Photoassimilation of aromatic compounds by *Rhodobacter sphaeroides* OU5

P. USHA KIRAN

Department of Plant Sciences

University of Hyderabad

Hyderabad 500 046

India

June 2009

Photoassimilation of aromatic compounds by *Rhodobacter sphaeroides* OU5

Thesis submitted for the award of

DOCTOR OF PHILOSOPHY

by

P. Usha Kiran

(Regd. No. 03LPPH06)

Department of Plant Sciences

University of Hyderabad

Hyderabad 500 046

India

June 2009

University of Hyderabad
(A Central University established in 1974 by act of parliament)
Hyderabad – 500046, INDIA

CERTIFICATE

This is to certify that Ms P.Usha Kiran has carried out the research work embodied in the present thesis under supervision and guidance of Dr. Ch. Venkata Ramana for a full period prescribed under the Ph.D ordinance of this University. We recommend her thesis **"Photoassimilation of aromatic compounds by *Rhodobacter sphaeroides* OU5"** for submission for the degree of Doctor of Philosophy of the University.

Dr. Ch. Venkata Ramana
(Research Supervisor)

Head,
Department of Plant Sciences

Dean,
School of Life Sciences

University of Hyderabad
(A Central University established in 1974 by act of parliament)
Hyderabad – 500 046, INDIA

DECLARATION

I here by declare that the work embodied in this thesis entitled **"Photoassimilation of aromatic compounds by *Rhodobacter sphaeroides* OU5"** has been carried out by me under the supervision of Dr. Ch. Venkata Ramana and this has not been submitted to any other Institute or University for the award of any degree or diploma.

P.Usha Kiran

Dr. Ch. Venkata Ramana
(Research Supervisor)

Acknowledgements

I thank my supervisor Dr. Ch. Venkata Ramana for his constant encouragement, support and for his diligent guidance throughout my doctoral research.

I thank the present and former Heads of the Department of Plant Sciences, Prof. A.R. Reddy, Prof. Appa Rao Podile, Prof. P. B. Kirti and Prof. M. N. V. Prasad; the present and former Deans of School of Life Sciences, Prof. A. S. Raghavendra and Prof. T. Suryanarayana for allowing me to use the facilities of the department and the school.

I thank Dr. Ch. Sasikala, JNTU for her guidance and also for extending her lab facilities.

I thank Prof. Appa Rao Podile and Prof. S. Dayananda for their help and cooperation.

I thank Prof. Basavaiah, School of Chemistry, for extending IR and LC-MS facilities.

I thank all the research scholars of the School of Life Sciences for their cooperation and help. The cooperation of the non-teaching staff is greatly acknowledged.

I sincerely acknowledge the infrastructural support provided by UGC-SAP and DST-FIST to the Dept. of Plant Sciences and the CSIR, Govt of India for the research fellowship (JRF/SRF) *in the project titled "Production of novel indole esters from 2-amino benzoate by Rhodobacter sphaeroides OU5 and evaluation of their biological activity" sanction No. …38[1085]\04\EMR- ll Dated 23-03-05.*

I wish to thank my friends Dr. Sripriya, Dr. Madhuri, Dr. Tripura, Anil, Vasu, Neeraja and Madhumita for their suggestions and help.

My special thanks to Dr. Sunayana and Rajini for their endless support and help.

I thank all my labmates Ranjith, Shobha, Aravind, Mujahid, Chakravarthy, Laxmi Prasuna, Prasad and Srinivas for their cooperation. I wish to thank my friends at JNTU, Dr. Archana, Dr. Aruna, Aparna and others for help.

My heartfelt thanks to my parents-in-law and other family members for their encouragement, help and support.

I express my gratitude to my parents, husband, sisters and brother-in-law for their unwavering love, endless patience, unconditional support and understanding. Without their blessings and best wishes I would not have been successful in completing this research work.

Finally I thank my daughter Vaishnavi for being there for me.

Usha kiran

Dedicated
to
my Parents &
Husband

LIST OF ABBREVIATIONS

ATCC	: American type culture collection
ATP	: Adenosine triphosphate
DEAE	: Diethyl aminoethyl
DOPA	: 3, 4-dihydroxyphenylalanine
ESI	: Electro spray ionization
HCL	: Hydrochloric acid
HPLC	: High pressure liquid chromatography
LC-MS	: Liquid chromatography mass spectroscopy
mmol	: Milli moles
mM	: Milli molar
NADH	: (Reduced) nicotinamide adenine dinucleotide
NADPH	: (Reduced) nicotinamide adenine dinucleotide phosphate
PAGE	: Polyacryl amide gel electrophoresis
PAL	: Phenylalanine ammonia lyase
PDA	: Photodiode array
PDAB	: Para dimethyl amino benzaldehyde
PLP	: Pyridoxal phosphate
SDS	: Sodium dodecylsulfate
TAL	: Tyrosine ammonia lyase
TFA	: Trifluro acetic acid
TPP	: Thiamine pyrophosphate
t_R	: Time of retention
UV	: Ultra violet
µg	: Micro gram

CONTENTS

Abstract

Purple bacteria metabolize aromatic compounds either by light dependent degradation of benzene ring, supporting growth by providing carbon source/electron donors or through transformations when they cannot cleave the benzene ring. Rhodobacter sphaeroides OU5, a purple non-sulfur bacterium lacks the capability to degrade aromatic compounds but has the ability to transform some of the aromatic compounds. The present thesis work extends an in-depth study on the metabolic potential of Rhodobacter sphaeroides OU5 in utilization of few aromatic compounds, though not as growth substrates.

Utilization of aromatic compounds as sole source of carbon or as electron donors by Rba. sphaeroides OU5 could not be demonstrated. However, assimilation of trans-cinnamate, 4-hydroxycinnamate, 3, 4-dihydroxycinnamate and 4-hydroxybenzoate was observed in Rba. sphaeroides OU5.

Inhibition in growth of Rba. sphaeroides OU5 and on the activity of 3-deoxy D-arabino-heptulosanate 7-phosphate (DAHP) synthase, (key enzyme in the shikimate pathway that leads to the biosynthesis of aromatic amino acids) in presence of aromatic compounds like trans-cinnamate and hydroxycinnamate was demonstrated.

trans-Cinnamate assimilation was light dependent and was observed by both growing and resting cells of Rba. sphaeroides OU5. Studies with whole cells and cell free extracts of Rba. sphaeroides OU5

indicate the transformation of trans-cinnamate to L-phenylalanine and L-tryptophan with intermediate phenylpyruvate. Involvement of the enzyme phenylalanine ammonia lyase (PAL) in the conversion of trans-cinnamate to L-phenylalanine could not be demonstrated in Rba. sphaeroides OU5. A ~ 43kD protein catalyzing this irreversible reaction was purified to electrophoretic homogeneity and was characterized from trans-cinnamate induced culture of Rba. sphaeroides OU5.

In addition to trans-cinnamate, Rba. sphaeroides OU5 assimilated hydroxycinnamates like 4-hydroxycinnamate, 3, 4-dihydroxycinnamate which are transformed to L-tyrosine and 3, 4-dihydroxyphenylalanine (DOPA), respectively. Among benzoates tested, 4-hydroxybenzoate was assimilated by Rba. sphaeroides OU5.

In conclusion, a novel metabolism of aromatic compound assimilation, with few biochemical modifications to an aromatic amino acid was suggested in Rba. sphaeroides OU5.

Introduction

General Introduction

1.1 Aromatic compounds and their release into the environment

Aromatic compounds are abundant in the biosphere as naturally occurring and man-made environmental pollutants. Pollutants include various kinds of pesticides, herbicides, insecticides, detergents and halogenated aromatic compounds, many of which are toxic, recalcitrant and accumulate in sediments. These compounds are mostly phenolic derivatives of a general phenylpropane or benzyl structure (Sleat and Robinson, 1984; Berry *et al.*, 1987). Naturally occurring aromatic compounds are products of secondary metabolism of plants (Gross, 1985), derived from bioconversion of aromatic amino acids (Balba and Evans, 1980) and as by-products of biological and chemical cleavage of lignin (Higuchi, 1985; Kirk and Farrell, 1987).

Lignin is the second most abundant carbon compound on the Earth and is an aromatic polymer of phenyl propanoid units connected by different C-C and C-O-C linkages, resulting in a complex structure. It contains guaiacyl, syringyl, *p*-hydroxyphenyl and biphenyl moieties. Many filamentous fungi degrade plant lignin by employing non-specific and extracellular enzymes. Fungal oxidases and peroxidases play a vital role in lignin degradation that releases several simple aromatic monomers (Orth *et al*., 1991). These monomers are mostly benzoates and phenylpropanoids like cinnamates, hydroxycinnamates, ferulate and sinapate suitable for further microbial mineralization.

1.2 Bacterial utilization of aromatic compounds

Aromatic compounds being abundant in nature, serve as important growth substrates for many microorganisms. By expressing different catabolic (biodegradative)

pathways, bacteria use a wide array of aromatic compounds as sole carbon, energy (Haryama and Timmis, 1992), electron (Lovley *et al.*, 1999) and as nitrogen sources (Esteve-Nunez *et al*., 2001). Aromatic compounds participate in energy metabolism by serving as electron acceptors as such (Mouttaki *et al*., 2008) or with modifications as aromatic ring substituents [the use of chlorinated aromatic compounds as electron acceptors in dehalorespiration] (Fetzner, 1998). Aromatic compounds like humic substances also serve as electron shuttles and are reduced by a number of facultative anaerobic bacteria such as *Paracoccus denitrificans* under anaerobic conditions (Lovley *et al.*, 1999). Reductive dehalogenation of aromatic and aliphatic compounds of haloaryl and haloalkyls serve as electron acceptors for the process of dehalorespiration in bacteria (Holliger *et al*., 1999). Schematic representation of the utilization of aromatic compounds by bacteria is shown in Fig 1.

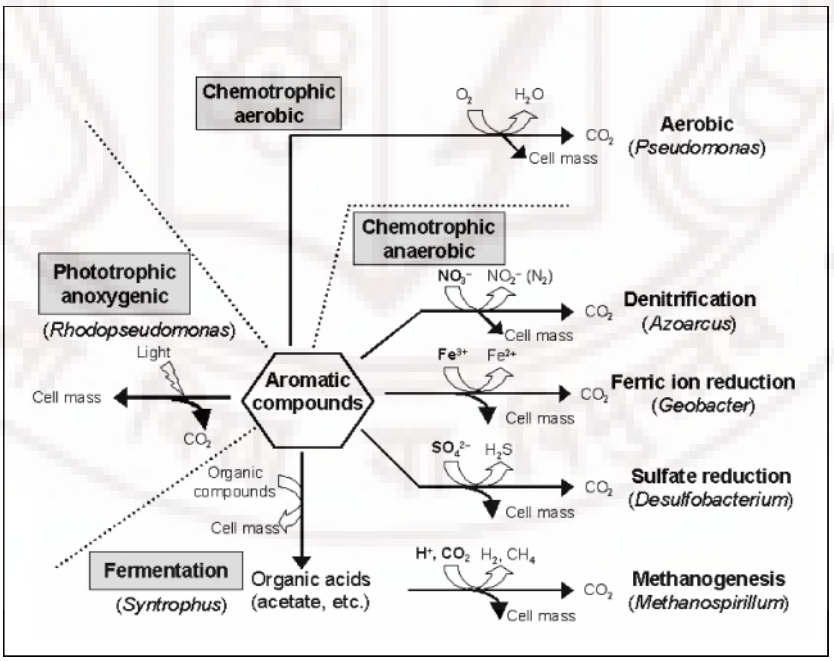


Fig 1: Bacterial utilization of aromatic compounds (Diaz, 2004)

1.3 **Degradation of aromatic compounds**

In the microbial catabolism of aromatic compounds, degradation of the benzene ring is an important step. The biochemistry of aromatic compound degradation can be aerobic, anaerobic or chimeric based on the use of oxygen.

1.3.1 **Aerobic degradation of aromatic compounds**

Aerobic degradation of aromatic compounds involves oxygen as terminal electron acceptor and for hydroxylation of the benzene ring. Oxygenases add hydroxyl substituents to the aromatic nucleus thus destabilizing the benzene ring for further degradation (Harayama *et al*., 1992). Benzene nucleus of the aromatic metabolite is cleaved by the incorporation of both atoms of oxygen into the substrate under the influence of dioxygenase enzyme, which catalyze the cleavage of the hydroxylated benzene ring to aliphatic and more assimilable compounds (*van der* Meer *et al*., 1992; Harayama and Timmis, 1992).

The oxygenase dependent biodegradation of aromatic compounds is channeled through the central aromatic metabolic intermediates like catechol, protocatechuate or gentisate. These intermediate compounds are the substrates of ring cleavage enzymes that use molecular oxygen to open the aromatic ring between two hydroxyl groups (ortho cleavage, catalyzed by intradiol dioxygenases) or proximal to one of the two hydroxyl groups (meta cleavage, catalyzed by extradiol dioxygenases) (Harayama and Timmis, 1992) (Fig 2A). *Pseudomonas* species and closely related organisms have been the most extensively studied owing to their metabolic versatality to aerobically degrade different aromatic compounds (Wackett, 2003).

1.3.2 **Anaerobic degradation of aromatic compounds**

Evans, was the first to establish that bacteria even under anoxic conditions metabolize aromatic compounds and attack the aromatic ring by reductive steps (reviewed in Evans, 1977; Evans and Fuchs, 1988). Anaerobic attack on the extremely stable structure of the benzene ring proceeds by a ring reduction mechanism, a biochemical process that is radically different from the oxygen requiring strategies (Harayama and Neidle, 1992). Oxygen was shown to influence the expression of anaerobic catabolic clusters (Carmona *et al*., 2009). In anaerobic environments, bacteria degrade aromatic compounds using alternative electron acceptors, such as nitrate (denitrifying bacteria), sulfate (sulfate reducers), ferric ions (ferric ion reducers), CO_2 (methanogens), other (chlorate, Mn, Cr etc) acceptors (Gibson and Harwood, 2002). In anaerobic pathways, monoaromatic compounds such as phenolic compounds, methylbenzenes and amino benzenes are metabolized via aromatic acids such as nitrobenzoates, hydroxybenzoates, or phenylacetate and then transformed to benzoate (Fuchs *et al*., 1994). One important but common step of these pathways is the addition of a coenzyme A (CoA) moiety to the carboxylic group of the aromatic acids by specific CoA ligases (Harwood *et al*., 1999). Key intermediates in the anaerobic aromatic metabolism are benzoyl-CoA and compounds with at least two meta-positioned hydroxyl groups like resorcinol, phloroglucinol and hydroxyhydroquinone. Benzoyl-CoA, an esterified compound is the major metabolic intermediate in almost all of the pathways proposed (Harwood and Gibson, 1997; Villemur, 1995). Benzoyl-CoA is further metabolized by benzoyl-CoA reductase, a key enzyme of anaerobic benzoate

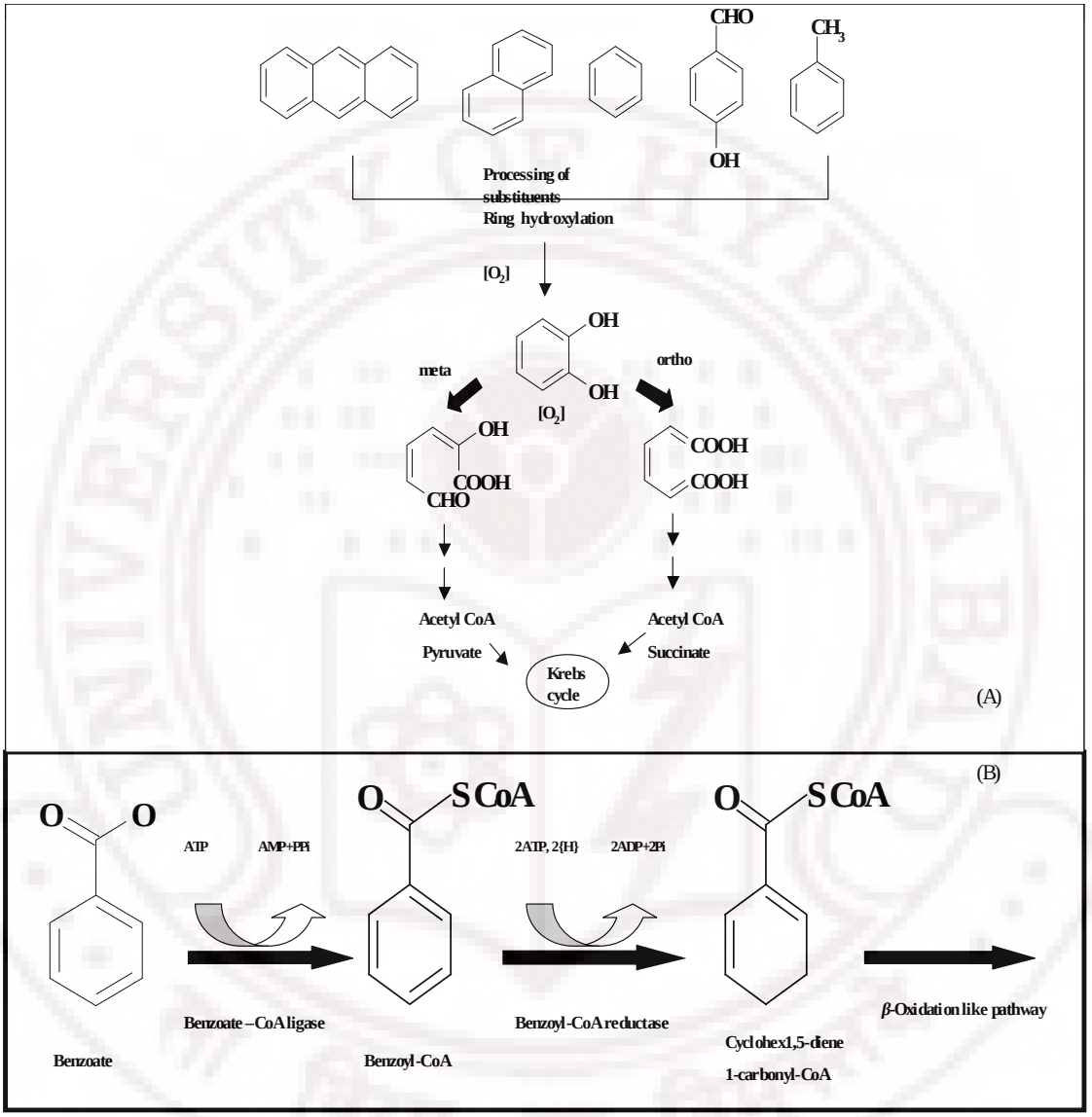


Fig 2: (A) The catabolic funnel for the aerobic degradation of aromatic compounds

(B) Anaerobic degradation of benzoate (Diaz, 2004)

degradation, which dearomatizes benzoyl-CoA via an ATP driven 2-electron reduction, yielding cyclohex-1, 5-diene-1-carbonyl-CoA. Further degradation involves hydrolytic ring cleavage, followed by conventional β-oxidation to acetyl-CoA (Fig 2B).

Pathways of anaerobic degradation of monoaromatic compounds have been mostly characterized in denitrifying bacteria *Thaeura aromatica*, *Azoarcus evansii* and in purple non-sulfur phototrophic bacterium *Rhodopseudomonas palustris* and also by some anaerobic microorganisms (Elder and Kelly, 1994; Londry and Fedorak, 1992). Another most studied anaerobic degradative mechanism in denitrifying bacteria is the addition of fumarate to hydrocarbons. This unique reaction catalyzing the initial step of anaerobic toluene metabolism is the addition of cosubstrate fumarate to the methyl group of toluene to yield the first intermediate benzylsuccinate, catalyzed by the enzyme benzyl succinate synthase (Leuthner *et al*., 1998; Krieger *et al*., 2001) and encoded by genes *bss*DCABE. This enzyme was well studied in toluene degrading sulfate reducers (Beller and Spomann, 1997), phototrophic bacteria (Zengler *et al*., 1999) and methanogenic consortia (Beller and Edwards, 2000).

1.3.3 **Chimeric metabolism of aromatic compounds**

Combining the features of both aerobic and anaerobic pathways, there exists chimeric pathway, as reported in *Pseudomonas* sp. (Altenschmidt and Fuchs, 1992). Similar to anaerobic degradation this pathway proceeds via CoA thioester formation and the aromatic ring reduction is coupled with simultaneous oxygen dependent hydroxylation of the benzene ring. This chimeric pathway was also observed in the degradation of phenylacetic acid, where combinations of anaerobic features (initial

formation of CoA derivatives) with typical aerobic reactions such as aromatic ring oxygenation (Fig 3) (Luengo *et al*., 2001) were reported.

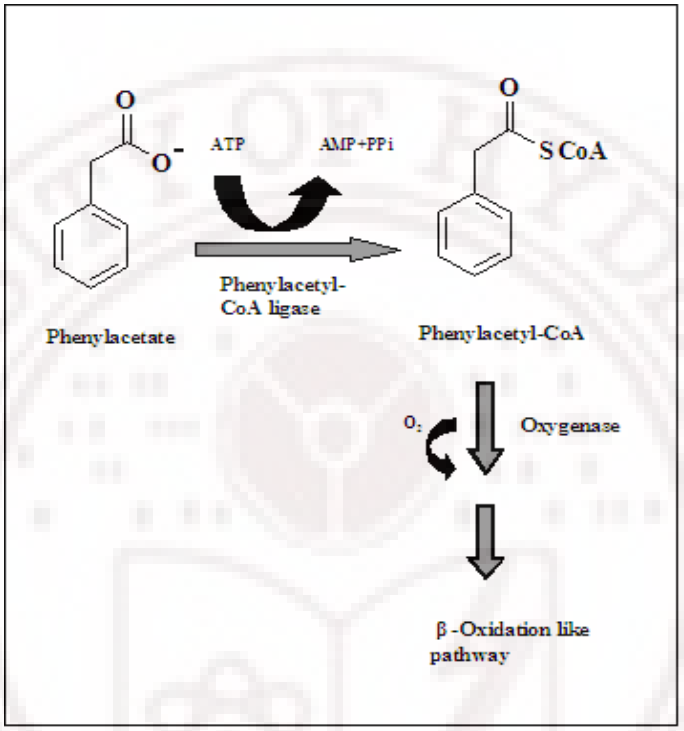


Fig 3: Chimeric pathway of phenylacetate catabolism (Diaz, 2004)

1.4 **Bacterial attacks on aromatic ring substituents**

Some microorganisms utilize aromatic ring substituents to their advantage while leaving the benzene ring untouched (Fig 4). The substituents attached to more complex aromatics may serve as carbon and energy, or even as nitrogen sources, for microorganisms that are unable to attack the benzene ring. Such modifications include:

(a) **Acyl side chain removal**: The side chains of phenylalkanoates, such as cinnamate are degraded by *β*-oxidation, yielding acetyl groups for biosynthesis or for energy e.g., *Rhodopseudomonas palustris* (Elder *et al*., 1992a).

(b) **Aromatic ester hydrolysis**: Bacteria hydrolyze aromatic esters and the ester group was further utilized. The mammalian urine constituent hippurate (benzoylglycine) was metabolized anaerobically by photosynthetic bacteria like *Rhodobacter capsulatus,* which uses glycine moiety after hydrolysis of benzoylglycine by the enzyme hippuricase (Madigan *et al*., 2001).

(c) **Demethylation**: Methoxylated aromatic molecules are major components of lignins. A number of acetogenic and other bacteria use the methyl group of phenyl methyl ethers for synthesis of acetic acid. The $-CH_3$ moiety was removed by reactions involving corrinoid and tetrahydrofolate cofactors, but the phenolic derivative was not used (Kreft and Schink, 1993). Some of the gram-positive bacteria like *Acetobacterium, Clostridium* and *Sporomusa* sp. use *o*-methyl groups as growth substrate. In addition, a thermophilic bacterium like *Desulfotomaculum thermobenzoicum*, utilize various methoxylated benzoate derivatives for growth by oxidizing the methoxy groups to CO_2 in the presence of sulfate as electron acceptor (Tasaki *et al*., 1992).

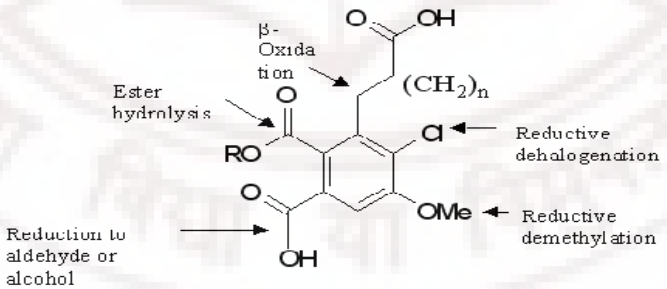


Fig 4: Commonly encountered attacks on aromatic ring substituents (Gibson and Harwood, 2002)

(d) **Aromatic compounds as sulphur and nitrogen sources**: Sulfonate moiety from the organosulfates was used as a sulfur source for growth by members of the genus *Clostridium* (Denger *et al*., 1996). Nitrogen containing substituents of aromatic compounds may also be utilized as nitrogen source by anaerobic bacteria. This had been observed for trinitrotoluene, which serves as nitrogen source for a *Desulfovibrio* sp. after amino reduction to triaminotoluene and further subjected to reductive deamination (Boopathy and Kulpa, 1992; Esteve-Nunez *et al.*, 2001).

1.5 **Bacterial transformation of aromatic compounds**

Many anaerobic bacteria cannot degrade benzene ring nor utilize the ring substituents of aromatic compounds, but carry out oxygen independent biotransformations. Biotransformation is a metabolic conversion process, which alters the structure of an original molecule. Such biotransformations by anaerobic bacteria involve specific substitutions on the aromatic ring through different strategies like (a) Oxidation (b) reductive transformation and (c) decarboxylation.

(i) Oxidation reactions: are involved in the conversion of aromatic amino acids to aryl acetates via aryl pyruvate. This type of biotransformation was reported in *Clostridium sticklandii* and *Clostridium bifermentans* (Baker, 1981). Aromatic amino acids with aliphatic side chains of three or more carbon atoms are oxidized to benzoyl CoA and phenyl acetyl-CoA, by *β*-oxidation in fatty acid degrading organisms (Elder and Kelly, 1994).

(ii) Reductive transformations: The purpose of reductive anaerobic transformations is to dispose off redox equivalents generated in oxidative reactions. These reactions are either involved in anaerobic respiratory chains or are part of fermentative pathways.

Reductive dehalogenation of halo aromatics has been observed in pure cultures of thiosulfate and sulfite- reducing bacteria eg: *Desulfomonile tiedjei* and several species of the genus *Desulfitobacterium* (Mohn and Tiedje, 1992). Acetogenic bacteria such as *Acetobacterium woodii* and *Peptostreptococcus productus*, convert aryl acrylates like ferurate, caffeate stoichiometrically to reduced compounds like hydroferulate and hydrocaffeate (Misoph *et al.,* 1996; Tschech and Pfennig 1984). *Clostridium sporogenesis* was able to transform all three aromatic amino acids to the corresponding aryl propionate derivatives (Baker, 1981). Nitro and azo groups are reduced to amino groups under anaerobic conditions (Sikkema and de Bont, 1994) by anaerobic and facultative bacteria like *Clostridium*, *Eubacterium* and *Veillonella* species. *Pseudomonas vesicularis* also transforms azo groups to amino groups (Haug *et al*., 1991).

(iii) **Decarboxylation:** Decarboxylation reactions of phenylacetate to toluene and hydroxyphenylacetate to *p*-cresol were catalyzed by amino acid fermenting gram-positive bacteria like C*lostridium* sp. (Barker 1981; Ward *et al.,* 1987). 4-Hydroxybenzoate to phenol and 3, 4-dihydroxybenzoate to catechol are reported transformations from *Clostridium hydroxybenzoicum* (Zhang and Wiegel, 1994). Some of the *Enterobacteriaceae* species and *Streptococcus gallolyticus* were capable of decarboxylating gallic acid to pyrogallol (Osawa *et al*., 1995). The physiological significance of these decarboxylations is to prevent acid accumulation in the medium and might probably to conserve energy (as membrane potential) from the reaction.

1.6 **Genes involved in aromatic ring catabolism**

Various studies on genes involved in aromatic ring degradation were shown to be clustered together (Brinkrolf *et al*., 2006; Dal *et al*., 2005). Regulation of these metabolic gene clusters is still under investigation. It was estimated that these regulatory mechanisms and their targets evolved independently and restricted to those bacteria, which are actively involved in catabolic pathways (Cases and de Lorenzo, 2001; de Lorenzo and Pe´rez-Martin, 1996). These regulators are divided into different families based on their target genes (Tropel and *van der* Meer, 2004).

Recent advances in bacterial genomics resulted in building the metabolic models integrating genomic data with available experimental data. Such kind of studies, known as, "metabolic reconstruction" were carried out in many bacteria for which genome information is available (Thiele *et al*., 2005; Feist *et al*., 2007; Oh *et al*., 2007). Metabolic reconstructions led to identification of all possible main ring-cleavage catabolic pathways for aromatic compounds in *Cupriavidus necator* JMP134 (Pérez-Pantoja *et al*., 2008). These reconstructions may help in deciphering the regulation of those enzymes that are involved in catabolism thus may help in genetic manipulation of catabolic pathways.

1.7 **Assimilatory metabolism**

Assimilatory processes include the various biochemical reactions that incorporate elements into biomass. By definition, assimilation includes primary and secondary production of organic matter and the formation of associated inorganic structural components. Since assimilation requires a source of energy to acquire, accumulate and metabolize nutrients, the availability of biochemically useful energy is a

major structuring agent for interactions between assimilatory processes and biogeochemical dynamics.

Inorganic compounds such as NO_3^-, SO_4^{2-}, and CO_2 are reduced by many organisms as sources of cellular nitrogen, sulfur and carbon, respectively. When an inorganic compound is reduced for use as a nutrient source, it is assimilated and the process is called assimilative metabolism. Table (1) summarizes some of features of the assimilatory and dissimilatory mechanisms. Simple aliphatic molecules such as organic acids, propane, glycerol and sugars are assimilated by bacteria which can easily be transported into the cell (George and Perry, 1984; Beam and Perry, 1974; Lueking *et al*., 1973). Lactic acid bacteria isolated from human gut assimilate cholesterol (Dora *et al*., 2002). Similarly, aliphatic amino acids were assimilated into protein and incorporation of exogenously supplemented aromatic amino acid, L-phenylalanine directly into proteins by *Escherichia coli* was reported (Miseta *et al*., 1996). *Rhodotorula glutinis* assimilates aromatic acids and dihydroxyphenols (Kocwa-Haluch and Lemek, 1995; Kocwa-Haluch, 1995).

1.8 **Microbial metabolism of cinnamate**

Cinnamic acid, formed from phenylalanine by the action of phenylalanine ammonia lyase (PAL) is a precursor for lignin biosynthesis. A series of enzymatic hydroxylations and methoxylations of cinnamic acid leads to coumaric acid (4-hydroxycinnamate), ferulic acid (4-hydroxy-3-methoxycinnamate) and sinapic acid (4-hydroxy-3, 5-dimethoxycinnamic acid). These aromatic monomers subsequently enter anaerobic environments by depolymerization of lignin and are subject to biodegradations or biotransformations. Cinnamic acid is used as a flavouring agent in baked foods,

Assimilative process	Dissimilative process
▪ Limited amount of the compounds are reduced to satisfy the needs of cell growth	Large amount of electron acceptor is reduced
▪ Reduction of nitrate to ammonium by nitrate reductase is assimilative process	Conversion of nitrate to dinitrogen is a dissimilative process
▪ Assimilatory nitrate reduction observed in cyanobacteria (Flores *et al*., 1983) *Klebsiella pneumonia* (Lin *et al.,* 1993), and *Bacillus subtilis* (Ogawa *et al.,* 1995).	Dissimilative nitrate reduction is observed in few microorganisms that include *Enterobacter amnigenus* (Fazzolari *et al*., 1990).
▪ Reduction of sulfate to sulfide is assimilative sulfate reduction and observed in *Beggiota*, *Thiobacillus* and *Sulfolobus*.	Dissimilative sulfate reduction observed in *Deulfovibrio* and *Desulfotomaculum* (Hamilton 1998a; Hamilton 1998b) where sulfate is the terminal electron acceptor.

Table 1: Characters of assimilative and dissimilative processes

sweets, ice cream, beverages and chewing gum (Roller and Seedhar, 2002). It inhibits the growth of several bacteria (Burt *et al*., 2004) and is an active compound in medicinal plants with anti *Helicobacter pylori* (Bae *et al*., 1998) and antituberculotic activity (Barnes *et al*., 2003). It also enhances the activity of drugs like isoniazid or rifampin against *Mycobacterium tuberculosis* (Rastogi *et al*., 1998).

1.8.1 **Cinnamic acid degradation:** Under anaerobic conditions degradation of cinnamic acid by methanogenic consortia was initiated by the conventional *β*-oxidation of the C_3-side chain, yielding a C_2-unit plus benzoate or benzoyl-CoA (Balba and Evans, 1979; Healy *et al.,* 1980; Evans and Fuchs, 1988). *Rhodopseudomonas palustris*, phototrophic bacteria degrade *trans*-cinnamate following benzoyl-CoA pathway [Fig 5(I)] (Harwood and Gibson, 1988). A syntropic co-culture of *Syntrophus buswelli* strain GA and *Desulfovibrio vulgaris* degrades cinnamic acid to produce acetate and H_2S without accumulation of benzoic acid (Aurburger and Winter, 1995).

1.8.2 **Biotransformations of cinnamic acid:** *Papillibacter cinnamivorans* (Defnoun *et al.,* 2000) a strictly anaerobic bacterium transforms cinnamic acid to acetic and benzoic acids, which accumulate without further degradation. Reductive transformation of *trans*-cinnamate to hydro-cinnamate involving reduction of the double bond in the side chain of some cinnamic acid derivatives was reported in methanogenic consortia (Nali *et al*., 1985; de la Torre and Gomez-Alarcon, 1991; Grbic-Galic and Young 1985), *Pseudomonas cepacia* (Andreoni *et al.,* 1984), *Pseudomonas putida* (Andreoni and Bestetti, 1986), *Wolinella succinogenes* (Ohmiya *et al.,* 1986), *Clostridium aerotolerans* DSM 5434, *Clostridium xylanolyticum* (Chamkha *et al.,* 2001) and *Corynebacterium glutamicum* (Labuda *et al.,*1993).

Pichia carsonii and yeast *Cryptococcus elinovii* converted cinnamic acid to styrene by non-oxidative decarboxylation (Shimada *et al.,* 1992; Middelhoven, 1995) and involved enzyme cinnamyl-alcohol dehydrogenase (Zeyen *et al*., 1995*)*. Transformation of cinnamic acids to their corresponding 4-vinyl derivatives by oxidative decarboxylation was reported in fungi, yeast and bacteria through oxidative

decarboxylation of the side chain (Cavin *et al*., 1997b; Edlin *et al.*, 1998). Cinnamic acid was converted to aromatic amino acid L-phenylalanine by the reverse action of Phenylalanine Ammonia Lyase (PAL) (Yamada *et al*., 1981; Evans *et al*., 1987a). Biotransformations of *trans*-cinnamate shown in Fig 5 (II).

1.9 **Metabolism of 4-hydroxycinnamate:** Phenolic acids occur in plants as hydroxycinnamic acids ester-linked to polysaccharides and are present as large amounts of simple phenolic acids, like *p*-coumaric, caffeic acid, ferulic and syringic acids. These are released during the aerobic catabolism of lignin (Kuwahara *et al*., 1980). *p*-Coumaric acid (4-hydroxybenzoic acid) was the central intermediate in the biosynthesis of many plant phenols and was present in esterified or free acid forms in many fruits, vegetables and graminaceous plants (Clifford, 1999). Utilization of diverse hydroxycinnamates as sources of carbon and energy was widely distributed among different microbial groups (Delneri *et al.*, 1995)

The aerobic catabolism of these compounds has been well studied while anaerobic microbial conversions of hydroxycinnamates were reported from anaerobic sewage sludge and anaerobic microphyte present in marshy sediments (Nali *et al*., 1985; de la Torre and Gomez-Alarcon, 1991). Bacteria such as *Streptomyces griseus* and filamentous fungi such as *Gliocladium deliquescens* convert 4-hydroxycinnamate to caffeic acid and related compounds (Torres *et al*., 2001).

Pycnoporus cinnabarinus, a fungus transformed 4-hydroxycinnamate into caffeic acid and also to *p*-hydroxybenzaldehyde (Alvarado *et al*., 2003; Alvarado *et al*., 2001). *Lactobacillus plantarum, Bacillus pumilis, Pseudomonas fluorescens* transformed 4-

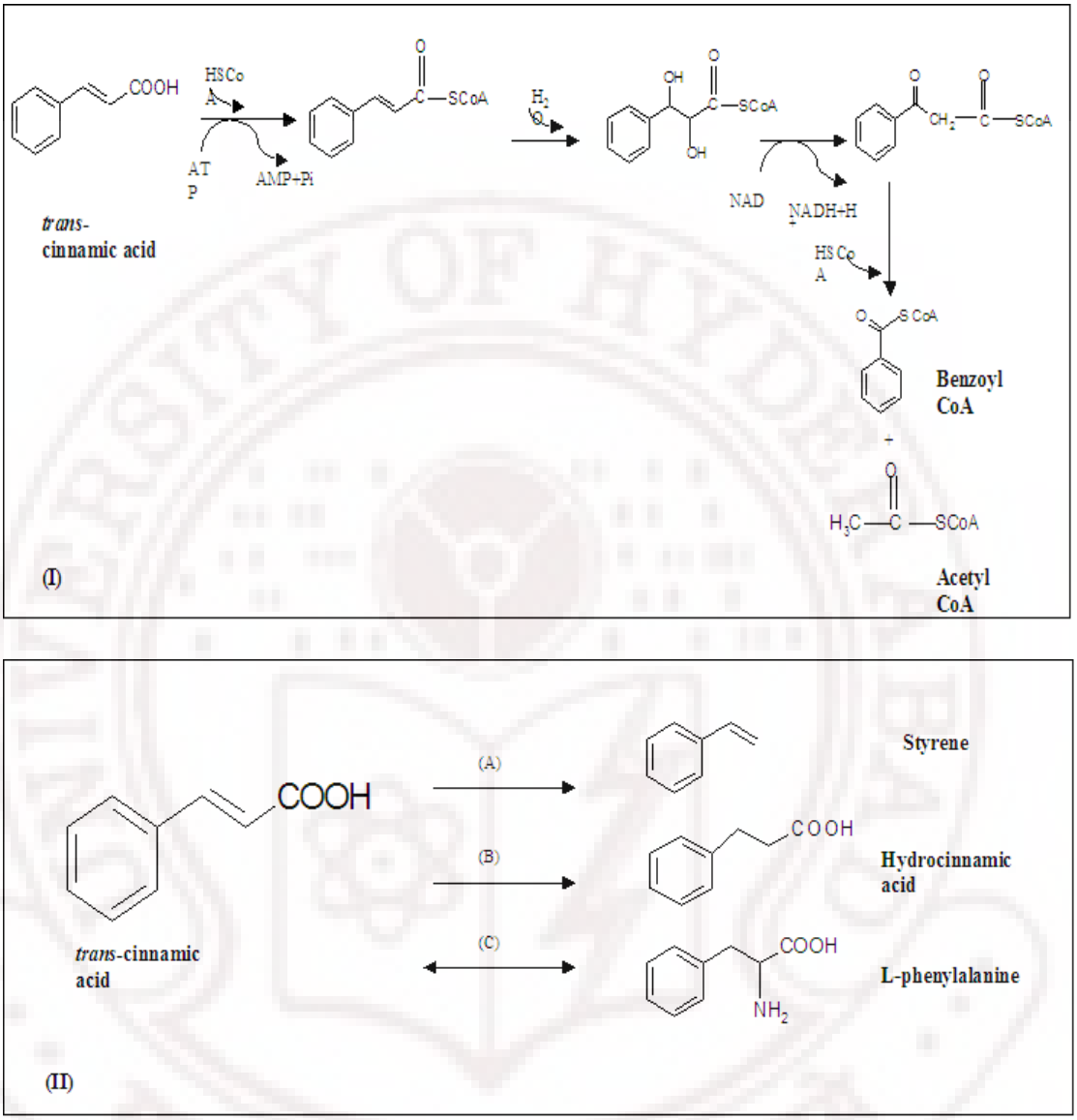


Fig 5: (I) Proposed mechanism for the anaerobic degradation of *trans*-cinnamic acid by *Rhodopseudomonas palustris* (Elder *et al*., 1992a**). (II) Biotransformation of *trans*-cinnamic acid to:** (A) Styrene by *Cryptococcus elinovii* (Middlehoven, 1995) (B) Hydrocinnamic acid by *Corynebacterium glutamicum* (Labuda *et al*., 1993) (C) L-phenylalanine by *Corynebacterium equii* (Evans, 1987a)

hydroxycinnamate to 4-vinyl derivatives that were further reduced to 4-ethyl derivatives (Cavin *et al.,* 1997a; Degrassi *et al*., 1995; Huang *et al.,* 1994).

These volatile phenols are potential contributors to the aroma of wine and other fermented foods (Potter and Fagerson, 1992). 4-Hydroxycinnamic acid was found to be the chromophore of photosensory protein PYP (photoactive yellow protein), which binds to the cysteine residue via a thioester bond (Hoff *et al.,* 1994). This PYP is a photoreceptor protein, found in the cytosol of *Ectothiorhodospira halophila* (Dux *et al*., 1998), in several purple bacteria (Kort *et al*., 1998) and in *Rhodobacter sphaeroides* (Haker *et al*., 2000).

1.10 **Metabolism of Ferulic acid:** Ferulic acid is an abundant hydroxycinnamic acid in the plant world, capable of being microbially transformed in to valuable aroma compounds (Rosazza *et al*., 1995a). It occurs in plants in its free form and is covalently linked to lignin and other polymers in the cell wall. It is also one of the widely reported antioxidant compounds (Vieira *et al.,* 1998). Ferulic acid is converted to vanillin (3-methoxy-4-hydroxy benzaldehyde) (Rosazza *et al*., 1995a) the most important and widely used flavour in the food industry and is responsible for the characteristic aroma of vanilla bean extracts. It is also a precursor for 4-vinyl guaiacol (3-methoxy 4-hydroxystyrene), the transformation being catalyzed by the enzyme ferulic acid decarboxylase (Donaghy *et al*., 1999). It is also converted to caffeic acid, a more potent antioxidant compound (Vieira *et al.,* 1998) by *Penicillium rubrum* (Tillet and Walker, 1990) and *Pseudomonas* sp. under anaerobic conditions (Taylor, 1983).

1.11 **Microbial metabolism of hydroxybenzoates**

Benzoates and hydroxybenzoates occur as natural components in plants (e.g., salicylic acid, gallic acid) or generated as intermediate metabolites in the microbial degradative pathways of various aromatic hydrocarbons, aromatic dicarboxylic acids and phenolic compounds. In bacteria, all three isomers (*o*-, *m*-, *p*-) of monohydroxylated benzoates are channeled into anaerobic metabolism by converting to benzoyl CoA (Goetz and Harmuth, 1992; Grund *et al*., 1990; Karegoudar *et al*., 1999). The carboxyl group was first activated to coenzyme A thioester and the product was then reductively dehydroxylated (Bonting *et al*., 1995, Brachmann *et al.,* 1993). 4-Hydroxybenzoate was an intermediate in the anaerobic degradation of phenol or *p*-cresol by the denitrifying bacterium *Thaeura aromatica,* photosynthetic species *Rhodopseudomonas palustris* (Diageri *et al*., 1993), while under aerobic conditions 3-hydroxybenzoic acid and 4-hydroxybenzoic acid was catabolized by the gentisate pathway (Jones and Cooper, 1990) or through protocatechuate (Karegoudar *et al*., 1999). 4-Hydroxybenzoate was the key intermediate in ubiquinone biosynthesis (Pennock and Threlfall, 1983).

1.12 **DEFINITION OF THE PROBLEM**

Among the anoxygenic phototrophic bacteria, purple non-sulfur bacteria degrade a wide range low molecular weight compounds including C-1 compounds, organic acids, amino acids, fatty acids, alcohols and carbohydrates (Berry *et al.,* 1987; Harwood and Gibson, 1988; Sasikala and Ramana, 1998). In addition to degradation, light dependent transformation of aromatic hydrocarbons was observed by

Rhodopseudomonas palustris (Noh *et al*., 2002), *Rubrivivax gelatinosus* (Willems *et al*., 1991), *Rhodobacter capsulatus* (Blasco and Castillo, 1992), *Rhodobacter blasticus* (Ahmed and Mohamed, 1994) and *Rhodobacter sphaeroides* (Rajasekhar *et al*., 1998).

Rhodobacter sphaeroides OU5, which occurs abundantly in paddy soil and waste waters (Sasikala and Ramana, 1998), lacks the ability to completely mineralize aromatic compounds for its growth, but can photobiotransform some of the aromatic compounds. *Rhodobacter sphaeroides* OU5 transforms 2-aminobenzoate to indole in the presence of fumarate (Nanda *et al*., 2000 and Sunayana *et al*., 2005) and aniline to indole derivatives (Vijay *et al*., 2006). It could also transform aromatic amino acids like phenylalanine to phenolic derivatives (Ranjith *et al*., 2007). An attempt was made to extend the study on exploring the metabolic capability of *Rhodobacter sphaeroides* OU5 in utilizing benzoates and phenyl propionates, which can meet some of its cellular demands though they cannot support growth. Hence, a detailed study on this topic was taken for Ph.D thesis with the following objectives.

OBJECTIVES

1. **To study the capability of *Rhodobacter sphaeroides* OU5 in utilizing aromatic compounds**
2. **To study the assimilation of *trans*-cinnamate by *Rhodobacter sphaeroides* OU5**
3. **To study the hydroxycinnamate and 4-hydroxybenzoate assimilation by *Rhodobacter sphaeroides* OU5**

Materials and Methods

2.1 **Glassware:** All the glassware used in the present experiments including test tubes, pipettes, measuring cylinders, culturing flasks, reagent bottles, petriplates and screw cap test tubes were of Borosil and Duran brand.

2.2 **Cleaning:** The glassware used in the experiments were initially soaked in dilute H_2SO_4 (20 % v/v) for 24 h and cleaned with tap water and teepol, a detergent. After removing all the traces of the detergent, the glassware was rinsed with single distilled water and kept in oven for drying at 100 °C.

2.3 **Water:** Single and double distilled water obtained from Milli-Q plant stored in white carboys was used for rinsing of glassware and for preparation of media, stock solutions and chemical analysis. Milli-Q water was used for HPLC analysis.

2.4 **Chemicals:** The chemicals used in this study were of analytical grade from Sigma-Aldrich, Lancaster, Ranbaxy, Merck, Qualigens, e-Merck and Himedia.

2.5 **Determination of pH:** pH was determined using a digital pH meter (Digisun electronics, India, model DI-707).

2.6 **Sterilization:** Sterilization of the culture media and glassware was done by autoclaving at 15 lbs for 15 min.

2.7 **Organism and growth conditions:** The purple non sulphur bacterium, *Rhodobacter sphaeroides* OU5 [ATCC-49885; DSM-7066] was obtained from the Bacterial Discovery Laboratory, Center for Environment, IST, JNTU, Hyderabad. The organism was grown photoheterotrophically (2,400 lux) on mineral medium (Table 2) with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen sources respectively, in fully filled (10x100 mm) screw cap test tubes or in reagent bottles (250/1000 ml) at 30±2 °C.

2.8 **Maintenance of stock culture:** Stock cultures of *Rba. sphaeroides* OU5 was maintained as agar stabs. Stabs were prepared by using 2 % (w/v) agar solidified heterotrophic medium, filled to ¾ volume of 5 ml capacity screw cap test tubes. The culture taken onto a sterile needle was stabbed into the agar and the culture tubes were illuminated (2,400 lux) and incubated at 30±2 °C. After 2-3 days of growth, the stab cultures were preserved under refrigeration at 4 °C until further use. The stabs were sub cultured every 90 days and contamination from other bacteria was checked periodically by streaking onto nutrient agar plates.

2.9 **Purity of the cultures:** Culture was checked for its purity before and after assay by streaking on nutrient agar plates ($g.l^{-1}$: peptone-5, beef extract-3 and agar-15 (Difco manual, 1998) and incubating aerobically under light (2400, lux) at 30±2 °C.

2.10 **ASSAYS**

2.10.1 **Assay with growing cells:** The logarithmically (24 h) growing culture of *Rba. sphaeroides* OU5 (0.48 O.D 660 nm) was transferred into screw cap test tubes (10x100 mm) containing photoheterotrophic medium and 0.5 mM of aromatic compound (*trans*-cinnamate/hydroxycinnamate/hydroxybenzoate) from the sterilized stock solution (100 mM) was added and incubated for 48 h under anaerobic/light (2, 400 lux) at 30±2 °C. After incubation the culture was harvested for analysis.

2.10.2 **Assay with resting cells:** The logarithmically growing culture (24 h) of *Rba. sphaeroides* OU5 (0.48 O.D 660 nm) was harvested by centrifugation (16,000 x g) and cell pellet was washed twice with saline before suspending into the basal medium containing aromatic compound. The culture was then dispensed into screw cap test tubes and incubated anaerobic/light (2, 400 lux) at 30±2 °C

Ingredients	**g. l^{-1}**
KH_2PO_4	0.5
$MgSO_4.7\ H_2O$	0.2
NaCl	0.4
NH_4Cl	0.04
$CaCl_2.2\ H_2O$	0.05
Organic carbon source/electron donor	3.0
Yeast extract	0.12
Ferric citrate (0.1 % w/v)	5 ml
Trace elements SL_7	1 ml

Mineral medium used for the growth of purple non-sulphur bacteria (after Biebl and Pfennig, 1981).

SL_7 ($mg.ml^{-1}$): HCl (25% v/v)-1ml; $ZnCl_2$-7; $MnCl_2.4H_2O$-100; H_3BO_3-60; $CoCl_2.6H_2O$-200; $CuCl_2.H_2O$-20; $NiCl_2.6H_2O$-20; $NaMoO_4.6H_2O$-40

Table 2: The composition of Biebl and Pfennig's medium (Biebl and Pfennig, 1981**)**

2.10.3 **Assay with cell free extracts:** *Rba. sphaeroides* OU5 culture grown in the presence of aromatic compound (0.5 mM) was harvested by centrifugation (16,000 x g) and cell pellet was suspended in Tris buffer (50 mM, pH 7.8) and sonicated [6x2 min, 8 cycles, probe MS 72 in Bandelin Sonoplus Sonicator]. This extract was centrifuged and supernatant collected was used as a source of enzyme for enzyme assays, while unsonicated cell debris was discarded.

2.11 **ANALYTICAL METHODS**

2.11.1 **Growth and biomass**: Growth was measured turbidometrically, in terms of optical density (O.D) using a Systronic colorimeter (mode 112) at 660 nm (filter 8) using uninoculated medium as blank. OD versus dry wt graph was plotted and the dry wt. was calculated by the empirical formula drawn from the graph

OD_{660} of 0.1 = 0 .15 mg dry wt.ml^{-1}

2.11.2 **Determination of 50 % inhibitory concentration (IC_{50}) of aromatic compounds on growth of *Rba. sphaeroides* OU5**

Fifty percent growth inhibitory concentration (IC_{50}) of aromatic compounds on growth/biomass of *Rhodobacter sphaeroides* OU5 was studied in the photoheterotrophic growth medium with malate (22 mM) as carbon and NH_4Cl (7 mM) as nitrogen source, along with aromatic compounds at various concentrations in fully filled screw cap test tubes (10x100 mm) incubated phototrophically (2,400 lux) at 30±2 °C.

2.11.3 **Estimation of metabolites by colorimetric analysis**

2.11.3.1 **Estimation of indoles:** Indole was estimated colorimetrically either by Salper's reagent (Gordon and Paleg, 1957) or by *p*-dimethylaminobenzaldehyde (PDAB) reagent (Kupfer and Atkinson, 1964).

(i) **Salpers method**: To 1 ml culture supernatant in ethylacetate, 2 ml of freshly prepared Salper's reagent [1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % (v/v) perchloric acid] was added and the absorbance was read at 535 nm against reagent blank.

(ii) **PDAB method**: To 1 ml culture supernatant made up to 5 ml with water, 1 ml of freshly prepared PDAB reagent [60 mg of *p*-dimethylaminobenzaldehyde in 1 ml of 3N H_2SO_4] was added and absorbance was read at 550 nm against reagent blank.

2.11.3.2 **Estimation of ammonia:** (Solorzano, 1969) Ammonia was estimated using NH_4Cl as standard. To 5 ml of the sample 0.2 ml of phenol alcohol (10 % w/v in 95 % ethanol), 0.2 ml of sodium nitroprusside (0.5 % w/v), 0.5 ml of oxidizing solution (20 % w/v trisodium citrate + 1% w/v NaOH + 25 ml 1.5 N NaOCl) was added. The colour intensity was read at 640 nm.

2.11.3.3 **Estimation of tryptophan**: To 1 ml of the aqueous extract made upto 5 ml with distilled water, 1 ml of freshly prepared PDAB (60 mg in 1ml of 3N H_2SO_4), 3 ml of chilled 30 N H_2SO_4, 0.1 ml of $NaNO_2$ (0.1% w/v) were added and after 15 min of incubation the absorption was read at 580 nm against reagent blank.

2.11.3.4 **Estimation of tyrosine**: (Ottaway, 1957) To 2 ml of solution containing 10-200 μg of tyrosine 0.1 ml of (0.3 % of 1-nitroso-2-naphthol in acetone) was added and kept in a water bath at 60 ^oC for 5 min. After removing from water bath, 4 drops of conc. HNO_3 was added, cooled in water bath for a minute and 6 ml of stabilizing reagent (0.12 M NaCl in 25 % acetone) was added. The colour intensity was read at 450 nm within 45 min.

2.11.3.5 **Estimation of amino acids:** Amino acids were estimated colorimetrically by adding 4 ml of ninhydrin (0.2 % w/v in acetone) to 1 ml of culture supernatant and the

mixture was kept in water bath at 60 ^{o}C for 15 min after which volume made up with acetone and the colour was read at 570 nm against reagent blank.

2.11.3.6 **Estimation of reducing sugars by DNS method**: Glucose consumption was determined as described by Miller 1958 using dinitrosalicylic acid as a chromogenic reagent.

2.11.3.7 **Estimation of 3, 4-dihydroxy phenyl pyruvate**: (Briggs method, 1922) One ml of assay mixture was deprotenized by the addition of 200 µl 40 % TCA and centrifuged. One ml of the supernatant was added to 500 µl of a mixture of equal volumes of 2.5 % ammonium molybdate in 5 N HCl and 3 % KH_2PO_4 and the colour complex so formed was read at 700 nm after 20 min.

2.11.3.8 **Estimation of proteins:** Proteins were estimated using Bradford's method (Bradford, 1976).

2.11.4 **Detection and estimation of metabolites by HPLC analysis**

Aromatic compounds were analyzed in HPLC performed at room temperature using a Shimadzu SPD-10AVP and LC-20AVT system, with a capcell pak Luna 5 µ C_{18} (2) 100A column (250 x 4.6 mm). The injection volume was 20 µl. Solvents like methanol, water (1:1) and methanol, Tris buffer (50 mM pH 7.8) in the ratio of 3:7 were used at a flow rate of 1.0 ml.min^{-1} and the compounds were detected using UV-VIS and PDA detectors. The metabolites were identified based on their retention time and by comparing with the standards.

The compounds like *trans*-cinnamate (270 nm), 4-hydroxycinnamate (300 nm) and indole (280 nm) were detected in methanol water solvent system (1:1) at retention time of 2.4, 3.0 and 4.5 min respectively.

Compounds like phenylalanine, phenylpyruvate, tyrosine, 3, 4-dihydroxycinnamate, 3, 4-dihydroxyphenylalanine, 4-hydroxybnezoate and fumarate were detected in methanol buffer (3:7) solvent system at retention time of 4.7, 7.8, 3.0, 2.9, 2.4, 2.85 and 2.0 min respectively.

2.11.5 **Identification of metabolites by LC-MS analysis**

Liquid chromatography (LC)-mass spectrometry (MS) analysis was performed with Shimadzu LC-1020A, equipped with an automatic injector. MS was performed using MS-ESI ion source (Nitrogen flow rate 0.5/h) using, both negative and positive ion mode. Analysis was performed at 40 ^{o}C (LC column oven) and 85 ^{o}C (MS ionization chamber). Methanol and water (1:1) was used as solvent at 0.2 ml.min^{-1} using a Luna 5µ C_{18} (2) 100A column (250 x 4.6 mm) and the compounds were detected (by way of LC) at 254 nm. The column effluent from the LC was nebulized into an atmospheric pressure chemical ionization region under nitrogen gas for generating molecular masses.

2.12 **ENZYME ASSAYS**

2.12.1 **Cinnamoyl Coenzyme A Synthetase activity:** (Elder *et. al.,* 1992)

The activation of aromatic acids to their respective CoA thioesters was measured spectrophotometrically at 30 ^{o}C by directly monitoring the formation of product at 311 nm (Cinnamoyl CoA). The reaction mixture (1 ml) contained 100 mM Tris-HCl buffer pH 8.5, 2 mM $MgCl_2$, 2 mM ATP, 0.25 mM *trans*-cinnamate and cell extract. Reaction was initiated by the addition of CoA (0.8 mM) to the sample cuvette.

2.12.2 **DAHP Synthase activity:** Enzyme activity was assayed by measuring the levels of erythrose 4-phosphate in the HPLC. The solvent system used was 0.002 N H_2SO_4 in HPLC grade water at a flow rate of 1 ml.min^{-1} and was detected at 200 nm in the UV-Visible detector.

2.12.3 **Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities** (Kyndt *et al*., 2002)

The activities of the enzymes were determined by measuring the formation of *trans*-cinnamate or 4-hydroxycinnamate from L-phenylalanine or L-tyrosine, respectively, using HPLC. The reaction mixture contained, in a final volume of 1 ml (Tris-HCl buffer 10 mM, pH 7.8), 100 µmol L-phenylalanine or L-tyrosine and an appropriate amount of cell free extract. The reaction was carried out in eppendorf test tubes (1.5 ml), incubated at 30 ºC for 20 min, activity was stopped by acidification with 1N HCl and analyzed by injecting 20 µl of clear supernatants in HPLC.

2.13 **Isolation and electrophoresis of proteins:**

- Wet packed cells of *Rhodobacter sphaeroides* OU5 suspended in 75 ml of Tris buffer (50 mM pH 7.8) with 10 % glycerol were subjected to sonication to complete cell lysis by MS-72 probe (Bandelin, Germany make, model-UW 2070) for 8-9 cycles. Cell debris was removed by centrifugation at 10,000 rpm for 20 min at 4 ^{o}C and the supernatant was used for ammonium sulfate saturation.
- To the supernatant (75 ml) solid ammonium sulfate was added until 30 % saturation is reached and the mixture was stirred for 1 hr. The suspension was centrifuged at 0 ^{o}C (10,000 rpm) for 20 min. To the supernatant ammonium sulfate was added to 60 % saturation and the suspension was treated as above. Finally to the supernatant obtained ammonium sulfate was added until 90 % saturation is reached. After stirring for 4 hrs the suspension was centrifuged at (10,000 rpm) for 20 min. The resulting precipitate was collected and dissolved in 10 ml of Tris buffer. The protein solution was dialyzed overnight in the 50 mM Tris buffer.

- The enzyme solution (9 mg protein) was loaded on to a DEAE-Cellulose column of 20 ml bed volume (4.2x2.5 cm) equilibrated with Tris buffer (50 mM pH 7.8) and different protein fractions were eluted using a gradient of 0-1 M NaCl.
- **PAGE analysis**: The presence of the enzyme in the induced culture was checked by comparing the protein pattern of the crude extracts from induced and uninduced cultures grown in the same medium with and without *trans*-cinnamate. The electrophoresis of the proteins was done on 8, 10 and 12 % SDS-PAGE, Native-PAGE and stained with coomassie blue or silver stain (Laemmeli, 1970).
- Native molecular weight of the protein was determined by FPLC (Bio-Rad model). The molecular masses of the enzyme polypeptides were determined by comparision with the mobilities of standard proteins of known molecular mass (Bio-Rad).

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.

Aromatic compound (0.5 mM)	IC_{50} (mM)	Assimilation
* *trans*-Cinnamate	4.5	+
4-Hydroxycinnamate	3.0	+
3, 4-Dihydroxycinnamate	2.5	+
* 4-Hydroxybenzoate	10.0	+
Phenylpyruvate	NT	+
L-Phenylalanine	NT	+
Benzoate	9.5	-
2-Hydroxybenzoate	2.0	-
3, 4-Dihydroxybenzoate	NT	-
4-Aminobenzoate	8.0	-
2-Aminobenzoate	4.0	-
Toluene	NT	-
Cresol	NT	-

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_4Cl (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 *trans*-cinnamate by *Rba. sphaeroides* OU5 (work with whole cells)**

3.2.1 **Effect of *trans*-cinnamate on growth of *Rba. sphaeroides* OU5**

Effect of different concentrations of *trans*-cinnamate (0.5-7.0 mM) on photoheterotrophic growth of *Rba. sphaeroides* OU5 was studied. Presence of *trans*-cinnamate did not alter the growth yield and biomass of *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 *Rba. sphaeroides* OU5 was inhibited at 7 mM. The 50 % inhibitory concentration (IC_{50}) of *trans*-cinnamate on photoheterotrophic growth of *Rba. sphaeroides* OU5 was approximately 4.5 mM.

3.2.2 **Influence of organic substrates on growth of *Rba. sphaeroides* OU5 at IC_{50} of *trans*-cinnamate.**

The influence of different organic substrates on the growth of *Rba. sphaeroides* OU5 in the presence of 4.5 mM of *trans*-cinnamate was studied (Fig 7). Growth of *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 **Influence of amino acids on the growth of *Rba. sphaeroides* OU5 at IC_{50} of *trans*-cinnamate.**

Growth of *Rba. sphaeroides* OU5 in the presence of *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 *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).

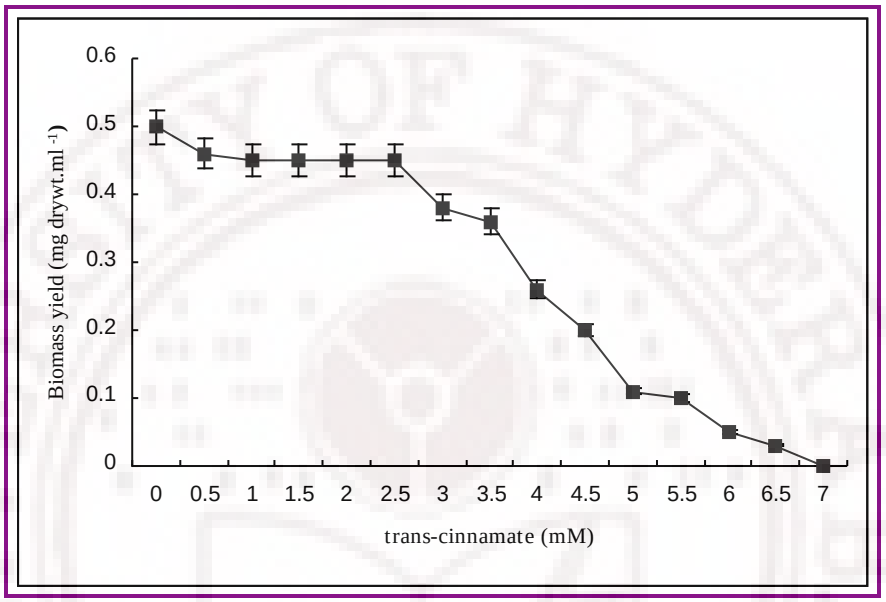


Fig 6: Minimum inhibitory concentration of *trans*-cinnamate on the photoheterotrophic growth of *Rba. sphaeroides* OU5

Assay was done with the growing cells of *Rba. sphaeroides* OU5 in photoheterotrophic medium with malate (22 mM) as sole carbon source and NH_4Cl (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 $^{\circ}$C in fully filled screw cap test tubes and growth was measured turbidometrically.

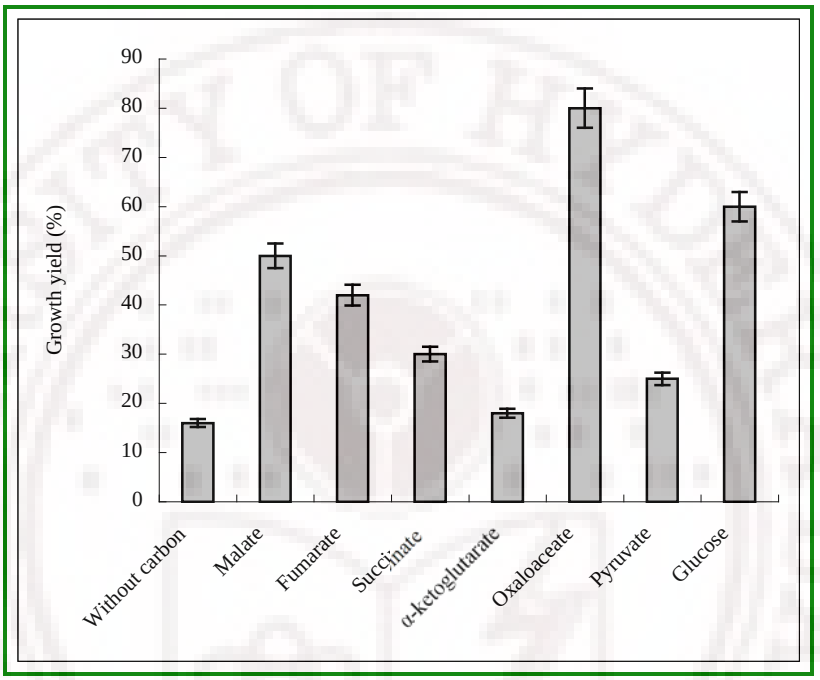


Fig 7: Influence of organic substrates on growth of *Rba. sphaeroides* OU5 at IC_{50} of *trans*-cinnamate

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.

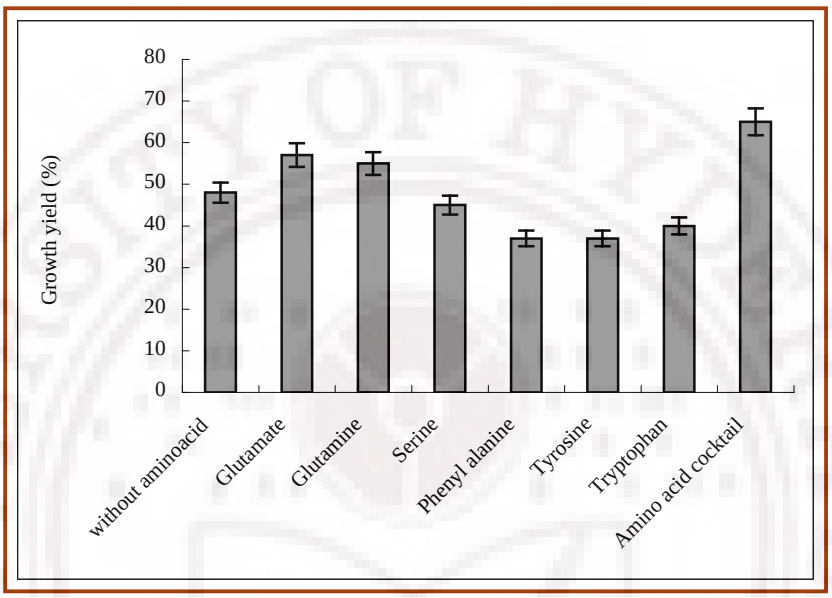


Fig 8: Influence of L-amino acids on growth of *Rba. sphaeroides* OU5 at IC_{50} of *trans*-cinnamate

(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-aminoacids 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).

Growth phase of cells	Biomass yield (mg drywt.ml^{-1})		*trans*-cinnamate consumption (mM)	
	Light	Dark	Light	Dark
Resting	0.48	0.48	0.5	0
Growing	0.27	0.05	0.4	0

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.

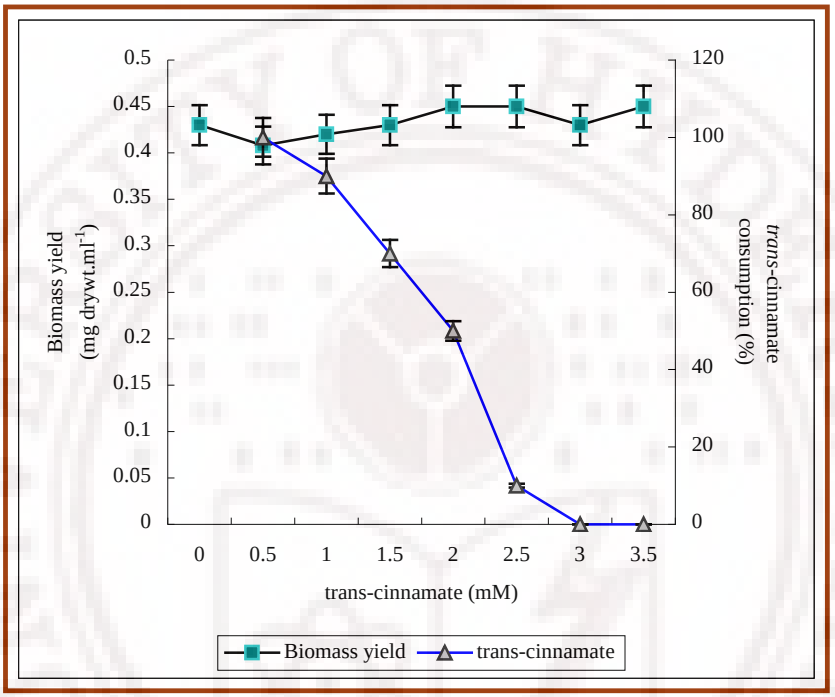


Fig 9: Assimilation of *trans*-cinnamate at different concentrations by resting cells of *Rba. sphaeroides* OU5

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.

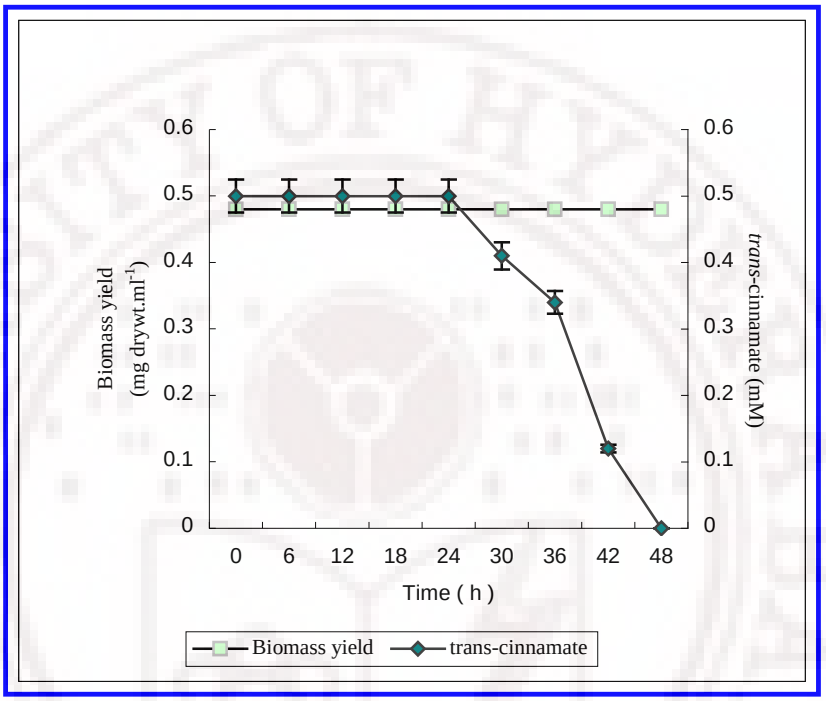


Fig 10: Photoassimilation of *trans*-cinnamate with time by resting cells of *Rba. sphaeroides* OU5

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.

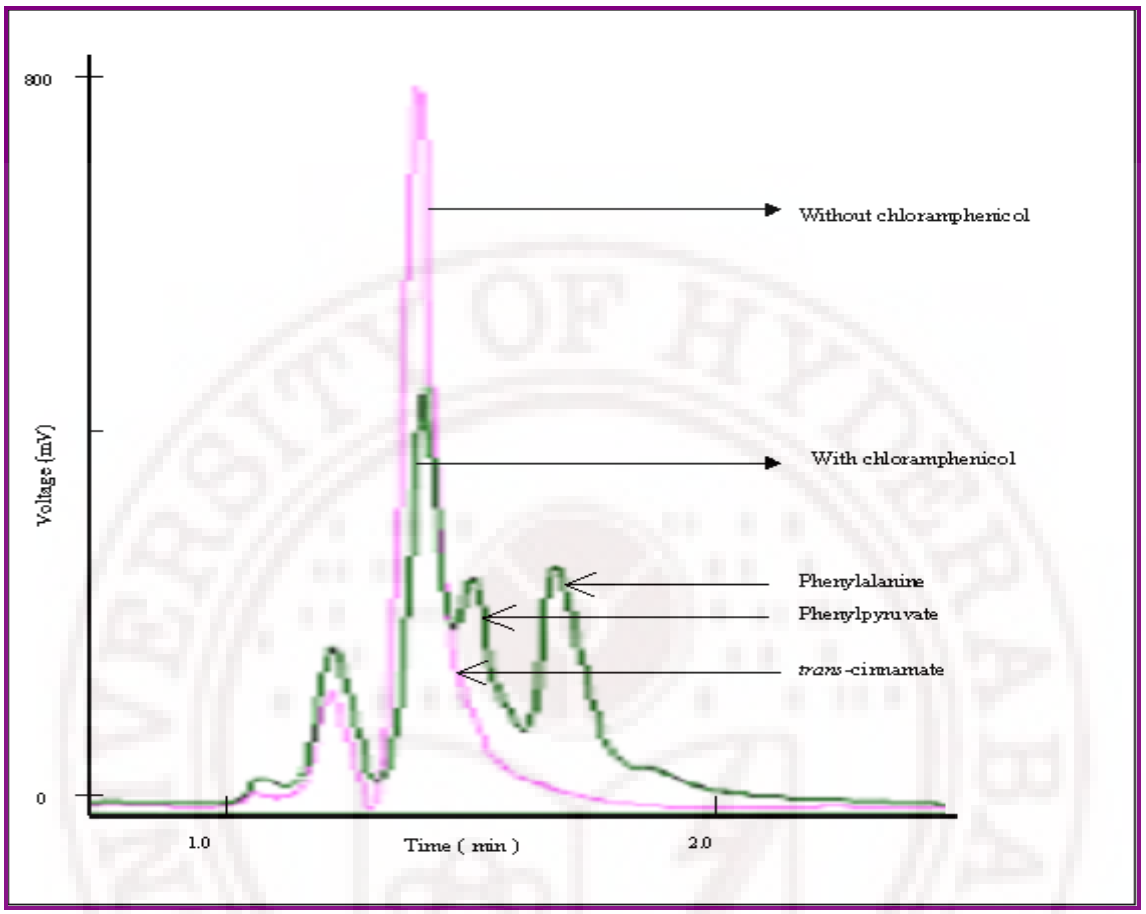


Fig 11: HPLC chromatogram showing *trans*-cinnamate assimilation by resting cells of *Rba. sphaeroides* OU5 treated with chloramphenicol

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).

Organic substrate (0.3 %)	Biomass yield (mg drywt.ml^{-1})		*trans*-cinnamate consumption (mM)	Metabolites (mM)			
	Without *trans*-cinnamate	With *trans*-cinnamate		Phe	Tyr	Trp	Indole
Control	0.05	0.03	0.15	-	-	-	-
Malate	0.52	0.50	-	-	-	-	-
Fumarate	0.60	0.56	-	-	-	-	-
Succinate	0.53	0.54	-	-	-	-	-
α-ketoglutarate	0.44	0.14	0.27	-	-	-	-
Oxaloacetate	0.48	0.50	-	-	-	-	-
Pyruvate	0.63	0.60	0.25	0.06	0.01	0.02	0.01
Glucose	0.35	0.34	0.45	0.1	-	-	0.08

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 NH_4Cl (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.

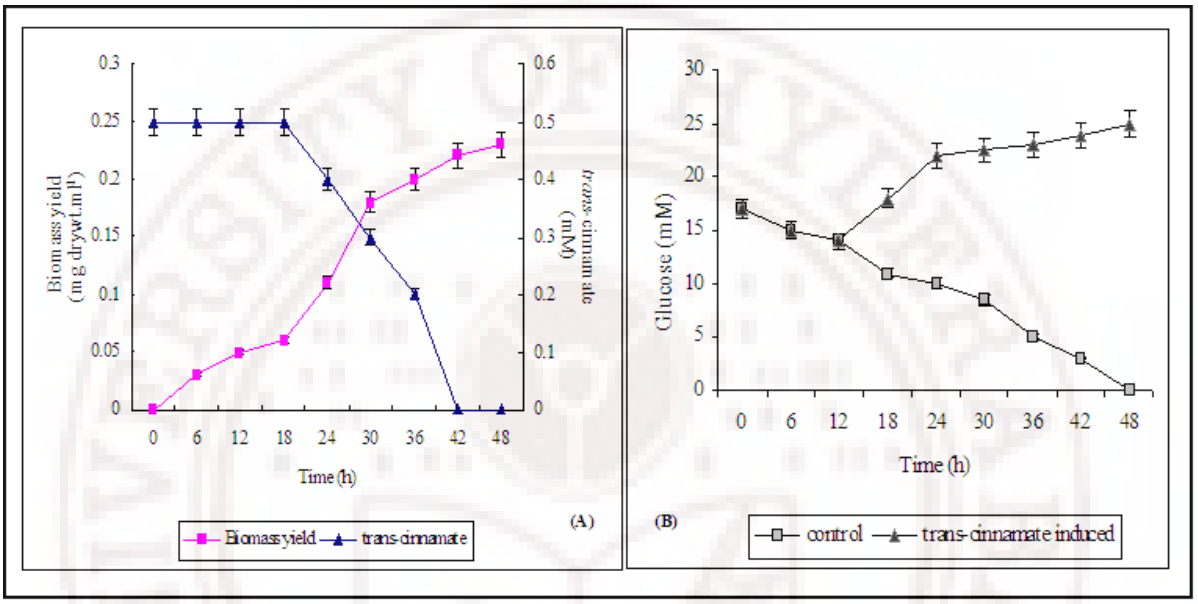


Fig 12: Time course of photoassimilation of *trans*-cinnamate by growing cells of *Rba. sphaeroides* OU5

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.

Nitrogen substrates (1mM)	*trans*-cinnamate consumption (mM)	Metabolites (mM)	
		Phenylalanine	Indole
NH_4Cl	0.40	0.08	0.09
NH_4OH	0.42	0.10	0.09
Glutamate	0.10	-	0.07
Glutamine	0.12	-	0.06
Serine	-	-	0.06
Phenylalanine	-	-	-
Tyrosine	-	-	-
Tryptophan	-	-	0.20
Control	-	-	-

Table 6: Assimilation of *trans*-cinnamate by growing cells of *Rba. sphaeroides* OU5 in the presence of different nitrogen substrates [Control = without nitrogen substrate]

Cells of *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 *trans*-cinnamate (0.5 mM). Levels of *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.

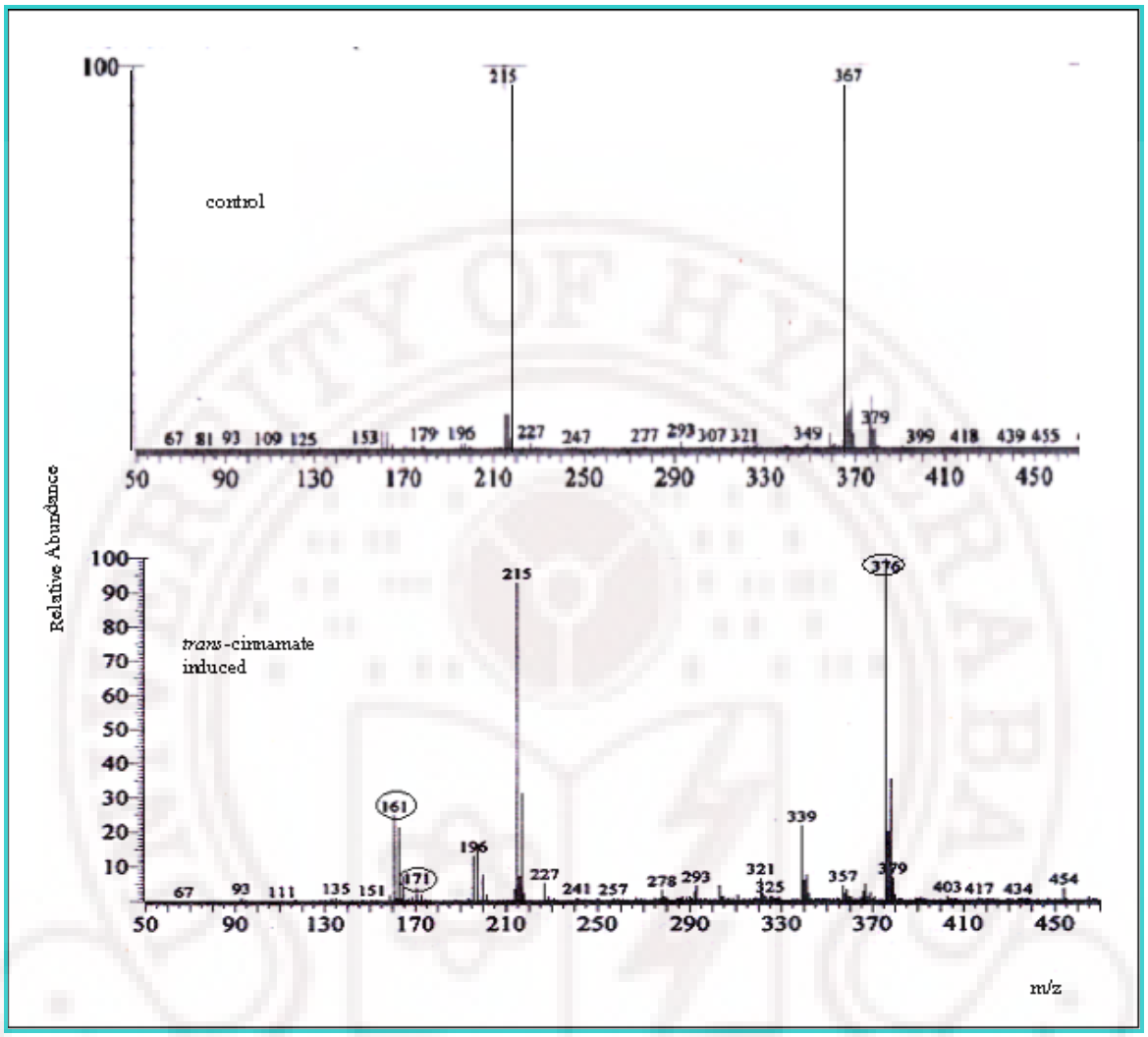


Fig 13: LC-MS metabolite profiling of control (uninduced) and *trans*-cinnamate induced culture supernatant of *Rba. sphaeroides* OU5

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).

Compound	DAHP synthase activity (% Inhibition)
Control (without *trans*-cinnamate)	0
trans-cinnamate	100

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 µmoles.mg protein^{-1})

The assay mixture (1 ml) contained 10 mM Tris HCl buffer (pH 7.8) with erythrose 4-phosphate at 100 µmoles, 50 µmoles of phosphoenolpyruvate along with *trans*-cinnamate at 50 µmoles 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 NH_4Cl (7 mM) as nitrogen source with 0.5 mM *trans*-cinnamate as supplement.

Cell free extract	*trans*-cinnamate consumption (µmoles.mg protein^{-1})
Control	30
Induced	100

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

Results expressed are an average of data done in triplicates.

Supplements	*trans*-cinnamate consumption (µmoles.mg protein^{-1})
Coenzymes	
Control (without coenzyme and aminodonor)	ND
NADH	100
NADPH	0
ATP	0
Amino donors	
Ammonia (NH_4OH)	200
Ammonium chloride	150
L-glutamate + PLP	130
L-glutamine + PLP	70

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 µmoles of *trans*-cinnamate, along with respective components like 25 µmoles of NADH, NADPH, ATP and 0.1 ml of 10 % NH_4OH, 0.1ml of 10 % NH_4Cl, L-glutamate, L-glutamine of 100 µmoles, PLP- 20 µg 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 µmoles at the end of 30 min of incubation (Fig 14).

Assay components	*trans*-cinnamate consumption (µmoles.mg protein^{-1})	L-phenylalanine formation (µmoles.mg protein^{-1})
Control	ND	ND
Ammonia	70	ND
Ammonia + NADH	100	70
Ammonium chloride + NADH	200	20
L-glutamate + PLP	300	50
L-glutamine + PLP	50	10

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 µmoles of *trans*-cinnamate, along with respective components like 25 µmoles of NADH, 0.1ml of 10 % NH_4OH, 0.1ml of 10 % NH_4Cl, L-glutamate, L-glutamine at 100 µmoles, pyridoxal phosphate (PLP)-20 µg 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_4Cl (7 mM) induced with *trans*-cinnamate (0.5 mM).

Assay components	Phenylpyruvate consumption (µmoles.mg protein^{-1})	L-phenylalanine formation (µmoles.mg protein^{-1})
Control	ND	ND
Ammonia	700	ND
Ammonia +NADH	1000	700
L-Glutamate + PLP	300	50

Table 11: Conversion of phenylpyruvate to L-phenylalanine by cell free extracts of *Rba. sphaeroides* OU5 (ND = not detected)

One ml of assay sample contained10 mM Tris-HCl buffer (pH 7.8), 280 µg of cell free extract, 100 µmoles of phenylpyruvate with respective components like 25 µmoles of NADH, 0.1 ml of 10 % NH_4OH, L-glutamate-100 µmoles, pyridoxal phosphate (PLP)-20 µg and incubated at room temperature. The reaction was stopped by acidifying with 1N HCl, filtered and analyzed in HPLC.

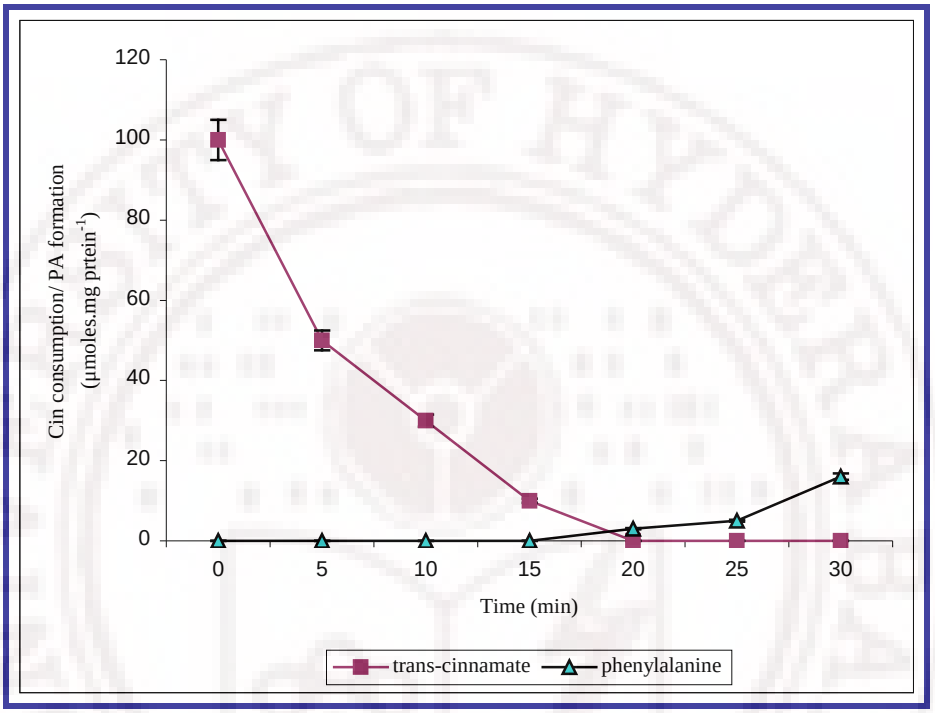


Fig 14: Consumption of *trans*-cinnamate with time by cell free extracts of *Rba. sphaeroides* OU5 [Cin = *trans*-cinnamate; PA = L-phenylalanine]

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 (NH_4OH), 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_R =3.2 min) in HPLC (Fig 17). Transformation of phenylpyruvate to L-phenylalanine was also observed in stoichiometric yields of L-phenylalanine (Fig 18) where as 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**

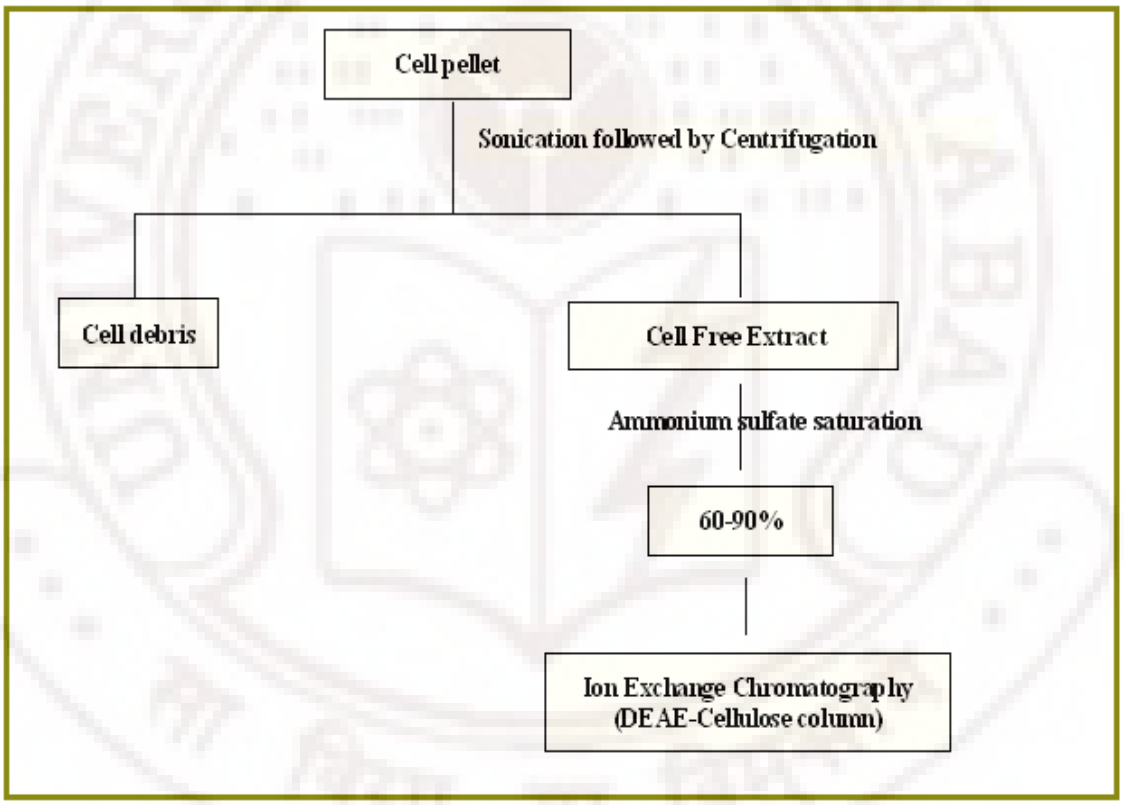
3.3.6.2.1 **Enzyme kinetics**: *trans*-Cinnamate consumption activity was studied with time. Activity was optimum at 5-10 min and then decreased beyond 10 min of incubation (19C).

3.3.6.2.2 **Calculation of K_m and V_{max} :** *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_{max} 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, Co-enzyme 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 ^{o}C was studied and *trans*-cinnamate consumption was maximum 28-30 ^{o}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.

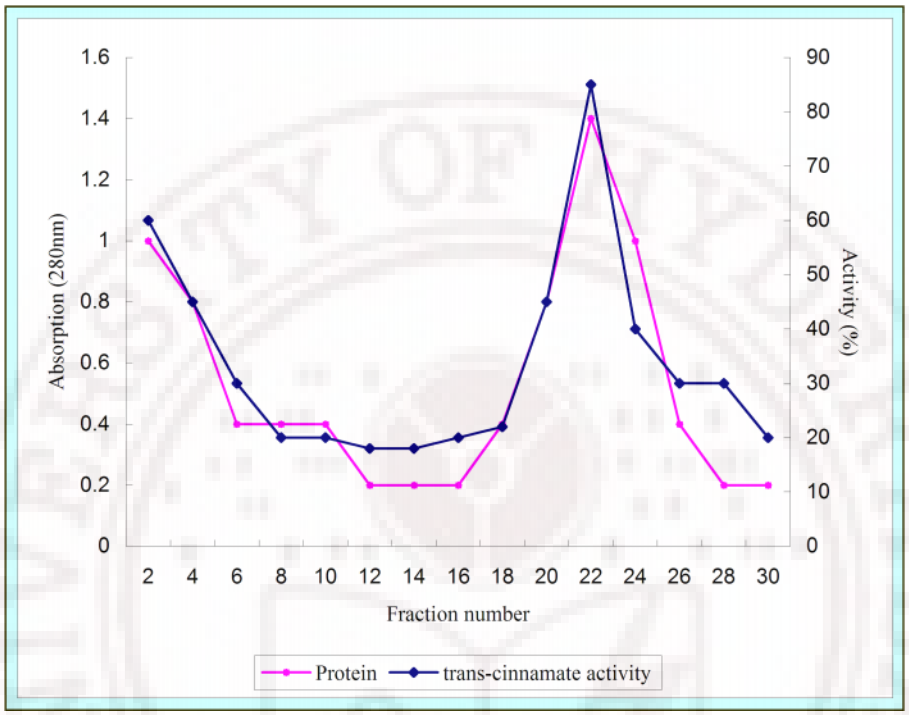


Fig 15: DEAE-Cellulose elution profile of protein

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.

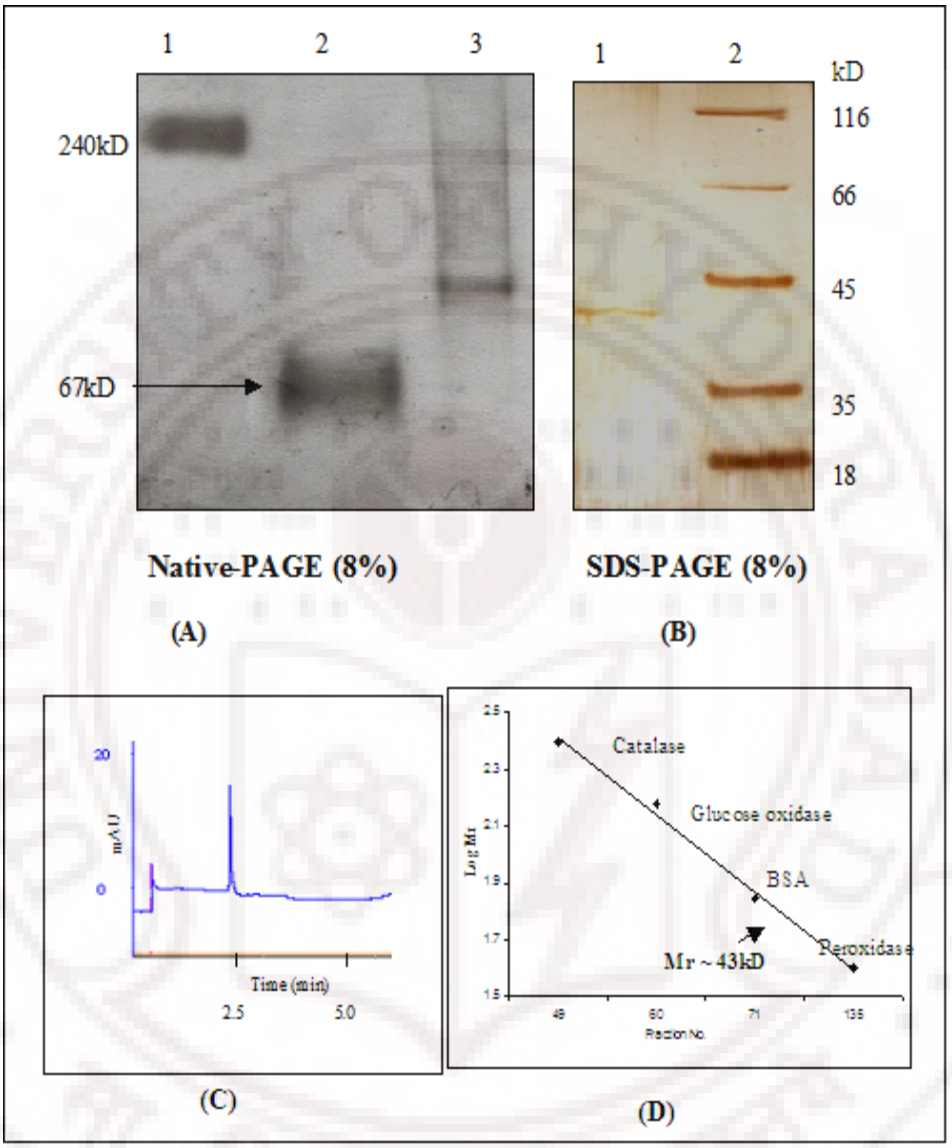


Fig 16(A): Purified protein in 8 % Native PAGE
Lane 1 = Catalase, 240 kD (native marker); Lane 2 = Bovine Serum Albumin, 67 kD (native marker);
Lane 3 = DEAE-Cellulose protein fraction
(B): Silver stained gel of purified protein in 8 % SDS-PAGE
Lane 1 = Purified protein; Lane 2 = Marker
(C): HPLC chromatogram showing the purity of the protein
(D): The molecular weight analysis of the protein using FPLC

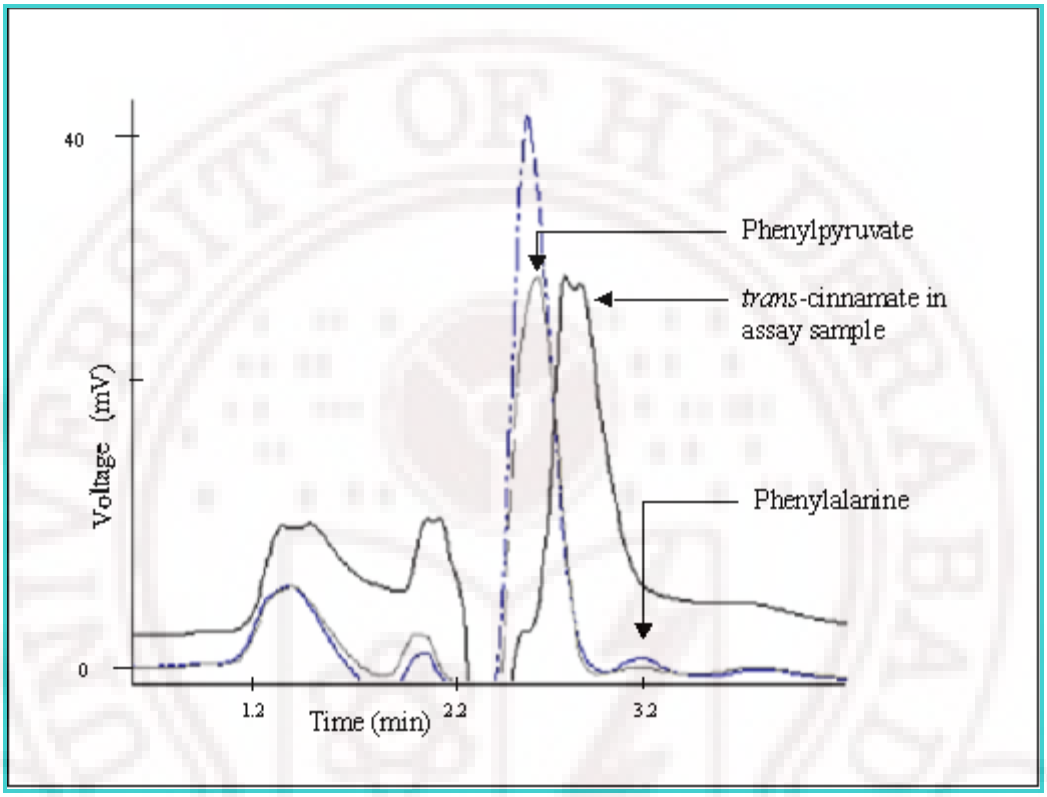


Fig 17: HPLC chromatogram showing the transformation of *trans*-cinnamate to phenylalanine by the purified protein

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.

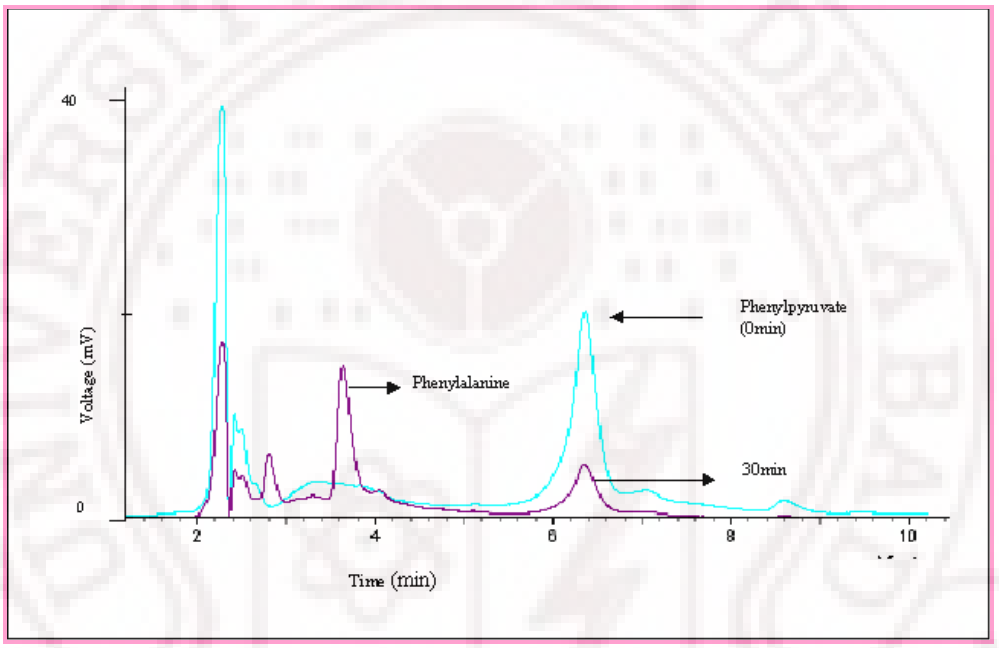


Fig 18: HPLC chromatogram showing the conversion of phenylpyruvate to phenylalanine by the purified protein

The chromatogram showing compounds in the assay sample at 0 min (blue coloured) and 30 min (pink coloured) were registered at 220 nm.

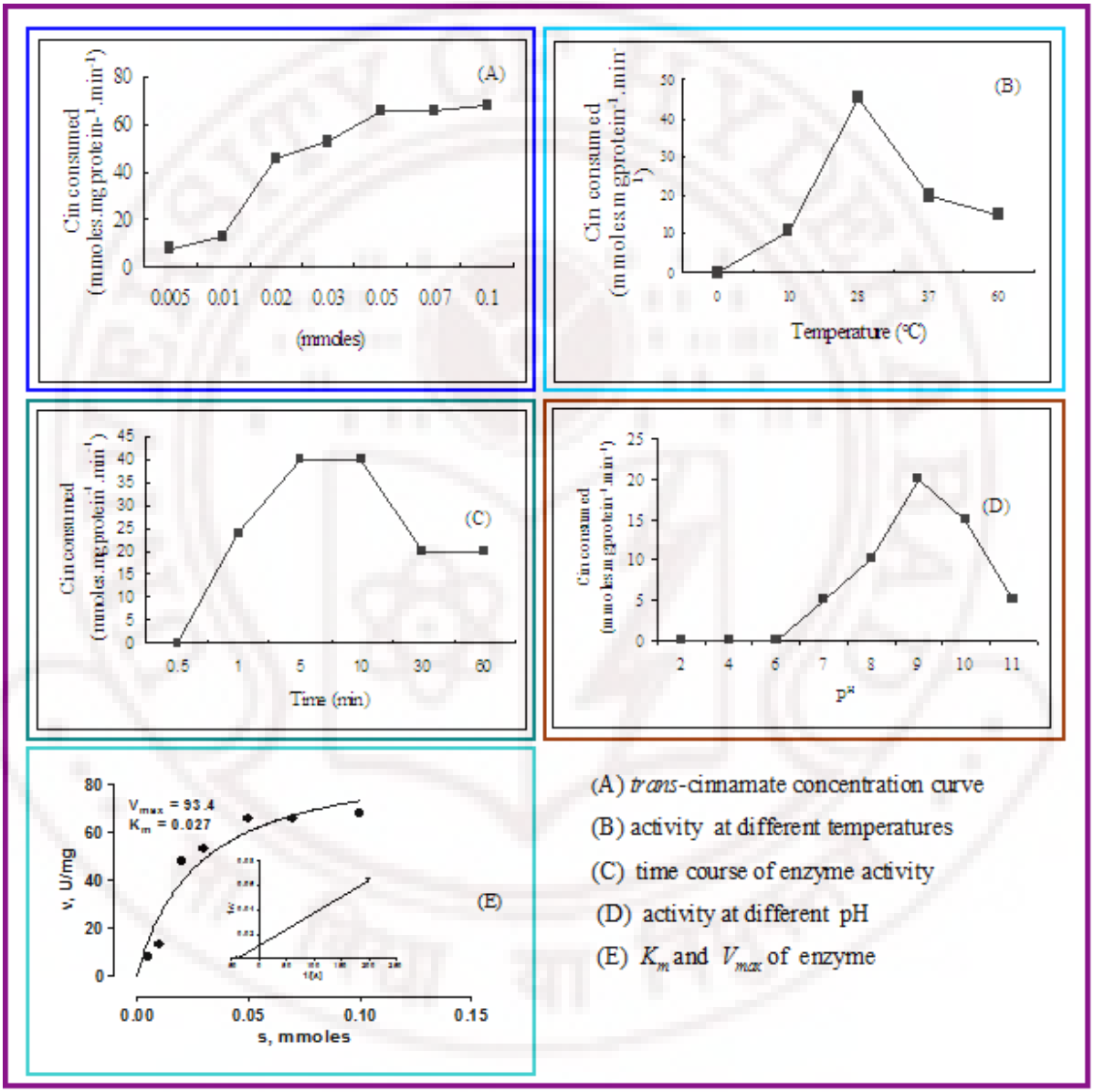


Fig 19: Characterization of enzyme catalyzing *trans*-cinnamate to phenylalanine

(Cin = *trans*-cinnamate)

Step	Volume (ml)	Total protein (mg)	Specific activity (Umg^{-1})	Yield (%)	Fold purification
Crude extract	70	245	0.6	100	1.0
60-90 % $(NH_4)_2SO_4$ fraction	6	0.17	2.4	56	4
DEAE-Cellulose fraction	4	0.03	8.8	8.5	14

Table 12: Summary of the protein yield from the major purification steps

Substrates	**Activity (%)**
trans-Cinnamate	100
4-Hydroxycinnamate	40
3, 4-Dihydroxycinnamate	40
Coenzymes	
NADH	85
NADPH	-
PLP	-

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

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 *Rhodobacter sphaeroides* OU5**

Metabolites like indole and tryptophan are released into *trans*-cinnamate induced culture supernatant of *Rba. sphaeroides* OU5 (Table 5 and 6 in chapter 2). Using cell free extracts of *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 (mH^{-2}) corresponding to phenyl acetaldehyde and 115 (mH^{+2}), 163 (mH^{+2}) corresponding to compounds, indole and phenylpyruvate respectively were detected and they were absent in the control assay mixture (without *trans*-cinnamate).

Assay components	*trans*-cinnamate consumption (µmoles.mg protein^{-1})	Products formed (µmoles.mg protein^{-1})	
		Indole	L-tryptophan
Control	ND	ND	ND
Ammonia	180	ND	ND
Ammonia +HA	140	50	ND
Ammonia + serine + PLP	130	ND	100

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 µmoles of *trans*-cinnamate, along with assay components like 0.1ml of 10 % NH_4OH (ammonia solution), L-serine at 100 µmoles, pyridoxal phosphate 20 µg with 280 µg of protein and incubated for 45 min at room temperature. Hydroxylamine was added at a concentration of 50 µmoles 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.

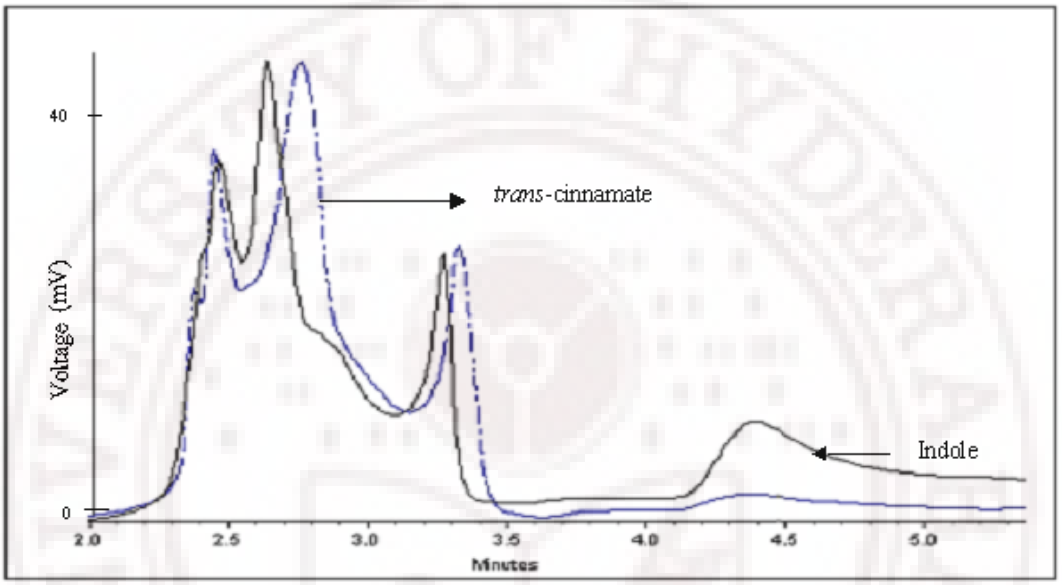


Fig 20: HPLC chromatogram of the enzyme assayed fraction showing *trans*-cinnamate and indole

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 µmoles of *trans*-cinnamate, along with 0.1 ml of 10 % NH_4OH, 50 µmoles of hydroxylamine with 280 µg of cell free extract and incubated for 45 min at room temperature.

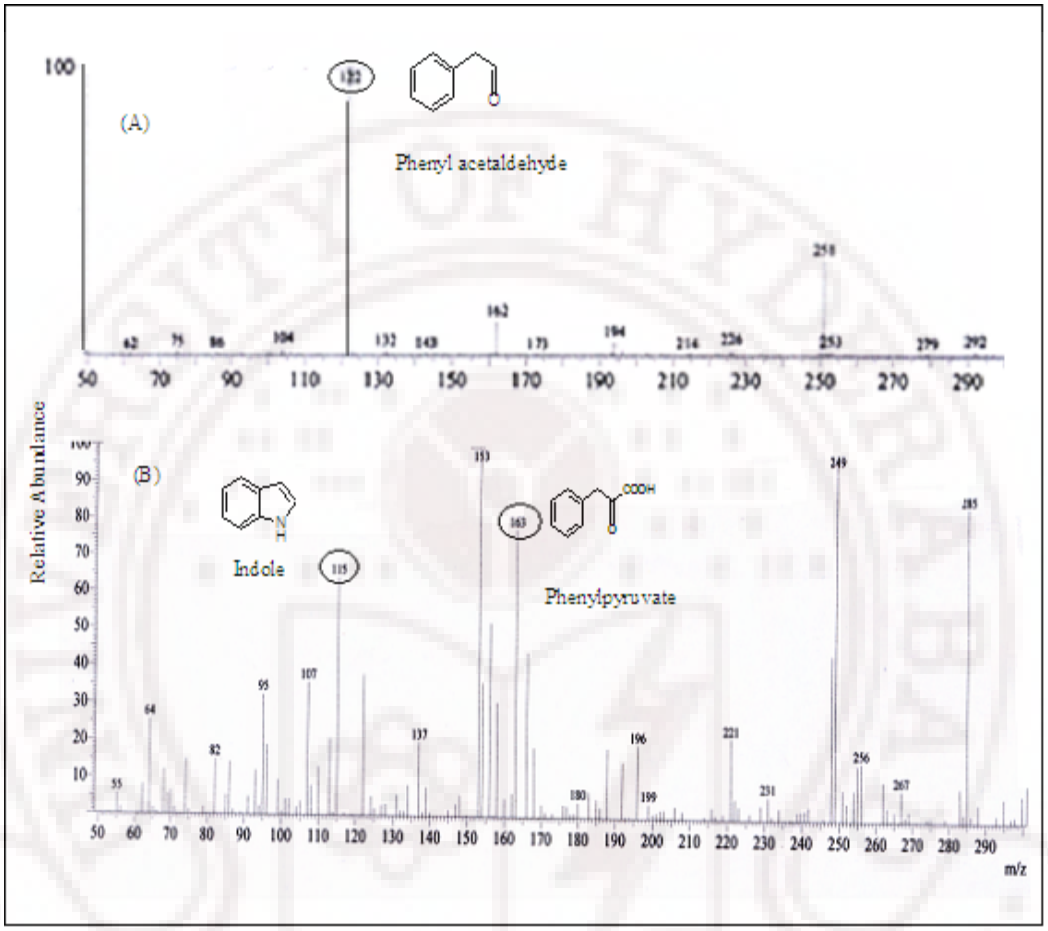


Fig 21: LC-MS metabolome profiling of the enzyme-assayed sample showing intermediates of *trans*-cinnamate to tryptophan transformation

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-

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.

Growth	Biomass yield (mg drywt.ml^{-1})	4-hydroxycinnamate consumption (mM)	3, 4-dihydroxy-cinnamate consumption (mM)
Resting cells	0.45	0.33	0.45
Growing cells	0.37	0.35	0.44

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

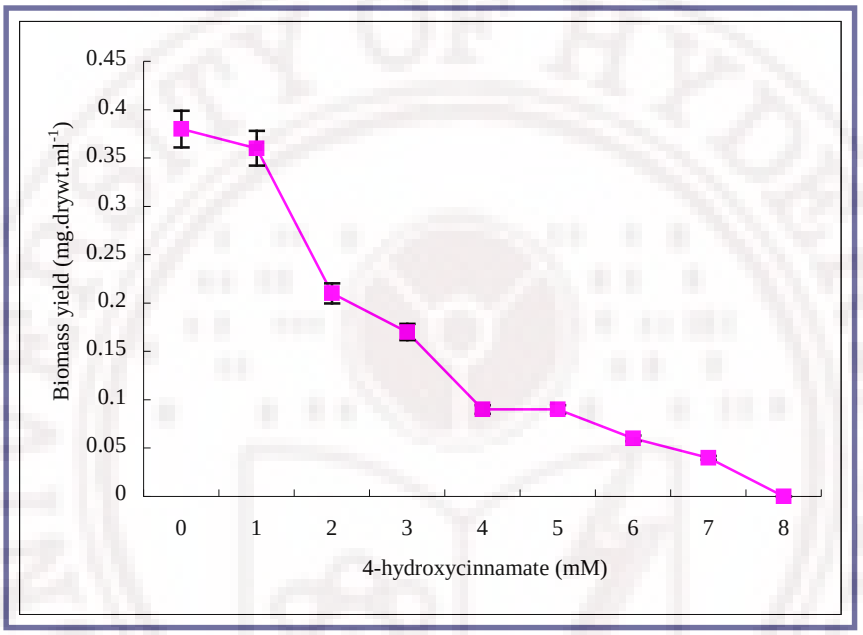


Fig 22: Minimum inhibitory concentration of 4-hydroxycinnamate on the photoheterotrophic growth of *Rba. sphaeroides* OU5

Assay was done with the growing cells of *Rba. sphaeroides* OU5 in photoheterotrophic medium with malate (22 mM) as sole carbon source and NH_4Cl (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 $^{\circ}$C in fully filled screw cap test tubes and growth was measured turbidometrically.

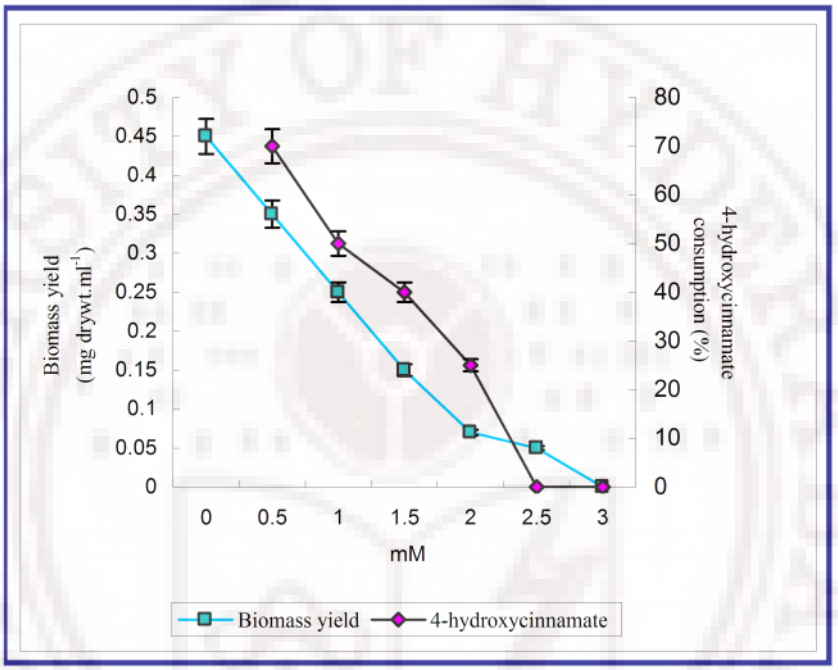


Fig 23: Assimilation at different concentrations of 4-hydroxycinnamate by growing cells of *Rba. sphaeroides* OU5

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 different concentrations of 4-hydroxycinnamate and assayed after 48 h incubation.

Organic substrate (0.3 % w/v)	Biomass yield (mg drywt.ml^{-1})		4-hydroxycinnamate consumption (mM)
	Without 4hydroxyci -nnamate	With 4-hydroxy cinnamate	
Control (With out organic substrate)	0.06	0.05	0.2
Glucose	0.45	0.42	0.45
Pyruvate	0.75	0.65	0.37
Acetate	0.4	0.30	0.2
Oxaloacetate	0.42	0.35	0.3
α-keto glutarate	0.44	0.30	0.3
Succinate	0.35	0.25	0.2
Fumarate	0.6	0.54	0.3
Malate	0.5	0.36	0.1

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 ^{o}C, in the presence of respective organic substrates (0.3 % w/v) as carbon sources and NH_4Cl (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.

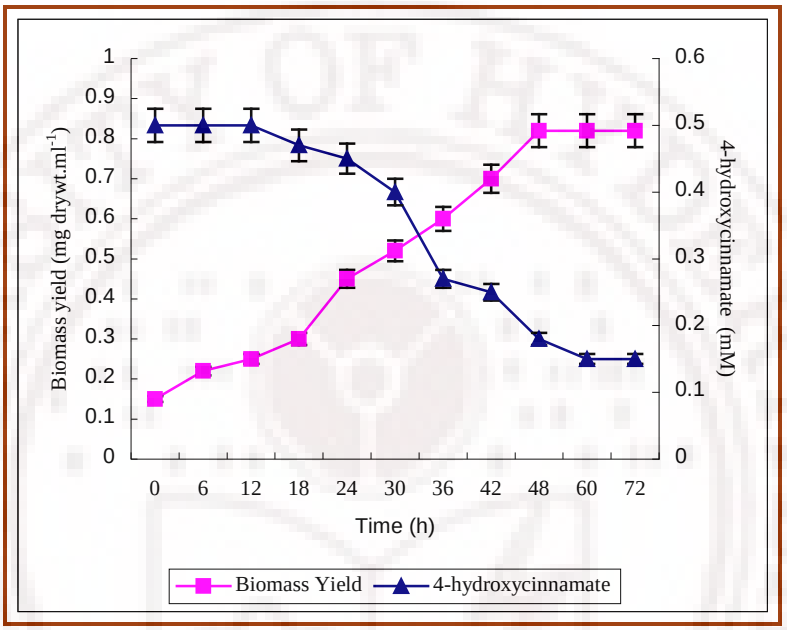


Fig 24: Assimilation and growth of 4-hydroxycinnamate by growing cells of *Rba. sphaeroides* OU5 with time

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.

Assay components	4-hydroxycinnamate consumption (µmoles.mg protein^{-1})	L-tyrosine formation (µmoles.mg protein^{-1})
Control (without Ammonia, NADH)	0	0
Ammonia + NADH	700	100

Table 17: Transformation of 4-hydroxycinnamate by cell free extracts of *Rba. sphaeroides* OU5

The assay mixture contained 1ml of 10 mM Tris HCl buffer (pH 7.8) with 100 µmoles of 4-hydroxycinnamate, along with 25 µmoles of NADH, 0.1ml of 10 % NH_4OH 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.

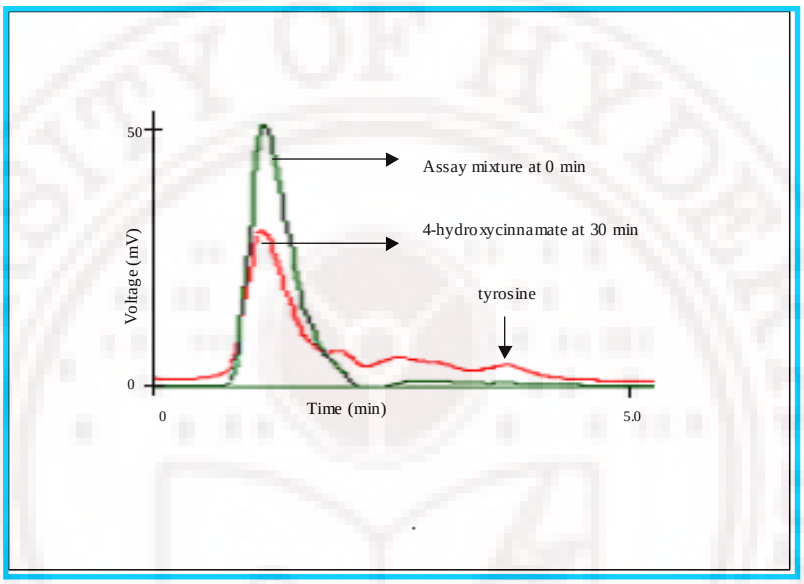


Fig 25: HPLC chromatogram showing the transformation of 4-hydroxycinnamate by cell free extracts of *Rba. sphaeroides OU5*

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 upto 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.

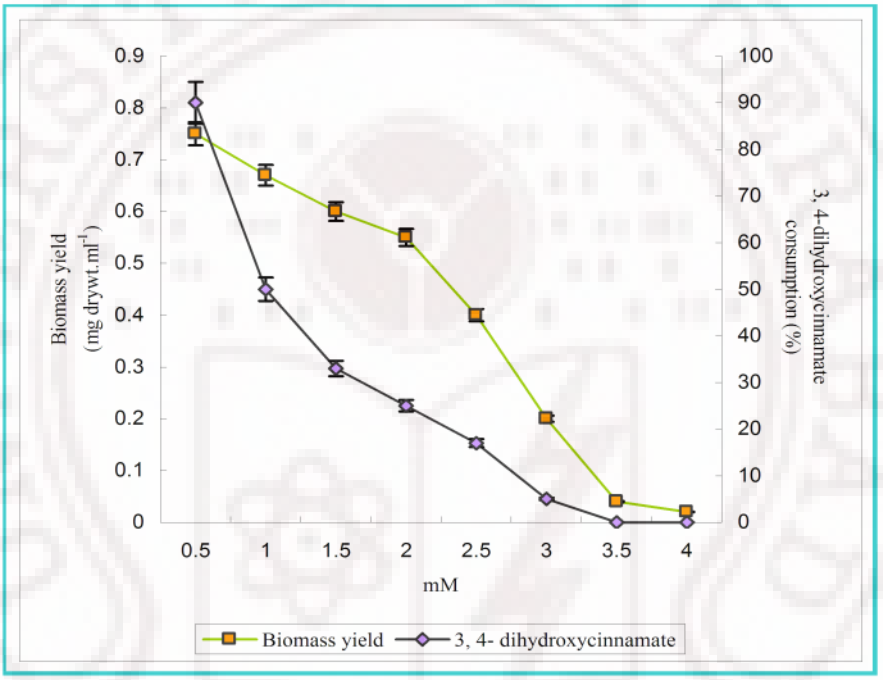


Fig 26: **Assimilation at different concentrations of 3, 4-dihydroxycinnamate by *Rba. sphaeroides* OU5**

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.

Organic Substrate (0.3 % w/v)	Biomass yield (mg drywt.ml^{-1})		3, 4-dihydroxy cinnamate consumption (mM)	3, 4-dihydroxy phenylalanine production (mM)
	Control	3, 4-dihydroxy cinnamate		
Control (without organic substrate)	0.06	0.02	0.3	ND
Glucose	0.44	0.60	0.1	ND
Pyruvate	0.75	0.75	0.47	0.16
Acetate	0.4	0.4	0.44	ND
Oxaloacetate	0.42	0.44	0.5	ND
α-keto glutarate	0.44	0.45	0.5	ND
Succinate	0.35	0.35	0.3	ND
Fumarate	0.57	0.55	0.36	ND
Malate	0.51	0.5	0.5	0.2

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_4Cl (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.

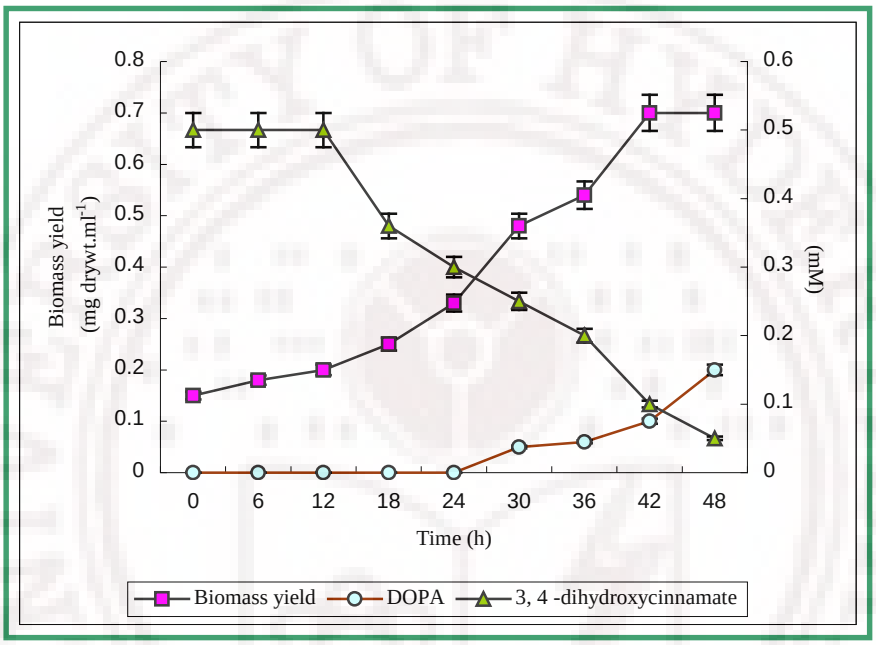


Fig 27: Photoassimilation of 3, 4-dihydroxycinnamate and production of DOPA by growing cells of *Rba. sphaeroides* OU5 with time

(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.

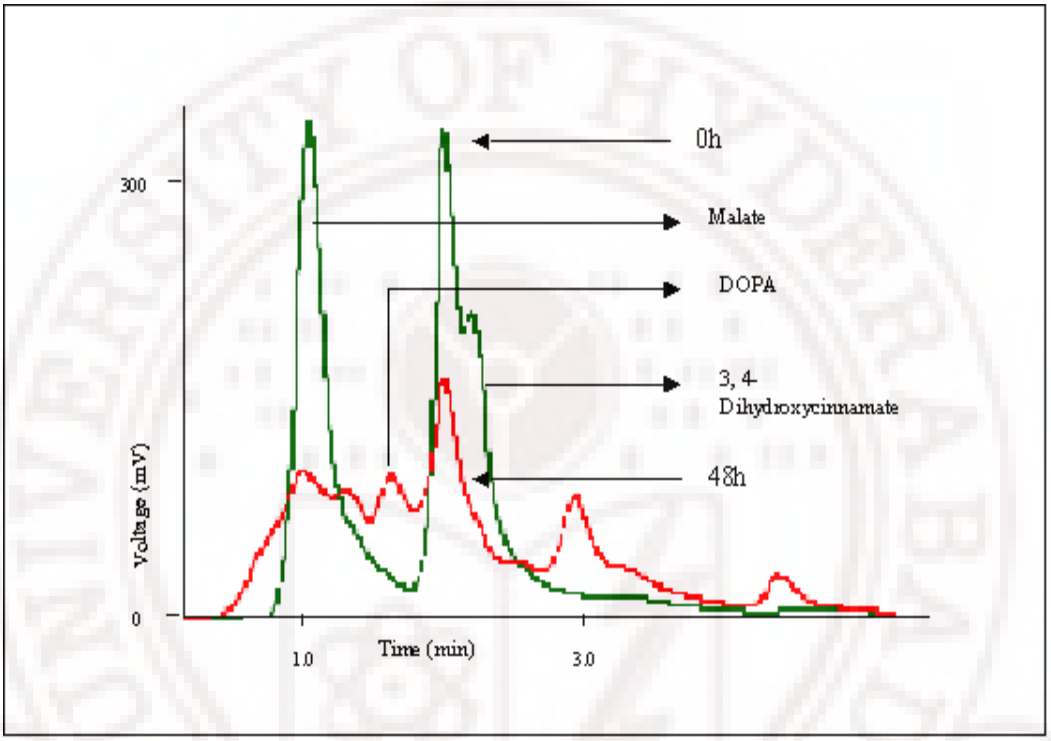


Fig 28: HPLC chromatogram showing the transformation of 3, 4-dihydroxycinnamate to 3, 4-dihydroxyphenylalanine by growing cells of *Rba. sphaeroides* OU5

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.

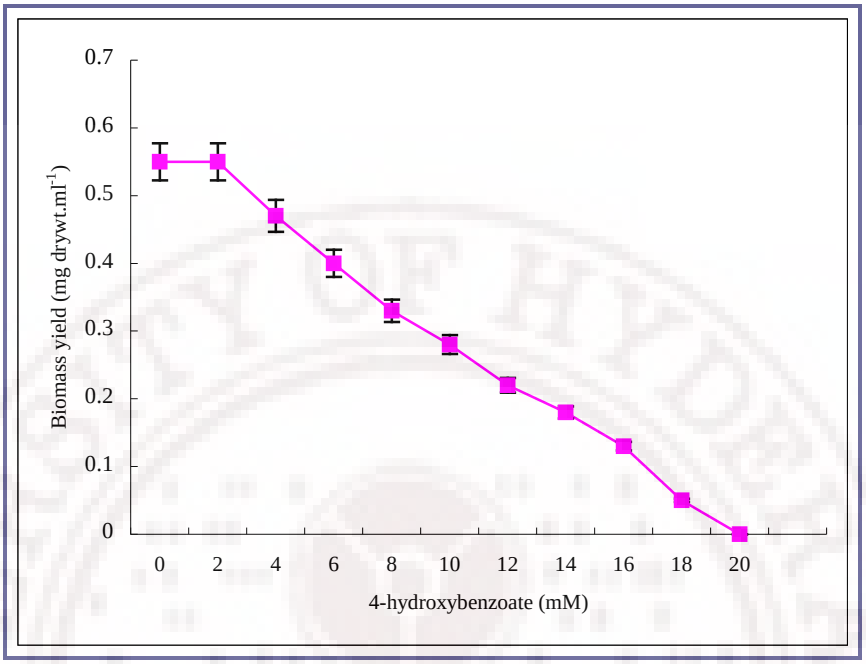


Fig 29: Minimum inhibitory concentration of 4-hydroxybenzoate on the photoheterotrophic growth of *Rba. sphaeroides* OU5

Assay was done with the growing cells of *Rba. sphaeroides* OU5 in photoheterotrophic medium with malate (22 mM) as sole carbon source and NH_4Cl (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 $^{\circ}$C in fully filled screw cap test tubes and growth was measured turbidometrically.

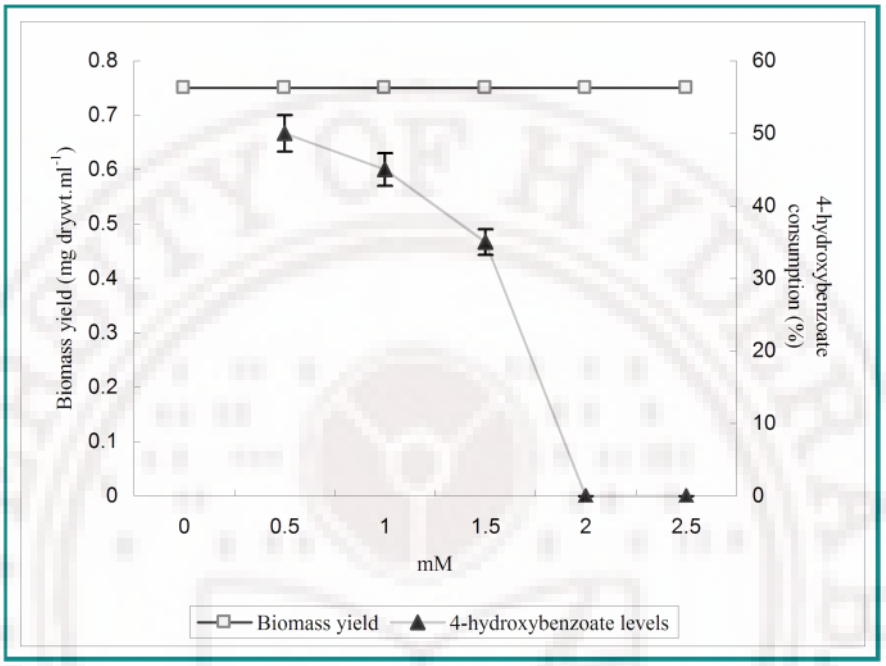


Fig 30: Assimilation at different concentrations of 4-hydroxybenzoate by growing cells of *Rba. sphaeroides* OU5

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.

Organic substrate (0.3 %)	Biomass Yield (mg drywt.ml^{-1})		4-hydroxybenzoate consumption (mM)
	Control	4-hydroxybenzoate	
Control (With out carbon)	0.06	0.07	0
Glucose	0.45	0.45	0
Pyruvate	0.75	0.7	0.2
Acetate	0.4	0.35	0
Oxaloacetate	0.42	0.5	0
α-keto glutarate	0.44	0.4	0
Succinate	0.35	0.4	0
Fumarate	0.6	0.56	0.25
Malate	0.5	0.50	0

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)

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.

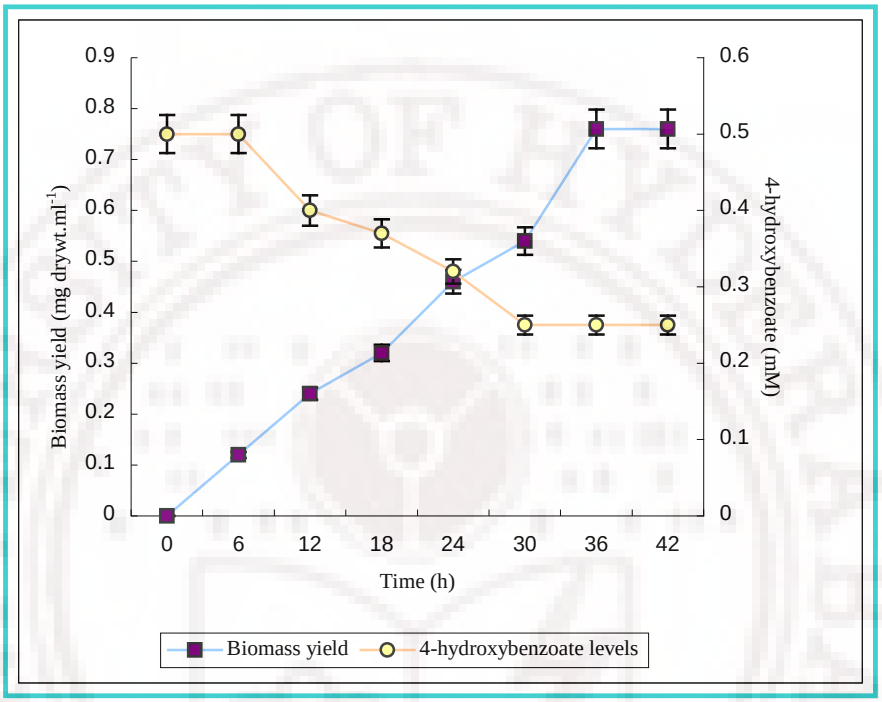


Fig 31: Photoassimilation of 4-hydroxybenzoate by growing cells of *Rba. sphaeroides* OU5 with time

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.

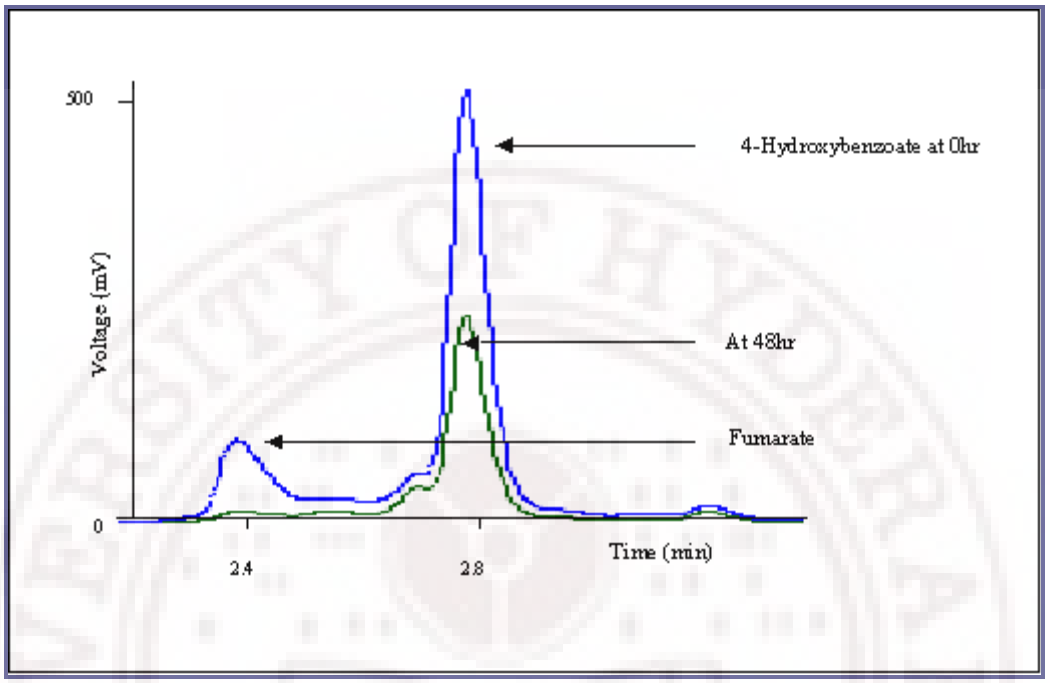


Fig 32: HPLC chromatogram showing 4-hydroxybenzoate assimilation by whole cells of *Rba. sphaeroides* OU5

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.

Assay components	4-hydroxybenzoate consumption (μmoles.mg protein^{-1})
None	ND
Fumarate	ND
Pyruvate	100
Pyruvate + Ammonia	50
Pyruvate + Ammonia +NADH	70

Table 20: 4-Hydroxybenzoate consumption by cell free extracts of *Rba. sphaeroides* OU5 (ND = not detected)

The assay mixture (1ml) contained 10 mM Tris-buffer (pH 7.8) with 100 μmoles of 4-hydroxybenzoate, along with the assay components like 25 μmoles of NADH, 0.1ml of 10 % NH_4OH (buffered ammonia), pyruvate 200 μmoles 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.

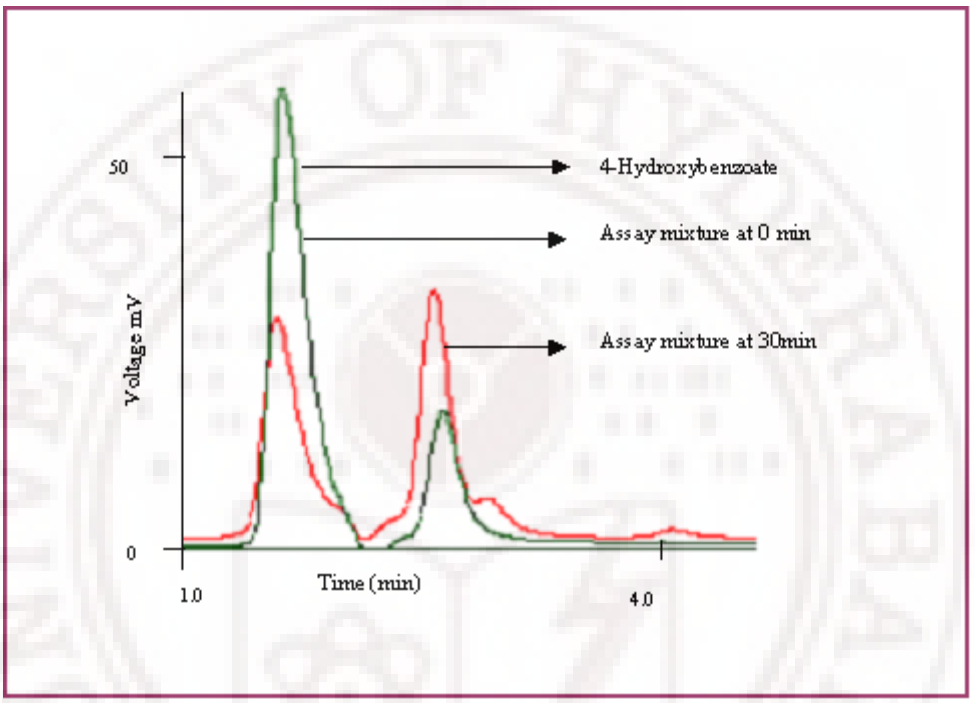


Fig 33: HPLC chromatogram showing the consumption of 4-hydroxybenzoate by cell free extracts of *Rba. sphaeroides* OU5

The assay was done with the cell free extract of *Rba. sphaeroides* OU5. The consumption of 4-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).

Discussion

4.1 **Background Introduction**

Aromatic compounds formed by microbial depolymerization of proteins and lignins are the second most abundant class of organic compounds in nature (next to carbohydrates) (Harwood and Parales, 1996; Boll *et al*., 2002). The biodegradation of monoaromatic compounds in particular phenyl propanoid side chain, is important for the global carbon cycle from an environmental point of view because these compounds are released from plant waste as breakdown products of lignin (Peng *et al*., 2003). Ubiquity and abundance of these aromatic compounds highlight the significance of their degradation for the maintenance of biogeochemical carbon cycle and in the sustainable development of the biosphere (Evans, 1977; Gibson and Harwood 2002). Aromatic compound degradation accomplished mainly by microorganisms indicate the growth-linked utilization of aromatic compounds as sole source of carbon or energy involving benzene ring cleavage and assimilation of the resulting aliphatic molecules (Harayama and Timmis, 1992). By definition assimilation means uptake of inorganic/organic elements into biomass and differs from dissimilatory process, which is discussed in the introduction (page 11-13).

Among the purple non-sulfur bacteria, *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* are the most widely distributed species among different habitats (Madigan, 1988). *Rps. palustris* utilizes aromatic compounds as growth substrates by aromatic ring cleavage (Harwood and Gibson, 1988), while *Rba. sphaeroides* OU5 lacks this capability. However, strain OU5 could utilize heterocyclic aromatic hydrocarbons like 4-dimethylaminopyridine, pyrazine, 2-aminopyrazine for growth (Rajashekar *et al*., 2000), indeed, the degradative pathways are not yet established in this strain. The present study deals with the assimilatory utilization of a few homocyclic aromatic compounds (including

trans-cinnamate and its hydroxy derivatives) by *Rba. sphaeroides* OU5 together with metabolic pathways and enzymes involved in the process.

4.2 **Toxicity of aromatic compounds**

Aromatic compounds like mineral oils (Singh and Kumar, 1991), polycyclic aromatic hydrocarbons, industrial solvents (Playne and Smith, 1983; Kilroy and Grey, 1992), agrochemicals (Perona *et al*., 1991; Cserhatieti *et al*., 1992), and surface-active compounds (Tubbing and Admiraal, 1991) are toxic to microbial cells, resulting from biochemical disturbances caused by these compounds in one or more metabolic pathways (Sikkema *et al*., 1995). Functions that were affected due to the toxic effect of aromatics in microbial cells include; growth, transport systems, CO_2 fixation (cyanobacterial) and gas production (by anaerobic bacteria) (Playne and Smith, 1983; Sierra-Alvarez and Lettinga, 1991). Inhibition in growth and leakage of macromolecules, altered ultra structure of the cells impaired membrane functions by pH dissipation across cytoplasmic membrane (Salmond *et al*., 1984) by lactic acid, benzoic acids (Warth, 1991) and other lipophilic compounds were also reported.

Toxicity of aromatic compounds on growth of *Rhodobacter sphaeroides* OU5 was analyzed in terms of IC_{50} (Table 3), which varied depending on structure and position of functional groups. Among the compounds analyzed (Table 3), hydroxy and aminobenzoates were inhibitory to the growth of strain OU5. Mono and dihydroxylated cinnamates were more toxic than cinnamic acid in *Rhodobacter sphaeroides* OU5 (Table 3) as observed earlier (Middelhoven and Gelpke, 1995; Hoskins, 1984) on the growth of several microorganisms (Hoskins, 1984; Tawata, *et al.*, 1996; Burt, 2004). The toxicity could be due to the inhibition of cytoskeleton development of the cell, which was affected by the calcium

gradient due to the presence of *trans*-cinnamate and its derivatives (Said *et al*., 2004; Neves *et al*., 2005). On the other hand, growth inhibition in *Rba. sphaeroides* OU5 could also be due to the inhibition of *denovo* synthesis of aromatic amino acids, since DAHP synthase activity (Table 7) was inhibited completely by *trans*-cinnamate in the assay with cell free extracts in contrast to partial inhibition with hydroxycinnamates. 3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (EC 2.5.1.54), a key enzyme in the shikimate pathway of aromatic amino acid biosynthesis that catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E-4-P) (Bentley, 1990; Gosset *et al*., 2001). Though there are no reports of *trans*-cinnamate inhibiting aromatic amino acid biosynthesis, glyphosate a non-selective herbicide inhibits EPSP synthase, an enzyme of shikimate pathway of aromatic amino acid biosynthesis. EPSP synthase EC 2.5.1.19 (5-enolpyruvylshikimate-3-phosphate synthase) catalyzes the condensation of shikimic acid and phosphoenolpyruvate (Steinrucken and Amrhein, 1980).

Cells of *Rba. sphaeroides* OU5 grown in the presence of *trans*-cinnamate or other aromatic compounds were viable on nutrient agar (data not presented in results chapter), which only indicated that these compounds are growth retardants and not lethal. Growth inhibition of *trans*-cinnamate on *Rba. sphaeroides* OU5 could either be reduced or enhanced due to the presence of organic molecules (Fig 7, Fig 8), which aid in reducing the toxicity and growth inhibition of xenobiotics on cells there by increasing the transformation rates of xenobiotics (Fakhruddin and Quilty, 2005). Glucose and oxaloacetate promoted growth, while presence of α-ketoglutarate, succinate and pyruvate increased the toxicity of *trans*-cinnamate (Fig 7). Amino acids like L-glutamate, glutamine and a mixture of aromatic amino acids promoted growth of *Rba. sphaeroides* OU5 (Fig 8), while a few (serine,

phenylalanine, tyrosine and tryptophan) had no effect. These carbon sources might act as an inducing agent for biodegradation/biotransformation enzymes or provide reducing power for degradation (Fakhruddin and Quilty, 2005; Chaudhuri and Wiesmann, 1995; Perkins *et al*., 1994).

4.3 **Assimilation of aromatic compounds by whole cells of *Rba. sphaeroides* OU5**

Cinnamate and hydroxycinnamates were utilized for growth by many bacteria, yeast and fungi, despite their toxic nature (Edlin *et al*., 1994). *trans*-Cinnamate was utilized as a sole source of carbon by a phototrophic bacterium *Rhodopseudomonas palustris* (Elder *et al*., 1992a) and the presence of this compound aided in the species selective enrichments (Madigan and Gest, 1988). Though growth of *Rba. sphaeroides* OU5 with *trans*-cinnamate as carbon/e^- donor could not be demonstrated, the light dependent utilization (Table 4) indicated the assimilatory process by both growing (Fig 12A) and resting cells (Fig 10) (Usha *et al*., 2007).

Among the aromatic compounds screened (Table 3), *Rba. sphaeroides* OU5 assimilated hydroxycinnamates, 4-hydroxybenzoate, phenylpyruvate and phenylalanine. Though assimilation of aromatic (phenylalanine; Miseta *et al*., 1996) and aliphatic amino acids (Gale and Folkes, 1962) was documented, the present study indicated the possibility of assimilation of other aromatic compounds by *Rba. sphaeroides* OU5. This process may be restricted only to a few aromatic compounds (cinnamates), since many others (toluene, cresol, amino and hydroxybenzoates) were not assimilated and one of the major reasons could be due to the lack of specific permeases or diffusional barriers across the cytoplasmic membrane limiting their entry into the cell.

4.4 **Influence of carbon substrates on assimilation of aromatic compounds**

Presence of glucose (Table 5, 16 and 18) along with ammonia (Table 6) promoted complete assimilation of *trans*-cinnamate and 4-hydroxycinnamate by whole cells of *Rba. sphaeroides* OU5. The reasons for acceralated assimilation in *trans*-cinnamate (Chamkha *et al*., 2001) and its derivatives (Oddou *et al*., 1999; Sachan *et al*., 2006) in the presence of glucose could not be predicted even from the present study. However, ammonia played a key role as an amino donor in the transformation of *trans*-cinnamate to phenylalanine (Table 7). Though glucose utilization was observed in control (without *trans*-cinnamate), its levels remained constant as initial in *trans*-cinnamate induced culture (Fig 12B) that indicates the prevention of supply of glucose to synthesize aromatic molecules. The inhibition of *trans*-cinnamate assimilation in *Rba. sphaeroides* OU5 in the presence of malate, fumarate, succinate and oxaloacetate (Table 5) is similar to those observed with *Pseudomonas* spp. (Basu *et al*., 2006) and are in contrary to the assimilation of 3, 4-dihydroxycinnamate (Table 18) and 4-hydroxybenzoate (Table 19). The summary of the substrates, which promoted and inhibited assimilation of aromatic compounds in *Rba. sphaeroides* OU5 is presented in Fig 34.

4.5 **Cinnamates and its hydroxy derivatives are photobiotransformed to aromatic amino acids by *Rba. sphaeroides* OU5**

Bacterial catabolism of cinnamates and hydroxycinnamates follows a *β*-oxidation pathway (Elder *et al*., 1992a). Most often they are also metabolized by different microorganisms into reduced products, whose mechanism involves reduction of the double

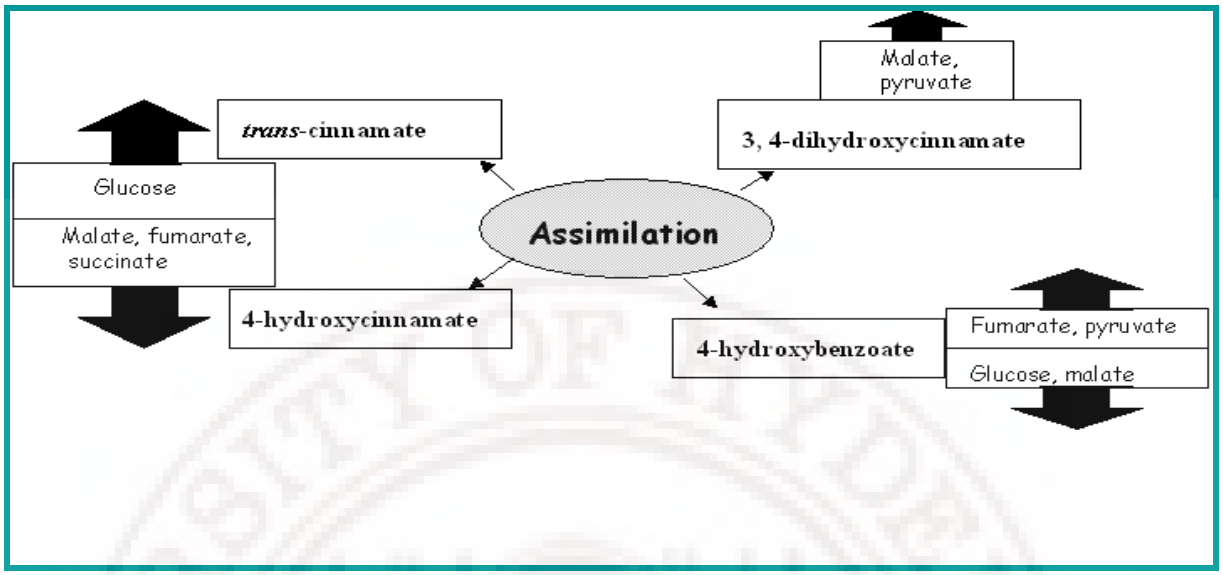


Fig 34: Influence of carbon substrates on the assimilation of aromatic compounds by *Rba. sphaeroides* OU5 (▲ = promoted assimilation; ▼ = inhibited assimilation)

bond in the C_3 aliphatic side chain (Nali *et al*., 1985; de la Torre and Gomez-Alarcon, 1991; Grbic-Galic and Young, 1985; Andreoni *et al*., 1984; Labuda *et al*., 1993). Decarboxylation of the propane side chain of cinnamic acids are the other metabolic patterns found in some microorganisms (Cavin *et al*., 1997a; Edlin *et al*., 1998). These are efficiently biotransformed to L-phenylalanine catalyzed by an enzyme phenylalanine ammonia lyase, also called in short as PAL (Yamada *et al*., 1981; Evans *et al*., 1987a; Evans *et al*., 1987b).

Rba. sphaeroides OU5 metabolized *trans*-cinnamate (Fig 11) and hydroxycinnamates (Fig 23) as evidenced by their loss from the culture supernatant. The transformed products of these compounds were identified using HPLC (Fig 11, 25) as aromatic amino acids and intermediates of the biosynthetic pathway. The pathway metabolites could be enhanced in the presence of chloramphenicol (Fig 13). Tyrosine, indole tryptophan and phenylalanine were the major metabolites identified (Table 5) from the

culture supernatant of *trans*-cinnamate induced *Rba. sphaeroides* OU5 after 48 hours of phototrophic assay. However, LC-MS analysis (Fig 13) indicated that there are many more metabolites than the HPLC identified ones from the *trans*-cinnamate induced cultures of *Rba. sphaeroides* OU5. These had the molecular masses (m/z) of 161, 171, 376 and do not match with the aromatic amino acids. The identification of these could not be confirmed in this study.

Using cell free extracts of *Rba. sphaeroides* OU5, transformation of 4-hydroxycinnamate to tyrosine (Table 16, 17 and Fig 25) and 3, 4-dihydroxycinnamate to L-DOPA (3, 4-dihydroxyphenylalanine; Table 19 and Fig 27) by whole cells of *Rba. sphaeroides* OU5 could be demonstrated. L-DOPA is a drug of choice for neurological injury and Parkinson's disease (Lee *et al*., 1996) and is mainly produced by plants (Haq *et al*., 2000). DOPA is also produced from L-tyrosine by the action of enzyme tyrosinase reported mainly in fungi (Rossaza *et al*., 1995; Ali and Haq, 2001) and its production by *Rba. sphaeroides* OU5 was demonstrated recently (Ranjith *et al*., 2007a). The downstream of DOPA in *Rba. sphaeroides* OU5 is catalyzed by at least 3 enzymes (Ranjith, 2008). (1) DOPA-reductive deaminase (EC 4.3.1.22) is a NADH dependent 234 kD heterotetramer (110, 82, 43 and 39 kD) catalyzed the formation of 3,4-dihydroxyphenylpropanoate (Ranjith *et al*., 2007a); (2) The 2-oxoglutarate dependent DOPA-transaminase (EC 2.6.1.49) catalyzed the formation of 3,4-dihydroxyphenylpyruvate and is PLP dependent (Ranjith *et al*., 2007a) and (3) An oxygen dependent DOPA-oxidative deaminase (EC 1.13.12.15) of 190 kD pentameric (54, 42, 34, 25 and 23 kD) enzyme catalyzed the formation of 3, 4-dihydroxyphenylpyruvate and ammonia from DOPA (Ranjith *et al*., 2008).

4.6 **Phenylalanine ammonia lyase (EC 4.3.1.5) is absent in *Rba. sphaeroides* OU5**

Phenylalanine ammonia lyase (PAL) is an ubiquitous enzyme in higher plants, catalyzing the non-oxidative deamination of L-phenylalanine to *trans*-cinnamic acid, ammonium ions and vice-versa (Ritter, 2004). Some PAL enzymes, in addition to phenylalanine, are also specific to tyrosine as substrate and are therefore called phenylalanine/tyrosine ammonia lyase (PAL/TAL) or TAL, depending on the relative activity towards these substrates (Xue *et al*., 2007). PALs were also identified in bacteria like *Streptomyces maritimus* (Xiang and Moore, 2005), *Photorhabdus luminescens* (Williams *et al*., 2005), *Sorangium cellulosum* (Hill *et al*., 2003) and *Streptomyces verticillatus* (Emes and Vining, 1970). In these bacteria, the PAL product cinnamic acid serves as an intermediate in the biosynthesis of antibiotic or antifungal compounds (enterosin, 3, 5-dihydroxy-4-isopropyl-stilbene, soraphan A and cinnamamide). PALs were also identified in cyanobacteria like *Anabaena veriabilis* and *Nostoc punctiforme*, although the ultimate fate of cinnamic acid in these cyanobacteria is unknown (Moffitt *et al*., 2007). PAL is abundantly present in yeast, especially in red yeast, *Rhodotorula glutinis* that has several commercial applications (D'Cunha *et al*., 1994). *Rodotorula* PAL is effective in the treatment of certain mouse neoplasms and is used to quantitatively analyze serum L-phenylalanine to monitor patients with phenylketonuria (Watanabe *et al*., 1992) and to prepare low phenylalanine diets (Yamada *et al*., 1981). Whole cells of *Rhodotorula* containing PAL have been used to synthesize L-phenylalanine (Yamada *et al*., 1981; Evans *et al*., 1987a; Evans *et al*., 1987b; Takac *et al*., 1995) and its methyl ester (D' Cunha *et al*., 1994) by reversing the physiological reaction.

Deamination of tyrosine catalyzed by enzyme TAL produces 4-hydroxycinnamate (also known as *p*-coumaric acid). The only confirmed bacterial sources of TAL are several species of purple phototrophic bacteria (*Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Halorhodospira halophila*) in which *p*-hydroxycinnamate is a precursor of the chromophore of photoactive yellow protein (PYP) (Kyndt *et al*., 2002; Watts *et al*., 2004) and the actinomycete *Saccharothrix espanaensis* where 4-hydroxycinnamate is used for saccharomycin antibiotic biosynthesis (Berner *et al*., 2006). The total genomic analysis of *Rba. sphaeroides* strains 2.4.1, ATCC 17025, ATCC 17029 (Choudhary *et al*., 2007) indicated the existence of TAL but not PAL (Xue *et al*., 2007) however, either TAL or PAL activity could not be demonstrated in *Rba. sphaeroides* OU5 in this study.

4.7 A novel enzyme involved in the transformation of *trans*-cinnamate to phenylalanine in *Rba. sphaeroides* OU5

Phenylpyruvate was identified as a stable intermediate in the transformation of *trans*-cinnamates in *Rba. sphaeroides* OU5 (Fig 17) and *Corynebacterium equii* (Evans *et al*., 1987a), however the enzyme catalyzing this reaction was not isolated. This enzyme differs from the PAL, in which phenylpyruvate was not the intermediate, suggesting the presence of an alternative enzyme in the synthesis of phenylalanine from *trans*-cinnamate in *Rba. sphaeroides* OU5. HPLC analysis (Fig 17, 18) using the cell free extracts confirmed the presence of an enzyme, which catalyzes the formation of phenylalanine from *trans*-cinnamate/phenylpyruvate (Table 10, 11 and Fig 14). The enzyme was purified (Flow chart 1) to near homogeneity and at every step the activity was confirmed by the conversion of *trans*-cinnamate to phenylalanine. The purified enzyme is a monomer of ~ 43 kD protein (Fig 16 A, B, C, D) which catalyzed the reductive amination of *trans*-cinnamate to phenylalanine (Fig 17, 18). This enzyme is partially characterized (Fig 19) and differs from

PAL, whose molecular weight ranges from 270-300 kD with 4 subunits (MacDonald and D'Cunha, 2007) and catalyzes a reversible reaction. The novel enzyme is named as cinnamyl amino reductase owing to its specificity for *trans*-cinnamate (Table 13) and catalyzing the reductive (NADH dependent) amination of *trans*-cinnamate. The novel enzyme also differs from phenylalanine dehydrogenase (EC 1.4.1.-; catalyzes the conversion of phenylpyruvate to phenylalanine and vice-versa) and the differentiating characters are summarized in table 21. A putative pathway of the *trans*-cinnamate transformation to phenylalanine is proposed in *Rba. sphaeroides* OU5 (Fig. 35) based on the metabolites identified. Incorporation of transformed products of assimilated *trans*-cinnamate (L-phenylalanine) into protein synthesis in terms of increased protein content could not be demonstrated in *Rba. sphaeroides* OU5. However, incorporation of exogenously supplemented L-phenylalanine directly into protein in *Escherichia coli* was reported earlier (Miseta *et al*., 1996).

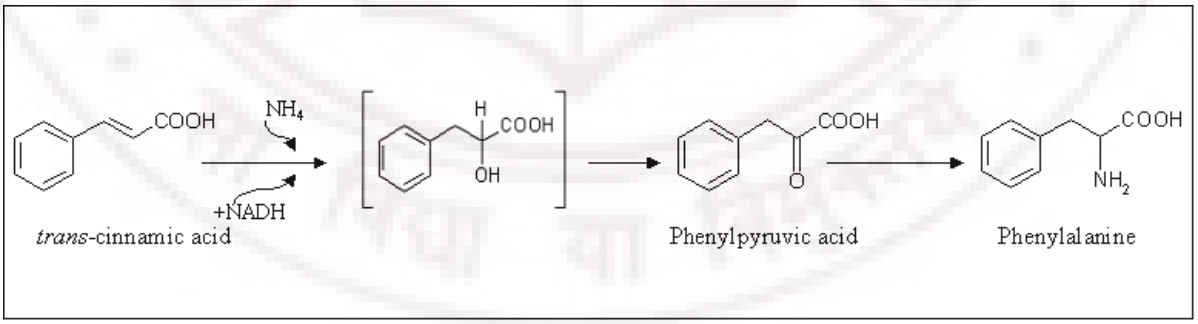


Fig 35: Hypothetical pathway of *trans*-cinnamate transformation to L-phenylalanine in *Rba. sphaeroides* OU5 (compound in parenthesis is the hypothetical compound)

Enzymes	Action	Coenzymes/ Cofactors	*Km* (mM)	M.wt (kD)	Sub-units	References
Phenylalanine ammonia lyase (PAL) (EC 1.4.3.1.5)	reversible	nil	~ 0.2-0.3	270-330	4	MacDonald and D'Cunha, 2007
Phenylalanine dehydrogenase (EC 1.4.1-)	reversible	NAD^+/ NADH	0.1-0.2 3.8	300-340 70	8 2	Asano *et al*., 1987 Misono *et al*., 1989
Cinnamyl amino reductase	unidirectional	NADH	0.27	43	-	Present study

Table 21: Differentiating characters of enzymes

4.7 Is *trans*-cinnamate to L-tryptophan transformation possible in *Rba. sphaeroides* OU5?

Based on the observation of increased indole and tryptophan levels in the supernatant of *Rba. sphaeroides* OU5 grown in presence of *trans*-cinnamate (Table 5, 6 and 14), possible transformation of *trans*-cinnamate to tryptophan was predicted and a putative pathway was proposed (Fig 36). Some of the molecular masses in the LC-MS spectrum (Fig 21) of the enzymatic analysis (of the cell free extract) matched with the predicted metabolite masses of the proposed pathway. Though there are reports on the supplementation of tryptophan inducing PAL in *Ustilago maydis* (Kim *et al*., 2001) no reports on transformation of *trans*-cinnamate to tryptophan are available. Bioproduction of styrene from phenylalanine by *Penicillium camemberti* involved catabolism of phenylalanine to a wide range of hydrophobic compounds (Pagot *et al*., 2006) which include some of the intermediates of the above proposed pathway.

4.8 **4-Hydroxybenzoate assimilation**

Though 4-hydroxybenzoate was assimilated by both whole cells (Table 19 and Fig 32) of *Rba. sphaeroides* OU5 and with cell free extracts (Table 20 and Fig 33), transformed product could not be identified and the work could not be extended in detail. The possible reason for assimilation of 4-hydroxybenzoate could be due to its role in ubiquinone biosynthesis (Pennock and Threlfall, 1983).

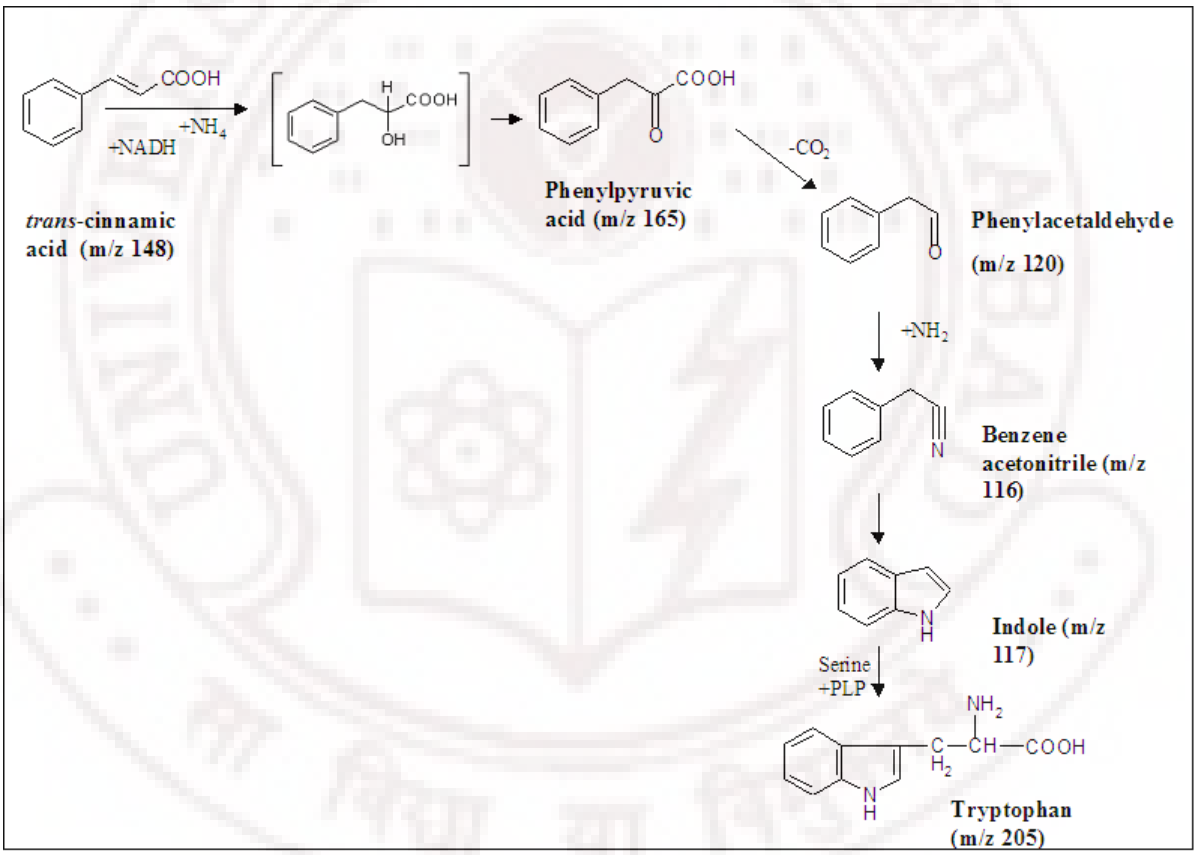


Fig 36: Hypothetical pathway of *trans*-cinnamate transformation to tryptophan in *Rba. sphaeroides* OU5 (compound in the parenthesis is the hypothetical compound)

4.9 **Status of aromatic compound metabolism by *Rba. sphaeroides* OU5**

Strain OU5 is a purple non-sulfur bacterium isolated by Sasikala (1990) and extensive work on the metabolic potential of this strain was done earlier (Nanda, 2003; Sunayana, 2007; Ranjith, 2008). The strain was characterized based only on the phenotypic characters and its molecular characterization needs to be established. On the basis of phenotypic characters strain OU5 belong to *Rhodobacter* closely related to *Rba. sphaeroides* OU5. The unique capability of the strain is its ability to transform aromatic molecules (Sunayana *et al.,* 2005a; Vijay *et al.,* 2006; Ranjith *et al.,* 2007a) leading to the discovery of novel terpenoids, which are potent phytohormones (rhodestrin, rhodethrin), cytotoxic and COX-1 inhibitors (Ranjith *et al.,* 2009) involving both basic and applied research (Sunayana *et al*., 2005b; Ranjith *et al.,* 2007b).

The present study adds to the knowledge of this strain to assimilate aromatic molecules without ring cleavage. Metabolism and toxicity of aromatic compounds studied with reference to *trans*-cinnamate and hydroxycinnamates whose mode of action is through the inhibition of DAHP synthase and are transformed to aromatic amino acids like phenylalanine, tyrosine and tryptophan. The study also suggested the involvement of a novel enzyme catalyzing reductive amination of *trans*-cinnamate to L-phenylalanine.

Conclusions

Conclusions

- Growth independent utilization of aromatic compounds like *trans*-cinnamate, 4-hydroxycinnamate, 3, 4-dihydroxycinnamate, 4-hydroxybenzoate, L-phenylalanine and phenylpyruvate (Table 3) was demonstrated in *Rba. Sphaeroides* OU5.

- Toxicity of aromatic compounds on growth of *Rba. sphaeroides* OU5 analyzed as IC_{50} varied depending on structure and position of functional groups on the aromatic ring.

- Inhibition of the DAHP synthase by *trans*-cinnamate and hydroxycinnamates indicated the possible mode of action of these compounds in inhibiting aromatic amino acid biosynthesis in *Rba. sphaeroides* OU5.

- Presence of organic molecules enhanced / suppressed growth inhibition in presence of *trans*-cinnamate.

- Light dependent assimilation of *trans*-cinnamate was demonstrated by both growing and resting cells of *Rba. sphaeroides* OU5 (Table 4).

- The process of assimilation is influenced in presence of organic molecules (Fig 34).

- In the process of assimilation, transformed products of *trans*-cinnamate and hydroxycinnamates were identified as phenylalanine, tryptophan, tyrosine and DOPA.

- Neither PAL nor TAL activity could be demonstrated in strain OU5.

- The enzyme catalyzing the transformation of *trans*-cinnamate to L-phenylalanine with intermediate phenylpyruvate was purified and characterized. This enzyme requires NADH as reducing power and ammonia as amino donor (Fig 16).

- The purified enzyme is a monomer of ~43 kD and was named as cinnamyl amino reductase.

- Transformation of *trans*-Cinnamate to L-tryptophan could be demonstrated in *Rba. sphaeroides* OU5 and a putative pathway for this transformation was proposed (Fig 35).
- 4-Hydroxybenzoate assimilation could be demonstrated in fumarate and pyruvate presence in *Rba. sphaeroides* OU5.
- The work on *Rhodobacter sphaeroides* OU5 is an update of the existing information on metabolism of aromatic compounds by this strain.

In Summary Major Findings

- ❖ *trans*-Cinnamate inhibits the enzyme DAHP synthase altering the *denovo* synthesis of aromatic amino acids.
- ❖ A novel protein of ~ 43 kDa catalyzing transformation of *trans*-cinnamate to L-phenylalanine was isolated and characterized.

Future Scope

- ➢ Isolated novel protein needs to be fully characterized.
- ➢ The genes involved in the transformation of *trans*-cinnamate to L-phenylalanine needs to be identified.
- ➢ Channeling of the *trans*-cinnamate into the protein synthesis needs to be established.
- ➢ Biosynthetic route of *trans*-cinnamate to L-tryptophan needs to be established in this strain.
- ➢ Work on 4-hydroxybenzoate needs a detail investigation.

Paper Published

Usha P., Sasikala, Ch. and Ramana, Ch.V. (2007). Light dependent assimilation of *trans*-cinnamate by *Rhodobacter sphaeroides* OU5. *Current Microbiology* **54:** 410-413.

References

Ahmed, S. A. M. and Mohamed, S. S. A. (1994). Utilization of aromatic compounds by phototrophic purple non-sulfur bacteria. *Biodegradation* **5**, 71-76.

Ali, S. and Haq, I. (2001). Studies on the microbiological transformation of L-tyrosine to L-dopa (3, 4-dihydroxyphenylalanine) by mutant strains of *Aspergillus oryzae*. *Pak. J. Plant Sci.* **6,** 97-113.

Altenschmidt, U. and Fuchs, G. (1992). Novel aerobic 2-aminobenzoate metabolism, purification and characterization of 2-aminobenzoate-CoA ligase, localization of the gene on a 8-kbp plasmid cloning and sequencing of the gene from a denitrifying *Pseudomonas* sp. *Eur. J. Biochem.* **205**, 721-727.

Alvarado, I. E., Lomascolo, A., Navarro, D., Delattre, M., Asther, M. and Lesage-Meessen, L. (2001). Evidence of a new biotransformation pathway of *p*-coumaric acid into *p*-hydroxybenzaldehyde in *Pycnoporus cinnabarinus*. *Appl. Microbiol. Biotechnol.* **57,** 725-730.

Alvarado, I. E., Navarro, D., Record, E., Asther, M., Marcel, A. and Lesage-Meessen, L. (2003). Fungal biotransformations of *p*-coumaric acid into caffeic acid by *Pycnoporus cinnabarinus*: an alternative for producing a strong natural antioxidant. *W. J. Microbiol. Biotechnol.* **19**, 157-160.

Andreoni, V. and Bestetti, G. (1986). Comparative analysis of different *Pseudomonas* strains that degrade cinnamic acid. *Appl. Environ. Microbiol.* **52**, 930-934.

Andreoni, V., Galli, E. and Galliani, G. (1984). Metabolism of ferulic acid by a facultatively anaerobic strain of *Pseudomonas cepacia*. *Syst. Appl. Microbiol.* **5**, 299-304.

Asano, Y., Nakazawa, A. and Endo, K. (1987). Novel phenylalanine dehydrogenases from *Sporosarcina ureae* and *Bacillus sphaericus*: purification and characterization. *J. Biol. Chem.* **262**, 10346-10354.

Aurburger, G. and Winter, J. (1995). Isolation and physiological characterization of *Syntrophus buswellii* strain GA from a syntrophic benzoate degrading, strictly anaerobic coculture. *Appl. Microbiol. Biotechnol.* **44**, 241-248.

Bae, E. A., Han, M. J., Kim, N. J. and Kim, D. H. (1998). Anti-*Helicobacter pylori* activity of herbal medicines. *Biol. Pharm. Bull.* **21,** 990-992.

Baker, H. A. (1981). Amino acid degradation by anaerobic bacteria. *Ann. Rev. Biochem.* **50**, 23-40.

Balba, M. T. and Evans, W. C. (1979). The methanogenic fermentation of *ω*–phenyl alkane carboxylic acids. *Biochem. Soc. Trans.* **7,** 403-405.

Balba, M. T. and Evans, W. C. (1980). Methanogenic fermentation of naturally occurring aromatic amino acids by a microbial consortium. *Biochem. Soc. Trans.* **8**, 1979-1981.

Barnes, C. C., Smalley, M. K., Manfredi, K. P., Kindscher, K., Loring, H. and Sheeley, D. M. (2003). Characterization of anti-tuberculotic resin glycoside from the prairic medicinal plant *Ipomoea leptophylla. J. Nat. Prod.* **66,** 1457-1462.

Basu, A., Apte, K. S. and Phale, S. P. (2006). Preferential utilization of aromatic compounds over glucose by *Pseudomonas putida* CSV86. *Appl. Environ. Microbiol.* **72**, 2226-2230.

Beam, H. W. and Perry, J. J. (1974). Microbial degradation and assimilation of n-alkyl-substituted cycloparaffins. *J. Bacteriol.* **118**, 394-399.

Beller, H. R. and Edwards, E. A. (2000). Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. *Appl. Environ. Microbiol.* **66,** 5503-5505.

Beller, H. R. and Spormann, A. M. (1997). Anaerobic activation of toluene and *o*-Xylene by addition of fumarate in denitrifying strain. *J. Bacteriol.* **179**, 670-676.

Bentley, R. **(**1990). The shikimate pathway: a metabolic tree with many branches. *Crit. Rev. Biochem. Mol. Biol.* **25,** 307–384.

Berner, M., Krug, D., Bihlmaier, C., Vente, A., Muller, R. and Bechthold, A. (2006). Genes and enzymes involved in caffeic acid biosynthesis in the actinomycete *Saccharothrix espanaensis*. *J. Bacteriol*. **188**, 2666-2673.

Berry, D. F., Francis, A. J. and Bollag, J. M. (1987). Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. *Microbiol. Rev.* **51,** 43-59.

Biebl, H. and Pfennig, N. (1981). Isolation of members of the family *Rhodospirillaceae.* In: The Prokayotes (Starr, M.P., Stolp, H., Truper, H. G., Balows, A. and Schlegel, H.G., Eds.), pp-167-273. Springer-Verlag, New York.

Blasco, R. and Castillo, F. (1992). Light dependent degradation of nitrophenols by the phototrophic bacterium *Rhodobacter capsulatus* E1F1. *Appl. Environ.Microbiol.* **58**, 690-695.

Bonting, C. F. C., Schneider, S., Schmidtberg, C. and Fuchs, G. (1995). Anaerobic degradation of *m*-cresol via methyl oxidation to 3-hydroxybenzoate by a denitrifying bacterium. *Arch. Microbiol.* **164**, 63-69.

Boll, M., Fuchs, G. and Heider, J. (2002). Anaerobic oxidation of aromatic compounds and hydrocarbons. *Curr. Opi. Chem. Biol*. **6**, 604-611.

Boopathy, R. and Kulpa, C. F. (1992). Trinitrotoluene (TNT) as a sole nitrogen source for a sulfate reducing bacterium resembling *Desulfovibrio* sp. (B-strain) isolated from an anaerobic digestor. *Curr. Microbiol.* **25**, 235-241.

Brackmann, R. and Fuchs, G. (1993). Enzymes of anaerobic metabolism of phenolic compounds. 4-Hydroxybenzoate-CoA reductase (dehydroxylating) from a denitrifying *Pseudomonas* species. *Eur. J. Biochem.* **213**, 563-571.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing principle of protein dye binding. *Anal. Biochem.* **72,** 248-254.

Briggs, A. P. (1922). A colorimetric method for the determination of homogentisic acid in urine. *J. Biol. Chem.* **51**, 453-454.

Brinkrolf, B. K. and Tauch, A. (2006). Transcriptional regulation of catabolic pathways for aromatic compounds in *Corynebacterium glutamicum*. *Genet. Mol. Res.* **5** (4), 773-789.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. *Int. J. Food Microbiol.* **94,** 223-253.

Carmona, M., Zamarro, M. T., Bla'zquez, B., Durante-Rodriguez, G., Juarez, J. F., Valderrama, J. A., Barragan, M. J. L., Garcia, J. L. and Diaz, E. (2009). Anaerobic catabolism of aromatic compounds: a Genetic and Genome Veiw. *Microbiol. Mol. Biol. Rev.* **73** (1), 71-133.

Cases, I. and de Lorenzo, V. (2001). The black cat/white cat principle of signal integration in bacterial promoters. *EMBO. J.* **20**, 1–1.

Cavin J. F., Barthelmebs, L., Guzzo, J., Van Beeumen, J., Bart, S., Trvers, J. F. and Divies, C. (1997a). Purification and characterization of an inducible *p*–coumaric acid decarboxylase from *Lactobacillus plantarum*. *FEMS Microbiol. Lett.* **147**, 291-295.

Cavin, J. F., Barthelmebs, L. and Divies, C. (1997b). Molecular characterization of an inducible *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*: gene cloning, transcriptional analysis, over expression in *Escherichia coli*, purification and characterization. *Appl. Environ. Microbiol.* **63**, 1939-1944.

Chamkha, M., Garcia, J. L. and Labat, M. (2001). Metabolism of cinnamic acids by some *Clostridiales* and amendation of the descriptions of *Clostridium aerotolerans*, *Clostridium celerecrescens* and *Clostridium xylanolyticum. Int. J. Syst. Evol. Microbiol.* **51**, 2105-2111.

Chaudhuri, B. K. and Weismann, U. (1995). Enhanced anaerobic degradation of benzene by enrichment of mixed microbial culture and optimization of the culture medium. *Appl. Microbiol. Biotechnol.* **43,** 178-187.

Choudhary, M., Zanhua, X., Fu, Y. X. and Kapkan, S. (2007). Genomic analysis of three strains of *Rhodobacter sphaeroides*: evidences of rapid evolution of chromosome II. *J. Bacteriol.* **189** (5), 1914-1921.

Clifford, M. N. (1999). Chlorogenic acids and other cinnamates-nature, occurrence and dietary burden. *J. Sci. Food Agric.* **79**, 362-372.

Cserhati, T., Illes, Z. and Nemes-Kosa, S. (1992). Effect of benzonitrile ester herbicides on the growth of some soil bacteria. *J. Appl. Bacteriol.* **72,** 523-528.

Dal, S., Trautwein, G. and Gerischer, U. (2005). Transcriptional organization of genes for protocatechuate and quinate degradation from *Acinetobacter* sp. Strain ADP1. *Appl. Environ. Microbiol.* **71**, 1025–1034.

D' Cunha, G. B., Satyanarayan, V. and Nair, P. M. (1994). Novel direct synthesis of L-phenylalanine methyl ester by using *Rhodotorula glutinis* phenylalanine ammonia lyase in an organic-aqueous biphasic system. *Enzy. Microbiol. Technol.* **16**, 318-322.

de Lorenzo, V. and Pe´rez-Martin, J. (1996). Regulatory noise in prokaryotic promoters: how bacteria learn to respond to novel environmental signals. *Mol. Microbiol.* **19**, 1177–1184.

Defnoun, S., Labat, M., Ambrosio, M., Garcia, J. L, Patel, B. K. (2000). *Papillobacter cinnamivorans* gen. Nov., a cinnamate-transforming bacterium from a shea cake digester. *Int. J. Syst. Evol. Microbiol.* **50**, 1221-28.

De'grassi, G., de Laureto, P. and Bruschi, C. V. (1995). Purification and characterization of ferulate and *p*-coumarate decarboxylase from *Bacillus pumilus*. *Appl. Environ. Microbiol.* **61**, 326-332.

Denger, K., Kertesz, M. A., Yock, E. H., Schon, R., Magli, A. and Cook, A. M. (1996). Anaerobic desulfonation of 4-toluoylsulfonate and 2-(4-sulfophenyl) butyrate by a *Clostridium* sp. *Appl. Environ. Microbiol.* **62**, 1526-1530.

De la Torre, M. A. and Gomez-Alarcon, G. (1991). Ferulic acid anaerobic degradation by marsh sediment microorganisms. *Microbios.* **68**, 119-127.

Delneri, D., Degrassi, G., Rizzo, R., Bruschi, C. V. (1995). Degradation of *trans*-ferulic acid and *p*-coumaric acid by *Acinetobacter calcoaceticus* DSM 586. *Biochem. Biophys. Acta.* **1244**, 363-367.

Diageri, T., Altenschmidt, U., Eckerskom, C. and Fuchs, C. (1993). Enzymes of anaerobic metabolism of phenolic compounds, 4-hydroxybenzoate CoA ligase from a denitrifying *Pseudomonas* species. *Eur. J. Biochem.* **213**, 555-561.

Diaz, E. (2004). Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. *Int. Microbiol.* **7,** 173-180.

Difco Manual. (1998). 11th Edition, different laboratories Sparks, Maryland, USA.

Donaghy, J. A., Kelly, P. F. and McKay, A. (1999). Conversion of ferulic acid to 4-vinyl guaiacol by yeasts isolated from unpasteurised apple juice. *J. Sci. Food Agric.* **79**, 453-456.

Dora, I., Pereira, A. and Gibson, G. R. (2002). Cholesterol assimilation by lactic acid bacteria and Bifidobacteria isolated from the human gut. *Appl. Environ. Microbiol.* **68**, 4689-4693.

Dux, P., Rubinstenn, G., Vuister, G. W., Boelens, R., Mulder, F. A., Hard, K., Hoff, W. D., Kroon, A. R., Crielaard, W., Hellingwerf, K. J., Kaptein, R. (1998). Solution structure and backbone dynamics of the photoactive yellow protein. *Biochem.* **37**(37), 12689-12699.

Edlin, D. A. N., Narbad, A., Gasson, M. J., Dickinson, J. R. and Lloyd, D. (1994). The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiol. Lett.* **125**, 311-316.

Edlin, D. A. N., Narbad, A., Gasson, M. J., Dickinson, J. R. and Lloyd, D. (1998). Purification and characterization of hydroxycinnamate decarboxylase from *Brettanomyces anomalus*. *Enzyme Microb. Technol.* **22**, 232-239.

Elder, D. J. and Kelly, D. J. (1994). The bacterial degradation of benzoic acid and benzenoid compounds under anaerobic conditions: unifying trends and new perspectives. *FEMS Microbiol. Rev.* **13**, 441-468.

Elder, D. J., Morgan, P. and Kelly, D. J. (1992a). Anaerobic degradation of *trans*-cinnamate and ω–phenylalkane carboxylic acids by the photosynthetic bacterium *Rhodopseudomonas palustris*: evidence for a β–oxidation mechanism. *Arch. Microbiol.* **157**, 148-154.

Elder, D. J., Morgan, P. and Kelly, D. J. (1992b). Evidence for two differentially regulated phenylpropenoyl-Coenzyme A synthetase activities in *Rhodopseudomonas palustris*. *FEMS Microbiol. Lett.* **98,** 255-260.

Emes, A. V. and Vining, L. C. (1970). Partial purification and properties of phenylalanine ammonia lyase from *Streptomyces verticillatus*. *Can. J. Biochem*. **48**, 613-622.

Esteve-Nunez, A., Caballero, A., Ramos, J. L. (2001). Biological degradation of 2,4,6-trinitrotoluene. *Microbiol. Mol. Biol. Rev.* **65**, 335-52.

Evans, W. C. (1977). Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. *Nature* **270**, 17-22.

Evans, W. C., Fuchs, G. (1988). Anaerobic degradation of aromatic compounds. *Annu. Rev. Microbiol.* **42**, 289–317.

Evans, C. T., Bellamy, W., Gleeson, M., Aoki, H., Hanna, K., Peterson, W., Conrad, D. and Misawa, M. (1987a). A novel efficient biotransformation for the production of L-phenylalanine. *Biotechnol.* **5**, 818-823.

Evans, C. Choma, C., Peterson, W. and Misawa, M. (1987b). Bioconversion of *trans*-cinnamic acid to L-phenylalanine in an immobilized whole cell reactor. *Biotechnol. Bioeng.* **30**, 1067-1072.

Fakhruddin, A. N. M. and Quilty, B. (2005). The influence of glucose and fructose on the degradation of 2-chlorophenol by *Pseudomonas putida* CP1. *World J. Microbiol. & Biotechnol*. **21,** 1541-1548.

Fazzolari, E., Mariotti, A. and Germon, J. C. (1990). Nitrate reduction to ammonia: a dissimilatory process in *Enterobacter amnigenus*. *Can. J. Microbiol.* **36**,779–785.

Feist, A. M., Henry, C. S., Reed, J. L., Krummenacker, M., Joyce, A. R., Karp, P. D., Broadbelt, L. J., Hatzimanikatis, V., Palsson, B. O. (2007). A genome-scale metabolic reconstruction for *Escherichia coli* K-12 MG1655 that accounts for 1260 ORFs and thermodynamic information. *Mol. Syst. Biol.* **3**,121.

Fetzner, S. (1998). Bacterial dehalogenation. *Appl. Microbiol. Biotechnol.* **50**, 633-657.

Flores, E., Guerrero, M. G. and Losada, M. (1983). Photosynthetic nature of nitrate uptake and reduction in the cyanobacterium *Anacystis nidulans*. *Biochim. Biophys. Acta* **722,** 408–416.

Fuchs, G., Mohamed, M. E. S., Altenschmidt, U., Koch, J., Lack, A., Brackmann, R., Lochmeyer, C. and Oswald, B. B. (1994). Biochemistry of anaerobic biodegradation of aromatic compounds, pp-513-553. *In* C. Ratledge (ed), Biochemistry of microbial degradation. Kluwer Academic Publishers. Dordrecht, Netherlands.

Gale, E. F. and Folkes, J. P. (1962). The assimilation of amino acids by bacteria. *Biochem. J.* **83** (430), 430-438.

George, M. L. and Perry, J. J. (1984). Assimilation of chlorinated alkanes by hydrocarbon-utilizing fungi. *J. Bacteriol.* **160**, 1171-1174.

Gibson, J. and Harwood, C. S. (2002). Metabolic diversity in aromatic compound utilization by anaerobic microbes. *Annu. Rev. Microbiol.* **56**, 345-369.

Goetz, F, E. and Harmuth, L. J. (1992). Gentisate pathway in *Salmonella typhimurium*: metabolism of *m*-hydroxybenzoate and gentisate. *FEMS Microbiol. Lett.* **97**, 45-50.

Gordon, S. A. and Paleg, L. G. (1957). Quantitative measurement of indole-3-acetic acid. *Plant Physiol.* **10,** 37-48.

Gosset, G., Bonner, C. A. and Jensen, R. A. (2001). Microbial origin of plant type 2-keto-3-deoxy-D-arabino-heptulosonate 7-phosphate synthases, exemplified by the chorismate- and tryptophan-regulated enzyme from *Xanthomonas campestris*. *J. Bacteriol.* **183,** 4061–4070.

Grbic-Galic, D. and Young, L. Y. (1985). Methane fermentation of ferulate and benzoate: anaerobic degradation pathways. *Appl. Environ. Microbiol.* **50**, 292-297.

Gross, G. G. (1985). Biosynthesis and metabolism of phenolic acids and mono-lignols. In: Higuchi, T. (ed) Biosynthesis and biodegradation of wood components. New York: Academic Press Inc., pp-61-179.

Grund, E., Knorr, C. and Eichenlaub, R. (1990). Catabolism of benzoate and monohydroxylated benzoates by *Amycolatopsis* and *Streptomyces* spp. *Appl. Environ. Microbiol.* **56**, 1459-1464.

Haker, A., Hendriks, J., Gensch, T., Hellingwerf, K., Crielaard, W. (2000). Isolation, reconstruction and functional characterization of the *Rhodobacter sphaeroides* photoactive yellow protein. *FEBS Lett.* **486**, 52-56.

Hamilton, W. A. (1998a). Bioenergetics of sulphate-reducing bacteria in relation to their environmental impact. *Biodegradation* **9** (3-4), 201-212 (12).

Hamilton, W. A. (1998b). Sulfate-reducing bacteria: physiology determines their environmental impact. *Geomicrobiol. J.* **15,** 19–28.

Harayama, S., Kok, M. and Neidle, E. L. (1992). Functional and evolutionary relationships among diverse oxygenases. *Ann. Rev. Microbiol.* **46**, 565-601.

Harayama, S. and Timmis, K. N. (1992). Anaerobic biodegradation of aromatic hydrocarbons by bacteria In: Sigel H, Sigel A (eds.) Metal ions in biological systems. Marcel Dekkar, New York, Vol **28,** pp-99-156.

Harwood, C. S. and Gibson, J. (1988). Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. *Appl. Environ. Microbiol.* **54**, 712-717.

Harwood, C. S. and Gibson, J. (1997). Shedding light on anaerobic benzene ring degradation: a process unique to prokaryotes. *J. Bacteriol.* **179**, 301-309.

Harwood, C. S., Burchhardt, G., Herrmann, H. and Fuchs, G. (1999). Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiol. Rev.* **22**, 439-458.

Harwood, C. S. and Parales, R. E. (1996). The ketoadipate pathway and the biology of self identity. *Ann. Rev. Microbiol.* **50**, 553-590.

Haug, W., Schmidt, A., Nortemann, R., Hempel, D. C., Stolz, A. and Knackmuss, H. J. (1991). Mineralization of the sulfonated azo dye mordant yellow 3 by a 6-aminonaphthalene-2-sulfonate degrading bacterial consortium. *Appl. Environ. Microbiol.* **57**, 3144-3149.

Haq, I., Ali, S., Manzoor, S., Qadeer, M. A. (2000). Optimization of cultural conditions for the production of L-dopa (3, 4-dihydroxy phenylalanine) from L-tyrosine by *Aspergillus oryzae*. *Pak. J. Botany* **32,** 255-258.

Healy, J. B. JR., Young, L. Y., Reinhard, M. (1980). Methanogenic decomposition of ferulic acid, a model lignin derivative. *Appl. Environ. Microbiol.* **39**, 436-444.

Higuchi, T. (1985). Biosynthesis of lignin. In: Higuchi, T. (ed) Biosynthesis and biodegradation of wood components. New York: Academic Press Inc., pp-141-160.

Hill, A. M., Thompson, B. L., Harris, J. P. and Segret, R. (2003). Investigation of the early stages in soraphan A biosynthesis. *Chembiochem.* **4**, 1358-1359.

Hoff, W. D., van Stokkum, I. H. M., van Ramesdonk, H. J., van Brederode, M. E., Brouwer, A. M., Fitch, J. C., Meyer, T. E., van Grondelle, R. and Hellingwerf, K. J. (1994). Measurement and global analysis of the absorbance changes in the photocycle of the photoactive yellow protein from *Ectothiorhodospira halophila*. *Biophys. J.* **67**, 1691-1705.

Holliger, C., Wohlfarth, G., Diekert, G. (1999). Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiol. Rev.* **22**, 383-398.

Hoskins, J. A. (1984). The occurrence, metabolism and toxicity of cinnamic acid and related compounds. *J. Appl. Toxicol.* **4**, 283-292.

Huang, Z., Dostal, I. and Rosazza, J. P. N. (1994). Purification and characterization of a ferulic acid decarboxylase from *Pseudomonas fluorescens*. *J. Bacteriol.* **176**, 5912-5918.

Jones, D. C. N. and Cooper, R. A. (1990). Catabolism of 3-hydroxybenzoate by the gentisate pathway in *Klebsiella pneumoniae* M5a1. *Arch. Microbiol.* **154**, 489-495.

Karegoudar, T. B., Chae, J. C. and Kim, C. K. (1999). Catabolism of 4-hydroxybenzoic acid by *Pseudomonas* sp. DJ-12. *J. Microbiol.* **37**, 123-127.

Kilroy, A. C. and Gray, N. F. (1992). The toxicity of four organic solvents commonly used in pharmaceutical industry to activated sludge. *Water Res.* **26,** 887-892.

Kim, S. H., Kronstad, J. W. and Ellis, B. E. (2001). Induction of phenylalanine ammonia-lyase activity by tryptophan in *Ustilago maydis*. *Photochem.* **58**, 849-857.

Kirk, T. K. and Farrell, R. L. (1987). Enzymatic 'combustion': the microbial degradation of lignin. *Annu. Rev. Microbiol.* **41**, 465-505.

Kreft, J. U. and Schink, B. (1993). Demethylation and degradation of phenylmethylethers by the sulfide-methylating homoacetogenic bacterium strain TMBS 4. *Arch. Microbiol.* **159**, 308-315.

Krieger, C. J., Roseboom, W., Albracht, S. P., Spormann, A. M. (2001). A stable organic free radicle in anaerobic benzylsuccinate synthase of *Azoarcus* sp. strain T. *J. Biol. Chem.* **276**, 12924-12927.

Kocwa-Haluch, R. (1995). Easy and inexpensive diffusion tests for detecting the assimilation of aromatic compounds by yeast-like fungi. Part II. Assimilation of dihydroxyphenols. *Chemosphere* **30,** 209-213.

Kocwa-Haluch, R. and Lemek, M. (1995). Easy and inexpensive diffusion tests for detecting the assimilation of aromatic compounds by yeast-like fungi. Part I. Assimilation of aromatic acids. *Chemosphere* **31,** 4333-4339.

Kort, R., Phillips-Jones, M. K., van Aalten, D. M., Haker, A., Hoffer, S. M., Hellingwerf, K. J. and Crielaard, W. (1998). Sequence, chromophore extraction and 3-D model of photoactive yellow protein from *Rhodobacter sphaeroides*. *Biochem. Biophys. Acta.* **1385** (1), 1-6.

Kupfer, D. and Atkinson, D. E. (1964). Quantitative method for determination of indole, tryptophan and anthranilic acid in the same aliquot. *Anal. Biochem.* **8**, 82-94.

Kuwahara, M. (1980). Metabolism of lignin-related compounds by bacteria in lignin biodegradation: Microbiology, Chemistry and Potential Applications, Vol 2, pp-46. CRC Press, New York.

Kyndt, J. A., Meyer, T. E., Cusanovish, M. A. and Van Beeumen J. J. (2002). Characterization of a bacterial tyrosine ammonia lyase, a biosynthetic enzyme for the photoactive yellow protein. *FEBS Lett.* **512**, 240-244.

Labuda, I. M., Keon, K. A. and Goers, S. K. (1993). Microbial bioconversion process for the production of vanillin. In *Progress in flavor Precursor Studies. Analysis-Generation-*

Biotechnology, pp-477-482. (Schreier, P. and Winterhalter, P. Stream, C., Eds.;) IL: Allured Publishing.

Laemmeli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London). **227**, 680-685.

Lee, S. G., Rao, H. S., Hong, S. P., Kim, E. H. and Sung, M. H. (1996). Production of L-Dopa by thermostable tyrosine phenol-lyase of a thermophilic *Symbiobacterium* spp. overexpressed in recombinant *Escherichia coli*. *J. Microb. Biotechnol.* **6,** 98-102.

Leuthner, B., Leutwein, C., Schulz, H., Horth, P., Haehnel, W., Schiltz, E., Schagger, H., Heider, J. (1998). Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalyzing the first step in anaerobic toluene metabolism. *Mol. Microbiol.* **28**, 615-628.

Lin, J. T., Goldman, B. S. and Stewart, V. (1993). Structures of genes *nasA* and *nasB* encoding assimilatory nitrate and nitrite reductases in *Klebsiella pneumoniae* M5al. *J. Bacteriol.* **175,** 2370-2378.

Londry, K. L. and Fedorak, P. M. (1992). Benzoic acid intermediates in the anaerobic biodegradation of phenols. *Can. J. Microbiol.* **38**, 1-11.

Lovley, D. R., Fraga, J. L., Coates, J. D., Blunt-Harris, E. L. (1999). Humics as electron donor for anaerobic respiration. *Environ. Microbiol.* **1**, 89-98.

Lueking, D., Tokuhisa, D. and Sojka, G. (1973). Glycerol assimilation by a mutant of *Rhodopseudomonas capsulata*. *J. Bacteriol.* **115**, 897-903.

Luengo, J. M., Garcia, J. L., Olivera, E. R., (2001). The phenyl acetyl-CoA catabolon: a complex catabolic unit with broad biotechnological applications. *Mol. Microbiol.* **39**, 1434-1442.

Mac Donald, M. J. and D'Cunha, G. B. (2007). A modern view of phenylalanine anmmonia lyase. *Biochem. Cell. Biol.* **85**, 273-282.

Madigan, M. T. and Gest, H. (1988). Selective enrichment and isolation of *Rhodopseudomonas palustris* using *trans*-cinnamic acid as sole carbon source. *FEMS Microbial Ecol.* **53**, 53-58.

Madigan, M. T., Jung, D. O. and Resnick, S. M. (2001). Growth of the purple bacterium *Rhodobacter capsulatus* on the aromatic compound hippurate. *Arch. Microbiol.* **175**, 462-65.

Middlehoven, W. J. and Gelpke, M. D. S. (1995). Partial converson of cinnamic acid into styrene by growing cultures and cell free extracts of the yeast *Cryptococcus elinovii*. *Antonie van Leeuwenhoek* **67**, 217-219.

Miller, G. L. (1958). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem*. **31**, 426-428.

Miseta, A., Csutora, P., Sipos, K. and Wheatley, D. N. (1996). Phenylalanine utilization for protein synthesis in beta-phenylpyruvic acid treated *Escherichia coli* cells. *Microbios.* **87**, 23-133.

Misono, H., Yonezawa, J., Nagata, S. and Nagasaki, S. (1989). Purification and characterization of a dimeric phenylalanine dehydrogenase from *Rhodococcus maris* K-18. *J. Bacteriol.* **171**, 30-36.

Misoph, M., Daniel, S. L. and Drake, H. L. (1996). Bi-directional usage of ferulate by the acetogen *Peptostreptococcus productus* U-1 CO_2 and aromatic acrylate groups as competing electron acceptors. *Microbiol.* **142**, 1983-1988.

Moffitt, C. M., Gordon, V. L., Bowman, E. M., Pence, J., Joseph, P. N. and Moore, S. B. (2007). Discovery of two cyanobacterial phenylalanine ammonia lyases: kinetic and structural characterization. *Biochem.* **46**, 1004-1012.

Mohn, W. W. and Tiedje, J. M. (1992). Microbial reductive dehalogenation. *Microbiol. Rev.* **56**, 482-507.

Mouttaki, H., Nanny, M. A. and McInerney, M. J. (2008). Use of benzoate as an electron acceptor by *Syntrophus aciditrophicus* grown in pure culture. *Appl. Environ. Microbiol.* **10** (12), 3265-3274.

Nali, M., Rindone, B., Tollari, S., Andreoni, V. and Treccani, V. (1985). Anaerobic microbial conversion of three hydroxycinnamic acids. *Experimentia* **41**, 1351-1353.

Nanda Devi, P. (2003). Photometabolism of homocyclic aromatic hydrocarbons by *Rhodobacter sphaeroides* OU5 and the newly isolated *Rhodobacter* sp. JA2. Ph.D thesis, JNTU, Hyderabad.

Nanda Devi, P., Sasikala, Ch. and Ramana, Ch. V. (2000) Light dependent transformation of anthranilate to indole by *Rhodobacter sphaeroides* OU5. *J. Ind. Microbiol. Biotechnol.* **24**, 219-221.

Neves, F. M., Kawano, C. Y. and Said, S. (2005). Effect of benzene compounds from plants on the growth and hyphal morphology in *Neurospora crassa*. *Braz. J. Microbiol.* **36**, 190-195.

Noh, U., Heck, S., Giffhorn, F. and Kohring, G. W. (2002). Phototrophic transformation of phenol to 4-hydroxyphenylacetate by *Rhodopseudomonas palustris*. *Appl. Microbiol. Biotechnol.* **58,** 830-835.

Oddou, J., Stentelaire, C., Lesage-Meessen, L., Asther, M. and Colonna Ceccaldi, B. (1999). Improvement of ferulic acid bioconversion into vanillin by high-density cultures of *Pycnoporus cinnabarinus*. *Appl. Microbiol. Biotechnol.* **53,** 1-6.

Ogawa, K-I., Akagawa, E., Yamane, K., Sun, Z-W., Lacelle, M., Zuber, P. and Nakano, M. M. (1995). The *nas* B operon and *nas* A gene are required for nitrate/nitrite assimilation in *Bacillus subtilis*. *J. Bacteriol.* **177**(5), 1409-1413.

Ohmiya, K., Takeuchi, M., Chen, W., Shimizu, S. and Kawakami, H. (1986). Anaerobic reduction of ferulic acid by *Wolinella succinogenes* from cow rumen. *Appl. Microbiol. Biotechnol.* **23**, 274-279.

Oh, Y-K., Palsson, B. O., Park, S. M., Schilling, C. H., Mahadevan, R. (2007). Genome scale reconstruction of metabolic network in *Bacillus subtilis* based on high-throughput phenotyping and gene essentiality data. *J. Biol. Chem.* **282** (39), 28791-2879.

Orth, A. B., Denny, M. and Tien, M. (1991). Over production of lignin-degrading enzymes by an isolate of *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **57**, 2591-2596.

Osawa, R., Fujisawa, T. and Sly, L. L. (1995). *Streptococcus gallolyticus* sp. nov.; gallate degrading organisms formerly assigned to *Streptococcus boviis*. *Syst. Appl. Microbiol.* **18**, 74-78.

Ottaway, J. H. (1957). A colorimetric method for the estimation of tyrosine. *Biochem.* **68**, 239-244.

Pagot, Y., Belin, J-M., Husson, F. and Spinnler, H. E. (2006). Metabolism of phenylalanine and biosynthesis of styrene in *Penicillium camemberti*. *J. Dairy Research* 1-6.

Pennock, J. F. and Threlfall, D. R. (1983). In: Biosynthesis of isoprenoid compounds (Porter, J. and Spurgeon, S. L. eds.) Vol 2. pp-191-203, Wiley, New York.

Peng, X., Misawa, N. and Harayama, S. (2003). Isolation and characterization of thermophilic bacilli degrading cinnamic, *p*-coumaric and ferulic acids. *Appl. Environ. Microbiol.* **69**, 1417-1427.

Perkins, P. S., Komisar, S. J., Puhakka, J. A. and Ferguson, J. F. (1994). Effects of electron donors and inhibitors on reductive dechlorination of of 2,4,6-trichlorophenol. *Water Res.***28,** 2101-2107.

Perona, E., Macro, E. and Orus, M. I. (1991). Effects of dimethoate on N_2-fixing cyanobacterium *Anabaena* PCC 7119. *Bull. Environ. Contam. Toxicol.* **47,** 758-763.

Playne, M. J. and Smith, B. R. (1983). Toxicity of organic extraction reagents to anaerobic bacteria. *Biotechnol. Bioeng.* **77,** 288-290.

Potter, T. L. and Fagerson, I. S. (1992). Phenolic compounds in Maple Sap. In *Phenolic Compounds and Their Effects on Health*; (Ho, C. T., Lee, C. Y., Huang, M. T., Eds.); ACS Symposium Series **506;** American chemical Society: Washington, D C, 1992; Part I.

Pérez-Pantoja, D., Rodrigo de la, I., Dietmar, H., Pieper, González, B. (2008). Metabolic reconstruction of aromatic compound degradation from the genome of the amazing pollutant-degrading bacterium *Cupriavidus necator* JMP134. *FEMS Microbiol.Rev.* **32** (5), 736-794.

Rajashekhar, N., Sasikala, Ch. and Ramana, Ch. V. (1998). Photobiotransformation of indole to its value added derivatives by *Rhodobacter sphaeroides* OU5. *J. Microbiol. Biotechnol.* **20,** 177-179.

Rajashekhar, N., Sasikala, Ch. and Ramana, Ch. V. (2000). Toxicity of N-containing heterocyclic aromatic compounds and their utilization for growth by a few purple non-sulfur bacteria. *Bull. Environ. Contam. Toxicol.* **65**, 375-382.

Ranjith, N. K. (2008). Catabolism of L-phenylalanine, L-tyrosine and L-tryptophan by *Rhodobacter sphaeroides* OU5. Ph.D thesis, HCU, Hyderabad.

Ranjith, N. K., Sasikala, Ch. and Ramana, Ch. V. (2009). A novel phenol terpenoid ester produced by *Rubrivivax benzoatilyticus* OU5 has cytotoxic and COX-1 inhibitory activity. *Nat. Prod. Commun.* **4,** 1-4.

Ranjith, N. K., Sasikala, Ch. and Ramana, Ch. V. (2007a). Catabolism of L-phenyalanine and L-tyrosine by *Rhodobacter sphaeroides* OU5 occurs through 3,4-dihydroxyphenylalanine. *Res. Microbiol.* **158**, 506-511.

Ranjith, N. K., Sasikala, Ch. and Ramana, Ch.V. (2008). Purification and characterization of 3,4-dihydroxyphenylalanine oxidative deaminase from *Rhodobacter sphaeroides* OU5. *Can. J. Microbiol***. 54**, 829-834.

Ranjith, N. K., Sasikala, Ch. and Ramana, Ch.V. (2007b). Rhodethrin: a novel indole terpenoid ether produced by *Rhodobacter sphaeroides* has cytotoxic and hormonal activities. *Biotechnology Lett.* **29,** 1399-1402.

Rastogi, N., Goh, K. S., Horgen, L. and Barrow, M. W. (1998). Synergistic activities of antituberculous drugs with cerulenin and *trans*-cinnamic acid against *Mycobacterium tuberculosis*. *FEMS Immunol. Med. Microbiol.* **21,** 149-157.

Roller, S. and Seedhar, P. (2002). Carvacrol and cinnamic acid inhibit microbial growth in fresh-cut melon and kiwi fruit at 4 degrees and 8 degrees C. *Lett. Appl. Microbiol.* **35,** 390-394.

Rosazza, J., Haung, Z., Dostal, L., Volm, T. and Rosseau, B. (1995a). Biocatalytic transformation of ferulic acid, an abundant aromatic natural product. *J. Ind. Microbiol.* **15**, 457-71.

Rosazza, K. C., Paul, G. M. and Wolf, S. (1995b). The microbial synthesis of L-dopa. *Biotechnol. Lett.* **16,** 210-215.

Sachan, A. Ghosh, S. and Mitra, A. (2006). Biotransformation of *p*-coumaric acid by *Paecilomyces variotii*. *Lett. Appl. Microbiol.* **42**, 35-41.

Said, S., Neves, F. M., Griffiths, A. J. F. (2004). Cinnamic acid inhibits the growth of the fungus *Neurospora crassa*, but is eliminated as acetophenone. *Int. Biodeg. Biodet.* **54** (1), 1-6.

Salmond, C. V., Kroll, R. G. and Booth, I. R. (1984). The effect of food preservatives on pH homeostasis in *Escherichia coli*. *J. Gen. Microbiol.* **130,** 2845-2850.

Sasikala, K. (1990). Studies on the biomass yield and photoproduction of hydrogen by a new isolate of purple non-sulfur bacteria, *Rhodobacter sphaeroides* O.U. 001. Ph.D thesis. Department of Microbiology, Osmania University, Hyderabad.

Sasikala, Ch. and Ramana, Ch V. (1998). Biodegradation and metabolism of unusual carbon compounds by anoxygenic phototrophic bacteria. *Adv. Microbial. Physiol.* **39,** 339-377.

Shimada, K., Kimura, E., Yasui, Y., Tanaka, H., Matsushita, S., Hagihara, H., Nagakura, M. and Kawahisa, M. (1992). Styrene formation by the decomposition by *Pichia carsonii* of *trans*-cinnamic acid added to a ground fish product. *Appl. Environ. Microbiol.* **58**, 1577-1582.

Sierra-Alvarez, R. and Lettinga, G. (1991). The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. *Appl. Microbiol. Biotechnol.* **34,** 544-550.

Sikkema, D. M. and De'bont, J. A. M. (1994). Degradation of nitroaromatic compounds by microorganisms. *Appl. Microbiol. Biotechnol.* **42**, 499-507.

Sikkema, J., Bont, J. A. M. and Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **59**, 201-222.

Singh, A. K. and Kumar, H. D., (1991). Inhibitory effect of petroleum oil on photosynthetic electron transport system in the cyanobacterium *Anabaena doliolum*. *Bull. Environ. Contam. Toxicol.* **47,** 890-895.

Solorzano, L. (1969). Determination of ammonia in natural waters by the phenol hypochlorite method. *Limnol. Oceanogr*. **14**, 799-801.

Steinrucken, H. C. and Amrhein, N. (1980). The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid 3-phosphate synthase. *Biochem. Biophys. Res. Commun.* **94,** 1207-1212.

Sunayana, M. R. (2007). Photometabolism of 2-aminobenzoate (Anthranilate) by *Rhodobacter sphaeroides* OU5. Ph.D thesis. H.C.U. Hyderabad.

Sunayana, M. R., Sasikala, Ch. and Ramana, Ch. V. (2005a). Production of novel indole ester from 2-amino-benzoate by *Rhodobacter sphaeroides* OU5. *J. Ind. Microbiol. Biotechnol*. **32**, 41-45.

Sunayana, M. R., Sasikala, Ch. and Ramana, Ch. V. (2005b). Rhodestrin: a novel indole terpenoid phytohormones from *Rhodobacter sphaeroides* OU5. *Biotech. Lett.* **27,** 1897-1900.

Takac, S., Akay, B. and Ozdamar, T. (1995). Bioconversion of *trans*-cinnamic acid to

Phenylalanine of *Rhodotorula glutinis*: Parameters and kinetics. *Enz. Microbiol. Technol.* **17,** 445-452.

Tasaki, M., Kamagata, Y., Nakamura, K. and Mikami, E. (1992). Utilization of methoxylated benzoates and formation of intermediates by *Desulfotomaculum thermobenzoicum* in the presence of absence of sulfate. *Arch. Microbiol.***157**, 209-212.

Tawata, S., Taira, S. Kobamoto, N., Zhu, J., Ishihara, M. and Toyama, S. (1996). Synthesis and antifungal activity of cinnamic acid esters. *Biosci. Biotechnol. Biochem.* **60**, 909-910.

Taylor, B. F. (1983). Aerobic and anaerobic catabolism of vanillic acid and some other methoxy-aromatic compounds by *Pseudomonas* sp. strain PN-1. *Appl. Environ. Microbiol.* **46**, 1286-1292.

Thiele, I., Vo, T. D., Price, N. D., Palsson, B. (2005). An expanded metabolic reconstruction of *Helicobacter pylori* (iIT341 GSM/GPR): An in silico genome-scale characterization of single and double deletion mutants. *J. Bacteriol.* **187** (16) , 5818-5830.

Tillet, R. and Walker, J. (1990). Metabolism of ferulic acid by a *Pencillium* sp. *Arch. Microbiol.* **154**, 206-208.

Torres, Y., Torres, J. L. and Rosazza, J. P. N. (2001). Microbial transformation of *p*-coumaric acid by *Bacillus megaterium* and *Curvularia lunata*. *J. Nat. Products* **64**, 1408-1414.

Tropel, D. and van der Meer, J. R. (2004). Bacterial transcriptional regulators for degradation pathways of aromatic compounds. *Microbiol. Mol. Rev.* **68**, 474–500.

Tschech, A. and Pfennig, N. (1984). Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. *Arch. Microbiol.* **137**, 163-167.

Tubbing, D. M. J. and Admiraal, J. (1991). Inhibition of bacterial and phytoplanktonic metabolic activity in the lower River Rhine by ditallow-dimethylammonium chloride. *Appl. Environ. Microbiol.* **57,** 3616-3622.

Usha, P., Sasikala, Ch. and Ramana, Ch. V. (2007). Light dependent assimilation of *trans*-cinnamate by *Rhodobacter sphaeroides* OU5. *Curr. Microbiol.* **54**, 410-413.

Van 'der Meer, J. R., de Vos, W. M., Harayama, S., Zehnder, A. J. B. (1992). Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol. Rev.* **56**, 677-694.

Vieira, O., Laranjinha, J., Madeira, V. and Almeida, L. (1998). Cholesteryl ester hydroperoxide formation in myoglobin-catalyzed low-density lipoprotein oxidation: concerned antioxidant activity of caffeic and *p*-coumaric acids with ascorbate. *Biochem. Pharmacol.* **55**, 33-340.

Vijay, S., Sunayana, M. R., Ranjith, N. K., Sasikala, Ch. and Ramana, Ch. V. (2006). Light dependent transformation of aniline to indole esters by the purple bacterium *Rhodobacter sphaeroides* OU5. *Curr. Microbiol.* **52**, 413-417.

Villemur, R., (1995). Coenzyme A ligases involved in anaerobic biodegradation of aromatic compounds. *Can. J. Microbiol.* **41**, 855-861.

Wackett, L. P. (2003). *Pseudomonas putida*-A versatile biocatalyst. *Nat. Biotechnol.* **21**, 136-138.

Ward, L. A., Johnson, K. A., Robinson, J. M. and Yokoyama, M. T. (1987). Isolation from swine faeces of a bacterium which decarboxylates *p*-hydroxyphenylacetic acid to 4-methylphenol (*p*-cresol). *Appl. Environ. Microbiol.* **53**, 189-192.

Warth, A. D. (1991). Effect of benzoic acid on glycolytic metabolite levels and intracellular pH in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **57,** 3415-3417.

Watanabe, S. K., Hemandez-Velazco, G., Iturbe-Chinas, F. and Lopez-Mungia, A. (1992). Phenylalanine ammonia lyase from *Sporidiobolus pararoseus* and *Rhodosporidium toruloides*: application for phenylalanine and tyrosine deamination. *World J. Microbiol. Biotechnol.* **8,** 406-410.

Watts, K. T., Lee, P. C. and Schmidt-Dannert, C. (2004). Exploring recombinant flavonoid biosynthesis in metabolically engineered *Escherichia coli*. *Chembiochem.* **5,** 500-507.

Willems, A., Gillis, M. and De' Ley, J. (1991). Transfer of *Rhodocyclus gelatinosus* to *Rubrivivax gelatinosus* gen. Nov. comb. Nov., and phylogenetic relationships with *Leptothrix, Sphaerotilus vatans, Pseudomonas saccharophila* and *Alcaligenes lactus*. *Int. J. Syst. Bacteriol.* **41**, 65-73.

Williams, J. S., Thomas, M. and Clarke, D. J. (2005). The gene *stlA* encodes a phenylalanine ammonia lyase that is involved in the production of a stilbene antibiotic in *Photorhabdus luminescens*. *Microbiol.* **151**, 2543-2550.

Xiang, L. and Moore, B. S. (2005). Biochemical characterization of a prokaryotic phenylalanine ammonia lyase. *J. Bacteriol.* **187**, 4286-4289.

Xue, Z., Mc Cluskey, M., Cantera, K., Sariaslani, F. S. and Huang, L. (2007). Identification, characterization and functional expression of a tyrosine ammonia lyase and its mutants from the photosynthetic bacterium *Rhodobacter sphaeroides. J. Ind. Microbiol. Biotechnol.* **34** (9), 599-604.

Yamada, S., Nabe, K., Izuo, N., Ngamichi, K. and Chibata, I. (1981). Production of L-phenylalanine from *trans*-cinnamic acid with *Rhodotorula glutinis* containing L-phenylalanine ammonia lyase activity. *Appl. Environ. Microbiol.* **42**, 773-778.

Zengler, K., Heider, J., Rossello-Mora, R., Widdel, F. (1999). Phototrophic utilization of toluene under anoxic conditions by a new strain of *Blastochloris sulfoviridis*. *Arch. Microbiol.* **172**, 204-212.

Zeyen, R. J., Bushnell, W. R., Carver, T. L. W., Robbins, M. P., Clark, T. A. and Boyles, D. A. (1995). Inhibiting phenylalanine ammonia-lyase and cinnamyl-alcohol dehydrogenase suppresses Mia1 (HR) but not mol 5 (non-HR) barley powdery mildew resistance. *Physiol. Mol. Plant Path.* **47**, 119-140.

Zhang, X. and Wiegel, J. (1994). Reversible conversion of 4-hydroxybenzoate and phenol by *Clostridium hydroxybenzoicum. Appl. Environ. Microbiol.* **60**, 4182-4185.

Current Microbiology Vol. 54 (2007), pp. 410–413
DOI: 10.1007/s00284-006-0440-8

Current Microbiology
An International Journal

Light-Dependent Assimilation of *trans*-Cinnamate by *Rhodobacter sphaeroides* OU5

P. Usha,[1] Ch. Sasikala,[2] Ch. V. Ramana[1]

[1]Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad, 500 046, India
[2]Environmental Microbial Biotechnology Laboratory, Center for Environment, IST, JNT University, Kukatpally, Hyderabad, 500 072, India

Received: 31 August 2006 / Accepted: 6 December 2006

Abstract. We present a novel light-dependent metabolism of an aromatic compound (*trans*-cinnamate) that is assimilatory rather than dissimilatory. Light-dependent assimilation of *trans*-cinnamate was observed by both growing and resting cells of *Rhodobacter sphaeroides* OU5. T*rans*-cinnamate assimilation could be correlated with simultaneous formation of both phenylalanine and tyrosine at near-stoichiometric ratios. *Trans*-cinnamate assimilation was promoted by carbon source and electron donors, such as glucose, pyruvate, or α-ketoglutarate, whereas malate, succinate, fumarate, and acetate were inhibitory.

Metabolism of aromatic compounds is well recognized in a few purple bacteria [6]. The current understanding of aromatic compound metabolism by purple bacteria is that it occurs either through light-dependent degradation of benzene ring, supporting growth by providing carbon source/e^- donors, or through transformations [6], in which the aromatic compound do not support growth. However, we report here that these bacteria have a third type of aromatic compound metabolism, i.e., assimilatory metabolism, in which the aromatic compound (*trans*-cinnamate) is assimilated with few biochemical modifications (L-phenylalanine, L-tyrosine) and probably incorporated directly into proteins.

Materials and Methods

Organism and growth conditions. *Rhodobacter sphaeroides* OU5 (American Type Culture Collection [ATCC] 49885; DSM 7066) was grown photoheterotrophically (anaerobic/light) (2,400 lux) in fully filled screw-cap test tubes (10 x 100 mm) or in reagent bottles (60 ml) on Biebl and Pfennig's [1] mineral medium with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen sources, respectively, at 30 ± 2°C. For chemoheterotrophic (aerobic/dark) growth, the organism was allowed to grow in 50 ml medium in a 250-ml conical flask and incubated in dark at 30°C under continuous agitation.

Correspondence to: Ch.V. Ramana; *email:* r449@sify.com or sasi449@yahoo.ie

Utilization of *trans*-cinnamate by growing cells. Logarithmic cultures of *Rba. sphaeroides* OU5 were inoculated (1% v/v) into basal medium containing *trans*-cinnamate (500 μM) along with other carbon sources/e^- donors (Table 1), with ammonium chloride as nitrogen source. Assay was done in fully filled screw-cap test tubes (10 x 100 mm; anaerobic) or 50 ml assay mixture in a 250-ml conical flask (aerobic), incubated for 48 hours at 30 ± 2°C, harvested by centrifugation (16,000 *x g* for 20 minutes). The supernatant was used for measuring *trans*-cinnamate using high-performance liquid chromatography (HPLC).

Preparation of resting cell suspensions and assay. Cells of *Rba. sphaeroides* OU5 grown photoheterotrophically with malate (22 mM) and ammonium chloride (7 mM) until mid–log phase (30 hours) were harvested by centrifugation (16,000 *x g* for 20 minutes). The pellet was washed twice and resuspended in basal salts medium [1] containing *trans*-cinnamate (0.5 mM). This suspension was then distributed into screw-cap test tubes (10 x 100 mm) to fill them fully (anaerobic) or into 50 ml assay mixture in a 250-ml conical flask (aerobic) and incubated under light (2,400 lux) or dark conditions at 30 ± 2°C.

Cell-free extracts. Resting cells of *Rba. sphaeroides* OU5 used for *trans*-cinnamate assay were harvested by centrifugation (16,000 *x g* for 10 minutes), and the pellet was washed (twice) with 0.05 M potassium phosphate buffer (pH 7.8) and resuspended in 10 ml of the same buffer. Cells were sonicated with an MS-72 probe (model-UW 2070; Bandelin, Germany) to complete cell lysis after 8 to 9 cycles. The sonicated sample was centrifuged (16,000 *x g* for 20 minutes), and the supernatant was used for further assays.

Table 1. Biomass yield, utilization of *trans*-cinnamate, and product formation by growing cells of *Rba. sphaeroides* OU5 in the presence of different carbon substrates[a]

Trans-cinnamate + other substrate (0.3% w/v)	Biomass yield (mg dry wt.ml^{-1})	*Trans*-cinnamate consumed (μM)	Products (μM)			
			Phenylalanine	Tyrosine	Total	Recovery (%)
Control (with out other substrates)	0.1	150	115	33	148	99
Malate	2.2	0	0	0	0	NA
Succinate	1.6	0	0	0	0	NA
Fumarate	2.3	0	0	0	0	NA
α-ketoglutarate	0.3	270	270	0	270	100
Pyruvate	1.8	250	164	83	247	99
Acetate	1.6	0	0	0	0	NA
Glucose	0.6	500	351	0	351	70

[a] Cells of *Rba. sphaeroides* OU5 were allowed to grow phototrophically for 48 hours in the presence of the substrates and assayed. NA = Not applicable.

Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities were determined by measuring the formation of *trans*-cinnamate or 4-hydroxycinnamate from L-phenylalanine or L-tyrosine, respectively, using HPLC. The reaction mixture contained a final volume of 1 ml (Tris-HCl buffer; 5 mM; pH 8.8), 100 μmol L-phenylalanine or L-tyrosine, and an appropriate amount of cell extract. The reaction was carried out in Eppendorf test tubes (1.5 ml) and incubated at 30°C for 20 minutes (unless other wise mentioned); proteins were eliminated by acidification with 10% (w/v; 100 mg.ml^{-1}) TCA, centrifuged (12000 *x g* for 5 minutes), and analyzed by injecting 20 μl clear supernatants in HPLC.

Analytic determinations. Growth was measured turbidometrically as optical density (OD) at 660 nm. Biomass yield was estimated using an OD-versus-dry weight graph (0.1 OD at 660 nm = 0.15 mg dry wt.ml^{-1}). HPLC analysis of substrates and products was performed at room temperature using a Shimadzu SPD-10AVP isocratic system. Methanol and water (1:1) was used as solvent at 2.0 ml.min^{-1} using a Luna 5-μ C_{18} (2) 100A column (250 x 4.6 mm), and the compounds were detected using an ultraviolet light–visible light detector at 300 nm for 4-hydroxycinnamate and at 200 nm for *trans*-cinnamate, L-phenylalanine, L-tyrosine, phenylpyruvate, and 4-hydroxyphenylpyruvate. The metabolites were identified based on their retention time and by comparing them with the standards. Identification of the metabolites was also based on their molecular masses determined through liquid chromatography–mass spectrometry (LC-MS) analysis. This was carried out on Shimadzu LC-MS (LCMS-2010A). Analysis was performed at 40°C (LC column oven) and 85°C (MS ionization chamber). Methanol and water (1:1) was used as solvent at 0.2 ml.min^{-1} using a Luna 5 μ C_{18} (2) 100A column (250 x 4.6 mm), and the compounds were detected (by way of LC) at 254 nm. The column effluent from the LC was nebulized into an atmospheric pressure chemical ionization region under nitrogen gas for generating molecular masses.

Results and Discussion

Growth on *trans*-cinnamate. Photoheterotrophic or chemoheterotrophic growth of *Rba. sphaeroides* OU5 was not observed when *trans*-cinnamate was used as sole source of carbon/e$^-$ donor. The 50% inhibition concentration of *trans*-cinnamate on photoheterotrophic (malate as carbon source/e$^-$donor) growth of *Rba. sphaeroides* OU5 was approximately 4.5 mM (data not shown).

Use of *trans*-cinnamate by resting cells. Growing cells of *Rba. sphaeroides* OU5 could not use *trans*-cinnamate when it was supplemented to the photoheterotrophic medium with malate (carbon source/e$^-$ donor) and ammonium chloride (nitrogen source). Therefore, resting cell suspensions were assayed for its use in a basal medium with out any supplements (carbon or nitrogen). Although the biomass remained constant, use of *trans*-cinnamate was observed after a lag period of approximately 24 hours, and the substrate (0.5 mM) was completely used with in another 24 hours (Fig. 1). Because the substrate did not support growth of *Rba. sphaeroides* OU5 as carbon source or e$^-$ donor, which occurs through a dissimilatory (degradation) process, we consider the use of *trans*-cinnamate to be assimilatory in nature. Under the assay conditions, complete (100%) use of *trans*-cinnamate was observed until 0.5 mM, at which point use gradually decreased to 10% at 4.5 mM. In addition to *trans*-cinnamate, assimilation was also observed with 4-hydroxycinnamate, whereas benzoate and its derivatives (2-hydroxybenzoate, 4-hydroxybenzoate, 2-carboxybenzoate, 2-aminobenzoate), L-phenylalanine, and phenylpyruvate were not assimilated by *Rba. sphaeroides* OU5 under the assay conditions used. Although we do not have any experimental evidence from our work or from that of others, this can be plausibly explained as being caused by diffusional barriers. In addition, *trans*-cinnamate assimilation was inhibited by the presence of organic substrates (substrates tested are listed in Table 1). *Trans*-cinnamate assimilation, however, could not be demonstrated under aerobic dark conditions.

Expected products of assimilation of *trans*-cinnamate by *Rba. sphaeroides* OU5, such as phenylalanine or tyrosine, could not be detected in the resting cell

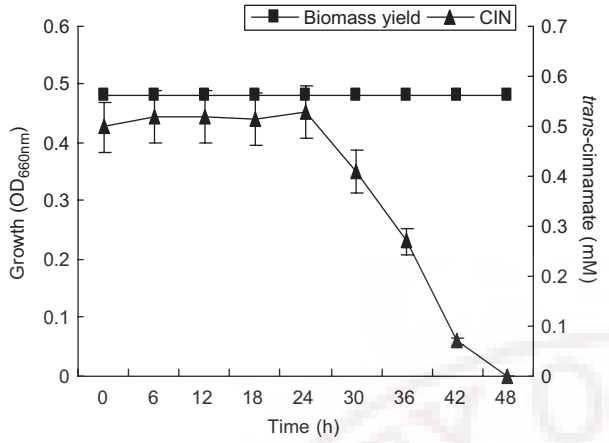


Fig. 1. Photoassimilation of *trans*-cinnamate by resting cells of *Rba. sphaeroides* OU5.

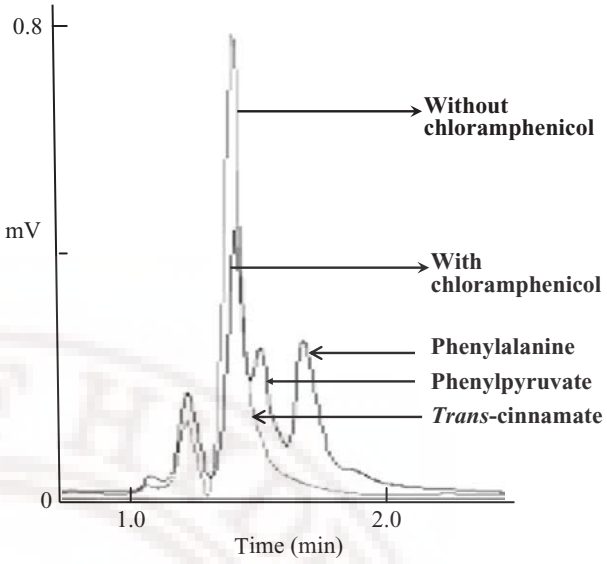


Fig. 2. HPLC chromatogram (at 200 nm) showing the products of *trans*-cinnamate by resting cells of *Rba. sphaeroides* OU5 when treated with and without chloramphenicol (20 ug.10ml^{-1}). Data pertains to the assay done after 48 hours, at which time phenylpyruvate and phenylalanine concentrations were 90 and 160 μM, respectively, with 50% loss of *trans*-cinnamate (250 μM).

assay supernatants. Therefore, another set of assays was conducted, with chloramphenicol included, and both phenylpyruvate and phenylalanine were detected in the assay supernatant (Fig. 2) in stoichiometry to the *trans*-cinnamate consumed. These results indicate that the product of *trans*-cinnamate, i.e., phenylalanine, may be incorporated directly into the proteins and thus support the assimilatory metabolism of *trans*-cinnamate in *Rba. sphaeroides* OU5.

Use of *trans*-cinnamate by growing cells. Because *trans*-cinnamate assimilation was not observed during growth of *Rba. sphaeroides* OU5 in photoheterotrophic medium with malate as carbon source/e^- donor, a number of other substrates were screened (Table 1). Growth of *Rba. sphaeroides* occurred on all the substrates tested. However, assimilation of *trans*-cinnamate varied and was dependent on the growth substrate used. The extent of assimilation was independent of the biomass yields obtained with the substrates tested. Among the substrates tested (Table 1), i.e., acetate, malate, succinate, and fumarate inhibited *trans*-cinnamate assimilation by *Rba. sphaeroides* OU5, whereas glucose, pyruvate, and α-ketoglutarate promoted such assimilation.

In the absence of *trans*-cinnamate, extracellular production of phenylalanine or tyrosine was not observed in the culture supernatants with respect to various substrates tested (data not shown; substrates tested are listed in Table 1). However, in the presence of *trans*-cinnamate, both phenylalanine and/or tyrosine were observed in the culture supernatants with various substrates in near stoichiometric ratios (Table 1). Compared with the resting cells, in which detection of phenylalanine and tyrosine was dependent on the presence of chloramphenicol, phenylalanine and tyrosine were observed in the culture supernatants of growing cells without addition of chloramphenicol (Table 1). Under normal growth conditions, in the absence of *trans*-cinnamate, *Rba. sphaeroides* OU5 cells do not overproduce phenylalanine or tyrosine, although in the presence of *trans*-cinnamate, the cells overaccumulate these compounds. We also demonstrated the products of *trans*-cinnamate metabolism using cell-free extracts of *Rba. sphaeroides* OU5 (Fig. 3). The 10-minute analysis shown in Fig. 3 resulted in nonstoichiomethic yields of phenylalanine and tyrosine compared with the complete loss of such in *trans*-cinnamate. The nonstoichimetric yields of products of *trans*-cinnamate could be explained as being caused by the accumulation of intermediates, i.e., phenylpyruvate and 4-hydroxyphenyl pyruvate, which were detected in the LC-MS chromatogram.

Enzymes involved in *trans*-cinnamate assimilation. Production of either *trans*-cinnamate or 4-hydroxycinnamate, from phenylalanine or tyrosine by the action of PAL or TAL, respectively, was not observed in *Rba. sphaeroides* OU5. These results indicated that neither of these enzymes may be involved in the assimilation of *trans*-cinnamate by *Rba. sphaeroides* OU5. *Trans*-cinnamate assimilation proceeds probably first through oxidation followed by transamination, resulting in the synthesis of

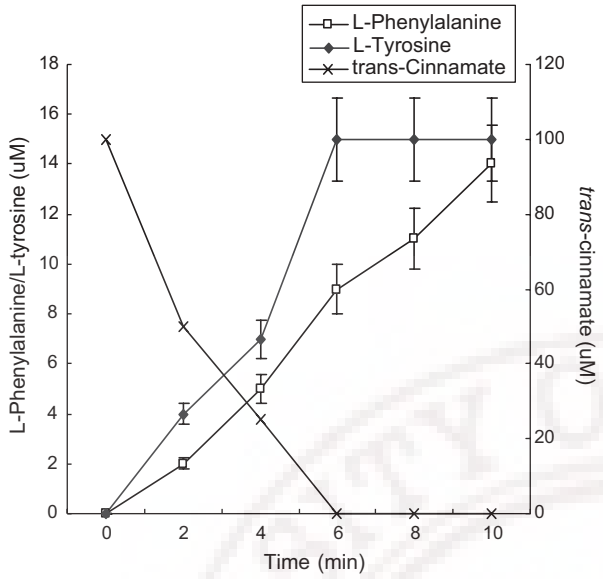


Fig. 3. Use of *trans*-cinnamate and production of products with time using cell-free extracts of *Rba. sphaeroides* OU5.

phenylalanine or tyrosine. Although TAL activity was reported earlier in a closely related species, *Rba. capsulatus* [3], so far there have been no reports of PAL among anoxygenic phototrophs, although the same was recently reported from a number of other bacterial species [8].

Conclusion

Trans-cinnamate use as sole carbon/e^- source by *Rba. palustris* has been previously reported [2, 7] and the compound has also been used for the selective enrichment of this bacterium [4]. Our study, for the first time (to the best of our knowledge), clearly indicated that aromatic molecules, such as *trans*-cinnamate, can be assimilated by *Rba. sphaeroides* OU5 directly into proteins rather than using the substrate as sole source of carbon/e^-. Incorporation of exogenously supplemented L-phenylalanine directly into proteins by *Escherichia coli* [5] (not observed in our study) can strongly support our study of *trans*-cinnamate being used for protein synthesis. Furthermore, *trans*-cinnamate could be a potential inhibitor of ''de novo'' synthesis of aromatic amino acids because the molecule is an analogue of phenylalanine and tyrosine. Thus, under the present assay conditions, *trans*-cinnamate is involved in the synthesis of phenylalanine and tyrosine. Our study widens the scope of investigation into diversified aromatic molecules supporting assimilatory aromatic metabolism, although thus far, investigations on aromatic hydrocarbon microbial metabolism have focused mainly on dissimilatory (degradation) metabolism.

ACKNOWLEDGMENTS

Council of Scientific and Industrial Research, Government of India, is acknowledged for financial support.

Literature Cited

1. Biebl H, Pfennig N (1981) Isolation of members of the family Rhodospirillaceae. In: Starr MP, Stolp H, Truper HG, Balows A, Schegel HG (eds) The prokaryotes, vol 1. New York, NY: Springer-Verlag, pp 167–273
2. Harwood CS, Gibson J (1988) Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. Appl Environ Microbiol 54:712–717
3. Kyndt JA, Meyer TE, Cusanovish MA, Van Beeumen JJ (2002) Characterization of a bacterial tyrosine ammonia lyase, a biosynthetic enzyme for the photoactive yellow protein. FEBS Lett 512:240–244
4. Madigan MT, Gest H (1988) Selective enrichment and isolation of *Rhodopseudomonas palustris* using *trans*-cinnamic acid as sole carbon source. FEMS Microbiol Ecol 53:53–58
5. Miseta A, Csutora P, Sipos K, Wheatley DN (1996) Phenylalanine utilization for protein synthesis in beta-phenylpyruvic acid treated *Escherichia coli* cells. Microbios 87:123–133
6. Sasikala Ch, Ramana Ch V (1995) Biodegradation and metabolism of unusual carbon compounds by anoxygenic phototrophic bacteria. Adv Microbial Physiol 39:339–377
7. Sasikala Ch, Ramana Ch V, Raghuveer Rao P (1994) Nitrogen fixation by *Rhodopseudomonas palustris* OU11 with aromatic compounds as carbon source/electron donors. FEMS Microbiol Lett 122:75–78
8. Xiang L, Moore BS (2005) Biochemical characterization of a prokaryotic phenylalanine ammonia lyase. J Bacteriol 187:4286–4289