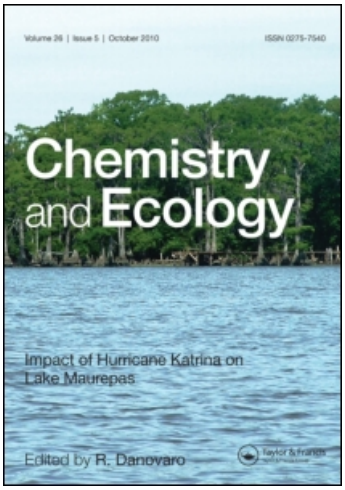

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Structure – biodegradation correlation of polyphenols for *Thauera aromatica* in anaerobic conditions

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The anaerobic degradation of phenolic compounds depends greatly on their molecular structure. We have undertaken a systematic study on several phenol derivatives which are commonly found in industrial waters with the aim of investigating the structure–biodegradation relationship for bacteria in anaerobic conditions. The *Thauera aromatica* strain was used; this bacterium is able to use phenol as a sole carbon source in liquid cultures in the absence of molecular oxygen. Nitrate is the electron sink with complete reduction to N_2. A systematic study has demonstrated the ability of this bacterial strain to use 3,4-dihydroxybenzoic acid, 4-hydroxycinnamic acid (*p*-coumaric acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,4-dimethoxybenzoic acid (veratric acid), and 3,4,5-trihydroxybenzoic acid (gallic acid) as substrate. The matching of the depletion of the concentration of such compounds with the increment in bacterial growth in the cultures demonstrates that the substrates are used as the sole carbon source. Also, a correlation between the rate of growth of bacteria and nitrite concentration has been observed. The progressive reduction in nitrate into nitrite and N_2 is monitored through analyses. The protein pattern extracted from bacteria grown on methylated substrates is now being compared with that of bacteria grown on non-methylated polyphenols that are structurally analogous in order to identify specific enzymes for demethylation processes.

Keywords: Polyphenols; Anaerobic metabolism; *Thauera aromatica*; Demethylation

1. Introduction

Phenolic compounds are plant constituents and very common organic pollutants in wastewater, soil, and sediments. They are produced by petroleum refineries, coke ovens, and wood industries, and in the manufacture of plastics, resins, dyes, pesticides, and pharmaceuticals [1]. Polyphenols are also present in high concentrations in olive mills and industrial sugar wastewaters. These compounds are considered toxic owing to their ability to inhibit microorganisms and are, in many cases, recalcitrant to biodegradation [2]. However, some species of bacteria, yeasts, and fungi [3] are able to metabolize polyphenols under aerobic conditions

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and some bacteria also under anaerobic conditions [4]. In aerobic metabolism, molecular oxygen is used to cleave the aromatic ring structures; instead, under anaerobic conditions, some microrganisms can completely degrade aromatic compounds to CO_2 and H_2O [5], and others can partially modify their structure [6].

It follows that there may be a correlation between the polyphenol molecular structure and resistance to biodegradation, but no study has used a single microrganism strain on a series of polyphenols with correlated structures and highlighted the role of the number and nature of ring substituents in modulating the bio-resistance of polyphenols. In the literature, it is reported that ferulic acid (see figure 1) is used by *Pseudomonas fluorescens* AN 103 [7], *Corynebacterium glutamicum* [8], *Pseudomonas cepacia* [9], *Lactobacillus plantarum* [10], *Brettanomyces anomalus* [11], and *Clostridium glycolicum* [12] as the sole source of carbon. *Streptococcus gallolyticus* [13] and *Acinetobacter calcoaceticus* DSM 586 [14] have been shown to grow on both ferulic acid and *p*-coumaric acid. Methoxylated compounds like syringic acid, veratric acid, and 3,4,5-trimethoxybenzoic acid have rarely been investigated for their degradation by aerobic microrganisms. Little is known about the biodegradation of such compounds in anaerobic conditions.

Sporomusa ovata metabolizes veratric acid aerobically [15], while *Sphingomonas paucimobilis* SYK-6 can use syringic acid [16] as a carbon source, and *Clostridium methoxyvorans* sp. nov. uses syringic acid and 3,4,5-trimethoxybenzoic acid [17] for its growth.

In our studies, we have used the denitrifying bacterium *Thauera aromatica* K172 [18] and tested its capacity to adapt to structurally related polyphenols and to use them as

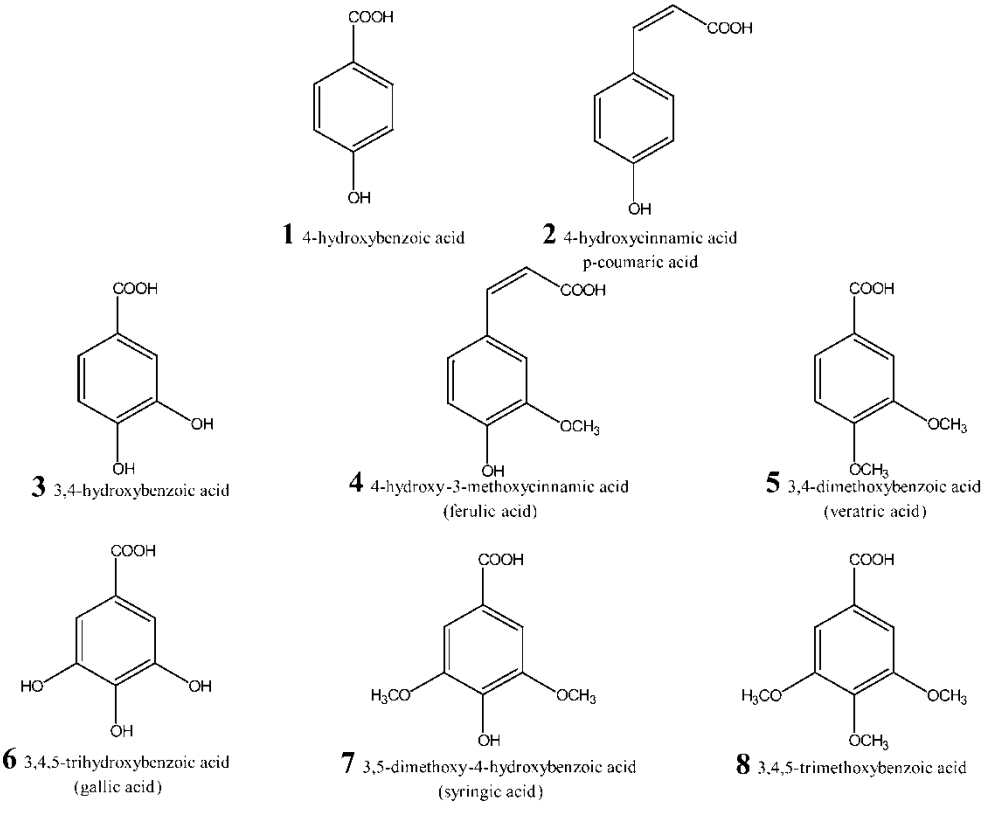

Figure 1. Substrates tested as the sole carbon source for *T. aromatica* under anaerobic conditions.

the sole carbon source. We have already described the degradation of 4-hydroxybenzoic acid [19]. In this paper, we report the results of the adaptation of *T. aromatica* to the correlated polyphenols: 4-hydroxycinnamic acid (*p*-coumaric acid), 3,4-dihydroxybenzoic acid, 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,4-dimethoxybenzoic acid (veratric acid), 3,4,5-trihydroxybenzoic acid (gallic acid), 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), and 3,4,5-trimethoxybenzoic acid (figure 1).

As shown in figure 1, compounds 1 and 2 have a single OH group in position 4 and differ in the substituent in position 1. Compounds 3 and 4 bear two OH groups. They differ in the group in position 1 in the ring (compare with 1 and 2) and in the degree of methylation of hydroxo groups in position 3 and 4. Compounds 6–8 have three OH groups in position 3–4–5: they differ in the degree of methylation. Using these substrates, we have been able to compare both the influence of the number of OH groups on biodegradation and the effect of the methylation of such groups.

2. Materials and methods

2.1 *Growth conditions*

Thauera aromatica K172 was cultivated at 28 °C under strictly anaerobic conditions in a basal medium containing (l^{-1}) Na_2KPO_4 0.8 g, KH_2PO_4 1.4 g, and K_2SO_4 0.052 g, using 0.5 l glass flasks sealed with latex rubber septa. After autoclaving and cooling under an N_2 atmosphere, the following components were added from sterile solutions: trace elements solutions (1 ml l^{-1}) containing HCl (25%, 7.7 M, 2.5 g l^{-1}); $FeCl_2 \cdot 4H_2O$ (1.5 g l^{-1}); $ZnCl_2$ (68 mg l^{-1}); $MnCl_2 \cdot 4H_2O$ (100 mg l^{-1}); H_3BO_3 (6 mg l^{-1}); $CoCl_2 \cdot 6H_2O$ (190 mg l^{-1}); $CuCl_2 \cdot 2H_2O$ (3 mg l^{-1}); $NiCl_2 \cdot 2H_2O$ (24 mg l^{-1}); $Na_2MoO_4 \cdot 2H_2O$ vitamins solution (0.5 ml l^{-1}) containing: cyanocobalamin (100 mg l^{-1}), tyamine hydrochloride (200 mg l^{-1}), pantothenate (100 mg l^{-1}), 4-aminobenzoate (80 mg l^{-1}), biotin (200 mg l^{-1}), nicotinic acid (200 mg l^{-1}) piridoxine (300 mg l^{-1}); Mg^{2+}/Ca^{2+} solution (0.5 ml l^{-1}), containing: $MgCl_2 \cdot 6H_2O$ (200 g l^{-1}) and $CaCl_2 \cdot 2H_2O$ (50 g l^{-1}); $NaHCO_3$ (42 g l^{-1}) solution (5 ml l^{-1}), KNO_3 (200 g l^{-1}) solution (1 ml l^{-1}). All these solutions were sterilized either by autoclaving (121 °C, 1 atm, 20 min) or by filtration using sterile syringe filter units (0.2 μm pore size).

Bacteria were grown on phenol, benzoate, or ferulic acid and then isolated and transferred with absolute exclusion of air using the vacuum-line technique for the cultures of the appropriate substrate, as detailed below.

2.2 *Substrate utilization*

All the experiments were performed in triplicate using an inoculum with 2% of an exponentially growing preculture. The substrates tested were sterilized by filtration (0.2 μm pore size) and injected from a concentrated solution to obtain a 5 mM final concentration.

The substrates used for the tests (figure 1) were: 4-hydroxybenzoic acid (1); 4-hydroxycinnamic acid (*p*-coumaric acid) (2); 3,4-dihydroxybenzoic acid (3); 4-hydroxy-3-methoxybenzoic acid (ferulic acid) (4); 3,4-dimethoxybenzoic acid (veratric acid) (5); 3,4,5-trihydroxybenzoic acid (gallic acid) (6); 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid) (7); and 3,4,5-trimethoxybenzoic acid (8). All the bottles containing the added substrates were compared with control bottles containing the medium without substrates.

2.3 *Analytical methods: Determination of growth parameters*

Bacterial growth was monitored by measuring the A_{578} corrected for the absorbance of the medium using 1 cm cuvettes. The consumption of the substrates was determined by taking UV-absorption spectra of the liquid cultures after centrifugation at 4500 g for 45 min after a 1:10 diluition. The maximum absorption wavelengths for the tested substrates were: 280 nm for 4-hydroxybenzoic acid ($\varepsilon_{280} = 19075\,\text{mol}\,\text{l}^{-1}\,\text{cm}^{-1}$); 286 nm for 4-hydroxycinnamic acid (*p*-coumaric acid) ($\varepsilon_{286} = 25513\,\text{mol}\,\text{l}^{-1}\,\text{cm}^{-1}$); 250 nm for 3,4-dihydroxybenzoic acid ($\varepsilon_{250} = 3655\,\text{mol}\,\text{l}^{-1}\,\text{cm}^{-1}$); 287 nm for 4-hydroxy-3-methoxycinnamic acid (ferulic acid) ($\varepsilon_{287} = 13567\,\text{mol}\,\text{l}^{-1}\,\text{cm}^{-1}$); 252 nm for 3,4-dimethoxybenzoic acid (veratric acid) ($\varepsilon_{252} = 9474\,\text{mol}\,\text{l}^{-1}\,\text{cm}^{-1}$); 259 nm for 3,4,5-trihydroxybenzoic acid ($\varepsilon_{259} = 8232\,\text{mol}\,\text{l}^{-1}\,\text{cm}^{-1}$); 262 nm for 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid) ($\varepsilon_{262} = 8985\,\text{mol}\,\text{l}^{-1}\,\text{cm}^{-1}$); and 253 nm for 3,4,5-trimethoxybenzoic acid ($\varepsilon_{253} = 7343\,\text{mol}\,\text{l}^{-1}\,\text{cm}^{-1}$). The nitrate or nitrite concentration was determined by colorimetric tests.

Additions of nitrate and substrate were periodically done to maintain a correct level of concentration of the two species, a fundamental condition for cellular growth. In particular, nitrate was added in small amounts, and the substrate was added when the concentration resulted fell below 2.5 mM.

3. Results and discussion

3.1 *Adaptation of* Thauera aromatica *strain to some substrates under test*

3.1.1 3,4-Dihydroxybenzoic acid. *Thauera aromatica* can conveniently grow on different substrates. We have developed techniques for their growth on phenol, benzoate, or ferulic acid as the sole carbon source. Cells from such cultures were used for adaptation to the new substrates.

As shown in figure 2, cells grown on benzoate (▲) are not able to metabolize 3,4-dihydroxybenzoic acid at a convenient rate. Induction of benzoate-grown cells for

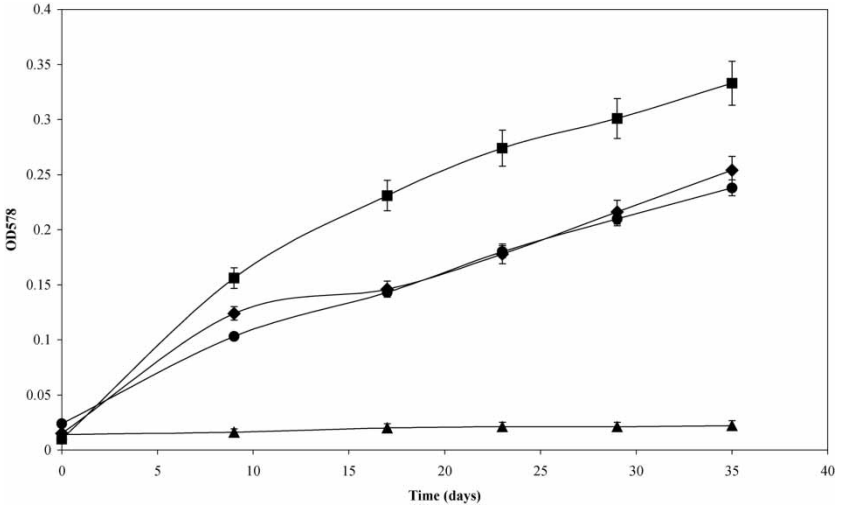

Figure 2. Turbidity of *T. aromatica* cultures on 3,4-dihydroxybenzoic acid using bacteria grown on different substrates. ▲: benzoate; ●: ferulic acid; ♦: phenol; ■: 3,4-dihydroxybenzoic acid.

3,4-dihydroxybenzoic acid in *Thauera aromatica* strain AR-1 has been reported [20]. This is probably due to a different expression of the enzymes involved in the degradation of this compound in *Thauera aromatica* strain K172 and *Thauera aromatica* strain AR-1.

Conversely, cells grown on phenol or ferulic acid readily use these substrates as carbon source; after 35 d, the turbidity increased 17-fold for the culture on phenol and 10-fold for the cultures on ferulic acid. Bacteria freshly isolated from 3,4-dihydroxybenzoic acid grow more rapidly on the same substrate: in particular, the turbidity increases 33-fold in 35 d.

3.1.2 Ferulic acid. Also, in the case of ferulic acid, bacteria grown on benzoate have a poor ability to degrade such a substrate; instead, cells grown on phenol show an easier adaptation to it (figure 3). The culture turbidity increased 50-fold in 20 d. Cells already adapted to ferulic acid increase the turbidity 123-fold in 20 d.

3.1.3 Veratric acid. As shown in figure 4, no bacterial growth is evident when *T. aromatica* grown on benzoate is used in a medium with veratric acid as the sole carbon source. Also, for *T. aromatica* grown on phenol, after a first phase of induction and growth during days 5–10, no relevant increase in OD_{578} is further observed. Instead, cultures on ferulic acid show a good ability to grow with veratric acid as the sole carbon source, with a 23-fold increase in turbidity. Cultures on veratric acid show a slightly higher degradative capacity than cultures on ferulic acid (24-fold increase in turbidity in 25 d).

A similar trend was observed with 4-hydroxycinnamic acid (*p*-coumaric acid) and 3,4,5-trihydroxybenzoic acid (gallic acid). Therefore, we have used *T. aromatica* grown on ferulic acid for faster growth on polyphenols.

3.1.4 4-Hydroxy-3-methoxycinnamic acid (syringic acid) and 3,4,5-trimethoxybenzoic acid. All the adaptation tests (using cells grown on benzoate, phenol, veratric acid, and ferulic acid) conducted on these substrates have shown a very slow growth.

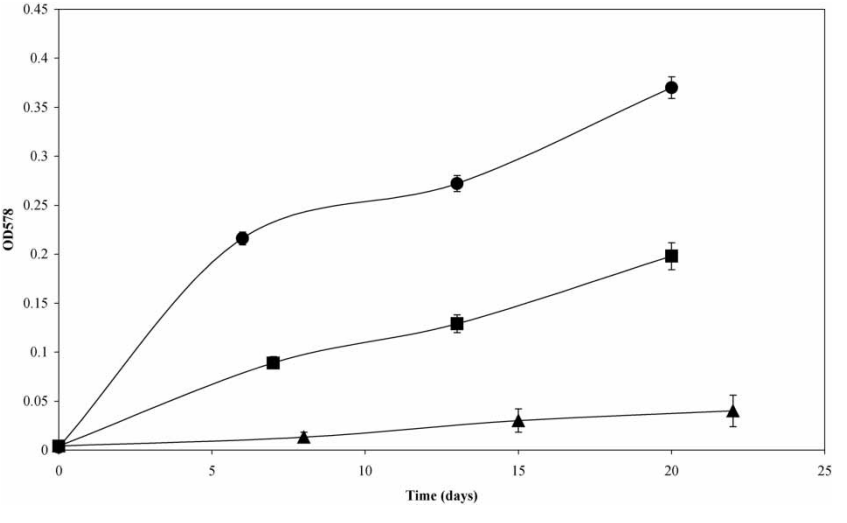

Figure 3. Turbidity of *T. aromatica* cultures on ferulic acid using bacteria grown on different substrates. ▲: benzoate; ●: ferulic acid; ♦: phenol.

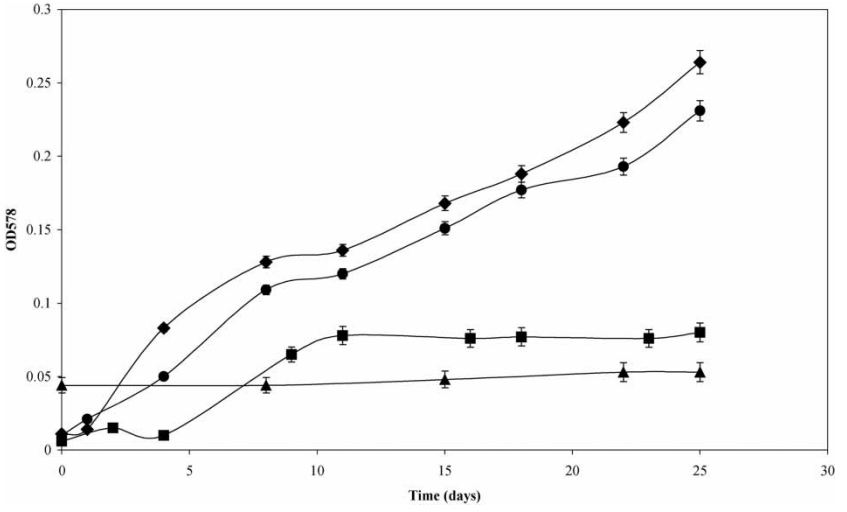

Figure 4. Turbidity of *T. aromatica* cultures on veratric acid using bacteria grown on different substrates. ▲: benzoate; ●: ferulic acid; ♦: phenol; ■; 3,4-dihydroxybenzoic acid.

3.2 *Phenolic compound degradation*

All compounds were used at a concentration of 5 mM. Figure 5 compares the depletion in concentration for the compounds tested.

It is evident that compounds 4-hydroxycinnamic acid (*p*-coumaric acid) (2), 3,4-dihydroxybenzoic acid (3), 4-hydroxy-3-methoxycinnamic acid (ferulic acid) (4), 3,4-dimethoxybenzoic acid (veratric acid) (5), and 3,4,5-trihydroxybenzoic acid (6) are used as the sole carbon source by *T. aromatica*, but at different rates of degradation. This type of bacterial strain shows a limited ability to degrade 4-hydroxy-3-methoxycinnamic acid (syringic acid) and 3,4,5-trimethoxybenzoic acid.

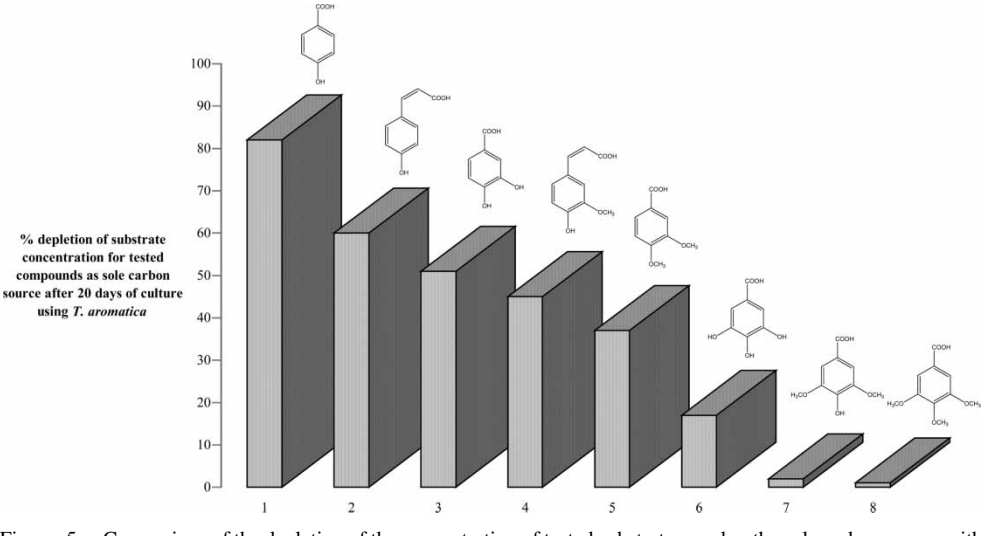

Figure 5. Comparison of the depletion of the concentration of tested substrates used as the sole carbon source with *Thauera aromatica* cultures at 20 d.

Comparing 1 with 2, it appears that substitution of the COOH group with the cinnamic group causes a deceleration of the rate of bacterial growth. For compounds 3, 4, and 5, one can observe as a general trend a lower degradability with respect to 1 and 2. Within the series 3, 4, and 5, the increase in substitution of OH with the OCH_3 group decreases the degradation ability. Moving from mono- to tri-substituted benzoic acid, the degradation process becomes more difficult. In particular, while 3,4,5 trihydroxybenzoic acid can be degraded very slowly, its methoxo congeners are used by *T. aromatica* as the source of carbon to a very limited extent: after 4 weeks of incubation, very limited bacterial growth and substrate consumption was detected.

Figure 5 shows that increasing the number of OH groups makes the compound more recalcitrant. Moreover, the substitution of a OH group with a methoxo group increases the resistance to biodegradation. A rationale for such a trend can be found in the fact that all compounds used are converted into benzoic acid, which follows the normal degradation path [21]. Such conversion requires the presence of dehydroxylating and demethylating enzymes. While the

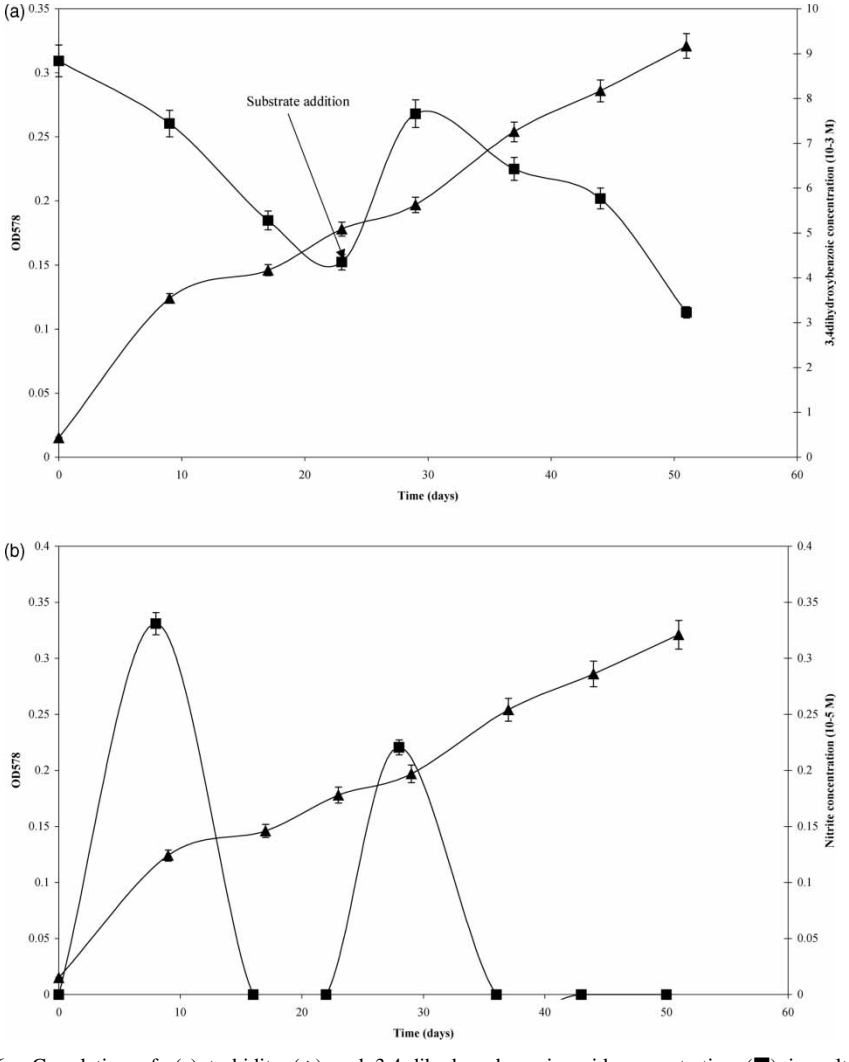

Figure 6. Correlation of: (a) turbidity (▲) and 3,4-dihydroxybenzoic acid concentration (■) in cultures of *T. aromatica*; (b) turbidity (▲) and nitrite concentration (■) in cultures of *T. aromatica.*

former are present in *T. aromatica*, which grows on phenol, the latter are not. Most likely, in order to adapt to methylated substrates, *T. aromatica* has to express a demethylating protein. Such enzymes have been reported recently for aerobic bacteria [22] but are not known for anaerobic microrganisms. Our attempts are now in the direction of the extraction of the protein pool from bacteria grown on methylated substrates in order to provide evidence for the presence of demethylating enzymes.

3.3 *Correlation of bacterial growth with nitrate reduction*

We have also investigated the correlation of biodegradation with the concentration of nitrate and nitrite in the medium. Figure 6a shows the substrate consumption (■) and turbidity (▲) increase for a culture of 3,4-dihydroxybenzoic acid.

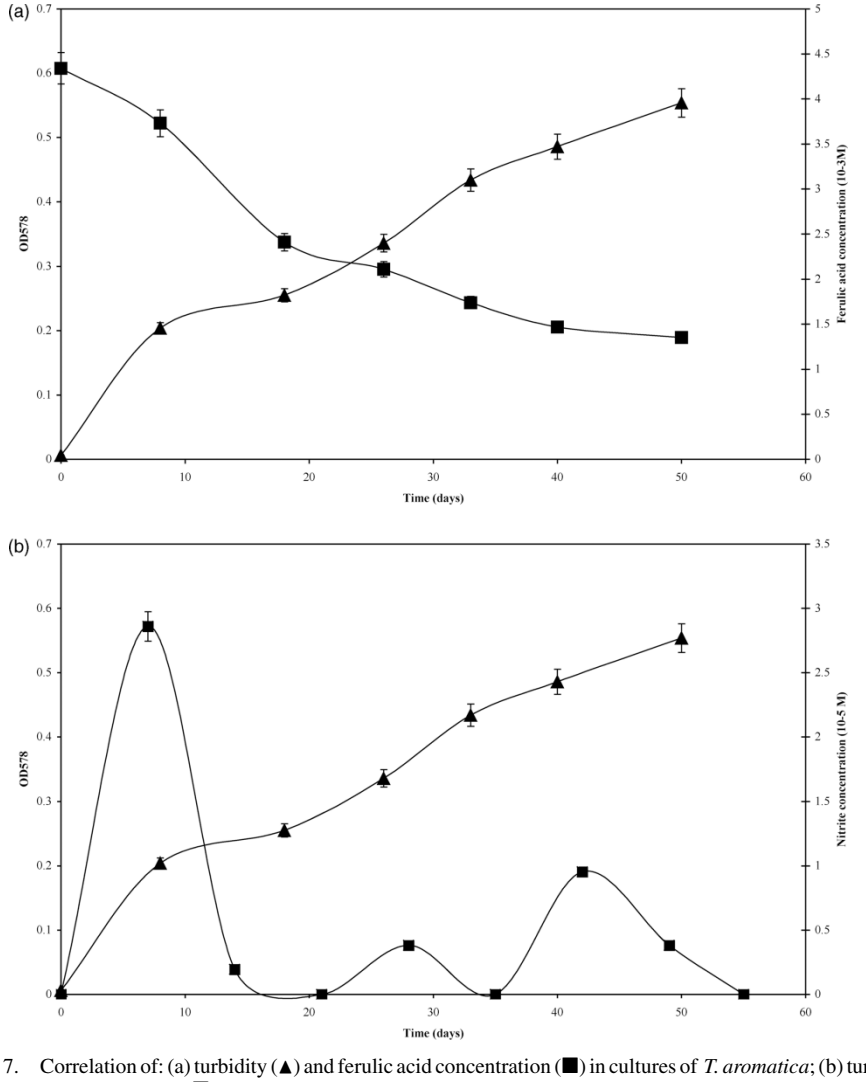

Figure 7. Correlation of: (a) turbidity (▲) and ferulic acid concentration (■) in cultures of *T. aromatica*; (b) turbidity (▲) and nitrite concentration (■) in cultures of *T. aromatica.*

The trends of the two curves correlate reasonably well. Figure 6b shows the correlation between bacterial growth and nitrite concentration. Nitrate is not reduced directly to N_2 but is more likely first converted into nitrite (equation (1)):

$$NO_3^- \longrightarrow NO_2^- \longrightarrow N_2, \tag{1}$$

which accumulates in the medium and is then reduced to N_2, as shown by the increase in gas pressure in the culture bottle. The accumulation of nitrite (figure 6b, day 10) causes a deceleration in the growth rate of the microrganisms. A plateau in the growing curve is observed once the nitrite concentration reaches its maximum. Nitrite accumulation is more evident within the first 30 d and tends to disappear with time. The origin of such trends is not is not currently clear. It has been suggested [23] that nitrite accumulation can depend on different factors such as inhibition of nitrite reductase or fluctuations in environmental

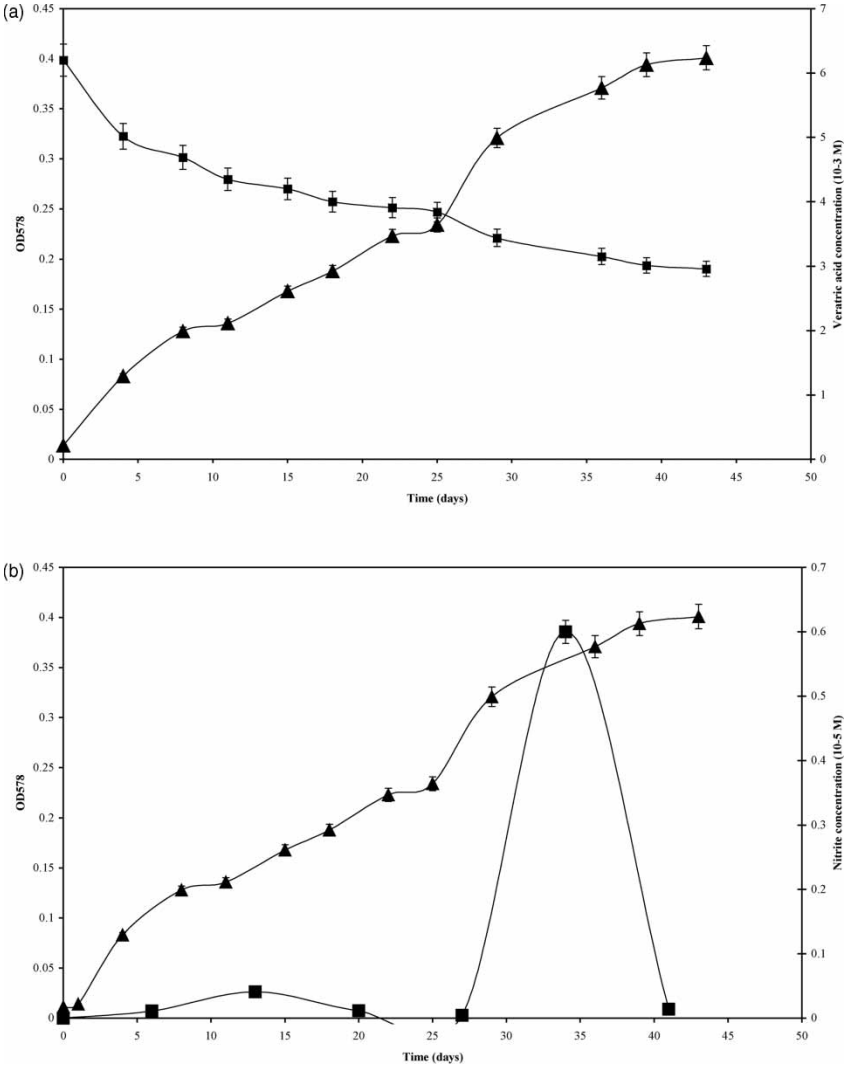

Figure 8. Correlation of: (a) turbidity (▲) and veratric acid concentration (■) in cultures of *T. aromatica*; (b) turbidity (▲) and nitrite concentration (■) in cultures of *T. aromatica.*

conditions in continuous cultures of a mixed population of denitrifying bacteria. Figure 7a shows the correlation of the bacterial growth to the depletion of the concentration of the substrate ferulic acid.

Also, in this case, the nitrate is reduced first to nitrite and the accumulation of the latter in the medium slows down bacterial growth. When nitrite is reduced to N_2, bacterial growth restarts. Nitrite formation is higher in the first 10 days and tends to decrease with time.

Veratric acid (figure 8b) almost does not show the accumulation of nitrite until day 30, when it appears at a considerable concentration with evident reduction in the growth rate (plateau in curve 8b). It is thus confirmed that the use of nitrate by the bacterial strain depends on the nature of the substrate [4].

4. Conclusions

The biodegradation of structurally related polyphenols under anaerobic conditions has been studied. We have demonstred that *Thauera aromatica* K172, under strictly anaerobic conditions, can use (as the sole carbon source) 4-hydroxycinnamic acid (*p*-coumaric acid), 3,4-dihydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid (ferulic acid), 3,4-dimethoxybenzoic acid (veratric acid), and 3,4,5-trihydroxybenzoic acid (gallic acid). For the first time, we have reported the trend of nitrite concentration towards growth rate for some of the substrates and shown that nitrate is not reduced directly to N_2 but first converted into nitrite (which may accumulate in the medium with a consequent slowing down of the growth rate), which is then converted into N_2. We have also shown that *T. aromatica* grown on benzoic acid or phenol may show a different adaptation to polyphenols. Moreover, the adaptation of bacteria to the new substrates is very much dependent on the molecular structure of the substrate. In particular, increasing the number of OH groups decreases the biodegradability. With a given number of OH groups in the molecules (max 3), the progressive methylation of such groups decreases the biodegradability. This can be related to the need to express specific proteins (dehydroxylating and demethylating enzymes) for the conversion of the initial polyphenol to benzoic acid, which seems to be a common end-product. While *Thauera aromatica* is known to have a dehydroxylating enzyme as it grows on phenol, it does not have a demethylating enzyme. Our work is now directed towards comparing the enzymatic pattern of bacteria grown on 3,4-dihydroxybenzoic acid with that of bacteria grown on the methylated form, 3,4-dimethoxybenzoic acid (veratric acid), with the aim of isolating and characterizing the enzyme responsible for demethylation, which is not known for bacteria grown under anaerobic conditions.

References

[1] S. Beszedits, M.D. Silbert. Treatment of phenolic wastewaters. B and L Information Services. Available online at: http://www.silbert.org/BandLlist.html (accessed September 1999).
[2] R. Capasso, A. Evidente, L. Schivo, G. Orru, M.A. Marcialis, G. Cristinzio. Antibacterial polyphenols from olive mill wastewaters. *J. Appl. Bacteriol.*, **79**, 393–398 (1995).
[3] L. Martirani, P. Giardina, L. Marzullo, G. Sannia. Reduction of phenol content and toxicity in olive oil mill waste waters with the ligninolytic fungus *Pleurotus ostreatus. Water Res.*, **30**, 1914–1918 (1996).
[4] M. Aresta, A. Dibenedetto, M. Narracci, I. Tommasi. A technology for the treatment of olive-mill waste water in a continuously fed plant. *Environ. Chem. Lett.*, **1**, 13–18 (2003).
[5] J. Koch, G. Fuchs. Enzymatic reduction of benzoyl-CoA to alicyclic compounds, a key reaction in anaerobic aromatic metabolism. *Eur. J. Biochem.*, **205**, 195–202 (1992).
[6] M. Chamkha, B.K.C. Patel, J. Garcia, M. Labat. Isolation of *Clostridium bifermentans* from oil mill wastewaters converting cinnamic acid to 3-phenylpropionic acid and emendation of the species. *Anaerobe*, **7**, 189–197, (2001).

[7] C. Civolani, P. Barghini, A.R. Concetti, M. Ruzzi, A. Schiesser. Bioconversion of ferulic acid into vanillic acid by means of a vanillate-negative mutant of *Pseudomonas fluorescens* strain BF13. *Appl. Environ. Microbiol.*, **66**, 2311–2317 (2000).
[8] I.M. Labuda, K.A. Keon, S.K. Goers. Microbial bioconversion process for the production of vanillin. In *Progress in Flavour Precursor Studies. Analysis-Generation Biotechnology*, P. Schreier and P. Winterhalter (Eds), pp. 477–482, Allured, Carol Stream, IL (1993).
[9] V. Andreoni, E. Galli, G. Galliani. Metabolism of ferulic acid by a facultatively anaerobic strain of *Pseudomonas cepacia. Syst. Appl. Microbiol.*, **5**, 299–304 (1984).
[10] L. Barthelmebs, C. Diviès, J. Cavin. Molecular characterization of the phenolic acid metabolism in the lactic acid bacteria *Lactobacillus plantarum. Lait*, **81**, 161–171 (2001).
[11] D.A.N. Edlin, A. Narbad, J.R. Dickinson, D. Lloyd. The biotransformation of simple phenolic compounds by *Brettanomyces anomalus. FEMS Microbiol. Lett.*, **125**, 311–316 (1994).
[12] M. Chamka, M. Labat, B.K.C. Patel, J. Garcia. Isolation of a cinnamic acid-metabolizing *Clostridium glycolicum* strain from oil mill wastewaters and emendation of the species description. *Int. J. Syst. Evolut. Microbiol.*, **51**, 2049–2054 (2001).
[13] M. Chamkha, B.K.C. Patel, A. Taore, J. Garcia, M. Labat. Isolation from a shea cake digester of a tannin-degrading *Streptococcus gallolyticus* strain that decarboxylates protocatechuic and hydroxycinnamic acid and emendation of the species. *Int. J. Syst. Evolut. Microbiol.*, **52**, 939–944 (2002).
[14] D. Delneri, G. Degrassi, R. Rizzo, V.C. Bruschi. Degradation of *trans*-ferulic acid and *p*-coumaric acid by *Acinetobacter calcoaceticus* DSM 586. *Biochim. Biophys. Acta*, **1244**, 363–367 (1995).
[15] E. Stupperich, R. Konle, C. Eckerskorn. Anaerobic O-demethylations of methoxynaphthols, methoxyfuran and fluoroanisols by *Sporomusa ovata. Biochem. Biophys. Res. Commun.*, **223**, 770–777 (1996).
[16] S. Nishikawa, T. Sonoki, T. Kasahara, T. Obi, S. Kubota, S. Kawai, N. Morohoshi, Y. Katayama. Cloning and sequencing of the *Sphingomonas* (*Pseudomonas*) *paucimobilis* gene essential for the *o*-demethylation of vanillate and syringate. *Appl. Environ. Microbiol.*, **64**, 836–842 (1998).
[17] T. Mechichi, M. Labat, B.K.C. Patel, T.H.S. Woo, P. Thomas, J. Garcia. *Clostridium methoxyvorans* sp. nov., a new aromatic *o*-demethylating homoacetogen from an olivemill wastewaters treatment digester. *Int. J. Syst. Bacteriol.*, **49**, 1201–1209 (1999).
[18] S. Breinig, E. Schiltz, G. Fuchs. Genes involved in anaerobic metabolism of phenol in the bacterium *Thauera aromatica. J. Bacteriol.*, **182**, 5849–5863 (2000).
[19] M. Aresta, E. Quaranta, L. Liberio, C. Dileo, I. Tommasi. Enzymatic synthesis of 4-hydroxybenzoic acid from phenol and CO_2: the first example of a biotechnological application of a carboxylase enzyme. *Tetrahedron*, **54**, 8841–8846 (1998).
[20] B. Philipp, D. Kemmler, J. Hellstern, N. Gorny, A. Caballero, B. Schink. Anaerobic degradation of protocatechuate (3,4-dihydroxybenzoate) by *Thauera aromatica* strain AR-1. *FEMS Microbiol. Lett.*, **212**, 139–143 (2002).
[21] A. Lack, I. Tommasi, M. Aresta, G. Fuchs. Catalytic properties of phenol carboxylase. *In vitro* study of CO_2: 4-hydroxybenzoate isotope exchange reaction. *Eur. J. Biochem.*, **197**, 473–479 (1991).
[22] B. Morawski, A. Segura, N. Ornston. Substrate range and genetic analysis of *Acinetobacter* vanillate demethylase. *J. Bacteriol.*, **182**, 1383–1389 (2000).
[23] M. Martienssen, R. Schops. Population dynamics of denitrifying bacteria in a model biocommunity. *Water Res.*, **33**, 639–646 (1999).