Biocatalytic decarboxylation and deacetylation studies on cinnamic acids and other phenylpropanoids

2.1 Introduction:
Decarboxylation is one of the oldest chemical transformations involving removal of a carboxyl group (-COOH), usually in the form of carbon dioxide (Scheme 1) and has been extensively used in organic synthesis.

\[
\begin{align*}
\text{Scheme 1}
\end{align*}
\]

2.2 Importance of decarboxylation:
Many pathways to natural products involve steps which remove portions of the carbon skeleton. Although two or more carbon atoms may be cleaved off via the reverse aldol or reverse Claisen reactions, by far the most common degradative modification is the loss of one carbon-atom by a decarboxylation reaction. Decarboxylation is a particular feature of the biosynthetic utilization of amino acids and various secondary metabolites are derived from amino acids via loss of the carboxyl group using specific enzymes termed as decarboxylases [Liu and Zhang (2006)]. Similarly, decarboxylation of \(\alpha\)-keto acids is a feature of primary metabolism in glycolysis and pyruvic acid to acetaldehyde or pyruvic acid to acetyl-CoA represents an example of overall oxidative-decarboxylation prior to the entry of acetyl-CoA into Krebs cycle [Dewick (2009)].

From the organic chemistry viewpoint, decarboxylation of cinnamic acids and their derivatives leads to the formation of immensely important bioactive compounds like styrene or hydroxystyrenes etc. Hydroxystyrenes, also known as vinylphenols, constitute one of the most extensively explored compounds [Kuwahara et al. (2004)] due to their wide ranging applications in food and flavors [Steffen (1994)], as intermediates in the preparation of other bioactive molecules [Stuart et al. (1994); Aslam et al. (1996)], and polymers/copolymers, electronics, ion exchange resins and photoresists [Lee et al. (1994); Atsushi et al. (1998)] etc. A burgeoning global demand coupled with the natural scarcity of several commercially important hydroxystyrenes, many of which are FEMA GRAS (Flavoring Extract Manufacturer Association; Generally Regarded As Safe) approved
flavoring agents such as 4-vinylguaiacol and 4-vinylphenol etc. (Figure 1) have provided an increased impetus for their synthesis which is by and large accomplished via decarboxylation of cinnamic acids.

Figure 1: Commercially important hydroxystyrenes

Thus, in view of the immense importance of decarboxylation reactions, numerous methodologies have been developed for accomplishing these reactions. As apparently known, the removal of carboxyl group is a tedious task and often requires prior activation by metal catalyst as well as the use of harsh organic base [Cohen and Schambach (1970)]. The most common approach for the decarboxylation of cinnamic acid derivatives involves refluxing at a very high temperature in the presence of quinoline (base) and anhydrous copper salt [Shepard et al. (1930)]. Also, a number of metal based catalysts such as Mn(III), Hg(OAc)$_2$, and AgOAc have been employed to carry out the reactions. Recently, some microwave and ionic liquid mediated green protocols and biotransformation processes have been developed for the decarboxylation of cinnamic acid derivatives into hydroxystyrenes. A brief description of some reported decarboxylation protocols has been presented below:

2.3 Reported methods for decarboxylation reactions:

2.3.1 Conventional approaches:
These approaches generally involve metal based catalytic systems and high temperature refluxing of cinnamic acids. Walling et al. studied the individual effects of both quinoline and copper salt towards decarboxylation of substituted cinnamic acids. It was found that decarboxylation occurred very slowly in the presence of Cu salt or quinoline alone [Walling and Wolfstrin (1947)]. However, addition of 10% copper sulphate to the mixture of cinnamic acid in boiling quinoline accelerated the decarboxylation process to yield the corresponding product (Scheme 2).
In another instance, Johnson et al. studied the acid-catalyzed (mixture of HBr and CH$_3$COOH) decarboxylation of cinnamic acids (Scheme 3). It was observed that rate of reaction increased with increase in the concentration of acid catalyst [Johnson and Heinz (1949)].

\[
\text{C}_6\text{H}_5\text{C}==\text{COOH} + \text{HA} \xrightleftharpoons{} \text{C}_6\text{H}_5\text{C}^+\text{CHCOOH} \rightarrow \text{C}_6\text{H}_5\text{C}==\text{CH}_2 + \text{CO}_2
\]

**Scheme 3**

Similarly, Overberger et al. synthesized p-acyloxy styrenes by the Cu/quinoline mediated decarboxylation of cinnamic acids. In the first step, p-hydroxy cinnamic acid was acetylated with long chain acid chlorides (C-1 to C-15) and the second step involved the decarboxylation reaction for the formation of acyloxy styrenes (Scheme-4) [Overberger et al. (1950)].

\[
\text{C}_6\text{H}_5\text{CH}=\text{CH}_2\text{COOH} \rightarrow \text{C}_6\text{H}_5\text{CH}=\text{CH}_2\text{COOH} + \text{RCOCl} \rightarrow \text{C}_6\text{H}_5\text{CH}=\text{CH}_2 + \text{RCO}_2\text{H}
\]

**Scheme 4**

In another report, *cis*-combretastatin A4 (an antimitotic compound) was synthesized via decarboxylation of corresponding α-phenyl cinnamic acid ((E)-3-(3'-Hydroxy-4'-methoxyphenyl)-2-(3'',4'',5''-trimethoxyphenyl)prop-2-enoic acid) using a combination of Cu/quinoline (Scheme 5) [Gaukroger et al. (2001)].

\[
\text{HOOC} \rightarrow \text{HOOC} \xrightarrow{\text{Quinoline, Cu powder, 200°C}} \text{HOOC} + \text{H}_3\text{CO}_3\text{OCH}_3 + \text{H}_3\text{CO}_3\text{OCH}_3
\]

**Scheme 5**

Thus, decarboxylation via conventional synthetic methods involves either the use of harsh chemicals [Cohen and Schambach (1970)] or protection-deprotection steps resulting in hazardous waste besides providing low yields of desired product due to tendency for the formation of several side reactions including polymerization [Locatelli et al. (2005); Bernini et al. (2007)].
Consequently, there have been efforts to design more benign decarboxylation processes with various improvements, however, still these are confined by the crucial use of metals [Moseley and Gilday (2006); Gooßen et al. (2006); Dickstein et al. (2007)].

2.3.2 Microwave and Ionic liquid assisted decarboxylation approaches:
In recent past, few green approaches utilizing microwave or ionic liquids have been accounted. Bernini et al. (2007) has developed a microwave assisted process for the decarboxylation of 4-hydroxycinnamic acids in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base and basic aluminum oxide as solid support (Scheme 6). The procedure was successfully extended to a natural sample of ferulic acid extracted from wheat bran to get 4-vinylguaiacol.

![Scheme 6](image)

Similarly, Nomura et al. reported the decarboxylation of hydroxylated cinnamic acids via microwave heating using amine bases (Scheme 7) [Nomura et al. (2005)].

![Scheme 7](image)

A microwave assisted decarboxylation of hydroxy substituted α-phenyl cinnamic acids into corresponding stilbenes using a combination of methylimidazole (MIm) and piperidine has been disclosed (Scheme 8) [Kumar et al. (2007)].

![Scheme 8](image)
Recently, Sharma et al. has reported a new platform for the metal- and quinoline-free decarboxylation of various \( N \)-heteroaryl and aryl carboxylic acids under microwave irradiation in aqueous condition employing ionic liquid [hmim]Br (Scheme 9). The addition of a mild base like aqueous sodium hydrogen carbonate (NaHCO\(_3\)) to [hmim]Br further improved the decarboxylation of hydroxylated cinnamic and aromatic acid substrates [Sharma et al. (2008)].

\[
\begin{align*}
R_1 & \quad R_2 \\
R_3 & \quad R_4
\end{align*}
\]

Scheme 9

All of these methods fit well into the context of Green Chemistry however, the increased health consciousness among consumers for foods and flavors comprising of all natural ingredients is still a major holdup with these procedures [Muheim and Lerch (1999)]. Thus, in this existing context of heightened environmental concerns along with ever increasing demand of natural flavors; microbial or biocatalytic decarboxylation of cinnamic acids has provided a panacean approach. The peculiar properties of biocatalysts, especially their high chemo-, regio- and enantioselectivity and their ability to work under mild reaction conditions appear to be particularly suitable for the production of styrenes. On top, the products from such bioconversion are considered natural under the European and American Legislation [Muheim and Lerch (1999); Topakas et al. (2003)].

2.3.3 Decarboxylation through biotransformation route:

Enzymatic or chemical catalysis is the basis of many industrial processes and often this technology compete with synthetic one for the production of important chemicals. Biotransformation processes represent a promising alternative to chemical synthesis as compounds produced by these are generally regarded as safe. Several studies employing microbial or plant cell cultures for the decarboxylation of cinnamic acids have been reported with prime focus on bioconversion of ferulic acid into commercially important 4-vinylguaiacol.

For example, Lee et al. used Bacillus pumilus as biocatalyst for the decarboxylation of ferulic acid in a two-phase system (Scheme 10). Higher biotransformation yields were
obtained when hydrocarbons such as $n$-pentane, $n$-hexane, $n$-heptane and $n$-octane were used as organic phase [Lee et al. (1998)].

\[
\text{Ferulic acid} \xrightarrow{Bacillus pumilus} \text{4-Vinylguaiacol}
\]

**Scheme 10**

Donaghy et al. has reported conversion of ferulic acid to 4-vinylguaiacol by yeast isolated from unpasteurised apple juice. The highest decarboxylase activity was associated with the washed resting cells of *Candida lambica* showing temperature and pH optima of 35°C and 6.5, respectively [Donaghy et al. (1999)]. Karmakar et al. has reported a new bacterial strain (*B. coagulans* BK07) isolated from decomposed wood-bark having ferulic acid decarboxylation properties. This strain rapidly decarboxylated ferulic acid to 4-vinylguaiacol which was immediately converted to vanillic acid via vanillin. Vanillic acid was further demethylated to protocatechuic acid [Karmakar et al. (2000)].

Similarly, Mathew et al. reported a yeast strain *Debaryomyces hansenii* for metabolism of ferulic acid to 4-vinylguaiacol (by the non oxidative decarboxylation of its side chain) and vanillin (Scheme 11). *Debaryomyces* produced 1470 mg/L of 4-vinylguaiacol after ten hours of incubation, corresponding to a molar yield of 95% while the production of vanillin reached a maximum of 169 mg/L [Mathew et al. (2007)].

\[
\text{Ferulic acid (2 g/L)} \xrightarrow{Debaryomyces hansenii} \text{4-Vinylguaiacol (1.47 g/L) and Vanillin (0.169 g/L)}
\]

**Scheme 11**

Decarboxylation of *trans*-cinnamic acids into corresponding styrenes has also been carried out through plant cell cultures of *Catharanthus roseus*, *Nicotiana tabacum*, *Daucus carota* and *Camellia sinensis* at room temperature in quantitative yields (Scheme 12) [Takemoto and Achiwa (2001)].
Scheme 12

Similarly, 4-vinylphenol has been synthesized through decarboxylation of \( p \)-coumaric acid with the help of \( p \)-coumaric acid decarboxylase (Scheme-13) [Barthelmebs et al. (2000)].

Scheme 13

Hashidoko et al. synthesized 4-vinyl phenol with the help of (E)-4-hydroxycinnamate decarboxylase (4-HCD) of \( Klebsiella \ oxytoca \). The presence of hydroxy group at \textit{para} position of the aromatic ring was found essential for decarboxylation (Scheme 14) [Hashidoko et al. (1993)].

Scheme 14

In majority of above reported methods, achieving excellent selectivity vis-à-vis biotransformation of ferulic acid into 4-vinylguaiacol still remains a tedious task due to frequently encountered problem of over oxidation [Karmakar et al. (2000); Mathew et al. (2007)] into corresponding acid/aldehyde/alcohol. Moreover, lower product yield and limited substrate applicability add to the constraints of these procedures.

Keeping in view the enormous potential for progress in the field of biotransformation on account of the unlimited reservoir of different enzyme activities existing in nature and the exciting achievements of modern biotechnology, there remains a scope for further improvement in the existing biocatalytic decarboxylation protocols of cinnamic acids.

2.4 Result and Discussion:

As a part of our ongoing efforts towards the production of important bioactive molecules like styrenes through green chemistry [Sinha et al. (2006, 2007); Kumar et al. (2007)], we have had interest in exploiting abundantly available natural raw materials such as ferulic
acid (from maize, wheat or rice bran) and related cinnamic acids for bioconversion into value added products; primarily styrenes, utilizing bacterial strains isolated from Western Himalayan region.

2.4.1 Screening of microbial strains:

Ferulic acid (1a) was chosen as a model substrate for the screening of microbial strains isolated from the soils of Western Himalayan region. The strains capable of decarboxylating 1a into vinylguaiacol (1b) were rapidly screened through thin layer chromatography (TLC) and then by high performance liquid chromatography (HPLC) against reference standard. The obtained product 1b was re-confirmed through GC-MS and NMR. Accordingly, two strains (KJLPB4 and KJPB2) showing good conversion were selected from a group of 38 strains screened. Based on 16S rDNA sequencing, strains KJLPB4 and KJPB2 were subsequently identified as Pantoea agglomerans species and hereafter named as P. agglomerans KJLPB4 (EMBL # FN263077) and P. agglomerans KJPB2 (EMBL # FN263076). The strains have been deposited with Institute of Microbial Technology, Chandigarh, India under MTCC 10409 and MTCC 10062, respectively.

2.4.2 Optimization of biotransformation procedure:

Optimization experiments were initially performed with strain KJLPB4 under three different sets – varying substrate concentration, incubation temperature and incubation time with 1a as model substrate. The effect of varying time and substrate concentration is shown in Figure 2. Initially, the reaction mixture was incubated up to 96 h at 28°C. A 200 µL of sample was taken at definite time intervals (after 12 h interval) and extracted with equal volume of ethyl acetate for analysis with RP-HPLC (Figure 3). It was found that optimum yield of about 98% was obtained at 48 h.

Figure 2. Effect of substrate concentration and incubation time on the yield of 4-vinylguaiacol by Pantoea agglomerans KJLPB4 at 28°C. Ferulic acid (FA) concentrations (g/L): 1.0 (♦), 2.0 (■), 2.5 (▲) and 3.0 (●).
Subsequently, ferulic acid concentration was optimized starting with 1.0 g/L and gradually increasing up to 3.0 g/L at 28°C. Optimum conversion was achieved from 2.0 g/L of substrate concentration for strain KJLPB4 at 48 h. With further increase in ferulic acid concentration, the yield declined correspondingly; 2.5 g/L of ferulic acid resulting in yield of around 50%. No bioconversion was observed when ferulic acid concentration was increased above 3.0 g/L.

Thus, it was ascertained that 2.0 g/L of substrate concentration, 28°C of incubation temperature and 48 h of incubation time was optimum for complete conversion of \(1a\) into \(1b\) with KJLPB4. Under similar set of conditions (2.0 g/L substrate concentration, 28°C and 48 h incubation time), bioconversion of \(1a\) to \(1b\) was 60% with strain KJPB2 along with 30% conversion into vanillic acid (\(1c\)) as a second product. However, if the reaction mixture was allowed to proceed for 72 h, yield of \(1c\) increased up to 85% (Scheme 15).
Scheme 15. Bioconversion of ferulic acid by newly isolated strains of \textit{P. agglomerans}

2.4.3 Determination of protein content and SDS page analysis:
The protein content determined by Bradford method [Bradford (1976)] was found to be 0.021 mg/mL and 2.9 mg/mL in case of KJLPB4 culture supernate and cell-free enzyme extract, respectively. In case of KJPB2 culture supernate, protein content was 0.036 mg/mL. The SDS page analysis of KJLPB4 culture supernate, cell free enzyme extract and KJPB2 culture supernate (Figure 4) showed that a number of proteins were secreted extracellularly by both the strains however, there was some qualitative and quantitative differences. As expected the numbers of proteins secreted extracellularly by KJLPB4 were less as compared to the number of proteins present in cell free extract which was in corroboration with their protein content.

Figure 4: SDS page analysis of various preparations from \textit{P. agglomerans} strains
2.4.4 Influence of co-solvent:
One of the shortcomings of biocatalysis in aqueous solution is the poor solubility of the substrates to be converted which is generally resolved by the use of co-solvent [Sorgedrager et al. (2008)]. In our case too, the poor solubility of substituted cinnamic acids in water posed a problem. Many reports are cited where basic medium with NaOH [Hua et al. (2007)], or NaHCO₃ [Kumar et al. (2007)] or organic solvent has been used [Nomura et al. (2005); Mathew et al. (2007)], besides the use of biphasic system [Lee et al. (1998)].

Pondering over the problem, the effect of various co-solvents (non-polar to polar solvents i.e. hexane, tert-butyl methyl ether, chloroform, N,N-dimethylformamide, tetrahydrofuran, acetonitrile, ethanol) on the biotransformation of ferulic acid was scrutinized. To our expectation, no conversion was there without using co-solvent and 1-2% (v/v) of water miscible organic solvent was found obligatory. However, further increase in the concentration of co-solvent led to decrease in reaction yields. Amongst the various organic co-solvents studied, best results were given by both N,N-dimethylformamide (DMF) and NaOH solution (pH 8.5). However, due to crucial pH adjustment step with NaOH [Hua et al. (2007)], DMF was preferred as co-solvent.

2.4.5 Decarboxylation in preparative scale:
To evaluate the practical use of isolated strains, preparative scale decarboxylation was carried out with 1a (50 mg x 5) dissolved in DMF (final concentration in medium 2%, v/v). Culture (25 mL x 5) of P. agglomerans KJLPB4 was added and the reaction was carried out at 28°C for 48 h in incubator shaker (220 rpm). Column chromatography of the crude sample (obtained from five batches) over silica gel with hexane: ethyl acetate (9:1, v/v) gave 179 mg of pure 4-vinylguaiacol with isolated yield of 93%.

Further, from the viewpoint of utilizing renewable natural sources, a sample of crude ferulic acid extracted from maize bran was subjected for bioconversion. Since ferulic acid is covalently bound to the polysaccharide fraction of plant cell wall in maize bran [Saulniet et al. (1995)], its recovery needs a chemical or enzymatic hydrolysis of the plant cell wall. Here, the chemical hydrolysis (with NaOH) was performed [Bunzel et al. (2005)] which resulted in 70% ferulic acid release from maize bran along with expected dimers and trimers of ferulic acid. This crude sample, without any further purification, was subjected to biotransformation with KJLPB4 resulting in the formation of 4-vinylguaiacol with quantitative yield.
2.4.6 Substrate spectrum:
Later on, to elucidate the scope and general applicability of the protocol under optimized conditions, a variety of structurally divergent substrates were subjected to decarboxylation for determining the structural requirements of substrates to be converted (Table 1).

Table 1. Decarboxylation of substituted cinnamic acids by *P. agglomerans* strains

<table>
<thead>
<tr>
<th>Entry</th>
<th>Cinnamic acid (a)</th>
<th>Product (b)</th>
<th>Time [h]</th>
<th>Yield %&lt;sup&gt;a&lt;/sup&gt; KJLPB4</th>
<th>Yield %&lt;sup&gt;a&lt;/sup&gt; KJPB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="cinnamic_acid_1.png" alt="Image" /></td>
<td><img src="product_1.png" alt="Image" /></td>
<td>48</td>
<td>98</td>
<td>60 + 30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(10 + 85&lt;sup&gt;b&lt;/sup&gt;)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td><img src="cinnamic_acid_2.png" alt="Image" /></td>
<td><img src="product_2.png" alt="Image" /></td>
<td>72</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td><img src="cinnamic_acid_3.png" alt="Image" /></td>
<td><img src="product_3.png" alt="Image" /></td>
<td>72</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td><img src="cinnamic_acid_4.png" alt="Image" /></td>
<td><img src="product_4.png" alt="Image" /></td>
<td>48</td>
<td>30</td>
<td>85</td>
</tr>
<tr>
<td>5</td>
<td><img src="cinnamic_acid_5.png" alt="Image" /></td>
<td><img src="product_5.png" alt="Image" /></td>
<td>96</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>6</td>
<td><img src="cinnamic_acid_6.png" alt="Image" /></td>
<td><img src="product_6.png" alt="Image" /></td>
<td>96</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>7</td>
<td><img src="cinnamic_acid_7.png" alt="Image" /></td>
<td><img src="product_7.png" alt="Image" /></td>
<td>72</td>
<td>30</td>
<td>nd</td>
</tr>
</tbody>
</table>

<sup>a</sup> On the basis of HPLC analysis; <sup>b</sup> formation of vanillic acid (1c) along with desired product 1b in 48 h of incubation; <sup>c</sup> formation of 1c got increased as major product after 72 h of incubation; nd = not detected.

It is clearly established from Table 1 that the best substrate for decarboxylation under optimized conditions with *P. agglomerans* strain KJLPB4 is ferulic acid (1a) while with KJPB2 it is *p*-coumaric acid (4a). It is relevant to mention here that such high yields for 1b and 4b has not been achieved earlier even by our group employing synthetic protocols [Sinha et al. (2007); Sharma et al. (2008)]. Both sinapic acid (2a) and caffeic acid (3a) showed lower conversion yields (Table 1, entries 2, 3). While for 2-hydroxy and 3-hydroxy cinnamic acid (Table 1, entries 5, 6) no conversion was observed. As reported earlier, decarboxylation of cinnamic acids is generally favoured by ortho- or para-hydroxy substitution at the aromatic ring as compared to meta-hydroxy counterpart [Nomura et al. (2005); Sinha et al. (2007)]. However, with our protocol even *m*-hydroxy counterpart of
ferulic acid i.e. isoferulic acid (7a) was decarboxylated into corresponding styrene (7b) by *P. agglomerans* strain KJLPB4 though the yield obtained was just 30% (Entry 7). Besides compounds 5a and 6a, some other substrates found unsuitable for decarboxylation with strains KJLPB4 and KJPB2 are listed in Table 2.

**Table 2.** Unsuitable substrates for decarboxylation with *Pantoea agglomerans* strains

<table>
<thead>
<tr>
<th>cinnamic acid</th>
<th>benzoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>p</em>-nitrocinnamic acid</td>
<td><em>p</em>-nitrobenzoic acid</td>
</tr>
<tr>
<td>3,4-dimethoxy cinnamic acid</td>
<td><em>p</em>-chlorobenzoic acid</td>
</tr>
<tr>
<td>4-bromocinnamic acid</td>
<td>phenylacetic acid</td>
</tr>
<tr>
<td>5-chloroindole-2-carboxylic acid</td>
<td>biphenylacetic acid</td>
</tr>
<tr>
<td>trans-3-indoleacrylic acid</td>
<td><em>p</em>-hydroxyphenylacetic acid</td>
</tr>
<tr>
<td>2-chloro-4-nitrobenzoic acid</td>
<td><em>p</em>-nitrophenylacetic acid</td>
</tr>
</tbody>
</table>

Inspired by the above success with 1a (conversion 98%), we attempted the decarboxylation of substituted ferulic acids as scanty literature is available on such conversions through microbes. For this, different derivatives of ferulic acid were prepared by known methods with consideration of following aspects (a) substitution on aromatic ring; (b) substitution pattern at the double bond; (c) substitution at acidic hydrogen; (d) substitution at hydroxy position as shown in Table 3. The prepared derivatives were subjected to bioconversion and it was found that substitution either at phenyl ring of ferulic acid or at acidic hydrogen hindered the decarboxylation by both strains (Table 3, entries 8,9,11). Similarly, substitution at double bond (12a) was met with limited success as the corresponding hydroxylated stilbene (12b) was obtained in traces (which might be attributed to its poor solubility).

In pursuit to access the industrially important acetylated styrenes [Kunitsky *et al.* (2005)], *O*-acetylation at hydroxy functional group of ferulic acid (13a) was carried out and subjected for bioconversion by *P. agglomerans* strain KJLPB4. To our utmost surprise, instead of decarboxylated product (13b) we predominantly got 4-vinylguaiacol (1b) along with minor deacetylated product 1a (Table 3, entry 13).

Similar results were shown by strain KJPB2 with acetylated *p*-coumaric acid (14a) where 4b was obtained in 80% yield. HPLC analysis of conversion at different time intervals revealed that in such cases both deacetylation and decarboxylation reactions were preceding in parallel and thus after 48 h majority of acetylated substrate was converted into deacetylated-decarboxylated product.
Inspired by the success with acetylated substrates 13a and 14a, we reasoned that acetylation of 12a (to enhance its solubility) might provide us with enhanced yield of corresponding hydroxylated stilbene. Therefore 15a was prepared and subjected for biotransformation where inquisitively we got only deacetylated product (12a) in 95% yield instead of expected 15b.

**Table 3.** Effect of substitution on ferulic acid (1a) decarboxylation by *P. agglomerans* strain KJLPB4

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate (a)</th>
<th>Product (b)</th>
<th>Time (h)</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Br-OCH COOH</td>
<td>Br-OCH AcO</td>
<td>96</td>
<td>nd</td>
</tr>
<tr>
<td>9</td>
<td>Br-OCH COOCH3</td>
<td>Br-OCH AcO</td>
<td>96</td>
<td>nd</td>
</tr>
<tr>
<td>10</td>
<td>Br-OCH COOH</td>
<td>Br-OCH AcO</td>
<td>96</td>
<td>nd</td>
</tr>
<tr>
<td>11</td>
<td>Br-OCH COOH</td>
<td>Br-OCH AcO</td>
<td>96</td>
<td>nd</td>
</tr>
<tr>
<td>12</td>
<td>HO OCH COOH</td>
<td>HO OCH AcO</td>
<td>72</td>
<td>traces</td>
</tr>
<tr>
<td>13</td>
<td>AcO-OCH COOH</td>
<td>AcO-OCH AcO</td>
<td>48</td>
<td>nd (13b)+95 (1b) +2 (1a)</td>
</tr>
<tr>
<td>14</td>
<td>AcO-OCH COOH</td>
<td>AcO-OCH AcO</td>
<td>48</td>
<td>nd (14 b)+80 (4 b) +2 (4a)</td>
</tr>
<tr>
<td>15</td>
<td>AcO-OCH COOH</td>
<td>AcO-OCH AcO</td>
<td>48</td>
<td>nd (15b)+95 (12a)</td>
</tr>
</tbody>
</table>

*strain KJPB2; nd= not detected

In order to further augment the applicability of the above protocol, different acetylated derivatives of substituted cinnamic acids were subjected to biotransformation with both strains and indeed got deacetylated products in good amounts in all cases with KJLPB4 (Table 4, entries 16-23) while low to moderate yields were obtained with KJPB2. Thus, in addition to decarboxylation, the isolated strain KJLPB4 have shown promise as an effective deacetylating agent [Parmar *et al.* (1998a, b)].
Table 4. Yield of deacetylated products with *P. agglomerans* strain KJLPB4

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate (a)</th>
<th>Product (b)</th>
<th>Time [h]</th>
<th>Yield [%]</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td></td>
<td></td>
<td>72</td>
<td>85+2 (3b)</td>
<td></td>
</tr>
<tr>
<td>17</td>
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*a* On the basis of HPLC analysis

2.5 Conclusion:

This study proves the usefulness of two newly isolated strains of *P. agglomerans* (KJLPB4 and KJPB2) for the production of hydroxystyrenes, many of which have high commercial potential. The simple and environment friendly method allows the preparative scale synthesis of 4-vinylguaiacol with KJLPB4 in good yield and excellent selectivity while strain KJPB2 is effectively exploited for production of vinyl guaiacol or vanillic acid depending upon the incubation time. In addition, strain KJLPB4 was effectively utilized for the chemoselective deacetylation over decarboxylation for acetylated α-phenylecinnamic acids.

2.6 Experimental:

2.6.1 Materials and methods:

The cinnamic acids (1-7, 13, 14) were of reagent grade (purchased from Merck, Sigma Aldrich and Alfa Aesar). The remaining cinnamic acid derivatives were synthesized in the
lab using appropriate methods [Trost (1991); Kumar et al. (2007)]. The structures and purity of the synthesized substrates was confirmed by NMR, GC-MS and HPLC. Solvents used were of analytical grade. $^1$H (300 MHz) and $^{13}$C (75.4 MHz) NMR spectra were recorded on a Bruker Avance-300 spectrometer wherever mentioned. GC-MS analysis was undertaken using a Shimadzu-2010 spectrometer.

2.6.2 Synthesis of substrates for decarboxylation (Table 3, 8a-15a) and deacetylation (Table 4, 16a-23a) studies:

2.6.2.1 Synthesis of trans 3-(5-Bromo-4-hydroxy-3-methoxyphenyl)-2-propenoic acid (8a):

5-Bromo-4-hydroxy-3-methoxybenzaldehyde (1.5 g, 7.5 mmol) and malonic acid (1.8 g, 17.5 mmol) were dissolved in pyridine (8 mL) using aniline (0.5 mL) as catalyst. The reaction mixture was stirred at room temperature for 24 h. Progress of reaction was monitored by thin layer chromatography (hexane/ethyl acetate 6:4). The reaction was then diluted with HCl 5 M and extracted with 3 x 5 mL of dichloromethane. The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered and concentrated until a residue was obtained. Compound 8a was purified by column chromatography (from ethyl acetate/hexane 6:4) and its spectral data was matched well with reported values [Gaspar et al. (2009)].

![Chemical structure of 8a](image)

White solid (Yield 42%) m.p. 233-234°C, $^1$H-NMR (DMSO-$_d_6$, 300 MHz): $\delta$ 7.52 (1H, d, $J = 15.5$ Hz), 7.32 (1H, d, $J = 1.9$ Hz), 7.14 (1H, d, $J = 1.9$ Hz), 6.35 (1H, d, $J = 15.4$ Hz), 3.91 (3H, s); $^{13}$C-NMR (DMSO-$_d_6$, 75.4 MHz): $\delta$ 168.2, 148.5, 146.2, 142.9, 126.3, 124.8, 117.2, 110.2, 109.2 and 56.3.

2.6.2.2 Synthesis of trans-methyl 3-(4-hydroxy-3-methoxyphenyl)propenoate (9a):

To the solution of ferulic acid (5 g) in anhydrous methanol (100 mL), passed HCl gas and left at room temperature for overnight. Further the mixture was heated at 50°C for 3 h. The reaction mixture (after removal of MeOH) was poured into chloroform (150 mL) and washed with water (100 mL x 2). The chloroform layer was washed again with a saturated aqueous solution of NaHCO$_3$ (100 mL). The obtained chloroform layer was dried over anhydrous Na$_2$SO$_4$ and evaporated. The residue was purified by silica gel column chromatography and eluted with hexane-ethyl acetate (3:1, v/v) to give methyl ferulate whose NMR spectra matched well with the reported values[Masuda et al. (2006)].
2.6.2.3 Synthesis of trans-3-(4-O-allyl-3-methoxyphenyl)propenoic acid (10a) and trans-3-(5-allyl-4-hydroxy-3-methoxyphenyl)-2-propenoic acid (11a):

(a) Synthesis of 4-O-allyl-3-methoxybenzaldehyde

A mixture of vanillin (131 mmol), anhydrous K₂CO₃ (267 mmol) was taken in anhydrous acetone (170 mL). Then added allyl bromide (25 mL) drop wise with constant stirring. The reaction mixture was refluxed for 5 h and the progress of the reaction was monitored by TLC. The crude product was purified by silica gel column chromatography using hexane:ethyl acetate (9:1) to give 4-O-allyl-3-methoxybenzaldehyde which was characterized by ¹H and ¹³C NMR and HRMS data.

(b) Synthesis of 5-Allyl-4-hydroxy-3-methoxybenzaldehyde:

The product from step (a) was taken in round bottom flask and subjected to microwave at 150 W for 15 min at 195-205°C temperature to undergo Claisen rearrangement. The progress of reaction was monitor with the help of TLC. Finally purification was done with column chromatography (hexane: ethyl acetate 9:1, v/v) and product was characterized by ¹H and ¹³C NMR and HRMS data.
= 6.63 Hz); $^{13}$C-NMR (CDCl$_3$, 75.4 MHz); $\delta$ 191.2, 149.6, 146.9, 135.1, 128.8, 127.8, 126.1, 116.7, 107.2, 56.1 and 33.1. HRMS-ESI: m/z [M+H]$^+$ for C$_{11}$H$_{12}$O$_3$, calculated 193.0786; observed 193.0713.

(c) **Synthesis of trans-3-(4-O-allyl-3-methoxyphenyl)propenoic acid (10a) and trans-3-(5-Allyl-4-hydroxy-3-methoxyphenyl)-2-propenoic acid (11a)**

A mixture of allylated aldehyde (2.5 mmol) obtained as described above, malonic acid (6 mmol), pyridine (15 mmol) in piperidine (0.25 mL) was heated on a water bath (90-95°C) for 3 h. The mixture was allowed to cool at room temperature and then poured into excess dilute HCl (50 mL, 2N). Precipitated solid was filtered, washed with cold water and dried to get the respective cinnamic acid which was further purified by column chromatography (hexane: ethyl acetate 6:4 v/v).

**trans-3-(4-O-allyl-3-methoxyphenyl)-2-propenoic acid (Table 3; 10a)**

![trans-3-(4-O-allyl-3-methoxyphenyl)-2-propenoic acid](image)

White solid (Yield 56%) $^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ 7.71 (1H, d, $J = 15.8$ Hz), 7.62-7.50 (2H, m), 6.82 (1H, s), 6.41 (1H, d, $J = 15.8$ Hz), 6.72-6.04 (1H, m), 6.06-6.38 (2H, m), 4.64 (2H, d, $J = 5.35$ Hz), 3.87 (3H, s); $^{13}$C-NMR (CDCl$_3$, 75.4 MHz): $\delta$ 170.8, 153.1, 149.8, 149.6, 135.7, 133.6, 127.9, 119.7, 117.3, 112.5, 109.7, 72.1 and 57.3. HRMS-ESI: m/z [M+H]$^+$ for C$_{13}$H$_{14}$O$_4$, calculated 235.2558; observed 235.2551.

**trans-3-(5-Allyl-4-hydroxy-3-methoxyphenyl)-2-propenoic acid (Table 3; 11a)**

![trans-3-(5-Allyl-4-hydroxy-3-methoxyphenyl)-2-propenoic acid](image)

White solid (Yield 42%) m.p. 184-187°C, $^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ 7.63 (1H, d, $J = 15.55$ Hz), 7.22 (1H, s), 7.04 (1H, s), 6.38 (1H, d, $J = 15.37$ Hz), 6.05-5.97 (1H, m), 5.11-4.99 (2H, m), 4.62 (1H, s), 3.91 (3H, s), 3.40 (2H, d, $J = 5.37$ Hz); $^{13}$C-NMR (CDCl$_3$, 75.4 MHz): $\delta$ 168.7, 148.7, 147.8, 146.5, 137.8, 127.6, 127.1, 124.9, 116.2, 109.5, 56.8 and 34.8. HRMS-ESI: m/z [M+H]$^+$ for C$_{13}$H$_{14}$O$_4$, calculated 235.2558; observed 235.2562.

**Synthesis of α-Phenyl-4-hydroxy-3-methoxycinnamic acid (Table 3; Compound 12a)** is described under section 2.6.2.5. Compound 13 and 14 were from commercial sources.

2.6.2.4 **Synthesis of acetoxylated α-phenyl-cinnamic acids via Perkin reaction (15a, 17a-21a):**
A mixture of phenylacetic acid (0.06 mol), triethylamine (35 mL) and acetic anhydride (70 mL) was taken in 250 mL round bottom flask and kept on stirring for 20 min. To this, benzaldehyde derivative (0.066 mol) was added and reaction mixture was refluxed for 9 h. The progress of the reaction was monitored by TLC and after completion the reaction mixture was cooled and water was added to it. The aqueous mixture was further acidified with dil HCl, ppt filtered, aqueous part extracted with ethyl acetate (3 x 20 mL), dried on sodium sulfate and concentrated under vacuum to obtain crude product which was further purified on Si gel column with a 1:1 mixture of ethyl acetate and hexane to obtain acetoxylated α-phenylcinnamic acids (15a, 17a-21a) whose spectral data was found matching with the reported values [Gaukroger et al. (2001); Solladié et al. (2003); Borrel et al. (2005)].

**α-Phenyl-4-acetoxy-3-methoxycinnamic acid (Table 3; 15a)**

\[
\begin{align*}
\text{AcO} & \quad \text{OCH}_3 \\
\text{COOH} & \quad \text{OH}
\end{align*}
\]

$^1$H NMR (DMSO-$d_6$, 300 MHz): δ 7.60 (1H, s), 7.30-7.25 (3H, m), 7.07 (2H, d, $J = 8.3$ Hz), 6.82 (1H, d, $J = 8.6$ Hz), 6.66 (1H, d, $J = 8.5$ Hz), 6.45 (1H, s), 3.19 (3H, s), 2.06 (3H, s); $^{13}$C NMR (DMSO-$d_6$, 75.4 MHz): δ 169.5, 169.0, 150.3, 140.1, 138.7, 136.6, 133.6, 133.5, 129.5, 129.2, 128.2, 124.1, 123.2, 113.8, 55.3 and 20.6.

**α-Phenyl-4-acetoxycinnamic acid (Table 4; 17a)**

\[
\begin{align*}
\text{AcO} & \quad \text{COOH} \\
\text{OCH}_3 & \quad \text{OH}
\end{align*}
\]

$^1$H NMR (DMSO-$d_6$, 300 MHz): δ 7.84 (1H, s), 7.31-7.28 (3H, m), 7.19-7.16 (2H, m), 7.03 (2H, d, $J = 8.3$ Hz), 6.84 (2H, d, $J = 8.4$ Hz), 2.18 (3H, s); $^{13}$C NMR (DMSO-$d_6$, 75.4 MHz): δ 173.1, 169.1, 151.4, 141.5, 135.1, 132.8, 131.7, 129.6, 128.6, 128.1, 121.6, 121.4 and 21.1.

**α-4'-Methoxyphenyl-4-acetoxy-3-methoxycinnamic acid (Table 4; Compound 18a)**

\[
\begin{align*}
\text{AcO} & \quad \text{OCH}_3 \\
\text{COOH} & \quad \text{OH}
\end{align*}
\]

$^1$H NMR (DMSO-$d_6$, 300 MHz): δ 7.51 (1H, s), 6.92 (2H, d, $J = 8.5$ Hz), 6.68-6.61 (3H, m), 6.54 (1H, d, $J = 8.5$ Hz), 6.33 (1H, s), 3.54 (3H, s), 3.16 (3H, s), 2.01 (3H, s); $^{13}$C NMR (DMSO-$d_6$, 75.4 MHz): δ 169.4, 168.4, 159.0, 150.1, 139.8, 138.7, 133.7, 132.5, 130.8, 128.2, 123.7, 122.3, 114.1, 113.5, 55.1 and 20.5.
α-4’-Methoxyphenyl-4-acetoxycinnamic acid (Table 4; Compound 19a)

![Chemical Structure](image)

$^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ 7.68 (1H, s), 7.11-7.05 (4H, m), 6.97-6.91 (4H, m), 3.75 (3H, s), 2.20 (3H, s); $^{13}$C NMR (CDCl$_3$, 75.4 MHz): $\delta$ 168.6, 168.3, 158.4, 150.3, 137.3, 132.6, 132.0, 130.9, 130.4, 127.7, 121.4, 113.7, 54.7 and 20.5. HRMS-ESI: m/z [M+H]$^+$ for C$_{18}$H$_{16}$O$_5$, calculated 313.3245; observed 313.3242.

α-4’-Acetoxyphenyl-4-acetoxy-3-methoxycinnamic acid (Table 4; Compound 21a)

![Chemical Structure](image)

$^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 7.72 (1H, s), 7.23 (2H, d, $J = 8.5$ Hz), 7.11 (2H, d, $J = 8.5$ Hz), 6.90 (1H, d, $J = 8.0$ Hz), 6.83 (1H, d, $J = 8.0$ Hz), 6.56 (1H, s), 3.34 (3H, s), 2.20 (3H, s), 2.11 (3H, s); $^{13}$C NMR (DMSO-$d_6$, 75.4 MHz): $\delta$ 168.8, 167.8, 150.4, 140.4, 138.5, 134.5, 133.5, 133.4, 130.9, 124.0, 122.8, 122.1, 113.3, 54.8, 20.0 and 19.5.

2.6.2.5 Hydrolysis of α-phenyl-acetoxycinnamic acids:
The acetoxylated α-phenyl-cinnamic acid 15a (0.007 mol) was dissolved in 10 mL solution of 10% NaOH and irradiated to microwave for 2 min. The reaction mixture was kept at room temperature for 1 h and then worked up. The ice-cooled reaction mixture was acidified with dil HCl, the obtained ppt filtered washed with water and dried to obtain hydroxylated α-phenyl-cinnamic acid derivatives (12a).

α-Phenyl-4-hydroxy-3-methoxycinnamic acid (Table 3; Compound 12a)

![Chemical Structure](image)

$^1$H NMR (CD$_3$COCD$_3$, 300 MHz): $\delta$ 7.71(1H, s), 7.39-7.18 (5H, m), 6.73-6.28 (2H, m), 6.43(1H, s), 3.31 (3H, s); $^{13}$C NMR $\delta$ (CD$_3$COCD$_3$, 75.4 MHz): 168.1, 148.0, 146.8, 140.2, 137.3, 129.9, 129.8, 128.6, 127.4, 126.4, 125.8, 114.7, 112.8 and 54.6.
2.6.2.6 General procedure for acetylation of compounds (Table 4; 16a, 22a and 23a):
A solution of substrate (5.5 mmol) and a catalytic amount of 4-dimethylaminopyridine (DMAP) in dry pyridine (5 mL) were cooled at 0°C. Acetic anhydride (12 mmol) was added drop wise over 10 min. After stirring overnight at room temperature, the solution was poured into cold water and the aqueous layer was extracted twice with ethyl acetate. The organic layer was successively washed with 1 M HCl and water, dried over anhydrous Na₂SO₄ and concentrated under in vacuo. The resulting solid was purified by recrystallization with ethyl acetate-hexane (8:2, v/v).

3,4-Diacetoxycinnamic acid (Table 4; Compound 16a)

<table>
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<th>Chemical Structure</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>White solid (Yield 86%)</td>
<td>( \delta ) 7.73 ( (1H, d, J = 15.8 \text{ Hz}) ), 7.44 ( (1H, dd, J = 8.5 \text{ and } 1.8 \text{ Hz}) ), 7.41 ( (1H, d, J = 1.8 \text{ Hz}) ), 7.26 ( (1H, d, J = 8.5 \text{ Hz}) ), 6.41 ( (1H, d, J = 15.8 \text{ Hz}) ), 2.32 ( (3H, s) ), 2.31 ( (3H, s) ); ( \delta ) 171.7, 168.5, 168.3, 145.5, 144.3, 142.9, 133.3, 127.1, 124.4, 123.4, 118.8, 21.1 and 21.0.</td>
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</tbody>
</table>

2.6.3 General procedure for biocatalytic decarboxylation:
Strains KJLPB4 and KJPB2 were isolated from the Western Himalayan soils and grown in nutrient broth (NB) medium at 28°C and 220 rpm for 24 h [Kasana et al. (2007)]. For the decarboxylation studies, Ferulic acid (1a) dissolved in minimum quantity of N,N-dimethylformamide (DMF) was added to 5 mL culture and incubated up to 96 h. Samples were periodically withdrawn, extracted with ethyl acetate and concentrated in vacuo at 40°C to remove solvent. 1 mL of methanol was added to the concentrated extract, filtered through 0.22 μM filter and subjected to HPLC analysis. The decarboxylated products were confirmed by matching their retention time and UV spectra with reference standards [Sharma et al. (2008)]. Similar procedure was followed for other compounds (2a-23a).

2.6.3.1 Spectral data of decarboxylated products:

4-Ethenyl-2-methoxyphenol or 4-vinyl guaiacol (Table 1; Compound 1b)

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Properties</th>
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<tbody>
<tr>
<td>Colorless viscous liquid, ( \delta ) 6.86-6.80 ( (3H, m) ), 6.62-6.52 ( (1H, m) ), 5.66 ( (1H, s) ), 5.70 ( (1H, d, J = 17.54 \text{ Hz}) ), 5.09 ( (1H, d, J = 10.92 \text{ Hz}) ),</td>
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</table>
3.83 (3H, s); $^{13}$C NMR (CDCl$_3$, 75.4 MHz) $\delta$ 146.4, 145.5, 136.6, 103.2, 120.1, 114.4, 111.5, 108.1 and 55.8.

**4-Hydroxy-3-methoxybenzoic acid (Table 1; Compound 1c)**

![Chemical structure of 4-Hydroxy-3-methoxybenzoic acid](image)

White solid, m.p. 209-212°C. $^1$H NMR (MeOD, 300 MHz): $\delta$ 7.58-7.56 (2H, m), 6.86-6.83 (1H, m), 3.89 (3H, s); $^{13}$C NMR (MeOD, 75.4 MHz): $\delta$ 170.2, 152.8, 148.8, 125.4, 123.2, 116.0, 113.9 and 56.6.

**4-Ethenylphenol or 4-vinylphenol (Table 1; Compound 4b)**

![Chemical structure of 4-Ethenylphenol or 4-vinylphenol](image)

Colorless liquid, $^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ 7.24 (2H, d, $J$ = 8.08 Hz), 6.74 (2H, d, $J$ = 8.89 Hz), 6.64-6.55 (1H, m), 5.54 (1H, d, $J$ = 17.38 Hz), 5.53 (1H, s), 5.07 (1H, d, $J$ = 10.52 Hz); $^{13}$C NMR (CDCl$_3$, 75.4 MHz): $\delta$ 155.5, 136.3, 130.6, 127.7, 115.4 and 111.7.

**α-Phenyl-4-hydroxy-3-methoxycinnamic acid (Table 3; Compound 12a)**

Spectral data same as described in 2.4.2.5

**α-Phenyl-4-hydroxycinnamic acid (Table 4; Compound 17b)**

White solid, m.p. 223-226°C. $^1$H NMR (MeOD, 300 MHz): $\delta$ 7.71 (1H, s), 7.31 (3H, m), 7.12 (2H, d), 6.83 (2H, d), 6.49(2H, d); $^{13}$C NMR (MeOD, 75.4 MHz): $\delta$ 170.2, 158.5, 140.4, 136.9, 132.3, 129.6, 129.5, 128.3, 127.2, 125.9 and 115.6.

### 2.6.4 Identification of Bacterial strain:

Strains KJLPB4 and KJPB2 showing ferulic acid decarboxylation activity were identified based on their 16S rDNA sequencing [Sambrook et al. (1989)]. The primers used for amplification were 5’-AGAGTTTGATCATGGCTCAGA-3’ and 5’-GGTTACCTTGTTACGACTT-3’. The amplified 16S rDNA gene was purified from agarose gel using Genelute Gel Extraction Kit (Sigma, USA), ligated into the cloning vector (pGEM-T easy vector) with the cloning kit (Promega, USA). The nucleotide sequencing of the gene was done by using Big Dye$^R$ Terminator Cycle Sequencing Kit (Applied Biosystems) and 3130xl Genetic Analyzer (Applied Biosystems). The BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD) was used for homology searches with the standard program default. These strains have been deposited in the
MTCC, IMTECH culture collection, Chandigarh, India under MTCC 10409 and MTCC 10062, respectively.

2.6.4.1 16S rDNA sequence of Pantoea agglomerans strains:
(a) KJLPB4:

AGTCGGACGATGTCGGCTGGTGGGGAACGGCTGGCATGGAGTAATGTCGGGGAATCAGACGAGGAGCTTGCTCCTTGGGGGACGAGTGGCGGACGGGTGAGTAATGTCTGGGGATTCTGCCCGATAGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTCACTATCGGATGAACCCAGATGGGATTAGCTAGTAGGCGGGGTAATGGCCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGAATGACCAGCCACGCTGGAACTGAGACACGGTCCATACTCCTACGGGAGGCAGCGGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAACCCTGCCGCGTGTATGAAGAACGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATGGGGTTAATAACCTTATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAATTCTGGCAGAGATGCCTTAGTGCCTTCGGGAACCGTGAGACAGGTGATGCATGGCTGTCGTCAGCTTGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGAGTAATGTCGGGAACTCAAAGGAGACTGCCGGTGAATAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATAACAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCACAAAGTGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAGGGTGTTGCAAAAGAAGTAGTGGTACATAACCTTCCGGAGTAAACGACCGCAGAAATAGCG

(b) KJPB2:

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAGAAGTCGGACGGTAGCACAGAGGAAGTTTGCTCCTTGGGGGACGAGTGGCGGACGGGTGAGTAATGTCTGGGGGTCTGCCCGATAGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTCACTATCGGATGAACCCAGATGGGATTAGCTAGTAGGCGGGGTAATGGCCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGAATGACCAGCCACGCTGGAACTGAGACACGGTCCATACTCCTACGGGAGGCAGCGGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATGGGGTTAATAACCTTATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAATTCTGGCAGAGATGCCTTAGTGCCTTCGGGAACCGTGAGACAGGTGATGCATGGCTGTCGTCAGCTTGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGAGTAATGTCGGGAACTCAAAGGAGACTGCCGGTGAATAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATAACAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCACAAAGTGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAGGGTGTTGCAAAAGAAGTAGTGGTACATAACCTTCCGGAGTAAACGACCGCAGAAATAGCG

2.6.5 Determination of protein content:
Cultures having OD$_{550}$ of 0.8 were harvested, centrifuged at 10000g for 15 min and supernatant was separated from the pellet. The pellet constituting resting cells was re-suspended in 50 mM tris HCl buffer (pH 7) and stored at 4°C till further experiments.
free enzyme extract was prepared from resting cells by cell disruption in lysis buffer followed by ultrasonication using a “Sonics Vibra Cell” sonicator equipped with a microtip. A pulse of 9.9 second bursts with 5 second cooling period between each burst was given for 8 minutes followed by centrifugation at 12000g for 15 min and filtration. The filtrate was kept at 0°C till further use. The protein content of culture supernate and cell free enzyme extract was determined according to Bradford method [Bradford (1976)] using BSA as standard.

2.6.6 Influence of co-solvent:
The stability towards different organic solvents was studied according to the procedure in which the amount of culture added is reduced and substituted by amount of organic solvents to make up final reaction volume 5 mL. The model substrate for this study was 1a.

2.6.7 Biotransformation studies with ferulic acid isolated from maize bran:
For the release of ferulic acid from maize bran alkaline hydrolysis was performed according to a reported method [Bunzel et al. (2005)]. In short, 100 g of maize bran was hydrolyzed using 2 M NaOH under nitrogen for 24 h and protected from light. After acidification (pH <2), liberated phenolic acids were extracted into ethyl acetate. The organic layer was dried over sodium sulfate and concentrated in vacuo resulting in ferulic acid enriched solution which was confirmed by HPLC analysis using reference standard.

2.6.8 Analysis of biotransformed products:
Analysis was performed using a Shimadzu HPLC (Model LC-20AT pump, DGU-20A5 degasser) equipped with auto sampler (SIL-20AC), photo diode array detector (CBM-20A; Shimadzu, Kyoto, Japan) and interfaced with IBM Pentium 4 personal computer. The separation was performed on a Purospher star RP218e column (150 x 4.6 mm id, 5 µM). Temperature of the column was set at 30°C. The mobile phase consisted of 0.05% TFA (Trifluoroacetic acid) in H₂O and methanol/acetonitrile (in 70: 30, v/v) with gradient elution (0-5 min, 40-70% B; 5-10 min, 70-100% B; 10-12 min, 100-40% B; 12-20 min, 40% B with a flow rate of 1 mL/min. Analysis wavelength was set at 254 and 280 nm. The quantification was performed using external standard method.
2.7 References:


dimethoxyphenol (canolol) isolated from canola oil. *Journal of Agriculture and Food Chemistry* **52**: 4380-87.


NMR spectra of some compounds

$^1$H NMR (in CDCl$_3$) spectrum of 4-Ethenyl-2-methoxyphenol or 4-vinyl guaiacol (Table 1; Compound 1b)

$^{13}$C NMR (in CDCl$_3$) spectrum of 4-Ethenyl-2-methoxyphenol or 4-vinyl guaiacol (Table 1; Compound 1b)
$^1$H NMR (in MeOD) spectrum of 4-Hydroxy-3-methoxybenzoic acid or vanillic acid (Table 1; Compound 1c)

$^{13}$C NMR (in MeOD) spectrum of 4-Hydroxy-3-methoxybenzoic acid or vanillic acid (Table 1; Compound 1c)
Biocatalytic decarboxylation and deacetylation...

Chapter 2

$\text{HO} \quad \text{OCH}_3$

$\text{COOH}$

$\text{H} \quad \text{NMR (in CD}_3\text{COCD}_3\text{) spectrum of } \alpha\text{-Phenyl-4-hydroxy-3-methoxycinnamic acid (Table 3; Compound 12a)}$

$\text{C NMR (in CD}_3\text{COCD}_3\text{) spectrum of } \alpha\text{-Phenyl-4-hydroxy-3-methoxycinnamic acid (Table 3; Compound 12a)}$

$\text{CD}_3\text{COCD}_3$

$13\text{C NMR (in CD}_3\text{COCD}_3\text{) spectrum of } \alpha\text{-Phenyl-4-hydroxy-3-methoxycinnamic acid (Table 3; Compound 12a)}$