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The Production, Separation and Stability of Pyoluteorin: A Biological Pesticide

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1. Introduction

Pyoluteorin (Plt, 4,5-dichloropyrrol-2-yl 2,6-dihydroxyphenyl), a polyketide antibiotic produced by certain strains of *Pseudomonas* spp., including the rhizobacteria *Pseudomonas fluorescens* CHA0 (Maurhofer et al., 1992), *Pseudomonas fluorescens* Pf-5 (Corbell & Loper, 1995), *Pseudomonas fluorescens* S272 (Yuan et al., 1998) and *Pseudomonas* sp. M18 (Hu et al., 2005) et al., was first identified and separated by Takeda from *Pseudomonas aeruginosa* (Takeda, 1958). Plt is a yellow crystal composed with a bichlorinated pyrrole linked to a resorcinol moiety (see Fig. 1), it can be completely dissolved in organic solvents such as methanol and chloroform (Wang et al., 2008). Plt can effectively inhibit phytopathogen fungi, including the plant pathogen *Pythium ultimum*, and suppress plant diseases caused by this fungus (Howell & Stipanovic, 1980; Maurhofer et al., 1992; Maurhofer et al., 1994). Moreover, in some instances, it contributes to the ecological competence of the producing strain within Rhizosphere (Howell & Stipanovic, 1980; Carmi et al., 1994; Yuan et al., 1998; Babalola, 2010).

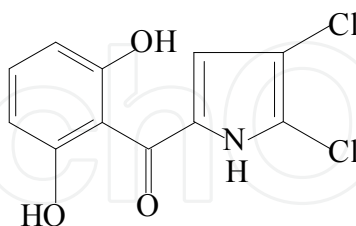


Fig. 1. Chemical structure of Plt

Many studies have focused on the metabolic regulation of Plt biosynthesis to enhance Plt production in cell cultivation. It was reported that the transcriptional activator gene *pltR* linked to Plt biosynthetic genes is required for Plt production (Nowak-Thompson et al., 1999). Whereas the regulator gene *pltZ*, which is located downstream of the Plt gene cluster in the genome of *Pseudomonas* sp. M18, could repress Plt production (Huang et al., 2004). Plt biosynthesis could be enhanced by the amplification of the housekeeping sigma factor *rpoD* in *Pseudomonas fluorescens* CHA0 (Schnider et al., 1995). The two component regulatory system GacS/GacA could positively regulate Plt production (Laville et al., 1992; Whistler et

al., 1998), and inactivation of *rpoS* (Sarniguet et al., 1995) or *lon* (Whistler et al., 2000) also resulted in the overproduction of Plt in *Pseudomonas fluorescens* Pf-5.

The culture conditions are usually important to the yield of any fermentation product. Carbon and nitrogen sources generally play a significant role because these nutrients are directly linked to cell proliferation and metabolite biosynthesis (Park et al., 2001; Casas Lopez et al., 2003; Li et al., 2008). In relation to this, Yuan et al. (Yuan et al., 1998) reported ethanol as a sole carbon source for Plt production by *Pseudomonas fluorescens* S272 cultivation in shake flasks. Duffy and Defago's study showed that Plt production was stimulated by glycerol but was repressed by glucose in cell culture of *Pseudomonas fluorescens* CHA0 (Duffy & Defago, 1999). The influence of other environmental factors on Plt production was also investigated by many researchers. In Brodhagen's study, Plt was found to be induced by itself in cell culture of *Pseudomonas fluorescens* Pf-5 (Brodhagen et al., 2004). In *Pseudomonas fluorescens* S272 cultivation, high NaCl concentration or heat shock could increase the production of Plt (Nakata et al., 1999). Chloride could also increase Plt biosynthesis in cell culture of *Pseudomonas fluorescens* YGJ3 (Matano et al., 2010). The minerals, such as zinc, could enhance Plt production in cell cultivation of both *Pseudomonas fluorescens* CHA0 (Duffy & Defago, 1999) and *Pseudomonas fluorescens* 4-92 (Saikia et al., 2009). However, there have been no reports on the effect of carbon and nitrogen ratio on Plt production through the fermentation of *Pseudomonas* spp. and no work was done on the scale-up fermentation for Plt production.

As a potential biological pesticide, understanding the stability of Plt under different environmental conditions as well as its residue in soil after incorporation into the environment was great important for its commercial use. Some analytical methods of Plt from the fermentation broth based on HPLC have been reported (de Souza & Raaijmakers, 2003; La Fuente et al., 2004). Kim et al. described a method for quantification analysis of Plt in fermentation broth with liquid chromatography-mass spectrometric (LC-MS) (Kim et al., 2003). Wang et al. developed a succinct quantitative method of capillary zone electrophoresis (CZE) for the determination of Plt in fermentation liquor of *Pseudomonas* sp. M18 (Wang et al., 2005). Trace determination of Plt in soil was done by Dong et al. using capillary electrophoresis (CE) (Dong et al., 2011). The degradation of Plt in water under different pH, temperature and light sources were studied by Zhang et al. (Zhang et al., 2010).

Pseudomonas sp. M18 is one of the plant growth promoting rhizobacteria (PGPR) selected in our lab, which can produce the secondary antifungal metabolites of Plt to suppress the root diseases caused by the soil-borne phytopathogens of crop plants (Hu et al., 2005). The chromosomally *rsmA*-inactivated and NTG mutation strain of *Pseudomonas* sp. M18R is a high Plt production strain obtained in our laboratory (Zhang et al., 2005). The identification of an effective medium formulation for Plt production is of great importance because it usually plays a pivotal role in cell growth and the production of metabolites. As a highly efficient anti-fungal metabolite, it is also important to develop the separation and purification method of Plt from its fermentation broth for large-scale preparation.

The medium optimization studies described in the literature have been conducted either by the "one-variable-at-a-time" technique or the "response surface method" (RSM) and "central composite design" (CCD) approach (Chang et al., 2002; Li et al., 2008). In this paper, the carbon or nitrogen sources and their initial concentrations were studied using the "one-variable-at-a-time" method for Plt production from *Pseudomonas* sp. M18R. Central composite design and response surface analysis were then applied to determine the optimal carbon/nitrogen ratio for Plt production. Influence of mineral amendment on Plt production was also investigated,

and the optimized medium was verified in both shake flask and bioreactor cultivation. The separation and purification method for Plt from the fermentation broth of M18R was investigated. The stability of Plt under different conditions were studied (Zhang et al., 2010), and a sensitive analytical method based on capillary electrophoresis (CE) for studying the degradation of trace amounts of Plt in soil samples was developed (Dong et al., 2011). The information obtained is considered fundamental and useful for the development of several *Pseudomonas* strain cultivation processes for efficient large-scale Plt production.

2. Materials and methods

2.1 Bacterial strain and seed culture conditions

The chromosomally *rsmA*-inactivated mutant strain of *Pseudomonas* sp.M18R was obtained as described by Zhang et al. (Zhang et al., 2005). The seed culture medium (with an initial pH of 7.2) consisted of the following components: glycerol, 18 g/L; peptone, 20 g/L; $K_2HPO_4 \cdot 3H_2O$, 0.732 g/L; and $MgSO_4$, 0.514 g/L. The stock culture was maintained on agar slants, which were inoculated with M18R, incubated at 28°C for 12 hours, and then used for seed culture inoculation. For the seed culture, about 5 mm² of the M18R bacterial slants was punched out with a sterilized cutter and was then transferred to a 250 mL Erlenmeyer flask containing 50 mL of the culture medium. The cultivation was maintained at 28°C on a rotary shaker (220 rpm) for 10.5 hours, reaching an OD₆₀₀ at about 1.1.

2.2 Experiments on M18R fermentation

The effects of carbon sources were studied using various carbon sources (18 g/L) such as glycerol, sucrose, glucose, fructose, lactose, maltose, and ethanol. For the investigation of nitrogen sources, 20 g/L of peptone, yeast extract, casein enzymatic hydrolysate, and casein acid hydrolysate were studied. For the investigation of initial carbon concentrations, different glycerol levels were used at 15, 18, 21, and 24 g/L. Furthermore, 12, 16, 20, and 24 g/L peptone was used for the study of initial nitrogen concentrations. In the experiments on the effects of carbon/nitrogen ratios, glycerol and peptone levels in the medium were changed, and a statistical approach was used. For the studies of mineral sources, 0.02 g/L $ZnSO_4$, $CuSO_4$, $CoCl_2$, $FeSO_4$, $MnCl_2$ or NaCl was added individually into the control medium. The cells were harvested on 72 h to analyze Plt production.

Inoculation was done by transferring 7.5 mL of the above seed culture broth to a 150 mL fermentation medium in a 500 mL shake flask. The fermentation medium was the same as the seed culture medium except for the conditions studied. The cultivation was conducted at 28°C on a rotary shaker at 220 rpm. Multiple flasks were run under each condition for all cultures. The cultivation data represent the mean values with the standard deviations from three independent flasks.

The scale-up fermentation was carried out in a 10 L SY-3000E bioreactor (Shiyou Company, Shanghai, China) with 6 L optimized medium. The fermentation was conducted at an aeration rate of 1.6 vvm and an agitation speed of 250 rpm for 96 h and the shake flask culture was done as control.

2.3 Central composite design

RSM was used to optimize the glycerol and peptone ratios for enhanced Plt production based on CCD (Li et al., 2008). The second-order model used to fit the CCD experimental results is shown in Eq. (1):

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where y is the predicted response, β_0 is a constant, β_i is a coefficient for the linear effect, β_{ii} is a coefficient for the quadratic effect, β_{ij} is a coefficient for the interaction effect, and x_i and x_j are the coded levels of variables x_i and x_j , respectively. The fitness of the second-order model was checked using the adjusted coefficient of determination R^2 , and its statistical significance was determined by the application of Fischer's F test. The software programs SAS (version 9.1 by SAS Institute Inc., NC, USA) and MatLab (version 7.1 by Mathworks, Inc.) were used for regression and graphical analyses of the data obtained, respectively.

2.4 Measurement of cell growth and Plt production

Cell growth was assayed in terms of optical density at 600 nm (OD600). A 1 mL culture broth was centrifuged at 12000 rpm for 5 min, and the sediments were re-suspended and diluted in distilled water.

The analytical procedures for Plt production were slightly modified as described by Zhang et al. (Zhang et al., 2005). For Plt extraction, 1 mL of each culture broth was mixed with the same volume of ethyl acetate. The upper layer was collected after complete agitation and centrifugation at 8000 g for 5 min. The water layer was then extracted once more with 0.5 mL ethyl acetate. The combined extracts were finally dried in a desiccated vacuum at 40°C and then dissolved in 1 mL of HPLC grade methanol. Using a Shimadzu LC-8A HPLC apparatus equipped with a variable-wavelength UV detector (Shimadzu, SPD-8A), 20 μ L sample was analyzed by reverse-phase HPLC. A Zorbax SB-C18 column (250×4.6 mm²; 5 μ m) was used at 25°C. The mobile phase consisted of 70% methanol (vol) and 30% water (vol), with the flow rate kept constant at 1 mL/min. Plt was monitored and quantified at 308 nm, and was then identified by comparison with its authentic sample.

2.5 Plt purification

Firstly, the fermentation broth was extracted with ethyl acetate and the crude Plt extract was obtained. Secondly, the crude Plt extract was separated by silica gel column chromatography with a glass column (700×50 mm²) packed with 500 g analytical grade silica of 100-200 mesh. The column was eluted with benzene-acetic acid (20:1, vol) and the elution which contained Plt was collected and evaporated at 40°C to dry, and the residue was dissolved in methanol (HPLC grade) for further purification.

Preparative HPLC was conducted for further purification of Plt using a Tigerkin C-18 column (300×20 mm²; 10 μ m) under 25°C with 50% methanol as the mobile phase. The flow rate was kept constant at 15 mL/min and the highest peak containing Plt was collected. Plt crystal was finally obtained after the elution was evaporated at 40°C to dry.

2.6 Trace analysis of Plt in soil samples (Dong et al., 2011)

A series of Gly-NaOH buffers with different pH values and concentrations were prepared. The stock solution of internal standard (IS) was prepared by adding 1.0 mg PCA into 10.0 mL methanol to fix its concentration at 100 μ g/mL. Standard solutions are prepared by dissolving Plt with different concentrations in methanol, forming a concentration gradient of 100, 75, 50, 25, 5, 2, 0.5 μ g/mL. All solutions and buffers were stored at 4°C.

Plt was extracted from soil samples (air dried, mixed and sieved through a 2-mm sieve) with ethanol. The extraction procedure was performed as follows: the working standard solution

was spiked into 10.0 g soil in an appropriate volume, which was ultrasonic extracted with 50 mL ethanol for 20 min. The extraction liquid was collected and the solid sample was transferred to the Soxhlet extractor for 4 h using another batch of 100 mL ethanol. After mixing and evaporating all organic phases, the dry residue was dissolved in 5 mL methanol and then centrifuged at 3000 rpm for 10 min. A portion of the 500 μ L methanol layer was distributed to one Eppendorf tube with an addition of 125 μ L PCA (25 μ g/mL). All solutions were stored at 4°C before injection into capillary.

The capillary-based experiments were carried out with an ACS 2000 HPCE apparatus (Beijing Cailu Scientific Inc., Beijing, China). The operating system is Windows XP professional SP2 with a HW-2000 chromatography work station (Qianpu Software Co. Ltd., Nanjing, China). Electrophoresis was performed in an untreated fused-silica capillary of 53 cm total length (44 cm effective length) \times 75 μ m I.D. (375 μ m O.D.) (Yongnian Optical Fiber Factory, Hebei, China). Before use, a new fused-silica capillary was pressure-rinsed with 0.1 M NaOH for 20 min, ultra-pure water for 20 min, 0.1 M HCl for 20 min, ultra-pure water for 20 min, and CE running buffer for 30 min orderly. The capillary was rinsed with CE running buffer for 5 min between injections and finally stored in water when not in use. The samples were injected in pressure mode at the inlet (13 mbar, for 45 s). The ultimate working voltage was 10 kV.

Plt degradation study in soil was carried out with samples of both near-surface soil (0~10 cm in depth) and rhizosphere soil (10 cm below surface), which were initially spiked with Plt at concentration of 500 μ g/kg. The extraction and analytical procedure were done as described above.

2.7 Degradation of Plt under different conditions

The degradation of Plt in water was done as describe by zhang et al. (Zhang et al., 2010). Plt solutions were prepared using our purified Plt and pure water, with the Plt concentrations varying between 121.5 mg/L and 626.5 mg/L. Temperature was thermostat-controlled, and irradiation was carried out in a Pyrex vessel, using natural sunlight. Samples were taken from the reactor periodically and were analyzed immediately by HPLC. The sample treatment procedure was as follows: 200 μ L ethyl acetate was added to the reactive solutions to stop the reaction and extract Plt. The organic solution was then dehydrated to obtain a powder residue of Plt. All experiments were preformed in duplicate.

3. Results and discussion

3.1 Optimization of M18R cultivation

3.1.1 Experiments on carbon sources and initial carbon concentrations

Carbohydrates are important carbon and energy sources for bacterial growth and metabolite biosynthesis. In the cell culture of *Pseudomonas fluorescens* S272, a high Plt production titer was obtained using ethanol as sole carbon source (Yuan et al., 1998). In the current work, we investigated the effects of ethanol as well as various common carbon sources, such as glycerol, sucrose, glucose, fructose, lactose, and maltose on M18R cultivation. The time profiles of cell growth (OD600) and Plt production are shown in Fig. 2. It can be seen that the cell grew well in glycerol, glucose, and fructose, and the maximum cell density was obtained in glucose after 72 hours of cultivation. A higher Plt production titer was obtained in glycerol and fructose for M18R. The order of maximum Plt production titer was 540.2 \pm 20.0, 401.2 \pm 24.8, 270.1 \pm 20.1, 212.6 \pm 12.6, 119.7 \pm 11.7, 37.2 \pm 3.2, and 45.7 \pm 3.7 mg/L in glycerol, fructose, ethanol, glucose, sucrose, lactose, and maltose media, respectively.

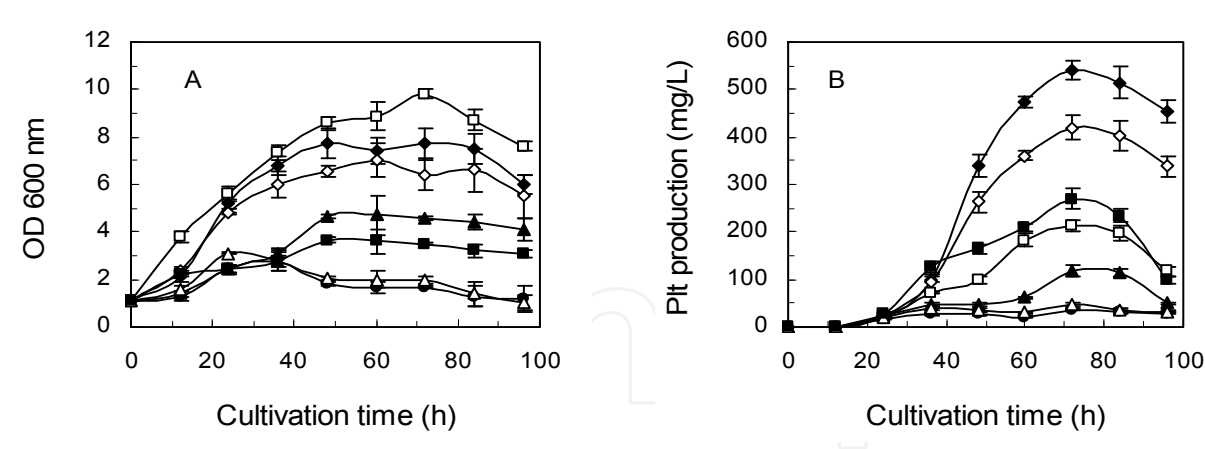


Fig. 2. Time profiles of cell growth (A) and Plt production (B) for the cell culture of *P. M18R* with different carbon sources (18 g/L). Symbols for different carbons: glycerol (dark diamond), fructose (open diamond), sucrose (dark triangle), maltose (open triangle), ethanol (dark square), glucose (open square), and lactose (dark circle). The error bars in the figure indicate the standard deviations from three independent samples.

Initial glycerol concentration (g/L)	Plt production (mg/L)	Plt productivity (mg/L per hour)	Plt yield on carbon (mg/g)
15 (72 h) ^a	460.3±27.8	6.39±0.39	30.7±1.9
18 (72 h)	568.3±30.1	7.89±0.42	31.6±1.7
21 (60 h)	464.3±31.9	7.74±0.53	22.1±1.5
24 (60 h)	423.7±19.8	7.06±0.33	17.7±0.8

Table 1. Effects of initial glycerol concentration on Plt production for *P.M18R* cultivation.
^a Cultivation time when the maximum Plt production was achieved.

Based on the above results, glycerol was selected as the carbon source for the cell culture of M18R. The effects of initial glycerol concentrations on Plt production are shown in Table 1. The highest production and productivity of Plt were obtained at an initial glycerol concentration of 18 g/L. The Plt production was decreased at high initial glycerol concentrations (21 or 24 g/L), the inhibitory effect of high initial carbon concentration on metabolite biosynthesis was also observed in ganoderic acid biosynthesis by *Ganoderma lucidum* (Fang & Zhong, 2002).

3.1.2 Experiments on nitrogen sources and initial nitrogen concentrations

In the cell culture, both carbon and nitrogen sources are very important for cell growth and metabolite production. Various organic nitrogen, including peptone, yeast extract, casein enzymatic hydrolysate, and casein acid hydrolysate, were studied in this paper. The time profiles of cell growth (OD600) and Plt production for the cell culture of M18R in different nitrogen were shown in Fig. 3. It is evident that the cells grew well in peptone, and a maximum Plt production titre of 553.2±27.2 mg/L was achieved after 72 hours of cultivation in the peptone medium. The effects of initial peptone concentrations on Plt biosynthesis were further examined, the results of which are shown in Table 2. The respective maximum Plt production and productivity of 568.3±30.1 mg/L and 7.89±0.42 mg/L per hour were obtained at an initial peptone concentration of 20 g/L.

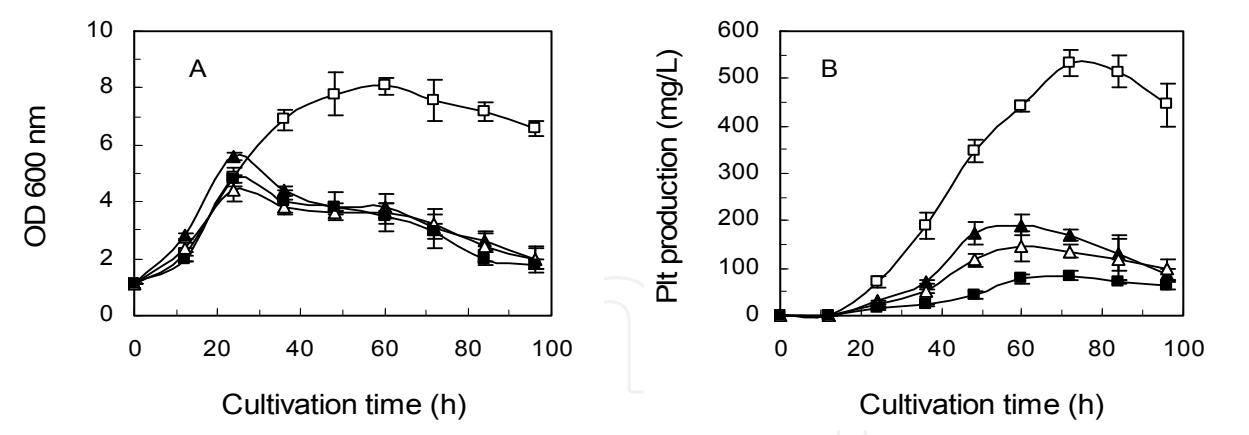


Fig. 3. Time profiles of cell growth (A) and Plt production (B) for the cell culture of *P. M18R* with different nitrogen sources (20 g/L). Symbols for different nitrogens: peptone (open square), yeast extract (dark square), casein acid hydrolysate (open triangle), and casein enzymatic hydrolysate (dark triangle). The error bars in the figure indicate the standard deviations from three independent samples.

Initial peptone concentration (g/L)	Plt production (mg/L)	Plt productivity (mg/L per hour)	Plt yield on carbon (mg/g)
12 (84 h) ^a	393.2±30.8	4.68±0.37	21.8±1.7
16 (72 h)	485.5±15.0	6.74±0.21	27.0±0.8
20 (72 h)	568.3±30.1	7.89±0.42	31.6±1.7
24 (60 h)	332.3±12.5	5.54±0.21	18.5±0.7

Table 2. Effects of initial peptone concentration on Plt production for the cultivation of *P.M18R*. ^a Cultivation time when the maximum Plt production was achieved.

3.1.3 Experiments on carbon/nitrogen ratio

The combined effect of carbon (glycerol) and nitrogen (peptone) was studied using the CCD because the concentrations of both carbon and nitrogen sources and their ratio are very important for metabolite production (Chang et al., 2002; Li et al., 2008). The levels of variables for CCD experiments were selected according to the results of the one-at-a-time strategy, and the coded (−1.414, −1, 0, 1, and 1.414) and real values of the variables at various levels are listed in Table 3. The experimental responses, along with the predicted response obtained from the regression equation, are also shown in Table 3.

Regression analysis was performed to fit the response function (Plt production) with the experimental data. From the variables obtained (Table 4), the model was expressed by Eq. (2), which represented Plt production (*y*) as a function of glycerol (*x*₁) and peptone (*x*₂) concentrations. Furthermore, the results of the F-test analysis of variance (ANOVA) in Table 5 showed that the regression was statistically significant (*P* < 0.05) at a 95 % confidence level. The model presented a high regression coefficient of 0.9694.

$$y(mg / L) = 577.732 + 71.327x_1 - 54.053x_2 - 46.802x_1^2 - 106.449x_2^2 - 24.010x_1x_2$$

(2)

Runs	x_1 (g/L) Glycerol	x_2 (g/L) Peptone	y (mg/L) Plt production	
			Observed	Predicted
1	21 (+1)	24 (+1)	444.8± 22.1 ^a	417.7
2	21 (+1)	16 (-1)	593.9± 35.2	573.9
3	15 (-1)	24 (+1)	311.8± 17.5	323.1
4	15 (-1)	16 (-1)	364.9±20.4	383.2
5	22.242 (1.414)	20 (0)	553.4± 10.9	585.0
6	13.758 (-1.414)	20 (0)	405.9± 25.0	383.3
7	18 (0)	25.696 (1.414)	278.9±14.1	288.4
8	18 (0)	14.304 (-1.414)	441.8± 19.4	441.3
9 ^b	18 (0)	20 (0)	548.1	577.7
10	18 (0)	20 (0)	573.6	577.7
11	18 (0)	20 (0)	602.1	577.7
12	18 (0)	20 (0)	589.6	577.7
13	18 (0)	20 (0)	575.1	577.7

Table 3. Experimental design and responses of the central composite design (CCD). ^a Samples were taken at 72h, and the standard deviation was calculated from three independent samples. ^b Runs 9-13 were replicates at the center point.

Parameters	Parameter estimate	Standard error	T value	Pr > t
Intercept	577.732	11.746	49.184	<0.0001
β_1	71.327	9.286	7.681	<0.0001
β_2	-54.053	9.286	-5.821	0.001
β_{11}	-46.802	9.958	-4.700	0.002
β_{22}	-106.449	9.958	-10.689	<0.0001
β_{12}	-24.010	13.133	-1.828	0.110

Table 4. Regression results from the data of central composite design (CCD) experiments x_1 : glycerol concentration; x_2 : peptone concentration

Regression	DF	Sum of squares	F value	Pr > F
Linear	2	64075	46.44	<0.0001
Quadratic	2	86495	62.69	<0.0001
Crossproduct	1	2306	3.34	0.110
Total model	5	152876	44.32	<0.0001

Table 5. ANOVA results for Plt production obtained from central composite design (CCD) experiments

The response surface plot obtained from Eq. (2) is shown in Fig. 4. Based on the canonical analysis produced by the SAS software, it is evident that Plt production reached its maximum at a combination of coded level 0.85 (x_1 , glycerol) and -0.35 (x_2 , peptone). The model predicted a maximum response of 617.6 mg/L Plt at 20.55 g/L glycerol and 18.6 g/L peptone levels as optimized medium components.

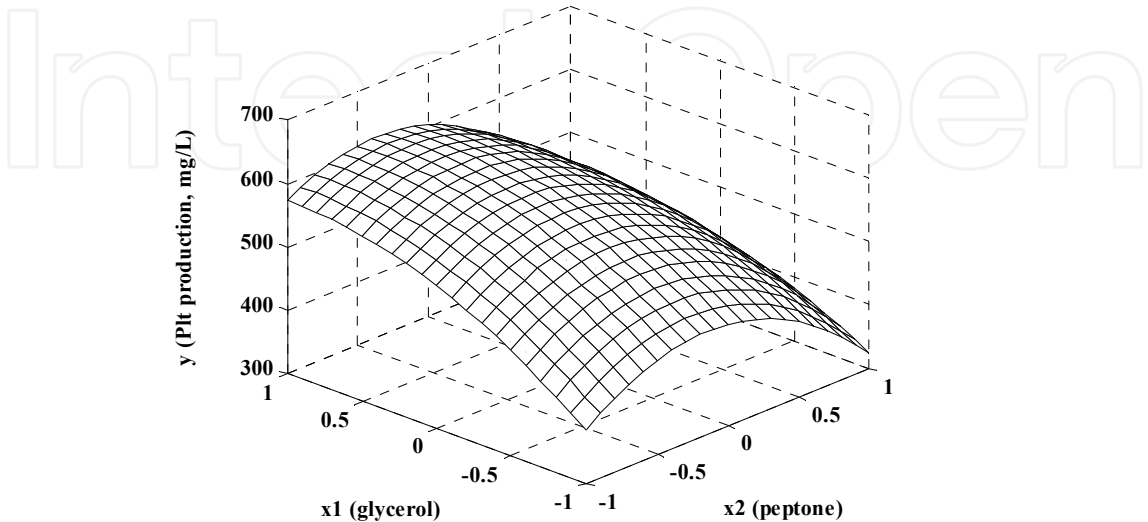


Fig. 4. The response surface curve of the effect of glycerol and peptone on Plt production by *P.M18R*.

To confirm the above prediction, experiments using both optimized (as predicted) and non-optimized media (18 g/L glycerol and 20 g/L peptone) were performed. The results are shown in Fig. 5. Similar time profiles of cell growth (OD600) were observed for both optimized and non-optimized media. A maximum Plt production titre of 648.3 ± 20.1 mg/L for 72 hours of cultivation was obtained for the cells cultured in the optimized medium, which was higher than that in the non-optimized medium.

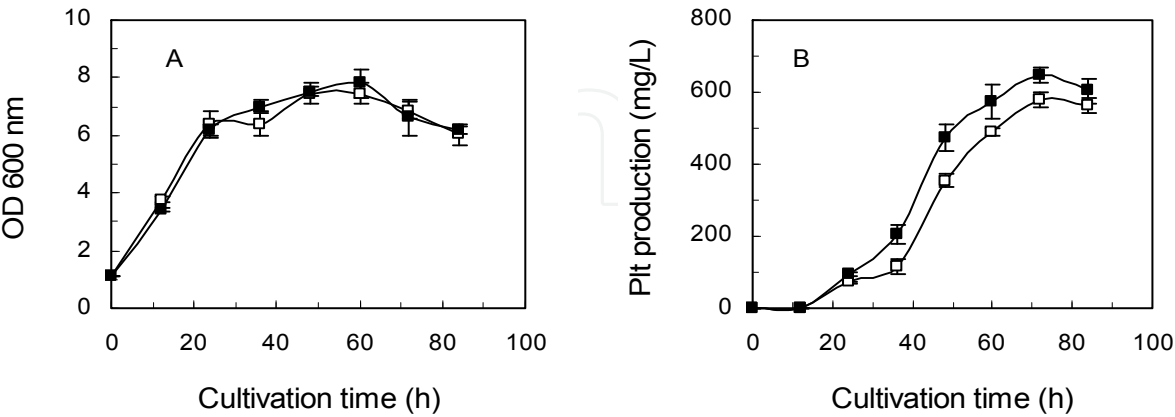


Fig. 5. Time profiles of cell growth (A) and Plt production (B) for the cell culture of *P. M18R* in different media. Symbols: non-optimized medium (open square), optimized medium (dark square). The error bars in the figure indicate the standard deviations from three independent samples.

The effects of major nutrients such as carbon/nitrogen sources as well as the ratios on Plt production were studied for the cell culture of *Pseudomonas sp.* M18R in order to obtain a suitable fermentation medium. The findings show that the most favorable carbon source for Plt biosynthesis in *Pseudomonas sp.* M18R was glycerol. The Plt production titre in a glycerol medium (540.2 ± 20.0 mg/L) was about 1.29, 2.00, 2.54, 4.51, 11.80, and 14.50 times of that in fructose, ethanol, glucose, sucrose, lactose, and maltose media for 72 hours of cultivation, respectively. The same results were obtained in the cell culture of the biocontrol strain *Pseudomonas fluorescens* CHA0. The respective Plt production titre in 48 hours was about 220, 50, and 10 ng/ 10^8 CFU in glycerol, fructose, and glucose media (Duffy & Defago, 1999). However, ethanol showed the highest Plt production for the cell culture of *Pseudomonas fluorescens* S272. The Plt production titre was about as twice of that in the ethanol medium (56 mg/L) as in the glycerol medium (26 mg/L) for 72 hours. Moreover, no Plt was observed in other carbons such as fructose, glucose, and sucrose, among others (Yuan et al., 1998). These results showed that the effect of carbon source on Plt production seemed to be strain dependent. For the organic nitrogen investigated, the Plt production titer was much higher in peptone than in yeast extract, casein enzymatic hydrolysate, and casein acid hydrolysate for our cell, and only a little Plt was observed in inorganic nitrogen such as ammonium nitrate (data not shown). In the cell culture of *Pseudomonas fluorescens* S272, similar result for the respective Plt production titer of 124, 88, and 31 mg/L in peptone, soybean, and ammonium nitrate medium was obtained (Yuan et al., 1998). According to the central composite design and response surface analysis, maximum Plt production (648.3 ± 20.1 mg/L) and productivity (9.01 ± 0.28 mg/L per hour) were successfully obtained using 20.55 g/L of glycerol and 18.6 g/L of peptone by the cell culture of *Pseudomonas sp.* M18R. The production titer of Plt for the cell culture of M18R obtained in this work increased about 10 times.

3.1.4 Experiments on mineral sources

The effects of mineral sources on Plt production in cell culture of M18R were investigated and the results were shown in Table 6. Plt production was influenced by addition of 0.02g/L $ZnSO_4$, $CuSO_4$, $FeSO_4$ or $CoCl_2$, whereas $MnCl_2$ or NaCl have no effects on it.

Culture medium	Plt Production (mg/L)
Control	609.5 ± 21.4
$ZnSO_4$	643.0 ± 26.9
$CuSO_4$	553.0 ± 25.1
$FeSO_4$	507.8 ± 17.8
$MnCl_2$	626.3 ± 33.2
$CoCl_2$	640.2 ± 30.9
NaCl	614.7 ± 28.4

Table 6. Effects of different mineral sources on Plt production for *P.M18R* cultivation on 72h. From Table 6 we could see that $ZnSO_4$ or $CoCl_2$ could enhance Plt production in *P.M18R*, the same results was obtained in *Pseudomonas fluorescens* CHA0 (Duffy & Defago, 1999). Plt production was decreased by the addition of 0.02 g/L $FeSO_4$ in *P.M18R*, but increased by

the addition of 0.15 g/L FeSO₄ in *Pseudomonas fluorescens* CHA0 (Duffy & Defago, 1999). High NaCl concentration (15 g/L) could increase the production of Plt in *Pseudomonas fluorescens* S272 (Nakata et al., 1999), however no effects was observed in our research. These results indicated that not only the mineral sources, but also the mineral concentration have obvious effects on Plt production. The optimal concentration for each mineral in cell culture of M18R is still under research.

3.1.5 Scale-up fermentation of M18R

A 10 L bioreactor was adopted to test the optimized medium conditions obtained in shake flask and the Plt production were shown in Table 7. Plt attained a maximal production of 708.1± 35.7 mg/L in 78 h, a little higher than that in flasks. The productivity of Plt in flask and bioreactor cultivation were nearly at the same level.

Culture condition	Plt production (mg/L)	Plt productivity (mg/L per hour)
Flask cultivation (72h)	673.8± 25.6	9.36± 0.57
Bioreactor cultivation (78h)	708.1± 35.7	9.08± 0.78

Table 7. Scale-up fermentation of *P. M18R* for Plt production.

3.2 Purification of Plt from the fermentation broth of M18R

3.2.1 Extraction of Plt from the fermentation broth

The solubility of Plt in different solvents was analyzed (see Table 8), and ethyl acetate was selected as the extract solvent.

Solvent	Plt solubility (g/L)
Methanol	761.7±26.1
Ethanol	750.8±34.5
Acetone	786.7±37.4
Ethyl acetate	520.7±12.8
Chloroform	90.1±3.1
Dichloromethane	13.82±0.06
Carbon tetrachloride	0.88±0.03
Water	0.75±0.01

Table 8. Solubility of Plt in different solvents at 293.2K.

The extract times and the volume ratio of the organic solvent used were studied, and the results were shown in Table 9. Every 5 mL fermentation broth was used for each condition and each experiment was carried out in triplicate. Plt content in the extract increased from 538.7 mg/L to 624.2 mg/L with the increasing of the volume ratio of ethyl acetate and fermentation broth from 0.5:1 to 2:1 for once extraction. When extracted twice, the volume ratio of organic solvent and culture broth have no influence on Plt extraction and all data for twice extraction were higher than once extraction.

Extract methods		Plt (mg/L)
Extract times	Volume ratio of ethyl acetate and fermentation broth	
Once	0.5:1	538.7±4.8
	1:1	597.6±33.9
	2:1	624.2±25.6
Twice	0.5:1	691.9±8.8
	1:1	698.0±60.0
	2:1	687.8±12.3

Table 9. Extract methods of Plt from the fermentation broth

From this result, 2 L fermentation broth was extracted with ethyl acetate twice and 1 L ester were used for each time. The extraction were combined and evaporated at 40°C to dry and 1.204 g of crude Plt was obtained.

3.2.2 Silica gel column chromatography

Samples of Plt extract (1.204 g) was mixed with 2.5 g silica gel and loading onto a glass silica gel column after grinding. The column was eluted with benzene-acetate acid (20:1, vol) and every 50 mL of the elution was collected and evaporated at 40°C to dry. The residues were dissolved in methanol and analyzed by HPLC, the fractions that contained Plt were combined and about 0.953 g of crude Plt was obtained. The HPLC chromatogram shown a purity of Plt was about 89% for this crude sample and further purification need to be done.

3.2.3 High performance liquid chromatography of Plt

Elution conditions of crude Plt were investigated on preparative HPLC column and the chromatogram were shown in Fig. 6. When 70% methanol was used as the mobile phase, the peaks of Plt and the main impurity were very near and their respective retention time were 7.26 min and 5.05 min. Decreasing methanol concentration from 70% to 50% could increase the retention time between Plt and the main impurity from 2.21 min to 17.04 min. Therefore, using 50% methanol as the mobile phase would be beneficial to the separation of massive samples on preparative column.

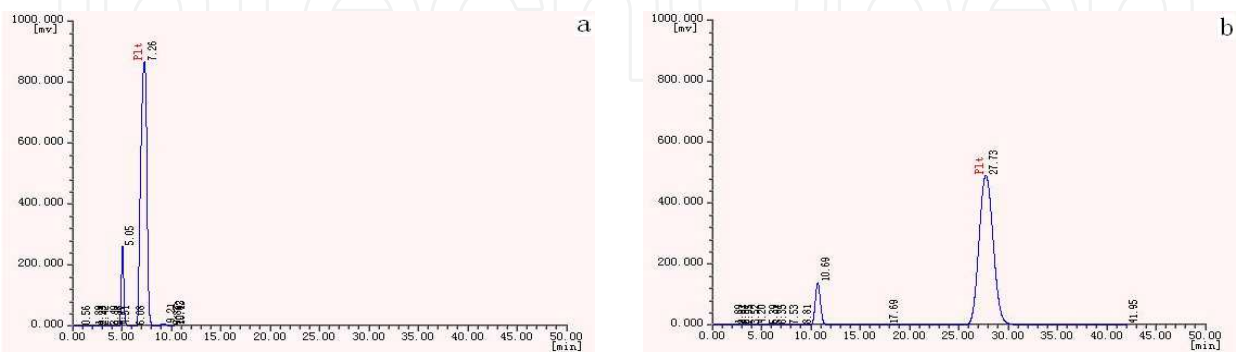


Fig. 6. Optimization of mobile phase on preparative HPLC. Conditions: C-18 column (300×20 mm², 5 μm); flow-rate: 15 mL/min; detection wavelength: 308 nm. a: 70% methanol; b: 50% methanol

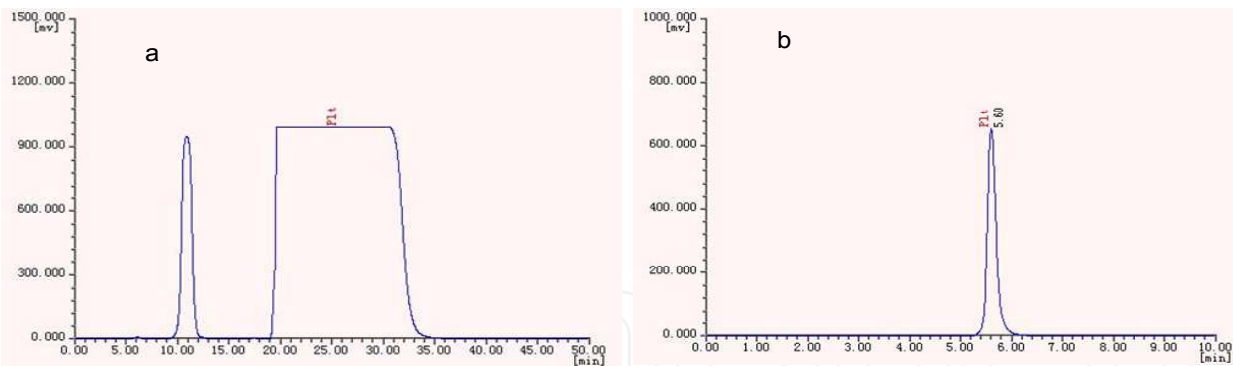


Fig. 7. a: Preparative HPLC chromatogram for Plt purification. Conditions: C-18 column (300 mm × 20 mm i.d., 5 μm); mobile phase: 50% methanol; flow-rate: 15 mL/min; detection wavelength: 308 nm. b: HPLC chromatogram of Plt after purification by preparative HPLC. Conditions: C-18 column (250 mm × 4.6 mm i.d., 5 μm); mobile phase: 70% methanol; flow-rate: 1 mL/min; detection wavelength: 308 nm.

Crude Plt (0.953g) was dissolved in 50% methanol to a total volume of 4 mL and then purified by preparative HPLC. The biggest peak (see Fig. 7a) was collected and evaporated to dry and 0.841g yellow crystal was finally obtained. Structure of the crystal was confirmed to be Plt by means of modern spectroscopic techniques, including UV, MS, ¹³C NMR and ²H NMR (data not shown). HPLC chromatogram of Plt crystal was shown in Fig. 7b and its purity was about 99%.

The results of Plt purification from the culture broth were shown in Table 10. From the table we could see that 0.841 g Plt was obtained from 2 L culture broth with a total recovery of about 69.8%.

Purification steps	Plt quantity (g)	Plt Purity (%)	Recovery (%)
Extract with ethyl acetate	1.204	38.8	100
Silica gel column chromatography	0.953	89.1	79.2
Preparative HPLC	0.841	98.9	69.8

Table 10. The results of Plt purification

3.3 Trace analysis of Plt by CE

3.3.1 Effect of applied voltage

For the trace analysis of Plt by CE, applied voltage (ranging from 5 to 25kV) has an obvious influence on migration time (Fig.8 (A)), peak height and peak area (Fig.10 (A)). With the decrease of the running voltage, the migration time of Plt increased and their corresponding peak height and peak area were heightened, which represented high detection sensitivity. Effect of applied voltage on separation of Plt and PCA (IS) was also investigated (Fig.9 (A)). The experiment showed no obvious variation on resolution. Considering the requirement for detection sensitivity, 10kV was selected as the optimum applied voltage in this experiment.

3.3.2 Effect of buffer pH values and buffer concentration

The pH of the electrolyte buffer (ranging from 8.3 to 9.0) plays an important role in both electroosmotic flow (EOF) and electrophoretic mobilities of the analytes, so as to impact the resolution (Fig.9 (B)). The experiment demonstrated that the increase of pH value led to a decrease in the migration time of PCA (Fig.8 (B)) and a certain increase in peak height but little change in peak area (Fig.10 (B)). However, the migration time of Plt (Fig.8 (B)) was reduced with pH values between 8.3 and 8.6, and then rose back when the pH value reached 8.6. Besides, the resolution of Plt and PCA decreased as the pH values increased, but Plt and methanol can not be separated with pH values below 8.4. For a comprehensive thought, the pH value of the background electrolyte buffer is controlled at 8.6, to get good resolutions of both Plt and PCA, and Plt and methanol.

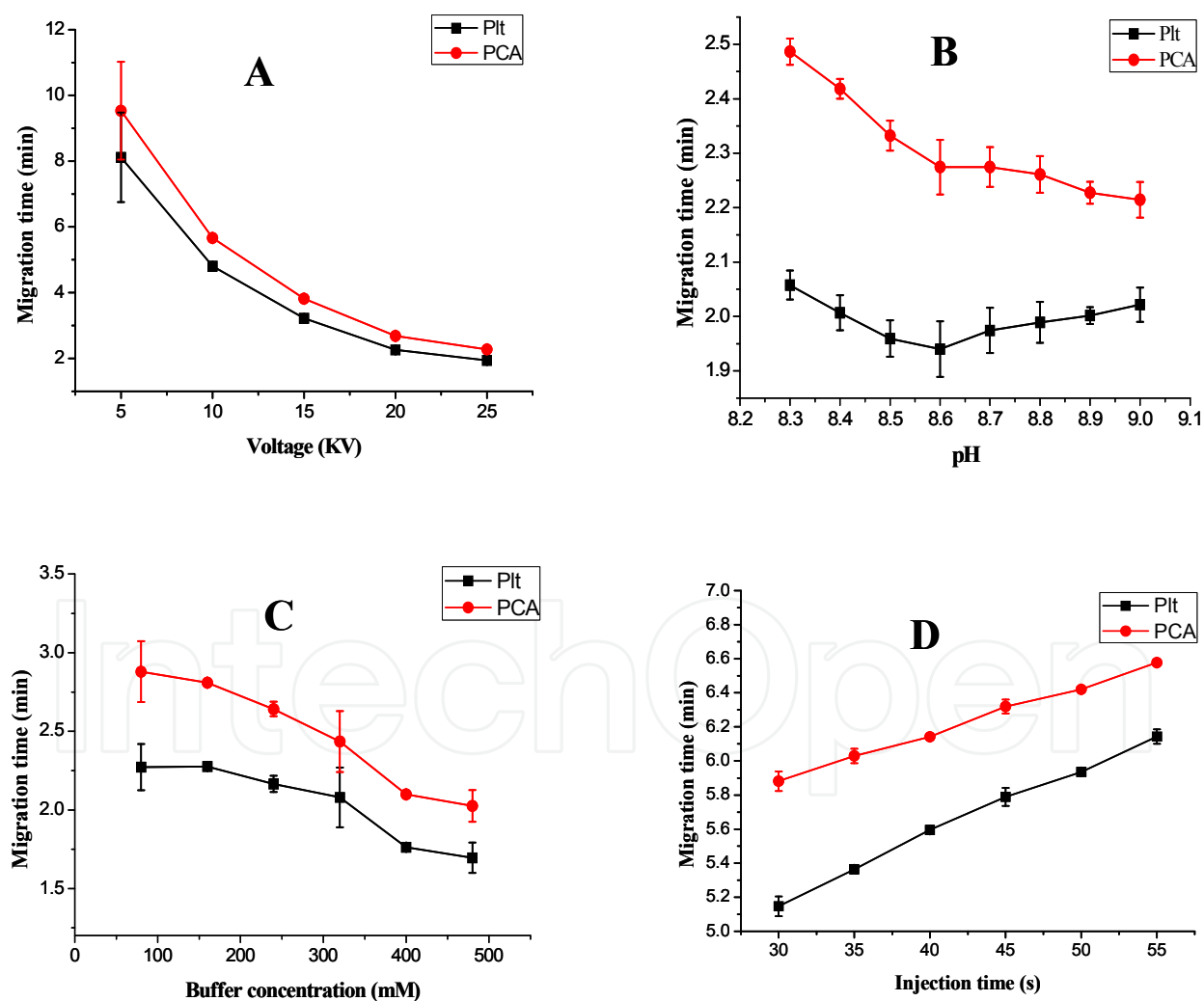


Fig. 8. Effects to the migration time of Plt and PCA in stacking CE by voltage (A), pH value (B), buffer concentration (C) and injection time (D)

3.3.3 Effect of buffer concentration

Different concentrations of Gly-NaOH buffer (ranging from 80 to 480 mmol/L) have evident influence on electric current, resolution and sensitivity. The results showed that the resolution of Plt and PCA (Fig.9 (C)) increased significantly with the increasing buffer concentrations. When the concentration was 240 mmol/L, the highest peak height and a relatively larger peak area of Plt (Fig.10 (C)) were gained. So, 240 mmol/L was selected as the optimum background buffer concentration in view of good detection sensitivity and resolution.

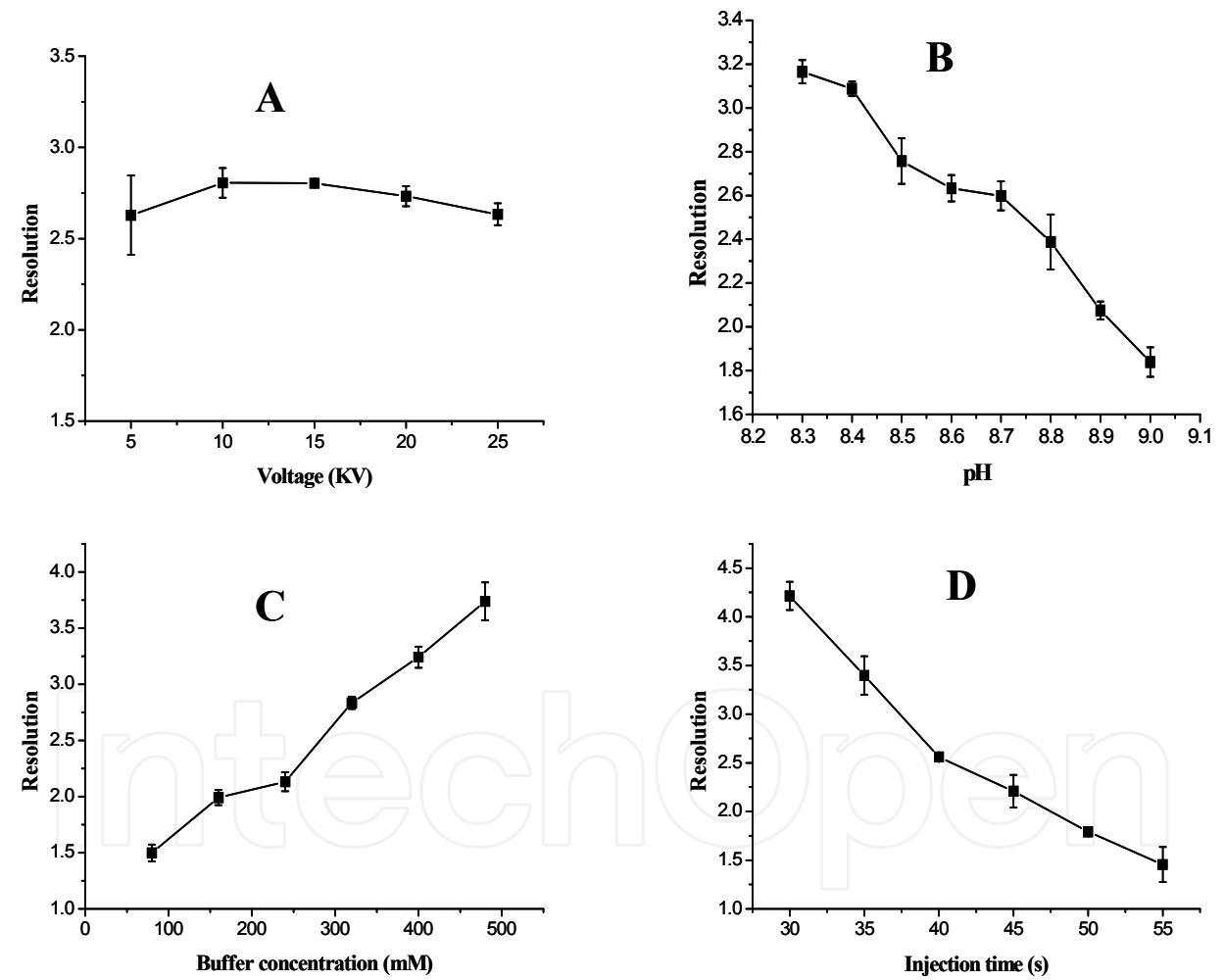


Fig. 9. Effects to the resolution of Plt and PCA in stacking CE by voltage (A), pH value (B), buffer concentration (C) and injection time (D)

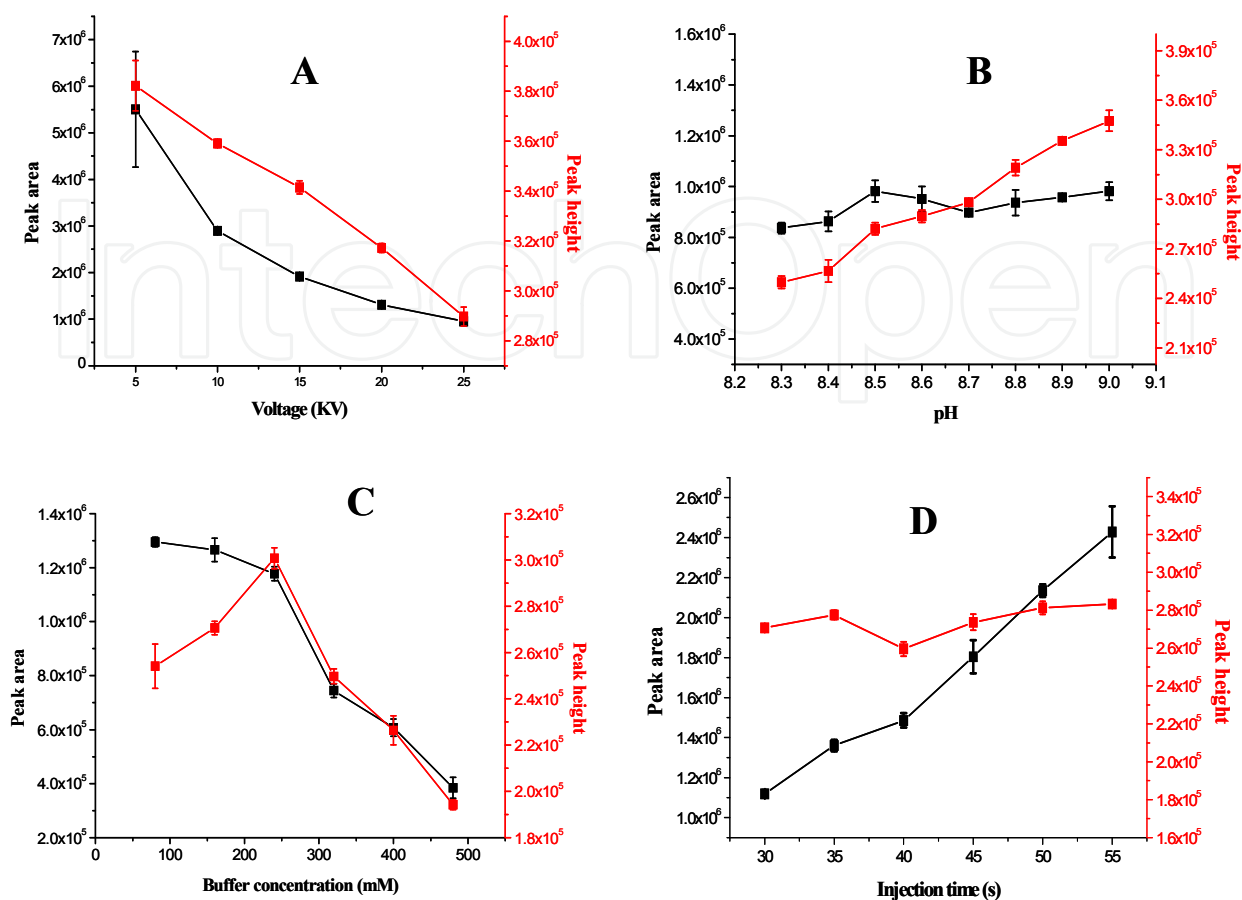


Fig. 10. Effects to the peak area and peak height of Plt in stacking CE by voltage (A), pH value (B), buffer concentration (C) and injection time (D)

3.3.4 Effect of injection time

The injection time (ranging from 30 to 55 s) impacts the sensitivity and resolution significantly. As the injection time increased, the resolution of Plt and PCA (Fig.9 (D)) got poorer (destroyed with injection time at 55 s), a great increase in peak area and little variation in peak height of Plt (Fig.10 (D)) was gained. Considering the requirement for both sensitivity and resolution, a 45 s injection time was selected.

3.3.5 Validity of the developed methods

Finally, we achieved the following optimized CE conditions: 240 mmol/L, pH 8.6 Gly-NaOH buffer, a fused-silica capillary of total length 53 cm \times 75 μ m ID, 375 μ m OD, with effective length 44 cm, 10 kV, 13 mbar 45 s pressure sample injection, and 25°C air-cooling for room temperature control. The method was validated for specificity, linearity and precision. The results showed that the method we developed had a good specificity and linearity for trace Plt analysis (Dong et al., 2011).

The precision of the developed method was evaluated by measuring inter-day and intra-day standard deviations (S.D.) and the relative standard deviations (R.S.D.) of both migration time and peak area ratios between the analyte and the IS. The intra-day values of S.D. and

R.S.D. were calculated based on six replicate injections of the four different concentrations within a day, while inter-day values were evaluated with six replicate injections of the same standard concentration in 6 days. The two precise results are listed in Table 11, which imply the stable analytic conditions and good repeatability of this method.

	Sample concentration	Migration time			Peak area ratio		
	($\mu\text{g/mL}$)	Mean (min)	S.D.	RSD (%)	Mean (10^6)	S.D. (10^6)	RSD (%)
Intra-day	100	6.295	0.223	3.54	4.585	0.298	3.64
	75	6.468	0.194	2.99	3.696	0.096	2.61
	25	6.323	0.068	1.08	1.171	0.039	3.36
	0.5	6.209	0.121	1.95	0.012	0.000	3.30
Inter-day	100	6.242	0.217	3.42	4.781	0.174	3.63
	75	6.309	0.297	4.58	3.411	0.125	3.65
	25	6.328	0.238	3.65	1.162	0.051	4.39
	0.5	6.002	0.080	1.33	0.014	0.001	4.77

Table 11. Intra-day and inter-day standard deviations of Plt determination (n=6)

CZE is a simple method for the monitor of Plt in fermentation liquor (Wang et al., 2005). However, it suffers from poor sensitivity due to the small sample amounts injected for detection. A field-amplified sample stacking advanced by Chien (Chien & Burgi, 1991) and Burgi (Burgi & Chien, 1991) was applied to overcome the limitation of CE. The analyte is dissolved in a diluted background electrolyte or pure solvent to form a sample solution with lower conductivity compared with CE buffer solution. When the high potential is applied to capillary, the sample zone generates an amplified electric field, which makes the analyte ions move faster until reaching the CE buffer zone. Thus, stacking could be achieved at the boundary between sample and CE zone. The method has been confirmed on sensitivity improvement of CE analysis for environmental samples (Albert et al., 1997; Palmarsdottir et al., 1997; Liu et al., 2008), and received approval in trace determination of pesticides and other environmental pollutants (Nunez et al., 2002; Carabias-Martinez et al., 2003; Lagarrigue et al., 2008). Herein, we developed a method of CE which combined field-amplified sample stacking to analyze trace amount of Plt. The limit of detection (LOD) of this method was 0.107 $\mu\text{g/mL}$, by setting the signal-to-noise ratio at 3:1, and the limit of quantification (LOQ) for Plt was 0.36 $\mu\text{g/mL}$ by setting the signal-to-noise ratio at 10:1. The LOD of our method was much lower than that of HPLC (10 $\mu\text{g/mL}$) (de Souza & Raaijmakers, 2003) and CEZ (0.66 $\mu\text{g/mL}$) (Wang et al., 2005). This result suggests that this method can meet with the requirement of pesticide residue analysis.

3.4 Degradation of Plt

3.4.1 Effect of light source

When a pesticide is used on farmland, it is exposed to sunlight, which includes both UV light and Vis light. Many compounds are light sensitive, such as aldicarb, parathion, mecoprop, linuron, and chlorpyrifos (Burrows & Canle, 2002; Anfossi et al., 2006). Thus, it is necessary to study the effect of UV/Vis irradiation on the degradation rate of Plt. We used a

fluorescent lamp (390-760 nm), an incandescent lamp (390-760 nm), a high-pressure mercury lamp (250-450 nm), and sunlight (280-800 nm) to investigate the effects of different irradiation on Plt degradation and the results is shown in Fig. 11. The photodegradation rate followed first-order kinetics. The half-life of Plt under fluorescent lamps (23.2 d) and incandescent lamp (23.5 d) showed little difference from that in the dark (24.9 d), whereas the high-pressure mercury lamp (3.1 d) and sunlight (3.5 d) greatly decreased its half-life.

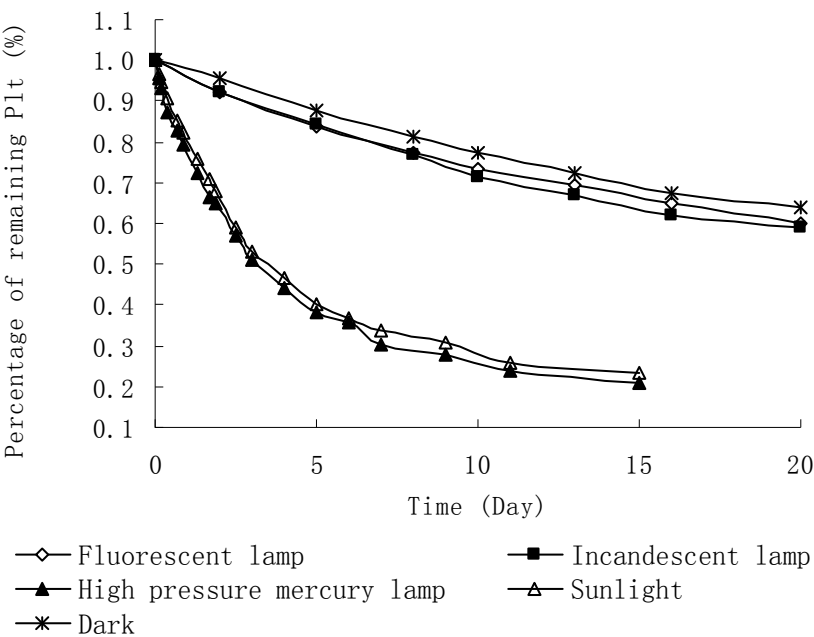


Fig. 11. The degradation of Plt on different light resources (stored at room temperature).

3.4.2 Effect of pH

Fig. 12 shows the photodegradation of Plt in buffered solutions at different pH levels. The degradation rate at different pH also followed first-order kinetics. The half-life of Plt decreased from 1.77 d to 0.42 d with the increasing pH increased from 5.8 to 7.8. In dark conditions, Plt is relatively stable at the pH range from 5.8~7.8 because the half-life were all

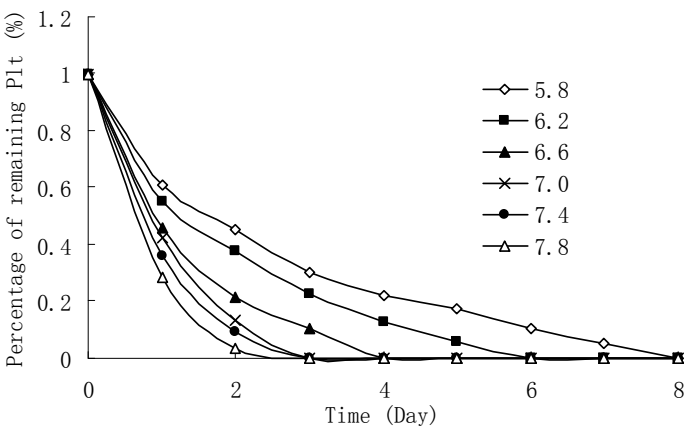


Fig. 12. The photodegradation of Plt at different pH values (room temperature, under the sunlight).

above 20 d (Zhang et al., 2010). This difference might be due to the change of UV-visible spectra of Plt at different solutions (Zhang et al., 2010). The higher efficiency of degradation of Plt under photo irritation at higher pH values can be due to the higher irradiation absorbance of higher-pH solutions.

3.4.3 Effect of water sources

A kinetic model was developed to study the degradation mechanisms and kinetics of Plt, and the results showed that Plt is relatively stable in pure water solutions and at room temperature (Zhang et al., 2010). Here, the stability of Plt in natural water at room temperature was investigated. The degradation of Plt in nature water also followed first-order reaction kinetics as that in pure water and the results were shown in Table 12. The half-life of Plt in nature water was much shorter (4.3 d in lake water and 5.2 d in rain water) than that in pure water (24.7 d), suggested that Plt is unstable in solutions of natural water.

Water source	Rate constant (d ⁻¹)	R ²	Half-life (d)
Lake water	0.1605	0.9955	4.3
Rain water	0.1343	0.9783	5.2
Pure water	0.0281	0.9564	24.7

Table 12. Effects of water sources on Plt degradation in the dark at room temperature

The stability of Plt for different treatment of lake water was shown in Table 13, the degradation also followed first-order reaction kinetics. The half-life of Plt in treated lake water (11.4 d in filtered lake water and 16.1 d in heated lake water) was much longer than that in untreated lake water (4.3 d), suggested that there might be Plt degrading bacteria in natural water.

Treat method	Rate constant (d ⁻¹)	R ²	Half-life (d)
Untreated	0.1605	0.9675	4.3
Filtered	0.0608	0.9483	11.4
Heated	0.0430	0.9964	16.1

Table 13. Degradation of Plt in treated lake water in the dark at room temperature

3.4.4 Plt degradation in soil (Dong et al., 2011)

Plt degradation study was carried out with samples of both near-surface soil (0~10cm in depth) and rhizosphere soil (10 cm below surface), which were initially spiked with Plt at concentration of 500 µg/kg. From the results shown in Fig. 13, it can be found that the degradation of Plt in rhizosphere soil was faster than that near-surface soil, which was possibly due to the higher microbial activity in rhizosphere soil. Plt degradation in studied soil tended to follow the first-order kinetics. The respective half-life values for Plt residue in near-surface and rhizosphere soil were 42.26 h and 32.84 h, much shorter than that in pure water.

On the whole the fast degradation of Plt under natural conditions (under sunlight, in soil, in natural water) demonstrated its excellent biological safety as an antimicrobial pesticide, but also revealed the disadvantage of not up to the requirement for a long control efficiency.

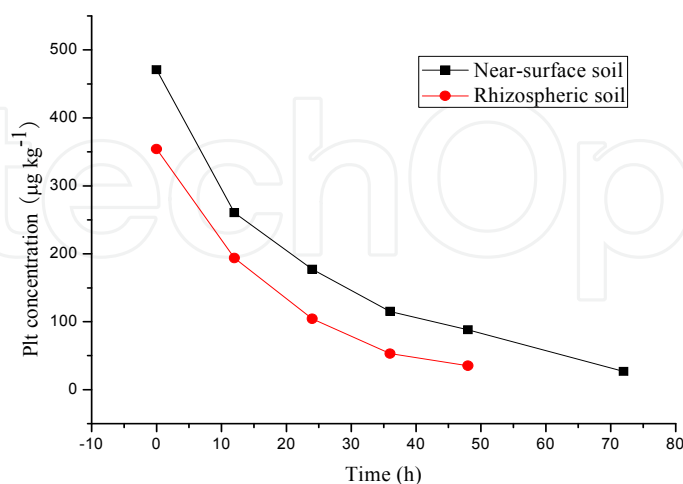


Fig. 13. Degradation curve of PCA in near-surface soil (■) and rhizosphere soil (●)

4. Colclusions

The effects of medium components on the production of Plt through cultivation of the *rsmA* inactivated mutant strain of *Pseudomonas sp.* M18R were investigated in shake flasks. The carbon sources examined were glycerol, sucrose, glucose, fructose, lactose, maltose, and ethanol, among which the 18 g/L glycerol was found to be the most favorable to Plt production. Meanwhile, the nitrogen sources examined were peptone, yeast extract, casein acid hydrolysate, and casein enzymatic hydrolysate, among which the 20 g/L peptone was found to be the most favorable to Plt production. To further enhance Plt production, the effects of carbon/nitrogen ratios were studied using central composite design and response surface analysis. The maximum Plt production titre of 648.3 ± 20.1 mg/L was achieved in a medium with optimized carbon and nitrogen (i.e., 20.55 g/L glycerol and 18.6 g/L peptone). The effects of inorganic components on Plt production were also studied. The optimized medium was conducted in bioreactor cultivation, and a maximal production of 708.1 ± 35.7 mg/L and productivity of 9.08 ± 0.78 mg/(L · h) was attained after 78 h cultivation. A simple and rapid method for Plt separation and purification from the fermentation broth of *Pseudomonas sp.* M18R was developed in this paper. Yellow Plt crystal with a purity of about 99% was obtained through ethyl acetate extraction, silica gel column chromatography and preparative HPLC with a total recovery of about 69.8%.

A sensitive analytical method based on CE with field-amplified sample stacking, Soxhlet's extraction and ultrasonic extraction for studying the trace amounts of Plt in soil samples was also developed. The results showed that this method was of high sensitivity, good linearity and good repeatability, and the whole processing procedures were proved with high recovery and convenience of analysis. This method can also be modified and developed to be applied in the determination of Plt in foods and drinking water.

The degradation of Plt under different conditions, such as water sources, pH value of solutions, UV intensity, soil et al., were investigated. Degradation of Plt followed first-order reaction kinetics, and it had a high degradation rate in soil, natural water and UV light. Plt

can decompose quickly in natural conditions suggested that it has good environmental compatibility. However, short residual time brings short reacting time. Thus, the method to improve its stability needs to conduct before its commercial use. The slow-release formulation of Plt in order to prolong its antifungal effects was achieved in our lab (Chen et al., 2010).

Overall, the findings of the study will be beneficial for the further development of the *Pseudomonas* spp. cultivation process in the large-scale production and the commercial use of Plt.

5. Acknowledgment

The financial support of the 973 Programs of China (No. 2009CB118906), the National Natural Science Foundation of China (NSFC project. 20706037), the 863 Programs of China (No. 2006AA10A209) and the Shanghai Leading Academic Discipline Project (Project Number: B203) are gratefully acknowledged.

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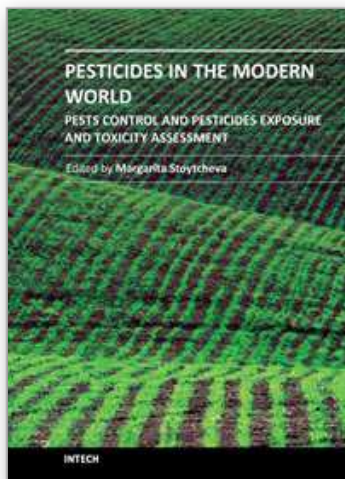
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Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment

Edited by Dr. Margarita Stoytcheva

ISBN 978-953-307-457-3

Hard cover, 614 pages

Publisher InTech

Published online 30, September, 2011

Published in print edition September, 2011

The present book is a collection of selected original research articles and reviews providing adequate and up-to-date information related to pesticides control, assessment, and toxicity. The first section covers a large spectrum of issues associated with the ecological, molecular, and biotechnological approaches to the understanding of the biological control, the mechanism of the biocontrol agents action, and the related effects. Second section provides recent information on biomarkers currently used to evaluate pesticide exposure, effects, and genetic susceptibility of a number of organisms. Some antioxidant enzymes and vitamins as biochemical markers for pesticide toxicity are examined. The inhibition of the cholinesterases as a specific biomarker for organophosphate and carbamate pesticides is commented, too. The third book section addresses to a variety of pesticides toxic effects and related issues including: the molecular mechanisms involved in pesticides-induced toxicity, fish histopathological, physiological, and DNA changes provoked by pesticides exposure, anticoagulant rodenticides mode of action, the potential of the cholinesterase inhibiting organophosphorus and carbamate pesticides, the effects of pesticides on bumblebee, spiders and scorpions, the metabolic fate of the pesticide-derived aromatic amines, etc.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Wei Wang, Hui Dong, Jingfang Zhang, Yuquan Xu and Xuehong Zhang (2011). The Production, Separation and Stability of Pyoluteorin: A Biological Pesticide, *Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment*, Dr. Margarita Stoytcheva (Ed.), ISBN: 978-953-307-457-3, InTech, Available from: <http://www.intechopen.com/books/pesticides-in-the-modern-world-pests-control-and-pesticides-exposure-and-toxicity-assessment/the-production-separation-and-stability-of-pyoluteorin-a-biological-pesticide>

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