Catabolism of L-phenylalanine, L-tyrosine and L-tryptophan by *Rhodobacter sphaeroides* OU5

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Ranjith Naik Kumavath

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500046
INDIA

Enrollment No. 03LPPH13

AUGUST- 2008

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad -500 046
INDIA

CERTIFICATE

This is to certify that **Mr. Ranjith Naik Kumavath** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D ordinance of this University. We recommend his thesis entitled **"Catabolism of L-phenylalanine, L-tyrosine and L-tryptophan by *Rhodobacter sphaeroides* OU5"** for submission for the degree of Doctor of Philosophy of this University.

This work has not been submitted for the award of any degree or diploma of any other University or Institute.

Dr. Ch. V. Ramana
Supervisor ----------

Head
Department of Plant Sciences ----------

Dean
School of Life Sciences ----------

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad -500 046
INDIA

CERTIFICATE

This is to certify that the thesis entitled "Catabolism of L-phenylalanine, L-tyrosine and L-tryptophan by *Rhodobacter sphaeroides* OU5 **",** embodies the results of the research carried out by **Mr**. **RANJITH NAIK KUMAVATH,** under my guidance and supervision for the award of the degree of **DOCTOR OF PHILOSOPHY IN PLANT SCIENCES** and that no part of the thesis has been submitted for any other degree. I further certify that such help or source of information as has been availed of in this thesis is duly acknowledged.

Place: HYDERABAD

Date: **(Dr. Ch. V. RAMANA)**

DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Catabolism of L-phenylalanine, L-tyrosine and L-tryptophan by *Rhodobacter sphaeroides* OU5"** has been carried out by me under the supervision of Dr. Ch. V. Ramana and this work has not been submitted for any degree or diploma of any other University or Institute earlier. All the assistance and help received during the course of investigation have been duly acknowledged

Ranjith Naik Kumavath
(Research Scholar)

Dr. Ch. V. Ramana
(Research Supervisor)

Dedicated
To
My
Beloved Parents

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LIST OF ABBREVIATIONS

HPLC	:	High performance Liquid chromatography
DAD	:	Diode array detector
LC/MS	:	Liquid chromatography - mass spectrometry
ESI	:	Electrospray ionization
APCI	:	Atmospheric pressure chemical ionization
IR	:	Infra red
NMR	:	Nuclear magnitic resonence
MALDI-TOF	:	Matrix assisted liquid desorption and ionization-time of flight
DEAE	:	Diethylanimo ethylamine
CM	:	Methyl cellulose
DOPA	:	3,4-dihydroxyphenylalanine
DOPLA	:	3,4-dihydroxyphenyllactic acid
DOPP	:	3,4-dihydroxyphenylpyruvate
DOPPA	:	3,4-dihydroxyphenylpropionic acid
DOPAATS	:	3,4-dihyroxyphenylalanine aminotransferase
DOPARDA	:	3,4-dihydroxyphenylalanine reductive deaminase
DOPAODA	:	3,4-dihydroxyphenylalanine oxidative deaminase
WAL	:	Tryptophan ammonia lyase
TAL	:	Tyrosine ammonia lyase
PAL	:	Phenylalanine ammonia lyase
HAL	:	Histidine ammonia lyase
IAA	:	Indole 3-acetic acid
EC	:	Enzyme code

CBB	:	Coomassie brilliant blue
PPM	:	Part per millian
PMSF	:	Phenyl methyl sulfonyl fluoride
EDTA	:	Ethylene diamine tetraacetic acid
TCA	:	Trichloroacetic acid
2-DE	:	Two-dimensional electrophoresis
MTT	:	3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide
COX	:	Cyclooxygenase
NADH	:	Nicotinamide adenine dinucleotide (Reduced)
NADPH	:	Nicotinamide adenine dinucleotide phosphate (Reduced)
NAD	:	Nicotinamide adenine dinucleotide (Oxidised)
FAD	:	Flovin adenosine dinucleotide
ATCC	:	American type culture collection
DTT	:	Dithioerythritol
MS	:	Mass spectrometry
ME	:	Beta-merchaptoethonol
PDAB	:	Para dimethylamino benzaldehyde
CID	:	Collision induced dissociation
TMPD	:	N, N, N^1, N^1 tetramethyl paraphenylenediamine
DMSO	:	Dimethly sulphoxide
TEMED	:	N,N,N',N'-tetramethylene diamine
CAPS	:	(3-[cyclohexylamino]-1-propanesulfonic acid)
CHCA	:	Cyano-4-hydroxycinnamic acid
BSA	:	Bovine serum albumin

Summary

Purple bacteria contribute significantly to the catabolic process in the oxic/anoxic phototrophic zones of several ecosystems. Catabolism of low molecular weight aromatic hydrocarbons under phototrophic anoxic conditions is restricted to a few purple non-sulfur bacteria. Photometabolism of a few benzenoid hydrocarbons was extensively studied in a few purple non-sulfur bacteria, which are capable of degrading the aromatic ring. On the contrary, a few incubation experiments indicated the photobiotransformation of aromatic hydrocarbons by purple bacteria incapable of degrading the benzenoid ring. Such light dependent transformations can be explored for biotechnology through inventerization of novel biomolecules by understanding the biochemical mechanisms involved. The present thesis work aimed in exploring the metabolic capabilities of a purple non-sulfur bacterium, *Rhodobacter sphaeroides* OU5 in transforming aromatic amino acids and inventerize novel biomolecules through metabolite mining and bioprospecting. This work has resulted in the discovery of several novel metabolites, enzymes and metablolic pathways in addition to the discovery of biotechnological potential of a couple of metabolites.

L-Phenylalanine, L-tyrosine and L-tryptophan were used as precursors, which supported growth of *Rhodobacter sphaeroides* OU5 only when used as sole source of nitrogen under phototrophic conditions.

Mining for metabolites was done after extracting the metabolites into organic solvents from the culture supernatant. The exometabolite (Foot printing) analysis was done using liquid chromatography and mass spectrometer (LC-MS) and the known metabolites were identified from their molecular masses. Nine phenols esters were identified in the presence of the precursor L-phenylalanine/L-tyrosine. Two of the purified phenolic esters were characterized based on infra-red (IR), ^{1}H & ^{13}C-NMR and mass analysis as 3,4-dihydroxy-benzoic acid 5-carboxy-4-hydroxy-3-methyl-pentyl ester and 3,4,5-dihydroxybenzoyl terpenoid ester having molecular masses of (m/z) 298 and m/z 304, respectively. Fifteen indole esters were also identified in the presence of the precursor L-tryptophan. Two were purified and characterized as 3-hydroxy-6- (1H-indol-3-yloxy)-4-methylhexanoic acid (Rhodethrin) and 4-aminocyclohex-4-ene-1, 2-diol having molecular mass (m/z) of 279 and 129 $[M+H]^{+1}$, respectively.

While the above metabolites were obtained from whole cells of *Rhodobacter sphaeroides* OU5, in total, six chromatographically distinct phenols and thirteen indolics were identified from the assay supernatant of cell free extracts incubated with L-phenylalanine/L-tyrosine. The products of the assay varied depending on the presence or absence of 2-oxoglutarate in the assay mixture. A few key enzymes identified in the aromatic amino acids catabolism of *Rhodobacter sphaeroides* OU5, include, 3,4-dihydroxyphenylalanine aminotransferase (EC 2.6.1.49), 3,4-dihydroxyphenylalanine-reductive deaminase (EC 4.3.1.22), 3,4-

dihydroxyphenylalanine-oxidative deaminase, L-tryptophan ammonia lyase (WAL) and L-tryptophan aminotrasferase (EC 2.6.1.27). Among these, 3,4-dihydroxyphenylalanine aminotransferase (EC 2.6.1.49) was purified to homogeneity and characterized. This enzyme (Mw. ~123kDa) in *Rhodobacter sphaeroides* OU5 was found to be a heterodimer of 60 and 63 kDa.

In the absence of 2-oxoglutarate, two novel enzymes were identified in *Rhodobacter sphaeroides* OU5, viz., DOPA-reductive deaminase and DOPA-oxidative deaminase whose products were confirmed as 3,4-dihydroxyphenylpropionic acid (DPPA), 3,4-dihydroxyphenyl-pyruvic acid (DOPP) and ammonia, respectively. These enzymes were purified to their homogeneity and characterized. DOPA reductive deaminase (Mw. ~274 kDa) has an obligate requirement for NADH, found to be a heterotetramer of 110,82,43 and 39 kDa. The novelty of the enzyme was confirmed through MALDI-TOF-TOF analysis and an enzyme code (EC 4.3.1.22) was assigned by NC-IUBMB (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology). An O_2 dependent DOPA oxidative deaminase (Mw. ~190kDa), a pentameric protein (54,42,34,25 and 23kDa) operates without the involvement of 2-oxoglutarate or NADH and is involved in the formation of ammonia from L-DOPA.

A novel enzyme, L-Tryptophan ammonia lyase (WAL) was identified for the first time in all life forms. This enzyme operates in the absence of

2-oxoglutarate and pyridoxal-5-phosphate (PLP) (as co-factor) and the products; indole-3-acrylic acid and ammonia were confirmed. The enzyme (Mw. ~ 238kDa) was purified to homogeneity and characterized. The heterotetrameric (98,55,45 and 35 kDa) protein was confirmed as a novel enzyme through MALDI-TOF-TOF analysis. L-Tryptophan aminotrasferase (EC 2.6.1.27) an enzyme involved in the conversion of L-tryrpophan to indole-3-pyruvic acid (IPA) and glutamate was also confirmed in *Rhodobacter sphaeroides* OU5.

The novel metabolites were screened for phytoharmonal activity, cytotoxicity against Sup-T_1 lymphoma and colo-125, Jurkat cells, U937 cancer cell lines and COX-1 & 2 inhibitory activities.

1. INTRODUCTION

1. Introduction

Anoxygenic phototrophic bacteria (APB) are a physiological group of photosynthetic prokaryotes, distributed in four different phyla that are able to grow under anaerobic conditions by photosynthesis with out oxygen liberation. These bacteria are widely distributed in anoxic habitats of various ecosystems (Bryantseva *et. al.,* 1999). They lack photosystem-II and carryout anoxygenic photosynthesis. They have metabolic versatility and depend on e^- donors which are more reduced than water, like reduced sulfur compounds, hydrogen and organic compounds (Widdel *et al.,* 1993). The purple non-sulfur bacteria are capable of metabolizing a wide range of aliphatic organic compounds and are versatile in inducing metabolic routes in response to nutritional changes in the environment. Metabolism of aromatic hydrocarbons on the other hand are restricted to a few low molecular weight molecules (Sasikala and Ramana, 1995). Benzoate and its derivatives support growth of a few of these bacteria (growth-supporting aromatic hydrocarbons). In contrast, some of the aromatic hydrocarbons although they do not support growth, can transform without aromatic ring cleavage (Sasikala and Ramana, 1995). The latter studies were mainly concentrated on the metabolism of nitrophenols (Roldan *et al.,* 1998; Witte *et al.,* 1998) and aromatic amino acids (Saez *et al.,* 1999) by *Rhodobacter capsulatus* and 2-aminobenzoate by *Rhodobacter sphaeroides* OU5 (Nanda *et al.,* 2000; Sunayana *et al.,* 2005a). However, these studies were concentrated through the conventional methods (like HPLC), which helped in the identification of a few metabolic intermediates. In the present thesis work, metabolite mining was done using LC-MS profiling, which helped in the identification of novel metabolites, pathways and

enzymes involved in the photometabolism of aromatic amino acids by *Rhodobacter sphaeroides* OU5.

1.1 *Bacterial metabolism of L-phenylalanine and L-tyrosine*

1.1.1 *Utilization for growth*

Aliphatic amino acids supported bacterial growth either as source of carbon or nitrogen (Evans and Fuchs, 1988). On the contrary, aromatic amino acids are not widely used as a source of carbon by bacteria (Saez et al., 1999). L-phenylalanine/L-tyrosine supported growth of *Rhodobacter capsulatus* (Saez *et al.,* 1999); *Pseuodomonas putida* (Herrera and Ramos, 2007) and *Brevibacterium lines* (Chang and Michel, 1985) as sole source of nitrogen but not as a source of carbon or carbon and nitrogen; modification of the aromatic rings was also observed in the process. In the anaerobic metabolism of L-phenylalanine by *Thauera aromatica*, the substrate was oxidized and severed as the sole source of carbon (Schneider *et al.,* 1997). On the other hand, species of *Clostridium* have apparently not modified the aromatic rings but used aromatic amino acids as oxidizing agents and reduced them to phenylpropionic acid or phenyl acetic acid (Barker *et al.,* 1981).

1.1.2 *Catabolism*

Catabolism of L-phenylalanine or L-tyrosine takes place through several aerobic or anaerobic routes. Catabolism of L-phenylalanine/L-tyrosine by bacteria is carried out by a peripheral pathway (similar to that of eukaryotes) resulted in the formation of homogensitate as a central intermediate (Fig. 1). Homogensitate dioxygenase was involved in the opening of the aromatic ring of homogensitate, producing maleylacetoacetate and fumarylacetoacetate. Finally these substrates were hydrolyzed by a specific hydrolase forming fumarate and acetoacetate (Elsa *et al.,* 2004). In plant and photosynthetic bacteria the catabolism of L-tyrosine is also crucial because of

homogentisate is a precursor of photosynthetic pigments biosynthesis (Serre *et al.*, 1999).

The initial step in the catabolism of L-phenylalanine or L-tyrosine is mediated through transamination or ammonia lyation process. L-Phenylalanine and L-tyrosine are converted by *Rhodobacter capsulatus* (Saez *et al.,* 1999) and *Pseudomonas putida* (Jimenez *et al.,* 2002) into phenyl pyruvate and 4-hydroxypyruvate, respectively, mediated by a non-specific aromatic aminotransferase. In most cases, L-phenylalanine was transformed to L-tyrosine by phenylalanine hydroxylase (Mastore *et al.,* 2005). *trans*-Cinnamic acid and para-hydroxycinnamic acid are the products of L-phenylalanine catalyzed by L-phenylalanine ammonia lyase/L-tyrosine ammonia lyase (PAL/TAL; EC: 4.3.1.25) (Panke *et al.,* 2004; Xiang and Moore, 2005; Cui *et al.,* 2008). *Para*-Hydroxycinnamic acid biosynthesis from L-tyrosine catalyzed by TAL was reported from *Rhodobacter capsulatus* (Kyndt *et al.,* 2002*)* and *Rhodobacter sphaeroides* (Watts *et al.,* 2004; Xue *et al.,* 2007). 3,4-Dihydroxyphenyalinine (DOPA) was biosynthesized from L-tyrosine by *Erwinia herbicola* (Koyanagi et al., 2005). Some of the methanogens converted L-tyrosine to p-cresol and phenol (Evans and Fuchs, 1988). The down stream of L-phenylpyruvate was through phenyl acetaldehyde (Asakawa *et al.,* 1968) leading to the formation of phenyl acetate, phenylglyoxylate and culminating in the biosynthesis of benzyl CoA (Fig. 1; Blanco *et al.,* 1990; Mohamed and Fuchs, 1993; Mohamed *et al.,* 1993; Schneider *et al.,* 1997).

1.1.3 ***Enzymes involved***

1.1.3.1 ***Ammonia lyase family*** **(EC 4.3.1.--)** Ammonia-lyases are a family of enzymes that catalyzes the deamination of amino acids. The most common and well studied

among this family of enzymes include, L-phenylalanine ammonia lyase (PAL), L-tyrosine ammonia lyase (TAL), PAL/TAL and L-histidine ammonia lyase (HAL).

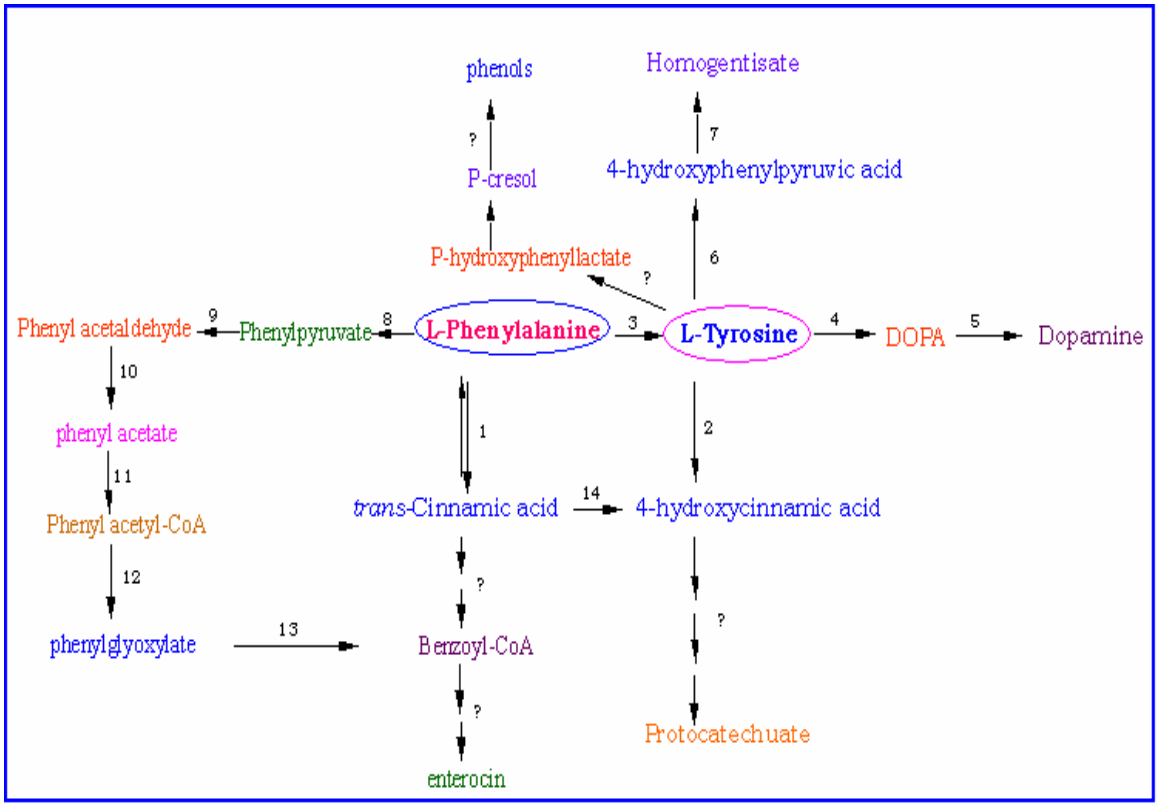


Fig. 1 General microbial catabolic pathways of L-phenylalanine and L-tyrosine

1) L-phenylalanine ammonia lyase (PAL; EC 4.3.1.24); 2) L-tyrosine ammonia lyase (TAL; EC 4.3.1.23); 3) phenylalanine hydroxylase; 4) tyrosine dehydrogenase; 5) 3,4-dihydroxyphenylalanine decarboxylase (DDC; EC 4.1.1.27); 6) tyrosine transaminase (TAT; EC 2.6.1.5); 7) 4-hydroxyphenylpyruvate dioxygenase; 8) phenylalanine aminotransferase (PATs; EC 2.6.1.5); 9) phenylpyruvate decarboxylase (PDC); 10) phenyl acetaldehyde dehydrogenase; 11) phenyl acetate Co-A ligase (EC 6.2.1.30); 12) phenyl acetate Co-A α-oxidizing enzyme; 13) phenylglyoxylate oxidoreductase; 14) cytochrome P450; DOPA=3,4-dihydroxyphenylalanine; ? = Indicates not known.

Deamination of tyrosine to *para*-hydroxycinnamic acid (pHCA) is catalyzed by a PAL/TAL enzyme. Phenylalanine ammonia lyase (PAL; EC 4.3.1.24) has been found in both higher plants (Lois *et al.,* 1989; Minami *et al.,* 1989) and various microorganisms (Langer *et al.,* 1997; Panke *et al.,* 2004; Xiang and Moore, 2005; Cui *et al.,* 2008). PAL enzyme are bifunctional in addition to L-phenylalanine, accept tyrosine as a substrate therefore called TAL. Among various microbial PAL/TAL enzymes, the fungi; *Rhodotorula glutinis* (RgTAL) had the highest TAL activity. All members of ammonia lyase family (PAL, TAL and HAL; Table. 1) contain a conversed ***"Ala, Ser, Gly"*** amino acids motif that undergoes autocatalytic cyclization to generate a 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO) group and acts as the catalytic electrophile for elimination of ammonia and a non-acidic β-proton from the amino acid substrate (Hernandez and Philips, 1993). This mechanism was supported by the X-ray crystal structure of the *Pseudomonas*-HAL (Schwede *et al.,* 1999) and *Rhodotorula glutinis*-PAL (Calabrese *et al.,* 2004). It is noteworthy that, most of the PAL and TAL enzymes are strongly inhibited by their products.

1.1.3.1.1 ***Phenylalanine ammonia lyase (PAL; EC 4.3.1.24)***

The enzyme from plant source performs the first reaction of the phenylpropanoids pathway and converts phenylalanine to *trans*-cinnamic acids (CA). Further hydroxylation of *trans*-cinnamic acid (CA) produces *para*-hydroxycinnamic acid (pHCA), which plays a pivotal role in the production of a diverse array of plant secondary metabolites. PAL from bacteria and plant deaminate phenylalanine to *trans*-cinnamic acid (CA), which is ultimately converted into secondary metabolites such as lignins, flavonoids and coumarins in plant (Winkel-shirley, 2001)- and several antibiotic compounds in bacteria (Williams *et al.,* 2005; Xiang and Moore, 2002; 2005). Humans recombinant phenylalanine ammonia lyase was explored for the

treatment of phenylketonuria (PKU) by metabolizing excess dietary phenylalanine (Sarkissian and Ganez, 2005).

1.1.3.1.2 ***Tyrosine ammonia lyase (TAL; EC 4.3.1.23)***

This enzyme is highly selective for L-tyrosine and synthesizes *para*-hydroxycinnamic acid (4-coumaric acid; pHCA) as a protein co-factor or antibiotic precursor in microorganisms (Kevin *et al.,* 2006). TAL enzyme from the photosynthetic bacterium *Rhodobacter sphaeroides* (RsTAL) was identified, cloned and functionally expressed in *E.coli* (Xue *et al.,* 2007). 4-coumaric acid serves as antibiotic precursor in microorganism (Kevin *et al.,* 2006) and co-factor for synthesis of a small 14 kDa water soluble protein designated as photoactive yellow protein (*PYP*) in *Ectothiorhospira halophilia, Rhodospirillum salexigens* and *Chromatium salexigenes* (Cusanovich and Meyer, 2003; Xue *et al.,* 2007).

Multiple research groups have used TAL for metabolic engineering of flavonoid and resveratrol biosynthesis pathways that require 4-coumaric acid as a precursor (Watts *et al.,* 2004; Jiang *et al.,* 2005; Zhang *et al.,* 2006; Qi *et al.,* 2007). Because of TAL forms 4-coumaric acid directly from L-tyrosine and uses in heterologous expression systems circumvents the need to express both PAL and 4-coumaric acid hydroxylase, a membrane bound cytochrome P450 enzyme for conversion of L-phenylalanine to 4-coumaric acid. Structural and functional studies of TAL identified a histidine in the active site as essential for controlling substrate preference for L-tyrosine over L-phenylalanine (Watts *et al.,* 2006; Louie *et al.,* 2006).

1.1.3.1.3 ***Phenylalanine ammonia lyase/Tyrosine ammonia lyase (PAL/TAL; EC 4.3.1.25)*** PAL/TAL was reported in a few microorganisms with possible involvement in the biosynthesis of secondary metabolites and similar to their plant counterpart. PAL/TAL enzymes from their involvents in conversion of aromatic amino acids

phenylalanine and tyrosine to *trans*-cinnamic acid (CA) and 4-coumaric acid, respectively. These compounds are of interest due to their potential as starting material for chemical and enzymatic conversion to a wide array of commercially valuable biomecules including, flavors, fragrances, pharmaceuticals, biocosmetics and other secondary metabolites (Xue *et al.,* 2007). However, two potential microbial routes from aromatic amino acids to pHCA can be envisioned (Fig. 1). In one route, (known to function in plants), 4-coumaric acid was formed from L-phenylalanine in a two-step process in which PAL removes the (pro-3S)-hydrogen and NH_3 from phenylalanine to yield *trans*-cinnamic acid (CA). In the next step, a cytochrome P450 enzyme system hydroxylates, CA to produces 4-coumaric acid (Werck *et al.,* 1990). The most natural PAL/TAL enzymes from either plants or microbial sources prefer to use L-phenylalanine rather than L-tyrosine as their substrate. In addition to their ability to convert L-phenylalanine to *trans*-cinnamic acid, also accept L-tyrosine as a substrate. In such reactions the enzyme is designated tyrosine ammonia lyase and reaction product is 4-coumaric acid. Exceptions to these rules are the recent reports have been described the identification, characterization, cloning and functional expression in *E. coli* of a TAL from anoxygenic phototrophic bacteria (Kyndt *et al.,* 2002; Watt *et al.,* 2004; Huang and Xue, 2006). In contrast to HAL, PAL/TAL is quite rare, identified thus far only in *Rhodobacter capsulatus* (integrated Genomics accession number; RRC01844) (Kyndt *et al.,* 2002), *Rhodobacter sphaeroides* (integrated genomics accession numbers; YP-355075) (Watt *et al.,* 2004) and very recently in *Saccharothrix espanaensis* (Gene bank accession numbers ABC88669) (Berner, 2006) (Table. 1). The sequence homologous to plant PAL such as from *Petroselinum crispum* (Rother *et al.,* 2002) (CAA57056; 30% identical and 48% similar) it rather shares greater homology to bacterial histidine ammonia lyase (HAL;

EC 4.3.1.3) such as from *Pseudomonas putida* (Schwede *et al.,* 1999) (A35251; 36% identical and 54% similar) and to TAL from *Rhodobacter sphaeroides* (Kyndt *et al.,* 2002). Historically, the amino acid ammonia lyases were thought to use a prosthetic dehydroalanine as an electrophile in the reaction mechanism but the three dimensional structure of PAL and HAL indicated that these enzymes contain MIO group for substrate activation (Schwede *et al.,* 1999; Calabrese *et al.,* 2004; Ritter and Schulz, 2004).

1.1.3.2 ***Deaminase family***

Deaminases are a group of enzymes that catalyze the elimination of ammonia from organic substitutes (Barker, 1981) and play an important role in the nitrogen cycle. Microbial L-amino acid deaminases identified so far include: L-tyrosine, L-phenylalanine (Xinag and Moore, 2005), arginine, glutamate, serine and cytosine. Based on the mechanism, they are further classified as: oxidative, reductive and hydrolytic deamination (David *et al.,* 2001).

1.1.3.3 ***3,4-Dihydroxyphenylalanine decarboxylase (EC 4.1.1.28)***

3,4-Dihydroxyphenylalanine decarboxylase purified from a mutant stain of *E. coli* is a homodimeric enzyme belonging to the α-family of pyridoxal-5-phosphate (PLP) enzyme. The catalytic activity consists in the generation of aromatic amine. DOPA decarboxylase catalyzes not only the decarboxylation of L-aromatic amino acids but also side reactions including half-transamination of aromatic amino acid and oxidative deamination of aromatic amines. Decarboxylation of L-aromatic amino acids, which is the main reaction the enzyme catalyzes was identified side reactions that is the oxidative deamination of aromatic amines (Bertoldi *et al.,* 2002; Bertoldi *et al.,* 1996) and the half transamination of aromatic amino acids accompanied by a pictet-spengler

reaction (Bertoldi *et al.,* 1999). The reaction specificity of DOPA decarboxylase does not change in the presence or absence of oxygen (Bertoldi and Borri, 2000).

1.1.3.4 ***3,4-Dihydroxyphenylalanine transaminase (EC 2.6.1.49)***

The enzyme catalyzes reaction between 3,4-dihydroxyphenylalanine (DOPA) and 2-oxoglutarate (an amino acceptor) to form 3,4-dihydroxyphenyl pyruvic acid (DOPP) and L-glutamate. This enzyme was reported from animal (Funnum and Larsen, 1965) and bacteria; *Alcaligenes faecalis* IAM 1015 (Nagasaki *et al.,* 1973) and *Enterobacter cloacae* (Nagasaki *et al.,* 1975).

1.1.3.5 ***Phenylpyruvate decarboxylase (EC4.1.1.43) and phenyl acetaldehyde dehydrogenase*** The enzyme catalyzes the reaction phenylpyruvate to phenyl acetate through phenyl acetaldehyde (Asakawa *et al.,* 1968) and also catalyzed the non-oxidative decarboxylation of phenylpyruvate in which, diphosphothiamin (DPT) and Mg^{2+} were co-factor. The compounds such as phenylpyruvate, indole pyruvate and keto acids with more than six carbons atoms in a straight chain served as substrate for the decarboxylase and subsequent step of the phenyl acetaldehyde catalyzed by the reaction of dehydrogenase (NAD as co-factor) from phenylacetate (Asakawa *et al.,* 1968).

1.1.3.6 ***Phenyl acetate-CoA ligase (EC 6.2.1.30)/phenyl acetate CoA α-oxidizing enzyme*** Phenyl acetic acids are common intermediate in the microbial metabolism of various aromatic substrates including phenylalanine. In *Thauera aromatica* (Sung and Fuchs, 1999); *Rhodococcus* sp. strain RHA1 (Juana *et al.,* 2005) phenyl acetate is oxidized under anoxic conditions, to a common intermediate benzyl CoA via phenyl acetyl CoA an enzyme catalyzes the reaction: phenyl acetyl CoA + 2 quinone +$2H_2O$ → phenylglyoxylate + 2quinone H_2 + CoASH (Sung and Fuchs, 1999). Phenyl acetyl CoA is formed from phenyl acetate by a specific phenyl acetate-coenzyme A (CoA)

ligase in both the aerobic and anaerobic pathways (Blanco *et al.,* 1990; Mohamed and Fuchs, 1993). Phenylglyoxylate is oxidized to benzyl CoA by the phenylglyoxylate: acceptor oxidoreductase complex enzyme, which is ultimately, transfers electrons to NAD^+ (Mohamed *et al.,* 1993; Schneider *et al.,* 1997).

1.1.3.7 ***Phenylalanine hydroxylase*** **(PhhA)**

The enzyme transformed phenylalanine to tyrosine. It is an iron-containing protein that requires the presence of the co-factor (6R)-L-erythro-5, 6,7,8-tetrahydrobiopterin (pterine) (Song and Jensen, 1996; Mastore *et al.,* 2005).

1.1.3.8 ***Other enzymes involved***

Tyrosine/phenylalanine aminotransferase (TAT; EC 2.6.1.5) from *Bacillus caldolyticus* catalyzes the conversion of tyrosine into 4-hydroxyphenylpyruvic acid and also phenylalanine to phenylpyruvate (Heilbronn *et al.,* 1999). The transformation of 4-hydroxyphenylpyruvate to homogentisate catalyzed by 4-hydroxyphenylpyruvate dioxygenase (Ruetschi *et al.,* 1992; Serre *et al.,* 1999). DOPA is synthesized by *Erwinia herbicola* (Koyanagi *et al.,* 2005) from L-tyrosine by a one-step oxidation reaction catalyzed by tyrosine phenol-lyase (TPL; EC 4.1.99.2) in *Escherichia coli* (Forrest *et al.,* 1993) (Mastore *et al.,* 2005).

1.2 ***Bacterial metabolism of Histidine***

1.2.1 ***Utilization for growth***

Streptomycetes, few enteric bacteria, *Pseudomonas* sp. and *Bacillus substilis* were capable of utilizing L-histidine as the sole source of carbon, nitrogen and carbon & nitrogen (Kroening *et al.,* 1987).

1.2.2 ***Histidine ammonia lyase (HAL; EC 4.3.1.3)*** Histidine ammonia lyase enzyme is apparently ubiquitous in bacteria. It is a red colored enzyme, which converts histidine in to urocanic acid and ammonia. The enzyme catalyzes the non-oxidative

elimination of the α-amino group of histidine and is closely related to the important plant enzymes phenylalanine ammonia lyase (Schwede *et al.,* 1999) and is further metabolized to glutamate (Poppe and Retey, 2005). It is widely distributed in higher plants (Whetten and Sederoft, 1992) and bacteria such as *Pseudomonas fluorescens, Pseudomonas aerusinosa, Pseudomonas putida, Pseudomonas testosteron* and A*erobacter aerogenes;* fungi (Godwin and Cunha, 2005) and yeast (Orndorff *et al.,* 1988). The whole genome sequence of photosynthetic bacterium *Rhodobacter sphaeroides* 2.4.1 indicated the presence of probable histidine ammonia lyase (Copeland *et al.,* 2005).

1.3 *Bacterial metabolism of L-tryptophan*

1.3.1 *Utilization for growth*

L-Tryptophan is metabolized by *Bacillus megaterium* (Bruknight and Sadoff, 1975), *Rodococcus erythropolis* (Suemori *et al.,* 1995) and several *Pseudomonas* (Keri *et al.,* 2005) as sole source of carbon, nitrogen and energy. L-Tryptophan has a variety of metabolic functions with in the cell. It is incorporated into the polypeptide chains of enzymes and proteins and a biosynthetic precursor of the co-factor NAD (Magni *et al.,* 1999).

1.3.2 *Catabolism*

Aerobic L-tryptophan degradation often referred as the kynurenine pathway is the major route to L-tryptophan catabolism in mammals. While a variety of chemical transformations related to the aerobic degradation of L-tryptophan and most for the genes and corresponding enzymes involved therein have been predominantly characterized in eukaryotes, relatively little was known about this pathways in bacteria (Fig. 2). In *E. coli* and many other bacteria the non-oxidative degradation of L-tryptophan to indole, pyruvate and ammonia precedes via pyridoxal-5-phospahte

(PLP) dependent tryptophanase (Tryptophan indole-lyase EC 4.1.99.1) (Cowell *et al.,* 1973; Vederas *et al.,* 1978). The oxidative degradation of exogenous L-tryptophan via the anthranilate pathway was implicated as a sole source of carbon and nitrogen, the gene encoding kynureninase (KYN; EC 3.7.1.3) was cloned from *Pseudomonas fluorescens* and corresponding enzyme homologous to eukaryotic KYN was characterized. The tryptophan 2,3-dioxygenase (TDO; EC 1.13.11.42) activity has been described in some bacteria, however the corresponding genes have been identified (Oleg *et al.,* 2000).

1.3.3 ***Enzymes involved***

1.3.3.1 ***Tryptophanase (EC 4.1.99.1)***

Tryptophan indole-lyase is a pyridoxal-5-phosphate (PLP) dependent enzyme found in many bacteria that catalyzes the reversible hydrolytic decomposition α, β-elimination of L-tryptophan leading to formation of indole, pyruvate and ammonia (Snell, 1975; Kulikova *et al.,* 2006). Tryptophanse is a homotetrametic protein in which (four identical 52 kDa monomers) each monomer binds to one molecule of PLP, which form an aldimine bound with a lysine residue (Snell, 1975). It was shown that the *tnaA* gene encoding tryptophanse is necessary for biofilm formation in *E.coli* (Martino *et al.,* 2003). It has been cloned from a virulent strain of *Haemophiles influenzae,* and the production of indole was found to be correlated with the ability of the strain to cause some infectious diseases includes; septicemia, epiglottitis and meningitis.Thus tryptophanase is convenient as a target for elaboration of efficient inhibitor, which could be used for treatment of meningitis.

1.3.3.2 ***Trytpohan aminotransferase (EC 2.6.1.27)*** This enzyme belongs to the family of transferase, catalyzes L-tryptophan into indole-3-pyruvic acid in the presence of 2-oxoglutarate (as the amino acceptor) and pyridoxal-5-phasphate (as co-

factor) from *Enterobacter cloacae* (Table.1) (Koga *et al.,* 1994) and also reported from plant and animal system (Niel *et. al.,* 1968; Stanley *et al.,* 1984)

1.3.3.3 ***Tryptophan 2,3-dioxygenase and Kynurenine formidase (EC 3.5.1.49)***

In the anthranilic acid pathway, tryptophan is first converted to formyl-kynurenine by tryptophan 2,3-dioxygenase and the latter to the formation of kynurenine and anthranilic acid by kynurenine formidase (Fig. 2) (Friedich and Mitrenga, 1981).

1.3.3.4 ***Indole-3-pyruvic acid decarboxylase***

Indole-3-pyruvic acid decarboxylase (IPDC) catalyzes the conversion of indole-3-pyruvic acid to indole-3-acetaldehyde has been isolated from *Azospirillum brasilense* and *Paenibacills polymyxa* E681 (Costacura *et al.,* 1994; Phi *et al.,* 2008) (Fig. 2). Indole-3-pyruvic acid can also be converted to indoleacetaldehyde non-enzymatically (Koga *et al.,* 1994).

1.3.3.5 ***Tryptamine oxidase (EC 1.4.3.4)/ tryptophan decarboxylase (EC 4.1.1.28)***

Tryptophan can also be converted to indole-3-acetaldehyde and IAA via the intermediate tryptapmine (Fig. 2). The initial metabolism of tryptophan to tryptamine is catalyzed by tryptophan decarboxylase (aromatic-L-amino acid decarboxylase) followed by conversion of tryptamine to indole-3-acetaldehyde by tryptamine oxidase (Patteen and Glick, 1996).

1.3.3.6 ***Indole lactate dehydrogenase (EC 1.1.1.110)*** This enzyme was isolated from *Clostridium saprogenic* (Jean and Demoss, 1968). It catalyzes indole-3-pyruvic acid to indole-3-lactic acid or reversible reaction of indole-3-lactate + NAD^+ gives rise to indole-3-pyruvate and $NADH+H^+$.

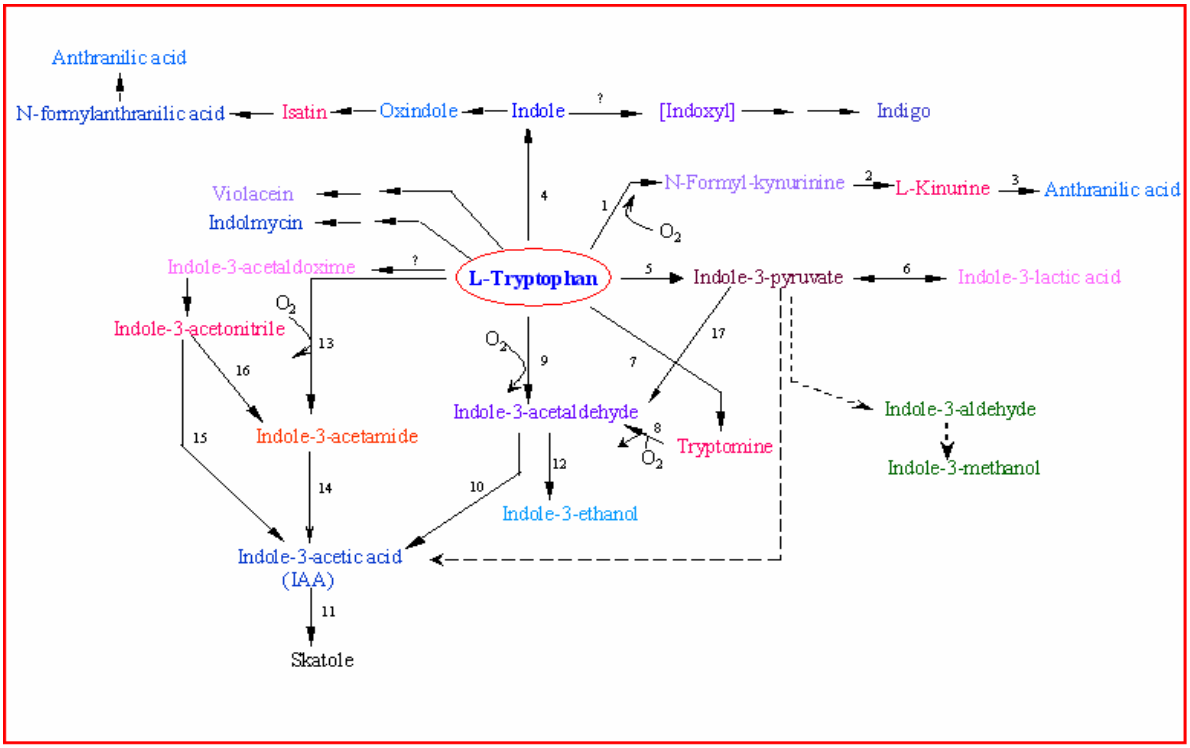


Fig. 2 Metabolism of L-tryptophan in bacteria. Tryptophan 2,3 dioxygenase (EC 1.13.11.11); 2) kynurenine formidase (EC 3.5.1.49); 3) kynureninase (EC 3.7.1.3); 4) tryptophanase (EC 4.1.99.1); 5) Tryptophan aminotransferase (EC 2.6.1.27); 6) indole lactate dehydrogenase (EC 1.1.1.110); 7) Tryptophan decarboxylase (EC 4.1.1.28); 8) tryptamine oxidase (EC 1.4.3.4); 9) Tryptophan side chain oxidase (EC 4.1.1.43); 10) indole acetaldehyde dehydrogenase (EC 1.2.1.3); 11) indole acetic acid oxidase; 12) indole acetaldehyde dehydrogenase; 13) Tryptophan 2-monooxygenase (EC 1.13.12.3); 14) indole acetamide hydrolase (EC 3.5.1.0); 15) Nitrilase; 16) Nitrile hydratase;17) indole-3-pyruvic acid decarboxylase. The dotted lines (---) indicate a spontaneous reaction.

1.3.3.7 *Other enzymes involved*

Indole-3-acetonitril is converted to IAA by the reaction of nitrilase has been identified in higher plants belonging to families Cruciferae (cabbage), Gramineae (Grasses) Musaceae (banana) (Bartling *et al.,* 1994; Kobayashi and Shimizu, 1994) and microbes (*Klebsiella ozonae*, *Alcaligenes faecalis* and *Rhodococcus rhodochrous* (Kobayashi and Shimizu, 1994). Nitriles can be hydrolyzed directly to their corresponding acids through the action of specific nitrilases or catalyzed via a two-step process involving an initial conversion to an amide by nitrile hydratase followed

by hydrolysis of the amide, to an acid by amidase or by acetamide hydrolase (Fig. 2) (Kobayashi *et al.,* 1992).

1.3.3.8 ***Indole-3-acetic acid (IAA) biosynthesis in bacteria***

IAA biosynthesis pathways in bacteria has been reviewed critically (Stijn *et al.,* 2007; Patten and Glick, 1996). The identification of intermediates led to five different pathways (Fig. 2) using L-tryptophan as a precursor for IAA. The discovery of IAA as a plant growth regulator coincided with the first indication of the molecular mechanisms involved in tumorigenesis induced by agrobacterium. It was later found that not only plants but also microorganisms including bacterial strain and fungi are able to synthesize IAA (Patten and Glick, 1996). The production of IAA by *Enterobacter* and *Pseudomonas* strains harboring the gene for indole-3-pyruvic acid decarboxylase were higher than those produced by these same strain with out the gene (Koga *et al.,* 1995). A number of studies have clearly shown that IAA can be a signaling molecule in microorganism in both IAA producing and IAA non-producing species (Stijin *et al.,* 2007). Catabolism of L-tryptophan to indole-3-acetic acid (IAA) occurs via five distinct routes; 1.Indole acetamide pathway catalyzed by tryptophan 2-monooxygenase (EC 1.13.12.3) and indole acetamide hydrolase (EC 3.1.1.51) (Comai and Kosuge, 1980). 2. Tryptophan side-chain oxidase pathway tryptophan side chain oxidase (TSO; EC 4.1.1.43) activity has demonstrated in *Pseudomonas fluorescens* CHA0. In this pathway tryptophan is directly converted to indole-3acetaldehyde by passing indole-3-pyruvic acid, which can be oxidized to IAA (Oberhansli *et al.,* 1991) 3. Indole-3-acetonitril pathway conversion of indole-3-acetonitrile to IAA by a nitrilase in *Alcaligenes faecalis* (Nagasawa *et al.,* 1990; Kobayashi *et al.,* 1993) nitrilase was detected with specificity for indole-3-acetonitrile.

Enzyme (EC.No.)	Organism	Co-factor	Substrate	Co-substrate	Products	Gene Bank No.	Reference
PAL (EC 4.3.1.24)	*Rhodotorula glutinis/R.rubra/ Rhodobacter capsulatus*	MIO	Phenylalanine	NA	*trans*-cinnamic acid	ABC88669	Godwin and Cunha, 2005; Xian *et al.*, 2005; Moffitt *et al.*, 2007
PAL/TAL (EC 4.3.1.25	*Rhodotorul rubra /Streptomycesmaritimus*	MIO	Phenylalanine/ Tyrosine	NA	CA/pHCA	AF254925/AAF81735	Watts *et al.*, 2006
TAL (EC.4.3.1.23)	*Rhodobacter sphaeroides/Rhodobacter capsulatus*	NA	Tyrosine	NA	PhCA	YP355075	Watts *et al.*, 2006; Xue *et al.*, 2007
HAL (EC 4.3.1.3)	*Rhodopseudomonas putida*	NA	Histidine	NA	Urocanic acid	RRC01844	Schwede *et al.*, 1999; Poppe and Retey, 2005
WAT (EC 2.6.1.27)	*Brevibacteriaum linens/Clostridium sporogenes/Enterrobacter cloacae*	PLP	Tryptophan	α-KGA	Indole pyruvic acid	AK102509	Koga et al., 1994
Phenylalanine/ Tyrosine aminotransferase (EC.2.6.1.5)	*Klebsiella pneumoniae*	PLP	Tyrosine	α-KGA	4-dihydroxyphenylpyruvic acid	NC.000913.1	Heilbronn *et al.*, 1999
DOPA transaminase (EC 2.6.1.49)	*Enterobacter cloacae/ Alcaligens faecalis/ Ervinia gerbicola*	PLP	L- DOPA	α -KGA	3,4-dihydroxyphenylpyruvic acid	D55724	Funnum *et al.*, 1965 Nagasaki *et al.*, 1973
Histidine transamiase EC 2.6.1.38)	*Pseudomonas acidovorans*	PLP	Histidine	α-KGA	Imidazol-5 yl pyruvate	AE001380	Coote *et al.*, 1969
TDC (EC 4.1.1.28)	*Streptococcus faecalis*	PLP	Tryptophan	NA	Tryptamine	M25151	Qiu-rong Li *et al.*, 2003;
DDC (EC 4.1.1.28)	*Streptococcus faecalis*	PLP	L- DOPA	NA	Dopamine	UO8597	Christenson *et al.*, 1972

Table.1 Few key enzymes involved in aromatic amino acids catabolism.
(PAL = phenylalanine ammonia lyase; TAL = tyrosine ammonia lyase; HAL = histidine ammonia lyase; WAT=tryptophan aminotransferase; DOPAATS = 3,4-dihydroxyphenylalanine aminotransferase; TDC= tryptophan decarboxylase; DDC= 3,4-dihydroxyphenylalanine decarboxylase; PLP= pyridoxal-5-phosphate; DOPA= 3,4-dihydroxyphenylalanine; pHCA = p-hydroxycinnamic acid; CA/pHCA = Cinnamic acid/p-hydroxycinnamic acid; α-KGA = 2-oxoglutarate; MIO = 3,5-dihydro-5-methylidene-4H-imidazol-4-one; NA= not applicable)

In *Agrobacterium tumefaciens* and *Rhizobium* sp., nitrile hydratase and amidase activity was identified, indicating the conversion of indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA) via indole-3-acetamide (IAM) (Kobayashi *et al.*, 1995). 4 Indole-3-pyruvate pathway the first step in this pathway is the conversion of tryptophan to indole-3-pyruvic acid by an aminotransferase (transamination). In the rate-limiting step, Indole-3-pyruvic acid is decarboxylated to indole-3-acetaldehyde

by indole pyruvate decarboxylase (IPDC). In the last step indole-3-acetaldehyde is oxidized in IAA Azospirillum *brasilense, Pseudomonas putida* and *Paenibacills polymyxa* E681 (Fig. 2)(Costacurta *et al.,* 1994; Brandl and Lindow, 1996; Patten and Glick, 2002; Phi *et al.,* 2008). 5.Tryptamine pathway was identified in *Azospirillum* and *Bacilluss cereus* conversion of exogenous tryptamine to IAA (Fig. 2) (Hartmann *et al.,* 1983; Perley and Stowe, 1996)

1.3.3.9 ***Tryptophan-independent pathway of IAA biosynthesis***

A bacterial tryptophan-independent pathway was demonstrated in *Azoperillum brasilense* by feeding experiments with labeled precursors (Prinsen et al., 1993).

1.3.3.10 ***IAA from anoxygenic phototrophic bacteria***

Indole-3-acetic acid production was reported in a few of anoxygenic phototrophic bacteria (*Rhodopseudomonas palustris*, *Rhodobacter sphaeroides*, *Rubrivivax gelatinosus* and *Rubrivivax tennius*) from L-tryptophan grown culture (Srinivas *et al.,* 2002) not only from L-tryptophan but also produced from indole and glycine by *Rhodobacter sphaeroides* (Rajashekar *et al.,* 1999).

1.4 ***Production of secondary metabolites from aromatic amino acids***

The metabolism of aromatic amino acids in microorganisms has been widely studied, many microorganisms have evolved secondary metabolic pathways with the capacity to produce compounds displaying an impressive array of pharmacological application includes, pigments, toxins, enzyme inhibitors, pesticides, herbicides, antiparasitics, mycotoxins, antitumer agents, antibiotics cytotoxicity activities and growth promoters of animal and plants.

1.4.1 ***Indole terpenoid ethers and esters***

The novel indole terpenoids viz., sphestrin (Sunayana *et al.,* 2005a) and rhodestrin (Sunayana *et al.,* 2005b) were isolated from the culture supernatants of *Rhodobacter*

sphaeroides OU5 grown in presence of 2-aminobenzoate. Indole terpenoid esters/ethers, are recent discoveries of biomolecules produced by a purple non-sulfur bacterium *Rhodobacter sphaeroides* OU5 having phytohormonal activity. They were produced in the presence of processor like, 2-aminobenzoate (Nanda *et al.,* 2006) or aniline (Vijay *et al.,* 2006).

There are a few reports of indole esters biosynthesis in plants system and microorganisms. 4-Chloroindole-3-acetic acid and its esters were chemically synthesized from 2-chloro-6-nitrotoluene as the starting material (Katayama, 2000). Esters of indole-3-acetic acid were extracted and purified from the liquid endosperm of immature fruits of various species of the horse chestrint (*Aesculus parviflora, Aesculus baumanni; Aesculus paviarubra and Aesculus paviahumulis*). The liquid endosperm contained at least twelve chromatographically distinct esters. One of these compounds were purified and characterized as an ester of indole-3-acetic acid and myoinositol (Domagaski *et al.,* 1987). Indole-3-acetyl-myo-inositol esters have been demonstrated as an endogenous component of etiolated *Zeamays* shoot tissue. The amount of indole-3-acetyl-myo-inositol eaters in the shoots was determined to be 74 nmoles.kg^{-1} fresh weight (Chisnell, 1984).

1.4.2 ***Biological applications of indole and its derivatives***

Indoles are extensively produced by the chemical industry for variety of applications including, pharmaceuticals, pesticides and dyes, they are widely used as analgesics (Magniez *et al.,* 1995), anti-inflammatory agents (Verma *et al.,* 1994), antihypertensive (Frishman, 1983), anti HIV compound (Britcher *et al.,* 1995) and phytohormones (Elsorra, 2003). Many indole esters were found as COX-2 selective enzyme inhibitors (Olgen *et al.,* 2007; Olgen and Nebioglu, 2002) they were also found to have anti-lipid peroxidation (LP) activity and antisuperoxid formation (SOD)

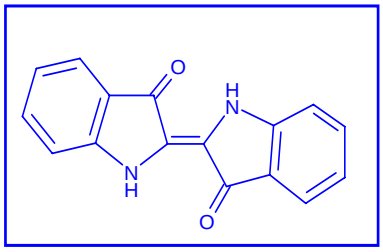
(Olgen and Coban, 2003, Olgen *et al.,* 2007). Indole esters were also found to have more phytoharmonal activity than their corresponding acids in the auxin bioassay (Katayama, 2000).

1.4.3 ***Indole-3-acetic acid (IAA)***

Light/horseradish peroxidase (HRP) activation has been suggested as a new photodynamic cancer therapy by forming free radicals (Dong *et al.,* 2006) such as indolyl, statolyl and perxoyl radicals (Folkes *et al.,* 2002), which can cause lipid peroxidation. The combination of IAA and HRP shows cytotoxic to mammalian cells includes, G361 human melanoma cells (Dong *et al.,* 2006) and human pancreatic cancer BXPC-3 cells (Chen *et al.,* 2005).

1.4.4 ***Indigo***

The blue dye indigo has been known since prehistoric times and is still one of the most economical important textile dyes, the first report of microbial indigo production



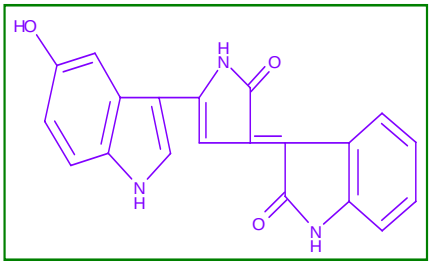
Indigo

was in 1928 (Gray, 1928) it is biosynthesized in bacteria via the oxidation of indole by a naphthalene dioxygenase and subsequent oxidation and dimerization (Ensley *et al.,* 1983). The desire to achieve a competitive, alternative to the chemical production of indigo rejuvenated interest in microbial indigo production (Murdock *et al.,* 1993) since many microorganisms expressing both monooxygenase (Allen *et al.,* 1997) and dioxygenase (Murdock *et al.,* 1993) during growth on aromatic hydrocarbons have been shown to transform indole to form indigo production have focused on the naphthalene dioxygenase from *Pseudomonas putida* PpG7 expressed in *Escherichia coli*. Some of the genes of indigo biosynthetic pathway have been cloned and used to construct “

engineering bacteria" with this kind of bacteria, more efficient fermentation systems for indigo production have been exploited (Han *et al.,* 2008)

1.4.5 ***Violacein***

Chromobacterium violaceum was first reported as an isolate from wet rice paste, one of the characteristics of this microorganism is the ability to produce a purple (deep violet) pigment known as violacein under aerobic conditions. The biological role of violacein in *Chromobacterium violaceum*, as well as its biosynthesis pathway was well reported (DeMoss and Evans, 1959) and the role of tryptophan and other indole derivatives (DeMoss and Evans, 1960; Hoshino *et al.,* 1987; Hoshino and Ogasawara, 1990; Duran *et al.,* 1994). Momen and Hoshino (2000). Tryptophan appears to be the only precursor molecule in violacein biosynthesis, its production is apparently essential for pigment production in *Chromobacterium violaceum* (Vasconcelos *et al.,* 2003; Regina *et al.,* 2004). The IUPAC name and molecular mass of violacein (3-[1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ylidene]-1,3-dihydro-2H-indol-2-one) and [m/z 343.34]. Violacein has attracted interest owing to its important multiple biological activities and pharmacological potentials such as antibiotic, bactericide (Duran *et al.,* 1983); tripanocide (Duran *et al.,* 1994); antitumaral (Melo *et al.,* 2003; Ferreira *et al.,* 2004; Saraiva *et al.,* 2004); antiviral (Kodach *et al.,* 2006); genotoxic (Andrighetti *et al.,* 2003) properties. The antioxidant efficiency against oxygen and nitrogen reactive species as a scavenger of hydroxyl, superoxide and nitric oxide radicals (Konzen *et al.,* 2006). In addition, it is capable of inducing apoptosis in cancer cell cultures (Duran and Menck, 2001) and

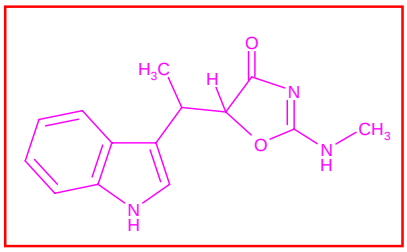


Violacein

effective against a panel of neoplastic cell lines, including leukemia lineage cancer diseases (Melo *et al.,* 2003).

1.4.6 ***Indolmycin***

Indolmycin is a secondary metabolite produced by *Streptomyces grisens* ATCC 1248 (formally *Syreptomyces albus* BA 3972A), which was isolated from a sample of African soil. Indolmycin completely inhibits bacterial TrpRS (Tryptophanyl tRNA Synthetase) enzyme (Makoto *et al.,* 2002) and it exhibits antimicrobial activity against gram-positive and gram-negative bacteria. Recently researchers have shown that indolmycin is active against *Mycobacteria* and *Helicobacter pylori.* Which is known as a major causative agent of chronic active gastritis.



Indolmycin

1.5 ***Phenols and its derivatives***

1.5.1 ***Alkyl esters of gallic acid***

The chief source for obtaining gallic acid is through the hydrolysis of plant based products like tannins (Inoue *et al.,* 1995). Microbial production of gallic acid was also reported using tannic acid as substrate (Kar *et al.,* 1999). The esters iso-amyl- (iAG), n-amyl- (nAG), iso-butyl- (iBG), n-butyl- (nBG) and isopropyl gallate (iPG) were synthesized from gallic acid were chemically synthesized (Christian *et al.,* 2007). Gallic acid (3,4,5-trihydroxybenzoic acid) is an industrially important phenol and finds its applications in various fields (Kar *et al.,* 1999).

Alkyl esters of gallate are an important group of biogenic molecules reported from plants (Yang *et al.,* 2003), bee propolis (Ahn *et al.,* 2004) and yeasts like candida (Stevenson *et al.,* 2007). These molecules are of biotechnological significance since they are known to have anti-oxidant (Chen *et al.,* 1997), anti cancer (Samaha *et al.,*

1997; Li *et al.,* 2003), anti HIV (Burke *et al.,* 1995) and anti-fungal/microbial (Tawata *et al.,* 1996) activities. Alkyl esters of gallic acid have antiviral, antibacterial antifungal properties (Fujita and Kubo, 2002) specifically against gram-positive bacteria (Kubo *et al.,* 2004).

1.6 *Mining for metabolites using metabolomics*

Microbial metabolomics has received much attention in the recent years (Natasa and Paul, 2003; Claudia *et al.,* 2005; Mlawule et al., 2007; Julia et al., 2007; Valeria *et al.,* 2008). It supports a wide range of microbial research from drug discovery efforts to metabolic engineering and pathways. Many researchers preferred the systematic investigations of various metabolites from enzyme based analysis (Mashigo *et al.,* 2007) using techniques like HPLC, LC-MS/MS, NMR and CE-MS (Capillary electrophoresis coupled to mass spectrometry) (Warwich, et al., 2005). Mining is a process of extraction, which helps in the identification of metabolites through liquid chromatography (LC)-mass spectrometry (MS) metabolic fingerprinting analysis. This lead to the discovery of several novel biomolecules (Natasa and Paul, 2003; Mikihisa, 2008), pathways (Wilkinson and Micklefied, 2007; Wolfram and Oliver, 2002), enzymes (Mashigo *et al.,* 2007) and genes (Mikihisa, 2008).

1.7 *Definition of the problem*

Metabolism of aromatic compounds was well recognized in a few photosynthetic bacteria (Harwood, 1999; Fuchs *et al.,* 1994; Sasikala and Ramana, 1998). The present understanding of aromatic compound metabolism by purple bacteria, either through light-dependent degradation of benzene ring, supporting growth by providing carbon source/electron donors, or through transformations. *Rhodobacter sphaeroides* OU5, a purple non-sulfur bacterium occurring abundantly in paddy soil and waste waters, lacks the ability to completely mineralize aromatic compounds for its growth,

but was able to photobiotransform some of the aromatic compounds. Transformation of 2-aminobenzoate to indoles (Nanda *et al.,* 2000; Sunayana *et al.,* 2005a) and aniline to indoles (Vijay *et al.,* 2006). Though an earlier study suggested that L-tyrosine was an intermediate and homogentisate as the end product of L-phenylalanine catabolism in *Rhodobacter capsulatus,* the aminotransferase activity and yields of homogentisate were low (Saez *et al.,* 1999). The hydroxyl group of L-tyrosine is in the 4th position while homogentisate is 2,5-dihydroxyphenylacetic acid. This shift in the hydroxyl group could be due to migration of the pyruvate side chain (Saez *et al.,* 1999). An attempt was made to extend the study, exploring the potential of *Rhodobacter sphaeroides* OU5 in aromatic amino acid metabolism. Hence a detailed study on this topic was taken for my Ph.D thesis with the following objective;

Objective

To investigate downstream process of L-phenylalanine, L-tyrosine and L-tryptophan metabolism in a purple bacterium *Rhodobacter sphaeroides* OU5 using metabolomics approach

2. METERIALS AND METHODS

2.Materials and Methods

2.1 ***Glassware*** All the glassware used in the present experiment including test tubes, pipettes, measuring cylinders, culturing flasks, reagent bottles, petriplates, screw cap test tubes were of Borosil or 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 detergent. After removing all the traces of the detergent, the glassware were rinsed with deionized water and kept in oven for drying at 100 ^oC.

2.3 ***Deionized water*** Deionized water obtained from deionizer plant (ion exchange India Ltd. Model-CA 20/U) was routinely used for rinsing of glassware after washing and for media preparation. Distilled water and double distilled water stored in white carboys was used for preparation of stock solutions and chemical analysis. Milli-Q water was sued for HPLC analysis (HPLC grade)

2.4 ***Chemicals*** The chemicals used in this study were of analytical grade obtained from Sigma- Aldrich, Lancaster, Qualigens or 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 were done by autoclaving at 15 lbs for 15 min at 120 ^oC.

2.7 ***Organisms and growth conditions*** A purple non-sulphur bacterium, *Rhodobacter sphaeroides* OU5 [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 (250/1000/5000 ml) on a mineral (Biebl and Pfennig's 1981) medium (Ingredients (g/L) KH_2PO_4 -0.5, $MgSO_4.7\ H_2O$ -0.2, NaCl -0.4,NH_4Cl -1.2,$CaCl_2.2\ H_2O$ -0.05, 3.0, Yeast extract -0.12, Ferric citrate (0.1%w/v)-5 ml and trace elements SL_7-1.0 ml ($mg.ml^{-1}$): HCl (25% v/v)-1ml; $ZnCl_2$-7; $MnCl_2.4H_2O$-100; H_3BO_3-60; $COCl_2.6H2O$-200; $CuCl_2.H_2O$-20; $NiCl_2.6H_20$-20; $NaMoO_4.6H_2O$-40) with malate (22 mM) and ammonium chloride (7 mM) as carbon e^- donor and nitrogen sources, respectively, at 30 ± 2 °C.

2.8 ***Maintenance of stock culture*** Stock cultures of *Rhodobacter sphaeroides* OU5 was maintained as agar stabs. Stabs were prepared by using 2% (w/v) agar solidified heterotrophic medium, filled to the ¾ volume of 5 ml capacity screw cap test tubes. The culture taken on a sterile needle was stabbed into the agar and the culture tubes were illuminated (2,400 lux) and incubated at 30 $\pm$ 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 60 days and contamination from other bacteria was checked periodically by streaking on nutrient agar plates.

2.9 ***Purity of the cultures*** Culture was checked for its purity before and after experiment by streaking on peptone agar plates ($g.l^{-1}$): Peptone-10, yeast extract-3, Agar-20 and NaCl-5, incubating aerobically at 30 $\pm$ 2 °C.

2.10 ***Growth and biomass*** Growth in terms of optical density (O.D) and the biomass as mg dry $wt.ml^{-1}$ was measured using a Systronic colorimeter (Mode-112) at 660 nm (Filter 8) against uninoculated media as blank.

2.11 ***Utilization of aromatic amino acids for growth*** Ammonium chloride was replaced with aromatic amino acids viz. L-tryptophan, L-phenylalanine or L-tyrosine (1 mM) when used as nitrogen source or malate was replaced when used as carbon source or both malate and ammonium chloride were replaced when used as source of carbon and nitrogen.

2.12 ***Bulk cultivation*** Culture was grown in 2 liter reagent bottle for bulk cultivation of *Rhodobacter sphaeroides* OU5 with 20 % (v/v) of initial inoculum the organism was grown on the photoheterotrophic growth medium (described above) till its logarithmic phase (~ 24-36 h) under light (2,400 lux), anaerobic conditions at 30 $\pm$ 2 ºC. a stock of sterilized L-tryptophan or L-phenylalanine were added to the culture giving a final concentration of 1mM.

HPLC profiling was carried out from the culture supernatant, which was extracted by ethyl acetate and after about 2 h, three distinct layers were seen in the extraction flask, the lower aqueous layer followed by an inter-phase lipid layer and the upper ethyl acetate layer. These layers were carefully separated using a separating funnel and excess water from the lipid and ethyl acetate layer was removed by adding anhydrous sodium sulfite. The ethyl acetate and aqueous layers were concentrated and used for further analysis.

2.13 ***Extraction and characterization of metabolites***

2.13.1 ***Whole cells***

2.13.2 ***Isolation of phenol terpenoid metabolites***

Ten litre of the L-phenylalanine induced (38h) culture was harvested and the supernatant was extracted thrice with ethyl acetate. The ethyl acetate extract was pooled and 1 g of anhydrous sodium sulphite was added to remove water molecules. The ethyl acetate layer

was evaporated to dryness under vacuum and resuspended in 5 ml of methanol (Extract-I). Further purification was done through column chromatography by loading on to silica gel (80-120 mesh) in 450 x 150 mm glass column followed by elution with hexane (Extract-Ia) and methanol (Extract-Ib). Extract-Ib was used for LC-MS analysis. The methanolic fraction (extract-Ib) was completely dried in a rota-vaporator at 20 ^{o}C, re-loaded on to a fresh silica gel column, dried and extracted with 50 ml hexane, chloroform, methanol (6:3:1v/v/v), which yielded a yellow colored metabolite (Extract-II), which had a R_f of 0.52 on a preparative TLC using solvents, methanol, chloroform (1:1v/v). Extract-II was used for LC-MS, ^{1}H & ^{13}C NMR, IR and UV analysis to determine the structure.

2.13.1.2 ***Isolation of indole terpenoid metabolites*** Ten liters of the L-tryptophan induced (6 h) culture was harvested by centrifugation (16,000 x g; 10 min) and the supernatant was extracted thrice with ethyl acetate, pooled, evaporated to dryness under vacuum and resuspended in 5 ml methanol (Fraction- A). Fraction-A was either directly used for LC-MS metabolite profiling or for further purification. The methanolic sample (Fraction-A) was loaded on to a silica gel (80-120 mesh) glass column (450 x 150 mm) and eluted first with 50 ml benzene (S1 fraction), followed by 50 ml methanol + benzene (7:3 v/v) (S2 fraction) and finally with 50 ml hexane (S3 fraction). Only fraction S1 and S2 gave positive test with indole reagents. These fractions were evaporated to dryness and resuspended in methanol. After confirming their purity by thin layer chromatography (TLC) under three different solvents, they were used for elucidation of structure using IR, NMR and mass analysis.

2.14 ***Studies using cell free extracts*** *Rhodobacter sphaeroides* OU5 adapted for (38 h) on L-phenylalanine or L-tryptophan (6 h) was used for the metabolite profiling studies using cell free extracts. The cells were harvested by centrifugation (16,000 x g for 10 min); pellet was washed (twice) with 0.05 M potassium phosphate buffer (pH 7.8) and resuspended in 50 ml of the same buffer. Cells were sonicated with MS-72 probe (Bandelin, Germany make, Model-UW2070) to complete cell lysis after 8-9 cycles. The cell suspension was centrifuged (16,000 x g for 20 min) and the supernatant was used as a source of enzyme. After enzyme assay the products were extracted in 10 ml ethyl acetate extracted twice, finally pooled and 5 mg of anhydrous sodium sulphite was added to remove water molecules. The ethyl acetate extract was evaporated to dryness under vacuum, resuspended in HPLC grade methanol and used for LC-MS profiling.

2.15 ***Analytical methods***

2.15.1 **C*hromatography*** Analytical thin layer chromatography was performed on glass plates (7 x 2cm) coated with Acme's silica gel GF-254 (254 mu) containing 13% calcium sulfate as a binder. The spots were visualized by short exposure to UV light or iodine vapor. Column chromatography was carried out using Acme's silica gel (60-120 mesh or 100- 200 mesh).

2.15.2 ***HPLC analysis*** Metabolites quantification was performed on HPLC (Shimadzu LC 20AT Japan) isocratic liquid chromatograph with DAD-SPDM20A using a reverse phase column Luna RP-C_{18} (2) 5 um (250 x 4.6mm) methanol 50% and water 50% was used at 0.5 ml/min; injection volume was 20 µl, the UV-spectra were recorded from 200 to 350 nm, with detection at 200, 254 and 350 nm.

2.15.3 ***Semi-preparative HPLC*** The concentrated phenolic compounds were purified using HPLC semi-preparative column Luna 5 µ C_8 (2) 100A column (250x10 mm) using UV-Visible detector at 200 nm and 420 nm methanol, water (1:1) was used as a solvent at 1.0 $ml.min^{-1}$ in isocratic mode. The HPLC purified compound from aqueous fraction (A2; PE-3 (phenol ester-3) was concentrated and purity was conformed with analytical C_{18} column with three different solvents and different wavelength, and it was used for elucidation of structure using IR, ^{1}H & ^{13}C NMR and Mass analysis.

2.15.4 ***LC-MS analysis*** Liquid chromatography (LC) - mass spectrometry (MS) analysis was performed with LC1020A from simadzu, equipped with an automatic injector. MS was performed using MS-ESI ion source (Nitrogen flow rate 0.5 / h). Working conditions were in ESI both negative and positive ion mode and the separation were performed using C_{18} column (Luna 5 µ C_{18} (2) 100A column (250 x 4.6 mm) analysis was performed at 40 ºC (LC column oven) and 85 ºC (MS ionization chamber). Mobile phase consisted of methanol and water (1:1) the column was equilibrated for 10 min prior to each analysis. Flow rate was 0.5 $ml.min^{-1}$ and injected volume was 10-40 µl and compounds were detected (LC) at 254 nm. The column effluent from the LC was nebulized into an Atmospheric Pressure Chemical Ionization (APCI) region under N_2 gas heated at 150 ^{o}C for generating molecular masses.

2.15.5 ***Fourier transform infrared spectroscopy (FT/IR)*** was recorded on a Jasco FT/IR-5300 spectrometer. All the spectra were calibrated against polystyrene absorption at 1601 cm^{-1} solid sample were recorded as KBr wafer and liquid sample as thin film between NaCl plates or solution spectra in CH_3Cl are reported in cm^{-1}. Spectrums were recorded on a Shimadzu FT/IR 8300 spectrophotometer.

2.15.6 ***^{13}C NMR*** NMR spectrum of the purified compound in 0.5 ml of $CDCl_3$ in a 5 mm NMR tube was recorded on Brucker AC200 (200 MHz) spectrometer, operating at 200 and 150 MHz for ^{13}C NMR, respectively.

2.15.7 ***^{1}H NMR*** proton magnetic resonance and ^{13}C NMR spectra were recorded on a Bruker-AC-200 spectrometer or Bruker Advance-400 spectrometer, ^{1}H NMR (200 MHz or 400 MHz) spectra for all the samples were measured in $CDCl_3$ unless other wise mentioned, with TMS (delta = 0 ppm) as internal standard.

2.5.8 ***Mass spectroscopy*** analyses was carried out with a VG 70-70H double-focusing spectrometer equipped with a combined EI/FI/FD ion source. Collision activation was carried out by introducing helium as target gas in the second field-free region. Samples were introduced via a direct inlet system with AI crucibles.

2.15.9 ***Colorimetric analysis***

2.15.9.1 ***Estimation of indoles*** were estimated colorimetrically either by Salper's reagent (Gordor and Paleg, 1957) or by p-dimethylaminobenzaldehyde (PDAB) reagent (Kupper and Atkinson, 1964).

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

2.15.9.3 ***PDAB Method*** To 1ml 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.

2.15.9.4 ***Ammonia*** Ammonia was estimated by the method of Solorzano (1969) using NH_4Cl as standard. To 5 ml of the sample 0.2 ml of phenol alcohol (10% w/v in 95%

ethanol) solution, 0.2 ml sodium nitroprusside (0.5 % w/v) solution and 0.5 ml of oxidizing solution [20 %(w/v) tri sodium citrate + 1% [w/v] NaOH + 25 ml 1.5 N NaOCl] were added and mixed well. The color intensity was read at 540 nm in a spectrometer (Biochrome Libra-12) against a reagent blank. The amount of ammonia present in the sample was calculated from a standard graph prepared using ammonium chloride.

2.15.9.5 ***Amino acids*** Amino acids were estimated colorimetrically by adding 4 ml of ninhydrin reagent (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 minutes after which the volume was made up with acetone and the color was read at 570 nm against reagent blank in a spectrophotometer. An optical density (OD) versus concentration, graph was prepared using alanine as standard.

2.15.9.6 ***L-Tryptophan*** was estimated colorimetrically using p-dimethylaminobenzaldehyde (PDAB) reagent (Kupfer and Atkinson, 1964) after removing the indole derivative with ethyl acetate. To 1 ml of the aqueous extract made up to 5 ml with distilled water. 1 ml of freshly prepared PDAB (60 mg in 1ml of 3 N 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 the absorption was read at 580 nm with against reagent blank.

2.15.9.7 ***Pyruvic acid*** was estimated according to the method (Umbreit et al., 1964) using sodium pyruvate as standard. To 3 ml of the sample, 1 ml of 2,4-dinitrophenylhydrazine (0.1% w/v in 2 N HCl) was added. After 10 min, 2 ml of 10% Na_2CO_3 and 4 ml of 2NaOH were added and the color was read at 520 nm in a systronic colometer against

blank. The amount of pyruvate present in the sample was calculated from a standard graph prepared using sodium pyruvate.

2.15.9.8 ***3,4-dihydroxyphenyl pyruvic acid*** was estimated according to the method Briggs (1922). 1 ml of sample was added to 0.5 ml of a mixture of equal volumes of 2.5% ammonium molybdate in 5 N HCl and 3% KH_2PO_4 and the color complex so formed was read at 700 nm after 20 min.

2.15.9.9 ***Total phenols*** was measured with an optimized Folin-Ciocalteu method (Swain and Hillis 1959) at 765nm in a Biochrom libra-12 spectrophotometer. Sample dilutions of 0.5% with water were made for assay determination.

2.16. ***Total Proteins***

2.16.1 ***Lowry method*** Protein content of the bacterial lysate as well as various sub-cellular fractions was estimated by the following procedure described by Lowry *et al*., (1951). 100 µl of sample was taken and mixed with 0.9 ml of water and 5 ml of alkaline solution (2% Na_2CO_3 and 0.5 % copper sulphate in 50:1 ratio). The contents were thoroughly mixed before allowing to stand at room temperature for 10 min. Immediately after incubation 0.5 ml of Folin-Ciocalteau reagent (diluted with distilled water in 1:1 ratio before use) was added and thoroughly mixed before allowing them to stand for 30 min at room temperature. After incubation blue color developed was measured at 750 nm. 5 ml of alkaline solution and 0.5 ml of Folin-Ciocalteau reagent served as blank.

2.16.2 ***Bradford's method*** (1976) Dissolve 100 mg of coomassie brilliant blue G-250 in 100 ml of absolute/ distilled ethanol or absolute methanol in on a shaker for ~ 60 min. added 100 ml 88% ortho-phosphoric acid, mixed well make the volume to 500 ml with distilled water. Filter through whatman No.1 and diluted 1:1 with water and check the

absorption at 550 nm against water blank (OD ~1.1). The increasing concentration of (10-100 µl) of BSA from a 0.2 mg/ml stock solution into clean dry test tubes. Added 1.5 ml of the dye solution to each tube and mix gently and incubated 5 min at room temperature. Finally read the color at 595 nm.

2.17 ***Enzyme assays***

2.17.1 ***Tryptophanase (EC 4.1.99.1)*** the enzyme assay was done according to Snell (1975). The assay mixture contained in a final volume of 1ml (0.05M potassium phosphate buffer pH 7.8) 100 µM of tryptophan, 0.25 µM of pyridoxal-5-phosphate (PLP) and appropriate amount of cell free extract was added. After incubation at 37 ^{o}C for 15 min. the assay mixture was chilled in an ice bath and extracted into 2 ml of ethylacetate. 1 ml sample of the ethylalacetate was assayed for indole using PDAB reagent.

2.17.2 ***Tryptophan aminotransferase (EC 2.6.1.27)*** was determined by measuring the loss of L-tryptophan using HPLC. The reaction mixture contained, in a final volume of 1 ml (0.05M potassium phosphate buffer; pH 7.5) 0.3 µmole 2-oxoglutarate, 100 µmole of L-tryptophan, 0.25 µmole pyridoxal-5-phosphate (PLP) and an appropriate amount of cell extract. After incubation at 30 ^{o}C for 5 min and reaction was terminated by acidification with 10% (w/v; 100 mg ml^{-1}) trichloroacetic acid (TCA). The sample was centrifuged (12,00 x g for 5 min) and analyzed by injecting 20 µl of clear supernatant of the initial and final assay samples in HPLC. A unit (U) of enzymatic activity is defined as the amount of enzyme that catalyzes the disappearance of 1 µmole tryptophan mg protein^{-1} min^{-1}.

2.17.3 ***Tryptophan ammonia lyase activity*** was measured as the amount of indole-3-acrylic acid and liberation of ammonia from L-tryptophan (100 µM) in the absence of pyridaxal-5-phaspate (PLP; as a co-factor). The reaction mixture contained in a final volume of 0.5 ml (0.05 M potassium phosphate buffer; pH 7.5), 100 µM of tryptophan and an appropriate amount of the cell free extract. The reaction was carried out in Eppendorf tubes (1.5ml), incubated at 30 ^{o}C. After 5 min (unless other wise mentioned), reaction was terminated by adding H_2SO_4 (5 N) and analyzed by injecting 20 µl of clear supernatant in HPLC. A unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 µmole of indole-3-acrylic acid and NH_3.mg protein^{-1}.min^{-1}.

2.17.4 ***3,4-Dihydroxyphenylalanine 2-oxoglutarate aminotransferase activity (EC 2.3.1.49)*** was determined by measuring the loss of DOPA using HPLC. The reaction mixture contained, in a final volume of 1 ml (0.05M potassium phosphate buffer pH 7.8), 0.3 µmole of 2-oxoglutarate, 0.3 µmole of DOPA, 0.25 µmole of pyridoxal-5-phosphate (PLP) and an appropriate amount of cell extract. The reaction was carried out in Eppendorf tubes (1.5 ml) and incubated at 30 ^{o}C. The reaction was terminated after 30 min (unless other wise mentioned) and proteins were denatured by acidification with 10% TCA.The sample was centrifuged (12,000 x g for 5 min) and analyzed by injecting 20 µl of clear supernatant in HPLC. A unit (U) of enzymatic activity is defined as the amount of enzyme that catalyzes the disappearance of 1 µmol DOPA mg protein^{-1} min^{-1}.

2.17.5 ***3,4-Dihydroxyphenylalanine reductive deaminase activity*** was determined by measuring the formation of ammonia from DOPA (100 µM) in the presence of NADH (10 µM) to a final volume of 0.5 ml of potassium phosphate buffer (0.05 M; pH 7.0). A

unit (U) of enzymatic activity is defined as the amount of enzyme that catalyzes the formation of 1 µmole ammonia.mg protein^{-1}.min^{-1}.

2.17.6 ***3,4-Dihydroxyphenylalanine oxidative deaminase activity*** was measured as the amount of ammonia liberated from DOPA (100 µM) in the absence of 2-oxoglutarate (amino acceptor) or NADH to a final volume of 0.5 ml of potassium phosphate buffer (0.05 M; pH 7.8). A unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1µmole NH_3.mg protein^{-1}.min^{-1} in the absence of 2-oxoglutarate or NADH**.**

2.17.6.1 ***Oxygen consumption*** was measured the O_2 uptake by the purified DOPAODA was monitored using a Clark type O_2 electrode (DW2, Hansatecch Ltd., King's Lynn, UK). The 1 ml (0.05 M potassium phosphate buffer; pH 7.8) reaction mixture contained 100 µmole L-DOPA and 25 µg protein (DOPAODA). Water at a constant temperature of 25 ^{o}C was circulated through the outer jacket of the reaction chamber. Calibration of the O_2 content in the electrode chamber was done with air-saturated water, assumed to contain 253 nmole O_2 ml^{-1} at 25 ^{o}C (Walker, 1988).

2.17.7 ***Esterase (EC 3.1.1.1)*** The standard protocol of Stoops (Stoops *et al.,* 1969) with minor modification was used for the confirmation of the ester bond of the metabolites. The assay mixture contained, in a final volume of 1 ml, 10 µmole of fraction-II or crude fractionated sample and 1(U) esterase (obtained from Sigma Aldrich) dissolved in 0.05M potassium phosphate buffer (pH.7.5). Adding the enzyme started reaction and the activity was measured after 5 min as loss in the initial peak height using HPLC or by confirming the samples using LC-MS and comparing with the esterase untreated sample.

2.18 ***Isolation and purification of enzymes*** The culture grown in L-tryptophan or L-phenylalanine/ L-tyrosine was centrifuged at (16,000 x g for 10 min) and the pellet was sonicated with the addition of 1mM EDTA and PMSF in phosphate buffer pH 7.8. It was centrifuged again at (16,000 x g for 20 min) to collect the supernatant.

2.18.1 ***Ammonium sulfate saturation of proteins*** The initial step in the purification of the enzymes was an ammonium sulfate saturation performed added with the crude enzyme extract and the protein precipitation between 10 to 75 % cutoff saturation was collected. This precipitation was dissolved in 2 to 5 ml of 0.05 M potassium phosphate buffer pH 7.8 and dialyzed for over night against three change of 3 litre of the same buffer.

2.18.2 ***Gel filtration column (Sephadex G-150) chromatography*** The dialyzed protein sample was applied to a column (1.6 x 95 cm) of Sephadex G-150, which had been equilibrated with 0.05 M phosphate buffer pH 7.8 and was eluted with the same buffer. Fractions of 1ml each were collected.

2.18.3 ***Ion/Anion exchange (DEAE/CM-Cellulose) column*** 10g of DEAE- cellulose was washed by suspending in 0.1 M NaOH, followed by neutralization to pH 7.8 with 0.05 mM potassium phosphate buffer, the DEAE-cellulose was resuspended in more buffer and packed so that the dimensions of the column were 2.2 x 16 cm. the column was placed at 4 ^{o}C overnight. Approximately 2 gm of protein containing the enzyme were submitted to the DEAE-cellulose column and the column was washed with 10 ml of buffer for equilibration. The enzyme was eluted from the column by using a linear gradient between equal volumes (50 ml each) of 0.05 mM potassium phosphate buffer,

pH 7.8 containing 0.5,1.0,1.5,2.0 M NaCl. The proteins were eluted at a flow rate of 30 ml per hour and fractions (2 ml) were collected until the proteins were completely eluted.

2.18.4 ***Affinity chromatography*** After the activation of sepharose-4B powder swelled matrix was packed onto the column; coupling with 1,4-butanedioldiglycidyl ether hydroxy spacer arm (coupling condition was pH 11-12 hydroxy ligands at 37 ^{o}C) the final bed volume of epoxide activated sepharose-4B was about 20 ml (35 x 45 mm) and ligands are immobilized directly to solid support material by formation of covalent chemical bands between functional group on the ligand and reaction group. The column was thoroughly washed with 0.1 M borate buffer more than 3 bed volumes, subsequently washed away excess ligand with coupling solution followed by double distilled water, finally equilibrated with 0.05M potassium phosphate buffer. Protein was eluted with gradient (10 mM, 50 mM and 100 mM) NaCl buffer.

2.19 ***PAGE analysis***

2.19.1 ***Native Polyacrylamide Gel Electrophoresis (Native PAGE)*** In Native PAGE, β-mercaptoethanol and SDS was not used while preparing the sample buffer, similarly in the preparation of electrode buffer SDS was not used, the resolving gel also did not contain SDS. The samples were added to the sample buffer and centrifuged for 5 min at 3,000 rpm, heating of the sample was avoided. The gels were run at 4 °C electrophoresis was carried out until the bromophenol blue dye marker reached about 4-5 mm and the gels were removed.

2.19.2 ***Electro elution of proteins*** After partially purified protein was obtained using various methods run the native gel and slice out the active band. The band was cut into pieces with a sharp razor and transfered into dialysis membrane and run with electro

elution buffer (25 mM tris-glycine, 15 mM tris acetic acid and 0.1M Sodium carbonate acetic acid pH 8.5) for three hours, to optimize the recovery of protein from unstained gel.

2.19.3 ***Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis*** SDS-PAGE was performed according to the method of Laemmli *et al*., (1970) with minor modifications. The separation of proteins was performed in 12% resolving gel with 4 % stacking gel. Both the resolving and stacking gel contained 1 % bis-acrylamide as a cross-linker and 0.1 % SDS.The final resolving buffer concentration was 0.375 M Tris-HCl (pH 8.8) in resolving gel and stacking buffer final concentration was 0.125 M Tris-HCl (pH 6.8) in stacking gel. Ammonium persulfate and N, N, N, N-Tetramethyl ethylenediamine (TEMED) was used as polymerizing agents in final concentrations of 0.05% and 0.1% respectively. The electrode buffer consists of 0.025M Tris, 0.192M glycine (3.025gm of tris, 14.4 gm of glycine and 1 gm of SDS in one litre of milli-Q distilled water, (pH 8.3) and 0.1% SDS. The samples buffer consists of 0.062M tris-HCl, 10 % glycerol, 2 % SDS, 5 % β-mercaptoethanol and 0.001 % bromophenol blue. The protein samples were incubated for 3 min in boiling water bath with the sample buffer and centrifuged at 3,000 x g for 2 min. The clear sample solutions were loaded into wells on the gels of 8 x 10 cm dimension, which was polymerized in glass plate fixed to mini-vertical slab gel apparatus. The gels were run at room temperature at 75 and 100 volts for stacking and resolving gels, respectively. Electrophoresis was carried out until the bromophenol blue dye marker reached the anode end of the gel and the gels were removed.

2.20 ***Staining methods***

2.20.1 ***Silver*** Silver staining was performed according to the method of Blum et al. (1987). The gel was fixed for more than 1 h in fixative and washed 3 times with 50 % ethanol for 3 times at every 20 min interval. The washed gel was pre-treated with 0.002 % sodium thiosulphate solution for exactly 1 min and rinsed 3 times in distilled water at every 20 sec. The pre-treated gel was impregnated in 0.2 % silver nitrate containing 0.028 % formaldehyde. The gel was rinsed 2 times with distilled water and developed for the proteins with the solution containing 6 % sodium carbonate, 0.018 % formaldehyde and 0.0004 % sodium thiosulphate. The gel was stopped for the development of protein bands in appropriate intensity with fixative solution for 10 min and was stored in 50 % methanol at 4 °C.

2.20.2 ***Coomassie*** Protein were visualized by staining with Coomassie brilliant blue R-250 after electrophoresis the gel put in to the sensitization solution (1% v/v acetic acid, 10% v/v ammonium sulfate) and stirred for an additional 2 h. the staining solution consists of 5 % v/v acetic acid 45% v/v ethanol and 0.125 (w/v) CBB R-250. Then gel placed into 20 volumes of staining solution and stirred for more than 4 h, finally transferred to the distaining solution-I (5% v/v acetic acid, 40% v/v ethanol) and then into the destining solution-II (3% v/v acetic acid, 30% v/v ethanol until the background was clear, the gel was preserved in 5% (v/v) acetic acid for several months (Wang *et al*., 2007).

2.21 ***Identification and sequencing of protein***

2.21.1 ***Electro blotting*** The SDS-PAGE was translated in to polyvinylidene difluoride (PVDF) membrane in semi- dry blot with (3-[cyclohexylamino]-1- propanesulfonic acid)

(CAP'S) buffer and band was excised and subjected to automated Edman degradation for N-terminal sequence.

2.21.2 ***Protein N-terminal sequencing*** the N-terminal amino acids from non denaturing-PAGE (6%) was electroblotted onto a polyvinylidene difluoride membrane (0.45 µm; Mille pore) in 25 mM trizma, 192 mM glycine and 10% methanol or CAP'S (3-[cyclohexylamino]-1- propanesulfonic acid) buffer (10 mM, pH.11) at 150 V for 12 h, stained with ponceau's and subjected to automated Edman degrade (Applied Biosystems-470)

2.21.3 ***In-gel digestion/mass spectrometry, Matrix-assisted laser desorption/ ionization-time-of-flight (MALDI-TOF) analysis*** The coomassie blue stained protein band was excised manually, chopped into small pieces and transferred into eppendorf tubes. A piece of protein-free acrylamide gel was taken in parallel as a negative control. The gel pieces were then destained, water washed, and reduced for 1 h at 57 °C using reduction buffer [100 mM NH_4HCO_3, 10 mM dithiothreitol (DTT)] to disrupt disulfide bonds. The bands were alkylated using alkylation buffer (100mM NH_4HCO_3 and 55 mM iodoacetamide) and were placed in the dark for 30 min at room temperature to prevent disulfide bonds from reforming (alkylation). The gel pieces were dehydrated with 50 µl of 100% acetonitrile, and then dried under vacuum using a Speedvac concentrator for 30 min.

Digestion solution (100 ng^{-1}µl trypsin) in 50 mM of NH_4HCO_3 was added to the dried gel pieces which were submerged in the digestion solution of 40 µl and incubated overnight at 37 °C. Peptides were extracted from the digested mixture by adding 100 µl of extraction buffer (50% acetonitrile containing 5% trifluoroacetic acid). This process

was repeated twice and the supernatant was collected after spinning at 10,000 x g for 5 min. The resulting peptide mixture was further concentrated in a speedvac concentrator for 1 h. The extracted peptide mixture was desalted using Millipore Ziptip C_{18} columns. The desalted tryptic peptide mixture was mixed with cyano-4-hydroxycinnamic acid (CHCA) matrix solution (CHCA matrix dissolved in 50% acetonitrile having 0.1% trifluoroacetic acid of 1 µl) and vortexed gently. A volume of 2 µl of the mixture containing CHCA matrix and the tryptic digest were loaded on a stainless steel plate and air-dried. Care was taken to prevent any keratin contamination. The sample was analyzed by Micro mass MALDI-TOF-TOF [Model MALDI-R (reflectron mode) Serial No. E RA076] in reflectron mode. A pulsed nitrogen laser of 337 nm was fired (voltage conditions: pulse voltage 3,120; source voltage 15,000; and reflectron voltage 500) to accumulate 100 shots per spectra and the peptide mass finger print profile of the sample was generated. The spectra width was narrowed to a range from 500 to 3,500 Da m/z. The spectra were processed (baseline correction, noise removal, deisotoping) by using Mass Lynx 3.5 version software. Proteins were identified using the public domain Mascot search engine by incorporating the standard parameters (http:// www.matrixscience.com). The database used was Swissprot and trypsin was used as proteolytic enzyme for limiting the miscleavages to one. Carbamidomethyl (C) and oxidation (M) of methionine were taken as fixed and variable modifications, respectively. The peptide mass values were $[MH]^+$ and monoisotopic and mass tolerance was limited to 100-200 ppm.

2.22 ***Properties of enzymes***

2.22.1 ***Effect of pH and T ^oC*** pH effect on the enzyme activity was studied by using 0.05 M potassium phosphate buffer in the pH ranges of 4-9. For optimum temperature the

enzyme activity was assayed varying range of temperature (0-80 ^{o}C) in potassium phosphate buffer (0.05 M, pH 7.0)

2.22.2 ***Effect of substrate concentration*** The effect of substrate concentration on reaction rate was studied using substrate in range of 10 to 150 µmole.

2.22.3 ***Kinetic parameter determination*** The K_m and K_{cat} (s^{-1}) or V_{max} for enzymes was determined using substrates consumption assays. The assay reaction mixture was incubated at varying concentration of substrates in 0.05 M potassium phosphate buffer, pH 7.5, at 30 ^{o}C and reaction initiated by the addition of the 100 µg/ml enzyme. The rate was measured as described above and analyzed using the double-reciprocal, non-linear curve method of Line Weaver and Burk.

2.23 ***Biological assays***

2.23.1 ***MTT- assay*** Reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (Mosman, 1883) (MTT, Sigma) is chosen as a cell viability measurement optimal endpoint. $SupT_1$ lymphoma and Colo-125 cancer cells in RPMI 1640, 10% FCS were seeded in 96 well plates. Increasing concentrations of compounds were added to the cells and incubated at 37 ^{o}C for 14 hrs in a CO_2 incubator with 5% CO_2. The media was replaced with a fresh growth medium along with 20 µl of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). After incubation for 4 hours in a humidified atmosphere, the media was removed and 200 µl of 0.1 N acidic isopropyl alcohol was added to the wells to dissolve the MTT-formazan crystals. The absorbance was recorded at 570 nm immediately after the development of purple color. Each experiment was conducted in triplicates and the data are represented as averages, with standard deviation.

2.23.2 ***Cyclooxygenase assay*** The enzyme activity was measured using chromogenic assay based on oxidation of N, N, N^1, N^1 tetramethyl paraphenylenediamine (TMPD) during the reduction of prostaglandin G2 to prostaglandin H_2 by COX-1 and COX-2 enzymes (Copeland *et al.,* 1994). COX-1 enzyme was from ram seminal vesicles and COX-2 was a recombinant human enzyme purified from sfg -cells.

10 mM stock solutions of compound were prepared by dissolving in DMSO. The final concentration of the compounds in 1 ml reaction mixture was 100 µM. The assay mixture consist of Tris buffer (pH 8.0), EDTA and hematin as cofactor. The activity of enzymes both COX-1 and COX-2 was checked initially, the assay mixture along with enzyme and test compound was incubated for 15 min. The enzyme activity was measured allowing the reaction for 1 min, celecoxib was used as the standard for COX-2

2.23.3 ***Auxin coleoptiles bioassay*** The coleoptiles of maize were cut and the explants were incubated in standard auxin solution. IAA (5 µM), rhodethrin and crude compounds (5 µM) incubated under light for 2-3 h, the increase in the length of coleoptiles were calculated as the increase in cm (%).

2.23.4 ***Phytohormonal activity*** The tissue culture plant of mulberry (*Mouras alba*) was transformed onto the MS medium containing various concentration (5nM-5µM) of the metabolites and also to the MS medium containing standard of indole-3-acitic acid (IAA), incubated under light for 10-15 days. The plant was observed for rooting after 15 days of incubation.

3. RESULTS

3.1 L-Phenylalanine catabolism *Rhodobacter sphaeroides* OU5

3.1.1 *Growth and simultaneous utilization of L-phenylalanine*

Rhodobacter sphaeroides OU5 could grow on L-phenylalanine or L-tyrosine only under phototrophic conditions and when used as sole source of nitrogen, but not as carbon or both carbon and nitrogen source. Figure. 3 shows the phototrophic growth and simultaneous utilization of L-phenylalanine and production of phenols, when used as sole source of nitrogen. *Rhodobacter sphaeroides* OU5 had a doubling time of about 24 h (as against 18 h with ammonium chloride) with 100 % consumption of L-phenylalanine observed with in 60 h. Increasing concentration of L-phenylalanine (1-5 mM) resulted in the production of total phenols which are near stoichiometry.

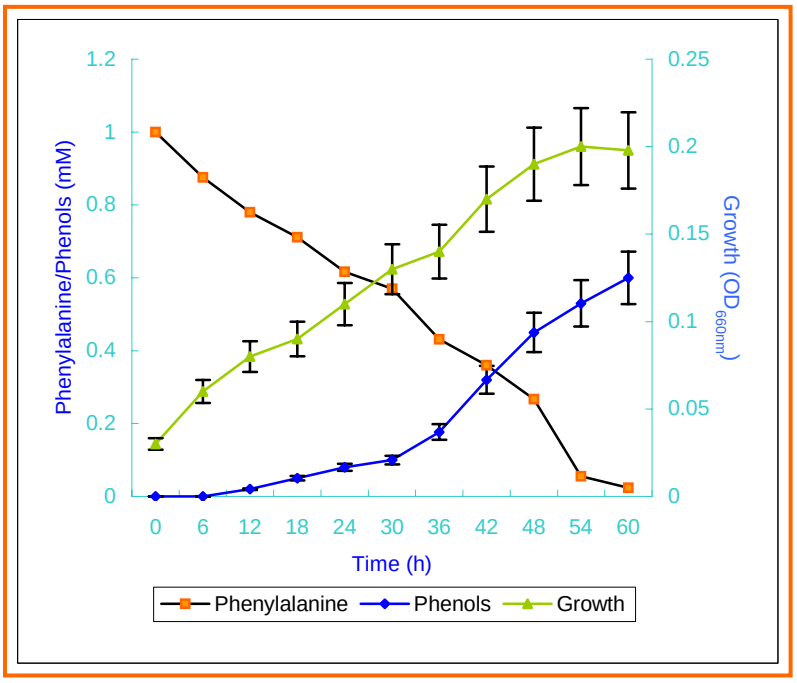


Fig. 3. Photoheterotrophic growth, simultaneous utilization of L-phenylalanine and production of phenols by *Rhodobacter sphaeroides* OU5.

Results expressed are average of two independent experiments done in triplicates. L-phenylalanine (1 mM) was used as sole source of nitrogen, while malate (22 mM) served as carbon source for heterotrophic growth under light (2,400 lux) anaerobic conditions performed at 30 ± 2 °C.

3.1.2 *Identification of the metabolites from the culture supernatant*

3.1.2.1 *Isolation and characterization of a metabolite from fraction-II*

A yellow colored metabolite was isolated from Fraction-II. The purity of the metabolite was confirmed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analysis. This metabolite was characterized based on ^{1}H (Fig. 4A), ^{13}C NMR (Fig. 4B), IR (Fig. 4C) analysis as 3,4-dihydroxy-benzoic acid 5-carboxy-4-hydroxy-3-methyl-pentyl ester (Fig. 4D), which we called as "rhodophestrol". Further, we confirmed the ester bond of the metabolite by hydrolyzing the bond by treating with the enzyme esterase, which resulted in the formation of 3,4-dihydroxybenzoate (protocatechuate) (Fig. 4E). UV absorption spectrum of the metabolite at 220 nm, confirms the aromatic nucleus and a peak at 419 nm indicates the presence of a terpenoid. The yield of rhodophestrol is 100 µM from 1 mM L-phenylalanine.

3.1.2.2 *Isolation and characterization of metabolites from aqueous fraction (A2)*

The metabolites isolated from aqueous fraction (A2) PE-3 was pure as confirmed from semi-preparative HPLC and LC-MS analysis. This metabolite was characterized based on ^{1}H (Fig. 5A), ^{13}C NMR (Fig. 5B), IR (Fig. 5C) analysis as 3,4,5-dihydroxybenzoyl terpenoid ester [1E, 3E)-7-hydroxy-3-methylocta-1, 3-dien-5-yn-1-yl 3, 4,5 trihydroxy-Benzoate] (Fig. 5D). UV absorption spectrum of the metabolite at 204 nm, confirms the phenol nucleus and a peak at 401 nm indicates the presence of a terpenoid. The ester bond was confirmed by treating the metabolite with the enzyme esterase, which resulted in the formation of 3,4,5-dihydroxybenzoate (gallate) as confirmed from the HPLC and LC-MS analysis (Fig. 6).

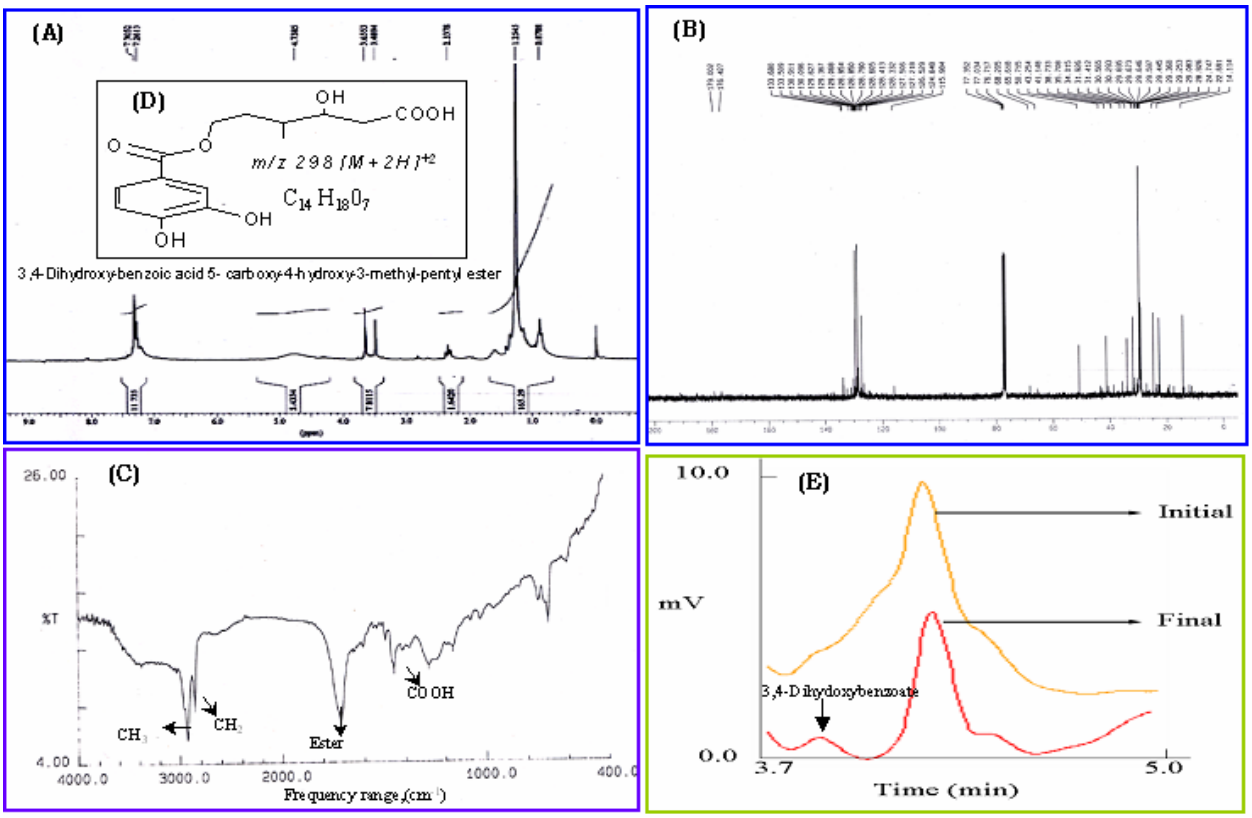


Fig. 4. Structural elucidation of one the metabolites isolated from the culture supernatant of *Rhodobacter sphaeroides* OU5 when grown on L-phenylalanine.
Culture grown on L-phenylalanine (1 mM) for 36 h in 5 L medium was used for the isolation of the metabolite. The compound was extracted from the hexane, chloroform and methanol (6:3:1v/v/v) extract (Fraction-II) (See materials and methods for more details). The metabolite is a bright yellow oily liquid and has UV-VIS peaks (nm) at 206, 220, 419.

(A) ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.30-7.26 (m, 3H, Ph =C-H), 4.73 (br s, 4H, - OH), 3.45 (t, 2H, O-CH_2), 3.40 (m, 1H, -CH-OH), 2.33 (d, 2H, OH-CH-CH_2), 1.25 (m, 3H, O-CH_2-CH_2-CH), 0.88 (d, 3H, CH-CH_3).

(B) ^{13}C NMR ($CDCl_3$, 150 MHz): δ 179.0 (Ph-C = O-O), 168.2 (Ph-C = O-O), 133.7 (Ph-CH=CH-CH-OH), 133.5 (Ph-CH-CH-OH-CH-OH), 126.0 (CH-C-CH), 124.0 (CH-C-CH), 115.98 (Ph-CH), 115.97 (Ph-CH), 68.7 (CH_2-OH), 65.6 (O-CH), 41.1 (OH-CH-CH_2), 35.7 (O-CH-CH_2), 34.0 (CH_2-CH-CH_3-CH), 14.1 (Me, H_2C-CH-OH-CH)

(C) Infra Red spectral analysis (KBr pellet; cm^{-1}) 3377, 2926, 2854, 1714, 1456, 1284,1163 and 700. EIMS m/z 298 $[M]^+$ and C 56.37 % and H 6.08 %, calcd. For $C_{14}H_{18}O_7$, C 56.37%, H 6.08%, O 37.55%. IUPAC name of the metabolite is 3,4-Dihydroxy-benzoic acid 5- carboxy-4-hydroxy-3-methyl-pentyl ester, we named as rhodophestrol (**rhodo** = from *Rhodobacter*; **phe** = phenol, **estrol** = ester terpenoid).

(D) Metabolite structure derived based on the NMR, IR and LC-MS analysis.

(E) Conformation of the ester bond of the metabolite by treating with esterase enzyme. Loss in the metabolite confirmed the ester bond with the release of 3, 4-dihydroxybenzoate.

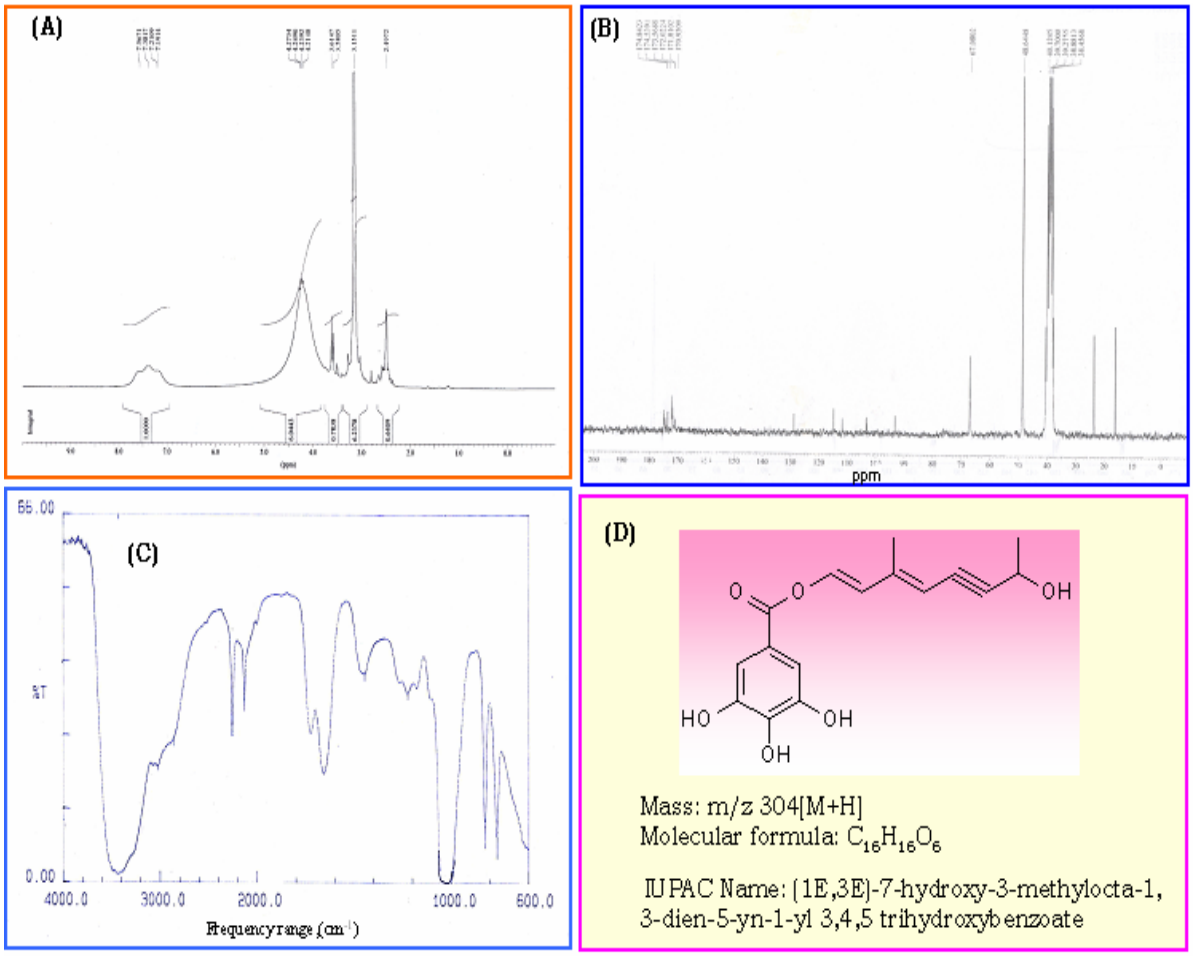


Fig. 5. Structural elucidation of PE-3 metabolite isolated from the culture supernatant of *Rhodobacter sphaeroides* OU5 when grown on L-phenylalanine.

(A) ^{1}H NMR ($CDCL_3$, 200MHz): δ 7.6-7.19 (m, 5H), 4.2-4.0 (br s, 4H), 3.6-3.58 (q, 1H), 3.15 (d, 3H), 2.4 (s, 3H)
(B) ^{13}C NMR ($CDCL_3$, 150MHz): δ 174.8 (ph-C=O-O), 174.5, 173.6, 172.0, 171.8(ph-CH_2-CH_2), 129.7(ph-CH-CH-O), 113.3(ph-CH_2=CH_2), 111.2(ph-CH), 108.7(CH=CH), 95.6(CH=CH), 76.3 (CH-CH), 58.7 (CH-CH-OH), 21.4(CH-CH_3), 14.7(CH-CH-CH_3)

(C) Infra Red (IR) spectral analysis (KBr pellet; cm^{-1}): 3433 (OH), 3022 (aromatic-CH), 2862 (aliphatic- CH), 2253, 2117 (alkynes), 1726 (esters) 1028, 823,761 (aromatic nucleolus) EIMS m/z 304$[M]^+$ and C 63.09 % and H 5.28 %, calcd. For $C_{16}H_{16}O6$, C 63.09%, H 5.28%, O 31.55%. IUPAC name of the metabolite is (1E, 3E)-7-hydroxy-3-methylocta-1, 3-dien-5-yn-1-yl 3,4,5 trihydroxybenzoate.

(D) Metabolite structure derived based on the NMR, IR and LC-MS analysis.

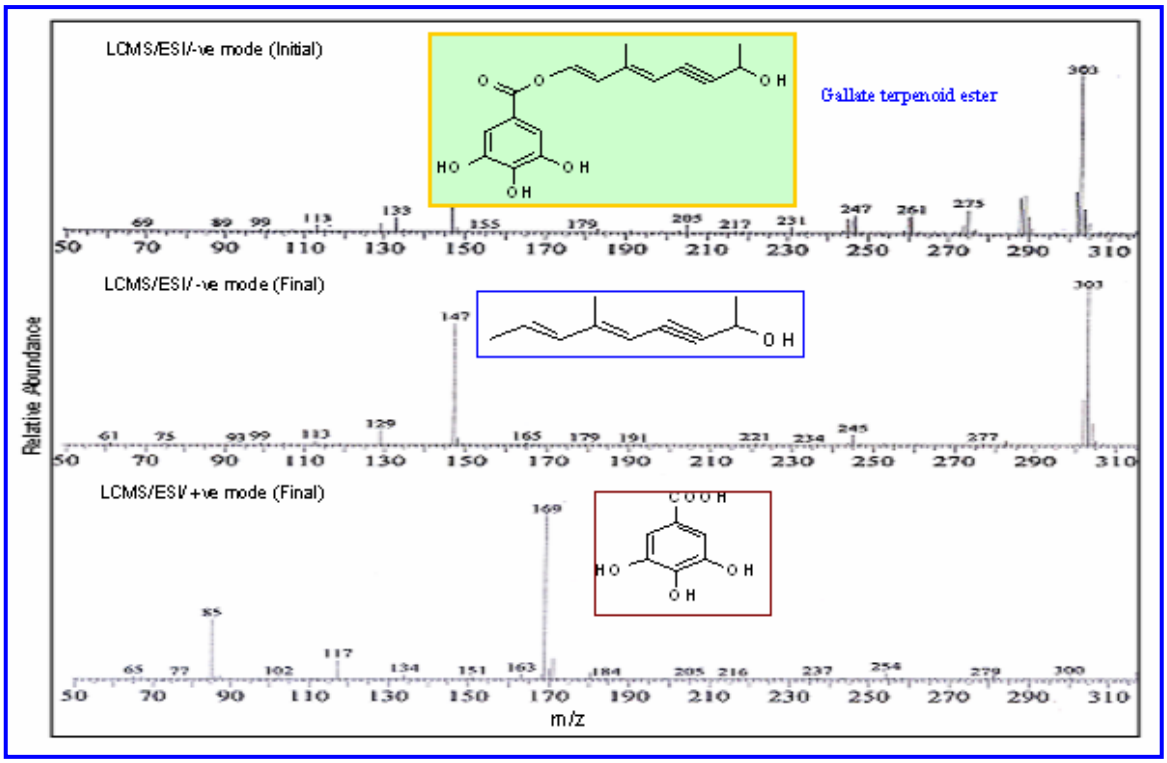


Fig. 6. Confirmation of ester bond of the molecule was done by hydrolyzing the ester bond using an esterase and the metabolites were analyzed using LC-MS.

3.1.3 ***HPLC profiling of culture supernatants***

Twenty µl of the clear fractions (A1, A2, B and C) were injected into HPLC and their respective profilings is shown in Fig. 7. Fraction-A1 had 9 chromatographically distinct peaks of which, R_t 2.5 min matched with that of standard gallate. From fraction- A2 profiling, in addition to gallate, protocatechuate (R_t = 3.4 min) was identified among the 9 chromatographically distinct peaks. Fraction-B had 5 chromatographically distinct peaks of which, two were identified as caffeate (R_t = 2.6) and protocatechuate (R_t = 3.4). While in fraction-C, which had 8 peaks, in addition to gallate and protocatechuate, catechol (R_t = 3.9 min) was identified. In summary, gallate (~0.5 mM) protocatechuate (~0.2 mM) and caffeate (~0.1 mM) were identified as major products in the presence of L-phenylalanine (1 mM).

3.1.4 ***LC-MS identification of phenolic esters*** Phenolic terpenoid esters were identified based on the LC-MS profiling analysis of the initial and esterase treated final samples of various fractions (Fig. 7) and the molecular masses were compared with the known phenolic esters (http//www.ncbi.nlm.nih.gov/sites/entrey-pubchemcompound). Gallate, caffeate, homogentisiate and protocatechuate esters were identified and the corresponding alcohols generated after esterase treatment are given in Table. 2. Since Gallate (~ 0.5 mM), protocatechuate (~0.2 mM) and caffeate (~0.1 mM) were the major phenols identified and constituted nearly 0.8 mM of the total phenols, the yield of the phenol terpenoid esters was ~ 0.2 mM.

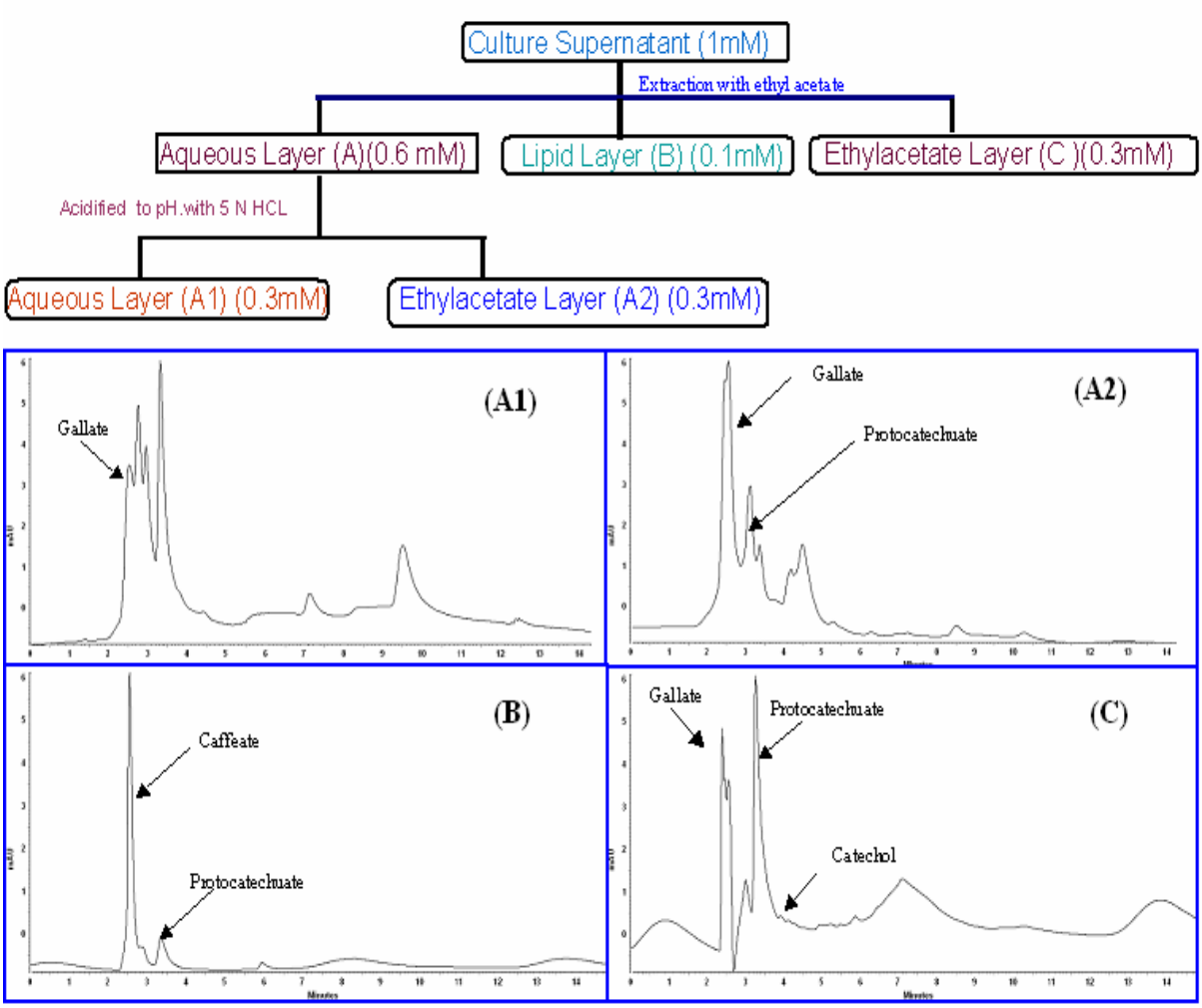


Fig. 7. Fractionation and HPLC profiling of various fractions of the culture supernatant of *Rhodobacter sphaeroides* OU5 grown in the presence of L-phenylalanine. Data given in the brackets as the yield of total phenols.

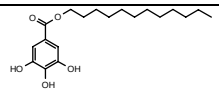
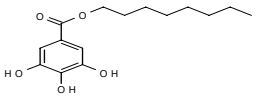
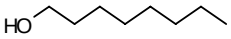
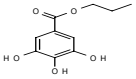
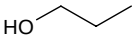
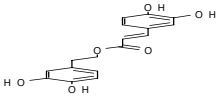
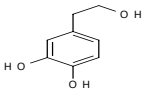
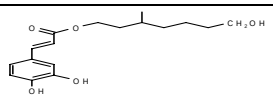
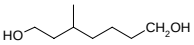
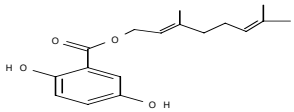
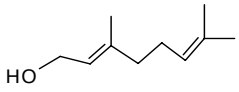
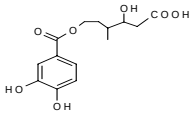
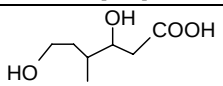
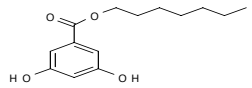
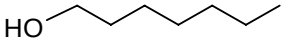
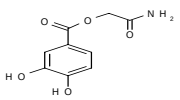
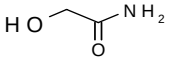
* Structure / IUPAC Names	Molecular mass of the metabolite (m/z)	Structure & Molecular mass (m/z) of the corresponding alcohols generated after esterase treatment
Gallate esters		
 Dodecy 3,4,5-trihydroxybenzoate	338 $[MH]^+$	 186$[MH]^{+1}$
 Octyl 3,4,5-trihydroxybenzoate	282$[MH]^+$	 130$[MH]^{+2}$
 Propyl 3,4,5-trihydroxybenzoate	212$[MH]^+$	 60$[MH]^+$
Caffeic acid esters:		
 2-(3,4-dihydroxyphenyl)ethyl (E)-3-(3,4-dihydroxyphenyl)prop-2-enoate	316$[MH]^+$	 154$[MH]^{+2}$
 7-hydroxy-3-methylheptyl(2E)-3-(3,4dihydroxyphenyl-acrylate	308$[MH]^{+2}$	 146$[MH]^{+1}$
Gentisiate & protocatechuate esters:		
 [(2E)-3,7-dimethylocta-2,6-dienyl] 2,5-dihydroxybenzoate	292$[MH]^+$	 154$[MH]^+$
 6-[(3,4-dihydroxybenzoyl) oxy]-3-hydroxy-4-methylhexanoic acid	298$[MH]^{+2}$	 162$[MH]^+$
 Heptyl 3,5-dihydroxybenzoate	252$[MH]^+$	 115$[MH]^{+1}$
 Carbamoylmethyl2,4dihydroxybenzoate	211$[MH]^{+1}$	 75$[MH]^+$

Table 2. LC-MS based identification of a few conjugated phenols form the various fractions (Fig.7).

*Metabolites were identified based on their molecular masses using the following web site: http//: www.ncbi/pubmed/chemcompound

3.1.5 *Cell free extract*

With cell free extracts of *Rhodobacter sphaeroides* OU5, nearly 90% of L-phenylalanine consumption was observed with simultaneous formation of phenols (Fig. 8A). The products were extracted into ethyl acetate and analyzed using LC-MS. In total, 6 different phenols were identified based on their molecular masses. These include, L-tyrosine (181m/z), 3,4-dihydroxyphenylalanine (DOPA; 197m/z), 3,4-dihydroxyphenylpyruvic acid (DOPP; 196 m/z), 3,4-dihydroxyphenyllactic acid (DOPL; 198 m/z), 3,4-dihydroxyphenylacetic acid (DOPAC; 168 m/z) and protocatechuic acid (PC; 154 m/z) (Fig. 8B). The qualitative and quantitative analysis through HPLC (Fig. 9) confirmed and supported the identity of L-tyrosine, DOPA and DOPP, which eventually might have given rise to DOPL, DOPAC and finally protocatechuate (PC). The overall stoichiometric yields of tyrosine, DOPA and DOPP as calculated from the HPLC analysis, after 30 min of assay, were as follows: L-Phenylalanine (1 mM) → Tyrosine (0.35 mM) → DOPA (0.27 mM) → DOPP (0.25 mM).

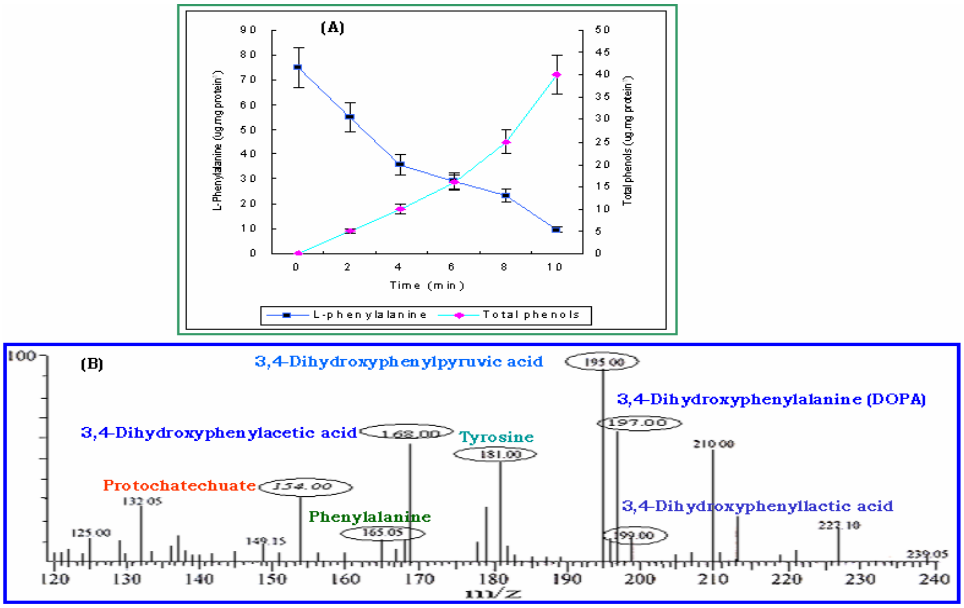


Fig. 8. (A) L-phenylalanine consumption and production of total phenols by cell free extract of *Rhodobacter sphaeroides* OU5 (B) LC-MS finger printing analysis of cell free extract by *Rhodobacter sphaeroides* OU5

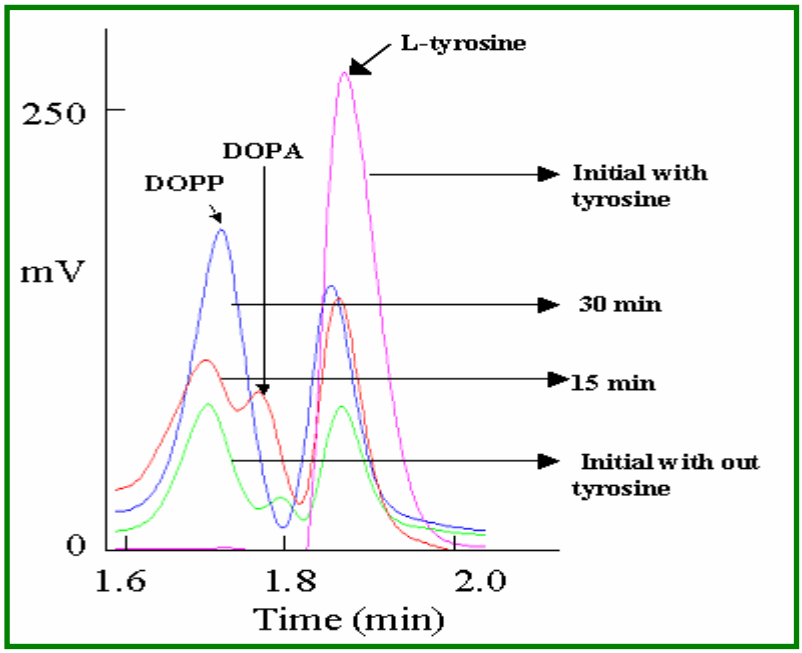


Fig. 9. HPLC chromatograms showing conversion of L-tyrosine to DOPA and DOPP by *Rhodobacter sphaeroides* OU5.

3.1.6 ***Transaminase activity***

The culture supernatants did not yield ammonia, which indicated that L-phenylalanine assimilation as nitrogen source may not be through deamination process and hence we looked for transaminase activities. Aromatic aminotransferase activity was measured in the presence of L-phenylalanine, L-tyrosine and DOPA as substrates in the presence of 2-oxoglutarate and the loss in the substrate was analyzed through HPLC. The respective activities (mg protein^{-1}.min^{-1}) were: 6, 10 and 21. The increase in transaminase activity with the substrates L-phenylalanine < L-tyrosine < DOPA suggest that transamination occurred at the level of DOPA. DOPA consumption stagnated in the absence of supplemented keto acceptor, which restored only in the presence of 2-oxoglutarate (other substrates tested were, pyruvate and oxaloacetic acid) (Fig.10A). The transaminated

product of DOPA was extracted (after acidifying to pH 4.0) into ethyl acetate, concentrated and analyzed using LC-MS. (Fig. 10B) A mass of 196 (m/z) indicate the product as 3, 4-dihydroxyphenylpyruvic acid (DOPP).

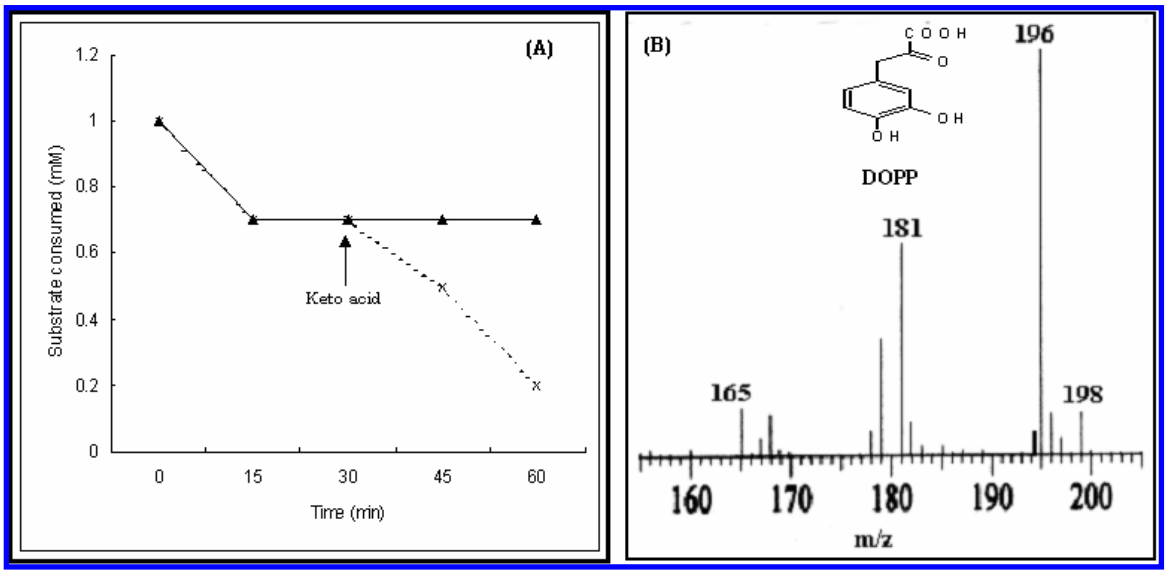


Fig. 10. (A) Loss of DOPA was observed on the presence of keto acid, maximum being with 2-oxoglutarate. (Less activity with pyruvate or OAA) (B) Product of transamination of DOPA as 3,4-dihydroxyphenyl pyruvic acid (DOPP) was confirmed using LC-MS analysis.

3.1.7 *Enzyme purification*

Enzymes were purified from *Rhodobacter sphaeroides* OU5, when L-phenylalanine was used as nitrogen source from cell free extract to apparent homogeneity by using the following steps (flow chart.1)

3.1.7.1 *3,4-dihydroxyphenylalanine 2-oxoglutarate aminotransferase (DOPAATS; EC 2.6.1.49)*

The DOPA 2-oxoglutarate aminotransferase partially purified through the ammonium sulfate 30-35% (saturation) precipitation was dissolved in 2 to 5 ml of 0.05 M potassium

phosphate buffer pH 7.8 and dialyzed for over night against three changes of 3 liters of the same buffer.

3.1.7.2 ***Gel filtration (Sephadex G-150)*** The dialyzed protein solution containing the DOPA 2- oxoglutarate aminotransferase was applied to gel filtration column (Sephadex G-150), which had been equilibrated with 0.05M-phosphate buffer pH 7.8 and was eluted with the same buffer. Fractions of 1ml each were collected. Fraction 18,19,20 were showed highest activity of DOPA 2-oxoglutarate aminotransferase (Fig. 11A) was recovered and followed by affinity purification

3.1.7.3 ***Affinity purification***

After gel filtration, the active fractions (18,19,20) were applied to Sepharose-4B column and protein was eluted with a linear gradient of NaCl (50 mM, 100 mM and 200 mM, pH 6.5) (Fig. 11B). Fractions 24,25,26,27 and 28 were pooled, concentrated and dialyzed 0.05M potassium phosphate buffer, pH 6.5) and used for the further characterization studies. The yield and purification factors of this enzyme at each step are summarized in Table. 3.The DOPA 2-oxoglutarate aminotransferase was purified approximately 30% recovery and specific activity being 2.0 U/mg.protein.

3.1.7.4 ***Native/ SDS PAGE***

After affinity purification protein fractions having the pure protein of DOPA2-oxoglutarate aminotransferase (Fig. 12B) which has given a single band and molecular mass of ~ 123kDa in a native-PAGE (8%) (Fig. 12A2). The enzyme could be a heterodimmer of 60 kDa and 63 kDa with reducing agent (β-mercaptoethanol) as per SDS-PAGE (12%) (Fig. 12B). The enzyme activities (%) with substrates DOPA, tyrosine phenylalanine, tryptophan, aspartate and DL-alanine were 100, 85, 69, 25, 17 and 29,

respectively (Table. 3). The K_m (μM) and V_{max} (s^{-1}) of the enzyme DOPA2-oxoglutarate aminotransferase were 0.35±0.045 and 0.29±3.0, respectively, with pH optimum of 6.0 (range 5-7) and the temperature at 35 ^oC (range 30-40 ^oC) (Table. 3).

3.1.7.5 ***In Gel assay of DOPA 2-oxoglutarate aminotransferase***

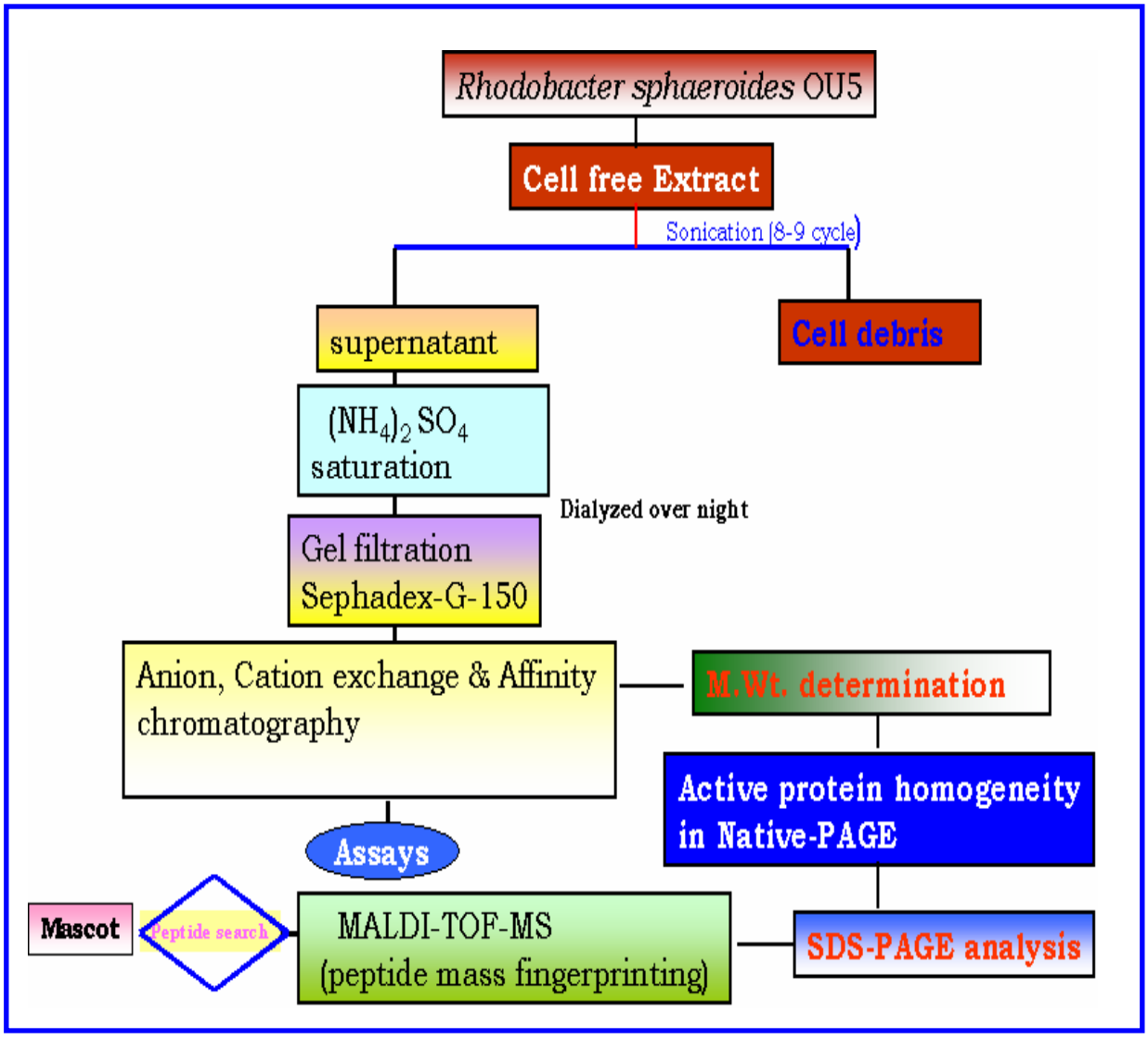
The DOPA 2-oxoglutarate aminotransferase was carried out in the non-denatured electrophoresis, after run the gel was incubated staining with ammonium molybdate reagent (Briggs, 1922). A blue color band was observed in 6h and product could be a is 3,4-dihydroxyphenylpyruvaic acid (DOPP) (Fig. 12A1).

3.1.7.6 ***Molecular weight determination***

The native molecular weight of the purified DOPA 2-oxoglutarate aminotransferase was determined by gel filtration (Sephadex G-150) column, the void volume of the column was determined using Blue Dextran and the column was calibrated with standard proteins: catalase (240 kDa), esterase (166 kDa), bovine serum albumine (BSA-67 kDa), ovalbumine (43kDa) and trypsinogen (24 kDa). It was confirmed a native mass of about Mwt.~123kDa (Fig. 11C).

3.1.8 ***Deaminase activity***

The deaminase product ammonia was observed only in the absence of added 2-oxoglutarate and product of DOPA was extracted after acidifying (pH 4.0) into ethyl acetate, concentrated and analyzed using LC-MS. A mass (Fig. 13) of 182 (m/z) indicated the product as 3,4-dihydroxyphenylpropionic acid (DPPA).



Flowchart 1. Schematic diagram of isolation and purification of enzymes involved in L-phenylalanine catabolism by *Rhodobacter sphaeroides* OU5

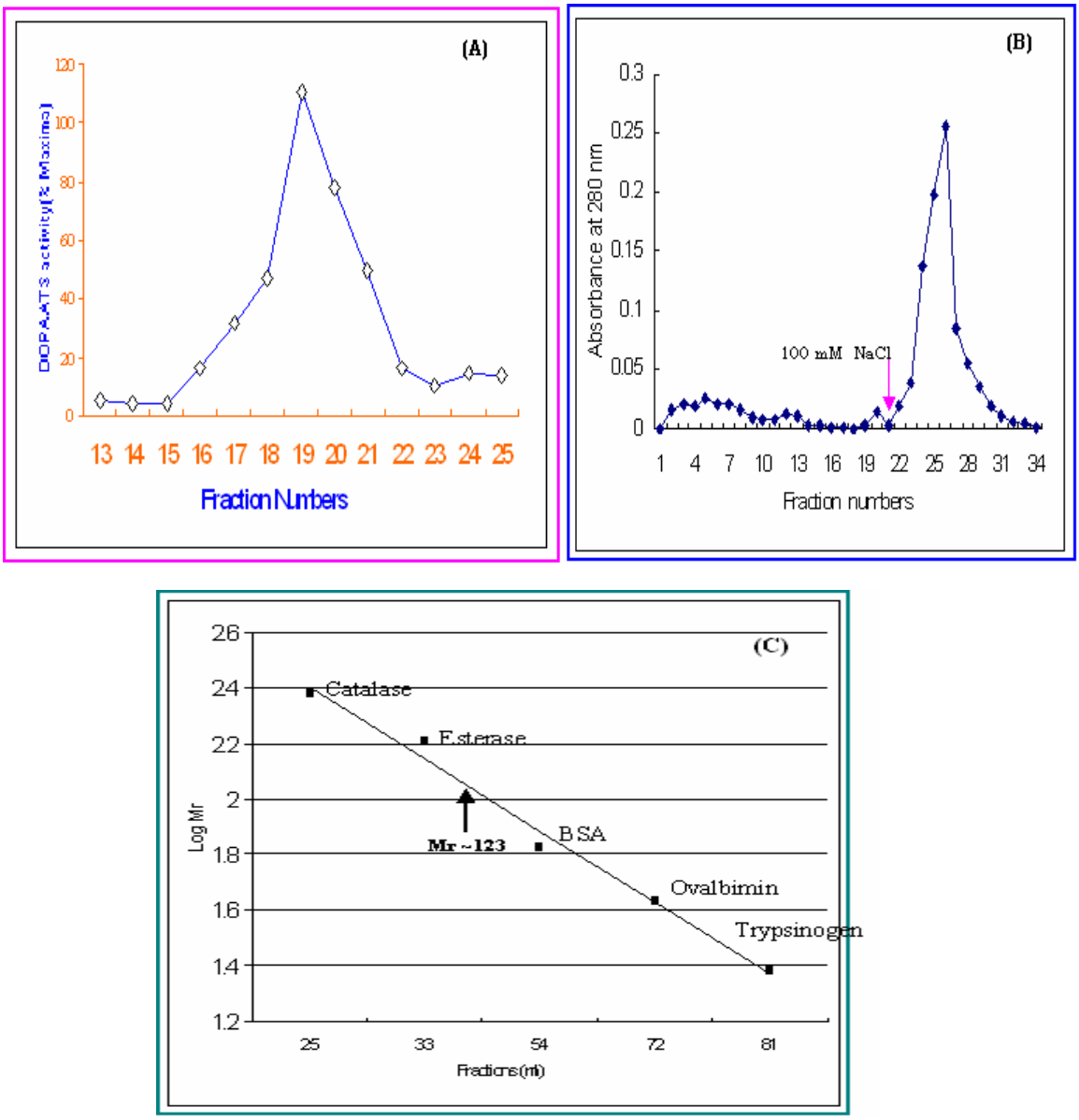


Fig. 11. (A) Gel filtration chromatography (Sephadex G -100) fraction of DOPA 2-oxoglutarate aminotrasferase from *Rhodobacter sphaeroides* OU5 and (B) further purified by affinity chromatography arrow indicates point eluted with 100 mM NaCl. (C) The molecular weight determined by using gel filtration (Sephadex G-150) column chromatography.

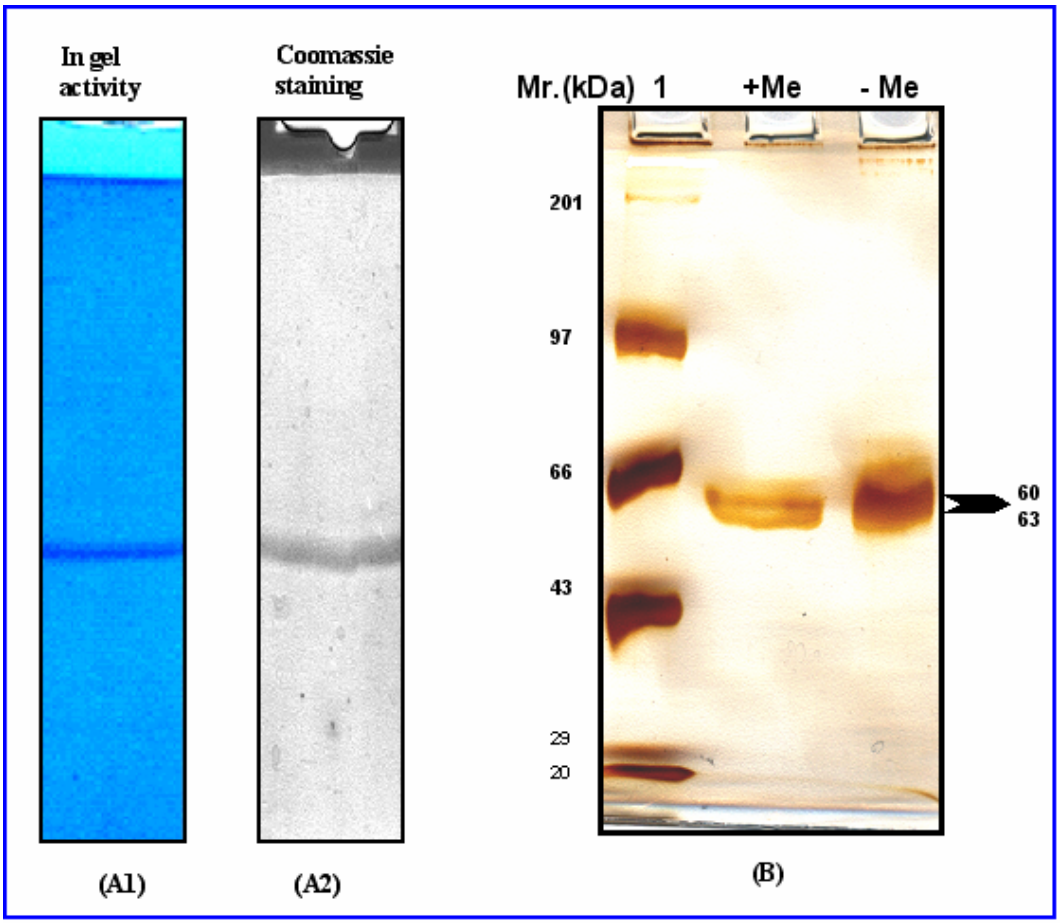


Fig. 12. (A1) In-gel activity of DOPA 2-oxoglutarate aminotrasferase and (8%) Native-PAGE (15µg protein) (A2) showing the purity of enzyme (B) (12%) SDS-PAGE analysis (35 µg protein); Lane-1, Protein molecular markes, with β-mercaptoethanol and with out β- mercaptoethanol.

Purification steps	Volume (ml)	Total protein (mg)	Total enzyme activity (µ mole.h^{-1})	Specific activity (µ mole.h^{-1}/mg protein)	Purification factors	Recovered activity (%)
Crude extract	50	360	22.8	0.28	1.0	100
$(NH_4)_2SO_4$ 35% (30-40) saturation	10	80	18.58	0.130	3-4	85
Gel filtration (Sephadex-G-100	60	30	10. 5	2.80	5-8	35
Affinity chromatography	3.5	2.0	2.0	1.85	2.0	30

Table. 3. Summary of the protein yield from the major purification steps while purifying DOPA 2-oxoglutarate aminotransferase of *Rhodobacter sphaeroides* OU5.

Parameters	DOPA 2-oxoglutarate aminotransferase (DOPAATS)
Substrate specificity (%)	: DOPA-100, Tyrosine-85, Phenylalanine-69, Tryptophan-25, Aspartate-17 and DL-alanine-29
Product	: 3,4-Dihydroxyphenylpyruvic acid (DOPP)
Co-substrate	: 2-oxoglutarate
Co-product	: Glutamate
Co-factor	: Pyridaxol-5-phasphate (PLP)
Optimal condition (range)	
PH	: 6.0 (5-7)
T ^{o}C	: 35 (30-40)
K_m (µM)	: 0.35±0.045
V_{max} (S^{-1})	: 0.29±3.0
Molecular weight (Determined by gel filtration Sephadex G-100)	: ~123kDa
Subunits (Determined by SDS-PAGE): 60, 63 kDa (Could be a dimmer)	

(Results are means±SD of three different determinations)

Table. 4. Characteristics of purified DOPA 2-oxoglutarate aminotrasferase (DOPAATS) from *Rhodobacter sphaeroides* OU5.

3.1.8.1 ***3,4-dihydroxyphenylalanine reductive deaminase***

The product of deaminase, ammonia, was observed only in the absence of added 2-oxoglutarate. The deamination product of DOPA was extracted (after acidifying; pH 4) into ethyl acetate, concentrated and analyzed using LC-MS. A mass of 182 (m/z) indicated the product as 3, 4-dihydroxyphenylpropionic acid (DPPA) and the enzyme as reductive deaminase. Though the enzyme L-phenylalanine ammonia lyase (PAL; EC. 4.3.1.24), which converts L-phenylalanine to *trans*-cinnamate is most commonly observed in plants and recently, also in prokaryotes, there are no reports so far (to the best of our knowledge) about the enzyme DOPA reductive deaminase, and hence we went for purification.

The enzyme deaminase was purified to its homogeneity through 25% ammonium sulphate saturation, followed by dialysis, Sephadex G-150 gel filtration chromatography (Fig. 16A) and finally using anion exchange chromatography (Fig. 16B). Fraction 6 of 1.0 M NaCl elution had the pure protein of reductive deaminase, which has a molecular mass of ~274 kDa in a native-PAGE (8%) (Fig. 17A). The enzyme 3,4-dihydroxyphenylalanine reductive deaminase (DOPARDA) could be a heterotetramer of 110, 82, 43 and 39 kDa subunits as per SDS-PAGE (10%) (Fig. 17B). This enzyme has an obligate requirement for NADH (Fig. 14), low activity (< 40%) with NADPH and no activity with FADH. LC-MS metabolite profiling of the enzyme reaction mixture (assayed in the presence and absence of NADH) was done in realizing the products of the enzyme. In the presence of NADH (Fig. 15A), molecular masses of 181, 182 $[M+H]^{+1}$ and 198 were identified as L-tyrosine, DOPPA and DOPLA, respectively. In the absence of NADH (Fig. 15B), a molecular mass of 197 was observed, which corresponds to

DOPA. These results further confirm the enzyme reductive deaminase, which is specific for DOPA, since DOPA accumulated in the absence of NADH, while it proceeded in the presence of NADH to the products DPPA and DOPLA, when L-tyrosine was the substrate. The enzyme activities (U) with the substrates DOPA, L-tyrosine and L-phenylalanine were 1, 0.8 and 0.4 respectively. The K_m (μM) and V_{max} (s^{-1}) of the enzyme 3,4-dihydroxyphenylalanine reductive deaminase (DOPARDA) was 0.21±0.23 and 0.065±0.07, respectively, pH optimum of 7.0 (range 6-7) and temperature at 40 ºC (range 30-50ºC). The characterization of the DOPA reductive deaminase was summarized in Table. 5.

The native molecular weight of the purified DOPA reductive deaminase was determined by gel filtration (Sephadex G-150) column (see materials and methods) it was confirmed a native mass of about Mwt. ~275kDa and the purity of the protein was confirmed using a reverse-phase HPLC (R_t = 2.15) with a C_{18} column (150 x 4.6 mm) and eluted with acetonitril in 0.3% TFA at 1 $ml.min^{-1}$ flow rate (Fig. 17C).

3.1.8.2 ***MALDI-TOF-TOF (peptide mass fingerprinting) PMF Analysis***

The protein band was selectively removed from the gel and trypsin digested sample was analyzed by MALDI-TOF-TOF peptide finger printing as described in materials and methods. Peptide mass fingerprint was used public domain database (NCBI, MSDB and SwissPort) search using MASCOT (http://www.matrixscience.com). There was no significant match found (Fig. 18A). Further the five major peptide peaks of parent mass 1270.722,1527.643,1662.708,2006.227 and 2000.200 (Fig. 18B) were analyzed by MS/MS, even for which no peptides significant match was found in database.

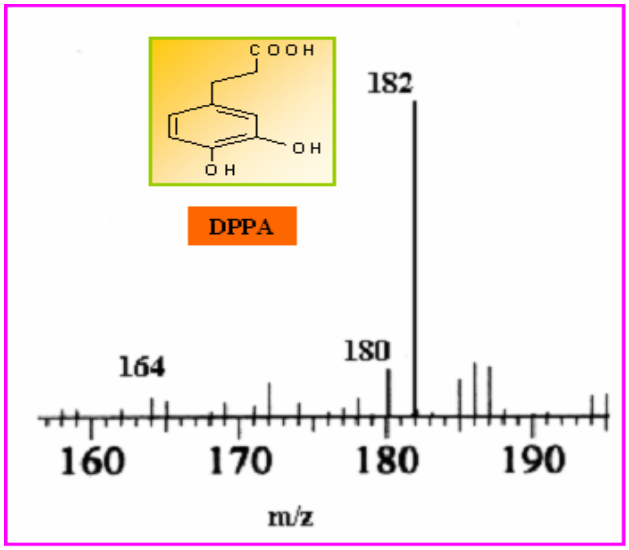


Fig. 13. 3,4 Dihydroxypropionic acid (DPPA) was observed in the enzyme assay supernatant indicated the presence of a reductive deaminase in *Rhodobacter sphaeroides* OU5.

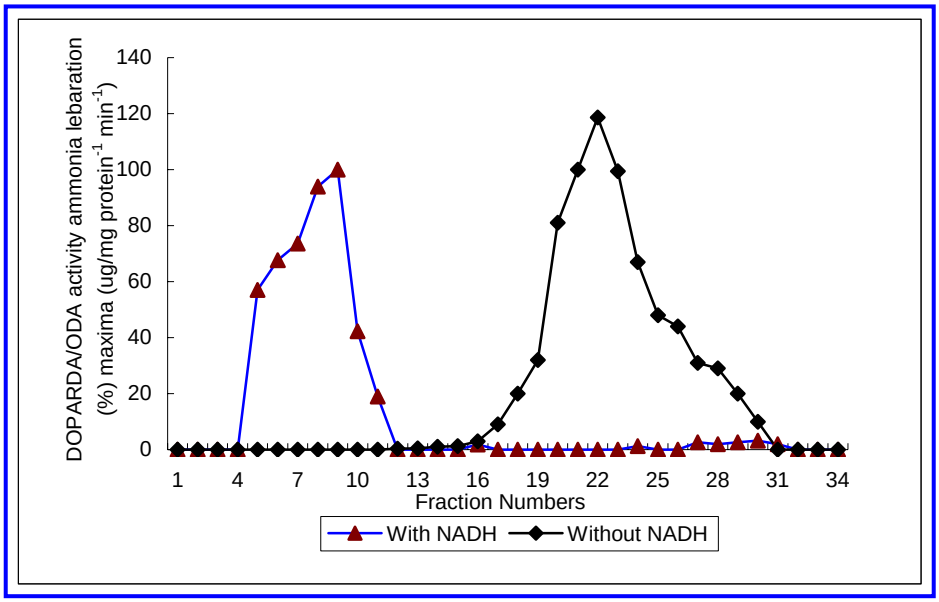


Fig. 14. Gel filtration chromatography (Sephadex-G-150) of DOPA reductive deaminase and DOPA oxidative deaminase.

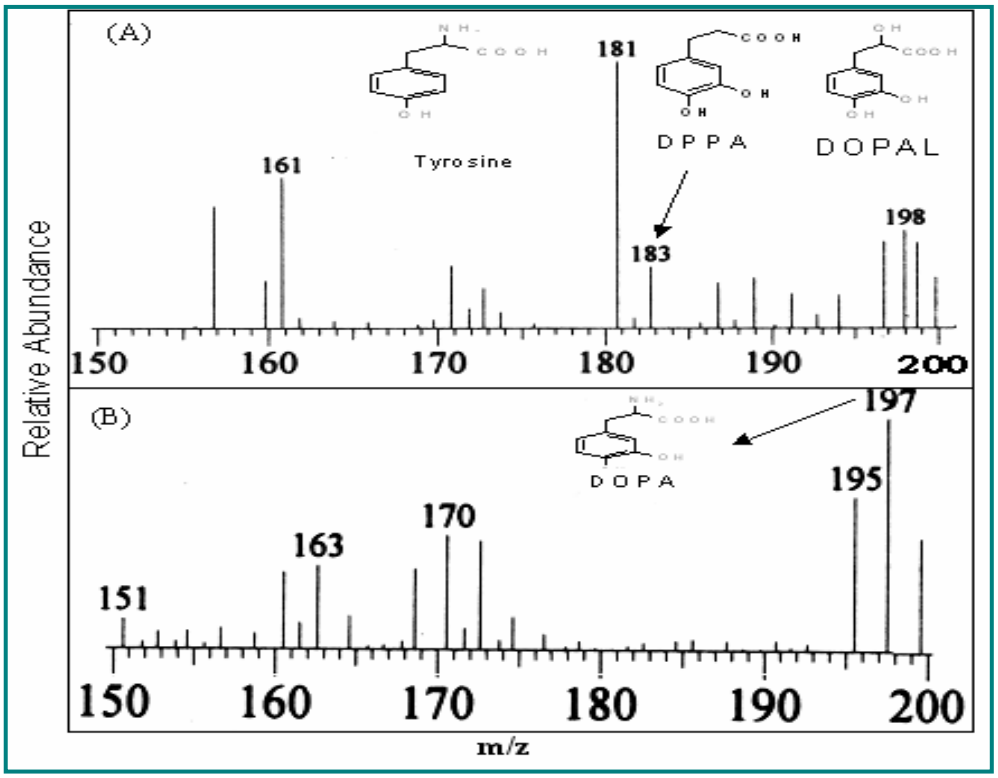


Fig. 15. LC-MS analyses of supernatant of 3,4-dihydroxyphenylalanine reductive deaminase (DOPARDA) enzyme activity assayed in the presence (A) and absence (B) of NADH.

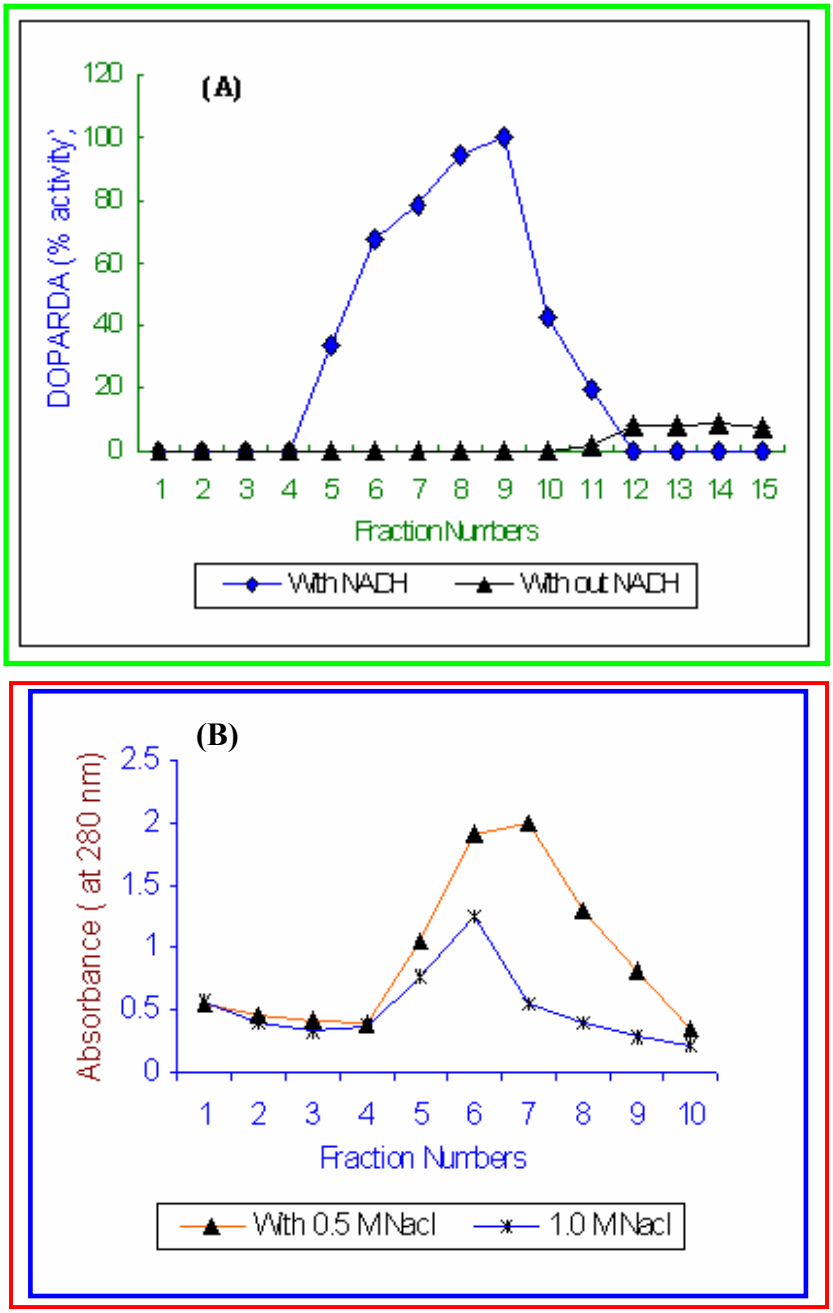
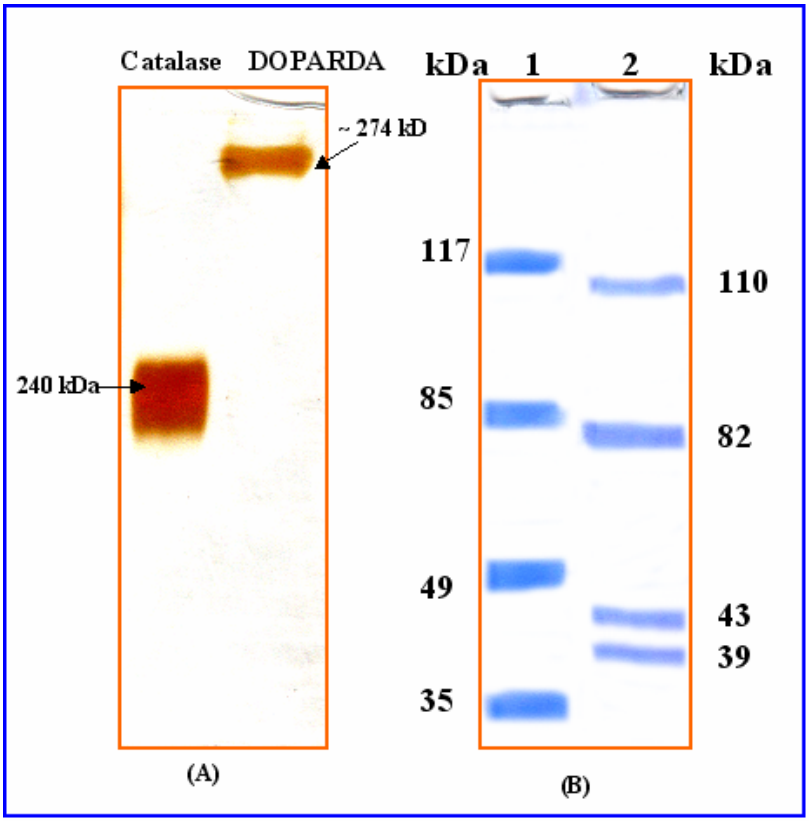


Fig. 16. DOPA reductive deaminase activity of Sephadex G-150 fraction (A). Protein fractions eluted through anion- exchange (DEAE-cellulose) (B).



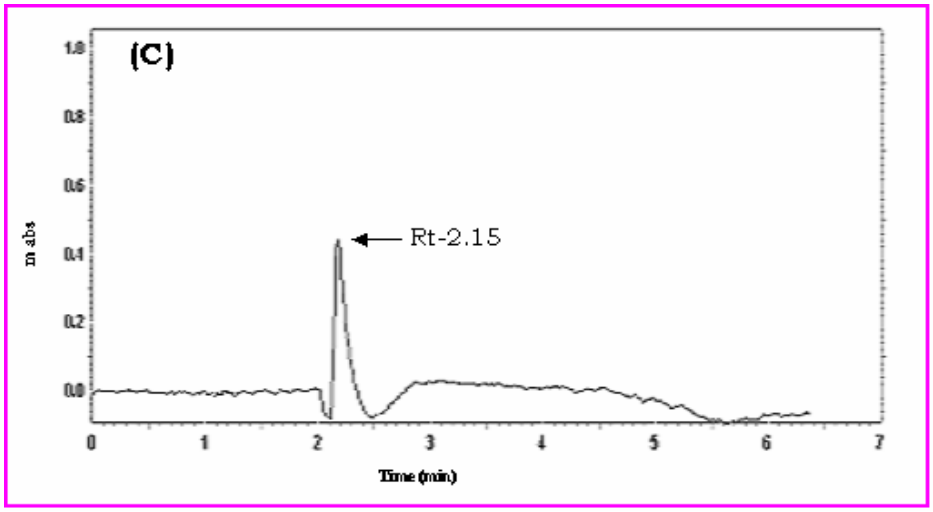
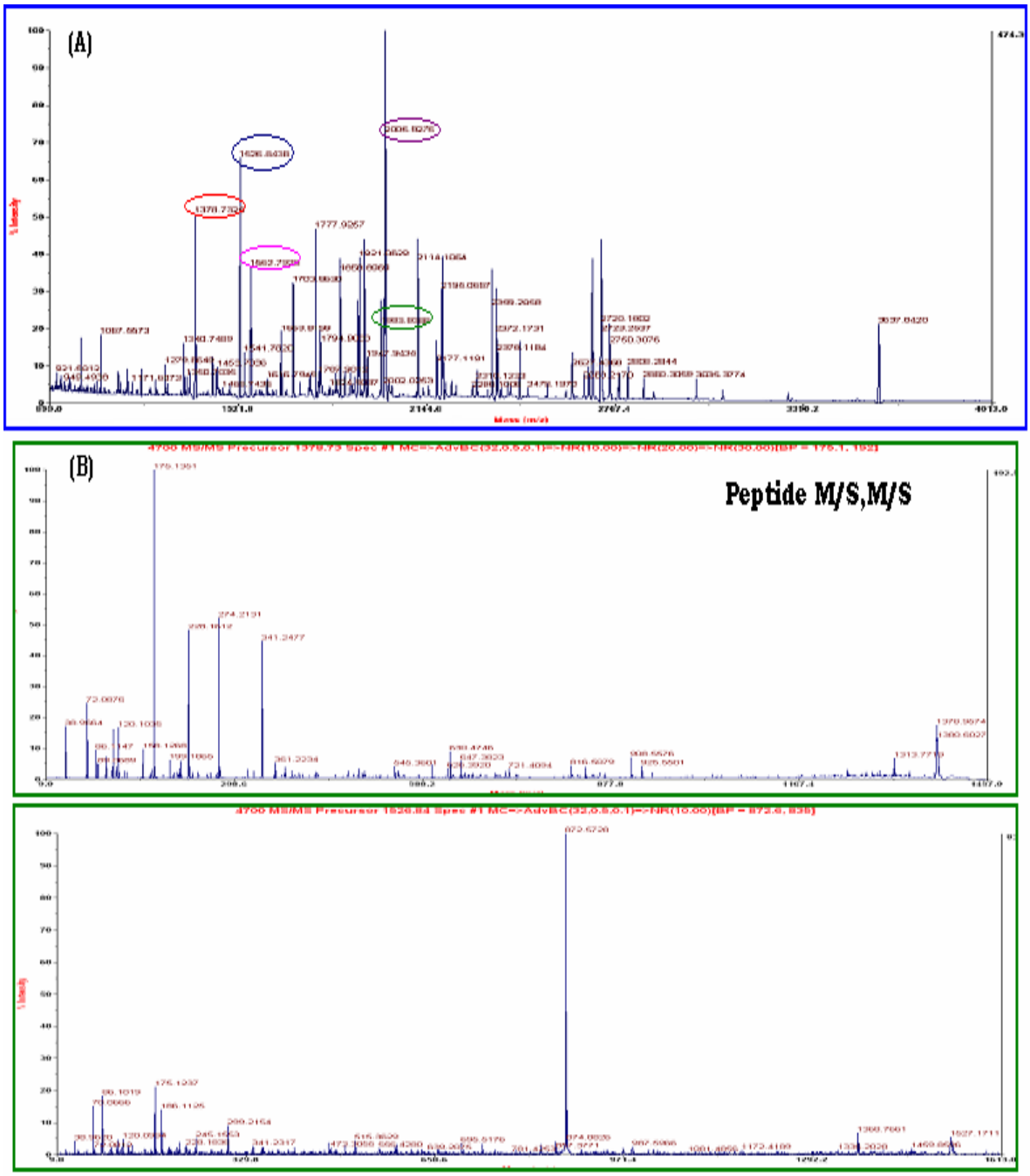


Fig. 17. (A) Fraction 6 of 1M NaCl elution was used for 8% Native-PAGE (20 µg protein) and (B) 10% SDS-PAGE (60 µg protein)(C) HPLC chromatogram showing the purity of DOPA reductive deaminase.


(A)
(B)
Peptide M/S,M/S

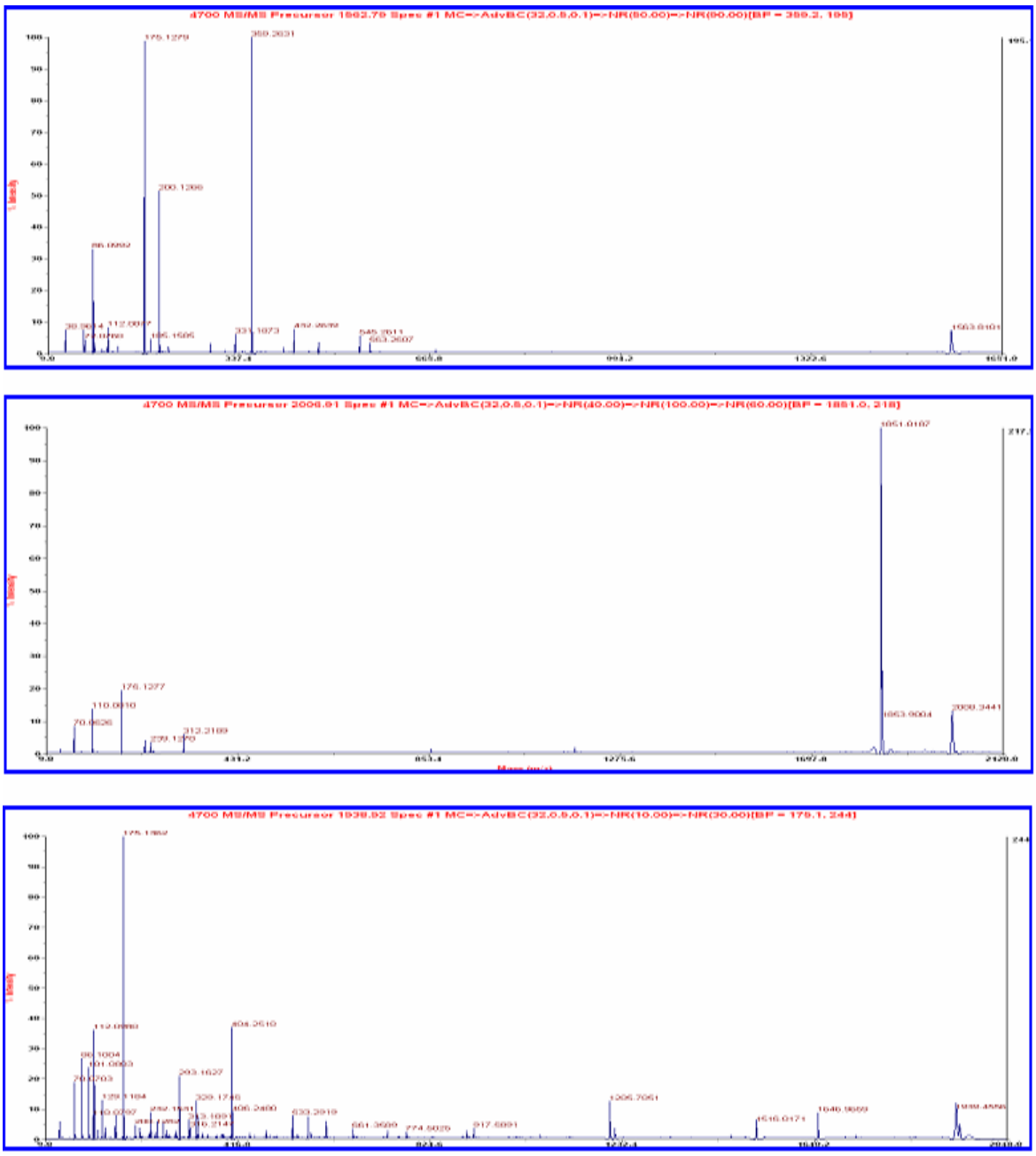


Fig. 18. (A) MALDI-TOF finger printing analysis of DOPA reductive deaminase

(B) MALDI- MS/MS peptide analysis of DOPA reductive deaminase.

Parameters	DOPA reductive deaminase (DOPARDA)
Substrate specificity (%)	: DOPA-100, Tyrosine-77, Phenylalanine-69, Glutamate-62, Glycine-40 and DL-alanine-42
Product	: 3,4-Dihydroxyphenylpropionic acid (DOPPA)
Co-substrate	: Nil
Co-factor	: NADH
Optimal condition (range)	
PH	: 7.0 (6-7)
T ^{o}C	: 40 (30-50)
K_m (μM)	: 21.23±0.09
V_{max} (S^{-1})	: 0.0636±3.0
Molecular weight (Determined by gel filtration Sephadex G-100)	: ~275kDa
Subunits (Determined by SDS-PAGE)	: 117,85,49,35 kDa (Could be a heterotetramer)

(Results are means±SD of three different determinations)

Table. 5. Characteristics of the purified DOPA reductive deaminase (DOPARDA) of *Rhodobacter sphaeroides* OU5.

3.1.9 *3,4-dihydroxyphenylalanine (DOPA) oxidative deaminase*

Sephadex G-150 gel eluted fractions 4 to 10 was dependent on NADH while, fractions 17 to 30 were independent of NADH which is identified as 3,4-dihydroxyphenylalanine oxidative deaminase (Fig. 14). Hence gone for further purification this enzyme.

3.1.9.1 *Isolation and purification of DOPA-oxidative deaminase*

3,4-Dihydroxyphenylalanine oxidative deaminase was partially purified through gradient ammonium sulphate saturation and the highest activity was achieved with 25% saturation, which was further purified by dialysis and gel filtration (Sephadex G-150) chromatography (see materials and methods). Fractions 16-29 (Fig.14) were subjected to cation exchange chromatography (CM cellulose) Fraction 7 & 8 of 1.5 M NaCl elution had the pure protein (Fig. 20A) of oxidative deaminase, which has a molecular mass of ~190 kD (Fig. 21A). The enzyme DOPA-oxidative deaminase could be a heteropentamer of 54, 42, 34, 25 and 23 kDa subunits as per SDS-PAGE (12%) (Fig. 21B). The native molecular mass of DOPA oxidative deaminase was confirmed (see more details in materials and methods) about ~190kDa (Fig.21C) and purity of the protein is also confirmed using a reverse-phase HPLC (R_t= 2.13) with a C_{18} column (150x 4.6 mm) and eluted with acetonitril in 0.3% TFA at 1 ml.min^{-1} flow rate (Fig. 20B).

3.1.9.2 *Characterization of DOPA-oxidative deaminase*

The enzyme specific activity with the substrates, DOPA, L-tyrosine, L-phenylalanine, L-tryptophan, glutamate, DL-alanine and glycine were 1.0, 0.8, 0.6, 0.5, 0.6, 0.2 and 0.3 µmole NH_3.mg protein^{-1} min^{-1}, respectively. The LC-MS analysis of the product when DOPA (Fig. 19A) or L-tyrosine (Fig. 19B) was used as substrates indicated the end

products as 3,4-dihydroxyphenylpyruvic acid (DOPP) and 4-hydroxyphenylpyruvic acid, respectively. The products, 3,4-dihydroxyphenylpyruvic acid and 4-hydroxyphenylpyruvic acid were confirmed by HPLC/LCMS, which had a R_t of 1.7 and 1.9 respectively (Fig. 22). Oxygen requirement for the formation of 3,4-dihydroxy phenylpyruvate from DOPA was confirmed using an oxygen electrode (Fig. 23). The K_m (µM) and K_{cat} (s^{-1}) of the enzyme DOPA oxidative deaminase was 11.84 ± 1.80 and 0.680 ± 0.023, respectively, The DOPA oxidative deaminase was inhibited by NADH (IC_{50} ~35 µM) and 2-oxoglutarate (IC_{50} ~25 µM). The pH optimum of 7.5 (range 6-9) and temperature optima at 40 ^oC (range 10-70 ^oC) the characterization of the enzyme was summarized in Table. 6.

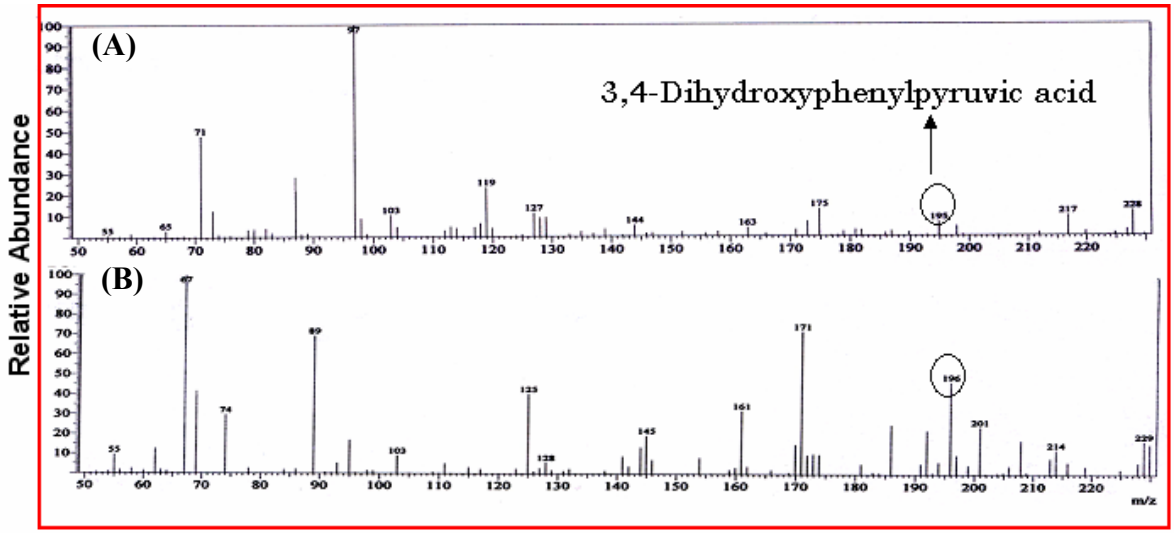


Fig. 19. Identification of product of DOPA oxidative deaminase using LC-MS analysis (A= DOPA; B=Tyrosine as substrates)

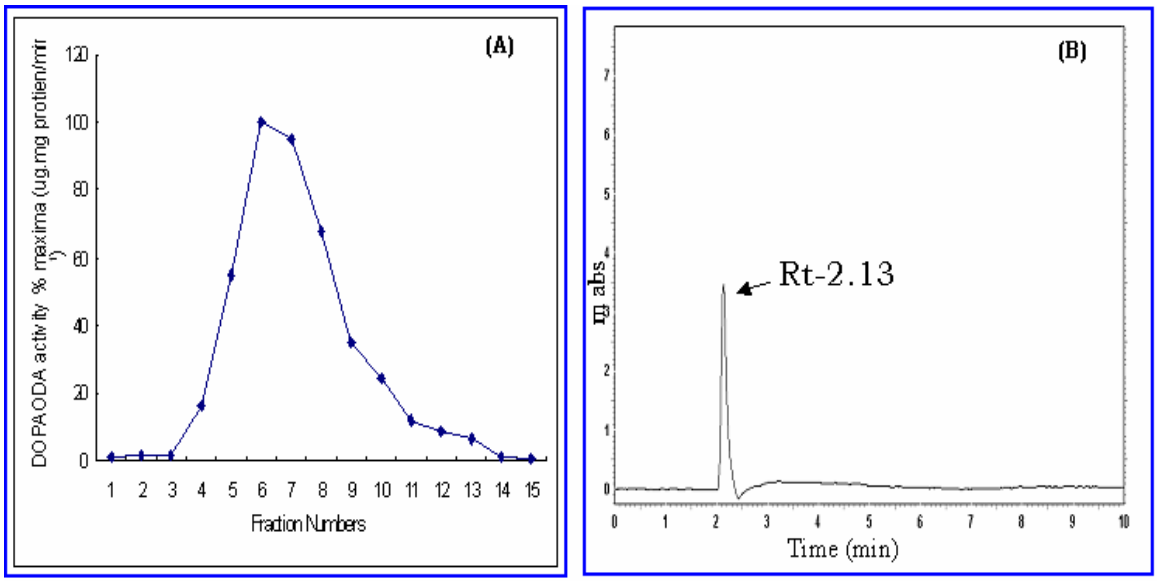


Fig. 20. Further purified by using C.M cellulose- ion exchange chromatography (A) and (B) the purity was checked by HPLC chromatography.

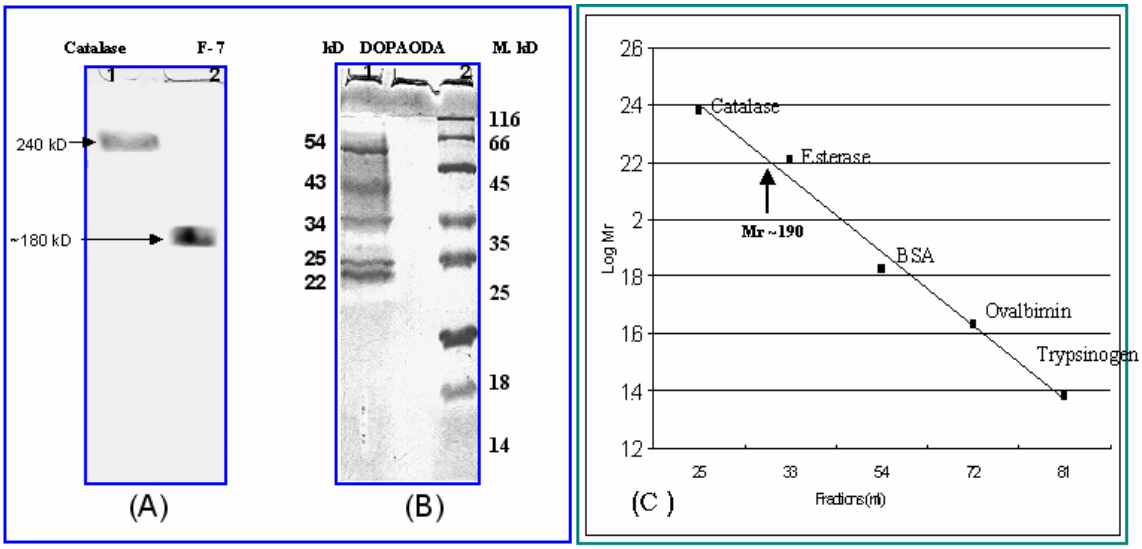


Fig. 21. (A) Native-PAGE (8%) (B) SDS-PAGE (12%) of DOPA oxidative deaminase (C) the molecular weight determined by using gel filtration (Sephadex G-150) column chromatography.

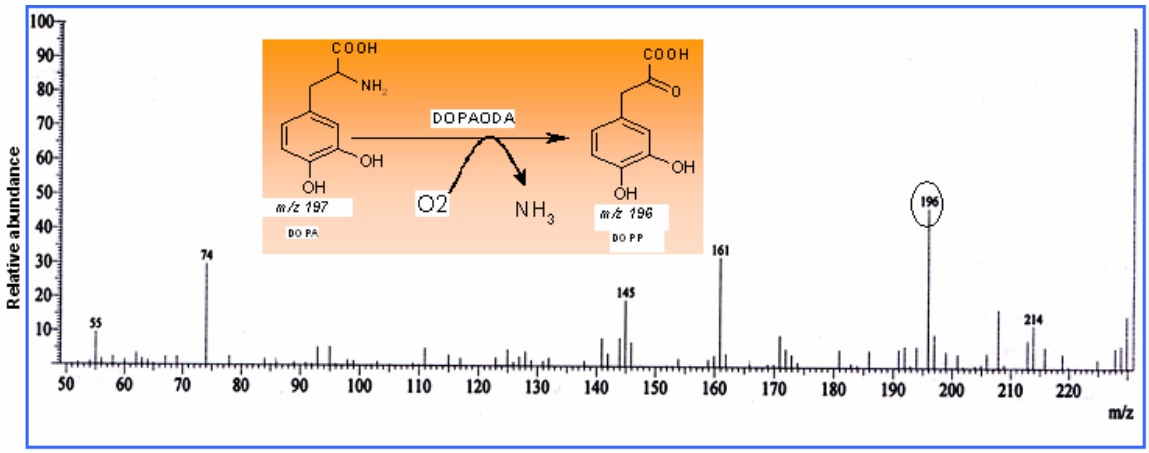


Fig. 22. LC-MS analysis of supernatant of DOPA oxidative deaminase enzyme activity assayed with substrate L-DOPA.

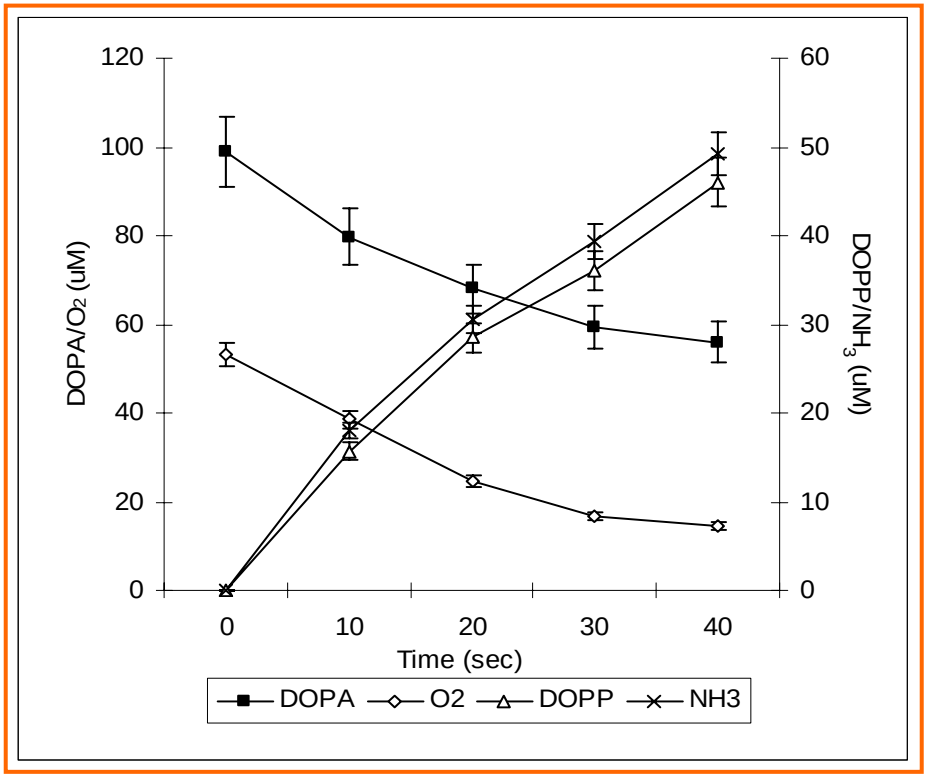


Fig. 23. Oxygen uptake by the purified enzyme was monitored using a Clark type O_2 electrode.

Parameters	DOPA oxidative deaminase (DOPAODA)
Substrate specificity (%)	: DOPA-100, Tyrosine-85, Phenylalanine-69, Glutamate-62, Glycine-40 and DL-alanine-20
Product	: 3,4-Dihydroxyphenylpyruvic acid (DOPP)
Co-substrate	: O_2
Co-factor	: Nil
Optimal condition (range)	
PH	: 7.5 (6-9)
T ^oC	: 40 (20-70)
K_m (μM)	: 11.84±1.80
V_{max} (S^{-1})	: 0.680±0.023
Molecular weight (Determined by gel filtration Sephadex G-100)	: ~190kDa
Subunits (Determined by SDS-PAGE): 54,43,34,25,22 kDa (Could be a pentamer)	

(Results are means±SD of three different determinations)

Table. 6 Characteristics of purified DOPA oxidative deaminase (DOPAODA) of *Rhodobacter sphaeroides* OU5.

3.2 L-Tryptophan catabolism by *Rhodobacter sphaeroides* OU5.

3.2.1***Growth and simultaneous utilization of L-tryptophan by Rhodobacter sphaeroides OU5*** L-Tryptophan supported growth of *Rhodobacter sphaeroides* OU5 when used as sole source of nitrogen. Growth was not observed when L-tryptophan was used as a sole source of carbon or carbon and nitrogen. When observed on L-tryptophan, as a nitrogen source, indoles production was increased from 0.05 to 1.0 mM concentration of L-tryptophan, remained constant till 5 mM (Fig. 24A). Figure-24B shows the phototrophic growth of *Rhodobacter sphaeroides* OU5 on L-tryptophan (1mM) as sole nitrogen source (malate as source of carbon/e^- donor), which has doubling time of about 11 h compared to 8 h on ammonium chloride. Complete utilization of L-tryptophan was observed within 6 h of phototrophic incubation of *Rhodobacter sphaeroides* OU5, with simultaneous production of indoles (Fig. 24B). Ammonia or amino acids could not be detected in the culture supernatant. Though entire L-tryptophan was consumed within 6 h, growth almost proceeded till 30 h and even utilization of indole was observed with time (Fig. 24B).

3.2.2 ***Identification and purification of metabolites in the culture supernatant of Rhodobacter sphaeroides OU5***

3.2.2.1 ***Identification of metabolites***

Rhodobacter sphaeroides OU5 could not produce indoles when grown on tryptone broth, however, replacing tryptone with L-tryptophan, as nitrogen source, it gave orange color reaction with PDAB reagent (Flow chart. 2) This color is in contrast to the normal rose-pink color for indole observed with *Escherichia coli* strain DH5α. Thin layer

chromatographic (TLC) analysis of the concentrated ethyl acetate extract (Fraction-A) yielded three major spots

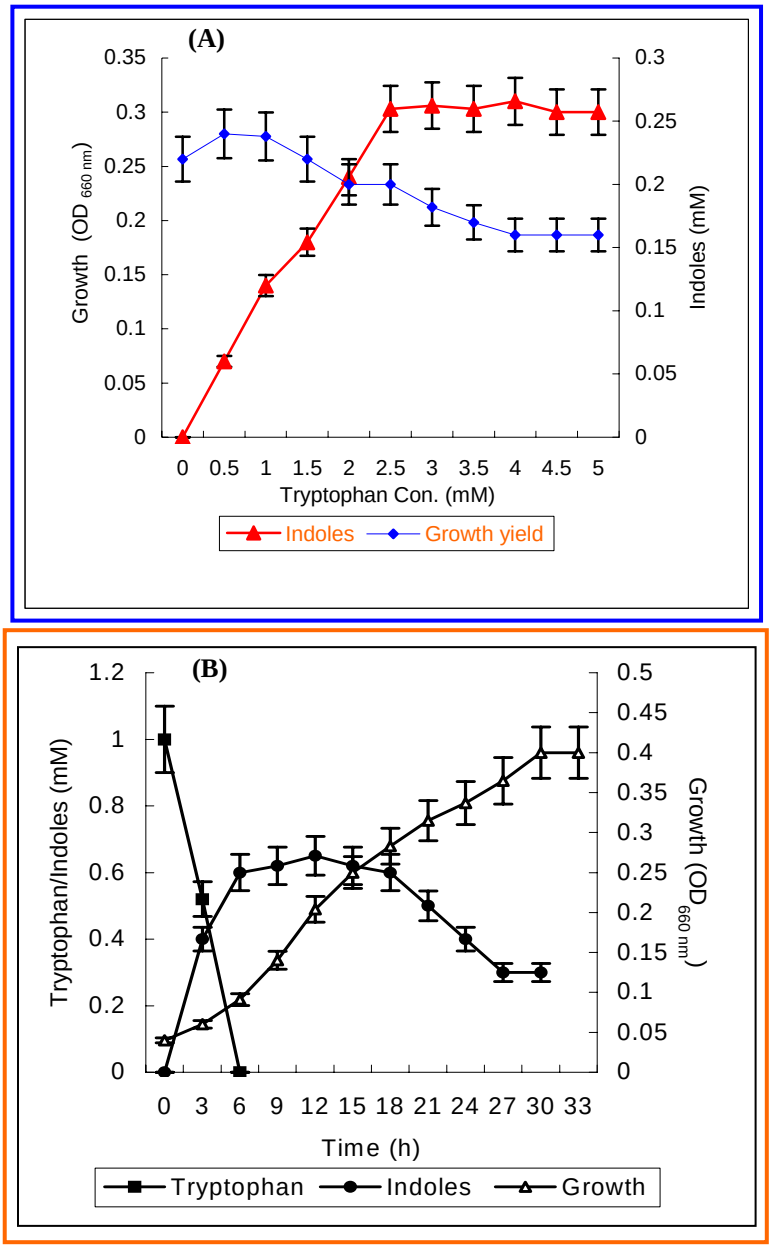


Fig. 24. Growth, L-tryptophan utilization and production of indoles by whole cells of *Rhodobacter sphaeroides* OU5.
(A= different concentrations of L-Tryptophan; B= Growth and utilization of L-tryptophan) L-tryptophan (1 mM) was used as sole source of nitrogen in the presence of malate (22 mM) as carbon source and incubated under phototrophic (2,400 lux) conditions at 30 $\pm$ 2 ^{o}C. Results expressed are average values of two independent experiments done in triplicates.

when run with chloroform and water (9:1) having R_F: 0.17, 0.37 and 0.94, which were yellow, purple and pink, respectively, when sprayed with PDAB reagent. The standard indole had a R_F of 0.98, with a pink spot and matched with the indole produced by *E. coli* strain DH5α. These results necessitated the purification and characterization of the indoles produced by *Rhodobacter sphaeroides* OU5 when grown on L-tryptophan.

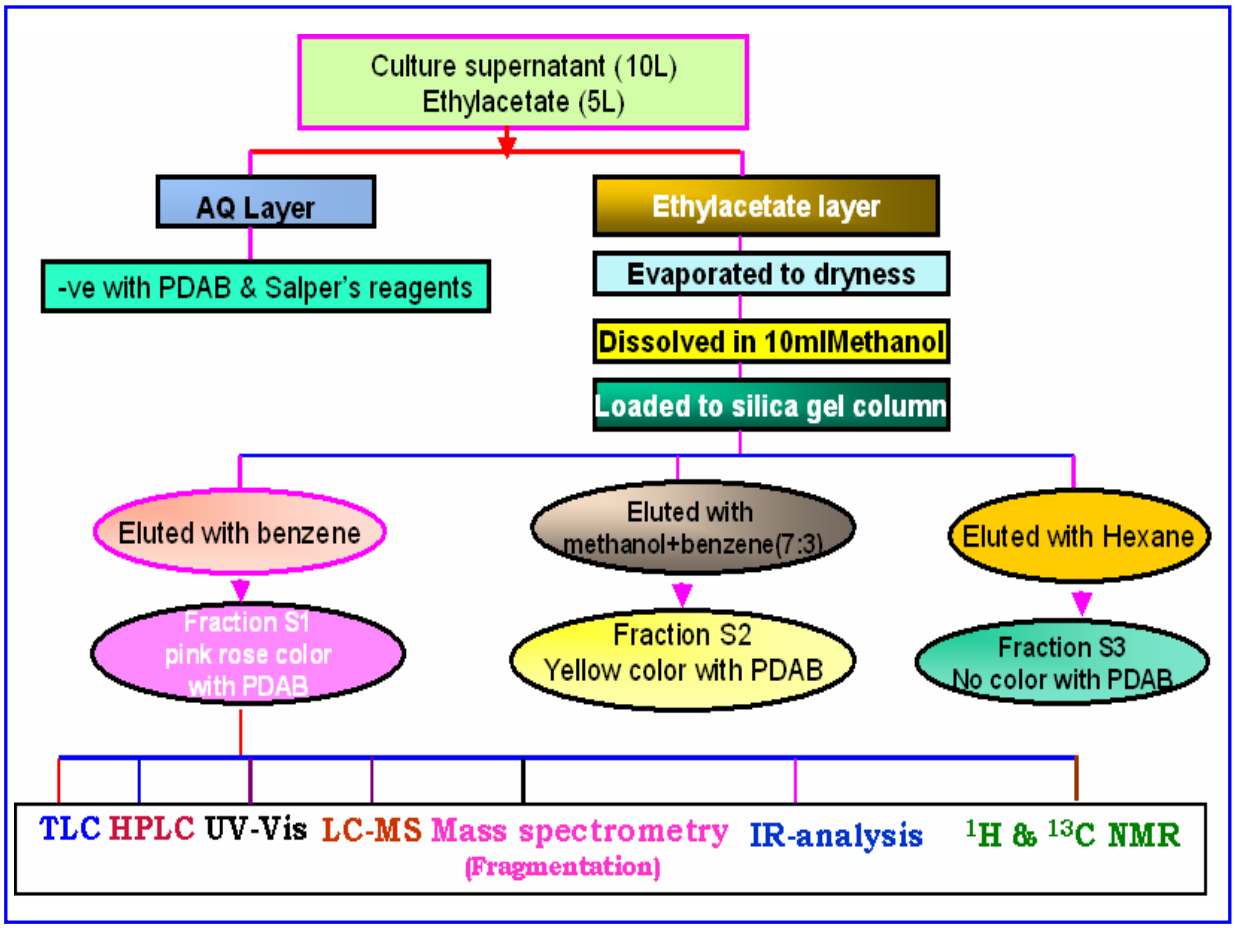
The benzene fraction (S1) yielded an orange-brown colored compound. The compound gave positive test to PDAB (pink color) and Salper's (orange) reagents. Only one spot was observed on thin layer chromatography (TLC) [solvent system: methanol+benzene+hexane (1:1:1)] having a R_F of 0.32. The compound had a mass of (m/z) 279 $[M+H]^+$ and the mass fragmentation of the compound is shows in Figure 25B. Confirmation of the structure comes through IR, ^1H and ^{13}C NMR analysis (given in foot notes of Fig. 25A,B; 25A; 26A,B) and the compound was identified as 3-hydroxy-6- (1H-indol-3-yloxy)-4-methyhexanoic acid.

S2 fraction was yellow in color and gave positive test with PDAB (yellow color) and negative to Salper's reagent. This compound had R_F of 0.45 in the above mentioned solvent. It has a mass of 129 $[M+1H]^{+1}$ and the mass fragmentation of the compound is shown in Figure. 27B. IR analysis NMR delta value (Fig. 27A,B) confirmed its structure as 4-aminocyclohex- 4-ene-1, 2 diol.

3.2.2.2 ***Metabolites of L-tryptophan photometabolism***

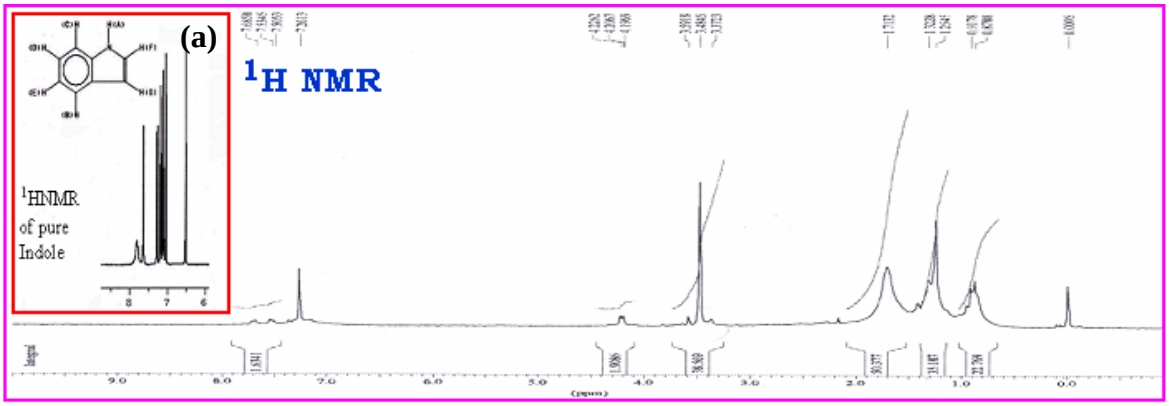
The LC-MS chromatograms of concentrated ethyl acetate extract of the 6 h culture supernatant indicated atleast 15 chromatographically distinct metabolites (Fig. 28). Four of these metabolites were identified as; pyruvic acid (R_t = 1.0; 98 m/z), indole 3-carboxaldehyde (R_t = 3.2; 145 m/z), indole 3-acetic acid (R_t = 4.1; 175 m/z), indole-3-

acrylaldehyde (R_t = 2.5 171m/z) and rhodethrin (R_t = 9.6; 279 m/z). Few of identified as (circle masses) known molecules using LC-MS analysis (Fig. 29A,B).



Flow chart 2. Schematic diagram showing fractionation of the culture supernatant (Purified S1, S2, compounds)

(AQ= Aqueous layer, EA=Ethayl acetate, PDAB = Para dimethyl aminobenzoldehyde)



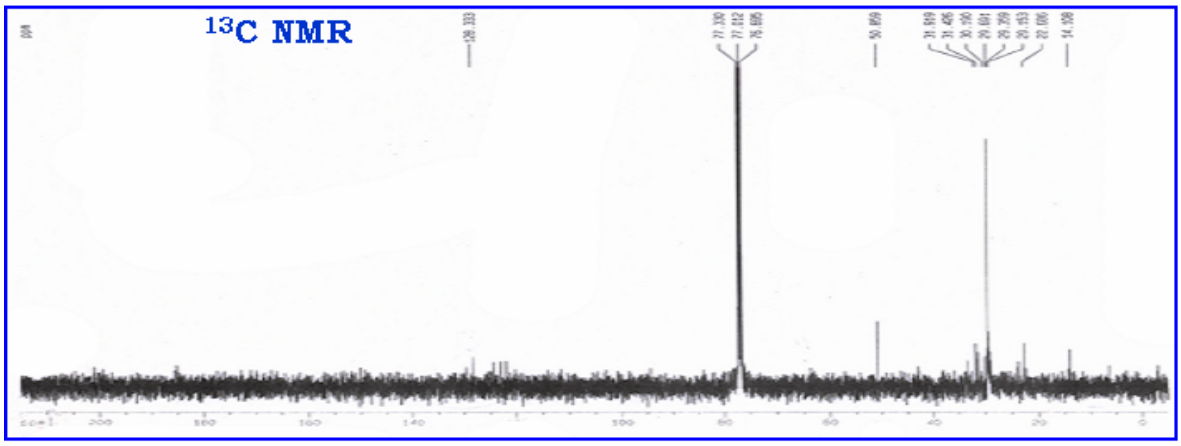


Fig. 25. ^{1}H and ^{13}C NMR of the purified metabolite isolated from the culture supernatant (S1 fraction) during growth on L-tryptophan by *Rhodobacter sphaeroides* OU5. [The metabolite is an orange brown solid]

^{1}H NMR (CDCl$_3$, 200 MHz) δ 8.93 (t, 2H), 7.00-6.97 (m, 4H), 5.65(m, 3H), 3.73(s, 3H), 2.00(m, 2H), 1.72-1.50(m, 2H), 1.25(m, 4H); 0.95(d, 2H); (a) ^{1}H NMR of standard indole.

^{13}C NMR (CDCl$_3$, 150 MHz) δ 132.7 (C-3′), 130.98 (C-5′), 129 (C-7), 118(C-1), 116(C-1′), 113 (C-6′), 112.9 (C-4′), 91.5 (C-1), 72 (C-1), 70(C-1), 42.2 (C-2), 38.0 (C-8), 35.7 (C-5), 32.1 (C-4), 19.6 (C-3);

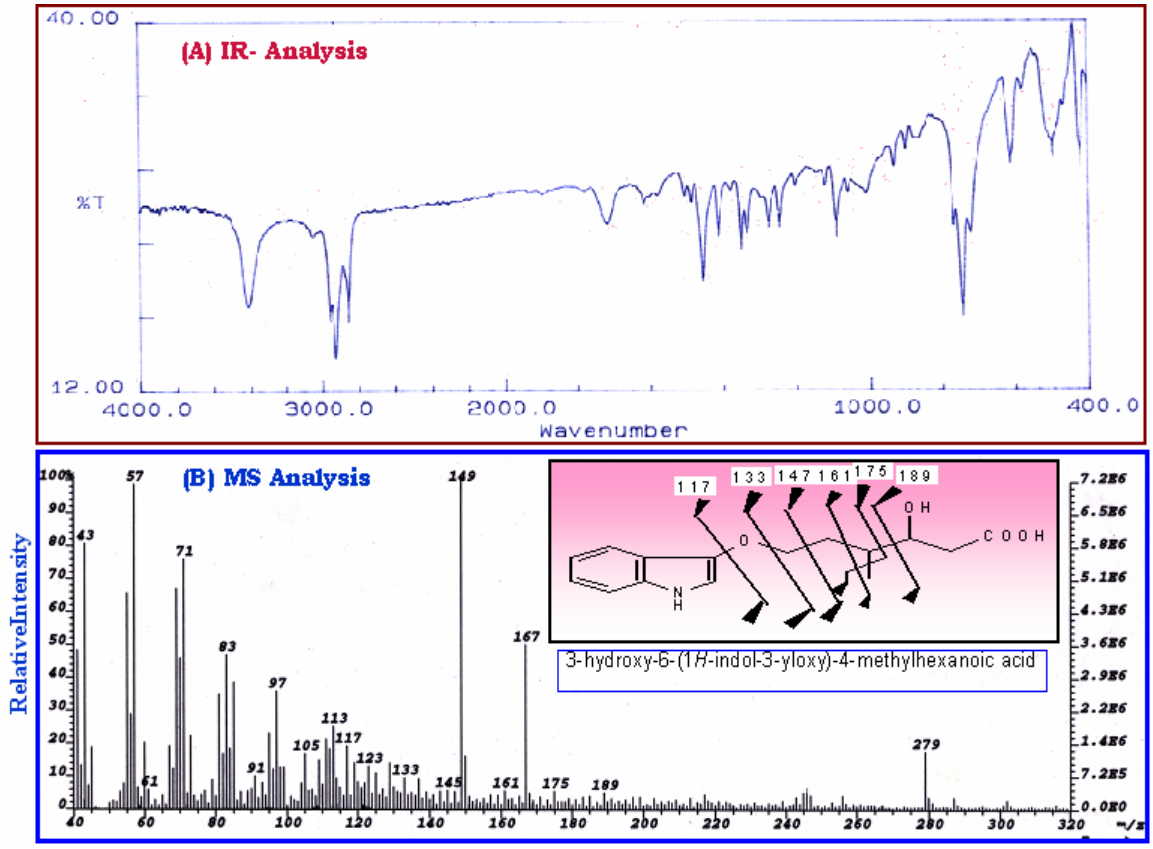


Fig. 26. (A) IR (KBr pellet; cm^{-1}) 3414, 2959, 2926, 1716, 1614, 1456, 1414, 1277, 1246, 1089, 742 and 497 (asymmetrical C-O-C stretches band at 1275-1200 [7.84-8.33 nm] confirm the aryl alkyl ether); EIMS m/z 279 $[M]^+$; anal. C 64.2 % and H 6.8 %, calcd. for $C_{15}H_{19}NO_4$, C 64.97%, H 6.91%. IUPAC name of the metabolite is 3-hydroxy-6-(1H-indol-3-yloxy)-4-methylhexanoic acid, which we named as rhodethrin (**Rhod** = from *Rhodobacter*; **ethr** = ether; **in** = indole).

(B) Mass analysis: Mass fragmentation and structure of purified metabolite.

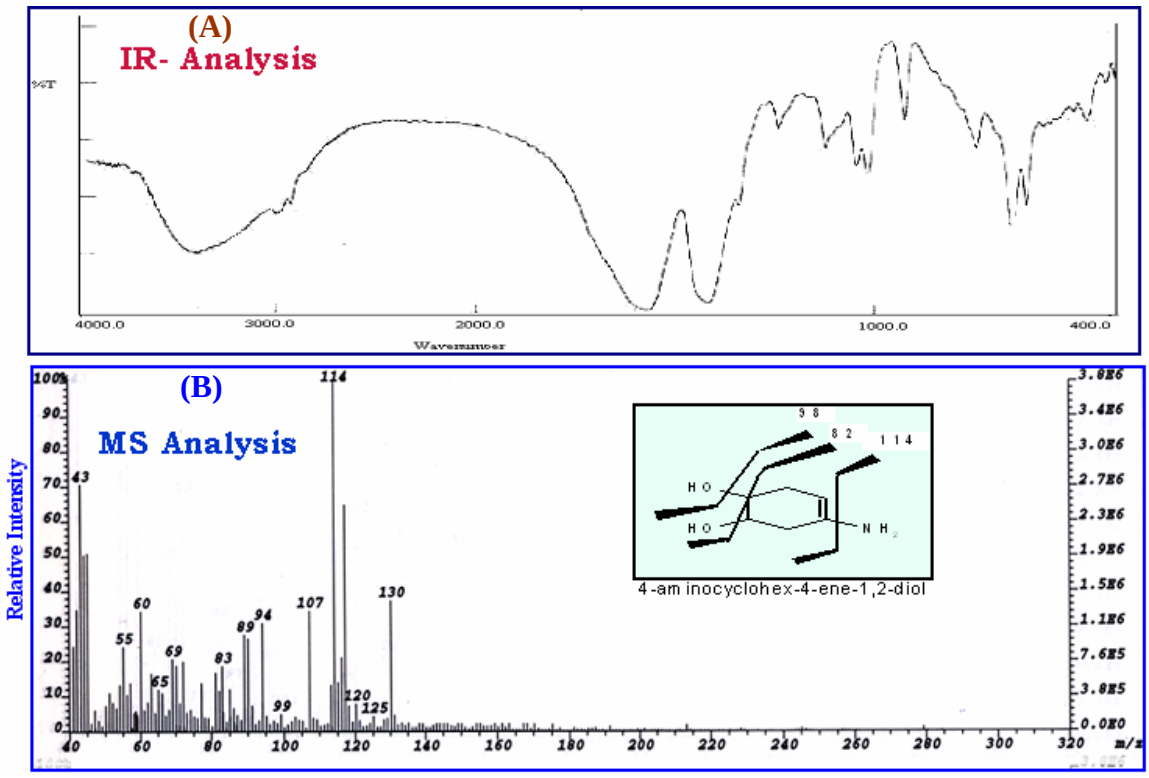


Fig. 27. Infra Red spectral analysis (KBr pellet; cm^{-1}) 3379, 1645, 1518 and 746 Mass-spectrum analysis of fraction S2 Showing fragmentation of the metabolite. Culture grown on L-tryptophan (1 mM) for 6 h in 10 L medium was used for the isolation of the metabolite. The NMR and IR data of the metabolites is given below.

(A) ^{1}H NMR spectrum of the metabolite. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.64 (d, 2H, $CH\text{-}NH_2$), 4.73(s, 1H,CH_2-CH), 3..80(d, 2H, OH-C-C-OH), 2.40 (s, 1H,CH_2), 2.30(m, 2H), 2.00(m, 2H,CH_2-CH), 1.25(s, 1H, OH-CH_2);

(B) ^{13}C NMR ($CDCl_3$, 150 MHz) δ147 (C-3′, $\underline{C}H\text{-}NH_2$), 102.9 (C-2′, $H_2N\text{-}\underline{C}H{=}CH$), 73(C- 6′, $\underline{C}H_2$-OH), 71.3 (C-5′ $\underline{C}H_2$-OH), 32.98(C-1′, $CH{=}CH\text{-}\underline{C}H_2$). 31.92 (C-4′, H_2N-$CH\text{-}\underline{C}H_2$),

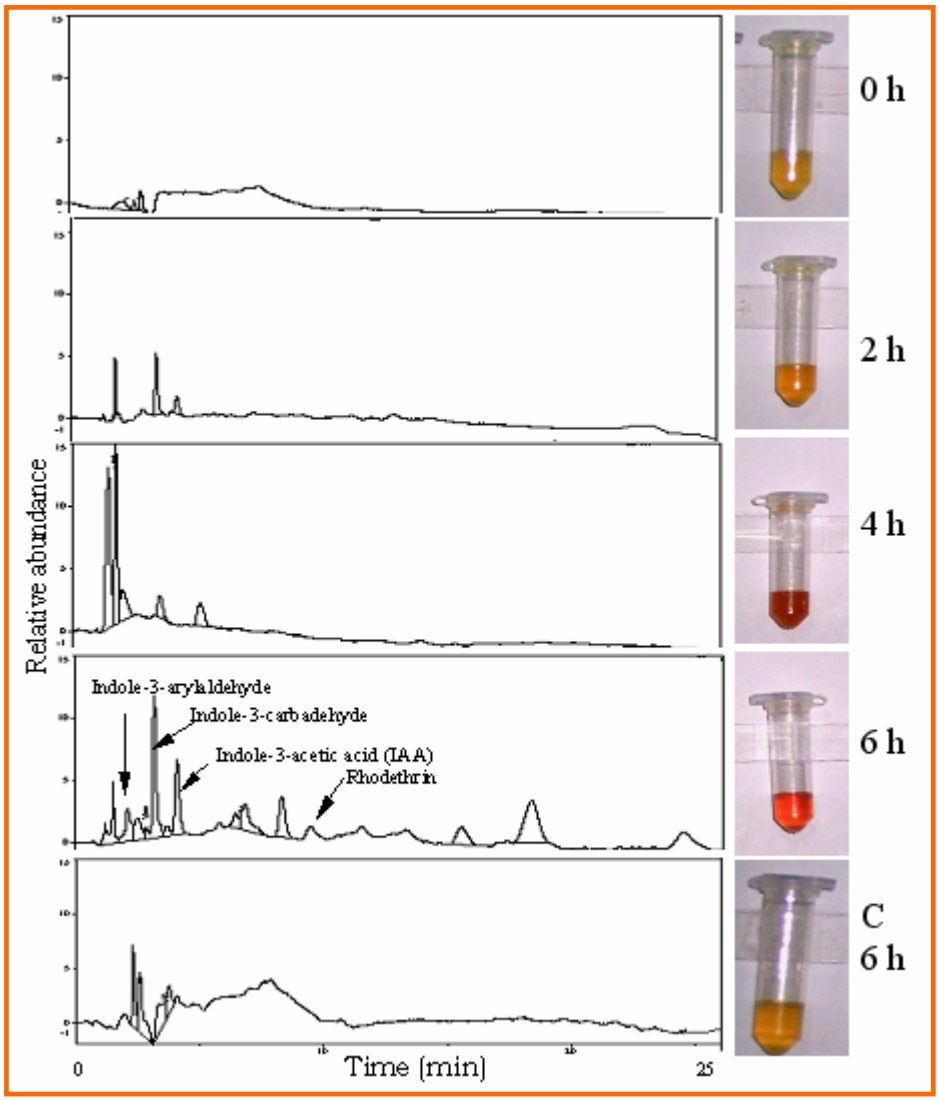


Fig. 28. Metabolic foot printing time course analysis of L-tryptophan grown culture supernatant using HLPC and color reactions with PDAB reagent.

(6h chromatogram represents 15 chromatographically distinct foot printing molecules)

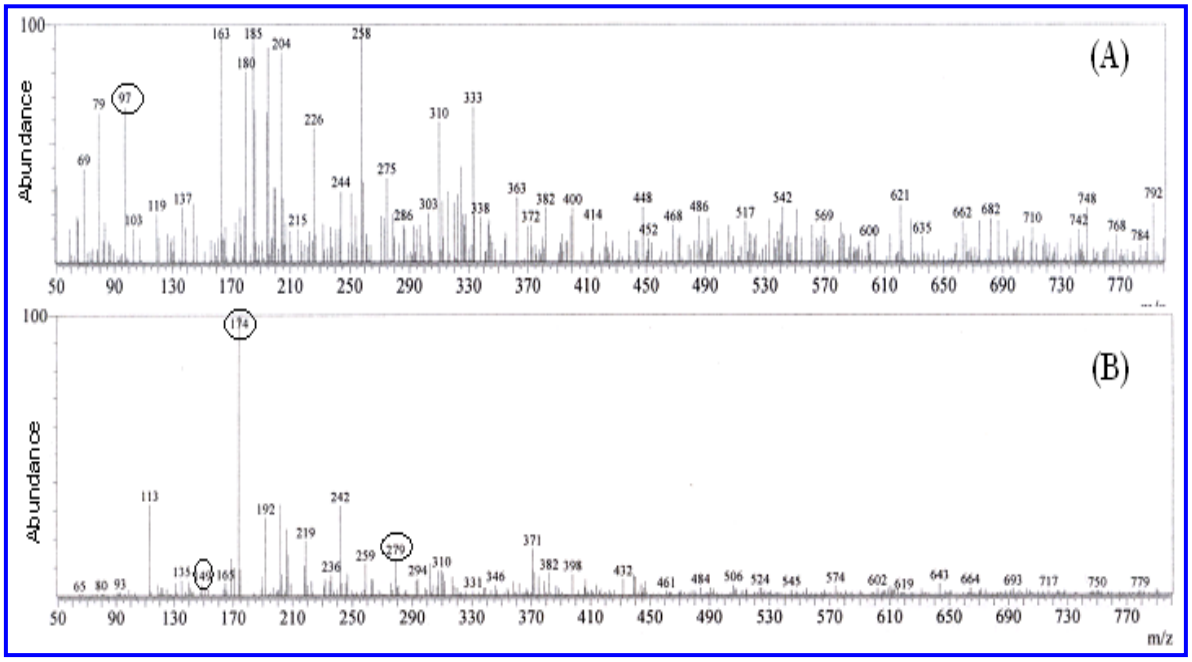


Fig. 29. (A) Represent positive mode of the ion masses of the metabolites in LC-MS analysis. (B) Represent negative mode of the ion masses in LC-MS chromatogram. The encircled ones are the masses of the metabolites of known compounds.

3.2.3 ***Cell free extracts***

With cell free extracts of *Rhodobacter sphaeroides* OU5, about 0.4 mM of L-tryptophan consumption was observed with simultaneous formation of indoles upto 0.4 mM (Fig. 30). The assays were done in the presence and absence of 2-oxoglutarate, which acts as an amino acceptor from L-tryptophan. The products were extracted into ethyl acetate and analyzed using LC-MS.

3.2.3.1 ***Metabolite profiling when 2-oxoglutarate was used in the assay***

Nearly 100% of L-tryptophan consumption was observed with simultaneous formation of indoles in 15 min. Indole 3-pyruvic acid (m/z 203), indole 3-acetaldehyde (m/z 159), indole 3-acetic acid, isatin (m/z 147), gallic acid (m/z 170), pyrogallol (m/z 126) and benzaldehyde (m/z 106) were identified from the assay supernatant based on their molecular masses. Indole-3-pyruvic acid (R_t = 16.9), isatin (R_t = 5.8), benzaldehyde (R_t =

8.9), gallic acid (R_t = 4.4) and pyrogallol (R_t = 5.0) were confirmed, however, indole 3-acetaldehyde and indole 3-acetic acid could not be detected in HPLC. These metabolites are the intermediates of a single pathway as confirmed from the studies using cell free extracts where L-tryptophan (Fig. 33A1) or IAA (Fig. 33A2) or isatin (Fig. 33A3) were used as precursor and the products were analyzed using HPLC/LC-MS

3.2.3.2 ***Metabolite profiling in the absence of 2-oxoglutarate in the assay***

Metabolites identified (Fig. 32B, B1, B2) from the assay include indole-3-acrylic acid (m/z 187); indole 3-acrylaldehyde (m/z 171), indole 3-carboxaldehyde (m/z 145), N-formylphenylacetic acid (m/z 178), N-formylanthranilic acid (m/z 165), anthranilic acid (m/z 137) and catechol (m/z 110). The products viz. indole 3-acrylic acid (R_t = 16.91); indole 3-carboxaldehyde (R_t = 8.7); anthranilic acid (R_t = 9.1) and catechol (R_t = 4.2) were confirmed through HPLC analysis (Fig. 33 B1, B2).

3.2.3.3 ***L-Tryptophan aminotransferase (WAT; EC 2.6.1.27)***

L-Tryptophan aminotransferase activity was detected by measuring the product, indole 3-pyruvic acid and glutamate using HPLC (Fig. 31A) and a mass of 203 m/z indicated that the product as indole-3-pyruvic acid confirmed using LC-MS analysis (Fig. 31B).

3.2.3. 4 ***L-Tryptophan ammonia lyase (WAL)***

The product of ammonia lyase was observed only in the absence of 2-oxoglutarate. A mass of 187 (m/z) indicated the product as indole 3-acrylic acid, which was confirmed using HPLC (Fig. 33B1a). The activity of this enzyme using cell free extracts indicated that this enzyme has 100 % affinity for L-tryptophan.

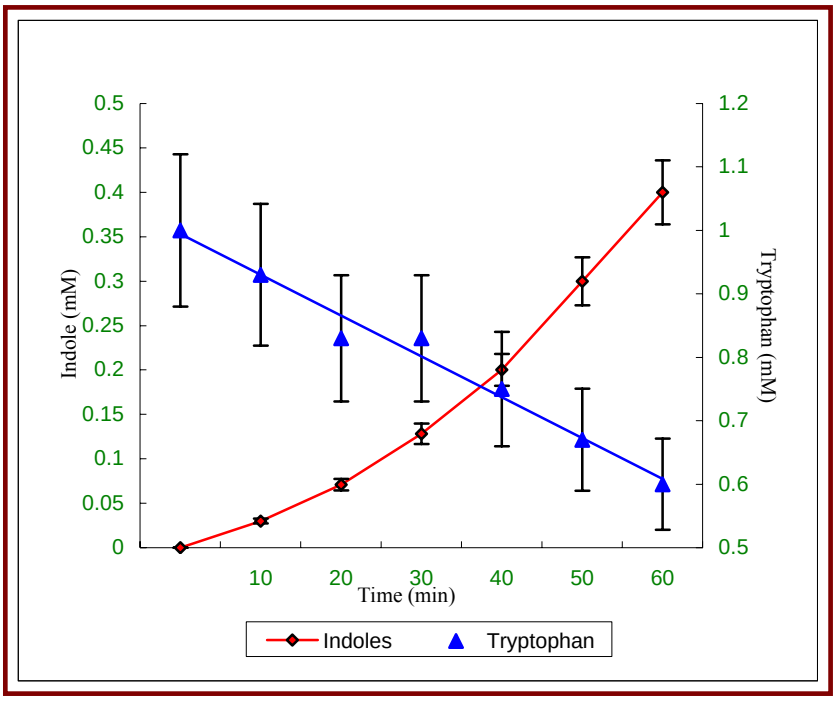


Fig. 30. Consumption of L-tryptophan and production of indoles using cell free extract of *Rhodobacter sphaeroides* OU5.

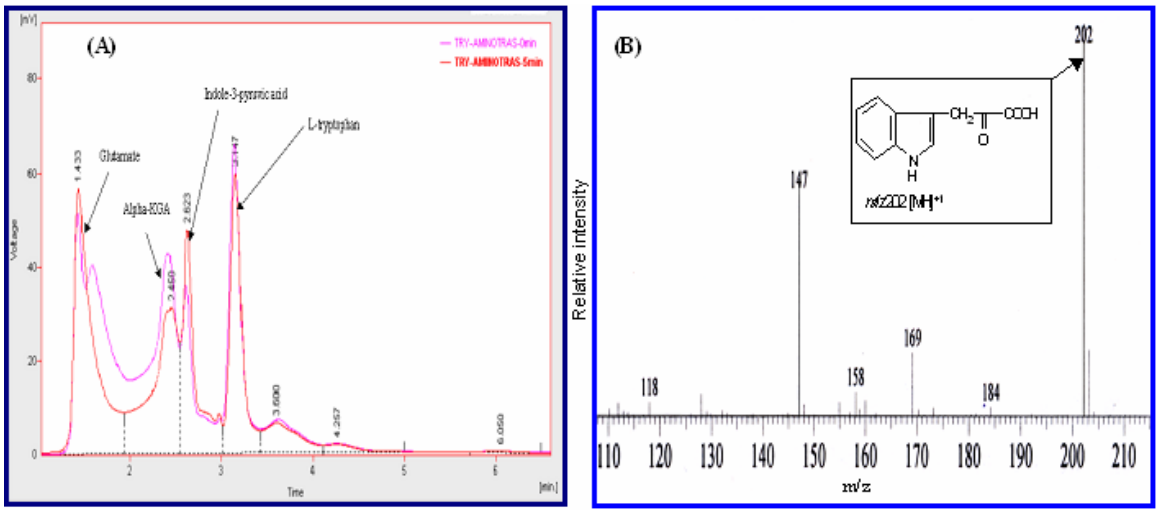


Fig. 31. (A) Tryptophan aminotransferase activity using HPLC, (B) product confirmation with LC-MS analysis.

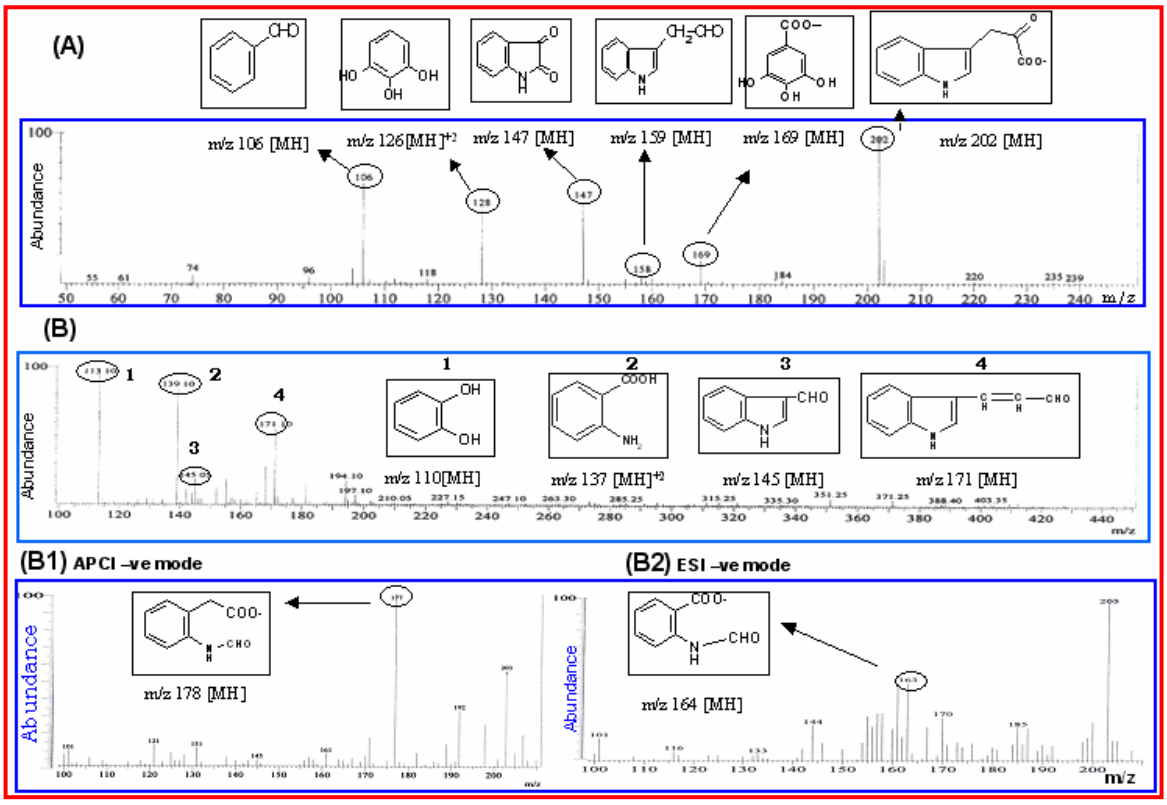


Fig. 32. LC-MS profiling of the cell free extract assay supernatant performed in the presence (A) or absence (B; B1; B2) of 2-oxoglutarate. Encircled molecular masses are those of identified metabolites.

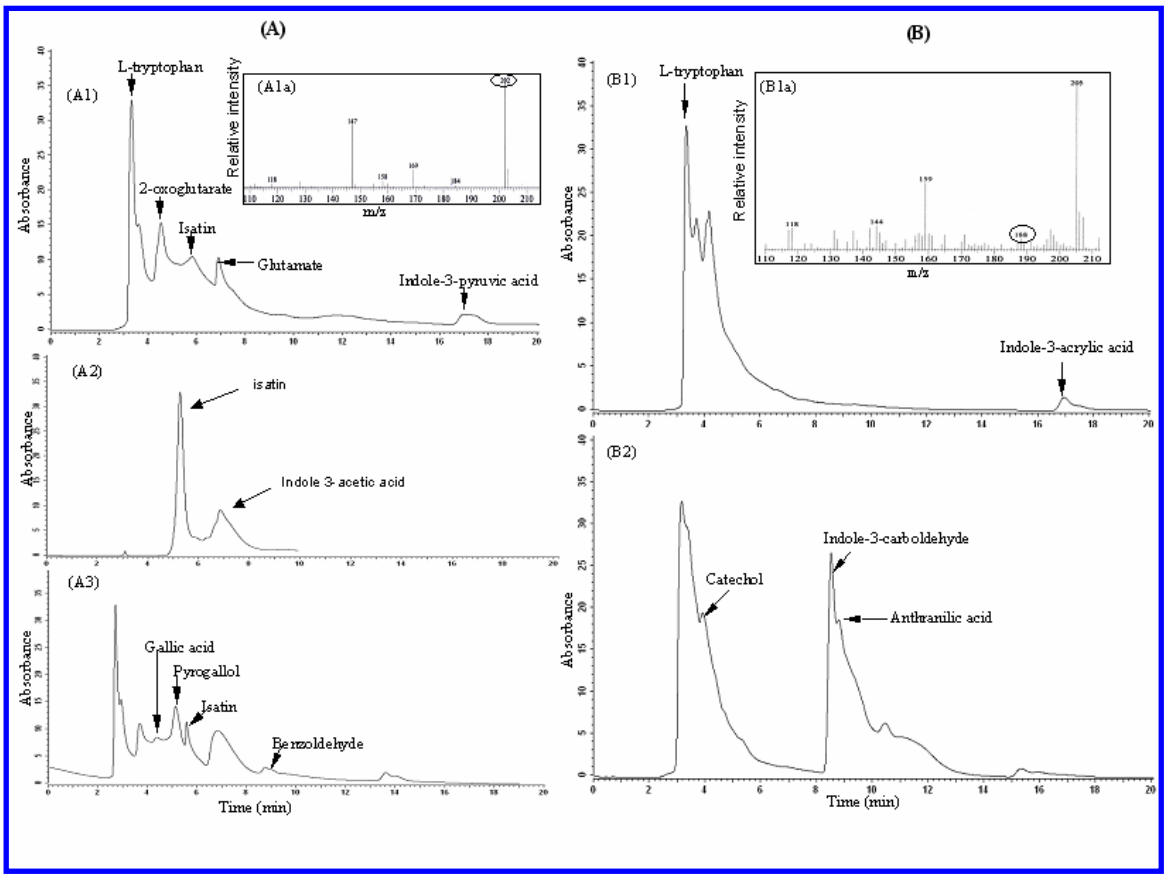


Fig. 33. HPLC chromatograms showing the metabolites of L-tryptophan catabolism by cell free extracts of *Rhodobacter sphaeroides* OU5.

A = Assay performed in the presence of 2-oxoglutarate; where L-tryptophan (A1), indole 3-acetic acid (A2) and isatin (A3) were used as precursors. A1a = LC-MS spectrum confirming indole 3-pyruvic acid (m/z 202).

B = Assay performed in the absence of 2-oxoglutarate; where L-tryptophan (B1) and indole 3-acrylic acid were used as precursors. B1a = LC-MS spectrum confirming indole 3-acrylic acid (m/z 187).

3.2.4 ***Enzyme purification***

Tryptophan ammonia lyase was purified from *Rhodobacter sphaeroides* OU5, when tryptophan was used as nitrogen source. From cell free extract to apparent homogeneity by using the following steps (Flow chart 3).

3.2.4.1 ***Identification of L-tryptophan ammonia lyase in Rhodobacter sphaeroides OU5***

L-Tryptophan catabolism in presence of 2-oxoglutarate yielded indole-3-pyruvic acid was confirmed the enzyme tryptophan aminotrasferase. In absence of 2-oxoglutarate the enzyme tryptophanase was not observed with cell free extract. There is no mass of 117 (m/z) was observed in the LC-MS profiling. It was observed that indole-3-acrylic acid and NH_3 formation from L-tryptophan was observed from the Sephadex G-150 gel eluted fractions 11 to 21 presence and absence of pyredoxyl-5-phosphate (PLP) there was no activity of tryptophanase was observed. It was observed In-gel assay activity when provided substrate with in the gel and stained with PADAB reagent. The product (indolic compound) was shows pink rose color band (Fig. 34).

3.2.4.2 ***Purification and characterization of L-tryptophan ammonia lyase (WAL)***

L-Tryptophan ammonia lyase was partially purified through gradient ammonium sulphate saturation and the highest activity was achieved with 30% saturation, which was further purified by dialysis and Sephadex G-150 gel filtration chromatography (see materials and methods) (Fig. 35A). These pooled fractions were subjected to anion-exchange chromatography (Fig. 35B). Fraction 16 and 17 of 1.5 M NaCl elution showed the pure protein of tryptophan ammonia lyase, molecular mass was determined by non-denaturing-PAGE (6%) was found to be ~233 kD (Fig. 36A). and subunits determined by SDS-PAGE (12%) indicated the enzyme WAL to be a hetero tetramer, 98, 55, 45,35 kD,

respectively (Fig. 36B). The purity of the tryptophan ammonia lyase enzyme was confirmed using a HPLC (R_t = 2.90), with RP- C_{18} column (150 x 4 mm) and eluted with acetonitril in 0.3% TFA at 1ml/min^{-1} flow rate (Fig. 35C).

3.2.4.2.1 ***pH and T ^oC effect***

The pH optimum of the tryptophan ammonia lyase was done using 0.05 M potassium phosphate buffer (pH 4.0 to 9.0) the enzyme exhibited activity at pH 6 to 8.0, with maximum (optimum) activity detected at pH 7.0, and temperature optimum at 40 ºC (range 30-60 ºC) (Table. 7).

3.2.4.2.2 ***Substrate specificity***

The substrate specificity of the purified WAL measured with L-tryptophan and various aromatic, aliphatic amino acids (Table. 7). In addition to the 100% specificity to the activity with L-tryptophan affinity for WAL, it also exhibited activity with L-phenylalanine (66 %), L-tyrosine (27 %), L-DOPA (19%), L-histidine (5%) glutamate (61%) glycine (27 %), and DL-alanine (26 %).

3.2.4.2.3 ***Kinetic constants***

The K_m (µM) and V_{max} of the enzyme WAL was determined the product ammonia formation with various concentrations of L-tryptophan showed a non-linear increase in the activity up to 60 and 100 µM the kinetic constant for tryptophan ammonia lyase are summarized in Table. 7. The K_m (µM) 40.4 $\pm$ 23.1 and V_{max} 0.9646 $\pm$ 0.2046.

3.2.4.2.4 ***Molecular weight determination***

The molecular weight of native WAL was determined by gel filtration (Sephadex-G-150) chromatography, the molecular mass was calculated using standard proteins catalase (240 kDa), esterase (166 kDa), BSA (67 kDa), Ovalbimine (43 kDa) and trypsinogen (24 kDa)

the void volume of the column was determined using Blue Dextran and calculated log Mwt. According their fraction eluted with standard proteins used as marker and confirmed is ~ 238 kDa (Fig. 35D).

3.2.4.2.5 ***N-terminal sequencing***

The N-terminal amino acid sequence WAL was determined to be **M, E, D, L, V** by automated Edman degradation. A BLAST search performed with the N-terminal sequence did not match with the N-terminal sequence of proteins in the (NCBI) database. Except for a probable histidine ammonia lyase in *Rhodobacter sphaeroides* 2.4.1

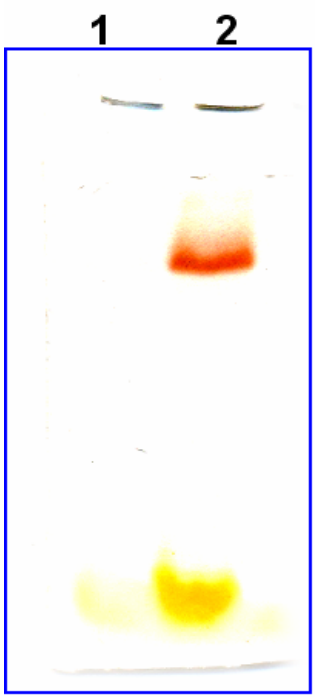
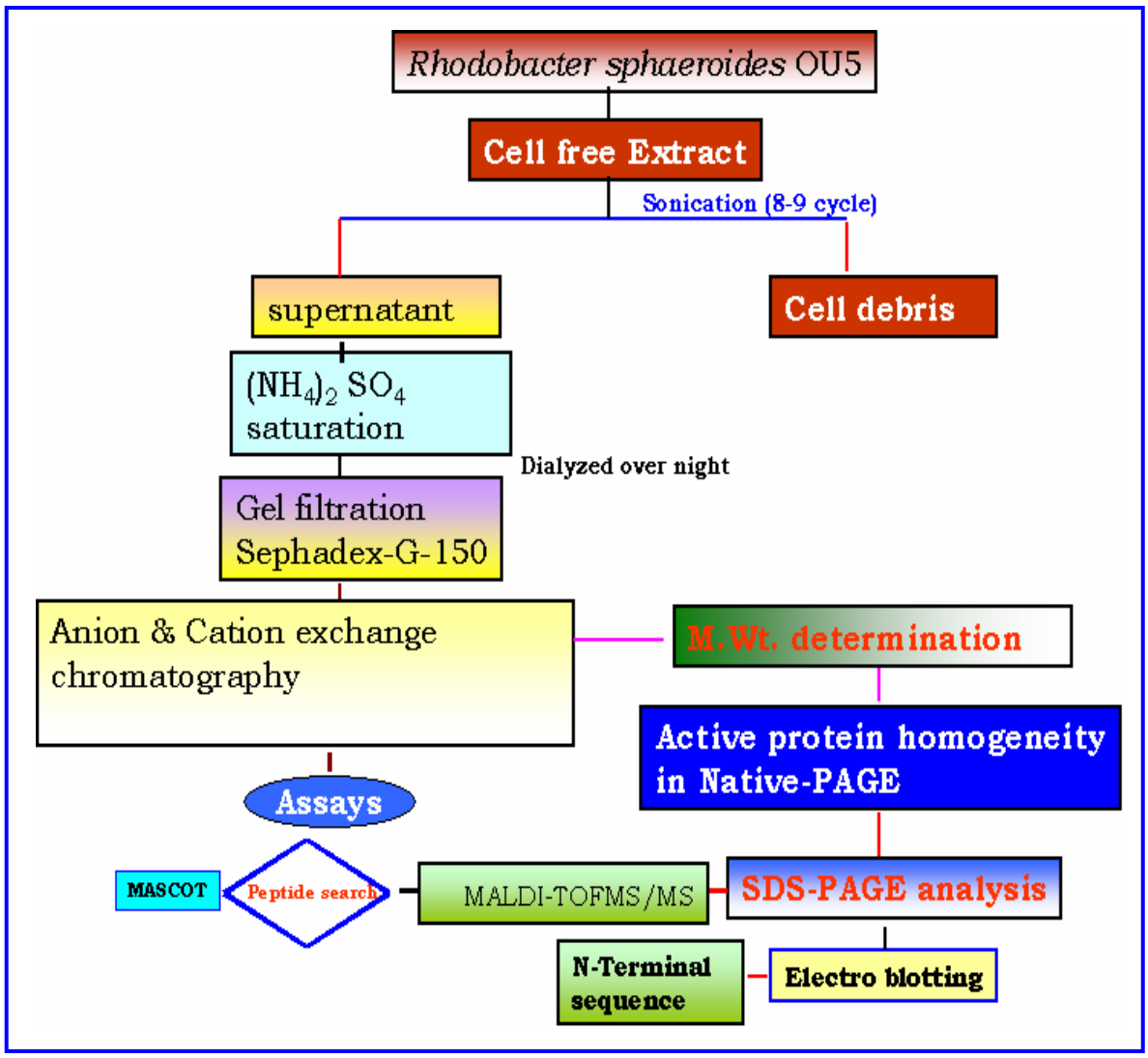


Fig. 34. Native ingel assay: treated with PDAB reagent. (Lane- 1: control, Lane- 2: tryptophan induced)



Flowchart 3. Schematic diagram of isolation and purification of enzymes involved in L-tryptophan catabolism.

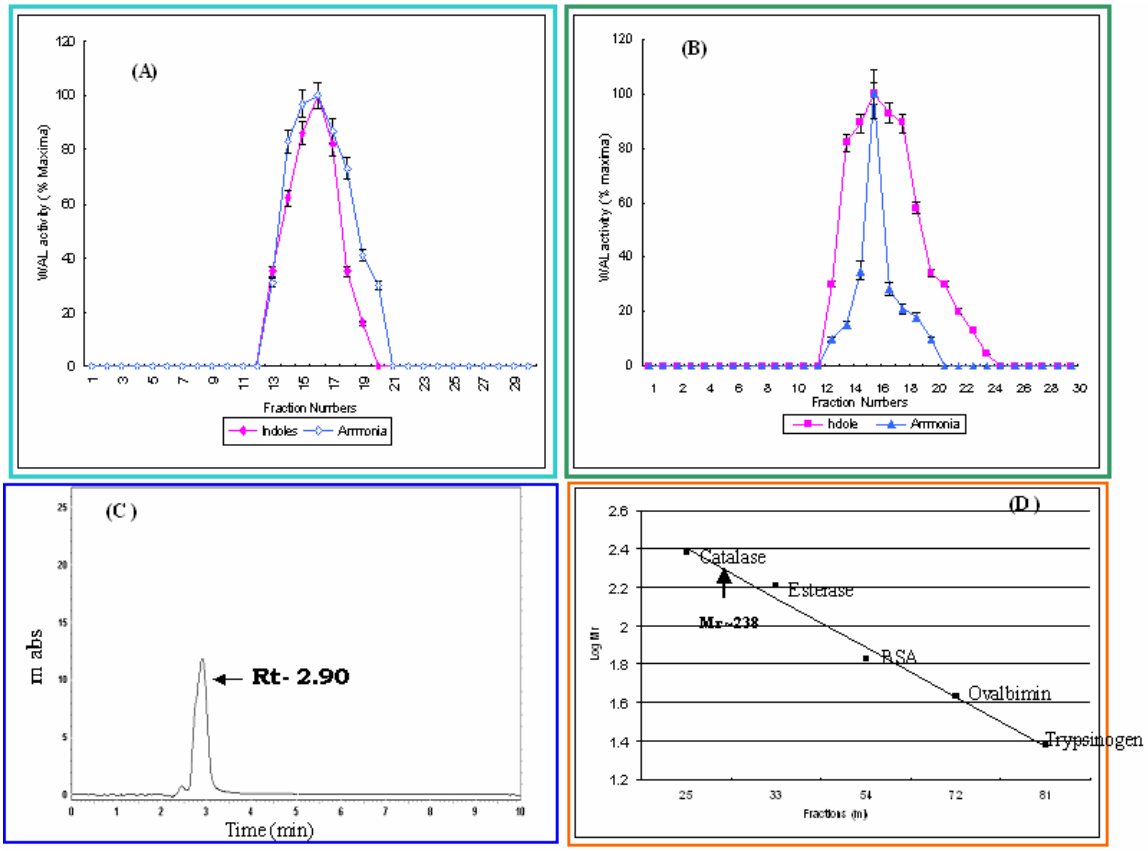


Fig. 35. (A) Elution profiling of tryptophan ammonia lyase activity from Sephadex G-150 gel filtration chromatography and anion exchange column using DEAE-Cellulose at gradient concentration of Nacl, (shown at 1.5 M) (B), Purity was determined by using HPLC eluted with acetonitril in 0.3% TFA at 1 ml.min^{-1} flow rate. (C), Molecular weight was determined by using Gel filtration (Sephadex G-150) column chromatography (D).

3.2.4.2.6 ***Product confirmation of tryptophan ammonia lyase***

L-Tryptophan ammonia lyase enzyme product was identified using LC-MS by their corresponding molecular masses as indole-3-acrylic acid or indole-3-acrylaldehyde (Fig. 38A, A1), and the same was confirmed by under the experimental HPLC conditions (L-tyrptophan Rt = 2.72, product is indole-3-acrylic acid R_t = 16.76, respectively) (Fig.38B, B1) indicating the presence of an active tryptophan ammonia lyase in *Rhodobacter sphaeroides* OU5. Activity of tryptophan ammonia lyase was shown in Figure. 37 with different time intervals.

3.2.4.2.7 ***Identification of WAL using MALDI-TOF Spectrometry***

The protein band 89,55,45 and 35 kDa were selectively removed from the gel slice subjected to MALDI-TOF-TOF as described in materials and methods. The protein was identified using public domain MASCOT (http://www.matrixscience.com) search by incorporating the standard parameters with three different databases were used (SwissPort, NCBI and MSDB). There was no significant score was observed with 89, 55, 45 and 35 molecular weight proteins respectively (Fig. 39A; 40A; 41A) it was found with 55 kDa protein about 54% score with that of hypothetical protein in *Rhodobacter sphaeroides* 2.4.1 (Table. 8) it could be the enzyme a probable protein as tryptophan ammonia lyase (Fig. 40A). Further each subunits major peaks of peptide mass from 89kDa, 882 and 786 peptides (Fig. 39A1, A2); from 55 kDa, 996 and 882 peptides (Fig. 40A1,A2); from 45 kDa, 606 peptide (Fig. 41A1) and from 35 kDa, 1386 and 786 peptides (Fig. 42A1; A2) were done by MS-MS even these peptides was also failed significant match found in database. Same of the probable enzymes with very low score and *de-novo* sequences are shown in Table. 9.

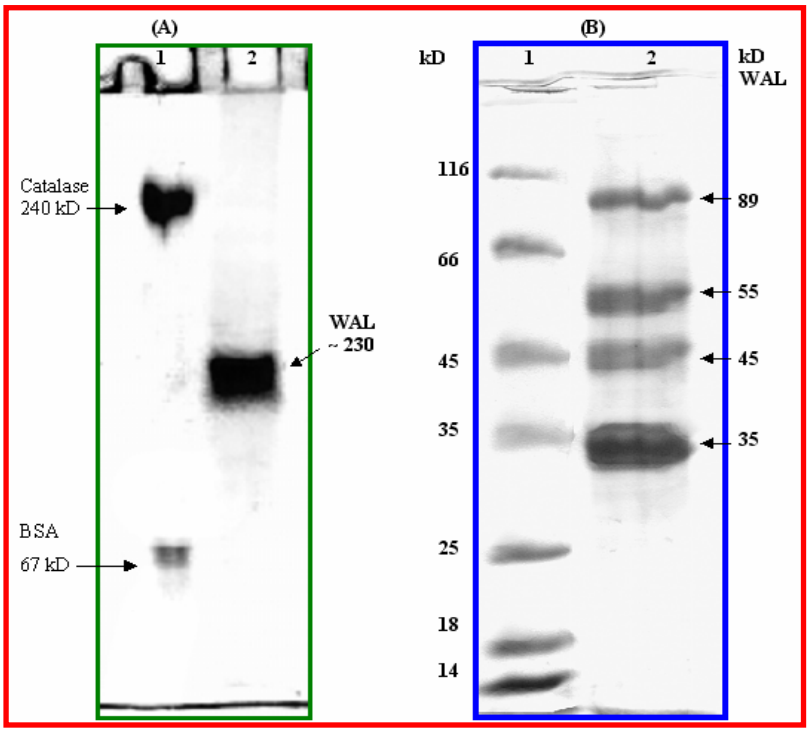


Fig. 36. (A) Native- PAGE (6%) of the purified Tryptophan ammonia lyase (Lane-1 Native Molecular marker 15 μg of Catalase and 8 μg of BSA and Lane-2 WAL). (B) SDS-PAGE (12%) analysis of Tryptophan ammonia lyase (WAL) Lane-1 standard molecular markers (116, 66,45,35,25,18,14 kDa) Lane-2. 65 μg of purified WAL (subunits 98,55,45,35kDa) in the presence of β-mercaptoethanol. The gel was stained using modified Coomassie blue R-250 (Wang *et al.*, 2007)

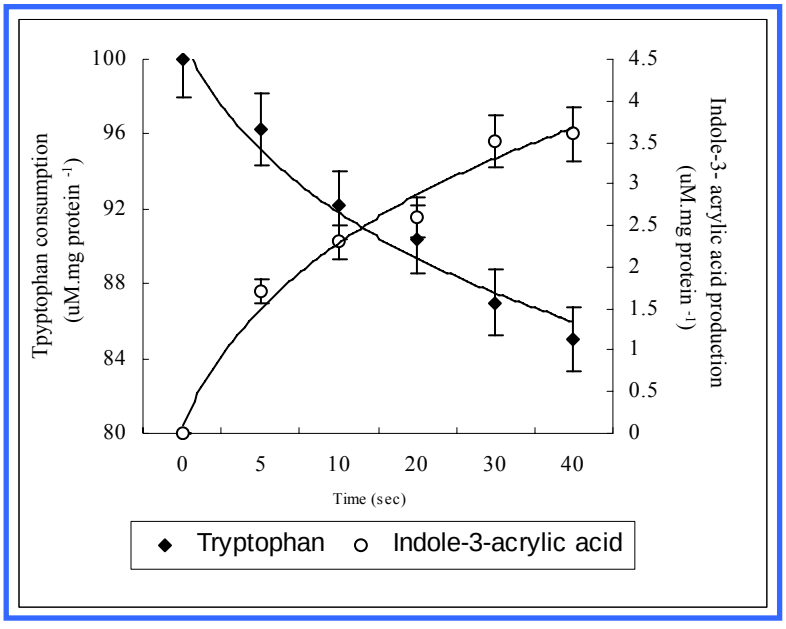


Fig. 37. Time course activity of L-tryptophan ammonia lyase of *Rhodobacter sphaeroides* OU5.

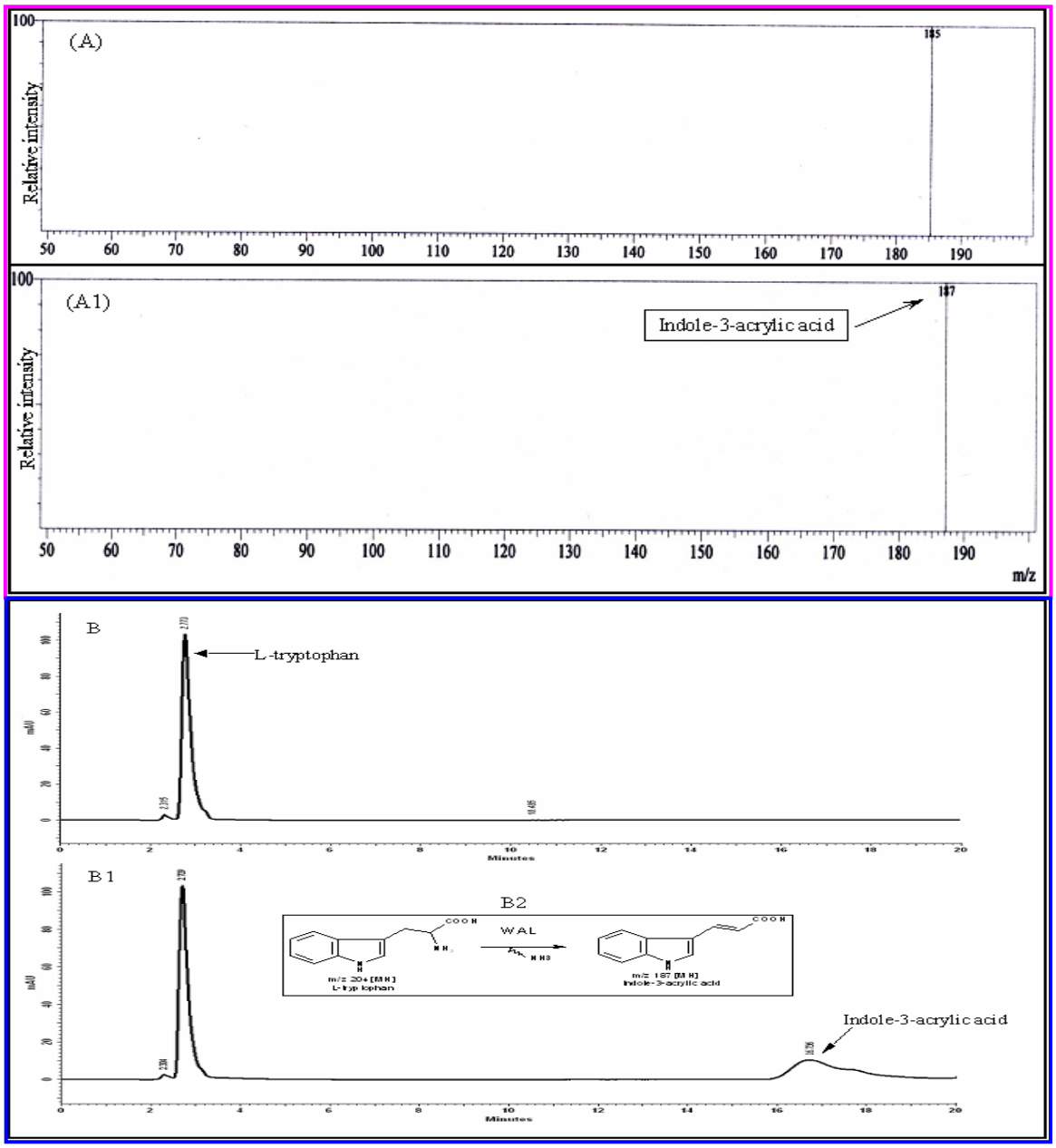


Fig. 38. LC-MS analysis using SIM mode both positive and negative modes the masses generated and the exact mass was 187 m/z. A) Negative mode; A1) Positive mode HPLC analysis of purified L-tryptophan ammonia lyase enzyme assayed supernatant; the product was confirmed indole-3-acrylic acid from substrate L-tryptophan. (B= Denatured enzyme assay (initial 0 min); B1) Final enzyme assay (5 min) and B2= enzyme biochemical reaction.

Parameters	Tryptophan ammonia lyase (WAL)
Substrate specificity (%)	: Tryptophan-100, Tyrosine-27, Phenylalanine-66, Glutamate-61, Glycine-27, DOPA-26, Histidine-5 and DL-alanine-20
Product	: Indole-3-acrylic acid (IAcryA)
Co-substrate	: Nil
Co-factor	: Nil
Optimal condition (range)	
PH	: 7.5 (6-8)
T ^{o}C	: 40 (30-60)
K_m (μM)	: 40.4±23.1
V_{max} (S^{-1})	: 0.964±0.2046
Molecular weight (Determined by gel filtration Sephadex G-100)	: ~225kDa
Subunits (Determined by SDS-PAGE)	: 89,55,45,35kDa (Could be a heterotetramer)
N-terminal sequence (partial)	: M, E, D, L, V..

(Results are means±SD of three different determinations)

Table. 7. Characteristics of purified L-tryptophan ammonia lyase (WAL) from *Rhodobacter sphaeroides* OU5.

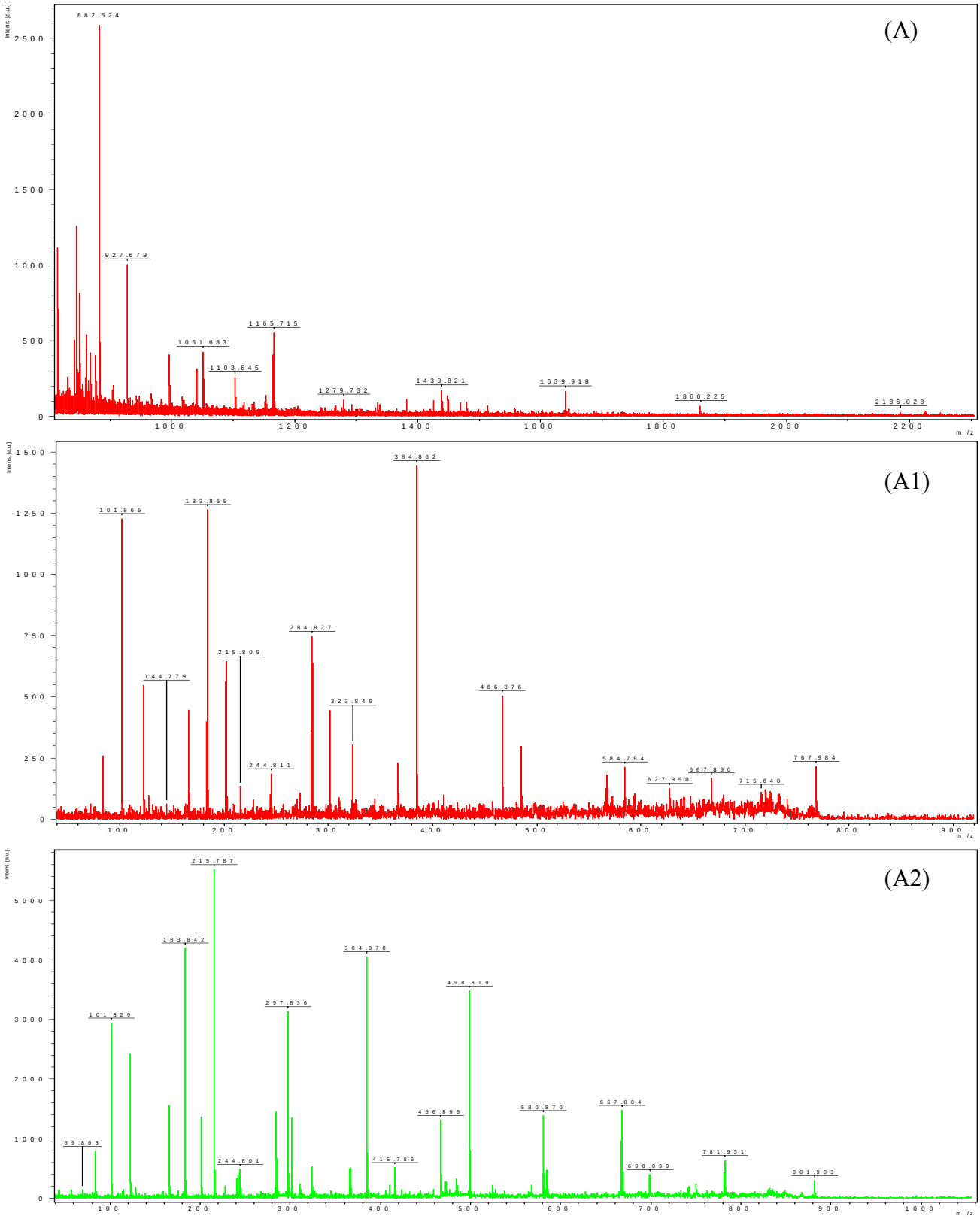


Fig. 39. MALDI-TOF-TOF (A) peptide fingerprinting, the subunit mass of the digested protein was 89 kDa; (A1) MS-MS analysis of 882 peptide and (A2) 786 peptide.

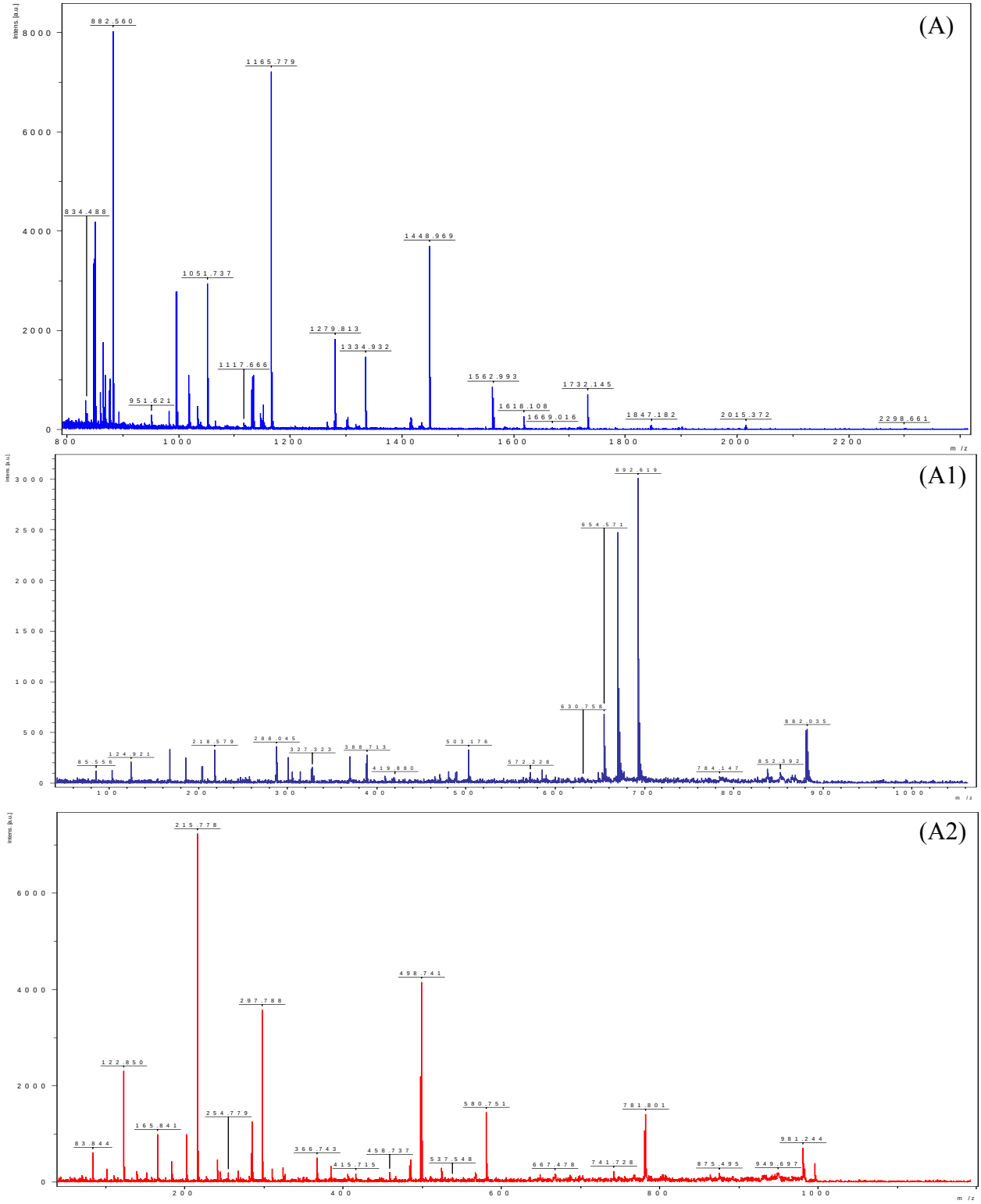


Fig. 40. MALDI-TOF-TOF (A) peptide fingerprinting, the subunit mass of the digested protein was 55 kDa; (A1) MS-MS analysis of 882 peptide and (A2) 891peptide.

Hypothetical protein - *Rhodobacter sphaeroides* (strain ATCC 17023 / 2.4.1 / NCIB 8253 / DSM 158).

MEGALNLSGA DRRKLERAMS ELDELVGTPP VETTHAEFVA SAACLPTLDA
LPPGEADLLA DLLAELGPTT PEDLVEAEAL NREIASMTPA EVAENYGTDA
DVRNAAAGAW LEQHQAERLE RFRKSGRNFY AEDKLKTTGQ PVRPYRRDLK
NLTPEERKAH RRAQKAKSKA NRSPEQVERE RAANKAHQQK RRGRERSATA
AAERANLALF

Mass Mr	Range	Protein sequence
*1264.5717	1-12	0 MEGALNLSGADR Dioxidation (M)
*1548.7678	1-14	2 MEGALNLSGADRRK Dioxidation(M)
*1783.9652	2-17	3 EGALNLSGADRRKLER
*847-5026	119-124	2 LERFRK
*1147.6574	119-127	3 LERFRKSGR
*2015.0337	119-134	4 LERFRKSGRNFYAEDK
*749.4296	122-127	2 FRKSGR
*1616.8059	122-134	3 FRKSGRNFYAEDK
*666.4037	158-162	2 KAHRR
*865.4994	159-165	2 AHRRAQK
*1279.7585	159-169	4 AHRRAQKAKSK
*700.4344	162-167	2 RAQKAR
*544.3333	163-167	1 AQKAK
*1598.8488	166-179	3 AKSKANRSPEQVER
*1435.7756	180-191	3 ERAANKAHQQKR
*1150.6319	182-191	2 AANKAHQQKR
*1585-8509	191-204	4 RRGRERSATAAAER
*672.3779	192-196	2 RGRER
*1429.7498	192-204	3 RGRERSATAAAER
*1903.0023	193-210	3 GRERSATAAAERANLALF

Table. 8 Tryptophan ammonia lyase (WAL) amino acid sequence (55 kDa subunit) based on peptide mass fingerprinting, nearest match found was of hypothetical protein *Rhodobacter sphaeroides* (strain ATCC 17023 / 2.4.1 / NCIB 8253 / DSM 158) and amino acid *de novo* sequence (red color) along with their masses are given above.

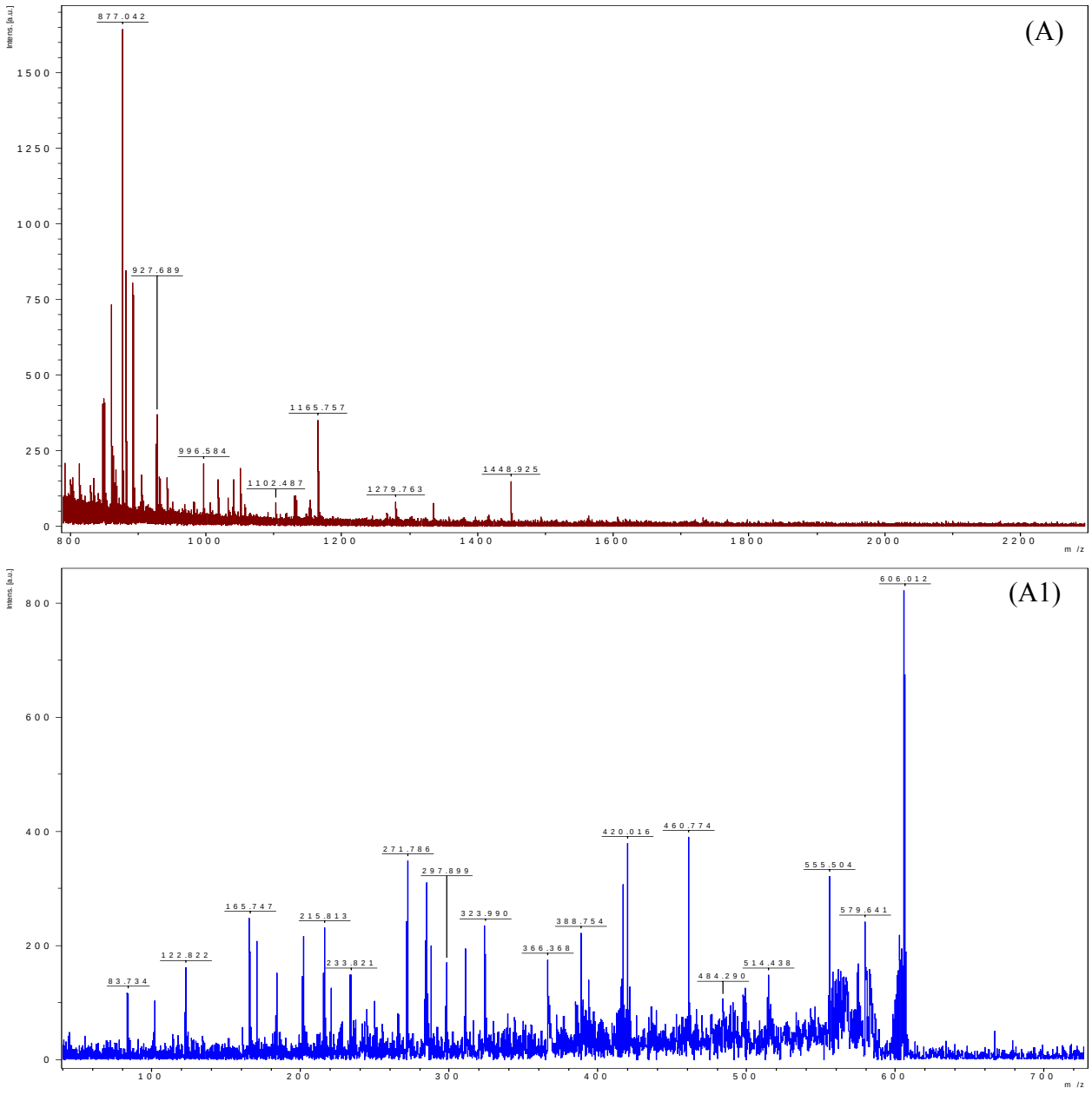


Fig. 41. MALDI-TOF-TOF (A) peptide fingerprinting, the subunit mass of the digested protein was 45kDa and (A1) MS-MS analysis of 606 peptide.

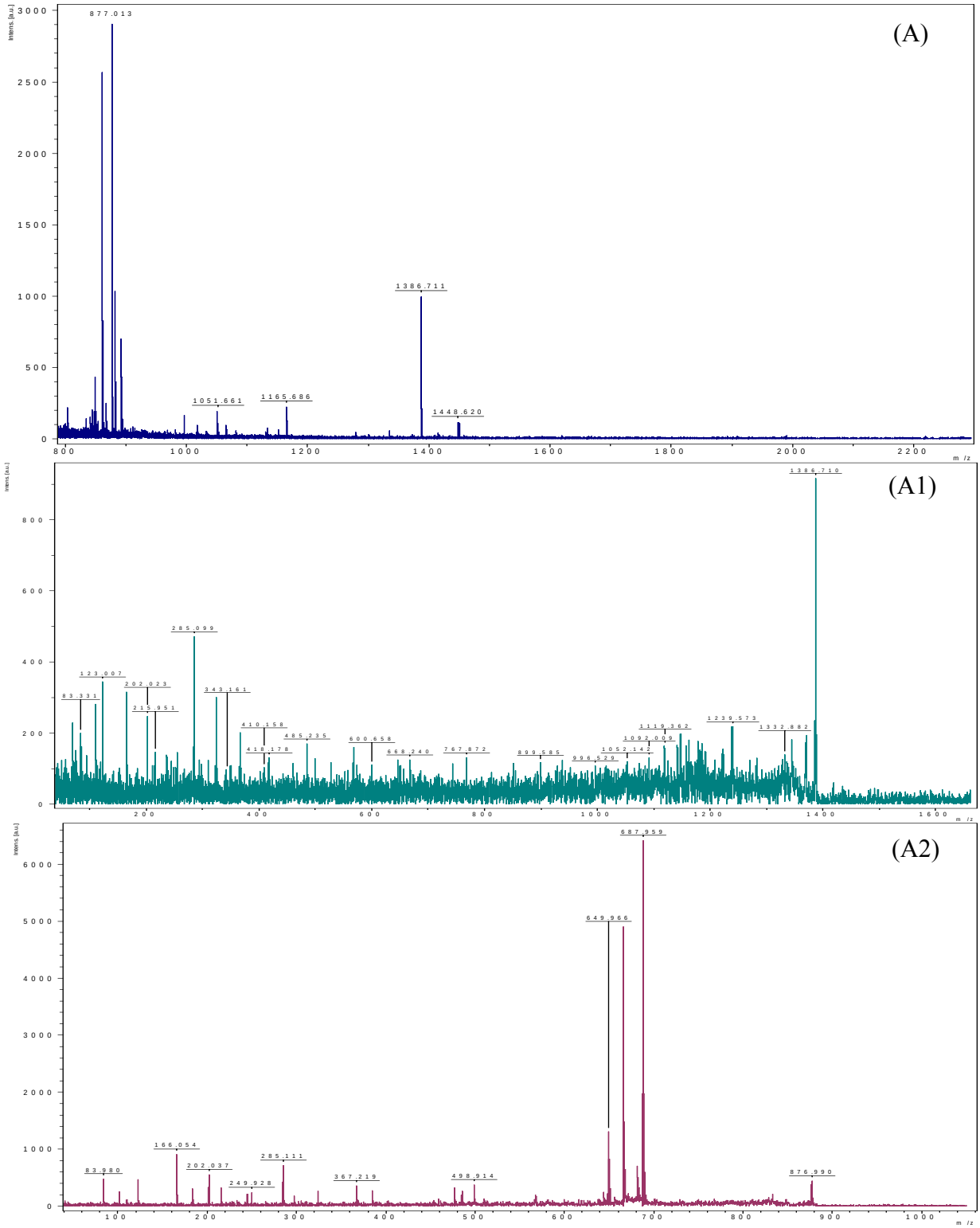


Fig. 42. MALDI-TOF-TOF (A) peptide fingerprinting, the subunit mass of the digested protein was 35kDa; (A1) MS-MS analysis of 1385 peptide and (A2) 876 peptide.

WAL Sub unit s (kDa)	Peptides Mass MS/MS	Probable protein	Sequence Coverage (%)	Database Accession No.	Amino acid sequences	Organism
89	882	D-alanine-lyase (EC6.3.2.4)	30	Q3EGD9ACTSC	QQKIAVLLGGTASER	*Bacillus clausii* KSM-K16
	786	Putative Gid B protein	20	Q3IYH5RHOS4	AGANAVLIGEQLMRK	*Rhodobacter sphaeroides* ATCC17023/2.4.1
55	981	Phenylacetaldoxime dehydratae	25	gi/170724581	LSPSLEGPMMEHGYSGAAR	*Shewanella woodyi* ATCC 51908
45	606	Putative 3,4-dihydroxyphenylacetate 2,3-dioxygenase	12	gi/39934733	VALLASGSLSHR	*Rhodopseudomonas palustris* CGA009
35	1386	30S Ribosomal protein S15	21	RS15 RHOSI	EGDTGSPEVQVAVLSSRIATLTEHFK	*Rhodobacter sphaeroides 2.4.9*
	786	Tryptophan repressor binding protein	10	gi/15837696	QIARGIAEVPGMAAR	*Xylella fastidiosa* 9a5c

Table. 9 Amino acids (*de-novo*) sequences found during MALDI-TOF MS/MS analysis of peptides having parent mass 882,786,981,606, 1386 and 786 along with probable protein and sequence coverage.

3.2.5 *Chemotrophic metabolism of L-tryptophan by Rhodobacter sphaeroides OU5*

3.2.5.1 *Growth and simultaneous utilization of L- tryptophan*

Rhodobacter sphaeroides OU5 could grow on L-tryptophan as sole source of nitrogen (malate served as carbon/e^- donor) under chemotrophic anaerobic (dark) conditions (Fig. 43) the organism had a doubling time of about 22 h (compared to 18 h on ammonium chloride). Indoles production was observed simultaneously during growth and the supernatant turned light pinkish color with ehrlich reagent.

3.2.5.2 *LC-MS identification of indole derivatives*

Indoles were extracted into ethyl acetate and analyzed using LC-MS. In total, 5 indole derivatives were identified based on their molecular masses includes, indole-3- acetic acid (IAA) (m/z 175), indoprofen (m/z 281), indobufen (m/z 295), ibogain (m/z 310) and gliotoxine (m/z 325) (Fig. 44).

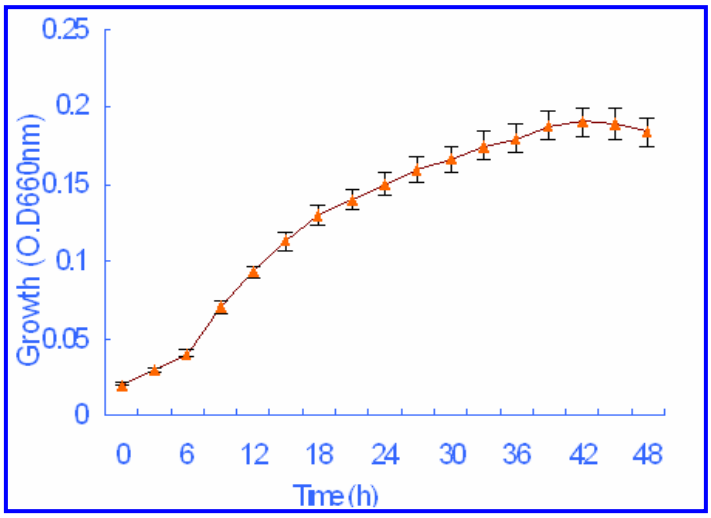


Fig. 43. Chemotrophic growth curve on L-tryptophan by *Rhodobacter sphaeroides* OU5.

L-tryptophan (1 mM) was used as sole source of nitrogen in the presence of malate (22 mM) as carbon source and incubated under chemotrophic (micro aerobic) conditions at 30 ± 2 °C.

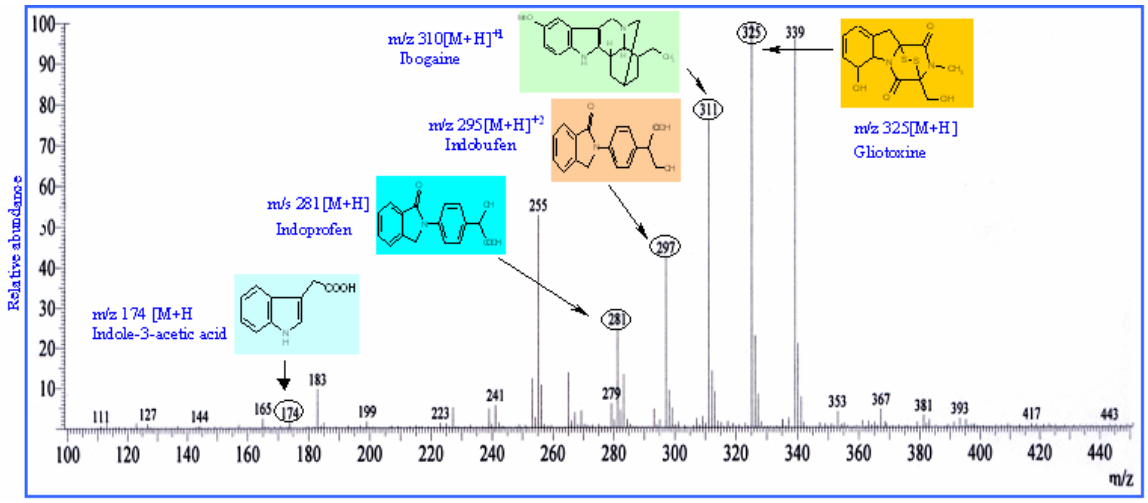


Fig. 44. LC-MS chromatogram of the ethyl acetate extracted culture supernatant after 48 h. Encircled molecular masses are those of identified metabolites.

3.3 Bioprospecting

3.3.1 **Rhodethrin**

Indole and its derivative are known to have phytoharmonal activity such as IAA and IPA are natural auxins. The esters of indole-3- acetic acid, indole myo-inositol and rhodestrin (Sunayana *et al.,* 2005b) since many indole esters are potential bioactive molecules (Olgen and Nebioglu, 2002) having cytotoxicity, anti-HIV, Cox-1 and 2 inhibitory activity and antimicrobial activity. Hence the metabolites of aromatic amino acids (L-tryptophan and L-phenylalanine or L-tyrosine) transformation product, which are also the indole and phenol derivatives, were screened for cytotoxicity, COX 1 and 2 inhibitory activities, and phytoharmonal as well as anti microbial activity.

3.3.1.2 ***Phytohormonal activity***

Rhodethrin gave a positive coleoptile test in an auxin bioassay. It promoted root initiation of mulberry (*Mouras alba*) in tissue culture. The compound induced rooting (Fig. 45) at 0.5 µM in comparison to indole-3-acetic acid (IAA) which induced at 5 µM. Further, root initiation was observed with in 8-10 days in rhodethrin treated plants, while with IAA it was observed only after 12-15 days. Rhodethrin treated plants showed increase in root length and thus differ from rhodestrin, where profuse rooting was observed.

3.3.1.3 ***Cell viability treated with crude extract and rhodethrin***

The percentage of inhibition of cell viability was computed with reference to the MTT reduction in control cells with out test compounds. While treated with different concentration of rhodethrin (10 pM, 50 nM and 100 µM) and crude extract. It was taken as percentage inhibition of various concentration of compound required for 50%

inhibition of cell viability (IC_{50}) was about 45 nM. Where as with crude extract almost all completely inhibition was observed (Fig. 46)

3.3.1.4 ***Cytotoxicity test***

The cytotoxicity of rhodethrin against Sup-T_1 (T cell lymphoma) and Colo-125 (Fig. 47) cancer cell lines was determined using the 3-(4,5-dimethylthylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Mosman, 1983). Both crude extract (fraction-A) and rhodethrin had maximum cytotoxicity on the cancer cell lines even at low concentrations tested (10 nM) (Fig. 47).

3.3.1.5 ***Morphological changes in Jurkat cells when induced by Rhodethrin***

Jurkat cells treated with various concentration of (10 µM, 10 nM and 20 pi M) or with out rhodethrin for 24 h were viewed under phase contrast microscope. Rhodethrin treated cells were shows morphological feature pertaining could be apoptotic cell death was clearly observed even 20 pM (Fig. 48)

3.3.1.6 ***Cyclooxygenase-2 inhibitory activity***

Rhodethrin (50 µM) inhibited COX-2 activity by 45%, while the crude extract (fraction A) by >60%. On the other hand, neither the crude extract nor rhodethrin inhibited COX-1 activity (Fig. 49).

Fig. 45. Phytoharmonal activity: Rooting in tissue culture plants.

Plants No: 1. Control; 2. IAA (5 µM); 3. Rhodethrin = 0. 5 µM; 4. Crude extract (1.0 mM) Photo showing the phytohormonal activity of the above compounds in mulberry plants cultured in MS medium pH 5.8.

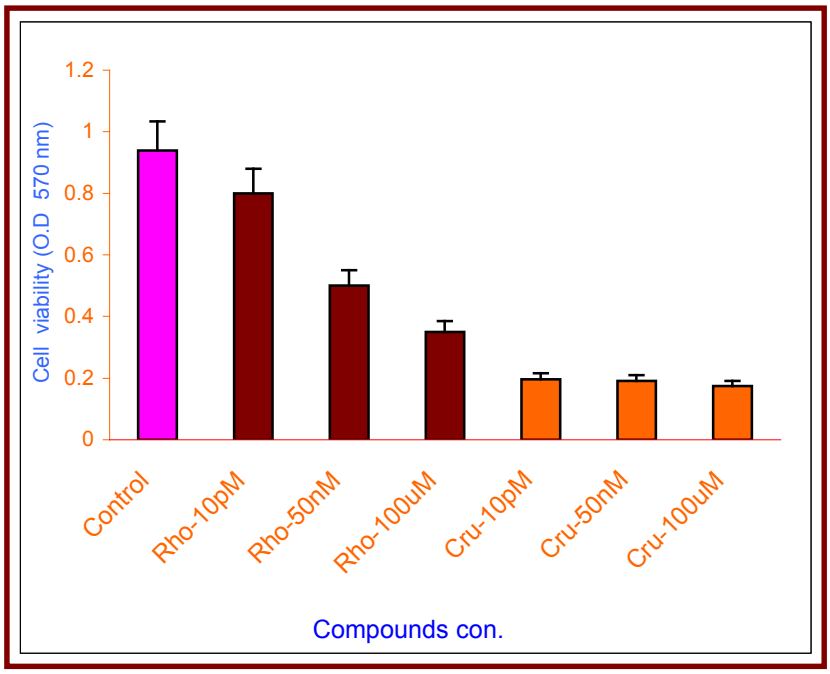


Fig. 46. MTT-assay for cell viability determination by using Rhodethrin.

(Control, Rho = Rhodethrin, Cru = Crude extract)

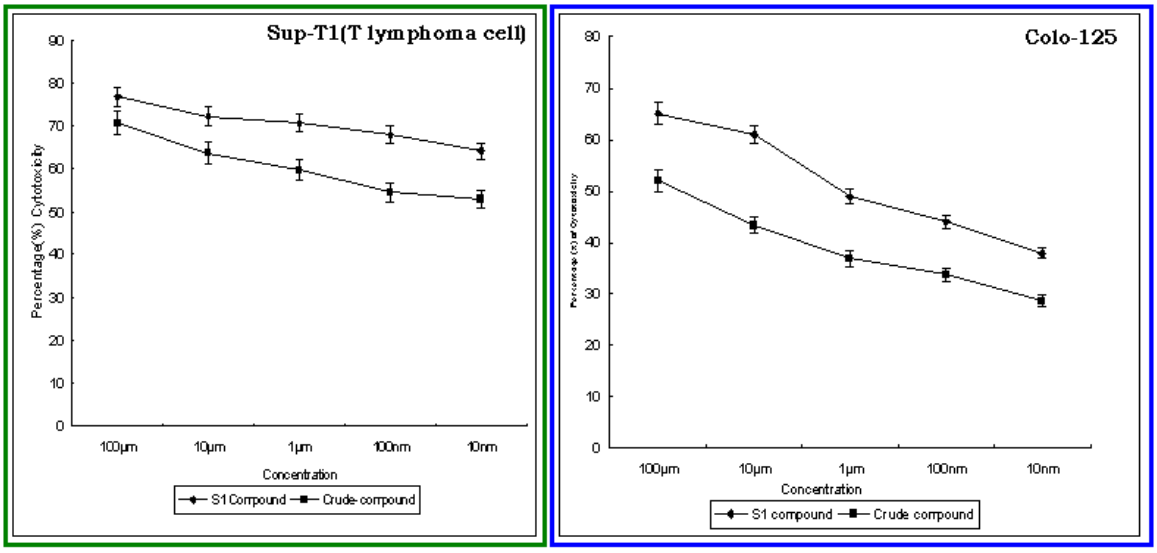


Fig. 47. Cytotoxicity of rhodethrin against Sup-T_1 and Colo-125 cancer cell line as determined by MTT method.

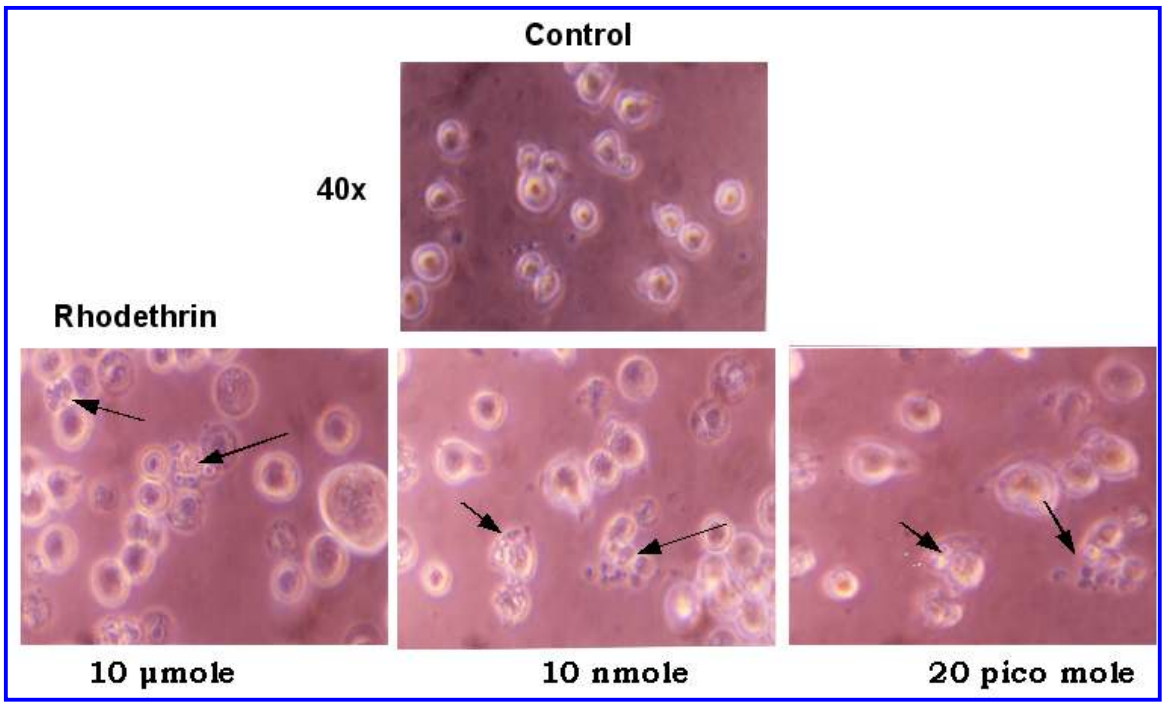


Fig. 48. Rhodethrin treated Jurkat cells showing cell death with various concentrations.

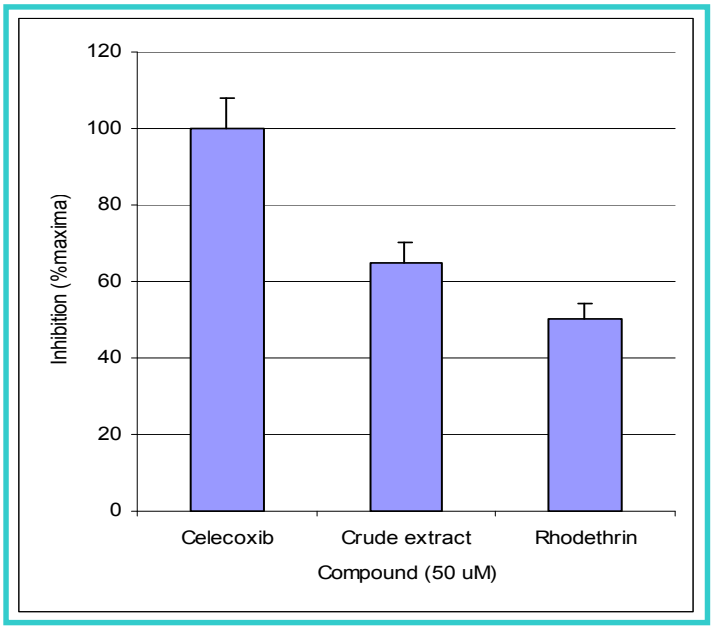


Fig. 49. Cox-2 inhibitory activity was observed with the crude and purified metabolites.

3.3.2 Rhodophestrol

3.3.2.1. ***Cytotoxicity against U937 Human leukemic monocyte lymphoma cells***

The cytotoxicity of rhodophestrol against U937 (Human leukemic monocyte lymphoma cell line) cancer cell line was determined using the 3-(4,5-dimethylthylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Mosman, 1983). Rhodophestrol had cytotoxicity (apoptosis bodies formation) on the cancer cell line even at low concentrations tested (50 nM) (Fig. 50A) compared with control (Fig. 50B)

3.3.2.2 ***Cyclooxygenase inhibitory activity***

Rhodophestrol had COX-I (~50%, Fig.51) and COX-II (~20%) inhibitory activity, as observed with rhodethrin.

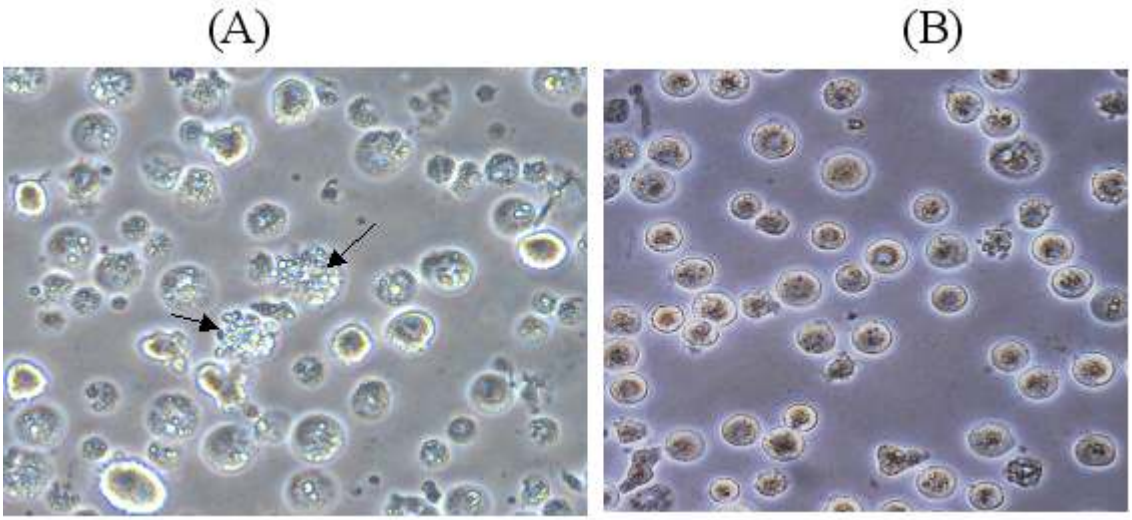


Fig. 50. Photograph showing the cytotoxicity (apoptosis) of rhodophestrol against U937 cancer cell line as determined by MTT method (Mosman, 1983).
(A) Treated cells and (B) control (untreated cell lines) Briefly, adherent cancer cell lines (U937) at the concentration of $0.2x10^6$ cells.ml^{-1} was seeded in 96-well microplates. The adherent cells were incubated for 18h to allow cell attachment. Rhodophestrol solution was added to the cell cultures at concentrations of 50µM and the cells were incubated for 24h. The MTT solution was added 3 h before the end of the incubation. Cell survival was evaluated with a multiwell scanning spectrophotometer at 570 nm.

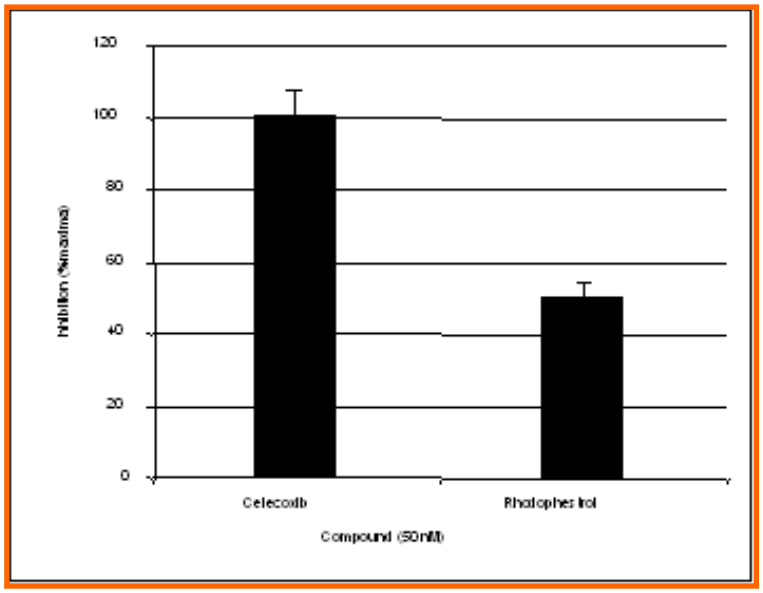


Fig. 51. Cox-1 inhibitory activity was observed with celecoxib and purified metabolite Rhodophestrol (50 nM).

4. DISCUSSION

4. Discussion

Few purple-non sulfur bacteria (PNSB) have the capability to degrade a wide range of low molecular weight aromatic hydrocarbons for growth (Gibson and Hard wood, 1994; Sasikala and Ramana, 1998; Harwood *et al.,* 1999) and thus help in the maintenance of bio-geochemical cycles. The most extensively studied species among PNSB is *Rhodopseudomonas palustris*, whose total genome analysis indicated the existence of at least five aromatic ring cleavage pathways, representing more aerobic (oxidative) mechanisms, than anaerobic (reductive; Larimer *et al.,* 2004). The other species of PNSB capable of utilizing benzoate for growth include; *Phaeospirillum fulvum* (Pfennig *et al.,* 1965), *Rubrivivax purpurens* (William *et al.,* 1991), *Rhodomicrobium vannielli* (Wright and Madigan, 1991) and *Rhubrivivax benzoatiliticus* (Ramana *et al.,* 2006). On the other hand, the aromatic utilization for growth was not well reported among other purple bacteria, some incubation results indicated light dependent transformation of aromatic hydrocarbons by *Rubrivivax gelatinosus* (Willems *et al.,* 1991), *Rhodobacter capsulatus* (Blasco- Castillo, 1992; Saez *et al.,* 1999); *Rhodobacter blasticus* (Ahmed and Mohamed, 1994) and *Rhodobacter sphaeroides* (Rajashekar *et al.,* 1998; Nanda *et al.,* 2000; Sunayana, *et al.,* 2005a,b; Vijay *et al.,* 2006).

4.1 ***L-Phenylalanine catabolism by Rhodobacter sphaeroides OU5***

Correlation between L-phenylalanine consumption and simultaneous production of phenols during growth as sole nitrogen source by *Rhodobacter sphaeroides* OU5 (Fig. 3) is similar to that observed with *Rhodobacter capsulatus* (Saez *et al.,* 1999) and differ

from anaerobic chemotrophic bacterial metabolism, which produced benzoate (Schneider *et al.,* 1997).

4.1.1 ***Work with whole cells***

Gallate was the major product of L-phenylalanine metabolism in *Rhodobacter sphaeroides* OU5 (Fig. 7). Gallate is a biotechnologically important compound (Kar *et al.,* 1999), mainly produced by the hydrolysis of tannins (Inoue *et al.,* 1995) and its microbial production by a few chemotrophic bacteria (Deschamps and Lebeault, 1984) and fungi (Kar *et al.,* 1999) was observed earlier. Other phenols identified from the culture supernatant of *Rhodobacter sphaeroides* OU5 includes, gallate, protocatechuate, catechol and caffeate (Fig. 7), while homogensitate was reported from *Rhodobacter capsulatus* (Saez *et al.,* 1999). Both gallate and caffeate had the same retention time under the experimental HPLC conditions used and their identity could be resolved and distinguished only through LC-MS analysis by their corresponding molecular masses.

Detailed characterization of two of the purified metabolites based on FT-IR (Fig. 4C; 5C), ^{1}H NMR (Fig. 4A; 5A), ^{13}C NMR (Fig. 4B; 5B) and mass analysis confirmed the structures as 3,4-dihydroxybenzoic acid 5-carboxy-4-hydroxy-3-methyl-pentyl ester (rhodophestrol) (Fig. 4D) and 2,3,4 tryhydroxybenzoyl terpenoid ester (Fig. 5D). The hydrolysis of the ester bond and of rhodophestrol (Fig. 4E) and 2,3,4-tryhydroxybenzoyl terpenoid ester (Fig. 6) by the enzyme esterase (EC 1.1.1.49) and identifying the corresponding phenol acid and alcohol through HPLC/LC-MS analysis helped in the confirmation of the novel metabolites, whose yield was about 100 µmole/ (65 µg)/ml^{-1} rhodophestrol and 0.2 µ mol 2,3,4-tryhydroxybenzoyl terpenoid ester.

Nine phenol esters were identified through hydrolysis of the ester bonds of the metabolites in different fractions (Fig. 7) using esterase enzyme and the corresponding acid and alcohol were analyzed using LC-MS. These are broadly categorized as gallate, caffeate, genisitate and protocatechuate esters (Table. 2). While the esterase hydrolyzed products represented the corresponding acids, the alcohols were hydroxyalkanoates, terpenols and phenols. Gallate alkyl esters have attracted the cosmetic and pharmaceutical industries as antioxidants (Nakayama, 1993). They are either chemically synthesized (Machado *et al.,* 2007) or extracted from plants (Yang *et al.,* 2003) and their microbial production is a discovery (Peng *et al.,* 2006). The marine bacterial isolate identified as belonging to the genus *Microbulbifer* could produce 4-hydroxybenoate alkyl esters (butyl, heptyl and nonyl 4-hydroxybenzoate esters; commonly called as "parabens"), which were antimicrobial (Peng *et al.,* 2006), which differ from the isolated conjugated metabolites from *Rhodobacter sphaeroides* OU5.

4.1.2 ***Work with cell free extracts***

3,4-Dihydroxyphenylalanine was identified as the major product of L-phenylalanine (L-tyrosine) metabolism of *Rhodobacter sphaeroides* OU5 (Fig 8; Ranjith *et al.,* 2007a). In the presence of 2-oxoglutarate, the increase in transaminase activity with the substrates (Page. 52) L-phenylalanine < L-tyrosine < L-DOPA suggests that transamination occurred at the level of 3,4-dihydroxyphenylalanine, whose product (3,4-dihydroxyphenylpyruvic acid) was confirmed from the molecular mass as 196 (m/z; Fig. 10). This result probably can explain the low aminotransferase activity of L-phenylalanine with the substrate 2-oxoglutarate in *Rhodobacer capsulatus* (Saez *et al.,* 1999). The enzyme 3,4-dihydroxyphenylalanine transaminase (DOPAATS; EC 2.6.1.49)

was reported earlier from animal system (pig brain; Funnum and Larsen, 1965) and bacteria (A*lkaligenes faecalis* [Nagasaki, 1973] and *Enterobacter cloacae* [Nagasaki, 1975]). The isolated ~123 kDa protein of DOPAATS from *Rhodobacter sphaeroides* OU5 is a heterotetramer (Table.10), which differ from the earlier reported homodimer (Funnum and Larsen, 1965). Enzyme code (EC 2.6.1.49) of DOPAATS was recently updated by the *Nomenclature Committee of International Union of Biochemistry and Molecular Biology* (NC-IUBMB; http://www.chem.qmul.qc.uk/iubmb/enzyme/EC2/6/1/49 based on the information of this enzyme published recently (Ranjith *et al.,* 2007).

Based on the product 3,4-dihydroxyphenylpropionic acid (DPPA; Fig. 13), in the absence of 2-oxoglutarate and formation of ammonia from DOPA confirms the absence of an active phenylalanine ammonia lyase (PAL) or tyrosine ammonia lyase (TAL) (Usha *et al.,* 2007) and indicated the presence of a NADH dependent reductive deaminase in *Rhodobacter sphaeroides* OU5 (Fig. 14, 15). The enzyme 3,4-dihydroxyphenylanaline reductive deaminase (DOPARDA) was isolated and characterized from *Rhodobacter sphaeroides* OU5 (Table. 5) and its novelty was conformed through MALDI-TOF (MS-MS) fingerprinting analysis (Fig. 21A, B). Ours is the only report of this enzyme, which was published recently (Ranjith et al., 2007a) and an enzyme code (EC 4.3.1.22) was assigned by NC-IUBMB (http://www.chem.qmul.qc.uk/iubmb/enzyme/EC4/3/1/22).

In the absence of either 2-oxoglutarate or NADH, ammonia formation was still observed (Fig. 14), which may be due to ammonia lyase or by an oxidative deaminase. We could not detect by HPLC and LC-MS either *trans*-cinnamate, 4-hydroxy *trans*-cinnamate or 3,4-dihydroxydroxy *trans*-cinnamate (caffeate) form the assay supernatant when substrates L-phenylalanine, L-tyrosine or DOPA, respectively were used. These

results indicate that ammonia formation in the absence of either NADH or 2-oxoglutarate was not due to the involvement of ammonia lyases in *Rhodobacter sphaeroides* OU5. However, tyrosine ammonia lyase (TAL) was reported from *Rhodobacter sphaeroides* (Xue *et al.,* 2007) and *Rhodobacter capsulatus* (Kyndt *et al.,* 2002). The enzyme L-phenylalanine ammonia lyase (PAL [EC 4.3.1.24]; converts L-phenylalanine into *trans*-cinnamic acid) and L-tyrosine ammonia lyase (TAL [EC 4.3.1.23]; converts L-tyrosine to *para*-hydroxyphenylpyruvic acid [4-HPP]), are commonly observed in plants (Minami et al., 1989) and recently also in the bacteria (Xiang *et al.,* 2005; Huang and Xue, 2003).

We have identified the product of the enzyme in the absence of 2-oxoglutarate or NADH using LC-MS as 3,4-dihydroxyphenypyruvic acid (DOPP; Fig. 19) and the same was confirmed using HPLC, indicating the presence of an active oxidative deaminase in *Rhodobacter sphaeroides* OU5. In a mutant strain of *E. coli* mutation of tyrosine332 to phenylalanine converted DOPA decarboxylase (EC 4.1.1.28) into an oxidative deaminase (Bertoldi *et al.,* 2002), which was pH dependent (Bertoldi and Voltattoni, 2001). 3,4-Dihydroxyphenylalanine oxidative deaminase (DOPAODA) of *Rhodobacter sphaeroides* OU5 had the highest activity with L-DOPA or L-tyrosine than with L-phenylalanine and L-tryptophan (Page. 70). The low K_m value reflects a strong affinity between (E+S) enzyme and L-DOPA (Table. 6). The DOPA oxidative deaminase from *Rhodobacter sphaeroides* OU5 did not exhibit any decarboxylase activity by changing pH and thus differ from the chimeric oxidative deaminase reported in *E.coli* (Bertoldi and Voltattoni, 2001; Bertoldi *et al.,* 2002). Formation of DOPP from DOPA was oxygen dependent (Fig. 23) and the enzyme (DOPAODA) activity was inhibited even at low concentration of NADH (~35 µM) or 2-oxoglutarate (~25 µM), which reflects DOPAODA's

physiological role in *Rhodobacter sphaeroides* OU5. The enzyme 3,4-dihydroxyphenylanaline oxidative deaminase was isolated and characterized from *Rhodobacter sphaeroides* OU5 (Table. 6). Ours is the only report of this enzyme, which was published recently (Ranjith *et al.,* 2008).

Bacterial metabolism of L-phenylalanine was well studied (see introduction page numbers 2-10). Our experimental evidences suggest that DOPA is the downstream product of L-phenylalanine or L-tyrosine in *Rhodobacter sphaeroides* OU5 (Fig. 8,9), thus differ from the homogentisate pathway observed in other bacteria (Bender, 1978; Mortimer, 1986). Based on the earlier evidence of production of DOPA by *Erwinia herbicola* from L-tyrosine (Koyanagi *et al.,* 2005) together with isolation of the enzyme DOPAATS, in this study and from other bacteria (Nagasaki *et al.,* 1973; Nagasaki *et al.,* 1975), we propose the bacterial DOPAATS pathway of L-phenylalanine catabolism (Fig. 52). The further downstream products of this metabolism were identified through the LC-MS analysis (Fig. 8). The role of alternative enzymes involved in the deamination of DOPA is also shown in the pathway (Fig. 52). The comparison of the three enzymes involved in ammonia metabolism of DOPA in *Rhodobacter sphaeroides* OU5 are shown in Table. 10.

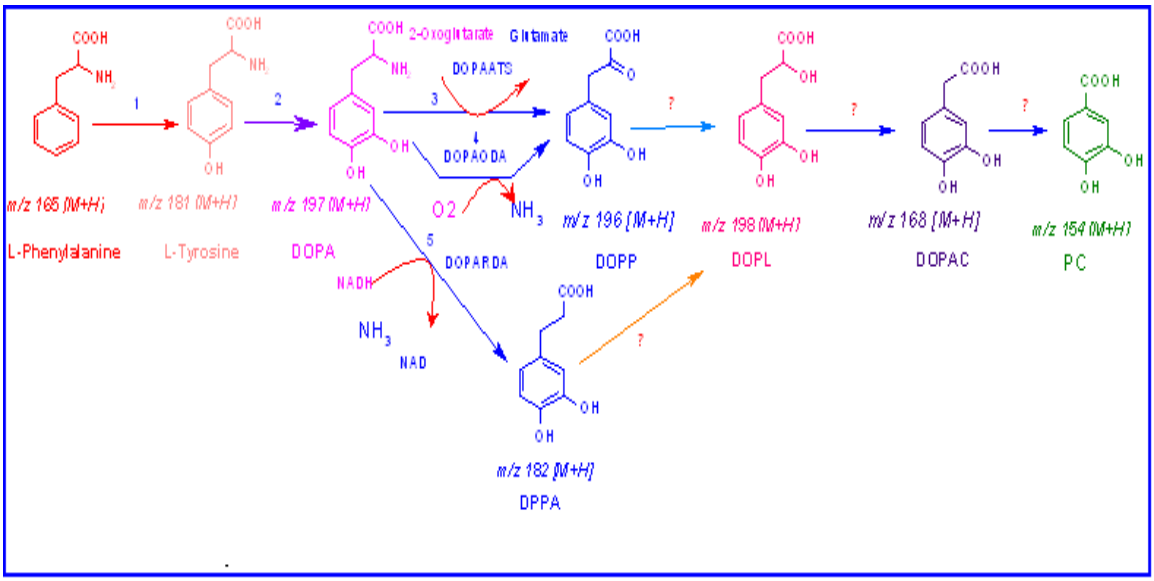


Fig. 52. Proposed intracellular catabolism of L-phenylalanine by *Rhodobacter sphaeroides* OU5.

The pathway is based on experimental evidence observed in the present study. [1= L-phenylalanine hydrogenase; 2=L-tyrosine dehydrogenase; 3= 3,4-dihydroxyphenylalanine 2-oxoglutarate aminotransferase (DOPAATS; EC 2.6.1.49); 4=3,4-dihydroxyphenylalanine oxidative deaminase (DOPAODA); 5= 3,4-dihydroxyphenyalanine reductive deaminase (DOPARDA; EC 4.3.1.22)? = indicated not known.3, 4-Dihydroxyphenylapyruvic acid (DOPP); 3,4-Dihydroxyphenylpropionic acid (DPPA); 3,4-Dihydroxyphenyllactic acid (DOPL); 3,4-Dihydroxyphenylacetic acid (DOPAC) 3,4-Dihydroxybenzoic acid or Protocatechuic acid (PC)]

Enzymes	Products	Co-substrates	Co-factors	K_m (µM)	K_{cat} (s^{-1})	M.Wt. (kDa)	Subunits (determined by SDS-PAGE) (kDa)	References
L-DOPA-oxidative deaminase	DOPP	O_2	Nil	11.84±1.80	0.680±0.023	~ 190	Pentamer (54, 43, 34, 25, 22)	Ranjith *et al.,* (2008)
L-DOPA-reductive deaminase	DPPA	Nil	NADH	21.23±0.09	0.0636±3.0	~ 274	Hetero-tetramer (117, 85, 49, 35)	Ranjith *et al.* (2007a)
L-DOPA-amino transferase	DOPP	2-KGA	PLP	4.1	ND	ND	Homodimer	*Fonnum *et al.,* (1965)
				0.35±0.045	0.29±3.0	~ 123	Heterodimer (60, 63)	Ranjith *et al.,* (2007a)

Table. 10. Differentiating characters of the three enzymes of *Rhodobacter sphaeroides* OU5 involved in ammonia liberation /transformation of DOPA, an intermediate in the catabolism of L-phenylalanine of *Rhodobacter sphaeroides* OU5.

[Results are means ± SD of three different determinations done in duplicates. *Isolated from Pig brain. α-KGA = 2-oxoglutarate; PLP = Pyridoxal-5-phosphate; NADH = Nicotinamide adenine dinucleotide; Nil = No requirement; kDa = Kilo Dalton; DOPP = 3,4-dihydroxyphenyl pyruvic acid; DPPA = 3.4-dihydroxyphenyl propionic acid; DOPA= 3,4-dihydroxyphenylalanine. ND = Not determined]

4.2 *L-Tryptophan catabolism by Rhodobacter sphaeroides OU5*

4.2.1 *Work with whole cells*

para-Dimethylaminobenzaldehyde reagent (Ehrlich reagent) is the most commonly and widely used reagents in the identification of indoles. This reagent gives pink to red color when reacted with indole and its derivatives, while yellow to brown with anthranilate. The culture supernatant of L-tryptophan induced *Rhodobacter sphaeroides* OU5 and *E.coli* (used for comparison) were tested with Ehrlich reagent. The orange and pink colored reactions were observed with *Rhodobacter sphaeroides* OU5 and *E. coli*, respectively, suggesting variations in the products of L-tryptophan metabolism. Two metabolites were isolated and characterized from the culture supernatant. Detailed characterization of a metabolite from Fraction S1, based on IR, ^{1}H and ^{13}C NMR and mass analysis (Fig. 25; 26A,B) confirmed its structure as 3-hydroxy-6-(1H-indole-33-yloxy)-4-methyl-hexanoic acid; it has a molecular mass of m/z 279 $[M+H]^{+}$ and was named as rhodethrin (**rhod**=from *Rhodobacter*; **ethr**=ether; **in**= indole) (Ranjith *et al.,* 2007b). This compound differs from sphestrin (Sunayana *et al.,* 2005a) or rhodestrin (Sunayana *et al.,* 2005b) in having an ether linkage rather than ester linkage and in the length of the terpenoid side chain. Esters of the myo inositol-indole acetate were only reported from plants (Domagalski *et al.,* 1987; Chisnell, 1984). The second metabolite was isolated from Fraction S2, which gave yellow color with PDAB reagent. This metabolite was also characterized based on IR, ^{1}H and ^{13}C NMR and mass analysis (Fig. 27) as 4-aminocylohex-4-ene-1, 2 diol, a probable intermediate of indole metabolism, since this metabolite was not a product of L-phenylalanine.

The LC-MS analysis revealed a more wide range of indoles (Fig. 29) including those purified (m/z 279 m/z and 129 $[M+1H]^{+1}$). The other metabolites identified include

indole-3-acrylaldehyde, indole-3-carboxaldehyde, indole-3-acetic acid and rhodethrin (Ranjith *et al.,* 2007b). We presume the molecular mass of 171 (m/z) as indole-3-acrylaldehyde; this compound is not available in the literature, but a related compound indole-3-acrylic acid in known (Marklova, 1999). Excretion of indole-3-acrylic acid in the urine and fecus of L-tryptophan fed animals was attributed to the intervention of intestinal microorganisms. Thus, indole-3-acrylaldehyde may be an intermediate of L-tryptophan metabolism in *Rhodobacter sphaeroides* OU5. Production of indole-3-carboxaldehyde was reported earlier from an *Acetenobacter* sp. (Olguin-Uribe *et al.,* 1997) and by an unidentified fungus (Kuniyoshi *et al.,* 2003). Microbial production of indole-3-acetic acid is well known (Stijn *et al.,* 2007; Patten and Glick, 1996) and was reported even from a few other purple bacteria (*Rhodopseudomonas palustris*, *Rhodobacter sphaeroides*, and *Rubrivivax tenuis*) when grown on L-tryptophan (Srinivas *et al.,* 2002) or from indole + glycine by *Rhodobacter sphaeroides* OU5 (Rajashekar *et al.,* 1999). Low levels of some of the metabolites observed in the L-tryptophan induced culture supernatant were also observed in the absence of L-tryptophan (control; Fig. 28), the notable one was indole-3-acetic acid.

4.2.2 ***Work with cell free extract***

Assay was done in the presence and absence of 2-oxoglutarate, which acts as an amino acceptor from L-tryptophan.The products were extracted into ethyl acetate and analyzed by LC-MS, which helped for further metabolome identification. Bacterial conversion of L-tryptophan to indole-3-pyruvic acid in the presence of 2-oxoglutarate by the enzyme tryptophan 2-oxoglutarate aminotransferase (EC 2.6.1.27) was well studied in plant, animal and microbial systems (Stanley *et al.,* 1984; Koga *et al.,* 1992). We also

confirmed the enzyme activity in *Rhodobacter sphaeroides* OU5 using cell free extract and the products, indole-3-pyruvic acid and glutamate were confirmed using HPLC (Fig. 31A,B; 32A). LC-MS metabolite profiling of the assay supernatant indicated the existence of metabolites other than indole 3-pyruvic acid; indole-3-acetaldehyde, indole-3-acetic acid, isatin, gallic acid, pyrogallol and benzaldehyde were identified from the assay supernatant based on their molecular masses. Indole-3-pyruvic acid, indole-3-acetic acid, isatin, benzaldehyde, gallic acid and pyrogallol were confirmed by HPLC. However, indole 3-acetaldehyde could not be detected in HPLC. These metabolites are the intermediates of a single pathway as confirmed from the studies using cell free extracts where L-tryptophan (Fig. 33A1) or IAA (Fig. 33A2) or isatin (Fig. 33A3) were used as precursor and the products were analyzed using HPLC and LC-MS. The results indicate the catabolism of L-tryptophan in the presence of 2-oxoglutarate by *Rhodobacter sphaeroides* OU5 proceeds through the indole-3-pyruvate pathway leading to the biosynthesis of indole-3-acetic acid with the intermediate indole 3-acetaldehyde (Lee *et al.,* 2004). The bioprocess generating isatin from L-tryptophan by methnogenic consortium (Gu and Berry, 1991), presence of benzoic acid and its conversion to gallate and to pyrogallol (Heider and Fuchs, 1997) strongly support the proposed L-tryptophan catabolic pathway in *Rhodobacter sphaeroides* OU5 (Fig. 53A).

While in the absence of 2-oxoglutarate, none of the above metabolites were observed in LC-MS profiling (Fig. 32 B, B1, B2). One normally expects the production of indole by the enzymatic action of tryptophanase (EC 4.1.99.1) encoded by *tna*A gene (Snell, 1975), which was not observed as confirmed through the LC-MS (Fig. 33B1a) and enzyme assay. The HPLC confirmed products were; indole 3-acrylic acid, indole 3-

carboxaldehyde, anthranilic acid and catechol (Fig. 33B2). The ammonia liberation was observed only in the absence of 2-oxoglutarate and the product indole-3-acrylic acid was confirmed by HPLC and LC-MS (Fig. 38A,A1, B, B1). Experiments using cell free extracts indicated that this enzyme has 100% affinity for L-tryptophan and decreased with L-phenylalanine, L-glutamate, L-tyrosine, DL-alanine/L-glycine and L-histidine (Table. 7). Based on some of the results (Fig. 32; 33B; 38) we propose the indole-3-acrylic acid pathway of L-tryptophan catabolism to occur in the absence of 2-oxoglutarate (Fig. 53B). To the best of our knowledge, the proposed indole 3-acrylic acid pathway is a novel biological pathway. Indole 3-acrylic acid was not listed as a phytoauxine (http://www.biologie.uni-hamburg.de/b-online/e31/31.htm) though its microbial origin was suspected (Marklova, 1999). Production of indole-3-acrylic acid and ammonia indicates that the enzyme as an ammonia lyase, since this family of enzymes cleaves at C-N bond of various substrates and results in the formation of ammonia and corresponding products. The study thus indicated the presence of a potential enzyme, tryptophan ammonia lyase (WAL), which adds to our knowledge of L-tryptophan metabolism and to the list of ammonia lyases (L-phenylalanine ammonia lyase (PAL; EC 4.3.1.24), L-tyrosine ammonia lyase (TAL; EC 4.3.1.23), PAL/ TAL (EC 4.3.1.25) L-serine ammonia lyase (SAL; EC 4.3.1.17) and L-histidine ammonia lyase (HAL; EC 4.3.1.3) reported so far, hence went for purification and characterization of tryptophan ammonia lyase enzyme.

Tryptophan ammonia lyase (WAL) was isolated, purified (Fig. 35) and characterized (Table. 7) as a ~225 kDa heterotetramer. The novelty of WAL was confirmed through MALDI-TOF (MS/MS) finger printing analysis (Fig. 39, 40). Among the four subunits, the 55 kDa subunit had ~54% score with a hypothetical protein in *Rhodobacter sphaeroides* 2.4.1 (Table. 8), while the remaining three had less significant match in database (NCBS, MSDB and SwissPort), which is summarized in Table 9. The partial N-terminal sequences of WAL matched with the probable histidine ammonia lyase of *Rhodobacter sphaeroides* 2.4.1 (Table. 7). The molecular evidence of this enzyme is in progress.

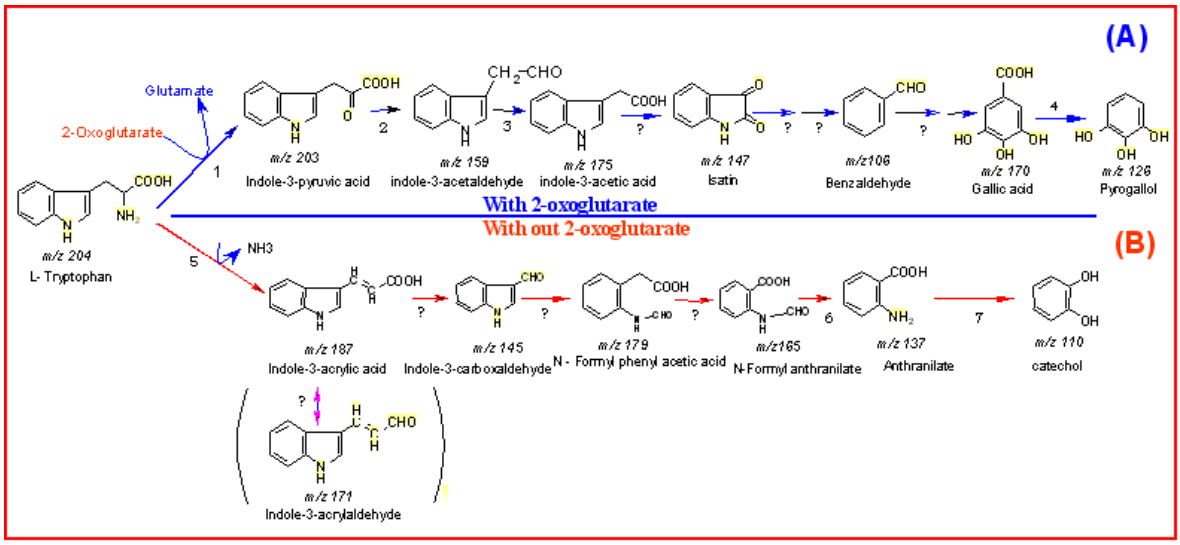


Fig. 53. Proposed pathways of L-tryptophan catabolism by *Rhodobacter sphaeroides* OU5. (**A** = in the presence of 2-oxoglutarate; **B**= in the absence of 2-oxoglutarate).

The proposed pathway is based on the metabolites identified in this study and the evidence for some of the enzymes is from the literature. 1=Tryptophan aminotransferase (EC2.6.1.27); 2=Indole-3-pyruvicacid decarboxylase (EC 4.1.1.74), 3= Aldehyde dehydrogenase (EC 1.2.3.1)/Aldehyde oxidase (EC 1.2.3.1); 4= Gallate decarboxylase (EC=4.1.1.59); 5= Tryptophan ammonia lyase; 6= N-Formylanthranilate deformylase (EC 3.5.1.68) and 7= Anthranilate dehydrogenase (EC 1.1.1.95). ? = Enzymes not identified.

4.2.3 ***Chemotrophic (Dark) metabolism of L-tryptophan*** Chemotrophic incubations of *Rhodobacter sphaeroides* OU5 with L-tryptophan resulted in the identification of some of the plant based secondary metabolites (Fig. 44). These were not reported so far from bacteria except from fungi (Nieminen *et al.,* 2002). Gliotoxine (GT) has antimicrobial, antifungal, antiviral, cytotoxic and immunosuppressive properties (Nieminen *et al.,* 2002, Waring and Beaver 1996). Ibogain is a naturally occurring indole alkaloid from tabermanthe ibogain and it is used for hallucinogenic properties during tribal ceremonies. Indoprofen and indobufen are non-steroidal anti-inflammatory drug used to treat rheumatoid arthritis (Bourinbaiar and Huang 1994) and cyclooxygenase (COX) inhibitory activities (Metchell *et al.,* 2004). Indican (Indoxyl-B-D-glucoside) indigo precursor produced from woad (*Isatis tinctoria L*) and chinese woad (Isatis *indigotica fort*) (Angelini *et al.,* 2007).

4.3 ***Bioprospecting*** Indole and its derivatives are known for antimicrobial activities (Babalola, 1998). In addition, molecules like indole-3-acetic acid (IAA) and indole-3-propionic acid (IPA), are natural auxins. Esters of indole acetic acid, indole myo-inositol esters (Domalgski *et al.,* 1987), sphestrin (Sunayana *et al.,* 2005a) and rhodestrin (Sunayana *et al.,* 2005b) were reported to have phytoharmonal activity. The isolated rhodethrin also showed phytoharmonal activity (Fig. 45; Ranjith *et al.,* 2007b) with an early and profused rooting in tissue culture plants even at very low concentrations in comparison to the standard IAA. Production of indole esters was reported from plants (Domagalski *et al.,* 1987; Chisnell, 1984) and by chemical synthesis (Katayama, 2000). However, microbial biosynthesis of indole terpenoids ester and ethers was reported in Rhodobacter sphaeroides OU5 (Sunayana *et al.,* 2005 a,b; Ranjith *et al.,* 2007b). The

recently reported novel strigolactones (Mikihisa, *et al.,* 2008) adds to the list of terpenoid phytohormones. The Cytotoxicity of rhodethrin against Sup-T_1 (T-cell lymphoma), Colo-125 (Fig.47) (10 nM) and Jurkat (20 pM) (Fig. 48) cancer cell lines and also has 45% COX-2 inhibitory activity (Fig. 49). Since many indole esters are potential bioactive molecules (Olgen and Nebioglu 2002) so far synthesized only through chemical routes (Katayama, 2000), the biological production of rhodethrin by *Rhodobacter sphaeroides* OU5 is worth exploiting.

The phenol esters are an important group of biogenic molecules reported from plants (Yang *et al.,* 2003), bee propolis (Ahn *et al.,* 2004), yeasts like candida (Stevenson *et al.* 2007) and from a marine bacterium; *Microbulbifer* (Peng *et al.,* 2006). Alkyl esters of phenols are of biotechnological significance since they have anti-oxidant (Chen *et al*. 1997), anti cancer (Samaha *et al.,* 1997; Li *et al.,* 2003), anti HIV (Burke *et al.,* 1995) and antimicrobial (Tawata *et al.,* 1996; Peng *et al.,* 2006) activities. The cytotoxicity of rhodophestrol against U937 (Human leukemic monocyte lymphoma cell line) was determined (apoptosis bodies formation) on this cancer cell line even at low concentrations (50 nM) (Fig. 50). It has COX-I (Fig. 51) and COX-II inhibitory activity. Rhodophestrol is a potential anticancer and thus worth exploiting from *Rhodobacter sphaeroides* OU5. The study also suggested production of a wide range of other phenols during the bioprocessing of an aromatic amino acid like L-phenylalanine by *Rhodobacter sphaeroides* OU5. Though most of these are representatives of free phenols, which many researchers have already identified as microbial products, the microbially produced phenol terpenoids and related conjugates (Table. 2) are novel bio-molecules, which are worth exploiting.

5.CONCLUSIONS

5. Conclusions

- 3,4-Dihydroxyphenylalanine (DOPA) was identified as an intermediate of L-phenylalanine metabolism through intermediate of L-tyrosine in *Rhodobacter sphaeroides* OU5.
- Three enzymes (2 are novel) were identified in the ammonia metabolism of DOPA:3,4-Dihydrophenylalanine 2-oxoglutarate aminotransferase; 3,4-Dihydroxyphenylalanine reductive deaminase and 3,4-Dihydroxyphenylalanine oxidative deaminase were purified to homogeneity and characterized.
- The pathway of L-phenylalanine catabolism is proposed based on the experimental evidences.
- Two enzymes were identified in the down stream of L-tryptophan in *Rhodobacter sphaeroides* OU5.
- In the presence of 2-oxoglutarate, L-tryptophan aminotransferase (WAT) and a novel enzyme L-tryptophan ammonia lyase (WAL) was identified in the absence of the (2-oxoglutarate) keto acid.
- L-Tryptophan ammonia lyase (WAL) was purified to homogeneity and characterized.
- A novel pathway of L-tryptophan catabolism is proposed based on the experimental evidences.
- Few novel metabolites were isolated and their biological activity was determined.
- Rhodethrin is one such novel molecule, which has Cox-2 inhibitory activity, cytotoxicity against cancer cell lines, phytoharmonal activity and antimicrobial activity.

- Rhodophestrol is novel phenol terpenoid molecule, which has Cox-1 inhibitory activity, cytotoxicity against cancer cell lines (U937; Human leukemic monocyte lymphoma cell line) and antimicrobial activity.

Major findings

- **Three novel pathways of microbial aromatic amino acid catabolism are proposed**
- **Four key enzymes (3 novel) involved in the catabolic process of L-phenylalanine and L-tryptophan were purified and characterized**
- **Bioactivity of Rhodethrin and Rhodophestrol were demonstrated**

Scope of future work

- **Several other enzymes of the proposed pathway needs to be isolated and characterized.**
- **Genes for the three novel enzymes already characterized are to be identified.**
- **Screening of other metabolites for biological activity.**
- **Exploitation of Rhodethrin and Rhodophestrol biological activities for human welfare.**

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7.PUBLICATIONS

7. Publications

Paper published/ Communicated

1. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2007) Catabolism of L-Phenylalanine and L-tyrosine by *Rhodobacter sphaeroides* OU5 occurs through 3,4-dihydroxyphenylalanine (DOPA). *Res Microbiol* **158:** 506-511.
2. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2007) Rhodethrin: A novel indole terpenoid ether produced by *Rhodobacter sphaeroides* OU5 has Cytotoxic and Phytohormonal activities. *Biotech Lett* **29:** 1399-1402.
3. Vijay, S., Sunayana, M.R., **Ranjith, N. K.,** Sasikala Ch and Ramana Ch (2006). Light dependent transformation of aniline to indole esters by a purple bacterium *Rhodobacter sphaeroides* OU5. *Curr Microbiol* **52:** 413-415.
4. **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* (Inpress).
5. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2008). Rhodophestrol: a novel phenol terpenoid ester produced by *Rhodobacter sphaeroides* OU5 has cytotoxic activity. *J Gen Appl Microbiol* (MS under revision).
6. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2008). Production of gallate and identification of phenol-esters from the culture supernatants of *Rhodobacter sphaeroides* OU5 grown on L-phenylalanine. *J Gen Appl Microbiol* (MS under revision).
7. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2008). Tryptophan ammonia lyase (WAL): an enzyme catalyzes the formation of indole 3-acrylic acid from L-

tryptophan isolated from *Rhodobacter sphaeroides* OU5 (To be communicated to *BBRC*).

8. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2008). Catabolism of L-tryptophan by *Rhodobacter sphaeroides* OU5 occurs through indole 3-pyruvic acid and indole 3-acrylic acid pathways. Curr. Microbiol. (Communicated)
9. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2008). Purification and characterization of 3,4-dihydroxyphenylalanine-2-oxoglutarate aminotransferase from *Rhodobacter sphaeroides* OU5. [MS under preparation]

Enzyme Code Number assigned by NC-IUBMB

1. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2007). A novel 3,4-dihydroxyphenylalanine reductive deaminase (DOPARDA) enzyme deposited in database as EC by Nomenclature Committee of the international Union of Biochemistry and Molecular Biology (NC-IUBMB) [**EC 4.3.1.22 created 2007**]
2. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2007). 3,4-dihydroxyphenylalanine 2-oxoglutarate aminotransferase (DOPAATS) enzyme updated in database as EC by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) [**EC 2.6.1.49 created 1972**] (Funnum and Larsen, 1965).
3. **Ranjith, N.K.,** Sasikala, Ch and Ramana, Ch.V. (2008). 3,4-dihydroxyphenylalanine oxidative deaminase (DOPAODA) enzyme applied for EC number by Nomenclature Committee of the international Union of Biochemistry and Molecular Biology (NC-IUBMB) [**EC 4.3.99 --**]

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Catabolism of L-phenylalanine and L-tyrosine by *Rhodobacter sphaeroides* OU5 occurs through 3,4-dihydroxyphenylalanine

N.K. Ranjith [a], Ch. Sasikala [b], Ch.V. Ramana [a,*]

[a] *Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500 046, India*
[b] *Bacterial Discovery Laboratory, Center for Environment, IST, J NT University, Kukatpally, Hyderabad 500 085, India*

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Abstract

Rhodobacter sphaeroides OU5 utilized L-phenylalanine as sole source of nitrogen for growth. The metabolites of L-phenylalanine catabolism, i.e. 4-hydroxy phenylalanine (L-tyrosine), 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenyl-pyruvic acid (DOPP), 3,4-dihydroxyphenyllactic acid (DOPLA), 3,4-dihydroxyphenyl-acetic acid (DOPAc) and 3,4-dihydroxybenzoic acid (PC), were identified using liquid chromatography-mass spectroscopy (LC-MS). With 2-oxoglutarate as an amino acceptor, DOPA aminotransferase activity was observed with cell-free extracts and the product DOPP was confirmed through mass analysis. Reductive deamination of DOPA also occurred in the absence of 2-oxoglutarate, whose products were 3,4-dihydroxyphenylpropionic acid (DPPA) and ammonia. The enzyme DOPA-reductive deaminase (DOPARDA) was purified to its homogeneity and characterized. DOPARDA has an obligate requirement for NADH and is functional at low concentrations of the substrate (<150 μM). The molecular mass of the purified enzyme was ≈274 kD and the enzyme could be a heterotetramer of 110, 82, 43 and 39 kD subunits as determined by SDS-PAGE.

Keywords: Biodegradation; Aromatic hydrocarbons; L-phenylalanine; 3–4 Dihydroxy phenylalanine (DOPA); DOPA aminotransferase (DOPAATS); DOPA reductive deaminase (DOPARDA); *Rhodobacter sphaeroides* OU5

1. Introduction

Purple non-sulfur bacteria are widely distributed in habitats rich in organic matter and are versatile in inducing metabolic routes in response to nutritional changes in the environment. While a wide range of aliphatic organic molecules are metabolized by these bacteria, the metabolism of aromatic hydrocarbons is restricted to a few molecules [18]. Low molecular weight aromatic hydrocarbons like benzoate and some of its derivatives support growth of a few of these bacteria (growth-supporting aromatic hydrocarbons). In contrast, some of the aromatic hydrocarbons, although they do not support growth, can be transformed without aromatic ring cleavage [18]. The latter studies were mainly concentrated on the metabolism of nitrophenols by *Rhodobacter capsulatus* [15,17,22]. Under phototrophic anaerobic conditions, *R. capsulatus* transformed 2,4-dinitrophenol to 2-amino, 4-nitrophenol [3], catalyzed by a cytosolic and homodimeric Flavin mononucleotide-linked 54 kD nitrophenol reductase [4]. L-phenylalanine and L-tyrosine were photometabolized by *R. capsulatus* through homogentisate [16]. In this report, we provide evidence that *Rhodobacter sphaeroides* OU5 metabolizes L-phenylalanine and L-tyrosine through the intermediate 3,4-dihydroxyphenylalanine (DOPA) resulting in the formation of protocatechuate and involves two key enzymes, DOPA transaminase and DOPA reductive deaminase.

* Corresponding author.
E-mail addresses: sasi449@yahoo.ie (Ch. Sasikala), r449@sify.com (Ch.V. Ramana).

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2. Materials and methods

2.1. Organism, growth conditions and preparation of cell-free extracts

R. sphaeroides OU5 (ATCC 49885; DSM 7066) was grown photoheterotrophically (anaerobic/light) (2400 lux) in fully filled screw cap test tubes (10 × 100 mm) or in reagent bottles (250/1000 ml) on a mineral medium [20] with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen sources, respectively, at 30 ± 2 °C. For growth on L-phenylalanine and L-tyrosine, ammonium chloride was replaced by the aromatic amino acid (1 mM) when used as nitrogen source, malate was replaced when used as carbon source, or both malate and ammonium chloride were replaced when used as sole sources of carbon and nitrogen.

Cells were harvested by centrifugation (16,000 × *g* for 10 min) at late logarithmic phase, 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 lysed by sonication using a MS-72 probe (Bandelin, Germany make, model-UW 2070) to complete cell lysis after 8–9 cycles. The cell suspensions were centrifuged (16,000 × *g* for 20 min) and the supernatant was used as a source of enzyme.

2.2. Enzyme activities

2.2.1. DOPA 2-oxoglutarate aminotransferase (DOPAATS) activity

This was determined by measuring the loss of DOPA using HPLC. The reaction mixture contained, in a final volume of 1 ml (0.05 M potassium phosphate buffer pH 7.8), 0.3 μmol of 2-oxoglutarate, 0.3 μmol of DOPA, 0.25 μmol of pyridoxal-5-phosphate (PLP) and an appropriate amount of cell extract. The reaction was carried out in Eppendorf tubes (1.5 ml) and incubated at 30 °C. The reaction was terminated after 30 min (unless otherwise mentioned) and proteins were denatured by acidification with 10% (w/v; 100 mg ml^{-1}) TCA. The sample was centrifuged (12,000 × *g* for 5 min) and analyzed by injecting 20 μl of clear supernatant in HPLC. A unit (U) of enzymatic activity is defined as the amount of enzyme that catalyzes the disappearance of 1 μmole DOPA mg $protein^{-1}$ min^{-1}.

2.2.2. DOPA reductive deaminase (DOPARDA) activity

This was determined by measuring the formation of ammonia from DOPA (100 μM) in the presence of NADH (10 μM) to a final volume of 0.5 ml of potassium phosphate buffer (0.05 M; pH 7.0). A unit (U) of enzymatic activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole ammonia mg $protein^{-1}$ min^{-1}.

2.3. Purification and identification of DOPARDA

The enzyme was purified from cell-free extracts of L-tyrosine-induced cells of *R. sphaeroides* OU5. Ammonium sulfate was added to the supernatant to achieve 25–30% saturation, keeping pH at 7.0. The proteins that precipitated were separated by centrifugation (30,000 × *g* for 20 min), resuspended (5 ml) in phosphate buffer (pH 7.0) and dialyzed against three changes of 3 liters of the same buffer for 3 h each. The enzyme was partially purified using Sephadex G-150 gel exclusion column chromatography (95 × 1.6 cm) by eluting at a flow rate of 1 ml per 15 min. In total, 50 fractions were collected and all fractions were assayed for enzyme activity. Fractions 7, 8 and 9 of gel-eluted proteins were applied to a DEAE-cellulose column (16 × 2.9 cm) for further purification. The bound proteins were eluted using NaCl gradients (0.5 M followed by a second one with 1.0 M NaCl) in 0.05 M potassium phosphate buffer (pH 7.0). The eluted fractions were assayed for enzyme activity and the highest active fraction eluted with 1.0 M NaCl was used for non-denaturing PAGE (8%) and SDS-PAGE (10%).

2.4. Extraction and purification of metabolites from cell-free extracts

After enzyme assay, the metabolites were extracted with 3 ml of ethyl acetate. The ethyl acetate layer was separated and 5 mg of anhydrous sodium sulfite was added to remove water molecules. The ethyl acetate extract was evaporated under a vacuum, resuspended in HPLC grade methanol and used for LC-MS analysis. Metabolites were identified based on their molecular masses.

2.5. Analytical determinations

Growth was followed turbidometrically at 660 nm. L-phenylalanine (0.2% [w/v] ninhydrin in acetone), phenols [21], proteins [5] and ammonia [19] were analyzed using standard methods. HPLC analysis of substrates/products was performed at room temperature using a Shimadzu SPD-10AVP isocratic system. Methanol plus water (1:1) was used as a solvent at 1.5 ml min^{-1}, Luna 5 μ C_{18} (2) 100A column (250 × 4.6 mm) and the compounds were detected using a UV-VIS detector at 200 nm. The retention times (t_R; min) for L-phenylalanine (2.4), L-tyrosine (1.9), DOPA (1.8) and DOPP (1.67) were used for qualitative analysis and compounds were quantified by comparison of the peak height with the corresponding standards. 2-Oxoglutarate (t_R 1.6 min) and glutamate (t_R = 1.7 min) were analyzed at 190 nm. Mass analysis was done using on a Shimadzu LC-MS-2010A. Analysis was performed at 40 °C for the LC column oven and at 85 °C for the MS ionization chamber. Methanol plus water (1:1) was used as a solvent at 0.2 ml min^{-1}, Luna 5 μ C_{18} (2) 100A was the column (250 × 4.6 mm) and the compounds were detected (LC) at 254 nm. The column effluent from the LC was nebulized into an atmospheric pressure chemical ionization (APCI) region under N_2 gas for generating molecular masses. Native-PAGE (8%) and SDS-PAGE (10%) were stained using silver and Coomassie brilliant blue (G-250), respectively, following the procedures of Laemmli [11].

3. Results and discussion

3.1. Growth and simultaneous utilization of L-phenylalanine

R. sphaeroides OU5 could grow on L-phenylalanine or L-tyrosine only under phototrophic conditions and when used as sole source of nitrogen. The compounds could not support growth as source of carbon or carbon and nitrogen. The organism had a doubling time of about 24 h (compared to 18 h with ammonium chloride) with 100% consumption of L-phenylalanine observed within 60 h.

3.2. Cell-free extracts

With cell-free extracts of *R. sphaeroides* OU5, nearly 90% of L-phenylalanine consumption was observed with simultaneous formation of phenols (Fig. 1). Phenols were extracted into ethylacetate and analyzed using LC-MS. In total, 6 different phenols were identified based on their molecular masses. These include L-tyrosine (181 m/z), 3,4-dihydroxyphenylalanine (DOPA; 197 m/z), 3,4-dihydroxyphenyl-pyruvic acid (DOPP; 196 m/z), 3,4-dihydroxyphenyllactic acid (DOPLA; 198 m/z), 3,4-dihydroxyphenylacetic acid (DOPAc; 168 m/z) and protocatechuic acid (PC; 154 m/z). HPLC analysis (Fig. 2 showing intracellular levels of these substrates) confirmed the identity of L-tyrosine, DOPA and DOPP, which eventually might gave rise to DOPLA, DOPAc and finally protocatechuate (PC). The overall stoichiometric yields of tyrosine, DOPA and DOPP as calculated from HPLC analysis extrapolated to 1 mM and after 30 min of assay were as follows:

$$\text{L-phenylalanine}\ (1\ \text{mM}) \rightarrow \text{L-tyrosine}\ (0.35\ \text{mM}) \rightarrow \text{DOPA}\ (0.27\ \text{mM}) \rightarrow \text{DOPP}\ (0.25\ \text{mM}).$$

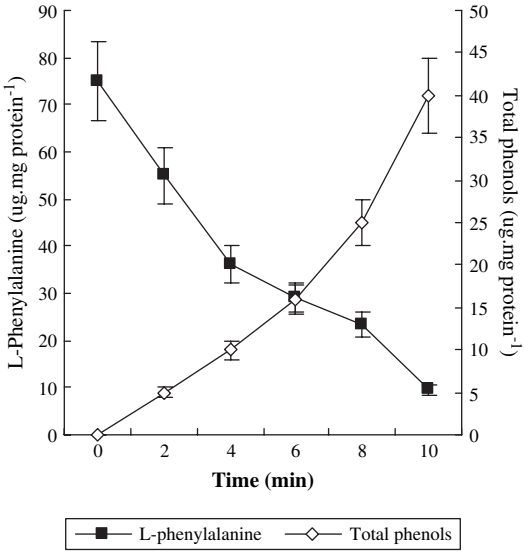

Fig. 1. L-Phenylalanine consumption and production of total phenols by cell-free extracts of *R. sphaeroides* OU5.

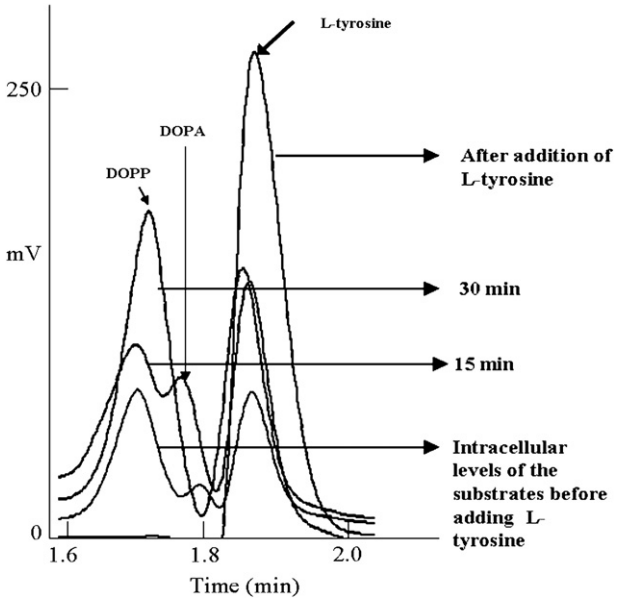

Fig. 2. HPLC chromatogram showing conversion of L-tyrosine to DOPA and DOPP by *R. sphaeroides* OU5.

3.3. Transaminase activity

The culture supernatant did not yield ammonia, which indicated that L-phenylalanine assimilation as nitrogen source may not be through a deamination process; hence, we looked for transaminase activities. Aromatic aminotransferase activity was measured in the presence of L-phenylalanine, L-tyrosine and DOPA as substrates in the presence of 2-oxoglutarate, and loss in the substrate was analyzed through HPLC. The respective activities were 6, 10 and 21 (μmoles mg protein^{-1} min^{-1}). Increased transaminase activity with substrates L-phenylalanine < L-tyrosine < DOPA suggested that transamination occurred at the level of DOPA. This result probably explains the low aminotransferase activity observed with substrates 2-oxoglutarate and L-tyrosine in *R. capsulatus* [16].

DOPA consumption stagnated in the absence of a supplemented keto acceptor, which was restored only in the presence of 2-oxoglutarate (other substrates tested were pyruvate and oxaloacetic acid). The transaminated product of DOPA was extracted (after acidifying to pH 4) into ethyl acetate, concentrated and analyzed using LC-MS. A mass of 196 (m/z) indicated the product as 3,4-dihydroxy phenylpyruvic acid (DOPP). Though DOPA transaminase (EC 2.6.1.49) was reported earlier from animal systems [9] and bacteria [*Alcaligenes faecalis* [13] and *Enterobacter cloaceae* [14]], to the best of our knowledge, this enzyme has not been reported thus far from anoxygenic phototrophic bacteria.

3.4. DOPA reductive deaminase

The product of deaminase, ammonia, was observed only in the absence of 2-oxoglutarate. The deamination product of DOPA was extracted (after acidifying; pH 4.0) into ethyl

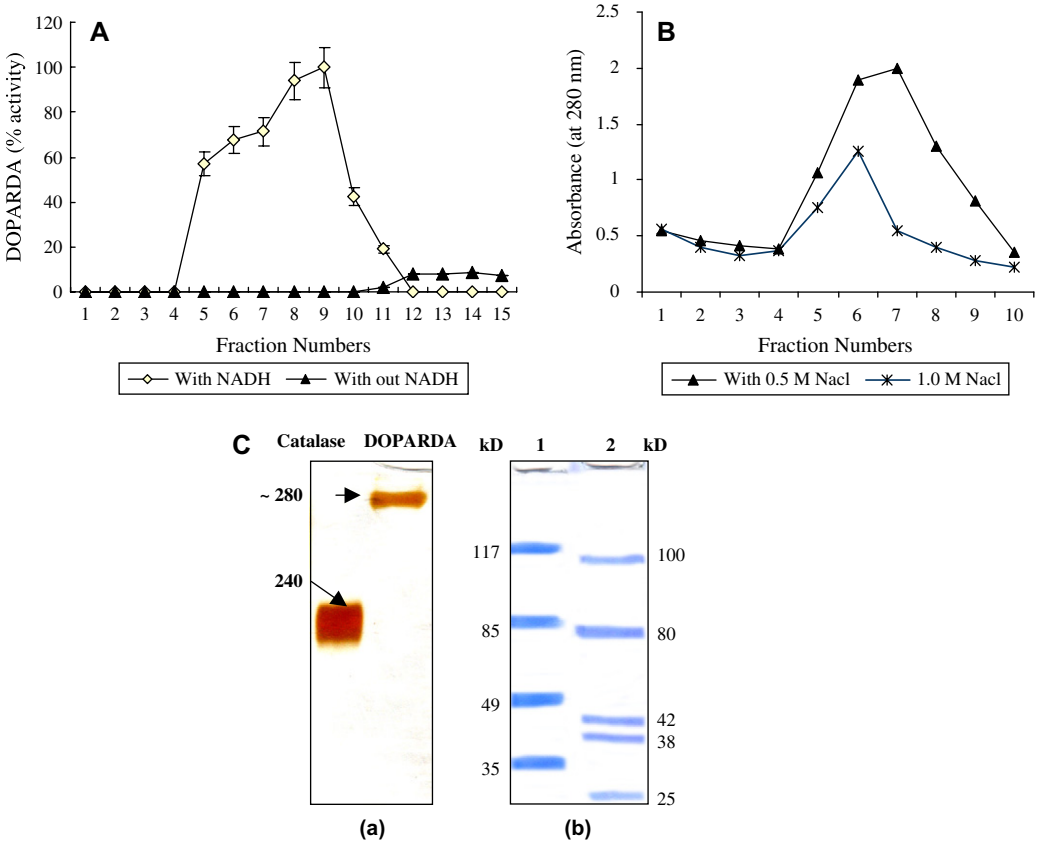

Fig. 3. DOPA-reductive deaminase activity of Sephadex G-150 fraction (A). Protein fractions eluted through anion-exchange (DEAE-Cellulose) (B). Fraction 6 of 1 M NaCl elution was used for 8% Native-PAGE (Ca; 20 μg protein) and 10% SDS-PAGE (Cb; 60 μg protein) analysis (C).

acetate, concentrated and analyzed using LC-MS. A mass of 182 (m/z) indicated the product was 3,4-dihydroxyphenylpropionic acid (DOPPA); this reaction was catalyzed by reductive deaminase. Though the enzyme L-phenylalanine ammonia lyase (EC. 4.1.1.5), which converts L-phenylalanine into *trans*-cinnamate, is most commonly observed in plants [6] and recently also in prokaryotes [23], there are no reports thus far (to the best of our knowledge) about the enzyme DOPA reductive deaminase; hence, we have gone ahead with purification and characterization of the enzyme.

The enzyme deaminase was purified to homogeneity through ammonium sulfate (25% saturation) precipitation, followed by dialysis, Sephadex G-150 gel filtration chromatography (Fig. 3A) and finally anion exchange chromatography (Fig. 3B). Fraction 6 of 1 M NaCl elution had the pure protein of reductive deaminase, which has a molecular mass of ≈274 kD in a native-PAGE (8%) (Fig. 3Ca). The enzyme DOPARDA could be a heterotetramer of 110, 82, 43 and 39 kD subunits, as per SDS-PAGE (10%) (Fig. 3Cb). This enzyme has an obligate requirement for NADH (Fig. 3A), low activity (<40%) with NADPH and no activity with FADH. LC-MS metabolite profiling of the enzyme reaction mixture (assayed in the presence and absence of NADH) was done by elucidating the products of the enzyme. In the presence of NADH (Fig. 4A), molecular masses of 181, 182 $[M + H]^{+1}$ and 198 were identified as L-tyrosine, DOPPA and DOPLA, respectively. In the absence of NADH (Fig. 4B), a molecular mass of 197 was observed, which corresponded to DOPA. These results further confirm that the enzyme reductive deaminase is very specific for DOPA, since DOPA accumulated in the absence of NADH, while it

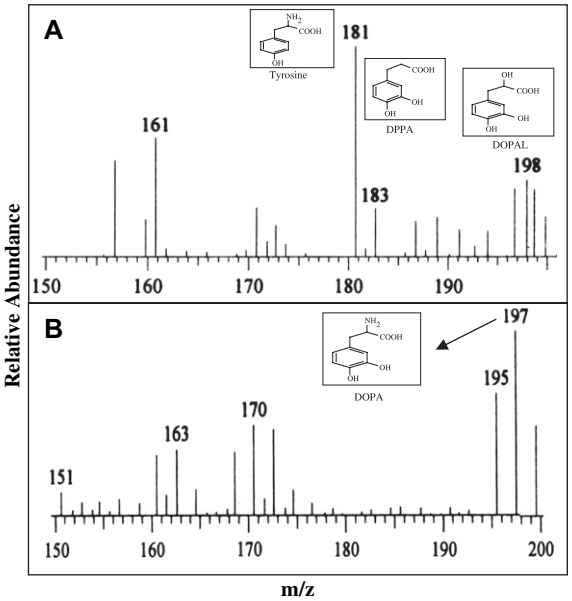

Fig. 4. LC-MS analysis of supernatants of DOPARDA enzyme activity assayed in the presence (A) and absence (B) of NADH.

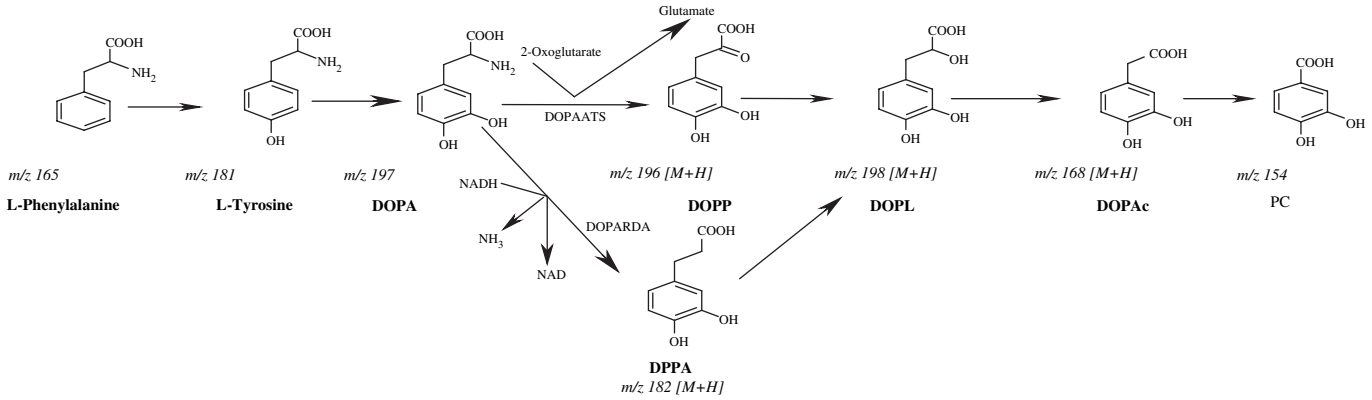

Fig. 5. Proposed intracellular catabolism of L-phenylalanine by *R. sphaeroides* OU5. The proposed pathway is based on experimental evidence observed in the present study.

proceeded in the presence of NADH to the products DPPA and DOPLA when L-tyrosine was the substrate. Enzyme activities (U) with substrates L-tyrosine, DOPA and L-phenylalanine were 1, 0.8 and 0.4 respectively. K_m (μM) and V_{max} of the enzyme DOPARDA were 0.21 ± 0.23 and 0.065 ± 0.07, respectively, with pH optimum at 7.0 (range 6–7) and the temperature at 40 °C (range 30–50 °C).

In conclusion, L-phenylalanine and L-tyrosine metabolism by bacteria has been clearly elucidated [1,7,8,12]. Though earlier studies [16] suggested that L-tyrosine was an intermediate and homogentisate as the end product of L-phenylalanine catabolism by *R. capsulatus*, the aminotransferase activity and yields of homogentisate were low. Furthermore, the hydroxyl group of L-tyrosine (4-hydroxyphenylalanine) is in the 4th position, while homogentisate is 2,5-dihydroxyphenylacetic acid. This shift in the hydroxyl group could be due to migration of the pyruvate side chain [16]. In the present investigation, the experimental evidence suggests that DOPA is the downstream product of L-tyrosine in *R. sphaeroides* OU5. It now remains to be investigated whether L-tyrosine is truly the intermediate in the homogentisate pathway of L-phenylalanine catabolism even in other bacteria [2,12]. In recent years, evidence of production of DOPA by *Erwinia herbicola* from L-tyrosine [10], together with isolation of the enzyme DOPAATS even from other bacteria [13,14], suggest wide distribution of the proposed DOPAATS pathway (Fig. 5) even among other bacteria. In addition, our study indicates the presence of a novel enzyme, DOPARDA, which contributes to our knowledge of L-phenylalanine and L-tyrosine microbial metabolism.

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ORIGINAL RESEARCH PAPER

Rhodethrin: a novel indole terpenoid ether produced by *Rhodobacter sphaeroides* has cytotoxic and phytohormonal activities

N. Kumavath Ranjith · Ch. Sasikala · Ch. Venkata Ramana

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Abstract A novel metabolite was isolated from the culture supernatants of *Rhodobacter sphaeroides* OU5 when grown on L-tryptophan as sole source of nitrogen under photoheterotrophic conditions. It was identified by IR, NMR (^{1}H, ^{13}C) and MS as an indole terpenoid ether [3-hydroxy-6-(1H-indol-3-yloxy)-4-methylhexanoic acid] and is named as rhodethrin. Rhodethrin at 0.5 μM gave positive test in auxin bioassay and initiated early rooting in tissue-cultured plants than IAA at 5 μM. Rhodethrin has cytotoxic activity against Sup-T_1 lymphoma and Colo-125 cancer cell lines at 10 nM.

Keywords Cytotoxicity · Indole terpenoid ether · Phytohormone · *Rhodobacter sphaeroides*

N. K. Ranjith · Ch. V. Ramana (✉)
Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500 046, India
e-mail: r449@sify.com

Ch. Sasikala
Environmental Microbial Biotechnology Laboratory, Center for Environment, IST, JNT University, Kukatpally, Hyderabad 500 085, India

Introduction

Anoxygenic phototrophic bacteria are a physiological group of photosynthetic prokaryotes, which are distributed in four different phyla. These bacteria are gaining importance with respect to the production of novel metabolites. Indole terpenoid esters, including sphestrin (Sunayana et al. 2005a) and rhodestrin (Sunayana et al. 2005b), have been reported from these bacteria. Sphestrin had no biological activity though rhodestrin had good phytohormonal activity, while neither compound had cytotoxicity. In the present communication, we report a novel molecule which has phytohormonal and cytotoxic activities that is produced by *Rhodobacter sphaeroides* OU5 when grown on a medium containing L-tryptophan as sole nitrogen source.

Materials and methods

Organisms and growth conditions

Rhodobacter sphaeroides OU5 (ATCC 49885; DSM 7066) was grown photoheterotrophically (anaerobic, with light at 2,400 lux) in fully filled (10 × 100 mm) screw-cap test tubes or in reagent bottles (250/1000 ml), using Biebl and Pfennig's (1981) mineral medium with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen sources, respectively, at 30 ± 2°C. For growth on L-tryptophan

as nitrogen source, ammonium chloride was replaced with L-tryptophan (1 mM).

Bulk cultivation, solvent extraction, isolation and purification of the indole derivatives

About 10 l of the L-tryptophan-induced (6 h) culture was harvested by centrifugation (16,000*g*; 10 min) and the supernatant was extracted three times with ethyl acetate, pooled and the extract was concentrated under vacuum (Fraction A). Fraction A was further purified using a silica gel (80–120 mesh) glass column (450 × 150 mm) and eluted first with 50 ml benzene (S1 fraction), followed by 50 ml methanol + benzene (7:3 v/v) (S2 fraction) and finally with 50 ml hexane (S3 fraction). Rhodethrin was purified from the S1 fraction.

Assay

Growth was followed turbidometrically at 660 nm. Tryptophan (Kupfer and Atkinson 1964) and indole were estimated by Ehrlich or Salper's reagent (Sunayana et al. 2005a). NMR spectrum of the purified compound in 0.4 ml $CDCl_3$ in a 5 mm NMR tube was recorded on Brucker AC 200 (200 MHz) spectrometer, operating at 50 and 200 MHz for ^{1}H and ^{13}C, respectively. Infra-red (IR) analysis was done on a Shimadzu FT/IR 5300 spectrophotometer and mass analysis using a Mass VG 70-70H analyzer. Auxin coleoptile bioassay and rooting effects in tissue culture (Sunayana et al. 2005b), cytotoxic activity against Sup-T_1 lymphoma and Colo-125 cancer cell lines (Mosman 1983) and COX-2 inhibitory (Copeland et al. 1994) studies were done using standard protocols.

Results and discussion

Rhodobacter sphaeroides OU5 was able to utilize L-tryptophan as sole source of nitrogen (malate served as carbon/e^- donor) under phototrophic conditions. Indole production was observed simultaneously during growth and the supernatant turned orange with Ehrlich reagent. Purification of the metabolites through fractionation yielded an orange-brown fraction S1, which became pink with Ehrlich reagent and orange with Salper's reagent. The compound proved to be pure as analyzed by preparative TLC [solvent system: methanol + benzene + hexane (1:1:1 by vol.)] having a R_F of 0.32 and thus differ red from standard indole, which has a R_F of 0.97. The purity of the compound was further confirmed using HPLC. The S2 fraction (see ''Methods'') turned yellow with Ehrlich reagent, which may be due to the presence of anthranilate and hence was not used for further studies.

The structure of the metabolite (Fig. 1) is based on IR, NMR (^{1}H, ^{13}C) and mass analysis. We named this newly isolated metabolite as rhodethrin. A UV spectral peak of the metabolite at 270 nm confirmed the indole nucleus and a peak at 419 nm indicated the presence of a terpenoid. This compound differs from sphestrin (Sunayana et al. 2005a) or rhodestrin (Sunayana et al. 2005b) in having an ether linkage rather than ester linkage and in the length of the terpenoid side chain. The yield of rhodethrin is 250 μM from 1 mM L-tryptophan.

Phytohormonal activity

Rhodethrin gave a positive coleoptile test in an auxin bioassay. It promoted root initiation of mulberry (*Mouras alba*) in tissue culture. The compound

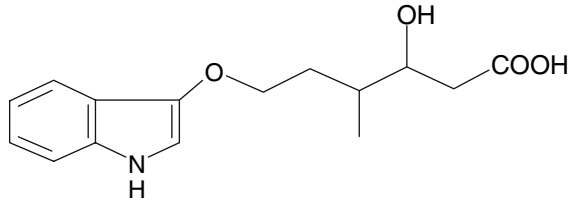

Fig. 1 Structure of the metabolite isolated from the culture supernatant (S1 fraction) during growth on L-tryptophan by *Rhodobacter sphaeroides* OU5. The metabolite is an orange brown solid; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.93(t, 2H), 7.00–6.97(m, 4H), 5.65(m, 3H), 3.73(s, 3H), 2.00(m, 2H), 1.72–1.50(m, 2H), 1.25(m, 4H); 0.95(d, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 132.7 (C-3′), 130.98 (C-5′), 129 (C-7), 118 (C-1), 116 (C-1′), 113 (C-6′), 112.9 (C-4′), 91.5 (C-1), 72 (C-1), 70 (C-1), 42.2 (C-2), 38.0 (C-8), 35.7 (C-5), 32.1 (C-4), 19.6 (C-3); IR (KBr pellet; cm^{-1}) 3414, 2959, 2926, 1716, 1614, 1456, 1414, 1277, 1246, 1089, 742 and 497 (asymmetrical C–O–C stretches band at 1275–1200 [7.84–8.33 nm] confirm the aryl alkyl ether); EIMS m/z 279 $[M]^+$; anal. C 64.2% and H 6.8%, calcd. for $C_{15}H_{19}NO_4$, C 64.97%, H 6.91%. The IUPAC name of the metabolite is 3-hydroxy-6-(1H-indol-3-yloxy)-4-methyl-hexanoic acid which is named as rhodethrin (rhod = from *Rhodobacter*; ethr = ether; in = indole)

induced rooting as well as shooting (Fig. 2A) at 0.5 μM in comparison to indole 3-acetic acid (IAA) at 5 μM. Further, root initiation was observed with in 8–10 days in rhodethrin-treated plants, while with IAA it was observed only after 12–15 days. Rhodethrin-treated plants had increased root length and thus it differs from rhodestrin, where profuse rooting was observed (Sunayana et al. 2005b).

Cytotoxicity test

The cytotoxicity of rhodethrin against Sup-T_1 (T cell lymphoma) (Fig. 2B) and Colo-125 (Fig. 2C) cancer cell lines was determined using the MTT method. Both the crude extract (fraction-A) and rhodethrin had maximum cytotoxicity on the cancer cell lines even at 10 nM (Fig. 2B, C).

Cyclooxygenase 2 inhibitory activity

Rhodethrin (100 μM) inhibited COX-2 activity by 25%, while the crude extract (fraction A) was >50%. On the other hand, neither the crude extract nor rhodethrin inhibited COX-1 activity.

Conclusions

Rhodethrin is the only molecule so far isolated as a product from anoxygenic phototrophic bacteria which has phytohormonal activity, cytotoxicity against cancer cell lines and also has some COX-2 inhibitory activity. Since many indole esters are potential bioactive molecules (Olgen and Nebioglu 2002) and are so far synthesized only through chemical routes

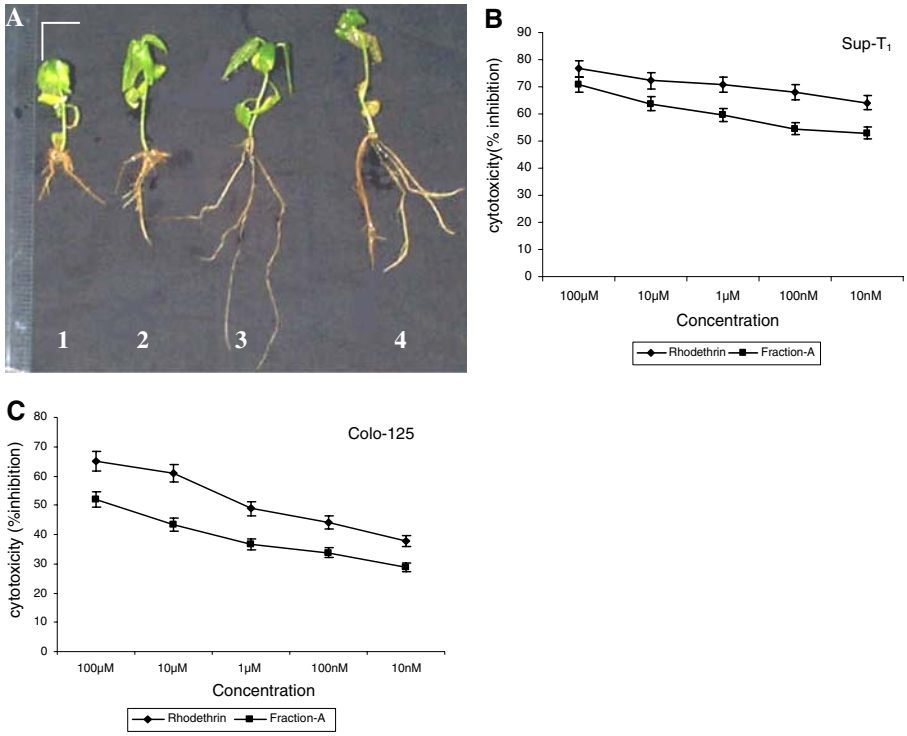

Fig. 2 (**A**) Photograph showing the phytohormonal activity (**A**) of rhodethrin along with standard phytohormones (Bar—see photograph 1 = 0.5 cm). 1 = control (without hormones); 2 = IAA (5 μM); 3 = rhodethrin (0.5 μM); 4 = crude extract (Fraction-A). Details of crude extract (Fraction A) and purified rhodethrin are given in Materials and methods. Photograph pertains to the samples harvested on the 15 day of assay on MS medium (pH 7.0), incubated in light (1500 lux) and at 26 ± 2°C; (**B** and **C**) cytotoxicity of rhodethrin against Sup-T_1 (T cell lymphoma) (**B**) and Colo-125 (**C**) cancer cell line as determined by the MTT method (Mosman 1983). Briefly, adherent cancer cell lines (Sup-T_1 & Colo-125) at 0.2 × 10^6 cells ml^{-1} was seeded in 96-well microplates. The adherent cells were incubated for 18 h to allow cell attachment. Rhodethrin solution was added to the cell cultures at 100 μM to10 nM and the cells were incubated for 24 h. MTT solution was added 3 h before the end of the incubation. Cell survival was evaluated with a multiwell scanning spectrophotometer at 570 nm

(Katayama 2000), biological production of rhodethrin by *Rhodobacter sphaeroides* OU5 is worth exploiting.

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Light-Dependent Transformation of Aniline to Indole Esters by the Purple Bacterium *Rhodobacter sphaeroides* OU5

Vijay Shanker,[1] Sunayana Mandala Rayabandla,[1] Ranjith Nayak Kumavath,[1] Sasikala Chintalapati,[2] Ramana Chintalapati[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, J NT University, Kukatpally, Hyderabad 500 072, India

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Abstract. In an attempt to understand the aromatic hydrocarbon metabolism by purple bacteria that do not grow at their expense, we earlier reported 2-aminobenzoate transformation by a purple non-sulfur bacterium, *Rhodobacter sphaeroides* OU5 (Sunayana et al., 2005, J Ind Microbiol Biotech 32:41–45), which is extended in the present study with aniline, a major environmental pollutant. Aniline did not support photo (light anaerobic) or chemo (dark aerobic) heterotrophic growth of *Rhodobacter sphaeroides* OU5 either as a sole source of carbon or nitrogen. However, light-dependent aniline transformation was observed in the culture supernatants and the products were identified as indole derivatives. The transformation was dependent on a tricarboxylate intermediate, fumarate. Five intermediates of the aniline biotransformation pathway were isolated and identified as indole esters having a mass of 443, 441, 279, 189, and 167 with unstoichiometric total indole yields of 0.16 mM from 5 mM of aniline consumed. The pathway proposed based on these intermediates suggest a novel xenobiotic detoxification process in bacteria.

Aniline, a nitrogen-substituted aromatic hydrocarbon, is widely used in the manufacturing of dyes, plastics, herbicides, and pesticides [9]. Owing to its wide usage, it is increasingly released into environments, contaminating soil and water [6]. The metabolism of aniline is restricted to a few microorganisms, which either grow at the expense of aniline aerobically [2]/anaerobically [13, 15] or transform it to products such as anthranilate, phenylhydroxyaniline, N-methylaniline, 2-methylquinone, or oxidized to nitrobenzene [1].

Our studies on the aromatic hydrocarbon transformations have focused mainly on indoles by *Rhodobacter sphaeroides* OU5 [10–12]. *R. sphaeroides* OU5 transforms 2-aminobenzoate to an indole ester [16] and, in the present communication, we report for the first time the transformation of aniline to an indole ester by *R. sphaeroides* OU5 in a light-dependent anoxic process.

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

Materials and Methods

Organism and growth conditions. *R. sphaeroides* OU5 (ATCC 49885; DSM 7066) was grown photoheterotrophically (anaerobic, 30°C; 2,400 lux) in fully filled reagent bottles (250/1000 ml) on Biebl and Pfennig's mineral medium [3] with malate (22 mM) and ammonium chloride (7 mM) as the carbon and nitrogen source, respectively, at 30 ± 2°C.

Photobiotransformation studies. *R. sphaeroides* OU5 was grown (fully filled 250 or 1000 ml reagent bottles) on the photoheterotrophic growth medium (described above) until reaching the late logarithmic phase (about 40–48 h). Aniline (25 mM; unless noted otherwise) was added and the phototrophic incubation continued for an additional 48 h. Photobiotransformation studies using cell suspensions were carried out as described previously [10].

Isolation and purification of the indole compound. The bacterial culture was harvested by centrifugation (16,000*g*, 10 min) and the supernatant used tor purification of the indoles. The ethyl acetate extract of the supernatant was dried in rota-vapprator at 40°C and suspended in ethanol (5 mL). The ethanol extract was loaded onto a 18 × 600 mm column packed with silica (80–120 mesh) and eluted with hexane:ethyl acetate (8:2), benzene, and n-butyl alcohol. Each fraction was eluted with benzene and with methanol to yield EA1 and

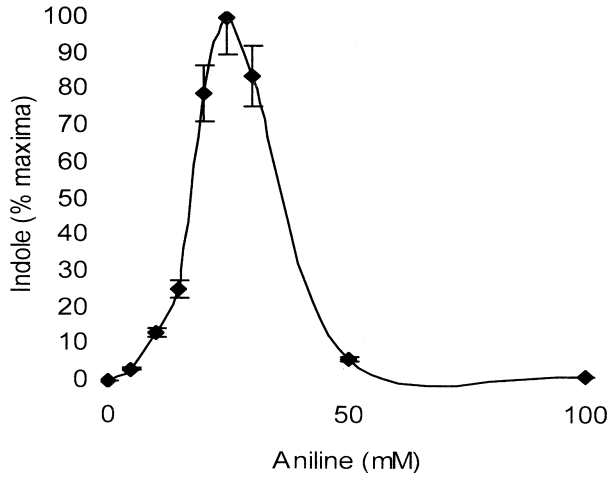


Fig. 1. Effect of aniline concentration on photoproduction of indole by *R. sphaeroides* OU5. Results expressed are the average values of two independent experiments done in duplicate using 48-h photoheterotrophic cultures (100 % indole = 0.16 mM).

X, EA2 and Y, EA and Z. The aqueous layer was acidified with 5N HCl, pH 4.0, extracted with ethyl acetate and dried. It was dissolved in ethanol and loaded onto the column and eluted with methanol and methanol: IN NaOH (9:1), respectively to yield AQ1 and AQ2.

Analytical determination. Growth was observed as an increase in optical density at 660 nm. Aniline produces a color reaction when combined with the Ehrlich reagent [8]. Therefore, Salper's reagent [5] was used for analysis of indole. Aniline was estimated with sodium hypochlorite reagent [10]. Infrared (IR) analysis was done on a Shimadzu FT/IR 8300 and mass analysis using a Mass VG.70-70H analyzer.

Results and Discussion

Aniline (0.5–3 mM) did not support phototrophic growth of *R. sphaeroides* OU5 (data not shown) as either a carbon or nitrogen source, although there are reports of aniline utilization for growth among the anoxygenic phototrophic bacteria, *Rhodopseudomonas palustris* [14]. Since phototrophic bacteria are known to transform many aromatic hydrocarbons [13], even though some do not support growth, it was our objective to look for the aniline transformation by *R. sphaeroides* OU5.

A 20% loss of aniline was observed (48 h) in the culture incubation, compared to the initial concentration. However, there was no disappearance in aniline in the uninoculated control cultures, confirming the enzymatic utilization of the substrate by *R. sphaeroides OU5.* A detailed investigation was conducted to examine aniline metabolism by *R. sphaeroides* OU5.

Detection and estimation of metabolites of aniline transformation by *R. sphaeroides* OU5. A culture supernatant of aniline (3 mM) incubated cells of *R. sphaeroides* OU5 produced a positive (orange) test when exposed to reagent, indicating the presence of indole in the culture supernatant. The yield of this indole was about 10 μM from 3 mM aniline. An increase in the product yield was observed when the aniline concentration was increased from 3 to 25 mM (Fig. 1). However, at 25 mM aniline, a 20% disappearance of aniline was observed, with the yield of 0.16 mM indole (Fig. 1). The indole yield was not in stoichiometry with the 20% of aniline (5 mM) consumed.

Table 1. Effect of substrates on growth (biomass) and photoproduction of indole from aniline by *R. sphaeroides* OU5

Substrate (0.15% w/v)	Biomass yield (mg dry wt·mL^{-1})	Indole photoproduction (mM)
Control (without substrate)	0.12	0.05
Fumarate (13 mM)	0.34	0.16
Malate (11 mM)	0.20	0.14
Succinate (13 mM)	0.20	0.11
Acetate (25 mM)	0.20	0.10
Oxaloacetate (11 mM)	0.20	0.13
Pyruvate (17mM)	0.30	0.13

Results (average of three replicates) using cell suspension and assayed after 48 h of light (2,400 lux) of anaerobic incubation.

The above data indicate that the increasing concentration of aniline increases the substrate consumption as well as the product yield, which may be attributed to the increase of aniline bioavailability, since aniline has limited water solubility [1]. The non-stoichiometric indole yield, even when high concentrations of aniline were used, may be a result of the presence of another class of metabolic intermediates that may not be reacting with Salper's reagent.

Attempts were made to increase the solubility of aniline in the media by dissolving aniline in ethanol (3:1) and by using ethanolic stocks. However, in doing so, no aniline was consumed or any indole product formed. In turn, the cells were lysed and were slimy due to the increasing hydrophobicity of aniline in ethanol, since these hydrocarbons are known to disrupt cell membranes [1]. The presence of an emulsifier such as Triton X-100 (aniline:Triton X-100 [17:1]) has increased indole yield to a certain extent (data not shown) confirming the bioavailability of aniline.

In an earlier study with 2-aminobenzoate [16], fumarate was suggested to be the conjugating carbon for indole photobiosynthesis. Similarly, in the presence of aniline, the high indole yield with fumarate (Table 1) in comparison to various carbon substrates also supports the possible role of fumarate as the conjugating molecule in this transformation. To confirm the role of fumarate in aniline transformation, different analogs of

Table 2. Analogues of fumarate used along with biomass and indole yields

	Fumarate	Maleate	Acrylate	Crotonate	Cinnamate	Control
Indole (mM)	0.16	0.08	0.07	0.04	0	0.05
Biomass yield (mg dry wt·mL^{-1})	0.34	0.09	0.21	0.15	0	0.12

Expermental details as in Fig. 2 except for the presence of analogues and aniline used at a 25-mM concentration.

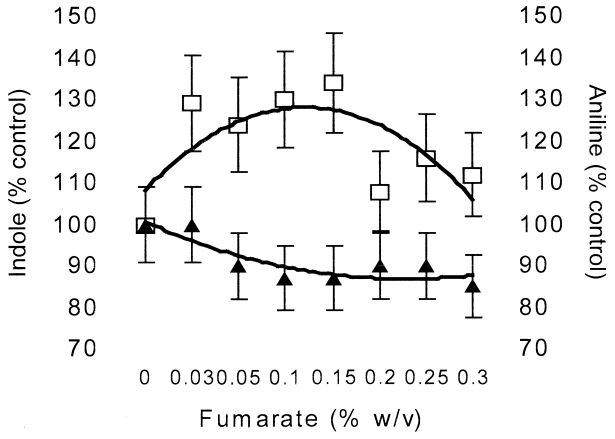


Fig. 2. Photoproduction of indole by *R. sphaeroides* OU5 with various concentrations of fumarate. 100 % control = Indole (□-□) 0.05 mM; Aniline (▲-▲) 25 mM.

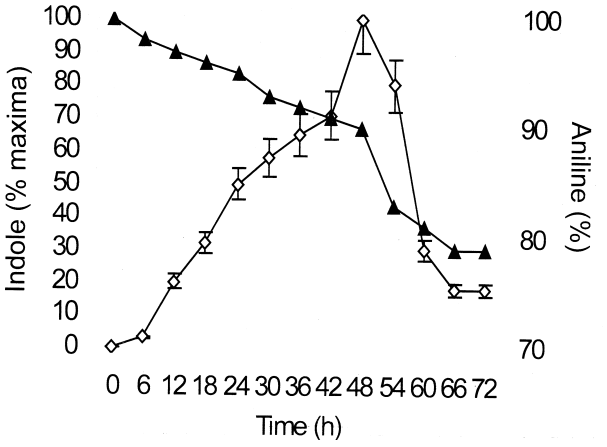


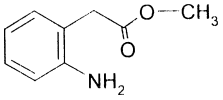
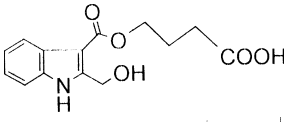
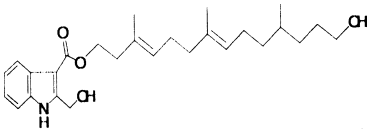
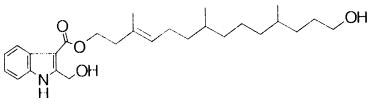
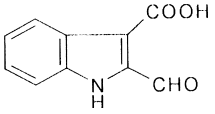
Fig. 3. Kinetics of indole production and aniline consumption by *R. sphaeroides* OU5. Results expressed are the average value of experiments done in duplicate using 48-h photoheterotrophic culture 100 % Indole (◇-◇) = 0.16 mM, 100 % Aniline (▲-▲) = 25 mM.

fumarate were used (Table 2), which partially or completely inhibited indole production. Increasing the concentrations of fumarate simultaneously increased indole yields and aniline consumption (Fig. 2). These data support the potential role of fumarate in aniline transformation too. Fumarate appears to be a very important carbon source not only for aniline transformation, but also for other aromatic hydrocarbon transformations such as 2-aminobenzoate [10], toluene, and xylene [7].

The transformation of aniline to indole by *R. sphaeroides* OU5 was observed only under anaerobic phototrophic conditions and was completely inhibited by aerobic dark/light conditions. Replacing aniline with its derivatives (0.5 mM), like 4-nitroaniline or 4-chloroaniline, did not result in indole, probably due to increased toxicity of these compounds compared to aniline. This is further supported by the complete growth inhibition by these compounds (data not shown) versus the increase in biomass yield in the presence of aniline (Table 1). A time course of indole photoproduction and simultaneous aniline consumption is shown in Figure 3. The data in this figure explain the production of indole during the first 48 h of phototrophic incubations and subsequently the drop in the indole levels indicating the breakage of the indole nucleus. The detailed analysis of isolated intermediates is presented in Table 3.

Based on the experimental evidence available, a possible pathway of photometabolism of aniline by *R. sphaeroides* OU5 is shown in Figure 4. Aniline conjugates with fumarate, yielding an indole derivative, 2-formyl-1H-indole-3-carboxylate. Subsequent esterification with a terpenoid alcohol (yet to be identified) yields sphestrin, ((3E,7E)-14-Hydroxy-3,7,11-trimethyl-3,5-tetradecadienyl 2-(hydroxymethyl)-lH-indole-3- carboxylate), which may be undergoing α-β oxidation (confirmation required) to yield propyl 2-(hydroxymethyl)-1H-indole-3-carboxylate. Finally, the indole ring is broken, forming methyl (2-aminophenyl) acetate.

Table 3. Color reactions, R_F, and mass of the compounds eluted from the supernatant along with structures and IUPAC names

Function	Color reaction P	Color reaction S	R_F[a]	IR (cm^{-1})	Structure	Mass (mZ)	IUPAC name and chemical formula
AQ1	Y	Y	1 = 0.51 2 = 0.54 3 = 0.34	3434 2926 1723		167 $[M+2H]^{+2}$	Methyl (2-amino phenyl) accetate $C_9H_{11}NO_2$
AQ2	PY	O	4 = 0.44	3436 2924 1599 1123		279 $[M+2H]^{+2}$	Propyl 2-(hydronymethyl) III indole-3-carboxylate $C_{14}H_{18}NO_5$
EA1	OB	YO	2 = 0.66 3 = 0.68 7 = 0.64	3456 2928 1771 1040		441 $[M]^+$	(3E,7E)-14-Hydrony-3,7,11-trimethyl-3, 5-tetradecadienyl 2-(hydronymethyl)-1H-indole-3-carbonylate $C_{27}H_{39}NO_4$
EA2	PY	O	1 = 0.57 2 = 0.84 5 = 0.84	3441 2928 1730 1109		443 $[M]^+$	(3E)-14-Hydroxy-3,7,11-trimethyl 3, 5-tetradecadienyl2-(hydroxymethyl)-1H-indole-3-carbonylate $C_{27}H_{41}NO_4$
EA3	O	DO	1 = 0.89 2 = 0.63 3 = 0.61 6 = 0.85	3399 3184 1412		189 [M]	2-Formyl-1H-indole-3-carboxylic acid $C_{10}H_7NO_3$

P = para-diaminobenzaldehyde; S = Salper's; Y = yellow; PY = pale yellow; O = orange; OB = orange brown; YO = yellow orange; DO = deep orange; R_F = retention factor.

[a]Solvents used for thin layer chromatography: 1 = methanol; 2 = methanol + benzene(2:1); 3 = methanol + ethyl acetate (1:1); 4 = methanol + benzene (2:8); 5 = n-butyl alcohol (NBA); 6 = NBA + methanol (1:1); 7 = benzene.

As aniline did not support growth, it is evident that the pathway must be a light-dependent detoxification process by *R. sphaeroides* OU5, that can be divided into three phases (Fig. 4). In phase I, a non-polar aromatic hydrocarbon is converted to an indole derivative with the help of fumarate, an important substrate involved in many anaerobic aromatic hydrocarbon detoxification/transformations [7]. While in phase II, esterification of the indole with a terpenoid lipid occurs, indicating the detoxification of indole, since indoles are known to interfere with the bacterial cell membranes [4]. Phase III results in the breaking of the indole nucleus yielding more polar compounds. Thus, the growth of *R. sphaeroides* OU5 in the presence of aniline (Table 2) explains the detoxification of aniline, which is otherwise inhibited with aniline derivatives (4-chloroaniline or 4-nitroaniline).

The proposed pathway is a novel pathway (Fig. 4) of aromatic hydrocarbon detoxification by microorganisms and is the first report of xenobiotic detoxication by anoxygenic phototrophic bacteria. The innovative adaptation of *R. sphaeroides* OU5 in overcoming the toxicity of aniline explains its ability to detoxify xenobiotics and opens the door for exploring various mechanisms of adaptations by anoxygenic phototrophic bacteria in detoxifying xenobiotics, thus allowing them to survive in polluted environments.

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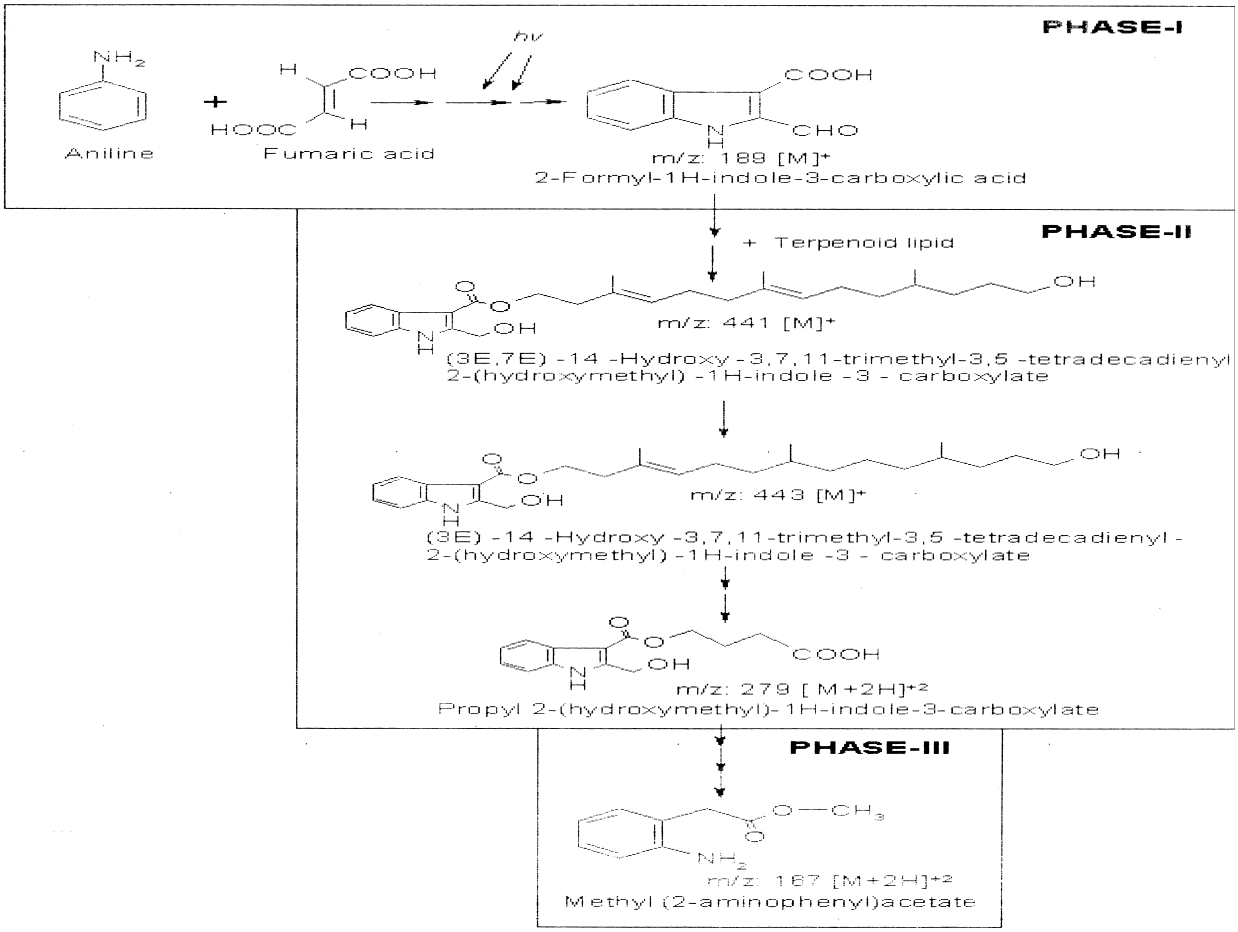

Fig. 4. Postulated pathway of aniline photometabolism by *R. sphaeroides* OU5.

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NOTE

Purification and characterization of 3, 4-Dihydroxyphenylalanine oxidative deaminase from *Rhodobacter sphaeroides* OU5

N.K. Ranjith, Ch.V. Ramana and Ch. Sasikala

N.K. Ranjith and Ch.V. Ramana. [1]Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500 046, India.

Ch. Sasikala. [2] Bacterial Discovery Laboratory, Center for Environment, IST, JNT University, Kukatpally, Hyderabad 500 085, India.

[*]**Corresponding author.** Ch.V. Ramana, e-mail: r449@sify.com; sasi449@yahoo.ie

Abstract

An enzyme involved in the catabolism of 3,4-dihydroxyphenylalanine (DOPA) is isolated from *Rhodobacter sphaeroides* OU5. The enzyme catalyzes the formation of 3,4-dihydroxyphenylpyruvic acid (DOPP) and ammonia from 3,4-dihydroxyphenylalanine (DOPA). Formation of NH_3 by 3,4-dihydroxyphenylalanine oxidative deaminase was O_2 dependent and the enzyme isolated to its homogeneity has 100% affinity for DOPA. 3,4-Dihydroxyphenylalanine oxidative deaminase is functional at low concentrations of the substrate (<100 μM) and is independent of NADH. The molecular mass of the purified enzyme is ≈190 kD and the enzyme could be a pentamer of 54, 42, 34, 25 and 23 kD subunits as determined by SDS-PAGE.

Keywords: L-Phenylalanine, L-tyrosine, 3, 4- Dihydroxyphenylalanine (DOPA), 3,4-Dihydroxyphenylalanine oxidative deaminase, *Rhodobacter sphaeroides* OU5.

Introduction

Deaminases are an important group of enzymes that catalyze the elimination of ammonia from organic substitutes (Barker 1981) and play an important role in the nitrogen cycle. L- Amino acid deaminases identified so far include; deaminases of arginine, glutamate, serine, cytosine (David et al. 2001), L-tyrosine and L-phenylalanine (Camm and Towers 1973; Xiang and Moore 2005). Recently, we have reported the occurrence of a NADH dependent enzyme, DOPA reductive deaminase (EC 4.3.1.22) from *Rhodobacter sphaeroides* OU5 grown on L-phenylalanine or L-tyrosine, where DOPA was the intermediate (Ranjith et al.2007). In addition, a 2-oxoglutarate (an amino acceptor) and pyridoxal-5-phosphate (PLP; as co-factor) dependent DOPA aminotransferase was also

reported from *Rba. sphaeroides* OU5 (Ranjith et al., 2007). In the present communication, we report for the first time a physiologically important enzyme, 3,4-dihydroxyphenylalanine oxidative deaminase catalyzing the reaction of 3,4-dihydroxyphenyalalanine (DOPA) + ½ O_2 → 3,4-Dihydrophenylpyruvic acid (DOPP) + NH_3, which is active under the limitation of a reducing equalent (NADH) or an amino acceptor (2-oxoglutarate).

Organism and growth conditions

Rhodobacter sphaeroides OU5 (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 (250 /2000 ml) on a mineral medium (Biebl and Pfennig 1981) with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen sources, respectively, at 30 ± 2 °C. For growth on L-phenylalanine or L-tyrosine, ammonium chloride was replaced with the respective aromatic amino acids (1 mM) used as nitrogen source, or malate was replaced when used as carbon source or both malate and ammonium chloride were replaced when used as sole source of carbon and nitrogen.

Cells were harvested by centrifugation (16,000 x g for 10 min at 4 ^{o}C) at logarithmic phase; pellet was washed (twice) with 0.05 M potassium phosphate buffer (pH 7.8) and resuspended in the same buffer. Cells were lysed by sonication using MS-72 probe (Bandelin, Germany make, model-UW 2070) to complete cells lyses after 8-9 cycles. The sonicated cell suspension was centrifuged (16,000 x g for 30 min at 4 ^{o}C) and the supernatant was used as the source of enzymes.

Analytical determinations

Protein (Bradford 1976), ammonia (Solórzano 1969) and 3,4-dihydroxyphenylpyruvate (Briggs 1922) were analyzed using standard protocols. Purity of the protein was confirmed using Schimadzu LC-20AT HPLC with florescence detector (acetonitril in 0.3% TFA at 1 $ml.min^{-1}$ was used as solvent). Mass analysis was done using Shimadzu LC-MS-2010A. Analysis was performed at 40 ºC for LC column oven and at 85 ºC for MS ionization chamber: methanol plus water (1:1) was used as solvent at 0.2 $ml.min^{-1}$, Luna 5 µ C_{18} (2) 100A was the column (250 x 4.6 mm) and the compounds were detected (LC) at 254 nm. The column effluent from the LC was nebulized into Electron Spray Ionization (ESI) or Atmospheric Pressure Chemical Ionization (APCI) region under N_2 gas for generating molecular masses. HPLC analysis of substrates/products was performed at room temperature using a Shimadzu SPD-10AVP isocratic system as described earlier (Ranjith et al. 2007).

DOPA oxidative deaminase activity

Enzyme activity wa**s** measured as the amount of ammonia or DOPP liberated from DOPA. The products ammonia and DOPP were quantified as described above. The reaction mixture contained, in final volume of 0.5 ml of potassium phosphate buffer (0.05 M; pH 7.8), 100 µM DOPA (with out 2-oxoglutarate [amino acceptor] or NADH) and appropriate amount of partially purified enzyme. The reaction was carried out at $30^{o}C$, and sample was taken every 50 sec (unless other wise mentioned). The protein was eliminated by acidification with 10% acetic acid (vol/vol, final concentration) and further centrifugation at 6000xg for 5 min. The supernatant was used for the analysis of substrate or product. A unit (U) of enzyme activity is defined as the amount of enzyme that

catalyzes the formation of 1 μmole NH_3.mg protein^{-1}.min^{-1} in the absence of 2-oxoglutarate or NADH.

The enzyme was purified from the cell free extracts of L-tyrosine-induced cells of *Rba. sphaeroides* OU5. Ammonium sulphate was added to the supernatants 10-70% saturation, keeping pH at 7.8. The proteins precipitated were separated by centrifugation (16,000 x g for 30 min at 4 ^{0}C), resuspended (5 ml) in 0.05 M potassium phosphate buffer (pH 7.5) and dialyzed against three-four changes of 2 litre of the same buffer for 2 hours each. The enzyme was partially purified using gel exclusion (Sephadex G-150) column chromatography (95 x 1.6 cm) by eluting at a flow rate of 1 ml per 5 minutes. In total, 35 fractions were collected and all the fractions were assayed for the enzyme activity.

Fractions 20, 21, 22, 23 & 24 of gel-eluted proteins were applied on a CM-Cellulose column (10 x 4.5 cm) for further purification. The bound proteins were eluted using NaCl gradients (0.5, 1.0, 1.5 M NaCl) in 0.05 M potassium phosphate buffer (pH 7.5). The eluted fractions were assayed for the enzyme activity and highest active fraction eluted with 1.5 M NaCl was used for (8%) non-denaturing PAGE. The molecular weight of the purified DOPA-oxidative deaminase was determined using the gel filtration (Sephadex G-150) column.The void volume of the column was determined using Blue Dextran that was calibrated with standard proteins: catalase (240 kD), esterase (166 kD), bovine serum albumine (BSA-67 kD), ovalbumine (43kD) and trypsinogen (24 kD). Purity of the protein was confirmed using a reverse-phase HPLC with a C_{18} column (150x 4.6 mm) and eluted with acetonitril in 0.3% TFA at 1 ml.min^{-1} flow rate. IC_{50} values for NADH and 2-oxoglutarate was determined using the purified DOPA-oxidative deaminase. The kinetic constant of the enzyme was done by non-linear curve fitting of the reciprocal plot.

Oxygen consumption

O_2 uptake by the purified DOPAoxidative deaminase was monitored using a Clark type O_2 electrode (DW2, Hansatech Ltd., King's Lynn, UK). The 1 ml (0.05 M Potassium phosphate buffer; pH 7.8) reaction mixture contained 100 µmole L-DOPA and 25µg enzyme. Water at a constant temperature of 25 °C was circulated through the outer jacket of the reaction chamber. Calibration of the O_2 content in the electrode chamber was done with air-saturated water, assumed to contain 253 nmole $O_2.ml^{-1}$ at 25 °C.

Extraction of metabolites

After enzyme assay, the metabolites were extracted (after acidified; pH 4.0) thrice into ethyl acetate. The ethyl acetate layers were pooled and 5 mg of anhydrous sodium sulphite was added to remove water molecules. The ethyl acetate extract was evaporated to dryness under vacuum, resuspended in HPLC grade methanol and directly used for LC-MS or HPLC.

Identification of 3,4-dihydroxyphenylalanine oxidative deaminase in *Rba. sphaeroides* OU5

It was observed (Fig. 1 A) that ammonia formation from DOPA by the Sephadex G-150 gel eluted fractions 4 to 10 was dependent on NADH, while fractions 17 to 30 were independent of NADH. In an earlier study, we reported (Ranjith et al. 2007) the characters of the NADH dependent DOPA-reductive deaminase together with the 2-oxoglutarate dependent DOPA-aminotransferase. Ammonia formation from DOPA in the absence of NADH is possible by two mechanisms, one by the ammonia lyase and the other due to an oxidative deaminase. The enzymes L-phenylalanine/L-tyrosine ammonia lyase (PAL/TAL; EC. 4.3.1.25) was commonly observed in plants (Camm and Towers

1973) and in a few prokaryotes (Xiang and Moore 2005) while DOPA ammonia lyase (EC 4.3.1.11) was recorded only from plants (MacLeod and Pridham 1963). We could not detect (by HPLC/LC-MS) either *trans*-cinnamate, 4-hydroxy *trans*-cinnamate or 3,4-dihydroxydroxy *trans*-cinnamate (caffeate) form the assay supernatant when substrates L-phenylalanine, L-tyrosine or DOPA, respectively were used. These results indicate that ammonia formation in the absence of either NADH or 2-oxoglutarate is not due to the involvement of ammonia lyases in *Rba. sphaeroides* OU5. There are reports of tyrosine ammonia lyase (TAL) from *Rba. sphaeroides* and *Rba. capsulatus* (Kyndt et al. 2002). We have identified the product of the enzyme assay using LC-MS as 3,4-dihydroxyphenypyruvic acid (DOPP) and the same was confirmed using HPLC, indicated the presence of an active oxidative deaminase in *Rba. sphaeroides* OU5. In a mutant strain of *E. coli,* mutation of tyrosine332 to phenylalanine converted DOPA decarboxylase (EC 4.1.1.28) into an oxidative deaminase (Bertoldi et al., 2002), which was pH dependent (Bertoldi and Voltattoni, 2001). Except for this enzyme, to the best of our knowledge, DOPA oxidative deaminase was not reported till date and hence we went for its isolation, purification and characterization.

Isolation & purification of DOPA-oxidative deaminase

3,4-Dihydroxyphenylalanine oxidative deaminase was partially purified through gradient ammonium sulphate saturation and the highest activity was achieved with 25% saturation, which was further purified by dialysis and gel filtration (Sephadex G-150) chromatography (see materials and methods) (Fig. 1A). These pooled fractions were subjected to cation exchange chromatography (CM cellulose) (Fig. 1B). Fraction 7 & 8 of 1.5 M NaCl elution had the pure protein (Fig. 2A; 2C) of oxidative deaminase, which

has a molecular mass of ≈190 kD (Fig. 2D). The enzyme DOPA-oxidative deaminase could be a heteropentamer of 54, 42, 34, 25 and 23 kD subunits as per SDS-PAGE (12%) (Fig. 2B).

Characterization of DOPA-oxidative deaminase

The enzyme specific activity with the substrates, DOPA, L-tyrosine, L-phenylalanine, L-tryptophan, glutamate, DL-alanine and glycine were 1.0, 0.8, 0.6, 0.5, 0.6, 0.2 and 0.3 μmole NH_3.mg protein^{-1} min^{-1}, respectively. The LC-MS analysis of the product when DOPA (Fig. 3A) or L-tyrosine (Fig. 3B) was used as substrates indicated the end products as 3,4-dihydroxyphenylpyruvic acid (DOPP) and 4-hydroxyphenylpyruvic acid, respectively. The products, 3,4-dihydroxyphenylpyruvic acid and 4-hydroxyphenylpyruvic acid were confirmed by HPLC which had a R_t of 1.7 and 1.9, respectively. Oxygen requirement for the formation of 3,4-dihydroxy phenylpyruvate from DOPA was confirmed using an oxygen electrode (Fig. 4). The K_m (μM) and K_{cat} (s^{-1}) of the enzyme DOPA oxidative deaminase was 11.84 $\pm$ 1.80 and 0.680 $\pm$ 0.023, respectively,.The DOPA oxidative deaminase was inhibited by NADH (IC_{50} ~35 μM) and 2-oxoglutarate (IC_{50} ~25 μM), which may explain the physiological role of the enzyme to be active under the limitation of these substrates. DOPA-oxidative deaminase isolated from *Rba. sphaeroides* OU5 did not exhibit any decarboxylase activity and thus differ from the enzyme earlier reported from *E. coli* (Bertoldi and Voltattoni 2001) and also differs (Table 1) from the DOPA aminotransferase and DOPA-reductive deaminase of *Rba. sphaeroides* OU5 (Ranjith et al. 2007), which contributes to our knowledge of L-phenylalanine and L-tyrosine microbial metabolism.

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Fig. 1. DOPA-oxidative deaminase activity of Sephadex G-150 fraction (A)Protein fractions eluted through cation-exchange (CM-Cellulose) (B).

Fig. 2. Fractions 7-8 of 1.5 M NaCl elution was used for Native-PAGE (8%) (A; 25 μg protein) and 12% SDS-PAGE (B; 60 μg protein). (C) HPLC chromatogram showing the purity of the protein; (D) Molecular weight was determined by gel filtration (Sephadex-G-150) method, using standard proteins (catalase-240 kD, esterase-166 kD, BSA-67 kD, ovalbimin-43 kD, trypsinogen-24 kD);

Fig. 3. The LC-MS analysis of supernatant of DOPA-oxidative deaminase activity assayed with the substrates L-DOPA (A) and L-tyrosine (B).

Fig. 4. Consumption of L-DOPA and O_2 and formation of ammonia and 3,4-dihydroxyphenylpyruvic acid (DOPP) during the reaction by the purified enzyme.

Table 1. Differentiating characters of the three enzymes of *Rba. sphaeroides* OU5 involved in ammonia liberation /transformation of DOPA, an intermediate in the catabolism of L-phenylalanine of *R. sphaeroides* OU5.

[%]Results are means ± SD of three different determinations done in duplicates. *Isolated from Pig brain. α-KGA = 2-oxoglutarate; PLP = Pyridoxal-5-phosphate; NADH = Nicotinamide adenine dinucleotide; Nil = No requirement; kD = Kilo Dalton; DOPP = 3,4-dihydroxyphenyl pyruvic acid; DOPPA = 3.4-dihydroxyphenyl propionic acid; DOPA= 3,4-dihydroxyphenylalanine. ND = Not determined

Enzyme	Product	Co-substrates	Co-factors	K_m (μM)	K_{cat} (s^{-1})	M.Wt. (kD)	Subunit (determined by SDS-PAGE) (kD)	References
%L-DOPA-oxidative deaminase	DOPP	O_2	Nil	11.84±1.80	0.680±0.023	~ 190	Pentamer (54, 43, 34, 25, 22)	In this study
L-DOPA-reductive deaminase	DOPPA	Nil	NADH	21.23±0.09	0.0636±3.0	~ 274	Hetero-tetramer (117, 85, 49, 35)	Ranjith et al. (2007)
L-DOPA-amino transferase	DOPP	2-KGA	PLP	4.1	ND	ND	Homodimer	*Fonnum et al., (1965)
				0.35±0.045	0.29±3.0	~ 123	Heterodimer (60, 63)	Ranjith et al. (2007)

Table-1

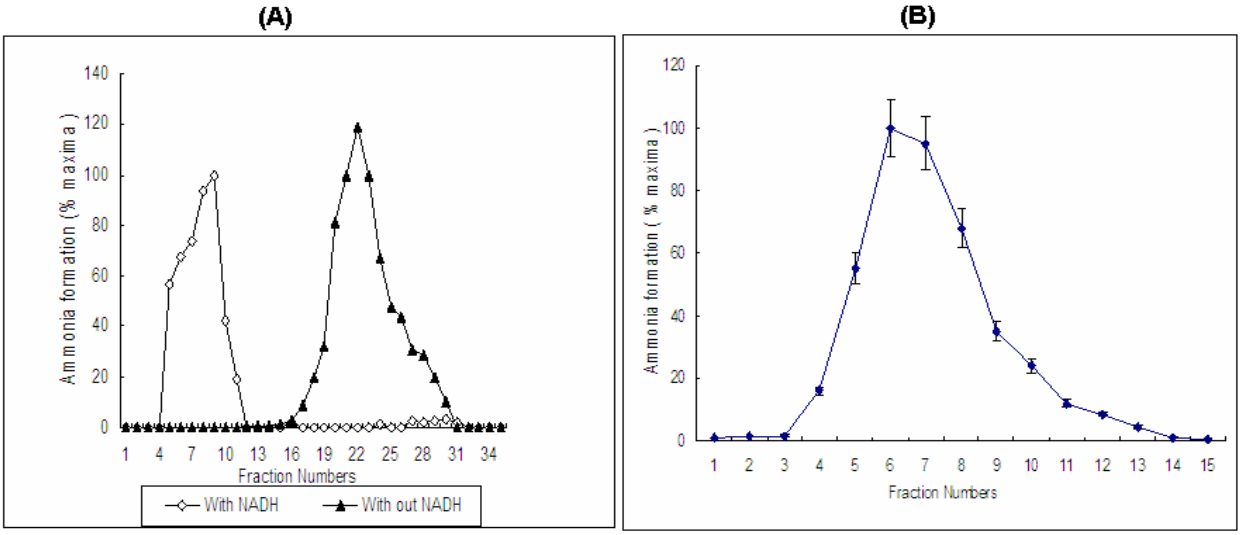
(A)
Ammonia formation (% maxima)
140
120
100
80
60
40
20
0
1 4 7 10 13 16 19 22 25 28 31 34
Fraction Numbers
With NADH
With out NADH
(B)
Ammonia formation (% maxima)
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Fraction Numbers

Fig-1

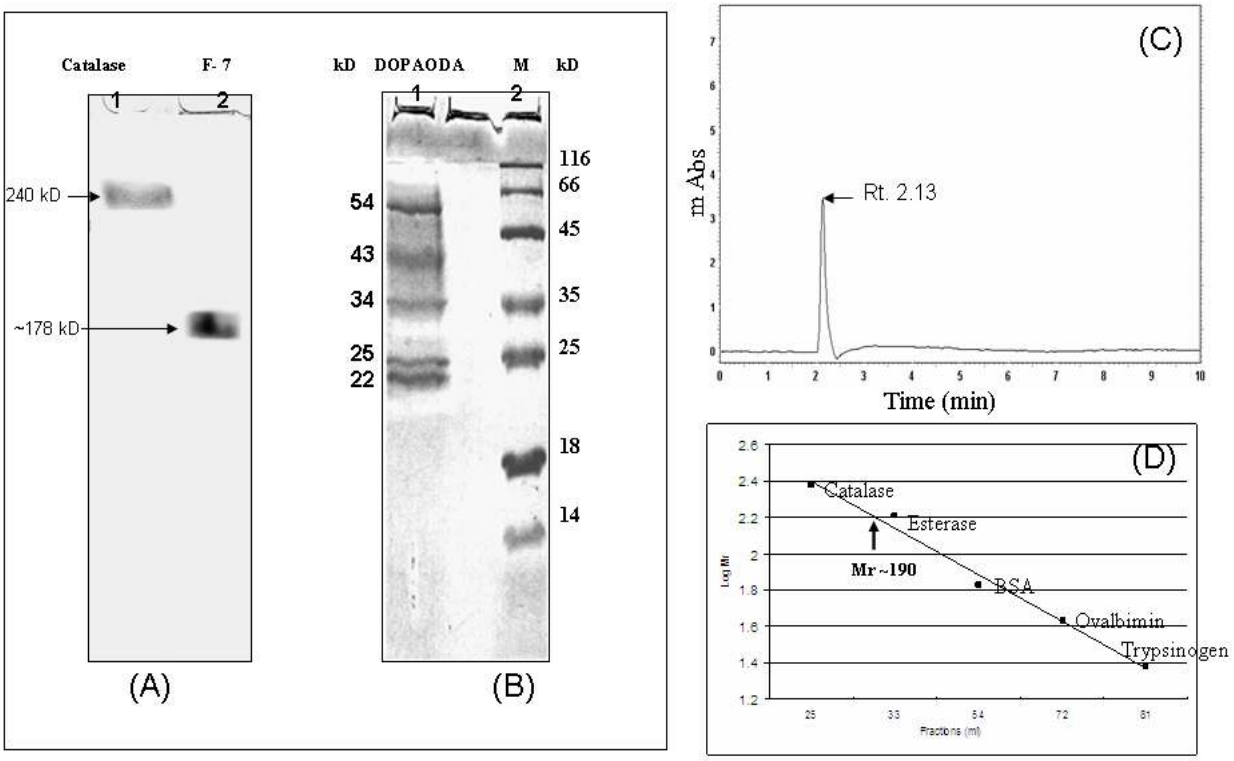
Catalase
F- 7
1
2
240 kD
~178 kD
(A)
kD
DOPAODA
M
kD
1
2
116
66
54
45
43
35
34
25
25
22
18
14
(B)
(C)
m Abs
Rt. 2.13
Time (min)
(D)
Catalase
Esterase
Mr ~190
BSA
Ovalbimin
Trypsinogen
Log Mr
Fractions (ml)

Fig-2

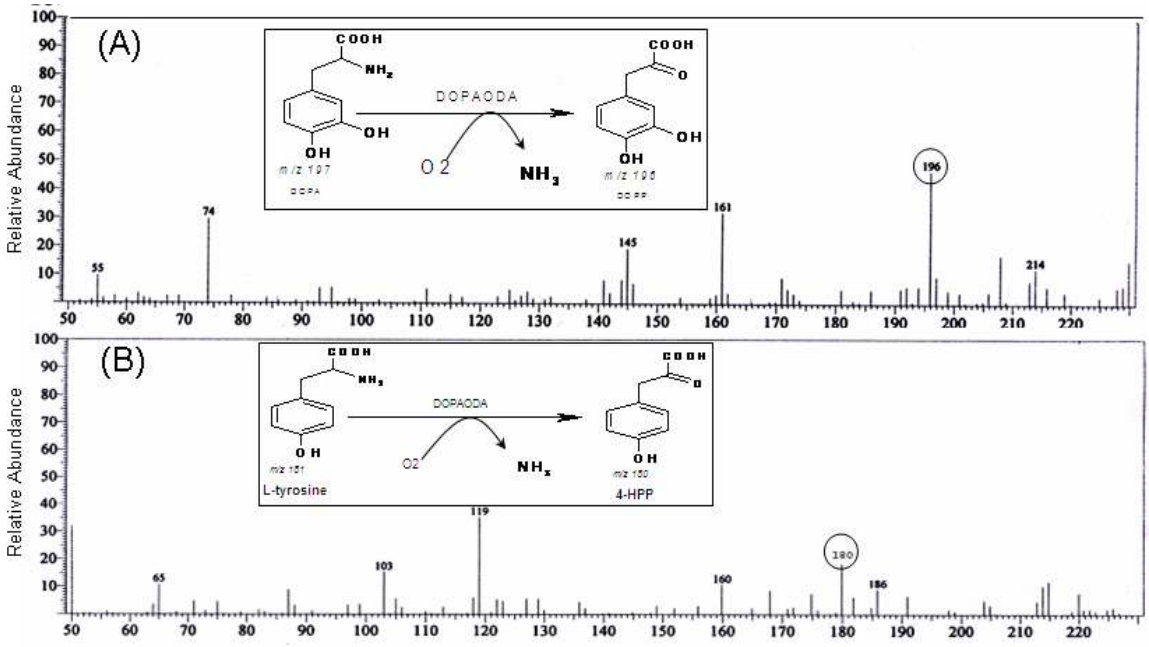
(A)
Relative Abundance
DOPAODA
O 2
NH3
m/z 197
DOPA
m/z 196
DOPP
74
55
145
161
196
214
(B)
Relative Abundance
DOPAODA
O2
NHx
m/z 181
L-tyrosine
m/z 180
4-HPP
119
65
103
160
180
186

Fig-3

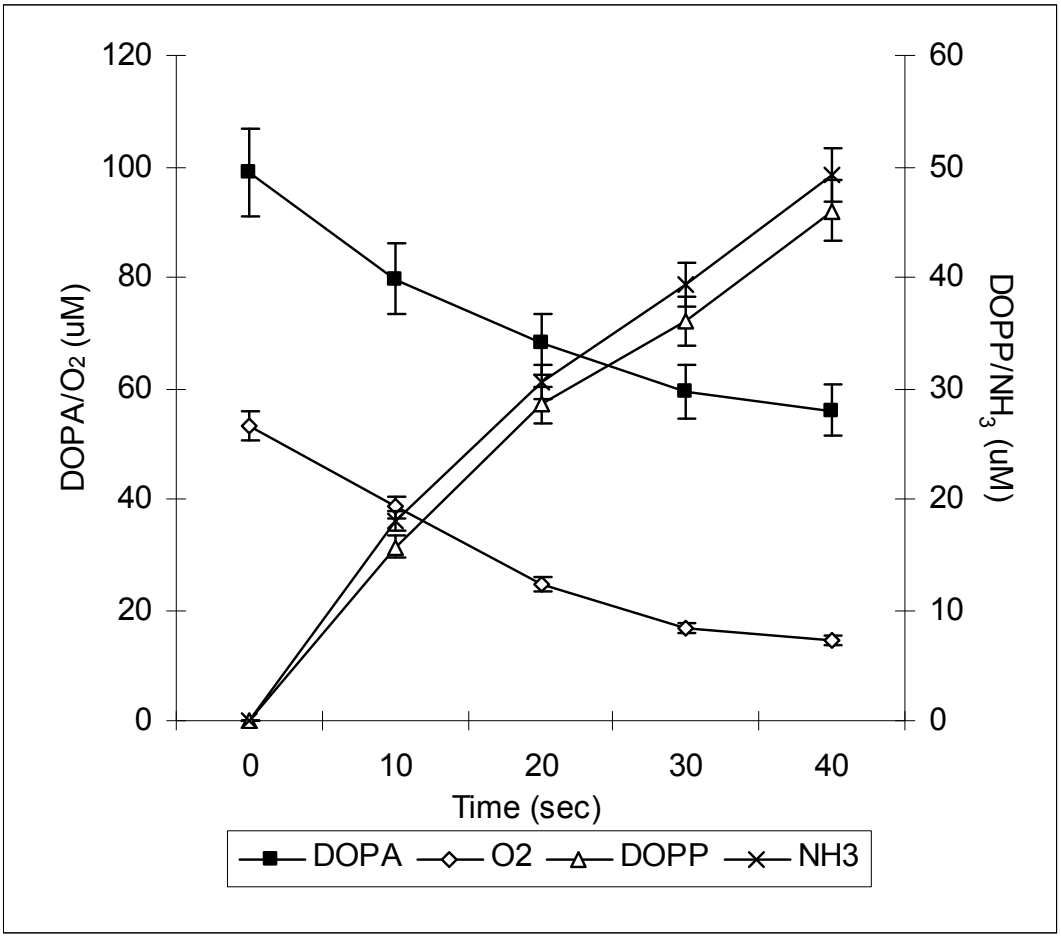
120
100
80
60
40
20
0
60
50
40
30
20
10
0
0
10
20
30
40
Time (sec)
DOPA/O2 (uM)
DOPP/NH3 (uM)
DOPA
O2
DOPP
NH3

Fig 4

Rhodophestrol: a novel phenol terpenoid ester produced by *Rhodobacter sphaeroides* OU5 has cytotoxic activity

Kumavath Ranjith N. [1], Sasikala Ch[2] and Ramana Ch.V[1*]

(1) Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500 046, India

(2) Bacterial discovery Laboratory, Center for Environment, IST, JNT University, Kukatpally, Hyderabad 500 085, India

*Author for Correspondence (E-mail: r449@sify.com)

Keywords Cytotoxicity, Phenol terpenoid ester, *Rhodobacter sphaeroides* OU5.

Abstract

A novel metabolite was isolated from the culture supernatants of *Rhodobacter sphaeroides* OU5 grown on L-phenylalanine as sole source of nitrogen under photoheterotrophic conditions. It was identified by IR, NMR (^{1}H, ^{13}C) and MS as a phenol terpenoid ester [6-[(3, 4-dihydroxybenzoyl)]-3-hydroxy-4-methylhexanoic acid] which has a molecular mass (m/z) of 298 and is named as rhodophestrol. It has cytotoxic activity against U937 (Human leukemic monocyte lymphoma) cell line.

Introduction

We have reported production of novel bio-molecules by a purple bacterium, *Rhodobacter sphaeroides* OU5. In the presence of L-tryptophan, indole terpenoids viz., sphestrin (Sunayana et al., 2005a), rhodestrin (Sunayana et al., 2005b) and rhodethrin (Ranjith et al. 2007) were isolated from the culture supernatants. Rhodethrin had excellent cytotoxicity and phytoharmonal activity. In the present communication, we report a novel phenol terpenoid ester isolated from the culture supernatant of *Rhodobacter sphaeroides* OU5 grown in the presence of L-phenylalanine, which has cytotoxic and cyclooxygenase-1 inhibitory activities.

Materials and methods

Organisms and growth conditions

Rhodobacter sphaeroides OU5 (ATCC 49885; DSM 7066) was grown photoheterotrophically (anaerobic, with light at 2,400 lux) in fully filled (10x100 mm) screw-cap test tubes or in reagent bottles (250/1000 ml), using Biebl & Pfennig's (1981) mineral medium with malate (22 mM) and ammonium chloride (7mM) as carbon and nitrogen sources, respectively, at 30±2°C. For growth on L-phenylalanine as nitrogen source, ammonium chloride was replaced with L-phenylalanine (1 mM).

Bulk cultivation, solvent extraction, isolation and purification of the phenol terpenoid metabolites

Ten litres of the L-phenylalanine assay supernatant was extracted thrice with ethyl acetate. The ethyl acetate extract was pooled and 1 g of anhydrous sodium sulphite was added to remove water molecules. The ethyl acetate layer was evaporated to dryness under vacuum and suspended in 5 ml of methanol (fraction I). Further purification was done through column chromatography by loading on to silica gel (80-120 mesh) in 450 x 150 mm glass column followed by elution with hexane (fraction Ia) and methanol (fraction Ib). The methanolic fraction (fraction Ib) was completely dried in a rota-vaporator at 30 ^{0}C, re-loaded on to a fresh silica gel column, dried and extracted with 50 ml hexane, chloroform, methanol (6:3:1v/v/v), which yielded a yellow colored metabolite (fraction II). The purity of the metabolite was confirmed using three different solvent systems. The metabolite had a R_F of 0.52 on TLC using the solvent methanol plus chloroform (1:1 v/v). Fraction II was used for ^{1}H & ^{13}C NMR, IR, UV and mass analysis to determine the structure.

Assay

Growth was measured turbidometrically as optical density at 660 nm. L-phenylalanine (0.2% [w/v] ninhydrin in acetone) and total phenols (Danish reagent; Swain and Hillis 1959) were quantified using standard protocols. NMR spectrum of the purified compound in 0.4 ml $CDCl_3$ in a 5mm NMR tube was recorded on Brucker AC 200 (200 MHz) spectrometer, operating at 50 and 200 MHz for ^{1}H and ^{13}C, respectively. UV analysis of the sample suspended in methanol was done in a Biochrom spectrophotometer. Infra red (IR) analysis was done on a Shimadzu FT/IR 5300 spectrophotometer. Cytotoxic activities against U937 (Human leukemic monocyte

lymphoma cell line) (Mosman 1983) and Cyclooxygenase-I/II inhibitory studies were done using standard protocols (Copeland *et al*., 1994).

Results and discussion

Rhodobacter sphaeroides OU5 was able to utilize L-phenylalanine only when used as sole source of nitrogen (malate served as carbon/e^- donor) under phototrophic conditions. Phenol production was observed simultaneously during growth and the supernatant gave blue color reaction with Danish reagent. Purification of the metabolites through fractionation yielded a yellow metabolite from Fraction II. The purity of the metabolite was confirmed using TLC and HPLC. This metabolite was characterized based on ^{1}H, ^{13}C NMR and IR analysis as 3,4-dihydroxybenzoyl terpenoid ester (Fig. 1). Further, we confirmed the ester bond of the metabolite by hydrolyzing the bond using the enzyme esterase, which resulted in the formation of 3,4-dihydroxybenzoate (protocatechuate). We named this newly isolated metabolite as rhodophestrol. UV spectral peaks of the metabolite at 206, 220 nm, confirms the phenol nucleus and a peak at 419 nm indicates the presence of a terpenoid. The yield of rhodethrin is 100 μM from 1 mM L-phenylalanine.

Cytotoxicity against U937 Human leukemic monocyte lymphoma cells

The cytotoxicity of rhodophestrol against U937 (Human leukemic monocyte lymphoma cell line) cancer cell line was determined using the 3-(4,5-dimethylthylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Mosman 1983). Rhodophestrol had cytotoxicity (apoptosis bodies formation) on the cancer cell line even at low concentrations tested (50 nM) (Fig. 2A,B).

Cyclooxygenase inhibitory activity

Rhodophestrol had COX-I (~50%, Fig. 2C) and COX-II (~20%) inhibitory activity.

Conclusions

Alkyl esters of phenols are an important group of biogenic molecules reported from plants (Yang *et al*. 2003), bee propolis (Ahn *et al.* 2004), yeasts like candida (Stevenson *et al.* 2007) and from a marine bacterium; *Microbulbifer* (Peng et al., 2006), however this is the first report (to the best of our knowledge) of a terpenoid ester of phenol isolated from a bacterium. Alkyl esters of phenols are of biotechnological significance since they have anti-oxidant (Chen *et al*. 1997), anti cancer (Samaha *et al.* 1997; Li *et al.* 2003), anti HIV (Burke *et al.* 1995) and antimicrobial (Tawata *et al.* 1996; Peng et al., 2006) activities. Rhodophestrol is a potential anticancer phenol terpenoid ester and thus worth exploiting from *Rhodobacter sphaeroides* OU5.

Acknowledgements RNK thanks the UGC, Govt. of India for the award of junior research fellowship. Financial support received from CSIR, Govt. of India is acknowledged. Prof. D. Basavaiah (LCMS, NMR, IR), Dr. J.Venkateswara Rao, IICT, Hyderabad (Cytotoxity studies) and Prof. P. Reddanna (Cox- 1& 2 inhibitory studies) are acknowledged for analytical support.

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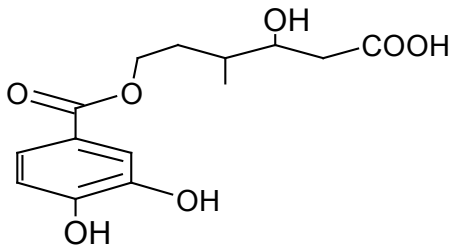


Fig 1

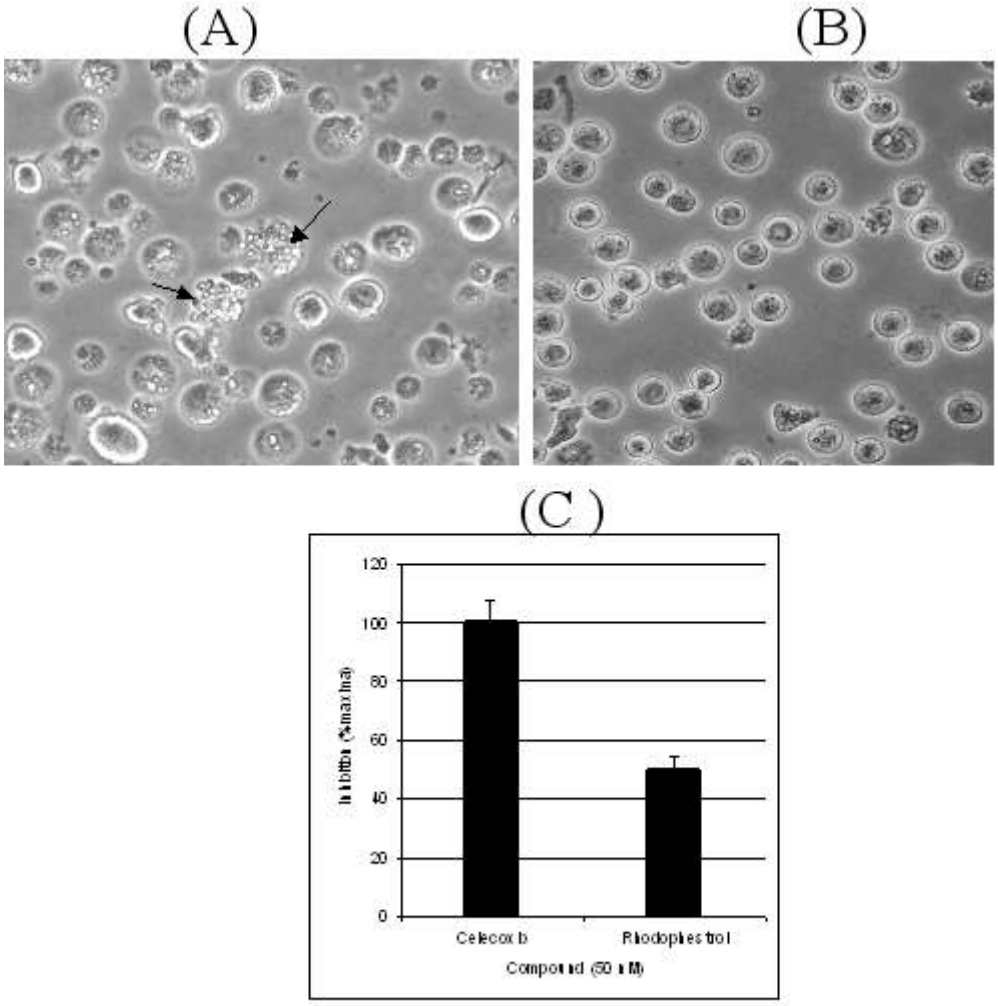

(A)
(B)
(C)
120
100
80
60
40
20
0
Celecoxib
Rhodoplestrol
Compound (50 nM)

Fig.2

Fig 1. Structural of the metabolite isolated from the culture supernatant of *Rba. sphaeroides* OU5 when grown on L-phenylalanine. Culture grown on L-phenylalanine (1 mM) for 36 h in 5 L medium was used for the isolation of the metabolite. The compound was extracted from the hexane, chloroform and methanol (6:3:1v/v/v) extract (Fraction II) (See materials and methods for more details). The metabolite is a bright yellow oily liquid and has UV-VIS peaks (nm) at 206, 220, 419.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.26-7.30 (m, 3H, Ph-OH-C=CH & Ph - HC=CH), 4.73 (s, 4H, O-CH_2), 2.33 (t, 2H, OH-CH-CH_2), 1.25 (m, 4H, O-CH_2-CH_2); 0.88(d, 3H, CH-CH_3).

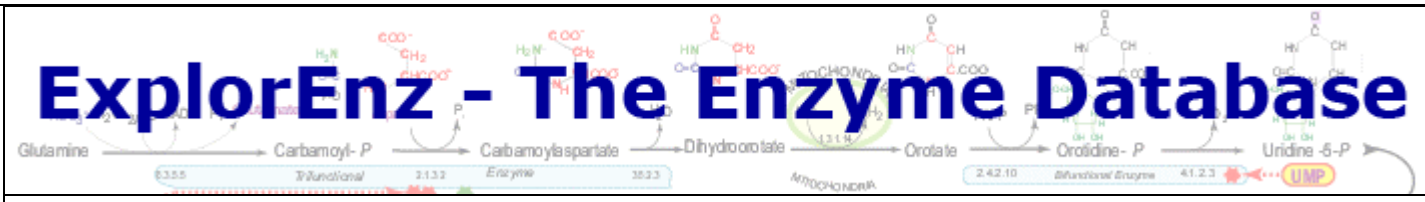
^{13}C NMR ($CDCl_3$, 150 MHz): δ133.7 (C-3′, Ph-CH=CH-CH-OH), 133.5 (C-4′, Ph-CH-CH-OH-CH-OH), 126 (C-1′, CH-C-CH), 124 (C-6′, CH-C-CH), 115.98 (C-5′), 115.98 (C-2′, O-CH=CH), 179.0 (C-6, Ph-C=O-O), 176.4 (C-1, CH2-C= O-OH), 168.2 (C-4, Ph-C=O-O), 65.6 (C-1, O-CH), 41.1 (C-5, OH-CH-CH_2), 35.7 (C-3, O-CH-CH_2), 34.0 (C-2, CH_2-CH-CH_3-CH), C3 Me, 14.1, H_2C-CH-OH-CH)

Infra Red spectral analysis (KBr pellet; cm^{-1}) 3377, 2926, 2854, 1714, 1456, 1284, 1163 and 700. EIMS m/z 298 $[M]^+$ and C 64.2 % and H 6.8 %, calcd. for $C_{14}H_{18}O_7$, C 56.37%, H 6.08%, O 37.55%. IUPAC name of the metabolite is [6-[(3, 4-dihydroxybenzoyl)]-3-hydroxy-4-methylhexanoic acid] which, we named as rhodethrin (rhodo = from *Rhodobacter*; phe = phenol, estrol = ester terpenoid).

Fig 2. Photograph showing the cytotoxicity (apoptosis) of rhodophestrol against U937 cancer cell line as determined by MTT method (Mosman, 1983). (A) Treated cells and (B) control (untreated cell lines) Briefly, adherent cancer cell lines (U937) at the concentration of $0.2x10^6$ $cells.ml^{-1}$ was seeded in 96-well microplates. The adherent cells were incubated for 18h to allow

cell attachment. Rhodophestrol solution was added to the cell cultures at concentrations of 50µM and the cells were incubated for 24h. The MTT solution was added 3 h before the end of the incubation. Cell survival was evaluated with a multiwell scanning spectrophotometer at 570 nm.

(C) Cox-1 inhibitory activity was observed with celecoxib and purified metabolite Rhodophestrol (50 nM)



Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)

Proposed to the Enzyme List

EC 4.3.1.22 3,4-dihydroxyphenylalanine reductive deaminase

EC	**4.3.1.22 –** accepted on 13 December 2007 [Last modified: 2007-11-15 04:02:28]
Accepted name:	3,4-dihydroxyphenylalanine reductive deaminase
Reaction:	3,4-dihydroxy-L-phenylalanine + **2** NADH = 3,4-dihydroxyphenylpropanoate + **2** NAD^+ + NH_3
Glossary:	DOPA = L-dopa = 3,4-dihydroxy-L-phenylalanine
Other name(s):	reductive deaminase; DOPA-reductive deaminase; DOPARDA
Systematic name:	3,4-dihydroxy-L-phenylalanine ammonia-lyase(3,4-dihydroxyphenylpropanoate-forming)
Comments:	Forms part of the L-phenylalanine-catabolism pathway in the anoxygenic phototrophic bacterium *Rhodobacter sphaeroides* OU5. NADPH is oxidized more slowly than NADH.
References:	1. Ranjith, N.K., Sasikala, Ch. and Ramana, Ch.V. Catabolism of L-phenylalanine and L-tyrosine by *Rhodobacter sphaeroides* OU5 occurs through 3,4-dihydroxyphenylalanine. *Res. Microbiol.* **158** (2007) 506–511. [PMID: 17616348]

[EC 4.3.1.22 created 2007]

Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)

In consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN)

Enzyme Nomenclature

Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes by the Reactions they Catalyse

http://www.chem.qmul.ac.uk/iubmb/enzyme/

World Wide Web version prepared by G.P. Moss
Department of Chemistry, Queen Mary University of London,
Mile End Road, London, E1 4NS, UK
g.p.moss@qmul.ac.uk

EC 2.6.1.49

Accepted name: **dihydroxyphenylalanine transaminase**

Reaction: 3,4-dihydroxy-L-phenylalanine + 2-oxoglutarate = 3,4-dihydroxyphenylpyruvate + L-glutamate

For diagram click here (mechanism).

Other name(s): dopa transaminase; dihydroxyphenylalanine aminotransferase; aspartate-DOPP transaminase (ADT); L-dopa transaminase; dopa aminotransferase; glutamate-DOPP transaminase (GDT); phenylalanine-DOPP transaminase (PDT); DOPA 2-oxoglutarate aminotransferase; DOPAATS

Systematic name: 3,4-dihydroxy-L-phenylalanine:2-oxoglutarate aminotransferase

Comments: A pyridoxal-phosphate protein.

Links to other databases: BRENDA, EXPASY, KEGG, ERGO, CAS registry number: 37277-98-8

References:

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[EC 2.6.1.49 created 1972]