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Enrichment of new alkane oxidizing bacterial strains for human drug metabolite production

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ABSTRACT

Human metabolites of active pharmaceutical ingredients (APIs) are of high interest for pharmacokinetic and toxicological studies. Moreover, some API metabolites are also under investigation as possible next generation drugs.

However, often highly selective chemical reactions, e.g. stereo- and regioselective hydroxylations are required to enable the production of these compounds and biocatalysis offers the only feasible synthetic approach. Screening microorganisms to discover new catalytic activities is usually a time consuming task and the outcome is unpredictable. Aerobic alkane oxidizing bacteria depend on their ability to hydroxylate hydrocarbons to introduce such non-activated carbon sources to their central metabolic pathways. Hence, we expected that alkane metabolizers generally might also enable hydroxylation of other substrates, e.g. APIs.

Based on this working hypothesis a collection of alkane metabolizing strains was established, roughly classified by 16S rDNA sequencing and screened for metabolite production of seven different pharmacological compounds. Several active strains were found, metabolizing dextromethorphan, harmine, phenacetin and tolbutamide. Overall, almost 30% of the new isolated strains showed activity with at least one of the 7 substrates.

1. Introduction

Alkane assimilation is a widespread feature which can be found in bacteria, yeast, fungi and algae and even animals. Microbes growing on alkanes as a sole carbon and energy source were commonly isolated from natural oil deposits, oil polluted soils or water. Some were even isolated from fuel tanks and from exhaust air filters [1,2].

Most alkane hydroxylating enzymes can be assigned to one of the following three groups, i.e. methane monooxygenase (MMO) related enzymes, enzymes related to *Pseudomonas putida* GPo1 alkane hydroxylase (AlkB) and cytochrome P450 enzymes [3–5]. While MMO and AlkB type enzymes utilize a non-heme oxo-diiron complex to oxidize the substrate, the oxidizing agent in cytochrome P450 enzymes is believed to be the extensively studied compound I [6]. Nevertheless all three share an enzymatic oxygen rebound mechanism [7,8].

Bioremediation is a major application of alkane degrading microbes [9–11]. In most biochemical pathways of aerobic alkane metabolizers the initial alkane functionalization is performed by monooxygenases yielding primary or secondary alcohols by hydroxylation. The ability of their hosts to utilize different chain length alkanes reflects the natural substrate specificity of the different enzyme classes. While organisms harboring MMO related enzymes usually grow on carbon chains up to C4 [12], AlkB and bacterial P450 (P450 class I) harboring strains prefer medium chain length alkanes (C5–C16) [13] whereas many yeasts and fungi harboring eukaryotic P450s (P450 class II) and other unknown oxygenases grow on medium to long chain alkanes (C10–C30) [14]. Usually, terminal hydroxylation followed by alcohol and aldehyde dehydrogenase mediated oxidation allows the microbes to assimilate simple aliphatic hydroxylated alkanes via β-oxidation. Subterminal oxidation yielding ketones after alcohol dehydrogenation is an alternative possibility. The ketones are then converted to esters by a Baeyer–Villiger monooxygenase and finally hydrolyzed by an esterase, yielding a fatty acid and a primary alcohol [15].

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Natural alkane hydroxylating P450 enzymes (e.g. CYP153) [16,17] and laboratory evolved P450 alkane hydroxylases such as, e.g. P450 BM3 variants [18] but also AlkB homologues [19] have been reported to also accept more bulky and complex substrates, making them interesting candidates for the synthesis of otherwise hardly accessible compounds such as fine chemicals or pharma compounds. Particularly, metabolites of mammalian cytochrome P450 enzymes need to be supplied for toxicological studies as well as new active pharmaceutical ingredients (APIs) for next generation drugs. In many cases classic chemical syntheses do not fulfill the demand of regio- and stereoselectivity in case of hydrocarbon activation. However, although often providing the only tool for such difficult synthetic applications, eukaryotic P450 enzymes are far from being perfect biocatalysts for industrial purposes. They are difficult to express large multi domain systems, need expensive cofactors and they show low turnover rates and lack of stability. Hence, there is a demand for new, alternative, efficient and robust enzymes catalyzing similar reactions as human cytochrome P450 enzymes. A library of alkane assimilating strains is supposed to be an ideal starting point for such screenings, promising a high fraction of organisms showing the desired activity.

Here we present the use of enrichment cultures from oil contaminated soil samples as natural sources for the selection and isolation of new microbial strains growing on linear and/or cyclic alkanes, i.e. *n*-hexane and cyclohexane. Individual alkane metabolizers were isolated, characterized and then screened for the conversion of seven APIs by high throughput HPLC/MS.

2. Experimental

2.1. Enrichment cultures

To isolate monooxygenase harboring strains from nature, soil samples from crude oil store area, decomposed oil units and an aeration tank of Jinling Petrochemical Limited Corp. (1–2 g of soil each, Nanjing, PR China) were mixed and inoculated into 300 mL sterile enrichment medium (AM1) (NH_4Cl 2 g/L, $MgSO_4$ 0.2 g/L, KH_2PO_4 2 g/L, pH 7.0; trace salts 0.1 mL/L (2.2 g $ZnSO_4{\cdot}7H_2O$, 1.1 g H_3BO_3, 0.5 g $MnCl_2{\cdot}4H_2O$, 0.8 g $Fe((NH_4)_2SO_4)_2{\cdot}7H_2O$, 0.1 g $CoCl_2{\cdot}6H_2O$, 0.16 g $CuSO_4$, 0.11 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, $Na_4EDTA{\cdot}4H_2O$), 0.25% cyclohexane, 0.25% hexane and 0.05‰ Tween-80). The cultivation was performed at 30 °C while shaking for 72 h in an Infors incubator at 200 rpm at a diameter of 2.5 cm with a feeding step (0.25% cyclohexane and 0.25% hexane per 24 h), then the culture was re-inoculated into a new enrichment medium and cultivated at 30 °C, 200 rpm for 72 h. The enrichment procedure was stopped after another re-inoculation and further 72 h cultivation step. A diluted enrichment culture was plated on AM1 agar plates for the isolation of the individual strains. The plates were incubated at 30 °C for several days and the colonies were divided into groups according to the colony morphology and the morphology checked by microscope. Totally 53 strains were selected, purified on isolation medium by streaking out for single colonies and stored at −70 °C.

2.2. Genomic DNA extraction

The microorganisms were harvested from LB plates, washed with 0.1× SSC (SSC: 150 mM NaCl, 15 mM Na_3Citrate) and resuspended in 1 mL lysis buffer (0.01% [w/v] Na_3PO_4, 20% [w/v] sucrose; pH 8.0). After addition of 50 μL of lysozyme (50 mg/mL), cells were lysed at 35 °C for 90 min. The lysed cells were resuspended in 9 mL TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and 900 μL of 10% SDS and 20 μL Proteinase K (manufacturer, concentration) were added. After incubation at 37 °C for 30 min, 2 mL of 5 M NaCl and 1.6 mL of CTAB/NaCl solution (10% [v/v] CTAB, 0.7 M NaCl) were added and the viscous solution was incubated at 60 °C for 10 min. The solution was cooled down and extracted with 1 volume ice-cold chloroform/isoamyl alcohol (24 + 1). Phases were separated by centrifugation at 3000 × *g* for 15 min. The aqueous phase was extracted with phenol/chloroform/isoamyl alcohol (25 + 24 + 1, ice-cold). Phases were again separated by centrifugation at 3000 × *g* for 15 min. The aqueous phase was mixed with 0.6 volumes ice-cold isopropanol and the DNA was precipitated at 4 °C for 8 h. The DNA was twice washed in 70% ethanol and then dried for 15 min at 37 °C. Afterwards, DNA was dissolved in 500 μL ddH_2O.

2.3. Characterization of strain morphology

Strain morphology was determined with a Carl Zeiss transmitted-light microscope at a 100× magnification.

2.4. Characterization of carbon source acceptance

To select the strains for their ability to grow on linear and/or cyclic alkanes they were grown on either *n*-hexane or cyclohexane vapor as a sole carbon source as described by Smits et al. [20,21].

2.5. Amplification of 16S rDNA

Primers described by Weidner et al. were used to amplify the 16S rDNA region from the genomic DNA: R1n: 5′-GCTCAGATTGAACGCTGGCG-3′, U2: 5′-ACATTTCACAACACGAGCTG-3′ [22]. All oligonucleotides used were manufactured by Invitrogen. The following conditions were used for PCR amplification: 20 ng genomic DNA, 0.5 μL primer R1n (10 pmol/μL), 0.5 μL primer U2 (10 pmol/μL), 5 μL dNTP mix (2 mM each), 5 μL 10× Taq Buffer, 1 μL Taq-Polymerase, filled up with ddH_2O to a total volume of 50 μL. Initial denaturation 120 s at 94 °C, 35 cycles of 80 s at 94 °C, 60 s at 52 °C and 90 s at 72 °C. Final elongation was done for 180 s at 72 °C. Amplified PCR products were purified using the WizardSV Gel and PCR clean-up system (Promega) and sequenced.

2.6. Cultivation of strains on alkane media

50 mL medium AM1 in baffled shaking flasks were inoculated with a single colony grown on an alkane vapor exposed plates. Cultures were incubated at 28 °C, 120 rpm and additional 0.25% cyclohexane + 0.25% *n*-hexane were fed every 24 h. On the 8th day, 2 h after the last feeding strains were harvested by centrifugation. The pellet was resuspended and shaken for 5 min in an ice-cold 1 + 4 suspension of diethyl ether and water. The phases were separated by centrifugation at 3000 × *g* for 10 min at 4 °C. The organic phase was subjected to GC analysis to detect formation of hexanol.

2.7. Biotransformation of APIs

4 mL LB-Miller broth were inoculated in test tubes with a single colony grown on an alkane vapor exposed agar plate. After 24 h incubation at 28 °C, 120 rpm, 1 mM of the substrate of interest was added for induction of and bioconversion by the hydroxylating enzyme(s). The cells were incubated for 4 more days at 28 °C while shaking at 120 rpm. One volume of ethanol was added to partially lyse the cells and extract eventual products. The cultures were left shaking for 1 more hour. The cultures were centrifuged at 6000 × *g* for 10 min to remove cell debris and the clear supernatant was subjected to HPLC- and HPLC/MS analysis, respectively.

Table 1
Classification of strains based on 16S RNA sequence and activities detected by HPLC–MS under screening conditions (Research Centre Applied Biocatalysis Culture Collection = AB-CC#)

Strain	AB-CC#	Strain with highest score	Score	Order	Growth *c*-Hexane	Metabolite
L1	5614	*Pseudomonas_sp._3C_12*	0.956	Pseudomonadales	+	M4
L3	5615	*Sphingobacterium sp. MG2*	0.882	Sphingobacteriales	–	M7
L4	5616	*bacterium_1B3*	0.901	Xanthomonadales	–	
L5	5617	*Pseudomonas plecoglossicida; R3*	0.814	Pseudomonadales	+	M2a
L6	5618	*Pseudomonas putida; OS-5*	0.880	Pseudomonadales	+	
L7	5619	*Pseudomonas sp. LAB-26*	0.973	Pseudomonadales	+	M4
L8	5620	*Pseudomonas sp. dcm7B*	0.590	Pseudomonadales	+	M5
L9	5621	*Klebsiella pneumoniae; CICC10072*	0.766	Enterobacteriales	+	
L10	5622	*Escherichia coli; T10*	0.812	Enterobacteriales	+	
L11	5623	*Bacterium #WM-B17*	0.758	Pseudomonadales	+	M7
L12	5624	*Bacterium SV70AB1-3; sv70ab1-3*	0.798	Sphingobacteriales	–	
Y5	5625	*Pseudomonas aeruginosa*	0.789	Pseudomonadales	+	
Z2	5626	*Rahnella sp. HX2*	0.929	Enterobacteriales	+	
Z3	5627	*Rahnella sp. 'CDC 2987-79'*	0.732	Enterobacteriales	+	M2a
Z4	5628	*Rahnella aquatilis*	0.929	Enterobacteriales	+	Unknown (S2)
Z5	5629	*Rahnella_sp._BIHB783*	0.931	Enterobacteriales	+	
Z6	5630	*Endophyte bacterium SS10*	0.896	Enterobacteriales	+	
Z7	5631	*Yersinia sp. TOHOCD5-6*	0.957	Enterobacteriales	+	
Z8	5632	*Serratia sp. AE2*	0.980	Enterobacteriales	–	M5
Z9	5633	*Yersinia enterocolitica*	0.865	Enterobacteriales	–	
Z10	5634	*Pantoea agglomerans CC-88166*	0.998	Enterobacteriales	+	
Z11	5635	*Bacillus simplex*	0.955	Bacillales	–	
Z13	5636	*Pseudomonas fluorescens*	0.987	Pseudomonadales	–	
Z15	5637	*Pantoea agglomerans CC-88023*	0.982	Enterobacteriales	+	
Z16	5638	*Alcaligenes_faecalis_subsp._faecalis*	0.951	Burkholderia	+	
Z18	5639	*Alcaligenes sp. STC1*	0.781	Burkholderia	+	M5
Z19	5640	*Pseudomonas putida J312*	0.866	Pseudomonadales	+	
Z20	5641	*Achromobacter xylosoxidans AU1011*	0.977	Burkholderia	+	
Z22	5642	*Pseudomonas sp. 4CBAI5;*	0.987	Pseudomonadales	+	
Z23	5643	*Pseudomonas sp. PHLL*	0.978	Pseudomonadales	+	
Z24	5644	*Pseudomonas putida; KL33;*	0.987	Pseudomonadales	+	
Z25	5645	*Pantoea agglomerans; CC-88172*	0.996	Enterobacteriales	+	
Z26	5646	*Pantoea agglomerans; AH16*	1.000	Enterobacteriales	+	M7
Z27	5647	*Pantoea agglomerans; 86350*	0.980	Enterobacteriales	+	
Z28	5648	*Pantoea agglomerans; GIST-CPs11*	0.990	Enterobacteriales	+	
Z29	5649	*Pantoea agglomerans; ChDC YP2*	0.984	Enterobacteriales	+	
Z31	5650	*Pantoea agglomerans; WAB1870*	0.977	Enterobacteriales	+	
Z33	5651	*Alcaligenes sp. A-13*	0.983	Burkholderia	–	
Z34	5652	*Achromobacter xylosoxidans_A1*	0.926	Burkholderia	+	
Z37	5653	*Alcaligenes sp. CCBAU10750*	0.969	Burkholderia	–	
Z38	5654	*Trabulsiella guamensis*	0.948	Enterobacteriales	+	
Z40	5655	*Achromobacter_sp. LQX8*	0.909	Burkholderia	+	

2.8. GC-analysis

All standards were purchased from Sigma–Aldrich. Analysis was done on an Agilent 6890N with FID. The temperature gradient was as follows: 8 min at 125 °C, heating 20 °C/min until reaching 200 °C, 200 °C hold for 5 min. Inlet and detector temperatures were 250 °C employing a HP 5, 60 m 320 μm × 1 μm column. The nitrogen flow was 1.2 mL/min.

2.9. HPLC/MS-analysis

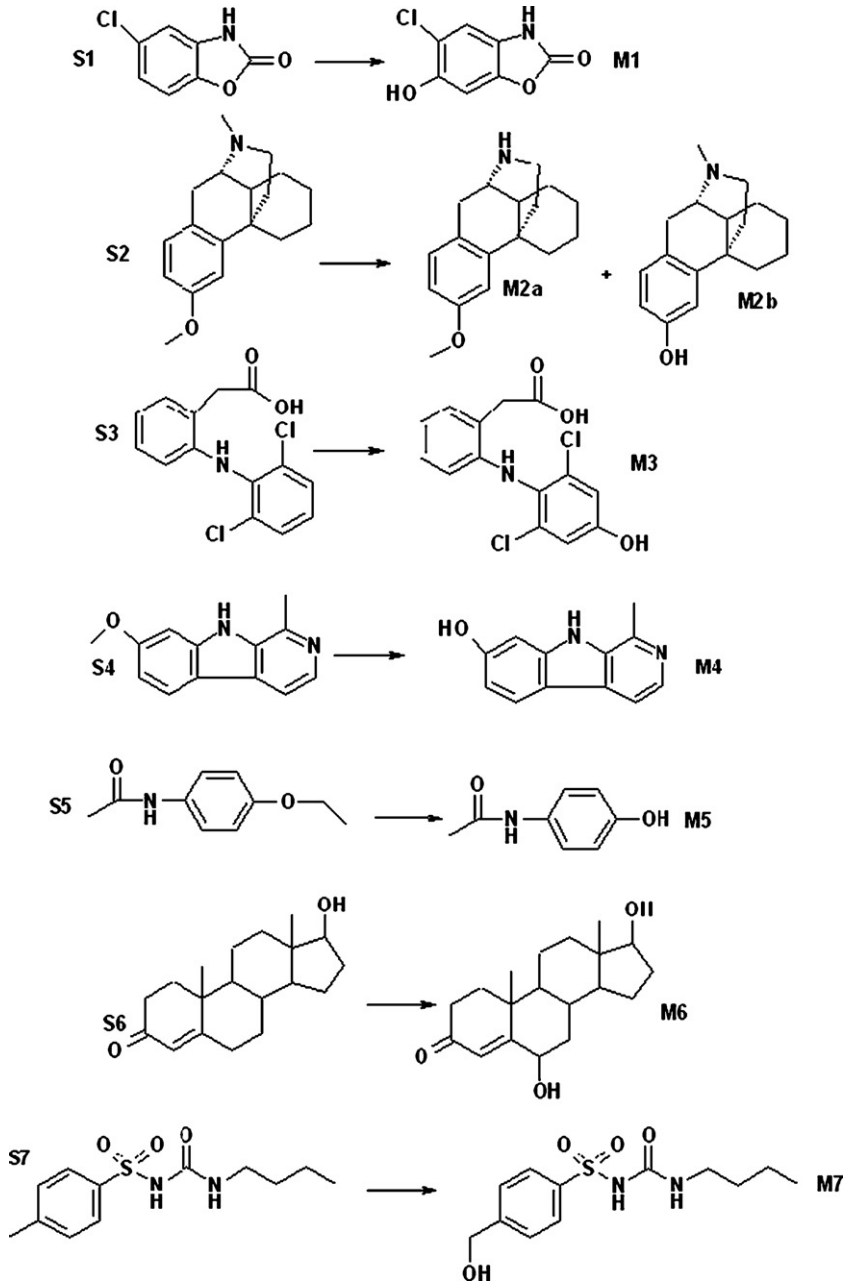
High performance liquid chromatography/mass spectrometry measurement was done on an Agilent 1200 with a UV detector and an Agilent G1956B mass detector in series. For separation, a Merck LiChroCART Purospher RP18 endcapped column with the dimensions 250 mm × 4.6 mm × 5 μm was employed. The mass detector settings were optimized for product detection with a single ion mode (SIM) at the expected *m*/*z* of the product in the preferred ionization mode (determined experimentally with an authentic standard). Additionally a scan with a range from 100 to 1000 *m*/*z* was recorded to monitor substrate depletion and possible by-products.

3. Results and discussion

3.1. Collection and characterization of microorganisms

Fifty-three alkane metabolizing strains were isolated from a mixture of various oil contaminated sources. After enrichment the strains were first characterized for their ability to grow on either *n*-hexane- or cyclohexane-vapor or on a mixture of both as a sole carbon source. All the strains grew on *n*-hexane but 9 of the 53 were not able to use cyclohexane as a sole carbon and energy source for growth (see Table 1). Since alkane utilizing strains usually have a good potential to also hydroxylate substrates different from alkanes, e.g. pyrrolidines and terpenes [23,24], we expected at least some of these strains to show hydroxylation of active pharmaceutical compounds, too.

Genomic DNA was isolated from all strains. 16S rDNA was amplified by PCR and sequenced for a first rough identification of the isolated strains. Sequencing revealed that 12 of the strains were identical to other isolates leaving 41 remaining diverse bacterial strains. The 16S rDNA sequence was assigned to the strain with the highest score using the sequence match search from the Ribosomal Database Project II [25]. The results are shown in Table 1. Of course,



Scheme 1. 7 APIs which were used as screening substrates and the main corresponding human metabolites; S1 chlorzoxazone, M1 6-hydroxychlorzoxazone; S2 dextromethorphane, M2a methoxyporphinan, M2b dextrorphan; S3 diclofenac, M3 4-hydroxydiclofenac; S4 harmine, M4 harmol; S5 phenacetin, M5 paracetamol; S6 testosterone, M6 6-hydroxytestosterone; S7 tolbutamide, M7 4-hydroxytolbutamide.

16S rDNA sequencing only allows a rough classification, especially if the similarity to fully characterized strains is quite low. However, a more detailed characterization of all 41 individual strains was out of scope of this study focusing on new API hydroxylating activities.

According to 16S rDNA sequencing results, all isolates except Z11 (*B. simplex*) belonged to the phylum of proteobacteria and 75% were gamma proteobacteria. The majority of the isolated proteobacteria consists of *Enterobacteriales* (~45% of all strains) and *Pseudomonadales* (~30% of all strains). Even more interesting, none of the characterized strains belonging to an order apart from *Burkholderia*, *Enterobacteriales* and *Pseudomonadales* was able to grow on cyclohexane as a sole carbon source.

3.2. Screening for API conversion

Microbial enzymes mimicking the selectivity of human CYPs could provide robust catalysts for drug metabolite production on gram-scale. Moreover, they could enable large-scale production of APIs for next generation drugs by activation of prodrugs to the final active pharmaceutical intermediate in vitro. This approach circumvents the difficulties caused by different metabolizer phenotypes when relying on the patient metabolism to activate drugs. Our collection of 41 newly isolated alkane metabolizing bacterial strains was screened for the conversion of 7 different APIs (chlorzoxazone, dextromethorphan, diclofenac, harmine, phenacetin, testosterone and tolbutamide, see Scheme 1) and analyzed by HPLC/MS. Table 1 shows the results of the API conversion reactions. With the excep-

tion of strain L3, which was not able to grow on cyclohexane but hydroxylated tolbutamide to 4-hydroxytolbutamide (see Table 1), exclusively strains of the group of cyclohexane metabolizers were able to convert the tested APIs. It came as no surprise that L3, as the only strain active on one of the APIs but unable to utilize cyclohexane for growth, showed activity on tolbutamide, which was one of the least bulky substrates tested and the only tested substrate where terminal hydroxylation of an aliphatic, linear alkane like side chain was observed. Although the yield of 4-hydroxytolbutamide produced by L3 was <1% of the substrate concentration as determined by HPLC–MS, amounts of the hydroxylated metabolite were significantly higher than the limit of quantitation. Under the same applied screening conditions the two cyclohexane metabolizing strains L11 and Z26 converted tolbutamide with yields of 5% and 6%, respectively. The retention times (RT) were 2.84 min for L3 and L11 and 2.86 min for Z26, being consistent with the RT of 2.83 min of an authentic standard of 4-hydroxytolbutamide. Microbial conversion of tolbutamide to 4-hydroxytolbutamide so far has only been reported for the fungus *Cunninghamella blakesleeana* [26] and thus to our knowledge it is the first time that this activity was observed in bacteria. Beyond its use as hypoglycemic drug, tolbutamide is used to test patients for their CYP2C subfamily metabolizer type, which is reflected by the ability to hydroxylate the drug to 4-hydroxytolbutamide [27]. The hydroxylated drug itself has a comparable hypoglycemic effect as tolbutamide but a more than 10-fold shortened serum half-life in human patients [28].

In addition to hydroxylation, API dealkylation is a major reaction in human phase 1 metabolism [29]. We screened our 41 new strains for dealkylation of the 3 APIs, dextromethorphan (O- and N-demethylation), harmine (O-demethylation) and phenacetin (O-deethylation). Dextromethorphan was converted by strains L5, Z3 and Z4. After 24 h 8% of the substrate was converted to the N-demethylated metabolite methoxymorphinan by strain L5. The retention time was 5.77 min. Strain Z3 yielded 11% of methoxymorphinan (RT 5.80 min). Both peaks co-eluted with an authentic reference of methoxymorphinan. The negligible variation of the retention times may be caused by small pH differences of the applied samples resulting in a varying degree of protonation of the amine function which in turn affects retention time. Strain Z4 showed an unidentified metabolite peak at 4.48 min which was not present in the negative control without dextromethorphan. Although no more dextromethorphan was detected after biotransformation the HPLC UV-peak area of the metabolite was only 7% of the peak area of dextromethorphan. The m/z value of the unidentified metabolite was 220 in positive ionization mode. We speculate that this mass refers to a fragment of $[C_{14}H_{21}NO+H]^+$, which could reflect a double demethylated, partially digested metabolite of dextromethorphan ($m/z = 272$, $[C_{18}H_{25}NO+H]^+$). This suggested metabolization of dextromethorphan for growth since no further unidentified peaks appeared. *C. blakesleeana* was reported to demethylate dextromethorphan to dextrorphane (O-demethylation) with a yield of 90% after 120 h [30]. In contrast, for the first time we have identified two bacterial strains performing the N-demethylation reaction of dextromethorphan to methoxymorphinan which is the second major metabolite produced by human CYP2D6. While dextromethorphan so far was mainly used as an antitussive, recent studies indicate that dextromethorphan and its metabolites might also be used as local anesthetics [31] or spinal anesthetics [32], with the metabolites showing strongly increased potencies in the latter case. Harmine was demethylated by L1 and L7, both resulting in metabolites showing a RT of 2.81 min (authentic standard of demethylated harmine: 2.81 min) but yields were very low for both. Harmine and other beta-carbolines are under investigation as anti cancer agents because of their cytotoxicity against various tumor cell lines [33,34].

Phenacetin was deethylated by strains L8, Z18 and Z31. In all those cases conversion to the expected products after 24 h was clearly approved by HPLC/MS-data. However, the product yields were also lower than 1%. The retention times of the metabolites were 2.57 min, 2.58 min and 2.57 min, corresponding to the RT of the authentic standard of paracetamol, which was 2.58 min. *Cunninghamella elegans* was reported to O-deethylate phenacetin and its O-alkyl homologs [35] but again, for the first time we identified a bacterial strain catalyzing this dealkylation reaction. While phenacetin itself is not used any more as an analgetic drug due to its nephrotoxicity, the O-dealkyl metabolite paracetamol (also known as acetaminophen) is one of the most popular and widely used drugs for the treatment of pain and fever [36].

No hydroxylation of chlorzoxazone, diclofenac or testosterone by any of the newly isolated strains was observed. However, testosterone hydroxylation using bacterial CYPs has been studied extensively by Agematu et al. previously and they already succeeded in hydroxylating testosterone at many different positions [37].

In total 11 out of 41 strains converted at least one out of the 7 APIs. All results were obtained employing standard screening conditions and yields can be expected to be significantly higher when optimizing conditions for preparative scale reactions. Disruption of genes of ADHs or Baeyer–Villiger monooxygenases of the alkane metabolization pathways or overexpression of the monooxygenases should help to increase the metabolite yields and will be the logic next step to enable preparative scale reactions.

4. Conclusion

In conclusion, the screening of alkane metabolizing strains for API conversion yielded a surprising high hit rate supporting our initial working hypothesis and cyclohexanone seems to be especially suited for the enrichment of new hydroxylating bacteria showing activity on other substrates, too. Moreover, the results are very encouraging to screen further substrates of interest with the now established collection of alkane metabolizers. Optimization of cultivation and biotransformation conditions and heterologous enzyme expression will reveal the true potential for preparative scale biotransformations using the individual enzyme activities found in this study.

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