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Transformation of *iso*-pentylbenzene by a biofilm-forming strain of *Candida viswanathii* TH1 isolated from oil-polluted sediments collected in coastal zones in Vietnam

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This work is aimed to assess the aerobic biotransformation of a branched side chain alkylbenzene, *iso*-pentylbenzene, by *Candida viswanathii* TH1. The yeast *Candida viswanathii* TH1 isolated from oil-polluted sediments collected in coastal zones in Vietnam exhibited as a strain that could better transform branched aromatic hydrocarbons in biofilm (pellicle) than in planktonic form. During incubation of TH1 as biofilm with *iso*-pentylbenzene, the seven intermediates produced were benzoic acid, phenylacetic acid, 2-methyl-4-phenyl-butan-1-ol, 2-hydroxy-phenylacetic acid, 2-methyl-4-phenylbutyric acid, succinic acid and *iso*-valerophenone as revealed by gas chromatography/mass spectra and high-performance liquid chromatography analyses. The occurrence of these intermediates showed that *iso*-pentylbenzene could be oxidized not only via mono- but also by a sub-terminal oxidation pathway. This is the first study on *iso*-pentylbenzene transformation by a biofilm-forming *Candida viswanathii* strain. The catabolic versatility of the biofilm-forming strain TH1 and its use for mono and sub-terminal oxidation during the transformation of *iso*-pentylbenzene enhance our understanding of the degradation of branched side chain phenylalkanes and give new insight into the potential role of such species in the transformation of other recalcitrant aromatic compounds.

Keywords: Aromatic cyclic hydrocarbon, biofilm, biotransformation, *Candida viswanathii*, *iso*-pentylbenzene.

Introduction

Alkylbenzenes are major constituents of oily wastes and pesticides, as well as of many synthetic detergents, and the biodegradation of such molecules by sewage organisms is therefore of environmental significance.[1] Although a number of publications are available on the biodegradation of alkyl-aromatic compounds, most of the studies are related to alkylaromatics with short linear alkyl chains.[2–5] It has been reported that aromatic hydrocarbons with branches and/or condensed ring structures, are more recalcitrant to biodegradation, i.e., fewer microorganisms can degrade these structures and only at rates much lower than those for simpler hydrocarbons found in petroleum products. The greater the complexity of the hydrocarbon structure, e.g., the higher the number of methyl branched substituents or condensed aromatic rings, the slower the rates of degradation and the greater the likelihood of accumulating partially oxidized intermediary metabolites.[6] Some polyalkylated benzene and some linear and branched alkanes were also shown to be degraded.[7,8] However, branched alkanes and polyalkylated benzenes are still significant environmental pollutants problems because of their resistance to degradation.

The degradation of alkylbenzenes may proceed via three different routes: (i) oxidation of the aromatic ring followed by ring cleavage without prior attack of the alkyl side chain, (ii) initial oxidation of the alkyl side chain or (iii) a combination of both. Aromatic rings generally are hydroxylated to form diols; the rings are primarily oxidized to substituted catechols, which are ring cleaved via *meta*-, or *ortho*- cleavages. When the alkyl chain length exceeds C7, the preferred route is attack on the alkyl chain via *ω*- and ß-oxidation. Generally, the terminal methyl group of a long *n*-alkyl side chain is initially oxidized to a carboxylic group, which is followed by classical ß-oxidation to form the carboxylic or acetic acid derivative, depending on whether there is an odd or even number of carbons in the alkyl side chain.[9]

In recent years, studies on the transformation of aromatic hydrocarbons and other recalcitrant compounds by biofilm-forming microorganisms have been conducted intensively. Several microorganisms are capable of degrading aromatic compounds better when grown as a biofilm rather than a planktonic culture.[10–11] Immobilization

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of a biofilm on a solid support can protect microorganisms from being damaged, while maintaining continuous cell growth and biodegradation activity. Biofilm formation is considered to be a general strategy that microorganisms use to survive in nature.[12] The cells in biofilms are usually encased in an extracellular polymeric matrix and are highly tolerant to physicochemical stresses. In addition, drastic changes in the cellular processes are observed in aiming for nutrient uptake, cell-to-cell communication, and elimination of potential competitors in the surrounding environment. Yamaga et al.[11] demonstrated that compared to free swimming cells, the immobilized *Acinetobacter calcoaceticus* P23 on plant surfaces showed better phenol degradation rates and could be exposed to higher concentrations of phenol without the loss of viability. Gilan et al.[13] reported a high capacity of *Rhodococcus ruber* C208 to form biofilms on polyethylene surfaces and to degrade up to 8% of the polyolefin within 30 days of incubation. On the basis of the results shown previously, the *Candida* genus was described as a good candidate for biotransformation of aromatic hydrocarbons.[9–14] However, thus far none of the published studies have reported on the transformation of aromatic compounds containing branched side chain by biofilm-forming yeast. Here, we investigated the transformation of *iso*-pentylbenzene by the biofilm-forming *Candida viswanathii* TH1 isolated from oil-contaminated sites in Vietnam by analyzing the produced intermediates to give a better understanding of this organism and the possibility of its use for industrial applications.

Materials

Microorganism strains

In this study, the *Candida viswanathii* TH4 and other hydrocarbon-degrading microorganisms were obtained from the strain collection at the Environmental Biotechnology Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology. These organisms were isolated from hydrocarbon-contaminated water and sediment samples in coastal areas in Vietnam such as Quang Ninh, Hai Phong and Thanh Hoa.

Cultivation media

Mineral salt medium and mineral salt agar,[15] nutrient broth medium (NB II) and nutrient agar II [16] supplemented with hydrocarbon substrates were used for cultivation of the bacterial strain.

Methods

Biofilm formation experiment

The experiment was performed according to the methods described by O'Toole and Kolter [18] and Morikawa et al.[17] In brief, an overnight culture was diluted to $OD_{600} = 0.3$ and inoculated (1%) into 300 μL of NB II medium in a 1.5-mL micro-centrifuge tube (TC131615, Nippon Genetics, Tokyo, Japan). The tube was kept standing at 30°C for 2 days and then the pellicles and the medium were removed. Afterward, the tube was gently rinsed with distilled water and filled with 500 μL of 1% crystal violet (CV) solution. After 25 min, the CV solution was removed, and the tube was washed with distilled water again. The CV attached to the biofilm was dissolved in 400 μL of acetone and was quantified by measuring its absorbance at 570 nm. The experiment was performed in triplicate and the results were expressed with standard deviation. The *Acinetobacter calcoaceticus* P23 was used as a positive control and the tube without any strain was used as a negative control.[11] The best biofilm-forming strain was selected for further experiments. Each data point is the average of triplicate experiments.

Identification of selected yeast

For the purpose of identification, the colony and cellular morphology of the strain was first analyzed under optical observation and scanning electron microscope. After that, whole-cell lysis PCR amplification was conducted to amplify the 18S rRNA gene using InstaGene Matrix (Bio-Rad, Hercules, CA, USA). Part of the 18S rRNA gene was amplified with standard PCR primers using an AccuPrep Gel Purification Kit (Bioneer, Korea). The primer sequences were ITS1 with a CG-clamp (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') in the ITS consensus region of the 18S rRNA genes.[19] The PCR products were purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The nucleotide sequence was determined by BigDye terminator cycle sequencing on an ABI 3100DNAsequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA, USA). The sequence data have been deposited in the public database under the accession numbers shown in Figure 1b. The 18S rRNA gene sequences of related species were retrieved from the public database to construct a phylogenetic tree. Distance trees based on multiple sequence alignments were generated with Clustal X version 2.0 using the neighbor-joining method with 1000 bootstrap trials. The TH1 strain was physiologically characterized with a 20C AUX API system (Biomérieux Japan Co., Ltd., Tokyo, Japan).

Biotransformation experiments

An overnight culture of *C. viswanathii* TH1 was diluted to $OD_{600} = 0.3$ and inoculated (1% vol) into 30 mL of NB II medium in a 100-mL flask. The flask was kept standing at 30°C for 1 day. TH1 formed a floating biofilm (pellicle) on the surface of the culture rather than a biofilm on the inner surface of the glass flask in this culture condition. The pellicle was used for further experiments. The medium

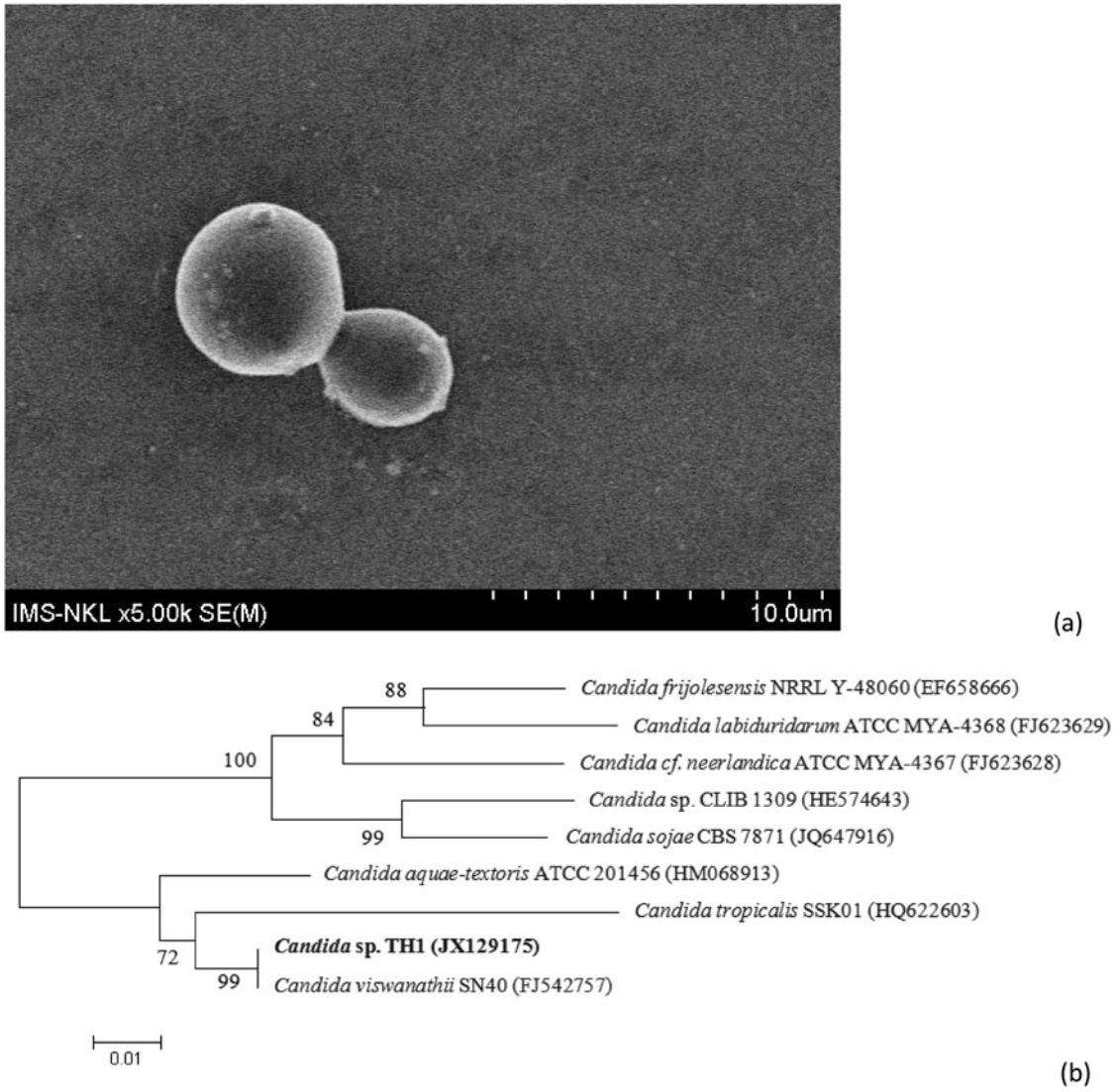


Fig. 1. (a) Scanning electron micrograph of TH1; (b) phylogenetic tree based on the 18S rRNA gene sequence of TH1 and its related species. The scale bar shows 0.01 nucleotide substitutions per site.

was removed from the flask, and the pellicle was gently rinsed with distilled water and filled with 50 mL of sterilized mineral salts medium supplemented with trace elements.[20] Next, *iso*-pentylbenzene (50 and 100 ppm) was added. To compare the degradation activities, it is important to use a similar number of planktonic cells with those of biofilms in MSM supplemented with 100 ppm *iso*-pentylbenzene. For controls, (i) cells in mineral salt medium (MSM) without the substrate and (ii) 50 ppm *iso*-pentylbenzene in MSM without cells were used.

After standing-incubation for 3 days at 30°C, the culture (0.1 mL of the culture supernatant) was analyzed by high-performance liquid chromatography (HPLC) for metabolic products. For kinetic studies, samples were taken every 12 h to analyze the products.

At the end of the experiment, the supernatants were filtered and extracted with diethylether and ethylacetate as described in a previous publication.[20] The obtained residues were dried under a nitrogen stream and dissolved in methanol or *n*-hexane and were used for gas chromatography-mass spectrometry (GC/MS) and HPLC analyses. All experiments were performed in triplicate.

Gas chromatography/mass spectrometric analyses (GC/MS)

GC/MS analyses of the metabolic products at the end of the transformation experiments were conducted by the Company of Fisons Instruments (Mainz-Kastell) with the following brief: A coupled system consisting of a GC 800 gas chromatograph equipped with a 30 m DB5-ms column (0.25 mm by 0.33 μm film; J&W Scientific, Folsom, CA, USA) and a mass selective detector MD 800 (Fisons Instruments) operating at 70 eV or a TSQ 700 (Finnigan

Corp., San Jose, CA, USA) triple quadrupole mass spectrometer operated in a single quadrupole mode (Q1) was used. Separation on the column was achieved by using a temperature programm from 60 to 290°C (10°C/min). Acid extracts were derivatized by methylation with diazomethane.[21] The samples were analyzed over a scan range of m/z (mass to charge ratio) 35–600 Da (Dalton). To confirm isolated metabolites, the authentic standard was purchased and added to the NIST library spectra. According to computer matching of the mass spectra and retention times with the database in the NIST library, the metabolites were identified.

High-performance liquid chromatography analyses (HPLC)

To determine the substrates and metabolites in the culture, 1 mL of the culture fluid was filtered or centrifuged to remove biomass. One hundred microliters of the supernatant were injected into the HPLC. The HPLC was performed on a Hewlett-Packard (Bad Homburg, Germany) HPLC apparatus 1050 M equipped with a quaternary pump system, a diode array detector 1040 M series I, and an HP Chemstation. The separation was achieved with a LiChroCart 125-4 RP-18 end-capped (5-μm) column (Merck, Darmstadt, Germany). An aqueous solution of methanol and H_3PO_4 (1 g L^{-1}) in bi-distilled water was used as the mobile phase with a linear gradient from 30 to 100% methanol within 14 min at a flow rate of 1 mL min^{-1}. All of the products were identified by comparison of the retention times and the UV/Vis spectra of the HPLC elution profiles of purchased authentic standards.

Results and discussion

Biofilm formation

With phenol, diesel oil or a mixture of naphthalene, pyrene and anthracene as the sole carbon and energy sources, we isolated six yeast strains from oil-polluted sediment samples collected from several coastal zones in Vietnam. The biofilm formation ability was examined for the six strains and a control without any strain. *Acinetobacter calcoaceticus* P23 was used as a positive control.[11] Each strain was grown in a 1.5 mL polypropylene tube without shaking for 24 h in NB II medium. The three strains P23, TH1 and TH15, formed robust biofilms, whereas the others formed marginal biofilms, and TH1 exhibited the best biofilm formation ability among the tested strains. Furthermore, when using a batch culture system for the biofilm formation, the biomass of the TH1 biofilms continued increasing even after 6 days (Table 1). The others formed only a small amount of biofilm under similar condition. Thus, we chose TH1 as a model yeast strain for further investigation.

Table 1. Biofilm formation of selected bacteria and yeast.

Strain	*Biofilm formation* (OD_{570})
Acinetobacter calcoaceticus P23 (Yamaga et al.[11])*	17.622
Negative control	0
Candida viswanathii TH1	24.044
TH6	7.956
TH13	11.822
TH15	15.947
TH16	12.243

(* *Acinetobacter calcoaceticus* P23 was the only bacterium that was used as a positive control; tube without any strain was used as a negative control).

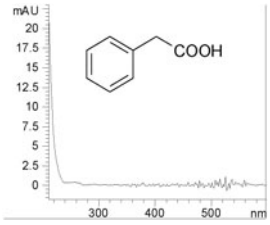
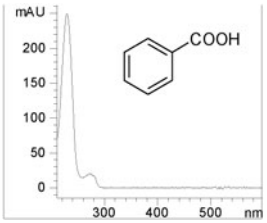
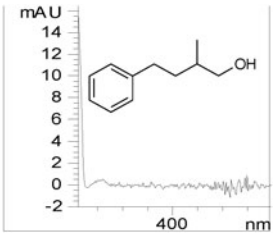
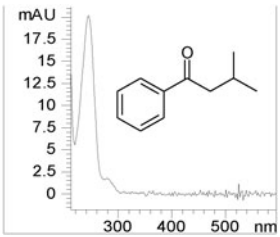
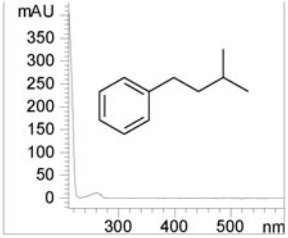
Identification

Yeast colonies were examined for morphological characteristics, such as color, shape, size, and surface properties on an NB II agar plate. Yeast cell morphology was observed by scanning electron microscopy (Fig. 1a). The microscopic morphology showed numerous globose, ovoid, or cylindrical budding yeast-like cells or blastoconidia (data not shown). Based on the analysis of the 500 nucleotides (nts) 18S rRNA sequence (ITS gene), strain TH1 shared 99% sequence identity with the type train of *Candida viswanathii* SN40 (FJ542757, four transitions difference), but significantly less sequence similarity with other strains of the genus: *Candida aquae-textoris* ATCC201456 (HM068913, 98% sequence identity, 10 nts sequence), *Candida tropicalis* SSK01 (HQ622603, 94% sequence identity, 26 nts sequence), *Candida sojae* CBS 7871 (JQ647916, 89% sequence identity, 42 nts sequence), *Candida labiduridarum* ATCC MYA-4368 (FJ623629, 87% sequence identity, 60 nts sequence), etc. (Fig. 1b). Furthermore, the results of the carbon assimilation tests using 20C AUX API showed that the TH1 was positive with: glucose, galactose, maltose, sucrose, trehalose, D-xylose, soluble starch, melezitose, glycerol, L-arabinose (delayed), D-mannitol, ribitol, salicin, citric acid and succinic acid; and was negative with: potassium nitrate, lactose, raffinose, L-sorbose, cellobiose, D-ribose, melibiose, galactitol, erythritol, inositol, D-glucitol, L-rhamnose, DL-lactic acid, D-arabinose. The *Candida viswanathii* MTCC 5158 was purchased to confirm the identification of the TH1;[22] therefore, the TH1 was identified as belonging to the species *Candida viswanathii.*

iso-*Pentylbenzene transformation*

After 3 days of incubation with *iso*-pentylbenzene at 30°C, from the culture supernatant of *C. viswanathii* TH1, four main metabolites I, II, III and IV, with retention times of 6.8 min, 7.0 min, 10.3 min and 11.3 min, respectively, were detected by HPLC analyses, which helped to follow their production and their degradation for the kinetics experiments (Table 2). Meanwhile, in the planktonic experiment, only the products I, II and VI were found. Therefore, in this article, we concentrated on the products formed by TH1 in the biofilm.

Table 2. Retention time and UV/Vis-spectrum of the identified metabolites produced by *C. viswanathii* TH1 cultured with *iso*-pentylbenzene (0.25 mL L^{-1}) in comparison with standard substances.

Met	*Name*	t_R [1] *(min)*	*UV/Vis-spectrum*	t_R [2] *(min)*	*m/z (%, 70 eV)*
I	phenylacetic acid	6.8		14.1 st	150 (28.4), 92 (7.8), 91 (100.0), 65 (15.8), 39 (8.1)
		6.7 st		14.1	150 (29.1), 92 (8.1), 91 (100.0), 65 (14.7), 39 (6.1) st
II	benzoic acid	7.0		12.38	136 (31.9), 106 (6.4), 105 (100.0), 77 (75.6), 51 (26.8), 50 (1397)
		7.0 st		12.3 st	136 (33.5), 106 (6.3), 105 (100.0), 77 (74.6), 51 (28.9), 50 (14.5)
III	2-methyl-4-phenyl-butan-1-ol	10.3		21.05	146 (43.1), 131 (62.3), 91 (97.6), 78 (11.6), 77 (20.4), 65 (14.7), 59 (100.0), 51 (10.8), 43 (15.5), 41 (8.7), 39 (9.5)
		10.3 st		21.35°st	146 (33.2), 131 (47.4), 91 (78.9), 78 (8.9), 77 (10.5), 65 (14.1), 59 (100.0), 51 (7.3), 43 (14.8), 41 (8.3), 39 (8.5) st
IV	*iso*-valerophenone	11.3		18.43	162 (9.3), 120 (28.9), 105 (100.0), 77 (46.6), 51 (15.3), 41 (7.3)
		11.3 st		18.43 st	162 (9.0), 120 (29.4), 105 (100.0), 77 (55.2), 51 (17.8), 41 (8.5) st
Sub	*iso*-pentylbenzene	16.6 st		14.1 st	148 (14.2), 105 (10.3), 93 (7.42), 92 (100.0), 91 (71.2), 77 (7.4), 65 (11.8), 57 (11.7), 41 (14.6), 39 (8.5) st

Met: metabolite; t_R [1]: Retention time in HPLC; t_R [2]: Retention time in GC/MS; Sub: substrate; st: purchased standard.

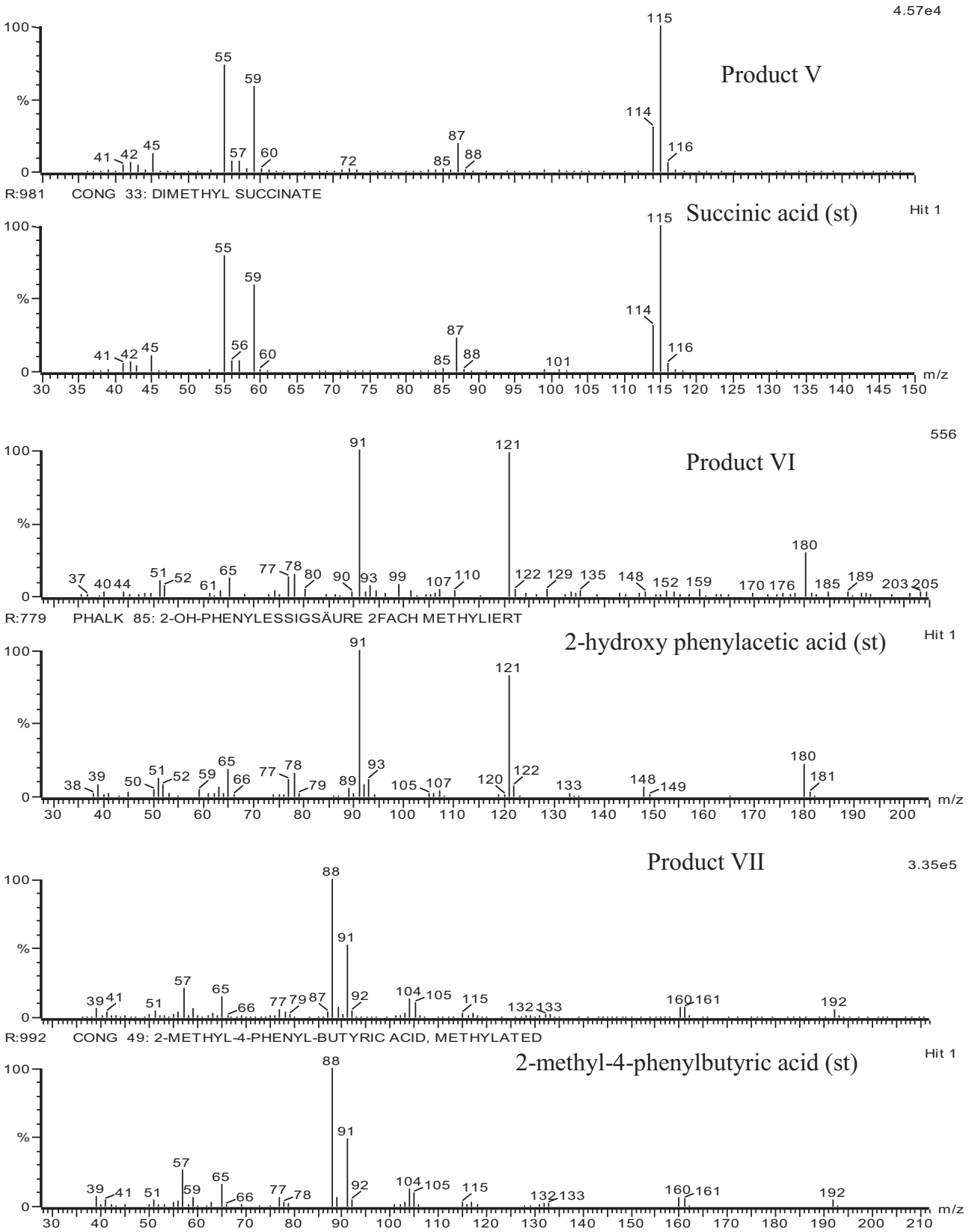


Fig. 2. Mass spectra of metabolites V, VI and VII as detected by GC/MS at retention times of 9.1, 20.1 and 20.8 min in comparison with the purchased standards (st). Structures were confirmed as succinic acid (product V), 2-hydroxy-phenylacetic acid (product VI) and 2-methyl-4-phenyl-butyric acid (product VII). All metabolites and standards were methylated.

All of these products were identified by comparison of the retention times and UV/Vis spectra of the HPLC elution profiles, as well as the retention times and mass spectra of the GC/MS, to those of veritable standards. Among the observed major metabolites detected by HPLC, metabolites I and II had UV/Vis-spectra and retention times that matched standard phenylacetic acid and benzoic acid, respectively. Accordingly, these products were confirmed to be phenylacetic acid and benzoic acid. Product III had a similar UV/Vis-spectrum and HPLC retention time (10.3 min) as 2-methyl-4-phenyl-butan-1-ol. Analysis by GC/MS identified product III as 2-methyl-4-phenyl-butan-1-ol in the alkaline extract at the same t_R of 21.05 min. Analysis by GC/MS and HPLC of the standard 2-methyl-4-phenyl-butan-1-ol confirmed product III as 2-methyl-4-phenyl-butan-1-ol.

This compound was proposed for product III based on the argument that this alcohol is carboxylated further to 2-methyl-4-phenyl-butyric acid and then to other shorter acid products that were detected in the culture broth of *C. viswanathii* TH1. Another major metabolite (IV) with a retention time of 11.3 min had a UV/Vis-spectrum corresponding to that of ketone compounds. Analysis by HPLC and GC/MS of the purchased standard *iso*-valerophenone confirmed product IV as *iso*-valerophenone.

Because of the limited sensitivity of analyzing aqueous samples by HPLC, the culture supernatants were extracted at different pH conditions, specifically pH 9, 7 and 2, and were analyzed by GC/MS. As a result, in addition to the four upper metabolites, three other products, V, VI and VII, were isolated. By comparison with the available standards, they were identified as succinic acid (t_R 9.1 min), 2-hydroxy-phenylacetic acid (t_R 20.1 min) and 2-methyl-4-phenyl butyric acid (t_R 20.8 min), respectively (Fig. 2).

Based on these identified metabolites, it was assumed that the transformation of *iso*-pentylbenzene by *C. viswanathii* TH1 started from the alkyl side chain, and then the chain shortened. A similar mode of catabolism has been previously demonstrated in other bacteria and yeasts, e.g., *Alcanivorax* sp. MBIC 4326.[23] *Mycobacterium neoaurum,*[24] *Nocardia salmonicolor* [8] and *Candida maltosa.*[9] However, the alkylbenzenes widely investigated were linear *n*-alkylbenzenes without branched side chain, whereas the *iso*-pentylbenzene used in this study has one branching point at the end of the alkyl chain. Moreover, to our knowledge, it is the first time that a biofilm-forming yeast was used to transform aromatic side chain hydrocarbons, which are considered recalcitrant compounds.

Kinetics analysis of the iso-*pentylbenzene transformation and its metabolites*

At the beginning of biotransformation, after 8 h of incubation, phenylacetic acid (product I) was excreted into the culture of TH1 with a content increasing from 0.01 μM to 0.018 μM. Benzoic acid (product II) was found later, after 12 h of culture with the amount increasing from 0.006 μM to 0.012 μM. *iso*-Valerophenone was found only in the reaction mixture of TH1 after 24 h at a trace amount and at the end of cultivation period (Fig. 3a). By HPLC analyses, product III (2-methyl-4-phenyl-butan-1-ol) and product VII (2-methyl-4-phenyl-butyric acid) were detectable at the same retention time (10.3 min) in the HPLC profiles. Therefore, the amount of these metabolites was not estimated.

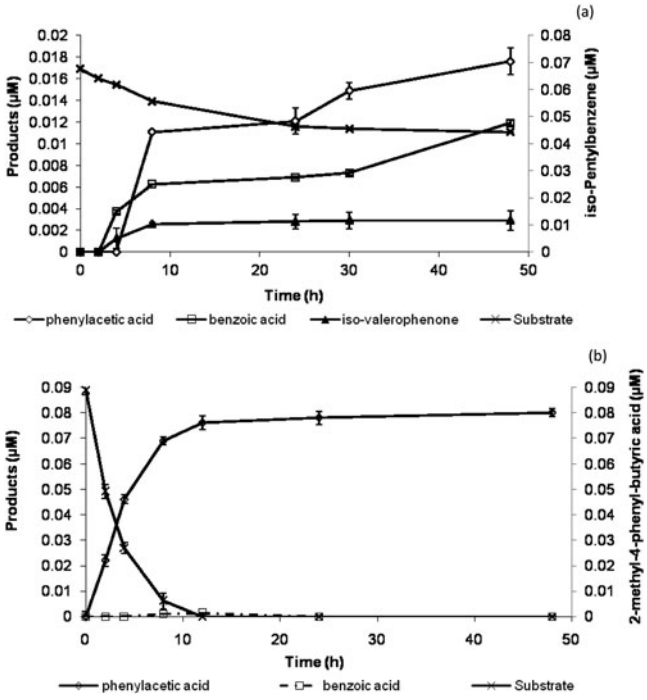


Fig. 3. (a) Kinetics of the transformation of *iso*-pentylbenzene by *C. viswanathii* TH1; (b) kinetics of the transformation of 2-methyl-4-phenyl-butyric acid by *C. viswanathii* TH1 (data are shown as the means ± SD of three independent cultures).

The ability of strain TH1 to further metabolize the metabolites I, II, III, IV and VII was evaluated by incubating a biofilm with 10 ppm of each of these compounds individually. No metabolites were detected when the cells were incubated with metabolites II or III. Metabolite I was converted to 2-hydroxybenzoic acid, which was detected as a sole metabolite. Metabolite IV was converted to benzoic acid and cultures incubated with metabolite VII produced principally phenylacetic acid which was detected after 2 h of incubation, but small amounts of metabolites II and VI were also produced (Table 3). However, TH1 was not able to use the other metabolites, such as benzoic acid (metabolite II) and 2-methyl-4-phenyl-butan-1-ol (metabolite III), as the sole carbon and energy sources for growth. The 2-methyl-4-phenyl-butyric acid (metabolite VII) was transformed mainly to phenylacetic acid, which could be detected in a significant amount after 2 h of cultivation (Fig. 3b). In addition, benzoic acid was obtained in a trace amount.

Table 3. Detected products by HPLC analysis of the cultures of *C. viswanathii* TH1 with products I, II, IV, V and VIII as substrates.

Substrate	*Formed metabolites from product I-VIII*	C. viswanathii *TH1*
Phenylacetic acid (product I)	2-hydroxy benzoic acid (VII)	+
Benzoic acid (product II)	n.d.	n.d.
2-methyl-4-phenyl-butan-2-ol (product III)	n.d.	n.d.
iso-valerophenone (product IV)	Benzoic acid (II)	+
2-methyl-4-phenylbutyric acid (product VII)	Phenylacetic acid (I)	++
	Benzoic acid (II)	+
	2-hydroxy benzoic acid (VII)	+

++, main metabolite; +, trace metabolite; n.d., not detectable.

Thus, according to biotransformation experiments with *iso*-pentylbenzene and its metabolites, this substrate was proposed to be transformed via two different pathways by *C. viswanathii* TH1 (Fig. 4). One pathway started by terminal oxidation of the methyl group in *iso*-pentylbenzene and further oxidation to form 2-methyl-4-phenyl-butan-1-ol, followed by the formation of 2-methyl-4-phenyl-butyric acid, which is then degraded via ß-oxidation to form phenylacetic acid. The latter is oxidized further to form (i) benzoic acid via oxidation and decarboxylation steps or (ii) (2-hydroxy-phenyl)-acetic acid a via hydroxylation step of the aromatic ring. The products benzoic acid and (2-hydroxy-phenyl)-acetic acid are then transformed by *ortho*- or *meta*-cleavage to succinic acid. In the second pathway, *iso*-valerophenone is formed from the corresponding secondary alcohol as a result of attack at carbon 4 of the branched alkyl side chain in *iso*-pentylbenzene. *iso*-Valerophenone undergoes further Bayer-Villiger-Oxidation to produce an ester intermediate (not detectable) which is then hydrolyzed to benzoic acid. The latter can then be cleaved to form succinic acid. Both of these proposed pathways are similar to that predicted for the metabolism of alkylbenzene by *M. neoaurum* SBUG 109,[24] as they are initiated by oxidation of the alkyl side chain, but these pathways differ from that of *Pseudomonas* sp.[25] where the metabolism is initiated by the oxidation of the aromatic ring followed by a ring fission.

In the former pathway, degradation of *iso*-pentylbenzene through 2-methyl-4-phenyl butyric acid, phenylacetic acid and benzoic acid suggested the involvement of a ß-oxidation mechanism operating on the branched alkyl side chain and a decarboxylation process of phenylacetic acid to yield benzoic acid. Sariaslani et al.[8] reported a similar mechanism for *Pseudomonas* sp. In degradation studies, 3-phenylbutyric acid was detected as an intermediate resulting from ω-oxidation and subsequent decarboxylation. 3-Phenylpropionic acid was then α-oxidized to produce phenylacetic acid, followed by a further decarboxylation step leading to benzoic acid. Chuchev and Belbruno [4] published the mechanism of the decarboxylation of *ortho*-substituted benzoic acids, and they concluded that aromatic decarboxylation processes play a significant role in biochemical reactions.

When 2-methyl-4-phenyl butyric acid was used as a substrate for biotransformation experiments, *C. viswanathii* TH1 produced phenylacetic acid as the major intermediate. Thus, mono-terminal oxidation, followed by ß-oxidation of the alkyl side chain to phenylacetic acid, has been confirmed in *C. viswanathii* TH1. Several hydrocarbon-utilizing bacteria and yeasts show a primary attack of the substrate's aliphatic side chain via mono-terminal

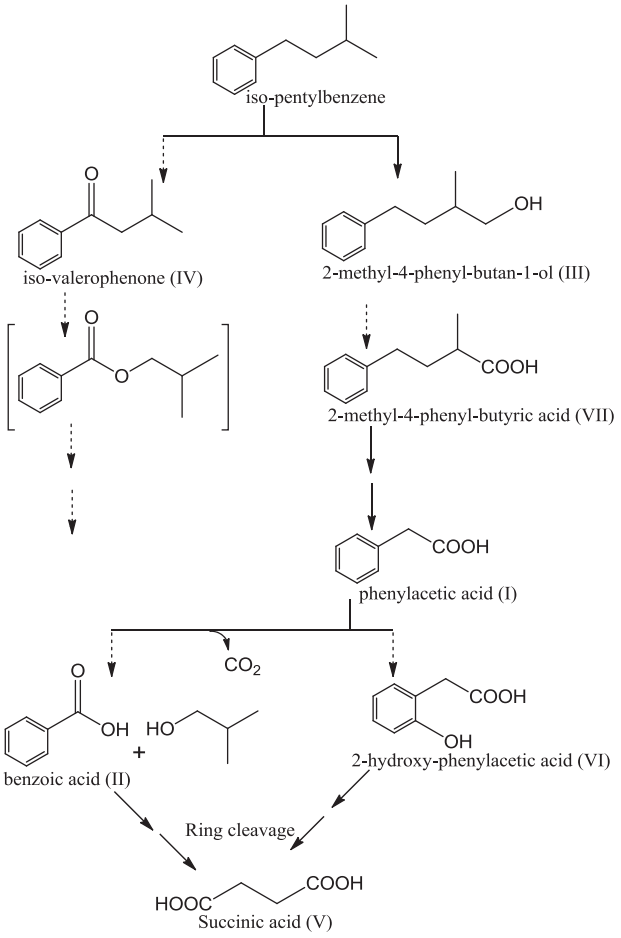


Fig. 4. Proposed pathways for the degradation of *iso*-pentylbenzene by *C. viswanathii* TH1*;* I, II, III, IV, V, VI, VII and VIII were isolated metabolites; [→], undetected metabolite→ not proven by experiments→, proven by experiments.

oxidation.[9–26] The presence of (2-hydroxy-phenyl)-acetic acid and succinic acid indicated that the proposed ring cleavage via an *ortho*- or *meta*-pathway was operative. The formation of a small amount of benzoic acid from 2-methyl-4-phenyl-butyric acid may be explained by an additionally integrated decarboxylation step.

Isolation of *iso*-valerophenone (metabolite V) and benzoic acid (metabolite II) in the cultures of *C. viswanathii* TH1 gave hints regarding the ability of this strain to degrade branched alkyl side chains via sub-terminal oxidation. Ketone metabolism by several microorganisms such as *M. neoaurum* SBUG 109,[20] *Marinobacter* sp.,[27] *Rhodococcus ruber* [28] and *Arthrobacter* sp.[29] occurs through by a sequence of reactions initiated by the insertion of an oxygen atom between the carbon atom of the *oxo* group and an adjacent carbon atom to form either an ester or a lactone. This pathway has been found in the degradation of *iso*-pentylbenzene by *M. neoaurum* SBUG 109.[20] However, bacterium was cultured in the planktonic form, whereas TH1 transformed this substrate in the biofilm form. Other transformation steps of ketone metabolism have been proposed. According to Cripps [30] and other references therein, with butan-2-one, three transformation steps of different metabolic pathways have been suggested: first, the ketone was converted into ethyl acetate by *Nocardia* sp., but further transformation was not described; second, the ketone was metabolized to propionic acid by *M. vaccae*, but further transformation was again not described; third, the ketone was catalyzed to 3-hydroxybutan-2-one by *Pseudomonas* sp., and further metabolism of this latter product proceeded via butan-2,3-dione followed by cleavage into two C_2 units.

Accordingly, it was reasonable to assume that *iso*-valerophenone was degraded by the initial Bayer-Villiger-Oxidation, which can be performed in two different ways resulting in phenol or benzoic acid as products. Previous studies on the metabolism of propane by *Mycobacterium vaccae* JOB-5 indicated that the major assimilatory pathway involves an initial sub-terminal oxidation.[31] However, to our knowledge, there is no information about the degradation of branched alkyl side chains of aromatics via the sub-terminal mechanism by *C. viswanathii* TH1 as a biofilm.

In conclusion, under aerobic and biofilm conditions, *iso*-pentylbenzene was degraded at the branched side chain via both mono- and sub-terminal oxidation. It is of particular interest that the cleavage of the benzene ring occurred in *C. viswanathii* TH1. These features could be potentially interesting for the biotransformation of other recalcitrant aromatic compounds.

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References

[1] Amund, O.O.; Higgins, I.J. The degradation of 1-phenylalkanes by an oil-degrading strain of *Acinetobacter lwoffi*. Anton. Van Leeuwen. **1985**, *51*, 45–56.

[2] Baggi, G.; Catelani, D.; Galli, E.; Treccani, V. The microbial degradation of phenylalkanes. 2-Phenylbutane, 3-phenylpentane, 3-phenyldodecane and 4-phenylheptane. Biochem. J. **1972**, *126*, 1091–7.

[3] Bains, J.; Boulanger, M.J. Structural and biochemical characterization of a novel aldehyde dehydrogenase encoded by the benzoate oxidation pathway in *Burkholderia xenovorans* LB400. J. Mol. Biol. **2008**, *379*, 597–608.

[4] Chuchev, K.; Belbruno, J.J. Mechanisms of decarboxylation of *ortho*-substituted benzoic acids. J. Mol. Struc. – Theochem. **2007**, *807*, 1–9.

[5] Zylstra, G.J.; Kim, E. Aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1. J. Ind. Microbiol. Biotechnol. **1997**, *19*, 408–414.

[6] Atlas, R.M. Petroleum biodegradation and oil spill bioremediation. Mar. Pollut. Bull. **1995**, *31*, 178–182.

[7] Lang, E. Diversity of bacterial capacities in utilizing alkylated benzenes and other aromatics compounds. Lett. Appl. Microbiol. **1996**, *23*, 257–260.

[8] Sariaslani, F.S.; Harper, D.B.; Higgins, I.J. Microbial degradation of hydrocarbons. Catabolism of 1-phenylalkanes by *Nocardia salmonicolor*. Biochem. J. **1974**, *140*, 31–45.

[9] Awe, S.; Mikolasch, A.; Hammer, E.; Schauer, F. Degradation of phenylalkanes and characterization of aromatic intermediates acting as growth inhibiting substances in hydrocarbon utilizing yeast *Candida maltosa*. Int. Biodeterior. Biodegr. **2008**, *62*, 408–414.

[10] Shimada, K.; Itoh, Y.; Washio, K.; Morikawa, M. Efficacy of forming biofilms by naphthalene degrading Pseudomonas stutzeri T102 toward bioremediation technology and its molecular mechanisms. Chemosphere **2012**, *87*, 226–233.

[11] Yamaga, F.; Washio, K.; Morikawa, M. Sustainable biodegradation of phenol by *Acinetobacter calcoaceticus* P23 isolated from the rhizosphere of duckweed *Lemna aoukikusa*. Environ. Sci. Technol. **2010**, *44*, 6470–6474.

[12] O'Toole, G.A.; Kaplan, H.B.; Kolter, R. Biofilm formation as microbial development. Ann. Rev. Microbiol. **2000**, *54*, 49–79.

[13] Gilan, I.O.; Hadar, Y.; Sivan, A. Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. Appl. Microbiol. Biotechnol. **2004**, *65*, 97–104.

[14] Hammer, E.; Kneifel, H.; Hofmann, K.; Schauer, F. Enhanced excretion of intermediates of aromatic amino acid catabolism during chlorophenol degradation due to nutrient limitation in the yeast *Candida maltosa*. J. Basic. Microbiol. **1996**, *36*, 239–243.

[15] Hornei, S.; Kohler, M.; Weide, H. Spectrum of fatty acids of a *Candida* strain following culture on *n*-alkanes. Z. Allg. Mikrobiol. **1972**, *12*, 19–27.

[16] Schauer, F. Abbau und Verwertung von Mineralölbestandteilen durch Mikroorganismen. Bodden **2001**, *11*, 3–31.

[17] Morikawa, M.; Kagihiro, S.; Haruki, M.; Takano, K.; Branda, S.; Kolter, R.; Kanaya, S. Biofilm formation by a *Bacillus subtilis* strain that produces gamma-polyglutamate. Microbiology **2006**, *152*, 2801–2807.

[18] O'Toole, G.A.; Kolter, R. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol. Microbiol. **1998**, *28*, 449–461.

[19] White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*; Innis, M.; Gelfand, D.; Sninsky, J.; White, T., Eds.; Academic Press: Orlando, FL, 1990; Chap. 3, 315–322.

[20] Nhi-Cong, L.T., Mikolasch, A.; Klenk, H.P.; Schauer, F. Degradation of the multiple branched alkane 2,6,10,14-tetramethylpentadecane (pristane) in *Rhodococcus ruber* and *Mycobacterium neoaurum*. Int. Biodeter. Biodegr. **2009**, *63*, 201–207.
[21] De Boe, T.D.; Backer, H.J. Diazonethane. Org. Synth. **1956**, *36*, 14–16.
[22] Kamble, A.L.; Soni, P.; Banerjee, U.C. Biocatalytic synthesis of S(−)-1-(1'-naphthyl) ethanol by a novel isolate of *Candida viswanathii*. J. Mol. Catal. B-Enzym. **2005**, *35*, 1–6.
[23] Dutta, T.K.; Harayama, S. Biodegradation of *n*-Alkylcycloalkanes and *n*-Alkylbenzenes via new pathways in *Alcanivorax* sp. strain MBIC 4326. Appl. Environ. Biotechnol. **2001**, *67*, 1970–1974.
[24] Nhi-Cong, L.T. *Degradation of branched chain aliphatic and aromatic petroleum hydrocarbons by microorganisms.* PhD thesis, University of Greifswald, Greifswald, Germany, 2008.
[25] Jigami, Y.; Omori, T.; Minoda, Y. The degradation of *iso*-propylbenzene and *iso*-butylbenzene by *Pseudomonas* sp. Agr. Biol. Chem. **1975**, *39*, 1781–1788.
[26] Herter, S.; Mikolash, A.; Schauer, F. Identification of phenylalkane derivatives when *Mycobacterium neoaurum* and *Rhodococcus erythropolis* were cultured in the presence of various phenylalkanes. Appl. Microbiol. Biotechnol. **2012**, *93*, 343–355.
[27] Rontani, J.F.; Gilewicz, M.J.; Michotey, V.D.; Zheng, T.L.; Bonin, P.C.; Bertrand, J.C. Aerobic and anaerobic metabolism of 6,10,14-trimethylpentadecan-2-one by a denitrifying bacterium isolated from marine sediments. Appl. Environ. Microbiol. **1997**, *63*, 636–643.
[28] Kostichka, K.; Thomas, S.M.; Gibson, K.J.; Nagarajan, V.; Cheng, Q. Cloning and characterization of a gene cluster for cyclododecanone oxidation in *Rhodococcus ruber* SC1. J. Bacteriol. **2001**, *183*, 6478–6486.
[29] Cripps, R.E.; Trudgill, P.W.; Whateley, J.G. The metabolism of 1-phenylethanol and acetophenone by *Nocardia* T5 and an *Arthrobacter* species. Eur. J. Biochem. **1978**, *86*, 175–186.
[30] Cripps, R.E. The microbial metabolism of acetophenone. Metabolism of acetophenone and some chloroacetophenones by an *Arthrobacter* species. Biochem. J. **1975**, *152*, 233–241.
[31] Johnson, E.L.; Smith, C.A.; O'Reilly, K.T.; Hyman, M.R. Induction of methyl tertiary butyl ether (MTBE)-oxidizing activity in *Mycobacterium vaccae* JOB5 by MTBE. Appl. Environ. Microbiol. **2004**, *70*, 1023–1030.