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COMPARATIVE STUDY OF TWO PURIFIED INULINASES FROM THERMOPHILE *THIELAVIA TERRESTRIS* NRRL 8126 AND MESOPHILE *ASPERGILLUS FOETIDUS* NRRL 337 GROWN ON *CICHORIUM INTYBUS* L.

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ABSTRACT

Thirty fungal species grown on *Cichorium intybus* L. root extract as a sole carbon source, were screened for the production of exo-inulinase activities. The thermophile *Thielavia terrestris* NRRL 8126 and mesophile *Aspergillus foetidus* NRRL 337 gave the highest production levels of inulinases I & II at 50 and 24 ºC respectively. Yeast extract and peptone were the best nitrogen sources for highest production of inulinases I & II at five and seven days of incubation respectively. The two inulinases I & II were purified to homogeneity by gel-filtration and ion-exchange chromatography with 66.0 and 42.0 fold of purification respectively. The optimum temperatures of purified inulinases I & II were 75 and 50 ºC respectively. Inulinase I was more thermostable than the other one. The optimum pH for activity was found to be 4.5 and 5.5 for inulinases I & II respectively. A comparatively lower Michaelis–Menten constant (2.15 mg/ml) and higher maximum initial velocity (115 µmol/min/mg of protein) for inulinase I on inulin demonstrated the exoinulinase's greater affinity for inulin substrate. These findings are significant for its potential industrial application. The molecular mass of the inulinases I & II were estimated to be 72 & 78 kDa respectively by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Key words: Inulinases, Production, Purification, Thermophile, Thermostability.

INTRODUCTION

Inulin is a potential source of fructose, or fruit sugar (22). It is present as a reserve carbohydrate in the roots and tubers of composite plants such as Jerusalem artichoke (*Helianthus tuberosus* L.), Chicory (*Cichorium intybus* L.), Sunflower (*Helianthus annuus* L.) and Dahlia (*Dahlia pinnata* Cav.). Chemically, it is a polymer (MM= 60000) of fructose units linked by β-2,1 bonds with a terminal glucose residue linked by α-1,2 bonds. This polymer is a potential feedstock for fuel ethanol production and fructose products. These fructo-oligosaccharides are widely used in many countries as food ingredients since they show many health benefits without any known harmful risks such as toxicity, carcinogenicity and mortality (3, 13, 24, 35). Moreover, crude inulin from chicory root extracts is hydrolyzed faster than pure inulin (36, 37).

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Chicory in its processed form serves as a coffee substitute in the manufacture of various coffee blends.

Inulin hydrolyzing enzymes (inulinases) are produced by plants, filamentous fungi, yeasts, and bacteria. Among the filamentous fungi, *Aspergillus* and *Penicillium* species are common inulinase producers (5, 9, 21, 25, 42, 43). Inulinases are classified as endo - or exoinulinases, depending on their mode of action. Endoinulinases (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) are specific for inulin and hydrolyse it by breaking the bonds between fructose units that are located away from the ends of the polymer chain, to produce oligosaccharides. Exo-inulinases (β-D-fructohydrolase; EC 3.2.1.80), split terminal fructose units in sucrose, raffinose and inulin to liberate fructose (14, 28, 32). Unfortunately, only a few of inulinases required for industrial applications have an optimum temperature of 50 °C or higher (14, 16, 19, 41). Therefore the search for inulinase fungal producers and the purification of these enzymes have received increasing attention. The purification and properties of inulinases have been studied in many fungal species (5, 7, 15, 18, 29, 30).

Thermophilic fungi can produce inulinases of higher optimum temperature which is an extremely important factor in the commercial industrial production of fructose or fructooligosaccharides from inulin. This is mainly because high temperatures ensure proper solubility of inulin and also prevent microbial contamination (9, 14, 46, 47). Moreover, higher thermostability of the industrially important enzymes also brings down the cost of production because lower amounts of enzyme are required to produce the desired product (16).

This work aims at the comparison between the optimum conditions for the production, purification and characterization of extracellular inulinases from the thermophile *Thielavia terrestris* NRRL 8126 and the mesophile *Aspergillus foetidus* NRRL 337. The aforementioned fungi were chosen after performing an initial screening of 30 fungal species belonging to 11 genera for their capability to produce inulinases when grown on *Cichorium intybus* root extract.

MATERIALS AND METHODS

Microorganisms and maintenance

All the micro-organisms used throughout this work were obtained from NRRL (Agricultural Research Service Culture Collection). They were kindly provided by United States Department of Agriculture (USDA), New Orleans, Louisiana 70179. The studied fungi were maintained in agar slants and in Petri dishes at 4 ºC in their respective maintenance media which were described by DSMZ Catalogue (8) in Table 1, i.e. maintenance medium no.129 (Potato Dextrose Agar), 90 (Malt extract Peptone Agar), 190 (YpSs-Yeast protein Soluble starch), 189 (Oat Flake) and 310 (V-8 vegetable juice agar).

Preparation of *Cichorium intybus* root extract

Chicory was gathered from Sharkeya governorate north east of Egypt and washed with cold water, grounded and blended by a mixer as a known weight of fresh roots (2%) with of 0.2 M citrate phosphate buffer (pH 6.0). The whole blend of Chicory extract was used as a sole carbon source throughout the study.

Inoculum preparation

Spores from the agar slants were suspended in sterile saline solution (0.85% NaCl) containing 0.01% Tween 80 to obtain 2.0 x 10^6 spore ml^{-1}. For all the experiments, 1 ml of this suspension was used.

Screening of fungal species for their potentiality to produce exo-inulinase

Fungal species were incubated for 6 days in 250 ml capacity Erlenmeyer conical flasks (triplicate) containing 50 ml of basal medium for the production of inulinases (2% chicory root extract, 0.05% $MgSO_4$ $7H_20$, 0.2% $NH_4H_2PO_4$ and 0.1% KH_2PO_4).The pH was adjusted at 6.0 (27, 51). The incubation was done at the ideal temperature for each fungus as previously

mentioned in Table 1. At the end of the fermentation process, the contents of each flask were gathered, and rapidly filtered using Whatman No:1 filter paper. The filtrate was then subjected to an enzyme assay for the determination of inulinases activity, in order to select the best fungi that were chosen for further studies.

Inulinase assay

One ml of Crude enzyme was added to 1ml of 0.5% (w/v) inulin (Sigma) in 0.2 M citrate phosphate buffer (pH 6.0) and incubated at 40 ºC for 20 min. The reaction was terminated by boiling for 5 min (19). The reducing sugars were subsequently analysed by 3.5 dinitrosalicylic-DNSA reagent (23). One unit of inulinase (U) was defined as the amount of enzyme, which produced 1 µmol min^{-1} of fructose under the assay conditions as described above.

Protein determination

Total protein was determined by using bovine serum albumin as protein standard (4). All the assay were carried out in triplicates.

Effect of different concentration of Chicory root extract on inulinases production.

Mixtures of basal medium (0.05% $MgSO_4$ $7H_20$, 0.2% $NH_4H_2PO_4$ and 0.1% KH_2PO_4) with Chicory root extract of different concentrations (2.0, 3.0, 4.0, 5.0%) were prepared. The pH was adjusted at 6.0, inoculated with spore suspension of the selected fungi and incubated as described before, then the inulinases activities were determined.

Effect of different nitrogen sources on inulinases production

Equimolecular amounts of four organic nitrogen source (asparagines, casein, peptone, yeast extract) and five inorganic nitrogen sources ($NaNO_3$, $(NH_4)_2HPO_4$, NH_4SO_4, NH_4NO_3, NH_4Cl) were replaced by the original nitrogen sources in the cultivation media of the selected fungi.

Effect of different incubation periods on inulinases production

Triplicate flasks were inoculated with each of the experimental fungi using the best concentration of Chicory root extract and the best nitrogen source from the previous experiments and incubated for different periods (3 – 8 days).

Enzyme purification and characterization

Aliquots of cell-free dialysate (CFD) were separately treated with $(NH_4)_2SO_4$ using range of saturation from 0.5 to 0.9; methanol or iso-propanol in a ratio of 1:1, 2:1, 3:1, 4:1 or 5:1 (alcohol : CFD). All samples were left overnight at 4 ºC. The precipitates were collected by centrifugation at 12 x 10^3 g for 15 min., dissolved in 5 ml acetate buffer (0.2 M, pH 6.0) and dialyzed overnight against the same buffer. Protein precipitates containing most of the enzyme activity were then fractionated on Sephadex G-100 column (2.5 x 82 cm) of Fraction Collector (Fra100, Pharmacia-Fin Chemicals) pre-equilibrated with acetate buffer. The column was eluted with the same buffer at 20 ml h^{-1}. Active fractions (5 ml each) were pooled, lyophilized and subjected for further purification on DEAE-cellulose column (Diethylaminoethyl-cellulose, fast flow, fibrous form-Sigma product) pretreated with distilled water followed by washing with 1 N HCl and water till the pH of the suspension was about 6.0 (31,34). It was then washed several times with 0.5 N NaOH until no more colour was removed. Column was eluted with gradient of 0 - 0.8 M NaCl prepared in 0.2 M acetate buffer (pH 6.0) at a flow rate of 10 ml h^{-1} and 5 ml fractions were collected and dialyzed once again to remove Na^+ and Cl^-. This enzyme was lyophilized and stored at 0 ºC for further investigations.

Characterization of the purified inulinases

Effect of temperature and thermal stability: The two purified inulinases were incubated with inulin at various temperatures ranging from 30 to 80 °C. Enzymes activity were measured as previously described. However, for determination

of thermal stability, the enzymes were incubated for variable durations (0 to 60 min) at fixed temperatures. The residual activity was estimated under standard assay conditions after incubation intervals and expressed as relative activity (%).

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 (0.1 M phosphate, pH 3.5-7.0; 0.2 M Tris-HCl, pH 7.0-9.0). To determine pH stability, the enzyme was incubated in the presence of pH values within the above cited for 60 min at room temperature (20 °C). The original pH value was then restored and the residual activity was estimated under standard assay conditions. The results were expressed as relative activity (%) referred to the activity observed before incubation.

Effect of substrate concentration: Inulinases were incubated with different concentrations of inulin (1-9 mg/ml) at 40 °C for 20 min. The reaction was stopped and the enzyme activity was measured under the standard assay conditions as previously described. The kinetic parameters (Michaelis–Menten constant, K_m and maximal reaction velocity, Vmax) were estimated.

Molecular mass determination: The molecular mass of the purified inulinases was estimated in sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) using standard protein markers (myosin, 205 kDa; B-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 67 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate, 36; carbonic anhydrase, 29 kDa; Sigma) (20).

Statistical validation of treatment effects: The mean, standard deviation, T-score and probability "P" values of 3 replicates of the investigated factors and the control were computed according to the mathematical principles described by Glantz (17). Results were considered highly significant, significant or non-significant where $P < 0.01$, > 0.01 and < 0.05, > 0.05 respectively. The comparison of all means was also carried out by Anova test for analysis of variance between groups (52).

RESULTS

Data are expressed in Table 1 as enzyme units produced in the culture medium filtrate of 30 fungal species belonging to 11 genera when grown on *Cichorium intybus* root extract under the conditions described in materials and methods. Throughout the text, the produced inulinases from the thermophile *Thielavia terrestris* NRRL 8126 and the mesophile *Aspergillus foetidus* NRRL 337 were given the numbers I & II respectively. The aforementioned fungi showed relatively good production of inulinases I & II (8.42 and 8.36 U ml^{-1}) after 6 days incubation at 50 and 24 ºC respectively (Table 1).

The results in Table 2 showed that the best concentration of Chicory root extract in media was 4% for the two inulinases. This result was statistically analyzed and confirmed by applying ANOVA, where P-values were $1.79^{X\ 10\text{-}7}$ & $1.01^{X\ 10\text{-}5}$ for inulinases I & II respectively which reflect the high significance of these results. The concentration (4%) was used as a control for the subsequent experiments.

When the effect of organic and inorganic nitrogen sources on inulinases production from the two selected fungi were investigated, it was observed that the yeast extract and peptone were the best organic sources which enhanced the production activity of inulinase I to 17.87 and inulinase II to 15.33 U ml^{-1} from *T. terrestris* and *A. foetidus* respectively (Table 3). The results in Table (4) showed that five and seven days of incubation were the best for inulinases I & II production respectively.

The first step of purification of the two enzymes is carried out by precipitation of protein from the cell-free dialysate. Isopropanol (1:1) and methanol (1:1) were found to be superior in obtaining protein fraction having the highest total activity of

inulinases I & II respectively. This resulted in 2.8 folds of purification in case of inulinase I (Table 5) and 2.7 folds in case of inulinase II (Table 6) with yields of 86.4 % and 94.9 % of the original activities respectively.

Table 1. Screening of fungal species for their potentiality to produce exo-inulinase in the culture medium filtrate after 6 days of incubation.

	Strains	NRRL no.	DSMZ catalogue no. of media* & ideal incubation temp.	Inulinase activity (U ml^{-1})
Mesophiles	*Actinomucor elegans* (Eidam) Benjamin & Hesseltine	1706	190 24 ° C	3.87 ± 0.0001
	Aspergillus aculeatus Iizuka	5094	129 24 ° C	6.15 ± 0.0025
	Aspergillus candidus Link	313	129 24 ° C	6.48 ± 0.0004
	Aspergillus chevalieri Thom & Church	79	189 24 ° C	6.85 ± 0.0025
	Aspergillus clavatus Desm	5811	129 24 ° C	7.21 ± 0.0003
	Aspergillus crystallinus Kwon-Chung & Fennell	5082	129 24 ° C	7.45 ± 0.0075
	Aspergillus foetidus Thom & Raper	341	129 24 ° C	8.36 ± 0.0613
	Aspergillus nidulans (Eidam) Vuillemin	187	129 24 ° C	4.33 ± 0.0001
	Aspergillus niger van Tieghem	326	129 30 ° C	8. 19 ± 0.0108
	Aspergillus ochraceus G. Wilhelm	398	90 24 ° C	7.11 ± 0.0273
	Aspergillus rhizopodus J.N. Rai, Wadhwani & S.C. Agarwal	6136	129 24º C	6.88 ±0.0004
	Aspergillus ustus (Bainier) Thom & Church	5077	129 24 ° C	4.23 ±0.0001
	Cladobotryum (Dactylium) dendroides (Bull.) W. Gams & Hooz	2903	90 20 ° C	5.98 ±0.0
	Curvularia inaequalis (Shear) Boedijn	13884	310 24 ° C	3.65 ± 0.0025
	Curvularia lunata (Wakker) Boedijn	2380	310 24 ° C	3.23 ±0.0001
	Fusarium oxysporum f. sp. *lycopersici*	26037	129 25 ° C	4.76 ± 0.0273
	Fusarium proliferatum (Matsushima) Nirenberg	26517	129 25 ° C	4.11 ±0.0001
	Mucor racemosus Fresen	1427	90 20 ° C	3.99 ±0.0004
	Penicillium asperosporum G. Sm.	3411	90 24 ° C	7.43 ±0.0001
	penicillium capsulatum Raper & Fennell	2056	129 24 ° C	7.32 ±0.0004
	Penicillium citrinum Sopp	1841	129 24 ° C	7.88 ±0.0
	Penicillium crustosum Thom	934	129 24 ° C	6.98 ±0.0072
	Penicillium variable Sopp	1048	129 24 ° C	7.14 ±0.0001
	Rhizomucor variabilis R.Y. Zheng & G.Q. Chen	28773	129 25 ° C	4.12 ±0.0108
	Aspergillus fumigatus Fresen.♦	163	129 24 ° C	8.11 ± 0.0001
			129 37 ° C	7.96 ± 0.0009
Thermophiles	*Humicola sp.* De Bert.	3135	129 40 ºC	6.24 ±0.0036
	Rhizopus homothallicus. Hess & Ellis	2538	129 35 ° C	4.55 ±0.0388
	Rhizomucor pusillus (Lindt) Schipper	2543	129 35 ° C	4.23 ±0.0
	Talaromyces thermophilus Stolk	2155	189 40 ° C	6.87 ±0.0183
	Thielavia terrestris Malloch & Cain	8126	129 50 ° C	8.42 ±0.0001(●)
	F value			957.9133
	P-value			1.21E-67

*maintenance media (numbers in parentheses correspond to the number of the medium in the DSMZ catalogue (8) and ideal incubation temperature for strain (♦) thermotolerent fungus. The obtained data was statistically analyzed using ANOVA (Analysis of variance between groups) the greater F value and $P < 0.01$ reflect a highly significant relations between results; (●)=Highest value to which other data was statistically compared using T-Test.

Table 2. Effect of different concentration of Chicory roots extract on the Production of inulinases by *Thielavia terrestris* and *Aspergillus foetidus*.

Concentration (%)	Inulinases activity U ml^{-1}	
	Inulinase I °	Inulinase II •
2.0	8.42 ± 0.0006	8.36 ± 0.0006
3.0	10.03 ± 0.0027	9.95 ± 0.0052
4.0	11.67 ± 0.0009(●)	12.32 ± 0.0532(●)
5.0	11.62 ± 0.0112	12.30 ± 0.0675
F value	529.0743	135.6306
P-value	1.7924 E-07	1.0134E-05

° inulinase I from *Thielavia terrestris*
• inulinase II from *Aspergillus foetidus*

Table 3. The effect of different inorganic and organic nitrogen sources on inulinases production from *Thielavia terrestris* and *Aspergillus foetidus*

N sources	Inulinases activity U ml^{-1}	
	Inulinase I	Inulinase II
Control	11.67 ±0.0005(●)	12.32 ± 0.0006(●)
Asparagines	14.11 ± 0.0273	12.77 ± 0.0004
Casein	15.76 ± 0.0001	14.01 ± 0.0
Peptone	15.97 ± 0.0112	15.33 ± 0.0432
Yeast extract	17.87 ± 0.0016	14.12 ± 0.0031
$(NH_4)_2HPO_4$	12.56 ± 0.0036	12.98 ± 0.0004
$NaNo_3$	13.11 ± 0.0076	13.11 ± 0.0273
NH_4Cl	12.58 ± 0.0244	12.54 ± 0.0004
NH_4NO_3	12.05 ± 0.0025	12.39 ± 0.0031
NH_4SO_4	11.98 ± 0.0016	12.56 ± 0.0508
F value	1445.3808	201.5735
P-value	1.1596E-23	5.1972E-16

Table 4. The effect of different incubation periods on inulinases production from *Thielavia terrestris* and *Aspergillus foetidus*

Days	Inulinases activity U ml^{-1}	
	Inulinase I	Inulinase II
3	4.34± 0.0002	3.81± 0.008
4	10.71± 0.0004	8.66± 0.0013
5	18.11± 0.0049(●)	13.67± 0.0067
6	17.95± 0.0001	14.33± 0.0657
7	17.22± 0.1092	15.67± 0.0056(●)
8	17.10± 0.0075	15.23± 0.0247
F value	1154.2975	603.3838
P-value	1.1668E-15	5.6412E-14

Value in the table represents the mean of 3 reading expressed as U ml^{-1} ± standard deviation. The obtained data was statistically analyzed using ANOVA (Analysis of variance between groups) the greater F value and $P < 0.01$ reflect a highly significant relations between results; (●)=value to which other data was statistically compared using T-Test (Tables 2-4).

Table 5. Precipitation of inulinase I from *Thielavia terrestris* cell-free dialysate by ammonium sulfate and low molecular weight alcohol

Treatment			Total protein (mg)	Total activity (U)	Specific activity (U/mg^{-1} protein)	Recovery %	Purification (folds)
Cell-Free Filtrate			112.6	435	3.86	100	1.0
Cell-Free Dialysate			112.0	411	3.67	94.5	0.95
$(NH_4)_2SO_4$ Saturation		0.5	31.5	302	9.58	69.4	2.4
		0.6	33.1	311	9.39	71.4	2.4
		0.7	38.6	342	8.86	78.6	2.3
		0.8	45.3	319	7.04	73.3	1.8
		0.9	49.8	305	6.12	70.1	1.5
Low molecular weight alcohol	Iso-propanol	1:1	34.3	376	10.96	86.4	2.8
		2:1	42.5	388	9.12	89.2	2.3
		3:1	46.0	401	8.71	92.2	2.3
		4:1	52.6	390	7.41	89.7	1.9
		5:1	65.3	376	5.75	86.5	1.5
	Methanol	1:1	30.0	290	9.66	66.6	2.5
		2:1	33.1	298	9.00	68.5	2.3
		3:1	35.6	307	8.62	70.5	2.2
		4:1	40.3	244	6.05	56.1	1.5
		5:1	43.5	227	5.21	52.2	1.3

Table 6. Precipitation of inulinase II from *Aspergillus foetidus* cell-free dialysate by ammonium sulfate and low molecular weight alcohol

Treatment			Total protein (mg)	Total activity (U)	Specific activity (U/mg^{-1} protein)	Recovery %	Purification (folds)
Cell-Free Filtrate			129.3	314	2.4	100	1.0
Cell-Free Dialysate			129.0	305	2.3	97.1	0.98
$(NH_4)_2SO_4$ Saturation		0.5	32.4	166	5.12	52.8	2.1
		0.6	35.2	174	4.94	55.4	2.0
		0.7	39.3	188	4.78	35.8	1.9
		0.8	43.7	181	4.14	57.6	1.7
		0.9	48.1	165	3.43	52.5	1.4
Low molecular weight alcohol	Iso-propanol	1:1	39.1	215	5.49	68.4	2.2
		2:1	42.4	221	5.21	70.3	2.1
		3:1	45.8	232	5.06	73.8	2.1
		4:1	49.1	205	4.17	65.2	1.7
		5:1	51.4	201	3.91	64.0	1.6
	Methanol	1:1	45.6	298	6.53	94.9	2.7
		2:1	52.6	300	5.70	95.5	2.3
		3:1	56.6	266	4.69	84.7	1.9
		4:1	58.1	204	3.51	64.9	1.4
		5:1	63.2	179	2.83	57.0	1.1

A summary of purification steps of inulinases I & II was recorded in Tables 7 and 8 respectively. The precipitated enzymes were then purified by gel filtration through Sephadex G-100. In this step, inulinases I & II were purified 15.1 and 13.7 fold with yields of about 69.2 and 77.1 % and specific activities of 59.02 and 32.9 U/mg protein respectively (Tables 7 & 8). The activities were located in peak 2 for both inulinases I & II (Figs.1 & 3). The purification procedure was completed by anion exchange chromatography on DEAE-Cellulose using a linear sodium chloride gradient. The results showed that inulinase I was purified 66.0 fold obtaining a final specific activity of 254.9 U/mg protein (Table 7), the highest activity was detected in peak 3 (Fig. 2) Inulinase II was purified 42.0 fold to a specific activity of 100.9 U/mg protein (Table 8), the highest activity was detected in peak 2 (Fig. 4). Inulinases I & II were separated at the concentration of 0.4 M of NaCl.

The optimum temperature of purified inulinase I was 75 ºC and 50 ºC for inulinase II (Fig. 5). Inulinase I retained its original activity after heating up to 75ºC for 1 hr. and lost 35% of its activity at 90ºC for 15 min (Fig. 6). However, inulinase II retained its original activity at 50ºC only for 45 min. and no activity was recorded after heating the enzyme (inulinase II) at 70 ºC for 1 hr (Fig. 7).

The optimum pH for activity was found to be 4.5 and 5.5 for inulinases I & II (Figs. 8 & 9) respectively. The pH stability exhibited by the inulinase I was between 4.5-5.0, but at 5.5 pH for inulinase II. The later enzyme retained only 94.4% of its original activity.

Effect of different inulin concentrations on the enzyme activity, as indicated by the initial rate of product formation, was studied. It was found that at low inulin concentrations, the enzymatic reaction was a first-order with respect to the substrate, but at higher concentrations it became zero-order and V_{max} (maximum initial velocity) of the purified inulinases I & II were recorded at 115 and 55 µmol/min/mg of protein) respectively. From these data the apparent K_m value of inulinases I & II for inulin was calculated to be 2.15 and 3.45 mg/ml respectively. The molecular mass of the inulinases I & II were estimated to be 72 & 78 kDa respectively by using SDS-PAGE (Fig. 10).

Table 7. A Summary of treatments used for the purification of inulinase I From *Thielavia terrestris* grown on Chicory roots with yeast extract.

Treatment	Total protein (mg)	Total activity (U)	Specific activity (U/mg^{-1} protein)	Recovery %	Purification (folds)
*C.F.F. (Volume = 200 ml)	112.6	435	3.86	100	1.0
**C.F.D.	112.0	411	3.67	94.5	0.95
***C.F.P. [1:1 isopropanol]	34.3	376	10.96	86.4	2.8
Gel filtration (Sephadex G-100)	5.1	301	59.02	69.2	15.1
Ion-exchange chromatography DEAE-Cellulose	1.02	260	254.9	59.8	66.0

* C.F.F. - Cell-Free Filtrate.
** C.F.D. - Cell-Free Dialysate.
*** C.F.P. - Cell-Free Precipitate

Table 8. A Summary of treatments used for the purification of inulinase II From *Aspergillus foetidus* grown on Chicory roots with peptone.

Treatment	Total protein (mg)	Total activity (U)	Specific activity (U/mg^{-1} protein)	Recovery %	Purification (folds)
*C.F.F. (Volume = 200 ml)	129.3	314	2.4	100	1.0
**C.F.D.	129.0	305	2.3	97.1	0.98
***C.F.P. [1:1 methanol]	45.61	298	6.5	94.9	2.7
Gel filtration (Sephadex G-100)	7.36	242	32.9	77.1	13.7
Ion-exchange chromatography DEAE-Cellulose	2.11	213	100.9	67.8	42.0

* C.F.F. - Cell-Free Filtrate.
** C.F.D. - Cell-Free Dialysate.
*** C.F.P. - Cell-Free Precipitate.

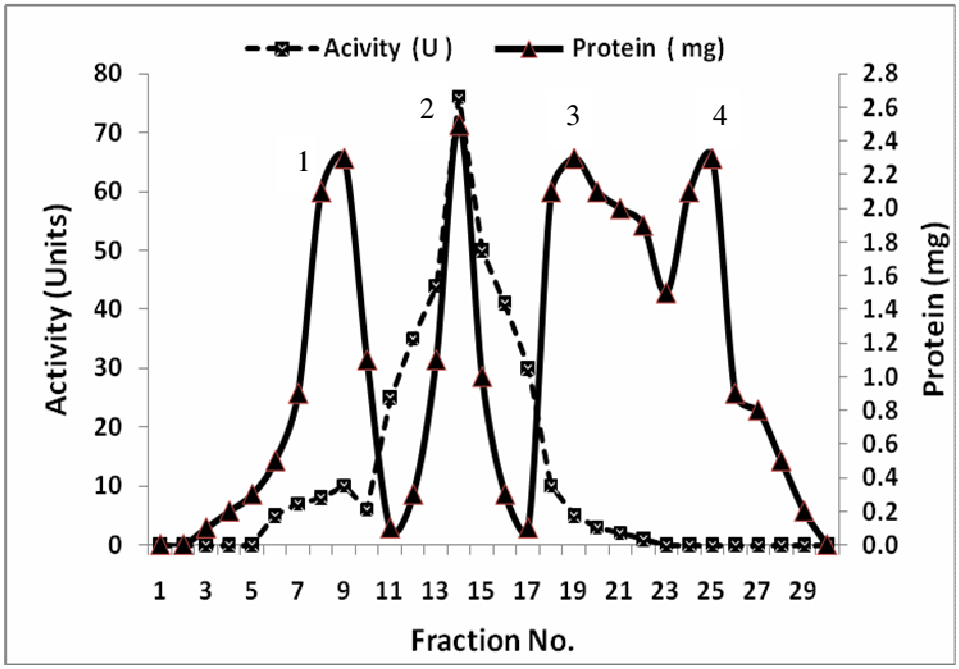

Figure 1. Purification of Inulinase I from *Thielavia terrestris* using gel filtration on Sephadex G-100

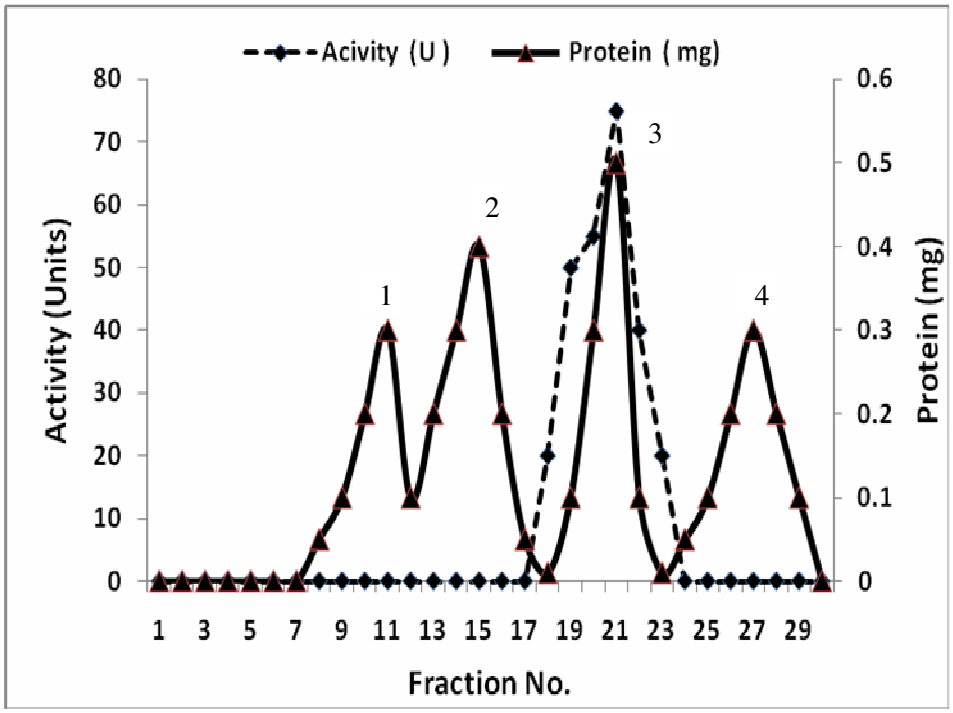

Figure 2. Purification of Inulinase I from *Thielavia terrestris* using ion exchange on DEAE-Cellulose.

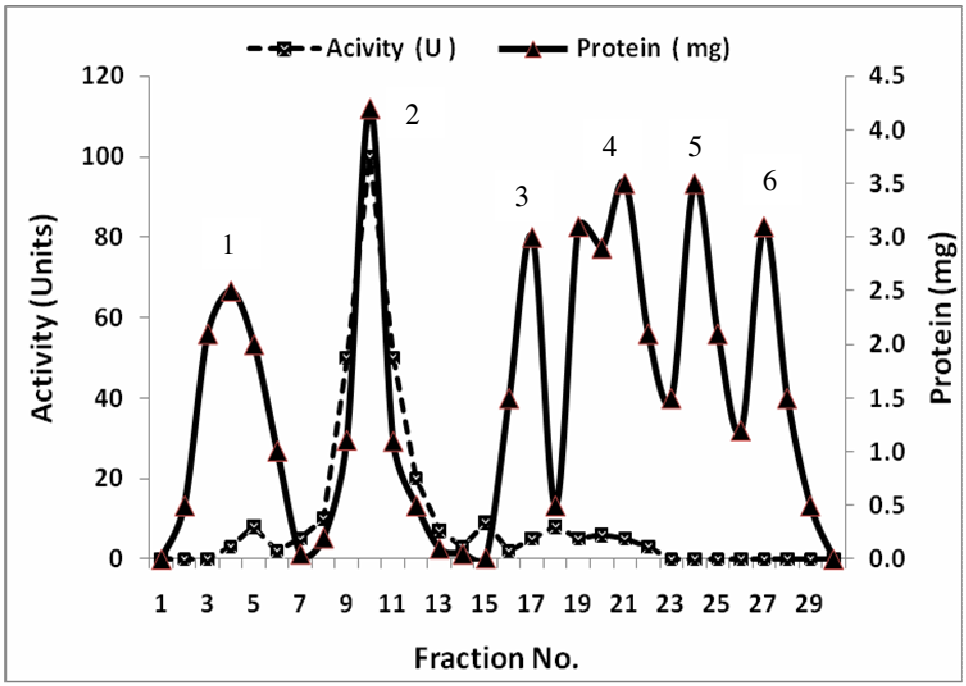

Figure 3. Purification of Inulinase II from *Aspergillus foetidus* using gel filtration on Sephadex G-100.

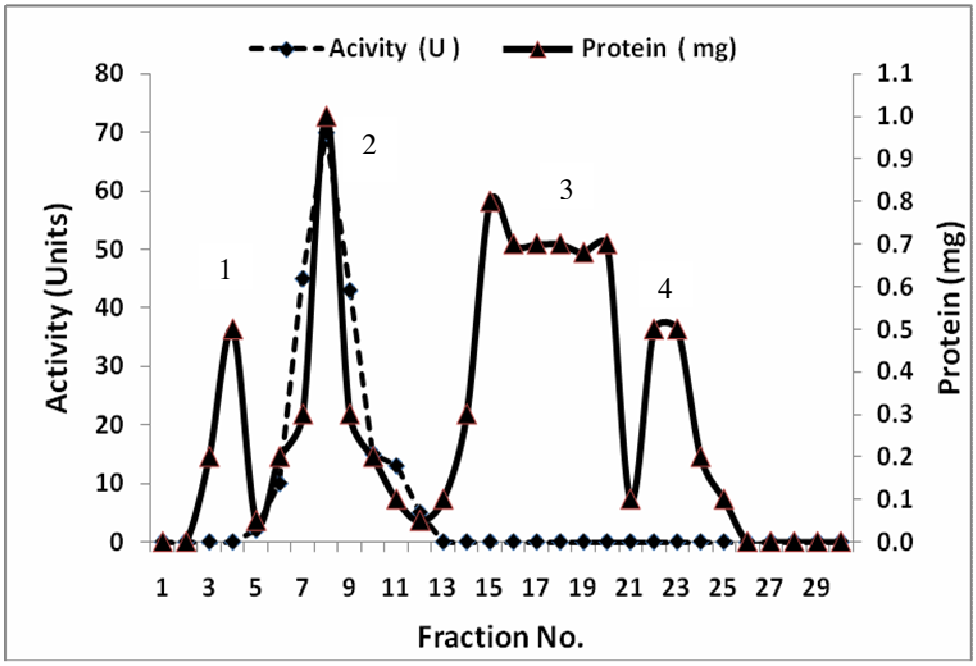

Figure 4. Purification of Inulinase II from *Aspergillus foetidus* using ion exchange on DEAE-Cellulose.

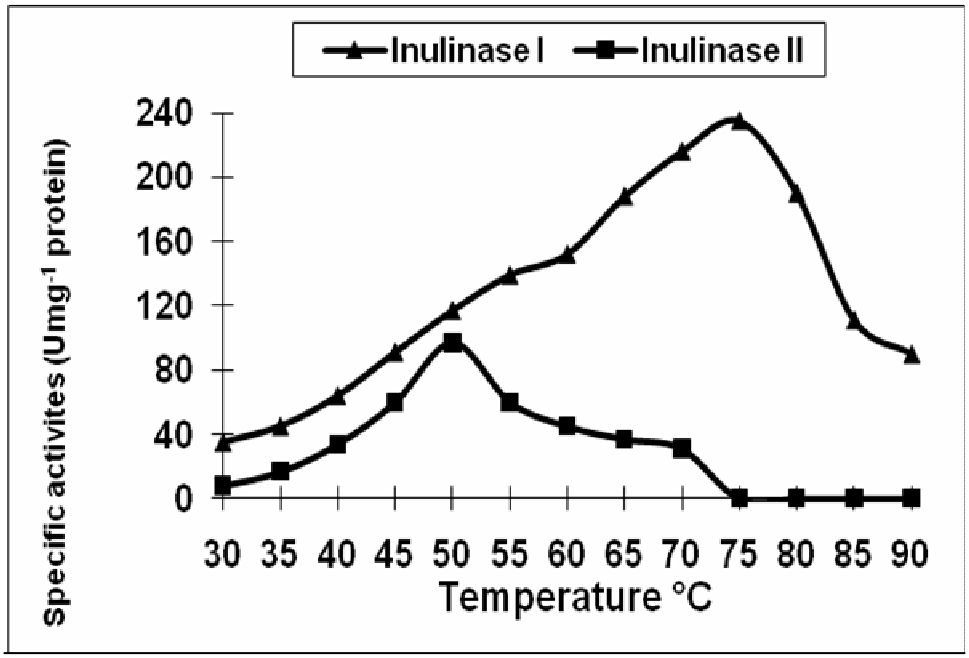

Figure 5. The effect of different temperature on the specific activities of inulinase I from *Thielavia terrestris* and inulinase II from *Aspergillus foetidus.*

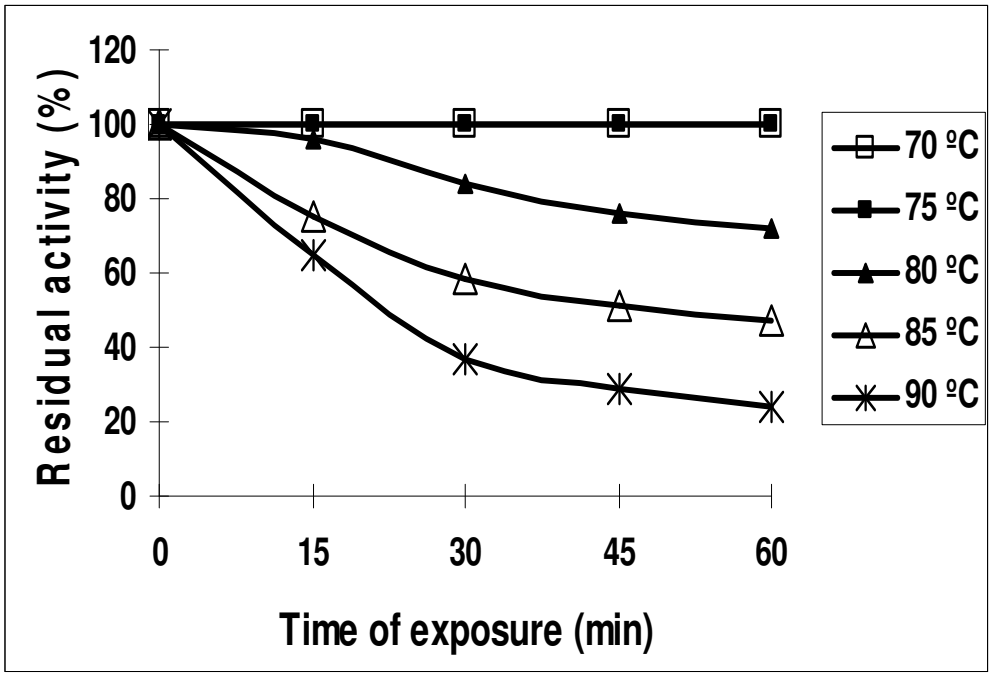

Figure 6. Thermal stability of the purified inulinase I from *Thielavia terrestris*

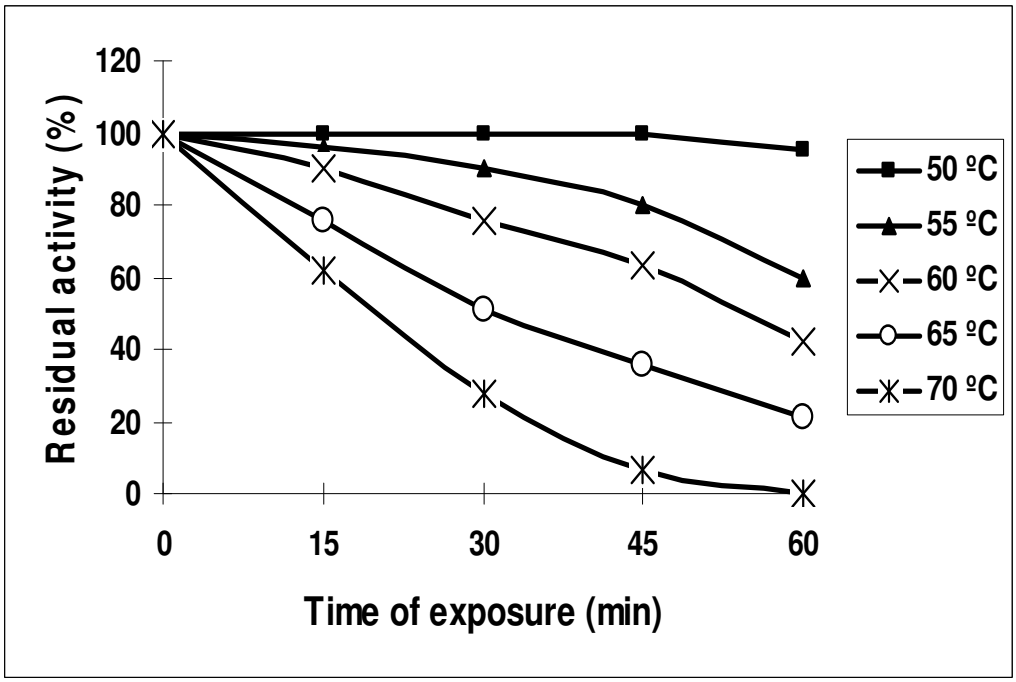

Figure 7. Thermal stability of the purified inulinase II from *Aspergillus foetidus*

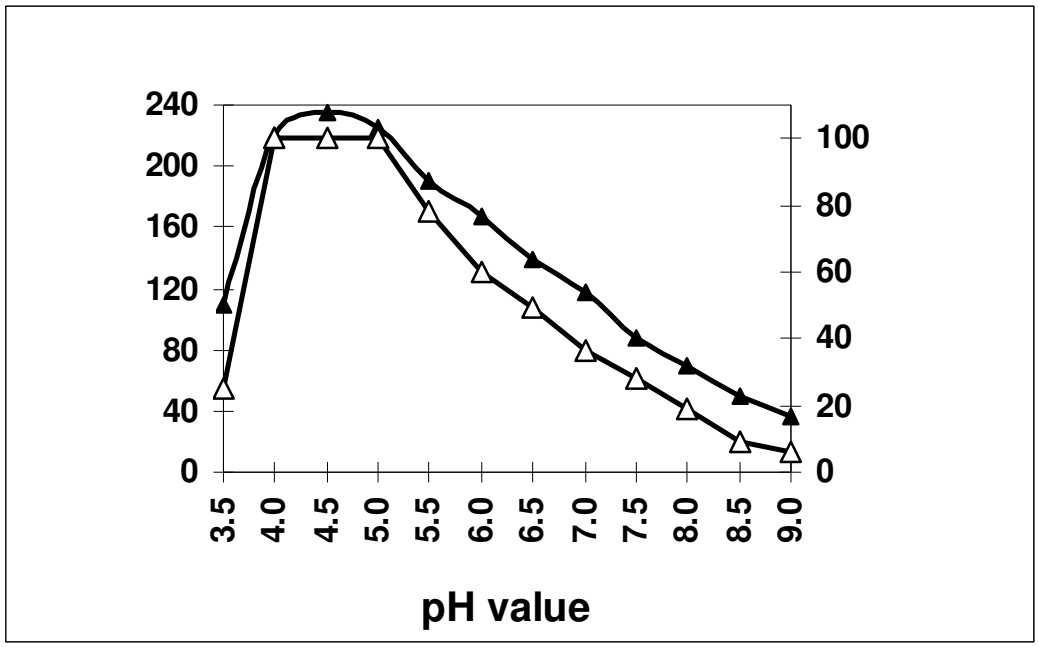

Figure 8. Effect of pH on the specific activity (-▲-) and stability (-Δ-) of the purified inulinase I from *Thielavia terrestris*. The enzyme preparation was held at the indicated pH for 20 min. for measuring the activity and for 1 hr. in case of stability.

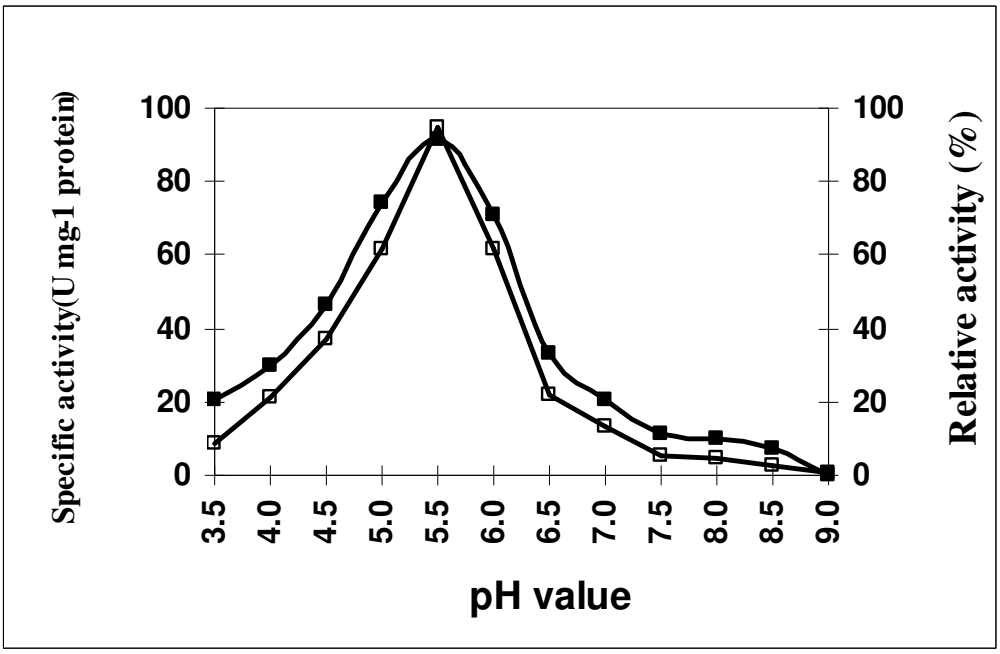

Figure 9. Effect of pH on the specific activity (-■-) and stability (-□-) of the purified inulinase II from *Aspergillus foetidus*. The enzyme preparation was held at the indicated pH for 20 min. for measuring the activity and for 1 hr. in case of stability.

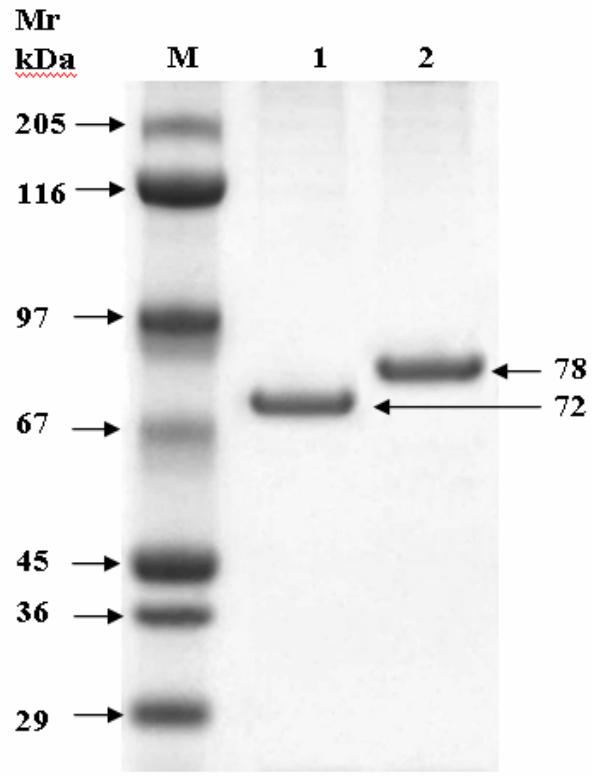

Figure 10. SDS-PAGE of the two purified Inulinase I & II eluted from DEAE-Cellulose column (Inulinase I from *Thielavia terrestris* = 72 kDa; Inulinase II from *Aspergillus foetidus* = 78 kDa).

DISCUSSION

The thermophile *Thielavia terrestris* NRRL 8126 and mesophile *Aspergillus foetidus* NRRL 337 were selected for the present study after screening 30 fungal species (24 mesophiles, 1 thermotolerent and 5 thermophiles) for their capacity to synthesis extracellular inulinases (8.42 and 8.36 U/ml respectively) after being grown on basal growth medium containing Chicory root extract 2% (w/v) as a sole carbon source (Table 1). However, in a previous study, *Aspergillus niveus* and *Penicillum purpurogenum* when grown on chicory root gave the highest inulinase activity at 7.0 and 9.00 U/ml respectively (26). The selection of the aforementioned two fungi aimed at throwing some light and comparing between the production and purification of inulinase from the two different fungi. *T. terrestris* and *A. foetidus* have an ideal temperature for growth of 50ºC and 24ºC respectively (8).

In this work, some experiments were performed to evaluate the effect of different concentrations of Chicory root extract, different organic & inorganic sources and different incubation periods on inulinases activity. The results were given in Tables 2, 3 & 4 respectively. The best concentration of chicory root extract in cultivation media for elevating the two inulinases activity was 4% w/v (Table 2). In this respect, other authors reported different optimum concentrations of composite plants extracts: 3% for Jerusalem artichoke (2, 12) and 5% for sunflower (11) as carbon sources for inulinases production. These different concentrations varied according to the type of plants (38, 39, 44, 45). Yeast extract and peptone showed superiority over inorganic nitrogen sources for the production of inulinases I & II from *T. terrestris* and *A. foetidus* respectively (Table 3), these results were also reported two authors (11, 12). It appears that each fungal species has a preference for a specific nitrogen source for optimum production of inulinase (6). Inulinase I from the thermophile *T. terrestris* achieved its higher activity after 5 days of incubation, while 7 days was the ideal incubation for inulinase II productivity from *A. foetidus* (Table 4). This finding was in accordance with previous authors (1, 9, 10, 16) who showed that thermophiles in general, possess a more faster rate of enzyme production than mesophiles.

Concerning the purification steps, the superiority of isopropanol and methanol in obtaining protein fraction having the highest total enzyme activity confirmed that inulinase has a particular structure which makes it resist the known denaturing effect of organic solvents (Tables 5, 6). Suitability of organic solvents in this regard was previously recorded by several authors (18, 48, 49).

Concerning the effect of temperature on the two inulinases activities (Fig 5), inulinase I was highly optimal at 75ºC, showing complete stability for one hour at the aforementioned

degree than inulinase II which was optimal at only 50ºC. Inulinase I also showed high stability up to 85ºC for 15 min. (Fig 6). This result is in agreement to a certain extent with the results obtained for other inulinases from *Aspergillus ficuum* (9), and *Aspergillus fumigatus* (16). Inulinase II showed less stability (Fig. 7) when compared with inulinase I from the thermophile *T. terrestris*. Thus, the higher thermostability of inulinase I makes it a potential candidate for commercial production of fructose (46).

The optimum pH lies in acidic range (4.5 & 5.5) for activity of inulinases I & II (Fig. 8 & 9), this pH range was identical to that recorded for inulinase (4.5) from *A. niger*-245 (7), 5.0 from *A. niger*, (50), and *P. janczewskii*, 5.0 (32, 33). 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. It is also clear that inulinase I was more stable than inulinase II where the original activity were not affected at 4.5 -5.0 pH for 60 min. while inulinase II retained only 94.4% of its original activity at its optimum pH value (5.5).

K_m value was calculated to be 2.15 and 3.45 mg/ml for inulinases I & II respectively. K_m values of inulinase of *Aspergillus versicolor* (19) and *Penicillium janczewskii* (33) were recorded at 3.8 and 4.4 mg/ml respectively. Moreover, comparatively lower k_m value (2.15 mg/ml) and higher maximum initial velocity (115 µmol/min/mg of protein) for inulinase I demonstrate the exoinulinase's greater affinity for inulin substrate. These findings are significant for its potential industrial application (31). The lower molecular mass (Fig. 10) of the inulinase I (72 kDa) than inulinase II (78 kDa) often evaluate the relative affinities of this enzyme (40).

Finally, thermophiles and especially thermophilic enzymes have gained a great deal of interest both as analytical tools, and as biocatalysts for application on a large scale. Utilization of these enzymes is up to our present day, despite many efforts, often limited by the cost of the enzymes (46). With an increasing market for the enzymes, leading to production in higher volumes, the cost is however predicted to decrease. Moreover, with a paradigm shift in industry moving from fossil fuels towards renewable resource utilization, the need of microbial catalysts is predicted to increase, and certainly there will be a continued and increased need of thermostable selective biocatalysts in the future.

CONCLUSION

Inulinases are promising candidates for use as complements in food ingredients and in the production of fermenting sugars. However, their utilization was limited due to the high cost of their production. Chicory is a very common wild plant in Egypt and so can be used as a cheap substrate for the commercial production of inulinase. The abundance of inulinases production by *Thielavia terrestris* and *Aspergillus foetidus* and the remarkable higher thermostability of inulinase I from *T. terrestris* can have a positive effect on enzyme industry.

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