Sustainable Resources Management Journal
2017, 2 (8) 01-12
http://doi.org/10.5281/zenodo. 1034650

Recycling Paper Waste for Sustainable Sugar Production / Kirkuk-Iraq

Asal Aziz Tawfeeq [1,*] **and Mohammad Aljaradin** [2]

[1]*Biotechnology Medical Laboratory Technique Dep., Technical College, Kirkuk, Iraq*

[2] *Department of Water Resources Engineering, Lund University, Sweden*

*Corresponding author email: dr.asal_asis@yahoo.com

ABSTRACT

In the work, fifty soil samples were cultivated on selective Gauze agar medium plates containing carboymethyl cellulose as the sole carbon source. Thirty-two samples showed similarity to the morphological characteristics of Streptomyces bacterium which were tested for cellulolytic activity using the 3,5-Dinitrosalicylic acid reagent. Four efficient Streptomyces isolates were selected for their high cellulolytic activity which was recorded after 12 hours of incubation in the early logarithmic phase of growth. The four isolates attained their optimum cellulose activity after 96 hours of incubation with an enzyme activity of (0.55 $\pm$ 0.1, 0.53$\pm$ 0.12 and 0.49 $\pm$ 0.22 U/mL) by the Streptomyces isolates AA-17-1, AA-17-19 and AA-17-22 respectively with recording a specific enzymatic activity of (0.68 $\pm$ 0.23 U/ml) of the Streptomyces isolate AA-17-32. The result showed, a supreme activity of the *Streptomyces purpureus* AA-17-32 to reduce (20 g) of paper waste to about (0.78 U/ml). After applying further morphological and biochemical analysis it's recognized that, the Streptomyces isolate AA-17-32 belonged to the species *Streptomyces purpureus*. It concludes that this method for the production of cellulose can be used for the sustainable development of sugar production from the degradable of waste materials.

Keywords: Paper waste; Sustainable sugar production; *Streptomyces purpureus* AA-17-32; Kirkuk.

1. INTRODUCTION

The ability of microorganisms to degrade polysaccharides such as cellulose present in waste paper is a characteristic growing a considerable interest in the terms of sustainable development since cellulose is by far the most abundant organic biopolymer in nature making about (50%) of the total organic carbon in the atmosphere where most of it is waste materials (Yassien et al., 2014, Barros et al., 2010 & Begum et al., 2009). Tracing cellulolytic microorganisms is preferential in the industrial process since an adult human requires about (250 g) of carbohydrate per day to maintain life, part of this demand could be met through the degradation of waste material by the high scalability of these microorganisms enzymes (Da Vinha et al., 2011, Yin et al., 2010 & Ponnambalam et al., 2011).

Since cellulases are the third most important industrial enzyme after proteases and amylases (Maki et al., 2012, Kirk, et al., 2002 & Kuhad et al., 2011). Therefore, many microorganisms with cellulolytic activity were isolated; among them were the streptomycetes as the most attractive candidate for tracing cellulase production due to their natural ability to secrete extracellular enzymes and antibacterial agents into the culture broth (Kirk, et al., 2002 & Kuhad et al., 2011). Members of the genus Streptomyces belong to order Actinomycetes, and was first described by the scientists Henrici and Waksman in 1940; they are filamentous gram positive bacteria, aerobic that produce a wide variety of secondary metabolites (Bibb, 1996 & Chakroabur et al., 1997). They are saprophytes, obtaining nutrients and energy by solubilizing organic materials in soil through the production of extra cellular hydrolytic enzymes (Da Vinha et al., 2011, Maki et al., 2012 & Van Wezel et al., 1997). In soil, the genus Streptomyces make up one of the most dominant groups where they find soil as the suitable conditions for growth and proliferation due to their ability to decompose lignocellulolytic materials (Da Vinha et al. 2011Yassien et al., 2014 & Da Vinha et al., 2011). Therefore, the present study aimed to isolate and characterize cellulolytic Streptomyces strains from soil and use these isolates for the degradation of paper waste.

2. MATERIALS AND METHODS:

2.1 Bacterial source:

Soil samples were collected in sterile clean glass bottles from different locations in Kirkuk Technical College campus during the period from March till June 2017 following the methodology recommended by (Mokni-Tlili *et al*., 2011).

2.2 Isolation of Streptomyces Species:

For each collected soil sample, one gram was diluted in (100 ml) of distilled water and one ml was plated on selective Gauze agar medium from Fisher Scientific/ UK supplied with (20g/1L) of carboymethylcellulose sodium C9481 from MERCK/ Germany as the sole carbon source and plates were incubated at 37 ˚C for 14 days. Characteristic colonies were selected and re-cultivated several times on new plates of Gauze agar selective medium according to (Tawfeeq, 2000).

2.3 Streptomyces identification and morphological characterization:

Visual observation of colony morphology and appearance on Gauze agar medium in addition to the microscopic characteristics examined under light microscope (Optika / Italy) after Gram – staining were all performed according to the procedure in (Taddei *et al*., 2006) as follows:

(i) Aerial mass colour: The mass colour of mature sporulation aerial mycelium was observed following Streptomyces isolates growth on Gauze agar plates and the aerial mass was classified colour series of grey, white, red, yellow, green, blue, violet and black.

(ii) Substrate mycelium: Distinctive colour of the substrate mycelium was recorded and was classified to the colour series of black, brown, pink, red, red-violet, tan, violet-purple, yellow and yellow-greenish.

(iii) Melanoid pigments: The production of melanoid pigments was also considered and grouped according to the colour series of: red, yellow and brown. Some isolates did not produce melanoid pigments and were highlighted with negative marks.

(iv) The consistency of aerial mycelia: Streptomyces isolates were also classified according to the consistency of the aerial mycelia in being leathery or chalky.

(v) Spore chain morphology: According to the shape of the spore chains observed under light microscopy, the isolates were grouped as follows: Mono Verticillate (MV), Spiral (S), Rectus Flexibilis (RF) and Retinaculum Aspertum (RA) spore chains.
(vi) Spore numbers in the chain. The number of spores in each chain was observed and recorded.

2.4 Cellulolytic activity assay of the Streptomyces isolates.

Cellulose activity of the Streptomyces isolates was assayed according to the method in (Tawfeeq, 2000 & Mokni-Tlili et al., 2011) using the 3, 5-Dinitrosalicylic acid (DNS-D0550 SIGMA) from MERCK / Germany for the quantitation of the enzymatically released reducing sugars. Results were defined in the chart of cellulose enzyme activity as unit per ml of reduced D- glucose for each Streptomyces isolate after incubation in liquid Gauze medium supplied with (20g/1L) of carboxyl methyl cellulose sodium C9481 from MERCK/ Germany as the sole carbon source for 168 hours at 37˚C.

2.5 Antimicrobial, physiologic and biochemical screening of the Streptomyces AA-17-32.

The antimicrobial activities of the Streptomyces isolate AA-17-32 against the microorganisms: Bacillus subtilis, Candida albicans and Aspergillus niger were all carried out according to the method documented in (Tawfeeq, 2000) where theses strains were kindly provided by the (Medical Laboratory Techniques Department) of the Technical College.
The physiological tests including urease, hemolysis and Lecithinase in addition to the resistance tests of the Streptomyces isolate AA-17-32 against NaCl 7%, Neomycin 0.01%, rifampicin and sodium acid 0.01% beside the isolate ability to grow on sucrose, inositol, Mannitol, Rhamnose, Raffinose and Xylose from (Merck / Germany) as substrates were applied for strain confirmation.

2.6 Comparison method.

A comparison was performed according to the method present in (Taddei *et al*., 2006) in order to establish similarities between the isolated *Streptomyces* isolate AA-17-32 and other Streptomycetes strains.

2.7 Preparation of spore suspension.

Spore suspensions of the isolated Streptomyces purpureus AA-17-32 were prepared according to the method recommended by (Tawfeeq, 2000) scrapping the culture in a slant with the loop with the addition of (10 ml) distilled water then filtering the suspension in the centrifuge for (10 minutes) at (300rpm). The precipitate was re-suspended in sterile distilled water.

2.8 Utilization of paper waste for sugar production by Streptomyces purpureus AA-17-32.

The same methodology used for the assaying of cellulolytic activity mentioned in step (4) above was applied but with the substitution of different weights of paper waste as the sole carbon source instead of Carboxymethyl cellulose in the growth liquid medium of gauze and flasks were inoculated with (108 - spore suspension of Streptomyces purpureus AA-17-32- calculated using a hemocytometer) and were incubated for 168 hours at 37˚C according to (Fatokum et al., 2016).

2.9 Preservation of Streptomyces purpureus AA-17-32.

A-Preservation for short-term: Slants were preserved at 4˚C in the refrigerator. B-Preservation for the long term:

(a): In soil: a suitable garden soil was prepared for preserving the spores. The soil was cleaned dried and sieved (0.05mm in diameter), dispensed in small Bijo bottles covered with cotton plugs. Then sterilized by autoclaving and dried at 105˚C for 3hr.The sterile soil was then inoculated with 1ml of dense spore suspension, placed in desiccators and left at room temperature (for a week till drying) then preserved at 4˚C.

(b): In 40% glycerol: Small Bijou bottles were prepared, each contained 5ml of 40% glycerol from (Merck / Germany), sterilized in the autoclave, inoculated with 5ml of spore suspension, mixed and then preserved at -20˚C.

2.10 Statistical analysis.

All experiments were run in triplicates and data obtained were analyzed for the average and standard deviation in Microsoft Excel spreadsheet according to the method in (Fatokum et al., 2016).

3. RESULTS AND DISCUSSION

3.1 Isolation and screening for cellulolytic streptomycetes

Fifty soil samples were collected in sterile test tubes from the upper depth of dry garden soil of about (5-10 cm) in depth with characterized earthy smell from different locations within the campus of the Technical College in Kirkuk during the period from March till June 2017. Screening for the cellulolytic streptomycetes was achieved by cultivating diluted soil samples on Gauze agar medium as a selective medium containing (20g/1L) of carboxymethyl cellulose as the sole carbon source for the isolation of cellulolytic streptomycetes combined with the antifungal (cycloheximide) which was added for the inhibition of other contaminating fungi in the collected soil samples and plates were incubated at 37˚C for 14 days. Many workers recommended soil and Gauze medium for the isolation of cellulolytic streptomycetes where they documented that, streptomycetes dominate largely soil micro flora numbering about (106-108) spores per gram of soil and Gauze medium gives excellent sporulation which is the main characteristic feature in these microorganisms (Yassin et al., 2014, Tawfeeq, 2000 & Taddei et al., 2006). Accordingly, thirty-two Streptomyces isolates were defined based on their morphological and microscopic features and results of streptomycetes isolate identification and morphological characterizations were shown in (table 1). Morphology has played a major role in distinguishing streptomycetes from other actinomycetes through their characteristic life cycle that included; production of aerial mycelium bearing spores, production of substrate mycelium and spore chain morphology and numbers.

Table 1: Main morphological and microscopic characteristics of the visually examined and microscopically examined stained spores of the streptomycetes isolates cultivated on Gauze agar medium incubated at 37˚C for 14 days.

Isolate number	Aerial mass color	Color of substrate mycelia	Melanoid pigments	Consistency of aerial mycelia	Type of Spore chain	Number of spores in chain
AA-17-1	White	Red-violet	-ve	Leathery	*MV	>5
AA-17-2	White	Pink	-ve	Chalky	MV	>10
AA-17-3	White	Violet	Red	Chalky	MV	> 10
AA-17-4	White	Red	-ve	Chalky	*S	> 10
AA-17-5	Gray	Yellow	-ve	Chalky	*RA	>10
AA-17-6	White	Tan	-ve	Leathery	MV	>5
AA-17-7	Red	Pink	-ve	Chalky	MV	> 10
AA-17-8	White	Pink	-ve	Chalky	*RF	> 10
AA-17-9	Gray	Brown	-ve	Chalky	S	>10
AA-17-10	Violet	Tan	-ve	Chalky	RF	>10
AA-17-11	White	Brown	Yellow	Chalky	RA	> 10
AA-17-12	White	Brown	Yellow	Chalky	S	> 10
AA-17-13	Blue	Tan	-ve	Chalky	S	>10
AA-17-14	White	Pink	-ve	Chalky	S	> 10
AA-17-15	Gray	Violet	Red	Chalky	RA	> 10
AA-17-16	White	Brown	Yellow	Chalky	RF	> 10
AA-17-17	Yellow	Brown	-ve	Chalky	RA	>10
AA-17-18	Green	Brown	Yellow	Chalky	RA	>10
AA-17-19	White	Yellow	Yellow	Chalky	S	> 10
AA-17-20	Gray	Tan	-ve	Chalky	S	> 10
AA-17-21	Gray	Brown	-ve	Chalky	S	>10
AA-17-22	White	Yellow	-ve	Chalky	RA	> 10
AA-17-23	Black	Black	-ve	Chalky	RA	> 10
AA-17-24	White	Brown	-ve	Chalky	S	| >10
AA-17-25	Gray	Brown	Brown	Chalky	S	> 10
AA-17-26	White	Yellow- greenish	-ve	Leathery	MV	>5
AA-17-27	White	Yellow	-ve	Chalky	S	> 10
AA-17-28	Blue	Violet-purple	-ve	Chalky	S	>10
AA-17-29	Black	Black	Yellow	Chalky	RA	>10
AA-17-30	Violet	Red	Red	Chalky	RF	> 10
AA-17-31	Violet	Red	Red	Chalky	RA	> 10
AA-17-32	Gray	Brown	Yellow	Chalky	RF	>10

3.2 Kinetics of cellulose production of the Streptomyces isolates:

On the other hand, the thirty-two Streptomyces isolates were tested for their cellulolytic activity and were subsequently subjected to a quantitative method based on the measurement of

reduced D-glucose in the solution of carboxymethyl cellulose using 3,5-Dinitrosalsylic acid as the reagent. Figure 1, shows the results of four isolates with high cellulolytic activity.

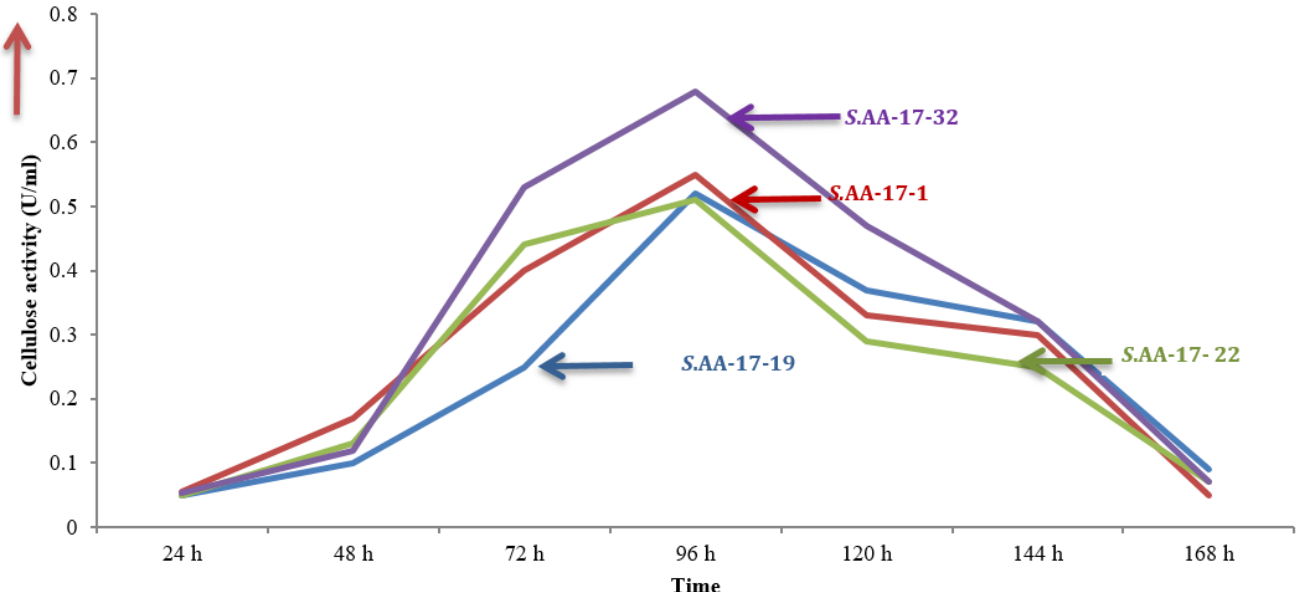

Figure 1: Quantitative determination with3,5-Dinitrosalsylic acid as reagent for the cellulolytic activity in Unit per ml of four selected Streptomyces isolates grown on liquid Gauze medium inoculated with (10^8) spore suspension and incubated at 37˚C for seven days.

These four Streptomyces isolates were selected to quantitatively determine their cellulolytic activity due to their heavy growth on Gauze selective medium and isolates were cultured under optimized conditions and monitored for growth in Gauze liquid medium with carboxymethyl cellulose as the sole carbon source for a period of 168 hours of incubation at 37˚C.

From the results of the figure above, it could be noticed that cellulose production was initiated at 12 hours of incubation, corresponding to early logarithmic growth phase of the four isolates. It was also indicated that the Streptomyces isolates attained their optimum cellulose production at 96 hours of incubation with an activity of (0.55 + 0.1, 0.53+ 0.12 and 0.49 + 0.22 U/mL) by the Streptomyces isolates AA-17-1, AA-17-19 and aa-17-22 respectively with specific productivity of 0.68 + 0.23 U/ml of Streptomyces isolate AA-17-32.

Moreover, it could be noted that cellulose production by the isolates was in tandem up until 120 hours of incubation, after which there was a decline in cellulose production, with respect to the late logarithmic growth phase. The same results were documented by (Fatokum et al., 2016, Das et al., 2014 & Wang et al., 2015) where their isolates showed the same cellulose productivity even under different conditions of temperature and pH.

3.3 Identification of the Streptomyces isolate AA-17-32

Due to the high cellulolytic activity of the Streptomyces isolate AA-17-32, it was selected for further assays for strain confirmation; results were shown in the table (2).

Table 2: Main morphological and biochemical features of the Streptomyces AA-17-32 isolate cultivated on Gauze agar medium incubated at 37˚C for 14 days and other the similar strain.

Characteristics	Streptomyces purpureus	Streptomyces isolate AA-17-32
Rectus flexuous Spore chain (RF)	+	+
Gray Spore mass	+	+
Brown substrate mycelium	+	+
Brown Pigmentation	+	+
Melanin production	+	+
Antibiosis against:		
Bacillus subtilis	-	-
Candida albicans	+	+
Aspergillus niger	-	-
Physiological test:		
Urease	+	+
Hemolysis	-	-
Lecithinase	+	+
Resistance to:		
NaCl 7%	-	-
Neomycin 0.01%	+	+
Rifampicin	+	+
Sodium azid 0.01%	-	-
Growth with:		
Sucrose, Rhamnose, Raffinose &xylose	-	-
Inositol & Mannitol	+	+

Attentive comparison between the Streptomyces isolate AA-17-32 and other strains of streptomycetes showed resemblances between the isolate and Streptomyces purpureus as

reflected in table (2). Thus, the isolated strain was named Streptomyces purpureus AA-17-32. (Where AA. was named after Asal Aziz, and number 17 is for being isolated during 2017 and 32 is the sample number).

3.4 Sustainable sugar production from waste papers

In this research, waste papers were collected from different departments at the Technical College campus in Kirkuk and stored at room temperature before use. Lignocellulosic wastes were pretreated with pure water at 190 °C for 15 min; then, fine chopped papers were added in (20g/ 100 ml w/v) in Gauze liquid medium without any other carbon source and cultures inoculated with (10^8) spore suspension of *Streptomyces purpureus* AA-17-32 incubated for 168 hours at 37˚C and sugar reduction was determined quantitatively using the 3,5-Dinitrosalicylic acid reagent. Results showed high cellulolytic activity encountered with this strain of about (0.78 $\pm$ 0.12 U/ml) after 96 hours of incubation (Figure 2).

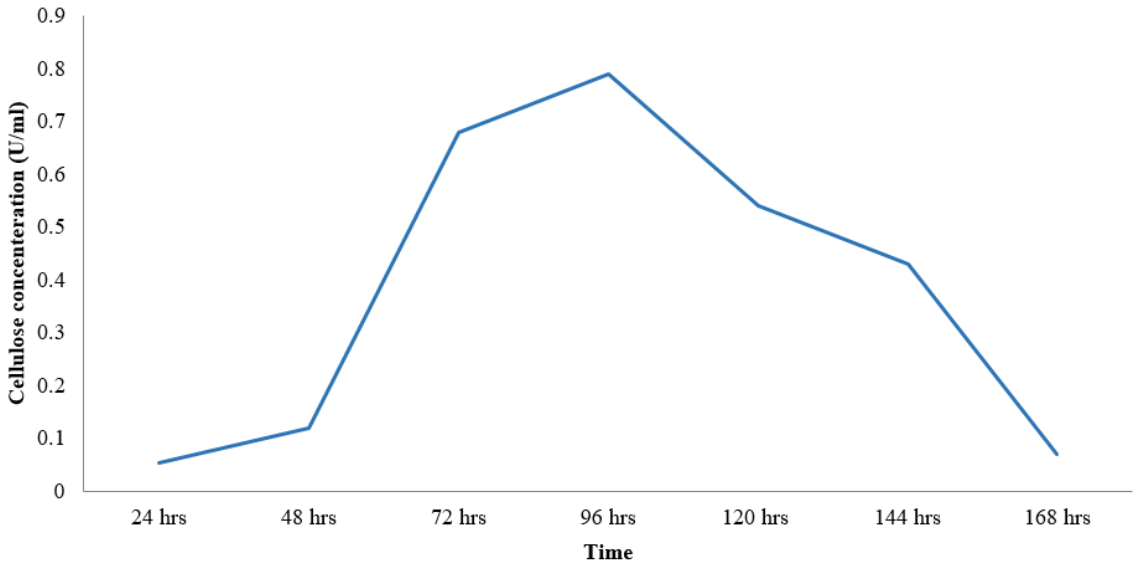

Figure 2: Quantitative determination with3,5-Dinitrosalsylic acid as reagent for the cellulolytic activity in unit per ml of *Streptomyces purpureus* AA-17-32 grown on liquid Gauze medium inoculated with (10^8) spore suspension and (20 g/ 100 ml) of paper waste as sole carbon source and incubated at 37˚C for seven days.

From the figure, we can see that the cellulose activity began to appear in the medium after 24 hours of incubation at 37˚C and pH (7.0). No detectable activities were measured before

this time probably because considerable amount of cellulose enzyme was produced by the isolate to degrade the complex chains of cellulose into smaller ones contained in the paper waste; then, after 24 hours of incubation, the smaller chains were broken into units of D- glucose which could be detected by the 3,5-Dinitrosalsylic acid reagent. However, the same results were obtained with two isolates mentioned in (Sun et al., 2014, Wen et al., 2014, Yassien et al., 2014 & Zhao et al., 2012).

4. CONCLUSIONS

Using urban cellulosic wastes for the sustainable production of sugar is very important in terms of sustainable development because of the globally increasing needs to food besides the ecological concerns. *Streptomyces purpureus* AA-17-32 could be a useful tool in solving environmental contamination problems associated with paper waste and could be used in food industry for the sustainable sugar production from paper waste.

5. RECOMMENDATIONS

Further studies are required on the genetic level for the investigation of cellulose enzyme kinetics *in vivo*. Also, further studies are required to study the effects of mutations on cellulose enzyme production.

6. REFERENCE

Barros, R.R.O., Oliveira, R.A., Gottschalk, L.F. and Bon, E.P.S., Production of cellulolytic enzymes by fungi *Acrophialophora nainiana* and *Ceratocystis paradoxa* using different carbon sources. Appl. Biochem. Biotechnol., 2010. 161:448-454.

Begum, F., Absar, N. and Alam, M.S., Purification and characterization of extracellular cellulase from *A. oryzae* ITCC-4857.01. J. Appl. Sci. Res., 2009, 5:1645-1651.

Bibb, M.J., The regulation of antibiotic production in Streptomyces coelicolor A3 (2). Mol. Microbiol., 1996, 142:1335-1344.

Chakroabur, R. and Bibb, M.J., The ppGpp Synthetase gene Cre (A) of Streptomyces coelicolor A3 (2), plays a conditional role in antibiotic production and morphological differentiation. J. Bacteriol., 1997, 179(18):5854-5861.

Das, P., Solanki, R. and Khanna, M., Isolation and Screening of cellulolytic Actinomycetes from diverse habitats. Int. J. Adv. Biotechnol. Res.(IJBR), 2014, 5(3): 438–451.

Da Vinha, F.N., Gravina-Oliveira, M.P., Franco, M.N., Macrae, A., da Silva Bon, E.P., Nascimento, R.P. and Coelho, R.R., Cellulase production by *Streptomyces viridobrunneus*

SCPE-09 using lignocellulosic biomass as inducer substrate. Appl. Biochem. Biotechnol., 2011, 164(3):256-267.

Fatokum, E.N., Nwodo, U.U. and Okoh, A.I., Classical Optimization of Cellulase and Xylanase : Production by a Marine Streptomyces Species Appl. Sci., 2016, 6: 286-300.

Kirk, O., Borchert, T.V. and Fuglsang, C.C., Industrial enzyme applications. Curr. Opin. Biotechnol., 2002, 13:345-351.

Kuhad, R.C., Gupta, R. and Singh, A., Microbial cellulases and their industrial applications. SAGA-Hindawi Acess to Research, Enzyme Research, 2011, volume 2011, Article ID 280696. 10 pages.

Maki, M.L., Idrees, A., Leung, K.T. and Qin, W., Newly Isolated and Characterized Bacteria with Great Application Potential for Decomposition of Lignocellulosic Biomass. J. Mol. Microbiol. Biotechnol., 2012, 22:156–166.

Mokni-Tlili S., Hafedh, B., Abdennaceur, H. and Ali, G.,Studies on the ecology of actinomycetes in an agricultural soil amended with organic residues: II. Assessment of enzymatic activities of Actinomycetes isolates. Journal of Microbiology & Biotechnology, 2011, 27(10): 2251-2259.

Ponnambalam, A.S. , Deepthi, R.S. and Ghosh, A.R., Qualitative display and measurement of enzyme activity of isolated cellulolytic bacteria. Biotechnol. Bioinf. Bioeng. 2011,1:33-37.

Sun, Y., Yi, X., Peng, M., Zeng, H., Wang, D., *et al*. ,Proteomics of *Fusarium oxysporum* race 1 and race 4 reveals enzymes involved in carbohydrate metabolism and ion transport that might play important roles in banana Fusarium, wilt. PLoS One, 2014, 9(12):e113818. https://doi.org/10.1371/journal.pone.0113818Tawfeeq, Asal Aziz, 2000, Isolation and identification of cellulolytic Streptomyces species from the local soil, Master thesis, Al Nahrain University, Iraq.

Taddei, A., Rodrı´guez, M., Ma´rquez-Vilchez,E. and Castelli, C., Isolation and identification of Streptomyces spp. from Venezuelan soils: Morphological and biochemical studies. I. Microbiological Research, 2006, (161) 222—231.

Van Wezel, G.P., White, J., Young, P., Postma, W.P. and Bibb, M.J., Substrate induction and glucose repression of maltose utilization by *Streptomyce coelicolor* A3 (2) is controlled by *mal* R family of regulatory genes. *Mol. Microbiol*., 1997, 23(3):537-549.

Wang, L., Xing, M., Di, R. and Luo, Y., Isolation, Identification and Antifungal Activities of *Streptomyces aureoverticillatus* HN6J Plant Pathol Microb 2015, 6:281.

Wen T, Huang X, Zhang J, Zhu T, Meng L, et al. . 2015, Effects of water regime, crop residues, and application rates on control of *Fusarium oxysporum* f. sp. cubense. J Environ Sci (China) 31: 30-7. doi: 10.1016/j.jes.2014.11.007.

Yassien, A.M., Jiman-Fatani, A.A.M. and Asfour, H.Z., Production, purification and characterization of cellulase from Streptomyces sp. Afr. J. Microbiol. Res., 2014, 8(4):348–354.

Yin, L., Lin, H. and Xiao, Z., Purification and characterization of a cellulase from *Bacillus subtilis* AJ1. J. Marine Sci. and Technol., 2010,18:466-471.

Zhao, S., Ren, F.E., Liu, J., Qin, J. and Pan, H., Screening, identification and optimization of fermentation conditions of an antagonistic actinomycetes strain to *Setosphaeria turcica* (in Chinese). Acta Microbiol. Sinica, 2012, 50: 1228-1236.