J Ind Microbiol Biotechnol (2005) 32: 41–45
DOI 10.1007/s10295-004-0193-y

ORIGINAL PAPER

M. R. Sunayana · Ch. Sasikala · Ch. V. Ramana

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

Received: 7 October 2004 / Accepted: 16 October 2004 / Published online: 22 February 2005

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.

M. R. Sunayana · C. V. Ramana (✉)
Department of Plant Sciences, School of Life Sciences,
University of Hyderabad, P.O. Central University,
Gachibowli, Hyderabad, 500046, India
E-mail: chvrsl@uohyd.ernet.in

C. Sasikala
Environmental Microbial Biotechnology Laboratory,
Center for Environment, Institute of Science and Technology,
JNT University, Kukatpally, Hyderabad, 500072, India

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.

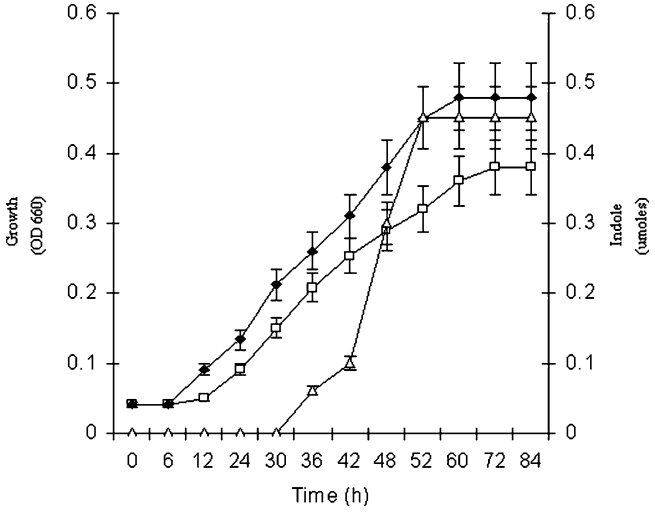

Fig. 1 Photoproduction of indole from 2-aminobenzoate simultaneous with growth by *Rba. sphaeroides* OU5. *White triangles* Indole photoproduction, *white squares* photoheterotrophic growth in the presence of 2-aminobenzoate, *black diamonds* photoheterotrophic growth in the absence of 2-aminobenzoate,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

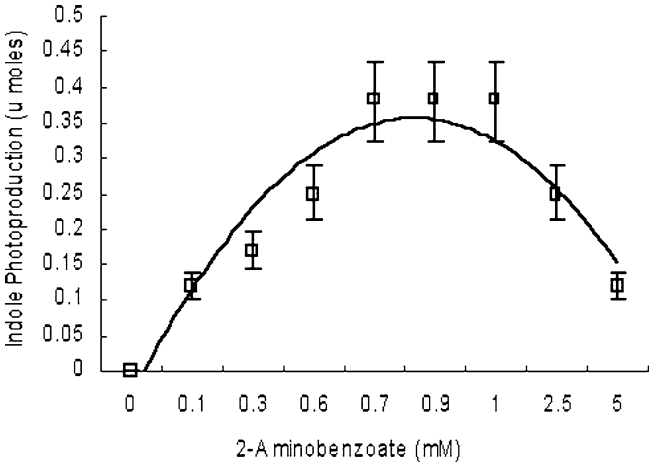

Fig. 2 Effect of 2-aminobenzoate concentration on the photoproduction of indole by *Rba. sphaeroides* OU5. Results expressed are for cell suspensions in the presence of different concentrations of 2-aminobenzoate and were assayed after 48 h. Error bars represent data from four replicates

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

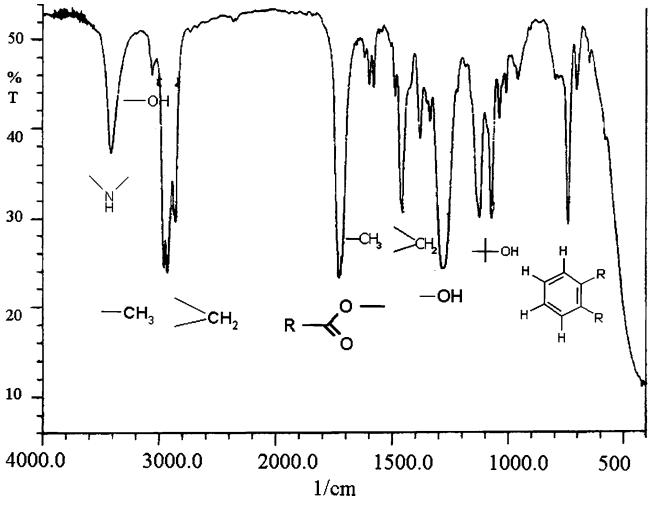

Fig. 3 IR spectral analysis of the purified compound

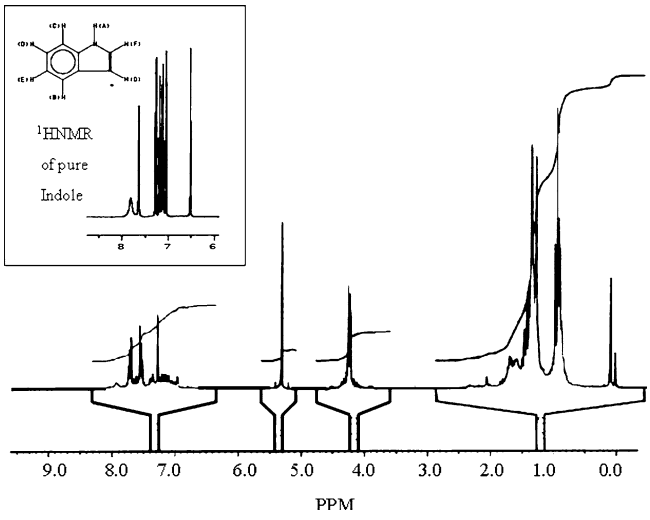

Fig. 4 ^{1}H NMR analysis of the isolated compound in $CDCl_3$. *Insert* Standard indole ^{1}H NMR spectra

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,

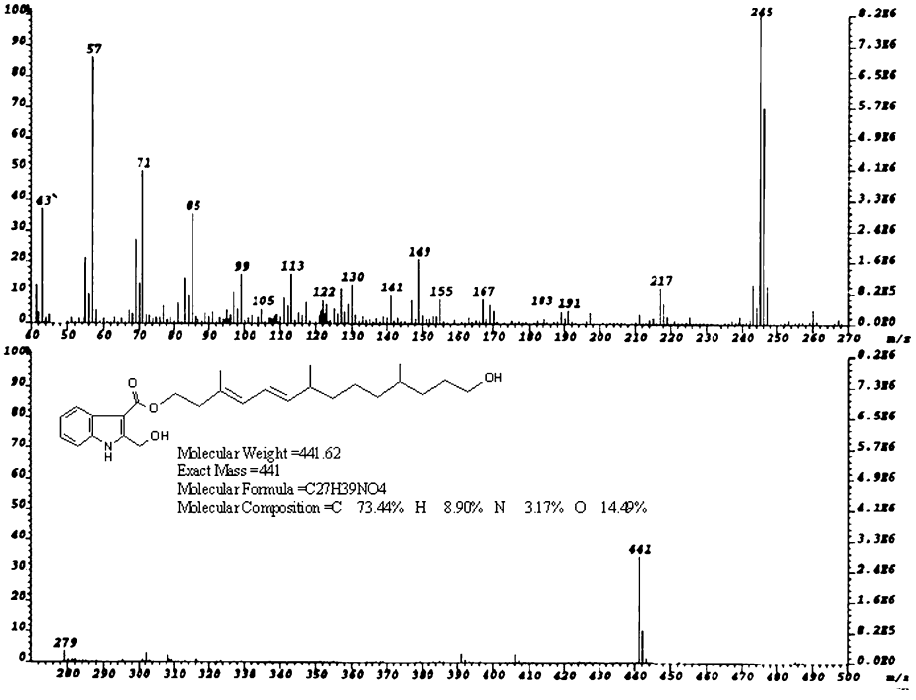

Fig. 5 Mass spectral analysis of the compound, showing the final structure and molecular composition

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].

Acknowledgements M.R.S. and Ch.V.R./Ch. S. thank the Council of Scientific and Industrial Research (CSIR), Government of India, for their support through a fellowship (JRF) and research grant, respectively. Ch.V.R. and Ch.S. thank the Department of Ocean Development (DOD) and the Department of Biotechnology, Government of India, for financial support, Professor D. Basavaiah for extending facilities for ^{1}H NMR and IR, IICT for mass analysis and the Department of Science and Technology, Government of India, for FIST support.

References

1. Anderson GM (1975) Quantification of tryptophan metabolites in rat feces by thin-layer chromatography. J Chromatogr 105:323–328
2. Biebl H, Pfennig N (1981) Isolation of members of the family Rhodospirillaceae. In: Starr MP, Stolp H, Truper HG, Balows A, Schlegel HG (eds) The prokaryotes. Springer, Berlin Heidelberg New York, pp 167–273
3. Blasco R, Castillo F (1997) Characterization of 2,4-dinitrophenol uptake by *Rhodobacter capsulatus*. Pest Biochem Physiol 58:1–6
4. Chisnell JR (1984) Myo-inositol esters of indole-3-acetic acid are endogenous components of *Zea mays* L. shoot tissue. Plant Physiol 74:278–283
5. Domagalski W, Schulze A, Bandurski RS (1987) Isolation and characterization of esters of indole-3-acetic acid from the liquid endosperm of the horse chestnut (*Aesculus* species). Plant Physiol 84:1107–1113
6. Garbe TR, Kobayashi M, Shimizu, N, Takesue N, Ozawa M, Hideaki Y (2000) Indolyl carboxylic acids by condensation of indoles with α-keto acids. J Nat Prod 63:596–598
7. Garbe TR, Kobayashi M, Yukawa H (2000) Indole-inducible proteins in bacteria suggest membrane and oxidant toxicity. Arch Microbiol 173:78–82
8. Heider J, Spormann AM, Beller HR, Widdel F (1999) Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol Rev 22:459–473
9. Imhoff JF (1992) Taxonomy, physiology and general ecology of anoxygenic phototrophic bacteria. In: Nicholas HM, Carr NG (eds) Photosynthetic prokaryotes. Plenum, New York, pp 53–92
10. Katayama M (2000) Synthesis and biological activities of 4-chloroindole-3-acetic acid and its esters. Biosci Biotechnol Biochem 64:808–815
11. Mollan RC, Harmey MA, Bonnelly DMX (1973) UV spectra of indoles in strong sulphuric acid. Phytochemistry 12:447–450
12. Nanda Devi, Sasikala Ch, Ramana Ch V (2000). Light-dependent transformation of anthranilate to indole by *Rhodobacter sphaeroides* OU5. J Ind Microbiol Biotechnol 24:219–221
13. Olgen S, Nebioglu D (2002) Synthesis and biological evaluation of *N*-substituted indole esters as inhibitors of cyclo-oxygenase-2 (COX-2). Farmaco 57:677–683
14. Rajasekhar N, Sasikala Ch, Ramana ChV (1998) Photobiotransformation of indole to its value-added derivatives by *Rhodobacter sphaeroides* OU5. J Ind Microbiol Biotechnol 20:177–179
15. Rajasekhar N, Sasikala Ch, Ramana ChV (1999) Photoproduction of indole-3-acteic acid by *Rhodobacter sphaeroides* from indole and glycine. Biotechnol Lett 21:543–545
16. Rajasekhar N, Sasikala Ch, Ramana ChV (1999) Photometabolism of indole by purple non-sulfur bacteria. Ind J Microbiol 39:39–44
17. Rajasekhar N, Sasikala Ch, Ramana ChV (2000) Toxicity of *N*-containing heterocyclic aromatic compounds and their utilization for growth by a few purple non-sulfur bacteria. Bull Environ Contam Toxicol 65:375–382
18. Ramana ChV, Sasikala Ch (2002) Light dependent reductive degradation of nitrobenzene by *Rhodopseudomonas palustri*. Ind J Exp Biol 42:229–232
19. Roldan MD, Blasco R, Caballero FJ, Castillo F (1998) Degradation of *p*-nitrophenol by the phototrophic bacterium *Rhodobacter capsulatus*. Arch Microbiol 169:36–42
20. Sasikala Ch, Ramana ChV (1998) Biodegradation and metabolism of unusual carbon compounds by anoxygenic phototrophic bacteria. Adv Microbiol Physiol 39:339–377
21. Wang D, Ding X, Rather PN (2001) Indole can act as an extracellular signal in *Escherichia coli*. J Bacteriol 183:4210–4216