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DEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID BY *Pseudomonas fluorescens* strain HH

Nguyen Thi Oanh, Ha Danh Duc*, Tran Dat Huy, Nguyen Gia Hien, Nguyen Thi Huynh Nhu

Dong Thap University, Vietnam

ABSTRACT

Pseudomonas fluorescens HH isolated from soil utilized 2,4-Dichlorophenoxyacetic acid (2,4D) as a sole carbon and energy source. The strain completely utilized 1.0 mM of 2,4D within 30 hr. The immobilized *Pseudomonas fluorescens* HH in alginate degraded 2,4D with higher rates compared to the rates of free-suspension cells. The determination of degradation and cell growth kinetics in exponential growth phase of bacteria showed that both fitted with the Edwards model, in which the maximal utilization rates and inhibition coefficient were 0.079 ± 0.008 mM/h and 0.820 ± 0.03 mM, respectively. The addition of glycerol as a cryoprotectant into alginate increased the survival of bacteria in beads during freeze-drying process, which resulted in reducing the adverse effects of bead lyophilization.

Keywords: Pseudomonas fluorescens HH, 2,4-Dichlorophenoxyacetic acid, Edwards model, maximal utilization rates, inhibition coefficient, lyophilization.

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**Corresponding author email*: hadanhduc@gmail.com

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INTRODUCTION

Herbicides are toxic to their intended target species and other organisms. 2,4D is one of the most commonly used herbicides to control broadleaf weeds in the world (Bortolozzi et al., 2004; Robles-González et al., 2006; González et al., 2012). 2,4D causes harmful effects on natural plants and animals (Cox 1999; Willemsen & Hailey 2001; Chinalia & Killham 2006). Moreover, the chemical causes serious health problems such as depression of the central nervous system, and damaging the liver and kidney of human and animals (Moody et al., 1992; Duffard et al., 1996; Kim et al., 1998; Kwangjick el al. 2001; Charles et al., 2001; Kim et al., 2005; Robles-González et al., 2006).

The wide use of 2,4D resulted in the accumulation of a considerable amount of 2,4D and its intermediates in soil, surface water, or groundwater. Even though the physical and chemical methods can be applied for cleaning up the chemical, biodegradation is the main process to remediate 2,4D in soil and water. There are a number of microorganisms utilizing 2,4D as the sole carbon and energy source described previously. They are *Pseudomonas cepacia* (Greer, 1990), *Azotobacter* sp. (Gauri et al., 2012), *Delftia* sp. (Hoffmann & Muller 2006; González et al., 2012), *Variovorax paradoxus* (Vallaeys et al., 1998), *Burkholderia cepacia* YK-2 (Cho et al., 2002). To increase the degradation rates, the isolation and augmentation of pure cultures in the contaminated sites should be carried out.

In Vietnam, 2,4D has been extensively used for weed control. Some 2.4D-degrading bacterial strains have been isolated in Vietnam (Nguyen Thi Phi Oanh et al., 2011; Huong et al. 2007). Also, 2,4D-degrading fungal strains were isolated countrywide from 10 Vietnamese soils (Itoh et al., 2013). However, the determination

of environmental conditions on the 2,4D degradation as well as the cell density necessary for practical use the contaminated soil bioremediation and water in Vietnam has not been investigated.

Immobilization techniques are preferable for bioremediation. The carriers protect microorganisms from the toxicity of chemicals, and improve the degrading efficiency of xenobiotics. Due to cheap, easy to handle and nontoxic property, alginate has been widely used in the encapsulation of bacteria. Because of the inconvenient storage, transportation and application of dried beads in field-scale bioremediation, a freeze-dried formulation should be developed. However, the lyophilization and rehydration processes may be harmful to immobilized cells. The use of cryoprotectants can enhance the survival of microorganisms during the process (Font de Valdez et al., 1983; Hubálek, 2003).

Thus, the aims of this study were to (1) isolate and identify 2,4D-degrading bacteria, (2) investigate the effects of environmental conditions such as pH and humic acid on the degradation rates, (3) determine the degradation by whole cells, and (4) examine the effects of cryoprotectants on microorganisms survival in alginate beads during lyophilization process.

MATERIALS AND METHODS

Chemicals and media

2,4D was dissolved in absolute ethanol as stock solutions prior to use. Mineral medium (MM) contained (in grams per liter) Na_2HPO_4, 2.79; KH_2PO_4, 1.00; $(NH_4)_2SO_4$, 1.00; $MgSO_4 \cdot H_2O$, 0.20 and 1.00 mL trace mineral solution. The trace mineral solution consisted of H_3BO_3, 0.30; $CoCl_2 \cdot 6H_2O$, 0.20; $ZnSO_4 \cdot 7H_2O$, 0.10; $Na_2MoO_4 \cdot 2H_2O$, 0.03; $MnCl_2 \cdot 4H_2O$, 0.03; $NiCl_2 \cdot 6H_2O$, 0.02; $CuCl_2 \cdot 2H_2O$, 0.01. pH of the MM medium was adjusted with HCl and NaOH solution. The medium was autoclaved at 121°C for 15 min. All chemicals were purchased from Sigma-Aldrich or Merck.

Enrichment, isolation and identification of the bacterial strain

Soil samples were collected from a a sugar cane field (at depths of 10–50 cm) in Tra Vinh province (South Vietnam) and stored at 5°C before the isolation of bacteria. The soils were air-dried at room temperature, mixed thoroughly, and sieved through 2 mm mesh to remove large debris to ensure homogeneous mixing. 5 g of the sample was transferred into a flask containing 200 mL of the MM medium. 0.5 mM of 2,4D was added into the solution. The incubation was carried out for 2 months, and 2,4D (0.5 mM) was added to the culture every week. The enrichment culture was incubated at room temperature (around 30°C) with continuous shaking at 150 rpm. The diluted samples were spread onto agar plates containing the MM medium and 0.5 mM of 2,4D. Each obtained bacterial strain was transferred to liquid MM medium to check the growth and 2,4D biodegradation ability of bacteria as a sole carbon and energy source. The bacterial isolate showing the most effective degradation was identified by *16S rRNA* sequencing as previously described by Duc (2017). The phylogenetic tree was constructed using the Tamura-Nei model in the MEGA version 6.0 software. The bootstrap values were expressed as percentages based on analysis of 1,000 resampled data sets.

Degradation of 2,4D by freely suspended and immobilized *Pseudomonas fluorescens* HH in liquid media

The degradation experiments in liquid media were carried out aerobically using a flask containing 100 mL of the MM medium supplemented with 2,4D. The cell inoculum (1.0 mL) was added to the reactor to give the original cell number of around 10^6 CFU/mL (based on the colony count method). For determination of the effects of co-substrates on biodegradation rates, glucose or humate (1.0 g/L) was supplemented to the media. All experiments were conducted at pH=7.0 except for the experiments on the effects of pH on chemical degradation.

For preparation of inoculum, bacteria were cultured in liquid MM medium supplemented with 0.5 mM of 2,4D and 1.0 g/L glucose for 18h. For the entrapped cell preparation, cells were collected by centrifugation at 10,000 rpm and 4°C for 10 min. Cell pellet was washed twice with sterile saline (0.85% NaCl) and resuspended in 2×MM medium. Sodium alginate

(6%, w/v) was dissolved and blended in a boiling water bath. The solution was then cooled to room temperature, mixed with concentrated bacteria (1:1, v/v), and gently stirred for 30 min. Glucose or glycerol was added as cryoprotectants at 10% and 20% (w/v) to the solution. The mixture was dripped into a solution containing 3% $CaCl_2$ (w/v) using a syringe. The contents were stirred with a magnetic stirrer for 60 min. With this procedure, the diameter of a bead was roughly 3 mm. The final alginate concentration and cell numbers in alginate beads were 3% and 3.5×10^9 CFUs/g, respectively. After storage for 24 hr in the $CaCl_2$ solution at 4°C, beads were then washed twice with sterile saline (0.85% NaCl).

The 2,4D biodegradation by immobilized cells was carried out in the MM medium. 2,4D was added at 1.2 mM as a sole carbon and energy source. The final number of cells was 3.5×10^9 CFUs/mL. Samples were taken during the incubation process for determination of remaining 2,4D. All degradation experiments in liquid media were carried out at room temperature with the shaking speed of 150 rpm.

Degradation and bacterial growth kinetics

The determination of the 2,4D degradation and bacterial growth kinetics was conducted in the MM medium supplemented with a range of 2,4D concentrations. Bacteria grew and utilized the chemical a sole carbon and energy source. The modified Michaelis-Menten equation of the Edward model with the specific degradation rates given by the equation: $V=K_{max}[\exp(-S/K_i)-\exp(-S/K_S)]$ (Edwards, 1970) was used to fit the degradation data. The kinetic parameters, including the maximum specific degradation and apparent half-saturation coefficient values of each substrate concentration were derived by linear regression fitting of the Lineweaver-Burk plot or double reciprocal plot (Lineweavear & Burk, 1934). The Dixon plot was used to determine the inhibition constant, in which the reciprocal of the velocity was plotted against the inhibitor concentrations (Dixon, 1953). GraphPad Prism 7.0 software (CA, USA) was used to solve the model equations.

Freeze-drying and rehydration process

The freeze-drying process was carried out using a bench-scale freeze-dryer (Labconco Co., USA) at (-)58°C and 0.1 mbar for 24 hours. The freeze-dried beads were stored at 4°C. Before use, the rehydration process was conducted. Beads were immersed in the sterile MM medium for 60 min with a rehydration volume of 50 mL/g bead. The rehydration process was carried out at room temperature, and beads were then carefully rinsed with saline before use.

The determination of cell survival in beads after rehydration was carried out by a modified method of Schoebitz et al. (2012). Twenty alginate beads were dissolved in 10 mL of sterile sodium citrate (6%, w/v) at room temperature with continuous shaking at 150 rpm until completely dissolved (about 30 min). Surviving cells released from the beads were diluted and spread on LB agar plates for counting the CFUs at 24th hr of incubation.

Analytical methods

The determination of 2,4D concentrations in liquid media was performed using reverse phase high performance liquid chromatography (HPLC) equipped with a UV detector (240 nm). The separation was performed at 40°C on C18 HPLC column (5 μm, 250 mm × 4.6 mm; Hyperclone, Phenomenex, USA). A 7:3 (v/v) ratio acetonitrile: ultrapure water mixture served as the mobile phase at a flow rate of 1 mL/min. Each peak was compared to those of the standard compounds of known concentration, where the retention time was used for identification and the peak area for quantitative estimation of the amount of that compound. Cell turbidity was determined at 600 nm (DU800, Beckman Coulter, Inc., USA) and the exponential growth rate was determined based on Zeyer et al. (1985) and was used for cell growth comparison.

Statistical analysis

The data are shown as the mean ± one standard deviation. Significant differences among means were statistically analyzed using one-way ANOVA with Duncan's test (Statistical Package for Social Sciences (SPSS) pro-

gram version 22.0). Microsoft Excel was used to analyze regression.

RESULTS AND DISCUSSION

Isolation and identification of the 2,4D-degrading bacterium

The isolated strain HH which utilized 2,4D as a sole carbon and energy source was a Gram-negative, rod-shaped bacterium. The 16S rRNA sequence had the highest degree of nucleotide sequence similarity with the genus *Pseudomonas* isolates (99% identity) of the sequences available in the NCBI GenBank database and the NJ based phylogenetic analysis placed the HH sequences within in the genus *Pseudomonas* with the closest similarity to *P. fluorescens* (Fig. 1). Accordingly, this strain is named *Pseudomonas fluorescens* HH hereafter.

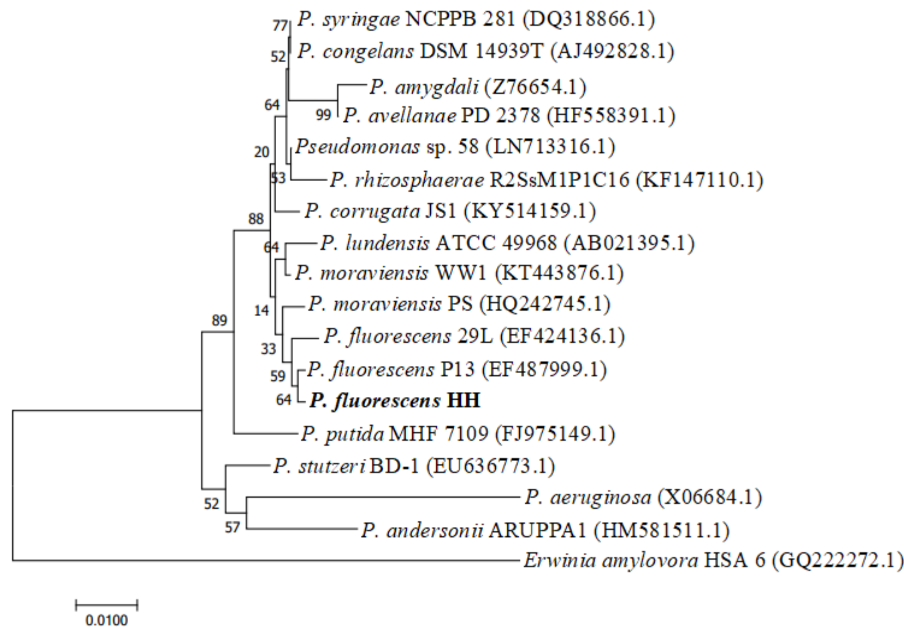

Figure 1. Phylogenetic tree based on 16S rRNA gene fragment shows the *Pseudomonas fluorescens* HH position. The scale bar (0.01) shows the number of nucleotide substitutions per base

Degradation of 2,4D by freely suspended and immobilized *Pseudomonas fluorescens* HH in liquid media

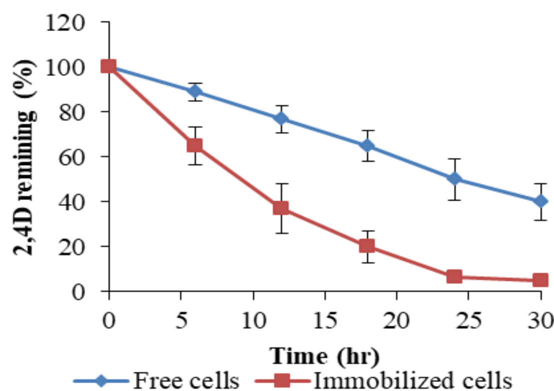

Figure 2. Degradation of 2,4D (1.2 mM) by freely suspended and immobilized *Pseudomonas fluorescens* HH in liquid media

Pseudomonas fluorescens HH grew and utilized 2,4D as the sole carbon and energy source in MM medium. The bacteria could utilize 1.0 mM 2,4D completely by 30 hr, which was similar to the degradation by *Achromobacter* sp. LZ35 (Xia et al., 2008). However, *Pseudomonas fluorescens* HH had no lag phase at these concentrations, which was different from some previous reports that the 2,4D degradation required required a lag phase (Xia et al., 2008; Greer et al., 1990).

The degradation by immobilized cells and freely suspended cells (with the same cell numbers and 2,4D concentration) was compared (Fig. 2). The immobilized *Pseudomonas fluorescens* HH showed better performances than freely suspended cells probably because the carrier materials protected cells from the surrounding environment, which was attributed

to the increased tolerance of the immobilized cells to 2,4D over freely suspended cells. Similarly, *Pseudomonas* sp. US1 immobilized in calcium alginate dehalogenated 2,4D with the rates comparable to those of free cells (Sahasrabudhe et al., 1991).

Degradation and bacterial growth kinetics

The kinetics of chemical utilization by *Pseudomonas fluorescens* HH and the cell growth under aerobic conditions were investigated with the 2,4D concentrations ranging from 0.1 to 1.5 mM at the exponential growth phase of bacteria. The degradation of the compound and cell growth curves of *Pseudomonas* HH followed the Edward model, and the substrate inhibition was observed (Fig. 3). The degradation and growth rates were highest at 0.7 mM, and decreased at higher concentrations. The 2,4D degradation and cell growth kinetics were calculated for various substrate concentrations, which were calculated and shown in table 1.

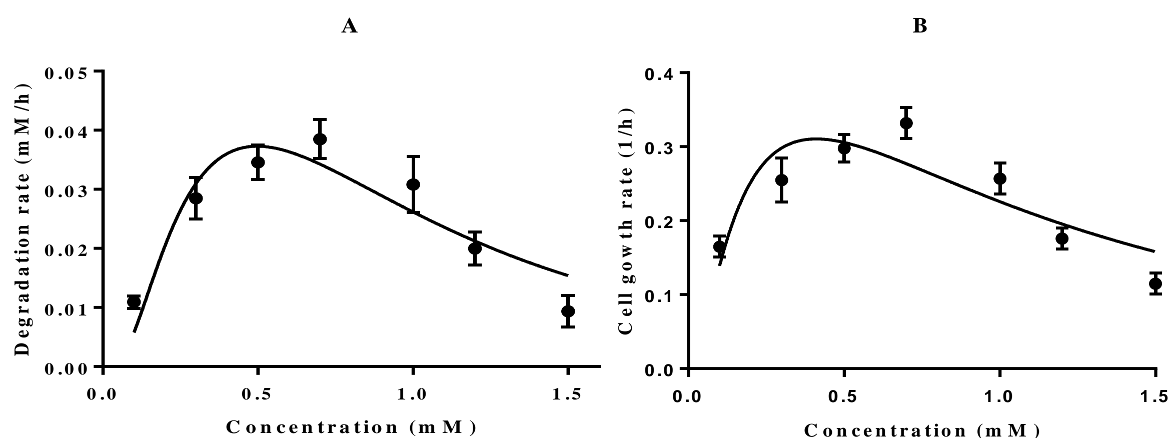

Figure 3. Relationship between the specific biodegradation rates and 2,4D concentrations (A), and growth rates and 2,4D concentrations (B) of *Pseudomonas fluorescens* HH. Data are shown as the mean ± SD, derived from at least three independent repeats

Table 1. Apparent kinetic parameters of 2,4D biodegradation by *Pseudomonas fluorescens* HH

Parameters	Degradation	Bacterial growth
Maximum of specific rate	0.079 ± 0.008 mM/h	0.060 ± 0.006 1/h
Apparent inhibition coefficient	0.820 ± 0.03 mM	0.712 ± 0.085 mM

Effect of cosubstrates and pH on 2,4D degradation

The addition of 0.5 and 1.0 g/L of glucose or humate resulted in the increase of degradation rates of suspended bacteria (Table 2). However, the increase of the cosubstrates to 1.5 g/L resulted in the reduction of the degradation rates. The decrease of degradation was probably because bacteria utilized glucose as the carbon source rather than 2,4D when glucose was added at a high concentration. For the growth rate, the addition of glucose and humate stimulated the growth, and the increase of concentrations resulted in higher growth rates.

A number of reports showed that the addition of exogenous carbons stimulated the degradation of 2,4D. The addition of glucose to stimulate the degradation of 2,4D was reported (Greer, 1990; Xia et al., 2017). Glucose was more preferred substrate and completely utilized before the degradation of 2,4D in anaerobic batch reactors (Celis et al., 2008). Humic substances usually present in cultivated soils, which probably affects the degradation of xenobiotics. In other studies, humic substances linked to the dechlorination of 2,4D by *Comamonas koreensis* CY01 (Wang et al., 2009) and stimulated the anaerobic degradation of 2,4D by *Corynebacterium humireducens* MFC-5 (Wu et al., 2013). In addition, humics were confirmed as terminal electron acceptors for microbial respiration (Lovley et al., 1996).

The pH determination on degradation

degradation was conducted in the medium without any cosubstrate. The pH level suitable for the activities of the isolate was around 7.0. The degradation rates of entrapped cells were around 20% higher than the rates of free cells at any pH level (Fig. 4).

Table 2. Effects of glucose and humate on degradation and cell growth of *Pseudomonas fluorescens* HH. Bacteria grew and degraded 2,4D (1.0 mM) in the MM medium supplemented with various concentrations of cosubstrates. Data were obtained 12 hr after incubation

Cosubstrates concentration (g/L)	Glucose		Humate	
	Degradation rate (mM/h)	Cell growth rate (1/h)	Degradation rate (mM/h)	Cell growth rate (1/h)
0	0.032 ± 0.002^{C}	0.026 ± 0.003^{D}	0.032 ± 0.002^{C}	0.026 ± 0.003^{C}
0.5	0.075 ± 0.005^{A}	0.035 ± 0.004^{C}	0.065 ± 0.005^{A}	0.032 ± 0.004^{C}
1.0	0.058 ± 0.005^{B}	0.048 ± 0.005^{B}	0.048 ± 0.003^{B}	0.042 ± 0.006^{B}
1.5	0.015 ± 0.001^{D}	0.066 ± 0.007^{A}	0.012 ± 0.001^{D}	0.060 ± 0.006^{A}

(*)Different superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a column using the one-way ANOVA with Duncan's test in SPSS software version

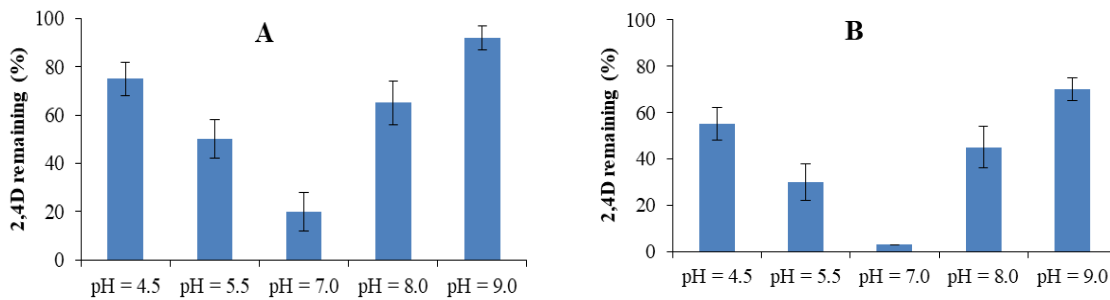

Figure 4. Effects of pH on 2,4D degradation by freely suspended (A) and immobilized (B) *Pseudomonas fluorescens* HH. The experiments were conducted at 1.0 mM of 2,4D, and data were obtained at 24th hr of incubation. Data are shown as the mean ± SD, derived from at least three independent repeats

Effects of cryoprotective agents on cell survival and 2,4D biodegradation of the freeze-dried, entrapped cells

The addition of protective solutes into the gel beads resulted in the decrease of 2,4D degradation rate except for 10% glycerol (Table 3). More cryoprotectants in beads resulted in lower biodegradation rates by entrapped cells before lyophilization. However, the biodegradation rates of bacteria in beads containing 10% and 20% cryoprotective additives were no statistically different after freeze-drying (Table 3). After the process, the biodegradation rates of entrapped cells were reduced with the highest reduction for beads without any cryoprotectant. The presence of glycerol in beads showed higher effectiveness than glucose on increase of cell survival during the freeze-drying process.

The presence of cryoprotectants reduced adverse effects of entrapped cells in this study. The 2,4D degradation rate of cells immobilized in the beads without any protective agent was sharply reduced after freeze-drying due to low cell survival. With the presence of cryoprotectants inside the cells, the osmotic difference with the external environment is reduced, and cold tolerance is improved (Kets et al., 1996). Previous reports showed that the survival of immobilized microorganisms was enhanced with the glycerol addition (Cui et al., 2006; Kearney et al., 1990; Wang et al., 2012; Zohar-Perez et al., 2002) probably because glycerol may prevent ice-crystal formation after penetrating into the cells (Madigan et al., 1997). Another report indicates that the glycerol addition protects the microorganism, increases pore size in beads, and controls the structure of the dried microcapsules (Zohar-Perez et al., 2002). Freeze-drying of immobilized cells has been applied for cell preservation for long-term storage (Kearney et al., 1990; Sompornpailin et al., 2014).

Table 3. 2,4D degradation by *Pseudomonas fluorescens* HH immobilized in alginate- cryoprotectant beads. The experiments were conducted for 24 hr of incubation, at initial concentration of 1.0 mM. The cell survival and degradation by cells in freeze-dried beads was determined immediately after rehydration

Cryoprotectant (%)	Fresh bead[(*)]	Freeze dried bead[(*)]	
	Degradation (%)	Degradation (%)	Cell survival (%)
None	93.3 ± 3.1^{A}	20.9 ± 5.2^{C}	18.8 ± 5.5^{C}
10 % glycerol	87.3 ± 5.9^{AB}	61.9 ± 9.5^{A}	80.5 ± 4.0^{A}
20 % glycerol	68.6 ± 6.6^{C}	65.7 ± 7.3^{A}	85.6 ± 6.6^{A}
10 % glucose	70.2 ± 5.1^{BC}	40.0 ± 6.9^{B}	51.4 ± 7.7^{B}
20 % glucose	55.3 ± 6.5^{D}	48.8 ± 3.9^{B}	58.8 ± 8.7^{B}

[(*)]Different superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within the same groups in a column using the one-way ANOVA with Duncan's test

CONCLUSION

This work describes the the 2,4D biodegradation by *Pseudomonas fluorescens* HH as a sole carbon and energy source. The immobilization of cells in alginate resulted in higher degradation of bacteria compared to suspended counterparts. The development of formulas for cell entrapment to enhance the survival of bacteria during lyophilization by the addition of cryoprotectant suggested that the immobilization technique described here has a potential for practical applications.

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