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Cellulase production by two *Streptomyces* species

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Abstract

Optimization of cellulase production by two actinobacterial species, identified as *Streptomyces auranticus* and *Streptomyces minoensis*, was studied on Hutchinson medium containing cellulose as carbon source. The optimal incubation period, temperature and initial pH recorded for maximum enzyme yield of *Streptomyces minoensis* were 6 days, 35ºC and 8.0 respectively. Meanwhile, *Streptomyces auranticus* showed different conditions for maximum enzyme production (8 days, 35ºC and pH 7.0). The best growth for the two isolates was recorded at 30ºC. The maximum cellulase production was observed in a medium containing carboxy-methylcellulose (CMC) and xylan as carbon sources for *Streptomyces auranticus* and *Streptomyces minoensis* respectively. The addition of tryptone as a nitrogen source exhibited a maximum cellulase activity for the two identified *Streptomycetes* species.

Keywords: Cellulase, Streptomyces minoensis and Streptomyces auranticus, Optimization

Introduction

A wide variety of bacteria are known for their hydrolytic enzymes production with streptomycetes being the best known enzyme producers (Vinogradova and Kushnir, 2003). They are capable of secreting an array of different extracellular enzymes including cellulase, chitinases and xylanase.

Cellulases are one of the most important industrial enzymes. They have attracted interest because of the diversity of their applications. In 2001, the world market for enzymes was over 1.5$ billion; this was doubled by the year 2008. The United States and Europe each consume 30% of the world output of enzymes. Approximately 75% of industrial enzymes are used for hydrolysis and depolymerization of complex natural substances (Kirk *et al*., 2002). Cellulase enzymes are produced from plant, animal and microbial sources. For commercial production, microbial enzymes have the enormous advantage of being scalable to high-capacity production by established fermentation techniques (Tahtamouni *et al*., 2006).

Industrial applications of cellulases are in the textile polishing named "biopolishing" of fabrics such as production of the stonewashed look of denims, and in household laundry

detergents to improve fabric softness and brightness (Hill *et al*., 2006). Moreover, they are used in animal feeds to improve nutritional quality and digestibility, in processing of fruit juices, and in baking; de-inking of paper is yet another emerging application (Ponnambalam *et al*., 2011). In addition, cellulase enzymes are involved in enzymatic hydrolysis of cellulose, one of the most abundant organic materials that can be converted to products with significant commercial interest. Bioconversion of cellulose to monomeric sugars has been intensively studied as researchers seek to produce bioethanol and biobased products, food and animal feeds, and many valuable chemicals (Barros *et al*., 2010).

The present work aims to isolate and identify two cellulase producing actinobacteria and optimize the conditions required for maximum cellulase production.

Material and Methods

Isolation technique

Soil samples collected from different localities in Egypt and Libya were subjected for actinobacteria-cellulase producers. Standard dilution plate technique was applied. Isolates were purified by streak-plating on starch nitrate agar plates following the method of Waksman, 1959. Colonies of actinobacteria were selected, isolated, purified and maintained as spore suspensions in 20% (v/v) glycerol at -20°C for subsequent investigation (Hopkins *et al*., 1985). The medium used for isolation, cultivation and stock maintenance of isolated strains was starch nitrate agar medium (Waksman, 1959). It contained (g/L): soluble starch, 20; KNO_3, 2; K_2HPO_4, 1; NaCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $CaCO_3$, 3; agar, 20; and distilled water up to 1L.

Screening for cellulase producing actinomycetes strains

A preliminary analysis of cellulolytic activity was conducted using grown strains on Hutchinson-medium has the composition (g/L): Ca $(NO_3)_2$, 2.5; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.3; NaCl, 0.1; $FeSO_4.7H_2O$, 0.01; $CaCl_2$, 0.1 and distilled water up to 1L, containing filter paper strip (1.0 X 10.0cm) as carbon source. The pH was adjusted to 7.0 with 1.0 N NaOH. A loopfull of streptomycetes cultures from agar plates was inoculated into glass tubes containing 5ml of the previous medium, and incubated at 30°C for up to 21 days. At the end of the incubation, the filter paper was examined if degraded and if any dark patches of growth are found in the filter paper.

Growth condition and enzyme production

Two starch-nitrate agar discs of each *Streptomyces* 5-7 old days culture grown at 30°C were inoculated in flasks containing 50ml of Hutchinson medium supplemented with 0.5 % (w/v) cellulose. The cultures were incubated under 150rpm shaking at 30°C for 14 days. The enzyme activity of the medium filtrate was assayed.

Enzyme assay

Cellulase activity was quantified according to Miller (1959) with some modifications. A reaction mixture composed of 0.2mL crude enzyme solution and 1mL of 1.0% cellulose dissolved in distilled water and an aliquot of diluted enzyme preparation. The mixture was incubated at 50°C in water bath for 15min. The determination of reducing sugar released during the incubation mixture was detected by the dinitrosalicylic acid method of Miller (1959). One ml of dinitrosalicylic acid reagent was added to 1ml of the clarified reaction mixture and standards. After mixing the mixtures were boiled in a water bath for 5min. After cooling to room temperature, the optical density of the coloured product at 550nm was recorded. Calibration curve constructed using D-glucose standard in the range of 0-5µmol ml^{-1} were used. One unit of cellulase activity was defined as the amount of enzyme that released 1µmol of glucose per minute under the above assay conditions. Enzyme and substrate controls were included routinely.

Identification of the selected Streptomyces isolates

The streptomycetes isolates used in this investigation was identified according to International *Streptomyces* Project (Shirling and Gottlieb, 1968a; 1968b; 1969; 1972; Pridham and Tresner, 1974a; 1974b; Bergey's Manual of Systematic Bacteriology, 1989).

Electron microscopy studies

Electron microscopy was performed using the cover slip technique. The cover slip was cut with a glass file and a suitable fragment with growth on

surfaces of starch nitrate agar cultures was chosen. It was mounted on a specimen-tube, coated with gold-palladium under vacuum and examined with a scanning electron microscope (Joel ISM-5300) operating at 10KV.

Optimization of culture conditions

An attempt was also made to determine the optimal culture conditions such as pH, temperature, incubation period and carbon and nitrogen source requirements for their maximum growth and activities. The biomass yield and cellulase production of the selected isolate was recorded. Microbial growth under different growth factors was assayed. Cell pellets were dried in hot air oven at 80°C to a constant weight. The dry cell weight per 50ml of culture broth was used to determine microbial growth.

Effect of incubation period: To determine the optimum incubation period of the isolates for maximum enzyme production, the supernatants were collected after 2, 4, 6, 8, 10 and 12 days of incubation and assayed as before. The growth was also recorded.

Effect of initial pH: To determine the optimum medium pH, for maximum enzyme production, selected medium of different pH (4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, and 9.5) was inoculated with the isolate. The growth and cellulase production were assayed as before.

Effect of temperature: To determine the optimum temperature for enzyme production the culture medium was incubated at 25°C, 30°C, 35°C and 40°C, at optimum pH and incubation period. The effect of temperature on growth and cellulase production was recorded as before.

Effect of carbon source: Different carbon sources; cellulose, CMC, xylan, glucose, maltose, sucrose, starch and xylose (5g/L) was added separately as a sole carbon source. The effects of these carbon sources in the production of cellulase, biomass yield, were recorded.

Effect of nitrogen source: For this purpose, a range of different nitrogen sources includes, $(NH_4)_2SO_4$, $Ca(NO_3)_2$, $NaNO_3$ and KNO_3, were added in equimolecular nitrogen weights equivalent to the nitrogen content of 2.5 gL^{-1} $Ca(NO_3)_2$ of Hutchinson medium.. Other nitrogen source like peptone (14% N), tryptone (13.5% N), yeast extract (09% N) and beef extract (12.5% N) were added separately. The effects of these nitrogen sources in the production of cellulase, biomass yield, were recorded.

Statistical analysis

Analysis of variance (one-way ANOVA) was used to identify statistically significant differences in cellulase activity and culture growth with incubation period, temperature, pH, carbon source and nitrogen source. All statistical analyses were performed using SPSS 18.0 software (SPSS, 2006).

Results

Screening of cellulase producers

Screening of actinomycetes was conducted using the filter paper if degraded and if any dark patches of growth are found on the filter paper as a preliminary study for choosing the best cellulase producers. After 21 days of incubation, two isolates out of five actinomycetes were selected for the highest rupture and dark patches on filter paper. The cellulolytic activity of the selected strains was confirmed under submerged fermentation indicating the highest cellulose degradation.

Isolates identification

The selected two actinobacterial isolates were identified as *Streptomyces auranticus* and *Streptomyces minoensis* according to their morphology, under light and scanning microscope (Fig. 1), and biochemical characteristics (Table 1).

Factors affecting the growth and cellulase activity

Different effects, such as the incubation period, pH, temperature, and different sources of carbon and nitrogen, were studied on growth and production of cellulase activities of *S. minoensis* and *S. auranticus* in Hutchinson medium containing cellulose (0.5%).

Effect of the incubation period

The time course for the production of cellulase activity is shown in Figure 2 for both *S. auranticus* and *S. minoensis*, respectively. The cellulase activity was highly significantly increased ($P< 0.0001$) during the growth of the organism, with the maximum production of enzyme detected at 8 days and 6 days (0.3286 U/ml and 0.2189 U/ml) and the maximum growth (0.329 g/50ml and

0.349 g/50ml) for *S. auranticus* and *S. minoensis*, respectively. After 8 days, cellulase activity was significantly declined by *S. minoensis* (0.0796 U/ml), while it reached the minimal level (0.15 U/ml) after 10 days by *S. auranticus*.

Table 1. Cultural, morphological and physiological characteristics of *S. auranticus* and *S. minoensis*

Characters		*S. auranticus*	*S. minoensis*
Colour and pigmentation	Aerial mass color	pink	grey
	Melanoid pigment on: tyrosine, peptone yeast and synthetic media	-	-
	Reverse side pigment	Pale yellow	Grey
	Soluble pigment	-	-
Spore morphology	Spore chain	flexuous	Straight
	Spore surface	Smooth	Smooth
Carbon source utilization	Arabinose	+	+
	Xylose	+	+
	Inositol	+	+
	Mannitol	+	+
	Fructose	+	+
	Rhamnose	+	+
	Sucrose	+	+
	Raffinose	±	+
Nitrogen source utilization	Potassium nitrate	+	+
	L-valine	+	+
	L-threonine	+	+
	L-serine	+	+
	L-Methionine	+	+
	L-histidine	+	+
	Hydroxy proline	+	+
	L-proline	+	+
	L-cysteine	+	±
	L-phenylalanine	+	+
Physiological properties	Milk coagulation	+	+
	Milk peptonization	+	+
	Starch hydrolysis	+	+
	Urea utilization	+	+
	Gelatin liquification	+	-
	Melanin/L-tyrosine	-	-
	Cellulose degradation	+	+
	Esculin degradation	+	+

(+) good, (±) Little, (-) nil.

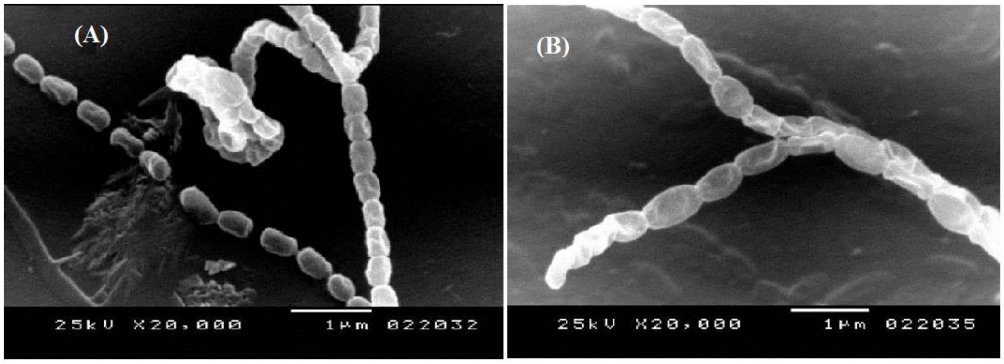

Fig 1. Scanning electron micrograph showing the two isolated streptomycetes growth after 7 days at 30°C. (A) *S. auranticus* and (B) *S. minoensis* isolates.

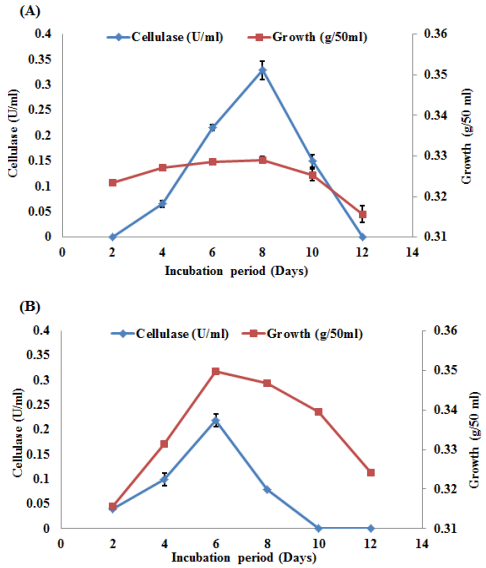

Fig 2. Effect of different incubation periods on cellulase production and growth of *S. auranticus* (A) and *S. minoensis* (B).

Effect of different initial pH

The optimum pH for cellulase production (Figure 3) was 7 and 8 with the maximum level of enzyme activity 0.2049 U/ml and 0.297 U/ml for *S. auranticus* and *S. minoensis*, respectively. On the other hand, the maximum growth (0.3139 g/50ml and 0.356 g/50ml) occurred highly significantly (P< 0.0001) at pH 7 and pH 8 for *S. auranticus* and *S. minoensis*, respectively. Cellulase activity was completely inhibited at pH 9.5 for *S. auranticus*.

Effect of temperature

The production of cellulase by *S. auranticus* and *S. minoensis* was highly affected by raising temperature of 25ºC up to 40ºC (Figure 4). The optimum temperature for the enzyme production was 35ºC (0.1568 U/ml and 0.233U/ml), while the maximum growth was at 30ºC (0.4866 g/50ml and 0.4502 g/50ml) by *S. minoensis* and *S. auranticus*, respectively. Cellulase production was gradually increased significantly (P< 0.0001) with increasing temperature for both *S. auranticus* and *S. minoensis*.

Effect of different carbon sources

The effect of a range of carbon sources on the growth and production of cellulase by *S. auranticus* and *S. minoensis* was varied (Figure 5). The best carbon sources for enzyme activity were found to be carboxymethylcellulose (CMC) for *S. auranticus* (0.207 U/ml) and xylan for *S. minoensis* (0.266 U/ml). The arrangement of different carbon sources in descending order according to its effect on cellulase production for *S. auranticus* was CMC, cellulose, maltose, glucose, xylan, starch, xylose and then sucrose, with activity ranging from 0.207 U/ml to 0.0278U/ml. In contrast, the carbon source arrangement for *S. minoensis* was xylan, cellulose, maltose, CMC, glucose, xylose, starch and sucrose, with activity varying from 0.266 U/ml to 0.0259 U/ml.

Effect of different nitrogen sources

The nitrogen sources effect on the production of cellulase by *S. auranticus* and *S. minoensis* isolates was investigated (Figure 6). The highest cellulase activity and growth (0.292 U/ml-0.0852 g/50ml for *S. auranticus* and 0.412 U/ml - 0.119 g/50ml for *S. minoensis*) were recorded with highly significantly (P< 0.0001) when tryptone was used as the nitrogen source. The arrangement of different nitrogen sources in descending order according to its effect on cellulase production by *S. auranticus* was tryptone, yeast extract, beef extract, peptone, potassium nitrate, sodium nitrate, calcium nitrate and finally ammonium sulphate. The descending order of the different nitrogen sources effect on cellulase production by *S. minoensis* was tryptone, beef extract, peptone, yeast extract, sodium nitrate, calcium nitrate, ammonium sulphate and then potassium nitrate.

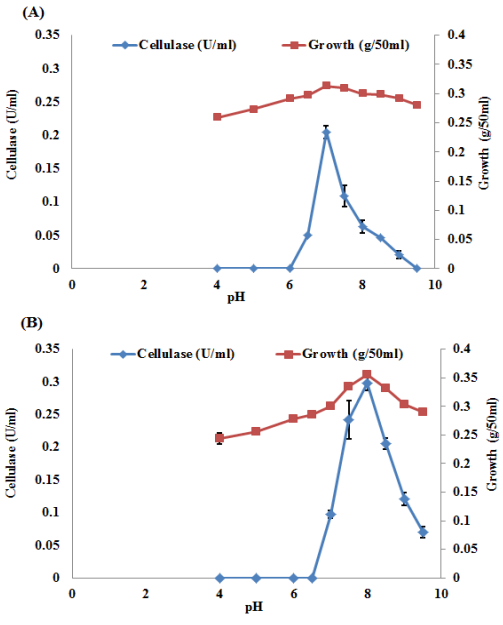

Fig 3. Effect of different pH on the cellulase production and growth of *S. auranticus* (A) and *S. minoensis* (B).

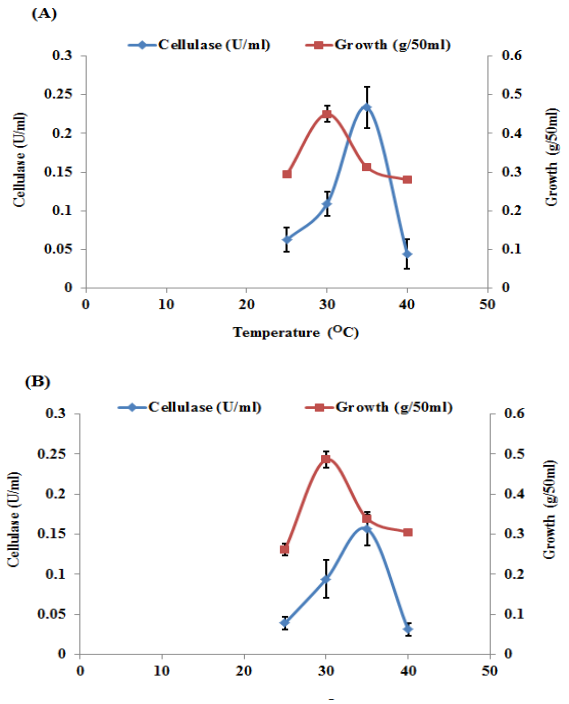

Fig 4. Effect of different temperatures on the cellulase production and growth of *S. auranticus* (A) and *S. minoensis* (B).

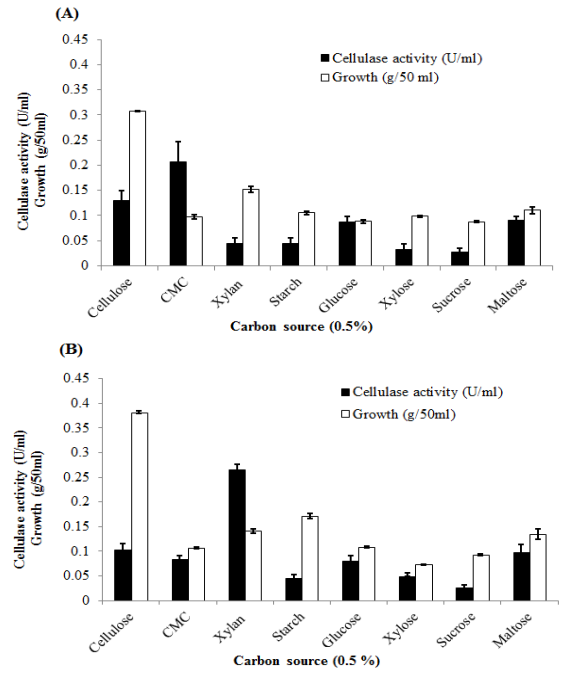

Fig 5. Effect of different carbon sources on the cellulase production and growth of *S. auranticus* (A) and *S. minoensis* (B).

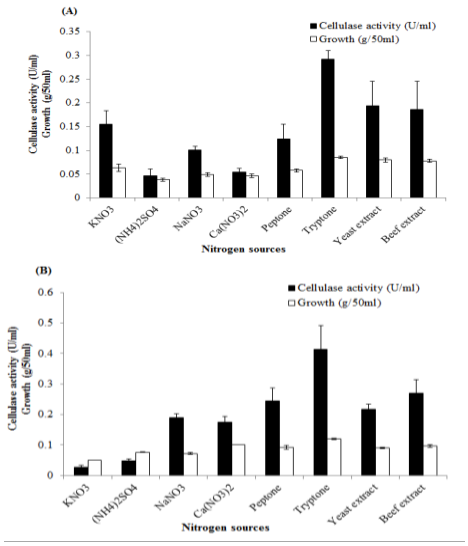

Fig 6. Effect of different nitrogen sources on the cellulase production and growth of *S. auranticus* (A) and *S. minoensis* (B).

Discussion

Streptomyces species have always been a source of thousands of bioactive compounds. Enzymes are one of the important products of this unusual group of bacteria. *Streptomyces* sp. with potential cellulolytic activity is subjected to produce cellulase in liquid culture (Chellapandi and Jani, 2008). *S. auranticus* and *S. minoensis* strains exhibited the highest ratio of dark patches or rupture to filter paper strip compared with the isolates, indicating a higher level of cellulose activity.

Cellulase production by *Streptomyces auranticus* and *S. minoensis* increased during the growth of the cultures in Hutchinson medium with the maximum production that detected after 8 and 6 days incubation, respectively. After this period, the activities of the enzymes decreased. The observed peaking and troughing of the production of extracellular enzymes might be attributed to the differences in the timing of induction of separate components of the cellulase system, the products of action of one component inducing the synthesis of another, differential inhibition by products of substrate hydrolysis, differential inactivation by proteases, or variation in the pH during cultivation conditions (Tuohy and Coughlan, 1992; Wang *et al.*, 1993).

Prolonged incubation periods (7, 8 days) required to obtain maximum enzymatic production by the studied Streptomyces isolates were in agreement with Arunachalam *et al.* (2010). Also, the maximum yield of endoglucanase activity was

obtained after 7 days (Azzedine *et al*., 2013). On the other hand, the production of the CMCase by *E. coli* JM109/DL-3 in submerged fermentations took only 3 days, which resulted in an increase in productivity of CMCase and decrease in its production cost (You-Jung *et al*., 2012). However, our results for *S. minoensis* are in agreement with that obtained by *Streptomyces griseorubens which exhibited high cellulase production after 6 days (Prasad et al., 2013).*

Temperature and pH values were found to be important parameters that influenced enzyme activities and production (Odeniyi *et al*., 2009). Cellulase enzyme from *S. auranticus* and *S. minoensis* was found to be active over a pH range of 7-9 with maximum activity at pH7 and pH8, respectively. This result is considerably similar to previous results reported by Azzedine *et al*. (2013) who found that cellulase enzyme from *Streptomyces* sp. (B-PNG23) was active over a pH7, also enzymatic activity was observed at alkaline pH (8-9). Similar results were reported by Rahna and Ambili (2011); Immanuel *et al*. (2006); GoKhan-Coral *et al*. (2002); Akiba *et al*. (1995); Prasetson and Doelle (1987); and Garcia-Martinez *et al*. (1980). However Solingen *et al*. (2001) studied the alkaline novel *Streptomyces* species isolated from east African soda lakes which showed an optimal pH of 8, while the *Cellulomonas* sp isolated by Irfan *et al*. (2012) recorded optimum activity at pH 7.5. *Trichoderma viridae* which produces cellulase presented an optimum activity at pH 8.0 as reported by Iqbal *et al*. (2011). Cellulase activity between pH 6.0 and 10.0 is useful in the textile industry (Kochavi *et al*., 1990) and in detergents (Suominen *et al*., 1993). The temperature has a great effect on the enzyme activity. *S. auranticus* and *S. minoensis* showed a maximum cellulase activity at 35°C within an optimum range 30°C-40°C. Alam *et al.* (2004) recorded a heavy growth and high cellulase activity by *S. omiyaensis* at 35°C-40°C. The maximum growth of mesophilic organisms at 35°C was reported by Shibli (2002). The enzyme CMCase showed a good production between 20°C to 40°C with maximum activity at 35°C for *Streptomyces* sp. strain NEAE-D by El-Naggar and Abdelwahed (2012). The optimum temperature recorded for maximum cellulase productivity at 35°C for *Bacillus subtilis* CBTK 106; *Bacillus* spp. B21 (Amritkar *et al*., 2004; Krishna, 1999) and *Pseudomonas fluorescense* (Bakare *et al*., 2005).

The best carbon sources of the enzyme activity and the growth of the organism were found to be CMC and xylan for *S. auranticus* and *S. minoensis*, respectively. The highest CMC-ase activity (233.56 U mL^{-l}) was recorded with the crude enzyme when CMC used as a carbon source and the lowest CMC-ase activity (11.11 U mL^{-l}) when sawdust and rice bran used as a carbon source reported by (Alam *et al*., 2004). The cellulase productivity by *S. auranticus* and *S. minoensis* was on maximum level when tryptone was used as the nitrogen source. This was in correlation with the findings of many other workers who found that maximum cellulase productivity was obtained by *Bacillus pumilus* BpCRI 6, *Pseudomonas flourescens*, *Monascus purpureus* and *Streptomyces* sp. BRC2 when tryptone was added as an organic nitrogen source to the production medium (Bakare *et al*., 2005, Chellapandi and Jani, 2008; Daniel *et al*., 2008). Also *Bacillus subtilis* KO strain gave maximum cellulase productivity when tryptone was added to the production medium (Shabeb *et al*., 2010).

The cellulases identified by both isolates would be fully characterized in a future work in order to investigate their usefulness in the industrial purposes.

Conclusion

It can be concluded that the two isolates *S. auranticus* and *S. minoensis* can be used for the production of useful substances by degrading cellulosic agro-wastes. Consequently, two goals would be achieved; getting rid of the continuously added agricultural wastes and recovery of bioenergy from degraded cellulose.

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الملخص العربي

عنوان البحث: إنتاج إنزيم السليوليز من نوعين من الإستربتوميسيس

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يهدف البحث الي عزل اكتينوبكتريا لها القدرة علي انتاج انزيم تحلل السليوليوز و دراسة تأثير ظروف النمو وتركيب الوسط الغذائي على إنتاج هذا الإنزيم. وتمت الدراسة بأستخدام العوامل المدروسه من درجات حراره و حموضة وغيرها علي بيئة هتشينسون الغذائية التى تحتوى على مادة السليلوز كمصدر وحيد للكربون. أظهرت النتائج أن الظروف المثلى لإنتاج إنزيم السليوليز للسلالتين كانت كالأتى: فترة التحضين المثلى هى ٦ و٨ أيام والأرقام الهيدروجينية كانت 7، ٨ لكل من إستربتوميسيس أورانتيكس وإستربتوميسيس مينوينسيس على التوالى. و كانت درجة الحرارة ٣٥م° هى المثلى لإنتاج الإنزيم للكائنين. أفضل المصادر الكربونية لإنتاج إنزيم السليوليز كان مادة الكربوكسي ميثيل سليلوز للسلالة استربتوميسيس اورانتيكس ومادة الزيلان للسلالة واستربتوميسيس مينوينسيس كمواد كيميائية نقية بتركيزات ٠,٥%. أما مادة التربتون كانت أحسن مصدر نيتروجيني لإنتاج إنزيم السليوليز لكلا الكائنين.