0273-2289/05/125/0001/$30.00

Bioconversion of Isoeugenol Into Vanillin by Crude Enzyme Extracted From Soybean

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Received February 3, 2004; Revised November 25, 2004; Accepted December 9, 2004

Abstract

Crude enzyme extracted from soybean was used to convert isoeugenol into vanillin. The effects of several factors on the bioconversion were studied. Conversion was affected by the amount of substrate and was also improved by the addition of absorbents, among which powdered activated carbon was the best. The effect of H_2O_2 concentration on the conversion was also studied. The optimum concentration of H_2O_2 was 1% (v/v). With 10 g/L of powdered activated carbon and 0.1% H_2O_2 added, vanillin reached a maximum concentration of 2.46 g/L after 36 h, corresponding to a molar yield of 13.3%.

Index Entries: Isoeugenol; vanillin; enzyme; bioconversion; soybean.

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most important flavors widely used in foods, confectionery, beverages, perfumes, cosmetics, and pharmaceuticals. Now it is produced mainly through chemical synthesis, which means that the vanillin obtained is not "natural." According to European and US regulations, natural products are those from plant or animal materials through physical, enzymatic, or microbiologic processes *(1–3)*. The increasing concern for health and nutrition stimulates a worldwide demand for natural vanillin, which is very expensive and can no longer be satisfied only by extracting from vanilla pods. Therefore, alternative processes for the production of natural vanillin have been

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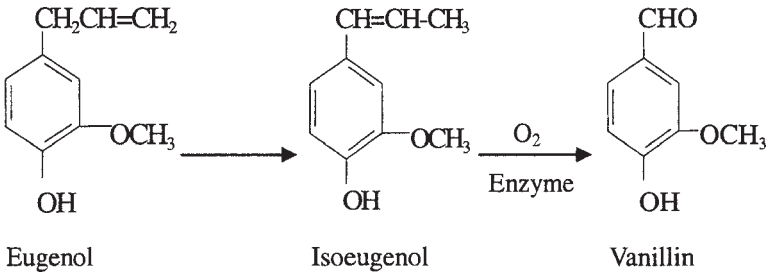

Fig. 1. Vanillin production from eugenol and isoeugenol.

studied. Among them biotransformation-based approaches become more and more attractive.

The production of vanillin by bioconversion has been studied in the past decade and biocatalysts employed have included fungi *(4,5)*, bacteria *(6)*, genetically engineered microorganisms *(7)*, plant cells *(8)*, and enzymes extracted from biologic materials *(9)*. This research was focused on vanillin's metabolic pathways (*de novo* synthesis and degradation pathway) in microorganisms and plant cells on a cellular and a genetic level. Recently, research has been gradually turned to the industrial production of vanillin. Laurence et al. *(4)* succeeded in establishing a two-step process to produce vanillin from ferulic acid: the first step was to transform ferulic acid into vanillic acid by *Aspergillus niger*, and the second was to reduce vanillic acid to vanillin by *Pycnoporus cinnabarinus*. Subsequently, this process was improved using maize bran as the source of ferulic acid, and the resulting vanillin values were US $600–1000/kg *(10)*. Our research group also succeeded in this way *(11)*. However, ferulic acid extracted from plant materials is rather expensive.

Eugenol isolated from clove oil on an industrial scale values only US $9/kg *(12)*, and it can be transformed to isoeugenol by simple isomerization. Hence, some scientists have recently begun to study the production of vanillin from eugenol and isoeugenol by microbial and enzymatic transformation *(6,13–15)*, as shown in Fig. 1.

In 1993, Markus and Paul *(14)* filed a patent related to the process of enzymatic production of vanillin from benzoe siam resin, isoeugenol, and eugenol. In this patent, commercial lipoxidase (Sigma L8383) was used as a catalyst. In a flask with 50 mL of a 50% (v/v) isoeugenol solution incubated for 4 d with 50 mg of lipoxygenase, the vanillin concentration reached 22.8 g/L. In a scale-up experiment to a 3-L reaction volume, only 7.3 g/L of vanillin was obtained from 50 g/L of isoeugenol after 120 h of incubation in the presence of 0.1 g/L of lipoxygenase. In 1998, Mane and Zucca *(15)* reported that the concentration of vanillin reached 2.85 g/L from 37.5 g/L of isoeugenol incubated for 3 d with 1 g/L of lipoxygenase in the presence of 2.5 g/L of Tween-80. However, the results were not ideal when enzymes directly extracted from soybean or potato or microbial enzymes were utilized *(14)*. There have been no further reports in this aspect in the past

decade, probably because commercially available lipoxygenase is too expensive to be acceptable in industry.

Lipoxygenase (EC l.13.11.12) is a class of iron-containing dioxygenase generally existing among plants, animals, algae, yeast, fungi, bacteria, and so on. It is well known that soybean has a high lipoxygenase content, compared with other materials. Hence, soybean is a potential source of lipoxygenase, and its extraction solution might be directly utilized for the bioconversion of isoeugenol into vanillin, instead of expensive commercial lipoxygenase.

In the present study, crude enzyme was extracted from soybean and then used to convert isoeugenol into vanillin. The effects of several factors on this bioconversion are discussed.

Materials and Methods

Chemicals

Vanillin and isoeugenol were purchased from Sigma (St. Louis, MO). Methanol was of high-performance liquid chromatography (HPLC) grade and other chemicals were of analytical grade.

Crude soybean enzyme was produced as follows. Soybean flour produced with soybeans from northeastern China (Jilin 35) was defatted with petroleum ether and then extracted for 1 h with 10 times the volume of water. Extracts were centrifuged for 30 min at 10,000*g*. After $(NH_4)_2SO_4$ was added to the suspension to reach 40% saturation, the mixture was kept still overnight and then centrifuged for 20 min at 6000*g*. With $(NH_4)_2SO_4$ added again to reach 60% saturation, the mixture was kept still overnight and centrifuged for 20 min at 6000*g*. Finally, the precipitate was dissolved in 0.1 *M* sodium borate buffer (pH 9.0), to be used as crude enzyme for bioconversion. All of these steps were generally performed at 4°C.

Bioconversion Process

All bioconversion operations were performed in 250-mL flasks containing 20 mL of crude enzyme solution shaken at 180 rpm. The concentration of vanillin was measured after 3 d. The factors affecting the bioconversion process included temperature, pH, and amount of substrate. The adopted temperature and pH for the bioconversion were 28°C and 9.0, respectively *(14,16)*.

Measurement of Enzyme Activity

Lipoxygenase activity was measured with linoleic acid as substrate *(17)*. One unit was defined as the amount of enzyme causing an increase of 0.001 in A_{234}/min in a 3.0-mL volume (1-cm light path).

Analysis of Vanillin and Isoeugenol

The concentrations of vanillin and isoeugenol were monitored by HPLC. Separation was achieved on a Lichrospher 100 C-18 reverse-phase

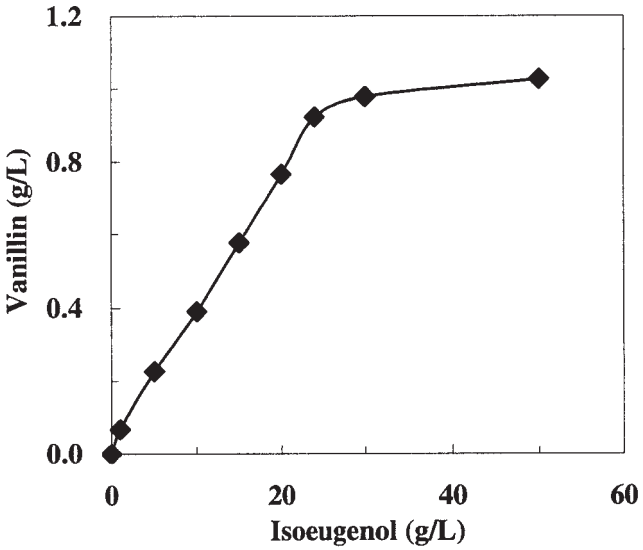

Fig. 2. Effect of amount of substrate on bioconversion of isoeugenol into vanillin. Bioconversion was carried out in 250-mL flasks containing 20 mL of crude enzyme solution at 28°C, pH 9.0, and 180 rpm.

column (250 × 4 mm id, 5-µm particle size) using an HPLC model HP-1100 (Agilent) equipped with a variable-VIS detector at room temperature. The mobile phase comprised a mixture of methanol and 0.01% acetic acid (65:35), and HPLC was performed isocratically at a flow rate of 1 mL/min. The eluate was continuously monitored by an ultraviolet detector at 270 nm.

After the bioconversion broth was extracted three times with ethyl acetate, the extract was dried over anhydrous Na_2SO_4, evaporated under vacuum, and then dissolved with methanol. The resultant solution was filtrated through a 0.45-µm nylon filter and subjected to HPLC analysis.

Results and Discussion

Effect of Amount of Substrate on Vanillin Production

Figure 2 shows the effect of the amount of substrate on conversion. The production of vanillin rose with the increase in the amount of substrate up to 25 g/L. However, the concentration of vanillin did not increase further when the amount of substrate exceeded 25 g/L, probably as a result of substrate inhibition or of the poor dispersibility of isoeugenol.

A total of 24 g/L of substrate was added to flasks in different modes, and the results are given in Table 1. It is suggested that there was no substrate inhibition because different substrate addition modes did not affect the bioconversion. Therefore, the poor dispersibility of isoeugenol was responsible for the tendency shown in Fig. 2.

Table 1
Effect of Addition Mode of Substrate
on Conversion of Isoeugenol Into Vanillin[a]

	Addition mode			
	24 g/L × 1	12 g/L × 2	8 g/L × 3	6 g/L × 4
Vanillin (g/L)	0.766	0.786	0.742	0.749

[a]Bioconversion was carried out in 250-mL flasks containing 20 mL of crude enzyme solution at 28°C, pH 9.0, and 180 rpm.

Table 2
Effect of Different Absorbents
on Bioconversion of Isoeugenol Into Vanillin[a]

Absorbent	Vanillin (g/L)
Control	0.702
Activated carbon	
Powdered	1.812
Granule	0.756
Resin	
D4020	1.163
X-5	1.013
HPD600	0.966
NKA-9	0.907
HZ816	0.907
DA201	0.895
HPD300	0.836
HPD100A	0.779
D-900	0.708
201x7	0.579

[a]Bioconversion was carried out in 250-mL flasks containing 20 mL of crude enzyme solution and 20 g/L of isoeugenol at 28°C, pH 9.0, and 180 rpm.

In the following experiments, the amount of substrate was kept at 20 g/L.

Screening for Absorbents

Various absorbents with a concentration of 10 g/L were added to improve substrate dispersibility and thus promote conversion. Except for 201 × 7, which is a strongly acidic cation, all the other tested resins are attributed to macroporous absorption. As shown in Table 2, powdered activated carbon was the best absorbent for vanillin production. Differences in the absorbents' dispersibility may be responsible for this result. Powdered activated carbon, with the best dispersibility, helped the enzyme contact with the absorbed substrate most effectively.

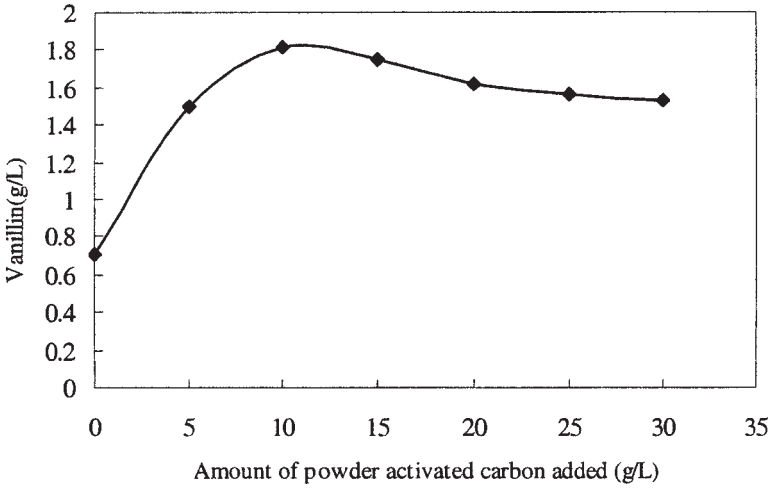

Fig. 3. Effect of amount of powdered activated carbon on bioconversion of isoeugenol into vanillin. Bioconversion was carried out in 250-mL flasks containing 20 mL of crude enzyme solution and 20 g/L of isoeugenol at 28°C, pH 9.0, and 180 rpm.

Effect of Amount of Powdered Activated Carbon on Vanillin Production

Conversion was performed with different amounts of powdered activated carbon added. The optimum amount of powdered activated carbon for conversion was found to be 10 g/L (Fig. 3).

Effect of H_2O_2 on Vanillin Production

A lack of oxygen is often an important limiting factor for aerobic processes. Oxygen supply is often improved through better reactor design and operational processes. Common measures taken include increasing stirring speed or aerating speed, using pure oxygen, or increasing reactor pressure. However, limited improvement has occurred because of the poor solubility of oxygen or of other mechanical and operational factors. Recently, some new methods have been used to improve oxygen supply, such as the addition of oxygen carrier and H_2O_2 *(18,19)*.

In this process, a high rotating speed can accelerate the flocculation of crude enzyme solution, so some other measures should be taken to improve oxygen supply. H_2O_2 can release oxygen by catalase or peroxidase. The oxygen molecules produced can be transferred rapidly owing to little resistance when the reaction rate is not very high. In addition, H_2O_2 is cheap, has low toxicity, and is convenient for the extraction of product from the reaction mixture. H_2O_2 may, however, be harmful to enzyme, which should be considered.

Effect of H_2O_2 Concentration on Enzyme Activity and Production of Vanillin

At different H_2O_2 concentrations, enzyme activity was tested after 7.5 h at 28°C, pH 9.0, and 180 rpm. As shown in Fig. 4, enzyme activity did not decrease when the concentration of H_2O_2 was <8.83 mmol/L.

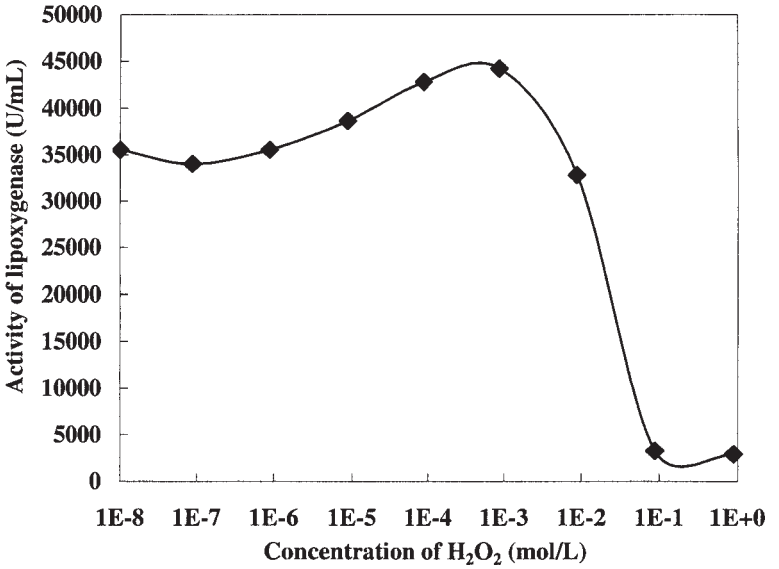

Fig. 4. Effect of H_2O_2 on activity of lipoxygenase.

Table 3
Effect of H_2O_2 Concentration on Bioconversion of Isoeugenol Into Vanillin[a]

	H_2O_2 (mmol/L)					
	0	8.83E-3	8.83E-2	0.883	8.83	88.3
Vanillin (g/L)	0.712	0.782	0.947	1.241	0.470	0.231

[a]Bioconversion was carried out in 250-mL flasks containing 20 mL of crude enzyme solution and 20 g/L of isoeugenol at 28°C, pH 9.0, and 180 rpm.

The concentrations of vanillin were tested after conversion with different amounts of H_2O_2 added, and the results are given in Table 3.

It can be concluded that H_2O_2 can accelerate conversion in a suitable concentration range. However, too much H_2O_2 is harmful to bioconversion because it can inactivate enzyme. The optimum concentration was 0.883 mmol/L (0.1% [v/v]).

According to Sheldon *(18)* and Joo et al. *(19)*, cytochrome oxidase P450 includes an iron-containing porphyrin group and can transfer electrons by the cycle of Fe^{2+} and Fe^{3+}. At the same time, it can insert an oxygen atom into its substrate. However, a complex coenzyme factor regeneration system is needed and can reduce the rate of oxidation reaction. It has now been found that cytochrome oxidase P450 can use an H_2O_2 shunt pathway to insert an oxygen atom into alkanes directly *(18)* (Fig. 5). This pathway is more effective and more rapid because the porphyrin complex in the enzyme transforms directly from the reductive state to the oxidative state without the need for coenzyme factor. Cytochrome oxidase P450 can function in this

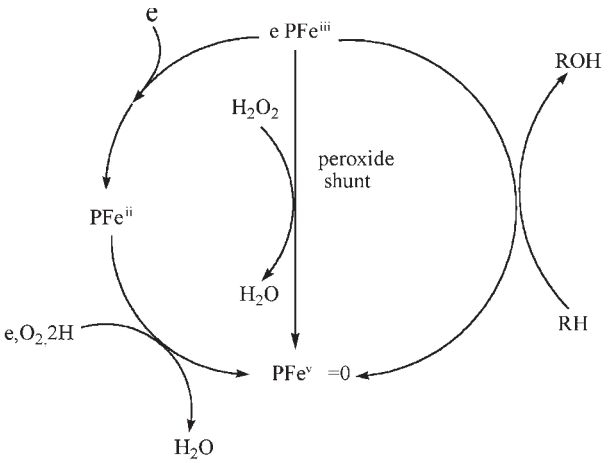

Fig. 5. Process of alkane oxidation catalyzed by cytochrome oxidase P450. (From ref. *18*.)

Table 4
Effect of Addition of Both Powdered Activated Carbon and H_2O_2 on Bioconversion of Isoeugenol Into Vanillin[a]

Sample	Control	0.1% H_2O_2	10 g/L of powdered activated carbon	0.1% H_2O_2 + 10 g/L of powdered activated carbon
Vanillin (g/L)	0.702	1.241	1.812	1.867

[a]Bioconversion was carried out in 250-mL flasks containing 20 mL of crude enzyme solution and 20 g/L of isoeugenol at 28°C, pH 9.0, and 180 rpm.

way with H_2O_2 added. However, this pathway is not very effective under normal conditions and may be a weakened pathway coexisting with mainstreams. The oxidation reaction can be accelerated in this effective and stable pathway only if this pathway is strengthened.

Lipoxygenase is also a kind of oxidase and has many properties in common with cytochrome oxidase P450. For example, it contains a nonporphyrin iron ion and is active only if the iron ion is of reductive state. Therefore, it is hypothesized that lipoxygenase also has an H_2O_2 shunt pathway though further research is expected.

It has already been found that the activity of lipoxygenase can be induced by H_2O_2 under some conditions *(20)*.

Effect of Addition of Both Powdered Activated Carbon and H_2O_2 on Production of Vanillin

The effect of the addition of both powdered activated carbon and H_2O_2 on bioconversion was studied. As shown in Table 4, vanillin concentration increased significantly after the addition of carbon and H_2O_2.

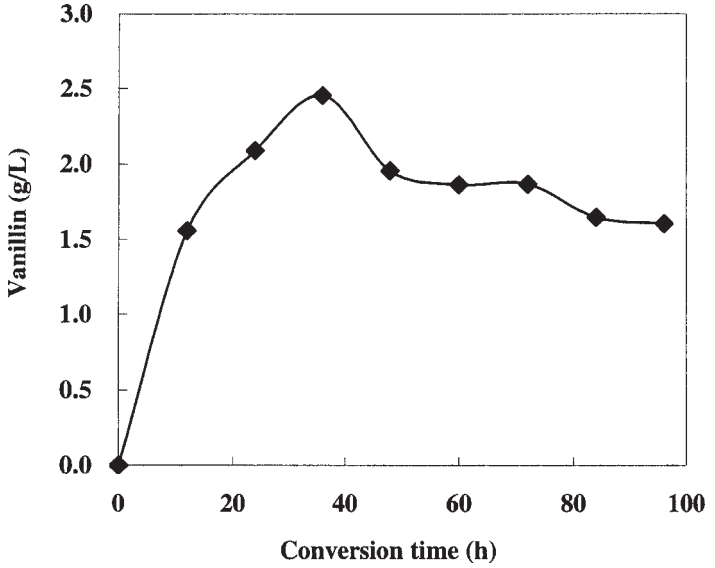

Fig. 6. Bioconversion of isoeugenol into vanillin by crude enzyme extracted from soybean. Bioconversion was carried out in 250-mL flasks containing 20 mL of crude enzyme solution and 20 g/L of isoeugenol at 28°C, pH 9.0, and 180 rpm with 10 g/L of powdered activated carbon and 0.1% H_2O_2.

Production of Vanillin From Isoeugenol

Using 20 g/L of isoeugenol as substrate and 20 mL of crude enzyme as catalyst, the conversion of isoeugenol into vanillin was performed in 250-mL flasks at 28°C, pH 9.0, and 180 rpm with 10 g/L of powdered activated carbon and 0.1% H_2O_2 added. Figure 6 shows the change in vanillin production in the conversion process. After 36 h, the concentration of vanillin reached 2.46 g/L, corresponding to a molar yield of 13.3%.

Conclusion

We studied the effect of several factors on the bioconversion of isoeugenol into vanillin by crude enzyme extracted from soybean. Bioconversion was performed in 250-mL flasks containing 20 mL of crude enzyme solution from soybean at 28°C, pH 9.0, and 180 rpm. The results revealed that the amount of substrate affected bioconversion. The addition of H_2O_2 and absorbent could also improve conversion. With 10 g/L of powdered activated carbon and 0.1% H_2O_2 added, vanillin reached a maximum concentration of 2.46 g/L after 36 h, corresponding to a molar yield of 13.3%.

For this bioconversion, purification steps of crude enzyme were determined and the cost of the enzyme was acceptable, with enzyme stability and conversion efficiency guaranteed. Furthermore, the substrate was cheap and the product concentration and yield were acceptable. Hence, vanillin obtained by this process is not very expensive and may be more competitive than those by other bioconversion processes.

It is worth mentioning that nonenzymatic oxidation from isoeugenol to vanillin was observed under strong acidity and basicity. However, the degree of unprompted oxidation is not high around neutral pH. Nonenzymatic oxidation should be avoided in enzymatic conversion. The mechanism of this conversion process is still under investigation.

Acknowledgment

We appreciate the financial support for this research from the State Key Basic Research and Development Plan of China (no. 2003CB716008).

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