Isolation and characterization of a novel Bacillus sp., strain YAS1, capable of transforming tyrosol under hypersaline conditions

Slim Abdelkafi a,b, Mohamed Chamkha a, Laurence Casalot b, Sami Sayadi a, Marc Labat b,*

a Laboratoire des Bio-procéédés, Centre de Biotecnologie de Sfax, Route de Sidi Mansour Km 4, 3038 BP ‘‘K’’, Sfax, Tunisia
b Biotechnologie microbienne des environnements chauds UR180, IRD, IFR-BAIM, Universités de Provence et de la Méditerranée, ESIL case 925, 163, Avenue de Luminy, 13288 Marseille cedex 9, France

Received 10 May 2005; received in revised form 19 August 2005; accepted 22 August 2005
First published online 6 September 2005
Edited by Dr. A. Oren

Abstract

A moderately halotolerant, Gram-positive, aerobic, motile, spore-forming bacterium, designated as strain YAS1, was isolated from an olive-brine fermentation rich in aromatic compounds, after enrichment on tyrosol. Strain YAS1 grew between 25 and 45 °C and optimally at 37 °C. It grew in the presence of 0–15% (v/w) NaCl, with an optimum of 3–6% (v/w) NaCl. The DNA G+C content was found to be 49.9 mol%. Phylogenetic analysis of the 16S rRNA gene revealed that this isolate was a member of the genus Bacillus. The newly isolated strain YAS1 represents the first moderately halotolerant bacterium transforming tyrosol to p-hydroxyphenylacetic acid (PHPA) in the presence of yeast extract.
© 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Tyrosol; Table-olive fermentation; Aromatic compounds; Moderately halotolerant; Bacillus sp.

1. Introduction

The worldwide production of table olives has been estimated at approximately 1 million tons per year with the Mediterranean countries being the main producers [1]. The transformation of table olives occurs during fermentation in the brining stage. Table olives contain a high proportion of phenolic compounds [2]. The specific composition depends on the variety of the olives [3]. Tyrosol (p-hydroxyphenylethanol) is one of the major phenolic compounds present in table olives [4] and exhibits toxicity toward several microorganisms [5]. Halotolerant bacteria are non-halophilic microorganisms that can tolerate high salt concentration [6]. Relatively few reports have addressed the degradation of aromatic compounds under highly saline conditions. Halomonas organivorans exhibited degradation of different aromatic pollutants in saline habitats of southern Spain [7]. A moderate halophilic bacterium, member of the genus Halomonas, was involved in biotreatment of saline phenolic waste-water and degrades phenol as the sole source of carbon and energy [8]. In addition, Halomonas campisalis, which was isolated near Soap Lake in central Washington, showed the capability to degrade phenol and catechol [9]. Aerobic degradation of tyrosol has been poorly studied. Pseudomonas aeruginosa has been reported to transform tyrosol into a significant amount of hydroxytyrosol and trace quan-
tities of \(p\)-hydroxyphenylacetic acid (PHPA) and 3,4-dihydroxyphenylacetic acid [10]. Tyrosol has been converted in aerobic conditions by a cell-free preparation of the white-rot basidiomycete \textit{Lentinus edodes} into dimeric tetracyclic ketone [11]. In this paper, we report the isolation from olive-brine fermentation and the characterization of a new aerobic tyrosol-converting, moderately halotolerant bacterium, strain YAS1 belonging to the genus \textit{Bacillus}.

2. Materials and methods

2.1. Source of strains

Samples were collected in sterile plastic bottles from a table-olive manufacturer (Chawatt factory) near Tunis and stored in the dark at 4 °C until use. Twelve strains were isolated from enrichment cultures inoculated with olive-brine fermentation wastewater. Different strains were routinely cultured and maintained in the basal medium supplemented with 5 mM tyrosol, as described below. Then one of them, named strain YAS1, was chosen for further investigation. Strain YAS1 belongs to the genus \textit{Bacillus}. In addition to the isolate, \textit{Bacillus firmus DSM 12} \textsuperscript{T}, \textit{Bacillus flexus DSM 1320} \textsuperscript{T}, \textit{Bacillus batavensis DSM 15601} \textsuperscript{T}, \textit{Bacillus niacin DSM 2923} \textsuperscript{T} and \textit{Bacillus megaterium DSM 32} \textsuperscript{T} were used as references for phenotypic characterization and utilization of aromatic compounds. These micro-organisms were cultivated under the same conditions.

2.2. Culture medium

The basal medium contained 50 g NaCl, 0.4 g \(\text{KH}_2\text{PO}_4\), 0.5 g \(\text{NH}_4\text{Cl}\), 0.33 g MgCl\(_2\) · 6 \(\text{H}_2\text{O}\), 0.05 g CaCl\(_2\) · 6 \(\text{H}_2\text{O}\) and 1 g yeast extract per liter of distilled water, which was supplemented with 1 ml trace element solution [12]. The pH of the medium was adjusted to 7.2 with 10 M KOH solution. Aliquots of 25 ml were dispensed into flasks and sterilized by autoclaving at 121 °C for 20 min. Substrates were injected from concentrated sterile stock solutions to obtain the desired final concentration. Strain YAS1 was routinely grown on basal medium containing 5 mM tyrosol.

2.3. Enrichment and isolation

A 2.5-ml sample from the table-olive fermentation was used to inoculate into 25 ml of basal medium containing 1 g l\(^{-1}\) yeast extract and 5 mM tyrosol as carbon sources. The culture was then incubated at 37 °C under agitation (150 rpm). The enrichment culture was subcultured several times under the same conditions until the substrate was completely metabolized. Non-inoculated tubes were run in the same conditions to verify that tyrosol was not partially transformed by abiotic ways in the aerobic medium. The disappearance of tyrosol was confirmed by HPLC. Aliquots (100 \(\mu\)l) of \(10^{-1}\)–\(10^{-10}\) dilutions were plated onto tyrosol (5 mM) agar basal medium and incubated overnight at 37 °C. Single colonies were picked and used for screening.

2.4. Strain characterization

For all experiments, basal medium containing 1 g l\(^{-1}\) yeast extract 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 weighted in flasks prior to dispensing 25-ml 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. [13]. For heat resistance, cells grown in basal medium containing yeast extract were exposed to temperatures of 80, 90 and 100 °C for 10 min. The cells were then cooled quickly to ambient temperature, inoculated into fresh glucose-containing medium and growth was recorded after 24 h incubation at 37 °C under agitation (150 rpm). Conditions for sporulation that were tested included growth in the presence of yeast extract or tyrosol, or without carbon source. Gram reaction was determined using the BiöMérieux Gram stain Kit according to the manufacturer’s instructions. Catalase activity was determined by bubble production in 3% (v/v) 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 pre-sterilized and concentrated stock solutions into flasks containing 25-ml pre-sterilized basal 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). The aromatic compound stock solutions were prepared, neutralized if necessary and sterilized by filtration (pore size 0.2 μm; Millipore). Aromatic compounds were tested with or without yeast extract (1 g l\(^{-1}\)). An increase in OD\(_{600}\) in substrate-containing cultures, compared with control tubes lacking substrates, was considered positive growth. Other phenotypic characteristics were determined by using API 20E and API 50CHB kits (BioMérieux, La Balme-Les-Grottes, France) following the methods of Logan and Berkeley [14]. Additionally, the API ZYM gallery (BioMérieux, La Balme-Les-Grottes, France) method was performed for determination of extracellular enzymatic activities.
2.5. Resistance to antibiotics

Resistance to antibiotics was determined on Mueller–Hinton agar (Difco 0252) using standard antibiotic disks (Biomérieux, Marcy l’Étoile, France). 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 intermediate sensitive to the antibiotics, was done according to the disk manufacturer’s instructions (BioMérieux). Tests were performed in triplicate.

2.6. G + C content, sequencing and phylogenetic analysis

The G + C content of DNA was determined by the DSMZ, using HPLC as described by Mesbah et al. [15]. The 16S rDNA gene of strain YAS1 was amplified by adding 1-μl 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 MgCl₂, 6 H₂O, 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 Fd1 and Rd1 (Fd1, 5’-AGAGTTTGATCCTGGCTCAG-3; Rd1, 5’-AAGAGG- TGATCCAGGCC-3’) were used to obtain a PCR product of approximately 1.5 kb corresponding to base position 8–1542, based on Escherichia coli numbering of the 16S rDNA gene [16]. The sample was placed in a hybrid thermal reactor thermocycler (BIOMetra), denatured by heating 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) according to the manufacturer’s protocol. Clone libraries were screened by direct PCR amplification from a colony using the vector-specific primers SP6 (5’-ATTAGGTGACACTATAGA-3’) and T7 (5’-TAATACGACTCCTATAG- GG-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 an insert of the expected length were isolated using the Wizard Plus SV Miniprep 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 [17]; base calling was examined and a contiguous sequence was obtained. The full sequence was aligned using the RDP Sequence Aligner program [18]. The consensus sequence was manually adjusted to conform to the 16S rRNA secondary structure model [16]. A non-redundant BLAST search [19] identified its closest relatives. Sequences used in the phylogenetic analysis were obtained from the RDP [18] and GenBank databases [20]. Positions of sequence and alignment ambiguity were omitted and pairwise evolutionary distances were calculated using the method of Jukes and Cantor [21]. A dendrogram was constructed by using the neighbor-joining method [22]. Confidence in the tree topology was determined by using 100-bootstrapped trees [23].

2.7. Analytical methods

Bacterial growth was measured at 600 nm by using a Shimadzu model UV 160A spectrophotometer. Aromatic compounds were measured by HPLC as described by Chamkha et al. [24]. The identity of aromatic compounds was confirmed by comparing retention times.

3. Results and discussion

3.1. Isolation and characterization of the isolate

To isolate different tyrosol-tolerant microorganisms, an enrichment culture method was used according to the protocol described in Section 2. After several dilutions and subculturing in the same liquid medium, a stable microbial consortium developed and confirmed the aerobic tyrosol biotransformation. The morphologically dominant bacterial population was a motile and spore-forming bacterium. Subsequently, this enrichment culture was serially diluted and used to inoculate Petri dishes. Twelve isolates were obtained. The 16S rRNA genes were compared by Amplified Ribosomal DNA Restriction Analysis (ARDRA) profiles. Three of these pure colonies (YAS1, YAS2, YAS3) showed both similar ARDRA (data not shown) profiles and a similar biotransformation of tyrosol. Strain YAS1 was selected for further characterization.

Strain YAS1 is a mesophilic, Gram-positive, motile and strictly aerobic bacterium. Cells are rod-shaped (0.4–0.9 μm in wide and 2.5–4.5 μm in long) in overnight culture on basal medium at 37 °C under agitation (150 rpm). The cells are found both singly and double, and can form chains. Colonies of the strain are smooth, circular, low-convex, semi-transparent and 2–3 mm in diameter after overnight culture at 37 °C. The temperature range supporting growth is from 25 to 45 °C, with an optimum at 37 °C. The pH range for growth is between 6 and 8.5 with an optimum at 7. Yeast extract was required for growth. Catalase and oxidase reactions are positive. Other characteristics of strain YAS1 in comparison with
the closely related species of genus *Bacillus* are shown in Table 1.

### 3.2. Properties of strain YAS1 in API-ZYM test

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

### 3.3. Antibiotic susceptibility

The growth behaviour of the YAS1 isolate was studied in the presence of a range of antibiotics. The strain was susceptible to penicillin (6 μg), streptomycin (10 UI), kanamycin (30 UI), neomycin (30 UI) and tetracycline (30 UI); weakly susceptible to chloramphenicol (30 μg) and colistin (10 μg), and resistant only to polymyxine (300 UI).

### 3.4. Phylogenetic position of the isolate

To analyze the phylogenetic position, the 16S rDNA sequence of YAS1 (comprising 1521 bp) was determined, and a phylogenetic tree was constructed (Fig. 1). The sequence was deposited in the GenBank database under the accession number AY880316. The phylogenetic analysis indicated that strain YAS1 is most closely related to species of genus *Bacillus*. No species in the genus *Bacillus* were higher than 97% in sequence similarity with the isolate. Therefore, this isolate could be further affiliated as a new species of genus *Bacillus*.

The G + C content of genomic DNA of strain YAS1 was 49.9 mol% as determined by the HPLC method. This value falls within the range described for species of the genus *Bacillus* [25].

### 3.5. Halotolerance of the isolate

The halotolerant properties of strain YAS1 were precisely determined by measuring the specific growth rate at different concentrations of sodium chloride under aerobic conditions and in the presence of 5 mM tyrosol. Growth occurs in the range of 0–15% (w/v) NaCl. This isolate is regarded as moderately halotolerant bacterium. Strain YAS1 did not require Na+ ion for growth, but grew optimally on media containing 3–6% (w/v) of NaCl. The same result has been reported with *Rheinheimera baltica* strain which did not require NaCl for growth, but which growth was stimulated by sodium ions [26]. No degradation took place in the presence of 20% NaCl.

### 3.6. Biotransformation of tyrosol to PHPA by strain YAS1

Tyrosol (p-hydroxyphenylethanol), well known as occurring naturally with other aromatic compounds in olive oil [27] and table-olive fermentation [4], can be
obtained mainly from olive leaves and fruits. Tyrosol was identified from olive pulp [27], olive stones [28], and with olive by-products inside olive cake [29] or olive mill wastewater [27,30]. A recent review [27] showed tyrosol being identified, in olive oil, as a free compound or linked with the dialdehydic form of elenolic acid, and with gluco-sylated forms in olive fruit. The formation of PHPA was measured along with the disappearance of 5 mM tyrosol after its addition to the aerobic medium inoculated with strain YAS1. Fig. 3 shows the time course of tyrosol depletion and accumulation of PHPA in the medium during the growth. During the first 3 h of incubation, the OD₆₀₀ remained constant. This adaptation phase, which varied from 3 to >10 h, depending on the initial concentration of tyrosol (data not shown), could be explained by the toxicity of tyrosol for the isolated strain. After this lag phase, the PHPA concentration in the medium increased until substrate utilization was complete, and consequently, an increase in the OD₆₀₀ was observed. Tyrosol completely disappeared after 10 h of incubation (Fig. 1). Now the only prominent compound belongs to PHPA, resulting in a yield of 100%.

PHPA was not metabolized by strain YAS1, even after more than one week of incubation. Similarly, the following aromatic compounds tested were not metabolized by strain YAS1: benzoate, hydroxylated benzoic acids (4-hydroxybenzoate, 3,4-, 3,5- and 2,4-dihydroxybenzoates and 2,4,6-trihydroxybenzoate), methoxylated benzoic acids (2,4-, 2,6-, 3,4- and 3,5-dimethoxybenzoates and 3,4,5-trimethoxybenzoate), mixed hydroxylated/methoxylated benzoic acids (4-hydroxy-3-methoxy-benzoate, 3-hydroxy-4-methoxybenzoate and 4-hydroxy-3,5-dimethoxybenzoate), 3,4-dimethylbenzoate, cinnamic acid, hydroxylated cinnamic acids (2-, 3-, and 4-coumaric acids and caffeic acid), methoxylated cinnamic acids (3- and 4-methoxy-cinnamic acids), mixed hydroxylated/methoxylated (ferulic acid), phenol and phenylacetic acid.

3.7. Growth at increasing concentrations of tyrosol: tolerance to PHPA

Addition of increasing amounts of tyrosol to the basal medium gradually inhibited the growth of strain YAS1. Biotransformation of tyrosol was not complete at concentrations greater than 14 mM, and whatever the original tyrosol concentration, PHPA produced did not exceed 19.3 mM (Fig. 2). With 30 mM of tyrosol, biotransformation decreased markedly, and at 50 mM, bioconversion and growth were completely inhibited. PHPA was not metabolized, but inhibited markedly growth of strain YAS1 at concentrations higher than 17.5 mM (data not shown). This observation could be explained by the toxicity of this compound. It was shown that accumulation of PHPA, which is highly bac-tericidal [31], generally inhibited further microbial activities (Fig. 3).

Acknowledgments

For the realization of this work, Slim Abdelkafi received a doctoral fellowship from the DSF (IRD, France), and facilities from CBS, Tunisia. We thank Pierre Thomas for his assistance in electron microscopy. We thank Adel Gargoubi and Jean Lorquin for their help in HPLC analyses.

References

maturation of the olive cultivar chemlali from Tunisia. J. Agric. Food Chem. 52, 5476–5481.


