CHAPTER 2

Isolation of lipid- and polysaccharide-degrading microorganisms from a subtropical forest soil, and analysis of the lipolytic strains *Bacillus* sp. CR-179 and *Rhodococcus* sp. CR-53

Figure C2.1 Iguazú rainfalls.

1 INTRODUCTION AND OBJECTIVES

Microorganisms or their enzymes are used in a wide range of biotechnological activities such as synthesis of antibiotics, decontamination of soils, and several industrial processes due to the fact that enzyme-catalyzed reactions are highly efficient and selective, are less polluting, and have a lower cost (Cherry & Fidantsef, 2003; see General introduction 3.3.1). Thus, there is an increasing interest for isolating new enzymes and new enzyme-producing strains for their use in biotechnology. Among these enzymes, "true" lipases, carboxylesterases, cellulases, xylanases and pectinases are some of the most important due to their wide applications (Cherry & Fidantsef, 2003; Gupta *et al*., 2004; see General introduction 3.3.1, 3.3.2 and 3.3.3).

Soils are a good source of novel enzyme-producing strains because they are nutrient-rich environments where there is a high proliferation of microorganisms. The diversity of these microorganisms depends on nutrient availability and several physicochemical properties related to the climate and type of soil: texture, pH, temperature, solar irradiation, aeration, water content, mineral composition, etc. Many soil microorganisms are highly active in nutrient recycling, mainly in the degradation of vegetable polysaccharides such as lignocellulose and its phenolic-derived compounds, and have therefore an enzymatic machinery very active (Atlas & Bartha, 1998; see General Introduction 3.3.4.3). Therefore, this chapter is focused on the isolation and characterization of new lipid- and polysaccharide-degrading soil microbial strains that could be of interest for biotechnological applications. The exact aims of this work were:

1. **To isolate the lipid- and polysaccharide-degrading microorganisms from three soil samples obtained from a subtropical forest of Puerto Iguazú (Argentina), and to determine the lipolytic activity of the most active strains.**

    a. To isolate in pure culture the native bacterial and fungal strains capable of growing aerobically at 20−42 ºC on CeNAN-olive oil agar, Horikoshi-I agar, LB agar, Nutrient agar, or Sabouraud-chloramphenicol agar, from three soil samples obtained from a subtropical forest of Puerto Iguazú (Argentina).

    b. To analyze the previously isolated microorganisms for the detection of the biotechnologically-interesting enzymatic activities: "true" lipase, carboxylesterase, cellulase, xylanase and pectinase.

    c. To select the microorganisms showing the highest activity on the substrates analyzed, to perform their cell-morphology description, and to store the microbial collection obtained at −80 ºC for further analyses.

    d. To determine the lipolytic activity of the strains more active on lipid substrates, and to select the most active ones for further assays.

2. **To analyze the morphological, biochemical and physiological properties of the lipolytic isolates *Bacillus* sp. CR-179 and *Rhodococcus* sp. CR-53.**

    a. To analyze the morphological, biochemical, physiological and molecular properties of the lipolytic isolates *Bacillus* sp. CR-179 and *Rhodococcus* sp. CR-53 in order to perform their preliminary identification.

    b. To analyze the lipolytic system of the lipolytic isolates *Bacillus* sp. CR-179 and *Rhodococcus* sp. CR-53.

    c. To identify the lipase-coding gene(s) of *Bacillus* sp. CR-179 by PCR amplifications using the primers for *Bacillus* lipases mentioned in Chapter 1.

♦ **NOTE:** The preliminary identification of strain *Rhodococcus* sp. CR-53, and the analysis of its lipolytic system was performed in collaboration with Dr. S. Falcocchio from the Università degli studi di Roma "*La Sapienza*".

2 MATERIALS AND METHODS

Unless otherwise stated, materials and methods used were those previously described at the General Materials and Methods section (see Tables M.1, M.2, and M.7 for more detailed explanation of the strains, culture media and primers used).

2.1 ISOLATION OF SOIL STRAINS

Three soil samples were collected by Dr. F.I.J. Pastor from a subtropical forest located at the Iguazú rainfalls area (Puerto Iguazú, Argentina). Soil no. 1 was a mixture of decomposing wood from a decaying tree and surface soil, soil no. 2 was highly compact clayey material buried 20 cm from the surface and containing small roots, and soil no. 3 was surface soil with a high content in vegetable material. The soil samples were stored at room temperature in axenic conditions and protected from direct sun light until the isolation was performed.

Isolation of the microorganisms from the soil samples was performed as described (General Materials and Methods 2.3), and it is summarized in Figure C2.2. Briefly, microorganisms were extracted by soil suspension in saline solution Ringer ¼, vigorous stirring and additional sedimentation. Two samples of the aqueous phase were collected, one of them was treated for 10 min at 80 ºC to isolate only spore former bacteria, and the other sample remained untreated in order to isolate spore former and non-spore former microorganisms. These samples were used for the isolation of aerobic and facultative anaerobic microorganisms by means of serial dilutions in Ringer ¼, spread on CeNAN-olive oil agar, Horikoshi-I agar, LB agar, Nutrient agar and Sabouraud-chloramphenicol agar, and subsequent incubation at 20 ºC, 30 ºC or 42 ºC under aerobic conditions. The resulting colonies that showed different morphological properties were selected and isolated in pure culture.

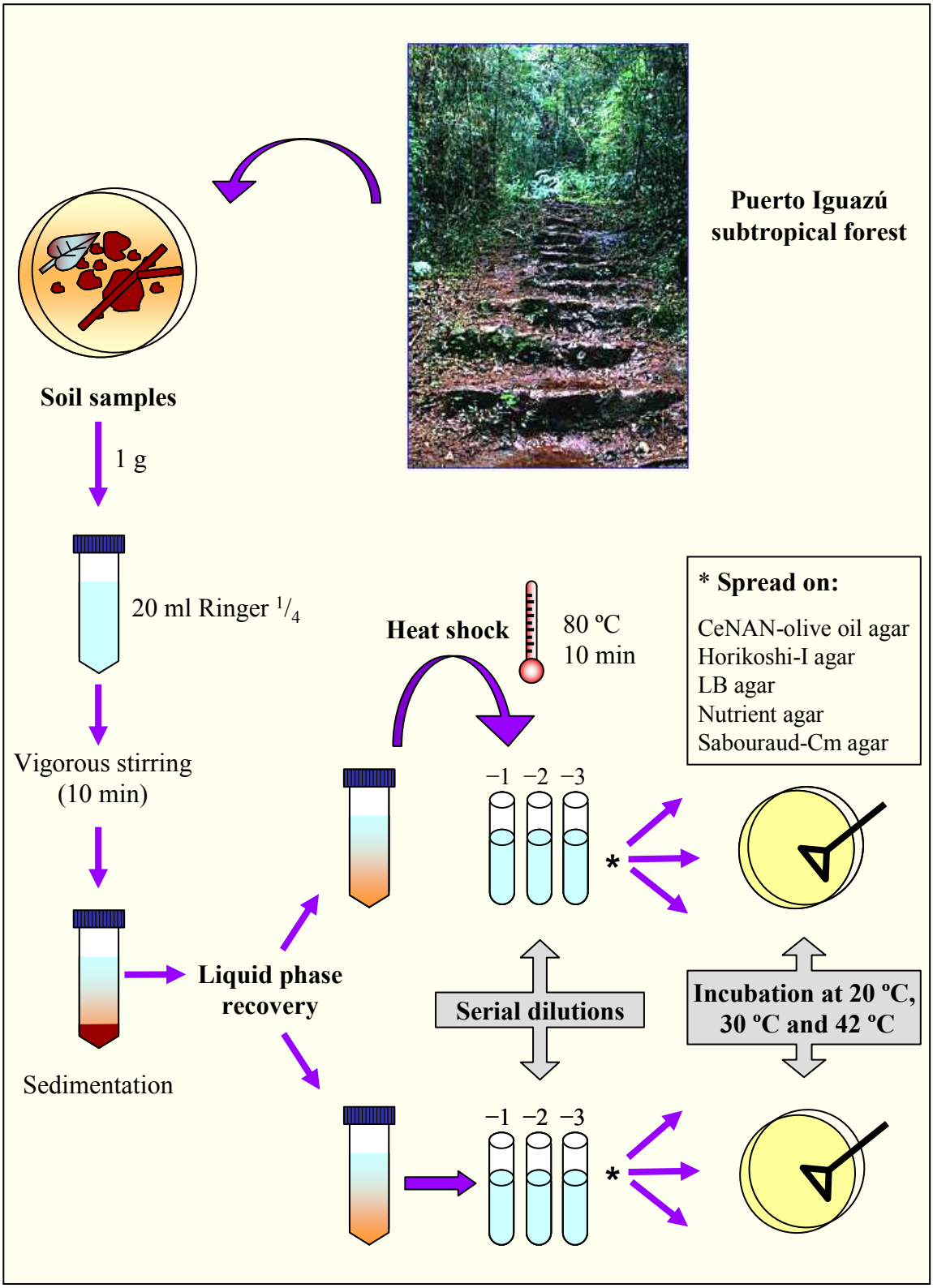

Figure C2.2 Isolation of microorganisms from a subtropical forest soil.

2.2 ENZYMATIC ASSAYS

The hydrolytic activities on olive oil, tributyrin, xylan, carboxymethyl cellulose and polygalacturonic acid (PGA) of the 724 strains isolated from the soil samples analyzed before were screened by means of a direct simple test based on streaking the isolated strains on agar plates supplemented with the desired substrate and incubated at the isolation temperature of each strain (see General Materials and Methods 5.1). The degree of hydrolytic activity produced by these strains was estimated by comparison of the fluorescence or hydrolysis haloes produced by these strains with respect to those produced by the control strains *E. coli* 5K (negative control), *Bacillus* sp. BP-7 (positive control on olive oil- and tributyrin-supplemented plates), and *Paenibacillus* sp. BP-23 (positive control on xylan-, carboxymethyl cellulose- and PGA-supplemented plates). The 76 strains bearing the highest hydrolytic activities were selected, analyzed by Gram or fungal stain, and stored at −80 ºC for further characterization.

Among these strains, fluorescence emission of the 48 strains that showed the highest activity in emulsified CeNAN-olive oil or tributyrin agar plates was assayed by fluorimetric paper assays using MUF-derivatives (see General Materials and Methods 5.4.1). The lipolytic activity on MUF-butyrate and MUF-oleate of the supernatants and crude cell extracts (prepared in 50 mM phosphate buffer (pH 7) as previously described in General Materials and Methods 4.1.1) from the 29 strains producing the highest fluorescence emission in the previous assays was subsequently determined at room temperature by classical fluorimetric liquid assay (General Materials and Methods 5.4.2.1).

Moreover, the lipolytic system of the lipid-degrading strains CR-179 and CR-53 was analyzed by zymogram assay (General Materials and Methods 5.2) of their supernatants and crude cell extracts after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) separation (General Materials and Methods 4.4.1 and 4.6, respectively).

2.3 IDENTIFICATION OF STRAINS CR-179 AND CR-53

2.3.1 Bacterial identification

Preliminary identification of the hydrolytic strains CR-179 and CR-53 was performed according to the microbiological tests recommended for the genus *Bacillus* and other Gram-positive bacteria (Gordon *et al.*, 1973) and, in the case of CR-53, for actinomycetes (Goodfellow *et al*., 1998). The morphological, physiological, biochemical and molecular assays were performed as previously described (General Materials and Methods 2.4). The molecular analysis of these strains was performed by amplification and sequencing of their 16S rDNA, followed by sequence analysis and computing of matrices of evolutionary distances (General Materials and Methods 3.5, 3.6 and 3.7, respectively).

2.3.2 Nucleotide sequence accession numbers

The 16S rDNA sequences of strain CR-179 and CR-53 were submitted to the EMBL under accession numbers AJ821280 and AJ786263, respectively.

2.4 DETECTION OF STRAIN CR-179 LIPASE-CODING GENES

Detection of strain CR-179 lipase-coding genes was performed by PCR amplifications under low-astringency conditions (Tm = 42−55 ºC), using consensus primers for the central region of *(Geo)Bacillus* lipase-coding genes (FWSUB/BWSUB and FWTHER/BWTHER), and using specific primers for the amplification of *B. subtilis lipA* (FWBSLA/BWBSLA) and *B. subtilis lipB* (FWYfiP/BWYfiP), as previously described in Chapter 1.

3 RESULTS

3.1 ISOLATION OF SOIL MICROORGANISMS

Three soil samples from a subtropical forest of the Iguazú rainfalls (Puerto Iguazú, Argentina) were used for the isolation of native microorganisms growing in an organic matter-rich environment, using the different culture conditions described in the Materials and Methods section. A total of 724 pure cultures of bacterial and fungal strains were obtained (Table C2.1). These strains were named "CR-*X*", being "*X*" the number of each isolate, and the morphological traits of some of them are shown in Figure C2.3.

Table C2.1 Number of CFU g^{-1} and isolates from each soil sample.

Soil	CFU* g^{-1} (Total)	CFU* g^{-1} (Fungi)	Isolates† (Total)	Isolates† (Fungi)
1	$2{\cdot}10^5$	$2{\cdot}10^4$	110	20
2	$6{\cdot}10^5$	$6{\cdot}10^4$	260	19
3	$6{\cdot}10^5$	$6{\cdot}10^4$	354	32
Total			724	71

*Colony forming units (CFU) per gram of soil. Maximum of isolates obtained among all the culture media and temperatures analyzed. Total refers to bacteria plus fungi.

†Number of different strains isolated in pure culture.

Fungi should be considered as putative.

All soil samples showed similar values of colony forming units per gram of soil (CFU g^{-1} = 2−$6{\cdot}10^5$; Table C2.1), independently from the 80 ºC treatment or the culture

medium and temperature used, although important differences were observed in the morphology of the isolated colonies (see Figure C2.3). Sabouraud-chloramphenicol medium was the exception because no growth was observed for the 80 ºC–treated samples, whereas the values obtained for the unheated samples were 10-fold lower than those obtained in the other culture media.

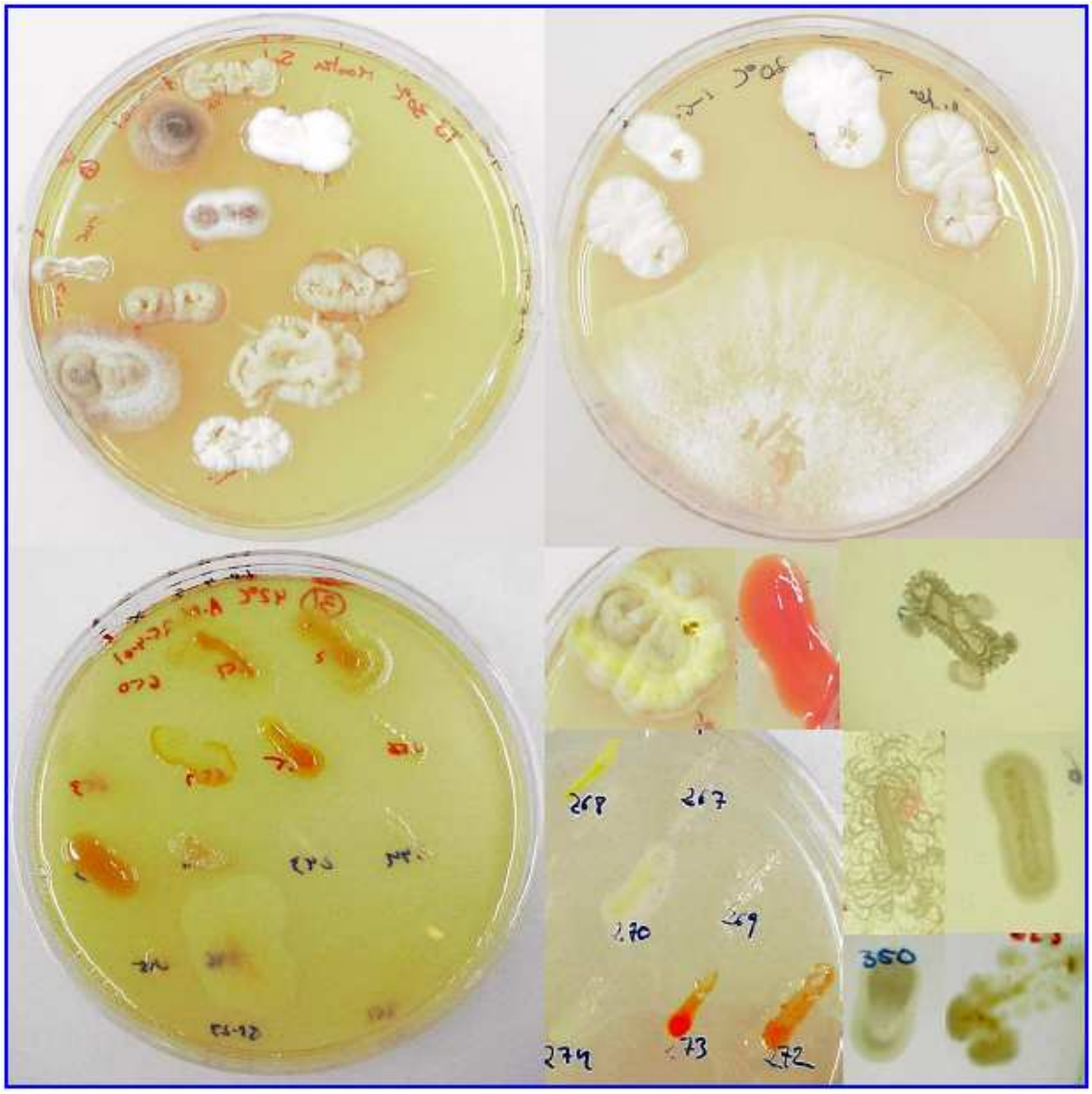

Figure C2.3 Examples of the morphological diversity found among the isolated strains.

3.2 SCREENING OF THE ENZYMATIC ACTIVITIES OF THE ISOLATED STRAINS

The hydrolytic activities of the 724 strains previously isolated were detected using a plate test for olive oil, tributyrin, xylan, cellulose and pectin degradation (see Materials and Methods). Among the isolates analyzed, 449 were active on one or more of the substrates evaluated, and 43 of them degraded all substrates. Table C2.2 shows the results with respect to each soil sample and substrate analyzed. Nearly half of the strains showed lipolytic activity (331 degraded olive oil and 360 hydrolyzed tributyrin), whereas the number of strains active on polysaccharides was lower (114, 116 and 82 degraded xylan, carboxymethyl cellulose and PGA, respectively). Almost no differences were found with respect to the type of hydrolytic activities or the relative number of active strains obtained among the isolation conditions used, with the exception of a slight predominance among the active strains of those isolated at 30 ºC.

Table C2.2 Screening of the hydrolytic activities of the soil isolates.

Soil	Isolates	Olive oil	Tributyrin	Xylan	CMCel*	PGA†
1	110	48	48	13	19	4
2	260	110	129	49	56	36
3	354	173	183	52	41	42
Total	724	331	360	114	116	82

*Carboxymethyl cellulose; †Polygalacturonic acid.

Table C2.3 shows in detail the substrates degraded by each isolate, with an estimation of the degree of hydrolytic activity produced by these strains, as well as the main features (temperature and medium of isolation, Gram or fungal stain, cellular morphology, etc) of these strains. To avoid too large table, only the 76 isolates (29 from soil no. 1, 21 from soil no. 2 and 26 from soil no.3) selected due to bearing the highest hydrolytic activities are shown.

Table C2.3 Hydrolytic activities of the most active soil isolates.

Strain	Isolation		Gram-morph.	Olive oil		TB	Xylan	CMCel	PGA
	ºC	Medium		Colour	Fluores.				
Controls									
BP-7 (C+)		NA	R+	+++	+++	$++_{+}$			
BP-23 (C+)		NA	R+				++++	$+++_{+}$	++++
5K (C−)		LB	R−	−	−	−	−	−	−
Soil no. 1									
CR-3	30	LB	R+ e	+++	−	+++	−	−	−
CR-6	30	LB	R+ e c	+	−	+	++++	+++	++++
CR-7	30	LB	R+ e c	+++	−	+++	−	−	−
CR-8	30	LB	R+	UD	UD	UD	++++	−	−
CR-12	30	LB	R+ e c	UD	UD	UD	++++	−	−
CR-17	42	LB	R+ c	UD	UD	UD	++++	−	−
CR-20	42	LB	R+ c	+	+	++	++	+	−
CR-26	20	LB	R+ e c	UD	UD	UD	−	++	−
CR-31	30	Sab-cm	F	+++	++++	UD	−	UD	−
CR-39	20	Sab-cm	F	−	++++	UD	+++	−	−
CR-40	20	Sab-cm	Y	+++	++++	−	−	−	−
CR-47	42	NA	RC+ e c	UD	UD	UD	+++	−	−
CR-53	20	LB	RC+	+	+++	+	−	+ −	−
CR-54	20	LB	R+ c	+	−	UD	−	++	−
CR-56	30	Sab-cm	Y	−	−	+	−	−	−
CR-64	20	Oil	I+ c	+++	++	+	−	+	−
CR-70	20	Sab-cm	F	+++	+++	+	+	−	−
CR-75	30	Sab-cm	F	−	−	+	−	+ −	−
CR-86	20	Hori-I	R+ e c	−	−	−	−	++	−
CR-89	20	Hori-I	Y	++ (++++h)	+ (+++h)	+++	−	−	−
CR-92	20	Hori-I	Y	++	+	++	+++	+	−
CR-93	20	Hori-I	R+ e c	−	−	−	−	−	++
CR-94	20	Hori-I	R+	UD	UD	−	−	−	++
CR-95	20	Hori-I	Y	++ (++++h)	+ (++h)	+++	−	−	−
CR-96	20	Hori-I	Y	++ (++++h)	+ (++h)	++	−	−	−
CR-97	20	Hori-I	F	+++	++++	+ −	−	−	−
CR-98	20	Hori-I	Y	++ (++++h)	++ (++h)	++	−	−	−

Strain	Isolation		Gram-morph.	Olive oil		TB	Xylan	CMCel	PGA
	ºC	Medium		Colour	Fluores.				
Soil no. 1									
CR-105	30	Oil	R+ e c	++	−	++	−	−	−
CR-109	30	Oil	R+ c	+++	+ −	+	−	−	−
Soil no. 2									
CR-158	30	Hori-I	RC+ e c	−	−	++++	−	−	−
CR-177	42	Hori-I	R+ e c	−	−	+	+++++	+++	+
CR-179	42	Hori-I	RC+ e c	++	++ (++++h)	++	++++	+++++	+++
CR-203	42	LB	R+ e c	++++	++++ (+++h)	+++	++	+++	+
CR-208	42	LB	R+ e c	++	−	+	+	++++	++
CR-237	42	LB	R+ e c	+++	++++ (++h)	++	++	++++	+
CR-246	42	LB	RC+ c	++++	++++ (+h)	+++	++	+++++	++++
CR-269	20	LB	R+ e c	+++	−	−	+++	++++	+++
CR-286	30	Oil	RC+ e c	+ −	−	++++	−	−	−
CR-290	42	Oil	RC+ c	++++	+++	++	++++	+++++	+++
CR-295	42	Oil	R+ c	++++	+++ (++h)	++	++++	+++++	+++
CR-301	42	Oil	R+ e c	++++	++++ (++h)	++	+++	++	−
CR-307	30	Oil	F	+++++	++++	−	+ −	−	−
CR-313	20	Oil	F	++++	++++	++	+ −	+	+++
CR-316	20	Oil	F	++++	++++	++	+ −	+	+++
CR-330	30	NA	R+ c	+	+	++++	−	−	−
CR-331	30	NA	R+ c	+	+	++++	−	−	−
CR-337	30	NA	R+ c	+	++	++++	−	−	−
CR-354	42	NA	R+ e c	UD	UD	UD	++++	+++	−
CR-358	42	NA	R+ e c	−	−	UD	++++	++	++
CR-362	42	NA	R+ e c	+	−	−	−	++++	−
CR-377	30	Hori-I	R+ e c	+	−	++++	+ −	−	−
Soil no. 3									
CR-407	30	LB	R+ e c	++	++++	UD	−	−	−
CR-445	30	LB	R± c	++	++++	++	+++	+++	++++
CR-451	42	LB	R+ e c	UD	UD	++++	−	−	−
CR-454	42	LB	R+ e c	+	++	++++	−	−	−
CR-478	42	LB	R+ c	UD	UD	UD	++++	+++	−
CR-479	42	LB	R+ e c	−	−	UD	++++	++	++

Strain	Isolation		Gram-morph.	Olive oil		TB	Xylan	CMCel	PGA
	ºC	Medium		Colour	Fluores.				
Soil no. 3									
CR-486	20	LB	R±	−	−	++++	−	−	−
CR-495	20	LB	R+ e c	−	−	−	++++	−	−
CR-518	20	Oil	R+	++++	++++	++	+++	++	++
CR-522	20	Oil	R+ e c	++++	++++	++	+++	++	++
CR-530	42	Oil	R+ e c	++++	++++ (++h)	++	++	++	+
CR-558	20	Sab-cm	F	++++	++++	++	+	−	−
CR-560	20	Sab-cm	F	++++	++++	+	+	−	++
CR-563	20	Sab-cm	F	++++	++++	−	−	−	−
CR-583	30	NA	R+ e c	++++	++++ (++h)	++	−	++	−
CR-611	30	NA	R−	−	++	++++	−	−	−
CR-618	30	NA	RC+ c	−	−	+	−	−	++++
CR-644	42	NA	RC+ c	−	+	+++	−	−	++++
CR-649	42	NA	R+ c	++++	++++ (++h)	+++	−	++	−
CR-653	42	NA	R+ c	−	−	++++	−	−	−
CR-654	42	NA	R+ e c	++	++++ (++h)	+++	++	++	−
CR-670	30	Hori-I	R+ c	++++	+++	+++	+	++++	+++
CR-680	30	Hori-I	R+ e	+	++	++++	−	−	−
CR-699	30	Hori-I	RC+ c	++	−	++++	−	−	−
CR-710	30	Hori-I	R+ c	++	−	++++	−	−	−
CR-716	20	Hori-I	R+ c	+++	−	−	+++	++++	+++

(1) Title abbreviations: ºC: temperature of isolation; Medium: culture medium of isolation; Gram-morph.: Gram or fungal stain and cellular morphology observed by optical microscopy; Fluores.: fluorescence emission observed on olive oil-supplemented plates after UV light irradiation; TB: tributyrin; CMCel: carboxymethyl cellulose; PGA: polygalacturonic acid.

(2) Control strain abbreviations: BP-7: *Bacillus* sp. BP-7 (positive control for olive oil and tributyrin degradation); BP-23: *Paenibacillus* sp. BP-23 (positive control for xylan, carboxymethyl cellulose and PGA degradation); 5K: *E. coli* 5K (negative control).

(3) Culture media abbreviations: Hori-I: Horikoshi-I agar; LB: Luria-Bertani agar; NA: Nutrient agar; Oil: CeNAN-olive oil agar; Sab-cm: Sabouraud-chloramphenicol agar.

(4) Gram-morphology abbreviations: +: Gram-positive; −: Gram-negative; ±: variable Gram-stain; C: coccus; F: putative fungus (fungal hyphae); I: Irregular morphology; R: rod; RC: rod-coccus; Y: putative yeast, c: putative capsulated microorganism; e: putative endosporulated microorganism.

(5) Enzymatic activity abbreviations: the number of + indicates the size of the degradation haloes obtained on the culture media containing tributyrin, xylan, carboxymethyl cellulose or PGA, or the intensity of the orange/pink colour or fluorescence obtained on olive oil-supplemented plates, whereas − indicates lack of such an activity. Fluorescence on olive oil refers to that of the streak (out of brackets) and to that of the haloes around the streak (in brackets and followed by "h"). UD: undetected activity, indicates that the strain did not grow on plates supplemented with that substrate.

The hydrolytic activity of some of the most active isolates described in Table C2.3 is shown below (Figure C2.4) as an example of the enzymatic activities evaluated.

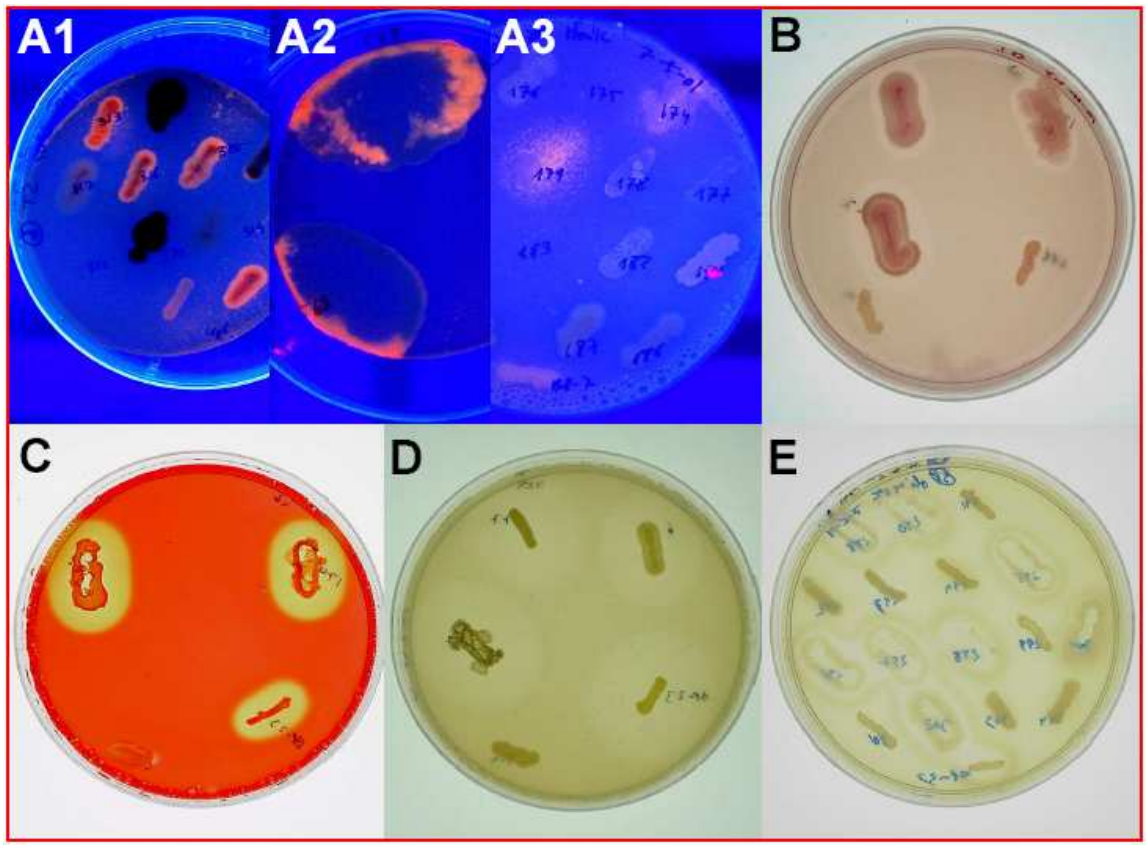

Figure C2.4 Hydrolytic activity of the most active isolates on the substrates evaluated.

Hydrolytic activity of some of the most active isolates growing on agar plates supplemented with olive oil (A1−3), tributyrin (B), carboxymethyl cellulose (C), xylan (D), and polygalacturonic acid (E). Orange-pink fluorescence emission (A1−3) and haloes around the streak (B, C, D, E) are indicators of the biotechnologically-interesting activities: "true" lipase, carboxylesterase, xylanase, cellulase and pectinase, respectively.

3.3 SELECTION OF THE MOST LIPOLYTIC ISOLATES

The lipolytic activity of the collection of 76 isolates selected due to their high hydrolytic activity was analyzed in more detail.

From these isolates, 48 strains showing a good performance in hydrolysis of olive oil and/or tributyrin were analyzed by fluorimetric paper assay on MUF-butyrate and MUF-oleate. Figure C2.5 shows an example of the lipolytic activity detected by these assays. The 29 strains producing the highest fluorescence emission in fluorimetric paper assays (Table C2.4) were chosen for further determination of their lipolytic activity.

Table C2.4 Isolates selected by fluorimetric paper assay.

Soil	Isolates analyzed	Isolates selected	Strains selected
1	20	13	CR-3, CR-6, CR-31, CR-39, CR-40, CR-53, CR-56, CR-64, CR-70, CR-89, CR-95, CR-105, CR-109
2	17	7	CR-179, CR-203, CR-208, CR-237, CR-290, CR-313, CR-377
3	21	9	CR-445, CR-479, CR-518, CR-522, CR-563, CR-611, CR-649, CR-653, CR-699
Total	**48**	**29**	

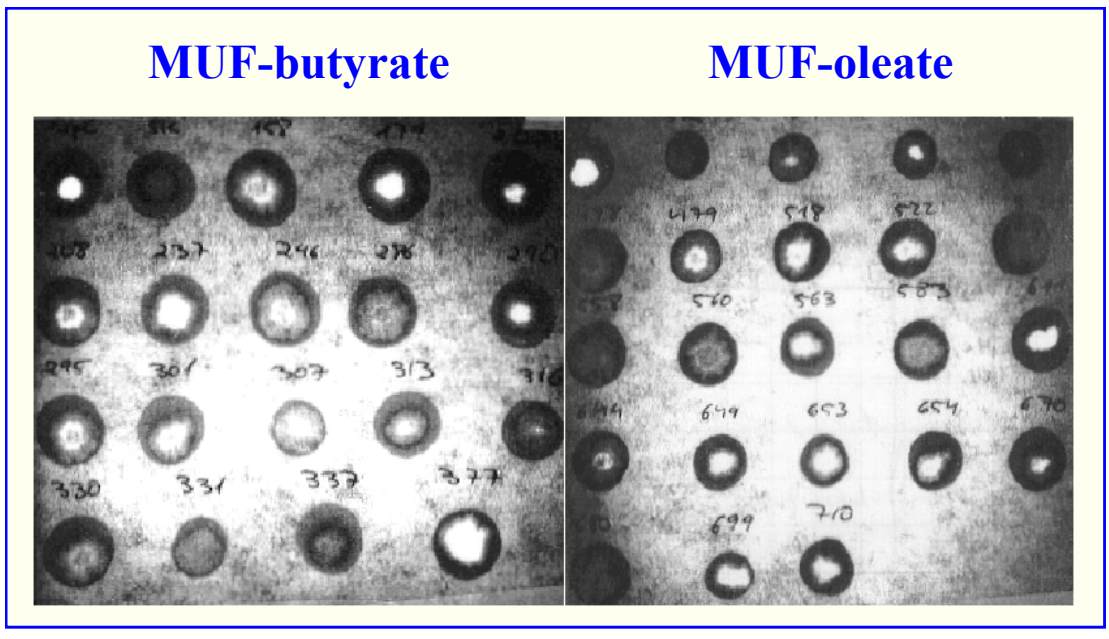

Figure C2.5 Fluorimetric paper assay of several selected lipolytic isolates.

Table C2.5 shows the activity results on MUF-butyrate and MUF-oleate produced by crude cell extracts and culture supernatants of the 29 lipolytic strains previously selected by fluorimetric paper assays. The activity of all strains on MUF-butyrate was higher than that found on MUF-oleate, and strain CR-179 was the most active isolate in general terms. This strain was by far the most active on MUF-butyrate (cell extracts: 1418.8 ± 7.5 mU mg^{-1} protein, and 6.9 ± 3.6·10^{-2} mU ml^{-1}; supernatants: 7.6 ± 0.1 mU ml^{-1} culture), and it was also very active on MUF-oleate (cell extracts). Other strains such as CR-611 (cell extracts) and the putative fungal isolates CR-31 (supernatants) and CR-563 (cell extracts) were very active on both MUF-butyrate and MUF-oleate. Strains CR-203, CR-445 and CR-522 (cell extracts) displayed also high activity on MUF-butyrate, whereas strains CR-53 and CR-377 (cell extracts) were among the most active on MUF-oleate (Table C2.5).

Table C2.5 Spectrofluorimetric determination of the lipolytic activity of the most active isolates on lipid substrates.

	MUF-butyrate			MUF-oleate		
	Cell extracts		SN*	Cell extracts		SN*
Isolates	mU mg^{-1} protein	mU ml^{-1}	mU ml^{-1}	mU mg^{-1} protein	mU ml^{-1}	mU ml^{-1}
Soil no. 1						
CR-3	12.9	0.8	1.2	0.910	0.050	0.004
CR-6	15.6	1.2	1.7	0.370	0.030	0.002
CR-31†	-	0.1	4.0	-	0.030	0.201
CR-39†	-	0.1	2.0	-	0.020	0.000
CR-40†	23.9	1.8	0.0	0.090	0.070	0.000
CR-53	9.7	0.3	1.1	1.230	0.040	0.038
CR-56†	23.6	2.0	0.8	0.960	0.080	0.000
CR-64	6.9	0.3	0.2	0.000	0.000	0.000
CR-70†	15.0	1.1	0.0	1.310	0.090	0.000
CR-89†	12.1	0.3	1.6	0.080	0.000	0.003

Isolates	MUF-butyrate			MUF-oleate		
	Cell extracts		SN*	Cell extracts		SN*
	mU·mg^{-1} protein	mU·ml^{-1}	mU·ml^{-1}	mU·mg^{-1} protein	mU·ml^{-1}	mU·ml^{-1}
Soil no. 1						
CR-95†	5.6	0.2	1.0	0.480	0.010	0.016
CR-105	36.2	2.7	2.8	0.000	0.000	0.021
CR-109	30.5	0.6	0.9	0.450	0.010	0.009
Soil no. 2						
CR-179	1418.8	6.9	7.6	4.650	0.020	0.003
CR-203	84.0	2.4	0.4	0.270	0.010	0.000
CR-208	65.0	1.1	0.3	0.420	0.010	0.000
CR-237	5.7	0.2	0.3	0.250	0.010	0.002
CR-290	364.6	3.9	7.0	1.100	0.010	0.002
CR-313†	18.7	0.0	0.1	0.000	0.010	0.000
CR-377	-	0.3	0.4	5.020	0.150	0.002
Soil no. 3						
CR-445	54.5	2.0	0.9	0.260	0.010	0.000
CR-479	25.9	0.8	0.3	0.000	0.000	0.000
CR-518	19.2	0.6	1.1	0.000	0.000	0.000
CR-522	24.6	0.6	1.1	0.000	0.000	0.015
CR-563†	22.5	2.1	0.8	9.770	0.910	0.006
CR-611	32.5	2.2	0.3	6.450	0.450	0.012
CR-649	27.2	0.4	0.4	0.720	0.010	0.002
CR-653	11.6	0.2	0.2	0.000	0.000	0.002
CR-699	15.8	1.2	0.3	2.980	0.230	0.008

*Culture supernatants; †putative fungal isolates.

The activity of all supernatants and some cell extracts (see "-" mark) is not expressed as mU mg^{-1} protein due to their low protein content, undetectable by the Bradford assay.

Results are the mean of two independent assays, each one performed in duplicate. The standard deviations obtained ranged from 0.5% to 10% of the corresponding mean values.

In view of the results obtained, strains CR-31, CR-53 and CR-105 isolated from soil no. 1, strains CR-179, CR-203, CR-290, CR-377 and CR-445 from soil no. 2, and strains CR-522, CR-563 and CR-611 isolated from soil no. 3 were selected as the most interesting strains with respect to lipid degradation. Moreover, strains CR-179, CR-203, CR-290 and CR-522 produced also a high degradation of xylan, carboxymethyl cellulose, and PGA, which increased the interest in this isolates. Strains CR-53 and CR-179 showed also singular colonial and cellular morphologies, which reinforced our interest in these strains.

Therefore, all these strains, as well as some of the most actives on polysaccharides, are being studied in our research group in order to identify them and to analyze their hydrolytic properties. Among them, the strains CR-53 and CR-179 have been preliminarily identified, and their lipolytic system analyzed in this work. The results obtained for these two isolates are shown in the next pages.

3.4 ANALYSIS OF STRAIN CR-179

3.4.1 Preliminary bacterial identification

Strain CR-179 was isolated from soil no. 2 after heat shock at 80 ºC and subsequent incubation on Horikoshi-I plates (pH 10) for 24 h at 42 ºC. This strain, showing a singular colonial and cellular morphology, producing high degradation of olive oil, triolein (not shown), tributyrin, xylan, carboxymethyl cellulose and PGA (Table C2.3; Figure C2.6), and being the most active strain on MUF-butyrate and also very active on MUF-oleate (Table C2.5), was the first strain chosen for further characterization.

The results of the morphological, biochemical, physiological and molecular test performed on this strain according to the microbiological tests recommended for the genus *Bacillus* and other Gram-positive bacteria (Gordon *et al.*, 1973) are detailed in the next pages.

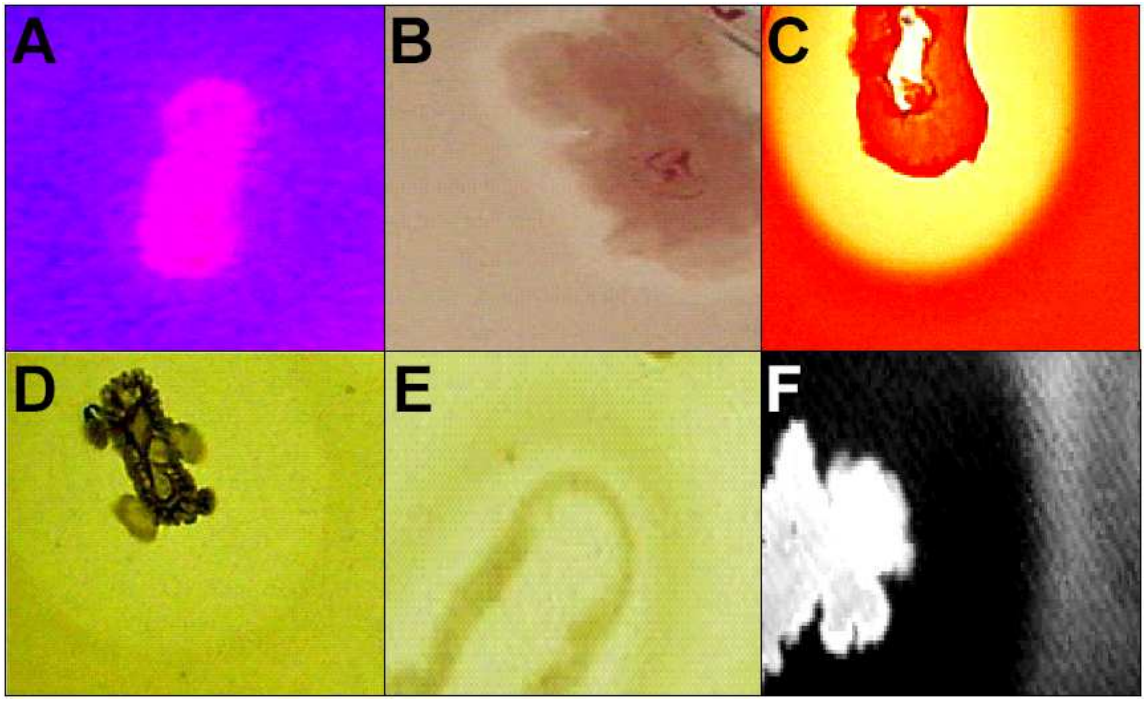

Figure C2.6 Hydrolytic activity of strain CR-179.

Hydrolytic activities of CR-179 growing on agar plates supplemented with olive oil (A), tributyrin (B), carboxymethyl cellulose (C), xylan (D), polygalacturonic acid (E) and casein (F). Pink fluorescence emission (A) and haloes around the streak (B, C, D, E and F) are indicators of the biotechnologically-interesting activities: "true" lipase, carboxylesterase, cellulase, xylanase, pectinase and protease, respectively.

3.4.1.1 Morphological analysis

Strain CR-179 produced white, round (diameter: 0.5 cm) but non-regular, crooked colonies with mucous material inside and covered by an external membranous coat. Gram (Figure C2.7A−B), spore and capsule (Figure C2.7C) stains, as well as scanning (Figure C2.7F) and transmission electron microscopy (Figure C2.7D−E) allowed us to observe Gram-positive rods with some pleomorphism. In general, these rods were 0.3−0.7 μm wide and 0.5−10 μm long, and were found singly or producing linear or branched chains. These chains were frequently bound to an abundant mucous matrix that retained the dyes used, mainly safranine. However, this matrix could not be distinguished by capsule straining, since only the cells and a tight area around them could be observed. Moreover, CR-179 showed also oval paracentrally endospores inside slightly swollen sporangia (Figure C2.7E).

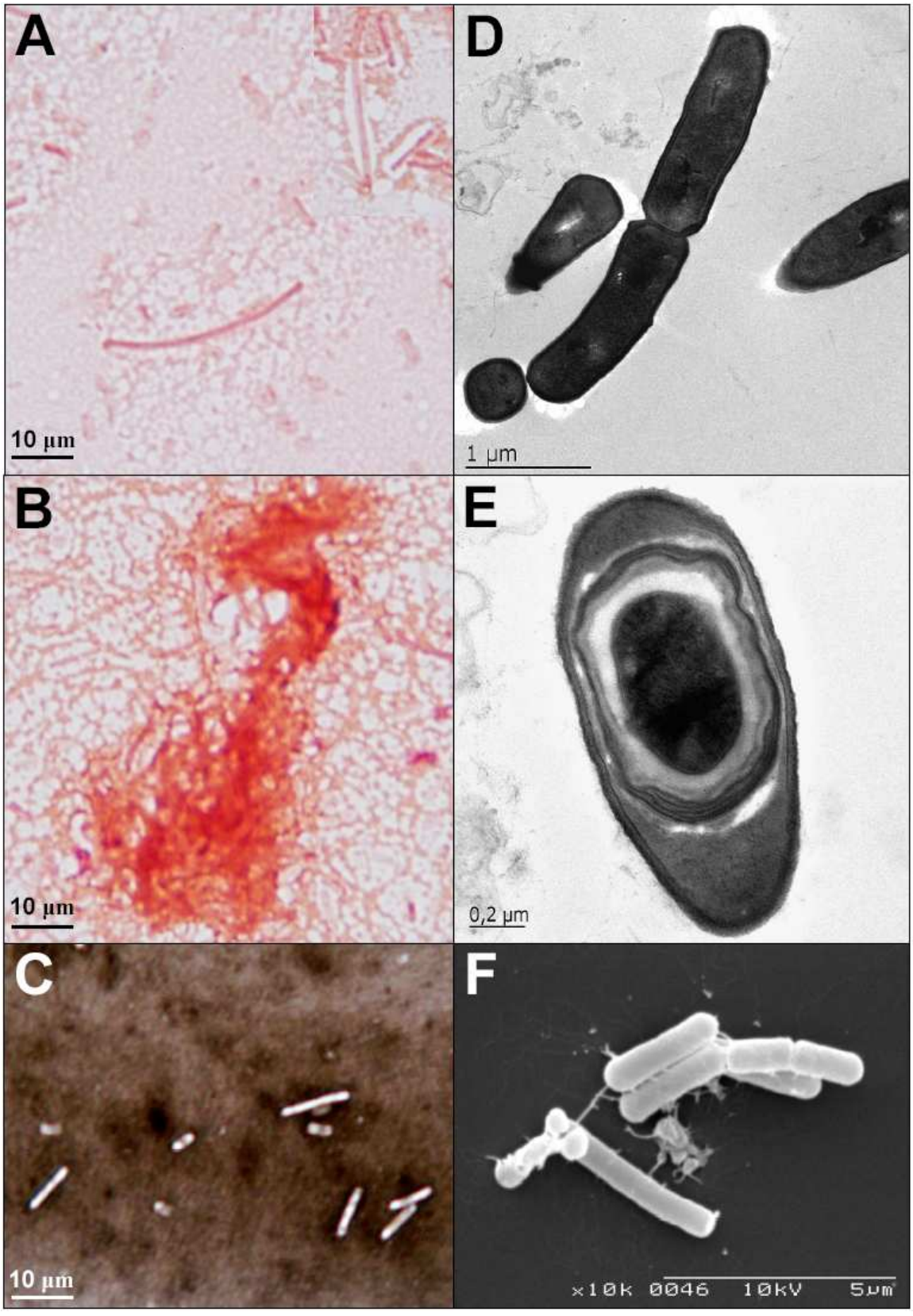

Figure C2.7 Microscopic analysis of strain CR-179 morphological properties.

Microphotographs of strain CR-179 obtained by optical (A and B: Gram stain; C: capsule stain), transmission (D, E) and scanning (F) microscopy. A certain degree of pleomorphism (A, C, D and F) can be observed. This strain produces an abundant mucous matrix that can be found around the cells (A), binding several cells (F), or accumulated in large aggregates (B). Details of cell division (D) and a spore inside a cell (E) are also shown.

3.4.1.2 Physiological, biochemical and molecular analysis

CR-179 was able to grow at 30 ºC and 42 ºC. It grew at 42 ºC on LB and Nutrient agar plates in the pH range from 4.8 to 10.3, but it was unable to grow on minimal media supplemented with olive oil, triolein or tributyrin. This strain was positive/positive in the oxidation/fermentation of glucose assay (facultative anaerobic), and it was positive for motility, catalase, β-galactosidase, gelatinase, nitrate reduction, oxidase, and Voges–Proskauer tests. Furthermore, it was very active in the degradation of casein (Figure C2.6F), indicating the presence of proteases among its hydrolytic enzymes. The strain was negative for the tests of methyl red, arginine dehydrolase, lysine and ornitine decarboxylase, urease, tryptophan deaminase, indole production, citrate utilization, hydrogen production from H_2S and growth on MacConkey medium.

Carbohydrate degradation at 30 ºC for 48 h was positive for D-arabinose, D-fructose, D-glucose, D-mannose, mannitol, sorbitol, amygdalin, esculin, cellobiose, and saccharose. It was negative for 2-keto gluconate, 5-keto gluconate, adonitol, arbutin, D-arabitol, D-fucose, D-lyxose, D-raffinose, D-tagatose, D-turanose, D-xylose, dulcitol, erythritol, galactose, glycerol, gluconate, glycogen, inositol, inulin, L-arabinose, L-arabitol, L-fucose, L-sorbose, L-xylose, lactose, maltose, melizitose, melobiose, N-acetyl glucosamine, rhamnose, ribose, salicylate, starch, trehalose, xylitol, α-methyl-D-mannoside, α-methyl-D-glucoside, β-gentiobiose and β-methyl xyloside (Table C2.6).

The results obtained through the morphological, physiological and biochemical tests assigned the strain CR-179 to the phenotypic group II of the genus *Bacillus* (*B. subtilis* group; Priest, 1993), although no complete match in carbohydrate degradation was obtained with any previously described *Bacillus* species. The closest species were *B. megaterium*, *B. lentus*, *Gb. stearothermophilus*, *B. subtilis* and *B. pumilus*, which differed from strain CR-179 in the degradation of 1 (D-arabinose), 2 (D-arabinose and sorbitol), 2 (D-arabinose and maltose), 3 (D-arabinose, ribose and maltose) and 3 (D-arabinose, arbutin and salicylate) carbohydrates, respectively (Table C2.6).

Table C2.6. Carbohydrate degradation of strain CR-179 and other *Bacillus*-related species showing a similar profile.

Carbohydrate	CR-179	*B. subtilis*	*B.mega*[*]	*B. lentus* 1	*B. pumilus*	*Gb. stearo*[†]
2-keto gluconate	–	–	–	–	–	–
5-keto gluconate	–	–	–	–	–	+ (6%)
Adonitol	–	+ (10%)	–	–	–	–
Amygdalin	+	+ (73%)	+ (50%)	+ (10%)	+ (86%)	+ (31%)
Arbutin	–	+ (84%)	+ (75%)	+ (5%)	+ (100%)	+ (31%)
Cellobiose	+	+ (89%)	+ (80%)	+ (10%)	+ (95%)	+ (31%)
D-Arabinose	+	+ (94%)	–	–	–	–
D-Arabitol	–	–	+ (15%)	–	–	+ (12%)
D-Fructose	+	+ (94%)	+ (83%)	+ (70%)	+ (100%)	+ (100%)
D-Fucose	–	–	–	–	–	–
D-Glucose	+	+ (100%)	+ (83%)	+ (70%)	+ (100%)	+ (100%)
D-Lyxose	–	–	–	–	–	–
D-Mannose	+	+ (94%)	+ (30%)	+ (80%)	+ (100%)	+ (87%)
D-Raffinose	–	+ (78%)	+ (83%)	+ (5%)	+ (18%)	+ (62%)
D-Tagatose	–	+ (5%)	–	–	+ (90%)	+ (6%)
D-Turanose	–	+ (68%)	+ (55%)	–	+ (59%)	+ (62%)
Dulcitol	–	+ (5%)	–	–	–	–
D-Xylose	–	+ (5%)	+ (83%)	+ (25%)	+ (72%)	+ (6%)
Erythritol	–	–	–	–	–	–
Esculin	+	+ (100%)	+ (94%)	+ (100%)	+ (100%)	+ (50%)
Galactose	–	+ (47%)	+ (70%)	–	+ (86%)	+ (50%)
Gluconate	–	–	–	–	–	–
Glycerol	–	+ (95%)	+ (85%)	–	+ (85%)	+ (62%)
Glycogen	–	+ (68%)	+ (83%)	–	+ (9%)	+ (50%)
Inositol	–	+ (94%)	+ (44%)	–	–	+ (6%)
Inulin	–	+ (84%)	+ (75%)	+ (5%)	+ (9%)	+ (5%)
Lactose	–	+ (15%)	+ (80%)	+ (60%)	+ (22%)	+ (5%)
L-Arabinose	–	+ (100%)	+ (83%)	+ (25%)	+ (90%)	+ (25%)
L-Arabitol	–	–	–	–	–	–
L-Fucose	–	–	–	–	–	+ (12%)
L-Sorbose	–	+ (10%)	–	–	+ (5%)	+ (25%)
L-Xylose	–	+ (5%)	–	–	–	–
Maltose	–	+ (100%)	+ (83%)	+ (60%)	+ (77%)	+ (100%)
Mannitol	+	+ (94%)	+ (83%)	+ (10%)	+ (95%)	+ (31%)
Melezitose	–	+ (5%)	+ (70%)	+ (5%)	+ (5%)	+ (50%)
Melibiose	–	+ (78%)	+ (83%)	–	+ (18%)	+ (56%)
N-Acetyl glucosamine	–	+ (21%)	+ (85%)	+ (80%)	+ (86%)	+ (5%)
Rhamnose	–	+ (26%)	–	+ (5%)	+ (1%)	–
Ribose	–	+ (73%)	+ (77%)	+ (40%)	+ (95%)	+ (31%)
Saccharose	+	+ (94%)	+ (83%)	+ (50%)	+ (100%)	+ (100%)
Salicylate	–	+ (78%)	+ (75%)	+ (25%)	+ (100%)	+ (31%)
Sorbitol	+	+ (99%)	+ (66%)	–	+ (4%)	+ (18%)
Starch	–	+ (84%)	+ (83%)	+ (5%)	+ (9%)	+ (62%)
Trehalose	–	+ (94%)	+ (83%)	+ (40%)	+ (99%)	+ (75%)
Xylitol	–	+ (5%)	–	–	–	+ (6%)
α Methyl-D-glucoside	–	+ (94%)	+ (30%)	–	+ (72%)	+ (75%)
α Methyl-D-mannoside	–	+ (1%)	–	–	+ (63%)	+ (6%)
β-Gentiobiose	–	+ (52%)	+ (55%)	–	+ (81%)	+ (12%)
β-Methyl xyloside	–	+ (5%)	–	–	+ (1%)	–

[*]*B. megaterium*; [†]*Gb. stearothermophilus*. The percentage of positive strains is in brackets.

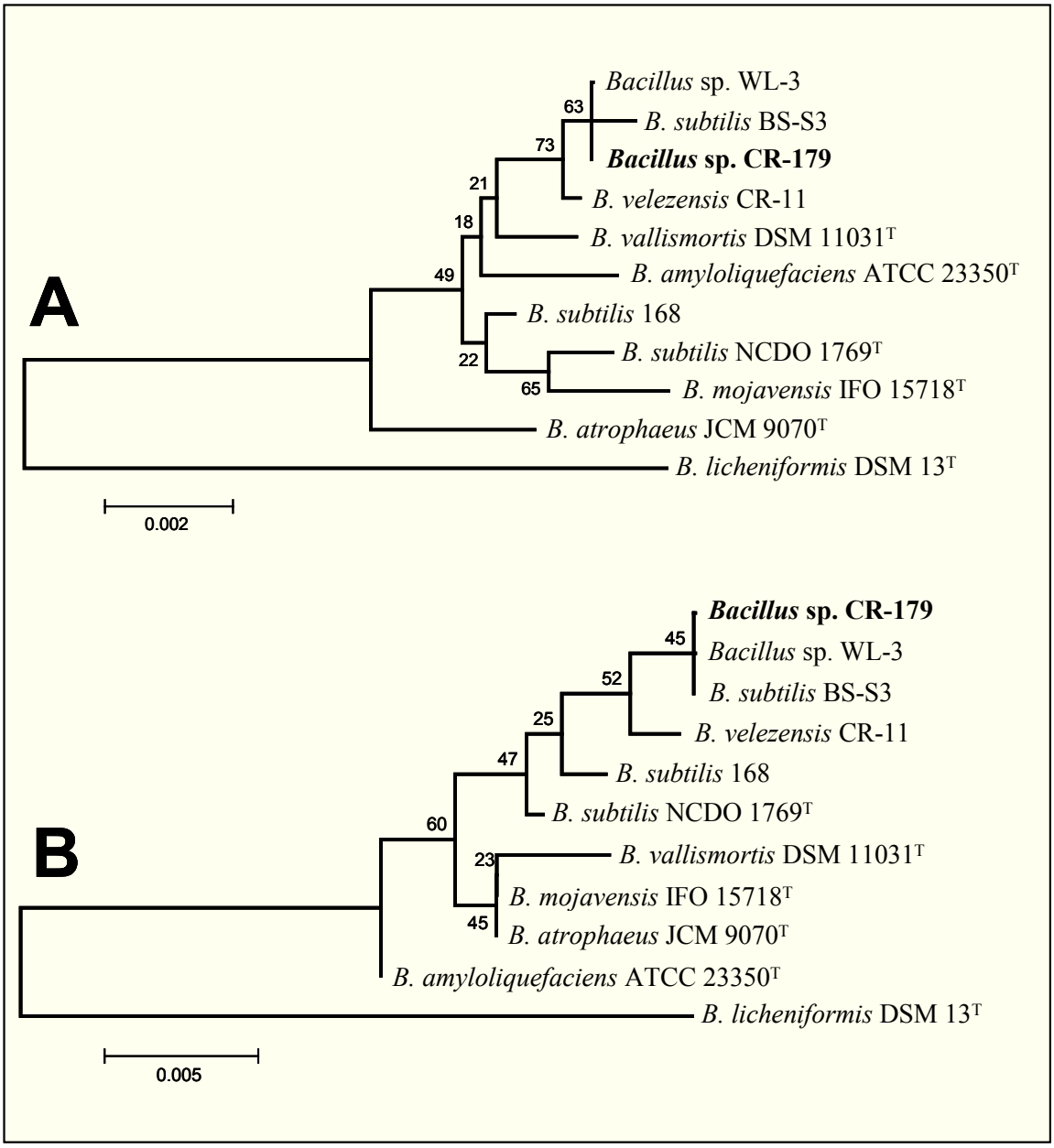

Figure C2.8 Phylogenetic trees based on the 16S rDNA sequence of strain CR-179.

Phylogenetic trees based on whole 16S rDNA sequences (A) and the hypervariable regions (HV) of the 16S rDNA sequences (B) of strain CR-179 and its closest strains and *Bacillus* type strain species. The bar indicates 0.2% (A) and 0.5% (B) estimated difference in nucleotide sequences. Numbers on the branches are bootstrap values. The accession numbers of the 16S rDNA sequences used to perform the phylogenetic trees are the following: *Bacillus* sp. WL-3 (AY601723), *B. subtilis* BS-S3 (AY583216), *B. subtilis* 168 (Z99104), *B. subtilis* NCDO 1769^T (X60646), *B. amyloliquefaciens* ATCC 23350^T (X60605), *B. vallismortis* DSM 11031^T (AB021198), *B. licheniformis* DSM 13^T (X68416), *B. mojavensis* IFO 15718^T (AB021191), *B. atrophaeus* JCM 9070^T (AB021181) and *B. velezensis* CR-11 (AY605932).

Therefore, traditional identification methods were followed by 16S rDNA sequence analysis. The nucleotide sequence of 1453 bp obtained displayed 100% identity to the 16S rDNA gene of *Bacillus* sp. WL-3, and 98−99% identity to the 16S rDNA of other strains belonging to the *Bacillus* rRNA group I (Ash *et al*., 1991). The closest type strain species (99% identity) were *B. vallismortis* DSM 11031^T, *B. amyloliquefaciens* ATCC 23350^T and *B. subtilis* NCDO 1769^T. The proximity of the isolate CR-179 to the mentioned strains was confirmed by the phylogenetic tree obtained by comparison of its 16S rDNA sequence to those of the closest *Bacillus* strains and type strain species (Figure C2.8A).

As stated by Goto *et al*. (2000), species of the genus *Bacillus* can be better classified using only the hypervariable (HV) region of the 16S rDNA (275 bp) because this region emphasizes better the phylogenetic distances. The 16S rDNA HV region of strain CR-179 showed 100% identity to those of *Bacillus* sp. WL-3 and *B. subtilis* BS-S3, and 99% identity to those of *B. subtilis* 168 and *B. subtilis* NCDO 1769^T. The phylogenetic tree obtained by comparison of the 16S rDNA HV regions of strain CR-179 and the closest *Bacillus* strains and type strain species confirmed *B. subtilis* NCDO 1769^T as the closest type strain species (Figure C2.8B).

The morphological, physiological, biochemical and 16S rDNA analyses performed did not allow us to definitively assign strain CR-179 to any known *Bacillus* species, although we could determine that it is closely related to *B. subtilis*. Nevertheless, further analyses are necessary to determine if this strain can be assigned to this species or corresponds to a new species. Thus, the strain was named *Bacillus* sp. CR-179.

3.4.2 Analysis of CR-179 lipolytic system

Bacillus sp. CR-179 showed a high activity on olive oil, triolein (not shown) and tributyrin (Figure C2.6A−B). Moreover, this strain was the most active isolate on MUF-butyrate (cell extracts: 1418.8 ± 7.5 mU mg^{-1} protein, and 6.9 ± 3.6·10^{-2} mU ml^{-1}; supernatants: 7.6 ± 0.1 mU ml^{-1} culture), and one of the most actives on MUF-oleate (cell extracts: 4.7 ± 6.8·10^{-2} mU mg^{-1} protein, and 2.0·10^{-2} ± 2.9·10^{-4} mU ml^{-1};

supernatants: $3.0 \cdot 10^{-3} \pm 2.9 \cdot 10^{-4}$ mU ml^{-1} culture) in the fluorimetric quantification assays (Table C2.5). Thus, the lipolytic system of this strain was studied in more detail by means of zymogram analysis and PCR detection of lipase-coding genes.

3.4.2.1 Zymogram analysis

The lipolytic activity of 50-fold concentrated crude cell extracts and supernatants from strain CR-179 was analyzed by SDS-PAGE and IEF followed by zymogram analysis on MUF-derivative substrates. The supernatants and cell extracts from this strain did not show activity bands on MUF-oleate, whereas two activity bands of ca. 52 and 33 kDa were detected in cell extracts separated by SDS-PAGE (Figure C2.9) when MUF-butyrate was used as substrate. However, no activity on this substrate could be detected in cell extracts separated by IEF or in the strain supernatants.

These results suggest that the lipolytic system of *Bacillus* sp. CR-179 includes at least one or two cell-bound lipolytic enzymes of 52 and 33 kDa, probably two carboxylesterases since they showed preference for short-chain substrates.

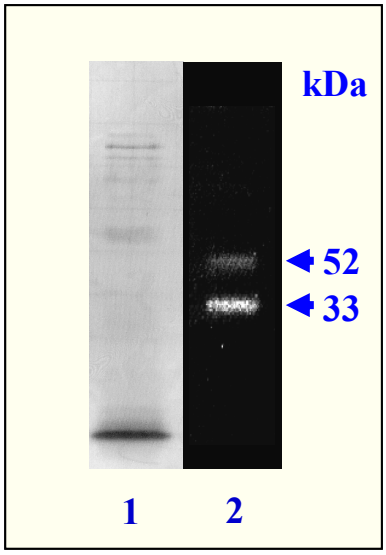

Figure C2.9 Zymogram analysis of *Bacillus* sp. CR-179 crude cell extracts.

The activity on MUF-butyrate of *Bacillus* sp. CR-179 crude cell extracts was analyzed by zymogram after SDS-PAGE separation. Although the Coomassie stain (1) shows that the cell extracts used were poorly concentrated, two activity bands could be observed on the zymogram (2).

3.4.2.2 PCR detection of lipase-coding genes

The lipolytic activity of strain CR-179, and its assignation to the genus *Bacillus*, led us to perform PCR experiments using the consensus primers for the central region of lipase-coding genes related to *B. subtilis lipA* (FWSUB/BKSUB) and *Gb.*

thermocatenulatus lipase gene (FWTHER/BKTHER) described in Chapter 1 in order to amplify CR-179 lipolytic enzymes. FWTHER/BKTHER-amplification produced no PCR bands, whereas FWSUB/BKSUB-amplification produced several bands with sizes ranging from 850 to 271 bp (not shown). Sequencing of these bands revealed that the band of 271 bp corresponded to a lipase-coding gene fragment showing 73% identity to *B. subtilis lipA* (Dartois *et al*., 1992) and 65% identity to *B. subtilis lipB* (Yamamoto *et al*., 1996; Eggert *et al*., 2000), whereas the other bands corresponded to unspecific amplifications. The deduced amino acid sequence of this band (Figure C2.10) showed 66.6% identity to *B. subtilis* LipA and 23.3% identity to *B. subtilis* LipB, and corresponded to the LipA amino acid positions from 111 to 201. However, the complete sequence of this gene could not be obtained using specific primers for *B. subtilis lipA*.

```
2      GCC AAC ACG CTC TAT TAC ATT AAA AAT CTT GAC GGC GGA GAC AAA   46
1       A   N   T   L   Y   Y   I   K   N   L   D   G   G   D   K   15

47     ATC GCC AAC GTC GTG ACA CTC GGC GGA GCG AAC GGC CTT GTG ACC   91
16      I   A   N   V   V   T   L   G   G   A   N   G   L   V   T   30

92     AAC CGG GCG CTT CCG GGG ACT GAT CCG AAC CAA AAG ATT TTA TAT   136
31      N   R   A   L   P   G   T   D   P   N   Q   K   I   L   Y   45

137    ACA TCG ATT TAC AGC TCA GCG GAC TTG ATC GTT CTG AAC CCG CTG   181
46      T   S   I   Y   S   S   A   D   L   I   V   L   N   P   L   60

182    TCA CGA CTG ATC GGC GGG AAA AAC GTG CAG ATT CAC GGT GTC GGA   226
61      S   R   L   I   G   G   K   N   V   Q   I   H   G   V   G   75

227    CAC ATC GGA CTT TTG ATG AAC AGC CAA GTA AAC GGG CTG ATT AAA   271
76      H   I   G   L   L   M   N   S   Q   V   N   G   L   I   K
```

Figure C2.10 Nucleotide and amino acid sequence of the lipase-coding gene fragment from *Bacillus* sp. CR-179 obtained by using the primers FWSUB/BWSUB.

A gene fragment of 271 bp from *Bacillus* sp. CR-179 was obtained by amplification using consensus primers for the central region of *B. subtilis lipA*-related lipases (FWSUB/BKSUB). The deduced amino acid sequence (90 residues) from this partial nucleotide sequence showed 66.6% identity to *B. subtilis* LipA at the amino acid positions from 111 to 201. Thus, comparative alignments with *B. subtilis* LipA allowed us to identify Asp^{53} and His^{76} (in red) as two putative members of the catalytic triad of this lipase, whereas the catalytic serine would be located 5 residues upstream of the amino acid sequence obtained.

When amplification using *B. subtilis lipB* specific primers (FWYfiP/BWYfiP) was performed, a band of 650 bp (not shown) was obtained. Surprisingly, sequencing of this band showed that *Bacillus* sp. CR-179 bears a lipase-coding gene 100% identical to *B. subtilis lipB* (Eggert *et al*., 2000) and *Bacillus* sp. BP-6 *lipA* (Chapter 1), and 99% identical to *B. megaterium lipA* (Chapter 1).

3.5 ANALYSIS OF STRAIN CR-53

3.5.1 Preliminary bacterial identification

Strain CR-53 was isolated from the soil sample no. 1 not treated at 80 ºC and incubated on LB plates (pH 7) for 4 days at 20 ºC. This strain showed a singular colonial and cellular morphology and produced high degradation of olive oil, triolein and tributyrin (Table C2.3; Figure C2.11), whereas it showed no activity on polysaccharides with the exception of carboxymethyl cellulose (Table C2.3). Moreover, this isolate was very active on MUF-oleate and moderately active on MUF-butyrate (Table C2.5). Therefore, it was also chosen for further characterization.

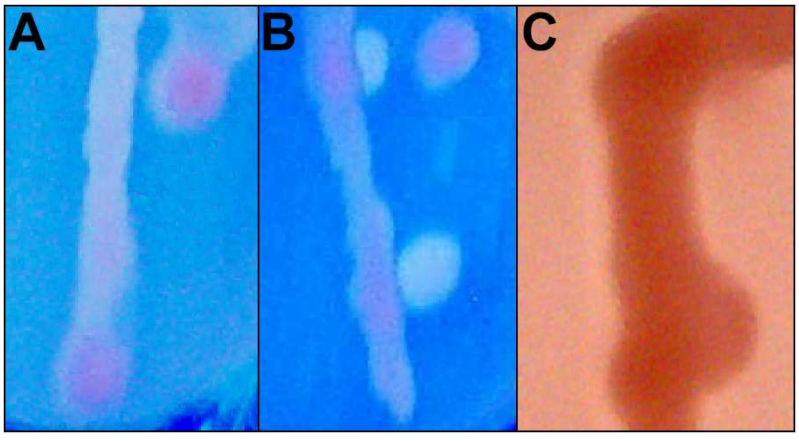

Figure C2.11 Activity of strain CR-53 lipid substrates.

Hydrolytic activity of CR-53 growing on agar plates supplemented with olive oil (A), triolein (B) and tributyrin (C). Pink fluorescence emission (A−B) and haloes around the streak (C) can be observed.

The results of the morphological, physiological, biochemical and molecular test performed on this strain according to the microbiological tests recommended for Gram-positive bacteria (Gordon *et al.*, 1973) and actinomycetes (Goodfellow *et al*., 1998) are detailed in the following pages.

3.5.1.1 Morphological analysis

Strain CR-53 produced pale-yellow white, round (diameter: 0.5 mm), creamy and very mucoid colonies that tended to spread on the plates when they were supplemented with lipid substrates (see Figure C2.11).

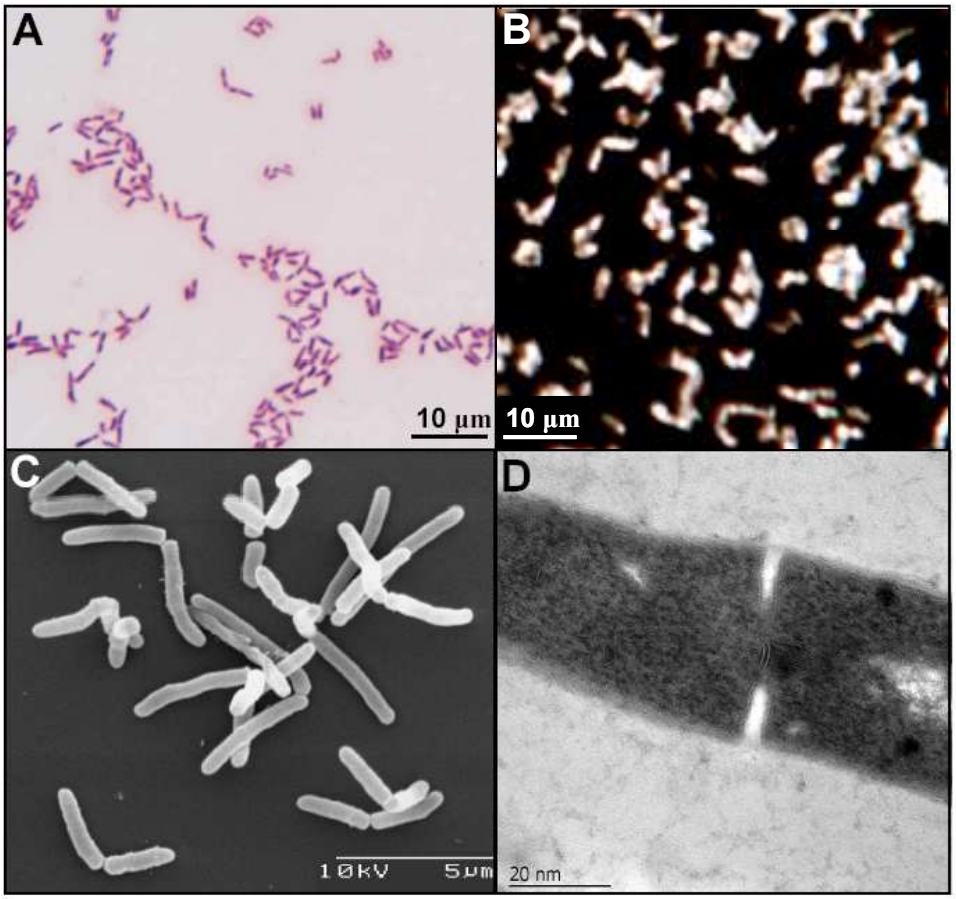

Figure C2.12 Cellular morphology of strain CR-53.

Microphotographs of strain CR-53 obtained by optical (A: Gram stain; B: capsule stain), scanning (C) and transmission (D) microscopy. Snapping division (C, D) and septum formation (D) can be observed.

After specific Gram, acid-fast and spore stains, strain CR-53 was identified as a non-sporulated, acid-fast negative, elementary-branching Gram-positive rod, although some coccoid forms were also observed. The rods were 0.2−0.5 μm wide and 2−5 μm long, and showed a certain degree of snapping division (Figure C2.12A−B), a frequent feature found among actinomycetes (Goodfellow *et al*., 1998). This morphology was confirmed by both, scanning and transmission electron microscopy, where the snapping division of the strain was clearly demonstrated (Figure C2.12C−D).

3.5.1.2 Physiological, biochemical and molecular analysis

Strain CR-53 was non-motile, catalase and urease positive, sensitive to lysozyme, and was negative for alcohol dehydrogenase, arylsulfatase, citrate, gas production from H_2S, gelatinase, indole, lysine and ornitine decarboxylase and Voges–Proskauer tests.

Carbohydrate degradation at 30 ºC for 48 h was positive D-arabitol,D-glucose, D-fructose, esculin, glycerol, inositol, mannitol, sorbitol, trehalose,and. It was negative for 2-keto gluconate, 5-keto gluconate, adonitol, amygdalin, arbutin, for cellobiose, D-arabinose, D-mannose, D-fucose, D-lyxose, D-raffinose, D-tagatose, D-turanose, D-xylose, dulcitol, erythritol, galactose, gluconate, glycogen, inulin, L-arabinose, L-arabitol, L-fucose, L-sorbose, L-xylose, lactose, maltose, melizitose, melobiose, N-acetyl glucosamine, rhamnose, ribose, saccharose, salicylate, starch, xylitol, α-methyl-D-mannoside, α-methyl-D-glucoside, β-gentiobiose and β-methyl xyloside.

The results obtained through these tests assigned the strain to the genus *Rhodococcus* (Tsukamura, 1974; Goodfellow & Alderson, 1977). However, no complete match in carbohydrate degradation was obtained with any previously defined *Rhodococcus* type strain species (Goodfellow *et al*., 1998). Table C2.7 shows the most relevant results obtained from sugar utilization of strain CR-53 in comparison with those found in close *Rhodococcus* type strain species. Among them, *R. erythropolis* ATCC 4277^T showed the highest similarity to strain CR-53, being only different for maltose and saccharose degradation.

Table C2.7. Characteristics that distinguish strain CR-53 from close *Rhodococcus* type strain species.

Carbohydrate	CR-53 - EB/R/C	*Rhodococcus erythropolis* ATCC 4277^T EB/R/C	*Rhodococcus marinonascens* DSM 43752^T H/R/C	*Rhodococcus rhodnii* DSM 43336^T EB/R/C	*Rhodococcus ruber* DSM 43338^T H/R/C	*Rhodococcus rhodochrous* DSM 43241^T EB/R/C
D-Glucose	+	+	+	+	+	−
D-Fructose	+	+	+	−	+	+
Esculin	+	+	+	−	−	+
Galactose	−	−	−	/	/	−
Glycerol	+	+	+	−	+	+
Inositol	+	+	+	−	−	−
Maltose	−	+	−	−	+	+
Mannitol	+	+	−	+	+	+
Rhamnose	−	−	−	−	−	−
Ribose	±	+	−	−	−	−
Saccharose	−	+	−	+	+	+
Sorbitol	+	+	+	+	+	+
Trehalose	+	+	−	−	+	+
β-methyl xyloside	−	−	w	w	−	−

Simbols: +: positive; −: negative; /: not determined; ±: variable; w: weakly positive; EB-R-C: elementary branching-rod-coccus growth cycle; H-R-C: hypha-rod-coccus growth cycle.

As previously stated by other authors, identification of rhodococci to species level is difficult by traditional methods (Goodfellow, 1989). For this reason, microscopic examination and the physiological and biochemical tests were followed by 16S rDNA sequence analysis. The 16S rDNA sequence of 1367 bp obtained from strain CR-53 displayed a high level (99%) of sequence identity to the 16S rDNA genes of several strains described as *R. erythropolis* (Katsivela *et al*., 1999), including the type strain *R. erythropolis* ATCC 4277^T, as well as to the 16S rDNA genes of several

unidentified *Rhodococcus* species. The phylogenetic trees obtained by comparison of the obtained sequence to those of other type strain species of the genus *Rhodococcus* confirmed the proximity of strain CR-53 to *R. erythropolis* (Figure C2.13).

Therefore, the analyses performed on strain CR-53 allowed us to determine that the isolated strain is closely related to *R. erythropolis* ATCC 4277^T and other *R. erythropolis* strains, although further analyses are necessary to determine if this strain can be assigned to this species. Thus, the isolated strain was named *Rhodococcus* sp. CR-53.

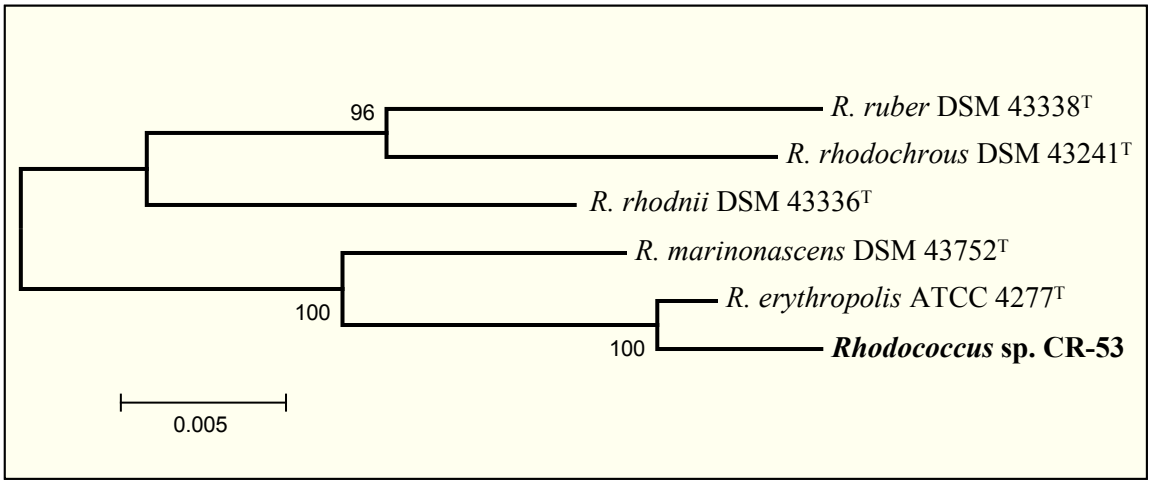

Figure C2.13 Phylogenetic tree based on the 16S rDNA sequence of strain CR-53.

Phylogenetic tree based on the 16S rDNA sequences of strain CR-53 and its closest *Rhodococcus* type strain species. The bar indicates the 0.5% estimated difference in nucleotide sequences. Numbers on the branches are bootstrap values. The accession numbers of the 16S rDNA sequences used to perform the phylogenetic tree are the following: *R. erythropolis* ATCC 4277^T (X81929), *R. marinonascens* DSM 43752^T (X80617), *R. rhodnii* DSM 43336^T (X80621), *R. ruber* DSM 43338^T (X80625) and *R. rhodochrous* DSM 43241^T (X79288).

3.5.2 Analysis of CR-53 lipolytic system

The lipolytic system of *Rhodococcus* sp. CR-53 was analyzed in more detail since this strain was active on olive oil, triolein, tributyrin (Figure C2.11) and MUF-butyrate (cell extracts: 9.7 ± 0.1 mU mg^{-1} protein, and 0.3 ± 3.0·10^{-3} mU ml^{-1}; supernatants: 1.1 ± 1.0·10^{-2} mU ml^{-1} culture; Table C2.5), and was one of the most

active isolates on MUF-oleate (cell extracts: $1.2 \pm 1.1 \cdot 10^{-2}$ mU mg^{-1} protein, and $4.0 \cdot 10^{-2} \pm 3.8 \cdot 10^{-3}$ mU ml^{-1}; supernatants: $3.8 \cdot 10^{-2} \pm 3.1 \cdot 10^{-3}$mU ml^{-1} culture; Table C2.5).

The lipolytic activity of 50-fold concentrated crude cell extracts and supernatants from strain CR-53 was analyzed by zymogram on MUF-derivative substrates after SDS-PAGE and IEF separation. The supernatants and cell extracts from this strain did not show any activity on MUF-oleate, whereas a band of ca. 60 kDa was detected in crude cell extracts separated by SDS-PAGE when MUF-butyrate was used as substrate (Figure C2.14). However, no activity on this substrate could be detected in cell extracts separated by IEF or in the strain supernatants.

These results suggest that the lipolytic system of *Rhodococcus* sp. CR-53 includes at least one cell-bound lipolytic enzyme of 60 kDa, probably a carboxylesterase since the enzyme showed preference for short-chain substrates.

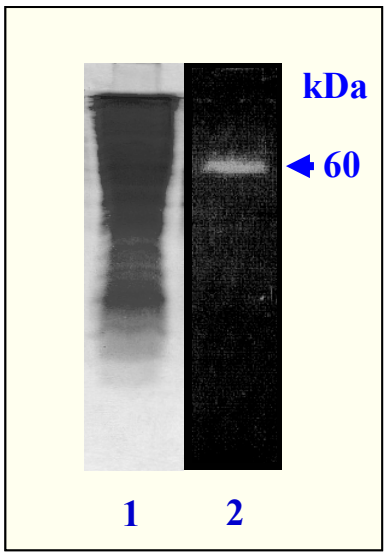

Figure C2.14 Zymographic analysis of crude cell extracts from *Rhodococcus* sp. CR-53.

The activity on MUF-butyrate of *Rhodococcus* sp. CR-53 crude cell extracts was analyzed by zymogram after SDS-PAGE separation. Coomassie stain (1) shows the high protein concentration of the strain cell extract that was necessary to detect the activity band of ca. 60 kDa on the zymogram (2).

4 DISCUSSION

4.1 ISOLATION OF SOIL MICROORGANISMS

Native microorganisms growing in an organic matter-rich environment were isolated, using different culture media and temperature conditions, from three soil samples collected from a subtropical forest of Puerto Iguazú (Argentina). The values of total colony forming units per soil gram (CFU g^{-1}) obtained were $2-6 \cdot 10^5$, 10-fold higher than those corresponding to fungal CFU g^{-1} (Table C2.1). From the isolated colonies considered as different on the basis of their morphology, 724 sporulated and non-sporulated, aerobic and facultative anaerobic, quimioheterotrophic, bacterial and putative fungal strains were obtained in pure culture.

Several limitations concerning the type and number of microorganisms isolated should be taken into account. On the one hand, sampling process and sample preparation can affect the results obtained (Alexander, 1977). For example, extraction and fragmentation of the microcolonies stuck to soil particles is an important factor affecting the quantitative results of an isolation process, and it could explain the lower amount of CFU g^{-1} found in soil no. 1 with respect to soils no. 2 and no. 3. Wood, the main component of soil no. 1, could not be completely disintegrated, which made more difficult the extraction and fragmentation of microcolonies. On the contrary, this problem was clearly reduced in soils no. 2 and no. 3, rich in clay. Moreover, clay is composed of smaller and more abundant particles than other materials, which favours the adhesion of a higher number of microorganisms to its surface. However, the higher diversity in the composition of soils no. 1 and no. 2 with respect to soil no. 1 could also explain the higher CFU g^{-1} values obtained in these two soil samples.

Furthermore, interpretation of quantitative results is even more unaccurated for fungi or actinomycetes since the extraction process can produce a different breaking of hyphae or sporangia, which would lead to significant differences in the number of colonies obtained.

On the other hand, there is not a culture medium or a temperature suitable for all microorganisms, thus five different media and three temperatures were used to reduce this limitation, although it is sure that these soil samples contained other microorganisms which could not grow at the assay conditions used. Moreover, small differences in pH, water content, the presence of a small radix, etc, which are difficult to reproduce in vitro, can produce high changes in the qualitative and quantitative results obtained in an isolation process (Alexander, 1977).

Despite of these limitations, the quantitative results obtained are in agreement with those previously reported for other soils (10^5–10^9 CFU g^{-1} dry weight; Atlas & Bartha, 1998). Moreover, it should be considered that the results of CFU g^{-1} are lower than those obtained for the same samples by microscopy (10^8–10^{10} cells g^{-1}) due to the fact that this method allows also the detection of microorganisms that can not be cultured. Nevertheless, the values of CFU g^{-1} obtained are relatively low considering that the samples were obtained from a subtropical forest soil rich in organic matter. One reason could be the fact that soil samples were stored for 3 months before being analyzed, which would be also in agreement with the high number of endosporulated microorganisms (more resistant) isolated. This fact could also explain the similar results of CFU g^{-1} obtained in the soil samples treated by heat shock at 80 ºC (only endosporulated or highly thermophilic microorganisms are able to grow after such treatment), with respect to those obtained for the untreated samples. Sabouraud-cm media was the only exception since this medium is specific for yeasts and fungi, whose spores and vegetative forms can not resist heat shock treatments at 80 ºC. Anyway, it is clear that many of the microorganisms of the soil samples analyzed were present as endospores and other inactive forms, although it is difficult to say if this phenomenon is typical of the soil analyzed, or if it is a consequence of the storage conditions of the samples and the time passed before the isolation was performed. With respect to this fact, it is known that from 60% to 100% of the soil isolates belonging to the genus *Bacillus* and other genera of the family *Bacillaceae* (the only endosporulated aerobic microorganisms) are found as endospores, which can remain as dormant structures for years, whereas the vegetative forms are only found in a high number under favourable and stable conditions (Priest, 1993).

Several limitations should be also taken into account with respect to the 724 isolates obtained in pure culture. First of all, it is possible that some of them were repeated because the same microorganism can produce a different morphology, pigmentation, etc, under different culture conditions (Alexander, 1977). This fact was confirmed during the screening of the hydrolytic activities, since many isolates showed different morphologies in the different culture media used for the screening. Moreover, the cellular and colonial morphologies of many soil microorganisms change at different stages of growth, or are different from those existing in the original environment (Alexander, 1977). It is also possible that some of the microorganisms isolated were not selected for pure culture due to showing a colonial morphology very similar to that of other strains previously obtained in pure culture.

In addition, some colonies that appeared very close to other colonies in the isolation process could not be obtained in pure culture, probably due to the fact that many soil bacteria require vitamins and other substances produced by other microorganism of their environment.

4.2 SCREENING OF THE ENZYMATIC ACTIVITIES OF THE ISOLATED STRAINS

All 724 isolated strains obtained in pure culture were analyzed to detect five biotechnologically-interesting enzymatic activities: “true” lipase, carboxylesterase, xylanase, cellulase and pectinase. Among the isolates, 43 showed all the activities and have, therefore, a high biotechnological potential, whereas 449 isolates showed one or more of these activities (Table C2.2). Almost no differences were found for the isolation conditions with respect to the type of hydrolytic activities or the relative number of active strains obtained.

When the hydrolytic strains were analyzed by Gram or fungal stain, most of them appeared as Gram-positive rods. However, some limitations concerning the microscopic observation of this isolates should be taken into account. First of all, it is

known that many soil strains display a different size, morphology or Gram-stain depending on if they are in their natural environments or in laboratory cultures, or depending on the culture conditions or if the cultures are fresh or old. For example, many Gram-negatives appear as Gram-positive in old cultures, the mycelia formed by some actinomycetes are disaggregated during Gram stain appearing as isolated bacteria, etc (Alexander, 1977). These facts justify in part the Gram-positive predominance among the hydrolytic isolates, although the most probable reason for this predominance is the high isolation of aerobic endosporulated microorganisms belonging to *Bacillus* and other related genera, as previously explained. These genera, being the most isolated, are also among the highest enzyme-producing (Priest, 1993), which could explain their predominance among the hydrolytic strains.

Studying the different activities separately, many strains were active on polysaccharides: 114 degraded xylan, 116 hydrolyzed carboxymethyl cellulose and 82 degraded PGA (Table C2.2), as is common in soils rich in vegetable biomass (López *et al*., 1998; Soares *et al*., 1999; Semedo *et al*., 2000). The lower amount of PGA-degrading strains could be explained by the fact that cellulose and xylan are more abundant in vegetable biomass than PGA or other pectin-derived polysaccharides.

The high number of lipolytic strains obtained with respect to polysaccharide-degrading strains (331 degraded olive oil, and 360 were active on tributyrin plates; Table C2.2) could seem surprising for soil samples rich in wood, dead leaves, etc, unless considering that vegetable polysaccharides are usually bound and covered by a high amount of very complex lipid compounds (named "pitch" in paper industry) such as waxes, phenolic compounds and others. These lipid compounds have to be degraded to obtain the cellulose and the other structural polysaccharides, and in some cases, act by themselves as a carbon and energy source or even as antimicrobial agents. Thus, many microorganisms growing in polysaccharide-rich environments display among their hydrolytic enzymes a wide range of esterases, including lipases, responsible for the degradation of the diverse lipids found in vegetable biomass (Williamson *et al*., 1998; Jaeger *et al*., 1999). In fact, soil strains with lipolytic activity are very frequent. For example, the screening of several thousands of microbial soil strains, including filamentous fungi, yeast and bacteria, has revealed that about 20% of them were lipase-producers (Jaeger & Eggert, 2002). Moreover, presence of lipolytic activity in soil

microorganisms has been correlated to degradation of toxic compounds (Margesin *et al*., 1999). In addition, the high number of lipolytic microorganisms obtained could be related to the use of CeNAN-olive oil plates in the isolation process, although the number of lipolytic microorganisms obtained from the other culture media used in the isolation process was also very high.

Considering the different hydrolytic activities of each isolate (see Table C2.3 for the most active ones), the results obtained indicate that the microbial community of the soil analyzed display a high complexity. Isolates showing the five enzymatic activities tested would be among the main responsible for the degradation of vegetal material, probably acting in cooperation with more specific strains hydrolyzing only defined lipids or polysaccharides, and with other non-isolated hydrolytic strains. All of them possibly perform a synergic degradation of vegetal biomass either by means of a simultaneous activity, or by changes in the predominant populations when microbial activity on simplest substrates produces an accumulation of compounds more difficult to degrade such as lignocellulose. Some of the strains without the enzymatic activities tested (isolated or non-isolated) could collaborate in vegetal material recycling by degrading simplest intermediate molecules produced by the lipid- and polysaccharide-degrading microorganisms, although probably many of them were active on other nutrients, or were present as spores or other inactive forms of resistance. Therefore, all these microorganisms probably constitute a robust community adapted to vegetal biomass degradation and capable of surviving under the presence of toxic compounds or under variable conditions of temperature, water availability, etc, as most natural microbial communities (Smith & Smith, 2001).

Since the aim of the isolation and screening process was to obtain a collection of novel hydrolytic strains whose enzymes could be of biotechnological interest, the 76 strains bearing the highest hydrolytic activities were selected and stored. Those isolates active on polysaccharides are being identified, and their enzymatic activities characterized in our research group. However, the degree of lipolytic activity is more difficult to differentiate by simple plate assays. For this reason, due to the fact that this PhD Thesis is focused on the study of lipolytic enzymes, and because of the enormous interest existing in lipases acting on vegetable compounds in several biotechnological processes (see General Introduction 3.3.3), such as the treatment of vegetable biomass

to liberate phenolic compounds used in flavour production and other processes (Williamson *et al*., 1998), the lipolytic activity of the lipid-degrading strains was subsequently analyzed in more detail to select those more active.

4.3 SELECTION OF THE MOST LIPOLYTIC ISOLATES

The search for the most lipolytic strains started with the selection, among the 76 strains chosen due to their high hydrolytic activity, of the 48 isolates producing the highest activity on olive oil- and/or tributyrin-supplemented plates. The lipolytic activity of these strains was subsequently analyzed by fluorimetric paper assay using MUF-derivative substrates, and those 29 strains producing the highest fluorescence emission in these assays (Table C2.4) were chosen for a further quantification of their lipolytic activity by fluorimetric liquid assay, using MUF-butyrate and MUF-oleate as substrates.

The activities obtained were higher on MUF-butyrate than on MUF-oleate for the 29 strains analyzed (Table C2.5), and they revealed some differences with respect to the results obtained by plate assays. The reason for these differences could be the lack of tributyrin or olive oil in the cultures used for the preparation of the cell extracts and supernatants analyzed. It is known that these lipids can modulate the expression of lipolytic genes in many microorganisms (see General introduction 3.3.1; Gupta *et al*., 2004), which would explain that the strains more active on plate assays were not the most active in the fluorimetric assays. However, it should be considered that no significant differences were found for the same strains when fluorimetric paper assays were performed using cell suspensions of streaks grown on LB plates or on lipid-supplemented CeNAN plates. Another reason for the differences observed could be the fact that the preparation of cell extracts releases the intracellular content, which is very rich in carboxylesterases even in secretory microorganisms. These intracellular carboxylesterases, whose activity is difficult to detect by plate assays, can easily be

detected by fluorimetric assay, and could be also responsible for the higher activity found on MUF-butyrate with respect to the activity found on MUF-oleate.

Among the strains analyzed, CR-179 was the most active in general terms since it was the most active on MUF-butyrate and one of the most active on MUF-oleate. Strains CR-105, CR-290, CR-611 and the putative fungal strains CR-31 and CR-563 were also very active on both MUF-butyrate and MUF-oleate, whereas other strains produced a high activity on MUF-butyrate (CR-203, CR-445, CR-522) or on MUF-oleate (CR-53 and CR-337) (Table C2.5). Thus, these 10 strains were selected as the most interesting ones with respect to lipolytic activity. Moreover, some of them produced also a high degradation of the polysaccharides analyzed (CR-179, CR-203, CR-290 and CR-522) or showed singular colonial and cellular morphologies (CR-53 and CR-179), which increased their interest. In addition, the fungal strains CR-31 and CR-563 were also very interesting due to fungal lipases are usually different from bacterial lipases with respect to their specificity, enantioselectivity, etc, and because they are widely used in food industry, as many fungi are considered as GRAS (generally regarded as safe) microorganisms (Jaeger *et al*., 1994; Benjamin & Pandey, 1998).

Therefore, these highly lipid-degrading bacterial and fungal strains and their lipolytic systems are being (or will be) identified and characterized in our laboratory. Among them, the strains CR-179 and CR-53 have been preliminary identified, and their lipolytic systems analyzed in this work.

4.4 ANALYSIS OF STRAIN CR-179

4.4.1 Preliminary bacterial identification

Strain CR-179 was isolated from soil sample no. 2, treated for 10 min at 80 ºC, and incubated on Horikoshi-I plates for 24 h at 42 ºC. This strain was the first strain characterized due to producing high degradation on the lipids and polysaccharides analyzed (Table C2.3; Figure C2.6), because it was the most active strain on MUF-

butyrate and also very active on MUF-oleate (Table C2.5), and because of its singular colonial and cellular morphology.

The morphological, physiological, biochemical and 16S rDNA analyses performed allowed us to identify it as a member of the phenotypic group II (*B. subtilis* group; Priest, 1993) and rRNA group I (Ash *et al*., 1991) of the genus *Bacillus*. The strain could not be assigned to any known *Bacillus* species although it is closely related to *B. subtilis* and other similar *Bacillus* species and strains. However, further analyses are necessary to determine if this strain can be assigned to this species or corresponds to a new species. In fact, wild isolates closely related to *B. subtilis* are phenotypically extremely similar, although they fall into several distinct groups based on protein-coding gene sequences and sexual isolation (Cohan *et al*., 1991; Roberts & Cohan, 1995). Two of these groups have been shown to represent the previously undiscovered species *B. mojavensis* (Roberts *et al*., 1994) and *B. vallismortis* (Roberts *et al*., 1996). The third group is *B*. *subtilis*, although this group has been recently divided on the basis of DNA relatedness, cell wall composition and partial sexual isolation into two subspecies, *B. subtilis* subsp. *subtilis*, that includes the type strain of *B. subtilis* NCDO 1769^T and those strains closely related to *B. subtilis* 168, and *B. subtilis* subsp. *spizizenii*, which includes the strains closely related to *B. subtilis* W23. Therefore, the strain remains named as *Bacillus* sp. CR-179, up to present.

Bacillus species are aerobic to facultative anaerobic, Gram-positive, endospore-forming, rod-shaped bacteria commonly found in soils, water sources or associated with plants (Priest, 1993), and frequently isolated by heat shock. They are an important source of industrial enzymes because they are well-known, most of them are considered GRAS microorganisms, and due to producing and secreting large amounts of homologous or heterologous enzymes, including lipases, xylanases, cellulases, pectinases, etc (Ferrari, 1993; see General Introduction 3.3.5). For this reason, there is an elevated interest in isolating new hydrolytic strains belonging to this genus, such as strain *Bacillus* sp. CR-179 isolated in this work. Moreover, several *Bacillus* lipases showing a high biotechnological potential have been recently described (see General Introduction 3.3.5). For this reason, and due to the high lipolytic activity displayed by *Bacillus* sp. CR-179, the lipolytic system of this strain was analyzed in more detail.

4.4.2 Analysis of CR-179 lipolytic system

Zymogram analysis revealed the production by this strain of two putative cell-bound carboxylesterases (CEs) of 52 and 33 kDa. The presence of a 52 kDa CE in *Bacillus* sp. CR-179 would be in agreement with the presence of cell-bound CEs in most *Bacillus*-related species, including PnbA from *B. subtilis* (Zock *et al*., 1994), EstA from *Paenibacillus* sp. BP-23 (Prim *et al*., 2000) and EstA1 from *Bacillus* sp. BP-7 (Prim *et al*., 2001), all of them showing 50−65 kDa MW and grouped into family VII of bacterial lipases (Arpigny & Jaeger, 1999), and with the presence of putative similar CEs in *B. megaterium* CECT 370 (43 kDa) and *Bacillus* sp. BP-6 (52 kDa) detected in Chapter 1. Moreover, the existence in strain CR-179 of a putative CE of 33 kDa would be also in agreement with the presence of the putative CEs of 27−37 kDa detected in *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 (Chapter 1).

Assignation of strain CR-179 to the genus *Bacillus* allowed us to analyze also the lipolytic system of this strain by PCR assays using primers for the amplification of lipase-coding genes from *Bacillus* and related genera. These experiments revealed the existence in this strain of two additional lipolytic enzymes belonging to subfamily I.4 of bacterial lipases (Arpigny & Jaeger, 1999), one which central region showed 66.6% identity to the amino acid sequence of *B. subtilis* LipA (Dartois *et al*., 1992), and a second enzyme 100% identical to *B. subtilis* LipB (Eggert *et al*., 2000) and *Bacillus* sp. BP-6 LipA (Chapter 1), and 99% identical to *B. megaterium* CECT 370 LipA.

Therefore, *Bacillus* sp. CR-179 lipolytic system involves at least two putative cell-bound CEs, and two secreted lipolytic enzymes. As will be further analyzed in the General Discussion, the presence of such a lipolytic system seems to be a conserved feature among *B. subtilis* and other related strains and species, mainly with respect to *B. subtilis* LipB-related CEs, which display a high conservation degree.

However, additional lipolytic enzymes could exist in *Bacillus* sp. CR-179 in view of its high lipid-degrading activity. In fact, PCR assays revealed the existence of lipolytic enzymes that could not by detected by zymogram analysis under our assay conditions, probably due to the fact that cell extracts and supernatants analyzed were poorly concentrated, or due to the expression of some lipolytic enzymes such as *B.*

subtilis LipB requires the presence of inducing lipids in the culture medium (Eggert *et al*., 2001), due to lipase inactivation by SDS or by some chemical agents present in the SDS-PAGE or IEF gels, or due to the assay conditions (temperature, pH, etc).

Therefore, further assays to perform a complete characterization of the lipolytic system of *Bacillus* sp. CR-179 are in progress. Also, its polysaccharide-degrading system, being one of the most active among all the isolates, will be investigated as far as the high biotechnological potential of the strain is concerned.

4.5 ANALYSIS OF STRAIN CR-53

4.5.1 Preliminary bacterial identification

Strain CR-53 was isolated from soil sample no. 1, not-treated by heat shock and incubated on LB plates for 4 days at 20 ºC. This strain, one of the most active on MUF-oleate, was selected among other microbial isolates because of its lipolytic activity on olive oil, triolein, tributyrin and MUF-derivatives (Table C2.3; Table C2.5; Figure C2.11), and due to its singular morphological properties.

The morphological, physiological, biochemical and 16S rDNA analyses performed allowed us to identify this strain as a member of the genus *Rhodococcus* (Tsukamura, 1974; Goodfellow & Alderson, 1977), closely related to the type strain *R. erythropolis* ATCC 4277^T (Goodfellow *et al*., 2002) and other *R. erythropolis* strains, although some differences in hydrocarbon utilization were found in CR-53 with respect to the type strains mentioned. Thus, further analyses will be necessary to determine if this strain can be assigned to this species. For this reason, the strain remains named as *Rhodococcus* sp. CR-53, up to present.

Rhodococci are aerobic, Gram-positive to Gram-variable, non-motile, catalase-positive actinomycetes of high G+C content capable. Many of them are able to form hyphae, sometimes branched, that fragment into rods and cocci since they are capable of morphological differentiation in response to their environment (Goodfellow *et al*.,

1998). They are widely occurring organisms frequently isolated from soils, as most actinomycetes, which represent from 10 to 30% of the total soil bacteria (Alexander, 1977). In general, actinomycetes are capable to degrade complex polymers, thus playing a relevant role in biomass conversion. The wide range of enzymatic activities they possess (Donadio et al., 2002), including the presence of lipases in members of the genera *Streptomyces*, *Mycobacterium, Propionibacterium* or *Arthrobacter* (Sommer *et al*., 1997; Miskin *et al*., 1997; Johri *et al*., 2001; Vujaklija *et al*., 2002), contribute to increase the capacity of colonization and adaptation of these microorganism to habitats rich in complex nutrients where other microorganisms using simpler substrates can not grow (Kanaly & Harayama, 2001; Murakami *et al*., 2004). Moreover, presence of lipase activity in these microorganisms has been generally correlated to their ability for biocide or toxic compound degradation (Bell *et al*., 1998).

Rhodococci share most of the general properties mentioned for the actinomycetes. In fact, *Rhodococcus* strains are of considerable environmental and biotechnological importance due to their broad metabolic diversity and array of unique enzymatic capabilities (Bell *et al*., 1998). They exhibit the ability to convert a wide range of organic compounds, producing many secondary metabolites and being able to degrade various chemical pollutants such as simple hydrocarbons, aromatic hydrocarbons, nitroaromatics, chlorinated polycyclic aromatics and other recalcitrant or toxic pollutants (Bell *et al*. 1998; Yoon *et al*., 2000) of interest to the pharmaceutical, environmental, chemical and energy sectors (Jones *et al*., 2004). Therefore, there is a high interest in isolating novel hydrolytic strains belonging to this genus, such as *Rhodococcus* sp. CR-53.

However, knowledge about the lipolytic activity of the members of this genus is very low if we consider that, although some *Rhocococcus* strains with lipolytic activity are used in synthesis processes, the serine esterase EstA from *Rhodococcus ruber* is the only lipolytic enzyme from this genus cloned, sequenced and expressed in *E. coli* (Schwab *et al*., 2003). This fact could seem surprising since some *Rhodococcus* species have shown to synthesize triacylglycerols (TAGs) and polyhydroxyalkanoates (PHAs) as storage materials (Wälterman et al., 2000; Kalscheuer et al., 2001). Production of PHAs is a common feature of many prokaryotes, whereas TAGs storage in prokaryotes is an exceptional feature found only in some Rhodococci and other actinomycetes. For

this reason, and in view of the high lipolytic activity of strain CR-53, we analyzed in more detail the lipolytic system of this isolate.

4.5.2 Analysis of CR-53 lipolytic system

Zymogram analysis revealed that the lipolytic system of *Rhodococcus* sp. CR-53 includes at least one cell-bound lipolytic enzyme of 60 kDa, probably a carboxylesterase (CE). However, additional lipolytic enzymes not detected due to our assay conditions (temperature, pH, presence of SDS, low protein concentration of the supernatants, etc), or due to the lack of inducing lipids in the cultures analyzed, could exist in this strain. The size, location and substrate preference of this enzyme are similar to those of the Gram-positive CEs of family VII of bacterial lipases, which includes the previously mentioned non-secreted *Bacillus*-related CEs, and the CEs from the actinomycetes *Arthrobacter oxydans* and *Streptomyces coelicolor* (Arpigny & Jaeger, 1999).

The enzyme described could contribute to the colonization of lipid-rich habitats such as those containing decomposing organic matter or oil-contaminated environments (Bell *et al*., 1998). Moreover, *Rhodococcus* sp. CR-53 or its putative CE could be used for field or biotechnological applications requiring the presence of an esterase, as well as in steroid modification or enantioselective synthesis (Larkin *et al*., 1998). Therefore, further studies are being conducted to perform the cloning and characterization of the enzyme in order to evaluate its biotechnological possibilities.

4.6 FUTURE PERSPECTIVES

The most active strains from the collection of hydrolytic strains obtained in this work are being identified, and their enzymatic activities characterized in our research group in order to evaluate the biotechnological potential of these strains and their

enzymes. For example, the hydrolytic strains CR-203 and CR-611 have been preliminarily identified as *Bacillus* sp. CR-203 (closely related to *B. pumilus*) and as *Pseudomonas* sp. CR-611 (closely related to *P. fluorescens*), and have shown to bear at least one lipolytic enzyme (Prim, 2002). Other strains such as the *B. pumilus*-related strain CR-522 (Falcocchio, S., personal communication), the xylanolytic strain CR-3, preliminarily assigned to the genus *Paenibacillus* (Roncero, B., personal communication), or the fungal strain CR-313, preliminarily identified as a member of the genus *Penicillium* and showing a high cellulolytic activity (Picart, 2004), have been also analyzed.

With respect to the strains studied in this work, *Bacillus* sp. CR-179 and *Rhodococcus* sp. CR-53, further assays are in progress in order to perform the complete identification of these strains. A more exhaustive analysis of the lipolytic system of these strains, using more concentrated cell extracts and supernatants, as well as different culture conditions and separation techniques will be also performed. Furthermore, the isolation and characterization of the lipolytic enzymes from these strains is also in progress, since *Bacillus* and *Rhodococcus* enzymes have an increasing application in biotechnological processes. For this reason, we have a special interest in isolating the lipolytic enzyme detected in strain CR-53, and in achieving the complete sequence of the lipase from strain CR-179 showing 66.6% identity to *B. subtilis* LipA: inverse PCR assays such as those performed in Chapter 1 would be a useful approach.

In addition, studying the polysaccharide-degrading system of *Bacillus* sp. CR-179 and the biotechnological potential of its corresponding enzymes, as well as analyzing the properties of the abundant mucous matrix produced by this strain, would be also very attractive.

5 CONCLUSIONS

I. The native microorganisms from three soil samples collected from a subtropical forest of Puerto Iguazú (Argentina) were isolated using different culture media and temperature conditions, and 724 strains were obtained in pure culture.

II. All 724 isolates were analyzed to detect five biotechnologically-interesting enzymatic activities: "true" lipase, carboxylesterase, cellulase, xylanase, and pectinase. Among them, 449 isolates showed one or more of these activities: 331 were active on olive oil, 360 degraded tributyrin, 114 degraded xylan, 116 hydrolyzed carboxymethyl cellulose and 82 degraded polygalacturonic acid.

III. The hydrolytic strains were analyzed morphologically, and those 76 bearing the highest hydrolytic activities and/or showing singular morphological properties were selected and stored.

IV. The lipolytic activity on MUF-derivative substrates of the 29 selected isolates showing the highest activity on lipid substrates was determined by fluorimetric liquid assay. Strains CR-179 (the most active isolate), CR-31, CR-53, CR-105, CR-203, CR-290, CR-337, CR-445, CR-522, CR-563, and CR-611 were selected as the most lipolytic isolates.

V. Strain CR-179, selected because of its high hydrolytic activity, and due to its singular morphological properties, has been preliminarily identified as a member of phenotypic group II and rRNA group I of the genus *Bacillus*, closely related to *B. subtilis*. Thus it has been named *Bacillus* sp. CR-179.

VI. Zymogram and PCR analyses performed on *Bacillus* sp. CR-179 have revealed that the lipolytic system of this strain involves at least two putative cell-bound carboxylesterases (one 32 kDa, and another of 52 kDa showing the typical features of the family VII of bacterial lipases), and two secreted lipolytic enzymes

belonging to subfamily I.4 of bacterial lipases and showing 66.6% and 100% identity to *B. subtilis* LipA and *B. subtilis* LipB, respectively.

VII. Strain CR-53, selected because of its high lipolytic activity and morphological properties, has been preliminarily identified as a member of genus *Rhodococcus* closely related to *R. erythropolis*. Thus it has been named *Rhodococcus* sp. CR-53.

VIII. Zymogram analysis has revealed that the lipolytic system of *Rhodococcus* sp. CR-53 includes at least one cell-bound carboxylesterase of 60 kDa with the typical features of family VII of bacterial lipases.

6 PUBLICATIONS

The work corresponding to this chapter has been published or submitted for publication in the following scientific articles (see Annex I for the full text):

- **Ruiz, C., Pastor, F.I.J. and Díaz, P.** (2005) Isolation of lipid- and polysaccharide-degrading microorganisms from subtropical forest soil, and analysis of lipolytic strain *Bacillus* sp. CR-179. *Lett. Appl. Microbiol.* 40: 218–22.
- **Falcocchio, S., Ruiz, C., Pastor, F.I.J., Saso, L. and Díaz, P.** (2005) Characterization of a new *Rhodococcus* soil isolate bearing lipolytic activity. *Can. J. Microbiol.* Submitted.