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Pyocyanin production in the presence of calcium ion in *Pseudomonas aeruginosa* and recombinant bacteria

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Abstract

P. aeruginosa (NRRL B-771) and its transposon mediated *vgb* transferred recombinant strain, PaJC, were used in the research. The optimization of pyocyanin production was carried out in the different conditions of cultivation (temperature, carbon sources and salt concentrations) in a time-course manner. For the cultures grown at 30 °C and 1 % sucrose in the presence of 50 mM $CaCl_2$, *P. aeruginosa*, 72 hours 4.53 mg/ml, PaJC is grown at 37 °C and 1 % sucrose in the presence of 250 mM $CaCl_2$, 72 hours 3.96 mg/ml with pyocyanin producing reached the highest value. The nutrient source, especially the carbon type, had a dramatic effect on pyocyanin production. This pyocyanin producing with *vgb* may be an effective method.

Keywords: *Pseudomonas aeruginosa*, pyocyanin, *Vitreoscilla* hemoglobin, calcium ion

Pseudomonas aeruginosa ve rekombinant bakterisinin kalsiyum iyonunun varlığında piyosiyanin üretimi

Özet

P. aeruginosa, (NRRL B-771) ve transpozon aracılı *vgb* geni aktarılan rekombinat suşu PaJC, araştırmada kullanılmıştır. Piyosiyanin üretiminde optimizasyonu, zaman bağlantılı bir şekilde farklı koşullar altında (sıcaklık, karbon kaynakları ve tuz konsantrasyonu) gerçekleştirilmiştir. *P.* aeruginos,a 50 mM $CaCl_2$ varlığında, 30 °C ve % 1 sükroz ortamında yetiştirilen kültürler 72 saat sonunda 4.53 mg / ml; PaJC ise, 250 mM $CaCl_2$ varlığında 37 °C ve % 1 sükroz ortamınada yetiştirilen kültürler 72 saat sonunda 3.96 mg / ml piyosiyanin üretiminde en yüksek değerlere ulaşmıştır. Özellikle değişik karbon kaynaklarında, piyosiyanin üretiminde dramatik bir etkisi vardı. *vgb* ile üreten Piyosiyanin üretiminde *vgb* geni etkili bir yöntem olabilir.

Anahtar Kelimeler: *Pseudomonas aeruginosa*, piyosiyanin, *Vitreoscilla* hemoglobini, kalsiyum iyonu

1. Introduction

Pyocyanin is the characteristic blue-green phenazine pigment produced by *Pseudomonas aeruginosa*. Pyocyanin is the most thoroughly studied of the phenazine pigments [1, 2]. Pyocyanin is a phenazine compound that occurs in nature in secretions of the pathogen *Pseudomonas* and affects the growth and viability of a wide range of microorganisms. Pyocyanin can be extracted from the broth in which cells producing pyocyanin are grown, and used as an antimicrobial agent [3]. They are the only Gram-negative rods, however, that produce pyocyanin [4, 5]. Pyocyanin also has antibiotic activity against a wide variety of microorganisms, which may benefit *P. aeruginosa* by elimination of competing microorganisms. It is of particular interest that *P. aeruginosa*, a "strict" aerobe, is itself insensitive to pyocyanin. *P.aeruginosa* survive overall oxidative stress [6, 7]. Pyocyanin is zwitterions that can easily penetrate biological membranes [8]. *P. aeruginosa* because of pyocyanin increased the oxygen uptake of *Streptococcus pneumoniae*, *Staphylococcus aureus*, and erythrocytes [9]. Pyocyanin, a blue redox-active secondary metabolite, is a member of a large family of tricyclic compounds known as phenazines.

Pyocyanin also inhibits microbial growth by initiating a redox cascade that can occur non-enzymatically via NADH or NADPH [5, 10]. Phenazine pigments such as pyocyanin are electron acceptors [11]. The synthesis of pyocyanin is affected by carbon and nitrogen sources in growth media [6, 9]. Further, it is thought that pyocyanin production may be coupled to intracellular ATP levels, resulting in increased pyocyanin production under carbon or nutrient-limited conditions. Thus, under limiting conditions, the growth of *P. aeruginosa* and the subsequent production of pyocyanin may alter the microbial community structure by inhibiting the growth of microorganisms sensitive to pyocyanin [9, 12]. Production of pyocyanin enhances *P. aeruginosa* virulence [13]. Virulence of *P. aeruginosa* is multifactorial and includes pyocyanin [14]. Pyocyanin is known to be a multifunctional metabolite [15]. Pyocyanin facilitates *P. aeruginosa* colonization of the environment [16]. Amounts of biosynthetic proteins for the extracellular virulence factor pyocyanin also increased with added calcium [10].

P. aeruginosa is Gram-negative bacterium found in almost every ecological niche, including soil, water and plants. It is frequently isolated from contaminated sites and is capable of producing metabolites (i.e., alginate, rhamnolipid, pyocyanin) that enhance its competitiveness and survival. The antimicrobial action of pyocyanin is bactericidal in nature. This pigment has been used diagnostically to describe both the physiology and the pathogenicity of this bacterium. Pyocyanin production may give *P. aeruginosa* a selective advantage in certain growth situations [17]. The natural habitat of *P. aeruginosa* is soil and water, although it is isolated frequently from the foliage of plants and from the stools of a small percentage of individuals [18].

In the aerobic bacterium *Vitreoscilla*, the synthesis of homodimeric hemoglobin (VHb), currently the only well-known prokaryotic hemoglobin can be induced under hypoxic conditions. The expression is maximally activated both in *Vitreoscilla* and *E. coli* under micro aerobic conditions, when the dissolved oxygen level drops below 10 % of air saturation. The *vgb* has been cloned into a variety of bacteria and eukaryotic organism mostly for the purpose of enhancing respiration, growth and productivity [19].

The aim purpose of this study is to determine the potential of VHb, protein known for its beneficiary effect on cell growth on metabolite production, on pyocyanin production, *P. aeruginosa* and PaJC. The presence of *vgb*/VHb may prove useful in this application.

2. Material and Methods

2.1. Bacterial Strains

Bacterial strains *P. aeruginosa* (NRRL B-771) and its transposon mediated *vgb* transferred recombinant strain, PaJC were used [20].

2.2. Growth Conditions

Cells were maintained on agar plates at 4 ºC with transfers at monthly intervals. The liquid media used throughout the study was Luria-Bertani (LB) broth medium (g/l); peptone (10), NaCl (10), and yeast extract (5). The final pH values of broth media was adjusted to 7. 100 µl of overnight cultures grown in 20 ml LB in 125 ml Erlenmeyer flasks was inoculated into 50 ml of the same medium in 150 ml volume flasks and incubated for different periods of time. The cultures were incubated at 200 rpm in flasks containing 50 ml medium. Shake-flasks were incubated at 30-37 °Cin a 200 rpm gyratory water-bath, drawing the samples at certain intervals (i.e., 24, 48, 72 and 96 h). *Pseudomonas* broth P base (PB) (modified) *Pseudomonas* broth P base (PB) (modified) (g/l); peptone (20), MgCl (1.4), K_2SO_4 (10) contained 1 % glucose, glycerol and sucrose added from a stock solution respectively, autoclaved. The pyocyanin production of both wild *P. aeruginosa* and its recombinants in the *Pseudomonas* broth P base was monitored at the given intervals during the course of the experiments.

In this study, $CaCl_2$ was used as a source of salt. The concentrations of $CaCl_2$ compound based in preliminary experiments; was 50 mM (low salt

concentration) and 250 mM (high salt concentration) were used in *Pseudomonas* broth P base (modified), respectively.

2.3. Pyocyanin Quantitation Assay

The pyocyanin assay is based on the absorbance of pyocyanin at 520 nm in acidic solution. A 5-ml sample of culture grown in PB to maximize pyocyanin production was extracted with 3 ml of chloroform and then re-extracted into 1 ml of 0.2 N HCl to give a pink to deep red solution. The absorbance of this solution was measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant, were determined by multiplying the optical density at 520 nm (OD_{520}) by 17.072 [1, 21].

3. Results

As the agitation rate and temperature are two leading factors are crucial for the cell growth and pyocyanin formation, *P. aeruginosa* and its *vgb* bearing strain (PaJC) were cultivated in shake flasks under agitation rates (200 rpm) and different temperature (30 and 37 °C). The intracellular pyocyanin level was determined cultures harvested at 96 h. Our preliminary studies showed that this compound was produced during the late (post-stationary) secondary phase of growth. In the presence of P *Pseudomonas* broth medium (not contain salt), *P. aeruginosa* and the production of PaJC pyocyanin bacteria 24, 48, 72, 96 hours have been identified.

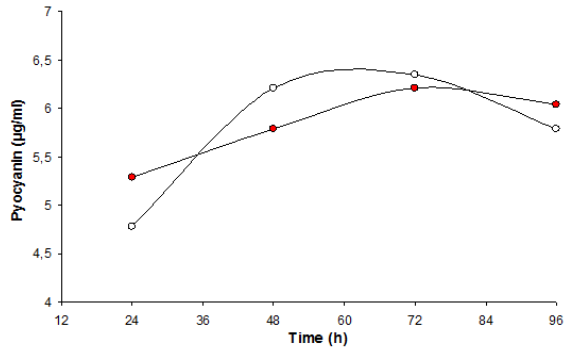
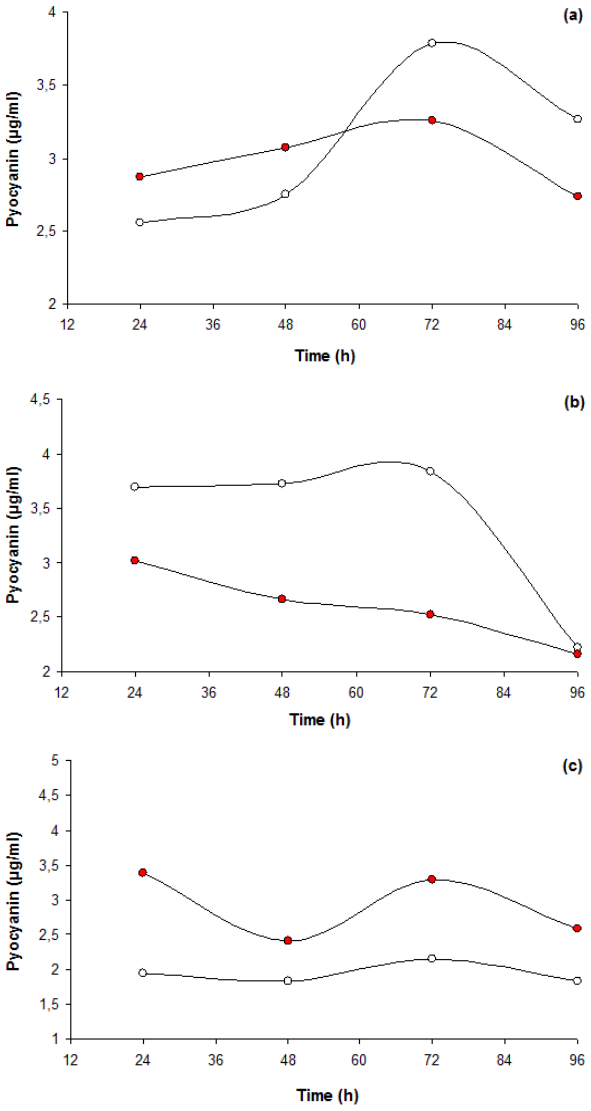


Figure 1. Pyocyanin levels of *P. aeruginosa* (○) and its *vgb* recombinant strain, PaJC (●), under grown in *Pseudomonas* broth media. Cells were grown a gyratory water-bath in150-ml volume flasks containing 50 ml culture medium.

P. aeruginosa 24 hours 4.78 mg/ml pyocyanin producing, 72 hours 6.35 mg/ml and reached the highest value. PaJC 24 hours 5.29 mg/ml and 72 hours 6.21 mg/ml was reached (Fig.1).



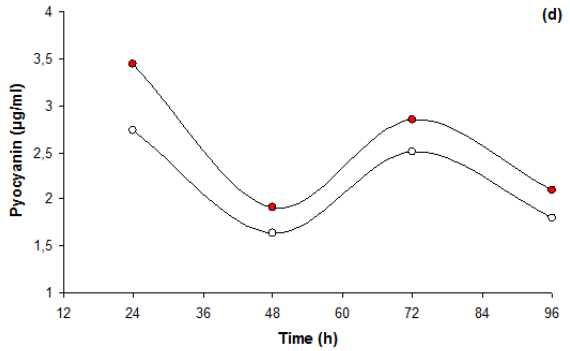
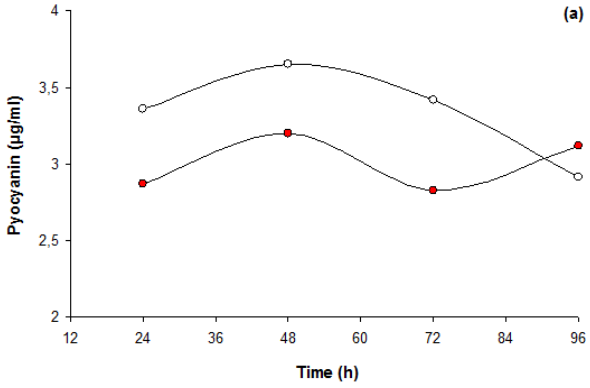


Figure 2. Pyocyanin levels of *P. aeruginosa* (○) and its *vgb* recombinant strain, PaJC (●), under different agitation conditions at (a, c) 30 °C 50 mM and (b, d) 37 °C 250 mM $CaCl_2$, grown in *Pseudomonas* broth supplemented 1 % glucose. Cells were grown agyratory water-bath in 150-ml volume flasks containing 50 ml culture medium.

3.1. Effect of Glucose

At 30 °C and in the presence of 1 % glucose, the average level of pyocyanin production in 50 mM $CaCl_2$ cultures were *P. aeruginosa* 3.09 and PaJC 2.98 mg/ml (Fig. 2a), while these values were 3.36 and 2.60 mg/ml for *P. aeruginosa* and PaJC (Fig. 2b), respectively, under 37 °C. The average level of pyocyanin in 250 mM $CaCl_2$ cultures was 1.94 and 2.92 mg/ml at 30 °C (Fig. 2c), 2.17 and 2.58 mg/ml at 37 °C for *P. aeruginosa* and PaJC (Fig. 2d), respectively.



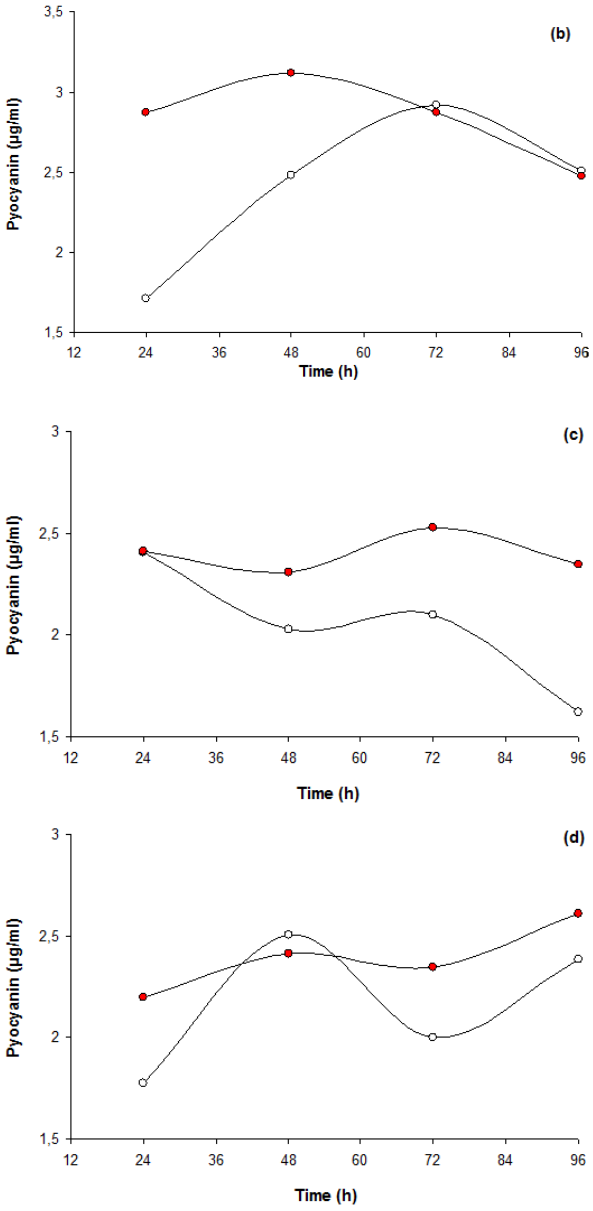


Figure 3. Pyocyanin levels of *P. aeruginosa* (○) and its *vgb* recombinant strain, PaJC (●), under different agitation conditions at (a, c) 30 °C 50 mM and (b, d) 37 °C 250 mM $CaCl_2$, grown in *Pseudomonas* broth supplemented 1 % glycerol. Cells were grown a gyratory water-bath in 150-ml volume flasks containing 50 ml culture medium.

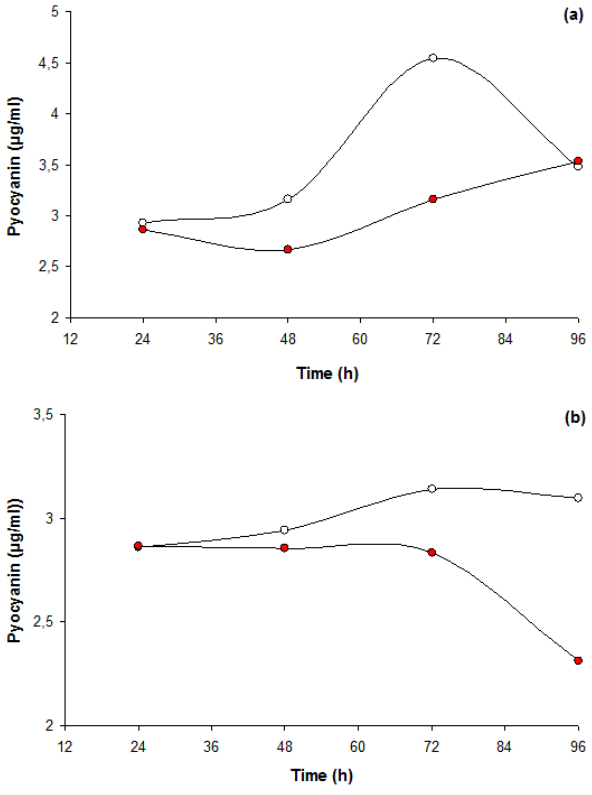
3.2. Effect of Glycerol

At 30 °C and in the presence of 1 % glycerol, the average level of pyocyanin production in 50

mM $CaCl_2$ cultures were *P. aeruginosa* 3.33 and PaJC 3.00 mg/ml (Fig. 3a), while these values were 2.40 and 2.83 mg/ml for *P. aeruginosa* and PaJC (Fig. 3b), respectively, under 37 °C. The average level of pyocyanin in 250 mM $CaCl_2$ cultures was 2.04 and 2.40 mg/ml at 30 °C (Fig. 3c), 2.17 and 2.39 mg/ml at 37 °C for *P. aeruginosa* and PaJC (Fig.3d), respectively.

3.3. Effect of Sucrose

At 30 °C and in the presence of 1 % sucrose, the average level of pyocyanin production in 50 mM $CaCl_2$ cultures were *P. aeruginosa* 3.53 and PaJC 3.06 mg/ml (Fig. 4a), while these values were 3.01 and 2.72 mg/ml for *P. aeruginosa* and PaJC, respectively (Fig. 4b), under 37 °C. The average level of pyocyanin in 250 mM $CaCl_2$ cultures was 1.66 and 2.51 mg/ml at 30 °C (Fig.4c), 2.22 and 2.65 mg/ml at 37 °C for *P. aeruginosa* and PaJC (Fig. 4d), respectively.



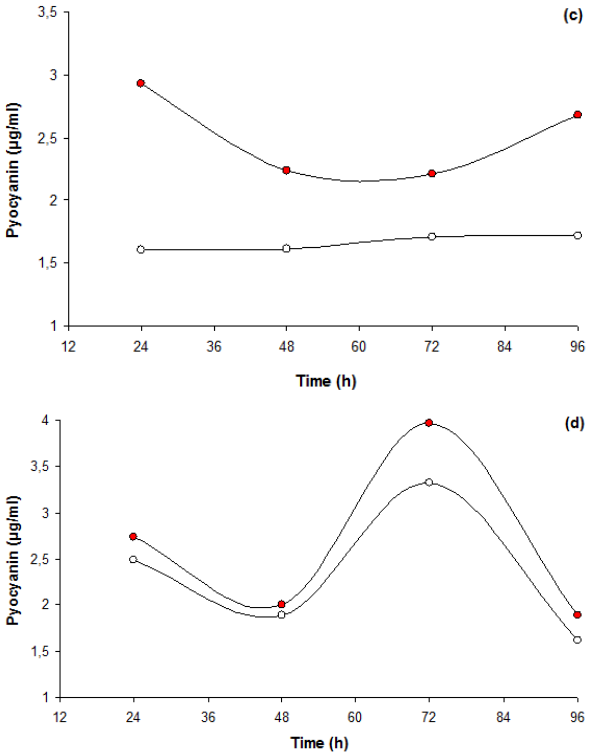


Figure 4. Pyocyanin levels of *P. aeruginosa* (○) and its *vgb* recombinant strain, PaJC (●), under different agitation conditions at (a, c) 30 °C 50 mM and (b, d) 37 °C 250 mM $CaCl_2$, grown in *Pseudomonas* broth supplemented 1 % sucrose. Cells were grown a gyratory water-bath in 150-ml volume flasks containing 50 ml culture medium.

4. Discussion

In particular, increase in the amount of pyocyanin production 72 hours after seen. *P. aeruginosa* in the presence of 50 mM $CaCl_2$ pyocyanin advantageous in terms of production when the concentration of 250 mM $CaCl_2$ pyocyanin PaJC more advantageous situation for the production of pass. Thus, at 72 h of incubation, where the PaJC strain showed slightly better pyocyanin production than *P. aeruginosa*. For the cultures grown at 30° C and 1 % sucrose in the presence of 50 mM $CaCl_2$, *P. aeruginosa*, 72 hours 4.53 mg/ml with pyocyanin producing reached the highest value. PaJC is grown at 37° C and 1 % sucrose in the presence of 250 mM $CaCl_2$, 72

hours 3.96 mg/ml with pyocyanin producing reached the highest value.

We have done so far in the literature for similar studies could not find work. Most pyocyanin were found to accumulate at the stationery stage of cell growth. Their accumulation in the supernatant started at the end of the logarithmic phase because pyocyanin are secondary metabolites. In the Pyocyanin production has been more than 37 °C. Therefore, it would be more economical to use 37 °C in practical applications. Sucrose was a much better substrate for pyocyanin production compared to glucose and glycerol carbon sources. In conclusion, a VHb-mediated increase in intracellular O_2 may occur, and in turn, lead to increase production of pyocyanin. The production of pyocyanin by both the recombinant and the host strain, however, seemed to be differently affected by carbon sources and salt concentrations. Sucrose was a much better substrate for pyocyanin production compared to glycerol and glucose. The PaJC strain showed a distinctly higher pyocyanin production than the parental wild-type strain throughout the whole incubation period when grown in *Pseudomonas* broth in glucose, sucrose and 250 mM $CaCl_2$.

Thus, a higher oxygen uptake afforded by the presence of the *vgb*/VHb may help the recombinant strain to better cope with this kind of pyocyanin production. The PaJC cells exhibit favorable properties including enhanced pyocyanin productivity over the wild strain. In this work glucose and sucrose were the most effective carbon sources for pyocyanin production. As a result, genetic engineering of pyocyanin producing strains with *vgb* may be an effective method.

5. Acknowledgement

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6. References

1. Essar, D.W., Eberly, L., Hadero, A. and Crawford, I.P. (1990). Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: Interchangeability of the two anthranilate synthases and evolutionary implications. Journal of Bacteriology, 172, 884-900.
2. Schwarzer, C., Fu, Z., Fischer, H. and Machen, T.E. (2008). Redox-independent activation of NF-κB by *P. aeruginosa* pyocyanin in a CF airway epithelial cell line. The Journal of Biological Chemistry, 283, 27144-27153.
3. Rao, Y.M. and Sureshkumar, G.K. (2005). Oxidative-stress-induced production of pyocyanin by *Xanthomonas campestris* and its effect on the indicator target organism, *Escherichia coli*. Journal of Industrial Microbiology and Biotechnology, 25, 266-272.
4. Daly, J.A., Boshard, R. and Matsen, J.M. (1984). Differential primary plating medium for enhancement of pigment production by *Pseudomonas aeruginosa*. Journal of Clinical Microbiology, 19, 742-743.
5. Mavrodi, D.V., Bonsall, R.F., Delaney, S.M., Soule, M.J., Phillips, G. and Thomashow L.S. (2001). Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. Journal of Bacteriology, 183, 6454-6465.
6. Hassett, D.J., Charniga, L., Bean, K., Ohman, D.E. and Cohen, M.S. (1992). Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase. Infection and Immunity, 60, 328-336.
7. Lee, J., Attila, C., Cirillo, S.L.G., Cirillo, J.D. and Wood, T.K. (2009). Indole and 7-hydroxyindole diminish *Pseudomonas aeruginosa* virulence. Microbial Biotechnology, 2, 75-90
8. Lau, G.W., Hassett, D.J., Ran, H. and Kong, F. (2004). The Role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends in Molecular Medicine, 10, 599-606.
9. Cox, C.D. (1986). Role of Pyocyanin in the acquisition of iron from transferin. Infection and Immunity, 52, 263-270.
10. Ran, H., Hassett D.J. and Lau, G.W. (2003). Human targets of *Pseudomonas aeruginosa* pyocyanin. Pnas Microbiology, 100, 14315-14320.
11. Warren, J.B., Loi, R., Rendell, N.B. and Taylor, G.W. (1990). Nitric oxide is inactivated by the

bacterial pigment pyocyanin. Biochemical Journal, 266, 921-923.

12. Norman, R.S., Moeller, P., McDonald, T.J. and Morris, P.J. (2004). Effect of pyocyanin on a crude-oil-degrading microbial community. Applied and Environmental Microbiology, 70, 4004-4011.
13. O'Malley, Y.Q., Reszka, K.J., Spitz, D.R., Denning, G.M. and Britigan, B.E. (2004). *Pseudomonas aeruginosa* pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells. American journal of Physiol Lung Cellular and Molecular Physiology, 287, 94-103.
14. Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. and Haas, D. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide and lipase. Molecular Microbiology, 24, 309-319.
15. Chieda, Y., Iiyama, K., Lee, J.M., Kusakabe, T., Yasunaga-Aoki, C. and Shimizu, S. (2008). Inactivation of pyocyanin synthesis genes has no effect on the virulence of *Pseudomonas aeruginosa* PAO1 toward the silkworm. *Bombyxmori*, FEMS Microbiology Letters, 278, 101-107.
16. Cheluvappa, R., Shimmon, R., Dawson, M., Hilmer, S.N. and Le Couteur, D.G. (2008). Reactions of *Pseudomonas aeruginosa* Pyocyanin with reduced glutathione. Acta Biochimica Polonica, 55, 571-580.
17. Onbasli, D. and Aslim, B. (2008). Determination of antimicrobial activity and production of some metabolites by *Pseudomonas aeruginosa* B1 and B2 in sugar beet molasses. African Journal of Biotechnology, 7, 4614-4619.
18. Janda, J.M. and Bottone, E.J. (1981). *Pseudomonas aeruginosa* enzyme profiling: predictor of potential invasiveness and use as an epidemiological tool. Journal of Clinical Microbiology, 14, 55-60.
19. Kahraman, H., Aytan, E. and Kurt, A.G. (2011) Production of methionine γ-lyase in recombinant *Citrobacter freundii* bearing the hemoglobin gene. BMB Reports, 44, 590-594.
20. Chung, J.W., Webster, D.A., Pagilla, K.R. and Stark, B.C. (2001). Chromosomal integration of the *Vitreoscilla* hemoglobin gene in *Burkholderia* and P*seudomonas* for the Purpose of producing stable engineered strains with enhanced bioremediating ability. Journal of Industrial Microbiology and Biotechnology, 27, 27-33.
21. Sarkisova, S., Patrauchan, M.A., Berglund, D., Nivens, D.E. and Franklin, M.J. (2005). Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. Journal of Bacteriology, 187, 4327-4337.