Isolation and characterization of *Halomonas* sp. strain IMPC, a *p*-coumaric acid-metabolizing bacterium that decarboxylates other cinnamic acids under hypersaline conditions

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Abstract

A moderately halophilic, mesophilic, Gram-negative, motile, nonsporulating bacterium, designated strain IMPC, was isolated from a table-olive fermentation rich in aromatic compounds, after enrichment on *p*-coumaric acid under halophilic conditions. Strain IMPC was able to degrade *p*-coumaric acid. *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid were detected as breakdown products from *p*-coumaric acid. Protocatechuic acid was identified as the final aromatic product of *p*-coumaric acid catabolism before ring fission. Strain IMPC transformed various cinnamic acids with substituent H, OH, CH_3 or OCH_3 in the *para*- and/or *meta*-position of the aromatic ring to the corresponding benzoic acids, indicating a specific selection. A β-oxidation pathway was proposed for these transformations. Phylogenetic analysis of the 16S rRNA gene revealed that this isolate was a member of the genus *Halomonas*. Strain IMPC was closely related to *Halomonas elongata* ATCC 33173^{T}and *Halomonas eurihalina* ATCC 49336^T.

Introduction

Table-olive fermentation contains a high concentration of polymers, including polyphenolic compounds, and constitutes a potential cause of pollution in the Mediterranean basin. The phenolic composition depends on the variety of the olives (Bouaziz *et al*., 2004). This agro-industrial wastewater was shown to be treated successfully in biological systems (Beltran-Heredia *et al*., 2000). A number of these aromatic compounds are degraded or transformed by bacteria and fungi (Sayadi *et al*., 2000; Chamkha *et al*., 2001; Allouche *et al*., 2004a; Abdelkafi *et al*., 2005). However, very little information is available about the degradation of aromatic compounds in saline conditions. Moderately, halophilic bacteria grow optimally between 0.5 and 2.5 M salt (Kushner, 1978). Among this heterogenous group of microorganisms, the genus *Halomonas* has been described as one of the few aromatic degraders. Indeed, *Halomonas organivorans* degrades different aromatic pollutants in saline habitats of southern Spain (Garcia *et al*., 2004). *Halomonas campisalis*, which was isolated near Soap Lake in central Washington, degrades both phenol and catechol (Alva & Peyton, 2003). In addition, a moderately halophilic strain of *Halomonas* is involved in the biotreatment of saline phenolic wastewater and degrades phenol as the sole source of carbon and energy (Hinteregger & Streichsbier, 1997).

This paper reports the isolation and characterization of a new *Halomonas* sp. strain IMPC from table-olive fermentation. This is the first report of a strain belonging to the genus *Halomonas* that specifically degrades *p*-coumaric acid. The strain also metabolizes other cinnamic acids by decarboxylating the C_3-aliphatic chain to produce the corresponding benzoic acids.

Materials and methods

Culture conditions

Bacteria were grown in a basal medium containing the following per liter: NaCl, 80 g; KH_2PO_4, 0.4 g; NH_4Cl, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.33 g; $CaCl_2$, 0.05 g; yeast extract, 0.1 g; and 1 mL trace-element solution (Widdel & Pfenning, 1981). The pH was adjusted to 7.2 with 10 M KOH. Aliquots of 25 mL were dispensed into flasks and sterilized by autoclaving at 121 °C for 20 min. The aromatic compound stock

solutions were prepared, neutralized if necessary and sterilized by filtration (pore size 0.2 μm; Millipore, Bedford, MA). Prior to inoculation, substrates were injected from concentrated sterile stock solutions to obtain the desired final concentration. Strain IMPC was routinely grown on basal medium containing 10 mM *p*-coumaric acid. Cultures (25 mL) were inoculated and incubated at 37 °C with agitation at 150 r.p.m.

Enrichment and isolation of *p*-coumaric acid degraders

A 2.5 mL sample from the table-olive fermentation was used to inoculate a 25 mL medium containing 10 mM *p*-coumaric acid. The culture was then incubated at 37 °C under agitation at 150 r.p.m. The enrichment culture was subcultured several times under the same conditions until the substrate was completely metabolized. Noninoculated cultures were performed under the same conditions to verify that *p*-coumaric acid was not partially transformed abiotically in the aerobic medium. The disappearance of *p*-coumaric acid was confirmed by high performance liquid chromatography (HPLC) analysis. Aliquots (100 μL) of 10^{-1}–10^{-10} dilutions were plated onto agar basal medium and incubated overnight at 37 °C. Single colonies were picked and used for screening.

Strain characterization

For all experiments, basal medium containing 10 mM *p*-coumaric acid was used. The pH of the medium was adjusted with 5 M HCl or 10 M KOH to obtain a range between 4 and 12. Different amounts of NaCl were directly weighed in flasks prior to dispensing 25 mL of medium to obtain the desired NaCl concentration (range 0–250 g L^{-1}). The temperature range for growth was analyzed between 5 and 55 °C (at 5 °C intervals). Light and electron microscopies were performed as described by Fardeau *et al.* (1997). For heat resistance, cells grown in a basal medium containing *p*-coumaric acid were exposed to temperatures of 80, 90 and 100 °C for 10 min. The cells were cooled quickly to ambient temperature, inoculated into a fresh glucose-containing medium and growth recorded after 24 h incubation at 37 °C with agitation at 150 r.p.m. The tested conditions for sporulation included growth in the absence of a carbon source or in the presence of yeast extract or *p*-coumaric acid. Gram reaction was determined using the BioMérieux Gram stain Kit according to the manufacturer's instructions. Catalase activity was determined by bubble production in a 3% (volume in volume) hydrogen-peroxide solution. Oxidase activity was determined by oxidation of 1% *p*-aminodimethylaniline oxalate. Experiments were performed in duplicate with an inoculum subcultured at least once under the same test conditions. The substrates tested for utilization were injected from presterilized and concentrated stock solutions into flasks containing a 25 mL presterilized medium. The following substrates were used: carbohydrates (20 mM) (glucose, fructose, galactose, maltose, mannitol and lactose); gelatin, peptone and yeast extract (2 g L^{-1}); glycerol (20 mM); and aromatic compounds (5 mM). Aromatic compounds comprised benzoic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), hydroxylated benzoic acids (*p*-, *m*- and *o*-hydroxybenzoic acids), methoxylated benzoic acids (*p*-methoxybenzoic acid; 2,4-, 2,5-, 3,4- and 3,5-dimethoxybenzoic acids), mixed hydroxylated/methoxylated benzoic acids (4-hydroxy-3-methoxybenzoic acid, 3-hydroxy-4-methoxybenzoic acid and 4-hydroxy-3,5-dimethoxybenzoic acid), methylated benzoic acids (*p*-methylbenzoic acid and 3,4-dimethylbenzoic acid), cinnamic acid, hydroxylated cinnamic acids (*p*-, *m*- and *o*-coumaric acids), caffeic acid (3,4-dihydroxycinnamic acid), methoxylated cinnamic acids (*p*- and m-methoxycinnamic acids), methylated cinnamic acids (*p*- and m-methylcinnamic acids), *p*-hydroxybenzaldehyde and *p*-hydroxyphenylacetic acid. Aromatic compounds were tested with or without yeast extract (0.1 g L^{-1}). An increase in $OD_{600\,nm}$ in substrate-containing cultures, compared with control tubes lacking substrates, was considered as positive growth. Other phenotypic characteristics were determined using API 20E and API 50CHB kits (BioMérieux, Marcy l'Etoile, France) following the methods of Logan & Berkeley (1984). Additionally, the API ZYM gallery (BioMérieux) method was performed for the determination of extracellular enzymatic activities.

Antibiograms

Resistance to antibiotics was determined on Mueller–Hinton agar (Difco 0252, Beckton Dickinson, Le Pont de Claix, France) using standard antibiotic disks (BioMérieux). The inhibition zone was noted after 48 h incubation. Inhibition diameters were recorded after 24 h of incubation at 37 °C under aerobic conditions. The classification of the strain as sensitive, not sensitive or intermediately sensitive to the antibiotics was according to the disk manufacturer's instructions (BioMérieux). Tests were performed in triplicate.

Analytical methods

Bacterial growth was measured at 600 nm using a Shimadzu model UV 160A spectrophotometer (Shimadzu, Duisburg, Germany). Aromatic compounds were measured by HPLC as described by Abdelkafi *et al.* (2005). Chemical structures of phenolic compounds were confirmed by gas chromatography-mass spectrometry (GC-MS) as described by Allouche *et al.* (2004b).

G+C content, sequencing and phylogenetic analysis

The G+C content of DNA was determined by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), using HPLC as described by Mesbah *et al.* (1989). The 16S rRNA gene of strain IMPC was amplified by adding 1 μL of cell culture to a thermocycler microtube containing 5 μL of 10 × *Taq* buffer, 0.5 μL of each 50 nM primers Fd1 and Rd1, 5 μL of 25 mM $MgCl_2 \cdot 6H_2O$, 0.5 μL of 25 mM dNTPs, 0.5 μL of *Taq* polymerase (5 U μL^{-1}) and 38 μL of sterilized distilled water. The universal primers Fd_1 and Rd_1 (Fd1, 5′-AGAGTTTGATCCTGGCTCAG-3 and Rd1, 5′-AAGGAGGTGATCCAGCC-3′) were used to obtain a PCR product of ∼1.5 kb corresponding to base positions 8–1542 based on *Escherichia coli* numbering of the 16S rRNA gene (Winker & Woese, 1991). The sample was placed in a hybrid thermal reactor thermocycler (BIOMetra, Leusden, The Netherlands), denatured for 1 min at 96 °C and subjected to 30 cycles for 20 s at 96 °C, 30 s at 55 °C and 2 min at 72 °C. This was followed by a final elongation step for 5 min at 72 °C. PCR products were cloned using the pGEM-T-easy cloning kit (Promega, Charbonnières-Les-Bains, France) according to the manufacturer's protocol. Clone libraries were screened by direct PCR amplification from a colony using the vector-specific primers SP6 (5′-ATTTAGGTGACACTATAGAA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) and the following reaction conditions: an initial denaturation for 2 min at 96 °C, then 40 cycles of denaturation, annealing and extension for 30 s at 96 °C, 30 s at 50 °C, 2 min at 72 °C and a final extension for 5 min at 72 °C. Plasmids containing inserts of the expected length were isolated using the Wizard Plus SV Minipreps DNA purification system (Promega), according to the manufacturer's protocol. Purified plasmids were sent for sequencing to Genome Express (Grenoble, France). Sequence data were imported into the sequence editor BioEdit version 5.0.9 (Hall, 1999); base calling was examined and a contiguous sequence was obtained. The full sequence was aligned using the RDP Sequence Aligner program (Maidak *et al.*, 2001). The consensus sequence was manually adjusted to conform to the 16S rRNA gene secondary structure model (Winker & Woese, 1991). A nonredundant BLAST search (Altschul *et al.*, 1997) identified its closest relatives. Sequences used in the phylogenetic analysis were obtained from the RDP (Maidak *et al.*, 2001) and GenBank databases (Benson *et al.*, 1999). Positions of sequence and alignment ambiguities were omitted and pairwise evolutionary distances were calculated using the method of Jukes & Cantor (1969). A dendrogram was constructed using the neighbor-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined using 100-bootstrapped trees (Felsenstein, 1985).

Results

Isolation of *p*-coumaric acid degraders

To isolate different *p*-coumaric-degrading microorganisms, an enrichment culture method was used. This enrichment culture was designed to select strains able to grow on *p*-coumaric acid as a sole carbon and/or energy source. The substrate transformation was followed by HPLC analyses. Based on their morphologies, three different *p*-coumaric-tolerant strains were isolated. These bacteria were then screened for their ability to degrade *p*-coumaric acid. One strain appeared to degrade the highest concentration of *p*-coumaric acid under halophilic conditions. This strain, IMPC, was selected for further characterization.

Morphology and physiology

Strain IMPC was isolated from a table-olive fermentation rich in aromatic compounds, after enrichment on *p*-coumaric acid. Cells of strain IMPC were Gram-negative rods (0.7–1.2 × 1.2–1.8 μm), motile and aerobic. Spores were not observed. The temperature range for growth was 25–45 °C, with optimal growth at 37 °C. The pH range for growth was 5–9, with an optimum at 7.2. Optimal growth occurred in the presence of 50–80 g L^{-1} NaCl. Growth occurred in the range of 0.5–150 g L^{-1} NaCl. This isolate was regarded as a moderately halophilic bacterium. Catalase and oxidase reactions were positive. ONPG hydrolysis was positive. Lysine decarboxylase, ornithine decarboxylase and tryptophane desaminase were not produced. Arginine dihydrolase was produced. Citrate was utilized. Gelatin was not hydrolyzed. Hydrogen sulfide was not produced. Nitrate was reduced to nitrite.

Properties of strain IMPC in API-ZYM test

The strain showed high enzyme activity for esterase (C4), leucine arylamidase, acid phosphatase, α-glucosidase, alkaline phosphatase, valine arylamidase, α-galactosidase, β-glucosidase and β-galactosidase. No activity was detected on cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α-fucosidase, esterase lipase (C8), lipase, trypsin, α-chymotrypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase and α-mannosidase.

Antibiotic susceptibility

The growth behavior of the IMPC isolate was studied in the presence of a range of antibiotics. The strain was susceptible to penicillin (10 μg), streptomycin (10 μg), kanamycin (30 μg), neomycin (30 μg), chloromphenicol (30 μg) and colistine (50 μg). It was resistant to tetracycline (30 μg) and polymyxin (25 μg).

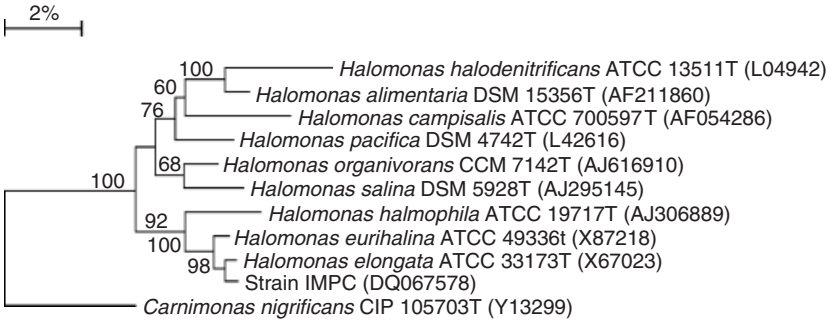

Fig. 1. Phylogenetic dendrogram based on 1301 unambiguous base pairs of the 16S rRNA gene sequences indicating the position of *Halomonas* sp. strain IMPC and its closest relative sequences validated at a species level belonging to the genus *Halomonas*. Reference type-strain organisms are included.

Phylogenetic analysis

To analyze the phylogenetic position, the 16S rRNA gene sequence of IMPC (comprising 1484 bp) was determined, and a phylogenetic tree based on 1301 unambiguous bp was constructed (Fig. 1). The 16S rRNA gene sequence of strain IMPC has been deposited in the GenBank database under accession number DQ067578. Phylogenetic analysis revealed that strain IMPC is a species of the genus *Halomonas*. Strain IMPC was found to be closely related to *Halomonas elongata* and *Halomonas eurihalina* (more than 99% similarity between the 16S rRNA gene sequences). Strain IMPC also shared 97.10% and 97.0% identity with *Halomonas halmophila* and *Halomonas salina*, respectively. The G+C content of strain IMPC was 62.6 mol% as determined by the HPLC method (Mesbah *et al*., 1989).

Metabolism of aromatic compounds by *Halomonas* sp.

To confirm that *Halomonas* sp. strain IMPC was able to grow aerobically on *p*-coumaric acid, time course growth experiments were undertaken and culture samples were taken at different times and analyzed using reverse-phase HPLC. Strain IMPC growing on basal medium supplemented with 10 mM *p*-coumaric acid reached the stationary phase in 12 h and the maximal optical density at 600 nm wavelength was 0.9–1.2. During the first 4 h of incubation, the $OD_{600\,nm}$ remained constant (Fig. 2). During this period, *Halomonas* sp. transformed *p*-coumaric acid into *p*-hydroxybenzoic acid without further ring degradation of the aromatic compound. *p*-hydroxybenzaldehyde appeared in the medium as an intermediate (Fig. 3). This pathway has been found in the white rot fungus *Pycnoporus cinnabarinus* (Estrada Alvarado *et al*., 2001). After the lag phase, the *p*-hydroxybenzoic acid concentration in the medium increased. The degradation of aromatic compounds started and, consequently, an increase in the $OD_{600\,nm}$ was observed. After 9 h, *p*-hydroxybenzoic acid was degraded and trace amounts of protocatechuic acid appeared in the medium as intermediates. The identities of these compounds were confirmed by GC-MS analysis. Aromatic compounds were completely removed from the culture after 14 h. Based on these results, a pathway for the degradation of *p*-coumaric acid was proposed, as shown in Figure 4. *p*-coumaric acid is firstly converted to *p*-hydroxybenzaldehyde, which is transformed to *p*-hydroxybenzoic acid and then to protocatechuic acid. This suggests that protocatechuic acid could be the final aromatic product of *p*-coumaric acid catabolism before ring fission.

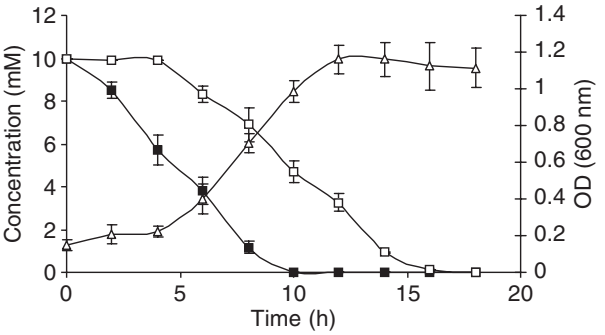

Fig. 2. Time course of the growth of *Halomonas* sp. strain IMPC in basal medium containing 10 mM *p*-coumaric acid (see Materials and Methods). △, $OD_{600\,nm}$; ■, *p*-coumaric acid; □, aromatic compounds.

Growth of *Halomonas* sp. strain IMPC on *p*-coumaric acid occurred only under aerobic conditions. The degradation of *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic, caffeic and protocatechuic acids was observed. The cinnamic compounds tested, namely cinnamic acid, *m*-coumaric acid, *m*- and *p*-methoxycinnamic acids, *m*- and *p*-methylcinnamic acids and ferulic acid were metabolized to their corresponding benzoic acid derivates, respectively, benzoic acid, *m*-hydroxybenzoic acid, *m*- and *p*-methoxybenzoic acids, *m*- and *p*-methylbenzoic acids and vanillic acid. The mechanism involved decarboxylation of the carboxyl group in the C_3-aliphatic chain. No degradation product was detected in the culture medium when these benzoic acids were used as the growth substrates.

In addition, the ability of strain IMPC to grow on phenylpropionic acid, *p*-hydroxyphenylpropionic acid, cinnamaldehyde, cinnamylalcohol and mandelic acid was tested. Cinnamaldehyde, cinnamylalcohol and mandelic acid were not metabolized. The metabolites detected by HPLC analysis in the culture of strain IMPC grown on phenylpropionic acid suggested that cinnamic acid, benzaldehyde and benzoic acid were the intermediates in phenylpropionic acid conversion. However, *p*-hydroxyphenylpropionic acid was degraded by strain IMPC via *p*-coumaric acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and protocatechuic acid.

Discussion

Strain IMPC, isolated from a sample of table-olive fermentation, after enrichment on *p*-coumaric acid, is a Gram-negative, aerobic, mesophilic, motile bacterium. The phenotypic, genotypic and phylogenetic characteristics of isolate IMPC indicated that it belongs to the genus *Halomonas*; it was found to be closely related to *Halomonas elongata* and *Halomonas eurihalina* (more than 99% similarity between the 16S rRNA gene sequences). Species of genus *Halomonas* have been isolated from saline soils, hypersaline lakes, marine water and other habitats (Ventosa, 1988; Javor, 1989). Our results extend the known ecosystems to include table-olive fermentation. Isolation of strain IMPC from table-olive fermentation on *p*-coumaric acid is not surprising as this biotope contains a wide range of aromatic compounds including *p*-coumaric acid. A strain of *Bacillus* sp. has been isolated from table-olive fermentation, after enrichment on tyrosol, and was found to be able to transform tyrosol to *p*-hydroxyphenylacetic acid (Abdelkafi *et al.*, 2005). Other studies showed that *Halomonas* species can degrade aromatic compounds (Hinteregger & Streichsbier, 1997; Alva & Peyton, 2003; Garcia *et al.*, 2004). However, to our knowledge, no previous study has reported the pathways for degradation of lignin-related compounds such as cinnamic acids under halophilic conditions. Traditionally, *p*-coumaric acid has been removed from industrial effluents by physicochemical methods (e.g. ozone treatment), but these treatments can be complex and expensive, as reported by Amat *et al.* (1999).

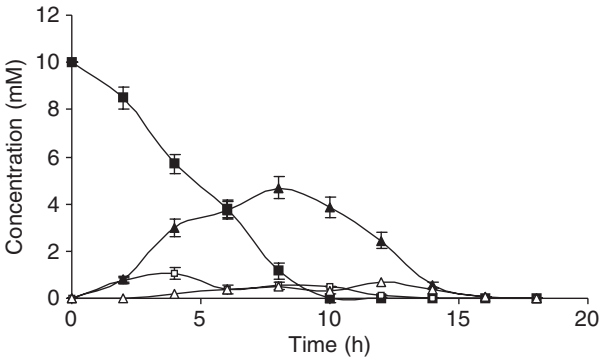

Fig. 3. Time course of *p*-coumaric acid degradation with *Halomonas* sp. strain IMPC; ■, *p*-coumaric acid; □, *p*-hydroxybenzaldehyde; ▲, *p*-hydroxybenzoic acid; △, protocatechuic acid.

GC-MS and HPLC analyses revealed that only cinnamic acids with a single hydroxy in the *para* position were fully degraded under aerobic conditions. The degradative routes taken in the aerobic metabolism of these compounds by *Halomonas* sp. strain IMPC are summarized in Figure 4. The metabolism of *p*-coumaric acid in *Halomonas* sp. strain IMPC proceeded via protocatechuic acid. Metabolism of *p*-coumaric acid involved the oxidation of the C_3-aliphatic side chain to produce *p*-hydroxybenzoic acid, followed by 3-hydroxylation to protocatechuic acid, which was also suggested in *Cetobacter calcoaceticus* DSM 586 (Delneri *et al.*, 1995) and *Sphingomonas paucimobilis* SYK-6 (Masai *et al.*, 2002). Many aerobic bacteria have been found to degrade *p*-coumaric acid via gentisic acid (Peng *et al.*, 2003). Strain IMPC also had the ability to transform the *p*-hydroxyphenylpropionic and phenylpropionic acids to *p*-coumaric and cinnamic acids, respectively, but they were not dead-end products. These intermediate products indicated that the decarboxylation mechanism involved a β-oxidative route. Among the aromatic substrates tested, only cinnamic acids with substituent H, OH, CH_3 or OCH_3 in the *para* and/or *meta* position of the aromatic ring were decarboxylated to the corresponding benzoic acids. No degradation products were detected in the culture medium when these benzoic acids were used as the growth substrates. Cinnamic acids were not metabolized when the aromatic ring was substituted in *ortho* position.

From these studies, it can be deduced that (i) cinnamic acids were completely metabolized when the ring was substituted only with a *para*-hydroxyl or *para*, *meta*-dihydroxyl groups, (ii) the decarboxylation of a cinnamic acid into the corresponding benzoic acid was strongly correlated to the position(s) of the substituent(s) and (iii) the mechanism

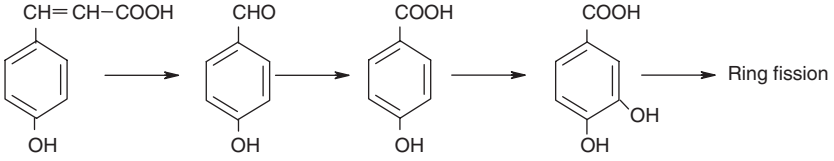

Fig. 4. Pathway for the biodegradation of *p*-coumaric acid by *Halomonas* sp. strain IMPC.

involved a β-oxidative decarboxylation of the carboxyl group in the C_3-aliphatic chain.

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