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FACTORS AFFECTING ON INULASE PRODUCTION BY *Aspergillus niger* AND PREPARATION OF FRUCTOSE SYRUP FROM INULIN

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ABSTRACT

Aspergillus niger has been shown to produce extracellular exo-and endo-inulase. This enzyme was used for inulin hydrolysis to produce syrups with more than 75% D-fructose. High fructose syrup can be used as low caloric sweetener. From this important point and others, *Aspergillus niger* was used in this study for inulase production and the results revealed that: maximal yield of enzyme is attained within 96 hours after inoculation. Inulin, dalhia tuber, chicory roots and artichoke tuber were found as the best carbon sources for enzyme production and to induce its productivity. Yeast extract and corn steep liquor stimulated the enzyme secretion. pH 5.5 and 35^{o}C were found as the pH and temperature optima for enzyme biosynthesis.

A rapid hydrolysis was observed as a result action of inulase on inulin for about 20 min at 55 ^{o}C, which appeared as the suitable conditions for substrate hydrolysis. pH 5.5 was found as the optimum pH for enzyme activity. *Aspergillus niger* inulase hydrolyzed sucrose, raffinose and inulin. Thus, this enzyme is not specific substrate degrading enzyme and belong to the group of fructano-hydrolyases. Mg^{+2} and Mn^{+2} at 1 mM activated the enzyme, while other ions were found as inhibitors.

Aspergillus niger inulase hydrolyzed inulin and Jerusalem artichoke with a higher degree of hydrolysis being 71.6 and 89.2%, respectively with 20 units of enzyme for 5 hours. Therefore, reducing sugars as fructose were raised and may be consumed as low caloric fructose-syrup sweetener and prevent sucrose-intolerant in diabetics.

Keywords: *Aspergillus niger*, Inulin, Jerusalem artichoke, Inulase production hydrolysis, fructose-syrup preparation.

INTRODUCTION

Fructose is receiving much attention regarding its use as sweetener in various fields of food and drink industries. Also it increases intestinal absorption of iron as well as improving in tolerance to dietary sucrose in sucrose-intolerant children. In this respect, fructose is of special interest for cold soft drinks. Since it is less cariogenic than sucrose. Moreover, its presence utilized by diabetics, and can be masks the bitter aftertaste of saccharin. Fructose is more soluble than sucrose, and more hygroscopic and as a result is more difficult to crystalize (Fleming & Grootwassink, 1979; Vandamme & Derycke, 1983; Gupta *et al*., 1989; Rashwan, 1996 and Shady *et al.*, 2000).

Inulase (2,1-β-D-fructan-fructanohydrolases, E.C.3.2.1.7) hydrolyze inulin to fructose in a single step from the non-reducing end of fructosidic chains. Inulase finds application in the production of high fructose syrup, alcohol, acetone and butanol (Vandamme & Derycke, 1983; El-Azhary, 1990; Nakamura *et al.*, 1995; Quirogo *et al.*, 1995 and Viswanathan & Kulkarni, 1995). Inulase also finds application as a diagnostic tool in diagnosing kidney problems (Kuehnle *et al.*, 1992).

Inulase is produced by a number of yeasts and filamentous fungi among them *Kluyveromyces fragilis* (Grootwassink & Hewitt, 1983 and El-Azhary, 1990); *K. marxianus* (Echeverrigaray & Tavares, 1985 and Shady *et al.*, 2000); *Aspergillus niger* and *Penicillium* spp (Nakamura *et al.*, 1978; Manzoni & Cavazzoni, 1988 and Viswanathan & Kulkarni, 1995).

The present studies were undertaken to study some factors affecting *A. niger* inulase production and activity. hydrolysis of different substrates and production of fructose syrup from inulin using enzymatic hydrolysis, was also done.

MATERIAL AND METHODS

Materials:

1- Inulin was obtained from BDH Chemicals LTd Pool England.

2- Jerusalem artichoke (*Helianthus tuberosus* L.):

Local cultivar Jerusalem artichoke was obtained from vegetable Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt. The tubers were harvested in the late fall season of 1999 and stored at –4°C until used. Tubers were peeled and cut into slices. The slices then vacuum dried at 30°C and packed in laminated polyethylene packs.

Organism and fermentation media:

Aspergillus niger strain was kindly obtained from Agric. Microbiol. Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt. The culture was maintained on a medium of Nakamura *et al.* (1978). The fungus was monthly transferred. The medium contains the following components: inulin, 3%; corn steep liquor, 2%; $(MH_4)H_2PO_4$, 1.2%; KCl, 0.07%; $MgSO_4.7H_2O$, 0.05%; $FeSO_4.7H_2O$, 0.001%and pH 4.5 for 5 days at 30°C ± 1.

Inoculum preparation:

Aspergillus niger was subcultured on Nakamura *et al.* (1978) agar and inoculated at 30°C for 72 h, spores from the slants were suspended in sterile 0.85 saline containing 0.01% tween 80. Spore suspension corresponing to approximately 2.5 x 10^6 spores/ml as determined by haemocytometer, was prepared and used in all the studies (Viswanathan and Kulkarni, 1995).

Fermentation technique

Erlenmeyer flask containing 45 ml liquid medium of Nakamura *et al.* (1978) was inoculated with 5% spore suspension prepared as above. Cultivation was carried out at 30°C for 12, 24, 48, 72, 96 and 120 hours on a rotary shaker at 200 rpm. After the incubation period, the mycelium was filtered through whatman No. 1 filter paper and the culture filtrate was centrifuged at 8000 rpm for 15 min then used as the source of extracellular inulase.

Enzyme assay:

Enzyme activity was estimated by determine reducing sugars according to Somogyi (1952). The reaction mixture contained 2 ml of 2% inulin, 0.5 ml of the enzyme solution and 2.0 ml of acetate buffer (pH 4.6)((Viswanathan and Kulkarni, 1995)). Incubation was performed at 50°C for 20 min. After incubation, the tubes were kept in a boiling water bath for 10 min to inactivate enzyme. Reaction mixture was assayed for reducing sugars and total sugars in the hydrolyzed products according to Somogyi (1952) and the anthrone method (Scott and Melvin, 1953), respectively.

The degree of hydrolysis was expressed as the percent of reducing sugar against the total sugar x 100.

One unit of inulase was considered as the amount of enzyme activity, which liberates 1 μg fructose equivalent from substrate/min under the assay conditions.

Inulin Hydrolysis

Twenty units of enzyme were added to 0.25 ml (4% w/v) of pure inulin or inulin containing Jerusalem artichoke (Rashwan, 1996), 0.75 ml of phosphate buffer (pH 5.5) and incubated at the optimum temmperature of the enzyme (55°C) for ¼, ½, 1, 2, 3, 4 and 5 hours. Reducing sugars were determined using fructose as standard.

RESULTS AND DISCUSSION

Factors affecing on enzyme production:

1- Incubation time:

Maximal yield of enzyme was attained after 96 hours of inoculation (Fig. 1), then, inulase productivity decreased. Also, the results show that, inulase was detected after 12 hours and increased steadily till it reached its maximum after 96 hours. El-Azhary (1990) and Shady *et al.* (2000) found that high yield of enzyme was found after 72 hours of the inoculation.

While, Workman and Day (1984) produced maximum inulase activity by *Kluyveromyces fragilis* after 48 hours of incubation.

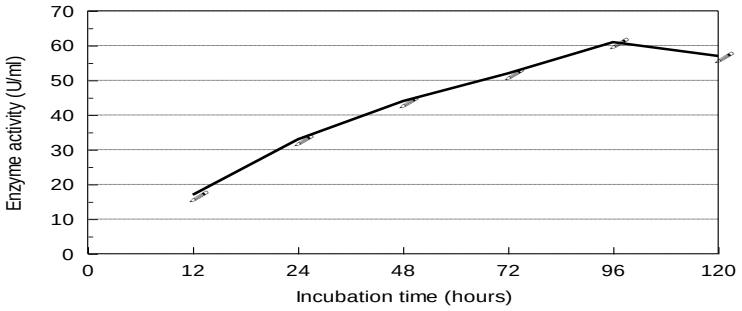

Fig. 1: Effect of incubation time on *A. niger* inulase biosynthesis.

2- Different carbon sources and agricultural wastes:

The effect of several carbohydrate and agricultural wastes were examined as carbon sources on extracellular inulase production by *Aspergillus niger.* These sources (1%) were added to the production medium instead of inulin to investigate their effect on enzyme production. The results (Table 1) indicated that *Aspergillus niger* has ability to degrade all agricultural wastes and hence produced inulase. Also, the enzyme was produced with all tested carbon sources used at varying levels. Highest enzyme yield was obtained with inulin, dahlia tuber, chicory roots and artichoke tuber, where enzyme activity reached 63.6, 63.2, 62.9 and 60.5 (U/ml), respectively. Very low level of enzyme was obtained with maltose, molasses (sugar cane) and glucose. Nakamura *et al.* (1978) concluded that inulin allowed much better enzyme formation than any other additional sugars. Also Beluche *et al.* (1980) found inulase activity only in *Debaryomyces cantarellii* cultures grown on inulin. These findings were similar to those obtained by Xiao *et al.* (1988); El-Azhary (1990); Fontana *et al*. (1994) and Shady *et al.* (2000).

Table 1: Effect of different carbon sources and some agricultural wastes on *A. niger* inulase biosynthesis.

Carbon source	Inulase Activity (U/ml)
Glucose	31.7
Maltose	21.3
Sucrose	57.0
Raffinose	58.9
Starch	44.3
Inulin	63.6
Fructose	47.1
Molasses (Sugar cane)	29.8
Molasses (Beet)	33.9
Dahlia tuber	63.2
Chicory roots	62.9
Beet pulp	42.8
Orange peel	47.7
Artichoke tuber	60.5

3- Nitrogen sources:

Nitrogen sources were added to the production medium instead of the initial nitrogen source (corn steep liquor and ammonium phosphate) with the same equivalent nitrogen content. It appears from the results (Table 2) that nitrogen sources greatly affected *A. niger* inulase biosynthesis. Also, enzyme activity differed with different of nitrogen sources used in the production medium. Yeast extract stimulated enzyme production and found to be the best nitrogen source resulting in accumulation of 79 U/ml. Also, corn steep liquor, peptone, tryptone and ammonium phosphate were found to be good promoters for enzyme biosynthesis. Similar data were obtained by Kim (1975), Nakamura *et al.* (1978) with an *Aspergillus niger*. Shady *et al*. (2000) found that inorganic nitrogen sources stimulated the enzyme biosynthesis.

Table 2: Effect of nitrogen sources on *A. niger* inulase formation.

Nitrogen sources	Inulase activity (U/ml)
Yeast extract	79
Peptone	73
Corn steep liquor	76
Urea	57
Tryptone	69
Casein	61
Gelatin	47
Ammonium phosphate	70
Sodium nitrate	47
Potassium nitrate	41
Ammonium chloride	52

4- Initial pH:

Results in Fig. (2) show that inulase was produced in a broad pH range (pH 4-8). Also it was observed that medium pH can affect inulase secretion by *Aspergillus niger.* Highest enzyme yield was found at pH 5.5, above or below this level, enzyme activity was reduced sharply. El-Azhary (1990) found that pH 6.5 was the optimum pH for *Kluyveromyces fragilis* inulase production. But, Shady *et al.* (2000) found that pH 7.5 was the optimum for *Kluyveromyces marxianus* inulase production.

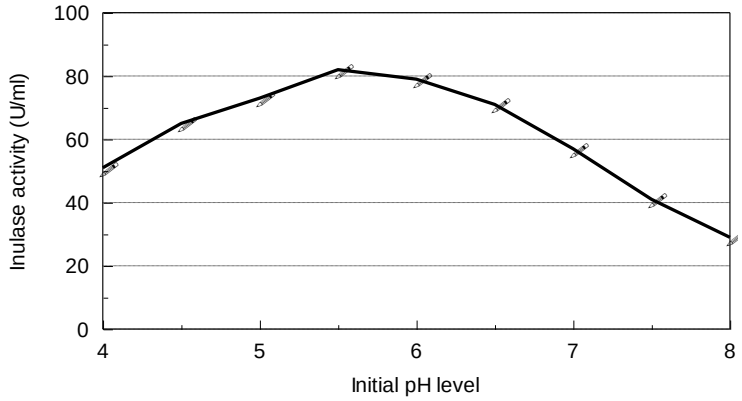

Fig. 2: Effect of initial pH on *A. niger* inulase production.

5- Incubation temperature:

The results obtained revealed that maximal yield of inulase production (90 units/ml) occurred at an incubation temperature of 35°C (Fig. 3). At higher or lower temperature a noteable decrease in inulase production was observed. High activity was found at 30 to 34°C (Grootwassink and Fleming, 1980). Shady *et al.* (2000) found that 30°C was the optimum temperature for *Kluyveromyces marxianus* inulase production.

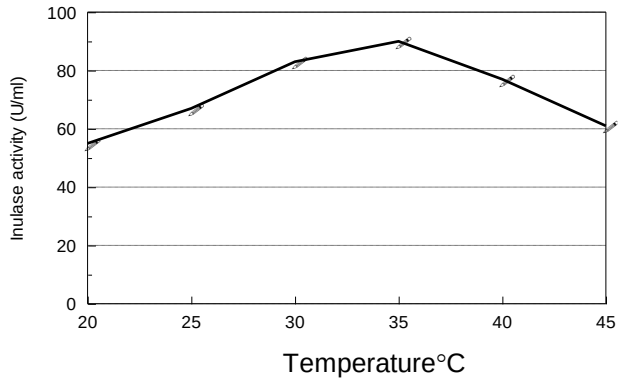

Fig. 3: Effect of incubation temperature on *A. niger* inulase secretion.

Enzyme properties:

1- Effect of temperature and time-course profile:

Using pH 4.6 in the reaction mixture, the optimum temperature was found to be 50-55ºC, above or below, enzyme activity decreased sharply (Fig. 4). Also, enzyme activty was varied from 5 to 30 min as reaction time-course, which, increased gradually with the progressive of time. A rapid hydrolysis was early observed as a result action of inulase on inulin for about 20 min, and then continued in a very slow rate. Therefore, the best reaction time and temperature for *Aspergillus niger* inulase activity were found to be 55ºC for 20 min. These results are in harmony with results obtained by Beluche *et al*. (1980) and Shady *et al*. (2000). While, Nakamura *et al*.(1995), found that 50ºC was the optimum temperature for *Aspergillus niger* inulase activity. But, Drent and Gottschal (1991) found 60ºC was the optimum temperature for inulase activity.

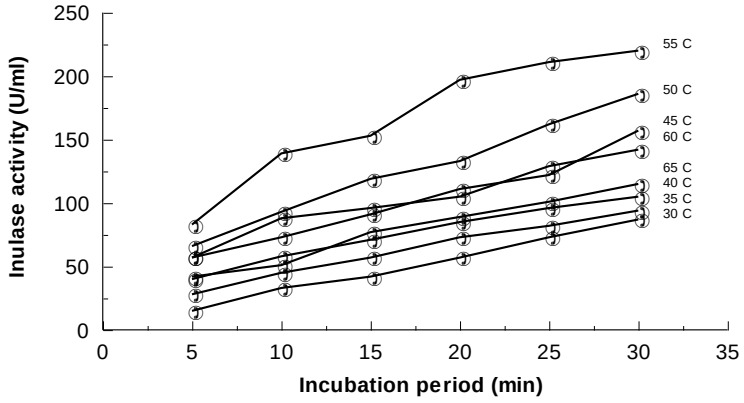

Fig. (4): Effect of incubation temperature on enzyme activity at different incubation periods.

2- Effect of pH on enzyme activity for different substrate hydrolysis:

The inulase activity was studied using citrate or phosphate buffer (10 mM). The pH optimum for enzyme activity was found in acidic region at pH 5.5 (Table 3). These results mean that this enzyme was acidic in nature. The results revealed that, sucrose, raffinose and inulin were an excellent substrates for enzyme activity. Inulase hydrolyzed the three substrates to good extent at different pH values. So, this enzyme is not specific substrate degrading enzyme, but, belong to the group of fructano-hydrolyases. Most of these enzymes act as β-fructosidases and liberate fructose, starting from the non reducing end of the fructoside chain. Similar results were obtained by Nakamura *et al*. (1995) and Shady *et al*. (2000). Beluche *et al*. (1980) found that pH 4.0 was the optimum pH for *Debaromyces cantarellii* inulase activity, while, Drent and Gottschal, (1991) found that inulase activity was optima at neutral pH.

Table 3: Effect of pH on enzyme activity for different substrate hydrolysis.

PH	Enzyme activity %		
	Inulin	Raffinose	Sucrose
2.0	77	70	63
2.5	83	77	70
3.0	87	82	77
3.5	91	89	85
4.0	94	95	97
4.5	100	100	100
5.0	109	103	107
5.5	112	109	110
6.0	108	105	104
6.5	98	94	91
7.0	87	77	71
7.5	75	60	50
8.0	64	57	30

3- Influence of effectors on enzyme activity:

Results in Table (4) indicated that *Aspergillus niger* inulase activity was stimulated by Mg^{+2} and Mn^{+2} ions, other ions reduced enzyme activity at different concentrations used, thus appear as inhibitors and reduced enzyme activity. $FeCl_3$ and $HgCl_2$ at 10 mM sharply reduced enzyme activity. On the other hand, in the presence of a lower concentrations of metal salts (1 μM), enzyme activity was not affected. These results are similar to those obtained by Nakamura *et al.* (1978) and Beluche *et al.* (1980).

Enzyme application:

Fructose syrup production during the hydrolysis of different substrates with *Aspergillus niger* inulase

Data presented in Table (5) show the effect of *Aspergillus niger* inulase on inulin and Jerusalem artichoke containing inulin in an effort to evaluate the suitability of this enzyme to be used for saccharifying these substrates and

produce fructose syrup. When these two substrates were treated with inulase, fructose was produced with some glucose. The degree of hydrolysis of two substrates was differed and increased as time proceeded. This may be due to the difference in affinity between two substrates and enzyme. The results also revealed that inulin containing tubers were hydrolyzed more rapidly than pure inulin, this is may be back to the difference in the degree of polymerization of inulin and the higher polyfructans in pure inulin (Workman & Day, 1984 and Manzoni & Cavazzoni, 1992). Similar results were also observed by Kim & Rhee (1989); Nakamura *et al.* (1995) and Shady *et al.* (2000).

Table (4): Effect of metal salts on *A. niger* inulase activity.

Salt	Enzyme activity %				
	10 mM	1 mM	0.1 mM	10 μM	1 μM
$CuCl_2$	52	69	91	100	100
$MnCl_2$	118	127	107	100	100
$MgCl_2$	99	106	101	100	100
$ZnCl_2$	82	100	100	100	100
$FeCl_3$	10	80	100	100	100
$AgNO_3$	32	40	50	84	100
$HgCl_2$	9	15	32	74	100
$Pb(NO_3)_2$	25	50	70	90	100

Table (5): Fructose syrup production during the hydrolysis of different substrates with *Aspergillus niger* inulase.

Time of hydrolysis (hours)	Hydrolysis %	
	Inulin	Jerusalem artichoke
¼	15.9	23.2
½	27.7	36.6
1	39.6	51.4
2	46.8	66.9
3	57.3	75.8
4	66.1	83.9
5	71.6	89.2

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العوامل المؤثرة على إنتاج إنزيم الإنيوليز من الأسبرجلس نيجر وإعداد شراب الفركتوز من الإنيولين

توفيق سعد محمد شادى[١] و فتحي إسماعيل علي حوقه[٢] و محمد علاء الدين أحمد الدمرداش[٣]

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هناك إتجاهات حديثة لإنتاج شراب الفركتوز المنخفض السعرات الحرارية والأكثر حلاوة من السكروز والآمن من ناحية الإستحدام لمرضى السكر وذلك بإستخدام إنزيم الإنيوليز وبصفة خاصة الميكروبى فى تحلل الإنيولين أو المصادر الطبيعية المحتوية عليه مثل درنات الخرشوف والداليا وغيرها ولقد ثبت أهمية إنزيم الإنيوليز المنتج من فطر الأسبرجلس نيجر ، ولذلك إتجهت هذه الدراسة نحو إنتاج هذا الإنزيم من الفطر ودراسة العوامل المؤثرة على الإنتاج وأهم خصائص هذا الإنزيم ، وقد أوضحت الدراسة الآتى :

- تم الحصول على أعلى كمية من الإنزيم بعد ٩٦ ساعة تحضين على البيئة المستخدمة فى الدراسة
- وجد أن الأنيولين ودرنات الداليا وجذور الشيكوريا ودرنات الخرشوف هما أفضل المصادر الكربونية لإنتاج الإنزيم حيث أحثت الفطر على إنتاج أعلى كمية من الإنزيم .
- حث مستخلص الخميرة ومنقوع الذرة كمصادر نيتروجينية على إنتاج الإنزيم وكانت أفضل المصادر النيتروجينية العضوية وغير العضوية حثاً على إنتاج الإنزيم .
- درجة pH ٥,٥ ودرجة حرارة ٣٥°م هما المثاليين كظروف بيئية لإنتاج الإنزيم .
- حدث تحلل سريع للإنيولين بإستخدام إنزيم الإنيوليز موضع الدراسة عند تحضينه على درجة ٥٥°م لمدة ٢٠ دقيقة كوقت تحلل ثم أعقب ذلك إستمرارية فى التحلل ولكن بمعدل بطئ .
- كانت درجة الـ pH ٥,٥ هى المثالية لنشاط الإنزيم حيث عندها تحلل السكروز والرافينوز والإنيولين بدرجة عالية . وقد أوضحت هذه التجربة عدم تخصص هذا الإنزيم فى تحلل مادة تفاعل واحدة ولذلك فقد صنف على أنه يتبع مجموعة الإنزيمات من نوع الـ fructano-hydrolases
- نشطت كل من أيونات المنجنيز والماغنسيوم سطح البروتين الإنزيمى وحثته على تحلل مادة التفاعل بمعدل أعلى فى حين أظهرت الأيونات الأخرى المستخدمة فى الدراسة فعل تثبيطى لنشاط الإنزيم وبصفة خاصة عند التركيزات العالية .
- أظهر تحلل الإنيولين النقى وإنيولين درنات الخرشوف معدل عالى من التحلل حيث بلغت نسبة التحلل ٧١,٦ ، ٨٩,٢% لهما على الترتيب بإستخدام ٢٠ وحدة إنزيم لمدة ٥ ساعات مما يعنى إمكانية إستخدامهما لإنتاج شراب الفركتوز المنخفض السعرات الحرارية والآمن من ناحية الإستخدام لمرضى السكر .