Results
3.1 Assimilation of aromatic compounds by *Rhodobacter sphaeroides* OU5

Growth of *Rhodobacter sphaeroides* OU5 could not be demonstrated on aromatic compounds as sole source of carbon or as electron donors, replacing malate in the Biebl and Pfennig’s medium. Among the various compounds tested, assimilation (utilization) of trans-cinnamate, 4-hydroxycinnamate, 3, 4-dihydroxycinnamate, 4-hydroxybenzoate, phenylpyruvate and L-phenylalanine from the culture supernatant was observed after 48 h of phototrophic incubation when added as supplement at a concentration of 0.5 mM (Table 3). The loss of compound in the supernatant could not support growth of *Rba. sphaeroides* OU5, since increase in the biomass (compared to control culture) could not be demonstrated. Other compounds like benzoate, 2-hydroxybenzoate, 2-aminobenzoate, 4-aminobenzoate, toluene and cresol levels in the supernatant remained same as the initial concentration (0.5 mM) and their loss by *Rba. sphaeroides* OU5 could not be demonstrated. Uninnoculated medium was used as control to check for possible photochemical reactions. However, no loss of compound was observed, which indicates loss is only due to biochemical reaction.

The toxicity of aromatic compounds on growth of *Rba. sphaeroides* OU5 was evaluated in terms of 50 % inhibitory concentration, IC$_{50}$ (Table 3). The IC$_{50}$ values of trans-cinnamate, 4-hydroxycinnamate and 3, 4-dihydroxycinnamate were 4.5, 3.0 and 2.5 mM, respectively. The IC$_{50}$ values of benzoate, 4-hydroxybenzoate, 2-hydroxybenzoate, 4-aminobenzoate and 2-aminobenzoate were 9.5, 10, 2.0, 8.0 and 4.0 mM, respectively. Assimilation of trans-cinnamate, hydroxycinnamates and 4-hydroxybenzoate was taken up for detailed study.
Table 3: IC$_{50}$ and photo assimilation of aromatic compounds by the whole cells of *Rhodobacter sphaeroides* OU5 [NT = Not tested; + = assimilated; - = Not assimilated]

* [for *trans*-cinnamate assimilation- glucose (0.3 % w/v) and for 4-hydroxybenzoate assimilation- fumarate (0.3 % w/v) were used as carbon sources]

* *Rhodobacter sphaeroides* OU5 culture was grown photoheterotrophically in Biebl and Pfennig’s medium (1981) with malate (22 mM) as sole carbon source and NH$_4$Cl (7 mM) as nitrogen source supplemented with aromatic hydrocarbons (0.5 mM) listed in the table for 48 h and assayed for assimilation. IC$_{50}$ values were calculated as mentioned in methodology.
3.2 Assimilation of \textit{trans}-cinnamate by \textit{Rba. sphaeroides} OU5 (work with whole cells)

3.2.1 \textbf{Effect of \textit{trans}-cinnamate on growth of \textit{Rba. sphaeroides} OU5}

Effect of different concentrations of \textit{trans}-cinnamate (0.5-7.0 mM) on photoheterotrophic growth of \textit{Rba. sphaeroides} OU5 was studied. Presence of \textit{trans}-cinnamate did not alter the growth yield and biomass of \textit{Rba. sphaeroides} OU5 upto 2.5 mM (compared to control) and beyond 2.5 mM gradual decrease in growth was observed (Fig 6). Growth of \textit{Rba. sphaeroides} OU5 was inhibited at 7 mM. The 50 \% inhibitory concentration (IC$_{50}$) of \textit{trans}-cinnamate on photoheterotrophic growth of \textit{Rba. sphaeroides} OU5 was approximately 4.5 mM.

3.2.2 \textbf{Influence of organic substrates on growth of \textit{Rba. sphaeroides} OU5 at IC$_{50}$ of \textit{trans}-cinnamate.}

The influence of different organic substrates on the growth of \textit{Rba. sphaeroides} OU5 in the presence of 4.5 mM of \textit{trans}-cinnamate was studied (Fig 7). Growth of \textit{Rba. sphaeroides} OU5 was increased by 30 and 10 \% (compared to control) in presence of oxaloacetate and glucose respectively, while other substrates like fumarate, succinate, pyruvate and α-ketoglutarate have inhibited the growth.

3.2.3 \textbf{Influence of amino acids on the growth of \textit{Rba. sphaeroides} OU5 at IC$_{50}$ of \textit{trans}-cinnamate.}

Growth of \textit{Rba. sphaeroides} OU5 in the presence of \textit{trans}-cinnamate at IC$_{50}$ concentration (4.5 mM) with different amino acids supplemented to the photoheterotrophic medium at a concentration of 1mM was studied. Growth increase over control (with out amino acid) was ~ 8 \% with L-glutamate and L-glutamine supplementation, while serine and aromatic amino acids added individually had rather inhibited the growth of \textit{Rba. sphaeroides} OU5. On supplementing, mixture of aromatic amino acids like L-phenylalanine, L-tyrosine and L-
tryptophan together at 1 mM, a 15 % increase in growth yield of *Rba. sphaeroides* OU5 over the control was demonstrated (Fig 8).

![Graph showing the minimum inhibitory concentration of trans-cinnamate on the phototrophic growth of *Rba. sphaeroides* OU5.](image)

**Fig 6: Minimum inhibitory concentration of trans-cinnamate on the phototrophic growth of *Rba. sphaeroides* OU5**

Assay was done with the growing cells of *Rba. sphaeroides* OU5 in phototrophic medium with malate (22 mM) as sole carbon source and NH₄Cl (7 mM) as sole nitrogen source in the presence of various concentrations of *trans*-cinnamate. The culture was incubated for 48 h anaerobically under light (2,400 lux) at 30±2 °C in fully filled screw cap test tubes and growth was measured turbidometrically.
Fig 7: Influence of organic substrates on growth of *Rba. sphaeroides* OU5 at IC$_{50}$ of *trans*-cinnamate

Actively growing cells of *Rba. sphaeroides* OU5 inoculated into photoheterotrophic medium containing respective organic substrate (0.3 % w/v) as carbon and ammonium chloride (7 mM) as nitrogen source supplemented with *trans*-cinnamate at 4.5 mM. Incubation conditions are same as in fig 6.
Fig 8: Influence of L-amino acids on growth of *Rba. sphaeroides* OU5 at IC₅₀ of *trans*-cinnamate
(Amino acid cocktail = mixture of phenylalanine, tyrosine, tryptophan together at 1 mM)

Actively growing cells of *Rba. sphaeroides* OU5 inoculated into photoheterotrophic medium with malate (0.3 % w/v) as carbon and ammonium chloride (7 mM) as nitrogen source supplemented with *trans*-cinnamate (4.5 mM) and respective L-amino acids at 1 mM. Growth was measured with respect to control (without amino acid). Incubation conditions are same as in fig 6.
3.2.4 *trans*-Cinnamate assimilation by growing and resting cells of *Rba. sphaeroides* OU5

3.2.4.1 Light dependent assimilation of *trans*-cinnamate by *Rba. sphaeroides* OU5

Assimilation of *trans*-cinnamate was observed only under light, anaerobic incubation by both growing and resting cells of *Rba. sphaeroides* OU5 (Table 4). Assimilation of *trans*-cinnamate (0.5 mM) could not be demonstrated when the culture of *Rba. sphaeroides* OU5 was incubated under dark anaerobic conditions even after 48 h of incubation.

3.2.4.2 Assimilation of *trans*-cinnamate at various concentrations by resting cells of *Rba. sphaeroides* OU5

Assimilation of *trans*-cinnamate at various concentrations was studied using resting cells of *Rba. sphaeroides* OU5. Assimilation was optimum at 0.5 mM concentration of *trans*-cinnamate and above 0.5 mM, assimilation decreased. However, the biomass of the culture remained constant at all concentrations of *trans*-cinnamate tested (Fig 9). 0.5 mM of *trans*-cinnamate was used for further studies.

3.2.4.3 Assimilation of *trans*-cinnamate with time by resting cells of *Rba. sphaeroides* OU5

Resting cell suspensions of *Rba. sphaeroides* OU5 were assayed with time for *trans*-cinnamate assimilation in basal medium with out any carbon or nitrogen source but with *trans*-cinnamate (0.5 mM) as a supplement. Assimilation of *trans*-cinnamate started with a lag period of 24 h and complete *trans*-cinnamate loss (0.5 mM) was observed at the end of 48 h of incubation, while the biomass of *Rba. sphaeroides* OU5 remained constant, (Fig 10).
3.2.4.4 Effect of chloramphenicol on the assimilation of trans-cinnamate

Assimilation of trans-cinnamate by the resting cells of *Rba. sphaeroides* OU5 in the presence of chloramphenicol was studied. In order to detect the metabolites released during trans-cinnamate assimilation, chloramphenicol a protein synthesis inhibitor was added to resting suspensions of *Rba. sphaeroides* OU5. 50% loss of trans-cinnamate (0.25 mM) was observed after 48 h incubation and in the HPLC analysis, two new peaks were detected along with trans-cinnamate in the supernatant, in which one peak matched with standard L-phenylalanine and the other with phenylpyruvate whose concentrations were 90 and 160 µM, respectively (Fig 11).

<table>
<thead>
<tr>
<th>Growth phase of cells</th>
<th>Biomass yield (mg drywt.ml⁻¹)</th>
<th>trans-cinnamate consumption (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>Resting</td>
<td>0.48</td>
<td>0.48</td>
</tr>
<tr>
<td>Growing</td>
<td>0.27</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 4: Light dependent assimilation of trans-cinnamate by resting and growing cells of *Rba. sphaeroides* OU5

Resting and growing cells of *Rba. sphaeroides* OU5 incubated under light and dark for 48 h in presence of trans-cinnamate (0.5 mM) as supplement and the consumption of trans-cinnamate was estimated using HPLC.
Fig 9: Assimilation of trans-cinnamate at different concentrations by resting cells of *Rba. sphaeroides* OU5

Experiment was done with resting cells of *Rba. sphaeroides* OU5 at different concentrations of trans-cinnamate (X-axis) and assay conditions are same as in fig 6. Biomass yield and trans-cinnamate consumption are shown on primary and secondary Y-axis respectively.
Fig 10: Photoassimilation of *trans*-cinnamate with time by resting cells of *Rba. sphaeroides* OU5

The basal medium supplemented with 0.5 mM *trans*-cinnamate was inoculated with resting cells of *Rba. sphaeroides* OU5 and incubated anaerobically for 48 h. At different time intervals *trans*-cinnamate levels in the supernatant were quantified using HPLC.
Fig 11: HPLC chromatogram showing trans-cinnamate assimilation by resting cells of *Rba. sphaeroides* OU5 treated with chloramphenicol

HPLC chromatogram (at 200 nm wavelength) showing trans-cinnamate assimilation by resting cells of *Rba. sphaeroides* OU5 when treated with chloramphenicol (20 µg.10 ml⁻¹ of basal medium). Initial (chromatogram in pink colour) and final levels (chromatogram in dark green) of trans-cinnamate are shown.
3.2.4.5 Effect of carbon substrates on trans-cinnamate assimilation by growing cells of *Rhodobacter sphaeroides* OU5

*trans*-Cinnamate assimilation was not observed during growth of *Rba. sphaeroides* OU5 in photoheterotrophic medium with malate as carbon source/electron donor, hence assimilation was studied with other organic substrates (Table 5). Growth of *Rba. sphaeroides* OU5 was observed on all the substrates tested. However, assimilation of *trans*-cinnamate varied and was dependent on the growth substrate used. Among the substrates tested (Table 5), maximum assimilation of *trans*-cinnamate was observed with glucose as carbon source. Pyruvate and α-ketoglutarate also promoted assimilation however, acetate, malate, succinate and fumarate inhibited *trans*-cinnamate assimilation. In addition to loss of substrate, metabolites like phenylalanine, tryptophan, tyrosine and indole were detected in the *trans*-cinnamate induced culture supernatant (Table 5).

3.2.4.6 *trans*-Cinnamate assimilation with glucose as carbon source by growing cells of *Rhodobacter sphaeroides* OU5

Growth and *trans*-cinnamate assimilation was studied with growing cells of *Rhodobacter sphaeroides* OU5 with glucose as carbon source. The assimilation started with a lag period of 18 h and was maximum during the logarithmic phase of growth (Fig 12A). By the end of 42 h, complete (0.5 mM) assimilation of *trans*-cinnamate was observed. Simultaneous presence of glucose and *trans*-cinnamate in the mineral medium reduced the rate of glucose consumption and its levels were almost same as the initial concentration (rather a slight increase of 7 % was observed), when compared with *Rba. sphaeroides* OU5 grown in mineral medium containing the same concentration of glucose alone (control) (Fig 12B).
3.2.4.7 Influence of nitrogen substrates on trans-cinnamate assimilation

Growth of *Rba. sphaeroides* OU5 was demonstrated on all the nitrogen substrates tested (biomass yield of 0.35 mg drywt.ml⁻¹). However, consumption of trans-cinnamate varied and was observed maximum with ammonia, followed by ammonium chloride, glutamate and glutamine (Table 6). While amino acids like serine, phenylalanine, tyrosine and tryptophan have rather inhibited trans-cinnamate assimilation. Metabolites like phenylalanine and indole were detected in the induced culture supernatant of *Rba. sphaeroides* OU5.

3.2.4.8 Metabolite profiling of *Rba. sphaeroides* OU5 grown on trans-cinnamate

In order to detect the metabolites produced in the presence of trans-cinnamate by *Rba. sphaeroides* OU5, the culture supernatant grown with and without trans-cinnamate was concentrated, extracted into methanol and was analyzed using LC-MS. The metabolites of masses (m/z) 161, 171, 376 were detected in the presence of trans-cinnamate while they were absent in the culture supernatant of *Rba. sphaeroides* OU5, grown without trans-cinnamate (control) (Fig 13).
Table 5: Assimilation of trans-cinnamate by growing cells of *Rba. sphaeroides* OU5 in presence of various organic substrates [control = without organic substrate; Phe = phenylalanine; Tyr = tyrosine; Trp = tryptophan]

Consumption of trans-cinnamate was studied with growing cells of *Rba. sphaeroides* OU5 grown on mineral medium with respective organic substrates (0.3 % w/v) as sole carbon source and NH₄Cl (7 mM) as sole nitrogen source, supplemented with trans-cinnamate (0.5 mM). Levels of trans-cinnamate and metabolites in the supernatant were measured using HPLC, after 48 h of phototrophic incubation.
Fig 12: Time course of photoassimilation of trans-cinnamate by growing cells of *Rba. sphaeroides OU5*

Mineral medium containing glucose (17 mM) as sole carbon source and ammonium chloride (7 mM) as sole nitrogen source supplemented with 0.5 mM *trans*-cinnamate was inoculated with growing cells of *Rba. sphaeroides OU5* culture and incubated anaerobically for 48 h. At different time intervals *trans*-cinnamate levels in the supernatant were quantified by HPLC as shown in fig (A).

Fig (B) shows the levels of glucose in the *trans*-cinnamate induced and control (without *trans*-cinnamate) culture supernatant of *Rba. sphaeroides OU5*. 
### Table 6: Assimilation of \textit{trans}-cinnamate by growing cells of \textit{Rba. sphaeroides} OU5 in the presence of different nitrogen substrates

<table>
<thead>
<tr>
<th>Nitrogen substrates (1mM)</th>
<th>\textit{trans}-cinnamate consumption (mM)</th>
<th>Metabolites (mM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phenylalanine</td>
<td>Indole</td>
</tr>
<tr>
<td>(\text{NH}_3\text{Cl}  )</td>
<td>0.40</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>(\text{NH}_4\text{OH}  )</td>
<td>0.42</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.10</td>
<td>-</td>
<td>0.07</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.12</td>
<td>-</td>
<td>0.06</td>
</tr>
<tr>
<td>Serine</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>-</td>
<td>-</td>
<td>0.20</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Cells of \textit{Rba. sphaeroides} OU5 grown on mineral medium with glucose (0.3 % w/v) as sole carbon source in presence of respective nitrogen source (1 mM) supplemented with \textit{trans}-cinnamate (0.5 mM). Levels of \textit{trans}-cinnamate and metabolites released in the supernatant were measured by HPLC, after 48 h of phototrophic incubation (2,400 lux) at 30±2 °C.
Fig 13: LC-MS metabolite profiling of control (uninduced) and trans-cinnamate induced culture supernatant of *Rba. sphaeroides* OU5

*trans*-Cinnamate induced and control (uninduced) culture supernatants of *Rba. sphaeroides* OU5 were concentrated and extracted with methanol. The methanol extract was concentrated and analyzed using LC-MS. Both mass spectra were recorded in negative mode. Metabolites encircled (with an estimated mass of 161, 171 and 376 respectively) are unique to *trans*-cinnamate induced culture and are absent in uninduced culture.
3.3 Biochemical mechanism involved in *trans*-cinnamate assimilation by *Rhodobacter sphaeroides* OU5

Assimilation of *trans*-cinnamate was observed by whole cells of *Rba. sphaeroides* OU5 (Table 3). The biochemical mechanism involved in this assimilation was studied with cell free extracts and purified enzyme preparations of *Rba. sphaeroides* OU5. Studies with whole cells of *Rba. sphaeroides* OU5 indicated consumption of *trans*-cinnamate and release of metabolites like indole, tryptophan, phenylalanine and tyrosine into *trans*-cinnamate induced culture supernatant (Table 5 and 6) and the same was studied using cell free extracts of *Rba. sphaeroides* OU5.

3.3.1 Effect of *trans*-cinnamate on DAHP synthase activity

Presence of *trans*-cinnamate in the medium reduced the consumption of glucose in contrast to the control (without *trans*-cinnamate) in which total glucose was consumed by 48 h (Fig 12 B). This indicates the possible effect of *trans*-cinnamate in preventing the entry of glucose into the shikimate pathway of aromatic amino acid biosynthesis by inhibition of some of the enzymes. Hence influence of *trans*-cinnamate on the first enzyme of this pathway was studied. *trans*-Cinnamate completely (100 %) inhibited the DAHP synthase activity i.e. condensation of erythrose 4-phosphate and phosphoenol pyruvate to 2-keto-3-deoxy arabino heptulosanate-7-phosphate (Table 7), which is committed toward synthesis of aromatic amino acids. DAHP synthase activity was also inhibited in the presence of 4-hydroxycinnamate, 3, 4-dihydroxycinnamate and 4-hydroxybenzoate and percentage inhibition was 40, 46 and 16 % respectively in comparison to control.
3.3.2 **trans-Cinnamate consumption with cell free extracts of *Rhodobacter sphaeroides* OU5**

Consumption of *trans*-cinnamate was studied using cell free extracts of *Rba. sphaeroides* OU5. *trans*-Cinnamate consumption was observed by both induced and uninduced cell free extracts of *Rba. sphaeroides* OU5. However, maximum consumption was observed with the *trans*-cinnamate induced cell free extracts (Table 8). Requirement of coenzymes and cofactors for *trans*-cinnamate consumption was studied. Among different coenzymes added, *trans*-cinnamate consumption was maximum with NADH while addition of ATP, NADPH had inhibited the consumption (Table 9). Specific metal cofactor requirement for *trans*-cinnamate consumption by the cell free extracts of *Rba. sphaeroides* OU5 could not be demonstrated.

3.3.3 **Amino donor and coenzyme requirement for *trans*-cinnamate transformation**

Amino donor was required for transformation of *trans*-cinnamate to phenylalanine. Hence, different amino donors like ammonia, ammonium chloride, glutamine, glutamate and serine with pyridoxal phosphate were added to the assay sample. Among them maximum *trans*-cinnamate consumption and transformation was observed with addition of ammonia, followed by ammonium chloride (Table 9).
Table 7: Effect of trans-cinnamate on 3-keto 2-deoxy-arabino heptulosanate 7-phosphate (DAHP) synthase activity

(The DAHP synthase activity in control was 26 moles.mg protein⁻¹)

<table>
<thead>
<tr>
<th>Compound (without trans-cinnamate)</th>
<th>DAHP synthase activity (%) Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>trans-cinnamate</td>
<td>100</td>
</tr>
</tbody>
</table>

The assay mixture (1 ml) contained 10 mM Tris HCl buffer (pH 7.8) with erythrose 4-phosphate at 100 moles, 50 moles of phosphoenolpyruvate along with trans-cinnamate at 50 moles with the cell free extract (350 µg) of protein and incubated for 20 min at 37 °C. After incubation the consumption of erythrose 4-phosphate was quantified using HPLC. Cell free extracts were obtained from *Rba. sphaeroides* OU5 grown with glucose (17 mM) as carbon source and NH₄Cl (7 mM) as nitrogen source with 0.5 mM trans-cinnamate as supplement.

Table 8: trans-cinnamate consumption activity by *Rba. sphaeroides* OU5

Results expressed are an average of data done in triplicates.
Table 9: Consumption of *trans*-cinnamate with cell free extracts of *Rba. sphaeroides* OU5

(NADH = reduced nicotinamide adenine dinucleotide; NADPH = reduced nicotinamide adenine dinucleotide phosphate; ATP = adenosine triphosphate; PLP = pyridoxal phosphate; ND = not detected)

The assay mixture contained (1 ml) of 10 mM Tris HCl buffer (pH 7.8) with 100 moles of *trans*-cinnamate, along with respective components like 25 moles of NADH, NADPH, ATP and 0.1 ml of 10 % NH₄OH, 0.1ml of 10 % NH₄Cl, L-glutamate, L-glutamine of 100 moles, PLP- 20 g with 280 µg of protein. The assay mixture was incubated for 30 min at room temperature. The reaction was stopped by acidifying with 1N HCl, filtered by 0.22 µm membrane filter and analysed in HPLC for the quantification of *trans*-cinnamate. Details of enzyme source were same as in table 7.
3.3.4 **Transformation of trans-cinnamate to phenylalanine with cell free extracts of *Rhodobacter sphaeroides* OU5**

Though there are reports of transformation of phenylalanine to *trans*-cinnamate and vice-versa, catalyzed by enzyme phenylalanine ammonia lyase (PAL). Activity of PAL could not be demonstrated with cell free extracts of *Rba. sphaeroides* OU5. This indicates that PAL may not be involved in the transformation of *trans*-cinnamate to phenylalanine in *Rba. sphaeroides* OU5. However, non-stoichiometric yield of phenylalanine was observed when cell free extract of *Rba. sphaeroides* OU5 was supplemented with ammonia (0.1ml of 10% ammonia solution) and NADH (Table 10). *trans*-Cinnamate to phenylalanine transformation activity was studied with other amino donors like ammonium hydroxide, ammonium chloride, glutamate and glutamine with and without NADH. The highest yield of phenylalanine from *trans*-cinnamate requires a reducing agent NADH (25 μmoles.ml⁻¹ of assay mixture) (Table 10). The reverse conversion of phenylalanine to *trans*-cinnamate was not observed with cell free extracts of *Rba. sphaeroides* OU5. Transformation of phenylalanine from phenylpyruvate was also demonstrated using cell free extracts of *Rba. sphaeroides* OU5 (Table 11). Phenylpyruvate was the intermediate involved in the transformation of *trans*-cinnamate to phenylalanine as observed in the assay with intact cells of *Rba. sphaeroides* OU5 (Fig 11).

3.3.5 **Kinetics of *trans*-cinnamate consumption with cell free extracts of *Rba. sphaeroides* OU5**

Time course of *trans*-cinnamate consumption was studied with *trans*-cinnamate induced cell free extracts of *Rba. sphaeroides* OU5. In the assay mixture with *trans*-cinnamate, NADH and ammonia, *trans*-cinnamate consumption started after 5 min and completed
by 20 min of incubation. While phenylalanine formation started after 15 min and reached a maximum of 20 moles at the end of 30 min of incubation (Fig 14).

<table>
<thead>
<tr>
<th>Assay components</th>
<th>trans-cinnamate consumption (moles.mg protein(^{-1}))</th>
<th>L-phenylalanine formation (moles.mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ammonia</td>
<td>70</td>
<td>ND</td>
</tr>
<tr>
<td>Ammonia + NADH</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>Ammonium chloride + NADH</td>
<td>200</td>
<td>20</td>
</tr>
<tr>
<td>L-glutamate + PLP</td>
<td>300</td>
<td>50</td>
</tr>
<tr>
<td>L-glutamine + PLP</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 10: Transformation of *trans*-cinnamate to L-phenylalanine by cell free extracts of *Rba. sphaeroides OU5* (NADH = reduced nicotinamide adenine dinucleotide; PLP = pyridoxal phosphate; ND = not detected)

The assay mixture contained (1ml) of 10 mM Tris HCl buffer (pH 7.8) with 100 moles of *trans*-cinnamate, along with respective components like 25 moles of NADH, 0.1ml of 10% NH\(_4\)OH, 0.1ml of 10% NH\(_4\)Cl, L-glutamate, L-glutamine at 100 moles, pyridoxal phosphate (PLP)-20 g with 280 μg of protein and incubated for 30 min at room temperature. The assay was stopped by acidifying with 1N HCl, filtered and analyzed in HPLC, for quantification of L-phenylalanine and *trans*-cinnamate. The source of cell free extract is *Rba. sphaeroides OU5* grown with glucose (17 mM) and NH\(_4\)Cl (7 mM) induced with *trans*-cinnamate (0.5 mM).
Table 11: Conversion of phenylpyruvate to L-phenylalanine by cell free extracts of *Rba. sphaeroides* OU5 (ND = not detected)

<table>
<thead>
<tr>
<th>Assay components</th>
<th>Phenylpyruvate consumption (moles.mg protein⁻¹)</th>
<th>L-phenylalanine formation (moles.mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ammonia</td>
<td>700</td>
<td>ND</td>
</tr>
<tr>
<td>Ammonia + NADH</td>
<td>1000</td>
<td>700</td>
</tr>
<tr>
<td>L-Glutamate + PLP</td>
<td>300</td>
<td>50</td>
</tr>
</tbody>
</table>

One ml of assay sample contained 10 mM Tris-HCl buffer (pH 7.8), 280 µg of cell free extract, 100 moles of phenylpyruvate with respective components like 25 moles of NADH, 0.1 ml of 10 % NH₄OH, L-glutamate-100 moles, pyridoxal phosphate (PLP)-20 µg and incubated at room temperature. The reaction was stopped by acidifying with 1N HCl, filtered and analyzed in HPLC.
Fig 14: Consumption of trans-cinnamate with time by cell free extracts of Rba. sphaeroides OU5  [Cin = trans-cinnamate; PA = L-phenylalanine]

Assay sample of 1 ml contained 10 mM Tris-HCl buffer (pH 7.8), 100 μmoles of trans-cinnamate, 25 μmoles of NADH, 0.1 ml of 10 % ammonia solution (NH₄OH), to which 280 μg of protein (cell free extract) was added and incubated at room temperature. At regular intervals of time aliquots were taken from the assay mixture and reaction was stopped by acidifying with 1N HCl, membrane filtered (0.22 µm) and analyzed using HPLC.
3.3.6 **Isolation and purification of the proteins involved in trans-cinnamate to phenylalanine transformation**

The protein involved in transformation of *trans*-cinnamate to phenylalanine by *Rba. sphaeroides* OU5 was isolated according to extraction procedure given in the Flow chart 1. The crude extract of *trans*-cinnamate induced culture of *Rba. sphaeroides* OU5 when subjected to ammonium sulfate fractionation resulted in an active *trans*-cinnamate consuming fraction between 60-90% saturation. This fraction was further purified by DEAE-Cellulose chromatography using step gradient of 0-1 M NaCl, active fractions were eluted in 0.6 M NaCl (Fig 15).

Active fractions (fraction 20 and 21) with *trans*-cinnamate consumption and phenylalanine formation were used for further characterization. Native and SDS-PAGE analysis (Fig 16A and 16B) of this fraction was detected by silver staining which showed a single protein band of ~42 kD. HPLC analysis indicated the purity of the protein (Fig 16C).

Native molecular weight of purified protein was determined using gel exclusion chromatography (Sephacryl G-100) by Fast Protein Liquid Chromatography (FPLC). Void volume of the column was determined using Blue Dextran and calibrated using standard protein markers; catalase (240 kD), glucose oxidase (90 kD), bovine serum albumin (67 kD) and peroxidase (40 kD) (Fig 16D). Based on the elution volume of protein with standard protein markers, molecular weight of the protein was found to be ~43 kD. Summary of the protein yield from the major purification steps is given in Table 12.
3.3.6.1 *trans*-Cinnamate to phenylalanine transformation by the purified protein

Reductive amination of *trans*-cinnamate to phenylalanine through phenylpyruvate was observed in presence of NADH, ammonia and the transformed product phenylalanine was detected as appearance of peak (t<sub>R</sub> = 3.2 min) in HPLC (Fig 17). Transformation of phenylpyruvate to L-phenylalanine was also observed in stoichiometric yields of L-phenylalanine (Fig 18) whereas the reverse conversion of L-phenylalanine to phenylpyruvate could not be demonstrated with the purified protein. This enzyme required no metal cofactor and reaction was competitively inhibited in presence of fumarate.

3.3.6.2 Characterization of protein

3.3.6.2.1 Enzyme kinetics: *trans*-Cinnamate consumption activity was studied with time. Activity was optimum at 5-10 min and then decreased beyond 10 min of incubation (19C).

3.3.6.2.2 Calculation of *K<sub>m</sub>* and *V<sub>max</sub>*: *trans*-Cinnamate consumption activity of protein was checked at varying concentrations (0.005-0.1 mmoles) of *trans*-cinnamate and was maximum at 0.02 mmoles (Fig 19A). The Michaelis constant for *trans*-cinnamate, calculated from lineweaver burk analysis was 0.027 mM and *V<sub>max</sub>* 93.4 (Fig 19E).

3.3.6.2.3 Substrate specificity of enzyme: The enzyme was specific towards analogues like 4-hydroxycinnamate and 3, 4-dihydroxycinnamate however, the activity was not observed with benzoate, 4-hydroxybenzoate, 2-hydroxybenzoate (Table 13). Activity was checked with addition of different coenzymes and was observed only with addition of NADH, however there was no activity in presence of coenzymes like NADPH, PLP, TPP, ATP, Coenzyme A and lipoic acid.
3.3.6.2.4 **Activity at different pH:** trans-Cinnamate consumption activity of the enzyme was studied at different pH (2-11). The enzyme had optimum activity at pH 9.0 (Fig 19D).

3.3.6.2.5 **Activity at different temperatures:** The activity of the enzyme at different temperatures from 0-60 °C was studied and trans-cinnamate consumption was maximum 28-30 °C (Fig 19B).

**Flow chart 1:** Steps involved in isolation and purification of protein

*trans*-Cinnamate induced culture of *Rba. sphaeroides* OU5 was harvested after 48 h of phototrophic incubation and cell pellet after sonication was subjected to further purification.
Fig 15: DEAE-Cellulose elution profile of protein

trans-cinnamate consumption activity was measured for all the fractions eluted with a linear gradient of 0-1.0 M NaCl and the active protein fractions eluted from DEAE column in 0.6 M NaCl were analyzed for trans-cinnamate consumption and the same were plotted on X-axis. Protein absorption and activity (in terms of trans-cinnamate consumption) were plotted on primary and secondary Y-axis respectively.
Fig 16(A): Purified protein in 8 % Native PAGE
Lane 1 = Catalase, 240 kD (native marker); Lane 2 = Bovine Serum Albumin, 67 kD (native marker);
Lane 3 = DEAE-Cellulose protein fraction
(B): Silver stained gel of purified protein in 8 % SDS-PAGE
Lane 1 = Purified protein; Lane 2 = Marker
(C): HPLC chromatogram showing the purity of the protein
(D): The molecular weight analysis of the protein using FPLC
Fig 17: HPLC chromatogram showing the transformation of *trans*-cinnamate to phenylalanine by the purified protein

The assay was done with the purified protein of *Rba. sphaeroides* OU5. The chromatogram showing assay sample at 0 min (solid line), sample after 10 min (dashed chromatogram) and the formation of phenylalanine at 30 min (dash-dot-dot) were recorded at 220 nm.
Fig 18: HPLC chromatogram showing the conversion of phenylpyruvate to phenylalanine by the purified protein

The chromatogram showing compounds in the assay sample at 0 min (blue coloured) and 30 min (pink coloured) were registered at 220 nm.
Fig 19: Characterization of enzyme catalyzing *trans*-cinnamate to phenylalanine

(*Cin = trans-cinnamate*)
Table 12: Summary of the protein yield from the major purification steps

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg(^{-1}))</th>
<th>Yield (%)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>70</td>
<td>245</td>
<td>0.6</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>60-90 % (NH(_4))(_2)SO(_4) fraction</td>
<td>6</td>
<td>0.17</td>
<td>2.4</td>
<td>56</td>
<td>4</td>
</tr>
<tr>
<td>DEAE-Cellulose fraction</td>
<td>4</td>
<td>0.03</td>
<td>8.8</td>
<td>8.5</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 13: Substrate specificity of the enzyme and its activity with coenzymes

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans-Cinnamate</td>
<td>100</td>
</tr>
<tr>
<td>4-Hydroxycinnamate</td>
<td>40</td>
</tr>
<tr>
<td>3, 4-Dihydroxycinnamate</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Coenzymes</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>85</td>
</tr>
<tr>
<td>NADPH</td>
<td>-</td>
</tr>
<tr>
<td>PLP</td>
<td>-</td>
</tr>
</tbody>
</table>

NADH = Reduced nicotinamide adenine dinucleotide; NADPH = Reduced nicotinamide adenine dinucleotide phosphate; PLP = Pyridoxal phosphate.
3.3.7 Transformation of trans-cinnamate to L-tryptophan by cell free extracts of \textit{Rhodobacter sphaeroides} OU5

Metabolites like indole and tryptophan are released into trans-cinnamate induced culture supernatant of \textit{Rba. sphaeroides} OU5 (Table 5 and 6 in chapter 2). Using cell free extracts of \textit{Rba. sphaeroides} OU5 transformation of trans-cinnamate to indole and L-tryptophan was studied, that could involve few intermediates like phenylpyruvate, phenyl acetaldehyde and benzoacetonitrile.

When the reaction mixture was supplemented with ammonia, trans-cinnamate consumption was detected. On supplementation of serine, pyridoxal phosphate to the assay mixture, tryptophan could be demonstrated (Table 14). With addition of hydroxylamine, an inhibitor of tryptophan biosynthesis, indole (immediate precursor of tryptophan biosynthesis) was demonstrated (Table 14). The intermediates involved in this conversion were identified based on the HPLC and LC-MS analysis of the enzyme assayed fraction. Indole was demonstrated in the presence of hydroxylamine in HPLC analysis (Fig 20). The assayed sample was lyophilized and the metabolites were extracted into methanol, analyzed in LC-MS (Fig 21). Mass \((m/z)\) 122 \((m\text{H}^2)\) corresponding to phenyl acetaldehyde and 115 \((m\text{H}^+2)\), 163 \((m\text{H}^+2)\) corresponding to compounds, indole and phenylpyruvate respectively were detected and they were absent in the control assay mixture (without trans-cinnamate).
Table 14: Conversion of trans-cinnamate to L-tryptophan with cell free extracts of *Rba. sphaeroides* OU5 (HA = hydroxylamine; PLP = pyridoxal phosphate; ND = not detected)

The assay mixture contained 1ml of 10 mM Tris HCl buffer (pH 7.8) with 100 moles of trans-cinnamate, along with assay components like 0.1ml of 10 % NH₄OH (ammonia solution), L-serine at 100 moles, pyridoxal phosphate 20 g with 280 μg of protein and incubated for 45 min at room temperature. Hydroxylamine was added at a concentration of 50 moles to 1 ml of reaction mixture. The reaction was stopped by acidifying with 1N HCl, filtered and analyzed in HPLC. The source of cell extract was same as given in Table 7.
Fig 20: HPLC chromatogram of the enzyme assayed fraction showing \textit{trans-}cinnamate and indole

The chromatogram was recorded at 280 nm with the assay sample at 0 min (the one in dash blue line) and after 30 min (black solid line). The assay sample contained 1 ml of 10 mM Tris HCl buffer (pH 7.8) with 100 moles of \textit{trans-}cinnamate, along with 0.1 ml of 10 % NH$_4$OH, 50 moles of hydroxylamine with 280 µg of cell free extract and incubated for 45 min at room temperature.
Fig 21: LC-MS metabolome profiling of the enzyme-assayed sample showing intermediates of trans-cinnamate to tryptophan transformation

Assay sample was lyophilized and extracted with methanol. The methanol extract was analyzed using LC-MS Shimadzu 2010. The mass spectrum ‘A’ taken in the negative mode shows the mass (m/z) 122 corresponding to phenyl acetaldehyde and ‘B’ taken in the positive mode shows masses (m/z) 115 and 163 corresponding to compounds indole and phenylpyruvate respectively.
3.4 Assimilation of hydroxycinnamates by *Rhodobacter sphaeroides* OU5

In addition to trans-cinnamate, its hydroxy derivatives like 4-hydroxycinnamate and 3, 4-dihydroxycinnamate were assimilated by *Rba. sphaeroides* OU5 (Table 1).

3.4.1 Assimilation of 4-hydroxycinnamate

Assimilation of 4-hydroxycinnamate (0.5 mM) was demonstrated with both resting and growing cells of *Rba. sphaeroides* OU5 under light anaerobic incubation (Table 15).

3.4.1.1 Effect of 4-hydroxycinnamate on growth of *Rba. sphaeroides* OU5

Effect of different concentrations of 4-hydroxycinnamate (1-8 mM) on photoheterotrophic growth of *Rba. sphaeroides* OU5 was studied. Growth of *Rba. sphaeroides* OU5 was inhibited completely at 8 mM. The 50 % inhibitory concentration (IC$_{50}$) of 4-hydroxycinnamate on photoheterotrophic growth of *Rba. sphaeroides* OU5 was around 3 mM (Fig 22).

3.4.1.2 Assimilation of 4-hydroxycinnamate at various concentrations

Assimilation of 4-hydroxycinnamate at various concentrations from 0-3 mM was studied using growing cells of *Rba. sphaeroides* OU5. Assimilation was optimum at 0.5 mM and observed upto 2.0 mM and of 4-hydroxycinnamate (Fig 23). The biomass of *Rba. sphaeroides* OU5 decreased beyond 2.5 mM of 4-hydroxycinnamate.

3.4.1.3 Effect of organic substrates on 4-hydroxycinnamate assimilation by *Rhodobacter sphaeroides* OU5

Assimilation of 4-hydroxycinnamate by *Rba. sphaeroides* OU5 in photoheterotrophic medium with malate as carbon source was low, hence influence of other organic substrates on 4-hydroxycinnamate assimilation was studied. Growth and consumption of 4-
4-hydroxycinnamate was observed with all the substrates tested. However, consumption of 4-hydroxycinnamate varied and was influenced by the carbon substrate used. Among the substrates tested (Table 16) glucose and pyruvate promoted maximum assimilation of 4-hydroxycinnamate followed by other substrates like fumarate, oxaloacetate, α-ketoglutarate and malate.

3.4.1.4 Assimilation of 4-hydroxycinnamate with time

Growth and 4-hydroxycinnamate assimilation at 0.5 mM was studied with time by growing cells of *Rba. sphaeroides* OU5. Assimilation started with a lag period of 12 h. 75 % of 4-hydroxycinnamate (0.5 mM) was assimilated by end of 48 h incubation and remained constant on further incubation (Fig 24). With resting cell suspensions, assimilation of 4-hydroxycinnamate followed a similar pattern with time while biomass yield remained constant (0.40 mg drywt.ml⁻¹) (data not shown).

3.3.1.5 Transformation of 4-hydroxycinnamate to L-tyrosine by cell free extracts of *Rhodobacter sphaeroides* OU5

Consumption of 4-hydroxycinnamate and formation of L-tyrosine was studied with cell free extract of the 4-hydroxycinnamate induced culture of *Rba. sphaeroides* OU5. Tyrosine ammonia lyase, TAL activity (involved in conversion of tyrosine to 4-hydroxycinnamate and vice versa) could not be demonstrated with 4-hydroxycinnamate induced culture of *Rba. sphaeroides* OU5. Amino donor is required for transformation of 4-hydroxycinnamate to tyrosine and among different amino donors tested, maximum consumption of 4-hydroxycinnamate and irreversible conversion to L-tyrosine was observed in the presence of ammonia and NADH in the assay mixture (Table 17). The fig 25 shows the HPLC chromatogram of enzyme assayed sample using cell free extracts of *Rba. sphaeroides* OU5.
Resting and growing cells of *Rba. sphaeroides* OU5 incubated for 48 h in presence of 4-hydroxycinnamate and 3, 4-dihydroxycinnamate (0.5 mM) as supplement and their consumption was estimated by HPLC.

Table 15: Assimilation of 4-hydroxycinnamate and 3, 4-dihydroxycinnamate by resting and growing cells of *Rba. sphaeroides* OU5

<table>
<thead>
<tr>
<th>Growth</th>
<th>Biomass yield (mg dry wt mL⁻¹)</th>
<th>4-hydroxycinnamate consumption (mM)</th>
<th>3, 4-dihydroxycinnamate consumption (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting cells</td>
<td>0.45</td>
<td>0.33</td>
<td>0.45</td>
</tr>
<tr>
<td>Growing cells</td>
<td>0.37</td>
<td>0.35</td>
<td>0.44</td>
</tr>
</tbody>
</table>
**Fig 22:** Minimum inhibitory concentration of 4-hydroxycinnamate on the photoheterotrophic growth of *Rba. sphaeroides* OU5

Assay was done with the growing cells of *Rba. sphaeroides* OU5 in photoheterotrophic medium with malate (22 mM) as sole carbon source and NH$_4$Cl (7 mM) as sole nitrogen source in the presence of various concentrations of 4-hydroxycinnamate. The culture was incubated anaerobically for 48 h under light (2,400 lux) at 30±2 °C in fully filled screw cap test tubes and growth was measured turbidometrically.
Fig 23: Assimilation at different concentrations of 4-hydroxycinnamate by growing cells of \textit{Rba. sphaeroides} OU5

Experiment was done with logarithmically grown cells of \textit{Rba. sphaeroides} OU5 inoculated into photoheterotrophic medium with glucose (17 mM) as carbon and ammonium chloride (7 mM) as nitrogen source supplemented with different concentrations of 4-hydroxycinnamate and assayed after 48 h incubation.
Table 16: Assimilation of 4-hydroxycinnamate by growing cells of *Rba. sphaeroides* OU5 in the presence of different organic substrates

Assay was done in triplicates with growing cells of *Rba. sphaeroides* OU5 after 48 h of phototrophic incubation (2,400 lux) at 30±2 °C, in the presence of respective organic substrates (0.3 % w/v) as carbon sources and NH₄Cl (7 mM) as nitrogen source in the medium supplemented with 4-hydroxycinnamate (0.5 mM). 4-Hydroxycinnamate levels in the supernatant were quantified using HPLC.
Fig 24: Assimilation and growth of 4-hydroxycinnamate by growing cells of *Rba. sphaeroides* OU5 with time

Experiment was done with logarithmically grown cells of *Rba. sphaeroides* OU5 inoculated into photoheterotrophic medium with glucose (17 mM) as carbon and ammonium chloride (7 mM) as nitrogen source supplemented with 4-hydroxycinnamate (0.5 mM). After 48 h phototrophic incubation samples were collected at specific intervals of time and estimated for 4-hydroxycinnamate consumption.
Table 17: Transformation of 4-hydroxycinnamate by cell free extracts of *Rba. sphaeroides* OU5

<table>
<thead>
<tr>
<th>Assay components</th>
<th>4-hydroxycinnamate consumption (μmol.mg protein⁻¹)</th>
<th>L-tyrosine formation (μmol.mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without Ammonia, NADH)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ammonia + NADH</td>
<td>700</td>
<td>100</td>
</tr>
</tbody>
</table>

The assay mixture contained 1ml of 10 mM Tris HCl buffer (pH 7.8) with 100 μmol of 4-hydroxycinnamate, along with 25 μmol of NADH, 0.1ml of 10 % NH₄OH with cell free extract (300 μg protein) and incubated for 30 min at room temperature. After incubation the consumption of 4-hydroxycinnamate and L-tyrosine formation was analyzed in HPLC. The source of cell free extract was 4-hydroxycinnamate induced culture of *Rba. sphaeroides* OU5.
Fig 25: HPLC chromatogram showing the transformation of 4-hydroxycinnamate by cell free extracts of *Rba. sphaeroides OU5*

The assay was done with the cell free extract of *Rba. sphaeroides OU5*. The consumption of 4-hydroxycinnamate ($t_R = 2.4$ min) and formation of tyrosine ($t_R = 3.3$ min) was detected in HPLC, by injecting respective standards. The chromatograms were registered at 220 nm with the assay sample at 0 min (chromatogram in green colour) and after 30 min (chromatogram in red colour).
3.4.2 Assimilation of 3, 4-dihydroxycinnamate by *Rhodobacter sphaeroides* OU5

Assimilation of 3, 4-dihydroxycinnamate was demonstrated with both resting and growing cells of *Rba. sphaeroides* OU5 under light anaerobic incubation (Table 15 in section 3.4.1).

3.4.2.1 Assimilation at various concentrations of 3, 4-dihydroxycinnamate

3, 4-Dihydroxycinnamate assimilation was studied at various concentrations, using growing cells of *Rba. sphaeroides* OU5. Assimilation of 3, 4-dihydroxy cinnamate was observed up to a concentration of 2.5 mM (Fig 26). Both assimilation of 3, 4-dihydroxy cinnamate and biomass of *Rba. sphaeroides* OU5 decreased beyond 2.5 mM of 3, 4-dihydroxycinnamate.

3.4.2.2 Effect of organic substrates on 3, 4-dihydroxycinnamate assimilation

Assimilation of 3, 4-dihydroxycinnamate by *Rhodobacter sphaeroides* OU5 was studied with various organic substrates. Among the organic substrates tested, all substrates promoted assimilation of 3, 4-dihydroxycinnamate and maximum was with malate (Table 18). However, with malate and pyruvate as carbon substrates, 3, 4-dihydroxyphenylalanine (DOPA) was detected in the induced (3, 4-dihydroxycinnamate) culture supernatant of *Rba. sphaeroides* OU5. DOPA production could not be demonstrated in presence of other organic substrates.

3.4.2.3 3, 4-Dihydroxycinnamate assimilation and DOPA production with time

Assimilation of 3, 4-Dihydroxycinnamate by *Rhodobacter sphaeroides* OU5 was studied with malate as carbon source and ammonium chloride as nitrogen source. Consumption started with a lag period of 12 h and was completed by the end of 48 h of incubation (Fig 27). However 3, 4-dihydroxyphenylalanine (DOPA) production started after 24 hrs of incubation (Fig 27). HPLC chromatogram of the induced 3, 4-dihydroxycinnamate culture supernatant showed the consumption of malate, 3, 4-dihydroxycinnamate and production of
3, 4-dihydroxyphenylalanine (Fig 28). Assimilation of 3, 4-dihydroxycinnamate was also demonstrated with resting cell suspensions of *Rhodobacter sphaeroides* OU5 but DOPA production could not be observed.

**Fig 26: Assimilation at different concentrations of 3, 4-dihydroxycinnamate by *Rba. sphaeroides* OU5**

Experiment was done with logarithmically grown cells of *Rba. sphaeroides* OU5 inoculated into photoheterotrophic medium containing malate (22 mM) as carbon, ammonium chloride (7 mM) as nitrogen source supplemented with different concentrations of 3, 4-dihydroxycinnamate and assayed after 48 h light anaerobic incubation.
Table 18: Assimilation of 3, 4-dihydroxycinnamate by growing cells of *Rba. sphaeroides* OU5 in the presence of different organic substrates  
(ND = Not detected; control = without 3, 4-dihydroxycinnamate)

Growing cells of *Rhodobacter sphaeroides* OU5 in the presence of different organic substrates (0.3 % w/v) as carbon and NH$_4$Cl (7 mM) as nitrogen source and 3, 4-dihydroxycinnamate (0.5 mM) as supplement were assayed after 48 h of phototrophic incubation. The levels of 3, 4-dihydroxycinnamate and 3, 4-dihydroxyphenylalanine (DOPA) were estimated by HPLC.
Fig 27: Photoassimilation of 3, 4-dihydroxycinnamate and production of DOPA by growing cells of *Rba. sphaeroides* OU5 with time

(DOPA = 3, 4-dihydroxyphenylalanine)

Experiment was done with logarithmically grown cells of *Rba. sphaeroides* OU5 inoculated into photoheterotrophic medium containing malate (22 mM) as carbon, ammonium chloride (7 mM) as nitrogen source and 3, 4-dihydroxycinnamate (0.5 mM) as supplement and was assayed at different time intervals.
Fig 28: HPLC chromatogram showing the transformation of 3, 4-dihydroxycinnamate to 3, 4-dihydroxyphenylalanine by growing cells of *Rba. sphaeroides* OU5

Culture supernatant of zero hour sample (chromatogram in green color) and 48 h light incubated sample (chromatogram in red color) were membrane filtered and analyzed using HPLC. The levels of 3, 4-dihydroxycinnamate and DOPA were estimated.
3.5 Assimilation of 4-hydroxybenzoate by *Rhodobacter sphaeroides* OU5

*Rba. sphaeroides* OU5 was tested for its capability to assimilate different benzoates like 2-hydroxybenzoate, 3, 4-dihydroxybenzoate, 4-hydroxybenzoate, 2-aminobenzoate and 4-aminobenzoate. Among them, only 4-hydroxybenzoate was assimilated by *Rba. sphaeroides* OU5 (Table 3 in chapter 1). Hence, its assimilation was studied by both growing and resting cells of *Rba. sphaeroides* OU5. 4-Hydroxybenzoate assimilation was demonstrated only with growing cells but not with resting cells of *Rba. sphaeroides* OU5.

3.5.1 Effect of 4-hydroxybenzoate on growth of *Rba. sphaeroides* OU5

Effect of different concentrations of 4-hydroxybenzoate (2-20 mM) on photoheterotrophic growth of *Rba. sphaeroides* OU5 was studied. Growth of *Rba. sphaeroides* OU5 was completely inhibited at 20 mM. The 50 % inhibitory concentration (IC$_{50}$) of trans-cinnamate on photoheterotrophic growth of *Rba. sphaeroides* OU5 was approximately 10 mM (Fig 29).

3.5.2 Assimilation at different concentrations of 4-hydroxybenzoate

Assimilation of 4-hydroxybenzoate was studied at different concentrations (0.5-2.5 mM) using growing cells of *Rba. sphaeroides* OU5 with fumarate as carbon source, ammonium chloride as nitrogen source and 4-hydroxybenzoate as a supplement. Assimilation was observed upto 1.5 mM and the biomass of *Rba. sphaeroides* OU5 remained constant irrespective of the concentration of 4-hydroxybenzoate used (Fig 30).

3.5.3 Effect of organic substrates on 4-hydroxybenzoate assimilation by growing cells of *Rhodobacter sphaeroides* OU5

Influence of organic substrates on 4-hydroxybenzoate assimilation was studied. Assimilation of 4-hydroxybenzoate by *Rba. sphaeroides* OU5 in photoheterotrophic
medium with either glucose or malate as carbon source/electron donor could not be demonstrated hence other organic substrates were screened (Table 19). Among the substrates tested, assimilation was observed with fumarate and pyruvate as carbon sources (Table 19) however, acetate, malate, succinate and glucose inhibited 4-hydroxybenzoate assimilation.

3.5.4 Assimilation of 4-hydroxybenzoate with time by growing cells of *Rhodobacter sphaeroides* OU5

Assimilation of 4-hydroxybenzoate by *Rba. sphaeroides* OU5 was studied with fumarate as sole carbon source. In the time course experiment (Fig 31) 50 % of 4-hydroxybenzoate (0.25 mM) was assimilated at 30 h of incubation and on further incubation remained constant upto 48 h. This assimilation in HPLC (Fig 32) was observed during logarithmic growth phase of *Rba. sphaeroides* OU5.

3.5.5 4-Hydroxybenzoate consumption by cell free extracts of *Rhodobacter sphaeroides* OU5

Consumption of 4-Hydroxybenzoate was studied using cell free extracts of 4-hydroxybenzoate induced culture of *Rba. sphaeroides* OU5. As observed with whole cells, 4-hydroxybenzoate consumption could not be demonstrated when fumarate was added to the assay mixture (in Tris buffer), but was observed with pyruvate. For unknown reasons addition of ammonia both with and without NADH had increased the consumption of 4-hydroxybenzoate (Table 20). However, the transformed metabolites could not be identified. The Fig 33 shows the HPLC chromatogram of the enzyme assay fraction showing 4-hydroxybenzoate consumption by cell free extracts of *Rhodobacter sphaeroides* OU5.
Assay was done with the growing cells of *Rba. sphaeroides* OU5 in photoheterotrophic medium with malate (22 mM) as sole carbon source and NH\(_4\)Cl (7 mM) as sole nitrogen source in the presence of various concentrations of 4-hydroxybenzoate. The culture was incubated anaerobically under light (2,400 lux) at 30±2 °C in fully filled screw cap test tubes and growth was measured turbidometrically.
Fig 30: Assimilation at different concentrations of 4-hydroxybenzoate by growing cells of *Rba. sphaeroides* OU5

Experiment was done with logarithmically grown cells of *Rba. sphaeroides* OU5 inoculated into photoheterotrophic medium with fumarate (22 mM) as carbon and ammonium chloride (7 mM) as nitrogen source with different concentrations of 4-hydroxybenzoate (0-0.25 mM) and assayed for its consumption.
Cells of *Rhodobacter sphaeroides* OU5 were allowed to grow phototrophically for 48 h in mineral medium in the presence of organic substrates (0.3 % w/v) supplemented with 4-hydroxybenzoate (0.5 mM) and assayed after incubation. The level of 4-hydroxy benzoate was estimated using HPLC.

### Table 19: Assimilation of 4-hydroxybenzoate by growing cells of *Rba. sphaeroides* OU5 in the presence of different organic substrates

(Control = without 4-hydroxybenzoate; ND = not detected)
Fig 31: Photoassimilation of 4-hydroxybenzoate by growing cells of *Rba. sphaeroides* OU5 with time

Experiment was done with logarithmically grown cells of *Rba. sphaeroides* OU5 inoculated into photoheterotrophic medium with fumarate (22 mM) as carbon and ammonium chloride (7 mM) as nitrogen source along with 4-hydroxybenzoate (0.5 mM) as supplement and was assayed at different time intervals.
Fig 32: HPLC chromatogram showing 4-hydroxybenzoate assimilation by whole cells of *Rba. sphaeroides* OU5

HPLC chromatogram (at 220 nm wavelength) showing 4-hydroxybenzoate assimilation by growing cells of *Rba. sphaeroides* OU5. Initial (chromatogram in blue colour) and final levels (chromatogram in dark green) of 4-hydroxybenzoate in supernatant were quantified using HPLC.
Table 20: 4-Hydroxybenzoate consumption by cell free extracts of *Rba. sphaeroides* OU5 (ND = not detected)

<table>
<thead>
<tr>
<th>Assay components</th>
<th>4-hydroxybenzoate consumption (μmoles.mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>ND</td>
</tr>
<tr>
<td>Fumarate</td>
<td>ND</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>100</td>
</tr>
<tr>
<td>Pyruvate + Ammonia</td>
<td>50</td>
</tr>
<tr>
<td>Pyruvate + Ammonia + NADH</td>
<td>70</td>
</tr>
</tbody>
</table>

The assay mixture (1ml) contained 10 mM Tris-buffer (pH 7.8) with 100 moles of 4-hydroxybenzoate, along with the assay components like 25 moles of NADH, 0.1ml of 10% NH₄OH (buffered ammonia), pyruvate 200 moles and 375 µg of protein (cell free extract) incubated for 30 min at room temperature. After incubation the consumption of 4-hydroxybenzoate was estimated using HPLC.
The assay was done with the cell free extract of *Rba. sphaeroides* OU5. The consumption of 4-hydroxybenzoate ($t_R = 2.8$ min) was detected in HPLC. The chromatograms were recorded at 220 nm with the assay sample at 0 min (the one in green colour) and at 30 min (in red colour).