RESEARCH LETTER

Bioconversion of tyrosol into hydroxytyrosol and 3,4-dihydroxyphenylacetic acid under hypersaline conditions by the new *Halomonas* sp. strain HTB24

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Abstract

This paper reports the characterization of a *Halomonas* sp. strain (named HTB24) isolated from olive-mill wastewater and capable of transforming tyrosol into hydroxytyrosol (HT) and 3,4-dihydroxyphenylacetic acid (DHPA) in hypersaline conditions. This is the first time that a halophile has been shown to perform such reactions. The potent natural antioxidant HT was obtained through a C3 hydroxylation on the ring cycle, whereas DHPA was synthesized via the 4-hydroxyphenylacetic acid (HPA) pathway, which has been well described from other bacterial sources. HT was produced first, and then DHPA was detected in the medium accompanied by traces of HPA. HPA involved another pathway resulting from the activity of an aryl-dehydrogenase, which is suggested to be responsible for both tyrosol and hydroxytyrosol oxidation. Maximal HT content (2.30 mM) and maximal DHPA (5.15 ± 0.42 mM) were obtained from a culture inoculated in the presence of 20 mM tyrosol and 0.5 g L^{-1} yeast extract. Following this, DHPA was quickly degraded into 5-carboxymethyl-2-hydroxymuconic semialdehyde by a 2,3-dioxygenase, finally resulting in succinate and pyruvate. Phylogenetic analysis of the 16S rRNA gene revealed that this isolate was a member of the genus *Halomonas*. Strain HTB24, with a G+C content of 55.3 mol%, is closely related to *Halomonas neptunia* DSM 15720^T, '*Halomonas alkaliantarctica*' DSM 15686^T and *Halomonas boliviensis* DSM 15516^T.

Introduction

Many industrial processes use salts and frequently release brine effluent into the environment. Another type of pollution for industrial effluents involves the production of large amounts of organic compounds (Oren *et al.*, 1992; Margesin & Schinner, 2001). Olive-mill wastewater (OMW) combines both of these types of pollution, and therefore represents a major environmental problem in the Mediterranean region (Sayadi & Ellouz, 1995). First, these polluted effluents carry large amounts of salt, related to olive conservation and olive-brine-fermentation processes. Second, OMW represents a source of pollution because it contains polyphenols, polyalcohols and a wide variety of toxic aromatic compounds resulting from olive cell-wall degradation during the oil-extraction process (Balice & Cera, 1984; Hamdi, 1993; Labat *et al.*, 2000; Lesage-Meesen *et al.*, 2001). Tyrosol is one of the major phenolic compounds present in OMW, and one that exhibits toxicity towards several microorganisms (Paredes *et al.*, 1986; Rodriguez *et al.*, 1988; Capasso *et al.*, 1992). Moreover, tyrosol and its derivatives are highly resistant to oxidation by air/oxygen and to bacterial and enzymatic degradation. Thus, halophilic bacteria, which are extremophilic microorganisms adapted to live in saline environments (Kushner *et al.*, 1978), are likely to be useful for the bioremediation of these hypersaline effluents containing high levels of tyrosol.

The microbial strategy involved in the aerobic metabolism of aromatic compounds, in almost all known cases, starts with hydroxylation reactions of the aromatic ring, mediated by mono- and di-oxygenases to destabilize the resonance of the benzene ring. These reactions generate mainly dihydroxylated aromatic compounds, which are of industrial interest (Salvo *et al.*, 1990). Recently, the

biotransformation of tyrosol [2-(*p*-hydroxyphenyl)-ethanol] into both hydroxytyrosol [2-(3,4-dihydroxyphenyl)-ethanol] and 3,4-dihydroxyphenylacetic acid was performed under various conditions, using resting cells or immobilized cells. A number of species of aerobic bacteria, for example *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas putida* F6, were described as able to metabolize tyrosol (Allouche *et al.*, 2004; Allouche & Sayadi, 2005; Bouallagui & Sayadi, 2006; Brooks *et al.*, 2006). Hydroxytyrosol has a range of properties, such as scavenging of free radicals, protection against oxidative DNA damage, and low-density-lipoprotein oxidation, and a strong antioxidant activity (Aruoma *et al.*, 1998; Visioli *et al.*, 1998). Moreover, it has been demonstrated, by mixing 3,4-dihydroxyphenylacetic acid and hydroxytyrosol, that a synergic action leading to higher antioxidant activity occurs (Fki *et al.*, 2005).

This paper reports, for the first time, a halophilic isolated strain capable of transforming tyrosol into both hydroxytyrosol and 3,4-dihydroxyphenylacetic acid, two potent natural antioxidants. This strain, named *Halomonas* sp. strain HTB24, was isolated from OMW.

Materials and methods

Culture conditions

Bacteria were grown in a basal medium containing (in $g\,L^{-1}$) NaCl, 50; KH_2PO_4, 0.6; K_2HPO_4, 0.6; NH_4Cl, 1.0; $MgCl_2 \cdot 6\,H_2O$, 10; $CaCl_2$, 0.1; and yeast extract, 0.5. The pH was adjusted to 7.5 with 10 M KOH solution. Aliquots of 25 mL were dispensed into flasks and sterilized by autoclaving at 121 °C for 20 min. When required, 2% agar (w/v) was added to the basal medium. The aromatic-compound stock solutions were prepared, neutralized if necessary, and sterilized by filtration (pore size 0.2 μm; Millipore). Prior to inoculation, substrates were added from concentrated sterile stock solutions to obtain the desired final concentration. Strain HTB24 was routinely grown on basal medium containing 5 mM tyrosol [2-(*p*-hydroxyphenyl)-ethanol]. Cultures (25 mL) were inoculated and incubated at 30 °C under agitation at 150 r.p.m. Non-inoculated controls were prepared in the same conditions to verify that tyrosol was not abiotically transformed in this aerobic medium. All culture experiments for studying the bioconversion of tyrosol by the selected strains were undertaken in triplicate. Three samples were withdrawn at the same time from each culture for analysis.

Enrichment and isolation of tyrosol degraders

A sample from an OMW evaporation pond in Marrakesh (Morocco) was used for bacterial isolation. The mud ($2\,g\,L^{-1}$) was used to inoculate a 25-mL basal medium containing 5 mM tyrosol. The culture was then incubated at 30 °C under agitation at 150 r.p.m. The enrichment culture was inoculated every day under the same conditions until the substrate was completely metabolized. The disappearance of tyrosol was measured by high-performance liquid chromatography (HPLC) analysis. Aliquots (100 μL) of 10^{-1} to 10^{-10} dilutions were plated onto tyrosol (5 mM) agar basal medium and incubated for 7 days at 30 °C. Single colonies were picked up and used for screening. The purity of the strains, and their shape, size and mobility were analysed using a photomicroscope (Nikon eclipse E600) with an oil-immersion objective (100 ×).

Strain characterization

Standard tests (Gram staining, oxidase and catalase activities) were performed as previously described (Smibert & Krieg, 1994). Growth was monitored on basal medium containing various concentrations of NaCl [0, 2, 5, 10, 15, 20, 25 and 30% (w/v)]. The temperature range for growth was monitored between 5 and 55 °C (at 5 °C intervals), and the pH range between 4 and 12 (with one-unit intervals) on basal medium and basal medium with 2% agar. For the sporulation test, cells grown in a basal medium containing tyrosol were exposed at temperatures of 80, 90 and 100 °C for 10 min. The cells were cooled quickly to ambient temperature, inoculated into a fresh basal medium, and growth was recorded after incubation for 24 h at 25 °C under agitation at 150 r.p.m. Experiments were performed in duplicate with an inoculum subcultured, at least once, under the same conditions. Further biochemical analyses were performed by inoculating API 20NE and API 50CH strips (BioMérieux, France) according to the manufacturer's instructions, except that the cultures were re-suspended in 3% (w/v) NaCl solution. The results obtained with the API tests were individually confirmed by testing various carbohydrates (20 mM) as sole carbon and energy sources in aerobic cotton-capped tubes filled with 5 mL of basal medium without yeast extract. The carbohydrates tested were D-glucose, D-fructose, D-sucrose, D-galactose, L- and D-arabinose, D-mannose, D-maltose, D-mannitol, D-sorbitol, D-trehalose, D-ribose, D-cellobiose, L-rhamnose, D-xylose, starch, gelatine, acetate, lactate, pyruvate and succinate. In addition, the API ZYM system (BioMérieux, France) was used as a determinative key to identify the activity of the main enzymes used (Humble *et al.*, 1977). Aromatic compounds were added (5 mM) from pre-sterilized and concentrated stock solutions (250 mM) into flasks containing 25 mL of basal medium, with or without yeast extract ($0.5\,g\,L^{-1}$), in order to study the ability of HTB24 to transform these compounds. The aromatic compounds used were catechol, phenol, tyrosol, benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), cinnamic

acid, hydroxylated cinnamic acids [*p*-coumaric (4-hydroxycinnamic acid), caffeic (3, 4-dihydroxycinnamic acid), ferulic (4-hydroxy-3-methoxycinnamic acid) acids], *p*-hydroxyphenylacetic acid and *p*-hydroxyphenylpropionic acid. An increase in the $OD_{600\,nm}$ of cultures containing substrates over that of cultures in control tubes lacking substrates was considered as positive growth.

G+C content, sequencing and phylogenetic analysis

The genomic DNA of HTB24 was extracted using a Wizard genomic DNA purification kit, according to the manufacturer's protocol (Promega). The universal primers Fd1 (5′CAGAGTTTGATCCTGGCTCAG-3′) and R6 (5′-TACGGTTACCTTGTTACGAC-3′) were used to amplify the small subunit rRNA gene. The 16S rRNA gene sequence was then aligned with reference sequences from the GenBank database (Maidak *et al.*, 1996) using the RDP Sequence Aligner program, and the alignment was manually verified to conform to the 16S rRNA secondary-structure model (Winker & Woese, 1991). Pairwise evolutionary distances, based on 1408 unambiguous nucleotides, were computed using the method of Jukes & Cantor (1969), and a dendrogram was constructed from these distances using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the dendrogram topology was determined by bootstrap analysis using 1000 re-samplings of the sequences (Felsenstein, 1985). All the used programs form part of the PHYLIP package (Felsenstein, 1993). The topology of the tree was supported using the maximum parsimony and maximum likelihood algorithms. The G+C content and the level of binding of the strain DNA were determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The DNA was isolated and purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977), and the G+C content was determined by HPLC (Mesbah *et al.*, 1989).

HPLC separation and chemical structure identification

In order to quantify the phenolic compounds, 1 mL of culture was centrifuged (10 000 ***g***, 5 min), and the clear supernatant was collected and acidified with 25 μL of glacial acetic acid and then analysed by reverse-phase HPLC.

In order to determine the chemical structures of the compounds, 5 mL of culture was centrifuged, and the supernatant was acidified with HCl until pH 3 and then extracted with ethyl acetate twice. After combining the organic phases and evaporation to dryness, pure methanol was added, and the solution was re-analysed by reverse-phase HPLC in the same conditions as in the routine experiments. Peaks were identified by comparing their retention time and respective UV spectra (diode array detection) with commercial standards. Definitive chemical structures were finally confirmed by gas chromatography-mass spectrometry (GC-MS).

HPLC was performed with Waters equipment comprising a 1525 binary pump, a 2996 diode array detector, a Rheodyne injector (model 7725i), fitted with a 20-μL loop, a temperature-control system and a degasser. Files were acquired with EMPOWER software. Separations were carried out on a Symetry C_{18} reversed-phase column (4.6 × 150 mm, ODS2, 5 μm) from Waters and protected with a guard cartridge. Elutions were performed at 30 °C, at a flow rate of 0.8 mL min^{-1} and using a linear gradient of acetonitrile (A) in water, acidified with 1% (v/v) acetic acid (B), in two steps : first, from 5 to 20% A for 25 min, and then from 20 to 100% A for 5 min (total 30 min). Phenolic compounds were visualised at 280 nm, and total spectra from each peak were analysed from 200 to 400 nm using the EMPOWER software. Data are averages from three determinations.

GC-MS analysis

For GC-MS analysis, HPLC fractions or ethyl acetate extracts were evaporated to dryness and derivatized using BSTFA [bis (trimethylsilyl)-trifluoroacetamide containing trimethylchlorosilane 1%, from Sigma, France] according to Ro *et al.* (1994), and then evaporated under nitrogen prior to the residue being dissolved in ethyl acetate. One microlitre of sample was then injected into a GC-MS instrument (from Agilent Technologies) equipped with a 6890N GC and a 5973 MSD system consisting of a quadrupole analyzer. The column was a capillary DB-1MS column from JW-Agilent Technologies (30 m × 0.25 inches), and the vector gas was helium at a flow rate of 1 mL min^{-1}. The temperature was increased from 100 to 260 °C at a rate of 4 °C min^{-1}, and then maintained at 260 °C for 10 min. Comparisons of mass spectra were undertaken using Wiley and NIST data banks.

Results

Isolation of hydroxytyrosol (HT)- and 3,4-dihydroxyphenylacetic acid (DHPA)-producing strains

To isolate tyrosol-degrading microorganisms, an enrichment culture was obtained according to the protocol described in the Materials and methods section. This enrichment culture was designed to select strains able to degrade tyrosol. The substrate transformation was confirmed by HPLC and GC-MS analysis. Based on their morphology, three distinct tyrosol-degrading strains, HTB24, TYRB22 and PHYP5, were isolated on the basis of their ability to produce several compounds during tyrosol degradation. Amongst them, HTB24 was found to produce

the highest levels of HT and DHPA, and therefore all studies were focused on this strain.

Physiology of strain HTB24

The organism used in this study was the moderately halophilic strain HTB24, isolated from an OMW rich in aromatic compounds, after enrichment on tyrosol. Cells are Gram-negative, motile, non-spore-forming rods, with variable lengths and widths (0.5–2.0 μm long and 0.2–0.5 μm in diameter). The temperature range for growth was 4–45 °C, with an optimum at 25 °C. The pH range was 5–11, with an optimum at 7.5. Growth occurred in the range 5–200 g L^{-1} NaCl, and optimal growth was between 20 and 50 g L^{-1} NaCl. This isolate is regarded as a moderately halophilic bacterium. Anaerobic growth was weak in the absence or presence of nitrate as final electron acceptor. Catalase and oxidase tests are positive. Acid is weakly produced from glucose, galactose, cellobiose, maltose, and lactose (API 50CH). According to API 20NE, the strain is positive for nitrate reduction to nitrite, glucose fermentation, β-galactosidase and utilization of glucose, mannitol, maltose, gluconate, adipate, malate and citrate. The strain is negative for indole production, arginine dihydrolase, urease production, aesculin, gelatine, and utilization of arabinose, mannose, and caprate. Acetate, lactate, citrate and succinate are utilized as sole carbon and energy sources. The strain showed high enzyme activity for alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, and β-glucosidase. No activity was detected on esterase lipase (C8), lipase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Growth occurred with benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, *p*-coumaric acid, caffeic acid and *p*-hydroxyphenylacetic acid and tyrosol as carbon sources. However, strain HTB24 showed a weak growth on vanillic and ferulic acid, and was unable to transform and grow on catechol, phenol, cinnamic acid and *p*-hydroxyphenylpropionic acid.

Phylogenetic analysis

In order to analyse the phylogenetic position, the 16S rRNA sequence of HTB24 (1508 bp) was determined, and a phylogenetic tree based on 1408 unambiguous basepairs was constructed (Fig. 1). The 16S rRNA gene sequence of strain HTB24 was deposited in GenBank under accession number EF422233. Phylogenetic analysis revealed that strain HTB24 could be identified as a species of the genus *Halomonas*. Strain HTB24 exhibited 16S rRNA gene sequence similarity values greater than 99% with the closely related species *Halomonas neptunia* DSM 15720^T, *Halomonas boliviensis* DSM 15516^T, '*Halomonas glaciei*' and '*Halomonas alkaliantarctica*'. Strain HTB24 also shared 98.83% and 97.78% identity with *Halomonas variabilis* and *Halomonas venusta*, respectively. The G+C content of HTB24 was 55.3 mol% as determined by HPLC (Mesbah *et al.*, 1989).

Metabolism of tyrosol and formation of *o*-diphenols

The formation of HT and DHPA by *Halomonas* sp. HTB24 was assessed by the addition of tyrosol (5 mM) to the basal medium. The inoculum for this experiment corresponds to cells grown on basal medium supplemented with tyrosol and harvested in stationary phase. The conversion was periodically monitored by HPLC analysis of samples collected from the medium. Figure 2 shows the time course of tyrosol depletion and the transient accumulation of HT and DHPA during growth from a culture inoculated with a dense pre-culture.

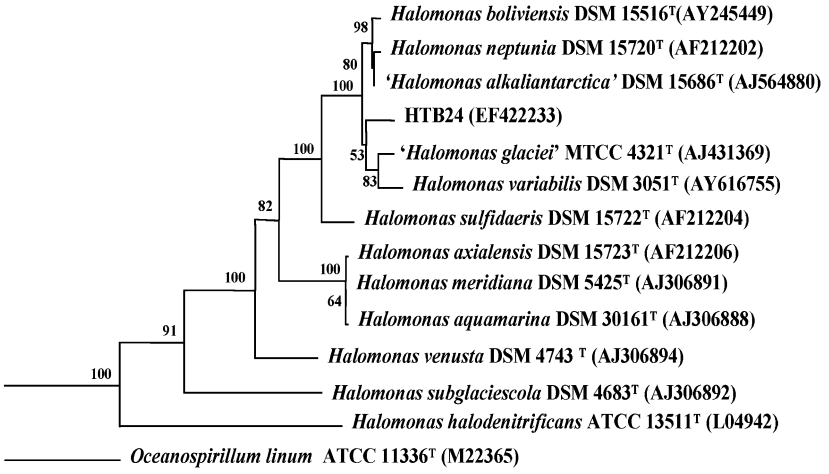

Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequence comparisons (1408 unambiguous basepairs) indicating the position of *Halomonas* sp. strain HTB24 among related members of the genus *Halomonas*. Reference type-strain organisms are included and accession numbers are in parentheses. Bootstrap values, expressed as percentages of 1000 replications, are shown at the nodes. Bar, 2 nucleotide substitutions per 100 nucleotides.

It was found that HT was easily quantified after 1 h of growth, and its concentration increased concomitantly with tyrosol depletion, reaching a maximum concentration at 12 h. HT concentration at this time was found to be 1.60 mM. After 8 h of culture, we recorded the formation of DHPA, accompanied by traces of 4-hydroxyphenylacetic acid (HPA), easily identified but not precisely quantified by HPLC. The maximum concentration of DHPA was reached after 20 h of growth, and was estimated to be 2.75 ± 0.48 mM. DHPA was subsequently degraded into a compound that exhibited a sharp yellow colour and was identified as the expected 5-carboxymethyl-2-hydroxymuconic semialdehyde (CHMS), as previously observed (Sparnins *et al.*, 1974; Cooper and Skinner, 1980). After purification of this compound by reverse-phase HPLC, UV data were recorded in an acidic (λ_{max} 322 nm) or alkaline (λ_{max} 380 nm) aqueous solvent and were consistent with the proposed structure of CHMS, suggesting the presence of a 2,3-dioxygenase (EC 1.13.11.15; Krishnan Kutty *et al.*, 1977).

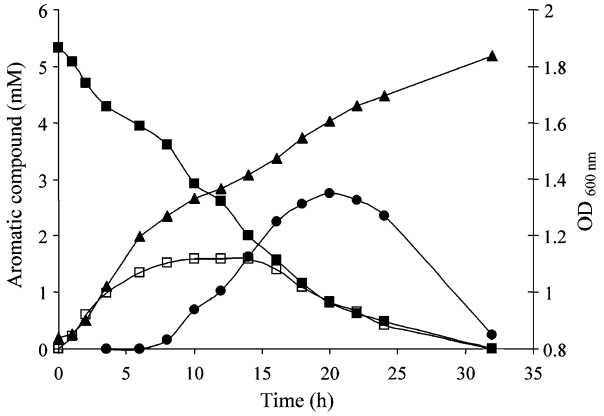

Fig. 2. Time course of *Halomonas* sp. strain HTB24 growth, tyrosol consumption and aromatic product formation in basal medium containing 5 mM tyrosol, 0.5 g L^{-1} yeast extract, and 50 g L^{-1} NaCl at 30 °C under agitation (150 r.p.m). (▲) $OD_{600\,nm}$; (■) tyrosol; (□) HT; (●) DHPA.

After 30 h, aromatic compounds had totally disappeared from the medium (see Fig. 2), concomitantly with the yellow colour of the culture. This observation was confirmed by the absence of CHMS in HPLC performed at 322 nm, and also by GC-MS analysis of the freeze-dried supernatant, which indicated the presence of both succinic and pyruvic acid. In addition, no other compounds were found in the medium. For example, we confirmed the absence of homogentisic acid (2,5-dihydroxyphenylacetic acid), frequently formed by a C1-hydroxylation from HPA, and 3,4-dihydroxymandelic acid, less frequently synthesized from DHPA (Hareland *et al.*, 1975; O'Connor *et al.*, 2001).

Inhibition effect of tyrosol concentration on *o*-diphenol production

In order to optimize HT and DHPA production by *Halomonas* sp. strain HTB24, increasing concentrations of tyrosol were used, and both growth and *o*-diphenol content were tested. Cells from routinely tyrosol-grown cultures were thus harvested at the end of the exponential phase and inoculated with a dense pre-culture in basal medium containing increasing concentrations of tyrosol (from 5 to 40 mM). Figure 4 shows that strain HTB24 can grow up to a maximum concentration of 30 mM tyrosol and that the growth rate decreases drastically after 20 mM. At this concentration, the growth rate was found to be 0.25 h^{-1}, whereas it was 0.35 h^{-1} when tyrosol was omitted. Furthermore, DHPA reached a maximal concentration of

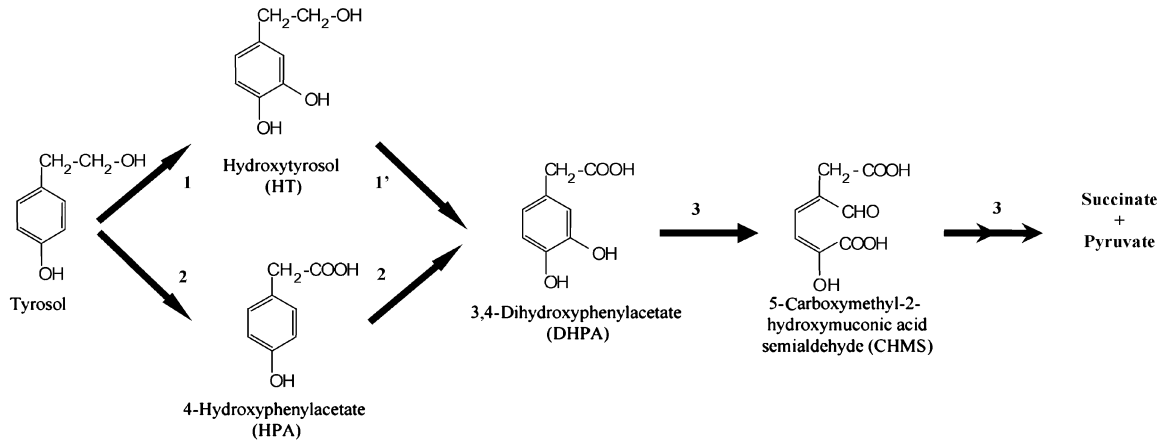

Fig. 3. Tyrosol metabolism pathway in *Halomonas* sp. strain HTB24. Route 1, hydroxytyrosol (HT) formation in the first 8 h by a C3-hydroxylation. Route 1′, hypothetical HT oxidation into DHPA. Route 2, HPA formation by oxidation and then DHPA synthesis by HPA hydroxylation (beyond 8 h). Route 3, formation of CHMS and then succinate and pyruvate.

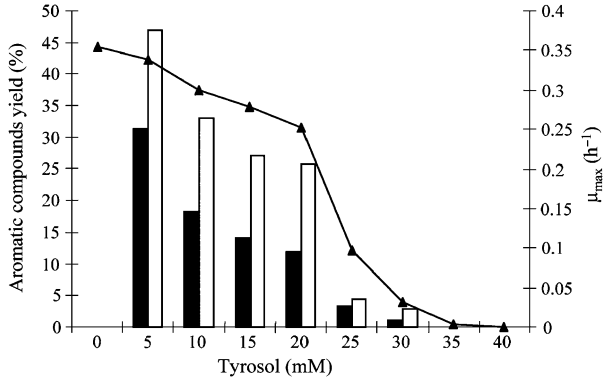

Fig. 4. HT and DHPA yield as a function of tyrosol concentration added to the basal medium containing 0.5 g L^{-1} yeast extract and 50 g L^{-1} NaCl at 30 °C under agitation (150 r.p.m). HT and DHPA levels were determined at 12 h and 20 h, respectively (see Fig. 2). (■) HT yield (%); (□) DHPA yield (%); (▲) μmax (h^{-1}).

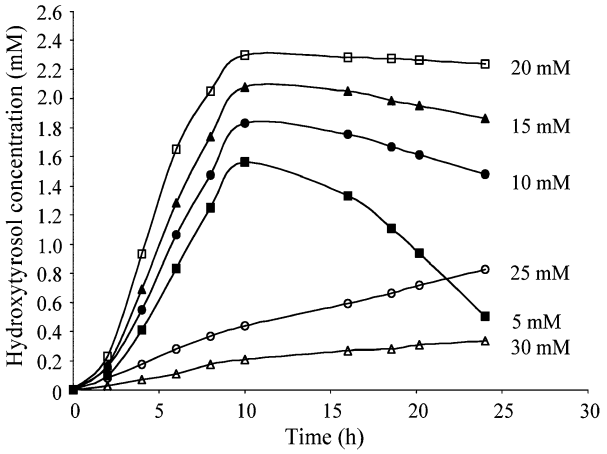

Fig. 5. Time course of HT production as a function of tyrosol concentration added to the basal medium containing 0.5 g L^{-1} yeast extract and 50 g L^{-1} NaCl at 30 °C under agitation (150 r.p.m). Tyrosol concentrations: (■) 5 mM; (●) 10 mM; (▲) 15 mM; (□) 20 mM; (○) 25 mM; (△) 30 mM.

5.15 ± 0.42 mM with 20 mM tyrosol (yield 26%) (Fig. 4). However, the maximal yield for DHPA (47%) was obtained at 5 mM tyrosol. Similarly, the maximal yield of HT (31%, i.e 1.56 mM) was obtained at 5 mM tyrosol. It was thus found that HT content reached a maximal value of 2.30 mM with a lower yield (12%) for 20 mM tyrosol. In the range 5 – 20 mM tyrosol, HT production increases in proportion to tyrosol concentration (Figs 4 and 5); and maximal HT content was routinely obtained in 12 h (Fig. 5), thus confirming our previous observations (see Fig. 2).

Discussion

Following enrichment on tyrosol, strain HTB24 has been isolated in our laboratory from an olive-mill wastewater. It was found to be a Gram-negative, motile, non-spore-forming rod, with pleiomorphic length and width (0.5–1.5 μm long and 0.2–0.5 μm in diameter). The phenotypic, genotypic and phylogenetic characteristics of HTB24 isolate indicated that it belongs to the genus *Halomonas*, and it was found to be closely related to *Halomonas neptunia*, '*Halomonas alkaliantarctica*', *Halomonas boliviensis*, and *Halomonas variabilis* (more than 99% of similarity between the 16S rRNA sequences). Species of the genus *Halomonas* have been isolated from saline soils, hypersaline lakes, marine water and other habitats (Ventosa, 1988; Javor, 1989). Isolation of this strain was not surprising in regard to olive-mill wastewater, which contains a wide range of aromatic compounds, including tyrosol. Other studies have shown that *Halomonas* species can degrade aromatic compounds (Hinteregger & Streichsbier, 1997; Garcia *et al.*, 2004). However, to our knowledge, no study has been reported on the hydroxylation, under hypersaline conditions, of tyrosol into hydroxytyrosol.

GC-MS and HPLC analyses revealed that tyrosol is metabolized into hydroxytyrosol (HT). Because no quinonic compound was found in the HTB24 medium after hydroxytyrosol formation, suggesting that hydroxylation would not be due to a tyrosinase activity (Espín *et al.*, 2001), we can assume that the hydroxylation is caused by a C3-monooxygenase-hydroxylase type. This has already been suggested for *Pseudomonas aeruginosa* (Allouche *et al.*, 2004; Bouallagui & Sayadi, 2006) and *Serratia marcescens* (Allouche & Sayadi, 2005). The synthesis and degradative routes taken in the aerobic metabolism of tyrosol by *Halomonas* sp. strain HTB24 are summarized in Fig. 3. HTB24 grows on yeast extract, and, during a first period of 8 h, hydroxylates tyrosol into hydroxytyrosol (route 1). A maximal concentration of HT of 2.3 mM was obtained with 20 mM tyrosol After 8 h of growth, we observed a second phase, during which tyrosol was also found to be catabolized by the HPA pathway (Sparnins *et al.*, 1974), leading to DHPA synthesis (route 2). The oxidation of tyrosol into HPA was probably caused by the presence of an aryl-dehydrogenase, which has been described as a non-specific enzyme (MacKintosh & Fewson, 1988). *Arthrobacter* sp. and *Bacillus* sp. have also been found to oxidize tyrosol into the corresponding HPA but, in both cases, and in contrast to our study, HPA was accumulated in the media (Knupp *et al.*, 1996; Abdelkafi *et al.*, 2005). Furthermore, the rapidly increasing concentration of DHPA cannot only be the result, in HTB24, of the transformation of HPA, but could also be the consequence of HT oxidation by the aryl-dehydrogenase. Subsequently, DHPA is metabolized into CHMS by a 3, 4-dihydroxyphenylacetate 2,3-dioxygenase, leading to the extradiol ring fission and the final metabolites succinate and pyruvate (Que *et al.*, 1981).

Maximum quantities of both HT and DHPA were obtained at a tyrosol concentration of 20 mM, and decreased drastically at higher concentrations. The same pattern was observed for HTB24, with higher growth rates for tyrosol concentrations up to 20 mM. These observations could be explained by the toxicity of this compound.

Using a halophilic strain growing in saline conditions, we have described, for the first time, the hydroxylation of tyrosol into hydroxytyrosol in a 12-h culture that produces 3,4-dihydroxyphenylacetic acid in the same time frame. These two compounds are known to act synergistically, therefore increasing the global anti-oxidant activity. These transformations could constitute an alternative and non-polluting procedure to obtain, in one step, molecules with properties that have been shown to be beneficial to health, for example antioxidant activity against free radicals.

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Statement

The Genbank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of HTB24 is EF422233. The strain HTB24 was deposited in the microorganism collection CIP (Institut Pasteur, France) and the deposit number was = CIP 109599.

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