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Production of vanillin by metabolically engineered *Escherichia coli*

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Abstract

E. coli was metabolically engineered to produce vanillin by expression of the *fcs* and *ech* genes from *Amycolatopsis* sp. encoding feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase, respectively. Vanillin production was optimized by leaky expression of the genes, under the IPTG-inducible *trc* promoter, in complex 2YT medium. Supplementation with glucose, fructose, galactose, arabinose or glycerol severely decreased vanillin production. The highest vanillin production of 1.1 g l^{-1} was obtained with cultivation for 48 h in 2YT medium with 0.2% (w/v) ferulate, without IPTG and no supplementation of carbon sources.

Introduction

Vanillin is a widely used flavor compound in food and personal products, with an estimated annual worldwide consumption of over 12 000 tons (Krings & Berger 1998, Lomascolo *et al.* 1999). Natural vanilla flavor from the orchid, *Vanilla planifolia*, supplies less than 1% of the total demand for vanillin (Prince & Gunson 1994). Therefore, there is a growing interest to produce "natural" vanillin from natural substrates by biotransformation. The value of vanillin extracted from vanilla pods is variously calculated as being between US$ 1200 kg^{-1} and US$ 4000 kg^{-1}, in contrast to the price of synthetic vanillin at under US$ 15 kg^{-1} (Lomascolo *et al.* 1999, Muheim & Lerch 1999).

Ferulic acid, which is abundantly available from several sources, is a potential substrate for bioconversion to vanillin and can be accomplished by several microorganisms including *Amycolatopsis* sp. (Achterholt *et al.* 2000), *Bacillus subtilis* (Peng *et al.* 2003), *Delftia acidovorans* (Plaggenborg *et al.* 2001), *Pseudomonas putida* (Plaggenborg *et al.* 2003), *Sphingomonas paucimobilis* (Masai *et al.* 2002) and *Streptomyces setonii* (Sutherland *et al.* 1983, Muheim & Lerch 1999). Ferulic acid can be converted to vanillin using the feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase (Figure 1). Ferulic acid is activated to the CoA thioester, catalyzed by *fcs*. Feruloyl-CoA is subsequently hydrated and cleaved to vanillin and acetyl-CoA. Both the reactions are catalyzed by *ech* genes. Vanillin, although produced from ferulic acid by the microorganisms, rapidly degrades after its formation. Therefore, *Escherichia coli* was investigated as vanillin producer by introducing the genes

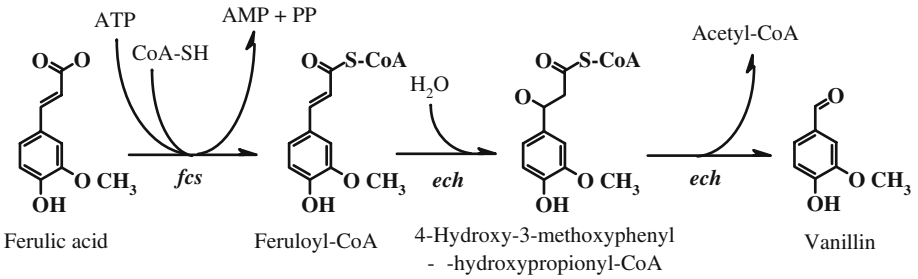

Fig. 1. Production pathway of vanillin from ferulic acid. Ferulic acid is converted to vanillin by feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase encoded by *fcs* and *ech*, respectively.

related to bioconversion of ferulic acid to vanillin, since it has no vanillin degradation pathway.

Materials and methods

Bacterial strains, plasmids, and culture conditions

E. coli strain DH5α was used for gene cloning and expression studies. *E. coli* was cultivated for 24 or 48 h at 37 °C and 180 rpm in 2YT medium (Sambrook *et al.* 2001), containing ampicillin of 100 μg ml^{-1} and ferulic acid (Sigma) at 0.2% (w/v). Glucose, fructose, galactose, arabinose, glycerol, lactose, and sucrose were added when indicated into the 2YT medium at 0.5% (w/v). Plasmid, pBAD24 (Guzman *et al.* 1995) and pTrc99A (Pharmacia) were used for gene cloning and vanillin production, respectively. IPTG induction for pTrc99A was carried out as indicated in Results.

PCR amplification and plasmid construction

The genes encoding feruloyl-CoA synthetase (*fcs*) and enoyl-CoA hydratase/aldolase (*ech*) were isolated by PCR from genomic DNA of *Amycolatopsis* sp. strain HR104 (DSM 9991). The sequences of primers for the genes were as follows: AHEF-F; 5′-GGCTAGCAGG AGCG**ATGCATGAGCACAGC**-3′ and AHEF-R; 5′-GGAATTCCCCTGG**TTAGCC GAAGCG**-3′. In these primer sequences, the coding regions are indicated by bold letters and the start or stop codons are underlined. Restriction sites, introduced to facilitate subcloning, are double underlined. The PCR was carried out using *pfx* DNA polymerase (Invitrogen) and a standard PCR protocol. The PCR product was digested with the *Nhe*I and *Eco*RI and cloned into pBAD24 to give pDAHEF. A DNA fragment containing *ech* and *fcs* was excised from pDAHEF with the *Nhe*I and *Bam*HI and introduced to the *Nco*I and *Bam*HI sites of pTrc99A to give pTAHEF. In this cloning, the *Nhe*I end of the DNA fragment and the *Nco*I end of the vector were treated with mung bean nuclease to make them bluntended. General procedures including restriction enzyme digestions, transformations, and other standard molecular biology techniques, were carried out as described by Sambrook *et al.* (2001).

HPLC quantitative analysis

Vanillin and ferulic acid were analyzed and quantitated by HPLC using a Symmetry C18 column (250×4.6 mm, 5 μm, Waters) with a multiphasic gradient at a flow rate of 1 ml min^{-1}. Solvent A was 20 mM sodium acetate, adjusted to pH 6, and solvent B was methanol. The proportion of solvent B rose from 0% at 0 min to 50% at 7 min and then decreased to 45% at 10 min, finally decreasing to 0% at 20 min. Quantitation was based on absorbance measurement at 260 nm.

Results and discussion

Effect of IPTG induction on vanillin production

Expression of *fcs* and *ech* genes under *trc* promoter of pTAHEF was controlled by IPTG. In order to know the optimal IPTG concentration for vanillin production, IPTG was initially added

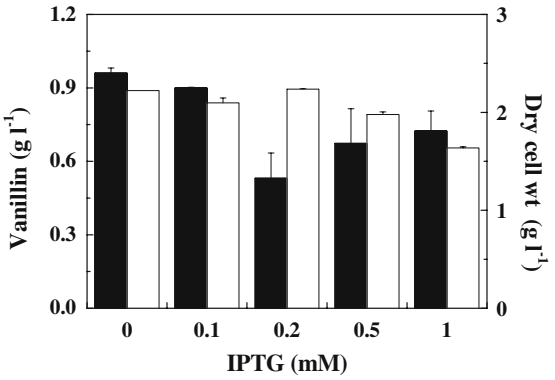

Fig. 2. Effect of concentration of inducer, IPTG, on vanillin production from the *E. coli* harboring pTAHEF in 2YT medium containing 0.2% (w/v) ferulic acid. Vanillin and cell growth are represented as solid and open bars, respectively.

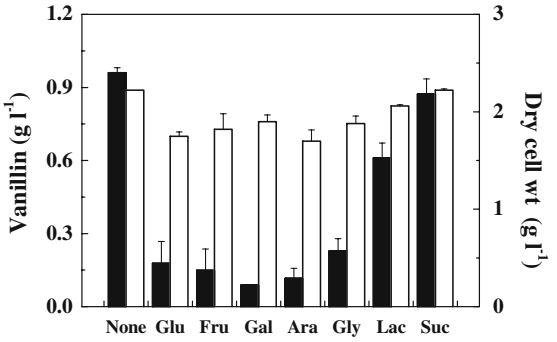

Fig. 3. Effect of carbon sources on vanillin production. Various carbon sources of glucose, fructose, galactose, arabinose, glycerol, lactose and sucrose were initially added into the culture medium at 0.5% (w/v). Vanillin and cell growth are represented as solid and open bars, respectively.

into 2YT medium up to 1 mM and the culture was carried out for 24 h (Figure 2). The highest vanillin production of 0.96 g l^{-1} was obtained without IPTG, which suggested the biotransformation of ferulic acid to vanillin was efficient and due to leaky expression of *fcs* and *ech* genes in 2YT rich medium. Cell growth was 2.2 g l^{-1} without IPTG, which was slightly decreased to 1.6 g l^{-1} at 1 mM IPTG. The decreased cell growth might be due to metabolic burden observed usually in highly induced condition. However, there was no significant difference on the amount of vanillin produced per gram of biomass with or without IPTG (0.43 and 0.44 g vanillin per gram dry cell wt, respectively).

Supplementation of carbon sources on vanillin production

Various carbon sources were added into 2YT medium at 0.5% (w/v) and the culture was carried out for 24 h (Figure 3). The highest production of vanillin was observed without supplementation of carbon sources. Supplementation of carbon sources, except lactose and sucrose, caused severe decrease of vanillin production. However, cell growth was not significantly affected by carbon sources. The severe decrease of vanillin production occurred only with metabolizable carbon source since *E. coli* DH5α cannot metabolize lactose or sucrose due to the absence of β-galactosidase and invertase genes. Further investigation should examine why the metabolizable carbon sources inhibited the biotransformation of ferulic acid to vanillin. The *fcs* and *ech* gene was also transferred to pBAD24 expression vector whose induction was controlled by arabinose. Maximum production of vanillin was 0.16 g l^{-1}, obtained with induction of 0.2% (w/v) arabinose (data not shown here). Based on the above results, it was concluded that vectors with sugar controllable promoter might not be appropriate for vanillin production from *E. coli*.

Time course of vanillin production

Vanillin production was carried out for 48 h without supplementation of carbon source and IPTG (Figure 4). Although cell growth ceased after 12 h, vanillin production continued until 48 h. Ferulate was rapidly consumed before 18 h and remained at 0.57 g l^{-1} after 48 h. Vanillin at 1.12 g l^{-1} was produced after 48 h with a conversion yield of 0.78 g vanillin per gram of ferulate. As far as we know, the used *E. coli* for vanillin production, with application of metabolic engineering techniques, has not been previously reported. It has only been reported that resting cell conversion using recombinant *E. coli* harboring *fcs* and *ech* genes produced 0.35 g vanillin l^{-1} (Achterholt *et al.* 2000). Therefore, we report here the highest vanillin production from *E. coli* with the application of metabolic engineering. Since *E. coli* has no vanillin degradation pathway and no formation of vanillin derivatives such as vanillic acid and vanillyl alcohol, *E. coli* might be

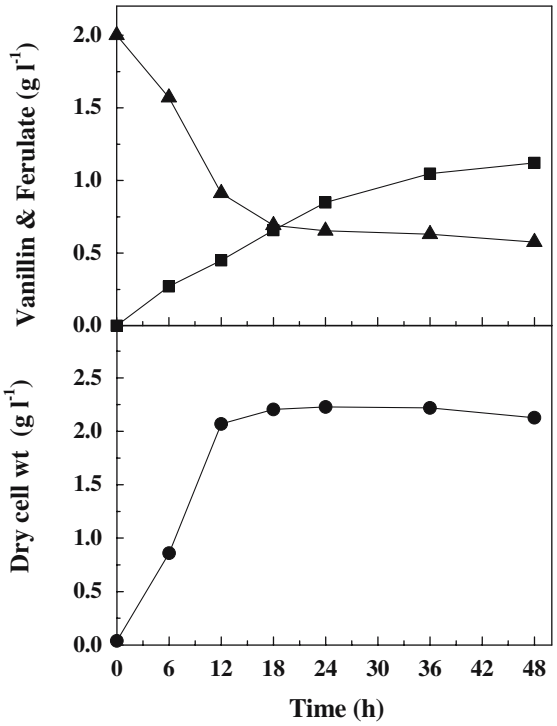

Fig. 4. Time-course of vanillin production (■), ferulate consumption (▲), and cell growth (●) in the recombinant *E. coli* in 2YT medium with 0.2% (w/v) ferulic acid, no IPTG, and no supplementation of carbon sources.

a good candidate host strain for production of high purity and quality vanillin in high yield.

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