

A comparative study of utilization of single and mixed phenolic compounds by individual and mixed culture

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Abstract Three bacterial strains; *Pseudomonas* sp. TRMK1, *Stenotrophomonas* sp. TRMK2 and *Xanthomonas* sp. TRMK3 were isolated from agro-industrial waste by enrichment culture technique that are capable of utilizing phenolic acids as sole source of carbon and energy. These strains were found to utilize *p*-coumaric, ferulic and caffeic acid. The individual strains utilized 5 mM of mixed phenolic acids within 20 h of incubation. The bacterial consortium composing these strains was prepared and studied the efficient degradation of phenolic compounds. The bacterial consortium showed the enhanced utilization of 30 mM individual and 25 mM mixed phenolic acids within 32 and 40 h of incubation, respectively. The degradation efficiency of these strains in all the above experiments was above 90%. The prepared bacterial consortium serves as a suitable method for the in situ application of sites contaminated with wide range of phenolic compounds.

Keywords Phenolic acids · *Pseudomonas* · *Stenotrophomonas* · *Xanthomonas* · Utilization · Consortium · Mixed · Substrates

Introduction

Utilization of natural phenolic compounds by microorganisms is predominant for the global carbon cycle from an ecological perspective (Peng et al. 2003). These

compounds are released as the breakdown products of lignin. They can be released into soil as products of plant tissue disintegration, leaf leachates and root exudates (Zhang et al. 2010). Major environmental pollution is due to the release of natural phenolic compounds from agroindustrial operations (Mendonça et al. 2004). These phenolics and their derivatives have toxic effects on the growth of the microorganisms at higher concentrations. Whereas, some microorganisms have the ability to utilize them as sole source of carbon and energy for their metabolic activities (Evans 1963). Further, phenolics hinder the growth of rhizospheric bacterial and fungal populations (Blum 2004; Blum and Gerig 2006).

Phenolic compounds such as cinnamic, *p*-coumaric, ferulic, caffeic, syringic, vanillic, protocatechuic and *p*-hydroxyphenyl acetic acids are released from agro-industrial operations in free and mixed form (Hamdi 1993; Labat et al. 2000). Plant aromatic compounds are degraded by many species of microorganisms which lead to the release of huge amount of carbon or otherwise locked away in plant secondary metabolites as lignin (Rosazza et al. 1995). Due to the abundance of these natural aromatic products, there is scientific interest in utilizing them as substrates in biotechnological processes for the production of flavoring compounds labeled as “natural” (Casey and Dobb 1992; Krings and Berger 1998). Limited studies have been documented on the utilization of mixed phenolic acids by both pure and mixed cultures (Di Gioia et al. 2001; Mendonça et al. 2004).

The degradation capability of individual strains, not necessarily contributes to the total degradation capability of the microbial consortium forming the association. The current study focuses on development of bacterial consortium and enhanced utilization of single and mixture of phenolic acids by individual and mixed strains.

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Materials and methods

Isolation, media composition and growth conditions

One gram of agro-waste was inoculated into 250 mL Erlenmeyer flask containing 50 mL of autoclaved mineral salts (MS) medium of pH 7 supplemented with 5 mM *p*-coumaric acid. The MS medium comprises (g/L): K₂HPO₄ 6.8; KH₂PO₄ 1.2; NH₄NO₃ 1.0; MgSO₄·7H₂O 0.1; MnSO₄·4H₂O 0.1; CaCl₂·2H₂O 0.1; FeSO₄·7H₂O 0.1; Na₂MoO₇·2H₂O 0.006. The flasks were incubated in rotary shaker with 180 rpm at 30 °C. Further, 5% of this inoculum was used for further sub-culturing which was carried out for six times. The purity of the culture was checked periodically by plating on Luria–Bertani (LB) agar plates.

Bacterial characterization and identification

The characteristics of all the three strains were performed by standard protocols. The 16S rDNA sequencing was carried out as per Lane (1991). The 16S rDNA was amplified from genomic DNA of bacterial strains TRMK1, TRMK2 and TRMK3 with universal 16S rDNA forward

27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') primers. The 16S rDNA sequencing was done by ABI PRISM 310 DNA sequencer and ABI PRISM Big Dye Terminator Cycle Sequencing Kit. DNA sequence analysis was then executed by BLAST network services at the National Center for Biotechnology Information (NCBI). The alignment of the sequences was done by CLUSTALQ pro V1.82 at the European bioinformatics site (<http://www.ebi.ac.uk/clustalw>). The sequence was polished manually after double-checking with the raw data to evacuate ambiguities and was submitted to GenBank. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Table 1 Morphological, physiological and biochemical characteristics of the strains TRMK1, TRMK2 and TRMK3

Characteristics	Results					
	TRMK1		TRMK2		TRMK3	
Cell morphology and grouping	Small rod, motile and short chains individual		Straight or slightly curved bacilli, single or in pairs		Straight rods, motile	
Growth temperature	30–40 °C		30–40 °C		30–40 °C	
Growth pH range	6–9		6–9		6–9	
Relation to oxygen	Aerobic		Aerobic		Aerobic	
Gram-stain	–		–		–	
Oxidase	–		–		–	
Gelatin liquefaction	–		–		–	
Voges–Proskauer (VP)	–		–		–	
Methyl red (MR)	–		–		–	
Endospore	–		–		–	
H ₂ S production	–		–		–	
Catalase	+		+		+	
lipase	+		+		+	
indole production	+		+		+	
Casein hydrolysis	+		+		+	
Starch hydrolysis	+		+		+	
Carbohydrate fermentation	A	G	A	G	A	G
Glucose	+	+	+	+	+	+
Lactose	–	+	–	+	+	+
Fructose	–	+	–	+	+	+

A acid, G gas

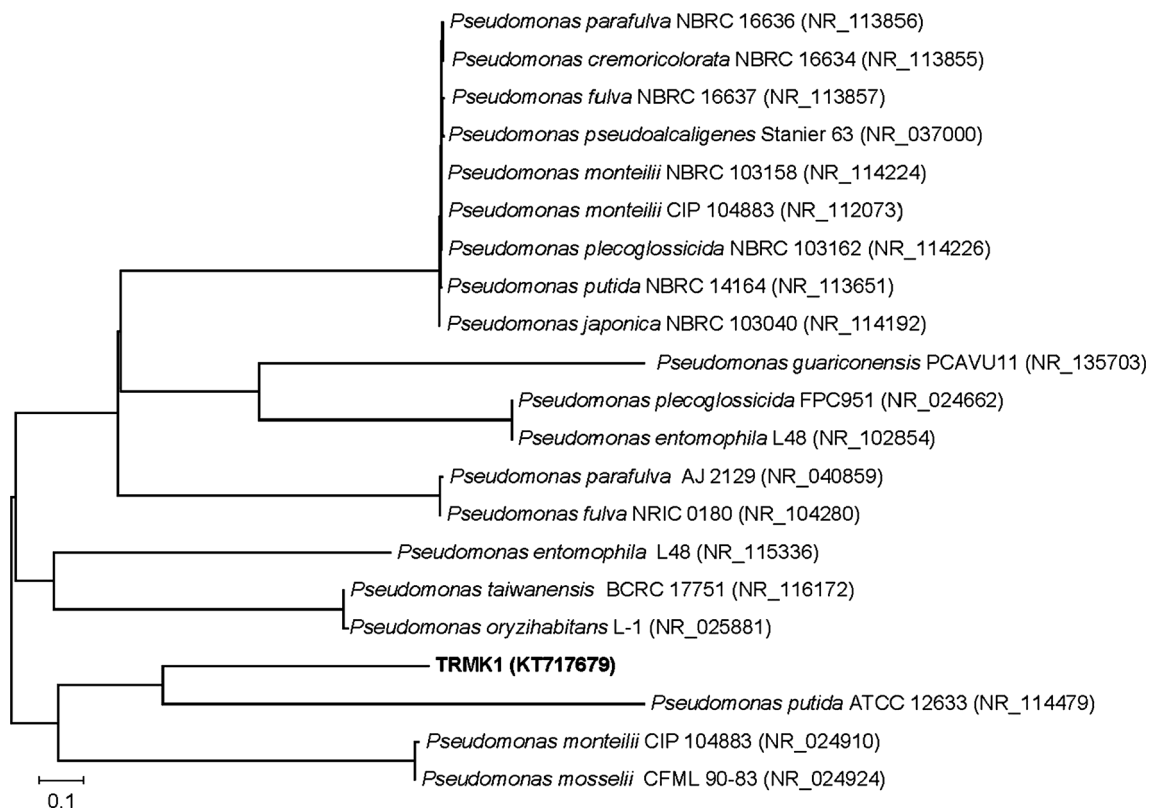


Fig. 1 Phylogenetic tree showing the position of isolate TRMK1 with reference to related strains. All 16S rDNA sequences of related strains have been retrieved from the NCBI database; 0.1 denotes the genetic distance. The strain is related to the *Pseudomonas putida* ATCC 12633 and named as *Pseudomonas* sp. TRMK1. NBRC

Biological Resource Center, ATCC American Type Culture Collection, BCRC Bioresource Collection and Research Center, NRIC NODAI Research Institute Culture Collection, CIP The Collection of Institut Pasteur, CFML Collection de la Faculté de Médecine de Lille

Preparation of bacterial consortium

Three different strains TRMK1, TRMK2 and TRMK3 were grown in a sterile MS medium in separate flasks supplemented with 5 mM *p*-coumaric acid as carbon and energy source. The inoculum size for each strain was 5% (v/v). Further, these strains were streaked separately on a MS-agar plates spread with *p*-coumaric acid and incubated at 30 °C for 48 h. Single colony from each plate was inoculated into 50 mL MS medium containing 5 mM *p*-coumaric acid and grown in orbital shaker at 180 rpm and 30 °C for 40 h. This mixed culture was used for consortium experiments.

Utilization of single substrate by individual strains and bacterial consortium

The utilization of individual phenolic acids was studied by individual strains as well as by the consortium prepared by using all the three isolates. Further, the inoculum size for utilization of individual phenolic acid by individual strains was 5% (v/v) whereas, 5% (v/v) inoculum of consortia was used for utilization of individual phenolic acid by bacterial consortium. Utilization of individual phenolic acids was

carried out in separate 250 mL flasks by inoculating strains TRMK1, TRMK2 and TRMK3 individually into 50 mL MS medium supplemented with 10 and/or 15 mM *p*-coumaric, ferulic and caffeic acids. Whereas, for bacterial consortium, the concentration of each phenolic acid was 30 mM. At regular intervals, 1 mL sample was withdrawn, centrifuged and the supernatant was filtered through 0.2 μm filter paper. The residual concentration of phenolic acids was estimated by HPLC. The % utilization of phenolic acids (η) at different time intervals of incubation was calculated by using the following expression (Mukram et al. 2016);

$$\text{Utilization ratio, } \eta (\%) = \frac{S_0 - S}{S_0} \times 100,$$

where S_0 represents the initial phenolic acid and S , the residual phenolic acid concentration.

Utilization of mixed substrate by individual strains and bacterial consortium

The phenolic acids were grouped into four sets, the first three sets comprise mixtures of two phenolic acids viz., *p*-coumaric + ferulic acid (set 1), *p*-coumaric + caffeic acid

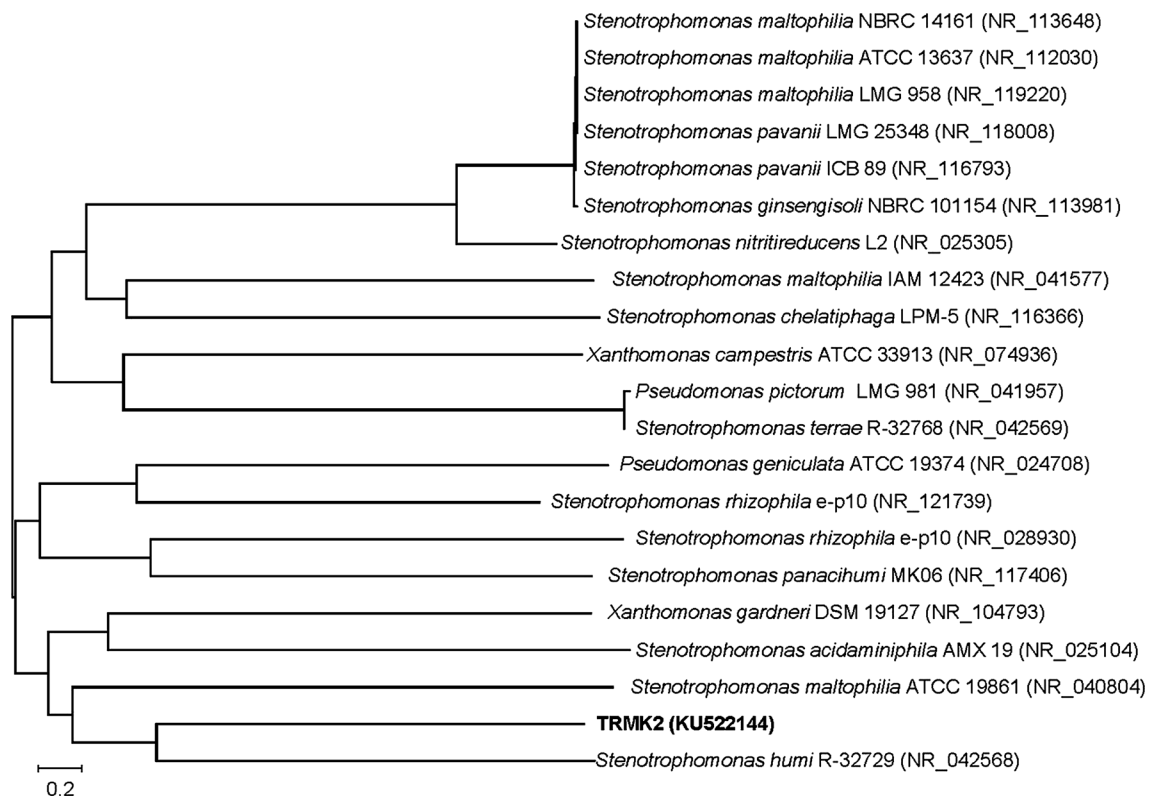


Fig. 2 Phylogenetic tree showing the position of isolate TRMK2 with reference to related strains. All 16S rDNA sequences of related strains have been retrieved from the NCBI database; 0.2 denotes the genetic distance. The strain is related to the *Stenotrophomonas humi* R-32729 and named as *Stenotrophomonas* sp. TRMK2. NBRC

(set 2), ferulic + caffeic acid (set 3) and the last set contains mixture of all the three phenolic acids viz., *p*-coumaric + ferulic + caffeic acid (set 4). The concentration of each phenolic acid in set 1, 2 and 3 was 2.5 mM for individual strain and 12.5 mM for mixed strain utilization experiments. The concentration of each phenolic acid in set 4 was 1.66 mM for individual and 8.33 mM for mixed strain utilization studies. Overall, 5 mM (individual strain) and 25 mM (consortium) phenolic acids were supplemented to different flasks containing 50 mL MS medium, inoculated with 5% (v/v) inoculum of individual strain and 5% (v/v) inoculum of bacterial consortium and incubated in rotary shaker (180 rpm at 30 °C). At regular time intervals, 1 mL culture was withdrawn, centrifuged and the supernatant was filtered through 0.2 µm filter paper and estimated the residual concentration by HPLC.

Analytical methods

The growth of strains on phenolic acids was determined spectrophotometrically by reading the absorbance at 600 nm (UV Specord 50). The phenolic acid concentration was estimated by HPLC (WATERS 2489, 515 binary

Biological Resource Center, ATCC American Type Culture Collection, BCRC Bioresource Collection and Research Center, DSM Deutsche Sammlung von Mikroorganismen und Zellkulturen, LMG LMG Culture Collection, IAM Institute of Molecular and Cellular Biosciences

pumps with C18 column 250 mm × 4.6 mm and ultraviolet detector). The mobile phase used was acetonitrile:water:acetic acid (30:69.5:0.5 v/v) at a flow rate of 1 mL/min (Chamkha et al. 2001).

Statistical analysis

The data were subjected to analysis of variance using SPSS21.0 software. The significance of differences between the treatments was judged by *F* test, while the treatment means were compared by least significant difference (LSD—Fisher's test) and honestly significant difference (HSD—Tukey's test) at *P* < 0.05. The results are taken from HSD only because of multiple variable comparison and least chances of type 1 error.

Results and discussion

Identification of bacterial strains

Three bacterial strains were isolated from agro-waste by selective enrichment culture technique. Various

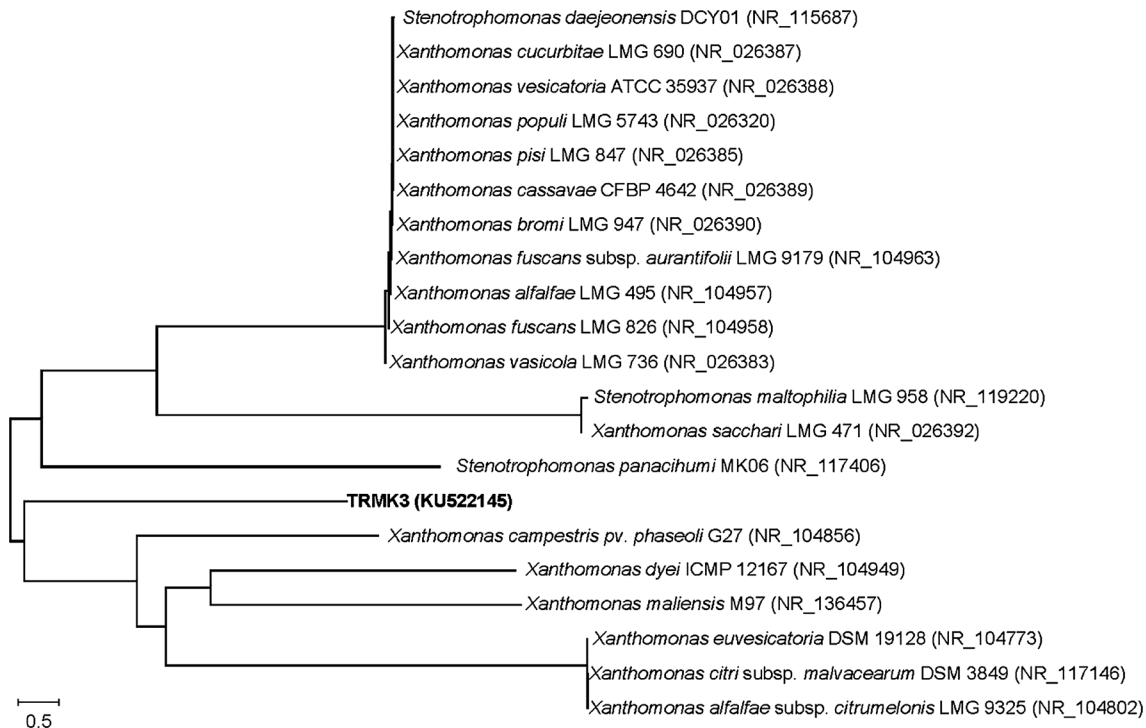


Fig. 3 Phylogenetic tree showing the position of isolate TRMK3 with reference to related strains. All 16S rDNA sequences of related strains have been retrieved from the NCBI database; 0.5 denotes the genetic distance. The strain is related to the *Xanthomonas campestris* G27 and named as *Xanthomonas* sp. TRMK3. ATCC American Type

Culture Collection, *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen, *ICMP* International Collection of Microorganisms from Plants, *CFBP* Collection Française de Bactéries associées aux plantes

Table 2 Utilization of individual phenolic acids by *Pseudomonas* sp. TRMK1, *Stenotrophomonas* sp. TRMK2 and *Xanthomonas* sp. TRMK3

Time (h)	<i>Pseudomonas</i> sp. TRMK1			<i>Stenotrophomonas</i> sp. TRMK2			<i>Xanthomonas</i> sp. TRMK3		
	<i>p</i> -CA (15 mM)	FA (10 mM)	CA (10 mM)	<i>p</i> -CA (15 mM)	FA (10 mM)	CA (15 mM)	<i>p</i> -CA (15 mM)	FA (10 mM)	CA (15 mM)
0	15 ± 0.04 ^{ab}	10 ± 0.01 ^{ab}	10 ± 0.01 ^{ab}	15 ± 0.04 ^{ab}	10 ± 0.7 ^{ab}	15 ± 0.02 ^{ab}	15 ± 0.05 ^{ab}	10 ± 0.02 ^{ab}	15 ± 0.02 ^{ab}
6	9 ± 0.01 ^{ab}	7 ± 0.07 ^{ab}	6.9 ± 0.1 ^{ab}	13.5 ± 0.3 ^{ab}	8.3 ± 0.1 ^{ab}	13.8 ± 0.06 ^{ab}	13.7 ± 0.05 ^{ab}	7.4 ± 0.2 ^{ab}	12.8 ± 0.15 ^{ab}
12	1.5 ± 0.01 ^{ab}	2 ± 0.04 ^{ab}	2 ± 0.1 ^{ab}	7.6 ± 0.2 ^{ab}	6.9 ± 0.7 ^{ab}	11.4 ± 0.1 ^{ab}	9.2 ± 0.05 ^{ab}	5.8 ± 0.15 ^{ab}	7.4 ± 0.11 ^{ab}
18	0.69 ± 0.02 ^{ab}	0.8 ± 0.07 ^{ab}	0.4 ± 0.04 ^{ab}	4.7 ± 0.2 ^{ab}	3.8 ± 0.06 ^{ab}	8.4 ± 0.09 ^{ab}	4.5 ± 0.2 ^{ab}	3.1 ± 0.15 ^{ab}	5.4 ± 0.04 ^{ab}
24	0.69 ± 0.02 ^{ab}	0.8 ± 0.07 ^{ab}	0.4 ± 0.04 ^{ab}	2.4 ± 0.2 ^{ab}	1.8 ± 0.08 ^{ab}	3.1 ± 0.05 ^{ab}	2.4 ± 0.2 ^{ab}	1.2 ± 0.05 ^{ab}	1.3 ± 0.08 ^{ab}
30	0.69 ± 0.02 ^{ab}	0.8 ± 0.07 ^{ab}	0.4 ± 0.04 ^{ab}	0.02 ± 0.01 ^{ab}	0.12 ± 0.01 ^{ab}	0.28 ± 0.02 ^{ab}	0.15 ± 0.05 ^{ab}	0.32 ± 0.04 ^{ab}	0.052 ± 0.01 ^{ab}
36	0.69 ± 0.02 ^{ab}	0.8 ± 0.07 ^{ab}	0.4 ± 0.04 ^{ab}	0.02 ± 0.01 ^{ab}	0.12 ± 0.01 ^{ab}	0.28 ± 0.02 ^{ab}	0.5 ± 0.05 ^{ab}	0.32 ± 0.04 ^{ab}	0.052 ± 0.01 ^{ab}

The values are represented as mean ± SE ($n = 3$)

p-CA *p*-coumaric acid, FA ferulic acid, CA caffeic acid

^a No significant difference between the individual substrates by all three strains utilization

^b Non-homogeneity of variance compared with bacterial consortium utilization (refer Table 3)

morphological, physiological and biochemical attributes of strains TRMK1, TRMK2 and TRMK3 were carried out. These strains are gram negative, aerobic and motile. The distinct attributes of all the strains are outlined in Table 1. 16S rDNA sequencing was used as an identification tool. The 16S rDNA sequence of the strains TRMK1, TRMK2 and TRMK3 were compared against the NCBI public

database. The 16S rDNA sequences of the type strains and the strains TRMK1, TRMK2 and TRMK3 were aligned and phylogenetic tree was constructed (Figs. 1, 2, 3). The comparison results showed that the isolated strains belong to *Pseudomonas*, *Stenotrophomonas* and *Xanthomonas* genera. Hence, the strains TRMK1, TRMK2 and TRMK3 were identified and named as *Pseudomonas* sp. strain

Table 3 Utilization of *p*-coumaric acid, ferulic acid and caffeic acid by bacterial consortium

Time (h)	<i>p</i> -CA (30 mM)	FA (30 mM)	CA (30 mM)
0	30 ± 0.01 ^{ab}	30 ± 0.02 ^{ab}	30 ± 0.01 ^{ab}
8	27.5 ± 0.1 ^{ab}	27.34 ± 0.2 ^{ab}	28.98 ± 0.6 ^{ab}
16	16.8 ± 0.2 ^{ab}	14.23 ± 0.09 ^{ab}	24.05 ± 0.1 ^{ab}
24	7.32 ± 0.1 ^{ab}	2.94 ± 0.2 ^{ab}	17.05 ± 0.1 ^{ab}
32	1.2 ± 0.1 ^{ab}	1.94 ± 0.3 ^{ab}	0.41 ± 0.02 ^{ab}
40	0.54 ± 0.04 ^{ab}	1.77 ± 0.1 ^{ab}	0.41 ± 0.01 ^{ab}

The values are represented as mean ± SE ($n = 3$)

p-CA *p*-coumaric acid, FA ferulic acid, CA caffeic acid

^a No significant difference between individual substrates utilization by bacterial consortium

^b Non-homogeneity of variance compared with individual strain utilization (refer Table 2)

TRMK1, *Stenotrophomonas* sp. Strain TRMK2 and *Xanthomonas* sp. Strain TRMK3. The 16S rDNA sequence of all the three strains were deposited and are accessible in NCBI GenBank database with accession numbers KT717679, KU522144 and KU522145, respectively.

Utilization of single substrate by individual strain and bacterial consortium

Pseudomonas sp. TRMK1 utilized 95.4% of 15 mM *p*-coumaric acid within 18 h, 92 and 96% of 10 mM each of ferulic and caffeic acid within 18 h of incubation and above this concentration, the strain restricted its growth. *Stenotrophomonas* sp. TRMK2 consumed 99.8 and 98.1% of 15 mM each of *p*-coumaric and caffeic acid, respectively. 98.8% of 10 mM ferulic acid is utilized by the strain within 30 h of incubation. Further, *Xanthomonas* sp. TRMK3 exhibited the utilization of 96.6% of 15 mM *p*-coumaric acid, 96.8% of 10 mM ferulic acid and 99.6% of 15 mM caffeic acid within 30 h incubation. Whereas, the reported strain, *Halomonas* sp. IMPC was able to utilize 10 mM *p*-coumaric acid (Abdelkafi et al. 2006). These three strains are capable of utilizing 15 mM *p*-coumaric acid within 30 h. Karmakar et al. (2000) reported that *Bacillus coagulans* BK07 utilized 95% of 2.13 mM ferulic acid. Yuan et al. (2013) isolated *Cupriavidus* sp. B-8 with the degradation efficiency of 96.84% for 1 mM ferulic acid. Whereas, the strains TRMK1, TRMK2 and TRMK3 utilized more than 92% of 10 mM of ferulic acid within 30 h of incubation. All the three strains were found to utilize 15 mM caffeic acid with the degradation efficacy of above 96%. The statistical analysis of Levene test showed homogeneity of variance between the utilization of all the substrates by these three strains. Tukey test showed no significant difference between the utilization of individual substrates by all the three strains. This means that all the strains have similar utilization pattern (Table 2). On the other hand, the bacterial consortium showed enhanced

utilization of higher concentration of all the phenolic acids. When 30 mM each of *p*-coumaric and ferulic acid was supplied, the consortium showed 98.2 and 94.1% utilization within 40 h of incubation. Further, the consortium took 32 h of duration to utilize 98.6% of 30 mM caffeic acid (Table 3). The Levene test showed homogeneity of variance between the individual substrates and in support, Tukey test also showed no significant difference between single substrate utilization by a bacterial consortium. Levene test has shown non-homogeneity of variance when compared with the utilization of single substrate by a bacterial consortium and individual strain.

Utilization of mixed substrates by individual strain and bacterial consortium

Pseudomonas sp. TRMK1 utilized 98 and 99.4% of 2.5 mM each of *p*-coumaric and ferulic acid (set 1), 99% each of 2.5 mM *p*-coumaric and 2.5 mM caffeic acid (set 2), 98.4 and 99.4% of 2.5 mM each of ferulic and caffeic acid (set 3) and 95, 99 and 98% of 1.66 mM each of *p*-coumaric, ferulic and caffeic acid (set 4) within 12 h of incubation. *Stenotrophomonas* sp. TRMK2 utilized 99.8 and 99% of set 1, 99.8 and 99.5% of set 2, 99 and 99.4% of set 3 and 99.6, 96.2 and 99.8% of set 4 with different phenolic acids within 20 h of incubation. Further, *Xanthomonas* sp. TRMK3 exhibited utilization of 99.9 and 98.8% of set 1, 99.7 and 99.2% of set 2, 99 and 98.4% of set 3 and 99.6, 99.5 and 99.3% of set 4 with phenolic acids in 16 h of incubation (Table 4). Limited studies have been carried out on the utilization of mixture of phenolic compounds. Di Gioia et al. (2001) reported the ability of two bacterial strains *Pseudomonas putida* DSM 1868 and *Ralstonia* sp. LD 35 to degrade a mixture of nine monocyclic aromatic compounds. Mendonça et al. (2004) reported the utilization of 350 mg of mixed phenolic compounds per liter within 8 h of incubation. In Table 4, Levene test showed the homogeneity of variance with

Table 4 Utilization of mixed phenolic acids by *Pseudomonas* sp. TRMK1, *Stenotrophomonas* sp. TRMK2 and *Xanthomonas* sp. TRMK3

Time (h)	<i>p</i> -CA + FA (set 1)		<i>p</i> -CA + CA (set 2)		FA + CA (set 3)		<i>p</i> -CA + FA + CA (set 4)		
	<i>p</i> -CA (2.5 mM)	FA (2.5 mM)	<i>p</i> -CA (2.5 mM)	CA (2.5 mM)	FA (2.5 mM)	CA (2.5 mM)	<i>p</i> -CA (1.66 mM)	FA (1.66 mM)	CA (1.66 mM)
<i>Pseudomonas</i> sp. TRMK1									
0	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	1.66 ± 0.01 ^{ab}	1.66 ± 0.01 ^{ab}	1.66 ± 0.01 ^{ab}
4	1.64 ± 0.04 ^{ab}	1.49 ± 0.03 ^{ab}	2.04 ± 0.05 ^{ab}	1.99 ± 0.04 ^{ab}	1.49 ± 0.05 ^{ab}	1.15 ± 0.04 ^{ab}	0.94 ± 0.02 ^{ab}	0.57 ± 0.06 ^{ab}	0.92 ± 0.05 ^{ab}
8	0.49 ± 0.04 ^{ab}	0.24 ± 0.02 ^{ab}	0.99 ± 0.09 ^{ab}	0.49 ± 0.04 ^{ab}	0.13 ± 0.02 ^{ab}	0.08 ± 0.01 ^{ab}	0.37 ± 0.02 ^{ab}	0.15 ± 0.02 ^{ab}	0.31 ± 0.02 ^{ab}
12	0.05 ± 0.01 ^{ab}	0.01 ± 0.05 ^{ab}	0.009 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.03 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.07 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.03 ± 0.01 ^{ab}
16	0.05 ± 0.02 ^{ab}	0.01 ± 0.01 ^{ab}	0.009 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.03 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.05 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.03 ± 0.01 ^{ab}
<i>Stenotrophomonas</i> sp. TRMK2									
0	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	1.66 ± 0.01 ^{ab}	1.66 ± 0.01 ^{ab}	1.66 ± 0.01 ^{ab}
4	1.59 ± 0.01 ^{ab}	1.91 ± 0.07 ^{ab}	1.52 ± 0.01 ^{ab}	1.43 ± 0.01 ^{ab}	1.36 ± 0.01 ^{ab}	1.87 ± 0.01 ^{ab}	1.28 ± 0.01 ^{ab}	1.26 ± 0.01 ^{ab}	1.22 ± 0.01 ^{ab}
8	0.8 ± 0.01 ^{ab}	1.53 ± 0.01 ^{ab}	0.98 ± 0.01 ^{ab}	0.98 ± 0.01 ^{ab}	0.65 ± 0.01 ^{ab}	1.06 ± 0.01 ^{ab}	0.95 ± 0.01 ^{ab}	0.90 ± 0.01 ^{ab}	0.94 ± 0.01 ^{ab}
12	0.47 ± 0.01 ^{ab}	0.99 ± 0.01 ^{ab}	0.60 ± 0.01 ^{ab}	0.46 ± 0.01 ^{ab}	0.49 ± 0.01 ^{ab}	0.77 ± 0.01 ^{ab}	0.39 ± 0.01 ^{ab}	0.37 ± 0.01 ^{ab}	0.32 ± 0.01 ^{ab}
16	0.03 ± 0.01 ^{ab}	0.12 ± 0.01 ^{ab}	0.08 ± 0.01 ^{ab}	0.07 ± 0.01 ^{ab}	0.16 ± 0.01 ^{ab}	0.07 ± 0.01 ^{ab}	0.03 ± 0.01 ^{ab}	0.15 ± 0.01 ^{ab}	0.04 ± 0.01 ^{ab}
20	0.005 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.004 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.006 ± 0.01 ^{ab}	0.07 ± 0.01 ^{ab}	0.002 ± 0.01 ^{ab}
24	0.005 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.004 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.005 ± 0.01 ^{ab}	0.06 ± 0.01 ^{ab}	0.002 ± 0.01 ^{ab}
<i>Xanthomonas</i> sp. TRMK3									
0	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	2.5 ± 0.01 ^{ab}	1.66 ± 0.01 ^{ab}	1.66 ± 0.01 ^{ab}	1.66 ± 0.01 ^{ab}
4	1.52 ± 0.03 ^{ab}	1.46 ± 0.01 ^{ab}	1.24 ± 0.01 ^{ab}	1.32 ± 0.01 ^{ab}	1.22 ± 0.01 ^{ab}	1.27 ± 0.01 ^{ab}	0.9 ± 0.01 ^{ab}	0.88 ± 0.01 ^{ab}	0.85 ± 0.01 ^{ab}
8	0.74 ± 0.01 ^{ab}	0.53 ± 0.01 ^{ab}	0.64 ± 0.01 ^{ab}	0.61 ± 0.01 ^{ab}	0.85 ± 0.01 ^{ab}	0.81 ± 0.01 ^{ab}	0.34 ± 0.01 ^{ab}	0.39 ± 0.01 ^{ab}	0.38 ± 0.01 ^{ab}
12	0.04 ± 0.01 ^{ab}	0.20 ± 0.01 ^{ab}	0.44 ± 0.01 ^{ab}	0.34 ± 0.01 ^{ab}	0.50 ± 0.01 ^{ab}	0.48 ± 0.01 ^{ab}	0.10 ± 0.01 ^{ab}	0.12 ± 0.01 ^{ab}	0.19 ± 0.01 ^{ab}
16	0.001 ± 0.01 ^{ab}	0.11 ± 0.08 ^{ab}	0.007 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.006 ± 0.01 ^{ab}	0.005 ± 0.01 ^{ab}	0.008 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}
20	0.001 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.007 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.02 ± 0.01 ^{ab}	0.005 ± 0.01 ^{ab}	0.005 ± 0.01 ^{ab}	0.008 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}

The values are represented as mean ± SE ($n = 3$)

p-CA *p*-coumaric acid, FA ferulic acid, CA caffeic acid

^a No significant difference within the sets and between the sets

^b Non-homogeneity of variance compared with bacterial consortium (refer Table 5)

Table 5 Utilization of mixed phenolic acids by bacterial consortium

Time (h)	p-CA + FA (set 1)		p-CA + CA (set 2)		FA + CA (set 3)		p-CA + FA + CA (set 4)	
	p-CA (12.5 mM)	FA (12.5 mM)	p-CA (12.5 mM)	CA (12.5 mM)	FA (12.5 mM)	CA (12.5 mM)	p-CA (8.33 mM)	FA (8.33 mM)
	CA (12.5 mM)	FA (12.5 mM)	p-CA (12.5 mM)	CA (12.5 mM)	FA (12.5 mM)	CA (12.5 mM)	p-CA (8.33 mM)	FA (8.33 mM)
0	12.5 ± 0.01 ^{ab}	12.5 ± 0.01 ^{ab}	12.5 ± 0.01 ^{ab}	12.5 ± 0.01 ^{ab}	12.5 ± 0.01 ^{ab}	12.5 ± 0.01 ^{ab}	8.33 ± 0.01 ^{ab}	8.33 ± 0.01 ^{ab}
8	9.6 ± 0.02 ^{ab}	9.09 ± 0.05 ^{ab}	10.5 ± 0.2 ^{ab}	10.25 ± 0.1 ^{ab}	5.19 ± 0.1 ^{ab}	5.50 ± 0.04 ^{ab}	7.6 ± 0.03 ^{ab}	7.63 ± 0.09 ^{ab}
16	0.03 ± 0.01 ^{ab}	0.19 ± 0.03 ^{ab}	0.65 ± 0.02 ^{ab}	0.54 ± 0.02 ^{ab}	0.94 ± 0.04 ^{ab}	0.48 ± 0.06 ^{ab}	0.37 ± 0.01 ^{ab}	0.35 ± 0.02 ^{ab}
24	0.001 ± 0.01 ^{ab}	0	0.013 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.006 ± 0.01 ^{ab}	0.016 ± 0.01 ^{ab}	0	0
32	0.001 ± 0.01 ^{ab}	0	0.013 ± 0.01 ^{ab}	0.01 ± 0.01 ^{ab}	0.006 ± 0.01 ^{ab}	0.016 ± 0.01 ^{ab}	0	0

The values were represented as mean ± SE (n = 3)

p-CA p-coumaric acid, FA ferulic acid, CA caffeic acid

^a No significant difference within the sets and between the sets

^b Non-homogeneity of variance compared with the sets of individual strain utilization (refer Table 4)

respect to the utilization within the sets 1, 2, 3 and 4 by all the three strains. Tukey test also showed no significant difference of utilization within all the four individual sets by all the three strains. On the contrary, the bacterial consortium utilized 99.9% of 12.5 mM p-coumaric acid and 100% of 12.5 mM ferulic acid from set 1. From set 2, 99.8 and 99.9% utilization of 12.5 mM each of p-coumaric acid and caffeic acid was witnessed. 99.9 and 99.8% of 12.5 mM each of ferulic and caffeic acid was utilized from the set 3. The bacterial consortium utilized the mixture of phenolic acids from set 4 viz., 100% of 8.33 mM each of p-coumaric and ferulic acid. 99.9% of 8.33 mM caffeic acid within 24 h of incubation. Wherein, Levene test also showed non-homogeneity of variance when compared with the utilization of substrates from sets 1, 2, 3 and 4 by individual strain and bacterial consortium, due to the ability of bacterial consortium to utilize high concentrations of phenolic acids (Table 5).

Pseudomonas sp. TRMK1, *Stenotrophomonas* sp. TRMK2 and *Xanthomonas* sp. TRMK3 are the potential strains for the degradation of different phenolic compounds. These strains were isolated by enrichment culture technique and identified based on 16S rDNA sequencing. These strains have the ability to utilize maximum concentration of 15 mM p-coumaric acid, ferulic acid and caffeic acid. On the other hand, the strains TRMK1, TRMK2 and TRMK3 utilized the different phenolic acids in the mixture in different combinations. All the three strains utilized more than 95% phenolic acid mixtures. Further, the bacterial consortium prepared was found to be more efficient in utilizing the mixed phenolic compounds in different combinations. These features make this bacterial consortium a potential method for effective in situ bioremediation of sites contaminated with various phenolic compounds.

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Compliance with ethical standards

Conflict of interest Authors declare that the manuscript does not contain any conflict of interest.

References

- Abdelkafi S, Labat M, Casalot L, Chamkha M, Sayadi S (2006) Isolation and characterization of *Halomonassp.* strain IMPC, a p-coumaric acid-metabolizing bacterium that decarboxylates other cinnamic acids under hypersaline conditions. *FEMS Microbiol Lett* 255:108–114

- Blum U, Gerig TM (2006) Interrelationships between *p*-coumaric acid, evapotranspiration, soil water content and leaf expansion. *J Chem Ecol* 32:1817–1834
- Blum U (2004) Fate of phenolic allelochemicals in soils—the role of soil and rhizosphere microorganisms. In: Macias FA, Galindo JCG, Molinillo JMG, Cutler HG (eds) *Allelopathy: chemistry and mode of action of allelochemicals*. CRC Press, Boca Raton, pp 57–76
- Casey J, Dobb R (1992) Microbial routes to aromatic aldehydes. *Enzyme Microbiol Technol* 14:739–747
- Chamkha M, Labat M, Bharat KCP, Garcia JL (2001) Isolation of a cinnamic acid-metabolizing *Clostridium glycolicum* strain from oil mill wastewaters and emendation of the species description. *Int J Syst Evol Microbiol* 51:2049–2054
- Di Gioia D, Fava F, Bertin L, Marchetti L (2001) Biodegradation of synthetic and naturally occurring mixtures of mono-cyclic aromatic compounds present in olive mill wastewaters by two aerobic bacteria. *Appl Microbiol Biotechnol* 55:619–626
- Evans WC (1963) The microbiological degradation of aromatic compounds. *J Gen Microbiol* 32:177–184
- Hamdi H (1993) Future prospects and constraints of olive mill wastewaters use and treatment: a review. *Bioprocess Eng* 8:209–214
- Karmakar B, Vohar RM, Nandanwar H, Sharma P, Gupta KG, Sobti RC (2000) Rapid degradation of ferulic acid via 4-vinylguaiacol and vanillin by a newly isolated strain of *Bacillus coagulans*. *J Biotechnol* 80:195–202
- Krings U, Berger RG (1998) Biotechnological production of flavours and fragrances. *Appl Microbiol Biotechnol* 49:1–8
- Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33:1870–1874
- Labat M, Augur C, Perraud-Gaime I, Roussos S, Sayadi S (2000) Biotechnological potentialities of polyphenolic compounds of coffee and comparison with olive. In: Sera T, Soccol CR, Pandey A, Roussos S (eds) *Coffee biotechnology and quality*. Kluwer, Dordrecht, pp 517–531
- Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. Wiley, Chichester
- Mendonça E, Martins A, Anselmo AM (2004) Biodegradation of natural phenolic compounds as single and mixed substrates by *Fusarium flocciferum*. *Electron J Biotechnol* 7:30–37
- Mukram I, Ramesh M, Monisha TR, Anand SN, Karegoudar TB (2016) Biodegradation of butyronitrile and demonstration of its mineralization by *Rhodococcus* sp. MTB5. *Biotech* 6:1–7
- Peng X, Misawa N, Harayama S (2003) Isolation and characterization of thermophilic bacilli degrading cinnamic, *p*-coumaric and ferulic acids. *Appl Environ Microbiol* 69:1417–1427
- Rosazza J, Huang Z, Dostal L, Rosseau B (1995) Review: biocatalytic transformation of ferulic acid: an abundant aromatic natural product. *J Ind Microbiol* 15:457–471
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci USA* 101:11030–11035
- Yuan CL, Wei-chun Y, Yong-hua Z, Zhi-hui Y, Yu Z, Yue-hui C (2013) Biodegradation of ferulic acid by a newly isolated strain of *Cupriavidus* sp. B-8. *J Cent South Univ* 20:1964–1970
- Zhang ZY, Pan LP, Li HH (2010) Isolation, identification and characterization of soil microbes which degrade phenolic allelochemicals. *J Appl Microbiol* 108:1839–1849