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New and highly active microbial phosphotriesterase sources

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One sentence summary: A simple fluorimetric screening allowed the selection of six wild-type microorganisms with high phosphotriesterase activity that could be used for the decontamination of organophosphorus compounds and for biotechnological applications.
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ABSTRACT

Many toxic insecticides used worldwide as well as some chemical warfare agents are phosphotriester derivatives. Therefore, detoxification of organophosphorus compounds has become the subject of many studies and in particular bioremediation, based on the phosphotriesterase catalysed hydrolysis of these compounds, has shown to be an effective and ecological methodology. In order to identify new bacterial phosphotriesterases, a simple and sensitive fluorimetric screening method on solid media was employed that allowed the selection of six strains with phosphotriesterase activity. Since pH and temperature are important parameters for bioremediation of contaminated soils and waters, the influence of these variables on the rate of the enzymatic hydrolysis was assessed. This study afforded notable results, being the most remarkable one the increased activity exhibited by *Nocardia asteroides* and *Streptomyces setonii* strains at 50°C, 7 and 30 times higher than at 30°C, respectively. Compared with the results obtained with *Brevundimonas diminuta*, whose activity is usually considered as reference, an increase of 26 and 75 times is observed, respectively.

Keywords: phosphotriesterase; organophosphorus compounds; bioremediation Nocardia asteroides; *Streptomyces setonii*; *Brevundimonas diminuta*

INTRODUCTION

Phosphotriesters, that belong to the group of organophosphorus compounds (OPs), have been synthesised since the late 1940s and extensively used as insecticides and in veterinary practice. They represent 38% of the pesticides used worldwide (Makkar *et al.* 2013) with 3 million cases of severe poisoning and around 200 000 deaths by exposure reported annually (Singh 2009). Their toxicity is due to the inhibition of the enzyme acetylcholine esterase, the enzyme responsible for the hydrolysis of acetylcholine neurotransmitter, causing overstimulation of the mammalian nervous system, paralysis and finally death (Mishra *et al.* 2012). Due to these hazardous properties, some OPs have been developed as chemical warfare agents like sarin and soman (Ghanem and Raushel 2005). (Fig. 1).

Over the last decade, an increasing amount of pesticides have been detected in soils, groundwater and surface waters (Rignarsdottir 2000). As a consequence, OPs detoxification has become the subject of many studies. In particular, efforts have been directed towards the enzyme-catalysed decontamination of OPs, since enzymatic hydrolysis of these compounds has considerable advantages over traditional physical and chemical methods such as bleach treatment, incineration or alkaline

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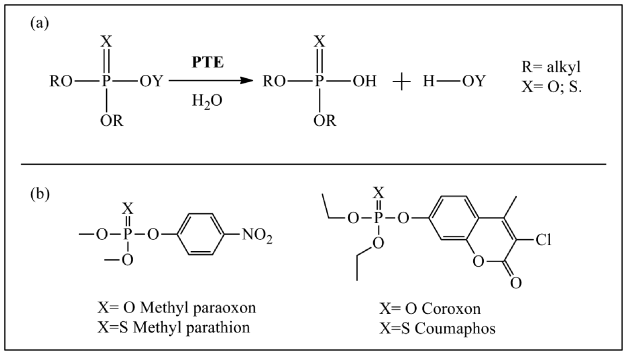

Figure 1. (**a**) Hydrolysis of phosphotriesters and phosphorothioesters biocatalysed by PTE (**b**) OPs employed in this work.

hydrolysis, the main one being the total transformation to innocuous substances (Niti *et al.* 2013).

The most important parameters that influence bioremediation of contaminated soils and waters are pH, nutritional content, microbial diversity, temperature and redox potential (Dua *et al.* 2002). In particular, pH is one of the principal variables since it affects directly the enzymatic activity (Singh, Walker and Wright 2006). This influence has been recently reported showing high chlorpyrifos and fenamiphos degradation (Singh *et al.* 2003a,b) at higher soil pH. On the other hand, temperature influences the rate of biodegradation by also controlling the rate of enzymatic reactions.

Phosphotriesterases (PTEs; E.C. 3.1.8.1), which carry out the hydrolysis of phosphotriesters (Lewis *et al.* 1988) (Fig. 1), are found in different organisms from bacteria to mammalian except insects. The most intensively studied mammal PTEs are human and rabbit serum paraoxonases. The enzymes from *Brevundimonas diminuta* (previously *Pseudomonas diminuta*) and *Flavobacterium sp.* are the best characterised bacterial PTEs (Sogorb *et al.* 2004) and hydrolyse a larger number of OP structures with higher efficiency than mammalian paraoxonases (Raushel 2002). Therefore, PTEs are promising candidates for bioremediation and in addition, their application to *in vivo* detoxification provides a possible catalytic antidote for therapeutic use (Ortiz-Hernández and Sanchez Salinas 2010).

In order to find new PTE activities, we screened our cell collection using a fluorimetric method on solid medium and the selected wild-type strains were evaluated as biocatalysts for the hydrolysis of phosphotriesters in liquid media at different pHs and temperatures in order to analyse the effect of different environmental conditions.

MATERIALS AND METHODS

Chemicals and microorganisms

Methyl-parathion (MP) and coumaphos (CP) were purchased from Sigma-Aldrich (St Louis, MO, USA). Methyl-paraoxon (MPO) and coroxon (CO) were obtained by oxidative desulfuration of MP and CP respectively following the previous report (Bielawski and Casida 1988). All other chemical reagents were commercially available and of the best analytical grade.

The culture media components were obtained from Merck (Darmstadt, Germany) and Difco (Sparks, MD, USA). Microorganisms were supplied by the *Colección Española de Cultivos Tipo* (CECT), Universidad de Valencia (Spain).

Growth conditions

The strains were cultured in liquid media at the optimum temperature and time, according to the American Type Culture Collection (ATCC). The culture broths were used directly for screening in solid medium. For quantification of PTE activity, they were cultured in liquid media for 72 h and centrifuged at 11 000 rpm for 5 min, and the resulting pellets were washed with physiological solution and recentrifuged. The obtained wet whole cell pastes were used as biocatalysts.

PTE fluorimetric detection on solid medium

Petri dishes (10 cm) containing solid low salt Luria Broth medium (NaCl 5 g L^{-1}, tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, Agar-Agar 15 g L^{-1} in distilled water) were divided into four quadrants. The culture broths of each tested strain (total 110) were streaked on individual quadrants, and the plates were incubated at optimal temperature and time, usually from 24 to 120 h. Then, sterile paper discs (diameter 1 cm and thickness 0.01 cm) impregnated with CO (0.8 μmol) were placed over the colonies and embedded with phosphate buffer (50 mM, pH 8, 20 μL). The plates were further cultured for 48 h at 30°C and the hydrolysis reaction was followed testing fluorescence by irradiation at 366 nm at different times (0, 2, 4, 6, 12, 24, 48 h).

Positive control was carried out using *B. diminuta* (Bd), chemical hydrolysis was assessed in absence of biocatalyst and intrinsic fluorescence was evaluated carrying out the reaction without substrate. All experiments were performed in duplicate.

Quantification of PTE activity in liquid medium

The corresponding PTE activities as initial reaction velocity were measured following a modified version of procedure previously reported (Munnecke 1976). The pellets, prepared as described above, containing 2 × 10^9 cells mL^{-1} were suspended in 1 mL of Tris-HCl buffer 50 mM pH 8 containing 2 mM MPO. Phosphotriester hydrolysis was carried out at 30°C in an orbital shaker at 200 rpm. Samples (50 μL) were taken at 0, 15, 30, 45, 60, 90, 120 and 150 min. Reaction products were assayed spectrophotometrically in 96-well plates by monitoring the production of *p*-nitrophenol at 405 nm.

Influence of experimental conditions on PTE activity

To evaluate the influence of temperature on PTE activity of the selected strains, the hydrolysis of MPO was assessed at 30°C, 40°C, 50°C and 60°C. The effect of pH was also evaluated, carrying out the reaction in 50 mM buffers: phosphate for pH 6 and 7, Tris-HCl for pH 8 and 9 or glycine-KOH for pH 10.

Statistical analysis

All experiments were performed in triplicate. Statistical significance was evaluated using Prism 6 statistical software (GraphPad, Inc. CA, USA). Results presented are expressed as mean values ± SD. For multiple comparisons between experimental groups, one-way ANOVA (followed by Dunnett or Tukey posteriori test) and two-way ANOVA (followed by mean 95% confidence interval (CI) comparison test) were performed. Significant levels were defined as $P < 0.05$.

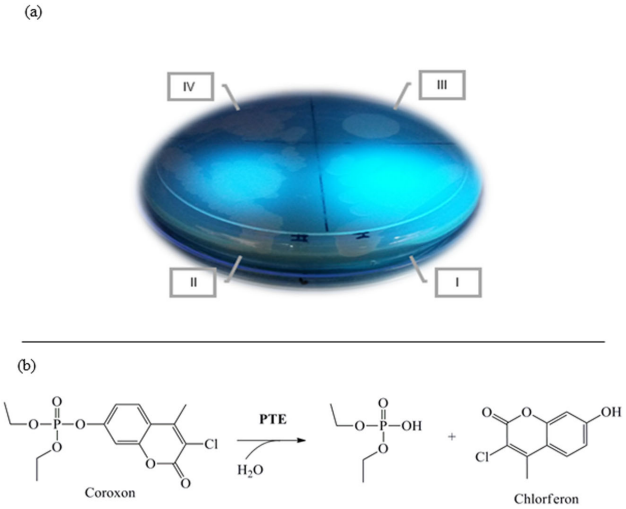

Figure 2. (**a**) Representative result obtained in the screening of PTE activity in solid media. (**b**) Enzymatic hydrolysis of coroxon.

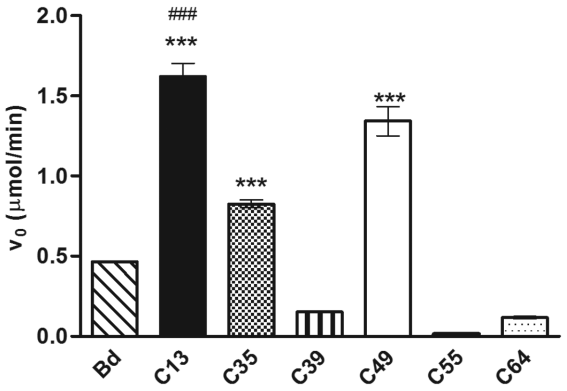

Figure 3. PTE activity of the selected microorganism. All tests were performed using 2 mM MPO, 30°C and pH 8. Results are expressed as mean ± CI one-way ANOVA followed by the Dunnett (***P < 0, 001) and Tukey post hoc test (###P < 0, 001).

RESULTS AND DISCUSSION

In order to identify new bacterial PTEs, different screening methodologies have been described. McDaniel and Wild (1988) employed filter pads impregnated with parathion applied to the surface of bacterial colonies, which developed yellow color due to the presence of *p*-nitrophenol. On the other hand, Harcourt *et al.* (2002) carried out a high-throughput fluorimetric microliter plate-based method using coumaphos hydrolysis as activity assay. Based on these previous works and considering that chlorferon (coumaphos and coroxon hydrolysis product) provides a more sensitive assay than *p*-nitrophenol and that the use as substrate of the more reactive coroxon speeds up the screening process (Chae, Postula and Raushel 1994), a sensitive and qualitative primary PTE screening that avoids the need of particular detection equipment was employed.

Using this methodology, our cell collection, composed of 110 microorganisms including mesophilic and thermophilic strains, was screened for PTE activity (Fig. 2). Six strains with PTE activity were selected: *Streptomyces phaeochromogenes* CCRC 10811 (C13), *S. setonii* ATCC 39116 (C35), *Nocardia corynebacterioides* ATCC 14898 (C39), *N. asteroides* ATCC 19296 (C49), *Arthrobacter oxydans* ATCC 14358 (C55) and *A. oxydans* ATCC 14359 (C64). After 2 h reaction, all of them displayed fluorescence. In no case, florescence was produced by chemical hydrolysis or by compounds present in the microorganisms. In Fig. 2a, a representative example is shown: quadrant I: Bd (positive control); quadrant II: strain C13; quadrant III: without biocatalyst (control for chemical hydrolysis); quadrant IV: strain C13 without substrate (control for intrinsic fluorescence).

In order to quantify the activities of the selected strains, an assay in liquid medium was performed monitoring the hydrolysis of MPO.

The reactions were followed throughout 150 min and *p*-nitrophenol concentration was determined spectrophotometrically. Determination of PTE activity, measured as initial rate (v_o) (Fig. 3), showed that strains C35, C49 and C13 exhibited two, three and four times higher activities than Bd, respectively. The other strains were less active than the microorganism selected as reference.

Influence of pH and temperature on PTE activity

Rochu *et al.* (2004) demonstrated that pH, temperature and the associated metal affect PTE activity; in addition, bacterial degradation of pesticides depends on soil properties and climatic conditions (Hindumanthy and Gayathri 2013). Therefore, to study the characteristics of the new bacterial PTEs, their activities were analysed at different pHs and temperatures. The results are shown in Fig. 4.

At pH 10, 7 and 8, strains C13, C35 and C49 showed respectively seven, sixteen and three times higher activities compared to Bd at the same pHs. For strains C13 and C35, an improvement in activity relative to standard conditions was also observed (twice and three times, respectively). Strain C35 showed high activity even at pH 6; therefore, it may have particular applications as proposed for *Lactobacillus brevis* that was selected as candidate for the decontamination of acidic raw food (Islam *et al.* 2010). The other strains, including the positive control (Bd), displayed low activities at the assayed conditions (Fig. 4a). The optimised pH for each strain is shown in Table 1.

Regarding the temperature profile, bacterial PTE activities were tested using the standard conditions for MPO hydrolysis but varying the temperature from 30°C to 60°C. The results are shown in Fig. 4b. In particular, C13 was at 40°C two times more active than at 30°C and 8 times more active than Bd at the same conditions, but much more remarkable, strains C49 and C35 exhibited at 50°C initial rates 7 and 30 times higher than at 30°C, respectively. When the results obtained with strains C49 and C35 are compared with Bd at 50°C, an increase in activity of 26 and 75 times was observed, respectively. The need of thermostable enzymes not only is of interest for bioremediation, but also because of their biotechnological industrial applications. The majority of the reported PTEs were mesophilic and few exceptions such as the PTE from the hyperthermophilic archaeon *Sulfolobus solfataricus* MT4 (Merone *et al.* 2005) were studied.

Finally, in order to optimise reaction conditions, further experiments were carried out using the combined pH and temperature that had afforded separately the highest activity for each strain (Table 1). Unlike the expected, the combination of these parameters did not produce a synergistic effect on the activity of the bacterial PTEs. Therefore, we selected those pHs and temperatures in which each strain showed the maximal activity (Table 2). Under such conditions, the degradation of different pesticides is in progress.

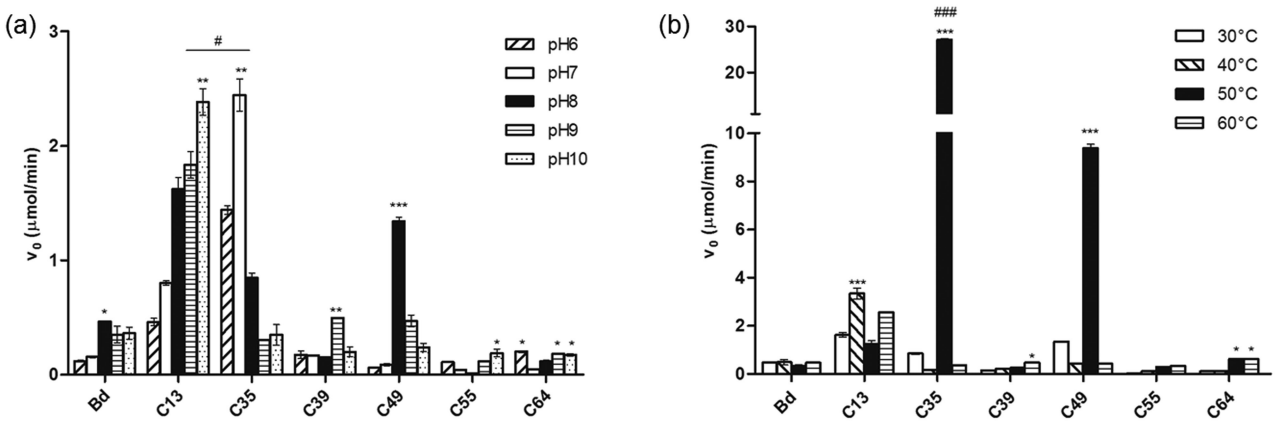

Figure 4. Variation of PTE activity according to (**a**) pH. All tests were performed using 2 mM MPO and 30°C (**b**) temperature. All tests were performed using 2 mM MPO and pH 8. Results are expressed as mean ± CI two-way ANOVA followed by the Tukey post hoc test. Statistical analyses were performed comparing different pHs or temperatures for each strain (***$P < 0,001$, **$P < 0.01$, *$P < 0.05$). Activities obtained for all the strain at different temperatures or at different pHs were also compared (###$P < 0.01$).

Table 1. PTE activity at optimised pHs and temperatures, and at combined conditions. Results are expressed as mean ± SD. Two-way ANOVA followed by mean 95% CI comparison post hoc (***$P < 0.001$, **$P < 0.01$). Statistical analyses were performed comparing pH and temperature with combined condition for each strain.

Microorganism	pH[a]	v_o (μmol/min)	Temperature[b] (°C)	v_o (μmol/min)	Combined conditions v_o (μmol/min)
Bd	**8**	0.46 ± 0.01	**60**	0.51 ± 0.02	0.51 ± 0.02
C13	**10**	2.38 ± 0.05	**40**	3.42 ± 0.09	2.70 ± 0.03
C35	**7**	2.55 ± 0.15**	**50**	27.2 ± 0.2***	14.9 ± 0.1
C39	**9**	0.50 ± 0.01	**60**	0.47 ± 0.02	0.23 ± 0.01
C49	**8**	1.34 ± 0.05**	**50**	9.3 ± 0.2	9.3 ± 0.2
C55	**10**	0.19 ± 0.02	**60**	0.33 ± 0.01	0.29 ± 0.02
C64	**6**	0.21 ± 0.01	**50**	0.63 ± 0.01	0.45 ± 0.02

[a]Experiments carried out at 30°C.
[b]Experiments carried out at pH8.

Table 2. Optimised reaction conditions for the biocatalysed hydrolysis of MPO. For all the tested strains the optimal pH was 8.

Microorganism	Optimal conditions (°C)	v_o
Bd	60	0.51 ± 0.02
C13	40	3.4 ± 0.1
C35	50	27.1 ± 0.2
C39	60	0.47 ± 0.02
C49	50	9.3 ± 0.2
C55	60	0.33 ± 0.01
C64	50	0.63 ± 0.01

In conclusion, employing a simple and sensitive fluorimetric screening method, six strains not reported so far as sources of PTE activity were selected. These microorganisms belong to the *Actinomycetes* family whose members usually show long-term persistence in soils.

The influence of pH and temperature on the enzymatic hydrolysis catalysed by these strains was evaluated obtaining interesting results, being the most remarkable ones the activities of strains *N. asteroides* ATCC 19296 and *S. setonii* ATCC 39116 that significantly increased their PTE activity by increasing the temperature. Moreover, *S. phaeochromogenes* CCRC 10811 is a versatile microorganism since it showed good PTE activities in the pH and temperature ranges analysed. Since OPs are lipophilic compounds that mainly stay on the soil surface (Singh, Walker and Wright 2006), the selected microorganisms may be of particular interest in those land areas that reach seasonally high temperatures (Land Surface Temperature, Earth Observatory, NASA [http://earthobservatory.nasa.gov/GlobalMaps]).

Therefore, a set of microorganisms that could be used in the decontamination of OPs in different environments, as well as for biotechnological applications, was obtained.

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Conflict of interest. None declared.

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