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PRODUCTION AND PROPERTIES OF INULINASE FROM *Arthrobacter* sp.

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ABSTRACT

Microbial inulinase has been proposed as the most promising approach to obtain fructose syrup from inulin which is used now, instead of sucrose, in many foods, pharmaceuticals, and beverages. Production and properties of inulinase from *Arthrobacter* sp. had been studied and the results revealed that, maximal yield of enzyme production is attained within 48 hours after inoculation, high inulinase production was observed when sucrose was used as additional carbon source, ammonium sulphate induced inulinase production by *Arthrobacter* sp., which was found as the best nitrogen source. Maximum inulinase production was obtained by using inoculum volume 5% (v/v), pH 7.0, at 30 ^{0}C. The properties of crude inulinase from *Arthrobacter* sp. was also tested. The enzyme showed maximum activity at pH 7.5 and 50 ^{0}C. The enzyme was stable more than 80% of its maximal activity at 30-50 ^{0}C after 30 min heat treatment.

Keywords: *Arthrobacter* sp*.,* inulinase, inulin, hydrolysis, high fructose syrup.

INTRODUCTION

An increasing demand for healthier and calorie-controlled food has forced many food industries to come up with a number of alternative safe sweeteners. Fructooligosaccharides (FOS) have also been accepted as functional sweeteners similar to other microbial oligosaccharides (Yun, 1996). Inulin is a naturally occurring polyfructan, which has been extensively explored for the production of high fructose syrup by either microbial or chemical processes. The enzymes degrading inulin (inulinase) belong to the group of fructoano-hydrolyses. Microbial inulinases can be divided into exo- and endo-acting enzymes according to their modes of action on inulin. 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, endoinulinases (2,1-β-d-fructan fructanohydrolase; EC 3.2.1.7) hydrolyse the internal β-2,1-fructofuranosidic linkages to yield inulooligosaccharides as the main products, inulotriose, inulotetraose, and inulopentaose (Vandamme and Derycke ,1983; Nakamura *et al*., 1997; Arand *et al* ., 2002; Ohta *et al*., 2002)..A number of fungal, yeast, and bacterial strains have been used for the production of inulinases among them, the strains belong to *Aspergillus* and *Kluyveromyces* genus were the most common and preferred choice for inulinase production. The production levels of inulinases in bacteria are not comparable to those of yeast and fungi. However, due to the ability of many bacteria to survive at high temperatures, attempts have been made to isolate bacterial strains which can produce high quantities of thermally stable inulinase. Data on inulinases biosynthesis using bacterial strains are scarce and mainly concern endo-inulinases (Singh and

Gill, 2006; Neagu and Bahrim, 2011). The present study was focused to study some factors affecting production and activity of inulinase by *Arthrobacter* sp.

MATERIALS AND METHODS

Bacterial strain and medium:

Arthrobacter sp. was obtained from Agric. Microbiol. Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt. Bacterial strain was grown in Allais *et al*. (1986) medium. The medium consists of (g/l): $(NH_4)_2SO_4$, 0.5; $MgSO_4$ $7H_20$, 0.2 ; KH_2PO_4, 3.0 ; Inulin, 2.0 ; Mineral salts solution (Atlas, 1995), 2 ml, pH of the medium was adjusted to 7.0.

Preparation of inoculum:

Agar slants were inoculated with the tested bacterial microorganism and incubated at 30 ^{0}C for 24 hr. The growth on the agar slants was scraped, using 5 ml sterilized distilled water, then transferred to a flask containing 50 ml of the fermentation medium and incubated for 24 hr on a rotary shaker operating at 150 rpm at 30 ^{0}C. The resulting cell suspension was used for inoculation of fermentation medium employed for inulinase production.

Fermentation technique:

Inulinase production was carried out by submerged fermentation using 250 ml Erlenmeyer flasks, each containing 50 ml of the production medium. After inoculation with 2.5 ml of the tested bacterial inoculum, the flasks were then incubated at 30 ^{0}C in a temperature controlled rotary incubator-shaker operated at 150 rpm. After an incubation time, depending on testing factor examined, the contents of the flasks were centrifuged at 5000 rpm for 20 min at 4 ^{0}C. Culture supernatant was used as a source of crude enzyme for further studies.

Enzyme assay:

The reaction mixture containing 0.1 ml crude enzyme and 0.9 ml of (0.1 M acetate buffer, pH 5) containing 0.2% inulin was incubated at 50 ^{o}C for 20 min. The reaction was inactivated immediately by keeping the reaction mixture at 100 ^{o}C for 10 min. The amount of reducing sugar in the reaction mixture was assayed by the method of Nelson (1944) modified by Somogyi (1952). One inulinase unit (U) was defined as the amount of enzyme that produces 1 µg of fructose per minute under the assay conditions used in this study.

Bacterial growth measurement:

The bacterial growth was measured as optical density at wave length 620 nm (Allais *et al*., 1987). Before reading, the suspensions always were diluted to give turbidity reading lower than 1.0.

pH determenation:

Values of pH were determined using a pH meter, model JENWAY 3505.

Effect of time course on inulinase production:

The crude enzyme preparations were taken after 6, 12, 24, 36, 48, 60, 72, and 84 hours for assaying inulinase activity.

Effect of additional carbon and nitrogen sources on inulinase production:

Seven carbon sources, namely, glucose; galactose; fructose; lactose; maltose; sucrose and starch were added to the basal production medium containing inulin as an inducer at concentration of 1 % (w/v). Also, initial nitrogen source was replaced with different nitrogen sources as a sole nitrogen source at the same nitrogen level to study their effect on inulinase production. These nitrogen sources namely, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$, NH_4Cl, KNO_3, peptone, beef extract and yeast extract.

Effect of initial pH, inoculum volume and incubation temperature on inulinase production:

For the pH study, the production medium was adjusted to pH ranging from 4 to 9 by using 1N NaoH and 1N HCL. Inoculation of the main cultures was performed with five different inoculum volumes (1, 2.5, 5, 7.5 and 10%). Four degrees of incubation temperatures (25°, 30°, 35° and 37°C) were tested to determine the optimum temperature for inulinase production.

Effect of the pH optimum on inulinase activity:

The effect of pH on inulinase activity was investigated by incubating the crude enzyme at pH range 4.0-9.0 with acetate buffer (0.1 M, pH 4.0-5.5), phosphate buffer (0.1 M. pH 6.0-8.0) and glycin-NaOH buffer (0.1 M, pH 8.5-9.0). The inulinase activity was measured after incubation at 50°C.

Temperature optimum and stability of on inulinase:

The effect of temperature on inulinase activity was investigated by incubating the enzyme reaction at different temperatures (30-70°C). The temperature stability of the crude enzyme was carried out by heating the crude enzyme at 30-٧٠°C in the absence of substrate for 30 min. Then the residual activity was determined at optimum conditions.

Statistical analysis:

The obtained experimental data were statistically analyzed using software of COSTAT.

RESULTS AND DISCUSSION

Effect of time course on inulinase production:

Maximal yield of enzyme was attained after 48 hours of incubation (Fig.1), then inulinase productivity decreased. Also, the results show that, inulinase was detected after 12 hours and increased steadily till it reached its maximum after 48 hours. These results are in agreement to those obtained by Kang *et al*. (1998). While, Haraguchi (2010) produced maximum inulinase activity by *Arthrobacter ureafaciens* D13-3 after 24 hours of incubation.

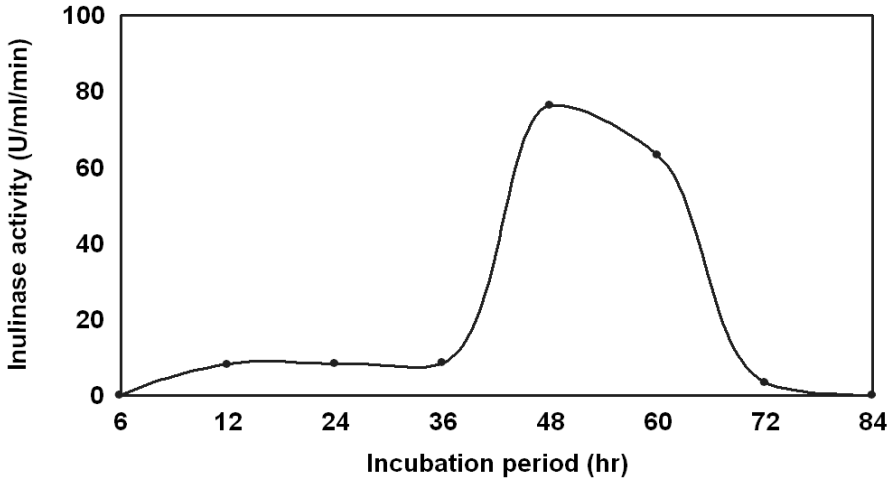

Fig. 1. Time course profile for inulinase activity.

Effect of additional carbon sources on inulinase production:

The effect of different carbon sources on inulinase production by *Arthrobacter* sp. was investigated (Table.1). These carbon sources were added to the production medium containing inulin as an inducer at concentration of 1 % (w/v). The obtained results showed that the kind of used additional carbon sources greatly affected the yield of inulinase produced in culture fluids of the producer microorganism. Addition of sucrose promoted the highest inulinase production (102.52 U/ml/min), the inulinase production was increased by (25.12 U/ml/min) over the value of the control. On the other hand, other additional carbon sources inhibit inulinase production. Similar observations were reported by several authors. Gill *et al*. (2003) reported that maximal inulinase production by *streptomyces* sp. GNDU 1 was found in the presence of inulin while, the presence of simple sugars inhibits inulinase production. Naidoo *et al*. (2009) reported that the highest inulinase activity was found in sucrose grown culture by *Xanthomonas campestris* pv. Phaseoli. Zherebtsov *et al*. (2002) found that *Bacillus polymyxa* 722 and *Bacillus polymyxa* 29 displayed the maximal activity on a starch-containing culture medium, while the maximal activity of *Bacillus subtilis* 68 was observed in the presence of sucrose.

Table.1. Effect of additional carbon sources on inulinase production by *Arthrobacter* sp.

Carbon Sources (1% w/v)	Final culture pH	Growth (O.D.)	Inulinase activity (U/ml/min)
Control	6.15	1.290	77.40
Glucose	4.48	2.430	3.73
Galactose	5.20	2.755	3.27
Fructose	4.61	1.900	0.52
Lactose	5.25	2.205	0.73
Maltose	5.20	2.333	1.21
Sucrose	4.57	2.183	102.52
Starch	5.78	2.943	47.23
LSD at 5%		**0.048**	**0.606**

Effect of nitrogen sources on inulinase production:

The original nitrogen source in the culture medium was replaced by different nitrogen sources for comparing each of them with other. These nitrogen sources were added to the basal medium at the same N-level of initial nitrogen source. The results (Table.2) clearly indicated that the source of nitrogen greatly affected the yield of inulinase, it is obvious that none of substituted nitrogen sources yielded more inulinase in comparison with control source. The highest values of inulinase production by *Arthrobacter* sp. were 102.52 U/ml/min in case of using $(NH_4)_2SO_4$, while peptone and yeast extract gave values of inulinase activity more than other inorganic nitrogen sources. Similar observations were reported by several authors. Zherebtsov *et al*. (2002) reported $(NH_4)_2HPO_4$ as the best source for inulinase production by *Bacillus polymyxa* 29, *Bacillus polymyxa* 722 and *Bacillus subtilis* 68. Naidoo *et al*. (2009) reported tryptone as the best source for inulinase production by *Xanthomonas campestris* pv. phaseoli KM 24. Gao *et al*. (2009) reported $(NH_4)H_2PO_4$ as the best source for inulinase production by *Bacillus Smithii* T7. Li *et al*. (2011) reported peptone as the best source for inulinase production by *Marinimicrobium* sp. LS-A18.

Table. 2. Effect of nitrogen sources on inulinase production by *Arthrobacter* sp.

Nitrogen sources	Final culture pH	Growth (O.D.)	Inulinase activity (U/ml/min)
Control	4.57	2.183	102.52
NH_4Cl	4.27	3.06٠	76.76
KNO_3	7.25	3.535	67.60
$(NH_4)_2HPO_4$	5.05	3.360	59.97
$NH_4H_2PO_4$	5.94	2.575	62.11
Peptone	6.85	2.103	85.92
Beef extract	6.87	1.946	69.09
Yeast extract	6.83	2.356	84.32
LSD at 5%		**0.041**	**0.963**

Effect of initial pH on inulinase production

The maintenance and control of pH is necessary for optimal enzyme formation. It is obvious from the results illustrated in (Fig.2) that pH of the growth medium greatly affected the inulinase production by *Arhrobacter* sp.. The optimum pH for inulinase production was observed at pH 7.0 (109.38 U/ml/min). The results showed also that there is gradual increase in enzyme production up to pH 7, then inulinase activity was decreased. There was negligible growth and enzyme production below pH 6. The obtained results are, indeed, in close agreement with the findings of Zherebtsov *et al*. (2002), Ayyachamy *et al*. (2007), Gao *et al*. (2009), Naidoo *et al*. (2009) and Li *et al*. (2011).

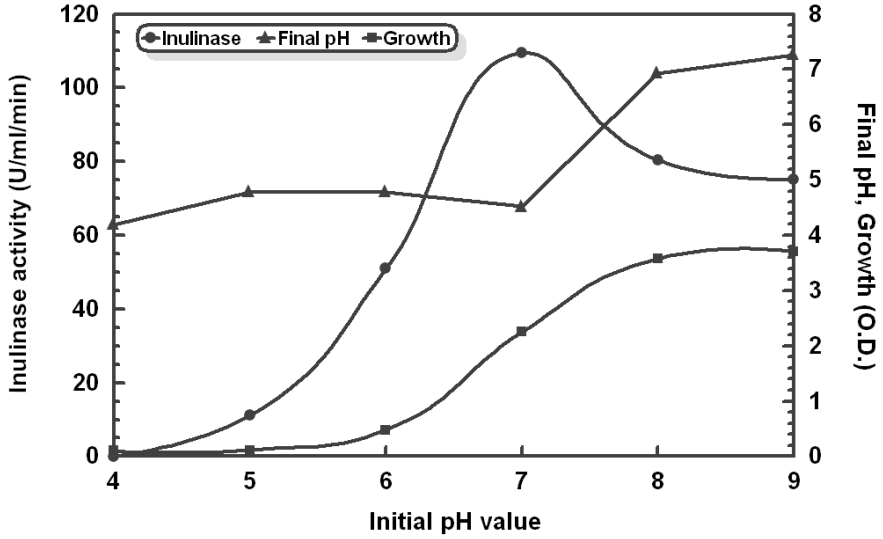

Fig. 2. Effect of medium initial pH on inulinase production by *Arthrobacter* sp.

Effect of inoculum volume on inulinase production

Fig.3 showes the effect of inoculum volume on inulinase production by *Arthrobacter* sp. Five different inoculum volumes (1, 2.5, 5, 7.5 and 10%) were testeded. The results showed that inulinase production reached its maximum value of 104.92 U/ml/min with 5% (v/v) inoculum. the inulinase production was gradually increased with the increase of inoculum volume up to 5% (v/v) and then decreased. Similary, Selvakumar and Pandey, (1999) found 3% inoculum volume to be sufficient for maximal extra-cellular inulinase production from *Staphylococcus* sp. While Ayyachamy *et al*. (2007) found 10% inoculum volume to be optimum for inulinase production from *Xanthomonas campestris* pv phaseoli.

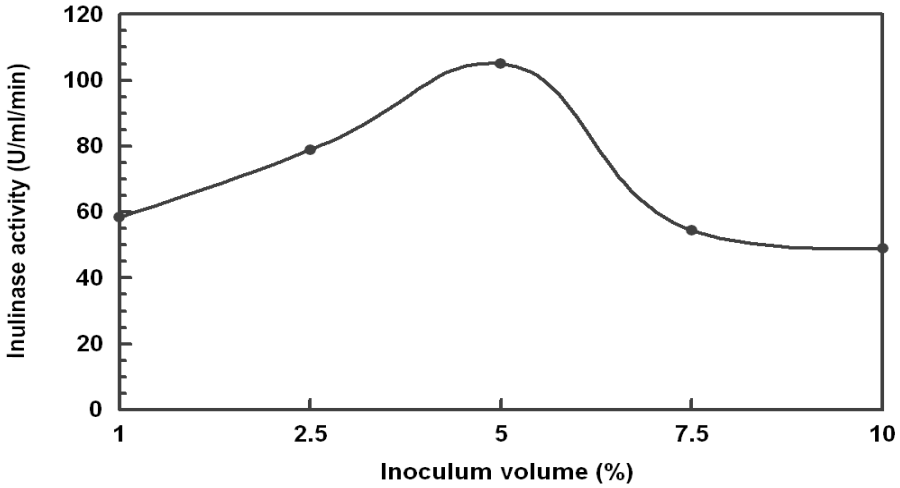

Fig. 3. Effect of inoculum volume on inulinase production by *Arthrobacter* sp.

Effect of incubation temperature on inulinase production

The obtained data illustrated by (Fig.4) show that the highest inulinase activity (101.35 U/ml/min) was reached at incubation temperature of 30°C. At higher or lower temperature a noteable decrease in inulinase production was observed. *Arthrobacter* sp. was able to grow at 25–37 °C with maximum growth at 25 °C. These results are in harmony with those obtained by Selvakumar and Pandey (1999), Zherebtsov *et al*. (2002), Gao *et al*. (2009), Naidoo *et al*. (2009) and Li *et al*. (2011).

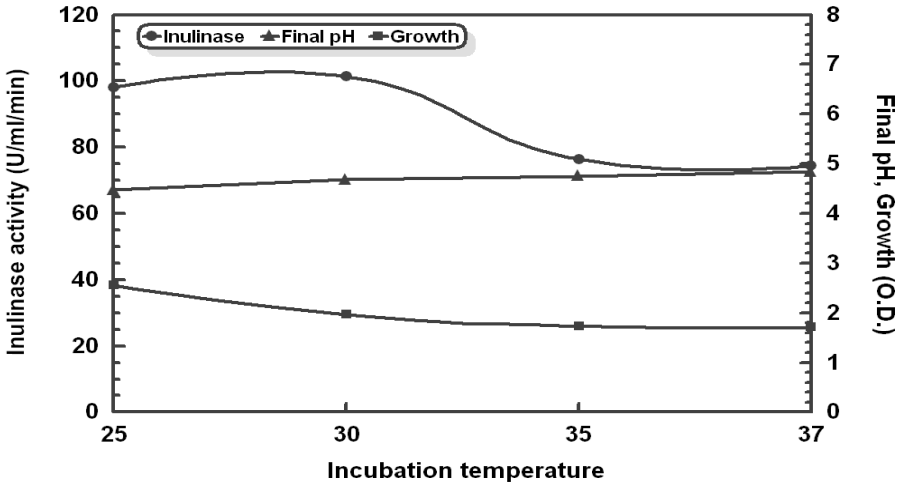

Fig. 4. Effect incubation temperature on inulinase production by *Arthrobacter* sp.

Effect of the pH of the reaction mixture on inulinase activity:

The effect of pH on inulinase activity was investigated by varying the reaction of the buffer solution in the experiment, acetate, phosphate and glycine-NaOH buffer were used to provide the pH range 4.0-9.0. The enzyme reaction mixture was incubated at 50°C. Data illustrated in (Fig.5) show that the pH range 5.0-7.5 seemed to be the optimal for activity of inulinase production by *Arthrobacter* sp. (83.80-100% relative activity). The maximum inulinase activity was found at pH 7.5. However, the enzyme activity decreases to about 55.16 % at pH 4.5. The enzyme retained 66.24 and 63.15% of its activity at pH 8.0 and 8.5, respectively. This values decreases to be 51.96% at pH 9.0. The obtained results are in agreement with those obtained by Kang *et al*. (1998) who reported that the enzyme produced by *Arthrobacter* sp. exhibited high activity at range of pH 5.0 – 10.5 and optimum was recorded at pH 7.5, while Haraguchi (2010) found that the maximum activity of the enzyme produced by *Arthrobacter ureafaciens* D13-3 was obtained at pH 5.5.

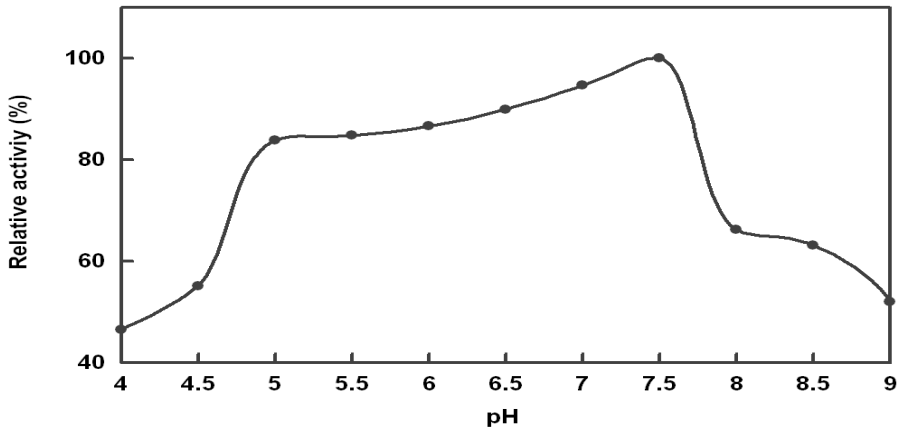

Fig.5. Effect of pH of the reaction mixture on the activity of inulinase *Arthrobacter* sp.

Effect of the temperature on inulinase activity:

The inulinase activities were examined in the temperature ranged from 30°C to 70°C using 0.1 M phosphate buffer pH 7.5. Data showed condesiderable enzymes activities at abroad range of temperatures from 30°C to 70°C with optimum at 50°C. Data illustrated in (Fig.6) show that enzyme activity increased with temperature within the range of 30°C - 50°C. A reduction in enzyme activity was observed at temperatures above 50°C. Inulinase activity showed the maximum value at 50°C, this value increased by 29.14%, 27.35%, 25.41% and 10.12% over that value obtained at 30°C, 35°C, 40°C and 45°C, respectively. The activity was then decreased up 70°C to record the lowest value with decrease percent equal to 30.62%. The obtained results are in agreement with those obtained by Kang *et al*. (1998) who found that the optimum temperature for the enzyme produced by *Arthrobacter* sp. was 50°C, also, Haraguchi (2010) found that 50°C was the

optimum temperature for enzyme activity produced by *Arthrobacter ureafaciens* D13-3.

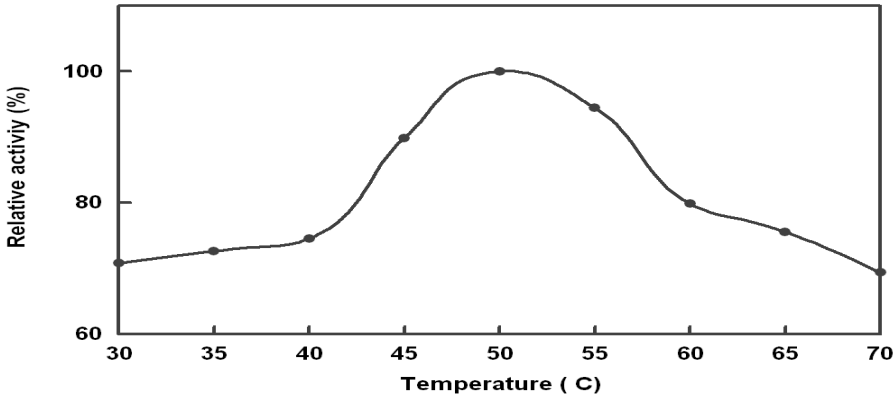

Fig.6. Effect of temperature on the activity of inulinase produced by *Arthrobacter* sp.

Effect of heat on inulinase stability:

The crude enzyme was heated at 30-70°C in the absence of substrate for 30 min. After cooling, the remaining activity was assayed in optimum assay conditions as mentioned before. The results (Fig.7) show that the enzyme did not affect by heating at 30°C for 30 min. The values are as the value of control. The enzyme was stable at 30–50°C more than 80% of their maximal activities, the enzyme retained 93.14% and 81.08% of the original activity after being heated at 40°C and 50°C for 30 min, respectively. The activity was then decreased up 70°C to record the lowest value with decrease percent equal to 55.80%. The obtained results are in agreement with those obtained by Kang *et al*. (1998).

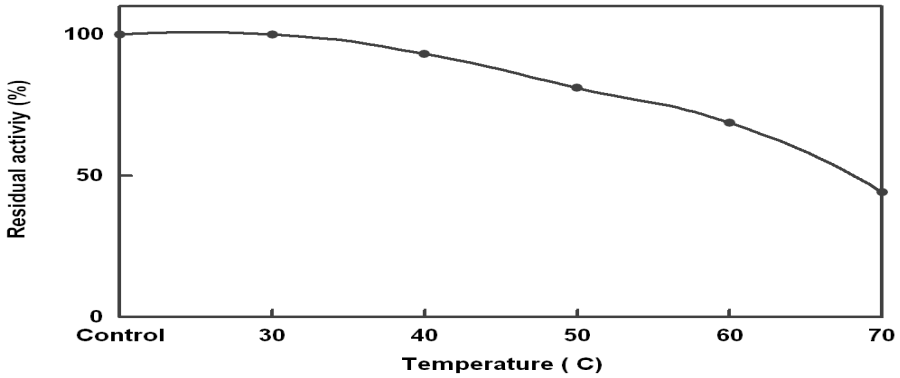

Fig.7. Effect of heat on the stability of inulinase produced by *Arthrobacter* sp.

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إنتاج وخصائص إنزيم الإنيولينيز من بكتريا الأرثروباكتر

محمد منصور قاسم – فتحى اسماعيل على حوقه – عايدة حافظ عفيفى عامر و أحمد محمود السواح.

قسم الميكروبيولوجيا – كلية الزراعة – جامعة المنصورة – المنصورة – مصر .

يعتبر الإنيولينيز الميكروبى من أكثر الوسائل المستخدمة حالياً للحصول على شراب الفركتوز من الإنيولين ، والذى يستخدم حالياً بدلاً من السكروز فى العديد من الأطعمة والأدوية والمشروبات ، استهدفت هذه الدراسة تعظيم إنتاج إنزيم الإنيولينيز من بكتريا الأرثروباكتر ، وكذلك دراسة بعض خصائص الإنزيم المنتج ، وقد أوضحت الدراسة الأتى : تم الحصول على أعلى كمية من الإنزيم بعد ٤٨ ساعة من التحضين ، لوحظ ارتفاع إنتاج إنزيم الإنيولينيز عندما استخدم السكروز كمصدر إضافى للكربون ، كانت كبريتات الأمونيوم هى أفضل مصادر النيتروجين حثاً على إنتاج الإنزيم.، تم الحصول على أعلى إنتاج للإنزيم باستخدام حجم لقاح ٥%(ح/ح) ، تركيز أيون هيدروجين ٧ ، عند درجة حرارة ٣٠ م ، كما تم دراسة خصائص الإنزيم المنتج حيث أظهر الإنزيم أقصى نشاط عند تركيز أيون هيدروجين ٧.٥ ، ودرجة حرارة ٥٠ م ، كما احتفظ الإنزيم المنتج بأكثر من ٨٠% من نشاطه بعد تسخينه لمدة ٣٠ دقيقة على درجات حرارة من ٣٠-٥٠ م.

قام بتحكيم البحث

أ.د / ساميه بيومى — كلية الزراعة – جامعة المنصورة

أ.د / حامد السيد ابو على — كلية الزراعة – جامعة بنها