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ORIGINAL PAPER

Biodegradation of karathane using adapted *Pseudomonas aeruginosa* in scale up process

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Abstract

Nowadays, one of the major problems facing the industrialized world is the contamination of soils, groundwater and sediments with pesticides. Despite the valuable contributions associated with the use of pesticides, many of these biologically active chemicals represent a potential hazard to humans and to nature.

In this study, biodegradation potential of adapted Pseudomonas aeruginosa S3 has been assessed on karathane (active ingredient dinocap), a contact fungicide and a non-systemic acaricide. The strain was isolated from contaminated soil and adapted to grow on karathane (up to 1%) as the only carbon source that is converted into less toxic and environment friendly compound. The media and process optimization was carried out in order to scale up the process. The higher biomass production was obtained on mineral salt media with corn flour 1% (nitrogen source) and glucose as additional carbon source. Also, the effect of initial pH and inoculum volume on biomass growth has been studied. The biodegradation potential of Pseudomonas aeruginosa S3 has been tested on soil contaminated with 0.2% karathane where the strain degradated 45.8% of pesticide.

Keywords: biodegradation of karathane, *Pseudomonas aeruginosa*, scale up process

Introduction

Wide-scale application of pesticides and herbicides is an essential part of augmenting crop yields, but excessive use of these chemicals leads to the microbial imbalance, environmental pollution and health hazard (7, 9). Soil is considered "the ultimate sink" of the pesticides applied in agriculture and their presence in significant quantities has direct effect on soil microbiological aspects, which in turn influence the plant growth. On the other hand, longer persistence of pesticides in soil lead to the increased adsorption of such toxic chemicals by plants to the level at which the consumption of plant products may prove hazardous to human beings as well as to livestock. Pesticides reaching to the soil are degraded by indigenous microorganisms, but the rate of degradation is very slow (1, 9). Therefore, this natural biodegradation may be improved by adapted microorganisms that convert contaminants into simpler non-toxic compounds.

In this paper, the biodegradation potential of adapted *Pseudomonas aeruginosa* S3 has been assessed on karathane (active ingredient dinocap), a dinitrophenyl crotonate fungicide. To scale up the process, the media and parameters were optimized in order to obtain a higher biomass production that biodegrades soil contaminated with karathane.

Materials and Methods

Microorganisms and culture conditions

Pseudomonas aeruginosa S3 was previously isolated from polluted soil and adapted to grow on karathane. The culture was maintained on nutrient agar slants.

The bacterial strains were inoculated onto Luria-Bertani (LB) liquid media with the following composition (g/L): bactotryptone 10.0, yeast extract 5.0, NaCl 10.0, pH 7.5 and incubated at 30^0C for 24h to obtain the inoculum. The bacterial growth was determined by measuring culture density, by the spectrophotometric absorption at 600nm. The composition of the basal mineral salt medium (MMP) used in this study was as follows (g/L): K_2HPO_4 1.0, KH_2PO_4 0.5, $MgSO_4x7H_2O$ 0.2, NaCl 1, $(NH_4)SO_4$ 1, pH 6.5. The different concentrations of karathane (0 - 1%) were added after sterilization. Experiments were carried out in 100 ml shaking flasks containing 20 ml MMP medium supplemented with karathane, different additional carbon and nitrogen sources. The shaking flasks were rotated at 250 rpm in constant temperature of 30^0C for 24 - 72h.

Analysis of karathane

The concentration of karathane in culture medium was determined using a method for nitrocompounds quantification (3). A mixture of 0.5 ml sample, 5 ml acetone and 1.5 ml NaOH 5% was used to observe the color reaction that was recorded as absorbance (OD) at 546nm. The color reaction formation of different intensities and stabilities were observed because the concentration of alkaline component and the presence of water influence the behavior of nitrocompounds. The quantitative expression was made using a standard curve (figure 1) that has a coefficient of regression (R^2) of 0.957.

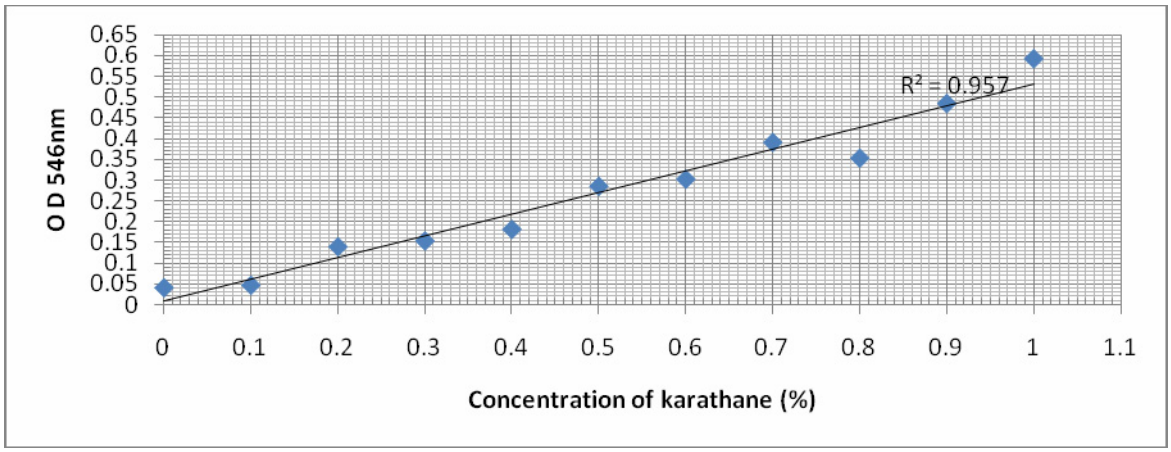


Figure 1. The standard curve for karathane determination

The effect of the karathane concentration on the growth of Pseudomonas aeruginosa S3

The bacterial strain was inoculated onto MMP medium with different concentrations of karathane up to 1% (0%, 0.2, 0.4%, 0.6%, 0.8%, 1%) and microbial growth (OD_{600}) and pH were recorded.

The effect of the carbon supplementation and nitrogen source on the biomass production.

Table 1 shows the additional carbon sources and nitrogen sources tested in the study. The nitrogen sources (triptone, urea, soybean meal, cornmeal, ammonium sulphate and magnesium nitrate) at the desired concentrations were added to MMP medium that contains karathane 0.5% (MMP-K 0.5%). The supplementary carbon sources (glucose, starch and sucrose) tested at different concentrations were added to MMP medium with cornmeal 1%

and karathane 0.5%. The biomass accumulation (OD_{600}), pH and karathane biodegradation were determined.

Table 1. Carbon supplementation and nitrogen sources tested

Culture medium	Concentration of carbon source	Concentration of nitrogen sources	Concentration of carbon supplementation
N1	Karathane 0.5%	Triptone 0.5%	-
N2		Urea 0.25%	-
N3		NH_4SO_4 0.5%	-
N4		$MgNO_4$ 0.5%	-
N5		Cornmeal 1%	-
N6		Soybean meal 1%	-
N7		Cornmeal 2%	-
C1=N5		Cornmeal 1%	-
C2			Glucose 0.1%
C3			Glucose 0.5%
C4			Starch 0.1%
C5			Sucrose 0.1%
C6			Glucose 0.3%

The effect of the inoculum size and the initial pH on the biomass production

To investigate the effect of inoculum concentration on the bacterial accumulation onto C2 medium, three different inoculum ratios (1%, 2.5% and 5%) were tested. The medium was modified with a NaOH 25% solution to obtain different pH values in experiments where the effect of initial pH on the bacterial growth was determined. Microbial density (OD_{600}), pH and karathane biodegradation were recorded.

Scale up process

The bioreactor Biostat C plus (Sartorius) was used to scale up the process of biomass production with adapted *Pseudomonas aeruginosa* S3 in optimized conditions (medium C6, inoculum size 1%, initial pH 7). The bacterial biomass accumulation was determined by spectrophotometer (OD_{600}), counting was performed with Thoma chamber and by plating method (UFC/ml) (2, 10).

Reproducibility of the data

Experiments were conducted in duplicate for each sample and the average results from representative experiments are shown. The real bacterial growth (ΔOD_{600}) was calculated by taking difference between the absorbance (OD_{600}) at the time of sampling (24h/48h/72h) and the optical density at 600 nm after inoculation. The karathane biodegradation was determined as a difference between the initial concentration of karathane and the value obtained after 48 hours of cultivation.

Results and Discussion

The effect of the karathane concentration on the growth of Pseudomonas aeruginosa S3

The bacterial strain *Pseudomonas aeruginosa* S3 tolerates karathane 1% but is able to grow in a medium with karathane up to 0.8%, using the toxic compound as a source of carbon and energy (figure 2). The maximum accumulation of biomass (ΔOD 600nm = 0.637) was

observed after 48 hours in a medium with 0.4% karathane. The pH values did not significantly change during 72 hours of cultivation. For the further experiments the concentration of karathane 0.5% was used in order to adapt the strain to a higher concentration of pesticides than the one usually recorded in the contaminated soil (0.2%).

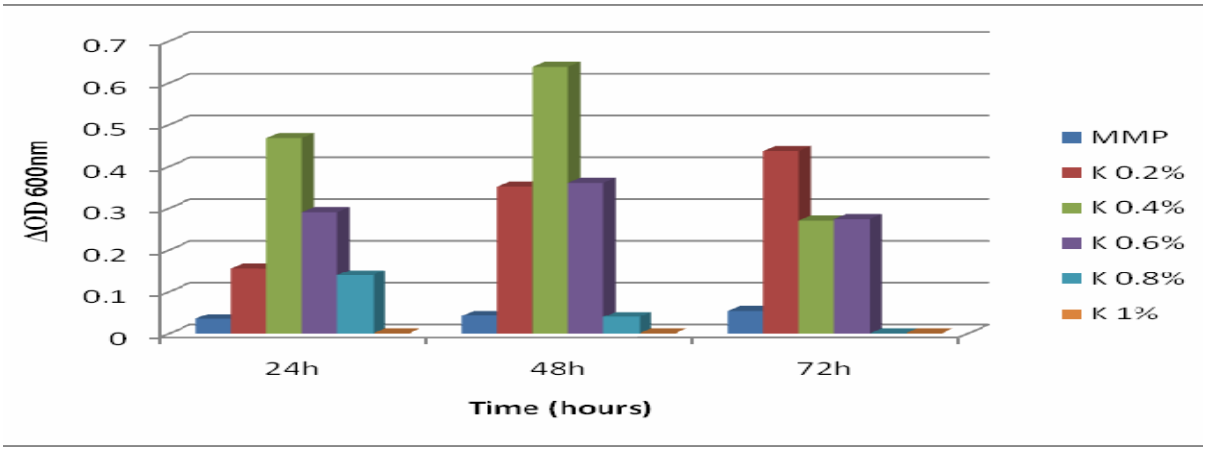


Figure 2. The effect of karathane concentration on the growth of *Ps. aeruginosa* S3

The effect of the carbon supplementation and nitrogen source on the biomass production

The medium optimization was carried out by testing the effect of nitrogen and carbon supplementation on the biomass production.

Firstly, several nitrogen organic (triptone, urea, soybean meal and cornmeal) and inorganic sources (ammonium sulfate and magnesium nitrate) were tested (figure 3). It has been noticed that the industrial nitrogen sources determined a significant increase of the bacterial biomass accumulation. Thus, the microbial density values are three times higher in the presence of cornmeal 1% (ΔOD_{600} = 1.74) and soybean meal (ΔOD_{600} = 1.82) than in medium without nitrogen source (ΔOD_{600} = 0.64). The inorganic nitrogen sources and urea do not stimulate the growth of *Pseudomonas aeruginosa* S3 strain. The pH increased to alkaline values in media with urea, triptone and soybean meal. The biomass production in medium N7 with cornmeal 2% was slightly higher than in medium N5 with cornmeal 1% but the karathane biodegradation was lower.

Kulkarni et al. (2006) isolated a *Pseudomonas putida* strain capable of metabolizing p-nitrophenol (PNP) as a sole source of carbon, nitrogen and energy in a minimal medium. Degradation of PNP and biomass production was significantly enhanced by glutamate (0.04 and 0.4 g/l), while the inorganic nitrogen supplement such as sodium nitrate and ammonium sulfate (0.04 and 0.4 g/l) showed no effect on the rate of PNP degradation. Moreover, glucose has been found to inhibit the PNP (300 ppm) degradation (6).

However, some studies proved that the presence of supplementary carbon source enhanced the biodegradation of nitrophenols (4). Thus, Schmidth et al. (1987) suggested that glucose improve the specific rate of 4-dinitrophenols degradation by a pure culture of *Pseudomonas* sp. (8). Similar, Hess et al. (1993) showed that glucose added to levels lower than 1 g/l enhanced the biomass accumulation of a bacterium consortium and the removal rate of 2,4-dinitrophenol (3).

In our study, we have tested the effect of carbon supplementation on the accumulation of biomass of *Pseudomonas aeruginosa* S3 and karathane biodegradation using glucose (0.1%, 0.3%, and 0.5%), starch 0.1% and sucrose 0.1% (figure 4). The maximum biomass

production (ΔDO_{600} = 2.02) was obtained after 48 hours of cultivation in the presence of glucose 0.3%. However, the karathane biodegradation was slightly higher in C2 medium with glucose 0.1% (0.375%) than in C6 medium with glucose 0.3% (0.345%). The highest level of glucose tested (0.5%) did not significantly enhance the growth of *Ps. aeruginosa* S3 and karathane biodegradation.

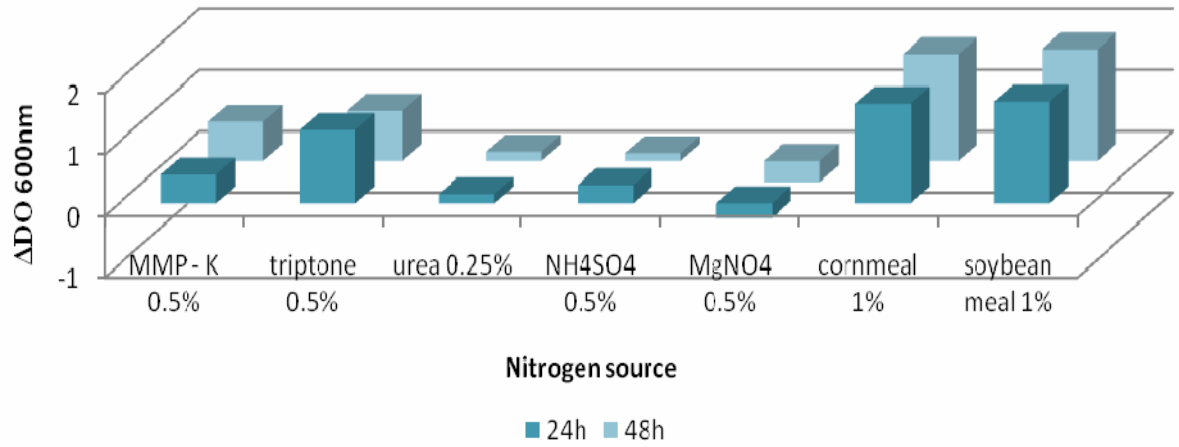


Figure 3. The effect of nitrogen sources on the biomass accumulation of *Pseudomonas aeruginosa* S3 in the presence of karathane 0.5%

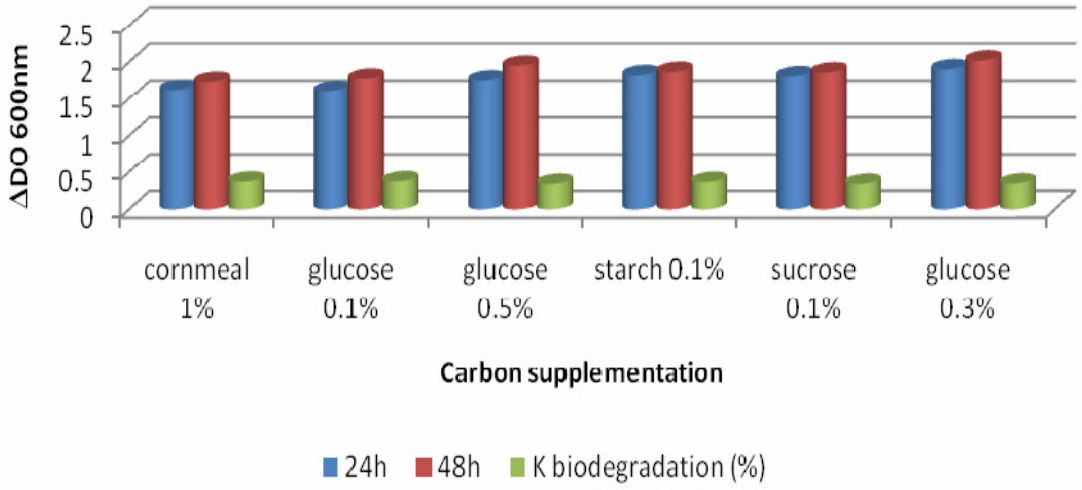


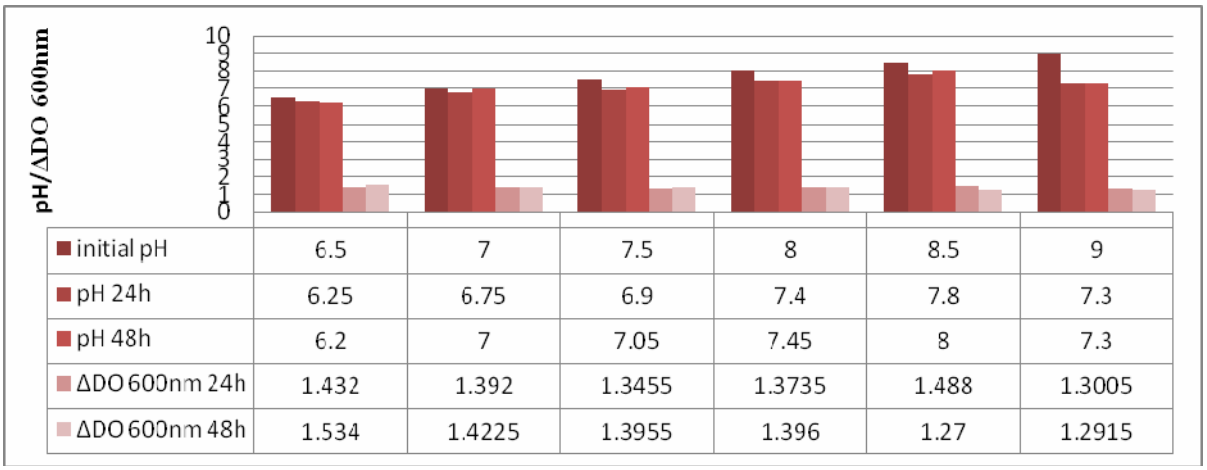
Figure 4. The effect of carbon supplementations on the biomass accumulation of *Pseudomonas aeruginosa* S3 and karathane biodegradation

The effect of the inoculum volume and initial pH on the biomass production

There are only few data regarding the effect of pH on the bacterial biomass production and biodegradation. Thus, removal of p-nitrophenol by *Ps. putida* has been found to be enhanced by alkaline pH values of 7.5 - 9.5 (5). However, our data showed that the increase of the inoculum concentration as well as the initial pH did not significantly influence the growth of the *Pseudomonas aeruginosa* S3 or karathane biodegradation (table 2, figure 5). Moreover, the pH values decreased with 0.5 - 2 units in media with alkaline initial pH.

Table 2. The effect of inoculation on the biomass accumulation of *Pseudomonas aeruginosa* S3

Inoculum ratio (%)	ΔDO 600nm 24h	ΔDO 600nm 48h	Karathane biodegradation (%)
1	1.432	1.534	0.25
2.5	1.532	1.530	
5	1.433	1.4525	



initial pH	6.5	7	7.5	8	8.5	9
pH 24h	6.25	6.75	6.9	7.4	7.8	7.3
pH 48h	6.2	7	7.05	7.45	8	7.3
ΔDO 600nm 24h	1.432	1.392	1.3455	1.3735	1.488	1.3005
ΔDO 600nm 48h	1.534	1.4225	1.3955	1.396	1.27	1.2915

Figure 5. The effect of initial pH on the biomass accumulation of *Ps. aeruginosa* S3

Scale up process

The higher biomass production (OD_{600} = 2.193, 10^9 CFU/ml) was obtained in a Biostat C Plus bioreactor using *Pseudomonas aeruginosa* S3 strain grown on three liters of medium with karathane 0.1% and glucose 0.3% as carbon and energy sources and cornmeal 1% as nitrogen source. The conditions of cultivations were:

- inoculation ratio 1%
- aeration ratio 1:5 (80%)
- temperature 30^0C
- initial pH: 7.0
- speed: 200 rpm
- time: 24h.

The karathane biodegradation with strain *Pseudomonas aeruginosa* S3 has been tested on soil contaminated with 0.2% karathane where the strain used 45.80% of pesticide.

Conclusions

The adapted strain *Pseudomonas aeruginosa* S3 has been used to biodegrade dinocap, the active dinitrophenyl coumpound from the fungicide karathane. The isolated strain can grow on karathane as a sole carbon and energy source, but the higher biomass production was obtained in an optimized medium with additional carbon source (glucose 0.3%) and cornmeal (1%). The inoculum size and initial pH do not have a significant effect on the biomass growth. The biodegradation potential of *Pseudomonas aeruginosa* S3 obtained in the bioreactor batch has been tested on soil contaminated with 0.2% karathane where the strain used 45.80% of the pesticide.

Acknowledgements

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