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Phenazine Production in The Presence of Heavy Metals in Recombinant *Erwinia herbicola* Bearing the Hemoglobin Gene

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Anahtar Kelimeler
Seconder metabolism
Erwinia herbicola
phenazine pigment
Vitreoscilla hemoglobin

Özet: In this study, from *Vitreoscilla* sp. recombinant strains were obtained from cloned bacterial hemoglobin (VHb) gene (*vgb*) Eh [pUC8:15] and its wild-type strain *Erwinia herbicola* phenazine production in the presence of heavy metals were investigated. Time-dependent production of phenazine in the wild bacteria not shows a significant change. In generally, at the end of 96 hours in the presence of Pb in terms of production of wild-type bacteria phenazine 2.91 µg/ml of recombinant bacteria is to produce fenazin production 9.5 µg /ml, respectively. The average phenazine highest values were 7, 8.8, 8 and 9.5 µg/ml Cd, Co, Fe and Pb for Eh [pUC8:15]. *E. herbicola* is 6.32, 3.33, 6.02 and 2.99 µg/ml Cd, Co, Fe and Pb, respectively. Genetic engineering *vgb*, may be an effective method to increase phenazine production.

Hemoglobin Geni Taşıyan Rekombinant *Erwinia herbicola'*da Ağır Metal Varlığında Fenazin Üretimi

Keywords
Sekonder metabolizma
Erwinia herbicola
fenazin pigmenti
Vitreoscilla hemoglobin

Özet: Bu çalışmada, *Vitreoscilla* sp. elde edilen bakteriyel hemoglobin (VHb) geni (*vgb*) aktarılmış yabanıl *Erwinia herbicola* ve rekombinat suş Eh [pUC8:15] da ağır metal varlığında fenazin üretimi araştırılmıştır. Yabanıl bakteri, zamana bağlı olarak fenazin üretiminde önemli bir değişim göstermemiştir. Genel olarak, Pb varlığında yabanıl bakteri, 96 saatin sonunda 2.91 mg/ml fenazin üretirken rekombinant bakteride 9.5 mg/ml dir. Fenazin üretimi en yüksek değerlere rekombinant bakteride sırasıyla, 7, 8.8, 8 ve 9.5 mg /ml olacak şekilde Cd, Co, Fe ve Pb varlığında gerçekleşirken; yabanıl bakteride; 6.32, 3.33, 6.02 ve 2.99 mg /ml olacak şekilde Cd, Co, Fe ve Pb varlığında gerçekleşmiştir. Genetik mühendisliği ile *vgb*, fenazin üretimi artırmak için etkili bir yöntem olabilir.

1. Introduction

Secondary metabolites are important factors for interactions between bacteria and other organisms. Bacterial secondary metabolites play critical roles in many aspects of bacterium-host interactions (Whistler, 2003). Secondary metabolites are chemical compounds historically defined as not being involved directly in "normal" growth, development, or reproduction. These compounds often are produced by the bacterium during the transition from exponential to stationary growth phase (Maddula et al, 2008). Phenazines are small, generally water-soluble, colored compounds and classified as secondary metabolites, i.e., compounds formed during the stationary phase and often having antibiotic properties (Selin et al, 2010; Warren et al, 1990). However, phenazines are produced by a wide variety of including both Gram-negative and Gram-positive species; include *Nocardia*, *Sorangium*, *Brevibacterium*, *Pseudomonas*, *Burkholderia*, *Erwinia*, *Pantoea agglomerans*, *Vibrio*, *Pelagiobacter* and members of the *Actinomycetes*, especially *Streptomyces* phenazine producers. Additionally, *Methanosarcina*, a member of the *Archaea*, was shown to contain a phenazine derivative. Bacteria are the only known source of natural phenazines. However, natural and synthetic phenazines are of significant interest because of their potential impact on bacterial interactions and biotechnological processes (Pierson and Pierson 2010; Selin et al, 2010). Almost all phenazines are broadly inhibitory to the growth of bacteria and fungi due to their ability to undergo cellular redox cycling in the presence of oxygen and reducing agents (including NADH and NADPH) and cause the accumulation of toxic

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superoxide and hydrogen peroxide. Several studies have demonstrated that phenazines are beneficial for the competitiveness and long-term survival of the producers in natural habitats (Mavrodi et al, 2010). To date, most ecological discussions of phenazines have focused on their crucial role in suppressing fungal pathogens of plants such as *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Gaeumannomyces graminis* var. *tritici* (Hernandez et al, 2004). Phenazines continue to be used for many diverse applications, including as electron acceptors and donors, as components of fuel cells, as environmental sensors and biosensors, and as central components of antitumor compounds. Phenazines are associated with antitumor activities. Cancer cells, having high levels of both topoisomerases, are more susceptible to this interference (Pierson and Pierson 2010).

2. Materials and Methods

2.1. Chemicals

NaCl, HCl, $Pb(NO_2)_3$, NaOH and $CoCl_2$ $6H_20$ were purchased from Merck. Yeast and peptone were purchased from Mast Diagnostics. $FeCl_3$, $Cd(NO_2)_3$ were purchased from Acros. Benzene was purchased from Riedel de Haen. All other chemicals used were of analytical grade.

2.2. Bacterial strains

Bacterial strains *Erwinia herbicola* (NRRL B-3466) and its *vgb* transferred recombinant strain of *Erwinia herbicola vgb* bearing the plasmid pUC8:15 were designated as 'Eh [pUC8:15]' was used in this study.

2.3. 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^{-1}); peptone (10), NaCl (10), and yeast extract (5). The final pH values of broth media was adjusted to 7.0. 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. Shake flasks were incubated at 37 °C in a 200 rpm gyratory water-bath, drawing the samples at certain intervals (i.e., 24, 48, 72 and 96 h). Stock heavy metal solutions were prepared in distilled water and were sterilized 121 ºC for 15 min. These solutions in various concentrations according to the metal tested were kept at 4 ºC for no longer than 1 month. The concentrations of heavy metal compounds; in LB broth cadmium (Cd), cobalt (Co), iron (Fe) and lead (Pb) and these heavy metal compounds 50, 100, 150 and 200 ppm were used, respectively.

2.4. Measurement phenazine pigment production

Phenazine was extracted from strain *Erwinia herbicola* 3466 and recombinant derivatives quantified by UV visible light spectroscopy. Briefly, triplicate 5 ml cultures grown overnight at 37 °C with shaking in LB broth were centrifuged, and the supernatants were acidified to pH 2 with concentrated HCl. Phenazines were extracted twice with an equal volume of benzene for 1 h. Following evaporation of the benzene under air, phenazines were resuspended in 0.5 ml of 0.1 N NaOH, and serial dilutions were quantified via absorbance of the pooled extracts was determined spectrophotometrically at 367 nm. The absorbance for each sample was normalized to total absorbance per initial 5 ml culture (Thomashow and Weller, 1988).

3. Results and Discussion

In this study, dissolution in water more easily used in the heavy metal cations. As the heavy metal type and concentration are two leading factors are crucial for the cell growth and phenazine formation, in 37 ºC temperature *E. herbicola* and its strain the 'Eh [pUC8:15]' were cultivated flasks under 200 rpm agitation rate. Especially after 72 hours of cultivation the productions of recombinant bacterium in LB fenazin increase in bacteria although they had not seen the same increase in the wild. Recombinant bacteria, phenazine produced 5-fold times more than in the wild bacteria at the end of 96 hours of (Fig 1).

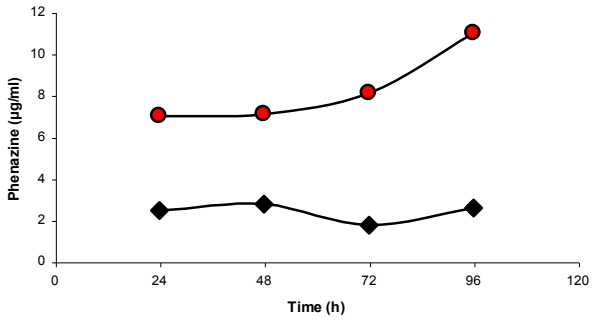
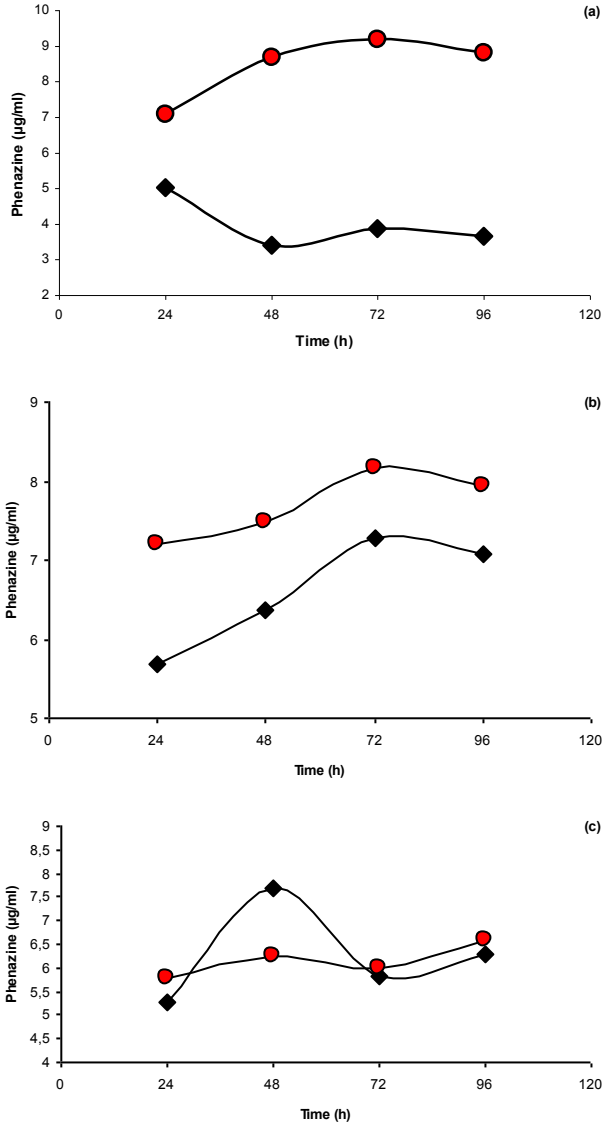


Figure 1. Erwinia herbicola (■), and recombinant 'Eh [pUC8:15]' (●) extracellular phenazine production in LB medium.

3.1. Phenazine production in the presence of Cd

Phenazine production of the highest, and 50 ppm Cd (Fig. 2 a) in the presence of recombinant bacteria observed that 72 hours (9.18 µg/ml). In the presence of wild bacteria 100 ppm Cd (Fig. 2 b) observed that 72 hours (8.16 µg/ml). In the presence of 50 ppm Cd and 150 ppm Cd 72 hours recombinant bacteria and

48 hours wild bacteria has been of the highest phenazine production (Fig. 2 c). Eh [pUC8:15] in Cd supplemented LB medium showed an average 3, 1.13, 1 and 1.16-fold higher phenazine values, 50, 100 150 and 200 ppm than their counterpats. Eh [pUC8:15] in 50 ppm Cd supplemented LB medium showed an average 3-fold higher phenazine value, however, showed similar level of phenazine 50, 100 150 and 200 ppm, than their counterpats. In generallly, 50, 100 and 150 ppm in the presence of Cd, a decrease is observed in the production of more than 72 hours after the phenazine, while in the presence of 200 ppm Cd (Fig. 2 d) an increase in the production phenazine.



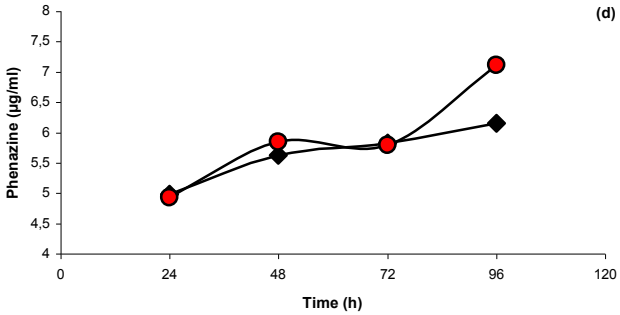
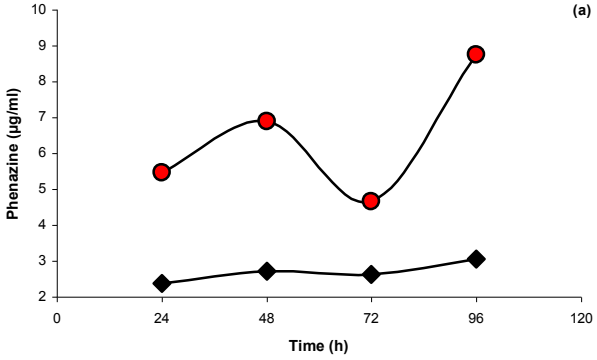


Figure 2. Erwinia herbicola (■), and recombinant 'Eh [pUC8:15]' (●) extracellular phenazine production in LB medium. a) 50 ppm, b) 100 ppm, c) 150 ppm and d) 200 ppm Cd.

3.2. Phenazine production in the presence of Co

Phenazine production of the highest, and 50 ppm Co (Fig. 3 a) in the presence of recombinant bacteria that 96 hours (8.75 µg/ml) and wild bacteria (3.05 µg/ml), while 100 ppm Cd (Fig. 3 b) in the presence of recombinant bacteria that 96 hours (9.45 µg/ml) and wild bacteria observed that 96 hours (3.74 µg/ml) was observed. Phenazine production in the presence of 150 ppm Co (Fig. 3 c) was the most appropriate. Recombinant bacteria produce 12.06 µg /ml phenazine. Wild-type bacteria produced 5.63 µg /ml phenazine. Difference was 2.15-fold.

Phenazine production in the presence of 200 ppm Co (Fig. 3 d) was the worst. A recombinant bacterium 72 hours 6.6 µg/ml of wild-type bacteria produces 3.3 µg/ml of highest production phenazine, and ultimately realized the difference was 3-fold. The most suitable Co concentration for the production of phenazine is in the presence of 100 ppm. In the presence of 150 and 200 ppm Co, wild bacteria after 72 hours phenazine production of recombinant bacteria an increase is observed. In generally, at the end of 96 hours in the presence of Co in terms of production of wild-type bacteria phenazine 4.16 µg/ml of recombinant bacteria to produce fenazin production 8.8 µg/ml. In the presence of Co recombinant bacteria and there is an increase in production of phenazine was approximately 2.12-fold.



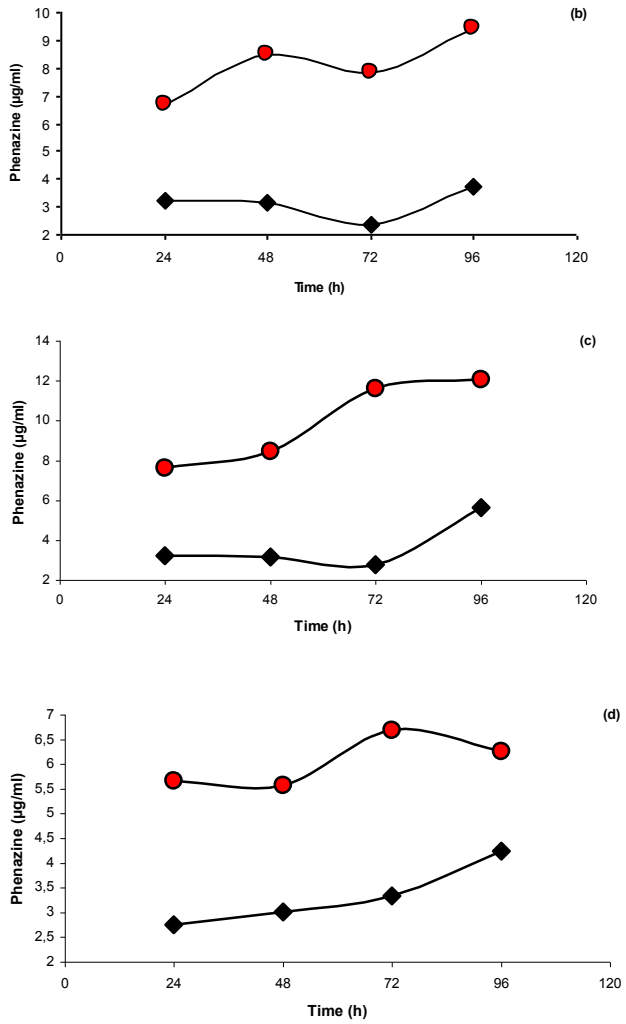


Figure 3. Erwinia herbicola (■), and recombinant 'Eh [pUC8:15]' (●) extracellular phenazine production in LB medium. a) 50 ppm, b) 100 ppm, c) 150 ppm and d) 200 ppm Co.

3.3. Phenazine production in the presence of Fe

Phenazine production of wild bacteria decreased with a time as the recombinant bacteria to increase in contrast to 3.12-fold and this increase is at the end of 96 hours (50 ppm Fe) (Fig. 4 a). Phenazine production of wild bacteria decreased with a time as the recombinant bacteria to increase in contrast to 2-fold and this increase is at the end of 96 hours (100 ppm Fe) (Fig. 4 b). In the presence 150 (Fig. 4 c) and 200 ppm (Fig. 4 d) Fe not seen a difference in terms of phenazine production. In generally, in the presence of Fe in terms of production at the end of 96 hours wild-type bacteria 6.2 µg/ml and recombinant bacteria 8 µg/ml was fenazin production.

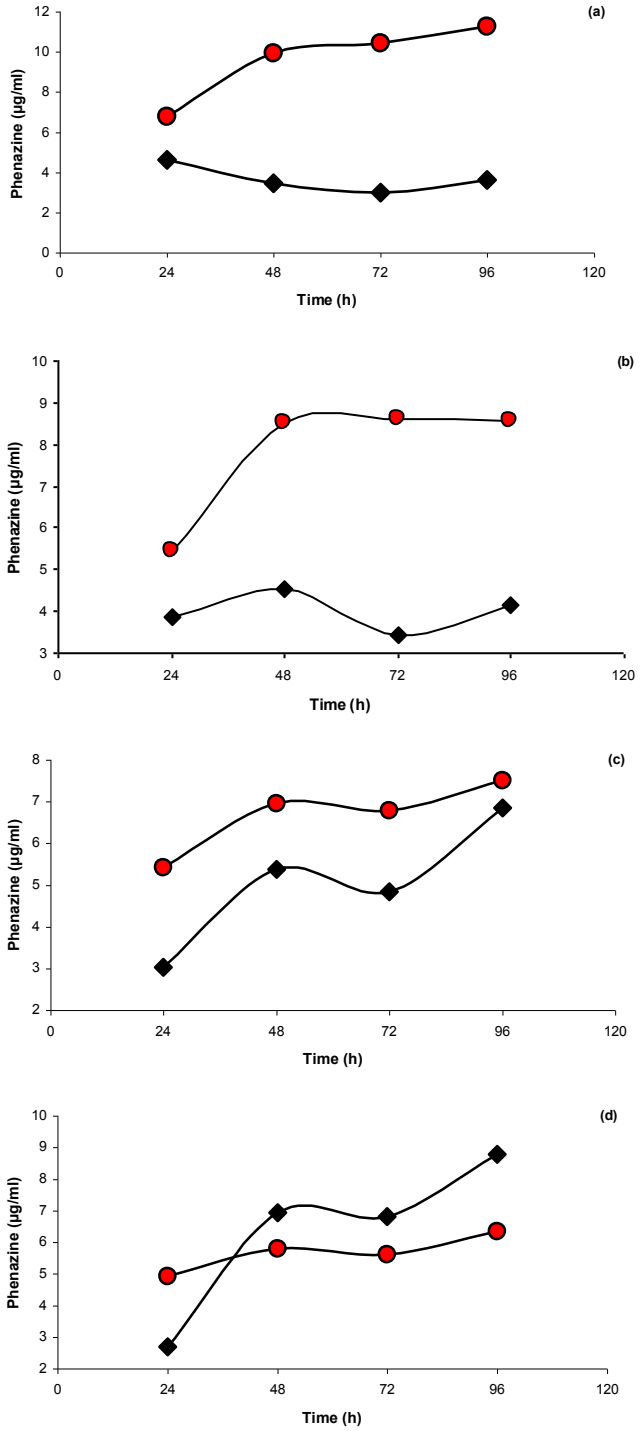
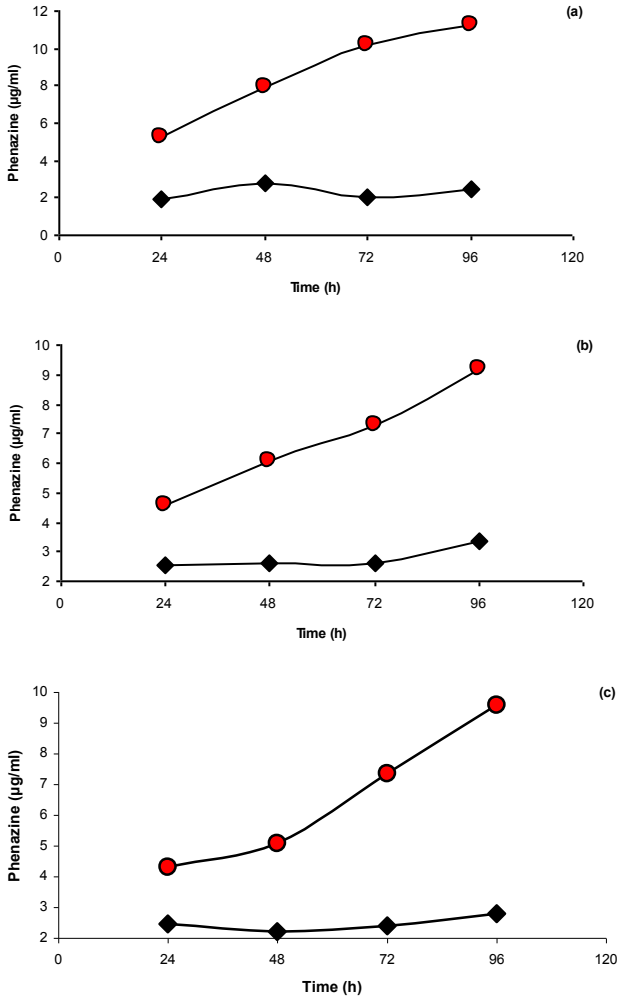


Figure 4. Erwinia herbicola (■), and recombinant 'Eh [pUC8:15]' (●) extracellular phenazine production in LB medium. a) 50 ppm, b) 100 ppm, c) 150 ppm and d) 200 ppm Fe.

3.4. Phenazine production in the presence of Pb

Phenazine production in the presence of 50 ppm Pb (Fig. 5 a), in recombinant bacteria 24 h and at the end of 96 h after the start hours later than wild-type shows an increase of 4.5 times. In the presence of 100 ppm Pb (Fig. 5 b) recombinant bacteria in LB medium, but this increase seen in time-dependent

increase at the end of 96 hour observed in only 2.7-fold. In the presence of 150 (Fig. 5 c) and 200 (Fig. 5 d) ppm Pb recombinant bacteria increased with time. These increases are seen within at the end of 96 h compared to wild-type bacteria were observed to be phenazine production of 3.5 and 3.1-fold. Time-dependent production of phenazine in the wild bacteria not shows a significant change. In generally, at the end of 96 hours in the presence of Pb in production of wild-type bacterium 2.91 µg/ml and recombinant bacterium 9.5 µg/ml has been fenazin production. The average phenazine highest values were 7, 8.8, 8 and 9.5 µg/ml Cd, Co, Fe and Pb for Eh [pUC8:15], while *E. herbicola* 6.32, 3.33, 6.02 and 2.99 µg/ml Cd, Co, Fe and Pb. Pb was a much better heavy metal substrate for phenazine production compared to other heavy metals (average, 10 µg/ml) for Eh [pUC8:15], while wild bacteria the worst (average, 2.91 µg/ml) for 96 h. 50 ppm Fe and Pb was much better heavy metal for phenazine production for, while *E. herbicola*, 150 ppm Cd and 200 ppm Fe.



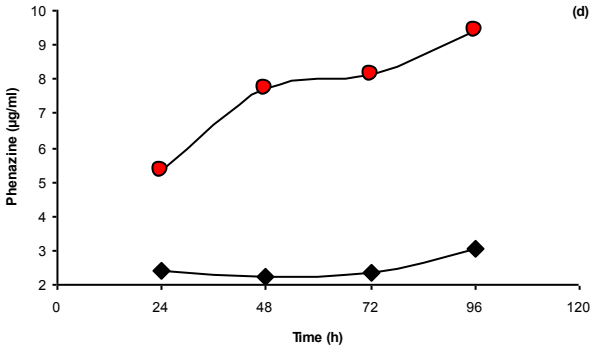


Figure 5. Erwinia herbicola (■), and recombinant 'Eh [pUC8:15]' (●) extracellular phenazine Production in LB medium. a) 50 ppm, b) 100 ppm, c) 150 ppm and d) 200 ppm Pb.

Many phenazine compounds are found in nature and are produced by bacteria and these phenazine natural products have been implicated in the virulence and competitive fitness of the parent organisms (Essar et al, 1990). There are multiple ways in which phenazines may affect ecological fitness. Phenazines can function as antibiotics, as accessory respiratory pigments (Hardison, 1998). Phenazine production has been well studied for a variety of organisms because of their function in the biocontrol of plant pathogens and because they serve as virulence factors in the human host. It is noteworthy that the environmental factors that are known to stimulate phenazine production make sense in the context of Fe (III) mineral reduction. These factors include low phosphate levels, oxygen limitation, and the presence of Fe (III) and organic components of root and/or seedling exudates such as fructose, ribose, and citric acid. Oxygen regulation of phenazine production may thus ensure that phenazines are produced under the most favorable conditions for mineral reduction (Hernandez et al, 2004).

Kaynaklar

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