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Degradation of chloropyrifos using free and sol-gel immobilized bacteria

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ABSTRACT

Chorpyrifos-degrading bacteria were isolated from Khan-Younes soil and identified as Burkholderia cepacia. The ability of the strain to degrade chorpyrifos were examine using both free and sol-gel immobilized bacteria using 200mg/L chorpyrifos. The concentration of the pesticides was lowered to less than 4mg/L and 58mg/L for free and immobilized respectively. Higher degradation rate of chorpyrifos was observed for free bacteria compared with immobilized bacteria. Optimum pH was found to be 7 for the degradation process for both immobilized and free bacteria. Temperature has an effect on the degradation of chorpyrifos by the two forms of bacteria, maximum degradation was observed at 30°C.

KEYWORDS

Chorpyrifos;
Degradation;
Sol-gel;
Burkholderia cepacia.

INTRODUCTION

Entrapment in crosslinked organic polymers is a well known method for the immobilization of enzymes and whole cells. Entrapped biomolecules are physically confined within the polymer matrix and can be reused several times. Organic polymers such as polyacrylamide gels are currently used in biotechnology but silica glasses could offer some advantages such as improved mechanical strength and chemical stability. Moreover they don't swell in aqueous or organic solvents preventing leaching of entrapped biomolecules. However glasses are made at high temperature and, up to now, enzyme immobilization can only be Performed *via* adsorption or covalent binding onto the surface of porous glasses[6]. The so-called sol-gel process opens new possibilities in the field of biotechnology[3]. Sol-gel glasses are formed at room temperature *via* the polymerization of molecular precursors such as metal alkoxides. Proteins can be added to the solution of precursors. Hydrolysis and condensation then lead to the formation of an oxide network in which biomolecules remain trapped. Small analytes can diffuse through the pores allowing bioreactions to be performed inside the sol-gel glass. Trapped enzymes still retain their biocatalytic activity and may even be stabilized within the sol-gel cage. A wide range of biological species such as antibodies and whole cells have been trapped within sol-gel matrices.

They usually retain their activity but weak interactions with the silica cage actually occur that can change their behavior.

SOL-gel confinement in silica matrices

Sol-gel silica can be synthesized at room temperature via the hydrolysis and condensation of [2].

TetraMethyl OrthoSilicate (TMOS), $Si(OCH_3)_4$. Hydrolysis gives reactive silanol groups whereas condensation leads to the formation of bridging oxygen as follows:

$$-Si\text{-}OCH_3 + H2O \rightarrow -Si\text{-}OH + CH_3OH \text{ (hydrolysis)}$$

$$-Si\text{-}OH + HO\text{-}Si\text{-} \rightarrow -Si\text{-}O\text{-}Si\text{-} + H_2O \text{ (condensation)}$$

The overall reaction is then

$$-Si(OCH_3)_4 + 2H_2O \rightarrow SiO_2 + 4CH_3OH$$

Chlorpyrifos is one of the most common insecticide used in Gaza Strip for agricultural purposes. Unwise use of chlorpyrifos and absence of official monitoring due to complex situation in occupied territories makes it pose serious environmental impact to the local fragile environment. Chlorpyrifos is a broad-spectrum organophosphate insecticide. It is a white crystal-like solid with a strong odor. While originally used primarily to kill mosquitoes, it is no longer registered for this use. Chlorpyrifos is effective in controlling cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice. It is used as an insecticide on grain, cotton, field, fruit, nut and vegetable crops, and well as on lawns and ornamental plants. It is also registered for direct use on sheep and turkeys, for horse site treatment, dog kennels, domestic dwellings, farm buildings, storage bins, and commercial establishments. Chlorpyrifos acts on pests primarily as a contact poison, with some action as a stomach poison. It is available as granules, wettable powder, dustable powder and emulsifiable concentrate Organophosphate pesticides are neurotoxins. These neurotoxins poison the nervous systems of unwanted insects by interfering with an enzyme in the brain, acetylchlolinesterase, which regulates signals between brain cells. Very similar enzymes are found in the human nervous system, and thus chlorpyrifos and other organophosphates can cause injury to the human brain[4].

Biodegradation

Studies of microbial degradation are useful in the development of strategies for the detoxification of the insecticides by microorganisms[7]. While there have been many reports of isolation and characterization of bacterial species cometabolically hydrolyzing organophosphorus insecticides[1], reports of bacterial species that utilize an insecticide as a sole source of carbon and energy for growth have been limited to date[8]. On the other hand, it is well known that plasmids can endow bacterial species with the ability to degrade various man-made organic compounds[9]. Catabolic plasmids have been thought to play an important role in the evolution of pesticide-degrading ability in microorganisms. For some organophosphates such as parathion, it has been relatively easy to isolate degrading bacteria: two different strains, Flavobacterium sp. Strain ATCC 27551 and Pseudomonas diminuta strain Gm, have been isolated from soils in the Philippines and United States, respectively[15] In other study[14] it was found that a strain of Pseudomonas putida could hydrolyze methyl parathion and use p-nitrophenol as a sole source of carbon. Although chlorpyrifos[O,O-diethylO-(3,5,6-trichlor-2-pyridyl) phosphorothioate] has been widely used for agricultural and household pest control since 1965, it has been problematic isolating a degrading strain for this organophosphate. Several attempts to isolate a chlorpyrifos-degrading microbial system by repeated treatments or enrichment of soils and other media with chlorpyrifos have not been successful[1].

The resistance of chlorpyrifos to enhanced degradation in soil was attributed for this failure. Chlorpyrifos has been reported to be degraded cometabolically in liquid media by Flavobacterium sp. and also by an escherichia coli clone with an opd gene[10,16]. However these microbes did not utilize chlorpyrifos as a source of energy from methyl parathion-enriched soil[1].

Instrumentation

A high performance liquid chromatography (Shimadzu/Japan) was used to determine chloropyrifos. pH meter (Hanna) HI 1280 was used to adjust the pH of the solutions. An Autoclave (Tutanuer) was used to Sterilize the samples and media. An Oven (Heraus) was used for incubation of the samples at different temperature, An autoclave (Tutanuer) was used to treat the samples and API 20E matrices through IdBact v. 1.1 software is applied for identification of the unknown

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organism.

Chemicals and reagents

Sodium chloride, Magnesium sulfate,Ammonium dihydrogen phosphate, Dipotassium hydrogen phosphate were obtained from Merck, Germany

Tetraethoxysilane (TEOS) and were purchased from(sigma) distilled water was used for preparation of all samples, standards and media

Media and reagents

Minimal Salt Media (MSM) was prepared from basic components, Nutrient Agar, MacConkey Agar, Triple Sugar Iron Agar (HiMedia, India). API 20 E (Biomeraux, France), Gram staining kit, oxidase reagent, 0.85% NaCl solutions.

Methods

Sources of chlorpyrifos-degrading microorganism

Soil samples from the surface upper 15cm of soil were taken from the different agricultural sites (TABLE 1) these sites were chosen on the basis that Khan younes soil has been subjected to this pesticide other sites were not. The samples were kept in plastic bags at ambient temperature until processing. The bags were kept open to prevent the increase of their temperature and subsequently death of bacteria. Twenty grams of thoroughly mixed soil sample from each site, was homogenized in 200ml sterile saline solution (0.85%) by shaking the preparation on a sonicator water bath shaker (model) at for 5 min.

Media preparation

Minimal salt medium (MSM) was used throughout the isolation phase. The contents are grams per liter of distilled water: K_2HPO_4(0.5), $(NH_4)_2SO_4$(0.5), $MgSO_4.7H_2O$ (0.5), $FeCl_3.6H_2O$ (0.01), $CaCl_2.2H_2O$ (0.01), $MnCl_2.4H_2O$ (0.01) and $ZnSO_4.7H_2O$ (0.0001). 500 ml of the medium was distributed into 5 flasks (200 ml), each contained 100 ml and the other 500 ml was placed in 1 liter flask. To each of the first 5 flasks, chlorpyrifos was added after boiling the medium for 10 minutes, autoclaving for 15 minutes at 121°C and cooling it to room temperature. Agar was added to the flask containing 500ml of MSM media, boiled until dissolved completely. The MSM agar was autoclaved and cooled to 50°C and the required concentration of chlorpyrifos was added, mixed and distributed into Petri dished, cooled to solidify and stored at refrigerator temperature until use.

TABLE 1 : Soil and wastewater samples used as sources for chlorpyrifos-degrading bacteria

Sample no.	Sample type	Source
1	Soil	Khan younes
2	Soil	Islamic University-Gaza, Garden
3	Wastewater	Islamic University-Gaza

Nutrient agar medium, triple sugar Iron Agar and MacConkey agar were prepared according to the manufacturer instructions.

Isolation of chlorpyrifos-degraders

For each sample, a 200ml Erlenmeyer flask containing 100ml of MSM, 200ppm chlorpyrifos was prepared. A volume of soil or sewage suspension (7.5ml) was transferred to each flask and incubated aerobically at room temperature with continuous agitation on Heidolph shaker.

After 6 days of incubation, subcultures were transferred onto MSM-chlorpyrifos Agar plates containing 100ppm chlorpyrifos. Plates were incubated at the same conditions for 7 days. Plates were checked for growth on daily basis. Visible growth was re-streaked on a fresh MSM-chlorpyrifos Agar plates containing 100ppm chlorpyrifos, Nutrient agar plates and Triple sugar Iron Agar. Pure colonies were preserved by freezing at -80°C in sterile 25% glycerol.

Immobilization of chloropyrifos-degrading bacteria

A bacterial colony was transferred to 50ml of sterile MSM (pesticide-free) and vortexed. A volume of 13.4ml of Tetraethyl-orthosilicat, a 3.4ml distilled water and 1.4ml (0.1 N) HCl were transferred into a conical flask. The flask was firmly corked and stirred by means of magnetic stirrer at room temperature for 3 hours. 17.8ml of the suspension solution which contain isolation bacteria and leave it until it became Gel. The monoliths obtained was left to dry at room temperature for two weeks, were powdered and washed with 0.85% saline solution[12].

Degradation studies using free isolate and immobilized bacteria

Effect of period of incubation

A sterile cotton swab was used to collect 3-4 colonies of chlorpyrifos-degrading isolate from the isolation plate. The swab was transferred to 10ml of sterile distilled water. The tube was vortexed for 2 minutes. One ml of the bacterial suspension or 0.20g of the sol-gel immobilized bacteria were transferred to two separate 250ml flask containing 100ml sterile MSM solution containing a weighted amount of chlorpyrifos to give 200mg/L of the pesticide. The flasks were incubated for 8 days at room temperature. The concentration of chlorpyrifos was monitored daily by HPLC.

1. The effect of pH variation

The pH of of two sets of solution each set contains 6 flasks each flask containing 50ml of MSM (200 ppm chlorpyrifos) was adjusted using either NaOH or HCl to obtain pH of 1, 3, 5, 7 and 9. One ml of the bacterial suspension (prepared as in the previous section) was added to each flask of one set, (it is assumed that each flask contains the almost same number of bacteria cells), A 0.10g of immobilized bacteria to each flask of the second set.

The test flasks were agitated for 4 days at room temperature. Samples were filtered at the end of incubation period and chloropyrifos was determined by the analytical method mentioned before.

2. The effect of temperature variation

Similar to the above preparation but keeping the pH=7.0(This value was found to be the optimum pH for degradation in this study).

Samples were incubated at varying temperatures(10, 20, 30 and 50°C)for four days. At the end of the incubation period, samples were filtered and chloropyrifos was determined.

3. The effect of temperature variation

A 0.1g sol-gel immobilized was transferred into 200 round flasks containing 200ppm chlorpyrifos dissolved in MSM solution and incubated at different temperature 10, 20, 30, 50^{0}C. Flasks were incubated for 4 days with continuous agitation. Chlorpyrifos concentration was measured at the end of the incubation period by HPLC.

4. Analytical method

All samples of chloropyrifos were determined using HPLC with UV-225nm. Detector and a 25cm column with i.d = 4.6mm, flow rate = 1.4mL/min., mobile phase acetonitrile 75% and water 25%, at room temperature.

Figure 1 : Chromatogram of chloropyrifos. HPLC with UV-225nm. Detector and a 25cm column with i.d = 4.6mm, flow rate = 1.4mL/min., mobile phase acetonitrile 75% and water 25%, at room temperature

In which 10, 30, 50,100, 150 and 200mg/L of chloropyrifos standard solution were prepared and A 20µL the standard solutions were injected with adjustable microsyringe. Chloropyrifos peak was identified as shown in figure 1 with a retention time 8.3 min.

A calibration plot was constructing by plotting peak area against concentration of chloropyrifos. The concentration of the sample solutions was found from the calibration.

RESULTS AND DISCUSSION

Isolation of chlorpyrifos-degrading bacteria

Soil samples

After one week of MSM-Chlorpyrifos enrichments (in broth media on shaker), subcultures were made on MSM-Chlorpyrifos agar plates. Colonies growing on plates were similar in each of three trials. One colony from each trial was picked and subcultured. The three isolates were designated as C1, C2 and C3.

During the initial screening of the isolates, C1 (isolated from Khan younes soil sample) exhibited the fastest growth as evident by the appearance of colonies after only 48 hours while C2 and C3 (isolated from Islamic university-Gaza soil) showed slower growth and thus eliminated.

Sewage sample

No isolate was recovered from any of the three

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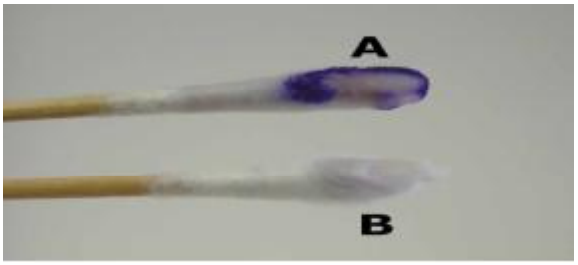

Figure 2: Oxidase test performed for the isolate (Positive result: purple color)

Figure 3 : API 20E results for C1 stain

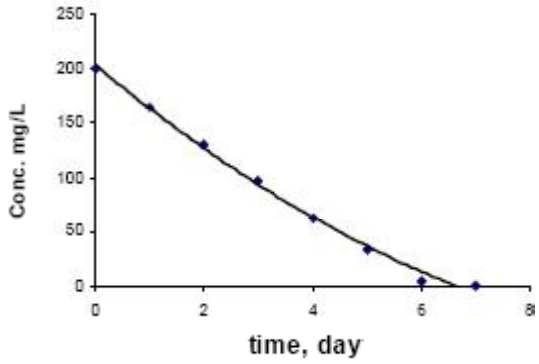

Figure 4: Concentration of of chlorpyrifos a by free bacteria at pH =7.0, at room temperature, and constant number of microorganisms

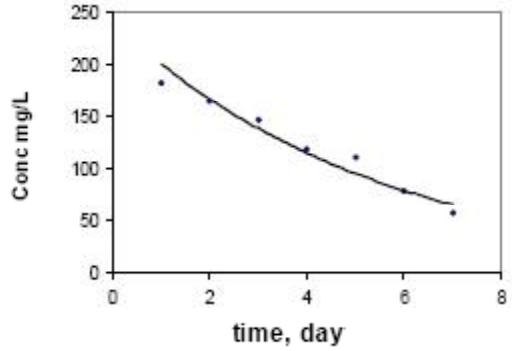

Figure 5: Degradation of chloropyrifos by sol-gel immobilized bacteria at pH = 7, at room temperature and constant number of microorganisms

sewage samples collected from the Islamic University-Gaza sewer.

Characterization of chlorpyrifos-degrading bacteria

C1 strain was subjected to morphological and biochemical investigation and the followings are the results of these investigation

Gram stain: Gram stained smear from C1 isolate exhibited Gram negative bacilli.

Oxidase test: C1 strain exhibited positive oxidase test results as shown in figure 2.

API 20 E

After 24 hours of incubating the API20 E tray, TDA, Indole and VP reagents were added as recommended by the manufacturer and the results of each biochemical test was recorded in the following TABLE.

Identification of the isolated strain

Based on gram stain results, API 20 E matrix was selected for the identification using IdBact v. 1.1 software. The result indicated that C1 strain is Burkholderia cepacia.

Degradation of chloropyrifos (200mg/L) by free bacteria was investigated at pH 7 and at room temperature over a period of time. As shown in figure 4 the concentration of chloropyrifos is dropped to a bout 150mg/L after 24 hours and degradation is continued till chloropyrifos concentration is lowered below limit of quantaion after seven days. As shown from the figure the half-life of degradation of chloropyrifos is about 3 days.

The same study was performed using sol-gel immobilized bacteria as shown in figure 5. Lower degradation rate of chloropyrifos was observed using trapped bacteria in which the half-life of degradation of chloropyrifos is about 4.2 days.

This trend could be microenvironment provided for immobilized bacteria may effect its Activity. It was concluded by Jacques Livage et al that bacteria in pure silica gels is not very active[13].

TABLE 2: API test results of C1 isolate

Test	Result	Test	Results
ONPG	-	GEL	+
ADH	+	CLU	-
IDC	-	MAN	-
ODC	-	INO	-
CIT	+	SOR	-
H_2O	-	RHA	-
URE	-	SAC	-
TDA	-	MEL	-
IND	-	AMY	-
VP	-	ARA	-

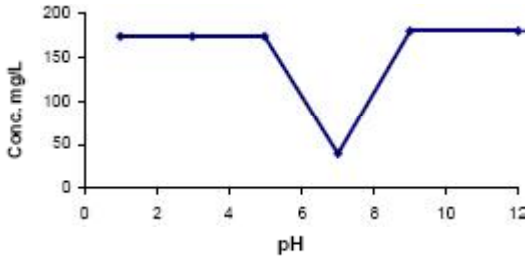

Figure 6: Effect of pH on the degradation of chlorpyrifos by free bacteria after 4days, at room temperature and constant number of microorganisms

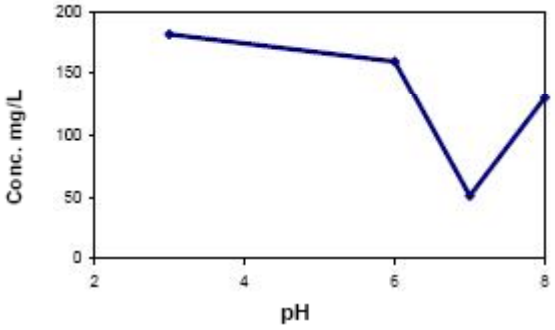

Figure 7: Effect of temperature on the degradation of chloropyrifos by immobilized bacteria, at pH 7, after 7.0 days and constant number of microorganisms

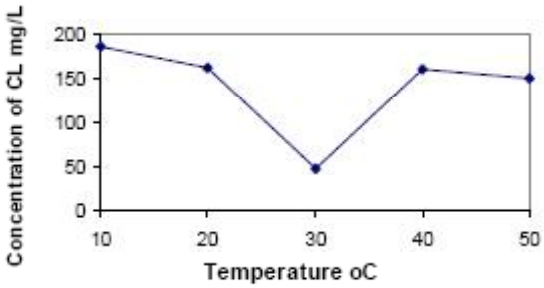

Figure 8: Effect of pH on the degradation of chloropyrifos by immobilized bacteria, at room temperature, after 7.0 days and constant number of microorganisms.

The effect of pH on the degradation of chloropyrifos was examined at room temperature over 3-12 pH range by free bacteria as demonstrated in figure 6 degradation process is low at acidic medium in 1-6 pH range and the same trend is observed in basic range pH 8-12 on the other hand maximum degradation was observed at pH 7.

The effect of pH on the degradation of chloropyrifos by immobilized bacteria was studied over a 3-8 pH range as shown in figure 7 (the range was not extended to higher values due to instability of sol-gel in highly basic medium).

Almost the same trend of degradation with immobilized bacteria was observed compared with free bacteria as a function of pH this could be the higher biological activity of the isolated strains at neutral medium. Miles et al. reported that faster degradation of chloropyrifos was observed in neutral soil[11].

The effect of temperature on the degradation of chloropyrifos by free and sol-gel immobilized bacteria was investigated at a temperature range (10-50°C) As demonstrated in figure 8 temperature has a significant effect on.

The degradation of chloropyrifos by the two forms of bacteria (frre and immobilized) after 4 days of inoccupation and at pH 7 that is, at 10°C the degradation is less than 15% and it is increasing with increasing temperature till maximum degradation was observed at 30°C in which almost 85% degradation was achieved, on the other hand degradation was much slower at 40 and 50°C.

This trend is exhibited by the two forms of bacteria. It was concluded in a work done by Frank et al. that the half-life for the disappearance of chlorpyrifos was 4.8 days at 21°C and 27 days at 4°C, indicating that temperature plays a major role in the degradation of chlorpyrifos in water[5].

CONCLUSION

The biological applications of sol-gel chemistry appear to be very promising method for immobilization of living cells like bacteria.

In this work chlopyrifos degrading bacteria were isolated from Khan-yonis soil sample that is historically subjected to this pesticide the isolate was identified as Burkholderia cepacia.

Degradation study of chlopyrifos was employed using free and sol-gel immobilized Burkholderia cepacia. It was found that the concentration of chlorpyrifos was dropped from 200mg/L to below limit of quantation using free bacteria and it is lowered to 58mg/L using immobilized bacteria.

It was found that temperature play a significant factor in the degradation of chlorpyrifos by the two forms of bacteria in which, maximum degradation was observed at 30°C. On the other hand pH effects the deg

radation of the mentioned pesticide by both free and immobilized Burkholderia cepacia that is it was found pH 7 is the optimum for the degradation of chlorpyrifos.

It is suggested to study degradation product that appear during the degradation by employing GC-MS which was not available in our laboratories.

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