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ORIGINAL PAPER

Polyester-degrading actinomycetes isolated from the Touchien River of Taiwan

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Abstract Actinomycete strains were isolated from upstream and downstream regions of the Touchien River in Taiwan and screened for the ability to degrade poly(ethylene succinate) (PES), poly(ϵ-caprolactone) (PCL) and/or poly(β-hydroxybutyrate) (PHB) by the clear-zone method. Out of 305 isolates 135 isolates were PHB-degraders (44.2%), 83 isolates were PCL-decomposers (27.2%), and 64 isolates could degrade PES (21.0%). Furthermore, 46 isolates could degrade both PHB and PCL (15%), 39 isolates could degrade both PHB and PES (12.8%), and 12 isolates could degrade the three polyesters used in this study. Based on the appearance of isolates, the major isolates belong to the *Streptomyces* genus (91.9%) and *Micromonospora* genus (8.1%).

Keywords Actinomycetes · Microbial degradation · Polyester · River

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Introduction

The worldwide annual production of synthetic polymers has reached about 14 billion metric tons (Shimao 2001). Treatment of plastic disposal is now an issue of great concern. To reduce the environmental impact of plastics, developed nations are beginning to impose strict controls or are mandating recovery, while industry is proactively developing biodegradable plastics as an alternative. Several biodegradable plastics with properties comparable to conventional plastics have been developed such as poly(hydroxyalkanoic acids) (PHA), poly(β-hydroxybutyrate) (PHB), poly (ϵ-caprolactone) (PCL), poly(ethylene succinate) (PES), poly(lactic acid) (PLA) and poly(tetramethylene succinate) (PTMS) (Tokiwa and Calabia 2004).

The distribution and abundance of PCL-, PHB-, PTMS-, and poly(propiolactone) (PPL)-degrading microorganisms in the environment have been investigated (Nishida and Tokiwa 1993a,b; Pranamuda et al. 1995). The distribution of aliphatic polyester-degrading microorganisms at different temperatures has also been studied (Nishida and Tokiwa 1993a; Pranamuda et al. 1995; Tansengco and Tokiwa 1998). Ecological studies on the diversity and abundance of such microorganisms in the natural environment are one of the essential elements for evaluating the biodegradability of aliphatic polyesters. The population of aliphatic polyester-degrading microorganisms in the environment can be efficiently evaluated by the plate count and clear-zone method (Nishida and Tokiwa 1993a; Pranamuda et al. 1995).

Most ecological studies concerning biodegradation of aliphatic polyesters have been focused on the general microbial population in the environment. There is little

information about the distribution of aliphatic polyester-degrading actinomycetes. Due to the slower growth rate of actinomycetes, they were always neglected during the isolation of microorganisms. Although the quantities of actinomycetes in the soil are lower than most microbes, the former would gain an advantage very easily in an environment with a lower nutrient level or with some materials which are hard to decompose. The actinomycetes would exhibit a special metabolic ability with respect to those materials because they could produce various secondary metabolic outcomes or enzymes. This study aimed to isolate actinomycetes which exhibit the ability to degrade each of the three polyesters and to determine the population of actinomycetes capable of degrading PHB, PCL, and PES.

Materials and methods

Materials

The aliphatic polyester samples used were: poly(ethylene succinate) (PES) with a number-average molecular weight ($\overline{Mn}$) of 1.0×10^4, poly(ϵ-caprolactone) (PCL, $\overline{Mn} = 6.8 \times 10^5$), and poly($\beta$-hydroxybutyrate) (PHB, $\overline{Mn} = 5.4 \times 10^5$). These polyesters were obtained from Aldrich Chemical Co.

Samples collection

River sediments were collected from five locations in both upstream and downstream regions of the Tou-Chien River system in Hsinchu, Taiwan between 1999 and 2000. These locations (in order) included Tieling, Hsin-le, Chutung, Dahua, and Hsi-chou. At each of the five locations, the depth range of all the sediment samples was about 5 cm. All samples were taken from the shoreline of the river, collected in sterile 50-ml tubes and kept at 4°C until they were processed.

Medium for isolating actinomycetes

The medium used for actinomycete isolation was starch-casein agar supplemented with (per liter) starch, 4.0 g; casein, 0.12 g; KNO_3, 1.60 g; NaCl, 1.60 g; K_2HPO_4, 1.60 g; $MgSO_4 \cdot 7H_2O$, 40 mg; $CaCO_3$, 16 mg; $FeSO_4 \cdot 7H_2O$, 8 mg; and agar, 18.0 g. All isolation media also contained cycloheximide (50 μg/ml) to minimize the growth of fungi and was autoclaved at 121°C for 15 min.

Isolation of actinomycetes

One ml of sediment sample was suspended in 9 ml of sterile water and treated at 50 °C for 60 min (Jensen et al. 1991), then serially diluted. 0.1-ml aliquots of 10^0–10^{-4} diluted suspensions were streaked on starch-casein agars (in triplicate) and cultivated at 25–28°C for 14 days. Actinomycetes representing all colonial morphologies observed from each sample were isolated and purified by repeated transfer on starch-casein agars. All isolated strains were cultivated on various agar media, including yeast extract-malt extract agar (International *Streptomyces* Project, ISP 2 medium), oatmeal agar (ISP 3 medium), inorganic salt-starch agar (ISP 4 medium), and asparagine-glycerol agar (ISP 5 medium).

Screening of aliphatic polyester-degrading actinomycetes

One gram of aliphatic polyester powder was dissolved in 50 ml of methylene chloride. The solution was emulsified into basal medium containing (per liter): yeast extract, 0.1 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1 g; $CaCl_2 \cdot 2H_2O$, 20 mg; NaCl, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg; $NaWO_4 \cdot 2H_2O$, 0.5 mg; $MnSO_4 \cdot H_2O$, 0.6 mg; and detergent ($CH_3(CH_2)_nCOONa$, $n = 6$–14, Nice Co., Taiwan), 50 mg. Methylene chloride was evaporated using a proctor laboratory hood. Agar (18 g) was added to the emulsified medium with a pH of 7.2, which was then sterilized in an autoclave at 121°C for 15 min and poured into Petri dishes. The purified isolates of actinomycetes were streaked out on emulsified PES, PCL, and/or PHB/agar plate, and incubated at 30°C for 7 days. The degradation ability of the isolates was determined by the formation of clear zones around the colonies.

Degradation of polyester films by actinomycetes

The isolates were grown in 250-ml Erlenmeyer flasks containing 100 ml of the basal medium and 100 mg of PES, PHB, or PCL films. Polymer films were prepared by the heat pressed method (Paranamuda et al. 1995). The film (about 180 μm thick) was sterilized with 75% (w/v) alcohol and irradiated with u.v. for 10 min. The flasks were incubated on a rotary shaker (180 rev/min) at 30°C. Culturing was done in triplicate, and the mean value for each experiment was used.

Identification of actinomycetes

Cultural characteristics were tested by using 14-day-old cultures grown at 28°C on various agar media, include yeast extract-malt extract agar (International Streptomyces Project, ISP 2 medium), oatmeal agar

(ISP 3 medium), inorganic salt-starch agar (ISP 4 medium) and asparagines-glycerol agar (ISP 5 medium). The ISCC-NBS Color-Name Charts (Kelly 1964) were used for determining color designations of substrate mycelium. The morphological characteristics were observed with a light microscope with a long working distance objective lens. Cell wall composition (DL- and LL-diaminopimelic acid isomer, A2 pm) was determined by the method of Hasegawa et al. (1983). One or two colonies were placed in a cryogenic vial (Evergreen Scientific) with 0.1 ml of 6 M HCl. The vial was heated by autoclaving at 121°C for 15 min. After cooling 1 µl of the hydrolysate was placed on a thin cellulose plate (Microcrystalline cellulose f, Tokyo Kasei Co., Ltd). One µl of 0.01 M DL-A2 pm (Sigma Chem. Co.) was spotted on the same plate as a standard. The plate was developed on the solvent system methanol–distilled water-6 M HCl–pyridine (80:26:4:10, v/v) for 3–4 h. After the plate had been dried, it was sprayed with Ninhydrin Spray Reagent (Merck Chem. Co.) and heated at 100°C for 5 min. The spots of A2pm appeared yellowish-green in color. The same procedure for A2pm was used to analyse the whole-cell sugar, except that the hydrolysis and development solvents were 0.25 M HCl and *n*-butanol–distilled water–pyridine–toluene (10:6:6:1, v/v), respectively, and the spraying reagent was acid aniline phthalate. The standard sugar solution contained galactose, glucose, mannose, arabinose, xylose, and ribose each at 1% concentration.

Substrate uptake

Basal medium (per liter) contained K_2HPO_4 1.5 g, (NH4) $_2SO_4$ 1.0 g, KH_2PO_4 5.0 g, KCl 0.5 g, $FeSO_4$ 5 mg, $MnSO_4$ 1.6 mg, $ZnSO_4$ 1.4 mg, $CoCl_2$ 2.0 mg, yeast extract 0.5 g, $MgSO_4$ 0.5 g and agar 20.0 g (pH 7.2). A carbon source, Tween 20, carboxymethylcellulose (CMC), xylan, starch, or casein, was added at a final concentration of 1%.

Water Bacterial Count: Spread Plate Method (Total viable counts)

Sediment sample (1 g) was mixed thoroughly with 9 ml of sterile water and allowed to stand for 30 min. A serial dilution was prepared by adding 1 ml of the sediment sample suspensions to 9 ml of sterile water. A 0.2 ml aliquot of the appropriate dilution was spread aseptically onto an agar plate. The plates were then incubated at 30°C for 48 h.

Results and discussion

Isolation of actinomycetes

To isolate actinomycetes effectively, the suspended sediment sample solutions and sediment samples obtained from five locations (Hsi-chou, Dahua, Tieling, Hsin-le and Chutung) along the Touchien River were treated at 50°C for 60 min (Jensen et al. 1991). A total of 305 actinomycetes strains were isolated from the collected samples. Actinomycetes colonies were recognized by a characteristically dry surface that looks leathery, powdery or cottony with rough edges or irregular shape (Lechevalier 1989) and by observation using a microscope. The total microbial population ranged from 0.71 to 8.71×10^4/ml of suspended sediment samples and 1.13 to 56.1×10^4/g of sediment samples.

Isolation of the aliphatic polyester-degrading actinomycetes

The isolated actinomycetes were screened for polymer-degrading capacity using the clear-zone method. The clear-zone method is the simplest method for investigating microbial degradation of aliphatic polyester, since the formation of a clear zone around the colony indicates solubilization of the polymer by enzyme(s) secreted from the microbes (Nishida and Tokiwa 1993a; Pranamuda et al. 1995). After 7 days of colony formation on the agar plates, a circular clear zone is formed around each colony containing a microorganism capable of degrading the polymer (Fig. 1). Results showed that 135 isolates were PHB-degraders (44.2%), 83 isolates were PCL-decomposers (27.2%), and 64 isolates were able to degrade PES (21.0%) (Table 1). Isolates that could degrade PHB are obviously more numerous than those that degrade PCL and PES. The ratio of polyester-degrading actinomycetes in the river-based sediment was apparently higher than in the soil (Paranamuda et al. 1995; Suyama et al. 1998). Among the 135 PHB-degraders, 46 could also degrade PCL and 39 isolates could degrade PES (12.8%). In addition 12 isolates were capable of degrading PHB, PCL and PES. Seven of these isolates originated in Tie-ling, 3 were from Hsi-chou and 2 were from Hsin-le (Table 2).

Distribution

The proportion of actinomycetes with polyester-degrading activity was in the order of Hsi-chou > Dahua > Tie-ling > Hsin-le > Chutung. Thus, there were

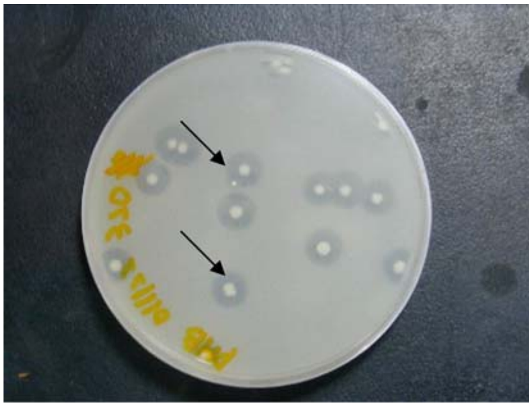

Fig. 1 The colonies of actinomycetes on agar plate with emulsified polyester

more actinomycetes isolates that could degrade polyesters in the less polluted areas of the Touchien River, Hsi-chou (medium-level of pollution) and Tie-ling (low-level pollution). Moreover, in Tie-ling it was possible to obtain strains that could degrade all 3 types of polyesters. We do not know if this is the general case. Further study is required to understand the relationship between the distribution of the actinomycetes capable of degrading polyester and the pollution level of the area.

Identification of actinomycetes

Most of the polymer-degrading isolates produced branched substrate mycelia on solid medium and aerial mycelia bearing chains of spores. The cell wall contained a major amount of L-A_2 pm and no diagnostic sugars were found in the whole cell hydrolysate. With a type I cell wall, these isolates, based on these characteristics, belong to the *Streptomyces* genus (91.9%). Some other isolates were orange to black in color with a leathery or moist surface. They only had substrate mycelia and no aerial mycelia, and single spores were found only on the substrate mycelia. The cell wall of these isolates contained *meso*-A_2pm, xylose and arabinose. These strains belong to the *Micromonospora* genus (8.1%).

Twelve isolates that could degrade all of the polymers belonged to the *Streptomyces* genus. The color of the aerial mass of these isolates was in the gray color-series on yeast extract–malt extract agar, oatmeal agar, and inorganic salt–starch agar. No soluble pigment was formed in media. Straight or flexible spore chains were found in most of these isolates. Spiral spore chains were formed on the mature aerial mycelia of isolate TH-11. The isolates of *Micromonospora* could only degrade PHB.

Substrates uptake

Each of these twelve isolates could use casein, carboxymethylcellulose, and starch as its carbon source, and all of these isolates, except isolate TH-11, could also use Tween 20. None of these isolates could use xylan as its carbon source. All isolates could produce amylase, protease and CMCase. In this study, since all of the isolates except isolate TH-11 could use Tween 20 as carbon source, these isolates may produce esterase to degrade polymers, though isolate TH-11 may use another enzyme to degrade polymers.

The morphological and physiological characteristics indicate that the majority of the isolates belong to either the *Streptomyces* genus (91.9%) or the *Micromonospora* genus (8.1%). PHB-degraders are widely distributed among the families of *Pseudonocardiacece* and related genera, including *Micromonosporaceae*, *Thermonosporaceae*, *Streptosporangiaceae* and *Streptomycetaceae* (Tokiwa and Jareat 2003).

Conclusion

The percentage of PHB-degrading microorganism in the ambient environment (soil samples) has been estimated to be 0.5–9.6% of the total colonies

Table 1 The distribution of polymer-degrading microorganisms and total number of actinomycetes at various places along the Tou-Chien River in Taiwan

Locations	TVC*10^5 /soil sample (CFU/g)	Number of actinomycetes			
		PES-degraders	PHB-degraders	PCL-degraders	Total isolated
Teiling	1.03 ± 0.17	16	36	20	83
Hsin-le	3.5 ± 0.4	15	21	23	72
Zhudong	4.0 ± 0.7	4	8	4	31
Da Hua	5.6 ± 0.65	8	16	10	32
Hsi-chou	3.8 ± 0.7	21	54	26	87
Total		64	135	83	305

TVC: Total Viable Count; PHB: Poly(β-hydroxybutyrate)

PCL: Poly(ϵ-caprolactone); PES: Poly(ethylene succinate)

Table 2 Clear-zone-forming ability of polyester-degrading microorganisms on agar plates emulsified with biodegradable polyesters[a] and their screening from various places along the Tou Chien

River locations	Strains	Clarity of clear-zone[b] on plate containing		
		PHB	PCL	PES
Teiling	*Streptomyces* sp. TH-31	+	+	++
	Streptomyces sp. TH-32	+	+++	++
	Streptomyces sp. TH-125	++	++	++
	Streptomyces sp. TH-130	+++	++	+
	Streptomyces sp. TH-274	++	+	+
	Streptomyces sp. TH-280	++	+++	++
	Streptomyces sp. TH-290	+	++	+
Hsi-chou	*Streptomyces* sp. TH-11	+++	+	+++
	Streptomyces sp. TH-63	++	++	+
	Streptomyces sp. TH-150	++	+++	+++
Hsin-le	*Streptomyces* sp. TH-28	+	++	++
	Streptomyces sp. TH-201	+	+++	++

[a]PHB: poly(hydroxybutyrate); PCL :poly(ϵ-caprolactone)

PES: poly(ethylene succinate)

[b]Clarily of clear-zone: + clear ++ clearer +++ clearest

(Suyama et al. 1998). PCL-and PHB-degrading microorganisms have been found to represent 0.2 to 11.4% and 0.8 to 11.0% of the total number of microorganisms in environmental soil samples (Paranamuda et al. 1995), respectively. In our study, it was surprising to find that the ratio of polyester-degrading actinomycetes in the river-based sediment is apparently higher than those in the soil mentioned above. 135 isolates were PHB-degraders (44.2%), 83 isolates were PCL-decomposers (27.2%), and 64 isolates could degrade PES (21.0%).

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