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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