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ORIGINAL PAPER

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Towards a high-yield bioconversion of ferulic acid to vanillin

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Abstract Natural vanillin is of high interest in the flavor market. Microbial routes to vanillin have so far not been economical as the medium concentrations achieved have been well below 1 g l^{-1}. We have now screened microbial isolates from nature and known strains for their ability to convert eugenol or ferulic acid into vanillin. Ferulic acid, in contrast to the rather toxic eugenol, was found to be an excellent precursor for the conversion to vanillin, as doses of several g l^{-1} could be fed. One of the isolated microbes, later identified as *Pseudomonas putida*, very efficiently converted ferulic acid to vanillic acid. As vanillin was oxidized faster than ferulic acid, accumulation of vanillin as an intermediate was not observed. A completely different metabolic flux was observed with *Streptomyces setonii*. During the metabolism of ferulic acid, this strain accumulated vanillic acid only to a level of around 200 mg l^{-1} and then started to accumulate vanillin as the principal metabolic overflow product. In shake-flask experiments, vanillin concentrations of up to 6.4 g l^{-1} were achieved with a molar yield of 68%. This high level now forms the basis for an economical microbial production of vanillin that can be used for flavoring purposes.

Introduction

The trend and demand for natural and healthy food in the past years has also had an impact on the flavor-producing industries, as the majority of today's flavors need to be natural. Chemically synthesized flavor chemicals under current US and European legislation are artificial or nature-identical and can thus not be used for the creation of natural flavors. Therefore physical processes such as extractions and distillations are used to yield the natural flavor building blocks. Very often key flavor chemicals cannot be obtained from nature via these routes at reasonable prices. As an alternative, fermentation and enzymatic reactions can be employed for the production of so-called natural aroma chemicals (Krings and Berger 1998). A good example is γ-decalactone, a peach-smelling ingredient, which is produced only in traces within the fruit. Starting from natural ethyl decanoate, γ-decalactone is produced by a microbial conversion (Häusler and Münch 1997). Such fermentatively produced flavor compounds are, in most cases, more expensive than the synthetic version.

A similar price situation holds for vanillin, the characteristic aroma component of vanilla pod extract. Starting from the waste product kraft lignin, vanillin can be obtained in a 10% yield (Fragues et al. 1996) with a market price of below U.S. $15/kg. In comparison, natural vanillin from vanilla beans has a market price 300 times higher than the synthetic one. The high market price of natural vanillin thus explains the recent research activities in this field (Gasson et al. 1998; Koseki et al. 1996; Falconnier et al. 1994; Casey and Dobb 1992) and the different patents taken by various flavor companies to produce vanillin by biotechnological means (Barraclough et al. 1994; Markus et al. 1992; Steinbüchel et al. 1998). It is interesting to note that the first patent claiming microbiologically produced vanillin dates back to 1990 (Rabenhorst and Hopp 1990). The inventors supplemented the cultures with 0.2 g l^{-1} eugenol to obtain 18 mg l^{-1} vanillin after a cultivation period of 13 days. The low concentration achieved reflects the toxic effect of eugenol, as only 0.2 g l^{-1} eugenol was fed. Therefore different precursors have been suggested, such as ferulic acid, curcumin or siam benzoin resin (Benz and Muheim 1996). Both eugenol and ferulic acid have been reported to be degraded via vanillin (Toms and Wood 1970; Rahouti et al. 1989; Tadasa 1977). However, the direct formation of vanillin has seldom been observed. Instead, vanillin was found to inhibit cell metabolism and thus, at low concentrations, most

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microbes either quickly oxidized or reduced the compound. On the other hand, basidiomycetes are known to be more tolerant to aromatic aldehydes, probably because of their ability to degrade wood compounds, and they have thus been used to reduce vanillic acid to vanillin. Vanillic acid can easily be obtained from ferulic acid (Barghini et al. 1998), and a two-step conversion resulted in 237 mg l^{-1} vanillin. In more recent studies, adsorbing the toxic vanillin to hydrophobic resins such as Amberlite XAD-2 was tried, resulting in a minor production increase (Asther et al. 1998). At present, however, the yields reported are far too low for any commercial production.

In this study we have screened different microorganisms for their ability to accumulate vanillin. This was done by isolating strains from nature and studying the pattern of their degradation of eugenol and ferulic acid. We have shown that different strains, such as *Pseudomonas putida* and *Streptomyces setonii*, use different metabolites as the metabolic overflow product. Whereas *P. putida* mainly produced vanillic acid, *S. setonii* accumulated vanillin and was thus found to be an ideal vanillin producer.

Materials and methods

Bacterial strains and culture conditions

P. putida (DSM 12585) was isolated from local forest soil and *S. setonii* (ATCC 391161) was purchased from ATCC. Both strains were kept at −80 °C as glycerol stock and cultured in shake flasks at 150 rpm. For all experiments, *P. putida* was grown in a medium (pH 7.3) containing $(NH_4)_2SO_4$ (1.5 g l^{-1}), KH_2PO_4 (1 g l^{-1}), Na_2HPO_4 (3 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (0.01 g l^{-1}), $ZnSO_4 \cdot 7H_2O$ (8.8 mg l^{-1}), $FeCl_3 \cdot 6H_2O$ (0.97 mg l^{-1}), $CuSO_4 \cdot 5H_2O$ (0.27 mg l^{-1}), $Na_2B_4O_7 \cdot 10H_2O$ (88 ng l^{-1}), $MnCl_2 \cdot 4H_2O$ (72 ng l^{-1}), and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (37 ng l^{-1}). *S. setonii* was grown in a medium (pH 7.2) containing glucose (1 g l^{-1}), Na_2HPO_4 (4 g l^{-1}), KH_2PO_4 (1 g l^{-1}), yeast extract (1 g l^{-1}), NaCl (0.2 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g l^{-1}), and $CaCl_2 \cdot 2H_2O$ (0.05 g l^{-1}). Ferulic acid (F-3500), purchased from Sigma, was added 16 h after inoculation, as a 10% solution (pH 7.2) to the desired concentration.

Preparation of crude extracts

Cells cultivated in 1-l shake flasks containing 250 ml medium were harvested by centrifugation (9000 rpm in a Sorvall rotor, type GSA; 15 min) and washed once with 50 ml 50 mM TRIS/HCl, pH 7.0. After resuspension in 2.5 ml washing buffer, the cells were passed through a French press. Alternatively, cells were opened by sonification (2 min at 20% intensity with 0.2-s intervals, using a Branson sonifier 450). The cell extracts were then centrifuged to remove the cell debris and kept at 4 °C until further use.

Enzymatic conversions

A 352-µl sample of crude cell extract was mixed with 40 µl 0.5 M TRIS-HCl buffer, pH 7.0 and 4 µl 25 mM NAD^+. The reaction was then initiated by the addition of 4 µl 25 mM substrate (ferulic acid or vanillin). Samples of 100 µl were taken at various intervals and mixed with 500 µl HPLC buffer A to inactivate the enzymes. After centrifugation the supernatant was analyzed by HPLC to quantify the various products formed.

Isolation of microorganisms from soil

Cultures were enriched by adding 10 g soil to 50 ml minimal medium (composition the same as *P. putida* medium described above) containing 0.1 g l^{-1} eugenol as sole carbon and energy source. After overnight incubation at 30 °C, 0.1 ml supernatant was plated on agar plates containing minimal medium plus 1 g l^{-1} eugenol. Single colonies were picked and used for screening. Actinomycetes were kindly provided by Dr. J. Watanabe from Nippon Roche K. K.

HPLC quantitative analysis of phenolic metabolites

Metabolites were analysed on a 4.6 × 250-mm C8 column (Brownlee, Applied Biosystems) by reverse-phase HPLC. A linear gradient from 0 to 100% in 15 min, using aqueous 0.1% trifluoroacetic acid as solution A and aqueous 80% acetonitrile supplemented with 0.1% trifluoroacetic acid as solution B, was applied at a flow rate of 1 ml min^{-1}. The absorbance of the separated metabolites was measured at 254 nm and quantified using standard stock solutions prepared with commercial ferulic acid, vanillin and vanillic acid.

Results

Eugenol, industrially isolated from clove oil with a current market price of around $5/kg, would be an excellent starting material for a microbial conversion to vanillin. Eugenol, however, is known to be rather toxic to microorganisms, inhibiting growth and metabolism. To screen different eugenol-tolerant microorganisms, we isolated soil bacteria using eugenol as sole carbon and energy source. Five different types of microbes were obtained, which were then analyzed for their ability to metabolize eugenol. At low concentrations (i.e. below 1 g l^{-1}) eugenol was degraded within 48 h in liquid cultures. Degradation of eugenol was observed with a time lag of several hours after its addition. Vanillic acid was identified as an intermediate degradation product, but no vanillin was found in the supernatant. Higher concentrations of eugenol proved to be toxic, resulting in the lysis of the cells. The most eugenol-tolerant bacterium was identified as *Pseudomonas putida* (DSMZ in Braunschweig, Germany). This appears to be a special strain of *P. putida* and most likely could be differentiated from the type strain by its eugenol tolerance.

The rather low eugenol degradation rate of the isolated *P. putida* prompted us to look for an alternative precursor for an efficient microbial production of vanillin. Ferulic acid has been reported as an intermediate in the metabolism of eugenol to vanillin (Tadasa 1977). This phenyl propenoic acid is commonly found in various corn brans (Herrmann 1976) where it is linked to sugar residues of the xylan fibers. The acid can be easily released and recovered by the use of ferulic acid esterases (Christov and Prior 1993). With the use of ferulic acid instead of eugenol, higher conversion rates were observed and feeding concentrations could be easily increased above 1 g l^{-1}. The isolated *P. putida* strain, grown overnight on a glucose medium, converted 1 g l^{-1} ferulic acid within 4.5 h (Fig. 1). Vanillic acid, as a

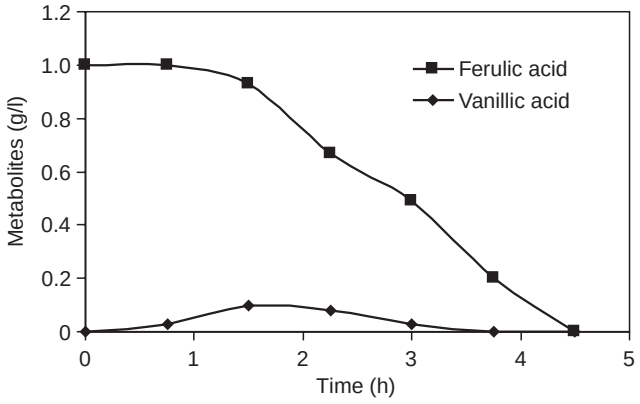

Fig. 1 *Pseudomonas putida* was pregrown on a glucose medium; 16 h after inoculation, 1 g l^{-1} ferulic acid was added, resulting in the transient accumulation of vanillic acid. The formation of vanillin as an intermediate was not observed

degradation product of ferulic acid, was found in the supernatant at a maximal concentration of 91 mg l^{-1}. To see whether vanillic acid was formed by oxidation of vanillin, *P. putida*, pregrown on ferulic acid, was treated with 1 g l^{-1} vanillin. The aldehyde was almost quantitatively converted to the acid within less than 2 h. The higher degradation rate of vanillin than that of ferulic acid might explain why no vanillin was found in the supernatant during the degradation of ferulic acid.

Additional evidence for vanillin being an intermediate of the conversion of ferulic acid to vanillic acid was obtained on the enzymatic level. Cell extracts prepared from ferulic-acid-induced *P. putida* cells completely converted 125 µmol vanillin within 5 min into vanillic acid, as followed by HPLC analysis. Ferulic acid was also converted to vanillic acid, though only incompletely and much more slowly than vanillin, with a 52% conversion after 24 h. Interestingly, addition of 200 µmol CoASH and 200 µmol ATP did not result in an increase of vanillic acid formation. Formation of vanillic acid as degradation product was confirmed by HPLC. Vanillin as an intermediate during the enzymatic conversion of ferulic acid was not observed.

The vanillin-converting enzyme could be identified as a NAD^+-dependent enzyme on the basis of the following criteria. First, the activity of an enzymatic fraction applied to a DEAE-Sepharose column at pH 8 and then eluted with 0.25 M NaCl had to be restored by the addition of NAD^+. Secondly, the formation of NADH was shown by an increase in absorbance at 560 nm when 2.5 µmol freshly prepared phenazinemethosulfate and 25 µmol *p*-nitroblue tetrazolium chloride (NBT) were also added to the reaction mixture. The NADH formed reduced the added NBT, resulting in an increase at 560 nm. Thirdly, flushing the enzyme solution with argon did not influence the conversion rate, ruling out an oxidase being responsible for the conversion.

The whole enzyme cocktail responsible for the conversion of ferulic acid to vanillic acid was most active at pH 7.0 and had a half-life of about 20 h at 4 °C. Freezing and thawing resulted in a minimal loss of activity. Interestingly, besides ferulic acid *p*-hydroxycinnamic acid was also converted to *p*-hydroxybenzoic acid. No conversion was observed when the phenyl ring was not hydroxylated in the *para* position as is the case for cinnamic acid or *p*-coumaric acid. In contrast, nonphenolic aromatic aldehydes, such as benzaldehyde or anisaldehyde, were rapidly oxidized by the crude enzyme extract to the corresponding acid. It appeared that the relative higher vanillin dehydrogenase activity, in comparison to the ferulic-acid-converting enzymes, prevented the formation of vanillin in the supernatant of *P. putida*.

A major drawback of using *P. putida* for producing vanillin by fermentation is the rapid degradation of vanillin to vanillic acid. We therefore extended our search to other microorganisms devoid of this characteristic. On the basis of the wide metabolic variety of actinomycetes, we have investigated over 120 isolated strains for their ferulic acid degradation pattern. Most of the strains tested did not degrade the acid at all; 12 strains were found to decarboxylate ferulic acid leading to vinylguaiacol and only 4 showed weak formation of vanillic acid without any accumulation of vanillin. Formation of minor traces of vanillin was found only with *S. setonii* as reported earlier (Sutherland et al. 1983). Feeding *S. setonii* with 1 g l^{-1} ferulic acid resulted in a maximal vanillin concentration of 124 mg l^{-1}. As seen in Fig. 2A, degradation of ferulic acid occurred quickly and vanillic acid was detectable earlier than vanillin. The latter observation can be explained by a rather fast oxidation of the very reactive aldehyde to the corresponding acid. This is in line with previous findings that vanillin is an intermediate of ferulic acid degradation to vanillic acid (Toms and Wood 1970). However, it is rather surprising that vanillic acid only accumulated to a certain level and that cellular regulatory mechanisms then somehow favored a continuous accumulation of vanillin. This was seen even more clearly when feeding higher amounts of ferulic acid to *S. setonii*. When 8 g l^{-1} ferulic acid was fed, vanillic acid reached a level of around 200 mg l^{-1} with a maximal vanillin accumulation of 3.8 g l^{-1} (Fig. 2B). Once ferulic acid had been depleted the vanillic acid concentration temporarily increased as the accumulated vanillin was now degraded. A level of around 200 mg l^{-1} vanillic acid was also observed when feeding between 4 g l^{-1} and 10 g l^{-1} ferulic acid was supplied, with a further transient increase of the vanillic acid concentration only when ferulic acid was depleted and the accumulated vanillin was degraded. The amount of vanillin accumulated depended on the amount of ferulic acid fed. Vanillin concentrations reached were 1.3 g l^{-1} with 4 g l^{-1} ferulic acid and 4.6 g l^{-1} with 10 g l^{-1} ferulic acid. The actual concentration of ferulic acid in the medium did not influence the accumulation rate of vanillin, as observed in fed-batch experiments. After the conversion of an initial 6 g l^{-1} ferulic acid, another 6 g l^{-1} was added, resulting in a further increase of the vanillin concentration (Fig. 3).

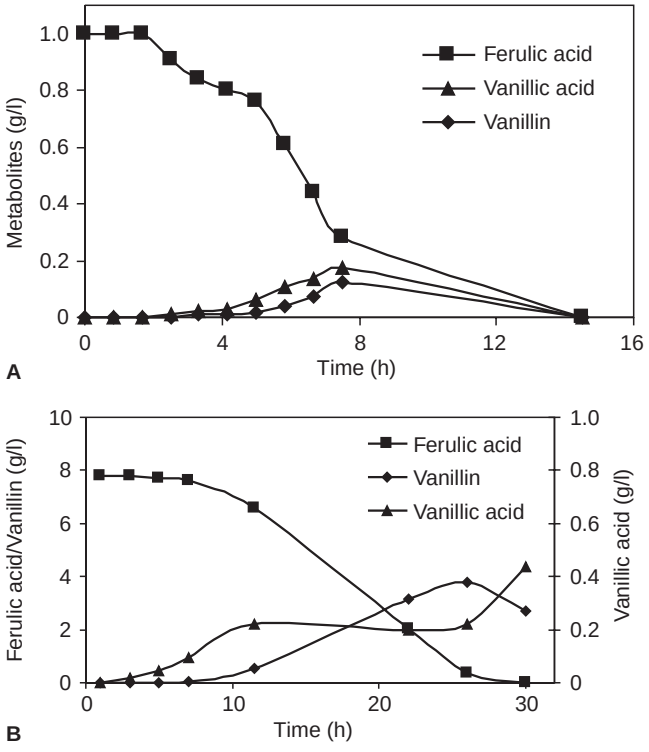

Fig. 2A, B Ferulic acid transformation to vanillin in cultures of *Streptomyces setonii* pregrown overnight on a glucose medium. The transient accumulation of vanillin was followed by HPLC analysis of the supernatant. Different amounts of ferulic acid were fed: **A** 1 g l^{-1}, **B** 8 g l^{-1}

The accumulation rate of vanillin remained constant throughout a 54-h cultivation period, reaching a maximal concentration of 6.41 g l^{-1} vanillin with a molar yield of 68%. After depletion of ferulic acid, both the vanillin formed and the residual vanillic acid were degraded.

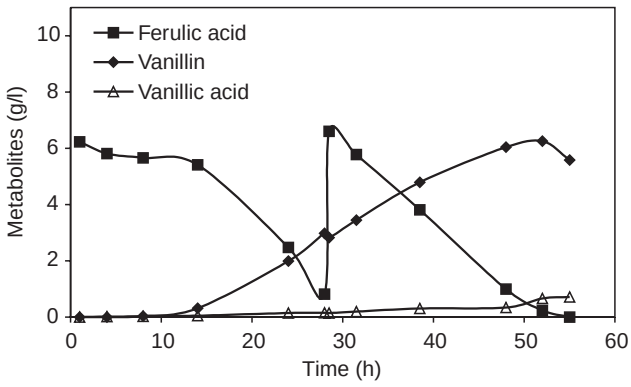

Fig. 3 Fed-batch experiment with cultures of *S. setonii*. 6 g l^{-1} ferulic acid was added to pregrown overnight cultures. After this initial ferulic acid feed was almost depleted, another 6 g l^{-1} was added. During this second feeding the vanillin concentration steadily increased whilst the vanillic acid concentration remained the same

It is interesting to note that the metabolic flux during the degradation of ferulic acid is completely different in *P. putida* and in *S. setonii*. As metabolic intermediate, *P. putida* used vanillic acid whereas *S. setonii* used vanillin. Interestingly, even small amounts of vanillyl alcohol could be detected in the supernatant of *S. setonii*. Other minor degradation products were identified as guaiacol and 3,4-dihydroxybenzoic acid. Both are degradation products of vanillic acid. Figure 4 schematizes the metabolic degradation pathway of ferulic acid in *S. setonii*.

Discussion

Different possibilities of producing vanillin by fermentation have been investigated in the past. Most of these efforts were hampered by the fact that, though vanillin

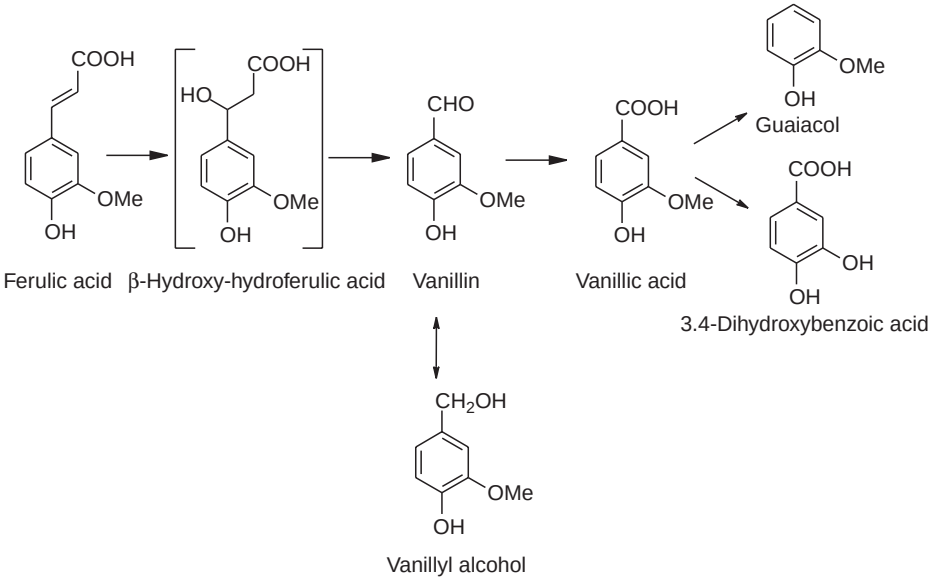

Fig. 4 Metabolic pathway of *S. setonii*. All compounds were directly observed in the culture medium, with the exception of the postulated hydroxylated intermediate (*in brackets*)

might have been formed as an intermediate, no accumulation was observed. We have therefore shifted our focus from the metabolic products to the enzymatic system that is responsible for converting ferulic acid. The presence of vanillin-oxidizing enzymes in ferulic-acid-induced cells was taken as a good indication that vanillin was truly formed. Especially in *P. putida*, where no vanillin was found in the supernatant, degradation pathways without vanillin as intermediate could be imagined. After the formation of the hydroxylated dihydroferulic acid (see also Fig. 4), this compound could be further oxidized to the corresponding ketone which, after the C—C bond cleavage, would yield vanillic acid and acetic acid. In that case, however, vanillin would not be an intermediate and thus no vanillin-oxidizing enzymes should have been induced. Such a β-oxidation-like pathway has been detected in *Lithospermum erythrorhizon* cell cultures (Yazaki et al. 1991).

The ferulic-acid-converting enzymes of *S. setonii* have been shown to harbor a broader substrate activity than those of *P. putida*. The enzymes from *S. setonii* also readily converted non-phenolic aromatic compounds like cinnamic acid, whereas *P. putida* enzymes appeared to convert only phenolic substrates with the hydroxy group in the *para* position. Similar results were obtained for a ferulic acid decarboxylase that was found to convert only *p*-hydroxylated acids (Edlin et al. 1998).

Pseudomonas sp. have been found to be excellent converters of ferulic acid into vanillic acid. Thus, several attempts have been made to inactivate or reduce the vanillin dehydrogenase activity. A Japanese patent describes the generation of mutant strains lacking this activity (Takasago Perfumery Co. Ltd. 1993). The mutants converted 1 g eugenol to 48 mg ferulic acid, 100 mg vanillyl alcohol and 280 mg vanillin within 9 h. This is a considerable amount of vanillin produced, but the unexpected formation of vanillyl alcohol points to severe changes in the metabolic flow, illustrating the limits of this system. Other investigations tried to reduce the vanillin dehydrogenase activity by adding different inhibitors such as dithiothreitol (Labuda et al. 1994). Addition of 5 mM dithiothreitol to *P. putida* cultures has been reported to lead to the formation of 210 mg l^{-1} vanillin after an incubation period of 54 days. It remains unclear whether this formation was due to a true inhibition of the vanillin dehydrogenase or only to a slow chemical reduction of the vanillic acid formed by dithiothreitol. In general, problems with the inactivation of the vanillin dehydrogenase activity might be attributed to the fact that several isoforms of the enzyme have been described, e.g. in *Pseudomonas fluorescens* BTP9 (Bare et al. 1996).

It appeared fairly clear that *P. putida* does not favor any accumulation of vanillin. Aldehydes, in general, are rarely accumulated in biological systems because of their rather high chemical reactivity (Lindahl 1992). In line with this, it was observed that *P. putida* fed with ferulic acid started to accumulate vanillic acid. The latter was tolerated at high concentrations without affecting the metabolism. Obviously, the bottleneck in the metabolic flow appeared to be the further degradation of vanillic acid. Such metabolic bottlenecks have been described in *Saccharomyces cerevisiae* with the production of ethanol (Sonnleitner and Hahnemann 1994).

The success of finding a high-yield vanillin fermentation process obviously is not based on changing the culture medium of *P. putida*, for example, or adding substances that interfere with the metabolism, but rather on the selection of an organism that has a metabolic bottleneck in the oxidation of vanillin to vanillic acid. At low concentrations of ferulic acid, it was not obvious that *S. setonii* has this specific characteristic, as both vanillic acid and vanillin were accumulated. However, at higher concentrations it became very clear that *S. setonii* has a special metabolic flow, as it allowed the accumulation of vanillin. Vanillin could be clearly observed as the metabolic overflow product.

S. setonii is remarkable not only for its tolerance towards vanillin, but also for its ability to accumulate vanillin close to its crystallization concentration of 1% at 20 °C (Clark 1990). This is the concentration region reached in our experiments (described elsewhere in Muheim et al. 1998).

In conclusion, the unique metabolism of *S. setonii*, producing vanillin as the metabolic overflow product during the degradation of ferulic acid, allowed the establishment of a production strategy for vanillin. The shake-flask experiments described form an ideal basis for the economical production of vanillin in bioreactors. Further productivity increases can be achieved by optimizing the medium composition in combination with a higher cell density.

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