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GENETIC ENGINEERING OF *ESCHERICHIA COLI* TO ENHANCE BIOLOGICAL PRODUCTION OF VANILLIN FROM FERULIC ACID

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Abstract. *Escherichia coli* is considered a good host for the production of vanillin from phytochemicals, such as ferulic acid. A major drawback of this vanillin-producing system is the genetic instability of *E. coli* recombinant strains bearing genes encoding feruloyl-CoA synthetase and feruloyl-CoA hydratase/aldolase. In this work, the possibility to develop a more stable strain was explored by integrating into the *E. coli* chromosome the *Pseudomonas fluorescens* genes encoding these two enzymes. The resulting strain, named FR13, was more efficient in vanillin production than strains expressing the same genes from a low copy plasmid vector.

INTRODUCTION

X Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major organoleptic component of the vanilla flavour, which is extracted, through a long and expensive process, from the cured beans of *Vanilla planifolia*. It is one of the most common flavouring compounds in the food industry and its production reaches about 12000 tons per year. The current market demand of this flavor is supplied mostly using synthetic vanillin, chemically produced from guaiacol and lignin, while natural vanillin obtained from *Vanilla* accounts for less than 1% of the global market [1].

Rising demand for natural ingredients and the fact that plant derived vanillin is relatively expensive (2000-4000 $/Kg), has led to the investigation of other biotechnological routes such as the microbial production of this flavour from phytochemicals, such as ferulic acid: 3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid [2].

Ferulic acid is the most abundant cinnamic-related compound in the plant world, occurs mainly in the cell walls and is involved in crosslinking lignin to polysaccharides. In several ferulic-degrading bacteria, ferulic acid is first activated to feruloyl-CoA by a feruloyl-CoA synthetase, encoded by *fcs* gene, and then the CoA thioester is subsequently hydrated and cleaved to vanillin and acetyl-CoA by a enoyl-CoA hydratase/aldolase, encoded by *ech* gene. A number of microorganisms, such as *Amycolatopsis* sp. strain HR167 [3], *Pseudomonas putida* [4], *Streptomyces setonii* [5, 6], have been proposed for the production of vanillin from ferulic acid. Recently, recombinant *Escherichia coli* has been considered a good candidate for vanillin production and a lot of biocatalysts able to convert ferulic acid into vanillin were developed [7, 8]. A major drawback of *E. coli* vanillin-producing systems is the genetic instability of the recombinant strains that causes rapid declines in levels of vanillin production. In this work, a considerably more stable strain was developed by integrating the ferulic catabolic operon of *P. fluorescens* BF13 into the *E. coli* chromosome. Some

parameters influencing the bioconversion process were optimized by employing resting cells of this recombinant *E. coli* strain.

MATERIAL AND METHODS

E. coli JM109 was used for all standard cloning procedures, and as a host for the ferulic catabolic genes. JM109 was grown in Luria-Bertani (LB) broth at 44°C in Erlenmeyer flasks on an orbital shaker at 180 rpm. For bioconversion experiments, cultivation was performed at 30°C. Kanamicyn was added at a final concentration of 25 µg/mL for the growth of recombinant strains. Plasmid used for metabolic engineering of *E. coli* JM109 was generated inserting a 7715-bp *Sst*I fragment, which contained the ferulic catabolic operon of *P. fluorescens* BF13 (GenBank accession number AJ536325), into a low-copy temperature-sensitive plasmid. The resulting plasmid, designated pFR2 was introduced by transformation into *E. coli* JM109 cells.

For bioconversion experiments, cells were collected by centrifugation (6000 x *g* at 4°C for 10 minutes), washed twice in M9 saline/phosphate buffer (4.2 mM Na_2HPO_4; 2.2 mM KH_2PO_4; 0.9 mM NaCl; 1.9 mM NH_4Cl), and suspended in the same buffer amended with 0.5 mg/L yeast extract) in order to obtain a final concentration of biomass of 4.5 g (wet weight)/L. Biotransformations were performed in 100 mL flasks containing 10 mL of cell suspension supplemented with a sterile solution of ferulic acid (5 mM) and incubated on a orbital shaker at 180 rpm. Vanillin was quantified after a 24-hour incubation by liquid chromatography. Reuse of resting cells was performed in bioconversion cycles of 24 hr with 4.5 g/L biomass and 5.0 mM ferulic acid. Once the bioconversion finished, the cells were collected by centrifugation, washed and immediately used for the next bioconversion cycle. The reaction volume was adjusted to have the fixed amount of cells (4.5 g/L).

RESULTS AND DISCUSSIONS

Ferulic catabolic genes encoding feruloyl-CoA synthetase and feruloyl hydratase/ aldolase from *P. fluorescens* BF13 were cloned into a plasmid with a temperature-sensitive replicon, designed for chromosomal integration into the *lacZ* gene of *Escherichia coli*. The resulting plasmid, pFR12, was used to transform strain JM109 and cells with an integrated pFR12 plasmid could be selected at 44° C using a plasmid-encoded kanamycin resistance. Sequencing across the integration sites of six transformants demonstrated that in all cases the plasmid integration occurred at the *lacZ* locus.

As shown in Figure 1, the strain with the integrated ferulic catabolic genes, named FR13, was more efficient in vanillin production than a JM109 derivative (JM109/pBB1) expressing the same genes on a low copy number plasmid (4 copies per genome).

The growth temperature and the physiological state of the cells employed in the bioconversion were found to influence vanillin production yield. An overnight culture grown at 44°C was diluted in fresh medium and incubated at different temperature for 3 hours.

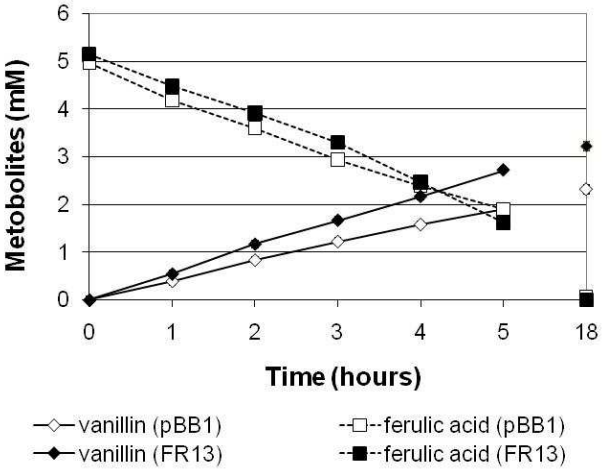

Fig. 1 Bioconversion of ferulic acid to vanillin using resting cells of *E. coli* JM109 derivatives carrying one (FR13) or four (pBB1) copies per genome of the ferulic catabolic genes from *P. fluorescens* BF13

Among the three temperature tested (44°C, 37°C and 30°C), the highest vanillin production was obtained with cells grown at 30°C, thus achieving a 75-fold increase in specific productivity with respect to corresponding values obtained with cells from cultures in stationary phase grown at 44°C (Table 1). The latter results were in agreement with Barghini *et al.* [8] who observed that a sub-physiological temperature of growth is an efficient strategy to increase vanillin production using *E. coli* strains expressing ferulic catabolic genes from a low-copy vector.

Table 1

Effect of the temperature of growth and the physiological state on the production of vanillin from ferulic acid using resting cells of *E. coli* FR13

Temperature of growth (°C)	Physiological state	Specific Productivity (mole/Kg/hour)
44	Stationary phase	0,004±0,001
44	Growth phase	0,050±0,001
37	Growth phase	0,056±0,001
30	Growth phase	0,299±0,004

Interestingly, pre-incubation of FR13 cells at low temperature (+4°C) prior using in bioconversion experiments allowed an increase in the vanillin production yield (Figure 2).

After 5 and 13 days of incubation at 4°C, a 12% to 30% improvement in the vanillin production was observed. This observation indicated that cells of *E. coli* FR13 can be stored at refrigerator temperature and used within two to three weeks, retaining their catalytic activity.

As the biomass production was costly and time-consuming, the possibility of reusing the applied resting cells was also evaluated. Resting cells, pre-incubated at 4˚C for 5 days, were reused four times achieving the vanillin production yields showed in Figure 3. Product yield remained over 50% until the fourth reuse.

Figure 3 also shows that the reuse of the cells permitted to obtain more than 6.6 Kg of vanillin per Kg of biomass.

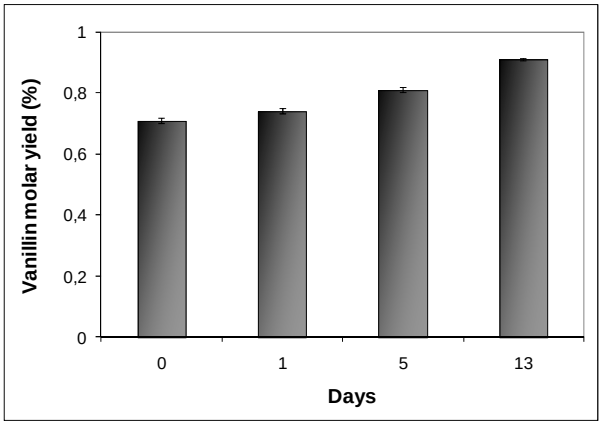

Fig. 2 Effect of low temperature treatments on vanillin production by resting cells of *E. coli* FR13

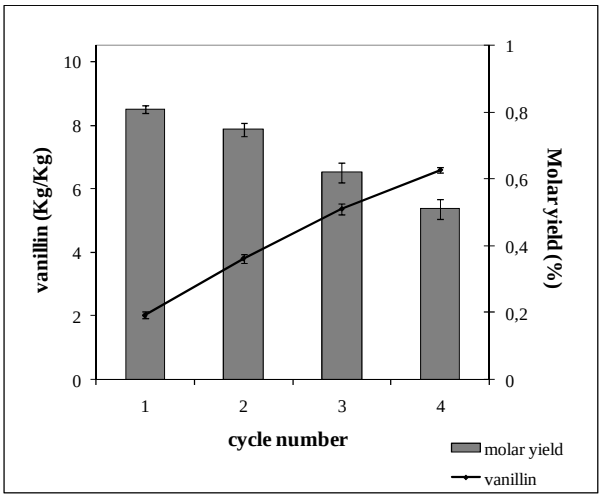

Fig. 3 Vanillin production after successive cells reutilization processes

CONCLUSIONS

An integrative vector was constructed to allow expression of genes encoding feruloyl-CoA synthetase and feruloyl-CoA hydratase/aldolase in *E. coli*. Optimisation of culture conditions to enhance the ability of the cells to convert ferulic acid to vanillin and of operational parameters, together with the reuse of the biomass, leaded to a final production of 6.6 Kg of vanillin per Kg (wet weight) of biomass, which is the highest found in the literature for *E. coli*.

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