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Endophytic bacterial communities colonizing the medicinal plant *Calotropis procera*: as resources of hydrolases

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Abstract

Calotropis procera (Aiton) W.T. Aiton is a shrub belongs to family Asclepiadaceae which known by its medicinal properties. It is a widely growing plant distributed in tropical and sub-tropical Africa, and America. This study is the first report which highlights the diversity of bacterial endophytes from *C. procera* as sources of numerous hydrolytic exo-enzymes. Endophytic bacteria were isolated from all plant parts such as; roots, stems, leaves, flowers, fruits and latex. *Bacillus* was the prevalent genus. At the species level, the bacterial diversity was high. Eight representative species were isolated including; *Citricoccus alkalitolerans* (Cps2) (NR025771), *Bacillus cereus* (Cps1) (NR074540), *B. pumilus* (Cps3) (NR112637), *B. firmus* (Cpl1) (NR025842), *B. niabensis* (Cpl3) (NR043334), *B. subtilis* (Cpl4) (NR113265), *B. amyloliquefaciens* (Cpl10) (NR041455) and *B. subtilis* subsp. *spizizenii* (Cpl13) (NR112686). Results of the current study emphasized that *C. procera* plant hosts diverse endophytic bacteria, which are potential producers of several economically important hydrolytic enzymes i.e., amylase, protease, cellulase, lipase and L-asparaginase. The aims of the current study were to identify the endophytic bacteria associated with the different organs of the medicinal plant *C. procera*, and to evaluate their potentialities to produce diverse extracellular hydrolytic enzymes.

Keywords: Endophytic bacteria, *Calotropis procera*, Hydrolases, Enzymes

1. Introduction

Endophytic bacteria are microorganisms that live either in a symbiotic, commensal or mutualistic relationship inside the internal living tissues of host plant (Ryan *et al*., 2008). A previous study of Schulz *et al*., (2002) revealed that endophytes originate from the plant rhizosphere, phyllosphere or may be transferred through the seeds, and they inhabit the internal tissues of their host plants without showing any deleterious effects. Bacterial endophytes have been isolated from many wild and crop species including monocotyledons and dicotyledons (Pundir *et al*., 2014), and comprise several genera and species (Pundir *et al*., 2014). Furthermore, Araujo *et al*., (2002a); Romero *et al*., (2014), reported that several

endophytic genera such as; *Azoarcus*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Bacillus*, *Burkholderia*, *Stenotrophomonas*, *Micrococcus* and *Microbacterium* were isolated from *Citrus sinensis* and *Solanum lycopersicum*.

Endophytic bacteria have excessive potential utilization in agriculture, industry and medicine. They produce a diverse array of natural bioactive metabolites; promote plant growth directly and\or indirectly, can fix atmospheric nitrogen, produce siderophores and phyto-hormones, solubilize minerals such as phosphorus, as well as they are effective biocontrol agents enhancing plant resistance against different pathogens (Patten and Glick, 1996; Schulz *et al*., 1999; Schulz *et al*., 2002; Schulz and Boyle, 2005; Ryan *et al*., 2008).

According to Nigam, (2013), extracellular hydrolytic enzymes are biological catalysts that are synthesized inside the microbial cell, and then excreted outside the cell to perform their functions in many biological processes. Several studies conducted by Gurung *et al*., (2013); Singh *et al*., (2016) highlighted that hydrolytic enzymes have wide range of applications in food, textile, medicine, pharmaceutical and dairy industries. Moreover, Khan *et al*., (2017) added that endophytic bacteria are potential sources of extracellular enzymes. Due to the easier culturing; extraction and purification of these hydrolases, and with the progress of modern biotechnology and protein engineering, these microbial enzymes have great biotechnological interest, as demonstrated by Jalgaonwala and Mahajan, (2011); Joshi and Kulkarni, (2014).

Calotropis procera (Aiton) W.T. Aiton is a member of the family Asclepiadaceae. It is abundant over the world. Earlier studies conducted by Akhtar *et al*., (1992); Orwa *et al*., (2009) revealed that this plant has many public names in different countries, and in Arabic it is known as Oshar. On the other hand, Ibrahim, (2013); Farahat *et al*., (2015) highlighted that the morphological nature of *C. procera* enables it to grow in harsh environments under drought and salinity conditions. A study of Rahman and Wilcock, (1991) documented that in tropical and subtropical Africa, *C. procera* natively exists in Egypt, Somalia, Libya, South Algeria, Morocco, Mauritania and Senegal. In Egypt, Aswan is among the popular phytogeographic regions that are characterized by abundant existence of this plant (Moustafa and Sarah, 2017). *C. procera* has a wide range of medicinal uses including; treatment of wounds, heart failure, cancer, fever, rheumatism, indigestion, cold, eczema, skin diseases, enlargements of abdominal viscera and intestinal worms (Abhishek *et al*., 2010).

The objectives of the current study were to identify the endophytic bacteria associated with the different organs of the medicinal plant *C. procera*, and to evaluate their potentialities to produce diverse extracellular hydrolytic enzymes.

2. Material and methods

2.1. Study area

This study was carried out on *C. procera* medicinal plant that survives in Aswan region, which has an extremely hot desert arid climate with less annual rainfall. Aswan is a governorate (24°5'26.95"N, 32°53'57.91"E) located in the country of Egypt, Africa. About 20 fresh healthy plant samples were collected from west of the Nile (Aswan university campus) and east of the Nile (Al khatara region) (Fig.1). Samples were immediately transferred to the bacteriology lab, Aswan University for further study.

2.2. Isolation of the endophytic bacteria

The endophytic bacteria were isolated from different organs of *C. procera* following the manual of Araújo *et al*., (2002a). Plant samples were washed under running tap water to remove dust and debris. They were cut into small pieces including; roots, stems, leaves, flowers and fruits. All pieces were surface sterilized with 5 % sodium hypochlorite for 5 min., followed by 70 % ethanol for 1 min., and then rinsed three times in sterile dist. water. For each plant piece, 1 g of tissue was aseptically macerated in 9 ml

sterile saline solution using a pestle and mortar. An aliquot of 1 ml of each suspension was spread on the surface of tryptic soy agar and nutrient agar plates, using a sterile spreader. Plates were incubated at 37°C for 72 h and observed daily for the appearance of bacterial colonies. For each plant sample, the growing bacterial colonies were counted and the population density was expressed as cfu\ ml. According to the colony morphology, the representative pure colonies were picked up, sub-cultured on nutrient agar plates and then stored at 4°C for further assays.

Fig. 1: Map showing the study area (province of Aswan) and both locations of *C. procera* samples collection

2.3. Phenotypic characterization

Morphological and biochemical characteristics of the selected endophytic bacterial isolates were investigated, according to the standard methods described in Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994). Colony morphology, Gram staining, spore formation, motility, hydrogen sulfide production, indole formation, citrate utilization, carbohydrate fermentation, methyl red and Voges Proskauer reaction (MRVP) of the isolates were studied.

2.4. Genotypic characterization

DNA extraction and 16s rRNA amplification were carried out following the methods described by Ausubel *et al*. (1995). Two primers; 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used as universal primers, in reference to Wilson *et al*., (1990). The Polymerase chain reaction (PCR) products were visualized on 1 % agarose gel using 100 bp nucleotide ladder as a molecular-weight size marker. Sequencing was performed in both sense and antisense directions

with dideoxynucleotides (dd NTPs) in the reaction mixture. The obtained sequences were analyzed using the National Center for Biotechnology Information (NCBI) Blast tool retrieved from the website (https://www.ncbi.nlm.nih.gov/). All nucleotide sequences were submitted to NCBI GenBank to assign accession numbers. Molecular evolutionary genetics analysis and construction of phylogenetic tree were performed using MEGA X software, according to Kumar *et al*., (2018).

2.5. Diversity analysis

At the species level, the diversity of endophytic bacteria accompanying *C. procera* was investigated using Simpson's Diversity Index (SDI), according to the following formula of Magurran, (2004):

$$SDI = 1 - \frac{\sum n(n-1)}{N(N-1)}$$

Where; n = number of colonies of each species; N = total number of colonies of all species.

The range is from 0 to 1, where: high scores (close to 1) indicate high diversity, and low scores (close to 0) indicate low diversity.

2.6. Assays of enzymatic activities of the bacterial isolates

Qualitative assessment of the extracellular amylase, protease, cellulase, lipase and L-asparaginase enzymes produced by the bacterial isolates was carried out using the agar-based methods as follow:

2.6.1. Amylase activity

The amylolytic potential was detected by inoculating the isolates in point on starch agar medium, and then the plates were incubated at 37°C for 72 h. Formation of clear zone was observed after the addition of 0.3 % (w/v) iodine solution (Cowan, 1991).

2.6.2. Protease activity

The proteolytic activity was detected following the method described by Jalgaonwala and Mahajan, (2011). Isolates were streak inoculated on gelatin agar medium and then incubated for 72 h at 37°C. Hydrolysis activity was revealed by the appearance of clear zones after adding acidic $HgCl_2$ solution.

2.6.3. Cellulase activity

The endophytic isolates were streaked on carboxymethyl cellulose (CMC) agar plates to estimate their cellulase potentialities. Plates were incubated for 72 h at 37°C. Hydrolysis zones were detected after adding 0.1 ml of aqueous Congo red to the plates, as described by Samanta *et al*., (1989). The excess stain was removed by adding 5 ml of 1 M NaCl. Formation of clear halos around the bacterial streak indicated positive cellulolytic potency.

2.6.4. Lipase activity

Qualitative lipase production was determined according to the method conducted by Sierra, (1957). A basal salt medium supplemented with 1 % (v/v) tributyrin, tween 40 or tween 60 was used. After incubating the plates for 72 h at 37°C, formation of whitish halos around the bacterial growth indicated lipolytic activity.

2.6.5. L-asparaginase activity

According to Gulati *et al*., (1997), L-asparaginase activity was estimated by streaking the bacterial isolates on M9 medium supplemented with 1 ml\ l of 2.5 % (w/v) phenol red solution. Plates were then incubated at 37°C for 72 h. Appearance of pink zones around the bacterial growth denoted L-asparaginase production.

3. Results and Discussion

To the best of our knowledge, this is the first report that detects the endophytic bacteria associated with the medicinal plant *C. procera*. Two common habitats of *C. procera* within province of Aswan including Aswan university campus representing west of the Nile and Al khatara village that is located at east of the Nile (Fig. 1) were chosen for samples collection.

Population density of the recovered endophytic bacteria from several plant organs is expressed in cells\ g of tissue, as shown in Table (1). Remarkably, the most heavily population density was recovered from the stem and leaf tissues, recording 14 and 15 cfu/ g; respectively, followed by root and flower. On the other hand, it was observed that latex and fruit were colonized with very low populations, which exhibited 2 and 1 cfu/ g, respectively. In accordance, recent studies conducted by Kandel *et al*., (2017); Verma and Sao, (2018) recorded the maximum density of endophytic bacteria in the leaves and stems tissues of wild rare medicinal plants including; *Acorus calamus*, *Andrographis paniculata*, *Clerodendrum erratum*, *Convolvulus microphyllous* and *Tephrosia perpuria*. During this study, about 8 different representative colonies were selected based on their morphological features and pigmentation. Currently, it is observed that *C. procera* hosted a few numbers of endophytic bacteria, this may be attributed to the antibacterial activity of the plant which limited growth of the endophytic bacteria, as stated in previous studies conducted by Nenaah, (2013); Muzammal, (2014).

Table 1: The population density (cfu/ g) of each endophytic bacterial isolate, recovered from each organ of *C. procera* plant

	***C. procera* plant organs**					
	Root	**Stem**	**Leaf**	**Fruit**	**Flower**	**Latex**
Isolates no.	**Population density (cfu\ g)**					
Cps1	10	4	2	2	3	1
Cps2	-	1	-	-	-	-
Cps3	-	2	-	-	-	-
Cpl1	-	-	3	-	-	-
Cpl3	-	-	1	-	-	-
Cpl4	-	5	5	-	-	-
Cpl10	-	1	2	-	5	-
Cpl13	-	1	2	-	-	-
Total density (cfu/ g)	10	14	15	2	8	1

Where; Cps1: isolate recovered from *C. procera* stem extract 1; Cps2: isolate recovered from *C. procera* stem extract 2; Cps3: isolate recovered from *C. procera* stem extract 3; Cpl1: isolate recovered from *C. procera* leaf extract 1; Cpl3: isolate recovered from *C. procera* leaf extract 3; Cpl4: isolate recovered from *C. procera* leaf extract 4; Cpl10: isolate recovered from *C. procera* leaf extract 10; Cpl3: isolate recovered from *C. procera* leaf extract 13, respectively.

Morphological and biochemical characteristics of the bacterial isolates are summarized in Table (2). The bacterial isolates are morphologically diverse, and exhibited different colony characteristics including; circular to irregular colonies, white to off white or yellow color, with entire or undulating margins and flat to convex textures. Cells of all the isolates are rods (bacilli), except for one isolate that has a spherical shape (coccus). All isolates have a Gram-positive reaction. Isolates were coded as Cps1, Cps2, Cps3, Cpl1, Cpl3, Cpl4, Cpl10 and Cpl13; where Cps; refer to a strain isolated from *C. procera* stems and Cpl; refer to a strain isolated from *C. procera* leaves.

Table 2: Morphological and biochemical characteristics of the endophytic bacterial isolates recovered from *C. procera* plant, in reference to Bergey and Holt, (1994)

Characteristics	**Bacterial isolates**							
	Cps1	**Cps2**	**Cps3**	**Cpl1**	**Cpl3**	**Cpl4**	**Cpl10**	**Cpl13**
Colony features	Entire, Circular, Raised	Entire, Circular, Convex	Irregular, Circular, Raised	Entire, Circular, Flat	Entire, Circular, Flat	Irregular, Circular, Flat	Undulate, Circular, Flat	Undulate, Circular, Flat
Cell shape	Rods	Cocci	Rods	Rods	Rods	Rods	Rods	Rods
Motility	Motile	Non	Motile	Non	Motile	Motile	Motile	Motile
Gram staining	+	+	+	+	+	+	+	+
Spore formation	+	-	+	+	+	+	+	+
hydrogen sulfide production	+	+	+	-	-	+	+	+
Indole formation	-	+	-	+	+	+	+	+
citrate utilization	+	-	+	+	-	+	+	+
Carbohydrate fermentation:	+	-	+	+	-	-	-	-
Glucose	+	-	+	+	+	+	-	+
Fructose	-	+	+	+	+	-	-	+
Sucrose	-	+	+	+	+	-	-	+
Maltose	+	+	+	-	+	-	-	+
Lactose	-	-	-	-	-	-	-	-
Dextrose	+	-	-	-	-	-	-	+
Galactose	+	-	+	-	-	+	+	+
Mannose	-	-	+	+	-	+	+	-
xylose								
Methyl red test	+	-	+	+	+	-	+	+
Voges Proskauer test	+	-	+	-	-	+	+	+

Where; Cps1, Cps2, Cps3, Cpl1, Cpl3, Cpl4, Cpl10 and Cpl13 were identifies as; *Bacillus cereus*, *Citricoccus alkalitolerans*, *B. pumilus*, *B. firmus*, *B. niabensis*, *B. subtilis*, *B. amyloliquefaciens* and *B. subtilis* subsp. *spizizenii*, respectively. (-): negative reaction; (+): positive reaction.

Using NCBI Blast tool (https://www.ncbi.nlm.nih.gov/), the isolates sequences were analyzed. The evolutionary history was inferred using the Neighbor-Joining method with 1000 bootstrap replicates, as demonstrated in Fig. (2). Blast results revealed high sequence matching of Cps1, Cps2, Cps3, Cpl1, Cpl3, Cpl4, Cpl10 and Cpl13 isolates with percent identity of 100 % to; *B. cereus* (NR074540), *Citricoccus alkalitolerans* (NR025771), *B. pumilus* (NR112637), *B. firmus* (NR025842), *B. niabensis* (NR043334), *B. subtilis* (NR113265), *B. amyloliquefaciens* (NR041455) and *B. subtilis* subsp. *spizizenii* (NR112686), respectively. The present 16S rRNA gene sequences of Cps1, Cps2, Cps3, Cpl1, Cpl3, Cpl4, Cpl10 and Cpl13 isolates are deposited in GenBank database, and accession numbers are assigned as; MN960268, MN960269, MN960270, MN960271, MN960272, MN960273, MN960274 and MN960275, respectively.

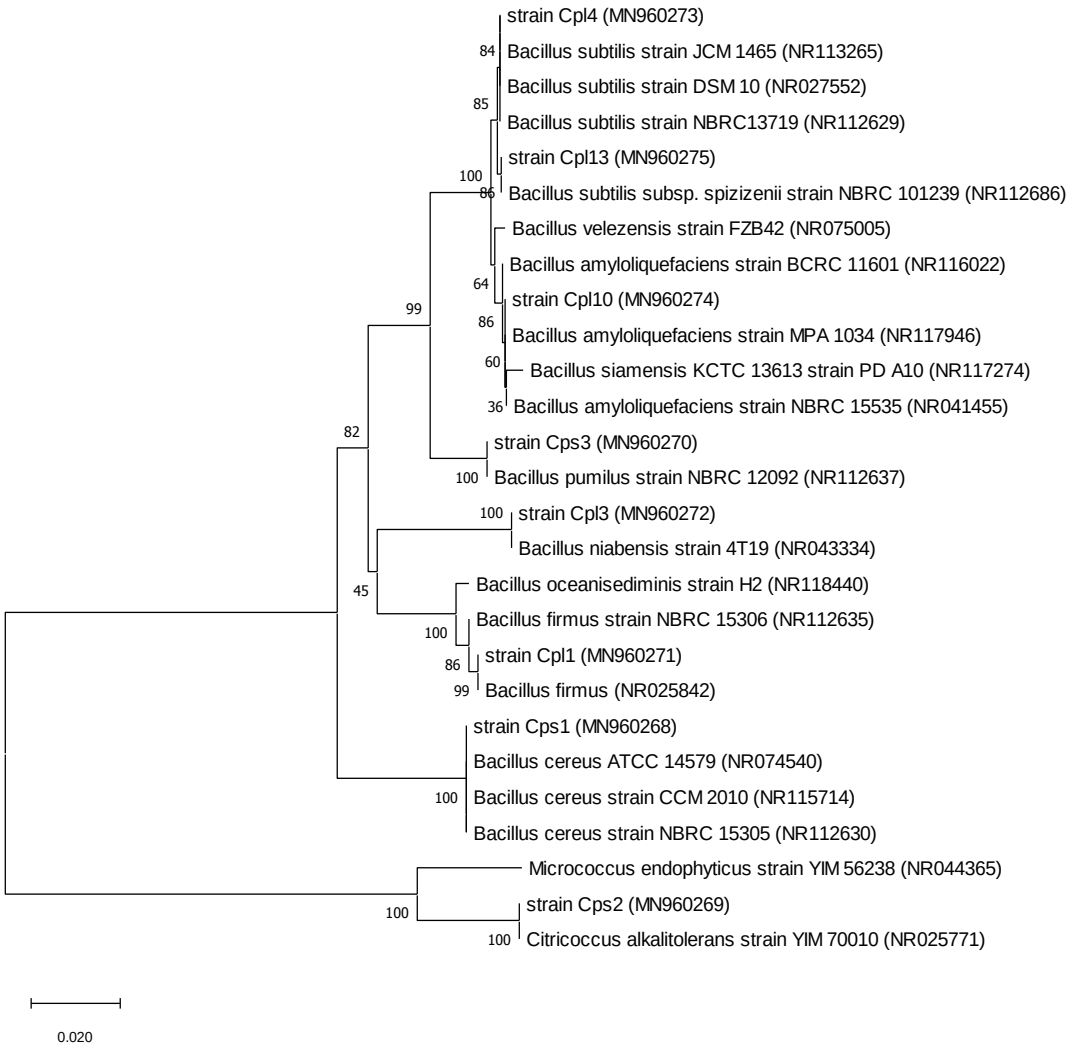

Fig. 2: Neighbor-joining tree with 1000 bootstrap replicates displaying the relationship between the endophytic bacteria associated with *C. procera* and the closely related bacteria derived from NCBI GenBank database using MEGA X software

Previous studies of Jalgaonwala *et al*., (2010); Kandel *et al*., (2017) isolated a variety of endophytic bacteria from several medicinal plants such as; *Azadirachta indica*, *Curcuma longa*, *Eucalyptus globulus*, *Musa paradiasica*, *Pongamia glabra*, *Aloe vera*, *Morrayo konengi* and *Osimum sanctum*. In the present study, the diversity of endophytic bacteria inhabiting *C. procera* was assessed at the species level, and results indicated that species diversity is remarkably high (SDI= 0.75). According to Nenaah, (2013); Muzammal, (2014), *Bacillus* is one of the most prevalent genera of endophytic bacteria associated with medicinal plant *C. procera*. Numerous species of genus *Bacillus* such as *B. subtilis*, *B. cereus*, *B. pumilus*, *B. megaterium* and *B. licheniformis* colonize the interior of medicinal plants, mainly; *Azadirachta indica*, *Pongamia glabra*, *Aloe vera* and *Morrayo konengi*, as reported by Jalgaonwala *et al*., (2010); Janardhan and Vijayan, (2012); Xia *et al*., (2015). In the current study, about 87.5 % of the representative endophytic bacterial species are related to the genus *Bacillus*. This may be attributed to the fact that this genus is characterized by its ability to form heat-resistant endospores, which can survive in the extremely hot climate of the province of Aswan, in the country of Egypt. Recently, Hagaggi, (2020); Hagaggi and Mohamed, (2020) reported that endophytic bacteria have been recognized as potential sources of bioactive natural products and hydrolytic enzymes. In the current study, all the bacterial isolates expressed potent capacity to produce a variety of extracellular hydrolytic enzymes such as; amylase, protease, cellulase, lipase and L-asparaginase, as demonstrated in Table (3).

Table 3: Extracellular hydrolytic enzymes produced by the endophytic bacterial isolates recovered from *C. procera* plant

Isolate	**Enzymatic activities**				
	Amylase	**Protease**	**Cellulase**	**Lipase**	**L-asparaginase**
Bacillus cereus (Cps1)	-	+++	+	+	+++
Citricoccus alkalitolerans (Cps2)	-	-	-	-	-
Bacillus pumilus (Cps3)	++	+	++	+	++
Bacillus firmus (Cpl1)	++	++	+	++	-
Bacillus niabensis (Cpl3)	++	+	+	++	-
Bacillus subtilis (Cpl4)	++	++	-	++	++
Bacillus amyloliquefaciens (Cpl10)	+++	+++	+++	-	-
Bacillus subtilis subsp. *spizizenii* (Cpl13)	++	++	++	+	++

Where; -: expresses no hydrolysis, +; expresses weak activity, ++; expresses moderate activity, +++; expresses strong activity

It is interestingly observed that amylase is produced by all isolates except *B. cereus* (Cps1) and *Citricoccus alkalitolerans* (Cps2); the highest production is exhibited by *B. amyloliquefaciens* (Cpl10). All isolates except *Citricoccus alkalitolerans* (Cps2) have proteolytic activities, where *B. cereus* (Cps1) and *B. amyloliquefaciens* (Cpl10) expressed potent potentialities. The best cellulase production is recorded by *B. amyloliquefaciens* (Cpl10) followed by *B. pumilus* (Cps3) and *B. subtilis* subsp. *spizizenii* (Cpl13). On the other hand, all the isolates showed moderate lipolytic activity, except for *Citricoccus alkalitolerans* (Cps2) and *B. amyloliquefaciens* (Cpl10) that do not produce lipase enzyme. Moreover, L-asparaginase enzyme is strongly produced by *B. cereus* (Cps1), and moderately by *B. pumilus* (Cps3), *B. subtilis* (Cpl4) and *B. subtilis* subsp. *spizizenii* (Cpl13), whereas the other isolates could not produce L-asparaginase. This is in accordance with the previous findings of Jalgaonwala *et al*., (2010); Gond *et al*., (2015); Hassan, (2017), which stated that the endophytic *Bacillus* species such as; *B. pumilus*, *B. megaterium*, *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* isolated from various medicinal plants including; *Pongamia glabra*, *Aloe vera*, *Morrayo konengi*, *Osimum sanctum* and *Teucrium polium*, displayed high potency of producing a variety of hydrolases. The hydrolytic enzymes of endophytes seem to be vital for colonization of the plants, as reported by Ruiz *et al*., (2002); Rivera *et al*., (2003); Guo *et al*., (2008). Therefore, the present isolates recovered from *C. procera* plant can be considered as natural resources for the production of hydrolytic enzymes, which can be exploited as candidates in many industries.

Conclusion

All parts of the medicinal plant *C. procera* inhabiting Aswan region, Egypt, were subjected to bacteriological analysis to assay the diversity of endophytic bacteria associated with the inner tissues of this plant. Moreover, the potentialities of the isolated bacteria for producing hydrolases were also investigated. All the isolates except *Citricoccus alkalitolerans* (Cps2) could produce a variety of extracellular hydrolytic enzymes including; amylase, protease, cellulase, lipase and L- asparaginase. As a supplement to this study, we recommend further optimization and purification of these enzymes, which may have pharmaceutical and medicinal importance.

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Conflict of interest

The authors declare that they have no conflict of interests.

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Ethical approval

Non applicable.

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