J. Agric. Sci. Mansoura Univ., 25 (10): 6493 - 6502, 2000.

ISOLATION AND CHARACTERIZATION OF PLANT GROWTH-PROMOTING RHIZOBACTERIA PRODUCING INDOLE-3-ACETIC ACID FROM PLANTS GROWING IN EGYPT

El-Khawas[1], H. M.; I. A.Ibrahim[2], Hala M. Anwar[2] and N. A. Hegazi[1]

[1] Department of Microbiology, Faculty of Agriculture, Cairo University, Giza, Egypt. [2] Tissue Culture Laboratory, Agriculture Development Systems Project, Ministry of Agriculture, Cairo, Egypt

ABSTRACT

The present work aims to surveying plant growth-promoting rhizobacteria (PGPR) for their ability to produce the plant growth regulator indole-3-acetic acid (IAA). One hundred and forty eight isolates from such bacteria were obtained representing the rhizosphere and rhizoplane of maize, wheat, barley, sorghum and sugar cane form different locations in Egypt (Giza, Faioum, Kafer El-Sheikh and Ismailia). Twenty isolates were selected according to their ability to produce high amount of microbial IAA. The determination of IAA was carried out by calorimetric method and chromatographic analysis (GLC). These isolates were grown on minimal medium with different carbon sources. Glucose was the best carbon source for growing the most isolates and the presence of tryptophan was necessary for production of IAA. Seven isolates which produce IAA more than others were purified and identified applying both conventional morphological, cultural, biochemical methods and API microtube systems. Accordingly, they were placed under the species of *Klebsieilla pneumomiae*, *Azospirillum* spp. *Enterobacter cloacae*, and *Pseudomonas fluorescens.*

INTRODUCTION

Many studies reported that, the bacteria provide some benefit to plants. There are two general types, symbiotic relationship with the plant and beneficial free living bacteria which living in the soil but are often near roots and are usually referred as plant growth promoting rhizobacteria PGPR (Kloepper *et al*. 1989 and Vanpeer and Schippers, 1989). Number of different bacteria may be considered to be PGPR, including *Azotobacter, Azospirillum*, *Pseudomonads*, *Acetobacter*, and bacilli (Bashan and Levanony 1990, and Glick 1995).

Plant growth promoting rhizobacteria have been isolated from crops and demonstrated the ability to promote plant growth under different growth conditions. Kapulnik *et al.* (1985) reported that inoculation with a mixture of *Azospirillum* strains of germinated wheat seeds significantly enhanced root elongation, surface area, and changes root morphology and root dry weight, as compared with non-inoculated controls. Also, i*n vitro* experiments using *Triticum durum* and different strains of *Azospirillum brasilense* were carried out to investigate the relative importance of the *Azospirillum* nitrogen fixing capacity and phytohormone production (Galli *et al.,* 1988 and El-Khawas 1990). Results suggested that an increase in the number and length of lateral

roots was due to the production of auxin-type substances by the microorganisms.

Forlani *et al*. (1995) and El-Khawas (1995) showed that several bacterial strains of the genera *Azotobacter*, *Azospirillum*, *Bacillus*, *Enterobacter, Klebsilla, Sarcina* and *Pseudomanads* were isolated from the rhizosphere of various crops and they were able to produce auxins. On the other hand, it was reported that IAA stimulate both rapid (increases in elongation) and long term (e.g., cell division and differentiation) responses in plants (Cleland 1990). Therefore, The present work aims to surveying plant growth-promoting rhizobacteria (PGPR) for their ability to produce the plant growth regulator indole-3-acetic acid (IAA) from crop roots in different location in Egypt.

MATERIALS AND METHODS

Plant samples and media

Representative root samples of growing maize, sugar cane and wheat were obtained from Agriculture Research Station, Faculty of Agriculture, Cairo University, Giza. In addition to wheat and barley from Kafr El-Sheikh, Maize and sorghum from Faioum and wheat and barley from Ismailia, were used as isolation sources for PGPR bacteria. The media used in the present study are listed in Table (1).

Isolation of plant growth-promoting rhizobacteria (PGPR)

The roots of representative crops were collected and shaken gently to remove loose attached soil. Roots with their adhering soil are considered as the rhizosphere samples. The initial dilution of each sample was prepared by adding root of each sample to a sampling bottle containing 100 ml of sterilized saline solution. Bottles were shaken for 10 min and further dilutions were prepared to isolate bacteria from rhizosphere. The roots were surface sterilized by dipping in sampling bottles containing 100 ml absolute ethanol and shaken vigorously for 3 min. Ethanol was decanted and added appropriate volume from saline solution. Roots were taken and crushed with appropriate volume of saline solution using blender and extracted root saps were used to isolate bacteria from rhizoplane.

Isolation of PGPR bacteria by pour plate method

This method was applied using the soil extract agar medium (Table 1). Five replicates were prepared from each dilution and colonies were obtained after 2-5 days of incubation at 30°C.

Isolation of *Azospirillum* by MPN technique

One-ml aliquots of suitable dilutions were transferred to each of 5 tubes containing 10 ml of selective NFM semi-solid medium (Table 1). Tubes were incubated at 30°C for 2-7 days. Positive most probable number (MPN) tubes of the higher dilutions were transferred several times into respective semi-solid NFM culture medium. For further purification, streaking on nutrient agar plates and single colony isolation were applied.

Table 1: Chemical composition of media used in the study (g/l).

Medium and source	Composition		
Soil extract Holm and Jensen (1972)	Soil extract		400 ml
	Tap water		600 ml
	Glucose		1.0
	Peptone		1.0
	Yeast extract		1.0
	K_2HPO_4		1.0
	Agar		20
NFM Day and Dobereiner (1976)	KH_2PO_4		0.4
	$MgSO_4$. $7H_2O$		0.2
	NaCl		0.1
	$FeCl_3$		10.0 mg
	Na_2 $MoO_4.2H_2O$		2.0 mg
	Yeast extract		0.1
	Biotin		2.0 mg
M_{56} EL-khawas (1990)	Origin solution:		
	K_2HPO_4		3.5
	KH_2PO_4		4.0
	NaCl		0.1
	(NH4) SO_4		1.0
	-Mg SO_4. $7H_2O$	(10%)	1.0 ml
	-Ca $(NO_3)_2$. $4H_2O$	(1%)	0.35 ml
	-Fe SO_4. $7H_2O$	(0.05%)	0.5 ml
	-Glucose	(20%)	20.0 ml
Nutrient agar	Peptone		5.0
	Beef extract		3.0
	Glucose		1.0
	Yeast extract		0.5
	Agar		20

All media were adjusted at pH 6.8-7.2.

One hundred and fourty eight isolates were obtained from which only twenty representative ones were selected for their fast growth and high production of auxins.

Determination of auxins by colorimetric method:

For rapid determination of auxins, the calorimetric Salkowski reaction was performed according to Tang and Bonner (1947). One ml of bacterial supernatant was added to 4 ml of Salkowski reagent (2.025 g $FeCl_3$ + 300 ml H_2 SO_4 + 500 ml H_2O). The mixture incubated in darkness 15-30 min. and rosy color means the presence of auxins, which measured by spectrophotometer at 530 nm.

Determination of IAA by using Gas-Liquid Chromatograph (GLC)

To determine the bacterial IAA, the isolated strains were grown in minimal medium with 0.1mg/ml tryptophane. After 3 days of incubation at 30 °C, bacterial cultures were centrifuged at 4000 rpm for 15 minutes. The aqueous residue was partially purified by partition with ethyl acetate. The acidic ethyl acetate fraction was then collected and dried under vacuum at 37°C to dryness to determine IAA using Gas-Liquid Chromatograph according to the method described by Shindy *et al*. (1975).

5. Effect of different carbon sources on growth of auxin producing bacteria

Minimal media were prepared by adding either of glucose, lactose or sucrose to study isolates growth by measuring optical density (OD) in spectrophotometer at 580 nm.

6. Identification and characterization methods

The superior seven strains in production of IAA were selected and identified by conventional tests such as Gram stain, cell morphology and motility in addition to nitrogenase activity determined for 24hr cultures (nmole $C_2H_4h^{-1}$ ml^{-1} culture). For strain identification, API microtube system Kit (API 20E) was used as a standard micromethod (Logan and Berkely, 1984).

RESULTS AND DISCUSSION

This research is designed to isolate and identify the auxins producing bacteria from rhizosphere and rhizoplane of different crops grown in different locations in Egypt. One hundred and forty eight isolates were collected, about 70.3% of such isolates were obtained from rhizosphere and 29.7% of isolates from rhizoplane. The results revealed that 55 isolates were from Giza, 45 isolates from Fauiom, 32 isolates from Kafr-El-Shikh and 26 isolates from Ismailia. The largest number of isolates was from wheat root while the lowest number of isolates was from sorghum being 46 and 16 isolates, respectively. In relation to auxin production, twenty isolates were produced high amount of auxins but only seven isolates gave very high response in Salkowisky reaction. The best seven isolates were isolates No. 1, 6, 16 and 25 from sugar cane rhizoplane, isolate No. 49 from wheat rhizoplane and No. 56 and 65 from maize rhizosphere and rhizoplane, respectively. Results indicated that presence of auxins producing bacteria with considerable production were higher in the rhizoplane than rhizosphere especially in sugar cane, wheat and maize.

Isolates that produce considerable amount of auxins were selected and grown in M_{56} minimal medium with different carbon sources Table (2). Differences were found in the growth among selected isolates and the best carbon source for most isolates was glucose. The highest growth rate (1.2 OD) was for isolate No. 41 and the lowest growth rate was for isolate No. 62 (0.11 OD). The isolate No. 16 was efficiently grown on various carbon sources.

Table 2: Effect of different carbon sources* on growth of auxins producing isolates.

Isolate number	Optical density (OD) at 580 nm		
	Lactose	Glucose	Sucrose
1	0.35	0.42	0.52
6	0.49	0.59	0.61
9	0.20	0.51	0.13
16	0.89	0.99	1.10
22	0.73	0.46	0.53
24	0.17	0.55	0.21
25	0.23	0.93	0.85
40	0.21	0.43	0.19
41	0.13	1.20	0.99
49	0.15	0.51	0.56
55	0.13	0.52	0.61
56	0.18	0.43	0.15
62	0.11	0.53	0.51
65	0.60	0.91	0.88
75	0.12	0.60	0.47
80	0.51	0.57	0.48
93	0.18	0.61	0.53
100	0.43	0.49	0.79
103	0.41	0.53	0.59
115	0.19	0.44	0.48

***Carbon sources were used as 10 g/l.**

The ability of selected isolates to produce auxins for different incubation time was measured using spectrophotometer and determined as µg/ml in M56 minimal medium supplemented with glucose and tryptophan (Table 3). The amounts of auxins produced by all strains were greater after 72 hr than 24 hr or 48 hr. The amounts of auxins ranged from 33 to 75 µg/ml after 72 hr. Isolate No. 16 produced the highest amount, but the lowest amounts resulted from isolates No. 40 and 93. Only seven isolates were selected to determine their ability to produce indole-3 -acetic acid (IAA) by gas-liquid chromatograph (GLC). Figure (1) revealed that IAA production ranged from 38µg/ml (isolate No. 65) to 71µg/ml µg/ml (isolate No. 16) which gave the largest amount of IAA. The results also showed that 48 hr. incubation time was more suitable than 24 hr for IAA production.

The different characteristics of the seven representative isolates are presented in Table (4). Three isolates were long spiral and motile (isolates No. 1, 6 and 49) and the other four isolates were short rods but only two isolates were motile. At the same time, all isolates were not able to form endospores and were Gram-negative. The nitrogenase activity of isolates No. 1, 6 and 49 was measured in NFM semi-solid medium aerobically after 24 hr of incubation at 30 ^{0}C while isolates No. 16, 25, 56 and 65 was measured in M_{56} liquid medium under anaerobic condition. The highest value was 33 nmoles C_2H_4 h^{-1} ml^{-1} culture for isolate No. 49.

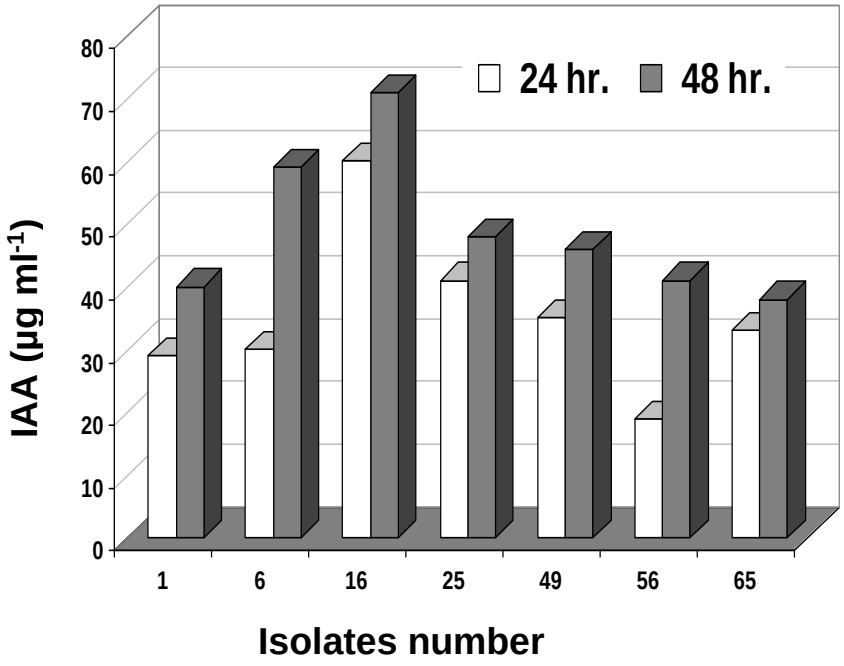

Figure 1: Production of IAA by selected PGPR isolates using Gas-Liquid Chromatograph (GLC).

Applying the API systems for further identification, it was found that isolate 56 was *Pseudomonas flurescens* while strains 16, 65 were related to *Klebsiella pneumoniae* but isolate 25 was belonged to *Enterobacter cloacae* (Table 5). Isolates 1, 6 and 49 were related to *Azospirillum* spp. based on biochemical characteristics and morphologycal properties.

Strains No. 16 *(KIebsieIla pneumoniae)* and No. 6 *(Azospirillum* spp.) that produce more IAA than other strains were isolated from rhizoplane of sugar cane. These findings are in the same line with Berge and Balandreau (1991) who reported that *Azospirillum* and *KIebsieIla* are very good colonizers of sugar cane and maize rhizospheres. Moreover, Kapulnik *et al.* (1985) and Whallon *et al.* (1985) indicated that *Azospirillum* colonization on the roots of wheat were located mainly on the cell elongation area and on the bases of root hairs, but fewer bacterial cells were present on the root cap or adsorbed to root hairs. In other study, Lutfu *et al.* (1981) characterized and isolated *Klebsiella oxytoca* from wheat roots. *Azospirillum* and other of PGPR bacteria are found to produce and release broad-spectrum of plant growth regulator; i.e. auxins, erveral gibberallins and cytoinins (El-Khawas *et al.* 1995; Beyeler *et al.* 1997)

Table 3: Production of auxins by selected isolates in M56 minimal medium supplemented with glucose and tryptophan.

Isolate number	Auxins* (µg/ml)		
	24 hr	48 hr	72 hr
1	35	41	51
6	38	51	61
9	19	35	40
16	69	73	75
22	19	35	40
24	22	29	38
25	43	45	59
40	16	25	33
41	18	23	37
49	27	51	57
55	28	37	43
56	35	49	52
62	26	31	41
65	39	46	49
75	19	36	42
80	17	33	39
93	21	25	33
100	13	26	34
103	17	37	43
115	15	27	39

***Auxins production was measured using spectrophotometer at 530 nm by the method of Tang and Bonmer (1947).**

Table 4: Characterization of selected IAA producing isolates.

Isolate number	Cell shape	Gram stain	Motility	N_2-ase activity nmoles $C_2H_4h^{-1}$ ml^{-1} culture
1	Long spiral	G^-	Motile	28
6	Long spiral	G^-	Motile	32
16	Short rods	G^-	Non-motile	21
25	Short rods	G^-	Motile	18
49	Long spiral	G^-	Motile	33
56	Short rods	G^-	Motile	13
65	Short rods	G^-	Non -motile	24

In other study, El-Khawas and Adachi (1999) showed that *Azospirillum brasilense* and *KIebsiella pneumoniae* produced high quantities of indole-3-acetic acid and tryptophol (indole-3-ethanol, TOL). Moreover, very low levels of indole-3- actaldhyde (IAAld) and indole-3- pyruvic acid (IpyA) were identified by *KIebsiella pneumoniae.* These results suggested that IAA could be synthesized from tryptophan (Trp.) through the indole-3-pyruvic acid pathway in *KIebsiella pneumoniae.* William *et al.* (1987) reported that different strains of *Pseudomonas* were capable to induce 1AA and additional indole compounds in liquid culture media supplemented with L-Tryptophan. On the other hand, Lin *et al.* (1983), E1-Khawas (1990) and Salamone *et al.* (1997) indicated that PGPR might have a positive effect on host growth due to production of plant growth substances.

Table 5: Identification of selected isolates using API 20E strip.

Characters	Isolate number			
	16	**25**	**56**	**65**
β-galactosidase	+	+	-	+
Arginine dihydrolase	-	+	+	-
Lysine decarboxylase	+	-	-	+
Ornithin decarboxylase	-	+	-	-
Citrate use	+	+	+	+
H_2S production	-	-	-	-
Urease	+	-	-	+
Trypotphane deaminase	-	-	-	-
Indole production	-	-	-	-
Acetoin production	+	+	+	+
Gelatine hydrolysis	-	-	+	-
Fermentation of:				
Glucose	+	+	+	+
Mannitol	+	+	-	+
Inositol	+	-	-	+
Sorbitol	+	+	-	+
Rhamnose	+	+	-	+
Saccharose	+	+	-	+
Melibiose	+	+	+	+
Amygdaline	+	+	-	+
Arabinose	+	+	+	+
Cytochrome oxidase	-	-	+	-
NO_2- production	+	+	+	+
N_2- production	-	-	-	-
Mobility	-	+	+	-
MacConkey	+	+	+	+
Identification	*Klebsiella pneumoniae*	*Enterobacter cloacae*	*Pseudomonas flurescens*	*Klebsiella pneumoniae*

REFERENCES

Bashan, Y. and H.Levanony (1990): Current status of *Azospirillum* inoculation techmology: *Azospirillum* as a challenge for agriculture. Can.J. Microbial. 36: 591-608.

Berge, T. H. and J. Balandreau (1991): Diversity of diazotroph populations in the rhizosphere of maize growing on different French soils. Boil. Fertil. Soils, 11: 210-215.

Beyeler, M; P. Michaux; C. Kell and D. Hass (1997): Effect of enhanced production of indole-3-acetic acid by the biological control agent *Psudomonas flurescens* CHAO on plant growth. In: Ogoshi A, Kobayashi K, Homma Y, Kodama F, Kondo N, Akino S (eds) Proceedings of the Fourth International Worksop on Plant Growth-promoting Rhizobacteria, Sapporo, Japan, pp 310-312.

Cleland, R.E. (1990): Auxin and cell elongation. In plant hormones and their role in plant growth and development. P.J. Davies(ed.), Kluwer Academic Publishers, Dordecht, the Netherlands, pp.132-148.

Day, J. M. and J. Doberiner (1976): Physiological aspects of N_2-fixation by a *spirillum lipoferum* from Digitaria roots. Soil Biol. Biochem. (8): 45-50.

El-Khawas, H.M. (1990): Ecological, physiological and genetic studies of *Azozpirillum*, Dissertation, Universitat Bayreuth, Genrmany.

El-Khawas, H.M. (1995): Indole acetic acid production by natural soil micro-residents Egypt. J. Appl. Sci., 10(1):575-582.

El-Khawas, H. and Adachi, K. (1999): Identification and quantification of auxins in culture medium of *Azospirillum* and *Kelbsiella* and their effect on rice roots. *Biol. Fertil. Soils*, 28: 377-381.

Forlani, G.; R.Pastorelli; M. Branzoni and F.Favilli (1995): Root colonization efficiency, plant growth-promoting activity and potentially related properties-associated bacteria. J. Gene. and Breed., 49: (4) 343-351.

Galli, E.; P.Barbieri; G.Zanetti and G. Wittmer (1988): Recent developments and perspectives of *Azospirllum*-gramineae association. Proceedings of the third International Symposium on Durum wheat. Foggia, Italy, 5-7 May, pp.335-342.

Glick, B. R. (1995): The enhancement of plants growth by free living bacteria Can. J. Microbial., 41: 109-117.

Holm, E. and V. jensen (1972): Aerobic chemoorganotrophic bacteria of a Danish beech forest. Oikos 23: 248-260.

Kapulnik, Y., Y.Okon and Y. Hens (1985): Changes in root morphology of wheat caused by *Azospirillum* inoculation. Can. J. Microb., 31 :881-887.

Kloepper, J.W., R.Lifshity and R.M.Zablotowicz (1989): Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol. 7: 39-43.

Lin W.Y; Y. Okon and R.W. Hardy (1983): Enhanced mineral uptake by *Zea maize* and *Sorghum bicolor* roots inoculated with *Azospirillum brasilense*. Appl. Environ. Microbiol., 45: 1775-1779.

Logan, N.A. and R.C.W. Berkeley (1984): Identification of *Bacillus* strains using the APi system. J. Can. Microbiol., 130 : 1871-1882.

Lutfu, Cakakcl, M.; H. J Evans and R. J. Seidler (1981): Characteristics of nitrogen fixing *Klebsiella oxytoca* isolated from wheat roots. Plant and soil 61:53-63.

Salamone, IEG, L. Nelson and G. Brown (1997): Plant growth promotion by *Psudomonas* PGPR cytokinin producers. In: Ogoshi A, Kobayashi K, Homma Y, Kodama F, Kondo N, Akino S (eds) Proceedings of the Fourth International Workshop on Plant Growth-Promoting Rhizobacteria, Sapporp, Japan, pp 316-319.

Shindy, W.W. and O.E. Smith (1975): Identification of plant hormones from cotton ovules. Plant physiol., 55; 550-554.

Tang, Y.W. and J. Bonner (1947): The enzymatic inactivation of indole acetic acid I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13 :11-25.

Vanpeer, R. and B. Schippers (1989): Plant growth 9 responses to bacteria with selected *Pseudomonas* strains rhizosphere microbial deial development in hydropnic cultures. Can. J. Microbial. 35: 456-463.

Whallon, J.H., G. Acker and H.M. El-Khawas (1985): Electron microscopy of yong wheat roots inoculated with *Azospirillum.* In: Klingmuller W. (ed) Azospirillum III, Genetic, Physiology, and Ecology. Springer, Berlin, Heidelberg, pp 222-229.

William, F., F.O.Stanley and G.D. Michale (1987): Auxin production by plant pathogenic *pseudomonas* and *xanthomonas*. Applied and Environmental Microbiology. Aug., 1839-1845.

عزل وتعريف البكتريا المشجعة لنمو النباتات و التى لها المقدرة على إنتاج اندول حمض الخليك من جذور بعض المحاصيل المزروعة فى مصر

حسين الخواص[١] - ا براهيم عبد المقصود[٢] -هالة انور[٢] - نبيل حجازى[١]

[١] قسم الميكروبيولوجى - كلية الزراعة- جامعة القاهرة و [٢] مشروع تطوير النظم الزراعية -مركز البحوث الزراعية

تهدف الدراسة علي حصر البكتريا المشجعة لنمو النباتات (PGPR) و التى لها المقدرة على إنتاج اندول حمض الخليك (IAA) من جذور بعض المحاصيل. تم الحصول على ١٤٨ عزلة نقية من أنواع البكتريا المختلفة وذلك من جذور بعض المحاصيل المختلفة (الذرة، القمح، الشعير، الذرة الشامي و قصب السكر) من أماكن متعددة فى مصر(الجيزة، الفيوم، كفر الشيخ و الأسماعيلية) ولقد تم إجراء العزل باستخدام طريقة الأطباق وبطريقة العد الأكثر إحتمالاً. بناءً على المقدرة العالية لإنتاج IAA تم اختيار عشرين عزلة وذلك عن طريق الكشف عن وجوده بطريقة لونية ثم بالتقدير الكمى له عن طريق التحليل الكروماتوجرافى. بعد تنمية العشرين عزلة على بيئات معدنية مع تغير مصدر الكربون فى البيئة وقياس درجة النمو ثبت أن الجلوكوز كان أفضل من السكروز والالكتوز بالنسبة لمعظم العزلات كذلك وجد أن وجود التربيتوفان فى البيئة ضرورى لإنتاج العزلات للهرمون. وقع الاختيار على أفضل سبع سلالات إنتاجاً للهرمون ليتم تعريفها بالطرق التقليدية بالإضافة إلى طريقة التقسيم الحديثة (API 20E-) لتصنيف العزلات المختارة والتى اتضح انها تنمتى إلى: *Azospirillum* spp., *Klebsiella pneumoniae*., *Enterobacter cloacae* and *Psudomonas flourescens*.