Pentachlorophenol degradation by *Pseudomonas fluorescens*

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**ABSTRACT**

Fluorescent Pseudomonads strains were considered as plant growth promoting bacteria. They exhibited antagonistic activities against phytopathogens and showed bio-fertilizing properties. The strain *Pseudomonas fluorescens* PsWw128, isolated from wastewater, can use the pentachlorophenol (PCP) as the sole source of carbon and energy. High-performance liquid chromatography (HPLC) and spectrophotometric methods were used to follow the PCP degradation and biomass PsWw128 formation. However, the removal efficiency of PCP was highly significant. Thus, PsWw128 was able to degrade more than 99% of PCP when this isolate was grown under a high concentration of PCP (250 mg L\(^{-1}\)) in a mineral salts medium (MSM). The simultaneous utilization of glucose and PCP indicates the diauxic growth pattern of PsWw128. PCP addition (100 mg L\(^{-1}\)) in the growth medium can contribute to a decrease of the antibiotic susceptibility, and increase the biofilm development. In the presence of the toxic pollutant PCP (100, 200 and 250 mg L\(^{-1}\)), the antibiotic sensitivity showed a decrease concerning the seven antibiotics tested. Furthermore, the biofilm formation appeared very low with OD\(_{600}\) = 0.075 in the Brain infusion broth supplemented with 25% of glucose, and developed a significant growth with an OD\(_{600}\) = 1.809 in the MSM supplemented with 250 mg L\(^{-1}\) of PCP.

**Key words** | antibiotic susceptibility, biodegradation, biofilm, fluorescent *Pseudomonas*, HPLC, pentachlorophenol

**INTRODUCTION**

Pentachlorophenol (PCP) is an environmental contaminant compound which has been extensively used in herbicides, fungicides, pesticides and as preservatives for wood and wood products (Crosby 1981; McLellan et al. 2007; Liping et al. 2012). PCP is listed as a priority pollutant in the USA and Europe, and its use has become severely restricted worldwide (Carvalho et al. 2016; Khessairi et al. 2014). PCP has been classified by USEPA as a B2 probably human carcinogen based on sufficient evidence from animal toxicity studies and inadequate human clinical data (Oubina et al. 1997). This pollutant, highly toxic and recalcitrant compound (Maszenan et al. 2011; Hui et al. 2015), can be biodegraded by a limited number of bacteria. PCP degradation has been studied in both aerobic and anaerobic systems (Tripathi & Garg 2013; Khessairi et al. 2014). Pseudomonads are ubiquitous in soil and water ecosystems and are known for their ability to degrade PCP under aerobic conditions (Field & Sierra-Alvarez 2008; Haritash & Kaushik 2009; Zhao & Wong 2009). The species of Pseudomonas, in general, grow rapidly and are particularly renowned for their ability to metabolize an extensive number of substrates, including toxic organic chemicals, such as PCP molecules. Therefore, *Pseudomonas* sp. (Bu34) was able to degrade around 4,000 mg L\(^{-1}\) of PCP (Lee et al. 1998). Santosh
et al. (2010) reported that P. stutzeri CL7 was able to grow up to 600 mg L\(^{-1}\) of PCP. Shah & Thakur (2002) recorded that P. fluorescens was able to remove around 72% PCP in 96 hours of incubation added at 100 mg L\(^{-1}\) PCP in the growth medium. The antibiotic sensibility of Pseudomonas in the environment was known to be influenced by some toxic pollutants. The resistance against the growth condition and antibiotics facilitates its use for various processes under stressed conditions (Nickel et al. 1985).

Besides, bioremediation procedures using microorganisms are considered an effective alternative to remove hazardous chemical compounds such as water, carbon dioxide, and inorganic elements (McGinnis et al. 1991; Langwaldt & Puhakka 2000). The bioremediation has been shown to be efficient in PCP degradation (Duncan & Deveral 1964; McGinnis et al. 1991; Borazjani & Diechl 1998; Cserjesi 1998; Prewitt et al. 2005). The chemical procedures used for toxicity decrease and lessening are followed by the bioremediation process, involving native microorganisms. In this situation, Pseudomonas is well known as an attractive candidate in bioremediation and biocontrol activities (Wayne et al. 1988).

The objectives of this work are: (1) the evaluation of the capacity of PsWw128 isolate to degrade the PCP at a high rate under different physicochemical conditions; (2) the determination of the PCP effect on biofilm formation; and (3) the assessment of PsWw128 behavior concerning the antibiotic susceptibility in the presence of PCP.

**MATERIALS AND METHODS**

**Bacterial strain used in this study**

The Pseudomonas strain used in this study was isolated from municipal wastewater and produced pyoverdine as fluorescent pigment on King B agar. Based on the BLAST analysis of 16S rRNA, PsWw128 was identified in previous investigations (Mehri et al. 2011) as P. fluorescens/P. thievervalensis (accession number: HM627582). The ISO-Electric Focusing analysis of pyoverdine and pyoverdine-mediated iron uptake methods confirmed the strain assignation to P. fluorescens bv. V(PL8) (Mehri et al. 2011). Furthermore, this strain showed multifunctional properties as phosphate solubilization, indole-3-acetic production and antagonistic activity against pathogenic and phytopathogenic Pseudomonas.

**Chemicals and reagents**

PCP (MW 226.34 and >99% purity) was obtained from Sigma-Aldrich (USA). All other chemicals used were of the highest purity commercially available.

**PCP degradation analyses**

The degradation study was performed by inoculating 1% of inocula of 10\(^{6}\) cfu mL\(^{-1}\) of PsWw128 in 250 mL Erlenmeyer flasks, containing 100 mL of liquid medium (MSM: mineral salts medium). The composition of MSM was in mg L\(^{-1}\) as follows: KH\(_2\)PO\(_4\), 800; Na\(_2\)HPO\(_4\), 800; MgSO\(_4\)\(\cdot\)7H\(_2\)O, 200; CaCl\(_2\) 2H\(_2\)O, 10; NH\(_4\)Cl, 500 and 1 mL of trace metal solution comprising, in (mg L\(^{-1}\)), Fe SO\(_4\) 7H\(_2\)O, 5; Zn SO\(_4\) H\(_2\)O, 4; Mn SO\(_4\) 4H\(_2\)O, 0.2; NiCl 6H\(_2\)O, 0.1; H\(_3\)BO\(_3\), 0.1; Co Cl\(_2\) 6H\(_2\)O, 0.5; Zn Cl\(_2\), 0.25; EDTA, 2.5. PCP (250 mg L\(^{-1}\)) was added to the medium after autoclaving. The flasks were incubated at 30 °C under constant shaking at a rate of 160 rpm min\(^{-1}\) using an incubator shaker (ZHWWY-2102 P) for 168 hours. The bacterial cell growth was determined by measuring the optical density at 600 nm (OD\(_{600}\)) (Edgehill & Finn 1983; Nancy & Gustavo 2001). The degradation of PCP in the filtrate culture was determined by high-performance liquid chromatography (HPLC) as described by Yang & Lee (2006). PCP removal by the strain was confirmed using HPLC analysis; Perkin Elmer Series YL9100 system fitted on Symmetry C18 columns and UV detectors at 280 nm. The column was eluted in an isocratic mode using a mobile phase (acetonitrile/orthophosphoric acid) at a flow rate of 1 mL min\(^{-1}\). The cell suspension was centrifuged to separate the biomass at 8,000 rpm for 5 min, and the supernatant was filtrated through 0.22 μm filters (Karn et al. 2010).

Microorganisms require optimum conditions for their growth, survival and metabolic activities in the environment. These parameters essentially include the nature of carbon sources, the ambient temperature of growth, the pH, some other growth factors such as the initial substrate concentration, etc. Therefore, the influence of temperature
on the PCP degradation was tested successively at 25, 30 and 37 °C. The effect of different concentrations of PCP on the growth of PsWw128 and the degradation ability of this strain was also studied. PsWw128 was inoculated with 1 mL of 10^6 CFU mL\(^{-1}\) in 250 mL Erlenmeyer flasks containing 100 mL of MSM supplemented with different concentrations: 20, 50, 150, 200, 250 and 300 mg L\(^{-1}\) of PCP corresponding to 0.076, 0.19, 0.53, 0.75, 0.95 and 1.14 mM of PCP. Furthermore, the effects of other carbon sources such as glucose on the PCP biodegradation were evaluated in this study, according to the following different treatments: MSM + PCP (20 mg L\(^{-1}\)), MSM + PCP (20 mg L\(^{-1}\)) + Glucose 1%, and MSM + Glucose 1%. Each treatment was conducted in a 250 mL flask that was incubated at 30 °C under constant shaking for 168 hours. The influence of the pH on the PCP degradation was studied in MSM supplemented with 100 mg L\(^{-1}\) of PCP as the sole carbon source at different pH values, namely: 4, 7, 7.5 and 8.5. The pH of the medium was adjusted with 0.5 M NaOH or 0.2 M H\(_3\)PO\(_4\) as recommended by Kao et al. (2005). The removal percentage was estimated using the following formula:

\[
\text{removal (\%)} = \frac{\text{area} - \text{area}}{\text{area}}
\]

(Thakur 1995; Khesairi et al. 2014). Three replicates were prepared for each.

**PCP effects on biofilm formation**

**Congo red agar method**

Congo red agar (CRA) medium was prepared with brain-heart infusion broth (BHI) composed in g L\(^{-1}\) as follows: sugar 36, agar 17, Congo Red indicator 0.8 and PCP 0 or 100 mg L\(^{-1}\). After autoclaving, the glucose 56 g · L\(^{-1}\) and PCP (100 mg L\(^{-1}\)) were added to BHI growth medium. The CRA plates were inoculated with PsWw128 and incubated under aerobic conditions at 30 °C for 48 hours (Rewatkar & Wadher 2013; Turki et al. 2014).

**Microtiter plate biofilm formation assay**

The strain PsWw128 of *P. fluorescens* was cultured for 24 h at 30 °C in 5 mL of BHI (Djordjevic et al. 2002), MSM + PCP (100, 200 and 250 mg L\(^{-1}\)) (Del Castillo et al. 2012). The cultures were diluted 1:20 in the same medium, and 200 μL of the final suspension was added to each well of a 96-well tissue culture-treated polystyrene plate (Becton Dickinson, Franklin Lakes, NJ). After 24 h of growth at 28 °C (Meliani & Bensoltane 2014), the plates were washed vigorously three times with phosphate-buffered saline (PBS) (1X, pH 7.4) to remove unattached bacteria and visualized by staining with 1% crystal violet for 15 min after washing with ethanol acetone (80:20). The biofilm was quantified in quadruplicate assays (Akhter et al. 2014), after adding 200 μL of 95% ethanol absorbance was measured at 585 nm with shaking (Kaczorek & Olszanowski 2011; Turki et al. 2014).

**Antibiotic susceptibility testing**

Antimicrobial susceptibility of the strain PsWw128 was performed by using the disk diffusion method on Mueller Hinton agar according to the Clinical & Laboratory Standards Institute (CLSI) criteria. The following antibiotics were tested (μg/disc): Ciprofloxacin (Cip: 5 μg), Amikacin (AKN: 30 μg), Meropenem (Mer: 10 μg), Cefepine (FEP: 30 μg), Gentamicin (Gen: 10 μg), Imipen (Imp: 10 μg) Tobramycin (Tob: 10 μg), Pipercillin (Pip: 10 μg), and Tazo-bactem (Taz: 10 μg). Inhibition diameters were measured after 24 h of incubation at 30 °C (Borriello et al. 2004; Turki et al. 2012; Akhter et al. 2014).

**RESULTS AND DISCUSSION**

**PCP degradation in bacterial culture**

As shown in Figure 1(a), the growth of the strain PsWw128 (*P. fluorescens*) is characterized by a fast step of acclimation and an exponential period of around 10 hours. The effects of carbon sources (glucose addition) on the PCP biodegradation by the strain of *P. fluorescens* clearly appeared. Glucose is used as the primary substrate in this experiment. Hence, the glucose activated the growth of the PsWw128 strain, and resulted in the degradation of PCP in only 1 day (Figure 1b). The best strain activity was observed in PCP MSM + glucose with an optical density (OD\(_{600}\) = 0.880) greater than the one registered in the presence of the PCP MSM medium as sole carbon source (OD\(_{600}\) = 0.156). The obtained results indicated that the glucose as co-substrate contributes effectively to the PsWw128 strain.
growth. In addition, the results showed that the growth of this strain was improved after the addition of glucose as co-metabolism phenomena (Premalatha & Rajakumar 1997).

The use of PCP as a growth carbon source in a liquid mineral medium and under aerobic conditions was revealed through HPLC analysis and OD measuring. On the other hand, the effect of PCP and an additional carbon source as glucose on the bacterial growth was tested in different culture conditions. The degradation capacity of this isolate of PsWw128 of *P. fluorescens* was improved in the presence of glucose. In this context, previous reports showed that *P. cepacia* could degrade PCP only in the presence of additional carbon sources (Karns et al. 1983).

Figure 2 shows that *P. fluorescens* utilizes PCP as the sole carbon source (primary substrate), since the biodegradation efficiency of the strain PsWw128 was evaluated every 24 h by HPLC analysis and OD measuring. A correlation was noted between the different growth phases and the degraded activity. Therefore, this strain presented three distinct growth phases: an acclimation or adaptation phase ranging between 0 and 96 h, exponential phase growth ranging between 96 and 120 h, and finally a latency phase ranging between 120 and 168 h. Thus, *P. fluorescens* strain growth was significantly increased up to 96 h and decreased thereafter, attaining a stationary phase at 120 h. It was observed that PsWw128 utilized more than 13% of PCP within 24 h, and above 99.9% within 120 h. In this study, we used the PCP as the sole growth carbon source in a liquid mineral medium and under aerobic conditions. In this context, several authors showed that aerobic bacteria were able to utilize PCP compounds as the sole carbon and
energy source (Thakur et al. 2001; Solyanikova & Golovleva 2004). A study conducted by Radehaus & Schmidt (1992) described the effect of *Pseudomonas* sp. strain RA2 on PCP degradation, portraying that this strain could achieve the mineralization of 160 mg L$^{-1}$ PCP in 7 days. Similar observations were reported by Shah & Thakur (2002) who studied *P. fluorescens* (TE3), which could mineralize around 72% of PCP (100 mg L$^{-1}$) in 96 hours.

Moreover, the effect of incubation temperature on the MSM liquid medium supplemented with 250 mg L$^{-1}$ of PCP was investigated. Three incubation temperatures ranging between 25 and 37°C were studied in this work. The results showed that the bacterial growth was directly influenced by the incubation temperature, and the optimum growth of this bacterium was observed at a temperature of around 30°C (Figure 3(a)).

Furthermore, the incubation temperature directly influenced the bacterial growth and the PCP degradation. Similar observations were reported by Chandra & Abhishek (2010), who showed that the degradation activity of organic pollutants usually requires an incubation temperature ranging between 28 and 30°C. In addition, Karn et al. (2010) investigations demonstrated that *P. stutzeri* isolated from the environment could degrade PCP at temperatures ranging between 25 and 35°C. Also, Yang & Lee (2006) showed that a strain of *P. resinovorans* could degrade PCP at a temperature of around 31°C.

Figure 3(b) presents the effect of liquid medium pH on the growth and the PCP degradation activity. Therefore, the isolate PsWw128 showed a maximum growth and degradation activity in the pH range of 4 and 7 with 100% of PCP degraded. However, the bacterial growth increased slightly at pH 8, but the degradation activity decreased strikingly to reach only 10% of PCP degradation, thereby at pH 8 the PCP appeared as toxic. On the other hand, the pH values around 8.5 allowed a maximum of PCP degradation and an activity of around 100%. The study conducted under optimal conditions of pH 8.5 and temperature
growth of 30 °C showed the change in PCP degradation efficiency and the energy yield under an increased PCP concentration in a single liquid medium, MSM. The variation of the PCP concentration in MSM slightly influenced the degradation activity of the strain PsWw128, and showed approximately 80% of PCP degradation when the PCP concentration varied from 20 to 250 mg L⁻¹. Nevertheless, the use of PCP above 200 mg L⁻¹ increases the PCP degradation rate of around 100% (Figure 3(c)).

Further, xenobiotic degradation by microorganisms was strongly influenced by the experimental conditions. Temperature, pH and toxic substances in the medium are expected to play a very important role in the removal efficiency (Dordio & Carvalho 2015). In this study, the maximum PCP degradation was observed at pH ranging from 4 to 7. These results differ from the conclusion of Karn et al. (2010), which portrayed that the peak of P. stutzeri CL7 PCP degradation was observed between 7.5 and 8.5. The optimal pH for P. mendocina NSYSU to biodegrade PCP determined at pH 6. Another study reported that a strain of P. aeruginosa degraded PCP at pH of between 7 and 8 (Wolski et al. 2006).

**PCP effects on the qualitative and quantitative biofilm formation**

Biofilm formation is a beneficial characteristic for a bacterial strain to be active in bioremediation. On the Congo red agar mucoid, white rose or colorless and convex colonies were observed in BHI agar, but in the presence of PCP at 100 mg L⁻¹, the PsWw128 showed a brown and mucoid colony (Figure 4). Briefly, attached bacterial cells were stained with crystal violet solution and crystal violet absorbance was measured using a microplate spectrophotometer. This biofilm formation appeared to be influenced by the increased PCP concentration of 100, 200 and 250 mg L⁻¹, to reach OD₅₈₅ values of 0.46, 1.50 and 1.80, respectively (Figure 5). The biofilm adherence to an abiotic surface increased when the amount of PCP was augmented. Thus, the increase in the medium’s toxicity has an effect of strengthening the development of the biofilm formation. It is known that the biofilm formation or production was considered as an important factor for survival in hostile and unfriendly environments. On the other hand, PsWw128 growing on BHI without PCP do not adhere to polystyrene surfaces (OD₅₈₅ ≤ 0.1).

On the other hand, the study of the P. fluorescens strain’s ability to produce a biofilm on contaminated PCP medium showed that the addition of the xenobiotic PCP in the growth medium at an increasing rate enhances the biofilm formation capacity. Meliani & Bensoltane (2014) reported that the P. fluorescens and P. aeruginosa isolates develop an important biofilm mass development, to protect cells from hostile environments. Therefore, the biofilm lifestyle can protect microorganisms against the host immune system and harmful environmental conditions. This protection enables microorganisms to survive and prosper (Anderson & Toole 2008). So, biofilm formation and development played an important role in the bioremediation of some waste contaminated sites (Del Castillo et al. 2012). Biofilm reactors or devices are very interesting for the remediation of xenobiotic compounds (Das et al. 2012). Thus, environmental stress conditions could affect biofilm production (Poole 2012), since biofilm development is considered as a natural strategy to maintain a favorable niche in stressful and unfriendly environments (Meliani & Bensoltane 2014). A number of studies suggested a close relationship between hydrocarbon degradation and biofilm formation (Dasgupta et al. 2013). Previous investigations have shown that the degradation of environmental pollutants is more efficient by microorganisms in biofilms than by free-living bacteria (Singh & Walker 2006; Verhagen et al. 2011). As an example, P. stutzeri biofilm-associated cells successfully degraded naphthalene and survived in petroleum contaminated soil, although biofilm cultures could
degrade the pesticide chlorpropham slower than planktonic cultures, and could not produce intermediate toxic compounds (Verhagen et al. 2011). The biofilm systems were efficient in removing toxic trace compounds in wastewater, according to various electrostatic and hydrophobic interactions and adsorption/desorption phenomena.

Antibiotic susceptibility testing

The antibiotic resistance of Pseudomonas strains isolated from the environment has been increasingly reported worldwide. In this study, we investigated the antimicrobial resistance character of the strain PsWw128 in hostile and unfriendly environments represented by the presence of xenobiotic elements such as PCP. This antibiotic susceptibility was determined by the agar disk diffusion method on Mueller Hinton agar according to the CLSI criteria. Results showed that PsWw128 was sensitive to the seven antibiotics tested in this experiment in the LB medium (Figure 6). In the presence of a concentration of PCP, 250 mg L\(^{-1}\), PsWw128 antibiotic sensibility was decreased for the six antibiotics tested (Ami, FEP, AKN, IPM, PIP, FOS) except Ciprofloxacin.
Besides, antibiotic resistance was very important for the selection of high-performance microorganisms. In this case, the PCP concentrations could induce antibiotic resistance as an environmental stress (Sardessai & Bhosle 2002). Muller et al. (2007) showed that an exposure to chemical stress results in a temporary decrease in the stress response genes, as resistance to antibiotics. Some studies showed that P. aeruginosa biofilm formation could slow the antibiotics sensitivity. Other cases have confirmed that biofilm production can increase resistance to various physical and chemical agents, such as antibiotics.

Therefore, this beneficial microorganism, with various properties, may be employed for bioremediation as inoculants in contaminated soil or mixed in wastewater to reduce PCP contamination. It has been shown that Pseudomonas sp. strain SR3 was able to bio-remediate soil and contaminated water with PCP (Rensnick & Chapman 1994). Tomasi et al. (1995) demonstrated that P. cepacia AC1100 metabolized PCP to the corresponding chlorohydroquinone. Pseudomonas sp. Bu34 was suggested to be able to biodegrade highly PCP contaminated soils, water, and wood products (Lee et al. 1998).

CONCLUSIONS

The present investigation was conducted to evaluate the ability of a saprophytic bacterial strain isolated from wastewater and identified as P. fluorescens, to metabolize PCP under various conditions. On the whole, results revealed that various factors could affect the ability and efficiency of PCP biodegradation. Hence in this study we have concluded that xenobiotic PCP molecules could affect the biofilm formation capacity. Pseudomonas biofilms are found to have an important implication for PCP bioremediation. Moreover, the highest PCP degradation was observed at pH ranging from 4 to 7, and with an optimum temperature of 30 °C. Furthermore, the availability of the PCP contaminant modulates antibiotic susceptibility. Thereby, these properties could be used in the case of decontamination areas polluted with PCP. Bioremediation using bioaugmentation of PCP contaminated soil, water or wood may be an alternative and attractive technological process and/or additives to conventional methods (physical and chemical), because they are economical and do not require energy demand. Consequently, the capacity of the P. fluorescens isolate to tolerate and effectively metabolize the PCP indicates that this saprophytic bacteria may be employed in bioremediation activities.

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