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Production of Vanillin from Ferulic Acid Using Recombinant Strains of *Escherichia coli*

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Abstract Vanillin is one of the world's principal flavoring compounds, and is used extensively in the food industry. The potential vanillin production of the bacteria was compared to select and clone genes which were appropriate for highly productive vanillin production by *E. coli*. The *fcs* (feruloyl-CoA synthetase) and *ech* (enoyl-CoA hydratase/aldolase) genes cloned from *Amycolatopsis* sp. strain HR104 and *Delftia acidovorans* were introduced to pBAD24 vector with P_{BAD} promoter and were named pDAHEF and pDDAEF, respectively. We observed 160 mg/L vanillin production with *E. coli* harboring pDAHEF, whereas 10 mg/L of vanillin was observed with pDDAEF. Vanillin production was optimized with *E. coli* harboring pDAHEF. Induction of the *fcs* and *ech* genes from pDAHEF was optimized with the addition of 13.3 mM arabinose at 18 h of culture, from which 450 mg/L of vanillin was produced. The feeding time and concentration of ferulic acid were also optimized by the supplementation of 0.2% ferulic acid at 18 h of culture, from which 500 mg/L of vanillin was obtained. Under the above optimized condition of arabinose induction and ferulic acid supplementation, vanillin production was carried out with four different types of media, M9, LB, 2YT, and TB. The highest vanillin production, 580 mg/L, was obtained with LB medium, a 3.6 fold increase in comparison to the 160 mg/L obtained before the optimization of vanillin production.

Keywords: vanillin, ferulic acid, metabolic engineering, recombinant *E. coli*

INTRODUCTION

Vanillin is a flavor compound which is widely used in food and personal products, with an estimated annual worldwide consumption of over 12,000 tons [1,2]. Moreover, vanillin displays antimicrobial and antioxidant properties and is used as a food preservative and for medicinal purposes [3-6]. Natural vanilla flavor from the *Vanilla planifolia* orchid supplies less than 1% of the total vanillin demand [7]. Therefore, because of the increasing interest in natural products, alternative processes are being developed to produce natural vanillin [2,8-13]. The value of vanillin extracted from *Vanilla* pods ranges from $1,200 per kilogram and $4,000 per kilogram, in contrast to the price of synthetic vanillin, the value of which is less than $15 per kilogram [2,14]. In recent years, a large number of studies have been performed to biosynthesize natural vanillin using microorganisms or isolated enzymes [2,9-13]. However, these bioconversions are not yet economically feasible. The most intensively studied process for vanillin production by bioconversion, the product of which then can be labeled "natural", is based on ferulic acid (4-hydroxy-3-methoxy-*trans*-cinnamic acid) as the substrate. Ferulic acid is the most abundant hydroxycinnamic acid in the plant world and occurs mainly in cell walls which are covalently linked to lignin and other polymers. Several different microorganisms, such as *Amycolatopsis sp.* strain HR167 [15], *Bacillus subtilis* [16], *Delftia acidovorans* [17], *Pseudomonas putida* [18], *Sphingomonas paucimobilis* [19], and *Streptomyces setonii* [14,20], have been proposed for the production of vanillin from ferulic acid. More than 10 g/L of vanillin was obtained with the actinomycetes, such as *Amycolatopsis sp.* strain HR167 [21] and *Streptomyces setonii* [22]. The major drawbacks of the actinomycetes fermentation process are the high viscosity caused by mycelial growth and the frequent formation of spores during cultivation, both of which have a negative effect on overall productivity. Vanillin-producing microorganisms, including the actinomycetes, are also able to use vanillin as a source of carbon and energy,

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which causes rapid degradation of vanillin following its formation. Therefore, since *Escherichia coli* has a well-developed fermentation process [23], a powerful genetic tool system for metabolic engineering [24,25], and no vanillin degradation pathway, the microorganism was investigated as a vanillin producer through the introduction of genes related to the bioconversion of ferulic acid to vanillin with exogenous supply of ferulic acid.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The microorganisms used in this study were purchased from the following companies: *Delftia acidovorans* (DSM 39) and *Amycolatopsis sp.* strain HR104 (DSM9991) from DSMZ of Germany, *Pseudomonas putida* KT2440 (ATCC47054) and *Streptomyces setonii* (ATCC39116) from ATCC of USA, and *Sphingomonas paucimobilis* (KCTC1870) from KCTC of Korea. *E. coli* strain DH5α (F^-ϕ80d*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44λ$^-$ *thi*-1 *gyr*A96 *rel*A1) was used for gene cloning and expression studies. For the cultivation of *D. acidovorans*, nutrient broth (NB) medium (BD Scientific, USA) was used. GYM medium (0.4% glucose, 0.4% yeast extract, and 1% malt extract, pH 7.2) was used for *Amycolatopsis* sp. strain HR104, Luria-Bertani (LB) medium was used for *P. putida* KT2440, and YEME [26] was used for *S. setonii*. *E. coli* was grown on a shaking water bath at 180 rpm and 37°C in M9 minimal salts, LB, 2YT, and terrific broth (TB) medium, prepared as described by Sambrook *et al.* [27]. If necessary, carbon sources of glucose, galactose, fructose, and glycerol were added into the growth medium at concentrations of 0.5%. Ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL) were also added as necessary. Ferulic acid (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide and added to the medium at desired concentrations [17]. pBluescriptIISK+ (Stratagene, USA) and pBAD24 [28] plasmids were used for gene cloning and vanillin production, respectively.

PCR Amplification and Plasmid Construction

The genomic DNA from each strain was prepared using the DNeasy tissue kit (Qiagen, USA). The genes encoding the two enzymes of the vanillin synthetic pathway from ferulic acid, feruloyl-CoA synthetase (*fcs*) and enoyl-CoA hydratase/aldolase (*ech*), were isolated from genomic DNA by PCR. The sequences of primers were as follows: DAEF-F; 5′-GGCTAGCAAGGAAGCGCA**ATGAGCACC**-3′ and DAEF-R; 5′-TTCCGCTTTCCCTTCCTCCAC GAATTCC-3′ for *D. acidovorans*, and AHEF-F; 5′-GGCTAGCAGGAGCGATGC**ATGAGCACAGC**-3′ and AHEF-R; 5′-**CGACCCGGAGAAGTAA**TGCGCAGGAT CCCG-3′ for *Amycolatopsis* sp. strain HR104. In these primer sequences, the coding regions are indicated by bold letters and the start and stop codons are underlined. Restriction sites, introduced to facilitate subcloning, are double-underlined. PCR was carried out using *pfx* DNA polymerase (Invitrogen, USA) and a standard PCR protocol. Plasmid pDDAEF contains two *fcs* and *ech* genes from *D. acidovorans*, inserted into the *Nhe*I and *Eco*RI sites of pBAD24 [28]. Plasmid pDAHEF was constructed with the *fcs* and *ech* genes from *Amycolatopsis* sp. strain HR104 by *Nhe*I and *Bam*HI of pBAD24. General procedures, including restriction enzyme digestions, transformations, and other standard molecular biological techniques, were carried out as described by Sambrook *et al.* [27].

HPLC Quantitative Analysis

Metabolites were extracted to ethanol from whole culture broth for reverse-phased HPLC analysis using a Symmetry C18 column (250 × 4.6 mm, 5 μm, Waters, USA) with a Sentry guard column (20 × 4.6 mm, Waters, USA). Analysis was carried out using a Shimadzu HPLC system model 10A*vp* (Shimadzu, Japan) equipped with an LC-10AD*vp* Solvent Delivery system, a SPD-10AV*vp* UV/VIS detector, a CTO-10A*vp* column oven, and a Shimadzu SIL-10AD*vp* auto-injector. The condition of chromatography was determined by modification of the previously described method [29]. Twenty μL extracts were eluted with 20 mM sodium phosphate of solvent A, adjusted to pH 6.0, and methanol of solvent B by a multiphasic gradient at a flow rate of 1 mL/min. 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 receding to 0% at 20 min. Typical approximate retention times were 4 min for vanillic acid, 12 min for ferulic acid, and 13 min for vanillin. The eluted metabolites were detected at 260 nm and 40°C. The compounds were identified by comparing them with the peaks of standards, ferulic acid, vanillin, and vanillic acid (Sigma-Aldrich, USA), analyzed by dissolving in ethanol.

RESULTS AND DISCUSSION

Comparison of Vanillin-Producing Microorganisms

The CoA-dependent, non-β-oxidative pathway was identified for conversion of ferulic acid to vanillin in *Amycolatopsis sp.*, *Delftia acidovorans*, *Pseudomonas putida*, and *Sphingomonas paucimobilis*. The genes which compose the CoA-dependent pathway, *fcs* and *ech*, encode feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase, respectively (Fig. 1). Ferulic acid can be converted to vanillin using these enzymes. Ferulic acid is activated to the CoA thioester, which is catalyzed by *fcs*. Feruloyl-CoA is subsequently hydrated and cleaved to vanillin and acetyl-CoA. Both reactions are catalyzed by *ech*. Vanillin is further oxidized to vanillic acid by *vdh*-encoded vanillin dehydrogenase and proceeds to complete degradation as a carbon and energy source. Alternatively, ferulic acid degradation may be initiated by the shortening of the side chain for a C2 unit [30,31]. The direct deacetylation, which would yield directly vanillin, was proposed for *Bacillus subtilis* [32] and *Streptomyces setonii* [20]. In order

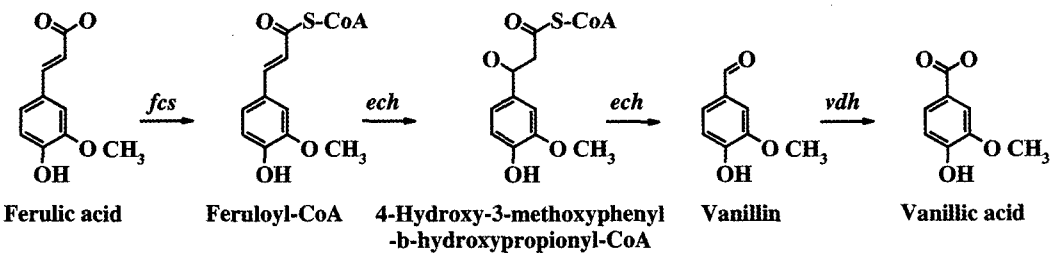

Fig. 1. Production pathway of vanillin from ferulic acid. Ferulic acid is catabolized to vanillin by the genes of *fcs*, feruloyl-CoA synthetase, and *ech*, enoyl-CoA hydratase/aldolase, *via* the route described as CoA-dependent deacetylation pathway. Vanillin is degraded to vanillic acid by the *vdh* (vanillin dehydrogenase) gene through the side chain reduction pathway.

Table 1. Production of vanillin from ferulic acid by various bacteria. Ferulic acid was added at a concentration of 0.2% in specific media for each bacteria, which were grown for 48 h

Strains	Ferulic acid (mg/L)	Vanillin (mg/L)	Vanillic acid (mg/L)
Amycolatopsis sp. strain HR104	-	-	-
Bacillus subtilis	220	-	7
Delftia acidovorans	-	69	650
Pseudomonas putida KT2440	520	-	-
Sphingomonas paucimobilis	150	-	-
Streptomyces setonii	17	488	46

to compare the vanillin production capabilities of those microorganisms, culture was carried out for 48 h under corresponding growth conditions with supplementation of 0.2% ferulic acid (Table 1). Ferulic acid was almost completely consumed by *Amycolatopsis* sp. HR 104, *D. acidovorans*, and *S. setonii*, whereas a significant amount of ferulic acid was left in cultures of *P. putida*, *B. subtilis*, and *S. paucimobilis*. This indicates that the former three bacteria have highly active genes which metabolize ferulic acid through vanillin. The highest amounts of vanillin and vanillic acid were observed in *S. setonii* and *D. acidovorans*, respectively. It was suspected that the activity of vanillin dehydrogenation in *D. acidovorans* was significantly higher than that of vanillin synthesis, and the opposite is true in the case of *S. setonii*. The activity of the complete degradation of ferulic acid beyond vanillin and vanillic acid in *Amycolatopsis* HR104 seemed the highest among the bacteria tested in this study, as there was no ferulic acid, vanillin, or vanillic acid was detected. Based on the above results, the vanillin production system of *Amycolatopsis* HR104, *D. acidovorans*, and *S. setonii* could be adopted for vanillin production in *E. coli*. By introducing genes related to vanillin synthesis into *E. coli* and excluding genes of vanillin degradation, high-yield, high-quality vanillin production could be achieved. However, the vanillin production pathway of *S. setonii* could not be used for the metabolic engineering of *E. coli*, because the pathway and related genes have not yet been identified.

Functional Expression of *fcs* and *ech* Genes in *E. coli*

The *fcs* and *ech* genes were amplified from *Amycolatopsis sp.* HR104 and *D. acidovorans* by PCR, and were transferred to pBAD24 expression vector, resulting in pDAHEF and pDDAEF, respectively. The expression of *fcs* and *ech* genes under the P_{BAD} promoter of the plasmids was controlled by arabinose. The P_{BAD} promoter is tightly regulated even in complex media, which is appropriate for metabolic engineering with precise control of gene expression [28]. Vanillin production from recombinant *E. coli* strains harboring pDAHEF or pDDAEF was investigated using 2YT medium containing 13.3 mM arabinose as an inducer and 0.1% ferulic acid as a sub-

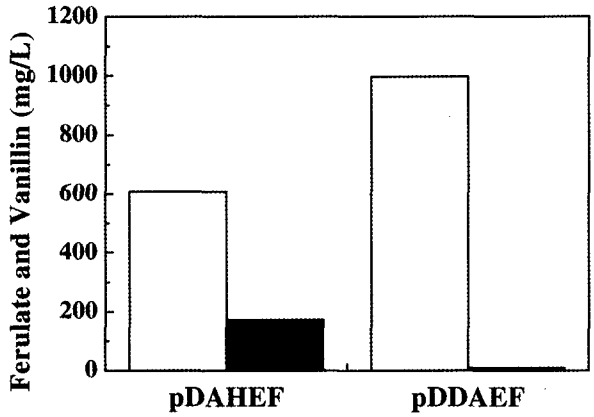

Fig. 2. Vanillin production of the recombinant *E. coli* harboring pDAHEF or pDDAEF. Culture was carried out in 2YT medium with 0.1% ferulic acid and 13.3 mM arabinose for 48 h. Ferulic acid and vanillin are represented as open and solid bars, respectively.

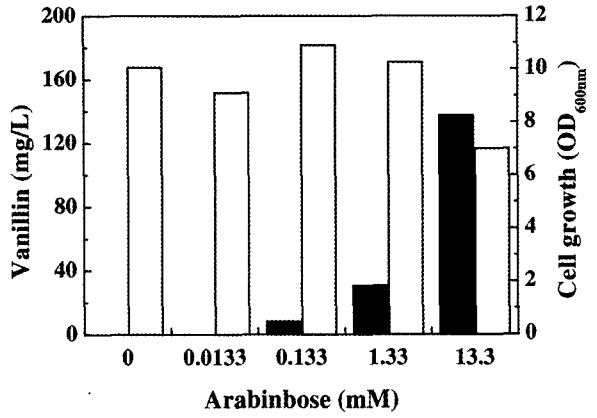

Fig. 3. Effect of concentration of an inducer, arabinose, on vanillin production from the *E. coli* harboring pDAHEF in 2YT medium containing 0.1% ferulic acid. Vanillin and cell growth are represented as solid and open bars, respectively.

strate (Fig. 2). After 48 h culture, 160 mg/L vanillin was obtained with pDAHEF, whereas a trace amount of 10 mg vanillin/L was obtained with pDDAEF. This finding suggests that *fcs* and *ech* genes of *Amycolatopsis sp.* HR104 are better for vanillin production in *E. coli* than those of *D. acidovorans*. Therefore, optimization of vanillin production was carried out with the recombinant *E. coli* harboring pDAHEF.

Effect of Induction Level and Time on Vanillin Production

In order to optimize induction conditions in the recombinant *E. coli* harboring pDAHEF, the effects of the concentration of inducer, arabinose, and the induction time on vanillin production were investigated. The recombinant *E. coli* was grown for 48 h at 37°C in 2YT medium containing 0.1% ferulic acid in the presence of different concentrations of arabinose, ranging from 0.013 mM to 13.3 mM (Fig. 3). Vanillin production was significantly increased as arabinose concentration increased to 13.3 mM to maximize induction of P_{BAD} promoter. Cell growth was slightly decreased at 13.3 mM arabinose, possibly be due to the metabolic burden caused by high level expression of *fcs* and *ech*. The optimal arabinose concentration for vanillin production was 13.3 mM. Induction time was optimized using 13.3 mM arabinose, which was supplied into the culture at different times between 0 and 24 h (Fig. 4). Even in complex media such as 2YT, there was no leaky expression of P_{BAD} promoter, and vanillin production commenced with the addition of arabinose. When arabinose induction was carried out at 12 to 24 h, vanillin was produced at an amount about 3-fold higher than that obtained with initial arabinose induction. Since vanillin has been reported to be toxic to microbial cells [33,34], it was suspected that arabinose induction at the initial growth phase was of no use to overall metabolism, including cell growth and vanillin production. When comparing cell growth at 12 h of culture, cell growth of

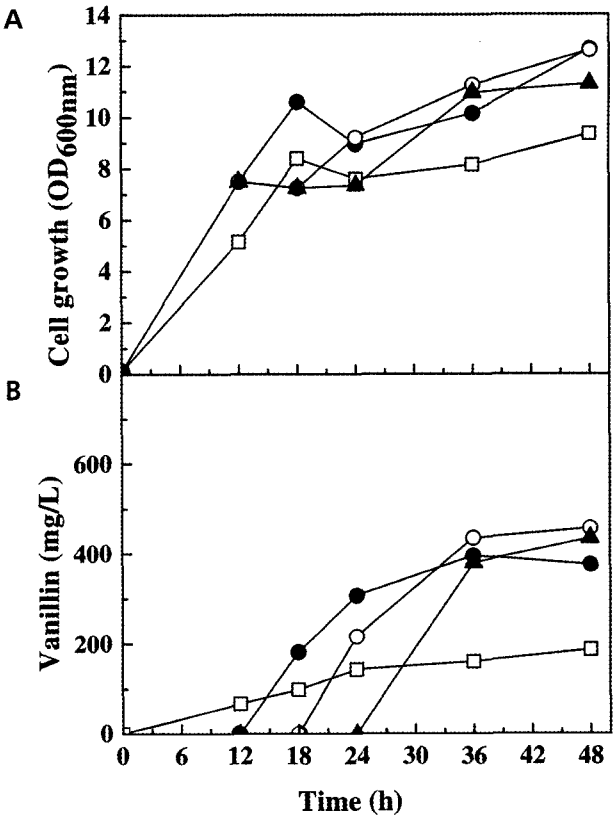

Fig. 4. Effect of induction time on cell growth (A) and vanillin production (B) of the *E. coli* harboring pDAHEF in 2YT medium containing 0.1% ferulic acid. 13.3 mM arabinose was added at a different times, 0 h (□), 12 h (●), 18 h (○), and 24 h (▲).

initial arabinose induction was significantly lower than cell growth at other times. Vanillin production of arabinose induction at 18 h was 450 mg/L, which was slightly higher than those obtained from induction at 12 and 24 h. Therefore, the optimal induction condition of vanillin synthetic genes was the addition of 13.3 mM arabinose at 18 h of cultivation.

Effect of Feeding Time and Concentration of Ferulic Acid on Vanillin Production

In order to determine the optimal feeding time of ferulic acid under the optimized condition of arabinose induction, it was fed initially, and at 18, 24, and 36 h (Fig. 5). Vanillin production was the highest with ferulic acid feeding at 18 h, which was also the arabinose induction time. Initial feeding with ferulic acid prior to arabinose induction was found to be useless. Ferulic acid feeding at 24 and 36 h also resulted in decreased vanillin production, because cells began to lose their metabolic activities, including vanillin synthesis in the stationary phase. With the use of ferulic acid at concentrations from 0.1 to 1.0%, fed at 18 h, culture was carried out for 48 h at 37°C. The vanillin production of 0.2% ferulic acid was 500 mg/L, which was

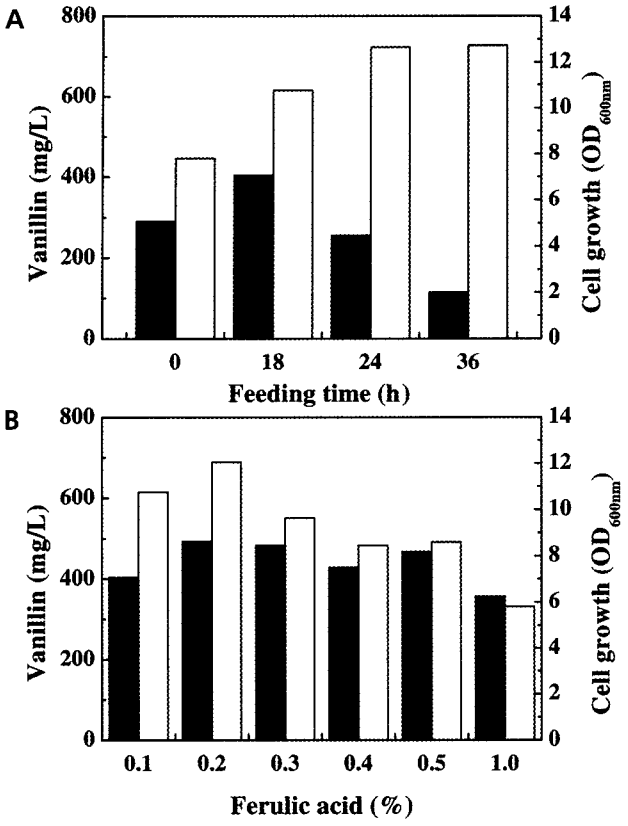

Fig. 5. Effects of feeding time and concentration of ferulic acid on vanillin production from the *E. coli* harboring pDAHEF under 13.3 mM arabinose induction at 18 h. Ferulic acid of 0.1% was fed at 0, 18, 24, and 36 h (A). Ferulic acid of 0.1 to 1.0% was fed at 18 h (B). Vanillin and cell growth are represented as solid and open bars, respectively.

slightly higher than the vanillin production at other concentrations. The lowest vanillin production was obtained with the addition of 1.0% ferulic acid, which was caused by the lowest cell growth. Since ferulic acid was dissolved in DMSO, causing the inhibition of cell growth at its high concentration, additions of high amounts of ferulic acid inevitably resulted in low cell growth. Increasing concentrations of ferulic acid did not increase vanillin production, which suggested that vanillin production might be limited by factors other than ferulic acid concentration.

Effect of Carbon Sources and Media on Vanillin Production

The effects of the addition of carbon sources on vanillin production were also investigated. A variety of carbon sources, such as glucose, fructose, galactose, and glycerol, were added to 2YT medium at concentrations of 0.5% (Fig. 6). Vanillin production was maximized due to increased cell growth with galactose as a carbon source. Approximately 550 mg/L of vanillin was obtained. However, with the supplementation of glucose, fructose, and glycerol, a significant decrease in vanillin production was

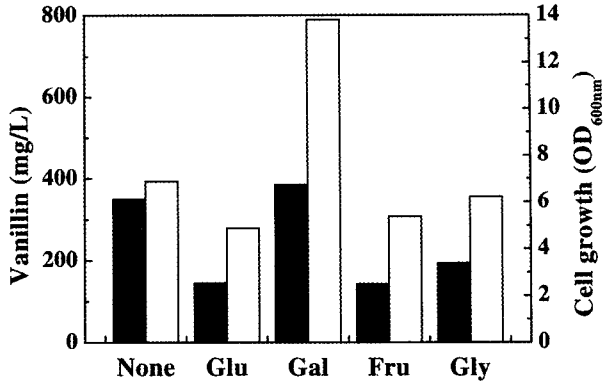

Fig. 6. Effect of carbon sources on vanillin production from the *E. coli* harboring pDAHEF with supplementation of 0.1% ferulic acid and 13.3 mM arabinose at 18 h. Various carbon sources of glucose, galactose, fructose, and glycerol were initially added into 2YT medium at concentrations of 0.5%. Vanillin and cell growth are represented as solid and open bars, respectively.

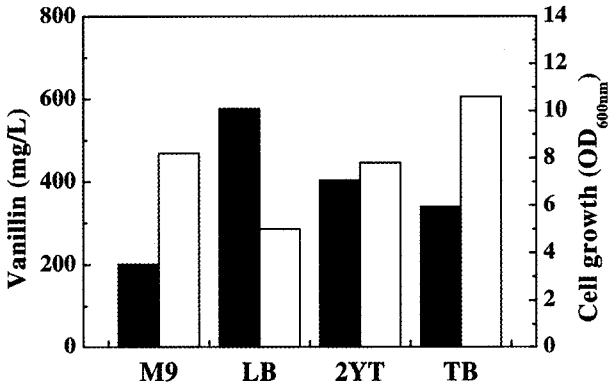

Fig. 7. Effects of media on vanillin production of the *E. coli* harboring pDAHEF. Ferulic acid and arabinose were added at 18 h as 0.1% and 13.3 mM, respectively. Vanillin and cell growth are represented as solid and open bars, respectively.

observed. Interestingly, the addition of some carbon sources significantly inhibited vanillin synthesis from ferulic acid. If we understood how the addition of carbon sources decreased the production of vanillin, this knowledge could be applied to increasing vanillin production in the opposite way.

With supplementation of 0.1% ferulic acid and 13.3 mM arabinose at 18 h, vanillin production was compared among M9, LB, 2YT, and TB media. Cell growth was the highest in TB, the richest medium, whereas the highest vanillin production of 580 mg/L was obtained with LB, the least rich medium among the complex media tested above (Fig. 7). The defined M9 medium did not produce adequate results on either cell growth or vanillin production. Therefore, if the optimization of culture media is carried out to balance cell growth and vanillin synthesis from the viewpoint of medium complexity, higher specific productivity of vanillin can be through optimization.

In this study, various vanillin-producing microorganisms were compared to clone genes which were efficient for vanillin production in *E. coli*. The recombinant *E. coli* harboring those vanillin synthetic genes might be good candidates for the commercial production of biovanillin. Since the pathway of vanillin synthesis from ferulic acid is a foreign pathway and there was no degradation pathway of vanillin, nor was there formation of byproduct derived from vanillin, it will be possible to get a high production yield of highly pure vanillin in *E. coli*. As far as we know, this is the first trial to use *E. coli* for the production of vanillin.

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