Metabolic Engineering of the Actinomycete *Amycolatopsis* sp. Strain ATCC 39116 towards Enhanced Production of Natural Vanillin

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ABSTRACT

The Gram-positive bacterium *Amycolatopsis* sp. ATCC 39116 is used for the fermentative production of natural vanillin from ferulic acid on an industrial scale. The strain is known for its outstanding tolerance to this toxic product. In order to improve the productivity of the fermentation process, the strain’s metabolism was engineered for higher final concentrations and molar yields. Degradation of vanillin could be decreased by more than 90% through deletion of the *vdh* gene, which codes for the central vanillin catabolism enzyme, vanillin dehydrogenase. This mutation resulted in improvement of the final concentration of vanillin by more than 2.2 g/liter, with a molar yield of 80.9%. Further improvement was achieved with constitutive expression of the vanillin anabolism genes *ech* and *fcs*, coding for the enzymes feruloyl-coenzyme A (CoA) synthetase (*fcs*) and enoyl-CoA hydratase/aldolase (*ech*). The transcription of both genes was shown to be induced by ferulic acid, which explains the unwanted adaptation phase in the fermentation process before vanillin was efficiently produced by the wild-type cells. Through the constitutive and enhanced expression of the two genes, the adaptation phase was eliminated and a final vanillin concentration of 19.3 g/liter, with a molar yield of 94.9%, was obtained. Moreover, an even higher final vanillin concentration of 22.3 g/liter was achieved, at the expense of a lower molar yield, by using an improved feeding strategy. This is the highest reported vanillin concentration reached in microbial fermentation processes without extraction of the product. Furthermore, the vanillin was produced almost without by-products, with a molar yield that nearly approached the theoretical maximum.

IMPORTANCE

Much effort has been put into optimization of the biotechnological production of natural vanillin. The demand for this compound is growing due to increased consumer concerns regarding chemically produced food additives. Since this compound is toxic to most organisms, it has proven quite difficult to reach high concentrations and molar yields. This study shows that improvements in the final vanillin concentrations and molar yields can be made through a combination of modification of the fermentation parameters and molecular strain engineering, without the need for methods such as continuous extraction from the fermentation broth. Using this approach, we were able to reach a final vanillin concentration of 22.3 g/liter, which is the highest vanillin concentration reported to date that was generated with *Amycolatopsis* sp. ATCC 39116 without additional extraction of the toxic product.

With an annual worldwide demand of more than 16,000 tons, vanillin is one of the most important flavor compounds (1). It is widely used in various applications, such as the production of fragrances, pharmaceuticals, foods, and beverages (2). The vast majority (99%) of the industrially used vanillin is chemically synthesized from lignin and crude oil derivatives, since the extraction of vanillin from the pods of *Vanilla planifolia* is highly cost- and labor-intensive (3). Although chemically synthesized vanillin can be produced at a very attractive price, there is growing interest in the production of natural vanillin by biotechnical approaches. Due to customers’ increasing concerns regarding “natural,” “healthy,” and “bio” products, natural flavors have greater economic value than the chemically produced versions. This has led to extensive research to produce valuable compounds in environmentally friendly processes based on renewable substrates.

In addition to its use as a flavoring agent, vanillin and other structurally related phenolic compounds exhibit antimicrobial and antioxidant properties and therefore are also used in various pharmaceutical applications and as preservatives. Such bioactive functions are dependent on the correct stereochemistry. Biocatalysis is highly stereospecific, regiospecific, and chemospecific, while synthetic production may lead to racemic mixtures and unintended by-products (4, 5). Furthermore, chemical approaches often face the problems of environmentally problematic reaction conditions and the production of hazardous waste materials.

The actinomycete *Amycolatopsis* sp. ATCC 39116 has outstanding tolerance to vanillin and therefore is used industrially for the production of natural vanillin from ferulic acid and other structurally related compounds (6, 7). Ferulic acid is the most abundant hydroxycinnamic acid in the plant world, as it is an important component of the cell walls of higher plants (2, 8). In this case, vanillin can be labeled a natural flavor since it is extracted...
TABLE 1

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amycolatopsis sp. ATCC 39116</td>
<td>Wild type</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>Amycolatopsis sp. ATCC 39116</td>
<td>vdh deletion mutant with replacement of vdh gene by kanamycin resistance gene</td>
<td>This study</td>
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<tr>
<td>Amycolatopsis sp. ATCC 39116</td>
<td>vdh deletion mutant of Amycolatopsis sp. ATCC 39116</td>
<td>This study</td>
</tr>
<tr>
<td>Amycolatopsis sp. ATCC 39116</td>
<td>Δvdh with additional copies of ech and fcs under control of permE* promoter</td>
<td>Invitrogen GmbH (Karlsruhe, Germany)</td>
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<td>E. coli Mach-1 T1</td>
<td>ara-14 leuB6 fluA31 aclV1 tsx78 glnV44 galK2 galT22 mcrA dcm6 hisG4 rfdD1 R(zgb210::Tn10) TetS endA1 rpsL136 dam13::Tn9 ylA5-5 mtl-1 thi-1 mcrB1 hisR2</td>
<td>New England Biolabs Inc. (Ipswich, MA, USA)</td>
</tr>
</tbody>
</table>

Plasmids

| p6apra                        | Apr’ from pMA5096, pAREp, oriV          | 18                            |
| pBlueScript SK (−)            | Ap’, LacPOZ’, T7 and T3 promoters       | Agilent GmbH (Waldbronn, Germany) |
| p6permE                       | p6apra with ermE* promoter             | 18                            |
| pSETGUS                      | Streptomyces integrative vector with gusA under control of tipA promoter | 21                            |
| p6sui                        | Nael/SacII-religated p6apra lacking essential part of pAREp, suicide plasmid | This study                   |
| p6suigusA                    | SpeI/EcoRV fragment of pSETGUS containing gusA cloned into Nhel/DraI sites of p6sui | This study                   |
| p6suigusA::Δvdh               | p6suigusA with flanking region of vdh; suicide plasmid for knockout of vdh | This study                   |
| p6suigusA::Δvdh::permE*:ech*  | p6suigusA::Δvdh with overexpression construct for ech and fcs under control of permE* promoter | This study                   |

from natural sources or “obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal or microbiological origin” (9). Unfortunately, microbially produced vanillin is often rapidly detoxified or degraded to serve as a source of carbon and energy. Thus, much research was done to establish vanillin production pathways in model organisms, like Escherichia coli or Saccharomyces cerevisiae, that are not able to degrade vanillin (1, 10–12). However, those approaches did not lead to an industrial application, because of the bacteria’s insufficient tolerance against the product.

To render microbial production economically attractive, first attempts were made to engineer the metabolism of Amycolatopsis sp. ATCC 39116 (7, 13) toward efficient accumulation of vanillin. The catabolism of ferulic acid in Amycolatopsis sp. ATCC 39116 proceeds via a coenzyme A (CoA)-dependent, non-β-oxidative pathway, similar to those in other ferulic acid-degrading strains. The necessary reactions are catalyzed by the enzymes feruloyl-CoA synthetase (Fcs) and enoyl-CoA hydratase/aldolase (Ech) (2, 6, 7, 14–17). Vanillin is then further converted to vanillic acid by the enzyme vanillin dehydrogenase (Vdh). Deletion of the encoding gene (vdh) in Amycolatopsis sp. ATCC 39116 led to strongly enhanced accumulation of vanillin and >90% reduction of its catabolism in biotransformation experiments (7).

To gain further access to efficient genetic engineering of Amycolatopsis sp. ATCC 39116, new molecular tools were developed (18). The use of suicide plasmids and expression vectors now facilitates genetic modifications of the genome of this promising production strain.

In this article, we report the genetic engineering of Amycolatopsis sp. ATCC 39116 toward more efficient accumulation of vanillin. A markerless vdh deletion mutant was generated and applied in fermentation processes. Moreover, an additional mutant that combined the vdh-negative genotype with efficient overexpression of the genes ech and fcs was constructed. These genes encode the enzymes feruloyl-CoA synthetase (fcs) and enoyl-CoA hydratase/aldolase (ech), which together catalyze the conversion of ferulic acid to vanillin. Both strains were examined for their suitability as new production platforms for natural vanillin and were able to provide major increases in vanillin concentrations and yields. Furthermore, the production process was optimized, aiming at higher product concentrations and molar yields, lower costs, and shorter runtimes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Cells of Escherichia coli were grown in lysogeny broth (LB) at 37°C (19). Cells of Amycolatopsis sp. ATCC 39116 were grown at 45°C in Caso medium (Merck, Darmstadt, Germany). For selection of plasmid-harboring strains, antibiotics were added to the medium as follows: ampicillin, 100 μg/ml for E. coli; apramycin, 50 μg/ml for E. coli and Amycolatopsis; kanamycin, 100 μg/ml for Amycolatopsis. Cell growth was measured by determination of the optical density at 400 nm (Amycolatopsis sp. ATCC 39116) or at 600 nm (E. coli).

DNA isolation and modification. Plasmid DNA was isolated from E. coli using thepeqGOLD plasmid miniprep kit I (PEQLAB GmbH, Erlangen, Germany). The DNA was digested by restriction endonucleases (Thermo Fisher Scientific GmbH, Schwerte, Germany) under the conditions described by the manufacturer. Phusion Hot-Start II high-fidelity
DNA polymerase, T4 DNA ligase, T4 DNA polymerase, and RevertAid reverse transcriptase (Thermo Fisher Scientific GmbH) were used according to the instructions of the manufacturer. Oligonucleotides were purchased from Eurofins MWG Synthesis GmbH (Ebersberg, Germany). DNA fragments were isolated from agarose gels or reaction mixtures using the peqGOLD gel extraction kit (PEQLAB GmbH).

**Transfer of DNA.** Plasmids were transferred into E. coli by employing the CaCl₂ method (19), whereas the transfer of plasmids into *Amycolatopsis* sp. ATCC 39116 was performed by direct transformation of mycelia, as described previously (20).

**Plasmid and mutant construction.** For the generation of a markerless gene deletion mutant of *Amycolatopsis* sp. ATCC 39116, a suicide plasmid was constructed. The previously reported *E. coli*-Amycolatopsis shuttle vector p6apra (18) was digested with Nael/SaclI to eliminate the essential part of the origin of replication for *Amycolatopsis*. The resulting fragment (3,575 bp) still contained oriV for replication in *E. coli*, the apramycin resistance gene, and several unique restriction sites for cloning. The fragment was religated, and *E. coli* was transformed with the resulting plasmid p6usi. Subsequently, the glucuronidase gene *gusA* was isolated as a SpeI/EcORV fragment from pSETGUS (21) and was cloned into the Nhel/Dral-linearized suicide plasmid p6usi, resulting in p6pusiusA.

Precise deletion of the *vdh* gene was accomplished via homologous recombination. The flanking regions upstream and downstream of the *vdh* gene were amplified using the following oligonucleotides: upstream region, *vdh*LF_for3 (5’-TCGTACCTCGGCCGTATCTGTT-3’); and *vdh*LF_rev_RF (5’-GGCGATTCAAGTGGACACGCGCCGGGGGGCG-3’); downstream region, *vdh*RF_for_LF (5’-CGCCCGCGCGCGCCGTGCACCTCTGGATGCGC-3’) and *vdh*RF_rev3 (5’-TGATCCTCTTGGTAGAGGCAGACC-3’). The resulting amplicons were purified and combined in a subsequent fusion PCR using primers *vdh*LF_for3 and *vdh*RF_rev3. The resulting fragment *Δvdh* combined the upstream and downstream regions of *vdh* and was cloned into the EcoRV site of the suicide plasmid p6usiA. After isolation of the resulting plasmid p6usiA::*Δvdh* from *E. coli*, the vector was transferred to *Amycolatopsis* sp. ATCC 39116::kmR. Transformants were selected on solid Caso medium containing 50 μg/ml apramycin. Additionally, the resulting colonies were overlaid with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), as described previously, to detect β-glucuronidase activity encoded by the suicide plasmid p6usiA. Mutants that showed the desired phenotype were first screened for the correct genomic integration of the suicide plasmid by colony PCR using primers *vdh*LF_for_diag (5’-GAAAA GCTGCTGCTAAGACGCGC-3’) and *vdh*RF_rev_diag (5’-GTGGCTTGCTC TGAAGCCAGCCGCGCCGCGCC-3’), which bind upstream and downstream of the recombination regions, respectively. Mutants that showed the correct genotype were then cultivated in liquid Caso medium for a second recombination event. For this, the cells were cultivated without antibiotics for at least 10 passages and were subsequently diluted and plated on Caso agar plates. Single colonies that did not show glucuronidase activity after being overlaid with X-Gluc were picked on agar plates with or without kanamycin. Mutants that had lost kanamycin resistance were finally analyzed via diagnostic PCR with different primer combinations, showing correct deletion of *vdh*. Furthermore, the resulting PCR fragments were analyzed via digestion and sequencing to verify the genotype of *Amycolatopsis* sp. ATCC 39116::*Δvdh*.

**Based on *Amycolatopsis* sp. ATCC 39116::*Δvdh*, another mutant strain, carrying additional copies of the genes *ech* and *fs* under the control of the strong constitutive promoter *permE* (18), was constructed. The genes *ech* and *fs* were amplified together from genomic DNA of *Amycolatopsis* sp. ATCC 39116 by using oligonucleotides *ech_for_SacI* (5’-AAAAAGAGCTC TAAAGGAGGTGACAACTGCTGCCGGCGGCGG-3’) and *fs*rev_Xbal (5’-AAACACACGAGTGAAGTGGCGGCGG-3’). The PCR product was digested with SacI and Xbal and subsequently cloned into SacI/Xbal-linearized pBlueScript SK’. The resulting plasmid pSK::echfs was transferred to *E. coli* Mach-1 T1, and enzyme activity was observed in ferulic acid biotransformation experiments. Next, pSK::echfs was digested with EcoCRI and HindIII, and the resulting fragment, which contained both genes, was ligated with the FspAI/HindIII-linearized expression vector p6permE, colinear to *permE* (18). For integration into the genome of *Amycolatopsis* sp. ATCC 39116::*Δvdh*, a fragment comprising *permE* and the genes *ech* and *fs* was amplified from the resulting plasmid p6permE::echfs by using the oligonucleotides p6apraMCS_for (5’-GGCGAGCGCGGGAGCGGAGCGG-3’) and p6apraMCS_rev (5’-CATTCTACGGGGTGTCACGAC-3’). The blunt PCR product was cloned in the HincII-linearized suicide plasmid p6usiA::*Δvdh* to achieve integration at the *Δvdh* locus. The resulting plasmid was designated p6usiA::*Δvdh*::p6permE::echfs and was transferred to *Amycolatopsis* sp. ATCC 39116::*Δvdh*::kmR. Screening of transformants, generation of the second recombination event, and verification of correct integration of the overexpression construct at the *Δvdh* locus were performed as described above.

**Glucuronidase enzyme assay.** To detect β-glucuronidase activity on agar plates, the colonies were overlaid with 1 ml of 1 mM X-Gluc solution. After a short incubation period, colonies containing hydrolytic β-glucuronidase activity turned blue because of the formation of 5,5′,4,4′-dibromo-4′,4′-dichloro-indigo.

**Fed-batch fermentation on 2-liter scale.** Fed-batch fermentation experiments were carried out in a 2-liter BioStat B Plus fermenter (Sartorius, Göttingen, Germany). Temperature, pH, dissolved oxygen (i.e., partial oxygen pressure [pO₂], and cell density (optical density at 850 nm) were monitored online during the fermentation. The cultivation was performed at 45°C with a fixed aeration rate of 2.0 volumes per minute per volume (vvm) and a constant stirrer speed of 600 rpm. The fermentation broth included 5 g/liter glucose and 5 g/liter yeast extract; 2 g/liter SAG 710 emulsion (Momentive Performance Materials Inc., Albany, NY, USA) was added to the medium as an antifoam agent. The cells were grown until they reached the stationary growth phase, whereupon the biotransformation was started through continuous feeding of ferulic acid (stock solution of 900 ml containing 55 g/liter ferulic acid [adjusted to pH 8.0 with NaOH], 5 g/liter glucose, and 5 g/liter yeast extract). The rate of feeding of ferulic acid was adjusted between 1.9 and 3.5 g/h to ensure an appropriate substrate supply during the biotransformation. The substrate concentrations, as well as the concentrations of intermediates of the catalabolism, were analyzed by high-performance liquid chromatography (HPLC) at the given time points. The fermentation was stopped after 20 h of biotransformation, when synthesis of vanillin had ceased.

**Determination of metabolic intermediates.** Excreted intermediates of the ferulic acid metabolism were analyzed by high-performance liquid chromatography using an Ultimate 3000 HPLC system ( Dionex, Idstein, Germany) without prior extraction. Culture supernatants were obtained by centrifugation (5 min at 16,000 x g) and diluted 1:10 to 1:100 with double-distilled water, if necessary. Intermediates were separated by reverse-phase HPLC using an Acclaim 120 C₄ column (particle size, 5 μm; column size, 250 by 2.1 mm), with a gradient of 0.1% (vol/vol) formic acid (eluant A) and acetonitrile (eluant B) in a range from 15 to 100% (vol/vol) eluant B. The run started with a flow rate of 0.1 ml/min, which was raised to 0.3 ml/min after 16 min, when eluent B reached 100%. For quantification, all intermediates were calibrated with external standards. The compounds were identified by their retention times and their absorption at a specific wavelength (259 nm, 280 nm, 285 nm, or 340 nm), as determined using a multiple-wavelength detector (MWD-3000; Dionex, Idstein, Germany). Data analyses and quantifications were performed with the associated software (Chromelone 6.8 Chromatography Data System; Dionex).

**Vanillin toxicity test.** Cells of *Amycolatopsis* sp. ATCC 39116 were cultivated in 300 ml of Caso medium at 45°C until the culture reached the stationary growth phase. Samples were withdrawn, diluted with sterile saline (0.9% NaCl), and plated on solid Caso medium in triplicate for counting of CFU for determination of the starting cell density (100%). Subsequently, the culture was separated into six equal aliquots of 45 ml. Vanillin (dissolved in dimethyl sulfoxide [DMSO]) was added in concentrations of 2, 4, 7, and 10 g/liter. In control experiments, an equivalent amount of DMSO was added to one of the cultures, while another culture was
monitored without any addition. The cultures were further incubated at 45°C, and samples were taken, diluted, and plated on agar plates after 1, 3, and 6 h. CFU were counted again, and percentages were calculated with respect to the starting cell density.

**RNA isolation and RT-PCR analysis.** For the extraction of total RNA, 1-ml samples of the fermentation broth were withdrawn and cells of wild-type *Amycolatopsis* sp. ATCC 39116 or the mutant strains were harvested by centrifugation (13,000 × g for 20 min at 4°C). The resulting cell pellets were resuspended in 800 μl TE buffer (50 mM Tris, 10 mM EDTA; pH 8.0), and RNA was isolated as described previously (22). Any remaining DNA contaminations were removed with the use of DNase I (Roche, Mannheim, Germany), and RNA was purified as described previously (22). Preparation of cDNA was accomplished by using RevertAid reverse transcriptase (Thermo Fisher Scientific GmbH, Schwerte, Germany) and the oligonucleotides *ech*_RT_for (5′-GAACCCCCACTGAAAG-3′) and *ech*_RT_rev (5′-GAGCTTCAGCCGCACCTC-3′). The final PCR was performed using Phusion Hot-Start II high-fidelity DNA polymerase. The possibility of DNA contamination in the isolated RNA was excluded in a control experiment with the addition of RNA without the prior reverse transcription (RT) step.

**RESULTS**

**Construction and characterization of vdh deletion mutant of *Amycolatopsis* sp. ATCC 39116.** The vanillin dehydrogenase from *Amycolatopsis* sp. ATCC 39116 (VDH<sub>ATCC 39116</sub>) has a substantial impact on the catabolism of vanillin in *Amycolatopsis* sp. ATCC 39116 and therefore is a promising target for engineering of the strain’s metabolism toward efficient accumulation. After replacing the encoding gene vdh with a kanamycin resistance cassette (7), we intended to generate a markerless deletion mutant as a new production strain. For this, a suicide plasmid that allowed the introduction of a suitable deletion construct and the screening of resulting heterogenotes was constructed. The origin of replication for *Amycolatopsis* in the shuttle vector p6apra (18) was removed by digestion, yielding the nonreplicating plasmid p6suigus. The suicide plasmid still contained the origin of replication for *E. coli*, the apramycin resistance gene, and several unique restriction sites for cloning of deletion constructs. To obtain the capability for phenotypic screening of homogenotes, the *gusA* gene of pSETGUS (21) was inserted into p6suigus. The *gusA* gene encodes β-glucuronidase, the activity of which can be easily identified on agar plates due to the strong blue color of the colonies after they are overlaid with X-Gluc. Homogenotes, which had lost the vector backbone, exhibited no color.

The plasmid for deletion of the vdh gene was successfully constructed and transferred to *Amycolatopsis* sp. ATCC 39116 Δvdh::kmR. After transformation, apramycin-resistant clones that exhibited glucuronidase activity could be observed. Unfortunately, we also detected transformants after transfer of p6suigusA without the deletion construct in a control experiment; however, significantly more recombination events were observed when p6suigusA::Δvdh was transformed.

Although a large number of clones in which illegitimate recombination events had occurred were found, we were able to find a clone in which the vector was integrated at the desired location. This clone was used for successful generation of the markerless deletion mutant, as described above. The deletion was confirmed by sequencing and restriction digestion.

After demonstration of a beneficial impact of the vdh deletion on the accumulation of vanillin by *Amycolatopsis* sp. ATCC 39116 in biotransformation experiments (7), the new markerless mutant *Amycolatopsis* sp. ATCC 39116 Δvdh was analyzed during fermentation.

Therefore, strain F33 was compared to the wild type to analyze the influence of VDH<sub>ATCC 39116</sub> on the catabolism of the desired product. The fermentation was performed on the 2-liter bioreactor scale, in triplicate. The initial volume of the starting culture was 1,000 ml. In contrast to previous studies (6) (J. Rabenhorst and R. Hopp, European patent application EP0761817), a very simple fermentation broth, consisting of 5 g/liter glucose and 5 g/liter yeast extract, was used. Since a significant effect of malt extract was not observable in previous growth experiments, malt extract was omitted from the basal medium, to reduce costs and to simplify the process. The fermentation was conducted in two stages. First, the cells were grown until they reached the stationary growth phase, which was obvious due to a significant increase in pO₂. Next, the biotransformation of ferulic acid to vanillin was started with continuous feeding of the substrate. The feeding rate was adjusted according to the concentration of ferulic acid in the medium, which was analyzed by HPLC at intervals of 1 h. Due to the toxic properties of phenolic compounds, we intended to keep the substrate concentration beneath 5 g/liter but not less than 1 g/liter, to ensure an appropriate supply for the cells. Glucose and yeast extract were added to the feeding solution since the supplementation was shown to positively influence the biotransformation in previous test fermentations.

The catabolism of ferulic acid and subsequent accumulation of vanillin started after an initial phase of adaption by the wild-type and F33 cells (Fig. 1B). During the first 2 h, the ferulic acid concentrations increased due to a continuous supply. After the cells had adapted to the biotransformation conditions, the substrate concentrations decreased in both fermentations until they were kept constant between 1 and 3 g/liter through adjustment of the feeding rate. After 16 h, the feeding was stopped and the substrate concentrations decreased to approximately 1.7 g/liter in the wild-type and mutant fermentations. The ferulic acid catabolism was not affected by the vdh deletion, as the wild type and the F33 mutant showed comparable behaviors (Fig. 1A). However, significant differences were observed for the accumulation of the desired product vanillin and the unintended by-product vanillic acid. The synthesis of vanillin by strain F33 was remarkably increased and resulted in a final concentration of 14.4 ± 1 g/liter after 20 h of biotransformation. In comparison, the wild-type *Amycolatopsis* sp. ATCC 39116 formed only 12.2 ± 2.3 g/liter vanillin in the same time. A further increase of the vanillin concentration was not observed in either fermentation, although ferulic acid was still available for the cells (Fig. 1A). Therefore, the fermentations were stopped after 29 h.

Concerning the catabolism of vanillin, strain F33 showed dramatically decreased formation of vanillic acid (final concentration of 0.26 ± 0.1 g/liter), while the wild type catalyzed vanillin continuously, resulting in 2.6 ± 1.7 g/liter vanillic acid at the end of the fermentation. Other intermediates of the catabolism, such as guaiacol or protocatechuic acid, and other by-products were detected in only trace amounts.

A total amount of 38.5 g ferulic acid was fed in both fermentations. With respect to the final vanillin concentration, strain F33 accumulated the desired product with a total molar yield of 87.8% ± 3.5%, while the wild type formed vanillin with a yield of only 73.9% ± 9.5% (Table 2).

**RT-PCR analysis of ech expression.** The fermentation experiments indicated inefficient ferulic acid degradation and vanillin accumulation by *Amycolatopsis* sp. ATCC 39116 and the mutant
F33 before the cells adapted to the feeding of ferulic acid within the first hours of the biotransformation phase (Fig. 1). RT-PCR analyses were conducted to determine whether this inducing effect occurred at the gene expression level. For this, samples of the fermentation broth from the F33 and wild-type experiments were taken (i) at the end of the growth phase, prior to the substrate feeding, and (ii) after 7 h of biotransformation, after the rate of vanillin synthesis had reached its maximum. The cells were harvested by centrifugation, and RNA was isolated as described in Materials and Methods. The enzymes feruloyl-CoA synthetase (Fcs) and enoyl-CoA hydratase/aldolase (Ech), which are responsible for the catabolism of ferulic acid to vanillin in *Amycolatopsis* sp. ATCC 39116 (17), are encoded by the genes *fcs* and *ech*, respectively. The open reading frames of the two genes are adjacent in the genome of *Amycolatopsis* sp. ATCC 39116, and the start codon of *fcs* (GTG) overlaps the stop codon (TGA) of *ech*. Along with the functions of the encoded enzymes, this genetic organization indicates that the two genes are cotranscribed under the control of the same promoter. To provide evidence that gene expression is induced after supplementation of ferulic acid, the transcription of *ech* was analyzed through RT-PCR. Therefore, an internal 529-bp fragment was amplified after preparation of cDNA by RT.

The RT-PCR analysis revealed a clearly visible band after supplementation of ferulic acid (Fig. 2, lane 3), whereas only a faint band could be detected before supplementation (Fig. 2, lane 1). The possibility of DNA contamination of the isolated RNA could be excluded in a control PCR experiment using RNA as the template without the previous RT reaction (Fig. 2, lanes 2 and 4). Additional RT-PCR analysis revealed that *ech* and *fcs* are transcribed as an operon in a single mRNA, since an overlapping fragment from the two genes could be amplified (data not shown).

**Construction and application of overexpression mutant of *Amycolatopsis* sp. ATCC 39116Δvdh::kmR.** To improve further the conversion of ferulic acid to vanillin, an *ech* and *fcs* overexpression mutant was generated. For this purpose, additional copies of

![FIG 1](https://example.com/image)  
**FIG 1** Biotransformation of ferulic acid by *Amycolatopsis* sp. ATCC 39116 and the related mutants F33 and F84. Cells were grown in 1,000 ml of fermentation broth in a 2-liter bioreactor at 45°C until they reached the stationary growth phase. Feeding with ferulic acid solution was then started. Biotransformation of ferulic acid (A) to vanillin (B), vanillic acid (C), and vanillyl alcohol (D) was observed at the given times using HPLC analyses, as described in Materials and Methods. Gray curves, total amounts of ferulic acid fed; black curves, concentrations of the intermediates in the fermentation broth. Triangles, *Amycolatopsis* sp. ATCC 39116; circles, mutant F33; diamonds, mutant F84.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total amount of ferulic acid supplied (g)</th>
<th>Amount of ferulic acid catabolized (g)</th>
<th>Final concentration of vanillin (g/liter)</th>
<th>Total amount of vanillin produced (g)</th>
<th>Molar yield (%)</th>
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<tr>
<td>F84</td>
<td>49.5</td>
<td>48.7 ± 0.2</td>
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<td>94.9 ± 0.4</td>
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<td>F33</td>
<td>38.5</td>
<td>35.5 ± 0.6</td>
<td>14.4 ± 1</td>
<td>24.4 ± 1.4</td>
<td>80.9 ± 4.6</td>
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<tr>
<td>ATCC 39116</td>
<td>38.5</td>
<td>35.7 ± 0.8</td>
<td>12.2 ± 2.3</td>
<td>20.7 ± 3.1</td>
<td>68.6 ± 10.4</td>
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* Yield of ferulic acid to vanillin.
expression vector p6permE (18), colinear to mid p6suigusA::vdh sette led to negative polar effects. Furthermore, the suicide plasmid p6suigusA::vdh could be used for targeted integration via homologous recombination.

First, the two genes were amplified together from the genome of Amycolatopsis sp. ATCC 39116 and cloned in the Amycolatopsis expression vector p6permE (18), colinear to permE*. In a second step, the genes were amplified together with the promoter, and the resulting fragment was cloned blunt in the vdh deletion construct p6suigusA::Δvdh. The resulting plasmid p6suigusA::Δvdh::ech::fcs was used to transform strain F33. Although apramycin-resistant clones were obtained, we faced the same problem of illegitimate integration of the suicide plasmid as mentioned above. Because of this, the integration of the suicide plasmid at the vdh locus was analyzed by amplification of the genomic area first. Correct integration of a 15.1-kb fragment that comprised the flanking regions of vdh and the suicide plasmid could be identified. After verification of the desired homologous recombination, the clone was further cultivated to achieve a second recombination event, as described for the vdh deletion. Accurate integration of the overexpression construct could be achieved in one mutant and was confirmed by diagnostic PCR (data not shown) and also by sequencing of the PCR product.

To characterize the mutant’s phenotype, strain F84 also was cultivated in three identical fermentation experiments on the 2-liter scale. In order to analyze the impact of the overexpression of ech and fcs on the catabolism of ferulic acid and the accumulation of vanillin, the wild-type Amycolatopsis sp. ATCC 39116 and the generated mutants F33 and F84 were cultivated in parallel experiments under the same conditions as described above. The effect of the enhanced expression of ech and fcs was obvious directly after the biotransformation phase was started with feeding of the ferulic acid solution (Fig. 1A). While the wild type and strain F33 exhibited a lag phase of about 3 h before the catabolism of ferulic acid increased and reached its maximum, conversion of ferulic acid to vanillin started in strain F84 directly after supplementation, with improved performance. Ferulic acid was fed at a significantly higher rate (up to 3.7 g/h), in comparison to the wild-type and F33 fermentations (up to 2.4 g/h), but levels started to decrease after 7 h. The higher feeding rate led to a total of 11 g more substrate, which could be fed in a shorter period of only 14 h (Fig. 1A).

The enhancing effect was also evident for the accumulation of vanillin, which was synthesized by strain F84 without the previously observed adaptation phase in the first hours (Fig. 1B). This resulted in a maximal production rate of 2.1 g/liter/h and a vanillin concentration of 8.4 g/liter after only 7 h of the biotransformation phase (wild type, 4.2 g/liter). As expected, the increased metabolism of ferulic acid to vanillin led to a higher final concentration of 19.3 g/liter, with a total molar vanillin yield of 94.9% with respect to the added substrate (Table 2). Concerning the ferulic acid consumed, the molar yield was even higher (96.4%), given that 0.4 g/liter of the substrate remained in the medium at the end, when no further synthesis of vanillin was occurring. Concerning the catabolism of vanillin, the new mutant F84 showed behavior comparable to that of strain F33, with slightly higher concentrations of vanillic acid and vanillyl alcohol (Fig. 1C and D).

**Vanillin toxicity test.** The fermentation experiments revealed an unknown limitation for the production of vanillin, although ferulic acid was still available for the cells (Fig. 1A). It is known that vanillin and other phenolic compounds exhibit antimicrobial properties (23). Although Amycolatopsis sp. ATCC 39116 is known to tolerate higher concentrations of vanillin than all other tested organisms, the fermentation parameters, which were recorded, clearly indicated that the cell metabolism is affected also in this bacterium by increasing concentrations of the desired product. The consumption of dissolved oxygen and the cell density were evidently decreasing when the process was approaching the final stage.

To elucidate the effect of vanillin on the viability of Amycolatopsis sp. ATCC 39116, the cells were cultivated in shake flasks until they reached the stationary growth phase, as in the fermentation experiments. Subsequently, six aliquots were prepared from this culture, and different concentrations of vanillin in the range of 2 g/liter to 10 g/liter were added. Samples were then withdrawn from the cultures at the given time points, diluted, and plated on agar plates for counting of CFU. The results represent the means of four independent replicates. The experiments clearly showed the antimicrobial effect of vanillin (Fig. 3). While a concentration of up to 4 g/liter was tolerated by the cells after an initial decrease of the viable cell concentration, 7 g/liter led to complete cell death within 6 h. Moreover, a vanillin concentration of 10 g/liter led to 90% cell death within only 1 h; no viable cells could be detected after only 3 h of incubation. Since a higher vanillin concentration could be detected in fermentation experiments, whether an adaption of the cells to the product might result in better tolerance was also tested. For this, we first added 2 g/liter vanillin to a culture, as this was shown to be tolerated by the cells in the initial experiment (Fig. 3). Subsequently, the concentration was increased to 10 g/liter after 1 or 3 h of cultivation. The results of this experiment showed that there was no adaption of the cells.
that often occur in actinomycetes (27), only 1% of the analyzed mutants showed a correct homologous recombination at the desired locus in the genome. Because integration events for the basic vector p6sui, lacking the gusA gene, were very infrequent, we assume recombination events between this gene and the genome of Amycolatopsis sp. ATCC 39116. Nevertheless, we employed this system for the phenotypic identification of heterogenotes and homogenotes, which considerably simplified the screening of potential mutants, as described previously for other actinomycetes (28). Finally, we were able to apply this system to generate a knockout of the targeted gene without introduction of heterologous DNA. Such a gene deletion system was hitherto missing and now can be used universally for the introduction of knockouts in Amycolatopsis sp. ATCC 39116, although it should be optimized in future studies for higher specificity and performance.

The gene deletion could be verified by diagnostic PCR, as well as by sequencing of the genomic locus (data not shown). Based on our knowledge of previous investigations, we examined the behavior of the new mutant strain Amycolatopsis sp. ATCC 39116 Δvdh (strain F33) regarding the accumulation and subsequent catabolism of vanillin in comparison to the wild-type strain, which has been used industrially until now.

The fermentation experiments confirmed the observations that we made during the previous biotransformation experiments (7). The catabolism of vanillin in mutant F33 was decreased by nearly 90%. While Amycolatopsis sp. ATCC 39116 accumulated vanillic acid up to a concentration of 2.6 ± 1.7 g/liter due to the continuous degradation of vanillin, the vdh deletion mutant showed only residual catabolism, which led to a vanillic acid concentration of 0.26 ± 0.1 g/liter (Fig. 1C). Further metabolic engineering might even improve this effect, since aldehyde dehydrogenase or oxidase activities seemed to be still present in Amycolatopsis sp. ATCC 39116 Δvdh. Similar observations were made for other production strains in which a comparable deletion had been introduced (14, 29, 30). However, it is evident that the knockout of vdh was the crucial step for consistent metabolic engineering to stop or at least to decrease the degradation of the desired product.

In accordance with these observations, vanillin was accumulated directly, and with an enhanced rate, by strain F33 (Fig. 1B). While vanillic acid is the first overflow product of ferulic acid catabolism in Amycolatopsis sp. ATCC 39116, the vdh deletion mutant F33 accumulates vanillin directly, without the initial synthesis of vanillic acid. This relationship between initial vanillic acid synthesis and subsequent vanillin accumulation was also apparent for the wild-type strain in other studies, in which the strain was used in fermentation experiments or in flask experiments in which vanillic acid was shown to enhance the synthesis of vanillin (6, 25). As expected, the reduced catabolism resulted in a significantly higher final concentration, with an even higher molar yield of vanillin. Strain F33 accumulated vanillin at a maximal rate of 1.9 g/liter/h (wild type, maximal rate of 1.5 g/liter/h). The enhanced synthesis led to a final concentration of 14.4 ± 1 g/liter (Table 2), which is an improvement of more than 2 g/liter in comparison with the wild type (12.2 ± 2.3 g/liter) (Table 2). Moreover, strain F33 metabolized ferulic acid to vanillin with a molar yield of 80.9% ± 4.6%, compared to 73.9% ± 9.5% for the wild type (Table 2). Given that we used a simplified fermentation broth without extraction of the product from the medium, this is
a very promising result for the implementation of this strain as a new production platform.

As shown previously (7), no influence of the knockout on the catabolism of ferulic acid was observed, as strain F33 and the wild type catabolized the substrate in comparable ways (Fig. 1A). However, both strains showed a phase of adaptation in the first 3 h, before efficient catabolism of ferulic acid occurred. Because we assumed the induction of gene expression by ferulic acid, RT-PCR analyses were carried out; the analyses clearly indicated that the expression of ech was induced after ferulic acid supplementation, as noted previously for other genes of the related catabolism (Fig. 2) (7, 29, 31). To overcome this effect and to optimize the metabolic network of Amycolatopsis sp. ATCC 39116 Δvhdh, additional copies of the ech and fcs genes were integrated into the strain’s genome under the control of the constitutive promoter permE.*. The genes encode the vanillin-anabolism-related enzymes feruloyl-CoA synthetase (fcs) and enoyl-CoA hydratase/aldolase (ech) (7, 17).

The overexpression construct was integrated at the former vdh locus by employing the same strategy, with the use of p6saurusA, as mentioned above. Although we faced the problem of illegitimate integration events involving the suicide plasmid, we finally generated the desired mutant, with integration of the permE::ech::fcs fragment in the genome. The genomic organization was verified by diagnostic PCR (data not shown) and sequencing of the amplification product obtained.

The new mutant Amycolatopsis sp. ATCC 39116 Δvhdh::permE::ech::fcs (strain F84) was then tested under the same fermentation conditions as strain F33 and the wild type. In comparison with the other two strains, the catabolism of ferulic acid by strain F84 was strongly enhanced, which was obvious because a larger amount of ferulic acid could be fed in a shorter time and still be nearly completely metabolized (Fig. 1A). Therefore, a greater amount of substrate could be fed before the catabolism of ferulic acid ceased at the end of the fermentation.

Furthermore, the constitutive expression of ech and fcs resulted in substantially enhanced synthesis of vanillin, especially during the first 8 h of the biotransformation phase. The previously observed adaptation phase of the wild type could be completely overcome, as 1.9 g/liter of vanillin was synthesized during the first 3 h of fermentation (Fig. 1B). The RNA analysis seems to support this, as the band corresponding to ech (Fig. 2, lane 3) was much more visible after the feeding began. However, we cannot completely rule out other explanations, such as increased stability of the mRNA during the production phase or increases in mRNA-degrading activities during growth. The total yield of the desired product could be increased to 19.3 g/liter, with a total molar vanillin yield of 94.9% with respect to added substrate. Given that 0.42 ± 0.1 g/liter of ferulic acid remained in the medium at the end of the fermentation, the strain metabolized the substrate with a molar yield of 96.4%, which nearly approaches the theoretical maximum.

Strain F84 showed increased accumulation of vanillic acid and vanillyl alcohol in comparison with strain F33, although both strains do not express VDHATCC 39116 due to the deletion of the corresponding gene (Fig. 1C). This is most likely correlated with the increased vanillin concentrations. This is obvious in the case of vanillyl alcohol, since the concentration remains about 8 to 10 mol% of the vanillin concentration at all times. For vanillic acid, the reason might also be found in the higher vanillin concentration, although the molar percentage in regard to vanillin was also higher (i.e., 1.6 mol% for strain F33 versus 3.8 mol% for strain F84). Another possible explanation could be that the increased supply of feruloyl-CoA enables or enhances a vanillin-independent competing pathway in Amycolatopsis that leads to the formation of vanillic acid. This pathway was shown to exist in Agrobacterium fabrum and was postulated for Amycolatopsis (32). In this coenzyme A-dependent β-oxidative deacetylation pathway, ferulic acid is first activated by Fcs and then further processed by a homologue of Ech. In contrast to the non-β-oxidative pathway that yields vanillin in Amycolatopsis sp. ATCC 39116, the common intermediate 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA is further converted via vanillyl-CoA to vanillic acid, without the formation of vanillin.

The fermentation data presented constitute the best overall productivity values for all strains shown, with respect to the final vanillin concentrations, the molar product yields, and the runtimes of the whole fermentation. By increasing the feeding rate, a shorter runtime could be achieved, yielding comparable final concentrations but lower molar product yields, considering the amount of ferulic acid that was fed. On the other hand, higher molar yields of up to 96.4% could be reached using strain F84 if the desired final vanillin concentration and therefore the amounts of supplied ferulic acid were decreased. Furthermore, we were able to produce 22.3 g/liter of vanillin if ferulic acid was supplied at a rate of more than 5.5 g/liter (Fig. 4). Unfortunately, this method of processing led to lower yields, because of the relatively large amount of ferulic acid that remained in the medium at the end of the fermentation.

In conclusion, the synthesis of vanillin in this kind of fermentation seems to be limited by the antimicrobial properties of the product, although higher final concentrations can be achieved, at the expense of lower total yields, with variation of some process parameters. The vanillin toxicity tests clearly showed the antibacterial effect of vanillin on the cells (Fig. 3). Although the cells seem to tolerate vanillin concentrations higher than 10 g/liter in the fermentation processes, the catabolism of ferulic acid stops at the end of the cultivation and the fermentation parameters clearly indicate that the cell metabolism collapses. A possible explanation for these differing observations might be that vanillin itself does not induce its own detoxification in Amycolatopsis but another metabolite is responsible for this. Ferulic acid might be a feasible candidate, since it also strongly induces the activity of ech and fcs. The activity of the vanillin-dehydrogenase-encoding gene vdh was shown to be induced by vanillin as well as ferulic acid, however (7). It could also be possible that, even with the induction of the cells, the concentration increase up to 10 g/liter was too drastic. During typical fermentations, this concentration is reached only after 9 h or more.

Although the cells tolerate higher vanillin concentrations during the fermentation, the toxicity of vanillin is most likely the reason for the decreasing production rates in the later stages of the process. Since the activation of ferulic acid is ATP and CoA dependent, further product formation might be limited by the availability of cofactors. This is supported by the finding that glucose supplementation of the feeding solution, in order to provide an additional source of carbon and energy, led to higher final concentrations and enhanced rates of catabolism.

Phenolic compounds, such as vanillin, have been shown to affect or even inhibit DNA, RNA, and protein synthesis, glucose uptake, pH homeostasis, and ion gradients, as they most likely
target the cytoplasmic membrane due to their hydrophobic nature (23, 33). Moreover, the aldehyde group has been shown to enhance these toxic effects, since it shows high reactivity and can form covalent bonds with DNA and proteins (34, 35). Therefore, increased vanillin formation is thought to be limited by the viability of the cells. Although the enzymes might still be active at the end of the fermentation, further catabolism could be hampered due to the unavailability of cofactors. The extraction of vanillin in order to maintain a nontoxic concentration was shown to enhance the overall yield of a production process (36) and might present an opportunity to overcome this problem. However, this kind of processing is associated with higher costs and more-complex equipment, which should be avoided on the industrial scale.

Future studies may focus on further engineering of the strain’s metabolism to direct the flow of intermediates to vanillin and to prevent the loss of substrate through competing or catabolic pathways. Further knockouts and directed expression of anabolism-related genes may lead to even higher yields of vanillin, while the discovery of a resistance mechanism against phenolic compounds might enhance the final concentrations. In this study, we clearly showed that consistent metabolic engineering of this high-value vanillin-producing strain can lead to even greater productivity, which renders the microbial production of natural vanillin much more economically attractive for the industry. To our knowledge, the final results that we achieved with the use of strain F84 represent the highest vanillin concentration, combined with the highest molar yield, that has been observed without extraction of the product from the fermentation broth.

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