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Arab Univ.
J. Agric. Sci.,
Ain Shams Univ.,
Cairo, 19(1), 163-170, 2011

CELLULASE PRODUCTION BY LOCAL BACTERIA ISOLATED FROM TAIF IN SAUDI ARABIA

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Keywords: Cellulase production, *Bacillus* sp.

ABSTRACT

Among 20 bacterial isolates isolated from the soil of El-hawia, El-hada, El-kaym and Karwa in Taif governorate in Saudi Arabia,two isolates had high efficacy in producing cellulase enzyme. They belonged to genus *Bacillus* (*Bacillus* 8 & 17). Some factors such as carbon source and wheat bran as a raw material, nitrogen source, pH and incubation temperature were investigated. Results indicated that CMC and cellulose were the most effective as they enhanced cellulases production .Sodium nitrate and ammonium chloride were the best nitrogen sources for cellulases production. Initial pH 7.0 was found to be optimal for growth and cellulase production. Incubation temperatures at 25 - 40ºC achieved high cellulases production by the two isolates.

INTRODUCTION

Cellulose biodegradation by cellulases and cellulosomes, produced by numerous microorganisms, represents a major carbon flow from fixed carbon sinks to atmospheric CO_2 (**Falkowski *et al* 2000; Melillo *et al* 2002 and Berner, 2003),** which is very important in several agricultural and waste treatment processes **(Angenent *et al* 2004; Das & Singh, 2004; Haight, 2005 and Schloss *et al* 2005),** and could be widely used to produce sustainable biobased products and bioenergy from less costly renewable lignocellulosic material to replace depleting fossil fuels and reduce environmental pollution (**Mohanty *et al* 2000; Mielenz, 2001; Galbe and Zacchi, 2002; Hoffert *et al* 2002; Lynd *et al* 2002; Wyman, 2003; Kamm and Kamm, 2004; Demain *et al* 2005; Moreira, 2005 and Reddy & Yang, 2005**).

Complete enzymatic hydrolysis of enzyme requires synergistic action of 3 types of enzymes, namely cellobiohydrolase (CBH), endoglucanase (EG) or carboxymethycellulase (CMCase) and β-glucosidases (**Bhat, 2000).** Cellulases are used in the textile industry for cotton softening and denim finishing; in laundry detergents for color care, cleaning and anti-deposition; in the food industry for mashing; in the pulp and paper industries for deinking, drainage improvement and fiber modification and they are even used for pharmaceutical applications **(Kirk *et al.*, 2002 and Cherry & Fidantsef, 2003).**

This work aimed to isolate some bacterial isolates, showed high efficacy in producing cellulases enzymes from Taif in Saudi Arabia and to find out some factors enhancing the cellulases production.

MATERIALS AND METHODS

Soil samples

Calcareous soil samples were obtained from El-hawia, El-hada, El-Kaym and Karwa in Taif governorate in Saudi Arabia for isolation of bacterial isolates.

(Taif area found in Eastern province of Saudi Arabia, the landscape between Makkah and Taif, is littered with high mountains).

(Received October 31, 2010)
(Accepted December 6, 2010)

Bacterial isolates

Twenty isolates were obtained from the soil of Taif governorate in Saudi Arabia.

Media used

Medium (1): Nutrient agar (**Difco Manual, 1984).** It was used for growth and maintenance of bacteria It has the following composition: (g/L) Peptone 5.0, Beef extract 3.0, Agar agar 20, Distilled water 1000 ml and pH 7.0.

Medium (2): Carboxymethyl cellulose medium (**Ray *et al* 2007)**. It was used for cellulase production. It has the following composition: (g/L) CMC 10.0,KH_2PO_4 4.0,$Na_2HPO_4$4.0, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.001, $FeSO_4.7H_2O$ 0.004, Tryptone 2.0, Distilled water 1000 ml and pH 7.0.

The above medium was modified by replacing glucose in the basal medium with addition of CMC in concentration of 10 g/L-1 as a substrate to produce cellulase enzyme.

Standard inoculum

For preparation of standard inoculum, both selected isolates of *Bacillus* were cultivated on nutrient broth individually at 30 ºC for 24 h where an average viable count of 3.5 - 4.3 $\times 10^6$ cells/ml culture broth was obtained. This was used as an inoculum for the production medium.

Fermentation

It was carried out in 250 ml Erlenmeyer flask containing 100 ml medium No. (2) for supplemented with CMC as a substrate to produce cellulase enzyme. The flasks were sterilized at 121°C for 15 min. The flasks were inoculated with 3% standard inoculum (v/v) transferred to the production medium, and then incubated on a rotary shaker (150 rpm). The broth after cultivation was used for enzyme studies**. (Dien *et al* 2006).**

Enzyme assays

Plate enzyme assay screening

At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 minutes. The Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulase activity producer. The largest ratio was assumed to contain the highest activity (**Howard *et al* 2003 & Ariffin *et al* 2006).**

Carboxymethyl cellulose (CMC ase) activity

CMCase activity was assayed using a method described by **Mandels and Weber (1969)**. The activity was estimated using 1 % solution of carboxymethlycellulose (CMC) in 0.05 M citrate buffer (pH 4.8) as substrate. The reaction mixture contained 1 ml citrate buffer, 0.5 ml of substrate solution and 0.5 ml of suitably diluted enzyme solution. The reaction was carried out at 50°C for 30 min. One unit of CMCase activity was expressed as 1 µ mol of glucose liberated per ml enzyme per minute.

Filter-paperase (FPase) activity

The activity of FPase was assayed according to the method explained by **Mandels and Weber (1969).** This method is similar to the CMCase assay method, but the substrate was Whatman No. 1 filter paper strip (1 x 6 cm) soaked in 1 ml 0.05 M sodium citrate buffer (pH 4.8). The samples were incubated with 0.5 ml enzyme solution at 50°C for 1 h. One unit of F Pase activity was determined as 1 µ mol of glucose liberated per ml enzyme per minute.

β-Glucosidase activity

One-tenth ml of the culture supernatant was incubated with 0.5 ml of 0.05 M acetate buffer (pH 5) containing 2.5 mg cellobiose. After incubation at 50ºC for 10 min, the glucose released was measured by the glucose oxidase peroxidase method **(Zaldívar *et al* 2001)**

Determination of sugars

The total amount of non-reducing sugars (as cellulose) was determined by the glucose oxidase peroxidase kit from (BIO-ADWIC) EL NASR PHARMACEUTICAL CHEMICALS Co. (Egypt).

Effect of different carbon sources

The appropriate carbon source was selected by replacing the original carbon substrate of the basal medium with equivalent carbon amount of each of the tested carbon sources (Glucose, Carboxymethycellulose, Cellobiose and Cellulose).

Pretreatment of wheat bran (raw material)

Wheat bran was pretreated with 4% solution of sodium hydroxide (2000 ml/ 100 g substrate), autoclaved at 121°C for 30 min. The material recovered by filtration was washed with distilled water until neutralilty (pH 7.0) and dried at 65°C to constant weight.

Effect of different nitrogen sources

To detect the proper nitrogen source for cellulase production by selected isolates, the prescribed nitrogen source of the fermentation medium was replaced by equivalent nitrogen amount of each of the tested organic [Yeast extract, Peptone, Urea] and inorganic nitrogen sources [$NaNO_3$, NH_4Cl & $(NH_4)_2SO_4$].

Effect of initial pH

Five values of pH ranged between 5.0 and 9.0 were chosen for studying their effects on cellulase enzyme to select the most suitable pH of the production medium.

Effect of incubation temperature

To determine the optimum temperature for cellulase production, fermentation was carried out at various temperatures in the range of 20 to 40ºC with 5ºC interval.

RESULTS AND DISCUSSION

Isolation of cellulase producing Bacteria

A number of 20 bacterial isolates were obtained from different sources of soil from Taif governorate in Saudi Arabia. Screening of bacterial isolates for cellulases activities was conducted by using Congo red test as a preliminary study for selecting the cellulases producers. After 72 hours of incubation, all 20 bacterial isolates showed signs of growth on CMC agar and demonstrated positive results in the Congo red test. Since the sole carbon source in CMC agar was carboxymethylcellulose (CMC), therefore the result of the test was a strong evident that cellulase was produced in order to degrade cellulose. They belonged to two genera *Bacillus* and *Micrococcus.* Data presented in **Table (1)** clearly show that *Bacillus* sp. (8) and *Bacillus* sp. (17) were the most efficient isolates selected according to the high ratio of clear zone diameter to colony diameter being 5.75 and 5.4 for *Bacillus* sp. (8) and *Bacillus* sp. (17), respectively. These results are on line with **Horikoshi, 1999 and Ozawa *et al* 2001** who found that the alkaliphilic properties of cellulases produced by *Bacillus* spp. due to the possible application of these enzymes in the detergent industry .Also, **Nakamura & Kappamura (1982) and Immanuel *et al* (2006)** studied the celluloytic property of bacterial species like *Pseudomonas, Cellulomonas, Bacillus, Micrococcus* and *Cellovibrio* spp.

An experiment was carried out to investigate the effect of different carbon sources such as glucose, carboxymethycellulose (CMC), cellobiose, cellulose and wheat bran (as a raw material) on the production of cellulase enzyme extracted by isolates of *Bacillus* sp.(8) and *Bacillus* sp (17). Five carbon sources were used as shown in **Table (2)**. Data presented in **Table (2)** clearly show that medium containing cellulose gave the highest yield of cellulase activity being 2.912 U/ml of CMCase, but medium containing CMC gave the highest FPase and β- glucosidases being 0.923 &0.542 U/ml by *Bacillus* sp.(8). While, the medium containing CMC gave the highest CMCase and β- glucosidases being 1.978 & 0.642 U/ml, respectively by *Bacillus* sp (17). Regarding the use of raw material as a sole source of carbon, the yield of CMCase enzyme was highest on wheat bran being o.761 &0.983 U/ml by *Bacillus* sp.(8) and *Bacillus* sp (17), respectively.

To evaluate the effect of nitrogen source on cellulase formation, the nitrogen source in the basal medium was replaced by different nitrogen sources. Data revealed that the supplementation of organic and inorganic nitrogen sources stimulated the cellulase yield and activity.

Results recorded in **Table (3)** clearly indicated that the sources of nitrogen greatly affected the production of cellulase. Sodium $nitrate_s$ was the best nitrogen source for *Bacillus* sp. (8) giving 3.197 U/ml of CMCase.

Ammonium chloride, however, gave the highest figures showing 0.923U/ml of FPase and 1.398 U/ml of β-glucosidases. Sodium nitrate also showed the best nitrogen source for *Bacillus* sp. (17) giving 2.594 U/ml of CMCase. Peptone as organic nitrogen source gave the highest values of cellulases enzymes being 2.190 U/ml of FPase and 2.902 U/ml of β-glucosidases.

These results are on line with **Krishna (1999) and Osono & Takeda (2001)** who reported that the source of nitrogen should be inorganic for better results.

Table 1. Hydrolysis ratio of carboxymethy cellulose (CMC) by bacterial isolates incubated at 28°C for 72 hours

Bacterial isolates	Growth diameter (cm)	*Cellulolysis diameter (cm)	**Cellulolysis ratio
Bacillus sp. (1)	0.8	2.7	3.38
Bacillus sp. (2)	0.7	2.0	2.86
Micrococcus sp. (3)	1.2	1.8	1.5
Micrococcus sp. (4)	0.6	1.5	2.5
*Micrococcus*_ sp. (5)	0.5	1.3	2.6
Bacillus sp. (6)	0.5	2.0	4.0
Bacillus sp. (7)	0.6	1.8	3.0
Bacillus sp. (8)	0.4	2.3	5.75
Bacillus sp. (9)	0.6	2.1	3.5
Micrococcus sp. (10)	0.9	1.9	2.11
Bacillus sp. (11)	0.8	1.9	2.38
Bacillus sp. (12)	0.6	1.3	2.17
Bacillus sp. (13)	0.6	2.1	3.5
Micrococcus sp. (14)	0.5	1.6	3.2
Bacillus sp. (15)	0.7	1.7	2.43
Micrococcus sp. (16)	0.6	2.0	3.33
Bacillus sp. (17)	0.5	2.7	5.40
Bacillus sp. (18)	0.7	1.7	2.43
Bacillus sp. (19)	0.5	2.5	5.0
Micrococcus sp. (20)	0.5	2.3	4.6

*** Cellulolysis diameter = Clear zone diameter – Growth diameter**

****Cellulolysis ratio = Cellulolysis diameter ÷ Growth diameter**

Table 2. Effect of carbon sources on production of cellulase enzyme by *Bacillus* sp. (8) and *Bacillus* sp. (17) using shake flasks (150 rpm) as a batch culture technique

Different carbon sources	*Bacillus* sp. (8)				*Bacillus* sp.(17)			
	Biomass g/100ml	Cellulase Activity (U)			Biomass g/100ml	Cellulase Activity (U)		
		CMCase	FPase	β-glucosidases		CMCase	FPase	β-glucosidases
Glucose	0.170	1.133	0.682	0.344	0.132	2.071	0.138	0.099
Carboxymethy cellulase (CMC) (Control)	0.254	1.653	0.923	0.542	0.197	1.978	0.793	0.642
Cellobiose	0.259	1.363	0.103	0.081	0.221	0.532	0.163	0.157
Cellulase	0.342	2.912	0.450	0.196	0.517	0.534	0.993	0.179
Wheat bran	0.132	0.761	0.093	0.064	0.118	0.983	0.197	0.123

Table 3. Effect of nitrogen sources on the production of cellulase enzyme by *Bacillus* sp. (8) and *Bacillus* sp. (17) using shake flasks (150 rpm) as a batch culture technique

Different Nitrogen Sources	*Bacillus* sp. (8)				*Bacillus* sp. (17)			
	Biomass g l 00ml	Cellulase Activity (U)			Biomass g l 00ml FPase	Cellulase Activity (U)		
		CMCase	FPase	B-glucosidases		CMCase	FPase	B-glucosidases
Yeast extract	0.131	2.618	0.551	1.287	0.201	1.380	2.015	2.227
Peptone	0.099	1.012	0.198	1.364	0.231	1.570	2.190	2.902
Urea	0.199	0.273	0.229	0.292	0.194	2.174	0.777	0.651
$NaNO_3$	0.136	3.197	0.353	0.446	0.198	2.594	0.809	0.748
NH_4Cl	0.098	0.434	0.923	1.398	0.138	1.178	0.527	0.442
$(NH_4)_2SO_4$	0.254	1.653	0.200	0.545	0.199	1.978	0.813	0.643

Table 4. Effect of initial pH on the production of cellulase enzyme by *Bacillus* sp. (8) and *Bacillus* sp. (17) using shake flasks (150 rpm) as a batch culture technique

Initial pH	*Bacillus* sp.(8)				*Bacillus* sp. (17)			
	Biomass g/100ml	Cellulase Activity (U)			Biomass g/100ml	Cellulase Activity (U)		
		CMCase	FPase	β-glucosidases		CMCase	FPase	β-glucosidases
5.0	0.119	1.132	1.176	0.196	0.077	0.463	0.131	0.098
6.0	0.188	1.271	0.534	0.313	0.105	0.193	0.151	0.139
7.0	0.254	1.652	0.912	0.592	0.197	1.978	0.793	0.642
8.0	0.203	1.093	0.463	0.273	0.117	0.867	0.298	0.121
9.0	0.050	0.615	0.239	0.099	0.053	0.751	0.233	0.111

Table 5. Effect of incubation temperature on the production of cellulase enzyme by *Bacillus* sp. (8) and *Bacillus* sp. (17) using shake flasks (150 rpm) as a batch culture technique

Incubation temperature °C	*Bacillus* sp. (8)				*Bacillus* sp. (17)			
	Biomass g/100ml	Cellulase Activity (U)			Biomass g/100ml	Cellulase Activity (U)		
		CMCase	FPase	β-glucosidases		CMCase	FPase	β-glucosidases
20	0.093	0.987	0.318	0.166	0.123	1.009	0.268	0.102
25	0.135	1.119	0.712	0.381	0.148	1.321	0.491	0.296
30	0.242	1.593	1.002	0.532	0.198	1.877	0.832	0.651
35	0.253	1.507	0.470	0.188	0.109	1.060	0.450	0.235
40	0.189	0.970	0.135	0.092	0.091	0.692	0.374	0.112

An increase in cellulase activity was observed when enriching medium with 1 % ammonium sulphate, but further increase in the concentration did not improve cellulase production. Also, **Rajoka (2004)** reported that KNO_3 and NH_4NO_3 were the best nitrogen sources for cellulase synthesis in *Cellulomonas flavigena*.

In Table (4), five values of pH ranged between 5.0 and 9.0 were chosen for studying their effects on cellulase production by *Bacillus* sp.(8) and *Bacillus* sp. (17). The highest yields of cellulase activity at pH 7.0 were 1.652 & 1.978U/ml of CMCase extracted by isolates *Bacillus* sp.(8) and *Bacillus* sp. (17), respectively. The respective values for FPase were 0.912 & 0.793 U/ml and for β-glucosidases were 0.592 &0.642 U/ml by *Bacillus* sp. (8) and *Bacillus* sp. (17), respectively.

These results are on line with **Ray *et al* (2007)** who found that pH 7.0 – 7.5 was more suitable for optimization of cellulase production by *Bacillus subtilis* and *Bacillus circulans*.

An experiment was constructed to find out the effect of different degrees of incubation temperatures ranged from 20 to 40ºC with 5ºC interval on cellulase production by *Bacillus* sp.(8) and *Bacillus* sp. (17). Results in **Table (5)** show that incubation on different degrees of temperatures from 20 to 40ºC achieved high cellulase production .These results are in agreement with **Immanuel *et al* (2006)** who recorded that endoglucanase activity in *Cellulomonas*, *Bacillus* and *Micrococcus* sp. reached maximum at the neutral pH and 40°C.

In the present study, it could be concluded that carbon and nitrogen sources, pH values and incubation temperatures play an important role in the production of cellulase activity by local bacilli isolated from Taif in Saudi Arabia.

Acknowledgement: The authors are pleased to acknowledge Taif University, Saudi Arabia for supporting this work during the project No.710- 431- 1 in Faculty of Science (women).

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