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Full Length Research Paper

Identification of pesticide-degrading *Pseudomonas* strains as poly-β-hydroxybutyrate producers

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Polyhydroxybutyrate (PHB) is an ecological promising substitute for polypropylene because it is biocompatible, biodegradable and can be produced by renewable sources. This study investigated PHB accumulation on pesticide-degrading *Pseudomonas*. 14 strains of pesticide-degrading *Pseudomonas* isolated from subtropical lowland soil in southern Brazil were analyzed using optical microscopy. Lipid inclusions were identified in four strains, and three of them, which degraded the pesticide carbofuran, had extensive granules accumulation which was detected by transmission electron microscopy. These strains were cultivated in a shaker at 28°C and the polymer was characterized by Fourier transform infrared (FTIR) spectroscopy. Strain CMM43 had the best accumulation after 48 h. The biopolymer was identified as poly-β-hydroxybutyrate.

Key words: Polyhydroxybutyrate (PHB), pesticide-degrading *Pseudomonas* sp., microscopy, subtropical lowland soil.

INTRODUCTION

The increasing search for polyhydroxyalkanoate (PHA)-producing bacteria is due to its potential applications in packaging (Kulkarki et al., 2011), as matrices for pesticides (Savenkova et al., 2001), and in suture lines (Volova et al., 2003). These polymers have properties that make them highly competitive with polypropylene (Reddy et al., 2003); and their replacement of petrochemical plastics is environmentally advantageous because they are biodegradable (Sudesh et al., 2000). Polyhydroxybutyrate (PHB) is the most studied PHA and is the one that is most frequently accumulated by bacteria. These polyesters work as carbon and energy reserves for the cell and can be produced from a large variety of substrates, including renewable resources (Madison and Huisman, 1999).

PHB are accumulated as granules by bacterial cell at up to 80% of the cell dry weight (Braunegg et al., 1998). Usually the granules formation occurs under conditions of nutritional limitation of N, P, O, or Mg and in excess carbon source (Anderson and Dawes, 1990) and in some bacteria the polymer accumulation occurs during growth in the absence of nutrient limitation (Lee, 1996).

Most *Pseudomonas* bacteria are capable of biosynthesizing medium chain length (MCL) PHAs (Haba et al., 2006). They seldom produce blends of PHAs consisting of PHB (short chain) and MCL PHAs (Hang et al., 2002), and they rarely accumulate PHB (Jiang et al., 2008; Mohan et al., 2010).

In this study, three strains of pesticide-degrading *Pseudomonas* from a culture collection were identified as PHB producers. An initial classification of the PHB-accumulating strains was performed using optical microscopy and transmission electron microscopy to determine the presence of granules in the bacterial cells. The selected strains were cultivated in a sucrose medium for later polymer extraction and characterization by Fourier transform infrared spectroscopy (FTIR).

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MATERIALS AND METHODS

Microorganisms

14 strains of pesticide-degrading *Pseudomonas* were used to identify lipid bodies. The strains were isolated from subtropical lowland soil in Rio Grande do Sul and conserved at the Culture Collection of Multifunctional Microorganisms at Embrapa Temperate Agriculture (CCMMETA). Six of the strains are known to degrade pyrazosulfuron-ethyl (CMM16, CMM17, CMM18, CMM19, CMM20 and CMM21), two to degrade glyphosate (CMM38 and CMM39), one to degrade clomazone (CMM1), and five to degrade carbofuran (CMM41, CMM42, CMM43, CMM44 and CMM45). *Bacillus megaterium* (CMM105) was used as a positive control for PHB production (Gouda et al., 2001).

Detection of lipophilic inclusions by Sudan Black

To detect lipid bodies by optical microscopy, the strains were cultivated on plates with nutrient agar and 5% sucrose in a bacteriological incubator at 28°C for 48 h. A preliminary screening for lipid bodies in the 14 strains was performed using Sudan Black B staining according to Schaad et al. (2001). Strains with positive Sudan Black B results were further assessed by transmission electron microscopy.

Detection of lipophilic inclusions by transmission electron microscopy

The quantities and sizes of lipid inclusions were characterized using transmission electron microscopy (TEM) for the strains previously selected by optical microscopy. For TEM analysis, cells were incubated on nutrient agar with sucrose for 48 h. The cells were washed with saline solution, bathed in two fixers, and then dehydrated in increasing concentrations of alcohol, 30, 50, 70, 90 and 95%(v/v), and P.A. acetone. Finally, the samples were embedded in Epon AB-DinP30 resin and cured for 5 days at 60°C. Ultra-thin sections were observed using TEM (ZEISS EM900).

PHB production in shaker flasks

Because strain CMM43 was identified by TEM as a good accumulator of lipid granules, it was used as a model for analyzing growth versus polymer accumulation. The inoculum was prepared by inoculating YM medium (Jeanes, 1974) with a loop of a pure culture of the organism grown for 24 h. The YM culture was grown in an orbital shaker for 18 h at 28°C and 150 rev.min^{-1}. 10% (v/v) of inoculum was added to nutrient broth with 12g.L^{-1} of sucrose (NB+SUC) and incubated in a shaker at 28°C and 150 rev.min^{-1}. Four time points (12, 24, 48 and 72 h) were assessed by TEM for the accumulation of inclusions. Cell dry weight (CDW) was determined at 24, 48 and 72 h by gravimetry and the amounts of PHB were measured in 10 mg of CDW which was subjected to acid methanolysis and analyzed by gas chromatography (Brandl et al., 1988). Residual sucrose was measured using dinitrosalicylic acid method (DNS); after the hydrolysis of samples (Miller, 1959).

The strains CMM41, CMM43 and CMM44, which were previously identified as accumulators of lipid inclusions, were grown in NB+SUC for 48 h, using the same inoculation, temperature and shaking methods previously described, for later extraction and polymer identification and characterization.

Polymer extraction

For PHB extraction, cells were centrifuged at 6300 *g* for 20 min, and pellets of harvested cells were bathed in 100 ml of chloroform. Samples were placed in closed tubes and heated at 58 to 59°C with magnetic shaking for 2 h. They were then centrifuged at 1100 *g* for 15 min, and the bottom organic phase in each tube was removed with a Pasteur pipette and transferred to a Petri plate. The plates were then covered by glass cubes for slow chloroform evaporation inside a gas exhaust chamber.

Identification of the accumulated polymer by Fourier transform infrared spectroscopy and characterization differential scanning calorimetry KBr tablets with ground polymer films and the PHB standard (Sigma) were used to identify the biopolymers produced by CMM41, CMM43 and CMM44 strains. A Fourier transform infrared spectrometer Shimadzu® model IR Prestige 21 was used to obtain the Fourier transform infrared (FTIR) spectra. 64 images were acquired over a wavelength range of 4500 to 500 cm^{-1} with a resolution of 4 cm^{-1}.

The thermal properties of PHB samples and standard PHB were investigated by differential scanning calorimetry (DSC). DSC was performed with a Perkin Elmer, model Pyris 6. It used a nitrogen atmosphere at a rate of 20 ml.min^{-1}. The samples were subjected to the following test conditions: isotherm 20°C for 1 min, first heating cycle of 20 to 200°C at a rate of 10°C min^{-1}, isotherm 200°C for 5 min; cycle cooling 200 to 20°C at a rate of 10°C min^{-1}, isotherm of 20°C for 5 min; second heating cycle of 20 to 200°C at a rate of 10°C min^{-1}. To assess the effect of thermal history in obtaining the samples, we observed values of melting temperature (Tm) in the first heating cycle. However, in order to eliminate the thermal history of the material, the Tm was taken in the second heating cycle. The crystallinity degree (Xc) was determined from the melting enthalpy of the sample (ΔHm) and the melting enthalpy of pure crystalline PHB (Δ 0mH = 146 J/g) (Jianchun et al., 2003; Gogolewski et al., 1993).

RESULTS AND DISCUSSION

Identification of lipophilic bodies

Among the 14 pesticide degrading strains assessed by optical microscopy for the presence of lipophilic bodies, four were positive: CMM41, CMM42, CMM43 and CMM44. All four belong to a group of carbofuran-degrading *Pseudomonas* spp. These strains were visualized using TEM.

TEM assessment of the bacterial cells showed that strains CMM41, CMM43 and CMM44 accumulated a high amount of intracytoplasmic granules (Figure 1a, c and d), which suggest that they were promising PHA biopolymer producers; hence, they were selected for further study. The general appearance of inclusions accumulated for these strains (Figure 1a, c and d) are very similar to granules stored for the positive control *Bacillus megaterium* (Figure 1e), though the micrographs suggested that the *Pseudomonas* spp. accumulated a higher numbers of granules. The granules were presented as dense electron bodies, very similar to PHA inclusions found in other bacteria (Loo and Sudesh, 2007). Granule diameters in strain CMM41 were between 0.37 and 0.75 µm; in strain CMM42, between 0.19 and 0.31 µm; in strain CMM43, between 0.6 and 1.1 µm; and in strain CMM44, between 0.4 and 0.8 µm. Granule diameters in *B. megaterium* were between 0.2 and 0.75 µm. According to Yoo et al. (1997), typical PHB granules

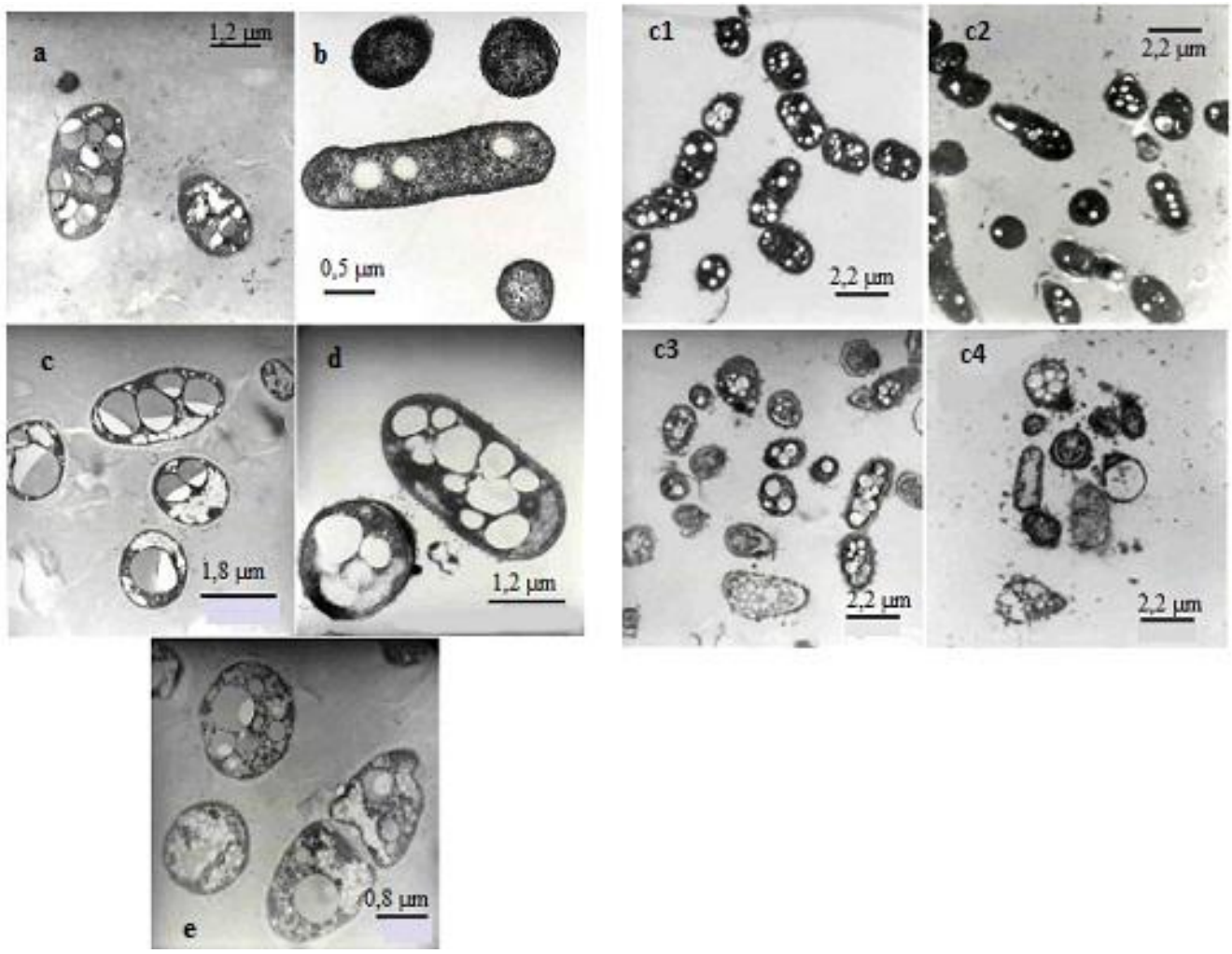

Figure 1. Transmission electron micrographs showing PHB inclusions in bacterial cells of *Pseudomonas* strains (a) CMM41, (b) CMM42, (c) CMM43, and (d) CMM44 and in cells of (e) the control bacteria *B. megaterium* cultivated in NB+SUC for 48 h and accumulation of inclusions as a function of cultivation time in strain CMM43 after (c1) 12 h, (c2) 24 h, (c3) 48 h and (c4) 72 h.

have diameters between 0.2 and 0.7 µm.

Cell growth and accumulation of PHB in strain CMM43

In Figure 1, it can be seen also that accumulation of lipophilic inclusions is a function of cultivation time of strain CMM43. The highest accumulations and largest inclusions were found after 48 h (c3), with the maximum CDW of 5.8 $g.L^{-1}$ and a PHB concentration of 29% CDW. The highest PHB accumulation in a short time was a result very relevant because generally PHB accumulation is a slow process (Jiang et al., 2008). It was observed that the carbon source was readily consumed during PHB accumulation registering a consumption of 8.6 $g.L^{-1}$ making 70% of initial sugar concentration. Most cells had not divided because in conditions of excess carbon, growth becomes unbalanced, and substrates are converted to intracellular polymer, which disfavors cellular synthesis (Beccari et al., 1998). At 12 h of incubation (c1), there was a substantial quantity of accumulated granules, but the cells were also in a process of dividing; the amounts of CDW, PHB and residual sugar were not analyzed. At 24 h (c2) there were evident reductions in the amount of inclusions and the extent of cell division; the CDW content of 2.14 $g.L^{-1}$ was found when strain CMM43 growth in this time and PHB content in the CDW was 9%. The sugar concentration obtained was 4.4 $g.L^{-1}$ which represents a consumption of 32%. At 72 h (c4), there were fewer inclusions than at other times, which could be due to the reserves being used by the cells (Sudesh et al., 2000). The quantitative PHB results demonstrated that the amounts of biopolymer in this time decreased to almost 4% of PHB content verified after 48 h. The results of CDW in this time were 6.3$g.L^{-1}$. The sugar consumption was almost the same as that in 48 h; it was 72% (8.8$g.L^{-1}$) showing

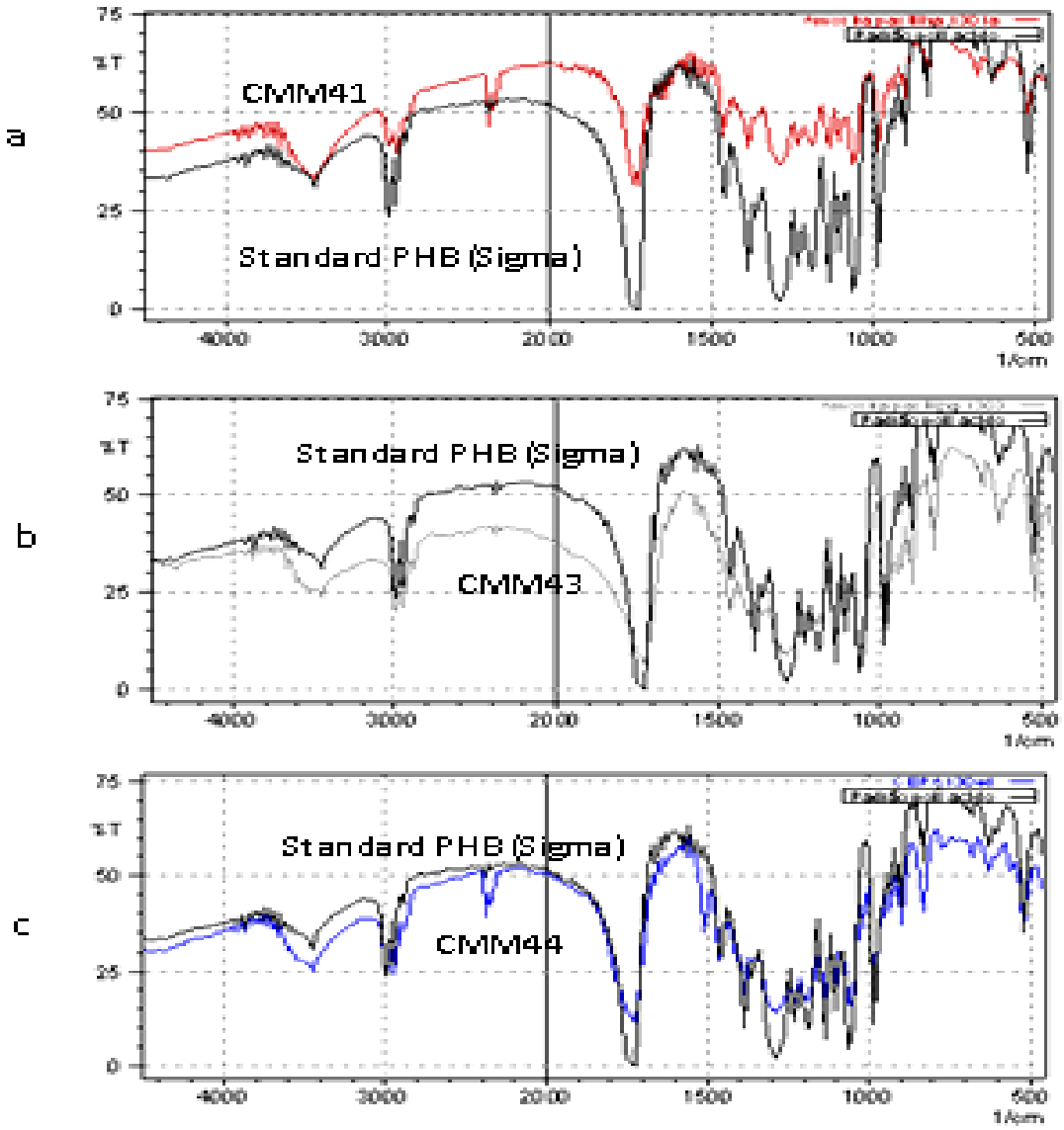

Figure 2. FTIR spectra of the polymers from strains (a) CMM41, (b) CMM43 and (c) CMM44 compared to the PHB standard.

the use of the polymer for the cell and not the carbon source.

Identification and characterization of accumulated biopolymer

Infrared spectra showed that the three strains had similar biopolymer compared to the PHB standard (Sigma) (Figure 2). All three samples analyzed displayed PHB characteristics. The band at 1454 to 1459 cm^{-1} corresponds to C-H asymmetrical stretching for CH_2 groups, while the band at 1378 cm^{-1} is equivalent to CH_3 group deformation. These bands are comparable to those found by Oliveira et al. (2007). The bands near 3000 cm^{-1} signify the asymmetrical stretching of C-H in aliphatic CH_3 and CH_2 groups (Khardenavis et al., 2006). The presence of strong absorption bands at 1724 to 1727 cm^{-1} and 1281 cm^{-1} is a consequence of the ester C-O bonds stretching, representing carboxylic (C=O) and carbonylic (C-O) bonds, respectively. The series of intense bands from 1250 to 950 cm^{-1} also correspond to the C-O enlargement for the ester group (Oliveira et al., 2007; Arun et al., 2009). The wide band located near 3440 cm^{-1} corresponds to the OH group (Oliveira et al., 2007; Arun et al., 2009). Some of the PHB bands produced by strain CMM41 were smaller compared to the standard spectrum pattern, which may be a result of differences in molecular weight or crystallinity (Lugg et al., 2008). In other study, we verified that these strains can accumulate mainfold

Table 1. Thermal properties of PHB.

Samples of PHB	T_m (°C) (1)	T_m (°C) (2)	(X_C) %
Standard	170	148	41
CMM41	168	131	27
CMM43	164	155	45
CMM44	166	156	37

PHAs. The polymer spectrum produced by strain CMM44 was almost identical to the standard PHB, possibly indicating that in this sample the PHB proporcion was higher than in other samples.

The results of thermal analysis are expressed in Table 1. The melting temperature of the different samples obtained from PHB synthesized by the strains and the standard PHB was a little lower to the values found in the literature such as 177.3°C described by Hong et al. (2008) and 177.2°C according to Chaijamrus and Udpuay (2008). These values may possibly be related with external contaminants like the residual biomass and inorganic elements from fermentation processes that could affect the thermal property of PHB (Kim et al., 2006) or with the technique of extraction. Valappil et al. (2007) reported the T_m of PHB extracted from the *Bacillus cereus* of 160.83°C using chloroform for the extraction.

The X_C obtained in the PHB produced by strains and the standard PHB were lower than the usual range of crystallinity value. El-Hadi et al. (2002) verified a X_C of 60% for the PHB from Copersucar/Brazil produced by the fermentation of molasses using *Alcaligens euthrophus*. The crystallinity degree obtained by the PHB produced by strain CMM43 was the closest compared to values found by Reddy et al. (2009) that varied according to the substrate used between 42 and 50%. These results are desirable because high levels of crystallinity made the polymer brittle and stiff resulting in a very poor mechanical properities (Savenkova et al., 2000).

Conclusions

This study showed that pesticide-degrading *Pseudomonas*, specially the carbofuran-degrading strains constitutes a promising group to investigate polyhydroxybutyrate (PHB) accumulation capability.

Pseudomonas sp. CMM41, CMM43 and CMM44, all of which degrade carbofuran pesticide, have the capacity to accumulate PHB from sucrose, an inexpensive substrate in Brazil, a trait that is not common in *Pseudomonas* sp. that, normally, accumulate MCL-PHA. The PHB accumulated by these strains was characterized as having low crystallinity degree, desirable feature for making the material less brittle.

Strain CMM43 had the best accumulation and largest inclusions just after shaking for 48 h compared to other cultivation times. Currently the strain CMM43 is being investigated to increase the production of PHB by optimization of fermentative parameters.

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