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PHB-degrading *Streptomyces* sp. SSM 5670: Isolation, Characterization and PHB-Accumulation

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New microbial bioprospecting has become an important way to find new polyhydroxyalkanoate (PHA) producers and degraders. Poly (3-hydroxybutyrate) (PHB), the best known member of the PHAs, has received much attention because it can be degraded completely in different environments without forming any toxic products. In this contribution an actinomycete, designated strain SSM 5670, showed the better response for PHB degradation by the clear-zone method. Nevertheless, it produces PHB in low amounts (5.6% dry cell weight). According to the phylogenetic analysis the strain most similar to the PHB-degrading isolate SSM 5670 was Streptomyces omiyaensis NBRC13449. The selected isolate was characterized by its cultural, morphological and physio-biochemical features and deposited in an Argentine culture collection under the name Streptomyces omiyaensis SSM 5670.

Key words: Streptomyces, isolation, characterization, poly(3-hydroxybutyrate), accumulation, 16S rDNA analysis.

Poly (3-hydroxybutyrate) (PHB), the best known member of the group of polyhydroxyalkanoates (PHAs), has received much attention because it can be degraded completely in different environments without forming any toxic products. The ability to degrade extracellular PHAs, widely spread among bacteria and fungi, depends on the secretion of specific extracellular PHA depolymerases (e-PHA depolymerases) although these microorganisms were not-necessarily PHA-accumulators[1].

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PHB-degrading actinomycetes (order Actinomycetales) have been firstly isolated from soil and compost by Mergaert *et al.*[2]. They represent one third of all the prokaryotic organisms presented in these media and comprise a large number of species and varieties with great differences in morphology, physiology, and biochemical activities. Particularly, in 2003 Tokiwa and Jarerat[3] determined the phylogenetic affiliation of PHB-degrading actinomycetes that were widely distributed among the families of *Pseudonocardiaceae* and related genera, *Micromonosporaceae*, *Thermonosporaceae*, *Streptosporangiaceae* and *Streptomycetaceae*. More recently, Tseng *et al.*,[4] isolated 105 PHB-degrading thermophilic actinomycetes from various environments in Taiwan, while Boyandin et al. determined the PHB-degrading actinomycetes of different genera *Actinomyces*, *Nocardia*,

Streptomyces[5] found in soil of different environments.

The identification of the *Streptomyces* species, has been performed according to well-known morphological[6-8], chemical[9] and molecular methods[10,11]. Although these techniques are classical methods, they are still used[12-15].

The microbial degradation of PHB by *Streptomyces* has been profusely studied not only in soil but also in other environments. In fact, Calabia and Tokiwa[16] investigated the thermophilic strain MG isolated from soil, capable of degrading PHB at 50 °C while Mabrouk and Sabry[17] isolated and studied *Streptomyces* sp. SNG9 from marine sediments.

The goal of this contribution is to isolate a PHB-degrading strain from PHB-enriched soil, characterize it and study the feasibility of the PHB accumulation. In fact, some Actinomycetes non only degrade but also produce PHB[18, 19]; PHB might act as a source of carbon reserve and be involved in the antibiotic production and sporulation[18]. Over the past years, *Nocardia*, *Rhodococcus*[20, 21], *Streptomyces* spp.[22] and *Kineosphaera limosa*[23] were identified as PHB-producers. More recently, Matias *et al*.,[20] reported the production of different types of PHAs by 34 strains of actinomycetes isolated from soil and described the morphological modification of hyphae and PHB granules.

MATERIALS AND METHODS

Source of microbial polyester

Bacterial PHB (trade name Biocycle®1000) was purchased from PHB Industrial SA, Brazil. PHB is a yellowish white powder, with a high degree of purity of over 99.5% and a molecular weight of approximately 200 kDa. 200 mm thick sheets were prepared by hot compression moulding of the PHB powder at (178±3)ºC and approximately (5±1) MPa. 1 cm×1 cm samples were cut from the sheets and used to isolate the PHB-degrading microorganism.

Isolation and screening of PHB-degrading actinomycetes

The modified method of isolation of PHB-degraders from compost was employed[24]. PHB samples were put into commercial stabilized compost, purchased under the name Bionatural® and set to biodegrade at room temperature during two weeks. The biodegraded PHB samples were washed with distilled water, making a suspension of the microorganisms. This suspension was diluted and spread over the surface of Actinomyces agar slants containing, in g/L distilled water: yeast extract, 0.2; starch, 1.0; agar, 1.5, pH 7.2 at 30ºC to enhance the isolation of actinomycetes. The organisms were purified by the dilution-streaking method; in order to determine their PHB-degrading ability, the isolated strains were plated in solid mineral medium supplemented with 0.1% (w/v) powdered PHB; this medium was composed of (g/L distilled water): K_2HPO_4, 1; $CaCl_2 \times 2H_2O$, 0.01; $FeSO_4 \times 7H_2O$, 0.01; $MgSO_4 \times 7H_2O$, 0.4; $(NH_4)_2SO_4$, 0.1; pH 7.0. The formation of a clear zone around each colony confirmed the presence of PHB-degrading actinomycetes; the extent of degradation was measured by the area of the clear zone.

Characterization of the PHB-degrading isolate

Cultural and morphological characteristics of PHB-degrading isolate were determined according to the methods and media of the International Streptomycetes Project (ISP)[25]. The morphology of the isolate of mature culture after 14 days was studied in starch agar with inorganic salts (ISP-1), yeast-malt extract agar (ISP-2) and glycerol-asparagine agar (ISP-3). The production of the melanoid pigment was observed on peptone-yeast-iron agar and tyrosine agar after 2 and 4 days. The color of the aerial and vegetative mycelia was determined according to the Tresner-Backus color series[26]. The form of the sporophores, the morphology of the spores and the spore chains on the mature culture were observed by Phillips PSEM 500 scanning electron microscope under potential accelerator of 10 kV, with magnification from 5000x up 20000x. The utilization of carbohydrates was investigated with a basal carbon nutrient medium with addition of the following sugars at 1% (w/v): D-glucose, D-xylose, D-fructose, D-galactose, D-mannitol, L-arabinose, L-rhamnose, L-inositol, raffinose, sucrose. Hydrolysis of starch, casein, gelatin, nitrate reduction, sodium chloride tolerance, lecithinase and lipase activity were studied according to Gerhardt and Murray´s methods[27]. The antagonistic properties were tested by the agar plate method and the sensitivity to antibiotics by paper disks method. Species description was according to ISP[28] and Bergey´s Manual of Determinative Bacteriology[29, 30].

16S rDNA analysis

DNA templates for the PCR were prepared using the InstaGene™ Matrix (Bio Rad, CA, USA) according to the supplier's instructions. Prepared DNA was amplified using primers 9F, 339F, 785F, 1099F, 536R, 802R, 1242R and 1510R[31] and PrimeSTAR® HS DNA polymerase (Takara Bio Inc., Japan) in a GeneAmp® PCR System 9700 (Applied Biosystems, USA) as instructed by the manufacturer. The PCR product was treated with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and applied to an ABI PRISM® 3100 genetic Analyzer (Applied Biosystems, CA, USA). The primers employed, BC2 or BC6, were assembled with ChromasPro 1.4 software (www.technelysium.com.au/Chromas Pro.html, accessed 30 February 2013). The phylogenetic analysis of the determined sequence was performed using the software Aporon 2.0 (TechnoSuruga Laboratory) and database information (Aporon DB-BA 3.0, GenBank/DDBJ/ EMBL). The genetic distances between sequencing were estimated by the Knuc values[32]. Finally, a phylogenetic tree was constructed by the neighbour-joining method[33].

PHB accumulation

To characterize the selected PHB-degrading isolate as a PHB producer, the strain was grown in 50 mL of mineral medium in 250 mL Erlenmeyers flasks. The medium contained, in g/L distilled water: glucose, 10.0; KH_2PO_4, 2.4; K_2HPO_4, 4.3; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 1.0; trace salts solution 1 mL 25]; pH 7.0. The medium was inoculated with 1.0 mL of freshly prepared spore suspension from a 14-day culture grown on Actinomyces agar slants. The culture was incubated at 30±1°C in a rotary shaker a 125 rpm during 110 h. The mycelial mass was harvested by centrifugation in a refrigerated centrifuge (Hermle Z323) at 10000*g* for 15 min, washed with distilled water, freezed at (-20)°C and then lyophilized for 24 h at (-53)ºC and 3×10^{-3} mbar (Labconco Corp., USA). The growth of the organism was measured by the weight of the liofilizated mycelial mass. Two lipophylic dyes were used to assess microscopically the ability of the isolate to produce PHB. On one hand, an alcoholic solution of 0.3% (w/v) Sudan Black B in 70% (v/v) ethanol was applied for 10 min before analysis by light optical microscopy; excess solution was taken out with xylol and fuchsine was used as the cellular wall dye[27]. On the other hand, for fluorescent microscopy, a solution of 1% (w/v) Nile Blue A was used at 55ºC for 10 min; excess solution was taken out with water followed by the addition of 8% (v/v) acetic acid solution for 1 min[34]. For extraction of PHB, the liofilizated mycelial mass was treated according to the method of Law and Slepesky[35] with NaClO at 37ºC for 12 h and subjected to a Soxhlet extraction for 24 h using chloroform. The chloroform extract was evaporated and converted to crotonic acid by treatment with concentrated H_2SO_4. The amount of PHB was quantified a 235 nm with a Beckman DV®530 UV/ Vis spectrophotometer.

RESULTS AND DISCUSSION

Isolation and screening of PHB-degrading actinomycetes

Thirteen cultures of PHB-degrading actinomycetes, differing in colonial morphology and diffusible pigments, were isolated from degraded compression moulded PHB samples following the dilution-plating method. Extracellular PHB depolymerases secreted by PHB-degrading microorganisms hydrolyzed the polymer extracellularly to water-soluble products that can be recognized by the appearance of transparent clearing zones around the colonies. The

Table 1. Classification of PHB-degrading actinomycetes cultures by the polymer over-layer method; the third column indicates the quantity of isolates, cultured in this work, according to this classification

Biodegradation	Clear zone area (A)	Number of cultures
High	$A > 20mm^2$	2
Moderate	$15mm^2 < A < 20mm^2$	4
Low	$A < 15mm^2$	7

Table 2. Cultural and morphological properties of the selected isolate SSM 5670

		Medium		
		ISP-1	ISP-2	ISP-3
Aerial mycelium	Growth	moderate	abundant	moderate
	Color	white, turns gray at maturity		
Vegetative mycelium	Growth	moderate	abundant	moderate
	Color	yellow-brown	dark gray-yellow	yellow-brown
Colony morphology	round, 4-8 mm diameters, dry aspect			
Sporulating structures	gray spore, undulated, smooth surface, sporophore *Rectus Flexibilis* type, number of spore 10 or more			

biodegradation of PHB by 13 different isolates was assessed using the polymer over-layer method during 12 days at 30ºC (Fig. 1). This method, based on the quantitative determination of the area of the clear zone (depolymerase activity), allowed to classify the cultures in three groups, as summarized in Table 1.

From the two isolates that produced the higher PHB degradation, the one with the greater clear zone area (24±2 mm^2) was selected for its detailed characterization and assessment of the PHB-accumulating ability. In what follows this isolate will be called SSM 5670.

Characterization of the PHB-degrading isolate SSM 5670

The selected isolate SSM 5670 was characterized based on its morphology (Table 2), physio-biochemical response, antagonistic properties and resistance to antibiotics (Table 3).

The isolate SSM 5670 had well developed branched mycelium. The vegetative mycelium was not fragmented. Aerial mycelium changed its color from white to grey while developed. Non-motile aerial spores arranged in long chains, forming *Rectus Flexibilis* (RF) type of sporophores. The sporophores contained more than 10 spores, each of them elongated with smooth surface. These characteristics, illustrated in Fig. 2, are summarized in Table 2.

The main physio-biochemical characteristics, presented in Table 3, allowed us to describe the isolate SSM 5670 as Gram-positive and aerobic in nature that hydrolyzes starch and casein but not gelatine. Furthermore, it reduces nitrates to nitrites. The growth kinetics depended on temperature with an optimal performance between 25 and 30 °C. A poor growth was shown on Czapek's solution agar while no growth was observed either in the presence of sodium chloride (>7%) or at 45 °C in any medium. The isolate SSM 5670 grew very well on mineral salts media, containing D-glucose, D-xylose, L-rhamnose, D-galactose, but it did not assimilate D- fructose, L-arabinose, raffinose, D-mannitol, L-inositol and sucrose. The isolate had no antibiotic activity against test-microorganisms (*Kocuria rhizophila* ATCC 9341, *Bacillus subtilis, Escherichia coli, Sacharomyces cereviseae*) but exhibited slight antimycotic activity against *Aspergillus niger*. It was sensitive to all antibiotics used in this investigation, especially to streptomycin.

According to Bergey´s Manual of Determinative Bacteriology[29,30], the characteristics presented in Tables 2 and 3 allowed us to claim that the PHB-degrading isolate SSM 5670 is a member of the genus *Streptomyces*. Furthermore, the grey to brownish color of the mature sporulated aerial mycelium, the *Rectus Flexibilis* type of spore chain morphology with smooth spore wall ornamentation and the lack of production of melanoid pigment clearly show that the isolate belonged to the chromogenic grey series of the genus *Streptomyces*.

Phylogenetic analysis

16S rDNA sequence study was performed in order to establish the phylogenetic similarity between the isolate SSM 5670 and representatives of the genus *Streptomyces*. The strain most similar to the isolate SSM 5670 was *Streptomyces omiyaensis* NBRC13449. In fact, it showed 98.6% similarity of nucleotide sequences, although *Streptomyces tanashiensis*, *Streptomyces narbonensis*, *Streptomyces wedmorensis* y *Streptomyces zaomyceticus* exhibited comparable levels of similarity (98.4%). Fig. 3 depicts a rooted phylogenetic tree based on the neighbor-joining

method. Therefore, the *Streptomyces sp.* SSM 5670 was presumably identified and deposited in the AGRAL FAUBA culture collection (Argentina) under the name *Streptomyces omiyaensis* SSM 5670.

PHB accumulation by *Streptomyces* sp. SSM 5670

Accumulation of PHB in *Streptomyces* sp. SSM 5670 was observed qualitatively by staining of cells with Sudan Black and fluorescent Nile Blue. The PHB production increased rapidly during the exponential phase of growth of the microorganism while the pH of the medium changes from neutral to slightly acid as illustrated in Fig. 4. The accumulation is associated to the bacterial need to preserve energy for development, spore formation and starvation[20]. However, 72 h after fermentation a maximum production of 5.6% dry cell weight was reached and then the PHB concentration diminished rapidly (Fig. 4). The beginning of the sporulation of the bacterium *Streptomyces* sp. SSM 5670 also took place in this phase. This decrement can be attributed to the intracellular utilization of the polymer during the stationary phase of the cellular growth and the sporulation process. Finally, 110 h after growing, when a good level of sporulation was observed, the PHB content reduced to 3.9% dry cell weight. These results agree with those by Kannan and Rehecek[18], who proposed that the intracellular polymer might be used as endogenous carbon and energy source to drive the process of sporulation. More recently Wu *et al*.[36] showed that another Gram positive bacterium, *Bacillus* sp. Jma5, produced low levels of PHB due to sporulation. Therefore, the

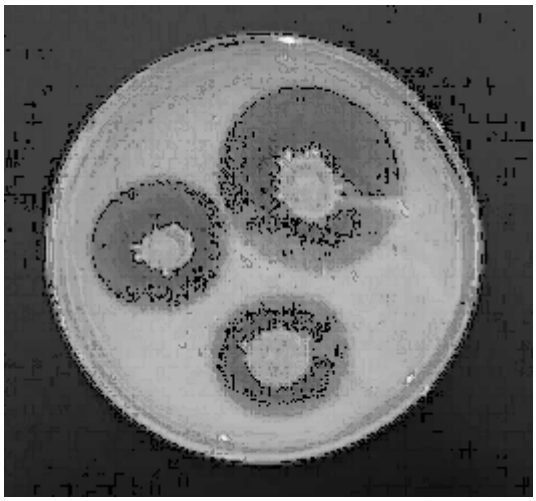

Fig. 1. Polymer over-layer method: The clear zone formation remarks the depolymerase activity of the PHB-degrading actinomycetes.

Table 3. Physio-biochemical and antagonistic properties of the isolate SSM 5670 and its sensitivity to antibiotics

Characteristics	Response
Gram reaction	Gram (+)
Production of catalase	+
Growth at 45 ºC	-
Optimum temperature for growth	25-30 °C
Growth under anaerobic condition	-
Growth on Mac Conkey agar	±
Growth on Czapek agar	±
Melanoid pigment on peptone-yeast-iron agar	-
Melanoid on tyrosine agar	-
Production of diffusible pigments	-
Growth on nutritive agar witch:	
5 % NaCl	+
7 % NaCl	-
Hydrolysis of: Casein	+
Starch	+
Gelatin	-
Enzyme activity: Urease production	+
Lecithinase production	+
Lipolysis	-
Nitrate reduction	+
Growth on sole carbon source 1% (w/v):	
D-glucosa	+
D-xilosa	+
D-fructosa	-
D-galactosa	+
D-manitol	-
L-arabinosa	-
L-ramnosa	+
L-inositol	-
Rafinosa	-
Sacarosa	-
Antibiosis to: *Kocuria rhizophila* ATCC 9341	-
Bacillus subtilis	-
Escherichia coli	-
Saccharomyces cereviseae	-
Aspergillus niger	+
Sensitivity to antibiotics[a]: Streptomycin (300 µg/disk)	28
Gentamycin (10 µg/disk)	19
Erythromycin (15 µg/disk)	21
Ampicillin (10 µg/disk)	9
Chloramphenicol (30 µg/disk)	9
Penicillin (10 µg/disk)	7

a: A sensitive response was characterized by the diameter of the inhibition zone (mm)

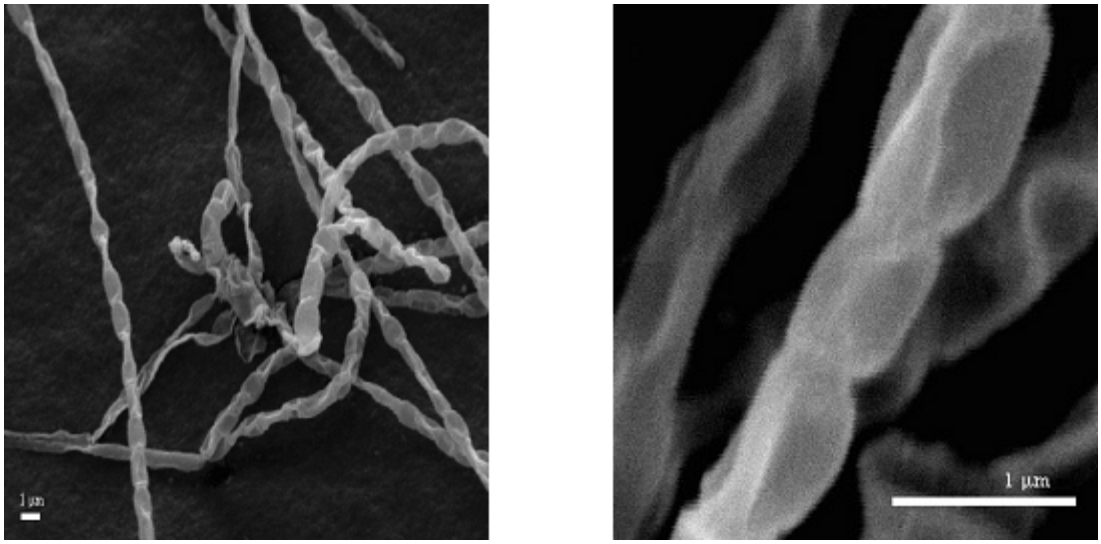

Fig. 2. Scanning electron micrographs of a spore chain (5000x) and a spore surface (10000x) of the isolate SSM 5670 (from left to right)

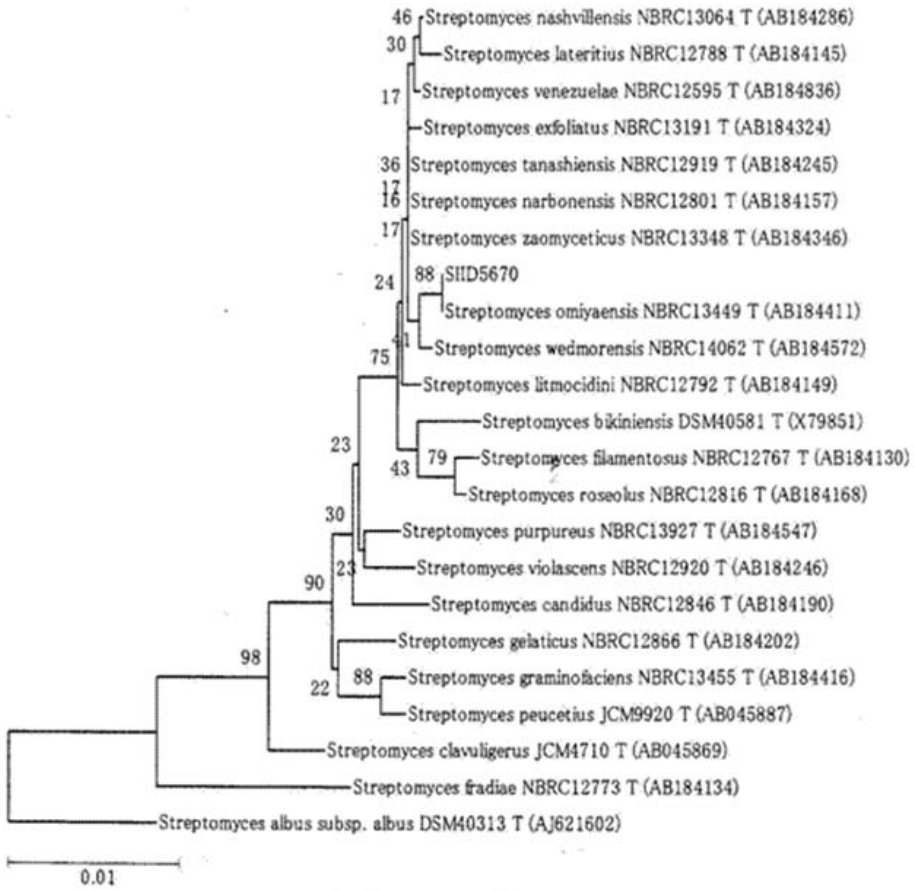

Fig. 3. Phylogenetic position of SSM 5670 strain (SIID 5670 in the list) among neighboring species. The scale bar represents one nucleotide substitution per 100 nucleotides

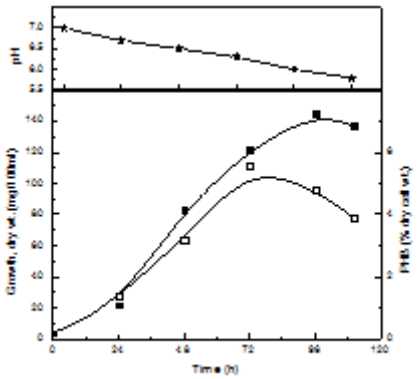

Fig. 4. Time dependence of: the growth of *Streptomyces* sp. SSM 5670 (▮), the PHB accumulation (□) and the pH (★) of the medium

prevention of sporulation might improve the bioreactor productivity of PHB in these strains.

In summary, a PHB-degrading organism isolated from soil ¯denoted as SSM 5670¯ agreed in 98.6% with *Streptomyces omiyaensis*, that is, one of the 17 strains of the chromogenic grey series of the genus *Streptomyces*. This strain was characterized according to its cultural, morphological and physio-biochemical features. It is noticeable that this isolate strain can produce PHB although in not very large amount. However, *Streptomyces* sp. SSM 5670 has a remarkable ability to degrade extracellular PHB; in fact, in a previous paper we have already presented the biodegradation of compression moulded samples of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) by this strain[37].

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