JJBS

Jordan Journal of Biological Sciences

Volume 1, Number 4, December. 2008
ISSN 1995-6673
Pages 141- 146

Influence of Culture Conditions on Cellulase Production by *Streptomyces* Sp. (Strain J2)

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Abstract

The purpose of this study was to determine the influence of growth conditions and medium composition on the cellulase enzyme production by *Streptomyces sp.* Production of cellulase enzyme by a *Streptomyces* strain (J2) was detected on cellulose agar (CA) medium after 4 days of incubation at 28 °C that exhibited a clear zone of 22 mm around the colony. Cellulase production was assayed by measuring the amount of glucose liberated in µmol/ml/min by using the dinitrosalicylic acid assay method. The highest crude enzyme activity (432 U/l) was observed after 3 days of incubation at pH 7 and 60 °C in a medium that was supplemented with 0.5% glucose, 0.2% starch, and 0.2% NH_4CL. However, enzyme production and activity were strongly decreased at 45°C and acidic pH. Enzyme production and activity were also inhibited when *Streptomyces* strain (J2) was grown in CMC broth supplemented with arabinose and yeast extract as a sole carbon and nitrogen source, respectively.

الملخص

كان الهدف من هذا البحث دراسة تأثير ظروف النمو وتركيب الوسط الغذائي على إنتاج إنزيم السليليز من بكتيريا الـ *Streptomyces*. تم الكشف على إنتاج انزيم السليليز من بكتيريا الـ *Streptomyces* بعد حضنها لمدة أربعة أيام على 28 درجة مئوية وفي وسط غذائي يحتوي على السليلوز وتم تقدير إنتاج الإنزيم بقياس الدائرة الشفافة حول مستعمرة البكتيريا والذي يعني أن الإنزيم افرز وانه قد حطم السليلوز المحيط بالمستعمرة. كذلك تم قياس نشاط الإنزيم المنتج بواسطة قياس كمية الجلوكوز المنتج عند استعمال طريقة DNSحيث سجل اكبر نشاط للأنزيم (432 وحدة) بعد ثلاثة أيام من الحضن على درجة حموضة 7 ودرجة حرارة 60 مئوية في وسط يحتوي على 0.5 % جلوكوز، و 0.2% نشأ، و 0.2 % NH_4CL . بدأ إنتاج ونشاط الإنزيم بالهبوط على درجة حرارة 45 مئوية ودرجة حموضة حامضية. لقد لوحظ كذلك أن نشاط الإنزيم قد انخفض بشدة عندما زرعت العزلة J2 في وسط يحتوي على سكر الارابينوز كمصدر وحيد للكربون ومستخلص الخميرة كمصدر وحيد للنيتروجين.

Keywords: Conditions; Culture; Cellulase; *Streptomyces* Sp

1. Introduction

A wide variety of bacteria are known for their production of hydrolytic enzymes with streptomycetes being the best known enzyme producers (Vinogradova and Kushnir, 2003). They are capable of secreting an array of different extracellular enzymes including cellulases, chitinases, and xylanases. Actinomycete's, one of the known cellulase-producers, has attracted considerable research interest due to its potential applications in recovery of fermentable sugars from cellulose that can be of benefit for human consumption and to the ease of their growth (Jang and cheng, 2003) compared to anerobic cellulase producers such as *Paenibacillus curdlanolyticus* (Pason *et.al*. 2006). The biotechnology applications of cellulases began in the early 1980s in animal feed followed by food applications (Harchand and Singh, 1997). Today, these enzymes account for approximately 20% of the world's enzyme market.

Few studies have been conducted on Streptomyces isolated from Jordan soil for their potential to produce enzymes of industrial importance. Rawashdeh et. al. (2005) isolated several Streptomyces isolates that were able to grow on tomato pomace. Upon further characterization, these isolates were able to produce cellulase, pectinase, and relatively large amount of xylanase. Tahtamouni et. al.(2006) isolated indigenous Streptomyces isolates that were capable of producing chitinase. These isolates exhibited fungicidal activity against sclerotia of the white cottony stem rot pathogen Sclerotinia sclerotiorum. Streptomycetes isolated from Jordanian habitats are poorly studied, especially the cellulase enzyme producers. Therefore, the present investigation is conducted to isolate soil streptomycetes from different habitats in Jordan, and to screen them for their ability to utilize cellulose as a sole source of carbon in an attempt to isolate a highly active isolate in cellulose production that cab used in the partial degradation of plant fibers, and thus cab be of industrial quality. The influence

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of different culture conditions on production of crude cellulase by the most active Sterptomyces isolate in submerged cultures is also studied.

2. Materials and Methods

2.1. Location, Sampling, Treatment of Soil Samples, and Isolation Technique

Twenty Soil samples were collected from 20 different regions in Jordan (Table 1). Enrichment of streptomycetes in the soil samples and isolation of *Streptomyces* spp. were performed as described by Saadoun *et. al.*(2008). Selected colonies were purified by repeated streaking. *Streptomyces*-like colonies were selected and screened for their ability to produce cellulase enzyme on cellulose agar medium (Carder, 1986).

2.2. Screening for Cellulase-Producing Streptomyces

Each isolate was suspended in sterile vial containing 3 ml distilled water, to create a spore suspension of 107 spores/ml. A drop (0.1 ml) from the suspension was cultured on the center of a cellulose agar (CA) plate (Carder, 1986). Pure Streptomyces isolates were cultured onto CA plates and incubated for 4 days at 28° C. Plates were then flooded with 0.1% Congo red for 15-20 min, then washed with 1ml NaCl, and kept over night at 5 °C. Bacterial colonies exhibiting clear zones against red color of non-hydrolyzed media were considered cellulase producers; and were tested again for confirmation (Carder, 1986).

2.3. Characterization of The Most Active Streptomyces Isolates

Streptomyces colonies that showed the largest clear zone were characterized morphologically and physiologically according to the International *Streptomyces* project (ISP) (Shirling and Gottlieb, 1966) and as described by Saadoun *et al.* (2008).

2.4. Cellulase Activity Assay

Cellulase activity was measured following the method of Miller (1959). Briefly, a reaction mixture composed of 0.2 ml of crude enzyme solution plus 1.8 ml of 0.5% carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 7.0) was incubated at 37 °C in a shaking water bath (GFL, Germany) for 30 min. The reaction was terminated by adding 3 ml of DNS reagent. The color was then developed by boiling the mixture for 5 min. Optical densities of samples were measured at 575 nm against a blank containing all the reagents minus the crude enzyme. Results were then compared to controls inoculated with an in active cellulytic streptomycete isolate. Results were interpreted in terms of enzyme activity in which one unit (U) of enzyme activity was defined as the amount of enzyme, which liberates 1μmol of glucose per minute under the above assay conditions (Miller, 1959).

2.5. Optimization of Growth Conditions and Cellulase Production

Erlenmeyer flasks containing 50 ml of CMC broth medium (Per liter: CMC: 10 g; KH_2PO_4: NaCl: 2 g; $MgSO_4.H_2O$: 1 g; $MnSO_4$:0.05 g; $FeSO_4.7H_2O$: 0.05 g $CaCl_2.2H_2O$: 2 g; NH_4Cl:2 g; pH 7-7.4) were inoculated with 1 ml of spore suspension (10^7spores/ml) of a 7 days old culture. Cultures were incubated in an orbital shaker incubator (TEQ, Portugal) at 28^{o}C for 5 days. Cellulase activity was then assayed daily by the DNS method as described above.

Table 1. Geographical locations from which soil samples were collected and designation of samples.

Location	Designation of samples	Latitude*	Longitude*
Safawi	Sa	32°10′N	37^{0}07′E
Mafrak	Ma	32°21′N	36^{0}12′E
Jafr	Ja	30°17′N	36^{0}20′E
Azraq	Az	31°50′N	36^{0}49′E
Ruwayshid	Ru	30°17′N	36^{0}07′E
Dayr abu sa'id	Da	32°^{0}30′N	35^{0}41′E
Mazar	Mz	31°04′N	35^{0}42′E
Marrow	Mr	32°37′N	35^{0}53′E
Tayiba	Ty	32°33′N	35^{0}35′E
Al-esheh	Esh	-	-
El-Ne`aemi	En	32°25′N	35^{0}55′E
En nabi	Na	32°04′N	35^{0}43′E
Hamamet e`lemat	Ha	32°24′N	35^{0}43′E
Kafr khall	KA	32°22′N	35^{0}53′E
Ba'un	Ba	32°23′N	35^{0}44′E
Raymun	Ra	32°17′N	35^{0}50′E
Jordan University of Science andTechnology	Just	32°34′N	36^{0}00′E
Turrah	Tu	32°38′N	35^{0}59′E
Shajarah	Sh	32°39′N	35^{0}56′E
Thnebeh	Th	32°40′N	35^{0}57′E
Total number of samples		20	

*Gazetteer of Jordan.

2.6. Effect of Ph and Temperature on Cellulase Production

To study the effect of pH and temperature on cellulase production, 250 ml Erlenmeyer flasks were prepared containing 50 ml of 0.5% (W/V) CMC broth, with pH values in the range of (4-9). Buffers including sodium acetate (pH 4), citrate buffer (5, and 6), phosphate buffer (pH 7), Tris buffer (pH 8 and 9) at final concentration of 50 mM were used to adjust the pH of the broth. Flasks were then incubated at 28 °C for the optimum incubation time. Samples from bacterial cultures growing in these broths were assayed daily for cellulase production using the standard DNS method (Miller 1959). The pH value giving the highest enzyme production was used for further enzyme assays.

The optimal temperature for enzyme production was determined by performing the standard assay procedure at range temperatures of 15 to 45 °C in an orbital incubator shaker (TEQ, Portugal). All further enzyme assays were performed at the determined optimum conditions (pH and temperature).

2.7. Effect of Various Carbon Sources on Cellulase Production.

To study the effect of various carbon sources on the enzyme production, 250 ml Erlenmeyer flasks were prepared containing 50 ml of mineral salts medium supplemented with 0.2% (W/V) of one of the following carbon sources: glucose, arabinose, starch, glycerol, citrus pectin, and CMC giving a total of 0.5% carbon source. Cultures were incubated at the determined optimum conditions (time, pH and temperature), and the activity of the enzyme was assayed as described above.

2.8. Effect of Various Nitrogen Sources on Cellulase Production

Different organic and inorganic nitrogen sources were tested for their effect on the enzyme production, following the same procedure used for testing the effect of carbon sources on cellulase production. Sources including KNO_3, NH_4Cl, peptone, asparagines, and yeast extract were used in this study. The activity of the enzyme was assayed as above.

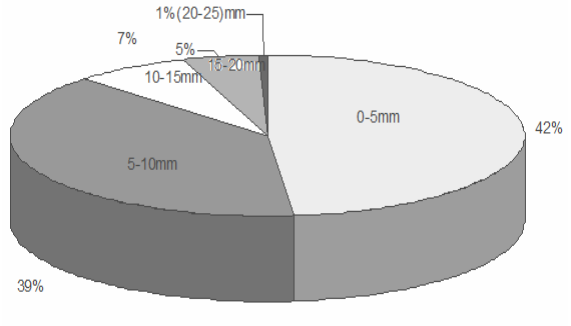


Figure 1. Distribution of the CMC degrading Streptomyces isolates.

2.9. Effects of The Combination of Ph and Temperature on Cellulase Production

This experiment was designed to test the effect of the combination of both temperature and pH on cellulase activity. Buffers of sodium acetate (pH 4 and 4.5), citrate (pH 5, 5.5 and 6), phosphate (pH 6.5 and 7), and Tris (pH 8 and 9) at 50 mM were used in this study to prepare 0.5% (W/V) CMC solution. Culture filtrates were used as crude enzyme source. Enzyme production was assayed as described eearlier at a wide range of temperatures from 4 to 100 °C.

2.10. Cellulase Production Under Optimum Conditions

The most active *Streptomyces* sp. (strain J2) was cultivated in CMC broth medium with the optimized incubation conditions (time, incubation temperature, pH, and carbon and nitrogen sources). Crude cellulase enzyme produced by this active strain under these optimal conditions was assayed as mentioned earlier.

2.11. Statistical Analysis

Analyses of variance for all data were performed using statistical analysis system (SAS Institute Inc., 2000). Means were separated by the least significant differences (LSD) at $\alpha = 0.05$.

3. Results

By employing enrichment methods, a total of 340 different *Streptomyces* isolates were recovered from 20 soil samples, which were collected from different habitats in Jordan. All of these isolates matched the genus description reported by Shirling and Gottlieb (1966), Nonomura (1974), and Williams *et al*., (1983).

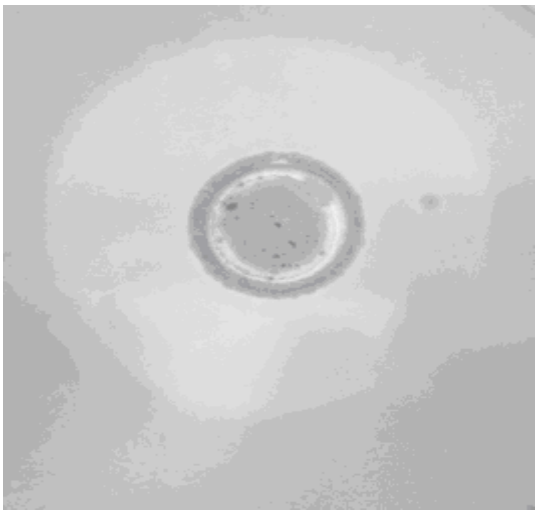


Figure 2. Cellulytic activity of *Streptomyces* sp. (strain J2) on cellulose agar (CA) indicated by clearing zone surrounding the colony.

Upon initial screening, it appeared that most of the isolates (94%) were able to produce cellulase enzyme (Figure 1) while only 6% of the isolates were unable to produce this enzyme. Cellulase producing isolates were categorized into 5 groups according to the width of inhibition zones; very strong (group 1), strong (group 2), moderate (group 3), weak (group 4), and very weak (group 5). Zones of inhibition were 20-25, 15-20, 10-15, 5-10, and 0-5 mm for the 5 groups, respectively. The first 3 groups were represented by 1, 5, and 7% activity, respectively, while groups 4 and 5 were represented by 39 and 42% activity, respectively (Figure 1).

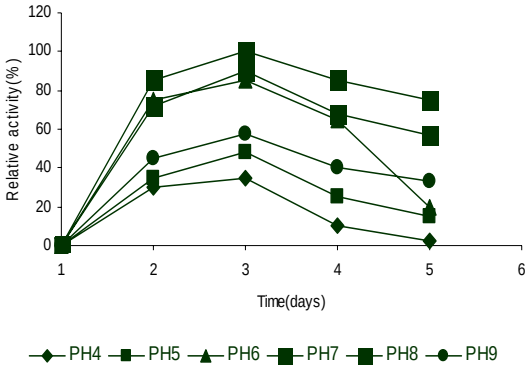


Figure 3. Determination of optimum pH for CMCase production by the J2 isolate. Enzyme activities at the different conditions are compared to the highest value, considered as 100%.

The isolate J2 was chosen as the most active cellulose-degrading isolated *Streptomyces*, which exhibited a 22 mm diameter of clear zone on CA plates (Figure 2).

Morphological and physiological characterization of this strain (J2) revealed that it belonged to the white color series with a distinctive reverse side color (light brown). In addition, this isolate did not produce diffusible and melanin pigments; and had a rectiflexible (RF) sporophore arrangement. The isolate was unable to utilize I-inositol and rhaminose. However, it utilizes other sugars such as D-glucose, L-arabinose, D-xylose, D-fructose, sucrose, D-mannitol, and raffinose.

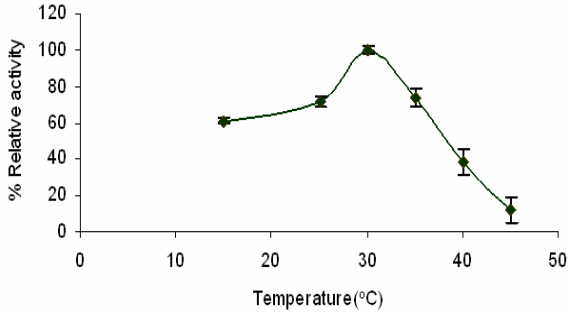


Figure 4. Determination of the optimum temperature for CMCase production by the J2 isolate. Enzyme activities at the different conditions are compared to the highest value, considered as.

Figure (3) shows that J2 strain minimally produce cellulase at pH 4 and 5 with relative activities of 35% and 48%, respectively, whereas more production was observed at pH 7 with relative activity of 100% after 72 h of incubation. Enzyme relative activity eaquals its activity at that specific condition when compared to the optimal (100%). Relative activities at pH 7 were not significantly different (P>0.05) from those activities at pH 8, but they were significantly (P>0.05) from the relative activities at pH 9 at, which the enzyme production barely exceeds 50%. Optimum enzyme production was observed after 3 days at 30°C (Figure 4) with 100% relative activity, which decreased considerably at 45 °C reaching only 12%.

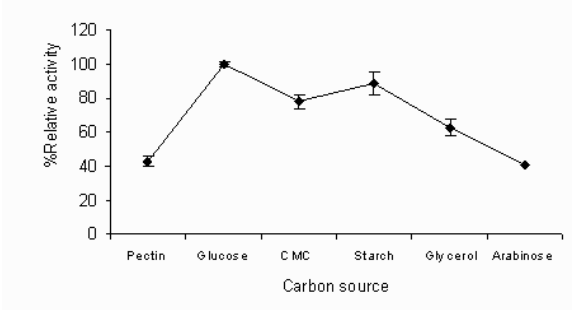


Figure 5. Effect of different carbon sources on CMCase production by J2 isolate. Enzyme activities at the different carbon sources are compared to the highest value, considered as 100%. Pars represent the standard errors for means at α=0.05.

Surprisingly, when observed in Figure (5), the maximal CMCase production by J2 was observed when 2% W/V glucose was used as a carbon source with a 100% relative activity. However, when pectin or arabinose was used, a significant decrease in the relative activity reaching 43%, 40% for pectin and arabinose, respectively was observed.

The highest level of enzyme production was achieved when NH_4CL was added to the CMC medium as sole source of nitrogen while the lowest yield was observed when yeast extract and asparagines were used as nitrogen sources yielding relative activities of 35% and 55%, respectively (Figure 6).

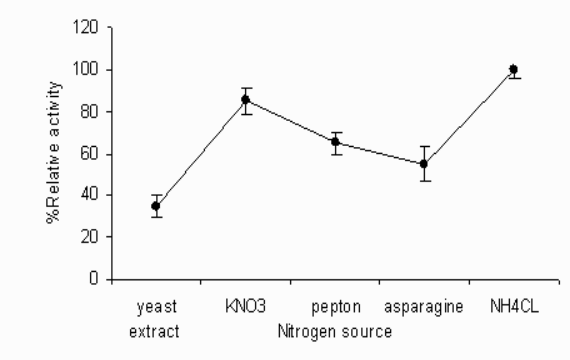


Figure 6. Effect of different nitrogen sources on CMCase production by J2 isolate. Enzyme activities at the different nitrogen sources are compared to the highest value, considered as 100%. Pars represent the standard errors for means at α=0.05.

The temperature and pH profiles are presented in (Figure 7). CMCase have shown more than 40% activity at all pH values tested with maximum production at pH 6 (Figure 7a). An optimum temperature (plateau) ranging from 45 to 60°C was observed (Figure 7b) with maximum production observed at 60°C. It is noteworthy that at 45°C the enzyme production still retained 80% of its activity. The highest level of cellulase activity (432 U/L) was observed under optimum conditions after 3 days of incubation at pH 7 (Figure 8).

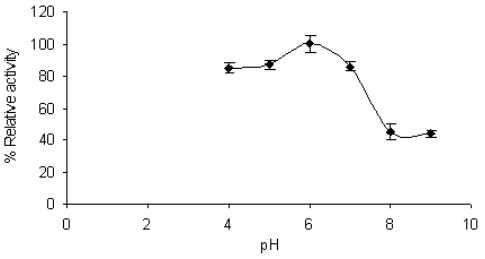
4. Discussion

Streptomyces species have been always a source of thousands of bioactive compounds. Enzymes are one of the important products of this unusual group of bacteria. Most of the Streptomyces isolates recovered from the different soils of Jordan produced fiber hydrolytic enzymes. Cellulase, one important hydrolytic enzyme, was produced by most of the isolates (94%).

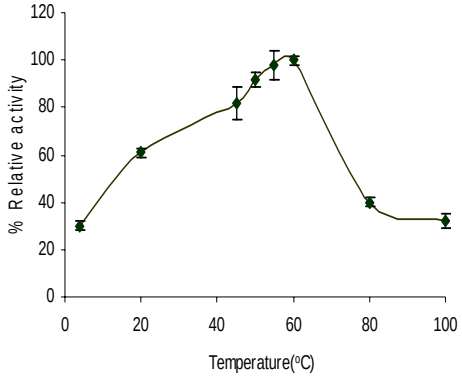
Figure 5. Effect of different carbon sources on CMCase production by J2 isolate. Enzyme activities at the different carbon sources are compared to the highest value, considered as 100%. Pars represent the standard errors for means at α=0.05.

CMCase enzyme from the active isolate J2 was found active over a pH range of 4-7 with maximum activity at pH 6. This result is considerably similar to what was reported by Theberge et. al. (1992) who showed that the optimum pH for endoglucanase from a strain of Streptomyces lividans was 5.5. However, the results appeared to contradict previous results reported by Solingen et al. (2001) of an alkaline novel Streptomyces species isolated from east African soda lakes that have an optimal pH of 8, highlighting the effect of alkaline environment on the adaptation of these Streptomyces. Furthermore, the maximum CMCase activity of isolate J2 was recorded at 60 °C with no significant difference ($p<0.05$) between 50 and 60°C. These results are in agreement with results reported by McCarthy (1987), who reported an optimal temperature for cellulase activity in the range of 40-55 °C for several Streptomyces species including S. lividans, S. flavogrisus, and S. nitrosporus. Jang and Chen (2003) described a CMCase produced by a Streptomyces T3-1 with optimum temperature 50 °C,

whereas Schrempf and Walter (1995) described a CMCase production by a S. reticuli at an optimum temperature 55 °C.



(a)



(b)

Figure 7 . Effect of pH (a) and temperature (b) on CMCase activities, Enzyme activities at the different conditions are compared to the highest value, considered as 100%.

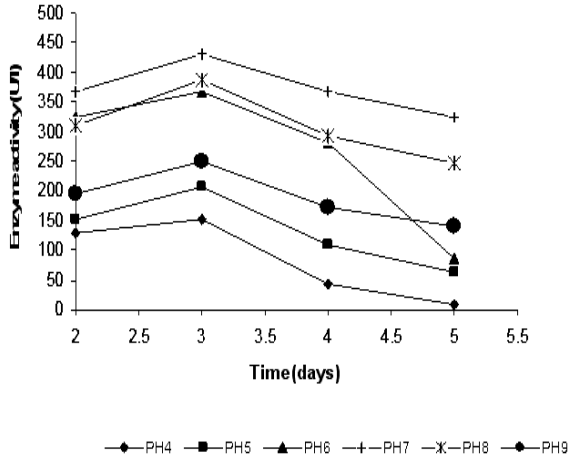


Figure 8. Course of cellulase production by J2 isolate at different pH values. Cellulase assayed when a mixture of glucose (0.5%) plus starch (0.2%) were used as a carbon sources and NH_4CL (0.2%) as a nitrogen source and temperature at 30 °C.

Contrary to the described pattern for other Streptomyces family members, our results indicates that cellulase production by J2 strain was not negatively affected by glucose. A result that may confer an economical advantage for this strain. However, there is no significant difference between glucose and CMC in the induction of cellulase production. It has been reported that the biosynthesis of cellulase is induced during growth on cellulose or other cellulose derivatives (Fernandez-Abalose et.al.. 1997; Godden et. al. 1989). In all cases, it has been found that it is essential to keep the required nutrients at low level to insure maximum accumulation of fermentation products (Priest, 1984). Overall, the study indicated that cellulase production from J2 isolate was constitutive in nature, as apparent from the very high number of Streptomyces isolates producing CMCase in Jordan soils.

Acknowledgments

Deanship of Scientific Research at Jordan University of Science and Technology funded this research (Grant No. 167/04).

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