Bioprocess Biosyst Eng (2014) 37:891–899
DOI 10.1007/s00449-013-1060-x

ORIGINAL PAPER

Effect of bioconversion conditions on vanillin production by *Amycolatopsis* sp. ATCC 39116 through an analysis of competing by-product formation

Xiao-kui Ma · Andrew J. Daugulis

Received: 20 June 2013 / Accepted: 9 September 2013 / Published online: 28 September 2013

Abstract This study investigated the effects of transformation conditions such as initial pH, the initial concentration of glucose and yeast extract in the medium, and the separate addition of ferulic acid and vanillic acid, on the production of vanillin through an analysis of competing by-product formation by *Amycolatopsis* sp. ATCC 39116. The extent and nature of by-product formation and vanillin yield were affected by initial pH and different initial concentrations of glucose and yeast extract in the medium, with a high yield of vanillin and high cell density obtained at pH 8.0, 10 g/l glucose, and 8 g/l yeast extract. High concentrations of ferulic acid were found to negatively affect cell density. Additional supplementation of 100 mg/l vanillic acid, a metabolically linked by-product, was found to result in a high concentration of vanillin and guaiacol, an intermediate of vanillin. Via an analysis of the effect of these transformation conditions on competing by-product formation, high concentrations of ferulic acid were transformed with a molar yield to vanillin of 96.1 and 95.2 %, by *Amycolatopsis* sp. ATCC 39116 and *Streptomyces* V1, respectively, together with a minor accumulation of by-products. These are among the highest performance values reported in the literature to date for *Streptomyces* in batch cultures.

Keywords Transformation condition · Vanillin production · *Amycolatopsis* sp. ATCC 39116 · An analysis of competing by-product formation · *Streptomyces* V1

X. Ma
Key Laboratory of Medicinal Resources and Natural Pharmaceutical Chemistry, Ministry of Education, National Engineering Laboratory for Resource Developing of Endangered Chinese Crude Drugs in Northwest of China, College of Life Science, Shaanxi Normal University, 199 Chang'an South Road, Xi'an 710055, Shaanxi, People's Republic of China
e-mail: biomarkuis@gmail.com

A. J. Daugulis (✉)
Department of Chemical Engineering, Queen's University, Kingston, ON K7L 3N6, Canada
e-mail: andrew.daugulis@chee.queensu.ca

Introduction

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is one of the major constituents of "natural vanilla" flavor responsible for its characteristic aroma [1] and is the most widely used flavor ingredient in the food and cosmetics industries. The price of "natural" vanillin is very high compared to the chemically synthesized form, mainly due to the limited availability of vanilla pods (currently only about 2,200 tons of cured pods per year [2]), which cannot meet the demand of the growing global market for natural vanillin. The production of natural vanillin is dependent on climate-associated fluctuations of harvest yields, economic and political disruptions, and expensive labor-intensive cultivation, pollination, harvesting and curing of vanilla pods [2]. Although vanillin has been mainly of synthetic origin, derived from raw materials such as eugenol, lignin, glyoxylic acid, parole or guaiacol, the difference between the prices of the chemically synthesized vanillin and cured vanilla pods combined with the increasing customer-led demand for natural flavors, has led to a growing interest to produce natural vanillin by biotransformations from natural substrates, including ferulic acid, phenolic stibenes, eugenol among others. This has also been supported by the fact that international

legislation specifies the term "natural products" to include materials produced from microbiological sources by living cells or their enzymes.

Microorganisms are known to be capable of producing hydroxybenzoates such as vanillin [3], from several naturally occurring or petroleum-based substrates via biodegradation or biotransformation processes. Recently, several species of microorganisms such as *Psychrobacter* sp. CSW4 [4], *Candida galli* PGO6 [5], *Pseudomonas resinovorans* SPR1 [6], *Bacillus pumilus* S-1 [7], *Streptomyces* [8] and *Brevibacillus agri* 13[9] have been studied for their capability to generate vanillin via biotransformation of ferulic acid or other structurally similar substrates. Among vanillin-producing genera, however, the actinomycetes, in particular several species of *Streptomyces* [8, 10], seem to be particularly efficient in utilizing hydroxycinnamic acids, especially ferulic acid, which is the most abundant hydroxycinnamic acid in the plant world and occurs mainly in cell walls covalently linked to lignin and other polymers [11]. This capability of the actinomycetes may be due to their saprophytic life cycle and also their proximity to plants in the soil environment, which may facilitate this bacterium to obtain plant genes responsible for the biosynthesis of vanillin [12]. Some early research using *Streptomyces* species [8, 13] showed significantly better results in vanillin production, in which the molecular yield and productivity have provided a clear advantage over organisms such as *Pseudomonas* [3] and *Psychrobacter* [4]. Some *Streptomyces* strains also exhibit a very high tolerance towards vanillin, which has far-reaching consequences for its downstream processing [2], as the crystallization concentration of vanillin is 1 % at 20 °C. Although a novel strain of *Bacillus fusiformis* was reported to produce 32.5 g/l vanillin over 72 h utilizing isoeugenol as substrate, its volumetric productivity was very low [14]. Overall, it appears as though the potential for a commercial biocatalytic process would be a route based on the biotransformation of ferulic acid by *Streptomyces* [4, 6, 8, 9, 13].

The biotransformation of ferulic acid to vanillin by *Streptomyces* has been studied previously [8, 10, 13]. Although fed-batch bioconversions using whole cells [8], enzymatic conversion with cell extracts [13] and in vitro bioconversion have attempted to obtain high-vanillin yields, some problems still remain with the accumulation of metabolites and the relatively low conversion efficiency of the substrate, ferulic acid. In most of the previously reported studies, the accumulated vanillic acid and other metabolites undergo rapid degradation or transformation to other intermediates in the pathway of vanillin synthesis by *Streptomyces* [8, 13]. As vanillin can be converted into by-products, intermediates (e.g. vanillic acid) or complex metabolites, therefore, lower molar conversion could be expected. These production processes have generated a variety of different by-products, including vanillic acid, vanillyl alcohol, 3,4-dihydroxybenzoic acid and guaiacol, which makes purification difficult, along with resulting in a low vanillin yield [15]. Although the great majority of production strategies have been based on increasing the yield of vanillin, increasing substrate consumption, and on strain improvements, there are few reports about the effects of transformation conditions on the metabolism of producer strains. In addition, apart from some preliminary information on vanillic acid and guaiacol metabolism during the transformation such as a report by Ghosh [10], there is a lack of information on the influence of transformation conditions through competing by-product metabolites of *Streptomyces*.

In this paper, the influence of transformation conditions such as initial pH, the initial concentration of glucose and yeast extract in the medium, and the separate addition of ferulic acid and vanillic acid on the transformation of ferulic acid to vanillin by *Amycolatopsis* sp. ATCC 39116, was investigated through an analysis of competing by-product formation. A resulting transforming protocol was developed for efficient vanillin production, and this protocol was then also applied to *Strptomyces* strain V1, an alternative high-vanillin producer.

Materials and methods

Chemicals

Ferulic acid (trans-4-Hydroxy-3-methoxycinnamic acid, ≥99 %), vanillin (4-Hydroxy-3-methoxybenzaldehyde, 99 %), vanillic acid (4-Hydroxy-3-methoxybenzoic acid, ≥97.0 %), 3, 4-dihydroxybenzoic acid and guaiacol were of analytical grade and of the highest purity (obtained from Sigma–Aldrich, USA). Glucose, yeast extract and others were obtained from Fisher Scientific. Solvents used were HPLC grade.

Organisms

Amycolatosis sp. ATCC 39116 and *Streptomyces* strain V1, which was a gift from Dr. Xu Ping (Shanghai Jiaotong University), were used for the biotransformation of ferulic acid. These two strains belong to the genus *Streptomyces*, and have been reported to be able to transform ferulic acid to vanillin [8, 13].

Shake flask cultures and transformations

The inoculum was prepared by adding 30 µl frozen *Amycolatopsis* sp. ATCC 39116 or *Streptomyces* V1 (CCTCC M 206065) stock culture to 30 ml basal medium in shake flasks

and cultivating at 28 °C, 220 rpm for approximately 16–18 h. Before transformation, the inoculum was transferred at a ratio of 1 % (v/v) to 30 ml fresh medium in a flask and incubated as above. Subsequently, the transformation of vanillin was conducted with supplementation of ferulic acid at 28 °C, 220 rpm. Ferulic acid was predissolved in a solution containing 37 g/l NaOH and 22 g/l NaH_2PO_4 (1:10, w/v) and filtered using 0.2 μm syringe filters.

The basal medium comprised the following ingredients as described previously [13] (g/l): glucose 1, Na_2HPO_4 4, KH_2PO_4 1, yeast extract 1, NaCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2 \cdot 2H_2O$ 0.05, pH 7.2. The influence of various transformation conditions such as initial pH, the initial concentration of glucose and yeast extract in the medium which are known to considerably influence the primary and secondary metabolisms of most microorganisms, and the separate addition of ferulic acid, and vanillic acid in the broth on transformation by *Amycolatopsis* sp. ATCC 39116 was investigated. The time course consists of approximately 18 h of cell growth, followed by the initiation of vanillin bioproduction. For pH evaluation, the initial pH of the medium was adjusted to the desired value by addition of either 1 M NaOH or 1 M HCl. The concentration range of glucose and yeast extract was chosen on the basis of the previous compositions of two media [8, 13] and separately supplemented to the basal medium at the beginning of the cultivation, while other components were kept constant. After 18 h of cultivation, a concentration of 6 g/l ferulic acid was added to the broth to initiate the transformations in all cases except for the evaluation of the influence of substrate concentration itself, in which different concentrations of substrate were provided to the broth after 18 h of cultivation. For the evaluation of the influence of vanillic acid supplementation, different concentrations of vanillic acid along with 6 g/l ferulic acid were provided in the broth after 18 h of cultivation. After transformation initiation, samples (taken at 24th h or later based on preliminary results) were assayed for vanillin, other metabolites, pH and OD_{600} of the culture and for shake flask transformation, the vanillin yield was monitored.

For shake flask transformations with a concentration of 12 g/l ferulic acid by *Amycolatopsis* sp. and *Streptomyces* V1, experiments were performed based on the optimized conditions including initial pH, the initial concentration of glucose and yeast extract in medium, and the addition of ferulic acid, and vanillic acid determined from the above except that inoculation was conducted with 4 % (v/v). And, samples were assayed for yield, vanillin and other metabolites, pH and OD_{600} of the culture.

Analytical methods

Growth was measured by optical density of the culture at 600 nm in a UV–Vis Spectrophotometer (Biochrom Ultrospec 3000 UV/Visible) or converted to CDW (cell dry weight) by means of a calibration curve.

Ferulic acid and the transformation products were analyzed by reverse phase HPLC on a C18 column (Polaris C18-A 150 × 4.6 mm, VARIAN, Inc. USA) using a Varian HPLC, equipped with dual pumps and a UV–Vis detector (320 nm). The mobile phase consisted of water, methanol and formic acid (80:20:1, v/v/v), and the flow rate employed was 1 ml/min. The samples were prepared by centrifuging at 5,000 rpm for 5 min and then filtered through 0.22 μm PTFE, membrane centrifuged, and filtered again before analyzing with HPLC. The standard deviations of the analyses were less than 5 % using external standards. The compounds eluted out in the following order: 3.4-dihydroxybenzoic acid (Retention time or RT, 3.4 min), vanillic acid (6.1 min), vanillin (6.8 min), *trans*-ferulic acid (8.4 min), and guaiacol (10.2 min) and two possible unidentified compounds (11, 13 min) [16, 17]. Vanillin yield (g/l) was calculated based on the volume of the fermentation broth, while molar yield (percent) was calculated as (mole vanillin accumulated × 100)/(mole substrate supplemented) [10]. Intermediate compounds such as vanillic acid, guaiacol and vanillin were measured during bioproduction for evaluation of influence of conversion conditions on vanillin production.

Results and discussion

The effect of initial pH of the medium on the transformation

To investigate the influence of the initial pH of the medium on the transformation, bioconversions of ferulic acid by *Amycolatopsis* sp. were initiated on broths after growing the cells for 18 h at different initial pH values from 7.0 to 8.5. During the transformations, *Amycolatopsis* sp. showed a constant and higher OD_{600} value for the broth cultivated at pH 8.0 than that at higher or lower pH values (data not shown). Analysis of competing by-product formation indicated that, although vanillin, vanillic acid, guaiacol, and 3,4-dihydroxybenzoic acid and the two other unknown compounds [16, 17] were detected during the transformations of broth cultivated at different initial pHs, the bioconversion of the broth cultivated at initial pH 8.0 generated much higher concentrations of products, especially vanillin, guaiacol and the two other unknown compounds. High concentrations of guaiacol indicate high concentration of vanillin produced during transformation, as guaiacol is thought to be a bioconversion product of vanillin [13], and the emergence of the two other unknown species was observed to be temporary as they were transformed into vanillin or other intermediates of vanillin with

increasing time. These results suggest that the broth cultivated at an initial pH 8.0 facilitates the transformation of ferulic acid to vanillin by *Amycolatopsis* sp.. All other transformations in this study were, therefore, conducted by cultivating the cells at an initial pH of 8.0, unless otherwise specified.

The effect of initial concentration of glucose on the transformation

To study the effect of initial glucose concentration in the medium on the transformation by *Amycolatopsis* sp., the basal medium was separately supplemented with glucose ranging from 1 to 15 g/l to generate cells for the transformations. As shown in Fig. 1a, higher initial concentrations of glucose (10 or 15 g/l) supported higher cell density, as anticipated, and even additional cell growth during the transformations, in contrast to 5 g/l glucose supplementation which showed little OD_{600} change. An analysis of competing by-product formation revealed a considerable difference in metabolism for the transformations using broths cultivated with different initial concentrations of glucose (Fig. 1b–d). Specifically, compared to the bioconversion of broth cultivated with 15 g/l glucose, the transformation with 10 g/l had enhanced formation of vanillin, vanillic acid, and guaiacol, at relatively higher concentrations of 0.31, 0.22, and 3.7 g/l, respectively. In the case of transformation of 5 g/l glucose, the final concentrations of these products in the broth were shown to be lower than that in the transformation with 10 g/l glucose supplementation. On the other hand, with respect to the transformation of cells cultivated with 1 g/l glucose possibly due to the lack of energy from the absence of additional glucose, the formed products were readily catabolized (data not shown).

The effect of yeast extract on the transformation

The effect of different initial concentrations of yeast extract in the medium on the cell density during transformation by *Amycolatopsis* sp. is seen in Fig. 2a, which shows that higher initial concentrations of yeast extract (10 and 8 g/l) supported cell growth even after 24 h, while the broth cultivated with low initial concentration of yeast extract in the medium shows relatively low OD_{600} after 24 h (Fig. 2a). An analysis of vanillin-metabolically linked products of *Amycolatopsis* sp. showed the different effects of yeast extract concentration on the metabolism during the transformations (Fig. 2b–d). In all cases, 3, 4-dihydroxybenzoic acid was observed to be present in very small amounts with minimal variation during transformation (data not shown). Cells grown with an initial 10 g/l of yeast extract in the medium were not observed to generate high yields of vanillin and other by-products during transformation, and the products formed at the beginning were observed to disappear quickly (Fig. 2b). The data in Fig. 2b also show that vanillin appears to be degraded prior to vanillic acid and guaiacol, which is in line with previous findings in which vanillin is thought to be an intermediate of ferulic acid degradation to vanillic acid [13]. The

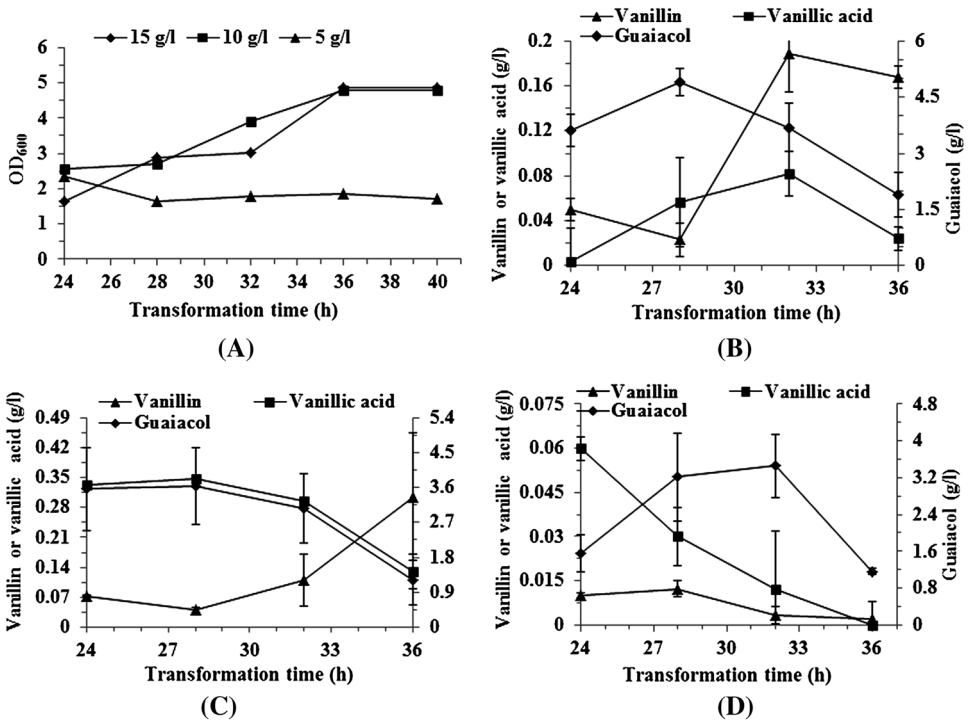

Fig. 1 Effect of initial concentration of glucose (g/l) in the medium on cell density and vanillin-metabolically linked metabolism of *Amycolatopsis* sp. ATCC 39116 during transformation. **a** Effect of different concentrations of glucose on cell density during transformation. **b** Effect of 15 g/l glucose in the medium on metabolism. **c** Effect of 10 g/l on metabolism. **d** Effect of 5 g/l on metabolism. The different concentrations of glucose were separately provided in the basal medium at the beginning of cultivation. After 18 h, 6 g/l of ferulic acid was added to the broth and transformations were then monitored by an analysis of vanillin-metabolically linked metabolites using HPLC

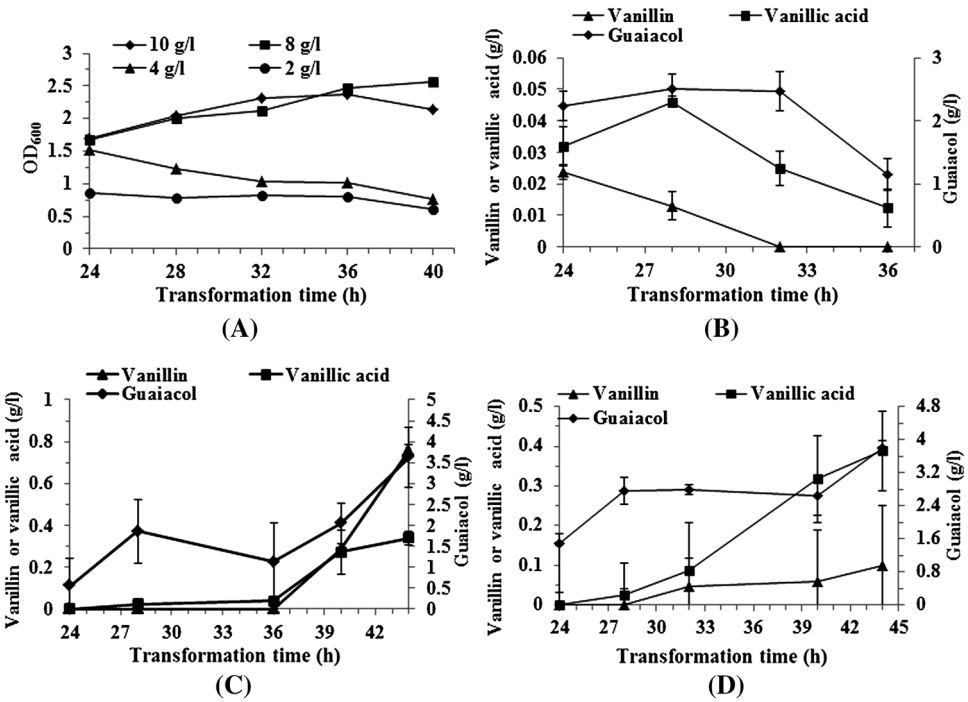

Fig. 2 Effect of the initial concentration of yeast extract in the medium on cell density and vanillin-metabolically linked compounds of *Amycolatopsis* sp. during transformation. **a** The effect of different initial concentrations of yeast extract on cell density during transformation. **b** Effect of 10 g/l yeast extract on metabolism. **c** Effect of 8 g/l on metabolism. **d** Effect of 4 g/l on metabolism. The different concentrations of yeast extract were separately provided in the basal medium at the beginning of cultivation

transformation using broth cultivated with 4 g/l yeast extract in the medium produced 0.10 g/l vanillin, along with low levels of by-products such as vanillic acid and guaiacol (Fig. 2d), while broth grown in medium containing 8 g/l yeast extract (Fig. 2c) generated 0.76 g/l vanillin and vanillic acid to a level of 0.39 g/l, together with relatively higher concentration of other by-products during transformation.

The effect of substrate concentration on the transformation

Various concentrations of the substrate, ferulic acid, were separately added to the broth of *Amycolatopsis* sp. after 18 h of cultivation to determine its influence on the transformations. As shown by OD_{600} with different substrate supplementations (Fig. 3a), a higher concentration of

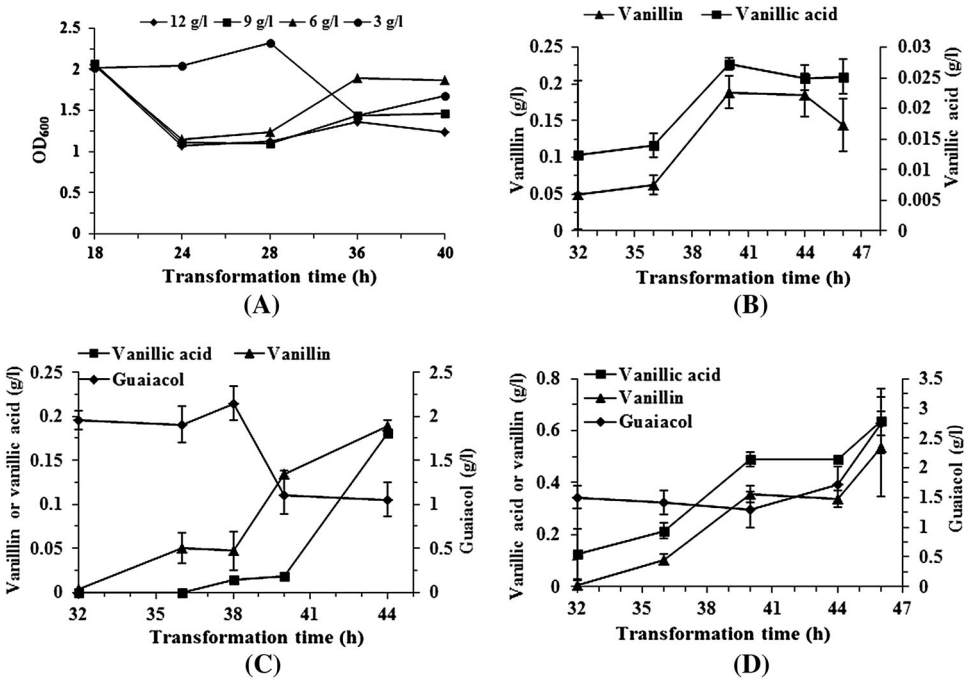

Fig. 3 Effect of the addition of different concentrations of ferulic acid on cell density and vanillin-metabolically linked compounds of *Amycolatopsis* sp. during transformation. **a** The effect of the addition of different concentrations of ferulic acid on cell density. **b** The effect of 12 g/l addition on metabolism. **c** The effect of 9 g/l addition on metabolism. **d** The effect of 6 g/l addition on metabolism. Different concentrations of ferulic acid were provided to the broth after 18 h of cultivation in the basal medium

substrate showed a negative effect on cell density during transformation. A ferulic acid concentration of 12 g/l exerted a more deleterious effect on cell density after the transformation was initiated, with a lower OD_{600} at 24 h compared to that with 3 g/l supplementation and a lower OD value at 40 h compared to that with other concentrations, as the OD_{600} in all cases after 18 h cultivation was almost identical. This is in line with previous reports that ferulic acid concentration should be limited to a relatively small amount, such as 5 or 9 g/l in batch transformations [8, 13].

An analysis of competing by-product formation confirmed the emergence of four products including vanillin, vanillic acid, guaiacol, and 3, 4-dihydroxybenzoic acid (only at very low and constant concentrations, data not shown in Fig. 3c, d), along with different amounts of two other unknown compounds. Specifically, when 12 g/l ferulic acid was provided to the broth, only traces of final vanillin and a small amount of other by-products were observed (Fig. 3b), in which no guaiacol was detected probably due to the small amount of vanillin in the broth, as guaiacol is thought to be generated from vanillin [13]. In contrast, the transformation with 9 g/l substrate generated relatively higher final concentration of vanillin (0.19 g/l), although still low, as well as higher concentration of other by-products such as guaiacol and vanillic acid (Fig. 3c). Interestingly, much large amounts of the two other unknown compounds were observed to be formed in this case, which suggests that 9 g/l substrate supplementation had relatively less detrimental influence on metabolism of this strain, along with relatively less damage to the cell growth (Fig. 3a) after the transformation was initiated. When 6 g/l ferulic acid was fed, vanillin reached a level of 0.54 g/l (Fig. 3d), with an accumulation of 2.54 g/l guaiacol and a small amount of the two other unknown compounds. In addition, the amount of vanillic acid reached a level of over 200 mg/l shortly after substrate supplementation, and this concentration has been deemed to favor the continuous accumulation of vanillin in *Amycolatopsis* sp. ATCC 39116 [13], possibly as a regulator for metabolism. In this case, the analytical results from samples taken before 32 h were not considered to be representative of any possible effects of substrate concentration on the vanillin transformation due to the increase of pH in the initial phase resulting from substrate supplementation.

The influence of ferulic acid on cell density and vanillin-metabolically linked metabolism of *Amycolatopsis* sp. may be connected primarily to the cell density in the culture itself, as it is assumed that a high cell density may more readily resist damage from ferulic acid supplementation. Thus, to increase the concentration of ferulic acid to be supplemented, it may be necessary to increase the cell density of this strain during the cell cultivation period. In addition, as *Streptomyces* can utilize ferulic acid as a sole carbon source for survival [10], glucose supplementation must be considered along with the concentration of ferulic acid provided in the broth, such as in fed-batch transformation.

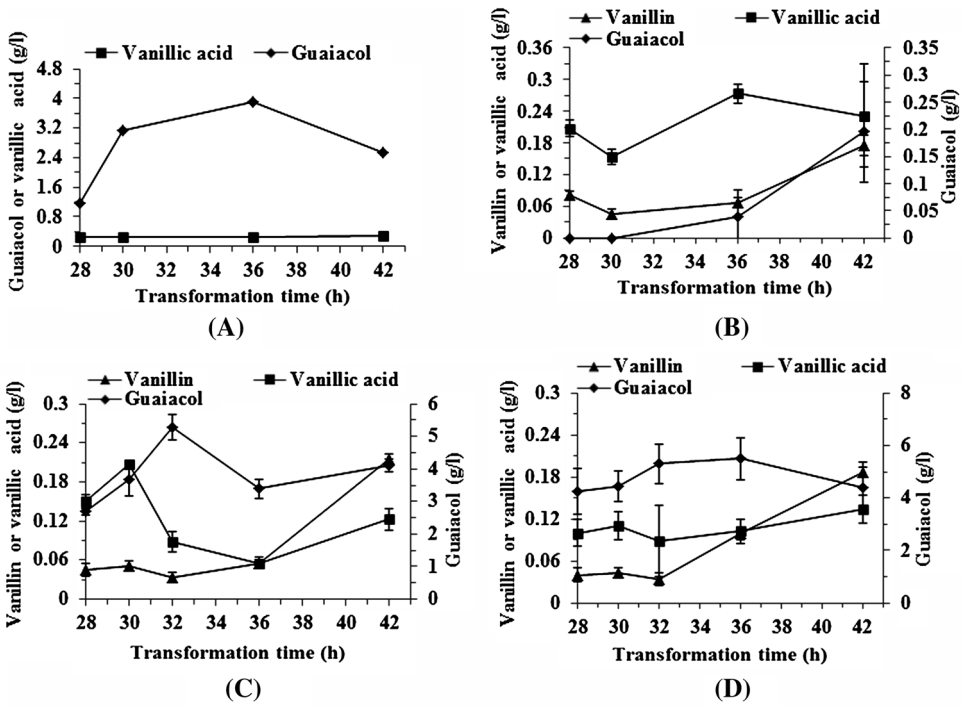

Fig. 4 Effect of the addition of different concentrations of vanillic acid on vanillin-metabolically linked compounds of *Amycolatopsis* sp. during transformation. **a** Effect of 200 mg/l vanillic acid addition on metabolism. **b** Effect of 150 mg/l addition on metabolism. **c** Effect of 100 mg/l addition on metabolism. **d** Effect of 50 mg/l addition on metabolism. Different concentrations of vanillic acid, along with 6 g/l ferulic acid, were provided to the broth after 18 h of cultivation in the basal medium

The effect of vanillic acid on the transformation

It has been reported that cellular regulatory mechanisms can favor the continuous accumulation of vanillin by *Amycolatopsis* sp. when vanillic acid accumulates to a level of 200 mg/l during transformation [13]. Therefore, different concentrations of vanillic acid were provided in the broth cultivated for 18 h simultaneously with ferulic acid feeding to investigate if addition of vanillic acid can enhance vanillin production. As shown in Fig. 4a, the addition of 200 mg/l vanillic acid in the broth imposed a strong inhibition on cell metabolism of *Amycolatopsis* sp., with no detectable vanillin and 3,4-dihydroxybenzoic acid, and also with no vanillic acid consumed. In addition, 2.5 g/l of guaiacol was formed by the end of the bioconversion, which suggests that *actinomycetes* are capable of utilizing ferulic acid in different ways depending on environmental conditions [10]. In contrast, supplementation of less than 200 mg/l (>50 mg/l) was seen to exert a favorable effect on the transformation (Fig. 4b, c). Transformation with 150 mg/l added in the broth produced 0.18 g/l vanillin by the end of the bioconversion (Fig. 4b). Encouragingly, the addition of 100 mg/l vanillic acid resulted in a higher concentration of vanillin, 0.22 g/l, a little lower than the yield in Fig. 3d, but a large amount of guaiacol (5.3 g/l) at the beginning of the transformation (Fig. 4c), a considerably higher value than the results in Fig. 3d, which indicates the additional supplementation of vanillic acid indeed influenced the vanillin-linked metabolism. Supplementation with 50 mg/l also showed an enhancement of vanillin production and metabolism, with 0.19 g/l vanillin formed and an accumulation of higher levels of vanillic acid and guaiacol (Fig. 4d). These results suggest that appropriate additional supplementation of vanillic acid at the beginning of the transformation can exert beneficial effects on vanillin synthesis by *Amycolatopsis* sp..

Transformation of high concentration of ferulic acid

Based on the above analysis of by-product formation and vanillin production under different conditions, the obtained "best" conditions were selected for the transformation of high concentrations of ferulic acid of 12 g/l by *Amycolatopsis* sp. and *Streptomyces* V1 as the transformation of this concentration value had been done by *S.setonii* (namely, *Amycolatopsis* sp. ATCC 39116) before [13] and which may provide a benchmark performance for comparison. Fig. 5a shows the biomass, vanillin and related by-products during bioconversion by *Amycolatopsis* sp., under the above transformation conditions (vanillic acid 100 mg/l, initial pH 8, yeast extract 8 g/l, glucose 10 g/l). The results revealed a high level of production of vanillin during the transformation of 12 g/l ferulic acid. More specifically, the

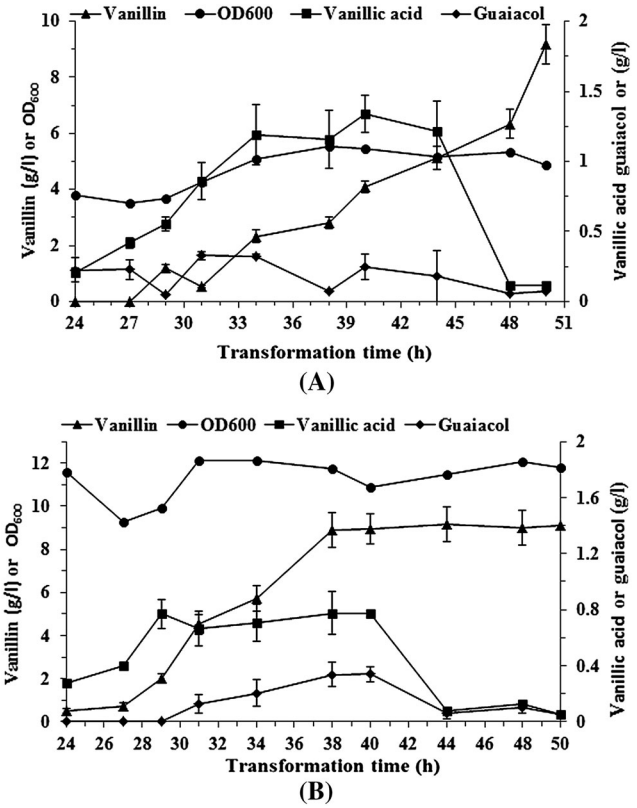

Fig. 5 Bioproduction of vanillin by *Amycolatopsis* sp. (**a**) and *Streptomyces* V1 (**b**). Shake flask transformations with a concentration of 12 g/l ferulic acid by *Amycolatopsis* sp. and *Streptomyces* V1, were performed based on the investigation of transformation conditions including initial pH, the initial concentration of glucose and yeast extract in the medium, and the addition of ferulic acid, and vanillic acid determined from the above

concentration of vanillic acid reached 0.21 g/l, a favorable level for the continuous accumulation of vanillin [13], approximately 6 h after the initiation of the transformation. The vanillic acid level continued to increase to its maximum of 1.34 g/l and then declined to its final value of 0.11 g/l when the bioconversion was stopped. This result was not contradictory with the above results of the effect of vanillic acid supplementation on the transformation as a too high initial concentration of vanillic acid, such as 200 mg/l, may just inhibit the initiation of transformation and its high level during transformation may just reflect high concentration of vanillin produced. During the transformation, guaiacol reached its maximum of 0.33 g/l in the mid-stage of the transformation and then declined to its minimum at the end; the reasons or mechanisms for this were not further investigated in this study, although it is in need of further study. Significantly, after a 15 h lag (about at 39th h), *Amycolatopsis* sp. began a rapid accumulation of vanillin, reaching a maximum of 9.18 g/l when the bioconversion was stopped, with a corresponding molar yield of 96.1 %. Interestingly, prior to the period of rapid

accumulation of vanillin, the cells also showed rapid synthesis of vanillic acid. The variation of pH during the transformation (after 18 h) was not significant, first decreasing from 8.5 to 8.0 (an initial increase of pH compared to the initial pH of the broth resulted from substrate supplementation.), and then increasing to 8.45 when the biotransformation was complete. This pH value has been validated to be beneficial to the inhibition of vanillin dehydrogenase in *Amycolatopsis* sp. [18].

When the optimum conditions for *Amycolatopsis* sp. were applied to the biotransformation by *Streptomyces* V1, a similarly enhanced performance was observed for vanillin production, as shown in Fig. 5b. Compared to *Amycolatopsis* sp., this strain started to quickly accumulate vanillin after 32–34 h, and reached a higher concentration of 4.50 g/l after 36 h of transformation, and finally obtained its maximum of 9.10 g/l with a corresponding molar yield of 95.2 % when the bioconversion was stopped. The concentration of vanillic acid sharply declined to 0.075 g/l when vanillin accumulation reached its plateau. In addition, guaiacol quickly increased after 35 h and declined after 44 h, which occurred almost at the same time as the sharp decrease in vanillic acid.

Although some authors have reported vanillin bioproduction in *Streptomyces* or related producers (Table 1) [8, 13, 14], this new and additional information concerning the interchange of vanillin intermediates and the effects of transformation conditions during transformation in this study have led to a better production strategy to synthesize vanillin by *Streptomyces*. This suggests that optimization of transformation conditions through metabolism analysis is an efficient strategy for the enhancement of the production of some metabolites, especially for metabolically linked products. Compared with previous studies [8, 14], the present work has a much higher yield but relatively lower product concentration in the broth, which should be enhanced in further study.

Significant interchange between some products was observed in the broth of *Streptomyces* such as *Amycolatopsis* sp. and *Streptomyces* V1, which also has been in part confirmed by other authors [8, 9, 13]. Further study is needed to analyze genome/proteome data from this genus to find molecular data supporting these results. Enhancement of vanillin production by additional supplementation of vanillic acid was, for the first time, observed both in *Amycolatopsis* sp. and *Streptomyces* V1, which may be ascribed to an inhibition of the transformation of vanillin to vanillic acid, as vanillin was thought to be an intermediate of vanillic acid in *Streptomyces* [13, 19]. In addition, when vanillin accumulated to a high concentration, the bioconversion of vanillin to vanillic acid was not observed, rather, vanillic acid declined under the optimized conditions in this study, which may be ascribed to the increased pH (8.45) of broth at this pH may exert deleterious effects on vanillin dehydrogenase of this strain [18]. The conversion of vanillic acid to vanillin was earlier reported in *Nocardia* sp. strain NRRL 5646 [20], but there were no data about this conversion in *Streptomyces*. In addition, as the presence of high concentrations of vanillin is reported to inhibit the growth of bacteria such as *Escherichia coli* DH5R and generate product inhibition [21], the bioproduction of vanillin utilizing the Two-Phase Partitioning Bioreactor platform (TPPB) is currently underway in our laboratory, as this approach has previously been shown to significantly enhance the bioproduction of another aroma compound, 2-phenylethanol, by *Kluyveromyces marxianus* [22].

Table 1 Comparison of transformation of vanillin with different strains. The substrate is ferulic acid unless specified otherwise

Organism	Final vanillin titre (g/l)	Substrate (g/l)	Yield (%)	Time (h)	Transforming style	Reference
Streptomyces sannanensis MTCC 6637	–	0.96	Vanillic acid	10	Cell extract	[10]
Brevibacillus agri 13	1.7	[b]	27.8	48	Biphasic system	[9]
Amycolatopsis sp. ATCC 39116	6.41	12	68	54	Enzymatic conversion	[13]
Streptomyces V1	5.24	9	74.6	18	Batch	[8]
	19.2	45	54.5	55	Fed-batch	
Bacillus fusiformis	32.5	[c]	–	72	Aqueous-organic system	[14]
Rhodococcus rhodochrous	0.004	0.03 (curcumin)	13.1	144	Whole cell	[18]
Recombinant *Escherichia coli*	1.98	3.0	83.0	48	Batch	[19]
Amycolatopsis sp. ATCC 39116	9.18	12	96.1	32	Whole cell batch	This work[a]
Streptomyces V1	9.09	12	95.2	32	Whole cell batch	This work[a]

– not specified

[a] Substrate supplementation along with vanillic acid at 100 mg/l

[b] Substrate is isoeugenol at a concentration of 10 %

[c] Substrate is isoeugenol at 60 % (v/v)

Conclusions

In this study, the final vanillin yield reached a maximum of 9.18 g/l with a corresponding molar yield of 96.1 % by *Amycolatopsis* sp. ATCC 39116, together with a minor accumulation of by-products. These results presented hereby suggest that optimization of transformation conditions through metabolism analysis is a significant and efficient strategy for metabolite production, especially for metabolically linked compounds. The present results may be extrapolated to the establishment of fed-batch process control strategies of vanillin bioproduction by *Streptomyces* such as substrate feeding and metabolism regulation such as the addition of vanillic acid.

Acknowledgments The Project was supported by Social Development and Scientific and Technological Plan in Shaanxi Province of China (Program No. 693102), Science and technology projects in Xi'an of China (No.694180) and the Fundamental Research Funds for the Central Universities of China (No.GK201003002). Many thanks to Julian Dafoe and Eric Peterson for their support during this study.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Webster TM (1995) New perspectives on vanilla. Cereal Foods World 40(4):198–200
2. Priefert H, Rabenhorst J, Steinbuchel A (2001) Biotechnological production of vanillin. Appl Microbiol Biotechnol 56(3–4):296–314
3. Toms A, Wood JM (1970) The degradation of trans-ferulic acid by Pseudomonas acidovorans. Biochemistry 9(2):337–343
4. Ashengroph M, Nahvi I, Zarkesh-Esfahani H, Momenbeik F (2012) Conversion of isoeugenol to vanillin by *Psychrobacter* sp. strain CSW4. Appl Biochem Biotechnol 166(1):1–12
5. Ashengroph M, Nahvi I, Zarkesh-Esfahani H, Momenbeik F (2011) Candida galli strain PGO6: a novel isolated yeast strain capable of transformation of isoeugenol into vanillin and vanillic acid. Curr Microbiol 62(3):990–998
6. Ashengroph M, Nahvi I, Zarkesh-Esfahani H, Momenbeik F (2011) Pseudomonas resinovorans SPR1, a newly isolated strain with potential of transforming eugenol to vanillin and vanillic acid. New Biotechnol 28(6):656–664
7. Su F, Hua DL, Zhang ZB, Wang XY, Tang HZ, Tao F, Tai C, Wu QL, Wu G, Xu P (2011) Genome sequence of bacillus pumilus S-1, an efficient isoeugenol-utilizing producer for natural vanillin. J Bacteriol 193(22):6400–6401
8. Hua DL, Ma CQ, Song LF, Lin S, Zhang ZB, Deng ZX, Xu P (2007) Enhanced vanillin production from ferulic acid using adsorbent resin. Appl Microbiol Biotechnol 74(4):783–790
9. Wangrangsimagul N, Klinsakul K, Vangnai AS, Wongkongkatep J, Inprakhon P, Honda K, Ohtake H, Kato J, Pongtharangkul T (2012) Bioproduction of vanillin using an organic solvent-tolerant Brevibacillus agri 13. Appl Microbiol Biotechnol 93(2):555–563
10. Ghosh S, Sachan A, Sen SK, Mitra A (2007) Microbial transformation of ferulic acid to vanillic acid by Streptomyces sannanensis MTCC 6637. J Ind Microbiol Biotechnol 34(2):131–138
11. Narbad A, Gasson MJ (1998) Metabolism of ferulic acid via vanillin using a novel CoA-dependent pathway in a newly-isolated strain of Pseudomonas fluorescens. Microbiology 144(Pt 5):1397–1405
12. Bode HB, Muller R (2003) Possibility of bacterial recruitment of plant genes associated with the biosynthesis of secondary metabolites. Plant Physiol 132(3):1153–1161
13. Muheim A, Lerch K (1999) Towards a high-yield bioconversion of ferulic acid to vanillin. Appl Microbiol Biotechnol 51(4):456–461
14. Zhao LQ, Sun ZH, Zheng P, Zhu LL (2005) Biotransformation of isoeugenol to vanillin by a novel strain of Bacillus fusiformis. Biotechnol Lett 27(19):1505–1509
15. Mathew S, Abraham TE, Sudheesh S (2007) Rapid conversion of ferulic acid to 4-vinyl guaiacol and vanillin metabolites by Debaryomyces hansenii. J Mol Catal B-Enzymatic 44(2):48–52
16. Mathew S, Abraham TE, Sudheesh S (2007) Rapid conversion of ferulic acid to 4-vinyl guaiacol and vanillin metabolites by Debaryomyces hansenii. J Mol Catal B Enzym 44(2):48–52
17. Karmakar B, Vohra RM, Nandanwar H, Sharma P, Gupta KG, Sobti RC (2000) Rapid degradation of ferulic acid via 4-vinylguaiacol and vanillin by a newly isolated strain of Bacillus coagulans. J Biotechnol 80:195–202
18. Fleige C, Hansen G, Kroll J, Steinbuchel A (2013) Investigation of the *Amycolatopsis* sp. strain ATCC 39116 vanillin dehydrogenase and its impact on the biotechnical production of vanillin. Appl Environ Microbiol 79(1):81–90
19. Sutherland JB, Crawford DL, Pometto AL III (1983) Metabolism of cinnamic, p-coumaric, and ferulic acids by Streptomyces setonii. Can J Microbiol 29(10):1253–1257
20. Li T, Rosazza JP (2000) Biocatalytic synthesis of vanillin. Appl Environ Microbiol 66(2):684–687
21. Yoon SH, Lee EG, Das A, Lee SH, Li C, Ryu HK, Choi MS, Seo WT, Kim SW (2007) Enhanced vanillin production from recombinant *E-coli* using NTG mutagenesis and adsorbent resin. Biotechnol Prog 23(5):1143–1148
22. Gao F, Daugulis AJ (2009) Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by kluyveromyces marxianus. Biotechnol Bioeng 104(2):332–339