Res. Microbiol. 152 (2001) 83–93

S0923-2508(00)01171-2/FLA

Biodegradation of hydroxylated and methoxylated benzoic, phenylacetic and phenylpropenoic acids present in olive mill wastewaters by two bacterial strains

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Received 3 August 2000; accepted 22 September 2000

Abstract – Two aerobic bacterial strains, a chlorophenol-degrading bacterium characterized in this work as a *Ralstonia* sp. LD35 on the basis of the sequence of the gene encoding for 16S ribosomal RNA, and *Pseudomonas putida* DSM 1868, capable of metabolizing 4-methoxybenzoic acid, were tested for their capacity to degrade monocyclic aromatic acids responsible for the toxicity of olive mill wastewaters (OMWs). Both strains possess interesting and complementary degradation capabilities in resting cell conditions: *Ralstonia* sp. LD35 was found to metabolize 4-hydroxybenzoic, 4-hydroxyphenylacetic, 3,4-dihydroxycinnamic and cinnamic acid, whereas DSM 1868 was capable of metabolizing 4-hydroxy-3-methoxybenzoic, 3,4-dimethoxybenzoic and 4-hydroxy-3,5-dimethoxybenzoic acid, as well as 4-hydroxybenzoic and 4-hydroxyphenylacetic acid. The kinetic parameters describing the growth of the two strains on the same compounds were determined in growing-cell batch conditions, and showed that both strains presented high affinity and high specific growth rates towards all assayed substrates. In addition, the two strains were capable of growing on and extensively biodegrading a mixture of monocyclic aromatic acids commonly found at high concentrations in OMWs, and of growing on a 20% dilution of a natural OMW. All these features make the two strains attractive candidates for the development of a biotechnological process for the biodegradation of aromatic compounds found in OMWs. © 2001 Éditions scientifiques et médicales Elsevier SAS

olive mill wastewaters / monocyclic aromatic compounds / biodegradation / bacteria

1. Introduction

Olive oil production gives rise to a large amount of effluents, usually referred to as olive mill wastewaters (OMWs), which have a high content of organic aliphatic and aromatic compounds [24]; for this reason, OMWs represent a problem of environmental concern in the countries of the Mediterranean area where olive oil is mainly produced. The phenol constituents, in particular, are responsible for the toxicity of these effluents towards bacteria, plants and animals [12, 22]; a significant amount of the so-called 'phenol fraction' is represented by monocyclic aromatic acid compounds, i.e. by hydroxylated and methoxylated benzoic, phenylacetic and 3-phenyl-2-propenoic acids [24].

It is known that the removal of the monocyclic aromatic compounds from OMWs greatly reduces the toxicity of this wastewater [13, 22]. In addition, as these compounds exert inhibitory effects towards methanogenic bacteria [11], a preliminary dephenolization of OMWs makes their treatment in conventional anaerobic digestors more efficient. Research studies on the biological degradation of OMW phenol fraction have been focused so far on the employment of fungi or on the use of oxidizing fungal enzymes [7, 10, 13, 22, 26]. Fungi have also been used for biological pretreatment of the waste before anaerobic digestion [11, 19]. Biological pretreatment based on the use of an uncharacterized aerobic sludge has also been explored [6]. However, no satisfactory results have been reached up to now and therefore the problem of the biological removal of the recalcitrant and toxic phenol fraction from OMWs needs further attention.

In this work, a preliminary study conducted in the perspective of using selected aerobic bacteria in the development of a biotechnological process for biodegradation of monocyclic aromatic acid compounds either occurring in OMWs or present in the

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effluents of anaerobic digestor plants fed with this waste was performed. Two bacterial strains, a *Ralstonia* sp. strain previously isolated from phenol-contaminated industrial wastewaters and taxonomically characterized in this work, and *Pseudomonas putida* DSM 1868, capable of growing on 4-methoxybenzoic acid and of O-demethylating several substituted benzoic acids [8], were tested for their capacity to degrade nine monocyclic aromatic compounds typically present at high concentrations in OMWs. In addition, since good knowledge of the kinetic parameters of microorganisms involved in a biotechnological process is generally required for its development and set-up [3, 18], kinetic parameters describing the growth of the two strains on each of the target compounds are studied. Finally, the capacity of each strain for growth on a synthetic mixture of the nine aromatic compounds and on a natural OMW was determined. To our knowledge, this is the first study in which selected aerobic bacterial strains are studied for their biodegradation capacities towards a large array of OMW monocyclic aromatic components.

2. Materials and methods

2.1. Chemicals

Monocyclic aromatic compounds, including monochlorophenols, as well as the constituents of minimum mineral medium MMM [18] were obtained from Aldrich (Milan, Italy). Constituents of tryptic soy agar (TSA) medium were purchased from Biolife Srl, Milan, Italy. The reagents employed for electrophoresis buffers and DNA manipulations were purchased from Sigma (Milan, Italy). HPLC solvents were purchased from J.T. Baker Italia (Milan, Italy).

2.2. Microorganisms and media

The LD35 strain was isolated from a chlorophenol-contaminated industrial wastewater during a previous work on the selection of microbial strains capable of degrading monochlorophenols; 2-chlorophenol was used as the selective substrate. The strain was found to be capable of using phenol (5.32 mM), 2-chlorophenol (1.56 mM), 3-chlorophenol (0.58 mM) and 4-chlorophenol (0.58 mM) as the sole carbon and energy sources (unpublished results). LD35 characterization was performed in this work for the first time using the API 20NE system (Montalieu Vercieu, France). Its genes encoding for 16S rRNA (16S rDNA) were amplified and sequenced as described in the next section. The aromatic ring cleavage mechanism was studied by growing the strain on MMM agar plates containing benzoic acid (4.09 mM) or 4-hydroxybenzoic acid (3.62 mM) and by using catechol or protocatechuate, respectively, as substrate according to Phillips [23]. LD35 was maintained on MMM supplemented with 2-chlorophenol (1.56 mM). *P. putida* DSM 1868 [8] was grown on MMM supplemented with 4-methoxybenzoic acid (6.57 mM).

2.3. Amplification and sequencing of 16S rDNA

Crude cell lysates were the source of template DNA. They were prepared as follows: after overnight growth on LB plates at 30°C, a single LD35 colony was picked up and suspended in 20 μL of sterile distilled water; the cell suspension was heated at 95°C for 10 min and then cooled to 4°C. Amplified 16S rDNA was obtained following the procedure described previously by Di Cello et al. [15]; 2 μL of the amplification mixture were analyzed by agarose gel (1.2% w/v) electrophoresis in TAE buffer (0.04 M Tris-acetate; 0.001 M EDTA) containing 0.5 μg/mL (w/v) ethidium bromide. Primers 27f (5′-GAGAGTTTGATCCT- GGCTCAG) and 1495r (5′-CTACGGCTACCTTGT- TACGA), which allow amplification of nearly all of the 16S rDNA, were synthesized by standard phosphoramidite chemistry, deprotected, dried and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The name of these and other primers used for DNA sequencing indicates the annealing position of their 3′ end to the *Escherichia coli* 16S rDNA in forward (f) or reverse (r) orientation. Amplified 16S rDNA was purified from the reaction mixture as described by Barberio and Fani [4] and cloned by means of the TOPO-TA cloning® kit (Invitrogen) in a linearized ‘pCR®II-TOPO’ vector. The ligase mixture was used to transform competent cells of the *E. coli* strain TOP10 [*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80 *lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R$^+$ *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] according to the supplier. Sequencing of 16S rDNA was performed by the enzymatic method [25] utilizing an automatic sequencer Stretch 373 (Perkin Elmer, USA). The reactions were performed using five specific primers, namely 27f, 1495r, 575f

(5′-AATTACTGGGCGTAAAG), 1114f (5′-GCAACGAGCCGCAACCC) and 559r (5′-CTTTACGCCCAGTAATT). The annealing temperature (T_a) was 50°C. The 16S rDNA nucleotide sequence obtained was aligned with the most similar ones of the Ribosomal Database Project (RDP) [21] using RDP utilities. The alignment was checked manually and used to construct a phylogenetic tree by the neighbor joining method according to the model of Jukes and Cantor with TREECON 2.2 [27]. The robustness of the inferred trees was evaluated by applying 100 bootstrap resampling. 16S rDNA nucleotide sequence obtained was submitted to GenBank and was assigned the accession number AF251026.

2.4. Resting cell experiments

In order to prepare resting cell experiments, LD35 cells were grown on MMM plus 2-chlorophenol (1.56 mM) and DSM 1868 on MMM plus 4-methoxybenzoic acid (6.57 mM) at 30°C and 150 rpm; after 48 h incubation (corresponding to an absorbance at 600 nm, A_{600}, of about 0.2 for LD35 strain and 0.8 for DSM 1868), cells were harvested by centrifugation (Beckman J2-HS centrifuge Beckman Coulter, USA), washed twice with 50 mM phosphate buffer and resuspended in the same buffer to obtain a suspension having an A_{600} in the range 1.2–1.4. The suspension was dispensed in 30-mL aliquots in 100-mL sterile bottles with Teflon coated screw caps and 50 mg/L (from a 5 g/L aqueous stock solution previously sterilized by filtration on a 0.22 µm filter) of the target compound were added. One-milliliter samples were periodically taken out; they were acidified with 50 µL of 2 M trichloroacetic acid, centrifuged at 14 000 *g* for 10 min and analyzed via HPLC. The substrates assayed (in duplicate) were: 4-hydroxyphenylacetic acid, 2,6-dihydroxybenzoic acid, 3,4-dimethoxybenzoic acid (veratric acid), 4-hydroxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), 3-phenyl-2-propenoic acid (cinnamic acid), 3,4,5-trimethoxybenzoic acid, 3,4-dihydroxycinnamic acid (caffeic acid), and 4-hydroxy-3-methoxybenzoic acid (vanillic acid). Heat-inactivated duplicate controls were prepared by the same procedure. The specific rate of compound uptake, defined as the rate of substrate uptake per unit of biomass, was calculated considering the time necessary to degrade 50% of the target compound and then dividing this value by the A_{600} of the cell suspension employed to set up the experiments.

2.5. Batch kinetic studies

A preliminary study aimed at determining the capacity of the two strains for growing on the target monocyclic aromatic compounds that were metabolized in resting cell conditions was performed. For this purpose, LD35 and DSM 1868 (pregrown on 2-chlorophenol and 4-methoxybenzoic acid, respectively, as reported above) were inoculated (3% v/v) into 100-mL Erlenmeyer flasks containing 30 mL of MMM with 100 mg/L of the target compound, and the bacterial growth in the cultures incubated at 30°C and 150 rpm was carefully followed through A_{600} measurements for 4 days. The main kinetic parameters describing the growth of LD35 and DSM 1868 on the target compounds that they could use as carbon and energy sources were studied in growing-cell batch culture conditions according to Armenante et al. [2]. Sterile 500-mL Erlenmeyer flasks containing 100 mL of MMM were inoculated with a fresh culture of LD35 or DSM 1868 (2% v/v) previously grown on 2-chlorophenol (1.56 mM) or 4-methoxybenzoic acid (6.57 mM) until the late log-phase of growth (LD35 and DSM 1868 cultures used as the inoculum displayed typically an A_{600} of about 0.180 and 0.750, respectively). The target compound was then added to each culture from a filter-sterilized aqueous stock solution (10 g/L) to obtain initial concentrations in a range that was chosen on the basis of the target compound typical occurrence in OMWs [26]: 4-hydroxyphenylacetic acid, 0.13–4.60 mM; 4-hydroxybenzoic acid, 0.15–7.24 mM; 3,4-dihydroxycinnamic acid, 0.11–2.77 mM; 4-hydroxy-3-methoxybenzoic acid, 0.12–5.95 mM; 3,4-dimethoxybenzoic acid, 0.11–7.68 mM. The cultures were then incubated at 30°C and 200 rpm. Samples were removed at regular intervals throughout the exponential growth phase and analyzed for biomass concentration (evaluated as A_{600} of the culture samples) and substrate concentration (via HPLC analysis). The data obtained were analyzed by means of the approach detailed in Armenante et al. [2]; the experimental plots describing the specific growth rate (μ) vs the substrate concentration (S) (calculated as the average substrate concentration in the time interval considered for the μ determination), were interpreted by using either the Monod kinetic model or the Andrews inhibitory kinetics [2]. The yield coefficient, Y, defined as the rate of substrate utilization per rate of biomass growth, was obtained for each microorganism on each substrate it degraded by dividing the variation of the A_{600} in the time interval considered for the μ determination in the cultures by the substrate concentration variation in the same period of time.

2.6. Growing cell experiments

They were performed in duplicate by using a synthetic mixture of the nine monocyclic aromatic compounds most commonly found in OMWs [24] as the sole carbon and energy source. The concentration of these compounds in the MMM employed for the experiments was: 4-hydroxyphenylacetic acid (1.15 mM), 2,6-dihydroxybenzoic acid (3.25 mM), veratric acid (1.92 mM), 4-hydroxybenzoic acid (2.17 mM), syringic acid (2.5 mM), cinnamic acid (0.84 mM), 3,4,5-trimethoxybenzoic acid (0.23 mM), caffeic acid (0.42 mM), vanillic acid (1.49 mM). These concentrations corresponded to 50% of those at which the used target compounds typically occur in OMWs [24]. However, a 4-fold more concentrated (with respect to that used in the experiments) filter-sterilized stock solution of this mix (prepared in 50 mM phosphate buffer, pH 7) was diluted in sterile MMM and used to prepare the sterile cultures. Five hundred-milliliter flasks containing 100 mL of this medium were employed. The cultures were inoculated (3% v/v) with LD35 or DSM 1868 (pre-grown on 2-chlorophenol or 4-methoxybenzoic acid, respectively) and incubated at 30°C and 200 rpm for 96 h. Three-milliliter samples were periodically removed for cell concentration measurements and HPLC analysis. An identical control flask, inoculated with heat-inactivated cells, was also prepared and sampled in the same way.

The capacity of LD35 and DSM 1868 for growth on a natural OMW was determined by inoculating the two strains (3% v/v) in 250-mL flasks containing 80 mL of sterile MMM and 20 mL of OMW. The used OMW, derived from an olive oil processing plant in Italy, had an acid pH (5.3), which was corrected to 7 by using 10 N NaOH. The flasks were incubated as previously described and the growth of the two strains was monitored both on TSA plates and on selective MMM agar plates.

2.7. Analytical methods

The cell concentration in the kinetic studies and in the cell suspension preparation was measured spectrophotometrically (A_{600}) by using a Varian Cary 100 scan UV visible spectrophotometer (Varian Instruments, TX, USA). LD35 and DSM 1868 growth on the synthetic mix of aromatic compounds was measured by using TSA plates, whereas growth of the two strains on natural OMW was determined by using both TSA and MMM agar plates with 2-chlorophenol (1.56 mM) or 4-methoxybenzoic acid (6.57 mM), respectively. Quantitative analysis of aromatic acids as well as identification of the produced metabolites was done via a HPLC system (Beckman Coulter, USA) equipped with a UV visible diode array detector operating at 260 and 235 nm and an Ultrasphere 4.6×25 mm ODS column. Column temperature was 35°C; injection volume was 20 μL. The eluents employed were: (A) water to which was added 1% (v/v) acetic acid and (B) methanol to which was added 1% (v/v) acetic acid. In isocratic conditions, 70% (A) and 30% (B) were employed. The solvent gradient method employed for the separation of the components of the mixture of monocyclic aromatic acids was: initial solvent composition, 90% (A) and 10% (B); isocratic elution for 23 min; solvent composition changed to 55% (A) and 45% (B) in 7 min; solvent composition changed to 35% (A) and 65% (B) in 7 min; isocratic elution for 5 min; back to initial solvent composition in 10 min. pH was measured by using a selective probe (81-04 model, ATI-Orion, Boston, MA).

3. Results

3.1. Characterization of the LD35 strain

LD35 strain had been previously isolated during a study on chlorophenol aerobic degradation; the taxonomy of this strain was characterized in this work. It was found to be a Gram-negative, non-sporulating, motile rod-shaped bacterium; it formed white round colonies having smooth margins when grown on TSA medium. The strain gave positive results for cytochrome oxidase; it reduced nitrate and did not form indole from tryptophan. It gave negative results for glucose fermentation, arginine dehydrogenase, urease, β-glucosidase, β-galactoxidase and gelatin hydrolysis tests. Assimilable compounds were gluconate, N-acetylglucosamine, malate and citrate. Unassimilable compounds were: arabinose, mannose, mannitol, maltose, phenylacetate, adipate and caproate. On the basis of these results, the API 20 NE test classified it as an *Alcaligenes* strain with

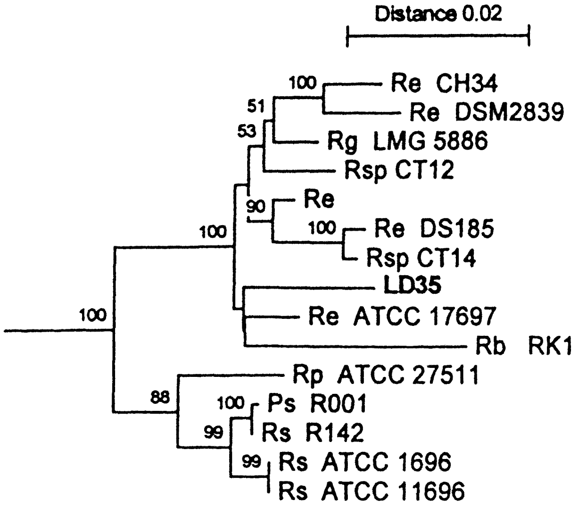

Figure 1. Phylogenetic tree showing the location of strain LD35 within the *Ralstonia* group. Branches are indicated by abbreviation of the genus/species followed by the strain name. Re, *Ralstonia eutropha*; Rb, *Ralstonia basilensis*; Rg, *Ralstonia gilardii*; Rp, *Ralstonia pickettii*; Rs, *Ralstonia solanacearum*; Rsp, *Ralstonia species*; Ps, *Pseudomonas syzygii*.

low discrimination. In order to better clarify the taxonomy of this strain, the nucleotide sequence of its 16S rDNA was determined and compared with those contained in the RDP [21], and the matrix obtained was used to draw a phylogenetic tree. As shown in *figure 1,* the LD35 strain was positioned in the *Ralstonia* group. Considering the 1201 alignment positions used to draw the tree, strain LD35 shared similarity values higher than 97% with six strains, four *R. eutropha* (Re, Re DSM 2839, Re ATCC 17697, Re CH34), the *Ralstonia* sp. strain CT12, and the *R. gilardii* strain LMG 5886. The highest similarity values (close to 98%) were shared with two *R. eutropha* strains (Re and Re DSM 2839). LD35 was found to cleave the aromatic ring of both catechol and protocatechuate in the *ortho* mode.

3.2. Metabolization of phenol compounds by *Ralstonia* sp. LD35 and *P. putida* DSM 1868 washed cells

Table I reports the specific uptake rates of the monocyclic aromatic compounds tested by *Ralstonia* sp. LD35, previously grown on 2-chlorophenol and *P. putida* DSM 1868, previously grown on 4-methoxybenzoic acid. Four compounds were metabolized by LD35 washed cells; 4-hydroxybenzoic acid was degraded via 3,4-dihydroxybenzoic acid, which was found to transiently accumulate, whereas 4-hydroxyphenylacetic acid was biodegraded without any intermediate production. Caffeic acid was biodegraded through the transient production of an aromatic metabolite, more hydrophilic than the target substrate. Cinnamic acid was biodegraded via benzoic acid, which was identified on the basis of its UV absorption spectrum recorded by the diode array detector and also by the coelution procedure. Benzoic acid was then metabolized by LD35 cells with no accumulation of further aromatic metabolites.

Six compounds were metabolized by DSM 1868 washed cells (*table I*). 4-hydroxybenzoic acid was degraded via 3,4-dihydroxybenzoic acid and 4-hydroxyphenylacetic acid was biodegraded without any intermediate metabolite production. Caffeic acid was slowly bioconverted to a more hydrophilic aromatic compound, which was not further degraded; it did not coelute with any of the molecules tested, such as 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, salicylic acid or benzoic acid. A dark product formation was also observed in this culture during incubation; 3,4-dimethoxybenzoic acid was quickly bioconverted into 4-hydroxy-3-methoxybenzoic acid, which was then completely degraded. 4-hydroxy-3,5-dimethoxybenzoic acid metabolization was slow and proceeded with the accumulation of a highly hydrophilic aromatic compound.

The concentration of the tested compounds in the sterile controls did not appreciably change with the incubation time, except for caffeic acid, which was found to slowly disappear along with the appearance of dark colored compounds in the culture.

3.3. Determination of kinetic parameters

4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid and 3,4-dihydroxycinnamic acid were found to be growth substrates for the LD35 strain. The plots describing the specific growth rate (μ) vs the substrate concentration for each of these substrates are reported in *figure 2,* whereas the equation type used to interpret the curves [2] and the obtained kinetic parameters are reported in *table II.* No inhibitory effects were found when 4-hydroxyphenylacetic acid was used as the substrate; in contrast, 4-hydroxybenzoic acid as well as 3,4-dihydroxycinnamic acid were able to inhibit LD35 growth when present in the medium at concentrations higher than 1.45 mM and 0.28 mM, respectively.

4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3,4-dimethoxybenzoic acid and 4-hydroxy-3-

Table I. Rates of substrate uptake by washed-cell suspensions of *Ralstonia* sp. LD35 and *P. putida* DSM 1868. The data presented are the average (± standard deviation) of two experiments.

Compound assayed (initial concentration = 50 mg/L[b])	Specific rate of compound uptake[a] [mmoles/(l × h × A_{600})]	
	LD35	DSM 1868
4-Hydroxyphenylacetic acid (0.33)	0.135 ± 0.015	0.024 ± 0.007
2,6-Dihydroxybenzoic acid (0.32)	0	0
3,4-Dimethoxybenzoic acid (0.27)	0	0.348 ± 0.024
4-Hydroxybenzoic acid (0.36)	0.196 ± 0.021	0.411 ± 0.030
4-Hydroxy-3,5-dimethoxybenzoic acid (0.25)	0	0.003 ± 0.001
Cinnamic acid (0.34)	0.018 ± 0.002	0
3,4,5-Trimethoxybenzoic acid (0.23)	0	0
Caffeic acid (0.28)	0.107 ± 0.013	0.016 ± 0.005
4-Hydroxy-3-methoxybenzoic acid (0.30)	0	0.010 ± 0.002

[a] h is the time (in hours) necessary to degrade 50% of the target compound; A_{600} is the absorbance of the cell suspensions employed.
[b] The mM concentration of each compound is indicated in parenthesis.

Table II. Kinetic parameters describing the growth of *Ralstonia* sp. LD35 and *P. putida* DSM 1868 on each of the target monocyclic aromatic acid compounds employed.

Strain	Growth substrate	Kinetic expression	$\mu_{\max}$ (1/h)	μ^* (1/h)	K_s (mM)	K_i (mM)	Y (A_{600nm}/mM)
LD35	4-hydroxyphenylacetic acid	Monod	0.3116	–	0.0110	–	0.2731 ± 0.0456
LD35	4-hydroxybenzoic acid	Andrews	–	0.4641	0.0484	25.8819	0.2019 ± 0.0316
LD35	caffeic acid	Andrews	–	0.2687	0.0148	3.5029	0.2793 ± 0.0896
DSM 1868	4-hydroxyphenylacetic acid	Monod	0.7381	–	0.1715	–	0.2085 ± 0.0695
DSM 1868	3,4-dimethoxybenzoic acid	Monod	0.4418	–	0.1035	–	0.2960 ± 0.0895
DSM 1868	4-hydroxybenzoic acid	Monod	0.6606	–	0.1844	–	0.1367 ± 0.0481
DSM 1868	4-hydroxy-3-methoxybenzoic acid	Monod	0.4358	–	0.2667	–	0.1135 ± 0.0414

methoxybenzoic acid were found to support the growth of DSM 1868. The growth kinetics on these substrates are reported in *figure 3*. No inhibitory effect was observed in any of the cases; therefore, the experimental data were analyzed with the Monod equation. The obtained kinetic parameters are reported in *table II*.

3.4. Growing cell experiments

Both strains were found to grow on the mixture of the nine monocyclic aromatic compounds previously tested in resting cell conditions as single compounds, here used at a concentration which corresponded to 50% of that at which they occur in natural OMWs. *Figure 4* shows the time course of the initial 30 h of the experiment; no substantial variations were observed in the remaining 66 h of incubation except for a continuous decrease in the cinnamic acid concentration to 0.12 mM in LD35 culture. The complete biodegradation of the same monocyclic aromatic acids which were metabolized as single compounds in resting-cell conditions (*table I*) was observed (*figure 4*). Significant caffeic acid depletion was also observed in the abiotic controls; thus, the data on caffeic acid degradation were corrected considering the abiotic concentration decrease detected in the control flask. The production of dark-colored compounds was revealed both in the DSM 1868 active cultures and in the sterilized control. The concentration of the two strains, which was measured as CFU/mL, increased rapidly in the initial 24 h (*figure 4*), after which the stationary phase of growth was reached both in LD35 and DSM 1868 cultures.

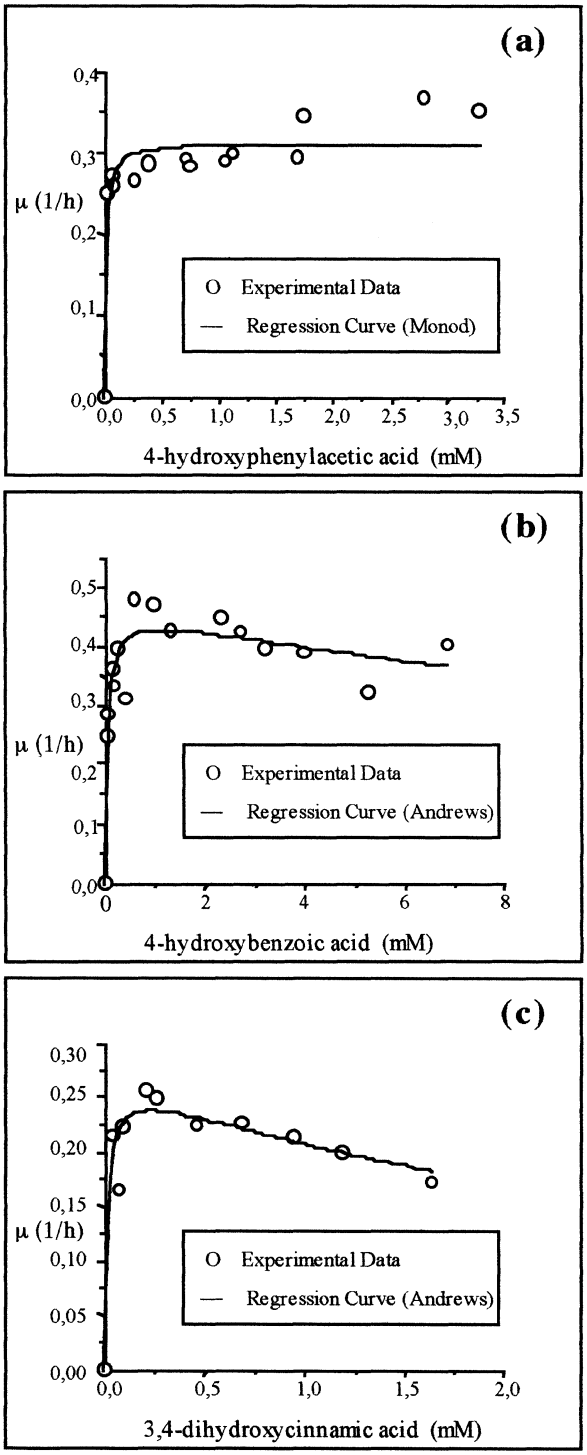

Figure 2. Specific growth rate of *Ralstonia* sp. LD35 strain as a function of the concentration of 4-hydroxyphenylacetic acid (a), 4-hydroxybenzoic acid (b) and 3,4-dihydroxycinnamic acid (c).

In addition, both strains were capable of growing on OMW constituents diluted in MMM (20% v/v): after an initial brief exponential phase, the stationary phase of growth was reached and the cell concentration (about 10^8 CFU/mL), measured by using MMM selective agar plates, remained almost constant until the end of the incubation time. Studies regarding changes in culture medium chemical composition due to bacterial growth on OMW were not performed.

4. Discussion

The main objective of this work was to select bacterial strains capable of degrading a large spectrum of the monocyclic aromatic compounds typically occurring in OMWs; this is the first step of a research project focused on the development of a biotechnological process for the removal of these toxic compounds from OMWs and/or from the effluents of OMW-fed anaerobic digestors, where they frequently persist after the treatment [5, 11]. Thus, in this work, two aerobic bacterial strains already available in our laboratory were analyzed for their biodegradation activities towards OMW monocyclic aromatic compounds; LD35, a monochlorophenol degrading bacterium, was also characterized as a *Ralstonia* sp. strain for the first time in this work.

The data obtained have shown that both LD35 and DSM 1868 possess interesting degradation capabilities towards several pure hydroxylated and methoxylated benzoic, phenylacetic and phenylpropenoic acids, which typically occur at high concentrations in OMWs [24]. *Ralstonia* sp. LD35 was capable of metabolizing (*table I*) and using as growth substrates (*figure 2*) 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid and 3,4-dihydroxycinnamic acid; their degradation proceeded with no accumulation of stable aromatic metabolites. Resting cells of LD35 metabolized cinnamic acid via benzoic acid (*table I*), even though cinnamic acid did not sustain this strain growth. The metabolization of unsubstituted 3-phenyl-2-propenoic acids is not common among bacteria [1, 14, 20]. A similar breakdown pathway for the propenoic side chain of substituted cinnamic acid molecules, such as ferulic acid and *p*-cumaric acid [3-(3-methoxy-4-hydroxyphenyl)-2-propenoic acid and 3-(4-hydroxyphenyl)-2-propenoic acid, respectively)], has been observed in cultures of *Pseudomonas* and *Acinetobacter* strains [1, 14, 28],

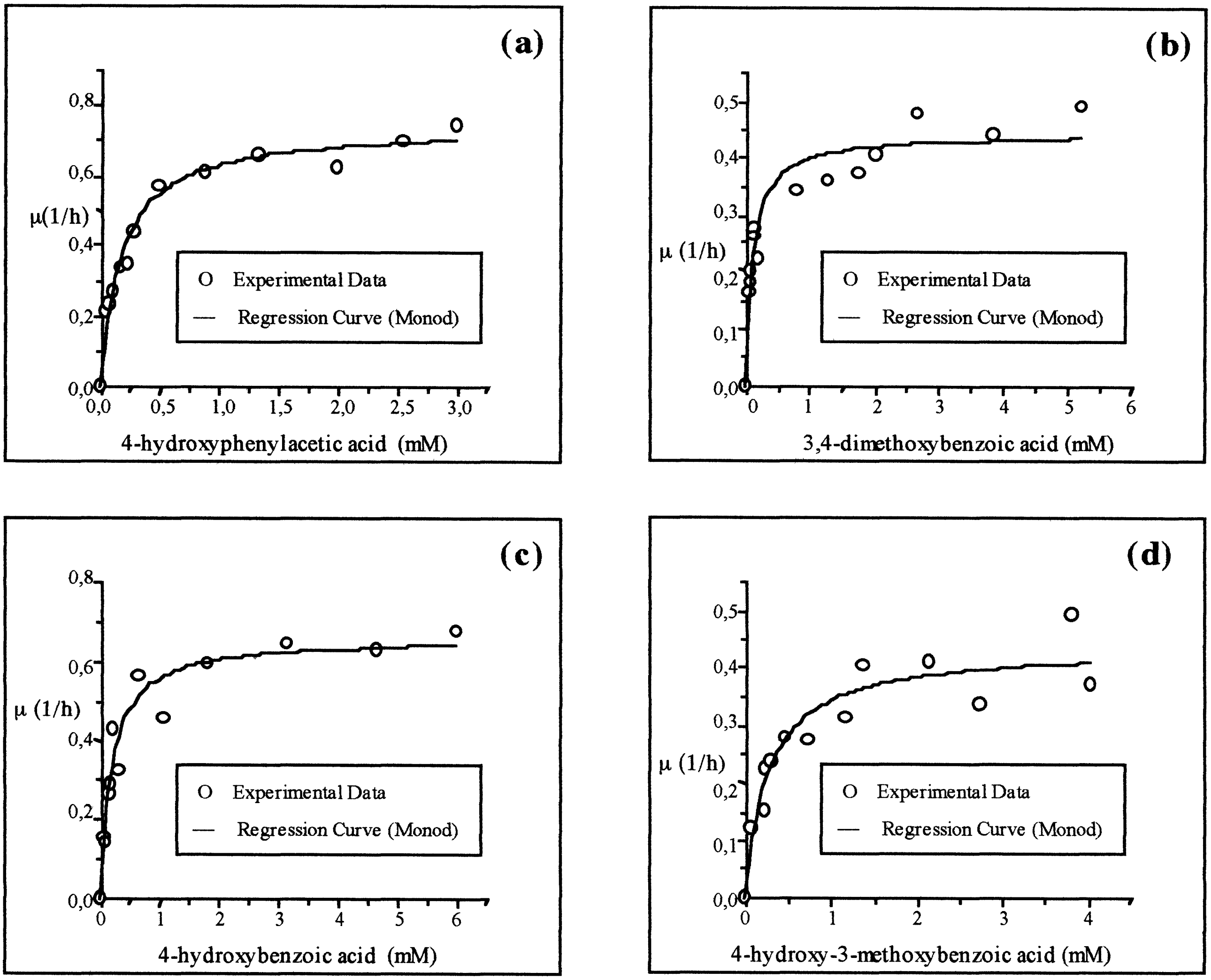

Figure 3. Specific growth rate of *P. putida* DSM 1868 strain as a function of the concentration of 4-hydroxyphenylacetic acid (a), 3,4-dimethoxybenzoic acid (b), 4-hydroxybenzoic acid (c) and 4-hydroxy-3-methoxybenzoic acid (d).

which could grow on these molecules but not, similarly to LD35, on cinnamic acid in the absence of other carbon and energy sources. Thus, it has been speculated that a hydroxyl group in the *para* position of the aromatic ring of 3-phenyl-2-propenoic acids has an important role in the substrate uptake [14] and/or in the deacetylating enzyme induction [1], i.e. in the biochemical processes required for the complete transformation of these compounds into Krebs cycle intermediates [1, 14]. The finding that LD35 could grow on 3,4-dihydroxycinnamic acid and not on cinnamic acid indicates that the occurrence of hydroxylic groups on the aromatic ring is also required by the LD35 strain for the proper metabolization of 3-phenyl-2-propenoic acids.

DSM 1868 was capable of metabolizing (*table I*) and using as growth substrates (*figure 3*) 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3,4-dimethoxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid. 3,4-dimethoxybenzoic acid was metabolized through a quick demethylation of the *para* methoxylic group with the production of 4-hydroxy-3-methoxybenzoic acid. The occurrence of a demethylating enzyme (referred to as 4-methoxybenzoate monooxygenase) in this strain has already been reported in the literature [8, 9]. The metabolization rate of 4-hydroxy-3-methoxybenzoic acid, both as an intermediate and as exogenous compound supplied to DSM 1868 cell suspensions as a target substrate, was lower than that of 3,4-dimethoxybenzoic acid (*table I*) and probably occurred by the slower O-demethylating activity of the 4-methoxybenzoate monooxygenase on *meta*-methoxylated groups with the production of 3,4-dihydroxybenzoic acid (protocatechuic acid). A low turnover rate for this substrate by purified DSM 1868 4-methoxybenzoate monooxygenase has already been evidenced [8]. However, protocatechuic acid did not accumulate either in resting or in growing cell conditions, probably because it was quickly metabolized. The present study has also shown that resting cells of DSM 1868 can slowly metabolize 4-hydroxy-3,5-dimethoxybenzoic acid, which has never been tested before as a substrate for this strain [8, 9]. This process can again be explained by the slow action of the 4-methoxybenzoate monooxygenase of DSM 1868 on this di-*meta*-methoxylated compound which, as a consequence, was not able to support the strain growth. DSM 1868 also metabolized and used as the sole carbon and energy source 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid probably via the corresponding dihydroxylated benzoic acid metabolites. 3,4-dihydroxycinnamic acid was slowly depleted in resting-cell conditions (*table I*) and it was unable to support DSM 1868 growth; this evidence, coupled with the observation that dark-colored compounds were produced during resting cell culture incubation, suggested that the disappearance of 3,4-dihydroxycinnamic acid (detected to a greater measure in the active culture with respect to the related sterile controls) was due more to oxidative polymerization processes than to biodegradation; on the other hand, the key role played by the bacterial oxidases in the oxidation and polymerization of hydroxylated monocyclic aromatic molecules has already been reported in the literature [17].

The biodegradation spectrum of the two strains did not change when they were growing on a mixture of the nine monocyclic aromatic acids tested separately in resting cell conditions (*figure 4*); this evidence suggests that: a) no specific enzyme induction phenomena were necessary for the target compound degradation; and b) no significant inhibition effects due to the simultaneous presence of the target compounds occurred during SM component biodegradation by the two bacteria. The two bacteria could also grow extensively on the constituents of a nonsterile OMW without exhibiting clear inhibition effects. On the basis of this evidence, LD35 and DSM 1868 appeared to be of special interest for the purpose of the present work. Considering the importance of kinetic parameters in the technological application of bacteria, growth kinetics of the two strains on each metabolized compound were studied. Generally, a high affinity towards all the assayed substrates was observed for both strains (*figures 2* and *3*; *table II*); this

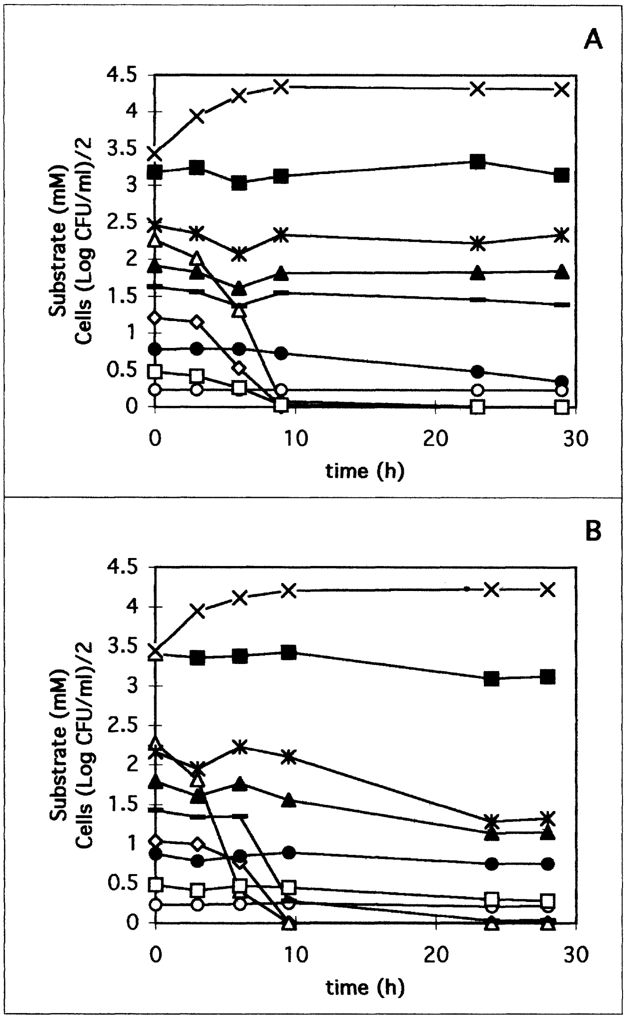

Figure 4. Depletion profiles of a mixture of monocyclic aromatic acids occurring in OMWs (at 50% of the concentration at which they occur in natural OMWs) by LD35 (A) and DSM 1868 (B) in the initial 30 h of batch incubation (◇: 4-hydroxyphenylacetic acid; ■: 2,6-dihydroxybenzoic acid; ▲: veratric acid; △: 4-hydroxybenzoic acid; *: syringic acid; ●: cinnamic acid; ○: 3,4,5-trimethoxybenzoic acid; □: caffeic acid; —: vanillic acid; X: CFU/mL). Each value is the average of the values obtained through duplicate experiments.

is evident, in particular, for LD35, which displayed K_s values which were generally much lower than those exhibited by DSM 1868. Both strains also presented a high $\mu_{\max}$ on the compounds tested: despite the fact that LD35 was found to be slightly inhibited by high concentrations of 4-hydroxybenzoic acid and 3,4-dihydroxycinnamic acid, its growth rates were generally comparable to those exhibited by other phenol-degrading bacteria [16]. DSM 1868 exhibited $\mu_{\max}$ values which were among the highest shown by monocyclic aromatic compound-degrading aerobic bacteria [2, 3, 16, 18].

In conclusion, both *Ralstonia* sp. LD35 and *P. putida* DSM 1868 have shown interesting degradation performances towards several recalcitrant monocyclic aromatic compounds of OMWs. Their average high affinity and high specific growth rate vs the target substrates make these strains attractive candidates for the development of a biotechnological process for OMW dephenolization and detoxification.

Acknowledgements

We would like to sincerely thank Dr C. Barberio and Dr S. Spagnesi (Dipartimento di Biologia Animale e Genetica, University of Florence, Italy) for their help in amplification, sequencing of 16S rDNA and taxonomical analysis of LD35, and the student, L. Zaniboni, who worked on the project. The project was supported by the Italian MURST (PRIN '98-Ex 40% Project).

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