ISOLATION, CHARACTERIZATION AND IMMOBILIZATION OFGLUCOSE OXIDASE FROM *ASPERGILLUS NIGER* 1- ISOLATION AND PURIFICATION

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ABSTRACT

Two strains of *Aspergillus niger* were propagated on Czapek broth medium using submerged culture in a shaker incubator at 30 °C and 125 rpm to produce glucose oxidase. The strain ATCC 166808 was selected because it gave higer enzymatic activity than the strain IMI 84305. The presence of glucose and sucrose in the medium at concentration of 20 gm/l for each enhanced maximum enzyme production after 3 days of incubation. The enzyme was found to be intracellular and the sonication time required to rupture the cellular walls was 15 minutes at 10 KHz. The enzyme was precipitated from the cell-free extract by the addition of 2 volumes of cold acetone and was partially purified by gel filtration chromatography using Sephadex G-150 followed by ion-exchange chromatography in a DEAE-cellulose column. A specific activity of 8528.6 units/mg protein and a purification of 53.2 folds with a yield of 47.6% was achieved.

INTRODUCTION

Glucose oxidase (β-D-glucose: oxygen oxidoreductase, E.C. 1.1.3.4) was discovered in 1928 by Muller in molds of the genera Aspergillus and Penicellium. After that, the enzyme was isolated from other fungi. This enzyme has a high specificity toward β-D-glucose which is oxidized -in the presence of molecular oxygen- into gluconic acid and hydrogen peroxide (Hayashi and Nakamura, 1976 and Rothberg *et al*., 1999). Glucose oxidase has a significant economical importance. It is used for the production of gluconic acid, for the removal of glucose and / or oxygen from some food products such as dried eggs, fruits, juices, meat and milk products in order to prevent oxidation and / or browing. It is also used – in conjugation with peroxidase – for specific determination of glucose and in biosensors manufacturing (El-Enshasy et al., 2001).

A.niger is considered to be the main producer of commercial glucose oxidase and many other enzymes, organic acids, growth regulators and other compounds which are generally recognized as safe (GRAS). Several carbon sources were used to propagate this mold such as hydrolyzed corn starch (Kona *et al*., 2001), glucose, sucrose, and molasses (Hatzinikolaou and Macris, 1995) and many other hexoses and pentoses (El-Enshasy *et al*., 2001). Many workers had studied the various methods of extraction and purification of the enzyme from cell-free extract (Fiedurek and Gromoda, 1996; Kalisz *et al*., 1997; Torres *et al*., 1998; Kim *et al*., 2001; Kona *et al*., 2001). The aim of the present work was to study some conditions required for the production of

glucose oxidase from the mold *A. niger* propagated on a submerged culture followed by isolation and partial purification of the enzyme.

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MATERIALS AND METHODS

Microorganisms: Two *Aspergillus niger* strains (ATCC 166808 and IMI 84305) were obtained from Food Sci. and Biotechnology Dept., College of Agriculture and Forestry, Mosul University.

Growth conditions: Cultures were propagated on potato dextrose agar slants at 30 °C for 7 days. Spore suspension was obtained by adding 5 ml of sterile distilled water to the slant surface and the spores were removed with a loop. The spore suspension was transferred to a sterile test tube and 1% of tween 80 was added. Spore count was determined using a hemocytometer. An inoculum size containing about 1.5×10^6 spores was transferred to 500 ml Erlenmeyer flasks containing 150 ml of sterile Czapek broth medium. The flasks were incubated at 30 C° in a shaker incubator operating at 125 rpm. The enzyme activity in the cell-free extract was assayed everyday throughout the incubation period.

To study the effect of the type and concentration of carbon sources on the enzyme production, glucose and sucrose were added to the growth medium at concentrations of (10 + 10), (20 + 20), (30 + 30), and (40 + 40) g/l. Also each sugar was used separately using the above concentrations.

Isolation and purification of the enzyme: At the end of the incubation period, the mold biomass was harvested by filtration through Whatmann No. 1 filter paper, washed by potassium phosphate buffer (0.1 M, pH 6) and suspended in the same buffer (75 g biomass + 25 ml buffer), The suspension was freezed at -15 C° for 24 hours and then subjected to sonication at 10 KHz to rupture the cellular walls, Cell-free extract was obtained by centrifugation at 9000×g for 10 minutes using a refrigerated centrifuge. The enzyme was then precipitated by adding cold acetone (1 – 2 volumes) with gentle stirring for 1 hour. The mixture was centrifuged at 9000 × g for 20 minutes and the precipitate was suspended in a small amount of phosphate buffer and applied on the top of a column (1.6 × 65 cm) of Sephadex G-150 that had been equilibrated with phosphate buffer (0.05 M, pH 6). The column was then eluted with the same buffer at a flow rate of 12 ml / hour. Enzyme contained fractions of 2 ml each were pooled and applied to the top of a column (1.6 × 25 cm) of DEAE-cellulose equilibrated with the same buffer. The column was washed with a linear NaCl gradient of 0 – 0.7 M in phosphate buffer at a flow rate of 24 ml / hour. Active fractions of 3 ml each were pooled.

Assay of glucose oxidase activity: The enzymatic activity was measured by the method described by Bergmeyer *et al.*(1970) by measuring the increase of absorbance at 436 nm due to the oxidation of guaiacol in the reaction mixture containing peroxidase. Enzyme unit is defined as the amount of the enzyme which liberate one micromole of hydrogen peroxide per minute under the experiment conditions.

Protein determination: Protein content was estimated according to the method of Lowry *et al*. (1951) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Choice of mold strain: The two *A. niger* strains (ATCC 166808 and IMI 84305)were tested for glucose oxidase productability through their propagation on Czapek broth medium in a submerged culture at 30 C° and 125 rpm for 3 days. At the end of the incubation period, the enzymatic activity was assayed in the cell-free extract and in the cultural filtrate of both strains. Also the wet biomass was measured.

Table (1): Glucose oxidase activity and biomass weight in two strains of *A. niger.*

Strain	Volume of cell–free ext.(ml)	Enz. activity in cell–free ext.(unit/ml)	Enz. activity (unit/l medium)	Wet biomass weight (g/l edium)	Enz.activity in cult. filtrate (unit/ml)
A.niger ATCC 166808	110	119	13090	260	4.3
A.niger IMI 84305	95	103	9785	216	2.4

As shown in the Table (1), the enzymatic activity in the cell-free extract and the biomass weight for the strain ATCC 166808 was higher than those for the strain IMI 84305. The first strain produced 13090 enzyme units/l of the medium, while the second strain produced 9785 enzyme units/l. It was also noticed that the enzyme concentration in the cultural filtrate of both strains was low so that it was neglected. This indicates that most of the enzyme was intracellular.

Effect of incubation time on enzyme production: This was estimated by the incubation of the strain ATCC 166808 on Czapek broth for 7 days and assaying glucose oxidase activity in the cell-free extract every day.

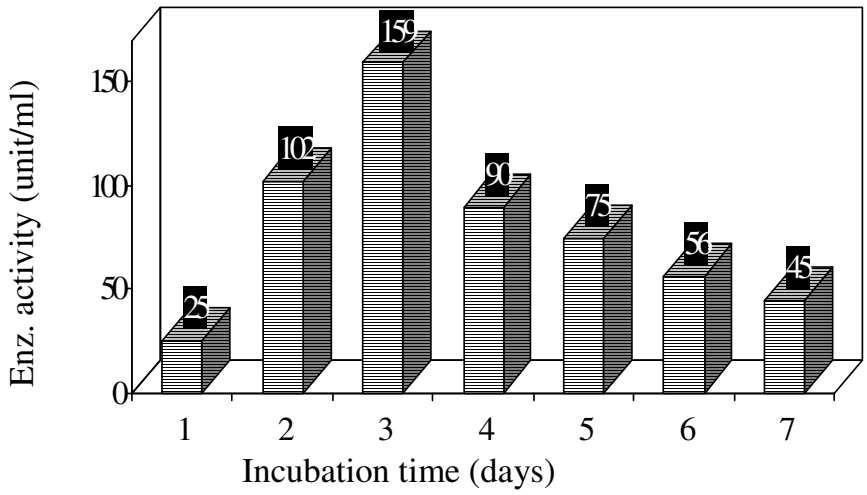

Fig. (1): Effect of incubation time on glucose oxidase production by *A. niger*

Fig. (1) shows that the activity after one day was very low and began to increase significantly through the second day of incubation (102 units/ml). Maximum production of the enzyme was reached after 3 days (159 units/ml) and after that it began to decrease gradually. This decrease may be due to the raising of the medium acidity and the accumulation of some toxic metabolites that may affect the enzyme production and the stability of the produced enzyme. The lysis of cells and the leakage of the enzyme to the cultural medium could be another reason because it was noticed that the decrease in enzyme activity in the cell-free extract was accompanied by increased activity in the cultural filtrate especially through the sixth and seventh days of incubation. However, a similar result was achieved by Hellmuth et al., (1995) who used a genetically engineered *A. niger* strain and found that maximum enzyme production was obtained after 60 hours of incubation. After 100 – 125 hours, the cells began to lyse gradually with increase of respiration, but they noticed that living cells utilize the components of the lysed cells and the produced gluconic acid untill 190 hours of incubation. In an other mutant strain of *A. niger*, the maximum glucose oxidase production was obtained after 3 – 4 days of incubation (fiedurek and gromoda, 1996).

Effect of carbon source and concentration on enzyme production: The effect of the addition of glucose, sucrose and their mixture on *A. niger* productivity of glucose oxidase was studied. After 3 days of incubation, the enzymatic activity in the cell-free extract was measured (Fig 2).

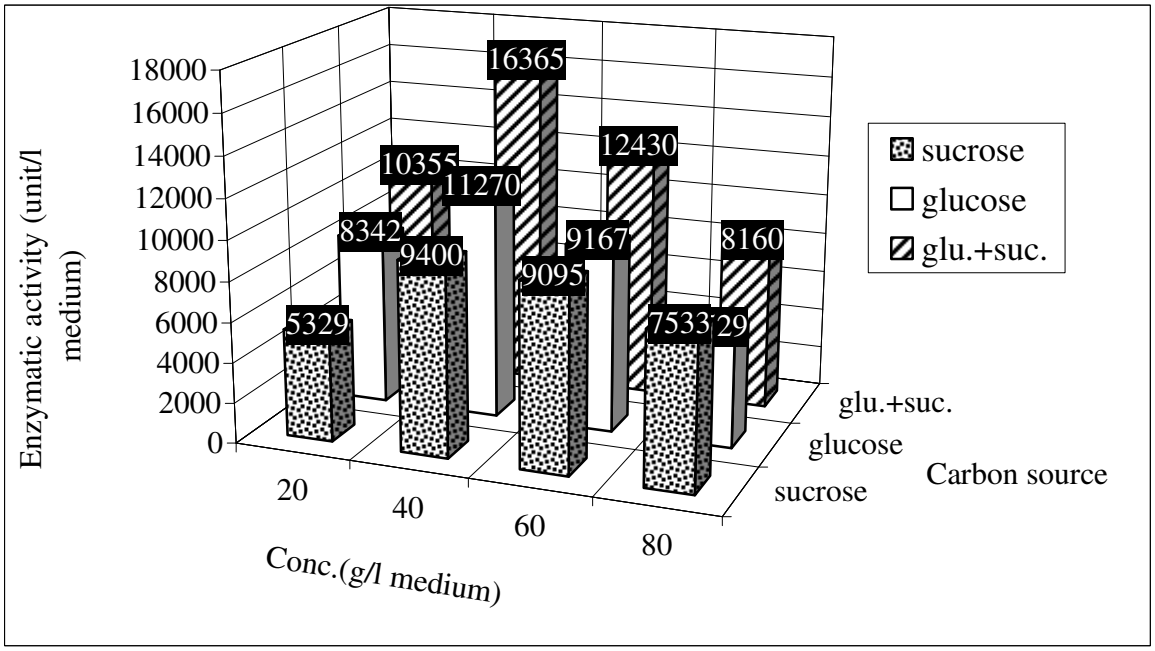

Fig. (2): Effect of carbon sources on glucose oxidase production by *A. niger*.

The highest enzyme production (16365 units/l) was achieved when a mixture of the two sugars was added to the propagation medium at concentration of (20 + 20) g/l. A lower production was obtained by the addition

of glucose alone (11270 units/l) followed by sucrose (9400 units/l) using the same concentration (40 g/l).

This may be explained by the fact that glucose acts as a rapid source of energy which enhances the growth of the cells at earlier stages untill the cells begin to produce their own enzymes and hydrolyze sucrose. However, the addition of glucose alone at high concentration in the early growth stages may inhibits the synthesis of the enzyme due to its catabolic repression effect on the mold cells. On the other hand, the use of sucrose as a unique carbon source may reduce growth rate especially at early growth stage due to absence of its hydrolyzing enzyme (invertase). Thus, the presence of glucose together with sucrose in the medium could be the best.

The effect of sugars concentrations on the enzyme production was also studied. The concentrations used were 20, 40, 60, and 80 g/l. The best concentration was found to be 40 g/l for all sugars used.

Previous studies did not mention the use of carbon source mixtures for glucose oxidase production. However, Hatzinikolaou and Macris (1995) used some carbon sourses such as molasses, sucrose, fructose and starch. They found that sucrose had given the highest enzyme productivity from *A. niger*. Kona *et al.*(2001) found that maximum glucose oxidase productivity was achieved when sucrose was added in a concentration of 70 g/l, but when glucose was used, 20 g/l was found to be the best concentration. In the case of *Penicillium pinophilium*, the highest productivity was obtained when sucrose was added at concentration of 40 g/l (Rando *et al.*, 1996).

Effect of sonication time on the efficiency of the enzyme extraction: The enzyme was extracted from cells by freezing the mold mycelium for 24 hours followed by sonication at 10 KHz.

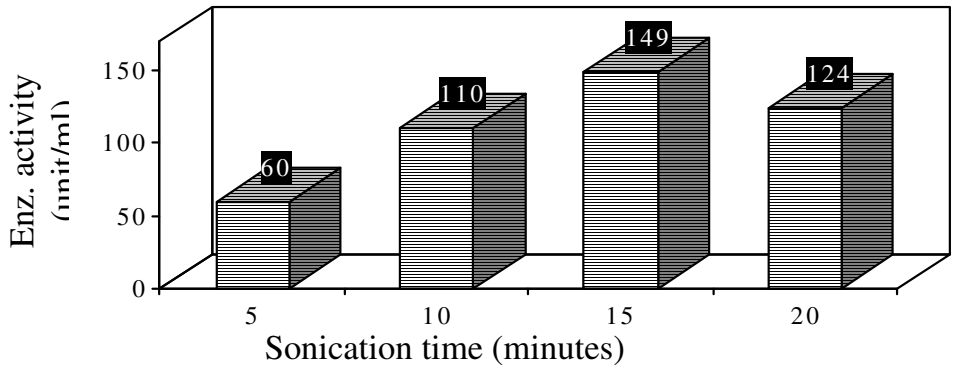

Fig. (3): Effect of sonication time on the extraction efficiency of glucose oxidase from *A. niger* cells

Fig (3) shows the effect of sonication time on the efficiency of rupturing the cellular walls which was expressed by assaying the enzymatic activity in the cell-free extract. 15 minutes was required to reach maximum activity. Upon exceeding this period, the activity began to decrease. This may be due to the denaturing effect of sonicator vibrations on the enzyme molecules. For this

reason, other assistant techniques, such as freezing and blending, could be used prior to sonication to reduce its denaturing effect (Hatzinikolaou and Macris, 1995; Pluschkell *et al*., 1996).

Isolation and purification of glucose oxidase: The enzyme was precipitated from the cell-free extract by adding cold acetone (-15 C°) with gentle stirring. Three concentrations of acetone were tested (1, 1.5, 2 volumes). The precipitate was dissolved in 10 ml of phosphate buffer and the enzymatic activity and protein content were assayed. The enzymatic activities in the precipitate were 517, 1003 and 1479 units/ml when acetone concentrations of 1, 1.5 and 2 volumes were used. Table 2 shows that the addition of two volumes of acetone gave the highest enzymatic activity in the precipitate (1479 unit/ml) with a specific activity of 2958 unit / mg protein. The enzyme yield of this stage was 81.9%.

Gel filtration and ion exchange chromatography: After acetone precipitation, the enzyme was subjected to partial purification through two steps. In the first, the enzyme solution was applied to a gel filtration column of Sephadex G-150.

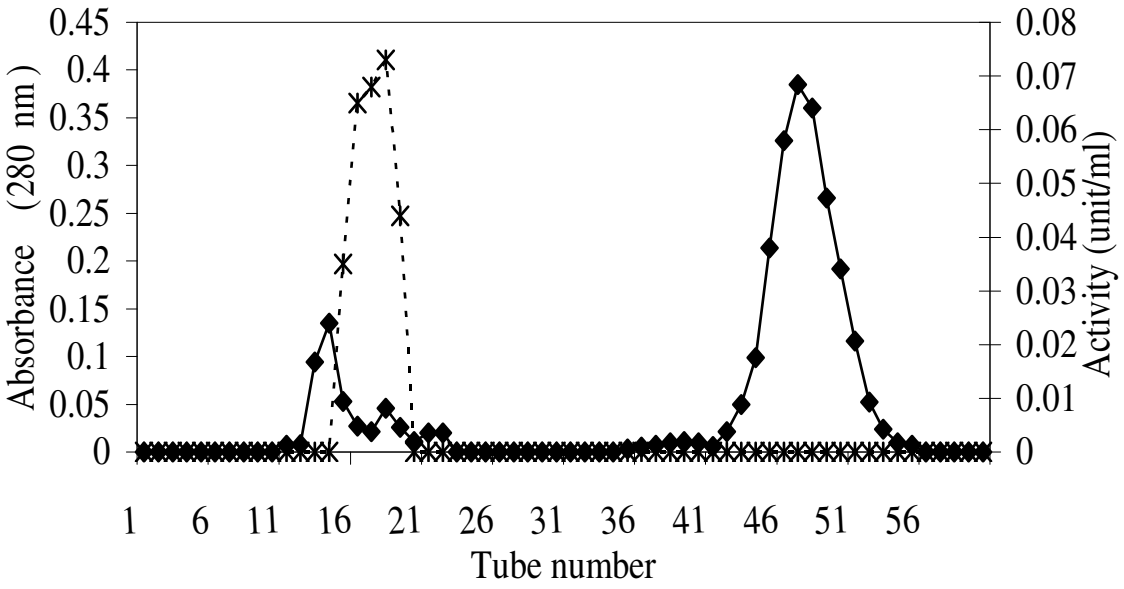

Fig. (4):Gel filtration of glucose oxidase on Sephadex G-150 with potassium phosphate buffer(0.05M,pH6).(◆—◆ protein, ✳--✳ activity).

As shown in the Fig. (4), the void volume of the column was 28 ml and the enzyme was eluted in the fractions 16 – 20 (each fraction was 2 ml). The enzymatic activity after this step was 198.5 unit/ml and the specific activity was 8270.8 units/mg protein with a yield of 55% and a purification of 51.6 folds. The second step of purification involved ion-exchange chromstography

in which the active fractionsthat pooled from the previous step were applied to a column of DEAE-cellulose and eluted with 50 ml phosphate buffer (column volume) followed by linear gradient elution with sodium chloride (0 – 0.7 M). The enzyme was eluted at 0.4 M of NaCl. Active fractions (24 ml) were pooled and the enzymatic activity and protein content were assayed. The activity was found to be 119.4 units/ml and the specific activity was 8528.6 units/mg protein. A purification of 53.2 folds with a yield of 47.6% was achieved (Table 2).

As shown in the Fig. (5), there is no protein peak other than that of the glucose oxidase. This may explain why the ion-exchange step did not give a significant increase in the specific activity and this might be due to that the gel filtration step was sufficient to bring the enzyme to a good degree of purification.

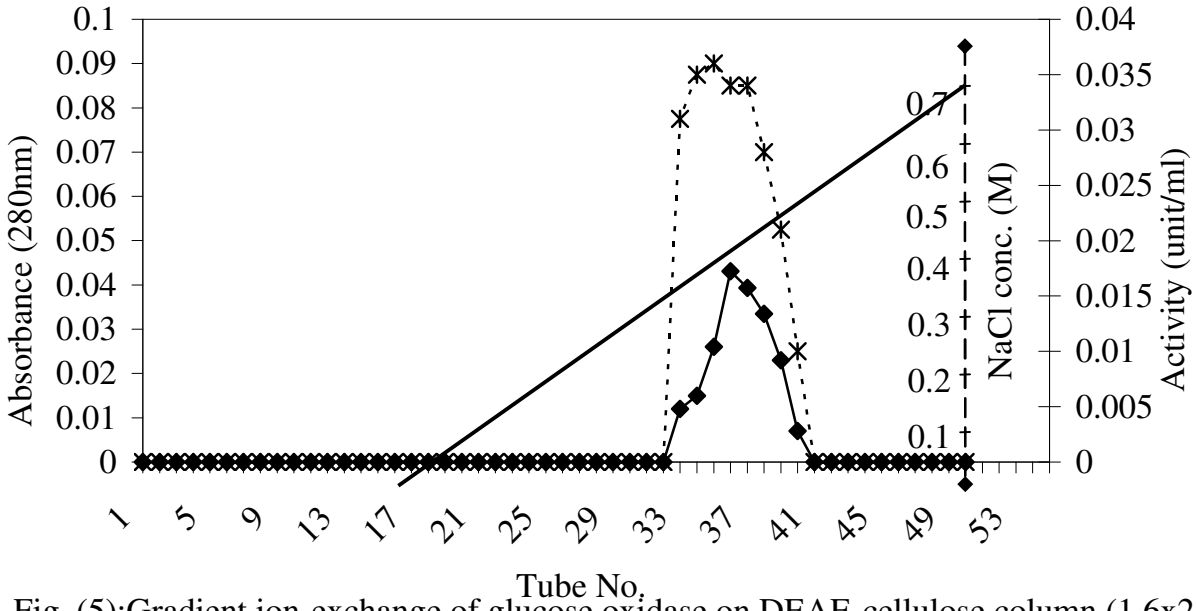

Fig. (5):Gradient ion-exchange of glucose oxidase on DEAE-cellulose column (1.6x25c with pot. phosph. buffer (0.05M,pH6) containing NaCl(0-0.7M).

Table (2): Purification steps of glucose oxidase from *Asp. niger*

Purification step	Volume (ml)	Enzymic activity (unit/ml)	Protein conc. (mg/ml)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Cell-free ext.	115	175	0.98	160.2	1	100
Acetone ppt	10	1479	0.5	2958	18.5	81.9
Sephadex G-150	50	198.5	0.024	8270.8	51.6	55
DEAE-cellulose	72	119.4	0.014	8528.6	53.2	47.6

عزل و توصيف و تثبيت أنزيم الكلوكوز أوكسيديز من فطر *Aspergillus niger*
١- العزل و التنقية

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الخلاصة

تم تنمية سلالتين من فطر *Aspergillus niger* (ATCC 166808 و IMI 84305) في مزرعة مغمورة من وسط زابك المغذي باستخدام محضن هزاز بدرجة حرارة ٣٠ مْ و سرعة ١٢٥ دورة في الدقيقة لغرض إنتاج أنزيم الكلوكوز أوكسيديز وقد أعطت السلالة الأولى (ATCC 166808) إنتاجا" إنزيميا" أفضل من السلالة الثانية لذلك تم اختيارها لإكمال الدراسة. أدت إضافة مزيج الكلوكوز و السكروز بتركيز (٢٠+٢٠) غم/لتر إلى زيادة الإنتاج. تم الحصول على أقصى إنتاج من الإنزيم بعد ثلاثة أيام من التحضين. كان معظم الأنزيم المنتج من النوع الداخل خلوي وتم استخلاصه من الخلايا باستخدام الموجات فوق الصوتية بذبذبة مقدارها ١٠ كيلو هيرتز ولمدة ١٥ دقيقة. رسب الانزيم من المستخلص الخلوي بإضافة حجمين من الأسيتون البارد. أجريت تنقية جزئية للانزيم باستخدام كروماتوكرافي الترشيح الهلامي في عمود من هلام السيفادكس (G-150) تلاه كروماتوكرافي التبادل الأيوني في عمود من DEAE-cellulose ، وقد أمكن الحصول على فعالية نوعية مقدارها ٨٥٢٨ وحدة انزيمية / ملغم بروتين ونقاوة ومقدارها ٥٣.٢ مرة وبحصيلة بلغت ٤٧.٦٪.

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