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Production of antimicrobial blue green pigment Pyocyanin by marine *Pseudomonas aeruginosa*

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ABSTRACT

This study focused on isolation and molecular identification of marine bacteria which producing bioactive pigment. For that, 10 pigmented bacteria were isolated from 3 water samples collected from Mediterranean Sea, Alex., Egypt. One isolate was had efficient value to produce blue green pigment. This one was identified using molecular biology techniques and recorded in GeneBank under title of *Pseudomonas aeruginosa* strain OSh1 with accession number KT032066. The produced pigment was extracted, purified and characterized as pyocyanin. The pathway of pyocyanin production contains many sequential genes, the important one is phzS. This gene was detected genetically in this study using specific primers and gave PCR product with 448 bp. To confirm that, the inducible proteins for pyocyanin synthesis genes were documented using SDS-PAGE. The results represent found 2 proteins with 36 and 43 kDa for phzM and phzS, respectively. The biological activity of the produced pyocyanin at different concentrations was investigated as antimicrobial agent. The antibacterial activity was ranged between 31- 42 mm of inhibition zone. Whereas, the inhibition zone for antifungal activity was ranged between 34- 35 mm. The results indicate the produced pyocyanin had high activity against multi-drug resistant microbes and we can use it as marine antibiotic.

Keywords: *Pyocyanin; Biosynthesis genes; Protein pattern; Marine antimicrobial agent.*

1. INTRODUCTION

Pseudomonas spp. is bacteria with specific characteristics; Gram-negative, rod-shaped and producing metabolites pigment [1]. *Pseudomonas* produces many extra-cellular bioactive pigments as phenazines. It is considered one of the largest groups for production of redox-active and nitrogen-containing heterocyclic compounds. These compounds have intensive studying because broad spectrum of their biological activity [2]. Pyocyanin (5-N-methyl-1-hydroxy- phenazine) is considered the most studied phenazine compound. It is a water-soluble blue-green pigment. The pyocyanin pigment is produced by nearly 95 % of *P. aeruginosa* isolates and it has different biological activities. The bioactivity is similar in some chemical structures as isoalloxazine, flavoproteins, flavin mononucleotide and flavin adenine dinucleotide compounds [3]. It is also used to control phytopathogens [4]. Phenazine as heterocyclic compound is produced naturally in form of deep red 5-methly-7-amino-1-carboxyphenazium betaine, following by converted to lemon yellow phenazine-1- carboxylic acid (PCA). In the final, it is converted to blue pyocyanin pigment [5]. The pyocyanin pigment has antitumor bioactivity because it has capability to generate reactive oxygen species [6]. As well as, it is used as biosensor for carrying out electron transfer between the electrode material and enzyme molecules based on its redox activity. Therefore, the biosensors based on pyocyanin can be applied to different fields such as agricultural, medicine and environment [7]. The pyocyanin pigment is composed of 2 molecules of N- methyl-1-hydroxyphenazine and it shows crystals in pure form [8]. In genetically synthesis of pyocyanin, seven genes have been identified. These genes are *phz C, D, E, F, G, M* and *S* and located in all *Pseudomonas* spp. [9]. However, *PhzM* and *phzS* are the main genes responsible for pyocyanin production. Two steps are recommended in pyocyanin synthesis from PCA. Firstly, it is catalyzed by *PhzM* enzyme and the PCA is converted to 5-methylphenazine-1-carboxylic acid betaine. In the second one, the pyocyanin is produced from hydroxylation and decarboxylation of 5-methylphenazine-1-carboxylic acid betaine by *PhzS* enzyme [10].

This study aims to produce pyocyanin using Egyptian *P. aeruginosa* strain OSh1 isolated from marine environment and molecular characterization of pyocyanin biosynthesis modified gene using PCR and SDS-PAGE. Also, we evaluate the antimicrobial activity of purified pyocyanin against pathogenic microorganisms for introducing to use it as marine drug.

2. MATERIALS AND METHODS

Isolation and Primary identification of marine *Pseudomonas* isolate. Three water samples were obtained from Mediterranean Sea, Alexandria governorate, Egypt, using sterile blue cap glass bottles (500 ml) and kept in ice box until transferred to the lab [11]. The isolation process was carried out in triplicate using the pour plate method. Ten-fold serial dilution of each sample was

prepared while 100µL of each dilution was spread onto sterilized marine agar plates and incubated at 28°C for 72h [12]. All the blue green pigmented colonies were isolated on marine agar plates and purified. The pure isolates were confirmed on cetrimide medium. Then the pure colonies were preserved on cetrimide agar slants at 4°C [13]. The primary identification of isolates was done based on procedures detailed in Bergey's Manual of Systematic Bacteriology [14].

Molecular identification of *Pseudomonas* isolate OSh1.

The total genomic DNA was isolated from the *Pseudomonas* isolate OSh1 by enzymatic lyses as methods detailed by Barakat *et al.* [15], and then purified [16]. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was developed using extracted DNA in the presence of reverse (16DG74) and forward (16RW01) primers which previously used by Kheiralla *et al.* [17]. The final 50 µl reaction mixture containing 1× PCR buffer (NEB, England), 1 unit Taq DNA polymerase (NEB, England), 1 pmol of 2 mM $MgSO_4$, 1 nmol of dNTPs, 0.25 pmol of forward and reverse primers and 10 µl template DNA was done. The PCR amplification was started by initial denaturation of DNA at 95°C for 5 min, followed by 34 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 45 sec and the mixture was stored for 10 min at 72 °C for complete extension followed by kept at 4°C until purified by QIAquick Gel Extraction Kit (QIAGEN, USA) [18]. The identification was completed by comparing the contiguous 16S rDNA sequence with the data available in GenBank databases using the BLAST program (National Centre for Biotechnology Information). The strain sequence was recorded in GenBank and obtained the accession number [19].

Production, extraction, purification and quantification of pyocyanin.

Pyocyanin was produced by king's medium as detailed by King *et al.* [20]. *P. aeruginosa* strain OSh1 was inoculated into the broth medium and incubated using shaker (120 rpm) at 28°C for 72h. After incubation and blue green pigment production, the culture cells were removed using centrifuge (10.000 rpm /10 min) and then filtered through 0.2 µm syringe filter [21]. Pyocyanin was extracted using serial chloroform extractions followed by acidification using 0.2 N HCl [12]. The pyocyanin concentration as micrograms per ml of culture supernatant was calculated using an extinction coefficient at 520 nm of 17.072 according to the following equation [22].

Pyocyanin Concentration (µg/ml) = $O.D_{520}$ * 17.072

After five extraction cycles, the pH of the isolated acidified water layer could be adjusted to pH 7.5 by a minimum volume of 0.1 M NaOH. Needlelike crystals formed in the chilled solution over the following 2 hours were trapped on a 0.45 µm filter and washed with deionized water, dried under vacuum and weighted [13; 23].

UV scanning of pyocyanin pigment.

Red colour pigment obtained by adding 0.2N HCl to blue chloroform extract was analyzed using UV-Vis spectrophotometer (Jenway UV/Visible-2605 spectrophotometer, England) and the range of scanning was 200-800 nm [3]. The maximum absorbance of pigment was detected.

Molecular detection of phenazine modifying gene (phzS).

Fifty milliliters of MSM liquid medium [24] was inoculated with single colony of *P. aeruginosa* or *E. coli* strains, and then incubated at 28 °C with shaking (120 rpm) for 72 h. The genomic DNA was extracted as previously presented [25]. The DNA purity and concentration were determined according to Barbas *et al.* [26] by measuring the absorbance at λ_{260nm} and λ_{280nm} using UV/VIS spectrophotometer (Jenway UV/Visible-2605 spectrophotometer, England) and confirmed on 1% agarose gel electrophoresis [27]. To detect the phenazine –modifying gene (phzS) involved in biosynthesis of pyocyanin in *P. aeruginosa* strain OSh1, the PCR amplification was conducted in 50 µL containing 2.5U of Dream-Taq DNA polymerase (Thermo scientific, formerly Fermentas, Lithuania), 1x Dream-Taq Buffer, 40 ng of extracted DNA, 0.2 mM of dNTPs, 1 mM $MgCl_2$ and 20 pmol of forward oligonucleotide primer (5'- TGCGCTACATCGACCAGAG-3') and the reverse primer (5'- CGGGTACTGCAGGATCAACT-3') [13]. The PCR reaction was performed with thermal cycler (MJ Research PTC-100, MJ Research Inc., MA, USA) by amplification conditions started with pre-denaturation step of double stranded DNA at 94°C for 5 min; followed 35 cycles of denaturation step 94°C for 40 sec, annealing step 48°C for 40 sec, extension step 72°C for 1 min; then one step of final extension 72°C for 10 min, and storage at 4°C. After amplification program was complete, the PCR product was electrophoresed on 1% agarose gel and visualized under UV light [28]. The gene was estimated in comparison to standard DNA ladder.

Protein detection of phenazine modified gene (phzS).

Single colony of *P. aeruginosa* was transferred into 100 ml of King's and LB broth media at 28 °C for 3 days on a shaker at 120 rpm. The cells from 3 ml of both cultures were collected by centrifugation (10000 rpm /5 min) and washed several times by 300 mM Tris buffer, pH 8. The produced cells were re-suspended in 300 µl of lysis buffer (0.32 mM sucrose, 1% SDS, 10 mM Tris-HCl) and left overnight at 4 °C. After that, unbroken cells and cell wall were removed by centrifugation (5000 rpm / 5 min). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the samples at ratio of 1:3 and boiled for 10 min. The cells proteins were separated by 10 % SDS-PAGE [24]. The molecular weight of the protein profile was estimated in comparison to marker (standard protein marker, 11-240kDa; Sigma, USA). The protein bands were appeared after staining with Coomassie Brilliant Blue G-250 (Sigma, USA). The electrophoresed proteins were documented using GelAnalyzer2010a program [29].

Determination of antimicrobial activity for produced pyocyanin at different concentrations.

The antimicrobial activity of pyocyanin at different concentrations was evaluated on Gram positive bacteria (*Staphylococcus aureus* ATCC-47077, Multi-resistance *Staphylococcus aureus* (MRSA), *Listeria monocytogenes* ATCC-35152, *Staphylococcus lentus*), Gram negative bacteria (*Vibrio cholera, Escherichia coli* ATCC-25922, *Aeromonas hydrophila, Salmonella ochrasius, Salmonella typhi* ATCC 15566, *Shigella* sp., *Flavobacter* sp. and *Pseudomonas aeruginosa*), Yeast (*Candida albicans* ATCC-10231 and *Candida tropica*) and Fungi (*Aspergillus niger* ATCC-16888 and *Fusarium oxysporum*). The pathogenic microbes were obtained from the American type culture collection (ATCC; Rockville, MD, USA), National Research Center (NRC), Microbiology Lab at National Institute of Oceanography and fishers, clinical specimens at El Salam International hospital [30]. The bacterial cells and fungal spore suspensions (10^6 CFU/ml) of

each tested microbes were spread onto the nutrient agar plates for bacteria and potatoes dextrose agar plates for fungi. The wells (7 mm diameter) were dug on the inoculated plates [31], and 70 µl of purified pyocyanin (Needle-like crystals) dissolved in DMSO at different concentrations (3.75, 7.5, 15, 30, 60, 75, 100, 120 µg/ml) were added to the wells. The plates were left 2h at 4 ^{o}C to allow the diffusion. The plates were incubated at 37 ^{o}C for 24 h except fungi which was incubated at 28 °C for 72 h, then, the inhibition zone diameter was measured expressed in millimeter and three replicates were averaged [32].

3. RESULTS

Isolation and primary identification of *P. aeruginosa.*

Out of 10 different pigmented bacterial colonies were isolated on marine agar medium, only one isolate was produced blue green diffusible pigment. This isolate was purified and subjected to classical identification. The results of morphological, cultural, biochemical and physiological characterizations of this isolate were represented in Table 1. This isolate was gram negative, short rods, producing blue green pigment on cetrimide agar (Figure 1). It was produced by oxidase and catalase enzymes and hemolysis blood samples. These results were compared with Bergey's Manual and leaded identification as *Pseudomonas aeruginosa*.

Table 1. Physiological and biochemical characteristics of *P.aeruginosa.*

Tests	Results
Colony	Circular, Raised, Smooth margin
Pigment on nutrient agar	Diffusible green pigment turning blue green
Pigment on cetrimide agar	Blue green after 24h
Gram stain	Negative
Motility	Motile
Cell shape and arrangement	Small rods , Mostly single
Growth at 50 ° C	+
Oxidation/fermentation test	Strictly aerobe
Citrate utilization	+
Catalase	+
Oxidase	+
Indol production	-
Methyl red fermentation	-
H_2S production	+
Starch hydrolysis	+
Lipid hydrolysis	+
Gelatin liquefaction	+
Blood hemolysis	+

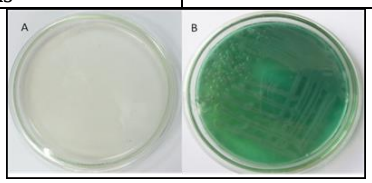

Figure 1. Production of blue green pigment on cetrimide medium by marine *P. aeruginosa.* Where A, un-inoculated cetrimide agar plate; B, blue green pigment produced on cetrimide.

Molecular Identification of *Pseudomonas aeruginosa* using16s rDNA analysis.

The identification of *Pseudomonas aeruginosa* was confirmed based on 16s rDNA technique. The size of PCR product was 370 Pb which detected on 1% agarose gel electrophoreses (Figure 2). The new sequence of *Pseudomonas aeruginosa* strain was compared with available 16S rDNA gene sequences from organisms in the Gen Bank databases. The sequence showed high similarity (99%) with *Pseudomonas aeruginosa*. This sequence was submitted to GeneBank and recorded as *Pseudomonas aeruginosa* strain OSh1with accession number of KT032066.

The chemical nature of pyocyanin was confirmed by reaching of red color (pyocyanin in acidic form), following by neutralizing of the mixture by 1N NaOH. The needle like crystals was formed in solution after chilling and then trapped onto syringe filter (0.45 µm), washed by deionized water, dried under vacuum and weighted. The concentration of pyocyanin quantification was calculated based on optical density at 520 nm of pyocyanin in acidic phase. The absorbance at 520 nm was then multiplied by 17.072 and the yield obtained was 60µg\ml.

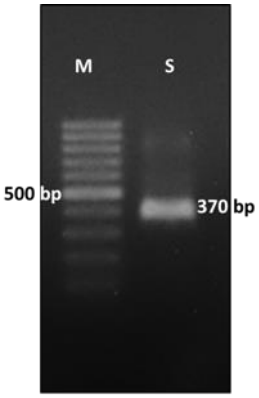

Figure 2. PCR amplification of 16S rRNA gene for marine *P. aeruginosa* (S) compared with 1Kb DNA marker (M).

Production, Extraction and purification of pyocyanin.

Blue green pigment suggestive to pyocyanin was produced during growth of *P. aeruginosa* on King's broth medium as picture represented in Figure 3. The pigment was started in appearing after 1d of incubation and increased with time until 3d. Pyocyanin pigment was extracted by the addition of chloroform to supernatant at ratio 3:5 v/v. The blue layer of pyocyanin/chloroform mixture was quoted to a new tube and the pyocyanin was purified by addition of 0.2N HCL.

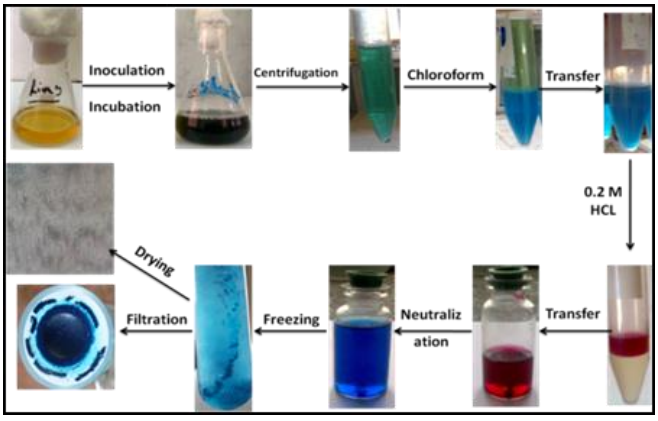

Figure 3. Flow chart for extraction and purification of pyocyanin steps.

Characterization of produced pyocyanin using UV/Vis spectrophotometer.

The absorbance spectrum of pyocyanin was scanned using UV/Vis spectrophotometer. The results of several mixtures scanning were represented in Table (2) and Figure (4). The mixture of pyocyanin /chloroform was given 6 peaks at 285, 310, 320, 325, 495 and 700 nm as maximum absorption. While 3 peaks out of 6 were lost after purification of the mixture by 0.2 N HCl and exhibited the

absorption maxima at 280,385 and 525 nm. However, four peaks were detected after complete purification and dissolving the needlelike in 15 % methanol.

Table 2. Maximum absorption of mixtures through pyocyanin purification.

Mixture	Maximum wavelength					
The mixture of pyocyanin/chloroform	285	310	320	325	495	700
Pyocyanin dissolved in 0.2 N HCl	280	385	525	-	-	-
Pyocyanin dissolved in 15% methanol	280	325	495	710	-	-

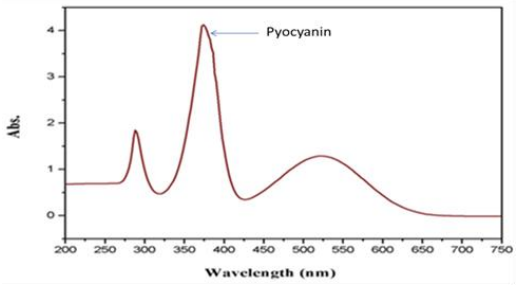

Figure 4. UV-Vis spectrum of pyocyanin dissolved in 0.2 M HCl (Acidic red form).

Molecular detection of the phenazine –modifying gene (phzS).

The PCR technique was developed to detect the phenazine–modifying gene (phzS) involved in biosynthesis of pyocyanin by *P. aeruginosa* strain OSh1 using phzs-R and phzs-F primers. The amplified fragment was separated on 1% agarose gel, and only one band at 448 bp for phenazine biosynthetic gene was detected in *P. aeruginosa* strain and not found in *E- Coli* strain that used as negative control (Figure 5).

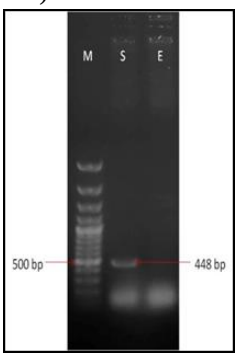

Figure 5. Agarose gel electrophoresis of PCR product amplified from DNA of *P. aeruginosa* strain OSh1 with primers phzs-F and phzs-R Where M, 1Kb DNA marker; S, PCR for DNA from *P. aeruginosa*; E, PCR for DNA from *E. coli*.

Protein detection of phenazine modified gene (phzS).

Cellular proteins of *P. aeruginosa* grown on King's and LB media were electrophorized by 10 % SDS-polyacrylamide gel. After staining of gel, the protein profile was detected under light conditions and the documentations were noted using GelAnalyzer2010a program and the results were represented in Table (3). The strain had protein bands of about 36 and 43 KDa that was corresponding to 2 enzymes encoded by 2 phenazine-modifying genes (PhzM and PhzS). These bands were not found when *P. aeruginosa* growing on LB medium. After analysis using, the results show that common 8 bands were detected when grow *P. aeruginosa* on King's medium and common 5 protein bands were detected with LB medium.

Table 3. Molecular weight, Number of bands, R_f and raw volume of bands on SDS-PAGE analyzed using GelAnalyzer 2010a program.

Source of protein	No. of bands	Molecular weight	R_f	Raw volume
***P. aeruginosa* OSh1 grown on King's medium**	8	61	0.201	1279
		49	0.258	2033
		43	0.298	2313
		36	0.353	3008
		28	0.431	3575
		23	0.51	2088
		21	0.553	1893
		18	0.61	1976
***P.aeruginosa* OSh1 grown into LB medium**	5	40	0.321	2092
		34	0.369	2926
		28	0.441	1565
		23	0.51	1617
		19	0.598	1234

Antimicrobial activity of different concentrations of pyocyanin.

The antimicrobial activity of purified pyocyanin (Needle-like crystals) at various concentrations ranged from 3.7 to 120 µg/ml was examined against Gram-negative and Gram-positive bacteria, yeast and fungi pathogenic strains. The results indicated that pyocyanin had high killing activity against all tested bacteria except *Shigella* sp. and *P. aeruginosa* and gave inhibition zone ranged between 31- 42 mm involved 7 mm of good diameter. As well as the pyocyanin was inhibiting growth of tested yeasts and gave inhibition zone 34 and 35 mm with *Candida tropica* and *Candida albicans* ATCC-10231, respectively. The growth of *Fusarium oxysporum* was decreased when used pyocyanin as antimicrobial agent and the inhibition zone was 15 mm (Table 4).

Table 4. Inhibition zone (mm) for antimicrobial activity of pyocyanin at different concentrations

Pathogenic Microorganisms		Different concentrations of pyocyanin (µg/ml)					
		3.7	**7.5**	**15**	**30**	**60**	**120**
Gram negative	*A. hydrophila*	0	17	18	19	32	46
	E. Coli	0	0	14	16	28	47
	Pseudomonas sp.	0	0	0	0	15	30
	V. cholera	0	10	12	20	30	46
Gram positive	*St.aureus* (MRSA)	0	15	21	28	33	38
	St. aureus	0	15	21	28	33	40
	L. monocytogenes	0	13	21	23	30	40
	St. lentus	0	17	21	28	34	40
Fungi	*C. albicans*	0	0	21	30	32	46
	C. tropica	0	0	18	26	32	45
	A. niger	0	0	0	0	0	0
	F. oxysporum	0	0	0	0	23	46

Where the inhibition zone diameter was involved 6 mm well diameter.

Discussion.

This study aims to isolate the marine pigmented bacteria and molecular characteristics of the most producer and biosynthesis genes. One isolate out of 10 different pigmented bacterial colonies isolated from marine environment at Mediterranean Sea, Alexandria, Egypt was the most efficient isolate in producing of blue green diffusible pigment. The identification of this isolate was *P. aeruginosa* strain OSh1 and recorded in GenBank under accession number: KT032066. This isolate showed intensive blue green pigment production on cetrimide agar medium and transferred to grow on king's broth medium to produce pyocyanin pigment. King's broth medium is considered the best medium to produce pyocyanin pigment. This medium improves pyocyanin

elaboration and inhibits the other pigments formation. Glycerol considered the source of energy and also helps pigment production [1]. Pyocyanin pigment was produced under optimal growth condition of pH, temperature, incubation period and shaking conditions. After cultivation and pyocyanin production, the bacterial cells were removed by centrifugation followed by possessing through 0.2 µm syringe filter. Pyocyanin into supernatant was extracted by solvent extraction technique based on the change of their solubility due to change in pH. The pyocyanin is soluble in chloroform (organic solvent) and has blue color, while an acidic pH, it turns reddish pink and become soluble in aqueous phase [23]. Pyocyanin quantification assay is based on the pocyanin's optical density at 520_{nm} at acidic phase. The concentration of pyocyanin was 60 µg/ml. Essar *et al.* [22] noted that pyocyanin concentration may be calculated using the acidity form of pyocyanin and multiplication of absorbance value at 520 nm with 17.072.

To characterize pyocyanin compound, the produced pyocyanin pigment was scanned from 200 to 700 nm using UV-Vis spectrophotometer. The results obtained in this branch were in agreement with previous findings by El-Fouly *et al.* [3] who found that Pyocyanin dissolved in 0.2 N HCl exhibited the maxima absorption at 300, 388 and 518 nm. Also it was in agreed with previous findings of Ohfuji *et al.* [33] who studied the UV/Vis spectrum of pyocyanin and found its characteristics peaks at 204.0, 242.5, 277.0, 387.5 and 521.5 nm when dissolved in 0.2 M HCl. Pyocyanin is a water soluble blue green bioactive pigment produced by *P. aeruginosa* species [34]. Genetically, this pigment produced based on seven genes, however, two genes namely; *phz*M and *phz*S are responded for pyocyanin production [10]. In present study, the pyocyanin biosynthetic modifying gene *(phzS)* was detected by PCR technique using specific primers and gave band on agarose gel at 448 pb. This gene is the most important one in pyocyanin production because it is considered the final gene in biosynthesis pathway and responsible for hydroxylation and decarboxylation of 5-methylphenazine 1-carboxylic acid betaine to pyocyanin. These clearly show the *P. aeruginosa* strain Osh1 contains phenazine biosynthetic operon and modifying genes which response to produce pyocyanin pigment [13]. Sequentially, the proteomic studies of pyocyanin biosynthesis modified genes were done on *P. aeruginosa* strain to confirm their having of the studied genes. Any active gene must be expressed to protein as the final product. *P. aeruginosa* was cultivated in King's and LB media to detect the *phz*M and *phz*S genes were constitutive or inducible genes. The total proteins were isolated and examined by SDS-PAGE. The results show the strain grown on King's medium had protein bands about 36 and 43 KDa that was corresponding to the two phenazine-modifying genes *phz*M and *phz*S, respectively. The results of this study were similar to the findings of Mavrodi *et al.* [9].

One of the most important goals of this study was obtained the natural antimicrobial agent which can be used as marine drug. In our study, the purified pyocyanin at different concentrations was assessed against gram positive and gram negative bacteria, yeast and fungi. The obtained results showed that pyocyanin has high antimicrobial activity by increasing their concentrations from 60 to 120 µg/ml. The antimicrobial activity is enhanced by almost 40% for all tested strains, thus the activity of pyocyanin as antibiotic is dependence on concentration. In the same trend, El-Shouny *et al.* [35] found that the growth of all tested G+ve bacteria and *Candida* sp. was completely inhibited by pyocyanin; whereas G-ve bacteria including *S. typhi* and *P. mirabilis* were intermediately affected and *K. pneumonia* was resistant to pyocyanin. Variation in the lipid content of Gram positive and Gram-negative bacteria cell wall may be responsible for the difference in the sensitivity of pyocyanin as antibiotic [36]. A study by El Feghali and Na'was [37] revealed that pyocyanin has a powerful inhibitory effect on the bacterial biofilm that can demonstrate valuable in developing new drugs for the treatment and prevention bacterial infections.

4. CONCLUSIONS

Blue green pigment, Pyocyanin, as antimicrobial agent was produced by marine *Pseudomonas aeruginosa*. Also, the molecular biology techniques were applied to know the pathway of this production. The produced pyocyanin was used as antimicrobial material against wide range of pathogenic microorganisms as broad spectrum. Finally, the produced pyocyanin may be used as antimicrobial drug in several environmental, agricultural and medical aspects.

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