JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Production of Natural Methyl Anthranilate by Microbial N-Demethylation of *N*-Methyl Methyl Anthranilate by the Topsoil-Isolated Bacterium *Bacillus megaterium*

MARCUS TAUPP,† DAG HARMSEN,‡ FRANK HECKEL,† AND PETER SCHREIER*,†

Lehrstuhl für Lebensmittelchemie, Universität Würzburg, Am Hubland, 97074 Würzburg, Germany, and Poliklinik für Paradontologie, Universität Münster, Waldeyerstrasse 30, 48149 Münster, Germany

Bacillus megaterium, isolated in a screening process from topsoil, was used for N-demethylation of natural *N*-methyl methyl anthranilate to produce natural methyl anthranilate. Maximal productivity of 70 mg/L/day was achieved under laboratory-scale conditions without further optimization. No byproducts were observed. Thus, production of "natural" methyl anthranilate using *B. megaterium* is a significant improvement over comparable already existing procedures.

KEYWORDS: ***Bacillus megaterium*; bioflavor; biotransformation; methyl anthranilate; N-demethylation; *N*-methyl methyl anthranilate**

INTRODUCTION

Twenty years after the introduction of bioflavors into the flavor market (*1*), the group of so-called "natural" flavor compounds (*2*) has reached a well-defined position. Nowadays, a number of industrially important natural flavor compounds are settled on the market (*3*). The continuing demand by consumers combined with the high economic value of natural flavor substances have led to permanent improvements of biotechnological processes, as well documentation of several "key compounds" such as γ-decalactone or vanillin (*4*, *5*).

Methyl anthranilate is the characteristic flavor compound of Concord grapes and also appears in several essential oils such as neroli and bergamot oils. The substance is also known as a characteristic constituent of wood strawberry (*6*). Methyl anthranilate is widely used in the industry for food flavorings and perfume compositions. A consumption of 27 tons/year by the flavor industry is reported but because of its very low concentrations in plants, no methyl anthranilate "ex plant" is available. A number of procedures to produce "natural" methyl anthranilate have been reported (*7*−*11*). However, their application is limited as the procedures suffer from low yields, long reaction times, and formation of byproducts. In this paper, we report an improvement of the microbiological production of methyl anthranilate using *Bacillus megaterium*.

MATERIALS AND METHODS

Chemicals. *N*-Methyl methyl anthranilate and methyl anthranilate were obtained from Lancaster (Lancashire, U.K.). Natural *N*-methyl methyl anthranilate was purchased from Aldrich (Schnelldorf, Germany). All organic solvents used for extraction were distilled prior use. Chemicals used for the preparation of liquid media were all of highest purity.

Microorganism. *B. megaterium* was isolated from topsoil in a screening process and characterized by 16S rDNA and DNA/DNA hybridization against the *B. megaterium* type strain ATCC 14581.

Isolation of Microorganisms from Topsoil. One gram of topsoil (collected from garden soil in Karlstadt, Germany) was stirred in sterile water for 20 min, and 100 μL of this solution was plated on Standard Methods Agar (Bio Mérieux), containing 10 μL of *N*,*N*-dimethylaniline, and incubated at 30 °C. Nitrogen-adapted microorganisms were singularized and cultivated on Standard Methods Agar plates. In total, 83 unknown cultures were obtained, and the isolated strains were used for substrate acceptability tests by incubation in liquid media.

Substrate Acceptability Test with the Isolated Strains. Liquid minimal medium (75 mL) was prepared according to the method of Dworkin and Foster (*20*). A trace element solution (375 μL) was added, and the culture medium was autoclaved (121 °C, 16 min) (*21*). The culture was maintained under sterile conditions during the addition of 750 μL of a sterile glucose solution (50%). This liquid medium was inoculated with freshly grown (on Standard Methods Agar plates) bacteria, and the culture was pregrown for 18 h at 30 °C and 120 rpm. Then, 100 μmol of *N*,*N*-dimethylaniline as substrate was added under sterile conditions, and the mixture was shaken for a further 18 h at 30 °C and 120 rpm. After incubation, the culture was worked up by sonication of the bacterial broth for 10 min and centrifugation at 15000*g* for 20 min. The supernatant was extracted four times each with 40 mL of diethyl ether; combined organic layers were dried over Na_2SO_4 and filtered, and the organic solvent was evaporated under reduced pressure. The residue was redissolved in 500 μL of dichloromethane and submitted to gas chromatographic−mass spectrometric (GC-MS) analysis to search for N-demethylated products. One strain, N-demethylating *N*,*N*-dimethylaniline, was incubated in further experiments with the substrate *N*-methyl methyl anthranilate.

RIDOM 16S rDNA Sequencing of the Isolated Bacteria. DNA preparation, DNA amplification, and DNA sequencing of the 16S ribosomal RNA gene were performed as previously described by Harmsen et al. (*12*). Briefly, the thermal cycling conditions consisted

* Author to whom correspondence should be addressed [telephone +49-931-888-5481; fax +49-931-888-5484; e-mail schreier@pzlc.uni-wuerzburg.de].
† Lehrstuhl für Lebensmittelchemie.
‡ Poliklinik für Paradontologie.

10.1021/jf0521395 CCC: $30.25
Published on Web 10/29/2005

of an initial denaturation (80 °C, 5 min) followed by 28 cycles of denaturation (94 °C, 45 s), annealing (53 °C, 1 min), and extension (72 °C, 90 s), with a single final extension (72 °C, 10 min). For amplification, the broad-range primers 16S-27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 16S-907r (5′-CCG TCA ATT CMT TTR AGT TT-3′) reported by Lane (*13*) were applied. The PCR product was purified by an enzymatic method using exonuclease I (New England Biolabs GmbH, Frankfurt-Hoechst, Germany) and shrimp alkaline phosphatase (Amersham Biosciences, Freiburg, Germany) (*14*). The amplicons were sequenced using the Prism BigDye Terminator v 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). The sequencing reaction required 0.5 μL of premix from the kit, 1.8 μL of Tris-HCl/$MgCl_2$ buffer (400 mM Tris-HCl; 10 mM $MgCl_2$), 10 pmol of sequencing primer, and 2 μL of the cleaned PCR product in a total volume of 10 μL. For 16S rDNA sequencing either of the primers 16S-27f or 16S-519r (5′-GWA TTA CCG CGG CKG CTG-3′) was used with an annealing temperature of 53 or 60 °C, respectively. All sequencing reactions were performed using a T1 thermocycler (Whatman Biometra, Göttingen, Germany) with 25 cycles of denaturation (96 °C, 10 s), annealing (5 s), and extension (60 °C, 4 min) and a thermal ramping of 1 °C/s. The sequencing products were purified with MultiScreen HV plates (Millipore, Billerica, MA) loaded with Sephadex G50 Superfine columns (Amersham Biosciences) according to the instructions of the manufacturer (Millipore Tech Note TN053), followed by preparation for running onto the ABI Prism 3100 Avant Genetic Analyzer. The region from base positions 54 to 510 (corresponding to *Escherichia coli* 16S rDNA positions) for the 16S rDNA was analyzed using Ridom TraceEdit Pro (version 1.0; Ridom GmbH, Würzburg, Germany) software. Sequences from primer regions were therefore not included in this analysis. Finally, a homology search of the sequence data against the RIDOM database was performed (*15*, *16*).

DNA/DNA Hybridization of the Isolated *B. megaterium* against *B. megaterium* ATCC 14581 Type Strain (DSM 32^T). The isolated strain, identified as *B. megaterium* by 16S rDNA analysis, was subjected additionally to a DNA/DNA hybridization analysis to reassess the correctness of the given species name. *B. megaterium* ATCC 14581 (DSM 32^T) type strain was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Deutschland) and cultivated in liquid media (medium 1, DSMZ) under the same incubation conditions as described above. Briefly, DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite according to the method of Cashion et al. (*17*). DNA/DNA hybridization was carried out as described by De Ley et al. (*18*) considering the modifications described by Huss et al. (*19*), using a Cary Bio UV−vis spectrophotometer equipped with a Peltier-thermostated 6 × 6 multicell changer and a temperature controller with in-situ temperature probe (Varian).

Deposition of the Soil Isolate. The isolated *B. megaterium* strain was deposited in the BCCM/LMG, Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent (RUG), B-9000 Gent, Belgium, under the LMG no. 23147.

Standard Incubation Method. Liquid minimal medium (75 mL) was prepared according to the method of Dworkin and Foster (*20*). A trace element solution (375 μL) was added, and the mixture was autoclaved (121 °C, 16 min) (*21*). The culture was maintained under sterile conditions during the addition of 750 μL of a sterile glucose solution (50%). This liquid medium was inoculated with freshly grown (on Standard Methods Agar plates) bacteria, and the culture was pregrown for 18 h at 30 °C and 120 rpm. Various amounts of *N*-methyl methyl anthranilate (10−100 mg) were added under sterile conditions, and the mixture was shaken for a further 18 h at 30 °C and 120 rpm. Control experiments without bacteria were carried out to verify the stability and authenticity of the starting material.

Variation of Incubation Broth. All media tested were prepared according to the method of Atlas and Parks (*21*). These were, in detail, as follows (g/L): *Bacillus* broth one-fourth strength (yeast extract, 2.5 g; pancreatic digest of casein, 1.0 g); beef extract with NaCl (beef extract, 10.0 g; NaCl, 5.0 g); diaminopimelic acid medium (pancreatic digest of casein, 5.0 g; beef extract, 3.0 g; diaminopimelic acid, 0.050 g); *Bacillus* medium 1 (peptone, 6.0 g; pancreatic digest of casein, 3.0 g; yeast extract, 3.0 g; beef extract, 1.5 g, $MnSO_4 \cdot 4\ H_2O$, 1.0 μg); *Bacillus* medium 2 [$(NH_4)_2HPO_4$, 1.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g, KCl, 0.2 g; yeast extract, 0.2 g]; *Bacillus* medium 3 (peptone, 10.0 g; lactose, 5.0 g; NaCl, 5.0 g; beef extract, 3.0 g; K_2HPO_4, 2.0 g). Finally, one medium was checked that was recommended by the DSMZ for the cultivation of *B. megaterium* (DSMZ medium 1: peptone, 5.0 g; meat extract, 3.0 g); preparation of DMSZ medium 1 was carried out without addition of $MnSO_4 \cdot H_2O$. All media were used in the same volume of 75 mL as described above.

Extraction for Subsequent Analysis of Biotransformation Products. After shaking, the culture was worked up by sonication of the bacterial broth for 10 min and centrifugation at 15000*g* for 20 min. The supernatant was extracted four times each with 40 mL of diethyl ether. The combined organic layers were dried over Na_2SO_4 (sicc) and filtered, and the organic solvent was evaporated under reduced pressure. The residue was redissolved in 2 mL of diethyl ether for subsequent HRGC-MS analysis and quantification using a calibration curve.

High-Resolution Gas Chromatography−Mass Spectrometry (HRGC-MS). HRGC-MS analysis was performed using an Agilent 6890 gas chromatograph (split injector 1:20) coupled to an Agilent 5973 mass selective detector. A J&W DB-Wax fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was employed. The temperature program was 3 min isothermal at 50 °C, then raised at 4 °C/min to 220 °C, using 1.0 mL/min helium constant flow. The MS operating values were as follows: ionization voltage, 70 eV (electron impact ionization); ion source and interface temperatures, 230 °C. Identification was carried out by comparison of mass spectrometrical and retention data of authentic methyl anthranilate with those of the sample.

RESULTS AND DISCUSSION

The used microorganism, isolated in a screening process from topsoil, was identified as *B. megaterium* by standard 16S rDNA analysis and DNA/DNA hybridization against *B. megaterium* ATCC 14581. A direct comparison of the 16S rDNA partial sequences indicated that the isolate belongs to the species *B. megaterium*, showing a similarity of 99.6% for 456 base pairs against the *B. megaterium* type strain (ATCC 14581, DSM 32^T). To complete the assignment of the isolate to the species *B. megaterium*, DNA/DNA hybridization against the *B. megaterium* type strain was conducted. A mean DNA/DNA similarity value of 83.2% was obtained. Therefore, the assignment of the isolate to *B. megaterium* is justified, and the results of the 16S rDNA analysis are supported by this technique.

B. megaterium was shown to be able to N-demethylate *N*-methyl methyl anthranilate to yield methyl anthranilate (**Scheme 1**). Byproducts, as previously reported in the course of fungal demethylation of *N*-methyl methyl anthranilate (*8*), were not observed.

A series of studies was performed to check the biotransformation capacity of *B. megaterium* using different amounts of *N*-methyl methyl anthranilate added to the incubation broth; they varied from 10 to 100 mg/75 mL of incubation medium.

The variation of the substrate concentration led to the following findings (**Figure 1**): Amounts of *N*-methyl methyl anthranilate lower than 20 mg/75 mL did not heighten the yields of methyl anthranilate; this means that a certain concentration of substrate had to be present in the incubation medium to start the microbial demethylation. Higher concentrations of *N*-methyl methyl anthranilate, however, did not further increase the

Scheme 1

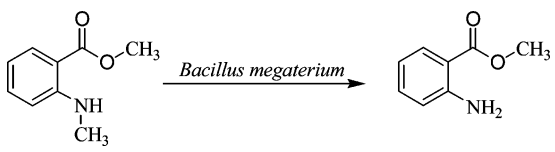

Table 1. Advantages and Disadvantages of Already Reported Biotechnological Processes To Produce Natural Methyl Anthranilate (MA) Compared with the *B. megaterium* Process [*N*-Methyl Methyl Anthranilate (NMMA)]

method	advantages	disadvantages	refs
enzymatic esterification of anthranilic acid	other natural aromatic esters such as methyl cinnamate or methyl salicylate can be obtained	max yields of 10%; methanol used for esterification denatures the enzyme; expensiveness of pure enzymes	*10*
de novo biosynthesis of MA	large amounts of the biocatalysts can be produced at anytime	very low yields and long incubation times; max yield of MA in 5 days = 18.7 mg/L	*7*, *9*
enzymatic N-demethylation of NMMA	short incubation times; up to 135 mg/L MA in 10 min of incubation time by use of pure soybean peroxidase	necessity of relatively pure enzymes; production of byproducts	*11*
microbial N-demethylation of NMMA by fungal microorganisms	large amounts of biocatalysts can be produced at anytime	low yields and long incubation times; production of the byproduct *N*-formyl methyl anthranilate; toxicity of NMMA and MA; max yield of MA after 24 days of incubation = 7 g/L, but production of *N*-formyl methyl anthranilate = 2.8 g/L	*8*, *22*
microbial N-demethylation of NMMA by *B. megaterium*	70 mg/L/day under lab-scale conditions; fast-growing and easy to handle microorganisms; no formation of byproducts; short incubation times	toxicity of NMMA and MA against *B. megaterium*	this work

production of methyl anthranilate; this is caused by the fact that both, substrate and product, exhibit toxic effects toward *B. megaterium*, as proven by measurement of the optical density (data not shown). Therefore, 20 mg/75 mL of *N*-methyl methyl anthranilate was the optimal substrate concentration for methyl anthranilate production under the mentioned laboratory conditions. Extension of the incubation time up to 48 h did not increase the yield of methyl anthranilate.

In addition, different incubation media were tested to investigate the influence of the medium on the N-demethylation activity of *B. megaterium* toward *N*-methyl methyl anthranilate. Among the media under study (see Material and Methods), *Bacillus* broth one-fourth strength, beef extract with NaCl, diaminopimelic acid medium, and DSMZ medium 1 were especially recommended for the growth of *B. megaterium*, whereas the others were media for *Bacillus* species in general (*21*).

As shown in **Figure 2**, remarkable differences were observed using various incubation media. At first glance, the differences in optical density are obvious. *Bacillus* medium 1, *Bacillus* medium 3, and DSMZ medium 1 provided good accommodation for the nutrition of *B. megaterium* witnessed through fast growth. *Bacillus* broth one-fourth strength and beef extract medium with NaCl showed lower growth of bacterial cells. Finally, *Bacillus* medium 2 led to descent of the bacterial cells maybe not having the important growth nutrients necessary for reproduction. In contrast to this, a low growth of bacterial cells resulted in a high amount of methyl anthranilate using *Bacillus* broth one-fourth strength. Interestingly, the highest growth of *B. megaterium* using *Bacillus* medium 1 yielded only 13% product. This clearly indicates not only that the demethylation is dependent on the number of bacterial cells but also that the composition of the incubation medium plays an important role in this process. The *Bacillus* broth one-fourth strength, containing only yeast extract and pancreatic digest of casein, seems to have some necessary content that is responsible for the microbial N-demethylation. Unfortunately, this medium led to the formation of a strong emulsion during solvent extraction. In the end, none of the tested media were able to heighten the yield of MA during the incubation process in comparison to the used defined standard medium (**Figure 1**) containing only various salts and glucose as carbon source. Advantageously, this medium is quite cheap to prepare and shows a well-defined composition, and the solvent extraction is easy to handle.

In comparison to other methods for the biotechnological production of natural methyl anthranilate (**Table 1**), the N-

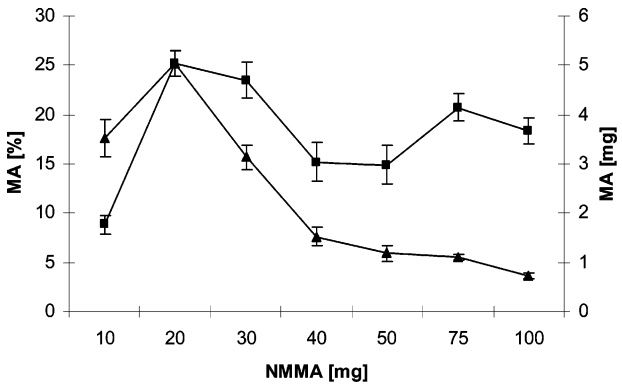

Figure 1. Influence of substrate concentration on yield of N-demethylation of *N*-methyl methyl anthranilate (NMMA) by *B. megaterium*. The highest yield of methyl anthranilate (MA) [■ (mg); ▲ (%)] was reached by addition of 20 mg of substrate to the incubation broth (75 mL), resulting in 5 mg of MA after 18 h.

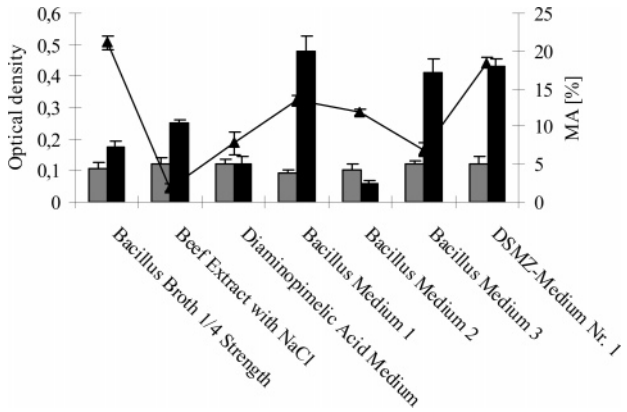

Figure 2. Influence of incubation medium on the production of methyl anthranilate (MA) by *B. megaterium*. Cells were grown to an optical density of 0.05. Optical density was measured 4 h (gray bars) and 24 h (black bars) after addition of 20 mg/75 mL of *N*-methyl methyl anthranilate. Highest yields [▲ (%)] were achieved by using *Bacillus* broth one-fourth strength medium. For incubation media, see Materials and Methods.

demethylation of *N*-methyl methyl anthranilate by *B. megaterium* has some considerable advantages.

Anthranilic acid, used as a natural precursor of methyl anthranilate, has been found in acid hydrolysates of casein and peptone, being cheap byproducts of the food industry. By the use of microbial enzymes in the presence of 10% methanol, anthranilic acid was esterified to methyl anthranilate. However, this enzymatic esterification of anthranilic acid to obtain methyl anthranilate suffers from problems such as low yields and the expensiveness of the biocatalysts. As the used methanol showed denaturation effects of the protein, maximum yields of 10% of MA were obtained. On the other hand, such a procedure allows the production of other important compounds such as methyl cinnamate or methyl salicylate (*10*).

The de novo synthesis of methyl anthranilate by two species of fungi belonging to the Polyporaceae, *Pycnoporus cinnabarinus* and *Poria cocos*, has been reported. Unfortunately, this method is afflicted with long incubation times and low yields. Best results were obtained with *P. cinnabarinus* I-397 at a low nitrogen concentration, the use of maltose as the carbon source, uncontrolled pH, and 1-day-old spores as the source of inoculum; 18.7 mg/L of methyl anthranilate was produced under these conditions after 5 days of cultivation (*7*, *9*).

In addition, different peroxidase preparations were used to demethylate *N*-methyl methyl anthranilate (*11*). One clear-cut benefit is the short incubation time using these enzyme preparations. However, the use of relatively pure enzymes and the lack of reusability of the biocatalyst are counterproductive for largeup-scale processes.

The application of a microbial system for the N-demethylation of *N*-methyl methyl anthranilate has also been reported (*8*). However, this patented N-demethylation process using fungal microorganisms such as *Trametes* and *Polysporus* species is expensive, incubation times are long, the yields are low, and an unwanted byproduct, *N*-formyl methyl anthranilate, is also produced (*22*). Despite maximum yields of 7 g/L/24 days, this process requires an additional time- and cost-expensive preincubation period of 13 days with a subsequent incubation period of a further 11 days. In contrast, the isolated *B. megaterium* is a fast-growing and easy to handle bacterium.

As byproducts in the N-demethylation of *N*-methyl methyl anthranilate by our isolated *B. megaterium* were not detectable and the productivity of 70 mg/L/day is quite acceptable under laboratory-scale conditions without any further optimization, the herewith reported procedure seems to be very promising. Trials to scale-up the process are under development.

LITERATURE CITED

(1) Schreier, P., Ed. *Bioflavour '87*; de Gruyter: Berlin, Germany, 1988.

(2) Council Directive of 22 June 1988 on the approximation of laws of the Member States relating to flavourings for use in foodstuffs and to source materials for their production (88/388/EEC).

(3) Freedonia Group. *Freedom Focus on Flavour and Fragrances, Market Research Report*; Freedonia Group: Cleveland, OH, 2004.

(4) Berger, R. G.; Krings, U.; Zorn, H. Biotechnology solutions for flavour production. In *State-of-the-Art in Flavour Chemistry and Biology*; Hofmann, T., Rothe, M., Schieberle, P., Eds.; DFA: Garching, Germany, 2005; pp 223−233.

(5) Schrader, J.; Etschmann, M. M. W.; Sell, D.; Hilmer, J. M.; Rabenhorst, J. Applied biocatalysis for the synthesis of natural flavour compounds—current industrial processes and future prospects. *Biotechnol. Lett.* **2004**, *26*, 463−472.

(6) VCF 2000. *Volatile Compounds in Food Database*; BACIS: Huizen, The Netherlands, 2000.

(7) Berger, R. G.; Drawert, F.; Hadrich, S. Microbial sources of flavour compounds. In *Bioflavour '87*; Schreier, P., Ed.; de Gruyter: Berlin, Germany, 1988; pp 415−434.

(8) Page, G. V.; Scire, B.; Farbood, M. I. Preparation of 'natural' methyl anthranilate with fungi. WO 88-US2262 1988; CAN 11: 5957; AN 1989:405957.

(9) Gross, B.; Yonnet, G.; Picque, D.; Brunerie, P.; Corrieu, G.; Asther, M. Production of methyl anthranilate by the basidiomycete *Pycnoporus cinnabarinus*. *Appl. Microbiol. Biotechnol.* **1990**, *34*, 387−391.

(10) Kittleson, J. R.; Pantaleone, D. P. Enzymic biphasic process for the synthesis of aromatic ester flavouring agents from the corresponding carboxylic acid and alcohol by esterification mediated by lipase from *Candida cylindracea*. US 94-236612, 1994; CAN: 123:227820; AN:772987.

(11) Van Haandel, M. J.; Saraber, F. C.; Boersma, M. G.; Laane, C.; Fleming, Y.; Weenen, H.; Rietjens, I. M. Characterization of different commercial soybean peroxidase preparations and use of the enzyme for N-demethylation of methyl *N*-methyl anthranilate to produce the food flavor methyl anthranilate. *J. Agric. Food Chem.* **2000**, *48*, 1949−1954.

(12) Harmsen, D.; Dostal, S.; Roth, A.; Niemann, S.; Rothgänger, J.; Sammeth, M.; Albert, J.; Frosch, M.; Richter, E. RIDOM: Comprehensive and public sequence database for identification of *Mycobacterium* species. *BMC Infect. Dis.* **2003**, *3*, 26.

(13) Lane, D. J. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*; Stackebrandt, E., Goodfellow, M., Eds.; Wiley: Chichester, U.K., 1991; pp 115−175.

(14) Dugan, K. A.; Lawrence, H. S.; Hares, D. R.; Fisher, C. L.; Budowle, B. An improved method for post-PCR purification for mtDNA sequence analysis. *J. Forensic Sci.* **2002**, *4*, 811−818.

(15) Harmsen, D.; Rothgänger, J.; Frosch, M.; Albert, J. RIDOM: ribosomal differentiation of medical microorganisms database. *Nucleic Acids Res.* **2002**, *30*, 416−417.

(16) Blackwood, K. S.; Turenne, C. Y.; Harmsen, D.; Kabani, A. M. Reassessment of sequence-based targets for identification of *Bacillus* species. *J. Clin. Microbiol.* **2004**, *42*, 1626−1630.

(17) Cashion, P.; Hodler-Franklin, M. A.; McCully, J.; Franklin, M. A rapid method for base ratio determination of bacterial DNA. *Anal. Biochem.* **1977**, *81*, 461−466.

(18) De Ley, J.; Cattoir, H.; Reynaerts, A. The quantitative measurement of DNA hybridization from renaturation rates. *Eur. J. Biochem.* **1970**, *12*, 133−142.

(19) Huss, V. A. R.; Festl, H.; Schleifer, K. H. Studies in the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst. Appl. Microbiol.* **1983**, *4*, 184−192.

(20) Dworkin, M.; Forster, J. W. Experiments with some microorganisms which utilize ethane and hydrogen. *J. Bacteriol.* **1958**, *75*, 592−603.

(21) Atlas, R. M., Parks, C. L., Eds. *Handbook of Microbiological Media*; CRC Press: Boca Raton, FL, 1993.

(22) Lomascolo, A.; Stentelaire, C.; Asther, M.; Lesage-Meessen, L. Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. *Trends Biotechnol.* **1999**, *17*, 282−289.

Received for review August 31, 2005. Revised manuscript received September 30, 2005. Accepted September 30, 2005.

JF0521395