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Synthesis of Hydroxytyrosol, 2-Hydroxyphenylacetic Acid, and 3-Hydroxyphenylacetic Acid by Differential Conversion of Tyrosol Isomers Using *Serratia marcescens* Strain

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We investigated to develop an effective procedure to produce the potentially high-added-value phenolic compounds through bioconversion of tyrosol isomers. A soil bacterium, designated *Serratia marcescens* strain, was isolated on the basis of its ability to grow on *p*-tyrosol (4-hydroxyphenylethanol) as a sole source of carbon and energy. During growth on *p*-tyrosol, *Ser. marcescens* strain was capable of promoting the formation of hydroxytyrosol. To achieve maximal hydroxytyrosol yield, the growth state of the culture utilized for *p*-tyrosol conversion as well as the amount of *p*-tyrosol that was treated were optimized. The optimal yield of hydroxytyrosol (80%) was obtained by *Ser. marcescens* growing cells after a 7-h incubation using 2 g/L of *p*-tyrosol added at the end of the exponential phase to a culture pregrown on 1 g/L of *p*-tyrosol. Furthermore, the substrate specificity of the developed biosynthesis was investigated using *m*-tyrosol (3-hydroxyphenylethanol) and *o*-tyrosol (2-hydroxyphenylethanol) as substrates. *Ser. marcescens* strain transformed completely *m*-tyrosol and *o*-tyrosol into 3-hydroxyphenylacetic acid and 2-hydroxyphenylacetic acid, respectively, via the oxidation of the side chain carbon of the treated substrates. This proposed procedure is an alternative approach to obtain hydroxytyrosol, 2-hydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid in an environmentally friendly way which could encourage their use as alternatives in the search for replacement of synthetic food additives.

KEYWORDS: Tyrosol, bioconversion, hydroxytyrosol, antioxidant, 3-hydroxyphenylacetic acid, 2-hydroxyphenylacetic acid, *Serratia marcescens*

INTRODUCTION

Hydroxylation of aromatic rings is a fundamental reaction used for the preparation of several active compounds. Compared with chemical production, hydroxylation of aromatic compounds by microorganisms is an interesting and promising method to synthesize the desired products in a single-step reaction, with a high regioselectivity and a stereoselectivity and under mild conditions (*1*). Furthermore, the products of such bioconversions are considered natural, since the European Community legislation includes products which occur in nature and that are produced by living cells or enzymes using starting materials from a natural source under the term "natural products" (*2*).

Hydroxylation of aromatic rings is a fundamental reaction used for the synthesis of several high-added-value compounds, including ortho-diphenols. Hydroxytyrosol (3,4-dihydroxyphenylethanol) is the most abundant ortho-diphenol compound occurring in olive oil (*3*) as well as in olive mill wastewaters (*4*, *5*). Several investigations reported that hydroxytyrosol is characterized by its highly antioxidant property and presents several interesting aspects for human health (*6*−*12*) that would allow its potential application in food, as well as in the cosmetic and pharmaceutical industries (*13*). Recent works demonstrated that the bioavailability of hydroxytyrosol makes it a beneficial addition to the diet (*14*, *15*). Several investigations developed various methods to produce hydroxytyrosol by means of chemical synthesis (*16*), through conversion of oleuropein (*17*, *18*), by enzymatic synthesis using tyrosinase as biocatalyst (*19*), and by bench-scale purification from olive mill wastewaters (*20*, *21*). Recently, we developed a continuous procedure for the extraction and the purification of hydroxytyrosol from olive mill wastewaters (*22*) and a microbial conversion procedure of *p*-tyrosol into hydroxytyrosol using *Pseudomonas aeruginosa* (*23*).

Besides hydroxytyrosol, 2-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid stand out as aromatic compounds of a high-added-value. These two products are frequently used in the pharmaceutical industry where they are used as intermediates in the preparation of some biologically active products such as antihypertensive agents (*24*). In a recent investigation, it was demonstrated that 3-hydroxyphenylacetic acid would be a starting material for the synthesis of three drugs which have relatively high affinity at gamma-hydroxybutyric acid (GHB) sites. (GHB) is a therapeutic and a neurotransmitter with a

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complex mechanism of action in vivo (*25*). 2-Hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid were chemically synthesized and commercially available products. 2-Hydroxyphenylacetic acid is a natural phenolic product found in the genus *Astilbe* and it derives from the shikimic acid pathway via phenylpyruvic acid (*26*). 3-Hydroxyphenylacetic acid is a major phenolic acid degradation product of proanthocyanidin metabolism in humans (*27*). These two phenolic compounds are synthesized by cultivating a fungus in the presence of phenylacetic acid (*24*, *28*).

The present work deals with the development of simple and highly convenient microbial transformations for the synthesis of hydroxytyrosol, 2-hydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid using, respectively, *p*-tyrosol (4-hydroxyphenylethanol), *m*-tyrosol (3-hydroxyphenylethanol), and *o*-tyrosol (2-hydroxyphenylethanol) as precursors. These substrates naturally occur in olive mill wastewaters (*29*). They can also be obtained from olive cake (*30*), olive stones (*31*), white wine (*32*), and beer (*33*). The developed biosynthesis methods could prove useful for laboratory applications as well as for a possible industrial exploitation.

MATERIALS AND METHODS

Chemicals. *p*-Tyrosol (4-hydroxyphenylethanol) and its isomers *m*-tyrosol (3-hydroxyphenylethanol) and *o*-tyrosol (2-hydroxyphenylethanol) were purchased by Fluka (France). Hydroxytyrosol, used as standard, was purfied from olive mill wastewaters as described by Allouche et al. (*22*).

Bacterial Strain Isolation. For bacterial isolation, an enrichment culture method was used. In this experimentation, soil (1 g), regularly irrigated with olive mill wastewaters (100 m^3/ha) for 5 years, was mixed with 100 mL minimal medium containing 0.1 g/L of *p*-tyrosol and 1 g/L of glucose both as carbon sources. The obtained culture was incubated overnight in an orbital shaker at 180 rpm and 30 °C. Each day, an aliquot (5 mL) from the incubated culture was used to inoculate a fresh medium of 100 mL in which the concentration of *p*-tyrosol was increased (0.1 g/L was added) while decreasing the glucose concentration. This procedure was repeated daily until the *p*-tyrosol concentration reached 1 g/L and the glucose was completely removed from the culture medium. Once *p*-tyrosol was completely metabolized, an aliquot (5 mL) from the last incubated culture was used to inoculate another fresh medium containing 1 g/L of *p*-tyrosol. This step was repeated four times. The disappearance of *p*-tyrosol was confirmed by thin-layer chromatography (TLC) analysis. Aliquots (0.1 mL) of 10^{-2} to 10^{-8} dilutions from the last acclimated culture were plated onto *p*-tyrosol (1 g/L) minimal agar medium and were incubated overnight at 30 °C. Single colonies were picked and used for screening.

Culture Conditions. Bacteria were grown in a minimal medium containing (g/L): Na_2HPO_4, 2.44; KH_2PO_4, 1.52; $(NH_4)_2SO_4$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.2; and $CaCl_2 \cdot H_2O$, 0.05 and 10 mL of trace-element solution which contains (% W/V): EDTA, 5; $ZnSO_4 \cdot 7H_2O$, 2.2; $CaCl_2$, 0.55; $MnCl_2 \cdot 5H_2O$, 0.5; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.11; $CuSO_4 \cdot 5H_2O$, 0.16; $CoCl_2 \cdot 6H_2O$, 0.16. The pH of the medium was adjusted to 7.2. The carbon substrates were filter sterilized. Solid media were prepared by the addition of 1.5% agar. Cultures (25 mL) were inoculated with 10^7 cells pregrown in Luria-Bertani broth and were incubated in an orbital shaker at 180 rpm and 30 °C. The growth rate was followed by measuring the changes in turbidity at 600 nm using a Shimadzu UV−visible-light spectrophotometer (UV-160A). All cultures used for studying the bioconversion of tyrosol isomers by the selected strain were undertaken in triplicate. Three samples were withdrawn at the same time from each culture for analysis, and standard deviation (SD) is indicated in each figure.

DNA Extraction and PCR Amplification. Total DNA was isolated from the isolated strain by using the alkaline-lysis method with certain modifications. The universal primers Fd_1 and Rd_1 were used to obtain a PCR product of approximately 1.5 Kb corresponding to base positions 8−1542 on the basis of *Escherichia coli* numbering of the 16S-DNA (*34*). A 50-μL reaction mixture contained 50 mg of genomic DNA, 1 μL of each primer, 5 μL of 10× buffer, 200 μL of deoxynucleoside triphosphate, 3.5 mM of $MgCl_2$, and 2.5 U of *Taq* polymerase (Promega). PCR was carried out by an initial denaturation at 94 °C for 1 min followed by cycles of annealing at 55 °C for 30 s, extension at 72 °C for 45 s (depending on the lengths of the amplified fragments), and denaturation at 94 °C for 30 s, followed by cycles of annealing at 55 °C for 30 s, extension at 72 °C for 45 s, and finally an extension cycle of 72 °C for 10 min.

HPLC and TLC Analyses. Aromatic compound identification and quantification were carried out by HPLC analysis as described by Akasbi et al. (*35*). It was performed on a Shimadzu C-R6A liquid chromatograph. The separation was carried out in a C_{18} column (length, 250 mm; internal diameter, 4.6 mm; Waters chromatography). The compounds were eluted with a gradient, acetonitrile (70%)-H_3PO_4 (0.1%), in which the concentration of acetonitrile varied as follows: 0 min, 10%; 0−20 min, increase to 50%; 20−25 min, 50%; 25−30 min, decrease to 10%. The column temperature was maintained at 40 °C and the flow rate was 0.5 mL/min. Sample detection was achieved at 280 nm with a Shimadzu SPD-6AUV detector connected to a Shimadzu C-R6A integrator. The injection volume was 20 μL.

Samples (1 mL) were withdrawn periodically from the culture medium and centrifuged at 7000*g* (10 min). The supernatant was analyzed directly by HPLC for substrate and intermediary-metabolite quantification. The compounds were identified and quantified by comparison of retention times and peak areas with those of authentic samples. The molar yields of produced compounds were calculated as follows: molar yield of produced compound = molar concentration of produced compound (mol/L)/initial molar concentration of tyrosol (mol/L) × 100.

The thin-layer chromatography (TLC) was monitored using silica gel sheets (Kieselgel 60 F_{254}; 0.2 mm layers; Merck) eluted with toluene-ethyl acetate-acetic acid (7:2:1, V/V/V). The constituents were visualized by exposure to UV light and were revealed by resublimed iodine fumes.

GC-MS Analysis. GC-MS was performed with a Hewlett-Packard model 5872A chromatograph apparatus, equipped with a capillary HP5MS column (length, 30 m; internal diameter, 0.32 mm; film thickness, 0.32 μm). The carrier gas was He, used at a 1.7 mL/min flow rate. The oven temperature program was as follows: 1 min at 100 °C, from 100 to 260 °C at 4 °C/min, and 10 min at 260 °C. A sample from the culture medium (1 mL) was extracted with ethyl acetate, and 100 μL of bis-(trimethylsilyl)-acetamide was added to 100 μL of the organic extract. The obtained solution was incubated 30 min at 60 °C. Ethyl acetate and bis-(trimethylsilyl)-acetamide were evaporated under a N_2 current, and the residue was redissolved in ethyl acetate (1 mL) and was analyzed by GC-MS.

RESULTS

Isolation of *p*-Tyrosol Degrading Strain. To isolate different *p*-tyrosol-tolerant microorganisms, an enrichment culture method was conducted according to the protocol described in the Materials and Methods section. This enrichment culture was designed to select strains able to grow on *p*-tyrosol as sole carbon source and energy. The substrate degradation was followed by TLC and HPLC analysis. Among several *p*-tyrosol tolerant strains, one microorganism was isolated for further studies on the basis of its capacity to produce hydroxytyrosol during *p*-tyrosol degradation. This strain was able to grow on high concentration of *p*-tyrosol (up to 4 g/L). However, it was unable to metabolize either *m*-tyrosol or *o*-tyrosol (data not shown). By means of the 16S-rDNA sequence analysis, the isolated strain was identified as *Serratia marcescens*.

Optimization of Conditions for Hydroxytyrosol Production by *Ser. marcescens* Growing Cells. To achieve maximal yield of hydroxytyrosol production by growing cells of *Ser. marcescens* strain, two parameters were optimized. For this purpose, the relationship between hydroxytyrosol production and

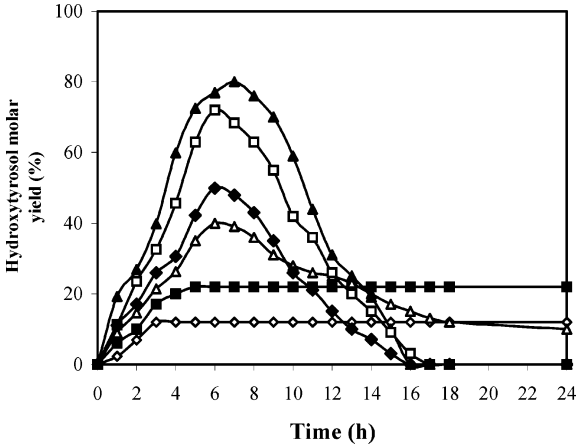

Figure 1. Time course of hydroxytyrosol formation by growing cells of *Ser. marcescens* strain with increasing concentrations of *p*-tyrosol. 1 g/L (◆), 1.5 g/L (□), 2 g/L (▲), 2.5 g/L (△), 3 g/L (■), 4 g/L (◇). Each point represents the mean of three determinations and three independent experiments (SD < 15% of the mean).

(i) the growth state of the used culture and (ii) the amount of treated *p*-tyrosol was examined.

Three *Ser. marcescens* cultures were prepared in minimal medium on *p*-tyrosol (1 g/L). These batch cultures were then supplemented with *p*-tyrosol (1 g/L) at different growth states: at the middle of the exponential phase (cell concentration = 0.6 g/L) for the first culture, at the end of the exponential phase (cell concentration = 1.14 g/L) for the second culture, and at the stationary phase (cell concentration = 1.24 g/L) for the third culture. These cultures were then incubated aerobically, in an orbital shaker at 180 rpm and 30 °C.

The depletion of *p*-tyrosol and the increase of hydroxytyrosol concentration in each medium were monitored by frequent HPLC sample analysis. The obtained results showed that the maximum level of hydroxytyrosol (0.5 g/L) was accumulated in the second culture where *p*-tyrosol was added at the end of the exponential phase of growth. This corresponded to a bioconversion molar yield of 44.8%. However, for the first and the third cultures, molar yields of 18% and 25% were obtained, respectively.

The effect of *p*-tyrosol concentration on the bioconversion rate was then examined. Six batch cultures pregrown on *p*-tyrosol (1 g/L) until the end of the exponential phase were supplemented, respectively, with different concentrations of *p*-tyrosol (1, 1.5, 2, 2.5, 3, 4 g/L). The optimal *p*-tyrosol concentration should be considered to be that which allows higher yields of hydroxytyrosol to be obtained, and afterwards, a delayed decrease in the metabolite. **Figure 1** shows the variation in the level of hydroxytyrosol produced with the treated *p*-tyrosol concentration. The maximum yield of the produced hydroxytyrosol increased with the amount of *p*-tyrosol used for concentrations up to 2 g/L. An excess of *p*-tyrosol concentration (over 2 g/L) was detrimental to the increase in the quantity of hydroxytyrosol produced. Furthermore, **Figure 1** shows that for tyrosol concentrations higher than 2.5 g/L, the bioconversion reaction is stopped after reaching its maximal product level. The consumption of the hydroxytyrosol produced did not start after more than 24 h. This may be explained by the toxicity of the *p*-tyrosol. Thus, the optimal *p*-tyrosol concentration would be 2 g/L which resulted in a bioconversion yield of 80%.

Kinetic Formation of Hydroxytyrosol by *Ser. marcescens* Grown Cells. The formation of hydroxytyrosol by *Ser. marcescens* strain was assessed by the addition of *p*-tyrosol (2 g/L) to a culture previously grown on *p*-tyrosol (1 g/L) until the end of the exponential phase. The bioconversion was monitored by HPLC analyzing samples withdrawn from the culture medium at different times. **Figure 2** shows the time courses of *p*-tyrosol depletion and transient accumulation of hydroxytyrosol in the medium during the growth of *Ser. marcescens* strain. During the first 4 h of incubation, OD_{600nm} remained constant (**Figure 2**). This lag-phase period could be explained by the toxicity of *p*-tyrosol toward the isolated strain. During this period, *Ser. marcescens* transformed 60% of *p*-tyrosol into hydroxytyrosol without aromatic compound consumption. After this lag phase, hydroxytyrosol concentration increased in the medium, but degradation of aromatic compounds started and consequently an increase of the OD_{600nm} was observed. After 7 h, hydroxytyrosol degradation started, and it was completely removed from the culture after 15 h. Maximal hydroxytyrosol concentration (1.78 g/L), resulting in a molar yield of 80%, was achieved at 7 h. The identification of hydroxytyrosol in the culture medium was achieved according to the comparison of the obtained GC-MS spectra with GC-MS apparatus library (**Table 1**). Moreover, the obtained mass fragments agreed with those described by Capasso (*36*).

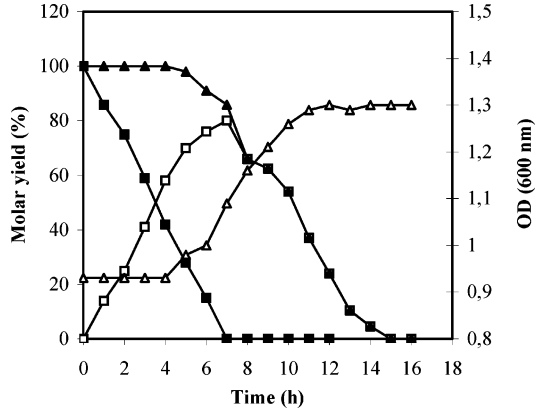

Figure 2. Time course of *Ser. marcescens* strain growth in minimal medium containing 2 g/L *p*-tyrosol. Optical density at 600 nm (△), *p*-tyrosol (■), hydroxytyrosol (□), aromatic compounds (▲). Each point represents the mean of three determinations and three independent experiments (SD < 12% of the mean).

Table 1. Abbreviated Mass Spectra of the Bioconversion Products of *p*-Tyrosol, *o*-Tyrosol, and *m*-Tyrosol by *Ser. marcescens* Strain

TMS derivatives of	mass spectra (*m*/*z* and % of the base peak)
hydroxytyrosol	370 (M^+, 39); 267 (90); 193 (25); 179 (12); 73 (100)
2-hydroxyphenylacetic acid	296 (M^+, 18); 281 (12); 253 (25); 164 (20); 147 (40);73 (100)
3-hydroxyphenylacetic acid	296 (M^+, 22); 281 (20); 253 (10); 164 (35);147 (25);73 (100)

Conversion of *m*-Tyrosol and *o*-Tyrosol by *Ser. marcescens.* To study the substrate specificity of the hydroxylation reaction by *Ser. marcescens* strain, the bioconversions of *m*-tyrosol (3-hydroxyphenylethanol) and *o*-tyrosol (2-hydroxyphenylethanol) were investigated. For this aim, 1 g/L of each compound was added to the corresponding culture pregrown on *p*-tyrosol (1 g/L). It should be stressed that *Ser. marcescens* strain did not grow on *m*-tyrosol and on *o*-tyrosol (data not shown). Using GC-MS analysis (**Table 1**), it was deduced that *m*-tyrosol and *o*-tyrosol were converted by *Ser. marcescens* into

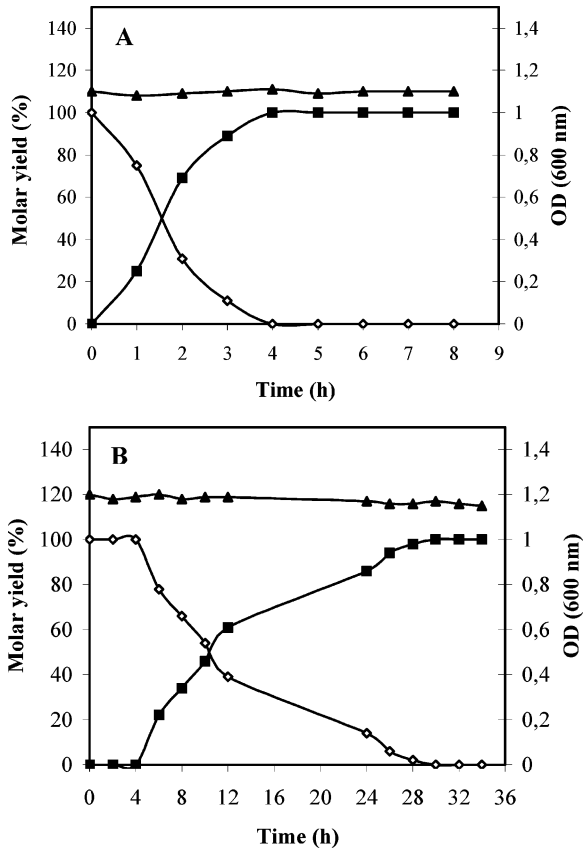

Figure 3. Transformation time courses of *m*-tyrosol and *o*-tyrosol into 3-hydroxyphenylacetic acid (**A**) and 2-hydroxyphenylacetic acid (**B**), respectively, using *Ser. marcescens* strain. *m*-Tyrosol or *o*-tyrosol (◇), 3-hydroxyphenylacetic acid or 2-hydroxyphenylacetic acid (■), optical density at 600 nm (▲). Each point represents the mean of three determinations and three independent experiments (SD < 15% of the mean).

3-hydroxyphenylacetic acid and 2-hydroxyphenylacetic acid, respectively. This result showed that *Ser.* marcescens hydroxylated the aromatic ring only when the existing hydroxyl group is in the *para* position (*p*-tyrosol). However, with the two other isomers (*m*-tyrosol and *o*-tyrosol), the side chain carbon was oxidized into acetic acid. Hence, the hydroxylation of *p*-tyrosol by *Ser. marcescens* strain is a substrate-specific reaction.

The conversion of *m*-tyrosol (1 g/L) and *o*-tyrosol (1 g/L) using *Ser. marcescens* strain pregrown on *p*-tyrosol was monitored by HPLC analyzing samples withdrawn from the corresponding cultures at different times. **Figure 3** shows the time courses of the *m*-tyrosol (**Figure 3A**) and the *o*-tyrosol (**Figure 3B**) depletion and the accumulation of the 3-hydroxyphenylacetic acid and the 2-hydroxyphenylacetic acid, respectively. During the incubation period, OD_{600nm} remained constant in the two-culture media. *Ser. marcescens* strain transformed entirely *m*-tyrosol and *o*-tyrosol to 3-hydroxyphenylacetic acid and 2-hydroxyphenylacetic acid, respectively, without further aromatic consumption. Maximal 3-hydroxyphenylacetic acid and 2-hydroxyphenylacetic acid concentrations (1.1 g/L), resulting in a molar yield of 100%, were achieved after 4 and 30 h, respectively. In addition, the bioconversion reactions started after 0 h in *m*-tyrosol and after 4 h in *o*-tyrosol. This could be explained by the hydrogen bond effect. This latter is more important in the chemical structure of 2-hydroxyphenylacetic acid than it is in the chemical structure of 3-hydroxyphenylacetic acid.

DISCUSSION

In the present study, we investigated the possibility of producing high-added-value products by microbial transformations using isomers of synthetic tyrosol as precursors. During the screening of microorganisms able to utilize *p*-tyrosol as a sole carbon and energy source, a strain, further characterized as *Ser. marcescens*, was isolated. This strain was capable of producing hydroxytyrosol using a high concentration (up to 2 g/L) of *p*-tyrosol. This result is in agreement with previous investigations demonstrating that *Ser. marcescens* strain tolerates high vanillin concentrations to be converted into vanillic acid (*37*). Furthermore, *Ser. marcescens* strain was capable of transiently accumulating a significant amount of hydroxytyrosol which resulted from an ortho-hydroxylation of *p*-tyrosol. Using optimal conditions, 1.6 g of hydroxytyrosol was obtained after a 7-h incubation of *Ser. marcescens* in 1 L of minimal medium in the presence of 2 g of *p*-tyrosol. This yield would be improved using the resting cell technique as was recently performed using *Pseudomonas aeruginosa* (*23*).

The produced hydroxytyrosol by the isolated strain *Ser. marcescens* resulted from an ortho-hydroxylation of *p*-tyrosol. In a previous investigation, Mason (*38*) noted that monooxygenases are commonly involved in the aromatic ring-hydroxylation of a wide range of aromatic compounds, and the most frequently encountered aromatic monooxygenases are of the *p*-hydroxybenzoate hydroxylase type. These enzymes add a single hydroxyl group to an already hydroxylated substrate to generate a product with vicinal hydroxyl groups.

The substrate-specificity of the performed hydroxylation reaction was examined. The obtained result suggested that the enzymatic system of the isolated strain *Ser. marcescens* was affected by the position of the hydroxyl group of the aromatic substrate. *Ser. marcescens* hydroxylated the tyrosol containing a hydroxyl group in the para position, but it oxidized the side chain carbon when the hydroxyl group was in the meta or ortho position. On the other hand, *Ser. marcescens* strain transformed completely *m*-tyrosol and *o*-tyrosol into, respectively, 3-hydroxyphenylacetic acid and 2-hydroxyphenylacetic acid by oxidizing the side chain carbon of the corresponding precursors. This result confirms the findings by De la Fuente et al. (*39*) who reported that grown and resting cells of *Ser. marcescens* oxidize a variety of aromatic aldehydes. The productions of 3-hydroxyphenylacetic acid and 2-hydroxyphenylacetic acid developed in the present study were achieved after 4 and 30 h of incubation time, respectively. Staudenmaier et al. (*24*, *28*) patented the preparation protocols of 3-hydroxyphenylacetic acid and 2-hydroxyphenylacetic acid via microbial hydroxylation of phenylacetic acid in the meta and the ortho positions, respectively. In these investigations, researchers used a fungus to convert phenylacetic acid entirely. However, compared with our procedure, the used protocols are very slow. Indeed, the performed reactions were accomplished after 7 days of incubation time with a molar yield of 100%. The produced 3-hydroxyphenylacetic acid and 2-hydroxyphenylacetic by the isolated *Ser. marcescens* strain would be used in the pharmaceutical industry as alternatives to the synthetic intermediates which are involved in the production of various medicinal products as was suggested in previous works (*24*, *28*).

In comparison with our previous study dealing with hydroxytyrosol production using *P. aeruginosa* (*23*), *Ser. marcescens* is a more promising strain for several reasons. Indeed, *Ser. marcescens* transformed entierly *m*-tyrosol and *o*-tyrosol into, respectively, 3-hydroxyphenylacetic acid and 2-hydroxyphenylacetic acid with molar yields of 100% while *P. aeruginosa* was

not able to accumulate either 3-hydroxyphenylacetic acid or 2-hydroxyphenylacetic acid which were completely metabolized at the end of the culture (*23*). Moreover, *Ser. marcescens* grew on high concentration of *p*-tyrosol (up to 4 g/L), whereas *P. aeruginosa* tolerated *p*-tyrosol at concentration up to 2 g/L (*23*). Furthermore, *Ser. marcescens* was able to transform high *p*-tyrosol concentration (5 g/L) into hydroxytyrosol with a high molar yield. Work is in progress to switch from batch cultures to continuous process development for potential industrial application.

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