estimation: The purified enzyme was homogeneoused by polyacrylamide gel electrophoresis. The molecular weight of the enzyme was estimated to be approximately 66.000-68.000 Da by both gel filtration (sephadex G-200) with standard protein markers and SDS-polyacrylamide gel electrophoresis[14] with standard

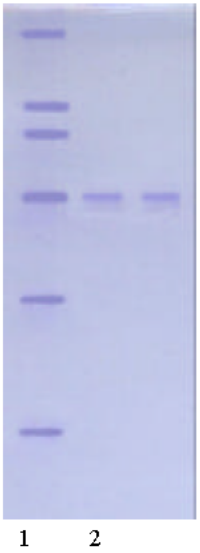

Fig. 2: The estimation of the molecular weight of the inulinase enzyme by SDS-PAGE
The enzyme was subjected to SDS-PAGE on an 10% (w/v) polyacrylamide slab gel at pH:8.3. Protein was visualed by Coommassie brillant blue R-250. Staining Lanes: 1, Standard proteins; 2, purified inulinase. The standard proteins were Myosin (205 kDa); β-Galactosidase (116 kDa); Phosphorylase B (97.4 kDa); Albumin bovine (66 kDa); Albumine egg (45 KDa); Carbonic anhydrase (29 kDa)

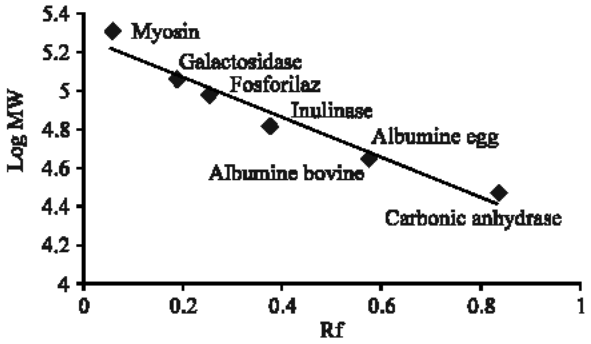

Fig. 3: SDS-polyacrylamide gel electrophoresis of purified inulinase from *A. alternata*

protein markers (Sigma). The findings showed that the enzyme protein was composed of a single polypeptide chain (Fig. 2 and 3).

Effects of pH and temperature and substrate concentrations on inulinase activity: The optimum pH was 5.0 and the optimum temperature was 30°C (Fig. 4). It can be seen in Fig. 5 that the inulinase activity was significantly lower at other pH values.

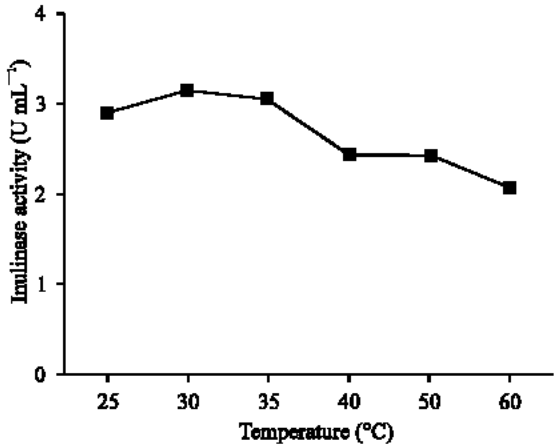

Fig. 4: The effect of temperature on the inulinase enzyme activity

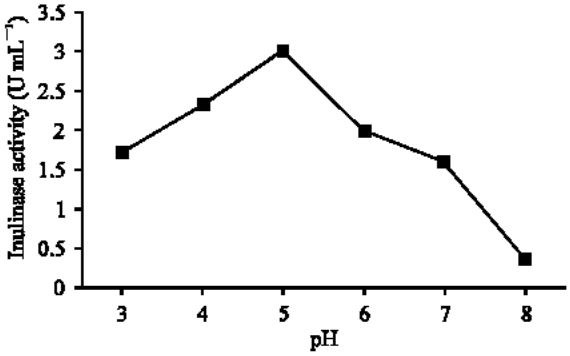

Fig. 5: The effect of pH on the inulinase enzyme activity

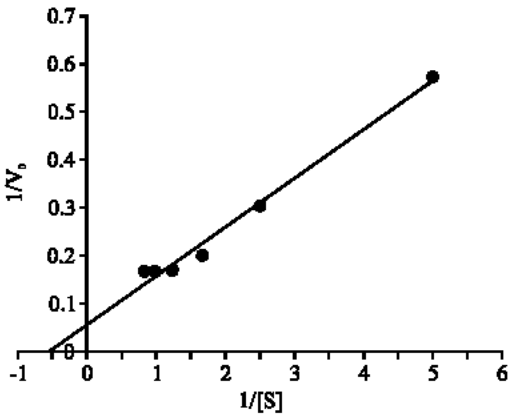

Fig. 6: Line- weaver Burk plots

Figure 6 summarises the effects of different concentrations of inulin on enzyme activity of *A. alternata*. When calculated by the Line weaver-Burk plot, the apparent K_m and V_{max} values for inulin hydrolysis under the standard assay conditions were 0.52 mM and 8.69 mMol mL^{-1} min^{-1}, respectively, assuming that the average molecular weight of inulin was 5000.

Table 4: The effects of some metals ions on the inulinase activity from *A. alternata*

Metal ions (1 mM)	Remaining activity (%)	Inhibition (%)	Metal ions (5 mM)	Remaining activity (%)	Inhibition (%)
-	100.00	-	-	100.00	-
Hg^{2+}	68.50	31.5	Hg^{2+}	37.10	62.90
Cu^{2+}	57.10	42.9	Cu^{2+}	45.71	54.30
Fe^{2+}	80.00	20.0	Fe^{2+}	74.20	25.80
Ba^{2+}	100.00	-	Ba^{2+}	100.00	-
Ca^{2+}	85.70	14.3	Ca^{2+}	74.20	27.80
Mg^{2+}	57.10	42.9	Mg^{2+}	51.40	48.60
Zn^{2+}	62.80	37.2	Zn^{2+}	51.40	48.60
Mn^{2+}	57.14	42.9	Mn^{2+}	-	100.00
Ag^{2+}	62.80	37.2	Ag^{2+}	57.10	42.90

Table 5: The effect of other chemicals on inulinase activity by *A. alternata*

Chemical (mM)	Remaining activity (%)	Inhibition (%)
(-)	100.00	-
β-Mercaptoethanol (1)	63.40	36.60
β-Mercaptoethanol (5)	55.70	44.30
KCN (1)	66.20	33.80
KCN (5)	27.17	72.83
Iodosobenzoate (1)	73.51	26.49
Iodosobenzoate (5)	70.03	29.97
DTNB (1)	26.82	73.18
DTNB (5)	18.11	81.89
pCMB (1)	66.20	33.80
pCMB (5)	62.71	37.29
EDTA (1)	69.68	30.32
EDTA (5)	52.26	47.74

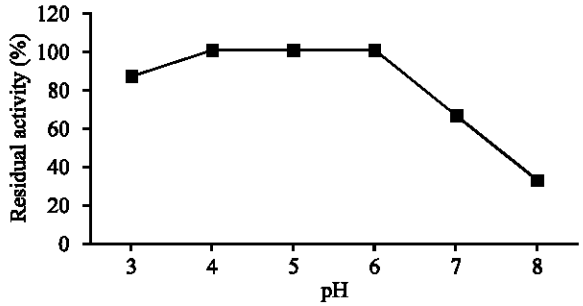

Fig. 7: pH stability of enzyme. The enzyme solution was incubated at various pH for 30 min at 4°C and then residual enzyme activity was measured at pH 5.0 and 30°C

pH and thermal stability: As shown in Fig. 7, the enzyme was stable in the pH range of 4.0-6.0, but its activity decreased below pH 4.0 and above pH.6.0. As shown in Fig. 8 the enzyme was stable up to 40°C, but activity decreased over 40°C.

Effects of metal ions on inulinase activity: The results of the enzyme reaction as performed in the reaction mixture containing 1 and 5 mM of each metal ion at pH 5.0 and 30°C were given in Table 4. Hg^{2+}, Cu^{2+}, Mg^{2+} and Ag^{2+}

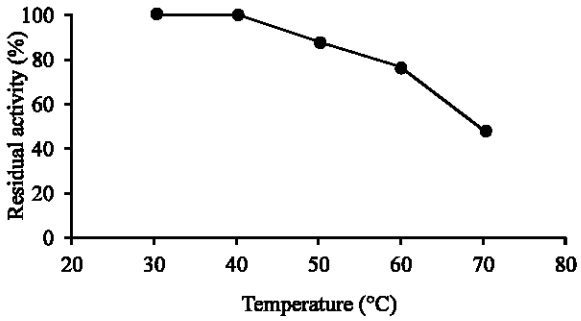

Fig. 8: Thermal stability of enzyme
The enzyme was incubated at various temperature at pH 5.0 for 20 min and then residual enzyme activity was measured at pH 5.0 and 30°C

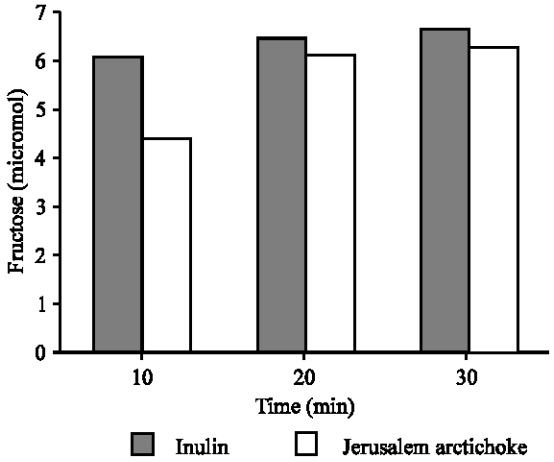

Fig. 9: The hydrolysis of inulin and jerusalem artichoke

inhibited the enzyme activities strongly (Table 4). Only 5 mM Mn^{2+} caused complete inhibition, whereas 5 mM Fe^{2+} and Ca^{2+} had only a slight inhibitory effect on activity.

Effect of some chemicals on inulinase activity: The results of experiments on the effects of chemicals (EDTA, KCN, iodosobenzoic acid, β-mercaptoethanol, pCMB (paracholoromercuribenzoate), DTNB (5,5'-dithio-bis(2-nitrobenzoicacid) at concentrations of 1 and 5 mM on the enzyme activity (each chemical was mixed with the enzyme in 1 mL of a 0.1 M sodium acetate buffer at pH 5.0) were as follows; β-mercaptoethanol (55.7%), KCN (27.17%), Iodosobenzoic acid (70.03%), DTNB (18.11%), pCMB (62.71%) and EDTA (52.26%) (Table 5). While the inhibitory effects of DTNB (1 and 5 mM) and KCN (5 mM) were high (between 72.8-81.9%) the inhibitions of β-mercaptoethanol (1 and 5 mM), Iodosobenzoic acid (1 and 5 mM) pCMB (1 and 5 mM) and EDTA (1 and 5 mM) ranged between 30.3-47.7%.

The hydrolysis of inulin and Jerusalem artichoke powder: When pure inulin 0.5% was hydrolysed (at 30°C, pH 5.0 and 30 min) by inulinase of *A. alternata*, the amount of the released fructose was assayed as 6.65 μ mol. Similarly, when jerusalem artichoke powder 0.5% was hydrolysed with the same enzyme, the amount of the released fructose was determined as 6.28 μ mol (Fig. 9).

DISCUSSION

Alternaria alternata was selected for the present study after screening several fungal strains for their capacity to synthesis extracellular inulinase after growing on Jerusalem artichoke powder based growth medium. We performed some of our experiments to evaluate the effects of different nitrogen sources added into the cultivation mediums on inulinase activity and the concerning results were given in Table 2. The original cultivation medium that consists of $NH_4H_2PO_4$ and NH_4NO_3 had a 2.30 U mL^{-1} enzyme activity. Firstly when we used 5 and 10 mM $NH_4H_2PO_4$, the enzyme activity was measured as 1.84 and 2.64 U mL^{-1}, respectively. Similarly, when we treated with 5 and 10 mM NH_4NO_3, the enzyme activity was measured as 2.64 and 2.76 U mL^{-1}, respectively. Moreover, when 10 mM $NH_4H_2PO_4$ and 10 mM NH_4NO_3 were used in combination, a higher enzyme activity was achieved (3.33 U mL^{-1}) compared to the ones obtained when these substances were used individually. Besides, when NH_4Cl which is not used in the original cultivation medium, was added to the medium as 5 and 10 mM, enzyme activity was measured as 2.53 and 2.64 U mL^{-1}. The enzyme activities were measured as 3.22 U mL^{-1} in combination of NH_4NO_3-NH_4Cl (10 mM) and 3.10 U mL^{-1} in combination of $NH_4H_2PO_4$-NH_4Cl (10 mM).

In this case, all concentrations of N sources which we used caused extremely high inulinase activities and it is appearent that using N sources in combination will be more effective when compared to their individual usages. In their studies on the effects of N sources on inulinase production of different fungi. Gupta *et al.*[2], Choi *et al.*[16] and Xiao *et al.*[17] reported different results patterns. Gupta *et al.*[2] have reported an increased production of inulinase with sodium nitrate as a nitrogen sources in the medium of *Fusarium oxysporium*, whereas addition of ammonium dihydrogen phosphate exerted an inhibitory effect on inulinase production by *Aspergillus niger*. Choi *et al.*[16] reported a reasonably high production of extracellular inulinase in *K. fragilis* (strain No:351) with $(NH_4)_2SO_4$[2]. Xiao *et al.*[11] showed that among inorganic nitrogen sources, the addition of 0.02 M $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, NH_4Cl or NH_4NO_3 gave a higher inulinase yield than $NH_4H_2PO_4$ or $NaNO_3$[11]. It appears that each fungal species has a preference for a specific nitrogen source for optimum production of inulinase[1-3,15,16].

When the effects of addition of different carbon sources on inulinase production were investigated, we found the maximum enzyme activity in the media containing 1% inulin. While 1% inulin among the tested C sources caused a high enzyme activity, 1% Jerusalem artichoke also led to a similar high activity of inulinase (Table 1). When 3% Jerusalem artichoke was used, an extremely high activity of enzyme was measured. On account of this, we suggest that 3% Jerusalem artichoke might be preferred over 1% inulin in the production of inulinase when it is evaluated economically. Gupta *et al.*[2] found for *K. fragilis* that an aqueous solution of chicory roots with 1% fructan was a better carbon source for inulinase production than inulin[2]. Thus, Jerusalem artichoke powder which is readily available cheaply seems to be a suitable fermentation substrate and it could provide a feasible alternative as a substrate, since pure inulin is only available in limited quantities and with high cost. In conclusion, although inulinase production in *Alternaria alternata* from Jerusalem artichoke was slightly lower than that from pure inulin, these results suggest a significant impact of its use in industrial processes, considering the high cost of commercially-pure inulin.

The inulinase from *A. alternata* was purified 65.89 fold by dialysis, concentrated with silica gel 60 and chromatographies on sephadex G-150 and DEAE cellulose. The molecular weight of purified enzyme was estimated to be 66.000 Da by SDS-PAGE and 68.000 Da by gel filtration. Similarly, the molecular weight of the inulinase from *Chysosporium pannorum* was 58.000 Da by SDS-PAGE. Moreover, the molecular weight of the same enzyme was estimated to be 56.000 Da by gel filtration (Sephacryl S-200) according to Xiao *et al.*[17]. The molecular weight of the inulinase from *A. ficuum* was determined to be 64.000 and 74.000 Da[18] . It can be seen that the molecular weight of inulinase from several *Aspergillus* species was estimated as 230.000 Da for *A. versicolor*[3] , as 54.000 Da for *A. candidus*[19], as 69.000 Da for *A. awamori*[20]. Gupta *et al.*[2] have reported that the molecular weight of *K. fragilis* inulinase was 250.000 Da. The molecular mass values of inulinase determined by SDS-PAGE were found to be 87-102 kDa for *K. Marxianus*[21], 66.000 Da for *A. ficuum*[22] and 57.000 Da for *K. marxianus bulgaricus*[23]. The relative molecular

weights of inulinase of *Penicillium* species, as determined by gel filtration and SDS-PAGE were in the range of 48.000-87.000 Da[7,24-26].

In this study using velocity data obtained with increasing concentration of inulin in the assay system Michaelis constant (K_m) and V_{max} values (as determined by Lineweaver -Burk and velocity us substrate concentration graphs) were found 0.52 mM and 8.69 mol mL^{-1} min^{-1}, respectively. The K_m of inulinase of *A. alternata* from the present study could be compared with K_m values of $1.2x10^{-2}$ mM from *C. acetobutylicum* ABKn8 by Efstathiou *et al.*[27], 0.21 mM from *P. purpurogenum* by Onodera and Shiomi[4], 0.42 mM from *P. trzebinski* by Onodera and Shiomi[25], 0.20 mM from *Penicillium* sp Tn-88 by Nakamura *et al.*[7]. K_m values of some *Aspergillus* species were found as follows; 0.12 mM for *A. versicolor*[3], 0.3 mM for *A. oryzae*[4] 0.48 mmol L^{-1} and 0.50 mmol L^{-1} for *A. niger*[28] and 0.003 mM for *A. awamori*[20]. In this study, the maximum inulinase activity was obtained at pH 5.0 and this was similar to the values reported for most fungal and bacterial inulinases. The data can be compared with values of 4.75, 5.2, 6.0, 6.1, 7.0 for inulinases from *K. marxianus* var. *bulgaricus*, *P. trzebinski*, *P. rugulosum*, *B. stearothermophilus* and *B. subtilis* 430A, respectively[23,25,29,30]. A quite wide range incubation pH (pH 4.1-7.0) were determined of nulinase enzyme in the studies of different authors[18,24,31]. Pessoni *et al.*[26] reported pH of 5.0, while Yun *et al.*[32], Kim *et al.*[33] and Gupta *et al.*[2] reported pH of 5.5.

We determined the maximum enzyme activity of *A. alternata* as 30°C. This temperature was also reported as the optimal temperature of inulinase activity for *C. acetobutylicum*, *C. pannarum* and *A. ficuum*, *F. oxysporum*[17,18,25,27,34,36]. However, the optimum temperature of inulinases from *A. niger*[31] and *A. oryzae*[4] were 50°C, while many other fungal inulinases's were found between 30-55°C[2,26,27-37]. However, it has been shown that denaturation of fungal inulinases (from *Penicillium, Aspergillus*) occurred above 40°C[38-40]. Inulinase from *A.* oryzae[4], some *Aspergillus* sp.[37] and some yeasts and *Penicillium* sp.[26] are more thermostable. They tolerate up to a temperature of 55°C.

In the present study metal ions were added at two different concentrations (1 and 5 mM) into the reaction mixtures and the enzyme activities were measured at pH 5.0 and 30°C. As can be seen in Tables 4 and 5, when effects of some metal ions and chemicals on inulinase activities were evaluated Hg^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+} and KCN and DTNB were found to have high inhibitory effects. On the other hand, Mn of the metal ions had a complete inhibitory effect. In the light of these findings, it can be suggested that as reported also by some other authors, some -SH groups might be essential for inulinase activity produced by moulds[18-38]. Present results can be compared with the study of Ongen Baysal *et al.*[9] who also similarly reported that the activity of inulinase from *A. niger* decreased in the presence of Ag^{2+}, Hg^{2+}, Cu^{2+} and Mn^{2+}. The high inhibitions obtained with pCMB, β-mercaptoethanol and DTNB are indicative of an implication of protein sulfidrils as functional groups in enzyme catalysis.

The hydrolysis ability of *A. alternata* inulinase on inulin and jerusalem artichoke powder was investigated. The enzyme was found to hydrolase both of the substrates in similar amounts. This finding led us to conclude that the industrial usage of raw jerusalem artichoke powder to obtain fructose could be important.

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