Photometabolism of 2-aminobenzoate (Anthranilate)

by *Rhodobacter sphaeroides* OU5

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that Mrs. M. R. Sunayana has carried out the research work embodied in the present thesis under supervision and guidance of Dr. Ch. Venkata Ramana for a full period prescribed under the Ph. D ordinance of this University. We recommend her thesis **"Photometabolism of 2-aminobenzoate (anthranilate) by *Rhodobacter sphaeroides* OU5"** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

I here by declare that the work embodied in this thesis entitled **"Photometabolism of 2-aminobenzoate (anthranilate) by *Rhodobacter sphaeroides* OU5"** has been carried out by me under the supervision of Dr. Ch. Venkata Ramana and this has not been submitted to any other Institute or University for the award of any degree or diploma.

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Sunayana

Dedicated to my
beloved parents
& husband

Contents

Abstract

Molecules which are alien to biological system are referred as xenobiotics. Xenotoxicity can be in terms of molecular structure or it can be dose dependent. Extensive work was done in the past in realizing the capability of microorganisms in degrading structure based xenobiotics. However, work related to molecules, which occur naturally but exceed physiological concentrations of biological systems is less studied. One such study reported earlier was on the metabolism of 2-aminobenzoate at physiologically elevated concentrations by a purple non-sulfur bacterium, *Rhodobacter sphaeroides* OU5. This organism lacks the capability to degrade aromatic hydrocarbons but has the ability to transform 2-aminobenzoate to indole. The present thesis work extends an in-depth study on the photometabolism of 2-aminobenzoate by *Rba. sphaeroides* OU5.

2-Aminobenzoate could not support growth of *Rba. sphaeroides* OU5 as a sole source of carbon, nitrogen or carbon and nitrogen. However, 2-aminobenzoate was transformed to an indole in a light dependent process by both growing and resting cells of *Rba. sphaeroides* OU5. In total 57 different metabolites were isolated of which, 25 were characterized as indole terpenoid esters based on UV, IR, ^{1}H, C^{13} NMR and mass spectroscopic analysis. All these esters appeared to be the

intermediates of indole terpenoid degradation pathway and based on the α, β oxidation of terpenoid side chain two putative indole terpenoid degradative pathways are proposed.

Evidences from the organic substrate screening and inhibitory studies using analogues confirm the role of fumarate as the conjugating molecule for indole biosynthesis from 2-aminobenzoate. A novel fumarate dependent indole biosynthetic pathway is proposed based on the experimental evidences obtained using cell free extracts. A novel protein involved in the biosynthesis of indole from 2-aminobenzoate and fumarate was isolated to its homogeneity and was characterized. The ~64 kD monomeric protein that catalyzes the formation of N-fumarylaniline (m/z 207) from 2-aminobenzoate and fumarate is named as N-fumarylaniline synthase.

In the presence of 2-aminobenzoate or indole no anti-oxidant proteins like superoxidedismutase, peroxidase, or stress-induced protein like sphaeroplast protein were detected. However, over expression of orange carotenoid proteins (OCPs) was observed and their role in indole detoxification was studied.

In conclusion, two distinct indole synthesizing pathways exist in *Rba. sphaeroides* OU5. At physiological concentrations of 2-aminobenzoate, the classical phosphoribosylpyrophosphate pathway is

operative and is cytosolic. While, at physiologically elevated concentrations of 2-aminobenzoate the fumarate dependent pathway of indole synthesis occurs, which is membrane bound and these two pathways appear to be distinctly delineated. The indole thus produced is detoxified through a process of terpenoid conjugation in which, OCPs play a major role. A hypothetical schematic representation of the major events of 2-aminobenzoate metabolism in *Rba. sphaeroides* OU5 is proposed. In addition, the work also extends in realizing the novel indole terpenoid esters as potential phytohormones.

Introduction

1.1 **General introduction**

Xenobiotics are the molecules foreign to biological system or they are the compounds that are released into the environment by the action of man (anthrapogenic) so that they occur in concentrations higher than natural (Ian Singlinton, 1994) or naturally present compounds administered by alternate routes or at unnatural concentrations (www. Bioanalytical.com/info/calendar/2000/index.html). The xenobiotic character of the molecules can be due the structural element that cannot be synthesized biochemically or they contain sterically oriented functional groups differing from the naturally existing compounds or due to their persistence at high concentration in the environment (Rieger *et al.,* 2002). Therefore the xenotoxicity of the compound can be structure based or dose dependent. Nitroaromatic compounds, poly chlorinated biphenyls, poly halogenated ethenes, azo dyes, pesticides and surfactants (Rieger *et al.,* 2002, Jain *et al*., 2005) are few structure based xenobiotics. While petroleum, oil spill, drugs such as antibiotics and preservatives like sodium benzoate are few dose dependent xenobiotics.

The ability of the microorganisms to metabolise xenobiotics, have received attention over years because of the persistence and toxicity of chemicals. The microbial degradation of xenobiotics is a cost effective method of removing pollutants from the environment and the process is known as bioremediation. A diverse group of microorganisms like bacteria and fungi were shown to degrade a wide range of xenobiotics like nitroaromatics, polychlorinated aromatics, poly halogenated aromatics, petro-products and pesticides and utilized them completely as growth substrates or mineralized them to harmless chemicals (Reviewed Jain *et al.,* 2005). Extensive work had been done on the biodegradation of structure-based xenobiotics. New genes, enzymes and metabolic routes involved in bacterial degradation of xenobiotics have been discovered and new strategies have been

developed which allow the discovery and flexibility of the microorganisms in environmental clean-up (Rieger *et al*., 2002; Jain *et al.,* 2005). However, limited work had been done on the dose dependent xenobiotics, especially in relation to the naturally occurring molecules, which occur at physiologically xenotoxic concentrations.

One such study done earlier on naturally occurring xenobiotic compounds at the physiologically elevated concentrations was, on the metabolism of 2-aminobenzoate by a purple non-sulphur bacterium *Rhodobacter sphaeroides* OU5. This organism lacks the ability to utilize the aromatic hydrocarbons for growth (Sasikala *et al.,* 1998) however, when 2-aminobenzoate was supplemented at physiologically elevated concentrations (1-3 mM) it was transformed to an indole derivative (Nanada *et al*., 2000).

1.2 **2-Aminobenzoate is a natural molecule**

2-Aminobenzoate has the molecular formula $C_7H_7NO_2$ and is an odorless white to yellow crystalline acid, which is freely soluble in water. It is an aromatic amine, which occurs physiologically as a metabolite of amino acid, tryptophan. It is used as an intermediate for the production of dyes, pigments and saccharin. It and its esters are used in preparing perfumes, pharmaceuticals and UV-absorber as well as corrosion inhibitors for metals and mold inhibitors in soya sauce. 2-Aminobenzoate is also known (unofficially) as "vitamin L", a vitamin required for lactation in human females.

1.3 **2-Aminobenzoate is widely distributed in various environments**

2-Aminobenzoate is a natural component of human urine and from 1 to 15 mg of it is excreted daily (BUA report 175; 1995, Germany). On account of its biodegradability under aerobic and anaerobic conditions and of its physicochemical properties, the compound is not geo-accumulated. Airborne 2-aminobenzoate is

photooxidatively degraded by -OH free radicals; the half-life for this reaction is 6.1 h (BUA report 175; 1995, Germany).

1.4 *Denovo* synthesis of 2-aminobenzoate and indole

2-Aminobenzoate and indole are the intermediates of the shikimate pathway, a pathway of aromatic biosynthesis (Coggins *et al*., 2003). Through this pathway, aminoacids like tryptophan, phenylalanine and tyrosine and many other aromatic compounds are biosynthesized. This pathway exists only in plants and microbial systems and is well studied in bacteria like *Escherichia coli, Salmonella typhimurium, Hafnia alvei* (Crawford, 1989). Hence, animals and human beings depend on microbial or plant systems to acquire the essential amino acids (Coggins *et al*., 2003).

1.4.1 Shikimate pathway:

The shikimate pathway starts with the formation of shikimate from erythrose 4-phosphate and phosphoenol pyruvate. The enzymatic conversion of shikimate 5-phosphate to 2-aminobenzoate has been investigated thoroughly in many microorganisms (Crawford, 1989). Shikimate reacts with enol pyruvate phosphate to yield 3-enol-pyruvylshikimate 5-phosphate. The latter compound is converted to chorismate by the elimination of orthophosphate. Chorismate is the branch point intermediate in aromatic biosynthesis. Chorismate is converted to 2-aminobenzoate an intermediate in tryptophan biosynthesis by anthranilate synthase. This enzyme was isolated and well studied in *Neurospora crassa, Aerobacter aerogenes* and *Escherichia coli* (Radwanski and Robert, 1995). In this conversion, the amino group of 2-aminobenzoate is derived from amide nitrogen of glutamine and amination occurs on the C_2 of the 2-aminobenzoate, the carboxyl of shikimate becomes the carboxyl of 2-aminobenzoate and aromatization of the ring occurs without rearrangement.

1.4.2 **Enzymes for indole biosynthesis**

1.4.2.1 **Anthranilate synthase:** Anthranilate synthase catalyses the first step of tryptophan synthesis in microorganisms and plants. In all organisms except for *Euglena gracilis*, this enzyme is an oligomer of non-identical protein chains, designated as AS-I and AS-II. AS-I catalyses the reaction**:**

$$Chorismate + NH_3 \rightarrow 2\text{-}Aminobenzoate + Pyruvate$$

AS-II binds glutamine and delivers the amide to AS-I for 2-aminobenzoate synthesis. The oligomeric composition of the enzyme and avidity of subunit association vary in different organisms. In *Euglena gracilis* the anthranilate synthase is a single polypeptide of 80 kD and it has both ammonia and glutamine dependent activity (Charles *et al.,* 1976). In *Acinetobacter calcoaceticus,* anthranilate synthase is a multimeric protein and is made up of two non-identical subunits of mass 86 kD. In *Enterobacteriaceae* the enzyme is made up of two equal sized protomers AS-I and AS-II however, in non-enteric species like *Pseudomonas* and in *Bacillus subtilis* they are of un-equal size (Sawula *et al*., 1973). The anthranilate synthase in *Salmonella typhimurium* is part of a multifunctional, tetrameric complex made up of two molecules each of the TrpE and TrpD polypeptides, the products of the first two genes *trp E* and *trp D* of the *trp* operon (Bauerle *et al*., 1987)

1.4.2.2 **Anthranilate phosphoribosyltransferase:** TrpD catalyzes the second step in tryptophan biosynthesis and belongs to the functional superfamily of phosphoribosyltransferases, which catalyze the transfer of the ribose-5-phosphate moiety from 5-phosphoribosyl-1-pyrophosphate (PRPP) to various acceptors. This reaction involves the practically irreversible replacement of the pyrophosphate moiety of PRPP by a nucleophile with accompanying anomeric inversion of the ribofuranose ring (Chelsky *et al*., 1974). In *Enterobacteriaceae*, three forms of this enzyme exist:

one form of enzyme has molecular weight of 45 kD and is separate from all other pathway enzymes eg: *Serratia.* Second form is also a single enzyme of 65 kD eg: *Erwinia* and the third form is of 65 kD but has covalently linked glutamine transferase, the activator of glutamine in the preceeding first reaction of the pathway (Largen and Belser, 1975). The consequence of the covalent linkage is that the anthranilate phosphoribosyltransferase and anthranilate synthase enzymes are combined into an aggregate. The genus *Escherichia* and *Salmonella* possesses the aggregates, in *Salmonella* the enzyme aggregate is of 280 kD with two equal components of 62 kD and it is encoded by *trp D* gene (Largen *et al*., 1978). One of the best-investigated *trp D* is from *Salmonella typhimurium* and of *Sulpholobus solfataricus* (Ivens *et al*., 2001), where it is part of a triple enzyme complex catalyzing the first three steps of Trp biosynthesis (Bauerle *et al.*, 1987; Edwards *et al*., 1988; Knochel *et al*., 1999).

1.4.2.3 **N-(5' phosphoribosyl) anthranilate isomerase and indole glyceraldehyde 3 phosphate synthase (Trp C):** The third and the fourth step in the pathway are catalyzed by these two enzymes. The conversion of phosphoribosylanthranilate (PRA) to enol-1-o-carboxyphenylamino-1-deoxyribulose phosphate (CDRP) is done by N-(5'phosphoribosyl) anthranilate isomerase and the conversion of CDRP into indole glyceraldehyde-3-phosphate is brought about by indole glyceraldehyde-3-phosphate synthase (Fig 1). These two activities are contained in a single polypeptide of 47-53 kD in most of the organisms like *Enterobacter cloacae, Clostridium freundii, Pseudomonas vulgaris, Serratia marcescens, Enterobacter liquifaciens* (Crawford, 1989).

1.5 **Utilization of 2-aminobenzoate for growth**

The existence of bacteria capable of utilizing 2-aminobenzoate as a sole source of carbon and e^- donor has been known for many years (Hayaishi *et al*., 1951). Bacteria

like *Pseudomonas* strain K172, *Azoarcus evansii*, *Rhodopseudomonas palustris Thauera aromatica* were able to grow anaerobically on 2-aminobenzoate as a source of carbon and energy (Schuhle *et al.,* 2001; Lochmeyer *et al.,* 1992; Harwood and Gibson, 1988; Schuhle *et al.,* 2003). Aerobically *Acinetobacter* sp. strain ADP1 was found to utilize 2-aminobenzoate as carbon source for growth (Bundy *et al.,* 1998). However, 2-aminobenzoate has also supported the growth of *Lactobacillus* as nitrogenous source and it was metabolized to indole like product (Rhuland and Bard, 1951).

1 .6 **Metabolism of externally supplemented 2-aminobenzoate by bacteria**

1.6.1 **Aerobic**

Aerobically the metabolism of 2-aminobenzoate occurs through three different pathways leading to the formation of catechol or gensitate (Fig 2A; reviewed by Lochmeyer *et al.,* 1992; Schuhle *et al.,* 2001) as central intermediates, which are further oxidized and cleaved. It was found that 2-aminobenzoate, benzoate and phenylacetate are metabolized aerobically in *Azoarcus evansii* via CoA thioester (Fig 2B). More specifically the 2-aminobenzoate is converted to 2-aminobenzyl CoA by 2-aminobenzyl CoA ligase, a monomeric protein of 65 kD. It is then hydroxylated and reduced to 2-amino-5–oxocyclohex-1-ene-1- carbonyl CoA and the reaction is catalysed by 2-aminobenzyl CoA monooxygenase/reductase (ACMR) then the compound undergoes β oxidations and ring cleavage (Schuhle *et al.,* 2001). In *Thauera aromatica*, a single benzoate CoA ligase appears to function in anaerobic benzoate and anaerobic 2-aminobenzoate metabolism as well as in aerobic benzoate metabolism. No specific 2-aminobenzyl CoA ligase could be identified (Schuhle *et al.,* 2003). The product of benzoate activation, the benzyl CoA, then acts as the inducer of separate anaerobic and aerobic pathways of benzyl CoA, depending on the presence or absence of oxygen (Schuhle *et al.,* 2003). In *Acinetobacter* 2-

aminobenzoate was degraded to catechol via the β-ketoadipate pathway (Fig 3). The studies with mutants of *Acinetobacter* sp. strain ADP1 facilitated the identification of the *antABC* genes, involved in 2-aminobenzoate degradation (Bundy *et al.*, 1998).

1.6.2 **Anaerobic metabolism of 2-aminobenzoate**

Anaerobically, metabolism of 2-aminobenzoate proceeds via activation of 2-aminobenzyl CoA and then ring reduction of benzyl CoA occurs to yield alicyclic acyl CoA as in *Thauera aromatica* and *Azoarcus evansii* (Fig 4C; Lochmeyer *et al.,* 1992).

1.6.3 **Chimeric pathway**

Apart from the above metabolisms, chimeric pathway of aerobic 2-aminobenzoate metabolism, combining the characteristics of both aerobic and anaerobic pathways was reported in *Pseudomonas* sp. The pathway proceeds via CoA thioester formation as in anaerobic degradation, but the aromatic ring reduction is coupled with simultaneously oxygen dependent hydroxylation reaction of the benzene ring, para position to the amino group. In *Pseudomonas* the thioester formed was anthronyl CoA and the genes for this are encoded on a 8.1kb pKB740 plasmid (Altenscyhmidt and Fuchs, 1992), whereas, anaerobically under denitrifying conditions (Fig 2B) 2-aminobenzoate was oxidized completely to CO_2 and NH_4^+ (Braun and Gibson, 1984).

1.6.4 **Biotransformation of 2-aminobenzoate**

The transformation of 2-aminobenzoate to indole was reported in mutants of *Escherichia coli* (Trudinger, 1955) and the production of dihydroxyindole, in the presence of fumarate was reported in *Rhodobacter sphaeroides* OU5 (Nanada *et al.,* 2000).

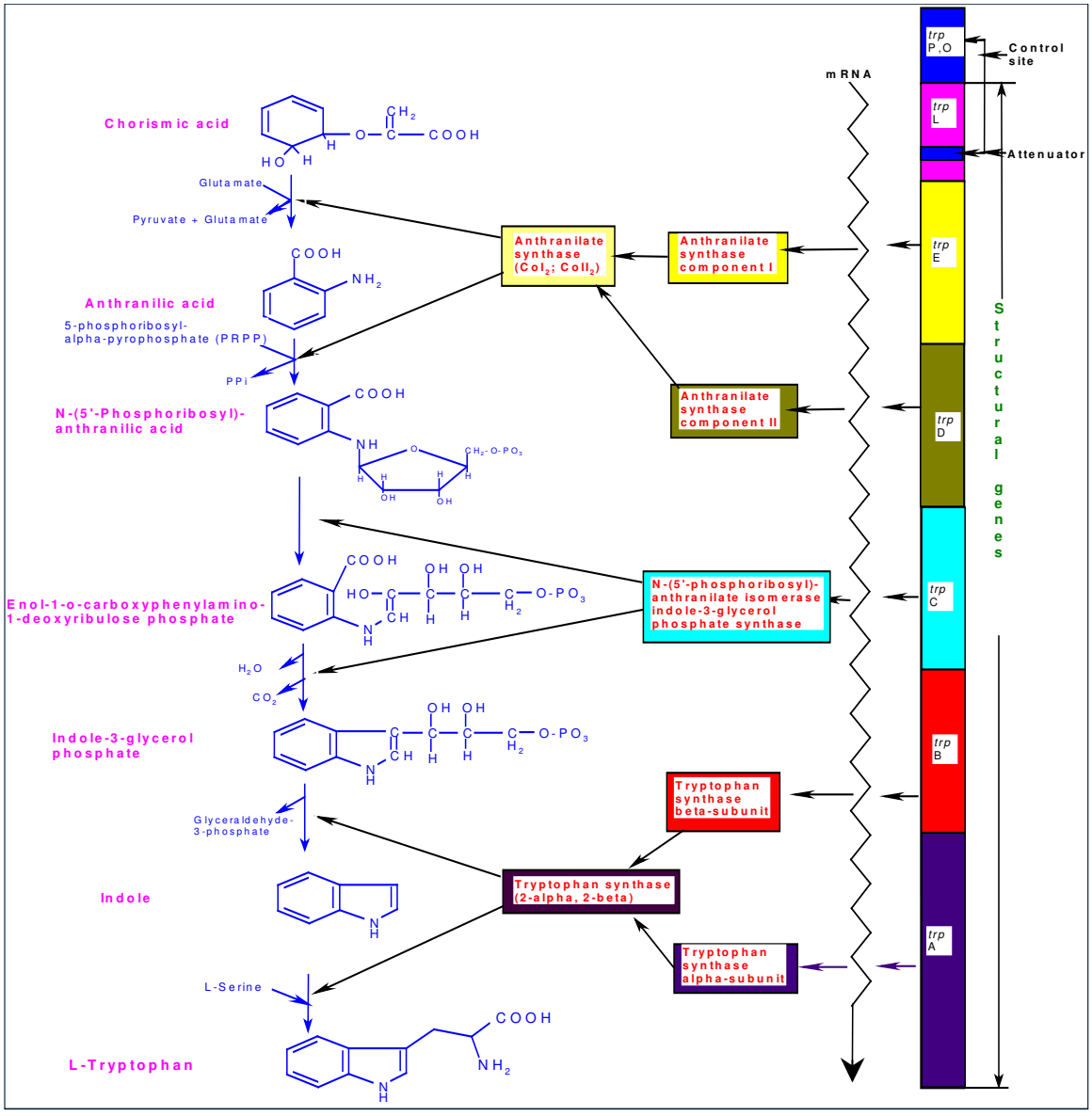

Fig 1: Tryptophan biosynthetic pathway

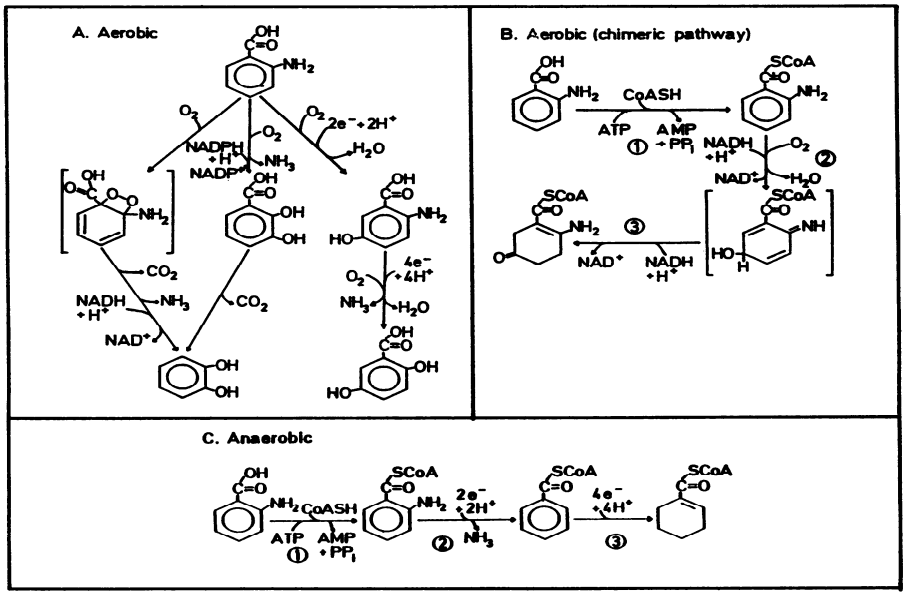

Fig 2: Bacterial metabolism of 2-aminobenzoate (Lochmeyer *et al*., 1992)

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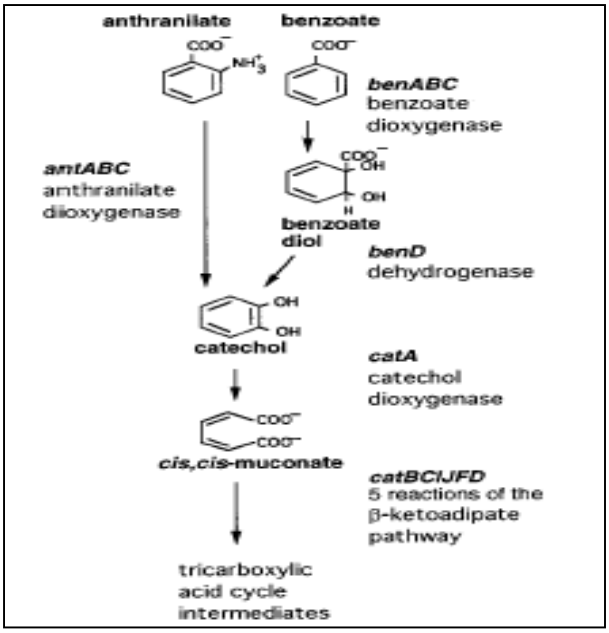


Fig 3: Degradation of 2-aminobenzoate and benzoate via the β-ketoadipate pathway in *Acinetobacter* sp. strain ADP1 (Bundy *et al.,* 1998)

1.7 **Indole toxicity**

Indole and its derivatives are toxic and mutagenic (Ochial *et al*., 1986). Indole inhibited the growth of few Gram –ve bacteria (Kubo, 1993), green algae like *Chlorella* (Tamiya *et al*., 1968) and fungi, *Aspergillus niger* (Kamath and Vaidyanathan, 1990). The growth inhibitory concentration of indole varies from organism to organism. Generally low concentration of indole (0.3-2 mM) inhibited the growth of microorganisms (Kamath and Vaidyanathan, 1990). Indoles inhibited growth of *Pseudomonas solanacearum* at concentration higher than 100 $\mu g.ml^{-1}$ (Matsuda *et al*., 1990). Antimicrobial activity of indole was explained as due to the aromaticity and nitrogen atom of the pyrrole nucleus and the mode of action is multifactorial (Matsuda *et al.,* 1990). In plants it inhibits anthraquinone biosynthesis (resulting in low pigmentation; Ei-Shangi *et al*., 1984) reduces porphyrins (Umrikhina and Krasnoyskii, 1969) and inhibits oxidative and photophosphorylation (Buechel and Draber, 1968). Indole inhibited glycolytic enzymes in *Aspergillus niger* (Kamath and Vidyanathan, 1990). The occurrence of indole inducible proteins suggests the membrane and oxidant toxicity of indole in *E. coli* (Garbe *et al.,* 2000). Indole is lipophilic and it gets dissolved in membrane lipids to cause membrane disarrangement, thus enabling direct interaction of redox cycling isoprenoid quinones and dioxygen resulting in the generation of superoxide.

Indole completely inhibits the growth of *E. coli* at 4.5 mM. The over production of alkyl hydroperoxide reductase and induction of sphaeroplast protein (spy) demonstrate oxidant toxicity of indole in *E. coli*. (Garbe *et al*., 2000). Generally, the anti-oxidant proteins induced in the presence of oxidants are alkylperoxidase (36 kD), superoxide dismutase (22 kD) and catalase (240 kD). Apart from these enzymes, in indole resistant *E. coli* mutants a new antioxidant protein was expressed that induced the formation of sphaeroplast and the isolated protein was found to be 16 kD and was

called as sphaeroplast protein (Garbe *et al*., 2000). A similar type of protein was also produced in mutants of *Brevibacterium* and it was found to be of 32 kD (Garbe *et al*., 2000).

1.8 **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 (Bru-Magniez *et al*., 1995), anti-inflammatory agents (Verma *et al.,* 1994), antihypertensive (Frishman, 1983), anti-HIV compound (Britcher *et al*., 1995) and phytohormones (Elsorra *et al*., 2003).

1.8.1 **Indole esters**

There are a few reports of indole ester biosynthesis in plant system and there are no reports of indole ester formation by microorganisms. 4-Chloroindole-3-acetic acid (4-Cl-IAA) 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 chestnut (*Aesculus parviflora*, *A. baumanni*, *A. pavia rubra* and *A. pavia humulis*). The liquid endosperm contained at least 12 chromatographically distinct esters. One of these compounds was purified and characterized as an ester of indole 3-acetic acid and myo-inositol (Domagalski *et al*., 1987). Indole 3-acetyl-myo-inositol esters have been demonstrated as an endogenous component of etiolated *Zea mays* shoots tissue. The amount of indole 3-acetyl-myo-inositol esters in the shoots was determined to be 74 ηmoles.kg^{-1} fresh weight (Chisnell, 1984).

1.8.2 **Biological activity and biotechnological potential of indole esters**

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 anti-superoxide formation (SOD) (Olgen & Coban, 2003; Olgen *et al*., 2007). Indole esters were also found to have more phytohormonal activity than their corresponding acids in the auxin bioassay (Katayama, 2000).

1.9 **Definition of the problem**

2-Aminobenzoate is a naturally occurring aromatic compound found as an intermediate in the metabolism of tryptophan biosynthesis. It plays a key role in the synthesis as well as in the degradation of many N-heterocyclic aromatic compounds. As a consequence of its wide occurrence, 2-aminobenzoate is a common growth substrate for many microorganisms that are able to cleave the aromatic ring. The metabolism of 2-aminobenzoate under aerobic and anaerobic conditions has been well studied. It has also acted as a precursor in the production of indole acetic acid in mutants of *Escherichia coli.* However, when 2-aminobenzoate was supplemented to a photosynthetic bacterium *Rhodobacter sphaeroides* OU5 that had no metabolic capability to utilize aromatic compounds as growth substrates, the transformation of 2-aminobenzoate to indole was observed. This transformation was found to be light dependent and the production of indole from 2-aminobenzoate was found to be dependent on TCA cycle intermediate, fumarate. Hence, a detailed study on the photometabolism of 2-aminobenzoate in *Rba. sphaeroides* OU5 was taken up for my Ph. D thesis with the following objectives.

1.10 **OBJECTIVES:**

1. **To study the capability of *Rba. sphaeroides* OU5 in utilizing 2-aminobenzoate for growth and production of indole**
2. **To study the metabolites of 2-aminobenzoate transformation by *Rba. sphaeroides* OU5**
3. **To understand the fumarate dependent indole biosynthesis from 2-aminobenzoate by *Rba. sphaeroides* OU5**
4. **Toxicological studies of 2-aminobenzoate and indoles on *Rba. sphaeroides* OU5**
5. **Bioprospecting the novel metabolites of 2-aminobenzoate biotransformation**

Materials and methods

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

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

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

2.1.4 **HPLC water:** Milli-Q water was used for HPLC analysis.

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

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

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

2.2.1 **Organism and growth conditions:** The purple non sulphur bacterium, *Rhodobacter sphaeroides* OU5 [ATCC-49885; DSM-7066] was obtained from the Bacterial Discovery Laboratory, Center for Environment, IST, JNTU, Hyderabad. The organism was grown photoheterotrophically (2,400 lux) on mineral medium (Table 1) with malate (22 mM) and ammonium chloride (7 mM) as carbon and

nitrogen sources respectively, in fully filled (15x125 mm) screw cap test tubes at 30 ±2 °C.

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

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

2.3 **ASSAYS**

2.3.1 **Assay with growing cells:** Logarthimically grown (24 h) culture of *Rba. sphaeroides* OU5 (0.48 $O.D_{660}$) was transferred into 15 x 32 mm screw cap test tubes and 1 mM stock of 2-aminobenzoate was added and incubated for 48 h under anaerobic/light (2, 400 lux) at 30 ± 2 °C. After incubation the culture was harvested for analysis.

2.3.2 **Assay with resting cells:** Logarthimically grown (24 h) culture of *Rba. sphaeroides* OU5 (0.48 $O.D_{660}$) was harvested by centrifugation at (16,000 x g, 10 min) and the cell pellet was washed thrice with the basal medium before suspending in the mineral medium containing 1 mM 2-aminobenzoate. The culture was then dispensed into screw cap test tubes and incubated anaerobic/light (2, 400 lux) at 30 ± 2 °C.

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

Mineral medium used for the growth of purple non-sulphur bacteria (after Biebl and Pfennig, 1981). SL_7 ($mg.ml^{-1}$): HCl (25% v/v)-1ml; $ZnCl_2$-7; $MnCl_2.4H_2O$-100; H_3BO_3-60; $CoCl_2.6H_2O$-200; $CuCl_2.H_2O$-20; $NiCl_2.6H_2O$-20; $NaMoO_4.6H_2O$-40

Table 1: The composition of Biebl and Pfennig's medium (Biebl and Pfennig's, 1981)

2.3.3 **Assay with cell free extracts:** *Rba. sphaeroides* OU5 grown in the presence of 2-aminobenzoate (1 mM) was harvested after 48 h of incubation by centrifugation (16,000 x g, 10 min) and the cell pellet was suspended in phosphate buffer (0.05 M, pH 7.8) and sonicated [6x2 min, 8 cycles, probe MS 72 in Bandelin Sonoplus Sonicator]. Cell free extracts were used for enzyme assays.

2.4 **Bulk cultivation for transformation studies:** Culture was grown in 2 liter reagent bottles for bulk cultivation of *Rba. sphaeroides* OU5. With 20 % (v/v) of initial inoculum, the organism was grown on the photoheterotrophic growth medium (described above) till its late logarithmic phase (about 36 to 40 h) under phototrophic (2,400 lux) conditions at 30 $\pm$ 2 ^{o}C. Ten ml stock of sterilized 2-aminobenzoate in ethanol (neutralized to pH 7) was added to the culture giving a final concentration of 1 mM. Phototrophic incubation was continued for another 48 h and later harvested for further analysis.

2.5 **ANALYSIS**

2.5.1 **Growth and biomass:** Growth was measured turbidometrically, in terms of optical density (O.D) using a Systronic colorimeter (mode 112) at 660 nm (filter 8) using uninoculated medium as blank.

2.5.2 **Determination of dry weight:**

Dry wt. of the culture was determined from O.D. versus dry wt. graph prepared specifically for this culture. An aliquot of the logarithmic culture was centrifuged at 10,000 rpm for 10 minutes and the pellet was washed [twice] with saline [0.1 % NaCl w/v] and re-suspended in distilled water. Known volumes of concentrated cell suspension at various dilutions were transferred to previously weighed aluminum boats and dried at 60 ºC. All weights were determined on a single pan balance. An

OD versus dry wt graph was plotted taking OD of cell suspensions at 660 nm. Calculation of the dry wt. was done by the empirical formula drawn from the graph.

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

2.5.3 **Determination of bacterial pigment:** Bacteriochlorophyll-a (Bchl-a) and the carotenoid content of the cultures was determined by the method of Cohen-Bazire *et al*., (1957). Five ml of bacterial culture was harvested by centrifugation (10,000 rpm for 10 min) and the pellet was washed thrice and suspended in 0.5 ml of distilled water and the pigments were extracted by the addition of 5 ml of methanol-acetone (7:2 v/v) mixture. The suspension was centrifuged to sediment the cells and the supernatant was used for pigment analysis.

A] **Estimation of Bacteriochlorophyll-a:** The O.D of Bchl-a extracted into methanol –acetone mixture was determined at 775 nm in Genesys spectrophotometer. The Bchl –content was calculated from the extinction of Bchl-a at 775 nm according to the following formula: Bchl-a (mg.100 ml^{-1}) = $O.D_{775}$ x 2.19

B] **Estimation of carotenoids:** The O.D of the carotenoids in the methanol–acetone mixture was measured at 456 and 510 nm. The carotenoid content was calculated after subtracting light absorption at these wavelengths caused by Bchl-a according to the following formula:

Chlorophyll correction at 456 nm = $O.D_{775}$ x 0.1

Chlorophyll correction at 510 nm = $O.D_{775}$ x 0.05

Yellow carotenoid content (mg.100 ml^{-1}) = (corrected $O.D_{456}$ x 0.367) - (corrected $O.D_{510}$ x 0.355)

Red carotenoid content (mg.100 ml^{-1}) = (corrected $O.D_{510}$ x 0.367) - (corrected $O.D_{456}$ x 0.355)

2.5.4 **Whole cell absorption spectrum:** Absorption spectrum of whole cells was measured by the sucrose method of Truper and Pfennig (1981). To 3.5 ml of the liquid culture, 5 g of sucrose was added and mixed thoroughly on a vortex spinner. The absorption spectrum from 300-1100 nm was measured on a Spectronic Genesys 2 spectrophotometer using sucrose in the medium blank.

2.5.5 **UV-spectroscopic analysis:** The spectral analysis of the carotenoid pigments in ethylacetate was done at 200-600 nm on Spectronic Genesys 2 spectrophotometer using ethylacetate as the blank.

2.5.6 **COLORIMETRIC ANALYSIS**

2.5.6.1 **Estimation of indoles:**

Indole was estimated calorimetrically either by Salper's reagent (Gordon and Paleg, 1957) or by p-dimethylaminobenzaldehyde (PDAB) reagent (Kupper and Atkinson, 1964).

2.5.6.1.1 **Salpers method:**

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

2.5.6.1.2 **PDAB Method:**

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

2.5.6.2 **Estimation of 2-aminobenzoate:** To 1 ml culture supernatant made up to 5 ml with water, 1 ml of freshly prepared PDAB reagent [60 mg of p-dimethylaminobenzaldehyde in 1 ml of 3 N H_2SO_4] was added and absorbancy was read at 420 nm against reagent blank.

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

2.5.7 **HPLC ANALYSIS**

2.5.7.1 **2-Aminobenzoate:** Detected in Shimadzu 10ATvp HPLC using UV-Vis detector at 210 nm on a C18 column with a mobile phase as methanol:water (1:1) or with methanol:phosphate buffer 0.05 M (30:70) at a flow rate of 1.5 $ml.min^{-1}$. The R_t of 2-aminobenzoate in this solvent is 3.93.

2.5.7.2 **Detection of indole:** The indole bound to the protein was extracted with ethyl acetate and detected in Shimadzu 10ATvp HPLC using UV-Vis detector at 270 nm, on a C18 column with mobile phase as methanol:water:acetonitrile (1:1:0.25) at a flow rate of 1.5 $ml.min^{-1}$. The R_t of indole in this solvent is 4.3.

2.6.1 **Isolation and purification of the metabolites:** Twenty-liter culture supernatant of *Rba. sphaeroides* OU5 grown on 2-aminobenzoate was harvested after 48 h of incubation. The culture supernatant was extracted thrice into ethylacetate and the aqueous layer was acidified with 5 N HCl and extracted thrice with ethylacetate. Both the extracts were dried in vacuo and the concentrated sample was used as crude extract for the isolation of various metabolites. The metabolites were purified from the crude extract with TLC and column chromatography using various organic solvents.

2.6.2 **Characterization of metabolites:** Purified metabolites were characterized using UV-Vis spectrophotometer, FT-IR, ^{1}H, C^{13} NMR and mass spectroscopic analysis.

2.6.2.1 **UV analysis:** The sample suspended in ethylacetate was analyzed in a Spectronic Genesys 2 spectrophotometer.

2.6.2.2 **Infra-red (IR) analysis:** Done on a Shimadzu FT/IR 8300 either as a NEAT sample or as a KBr pellet.

2.6.2.3 **Mass analysis:** Done using a mass VG 70-70H analyzer.

2.6.2.4 **NMR:** Purified compound suspended in $CDCl_3$ used for ^{1}H and ^{13}C NMR using a Brucker AC200 (200 MHz) analyzer.

2.6.2.5 **LC-MS analysis:** Done with the crude extracts isolated from the culture supernatant into ethylacetate. The compound was re-dissolved in methanol and analyzed on LC-MS (Shimadzu 2010 made in Japan) with methanol and water (1:1) as the mobile phase with a flow rate of 0.2 $ml.min^{-1}$, using coil with the positive mode on ESI probe.

2.6.2.6 **GC-MS analysis:** The metabolites were extracted from the enzyme assay sample into methanol was analyzed on GC-MS Shimadzu GC-MS QP 2010, with the injection volume of 2 µl on EI probe with column oven temperature of 300 °C, injection temperature 310 °C, column flow 5.2 $ml.min^{-1}$ split ratio was 5.0 with Helium as the carrier gas and methanol as the solvent and the molecular masses of the column effluent from the GC was analyzed through GC 2010 mass analyzer.

2.7 ENZYME ASSAYS

2.7.1 Tryptophanase:

Enzyme assay was done according to Lester and Yanofsky (1961). To 1 ml sonicated culture sample, 1 ml of 0.05 M phosphate buffer (pH 7.8) containing 0.4 µmoles tryptophan and 10-µg pyridoxal phosphate was added. After incubation at 37 ºC for 30 min with agitation, the assay mixture was chilled in an ice bath and extracted into 4 ml of ethylacetate. One ml sample of the ethylacetate extract was assayed for indole using PDAB reagent.

2.7.2 **Anthranilate synthase:**

Enzyme assay was done with 50 µl cell free extracts in phosphate buffer 0.05 M (pH 7.8) containing 100 µmoles 2-aminobenzoate, 10 µmoles PRPP, 10 µg magnesium chloride ($MgCl_2$) and incubated (lux 2,400) at 30 ^oC for 30 min. After incubation the consumption of 2-aminobenzoate and PRPP were analysed in HPLC using UV-Vis detector at 220 nm on a C18 column with a mobile phase as methanol:water (1:1) at a flow rate of 1.5 $ml.min^{-1}$. The R_t of 2-aminobenzoate in this solvent is 3.93 and PRPP is 2.74.

2.7.3 **Assay for fumarate consumption:**

To 1 ml of 0.05 M phosphate buffer pH 7.8 containing 0.1 mmole 2-aminobenzoate, 0.1 mmole fumarate, 20 µg pyridoxal phosphate, and 5 µg magnesium chloride, 5µg NADP, 10 µl of enzyme sample is added and incubated at 28 ºC for 10 min. The reaction was stopped and the consumption of fumarate was analyzed in HPLC at 220 nm in a C18 column with potassium phosphate buffer: methanol (70:30) as a mobile phase with a flow rate of 1 $ml.min^{-1}$. The R_t of fumarate is 2.45.

2.7.4 **Catalase:**

Catalase activity was measured spectrophotometrically by measuring the rate of H_2O_2 disappearance at 240 nm, taking $\Delta\varepsilon$ at 240 nm as 43.6 $mol.L^{-1}$ c^{-1} (Chance and Meahly, 1955; Patterson *et al*., 1984). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 19 mM H_2O_2 and 100 µg protein in a final volume of 3 ml. The activity was determined by the oxidation of H_2O_2 at 240 nm. The change of absorbance of 1 O.D. represents the oxidation of 25 µmoles of H_2O_2.

2.7.5 **Superoxide dismutase:**

Superoxide dismutase activity was determined by the method of Beauchamp and Fridovich, 1977. The required cocktail for SOD activity was prepared by mixing 27

ml of sodium phosphate buffer (pH 7.8), 1.5 ml of methionine (300 mg ml^{-1}), 1 ml of NBT (Nitroso blue tetrazolium) (14.4 mg 10 ml^{-1}), 0.75 ml of Triton X-100 and 1.5 ml of 2 mM EDTA. To 1 ml of this cocktail, 10 µl of riboflavin (4.4 mg.100 ml^{-1}) and 50 µg of protein were added. After mixing, the contents were illuminated for 8 min under light (2,400 lux). The temperature was maintained at 25 ºC using a water bath. A tube with protein kept in dark served as blank, while the control tube without enzyme was illuminated. The increase in absorbance was measured at 560 nm. The reduction of NBT under illumination was measured without enzyme and also in the presence of the enzyme. Activity of SOD is the measure of NBT reduction in light without protein minus NBT reduction with protein. One unit of activity is the amount of protein required to inhibit 50 % initial reduction of NBT under light.

2.7.6 **Peroxidase:**

Ascorbate peroxidase was assayed by the method of Nakano and Asada, 1981. The reaction mixture for measuring ascorbate peroxidase activity contained 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 250 mM H_2O_2 and 50 µg of protein. The activity was recorded as decrease in absorbance at 290 nm for 1 min and the amount of ascorbate oxidized was calculated from the extinction coefficient-2.8 mM^{-1} cm^{-1}.

2.7.7 **Esterase:**

To 1 ml phosphate buffer pH 7.8 (0.05 M) containing 10 U of enzyme esterase, 10 µl of the metabolite (indole ester 5 ηM) was added and incubated for 5 min. The esterase activity was known as the decrease in the compound peak height in HPLC, due to the breakage of the ester bond in the compound. The decrease in the peak height of the compound was detected in (Shimadzu 10ATvp) HPLC using UV-Vis detector at 250 nm on a C18 column with a mobile phase as methanol:water (1:1) at a flow rate of 1.5 $ml.min^{-1}$.

2.8 **Isolation and electrophoresis of proteins:**

The isolation of proteins involved in indole biosynthesis from 2-aminobenzoate and fumarate and orange carotenoid proteins (OCPs) are described in the results. The electrophoresis of the proteins was done on 12 %, 10 % SDS-PAGE and 6 % Native-PAGE and stained with coomassie blue or silver stain (Lammeli, 1970).

2.9 **Gel elution of proteins:**

2.9.1 **Electroelution:** The protein bands from the PAGE gels were cut and extracted by electro-elution in Tris–glycine buffer (1.5 M) at 70 V for 3 h in horizontal electrophoresis tank.

2.9.2 **Elution with buffer**: The protein bands were crushed and incubated in elution buffer (Tris/phosphate buffer pH 7.5 containing Triton X-100 (0.1 % v/v) at 4 °C for 20 h.

2.10 **Protocols used in bioprospecting the metabolites**

2.10.1 **Auxin bioassay:** The coleoptiles of root and shoot tips of maize were cut and the explants were incubated in the standard auxin solutions of NAA (5 µM), IAA (5 µM) and also the metabolites (5 ηM) under light for 24 h. The increases in the length of coleoptiles were calculated as the percentage increase of length.

2.10.2 **Phytohormonal activity:** The tissue culture plants of mulberry and tobacco were transferred onto the MS medium containing various concentrations (5ηM-5µM) of the metabolites and also to the MS medium containing standards of NAA, IAA, 2, 4-D (5µM) and incubated under light for 15-20 days. The plants were observed for rooting after 15 days of incubation.

2.10.3 **Cyclooxygenase-1 and 2 inhibitory activities**:

The metabolites were assayed for cyclooxygenase-1 and 2 inhibitory activities according to the method of Copeland *et al.,* 1994.

Results

3.1 **Utilization of 2-aminobenzoate for growth and production of indole**

3.1.1 **Utilization of 2-aminobenzoate for growth**

Rhodobacter sphaeroides OU5 was unable to utilize 2-aminobenzoate (3 mM) as a sole source of carbon or nitrogen or carbon and nitrogen or as e^- donor, replacing either malate or ammonium chloride or both respectively. Even reducing the concentration of 2-aminobenzoate (0.1 mM) and addition of bicarbonate (0.1 % w/v) did not support growth. Fig 4 shows photoheterotrophic growth of *Rba. sphaeroides* OU5 on malate and ammonium chloride; it has a doubling time of about 8 h. Presence of 2-aminobenzoate (1 mM) has not altered the doubling time of this strain (Fig 5) when supplemented to the photoheterotrophic growth medium. Loss of 2-aminobenzoate from the culture supernatant was observed after 30 h of phototrophic incubation. The culture supernatant when analyzed with Salper's reagent and with PDAB gave positive test indicating the presence of indole. Indole production was observed by both growing and resting cells of *Rba. sphaeroides* OU5 .

3.1.2 **Light and dark production of indole**

Production of indole from 2-aminobenzoate was not observed when the culture was incubated in dark aerobic conditions. Indole production was observed only under phototrophic incubations by both growing (Fig 5) and resting cells (Fig 6). With growing cells indole was produced after 30 h of incubation (Fig 5) and maximum indole yield was observed after 48 h. While with the resting cells indole production started with a lag period of 6 h and reached a maximum within 42 h (Fig 6). Maximum indole yield of 0.7 mM was obtained with the growing cells from 1 mM of 2-aminobenzoate. The conversion efficiency in terms of total indole yield was 70 %.

3.1.3 **Indole production at various cell densities**

Indole production was checked with various cell densities, using resting cell suspensions. Indole production was optimum at cell densities of 0.4-0.7 $O.D_{660}$ (Fig 7). The indole yield declined with increasing cell density from 0.5-1.5 ($O.D_{660}$) tested.

3.1.4 **Effect of 2-aminobenzoate concentration on indole production**

Indole yields were checked with different concentrations of 2-aminobenzoate and a graph was plotted (Fig 8). Indole yields increased with increasing concentration of 2-aminobenzoate reaching optima around 0.7 mM to 1.0 mM and declined at higher concentrations.

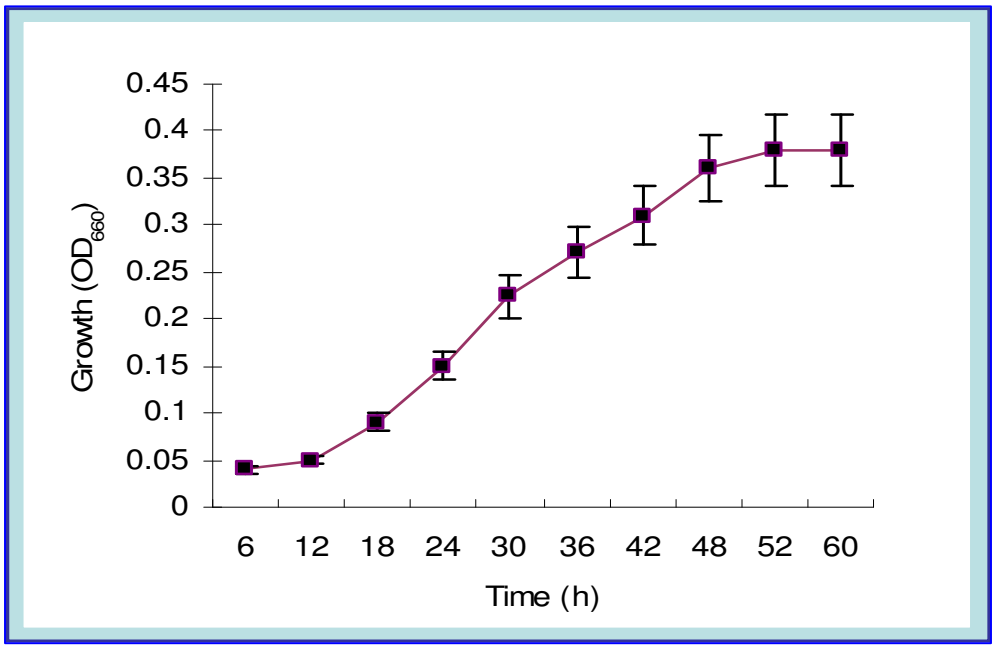


Fig 4: Photoheterotrophic growth of *Rba. sphaeroides* OU5

Experiment was done with logarithmically grown cells of *Rba. sphaeroides* OU5 inoculated into photoheterotrophic growth medium containing malate (22 mM) as carbon and ammonium chloride (7 mM) as nitrogen source incubated in fully filled screw cap test tubes (15x125 mm), exposed to light (2, 400 lux) anaerobically at 30±2 °C. Results expressed are average of an experiment done in triplicates.

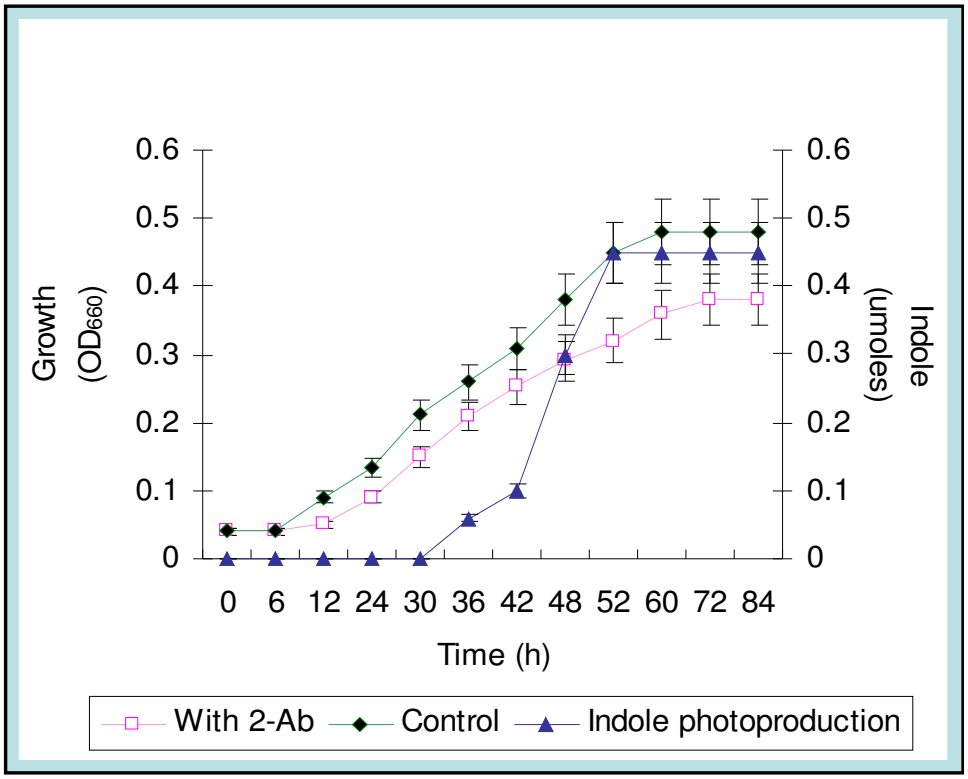


Fig 5: Photoheterotrophic growth and simultaneous production of indole by *Rba. sphaeroides* OU5

Experimental conditions are same as in fig 4.

With 2-Ab = Photoheterotrophic growth in the presence of 2-aminobenzoate;

Control = Photoheterotrophic growth in the absence of 2-aminobenzoate.

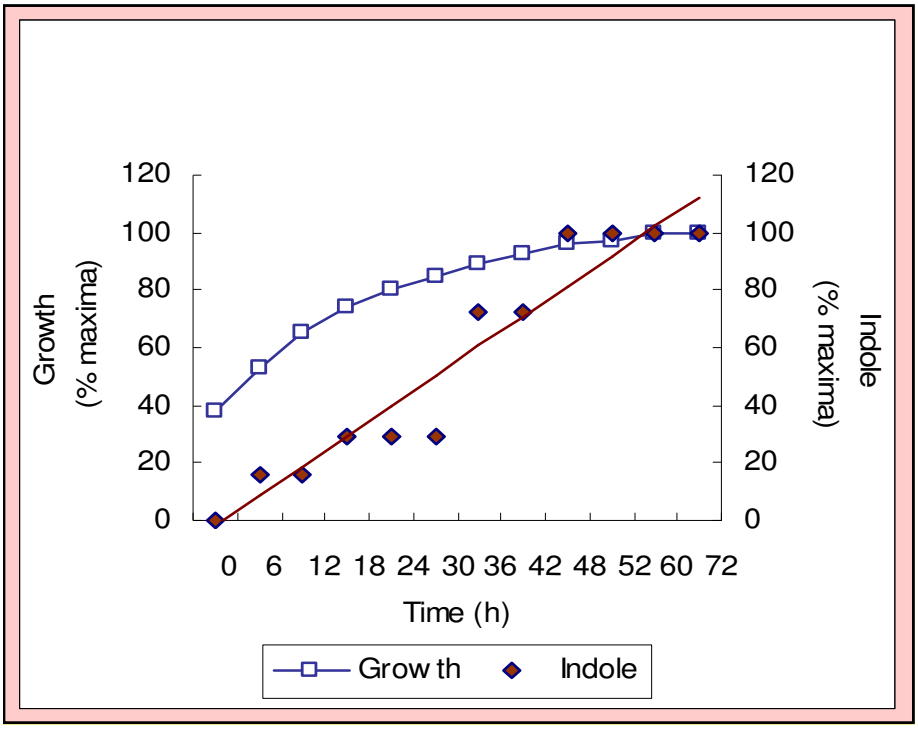


Fig 6: Photoproduction of indole from 2-aminobenzoate by resting cells of *Rba. sphaeroides* OU5

Resting cells showed a lag of 6 h for indole photoproduction. Biomass 100 % = 0.57 mg dry wt.ml $^{-1}$; 100 % indole = 0.35 µM.

Experiment was done with resting cell suspension inoculated into the mineral medium containing 1 mM 2-aminobenzoate and 13 mM fumarate and incubated anaerobically in fully filled screw cap test tubes (15x125 mm) at (2, 400 lux) and 30±2 °C. Results expressed are average of the experiment done in triplicates.

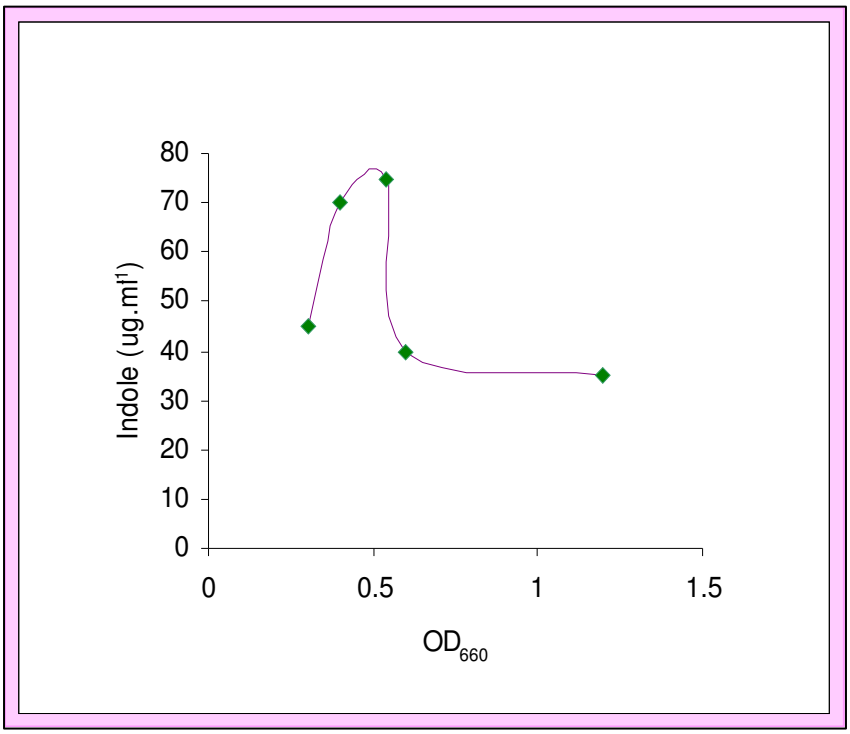


Fig 7: Indole production by *Rba. sphaeroides* OU5 at various cell densities

Experiment was done with resting cell suspensions at different cell densities and the assay conditions are same as in fig 6.

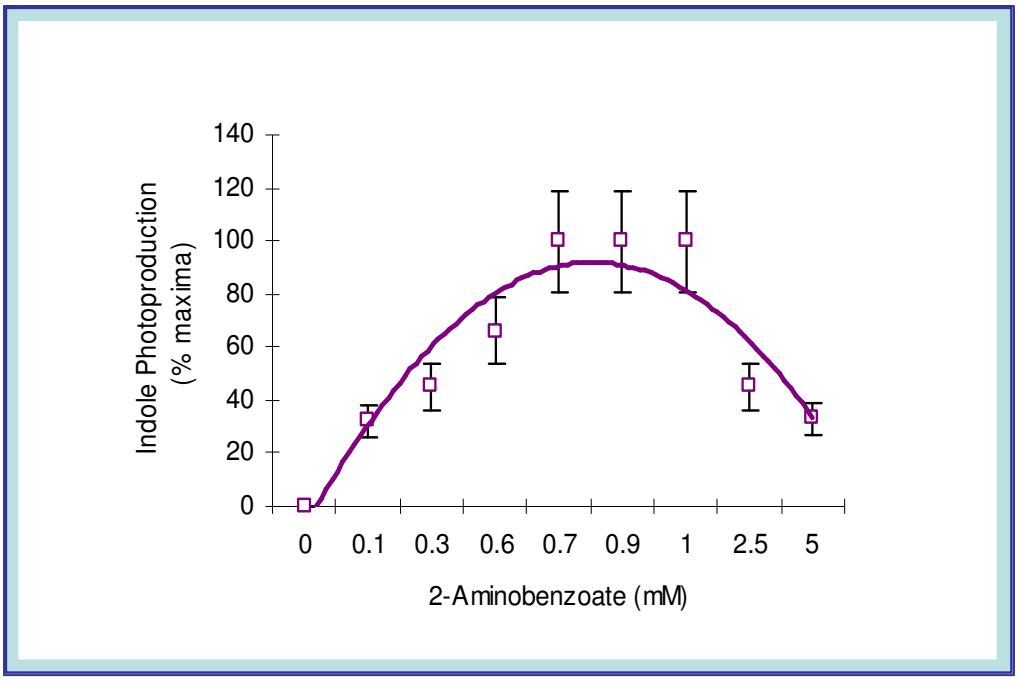


Fig 8: Effect of various concentrations of 2-aminobenzoate on indole production by *Rba. sphaeroides* OU5

Experimental details are as in fig 6 except that 2-aminobenzoate stock was added to get different concentrations in the culture tubes before incubation. Results pertain to the data analyzed after 48 h and the error bars represent the data from quadruplicates.

3.2 **Metabolomics of 2-aminobenzoate transformation by *Rhodobacter sphaeroides* OU5**

3.2.1 **Lipid associated indole**

In the presence of 2-aminobenzoate, lipid (non-polar) content was found to be more than that of the control as observed in the culture supernatant of *Rba. sphaeroides* OU5, when extracted with ethylacetate (not quantified but observed visually). The indole produced from 2-aminobenzoate was found in the lipid fraction and it varied with cell density. With low cell density the lipid indole fraction was high and it decreased at high cell density (Fig 9).

3.2.2 **Metabolite profiling of culture supernatant of *Rba. sphaeroides* OU5 grown in the presence of 2-aminobenzoate**

In order to know the range of metabolites produced in the presence of 2-aminobenzoate by *Rba. sphaeroides* OU5, the culture supernatant of *Rba. sphaeroides* OU5 grown with and without 2-aminobenzoate was extracted with ethylacetate and the concentrated extract was analyzed using LC–MS. The metabolites of masses (*m/z*) 169, 189, 205, 227, 281, 386, 405, 413, 441, 489, 530 and 595 (Fig 10) were detected in the presence of 2-aminobenzoate, while they were absent in the culture supernatant of *Rba. sphaeroides* OU5 grown without 2-aminobenzoate (data not shown).

3.2.3 **Detailed analysis of A14 (Initially isolated and characterized metabolite of 2-aminobenzoate transformation by *Rba. sphaeroides* OU5)**

The A14 metabolite was obtained from the ethylacetate extract of the culture supernatant of *Rba. sphaeroides* OU5 grown in the presence of 2-aminobenzoate. The ethylacetate extract was eluted with benzene in the column chromatography to get B1, B2 and B3 fractions. The benzene extract of B1 on further elution gave A14. The A14 gave orange red color with Salper's and Ehrlich reagent, suggesting that the compound is an indole derivative.

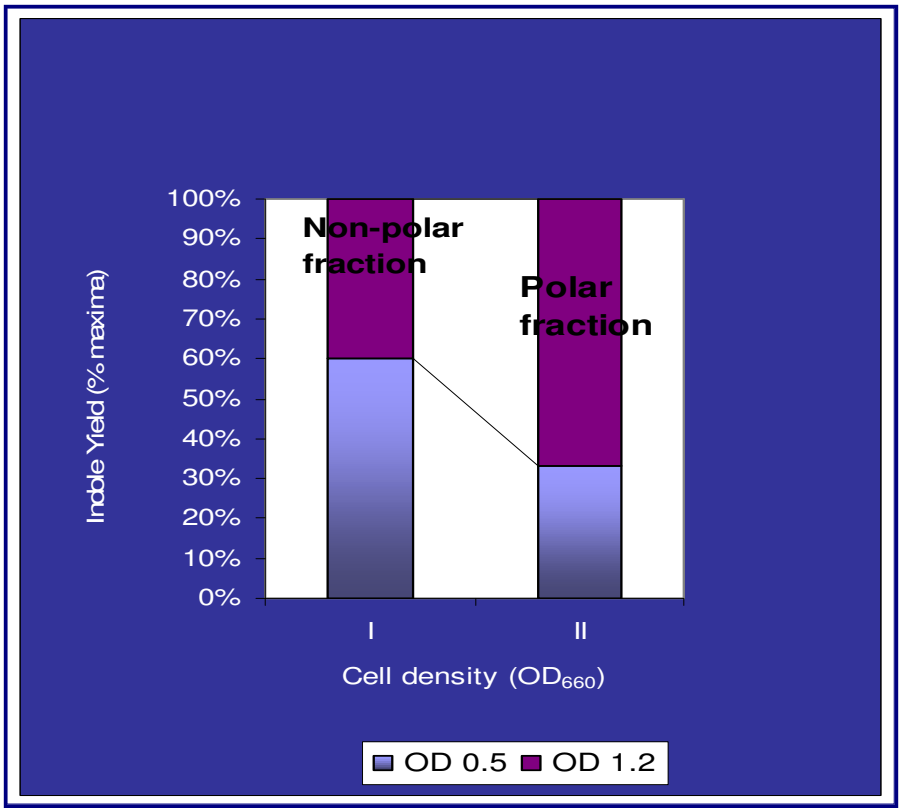


Fig 9: Distribution of indole produced between the polar and non-polar fractions at two different cell densities of *Rba. sphaeroides* OU5 grown in the presence of 2-aminobenzoate

Experiment was done with resting cell suspensions at two different cell densities to which 1 mM 2-aminobenzoate and 13 mM fumarate was added and incubated anaerobically for 48 h at (2,400 lux) and at 30±2°C. Results are average of data obtained from triplicates.

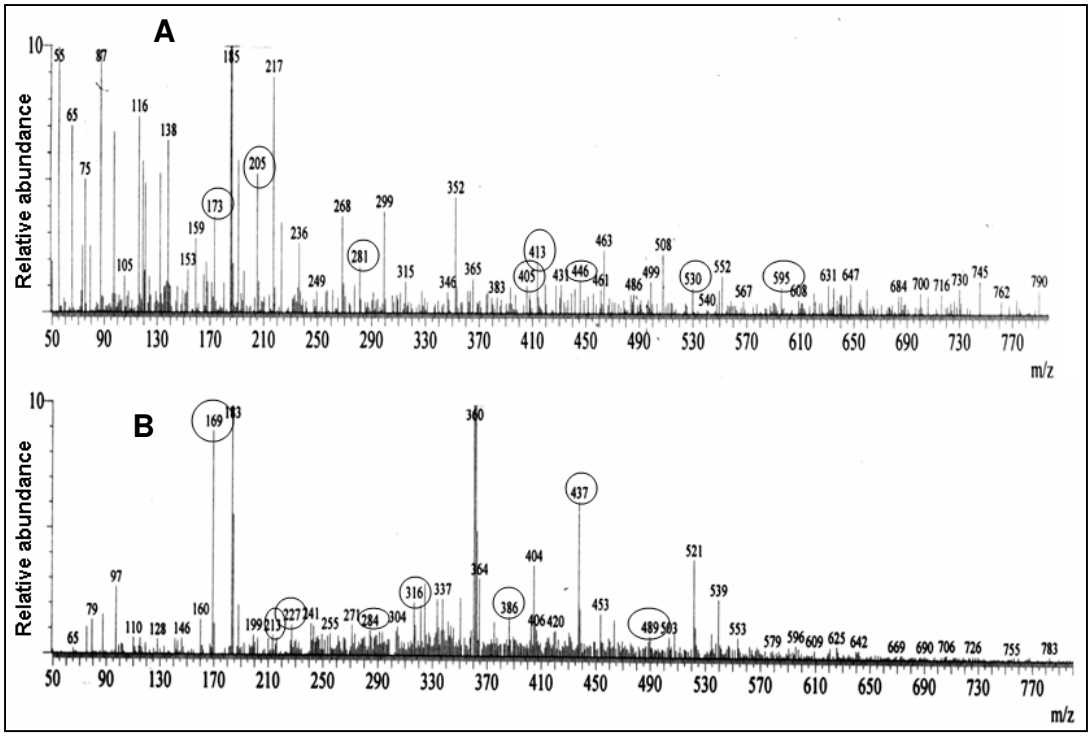


Fig 10: LC-MS metabolite profile of the culture supernatant of *Rba. sphaeroides* OU5 grown with 2-aminobenzoate

A-Represent the ion masses of the metabolites of the peak one integration of LC chromatogram (data not shown); B-Represent the ion masses of the metabolites of the second peak integration of LC chromatogram. The encircled ones are the masses of the metabolites appeared in the culture supernatant of *Rba. sphaeroides* OU5 when grown with 2-aminobenzoate and these were absent in the culture without 2-aminobenzoate.

The compound was found to be pure and had a R_f of 0.64, 0.63, 0.7 with benzene; benzene:chloroform (1:1 v/v) and benzene:ethyl acetate (8.5:1.5 v/v) respectively. UV absorption maxima of the compound at 251, 275 and 320 nm in ethyl acetate indicate the basic indole nucleus, which has absorption at 275 nm. Infrared analysis of the compound (Fig 11) confirms the presence of indole nucleus with peaks (cm^{-1}) at 3417 corresponding to N-H and 740 to the benzene. Further, the compound has a very strong ester peak at 1728. Strong peaks at 2960 – 2850 and medium peaks at 1470 – 1430 strongly suggest that there are $–CH_3$ and $–CH_2$- groups. Strong peaks at 1070, 1276 and a weak peak at 3049 indicate the presence of hydroxyl groups.

The presence of ester linkage in the metabolite was confirmed through esterase activity. The decrease in the peak height of the compound in the presence of esterase enzyme confirmed the presence of ester linkage in A14 (Fig 12)

Fig 13A shows the ^1H NMR spectrum of the isolated compound in $CDCl_3$. The basic skeleton of indole matches with the standard indole ^1H NMR (Fig 13B; insertion). Fig 14 shows the mass fragmentation of the compound with a molecular mass of 441. The molecular formula of the compound is $C_{27}H_{39}NO_4$. The molecular composition of C 73.44 and H 8.9 matches with the analysis C 72.24 and H 8.58. The IUPAC name of the compound is (3E)-13-hydroxy-1, 1, 5, 9-tetramethyl trideca-3,7-dienyl 2-(hydroxymethyl)-1H-indole-3-carboxylate. We call this compound with a common name as sphesterin (**sph**aeroides **ester** **in**dole). Sphesterin appears orange in benzene, yellow in ethylacetate and completely evaporated and dried sample is orange brown. The compound has no prominent peak in the visible region of absorption spectrum. The aliphatic moiety of the structure (Fig 14) suggests that probably spheroidene group of terpenoid may be conjugating with the indole acid. The change in the chrome is the typical feature of spheroidene (yellow), which on oxidation forms spheroidenone (yellow red) found in *Rba. sphaeroides* OU5. We suspect that sphesterin may be an intermediate formed due to the degradation of spheroidene moiety of indole ester.

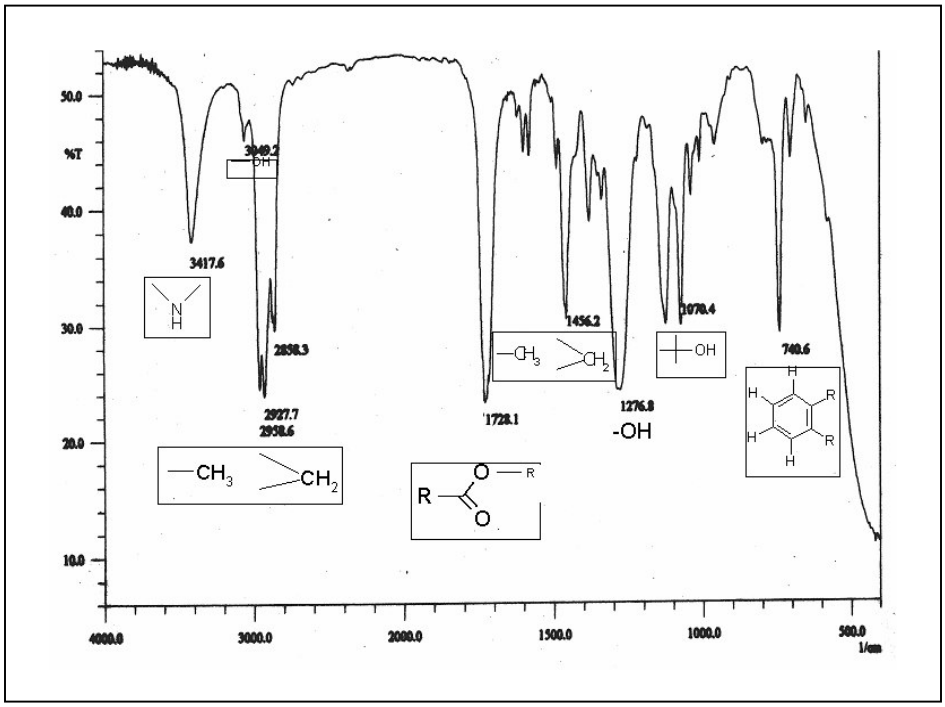


Fig 11: Infra-red spectral analysis of the purified compound sphesterin

Sphesterin dissolved in dichloromethanol was analyzed on a Shimadzu FT/IR 8300

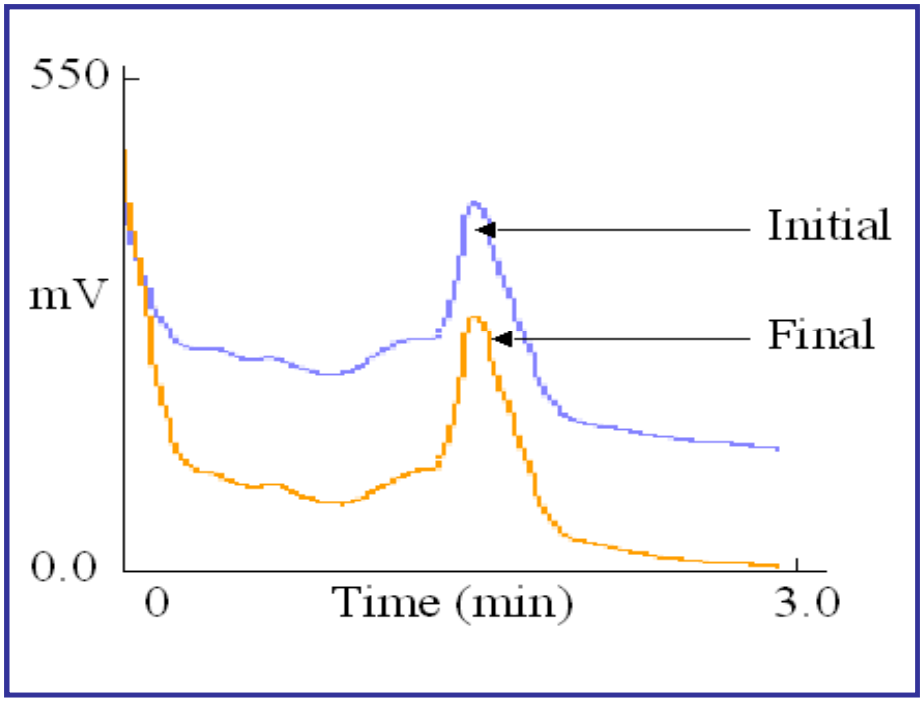


Fig 12: Action of esterase enzyme on indole terpenoid ester (sphesterin)

Loss of the metabolite confirms the presence of ester bond in the metabolite. Assay was done in 1 ml phosphate buffer 0.05 M (pH 7.8) containing 10 U of esterase enzyme and 10 µl metabolite (5 ηM); incubated for 5 min.

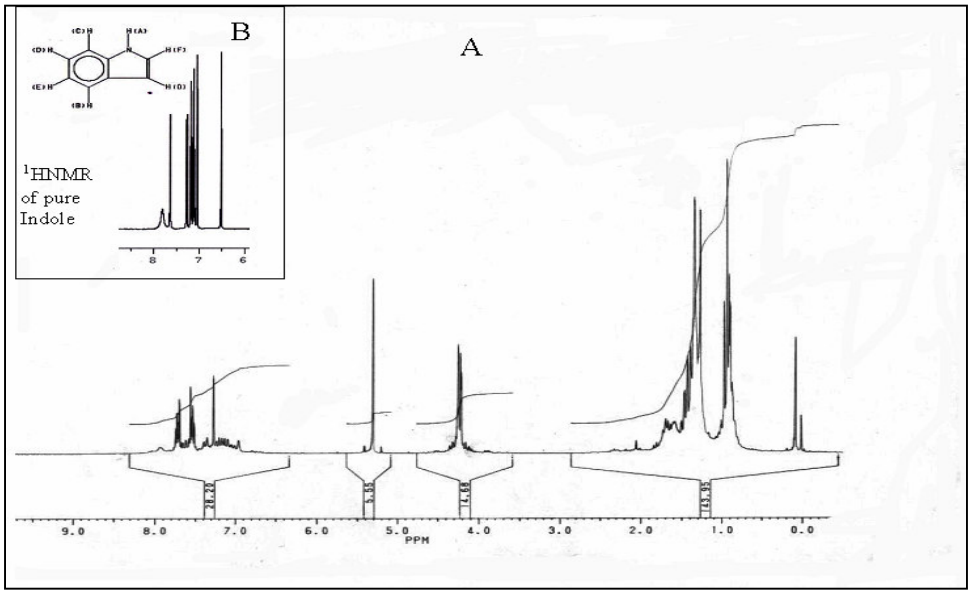


Fig 13: ^{1}H NMR analysis of the isolated compound sphesterin in$CDCl_3$ (A) B) Insertion is the standard indole ^{1}H NMR spectrum

Sphesterin dissolved in 1ml $CDCl_3$ in a NMR tube was analyzed on Brucker AC200 (200 MHz) analyzer

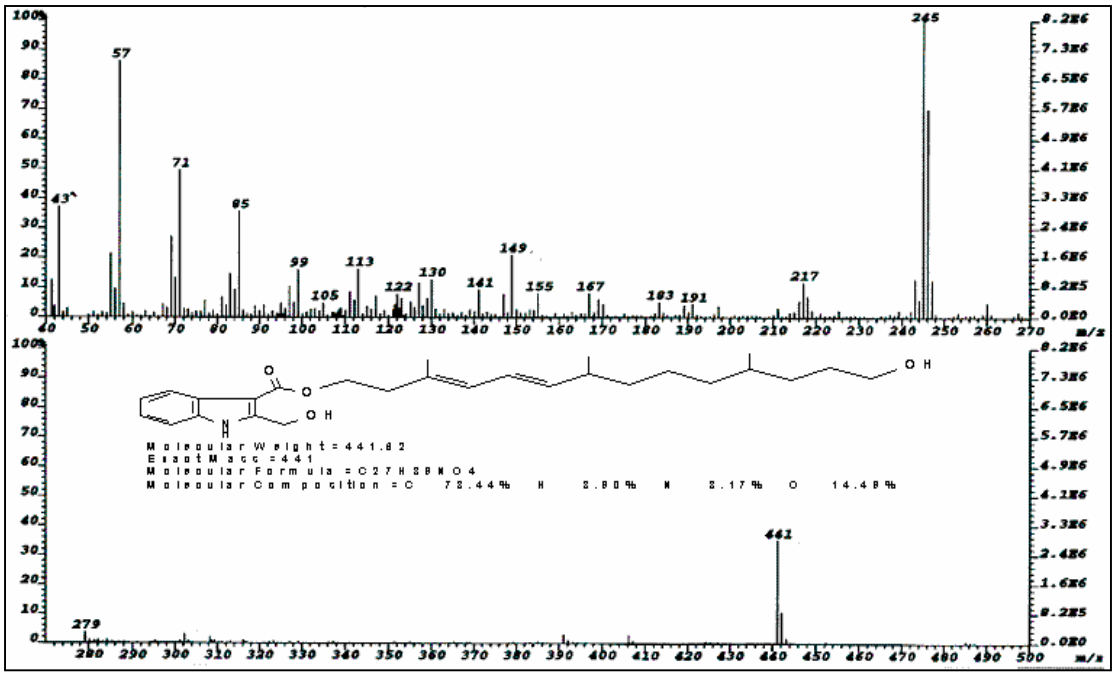


Fig 14: Mass spectral analysis of the compound showing the final structure and the molecular composition.

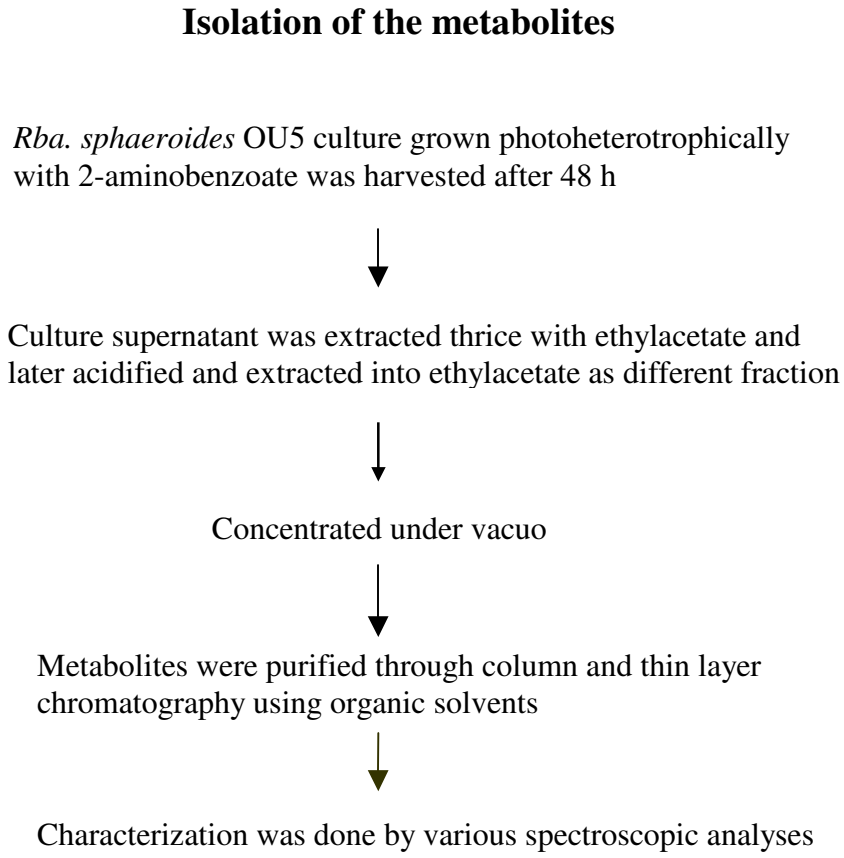
Sphesterin dissolved in methanol was analyzed on mass VG 70-70H analyzer.

3.2.4 **Isolation of few more metabolites of 2-aminobenzoate transformation by *Rba. sphaeroides* OU5**

Twenty liter culture of *Rba. sphaeroides* OU5 grown in the reagent bottles containing photoheterotrophic medium with 2-aminobenzoate (1 mM), was used for extraction of the metabolites. The metabolites of 2-aminobenzoate transformation were isolated from the culture supernatant after 48 h of phototrophic incubation. The supernatant was extracted using ten-liter ethylacetate and the aqueous layer was acidified and re-extracted with (10 L) ethylacetate (Flow chart 1) both these extracts were dried in vacuo independently. The metabolites from the crude extracts of ethylacetate layer and aqueous layer were purified in various steps (shown in flow chart 2, 3, 4, 5, 6 and 7) by TLC and column chromatography using various organic solvents. At each step of purification, the compounds were tested for the presence of indole nucleus with PDAB and Salper's reagent. The purity of the compound was finally confirmed on TLC with three different solvent systems. The compounds in the flow chart 8 are the metabolites extracted from the culture supernatant of *Rba. sphaeroides* OU5 grown in the presence of 2-aminobenzoate in the early hours of incubation (after 12 h).

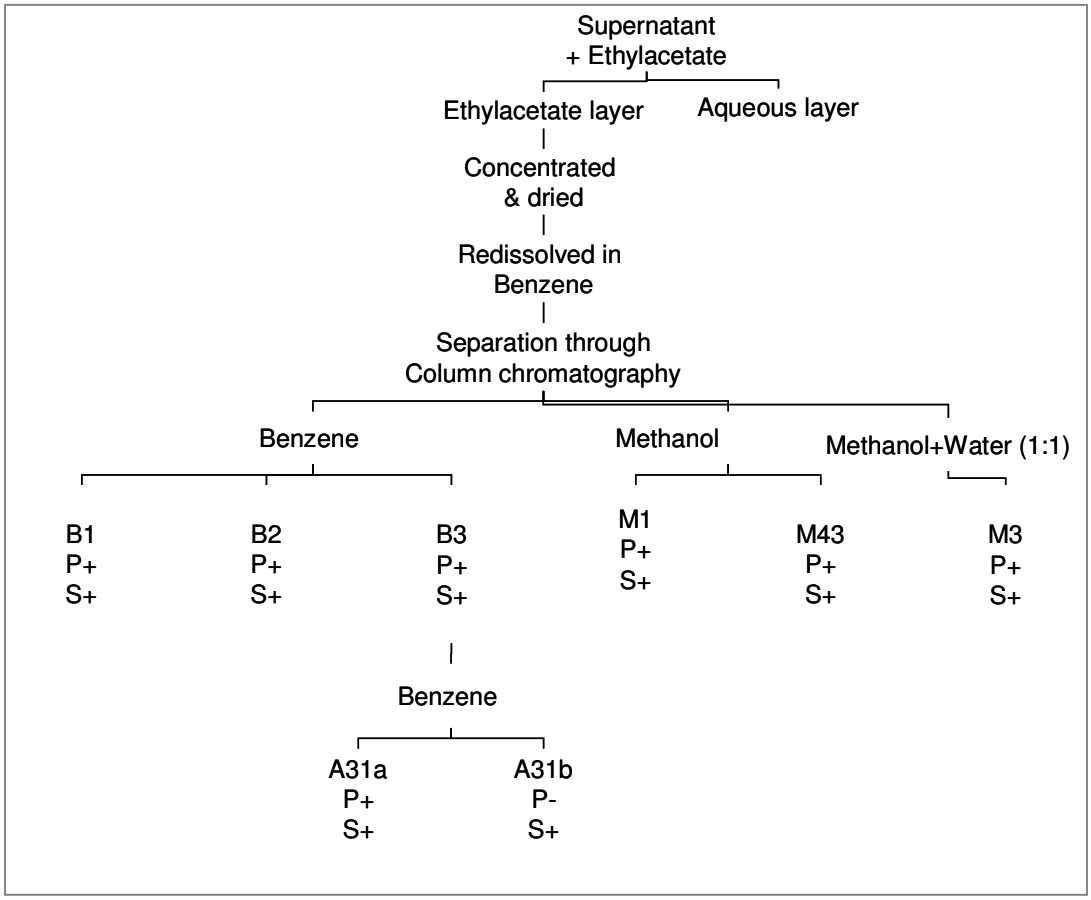
3.2.5 **Characterization of the metabolites**

Purified metabolites were characterized using UV, IR, ^{1}H, ^{13}C NMR and mass spectroscopic analyses and based on these results the structures of the compounds were elucidated and they were identified as indole terpenoid esters (Fig 15a, b, c, d, e, f, g). In total 57 different compounds were isolated out of these 29 were characterized. The compound color, the color reactions with PDAB and Salper's reagent, yield, mass and other analytical properties are given in the table 2 and 3.



Flow chart 1: Isolation of the metabolites of 2-aminobenzoate transformation

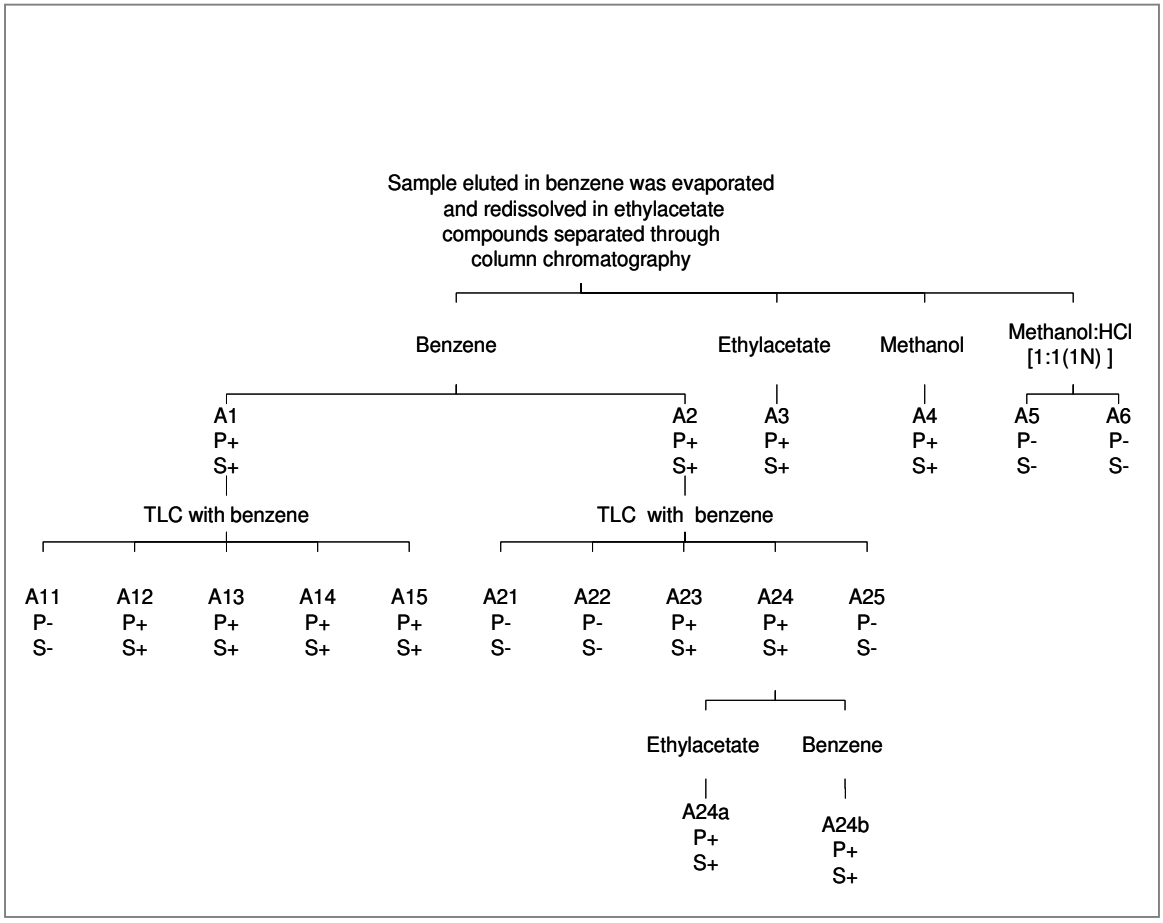
Extraction and purification of metabolites from the culture supernatant of *Rba. sphaeroides* OU5



Flow chart 2: Purification of metabolites of 2-aminobenzoate transformation from the ethylacetate layer

P+ = Positive to PDAB reagent; P- = Negative to PDAB reagent; S+ = Positive to Salper's reagent; B1, B2, B3, M1, M43, M3, A31a, A31b are different fractions separated during elution through column chromatography.

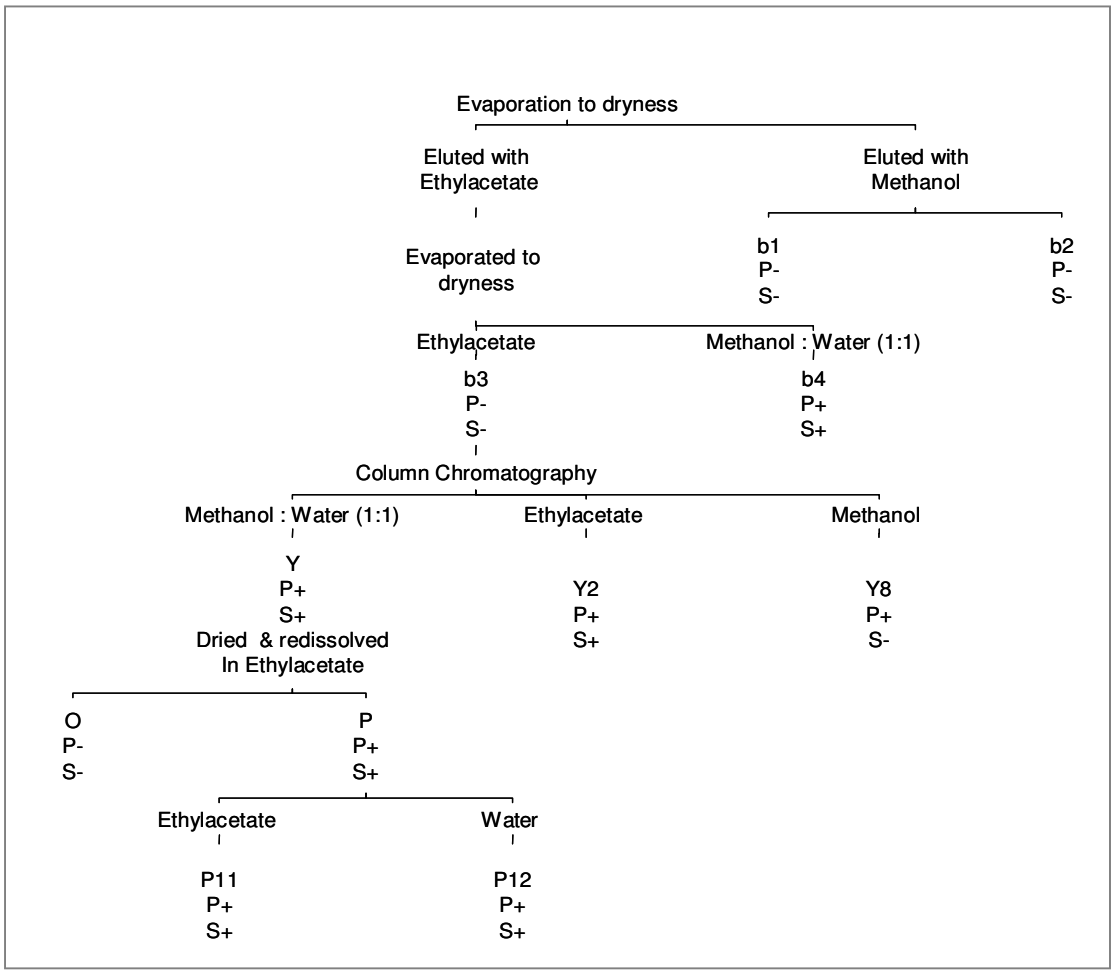
Isolation of metabolites from fraction B1



Flow chart 3: Purification of metabolites from B1 fraction of the ethylacetate layer

P+ = Positive to PDAB reagent; P- = Negative to PDAB reagent; S+ = Positive to Salper's reagent; S- = Negative to Salper's reagent; A1, A2, A3, A4, A5, A6, A11, A12, A13, A14, A15, A21, A22, A23, A24, A25, A24a and A24b are different fractions separated during elution through column chromatography.

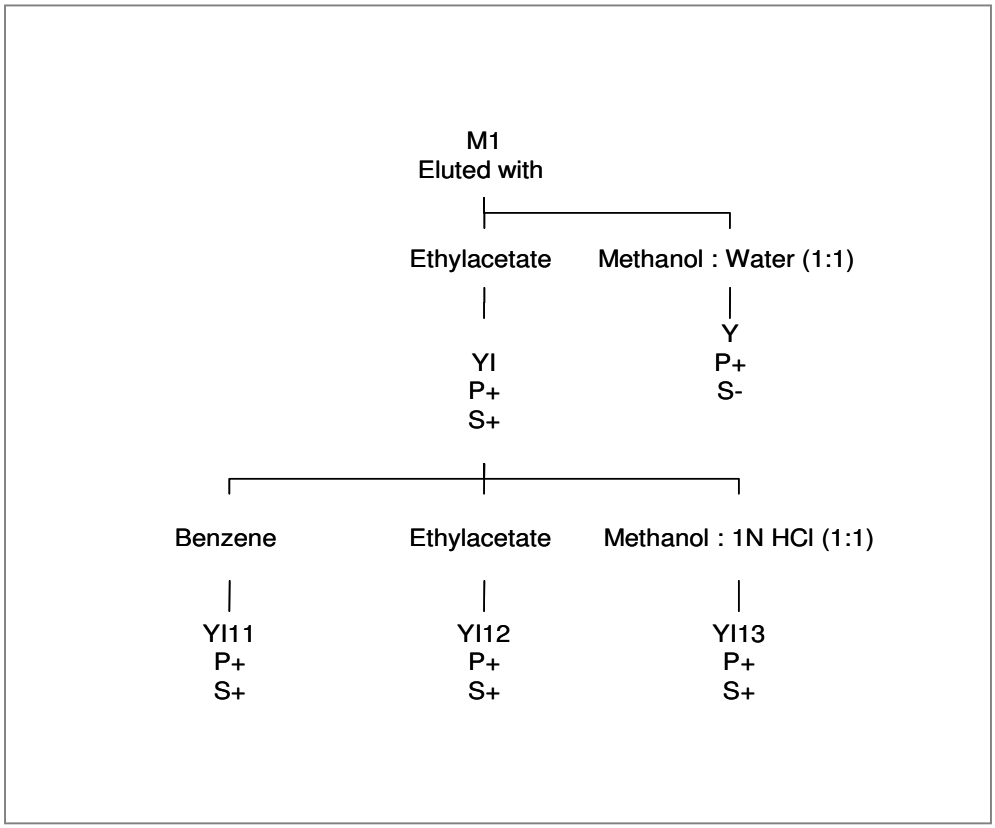
Isolation of metabolites from fraction B2



Flow chart 4: Purification of metabolites from B2 fraction of the ethylacetate layer

P+ = Positive to PDAB reagent; P- = Negative to PDAB reagent; S+ = Positive to Salper's reagent; S- = Negative to Salper's reagent; b1, b2, b3, b4, Y, Y2, Y8, O, P, P11 and P12 are different fractions eluted from B2 fraction of the ethylacetate layer.

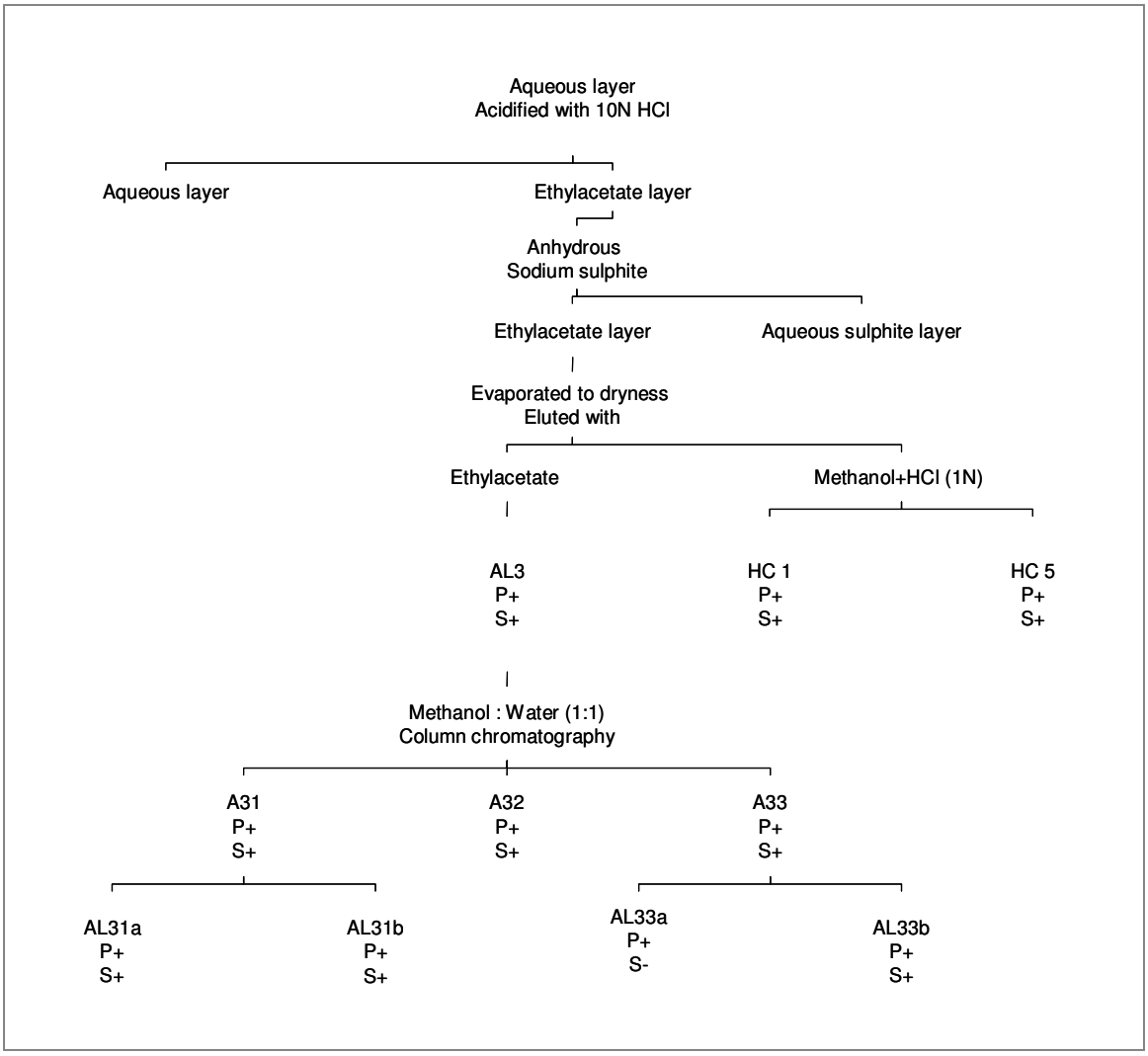
Isolation of metabolites from fraction M1



Flow chart 5: Purification of metabolites from M1 fraction of the ethylacetate layer

P+ = Positive to PDAB reagent; P- = Negative to PDAB reagent; S+ = Positive to Salpers reagent; S- = Negative to Salpers reagent; YI, Y, YI11, YI12 and YI13 are different fractions eluted from fraction M1 of the ethylacetate layer.

Isolation of metabolites from Aqueous layer

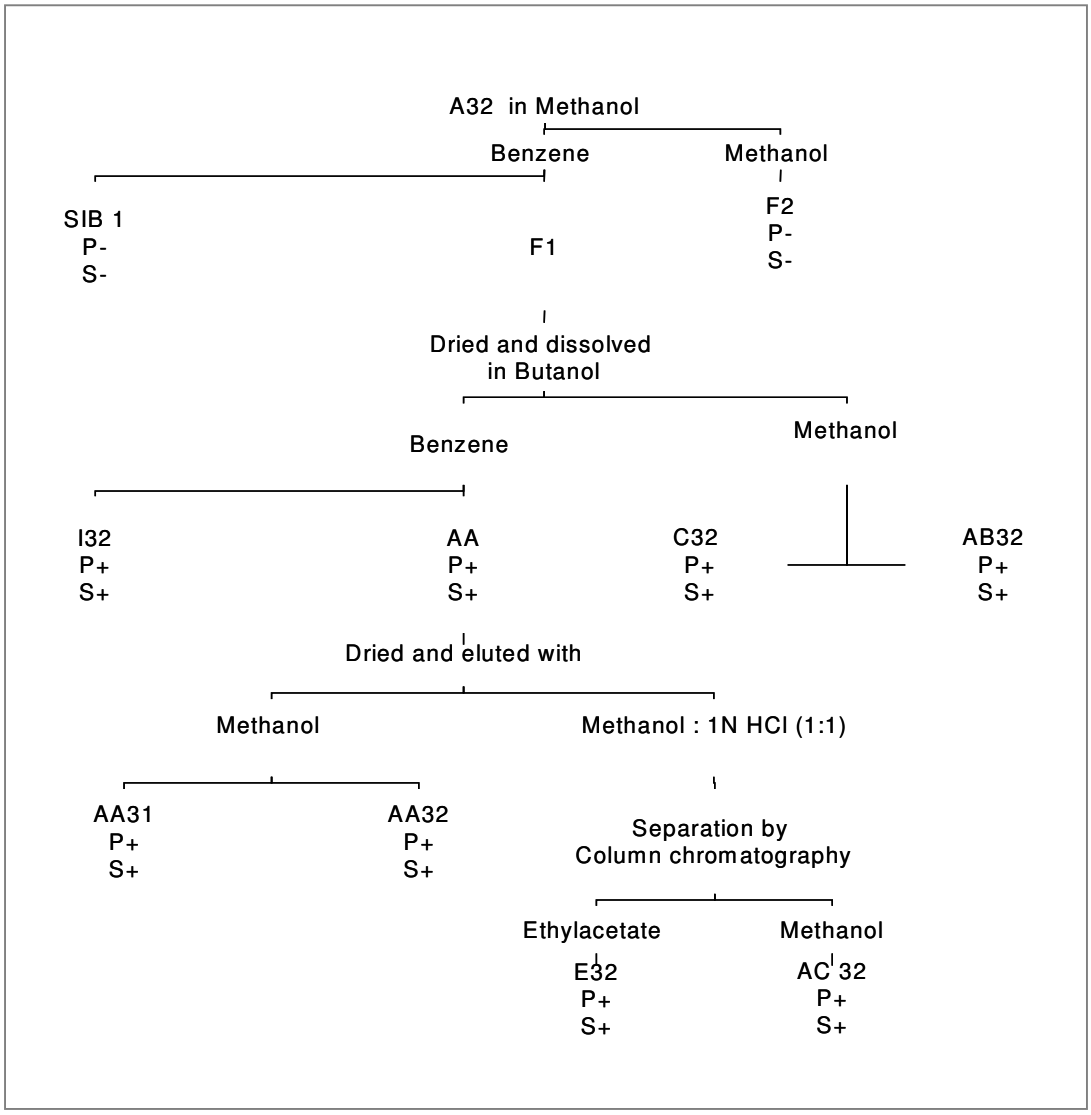


Flow chart 6: Purification of metabolites from the aqueous layer of the culture supernatant of *Rba. sphaeroides* OU5

Details are same as in the flow chart 5.

AL3, HCL 1, HCL 5, A31, A32, A33, AL31a, AL31b, AL33a and AL33b are different fractions eluted from aqueous layer using various organic solvents.

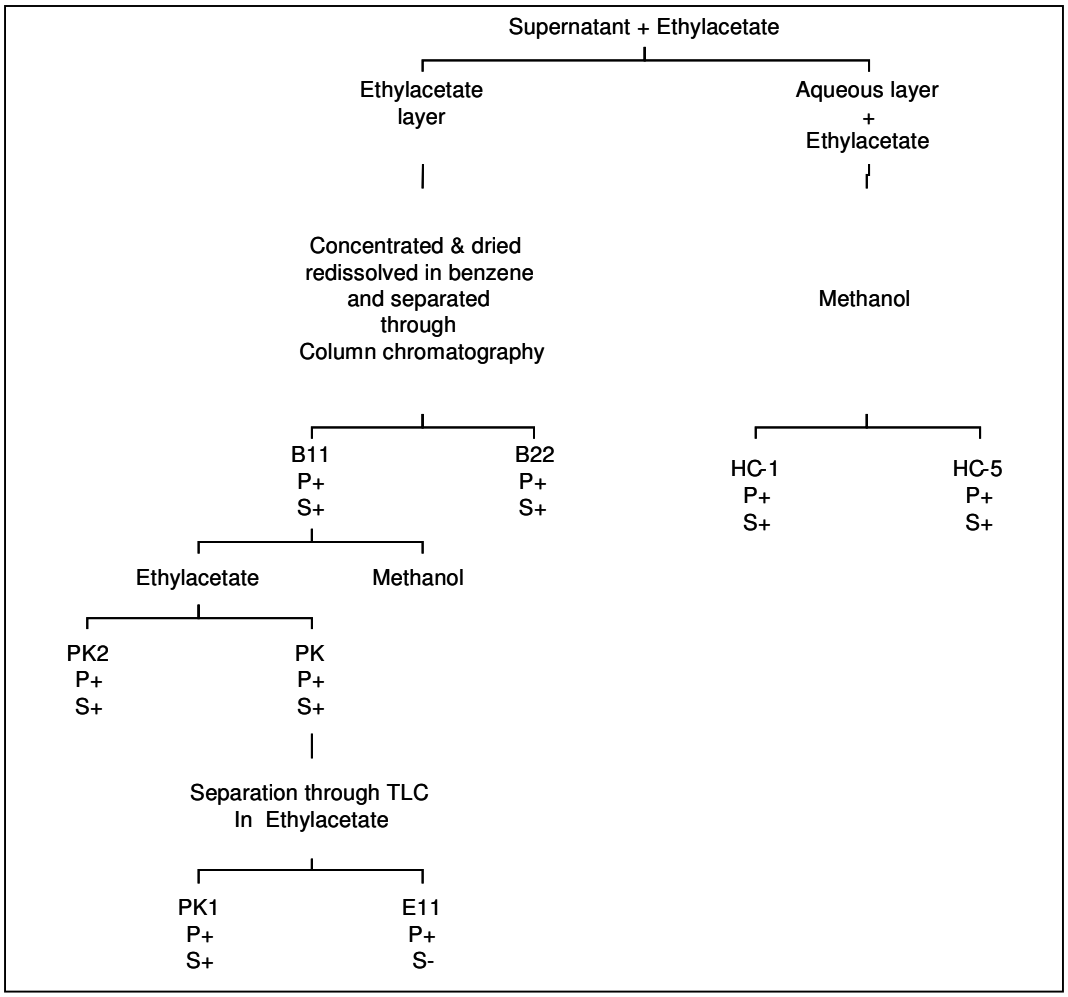
Isolation of metabolites from fraction A32



Flow chart 7: Purification of metabolites from A32 fraction of the aqueous layer.

Details are same as in the above flow chart. SIB 1, F1, F2, I32, AA, C32, AB32, A31, AA32, E32 and AC32 are different fractions eluted from A32 fraction of the aqueous layer.

Isolation of metabolites from the culture supernatant at 12 h



Flow chart 8: Purification of metabolites from the culture supernatant at 12 h of phototrophic incubation

Details are same as in the above flow chart.

B11, B22, PK, PK1, PK2 and E11 are fraction eluted from the ethylacetate layer; HC-1 and HC-5 are eluted from aqueous layer.

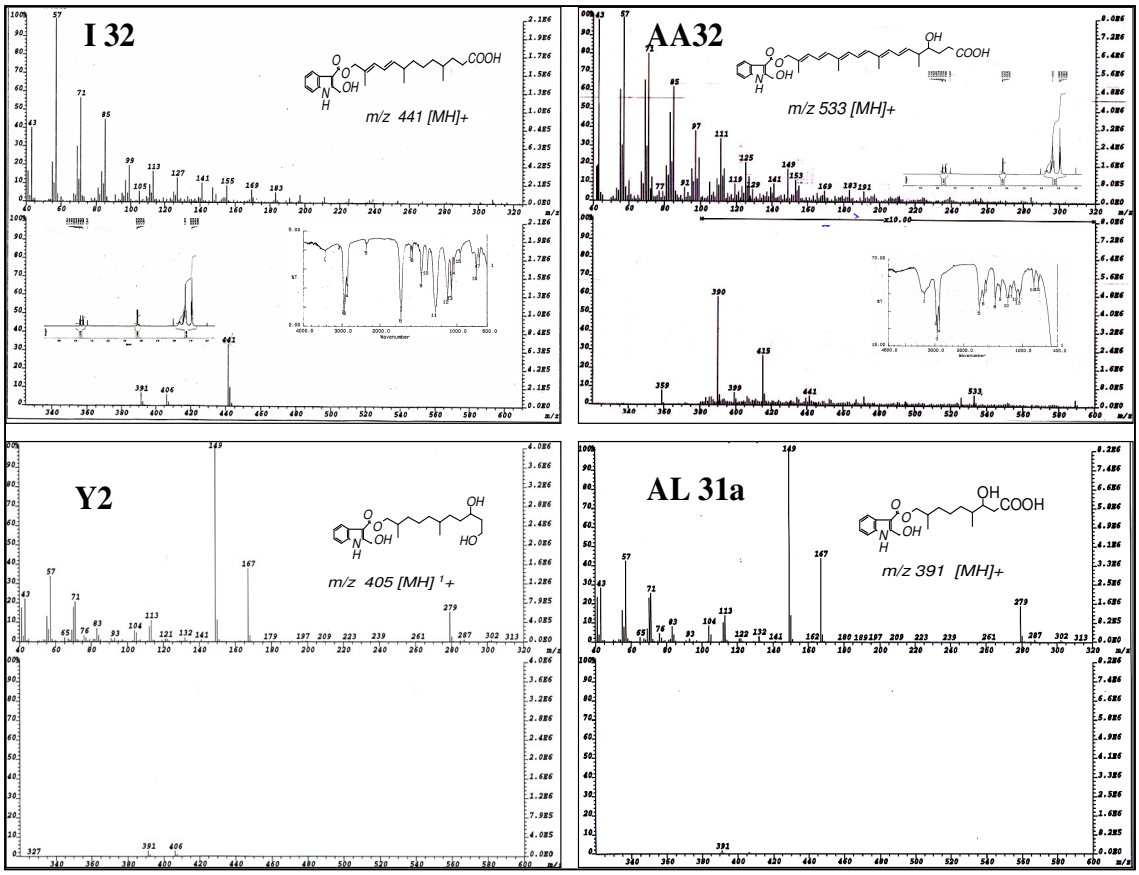


Fig 15a: IR, NMR, mass spectroscopic analyses and structure of the compounds I32, AA32, Y2, AL31a.

IR, NMR and mass spectroscopic analyses were done as given in materials and methods 2.6.2.

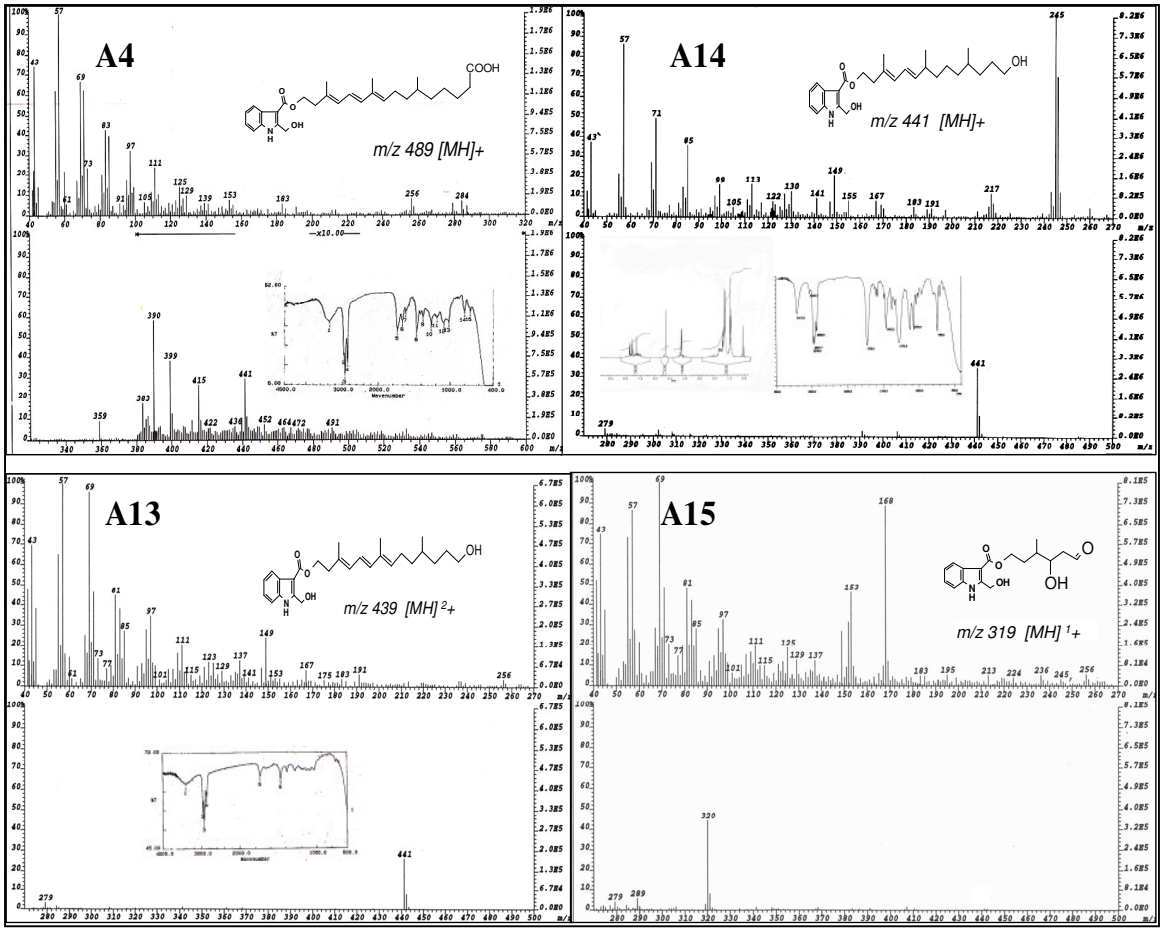


Fig 15b: IR, NMR, mass spectroscopic analyses and structure of the compounds A4, A14, A13 and A15

IR, NMR and mass spectroscopic analyses were done as given in materials and methods 2.6.2.

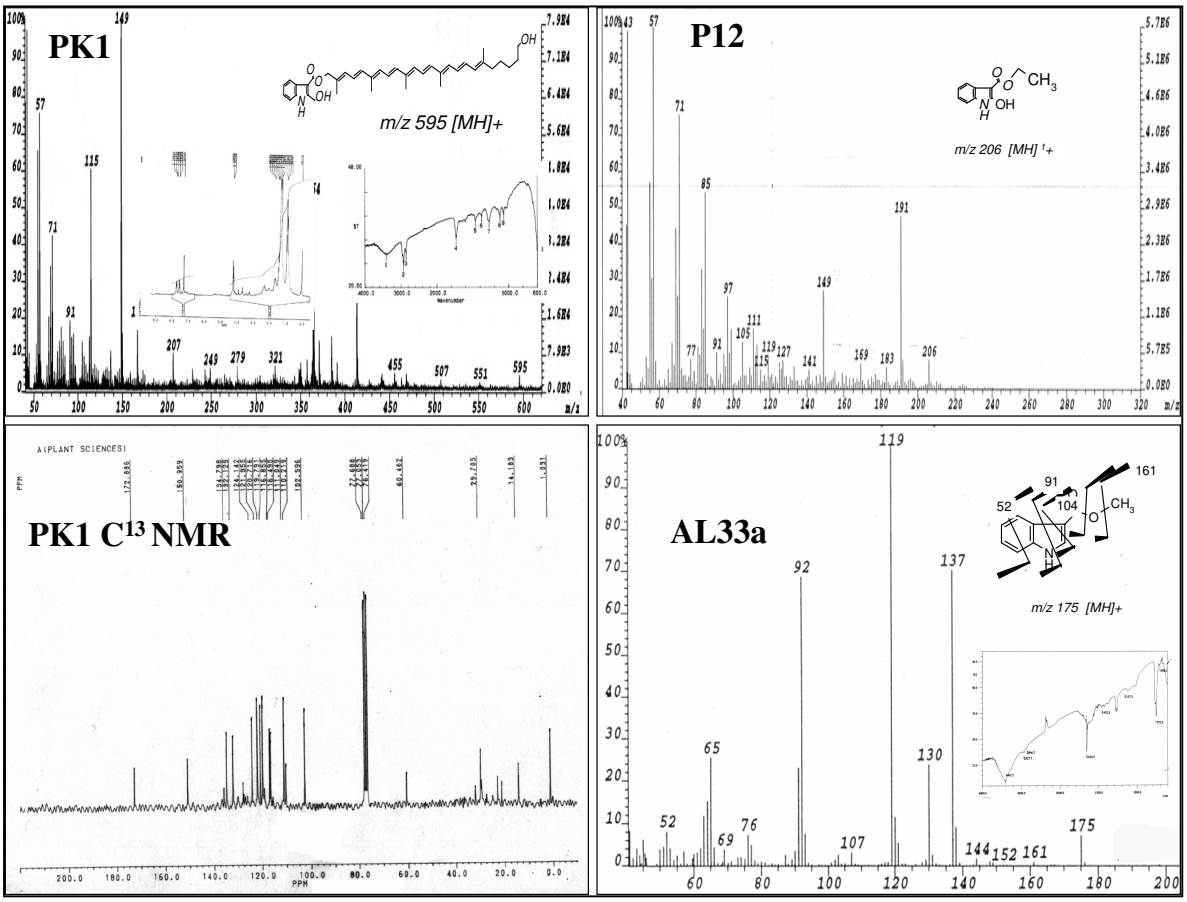


Fig 15c: IR, NMR, mass spectroscopic analyses and structure of the compounds PK1, P12, AL33a

IR, NMR and mass spectroscopic analyses were done as given in materials and methods 2.6.2.

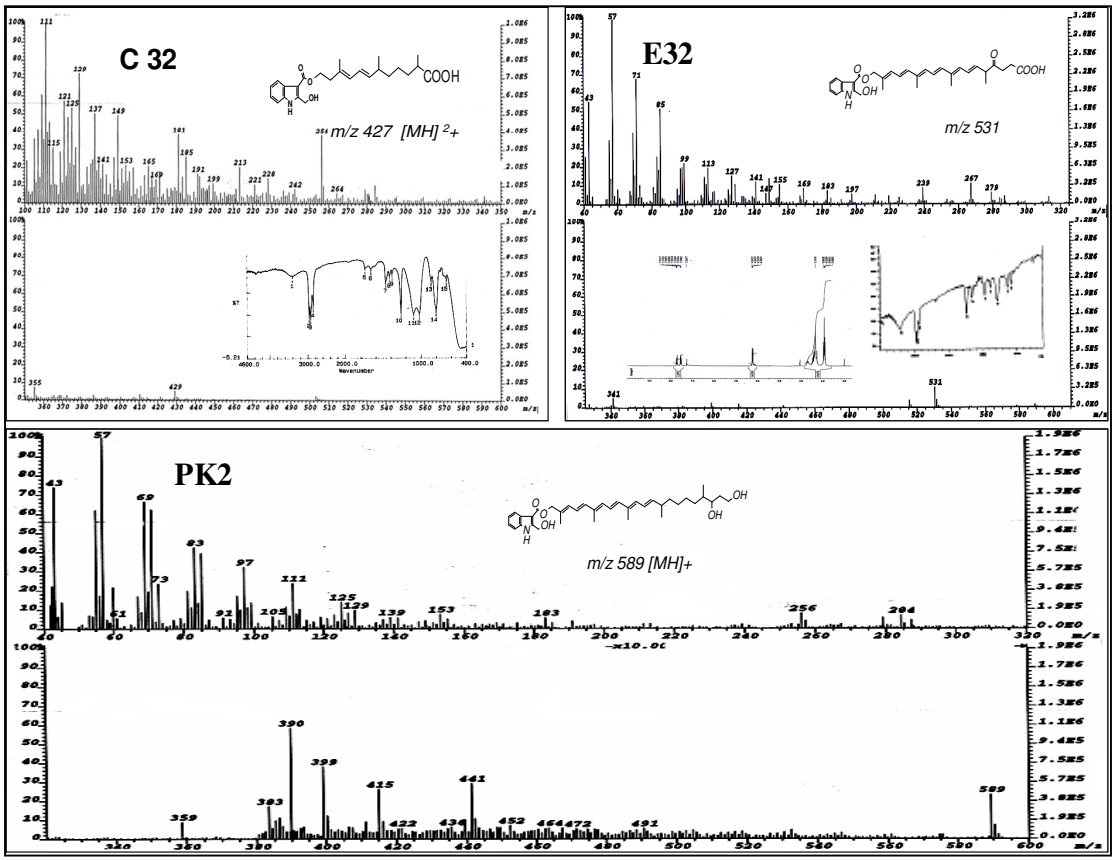


Fig 15d: IR, NMR, mass spectroscopic analyses and structure of the compounds E32 C32 and PK2

IR, NMR and mass spectroscopic analyses were done as given in materials and methods 2.6.2.

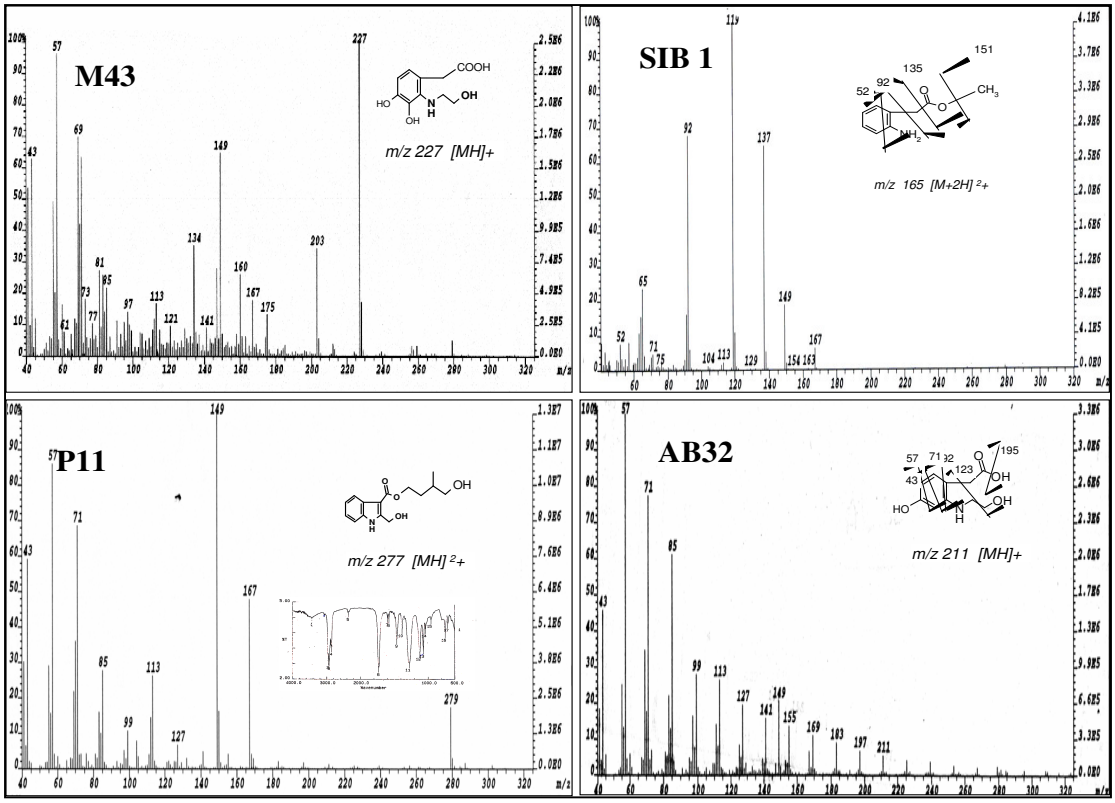


Fig 15e: IR, NMR, mass spectroscopic analyses and structure of the compounds M43, SIB 1, P11 and AB32

IR, NMR and mass spectroscopic analyses were done as given in materials and methods 2.6.2.

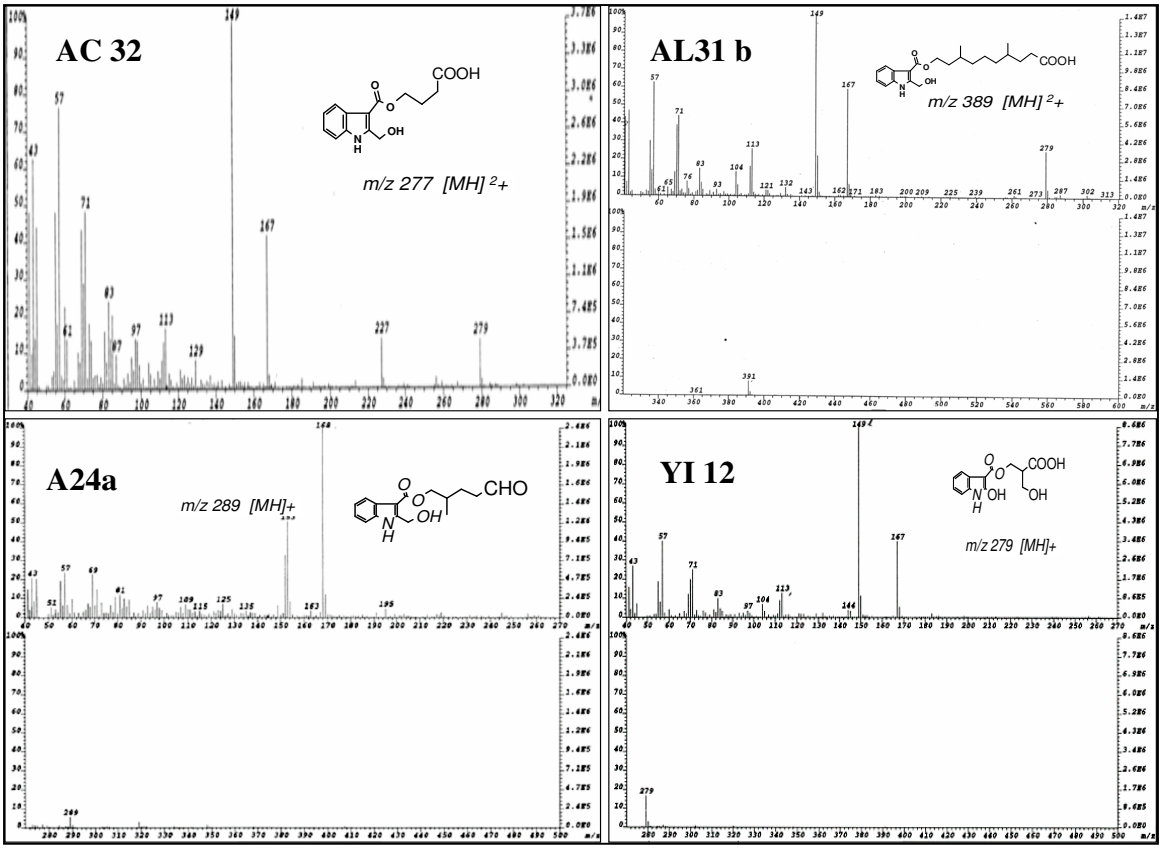


Fig 15f: IR, NMR, mass spectroscopic analyses and structure of the compounds AC32, AL31b, A24a, YI12

IR, NMR and mass spectroscopic analyses were done as given in materials and methods 2.6.2.

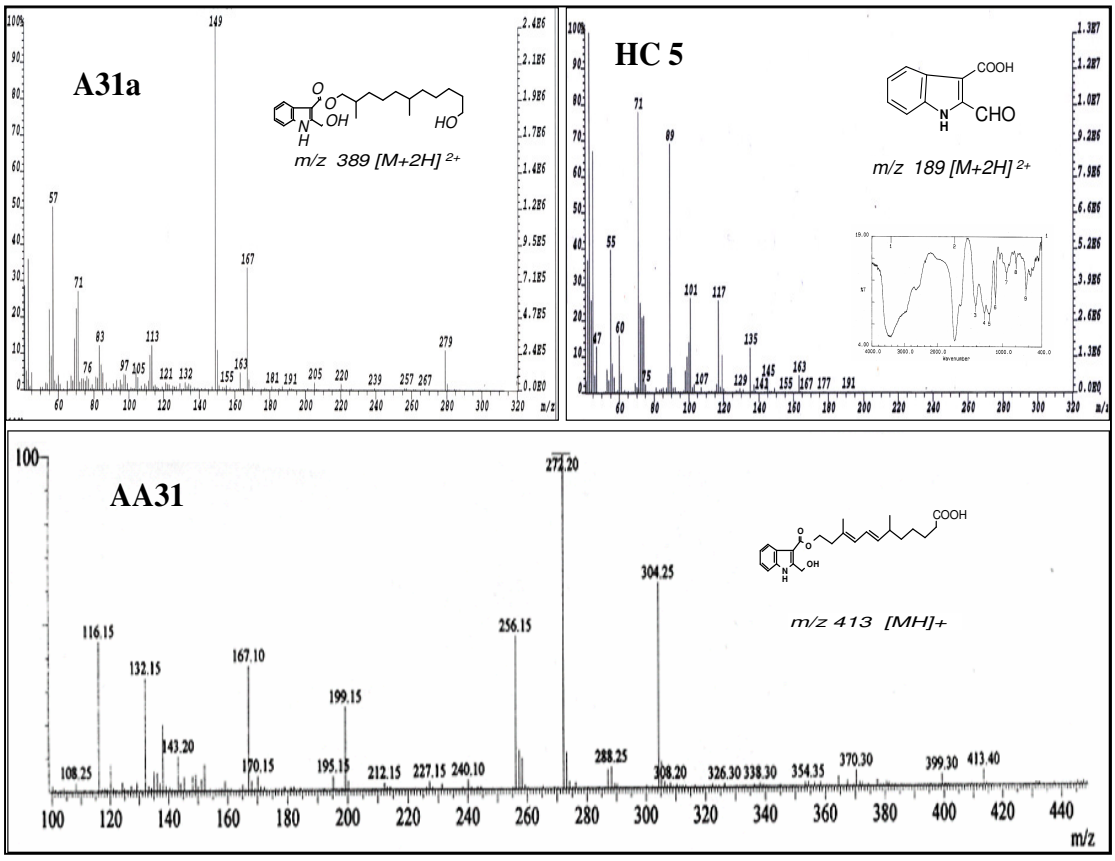


Fig 15g: IR, NMR, mass spectroscopic analyses and structure of the compounds A31a, HC 5 and AA31

IR, NMR and mass spectroscopic analyses were done as given in materials and methods 2.6.2.

***Sample code	Metabolite color	Color reaction with:		**R_f	Yield	Molecular mass
		PDAB	Salper`s		(µmoles)	(*m/z*)
Fraction-1						
*PK1	Rose pink	P	P	4 = 0.68; 16 = 0.87; 2 = 0.76	0.5	595
A4	Orange red	P	P	4 = 0.8; 2 = 0.78; 1 = 0.75	3.7	489
PK2	Orange red	OR	OR	4 = 0.7; 2 = 0.82; 1= 0.68	3.5	589
A15	Orange red	LP	OR	7 = 0.93; 4 = 0.5; 15 = 0.8	2.6	319
A14	Orange red	OY	OY	1 = 0.64; 2 = 0.75; 3 = 0.63	6.5	441
A13	Orange red	O	O	1 = 0.54; 3 = 0.48	6.5	439
A24a	Pale yellow	OY	O	6 = 0.8; 1 = 0.82; 3 = 0.79	1.3	289
A31a	Pale yellow	YR	O	11 = 0.8; 12 = 0.78	4.8	389
A31b	Yellow	Y	OY	6 = 0.8; 1 = 0.82	1.8	-
Fraction-2						
P11	Orange yellow	OY	OY	3 = 0.7; 2 = 0.78; 4 = 0.69	1.3	277
P12	Pale yellow	R	O	4 = 0.69: 1= 0.71; 3 = 0.68	1.4	206
Fraction-3						
M43	Light green	OY	OY	7 = 0.7; 9 = 0.68	3.0	227
YI12	Orange yellow	Peach	O	11 = 0.53; 4 = 0.3; 5 = 0.34	9.1	279
Y2	Yellow	R	R	7 = 0.5; 8 = 0.48; 9 = 0.52	1.8	405

Table 2: Color reactions and mass analysis of various metabolites isolated from the ethylacetate layer

Y = Yellow; O = Orange; OY = Orange yellow; P = Pink; LP = Light pink; YR = Yellow red; OR = Orange red; R = Red; PDAB = p-dimethylaminobenzaldehyde;
**R_f of metabolite on TLC and the whole numbers represent different solvent systems (1 = benzene; 2 = benzene + ethyl acetate [1:1]; 3 = benzene + chloroform [1:1]; 4 = methanol; 5 = isopropanol; 6 = toluene; 7 = methanol + ethyl acetate [4:1]; 8 = ethyl acetate + isopropanol [4:1]; 9 = ethyl acetate + ethanol [4:1]; 11 = methanol + 1N HCl [1ml]; 12 = methanol + H_2O [1:1]; 15 = ethanol; 16 = ethyl acetate.
- = Analysis not done.

***Sample code = Codes of the compounds purified as in flow chart 2-7.

***Sample code	Metabolite color	Color reaction with: PDAB	Salper`s	**R_f	Yield (µmoles)	Molecular mass (*m/z*)
Fraction–1						
HC-5	Brown	OR	Pink	11 = 0.62; 13 = 0.71; 5 = 0.5	0.6	189
HC-1	Brown	-ve	Pink	11 = 0.3; 13 = 0.5; 5 = 0.47	0.6	161
AL31b	Pale yellow	OY	OY	11 = 0.8; 13 = 0.7; 5 = 0.69	1.8	389
AL31a	Yellow	OY	Pink	7 = 0.6; 10 = 0.59; 13 = 0.6	49.8	391
AC32	Colorless	Y	OY	4 = 0.78; 14 = 0.70	3.6	277
AL33a	Pale yellow	LY	-ve	4 = 0.85; 15 = 0.79	22.2	175
AL33b	Colorless	PY	LY	12 = 0.98; 4 = 0.86	1.3	-
AL 41	Orange Yellow	LY	-ve	16 = 0.67; 15 = 0.7 4 = 0.68	1.2	-
AA32	Orange yellow	OY	OY	7 = 0.46; 4 = 0.5; 15 = 0.48	1.2	533
AA31	Orange yellow	OY	P	7 = 0.9; 4 = 0.47; 15 = 0.5	1.2	413
C32	Brown	P	P	1= 0.8; 2 = 0.78; 3 = 0.82	3.6	427
E32	Orange brown	YR	P	7 = 0.85; 2 = 0.66; 9 = 0.75	1.2	531
AB32	Colorless	LY	-ve	16 = 0. 2; 4 = 0.77	3.6	211
SBI-1	Pale white crystals	Y	OY	4 = 0.7; 7 = 0.69; 8 = 0.67	1.2	165
I32	Colorless	LP	LP	10 = 0.75; 12 = 0.85	12.6	441

Table 3: Color reactions and mass analysis of various metabolites isolated from the aqueous layer

Details of the abbreviations used are as given in table 1, except; -ve = negative color reaction. Solvent 10 = methanol + 1N NaOH (1 ml); 13 = methanol + acetic acid (1 ml); 14 = acetone.

3.3 **Fumarate dependent indole biosynthesis from 2-aminobenzoate**

3.3.1 **Work with whole cells**

3.3.1.1 **Effect of different carbon sources on indole production**

Since the transformation of 2-aminobenzoate requires an additional carbon for indole synthesis, experiments were done to identify the possible carbon involved in the indole synthesis. Different precursors like acetate, α-ketoglutarate, succinate, malate, fumarate, oxaloacetic acid, glucose, fructose, lactate, arabinose and glycine were used along with 2-aminobenzoate and observed for indole yield. The substrates used are mostly the intermediates of citric acid cycle and a few are carbohydrates and some fatty acids. Among the substrates screened, highest yields were observed with the citric acid cycle intermediates, i.e. fumarate, succinate and malate (Table 4). The high indole yields with fumarate, malate and succinate indicate the role of these substrates as the conjugating molecules with 2-aminobenzoate in photobiotransformation by *Rba. sphaeroides* OU5.

3.3.1.2 **Studies with 2-Fluoroacetic acid**

To further confirm the role of citric acid cycle intermediates as conjugating molecules, studies were done using 2-fluoroaceticacid (FAA). FAA is a known inhibitor of TCA cycle and it has inhibited indole production from 2-aminobenzoate with glucose, although biomass yield was not affected (Table 4). FAA has inhibited indole production with all other carbon substrates except for citric acid cycle intermediates (Table 4). Highest yields were observed in the presence of fumarate and malate compared to succinate and α-ketoglutarate indicating the role of fumarate or malate as the conjugating molecule with 2-aminobenzoate, resulting in the formation of an indole by *Rba. sphaeroides* OU5.

3.3.1.3 **Effect of fumarate analogues on indole production**

To confirm the possible molecule among TCA cycle intermediates that conjugate with 2-aminobenzoate in indole formation; experiments were done using the analogues of fumarate. Production of indole from 2-aminobenzoate was completely inhibited in the presence of fumarate analogues. The analogues like cinnamate and acrylate have inhibited both the biomass and indole yield while maleate and crotonoate have inhibited only the indole yield. These results indicate fumarate as the conjugating carbon with 2-aminobenzoate in indole photoproduction by this strain (Table 5).

3.3.1.4 **Influence of fumarate concentration on indole photoproduction**

To optimize the indole yield studies were done using resting cells of *Rba. sphaeroides* OU5 at different concentrations of fumarate and with 2-aminobenzoate at 1 mM. *Rba. sphaeroides* OU5 has produced maximum indole yield of 0.34 mM at 0.15 % (w/v) of fumarate (Fig 16).

3.3.1.5 **Indole production from various aromatic compounds**

Indole production from various aromatic compounds like benzoate, pthalate, salicylate, aniline, 2-nitroaniline, 2-nitrotoluene, xylene, catechol, p-aminobenzoate, tryptophan and 3-aminobenzoate were studied in order to know the role of these aromatic compounds in indole production. Indole production was observed only from aniline, tryptophan and 2-aminobenzoate and not from other aromatic compounds tested (Table 6).

Substrate (0.1 % w/v or v/v)	Biomass yield (mg dry wt.ml^{-1})		Indole Photoproduction (µmoles)	
	-FAA	+FAA	-FAA	+FAA
Control (with out substrate)	0.2	0.2	0.1	0.1
Acetate	0.5	0.2	0.1	0.0 (-)
α– ketoglutarate	0.3	0.3	0.2 (-)	0.2 (+)
Succinate	0.3	0.3	0.3 (+)	0.3 (+)
Fumarate	0.5	0.3	0.4 (+)	0.3 (+)
Malate	0.4	0.3	0.4 (+)	0.3 (+)
Oxaloacetate	0.2	0.2	0.2 (+)	0.2 (+)
Pyruvate	0.6	0.3	0.1	0.1
Arabinose	0.1	0.2	0.0 (-)	0.0 (-)
Fructose	0.2	0.3	0.1	0.0 (-)
Dextrose	0.3	0.2	0.1	0.0 (-)
Lactate	0.1	0.0	0.1	0.0 (-)
L-Glycine	0.1	0.0	0.0 (-)	0.0 (-)
L-Serine	0.2	0.0	0.1	0.0 (-)
DL-Alanine	0.0	0.0	0.0 (-)	0.0 (-)
L-Glutamine	0.2	0.1	0.3	0.0 (-)

Table 4: Effect of organic substrates on photoproduction of indole by *Rba. sphaeroides* OU5

-FAA = Absence of 2-fluoroacetate; +FAA = With 2-fluroacetate; (+) = Promoted indole photoproduction; (-) = Repressed indole photoproduction.

Assay was done in triplicates with cell suspensions after 48 h of phototrophic incubation (lux 2,400) at 30±2°C, in the presence of different organic substrates (0.1 % w/v, v/v).

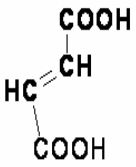
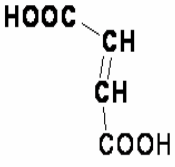
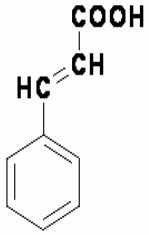
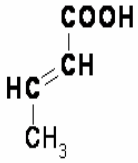
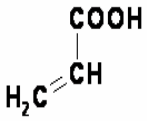
	 Fumarate	 Maleate	 Cinnamate	 Crotonate	 Acrylate
Indole (mM)	0.16	0	0	0	0
Biomass yield (mg dry wt.ml^{-1})	0.34	0.34	0	0.34	0

Table 5: Effect of analogues of fumarate on indole photoproduction

Assay was done in triplicates with cell suspensions after 48 h of phototrophic incubation in the presence of different substrates (13 mM).

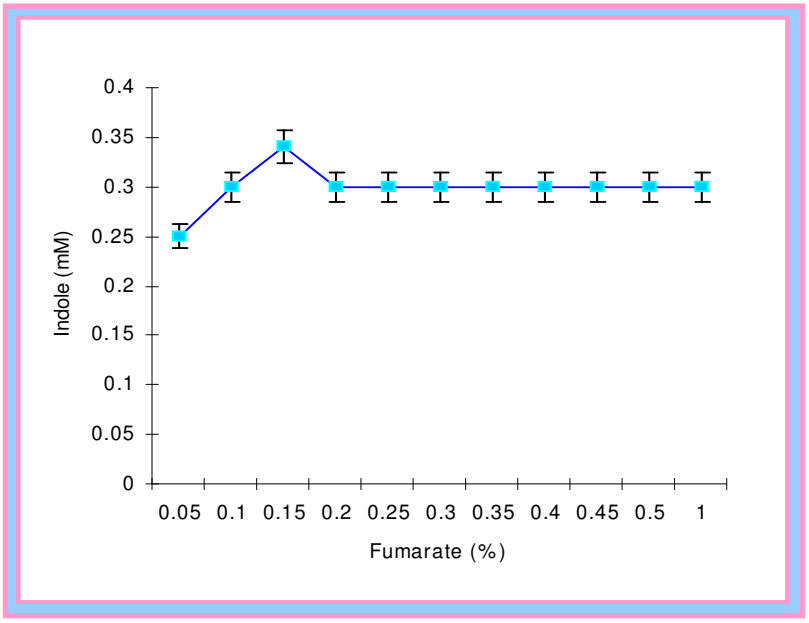


Fig 16: Photoproduction of indole by *Rba. sphaeroides* OU5 with various concentrations of fumarate

Maximum indole yield of 0.34 mM was obtained from 0.15 % (w/v) of fumarate and 2-aminobenzoate at 1 mM.

Assay was done with cell suspensions after 48 h of phototrophic incubation and the vertical bars on the graph indicate the standard deviation.

Aromatic compounds (0.1 % w/v, v/v)	Indole photoproduction (µmole)
Control (2-aminobenzoate)	0.3
Benzoate	0.0
Pthalate	0.0
Salicylate	0.0
Aniline	0.3
2-Nitroaniline	0.0
2-Nitrotoluene	0.0
Xylene	0.0
Catechol	0.0
p-aminobenzoate	0.0
Tryptophan	1.0
3-aminobenzoate	0.0

Table 6: Photoproduction of indole from different aromatic compounds

Assay with cell suspensions after 48 h of phototrophic incubation in the presence of malate (0.1 % w/v) and aromatic compound (0.1 % v/v, w/v).

3.3.1.6 **Effect of different analogues of 2-aminobenzoate on indole production**

To confirm the substrate specificity of 2-aminobenzoate in indole production, studies were done using different analogues of 2-aminobenzoate. Production of indole from 2-aminobenzoate in the presence of different analogues of 2-aminobenzoate like benzoate, pthalate, salicylate, aniline, 2-nitroaniline, 2-nitrotoluene, xylene, catechol and p-aminobenzoate was studied, with fumarate as the conjugating molecule (Table 7). The production of indole was completely or partially inhibited in the presence of different analogues except for aniline confirming the substrate specificity of 2-aminobenzoate in indole production.

3.3.1.7 **Indole production in the presence of hydroxylamine**

The indole production was studied in the presence of hydroxylamine both in the presence and absence of 2-aminobenzoate. The indole yield was more in the presence of both hydroxylamine and 2-aminobenzoate after 48 h of phototrophic incubation (Fig 17).

3.3.1.8 **Is photobiotransformation of 2-aminobenzoate; an inducible or constitutive metabolism?**

Experiments were done using chloramphenicol (0.2 mg/ml) to understand the inducible or constitutive nature of this biotransformation. The results of this study indicate that chloramphenicol has completely inhibited 2-aminobenzoate photobiotransformation (data not shown). Thus the process of biotransformation of 2-aminobenzoate is predicted to be an inducible metabolism.

Analogues of 2-aminobenzoate (0.1 % w/v, v/v)	Indole photoproduction (µmole)
Control (2-aminobenzoate)	0.3
Benzoate	0.1
Pthalate	0.0
Salicylate	0.0
Aniline	0.3
2-Nitroaniline	0.2
2-Nitrotoluene	0.0
Xylene	0.2
Catechol	0.1
p-aminobenzoate	0.1

Table 7: Effect of analogues of 2-aminobenzoate on photoproduction of indole by *Rba. sphaeroides* OU5

Assay was done with cell suspensions after 48 h of phototrophic incubation in the presence of malate (0.1 % w/v), aromatic compound (0.1 % w/v) and 2-aminobenzoate (1 mM).

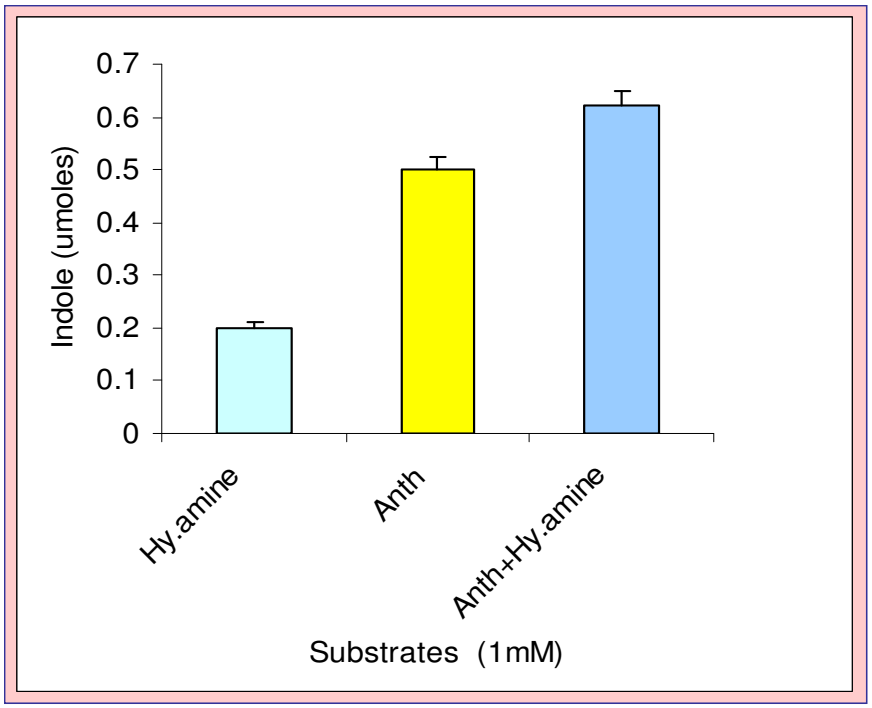


Fig 17: Production of indole by *Rba. sphaeroides* OU5 from 2-aminobenzoate and hydroxylamine

Hy-amine = Hydroxylamine; Anth = 2-aminobenzoate; Anth+Hy-amine = 2-aminobenzoate and hydroxylamine

Experiment was done with growing cells in the presence of 1 mM hydroxylamine, 2-aminobenzoate and hydroxylamine+2-aminobenzoate and assayed after 48 h of phototrophic incubation. The presence of hydroxylamine with 2-aminobenzoate has increased the indole yield.

3.3.2 **Work with cell free extracts**

3.3.2.1 **Assay for indole production**

To 1 ml of 0.05 M phosphate buffer (pH 7.8) containing 1 mmole 2-aminobenzoate, 1 mmole fumarate, 20 µg pyridoxal phosphate (PLP) and 10 µg magnesium chloride, 0.5 ml cell free extract (100-200 µg protein) was added and incubated for 1 h under light at 30 ºC. It was chilled in an ice bath and extracted into 4 ml ethylacetate. One ml sample of the ethylacetate extract was dried in a water bath and assayed for indole using PDAB reagent.

3.3.2.1.1 **Photoproduction of indole:** In order to confirm the light dependent indole production, assay for indole production was done as above in dark and light conditions using cell free extracts of 2-aminobenzoate induced and uninduced *Rba. sphaeroides* OU5. Indole was detected only in light exposed samples and the indole production was observed with cell free extracts of both control and 2-aminobenzoate induced cultures.

3.3.2.1.2 **Requirement of carbon source for 2-aminobenzoate transformation:**

Requirement of an additional carbon source for transformation of 2-amino benzoate was already known from the earlier experiments with the whole cells. However, the same was confirmed with the enzymatic assay using the cell free extracts. The substrates used are mostly the intermediates of citric acid cycle, carbohydrates and some fatty acids. Among all, highest yields were observed with the citric acid cycle intermediates, i.e. fumarate and malate (Fig 18). The high indole yields in the presence of fumarate and malate indicated the role of these substrates in 2-aminobenzoate transformation by *Rba. sphaeroides* OU5.

3.3.2.1.3 **Effect of analogues of fumarate on indole production:** To confirm the role of fumarate as the conjugating molecule, studies with cell free extracts were done using analogues of fumarate like cinnamate and crotonoate. However, the presence of these analogues has not inhibited the transformation with cell free extracts instead their presence has increased the indole yield fig 19.

3.3.2.1.4 **Effect of analogues of 2-aminobenzoate on indole production:**

Effect of different concentration of salicylate and pthalate (0.1-1 mM) on 2-aminobenzoate transformation was studied with whole cells. Salicylate at 0.1 mM was found to act as an activator of 2-aminobenzoate transformation and the indole yield was high with the whole cells. However, in the enzymatic assay done with the cell free extracts, presence of salicylate has inhibited indole yield from 2-aminobenzoate although salicylate alone has produced highest indole yield fig 20.

3.3.3 Effect of 2-aminobenzoate on the enzyme tryptophanase of *Rba. sphaeroides* OU5

The enzyme tryptophanase plays a key role in the down stream of tryptophan metabolism producing indole, pyruvic acid and ammonia. To know the role of this enzyme in 2-aminobenzoate metabolism, the culture of *Rba. sphaeroides* OU5 was grown in the presence of tryptophan, 2-aminobenzoate and tryptamine. The presence of 2-aminobenzoate has induced the production of tryptophanase in *Rba. sphaeroides* OU5 however, the activity is 13% in comparison to the culture grown with tryptophan (Table 8).

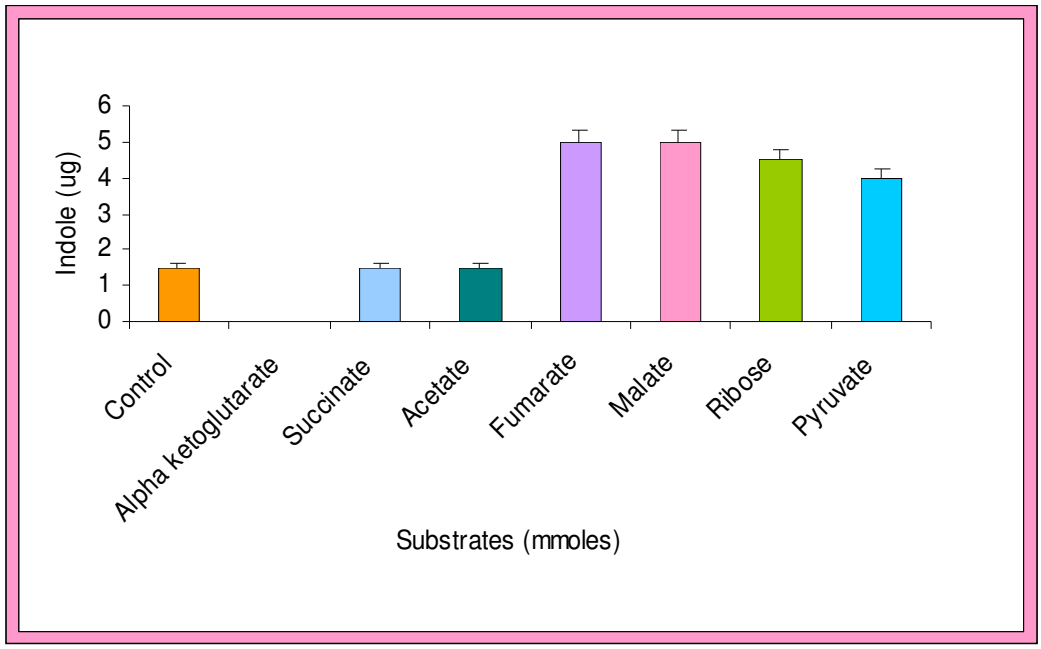


Fig 18: Photoproduction of indole from various carbon sources with cell free extracts of *Rba. sphaeroides* OU5.

Experiments were done with cell free extracts of 2-aminobenzoate induced culture of *Rba. sphaeroides* OU5 with 1 h of light incubation (2,400 lux) at 30±2 °C. The assay mixture contained 1 ml of 0.05 M phosphate buffer (pH 7.8) with 1 mmole 2-aminobenzoate, 1 mmole substrate, 20 µg pyridoxal phosphate, and 10 µg magnesium chloride and 0.5 ml cell free extract (100-200 µg protein). The indole produced was extracted with ethylacetate and estimated using PDAB reagent.

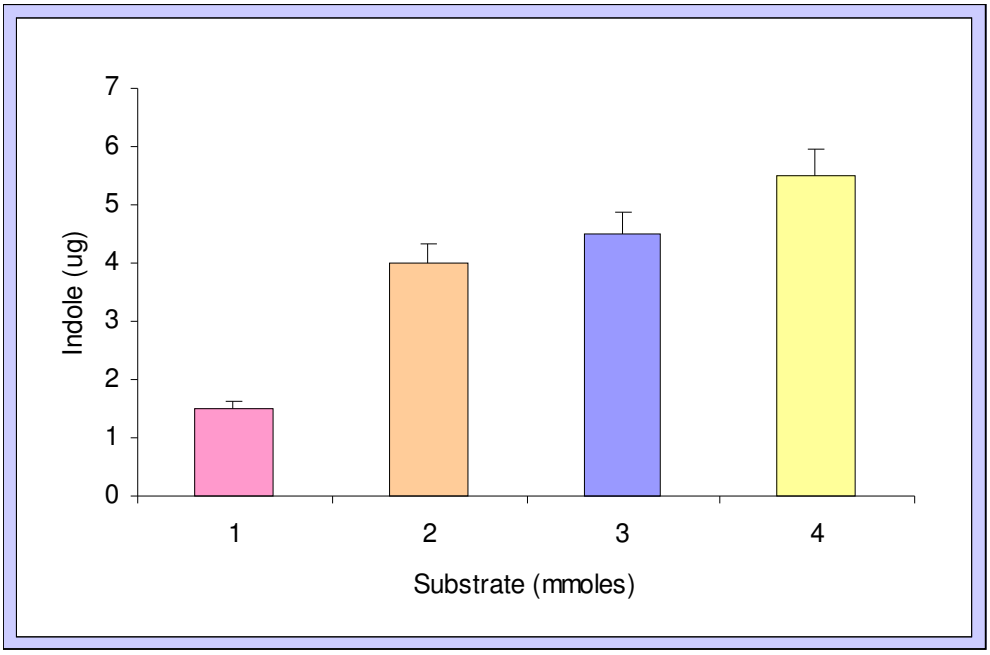


Fig 19: Photoproduction of indole with analogues of fumarate using cell free extracts of *Rba. sphaeroides* OU5

1 = Control; 2 = Fumarate + 2-aminobenzoate; 3 = Fumarate + 2-aminobenzoate + cinnamate; 4 = Fumarate + 2-aminobenzoate + crotonoate

Experiments were done with cell free extracts of 2-aminobenzoate induced culture of *Rba. sphaeroides* OU5 with 2 h of light incubation (2,400 lux) at 30±2 °C. Assay conditions are same as given in fig 18, except that analogues (1 mmole) of fumarate are added extra.

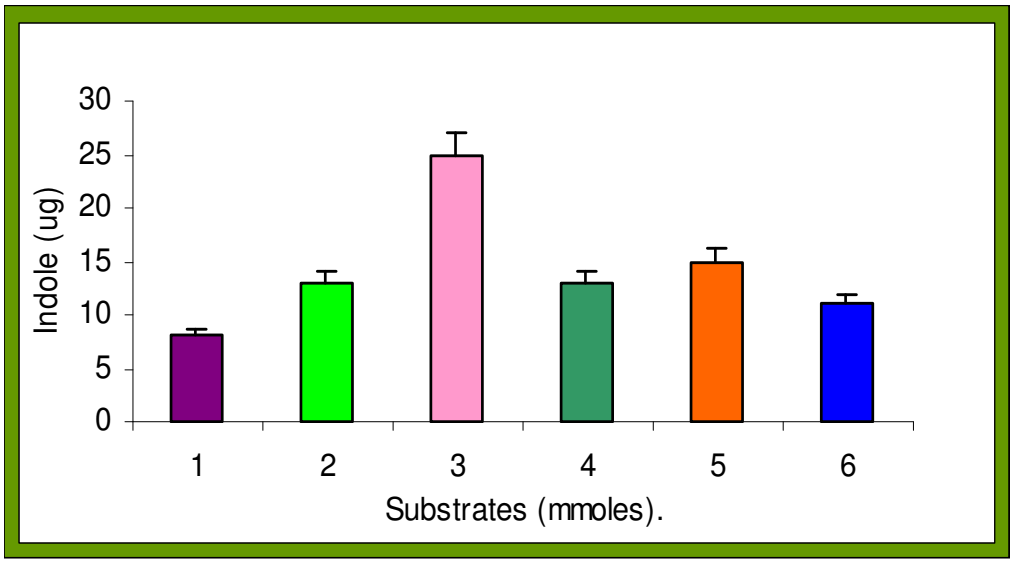


Fig 20: Photoproduction of indole in the presence of analogues of 2-aminobenzoate with cell free extracts of *Rba. sphaeroides* OU5

1 = Control; 2 = 2-aminobenzoate; 3 = Salicylate; 4 = 2-aminobenzoate+ salicylate; 5 = Pthalate; 6 = Pthalate+2-aminobenzoate

Experimental conditions are same as given in fig 18, except that analogues (1 mmole) of 2-aminobenzoate are added to the assay mixture.

Culture grown in the presence of various substrates (0.1 % w/v)	Tryptophanase n moles mg dry $wt^{-1}min^{-}$	% maxima of tryptophanase in induced culture
Tryptophan	4.5	100
Tryptamine	0.6	13
2-Aminobenzoate	0.58	13
2-Aminobenzoate+Tryptophan	2	44
Indole	-	0
Control	-	0

Table 8: Tryptophanase activity of *Rba. sphaeroides* OU5 grown with different substrates

Rba. sphaeroides OU5 was incubated photoheterotrophically for 48 h in the presence of various substrates (0.1 % w/v). Enzyme assay was done with 50 µl cell free extracts (20-50 µg protein) in 1ml phosphate buffer 0.05 M (pH 7.8) containing 0.4 µmole tryptophan, 10 µg pyridoxalphospahate and incubated at 30 ºC for 30 min. After incubation the indole was extracted with ethylacetate, dried and estimated with PDAB reagent.

3.3.4 **Effect of 2-aminobenzoate on anthranilate synthase**

From the earlier result it is known that the contribution of tryptophanase in indole production from 2-aminobenzoate was in-significant; hence enzyme assay was done using various fractions of the cell free extracts to check the activity of anthranilate synthase (protocol given in the materials and methods 2.7). Anthranilate synthase activity was found in the cytosolic fraction of both control and 2-aminobenzoate induced culture and it was high in the 2-aminobenzoate induced culture of *Rba. sphaeroides* OU5 table 9. The results indicate that the enzyme anthranilate synthase is localized in the cytosolic fraction of the cell.

3.3.5 **Electrophoresis and protein separation**

Earlier work with chloromphenicol has indicated the transformation of 2-aminobenzoate as an inducible process. Further work was done to know the difference in the protein profiles of the normal and 2-aminobenzoate induced cultures of *Rba. sphaeroides* OU5. The cell lysate fractionated into cytosol, membrane fraction and debris (the membrane fraction was fractionated based on the release of proteins from the membrane pellet with the detergent Triton X-100 into membrane fraction I & II and debris) was analysed on the SDS-PAGE. It was observed that in the presence of 2-aminobenzoate the proteins of 100, 64, 47, 37, 35 kD proteins were found to be over expressed and 90, 67, 59 kD were found to be new in comparison to the control culture fig 21.

3.3.6 **Isolation and purification of the proteins involved in 2-aminobenzoate transformation**

Production of indole from 2-aminobenzoate and fumarate was already known from the work with cell free extracts. Hence, work was done to isolate the proteins involved in indole production from 2-aminobenzoate. The proteins were isolated from the pellet fraction (membrane) and the extraction was done as given in flow chart 9.

Fractions	Anthranilate synthase activity (2-aminobenzoate consumption) (mmoles.mg protein^{-1})	
	Control	Induced
Cytosol	0.2	2.1
Membrane	0.0	0.15

Table 9: Anthranilate synthase activity of *Rba. sphaeroides* OU5

Results expressed are an average of data from triplicates. Enzyme assay was done with 1 ml phosphate buffer 0.05 M (pH 7.8) containing 100 µmoles 2-aminobenzoate, 10 µmoles PRPP, 10µg $MgCl_2$, to which 50 µl of protein sample was added and incubated under light (lux 2,400) at 30 ºC for 30 min. After incubation the consumption of 2-aminobenzoate was analysed in HPLC.

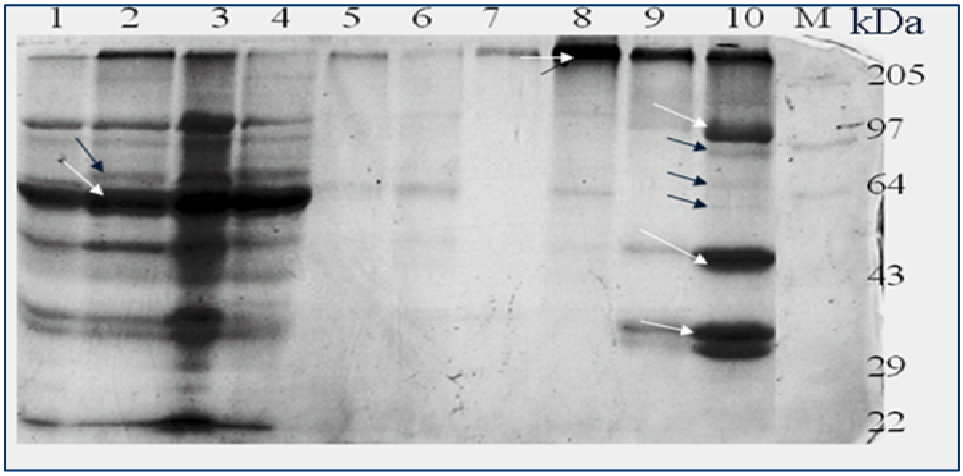
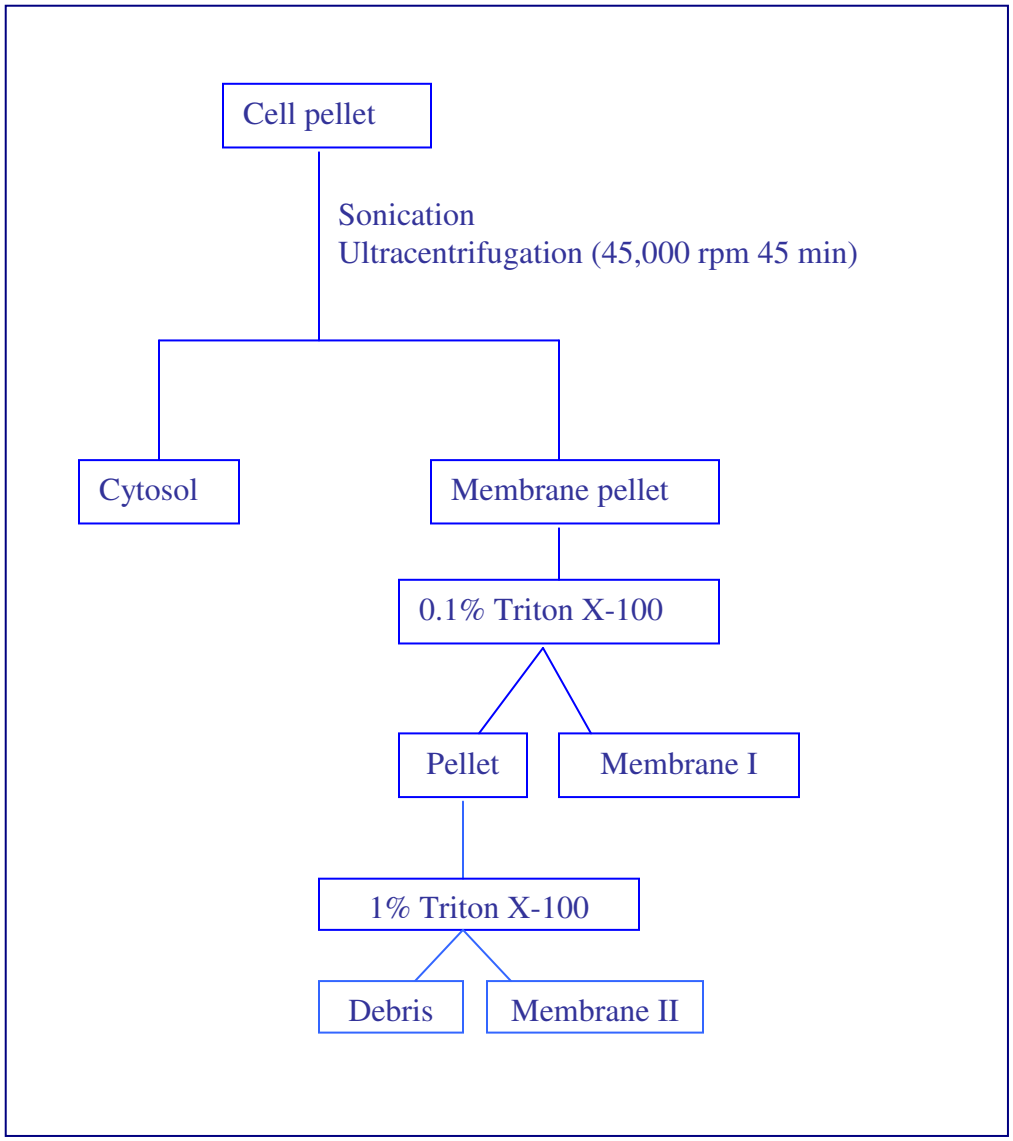


Fig 21: Protein profiles of different fractions of *Rba. sphaeroides* OU5 cell lysate grown with and without 2-aminobenzoate

10 % SDS-PAGE stained with coomassie blue; **lanes:** 1= control sonicate; 2 = induced sonicate; 3 = control cytosol; 4 = induced cytosol; 5 = control 0.1 % membrane fraction; 6 = induced 0.1 % membrane fraction; 7 = control 1 % membrane fraction; 8 = induced 1 % membrane fraction; 9 = control debris; 10 = induced debris.

Arrows in white represent new proteins- 90, 67, 59 kD.

Arrows in black represent the over expressed proteins-100, 64, 47, 37, 35 kD.



Flow chart 9: Isolation of protein involved in 2-aminobenzoate and fumarate conjugation

2-Aminobenzoate induced *Rba. sphaeroides* OU5 was harvested after 48 h of phototrophic incubation and the cell pellet after sonication was fractionated by ultracentrifugation.

3.3.6.1 **Enzyme activities for 2-aminobenzoate consumption**

The protein fractions separated as above were analyzed for 2-aminobenzoate and fumarate consumption (Table 10). The fraction showing maximum activity of 2-aminobenzoate consumption mainly in the presence of fumarate was taken for isolating the pure protein. The enzymatic assay for the consumption of 2-aminobenzoate and fumarate was checked in both control and 2-aminobenzoate induced cultures of *Rba. sphaeroides* OU5. The consumption of fumarate and 2-aminobenzoate was found in both induced and uninduced cultures and the activity was high in the induced culture (Table 10). These results indicate the existence of a novel pathway of fumarate dependent indole biosynthesis in *Rba. sphaeroides* OU5. The membrane fraction I showing the highest activity was considered as the crude enzyme and was used to isolate the pure protein.

3.3.6.2 **Transformation of 2-aminobenzoate and product formation**

Enzyme assay was done with the membrane fraction I (crude enzyme) and the product of 2-aminobenzoate transformation was detected in HPLC. The consumption of 2-aminobenzoate was observed only in the presence of fumarate and the transformed product was detected as appearance of a new peak (Fig 22).

3.3.6.3 **Detection of the intermediates of indole biosynthesis from fumarate and 2-aminobenzoate**

The intermediates in indole biosynthesis from 2-aminobenzoate and fumarate were isolated from the assay sample done with the crude enzyme. The assayed sample was lyophilized and the metabolites extracted into methanol were analyzed in LC-MS. The encircled ones of mass (*m/z*) 187 and 205 in the fig 23 are the novel metabolites detected and they were absent in the control.

Fraction	2-aminobenzoate consumption m moles.mg protein^{-1}	Fumarate Consumption m moles.mg protein^{-1}
Control culture		
Cytosol	2.6	11.4
Membrane	2.2	14
2-aminobenzoate Induced culture		
Cytosol	1.8	25
Membrane	2.8	23
Membrane I	1.4	30.3
Membrane 11	1.3	13.3

Table 10: Consumption of 2-aminobenzoate and fumarate by different fractions of *Rba. sphaeroides* OU5 cell lysate

Assay was done in 1 ml phosphate buffer containing 100 µmoles 2-aminobenzoate, 100 µmoles fumarate, 10 µg PLP, 5 µg $MgCl_2$, 5 µg NADP to which 50 µl of enzyme sample was added and incubated for 10 min at 30 °C. After incubation the fumarate and 2-aminobenzoate consumption was analysed in HPLC.

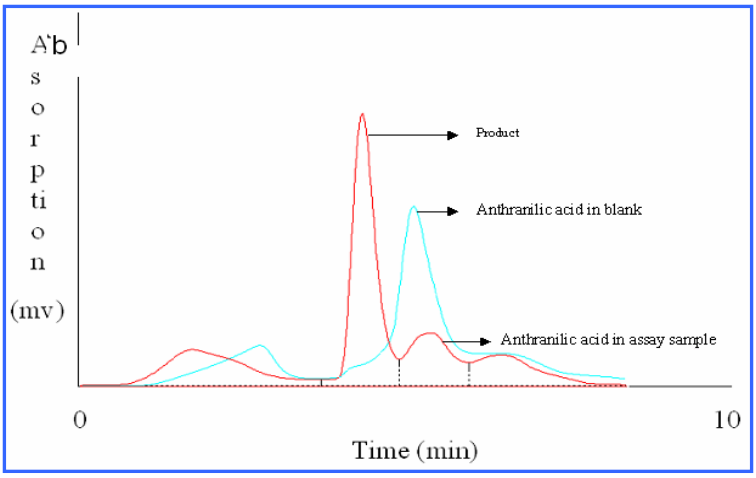


Fig 22: 2-Aminobenzoate transformation and product formation by crude protein of *Rba. sphaeroides* OU5

The assay was done with the crude protein (membrane fraction I) of *Rba. sphaeroides* OU5. The consumption of 2-aminobenzoate and product formation was detected in HPLC.

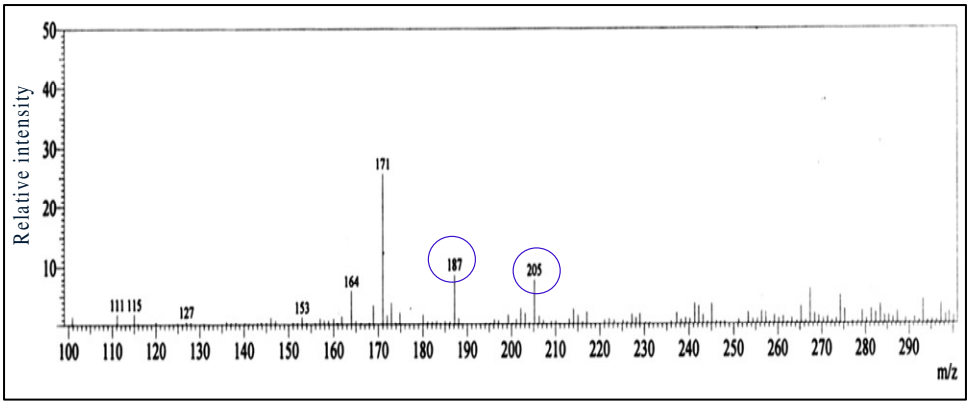


Fig 23: LC-MS metabolome profiling of the crude enzyme showing 2-aminobenzoate and fumarate consumption activity

Assay sample was lyophilized and extracted with methanol. The methanol extract was analyzed on LC–MS Shimadzu 2010.

3.3.7 **Isolation of the protein involved in 2-aminobenzoate and fumarate conjugation**

For the isolation of pure protein, Sephadex G-100, DEAE columns were employed, however we were unsuccessful in isolating a pure protein. Hence gel elution method was employed to isolate the protein involved in 2-aminobenzoate and fumarate conjugation. The membrane fraction I (crude enzyme) showing positive enzyme activity for both fumarate and 2-aminobenzoate was analyzed on a Native PAGE. The protein bands obtained were gel eluted and assayed; out of 3 bands as in fig 24, one has shown positive activity towards fumarate and 2-aminobenzoate consumptions. The protein P1 showing positive towards 2-aminobenzoate and fumarate consumption was extracted from the gel using semi-preparative gel electrophoresis and the purity of the isolated protein was checked by reloading it on 10 and 6 % Native PAGE and detection was done by silver staining. The protein was found to be a monomer of ~64 kD in both native and SDS-PAGE (Fig 25 & 26).

3.3.7.1 **Enzyme activity of P1:**

The purified protein P1 has shown activity for both 2-aminobenzoate and fumarate consumption. The assay sample was lyophilized and the product of its activity was extracted with methanol and analysed on GC-MS. The metabolite of molecular mass (*m/z*) 207 was detected in GC-MS (Fig 27) and it is N-anilinefumarate hence, we call this protein as N-anilinefumaryl synthase (P1).

3.3.7.2 **Protein bound substrates:**

The acid hydrolysed protein P1 when analysed in HPLC, protein bound metabolites were detected at an R_t of 2.5 and 3.8 and these metabolites matched exactly with standard 2-aminobenzoate and fumarate (Fig 28) confirming the role of this protein in fumarate and 2-aminobenzoate conjugation.

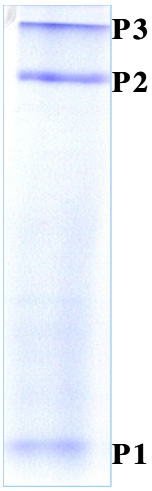


Fig 24: Crude proteins with three protein bands P1, P2 and P3

6% Native PAGE, stained with coomassie blue.

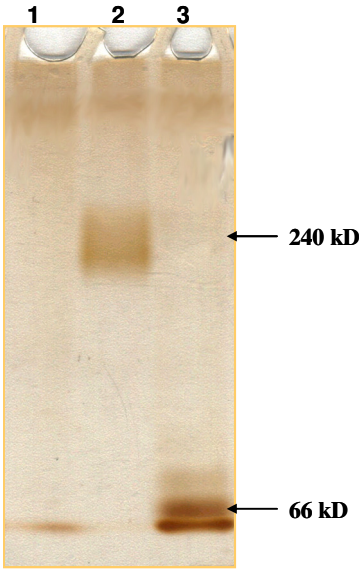


Fig 25: P1 protein in the Native PAGE

P1 matches with the standard marker BSA of 66 kD; Lane 1 = P1 protein purified; Lane 2 = 240 kD Catalase (native marker); 3 = 66 kD BSA (native marker).

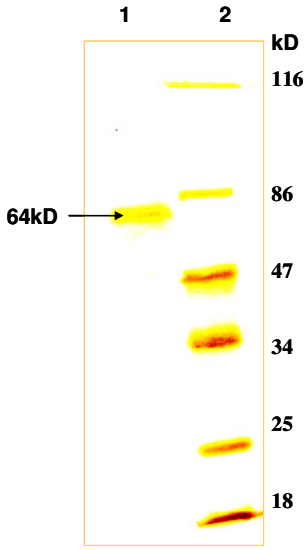


Fig 26: 10%SDS PAGE, silver stained showing P1 protein of 64 kD

Lane 1 = Purified protein P1; Lane 2 = Marker

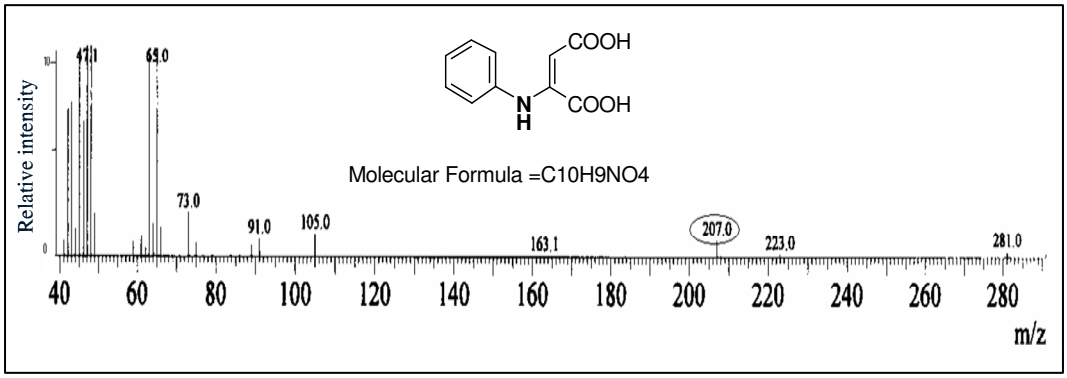


Fig 27: Product of P1 protein activity in GC-MS

Assay sample was lyophilized and extracted with methanol. The methanol extract was analysed on GC–MS Shimadzu 2010.

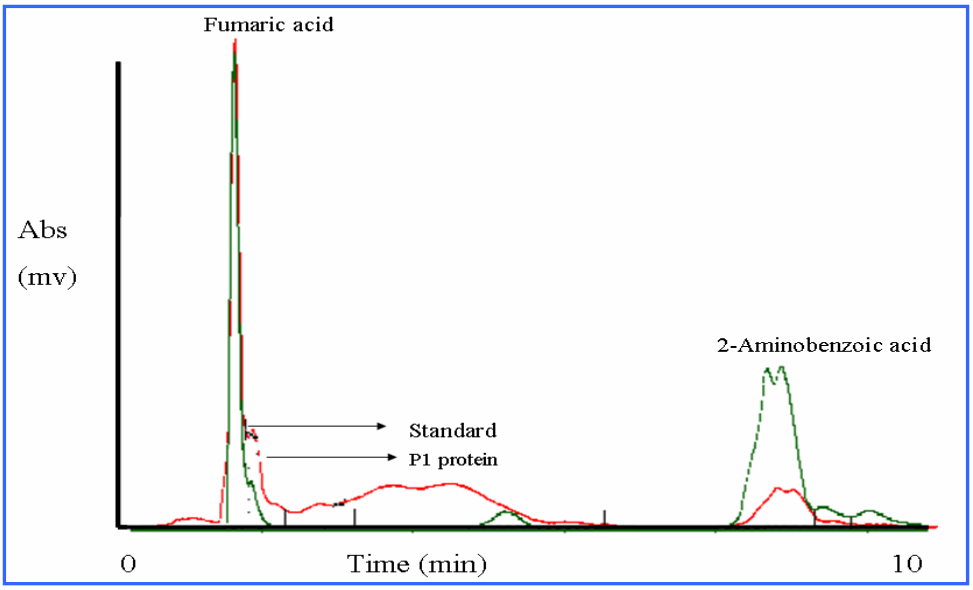


Fig 28: P1 protein bound metabolites

Acid hydrolysed protein was analyzed in HPLC and 2-aminobenzoate and fumarate are the protein bound metabolites detected.

3.3.7.3 **Characterization of protein N-anilinefumaryl synthase (P1)**

3.3.7.3.1 **Enzyme kinetics:** Fumarate and 2-aminobenzoate consumption activity of the protein P1 was done with time. Increase in activity of fumarate and 2-aminobenzoate consumptions was observed with time and the reaction was completed within one minute (Fig 29A).

3.3.7.3.2 **Calculation of K_m and V_{max}:**

The activities of the enzyme were checked at varying concentrations of fumarate and 2-aminobenzoate. The protein showed maximum activity at 80 µmoles of 2-aminobenzoate and fumarate at 600 µmoles (Fig 29B, C). The K_m of the enzyme P1 is 0.059 and V_{max} 86.9 for 2-aminobenzoate consumption activity and for fumarate consumption activity the K_m is 0.38 and V_{max} is 4.09 (29E, F).

3.3.7.3.3 **Activity at different pH:**

The 2-aminobenzoate consumption activity of the enzyme was studied at different pH. The enzyme has optimum activity at pH 6.5 (Fig 29G).

3.3.7.3.4 **Activity at different temperatures:**

The activity of the enzyme at different temperatures was studied and the assay was done for 2-aminobenzoate consumption. The P1 protein has maximum activity at 30-40°C (Fig 29D).

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3.3.7.3.5 **Enzyme activities of P1 at various protein concentrations:**

The enzyme activity of P1 was studied with increasing concentration of the protein. The correlation in the enzyme activity was observed with increasing protein concentration (Fig 29H).

3.3.7.3.6 **Enzyme activity of P1 with various substrate analogues:**

In the presence of fumarate analogues like succinate, oxaloacetate, α-ketoglutarate, cinnamate, salicylate and PRPP, the 2-aminobenzoate consumption activity of P1 was studied. The 2-aminobenzoate consumption activity was not observed with any of the above substrates. Presence of cinnamate along with fumarate has inhibited 2-aminobenzoate consumption activity (Table 11). In presence of 2-aminobenzoate analogues like 3-aminobenzoic acid, benzoic acid and aniline; the fumarate consumption activity was studied. Fumarate consumption was observed in the presence of 2-aminobenzoate and aniline. Presence of salicylate along with 2-aminobenzoate has inhibited fumarate consumption activity (Table12). The characteristics of P1 protein are given in table 13.

Substrates	**2-aminobenzoate consumption by P1**
Fumaric acid	+
Sodium succinate	-
α –KGA	-
OAA	-
PRPP	-
Glucose	-
Cinnamic acid	-
Salicylic acid	-
Fumaric acid+cinnamic acid	-
Cofactors	
NAD	-
NADP	+
PLP	+
TPP	-
FTP	-
ATP	-
CoA	-
Lipoic acid	-

Table 11: 2-Aminobenzoate consumption by P1 in the presence various substrates

α-KGA = α-ketoglutarate; OAA = Oxaloacetic acid; PRPP = Phosphoribosyl pyrophosphate; NAD = Nicotinamide adenine dinucleotide; NADP = Nicotinamide adenine dinucleotide phosphate; PLP = Pyridoxyl phosphate, TPP = Thiamine pyrophosphate; FTP = Flavoadenine triphosphate; ATP = Adenine trinucleotide phosphate; CoA = Coenzyme A

+ = Activity observed; - = Activity not observed

Substrates	Fumarate consumption by P1
2-Aminobenzoic acid	+
3-Aminobenzoic acid	-
Benzoic acid	-
Aniline	+
2-aminobenzoic acid+salicylic acid	-

Table12: Activity in terms of fumarate consumption by the proteins in the presence of various substrates

+ = Activity observed; - = Activity not observed

Properties	Character
K_m	2-Aminobenzoate 0.059 (mM) Fumarate 0.38 (mM)
V_{max}	2-Aminobenzoate 86.9 ($U.mg^{-1}$) Fumarate 4.09 ($U.mg^{-1}$)
pH optima	6.5
Temperature effect	30-40 °C
Cofactors and coenzymes required Metal co factor	NADP, PLP Not required
Molecular weight	~64 kD
Inhibitors for fumarate consumption	Cinnamate
Inhibitors for 2-aminobenzoate consumption	Salicylate

Table 13: Characters of P1 protein

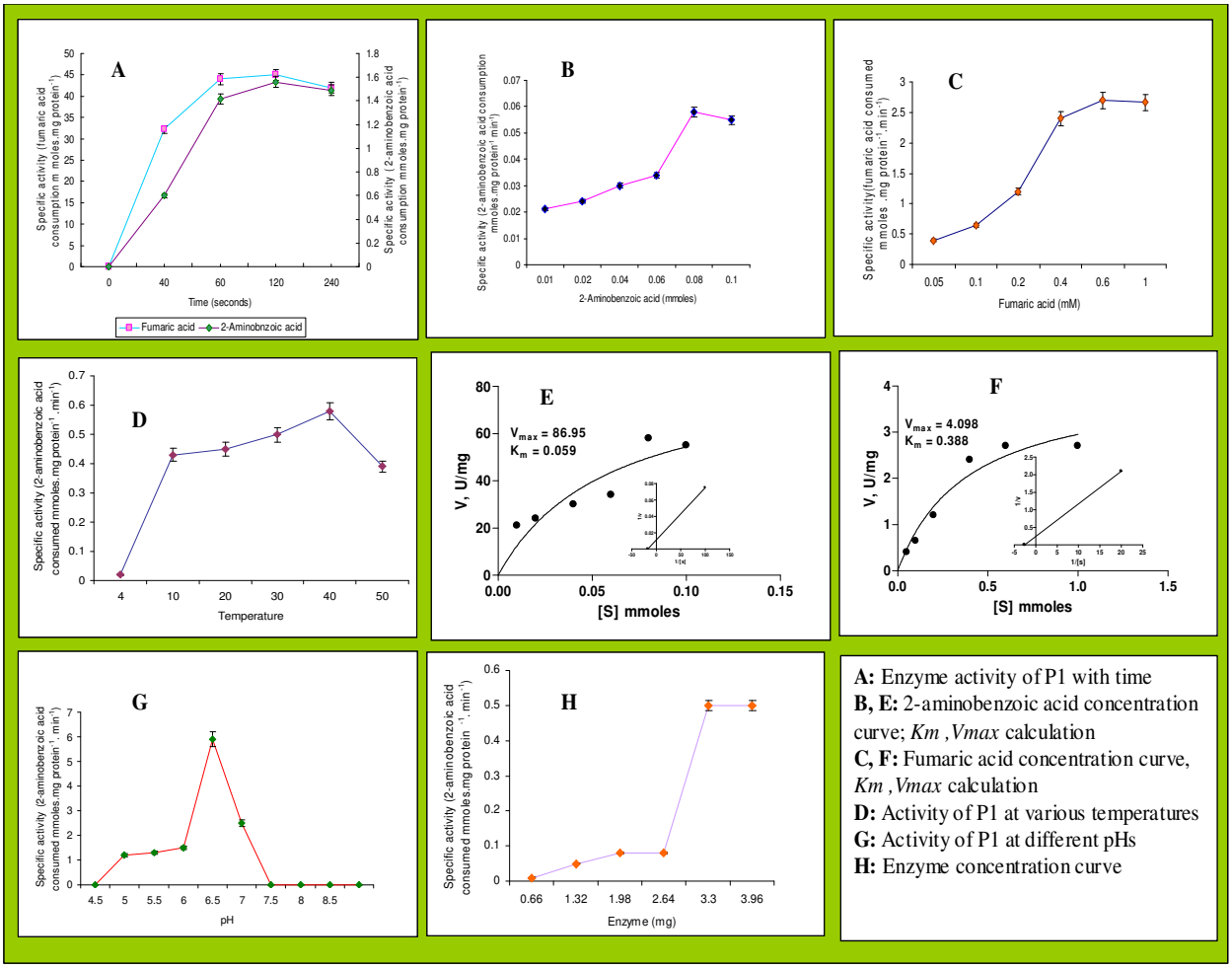


Fig 29: Characterization of P1 protein

3.4 **Toxicological studies of indole and 2-aminobenzoate on *Rba. sphaeroides* OU5**

Indole is known to be growth inhibitory to microorganisms (Claus and Kutzner, 1983). The inhibitory concentration of indole is 0.3-2 mM. The 4.5 mM indole was growth inhibitory in *Escherichia coli*. Generally the anti-oxidant proteins induced in the presence of oxidants are alkylperoxidase (36 kD), superoxide dismutase (22 kD) and catalase (240 kD). Apart from these enzymes, in indole resistant *E. coli* mutants; a new antioxidant protein was expressed that induces the formation of sphaeroplast and it is a 16 kD sphaeroplast protein (Garbe *et al.,* 2000). Similar type of protein was also produced in mutants of *Brevibacterium* and it was found to be of 32 kD (Garbe *et al.,* 2000). Since the indole and its derivatives are toxic and mutagenic, and since the isolated products of 2-aminobenzoate transformation in *Rba. sphaeroides* OU5 are also the indole derivatives, a study has been taken up, to know the toxic effects of 2-aminobenzoate and indole on *Rba. sphaeroides* OU5. The following parameters were studied under toxicity of 2-aminobenzoate and indole:

- Effect of 2-aminobenzoate and indole on growth of *Rba. sphaeroides* OU5.
- Their effect on pigment content and
- Proteins

3.4.1 **Effect of 2-aminobenzoate and indole on growth of *Rba. sphaeroides* OU5**

Effect of different concentrations of 2-aminobenzoate and indole (0.1-8 mM) on growth of *Rba. sphaeroides* OU5 was studied. Presence of 2-aminobenzoate (0.1-8 mM) has neither altered the growth nor has inhibited the biomass yield and there was no change in the culture color. However, with indole

at 4.5 mM the growth of the culture was less when compared to control and there was a change in the culture color from brown to green (Fig 30). When the culture that has turned green was streaked onto a nutrient agar plate, there was no visible colony appeared, but the culture appeared viable when observed under the microscope (Fig 31). Therefore, the culture exposed to indole of 3 mM and above was considered to be under the state of VBNC (viable but non-culturable). However, no such change in the culture color was observed with indole derivatives like IPA, IBA, IAA and indole acetamide at 4.5 mM concentration (Fig 32).

3.4.2 **Effect of 2-aminobenzoate and indole on whole cell absorption spectra of *Rba.* sphaeroides OU5**

The whole cell absorption spectrum of *Rba. sphaeroides* OU5 showed an absorption max at 380, 450 nm reflecting to the carotenoids and 806, 890 nm to bacteriochlorophyll-a however, in the presence of 2-aminobenzoate the spectrum was same but significant reduction in the peak heights was observed. Whereas with indole at 3 mM no other absorption peaks were observed except for a peak at 860 nm (Fig 33). An additional peak at 680 nm was observed which is supposed to be a plant chlorophyll absorption peak. The disappearance of 800 nm peak and appearance of 680nm peak gives an indication of an altered bacteriochlorophyll-a.

3.4.3 **Effect of 2-aminobenzoate and indole on pigments of *Rba. sphaeroides* OU5**

Effect of 2-aminobenzoate and indole on the pigments of *Rba. sphaeroides* OU5 was studied. It was observed that with increasing concentration of 2-aminobenzoate bacteriochlorophyll-a increased and the red and yellow carotenoids decreased (Fig 34). While with indole, the total pigment content decreased with increasing indole concentration (Fig 35), indicating the effect of indole on the pigments of *Rba. sphaeroides* OU5.

Fig 30: *Rba. sphaeroides* OU5 with different concentrations of indole

Assay was done with the growing cells after 24 h of phototrophic incubation in the presence of various concentrations of indole. Change in the culture color was observed at 3-8 mM indole.

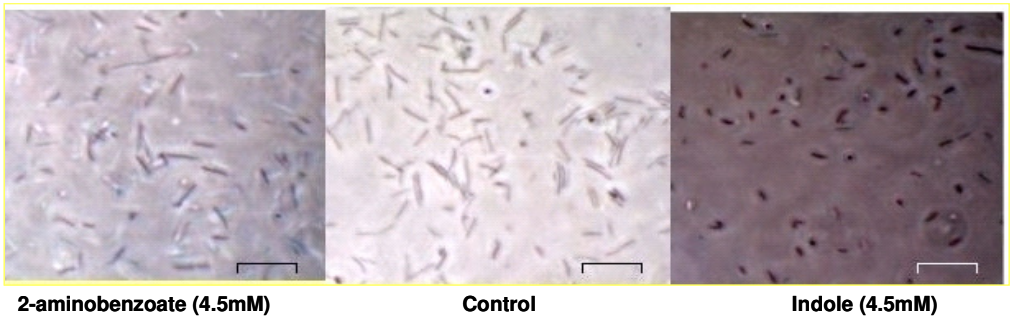


Fig 31: Cell morphology of *Rba. sphaeroides* OU5 induced with 2-aminobenzoate, indole and uninduced culture

Assay was done with growing cells after 24 h of phototrophic incubation in the presence of 2-aminobenzoate and indole. In the figure bar = 10 micrometers

The cell size got decreased with indole at 4.5 mM.

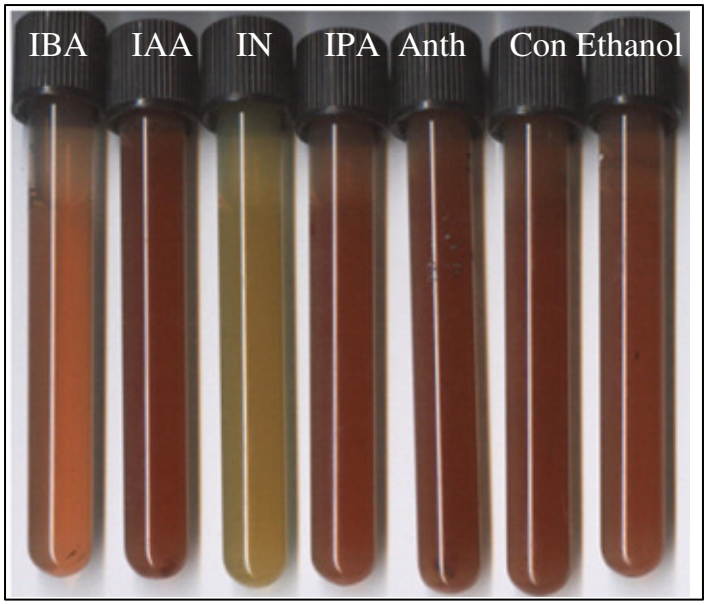


Fig 32: *Rba. sphaeroides* OU5 with different indole derivatives (4.5 mM)

IBA = Indole butyric acid; IAA = Indole acetic acid; In = Indole; IPA = Indole propionic acid; anth = 2-aminobenzoate; Con = Control; Ethanol = Culture to which ethanol was added to equate the ethanol present in the indole stocks.

Assay was done with the growing cells after 24 h of phototrophic incubation in the presence of indole derivatives (4.5 mM). Stock of indole and its derivatives prepared in ethanol are added to the culture before incubation.

Change in the culture color was observed with indole alone.

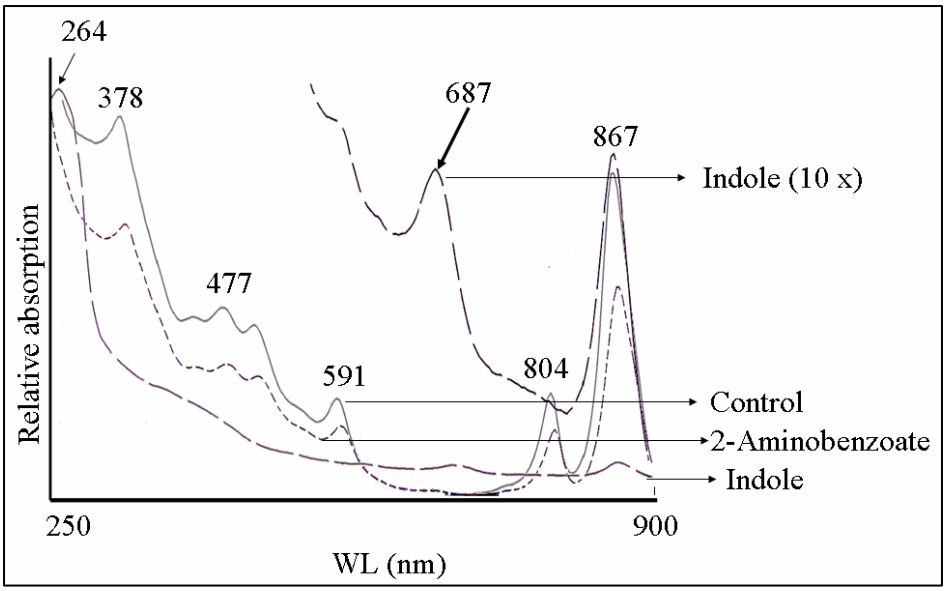


Fig 33: Whole cell absorption spectrum of *Rba. sphaeroides* OU5 grown with indole (4.5 mM) and 2-aminobenzoate (4.5 mM)

Rba. sphaeroides OU5 was harvested for analysis after 24 h of phototrophic incubation in the presence of 2-aminobenzoate and indole (4.5 mM). Assay was done with the cells in sucrose solution and measured at 250-900 nm in Genesys 2 spectrophotometer. Indole (10x) = Indole spectrum was 10 times amplified.

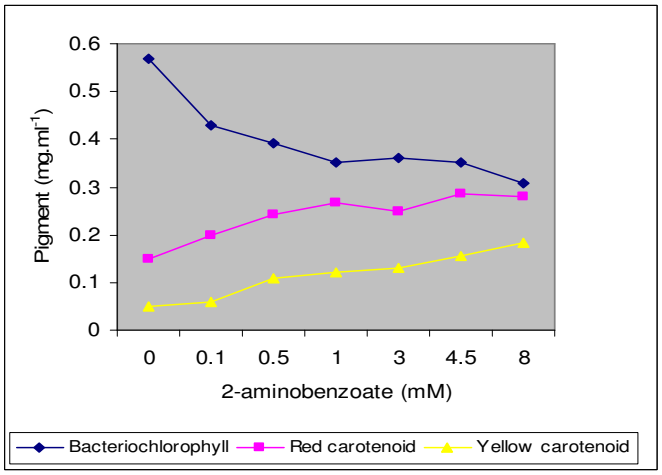


Fig 34: Effect of various concentrations of 2-aminobenzoate on pigments of *Rba. sphaeroides* OU5

With increasing concentrations of 2-aminobenzoate, bacteriochlorophyll decreased but red and yellow carotenoids increased. Pigments were extracted from the culture pellet using 5 ml of methanol: acetone (7:2) mixture. Analyses were done at 456, 510 and 775 nm in Genisys 2 spectro-photometer.

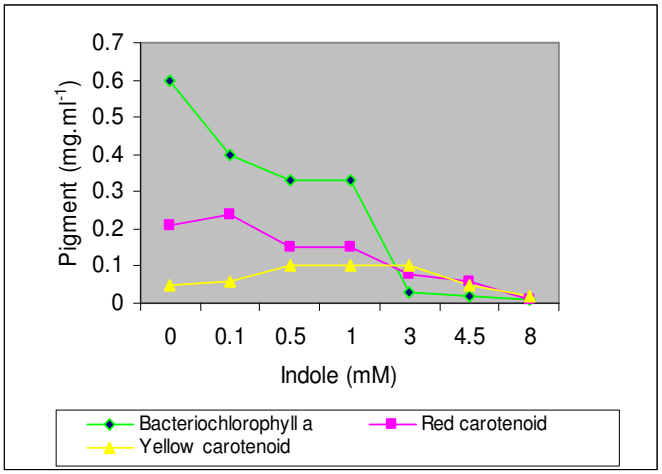


Fig 35: Effect of various concentrations of indole on pigments of *Rba. sphaeroides* OU5

With increasing concentrations of indole the pigment content decreased. Experimental conditions are as in the above figure.

3.4.4 **Effect of indole and 2-aminobenzoate on proteins of *Rba. sphaeroides* OU5**

The presence of oxidants like indoles induces the production of reactive oxygen species (ROS). In response to ROS, anti-oxidant proteins like catalase, alkylperoxidase, superoxidedismutase are generally produced. Sometimes stress induced proteins called as sphaeroplasts are also produced in *E. coli*. Hence, experiments were conducted to know the production of anti-oxidant and stress induced proteins in *Rba. sphaeroides* OU5 induced with indole and 2-aminobenzoate. However, no such stress induced proteins and anti-oxidant proteins were detected in *Rba. sphaeroides* OU5. But the culture has shown low catalase activity, in the presence of 2-aminobenzoate and indole. However, over expression of certain orange colored proteins (OCPs) were observed in the presence of 2-aminobenzoate and indole (Fig 36, 37).

3.4.5.1 **Orange carotenoid proteins (OCPs) in *Rba. sphaeroides* OU5**

Orange colored proteins (OCPs) are water soluble carotenoid proteins well studied in plants and in cyanobacteria however, there are no reports in anoxygenic phototrophic bacteria. These proteins are found to over express in *Rba. sphaeroides* OU5 in the presence of 2-aminobenzoate and indole (Fig 36, 37). In the presence of 2-aminobenzoate the over expression of these proteins was more at 0.5-3 mM. (Fig 36) may be because the indole production from 2-aminobenzoate was optimum at that concentration. In the presence of indole at 0.1-1 mM the expression of these proteins was more (Fig 37) in comparison to the control. While with increasing indole concentration beyond 1 mM the OCPs decreased. The green colored proteins (GCPs) were observed with 3 mM indole (Fig 37). The protein bands were tested for the presence of protein bound indole using PDAB reagent. With PDAB reagent the bands turned to dark pink indicating the presence of indole (Fig 36, 37).

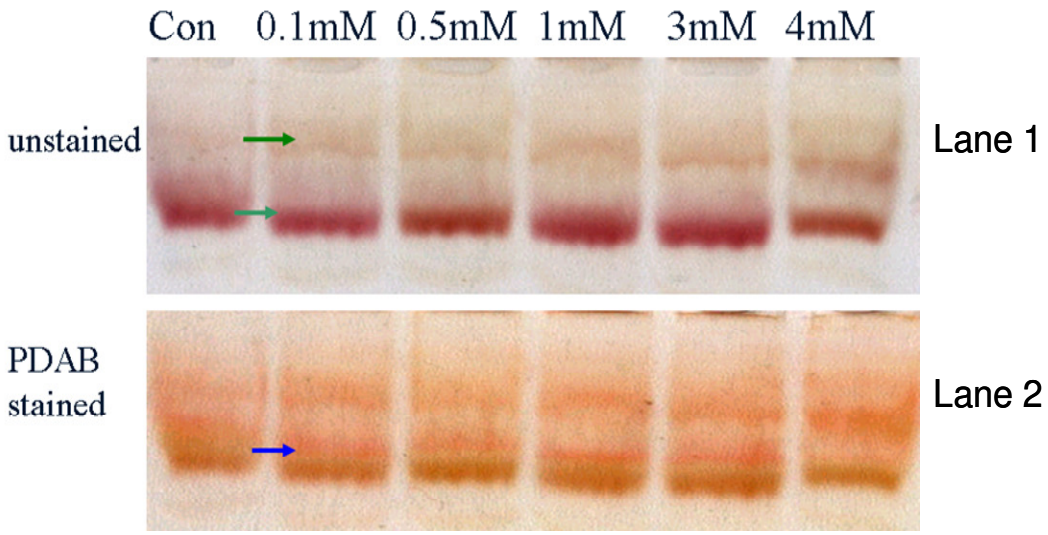


Fig 36: Orange carotenoid proteins (OCPs) in *Rba. sphaeroides* OU5 at different concentrations of 2-aminobenzoate (0.1-4 mM)

6 % Native gel showing colored proteins in lane 1. The gel was stained with PDAB reagent for 5 min and the proteins turned to dark pink in lane 2. Arrows represent the over expressed proteins in comparison to the control.

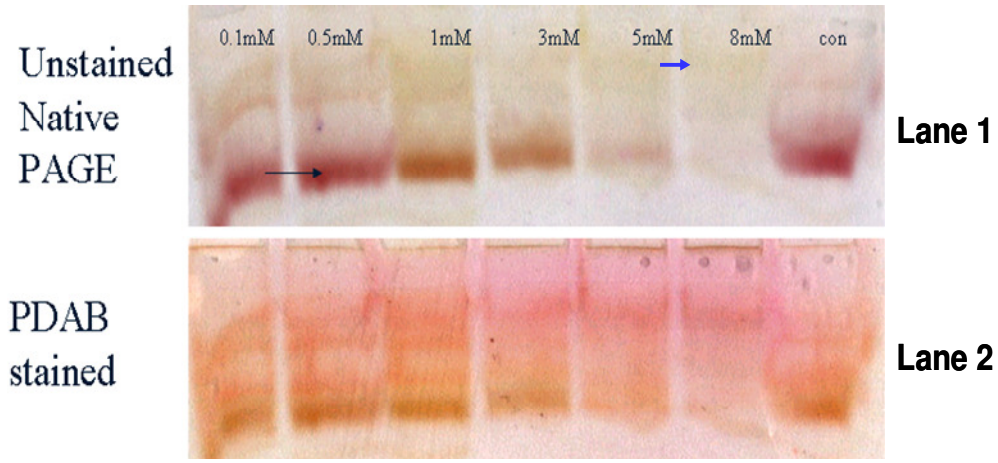


Fig 37: Orange carotenoid proteins (OCPs) in *Rba. sphaeroides* OU5 at different concentrations of indole

6 % Native gel showing colored proteins in lane 1. The gel was stained with PDAB reagent for 5 min and the proteins turned to dark pink in lane 2. Black arrows represent the over expressed proteins in comparison to the control. Blue arrow indicates the green colored protein (GCP).

3.4.5.2 **Isolation of orange carotenoid proteins (OCPs)**

Rba. sphaeroides OU5 was grown in the presence of 2-aminobenzoate (1 mM) for 48 h before harvesting. The pellet was washed twice with 0.05 M phosphate buffer (pH 7.8) and sonicated in the same buffer containing 0.1 % Triton X–100, the cell free extract was analysed on the native gels. The protein bands were crushed and incubated in the gel elution buffer (Tris buffer 0.1 M pH 8 with 0.1% Triton X-100). The protein obtained after centrifugation was dialyzed in the phosphate buffer for further analysis.

3.4.5.3 **SDS-PAGE analysis of OCPs:** In SDS-PAGE, the OCPs of *Rba. sphaeroides* OU5 extracted from the uninduced, 2-aminobenzoate and indole induced cultures appeared as the protein bands of 33.5, 35.6, 53.4, 77.6 and 110 kD. No change in protein profile of OCPs in the presence and absence of 2-aminobenzoate and indole was observed (Fig 38).

3.4.5.4 **Detection of Indole:** The binding of indole to OCPs was also confirmed in HPLC. The homogenized OCPs solution in Tris buffer (pH 8) showed indole peak. The indole bound to OCP was matching with standard indole and also with the indole terpenoid esters (Fig 39). Further to know the nature of the indole bound to OCPs, the OCP bound indole was extracted with ethylacetate, dried and re-dissolved in methanol and then analysed using IR and GC-MS. The IR indicated the ester peak of the compound and from the GC-MS analysis (Fig 40) the molecular mass of the compound was (*m/z*) 741. From these results the compound is predicted as an indole terpenoid ester.

3.4.5.5 **To identify the indole binding property of OCPs:** The indole consumption activity of OCP protein was done in HPLC. The decrease in the indole content in the sample containing the protein indicated the role of OCPs in

indole binding, however the decrease in the concentration of other substrates like 2-aminobenzoate and benzoate was not observed when added to the protein (Fig 41).

3.4.5.6 **The carotenoids of OCPs:**

The carotenoids of OCPs were extracted with ethylacetate and the absorption maxima were read in spectrophotometer. The sample has given maximum absorption peaks at 375, 477 and 591 nm reflecting to the carotenoid sphaeroidene of purple non sulphur bacterium *Rba. sphaeroides* OU5. The remaining aqueous fraction containing the OCP protein showed absorption at 498, 639 and 780 nm reflecting to carotenoid. OCP protein has maximum absorption peak at 225 nm (Fig 42).

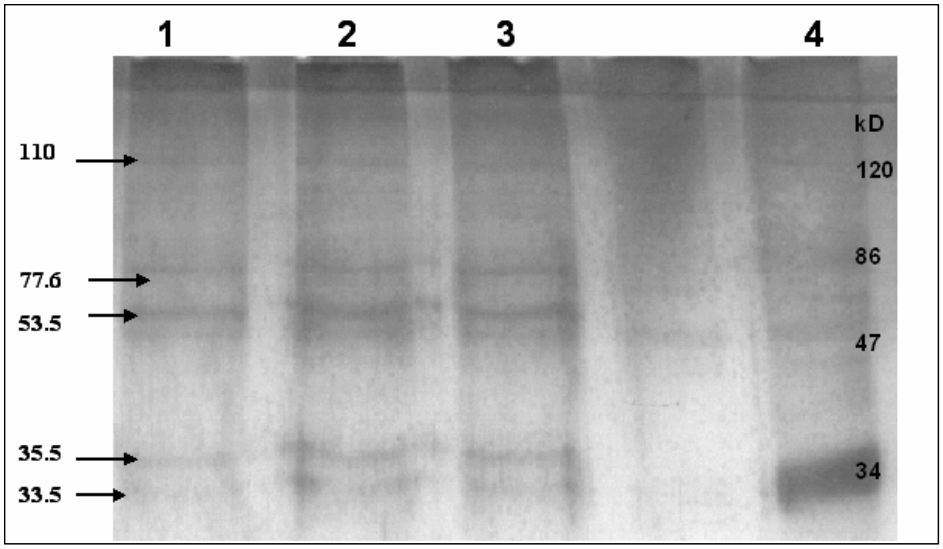


Fig 38: OCPs of *Rba. sphaeroides* OU5 extracted from uninduced, 2-aminobenzoate and indole induced cultures

Lane 1: OCP from 2-aminobenzoate induced culture; lane 2: OCP from indole induced culture; lane 3: OCP from uninduced culture of *Rba. sphaeroides* OU5; lane 4 : Marker.

10 % SDS gel was stained with silver stain and it indicates 110, 77.6, 53.5, 35.5 and 33.5 kD protein bands of OCP protein extracted from *Rba. sphaeroides* OU5 grown with and without 2-aminobenzoate and indole.

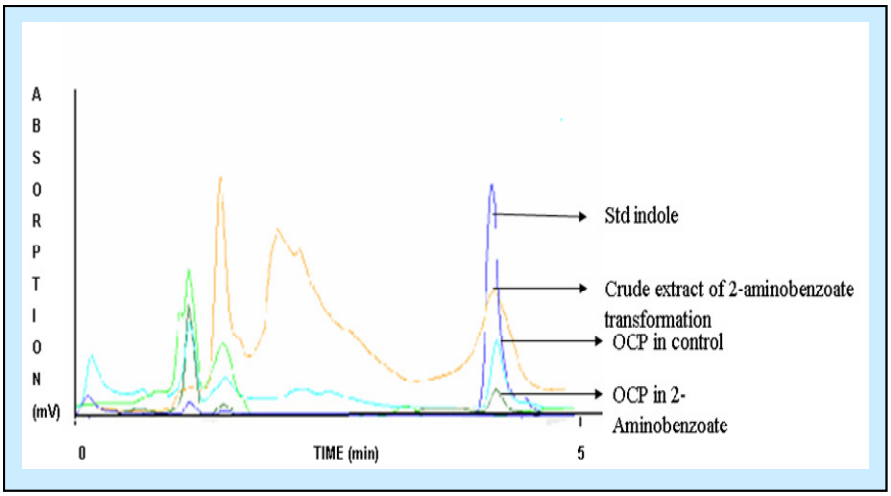


Fig 39: Indole of OCPs was matched with the standard indole and indole terpenoid esters

OCP in phosphate buffer (pH 7.8) was analyzed in HPLC at 269 nm using UV-Vis detector on a C18 column using methanol as mobile phase, at a flow rate of 1.5 ml. min^{-1}.

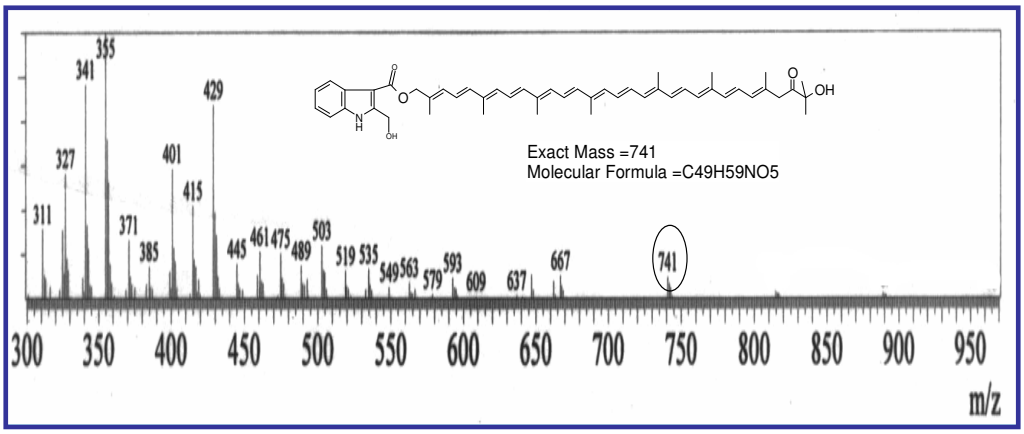


Fig 40: GC-MS analysis of OCP bound indole

The metabolite structure, mass and molecular composition is inserted in the figure. OCP bound indole was extracted with ethylacetate, dried and re-dissolved in methanol and analyzed in GC-MS using Eq probe, column temperature 300 °C; oven temperature 310 °C, injection volume 2µl, using Helium as the carrier gas.

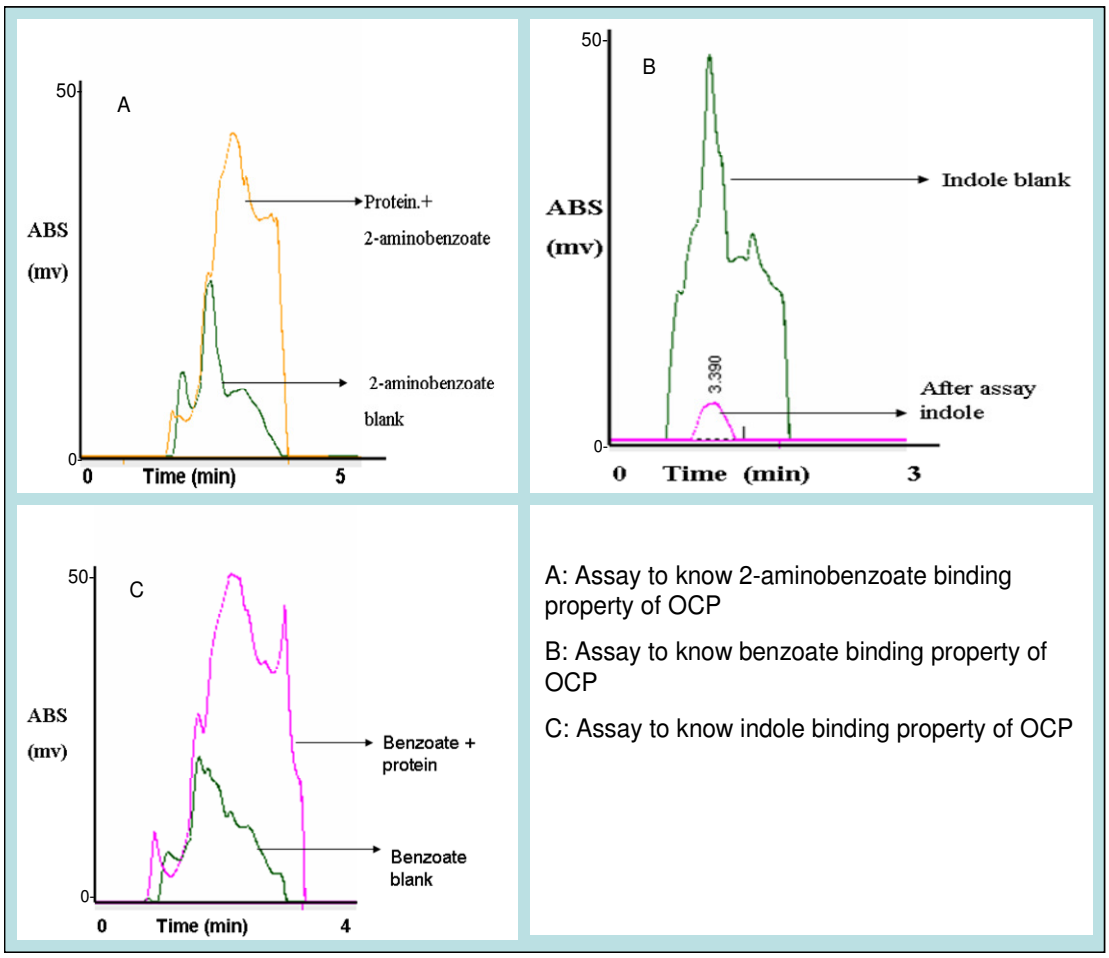
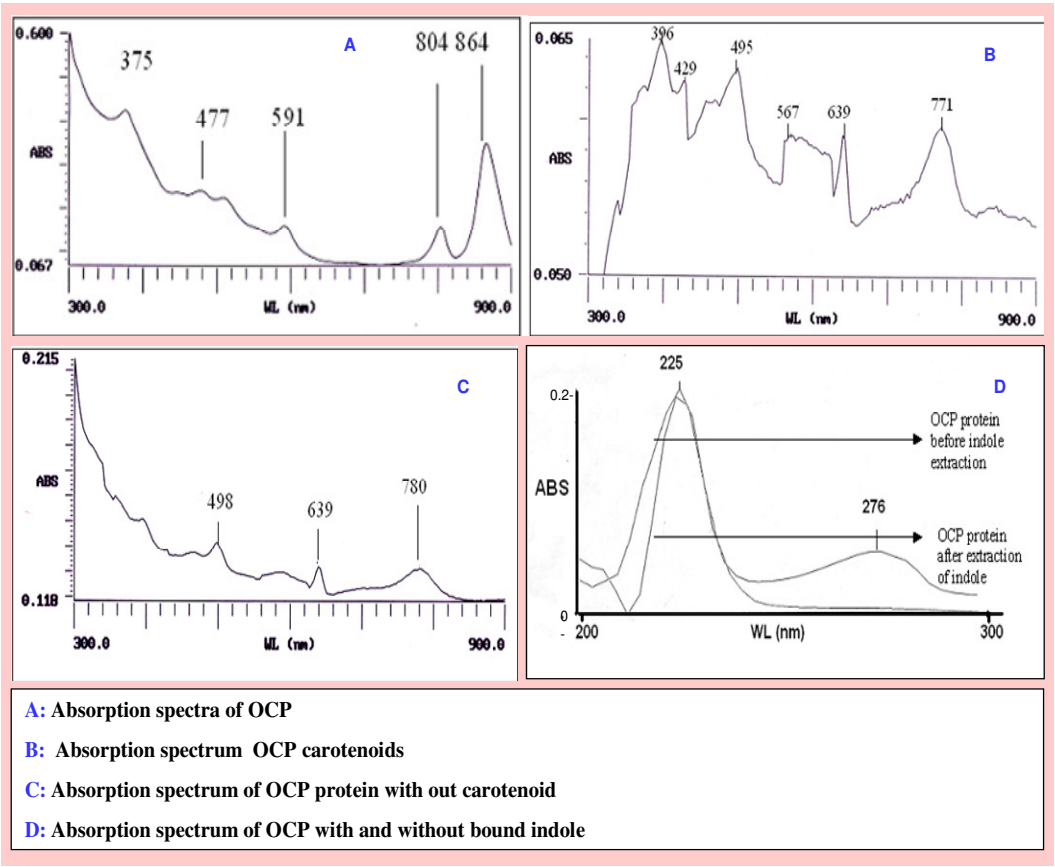


Fig 41: Assay to know the substrate binding property of OCP

Assay was done in 200 µl phosphate buffer pH 7.8 to which 50 µl of protein sample and 10 µmoles of the substrate was added and incubated for 10 min. The decrease in the substrate was analyzed in HPLC at 268 nm for indole and at 220 nm for 2-aminobenzoate and benzoate, using methanol as mobile phase on C18 column at a flow rate of 1 $ml.min^{-1}$.



A: Absorption spectra of OCP

B: Absorption spectrum OCP carotenoids

C: Absorption spectrum of OCP protein with out carotenoid

D: Absorption spectrum of OCP with and without bound indole

Fig 42: Absorption spectra of different samples of OCP

The absorption spectrum of OCP in buffer was observed in UV-Visible region, after extraction of carotenoids from the protein into ethylacetate, the absorption was taken for both the protein as well as for the carotenoid extract. The protein has maximum absorption peak at 225 nm in UV region.

3.5 **Bioprospecting the metabolites of 2-aminobenzoate transformation**

Indole and its derivatives are known to have antimicrobial activity (Matsuda *et al*., 1990). Unlike indole acetic acid and indole propionoic acid; the natural auxins, the esters of indole acetic acid and indole myo-inositol are also known to have phytohormonal activity. Hence the metabolites of 2-aminobenzoate transformation, which are also the indole derivatives, were screened for the phytohormonal as well as anti-microbial activity.

3.5.1 **Anti-microbial activity**

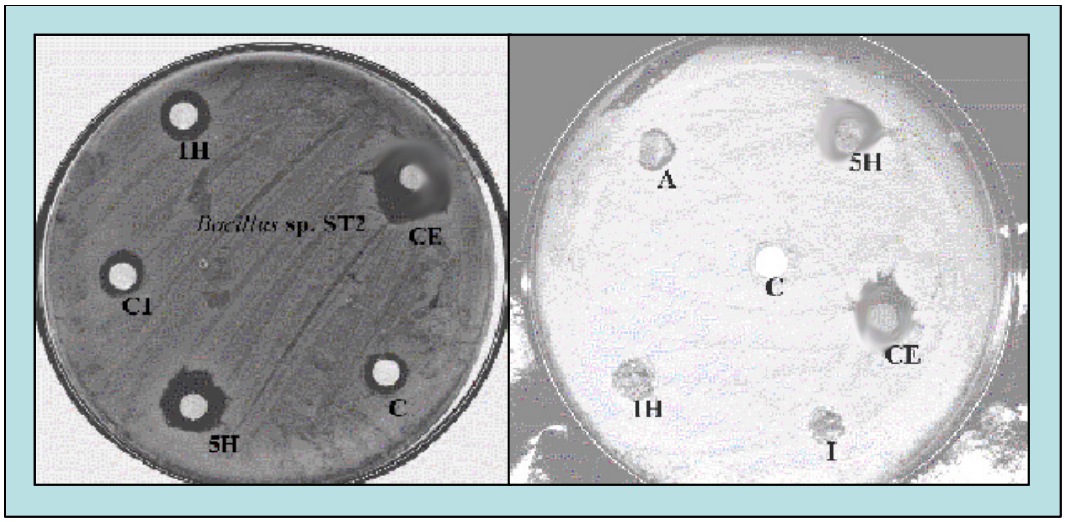
Crude extract as well as sphesterin [indole ester with molecular mass 441which was characterized initially was called as sphesterin] were screened for antimicrobial activity against the Gram +ve and Gram -ve bacteria. The zone of inhibition was seen with the crude extract as well as with the sphesterin (Fig 43). The activity was shown against few gram +ve organisms tested.

3.5.2 **Phytohormonal activity**

The crude extracts extracted from culture supernatant as well as the purified compounds of 2-aminobenzoate transformation were screened for phytohormonal activity. Among all, the compound of mass 595 and the crude extracts of both ethyl acetate and acidified extracts gave positive reaction for auxin bioassay and induced profussed and early rooting in tissue culture plants of mulberry, tobacco and groundnut in comparison to the standards (NAA and 2,4-D). The amount of these compound required for showing the activity was 5ηM and it was very less, in comparison to the standards (5 µM). The compounds also supported healthy development and multiple shooting in the tissue cultured plants of tobacco, mulberry and groundnut (Fig 44, 45, 46 and 47).

3.5.3 **COX-1 & 2 inhibitory studies**

The crude extract and purified compounds at 2 ηM was checked for COX-1 and 2 inhibitory activities. The compounds found to have no COX-1 and 2 inhibitory activities.



Bacillus sp *Serratia* sp

Fig 43: Anti-microbial activity with the metabolites of 2-aminobenzoate transformation

CE = Crude extract of ethylacetate layer in ethanol; H1 = 1N HCl acidified aqueous crude extract in ethanol with pH 5; C1 = Ethanol control for 1H; 5H = 5N HCl acidified crude extract in ethanol with pH 6; C = Ethanol control for 5H. Nutrient agar plates were spreaded with culture of *Bacillus* and *Serratia* and the filter paper discs containing either the crude extract or purified metabolites of 2-aminobenzoate transformation were placed on the agar and incubated for 24 h at 30 °C.

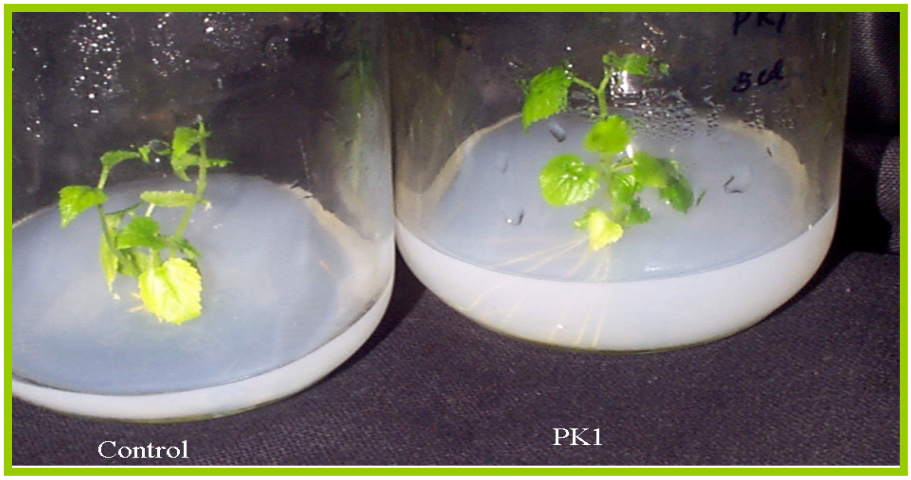


Fig 44: Mulberry plants showing multiple shooting

Control = Without any phytohormone; PK1 = Purified metabolite of 2-aminobenzoate transformation. Plants were incubated for 10 days on the tissue culture medium, supplemented with PK1 (5 ηM).

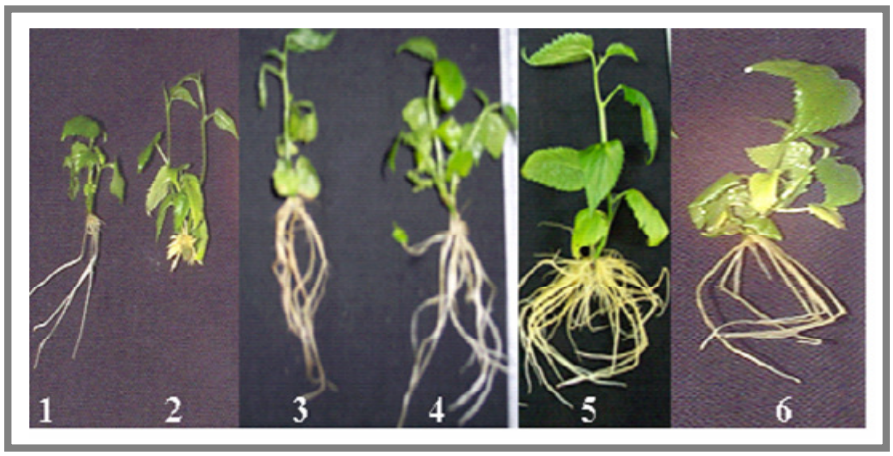


Fig 45: Photograph showing the phytohormonal activity with the supplemented compounds in mulberry plants

Plants no: 1 = Control; 2 = NAA (5µM); 3 = 2,4-D (5µM); 4 = Crude extract (0.05µM); 5 = Acidified crude extract (0.05µM); 6 = Rhodestrin (0.005µM). Experiment was done in duplicates and the results were analysed after 15 days of incubation.

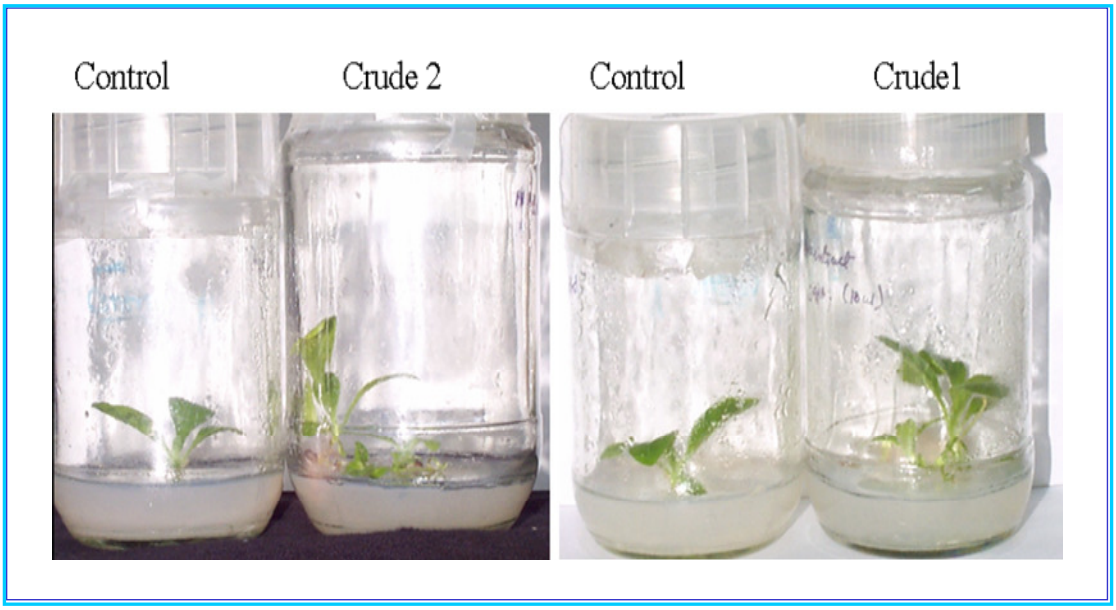


Fig 46: Tobacco plants showing multiple shooting and healthy development

Control = Without any phytohormone; Crude 2 = Metabolites of the aqueous layer; Crude 1 = Metabolites of ethylacetate layer from the culture supernatant of *Rba. spaheroides* OU5 grown with 2-aminobenzoate.

Plants were incubated for 10 days on the tissue culture medium, supplemented with crude samples of indole terpenoid esters instead of NAA.

Fig 47: Photograph showing the phytohormonal activity with the supplemented compounds in tobacco plants

Control = Without any phytohormone; HCl extract = Metabolites of the aqueous layer; crude extract = Metabolites of ethylacetate layer from culture supernatant of *Rba. sphaeroides* OU5 grown with 2-aminobenzoate. Experiment was done in duplicates and the results were analysed after 15 days of incubation.

Discussion

4.1 **Background introduction**

Few Purple Non-Sulfur Bacteria (PNSB) (a major physiological group among Anoxygenic phototrophic bacteria [APB]) have the capability to degrade a wide range of low molecular weight aromatic hydrocarbons for growth (Gibson and Hardwood, 1994; Sasikala and Ramana 1998; Harwood *et al*., 1999; Semple, 1999) and thus help in the maintenance of bio-geochemical cycles. In most cases, aromatic ring cleavage by PNSB was light dependent and this bioprocess is commonly referred as "photometabolism" (Harwood and Gibson, 1988). Photometabolism is one among the anaerobic energy yielding aromatic metabolisms, while the others are; nitrate reduction (denitrification), sulfate reduction, iron reduction, methanogenesis and fermentation (Heider and Fuchs, 1997). However, the role of light in photometabolism of aromatic compounds is not understood (Sasikala and Ramana, 1998).

While the initial studies of photometabolism were concentrated on the naturally occurring and low molecular weight aromatic hydrocarbons (Evans, 1977), more emphasis is given in the recent years on the photobiodegradation and transformation of recalcitrant and xenobiotic molecules (Sasikala and Ramana, 1998; Reiger *et al*., 2002). The most extensively studied species among the PNSB is *Rhodopseudomonas palustris*, whose total genomic analysis indicated the existence of at least 5 aromatic ring cleavage pathways, representing more aerobic mechanisms, than anaerobic (Larimer *et al*., 2004). The other species of PNSB capable of degrading benzoate include; *Phaeospirillum fulvum* (earlier *Rhodospirillum fulvum*, Pfennig *et al*., 1965), *Rhodocyclus purpureus* (Pfennig, 1978), *Rhodomicrobium vannielli* (Wright and Madigan, 1991) and *Rubrivivax benzoatiliticus* (Ramana *et al.*, 2006). On the other hand, some incubation results indicated light dependent transformation of aromatic hydrocarbons by *Rubrivivax gelatinosus* (Willems *et al*., 1991), *Rhodobacter capsulatus* (Blasco and Castillo, 1992), *Rhodopseudomonas*

blastica (Ahmed and Mohamed, 1994) and *Rhodobacter sphaeroides* (Rajasekhar *et al., 1998*). The most recent discovery on the aromatic hydrocarbon metabolism by PNSB is the light dependent assimilatory metabolism of *trans*-cinnamate observed in *Rhodobacter sphaeroides* OU5 (Usha *et al*., 2007). The discovery of a novel fumarate and light dependent transformation of 2-aminobenzoate to indole by *Rba. sphaeroides* OU5 (Nanda *et al*., 2000) was taken up as a detailed study for the present Ph.D thesis.

4.2 **Utilization of 2-aminobenzoate for growth by *Rba. sphaeroides* OU5**

Among PNSB, the most widely and abundantly distributed species are *Rps. palustris* and *Rba. sphaeroides* (Madigan, 1988). While *Rps. palustris* is known for aromatic ring cleavage and for utilization of aromatic hydrocarbons as growth substrates, *Rba. sphaeroides* lacks this capability as observed also for 2-aminobenzoate in the present study. Though homocyclic aromatic hydrocarbon utilization for growth was not observed in *Rba. sphaeroides* OU5, heterocyclic aromatic hydrocarbons viz., 4-dimethylaminopyridine, pyrazine, 2-aminopyrazine has supported the growth (Rajashekar *et al.,* 2000), although the degradation pathways are not yet established.

4.3 **Transformation of 2-aminobenzoate by *Rba. sphaeroides* OU5**

Light dependent transformation of indole to tryptophan, tryptamine, indole-lactic acid and indigo was the first observation made with *Rba. sphaeroides* OU5 (Rajasekhar *et al*., 1998). The extended work with strain OU5 indicated the formation of indole 3-acetic acid (Rajasekhar *et al*., 1999b) and tryptophan (Rajasekhar *et al*., 1999a) from indole and glycine. Some incubation experiments also indicated the capability of this strain in the degradation of indole nucleus and the production of 2-aminophenol acetate and 2-aminobenzoate into the medium (Rajasekhar *et al*., 1999c). The transformed metabolite 2, 3-dihydroxyindole was identified from culture

supernatant of *Rba. sphaeroides* OU5 incubated in the presence of 2-aminobenzoate (Nanda *et al*., 2000). Production of indole into the medium by *Rba. sphaeroides* OU5 in the presence of 2-aminobenzoate was also confirmed in the present study by both growing (Fig 5) and resting cells (Fig 6). The indole thus produced, was greatly dependent on the initial cell density (Fig 7) and 2-aminobenzoate concentration (Fig 8).

4.4 **Characterization and identification of indoles from the culture supernatant of *Rba. sphaeroides* OU5 grown in the presence of 2-aminobenzoate**

Indole and a number of its derivatives are conventionally identified by the formation of color reaction with p-dimethylaminobenzaldehyde (PDAB) in the presence of an acid (Kuper and Atkinson, 1964), with several modifications in the protocols; they are being called as different reagents. The most common among them is the Kovac's reagent, which gives rose indole when reacted with indole. However, most of the times, the color reactions does not discriminate the difference in the molecules and the complexity increases when small changes occur on the nucleus. Thus, indole and 2, 3-dihydroxyindole (Nanda *et al*., 2000) give similar color reaction with PDAB reagent. These cryptic indoles can be identified only using polychemical analysis, which is possible only when they are extracted and isolated to their purity.

The formation of an orange pink color when the culture supernatant was reacted with Kovac's reagent indicated the existence of an indole derivative rather than pure indole, which gives rose color (Kuper and Atkinson, 1964). This necessitated a detailed investigation in isolating, characterizing and identifying the 2-aminobenzoate phototransformed products in *Rba. sphaeroides* OU5. Ethylacetate extraction of the culture supernatant of 2-aminobenzoate induced culture resulted in accumulation of indole at the inter-phase of water and ethylacetate, which was

saturated with lipids, indicating that the indoles produced are of non-polar in nature (Fig 9). The LC-MS profiling (Fig 10) of the ethyl acetate extract had at least 16 molecular masses, which were not observed in the control. In order to isolate these metabolites in good quantities, extractions were done from 20 L culture supernatant of *Rba. sphaeroides* OU5.

As many as 57 different metabolites were isolated through fractionation (Flow chart 1-7) of which, 29 gave positive color reaction with PDAB reagent (Table 2 and 3). The R_f values of the isolated metabolites, in three different solvent systems, were different for different metabolites, which distinguish the metabolites from one another. The yields of these metabolites ranged from 0.5 to 50 µmoles and the total yield of all metabolites is about 150 µmoles.

Detailed characterization of one of the metabolite based on IR (Fig 11), ^1H (Fig 13), and mass (Fig 14) analysis confirmed the structure as (3E)-13-hydroxy-1, 1, 5, 9-tetramethyl trideca-3,7-dienyl 2-(hydroxymethyl)-1H-indole-3-carboxylate; it has a molecular mass of 441 (*m/z*) and was named as sphesterin (Sunayana *et al.,* 2005a). Rhodestrin was an additional metabolite that was published recently (Sunayana *et al*., 2005b). The ester linkage of the metabolites was confirmed through the hydrolysis of the ester bond using esterase enzyme (Fig 12). The structures of the other metabolites were determined in a similar way (Fig 15a-f) and the representative molecular masses are given in table 2 and 3. Most of the purified metabolite masses matched with those observed in the LC-MS spectrum (Fig 10).

The color reactions of some of the metabolites with PDAB were pink to red indicating that these are the indole derivatives, while the yellow to brown indicated the metabolites as indole ring cleaved products. Since most of the metabolites are terpenoid conjugates, they are colored (table 2, 3). Wide range of indole-conjugated terpenoids isolated in the present study is novel. However, indole esters of myo-

inositol-indole acetates were reported in plants (Domagalski *et al.* 1987, Chisnell 1984).

Terpenoids (isoprenoid compounds) are one of the major and widely distributed biogenic molecules in the geosphere. Anoxygenic phototrophic bacteria contribute significantly to the production of these molecules and the relatively stable isoprenoid skeletal unit helped in the isolation of a new aromatic carotenoid biomarker, okenane, giving evidences of the existence of these bacteria in the Paleoproterozoic ara (~1.5 Gyr) (Brocks *et al*., 2005). The well-known terpenoid molecules of anoxygenic phototrophic bacteria like carotenoids, bacteriochlorophylls, quinones and hopanoids significantly contribute to various cellular activities, while simultaneously they are also recognized for their biotechnological applications (Sasikala and Ramana, 1995). In addition to these terpenoid molecules, the present study indicates the production of several novel indole terpenoid esters by a purple non-sulfur bacterium *Rba. sphaeroides* OU5 when grown on anthranilate (Sunayana *et al*., 2005a) or aniline (Vijay *et al.,* 2006). Terpenoid wax esters on the other hand are produced from substrates like n-alkanes (Ishige *et al*., 2002; Bredemeir *et al.,* 2003) or other terpenoids (Rontani *et al*., 1997, 2003) by bacteria other than anoxygenic phototrophic bacteria. Evidence suggests that these compounds serve as ATP-generating substrates during starvation (Rontani *et al*., 1999, 2003). In contrast to the terpenoid wax esters, the function of indole terpenoid esters in anoxygenic phototrophic bacteria is still not known.

Though esterification of phytol was suggested (Brooks and Maxwell, 1974; Brooks *et al.,* 1978) to be a significant process in microbially active sediments and phytol esters were detected in some marine (Boon and de Leeuw, 1979) and lacustrine sediments (Cranwell, 1986), their origin is not satisfactorily explained. Terpenoid esters were reported in microbially active sediments (Boon and de Leeuw,

1979; Cranwell, 1986; Grossi *et al*., 1998), pure microbial cultures (Rontani *et al*., 1999), higher plants (Csupor, 1971), bryophytes (Buchanan *et al.,* 1996), mosses (Leeuw *et al*., 1977), dinoflagellates (Withers and Nevenzel, 1977) and marine zooplankton (Sargent and Fali-Petersen, 1981). Our work adds to the diversity of bacteria capable of producing such esters.

Based on the following evidences: 1. terpenoid biodegradation proceeds through α, β oxidations (Rontani and Volkman, 2003) (Fig 48) 2. methyl terminal is first oxidized to an alcohol, then converted to an aldehyde and finally to an acid (Rontani and Volkman, 2003; Van den Brink *et al*., 2005) and 3. the sub-terminal carbons are oxidized before the terminal carbons are lost in the oxidations (Rontani *et al*., 1999), we established the relationships between the metabolites and propose a putative pathway (Fig 49) of the different metabolites isolated from the culture supernatant of *Rba. sphaeroides* OU5 when grown in the presence of 2-aminobenzoate.

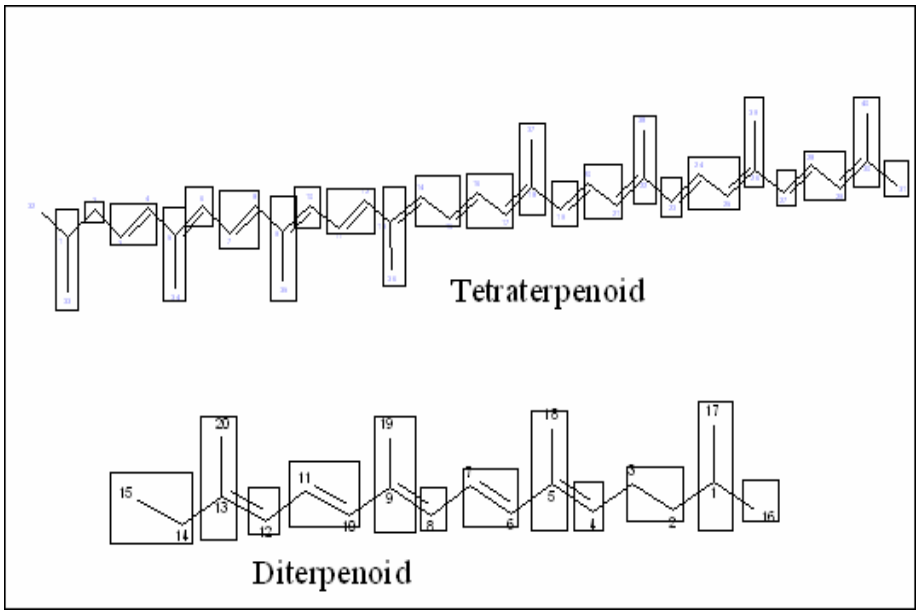


Fig 48: Di and tetraterpenoids indicating α, β oxidations

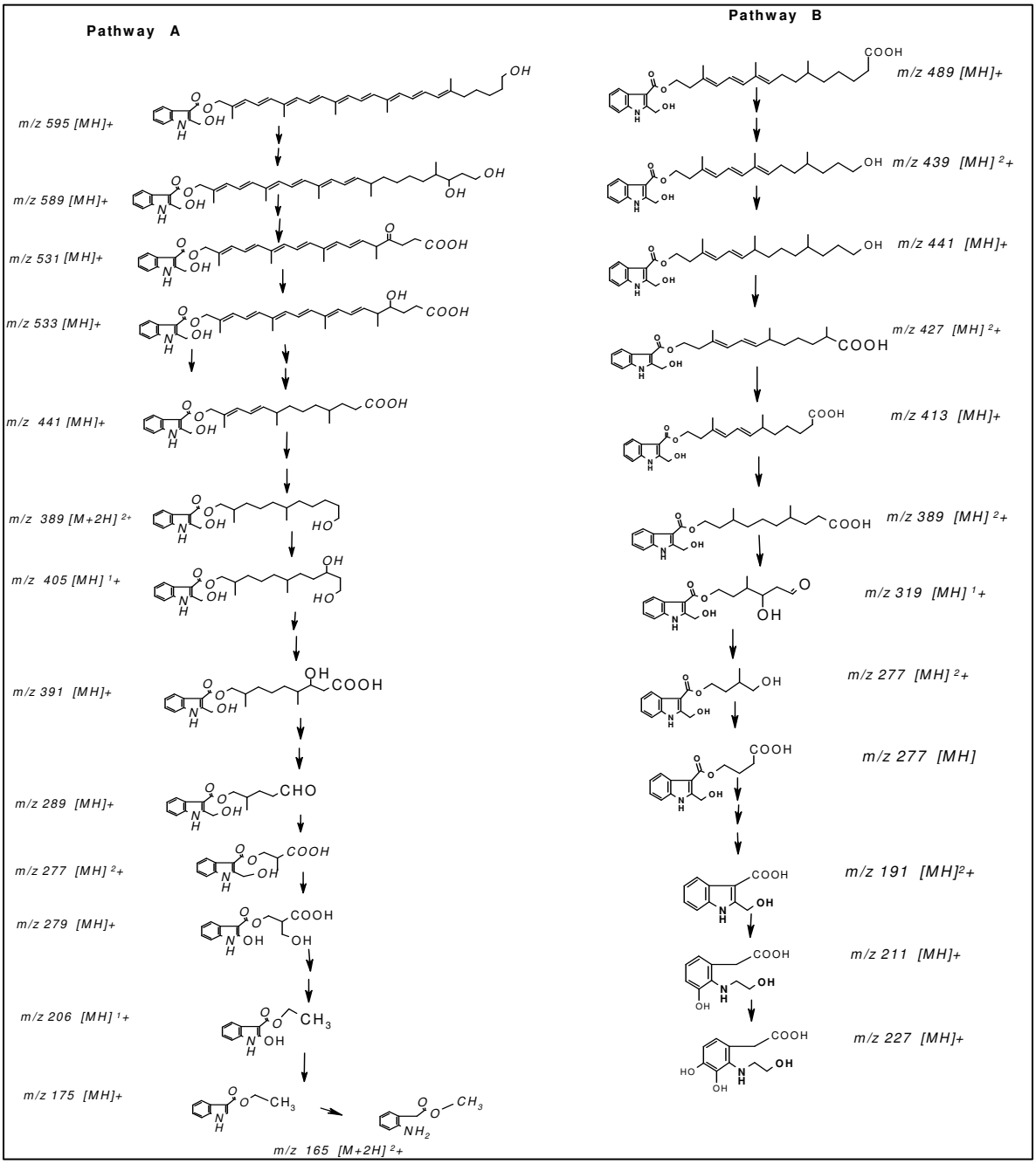


Fig 49: Indole terpenoid degradation pathways in *Rba. sphaeroides* OU5

4.5 **Indole synthesis from 2-aminobenzoate and fumarate by *Rba. sphaeroides* OU5**

Only plants and microorganisms have the capability to synthesize the indole nucleus (Crawford *et al*, 1989) and this *denovo* synthesis occurs from the precursor 2-aminobenzoate, which inturn is synthesized through shikimate pathway (Radwanski and Robert, 1995). Phospho-ribosyl-pyrophosphate (PRPP) is the conjugating molecule and this reaction is catalysed by the enzyme anthranilate synthase (AS-II) coded by *trpE(D)* gene. The further conversion of phospho-ribosyl anthranilate to indole is brought about by phospho-ribosyl anthranilate isomerase and indole glyceraldehyde phosphate synthase (Fig 1). Most of these indole biosynthesizing enzymes were reported to be cytosolic and the enzymes/genes of the *trp* operon is well known and extensively studied in various microorganisms (Gary Xie *et al*., 2003).

The total genomic analysis of *Rba. sphaeroides* strains 2.4.1 , ATCC 17025, ATCC 17029 (Choudhary *et al*., 2007) indicated the existence of *trp* operon genes (*trpAa/yibQ/trpAb/trpB/trpD* and *trpC/aroR/trpEb*) residing on two separate chromosomes (Chris *et al*., 1999). Despite the presence of these genes, our observations with whole cells (Table 4) and cell free (Fig 18) extracts indicated that the indole synthesis from 2-aminobenzoate in *Rba. sphaeroides* OU5 greatly dependent on the TCA cycle intermediates (Table 4) rather than the common precursors of PRPP like glucose and other sugars (Table 4; Fig 18). Further, the inhibition of indole production from sugars in the presence of 2-fluoroacetic acid (Table 4) confirmed the role of TCA cycle intermediates in an alternative pathway of indole synthesis in *Rba. sphaeroides* OU5. The fact that the high indole yield with fumarate in comparison with other TCA cycle intermediates (Table 4; Fig 18) and the confirmation using fumarate analogues (Table 5) strongly supports the existence of an alternate fumarate dependent indole biosynthetic pathway. Indole production was not

observed in the absence of 2-aminobenzoate either with or without carbon substrate suggests the inducible nature of indole production by the precursor, rather than due to carbon starvation, as observed in *Escherichia coli* (Wang *et al*., 2001). Inhibition of indole production by some of the 2-subtituted benzenes (Table 6, 7 and Fig 20), which act as analogues of 2-aminobenzoate, further supported the novel mechanism of indole biosynthesis.

In anaerobic aromatic metabolism, the conjugation of fumarate with methylated benzenes like toluene, xylenes resulted in the formation of benzylsuccinate and the reaction was catalyzed by benzylsuccinate synthase (Heider *et al*., 1999). However, the present study adds to the knowledge of fumarate conjugation in aromatic metabolism of 2-aminobenzoate. Microbial production of indole in most of the times is due to the down stream of tryptophan and the catabolic products indole, pyruvate and ammonia were observed due to the enzymatic action of tryptophanase (Elsorra *et al*., 2007). The low activity of the enzyme tryptophanase observed in the 2-aminobenzoate induced culture (Table 8) suggests that the indole production due to tryptophanase is low. On the other hand, the important enzyme in the upstream reaction of indole synthesis is anthranilate synthase and the observations of anthranilate synthase activity in the cytosolic fraction (Table 9) and fumarate dependent indole synthesizing enzyme activity in the membrane fraction (Table 10) clearly distinguishes the two pathways as distinct and delineated from each other.

4.6 **Fumarate dependent pathway of indole biosynthesis in *Rba. sphaeroides* OU5**

In order to elucidate the metabolic pathway of fumarate dependent indole biosynthesis we have first proposed a theoretical pathway (Fig 50) based on the above physiological and biochemical evidence (Fig 22). Some of the molecular masses in the LC-MS spectrum (Fig 23) of the membrane fraction of the enzymatic analysis matched with the predicted metabolite masses of the proposed pathway. Further, the

isolation of a ~64 kD protein (Fig 24, 25, 26), which catalyzed the conjugation of fumarate with 2-aminobenzoate, and its product which had a molecular mass (*m/z*) 207 with IUPAC name N-aniline fumarate that matched with the first intermediate of the pathway (Fig 27), confirms the proposed pathway. The membrane associated ~64 kD protein was characterized (Fig 29 and table 11-13). The only enzyme known so far catalyzing the fumarate aromatic conjugation is benzylsuccinate synthase (BSS). BSS is a ~220 kD protein, which has 3 subunits of 98, 8.5 and 6.4 kD (Heider *et al.,* 1999; Achong *et al*., 2001). However, the isolated ~64 kD protein is a monomer catalyzing the fumarate, 2-aminobenzoate conjugation reaction, thus is a novel enzyme. The novelty of this enzyme is further confirmed through MALDI analysis, which has not scored more than 53 % in BLAST search (Fig 51). The high substrate specificity and the metabolite release from the hydrolyzed protein (Fig 28) confirms the role of this enzyme, which was characterized (Fig 29), and named as "N-fumarylaniline synthase". The N-terminal sequencing of this enzyme is in progress.

4.7 **Indole toxicity in *Rba. sphaeroides* OU5**

The toxicity of indole and its multiple mode of action in biological system have already been discussed in the introduction (page12 -13). The MIC of indole on *Rba. sphaeroides* OU5 was 4 mM and the IC 50 was 0.6 mM as reported by Rajshekar *et al*., 2000 however, the investigator had not made attempts in understanding the mode of action of indole. In contrast, to indole inhibition at 4.5 mM (Fig 32, 35), 2-aminobenzoate had not inhibited the growth and the pigment production in *Rba. sphaeroides* OU5 at the similar concentration (Fig 30, 31, 34). Though the cells were found viable at elevated levels of indole (Fig 31), they were not found to be culturable, which could be attributed as due to the reduction in the cell size (Fig 31). This is a common phenomenon observed in VBNC (viable but non culturable) (Yogita, 2005; Oliver, 1995). In the presence of indole both quantitative and

qualitative changes were observed in the pigments of *Rba. sphaeroides* OU5 (Fig 33, 34). Most interesting and notable observation is the disappearance of short peak of bacteriochlorophyll-a at 804 nm and appearance of a peak at 687 nm (Fig 33) indicated an alternation in the bacteriochlorophyll-a in the presence of elevated concentration of indole.

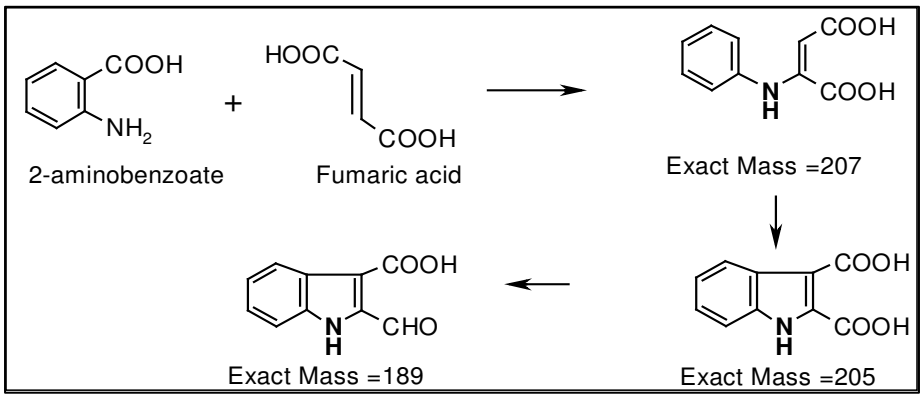


Fig 50: Fumarate dependent indole biosynthetic pathway in *Rba. sphaeroides* OU5

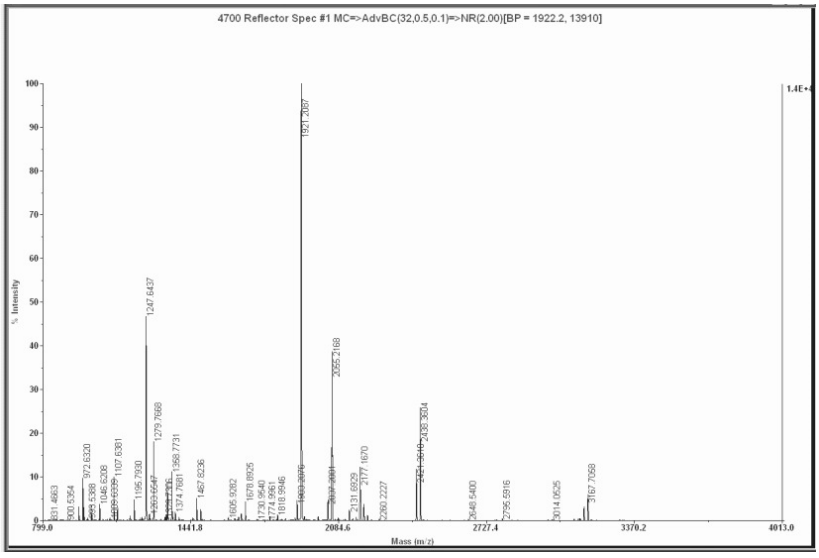


Fig 51: MALDI TOF analysis of the protein N-aniline fumaryl synthase

4.8 **Anti-oxidant proteins in *Rba. sphaeroides* OU5 to combat indole toxicity**

Indoles are general oxidants and their presence causes the production of reactive oxygen species (Garbe *et al*., 2000). However, catalase, superoxidedismutase, peroxidase are produced to combat oxidant toxicity. Stress induced proteins like sphaeroplast are also produced in *E. coli* (Garbe *et al*., 2000). In *Rba. sphaeroides* OU5 the anti-oxidant proteins like superoxidedismutase, peroxidase were not observed except for catalase. However orange carotenoid proteins (OCPs) (Fig 36 and 37) were found to be over expressed in the presence of indole and 2-aminobenzoate.

The SDS proteome profile of 2-aminobenzoate and indole induced culture of *Rba. sphaeroides* OU5 when compared to the control had no major differences (Data not shown). However, in the native profiling, there was a difference observed with respect to some of the colored proteins (Fig 36, 37). The studies pertaining to the in-gel indole assay of colored proteins and the data of HPLC (Fig 39) and GC-MS (Fig 40) analysis indicated the indole tagging nature of the colored proteins. Such colored proteins were also observed in some of the oxygenic phototrophic bacteria and are commonly referred as orange carotenoid proteins (OCPs). However, OCPs were not reported from anoxygenic phototrophic bacteria and the observation of such colored proteins in the present study indicate the possibility of OCPs in *Rba. sphaeroides* OU5 also.

OCPs are orange carotenoid proteins; there are water-soluble proteins unlike pigment carotenoids, which are fat-soluble. OCPs are well studied in plants as a protein of 32 kD (Deruere *et al*., 1994). In cyanobacteria these are of 35 kD and they function in photooxidation (Kerfeld *et al*., 2004a), and it contains a single non-covalently bound keto-carotenoid, 3-hydroxyechinone (Holt and Krogmann, 1981; Wu and Krogmann, 1997; Kerfeld, 2004a, b). OCPs were found to vary, with regard to their location in the cells, solubility in water along with usage of detergents and in

the type of carotenoid association. These proteins are highly conserved among cyanobacteria, their structure, amino acid composition and the gene along with their operon is well studied (Wu and Krogmann, 1997; Kerfeld, 2004a, b).

Apart from OCPs, Red carotenoid proteins (RCPs) were reported as a 16 kD proteolytic fragment of the OCP (Wu and Krogmann, 1997; Kerfeld, 2004a, b). Several function for the OCP in photoprotection have been suggested based on its structure and on *invivo* experiments, such as quencher of reactive oxygen species and singlet oxygen species, carotenoids transporter protein and also as a photo protective shield (Kerfeld, 2004a, b) and play a major role in phycobilisome – associated NPQ mechanism (Adjele *et al*., 2006).

In addition to OCPs and RCPs, water-soluble chlorophyll proteins (WSCPs) were reported in higher plants (Christane Reinbothe *et al*., 2004). They differ from normal chlorophyll binding proteins that function in photosynthesis. WSCP family proteins are induced after drought and heat stress as well as after leaf detachment. Their novel role in chlorophyll degradation pathways and their role as pigment carriers during light induced chloroplast development have been proposed (Christane Reinbothe *et al*., 2004). In contrast to these functions our results prove the indole tagging property of OCPs and predict their role in indole detoxification. The SDS profiling of the OCPs have shown the protein bands of 110, 77.6, 53.5, 35.5 kD (Fig 38).

4.9 **Indole terpenoid esters have phytohormonal activity**

Indole and its derivatives are antimicrobial (Matsuda *et al*., 1990; Babalola, 1998). In adition the molecules like indole acetic acid and indole propionic acid; the natural auxins, esters of indole acetic acid and indole myo-inositol esters were reported to have phytohormonal activity (Domalgski *et al*., 1987). The result from fig

44 to 46 indicates the phytohormonal activity of indole terpenoid esters. These esters have induced early and profused rooting in tissue culture plants at very low concentrations in comparison to the standards of IAA and NAA. The production of indole esters was reported in plants (Domagalski *et al*. 1987, Chisnell, 1984) and by chemical synthesis (Katayama, 2000). However, microbial synthesis of indole terpenoid esters was reported recently (Sunayana *et al*., 2005a, b).

4.10 **Are indole terpenoid esters the products of detoxification?**

Microorganisms developed different strategies to combat stress induced by the xenobiotics and detoxification is one such process developed by few microorganisms. The conjugation mechanism is one among the detoxification reactions in which, molecules of metabolic intermediates like sugars, amino acids and small peptides conjugate with the xenobiotic molecules and are eliminated from the cell (De Ann, 1998). In microorganisms and plants this process is carried out in two or three different phases, which includes the conjugation and localization reactions. In *Rba. sphaeroides* OU5 this phenomenon is observed (Fig 52), in which the molecules like 2-aminobenzoate, which is probably a physiological xenobiotic, is transformed to an indole derivative by conjugation with fumarate in phase I. While in the phase II, the indole thus produced, might be localized by terpenoid conjugation and excreted out of the cell. The catabolic phase is the phase III in which the degradation of indole terpenoid esters occurs resulting in the formation of catechol like products. This process of detoxification probably helps the cell to over come the toxicity of 2-aminobenzoate, which was supplemented to the cells at physiologically elevated concentrations.

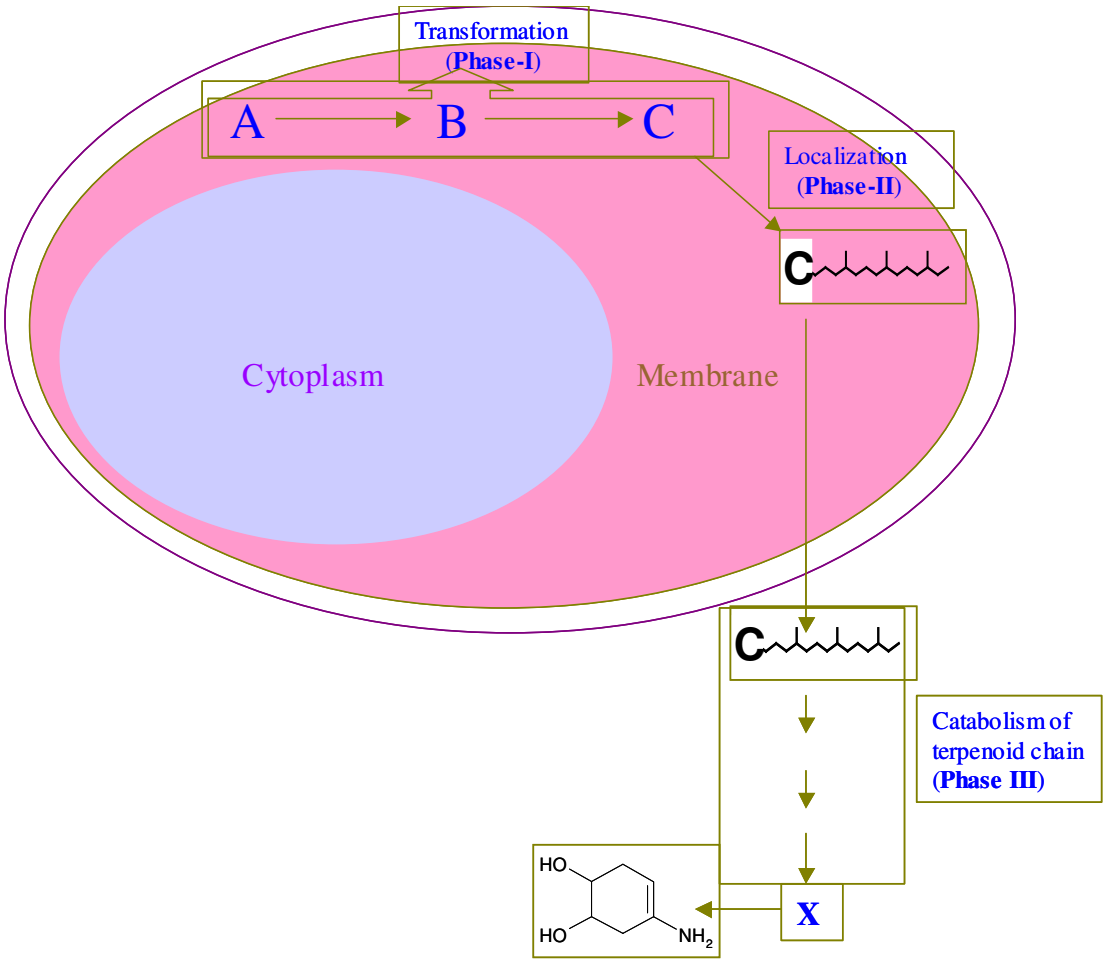


Fig 52: Diagrammatic representation of over all process of 2-aminobenzoate metabolism in *Rba. sphaeroides* OU5.

Conclusions

- *Rhodobacter sphaeroides* OU5 has a doubling time of about 8h in malate and ammonium chloride medium.
- *Rba. sphaeroides* OU5 lacks the ability to utilize 2-aminobenzoate for growth. However, it transforms 2-aminobenzoate to indole and the process is light dependent.
- Indole production was observed by both growing and resting cells, maximum indole yield was 0.7 mM from 1 mM 2-aminobenzoate.
- Indole production was optimized for cell density (OD_{660} 0.4-0.7) and 2-aminobenzoate concentration (0.7-1.0 mM).
- With LC-MS metabolite profiling, various metabolites of 2-aminobenzoate transformation were identified.
- Twenty-nine metabolites were isolated, purified and characterized by various spectroscopic analyses as indole terpenoid esters.
- Indole terpenoid esters varied in their side chain attachment. And based on the masses and on the α, β oxidations of the terpenoid side chains, two putative pathways of indole terpenoid degradation are proposed.
- Indole terpenoid esters can be produced from other aromatic compounds like aniline and tryptophan.
- Indole production from externally supplemented 2-aminobenzoate by *Rba. sphaeroides* OU5 was fumarate dependent.
- Work with cell free extracts has demonstrated the presence of two different pathways of indole synthesis in *Rba. sphaeroides* OU5, i.e. classical PRPP dependent and the novel fumarate dependent pathways.
- The fumarate dependent pathway appears to be membrane bound and a ~64 kD protein catalyzing the transformation of 2-aminobenzoate and fumarate to indole was isolated and characterized.

- 2-Aminobenzoate did not influence the cell size and viability of *Rba. sphaeroides* OU5 at concentrations tested. However, indole influences the culture color, pigments, cell size and culturability.
- Commonly induced antioxidant proteins were not observed in *Rba. sphaeroides* OU5 but indole bound OCPs were detected and their probable role in indole detoxication through esterification was predicted.
- Biological activity was demonstrated with the novel metabolites.

In summary the major findings are:

- A novel light dependent, fumarate mediated pathway of indole synthesis from 2-aminobenzoate was discovered in *Rba. sphaeroides* OU5.
- A novel mechanism of indole detoxification through terpenoid conjugation aided by colored proteins was identified.
- Potential of indole esters as phytohormones needs to be exploited.

Scope for future work:

- Proteomics and genomics of PRPP independent (fumarate dependent) indole biosynthesis, indole esterification and indole terpenoid degradation pathway(s) need to be studied and

- Bioprospecting of the novel metabolites.

List of papers published/under preparation:

1. **Sunayana, M. R.**, Sasikala, Ch., and Ramana, Ch. V., (2005). Production of a novel indole ester from 2-aminobenzoate by *Rhodobacter sphaeroides* OU5. **J. Ind. Microbiol. Biotechnol. 32:**41-45.
2. **Sunayana, M. R**., Sasikala, Ch., and Ramana, Ch. V., (2005). Rhodestrin: A novel indole terpenoid phytohormones from *Rhodobacter sphaeroides*. **Biotechnol. Lett. 27:**1897-1900.
3. Vijay, S., **Sunayana, M. R.**, Ranjith, N. K., Sasikala, Ch., and Ramana, Ch., (2006). Light dependent transformation of aniline to indole esters by the purple bacterium *Rhodobacter sphaeroides* OU5. **Current Microbiol. 52:**413-41.
4. **Sunayana, M. R**., Sasikala, Ch., and Ramana, Ch., (2007). Metabolite profiling and range of indole terpenoid esters produced by *Rhodobacter sphaeroides* OU5 grown on 2-aminobenzoate indicate a novel mechanism of indole degradation [**Appl. Environ. Microbiol. MS under preparation**].
5. **Sunayana, M. R**., Sasikala, Ch., and Ramana, Ch., (2007). Involvement of Orange carotenoid proteins (OCPs) of *Rhodobacter sphaeroides* in the metabolism of 2-aminobenzoate and synthesis of indole esters [**FEMS Microbiol. Lett. MS under preparation**].
6. **Sunayana, M. R**., Sasikala, Ch., and Ramana, Ch., (2007). Novel metabolism of fumarate dependent indole biosynthesis in *Rhodobacter sphaeroides* OU5. [**MS under preparation]**.

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ORIGINAL PAPER

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Production of a novel indole ester from 2-aminobenzoate by *Rhodobacter sphaeroides* OU5

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Abstract Culture supernatants of *Rhodobacter sphaeroides* OU5 grown in the presence of 2-aminobenzoate gave an orange-red color-reaction with Salper's reagent, suggesting the presence of an indole derivative. This production was light-dependent and inducible only with 2-aminobenzoate. Replacement of 2-aminobenzoate with other 2-substituted benzoates did not result in the formation of indole. Fumarate appeared to be the conjugating molecule with 2-aminobenzoate, resulting in the formation of an indole derivative. The purified indole derivative was orange-brown in color, with a yields 0.34 mM from 1 mM 2-aminobenzoate. Infrared analysis suggested an indole ester and ^{1}H NMR analysis indicated an indole carboxylate, esterified with a terpenoid alcohol. The indole ester has a mass of 441 with the molecular formula $C_{27}H_{39}NO_4$. The IUPAC name of the compound is (3 *E*,5 *E*)-14-hydroxy-3,7,11-trimethyl-3,5-tetradecadienyl 2-(hydroxymethyl)-1 *H*-indole-3-carboxylate; and the common name given to this compound is sphestrin.

Keywords Aromatic hydrocarbons · Photobiotransformation · Indole-ester · 2-aminobenzoate · *Rhodobacter sphaeroides* OU5

Introduction

Purple non-sulfur bacteria metabolize a few low-molecular-weight aromatic hydrocarbons for growth under phototrophic, anoxic [20] or microxic [19] conditions. These include nitro, halogenated, carboxylated, phenyl alkanoated, methoxylated and hydroxylated benzenes and a few *N*-substituted heterocyclic aromatic hydrocarbons [20]. Although most of these compounds support growth, as carbon and/or nitrogen source, a few are simply photobiotransformed [20]; and indole photobiotransformation by *Rhodobacter sphaeroides* OU5 is one such transformation extensively studied by our group [14–16].

On the one hand, indole degradation is of major environmental concern because of pollution problems [15] and on the other, these compounds are of industrial (dyes), pharmaceutical (antioxidants) and agricultural (phytohormones) importance [14]. Indole is a known antimicrobial compound [7] and its effect (along with other *N*-heterocyclic aromatic hydrocarbons) on a few purple non-sulfur bacteria has been studied [17]. When indole was used as sole source of carbon or nitrogen, its degradation by *Rba. sphaeroides* OU5 resulted in the formation of 2-aminobenzoate [15]. In contrast, indole was biotransformed to various derivatives in the presence of other organic compounds (precursors) [14]. In the presence of serine and other precursors, tryptophan was the major product [14], while indole-3-acetate was the product of tryptophan photobiodegradation [16]. Recent studies indicated the synthesis of indole itself from 2-aminobenzoate by *Rba. sphaeroides* OU5, in a light- and tricarboxylic acid cycle-dependent transformation [12]. In this paper, we report the photoproduction of a novel indole ester by *Rba. sphaeroides* OU5 from the precursor, 2-aminobenzoate.

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Materials and methods

Organism and growth conditions

Rba. sphaeroides OU5 (ATCC 49885, DSM 7066) was grown photoheterophically (anaerobic, with light at 2,400 lux) in fully filled (15×150 mm) screw-cap test tubes, using Biebl and Pfennig's [2] mineral medium with

malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen sources, respectively at 30 ± 2°C

Photobiotransformation studies

2-Aminobenzoate (1 mM) was added to the photoheterotrophic growth medium for transformation studies using growing cultures. A logarithmic culture [optical density at 660 nm (OD_{660}) = 0.3] was inoculated (20% v/v) into 15×150 mm screw-cap test tubes, incubated at 30 ± 2°C under illumination (2,400 lux) and harvested at different time-intervals. Photobiotransformation studies using cell suspensions were carried out as described earlier [12].

Bulk cultivation and transformation studies

Each culture was grown in a 2-l reagent bottle containing 1,600 ml of photoheterotrophic medium with malate (22 mM) and ammonium chloride (7 mM) as carbon/e− donor and nitrogen source, respectively. A logarithmically growing culture (400 ml, 20% v/v, OD_{660} = 0.3) was used as the inoculum and the culture was allowed to grow for 48 h under phototrophic (2,400 lux) conditions at 30 ± 2°C. A stock (10 ml) of sterilized 2-aminobenzoate in ethanol (neutralized to pH 7) was added to the culture (giving a final concentration of 1 mM) and after 48 h of incubation, the culture was harvested for analysis.

Isolation and purification of the indole compound

After incubation, the culture was centrifuged (16,000 *g*, 10 min) and the supernatant was used for the purification of indole. Culture supernatant (2 l) was extracted thrice with ethyl acetate; and the ethyl acetate extract was completely dried in a rota-vaporator at 40°C and resuspended in benzene. The benzene extract was loaded onto an 18×600 mm column packed with silica (80–120 mesh) and eluted with different organic solvents. Elution was done first with benzene, followed by methanol and finally with methanol:water (1:1 v/v). Two compounds were separated during benzene elution (B1, B2). Sample B1 gave a positive indole test with the indole reagents and was used for identification after its confirmation as a single spot on a thin-layer chromatograph (TLC), using three different solvents (benzene, benzene:chloroform at 1:1 v/v, benzene:ethyl acetate at 8.5:1.5 v/v).

Assay

Growth was followed as an increase in OD_{660} [15]. Since 2-aminobenzoate interferes with the Ehrlich reagent (60 mg *para*-diaminobenzaldehide in 10 ml of 3 N H_2SO_4), Salper's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% perchloricacid) was used for the routine analysis of indole production [14]. Purified indole suspended in deuterated chloroform ($CDCl_3$) was used for ^1H NMR, using a Brucker AC200 analyzer at 200 MHz. UV analysis of the sample suspended in ethyl acetate was done in a Spectronic Genesis2 spectrophotometer. Infrared (IR) analysis was done on a Shimadzu FT/IR 8300 and mass analysis was done using a VG 70-70H mass analyzer. The following web sites were used for interpretation of the structure: http://www.chem.unipotsdam.de/tools, http://www.spectroscopy

Controls

The purity of the culture was routinely checked before and after assay on nutrient agar plates. Cultureless controls were used to check against any possible photochemical transformations. An ethanol control was also run in parallel with all experiments. The purity of the isolated compound was confirmed with more than one solvent system on TLC plates. All results were highly reproducible, were performed in duplicates and were repeated at least twice.

Results and discussion

Nitrogen-substituted aromatic hydrocarbons like nitrobenzene [18] and nitrophenols [3] support the growth of the purple bacteria *Rhodopseudomonas palustris* and *Rba. capsulatus*, respectively. Although purple bacteria are not known to utilize 2-aminobenzoate for growth as a source of nitrogen or carbon, its transformation into an indole derivative has been reported [12]. The transformation of 2-aminobenzoate to indole by *Rba. sphaeroides* OU5 was a light-dependent process and such transformation could not be observed under either aerobic or anaerobic dark conditions (data not shown). The culture supernatant of *Rba. sphaeroides* OU5 grown photoheterotrophically in the presence of 2-aminobenzoate gave an orange-red color with Salper's or Ehrlich reagent, confirming the presence of an indole derivative. Both of these reagents give characteristic colors with indoles [1]. Pure indole gives a cherry-red color with Ehrlich reagent, due to the formation of a rose indole. The formation of an orange-red color with the culture supernatant of *Rba. sphaeroides* OU5 indicates the presence of an indole derivative rather than indole itself. Replacing 2-aminobenzoate with other 2-substituted benzoates, viz. 2-hydroxybenzoate (salicylate), 2-carboxybenzoate (pthalate), 2-nitrobenzene, 2-chlorobenzene, did not yield an indole, suggesting substrate-specificity for this indole synthesis. In addition, the presence of these 2-substituted benzoates, along with 2-aminobenzoate, inhibited indole photoproduction, probably due to competitive inhibition. Indole production was not observed in the absence of 2-aminobenzoate either with

Table 1 Effect of organic substrates on the photoproduction of indole by *Rba. sphaeroides* OU5. Results are averages from four replicates using cell suspensions assayed after 48 h of anaerobic incubation with light (2,400 lux). *FAA* Fluoroacetate, + indole photoproduction promoted, − indole photoproduction inhibited

Substrate (0.1% w/v or v/v)	Indole photoproduction (mM)	
	Without FAA	With FAA
Control (without substrate)	0.1	0.1
Pyruvate	0.1	0.1
Acetate	0.1	0.0 (−)
α-Ketoglutarate	0.1	0.1
Succinate	0.3 (+)	0.3 (+)
Fumarate	0.4 (+)	0.4 (+)
Malate	0.4 (+)	0.4 (+)
Oxaloacetate	0.2 (+)	0.2 (+)
Fructose	0.1	0.0 (−)
Dextrose	0.1	0.0 (−)

or without carbon substrate, suggesting the inducible nature of indole production by the precursor, rather than due to carbon starvation as observed in *Escherichia coli* [21]. In addition, indole production was always associated with the disappearance of 2-aminobenzoate in the medium, thus confirming the production of indole through 2-aminobenzoate biotransformation. The presence of a few tricarboxylate cycle (TCA) intermediates enhanced the yield of indole; and this was not inhibited by fluoroacetate (Table 1). The inhibition of indole photoproduction with fluoroacetate from acetate, dextrose and fructose supports the role of TCA cycle intermediates in photobiotransformation. Among all the TCA intermediates, fumarate was suggested as the conjugating molecule, based on the molar yields [12]. In the present study, cinnamate completely inhibited the photoproduction of indole, while benzoate had no effect. These results further confirm that fumarate is the conjugating molecule with 2-aminobenzoate, since cinnamate is an analogue of fumarate.

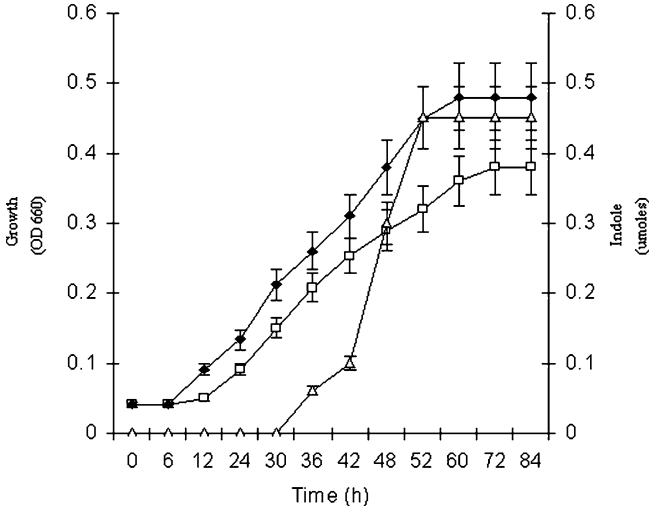

Fig. 1 Photoproduction of indole from 2-aminobenzoate simultaneous with growth by *Rba. sphaeroides* OU5. *White triangles* Indole photoproduction, *white squares* photoheterotrophic growth in the presence of 2-aminobenzoate, *black diamonds* photoheterotrophic growth in the absence of 2-aminobenzoate, *umoles* micromoles. The error bars represent data from four replicates

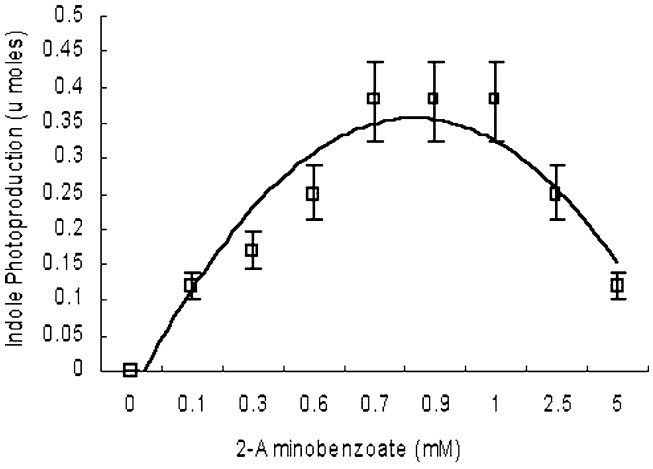

Fig. 2 Effect of 2-aminobenzoate concentration on the photoproduction of indole by *Rba. sphaeroides* OU5. Results expressed are for cell suspensions in the presence of different concentrations of 2-aminobenzoate and were assayed after 48 h. Error bars represent data from four replicates

Figure 1 shows indole photoproduction by growing cells of *Rba. sphaeroides* OU5. A lag period of 8 h for indole production was observed with cell suspensions [12]. In contrast, with growing cultures, indole production could be detected only after a lag period of 36 h (Fig. 1), which later stopped with the cessation of growth. Indole photoproduction increased with increasing concentrations of 2-aminobenzoate, reaching an optimum at 0.7–1.0 mM (Fig. 2).

The benzene extract from the culture supernatant yielded two compounds, B1 and B2. Sample B1 gave an orange-red color with both the Salper's and Ehrlich reagents, suggesting that the compound is an indole

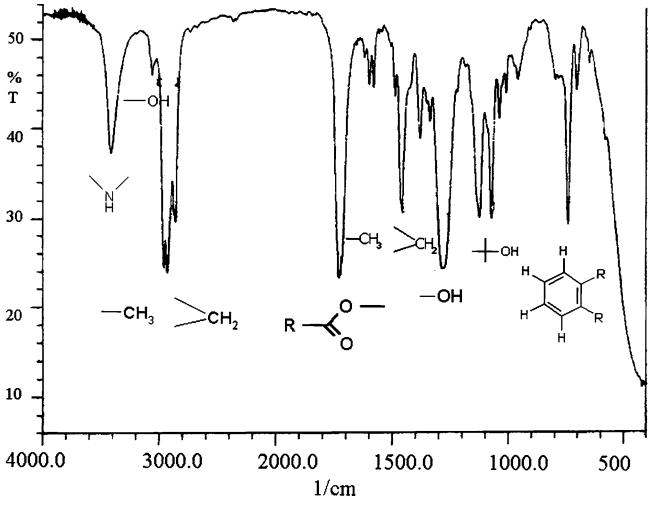

Fig. 3 IR spectral analysis of the purified compound

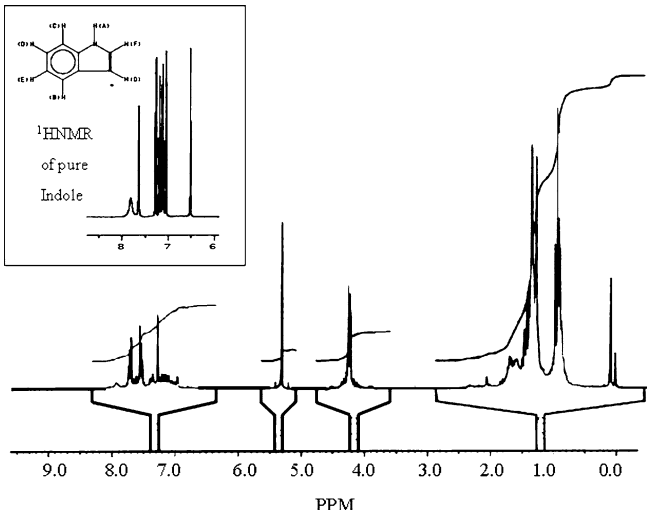

Fig. 4 ^{1}H NMR analysis of the isolated compound in $CDCl_3$. *Insert* Standard indole ^{1}H NMR spectra

derivative. The compound was found to be pure and had R_F values of 0.64, 0.63 and 0.7 with benzene, benzene:chloroform (1:1 v/v) and benzene:ethyl acetate (8.5:1.5 v/v), respectively. The UV absorption maxima of the compound in ethyl acetate were 251, 275 and 320 nm, indicating the basic indole nucleus, which has an absorption around 275 nm [11]. IR analysis of the compound (Fig. 3) confirmed the presence of an indole nucleus with a peak (1/cm) at 3,417 corresponding to N–H and another at 740 corresponding to the benzene ring. Further, the compound had a very strong ester peak at 1,728. Strong peaks at 2,960–2,850 and medium peaks at 1,470–1,430 strongly suggested that there are $–CH_3$ and $–CH_2$ groups. Strong peaks at 1,070, 1,276 and a weak peak at 3,049 indicated the presence of hydroxyl groups.

Figure 4 shows the ^{1}H NMR spectra of the isolated compound in $CDCl_3$. The basic skeleton of indole matches the standard indole ^{1}H NMR (Fig. 4, insert) and that reported earlier [21]. Figure 5 shows the mass fragmentation of the compound, with a molecular mass of 441. The molecular formula of the compound is $C_{27}H_{39}NO_4$ and the molecular composition of C 73.44 and H 8.9 matches the elemental analysis: C 72.24 and H 8.58. The IUPAC name of the compound is (3E,5E)-14-hydroxy-3,7,11-trimethyl-3,5-tetradecadienyl 2-hydroxymethyl-1H-indole-3-carboxylate. We gave this compound the common name of sphestrin (*sph*aeroides *est*e *r in*dole). Sphestrin appears orange in benzene and yellow in ethyl acetate; and the completely dried sample is orange-brown. The compound has no prominent peak in the visible region of its absorption spectrum. The aliphatic moiety of the structure (Fig. 5) suggests that a terpenoid is a conjugating molecule with the indole acid. The change in the color is a typical feature of the spheroidene (yellow-colored carotenoid) found in this organism, which on oxidation forms spheroidenone (yellow-red) [9]. Sphestrin appears to be an intermediate formed due to the degradation of the terpenoid moiety of an indole ester. The yield of sphestrin is 0.34 mM out of the total indole yield of 0.7 mM from 1 mM of 2-aminobenzoate consumed. The sphestrin yield is not in stoichiometry with the consumed precursor because of the other intermediates of the pathway, whose analysis is in progress.

The present study demonstrates the ability of *Rba. sphaeroides* OU5 to biotransform 2-aminobenzoate into an indole ester in a light-dependent process. However,

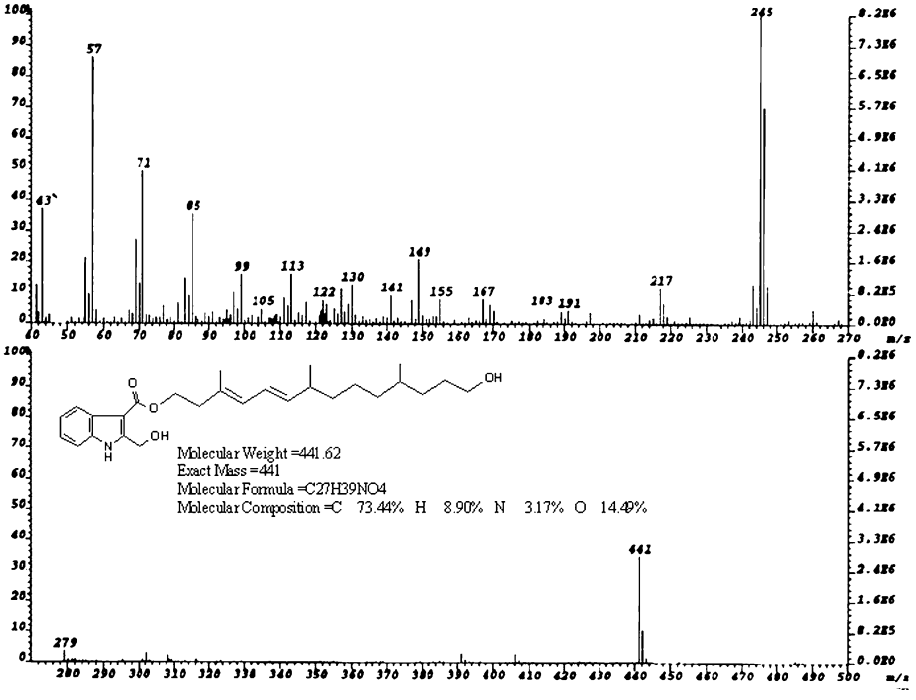

Fig. 5 Mass spectral analysis of the compound, showing the final structure and molecular composition

since the indole identified in an earlier study [12] (2,3-dihydroxyindole) and the present indole ester are different, it has to be investigated whether these are the intermediates of a single pathway or the products of independent pathways. For this, a further detailed study using larger volumes of the culture and also looking for the intermediates within the cell is warranted. The first conjugating molecule with 2-aminobenzoate appears to be fumarate. Fumarate is known to detoxify several methylated benzenes [8], forming benzyl succinate, which on beta-oxidation and further metabolism is mineralized. Thus, the isolated indole ester may be a product of such a process of detoxification where 2-aminobenzoate conjugates with fumarate to form an indole carboxylate (a hypothetical compound, requiring evidence). This may further conjugate with a terpenoid alcohol (probably spheroidene), resulting in the formation of an indole ester. These results also suggest that the organism is trying to convert aromatic polar compounds (2-aminobenzoate, hypothetical indole carboxylate) into a more non-polar compound (sphestrin). As a result, the compounds do not interfere with the cellular metabolic activities and cause membrane toxicity, as do indoles [7]. This could be the reason why *Rba. sphaeroides* OU5 is able to grow even in the presence of 2-aminobenzoate (Fig. 1), while no growth occurs on indole [17]. Further work is required to confirm the hypothetical intermediate, indole carboxylate, the complete esterifying alcohol and the pathway leading to the formation of sphestrin. This work also has an interesting biotechnological significance, since indole esters are very useful and effective in inhibiting cyclo-oxygenase-2 [13] and thus have an application in cancer therapy. Since many indole esters are synthesized only through chemical routes [10], the present process can be an alternative for chemical synthesis. So far, indole esters have been reported only from plant systems [4, 5] and this is the first report of their production by a microbial system, although microbially produced indole carboxylates were reported earlier [6].

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

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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.

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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

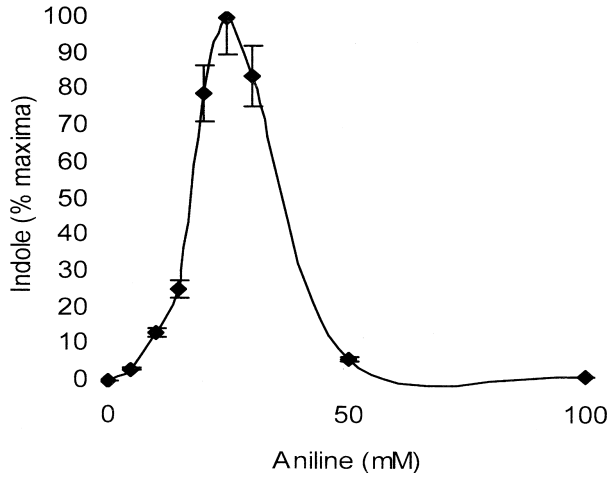


Fig. 1. Effect of aniline concentration on photoproduction of indole by *R. sphaeroides* OU5. Results expressed are the average values of two independent experiments done in duplicate using 48-h photoheterotrophic cultures (100 % indole = 0.16 mM).

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.

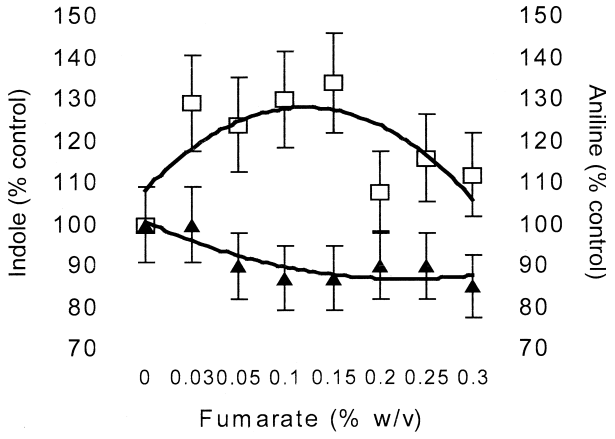


Fig. 2. Photoproduction of indole by *R. sphaeroides* OU5 with various concentrations of fumarate. 100 % control = Indole (□-□) 0.05 mM; Aniline (▲-▲) 25 mM.

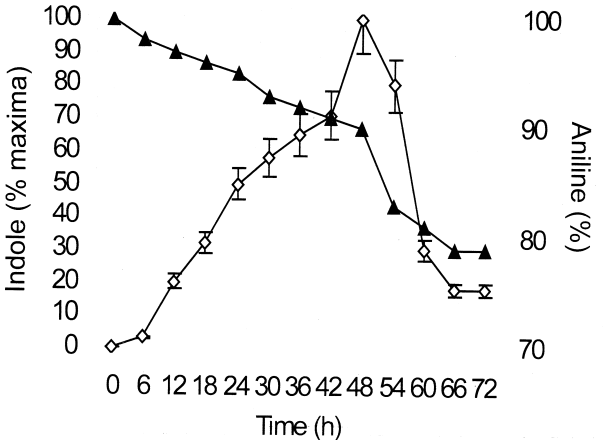


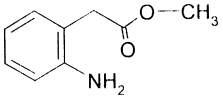
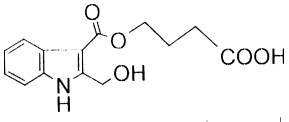
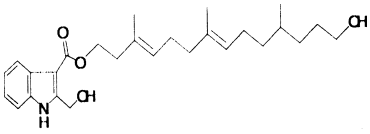
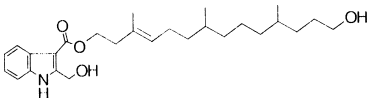
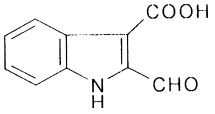
Fig. 3. Kinetics of indole production and aniline consumption by *R. sphaeroides* OU5. Results expressed are the average value of experiments done in duplicate using 48-h photoheterotrophic culture 100 % Indole (◇-◇) = 0.16 mM, 100 % Aniline (▲-▲) = 25 mM.

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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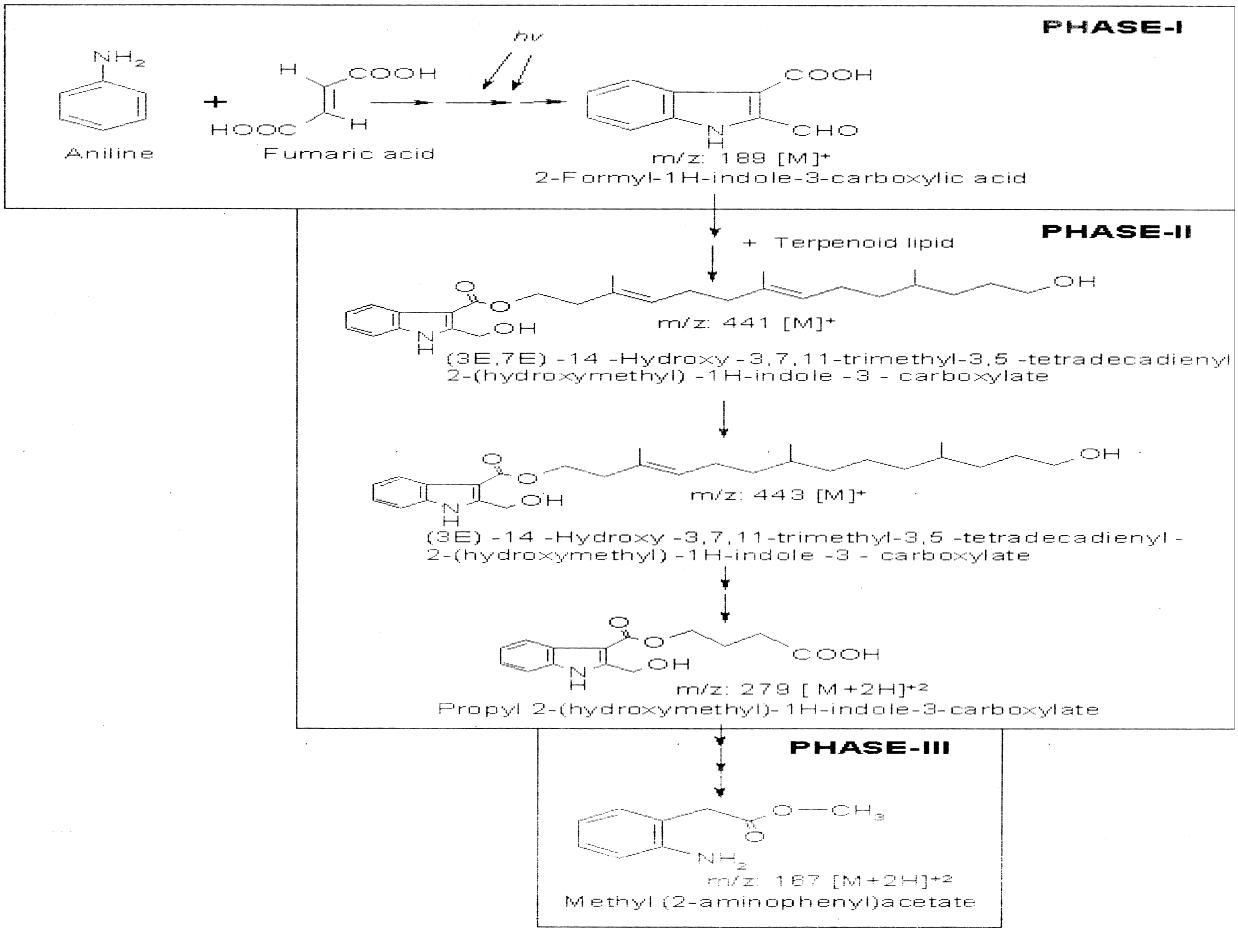

Fig. 4. Postulated pathway of aniline photometabolism by *R. sphaeroides* OU5.

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Rhodestrin: a novel indole terpenoid phytohormone from *Rhodobacter sphaeroides*

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Abstract

A new phytohormone was isolated as a metabolite of anthranilate photobiotransformation by *Rhodobacter sphaeroides* OU5. It was identified by MS and NMR (^{1}H, ^{13}C) as an indole terpenoid ester [(2E,4E,6E,8E,10E,12E,14E,16E,18E)24-hydroxy-2,6,10,14,19 pentamethyl tetrecosa-2,4,6,8,10,12,14,16,18 nonenyl-2(hydroxy methyl)-1H-indole-3-carboxylate] and is named as rhodestrin. Rhodestrin at 50 nM gave positive test in auxin bioassay and initiated more profuse and early rooting in tissue-cultured plants than other auxins at 5 μM.

Introduction

Anoxygenic phototrophic bacteria are well recognized in biotechnology (Sasikala & Ramana 1995a, b). Like many other bacteria (Costacura & Vanderleyden 1995), a few purple non-sulfur bacteria produce substantial quantities of phytohormones (Sasikala & Ramana 1995a). Such phytohormones include: (4,6-hydroxy-3-methyl-2-trans-2-butylamino)-9-β-D-ribofuranosyl purine [zeatinriboside] from *Rhodospirillum rubrum* (Serdyuk *et al*. 1993), kinetin and zeatin (both cytokinins) from *Rhodobacter sphaeroides* (Kuroda 1990), while auxins, indole 3-acetic acid (Yokoyama 1990, Rajasekhar *et al*. 1999) and indole 3-butyric acid (Yokoyama 1990) from *R. sphaeroides*, are well documented. In addition to the production of hormones, the property of promoting plant growth helped in recognizing them as plant growth promoting rhizobacteria (Sasikala & Ramana 1995b). In the present communication we report a new indole terpenoid ester produced by *R. sphaeroides* with high physiological activity in auxin bioassay. Natural indole esters reported so far are mostly of plant origin and these are the esters of myo-inositol-indole acetates (Domagalski *et al*. 1987, Chisnell 1984) and for the first time we reported recently (Sunayana *et al*. 2005) an indole terpenoid ester (sphestrin) production from anthranilate by *R. sphaeroides*, which had low auxin activity (unpublished data).

Materials and methods

Organism and growth conditions

Rhodobacter sphaeroides OU5 (ATCC 49885, DSM 7066) was grown photoheterotrophically (anaerobic, with light at 2400 lux) in fully filled (15×150 mm) screw-cap test tubes, using Biebl & Pfennig's (1981) mineral medium with malate (22 mM) and ammonium chloride (7 mM) as

carbon and nitrogen sources, respectively at 30 ± 2 °C.

Photobiotransformation studies

Photobiotransformation of anthranilate was studied in cell suspensions as described earlier (Sunayana *et al*. 2005).

Bulk cultivation, solvent extraction, isolation and purification of the indolic compound

Bulk cultivation (2 l) and other growth parameters of *R. sphaeroides* were described elsewhere (Sunayana *et al*. 2005). The culture supernatant (2 l) (pH 7.5) after (12 h) assay was extracted with ethyl acetate and the extract was concentrated under vacuum (crude ethyl acetate extract, – Extract I). The aqueous layer was acidified (pH 4, with 5 M HCl), extracted with ethyl acetate and concentrated under vacuum (acidified ethyl acetate extract – Extract II). Rhodestrin was purified from extract I.

Assay

Growth was followed turbidometrically at 660 nm. Indole was estimated by Ehrlich or Salper's reagent (Sunayana *et al*. 2005). NMR spectrum of the purified compound in 0.6 ml $CDCl_3$ in a 5 mm NMR tube was recorded on Brucker AC200 (200 MHz) spectrometer, operating at 200 and 120 MHz for ^{1}H and ^{13}C, respectively. UV analysis of the sample suspended in ethyl acetate was done in a Spectronic Genesis2 spectrophotometer. Infra-red (IR) analysis was done on a Shimadzu FT/IR 8300 and mass analysis using a Mass VG 70-70H analyzer. Auxin coleoptiles bioassay and rooting effects in tissue culture were done using standard protocols.

Results and discussion

Rhodobacter sphaeroides was unable to grow on anthranilate, either as sole source of carbon or nitrogen (Nanda *et al*. 2000, Sunayana *et al*. 2005). However, it transformed anthranilate in a light-dependent process and, so far, two metabolites have been characterized. A dihydroxylated indole was extracted into ethyl acetate from the culture supernatants of *R. sphaeroides* OU5 grown for 72 h in the light under anaerobic conditions in the presence of anthranilate (Nanda *et al*. 2000), while 48 h incubations yielded an indole terpenoid ester, sphestrin, which was extracted into benzene (Sunayana *et al*. 2005).

TLC analysis of the initial ethyl acetate extract (Extract I) of anthranilate-supplemented culture supernatants of *R. sphaeroides* after 12 h of incubation yielded a bright pink colored spot having an R_F 0.87 (benzene). This compound was different from standard indole (R_F 0.97), sphestrin (R_F 0.64; Sunayana *et al*. 2005) and dihydroxylated indole (R_F 0.96; Nanda *et al*. 2000). The metabolite gave positive test with the indole reagents, thus necessitating its purification, which was achieved on silica gel 120 mesh column chromatography followed by preparative TLC using benzene:chloroform (1:1 v/v) as solvent. After its confirmation as a single spot on TLC, the metabolite was analysed.

Figure 1 gives the structure of the metabolite based on UV, IR, NMR (^{13}C, ^{1}H) and mass analysis. Spectral peaks at 456 and 530 nm, characteristic feature of some carotenoid molecules, reflects pink color of the metabolite. The metabolite is an indole terpenoid ester (Figure 1) and is different in molecular mass and in terpenoid side chain length of the indole ester reported earlier (sphestrin), which had a molecular mass of 441 (Sunayana *et al*. 2005). Our results indicate that these indole esters could be the metabolic intermediates of a pathway, with 595 molecular mass metabolite as an earlier intermediate of 441 and dependent on fumarate and a terpenoid molecule for their biosynthesis. We named this newly isolated metabolite as rhodestrin. The yield of rhodestrin is 12 μM from 200 μM anthranilate.

Phytohormonal activity

Rhodestrin gave a positive coleoptiles test in an auxin bioassay (data not shown). It promoted root initiation of mulberry in tissue culture. The compound induced profuse rooting (Figure 2) at 50 nM in comparison to three other auxins at 5 μM. Further, root initiation was observed with in 6–8 days with rhodestrin-treated plants, while with the other auxins it was observed only after 12–15 days. Moreover, the root-to-shoot ratio was high in the plants supplemented with rhodestrin.

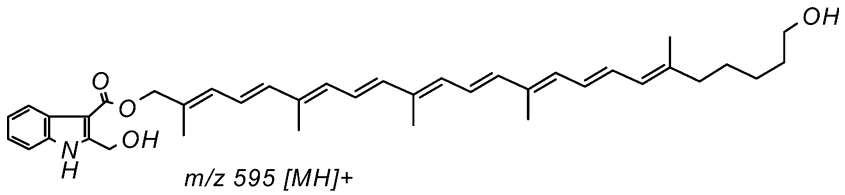

Fig. 1. Structure of the metabolite isolated from the culture supernatant during photobiotransformation of 2-aminobenzoate by *Rhodobacter sphaeroides* OU5. The metabolite is a pink amorphous solid, UV (MeOH) λ_{max} (log $\in$) 2 21 (3.05), 270 (2.64), 470 (2.20), 536 (2.51) nm; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.8-7.2(m, 4H), 4.21(m, 13H), 2.5(m, 4H), 1.6-0.88(m, 23H); ^{13}C NMR ($CDCl_3$, 120 MHz)d 150.2 (C-2′), 102.5 (C-3′), 125.4 (C-4′), 116.4 (C-5′), 119.7 {C-6′, C-7′), 110.2 (C-8'), 130.9 (C-9′), 50 (C-10′), 172.8 (C-11′), 60.4 (C-1),134.7 (C-2), 124.1 (C-3), 121.9 (C-4), 120.7 (C-5), 134.6 (C-6), 124.0 (C-7), 121.8 (C-8), 120.6 (C-9), 132.1 (C-10),121.7 (C-11), 116.8 (C-12), 110.0 (C-13), 132.0 (C-14), 121.6 (C-15), 116.9 (C-16), 116.7 (C-17), 110.1 (C-18), 135.7 (C-19), 32.8 (C-20), 29.0 (C-21), 28.5 (C-22), 29.7 (C-23), 55.8 (C-24), 21.8 (C_{19}-Me,), 20.5 (C_2-Me), 14.1 (C_6-Me), C_{10}-Me, C_{14}-me); EIMS m/z 595$[M]^+$; anal. C 79.24% and H 8.58%, calcd. for $C_{39}H_{49}NO_4$, C78.62%, H8.29%. IUPAC name of the metabolite is (2E,4E,6E,8E,10E,12E,14E,16E,18E)24-hydroxy-2,6,10,14,19 pentamethyltetrecosa-2,4,6,8,10,12,14,16,18nonenyl-2(hydroxymethyl)-1H-indole-3-carboxylate which, we named as rhodestrin (rhod = from *Rhodobacter*; estr = ester; in = indole).

Fig. 2. Photographs showing the phytohormonal activity of rhodestrin (6) along with other phytohormones (Bar-see Photograph 1 = 0.5 cm). 1 = control (with out hormones); 2 = naphthalene acetic acid (NAA) (5 μM); 3 = 2,4 dichloro phenoxy acetic acid (2,4-D) (5 μM); 4 = crude extract I; 5 = acidified crude extract II and 6 = rhodestrin (0.05 μM). Details of crude extract I, acidified crude extract II and purified rhodestrin are given in Material and methods. Photographs pertain 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.

On the contrary, the phytohormonal activity of sphestrin was negligible, while there was no activity observed with dihydroxy indole, which were both reported previously from *R. sphaeroides* OU5 (Nanda *et al.* 2000, Sunayana *et al.* 2005).

Conclusions

Indole esters have a greater phytohormonal activity than their corresponding acids in the auxin bioassay (Katayama 2000). Our data confirms this and opens scope for their production from anoxygenic photosynthetic bacteria. While many other bacteria produce auxins, like indole 3-acetic acid from tryptophan (Patten & Glick 1996), the novelty of *R. sphaeroides* OU5 is to produce indole terpenoid esters directly from anthranilate.

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