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Research Article

OPTIMIZATION OF CARBOFURAN HYDROLASE PRODUCTION BY A PSEUDOMONAS CEPACIA STRAIN

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ABSTRACT

The enzymatic degradation of carbofuran derivative, namely, 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate was studied in a strain of the bacterium *Pseudomonas cepacia*(Fsv), isolated from a soil that had undergone successive treatments for many years with this insecticide. So, approximately 84 % of the activity was extracellular and 16 % was localized in the insoluble fraction after cell lysis. It is found that the maximum enzymatic activity was obtained at pH 8.5 and 40 °C. It is reached 128 and 112 µmol mL^{-1} min^{-1} at 4 g of ammonium sulphate as sole source of nitrogen and 4.5 g of glucose as sole source of carbon, respectively. It is found also that the enzymatic activity reaches 116 µmol mL^{-1} $min^{-1}$with the presence of mixture of two sources. At the same time, the biomass linked to enzymatic activity increased to reach 2.42, 2.12 and 2.61, respectively.

INTRODUCTION

The enzymes are less toxic, more ecological and economical tools than the chemical substances during the various stages of production in different sectors such as the agri-food, pharmaceutical, textile, brewery and detergent industries (Kennedy, 1987).

However, the traditional industrial production of enzymes in large quantities was related to intensive exploitation of resources and large quantities of waste. Today, many enzymes were produced using genetically modified bacteria or yeasts in large fermenters. This technique enables, compared to conventional manufacturing processes, to greatly reduce the need for raw materials, especially water and energy, emissions of air pollutants and waste. So, 80 % of the industrially manufactured enzymes were currently produced using genetically modified microorganisms, beside which, another process of depollution, minimally hazardous, economical, versatile and environment-friendly. This technique was involved in exploring the genetic diversity of natural populations; It is known as bioremediation (Finley *et al*, 2010), which refers to a promising treatment method that exploits the ability of microorganisms to remove pollutants from contaminated sites (Boopathy, 2000).

Several studies have focused on the isolation and identification of strains with high performance in degrading a wide range of pesticides that have become ineffective in controlling their biological target.

The Carbofuran, which is the insecticide of the N-methylcarbamate family, was a highly toxic pesticide that is heavily used in agriculture due to its high effectiveness. It has been the subject of many studies concerning its biodegradation by microorganisms. In fact, we have isolated from a soil with a previous history of treatment with carbofuran, a fungus (*Gliocladium sp*) capable of degrading it with a rate of 81 % (Slaoui *et al*, 2007). Similarly, another strain of *Pseudomonas sp*. (Bano and Musarrat, 2004), *Pseudomonas putida* KT2440

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(Ting *et al*, 2016), *Arthrobacter globiformis, Streptomyces sp*., *Bacillus Beijingensis, Bhargavaea indica*, able to degrade carfuran and even other pesticides, have been isolated (Shalini *et al*, 2017) .....

The characterization of the enzymes responsible for degrading pesticides allows the mitigation of economic losses caused by their extremely rapid degradation by microorganisms, but mainly it facilitates the use of these enzymes in simple and economical processes of detoxification and elimination of agrochemical waste (Blashek, 1992).
In our previous work (Slaoui *et al*, 2001), we have isolated from a soil loaded with carbofuran (2,2-dimethyl-2,3-dihydro-1-benzofuran-7-yl methylcarbamate) a bacterium identified as *Pseudomonas cepacia* (Fsv) with a great ability to degrade the insecticide and nematicide, widely used in agriculture to fight diseases in vegetables, fruits and crops and whose potential risks to human health from its excessive use were approved because it is a potent inhibitor of AChE (Nguyen *et al*, 2014). So, we have identified the appearance of clear zones around the colonies on solid synthetic medium suggesting the presence of an extracellular enzyme system (Slaoui *et al*, 2001).

The objective of this study is the characterization of carbofuran hydrolase produced by *Pseudomonas cepacia* strain.

MATERIALS AND METHODS

The Fsv strain corresponding to *Pseudomonas cepacia*, exhibiting the highest abilities to use carbofuran as a sole source of carbon and nitrogen (Slaoui *et al*, 2001), was cultured on minimal medium (Derbyshire *et al*, (1986) containing (g/L): $MgSO_4$, 0,01; K_2HPO_4, 1.0; Na_2HPO_4, 2.1; $CaCl_2$. $2H_2O$, 0.1; $CuSO_4$, 0.04; $FeSO_4.7H_2O$,0.001; Na_2MoO_4, 0.002, and 200 µL/mL carbofuran (99 % pure analytical grade) as sole source of carbon and nitrogen, the pH was adjusted to 7.5. 20 mL of culture medium were placed in 100 mL flasks, the cultures were incubated at 30 °C with constant shaking at 150 rpm for 48 hours

Demonstration and localization of enzymatic activity

Cell suspensions were stopped at the end of the exponential growth phase at 48 h. After centrifugation (12000 rpm, 20 min, 4 °C), the culture medium (fraction I) and a pellet containing the intact cells (fraction II) are obtained. A part of these cells was resuspended in phosphate buffer (pH=7.5) and then lysed by sonication (ultrason). After a new centrifugation (12000 rpm, 20 min, and 4 °C), a soluble and an insoluble fractions (fractions III and IV, respectively) were obtained.

The measurement of the enzymatic activity of carbofuran hydrolase in each fraction was carried out by spectrophotometry according to the protocol described by Derbyshire *et al*, (1987) and (Karns *et al*, 1986), preferably adopted by several authors (Chaudhry *et al*, 2002). It was based on measuring the increase in absorbance due to the appearance of o-nitrophenol in the reaction medium following hydrolysis of o-nitrophenyl dimethyl carbamate, a colorimetric substrate similar to carbofuran.

400 µL of o-nitrophenyl dimethyl carbamate dissolved in methanol (99.5%) are introduced into a hemolysis tube and 200 µL of phosphate buffer (pH=7.5) are added. The volume of the reaction mixture is made up to 1 mL by the eventual addition of each fraction (I, II, III, and IV).

In a bath-Marie, the reaction medium was heated at 40 °C for 15 min. After homogenization, the absorbance at 413 nm of each fraction was measured against a control (containing all the elements of the reaction mixture except the crude enzyme extract which is replaced by 400 µL of distilled water). The enzyme reaction is stopped by adding 4 µL of concentrated sulfuric acid and the mixture is then homogenized and heated in a bath-Marie for 5 min and then cooled for 15 min at room temperature.

The calibration curve of o-nitrophenol was established. The enzymatic activity corresponds to the number of micromoles (µmol) of o-nitrophenol released in one minute in the reaction medium under the conditions described above.

pH effect

In order to estimate the optimum pH of the enzyme, each fraction (I, II, III, and IV) was diluted in 10 mM of K_2HPO_4 (phosphate buffer) at different pH values : 7; 7.5; 8; 8.5; 9; 9.5 and 10. The o-nitrophenyl dimethyl carbamate substrate solutions were prepared in the same buffer solutions. The enzymatic activity was determined at 40 °C for 24 hours. The biomass was determined by measurement of turbidity at 600 nm on mineral medium supplemented with carbofuran as sole source of carbon and nitrogen.

Temperature effect

The temperature was tested on the growth of the Fsv strain and on the appearance of the carbofuran hydrolase activity at 20; 30; 37; 40; 45 and 50 °C. The reaction mixture contains the o-nitrophenyl dimethyl carbamate substrate, the phosphate buffer at optimum pH and each fraction (I, II, III, and IV).

The biomass and the enzymatic activity of each fraction were determined at the end of growth after 24 hours of incubation on a mineral medium containing only carbofuran as sole source of carbon and nitrogen.

Effect of carbon and/or nitrogen supply

The influence of a carbon and/or nitrogen supply to the mineral medium (M1) was studied in the presence of 200 µL/mL of carbofuran such as indicated in Table I.

Table I Different nutritional intakes tested for the cultivation of *Pseudomonas cepacia*on a minimal medium containing 200 µg/mL of carbofuran.

Medium	Nutritional supply
M_1	Control (without addition)
M_2	M_1 + $(NH_4)_2SO_4$ (4 g)
M_3	M_1 + NH_4NO_3 (1,5 g)
M_4	M_1 + NH_4Cl (1,5 g)
M_5	M_1 + glucose (4,5 g)
M_6	M_1 + $(NH_4)_2SO_4$ (4 g) + glucose (4,5 g)

The cultures were carried out under optimum conditions of temperature and pH for 24 hours with stirring rate (105 rpm). In parallel, in order to verify that the enzymatic system of the Fsv strain is an hydrolytic type, a culture was carried out at 40 °C, pH 8.5 and in the dark on mineral medium, deprived of glucose and $(NH_4)_2SO_4$, containing 2 g/L of methylamine

(CH_3NH_2), a degradation product of carbofuran, as a source of carbon and nitrogen.

RESULTS

Localization of enzymatic activity

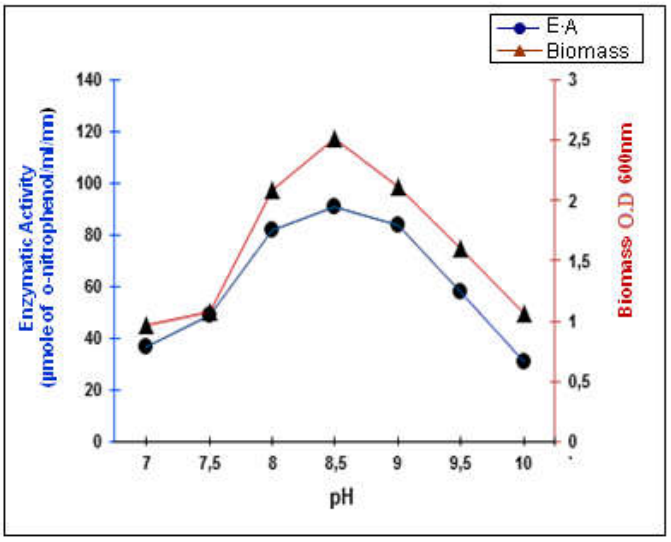
The 84 % of the carbofuran hydrolase activity of the *Pseudomonas cepacia* is extracellular and absent in intact cells (Table II). A low activity was found in fractions III (6 %) and IV (10 %).

Table II Localization of carbofuran hydrolase on four fractions of cell suspensions of *Pseudomonas cepacia* after 48 h of culture with stirring rate of 105 rpm, 40 °C and pH 7.5 on mineral medium containing 200 µg/mL of carbofuran.

Enzymatic fractions	Enzymatic Activity µmole of nitrophénol/mL/min	%
Fraction I : culture medium	42	84
Fraction II : intact cells	0	0
Fraction III (insoluble fraction)	3	6
Fraction IV (soluble fraction)	5	10

pH effect

The variations in enzymatic activity (EA) and biomass (B) with pH are presented in Figure 1. It is observed that the EA and B reach their maximum values of 91 µmol mL^{-1} min^{-1} and 2.51, respectively, at pH 8.5.



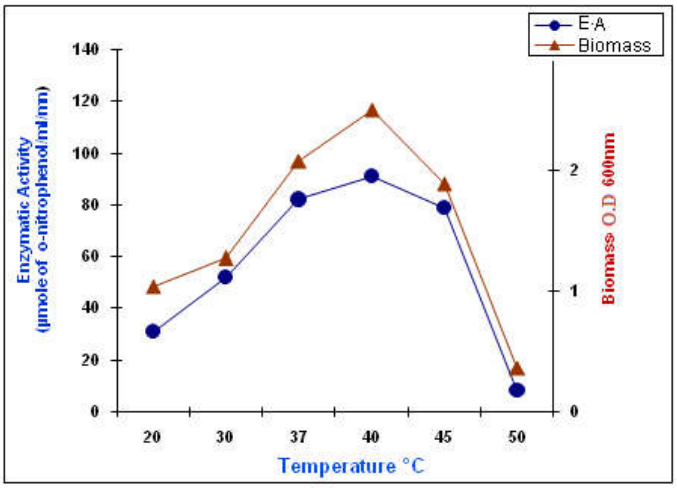
Graph 1 Effect of pH on the growth (biomass at 600 nm) and the carbofuran hydrolase activity of the *Pseudomonas cepacia* strain after 24 hours of culture at 40 °C and 105 rpm in a shaker on mineral medium containing 200 µg/mL of carbofuran.

Influence of temperature

The variations in enzymatic activity (EA) and biomass (B) with temperature are presented in Figure 2. It is observed that the EA and B are important at 37 °C where their values are 82µmol mL^{-1} min^{-1} and 2.08, respectively. It can be seen that their maximum values of 91µmol mL^{-1} min^{-1} and 2.5, respectively, were obtained at 40 °C. At 50 °C, the growth and enzymatic activity tend towards zero.

Effect of a carbon and/or nitrogen supply

The Biomass and carbofuran hydrolase activity of *Pseudomonas cepacia* strain after 24 hours of culture under six different nutritional conditions were presented in Table 3.



Graph 2 Temperature effect on growth (biomass at 600 nm) and the activity of carbofuran hydrolase of *Pseudomonas cepacia* strain Fsv after 24 hours of culture at pH 8.5 and stirring rate of 105 rpm on the mineral medium containing 200 µg/mL of carbofuran.

Table 3 Biomass and carbofuran hydrolase activity of *Pseudomonas cepacia* strain after 24 hours of culture under six different nutritional conditions at 40 °C and pH 8.5 and under 105 rpm, in the presence of 200 µg/mL of carbofuran.

Medium	Biomass ($A_{600\ nm}$)	Enzymatic Activity (µmol of *o*-nitrophenol/mL/min)
M_1	1.56	42
M_2	2.42	128
M_3	1.71	18
M_4	1.62	12
M_5	2.12	112
M_6	2.61	116

Compared to the control, the growth and enzymatic production are markedly increased by the addition of ammonium sulphate. In addition, glucose as a source of carbon, whether or not associated with an ammonium sulphate supply, also gives good results in inducing the production of the enzyme (116 µmol mL^{-1} min^{-1}). It is observed that the nitrate or ammonium chloride does not decrease growth but negatively affects the production of the enzyme.

The *Pseudomonas cepacia* strain Fsv was able to grow well on the mineral medium free the glucose and ammonium sulphate with methylamine, the hydrolysis product of carbofuran, as the sole source of carbon and nitrogen. After 24 hours of culture at pH 8.5 and 40 °C, biomass (A 600 nm = 1.46) and an enzymatic activity of 40 µmol mL^{-1} min^{-1} were obtained.

DISCUSSION

The *Pseudomonas cepacia* production of carbofuran hydrolase is related to cell growth when the mineral medium was used at different pH and temperature values. It is found that the carbofuran hydrolase activity was about 84 % extracellular and 16 % of the activity was located in the extract after cell lysis, suggesting that a minimal amount of the enzyme was either cytosolic or membrane bounded. In *Pseudomonas sp.*50432, carbofuran hydrolase was essentially cytoplasmic (86 %) and only (14 %) was associated with the membrane (Chaudhry *et al*, 2002). Whereas in the *Pseudomonas* sp.CRL-OK strain the carbofuran hydrolase activity was totally cytosolic (Mulbry and Eaton, 1991). It should also be noted that, in the ER2

bacterium, which is capable of rapidly degrading several N-methylcarbamate compounds and especially carbofuran as the sole source of carbon and nitrogen, no extracellular carbofuran hydrolase activity has been detected (Topp *et al*, 1993). Similarly, carbofuran hydrolase in *Achromobacter sp* was both extracellular (77 %) and intracellular (23 %) (Duquenne *et al*, 1998). The localization of carbofuran hydrolase varies according to the species. However, the maximum enzymatic activity at pH 8.5 was in agreement with the optimal pH of *Pseudomonas* CRL-OK (Mulbry and Eaton, 1991) as well as that of *Pseudomonas sp*.50432 (Chaudhry *et al*, 2002). While, this pH was seven for *Pseudomonas sp* (Yu *et al*, 2009) and a strain of *Bacillus subtilis* (Li *et al*, 2010). The *Achromobacter strain* WM111, on the contrary has a better carbofuran hydrolase activity at pH 9-10.5 (Hayatsu et al, 2001).

We have found that the optimum production temperature of carbofuran hydrolase enzyme is 40 °C. In the literature, the optimum temperature for its production was generally above 37 °C. It is 45 °C in *Pseudomonas sp*.50432 (Chaudhry *et al*, 2002)and at a group of carbofuran-degrading bacterium as the sole source of carbon. In addition, the temperature reaches even 50 °C for the bacterial strain *Achromobacter*WM111 (Hayatsu, 2001) and 60 °C for *Pseudomonas* CRL-OK (Mulbry and Eaton, 1991).Furthermore, for the *Pseudomonas* sp (Yu *et al*, 2009) the optimal degradation temperature of carbofuran was only 30 °C, this result is consistent with that found by Li *et al*, (2010) in a strain of *Bacillus subtilis.*

As according to Hirose *et al*,(1996), each organism has optimal conditions for its growth and the production of metabolites, so we have studied the influence of a carbon and/or nitrogen supply on the production of carbofuran hydrolase in *Pseudomonas cepacia.* The results showed that a ammonium sulphate and glucose intake improve cell yield and carbofuran hydrolase production. Our results are in agreement with those obtained by Parekh *et al* (1994), al which they reported that the addition of glucose to a mineral medium containing carbofuran leads to an increase in enzymatic activity. This result can be explained by the rapid assimilation of glucose by microorganisms. The supply of glucose in the medium causes proliferation of carbofuran degraders bacteria (Duquenne *et al,* 1998) and the presence of metabolizable carbon in the environment has a positive effect on the synthesis of enzymes necessary for the hydrolysis of carbofuran (Topp, 1993).

The ability of the Fsv strain to use methylamine suggests that it is a metabolic intermediate for carbofuran degradation. The enzymatic system involved in the degradation of carbofuran is the hydrolytic type. By this pathway, the methylamine would be oxidized to formaldehyde which would itself be oxidized to formate and to CO_2 (Anthony, 1988). Our results are consistent with those of Kim *et al*, (2004) who reported that the strain *Sphingomonas sp*.SB5 degrades carbofuran hydrolytically and uses methylamine as a source of carbon and nitrogen. The same result was found by Castellanos *et al*, (2014).Likewise, Topp *et al*, (1993) have shown that the strain ER2 has the ability to hydrolyze carbofuran to carbofuran-7-phenol and methylamine using it as the only source of carbon and nitrogen, whereas Chaudhry *et al*, (2002) reported that *Pseudomonas sp*.50432 degrades carbofuran according to the both pathways, hydrolytic and oxidative.

CONCLUSION

Acomparison of the carbamate hydrolase enzymes already studied, plus the one we have characterized, shows a variation in localization, substrate specificity, biochemical and physical characteristics, indicating a diversity of hydrolase genes in different microorganisms. The wide variation in optimum pH and temperature values of carbamate hydrolases can be a valuable tool for their use in soil bioremediation processes for the detoxification of wastes from different types of pesticides.

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