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ORIGINAL PAPER

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Biodegradation of synthetic and naturally occurring mixtures of mono-cyclic aromatic compounds present in olive mill wastewaters by two aerobic bacteria

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Abstract Two bacterial strains, *Ralstonia* sp. LD35 and *Pseudomonas putida* DSM 1868, were assayed for their ability to degrade the monocyclic aromatic compounds commonly found in olive mill wastewaters (OMWs). The goal was to study the possibility of employing the two strains in the removal of these recalcitrant and toxic compounds from the effluents of anaerobic treatment plants fed with OMWs. At first, the two strains were separately assayed for their ability to degrade a synthetic mixture of nine aromatic acids present in OMWs, both in growing- and resting-cell conditions. Then, due to the complementary activity exhibited by the two strains, a co-culture of the two bacteria was tested under growing-cell conditions for degradation of the same synthetic mixture. Finally, the degradation activity of the co-culture on two fractions was studied. Both fractions one deriving from natural OMWs through reverse osmosis treatment and containing low-molecular weight organic molecules, and the other obtained from an anaerobic lab-scale treatment plant fed with OMWs, were rich in monocyclic aromatic compounds. The co-culture of the two strains was able to biodegrade seven of the nine components of the tested synthetic mix (2, 6-dihydroxybenzoic acid and 3, 4, 5-trimethoxybenzoic acid were the two undegraded compounds). In addition, an efficient biodegrading activity towards several aromatic molecules present in the two natural fractions was demonstrated.

Introduction

Olive oil mill wastewaters (OMWs), i.e. the aqueous effluent obtained from olive oil milling, have a high organic load due to the presence of sugars, lipids, proteins, and a number of other organic compounds, including monomeric and polymeric aromatic molecules, generally referred to as phenolic compounds (Ronchero et al. 1974; Ehaliotis et al. 1999). Phenolic compounds, which consist of mono-cyclic aromatic molecules, such as hydroxylated- and/or methoxylated-benzoic, phenylacetic and phenylpropenoic acids, and higher molecular weight compounds obtained through their polymerisation (Ronchero et al. 1974; Toscano et al. 1997), are recalcitrant to biodegradation and are the main reason for the toxicity of OMWs (Capasso et al. 1995; Beccari et al. 1996; Martirani et al. 1996). Therefore, OMWs cannot be released in the environment, but have to be adequately treated before being discharged.

The treatment of OMWs has been the object of several lab- and large-scale investigations, but no satisfactory solution has been found up to now (Andreozzi et al. 1998). One of the most studied and promising OMW treatment technologies is anaerobic biological digestion; however, the effectiveness of this treatment is not always satisfactory, in particular with respect to the OMW phenolic fraction, which can inhibit anaerobic microflora (Borja et al. 1995). As a consequence, some of the phenolic compounds tend to persist in the effluents of the anaerobic treatment (Borja et al. 1995; Beccari et al. 1996, 1998). In order to improve the anaerobic process, anaerobic pre-treatment with *Aspergillus* strains capable of removing most of the phenolic compounds prior to anaerobic treatment has been proposed (Hamdi and Ellouz 1993; Borja et al. 1995). The possibility of pre-treating OMWs with a microbially uncharacterised aerobic sludge taken from a municipal wastewater treatment plant has also been recently explored by Benitez et al. (1997). Finally, the possibility of enhancing the anaerobic removal of biorecalcitrant phenols by pre-treating OMWs with $Ca(OH)_2$ and bentonite was studied by Beccari et al. (1999). Nonetheless, the problem of the persistence of aromatic compounds in the effluents of conventional anaerobic treatments plants fed with OMWs remains and needs to be further investigated. To our knowledge, no attempts to remove these compounds from the effluents of the anaerobic process by using an

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integrated post-treatment with specialised microorganisms have been made up to now. Due to their high growth rate and metabolic versatility, aerobic bacteria could be used in this type of post-treatment facility.

A previous study conducted in our laboratory showed that two bacterial strains possess interesting degradation capabilities towards several compounds that constitute most of the mono-aromatic fraction of OMWs (Di Gioia et al. 2000): *Ralstonia* sp. LD35, isolated from industrial wastewaters and able to use phenol and monochlorophenols as the sole carbon and energy sources (Di Gioia et al. 2000), and *Pseudomonas putida* DSM 1868, which is able to grow on 4-methoxybenzoic acid and of O-demethylating several substituted benzoic acids (Bernhardt et al. 1988). In order to explore the potential of employing the two strains in the removal of these compounds from effluents of anaerobic treatment plants fed with OMWs, this work was aimed at studying the ability of *Ralstonia* sp. LD35 and *P. putida* DSM 1868 to biodegrade a synthetic mixture of nine monocyclic aromatic acids present in OMWs as well as two fractions rich in monocyclic aromatic compounds, one derived from natural OMWs through reverse osmosis treatment and containing low-molecular weight organic molecules and the other consisting of the effluent of an anaerobic lab-scale treatment plant. The results obtained indicate that the two strains complemented each other with respect to their ability to degrade mono-aromatic compounds present in OMWs. Moreover, when combined in a co-culture, they were able to biodegrade completely a large array of aromatic compounds present in the OMW-derived fractions assayed.

Material and methods

Chemicals

Mono-cyclic aromatic compounds, including 2-chlorophenols and 4-methoxybenzoic acid, as well as the constituents of the mineral medium MMM described by Fava et al. (1996) were obtained from Aldrich Chemical (Milwakee, Wis.). The constituents of the tryptic soy agar (TSA) medium were purchased from Biolife Srl (Milan, Italy). The solvents used in the HPLC analyses were purchased from Baker Italia (Milan, Italy).

Microorganisms and media

Ralstonia sp. LD35 strain (Di Gioia et al. 2000; 16S rDNA nucleotide sequence deposited into GenBank with the accession number AF251026) was maintained in liquid cultures of MMM (Fava et al. 1996) supplemented with 2-chlorophenol (1.56 mM); *P. putida* DSM 1868 (Bernhardt et al. 1988) was cultured in MMM with 6.57 mM 4-methoxybenzoic acid as the sole carbon and energy source. The aqueous synthetic mixture (SM) of the mono-aromatic acid compounds employed to test the biodegrading activity of the two strains was prepared by dissolving the following acids in 50 mM phosphate buffer (pH 7): 4-hydroxyphenylacetic acid (4.60 mM), 2, 6-dihydroxybenzoic acid (12.99 mM), 3, 4-dimethoxybenzoic acid (veratric acid, 7.69 mM), 4-hydroxybenzoic acid (8.69 mM), 4-hydroxy-3, 5-dimethoxybenzoic acid (syringic acid, 10 mM), 3-phenyl-2-propenoic acid (cinnamic acid, 3.37 mM), 3, 4, 5-trimethoxybenzoic acid (0.94 mM), 3, 4-dihydroxycinnamic acid (caffeic acid, 1.66 mM), 4-hydroxy-3-methoxybenzoic acid (vanillic acid, 5.95 mM). The SM was filter-sterilized (0.22-μm cellulose nitrate filters); the final pH was around 6. The concentration of each compound in the SM was twice that at which it typically occurs in natural OMWs [according to Ronchero et al. (1974)]. The sterile SM solution was then diluted either with autoclaved sterilised phosphate buffer or MMM to obtain cultures with the desired final concentrations of SM compounds. The mixture solution containing the mono-aromatic acid compounds at the concentration reported by Ronchero et al. (1974) was obtained by diluting SM 1:1 with sterile buffer or MMM and is referred to as “100% SM”.

Resting-cell experiments

For resting-cell experiments, *Ralstonia* sp. LD35 cells were grown on MMM plus 2-chlorophenol (1.56 mM) and *P. putida* 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 cultures and 0.8 for DSM 1868], cells were harvested by centrifugation (Beckman J2-HS centrifuge, Beckman Coulter, Calif.), washed twice with 50 mM phosphate buffer (pH 7) and re-suspended in the same buffer to obtain a suspension with an A_{600} of 2.4–2.8. Fifteen ml of the cell suspension were dispensed into 100-ml sterile bottles with Teflon-coated screw caps; 15 ml of SM (and phosphate buffer, when necessary) were added to obtain cell suspensions with SM at 100, 50, 20, and 10%. The bottles were incubated at 150 rpm and 30 °C for 96 h. One-ml samples were periodically removed, acidified with 25 μl of 2 M trichloroacetic acid, centrifuged at 10,000 *g* for 10 min and analysed via HPLC. Heat-inactivated controls were prepared by the same procedure. The initial metabolic rate of each compound, expressed as mM/h, was calculated by dividing 50% of its initial concentration by the time required to biodegrade this amount.

Growing-cell experiments

Five hundred-ml Erlenmeyer flasks containing 100 ml culture broth were employed to test the biodegrading activity of the two strains under growing-cell conditions. SM was added to MMM, as reported above, to obtain duplicate cultures with a final SM concentration of 100, 50, 20, and 10%. *Ralstonia* sp. LD35 and *P. putida* DSM 1868 (pre-grown on 2-chlorophenol and 4-methoxybenzoic acid, respectively, as reported above) were then inoculated (3% v/v). In experiments employing the co-culture LD35-DSM 1868, the inoculum was prepared by mixing the two pure cultures at a ratio of 1:1. The inoculated cultures, prepared in duplicate, and control flasks inoculated with heat-inactivated cells were incubated at 30 °C and 150 rpm for 96 h. Three-ml samples were periodically removed for cell concentration measurements and HPLC analysis. The initial rate of metabolism of each compound was calculated as reported before for each culture; the data reported are the average of the values obtained from duplicate experiments.

Experiments with the OMW “reverse osmosis” fraction

The “reverse-osmosis” (RO) fraction of a natural OMW was kindly supplied by Prof. Canepa (University of Genova, Italy). It was obtained through reverse osmosis treatment of an OMW, which was produced in an olive oil processing plant in Italy. The RO fraction contained aliphatic and aromatic compounds naturally occurring in OMWs; the molecular weights of the compounds were < 350. The RO process increased the concentration of this fraction, so that the concentration at the end of treatment was 9.6 times higher than in the original OMW (Canepa, personal communication). A partial characterisation of the fraction, which had a pH of 5.3, was carried out by HPLC by comparing the UV absorption spectrum and the retention time of each peak with several

Table 1 Degradation percentages of the synthetic mixture (*SM*) constituents in resting-cell (*RC*) and in growing-cell (*GC*) conditions after 96 h incubation. Initial metabolic rates, expressed as mM/h, are indicated within *parentheses*. *ND* Not determined, as 50% degradation of the compound was not achieved in the experiment

	LD35 (RC)	DSM 1868 (RC)	LD35 (GC)	DSM 1868 (GC)	LD/DSM (GC)
10%SM					
4-Hydroxyphenylacetic acid (0.23 mM)	100 (0.049)	100 (0.027)	100 (0.035)	100 (0.063)	100 (0.069)
2,6-Dihydroxybenzoic acid (0.65 mM)	0	0	0	0	0
Veratric acid (0.39 mM)	0	100 (0.108)	0	86 (0.051)	100 (0.021)
4-Hydroxybenzoic acid (0.44 mM)	100 (0.147)	100 (0.281)	100 (0.038)	100 (0.090)	100 (0.089)
Syringic acid (0.5 mM)	0	50 (0.001)	0	42 (ND)	42 (ND)
Cinnamic acid (0.17 mM)	100 (0.022)	0	91 (0.005)	0	73 (0.003)
3,4,5-Trimethoxybenzoic acid (0.05 mM)	0	0	0	0	0
Caffeic acid (0.08 mM)[a]	100 (0.027)	65 (0.003)	100 (0.009)	67 (0.002)	100 (0.008)
Vanillic acid (0.3 mM)	0	100 (0.051)	0	100 (0.068)	100 (0.052)
20% SM					
4-Hydroxyphenylacetic acid (0.46 mM)	100 (0.079)	100 (0.104)	100 (0.058)	100 (0.104)	100 (0.071)
2,6-Dihydroxybenzoic acid (1.30 mM)	0	0	0	0	0
Veratric acid (0.77 mM)	0	100 (0.156)	0	74 (0.047)	90 (0.028)
4-Hydroxybenzoic acid (0.87 mM)	100 (0.158)	100 (0.421)	100 (0.062)	100 (0.153)	100 (0.147)
Syringic acid (1.00 mM)	0	36 (ND)	0	41 (ND)	48 (ND)
Cinnamic acid (0.34 mM)	100 (0.015)	0	93 (0.013)	0	78 (0.008)
3,4,5-Trimethoxybenzoic acid (0.09 mM)	0	0	0	0	0
Caffeic acid (0.17 mM)[a]	100 (0.029)	65 (0.013)	100 (0.013)	66 (0.010)	100 (0.037)
Vanillic acid (0.60 mM)	0	100 (0.142)	0	100 (0.150)	100 (0.072)
50% SM					
4-Hydroxyphenylacetic acid (1.15 mM)	100 (0.059)	100 (0.027)	100 (0.172)	100 (0.224)	100 (0.108)
2,6-Dihydroxybenzoic acid (3.25 mM)	0	0	0	0	0
Veratric acid (1.92 mM)	0	80 (0.023)	0	34 (ND)	84 (0.013)
4-Hydroxybenzoic acid (2.17 mM)	100 (0.112)	100 (0.247)	100 (0.168)	100 (0.259)	100 (0.331)
Syringic acid (2.50 mM)	0	36 (ND)	0	38 (ND)	43 (ND)
Cinnamic acid (0.84 mM)	54 (0.006)	0	84 (0.019)	0	55 (0.013)
3,4,5-Trimethoxybenzoic acid (0.24 mM)	0	0	0	0	0
Caffeic acid (0.42 mM)[a]	100 (0.039)	65 (0.010)	100 (0.030)	62 (0.027)	100 (0.070)
Vanillic acid (1.49 mM)	0	100 (0.030)	0	100 (0.165)	100 (0.103)
100% SM					
4-Hydroxyphenylacetic acid (2.30 mM)	59 (0.021)	83 (0.027)	100 (0.125)	100 (0.184)	100 (0.132)
2,6-Dihydroxybenzoic acid (6.50 mM)	0	0	0	0	0
Veratric acid (3.85 mM)	0	0	0	6 (ND)	15 (ND)
4-Hydroxybenzoic acid (4.35 mM)	64 (0.049)	100 (0.044)	100 (0.147)	100 (0.220)	100 (0.292)
Syringic acid (5.00 mM)	0	0	0	21 (ND)	20 (ND)
Cinnamic acid (1.69 mM)	7 (ND)	0	38 (ND)	0	22 (ND)
3,4,5-Trimethoxybenzoic acid (0.47 mM)	0	0	0	0	0
Caffeic acid (0.83 mM)[a]	37 (0.015)	40 (ND)	100 (0.059)	38 (ND)	100 (0.002)
Vanillic acid (2.98 mM)	0	5 (ND)	0	100 (0.094)	100 (0.093)

[a] Data corrected by considering the compound loss detected in the control

pure, mono-cyclic aromatic compounds. The co-elution procedure was also used in the characterisation.

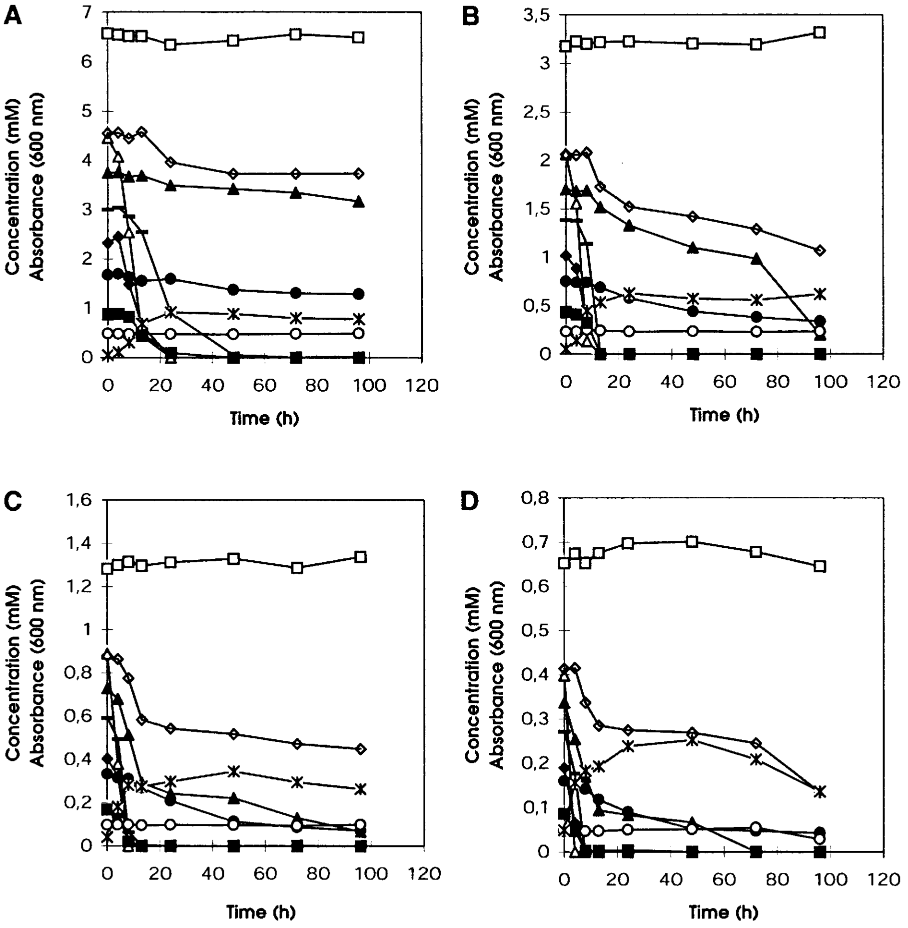
The ability of the co-culture to biodegrade the aromatic constituents of the RO fraction in the absence of other carbon and energy sources was determined through duplicate growing-cell batch experiments. Filter-sterilised RO fraction was added at 20% (v/v) to sterile MMM (total volume=100 ml, final pH=6.7) in 250-ml flasks. The flasks were then inoculated with equal amount of *Ralstonia* sp. LD35 and *P. putida* DSM 1868 separately pre-grown as previously reported. The inoculated cultures, as well as a sterile control prepared with heat-inactivated cells, were incubated for 1 week under the conditions described above.

Experiments with the anaerobic effluent

The anaerobic effluent was supplied by Prof. Beccari's group (University of Rome, Italy). The effluent was produced by a lab-scale methanogenic reactor continuously fed with a diluted OMW (88.8 g COD/l); this effluent contained 8.1 g COD/l, 0.1 g lipids/l and 1.5 g phenols /l; its pH was 8.85 (see Beccari et al. (2000) for further details).

The ability of the co-culture to metabolise this fraction in the absence of other carbon and energy sources was determined through growing-cell batch experiments. The filter sterilised anaerobic effluent fraction was added at 25% (v/v) to sterile MMM (total volume=25 ml, final pH=7.5) in a 100-ml flask; the pH was adjusted to 7 with a few drops of 2 N HCl. The flask was then inoculated with equal concentrations of *Ralstonia* sp. LD35 and *P. putida* DSM 1868 cells, separately pre-grown as described above. The inoculated culture and the related control inoculated with heat-inactivated cells were incubated for 1 week under the conditions described above. At 24-h intervals, the pH of the culture was measured and corrected to 7 with 2 N HCl when it was higher than 7.5. One-ml samples were periodically removed and used for cell counts on TSA and selective MMM plates, and for HPLC analyses.

Fig. 1A–D Depletion profiles of the mono-cyclic aromatic acids present in the SM at 100% (**A**), 50% (**B**), 20% (**C**) and 10% (**D**) (see Materials and methods) by a co-culture of LD35 and DSM 1868 strains. ◆ 4-hydroxyphenylacetic acid, □ 2, 6-dihydroxybenzoic acid, ▲ veratric acid, Δ 4-hydroxybenzoic acid, ◇ syringic acid, ● cinnamic acid, ○ 3, 4, 5-trimethoxybenzoic acid, ■ caffeic acid, – vanillic acid, *absorbance at 600 nm. The values presented are the average of duplicate experiments



Analytical methods

Cell concentration was determined by measuring the A_{600} with a Varian Cary 100 Scan UV-Visible spectrophotometer (Varian Instruments, Tex.) or by determination of the colony-forming units/ml (CFU/ml) on TSA plates and/or on MMM selective plates (i.e. with 1.56 mM 2-chlorophenol added for the LD35 strain, and 6.57 mM 4-methoxybenzoic acid for the DSM 1868 strain). SM mono-cyclic aromatic acids were qualitatively and quantitatively analysed with a HPLC system equipped with an UV-visible diode array detector operating at 260 and 235 nm, and a Ultrasphere 4.6×25 mm ODS column (Beckman Coulter). The column temperature was 35 °C; the injection volume was 20 µl. The solvents employed were: water acidified with 1% (v/v) acetic acid (A) and methanol acidified with 1% (v/v) acetic acid (B). The solvent gradient method employed for the mono-cyclic aromatic acid separation 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. The flow rate was 1 ml/min. The RO fraction and the anaerobic effluent fraction were analysed via the same HPLC system by lengthening the last isocratic elution from 5 to 16 min for the RO fraction analyses.

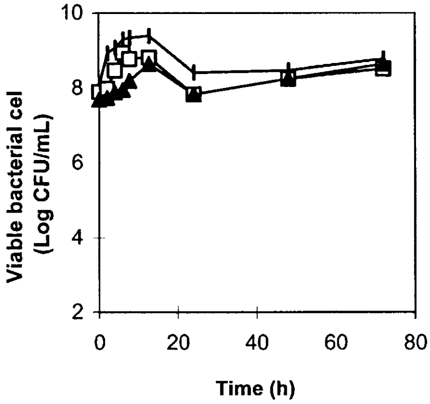


Fig. 2 Cell counts of the members of the LD35-DSM 1868 co-culture grown in the presence of SM as the carbon source at 50% (see Materials and methods). ■ TSA plates, □ MMM plus 4-methoxybenzoic acid agar plates, ▲ MMM plus 2-chlorophenol. The values presented are the average of duplicate experiments

Results

Degradation of the SM components in resting-cell conditions

Ralstonia sp. LD35 washed cells, pre-grown on 2-chlorophenol, and *P. putida* DSM 1868 washed cells, pre-grown on 4-methoxybenzoic acid, were separately assayed for their ability to metabolise the synthetic mixture SM, used at initial concentrations of 100, 50, 20 and 10% of those at which the mono-cyclic aromatic constituents are present in natural OMWs (Ronchero et al. 1974). The LD35 strain metabolised 4-hydroxyphenylacetic, 4-hydroxybenzoic, cinnamic and caffeic acid,

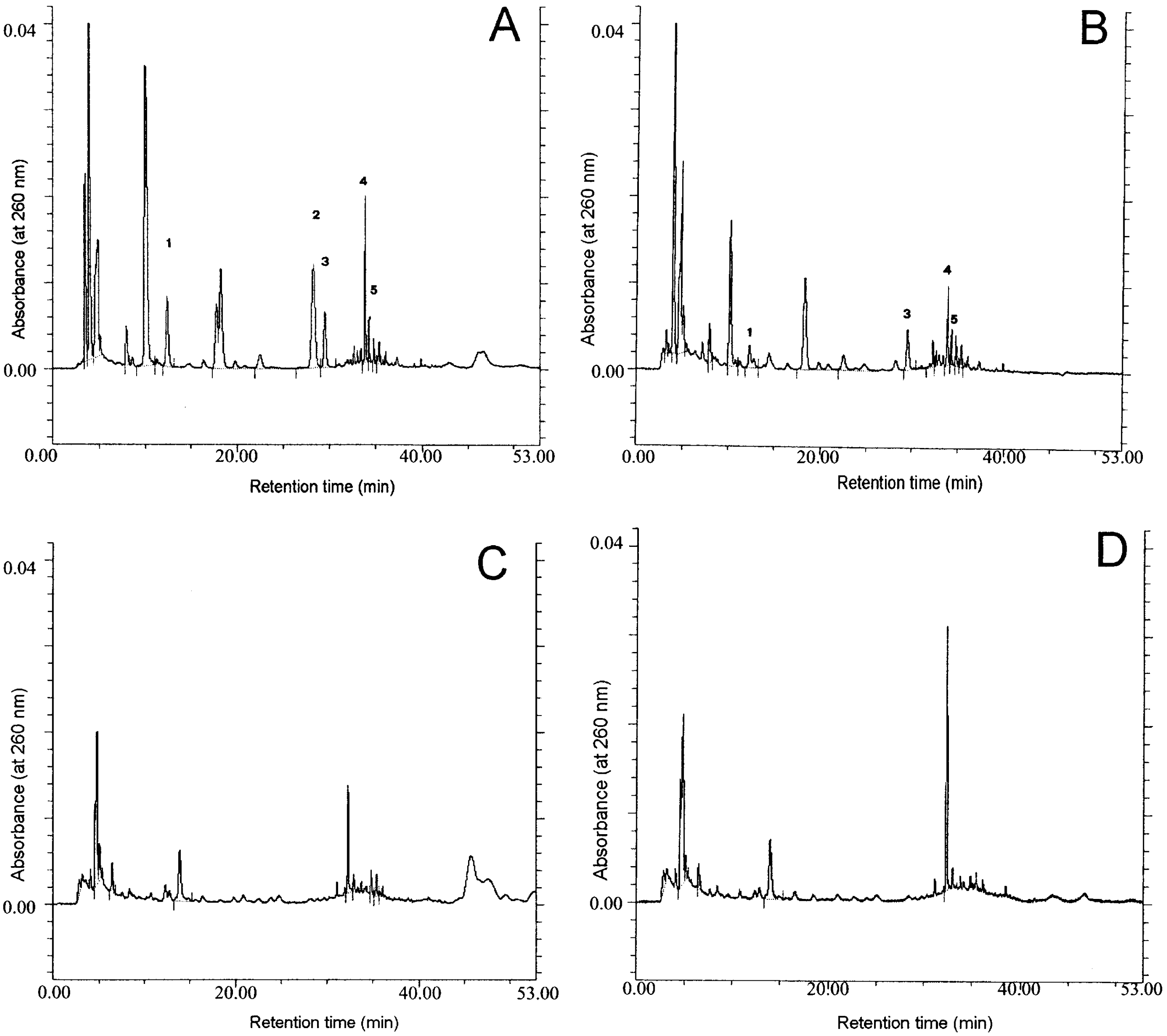


Fig. 3A–D HPLC chromatograms of the RO fraction at 20% concentration (v/v) inoculated with the LD35-DSM 1868 co-culture at the beginning of the incubation (**A**), and after 24 h (**B**), after 48 h (**C**) and after 1 week (**D**) incubation. Identified peaks: *1* catechol, *2* vanillic acid, *3* caffeic acid, *4* coumaric acid, *5* ferulic acid

whereas DSM 1868 metabolised 4-hydroxyphenylacetic, veratric, 4-hydroxybenzoic, syringic, caffeic and vanillic acid (Table 1). Initial metabolic rates were also calculated (Table 1). The DSM 1868 resting-cell samples took on a dark colour which was not observed in the LD35 samples. The only SM compound that decreased appreciably in the heat-inactivated controls (about 40–45% after 96 h) was caffeic acid; thus, the data related to caffeic acid biodegradation presented in Table 1 have been corrected by considering the amount of loss in the abiotic control.

Degradation of the SM components in growing-cell conditions

Biodegradation of the SM components (at concentrations of 100, 50, 20, and 10%) was also tested in batch growing-cell conditions. The pH of the culture broth, measured after SM addition, was in the range 6.8–7.2 for all SM concentrations employed. The biodegradation percentages obtained after a 96-h incubation, as well as the initial metabolic rates, are reported in Table 1. Metabolism of 4-hydroxybenzoic, 4-hydroxyphenylacetic and caffeic acid started at the beginning of the incubation, whereas cinnamic acid was metabolised only after a 10-h lag phase and when growing cells had already reached the late-exponential growth phase (data not shown). Metabolism of 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, veratric acid and vanillic acid by *P. putida* DSM 1868 began at the start of the incubation, whereas syringic acid was metabolised at the end

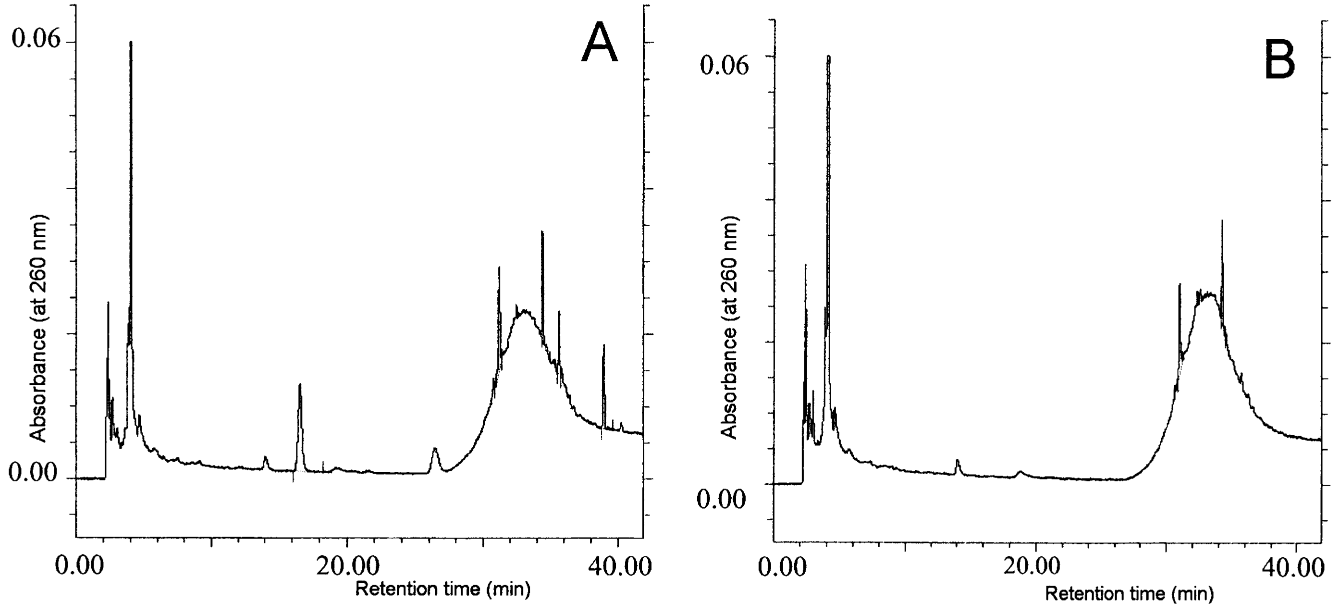


Fig. 4A, B HPLC chromatograms of the anaerobic effluent used at 25% concentration (v/v) in MMM inoculated with the LD35-DSM 1868 co-culture at the beginning of the incubation (**A**) and after 1 week (**B**)

of the logarithmic growth phase (data not shown) and its metabolism was partial (Table 1). Caffeic acid was slowly metabolised, and its initial concentration decreased by about 40–45% in the control samples; the data reported in Table 1 have therefore been corrected accordingly. In addition, the colour of the DSM 1868 cultures darkened beginning 24 h after the start of incubation.

Biodegradation experiments were also carried out with a co-culture of the two strains (inoculated at identical initial cell concentrations) on MMM containing SM at 10, 20, 50 and 100% as the sole carbon and energy source. The depletion profiles of each of the nine constituents of the SM and the evolution of the A_{600} in the cultures throughout the experiment are reported in Fig. 1. The biodegradation percentages (at day 96 of incubation) and the rate of metabolism of the SM compounds are summarised in Table 1. The A_{600} of the culture broth was found to increase quickly during the initial 25 h of incubation, due to the extensive metabolism of 4-hydroxybenzoic, 4-hydroxyphenylacetic, veratric, caffeic and vanillic acid (Fig. 1). Samples of the cultures were periodically subjected to plate counts on TSA and on selective media during the initial 72 h of incubation. The two pure cultures grew successfully on the SM constituents and exhibited similar growth curves at all the SM concentrations tested. After an initial increase of the cell concentration, a significant CFU/ml decrease was observed 14 h after the start of incubation; then, the cell number remained almost constant until the end of the incubation time. As an example, the profile obtained with 50% SM is shown in Fig. 2.

Biodegradation of the constituents of the RO fraction

A qualitative and quantitative characterisation of the mono-cyclic aromatic compounds of the RO fraction was attempted through HPLC analysis. The fraction presented several UV-detectable peaks tentatively ascribed to monomeric and polymeric aromatic compounds; only five peaks were clearly identified by co-elution with pure mono-cyclic aromatic compounds, i.e. catechol (1.36 mM), vanillic acid (0.17 mM), caffeic acid (0.11 mM), coumaric acid (4-hydroxycinnamic acid, 0.13 mM), and ferulic acid (4-hydroxy-3-methoxycinnamic acid, 0.06 mM) (Fig. 3A). The ability of the *Ralstonia* sp. LD35-*P. putida* DSM 1868 co-culture to biodegrade the HPLC UV-detectable compounds present in the RO fraction was assayed under batch growing-cell conditions by using the RO fraction at 20% concentration (v/v) as the sole carbon and energy sources. This dilution was chosen on the basis of preliminary experiments, which showed growth inhibition of both strains in the presence of the RO fraction at higher concentrations. The pH at the beginning of the incubation was 6.5 and increased to 7.8 after a 1-week incubation. HPLC analyses of the culture broth at different incubation times (Fig. 3) showed that most of the peaks present at the beginning of the incubation disappeared after 1 week, whereas two peaks, which were probably stable intermediates of the biodegraded RO compounds, appeared in the broth and increased starting from the second day of incubation. The highest of the two peaks was characterised as 4-hydroxyacetophenone.

Cell growth was monitored on selective agar plates (data not shown). The data showed that both strains grew on the RO fraction [initial concentrations: 1.5×10^7 CFU/ml (DSM 1868) and 3.3×10^7 CFU/ml (LD35); final concentrations (after 1 week): 2.8×10^9 CFU/ml (DSM 1868) and 3.4×10^9 CFU/ml (LD35)].

Biodegradation experiments employing the anaerobic effluent

The effluent employed had a COD of 8.1 g/l (Beccari et al. 2000) and contained a number of aromatic compounds detectable by HPLC analyses (Fig. 4A). The ability of the *Ralstonia* sp. LD35-*P. putida* DSM 1868 co-culture to grow on this fraction, previously sterilised by filtration, was assayed by using the effluent at 25% concentration (v/v) as the sole carbon and energy source. The pH of the active culture was 7.5 at the beginning of the incubation and was corrected to 7 during the incubation, as it tended to increase as metabolism proceeded. Plate counts on TSA and selective plates showed that both strains could grow on the fraction and, after the initial growth phase, their concentrations remained stable throughout the incubation [initial concentrations: 1.5×10^7 CFU/ml (DSM 1868) and 7.0×10^6 CFU/ml (LD35); final concentrations (after 1 week): 1.6×10^8 CFU/ml (DSM 1868) and 7.0×10^7 CFU/ml (LD35)]. By comparing the results of the HPLC analyses of the culture broth at the beginning and at the end of the incubation (Fig. 4), it could be evidenced that about four of the effluent aromatic compounds disappeared after 1 week. In the abiotic control, the levels of the aromatic constituents of the fraction assayed did not decrease.

Discussion

In this work, the ability of two aerobic bacterial strains, *Ralstonia* sp. LD35 and *P. putida* DSM 1868, to degrade a synthetic mixture of the mono-cyclic aromatic acids most commonly present in OMWs, as well as two OMW-derived fractions rich in low-molecular-weight aromatic compounds, was studied. The goal was to explore the possibility of employing the two strains in the removal of these compounds from the effluents of anaerobic treatment plants fed with OMWs. To our knowledge, this is the first study aimed at the removal of aromatic compounds from this effluent. Previous studies have focused on the reduction of the recalcitrant aromatic compound concentration in OMWs before their treatment in anaerobic digestors (Hamdi and Ellouz 1993; Borja et al. 1995; Benitez et al. 1997; Beccari et al. 1999).

In resting-cell conditions, the two bacteria tested as pure cultures degraded the SM mono-cyclic aromatic compounds that they biodegraded as pure compounds in our previous work (Table 1; Di Gioia et al. 2000). This indicates that the degradation spectrum of the two strains was not affected by the occurrence of other mono-cyclic aromatic acids in the culture media. Generally, the highest metabolic rates were observed in the presence of 20% SM, and these values tended to significantly decrease by increasing the SM concentration (Table 1), probably as a consequence of substrate inhibition phenomena.

In growing-cell conditions, both strains exhibited the same biodegradation capabilities vs the SM constituents that they displayed in resting-cell conditions (Table 1). Some of the SM compounds, such as cinnamic acid (in LD35 cultures) and syringic acid (in DSM 1868 cultures), were not degraded at the beginning of the incubation, but only after a certain A_{600} had been reached in the cultures. As these compounds are not growth substrates for the corresponding strains (Di Gioia et al. 2000), it was speculated that they were metabolised through cometabolism by LD35 or DSM 1868 cells grown on other metabolised SM constituents. The highest metabolic rates obtained under growing-cell conditions were at 50% SM, i.e. at a higher concentration than for resting-cell cultures, and, generally, the biodegradation rates were less affected by substrate (SM) concentration compared to resting-cell conditions (Table 1). The lower sensitivity of LD35 and DSM 1868 growing-cells to substrate inhibition may be due to a larger availability of energy and reducing power [NAD(P)H] in these cells compared to the corresponding suspended cells, as already hypothesised by other authors to explain the stimulation of aerobic biodegradation of aromatic compounds during microorganism growth (Kohler et al. 1988; Commandeur et al. 1996). The decrease in caffeic acid observed in the heat-inactivated controls, accompanied by the appearance of a dark colour, could be ascribed to the spontaneous oxidation and polymerisation of this compound. This is in accordance with previous observations made with other dihydroxylated aromatic molecules (Fava et al. 1993), The dark colour that was also observed in DSM 1868 active resting- and growing-cell cultures can be ascribed to the same phenomenon.

The biodegradation spectrum of each single strain complemented that of the other; thus, the effect of a co-culture, prepared by combining equal concentrations of cells of the two strains, on SM biodegradation under growing-cell conditions was examined. The metabolic rates and percent biodegradation of the SM constituents were similar to those obtained with each single strain with the same culture conditions (Table 1). This indicates that the specific biodegradation spectrum exhibited by each pure culture was maintained when the two strains were combined in a co-culture (Table 1, Fig. 1). Interestingly, veratric acid degradation, which is mediated by solely by DSM 1868, was greater (at all SM concentrations tested) when DSM 1868 was used in combination with LD35 (Table 1). This may be ascribed to the degradation by LD35 of some SM components (or related metabolites) capable of inhibiting the DSM 1868 strain. The dark colour observed in the co-culture flasks was generally lower than in the corresponding DSM 1868 cultures, probably due to rapid metabolism of caffeic acid by LD35 (Fig. 1).

Cell concentration was monitored through plate counts and A_{600} measurements; however, the two profiles differed from each other (Figs. 1, 2), probably because the A_{600} values reflected both the bacterial cell concentration and the pigment produced in the cultures. The cell count decreases observed after 14 h of incubation (see Fig. 2 for 50% SM supplemented cultures) could be ascribed to the presence of toxic/inhibitory metabolites in the culture broth. In particular, since it occurred in parallel to the darkening of the broths, it is possible to speculate that oxidative polymerisation of dihydroxylated compounds present in the SM, whose formation typically occurs through reactive and toxic radicals or ionic intermediates (Fava et al. 1993), could be responsible for the decreased cell count.

The co-culture was capable of extensively metabolising seven of the nine SM compounds; only 2, 6-dihydroxybenzoic acid and 3, 4, 5-trimethoxybenzoic acid were not degraded by the co-culture, as already evidenced in experiments previously conducted with pure compounds (Di Gioia et al. 2000). The difficulty in degrading the two compounds is not surprising; in fact, *ortho*-substituted compounds are generally highly recalcitrant to biodegradation (Reineke and Knackmuss 1988), and *meta*-methoxylated positions are attacked with difficulty by demethylating enzymes (Bernhardt et al. 1988).

In order to test biodegradation by the LD35-DSM 1868 co-culture under conditions closer to those in practical use, the co-culture was investigated for its ability to grow on the organic constituents of two OMW-derived fractions, i.e. a RO fraction, containing molecules with molecular weights in the range <350, and the effluent of an an aerobic pilot plant. The co-culture grew successfully on the two fractions, which supported comparable growth of the two co-culture members. The LD35-DSM 1868 co-culture rapidly biodegraded the monocyclic aromatic compounds (catechol, vanillic acid, caffeic acid, coumaric acid, ferulic acid, and other non-identified compounds) in the RO fraction (Fig. 3). In addition, the co-culture removed some of the low-molecular-weight aromatic compounds detected in the anaerobic effluent assayed (Fig. 4). To our knowledge, this is the first time that aerobic bacteria have been assayed for their biodegradation potential vs these types of OMW-derived fractions.

In conclusion, the data presented in this work suggest that the *Ralstonia* sp. LD35-*P. putida* DSM 1868 co-culture can be used in the development of an aerobic biotechnological process aimed at removing recalcitrant low-molecular-weight aromatic compounds present in OMWs, and in particular in the effluent of anaerobic treatment plants fed with OMWs.

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