

การแยกและการศึกษาสมบัติทางเคมีของไพโอไซยานินที่ผลิตได้จากแบคทีเรีย *Pseudomonas aeruginosa* TISTR 781 (ATCC 9027)

Isolation and study of chemical properties of pyocyanin produced from *Pseudomonas aeruginosa* TISTR 781 (ATCC 9027)

กานดา เสาสุง (Kanda Saosoong)¹ สันติ ทองธรรมมา (Santi Tongtumma)¹
ศักดิ์สิทธิ์ จันทร์ไทย (Saksit Chanthai)² วิรัช ว่องพัฒน์กุล (Wirach Wongphathanakul)²
วรรณดี บุญญัตริรัชต์ (Wandee Bunyatratthata)³ เฉลิม เรืองวิริยะชัย (Chalerm Ruangviriyachai)^{4*}

บทคัดย่อ

แบคทีเรีย *Pseudomonas aeruginosa* TISTR 781 (ATCC 9027) สามารถผลิตสารปฏิชีวนะสีน้ำเงินได้เป็นส่วนใหญ่ในอาหารเหลวสูตร King's A medium (KA) หลังผ่านกระบวนการแยกและทำให้บริสุทธิ์โดยใช้คอลัมน์โครมาโทกราฟีที่บรรจุด้วยเรซินชนิดแอมเบอร์ไลต์ เอ็กซ์เอดี-16 และซิลิกาเจล ตามลำดับ แถบที่ปรากฏบนคอลัมน์ชนิดซิลิกาเจล ประกอบด้วยแถบสีเหลือง สีน้ำเงินเข้ม และสีเขียวเข้ม ตามลำดับ สารปฏิชีวนะสีน้ำเงินที่ได้มีความบริสุทธิ์เมื่อตรวจสอบเบื้องต้นด้วย เทคนิค TLC และ HPLC และจากข้อมูลการวิเคราะห์ด้วยเทคนิค UV พบว่าสารสีน้ำเงินที่แยกได้อาจเป็นไพโอไซยานิน ส่วนสารสีเหลืองอาจเป็น 1-ไฮดรอกซีฟีนาซีน ส่วนการศึกษาสมบัติความเป็นขั้วของไพโอไซยานิน โดยใช้ตัวทำละลายที่เหมาะสมในทาง TLC ก็คือ สารผสมของไดคลอโรมีเทน-เมทานอล (1:1) โดยปริมาตร และยังได้ศึกษาอิทธิพลของช่วงเวลาเก็บสารต่อความเสถียรของโครงสร้าง และ pH ต่อการเปลี่ยนแปลงค่าการดูดกลืนแสงสูงสุดของไพโอไซยานินอีกด้วย

Abstract

Blue antibiotic can be produced mainly by *Pseudomonas aeruginosa* TISTR 781 (ATCC 9027) when grown in King's A medium (KA). After isolation and purification by both Amberlite XAD-16 resin column and subsequently silica gel column chromatography, bands on the silica gel column that appeared were bright yellow, dark blue and dark green. The purified blue pigment was examined for its homogeneity by TLC and HPLC. According to UV data, the isolated blue band could be pyocyanin whereas the bright yellow could be 1-hydroxyphenazine. A study on the polarity of pyocyanin by varying solvents for TLC indicated that the mixture of dichloromethane-methanol (1:1, v/v) is an optimum solvent. The effects of storage time on structural stability and pH on maximum absorption of pyocyanin have also been studied.

คำสำคัญ: ไพโอไซยานิน *Pseudomonas aeruginosa* อาหารสูตรคิงก์ เอ

Keywords: Pyocyanin, *Pseudomonas aeruginosa*, King's A medium

¹นักศึกษาระดับบัณฑิตศึกษา สาขาวิชาเคมีวิเคราะห์ คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น

²ผู้ช่วยศาสตราจารย์ ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น

³ผู้ช่วยศาสตราจารย์ ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น

⁴รองศาสตราจารย์ ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น

* corresponding author, e-mail: chal_ru@kku.ac.th

Introduction

Most natural products from a variety of microorganisms have been extensively studied. Bacterial strains of fluorescent Pseudomonads have been investigated widely as potential antibiotic agents (Dwivedi and Johri, 2003). Phenazine compounds, one group of bacteria natural products, are small, nitrogen-containing heterocyclic pigments, water-soluble, colored compounds and secondary metabolites primarily obtained from soils or marine habitats (Laursen and Nielsen, 2004). Strains of *Pseudomonas aeruginosa* produce a variety of redox active phenazines including pyocyanin (PYO), phenazine-1-carboxylic acid (PCA), 1-hydroxyphenazine (1-OH-PHZ) and phenazine-1-carboxamide (PCN) (Chin-A-Woeng et al., 2003). Pyocyanin (*N*-methyl-1-hydroxyphenazine) is a blue phenazine pigment of low molecular weight. It is chloroform soluble and exhibits as a redox cyclic compound (Cox, 1986). Pyocyanin gives absorption maximum wavelength at 278 nm in 0.2 M HCl and has a melting point in the range of 132-134 °C (Fernández and Pizarro, 1997). Only *P. aeruginosa* among various *Pseudomonas* spp. can produce pyocyanin in several media such as King's A medium (KA) (Byng et al., 1979), brain-heart infusion medium (BHI) (Kerr et al., 1999) and glycerol-alanine medium (Reszka et al., 2004). The isolation and purification processes for pyocyanin require several steps including liquid-liquid extraction (Stead et al., 1996), ion exchange chromatography (Kanner et al., 1978) and adsorption chromatography (Ohfuji et al., 2004). The methods used for homogeneity test of pyocyanin are thin-layer chromatography (TLC), gas chromatography (GC) (Gurusiddaiah, 1986) and reverse phase-high performance liquid chromatography (RP-HPLC) (Ge et al., 2004). Moreover, UV, IR, NMR and

GC/MS techniques have also been described in the literature for pyocyanin characterization (Brisbane et al., 1987; Kumar et al., 2004; Vukomanovic et al., 1997; Mavrodi et al., 2001; Usher et al., 2002). Pyocyanin is also shown as a mediator of a glucose sensor (Ohfuji et al., 2004). Furthermore, some evidence suggests that pyocyanin has antibiotic properties against a range of microorganisms (Baron and Rowe, 1981). Therefore, according to these properties, it is an interesting organic compound for applications. In this research, isolation, purification, analysis and some chemical properties of pyocyanin produced from *P. aeruginosa* TISTR 781 (ATCC 9027) were studied.

Materials and methods

Bacterial strain and culture conditions:

P. aeruginosa TISTR 781 (ATCC 9027) was purchased from the Thailand Institute of Scientific and Technological Research (TISTR) and was used for pyocyanin production. It was streaked on Luria-Bertani (LB) agar plates (Hernández et al., 2004) and incubated at room temperature for 1-2 days. A single colony from a LB agar plate was transferred to 100 ml of KA medium [Bactopeptone 10 g (Oxoid, England), NaCl 5 g, glycerol (Carlo Erba, Italy) 10 ml and K₂SO₄ 1.4 g (Carlo Erba, Italy) in 1 liter distilled water] and incubated at room temperature with an orbital shaker for a day and was used as a starter. To increase pyocyanin production, the starter was transferred into KA medium with 1:100 bacterial dilutions. Batch fermentation was performed in 500 ml shaking flasks containing 200 ml of the growth medium, which were stirred on a rotary shaker at 150 rpm at room temperature (~28 °C) for 2 days.

Isolation and purification of pyocyanin:

The polymeric adsorbent of Amberlite XAD-16 resin (Fluka, France) column (55 cm x 5.5 cm, i.d.) was used for pyocyanin isolation (Stead et al., 1996), eluting with 70% (v/v) acetonitrile (AR grade, Lab Scan, Ireland) in water. Pyocyanin was purified by dichloromethane extraction and silica gel column (60 cm x 3 cm, i.d.) chromatography, gradually eluted with dichloromethane (AR grade, Carlo Erba, Italy), followed by 10% (v/v) methanol (AR grade, Carlo Erba, Italy) in dichloromethane, as previously reported (Ohfuji et al., 2004). For monitoring of pyocyanin, the solution was detected by Agilent 8453 UV-Visible spectrophotometer range of 200-500 nm. The purity of pyocyanin was determined by TLC with optimum solvent system used and also by RP-HPLC under gradient elution using the Waters HPLC system with a C_{18} reverse phase column. Elution was undertaken on solvent A; water:TFA (HPLC grade, BDH chemicals, England) (100:0.01, v/v) and solvent B; water: acetonitrile (HPLC grade, Lab Scan, Ireland): TFA (10:90:0.01, v/v) (Fernández and Pizarro, 1997) as mobile phase. A 1 ml min^{-1} flow rate was used and monitored at 254 nm with photodiode array detection.

Analysis of pyocyanin: Full UV spectra were obtained in 0.2 M HCl, methanol and dichloromethane (Fernández and Pizarro, 1997). The solutions were detected by UV-Visible spectrophotometer over the range of 200-500 nm.

Chemical properties of pyocyanin: Some chemical properties of pyocyanin have been studied, including optimization of TLC solvent, storage time and pH effects. Based on TLC, it has been studied with several solvent systems; these are dichloromethane,

dichloromethane-ethylacetate (9:1), dichloromethane-acetone (4:1), dichloromethane-methanol (9:1) and dichloromethane-methanol (1:1). For pH effect, six solutions of purified pyocyanin (1.03 mM) had pH adjusted to 2, 4, 6, 9, 10 and 11. These were monitored using the UV-Visible spectrophotometer in the range 220-550 nm. For the effects of storage time on structural stability, solutions were kept in the dark and sampled at 6 time intervals of 0, 4, 17, 23, 42 and 52 hours storage times with various pH values, which were monitored using the same conditions as the pH study.

Results and discussion

P. aeruginosa TISTR 781 (ATCC 9027) can be grown on a LB agar plate after 1 day and single colonies obtained. After bacteria were grown in KA medium at room temperature, the blue solution appeared preliminarily indicating pyocyanin production.

After isolation of phenazine derivatives using Amberlite XAD-16 resin column, two bands (dark yellow and blue) appeared on the column and the interesting blue fraction was collected. When the eluate was evaporated and lyophilized, crude pyocyanin was approximately 1.05 g/l of bacterial culture. The dark blue color was extracted into dichloromethane. The organic phase was evaporated and purified with a silica gel column. The bright yellow band was obtained in the first fraction after elution with dichloromethane. The dark blue band was then eluted with 10% methanol in dichloromethane. A dark green band still remained at the top of the column. Finally, blue crystals were obtained after removing solvent from the dark blue

solution. For phenazine purity determination, the purified blue phenazine compound gives single spot with R_f 0.53 on TLC plate when developed with dichloromethane-methanol (1:1), indicating our purified blue phenazine compound was free from other contaminants.

P. aeruginosa can produce more than one type of phenazine (Kumar et al., 2004) and UV technique was used for analysis of the purified fraction obtained from the silica gel column. The appearance and absorption wavelengths of the pigments are shown in Table 1. Both pigments were identical to those previously described by Fernández and Pizarro (1997). The crude and purified blue pigments were analyzed under gradient HPLC by the modified method (Fernández and Pizarro, 1997) and chromatograms are shown in Figs. 1(A) and 1(B). The results show that bacterial phenazines including pyocyanin (I) and other phenazines [(II), (III)] could be separated with retention times of 12.7, 20.4 and 22.7 min respectively, as shown in Fig. 1(A). The phenazine derivatives were further purified by silica gel column chromatography and a single peak with retention time of 12.7 min obtained as shown in Fig. 1(B). The maximum absorption of purified blue pigment was at 278 nm in 0.2 HCl, 308 nm in CH_2Cl_2 and 319 nm in methanol and the maximum absorption of purified bright yellow pigment was at 274 nm in 0.2 HCl, 264 nm in CH_2Cl_2 and 263 nm in methanol (Table 1), which corresponds to a previous report (Fernández and Pizarro, 1997). Therefore, as shown from UV data, the purified blue pigment should be pyocyanin and the purified bright yellow pigment should be 1-hydroxyphenazine, with chemical structures as shown in Fig. 2.

Some chemical properties of the blue pigment obtained from *P. aeruginosa* TISTR 781 (ATCC 9027) are as follows. In a previous report, the purity test of phenazine antibiotic by the TLC method has been studied (Gurusiddaiah et al., 1986). The phenazine compound has proven to be pure by TLC under various solvent systems. In this study, we also optimized TLC solvents for pyocyanin, as shown in Table 2. We found that the single blue spot of pyocyanin pigment appeared on the TLC plate in all cases of solvent system. However, the selected solvent for the TLC method was dichloromethane-methanol (1:1) since the R_f of the studied pigment is not too low or too high. This solvent system can also separate both yellow and blue pigments with good separation. The color of pyocyanin solutions changed depending on pH variation. They appeared red at pH 2-4, purple at pH 5 and blue at $\text{pH} \geq 6$, respectively. Correlation between color variation and pH of pyocyanin solutions has been reported (Ohfujii et al., 2004). Maximum absorption wavelengths of various pH of the solutions are shown in Fig. 3. At 279 nm the absorbance decreased with pH varying from strong acidic to strong basic solutions. However, it increased with pH varying from strong acidic to strong basic solutions at 311 nm at time 0 hour. Since the structure of pyocyanin contains an OH group on its C_1 , this is the phenolic character ($\text{pK}_a \sim 4.8$) (Reszka et al., 2004). The deprotonation of pyocyanin with increasing pH affects its absorbance (Hypochromic shift at 279 nm and Hyperchromic shift at 311 nm) and maximum absorption wavelengths (Bathochromic shift or Red shift). The absorbance and maximum absorption wavelengths of the solutions at pH lower than 10 were independent of time. On the other hand,

the color of the solution at pH 11 started appearing from blue to red-violet in basic, and also changed to yellow in acidic solution from 5 to 52 hours. Furthermore, the maximum absorption wavelength of pyocyanin in pH 11 shifted from 311 nm to 293 nm (Fig. 4). The storage time and pH had a close correlation with the structural changes of pyocyanin to be 1-hydroxyphenazine. It was reported that 1-hydroxyphenazine can be generated from pyocyanin by alkaline hydrolysis (Wilson et al., 1987). In addition, the acidic yellow solution in this study with maximum absorption wavelength at 273 nm is also consistent with a previous report (Watson et al., 1986).

Conclusions

Phenazine derivatives were produced by *P. aeruginosa* TISTR 781 (ATCC 9027) in KA medium at ambient temperature with vigorous shaking incubation for 2 days. The produced phenazine was isolated by Amberlite XAD-16 resin column eluting with 70% acetonitrile in water. Dark yellow and blue fractions were obtained and only the interesting blue fraction was collected and lyophilized to give crude pyocyanin. The approximately yield of crude pyocyanin was 1.05 g/l of bacterial culture. In purification by mean of gradual elution, a yellow pigment was achieved when eluting with dichloromethane and a blue pigment was achieved when eluting with 10% MeOH in dichloromethane. Chemical structures of the phenazine derivatives were preliminarily confirmed by UV spectra, and homogeneity tested by TLC and gradient HPLC. The obtained blue pigment showed a single spot when

tested with a suitable TLC solvent system, which is dichloromethane-methanol (1:1, v/v), indicating that it was free of other contaminants. Furthermore, when testing the purity with HPLC, a single peak of blue pigment with retention time of 12.7 min was found. Based on color compounds and UV data, the blue pigment should be pyocyanin. At pH 7.0, pyocyanin solution is blue in color with maximum absorption wavelengths at 379, 311 and 238 nm in 0.2 M HCl. The color of the pigment solution changed depending on the pH. Correlation of storage time and pH variation was found causing structural changes of pyocyanin. At pH 11, the color of pyocyanin solution started appearing from blue to red-violet in basic solution with absorption maxima shifted from 311 to 293 nm. The red-violet solution changed to yellow in acidic with maximum absorption wavelengths at 384 and 272 nm that should be from 1-hydroxyphenazine. On the other hand, the absorption and maximum absorption wavelengths of the solutions at pH lower than 10 were independent of time within 52 hours at room temperature.

Acknowledgements

The authors would like to thank the Department of Chemistry and the Department of Microbiology, Faculty of Science, Khon Kaen University for instruments and experiment facilities. Financial support from the Center for Innovation in Chemistry: Postgraduate Education and Research Program in Chemistry (CIC-PERCH), Commission on Higher Education, Ministry of Education is gratefully acknowledged. We also thank to the National Research Council of Thailand (NRCT) for chemical.

References

- Baron, S.S. and Rowe, J.J. 1981. Antibiotic action of pyocyanin. *Antimicrob Agents Chemother* 20: 814-820.
- Brisbane, P.G., Janik, L.J., Tate, M.E. and Worren, R.F.O. 1987. Revised structure for the phenazine antibiotic from *Pseudomonas fluorescens* 2-79 (NRRL B-15132). *Antimicrob Agents Chemother* 31: 1967-1971.
- Byng, G.S., Eustice, D.C. and Jensen, R.A. 1979. Biosynthesis of phenazine pigments in mutant and wild-type cultures of *Pseudomonas aeruginosa*. *J Bacteriol* 138: 846-852.
- Chin-A-Woeng, T.F.C., Bloemberg, G.V. and Lugtenberg, B.J.J. 2003. Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytol* 157: 503-523.
- Cox, C.D. 1986. Role of pyocyanin in the acquisition of iron from transferrin. *Infect Immun* 52: 263-270.
- Dwivedi, D. and Johri, B.N. 2003. Antifungals from fluorescent Pseudomonads: Biosynthesis and regulation. *Curr Sci* 12: 1693-1703.
- Fernández, R.O. and Pizarro, R.A. 1997. High-performance liquid chromatographic analysis of *Pseudomonas aeruginosa* phenazines. *J Chromatogr A* 77: 99-104.
- Ge, Y., Huang, X., Wang, S., Zhang, X. and Xu, Y. 2004. Phenazine-1-carboxylic acid is negatively regulated and pyoluteorin positively regulated by *gac A* in *Pseudomonas* sp. M 18. *FEMS Microbiol* 237: 41-47.
- Gurusiddaiah, S., Weller, D.M., Sarkar, A. and Cook, R.J. 1986. Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp. *Antimicrob Agents Chemother* 29: 488-495.
- Hernández, M.E., Kappler, A. and Newman, D.K. 2004. Phenazines and other redox-active antibiotics promote microbial mineral reduction. *J Appl Microbiol* 70: 921-928.
- Kanner, D., Gerber, N.N. and Barthe, R. 1978. Pattern of phenazine pigment production by a strain of *Pseudomonas aeruginosa*. *J Bacteriol* 134: 690-69.
- Kerr, J.R., Taylor, G.W., Hoiby, N., Cole, P.J. and Wilson, R. 1999. *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. *J Clin Pathol* 52: 385-387.
- Kumar, R.S., Ayyadurai, N., Pandiaraja, P., Reddy, A.V., Venkateswarlu, Y., Prakash, O. and Sakthivel, N. 2004. Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broad-spectrum antifungal activity and biofertilizing traits. *J Appl Microbiol* 10: 1-10.
- Laursen, J.B. and Nielsen, J. 2004. Phenazine natural products: biosynthesis, synthetic analogues, and biological activity. *Chem Rev* 104: 1663-1685.
- 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. *J Bacteriol* 183: 6454-6465.
- Ohfuji, K., Sato, N., Hamada-Sato, N., Kobayashi, T., Imada, C., Okuma, H. and Watanabe, E.

2004. Construction of a glucose sensor based on a screen-printed electrode and a novel mediator pyocyanin from *Pseudomonas aeruginosa*. *Biosens Bioelectron* 19: 1237-1244.
- Reszka, K.J., Malley, Y.O., McCormick, L.M., Dening, G.M. and Britigan, B.E. 2004. Oxidation of pyocyanin, a cytotoxic product from *Pseudomonas aeruginosa*, by microperoxidase 11 and hydrogenperoxide. *Free Radic Biomed* 36: 1448-1459.
- Stead, P., Rudd, B.A.M., Bradshaw, H., Noble, D. and Dawson, M.J. 1996. Induction of phenazine biosynthesis in cultures of *Pseudomonas aeruginosa* by L-N-(3-oxohexanoyl) homoserine lactone. *FEMS Microbiol* 140: 15-22.
- Usher, L.R., Lawson, R.A., Geary, I., Taylor, C.J., Bingle, C.D., Taylor, G.W. and Whyte, M.K.B. 2002. Induction of Neutrophil apoptosis by the *Pseudomonas aeruginosa* exotoxin pyocyanin: a potential mechanism of persistent infection. *J Immun* 168: 1861-1868.
- Vukomanovic, D.V., Zoutman, D.E., Stone, J.A., Marks, G.S., Braien, J.F. and Nakatsu, K. 1997. Electrospray mass-spectrometric, spectrophotometric and electrochemical methods do not provide evidence for the binding of nitric oxide by pyocyanine at pH 7. *J Biochem* 322:25-29.
- Watson, D., Macdermot, J., Wilson, R., Cole, P.J. and Taylor, G.W. 1986. Purification and structural analysis of pyocyanin and 1-hydroxyphenazine. *J Biochem* 159: 309-313.
- Wilson, R., Pitt, T., Taylor, G., Watson, D., Macdermot, J., Sykes, D., Roberts, D. and Cole, P. 1987. Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of Human Respiratory cilia in vitro. *J Clin Invest* 79: 221-229.

Table 1. The appearance and major peaks in ultraviolet spectra of both yellow and blue pigments

	Compound			
	Blue pigment		Yellow pigment	
	This study	Fernández and Pizarro, 1997	This study	Fernández and Pizarro, 1997
Appearance	Dark blue needle	Dark blue needle	Yellow fine needle	Yellow fine needle
Absorption maxima (nm)				
in 0.2 M HCl	388, 278*	388, 278*	383, 364, 274*, 239	383, 364, 274*, 242
in CH ₂ Cl ₂	325, 308*, 238	327, 309*, 242	368, 360, 353, 264*	368, 360, 350, 264*
in methanol	368, 319*, 238	368, 318*, 238	368, 363, 350, 263*	368, 360, 350*, 263*

* Maximum absorption wavelength

Table 2. Silica gel thin-layer chromatographic migration of blue pigment

Solvent system	R _f	
	Blue pigment	Yellow pigment
Dichloromethane	0.00	0.44
Dichloromethane -ethyl acetate (9:1)	0.05	0.66
Dichloromethane -acetone (4:1)	0.05	0.68
Dichloromethane -methanol (9:1)	0.49	0.70
Dichloromethane -methanol (1:1)	0.53	0.77

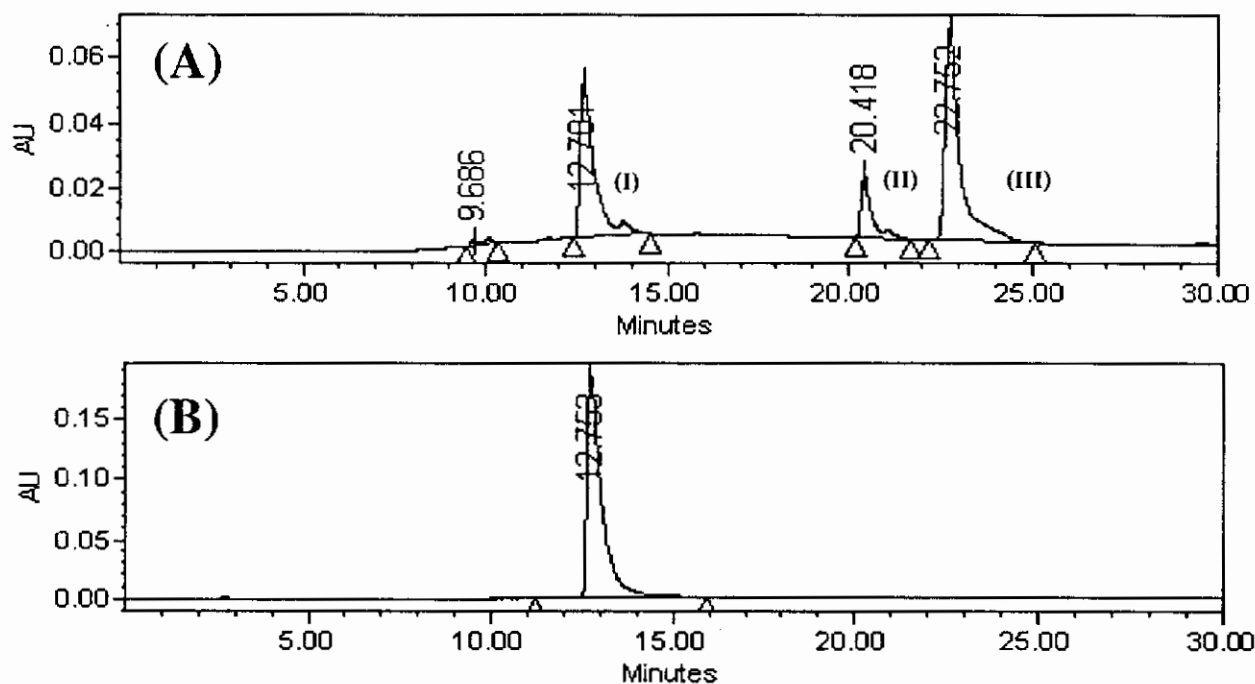


Figure 1. The chromatograms of crude (A) and purified pyocyanin (B): Chromatogram in Fig. (A) showing peaks of pyocyanin (I) and other phenazines [(II), (III)] using RP-HPLC under gradient elution system with C18 column and eluting with gradually changing composition of solvent A; water: TFA (100:0.01, v/v) and solvent B; water: acetonitrile: TFA (10:90:0.01, v/v), 1 ml min⁻¹ of flow rate and monitoring at 254 nm with photodiode array detector.

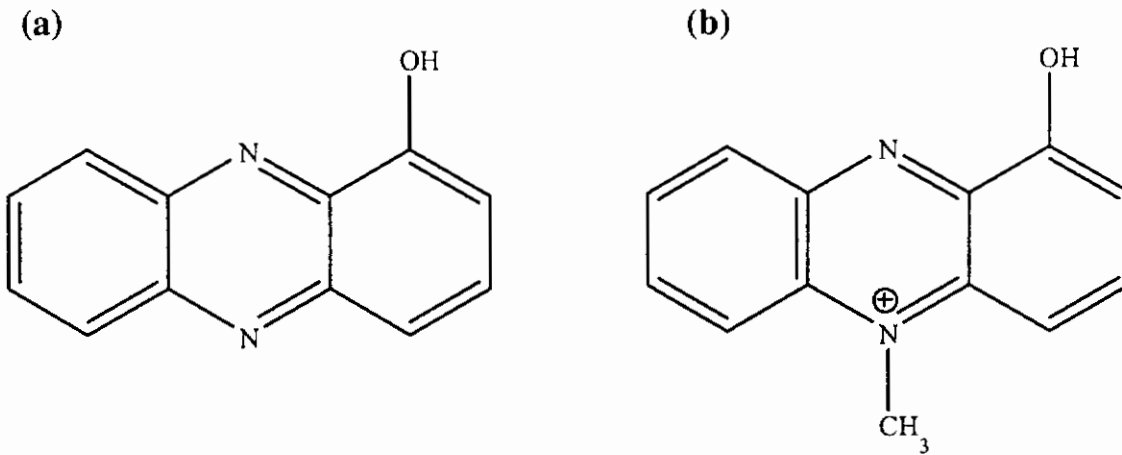


Figure 2. Chemical structures of 1-hydroxyphenazine (a) and pyocyanin (b) (Mavrodi et al., 2001)

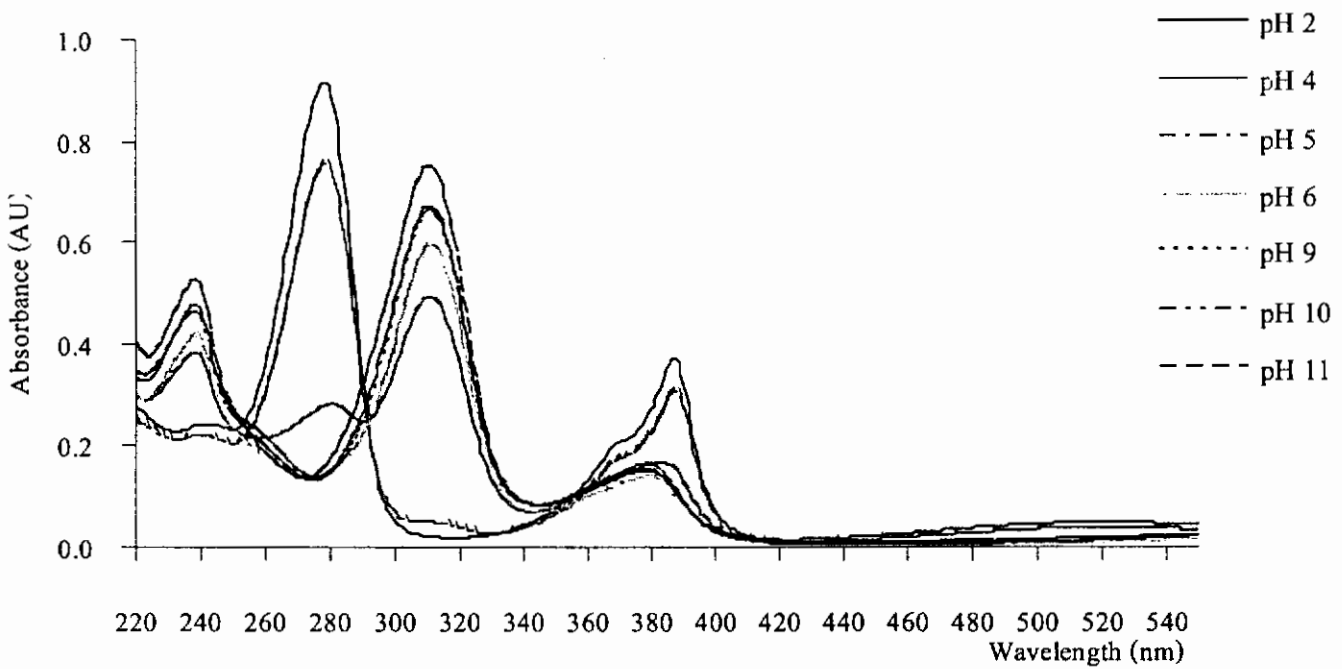


Figure 4. Effect of storage time on maximum absorption wavelength of pyocyanin at pH 11