Materials & Methods
Material and Methods

Bacterial Strains

Microorganisms used in this study for vanillin and related compounds production were isolated in our laboratory from decomposed wood-bark samples, obtained from northern Himalayan range. The most potent one (Isolate No. 07, designated as BK07), which showed maximum bioconversion of ferulic acid within minimum period of time was selected for this study and was identified as *Bacillus coagulans* at MTCC (Microbial Type culture collection), Institute of Microbial Technology, Chandigarh, India. The strains of *Bacillus subtilis* BD170 (Rec') and BD 221 (Rec') used for rec-assay of vanillin were obtained from Bacillus Genetic Stock Centre, Columbus, USA.

Standard Chemicals

Ferulic acid (4-Hydroxy-3-methoxycinnamic acid), Vanillin (4-Hydroxy-3-methoxy benzaldehyde), vanillic acid (4-Hydroxy-3 methoxy benzoic acid), Protocatechuic acid (3,4-Dihydroxybenzoic acid) were purchased from Sigma Chemical Company, USA. 4-Vinylguaiacol (4-Hydroxy-3-methoxystyrene) was purified from the ferulate-grown culture extracts of *Bacillus coagulans* BK07.

Solvents

Chloroform, methanol, diethyl ether and ethyl acetate *etc.* used were of GR and HPLC grade and were purchased from E. Merck, Germany.

TLC plates

Silica gel GF254 plates of 0.25 mm. thickness were obtained from E. Merck, Germany.
**Bacteriological Media** and their constituents were purchased locally from Himedia, India.

**Other Chemicals and Biochemicals**

2,4-Dinitrophenylhydrazine was purchased from BDH Ltd, London, Q-Sepharose and Sephadex G-200, were purchased from Pharmacia Fine chemicals, Uppsala, Sweden. Diazotized sulfanilic acid, Coomassie brilliant blue R250, Tris, SDS, Glucose-6-phosphate, NADP, ATP, NTG, etc. were purchased from Sigma Chemical Company USA. All other Chemicals/ Biochemical, if otherwise specified, used were of AR/GR grade and were purchased from local commercial sources.

**Buffers**

**PBS**

- 50 mM sodium phosphate buffer pH 7.0
- 150 mM sodium chloride

**PB**

- 50 mM sodium phosphate buffer pH 7.0
- 10 mM sodium phosphate buffer pH 7.2.
- 200 mM sodium phosphate buffer pH 7.2

**Cofactor Buffer Solution (Hirano et al., 1982)**

- MgCl₂ : 8 μM
- KCl : 33 μM
- Sodium phosphate buffer : 10 mM (pH 7.2)
Reagents

**M9-Minimal Salt Solution** (Sambrook *et al.*, 1989)

- Na$_2$HPO$_4$·7H$_2$O : 64 gm
- KH$_2$PO$_4$ : 15 gm
- NaCl : 2.5 gm
- NH$_4$Cl : 5 gm
- 1 M MgSO$_4$ : 10 ml
- 100 mM CaCl$_2$ : 5 ml
- Distilled Water : 985 ml

Total : 1000 ml

pH : 7.2

M9-Minimal Salt Solution was diluted 5 times with distilled water before use.

**Minimal Salt Solution (MM)** (Hirano *et al.*, 1982)

- (NH$_4$)$_2$SO$_4$ : 1 gm
- KH$_2$PO$_4$ : 10 gm.
- MgSO$_4$·7H$_2$O : 0.5 gm
- Distilled water : 1000 ml

Total : 1000 ml

MM solution was neutralized with 1M KOH solution.
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Cofactor Solution (Hirano et al., 1982)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose - 6 - phosphate</td>
<td>20 mg</td>
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<tr>
<td>NADP</td>
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<tr>
<td>Cofactor Buffer solution</td>
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<td>Total</td>
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All the above solutions were sterilized at 15 lb pressure for 15 minutes, except the cofactor solution, which was, filter sterilized by using 0.2 μ Millipore filter.

Media

Nutrient Broth

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<th>Component</th>
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<td>Peptone</td>
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<td>Sodium chloride</td>
<td>5.0 g</td>
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<tr>
<td>Beef extract</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5 g</td>
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<tr>
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<td>Total</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
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Nutrient Agar

<table>
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<td>Nutrient broth</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Total</td>
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</tr>
<tr>
<td>pH</td>
<td>7.2 - 7.4</td>
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**Material and Methods**

**Enrichment liquid medium (for Screening)**

<table>
<thead>
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<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>M9 - minimal salt solution</td>
<td>200 ml</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>800 ml</td>
</tr>
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</table>

**Total**: 1000 ml  
**pH**: 7.2

**Enrichment agar medium**

<table>
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<th>Component</th>
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<tr>
<td>Agar</td>
<td>18 g</td>
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**Total**: 1000 ml  
**pH**: 7.2

**Seed medium (M9-yeast extract)**

<table>
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<th>Component</th>
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<tbody>
<tr>
<td>M9 - minimal salt solution</td>
<td>200 ml</td>
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<tr>
<td>Yeast extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>800 ml</td>
</tr>
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**Total**: 1000 ml  
**pH**: 7.2

**Production Medium (M9-yeast extract-ferulic acid)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9- minimal salt solution</td>
<td>200 ml</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>1 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>800 ml</td>
</tr>
</tbody>
</table>

**Total**: 1000 ml  
**pH**: 7.2
Modified Schaeffer's medium (Leighton and Doi, 1971)

Difco nutrient broth : 16 gm
KCl : 2 gm
MgSO4.7H2O : 0.5 gm
MnCl2 : 10.8 mg
FeSO4.7H2O : 278 µg
Ca(NO3)2.4H2O : 236 mg
Glucose : 1 gm
Bacto Agar : 15 gm
Distilled Water : 1000 ml

Total : 1000 ml
pH : 7.0

Liquid Broth medium (Hirano et al., 1982)

Beef wet extract : 10 gm
Poly peptone powder : 10 gm
Sodium Chloride : 5 gm
Distilled water : 1000 ml

Total : 1000 ml
pH : 7.0

All the above media/medium constituents were sterilized at 15 lb pressure for 15 minutes except ferulic acid solution which was filter sterilized using 0.2 µ Millipore filter.
METHODS

SCREENING AND IDENTIFICATION OF THE ISOLATE

Screening

Soil and decomposed wood-bark samples from various environmental niches e.g., pine and spruce growing areas in northern Himalayan range like Leh, Ladakh, Chamba, Kulu-Manali, Dalhousie and Dharamshala and from various locations in plains such as Calcutta, Assam, Panjab, Haryana and areas near Chandigarh, India were collected. Papermill waste, which are lignin rich were also collected from Star Paper Mill, Shaharanpur; Indian Paper and Pulp Research Institute, Yamunanagar and Gurunanak Paper Mill, Mohali etc.

The samples were enriched for isolation of ferulic acid degrading organisms by mixing the samples (1.0 gm%) with enrichment liquid medium and incubating at 37°C for 48-72 h in a rotary shaker at 180 rpm. The enriched cultures were plated on enrichment agar medium, after proper dilution with sterile distilled water and the plates were incubated at 37°C for 48-72 h. The microorganisms growing on these plates were then purified to single colonies by repeated streaking on enrichment agar medium.

The isolates obtained were individually screened for their ability to degrade ferulic acid. Each individual isolate was inoculated in 50 ml of enrichment liquid medium in a 250 ml flask and incubated in an orbital shaker at 180 rpm at 37°C. The samples were withdrawn at regular intervals of 4 hrs, centrifuged (10,000 x g for 10 min) and supernatants were diluted appropriately with sterile distilled water and scanned at λ200 -λ400nm with Beckman DU640B Spectrophotometer, to observe any change in UV absorption pattern of ferulic
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acid. 0.1ml of the culture medium, which showed changes in UV absorption pattern, was plated on enrichment agar medium to check any contamination. Whenever any contamination was observed, each individual pure colony was again checked for its ability to bring changes in UV-absorption pattern of ferulic acid. This process was repeated a number of times to select the most potent organism showing fastest degradation of ferulic acid. Microbial cultures were purified by restreaking individual colonies on the enrichment agar medium, maintained on nutrient agar plates/slants and stored in 10% glycerol at -70°C for long term storage.

Assay of degradation products for screening

Culture broth (50ml) of different organisms, capable a degrading ferulic acid was centrifuged at 10,000xg for 10 min and supernatants thus obtained were extracted with equal volume of chloroform. After evaporating chloroform of each extract under vacuum at 40°C in a rotavapour (Buchi, Switzerland), 20 μl of each evaporated sample was applied onto a TLC plate with the help of a calibrated capillary and air dried. TLC plate was developed in Chloroform: Methanol: 80:20 solvent system and air-dried. Formation of vanillin as metabolic intermediate was detected by spraying with 2,4-dinitrophenylhydrazine (0.1% solution made in 30% perchloric acid), which developed vanillin as a orange - red band at Rf. of 0.89, same as that of standard vanillin. Also plates were visualized under UV light (λ254nm) before spraying with 2,4-dinitrophenylhydrazine to observe vanillin and other related compounds as metabolic intermediates of ferulic acid. The organism which showed fastest degradation of ferulic acid and developed most intense orange - red colour on TLC plate was selected for further study.
Identification of the isolate BK07

The isolate BK07 was identified on the basis of morphological, physiological and biochemical characteristics as described in Bergey's Manual of Systematic Bacteriology (Sneath, 1994) at Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

OPTIMIZATION OF CULTURE CONDITIONS FOR FERULIC ACID BIOTRANSFORMATION TO VANILLIN AND RELATED COMPOUNDS BY B. COAGULANS BK07

Various medium constituents as well as conditions e.g. nitrogen and carbon sources, metal ions, inoculum size, temperature, pH, agitation and substrate concentrations etc. were optimized for obtaining maximum biotransformation of ferulic acid to vanillin and related compounds by B. coagulans BK07 in shortest period of time.

Nitrogen Sources

Both organic and inorganic nitrogen sources were used to study their effect on ferulic acid biotransformation by B. coagulans BK07. These nitrogen sources were added at a concentration of 0.1% in the production medium, in replacement of NH₄Cl which was already present in M9-minimal salt solution.

Organic nitrogen sources:

The various organic nitrogen sources e.g. yeast extract, peptone, beef extract, tryptone, urea, gelatin, casein and soybean meal etc. were tried at 0.1% concentration in the production medium to obtain maximum growth as well as biotransformation products of ferulic acid in the shortest possible time. These organic nitrogen sources were sterilized along with M9-minimal salt solution at
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15 lb pressure for 15 min and ferulic acid was filter sterilized before addition to a final concentration of 0.1% in production medium. Eight hours old inoculum was added @2% in the production medium and incubated at 37°C and 200 rpm for 10 hrs. Growth of the strain BK07 as well as ferulic acid degradation and metabolites formation were monitored as described earlier.

Inorganic nitrogen sources:

Different inorganic nitrogen sources like NH₄Cl, (NH₄)₂HPO₄, (NH₄)₂SO₄, NH₄NO₃ and KNO₃ were used to study their effect on ferulic acid biotransformation by B.coagulans BK07. After inoculation (@2%) with 8 hrs old inoculum, media were incubated at 37°C and 200 rpm for 10 hrs. Growth of strain BK07 as well as ferulic acid degradation and metabolites formation were observed as described earlier.

Effect of Different Concentrations of Nitrogen Source

To the production medium, yeast extract as the nitrogen source was added at different concentrations (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5%). Inoculum @2% was added and flasks were incubated at 37°C and 200 rpm for 10 hrs. Growth of the strain BK07 as well as ferulic acid biotransformation and metabolites formation were measured as described earlier.

Carbon Sources

Effect of different carbon sources viz. glucose, xylose, arabinose, sucrose, lactose, maltose, cellobiose, xylobiose, molasses, cellulose and soluble starch etc. on growth of strain BK07 as well as on ferulic acid biotransformation was studied. All the above carbon sources were added at the final concentration
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of 0.5% each in the production medium with or without yeast extract, after sterilizing separately at 10 lb pressure for 30 min. Ferulic acid was filter sterilized by using 0.2 µ Millipore filter and added at the final concentration of 0.1% in the production medium. The medium was then inoculated with 2% inoculum and incubated at 37°C and 200 rpm for 10 hrs. The growth was determined by taking O.D. of the culture broth at λ600 nm. Ferulic acid degradation as well as metabolites formation was monitored by HPLC analysis of the centrifuged culture supernatant as well as evaporated culture extracts after, regular intervals of time.

Metal Ions

The effect of various metal ions on the biotransformation of ferulic acid was studied by addition of their salts in the production medium at a concentration of 1 mM. These salts included AgNO₃, BaCl₂, CaCl₂, CuSO₄, FeCl₂, HgCl₂, MgSO₄, MnSO₄, ZnCl₂ and NaCl, etc. Each of these metal ions was added to the M9-yeast extract - ferulic acid medium in replacement of the existing NaCl, MgSO₄ and CaCl₂, present in the M9 - minimal salt solution. A control was put up in which no metal ions except Na₂HPO₄·7H₂O and KH₂PO₄ were added. Then 2% inoculum was added and incubated at 37°C and 200 rpm for 10 hrs. Growth of the strain BK07 as well as ferulic acid degradation and metabolites formation were monitored as described earlier.

Effect of Inoculum Size

After the production medium was optimized with M9 minimal salt solution containing 0.1% yeast extract and 0.1% ferulic acid, the effect of inoculum size on ferulic acid biotransformation was studied. 50 ml of the
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optimized production medium was dispensed in 250 ml Erlemeyer flasks and the effect of inoculum size was studied by incorporating 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% of the 8 hrs grown seed culture. The flasks so inoculated were incubated at 37°C and 200 rpm for 10 hrs. Growth of the strain as well as ferulic acid biotransformation and metabolites formation were observed as described earlier.

Effect of Temperature

The 250 ml Erlenmeyer flasks containing 50 ml optimized production medium were inoculated with 2% seed culture of 8 hrs old inoculum. The flasks thus inoculated were incubated at different temperatures (30, 37, 40, 45, 50, 60 and 70°C) and 200 rpm for 10 hrs. Growth of the strain as well as ferulic acid biotransformation and metabolites formation were monitored as described earlier.

Effect of pH

The effect of initial pH of the optimized production medium containing 0.1% ferulic acid was studied by adjusting the pH of the medium at 5.0, 5.5, 6.0, 6.5 and 7.0 by addition of 1N HCl and at 7.2, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 11 and 12 by addition of 1N NaOH. 8 hrs old inoculum was added @2% to each flask and incubated at 37°C and 200 rpm for 10 hrs. The growth of the strain as well as ferulic acid biotransformation and metabolites formation were observed as described earlier.

Effect of Agitation Conditions

The 500 ml Erlenmeyer flasks containing 100 ml optimized production medium, were inoculated @2% with 8 hrs grown seed culture. The inoculated
flasks were incubated at 37°C for 10 hrs at 100, 150, 180, 200, 250 and 300 rpm. The growth of the strain BK07 as well as ferulic acid biotransformation and metabolites formation were monitored as described earlier.

**Effect of Substrate Concentrations**

The effect of initial substrate (ferulic acid) concentrations in the optimized production medium was studied by adding different concentrations (viz. 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5%) of ferulic acid in the production medium after filter sterilization through 0.2 μ Millipore filter. Then, 8 hrs old inoculum was added @2% in the production medium containing different concentrations of substrate and incubated at 37°C and 200 rpm for 10 hrs. The growth of the strain as well as ferulic acid biotransformation and metabolites formation were observed as described earlier.

**GROWTH OF STRAIN BK07 AND SUBSTRATES DEGRADATION:**

**Inoculum preparation**

A portion of a single colony from enrichment agar medium was inoculated in M9-yeast extract medium and grown for 8 hrs. at 37°C and 200 rpm.

**Seed culture preparation**

A loopful of above inoculum was added to the seed medium and incubated for 8 hours at 37°C and 200 rpm.

**Growth of the strain BK07**

Two ml seed culture was introduced into 100 ml production medium and grown at 37°C and 200 rpm in an orbital shaker. Samples (2 ml) were
withdrawn at regular intervals of 2 hours and growth was measured as O.D at \( \lambda_{600} \) nm.

**Ferulic Acid Degradation by Strain BK07**

Seed culture @ 2% was added to the 500 ml production medium containing 0.1% ferulic acid and grown at 37°C and 200 rpm in a rotary shaker. Samples (2ml) were withdrawn at regular intervals of 2 hours. Growth was measured as OD at \( \lambda_{600} \) nm. The culture samples were then centrifuged at 12,000xg for 10 min at 4°C in a 17RS Centrifuge (Heraeus Sepatech, Germany). The degradation patterns of ferulic acid in the culture media were observed by scanning the centrifuged culture supernatant at \( \lambda_{200-400} \) nm, after 50-fold dilution with distilled water (Otuk, 1995). Whereas decrease in ferulic acid concentration as well as various metabolites formation were quantified by HPLC analysis of the centrifuged culture supernatant. The remaining culture media at different intervals of time were used for extraction of ferulic acid degradation products.

**Degradation of 4-vinylguaiacol by strain BK07**

Two ml inoculum from 8 hours grown seed culture was introduced into 100 ml production medium containing 0.1% purified 4-vinylguaiacol instead of ferulic acid and grown at 37°C and 200 rpm. Samples (2ml) were withdrawn at regular intervals of 2 hours. Growth was measured as O.D at \( \lambda_{600} \) nm. The culture samples were then centrifuged at 12,000xg for 10 minutes at 4°C. The degradation of 4-vinylguaiacol as well as metabolites formation were observed by HPLC analysis of the centrifuged culture supernatant.
Degradation of vanillin by strain BK07

Two ml inoculum pregrown in seed medium for 8 hours was introduced into 100 ml production medium containing 0.1% pure vanillin instead of ferulic acid and grown at 37°C and 200 rpm. Samples (2 ml) were withdrawn at regular intervals of 2 hours and growth was measured as O.D. at λ600 nm. The degradation of vanillin as well as metabolites formation was monitored by HPLC analysis of the centrifuged culture supernatant.

Preparation of Cell-Free Extract

Cells grown for 7 hours in one litre flasks containing 250 ml production medium were harvested by centrifugation at 10,000xg for 10 min at 4°C in a GSA, rotor (Sorvall, DuPont, Inc. wilmington, Del.). The supernatant was removed, and the pellet was washed once with 50 mM Tris. HCl (pH 7.2) buffer. The pellet was resuspended in 5 ml of washing buffer per gram of wet cells and cells were then disrupted by ultrasonication in an ice bath for a total of 4 minutes (at 20% intensity with 30 second intervals), using a sonicator (Heat system Inc., USA). The crude cell extracts were then centrifuged at 15,000 x g for 10 min at 4°C in a 17RS Contifuge (Heraeus Sepatch, Germany) and the supernatant was stored at -20°C for later use.

Ferulic acid degradation by cell-free extract and ferulate-CoA ligase activity

Ferulate-CoA ligase was assayed spectrophotometrically at 37°C; the method was based on that described by Rhodes & Wooltorton (1973). The reaction mixture (1ml) contained 50 mM Tris. HCl (pH 7.2), 2.5 mM MgCl₂, 2.5 mM ATP, 0.5 mM ferulic acid, 0.2 mM CoASH and 100 μl cell free extract.
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The assay was started by addition of the CoASH and the formation of feruloyl-CoA was measured at λ₃₄₅ nm in a Beckman DU 640B Spectrophotometer. The molar absorption coefficient value used for feruloyl-CoA was 1.9x10⁴M⁻¹cm⁻¹ as established by Zenk et al. (1980). The degradation of ferulic acid in this reaction mixture was observed by HPLC analysis of the centrifuged reaction mixture at regular intervals of 30 minutes.

Extraction of Ferulic Acid Degradation Products

Metabolic products were assayed when at least half of the aromatic growth substrate had been metabolized as observed by the UV absorption spectrum or HPLC analysis of the culture supernatant. Culture broth (500 ml), taken at different intervals of time (5, 7 and 9 hr) based on degradation pattern (60, 95 and 99% respectively as measured by UV absorption pattern or HPLC analysis), were centrifuged at 10,000 x g for 10 min at 4°C in a GSA, rotor (Sorvall, DuPont Inc. Wilmington, Del.). The supernatants were extracted with equal volume of chloroform by mixing vigorously in a 2000 ml separating funnel and kept overnight for separation of two layers. The lower (chloroform) layer was collected from separating funnel leaving behind the upper supernatant layer. The upper layer was again analysed by HPLC or scanned at λ₂₀₀-₄₀₀ nm to see remaining undegraded ferulic acid, if at all present in this layer. This upper (supernatant) layer was kept at 50°C for overnight, so that the remaining chloroform gets evaporated. The cells obtained after centrifugation were sonicated after mixing with 50mM sodium phosphate buffer (pH 7.0) to a density of 1 gm/5ml (wet weight basis). After centrifugation the cell free extracts were added back to the respective upper (supernatant) layers of
MatviiaL and MetAacb

chloroform extracts and incubated at 37°C for one day. After incubation with cell free extracts for one day the supernatants were again analysed by HPLC or scanned at λ200-400nm to observe any further degradation of the remaining ferulic acid. After observing further degradation of ferulic acid in the supernatants of different time intervals, the supernatants were again extracted with equal volume of diethyl ether. The ether-supernatant mixtures were mixed vigorously in a 2000 ml separating funnel and kept overnight for better separation of two layers. The upper (ether) layer was separated out discarding the lower supernatant layer, thus extracting the supernatant first with chloroform and subsequently with diethyl ether, which gave maximum recovery of ferulic acid degradation products.

Separation of Ferulic Acid Degradation Products

The extracted solvents were removed by evaporation at 40°C under vacuum on a rotary vacuum evaporator (Buchi, Switzerland). Evaporated culture extracts of both chloroform and diethyl ether of respective time intervals were mixed together and applied separately onto preparative silica gel GF254 plates as bands by use of a Hamilton injector. Chloroform: methanol (80:20 and 99.5 : 0.5) solvent systems were used respectively to separate bands. The aromatic compounds as bands on TLC plates were visualized under λ254 and λ365 nm UV light. The bands were marked under UV light, scraped off separately and extracted with chloroform or diethyl ether. After evaporation of chloroform or diethyl ether metabolic products were identified by 1H- NMR spectroscopy. Also evaporated culture extracts of both chloroform and diethyl ether of different time intervals (5, 7 & 9 hr) were mixed together and loaded onto a
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silica gel (100-120 mesh) column (2 x 50 cm) (Huang et al., 1993). Elution was done with chloroform : methanol (99.9:0.1) solvent system in the first phase, then with chloroform : methanol (80:20) solvent system in second phase. Fractions of 5 ml each were collected at a flow rate of 1 ml/min. The fractions were evaporated to dryness in vacuum at 40°C and TLC was done to confirm the fractions containing different degradation products of ferulic acid. The metabolites were then analysed by HPLC and characterized by $^1$H- NMR.

Analysis and Identification of the Ferulic Acid Degradation Products

TLC Analysis

Thin layer chromatography was carried out using 0.25 mm thick layers of silica gel GF$_{254}$ plates, either obtained from E.Merk, Germany or, prepared on glass plates with a Quickfit Industries (London) spreader. The silica gel plates, so prepared were air dried and activated at 100°C for 2 hours before use. Samples were loaded onto silica gel plates with the help of 50 l capillary tube or hamilton injector. The TLC plates were developed with two different solvent systems: (i). chloroform : methanol (80:20) and (ii) chloroform : methanol (99.5 : 0.5) as per requirement.

The developed chromatographs were then directly visualized under $\lambda_{254}$ and $\lambda_{365}$ nm UV light to observe the quenching of fluorescence by aromatic compounds. Aromatic aldehyde (vanillin) was detected by spraying the plates with 2,4-dinitrophenylhydrazine (0.1% solution made in 30% perchloric acid), which developed as a orange-red spot. Aromatic acids and other phenolic compounds were detected by spraying the plates with diazotized sulfanilic acid solution, made in 1N HCl. The unsprayed spots on TLC plates with appropriate
Rf values, matching with standard vanillin, vanillic acid and protocatechuic acid were marked under UV light, scraped off separately and extracted with chloroform or diethyl ether. After evaporation of chloroform or diethyl ether under vacuum at 40°C, the ferulic acid degradation products were identified by $^1$H-NMR spectroscopy and quantified by HPLC analysis. A large unknown spot on TLC plate, presumed to be 4-vinylguaiacol at Rf value 0.95 was scrapped off and extracted with chloroform, and after evaporation of chloroform it was identified by $^1$H-NMR spectroscopy.

**NMR Spectroscopy**

$^1$H-NMR spectra were recorded on a Bruker AC 300F NMR Spectrometer (300 MHz), using carbontetrachloride with tetramethylsilane as internal standard. Chemical shift values (ppm) and coupling constants were given in Hertz.

**HPLC Analysis**

The samples (10 μl each) were analysed using a Beckman 342 dual pump liquid chromatograph, equipped with a Model 165 detector set at λ$_{279}$ nm. The detector signals were recorded on a Shimadzu (Kyoto, Japan) CR3A integrator. Altex ultrasphere ODS Column (particle size 5μm; 25cm x 4.6 mm) and mobile phase methanol: water: acetic acid (30:70:1 vol./vol./vol.) were used (Rahouti et al., 1989). The flow rate was 1.0 ml per minute. The compounds were identified as well as quantified by comparison with the peaks of standards. HPLC analysis of the centrifuged culture supernatant at different intervals of time as well as evaporated culture extracts were performed to quantify the metabolites formed during ferulic acid biodegradation.
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Pilot Scale Biotransformation of Ferulic Acid in 7 L Fermenter

A loopful of the actively growing culture was inoculated into 100 ml conical flask containing 20 ml of sterile M9-yeast extract medium. The culture flask was incubated at 37°C in an orbital shaker at 200 rpm for 8 hrs. Then 2ml of this culture was used to inoculate 100 ml of sterile seed medium in 500 ml conical flask and was incubated at 37°C and 200 rpm in an orbital shaker for 8 hrs. This 8 hrs old seed culture was used to inoculate 5-liter production medium containing M9-minimal salt solution, 0.1% yeast extract and 0.1% ferulic acid in a 7 litre fermenter (Chemap AG, Switzerland). Fermentation was carried out at 37°C and 200 rpm with 0.2v/v/m aeration. Growth was monitored at regular intervals of 2 hrs by taking the O.D of the fermentation broth at \( \lambda_{600} \) nm. Degradation of ferulic acid as well as metabolites formation were monitored by HPLC analysis of the centrifuged culture supernatant at different intervals of time. The fermentation was terminated after 9 hrs when more than 99% substrate degradation was achieved, as measured by HPLC analysis of the culture supernatant. The fermentation broth was centrifuged at 10,000 x g for 20 min. The bacterial cells separated were stored at -20°C for enzyme purification later on, while the supernatant was extracted immediately with equal volume of chloroform. After evaporation of chloroform under vacuum at 40°C, the metabolites were analysed and identified as described earlier.
PARTIAL PURIFICATION OF FERULIC ACID DECARBOXYLASE ENZYME FROM \textit{B. COAGULANS BK07}

Culture condition for enzyme production

The bacterium was grown at 37°C and 200 rpm in seed medium for 8 hrs and then inoculated @2% into 1 litre flasks, each containing 250 ml production medium consisting of M9-minimal salt solution, 0.1% yeast extract and 0.1% ferulic acid. The flasks were then incubated at 37°C and 200 rpm in a rotary shaker. Cells were harvested after 7 hrs of incubation by centrifugation at 10,000xg for 10 minutes at 4°C in a GSA rotor (Sorvall, DuPont, Inc. Wilmington Del.).

Preparation of Cell-Free Extract

Cell pellets were washed with cold 50 mM sodium phosphate buffer (pH 7.0) and collected by centrifugation at 12,000 x g for 10 min at 4°C and suspended in cold 50 mM sodium phosphate buffer (pH 7.0) to a density of 200 gm/litre (wet weight basis). The washed cells were then disrupted by ultrasonication in an ice bath for 4 minutes (at 20% intensity with 30 second intervals) using a sonicator (Heat System Inc., USA). The crude extracts were centrifuged at 15,000 x g for 10 min at 4°C in a 17RS Contifuge (Heraeus Sepatech, Germany) and the supernatant was stored at -20°C after adding 0.5 mM phenylmethylsulfonylfluoride as proteinase inhibitor. These cell free extracts were used as the source of enzyme in the following enzyme purification steps.
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Protein estimation

The protein contents in all the samples were estimated according to Bradford's dye binding assay (Bradford, 1976), using bovine serum albumin (BSA) as a standard. All these assays were carried out in triplicate and appropriate buffer and reagent controls were also used for each samples. The fractions from each column during enzyme purification by chromatography were monitored by taking O.D at $\lambda_{280}$ nm.

Enzyme Assay

Ferulic acid decarboxylase activity was assayed by measuring the formation of enzymatic product 4-hydroxy-3-methoxystyrene (4-vinylguaiacol) by HPLC (Huang et al., 1994) or by UV spectrophotometer. In routine work assay was done by monitoring the appearance of new peak at $\lambda_{258}$ nm (Degrassi et al., 1995). The assay mixture consisted of 50mM sodium phosphate buffer (pH 7.0) and 0.5 gm of ferulic acid per litre. Reactions were initiated by adding cell free extract (5% vol./vol.) into the assay mixture and incubating the mixture for 60 min at 37°C with shaking at 200 rpm. 1U of activity was defined as the degradation of 1 pmol of ferulic acid per min (Degrassi et al., 1995).

Enzyme Purification

All the steps of enzyme purification were performed at 4°C and buffers used were of pH 7.0 unless specified otherwise. The cell-free extract (6 ml, 41.3 mg protein per ml) obtained from 2.5 liters of bacterial culture was loaded onto a Q-Sepharose Fast Flow ion exchange chromatography column (1.4 x 14 cm, 11 ml, Pharmacia LKB Biotechnology, Uppsala, Sweden), equilibrated with 50mM sodium phosphate buffer. The unbound proteins were washed off the
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Column with 30 ml of the same buffer at a flow rate of 1 ml/min. The proteins were eluted at a flow rate of 1ml/min by a 60 ml linear gradient (from 0 to 1 M) of NaCl in 50 mM sodium phosphate buffer (pH 7.0) and fractions of 2.0 ml were collected. After collecting all the fractions the protein content and decarboxylase activity in each fraction was assayed as described earlier. Fractions 7 to 10 containing enzymatic activity, were pooled together (8 ml), concentrated by freeze drying in a Drywinner 3 (HetoFD3, Heto Lab Equipment, Denmark) and reconstituted to 5 ml with 50 mM sodium phosphate buffer (pH 7.0) for loading onto gel filtration column.

Gel Filtration Column Chromatography

The concentrated enzyme solution (5 ml) obtained from active fractions of Q-Sepharose column was loaded onto a Sephadex G-200 (medium particle size, sigma) gel filtration (GF) chromatography column (2.5 x 50 cm, 233 ml), equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.9% NaCl. The proteins were eluted with the same buffer at a flow rate of 0.5 ml/min and fractions of 3.0 ml were collected. After collecting all the fractions the protein content and decarboxylase activity of each fraction assayed as described earlier. The fractions 40 to 52 containing enzymatic activity, were pooled together and concentrated by freeze drying in a Drywinner 3 (Heto FD3, Heto Lab Equipment, Denmark) for 12 hrs and reconstituted to desired volume before resolving by gel electrophoresis.

Gel Electrophoresis

The proteins in the crude cell extract, active pooled together fractions of Q-Sepharose column and Sephadex G-200 column were resolved by denaturing
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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 5% stacking gel and 15% resolving gel) by the method of Sambrook et al. (1989) on a minigel (Sigma Chemical Co., St. Louis, USA). Molecular weight was determined by use of low-molecular weight protein markers ranging from 14.2 to 94 KDa (SDS-7; Sigma). Protein bands were stained with 0.1% Coomassie blue R-250 after electrophoresis. SDS-PAGE was performed at 15°C in a 15% gel by the method described above and was used to ascertain the degree of protein purity.

Characterization of Partially Purified ferulate decarboxylase enzyme

Partially purified ferulic acid decarboxylase enzyme (from Sephadex G-200 gel filtration column) was used for characterization purpose.

Effect of temperature on ferulate decarboxylase activity

For determination of enzyme activity versus incubation temperature, enzyme incubations were conducted in the temperature range of 4°C to 80°C in 50mM sodium phosphate buffer (pH 7.0). The enzyme incubations were carried out as described earlier for 60 min before HPLC analysis. The results were expressed as relative activities versus temperature.

Effect of pH on ferulate decarboxylase activity

The influence of pH on enzyme reaction rates was determined by incubating the partially purified enzyme as usual in 50mM sodium phosphate buffers at pH ranging from 5 to 12. The enzyme incubations were carried out as described earlier for 60 min at 37°C. The results were expressed as relative activities versus pH.
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Effect of metals and inhibitors on ferulate decarboxylase activity

For determination of effects of metal ions and inhibitors on enzyme activity, enzyme was preincubated with inhibitors at 0.1 mM or metal ions at 1.0 mM in 50 mM sodium phosphate buffer (pH 7.0). The enzyme incubations were carried out as described earlier for 60 min at 37°C. In case of inhibitors, after preincubation, the remaining activity was assayed by incubating the mixture with ferulic acid. Samples containing only enzyme in phosphate buffer were used as controls. Enzyme activities in the presence of metal ions or inhibitors were expressed as relative activity as against controls (100%).

Substrate specificity of ferulate decarboxylase

The assay mixture consisted of 50 mM sodium phosphate buffer (pH 7.0) and 0.5 gm of individual substrate (e.g. Ferulic acid, 2-hydroxy cinnamic acid, 4-hydroxy cinnamic acid, 4-carboxy cinnamic acid, p-coumaric acid, trans-cinnamic acid and cis-2-methoxy cinnamic acid) separately per litre. Reactions were started by adding 50 μl of enzyme solution (from Sephadex G-200 gel filtration, enzyme activity was 2.15 μmol/min) in 450 μl of reaction mixture of each substrate in reaction vials. After incubation for 60 min at 37°C with shaking at 200 rpm the reaction was stopped by adding 2 ml of methanol to the mixture. The reaction mixtures were then analyzed by HPLC for the formation of styrene derivatives, which indicated the decarboxylation of substrates.
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TOXICITY STUDY OF VANILLIN

Bacillus subtilis Spore Rec-Assay With and Without Metabolic Activation.

Spore Preparation

Spores of both the strains (BD170 Rec' and BD 221 Rec') were prepared by spreading broth cultures on modified Schaeffer's agar medium plate and incubating at 37°C for 3 days (BD170 Rec') or 5 days (BD 221 Rec'). Spores were then scraped up, washed twice with minimal salt solution (MM), resuspended in the same solution and incubated at 80°C for 10 min. The spores were then washed twice by centrifugation with sterile distilled water and resuspended in sterile distilled water for storage at 4°C in refrigerator.

Rat-Liver Homogenate Preparation

Rat-liver homogenate was prepared according to the original method of Ames et al. (1973), from a phenobarbitone treated male rat weighing 210 gm. This male rat was given 21mg phenobarbitone (@100 mg/kg) in saline, intraperitoneally at 72, 48 and 24 hrs prior to sacrifice. On third day i.e 24 hrs after the last dose of phenobarbitone, the animal was sacrificed by cervical dislocation, placed on an autopsy board with feet secured with pins. The animal was swabbed with 95% ethanol. Incision was made in skin and the skin was tilted flap open with the help of pins. The muscle layer was also swabbed with 95% ethanol. Then the incision in muscle layer was made and liver was excised and placed in a preweighed beaker containing sterile, chilled 0.15M KCl (1 ml/gm of wet liver). The beaker containing chilled KCl solution and liver was
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times (4-5) in fresh sterile 0.15 M KCl solution and finally it was transferred to a beaker containing 3 volume of 0.15 M KCl (3ml/gm of wet liver). The liver was minced with sterile scissors and homogenized in a Potter Elvehgen apparatus, centrifuged at 9,000 x g for 10 min. The supernatant solution (S9) after centrifugation at 9,000 x g, was aliquoted in 1 ml fractions and frozen quickly in bed of crushed dry ice and stored at -70°C for further use. Before storage the sterility was checked by spread plating 0.1 ml of supernatant on nutrient agar plate.

Spore Rec-Assay Procedure (with metabolic activation) : 0.1 ml rat liver homogenate (S9) and spores (0.1 ml of suspension of 2x10^7 spores per ml) of BD 170 Rec+ or BD 221 Rec- strain of Bacillus subtilis were placed in an empty sterile petridish (90 mm diameter), then 10 ml of molten broth medium (autoclaved with 0.8% Difco agar and kept at 43°C) was poured in and mixed well. When the agar medium was well solidified, it was kept at 4°C for 30 min in a refrigerator. The plates were dried and 20 µl of the cofactor solution each was plated as drops on a single spot (four spots in one petridish). After few minutes, when the drops of cofactor solution become dry, another 20 µl vanillin solution of different concentrations (0.0001, 0.001, 0.01, 0.1, 0.5, 1.0, 2.5 and 5 mg per 20 µl) were plated as drops exactly on the spots of the drops of cofactor solution and allowed for 5-10 minutes to dry the drops. After that, the petridishes were incubated at 37°C (Hirano et al., 1982). After 48 hrs incubation the diameters of inhibition zones appearing around the spots were measured and the values of the BD170 Rec+ strain were compared with those of the BD221 Rec- strain. The procedure for spore rec- assay without metabolic activation is same as above;
the only difference is that no rat liver homogenate (S9) and cofactor solution was added (Hirano et al., 1982).

**ATTEMPTS FOR IMPROVEMENT OF STRAIN *B. COAGULANS* BK07 TO INCREASE PRODUCTION OF VANILLIN FROM FERULIC ACID BY MUTAGENESIS**

For obtaining hyper vanillin producing mutants from ferulic acid biodegradation, we have used both the physical and chemical mutagenic agents to carry out mutation experiments.

**Induction of Mutation Using Ultra Violet (UV) Light**

The strain BK07 was grown at 37°C in M9 - yeast extract medium (50 ml) for 6 hrs, so as to achieve the log phase and cell density of $1.9 \times 10^8$ colony forming units per ml. The cells were then chilled on ice bath for a few minutes to prevent further growth, and the culture divided into 5 ml portions and centrifuged for 5 minutes at 12,000xg to pellet the cells. Each pellet was then resuspended in 1.0 ml of sterile 50 mM sodium phosphate buffer (pH 7.0). One of these samples at $10^{-6}$ and $10^{-8}$ final dilutions was plated on nutrient agar plate as unirradiated control. The remaining undiluted cultures were transferred to sterile glass petri-plates of 90 mm diameter. A UV lamp (1.875 J.m$^{-2}.S^{-1}$) was properly prewarmed for at least 30 minutes. The sterile petriplates containing 1 ml each undiluted cultures suspension were placed under the UV lamp after removing the lid of the plates. The plates were tilted slightly from side to side to allow uniform exposure. Each of the plate was exposed for a specific time period, ranging from 15 seconds to 105 seconds in order to obtain upto 99% killing.
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The cells surviving the irradiation (in each plates) were immediately diluted appropriately in sterile distilled water blanks and spread plated on nutrient agar plates for determination of survival rates. The plates were then incubated at 37°C for 12-24 hrs in dark, along with unirradiated controls (appropriately diluted) and colony counts were taken. The remainder of each of the undiluted irradiated samples were centrifuged (12,000xg; 15 min; 4°C) and the cells resuspended in 10 ml of nutrient broth. The exposure time giving 0.1 - 1% survival fraction (99 -99.9% killing) was chosen in order to induce mutation in this bacterial strain.

Induction of Mutation Using N-methyl-N'-Nitrosoguanidine (NTG):

The strain BK07 was grown at 37°C in M9-yeast extract medium (50 ml) for 6 hrs, so as to achieve the log phase and cell density of 1.9 x 10^8 colony forming units per ml. The cells were then chilled on ice bath for a few minutes, to prevent further growth and culture divided into 5 ml portions and centrifuged for 5 minutes at 12,000 x g to pellet the cells. Each pellet was then resuspended in 950 µl of sterile 50 mM sodium phosphate buffer (pH 7.0), and transferred into sterilized 30 ml test tubes. In one of these samples another 50 µl of sodium phosphate buffer (pH 7.0) was added and its 10^-6 and 10^-8 final dilutions were plated on nutrient agar plate as untreated control. Then 50 µl of a stock solution of NTG, having concentration of 1 mg/ml (prepared in sterilized distilled water at pH 7.0 ± 0.2), was added to the remaining cell suspensions to achieve the final concentration of 50 µg/ml of NTG. The tubes containing reaction mixtures were incubated at 37°C and shaken intermittently. Each reaction tubes were
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withdrawn after different intervals of reaction time, ranging from 4 minutes to
20 minutes in order to obtain up to 50% killing.

In order to stop the action of NTG after different intervals of reaction
time 20 mg of cysteine was dissolved in the first dilution blank of each set of
reaction mixture. The cells surviving NTG treatment (in each test tube of
different time interval) were immediately diluted in sterile distilled water blanks
to an appropriate series of dilutions and spread plated on nutrient agar plates for
determination of survival rates. The plates were then incubated at 37°C for 12-
24 hrs in dark along with untreated control of appropriate dilutions, and colony
counts were taken. The remainder of each of the undiluted NTG treated samples
were centrifuged (12,000xg, 15 min., 4°C) and the cells resuspended in 10 ml of
nutrient broth. The exposure time which gave 50% or less survival fraction
(50% or more killing) was chosen in order to induce mutations with NTG, in this
bacterial strain.

Screening of Mutants

For screening of mutants, the colonies from nutrient agar plate were
spotted onto test plates containing M9-minimal salt solution, 0.1% yeast extract,
0.1% ferulic acid and 2% agar agar, with a pH of 7.2 . Each plate was also
spotted with a control (unirradiated or not treated with NTG). The replica of
each colony was maintained on nutrient agar plates by spotting with a sterile
tooth pick. The plates were incubated at 37°C for 8 hrs. After incubation, the
test plates were sprayed with 0.1% solution of 2,4- dinitrophenylhydrazine,
made in 30% perchloric acid and again incubated at room temperature for 1-2
hrs. The intensity as well as diameter of orange-red zones, developed around the
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colonies were observed and compared with that of untreated control. The colonies showing more intensity and larger diameter of coloured zone were chosen for further investigation.