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USE OF PLANT MATERIALS AS SOURCES FOR INULINASE PRODUCTION BY *Aspergillus* SP. STRAINS

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ABSTRACT

Five fungal strains namely *Aspergillus niger* 6A, *A. niger* NRRL, *A. niger* NRC, *A. fumigatus* and *A. oryzae* were used in this study. Five different plant materials which underground roots and tubers were directly used in powder form as natural sources. They were chicory (*Cichorium intibus*), dahlia (*Dahlia pinnata*), Girasole or jurusalem artichoke (*Helianthus tuberosus*), suger beet (*Beta vulgaris*) and artichoke or Alkhrishv (*Cynara cardunculus*). Dahlia tubers and Girasole additions were the best inducer for inulinase production amongst other plant materials used. On the other hand, chicory, alkhrishv and sugar beet additions had negligible and/or negative effects on inulinase production by the tested fungal srtains.

Keywords : *Aspergillus* sp., Inulinase activity, Chicory, Dahlia, Girasole, Suger Beet and Alkhrishv.

INTRODUCTION

Inulinase is one of the key enzyme in the production of food industry such as high fructose syrup processing. The traditional production requires at least two enzymes to complete all processes and provide only 75% yield of product. However, if inulinase is used in this process, it can reduce both steps and gave 95% yield. Another important factor is the difficulty in harvesting enzyme from fungi. Fungal enzyme production is more complicated than bacteria due to hyphae. In addition, there is interest in ethanol production as alternative energy in the present by using inulinase producing bacteria and inulin-containing plant as substrate in fermentation process (Bonciu *et al*., 2010).

After starch, fructans are the most abundant non-structural polysaccharides found in a wide range of plants. Inulin is a polydispersed fructan consisting mainly of β (2,1) fructoysl-fructose links terminated by a sucrose residue. It serves as a storage polysaccharide in many members of Liliaceae, Amaryllidaceae, Gramineae, Asteraceae, Compositae etc. and is accumulated in the underground roots and tubers of several plants including Jerusalem artichoke (*Helianthus tuberosus*), chicory (*Cichorium intibus*), dahlia (*Dahlia pinnata*) Jerusalem artichoke (*Asparagus officinalis*), and Dandelion (*Taraxacum officinale*) (Sharma & Gill, 2007; Kango, 2008; Singh & Bhermi, 2008 and Chi *et al*., 2011).

Microbial inulinases can be divided into exo- and endo-acting enzymes according to their modes of action on inulin. Endoinulinases (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) are specific for inulin and hydrolyse the internal β-2,1-fructofuranosidic linkages to yield inulooligosaccharides as the main products, e.g. inulotriose, inulotetraose, and inulopentaose. Exoinulinases (β-D-fructan fructohydrolase; EC 3.2.1.80) successively split off terminal fructose

units from the non-reducing end of inulin, and also hydrolyse sucrose and raffinose (Chen *et al*., 2009)

A number of fungal, yeast and bacterial strains have been reported for the production of inulinases. Amongst the filamentous fungi, *Aspergillus* species are the common inulinase producers and high inulinase producers (Singh and Singh, 2010).

The plant inulin is a renewable and abundant substrate for the microbial production of high fructose syrup, which has gained importance in food, drink and neutraceutical industries. Fructose is the sweetest natural sweetener and is 1.5–2 times sweeter than sucrose and is less cariogenic and has no bitter aftertaste of saccharin and hence can be used as an alternative sweetener for diabetics. Conventional fructose preparation from starch needs at least three enzymatic steps involving a-amylase, amyloglucosidase and glucose isomerase activities and maximal yields are reported to be 45% fructose solutions. An easier, direct, cheap and quicker alternative could be enzymatic hydrolysis of polydispersed reserve fructan, inulin (Kango, 2008).

This work aims to study the potent inulinase producing fungal strain on some vegetal substances.

MATERIALS AND METHODS

Plant materials used

Five different plant materials which have underground roots and tubers were collected from local markets were used in this study. Chicory (*Cichorium intibus*), dahlia (*Dahlia pinnata*), Girasole or jurusalem artichoke (*Helianthus tuberosus*), suger beet (*Beta vulgaris*) and artichoke or Alkhrishv (*Cynara cardunculus*).

Fungal strains used:

Five different fungal strains were used in this study. Two local fungal strains, namely *Aspergillus oryzae* and *A. fumigatus*; were kindly obtained from plant pathology Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt. One strain, *A. niger* NRRL 2270; was obtained from Microbial Properties Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, USA. Another one, *A. niger* NRC was kindly obtained from National Research Center (NRC) Giza, Egypt. The last one, namely *A. niger* 6A was kindly obtained from Agricultural Research Center (ARC) Giza, Egypt. These fungal strains were recommended as high producers for inulinase.

Cultivation medium for inulinase production :

The cultural medium was used as basal medium for inulinase production. The chemical composition of this medium was as following (g/l): $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 3.0; $NaNO_3$, 1.5; $MgSO_4.7H_2O$, 0.01 and inulin, 3.0 with pH 7.0. Inulin was sterilized separately and added to the medium before inoculation (Kumar *et al*., 2005).

Microbiological Methods:

Pretreatment of plant materials as a source of inulin

The plant materials were washed in running tap water, sliced, and then dried at 70◦C for 72 h. The dried slices were then milled to a fine powder with a hammer mill. After milling, the resultant powder was directly used as carbon source and inulin source (Dilipkumar *et al*., 2011).

Maintainance of fungal strains:

The fungal strains were maintained on potato dextrose agar medium (PDA) slants at 5°C and monthly subcultured.

Preparation of fungal spores suspension:

Spores appeared on PDA slant after 7 days were scraped by using 5 mL sterilized saline solution containing 8 g NaCl/L and suspended in 45 mL of the same solution. Spores count was performed in a Hematocytometer (model Buerker MOM BUDA pest) direct hemocytometer counting. Spores suspention corresponding to approximately $5x10^6$ spores per ml (Pintado *et al*., 1997).

Inoculum size used:

The inoculum size used was 1% for inoculating the cultivation medium for all experiments and the inoculum value was ontianed about $5x10^6$ spores per ml.

Inulinases production:

Inulinases production was carried out by submerged fermentation using 250 ml Erlenmeyer flasks each containing 50 ml of cultivation medium. After inoculation with 0.5 ml of the tested fungal standard inoculum of spores suspension, the flasks were then incubated at 30°C for 6 days in a temperature controlled rotary incubator-shaker operated at 200 rpm. The fermented broth was filtered through double-layered Whatman paper filter. After filtration, the supernatant was collected as the crude enzyme solution for further studies (Chen *et al*., 2009).

Biochemical determinations :

Final pH:

Values of pH were determined in the cultural filtrate using a pH meter, model JENWAY 3505. The cultural filtrates were used for final pH determinations

Determination of reducing sugar

Reducing sugar in the fermented medium was determined by the Nelson–Somogyi method. Total sugar was measured as reduction of sugar after hydrolysis of the fermented medium (Cui *et al*., 2011). The residual reducing sugars were determined as fructose after Nelson, (1944). Standard curve of fructose was performed and the equation of inulinase activities was as follow : X (μg/ml) = (Y – 0.0021) / 0.0591

Enzymatic activity

The inulinase activity of the supernatant was determined as following: 0.5 ml cultural filtrate was incubated with 2.0 ml of 0.2% inulin, 2.0 ml acetate buffer (pH 4.6) at 40°C for 20 min. (Cazetta *et al*., 2005). One unit of inulinase activity was defined as the amount of enzyme that hydrolyses 1 μg fructose per min under the above conditions.

RESULTS AND DISCUSSION

Five fungal strains namely *Aspergillus niger* 6A, *Aspergillus niger* NRRL, *Aspergillus niger* NRC, *Aspergillus fumigatus* and *Aspergillus oryzae* were used in this study. Five different plant materials which underground roots and tubers collected from local markets were used directly in powder form as a natural source of inulin. They were Chicory (*Cichorium intibus*), Dahlia (*Dahlia pinnata*), Girasole

or jurusalem artichoke (*Helianthus tuberosus*), suger beet (*Beta vulgaris*) and artichoke or Alkhrishv (*Cynara cardunculus*).

Effect of dahlia tubers addition on inulinase production by selected fungal srtains.

Table 1 show the effect of adding dahlia tubers on inulinase production by selected fungi. The highest values of inulinase production were 121.4, 112, 86.6, 45.8 and 56.4 µg fructose/ml after 144, 120, 96, 144 and 96 h of incubation period by *Aspergillus niger* 6A, *A. niger* NRC, *A. niger* NRRL, *A. oryzae* and *A. fumigatus*, respectively. These inulinase activities were correlated with,the final pH to be 6.11, 6.20, 4.18, 6.73 and 7.08 (Fig.1).

Table 1: Inulinase activities by selected fungal strains as induced by dahlia tubers.

Fungal srtains	Incubation period (hr)				
	48	72	96	120	144
Aspergillus niger 6A	43.4	54.4	70.8	88	121.4
Aspergillus niger NRC	10.6	52.4	54.6	112	81.2
Aspergillus niger NRRL	60.4	71.2	86.6	78	72.8
Aspergillus oryzae	10.4	14.6	25.6	44.4	45.8
Aspergillus fumigatus	35	54.4	56.4	48.2	27

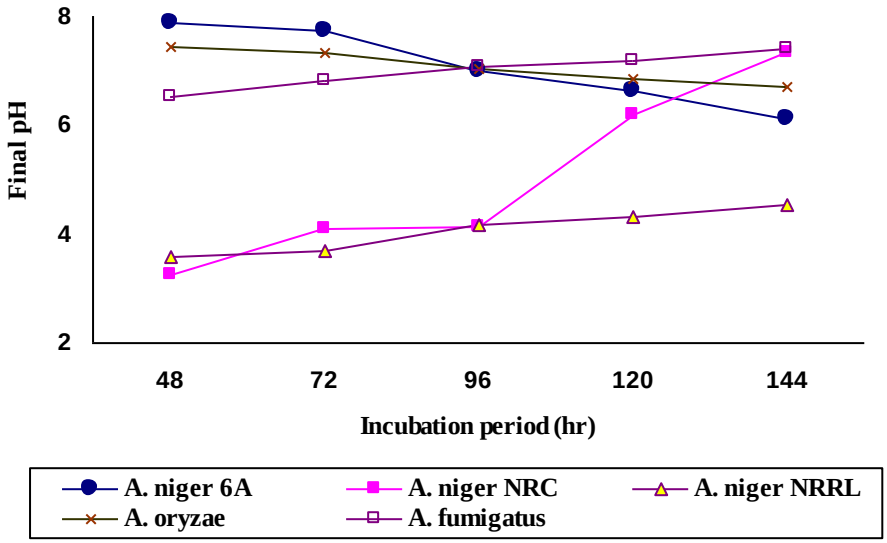

Fig.1: Effect of dahlia tubers addition on final pH of inulinase production by selected fungal srtains.

The more effect was found with *A. niger* NRC and *A. niger* 6A since inulinase activity was increased by 118.75% and 93.93%, respectively. The lowest values of inulinase activities were found with *Aspergillus niger* NRRL, *A. fumigatus* and *A. oryzae* since the increase percent were found to be 51.39%, 27.03% and 4.09%, respectively. These results are in agreement with those obtained by Sharma *et al*., (2006) who reported that, the enzyme

activity was about 1.6-fold higher than activity that obtained by using pure inulin as a carbon source.

Complex substrates from plant materials, plant materials vegetal substrate are showing very interesting results for inulinase production. Roots and tubers of several plants belonging to families Compositae and Liliaceae have been reported to be good sources of inulin. Among these, Dahlia tubers and dahlia extract were used as a substrate for both cell cultures and inulinase production by several authors (Gill *et al*., 2006; Sharma *et al*., 2006; Singh *et al*., 2006; Singh *et al*., 2007; Kango, 2008; Singh & Bhermi, 2008 and Chen *et al*., 2009).

Effect of Girasole addition on inulinase production by selected fungal srtains.

Table 2 show the effect of Girasole addition on inulinase production by selected fungi. The highest values of inulinase production were by *A. niger* NRC, *A. niger* 6A, *A. niger* NRRL, *A. oryzae* and *A. fumigatus* were 110, 95.4, 83.6, 54.6 and 48.4 µg fructose/ml, respectively. The determined inulinase activities were found to correlate with 120 h of incubation except *A. fumigatus* was found at 96 h. These values of inulinase activities were found to correlate with the final pH values being 6.41, 4.33, 3.17, 6.43 and 6.52, respectively (Fig. 2).

Table 2: Inulinase activities by selected fungal strains as induced by Girasole.

Fungal srtains	Incubation period (hr)				
	48	**72**	**96**	**120**	**144**
Aspergillus niger 6A	75.6	76.2	94	95.4	96.2
Aspergillus niger NRC	24	34	41.2	110	67.2
Aspergillus niger NRRL	44.8	45.4	48.2	76.4	83.6
Aspergillus oryzae	23.8	32	42.2	54.6	30
Aspergillus fumigatus	29	40.6	48.4	40	38.8

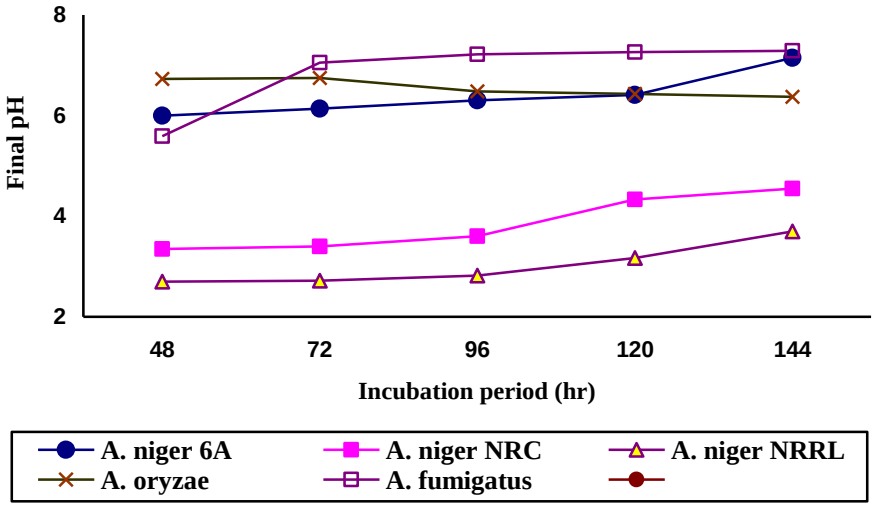

Fig. 2: Effect of Girasole addition on final pH of inulinase production by selected fungal srtains.

The effects of Girasole addition on inulinase production had the same effect which occured with Dahlia tubers addition compared with crude inulin and it also varied according to the fungal srtains. The highest effect was also found with *A. niger* NRC 121.4 and the lowest values were 52.40, 33.57, 24.09 and 9.01 for *A. niger* 6A, *A. niger* NRRL *A. oryzae* and *A. fumigatus*, respectively.

Effect of chicory addition on inulinase production by selected fungal srtains.

Table 3 show the effect of chicary addition on inulinase production by selected fungi. The highest values of inulinase activities obtained by *A. niger* 6A, *A. niger* NRRL, *A. niger* NRC, *A. fumigatus* and *A. oryzae* were 64.2, 55.8, 40.4, 35.4 and 24.2 µg fructose/ml, respectively. These values of inulinase activities were detected after 144, 120, 120, 48 and 144 h of incubation. The determined inulinase activities were found to correlate with the final pH being 6.25, 6.20, 3.65, 6.54 and 6.66 (Fig. 3).

Table 3. inulinase activity by selected fungal strains as induced by Chicory.

Fungal srtains	Incubation period (hr)				
	48	**72**	**96**	**120**	**144**
Aspergillus niger 6A	40.4	41.4	45.2	54.2	64.2
Aspergillus niger NRC	16.2	19.8	25.4	40.4	40
Aspergillus niger NRRL	40.6	44.8	51.4	55.8	54.4
Aspergillus oryzae	8	12	14	17	24.2
Aspergillus fumigatus	35.4	30.2	18.8	3	0.88

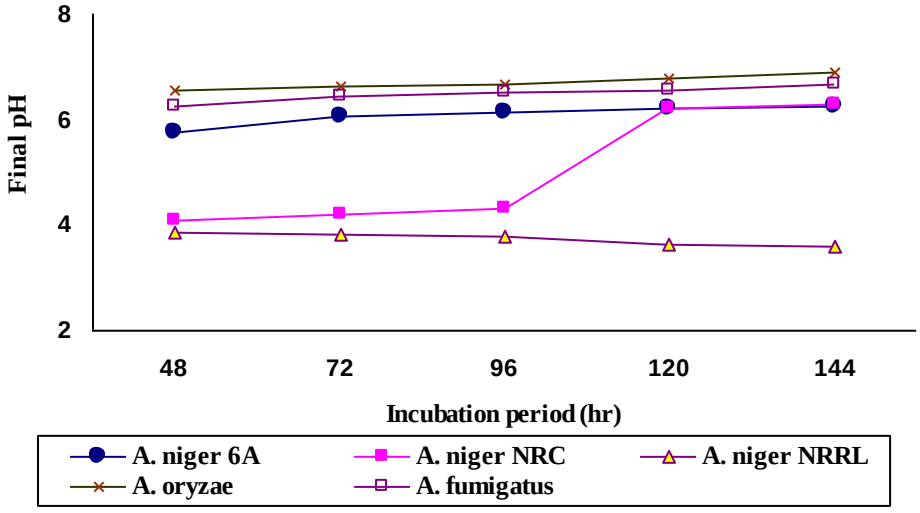

Fig. 3: Effect of chicory addition on final pH of inulinase production by selected fungal srtains.

Effect of alkhrishv addition on inulinase production by selected fungal srtains.

Table 4 show the effect of alkhrishv addition on inulinase production by selected fungi. The highest values of inulinase activities produced by *A. niger* NRRL, *A. niger* 6A, *A. fumigatus*, *A. oryzae* and *A. niger* NRC were 77.6, 64.2, 44.4, 41.4 and 46 µg fructose/ml, respectively. These activities were detected after 144, 96, 120, 96 and 96 h of incubation. The inulinase activities were found to correlate with the final pH were 6.10, 4.13, 4.60, 7.66 and 7.60 (Fig. 4).

Decrease in inulinase activities can be due to catabolic repression of the enzyme synthesis by high concentration of simple sugars obtained in cultivation medium as a result of inulinase activities. Alkhrishv is commonly produced high levels of fermentable sugars. High glucose concentration repressed fermentation activity dramatically (Singh *et al*., 2006; Sirisansaneeyakul *et al*., 2006; Kango, 2008; Singh &Bhermi, 2008; Ge *et al*., 2009; Bonciu *et al*., 2010, and Zhang *et al*., 2010).

Table 4. Inulinase activities by selected fungal strains as induced by alkhrishv

Fungal srtains	Incubation period (hr)				
	48	72	96	120	144
Aspergillus niger 6A	18.6	27	31.4	54.2	64.2
Aspergillus niger NRC	10.6	36.6	46	31.8	25.6
Aspergillus niger NRRL	27.8	37.8	74.6	77.6	59.8
Aspergillus oryzae	5.2	26	41.4	28.6	14.4
Aspergillus fumigatus	27.8	34.4	44.4	36	34.6

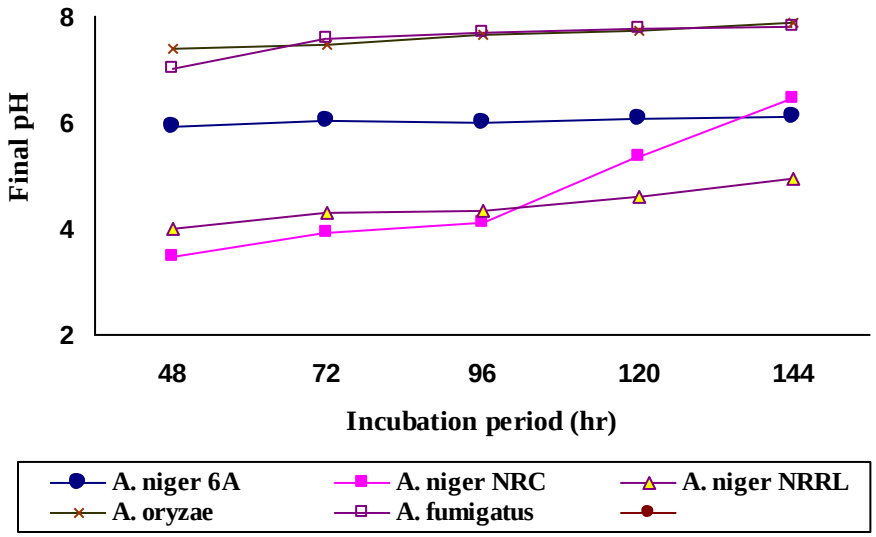

Fig. 4: Effect of alkhrishv addition on final pH of inulinase production by selected fungal srtains.

Also, obtained results are in a good line with those results obtained by the previous authors, where alkhrishv addition had two effect on inulinase production compared with crude inulin. The first was caused a good effect on *A. niger* NRRL and *A. niger* 6A since the activity increase by 35.66% and 2.56% compared with crude inulin, respectively. The second was caused no effect and obtained values were 0.0, -6.76 and -10.16% for *A. fumigatus*, *A. oryzae* and *A. niger* NRC, respectively.

Effect of sugar beet addition on inulinase production by selected fungal srtains.

Table 5 show the effect of sugar beet addition on inulinase production by selected fungi. The highest values of inulinase produced by *A. niger* NRRL, *A. niger* 6A, *A. niger* NRC, *A. oryzae* and *A. fumigatus*, were 68.2, 59.2, 35.6, 32.8 and 28.6 µg fructose/ml, respectively. These activities were found after 120, 48, 144, 48 and 48 h of incubation. These inulinase activities were found to correlate with the final pH of the cultivation medium to be 6.55, 4.18, 3.46, 6.80 and 5.53 (Fig. 5).

Table 5: Inulinase activity by selected fungal strains as induced by sugar beet.

Fungal srtains	Incubation period (hr)				
	48	72	96	120	144
Aspergillus niger 6A	59.2	51.2	43.4	34.6	24.8
Aspergillus niger NRC	14.4	17.8	21.4	22.2	35.6
Aspergillus niger NRRL	37.6	39.6	57.2	68.2	64.8
Aspergillus oryzae	32.8	25.2	19	3.2	3
Aspergillus fumigatus	28.6	17	13	7.4	3.4

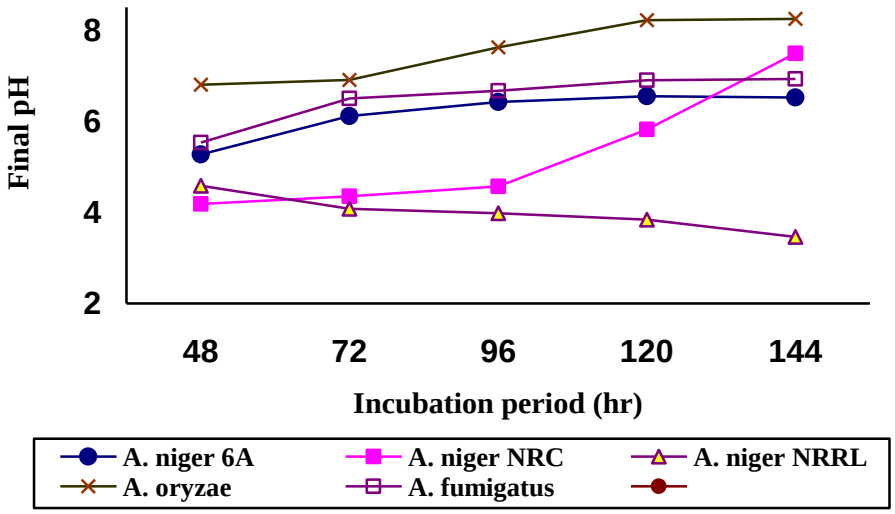

Fig. 5: Effect of sugar beet addition on final pH of inulinase production by selected fungal srtains.

The effects of sugar beet addition on inulinase production had the same pattern which occured with alkhrishv addition and it also caused both decrease and increase of inulinase activities produced. The first was caused a good effect on *A. niger* NRRL 19.23% compared with crude inulin. The second was no effect and values were -5.43, -25.45, -30.47 and -34.70% for *A. niger* 6A, *A. oryzae*, *A. niger* NRC and *A. fumigatus*, respectively.

It could be observed from obtained results that, dahlia tubers and Girasole additions were the best inducer for inulin as production amongst other plant materials used. On the other hand, chicory, alkhrishv and sugar beet additions had negligible and/or negative effects on inulinase production by the tested fungal strains.

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استخدام مواد نباتية كمصادر لإنتاج إنزيم الإنيولينيز من سلالات فطر الاسبرجيلس

فتحى إسماعيل على حوقه* ، عبد الله العوضى إبراهيم سليم* ، محمود محمد عوض الله السواح* ، أحمد زكى محمد على ، محمد كامل محمد محمود**.**

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استخدم فى هذا البحث خمس سلالات من فطر الأسبرجيلس وهى *Aspergillus niger* 6A ، *A. niger* NRRL ، *A. niger* NRC ، *A. fumigatus* ، *A. oryzae* ، كما استخدم السريس ، الداليا ، الطرطوفة ، بنجر السكر والخرشوف كإضافات نباتية فى عمل بيئات غذائية لإنتاج إنزيم الإنيولينيز حيث أعطت درنات الداليا والطرطوفة أعلى إنتاجية للإنزيم بينما إضافة السريس وبنجر السكر والخرشوف أظهرت نتائج اقل فى إنتاج الإنزيم.

قام بتحكيم البحث

أ.د / محمد منصور قاسم	**كلية الزراعة – جامعة المنصورة**
أ.د / حسين عبد الله محمد الفضالي	**كلية زراعة دمياط – جامعة المنصورة**