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In Vitro Study to Recognize the Optimum Conditions Affecting the Endophytic Cellulase Production

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ABSTRACT

In this study traditional microbiological isolation techniques were used to recognize cellulolytic activity of most potent isolates. The isolates were identified using both morphological and molecular methods. Functional studies were carried out to determine the optimum pH, temperature, incubation period, inoculum size, carbon and nitrogen sources for screened isolate. High cellulase producing endophytic bacteria *Achromobacter spanius, Bacillus amyloliquefaciens* and *Stenotrophomonas maltophilia* isolated from leaves, stems and roots of; Clover, Wheat, Lettuce, Spinach, *Vicia Faba*, Garlic, Dill, Olive, Acacia and Prosopis trees were subjected to controlled conditions in laboratory. Tested factors which believed to be effective on improving growth conditions were studied in vitro to achieve the highest production of cellulase enzyme. The highest values of cellulase were produced by *Achromobacter spanius* (2.50 mg/ml) followed by *Bacillus amyloliquefaciens* (2.26 mg/ml) after 6 days of incubation. The optimum conditions resulted in optimum cellulase production were 30°C at pH 6 for *Achromobacter* and *Bacillus* while the optimum conditions for *Stenotrophomonas* was 40°C at pH 7. Isolates produced the highest values of cellulase with ammonium sulphate and 30 g/l glucose as nitrogen and carbon sources, respectively. *Achromobacter* was more effective in producing cellulase than *Bacillus* and *Stenotrophomonas*. This was true under all the tested conditions.

KEYWORDS

Cellulase Enzyme, Endophytic Cellulolytic Bacteria, Culture Conditions.

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INTRODUCTION

In vitro series of microbiological tests were carried out to recognize the most effective aseptic conditions that improved the efficiency of endophytic bacteria in producing cellulose enzyme. Lignocellulosic biomass is the most abundant organic material in nature with high biotechnological potential **(Rastogi *et al*., 2010)**. The large volume of lignocellulolytic waste could cause serious environmental pollution **(Vivek *et al*., 2008).** The cellulose conversion to simple sugars by microorganisms plays an important role in the carbon cycle **(Romano *et al*., 2013).** A set of cellulases such as endoglucanase (1, 4-ß-D-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1, 4-ß-D-glucan-gluco-hydrolase; EC3.2.1.74) and ß-glucosidase (ß-D-glucoside-gluco-hydrolase; EC3.2.1.21) is required for complete hydrolysis of cellulosic material. Lastly, the cellobiose is converted to glucose by ß-glucosidase **(Lynd *et al*., 2002).** Some bacterial genera such as *Bacillus* and *Paenibacillus* are capable of degrading both Carboxymethyl cellulose (CMC) and Avicel (carbon source) **(Afzal *et al*., 2010),** as well as lignocellulosic waste from the agro-industry **(Macedo *et al*., 2013).** Low cost agricultural residues such as sugarcane bagasse, wheat bran, rice bran and others, which have been found to be good sources of cellulase production moreover; various carbon sources in the growth medium influenced the bacterium's ability to produce cellulose **(Techapun *et al.*, 2003)**.

Cellulose, a crystalline polymer of D-glucose residues connected by β-1, 4 glucosidic linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature **(Saha *et al.,* 2006)**. Therefore, it has become considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. Cellulose enzyme is responsible of cellulose degradation and produced by several microorganisms, commonly by bacteria and fungi. **Immanuel *el al.,* (2006)** explored that *Cellulomonas, Bacillus* and *Micrococcus spp.* were more effective in producing endoglucanase enzyme at pH ranged from 5-7. Microorganisms are important in conversion of lignocellulose wastes into valuable products like biofuels produced by fermentation **(Lynd *et al.,* 2002)**. Consequently, successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes **(Alam *et al.,* 2004)**.

In this respect, some studies have been focused on the cellulase producing fungi **(Lan *et al.,* 2013; Saini *et al*., 2015; Sharma *et al*., 2015; Callow *et al.,* 2016),** bacteria **(Rastogi *et al.,* 2010; Assareh *et al.,* 2012; Shanmugapriya *et al*., 2012)** and actinobacteria **(Cirigliano *et al*., 2013; Sarita *et al.,* 2013).** Enormous amounts of agricultural, industrial and municipal cellulosic wastes are accumulating or used inefficiently due to their high cost towards utilization processes **(Lee *et al.*, 2008).** Therefore, this problem is arising as a topic of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. **Nandimath *et al.,* (2016)** found that, increasing glucose in addition to optimum pH, temperature, time and nutritional requirements resulted in high cellulase production. Therefore, the present work aimed at recognizing the optimum conditions for improvement of enzymatic hydrolysis of organic residues specially rice straw to protect the environment from expected pollution.

MATERIALS AND METHODS:

Sample Collection and Isolation of Endophytic Bacteria:

Roots, stem and leaves of clover and wheat under clay soils from Sohag City -Egypt, lettuce, spinach, vicia faba, garlic, Acacia and Prosopis trees under sandy soils from Anshas -Elsharkia City- Egypt, Wheat, Dill and Olive under calcareous salt-affected

soils from Ras Sudr - South Sinai City-Egypt, and Acacia and Prosopis trees from Desert Research Center in Mataria - Cairo City- Egypt, were transferred to the Lab. in sterile sampling bags and processed within 1 hour after collection. The plant organs were washed with running water to remove soil particles and were surface sterilized by sequential immersion in 70% ethanol for 5 min and solution of hypochlorite (0.9% available chlorine) for 20 minutes, then washed three times with sterile distilled water to remove surface sterilizing agents before being soaked in 10% $NaHCO_3$ solution to disrupt the plant tissues and to inhibit the growth of fungi **(Cao *et al.*, 2003)**. Surface sterilized organ was divided into small fragments (1-3) cm under aseptic conditions and was placed on Luria Bertani agar media (LB-agar) used for isolation of endophytic bacteria **(Gordon and Weber, 1951)**. This medium contains; tryptone 10g/l, yeast extract 5g/l, sodium chloride 5g/l, agar 15g/l). The LB-agar plates were incubated at 37°C for 2-3 weeks.

Screening the endophytic cellulase producing bacteria:

Qualitative assay

Qualitative assay for cellulolytic activity of 58 bacterial isolates were processed using cellulose Congo red agar (CCA) contains (g/L): K_2HPO_4 0.50; $MgSO_4$ 0.25; CMC 1.88; Gelatin 2.00; Congo red 0.20; trace salts solution 1.00 mL. Trace salt solution was composed of, $FeSO_4 \cdot 7H_2O$ 0.1 g, $MnCl_2 \cdot 4H_2O$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, distilled water 100.0 ml and agar 20.0 g as proposed by **Hendricks *et al.*, (1995)**. After sterilization, the clear opaque zone is measured using zone scale 3 mm in diameter. The plates were incubated at 37°C for five days. The plates were flooded with 1M NaCl to detect the cellulolytic activity of isolates. The formation of a clear zone of hydrolysis indicated the CMC degradation. Clear zone diameter /colony diameter ratio was measured to select the highest cellulase producer **(Ariffin *et al.*, 2006).**

Quantitative assay

The most efficient seventeen isolates elected after qualitative assay were subjected to quantitative assay. Quantitative screening of cellulase producing seventeen bacterial isolates was carried out using the modified medium after **Hendricks *et al.* (1995)**, contains (g/L): K_2HPO_4, 0.50; $MgSO_4$, 0.25; CMC, 2.00; Trace salts solution, 1 mL. pH of the medium was adjusted to 7.0 using 1 N NaOH before autoclaving. About 10^{-3} CFU / ml of culture from the CCA slant was transferred to 250 mL Erlenmeyer flask containing 50 mL of cellulase production medium. The culture was incubated at 28 ± 2°C with 200 rpm for five days. The culture broth was centrifuged at 10,000 rpm for 15 min at 4°C to separate the bacterial biomass. The cell free supernatant was analyzed for cellulase enzyme activity.

*Identification of the most potent cellulase producing bacteria***:**

The most potent isolates were identified using 16S rRNA gene sequences to provide genus and species for isolates which processed by Sigma Scientific Services Co. The most potent isolates were identified as, *Achromobacter spanius*, *Bacillus amyloliquefaciens*, and *Stenotrophomonas maltophilia*.

- *DNA Extraction of Endophytic Bacteria***:**

DNA extraction was carried out by using protocol of Gene Jet genomic DNA purification Kit, then PCR clean up to the PCR product was done using Gene JET™ PCR Purification Kit (Thermo K0701) according to **Boom *et al.* (1990)**.

- *Phylogeny Tree Construction***:**

Sequences of 16S rRNA was matched with sequences of reference strains in *GenBank* database (http://www.ncbi.nlm.nih.gov) and was aligned through *Clustal W Multiple Alignment* tool. Phylogeny tree was constructed using phylogeny tool in *MEGAV.6 program*, inference using *Maximum Likelihood* method, and analyzed with evolutionary

distance using *Tamura-Nei* model **(Tamura *et al.*, 2011).**

Bacterial strains:

Endophytic bacteria *Achromobacter spanius, Bacillus amyloliquefaciens* and *Stenotrophomonas maltophilia* were maintained on carboxymethyl cellulose (CMC) broth media **(Yin *et al*, 2010),** (g/l): peptone 10.0; carboxymethyl cellulose (CMC) 10.0; K_2HPO_4 2.0; ($MgSO_4.7H_2O$) 0.3; $(NH_4)_2SO_4$ 2.5 and gelatin 2.0; media is adjusted at pH 7 for 5 days of incubation in shaker with 200 rpm at 30˚C.

Optimum culture conditions for optimizing cellulase production:

The investigated parameters temperature, pH, incubation periods, inoculum size, carbon sources and nitrogen sources affecting cellulase enzyme productivity were carried out on (CMC) broth media. Cellulase enzyme was determined in filtrates obtained from culture media according to **Miller, (1959).**

- *Temperature, pH, incubation period, inoculum size, nitrogen source, Carbon source*:

Carboxymethyl cellulose (CMC) broth media was used to determine the optimum temperature for cellulase production, at pH 7 and inoculum size (10^{-3}) and various temperatures i.e. 30, 35, 40, 45, 50, 55 and 60ºC and incubated for five days. Cell-free culture filtrate was obtained and used as enzyme source. Similar broth media were subjected to different pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.5, 8.5 to 9.0) for five days in flasks using 6N HCl or 6N NaOH. The cultures were incubated at 30˚C. Different incubation periods and regular intervals of 2, 3, 4, 5, 6, and 7 days at 30˚C were examined. Culture media was incubated under different incubation periods and regular intervals of 2, 3, 4, 5, 6, and 7 days at 30˚C. The flasks inoculated with different inoculums size i.e. (10^{-1}), (10^{-3}), (10^{-5}) and (10^{-6}). Amm. sulphate, yeast extract and urea were used as N sources instead of peptone in (CMC) broth media. Glucose, soluble starch, insoluble starch, fructose, maltose, and sucrose) at different concentrations of 10, 30 and 50g/L were examined in carboxymethyl cellulose (CMC) media instead of carboxymethyl cellulose.

RESULTS AND DISCUSSION

Screening of cellulolytic endophytic bacteria by qualitative assay:

About more than 58 microorganisms isolated, all microorganisms are able to grow on LB agar media but only some of them were able to produce clearing zone which indicated cellulolytic activity on CMC agar plates and proved positive to cellulolytic activity by cellulose-Congo red agar (CCA). About 17 isolates were active in producing clearing zone. Pure colonies of halo zone produced isolates were stored on slants.

Selection of the most potent cellulase productivity bacteria (quantitative assay):

17 isolates showed clear zone on cellulose Congo red agar media and exhibited remarkable enzyme activity were quantitatively screened for cellulolytic activity. Among them only *Achromobacter spanius, Bacillus amyloliquefaciens,* and *Stenotrophomonas maltophilia* were identified as the most potent isolates.

Identification of the most potent cellulase producing bacteria:

Amplicon 16S rRNA sequences of isolates *Achromobacter spanius, Bacillus amyloliquefaciens* and *Stenotrophomonas maltophilia* was applied. On 1% agarose gels showed in 1500 bp. Amplification 16S rRNA sequences of bacteria can produce 1500 - 1600 bp amplicon. Analysis of DNA sequences showed that isolates was identified as *Achromobacter spanius* (Fig. 1), *Bacillus amyloliquefaciens* (Fig. 2) and *Stenotrophomonas maltophilia* (Fig. 3).

Phylogeny Tree Construction:

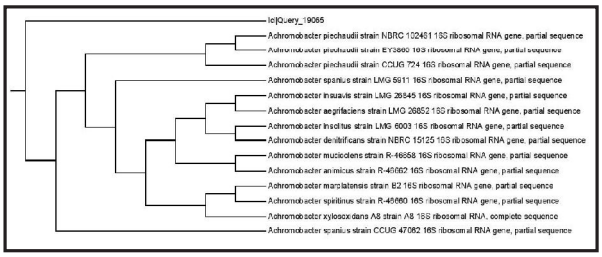

Fig. (1): Phylogeny tree of isolate No. (1) Was identified as *Achromobacter spanius*.

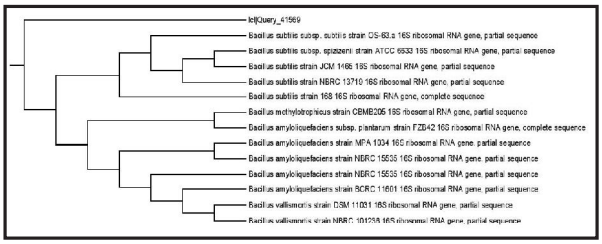

Fig. (2): Phylogeny tree of isolate No. (2) Was identified as *Bacillus amyloliquefaciens*.

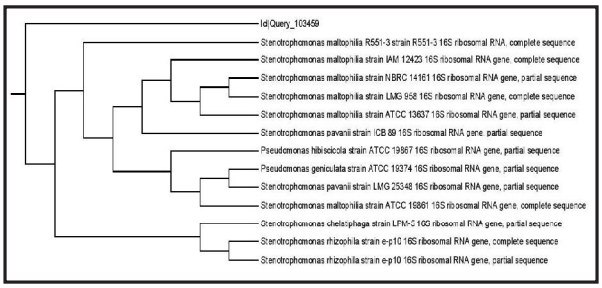

Fig. (3): Phylogeny tree of isolate No. (3) Was identified as *Stenotrophomonas maltophilia*.

***Optimization of cellulase production*:**

The activity of enzyme excreted by *Achromobacter spanius, Bacillus amyloliquefaciens,* and *Stenotrophomonas maltophilia*. Under different investigated parameters were determined (Tables 1-6).

Table (1) : *Effect of temperature on cellulase enzyme activity.*

Temperature °C	Enzyme activity Units (mg/ml)			
	Achromobacter spanius	***Bacillus amyloliquefaciens***	***Stenotrophomonas maltophilia***	**Mean**
30	1.38	1.34	0.89	1.20
35	1.26	1.29	1.27	1.29
40	1.21	1.17	1.30	1.23
45	0.98	0.91	0.74	0.88
50	0.96	0.90	0.61	0.82
55	0.70	0.73	0.61	0.68
60	0.59	0.65	0.60	0.61
Mean	1.01	1.00	0.86	

L.S.D. at 0.05: *Bacteria 0.019, Temperatures, 0.030, Bacteria x Temperatures, 0.017.*

Gradual increase in incubation temperature resulted in decrease of cellulase production for *Achromobacter* and *Bacillus* respectively, while *Stenotrophomonas* reflected fluctuated response to different temperature affected cellulase production (Table.1). Cellulase activity of all isolates was decreased at 60°C. The optimal temperature for growth was 30°C for *Achromobacter spanius* (1.38 mg/ml) and *Bacillus amyloliquefaciens* (1.34 mg/ml), recording the highest enzyme activity while *Stenotrophomonas maltophilia* produced the highest value of 1.30 mg/ml at 40°C after incubation for five days. Regardless temperatures, the mean average indicated that ~~it~~ *Achromobacter and Bacillus* produced high enzyme quantities. Our results are in agreement with **Nandimath *et al.* (2016)** who reported that, both. *Bacillus* sp. and *Pseudomonas* sp. produced maximum cellulase production at 30°C, the optimum reaction rate was observed at 30°C. Also, **Immanuel *et al.* (2006)** mentioned that, the enzyme activity was carried out by using various temperature 20-50°C. The enzyme activity was minimum at 20°C by the

bacterial strains such as *Cellulomonas, Bacillus* and *Micrococcus* sp., respectively. Maximum production enzyme was reached at 40°C, this results is approved with our result with *Stenotrophomonas* where it produced (1.30 mg/ml) at 40°C. Further increase of temperature, reduced the enzyme activity considerably at 50°C by the tested bacterial strains. **Shanmugapriya *et al.* (2012)** mentioned that, optimized conditions of CMC and Coir waste as substrates for *Bacillus* sp. were at 40ºC.

Table (2): *Effect of pH on cellulase enzyme activity.*

pH	Enzyme activity Units (mg/ml)			
	Achromobacter spanius	*Bacillus amyloliquefaciens*	*Stenotrophomonas maltophilia*	Mean
5.0	1.31	1.02	0.66	0.99
5.5	1.31	1.07	0.80	1.06
6.0	1.61	1.48	0.94	1.34
6.5	1.48	1.41	1.28	1.39
7.0	1.41	1.42	1.43	1.42
7.5	1.37	1.30	1.31	1.33
8.0	1.17	1.09	1.15	1.14
8.5	1.13	0.95	0.89	0.99
9.0	1.06	0.89	0.79	0.91
Mean	1.70	1.52	1.11	

L.S.D. at 0.05: Bacteria 0.017, pH 0.029, Bacteria x pH 0.051.

Achromobacter and *Bacillus* showed the best result of cellulase production at pH 6 (1.61 and 1.48 mg/ml, respectively) while the best one for *Stenotrophomonas* was observed at pH 7 (1.43 mg/ml) (Table. 2). The mean average of pH values indicated that pH 6.5 and pH 7 were better in enzyme production for all isolates (1.39 and 1.42). Mean average of cellulase production showed the superiority of *A. spanius* and *B. amyloliquefacien*s over *S. maltophilia*. The enzymatic activity of *Achromobacte*r and *Bacillus* was stable at pH 5.0 and 5.5 then increased at pH 6.0. The results showed a decrease in enzyme activity at pH 9.0. On the other hand *Stenotrophomonas* was demonstrated the highest enzymatic activity at pH 7.0 (1.43mg/ml) and showed progressive decrease until pH 9.0 (0.79 mg/ml). From the above results, it was found that, the best enzymatic activity of *Achromobacter* and *Bacillus* was at pH 6.0 while the best enzymatic activity of *Stenotrophomonas* was at pH 7.0. Our results are in agreement with **Ibrahim and El-diwany (2007)** who mentioned that, the optimal production of cellulases at pH 7.5 which is close to the optimum pH value of most Bacillus cellulases. Also**, Immanuel *et al.* (2006)** reported that, the endoglucanase enzyme activity by *Cellulomonas* sp at varying pH level showed that the activity was increased from pH 5.0 to 7.0. Further increase in pH level to 8.0 and 9.0 resulted in considerable decrease in enzyme activity.

A. spanius and *B. amyloliquefaciens* produced 2.50 and 2.26 mg/ml at 6 days incubation period while *S. maltophilia* produced 1.43 mg/ml after 7 days of incubation (Table. 3). The lowest enzyme activity for cellulase produced by the isolated microorganisms was 1.44 mg/ml after 2 days incubation period. Also, cellulase enzyme activity on mean average basis was gradually increased with increasing the incubation period ~~to~~ where the highest value detected after 6 days (2.07mg/ml). According to mean average of isolates, it could be ranked as, *A. Spanius* > *B. amyloliquefaciens* > *S. maltophilia*.-The enzyme activity of *Achromobacter* ,*bacillus* and *Stenotroph-*

omonas were increased gradually with increased incubation periods our results are in agreement with **Shaikh *et al.* (2013)** who found that, increased cellulase production by *Bacillus Sp*. and *Pseudomonas Sp* fermentation was carried out at different incubation periods ranging from (2-5days), The incubation period of 4 days achieved the highest cellulase enzyme production on the other hand in our study the maximum production of enzyme activity was after incubation for 6 days (2.5 and 2.26 mg/ml), respectively.

Table (3): *Effect of incubation periods on cellulase enzyme activity.*

Incubation period (days)	Enzyme activity Units (mg/ml)			
	Achromobacter spanius	***Bacillus amyloliquefaciens***	***Stenotrophomonas maltophilia***	**Mean**
2	1.62	1.61	1.08	1.44
3	1.69	1.65	1.02	1.46
4	1.92	1.72	1.23	1.63
5	2.35	1.86	1.40	1.87
6	**2.50**	**2.26**	1.44	2.07
7	2.44	2.25	**1.43**	2.04
Mean	1.79	1.62	1.09	

L.S.D. at 0.05: Bacteria 0.021, Incubation periods 0.030, Bacteria x Incubation periods 0.052.

Table (4): *Effect of inoculum size on cellulase enzyme activity.*

Inoculum size c.f.u/ml	Enzyme activity Units (mg/ml)			
	Achromobacter spanius	*Bacillus amyloliquefaciens*	*Stenotrophomonas maltophilia*	Mean
(10^{-1})	1.57	1.35	1.07	1.49
(10^{-3})	2.51	1.83	1.79	1.91
(10^{-5})	2.09	1.57	1.57	1.22
(10^{-6})	1.98	1.43	1.39	1.14
Mean	2.04	1.54	1.45	

L.S.D. at 0.05: Bacteria 0.037, Inoculum size 0.043, Bacteria x Inoculum size 0.075.

Inoculum size of 10^{-3} was the optimum inoculum for best growth of tested isolates to produce the best cellulase enzyme (1.91mg/ml). In this regard, *A. spanius* produced the highest cellulase production (2.51mg/ml) followed by *B. amyloliquefaciens* (1.83mg/ml), then *S. maltophilia* (1.79 mg/ml) at inoculum size 10^{-3} (Table 4). Our results showed that the highest cellulase activity was resulted in inoculum size 10^{-3} for the three isolates. **Shaikh *et al.*, (2013)** mention that, the inoculum size of 2.0 % achieved the highest cellulase enzyme production in *Bacillus sp.*

Table (5): *Effect of nitrogen sources on cellulase enzyme activity.*

Nitrogen sources	Enzyme activity Units (mg/ml)			
	Achromobacter spanius	*Bacillus amyloliquefaciens*	*Stenotrophomonas maltophilia*	Mean
Ammonium. Sulphate	1.44	1.29	1.19	1.31
Urea	1.09	1.05	1.05	1.06
Yeast extract	1.39	1.08	1.07	1.18
Mean	1.31	1.14	1.10	

L.S.D. at 0.05: Bacteria 0.031, Nitrogen Sources 0.032, Bacteria x Nitrogen Sources 0.057.

Ammonium sulphate was the best nitrogen source resulted in the highest enzymatic activity of cellulase for the three isolates (1.31 mg/ml) followed by yeast extract (1.18 mg/ml) then urea (1.06 mg/ml) (Table.5). In this respect, *A.spanius* produced the highest cellulase activity (1.44 mg/ml), followed by *B. amyloliquefaciens* (1.29mg/ml) and *S.maltophilia* (1.19 mg/ml) came to the next.⁻In general, ammonium sulphate and yeast extract as nitrogen source were used in the medium where produced the highest cellulase enzyme for *Achromobacter* (1.44 and 1.39mg/ml), respectively. On the other hand only ammonium sulphate was the best source of N to produce the maximum production of enzyme, at the same time urea and yeast extract did not effect on cellulase production by *Bacillus* and *Stenotrophomonas* isolates (Table. 5).Our results in the same line with **Sethi *et al.* (2013)**, who mentioned that, among the various nitrogen sources tested, ammonium sulphate was found to be the best nitrogen source for cellulase production by *Pseudomonas fluorescens, Bacillus subtilus, E. coli,* and *Serratia marscens* due to their direct entry in protein synthesis.

The results in Table. (6) indicated that, 30 g/l glucose, based on mean average, produced 2.09 mg/ml better than 10 g/l and 50 g/l (1.95mg/ml). In the same way, 30 g/l soluble starch produced 1.22 mg/ml followed by 10 g/l (1.17 mg/ml) and 50 g/l (1.03 mg/ml). While, *S. maltophilia* produced 0.94 mg/ml in case of 30 g/l insoluble starch addition in (CMC) broth media instead of carboxymethyl cellulose. Increasing the quantity of fructose up to 30 g/l led the tested isolates to produce more enzyme which accounted for 1.23, 1.12and 0.94mg/ml for same sequence. In the same direction, increasing fructose up to 50 g/l resulted in decrease in enzyme activity ~~at~~ of all isolates. Mean average of C source levels, 30 g/l maltose produced 0.87 mg/ml followed by addition of 10 g/l (0.86 mg/ml) and 50 g/l (0.85 mg/ml). Addition of 30 g/l maltose compensated 0.92, 0.81 and 0.85 mg/ml cellulose by *A.spanius*, *B. amyloliquefaciens* and *S. maltophilia*, respectively. Mean average of carbon sources reflected the superiority of 30 g/l of sucrose (0.94 mg/ml) over 50 (0.92 mg/ml) and 10 g/l (0.89 mg/ml). Comparison held between isolates indicted the superiority of *A. spanius* (1.04 mg/ml) over *B. amyloliquefaciens* (0.89 mg/ml) and *S. maltophilia* (0.81mg/ml) with addition of 30 g/l of sucrose. The results indicated that, concentrations of glucose, soluble starch, insoluble starch, fructose, maltose and sucrose (30 g/l) as a carbon source were better than (10 g/l) and (50 g/l), respectively in all the three isolates. *Achromobacter* was produced cellulase activity more than *Bacillus* and *Stenotrophomonas* with the use of all carbon sources and the highest production of cellulase enzyme was induced by glucose (30 g/l) as carbon source. Our results in the same line with **Sethi *et al.* (2013),** who mentioned that, glucose was found to be the best source for cellulase production. Also, the highest cellulase production compared to other carbon sources by *Pseudomonas fluorescens, Bacillus subtilus, E.*

coli, and *Serratia marscens*. At the same time **Shanmugapriya *et al.* (2012)** mentioned that, among the various carbon sources tested, glucose was found to be the best carbon source for the substrates CMC and coir waste by *Bacillus species*, because glucose is the good inducer of the cellulase production.

Table (6): *Effect of carbon sources on cellulase enzyme activity.*

Enzyme activity (mg/ml)					
Carbon source	**Quantity by g/l**	***Achromobacter Spanius***	***Bacillus amyloliquefaciens***	***Stenotrophomonas Maltophilia***	**Mean**
Glucose	10	2.06	2.04	1.76	1.95
	30	2.23	2.14	1.89	2.09
	50	2.09	1.98	1.78	1.95
	Mean	2.13	2.05	1.81	
	L.S.D. at 0.05		Bacteria 0.032, Glucose 0.030, Bacteria x Glucose0.054		
Soluble starch	10	1.23	1.18	1.10	1.17
	30	1.27	1.24	1.14	1.22
	50	1.05	0.96	1.07	1.03
	Mean	1.18	1.13	1.10	
	L.S.D. at 0.05		Bacteria 0.031, Soluble starch 0.031, Bacteria x Soluble starch 0.057		
Insoluble starch	10	1.07	1.03	0.91	1.00
	30	1.23	1.12	0.94	1.09
	50	1.13	1.09	0.89	1.03
	Mean	1.14	1.08	0.91	
	L.S.D. at 0.05		Bacteria 0.032, Insoluble starch 0.031, Bacteria x Insoluble starch 0.055		
Fructose	10	1.01	0.87	0.86	0.92
	30	1.28	1.06	1.06	1.13
	50	1.17	1.01	0.96	1.05
	Mean	1.15	0.98	0.96	
	L.S.D. at0.05		Bacteria 0.03, Fructose 0.04, Bacteria x Fructose 0.54		
Maltose	10	0.92	0.79	0.89	0.86
	30	0.93	0.85	0.83	0.87
	50	0.92	0.81	0.83	0.85
	Mean	0.92	0.81	0.85	
	L.S.D. at0.05		Bacteria 0.031, Maltose 0.033, Bacteria x Maltose 0.056		
Sucrose	10	1.02	0.86	0.81	0.89
	30	1.07	0.93	0.82	0.94
	50	1.04	0.90	0.81	0.92
	Mean	1.04	0.89	0.81	
	L.S.D. at0.05		Bacteria0.032, Sucrose 0.030, Bacteria x Sucrose 0.054		

In conclusion, *Achromobacter spanius* was superior over *Bacillus amyloliquefaciens* and *Stenotrophomonas maltophilia.* This holds true with all carbon sources and the highest production of cellulase enzyme was induced by 30 g/l glucose which considered the optimum level. *Achromobacter spanius* was superior over other isolates

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دراسة معملية لتمييز الظروف المثلى المؤثرة على انتاجية السليوليز من البكتيريا الداخلية

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عزلت البكتريا الداخلية والتي انتجت اعلي قيمة لإنزيم السليوليز وهي اكروموباكتر سبانيس، باسيلس أميلوليكيفاسيانز وستينوتروفوموناس مالتوفيليا من الأوراق، السيقان والجذور لنبات البرسيم، القمح، الخس، السبانخ، الفول البلدي، الثوم، الشبت، الزيتون، وشتلات أشجار الأكاسيا والبروسوبس تمت الدراسة تحت ظروف محكمة في المعمل. أختبرت العوامل المؤثرة على ظروف النمو في الوسط الغذائي لإنتاج أعلى قيمة لإنزيم السليوليز مثل درجة الحرارة، درجة الحموضة، فترة التحضين، حجم اللقاح، مصادر النيتروجين والكربون. أنتجت أعلى قيمة من إنزيم السليوليز بواسطة اكروموباكتر سبانيس (٢,٥٠ مجم/مل) يليها باسيلس أميلوليكيفاسيانز (٢,٢٦ مجم/مل) بعد ٦ ايام من فترات التحضين. وجد أن الظروف المثلي لإنتاج السليوليز ٣٠ درجة مئوية عند درجة حموضة ٦ لاكروموباكتر سبانيس وباسيلس أميلوليكيفاسيانز بينما وجد أن الظروف المثلي لستينوتروفوموناس مالتوفيليا هى ٤٠ درجة مئوية عند درجة حموضة ٧.

أنتجت العزلات الثلاث أعلي قيمة من إنزيم السليوليز مع كبريتات الأمونيوم كمصدر نيتروجيني و ٣ جم/لتر جلوكوز كمصدر كربوني نسبيا. أنتج أكروموباكتر انزيم السليوليز أعلي من باسيلس وستينوتروفوموناس تحت كل الظروف المثلي.

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