Effect of cultural conditions on the production of glucoamylase from *Aspergillus ustus*

تأثير الظروف المزرعية في إنتاج إنزيم الجلوكواميليز من الفطر *Aspergillus ustus*

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Abstract
A strain of *Aspergillus ustus* was able to produce glucoamylase in a medium composed of date juice as the main carbon source supplemented with maltose as an inducer and additional carbon source, NH_4CL or $(NH_4)_2SO_4$ as a nitrogen source and KH_2PO_4 and $MgSO_4.7H_2O$ as nutrients. The effect of cultural conditions on the glucoamylase production was studied using this medium.Results revealed that date juice of 0.1% total carbohydrates, maltose 1.5% (W/V), NH_4Cl 0.2% supplemented with the above mentioned nutrients, adjusted at pH 4.5-5.0 and incubated at 30 ºC for 72 hrs under static conditions was the most efficient and gave higher enzyme yield.
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المستخلص
أظهرت عزلة من الفطر *Aspergillus ustus* قابلية عالية على إنتاج إنزيم الجلوكوأميليز باستخدام وسط مكون من عصير التمر كمصدر رئيسي للكربون مدعم بالمالتوز كمادة حاثة وكمصدر كربوني إضافي ، وكلوريد الامونيوم أو كبريتات الامونيوم كمصدر نيتروجيني وفوسفات البوتاسيوم ثنائية الهيدروجين وكبريتات المغنسيوم المائية كمغذيات.
درس تأثير الظروف المزرعية في إنتاج إنزيم الكلوكوأميليز باستخدام هذا الوسط ، وأظهرت النتائج إن عصير تمر ٠.١% (كربوهيدرات كلية) المدعم بالمالتوز ١.٥% (وزن/حجم) وكلوريد الامونيوم ٠.٢% والمغذيات المشار إليها أعلاه ، ذو رقم هيدروجيني ٤.٥-٥.٠ والمحضن على درجة حرارة ٣٠ º م لمدة ٧٢ ساعة تحت ظروف ساكنة كان أكثر كفاءة وأعطى أعلى حصيلة من الأنزيم .

Introduction

Glucoamylase (α-1,4 glucan glucohydrolase, EC 3.2.1.3) is one of the most important enzymes used in food processing as well as in commercial production of glucose from starch (1).Although, it is produced by various microorganisms, including bacteria, yeast, it is common in fungi such as *Aspergillus sp*., *Rhizopus sp., Endomyces sp.*, *Monascus sp.*, *Mucor rouxianus* and *Penicillium oxalicum*.Although, genetic manipulation has received considerable attention due to its role in increasing the expression levels of a large number of microbial enzymes, including glucoamylase, traditional screening procedures make possible to find new attractive wild microorganisms able to produce useful enzymes (2).

In addition to that, cultural conditions play an important role in changing quantity and type of fermentation products. Enzyme substrates are typical inductors with different potentials for induction, as in the case of glucoamylase synthesis induced by maltose, when compared with induction mediated by starch (3, 4, 5).

Optimisation of composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* was studied (6). Another studies indicated that urea was a suitable nitrogen source for glucoamylase synthesis in submerged and solid state fermentation (4, 7).

The purpose of this work was to investigate the ability of *A. ustus* to produce glucoamylase as well as the effect of cultural conditions on the enzyme production from this fungus.

Materials and Methods

Microorganism:

Aspergillus ustus was obtained from Dep. of Biology, College of Science, University of Karbala. It was maintained on slants and plates of Sabouraud's dextrose agar and reactivated on the same medium at 30 ºC for 2 weeks.

Preparation of medium, cultivation and production of glucoamylase

Date juice was prepared by adding one liter of distilled water (70-80 ºC) to one Kilogram of Zahdi dates and left overnight at room temperature (8). The obtained juice was filtered and the filtrate was diluted to the desired concentration (total carbohydrates) using the phenol-sulfuric acid method (9). The diluted juice was used in the subsequent experiments.

The medium used in checking the ability of the fungus *A. ustus* to produce glucoamylase was: date juice 0.5%, maltose 1%, NH_4Cl 0.2%, KH_2PO_4 0.2% and $MgSO_4.7H_2O$ 0.2%. It was adjusted to pH 4.5 and then divided into 250 ml flasks containing 50 ml medium. After autoclaving, the medium was inoculated with one 6 mm diameter core (≈ 1 x 10^{7} spores/ml) from the reactivated fungus and incubated at 30 ºC for 72 hours in a rotary shaker at 120 rpm. At the end of incubation, flasks were drawn and glucoamylase activity and protein were determined. Then the following parameters were studied:

Effect of growth mode: *A. ustus* was grown on the production medium described above at static condition, 40, 80 and 120 rpm for 72 hours.

Effect of incubation period: Fermentation was allowed to proceed for 5 days under static conditions to monitor glucoamylase production. Flasks were harvested at 24 hrs intervals.

Effect of carbon source: Date juice and maltose were used as carbon sources for enzyme production from *A. ustus*.

Concentrations of 0.1, 0.3, 0.5, 1.0, 1.5, 2 and 2.5% total carbohydrates were used from date juice, while concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v) were used from maltose.

Effect of nitrogen source: NH_4Cl and $(NH_4)_2SO_4$ were supplemented as individual components to the production media at concentrations of 0.1, 0.2, 0.4 and 0.6% (w/v) to check their effect on glucoamylase production.

Effect of initial pH: The production medium was adjusted to pH 3.0, 3.5, 4.0, 4.2, 4.5, 4.7, 5.0, 5.5, 6.0 and 6.5 using HCl or NaOH as required.

Glucoamylase assay: Enzyme activity was measured according to the method described by Specka *et al.* (1991) (10) with some modification. Substrate was maltose 2% (w/v) in sodium acetate buffer (0.1 M, pH 5.0). After incubation the mixture (0.3 ml culture filtrate and 0.3 ml substrate) for 15 min at 45º C, the reaction was stopped by boiling for 15 min., too. Glucose liberated by glucoamylase action was measured enzymatically using glucose oxidase/ peroxidase kit according to the instructions of the supplied company.

One unit of glucoamylase is defined as the amount of enzyme that liberates 1 μmole of glucose/min. under the assay conditions.

Protein determination: Protein concentration in culture filtrate was determined by Bradford method (11) using bovine serum albumin as a standard.

Specific activity is expressed as units/mg of protein.

Results and Discussion

In order to check the ability of *A. ustus* for glucoamylase production, a medium of date juice supplemented with additional carbon, nitrogen source and some nutrients was used. Results revealed that the fungus was able to produce glucoamylase with specific activity of 20.23 units/mg protein. The result indicates also the efficiency of the above medium in the production of the fungal enzymes.

Effect of growth mode: The growth mode was determined using static and shake flask techniques. Specific activity of glucoamylase obtained by static flask was 1.97, 3.33 and 3.38 fold than that with 40, 80 and 120 rpm, respectively (Fig. 1). For this reason, static conditions were used in all following experiments. This growth mode led to significantly higher yields of glucoamylase from *Aspergillus tamarii* than those obtained using shaking culture (2).

Static conditions were also used in the enzyme production from *Scytalidium thermophilum* 15.8 (12). While shaking at 170 and 130 rpm were used in glucoamylase production from *Aspergillus niger* and *Humicola grisea*, respectively (13, 14).

Effect of incubation period: A typical time course for the production of glucoamylase is presented in Fig. 2. Enzyme production started at 24 hrs, reaching its maximum after 72 hours of incubation, which decreased with further incubation.

Glucoamylase specific activity obtained after 72 hrs was 1.94, 1.17, 1.64 and 2.3 fold than that with 24, 48, 96 and 120 hrs of incubation, respectively. Short and long incubation periods were used in glucoamylase production. *Aspergillus awamori* was used to produce the enzyme at maximum level after 40 hrs of fermentation (15). While maximum production of the enzyme from *Aspergillus niger* and *Scytalidium thermophilum* occurred after 96 and 168 hrs respectively (12, 13).

The present study included also monitoring pH changing during incubation period. pH dropped to 2.77 after the first 24 hrs and became nearly constant during the four subsequent days of incubation (Fig. 2). The decrease in pH value may be attributed to the formation of some organic acids resulted from high consumption of the available carbohydrates in the medium used in this study (16).

Effect of carbon source:

Effect of date juice concentration: Fig. 3 illustrates the effect of date juice concentration on glucoamylase production from *A. ustus*. However, it is clear that enzyme specific activity increases with the decrease of date juice concentration. Date juice of 0.1% total carbohydrates exhibited highest specific activity with 48.46 units/mg protein, so it was chosen as the best concentration and used in all the following experiments.

Zahdi dates contain 67.1% total sugars calculated as 57.5% monosaccharides and 9.6% disaccharides (17).

The consumption of monosaccharides enables the fungus to form good growth which would help it in maltose degradation and then glucoamylase production.

Studies about the use of date juice in glucoamylase production from fungi are not available, but there are more than one study about the use of this carbon source in the production of other fungal enzymes such as polygalacturonase from *Mucor pucillus* (18) and protease (19).

Effect of maltose concentration: Maltose was supplemented to the date juice medium in order to stimulate glucoamylase production. Highest production was observed with maltose 1.5% where specific activity obtained at this concentration was 3.63, 2.28, 1.15, 1.21 and 1.22 fold than that with control, 0.5, 1.0, 1.5, 2.0 and 2.5%, respectively (Fig. 4).

Many studies mentioned the use of maltose as an inducer in the production of glucoamylase. Maltose 1% was used in the production of this enzyme from *Aspergillus tamari* (2). In contrast, high production of the enzyme from *Thermomyces lanuginosus* ATCC 34626 was obtained by using dextrin (6).

Effect of nitrogen source: Two nitrogen sources [NH_4Cl and $(NH_4)_2SO_4$] were used at various concentrations to compare their influence on glucoamylase production from *A. ustus*. Results revealed that NH_4Cl was the best in comparison with $(NH_4)_2SO_4$.

The best concentration of NH_4Cl was found to be 0.2% followed by 0.1%, control, 0.4% and 0.6%, respectively (Fig. 5). Highest concentrations of NH_4Cl used in this study (0.4 and 0.6%) gave specific activity less than that with control. As regards$(NH_4)_2SO_4$, low concentrations of this nitrogen source (0.1 and 0.2%) have no influence on the enzyme production in comparison with control. While concentrations of 0.4 and 0.6% gave specific activity less than that with control.

The result obtained from the present study was in agreement with previous study which demonstrate the efficiency of using NH_4Cl 0.2% in glucoamylase production from *A. niger* (13). In contrast, the obtained result was not in agreement with the finding of Nguyen *et al.* (2000) (6) who reported that among seven nitrogen sources, L-asparagine 0.75% was found to be the best in glucoamylase production from *Thermomyces lanuginosus* ATCC 34626.

Nitrogen may be supplied in a number of different forms such as NO_3, NO_2^-, N_2, NH_4^+ and R-NH_2 and microorganisms vary in their abilities to assimilate nitrogen. Moreover, most microorganisms can use NH_4^+ as a sole nitrogen source (20).

Effect of initial pH: Studies on the effect of initial pH on the enzyme production were carried out within the pH range 3 to 6.5. The results illustrated in Fig. 6 indicate that maximum enzyme production occurred within the pH range 4.5-5.0.

Similar results were obtained during glucoamylase production by *Thermomyces lanuginosus* where optimum pH was found to be 4.9 (6).

Another study demonstrated that the production of amylase from *Aspergillus tamarii* was tolerant to a wide range of initial culture pH values (from 4 to 10) (2).

Most organisms have a fairly narrow optimal pH range. The optimal pH must be empirically determined for each species (20).

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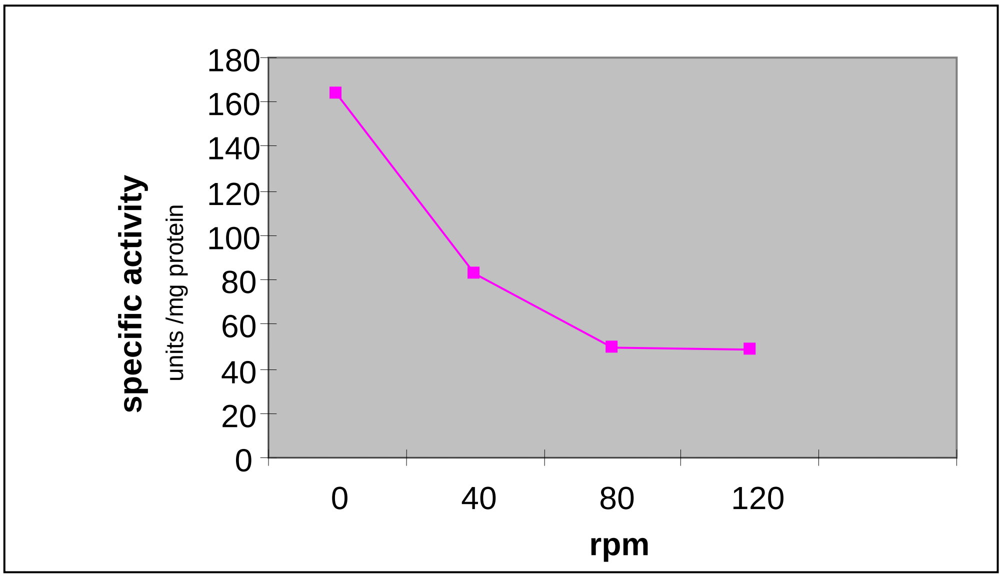

Fig.1. Effect of growth mode on glucoamylase production from *A. ustus*

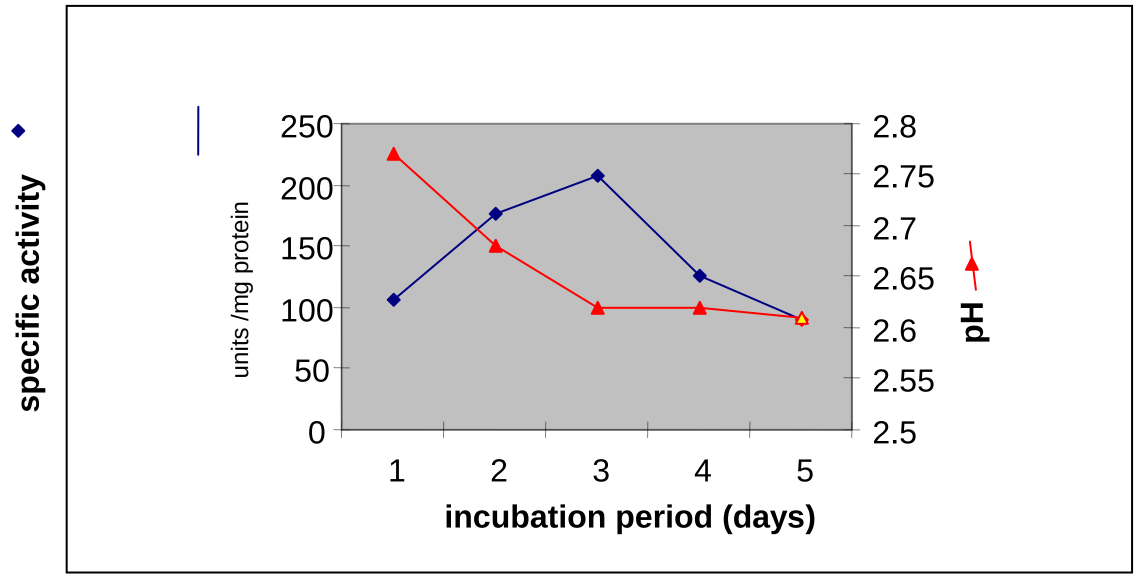

Fig.2. Effect of incubation period on glucoamylase production from *A. ustus*

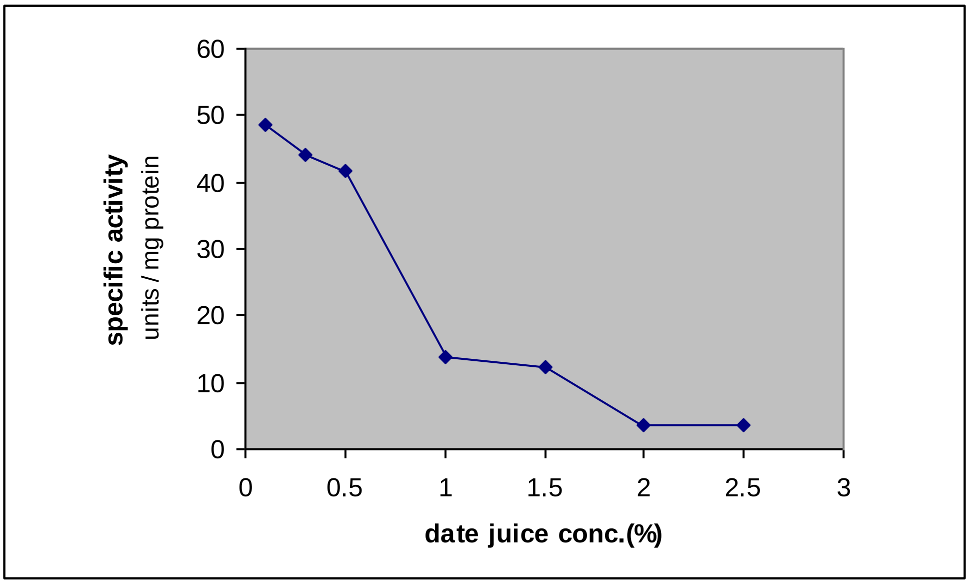

Fig.3. Effect of date juice concentration on glucoamylase production from *A. ustus*

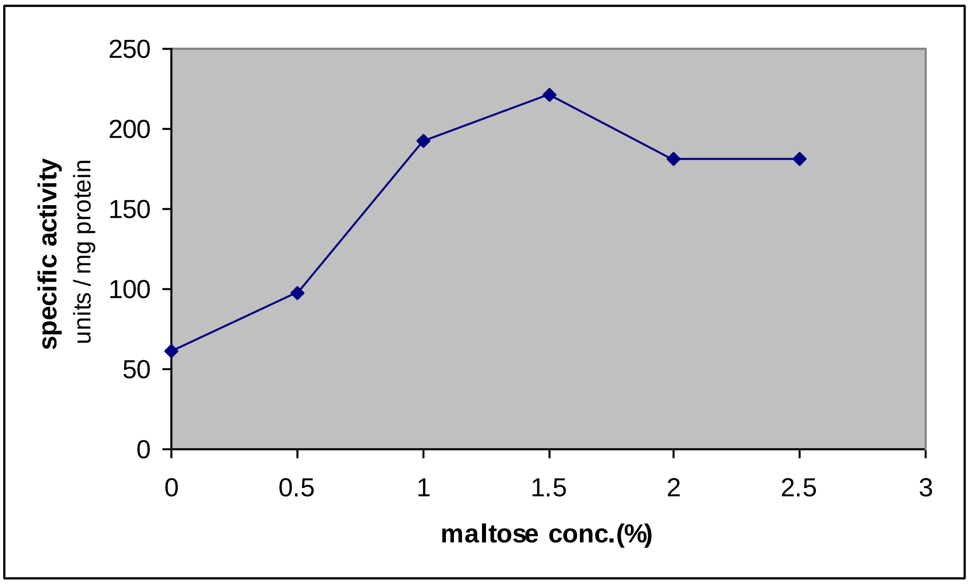

Fig.4. Effect of maltose concentration on glucoamylase production from *A. ustus*

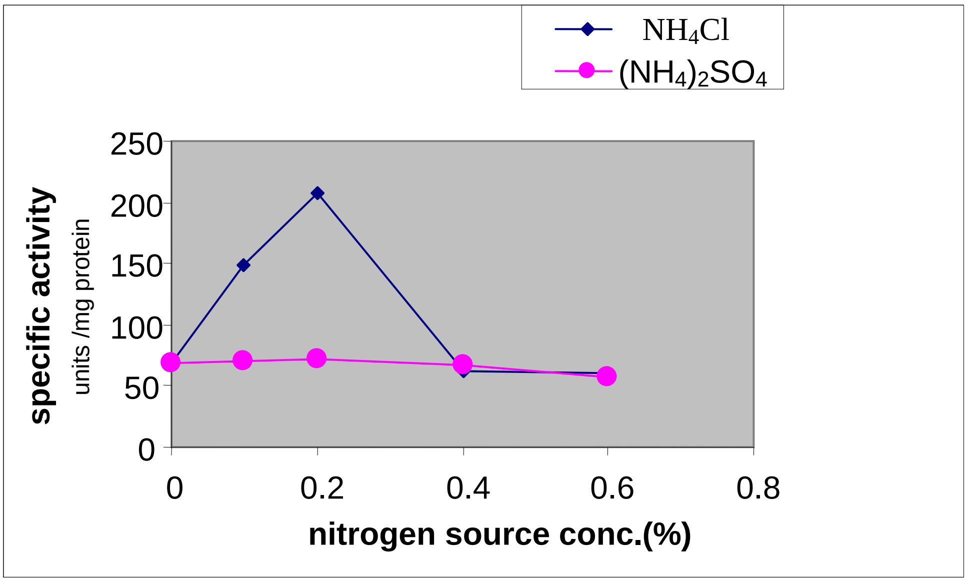

Fig.5. Effect of nitrogen source concentration on glucoamylase production from *A. ustus*

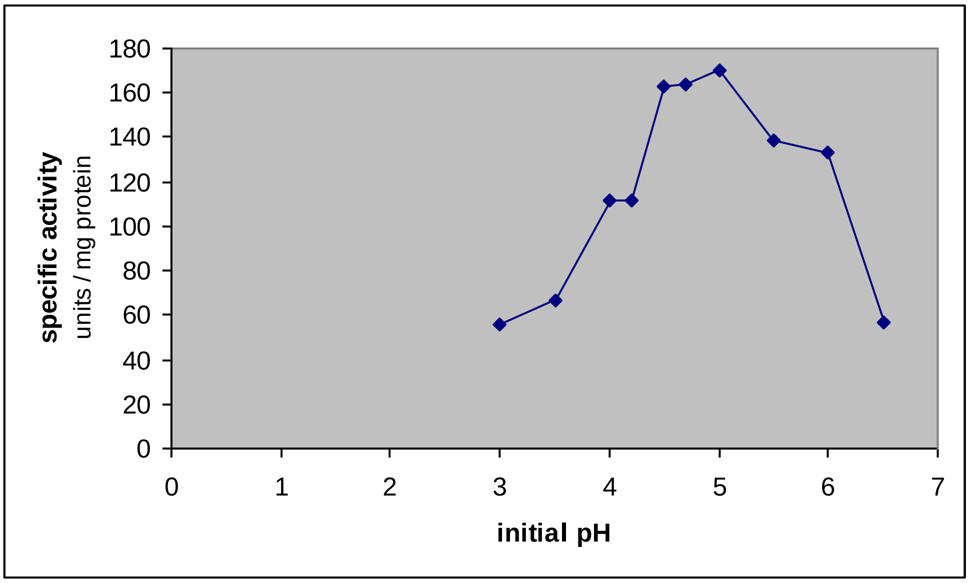

Fig.6. Effect of initial pH on glucoamylase production from *A. ustus*