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OPTIMIZATION OF THE CELLULASE ENZYME PRODUCTION BY *BREVIBACTERIUM HALOTOLERANS* ISOLATED FROM WADI EL-NATRUN, EGYPT

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The present work was carried out to isolate the cellulose-decomposing bacteria (CDB) from Wadi El-Natrun, Egypt. Totally 40 isolates were isolated from saline mud samples. Eight out of 40 isolates exhibited high hydrolysis capacity of the Congo-Red screening method. The highly producer isolate (CDB2) was selected to optimize some nutritional and environmental factors for improving cellulase production. The bacterial isolate was identified on the basis of molecular characteristics using 16srRNA. The sequence analysis showed 99% similarity to *Brevibacterium halotolerans*, which is then deposited in the GenBank in NCBI under the accession number MN148622. The bacterium showed highly cellulase production by using carboxymethyl cellulose (CMC) as a sole carbon source. The results showed that the optimum conditions for cellulase production by the selected isolate were noticed when grown on CMC (20 g/L) at pH 8.0 incubated at 35^{o}C in batch conditions with shaking. The results revealed that, the bacterial isolate showed potential cellulase production at stationary phase of growth corresponding to an incubation period of 3-5 days. The cellulase enzyme has high specificity to CMC considered to be an endo-1,4-glucanase.

Keywords: Cellulose-decomposing bacteria, Cellulase, *Brevibacterium halotolerans.*

INTRODUCTION

Plant represents the major source of cellulose on Earth. Cellulose polymer is very hard to be degraded and its accumulation in nature as waste represents one of the major sources of the environmental pollution (Yousef et al. 2019). Microbial activity is responsible for most of the turnover of the carbon in cellulose that is commonly degraded by an enzyme called cellulases. Microorganisms commonly bacteria and fungi

produce cellulases as well as some insects, mollusks, nematodes, and protozoa also produce cellulases (Sheng *et al*., 2016).

The cellulase is a system consisting of endoglucanases, exoglucanases, and β-D-glucosidases, all of which hydrolyze crystalline cellulose synergically. The cellulases endoglucanases are found only in bacteria. These polysaccharide hydrolyzing enzymes also include alkaline cellulase with high potential as laundry detergent additives (Yin et al. 2010).Microorganisms especially bacteria are the main producers of enzymes that decompose cellulose in soils, which make cellulase the most important role in plant biomass decomposition (Gupta et al. 2012).The cellulose-decomposing bacteria include aerobic, anaerobic, mesophilic and thermophilic strains, inhabiting a great variety of environments, including the most extreme temperature, pressure and pH. Cellulases from bacteria are widely exploited because of high growth rate and inhabit a wide variety of environmental and industrial niches that are extreme environments such as thermophilic or psychrophilic, alkaliphilic or acidiophilic and halophilic strains (Sadhu and Maiti, 2013).

Nowadays, investigation on the production of extremozymes from halophilic bacteria has received more attention. This interest is due to their capacity to efficiently catalyze a process and show optimal activities at different salt concentrations. Halophiles are the most probable source of extremozymes, since they are also capable of tolerating alkaline pH and high temperatures (Yin et al. 2010). Halophilic bacteria are able to grow optimally in media containing NaCl concentration of 3-15% (Yu and Li, 2015). Cellulase yields appear to depend on a complex relationship involving a variety of factors such as temperature, pH, incubation period, cations, carbon and nitrogen sources (Immanuel et al., 2006).Therefore, the objectives of this study were to isolate and screen the halophiliccellulase producing bacteria from lake sediments (mud) samples obtained from Wadi El-Natrun lakes that has reported issues of high salinity problems. Also, maximize the capability of the most efficient cellulose bacterial strain (*Brevibacterium halotolerans*) for cellulase production was evaluated.

MATERIALS AND METHODS

Sample collection and bacterial growth conditions

Twenty saline mud samples were collected from different locations of Wadi El-Natrun lakes. Halophilic bacteria were isolated from mud samples using serial plate dilution. All samples were cultured in nutrient broth medium containing 5 or 10% NaCl. The pH of culture media was adjusted to 7.5 before autoclaving. Cultures were incubated at 37°C in an orbital shaker, at 140 rpm for 3 days. The bacterial growth showing turbidity was purified for isolation of single bacterial colony.

Screening of Cellulolytic bacterial isolates

Cellulose-decomposing ability of bacterial isolates was performed by streaking the isolates on the cellulose Congo- Red agar medium (KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, cellulose 2 g, agar 15 g, Congo-Red 0.2 g, gelatin 2 g; agar 15 g, distilled water 1 L) at pH 7.2 (Garder 1986). The bacterial colonies with the ability to form clear zone around its colony were picked up for further using. Then, the Cellulose-degrading potential of positive results was estimated by calculating hydrolysis capacity (HC): the ratio of diameter of clear zone and colony (Hendricks *et al.*, 1995). Cellulolytic activity of isolates was evaluated according to the extent and intensity of the hydrolytic clearing zones. The highly potential isolate was used for further study.

Molecular identificationof the cellulolytic bacterial isolate

The bacterial isolate that showed the highest enzyme activity was selected as a cellulose- producing bacteria for molecular identification by partial sequencing of the 16s rRNA gene. PCR product was sequenced in both directions using 27F and 1500R primers.A PCR product for sequencing was conducted by using the following two primers, 27F (5'-GAG TTT GAT CCT GGC TCA G-3', positions 9-27 on 16S rDNA) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3', position 1509-1492 on 16S rDNA). The obtained 16S rRNA gene sequences were aligned with known 16S rDNA sequences in the GenBank database using the basic local alignment search tool (BLAST) at the National Center for

Biotechnology Information (http://www.ncbi.nlm. nih.gov/BLAST).The sequence of the strain has been deposited in the GenBank nucleotide sequence database

Quantitative assay of cellulase production

The bacterial isolates were also assayed for cellulose-degrading potentials in a modified basal salt medium containing 5 NaCl with composition (gL^{-1}): K_2HPO_4, 0.8 $(NH_4)_2SO_4$, 1.0 KH_2PO_4, 0.2 $MgSO_4.7H_2O$, 0.2 $CaCl_2.H_2O$, 0.1 2 and $FeSO_4$, $7H_2O$, 0.005 (Gupta et al. 2012). The medium was supplemented with 10g/L of cellulose as a sole carbon source. The pH of culture media was adjusted to 7.5 before autoclaving. Cultures were incubated at 37°C in an orbital shaker, at 140 rpm for 3–7 days. The bacterial isolates subjected to cellulase production in 250 ml Erlenmayer flask using basal medium with selected carbon source for 48h of fermentation period at 35°C with agitation speed of 140rpm. Bacterial growth was monitored by turbidity at OD_{600}.The quantitative assay method was used to determine the cellulase activity of the selected bacterial isolate in liquid medium. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a DNS method (Miller 1959). A bacterial isolate with the highest activity was selected for optimization of cellulase production.

Optimizing of enzyme production

The growth conditions of higher cellulase producer bacteria were assessed. Then, the optimization factors for maximum enzyme activity were investigated. Inocula were prepared from the bacterial grown on cellulose medium at 37°C, for 3 days.

Shaking and static condition

The effect of shaking and static conditions on the cellulase production was assessed. The bacterial culture was allowed to grow under shaking at 100 rpm or in static condition.

Salt concentration

The effect of salt concentration on the cellulase production was assessed. The bacterial culture was allowed to grow in media of different NaCl concentration ranging from 1% to 15%.

Carbon sources.

The effect of various carbon sources such as cellulose, carboxy methyl cellulose (CMC) and filter paper (FP) at the concentration of 1% was examined in the cellulase production medium.

Temperature optimum

Production medium at pH 7 was inoculated with overnight grown selected bacterial strain. The inoculated broth medium was incubated at different temperatures (20, 30, 35, 40and 50^{o}C) for 48 h. At the end of incubation period the cell-free culture filtrate is obtained and used to determine the cellulase activity.

Initial pH values

To determine the best pH value for enzyme activity, the isolate was grown on basal broth medium containing 20 g per liter cellulose as a sole carbon source adjused to different pH values (5, 6, 6.5, 7, 7.5, 8 and 9) and tested after 3 days of incubation. The initial pH was adjusted by 0.1 HCl or 0.1 NaOH.

Substrate concentrations

Different concentrations of cellulose (10, 20, 30, 40 and 50 g per liter) were incorporated into basal salt broth medium in 100 ml flasks. Media were inoculated with the bacterial isolate under investigation and the flasks were incubated at 30^{o}C for 48 h.

Incubation periods

To investigate the effect of different incubation periods on bacterial biomass, the culture flasks inoculated with bacterial isolate were kept grown for a period of 7 days and enzyme activity was assessed after 1, 2,

3, 4, 5, 6 and 7 days of incubation. The activity of free crude cellulase was assessed.

RESULTS AND DISCUSSION

Isolation and screening of cellulase production

Forty halophilic bacteria were isolated by inoculating the diluted mud samples in nutrient broth medium containing 5 or 10 % NaCl. The bacterial isolates were individually screened to determine their cellulase production capability by streaking onto Congo-Red agar. The bacterial isolate was found to be positive on medium (cellulose Congo-Red agar) producing clear zone as shown in Fig.(1a) during aerobic incubation (Gupta et al.2012). The results revealed that eight bacterial isolates were 7-16 mm of clear zone radius. The obtained results showed also that clearing zone and HC value ranged to be between 2 to 7 (Fig. 1b). The range of HC value obtained is nearly similar to that reported by Gupta et al. (2012) and Lu et al. (2006). Whereas, Hatami et al. (2008) found that the hydrolytic value of cellulolytic aerobic bacterial isolates from farming and forest soil, ranged between 1.38 to 2.33 and 0.15 to 1.37 respectively. The results represent in figure 1 revealed that, the bacterial isolate CDB2 exhibited the highest extracellular cellulase activities compared to other isolates.

Congo-red interacts with (1, 4) - β–D- glucans and (1, 3) β D-glucans and a clearing zone around the colony on the agar medium indicates the hydrolysis of cellulose. The bacteria secrete cellulase enzyme degrade cellulose into cellobiose, and then break down cellobiose to form glucose and finally metabolize glucose to organic acids (Lactic acid, acetic acid etc.). The organic acids thus formed lower the pH of medium. The pH difference affected the color of the medium and the formation of the yellow clear zone around the colony would finally appear indicating the cellulase enzyme production.

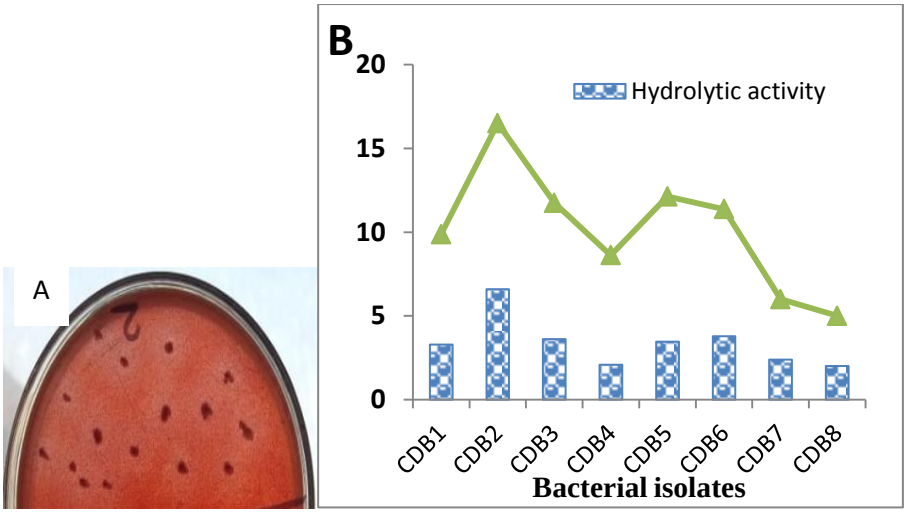

Fig 1 A: Zone of clearance of CDB2 on cellulose Congo Red agar plates. **B**: hydrolytic activity of the bacterial isolates on cellulose Congo Red agar plates.

Molecular identification of the selected bacterial isolate

The selected bacterial strain was found to have ability to degrade cellulose subjected to molecular identification by partial sequencing of the 16s rDNA gene and the result demonstrate 98% homology *Brevibacterium halotolerans*. Furthermore, its nucleotide sequence was deposited in the GenBank in NCBI under the accession number MN148622.

Optimization of cellulase production by selected bacterial isolate (CDB2)

1-Effect of shaking and static conditions

The effect of shaking on growth and cellulase production of CDB2 were presented in figure 2. The obtained results revealed that incubation of *Brevibacterium* under shaking conditions exhibited more cellulase activity than static incubation. Previous studies found similar result as agitation speed is an important factor which governs the dissolved oxygen level in the culture broth that affects cell growth of cellulase producing microorganisms (Jo et al. 2008). Hussain et al. (2017) found that *B. megaterium* BMS4, *B. subtilis* BTN7A, and *B. amyloliquefaciens* SA5 exhibited more cellulase activity than static incubation under shaking

conditions. In contrast to this finding, Jo et al. (2008) found that, *A. flavithermus* BTN7B produced more cellulase in the static state as compared with shaking. Also, higher agitation speed has been shown to inhibit cellulase activity (Li et al. 2008).

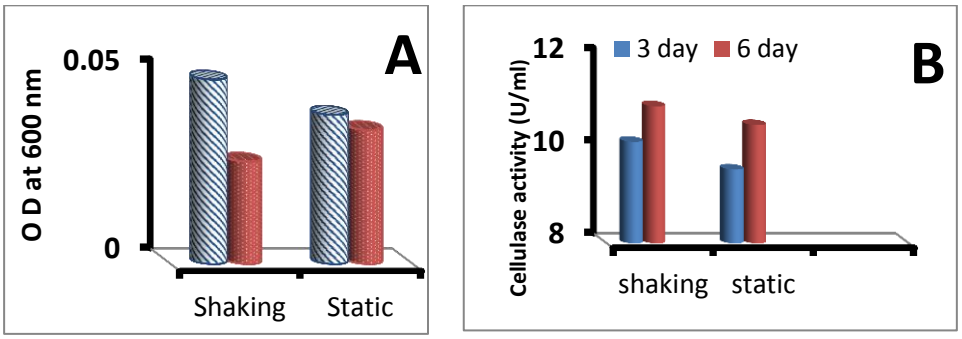

Fig. 2: Effect of shaking and static conditions on the growth (A) and cellulase production (B) of *Brevibacterium halotolerans* .

2-Effect of salt concentration

The selected bacterial isolate (CDB2) was grown in the media of different salt concentration ranging from 1% to 15%. The maximum enzyme activity was observed in salt concentration range 6- 15% (Figure 3). Cellulase production by *Brevibacterium* was dependent on NaCl concentration. The bacterium was able to grow up to a salt concentration of 15% with an optimum growth at 6%. Bacterial growth was observed even at 0% NaCl showing its no dependency on salt for its moderate growth, thus indicating the halophilic nature of the bacterium. The same finding was noticed by Thaz et al. (2015) on the growth and cellulose production by halotolerant bacteria. Halotolerant strain *Salinivibrio* sp. (Chung-Yi *et al.*, 2009) reported a clear dependence on Na+ ion for maximum enzyme activity and at 5% of NaCl concentration the activity was more, but enzyme was active over a range of 1-15% of NaCl and bacterium was able to grow even in the absence of NaCl. Rachamontree (2017) noticed that the cellulase activity of halophilic bacteria not effected by using different NaCl concentration (3-10%).

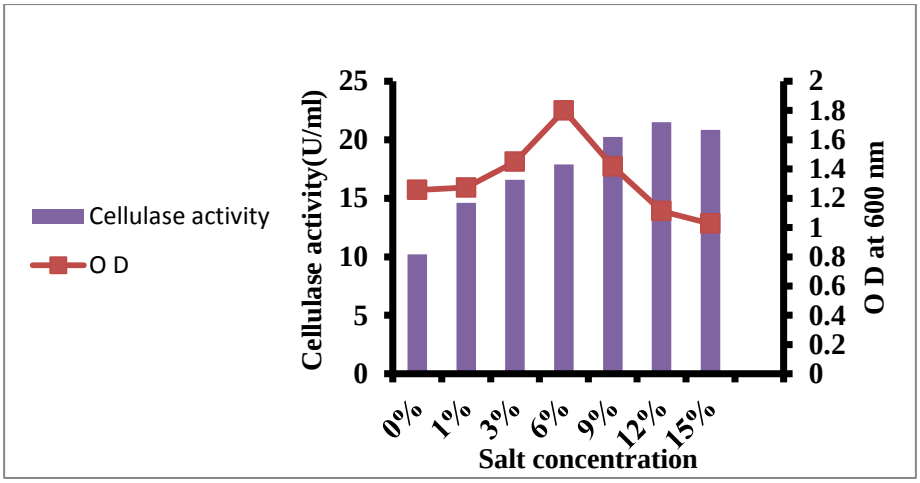

Fig. 3: Effect of NaCl on the growth and cellulose production of *Brevibacterium halotolerans* after 3 days.

3- Effect of different carbon sources

Ten gram|L from each of the following; filter paper (FP), carboxy methyl cellulose (CMC) and cellulose powder were separately inoculated with bacterial isolate and incubated at 30°C for 7days.The results showed that the highly bacterial growth in CMC substrate followed by cellulose (figure 4). The purified cellulase of *B. subtilis* YJ1 has high specificity to CMC and was considered to be an endo-1,4-glucanase (Yin et al. 2010). Ekperigin (2007) reported that the culture supernatant of *Branhamella* sp. Induced cellulase enzyme when CMC used as a sole carbon source. Bhat and Maheshwari (1987) also reported that *Sporotrichum Thermophile* revealed the low activity of endoglucanase compared to *Trichoderma reesei* when cellulose was used as a substrate.

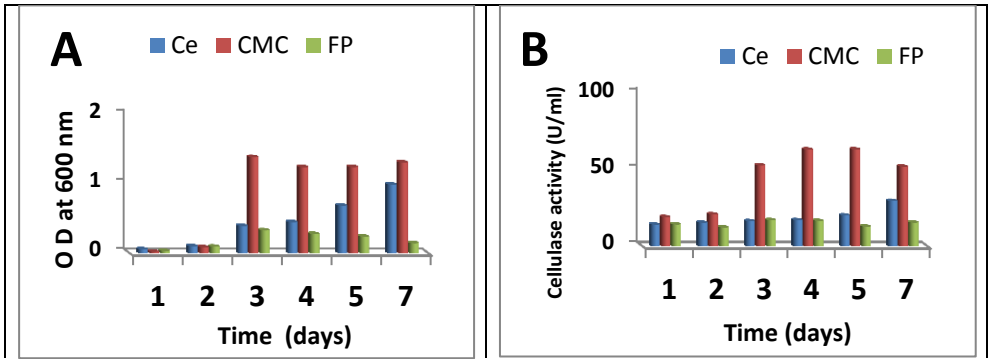

Fig 4: Effect of carbon source on the growth (A) and cellulose production (B) of *Brevibacterium halotolerans*.

4-Effect of temperature

It is clear that, the optimum temperature for maximum enzyme activity was achieved at 35^{o}C as depicted in figure 5. A number of enzymes with temperature optima of 40°C were reported previously by Nasir et al. (2011).Optimum temperature for maximum growth of *Bacillus subtilis* was 40^{o}C (Sethi et al. 2013). These results were quite similar to those of Bakare et al. (2005) who found that the cellulase enzyme produced by *Pseudomonas fluorescence* was activated at 30 to 35^{o}C showing the optimum temperature at 35^{o}C. Ray et al. (2007) reported that minimum cellulase yield was observed when fermentation was carried out at 45^{o}C, while maximum yield was obtained at 40^{o}C by *Bacillus subtilis* and *Bacillus circulans*. Immanuel et al. (2006) also recorded maximum endoglucanase activity in *Cellulomonas*, *Bacillus,* and *Micrococcus* sp. at 40^{o}C and neutral pH.

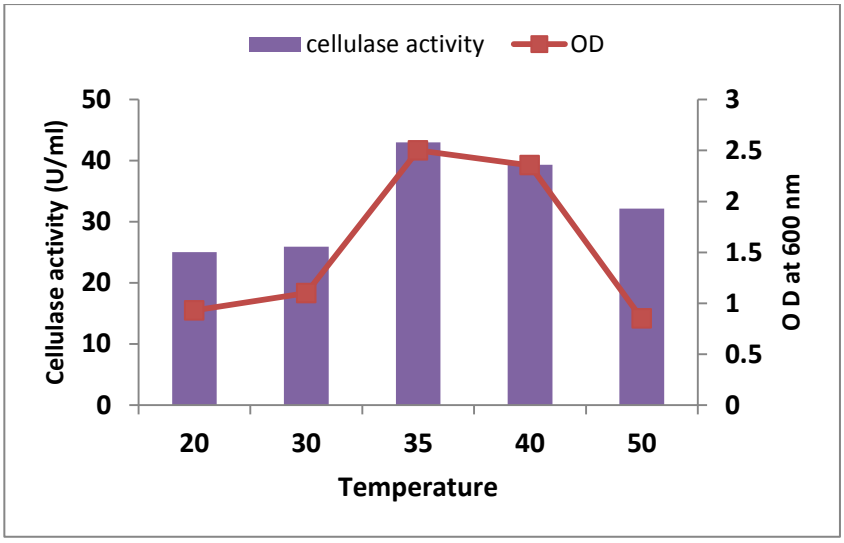

Fig 5: Effect of temperature on the growth and cellulase production of *Brevibacterium halotolerans*.

5-Effect of pH values

The results showed that the bacterial strain could grow and produce extracellular cellulase in a wide range of pH (5.0 -10.0). The initial pH value of the medium is one of the most important parameters affecting cell growth and enzyme productivities (**Yousef and Aldaby 2016**; **Bacha*et al.*, 2015**).The results showed that pH 8 was the optimum pH for the maximum yield of bacterial growth and cellulase production (fig. 6). Cellulase was produced maximum value at pH 8.0 indicating that pH has a direct effect on the uptake of mineral nutrients by bacteria from the medium (Yin et al. 2010).The data were come in agreement with other studies stated that most bacterial enzymes show optimal activity between pH 6 and 8 (Daniel et al. 2010).This result was in correlation with the finding of other workers for different *Bacillus subtilis* strains (Abdel-Mawgoud et al. 2008, Sethi et al. 2013).

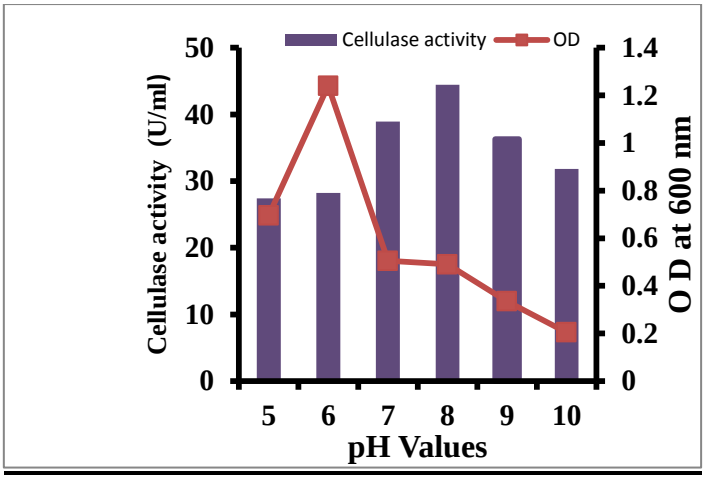

Fig 6: Effect of pH on the growth and cellulase production of *Brevibacterium halotolerans*.

6-Effect of substrate concentration

Cellulose was used as a carbon source utilizing by bacterial strain. It was prepared in serial concentration (5, 10, 15, 20, 25, 30glL), at pH7. It was found that the maximum product of cellulase was obtained at concentration 20 g cellulose per liter (Fig 7). Shadrin et al. (2016) found that 1.8% CMC was optimum for cellulase production from *Bacillus subtilis* AS3.

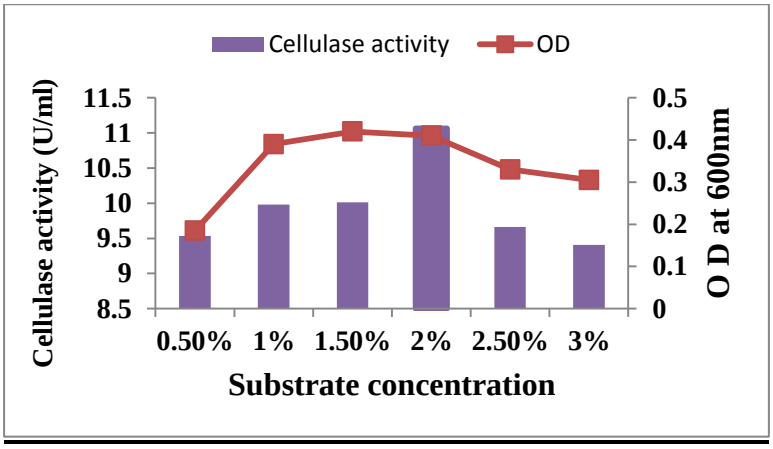

Fig. 7: Effect of substrate concentration on the growth and cellulase production of *Brevibacterium halotolerans*.

7-Effect of inoculum concentration

The inoculums concentration is one of the greatest important parameters which effects cellulase production by bacteria. Different volumes of overnight bacterial inocula (0.25, 0.5, 1.0, 2.0 and 3.0 ml) were distributed into 50 ml broth medium. The flasks were incubated at 37^{o}C for 48 h on a rotary shaker (120 rpm). The results showed that the 2 ml inoculum exhibited the highest bacterial growth and cellulase production (fig 8). While, further increase or decrease in inoculum concentration lead to decrease in cellulase production. The initial microbial size may be affects the growth and metabolic pathways. The lower inoculums percent may lengthen the lag phase of bacterial growth, on the other hand the high inoculums size may be stimulating the growth but reduce some metabolic activities of the culture (Aboseidah et al. 2017).

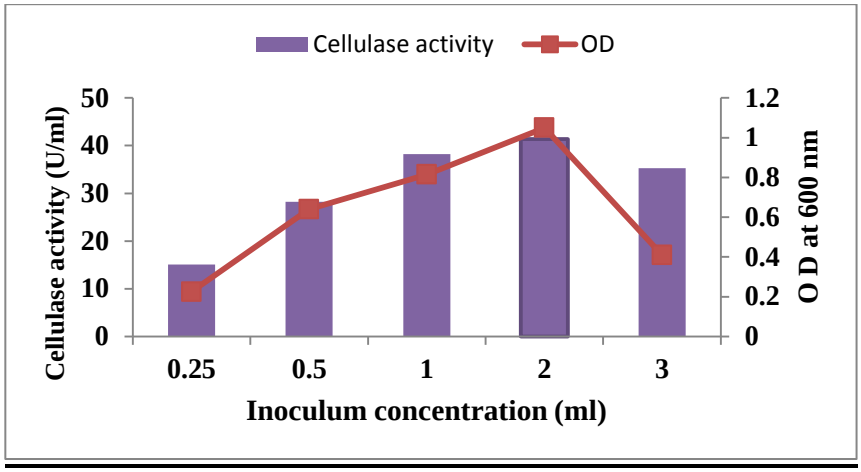

Fig. 8: Effect of the bacterial ioncula on the growth and cellulase production of *Brevibacterium halotolerans*.

8- Effect of incubation periods

Bacterial growth was studied in order to identify the suitable time period for cellulase production. The growth rate of the bacterial strain was measured as OD_{600} using 20 g/L CMC as a sole carbon source at pH 7. The results represented in Figure 9 revealed that, cellulase production was not observed in the first two days of growth and thereafter production increased significantly with a maximum growth after 4 days. The

secretion of cellulase enzyme in the stationary phase can be compared with the other reports (Shankar and Isaiarasu 2011; Bajaj et al., 2009; Bansal et al., 2012, Thaz et al.2015) where the maximum enzyme production was obtained in the stationary phase corresponding to an incubation period of 72 h.

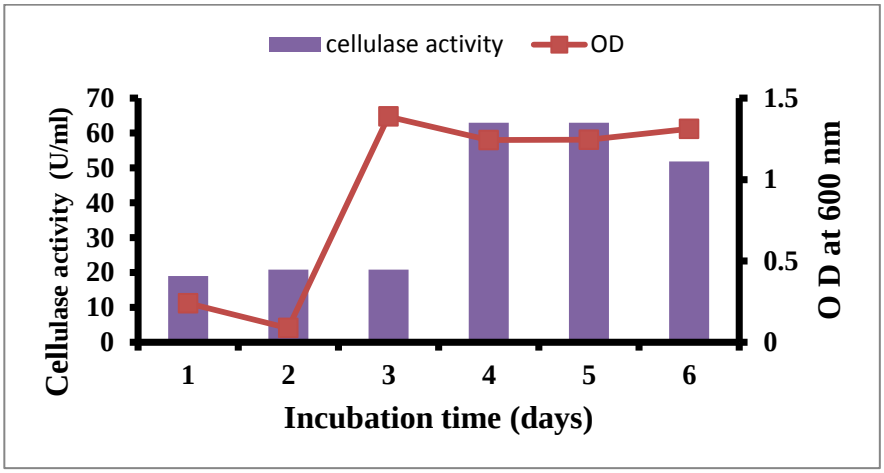

Fig. 9: Effect of incubation period on the growth and cellulase production of *Brevibacterium halotolerans.*

CONCLUSION

The present study showed that the production of hallophilic cellulase by *Brevibacterium halotolerans* strain. These hallophilic cellulase-producing bacteria can be used for bioconversion of cellulosic materials to useful products in hyper saline of polluted environments and offer important biotechnological potential.

Conflict of interest: We have no conflict of interest with this publication

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تعظيم الاستفادة من إنتاج أنزيم السليوليز من بكتيريا *Brevibacterium halotolerans* المعزولة من وادى النطرون- مصر

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تم تنفيذ العمل الحالي لعزل البكتيريا المحللة للسليلوز (CDB) من وادي النطرون ، مصر. تم عزل ٤٠ عزلة بكتيرية و ٨ عزلات لديها قدرة عالية على التحلل المائي لطريقة صبغ الأحمر الكونغو. ثم تم اختيارالبكتريا عالية الإنتاجية (CDB2) لتحسين بعض العوامل الغذائية والبيئية لتحسين إنتاج السليوليز عن طريق العزلة البكتيرية المنتخبة. تم التعرف على السلالة البكتيرية المعزولة على أساس الخصائص الجزيئية باستخدام srRNA١٦. أظهر تحليل التسلسل تشابهاً بنسبة ٩٩ ٪ مع بكتريا *Brevibacterium halotolerans* ، والتي تم وضعها بعد ذلك في بنك الجينات في NCBI تحت رقم MN148622. أظهرت هذه البكتيريا إنتاج عالي للسليوليز عن طريق استخدام السليلوز كربوكسي الميثيل (CMC) كمصدر وحيد للكربون. أظهرت النتائج أن الظروف المثلى لإنتاج السليوليز من قبل العزلة المختارة قد لوحظت عندما نمت على ٢٠ جم / لتر CMC عند درجة الحموضة ٨.٠ المحضنة عند ٣٥ درجة مئوية في ظروف اهتزاز. أوضحت النتائج أن العزلة البكتيرية أظهرت إنتاج السليوليز المحتمل في المرحلة الثابتة من النمو المقابلة لفترة الحضانة من ٣-٥ أيام. يحتوي إنزيم السليوليز على خصوصية عالية لـ CMC التي تعتبر إندو -١،٤-جلوكاناز.