Expression and characterization of a 9-cis-epoxycarotenoid dioxygenase from *Serratia* sp. ATCC 39006 capable of biotransforming isoeugenol and 4-vinylguaiacol to vanillin

Jiao Tang, Lei Shi, Lulu Li, Liangkun Long, Shaojun Ding*

The Co-Innovation Center of Efficient Processing and Utilization of Forest Resources, Jiangsu Key Lab for the Chemistry & Utilization of Agricultural and Forest Biomass, College of Chemical Engineering, Nanjing Forestry University, Nanjing, 210037, China

A B S T R A C T

A 9-cis-epoxycarotenoid dioxygenase gene from *Serratia* sp. ATCC 39,006 (*SeNCED*) was overexpressed in soluble form in *E.coli*. SeNCED showed the maximum activity at 30 °C and pH 8.0, and it was stable relatively at range of pH 5–10 and temperature of 20 °C to 30 °C. SeNCED effectively catalyzes the side chain double bond cleavage of isoeugenol and 4-vinylguaiacol to vanillin. The kinetic constant K_m values toward isoeugenol and 4-vinylguaiacol were 18.92 mM and 6.31 mM and V_{max} values were 50.73 IU/g and 4.77 IU/g, respectively. Moreover, the SeNCED exhibited an excellent organic solvent tolerance and the enzyme activity was substantially improved at presence of 10% of trichloromethane. The produced vanillin was achieved at an around 0.53 g/L (3.47 mM) and 0.33 g/L (2.17 mM) after 8 h reaction at 4 mM of isoeugenol and 4-vinylguaiacol, respectively, using transformed *Escherichia coli* cells harboring SeNCED in the presence of trichloromethane.

1. Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most important flavor and fragrance compounds, which is widely used in beverages, confectionery, foods, perfumes, and cosmetics industry. Historically, its production was almost dependent on extracting from vanilla beans directly, by which the accessible vanillin was seriously restrictive and at a high market price calculated as being between 1200 and 4000 $kg^{-1}[1]. Whereas the products obtained through chemical synthesis was at the price of less than 15 $kg^{-1}, which satisfied the constantly increasing markets. However, many unexpected environmentally unfriendly by-products often appeared during the chemical synthetic processes. Besides, the increasing concerns for health and nutrition stimulated a worldwide demand not for those synthesized chemically but natural vanillin. The vanillin produced from raw materials by biotechnology was equal to those extracted directly from vanilla beans on quality, and it was identified as "nature" vanillin by the FDA and European legislation [2–4]. Hence, biotransformation-based approaches for vanillin production in the filed green chemistry become more and more attractive for flavor industry to replace conventional chemical syntheses.

As we all know, ferulic acid (FA) is an abundant phenolic acid and can be recovered from agro-industrial wastes [5–7]. Several metabolism pathways from FA to vanillin have been reported in microbes [8,9]. Of those, in the coenzyme-dependent deacetylation pathway, the FA is converted to feruloyl-CoA catalyzed by feruloyl-CoA-synthetase (Fcs), and subsequently transferred to vanillin by enoyl-CoA-hydrolase (Ech). The engineered *Escherichia coli* and other bacterial cells harboring Fcs and Ech effectively converted FA to vanillin [10,11]. The engineered strains possess high potential for biosynthesis of vanillin, but the fact that Fcs requires expensive ATP and CoA as coenzymes makes the synthetic route complicate and high-cost. Assuredly, if the vital enzymes could be substituted by coenzyme independent proteins, the biosynthesis process of vanillin will be more efficient and economical.

Isoeugenol is the main constituent of essential oil of clove tree, and a variety of microbial species that metabolize isoeugenol to vanillin or vanillic acid have been isolated in succession [12–17]. The *Pseudomonas putida* IE27 cells produced 16.1 g/L vanillin from 150 mM isoeugenol, with a molar conversion yield of 71% [18]. While in strain I58, the produced vanillin was continuously converted to vanillic acid with a molar yield of 98%, which leads to an extremely low accumulation of vanillin [14]. The enzymes responsible for the transformation of isoeugenol to vanillin have been characterized, of which the sequence was similar to some of

the carotenoid cleavage oxygenases (CCOs), and they also had been reported to possess the potential for transforming 4-vinylguaiacol, a vital intermediate observed in microbial metabolism of FA to vanillin, but the activity was extremely low [16,19].

Recently, a novel CCO protein from *Caulobacter segnis* ATCC 21,756 (Cso2) was characterized capable of transforming both isoeugenol and 4-vinylguaiacol to vanillin without any coenzymes. A two-step biosynthetic pathway was constructed in *E.coli*, in which FA was firstly decarboxylated to 4-vinylguaiacol by phenolic acid decarboxylase and then oxidized to vanillin via oxygenase (Cso2) at an 80% of overall conversion [20]. Furthermore, the cascade synthesis of vanillin from ferulic acid via 4-vinylguaiacol was also achieved by the immobilized phenolic acid decarboxylase and Cso2 [21]. In fact, many organisms such as *Paecilomyces variotii*, *Pestalotia palmarum* [22], *Bacillus coagulans* [23] and *Enterobacter* sp. Px6-4 [24] had been reported to metabolize ferulic acid to vanillin via 4-vinylguaiacol. The enzymes catalyzing the first reaction have been well studied but the biotransformation of 4-vinylguaiacol to vanillin had been rarely reported before. The Cso2 protein was actually able to catalyze this reaction effectively; however, the insoluble expression reduced its application value. In order to increase the solubility, a molecular chaperone protein is indispensable to co-expressed with the target protein, which consequently led to a complexity of operation.

In order to mine superior catalysts useful for vanillin production from lignin-related phenylpropanoids, a gene mining method was carried out in this study and a new CCO protein named SeNCED from *Serratia* sp. ATCC 39,006 was functionally cloned and overexpressed in a large proportion of soluble form in *E. coli*. It cleaves isoeugenol, noticeably as well as 4-vinylguaiacol to vanillin independent on coenzyme, similar as Cso2 mentioned above. The enzymatic properties were studied and the potential of application for vanillin synthesis from isoeugenol or 4-vinylguaiacol was investigated in this study.

2. Materials and methods

2.1. Chemicals

Isoeugenol and ferulic acid from Sinopharm Group (Beijing, China), 4-vinylguaiacol and vanillin from Sigma-Aldrich (St. Louis, MO, USA) were all dissolved in dimethyl sulfoxide as stock solutions of 500 mM. The dimethyl sulfoxide, trichloromethane and other organic compounds were obtained from Sinopharm Group (Beijing, China). Standard protein marker for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), polymerase chain reaction (PCR) buffers, FastPfu DNA polymerase, and restriction enzymes were all obtained from Takara (Japan). The kits used for bacterial plasmid extraction, gel extraction and PCR purification were all purchased from Transgene (Beijing, China). All other chemicals were analytical grade.

2.2. Strains and culture conditions

Serratia sp. ATCC 39,006 purchased from the American Type Culture Collection (Manassas, VA, USA) was grown in LB medium formulated with 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl and incubated with shaking (200 rpm) at 37 °C. *E. coli* Top 10 and *E. coli* BL21 (DE3) strains (TransGene, Beijing, China) were used for gene cloning and expressing hosts, respectively. The pET-28a (+) (Novagen, Copenhagen, Denmark) was used as expression vector. The recombinant *E. coli* cells were cultured with shaking (200 rpm) at 37 °C in LB medium supplemented with 100 μg/mL kanamycin.

2.3. Cloning and expression of SeNCED

According to SeNCED sequence (WP_021015152.1), two oligo-nucleotide primers, *SeNCED*-F: 5′-CATGCCATGGCCATGAGTCT-GAAATTTCC-3′ (the *Nco*I restriction site underlined) and *SeNCED*-R: 5′-CCGCTCGAGTCAATGATGATGATGATGATGCCGGCCG-GATTGGGTAC-3′ (the *Xho*I restriction site underlined) were designed to amplify the *SeNCED* gene by PCR. The amplified DNA fragment digested with *Nco*I and *Xho*I was subsequently inserted into pET-28a (+) digested with the same enzymes. The constructed plasmid, pET-28a (+)-*SeNCED* was cloned into *E. coli* Top 10 strain and the target gene was confirmed by sequencing. The pET-28a (+)-*SeNCED* was then transformed into *E. coli* BL21 (DE3). The recombinant *E. coli* BL21 (DE3) cells were cultured at 37 °C in LB medium supplemented with 100 μg/mL kanamycin for 3 h (OD600 = 0.6–1.0), then a certain amount of IPTG and 1 mM FeCl$_2$ was added and cultured at 28 °C for 12 h for the over-expression of SeNCED protein.

2.4. Purification of SeNCED

The expressed cells were harvested by centrifugation (6000 × g, 30 min, 4 °C). The pellets resuspended in lysis buffer (50 mM NaH$_2$PO$_4$; 300 mM NaCl pH 8.0) were disrupted by sonication at an ice bath. After centrifugation (10,000 g, 4 °C, 10 min), the supernatant was applied onto a Ni-NTA agarose gel equilibrated in the lysis buffer containing 1 mM imidazole for the recombinant enzyme purification according to the manufacturer's manual and the concentration of obtained protein was measured with BCA Protein Assay Kit (Sangon, Shanghai, China). The purified recombinant enzymes were maintained at 4 °C and used for the following research as soon as possible. The enzyme homogeneity and the molecular of purified SeNCED were estimated using SDS-PAGE.

2.5. Enzyme assay

The catalytic activity of recombinant SeNCED was assayed with isoeugenol or 4-vinylguaiacol as substrates. The standard assay mixture contains 2 mM substrate and 10% v/v of glycerol in potassium phosphate buffer (100 mM, pH 8.0), and an appropriate amount of enzyme in a total volume of 0.5 mL. The reactions were performed at optimal temperature with vigorous shaking for 24 h and then terminated by adding 0.5 mL methanol. After centrifugation at 10,000 g for 10 min, the supernatant was analyzed using high-performance liquid chromatography (HPLC). One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of vanillin per minute. Kinetic constants were determined at optimal condition with different substrate concentrations ranging from 0 to 20 mM. The maximal reaction rate (Vmax) and apparent Michaelis–Menten constant (Km) were calculated by Origin 8.0 software (http://www.originlab.com/) using nonlinear regression.

2.6. Effects of pH, temperature, and chemicals on SeNCED activity and stability

Optimal pH for enzyme reactions were estimated using isoeugenol as the substrate in different buffers at a range of pH 6.0–10.0 (0.1 M potassium phosphate buffer pH 6.0–8.5, glycine-NaOH buffer pH 8.0–10.0). Optimal temperature for the SeNCED activity was determined by standard assay at a range of temperature from 20 °C to 45 °C in 0.1 M potassium phosphate buffer (pH 8.0). The results were expressed as percentage of the activity values determined under either the optimal pH or temperature.

Thermal stability was analyzed by determining the residual activity of purified SeNCED to transform isoeugenol to vanillin after preincubation at a range of 20 °C–45 °C for a specific time as indicated. To evaluate the pH stability, enzyme was preincubated at universal buffer containing 50 mM phosphoric acid, acetic acid and boric acid at a range of pH 3.0 to pH 12.0 for 2 h, and then the residual activity was measured under the standard reaction condition. The results were expressed as percentage of the activity value determined via the purified enzyme without preincubation.

To investigate the effects of various chemicals containing metal ions, metal chelators and detergents on SeNCED activity, Ca^{2+}, Co^{2+}, Mg^{2+}, Li^+, Cu^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Na^+, SDS, Triton X-100, 2,2-bipyridine, 10-phenanthrolase and ethylene diamine tetracetic acid (EDTA) were assayed at a final concentration of 5 mM in the reaction mixture. Activity was determined as described above and was expressed as percentage of the enzyme activity obtained in the reaction without any chemicals added.

2.7. Effect of organic solvents on SeNCED activity and stability

The effect of organic solvents on enzyme activity to two substrates was also analyzed in the presence of various organic solvents at 10% (v/v). The reaction was performed at standard assay condition in 10-mL glass bottles with screw caps (Teflon seals), and the product was quantified using HPLC. To investigate its stability to organic solvent, the purified SeNCED was co-shaked with 20% or 50% (v/v) of various organic solvents at 200 rpm at 20 °C for 2 h or 24 h in 10-mL sealed bottles. After incubation, the water phase containing SeNCED were sufficiently separated by centrifugation (10,000 g at 4 °C for 10 min), and the residual activity was determined under standard condition.

2.8. Vanillin biosynthesis via the purified SeNCED and whole cells harboring SeNCED

The biotransformtion of isoeugenol and 4-vinylguaiacol to vanillin was conducted by both purified SeNCED and transformed E.coli cells harboring SeNCED. The catalytic reaction initiated by purified SeNCED was proceeded in the reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0), 10% (v/v) glycerol, 4 mM substrate and 0.2 mg/mL purified enzyme. The reactions were performed at 30 °C with vigorous shaking for 72 h.

The reaction catalyzed by the transformed E.coli cells was performed in a 50-mL conical flask at 30 °C with vigorous shaking for 72 h. The reaction mixture (2 mL) contained 100 mM potassium phosphate buffer (pH 8.0), 10% (v/v) glycerol, 4 mM substrate, 5% (v/v) trichloromethane and the E. coli whole cells at a final concentration of OD_{600} 3.0. After reaction, the mixtures were diluted with methanol to constant volume of 4 mL, and the product was quantified using HPLC. For preparation of the whole cells, the transformed E. coli cells were harvested by centrifugation (6000 g, 30 min, 4 °C) and washed triples with potassium phosphate buffer (100 mM, pH 8.0) containing glycerol (10% v/v) after 12 h induction at 28 °C as described above.
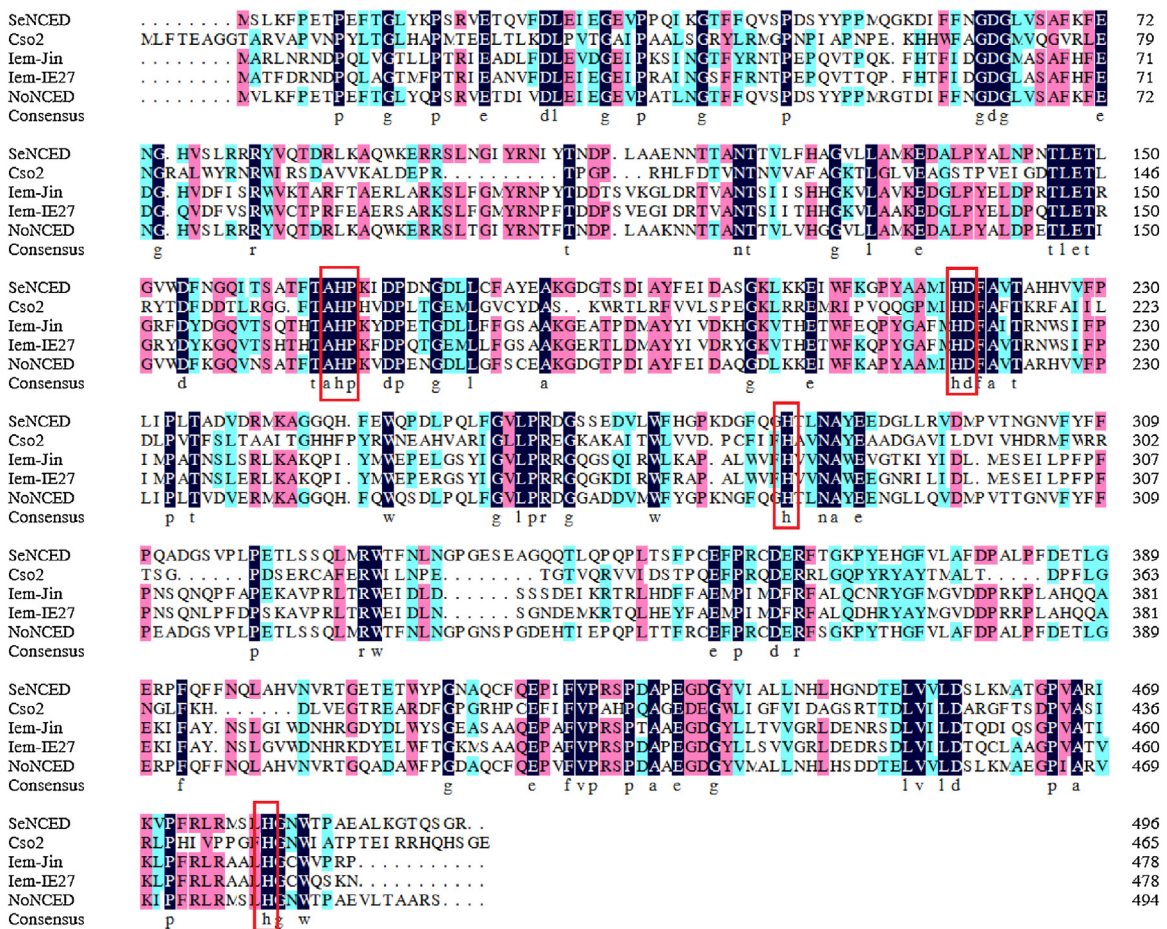

Fig. 1. Multiple alignment of the amino acid sequences of SeNCED and other CCOs enzymes from different bacteria. Identical residues are shaded in black and conserved residues are shaded in other colors. SeNCED: 9-cis-epoxycarotenoid dioxygenase from Serratia sp. ATCC 39,006 (WP_021015152.1), Cso2: CCO from Caulobacter segnis 21,756 (WP_013079514.1), Iem-IE27 and Iem-JIN1: Isoeugenol monooxygenase (Iem) from Pseudomonas putida strain IE27 (GenBank: BAF62888.1) and strain Jin1 (GenBank: ACP17973.1), respectively, NovoNCED: 9-cis-epoxycarotenoid dioxygenase from Novosphingobium sp. Rr 2–17 (GenBank: EIZ77617.1).

2.9. Analytical methods

The oxidized product was analyzed using HPLC as described previously by Hu et al. [25]. Identification of the oxidized product was performed by gas chromatography–mass spectrometry (GC–MS) (TRACE DSQ, Thermo Fisher Scientific) with a DB-5 column (length, 30 m; diameter, 0.25 mm;Agilent), the column temperature was initially held at 50 °C for 3 min, then programmed to 260 °C at a rate of 10 °C/min, with a final hold time of 5 min. The components were identified based on the comparison of their relative retention times and mass spectra with those of the established standards (NIST05 library data of the GC–MS system) and previous literature data.

3. Results and discussion

3.1. Cloning and analysis of SeNCED

By analysis of the genome sequence of the *Serratia*.sp ATCC 39,006 published on National Center of Biotechnology Information (NCBI) (Aseembly: GCA_000463345.2), a protein, defined as 9-cis-epoxycarotenoid dioxygenase (SeNCED) (WP_021015152.1), shares high sequence similarity (59%) with Cso2 from *Caulobacter segnis* ATCC 21,756 (WP_013079514.1), belonging to CCO family, consists of 1488 bp fragment encoding 496 amino acids with theoretic pI and molecular weight values of 5.04 and 55.59 KDa. It also showed 83.67% similarity with the 9-cis-epoxycarotenoid dioxygenase of *Novosphingobium* sp. Rr 2–17 (GenBank: EIZ77617.1), and 39.44% and 39.03% similarity with the isoeugenol monooxygenases (Iem) from *Pseudomonas putida* IE27 (GenBank: BAF62888.1), and *Pseudomonas nitroreducens* Jin1 (GenBank: ACP17973.1), respectively. The CCOs were identified as iron-contained proteins and a Fe^{2+}-4-His arrangement acting as a vital role at the oxidative reactions was existed in these proteins [20]. Alignment of the amino acid sequences of SeNCED and these CCOs showed that four histidines His^{167}, His^{218}, His^{283} and His^{480} was extremely conversed as shown in the red frame (Fig. 1), which might be responsible for combining Fe^{2+}. To get a further insight into the evolutionary relationship between SeNCED and other CCOs, phylogenetic trees of the 20 amino acid sequences were constructed using the Neighbor-Joining and Maximum Parsimony method (Fig. 2). As a consequence, a near relationship among SeNCED, NCED from *Novosphingobium*, Dioxygenase from *Kaistia soil* and *Rhodobacteraceae* was observed. When to investigate the ability of *Serratia* sp. ATCC 39,006 for metabolizing aromatic compounds, a small amount of vanillin was detected in the culture during its metabolic process of isoeugenol, 4-vinylguaiacol and ferulic acid as substrates (Figs. S1–S3 in the Supplementary material). Based on the sequence and metabolite analysis, we supposed that SeNCED acts as the vital enzyme in the vanillin synthesis procedures from those aromatic compounds.

3.2. Expression and purification of the recombinant SeNCED

The *E.coli* BL21 (DE3) strain containing recombinant plasmid pET-28a (+)-SeNCED was used as heterologous host to obtain the overexpression of SeNCED. The recombinant protein was induced by 0.4 mM IPTG and 1 mM Fe^{2+} at 28 °C for 12 h after a preincubation at 37 °C to a cell concentration of OD_{600} 0.6–1 (Fig. 3A–C). When 1 mM Fe^{2+} was added together with 0.4 mM IPTG in the medium, the transformed *E.coli* cells showed a 1.8-fold higher activity than those cultured without Fe^{2+} (Fig. 3 D). It was in good agreement with catalytic mechanism of the Cso2 and other CCOs, which regarded Fe^{2+} as prosthetic group [26,27]. This result indicated that SeNCED was also a Fe^{2+} contained protein.

The recombinant SeNCED was then purified via affinity chromatography using Ni-NTA agarose gel. In order to verify the expression of target protein and its solubility, the non-induced whole cells, the induced whole cells, and its cell debris and supernatant were applied to SDS-PAGE. It showed a strong band of SeNCED in the supernatant of induced whole cells, dissimilar to the Cso2 shown in the cell debris [21], declaring that the recombinant
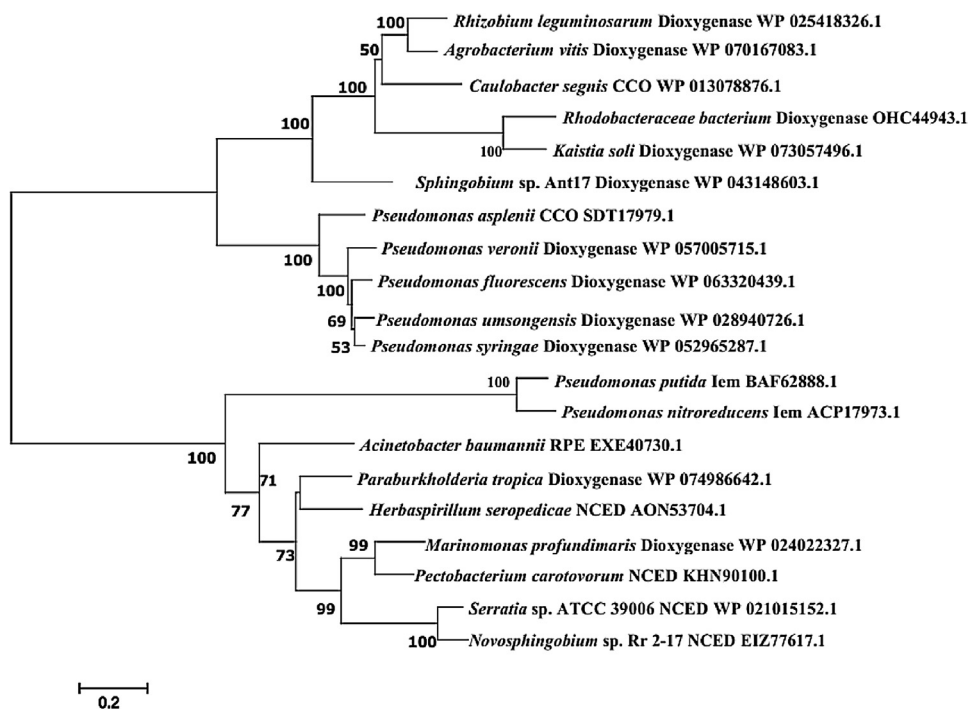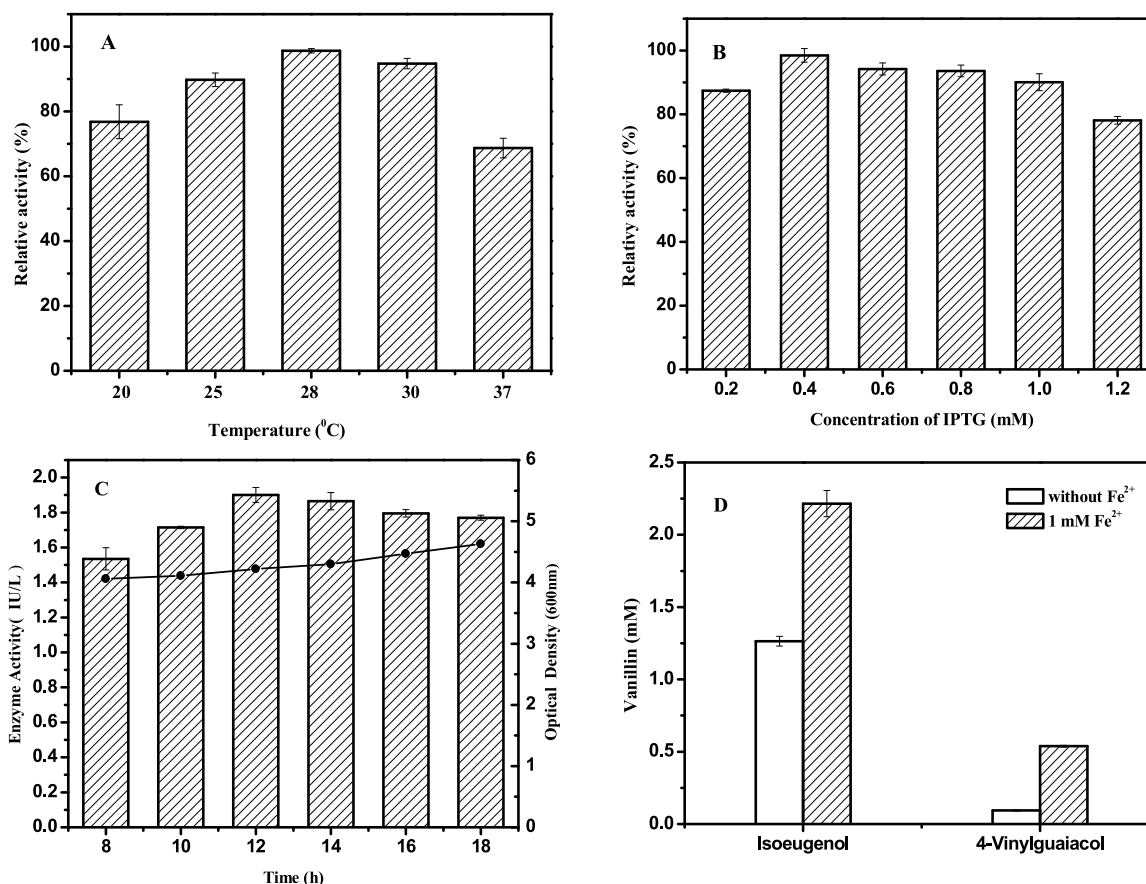

Fig. 2. The phylogenetic trees resulting from analysis of carotenoid cleavage oxygenases of 20 amino acid sequences using Neighbor-Joining method. Numbers on nodes correspond to percentage bootstrap values for 1000 replicates.

Fig. 3. Effects of induction temperature (A), dosage of IPTG (B), induction time (C), and Fe^{2+} (D) on the recombinant SeNCED production. Filled circles in (C) represent the variation of cell concentration.

protein was expressed in a large proportion of soluble form without co-expression of chaperon proteins. The purified protein was shown as a single band at molecular mass of about 55 KDa, which coincided perfectly with the theoretical molecular mass as shown in Fig. 4.
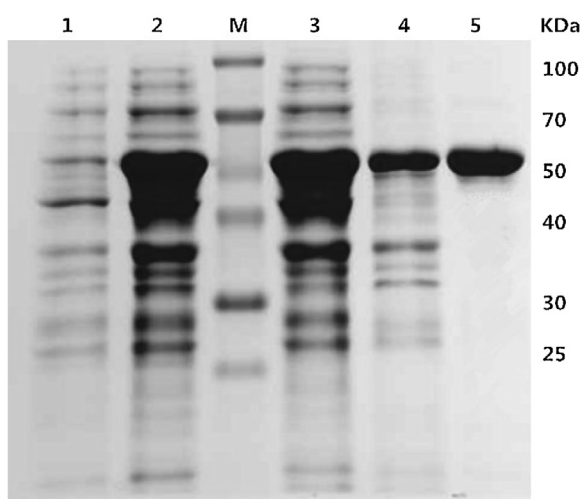

Fig. 4. SDS-PAGE analysis of SeNCED solubility and purified protein. Lane 1 and 2: whole *E.coli* BL21 (DE3) cells without induction and with a 12 h induction, respectively; Lane 3: supernatant of induced cell lysate; Lane 4: induced cell debris; Lane 5: purified SeNCED. Lane M: protein marker.

3.3. Effect of temperature and pH on SeNCED activity

To study the effects of temperature and pH on SeNCED activity, isoeugenol was used as substrate for the enzymatic reactions. The maximum activity was detected at 30 °C (Fig. 5A). The enzyme presented over 80% of relative activity under a temperature range from 20 °C to 40 °C, but almost totally inactivation as the temperature is above 45 °C. The SeNCED was relatively stable at 20 °C and 30 °C, more than 90% of initial activity residue was detected after 150 min of incubation (Fig. 5C). However, its activity was decreased to a certain degree when incubated at the temperatures higher than 40 °C, only about 50% of its initial activity was detected at 40 °C after 30 min of incubation and almost inactivated at 50 °C. The optimum pH was observed at 8.0 and over 80% of relative activity was detected under a pH range from 7.0 to 9.0 (Fig. 5 B), indicating a preference for slight alkaline environment, which was similar to that seen for other CCOs [19,28,29]. The SeNCED also showed a high stability at pH between 5.0 and 11.0, over 80% of initial activity was maintained after 2 h of incubation at universal buffers (Fig. 5D).

3.4. Effect of various chemicals on SeNCED activity

SeNCED activity was examined in the presence of several chemicals as shown in Table 1. No significant changes of SeNCED activity were observed with the addition of Ca^{2+}, Co^{2+}, Mg^{2+}, Li^+, Mn^{2+} and Na^+ or detergents. And a slight activity decrease by over 20% was detected in the presence of Zn^{2+}, Ni^{2+}, Fe^{2+} and Fe^{3+}. Moreover, a severe inhibition was appeared due to the Cu^{2+} addition. The loss of SeNCED activity might indicate some critical
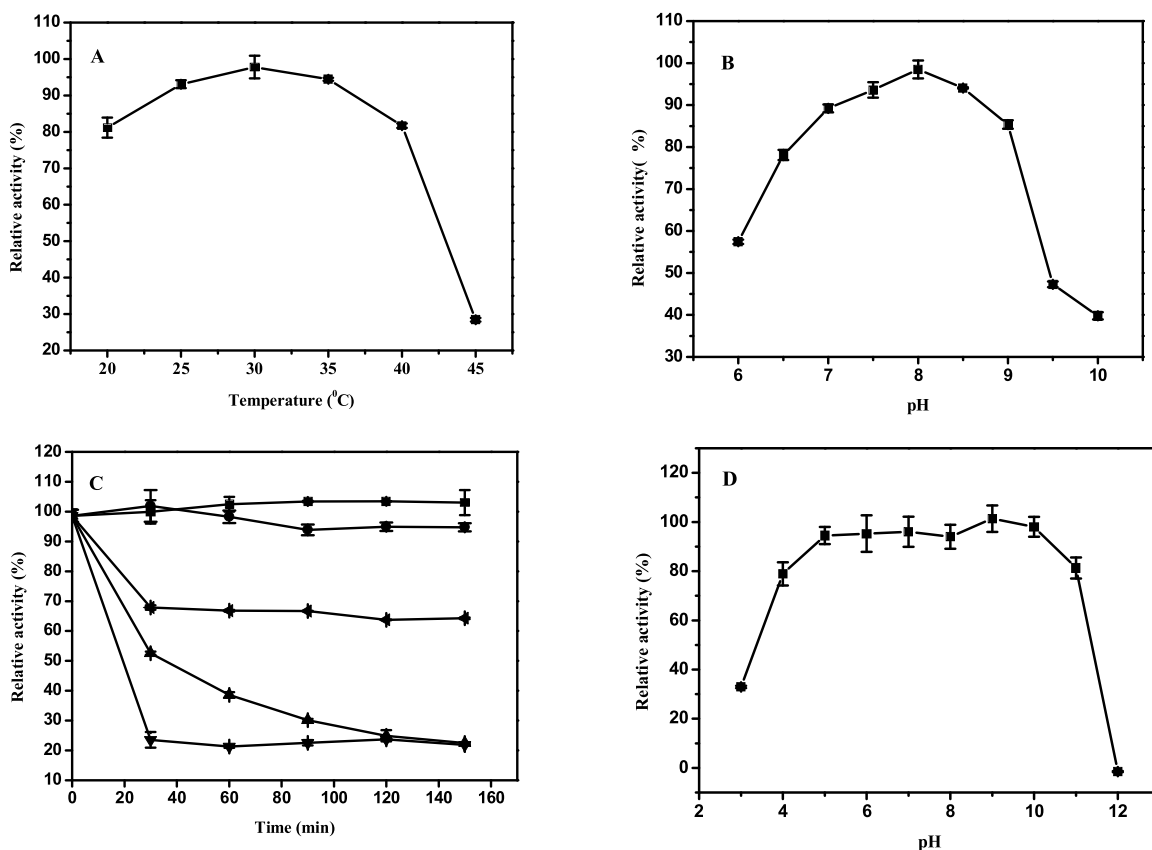
Fig. 5. Effects of temperature (A) and pH (B) on SeNCED activity, and thermal (C: ■, 20°C; ●, 30°C; ◄, 35°C; ▲, 40°C; ▼, 45°C) and pH (D) stability. Values shown are means of duplicate means ± standard error (SE).

Table 1
Effect of various chemicals on the recombinant SeNCED activity.

	Chemicals (5 mM)	Relative activity (%)
Control	None	99.84 ± 0.23
Metal ions	Ca^{2+}	93.77 ± 2.46
	Co^{2+}	82.38 ± 4.16
	Mg^{2+}	91.11 ± 3.82
	Li^+	94.53 ± 2.73
	Cu^{2+}	3.17 ± 0.17
	Zn^{2+}	77.07 ± 5.38
	Ni^{2+}	74.61 ± 0.64
	Fe^{3+}	62.51 ± 0.01
	Fe^{2+}	70.55 ± 5.62
	Mn^{2+}	98.24 ± 0.44
	Na^+	97.27 ± 0.44
Detergents	SDS	90.28 ± 0.67
	Triton X-100	66.54 ± 1.46
Metal chelators	2,2-bipyridine	86.05 ± 0.13
	1,10-phenanthrolase	26.53 ± 2.99
	EDTA	64.77 ± 2.16

The relative activity was expressed at the percentage of the activity without addition of compound. All values shown are duplicate means ± standard error (SE).

changes of enzyme structure in the presence of these metal ions. Unexpectedly, the Fe^{2+} cannot stimulate the SeNCED activity when it was added in the reaction mixture, which was not similar to the CCD4 from *Osmanthus Fragrans* [29]; but an obvious increase of enzyme activity was detected when the Fe^{2+} was added during the incubation of transformed *E.coli* cells (Fig. 3D). These results might indicate that the Fe^{2+} was involved in the formation of SeNCED enzyme activity center and it was no more needed if the protein is folded rightly. In addition, metal chelators 2, 2-bipyridine, 10-phenanthrolase and EDTA showed inhibition to the enzyme

activity at some level, which might verified that the Fe^{2+} was typically involved in catalytic reaction of SeNCED.

3.5. Kinetic parameters of SeNCED and ability for vanillin synthesis

Kinetic constants of SeNCED were tested under the optimized condition. K_m values were 18.92 ± 3.26 and 6.31 ± 1.98 mM, while V_{max} values were 50.73 ± 5.28 and 4.77 ± 0.73 IU/g toward isoeugenol and 4-vinylguaiacol respectively. Apparently, the ability for transforming isoeugenol is over 10-folds higher than 4-vinylguaiacol, which indicated that the SeNCED showed a substrate preference for isoeugenol rather than 4-vinylguaiacol. It was similar to the Iem from *Pseudomonas putida* IE27, an enzyme also capable of catalyzing those two substrates to vanillin, but its activity to 4-vinylguaiacol is low at only 1% of that to isoeugenol [28]; while to the Cso2, the ability for transforming those two substrates was roughly equal [21]. The precursors isoeugenol and 4-vinylguaiacol were catalyzed by purified SeNCED at the optimal condition and the vanillin was synthesized from those two substrates during a given time. The recombinant SeNCED produced approximately 0.57 g/L and 0.2 g/L of vanillin after 72 h reaction from 4 mM isoeugenol and 4-vinylguaiacol, respectively. Approximately 92% of isoeugenol and 32% of 4-vinylguaiacol was finally converted to vanillin (Fig. 6).

3.6. Effects of organic solvents on activity and stability of SeNCED

The effects of various water-immiscible organic solvents on activity and stability of SeNCED were investigated. The enzyme activity was substantially increased at the presence of trichloro-methane by 1.7 and 2.0 folds of its initial activity for both
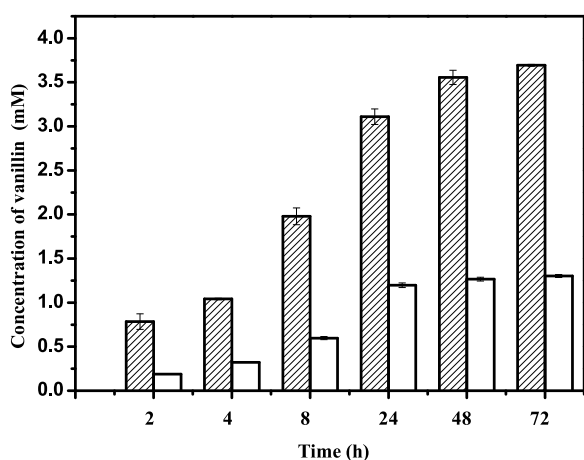
Fig. 6. Vanillin synthesis from isoeugenol (filled bars) and 4-vinylguaiacol (blank bars) by purified SeNCED.

Table 3
Effect of various organic solvents on recombinant SeNCED stability.

Organic solvents	Concentration (%,v/v)	Relative activity (%)	
		2 h	24 h
Dichloromethane	20	85.88 ± 1.4	87.94 ± 2.39
	50	88.64 ± 0.64	85.73 ± 1.66
Trichloromethane	20	88.74 ± 2.15	95.30 ± 1.52
	50	85.81 ± 0.18	112.34 ± 0.34
n-Caprylic alcohol	20	87.14 ± 1.32	84.99 ± 2.36
	50	87.78 ± 1.69	84.73 ± 0.44
Toluene	20	84.45 ± 2.18	86.42 ± 2.82
	50	90.75 ± 1.24	81.33 ± 1.47
Ethyl acetate	20	85.54 ± 3.66	78.11 ± 2.51
	50	80.27 ± 1.61	70.46 ± 2.04
Cyclohexane	20	86.20 ± 3.25	97.47 ± 0.82
	50	98.79 ± 0.55	85.96 ± 5.89
n-Hexane	20	84.01 ± 1.34	90.54 ± 2.30
	50	86.63 ± 0.36	83.78 ± 1.34
Petroleum ether	20	79.81 ± 0.59	68.68 ± 1.67
	50	85.06 ± 4.88	68.92 ± 0.59
n-Butyl alcohol	20	46.41 ± 0.87	57.61 ± 1.26
	50	64.01 ± 6.74	54.27 ± 0.36
n-Octane	20	80.78 ± 1.39	84.37 ± 1.03
	50	86.18 ± 3.15	85.51 ± 1.60

The relative activity was expressed at the percentage of the activity without co-incubation with any solvents. All values shown are duplicate means ± standard error (SE).

isoeugenol and 4-vinylguaiacol, respectively (Table 2). Meanwhile, a light increase of enzyme activity was detected in the presence of dichloromethane, n-caprylic alcohol or cyclohexane when 4-vinylguaiacol was used as substrate, but a mild decrease was observed when isoeugenol was used as substrate. However, an obvious decrease of SeNCED activity was observed by approximately 20% in the presence of n-hexane, and even over 60% in the presence of toluene, ethyl acetate or n-butyl alcohol using either isoeugenol or 4-vinylguaiacol as substrates. Moreover, the activity was decreased by about 50% when the n-octane and petroleum ether was added using isoeugenol as substrate, but only 10%–30% reduction was observed to 4-vinylguaiacol. The effect mechanisms of those organic solvents on SeNCED activity were not clear now due to the complexity arising from different organic solvents. As reported, the three-dimensional structure and flexibility of the enzyme active site might be affected by organic solvents, leading to an enzymatic activity and specificity variation [30,31].

The biphasic organic/aqueous system has many advantages for enzymatic reactions or biotransformations [32], therefore, those enzymes or microbes exhibiting remarkable organic solvent tolerance can be effectively used as catalysts in the organic-aqueous biphasic reaction system [25,33]. However, most native enzymes exhibit lower activities or stabilities as the organic solvents were co-presented in the reaction mixture. Thus, the sufficient organic solvent tolerance for the enzymes is highly demanded in the biphasic system reactions. Several enzymes mostly lipases, proteases, and esterases have been reported to be tolerant to organics, but rarely for CCOs [34–37]. In our study, an

admirable stability of SeNCED to organic solvent was observed (Table 3). After co-incubation with solvents in 20°C for 24 h, the enzyme still remained more than 80% of its initial activity in most used solvents even at the concentration up to 50%. This result implied that SeNCED can be effectively used for the vanillin synthesis from aromatic compounds in organic-aqueous biphase or even organic phase. The phenolic acid decarboxylase from *Bacillus licheniformis* [25] and *Bacillus amyloliquefaciens* [38] showed high solvent tolerant and efficient in conversion of hydroxycinnamic acids to vinyl phenol derivatives when using the organic-aqueous biphasic system. The SeNCED might be a potential candidate for vanillin synthesis from ferulic acid by combined using with these phenolic acid decarboxylases in a two-step biosynthetic reaction system, owing to its wonderful tolerance to organic solvents.

3.7. Bioconversion of isoeugenol and 4-vinylguaiacol to vanillin by transformed E.coli

Bioconversions of isoeugenol and 4-vinylguaiacol using transformed *E.coli* cells harboring SeNCED were investigated. According to our experimental results, trichloromethane was added as organic solvent for vanillin synthesis. The molar conversion was achieved up to 90% with an 8 h reaction in the presence of 5% trichloromethane using isoeugenol as substrate; but only about 36% was obtained in the reaction without trichloromethane. Similarly, the molar conversion was achieved up to 54% after 8 h incubation in the presence of 5% trichloromethane but only around 16% was detected in trichloromethane non-contained reactions when used 4-vinylguaiacol as substrate. This data indicated that the addition of trichloromethane could significantly reduce the reaction time which was beneficial for the biosynthesis of vanillin. The produced vanillin was achieved at around 0.53 g/L and 0.33 g/L after 8 h, and 0.60 g/L and 0.43 g/L after 72 h reaction at 4 mM of isoeugenol and 4-vinylguaiacol, respectively, in the presence of trichloromethane (Fig. 7). Water immiscible organic solvents might be beneficial for the enzyme reactions by alleviating the inhibitory and toxic effects of the substrate and product on enzymes due to the extraction of hydrophobic substrate or product [26]. There have been several studies exhibited the improvement

Table 2
Effect of various organic solvents on recombinant SeNCED activity.

Organic solvents	Relative activity (%)	
	Isoeugenol	4-Vinylguaiacol
Dichloromethane	93.83 ± 1.94	121.97 ± 2.21
Trichloromethane	176.16 ± 8.37	208.47 ± 1.82
n-Caprylic alcohol	74.73 ± 3.5	144.66 ± 5.48
Toluene	39.56 ± 0.75	29.36 ± 0.58
Ethyl acetate	19.35 ± 0.39	35.06 ± 0.13
Cyclohexane	91.01 ± 3.6	122.72 ± 6.81
n-Hexane	84.29 ± 6.15	86.15 ± 4.22
Petroleum ether	46.05 ± 1.27	92.95 ± 2.89
n-Butyl alcohol	38.19 ± 0.20	21.44 ± 1.91
n-Octane	55.34 ± 2.86	70.45 ± 2.61

The relative activity was expressed at the percentage of the activity without addition of any solvents. All values shown are duplicate means ± standard error (SE).

Fig. 7. Biotranformations of isoeugenol and 4-vinylguaiacol to vanillin by whole cells of recombinant *E.coli* BL21(DE3) harboring SeNCED. Symbols: reactions used isoeugenol as substrate in the presence of 5% (v/v) of trichloromethane (■) or not (□) and used 4-vinylguaiacol as substrate with 5% of trichloromethane (●) or not (○).

of productivity in the presence of specific organic solvents [32,38,39]. In previous study, the isoeugenol at a concentration of 200 mM was transformed to vanillin by *Pseudomonas putida* cells [18]; while 4-vinylguaiacol at concentration of 75 mM was transformed to vanillin efficiently using recombinant *E.coli* cells [26]. In the future, the substrate feeding strategies or an organic/aqueous biphasic system still need to be optimized for a further improvement of vanillin synthesis.

4. Conclusion

In this study, SeNCED from *Serratia* sp. ATCC 39,006 was successfully overexpressed and characterized. The recombinant protein was expressed in a large proportion of soluble form independent on chaperon proteins. Moreover, it showed an excellent organic solvent tolerance especially for trichloromethane. The addition of trichloromethane could significantly increase the enzyme activity and reduce the reaction time beneficial for vanillin biosynthesis. The transformed *E.coli* cells converted isoeugenol and 4-vinylguaiacol at molar conversions of 90% and 65% and the maximum vanillin concentrations were up to 0.60 g/L and 0.43 g/L, respectively. This study provided a promising CCO enzyme, which may be used for the "nature vanillin" production from isoeugenol and 4-vinylguaiacol.

Conflicts of interest

None declared.

Acknowledgements

Appendix C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.btre.2018.e00253

References

[1] S. Serra, C. Fuganti, E. Brenna, Biocatalytic preparation of natural flavours and fragrances, Trends Biotechnol. 23 (2005) 193–198.
[2] H. Priefert, J. Rabenhorst, A. Steinbüchel, Biotechnological production of vanillin, Appl. Microbiol. Biotechnol. 56 (2001) 296–314.
[3] L. Yan, P. Chen, S. Zhang, S.Y. Li, X.J. Yan, N.B. Wang, N. Liang, H.Y. Li, Biotransformation of ferulic acid to vanillin in the packed bed-stirred fermenters, Sci. Rep. 6 (2016) 34644.
[4] C. Fleige, F. Meyer, A. Steinbuchel, Metabolic engineering of the *Actinomycete Amycolatopsis* sp strain ATCC 39116 towards enhanced production of natural vanillin, Appl. Environ. Microbiol. 82 (2016) 3410–3419.
[5] H.D. Shin, S. Mcclendon, T. Le, F. Taylor, R.R. Chen, A complete enzymatic recovery of ferulic acid from corn residues with extracellular enzymes from *Neosartoryaspinosa* NRRL185, Biotechnol. Bioeng. 95 (2006) 1108–1115.
[6] S.Q. Zhao, S.W. Yao, S.Y. Ou, J. Lin, Y. Wang, X.C.A.J. Li, B. Yu, Preparation of ferulic acid from corn bran: its improved extraction and purification by membrane separation, Food Bioprod. Process. 92 (2014) 309–313.
[7] K.K. Jiang, L.L. Li, L.K. Long, S.J. Ding, Comprehensive evaluation of combining hydrothermal pretreatment (autohydrolysis) with enzymatic hydrolysis for efficient release of monosaccharides and ferulic acid from corn bran, Ind. Crop Prod. 113 (2018) 348–357.
[8] S. Mathew, T.E. Abraham, Bioconversions of ferulic acid, an hydroxylcinnamic acid, Crit. Rev. Microbiol. 32 (2006) 115–125.
[9] B. Kaur, D. Chakraborty, Biotechnological and molecular approaches for vanillin production: a review, Appl. Biochem. Biotechnol. 169 (2013) 1353–1372.
[10] W. Yang, H. Tang, J. Ni, Q. Wu, D. Hua, F. Tao, P. Xu, Characterization of two *Streptomyces* enzymes that convert ferulic acid to vanillin, PLoS One 8 (2013) e67339.
[11] D. Chakraborty, A. Selvam, B. Kaur, J.W.C. Wong, O.P. Karthikeyan, Application of recombinant *Pediococcus acidilactici* BD16 (fcs (+)/ech (+)) for bioconversion of agrowaste to vanillin, Appl. Microbiol. Biotechnol. 101 (2017) 5615–5626.
[12] H. Furukawa, H. Morita, T. Yoshida, T. Nagasawa, Conversion of isoeugenol into vanillic acid by *Pseudomonas putida* I58 cells exhibiting high isoeugenol-degrading activity, J. Biosci. Bioeng. 96 (2003) 401–403.
[13] L.Q. Zhao, Z.H. Sun, P. Zheng, L.L. Zhu, Biotransformation of isoeugenol to vanillin by a novel strain of *Bacillus fusiformis*, Biotechnol. Lett. 27 (2005) 1505–1509.
[14] H. Furukawa, H. Morita, T. Yoshida, T. Nagasawa, Conversion of isoeugenol into vanillic acid by 32 *Pseudomonas putida* I58 cells exhibiting high isoeugenol-degrading activity, J. Biosci. Bioeng. 96 (33) (2003) 401–403.
[15] M. Ashengroph, I. Nahvi, H. Zarkesh-Esfahani, F. Momenbeik, Conversion of isoeugenol to vanillin by *Psychrobacter* sp. strain CSW4, Appl. Biochem. Biotechnol. 166 (2012) 1–12.
[16] J.Y. Ryu, Seo J, S. Park, J.H. Ahn, Y. Chong, M.J. Sadowsky, H.G. Hur, Characterization of an isoeugenol monooxygenase (Iem) from *Pseudomonas nitroreducens* Jin1 that transforms isoeugenol to vanillin, Biosci. Biotechnol. Biochem. 77 (2013) 289–294.
[17] M. Ashengroph, J. Amini, Bioconversion of isoeugenol to vanillin and vanillic acid using the resting cells of Trichosporon asahii, 3 Biotech 7 (2017) 358.
[18] M. Yamada, Y. Okada, T. Yoshida, T. Nagasawa, Biotransformation of isoeugenol to vanillin by *Pseudomonas putida* IE27 cells, Appl. Microbiol. Biotechnol. 73 (2007) 1025–1030.
[19] M. Yamada, Y. Okada, T. Yoshida, T. Nagasawa, Purification, characterization and gene cloning of isoeugenol-degrading enzyme from *Pseudomonas putida* IE27, Arch. Microbiol. 187 (2007) 511–517.
[20] T. Fufuya, M. Miura, K. Kino, A coenzyme-independent decarboxylase/oxygenase cascade for the efficient synthesis of vanillin, ChemBioChem 15 (2014) 2248.
[21] T. Furuya, M. Kuroiwa, K. Kino, Biotechnological production of vanillin using immobilized enzymes, J. Biotechnol. 243 (2017) 25–28.
[22] M. Rahouti, F. Seigle-Murandi, R. Steiman, K.E. Eriksson, Metabolism of ferulic acid by *Paecilomyces variotii* and *Pestalotia palmarum*, Appl. Environ. Microbiol. 55 (1989) 2391–2398.
[23] B. Karmakar, R.M. Vohra, H. Nandanwar, P. Sharma, K.G. Gupta, R.C. Sobti, Rapid degradation of ferulic acid via 4-vinylguaiacol and vanillin by a newly isolated strain of Bacillus coagulans, J. Biotechnol. 80 (2000) 195–202.
[24] X. Li, J. Yang, X. Li, W. Gu, J. Huang, K. Zhang, The metabolism of ferulic acid via 4-vinylguaiacol to vanillin by *Enterobacter* sp. Px6-4 isolated from Vanilla root, Process Biochem. 43 (2008) 1132–1137.
[25] H. Hu, L. Li, S. Ding, An organic solvent-tolerant phenolic acid decarboxylase from *Bacillus licheniformis* for the efficient bioconversion of hydroxycinnamic acids to vinyl phenol derivatives, Appl. Microbiol. Biotechnol. 99 (2015) 5071–5081.
[26] T. Fufuya, M. Miura, M. Kuroiwa, K. Kino, High-yield production of vanillin from ferulic acid by a coenzyme-independent decarboxylase/oxygenase two-stage process, New Biotechnol. 32 (2015) 335–339.
[27] G. Moiseyev, Y. Takahashi, Y. Chen, S. Gentleman, T.M. Redmond, R.K. Crouch, J. X. Ma, RPE65 is an iron (II)-dependent isomerohydrolase in the retinoid visual cycle, J. Biol. Chem. 281 (2006) 2835–2840.
[28] J.Y. Ryu, J. Seo, S. Park, J.H. Ahn, Y. Chong, M.J. Sadowsky, H.G. Hur, Characterization of an isoeugenol monooxygenase (Iem) from *Pseudomonas nitroreducens* Jin1 that transforms isoeugenol to vanillin, Biosci. Biotechnol. Biochem. 77 (2) (2013) 289–294.

[29] X. Zhang, J. Pei, L. Zhao, F. Tang, X. Fang, J. Xie, Overexpression and characterization of CCD4 from *Osmanthus fragrans* and β-ionone biosynthesis from β-carotene in vitro, J. Mol. Catal. B Enzym. 134 (2016) 105–114.

[30] T. Gerhards, U. Mackfeld, M. Bocola, E.V. Lieres, W. Wiechert, M. Pohl, D. Rother, Influence of organic solvents on enzymatic asymmetric carboligations, Adv. Synth. Catal. 35 (2012) 2805–2820.

[31] A.M. Klibanov, Improving enzymes by using them in organic solvents, Nature 409 (2001) 241–246.

[32] N. Wangrangsimagul, K. Klinsakul, A.S. Vangnai, J. Wongkongkatep, P. Inprakhon, K. Honda, Bioproduction of vanillin using an organic solvent-tolerant *Brevibacillus agri* 13, Appl. Microbiol. Biotechnol. 93 (2) (2012) 555–563.

[33] Z.Q. Liu, S.C. Dong, H.H. Yin, Y.P. Xue, X.L. Tang, X.J. Zhang, J.Y. He, Y.G. Zheng, Enzymatic synthesis of an ezetimibe intermediate using carbonyl reductase coupled with glucose dehydrogenase in an aqueous-organic solvent system, Bioresour. Technol. 229 (2017) 26–32.

[34] N. Doukyu, H. Ogino, Organic solvent-tolerant enzymes, Biochem. Eng. J. 48 (2010) 270–282.

[35] H. Guo, Y. Zhang, Y. Shao, W. Chen, F. Chen, M. Li, Cloning, expression and characterization of a novel cold-active and organic solvent-tolerant esterase from *Monascus ruber* M7, Extremophiles 20 (2016) 451–459.

[36] V.U. Yele, K. Desai, A New thermostable and organic solvent-tolerant lipase from *Staphylococcus warneri* ; Optimization of media and production conditions using statistical methods, Appl. Biochem. Biotechnol. 175 (2015) 855–869.

[37] T. Maruthiah, A. Palavesam, Characterization of haloalkalophilic organic solvent tolerant protease for chitin extraction from shrimp shell waste, Int. J. Cast Met. Res. 97 (2017) 552–560.

[38] D.H. Jung, W. Choi, K.Y. Choi, E. Jung, H. Yun, R.J. Kazlauskas, B.G. Kim, Bioconversion of *p*-coumaric acid to *p*-hydroxystyrene using phenolic acid decarboxylase from *B. amyloliquefaciens* in biphasic reaction system, Appl. Microbiol. Biotechnol. 97 (2013) 1501–1511.

[39] J. Yang, S. Wang, M.J. Lorrain, D. Rho, K. Abokitse, P.C. Lau, Bioproduction of lauryl lactone and 4-vinylguaiacol as value-added chemicals in two-phase biotransformation systems, Appl. Microbiol. Biotechnol. 84 (2009) 867–876.