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Isolation and identification of cellulases producing thermophilic bacteria and their ability to produce xylanase enzymes

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Abstract

Forty four thermophilic cellulases producing bacterial isolates were isolated at 50°Cfrom compost, soil and manure samples which collected from Qalubia Governorate, Egypt. Tow isolates termed C27 and C30which gave the highest results in all determinations were identified as *Bacillus circulans* and *Cellulomonas flavigena* respectively. The highest hydrolysis clear zones on CMC medium at 50°C by the two identified isolates were (1.8 cm). Fp-ase activities were 1.9 and 1.6 U/ml, CMC- ase activity were 2.1 and 2.5 U/ml by *B. circulans* and *C. flavigena*, respectively. In addition ,their ability to produce Xylanase enzymes on oat spelt agar medium at 50°C was studied.

Keywords: CMC, Xylan , *Bacillus circulans* ,*Cellulomonas flavigena* and Congo red.

Introduction

Cellulose is commonly degraded by an enzyme called cellulose, this enzyme produced by several microorganisms, commonly by bacteria and fungi **(Immanuel *et al*., 2006).** Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials **(Leeand Koo, 2001).**The complete enzymatic hydrolysis of cellulosic materials needs different types of cellulase; endoglucanase,exocellobiohydrolase and β-glucosidase (**(Yi*et al*., 1999).** The endoglucanase randomly hydrolyzes the α-1, 4 bonds in the cellulose molecule, and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Lastly, the cellobiose is converted to glucose by â-glucosidase**(Bhat and Bhat, 1997).** On the other hand,Xylans are hemicellulose compounds and is the second most abundant natural polysaccharide behind cellulose **(Collins *et al*., 2005).** These compounds are present in the cell wall and in the middle lamella of plant cells. Xylanolytic enzymes are a group of enzymes that are involved in the hydrolysis of xylan and arabinoxylan polymers. These enzymes include endo-1, 4- β-xylanase, β-gylosidase, α-arabinofuranosidase and acetylxylan esterase **(Biely, 1993).** In natural environment, xylanases are produced mainly by microorganisms, marine algae, protozoans, crustaceans, insects and snails. Among microbial sources, filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase activities are much higher than those found in yeasts and bacteria **(Krisana*et al*., 2005).**

The aim of the present study is to isolate and identify cellulases producing thermophilic bacteria and study their ability to produce xylanase .

Materials and Methods

Enrichment, isolation and screening for cellulases producing thermophilic bacteria

Compost, soil and manure samples were collected from Qalubia Governorate, Egypt.

Isolation of thermophilic bacteria

Isolation of cellulolytic thermophilic bacteria from compost, soil and manure was done by serial dilution technique using nutrient agar medium **(Difco Manual, 1984).** The plates were incubated at 50 ± 2 °C for 2 days .The thermophilic bacterial isolates were maintained and sub-cultured on nutrient agar slant and stored at 4 ºC.

Screening for cellulases producing thermophilic bacteria

Plate Cellulase enzyme assay screening

Each isolate was inoculated on carboxymethyl cellulose (CMC) medium **(Ray *et al*., 2007)** and incubated at 50 ± 2°C for 3 days. After that, CMC 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 1*M*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 **(Ariffin*et al*., 2006).**

Determination of Cellulase activity using CMC

Cellulase activity was determined by CMC activity using the method described by **Mandels and Weber (2006).** The activity was estimated using 1%

solution of carboxymethlycellulose (CMC) as a substrate in 0.05 *M* citrate buffer (pH 4.8) Optical density was taken against isopropyl solution at 420 nm.

Determination of cellulase activity using filter paper

An activity of cellulase using filter paper was assayed according to the method explained by **Mandels and Weber (2006).** This method is similar to the CMC assay method, but the substrate was Whatman No. 1 filter paper strip (1 x 6 cm) soaked in 1 ml of 0.05 *M* sodium citrate buffer (pH 4.8) Optical density was taken against isopropyl solution at 420 nm.

Screening for Xylanase producing thermophilic bacteria

The bacteria isolates which gave positive results in cellulose enzyme assays were inoculated on oat spelt agar medium plates **(Muthezhilan *et al.*, 2007)** for xylanase enzymes production. The plates were incubated at 50 ± 2°C for 3 days. After that, xylanase enzymes assay was similar to described in plate cellulases enzymes assay.

Identification of the most potent isolates

The most potent isolates which showed highly results in all previous examined parameters were identified according to **Bergey's Manual of Systematic Bacteriology (2004).**

Statistical analysis

The collected data were statistically analyzed using SPSS computer analysis programs **(Foster, 2001).**

Results and discussion

Isolation of cellulases producing thermophilic bacteria

One hundred and seventeen thermophilic bacterial isolates were isolated from different sources on nutrient agar medium. Forty five of them are found to be cellulases producing bacteria while the rest are non-cellulase producing bacteria on CMC agar medium. The percentages of both cellulases and non-cellulases producing thermophilic bacteria are represented in **Fig (1).**

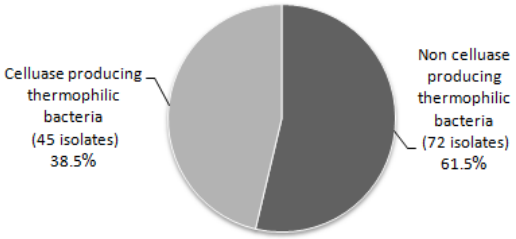

Fig.1. Diagram illustrates the percentages of cellulase and non cellulase producing bacteria.

The primary screening: data presented in **Table (1)** illustrated the types of the different sources for isolation and their ability in cellulose decomposition. Also, data indicated that forty five isolates are from compost, twenty four isolates are from soil and the rest are from farm manure.

Table 1. Primary screening of cellulose decomposers.

Source	Isolates	Results.	Source	Isolates	Results.	Source	Isolates	Results.	Source	Isolates	Results
Compost	**C1**	+	Compost	C31	-	Manure	S61	-	Manure	**M91**	+
	C2	-		**C32**	+		**S62**	+		**M92**	+
	C3	+		**C33**	+		S63	-		**M93**	+
	C4	-		**C34**	+		S64	-		**M94**	+
	C5	-		C35	-		S65	-		M95	-
	C6	+		C36	-		**S66**	+		M96	-
	C7	-		C37	-		S67	-		**M97**	+
	C8	-		C38	-		S68	-		**M98**	+
	C9	+		C39	-		S69	-		**M99**	+
	C10	-		C40	-		**M70**	+		M100	-
	C11	-		C41	-		**M71**	+		M101	-
	C12	+		C42	-		**M72**	+		**M102**	+
	C13	-		C43	-		M73	-		M103	-
	C14	-		C44	-		M74	-		**M104**	+
	C15	-		C45	-		M75	-		M05	-
	C16	-	Soil	**S46**	+		M76	-		**M106**	+
	C17	-		**S47**	+		**M77**	+		**M107**	+
	C18	-		S48	-		M78	-		**M108**	+
	C19	+		**S49**	+		**M79**	+		**M109**	+
	C20	+		S50	-		M80	-		**M110**	+
	C21	+		S51	-		M81	-		**M111**	+
	C22	-		S52	-		**M82**	+		M112	-

C23	-	S53	-	**M83**	+	M113	-
C24	-	S54	-	**M84**	+	M114	-
C25	-	**S55**	+	**M85**	+	M115	-
C26	-	S56	-	M86	-	M116	-
C27	+	**S57**	+	M87	-	M117	-
C28	-	S58	-	**M88**	+		
C29	-	S59	-	M89	-		
C30	+	S60	-	M90	-		

(+) refers to positive cellulases producing isolates. (-) refers to negative cellulases producing isolates.
*letter C refer to the source of isolate (compost), S (Soil) and M (Manure)

The numbers and percentages of cellulolytic thermophilic bacteria isolated from the different sources are represented in **Fig (2).** The highest proportion of cellulolytic thermophilic isolates are found in samples obtained from manure with percentage of 56% followed by isolates collected from compost samples with percentage of 29 %. The lowest percentage was found in soil samples which is 15% of the total number of bacterial isolates.

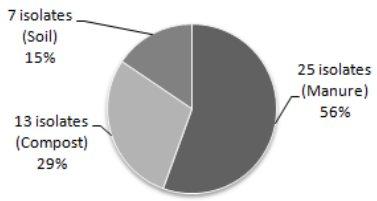

Fig2. Diagram showing the percentage of cellulolytic thermophilic bacteria from different isolation sources.

These results are in harmony with **Bayer *et al.* (1998)**who reported that the bacterium degraded cellulose by secreting several enzymes organized in a tight complex termed a cellulosome.

The secondary screening was carried out to investigate from the positive cellulases producing isolates appending that determine the growth diameter(cm) on CMC agar medium to be a strong evidence for proceed the results**.**

Data illustrated in **Fig (3)**indicated the first step in the secondary screening determine the growth diameter clear zone of hydrolysis (cm). The obtained results clearly showed that the isolates number C27 and C30 gave the highest growth diameter, clear zone of hydrolysis commonly isolates C27 and C30 consider the most potent isolates to produce cellulases enzymes (CMC-ase,Fp-ase) under thermophilic conditions.

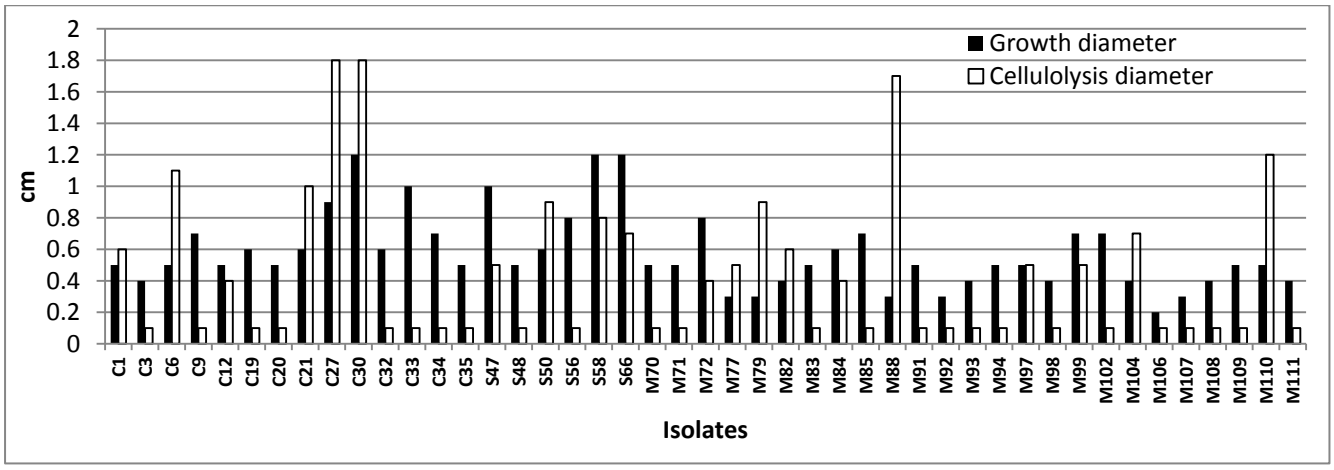

Fig 3.Histogram showing the primary screening of cellulose decomposers.
*letter C refer to the source of isolate (compost), S (Soil) and M (Manure)

Similar results were obtained by **Mandels *et al.*, (2006)** who reported that the thermophilic or extremely thermophilic microorganisms isolated from compost were able to produce cellulases. Also, **Ariffin *et al*. (2006)** reported that the largest clear zone of hydrolysis was assumed to contain the activity and the highest cellulase activity producer

Data in **Fig (4)** showed that the selected isolates were able to produce cellulases under thermophilic condition to come to 50 $\pm 2^0$. Generally, the cellulase enzymes were decreased in soil isolates follow by manure isolates. On the other hand compost isolates recorded the highest amount of cellulases enzymes. On the contrary the distribution of thermophilic isolate which produce cellulases enzymes localized in manure. These results are in accordance with those

obtained by **Kim (1995)** who reported that *B.circulans* cellulase was more stable than the other studied enzymes, which was stable up to 50°C. Seventy-eight % of its activity remained after 72 hrs, and the enzyme was inactive at 80°C

Also, data graphically illustrated in **Fig (4)** showed that the isolates number C27 and C30 were the highest producers of cellulases enzymes (CMC-ase, Fp-ase).Whereas, the isolate number S55 was the lowest producer of cellulases enzymes (CMC-ase, Fp-ase).

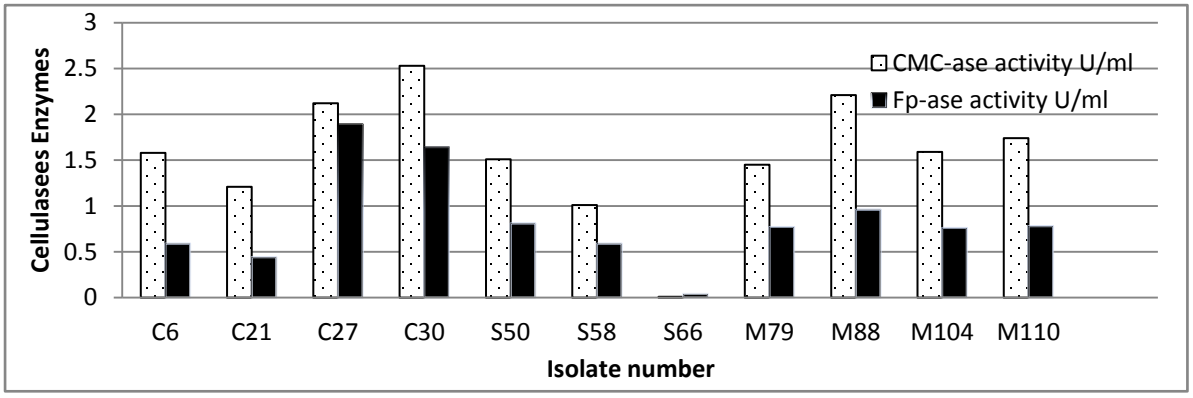

Fig 4.Histogram showing secondary screening of bacteria isolates based on cellulose decomposition
*letter C refer to the source of isolate (compost), S (Soil) and M (Manure)

Correlation coefficient between cellulolysis diameter and CMC enzyme

Data presented in **Table (2)** estimated the correlations between these three variables mutually; celluolysis ratio and CMC-ase; celluolysis ratio and Fp-ase; CMC-ase and Fp-ase. It is evidence that the correlation between celluolysis diameter and CMC enzyme is positive and quite strong for both types of bacteria. The correlation celluolysis diameter and filter paper (FP) enzyme is positive in both cases. In spite of this, the correlation is quite strong for the C27 while it is less strong in the other bacterial strain of C30.

Table 2. Correlation coefficient between celluolysis diameter and carboxymethyl cellulose enzyme.

Stains	No. of replicates	celluolysis diameter (cm)	CMC-ase U/ml	FP - ase U/ml	Correlation1	Correlation2	(CMC-FP) ase correlation
	1	1.9	2.4	1.67			
C27	2	1.7	2.3	1.6	1	1	1
	3	1.8	2.35	1.64			
	1	1.5	2.1	1.86			
C30	2	1.8	2.12	1.87	0.8	0.6	1
	3	1.8	2.16	1.94			

Correlation 1: Correlation coefficient between celluolysis diameter and carboxymethyl cellulose enzyme
Correlation 2: Correlation coefficient between celluolysis diameter and filter paper (FP) enzyme
(CMC-FP) ase correlation coefficient between carboxymethyl cellulose enzyme and filter paper (FP) enzyme

These results are in agreement with **Immanuel *et al.* (2006)**who reported that cellulolytic property of bacterial species like *Pseudomonas*, *Cellulomonas*, *Bacillus*, *Micrococcus*, *Cellovibrio* and *Sporocytophaga* spp.

Screening for xylanase producing thermophilic bacteria using plate cellulase enzyme assay screening

The last step of screening was carried out to investigate the ability of the thermophilic cellulosolytic isolates to produce xylanase. Appending that determine the growth diameter clear zone of hydrolysis (cm).Data graphically illustrated in **Fig (5)** showed that out of forty five isolates, nineteen isolates gave positive results which achievement 42% the rest isolates which equality 57.7% gave negative results.

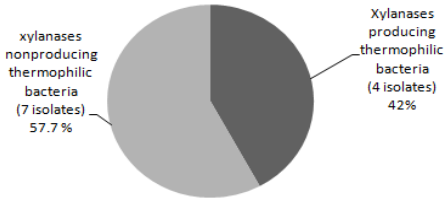

Fig 5. Diagram illustrated the percentage of the total xylanase producing and non-producing thermophilic bacteria.

Data illustrated in **Fig (6)** showed the growth diameter and clear zone of xylan hydrolysis. The obtained results clearly showed that isolates number C27 and C30 gave the highest growth diameter clear zone of xylan hydrolysis.

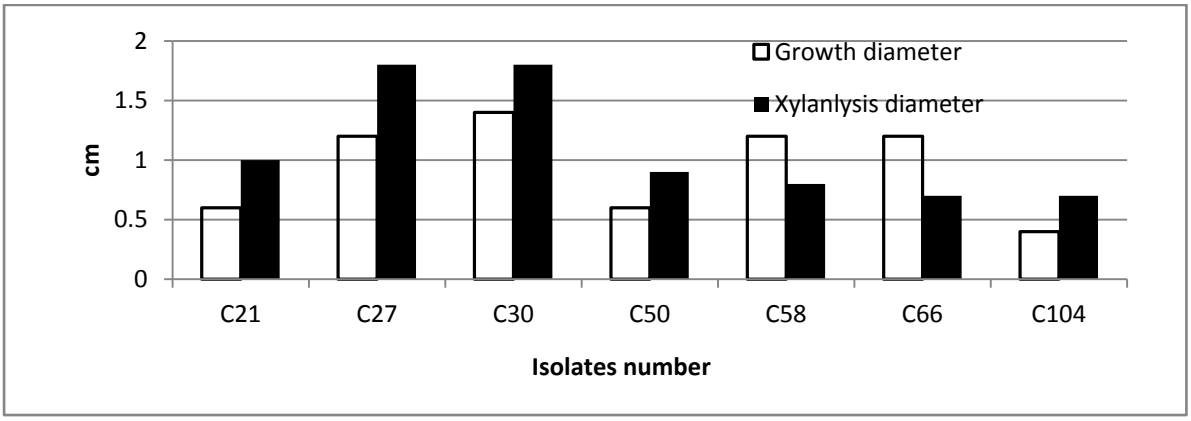

Fig 6. Histogram showing the growth of xylan lysis diameter of the selected isolates.

As a result of the all previous determination, it was clear that the isolates number C27 and C30were the highest producers for cellulases and xylanase enzyme . Therefore, these two isolates were selected and identified to be used as bioaccelerators in composting.

Identification of bacterial isolates

Table (11) illustrated the morphological and physiological studies, the more potent isolates were identified as *Bacillus circulans*(C27) and *Cellulomonas flavigena* (C30) according to **Bergey's Manual of Systematic Bacteriology (2004).**

This result is agreeing with **Kotchoni and Shonukan (2002)** who reported that the production of cellulase by wild type cells of *Bacillus* and *Cellulomonas* .Also, **Immanuel *et al*., (2006)** indicated that isolated thermophilic *Bacillus* strains with cellulolytic activities, similarly cellulolytic properties of bacterial species like *Pseudomonas*, *Cellulomonas*, *Bacillus*, *Micrococcus*, *Cellovibrio* and *Sporocytophaga* spp.

Table 3. Morphological and physiological characteristics of bacterial isolates.

characteristics	Strains	
	C27	C30
Shape	**long rods**	**irregular rods**
motility	+	-
Gram stain	G+	G+
Spore formation	+	-
Position of spores	central	-
Hydrolysis of:		
Casein	+	+
Starch	+	+
Growth on:		
Glucose	+	+
Arabinose	+	+
Mannitol	+	+
Fructose	+	+
Xylose	+	+
Galactose	+	-
Lactose	+	+
Sucrose	+	+
Lactose 10 %	+	+
Glucose 10 %	+	+
Citrate	-	-
Ashby's medium	+	+
NaCl 10 %	-	-
Glucose fermentation	+	+

Growth at		
5 °C	-	-
50 °C	+	+
Cellulose degradation	+	+
Nitrate reductase	+	+
Urease	-	-
Gelatinase	+	+

Conclusion

The results of this work indicated that the compost and manure are rich sources of many thermophilic bacteria which could produce cellulases and xylanase enzyme and could be considered good bioaccelorator to decompose complex organic materials and further studies are recommended to use these stains in composting process acceleration.

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عزل وتعريف سلالات بكتيرية محبة للحرارة ومحلله للسليولوز وقادرة على إنتاج الإنزيمات المحللة للزيلان

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أجرى هذا البحث بهدف عزل وتعريف ميكروبات محبة للحرارة ومحللة للسليولوز وذلك من مصادر مختلفة ودراسة مقدرتها على تحليل الزيلان وقد أسفرت الدراسة عن النتائج التالية:

- تم الحصول على ٤٥ عزلة محبة للحرارة ومحللة للسليولوز وذلك عند التحضين على ٥٠ درجة مئوية وذلك من مصادر مختلفة مثل الكمبوست والتربة والروث والتى تم تجميعها من محافظة القليوبية بجمهورية مصر العربية.
- ولقد أظهرت العزلتين C27 و C30 أعلى النتائج وتم تعرفهما على أنهما ***Bacillus circulans*** **and** ***Cellulomonas flavigena*** على الترتيب، حيث سجلا أعلى مساحة للتحلل على بيئة أجار CMC والتى قدرت بـ ١,٨ سم.
- كذلك عند تقدير نشاط إنزيم Fp –ase سجل ١,٩و١,٦ U/ml على التوالى
- كذلك سجل تقدير نشاط إنزيم CMC-ase للعزلتينب٢,١ و٢,٥ U/ml على التوالى.
- بالإضافة لذلك فقد أظهرت النتائج قدرة العزلتين على تحليل الزيلان عند التحضين درجة حرارة ٥٠ درجة مئوية.