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# Characterization of the *p*-Coumaric Acid Decarboxylase from *Lactobacillus plantarum* CECT 748$^T$

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It was previously reported that cell cultures from *Lactobacillus plantarum* CECT 748$^T$ were able to decarboxylate phenolic acids, such as *p*-coumaric, *m*-coumaric, caffeic, ferulic, gallic, and protocatechuic acid. The *p*-coumaric acid decarboxylase (PDC) from this strain has been overexpressed and purified. This PDC differs at its C-terminal end when compared to the previously reported PDC from *L. plantarum* LPCHL2. Because the C-terminal region of PDC is involved in enzymatic activity, especially in substrate activity, it was decided to biochemically characterize the PDC from *L. plantarum* CECT 748$^T$. Contrarily to *L. plantarum* LPCHL2 PDC, the recombinant PDC from *L. plantarum* CECT 748$^T$ is a heat-labile enzyme, showing optimal activity at 22 °C. This PDC is able to decarboxylate exclusively the hydroxycinnamic acids *p*-coumaric, caffeic, and ferulic acids. Kinetic analysis showed that the enzyme has a 14-fold higher $K_M$ value for *p*-coumaric and caffeic acids than for ferulic acid. PDC catalyzes the formation of the corresponding 4-vinyl derivatives (vinylphenol and vinylguaiacol) from *p*-coumaric and ferulic acids, respectively, which are valuable food additives that have been approved as flavoring agents. The biochemical characteristics showed by *L. plantarum* PDC should be taken into account for its potential use in the food-processing industry.

**KEYWORDS: Phenolic acid decarboxylase; hydroxycinnamic acids; decarboxylation; vinylphenols; flavoring agents**

## INTRODUCTION

*Lactobacillus plantarum* is a lactic acid species that is most frequently encountered in the fermentation of plant materials, where phenolic acids are abundant (*1*). Phenolic acids account for almost one-third of the dietary phenols, and there is an increasing awareness and interest in the antioxidant behavior and potential health benefits associated with these simple phenolic acids. It is their role as dietary antioxidants that has received the most attention in recent literature (*2*).

We have previously reported that cultures of *L. plantarum* CECT 748$^T$ metabolize some phenolic acids, such as *p*-coumaric, *m*-coumaric, caffeic, ferulic, gallic, and protocatechuic acids (*3*). The gene encoding a *p*-coumarate decarboxylase (PDC) from *L. plantarum* LPCHL2 has been cloned (*4*) and the recombinant protein (174 amino acid residues) overexpressed in *Escherichia coli*. Cavin et al. (*5*) have analyzed the substrate specificity of the purified PDC using 10 hydroxycinnamic acids, concluding that only the acids with a para hydroxyl group with respect to the unsaturated side chain and with a substitution of −H or −OH in position meta were metabolized (*5*).

In 2003, the complete genome sequence of *L. plantarum* WCFS1 was available (*6*). This strain possesses a PDC enzyme that differs, mainly in its C-terminal region, from the enzyme previously purified from *L. plantarum* LPCHL2. Recently, to crystallize PDC from *L. plantarum* type strain CECT 748$^T$ (*7*), we determined that its amino acid sequence is identical to that of *L. plantarum* WCFS1.

By expressing in *E. coli* native and chimeric phenolic acid decarboxylases, it was suggested that the C-terminal region in bacterial PDC is involved in enzymatic activity, especially in substrate specificity (*8*). Because (i) we have determined that *L. plantarum* CECT 748$^T$ is able to decarboxylate several cinnamic acids as well as several benzoic acids, (ii) some of these acids were not previously tested by using purified PDC from *L. plantarum* LPCHL2, and (iii) *L. plantarum* CECT 748$^T$ possesses a PDC enzyme that differs from *L. plantarum* LPCHL2 in its C terminus, we decided to express and biochemically characterize the PDC from *L. plantarum* CECT 748$^T$.

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WCF   MTKTFKTLDDFLGTHFIYTYDNGWEYEWYAKNDHTVDYRIHGGMVAGRWVTDQKADIVML  60
748   ************************************************************  60
LPC   ************************************************************  60

WCF   TEGIYKISWTEPTGTDVALDFMPNEKKLHGTIFFPKWVEEHPEITVTYQNEHIDLMEQSR 120
748   ************************************************************ 120
LPC   ************************************************************ 120

WCF   EKYATYPKLVVPEFANITYMGDAGQNNEDVISEAPYKEMPNDIRNGKYFDQNYHRLNK   178
748   ********************************************************     178
LPC   ********************E-************************LIKTTIV      174
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**Figure 1.** Comparison of protein sequence of *L. plantarum* PDC from strains WCFS1 (WCF) (accession no. CAD65735), CECT 748$^T$ (748), and LPCHL2 (LPC) (accession no. AAC45282). Asterisks, amino acid identity; dashes, gaps introduced to maximize similarities.

## MATERIALS AND METHODS

**Materials.** *L. plantarum* CECT 748$^T$ (ATCC 14917, DSMZ 20174) was purchased from the Spanish Type Culture Collection. The 19 phenolic acids analyzed in this study were 7 cinnamic acids, 9 benzoic acids, and 3 other food phenolic acids, such as phloretic acid (Aldrich H524006), chlorogenic acid (Sigma C3878), and ellagic acid (Sigma E2250). The cinnamic acids were *p*-coumaric acid (Sigma C-9008), *o*-coumaric acid (Fluka 28170), *m*-coumaric acid (Aldrich H23007), cinnamic acid (Aldrich C8, 085-7), caffeic acid (Sigma C0625), ferulic acid (Sigma F3500), and sinapic acid (Sigma D7927). The benzoic acids assayed were benzoic acid (Merck 6391513), syringic acid (Fluka 86230), gallic acid (Fluka 48630), salicylic acid (Merck 631), gentisic acid (Aldrich 149357), veratric acid (Fluka 94872), *p*-hydroxybenzoic acid (Fluka 54630), protocatechuic acid (Sigma P5630), and vanillic acid (Fluka 94770).

The phenolic acid derivatives 4-vinylphenol (Lancaster L10902) and 4-vinylguaiacol (Lancaster A13194) were used as standards for the identification of the decarboxylated compounds from *p*-coumaric and ferulic acids.

**Expression and Purification of the *p*-Coumaric Acid Decarboxylase.** The cloning and overexpression of the *pdc* gene from *L. plantarum* have been previously described (*7*). Briefly, the *pdc* gene (coding for *p*-coumaric acid decarboxylase) from *L. plantarum* CECT 748$^T$ was PCR-amplified and inserted into the pURI3 vector by using a restriction enzyme- and ligation-independent cloning strategy (*9*).

**Protein Assay.** Protein concentration was measured according to the method of Bradford using a protein assay kit purchased from Bio-Rad Laboratories (Germany) with bovine serum albumin as standard.

**Enzyme Activity Assay.** PDC activity was assayed by measuring the amount of 4-vinylphenol produced from *p*-coumaric acid. The standard reaction was performed by adding 30 $\mu$L of purified PDC enzyme (3 $\mu$g/mL) into 1 mL of reaction solution containing substrate at 4 mM in 25 mM phosphate buffer (pH 6.5) and incubating at 30 °C for 20 min. The assay time was under the linear range of enzyme reaction. The reaction was terminated by extracting twice with ethyl acetate. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 $\mu$mol of 4-vinylphenol per minute. Substrate and enzyme blanks were also prepared in which the enzyme or substrate was incubated with the buffer.

Kinetic analysis was performed under conditions of pH 6.5 and 30 °C for 20 min in 25 mM phosphate buffer containing substrate (*p*-coumaric, caffeic, or ferulic acid) at different concentrations ranging from 0.125 to 48 mM. Values of $K_M$ were calculated by fitting the initial rates as a function of substrate concentration to the Michaelis−Menten equation.

To determine the optimal pH of the PDC, the purified enzyme was incubated within different pH values (3−10) at 30 °C for 20 min using *p*-coumaric acid (4 mM) as the substrate. Citric acid−sodium citrate buffer (100 mM) was used for pH 3−5, phosphate buffer (100 mM) for pH 6−7, Tris−HCl buffer (100 mM) for pH 7−8, and 100 mM glycine−KOH buffer for pH 9 and 10. The optimal temperature was assayed by incubating the purified PDC in 25 mM phosphate buffer (pH 6.5) at different temperatures (4−90 °C) for 20 min using *p*-coumaric acid (4 mM) as substrate.

The temperature stability was assayed by preincubating the purified PDC in 25 mM phosphate buffer (pH 6.5) at different temperatures during 1, 2, 3, 5, 12, 24, and 48 h at 17, 22, 30, 37, or 52 °C without the substrate, and then 4 mM *p*-coumaric acid was added as substrate for residual activity assay.

To assay the effects of metals and other additives on *L. plantarum* PDC activity, the enzyme (90 ng of protein) was incubated with a 1 mM concentration of different metals and inhibitors and 4 mM *p*-coumaric acid in 1 mL of 25 mM phosphate buffer (pH 6.5) at 30 °C for 20 min. The activity was calculated as relative to the sample containing no additives.

To validate the method the MINITAB Student test was used. Three replicate determinations were carried out for each experiment. Relative standard deviations were ≤5%.

**HPLC Analysis of the Degradation Products from Phenolic Acids.** PDC in phosphate buffer (25 mM, pH 6.5) was incubated during 4 h at 30 °C in the presence of each phenolic acid at 1 mM final concentration. As control, phosphate buffer containing the phenolic acid was incubated in the same conditions. The reaction products were extracted twice with ethyl acetate (Laboratory-Scan, Dublin, Ireland) and analyzed hy HPLC-DAD.

A Thermo (Thermo Electron Corp., Waltham, MA) chromatograph equipped with a P400 SpectraSystem pump, an AS3000 autosampler, and a UV6000LP photodiode array detector was used. A gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reversed-phase Nova-Pak $C_{18}$ (25 cm × 4.0 mm i.d.) 4.6 $\mu$m particle size, cartridge at room temperature as follows: 0−55 min, 80% B linear, 1.1 mL/min; 55−57 min, 90% B linear, 1.2 mL/min; 57−70 min, 90% B isocratic, 1.2 mL/min; 70−80 min, 95% B linear, 1.2 mL/min; 80−90 min, 100% linear, 1.2 mL/min; 100−120 min, washing 1.0 mL/min, and reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm (*10*). Samples were injected in duplicate onto the cartridge after being filtered through a 0.45 $\mu$m PVDF filter (Teknokroma, Spain).

The identification of degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers or by LC-DAD/ESI-MS.

## RESULTS AND DISCUSSION

**Enzymatic Activity of *L. plantarum* PDC.** *L. plantarum* CECT 748$^T$ isolated from pickled cabbage has been shown to produce a *p*-coumarate decarboxylase identical to that produced by *L. plantarum* WCFS1 strain, isolated from saliva (*7*). Strikingly, the purification of another PDC from *L. plantarum* LPCHL2 strain, with a different amino acid sequence, has been previously reported. **Figure 1** shows an alignment of the available *L. plantarum* PDC protein sequences. PDCs from *L. plantarum* WCFS1 and CECT 748$^T$ were identical and showed

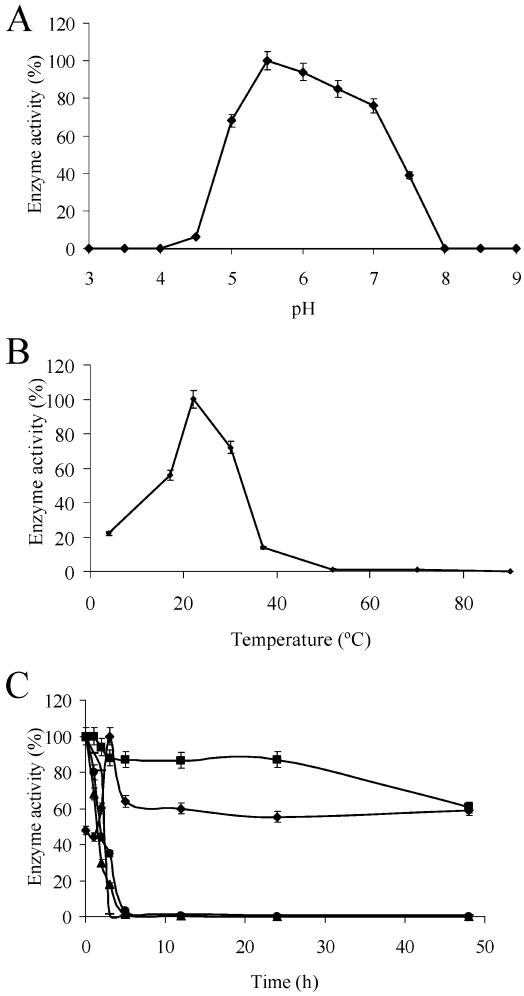


**Figure 2.** Biochemical characterization of the *L. plantarum* CECT 748$^T$ purified PDC: (**A**) optimum pH for activity (PDC reactions were performed at pH ranging from 3 to 9); (**B**) optimum temperature for PDC activity (purified PDC was incubated at temperatures ranging from 4 to 90 °C); (**C**) thermal stability of PDC [purified PDC was incubated at 17 °C (◆), 22 °C (■), 30 °C (—), 37 °C (●), or 52 °C (▲) in phosphate buffer (50 mM, pH 6.5); at indicated times, aliquots were withdrawn, and analyzed as described under Materials and Methods. The experiments were done in triplicate, and the mean value is shown.

a 95% identity to PDC from *L. plantarum* LPCHL2. As shown in **Figure 1**, the differences among these proteins were located in their C-terminal ends, a region that has been suggested to be involved in determining PDC substrate specificity and catalytic capacity (*8*). Thus, we decided to characterize the PDC from *L. plantarum* CECT 748$^T$ in terms of enzymatic activity as well as substrate specificity.

It was previously reported that purified PDC from *L. plantarum* LPCHL2 showed full enzyme activity at 25 mM phosphate buffer (pH 6) without any exogenously added cofactors or metal ions and was independent of the presence of oxygen. The *L. plantarum* LPCHL2 PDC enzyme was stable in 25 mM phosphate buffer, with >80% of the activity being conserved after 10 h of incubation at 30 °C. The optimal pH and temperature were 5.5−6 and 30 °C, respectively (*5*). We have purified recombinant PDC enzyme from *L. plantarum* CECT 748$^T$ (*7*) that showed an optimal activity at pH 5.5 (**Figure 2A**). The enzyme appeared to be slightly acid, which is in accordance with PDC from *Cladosporium phlei* (*11*) and *Bacillus pumilus*, which exhibited almost 100% of total activity between pH 5.0 and 6.0 (*12*).

**Table 1.** Effect of Additives on *L. plantarum* CECT 748$^T$ PDC Activity

| addition (1 mM) | residual activity (%) |
|---|---|
| control | 100 |
| $MgCl_2$ | 94 |
| KCl | 110 |
| $CaCl_2$ | 86 |
| SDS | 10 |
| Triton X-100 | 41 |
| urea | 102 |
| EDTA | 65 |
| DMSO | 57 |
| $\beta$-mercaptoethanol | 18 |

From the temperature range assayed, it can be concluded that the enzyme had an optimal activity at 22 °C (**Figure 2B**) and retained virtually all of its activity at this temperature for 24 h (**Figure 2C**). Unexpectedly, the enzyme is very active only up to 30 °C, as at 37 °C it showed only 15% of the maximal activity (**Figure 2B**). In addition, and contrarily to what had been previously reported, the enzyme was almost inactivated by preincubation at 30 °C during 12 h (it retained only 1% of the original activity) (**Figure 2C**). However, incubation during 48 h at 17 or 22 °C reduces to only 20% the maximal activity of PDC. Although it was previously reported that *L. plantarum* LPCHL2 PDC conserved >80% activity after 10 h of incubation at 30 °C (*5*), the temperature results herein obtained on recombinant PDC from *L. plantarum* CECT 748$^T$ seems to indicate that it is a heat-labile enzyme. The differences observed on temperature resistance between both *L. plantarum* PDC proteins could be due to their differences in the C-terminal amino acid sequences. Similarly to *L. plantarum* CECT 748$^T$ recombinant PDC, it was shown that PDC from *C. phlei* was also a heat-labile enzyme (*11*) as the activity was reduced to about three-fourths even for incubation at 35 °C for 5 min. In addition, the activity of PDC from *B. pumilus* is rapidly lost above 37 °C and is completely abolished above 42 °C (*12*). In *Bacillus* sp. BP-7, on the basis of the results obtained after incubation at 55 °C, Prim et al. reported that the PDC is a mesophilic enzyme (*13*).

The effects of various metal ions (such as $Mg^{2+}$, $K^+$, and $Ca^{2+}$) surfactants (SDS and Triton X-100), denaturants (urea), chelators (EDTA), and inhibitors (DMSO, $\beta$-mercaptoethanol) on enzymatic activity were also investigated (**Table 1**). The purified PDC from *L. plantarum* LPCHL2 was completely inhibited by 0.3% (w/v) of sodium dodecyl sulfate (*5*). Our results corroborate previous ones, as 1 mM SDS reduces *L. plantarum* CECT 748$^T$ recombinant PDC activity to 10% of its original activity. Results obtained with *C. phlei* PDC indicated that metal ions are not involved in their activity (*11*); similarly to *C. phlei* PDC, the activity of *L. plantarum* CECT 748$^T$ recombinant PDC was not significantly affected by metal ions (**Table 1**).

**Substrate Specificity of *L. plantarum* PDC.** We have studied previously the ability of *L. plantarum* CECT 748$^T$ cultures to decarboxylate 19 different cinnamic and benzoic acids. From these acids, only *p*-coumaric, *m*-coumaric, caffeic, ferulic, gallic, and protocatechuic acids were metabolized. All of these acids were decarboxylated, and some of them were also reduced (*3*). To ascertain our previous results, the same 19 phenolic acids were assayed by using recombinant PDC from *L. plantarum* CECT 748$^T$. As expected, the phenolic acids that were not metabolized by *L. plantarum* cultures were not metabolized by the purified enzyme. However, differences were found in relation to the phenolic acids metabolized. The cinnamic acid *m*-coumaric acid and the benzoic acids gallic and protocatechuic

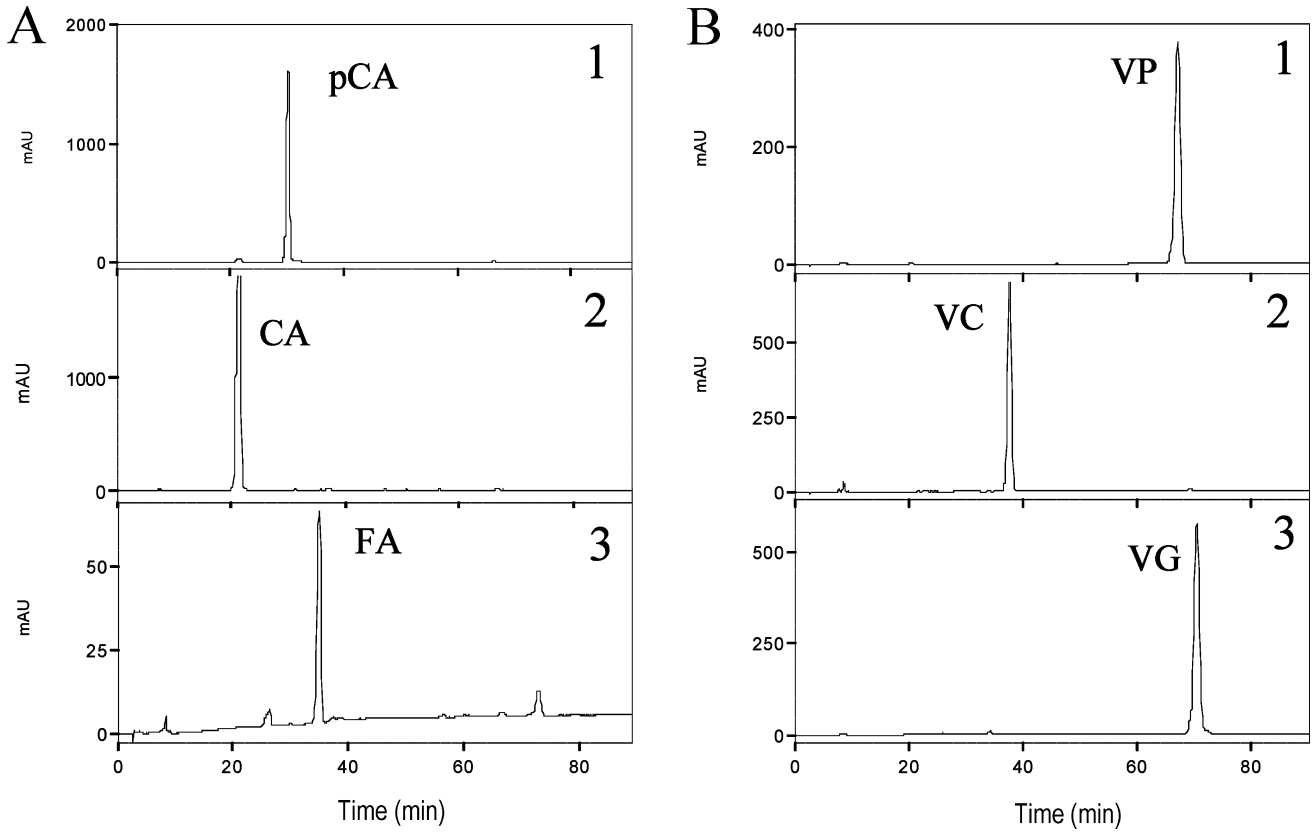


**Figure 3.** (**A**) HPLC chromatograms of the decarboxylation of *p*-coumaric, caffeic, and ferulic acids by *L. plantarum* PDC. Purified PDC from *L. plantarum* CECT 748$^T$ was incubated for 4 h in presence of *p*-coumaric (1), caffeic (2), and ferulic gallic acid (3) (**B**). HPLC chromatograms from the control samples are also indicated (**A**). The chromatograms were recorded at 280 nm. pCA, *p*-coumaric acid; CA, caffeic acid; FA, ferulic acid; VP, vinylphenol; VC, vinylcatechol; VG, vinylguaiacol.

**Table 2.** Kinetic Parameters of *L. plantarum* CECT 748$^T$ PDC

| substrate[a] | $K_M$ (mM) | $V_{max}$ ($\mu$mol/h mg) |
|---|---|---|
| *p*-coumaric acid | 1.12 | 711 |
| caffeic acid | 1.07 | 742 |
| ferulic acid | 0.08 | 54 |

[a] Kinetics analyses were carried out under conditions of pH 6.5 and 30 °C for 20 min with phenolic acids at concentrations ranging from 0.125 to 48 mM.

acids, which were decarboxylated by cell cultures, were not used as substrate by purified PDC. As previously reported, PDC decarboxylates only *p*-coumaric, caffeic, and ferulic acids (**Figure 3**) (*8*). Commercial 4-vinylphenol and 4-vinylguaiacol, used as standards, were co-injected with the reaction mixtures for the identification of the products released after PDC decarboxylation; 4-ethylcatechol was identified by LC-DAD/ESI-MS (data not shown). Therefore, these results indicated that a still unknown decarboxylase enzyme(s) must be responsible for the decarboxylation of *m*-coumaric, gallic, and protocatechuic acids by *L. plantarum* CECT 748$^T$. These results are in agreement with those reported previously by Barthelmebs et al., who showed, by knockout of the PDC gene, that *L. plantarum* has a second acid phenol decarboxylase enzyme, better induced with ferulic than with *p*-coumaric acid (*14*).

Kinetic parameters of PDC were investigated at pH 6.5 and 30 °C for 20 min using *p*-coumaric, caffeic, or ferulic acids as substrates (**Table 2**). The PDC enzyme has $K_M$ values of 1.12 and 1.07 mM for *p*-coumaric and caffeic acids, respectively, being only 0.08 mM for ferulic acid. By using purified PDC from *L. plantarum* LPCHL2, at the optimal pH and temperature, the PDC enzyme has a $K_M$ of 1.4 mM, a maximum velocity of 766 $\mu$mol min$^{-1}$ mg$^{-1}$, and a $K_{cat}$ of about 103 s$^{-1}$ for *p*-coumaric acid, and it was reported that only *p*-coumaric and caffeic acids were metabolized, at the same rate and $K_M$ (*5*). In *Bacillus* sp. BP-7, under the conditions used, the conversion rates were 13.4, 7.2, and 11.8 $\mu$M/min for *p*-coumaric, caffeic, and ferulic acid, respectively (*13*). However, although *L. plantarum* PDC shows a low $V_{max}$ by ferulic acid, this acid was completely decarboxylated to vinylguaiacol by *L. plantarum* CECT 748$^T$ PDC (**Figure 3**). The activity of *L. plantarum* PDC on the hydroxycinnamic acids, *p*-coumaric and ferulic acids, resulted in the production of 4-vinylphenol and 4-vinylguaiacol, which are valuable intermediates in the biotechnological production of new fragrance chemicals. In addition, both compounds are considered to be food additives and are approved as flavoring agents by regulatory agencies (4-vinylphenol, JECFA 711 and FEMA 3739; and 4-vinylguaiacol, JECFA 725 and FEMA 2675) (*15*).

In conclusion, kinetic parameters determined for *L. plantarum* CECT 748$^T$ PDC against *p*-coumaric, caffeic, and ferulic acids indicate that at high substrate concentrations ([S] $\gg K_M$), both *p*-coumaric and caffeic acids are much more efficiently decarboxylated than ferulic acid, due to the much higher $V_{max}$ value. On the contrary, in conditions where [S] $\ll K_M$ ferulic acid is more rapidly decarboxylated due to the much higher relative affinity (lower $K_M$). This markedly different kinetic behavior may be relevant biologically. In addition, these results also indicate that the presence of bulky moieties in the meta position of the aromatic ring of the substrate (a methyl group in ferulic acid) clearly affects the binding step to the enzyme. On the other hand, this PDC seems to be a heat-labile enzyme, as compared to the previously described PDC from *L. plantarum* LPCHL2. Knowledge of the biochemical characteristics of this protein

could be useful for the enzymatic synthesis of the flavoring agents 4-vinylphenol and 4-vinylguaiacol.

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