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Production, Purification and Characterization of Cellulose from Local Isolate of *Pantoea* spp

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Abstract
Soil samples from fields cultivated with barley and wheat in addition to samples from spoiled orange and apple fruits and carrot roots were collected with the aim to isolate cellulase producing bacterial strains. Bacterial isolates obtained from these samples were grown on a selective medium containing carboxymethyl cellulose (CMC) as a sole source for carbon and energy. Results showed that nine isolates out of fifty were able to produce cellulase.The specific activity of cellulase in culture filtrate of the most efficient isolate was 1.601 u/mg protein.This isolate was identified according to its morphological characteristics and biochemical tests, and then by using Api 20-E and VITEK-II identification systems and was identified as *Pantoea dispersa*. Cellulase produced by *P. dispersa* was purified throughout four purification steps including ammonium sulfate precipitation, dialysis followed by purification with ion exchange chromatography using DEAE-cellulose, and then gel filtration throughout sephadex-G200. Purification results showed that the specific activity of the purified enzyme was 1181.8 u/mg protein with a purification fold and yield 115.2 and 26.6% respectively. Purified cellulase from *Pantoea dispersa* was well characterized by studying some enzyme characteristics. The molecular weight of cellulase was 15148 dalton, and pH 7.0 was the optimum for enzyme activity and stability, while 30 ºC was the optimum for enzyme activity and stability.

Keywords: Cellulase, *Pantoea dispersa*, cellulose degrading bacteria.

انتاج وتنقية وتوصيف انزيم السيليليز من العزلة المحلية *Pantoea spp*

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الخلاصة :
جمعت عينات من التربة المزروعة بالحنطة والشعير بالإضافة إلى عينات من فاكهة البرتقال والتفاح وجذور الجزر التالفة، بهدف عزل سلالات بكتيرية منتجة للسيليليز . نميت العزلات البكترية المعزولة من هذه العينات على أوساط انتخابية متضمنة carboxymethyl cellulose كمصدر وحيد للكربون والطاقة . أظهرت النتائج إن 9 عزلات من بين 50 عزلة قادرة على إنتاج السيليليز. كانت الفعالية النوعية للسيليليز في الراشح البكتيري للعزلة الأكثر كفاءة هو 1.601 وحدة/ملغم بروتين. شخصت العزلة اعتمادا على الصفات المظهرية والاختبارات الكيميوحيوية ثم باستعمال Api 20-E و VITEK II وكانت العزلة هي *Pantoea dispersa* . تمت تنقية السيليليز المنتج من قبل هذه العزلة بأربع خطوات تنقية وهي الترسيب بكبريتات

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الامونيوم والديلزة ثم التنقية بالمبادل ألايوني DEAE-Cellulose ثم بالترشيح الهلامي خلال Sephadex G200 . اظهرت النتائج فعالية نوعية للإنزيم المنقى 1181.8 وحدة/ملغم بروتين مع عدد مرات تنقية 115.2 وحصيلة 26.6%. تم توصيف الإنزيم المنقى للعزلة *Pantoea dispersa* من خلال دراسة بعض صفات الإنزيم إذ كان الوزن الجزيئي للإنزيم هو 15148 دالتون، والرقم الهيدروجيني الامثل لفعالية وثبات الإنزيم هو 7، بينما درجة الحرارة 30 °م هي الأمثل لفعالية وثبات الإنزيم .

Introduction

It is well known that plants are the most abundant source of renewable carbon and energy on the earth and cellulose is the most abundant biomass on the earth. Cellulases are inducible enzymes, which are synthesized by large number of microorganisms either cell-bound or extracellular during their growth on cellulosic materials [1]. Chiefly fungi, bacteria, and protozoa that catalyze cellulolysis produce cellulases. However, there are also cellulases produced by a few other types of organisms, such as some termites and the microbial intestinal symbionts of other termites [2]. They annually produce about 4×10^9 tons of cellulose which is a highly stable polymer consisting of β-1,4-linked glycosyl residues, along with other polysaccharides [3]. Cellulases are found within 12 GH families with two enzyme commission numbers: EC 3.2.1.4 (endoglucanase) and EC 3.2.1.91 (cellobiohydrolase) [4, 5]. The biological degradation of cellulose has been studied for many years, and a number of cellulolytic enzymes, especially cellulases produced by fungi and bacteria. Cellulases have been isolated ,characterized [6], Because of the diversity of their application, the major industrial applications of cellulases are in textiles, food, detergents, leather and paper industries, biofuel, fiber modification and pharmaceutical applications [7]. For many years, cellulose-degrading bacteria have been isolated and characterized from a variety of sources such as soil, decayed plant materials, hot springs, organic matters, feces of ruminants and composts [8]. Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms. It has been widely used for the production of cellulase for industrial purposes like extraction of olive oil, improving the quality of bakery products, extraction and clarification of fruit and vegetable juices [9]. The bioconversion of cellulosic materials mainly depends on the nature of cellulose sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes. Cellulose quality, temperature, aeration, carbon source, incubation period, medium additives, pH of the medium and the presence of inducers are important parameters for the optimized production of cellulase enzymes [10].The present investigation therefore was focused on the production of cellulase from local isolates, identification of the most producible strain, then purification and characterization of the enzyme.

Materials and methods

Isolation of bacterial isolates

For isolation of bacterial isolates, samples from soil cultivated with barley and wheat in addition to samples from spoiled orange and apple fruits and carrot roots were collected, and cultured into a medium containing 1% CMC (for cellulase induction), 0.1% KNO_3, 0.1% KH_2PO_4 and 0.5% $MgSO_4.7H_2O$, and incubation for 24 hrs at 35 ºC with shaking at 140 rpm . After incubation, cultures were centrifuged at 8000 × g for 20 min., and regarded as a crude enzyme [11].

Identification of bacterial isolates

Bacterial isolates were identified according to Selvakumar *et al* [12] by studying their cultural, morphological characteristics and biochemical tests. Then identification was confirmed by using Api-20E and VITEK-II.

Enzyme assay

Cellulase endo-β-1,4-glucanase activity was measured according to Samira [13] by mixing 0.1 ml of enzyme (crude filtrate) with 0.1 ml of 1% (w/v) CMC in 10 mM sodium phosphate buffer, pH was adjusted to 7.0 at 37 °C for 60 min. Then the reaction was stopped by adding 1ml of 3,5- dinitro salicylic acid (DNS) reagent, and the mixture was placed in boiling water bath for 10 min. After cooling to room temperature, optical density at 540 nm was measured. Cellulase activity was calculated throughout glucose standard curve. Enzyme activity unit was defined as the amount of enzyme that releases 1μmol of glucose per min [13].

Determination of protein concentration

Protein concentration in culture filtrates was determined according to Bradford's method using bovine serum albumin as a standard protein [14].

Purification of cellulase

Cellulase in culture filtrates of *Pantoea* sp. was purified according to Yin *et al* [1] throughout three purification steps, first by ammonium sulfate precipitation then dialyses against 20 mM Tris-HCl (pH 8) for 24 hrs, and ion exchange chromatography through DEAE-cellulose (175 × 20 cm) with elution by using 20 mM Tris-HCl (pH8) at a flow rate of 30 ml/hr., and finally by gel-filtration chromatography using sephadex G-200 (1.25 × 30 cm).

Characterization of cellulase

Molecular weight of the pure cellulase was determined by gel filtration chromatography by using Sephadex G-200**.** Optimal pH and temperature for cellulase activity and stability were examined according to Yin *et al* [1].

Results and Discussion

Collection of cellulase producing bacteria

Fifty isolates were obtained and screened to examine their ability in cellulase production.

Identification of bacterial isolates

The selected *Pantoea* sp. isolate was identified according to morphological, cultural and biochemical tests. Additional confirmation was carried out using Api E 20 and VITEK 2. Different morphological characteristics were studied to identify the selected isolate. It appeared as straight rods in shape, motile, non- hemolytic, non-capsulated, non-sporeforming. Colonies of this isolate were smooth, mucoid, yellow pale pigmented, irregularly round, rough and wrinkled that are difficult to remove with a platinum wire.

On the other hand different biochemical tests were applied to identify the isolate according to Selvakumar *et al* [12].Our findings are in accordance with those of Selvakumar *et al.* [12] who mentioned that this biochemical characteristics table 1 is applied to *P. dispersa*.

Table 1- Biochemical tests used for identification of the selected isolate

Biochemical test	**Result**
Gram stain	-
Yellow pigment	+
Catalase	+
Oxidase	-
Motility	+
VP (Acetoin production)	**+d**
Indole production	-
Urease	+
Gelatinase	+
Citrate utilization	+
Fermentation / Oxidation of	
Mannitol	+
Lactose	+
Rhamnose	+
Maltose	+
Sucrose	+
Salicin	+
Sorbitol	-
Growth at	
30°C	+
41°C	+
4°C	-
44 °C	-

Positive test (+), negative test (-), positive test 1-4 day (+d).

Ability of local isolates in producing cellulase

Screening of cellulase producing bacteria

Screening of cellulase production was achieved according to the well diffusion agar method by transferring 100µl crude filtrate of overnight culture of each bacterial isolate into wells done in CMC-agar medium. Results table 2 showed a clear zone of hydrolysis around each well in the CMC–agar medium due to the activity of cellulase produced by each isolate as reported by Raju and Divakar [15].

Table 2- Diameter of zone of hydrolysis around well in CMC-agar medium caused by crude filtrates of bacterial isolate.

Isolate symbol	Diameter of clear zone (mm)
A3	**20**
A2	11
S1	8
S3	9
O1	14

Cellulase production assay

Quantitative screening for cellulase production was achieved by determining enzyme specific activity in culture filtrate for these isolates after culturing in cellulase production medium at 35 °C for 24 hrs. Results indicate that all bacterial isolates were cellulase producers with variable degrees table 3. Among these isolates, A3, which isolated from spoiled apple fruits was the most efficient in cellulase production because of the specific activity of cellulase in crude filtrate of this isolate recorded to 1.601 unit/mg. According, A3 isolate was selected for further studies.

Table 3- Specific activity of cellulase produced by different bacterial isolates

Isolate symbol	Protein conc. (mg/ml)	Activity (unit/ml)	Specific activity (unit/mg)
A3	0.122	0.195	**1.601**
A2	0.162	0.072	0.450
S1	0.150	0.096	0.641
S3	0.134	0.100	0.760
O1	0.140	0.205	1.471

Purification of cellulase

To purify cellulase produced by the locally isolated *P. dispersa* A3 under the optimum conditions, four purification steps were used to obtain the purified enzyme. The purification steps included the following [1].

Ammonium sulfate precipitation

Ammonium sulfate precipitation was used for cellulase purification, as a first step using gradual saturation ratios ranged between 60 and 80% for precipitating the crude enzyme. Results showed that 80% of ammonium sulfate was completely precipitated the enzyme under the optimum conditions. The specific activity of cellulase decreased with increasing of ammonium sulfate precipitation ratio, and the optimum precipitation ratio of ammonium sulfate was 70%, where at this ratio the specific activity of cellulase reached 11.81 u/mg with a purification fold of 1.15, while the higher ammonium sulfate saturation ratio decreased the enzyme specific activity.

After ammonium sulfate precipitation, the precipitate was redissolved in a small amount of 0.02 M Tris-HCl buffer pH 8 and dialyzed against the same buffer. Results showed that after dialysis, cellulase specific activity increased to 15.71 u/mg and activity was increased to 9.43 u/ml.

Ion exchange chromatography

One protein peak appeared in the washing step, while there were two protein peaks appeared after elution by the gradient concentration of sodium chloride figure 1. Three protein peaks were detected by measuring the absorbance at 280 nm of each eluted fraction. One protein peak was assayed to detect cellulase activity. Protein fractionation distributed in fraction numbers 23 to 34 with a cellulase activity reached 18.86 u/ml. These fractions were pooled and concentrated to 5ml, then protein concentration, cellulase activity and specific activity were measured.

These results indicated that cellulase has a positive net charge, since it was not bound with anionic ion exchanger of DEAE-cellulose. Results indicated in table (4) showed that protein concentration and cellulose activity and specific activity in this step were 0.024 mg/ml, 18.86 u/ml, 785.8 u/mg protein respectively, with a purification fold of 76.6 and enzyme yield of 27.5%. Results were in agreement with Yin *et al* [1] who reported that enzyme was washed from this ion exchanger. Cellulase purified by the ion exchanger chromatography technique was further purified using the gel filtration chromatography technique to get a purified enzyme.

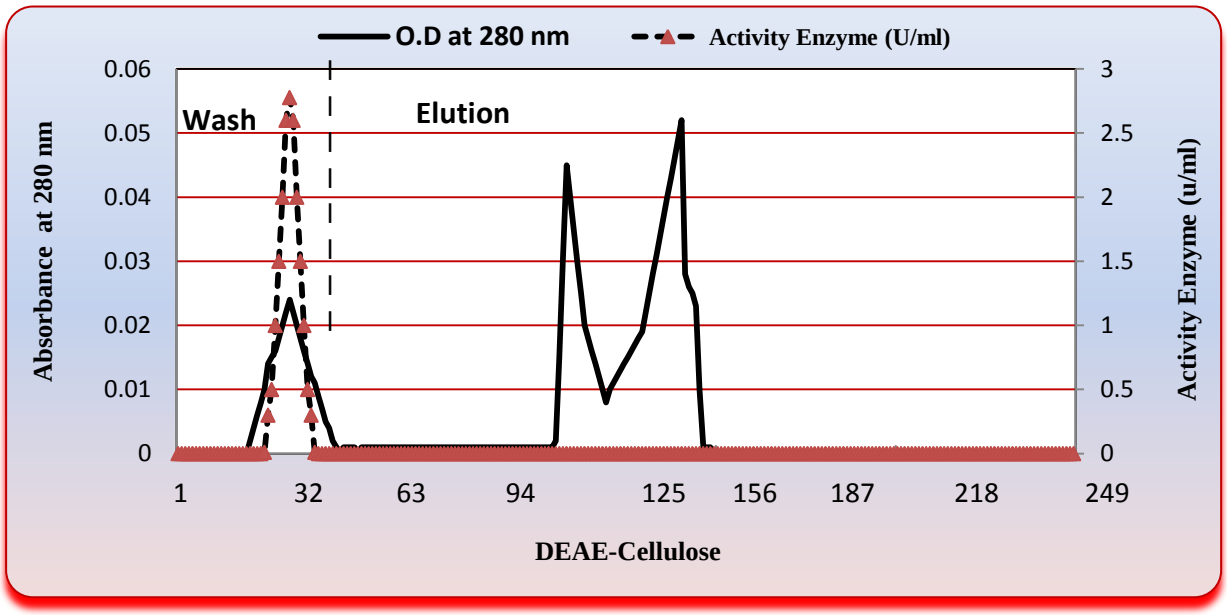

Figure 1- Ion exchanger chromatography of cellulase produced by locally isolated *P. dispersa* A3 using DEAE-Cellulose column (1.75 x 20 cm) with a flow rate of 30 ml/hour

The next step of the purification, it was achieved using gel filtration chromatography by applying 3 ml of (3 mg/ml) of the sample to sephadex-G200 previously equilibrated with 0.02 M Tris-HCl buffer pH 8. Sephadex-G200.

Two peaks represented cellulase activity appeared after elution with Tris-HCl buffer at fraction number 36. Fractions represented cellulase activity were pooled and concentrated by sucrose. Protein concentration, activity and specific activity were measured in 7 ml of concentrated enzyme.

An increase in the specific activity of the purified enzyme (1178.3 unit/mg) and of purification fold (114.9) and of yield for cellulase was 43.3% was reported table 5.

Mirzaakhmedov *et al* [16] found that cellulase was purified by gel filtration to get purified enzyme with a specific activity of 1.70 u/mg and 2.47 for a purification degree.

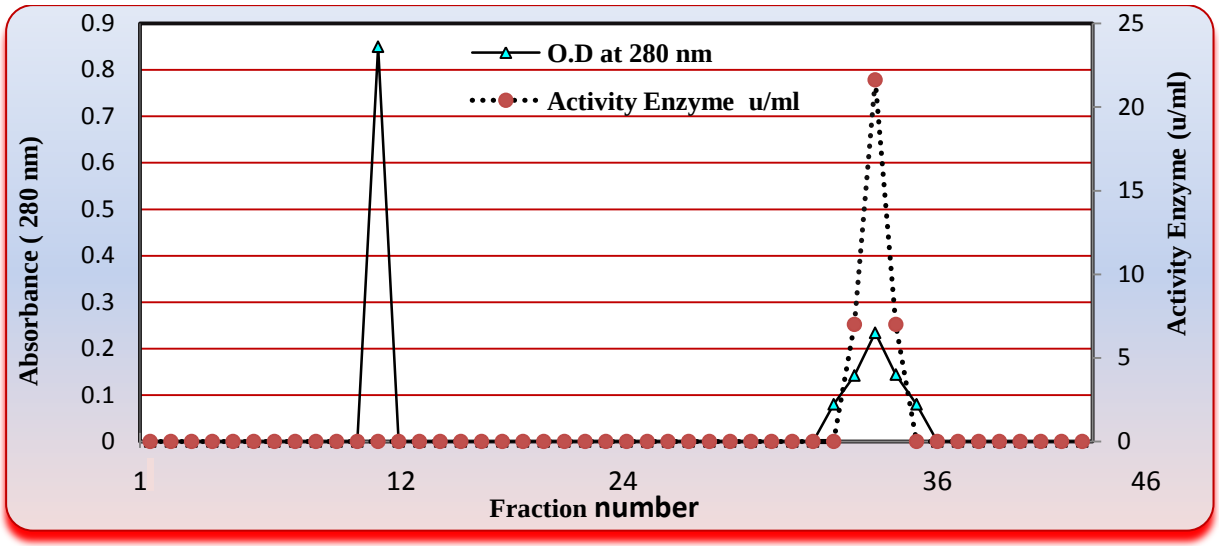

Figure 2- Gel filtration chromatography of cellulase produced by locally isolated *P. dispersa* A3 using sephadex-G200 column (1.25 cm x 30 cm) equilibirated with Tris-HCl buffer solution pH, fraction volume was 3 ml at flow rate of 30 ml/hours

Table 4- Purification steps for cellulase produced by locally isolated *P. dispersa* A3.

Purification steps	Volume (ml)	Enzyme Activity (u/ml)	Protein concentration (mg/ml)	Specific activity (u/mg)	Total activity (u)	purification (fold)	Yield (%)
Crude Enzy.	156	2.193	0.214	10.25	342.1	1	100
Amm. sulphate	30	2.54	0.215	11.81	76.2	1.15	22.27
Dialysis	19	9.43	0.6	15.71	179.1	1.53	52.3
Ion exchanger	5	18.86	0.024	785.8	94.3	76.6	27.5
Gel filtration	7	21.21	0.018	1178.3	148.4	114.9	43.3

It is concluded from the current experimental work the possibility to achieving fully purify and characterize cellulase produced from a bacterial strain from spoiled apple using four steps of precipitation, dialysis, ion exchanger chromatography and gel filtration chromatography.

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