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DETECTION, PURIFICATION, AND IDENTIFICATION OF SIDEROPHORES PRODUCED BY *Pseudomonas fluorescens* ISOLATES USING SDS-PAGE AND HPLC.

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ABSTRACT

Three isolates of *Pseudomonas fluorescens* were isolated from the soil rhizosphere of potato growing fields and identification was confirmed by comparison of the morphological traits and physiological characteristics according to Murray *et al*., 1984 and Klement *et al*.,(1990). *P. fluorescens* isolates were tested to prove their ability in production of siderophores. Chrome Azurol Sulfonate assay (CAS assay) test was used to detect the presence of siderophores. Formation of an orange halo around a well bored into the CAS plate, was taken as an indication for the presence of siderophores. Concerning the optimization of conditions for highest production of siderophores on different media, the results revealed that king's B medium, exhibited the highest production of sidrophores. Also, data showed that pH 7 and 27 °C were the best in this respect while at pH 6 and 8 sidrophores production completely inhibited. One of the siderophores gene was detected by specific primer in *P. fluorescens* and one band at molecular weight 520 bp was observed. SDS–PAGE analysis was used to detect the possible outer membrane receptor protein (OMRP), in both whole cell pellet and membrane pellet at no iron and high iron concentration. The results revealed the existence of one band at 84 KDa in case of no iron concentration. Purification of siderophores in culture supernatant was done by a sephadex G -25 gel filtration column. Nine fractions were obtained and they tested for siderophores activity by CAS assay. There were two types of siderophores in *P. fluorescens* culture supernatant (pyoverdin and pyochelin) and they were detected by high performance liquid chromatography (HPLC).

INTRODUCTION

Iron is a vital element required by virtually all living organisms, including bacteria. At low iron concentrations, bacteria are known to produce iron chelators called siderophores for ensuring their iron availability (Kathleen, 2007). The term "siderophore" is Greek for "iron carrier" and is so named because these molecules produced by micro organisms have an extremely high affinity for ferric iron (Landford,1973), thus siderophores bind iron and transport them to the cell. *P. fluorescens* as an example has the ability to suppress *R. solanacearum*. One of the mechanisms involved in disease suppression of potato is the production of antibiotic and siderophores. There are over 500 described siderophores had low molecular weight (350-1500 Daltons) as organic molecules (Wandersman and Deleplaire, 2004). Because of the high concentration of iron inside the bacterial cell and extremely low concentrations outside the cell, siderophores complexes are actively

transported across membrane via outer membrane receptor protein (OMRP) (Buchanan *et al*., 2003). In general, the first step of entry of ferric siderophores into Gram-negative bacteria is mediated by specific outer membrane receptors. This transport into the periplasm requires the proton motive force (pmf) of the cytoplasmic membrane and an energy transduction complex, which includes the cytoplasmic membrane proteins TonB, ExbB and, ExbD (Erin, 2005). Although most siderophores are either hydroxamates or catechols, the chrome azurol sulfonate assay (CAS assay) has become widely used and may be applied on agar surfaces or in solution (Abigail, 2007). It is based on the colour change that accompanies transfer of the ferric ion from its intense. Raaska *et al*., (1992) detected the siderophores produced by *P. fluorescens* and *P. chlororaphis* from the culture supernatants by the universal Chrome azurol sulfonate assay (CAS assay) at wave lengths 620-690 nm. The CAS assay was applied to detect *Pseudomonas* siderophores directly in situ, and the most common detection method for siderophores production is the universal assay of Schwyn and Neilands (1987). The ability of *Pseudomonas* to grow and to produce siderophores is dependent on both the iron content and the type of carbon sources in the medium. Under conditions of low iron concentration the *Pseudomonas* isolates studied produced yellow-green fluorescent iron binding peptide siderophores, (Qarah *et al*., 2005). In addition, Djibaoui and Bensoltana (2005) reported that the highest siderophores concentrations were obtained from *Pseudomonas* culture in succinate medium. Similarly, Huiming *et al*., (1991) cleared that addition of iron to the culture medium resulted in increased culture growth with markedly decreased yield of siderophore. Also there are about six genes which were found to be responsible for siderophores production. Generally, *P. fluorescens* produces two types of siderophores which are pyoverdin and pyochelin. (Isabelle *et al*., 2001). *P. aeruginosa* and *P*. *fluorescens* produce an extracellular compound with yellowish green fluorescence, called pyoverdin, which functions as a siderophore. The production of pyoverdin, formerly called fluorescein, is concomitant with the production of another siderophore, pyochelin. Pyoverdin is produced by *P. aeruginosa* in several forms, some of which were separated on gel filtration columns and on reverse-phase, high-pressure liquid chromatography columns. An active form of iron-free pyoverdin was purified to homogeneity, Dany and Jean-morie, (1988)**,** Alain *et al*., (2003) and Laurent *et al*., (2007). In addition, Huiming *et al*., (1991) purified siderophores that produced by *P. fluorescens* strain V1 using gel filtration chromatography, the molecular weight of the siderophores they identified estimated to be 1,000 Daltons. The objectives of this study were detection of siderophores produced by *P. fluorescens*, optimization of conditions for siderophores production by *P. fluorescens*, detection of one of the siderophores genes, and of possible outer membrane receptor protein (OMRP), purification of siderophores, identification of siderophores types by High performance liquid chromatography. It was hopped that results obtained from the present study could help in revealing the best antagonistic bacterial product to suppress variety of plant diseases.

MATERIALS AND METHODS

Preparation of fluorescent pseudomonads cultures and identification:-

Fluorescent pseudomonads were isolated by serial dilution in king's B medium (KB) (proteose peptone, 1 g; $K_2HPO_4.3H_2O$, 0.15 g; $MgO_4.7H_2O$, 0.15 g; Glycerol, 1.5 ml; Agar, 2g; D.W., 100 ml., pH 7.2-7.4) (King *et al*., 1954) from potato rhizosphere. Cells were selected on king's B medium and incubated at 30ºC for 24 hr. Then cultures were examined under ultraviolet (UV) lamp at 430 nm to detect the presence of fluorescent colonies. A fluorescent pseudomonads bacterium was identified to species according to morphological and physiological tests recommended by Murray *et al*., (1984) and Klement *et al*., (1990) Table (1).

Detection of siderophores production by *P. fluorescens*

Chrome Azurol S (CAS) Assay

The CAS Assay (Schwyn and Neilands, 1987) is the universal chemical assay for siderophores detection and is based on a siderophore's high affinity for ferric ion. CAS plates are blue in colour because chrome Azurol S dye is complexed with ferric ion. When siderophores is present, an orange free dye is released (Erin, 2005).

Fe^{3+} -dye (blue) + siderophore $\rightarrow$ Fe^{3+} - siderophore + dye (orange)

P. fluorescens isolate (G) which was the most antagonistic against *R. solanacearum* isolates according to previous study was chosen for further studies. This isolate was grown in King's B medium without phosphate. The medium consisted of (magnesium sulphate 1.5 g/l, Merk, Trypton 10 g/l, Difco, proteose peptone 10 g/l, Difco, glycerol 10 m/l, BDH) and free from iron. KB medium supplemented with 0.5 µM $Fe_2(so_4)_3$ (low iron) was used as well as with 20 µM $Fe_2(so_4)_3$ (high iron). Cultures of the tested isolate supplemented with iron concentrations were grown for 48 hr on a rotary shaker and the supernatant from each was collected by centrifugation at 1000 rpm for 10 min using a $\pm$ 2 cork borer, well were bored into a CAS plate and 60 µl a liquots of each culture supernatant was pipetted into a separate well. Sterile media was also added to a well as a control. The plate was then incubated at room temperature. Depending on the culture, colour formation may take 30 minutes to 5 hr. Formation of an orange halo around the well indicate that culture is producing a siderophore.

Optimization of conditions for siderophore production:

Effect of iron concentrations and various carbon sources on bacterial growth and siderophores production:

Cultures were grown for 48 hr at 27ºC with shaking (150 rpm) in dark. The bacterial inocula were prepared by suspending a loopful of an actively growing in saline. The inoculum level was calibrated to give a turbidity of 0.04. Three hundred µl of these inocula were suspended in 30 ml of media. To remove traces of iron, glassware was cleaned with 6 M HCl and with double distilled water. Three basal media were employed with $FeCl_3$ added in increasing amounts (0, 10, 70, 140, 200, 260 µg/ml). The media contain the following components according to (Djibaoui and Bensoltana, 2005)

- **Succinate medium**:- KH_2PO_4 6 g/l, K_2HPO_4 3 g/l $(NH_4)_2$ SO_4 1 g/l, $MgSO_4.7H_2O$ 0.2 g/l sodium succinate., **King's B medium**:- magnesium sulphate 1.5 g/l, tryptone 10 g/l proteose peptone 10 g/l, glycerol 10 ml/L., **Asparagine medium**: asparagine 5 g/l, $MgSO_4$ 0.1 g/l, and K_2HPO_4 0.5 g/l. Bacterial growth was determined using plate count technique.

Effect of temperature on bacterial growth and siderophores production:

While most of *P. fluorescens* isolates grow at 25º-35ºC, this may not the optimal temperature for siderophores production. Five flasks were prepared with 50 ml KB medium and inoculated with *P. fluorescens* isolate (G). The flasks containing the inoculated medium by *P*. *fluorescens* tested isolate were incubated at different temperatures (20, 25, 27, 30, and 35 ºC).

Effect of pH on bacterial growth and siderophores production:

Six flasks containing KB medium were inoculated with *P. fluorescens* isolate at various pH degrees (6, 6.5, 6.8,7, 7.3, and 8).

Detection of one of siderophores genes by specific primer.

PCR amplification was carried out in the bacterial genomic DNA of the two isolates of *P*. *fluorescens* (one siderophores producer and the other non-producer for siderophores) to study the presence/absence of siderophores using specific primers; the forward 5\ CGC AAA TGC AGC AAC CGC AT 3\ and the reverse 5\ ACC TGG ACG AAG GTG GCC AT 3\. The PCR was performed as follows: initial cycle with 95ºC for 5 minutes and 34 cycles of 95ºC for 1 minute, 60ºC for 1 minute and 72ºC for 10 min. The PCR product was visualized on agarose gel 1% and photographed using gel documentation system. (Youard, *et al*., 2007).

Detection of possible outer membrane receptor protein (OMRP):

Preparation of sample for SDS-PAGE analysis:

P. fluorescens isolate (G) was grown in KB medium for 48 hr at 27ºC in two shake flasks (150 rpm) one with no added iron and other with 20 µM $FeCl_3$. Added cells were harvested by centrifugation at 10,000 rpm for 10 min and supernatant was discarded. Some of the whole cell pellet was saved and stored in an eppendorf tube at -8ºC. The remaining pellet was resuspended in lysis buffer (PMSF 1 mM, 2-BMES mM, Tris HCl 50 mM pH 8.8) and sonicated in an ice bath using a large probe (9.0, 9.0 second bursts of one minute with one minute pauses). Sonicated samples were centrifuged at 10,000 rpm for 10 min and the supernatant was poured into ultracentrifuge tube. These were centrifuged at 30,000 rpm for 90 min and resulting membrane pellets were stored at -20ºC (Buchanan *et al*., 2003).

SDS-PAGE analysis of samples:

Electrophoresis stock solutions

The stock solutions used for protein electrophoresis were as follows: **A. 30% Acrylamide stock solution (kept in dark at 4°C):**

Acrylamide (29 g), Bis-acrylamide (1 g), Deionized distilled water (Up to 100 ml). Any insoluble materials were removed by filtration through Whatman filter paper No.1. **B. Sodium dodecylsulfate (10% w/v SDS):** Ten grams of sodium dodecylsulfate were dissolved in 100 ml deionized distilled water. **C. Ammonium persulphate solution (10% w/v):** One gram of ammonium persulphate was dissolved in 10 ml deionized distilled water and kept at 4°C. The solution unstable and must be made just before use. **D. Buffers: I)**

Separating gel buffer (1.5 M Tris-HCl, pH 8.8, kept in dark at 4°C)**:** Tris-base (18.15 g) was dissolved in 50 ml deionized distilled water and adjusted to pH 8.8 using concentrated HCl. The final volume was made up to 100 ml.
II) Staking gel buffer (0.5 M Tris-HCl, pH 6.8, kept in dark at 4°C)**:** Tris-base (6.05 g) was dissolved in 50 ml deionzed distilled water and adjusted to pH 6.8 using concentrated HCl. The final volume was made up to 100 ml.
III) Electrophoresis buffer (pH 8.3-8.5)**:** The tank buffer consists of 3 g Tris-base, 14.4 g glycine and 1 g sodium dodecylsulphate dissolved in 1000 ml deionized distilled water.
Separating and staking gel preparation: Vertical slab (16 x 18 cm) gel electrophoresis apparatus (Bio-Rad, USA) was used. All glass plates were washed with deionized distilled water, then surface sterilized with ethanol. Spacers of 1.5 mm were used. Gels were prepared according to the protocol of Laemmli (1970). **a] 12% Separating gel (30 ml solution):** 30% Acrylamide stock solution (12 ml)**;** 1.5 M Tris-HCl, pH 8.8 (7.5 ml)**;** 10% (w/v) SDS (0.3 ml)**;** 10% Ammonium persulphate solution (0.3ml)**;** Deionized distilled water (9.9 ml)**;** TEMED added last (12 µl). Separation gel solution was instantly swirled, then poured simultaneously to a height of 1.5 cm below the bottom of the comb and left to polymerize for at least 30 min. Separating gels were overlaid with 1 ml of water which removed before the stacking gel solution was poured. **b] 5% Stacking gel (10 ml solution):** 30% Acrylamide stock solution (1.7 ml)**;** 0.5 M Tris-HCl (pH 8.8) 1.25 ml**;** 10% (w/v) SDS 0.1 ml**;** 10% Ammonium persulphate solution 0.1 ml**;** Deionized distilled water (6.8 ml) TEMED (10 µl). Stacking gels solution was quickly poured over the separating gel, and combs were used. Gels were left to polymerize for 30 min before running.
Protein sample preparation:

Sodium dodecyle sulphate (SDS) was add to the sample at a rate of 4 mg SDS/1 mg protein, mixed with 50 µl 2-mercaptoethanol then boiled at 100°C in water bath for 3-5 min.

Loading and running of the samples:

Twenty microlitters of each protein sample were loaded in the wells of the staking gel. The samples were covered with electrode buffer. Few drops of bromophenol blue (4 mg/100 ml deionized water) were added to the electrode buffer (tracking dye). Protein marker with molecular weight ranged from 1.4.4 to 116.6 KDa was used as standard. Electrophoresis was carried out at constant voltage of 100 V for 3 h in electrophoresis running buffer (pH 8.3-8.5).

Staining and distaining of the gel:

The gel was stained in 50 ml of staining solution (0.125% coomassie blue R-250, 50% methanol and 10% acetic acid) and then the gel was destained in a distaining solution (20% methanol, 10% acetic acid, and 70% H_20). The gel was placed between two sheets of cellophane membrane and dried on gel drier for 2 hr. and photographed by digital camera.

Purification of siderophores:

Culture supernatant (1 liter) from strain U7 was lyophilized; the residue was solved in 5 ml of deionized water and centrifuged to remove the

undissolved material. A 2-ml sample of this material was applied to a Sephadex G-25 (Pharmacia) gel filtration column (1.5 by 70 cm) equilibrated with water-methanol (10:1, vol/vol) as a solvent. Fractions of 3 ml were collected and assayed for siderophores by CAS assay. The positive fractions were pooled, lyophilized, and resuspended in 1.0 ml of water. Approximately 5 mg of partially purified siderophore was obtained from 1 liter of culture supernatant. Fractions were also assayed for siderophore activity with CAS solution. (Kathleen 2007).

Identification of siderophores types by High Performance Liquid Chromatography (HPLC).

Liquid cultures were grown for 3 days. Culture was shaken for 20 min, and the bacteria were removed by centrifugation (22 min, 10,000 rpm) and filtration through a 0.2-µm-pore-size membrane filter. The HPLC analyses were carried out by using the filtered culture media, the pyoverdin production was estimated by measuring the absorbance at 403 nm in order to determine the injection volume for the strain. The aim was to detect, by using HPLC, the retentions time (RT) of peaks with comparable heights. The HPLC analyses were performed with Nucleosyl C_{18} columns and a Waters 2190 system. The difference in RT (ΔRT) (wavelength, 403 nm) the culture medium of the strain was injected for a maximum of three analyses before or after each investigated strain. When the heights of the peaks were found to differ too much, the analyses were carried out again after readjustment of the injected volumes. Pyoverdin detection was performed by using the spectra of the molecules between 200 and 500 nm obtained with a Waters PDA 996 photodiode array detector. Two HPLC programs were used, in which solution A was a 17 mM NaOH-acetic acid buffer (pH 5.3) and solution B was acetonitrile. HPLC program 1 was as follows (flow rate, 1 ml/min): 100% solution A, 1 min; from 100% solution A to 97% solution A, 2 min; 97% solution A, 9 min; and from 97% solution A to 30% solution A, 25 min. HPLC program 2 (flow rate, 0.9 ml/min) was as follows: 100% solution A, 8 min; from 100% solution A to 98% solution A, 2 min; 98% solution A, 10 min; from 98% solution A to 95% solution A, 5 min.; from 95% solution A to 30% solution A, 15 min; and 30% solution A, 5 min. A pyoverdin production by *P. fluorescens* (G) was initially carried out in King's B medium by using HPLC program 1 (Alain *et al*., 2003).

RESULTS

Identification of *Pseudomonas fluorescens* isolates

Three isolates were identified as *Pseudomonas fluorescens* on the basis of their morphological and physiological characteristics. Data presented in Table (1) revealed that all the isolates were gram negative, non sporulating and motile. Meantime, the isolates exhibited positive reaction for *fluorescence* on KBA medium, growth at 4ºC, pH 7, levan formation and gelatin liquefaction. However, the isolates showed negative reaction for starch hydrolysis, growth at 41ºC and in 7 % Nacl.

Detection of siderophores production by *P. fluorescens*

CAS assay was used to determine the ability of *P. fluorescens* to produce siderophores. Formation of an orange halo around a well bored into the CAS plate indicates that siderophores is present in the supernatant of cultures. These results indicated that *P. fluorescens* produces siderophores under no iron and low iron respectively, while siderophores were not produced when sufficient iron was available in the medium for the bacterium tested Fig (1).

Table (1): Morphological and physiological characteristics of the antagonistic *Pseudomonas fluorescens* isolates.

Characteristics	G	H	I
Gram Staining	-	-	-
Sporulation	-	-	-
Fluorescene on KBA	+	+	+
Motility	+	+	+
Growth at 41°C	-	-	-
Growth of 4°C	+	+	+
Growth at pH 7	+	+	+
Growth in 7% NaCl	-	-	-
Levan formation	+	+	+
Gelatin liquefaction	+	+	+
Starch hydrolysis	-	-	-

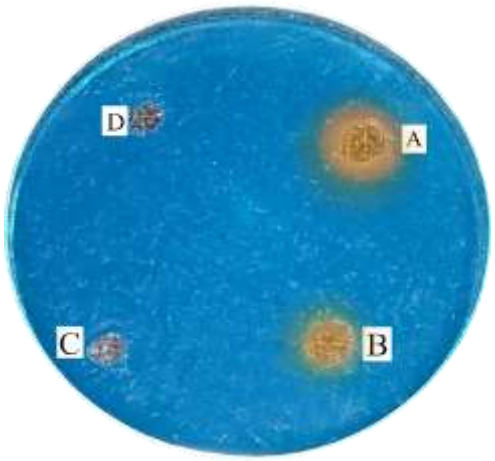

Fig. (1): CAS assay plate for *P. fluorescens* isolate tested A) no added iron. B) 0.5µM $Fe_2(So_4)_3$ (low iron). C) 20.0 µM $Fe_2(So_4)_3$ (high iron). D) un-inoculated media (control).

Optimization of conditions for siderophores production:

Effect of iron concentrations and various carbon sources on bacterial growth and siderophores production:

Iron concentration is probably the single most important factor in how much an organism produces siderophores, as genes for siderophores biosynthesis are expressed under the direct control of iron concentration in

the environment. Data presented in Table (2) showed that King's B was the most suitable for bacterial growth with average of 1.7, followed by succinate (1.13) and Asparagine medium (0.96) respectively. The maximum bacterial growth was observed at 200 mg/L on King's B, followed by Succinate and Aspargine where exhibited values (2.07), (1.40) and (1.31) respectively. However, at 0.0 mg/L iron concentration, bacterial growth was noticed, but, markedly less than the other concentrations. Significant differences were observed between King's B medium and the two other media i.e Succinate and Asparagine, however, significant differences were not detected between Succinate and Asparagine medium on bacterial growth. Results revealed that the increase in iron concentration was correlated with decrease in siderophores production even completely suppressed at 260 mg/L concentration of iron on the three media tested. King's B medium exhibited the highest production of siderophores at (0.0) iron concentration where induced value (35.04) followed by Succinate (31.04) and Asparagine (28.96). Significant differences were not observed between King's B (23.55) and Succinate medium (21.10), but Aspargine medium (15.16) significantly differed from the other media. Generally, King's B medium exhibited the highest production of sidrophores followed by Succinate and Asparagine respectively.

Table (2): Effect of iron concentrations and various carbon sources on bacterial growth and siderophores production.

Fe^{3+} Conc mg/L	Bacterial growth*				Siderophores production			
	King's B	Succinate	Aspargine	Mean	King's B	Succinate	Aspargine	Mean
0	1.50	0.98	0.86	1.113	35.04	31.04	28.96	31.86^{a}
10	1.88	0.95	0.82	1.216	31.51	29.51	28.0	29.67^{ab}
70	1.90	1.25	1.24	1.463	29.91	29.06	20.0	26.32^{ab}
140	1.96	1.33	1.14	1.476	24.82	26.0	14.0	21.60^{b}
200	2.07	1.40	1.13	1.593	20.0	11.0	0.01	10.34^{c}
260	0.87	0.87	0.40	0.713	0.0	0.0	0.0	0.0^{d}
Mean	1.70^{a}	1.13^{b}	0.96^{b}		23.55^{a}	21.10^{a}	15.16^{b}	
$L.S.D_{0.05}$ for carbon sources	0.211				4.99			
$L.S.D_{0.05}$ F^{+3} conc.	0.297				7.06			

- **Means followed by the same letter (s) are not significantly different according to $L.S.D_{0.05}$ values. * each value X 10^{7} estimated as colony forming unit (cfu).**

Effect of temperature on bacterial growth of *P. fluorescens* and siderophores production*:*

Data in Table (3) showed that both growth and siderophores production are highest at 27°C, where that degree exhibited values 1.9 and 38.06 respectively. Such results cleared that 27°C was the most suitable for bacterial growth and siderophores production. Results also revealed that the higher temperature, the lowest growth as well as siderophores production even completely suppressed at 35ºC.

Table (3): Effect of temperature on bacterial growth of *P. fluorescens and* siderophores production.

Temperature °C	Bacterial growth*	Siderophores production
20	0.00	0.00
25	1.15	29.45
27	1.90	38.06
30	1.01	0.01
35	0.00	0.00
x ± S.D.	**0.81 ± 0.81**	**13.50 ± 18.74**

*** each value X 10^7 .**

Effect of pH on bacterial growth of *P. fluorescens* and siderophores production.

Data presented in Table (4) revealed that the highest production of siderophores by *P. fluorescens* was induced at pH 7, however, this value of pH did not exhibit the highest growth of bacterial isolate tested. Bacterial growth and siderophores production completely inhibited at pH 8 as well as pH 6.

Table (4): Effect of pH on bacterial growth of *P. fluorescens* and siderophores production.

pH	Bacterial growth*	Siderophores production
6	0.00	0.00
6.5	0.65	0.00
6.8	1.02	15.22
7	1.70	39.28
7.3	2.01	24.55
8	0.00	0.00
x ± S.D.	**0.90 ± 0.84**	**13.18 ± 16.34**

*** each value X 10^7 .**

Detection of one of siderophores genes by specific primer

Specific primer was used to study the presence of one of siderophores gene in *P. fluorescens* isolate (G) which known to produce siderophores and another bacterial isolate (O) non producer for siderophores. Date illustrated in Fig (2) showed that one band at molecular weight 520 bp was present in *P. fluoresens* isolate (G) and absent in another isolate (O).

SDS/PAGE analysis was performed on whole cell pellets and membrane pellets of cultures of the tested *P. fluorescens* isolate grown in a no-added iron medium as well as a high iron medium to detect a possible OMRP involved in siderophores transport. Results revealed that the protein was expressed in the no-added iron pellets, while it repressed in high iron concentrations. The molecular weights for most described OMRPs are in the range of 80-85 kilodaltons (kDa). The SDS/PAGE gel in Fig (3) shows the presence of a band in this molecular weight range that is only present in the no added iron cultures, indicating that it is regulated by the amount of iron in the medium. This protein is likely involved in siderophores transport

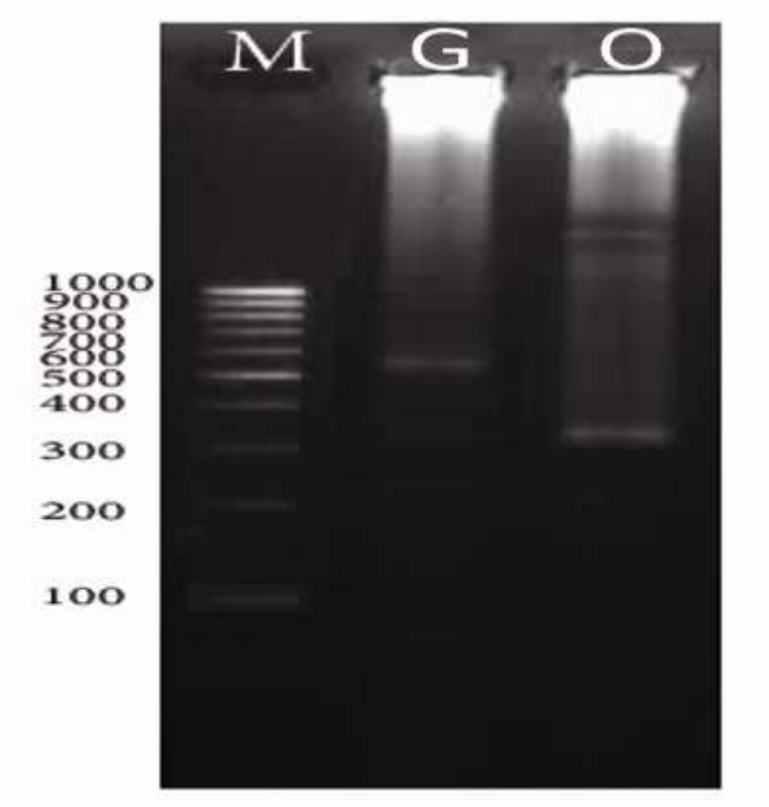

Fig. (2) **e by**
plate
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Detecti

Purification of siderophore:

The siderophores were purified from *P. fluorescens* culture supernatant by Sephadex G-25 gel filtration column. Data showed that 9 fractions were collected and assayed for CAS activity. Two fractions were positive to CAS assay and others were negative.

Identification of siderophores types by High Performance Liquid Chromatography (HPLC):

The two positive fractions were analyzed by HPLC. The HPLC method of analyzing siderophores production presented in this study proved to be a very powerful method. Data presented in Fig (4) indicated that there were two types of siderophores pyochelin and pyoverdin.

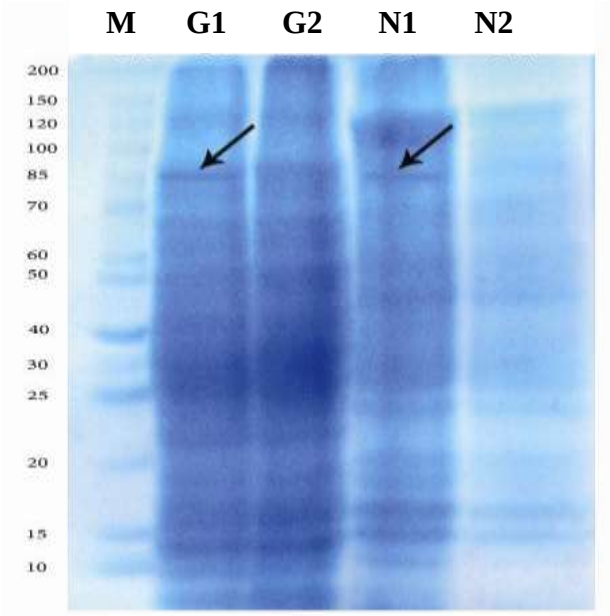

Fig. (3) SDS-PAGE analysis of whole and membrane pellets of *P. fluorescens* : M) molecular weight standard G_1: whole cell pellet in case of *P. fluorescens* grown in a (no added iron medium), G_2: *P. fluorescens* grown in a high iron medium.
N_1: Membrane pellet in case of *P. fluorescens* grown in a (no added iron medium), N_2: *P. fluorescens* grown in a high iron medium.

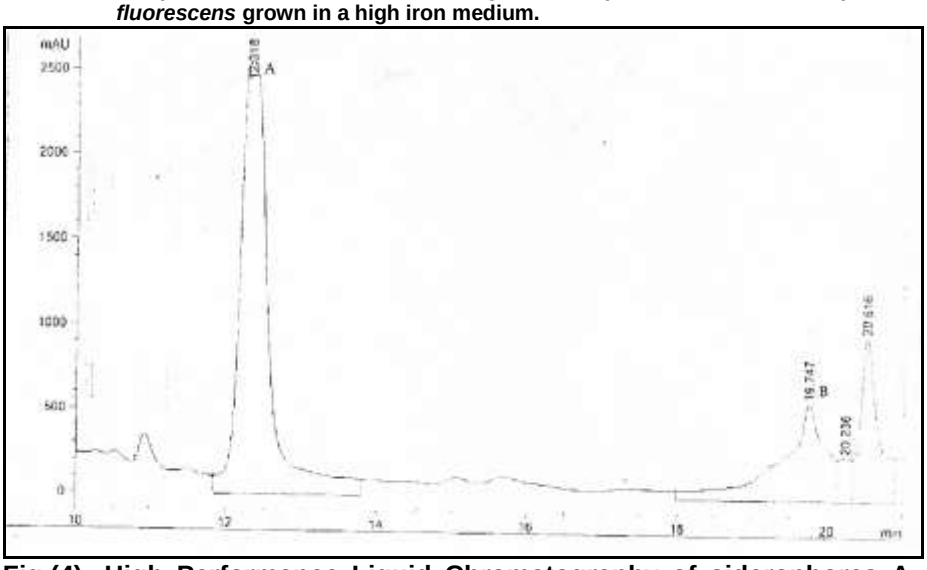

Fig.(4): High Performance Liquid Chromatography of siderophores A: pyoverdin and B: pyochelin) produced by *P. fluorescens* (G) isolate.

DISCUSSION

On the basis of the results obtained, three isolates of *P*. *fluorescens* were isolated from rhizosphere of potato fields and identified according to morphological and biochemical characteristics recommended by Murray *et al*., (1984) and Klement *et al*., (1990). Results indicated that the three isolates of *P*. *fluorescens* were gram negative, non sporulating and exhibited negative reaction for starch hydrolysis, growth at 41ºC and in 7 % Nacl. However, the isolates induced positive reaction in case of fluorescence on KBA medium, growth at 4ºC, pH 7, levan formation and gelatin liquefaction. *P. fluorescens* had the ability to synthesize a wide range of secondary metabolites. Among those were iron-chelating compounds called siderophores. Our results indicated that an orange halo around the well bored in the solid medium was noticed, and the CAS plate assay was blue in colour because chrome Azurol S dye is complexed with ferric ion. When the siderophores are present, they react with iron and the dye released which is orange colour. This result agreed with Huiming *et al*., (1991), Erin, (2005), and Abigail, (2007). Concerning, conditions that optimized for siderophores production were temperature, pH, iron concentration in the media and various carbon sources. Siderophores are iron-specific compounds which are secreted under low iron stress and the production of siderophores in the medium employed was inversely proportional to the iron concentration in the medium. Data in the present study indicated that the medium free from iron induced the higher production of siderophores. Besides, data presented indicated that the higher the iron concentrations, the lowest growth and sidrophore production. Our results indicated that siderophores production was the highest at (0.0) iron concentration and decreased as the iron concentration increased even completely depressed at 260 mg/L. These results are in agreement with Budzikiewicz, (1993), Qarah *et al*., (2005), Djibaoui and Bensoltana, (2005) and Katheleen (2007), where they reported that siderophores production positively induced under iron-stress. When *P. fluorescens* was grown in three different media, *i.e.* Succinate medium, Asparagine medium and king's B medium it could be concluded from our results that King's B medium induced the highest siderophores production. Significant differences were detected between King's B and the other two media *i.e.* Succinate and Asparagine. Such results were in contradictory with Djibaoui and Bensoltana **(**2005**)** where they reported that the highest siderophores production was obtained in Succinate medium while, were in harmony with them where they showed that iron increased the growth yield of bacteria and repressed siderophores production at levels above 200 µg/l, but had a positive effect below 160 µg/l. Data showed that the highest production of siderophores was induced at 27 ºC and the higher the temperature, the lowest growth as well as siderophores production even completely inhibited at 35ºC. Also, our results revealed that the highest production of siderophores at pH 7 (neutral), and depressed at pH 8 as well as pH 6. Concerning the detection of one of siderophores gene in *P. fluorescens* isolate which is known to produce siderophores and in another bacterial isolate that did not produce siderophores, our results showed that

one band at 520 bp was found in *P. fluorescens* isolate (G) and absence in the another isolate (O). These results somewhat agreed with those reported by Isabelle *et al*., (2001), Michelle *et al.,* (2004), and Youard *et al*.,.(2007). We have also detected a possible outer membrane receptor gene protein (OMRP) involved in the transport of siderophores in *P. fluorescens*. This possible receptor is detected only under no iron concentration and falls into the molecular weight range (84kDa) and this band did not found in the whole cell pellet or membrane pellet in high iron concentration. This was in agreement with Dimitris *et al*. (1999), Van der Helm and Chakraborty, (2001), Ferguson *et al*., (2002), and Buchanan *et al*., (2003). The HPLC method of analyzing siderophores production presented proved to be a very powerful method for rapidly determining. Results in this study revealed that existence of two types of siderophores and this result were similar to those reported by Dany and Jean-morie (1988), Alain *et al*., (2003), and Laurent *et al*., (2007).

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اكتشاف وتنقية وتعريف السيدروفورز المفرزة بواسطة البكتيرة سيدوموناس فلورسنس باستخدام الـ SDS-PAGE والفصل الكروماتوجرافي عالي الكثافة.

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- تم عزل ثلاث عزلات من البكتيرة سيدوموناس فلورسنس من الريزوسفير من مزارع بطاطس وتم تعريف هذه العزلات طبقا للصفات المورفولوجية والفسيولوجية والبيوكيميائية .
- تم الكشف عن السيدروفورز في راشح أقوي عزلة من هذه العزلات وذلك عند تنميتها على بيئة كينج B ووجد أن هذه البكتيرة تفرز هذه المركبات (سيدروفورز).
- تم دراسة الظروف المثلى لإنتاج السيدروفورز من العزلة G ووجد أن درجة الحرارة المثلى لنمو البكتيرة وانتاج السيدروفورز هي 27º م، وأن درجة الـ pH المثلى للنمو وانتاج السيدروفورز هي 7.
- كذلك أثبتت النتائج أن بيئة كنج B أكثر البيئات ملاءمة لنمو هذه البكتيرة سيدوموناس فلورسنس وانتاج السيدروفورز . علاوة على ذلك فقد وجد أن تركيز الحديد الأمثل لانتاج السيدروفورز هو صفر(أي في حالة عدم وجود حديد).
- وقد أسفرت الدراسات عن عزل أحد الجينات المسئولة عن افراز السيدروفورز بواسطة بادئ متخصص ووجد انه يظهر باند (شريط بروتيني) عند وزن جزيئي 520 bp وذلك عند المقارنة بعزلة بكتيرية أخرى لا تفرز السيدروفورز فلم يظهر هذا الباند.
- تم أيضا من خلال البحث والدراسة الكشف عن وجود المستقبلات البروتينية للسيدروفورز في كل من الغشاء البلازمي الكامل السليم للخلية في العزلة المختبرة وأيضا بعد تكسير الغشاء البلازمي بواسطة جهاز السونيكيتور في ظل عدم وجود حديد وكذلك في حالة وجود تركيز عالي من الحديد وقد وجدت باند عند وزن جزيئي 84 KDa في كل من الغشاء البلازمي كاملا وكذلك بعد تكسيره فقط في ظل عدم وجود الحديد.
- إضافة إلى انه تم تنقية راشح الخلية البكتيرية المنماه على بيئة كنج B للحصول على السيدروفور بواسطة عامود الكروماتوجرافي سيفاديكس G-25 ونتج عن ذلك 9 أجزاء تم اختبارهم مرة أخرى للكشف عن وجود السيدروفور باستخدام اختبار الكرومو أزورول S.
- كذلك تم حقن جهاز التحليل الكروماتوجرافي عالى الكثافة لمعرفة نوع السيدروفورز الموجودة في راشح الخلية البكتيرية سيدوموناس فلورسنس المنماه على بيئة كنج B السائلة وأسفرت النتائج عن وجود نوعين من السيدروفورز هما بيوفيردين ، بيوكيلين .

قام بتحكيم البحث

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