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Original Research Article

Isolation, screening, identification and improvement the production of cellulase produced from Iraqi soil

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A B S T R A C T

Fifty local isolates were collected from soil samples of fields cultivated with wheat and barley crops located in Al-Jaderia, Baghdad University. Other samples were collected from spoiled fruit and rot of apple, orange and carrot. The isolates were screened cellulase production using quantitative method. Results indicated the presence of variable degrees in the production between the isolates, but the highest producer was A3 isolated from the spoiled apple fruits, which was identified using morphological, cultural and biochemical tests, then identified by using Api 20E and VITEK2. Results showed that the isolate belongs to *Pantoea dispersa* (A3). Cellulase production was induced by using CMC as a sole carbon source. To increase the production of cellulase in *Pantoea dispersa* A3, the local isolate was subjected to UV-rays as a mutagenic agent then screening for an efficient cellulase producer strain. Results exhibited a potential variation among strains in the production of cellulase. Some 9% an increase in cellulase production 7.79–34.75 folds compared with the wild type. On the other hand, 90% of mutants showed lower cellulase production, while only 1% with the same productivity as the wild type. The LD90 was achieved after 3 seconds of irradiation under UV-light, and most of A3 cultivability was lost after 5 seconds of irradiation.

Keywords

Cellulase, *Pantoea dispersa*, Mutagenesis, UV

Introduction

Cellulose is the most abundant renewable resource on the earth (100 billion tons/year). It is the primary product of photosynthesis in the environment. Cellulase an enzyme degrades cellulose, is produced by various microorganisms such as bacteria and fungi (Atlas *et al.*, 1985). About 200 gigatons of CO_2 are fixed of earth every year and the equivalent amount of organic material has to be degraded representing 30% by plants and animals and 70% by microorganisms (Gottschalk, 1988). Cellulose accounts as 50% of the dry weight of plant biomass that is foreseeable sustainable source of fuels and materials available to humanity (Dashtban *et al.*, 2010). Catalytic domains of cellulases were first classified into six families with alphabetical letters, i.e. families A to F, based on amino acid sequence homology and hydrophobic cluster analysis. Six families were later added to the classification. Using hydrophobic cluster

analysis, all the available sequences of glycosyl hydrolases (E.C. 3.2.1.X) were compared and classified into families with Arabic numerals, currently families 1 to 58 (Henrissat *et al.,* 1989).

Cellulases are produced naturally by different symbiotic fungi, protozoa, and bacteria that have the ability to breakdown cellulose, but they are manifested in fungi and microbial sources (Almin *et al.,* 1975). Bacteria such as *Pantoea* spp, *Clostridium thermocellum, Pseudomonas fluorescens, Ruminococcus albus* and *Streptomyces* spp are cellulase producers. Some *Actinomycetes* produce these enzymes as well like *Streptomyces* spp, *Thermoactinomyces* spp and *Thermomonospora curvata*. Many fungi produce these enzymes like *Chrysosporium lignorum, Schizophyllum commune, Penicillium notatum, Sporotrichum pulverulentum, Trichoderma reesei, Acremonium cellulolyticus, Aspergillus acculeatus, Aspergillus fumigatus, Aspergillus niger, Fusarium solani, Irpex lacteus, Penicillium funmiculosum, Sclerotium rolfsii, Sporotrichum cellulophilum, Talaromyces emersonii, Thielavia terrestris, Trichoderma koningi* and *Trichoderma viride* (Agustini *et al.,* 2012; Chudasama and Thaker, 2012).

However, there are also cellulases produced by a few other types of organisms, such as some termites and microbial intestinal symbionts of other termites. Screening for bacterial cellulase activity in microbial isolates is typically performed on plates containing crystalline cellulose or microcrystalline cellulose such as Avicel in the agar at a final concentration of 0.1–0.5% (w/v). After incubation for a suitable period, a zone of clearing surrounds the colonies indicates cellulase producer by using Congo red as indicator (Sadhu and Maiti, 2013).

UV radiation may be a hazard to the human population but it is also an environmental stress for other organisms such as bacteria. Such environmental stress caused by UV may in some way induce different evolutionary changes on bacteria that would have otherwise not been selected for. This area thus provides avenues of physiological, ecological, and genetic investigation because mutations play a key role in biological processes such as evolution, carcinogenesis, and generation of somatic genetic diversity (Abdel-Aziz *et al.,* 2011). Strain improvement for cellulase production via mutagenic agents has attracted great attention owing to their efficiency (Baron and Finegold, 1998). Many of mutagenesis tests by using UV light are excrete over-production of cellulase in *Cellulomonas* spp. Over-producers for xylanase (161% production improved) was achieved in *Streptomyces* spp by using UV light (Collee *et al.,* 1996). Thus, the aim of the current work is to isolate and characterize a bacterial strain having high cellulase yield from local Iraqi environment.

Materials and Methods

Isolation of cellulolytic bacteria

Cellulolytic bacterial isolates were isolated from the soil and spoiled fruit and rot of carrot, apple and orange by using a serial dilutions and pour plate technique. Nutrient agar used for isolating cellulolytic bacteria at 30 °C after 24 hrs of incubation (Irfan *et al.,* 2012). Isolates were purified by repeated streaking, and then preserved at 4 °C for further studies.

Screening of cellulolytic bacteria

Pure cultures of bacterial isolates were individually transferred into CMC agar plates. After incubation for 24 hrs, plates were flooded with 1% Congo red and allowed to stand for 15 min at room

temperature, and then 1 M of NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis (Irfan *et al.*, 2012).

Quantitative screening (Samira *et al.*, 2011)

Quantitative screening was achieved by determining cellulase activity in culture filtrate for each bacterial isolate after propagation in CMC broth medium at 35 ºC for 24 hrs. Cellulase activity was measured by mixing 0.1 ml of enzyme (crude filtrate) with 0.1 ml of 1% (w/v) CMC in 10 mM sodium phosphate buffer, the pH was adjusted to 7.0 at 37 °C for 60 min.

The reaction was stopped by adding 1.0 ml 3, 5- dinitro salicylic acid (DNS) reagent. The mixture was placed in a boiling water bath for 10 min, left to cool at room temperature, and the optical density at 540 nm was read. Cellulase activity was calculated in accordance with glucose standard curve.

Cellulase activity (u/ml) = OD at 540 nm / Slope of glucose standard curve ×1000 / MW of glucose.

Enzyme activity unit was defined as the amount of enzyme that releases 1 μmol of glucose per min.

Identification of bacterial isolates: To identify bacterial isolates, cultural and morphological characteristics (size, shape, edge, gram staining, and color) were examined according to Atlas *et al.* (1995).

Mutagenesis of the selected isolate

Mutagenesis of the selected bacterial isolate was achieved according to Krootdilaganandh (2000) by subjecting fresh cultures of bacterial growth to UV light in a dark place. A suspension of 1 ml of overnight bacterial isolate culture was inoculated into 100 ml of nutrient broth for 6 hrs at 37 ºC. Then 10 ml of the cell suspension was transferred into a sterilized Petridish and placed in a tray inside the irradiation unit (15× 25 cm). The distance between tray and source of UV ray was 11 cm. Cell suspension was subjected to UV irradiation for different periods with time intervals of 1 sec for 15 sec. For each time interval, aliquot of 100 µl of cell suspension was taken, serially diluted and spread on a nutrient agar plate and incubated in dark at 37 ºC for 24 hrs to estimate survivals and detection of mutants at the killing percentage of more than 90%. Suspected mutants were screened for their ability in cellulase production.

Results and Discussion

Isolation of cellulase producing bacteria

Results indicated in Table 1 showed that 19 bacterial isolates were obtained from soil samples, and 31 bacterial isolates were obtained from spoiled fruit samples (Table 1).

These isolates were screened to examine their ability in producing cellulase and pectinase enzymes.

Qualitative screening

Qualitative screening for cellulase production by local isolates was achieved by detecting their ability to grow on CMC agar medium. Results showed that these isolates were able to grow on cellulase agar medium with variable degrees according to the formation of clear halo around the colonies of each bacterial isolate (Table 2). The

diameters of hydrolysis zone ranged between 10 and 14 mm for different isolates. A3 isolate was the most efficient in cellulase production since it gave the widest halo zone of hydrolysis on cellulose agar medium (14 mm). This result indicates that local isolates were able to produce cellulase which converts the cellulosic materials at pH 7 to soluble reducing sugars. Congo red is an indicator for attachment to the cellulosic material forming clear halo zones (Krootdilaganandh, 2000; Nurachman, 2010).

Quantitative screening for cellulase production

Quantitative screening for the ability of local isolates in cellulase production was achieved by determining enzyme specific activity in culture filtrates for these isolates after culturing in cellulase production medium at 35 °C for 24 hrs.

All bacterial isolates were cellulase producers with variable degrees (Table 3) because of the specific activity of cellulase in the culture filtrates ranged between 0.45–1.601 unit/mg. Among these isolates, A3 isolated from spoiled apple fruits was the most efficient in cellulase production since the specific activity of the cellulase in the crude filtrate for this isolate reached 1.601 unit/mg. According to these results, A3 isolate was selected and subjected for further investigations.

Identification of bacterial isolates

The selected isolate was identified according to morphological, cultural and biochemical tests. Additional confirmation was carried out using Api E 20 and VITEK 2.

Morphological characteristics

Different morphological characteristics were studied to identify the selected isolate. It appeared as straight rods in shape, motile, non- hemolytic, non-capsulated, non-sporeforming. Colonies of this isolate were smooth, mucoid, yellow pale pigmented, irregularly round, rough and wrinkled that are difficult to remove with a platinum wire.

Biochemical identification

The isolate was positive for catalase, Voges Proskauer, urease and gelatinase tests (Table 4). It was also able to produce yellow pigment, grows at 30 and 41 ºC, utilizes citrate and motile, while it was negative for Gram staining, oxidase and indole production tests, and unable to grow at 4 ºC and 44 ºC.

Table.1 Bacterial isolates obtained from soil samples and spoiled fruits

Sample source	Type of sample	No. of isolates
Soil	Wheat crop	9
	Barley crop	10
Spoiled fruit and rot	Apple	13
	Orange	8
	Carrot	10
Total		50

Table.2 Ability of local isolates to cellulase production expressed by zone of hydrolysis on CMC agar medium after incubation for 24 hours at 30 ºC

Isolate symbol	Source	Diameter of clear zone (mm)	Isolate symbol	Source	Diameter of clear zone (mm)
S1	Soil	11	O1	Orange	13
S2	Soil	10	O2	Orange	-
S3	Soil	12	O3	Orange	-
S4	Soil	-	O4	Orange	-
S5	Soil	-	O5	Orange	-
S6	Soil	10	O6	Orange	-
S7	Soil	-	O7	Orange	-
S8	Soil	-	O8	Orange	-
S9	Soil	-	A1	Apple	-
S10	Soil	-	A2	Apple	12
S11	Soil	-	A3	Apple	**14**
S12	Soil	-	A4	Apple	10
S13	Soil	-	A5	Apple	-
S14	Soil	-	A6	Apple	-
S15	Soil	-	A7	Apple	-
S16	Soil	-	A8	Apple	-
S17	Soil	-	A9	Apple	-
S18	Soil	-	A10	Apple	-
S19	Soil	-	A11	Apple	-
C1	Carrot	-	A12	Apple	-
C2	Carrot	10	A13	Apple	-
C3	Carrot	-	-	-	-
C4	Carrot	-	-	-	-
C5	Carrot	-	-	-	-
C6	Carrot	-	-	-	-
C7	Carrot	-	-	-	-
C8	Carrot	-	-	-	-
C9	Carrot	-	-	-	-
C10	Carrot	-	-	-	-

Table.3 Specific activity of cellulase produced by different bacterial isolates

Isolate symbol	Protein conc. (mg/ml)	Activity (unit/ml)	Specific activity (unit/mg)
A3	0.122	0.195	**1.601**
A2	0.162	0.072	0.450
S1	0.150	0.096	0.641
S3	0.134	0.100	0.760
O1	0.140	0.205	1.471

Table.4 Biochemical tests for identification of the selected bacterial isolate

Biochemical test	Result
Gram stain	-
Yellow pigment	+
Catalase	+
Oxidase	-
Motility	+
VP (Acetoin production)	+d
Indol	-
Urease	+
Gelatinase	+
Citrate utilization	+
Fermentation / Oxidation of	
Manitol	+
Lactose	+
Rhammnose	+
Maltose	+
Sucrose	+
Salicin	+
Sorbitol	-
Growth at	
30 ℃	+
41℃	+
4 ℃	-
44 ℃	-

(-): Negative result; (+): positive result; (+d): positive during 1–4 days;
(+): strains positive during 1–2 days; (d): Positive during 4–5days.

Figure.1 Survivals of *P. dispersa* A3 after UV irradiation for different times of period

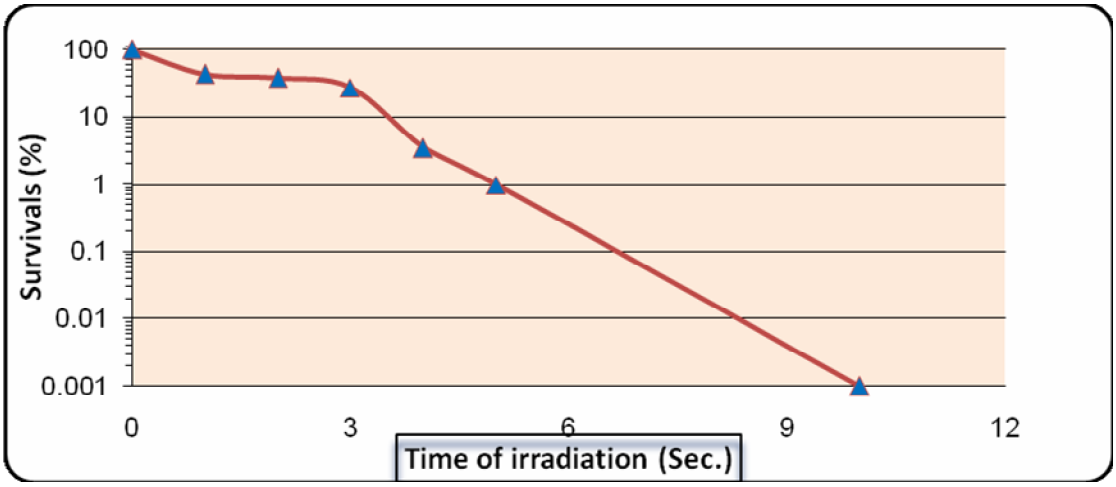

Table.5 Identification of locally isolated *Pantoea* spp using Api 20E identification system kit

Test	Result
β-Galactosidase	+
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithin decarboxylase	-
Citrate utilization	-
Hydrogen sulphide	-
Urease	-
Tryptophan deaminase	-
Indole	-
Voges-Proskauer	+
Gelatinase	-
Glucose fermentation	+
Inositol fermentation	+
Sorbitol fermentation	-
Rhamnose fermentation	+
Sucrose fermentation	+
Melibiose fermentation	+
Amygdalin fermentation	-
Arabinose fermentation	+
Manitol fermentation	+

Table.6 Cellulase specific activity for mutants and wild type

Mutant No.	Specific activity (u/mg)
Wild type	`1.601
Pantoea dispersa A3 M40	31.20
Pantoea dispersa A3 M41	24.10
Pantoea dispersa A3 M53	28.50
Pantoea dispersa A3 M69	66.83
Pantoea dispersa A3 M71	55.65
Pantoea dispersa A3 M72	85.33
Pantoea dispersa A3 M74	51.25
Pantoea dispersa A3 M86	124.57
Pantoea dispersa A3 M87	221.94

Results of carbohydrate fermentation tests showed that this isolate was able to ferment manitol, lactose, rhamnose, maltose, sucrose and salicin, while it was unable to ferment sorbitol as indicated in table (Henrissat *et al.*, 1989). These results confirm that this isolate belongs to *Pantoea* spp as it was mentioned by

Deletoile *et al.* (2009) and Alexandra *et al.* (2010). Identification of the bacterial isolate as *Pantoea* spp was confirmed by using Api 20E and VITEK2 identification system.

The isolate was positive for β-galactosidase, ferment glucose, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose and manitol, while it was negative for arginine dehydrolase, lysine decarboxylase, ornithin decarboxylase, hydrogen sulfide production and tryptophan deaminase (Table 5). According the results, it could be concluded that this isolate is characterized as *Pantoea* spp, and then it was differentiated from other *Pantoea* spp as *Pantoea dispersa* by its ability to metabolize malonate, melibiose, 5-ketoglucanate and growth at 41ºC but not at 44 ºC. Our findings are in accordance with those of Selvakumar (Gavini *et al.*, 1989) who mentioned that these biochemical characteristics are applied to *P. dispersa*.

The isolation of *P. dispersa* from plant products is so important, since it produces chitinase, glucanase and protease causing crop disorders and damage for instance a reduction in cotton yield up to 10–15% was reported due to plant cell wall degrading enzymes. They cause medical disorders for humans like urinary tract infection and arthritis (Gavini *et al.,* 1989; Champs *et al.,* 2000; Gohel *et al.,* 2004; Medrano and Bell, 2007).Optimum conditions for cellulase production under different growth conditions were examined.

Mutagenesis of *Pantoea dispersa* A3

Physical mutagenesis was achieved by subjection the cell suspension of *P. dispersa* A3 to UV-light at a wave length of 254 nm for 1, 2, 3, 4, 5, 10 and 15 seconds in attempt to enhance the productivity of the isolate. LD90 was reached after 3 seconds of irradiation with UV-light, and most of A3 cultivability was lost after 5 seconds (Fig. 1). Survivals of irradiated bacterial cells were screened and selected to produce cellulase. Results indicated that only 9 out of 100 mutated cells exhibited increase in cellulase production compared with the wild type (Table 6). On the other hand, 90 mutants showed lower cellulase production, while only 1 out of 100 had the same productivity as the wild type. UV-irradiation causes miss repair or damaged DNA by SOS repair system and is termed indirect mutagen. It was applied as mutagens for the halo tolerant *Micrococcus* sp., cell survival and mutability of *p. aerugenosa* and *P. syringae*, survivals of *Shewanella oneidensis* were determined after UV radiation (Sadhu and Maiti, 2013). It is concluded from the current experimental work the possibility of isolating highly produced of cellulase bacterial strain from spoiled apple after exposure to UV-c as a source for mutagenesis.

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