Optimization of Paraquat Degradation by Microbial Consortium from Rhizosphere Soil

การหาสภาวะที่เหมาะสมในการย่อยสลายพาราควอทโดยใช้กลุ่มจุลินทรีย์ที่คัดแยกจากดินบริเวณรอบ ๆ รากพืช

Mullika Teerakun[1], Piyawadee Saraphirom[2] and Alissara Reungsang[3]
มัลลิกา ธีระกุล[1], ปิยะวดี สราภิรมย์[2] และอลิศรา เรืองแสง[3]

[1]Faculty of Agro-Industrial Technology, Kalasin University
[1]คณะเทคโนโลยีอุตสาหกรรมเกษตร มหาวิทยาลัยกาฬสินธุ์
[2]Faculty of Science and Technology, Rajabhat Maha Sarakham University
[2]คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยราชภัฏมหาสารคาม
[3]Faculty of Technology, Khon Kaen University
[3]คณะเทคโนโลยี มหาวิทยาลัยขอนแก่น

Abstract

The optimization of paraquat degradation by microbial consortium from rhizosphere soil was investigated. The ability of paraquat degradations was evaluated under the variation of media compositions, incubation temperature and initial pH levels. The microbial consortium was acclimatized by being cultured in LB (Luria-Bertani) broth and supplemented with 10 mg/L paraquat before used as seed inoculums. In different paraquat mineral salt medium (PMS), paraquat acts as a carbon and nitrogen source (PMS1), as a carbon source (PMS2) and paraquat act as a nitrogen source (PMS3). All three were used in this study. The media that gave the percentage of paraquat degradation was used for examining of temperature (15, 20, 25, 30, 35, 40 and 45 °C) and pH (5, 6, 7, 8 and 9) to degrade paraquat by microbial consortium. The maximum percentage of paraquat degradation (91%) was found in PMS3 and it was significantly different from PMS1 and PMS2 ($p < 0.005$). It indicated that paraquat was used as nitrogen source for culturing. The highest percentage of paraquat degradation (>90%) was found in PMS3 cultivation during 28 days at 30 °C and pH 7. The microbial consortium observed paraquat degrader was performed by serial dilution techniques and spread on PMS3 medium. Three strains of paraquat degrader were obtained as JP1, JP2 and JP3. The morphological characteristic of these strains was investigated under the microscope. JP1 and JP3 were gram positive rod while JP2 was gram negative cocci.

Keywords: paraquat, degradation, microbial consortium, rhizosphere soil

บทคัดย่อ

การศึกษาหาสภาวะที่เหมาะสมในการย่อยสลายพาราควอทโดยใช้กลุ่มจุลินทรีย์ที่มีความสามารถย่อยสลายสารพาราควอทที่คัดแยกจากดินบริเวณรอบ ๆ รากต้นมันสำปะหลัง เพื่อทดสอบความสามารถในการย่อยสลาย

พาราควอทภายใต้สภาวะต่าง ๆ คือ สูตรอาหารเลี้ยงเชื้อ อุณหภูมิในการเพาะเลี้ยง (15, 20, 25, 30, 35, 40 และ 45 °C) และความเป็นกรด-ด่างเริ่มต้นของอาหารเลี้ยงเชื้อ (5, 6, 7, 8 และ 9) ตามลำดับ โดยจุลินทรีย์ถูกเพาะเลี้ยงในอาหารเหลว LB ที่เติมพาราควอท 10 มก./ล. เพื่อทำให้คุ้นเคยกับสภาพแวดล้อมที่มีพาราควอทก่อนถูกนำไปใช้เป็นหัวเชื้อในการทดลองต่อไป จากนั้นศึกษาการย่อยสลายพาราควอทในอาหารเลี้ยงเชื้อ paraquat mineral salts medium (PMS) ที่มีสูตรอาหารแตกต่างกัน คือ ใช้พาราควอทเป็นแหล่งคาร์บอนและแหล่งไนโตรเจน (PMS1) ใช้พาราควอทเป็นแหล่งคาร์บอน (PMS2) และใช้พาราควอทเป็นแหล่งไนโตรเจน (PMS3) พบว่าเปอร์เซ็นต์การย่อยสลายพาราควอทโดยกลุ่มจุลินทรีย์ที่เพาะเลี้ยงในอาหาร PMS3 (91%) แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) กับการย่อยสลายพาราควอทโดยกลุ่มจุลินทรีย์ที่เพาะเลี้ยงใน PMS1 และ PMS2 เมื่อศึกษาอุณหภูมิและความเป็นกรด-ด่างที่เหมาะสมในการย่อยสลายพาราควอท พบว่ากลุ่มจุลินทรีย์ที่เพาะเลี้ยงใน PMS3 สามารถย่อยสลายพาราควอทได้ดีที่อุณหภูมิ 30 °C และความเป็นกรดด่างเริ่มต้นของอาหารเท่ากับ 7 โดยสามารถย่อยสลายพาราควอทได้มากกว่า 90% ภายในระยะเวลา 28 วัน จากนั้นกลุ่มจุลินทรีย์ที่มีความสามารถในการย่อยสลายพาราควอทถูกคัดแยกด้วยวิธี serial dilution spread plate technique บนอาหารแข็ง PMS3 โดยสามารถคัดแยกจุลินทรีย์ได้ 3 ไอโซเลท คือ JP1, JP2 และ JP3 จากนั้นนำไปศึกษาลักษณะภายใต้กล้องจุลทรรศน์ พบว่า JP1 และ JP3 มีลักษณะเป็นแท่ง และย้อมติดสีซาฟรานินบ่งชี้ว่าเป็นแกรมลบ ในขณะที่ JP2 มีลักษณะกลม และย้อมติดสีคริสตัลไวโอเลตบ่งชี้ว่าเป็นแกรมบวก

คำสำคัญ: พาราควอท, การย่อยสลาย, กลุ่มจุลินทรีย์, ดินบริเวณรอบ ๆ รากพืช

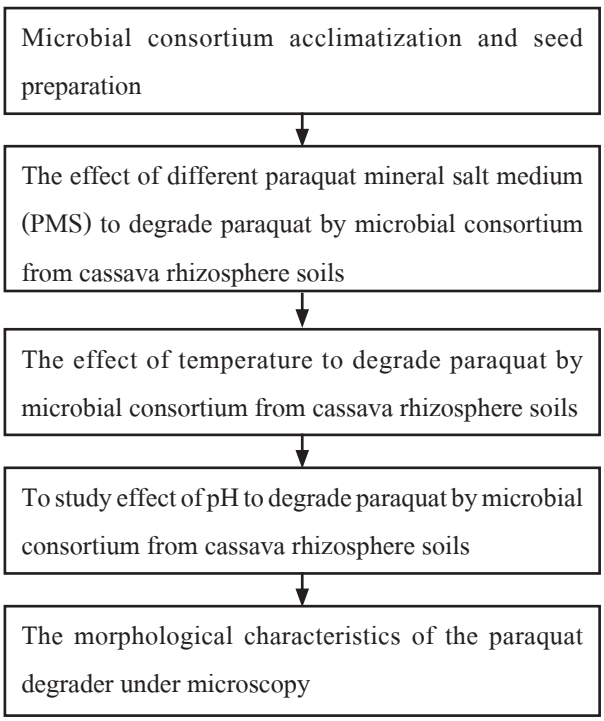
Introduction

The current, pesticide was widely use in agriculture as a result of being fast, convenient and low cost. Increasing the uses of pesticides in agriculture and domestic activities is polluting soil and water resources day by day. Pesticides, when applied could then accumulate to toxic levels in the soil and become harmful to microorganisms, plant, wild life and man (Amakiri, 1982; Hamadi, et al., 2004; Stanley, et al., 2013). Paraquat (1,1-dimethyl-4,4-bipyridyl dichloride) is a group of bipyridylium herbicides. It is quick-acting, non-selective contact herbicide that leaves no residues because of its rapid, irreversible adsorption to soil (Kreisig, et al., 1997; Pavlović, et al., 2014). It destroys plant tissue by disrupting photosynthesis and rupturing cell membranes, which allows water to escape leading to rapid desiccation of foliage (Dinis-Oliveira, et al., 2006; Watts, 2011). The paraquat usage has become abusive and has generated a great concern due to its damage for aquatic environment and human health, as a consequence of its large availability, low toxic dose and relatively low cost. Although this herbicide was banned in Europe since 2003, it is still utilized as a main herbicide in Thailand. The pesticides import statistics in Thailand reported that paraquat is the quantity imported in the top ten. It was imported into the second and volume reached 26,729,936 kg in A.D. 2012 (The office of Agricultural Regulation, 2012). Paraquat has high water solubility (700 g/l at 20 °C), making it is easily to be absorbed into the soil and potentially into drinking water supplies (Lock & Wilks, 2010) thus can spread easily in the environment. Paraquat strongly binds to soil particles and tends to strongly remain to for a long time in an inactive state, although it can also desorb again and become biologically active (Extension Toxicology Network, 1996). Pesticide contamination in soil was caused by high loading use, poor storage, washing and rinsing of equipment in agricultural. This factors leads to the problem of pesticide contamination in surface water and groundwater, subsequently (Struthers, et al.,

1998). The survey reported that paraquat was found to be contaminated in groundwater of central plains area in Thailand include Sing Buri, Ang Thong, Lop Buri and Chai Nat province (1.5-18.9 µg/L). A paraquat residue in surface water and sediment of Songkhram River was 9.26-87 µg/L (Division of Agricultural Toxic Substances, 2000). The detected paraquat are classified as at risk for the health of the population due to the amount allowed in drinking water (nearby 40 µg/L) (Gustafson, 1993). It is important to prevent the release of these contaminants into the environment.

Although several physical and chemical processes are used to remove paraquat in environment such as photocatalytic process, adsorption, photo-fenton process (Kanchanatip, et al., 2011; Tantriratna, et al., 2011; Trovó, et al., 2013; Pavlović, et al., 2014; Sieliechi & Thue, 2015). Bioremediation has the main role in pesticide biodegradation in the soil (Forouzangohar, et al., 2005; Ranjbar, et al., 2009; Ansari Shiri, et al., 2016). Bioremediation is considered as a harmless, efficient, and economical biotechnological pathway for the removal of herbicides (Reyad, et al., 2014). The use of microorganisms in the degradation and detoxification of many toxic xenobiotics, especially pesticides, is an efficient tool for the decontamination of polluted sites in the environment (Mohamed, 2009). Biological decontamination methods are preferable to conventional approaches because in general, microorganisms degrade numerous environmental pollutants without producing toxic intermediates (Pieper & Reineke, 2000; Furukawa, 2003). Paraquat is biodegradable, and that photo-degradation and hydrolysis were not major environmental degradation processes for paraquat. Several studies reported the biodegradation of paraquat by various microorganisms such as *Rhizoctonia solani*, *Lipomyces starkeyi*, *Pseudomonas putida*, *Achromobacter* sp., *Aerobacter oerogenes*, *Agrobacterium tumefaciens*, *Clostridium pasteureanum*, *Pseudomonas fluorescens* (Rodriguez-kabana, et al., 1966; Carr, et al., 1985; Hata, et al., 1986; Kopytko, et al., 2002). Furthermore, environmental parameters such as temperature, pH and nutrient can affect the level of toxicity of pesticide to microorganisms. Microorganism's activities directly related to the availability of nutrients required. Soil organic matters enhance the stimulation of microbial activity in soil and facilitate the biodegradation process (Pal, et al., 2005). The nutrients will allow the microbes produce the necessary enzymes, which will degrade the contaminants. Carbon is the most needed nutrient, followed by nitrogen, oxygen, hydrogen and phosphorus (Shahgholi, 2014). The grade of the degradation is increasing with the rise of the temperature, as proved in studies completed under tropical circumstances. The number of the biochemical reactions rise with the temperature rise, although above a certain temperature the microbial cells decease (Walker, et al., 2001). The objectives of this study were to evaluate the ability of microbial consortium to paraquat degradations under the variation of media compositions, incubation temperature and initial pH.

Scope of research



Materials and Methods

Soil sample

Soil samples were collected from 5 different rhizosphere soils in the cassava field with a history of using herbicide. It is expected that this will have on soil microorganisms adapted to degrade paraquat. The paraquat concentration in soil sample was analyzed by spectrophotometric method. It was found that no paraquat detected. Soil sample was passed through a 2-mm sieve (pore size) and air dried until the moisture content about 8% kept at 4°C. Before use, soil was dissolved in 0.1 M phosphate solution buffer (pH 7.5) and centrifuged at 4,000 rpm for 15 min to eliminate the residual nitrogen in the soil (Mandelbaum, et al., 1995).

Medium for microorganism growth

Paraquat mineral salt medium (PMS) was supplied with 10 mg/L paraquat and contained (per liter of distillation water) 1.6 g K_2HPO_4; 0.4 g KH_2PO_4; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl; 0.02 g $CaCl_2$; 1 mL of a trace element solution; 1 mL of a vitamin stock solution and 1 mL of $FeSO_4.7H_2O$ stock solution (5 g/L). The trace element solution contained 2 g/L boric acid; 1.8 g/L $MnSO_4.H_2O$; 0.2 g/L $ZnSO_4$; 0.1 g/L $CuSO_4$; 0.25 g/L Na_2MoO_4. The vitamin stock solution contained 100 mg/L of thiamine and 40 mg/L of biotin. The $FeSO_4.6H_2O$ stock solution and vitamin stock solutions were filter sterilized, kept at 4°C and added to the medium after autoclaving (Ansari Shiri, et al., 2016).

Microbial consortium acclimatization and seed preparation

The microbial consortium was acclimatized by Luria-Bertani (LB) broth supplemented with paraquat. Ten gram of cassava rhizosphere soils was added with 90 mL LB broth supplemented with 10 mg/L paraquat in a 250 mL Erlenmeyer flask. All experiments were incubated under aerobic conditions at room temperature using obital shaker at 150 rpm for 7 days. Ten milliliter of broth was transferred into the same formula of fresh media for 4 times. The culture acclimatization was performed to enhance paraquat degradation ability of microorganisms. The reduction of paraquat concentration in broth was checked in every time of transferation to confirm the degradable capability of microorganisms. After acclimatization step, the microbial consortium in broth were harvested using centrifugation at 4,000 rpm for 15 min under 4°C and the pellets were re-suspended in autoclaved minimal salt medium before using as the seed inoculums in next experiment.

The effect of paraquat degradation on different environmental conditions

The effect of different paraquat mineral salt medium (PMS), temperature and pH on paraquat degradation by microbial consortium were determined. The microbial consortium to degrade paraquat were carried out as a function of different paraquat mineral salt medium (PMS) supplemented with 10 mg/L paraquat; PMS1 is paraquat using carbon and nitrogen source, PMS2 is paraquat using carbon source and PMS3 is paraquat using nitrogen source. Experiment was conducted in a 250 mL Erlenmeyer flask containing 90 mL different paraquat mineral salt medium (PMS1, PMS2 and PMS3) and 10^6 CFU/mL of seed inoculums. It was incubated under aerobic conditions at room temperature, 150 rpm for 28 days. Culture broth was sampled at days 0, 3, 5, 7, 14, 21 and 28 to determine the paraquat residue. The media that gave the percentage of paraquat degradation would be used for examine the effect of temperature (15, 20, 25, 30, 35, 40 and 45°C) and pH (5, 6, 7, 8 and 9) to degrade paraquat by microbial consortium. Control experiment was conducted using the same experiment without inoculums.

Analysis of paraquat residue

Five milliliter of sample was taken and centrifuged at 4,000 rpm for 15 min. Supernatant were added with 1 mL of 2% $Na_2S_2O_4$ in 0.3 M NaOH. Paraquat residue was determined in spectrophotometric method by UV-1800 Spectrophotometer (SHIMADZU, Japan) at 600 nm (Rai, et al., 1997; AOAC, 2000). The percentage of recovery for this method is 93.

Statistical analyses

All experiments were carried out in triplicate, and the results were expressed as the mean. The significance analyses of experimental data were assessed using ANOVA with Duncan's New Multiple Range Test (DMRT) post-hoc pairwise comparison.

Results and Discussion

The effect of paraquat degradation on different environmental conditions

The effect of different paraquat mineral salt medium (PMS) on paraquat degradation by microbial consortium was determined. The function of different paraquat mineral salt medium (PMS) including; PMS1 is paraquat using carbon and nitrogen source, PMS2 is paraquat using carbon source and PMS3 is paraquat using nitrogen source. The result was shown in Table 1. The percentage of paraquat degradation by microbial consortium in PMS3 (91%) was significant different from PMS1 and PMS2 ($p < 0.005$). It indicated that paraquat was used as nitrogen source for culturing. Similar to reports from many researchers that paraquat used as sole nitrogen source by yeast *L. starkeyi* (Baldwin, et al., 1966; Yang & Funderburk, 1978; Carr, et al., 1985) and bacterial of *Aerobacter aerogenes*, *Agrobacterium tumefaciens*, *Pseudomonas fluorescens* and *Bacillus cereus* (Tu & Bollen, 1968). Funderburk (1969) reported that nitrate is the end product of paraquat when it is completely metabolized by yeast. Frequently found that microorganism isolated from contaminated soil with herbicide can utilize the herbicide as carbon or nitrogen source in synthetic media (Bozarth, 1966).

Table 1

Percentage of paraquat degradation on different formula paraquat mineral salt medium (PMS) by microbial consortium at day 28

Paraquat mineral salt medium (PMS)	Paraquat degradation (%)*
PMS1	56.65 ± 3.15b**
PMS2	59.99 ± 1.02b
PMS3	91.08 ± 1.80a
Control	6.90 ± 1.32c

*Mean value ± standard diviation of three replicates

**Different character indicates significant differences at $p < 0.05$ (DMRT)

The PMS3 media that gave the percentage of paraquat degradation would be used for examine the effect of temperature to degrade paraquat by microbial consortium. The highest effect in degradation of paraquat in medium was 93% when it was cultivated in PMS3 30°C during 28 days (Table 2). The limitation on degradation at temperatures below 15°C was found after 14 days. While temperature of 30-35oC, the microbial consortium was significant different to degrade paraquat after 28 days of incubation. It was found that paraquat degrader was capable of degrading paraquat over a wide range of temperatures, but the degradation occurred only after a protracted lag period (Carr, et al., 1985). Farahani, et al. (2008) also reported that temperature significantly affected on degradation kinetics of organic contaminant in soil. In addition, increasing of 10°C in temperature was enhancement of pesticide degradation while decreased

the half-life of pesticide by factor of 2-3 times because of high temperature is also favorable for microbial growth and enhances biological activity of pesticide (Lehman, et al., 1992; Singh & Kulshrestha, 1995).

Table 2

Effects of temperature on ability of the microbial consortium to degrade paraquat.

Temperature (°C)	Paraquat degradation (%)*
15	28.18 ± 1.54e**
20	41.47 ± 4.00d
25	73.75 ± 3.22b
30	92.88 ± 4.86a
35	89.77 ± 2.38a
40	64.13 ± 1.07c
45	40.72 ± 2.19d
Control	6.68 ± 0.97

*Mean value ± standard diviation of three replicates
**Different character indicates significant differences at $p < 0.05$ (DMRT)

Paraquat degradation using microbial consortium was carried out as a function of different pH values in PMS3 adjusted to pH 5, 6, 7, 8 and 9 at 30°C (Table 3). Optimum paraquat biodegradation occurred at pH 7, where the degradation process about 90% within 21 day, whereas in a culture with pH 6 and 8 the degradation of paraquat was reduced by 81 and 76%, respectively within 28 days. At pH 5 and 9, paraquat degradation after 28 day was only about 40%. According to their report, the organism was growth and also degraded paraquat over a wide range of pH values; in addition, the increasing of pH value supported the growth of microorganism while, the reduction in pH of the culture media would be effect from chemical changes of substrates which must have been precipitated by microbial enzymes (Atlas & Bartha, 1972; Margino, et al., 2000). Anderson and Drew (1972) found that the optimum pH range for cell proliferation between pH 5.0 and 6.5, whereas high paraquat degradation was obtained between pH 3.6 and 7.8.

Table 3

Effects of pH on ability of the microbial consortium to degrade paraquat.

pH	Paraquat degradation (%)*
5	41.17±3.36d**
6	81.31±2.16b
7	91.93±2.74a
8	75.77±3.17c
9	42.90±2.01d
Control	6.30 ± 1.37

*Mean value ± standard diviation of three replicates
**Different character indicates significant differences at $p < 0.05$ (DMRT)

Characteristic of paraquat degrader under microscopy

Figure 1 showed the characteristic of microbial consortium capable of degrading paraquat, three strains of microorganisms coded as JP1, JP2 and JP3 that can grow on PMS3 agar were obtained by serial dilution spread plate technique. The morphological characteristics of the isolates under microscopy were investigated. JP1 and JP3 was gram positive rod while JP2 was gram negative cocci.

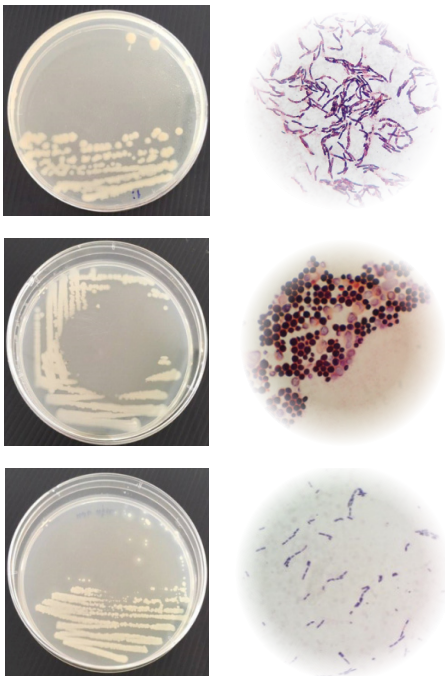


Figure 1 The morphological characteristics of the isolates under microscopy (a) JP1; (b) JP2 and (c) JP3.

Conclusion

1. The percentage of paraquat degradation by microbial consortium in PMS3 (91%) was significant different from PMS1 and PMS2 ($p < 0.005$). It indicated that paraquat was used as nitrogen source for culturing.

2. The highest effect in degradation of paraquat in medium was 93% when it was cultivated in PMS3 30°C within 28 days of incubation.

3. The optimum condition for degrading paraquat by microbial consortium was pH 7.

4. Three strains of microorganisms with paraquat degradable activity were JP1, JP2 and JP3. The morphological characteristics of the isolates under microscopy were gram positive rod (JP1 and JP3) and gram negative cocci (JP2).

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