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ORIGINAL PAPER

Lorena M. Tobella · Marta Bunster · Amalia Pooley
José Becerra · Felix Godoy · Miguel A. Martínez

Biosynthesis of poly-β-hydroxyalkanoates by *Sphingopyxis chilensis* S37 and *Wautersia* sp. PZK cultured in cellulose pulp mill effluents containing 2,4,6-trichlorophenol

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Abstract Poly-β-hydroxyalkanoates (PHA) polymer is synthesized by different bacterial species. There has been considerable interest in the development and production of biodegradable polymers; however, the high cost of PHA production has restricted its applications. Kraft cellulose industry effluents containing 2,4,6-trichlorophenol (10 or 20 μg ml^{-1}) were used by the bacteria *Sphingopyxis chilensis* S37 and *Wautersia* sp. PZK to synthesize PHA. In this condition, *S. chilensis* S37 was able to grow and degrade 2,4,6-trichlorophenol (ca. 60%) and 80% of these cells accumulated PHA. *Wautersia* PZK completely degraded 2,4,6-TCP and more than 90% of the cells accumulated PHA in 72 h. The PHA detection was performed by flow cytometry and polyester composition was characterized by gas chromatography-mass spectroscopy (GC-MS), indicating that these polymers are made by 3-hydroxybutyric acid and 3-hydroxyhexadecanoic acid for S37 and PZK strains, respectively. Results demonstrated that strains' growth and PHA production and composition are not modified in cellulose effluents with or without 2,4,6-TCP (10–20 μg ml^{-1}). Therefore, our results indicate that *S. chilensis* S37 and *Wautersia* sp. PZK are able to degrade a toxic compound such as a 2,4,6-TCP and simultaneously produce a valuable biopolymer using low-value substrates.

Keywords Kraft cellulose effluent · Biosynthesis · Poly-β-hydroxyalkanoate · Degradation · 2,4,6-Trichlorophenol

L. M. Tobella · F. Godoy · M. A. Martínez (✉)
Laboratorio de Biorremediación,
Departamento de Microbiología,
Facultad de Ciencias Biológicas,
Universidad de Concepción, Casilla, 160-C Chile
E-mail: mimartin@udec.cl

M. Bunster
Departamento de Biología Molecular,
Facultad de Ciencias Biológicas,
Universidad de Concepción,

A. Pooley
Departamento de Polímeros,
Facultad Química Universidad de Concepción,

J. Becerra
Laboratorio Fitoquímica Facultad
de Ciencias Naturales y Oceanográficas,
Universidad de Concepción,

Introduction

Nutrients frequently vary in the aquatic environment and as a consequence bacterial life cycle alternates between periods of growth and non-growth [14]. Many bacteria show different strategies of survival in this condition, such as dormancy, physiologic flexibility and synthesis of reservoir substances. These reservoir substrates include granules such as glycogen, polyphosphate or poly-β-hydroxyalkanoate (PHA) [1].

The PHAs are synthesized by many bacteria as intracellular endogenous reserves and are accumulated under nutrient limitation and carbon excess [18]. It has been demonstrated that bacteria synthesize and accumulate PHA as carbon and energy storage, to use it during carbon source starvation periods [10]. These PHA granules have acquired industrial interest because their physical and mechanical properties are similar to those of conventional polypropylene plastics [12]. The PHA polymers can be used in the production of biodegradable plastics for a wide range of applications. Nevertheless, production of PHA is more expensive than conventional plastic material, limiting its current applications [5].

On the other hand, Kraft cellulose industry effluents contain different kinds of carbon sources that bacteria can use for growth [4], while simultaneously containing toxic compounds including chlorophenols [11], which can interfere in the metabolism of bacterial cells [17]. Nevertheless, several heterotrophic bacteria have been isolated for their ability to degrade these compounds [7, 8, 15]. In previous works, in our laboratory, we isolated bacterial strains able to degrade chlorophenols and

accumulate PHA [2, 7, 8,]. The aim of this work was to evaluate the capability of the *Sphingopyxis chilensis* S37 and *Wautersia* sp. PZK (ex *Ralstonia*) [19] strains to synthesize PHA using organic matter present in a Kraft cellulose industry effluent containing 2,4,6-trichlorophenol (2,4,6-TCP).

Materials and methods

Bacterial strains

S. chilensis S37 and *Wautersia* sp. PZK bacterial strains were isolated in previous works [6, 15] from a superficial sediment of a river (Biobío River, central Chile), 1000 m downstream from where the river receives a non-treated Kraft cellulose pulp mill effluent. Both strains possess the ability to degrade 2,4,6-TCP and accumulate PHAs.

Bacterial viability and degradation of 2,4,6-trichlorophenol

Cells of S37 or PZK strains were grown in R_2A broth (72 h, 25°C, orbital shaking at 150 rpm), collected by centrifugation, washed three times with saline solution and suspended at 1×10^5 CFU ml^{-1} in 50 ml Erlenmeyer flasks containing 25 ml of Kraft cellulose effluent plus saline solution (K_2HPO_4 $\cdot 3H_2O$ 3.6 g l^{-1}, $Na_2H_2PO_4$ $2H_2O$ 2.86 g l^{-1}, KH_2PO_4 1.46 g l^{-1}, $(NH_4)2SO_4$ 1 g l^{-1}, $MgSO_4$ $7H_2O$ 0.19 g l^{-1}) in a 3:1 ratio.

Erlenmeyer flasks containing the same effluent plus saline solution in a 3:1 ratio were supplemented with 2,4,6-TCP (10, 20 or 50 μg ml^{-1}). The cultures were incubated at 30°C (125 rpm) and monitored daily during 5 days. Viable bacteria were determined each day by the Milles and Misra method [9] in R_2A agar plates. Degradation of 2,4,6-TCP was determined spectrophotometrically at 310 nm ($A_{310\ nm} = 4.56\times10^3$) [2].

Kraft cellulose effluent analysis

Analysis were carried out by gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard 5972 mass selective detector and an Agilent technologies Hewlett Packard HP-5MS capillary column (30 m×0.25 mm internal diameter, film thickness 0.25 μm). Carrier He (30 psi) splitless 1:100, injection temperature 250°C, initial oven temperature 100°C (5 min), temperature increase 10°C per minute, final isotherm 275°C (28 min). Samples of 100 ml were extracted with ethyl acetate three times and evaporated until 1 ml in a rotatory evaporator; 1 μl was used for injection in GC-MS.

Analysis of organochlorine compounds was carried out by GC with electron capture detector (GC-EDC) in a Shimadzu (Kyoto, Japon) 9-A instrument linked to a Shimadzu C-R7A chromatic integrator.

Total organic carbon (TOC) in Kraft effluent was determined using a TOC-5000 Shimadzu; for this propose, 50 ml of the effluent was analysed. After incubation for 5 days with the bacterial strains, TOC of Kraft effluent samples was filtered through an acetate filter with 0.2 μm pores, prior to analysis.

Detection of PHAs

The PHA detection was performed by flow cytometry [10] in a Facscalibur Flow Cytometer (Becton Dickinson), using bacterial cells incubated during 72 h in a Kraft cellulose effluent with 2,4,6-TCP added (10, 20 or 50 μg ml^{-1}). The results of each sample were based on the analysis of 20,000 events.

Polyester analysis

The polyester of both strains was characterized according to [3], using methanolysis of 3–5 mg lyophilized cells in the presence of 15% v/v sulphuric acid. The resulting methyl ester of constituent hydroxyalkanoic acid were characterized using the same previously used GC-MS.

Results and discussion

Bacterial viability and degradation of 2,4,6-trichlorophenol

The results indicate that the carbon source present in cellulose effluents allows bacterial cell growth (Fig. 1a, b) with a maximum viable cell count of 1×10^8 CFU ml^{-1} at 72 h in both strains. In cellulose effluent supplemented with 10 or 20 μg ml^{-1} of 2,4,6-TCP, both strains grow. However, with 50 μg ml^{-1} of 2,4,6-TCP (Fig. 1a, b) the culture could be considered dead after 48 h of incubation according to the level of lethality proposed by [16]. In previous works [2, 6], we demonstrated that both strains have the ability to tolerate concentrations equal to or higher than 500 μg ml^{-1} of 2,4,6-TCP in the presence of an easily metabolizable carbon source. The presence of 50 μg ml^{-1} 246-TCP added to the other organochlorine compounds present in the effluents, detected by CG-EDC (data not shown), may be increasing the effluent antibacterial activity affecting their viability.

On the other hand, at 48 h of incubation, both strains were able to degrade a similar proportion, ca. 50%, of 2,4,6-TCP, when 10 or 20 μg ml^{-1} was added (Table 1). Additionally, after 72 h, the PZK strain was able to completely degrade 2,4,6-TCP. Previous studies demonstrated that the PZK can grow using 2,4,6-TCP as a unique carbon source [2, 15]. On the contrary, the S37 strain depends on other easily metabolizable compounds to grow and degrade 2,4,6-TCP [2, 7, 8] [13] reported two types of 2,4,6-TCP degradation. One type degrades

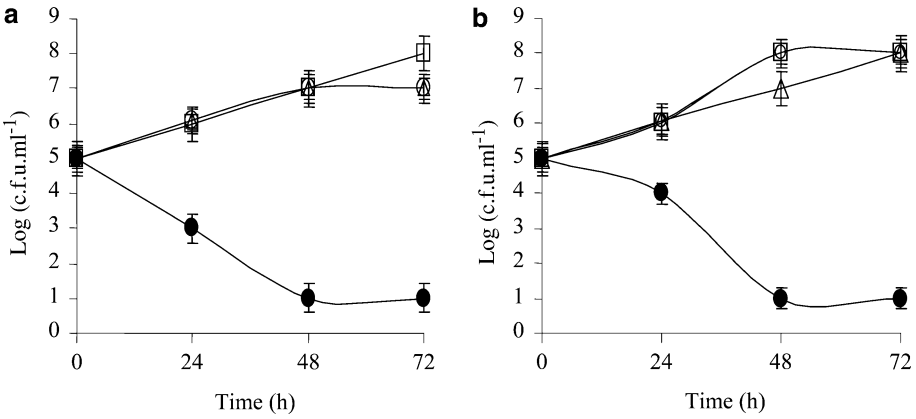

Fig. 1 Viable bacterial counts of *S. chilensis* S37 (**a**) and *Wautersia sp*. PZK (**b**) incubated in a Kraft cellulose effluent with different concentrations of 2,4,6-TCP. Panel **(a)** S37 strain, control (□), 10 μg ml^{-1} (△), 20 μg ml^{-1} (○) and 50 g ml^{-1} (●). Panel **(b)** PZK strain control (□), 10 μg ml^{-1} (△), 20 μg ml^{-1} (○) and 50 μg ml^{-1} (●). Values are means of three replicates

Table 1 Percentage degradation of 2,4,6-trichlorophenol (μg ml^{-1}) by *S. chilensis* S37 and *Wautersia* sp. PZK over 48 and 72 h in R2A broth and Kraft cellulose effluent with 2,4,6-trichlorophenol

Strains	2,4,6-TCP	R2A broth		Cellulose effluent	
		48 h	72 h	48 h	72 h
S. chilensis	10	45	100	60	65
S. chilensis	20	20	44	38	56
Wautersia sp.	10	56	100	66	100
Wautersia sp.	20	20	100	42	100

low concentrations of this compound and is unable to use it as carbon source to grow (mainly *S. chilensis* S37). The other type (mainly *Wautersia*) is able to degrade higher concentrations of 2,4,6-TCP using it as sole carbon source. These differences in degradative activity between strains may account for the results obtained in this work.

The TOC determined by TOC in the cellulose effluent was 277 μg l^{-1}, and TOC measured after 5 days in effluent inoculated with the bacterial strains was 181 μg l^{-1}, indicating that both strains are able to consume ca. 65% of this carbon source.

Ten signals were detected by CG-MS in the cellulose effluents (Fig. 2a); five of them, with a match quality equal to or higher than 85 were identified as 2-(2-butoxyethoxy-ethanol), 3-hydroxy-2,4,4,4-trimethylpentyl ester propanoic acid, 1,1,3,3-tetramethylbutil-phenol, nonyl-phenol, *n*-hexadecanoic acid, respectively. Other compounds, including chloroaromatic, also were detected by GC-MS and GC-EDC (data not shown). In a cellulose effluent inoculated with those strains, all these compounds disappeared after 5 days of bacterial incubation (Fig. 2b). These results indicate that bacteria strains are able to utilize organic matters present in the Kraft effluent. Similar results were reported by [4]. They indicated that *Ancylobacter*,*Pseudomanas* and *Methylobacterium* strains isolated from cellulose effluent grow and remove organic compounds, measured by adsorbable organic halogen (AOX).

PHA detection and characterization

Both strains were able to accumulate PHA when they were cultured in cellulose effluents with or without 10 or 20 μg ml^{-1} 2,4,6-TCP. The percentage of cells containing PHA granules was larger when cultured in cellulose effluents rather than in R_2A broth (Table 2). This result can be explained because this polymer is usually produced under unbalanced growth conditions such as nitrogen or phosphorus limitation [12], conditions detected in Kraft cellulose effluents studied (data not shown) but not in the R2A broth [12] described two groups of bacteria able to synthesize PHA; the first group requires the limitation of essential nutrients such as a N, P, Mg or S for efficient synthesis of PHA from excess carbon source. The second group does not require nutrient limitation for PHA synthesis. The higher PHA accumulation in cells cultured in cellulose effluent rather than R_2A medium observed in this work can be explained if S37 and PZK strains behave as the first group of bacteria. The addition of 2,4,6-TCP (10 or 20 μg ml^{-1}) did not affect biopolymer accumulation in bacterial cells incubated in the cellulose effluent (Table 2). The constituents of PHA in *S. chilensis* S37, cultured in cellulose effluents or R_2A broth, were 3-hydroxybutyric acid and 3-hydroxyhexadecanoic acid. A similar result was previously reported by [7, 8] for *S chilensis* S37. On the other hand, in *Wautersia* sp. PZK, monomers of medium-chain lengths, corresponding to 3-hydroxyhexadecanoic acid, were detected. In both strains, the presence of 2,4,6-TCP did not affect PHA compositions.

This article reports that *Wautersia* sp. PKZ and *S. chilensis* S37 are able to grow in cellulose effluents and simultaneously produce PHA polymers. Bacteria able to degrade toxic compounds like 2,4,6-TCP and synthesize PHA using a carbon source present in industrial effluents also provide an interesting alternative for toxic compound removal, significantly reducing biopolymer

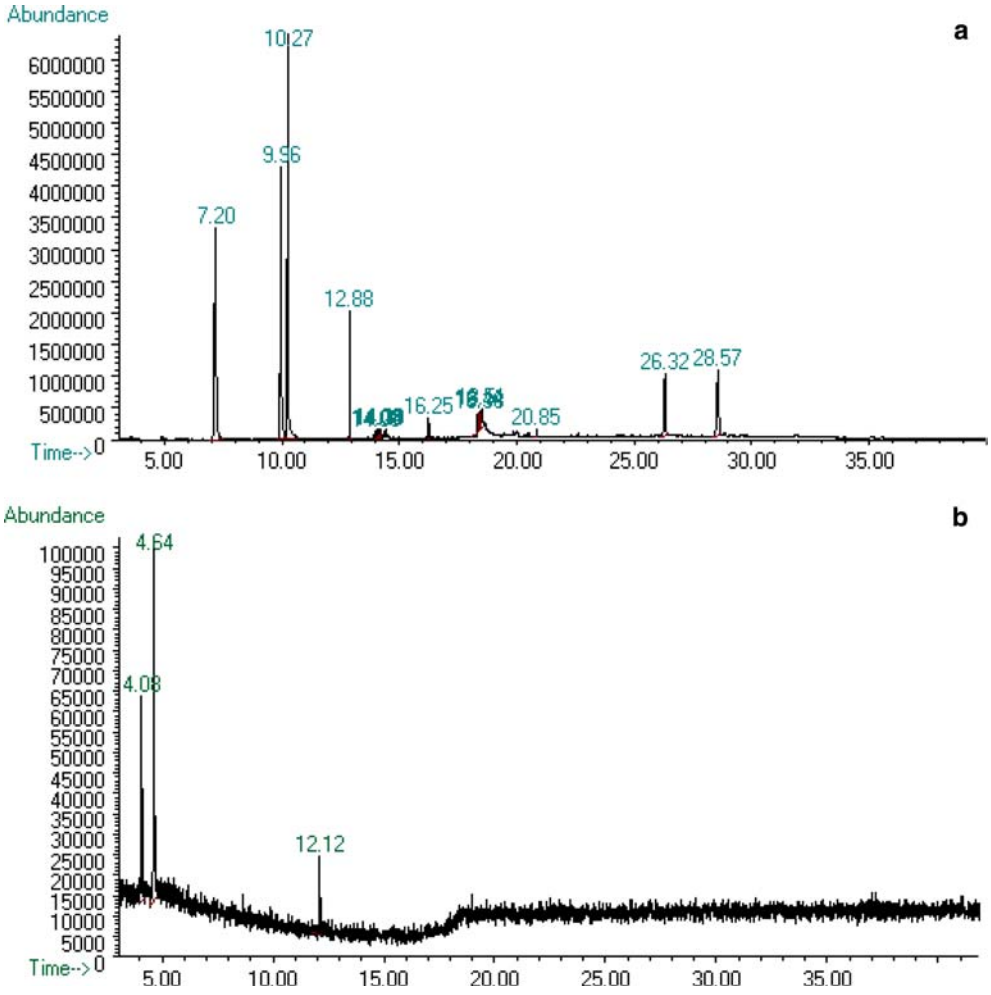

Fig. 2 Effluent cellulose chromatogram (**a**), Effluent cellulose chromatogram after 5 days inoculation with *Wautersia* sp. PZK (**b**)

Table 2 Percentage of bacterial cells with poly-ß-hydroxyalkanoates after 72 h incubation in R_2A and cellulose effluent supplemented with 2,4,6-trichlorophenol (μg ml^{-1}), measured by flow cytometry

Strains	2,4,6-TCP	R_2A broth	Cellulose effluent
S. chilensis (control)	0	70	82
S. chilensis	10	63	84
S. chilensis	20	68	80
S. chilensis	50	21	38
Wautersia sp. (control)	0	65	94
Wautersia sp.	10	68	93
Wautersia sp.	20	72	91
Wautersia sp.	50	35	45

production costs and diminishing the environmental contamination by Kraft cellulose mill effluents.

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