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Some Properties of Inulinase from *Aspergillus fumigatus*

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Abstract: A local isolate identified as *Aspergillus fumigatus* was tested for inulinase and invertase production. Different carbon sources alone or in combination with inulin were tested. About 91% inulinase was found as extracellular enzyme in culture medium with inulin and wheat bran as sole C-source (1% for each). The activity towards sucrose (invertase enzyme) was detected only in the supernatant and represents about 26% from the inulinase activity. The production of both enzymes was inhibited with ammonical compounds as nitrogen source, whereas the maximum level (3.72, 0.35 U/ml for inulinase and invertase respectively) was observed when 0.38% KNO_3 was used as nitrogen source. Beef extract, yeast extract, peptone and tryptone reduced the amount of inulinase by about 83-91%. Both enzymes were produced in a broad pH range between 4.0-8.0 as initial pH. Optimum temperature was found to be 30°C for inulinase and a dramatic reduction was observed above 35°C, while 40°C was observed for invertase. The properties of crude inulinase were also studied. The enzyme has optimum pH 5.5 in acetate buffer and optimum temperature 45°C. Fructose at concentrations higher than 0.2mg/reaction mixture inhibited the enzyme activity. The enzyme was stable for 30 min at 30°C and 40°C in pH 5.5 and for 24h at 4°C in pH 6 - 6.5. Addition of 10% glycerol protected the enzyme from deactivation at 45°C and 50°C for 30 min. The enzyme was completely inhibited by 1mM Ag^+ and Hg^{2+} and activated by the presence of 1mM Ca^{2+} or Mn^{2+}.

Key words: Inulinase, *Aspergillus fumigatus*, invertase, wheat bran, and inulin

Introduction

Inulin accumulates as a reserve polymer in several interesting agricultural crops such as Jerusalem artichoke, chicory, dahlia and several others. Inulin represents a source for the production of ultra-high-fructose syrups by enzymatic hydrolysis. D-fructose is occupying an increasingly important position in the modern world as a sweetener because it offers several advantages, such as its natural occurrence both as a free sugar in the majority of fruits and as a polymer in several plants, its higher sweetening value, its physiological metabolism in the human body, and its insignificant insulinogenic effects (Pandey *et al.*, 1999b).

Sucrose has been commonly used as a sweetener in the food industry for a long time. However, because of its medicinal and nutritional properties, D-fructose has gained popularity in the food and beverage industries. This sugar has emerged as a safe alternative sweetener to sucrose, which causes problems related to carpulence, cariogenicity and diabetes (Vandamme & Derycke, 1983). Chemical hydrolysis of inulin was used originally but proved to be more costly and gives more undesirable degradations and by-products (such as difructose anhydride) than enzymatic hydrolysis (Vandamme & Derycke, 1983 and Toran-Diaz *et al.*, 1985).

Enzymes that degrade inulin were first isolated and characterized in plants (Rutherford & Deacon, 1972). Inulinase (EC. 3.2.1.7) and invertase (EC. 3.2.1.26) both catalyze the hydrolysis of inulin and sucrose but the inulinase enzyme has a higher specificity for inulin than invertase. Unlike plant enzymes, microbial inulinase showed high sucrose hydrolyzing activity. Most of them were more active on sucrose than inulin (Ettalibi & Baratti, 1990).

In industry the bulk sucrose hydrolysis for obtaining the inverted sugar syrup is carried out at 75°C in the presence of HCl. The use of invertase, constitutes an alternative to acid hydrolysis, because the enzyme acts under mild pH and temperature.

Microbial inulinase are usually inducible and exoacting enzymes, which catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose) (Vandamme & Derycke, 1983). These enzymes have been reported in several microorganisms (Negoro, 1978 and Xiao *et al.*, 1988). The present work reports on the production of inulinase and invertase enzymes by *Aspergillus fumigatus* strain.

Materials and Methods

Microorganism: *Aspergillus fumigatus* was locally isolated from the flax retting water, Linen company, Tanta, Egypt. It was identified by the "Central–Bureau voor Schimmel Cultures, Baarn/Holland". The fungus was maintained on glucose - peptone slants and stored at 4°C.

Cultivation: The strain was cultivated in 50 ml of liquid medium in 250 ml flasks. The medium contained (g/l) 10 inulin, 2 $NaNO_3$, 0.5 Mg $SO_4.7H_2O$, 0.5 KCl, 0.5 KH_2PO_4, 0.01 $FeSO_4.7H_2O$ and 0.2 yeast extract. The initial pH was adjusted to 6.0. The medium was autoclaved at 121°C for 20 min without inulin. Inulin was sterilized by filtration and then added to autoclaved medium. All experiments were done under static conditions for 9 days at 30°C (except the effect of temperature).

Effect of carbon and nitrogen sources on enzyme production: Twelve carbon sources (inulin, fructose, sucrose, sugarcane molasses, wheat bran, pectin, glucose, lactose, xylan, maltose, starch and xylose) were tested as substrates for the production of inulinase and invertase enzymes at 1% (w/v).

To examine the effect of nitrogen sources on inulinase production, sodium nitrate and yeast extract, found in the medium were replaced on equal nitrogen basis by beef extract, yeast extract, peptone and tryptone (organic nitrogen) and ammonium sulfate, ammonium dihydrogen phosphate, ammonium chloride, ammonium nitrate, sodium nitrate and potassium nitrate (inorganic nitrogen).

Effect of medium pH on enzymes production: The initial pH of culture medium was adjusted to different values with 1N HCl or NaOH solution. The medium was inoculated and incubated under test conditions.

Effect of incubation temperature on enzymes production: The optimized medium was inoculated with the fungus under test and incubated at different temperatures ranged from 20 to 45°C.

Crude enzyme preparation: Three flasks were taken at regular intervals and filtered to remove mycelia. The supernatants were used as the source of crude extracellular inulinase and invertase enzymes. The mycelia were washed with chilled water and extracted in a mortar with 0.1M acetate buffer (pH 5.6) in the presence of two times acid-washed sand. The extracts were then filtered and centrifuged at 5000 rpm in cold centrifuge for 20 min. The supernatant was assayed for intracellular inulinase and invertase enzyme.

Enzyme assays: To assay inulinase activity, a mixture of 0.1 ml of 1.0% inulin in 0.1M sodium acetate buffer (pH 5.6) and 1 ml of diluted crude enzyme was incubated for 15 min at 45°C. Fructose release was estimated by 3,5-dinitrosalicylic acid method (Miller, 1959). One unit of inulinase activity was defined as the micromole of fructose per min under the assay conditions.

The invertase activity was also assayed according to Miller (1959). The reaction mixture and conditions were similar as in inulinase

activity, but sucrose was used as substrate. One invertase unit was defined as the amount of enzyme which formed one micromole of reducing sugar per min under the assay conditions.

Protein determination: Protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard. All the results obtained in this work are the average of three sets of the experiments.

Effect of pH and temperature on inulinase activity: In order to choose the best buffer for enzyme activity, acetate, citrate-phosphate and phosphate buffer (0.1M, pH 5.6) were tested. To determine the optimum pH, the enzyme activity was measured in 0.1M acetate buffer (pH 3.0 – 6.0) and 0.1M phosphate buffer (pH 5.0–7.5).The effect of temperature was determined by performing the reaction at a temperature range of 20 to 70°C.

Effect of substrate concentration: The enzyme was incubated with different inulin concentration (0.1 – 2%) and the activity was measured under the standard assay conditions.

pH and temperature stability of inulinase: The effect of pH on the stability of inulinase activity was evaluated by incubating the enzyme in acetate buffer pH (5.0-6.5) and in phosphate buffer pH (7.0-7.5) for 24h at 4°C and for 4h at 30°C. To test the stability at different temperatures, the filtrate was incubated without substrate for 15 and 30 min at pH 5.5 and the residual activity was determined.

Inhibition of inulinase activity by fructose: Different fructose concentrations range (0.02-0.3 mg) was added to the reaction mixture and the activity was estimated under the assay conditions.

Effect of some metal ions: The effect of different ions was determined by incubating the enzyme for 15 min at 45°C in 1mM of the metals solutions, then the residual activity was determined under the standard assay conditions.

Results and Discussion

Enzyme production in shaken and static cultures: The organism was cultivated in submerged culture and incubated under shaken and static conditions at 30°C. After different intervals, samples were withdrawn for enzymes assays. The maximum inulinase activity for both extracellular and cellular was obtained after 4 days in shaken conditions and after 9 days in static conditions (about 11% only was found as cellular enzyme) (Table 1).
The assay of reducing sugars in the culture filtrates in both experiments indicate the presence of high sugar concentration after 2 days (data not shown). With increasing the incubation time, the sugars disappeared faster in shaken than in static conditions. It may be concluded that the enzyme was inhibited by the presence of high concentration of reducing sugars. This finding may explain why the enzyme reached its maximum value after 9 days in static condition, when the sugars were utilized by the organism. Vandamme & Derycke (1983) reported that the presence of glucose or fructose repressed the inulinase formation. Maximum extracellular invertase enzyme was reached after 7 days in both conditions, while the cellular was not significant. Although the maximum inulinase enzyme was obtained after 4 days in shaken and after 9 days in static condition, the static condition was chosen for the rest experiments, because the amount of enzyme in static was 5.5 times more than in shaken. The protein content ranged from 0.01 – 0.03 mg/ml in static condition and from 0.05 – 0.07 mg/ml in shaken condition.

Effect of carbon sources: Among the twelve tested carbon sources, only six gave positive results (Table 2). Inulin was found to be the best C-source for inulinase production (0.85 and 0.10 U/ml for extracellular and cellular enzyme respectively), while wheat bran and sugar cane molasses were the best C-sources for extracellular invertase (0.23 and 0.16 U/ml respectively). Fructose, sucrose, sugar cane molasses, wheat bran and pectin were also found as fair C-sources for inulinase production. When 1% inulin was replaced by 1% glucose, lactose, xylan, maltose, starch or xylose, no significant inulinase or invertase was detected (data not shown).
The combination between inulin and fructose, sucrose, sugar cane molasses or pectin gave no stimulating effect on inulinase production and only the addition of 1% wheat bran to inulin (1%) increased the extracellular inulinase 1.5 times in comparison with that when inulin was used alone (Fig. 1). In case of invertase, the combination between inulin and sugar cane molasses or wheat bran increased the enzyme production by about 4.4 times in comparison with that when inulin was used as the sole carbon source in the medium. These results may be due to the fact that wheat bran and sugar cane molasses contain some growth factors. The protein content of the supernatant was increased 2-times (0.06 mg/ml) by the addition of wheat bran to inulin. Snyder and Phaff (1960) examined the effect of different carbon sources on extracellular inulinase produced by *Kluyveromyces fragilis* . Highest enzyme yields were obtained with inulin (0.3U/ml), medium level was observed with fructose (0.1U/ml) and only very low level with sucrose as carbon source. Many other investigators have also observed higher inulinase activity with medium containing inulin as the sole carbon source (Xiao *et al.*, 1988, Yokota *et al.*, 1991 and Pandey *et al.*, 1999a). Negoro and Kito (1973) and Nakamura *et al.* (1978) also concluded that inulin allowed much better inulinase production than any other carbon source. Pandey *et al.* (1999a) tested the combination of inulin with different C-sources on inulinase production and concluded that none of the tested combinations showed any stimulating effect on enzyme production in comparison with inulin as the sole C-source. Efstathiou *et al.* (1986), on the other hand, observed that the combination of inulin with fructose, xylose, glucose or sucrose decreased the amount of the produced enzyme. The findings in this publication are similar.
The effect of inulin concentration in medium containing 1% wheat bran was tested. There was a marginal increase in enzyme production with 1.5% inulin concentration in comparison with 1%, higher concentrations were apparently not useful (Fig. 2). With 3% inulin, the rate of enzyme production was slower by about 18% than that with 0.5% inulin. Similar results were obtained by Pandey *et al.* (1999a).

Effect of nitrogen sources: All the organic nitrogenous compounds reduced the amount of the enzyme produced (Table 3). A reduction of 83% was obtained with beef extract as nitrogen source and about 89 – 91% reduction was observed when tryptone, yeast extract or peptone were used as nitrogen sources. These results are in contradiction with the results obtained by many authors. Snyder and Phaff (1960) found that yeast extract performed better than yeast nitrogen base on inulinase produced by *Kluyveromyces fragilis*. Kim (1975) tested a wide range of organic nitrogen sources as to their effectiveness for inulinase production by *Penicillium* sp1. and observed that peptone and corn steep liquor stimulated enzyme production whereas urea and yeast extract had less influence.
The best results in this work for both inulinase and invertase were obtained when sodium or potassium nitrate was used as nitrogen source. These results are in agreement with the findings of Gupta *et al.* (1990) who found that maximum inulinase was observed with $NaNO_3$ after 9 days of fungal growth. On the other hand, best result was obtained with $(NH_4)_2SO_4$ as nitrogen source (Derycke and Vandamme 1984). All compounds with ammonical nitrogen used in this work showed inhibitory effect on enzymes production (data not shown). It appears that each fungal species has a preference for a specific nitrogen source for optimum production of inulinase (Gupta *et al.*, 1990).
In order to optimize the production of enzyme, different KNO_3 concentrations were tested. The results obtained showed that 0.38% KNO_3 gave the maximum enzyme production. Increasing the amount of nitrogen source above this value, decreased the amount of enzyme produced. Highest inulinase activity recorded was obtained in medium with 10 g/l inulin and 5g/l yeast extract (Yokota *et al.*, 1991). They also tested different meat extract concentrations on enzyme production and found that the enzyme activity declined with increasing concentration of meat extract.

Table 1: Production of inulinase and invertase under shaked and static conditions.

Time (days)	Shaked (U/ml)			Static (U/ml)		
	Extracellular inulinase	Cellular inulinase	Extracellular invertase	Extracellular inulinase	Cellular inulinase	Extracellular invertase
2	0.09	0.000	0.000	0.00	0.000	0.000
4	0.16	0.025	0.046	0.21	0.063	0.000
7	0.12	0.017	0.290	0.46	0.070	0.090
9	0.08	0.007	0.230	0.87	0.100	0.063
11	-	-	-	0.62	0.08	0.032

- Not determined

Table 2: Effect of carbon sources on enzymes production under static conditions

C- sources (1%)	Enzyme		
	Inulinase (U/ml)		Invertase (U/ml)
	Extracellular	Cellular	Extracellular
Inulin	0.85	0.10	0.08
Fructose	0.33	0.10	0.01
Sucrose	0.21	0.05	0.03
Sugar cane molasses	0.35	0.00	0.16
Wheat bran	0.34	0.00	0.23
Pectin	0.23	0.00	0.08

Incubation time: 9 days

Table 3: Effect of nitrogen sources on enzymes production

Nitrogen sources	Enzyme		
	Inulinase (U/ml)		Invertase (U/ml)
	Extracellular	Cellular	Extracellular
Yeast extract +$NaNO_3$*	0.85	0.10	0.08
Beef extract	0.41	0.10	0.06
Yeast extract	0.21	0.00	0.09
Peptone	0.20	0.00	0.11
Tryptone	0.27	0.00	0.00
$NaNO_3$	2.21	0.26	0.32
KNO_3	2.42	0.30	0.41

* The control

Table 4: pH stability of crude inulinase

pH	Relative activity (%)	
	4°C*	30°C**
5.0	76.44	54.17
5.5	78.05	70.53
6.0	100.00	100.00
6.5	100.00	100.00
7.0	73.17	65.85
7.5	42.17	32.66

* The enzyme was preincubated for 24h at 4°C
** The enzyme was preincubated for 4h at 30°C

Table 5: Effect of some metals ions on the activity of crude inulinase

Metal ions (1mM)	Relative activity* (%)
Control	100.00
Na^{+}	83.14
Ca^{2+}	145.92
Mn^{2+}	118.88
Ag^{+}	0.00
Hg^{2+}	0.00
EDTA	6.15
Cu^{2+}	53.34
Zn^{2+}	85.47

* The enzyme was preincubated with the metal solutions in acetate buffer pH (5.5) for 15 min at 45°C and the residual activity was measured as % from the control.

The same results were also obtained with invertase enzyme.

Effect of medium pH: The pH of the medium changed with the growth of fungus and tended to reach a pH in range of 7.0 – 8.8 as final pH. The organism produced both enzymes in a broad pH range between 4.0–8.0 as initial pH and there is no great difference in the amount of enzyme with medium pH . The maximum was obtained at initial pH 6.0 (3.81 and 0.45 U/ml for inulinase and invertase respectively). Shake flask fermentation of *Penicillium* sp.1 in unbuffered medium gave highest yields at pH 5.0 (Kim, 1975). Nakamura *et al.* (1997) observed higher inulinase production by *Penicillium* sp. TN88 when the pH of fermentation medium was highly acidic (3.0 – 4.0). Maximum yield was obtained after 9 days with *Fusarium oxysporum* in a medium having an initial pH of 5.5 (Gupta *et al.*, 1990).

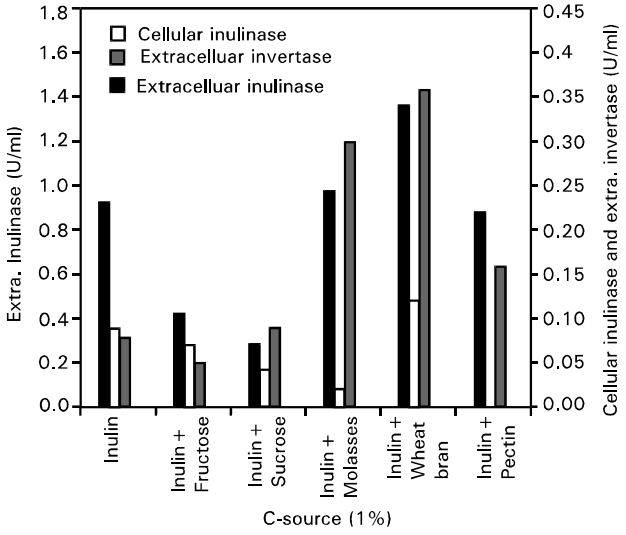

Fig. 1: Effect of combination between inulin and some C-sources on enzymes production

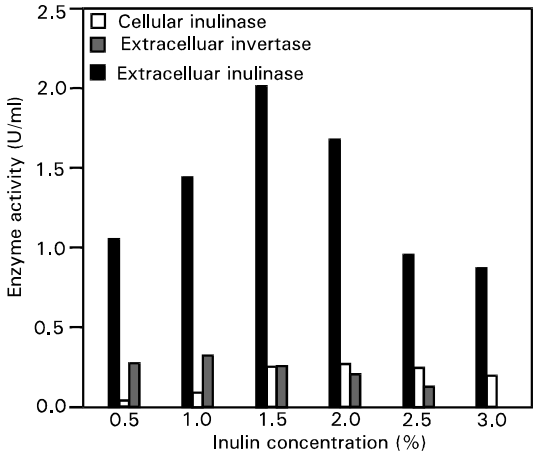

Fig. 2: Effect of inulin concentration on enzymes production

Effect of incubation temperature: Optimal temperature for inulinase enzyme was found to be 30°C. Higher temperatures resulted in a dramatic decrease in the amount of enzyme, especially the cellular enzyme (Fig. 3). On the other hand, maximum extracellular invertase was obtained at higher

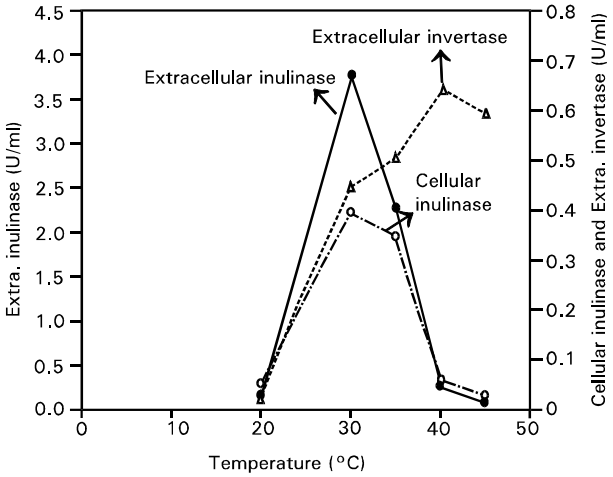

Fig. 3: Effect of incubation temperature on enzymes production

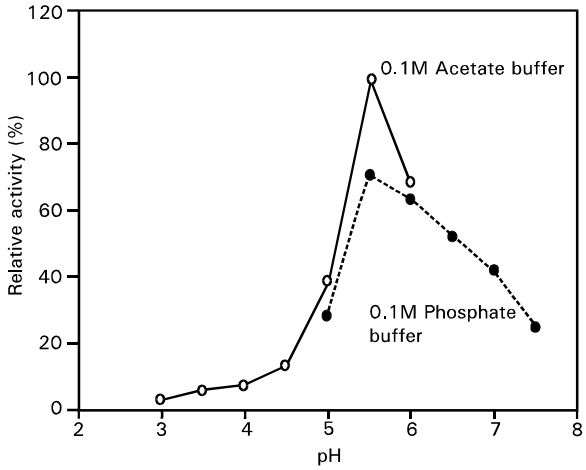

Fig. 4: Effect of pH on crude inulinase activity

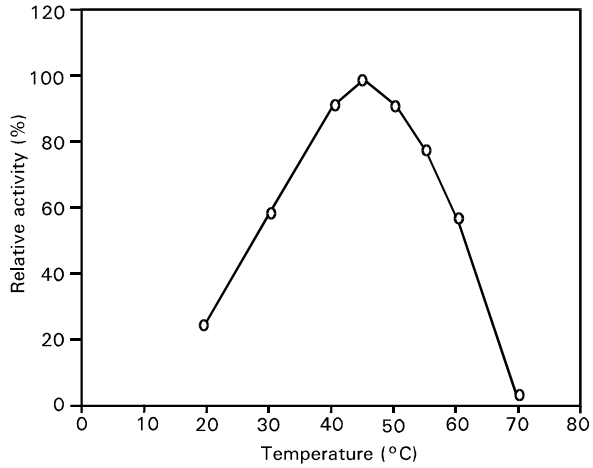

Fig. 5: Effect of temperature on activity of crude inulinase

temperature between 40 - 45°C. Optimal activity was obtained at 30 - 33°C with *Penicillium* sp.1 (Kim, 1975). Xiao *et al.* (1988) found that maximum inulinase was obtained at 22°C and there was no activity at 32°C. With *Kluyveromyces fragilis*, high activity

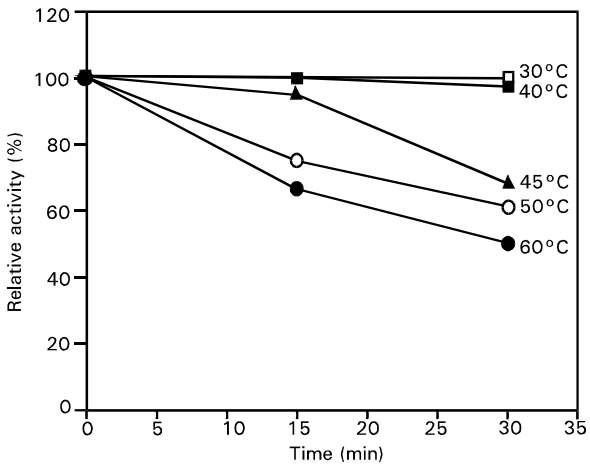

Fig. 6: Temperature stability of crude inulinase

was observed at 30 - 34°C (Grootwassink and Fleming, 1980).

Properties of crude inulinase: Some properties of the crude extracellular inulinase were investigated.

Effect of pH and temperature on the activity: The best results were obtained with acetate buffer. A reduction of 86 and 45% were observed with citrate-phosphate and phosphate buffer respectively. Maximal activity was observed at pH 5.5 in acetate buffer (Fig. 4). Xiao *et al.* (1988) and Yokota *et al.* (1991) obtained the same results with inulinase from *Chrysosporium pannorum* and *Arthrobacter* sp. H65-7 respectively. This low pH value is advantageous for industrial preparation of sugar syrups because of reduced color formation at low pH values (Vandamme and Derycke, 1983). Many other authors observed that inulinase enzyme have an optimum pH in the range of 4.5-5.0 (Snyder and Phaff, 1960; Negoro and Kito, 1973; Nakamura and Nakatsu, 1977 and Zittan, 1981). The effect
of temperature on the activity was measured as described under materials and methods. From the results obtained maximal activity was observed at 45°C (Fig. 5). Similar results were obtained for *Penicillium* sp. 1 (Nakamura and Nakatsu, 1977) and for *Aspergillus niger* 12 (Nakamura *et al.*, 1978). A reduction of 97% was obtained at 70°C but only 42% reduction occurred at 60°C.

Effect of substrate (inulin) concentration: Maximum activity was observed at inulin concentration of 1.2% (2.4 mM). The K_m value of the enzyme was estimated to be 2.2 mM, assuming that the molecular weight of inulin is 5,000. This result was approximately near the K_m obtained for *Aspergillus niger* (Nakamura *et al.*, 1978). Other K_m values ranged from 0.17 to 17 were reported for different inulinase (Vandamme and Derycke, 1983).

pH and temperature stability: From the data shown in Table 4, the enzyme was stable at pH 6.0 and 6.5. A reduction of 22% and 27% occurred after 24h at 4°C (pH 5.5 and 7.0 respectively) in comparison with 30 and 35% reduction at the same pH after 4h at 30°C. The enzyme was stable for 30 min at 30 and 40°C (Fig. 6). At higher temperatures the enzyme lost about 39 and 50% activity after 30min at 50 and 60°C respectively. In presence of 10% glycerol at 45°C the enzyme retained its full activity for 30 min, whereas in the absence of glycerol only 69% of the original activity remained after the same time (data not shown). Gupta *et al.* (1990) found that 82% of the enzyme activity was obtained in presence of 10% glycerol at 45°C, whereas only 49% was observed in the absence of glycerol. Inulinase from *Clostridium acetobutylicum* lost its activity completely when heated for 20 min at 55°C (Efstathiou *et al.*, 1986). Nakamura *et*

al. (1978) described that 25% reduction occurred when *Aspergillus niger* inulinase was treated for 30 min at 50°C.

Inhibition by fructose as end product: No inhibition was observed at concentrations lower than 0.2mg/reaction mixture. A reduction of 53% was observed with fructose concentration of 0.25mg/reaction mixture. Total inhibition was obtained for inulinase from *Bacillus subtilis* at 0.25 mg fructose (Vullo *et al.*, 1991).

Effect of some metal ions: Complete inhibition occurred with Ag^+ and Hg^{2+} and only 6% of the activity was obtained in presence of 1mM EDTA. Ca^{2+} and Mn^{2+} were activating the enzyme by 46 and 19% respectively (Table 5). The strong effect observed with Hg^{2+} or Ag^+ suggested that some SH groups in the proteins were essential for the activities. Ettalibi and Baratti (1990) obtained similar results for Hg^{2+}, Ag^+ and Ca^{2+}. Nakamura and Nakatsu (1977) reported the activation of inulinase by Mn^{2+}. In this study, *Aspergillus fumigatus* was tested for the ability to degrade inulin into a valuable monosaccharide (fructose).

It can be concluded that the best conditions for inulinase production are inulin and wheat bran as carbon source, KNO_3 as nitrogen source, initial pH 6.0, and a temperature 30°C.

The produced inulinase has optimum pH 5.5 and temperature 45°C. The addition of glycerol protect the enzyme at high temperature and increased its stability. Inhibition of the enzyme with Hg^{2+} or Ag^+ indicate the presence of SH groups in the proteins.

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