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Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*

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ABSTRACT

Sixty-three isolates belonging to the genus *Pseudomonas* were isolated from different environmental sources including; soil, water and clinical specimens. Twenty out of them were identified as *Pseudomonas aeruginosa* and individually screened for pyocyanin production. *P. aeruginosa* R_1, isolated from rice-cultivated soil and *P. aeruginosa* U_3 selected from clinical specimen (Urinary tract infection) were the highest pyocyanin producers; pyocyanin production reached 9.3 and 5.9 μg/ml, respectively on synthetic glucose supplemented nutrient medium (GSNB). The identification of both selected strains (*P. aeruginosa* R_1 and *P. aeruginosa* U_3) was confirmed by 16S rRNA, the similarity with other strains available in database was 97% (with *P. aeruginosa* FPVC 14) and 94% (with *P. aeruginosa* 13.A), respectively. *P. aeruginosa* R_1 and *P. aeruginosa* U_3 are accessed at gene bank with accession numbers KM924432 and KM603511, in the same order. Pyocyanin was extracted by standard methods, purified by column chromatography and characterized by UV-Vis absorption, mass spectrometry and nuclear magnetic resonance. The antimicrobial activity of purified pyocyanin against multi-drug resistant microbes was investigated; the efficiency of pyocyanin was more obvious in Gram +ve bacteria than Gram−ve bacteria and yeast. To reduce the cost of pyocyanin production, a new conventional medium based on cotton seed meal supplemented with peptone was designed. The pyocyanin production of both selected strains *P. aeruginosa* R_1 and *P. aeruginosa* U_3 using the new medium is increased by 30.1% and 17.2%, respectively in comparison with synthetic GSNB medium, while the cost of production process is reduced by 56.7%.

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1. Introduction

Genus *Pseudomonas* produces a variety of extra-cellular pigments of which phenazines comprise the most significant one. The most characteristic feature of *Pseudomonas aeruginosa* is the production of soluble pyocyanin pigment: a water soluble blue green phenazine compound. From the beginning, pyocyanin had been used as a reversible dye with a redox potential similar to that of menaquinone. Pyocyanin has various pharmacological effects on prokaryotic cells; its biological activity is related to similarity in the chemical structure to isoalloxazine, flavoproteins, flavin mononucleotide and flavin adenine dinucleotide compounds (Ohfuji et al., 2004). It is also used to control phytopathogens (Sudhakar, Karpagam, & Shiyama, 2013). In addition, the bioprocess and downstream processing of pyocyanin for aquaculture applications have been reported (Priyaja et al., 2014).

The phenazine-based pyocyanin pigment has a particular interest for its capability to generate reactive oxygen species (ROS). Tumor cells are susceptible to reactive oxygen species produced by pyocyanin since it interferes with topoisomerase I and II activities in eukaryotic cells (Hassani, Hasan, Al-Saadi, Ali, & Muhammad, 2012). Pyocyanin also has got application in biosensors as a redox compound for carrying out electron transfer between enzyme molecules and the electrode material. Therefore, the biosensors based on pyocyanin – were also expected to apply to different fields such as agricultural, medicine and environment (Priyaja, 2013).

Pyocyanin can be used as electron shuttle in microbial fuel cells by enabling bacterial electron transfer towards the microbial fuel cells (MFC) anode (Pham et al, 2008). It was observed the addition of pyocyanin to MFC-containing *Brevibacillus* sp. PTH1 doubled the rate of electron transfer (Rabaey, Boon, Hofte, & Verstraete, 2005). In addition, pyocyanin could conjugate to organic compounds and forming new complexes those are used in organic light emitting devices (OLED). These devices were gaining importance due to their low voltage requirements, wide color range, and light weight (Chen & Xiao-Chang, 2004).

Despite the various applications of pyocyanin, it remains a costly compound in the market since the cost of five micrograms of available pyocyanin in market (HPLC grade, purity > 98%) is 82 EUR (www.sigmaaldrich.com). The objectives of this study were: (1) to isolate different *P. aeruginosa* strains from various environmental sources; (2) purify and characterize the produced pyocyanin by standard techniques; (3) reduce the cost of pyocyanin production; (4) and study the functional activity of pyocyanin against multi-drug resistant microbes.

2. Material and methods

2.1. *Microorganisms and* Pseudomonas *spp.*

A total of one hundred clinical specimens were kindly donated by patients under clinical investigations in El-Mattaria Learning Hospital, El-Kasr Al-Ainy Hospital, National Institute for Kideny and Urinary tract, as well as private labs; in which sampling ethics are followed during sampling process. Sterile cotton swabs or sterile screw capped containers were used in sampling process according to the nature of the clinical specimen. Collected samples were cultured onto blood and MacConkey's agar media; those were incubated overnight at 37 °C to isolate the microbe causes infection. Non-lactose fermented colonies were selected and their oxidase ability was tested (Vandepitte et al., 2003).

Two water samples were collected from local water-drainage systems located in Dakahlia and Sharkia governorates. Sampling procedures as well as microbiological analysis were carried out according to standard methods for water and wastewater examination (APHA, 2005). Seven rhizosphere soils were collected at a depth of 30 cm from different governorates in Egypt (Assuit, Dakahlia and Sharkia) and kept in polyethylene bags at 4 °C until isolation process. Fifty grams of each soil sample was placed in 100 ml of sterile saline solution (0.9%) and vigorously shaken at 300 rpm for one hour. One milliliter of each soil suspension was added to 9 ml of Ampicillin Chloramphenicol Cycloheximide broth medium (ACC) and incubated at 37 °C for 24 h. Growing colonies were plated onto nutrient agar medium and pigmented colonies were selected and primarily identified by standard biochemical tests including; oxidase, catalase, gelatin liquefaction, citrate utilization as well as their capability to grow at 42 °C (Palleroni, 1992).

All Multi-drug resistant bacteria used in the present study (*Escherichia coli*, *Klebsiella* sp., *Shigella* sp., *Salmonella typhi* and *Staphylococcus aureus*, as well as *Candida albicans*) were kindly provided by El-Mattaria Learning Hospital.

2.2. *Molecular identification of* P. aeruginosa *strains*

DNA extraction, design of specific primers and sequencing were done using the protocol of GeneJet genomic DNA purification Kit (Thermo). Polymerase chain reaction was performed by Maxima Hot Star PCR Master Mix (Thermo). PCR reaction mixture consists of; 5 μl template DNA, 25 μl Maxima Hot Start PCR Master Mix (2×), 20 μM of each primer and 18 μl of nuclease-free water. The amplification conditions were as the following; one cycle represents initial amplification at 95 °C for 10 min followed by 35 cycles of denaturation (95 °C for 30 s), annealing (65 °C for 1 min) extension (72 °C for 90 s) and finally the process ends by final extension at 72 °C for 10 min. PCR mixture (4 μl) was loaded on 1% agarose gel to examine the PCR product against 1 Kb plus ladder (Thermo). PCR product was cleaned up using GeneJET™ PCR Purification Kit (Thermo) and finally sequenced by the aid of ABI 3730×l DNA sequencer (GATC Company). The sequences were compared with 16 S rRNA genes sequencing in Genebank using the blast function and the phylogenetically tree was drawn according to MEGA program version 6.

The 16S rRNA primers used in the present study were:

Forward: AGAGTTTGATCCTGGCTCAG
Reverse: GGTTACCTTGTTACGACTT

2.3. *Preparation of raw materials and wastes*

Corn steep liquor was prepared by soaking 500 g of well-washed fresh maize grains in one liter of distilled water for two days at −4 °C. The mixture was grounded by grinder and allowed to stand for another two days at −4 °C. Then, the mixture was filtered through four layers of cheese cloth and

stored at −20 °C until use (Obayori et al., 2010). Potato washing-water was collected from local restaurant, selling fried potato, and stored at −20 °C.

Cotton seed meal used in the present study was provided from El-Mahla Company for Soap and Oil located in Egypt. Grape seeds, pea pods and taro leaves were washed, sieved and dried in an oven at 60 °C overnight. They were crushed with grinder and kept in polyethylene bags at −4 °C until use. Olive wastes were purchased as dry powder from local markets in Sinai governorate and peat moss was purchased from Ministry of Agriculture in Egypt. These six raw materials and wastes were mixed with 1.5% sulfuric acid (1:5 W/V) and autoclaved at 121 °C for 2 h; their extracts were filtered to remove solid debris, and stored at −20 °C until use.

Vegetables frying oil and Motor oil were collected and hydrolyzed with water at 121 °C and 1.5 Psi for two hours to hydrolyze the waste oil to glycerol and fatty acid (Holliday, King, & List, 1997).

2.4. Pyocyanin pigment production

Different *P. aeruginosa* strains were cultured in 250 ml conical flasks containing 50 ml of glycerol supplemented nutrient broth medium (GSNB) and statically incubated at 37 °C (Rahman, Pasirayi, Auger, & Ali, 2009). Pyocyanin pigment production was estimated after four days.

For comparison, three different synthetic artificial media were used for *Pseudomonas aeruginosa* proliferation and pigment production. These media are King's A media (King, Ward, & Raney, 1954), mineral medium (Baron & Rowe, 1981) and GSNB.

To reduce the cost of pyocyanin production, the low-cost raw materials and wastes were aseptically added to basic mineral salt medium containing potassium sulfate (10 g/l), and magnesium chloride (3 g/l). Peptone, yeast extract and glycerol were individually added to cotton seed meal medium to enhance pyocyanin production according to their percentages in GSNB medium (control). In another experiment, the cotton seed meal medium was supplemented with different concentrations of peptone (2.5, 5, 7.5, 10 and 12.5 g/l), the pyocyanin production and *P. aeruginosa* growth were determined. The cost of pyocyanin production on new designed conventional medium was calculated using a Microsoft office project program (Microsoft® office project, 2003) and compared to that produced on GSNB.

2.5. Analytical analysis

At the end of incubation period, the biomass of *P. aeruginosa* was harvested by centrifugation, washed with distilled water three times and dried in an oven at 70 °C for 24 h. The growth yield was determined as dry weight per volume.

Pyocyanin was extracted from cell-free filtrate using ch1oroform according to procedure previously described (Saha, Thavasi, & Jayalakshmi, 2008). Pyocyanin was quantitatively assay based on measuring the absorbance of pyocyanin in the acidic form at 520 nm according to the following equation (Essar, Eberly, Hadero, & Crawford, 1990).

$$\text{Concentration of pyocyanin } (\mu g/ml) = O.D_{520} \times 17.072$$

For raw materials and wastes, the total carbohydrates were estimated by phenol-sulfuric acid method (Dawson, Elliot, & Jones, 1986) using glucose as standard whereas the total nitrogen content was determined by Kjeldahl procedure (Reddy, Palmer, Pierson, & Bothast, 1983).

For rhamnolipid production, cultures of *P. aeruginosa* were inoculated into a 100 ml flask containing 50 ml of liquid culture medium either GSNB or cotton seed meal medium and incubated at 150 rpm for 96 h. Afterward, rhamnolipid concentration was estimated by the phenol-sulfuric acid method and elucidated by a colorimetric assay at 490 nm. (Rikalovic, Gordana-Cvijovic, VrviĆ, & KaradŽiĆ, 2012).

The swarm plates were prepared using 0.5% Bacto agar, 0.5% peptone, 0.2% yeast extract and 1.0% glucose, per 100 ml distilled water. Ten microliters of each of tested strains was inoculated at the center of the agar surface. Inoculated plates were incubated for 16 h at 37 °C before visual observation (Krishnan, Yin, & Chan, 2012).

The antimicrobial activity of different concentrations of pure pyocyanin (20, 50 and 100 μg/ml) was studied against 18 h-old cultures of pathogenic microbes mentioned earlier by MIC technique on nutrient broth medium for bacteria and peptone dextrose broth medium for yeast (Turnidge, Ferraro, & Jorgensen, 2013).

2.6. Purification and characterization of pyocanin pigment

The crude pyocyanin pigment previously stored in sterile containers at 4 °C was re-dissolved in 5 ml of chloroform and absorbed onto small quantity of silica gel (mesh size 200–500). Silica gel absorbed crude pigment was loaded on column (30 cm length × 2 cm diameter) that had been equilibrated with 1% methanol in chloroform. Purified pyocyanin was eluted with 15% methanol in chloroform. The eluted fractions were examined by scanning UV-Vis spectrophotometer; fractions have the same λ-max were collected together and dried on a rotary evaporator at 37 °C. The purified pyocyanin was subjected to spectroscopic analysis. Ultraviolet and visible absorption spectra of purified pyocyanin dissolved in chloroform or 0.1 N HCl was recorded over a range of 200–700 nm. Mass spectrometric analysis of purified pyocyanin was performed on Shimadzu mass spectrometry GCMS-QP 1000 EX mass spectrometry at 70 eV, Micro Analytical center, faculty of science, Cairo University, Egypt. ^{1}H NMR spectra of purified pyocyanin was performed at 25 °C using a 300 MHz NMR instrument (Located at Micro Analytical center) using HPLC grade $CdCl_3$ as a solvent system. The typical acquisition parameters utilized were repetition time 2 s and line broadening frequency 0.5 Hz.

3. Results and discussion

3.1. Isolation of P. aeruginosa from different environmental sources and selection the highest pyocyanin producers

The genus *Pseudomonas* has received widespread attention due to its medical and phytopathogenic importance, as well as

Table 1 – Classification of bacterial isolates selected from different clinical specimens.

Source of clinical specimens	Number of characterized bacterial isolates				Number of *P. aeruginosa* strains[a]
	Hemolysis	Lactose fermentation	Growth at 42 °C	Grape-like odor	
Sputum	22	11	2	2	2
Urine	40	22	5	5	5
Wounds	38	16	4	4	4

[a] Expected *P. aeruginosa* strains.

its catabolic versatility (Rubilar, Diez, & Gianfreda, 2008 and Ananthakrishnan, Kumarasamy, Raja, & Malini, 2012). *P. aeruginosa* is an opportunistic pathogen of humans, belonging to the bacterial family Pseudomonadaceae which is widespread in the environment; in soil, fresh water, marine environment, most man-made environment etc. It has also been widely isolated from hospitals, clinical instruments, cosmetics and medical products (Franzetti & Scarpellini, 2007). Bacterial isolates selected from different clinical specimens were cultured on blood agar medium, isolates that showed positive hemolysis activity were chosen and re-cultured on MacConkey's agar medium. Bacterial isolates could not ferment lactose and characterized by grape-like odor derived from the production of 2-aminoacetophenone were selected and their oxidase activity were tested. Their capabilities to produce blue-green phenazine pigment and grow at 42 °C were also examined; the selected bacterial isolates are expected to be *P. aeruginosa*.

Out of forty bacterial isolates selected from various urine samples, only five strains are expected to be *P. aeruginosa*. As for the sputum specimens, twenty-two bacterial isolates were selected, however only two belong to species *P. aeruginosa*. Thirty-eight wound swabs have been taken from different patients and characterized-as previously described to identify the causing microbe; four isolates were identified as *P. aeruginosa* (Table 1). According to origin of infection, the results were calculated in percentages and showed that *P. aeruginosa* was the most common cause of urinary tract infection (12.5%), followed by burning wounds (10%) and finally sputum infection (9%). Another study showed that *P. aeruginosa* is the most common cause of burn infection in Egypt (66.7%), followed by urinary tract infection (26.7%) and finally sputum infections (16.7%) (Mansour, Eldaly, Jiman-Fatani, Mohamed, & Ibrahim, 2013).

Seven rhizosphere soil and two agricultural wastewater samples were collected from different governorates located in Egypt including Assuit, Dakahlia and Sharkia. These soil samples were adhering to the root of some crops (Corn, Rice and Wheat) and vegetables (Cucumber, Pepper and Potatoes). The isolation of *Pseudomonas* genus from rhizosphere soil and two agricultural wastewater samples are based on its high level of intrinsic and acquired multiply resistance against antibiotics. All samples were cultured on ACC medium containing ampicillin, chloramphenicol and cycloheximide antibiotics to exclude the most bacterial flora and to select a resistant one. Fifty-two bacterial isolates were chosen and cultured on kings A medium to enhance the pigment production. Nine bacterial isolates could produce blue-green pigment on kings A medium and developed yellow-green fluorescent pigment under UV light (365 nm). Three of which were isolated from rice-cultivated soil, two from agricultural wastewater, two from uncultivated soil, one from cucumber-cultivated soil and one from potatoes-cultivated soil (Table 2), they are belong to *Pseudomonas* genera. Al-Hinai et al. (2010) selected one hundred bacterial isolates from different soil samples; most of them developed pale green to dark green pigmentation on asparagine medium and released a sweet grape-like odor; these isolated bacteria were potentially Pseudomonas.

Twenty bacterial strains -expected to be *P. aeruginosa*-were selected and individually screened for pyocyanin production; eleven of them produce less than 1 μg/ml (Data not shown). Table 3 revealed that *P. aeruginosa* (R_1) isolated from rice-cultivated soil (Dakahlia governorate) followed by *P. aeruginosa* (U_3) isolated from clinical specimen (Urinary tract infection) were the highest pigment producers. The crude pyocyanin production titer was 6.3 and 5.9 μg/ml, respectively; whereas the pyocyanin production of the rest *P. aeruginosa*

Table 2 – Classification of bacterial isolates selected from different rhizosphere soil and agricultural wastewater samples.

Source of isolation	Number of pigmented bacterial isolates	Number of *P. aeruginosa* strains[a]
Corn-cultivated from Dakahlia governorate	5	–
Cucumber-cultivated from Dakahlia governorate	13	1
Peper-cultivated from Dakahlia governorate	2	–
Potatoes-cultivated from Sharkia governorate	4	1
Rice-cultivated from Dakahlia governorate	13	3
Uncultivated soil from Assuit governorate	5	2
Wheat-cultivated from Sharkia governorate	2	–
agricultural wastewater from Dakahlia governorate	7	2
agricultural wastewater from Sharkia governorate	1	–

[a] Expected *P. aeruginosa* strains.

Table 3 – Screening different *Pseudomonas aeruginosa* strains for pyocyanin production.

Symbols of *P. aeruginosa* strains[a]	Source of isolation	Pyocyanin production (μg/ml)
A_4	agricultural wastewater from Dakahlia governorate	4.2
M_1	Uncultivated soil from Assuit governorate	5.7
M_2	Uncultivated soil from Assuit governorate	4.5
P_2	Potatoes-cultivated from Sharkia governorate	3.5
R_1	Rice-cultivated from Dakahlia governorate	6.3
R_7	Rice-cultivated from Dakahlia governorate	1.8
S_{13}	Clinical specimen (sputum)	2.1
U_3	Clinical specimen (urine)	5.9
W_3	Clinical specimen (infected wound)	1.7

[a] Expected *P. aeruginosa* strains.

strains was ranged from 1.7 μg/ml to 5.7 μg/ml. The variation in pyocyanin production among different strains could be attributed to regulators. Liang, Duan, Sibley, Surette, and Duan (2011) identified a novel regulator of the quorum sensing system in *P. aeruginosa* and called it QteE; the over-expression of QteE in *P. aeruginosa* significantly reduced the accumulation of homoserine lactone signals and affected the pyocyanin production. The higher pigment producers (R_1 and U_3) were selected and their identification was confirmed by molecular tools, as well as their pigments were purified and characterized.

3.2. *Molecular identification of selected* Pseudomonas *strains*

16S rRNA sequence has long been used as a taxonomic gold standard in determining the phylogenies of bacterial species (Weose, 1987). The comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including the species and subspecies levels. The new sequence of *P. aeruginosa* R_1 was found to share 97% similarity with those of *P. aeruginosa* FPVC 14; this strain was accessed in gene bank for KM924432. The new sequence of *P. aeruginosa* U_3 was found to share 94% similarity with those of *P. aeruginosa* 13.A; this strain was accessed in gene bank for KM603511. Phylogenetic trees of both selected strains match with other strains available in public database (Fig. 1A and B). Interestingly, the percentage of similarity was found to be higher in *P. aeruginosa* R_1 than *P. aeruginosa* U_3, since the former strain produces higher pyocyanin than the latter. On contrary, Priyaja et al. (2014) found the 16S rRNA gene sequence analysis of five isolates of *Pseudomonas* (MCCB102, 103, 117, 118 and 119) possessed 99% nucleotide sequence identity to *P. aeruginosa* (99%) although they varied significantly in salinity-dependent pyocyanin production.

3.3. *Purification and characterization of pyocyanin pigment*

Like most low-molecular weight compounds, the crude pyocyanin of selected *P. aeruginosa* strains (R_1 and U_3) were purified using column chromatography. When pyocyanin was eluted with 15% methanol in chloroform; crude pigment fractions on the silica gel column appeared in yellow-green, light blue and dark blue bands. The blue fractions were collected, dried and stored at −20 °C for characterization. Following the same trend, Ohfuji et al. (2004) purified pyocyanin produced by *P. aeruginosa* using silica gel column equilibrated with 1% methanol in chloroform. Pyocyanin was fractionated into yellow, red, light blue and dark blue bands, a yellow-green band tightly bound to stationary phase even after elution by 15% methanol in chloroform; whereas blue band easily eluted as pure pyocyanin.

The physicochemical properties of the pure pyocyanin were investigated; results revealed that the pigment easily dissolved in chloroform, HCl, and hot water not in cold water. Several solvents including chloroform, toluene, n-heptane, petroleum ether, hexane, benzene, diethyl ether and methylene chloride were examined to completely achieve pyocyanin extraction. It was found that pyocyanin was successfully extracted from the fermentation broth medium using benzene, chloroform and methylene chloride. However, chloroform and methylene chloride were more efficient than benzene.

The absorbance spectrum of pyocyanin was monitored from 300 to 700 nm using UV-Vis spectrophotometer. Pyocyanin dissolved in methanol exhibited absorption maxima at 316, 367 and 700 nm; whereas pyocyanin dissolved in 0.2 N HCl exhibited the maxima at 300, 388 and 518 nm (Fig. 2A and B). These results were in agreement with previous findings. Ohfuji et al. (2004) studied the UV-Vis spectrum of pyocyanin and found its characteristics peaks at 201.0, 238.0, 318.5, 710.5 and 886.5 nm when dissolved in methanol whereas it exhibited a maxima at 204.0, 242.5, 277.0, 387.5 and 521.5 nm when dissolved in 0.2 M HCl.

The structural elucidation of the pigment was determined using mass spectrometry and nuclear magnetic resonance. Fig. 3 illustrated the mass spectrum analysis of pyocyanin; the purified compound demonstrated a protonated molecular ion at *m/z* 211 whereas the calculated one is 211.09 for $C_{13}H_{11}N_2O$. The purified pyocyanin was dissolved in $CdCl_3$ and injected into ^1H NMR spectroscopy for conformation. NMR spectrum shows two characteristic peaks from δ 3.3–4.3 ppm, they are indicative for the methyl group linked to nitrogen molecule whereas six condensed peaks that appeared at δ 7–9 ppm represented aromatic ring region (Fig. 4). These results confirm the purity of produced pyocyanin and they are in accordance with the previous reports (Karpagam, Sudhakar, & Lakshmipathy, 2013 and Priyaja, 2013).

The functional activity of purified pyocyanin (Needle-like crystal) at different concentrations (20, 25, 30, 35, 40, 45, 50 μg/ml) was monitored against Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*E. coli*, *Klebsiella* sp., *S. typhi*, *Shigella* sp.) and yeast (*C. albicans*). Results shown in Fig. 5 indicate that pyocyanin has a high antimicrobial activity against all tested

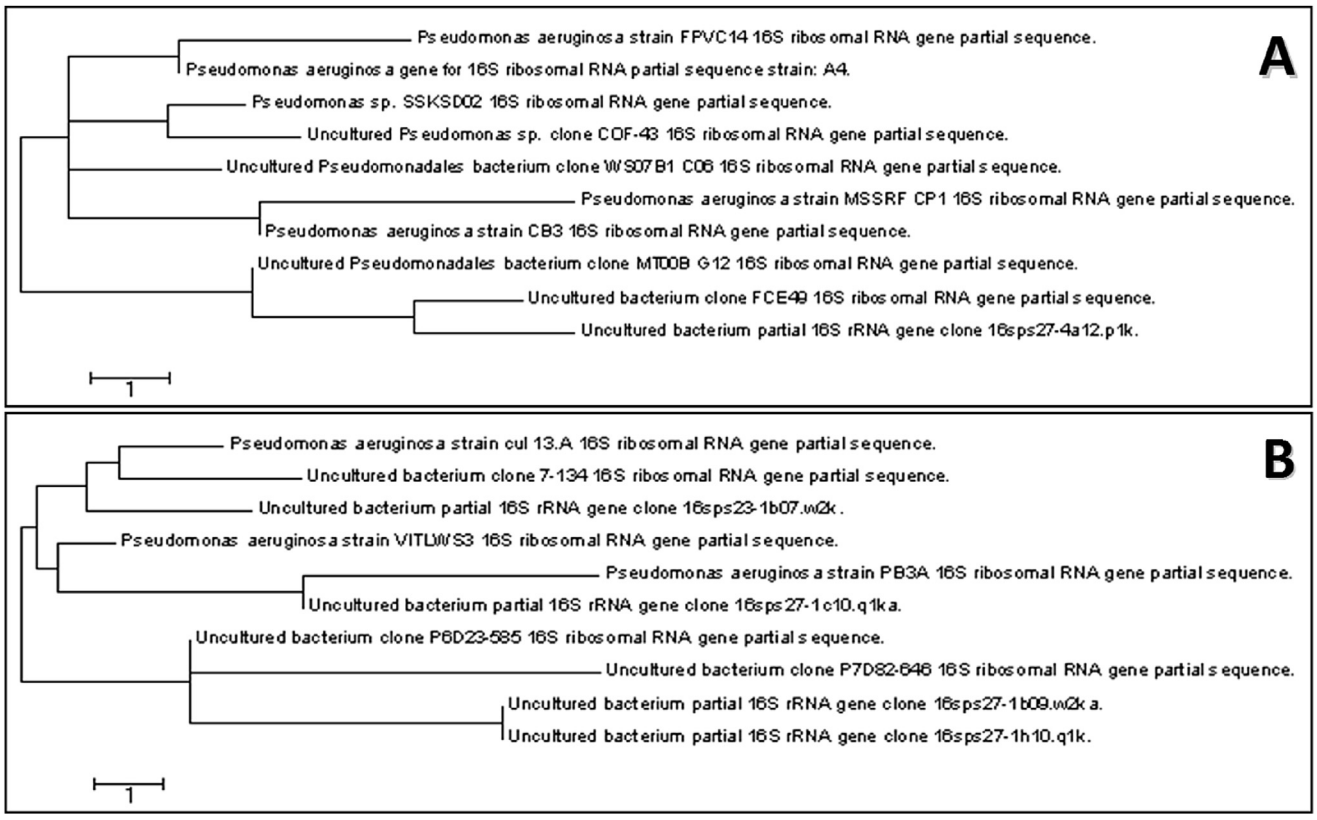

Fig. 1 – The BLAST database of National Center for Biotechnology Information (NCBI) was used to compare resolved sequence of the selected *P. aeruginosa* strains with known 16S rRNA sequences. A: *P. aeruginosa* R_1 B: P. *aeruginosa* U_3.

pathogenic microbial strains and acts as broad spectrum antibiotic. The lowest MIC of purified pyocyanin (20 μg/ml) was exhibited by *S. aureus*; whereas the highest MIC (50 μg/ml) was recorded by *E. coli*. i.e the highest antimicrobial activity of pyocyanin was observed against G+ve bacteria (*S. aureus*) comparing to other tested strains (either G-ve bacteria or yeast). By increasing pyocyanin concentration from 50 μg/ml to 100 μg/ml, its antimicrobial activity is enhanced by nearly 40% for all tested strains (Data not shown), thus the activity of pyocyanin as antibiotic is concentration dependent. In the same trend, El-Shouny, Al-Baidani, and Hamza (2011) found that the growth of all tested G+ve bacteria and Candida spp. were completely inhibited by pyocyanin; whereas G−ve bacteria, including *S. typhi* and *Pseudomonas mirabilis*, were intermediately affected and *K. pneumonia* was resistant to pyocyanin.

Pyocyanin, a water soluble bio-active compound, produced by *P. aeruginosa*, has the capacity to arrest the electron transport chain of the fungi and exhibits antifungal activity (Wilson et al., 1987). Variation in the lipid content of cell wall of Gram-positive and Gram-negative bacteria may be responsible for the variation in the sensitivity of pyocyanin antibiotic.

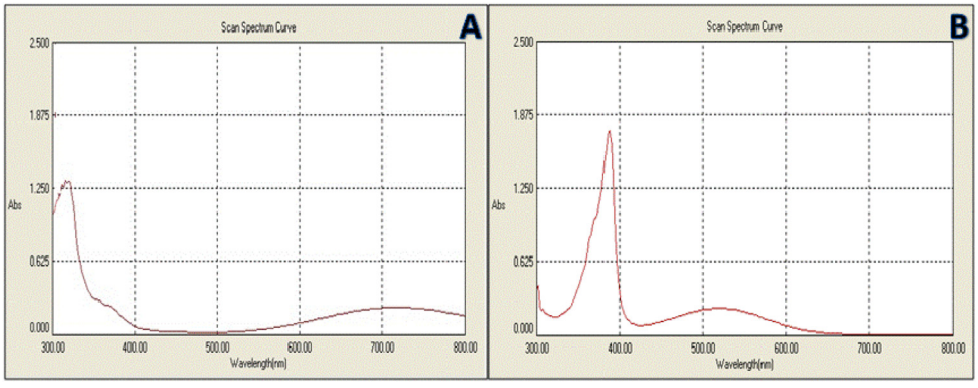

Fig. 2 – UV-Vis absorption spectrum of purified pyocyanin pigment. A: Pyocyanin dissolved in methanol (Neutral alkaline blue form). B: Pyocyanin dissolved in 0.2 M HCl (Acidic red form).

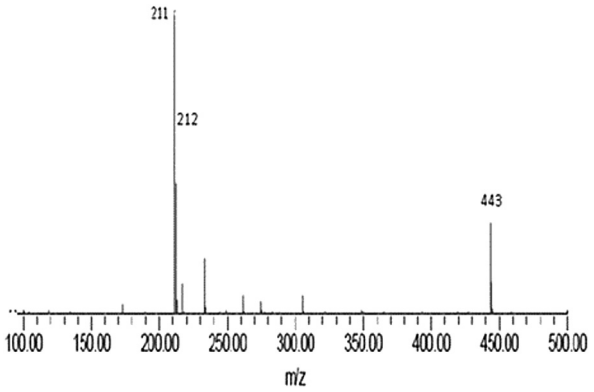

Fig. 3 – Mass spectrometry analysis of purified pyocyanin pigment.

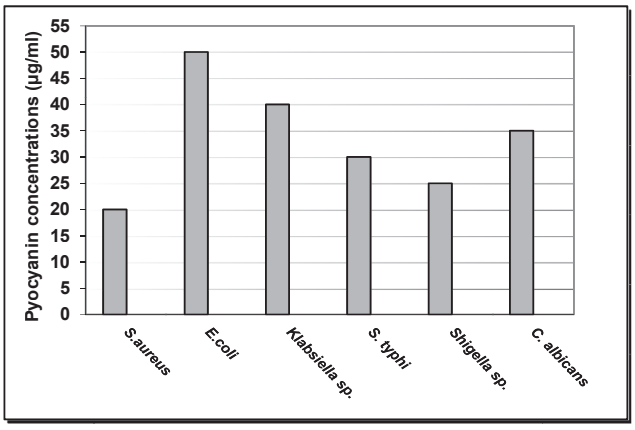

Fig. 5 – MIC of pyocyanin against different multi-drug resistant microbial strains.

Pyocyanin increases intracellular oxidant stress and exhibits a redox cycle under aerobic condition. This leads to reactive oxygen species (ROS) production such as superoxide and hydrogen peroxide; these ROS compounds are capable of inhibiting microbial growth (Denning et al., 1998 and Das & Manefield, 2012). Another study based on denaturant gradient gel electrophoresis analysis revealed that the resistance or sensitivity to pyocyanin is related to level of catalase and superoxide dismutase enzymes (Norman, Moeller, McDonald, & Morris, 2004).

3.4. *Reducing the cost of pyocyanin production*

Many synthetic media are previously recommended for *P. aeruginosa* proliferation and pyocyanin production such as; king's A medium, glycerol supplemented nutrient broth (GSNB) and mineral medium (Kavitha et al., 2005). These media were prepared with standard methods. The growth of selected *P. aeruginosa* strains and pyocyanin pigment production was determined after 4 days. Results revealed that the three tested media enhanced the growth of both *P. aeruginosa* strains (R_1 and U_3) regard less to type of aeration condition (shaking or static) but the supporting order is different (Table 4). The highest increase in the growth yield for *P. aeruginosa* (R_1) strain is achieved by mineral medium; then king's A medium and finally GSNB medium. On the other hand, king's A medium supported the highest increase in the growth yield for *P. aeruginosa* strain (U_3). Under shaking condition, king's A medium resulted in the highest growth yield (88.5 and 84.9 mg/50 ml) for both *P. aeruginosa* strains (R_1 and U_3, respectively.

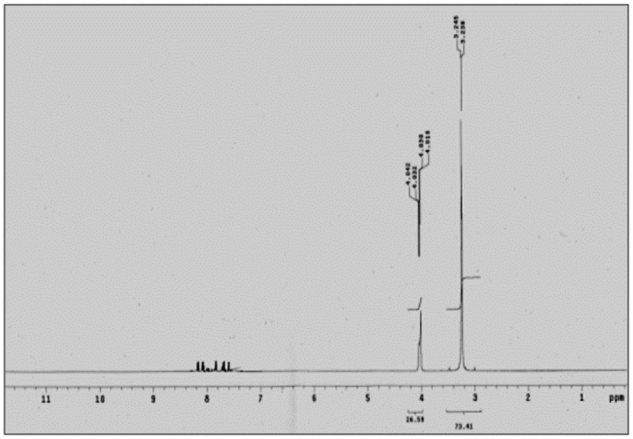

Fig. 4 – ^{1}H-NMR spectrum of purified pyocyanin pigment.

Data shown in Table 4 also demonstrated that king's A and GSNB media manage to support the pyocyanin production for both *P. aeruginosa* strains (R_1 and U_3) either under shaking or static condition. The enhancement effect of king's A medium on pyocyanin production was previously mentioned (Vinckx, Wei, Matthijs, & Cornelis, 2010). They induced *P. aeruginosa* mutant that couldn't grow in LB medium and lost its pigmentation ability; whereas it could grow as the wild type and produce pyocyanin on king's A medium. This observation could be explained by the protective action of the redox-active pyocyanin, and was also confirmed by the restoration of the capacity to grow in LB medium upon addition of pure pyocyanin.

Results also indicate that GSNB medium is generally the most favorable medium for pyocyanin production. Under static condition, GSNB nearly doubled the pyocyanin production for *P. aeruginosa* strain (U_3) whereas it increased the pyocyanin production for *P. aeruginosa* strain (R_1) by nearly three folds. Similarly, Rahman, Pasirayi, Auger, and Ali (2010) developed a microfluidic system that handles a small population of cells of a model microorganism, *P. aeruginosa* DS10-129. Under the conditions of the microbioreactor, the organism produced extracellular secondary metabolites when grown on modified GSNB medium.

In the present study, stationary phase favored pyocyanin compared to the agitation condition. Price-Whelan, Dietrich, and Newman (2007) studied the production of pyocyanin pigment in stationary-phase and investigated the physiological consequences of pyocyanin reduction by assaying intracellular concentrations of NADH and NAD^+ in a wild-type strain of *P. aeruginosa* and a mutant defective in phenazine production. They found that mutant accumulated more NADH in stationary phase than the wild type; this increase is

Table 4 – Proliferation of the selected *Pseudomonas aeruginosa* strains and their pyocyanin production on the most recommended synthetic media.

Selected *Pseudomonas aeruginosa* strains	Type of fermentation medium	State of incubation	Growth yield (mg/50 ml)	Pyocyanin production (μg/ml)
P. aeruginosa R_1	King's A	Static	46.7	6.3
		Shaking	88.5	4.5
	Mineral medium	Static	81.1	ND[a]
		Shaking	75.5	ND
	GSNB[b]	Static	33.1	9.3
		Shaking	45.9	3.2
P. aeruginosa U_3	King's A	Static	39.7	5.8
		Shaking	84.9	2.4
	Mineral medium	Static	28.2	ND
		Shaking	26.6	ND
	GSNB	Static	27.0	5.9
		Shaking	16.1	3.7

[a] ND = Not Detected.
[b] GSNB = Glycerol supplemented mineral medium.

correlated with a decrease in oxygen availability and phenazine production.

Since microorganisms have superiority to synthesize value-added products including pigments using a variety of raw carbohydrates under varying cultivation processes (Singh & Kumar, 2007). A new conventional medium based on raw materials, or wastes available in Egypt, have been designed for reducing the cost of pyocyanin production. Most selected raw materials or wastes (Agricultural or oil origin) were hydrolyzed by acid in order to decompose them into simple materials that can be metabolized by bacteria. The total carbohydrate content of the tested raw materials or wastes increased in the following order: motor oil, vegetative frying oil, taro leaves, cotton seed meal, peat moss, olive waste, potato-washing water, corn steep liquor, pea pods and finally grape seed.

The synthetic medium used, GSNB resulted in the highest pyocyanin yield was selected for comparison purpose in upcoming experiments. The C:N ratio of selected materials under investigation was adjusted to be equal to C:N ratio of standard medium (GSNB). Carbon-to-nutrient ratio determines whether nutrients are immobilized in the microbial biomass at excess carbon availability or mineralized to become available for uptake at excess nitrogen availability (Wang, Law, & Pak, 2010). The C:N ratios of all materials decreased in the following order: potato washing water, peat moss, corn steep liquor, olive waste, grape seed, potato frying oil, pea pod, motor oil, taro leaves and finally cotton seed meal.

The low-cost conventional media based on raw materials or wastes were individually screened to enhance the growth of selected *P. aeruginosa* strains and pyocyanin production. The data evaluated in Table 5 showed the incapability of all tested wastes to support *P. aeruginosa* strains growth in the same manner achieved by control synthetic medium. *P. aeruginosa* (R_1) strain could grow in all tested wastes; whereas *P. aeruginosa* (U_3) strain couldn't use peat moss and motor oil-based media for energy generation and cell building. Cotton

Table 5 – Screening different low-cost conventional media for supporting *Pseudomonas aeruginosa* proliferation and pyocyanin production in comparison with synthetic mediun (GSNB).

Low-cost conventional media	Selected *Pseudomonas aeruginosa* strains			
	P. aeruginosa R_1		*P. aeruginosa* U_3	
	Growth yield (mg/50 ml)	Pyocyanin production (μg/ml)	Growth yield (mg/50 ml)	Pyocyanin production (μg/ml)
Control synthetic medium (GSNB)	29.2	9.2	23.8	5.6
Corn steep liquor	12.2	–	12.0	–
Cotton seed meal	18.3	4.0	15.8	2.2
Grape Seed	11.1	3.1	11.9	1.4
Motor oil	6.0	–	–	–
Olives waste	17.0	1.3	14.0	–
Pea pods	19.0	1.6	14.6	–
Peat moss	8.1	–	–	–
Potato-washing water	17.0	–	12.3	–
Taro leaves	13.2	2.5	13.1	–
Vegetables frying oil	6.9	–	3.9	–

seed meal-based medium supported a higher growth yield 18.3 and 15.8 mg/50 ml for *P. aeruginosa* strains; R_1 and U_3, respectively in comparison to other wastes and raw materials. This means the growth yield of *P. aeruginosa* strains R_1 and U_3 decreased by 40 and 20%, respectively in comparison to GSNB synthetic medium. On the other hand, the lowest growth yield of *P. aeruginosa* (R_1) strain was supported by motor oil-based medium. In all studied media; the production of pyocyanin pigment was lower than that achieved by the synthetic control medium. Table 5 confirmed that cotton seed meal-based medium achieved the highest pyocyanin production in comparison with all tested raw materials or wastes, since it supported 4.0 and 2.2 μg/ml of pyocyanin for *P. aeruginosa* strains R_1 and U_3, respectively. Afterwards, the grape seed-based medium could accomplish 3.1 and 1.4 μg/ml of pyocyanin in the same order. Taro leaves, pea pod and olives wastes-based media were able to support pyocyanin production by *P. aeruginosa* (R_1) strain 2.5, 1.6 and 1.3 μg/ml respectively, but they failed to support pyocyanin production by *P. aeruginosa* strain U_3.

Cotton seed meal is the only promising waste that enhanced *P. aeruginosa* proliferation and pyocyanin production. Cotton is grown primarily for its boll that produces the lint or fiber that is the raw material for numerous textile products. The cotton seed is squeezed to produce cotton seed oil, cotton seed meal (cake) and hulls. One ton of seed yields about 200 kg of oil, 500 kg of cotton seed meal and 300 kg of hulls according to Egyptian Ministry of Agriculture report in 2012/2013. The cotton seed meals are important protein concentrated for livestock, fish farmers and proliferation of microorganisms (Soltan, Fath El-Bab, & Saudy, 2011).

Many authors reported the use of conventional cheap carbon sources for *Pseudomonas* proliferation and pyocyanin production. Onbasli and Aslim (2008) reported that molasses can be used as substrate for *Pseudomonas* proliferation, exopolysaccaride biosynthesis and pyocyanin production. Onbasli, Aslim, and Yuvalicelik (2011) tested twenty strains of *Pseudomonas* spp. for their secondary metabolite (pyocyanin and rhamnolipid) production, as well as their ability to degrade some organic pollutants (1% benzene and 2% gasoline). All tested strains exhibited microbial growth, pigment and rhamnolipid production in presence of hazardous organic pollutants as a sole carbon source; the pyocyanin production ranges between 0.02 and 1.30 μg/ml. Low cost organic nitrogen sources such as: pea pods, soy-okra, taro leaves and flours like black gram, soya meal, green gram, moth and lentils, as well as corn steep liquor were assayed for *P. aeruginosa* strains proliferation and valuable compounds production, pea pods and soy-okra were found to be the appreciate choice suitable to the highest production (Patel & Desai, 1997 and Panesar, Panesar, & Bera, 2011).

The synthesis of pyocyanin is affected by carbon and nitrogen sources present in the fermentation media; it was observed that pyocyanin couldn't be produced in the organic medium prepared from natural materials such as: bouillon, malt extract and different enzymatic preparations of commercial peptone. In addition, high phosphate concentrations have an inhibitory effect on pyocyanin production (Ramalho, Cunha, Teixeira, & Gibbs, 2002). Enriching the low-cost conventional medium based on cotton seed meal (C:N~3:1) with complex nutrients, such as glycerol, peptone and yeast extract, was the aim of the following experiment. Table 6 represents pyocyanin production using cotton seed meal based medium -supplemented with peptone-enhanced the growth yield to 21.0 and 18.3 mg/50 ml i.e. growth yield was enhanced by nearly 14 and 20% for both *P. aeruginosa* R_1 and U_3 strains, respectively. On the other hand, a clear decrease in the growth yield was observed when a conventional low-cost medium was supplemented with yeast extract and glycerol. Table 6 also showed that cotton seed meal medium supplemented with peptone (5%) resulted in the highest pyocyanin production for both *P. aeruginosa* strains in comparison with synthetic control medium. The percentages of increase are 30 and 16 for *P. aeruginosa* (R_1) and *P. aeruginosa* (U_3) strains, respectively. Glycerol inhibited pyocyanin production for both *P. aeruginosa* strains whereas supplementation of cotton seed meal-based medium with yeast extract had a positive effect on pyocyanin production; this enhancement was more obvious in *P. aeruginosa* (R_1) than in *P. aeruginosa* (U_3).

Peptone is an enzymatic digest of animal protein and contains only a negligible quantity of proteases and more complex constituents; the nutritive value of peptone is largely dependent on the amino acid content. Nutrients, such as amino acids, can stimulate the production of secondary metabolites, either by increasing the amount of limiting precursors or by inducing a biosynthetic enzyme or both (Demain, 1998). Peptone is an essential material not only for bacterial growth but also for pyocyanin formation as a source of amino acid or the presence of substances promoting pigmentation. Lundgren et al. (2013) reported that some *P. aeruginosa* strains were able to produce pyocyanin when grown in media containing amino acid, especially alanine.

The effect of different concentrations of peptone (2.5, 5, 7.5, 10 and 12.5 g/l) on the proliferation of *P. aeruginosa* strains and pyocyanin production using cotton seed meal-based medium was investigated. Fig. 6 shows that increasing peptone concentrations in cotton seed meal-based medium has a positive effect on the proliferation of both tested strains. Elevating the peptone concentration from 2.5 to 12.5 g/l, increased the growth yield of *P. aeruginosa* R_1 by nearly 5–84 % and by 10–83% for *P. aeruginosa* U_3 in comparison with medium lacking peptone (Table 5). Fig. 6 also illustrated that increasing peptone concentration from 2.5 g/l to 5 g/l enhanced pyocyanin yield from 11.4 μg/ml to 12.0 μg/ml for *P. aeruginosa* R_1, and then decreased. Similarly, pyocyanin yield increased from 4.9 μg/ml to 9.3 μg/ml by elevating peptone concentration from 2.5 g/l to 7.0 g/l for *P. aeruginosa* U_3. Turner and Messenger (1986) found that the addition of ammonium sulphate to fermentation medium dramatically reduced phenazine production without affecting growth of different *Pseudomonas* spp. This concept is true for inorganic nitrogen source, unlike the organic one, since bacterial strains can also utilize them as additional carbon sources. In the same trend, Van Rij, Wesselink, Chin, Bloemberg, and Lugtenberg (2004) reported that the addition of different concentrations of aspartic acid, glutamine, phenyl alanine and tyrosine to the fermentation medium could improve phenazine production by *Pseudomonas chlororaphis* PCL 1391.

The ability of cotton seed meal-based medium-supplemented with peptone- to support secondary metabolites,

Table 6 – Enhancing *P. aeruginosa* proliferation and pyocyanin pigment production by enriching the low-cost conventional medium based on cotton seed meal with complex nutrients.

Low-cost conventional media	Selected *Pseudomonas aeruginosa* strains			
	P. aeruginosa R_1		*P. aeruginosa* U_3	
	Growth yield (mg/50 ml)	Pyocyanin production (μg/ml)	Growth yield (mg/50 ml)	Pyocyanin production (μg/ml)
Control synthetic medium (GSNB)	29.4	9.3	22.0	5.8
Cotton seed meal	18.4	4.0	15.3	2.4
CS supplemented with peptone	21.0	12.1	18.3	6.8
CS supplemented with yeast extract	11.5	5.3	10.5	4.0
CS supplemented with glycerol	15.0	–	13.9	–

other than pyocyanin or maintaining the physiological characteristics of producer organism (*P. aeruginosa*) was also investigated. Many microorganisms are able to synthesize different types of bio surfactants when grown on various carbon sources. These bio surfactants include low molecular weight glycolipids, lipopeptides and high molecular weight lipid-containing polymers such as: lipoproteins, lipo-polysaccharide-protein complexes and polysaccharide-protein-fatty acid complexes. *P. aeruginosa* is capable of using different carbon sources including glycerol, vegetable oils, hydro-carbons and others to produce rhamnolipids (Sidal & Yilmaz, 2012). Results proved that the recommended cotton seed meal-based medium-supplemented with peptone-succeeded to produce rhamnolipid biosurfactant, but the production was less than the control synthetic medium (GSNB). Rhamnolipid production decreased from 394 to 131 mg/l for *P. aeruginosa* R_1 strain, and from 379 to 171 mg/l for *P. aeruginosa* U_3 strain. In the same trend, Rashedi, Mazaheri Assadi, Bonakdarpour, and Jamshidi (2005) used fermentation media containing ethanol, glucose, vegetable oils and molasses individually as sole carbon source to produce rhamnolipid; in addition to monitoring the growth yield of *P. aeruginosa*. The rhamnolipid production and the growth yield increased with increasing the concentration of molasses; whereas the maximum production occurred when 7% (v/v) of molasses were used. Further increase in the concentration of molasses did not have a significant effect on rhamnolipid production.

Bacterial motility plays a key role in the surface of bacteria colonization and the subsequent formation of resistant communities of bacteria is called biofilms. *P. aeruginosa* is capable to do two well-known types of motilities; swimming motility

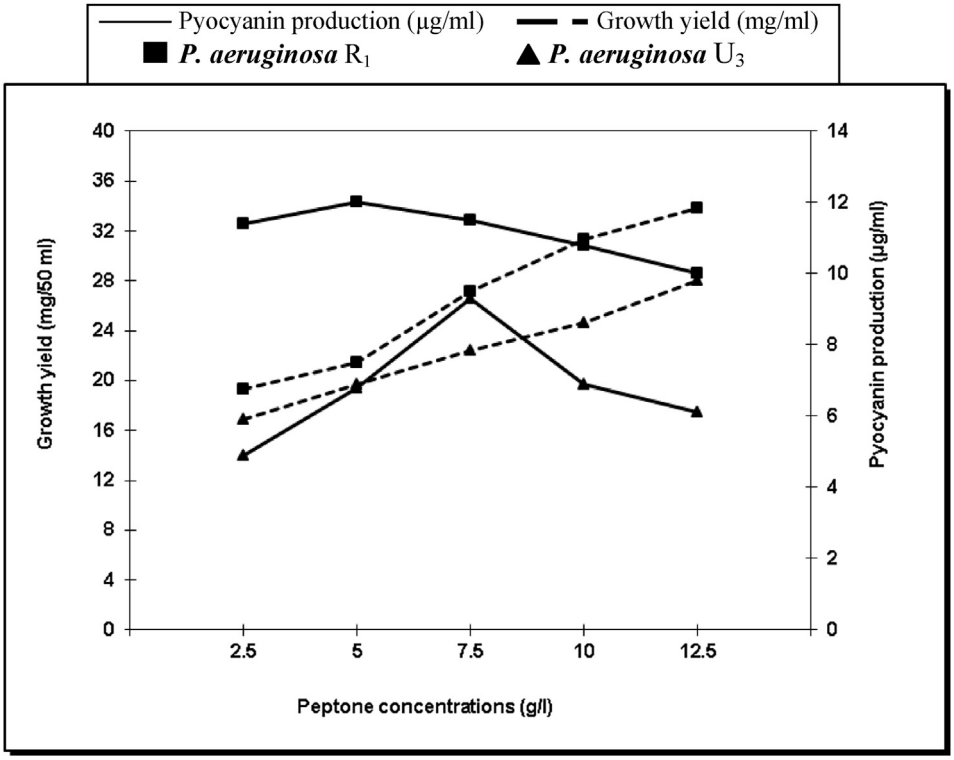

Fig. 6 – Effect of increasing concentrations of peptone supplemented cotton seed meal-based medium on the proliferation of *Pseudomonas aeruginosa* strains and pyocyanin production.

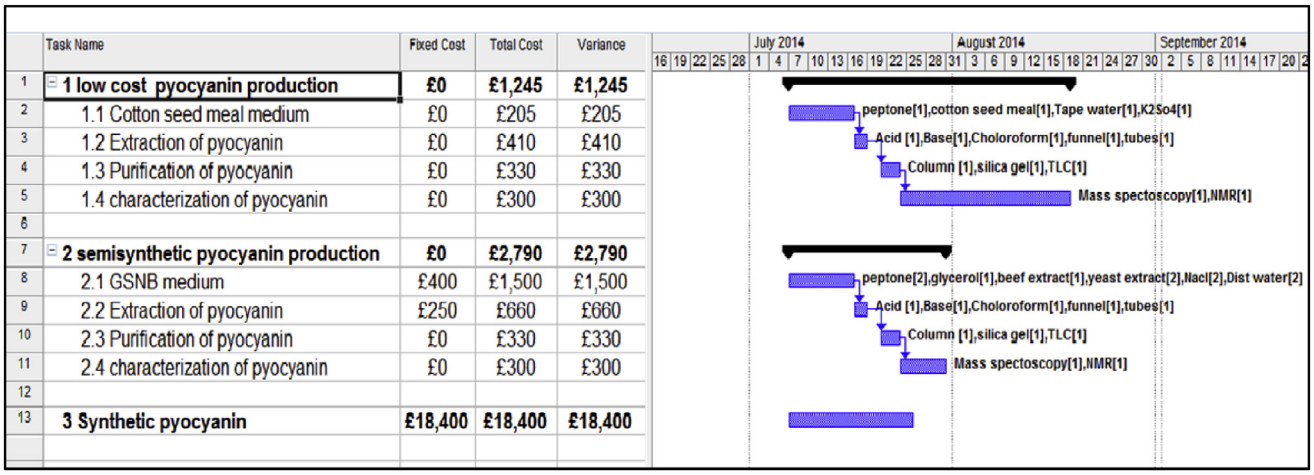

Fig. 7 – Comparison between the cost of individual resources of promising cotton seed medium and synthetic standard GSNB medium for pyocyanin production.

based on the flagellum and twitching motility relying on type IV pili. A third less well understood motility behavior (known as swarming) is dependent on the combination of the polar flagellum, type IV pili and rhamnolipid production (Caiazza, Shanks, & O'Toole, 2005). When *P. aeruginosa* cells were grown on the center of the agar surface, the regular colony was formed and followed by armed-like shape; appeared due to activation of flagella production. Both *P. aeruginosa* strains were swarmed on the control synthetic medium, as well as cotton seed meal-based medium. However; the swarming motility was more obvious in control medium than low-cost conventional medium. The chemotaxis-regulated motion mediated by the polar flagellum, twitching and swarming may be affected by the components of cotton seed meal-based medium. Vinckx et al. (2010) deduced an inverse relation between pyocyanin production and swarming motility. They selected pyocyanin hyper producer mutant with a very weak swarming motility and no rhamnolipid production capability.

From the industrial point of view, the medium that suppress the swarming motility and rhamnolipid production is the best choice for pyocyanin production. The most interesting result of the present study, the capability of cotton seed meal-based medium to support a higher pyocyanin yield, in comparison with classic described medium GSNB by 40%. By assessing the cost of pyocyanin production, it is found that the purified pyocyanin extracted from cotton seed meal-based medium-supplemented with peptone-shows a great reduction in the cost compared to the synthetic control medium (GSNB) in addition to chemical synthesized pyocyanin available in market (Sigma Aldrich Life Sciences) (Fig. 7). The cost of purified gram of pyocyanin produced using low-cost recommended conventional medium based on cotton seed meal is not exceeding 1300 LE, whereas the purified pyocyanin formed using the synthetic control medium costs approximately 3000 LE, while the cost of commercially available pyocyanin (1 g) is 18400 LE. These results revealed how the biological production of pyocyanin is critically on demand using the promising cotton seed meal-based medium.

In conclusion, the present study recommends the biological synthesis of pyocyanin using a new designed medium based on cotton seed meal and using the produced pyocyanin in unlimited industrial applications.

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