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Metabolic engineering of *Pediococcus acidilactici* BD16 for production of vanillin through ferulic acid catabolic pathway and process optimization using response surface methodology

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Abstract Occurrence of feruloyl-CoA synthetase (*fcs*) and enoyl-CoA hydratase (*ech*) genes responsible for the bioconversion of ferulic acid to vanillin have been reported and characterized from *Amycolatopsis* sp., *Streptomyces* sp., and *Pseudomonas* sp. Attempts have been made to express these genes in *Escherichia coli* DH5α, *E. coli* JM109, and *Pseudomonas fluorescens*. However, none of the lactic acid bacteria strain having GRAS status was previously proposed for heterologous expression of *fcs* and *ech* genes for production of vanillin through biotechnological process. Present study reports heterologous expression of vanillin synthetic gene cassette bearing *fcs* and *ech* genes in a dairy isolate *Pediococcus acidilactici* BD16. After metabolic engineering, statistical optimization of process parameters that influence ferulic acid to vanillin biotransformation in the recombinant strain was carried out using central composite design of response surface methodology. After scale-up of the process, 3.14 mM vanillin was recovered from 1.08 mM ferulic acid per milligram of recombinant cell biomass within 20 min of biotransformation. From LCMS-ESI spectral analysis, a metabolic pathway of phenolic biotransformations was predicted in the recombinant *P. acidilactici* BD16 (fcs^+/ech^+).

Keywords *Pediococcus acidilactici* BD16 · Ferulic acid · Vanillin · Feruloyl-CoA synthetase · Metabolic engineering · LCMS-ESI

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Introduction

With the increasing consumer demand for natural food additives, biotechnological approaches for vanillin production have gained more interest in recent years, since they offer alternative way to produce this aromatic substance in a "natural" way. Until today, bioprocess for ferulic acid (FA) to vanillin biotransformation has been developed to an economically feasible scale (Overhage et al. 2003; Kaur and Chakraborty 2012). Vanillin production from FA has been reported in several microbial strains like *Amycolatopsis* sp. ATCC 39116 (Muhim et al. 2001; Devis et al. 2012), *Amycolatopsis* sp. HR167 (Achterholt et al. 2000), *Pediococcus acidilactici* (Kaur et al. 2013a, b), *Pseudomonas fluorescens* AN103 (Gasson et al. 1998), *Pseudomonas* sp. HR199 (Overhage et al. 1999b), *Rhodococcus* strains (Plaggenborg et al. 2006), *Sphingomonas paucimobilis* SYK-6 (Masai et al. 2002), *Streptomyces setonii* (Muhim and Lerch 1999), and *Streptomyces* sp. V1 (Yang et al. 2013). These strains generally use either coenzyme A-dependent, non-β-oxidative pathway or reductive pathway for FA bioconversion, which involves activity of feruloyl-CoA synthetase (Fcs) and enoyl-CoA hydratase/aldolase (Ech) enzymes. Vanillin production pathway was recently predicted in native *P. acidilactici* BD16 that indicates a key role of carboxylic acid reductase (Car) in vanillin production. Earlier, authors have reported use of ferulic acid esterase (Fae) producing *P. acidilactici* for the extraction of FA from rice bran and its further biotransformation to phenolic derivatives like vanillin (Kaur et al. 2013a, b). Native *P. acidilactici* BD16 is able to produce 1.26 g L^{-1} crude vanillin on RBM medium containing 150 g L^{-1} rice bran and 50 μg L^{-1} FA which is the highest yield reported so far in case of lactic acid bacteria (LAB) (Kaur and Chakraborty 2013). After extraction of the vanillin from the biotransformation medium, it was concentrated by rotary vacuum evaporation and crystallized. Using native *P. acidilactici*

BD16, 1.04 g L^{-1} crystal vanillin can be recovered from the biotransformation medium containing FA (unpublished data).

Earlier, several engineered *Escherichia coli* strains were adopted for vanillin production at laboratory scale, but genetic instability of the recombinant strains is a major drawback as it causes rapid decline in levels of vanillin produced. Recently, native *P. acidilactici* BD16 was shown to exhibit vanillin tolerance up to 7 g L^{-1} and was reported to possess high FA retention capacity which means FA as well as vanillin are not metabolized in other native metabolic pathways of the strain as the strain is lacking vanillin dehydrogenase (Vdh) activity also (unpublished data). Vdh enzyme causes rapid decline in the levels of vanillin produced in many native microbial strains which overcome vanillin toxicity by converting it into vanillyl alcohol and other phenolic products (Di Gioia et al. 2011). The above-stated property of many microbial systems is another major drawback of their use at an industrial scale.

Therefore, present study was undertaken with the aim to engineer a natural vanillin tolerant strain of *P. acidilactici* BD16 by introducing synthetic vanillin gene cassette bearing *fcs* and *ech* genes using shuttle vector pLES003 and optimization of FA to vanillin biotransformation process using cell extracts of recombinant *P. acidilactici* BD16 (fcs^+/ech^+). Some important process parameters that influence bioconversion process (like substrate concentration, biomass concentration, temperature, and time) were statistically optimized using response surface methodology (RSM). Finally, optimized process conditions were used for scale-up of FA to vanillin biotransformation, using enzyme extracts of 1 mg biomass and cell-free supernatant for intracellular and extracellular vanillin biotransformation in the recombinant system.

Materials and methods

Microorganism and growth conditions

P. acidilactici BD16 MTCC 10973 was used as a host for expression of FA catabolic genes viz. *fcs* and *ech*. Recombinant *P. acidilactici* BD16 (fcs^+/ech^+) was grown in de Man's Rogosa Sharpe Medium (MRS) broth (20 g L^{-1} dextrose, 10 g L^{-1} beef extract, 10 g L^{-1} peptone, 5 g L^{-1} sodium acetate, 5 g L^{-1} yeast extract, 2 g L^{-1} triammonium citrate, 2 g L^{-1} dipotassium hydrogen phosphate, 0.1 g L^{-1} magnesium sulfate, 0.05 g L^{-1} manganous sulfate, and 1 mL L^{-1} Tween 80, pH 5.6) under shaking conditions at 37 °C for 24 h. Erythromycin was added in the culture medium at a final concentration of 25 μg mL^{-1} for selection of recombinant strains.

Designing of synthetic vanillin gene cassette

Synthetic vanillin gene cassette containing genes encoding Fcs (converts FA to feruloyl-CoA) and Ech (converts feruloyl-CoA to vanillin) was designed by computer-assisted protocol using reference *ech* and *fcs* genes of *Amycolatopsis* sp. HR 167 vide GenBank accession number AJ290449.1 (Achterholt et al. 2000) and reverse translated as per codon choice of *P. acidilactici* by applying sequence manipulation suite 2 tools. In order to increase the activities of Fcs and Ech in recombinant *P. acidilactici* BD16 (fcs^+/ech^+) strain, an autoinducible *Pediococcus* promoter P_{289} (GenBank accession no. GQ214404) was integrated upstream to *fcs* gene. Other control regions such as RBS of *Pediococcus* sp., stop codons, and *Eco*R1 linkers were attached to the designed construct. Full-length sequence of vanillin biosynthesis gene cassette sent to GenScript Private Limited, USA, for its synthesis. Synthetic vanillin gene cassette (2,485 bp) was provided as a cloned construct on pCC1 vector in *E. coli* top10. It was deposited with GenBank database vide accession number KJ543568.

Subcloning of vanillin biosynthetic gene cassette and construction of recombinant plasmid pLES003 ($P_{289}/fcs^+/ech^+$)

The shuttle vector pLES003 used for subcloning vanillin biosynthetic gene cassette was constructed in a previous report by Wada et al. (2009). Plasmid pCC1 ($T_7/ech^+/fcs^+$) was isolated from *E. coli* top10 using Geneaid High-Speed Plasmid Mini Kit. Further, a 2,485-bp vanillin biosynthetic gene cassette ($P_{289}/ech^+/fcs^+$) containing synthetic *fcs* (1,473 bp) and *ech* (861 bp) genes flanked by *Eco*RI sites was subcloned into a shuttle vector pLES003 provided by Masafumi Noda (Assistant Professor, Hiroshima University, School of Life Science, Japan). Procedure involved simple *Eco*RI digestion of pCC1 ($T_7/ech^+/fcs^+$) and pLES003 using a reaction mixture containing 50 μg of plasmid DNA, 50 U of restriction enzyme and 1× *Eco*RI reaction buffer. Digestion was carried out at 37 °C for 3 h. Fragments of digested pCC1 ($T_7/ech^+/fcs^+$) were separated on 2 % agarose gel which was prepared by dissolving 1 g of agarose in 50 mL of 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) using 1× TBE as running buffer. Vanillin biosynthetic gene fragment of 2,485 kb was extracted from gel by using Genei gel extraction kit. It was further ligated into *Eco*RI linearized pLES003 using T_4 DNA ligase using 1 U enzyme per microgram of DNA at 25 °C overnight. Ligation was confirmed on 2 % agarose gel and visualized on UV transilluminator (Bio-Rad).

Preparation of competent cells of *P. acidilactici* BD16 and their transformation

P. acidilactici BD16 was inoculated in MRS broth and incubated at 37 °C for 24 h. After two subculturings, 1 %*v*/*v* of overnight grown pre-culture was transferred to 100-mL MRS medium. Cells were multiplied in MRS broth containing 3 %

glycine and 50 mM DL-threonine for 2 to 4 h at 37 °C to achieve absorbance (OD_{600}) of 0.4–0.6. Cultures were then kept on ice for approximately 15 min. Cells were harvested from 4 mL culture broth by centrifugation at 4,000 rpm for 5 min at 4 °C, washed twice in 2-mL washing solution (0.5 M sucrose and 10 % glycerol), and resuspended in 1 mL solution containing 0.6 M sucrose, 1 mM K_2HPO_4, 1 mM $MgCl_2$, pH 7.0 for 30 min. After centrifugation at 5,000 rpm, cells were resuspended 1 mL of $CaCl_2$ containing 15 % glycerol for storage at −70 °C in deep freezer (Caldwell et al. 1996).

One-hundred-microliter aliquot of competent cells were thawed and were mixed with 10 μL of recombinant plasmid DNA pLES003 (P_{289}/*ech*$^+$/*fcs*$^+$) and incubated on ice for 20–30 min. Eppendorf was placed at 45 °C in water bath for 2 min to provide heat shock for facilitating transformation of pLES003 (P_{289}/*ech*$^+$/*fcs*$^+$) into *P. acidilactici* BD16. One-milliliter outgrowth medium (MRS containing 0.5 M sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$) was added to eppendorf after heat shock and incubated at 37 °C for 1 h to active and to repair the cells. Recombinant *P. acidilactici* BD16 (*ech*$^+$/*fcs*$^+$) strains were selected on MRS agar media containing 25 μg/mL erythromycin.

Preparation of enzyme extracts

Recombinant strain of *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$) was grown in MRS broth containing 25 μg mL^{-1} erythromycin for 24 h at 37 °C. Cells were harvested at regular intervals viz. 6 h (early log phage of 0.5 OD), 8 h (log phage of 1 OD), 12 h (late log phage of 1.5 OD), 16 h (stationary phase of 2 OD), and 18 h (late stationary phase of 2.5 OD) by centrifugation and pellets were resuspended in 20 mM phosphate buffer (pH 7). Cell-free extracts from these pellets were prepared by disrupting cells by sonication (1 min/mL of cell suspension with maximum amplitude of 100 μm) with Sonics Vibra Cell Ultrasonic Disintegrator for subsequent enzyme assay. Each cell-free extract contained 100 μg mL^{-1} soluble protein (adjusted after suspending in 20 mM phosphate buffer, pH 7), which were taken for intracellular enzymatic assays and vanillin production. Similarly, cell-free supernatant (CFS) containing 100 μg mL^{-1} soluble protein (prepared in 20 mM phosphate buffer, pH 7) was taken for extracellular enzymatic assays and vanillin production. Formation of feruloyl-CoA by heterologously expressed Fcs and Ech enzymes was measured using optical enzymatic test as described previously (Narbad and Gasson 1998). Vanillin production was estimated in each extract using standard protocol of Converti et al. (2010).

Optimization of vanillin production using RSM

RSM software (Statacase Design Expert version 8.0.7.1) was used to optimize the bioconversion process by studying the effect of biomass, temperature, time, and substrate concentration (FA). Four studied parameters, (a) substrate as FA, (b) biomass, (c) time of assay, and (d) temperature, were entered into the central composite design (CCD) which generated a full-factorial screening design with a total of 30 experiments including 6 replicate center points (Table 1). Results obtained in CCD were analyzed in terms of three vanillin responses as extracellular, intracellular, and total yield of vanillin (nM).

Scale-up studies

Runs giving maximum vanillin responses in all the three sets were selected from CCD design. Components were scaled up to 1 L to validate vanillin responses in recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$). Biotransformation medium contained 100 mM potassium phosphate buffer (pH 7.0), 2.5 mM $MgCl_2$, 2.5 mM ATP, and 0.2 mM coenzyme A. Extracellular activity was carried out according to run 11 (shown in Table 1) and intracellular activity carried out according to run 25 (shown in Table 1). For extracellular assay, 200 μg mL^{-1} FA, and for intracellular assay, 10 μg mL^{-1} FA was added in the reaction mixture containing 100 μg mL^{-1} protein containing CFS and cell-free extract of recombinant culture harvested after 16 h at 35 °C and 18 h at 25 °C for respective assays. Amounts of feruloyl-CoA/vanillin were estimated at 346 nm at 10-min intervals for 1 h. Confirmatory experiments were carried out in triplicate, and final data was presented taking mean of all the three responses.

Enzymatic assay

Enzymatic activity was measured in the reaction mixture (1 mL) containing 100 mM potassium phosphate buffer (pH 7.0), 100 mM tris-hydrochloric acid, 2.5 mM $MgCl_2$, 10 μg mL^{-1} FA, 2.5 mM ATP, and 0.2 mM coenzyme A. As an enzyme source, 100 μg mL^{-1} protein extract containing 4.5 μL of supernatant and 7 μL of cell-free extract for extracellular and intracellular assays, respectively. The activity assay was started by the addition of ATP, and the initial increase in absorbance reflecting formation of feruloyl-CoA was measured at 346 nm using a UV-visible Thermo Fisher Scientific spectrophotometer using modified Narbad and Gasson 1998.

Kinetic study of Fcs

Specificity of enzyme toward different substrates is different. Some enzymes demonstrate high degree of specificity and some catalyze a reaction in only one direction (e.g., dehydrogenation), but some will carry out in both forward and reverse directions usually at different rates (e.g., hydrogenation in addition to dehydrogenation). That is why Km and Vmax, which represent specificity and rate of catalysis, are important

Table 1 Tabular representation of CCD run order

Run	Factor 1 A: Substrate (μg mL^{-1})	Factor 2 B: Temperature (°C)	Factor 3 C: Time (min)	Factor 4 D: Biomass (OD_{600})
1	10	25	10	1
2	105	15	35	1.5
3	105	35	35	2.5
4	105	35	35	1.5
5	200	25	10	2
6	105	55	35	1.5
7	10	25	10	2
8	200	45	60	2
9	105	35	35	1.5
10	10	45	60	2
11	200	25	60	2
12	105	35	35	1.5
13	105	35	0	1.5
14	200	25	10	1
15	10	45	10	2
16	105	35	35	1.5
17	10	45	60	1
18	295	35	35	1.5
19	200	45	10	2
20	105	35	35	1.5
21	105	35	85	1.5
22	10	25	60	2
23	200	25	60	1
24	10	45	10	1
25	10	25	60	1
26	105	35	35	0.5
27	105	35	35	1.5
28	0	35	35	1.5
29	200	45	60	1
30	200	45	10	1

factors for characterizing an enzymatic reaction. The above-stated reaction mixture containing different concentrations of FA (10 to 650 μg mL^{-1}) were used to determine kinetic parameters (Km and Vmax) of cloned Fcs/Ech enzymes in triplicate. CFS or cell-free extracts containing 100 μg mL^{-1} protein were used in each assay which was carried out at 30 °C. From the slope of Lineweaver Burk plots, Km and Vmax values were calculated (Yang et al. 2013).

Extraction of phenolics and metabolic profiling of recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$) culture by LCMS-ESI

After 20 min of biotransformation, biotransforming medium was acidified to pH 2 with 6 N HCl and phenolics were extracted from 1 L culture supernatant of recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$), and metabolites were partitioned into an equal volume of ethyl acetate (1:1, *v*/*v*). Ethyl acetate fraction was separated and concentrated by rotary vacuum evaporation at 50 °C. Solvent was completely evaporated, and phenolics were recovered in 5 mL 80 % (*v*/*v*) methanol and then used for metabolite analysis. Phenolic characterization was carried out using liquid chromatography/mass spectrometry on a LCMS instrument (Thermo, model LTQ-XL) using positive electron spray ionization software (Kaur et al. 2013b).

Results

Designing of synthetic gene construct and construction of recombinant plasmid pLES003 (P_{289}/*fcs*$^+$/*ech*$^+$)

From the recombinant vector pCC1 (T_7/*fcs*$^+$/*ech*$^+$), synthetic vanillin gene cassette of 2,485 bp bearing *fcs* and *ech* genes was amplified by PCR and digested with *Eco*RI to generate sticky ends. Recombinant plasmid pLES003 (P_{289}/*fcs*$^+$/*ech*$^+$) was constructed by ligating synthetic vanillin gene cassette with 6,134-bp long *Eco*RI linearized plasmid pLES003 (Fig. 1). During subcloning, a spontaneous mutation involving single base pair change (A to G) at nucleotide position 33 occurred in the promoter region of the synthetic construct. The sequence of the mutated vanillin gene construct in pLES003 (P_{289}/*fcs*$^+$/*ech*$^+$) was deposited in GenBank database (GenBank accession no. KJ543569).

For genomic mapping, recombinant plasmid pLES003 (P_{289}/*fcs*$^+$/*ech*$^+$) was digested with *Eco*RI and after electrophoresis, 2,485 bp synthetic fragment, native vector 6,134 bp, and total 8,619 bp recombinant vector were visualized in 0.8 % agarose gel (Fig. 2).

Optimization of vanillin production using RSM

Three-dimensional response surface curves were plotted for the vanillin in responses obtained in CCD design to study interaction between four response variables selected and to determine the optimum concentration of each for maximum vanillin yield.

Intracellular vanillin biosynthesis

Maximum intracellular vanillin yield obtained was 104.2 nM (run 25, containing 10 μg mL^{-1} FA and cell extract from biomass of 1 OD/mL at 25 °C for 60 min). Time has maximum influence on intracellular vanillin production, and highest production was achieved after 1 h. Vanillin production increased as incubation proceeded up to 85 min, and 66.8 nM vanillin was estimated during biotransformation reaction

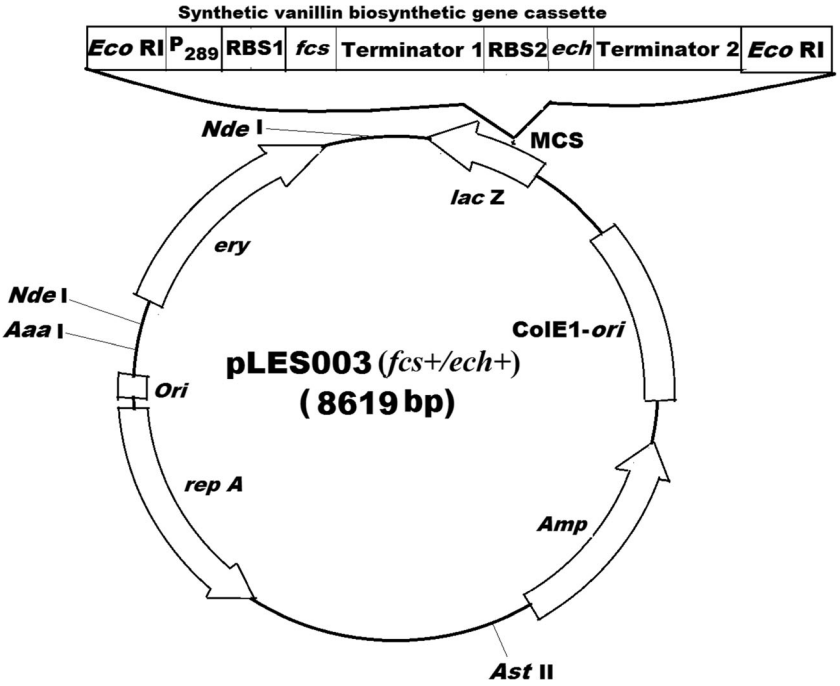

Fig. 1 Genetic features of recombinant pLES003 ($P_{289}/fcs^{+}/ech^{+}$)

performed as per run 21 (Table 2). Substrate concentration, temperature, and biomass influenced inversely but significantly in vanillin production as with increasing substrate concentration to 200 μg mL^{-1}, temperature to 45 °C, and biomass of 2 OD/mL, the intracellular vanillin levels dropped to 17.6 nM (run 23), 31.5 nM (run 17), and 15.7 nM (run 22), respectively (Table 2, Fig. S1).

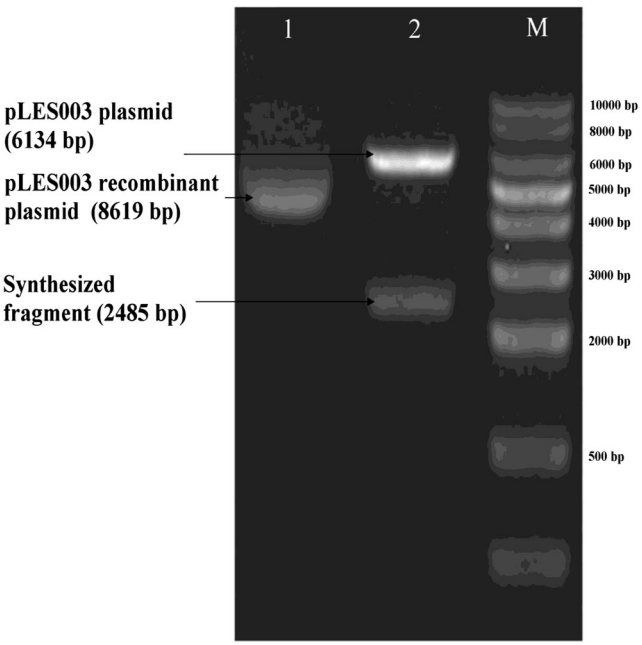

Fig. 2 Gel analysis of recombinant construct pLES003 ($P_{289}/fcs^{+}/ech^{+}$)—recombinant pLES003 (*lane 1*), recombinant pLES003 digested with *Eco*RI (*lane 2*), and KB ladder (*lane M*)

ANOVA analysis of intracellular vanillin response

The model *F* value of 4.17 implies that the model is significant. There is only a 0.48 % chances that a "model *F* value" this large could occur due to noise. Value of "prob>*F*" less than 0.05 indicates that the model terms are significant. In this case, model terms A, C, D, A^2, C^2, and D^2 are significant as they have profound influence on vanillin synthesis. The "lack of fit *F* value" is significant at a level of 76.46 (Table S1).

Extracellular vanillin biosynthesis

Highest vanillin molar yield was achieved by extracellular activity of the cloned FA catabolism enzymes, namely, Fcs and Ech which accounted for the production of 984.4 nM of vanillin (run 11, containing 200 μg mL^{-1} FA, enzyme preparation extracted from biomass 2 OD/mL at 25 °C for 60 min). The temperature at which bioconversion assay was carried out markedly influenced vanillin yields. Among the three temperatures tested, highest vanillin synthesis was achieved at 25 °C after 1 h of incubation. Time, FA concentration, and biomass concentration are positively and significantly correlated with vanillin production. With decrease in time from 60 to 10 min (run 5), biomass at 1 OD (run 23), and substrate concentration of 10 μg mL^{-1} (run 22), vanillin levels dropped to 5, 365.2, and 29 nM, respectively. Temperature has a negative influence on vanillin production as with increase in incubation temperature from 25 to 45 °C; vanillin production decreased to 199 nM (Table 2, Fig. S2).

Table 2 Vanillin responses obtained during CCD analysis

Run	Response 1		Response 2		Response 3	
	Extracellular activity (nM)		Intracellular activity (nM)		Total activity (nM)	
	Actual value	Predicted value	Actual value	Predicted value	Actual value	Predicted value
1	0	16	19.5	27.5	19.5	43.5
2	815	644.6	46	50.6	861	695
3	551	248	22.6	11.6	573.6	259.6
4	**779.6**	778	**70**	68.5	**846.5**	846.4
5	5.0	378.6	0	0	5.0	372.6
6	447.3	347.1	98.5	76.6	545.8	423.7
7	28.4	0	4.4	0	32.7	0
8	199	458.7	0	18.7	199	477.4
9	**780.6**	777.8	**70**	68.5	**850.6**	846.4
10	62.5	222.5	24.3	23.2	86.8	245.7
11	**984.4**	861.3	0	11.3	**984.4**	872.6
12	**774.7**	777.8	**70**	68.5	**844.7**	846.4
13	4.4	2.9	0	7.1	4.4	10
14	493	327.9	3.72	0	496.7	323.3
15	12.6	64.5	34.12	35.4	46.8	99.9
16	**775.5**	777.8	**68**	68.5	**843.6**	846.4
17	155.4	57.5	31.5	64.3	186.9	121.8
18	555.4	416.5	0	0	555.4	389.5
19	215.4	147	22.5	36.5	237.9	183.5
20	**778.3**	777.8	**69.5**	68.5	**847.8**	846.4
21	691.2	422	66.8	42.5	758	464.5
22	29	304.6	15.7	22.1	44.7	326.6
23	365.2	589.1	17.6	43	382.8	632.1
24	3.1	121.1	66.9	46.2	70	167.3
25	60	123.3	**104.2**	80.8	164.15	204
26	0	32.3	60.4	54.1	60.4	86.5
27	**778**	777.8	**66.8**	68.5	**844.8**	846.4
28	0	0	0	9.70	0	0
29	155.4	202.9	36.9	32.8	192.2	235.6
30	112.4	112.6	0	20.3	112.4	132.9

Values in bold represent the maximum vanillin production by intracellular, extracellular and total enzymatic response

ANOVA analysis for extracellular vanillin response

F value of 4.16 indicates a significant CCD model of extracellular response. There is only a 0.48 % chances that a model *F* value this large could occur due to noise. In this case, A, C, A^2, C^2, and D^2 are significant model terms as prob>*F* values of these terms found to be less than 0.05. The lack of fit *F* value of 13,045.5 is identified as significant (Table S2).

Total molar vanillin yields in recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$)

A very high extracellular enzymatic activity of cloned genes was credited for maximum vanillin synthesis (984.4 nM) in recombinant cell extracts as obtained in run 11 (Table 2). The total extracellular and intracellular vanillin yields were also comparable in case of run 2, 4, 9, 12, 16, and 20 (861, 846.4, 850.6, 844.7, 843.6, and 847.8 nM, respectively) containing 105 μg mL^{-1} FA using enzyme preparation extracted from biomass of 1.5 OD/mL at 35 °C for 35 min of incubation. A higher cell density (1.5 OD/mL), when the cells are in log phase, was found to generate maximum vanillin. A decrease in vanillin formation by extracellular activity (551 nM) (run 3) was observed when the cells were harvested at higher OD_{600} (2.5; cells under stationary phase), and no vanillin formation was observed at lower OD_{600} (0.5; cells in early exponential growth phase) as observed in run 26 (Table 2, Figs. S1, S2 and 3). Intracellular activity of cloned enzymes was totally suppressed in the presence of 200 μg mL^{-1} FA whereas extracellular activity at this time was found to be maximum

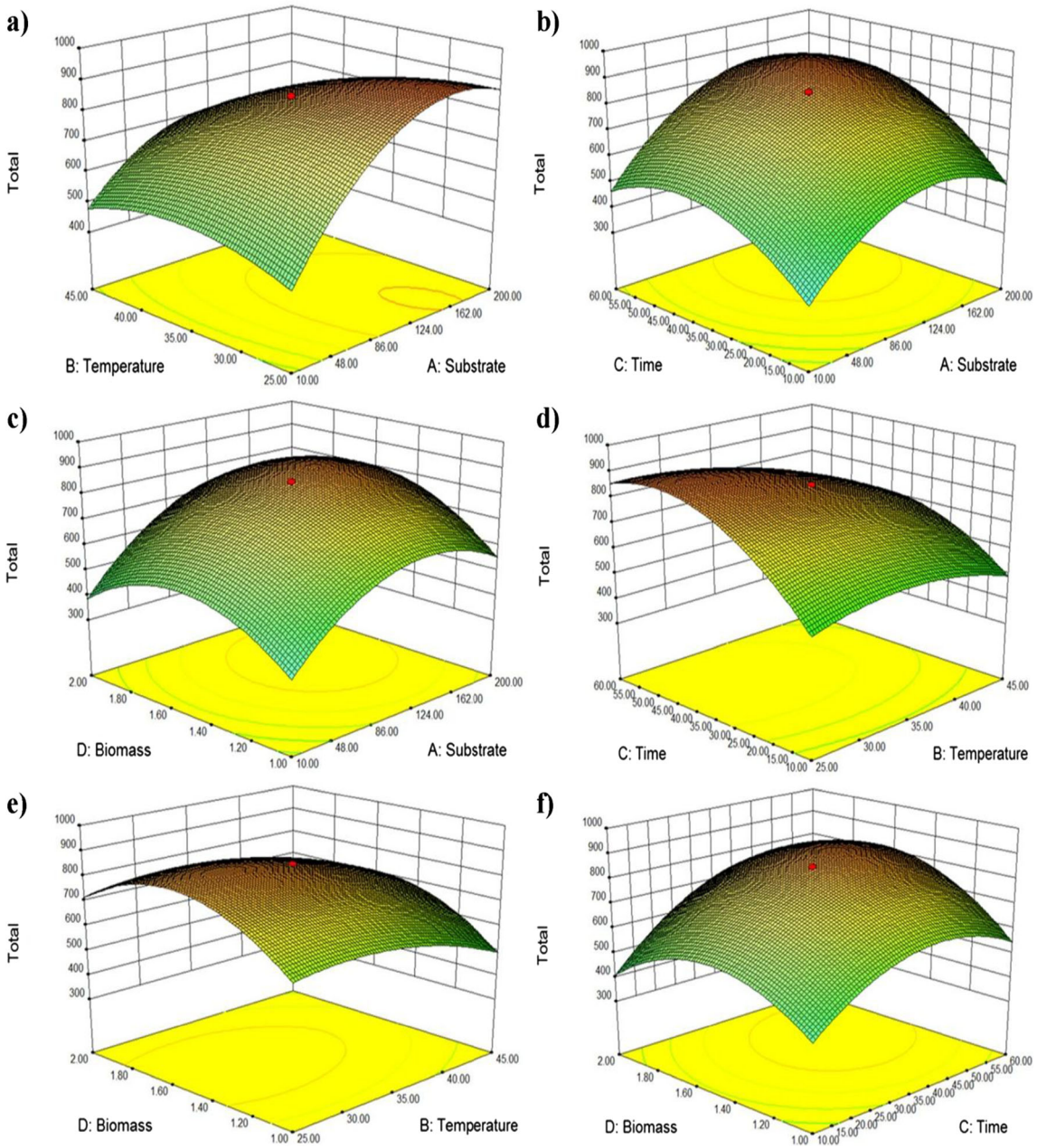

Fig. 3 Responses showing effect of variables on total vanillin production in recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$)

as indicated in run 11 (Figs. S1, S2, and 3). These results show that extracellular enzymatic activities of cloned genes have higher affinity for the substrate than enzyme expressed intracellularly. That is why vanillin production using extracellular enzymes extract was more than vanillin production using intracellular enzyme extract (Table 2).

ANOVA analysis of the final response

F value of 4.26 implies the model as significant and there is only a 0.43 % chances that a model *F* value this large could occur due to noise. Prob>*F* less than 0.05 value indicate significant model terms which are A, C, A^2, C^2, and D^2 in this case. The lack of fit *F* value of 10,767.79 is significant as there is only a 0.01 % chance of getting a random response (Table 3).

Scale-up studies and validation of the model

In recombinant *P. acidilactici* (*fcs*$^+$/*ech*$^+$), optimized conditions for vanillin biosynthesis were scaled up and 2.62 mM vanillin was obtained after 10 min of incubation with CFS (extracellular), and 0.57 mM vanillin was obtained after 20 min of incubation with cell-free extracts (intracellular).

Table 3 ANOVA analysis of total vanillin response obtained during CCD analysis

ANOVA analysis of CCD response variables

Source	Sum of Squares	CE	*df*	Mean square	SE	95 % CI Low	95 % CI High	*F* Value	Prob > *F*
Model	2.859E+006		14	2.042E+005				4.26	**0.004**
Intercept		846.35			89.42	655.75	1036.95		
A-FA	3.926E+005	127.90	1	3.926E+005	44.71	32.60	223.20	8.18	0.012
B-Temperature	1.105E+005	−67.85	1	1.105E+005	44.71	−163.15	27.45	2.30	0.15
C-Time	3.099E+005	113.63	1	3.099E+005	44.71	18.33	208.93	6.46	0.02
D-Biomass	44968.79	43.29	1	44968.79	44.71	−52.01	138.59	0.94	0.35
AB	98720.07	−78.55	1	98720.07	54.76	−195.27	38.17	2.06	0.17
AC	21968.43	37.05	1	21968.43	54.76	−79.66	153.77	0.46	0.51
AD	13903.36	29.48	1	8266.45	54.76	−87.24	146.19	0.29	0.6
BC	42470.00	−51.52	1	42470.00	54.76	−168.24	65.20	0.89	0.36
BD	1.70	0.33	1	1.70	54.76	−116.4	117.04	3.536E−005	1
CD	36579.43	47.81	1	36579.43	54.76	−68.9	164.53	0.76	0.4
A2	8.706E+005	−178.16	1	8.706E+005	41.82	−267.3	−89	18.15	0.0007
B2	1.411E+005	−71.73	1	1.411E+005	41.82	−160.87	17.4	2.94	0.11
C2	6.361E+005	−152.28	1	6.361E+005	41.82	−241.43	−63.1	13.26	0.002
D2	7.771E+005	−168.33	1	7.771E+005	41.82	−257.47	−79.2	16.20	0.001
Residual	7.197E+005		15	47,977.28					
Lack of Fit	7.196E+005		10	71,962.6				10,767.8	<0.0001
Pure error	33.42		5	6.7		Coefficient of determination (R^2)=0.80			
Cor Total	3.579E+006		29						

Value in bold shows that the model is significant

CE coefficient estimate, *SE* standard error, *CI* coefficient interval, *SS* sum of squares, *df* degrees of freedom, *MS* mean square

Intracellular vanillin yield increased from 0.2 to 0.57 mM whereas extracellular vanillin yield increased from 0.92 to 2.58 mM as observed in Fig. 4. Overall, 3.14 mM of vanillin was obtained from 1.08 mM FA within 20 min using combined extracellular and intracellular responses which was 14.9 times higher than the native *P. acidilactici* strain BD16 (with 0.21 mM vanillin in 1 h and 20 min) and seven times more than recombinant *E. coli* top10 (*fcs*$^+$/*ech*$^+$) (with 0.45 mM vanillin within 20 min). Approximately, threefold higher vanillin production was reported in the recombinant *P. acidilactici* (*fcs*$^+$/*ech*$^+$) after scale-up of the process.

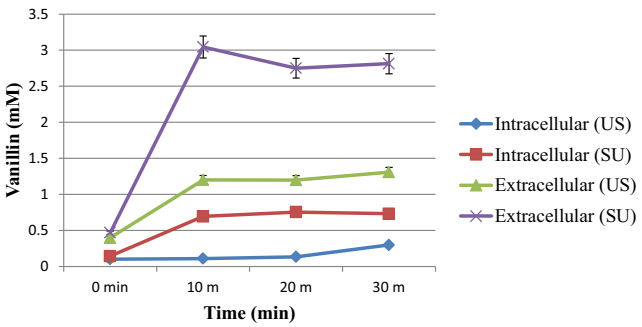

Fig. 4 Comparison of vanillin production in recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$) during scale-up

Enzyme activity of Fcs

Fcs enzyme activity was measured in cell suspension of recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$) grown for 12 h, and extracellular and intracellular enzyme activities of Fcs were assayed in triplicate as per standard protocol (Narbad and Gasson 1998). An increase in absorbance at 346 nm was observed due to the formation of feruloyl-CoA with concomitant vanillin production reported after 30 and 10 min of incubation for intracellular and extracellular activity, respectively. Extracellular Fcs activity (12.63 IU mL^{-1}) was found to be higher than intracellular activity (4.63 IU mg^{-1}) as observed in this recombinant system.

Kinetic study of Fcs

Km and Vmax of extracellular and intracellular enzyme preparations were calculated through Lineweaver Burk plots. Km (16.2 μM) and Vmax (352 U mg^{-1}) of intracellular enzyme was higher than extracellular enzyme (Km 4.07 μM and Vmax 274 U mg^{-1}). Extracellular enzyme has lesser Km than intracellular enzyme which indicates its higher access to substrate provided than intracellular enzyme. That is why extracellular enzyme showed more activity than intracellular enzyme (Fig. S3).

LCMS-ESI-based metabolic profiling of recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$)

Functional identification of the metabolites generated due to the activity of cloned Fcs and Ech enzymes was carried out using LCMS-based metabolic profiling technique. LCMS of methanolic reaction mixtures was performed using positive electron spray ionization, and phenolic metabolites were detected within mass range of 100 to 300. Vanillin with a mass of 156 kD (153 vanillin+3H$^+$) (80 % relative abundance; retention time 0.34 min) was detected along with its fragmentation product at 79.27 kD (90 % relative abundance, retention time 0.12 min) in the extracellular extracts which was comparatively more than vanillin peak (58 % by relative abundance, retention time 0.35 min) and its fragmentation product (100 % relative abundance, retention time 0.12 min) detected in intracellular extract. Major products such as methoxycinnamic acid (100 % relative abundance, retention time 0.5 min) and fragmentation products of FA, caffeic acid, and trans-cinnamic acid were also found in abundance along with pyrogallol, caffeic acid, and trans-cinnamaldehyde in the both reaction mixtures. Sinapic acid was found in extracellular extract, but it was absent in intracellular extract. 4-Ethylphenol was not detected in any of the extract tested which symbolizes that the strain is lacking ferulic acid decarboxylase (fdc) activity as shown in Fig. 5.

Depending upon the metabolites characterized by LCMS-ESI, we can predict a metabolic pathway of FA to phenolic biotransformation which involves an intricate network of decarboxylation, hydroxylation, dehydroxylation, methylation, and demethylation reactions as summarized in Fig. 6. FA to pyrogallol formation takes place through different steps involving hydroxylation, methylation, demethylation, and decarboxylation, where 5-hydroxy ferulate, sinapic acid, and gallic acid were produced as main intermediates. Metamethoxycinnamic acid was the most abundant and stable metabolite found in the reaction mixture which confirms FA dehydroxylation as a frequent process during FA biotransformation. This compound after demethylation and dehydroxylation forms cinnamic acid. Bioconversion of FA to cinnamic acid proceeds through a separate pathway in which demethylation and dehydroxylation are found as key steps with the formation of caffeic acid and p-coumaric acid as key intermediates. Caffeic acid to cinnamic acid conversion involves dehydroxylation reaction that yields m-coumaric acid as an intermediate. p-Coumaric acid fragmentation and cinnamic acid to cinnamaldehyde reductions have been identified from their fragmentation product detected in the reaction mixture during LCMS-ESI-based metabolic profiling. FA to vanillin biotransformation through coenzyme A-dependant pathway has been confirmed in the metabolically engineered *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$). The cloned enzymes Fcs and Ech successfully converted FA to vanillin through feruloyl-CoA and 4-hydroxy-3-methoxyphenyl-β-hydroxy propionyl-CoA as reaction intermediates. Decarboxylase activity is common in the wild-type LAB strain that results in formation of 4-vinylphenol followed by 4-ethylphenol as described previously by Kaur et al. (2013a, b). Formation of 4-vinylguiacol was not observed during biotransformation of FA into vanillin in both native as well as recombinant *P. acidilactici* BD 16 strains. 4-Vinylguiacol production during phenolic biotransformation was earlier reported only in few LAB strains like *Pediococcus* sp. (Bloem et al. 2007) and *P. pentosaceus* (Di Rivas et al. 2009).

Discussion

An increase in customer demand for natural vanillin has led to a growing interest of scientists for metabolic engineering of starter culture strains. Earlier, vanillin biosynthetic genes, namely, *ech* and *fcs* of *Amycolatopsis* sp. HR167, *Amycolatopsis* sp. HR104, *D. acidovorans*, *Pseudomonas* sp. HR199, and *P. fluorescens* BF13 have been cloned in *E. coli* using inducible expression systems (Table 4). Using metabolic engineered strains, vanillin production was found to have limited success due to longer bioconversion time required, genetic instability of the recombinants, and complicated downstream processing, etc. (Kaur and Chakraborty 2013).

In our laboratory, a natural vanillin producing *P. acidilactici* BD16 was isolated from a milk product and characterized for its biochemical and molecular properties (Kaur et al. 2013a). The wild-type strain was deposited with MTCC culture collection center, Institute of Microbial Technology, Chandigarh, India, vide culture deposit no. MTCC10973. *P. acidilactici* has already got GRAS status (GRN no. 000171, awarded by Food and Drug Administration); thus, it could be utilized as a probiotic food additive as per WHO guidelines on food and feed additives. A vanillin biosynthetic cassettes has been designed and cloned in vector pLES003 (pLES003-P_{289}/*fcs*$^+$/*ech*$^+$) for expression in competent cells of *P. acidilactici* BD16 MTCC10973. Using the strategy of metabolic engineering, 2.58 mM extracellular vanillin was recovered within 10 min and 0.57 mM intracellular vanillin after 20 min of incubation in the recombinant *P. acidilactici* (*fcs*$^+$/*ech*$^+$) cell extracts. Overall, 3.14 mM vanillin was recovered from 1.08 mM FA with a molar conversion rate of approximately 100 %. Its native strain however did not convert FA efficiently into vanillin in which only 5.5 % molar conversion rate was observed. Engineered *P. acidilactici* (*fcs*$^+$/*ech*$^+$) showed higher affinity for FA, as well as a higher FA to vanillin molar conversion rate (approximately 100 %) in extracellular

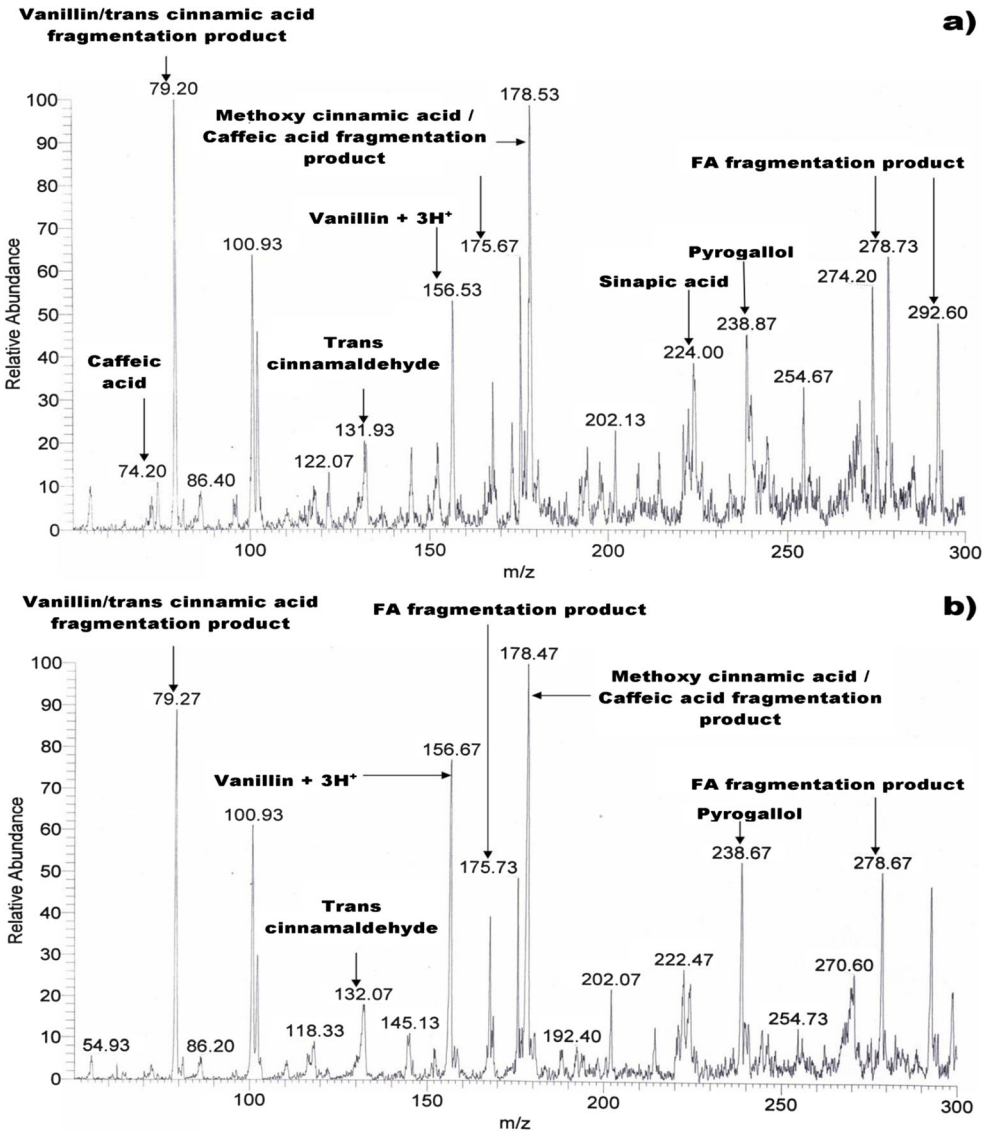

Fig. 5 Metabolites detected during LCMS-ESI of recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$) cultures; **a** intracellular extract and **b** extracellular extract

extracts, whereas only 22.63 % FA to vanillin molar conversion was achieved using intracellular extracts after scale-up. During LCMS-ESI-based metabolic profiling, vanillin degradation products like vanillyl alcohol and protocatechuic acid were not detected in cell extracts of recombinant *P. acidilactici* (*fcs*$^+$/*ech*$^+$). Results indicate its better suitability and acceptability than other recombinant microbial strains like *Pseudomonas* sp. HR199 (Overhage et al. 1999b) and *E. coli* JM109 (pBB1) (Barghini et al. 2007) as vanillin producing starter cultures for industrial processes. Recombinant *P. acidilactici* (*fcs*$^+$/*ech*$^+$) strain shows sevenfold more efficient FA to vanillin biotransformation than recombinant *E. coli* top10 strain (*fcs*$^+$/*ech*$^+$). Recently, Yang et al. (2013) reported that the cloned Fcs enzyme in recombinant *E. coli* (Km 0.35 mM mg^{-1} FA) is less substrate-specific than cloned Fcs enzyme in recombinant *P. acidilactici* (*fcs*$^+$/*ech*$^+$) (Km 4.07 μM mg^{-1} FA extracellular and 16.2 μM mg^{-1} FA intracellular). After statistical optimization of bioconversion reaction using CCD model of RSM, a 100 % molar conversion of FA to vanillin was reported for extracellular response (run 11).

In previous reports, *Oenococcus oeni* or *Lactobacillus* sp. (Bloem et al. 2007), *E. coli* strains (Yoon et al. 2005a, b; Lee et al. 2009; Luziatelli and Ruzzi 2008), *Pseudomonas* sp. (Plaggenborg et al. 2003), *Streptomyces* sp. (Achterholt et al. 2000; Hua et al. 2006), or *Amycolatopsis sp*. HR167 (Overhage et al. 2006) were used to optimize vanillin production in which longer incubation period and higher biomass utilization were affecting economic viability of the process (as indicated in Table 4). Production levels of vanillin in recombinant microbial systems ranged from 0.022 mM min^{-1} in *E. coli* (*fcs*+/*ech*+) to highest yield 0.16 mM min^{-1} in recombinant *P. acidilactici* (*fcs*$^+$/*ech*$^+$).

There are few metabolic engineering approaches reported so far in the literature utilizing host organisms other than

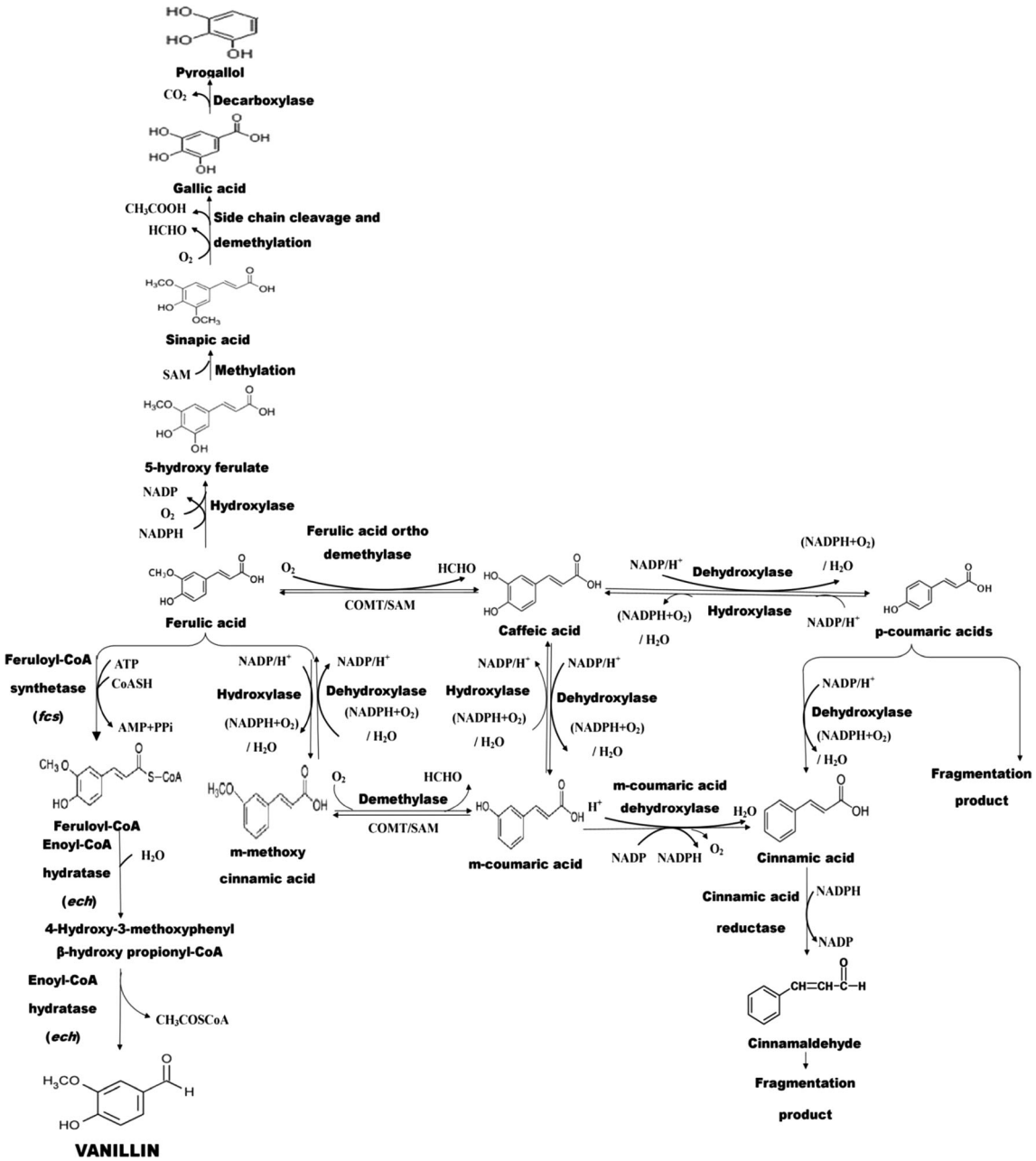

Fig. 6 FA degradation and vanillin biosynthetic pathways identified in recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$) in scale-up medium

E .coli. Bioconversion of FA to vanillin and vanillic acid was investigated by Narbad and Gasson (1998), and they found that 32 % of added FA (1 pmol) was converted to vanillin (molar conversion rate 18 %) and vanillic acid (molar conversion rate 14 %) within 4 h using crude extracts prepared from *P. fluorescens* AN103 cells. Supplementation of NAD in the incubation reaction mixture increased molar conversion rate of FA to vanillin by 68 %. When the incubation was prolonged, vanillin degradation to vanillic acid was reported. CoASH, ATP, and $MgCl_2$ were found as absolute cofactors for bioconversion for FA biotransformation of FA to vanillin.

Another study reported that vanillin yields in *P. fluorescens* AN103 could be improved by disrupting *vdh* gene (Martinez-Cuesta et al. 2005). Barbosa et al. (2008) statistically optimized vanillin production by solid-state fermentation on green coconut husk in *Phanerochaete chrysosporium*. Plackett Burmen design was implemented to evaluate role of 19 variables including different N sources and 52.5 μg g^{-1} of vanillin was recovered after 24 h of incubation. In another report, *vdh* gene of *P. fluorescens* AN103 was disrupted and cloned it with *fcs* and *ech* genes on a low-copy plasmid pBB1 which produced 8.41 mM vanillin from FA acid (Di Gioia et al. 2011). In these cases, genetic instability of the recombinant

Table 4 Comparison of vanillin production using various metabolic engineered strains utilizing FA as a substrate

Sr. No.	Source strain	Host strain	Plasmid used	Vanillin production	Biotransformation time	References
1	*Pseudomonas* sp. HR199 (*fcs*$^+$/*ech*$^+$)	*E. coli*	pSK*ech*E/H*fcs*	22 μM	1 min	Overhage et al. (1999a)
2	*Amycolatopsis* sp. HR167 (*fcs*$^+$/*ech*$^+$)	*E. coli*	pBluescript SK$^-$	3,099 μM	23 h	Achterholt et al. (2000)
3	*Pseudomonas* sp. HR199 (*fcs*$^+$/*ech*$^+$)	*E. coli*	pSKechE/Hfc	1,971 μM	2 h	Overhage et al. (2003)
4	*Amycolatopsis* sp. HR104 (*fcs*$^+$/*ech*$^+$)	*E. coli* DH5α	pTAHEF	7,228 μM	48 h	Yoon et al. (2005a)
5	*Amycolatopsis* sp. HR104 (*fcs*$^+$) and *D. acidovorans* (*ech*$^+$)	*E. coli*	pDAHEF	3,811 μM	18 h	Yoon et al. (2005a)
6	*P. fluorescens* BF13 (*fcs*$^+$/*ech*$^+$)	*E. coli* M109	pBB1	3,483 μM	6 h	Barghini et al. (2007)
7	*Amycolatopsis* sp. HR104 (*fcs*$^+$*ech*$^+$)	*E. coli* DH5α	pTAHEF	6,571 μM	48 h	Yoon et al. (2007)
8	*Pseudomonas* sp. (*fcs*$^+$/*ech*$^+$)	*E. coli*	pFR2	6.6 kg kg^{-1} biomass	24 h	Ruzzi et al. (2008)
9	*Amycolatopsis* sp. HR104 (*fcs*$^+$/*ech*$^+$)	*E. coli* DH5α (pTAHEF-*glt*A$^+$/*icd*$^-$)	pTAHEF-gltA	13,011 μM	48 h	Lee et al. (2009)
10	*Amycolatopsis* sp. HR104 (*fcs*$^+$/*ech*$^+$)	*E. coli*	pTBE-FP	13,800 μM	30 h	Song et al. (2009)
11	*P. fluorescens* BF13 (*fcs*$^+$/*ech*$^+$)	*P. fluorescens* (*vdh*$^-$)	pBB1	8,543 μM	24 h	Di Gioia et al. (2011)
12	*fcs*$^+$/*ech*$^+$ synthetic fragment	*Pediococcus acidilactici* BD16 (*fcs*$^+$/*ech*$^+$)	pLES003	Extracellular yield 2,576 μM Intracellular yield 565 μM	10 min 20 min	This study
13	*fcs*$^+$/*ech*$^+$ synthetic fragment	*E. coli* top10 (*fcs*$^+$/*ech*$^+$)	pCC1 BAC	Extracellular yield 296 μM Intracellular yield 151 μM	30 min 30 min	Unpublished data

strain was adjudged as a major drawback, and moreover, the use of *Pseudomonas* sp. is restricted in food products.

After critical analysis of the previous reports on vanillin production from FA (Table 4), higher FA to vanillin bioconversion rate was observed when enzyme extracts were used instead of whole cells. Extracellular extracts of recombinant *P. acidilactici* (*fcs*$^+$/*ech*$^+$) facilitated production of 2,576 μM vanillin within 10 min of incubation, and intracellular extracts yielded 565 μM vanillin within 20 min of incubation with the substrate, i.e., FA. This is the most efficient FA to vanillin bioconversion system reported so far using a recombinant microbial system. Earlier, there is a single report on recombinant *E. coli* (*fcs*+/*ech*+) where cloned Fcs and Ech enzymes resulted in production of 3.34 mg L^{-1} vanillin from 3.7 mM FA per minute with a molar conversion rate of 0.022 Mm min^{-1} mL^{-1} (Overhage et al. 1999a).

In our previous study, whole cells of native *P. acidilactici* BD16 were used for vanillin production on rice bran medium containing 150 g L^{-1} rice bran and 50 μg mL^{-1} FA and 1.269 g L^{-1} of crude vanillin was obtained (Kaur and Chakraborty 2013). However, 3.14 mM (478 mg L^{-1}) vanillin was obtained in the present study using 1.08 mM (210 μg mL^{-1}) of FA and enzyme extracts from 1 mg biomass of *P. acidilactici* BD16 (*fcs*+/*ech*+) cells. As rice bran is a rich source of FA and cinnamic acid derivatives, a higher production was reported in this biochemically unspecific media than synthetic medium used in the present study, where only FA was available for supporting growth as well as vanillin production in recombinant cells. If we follow a similar strategy in case of recombinant *P. acidilactici* BD16 (*fcs*$^+$/*ech*$^+$) also, vanillin productions will definitely increase. In our preliminary study, vanillin production was observed after 8 h in native *P. acidilactici* BD16, whereas in the present strategy, biotransformation period is reduced to 20 min. This work is still under progress. We can exploit this recombinant microorganism, especially because of its probiotic nature, as a starter culture in various dairy products, and process could also be optimized for in situ vanillin production in distillery, candies, chocolates, choco-eclairs, ice creams, yogurts, desserts, etc.

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Conflict of interest We do not have any conflict of interest.

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