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ORIGINAL PAPER

L-Tryptophan catabolism by *Rubrivivax benzoatilyticus* JA2 occurs through indole 3-pyruvic acid pathway

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Abstract *Rubrivivax benzoatilyticus* JA2 utilizes L-tryptophan as the sole source of nitrogen for growth, and it has a doubling time of ~11 h (compared to 8 h with ammonium chloride). With cell free extracts in the presence of 2-oxoglutarate, indole-3-pyruvic acid, indole-3-acetaldehyde, indole-3-acetic acid, isatin, benzaldehyde, gallic acid and pyrogallol were identified using high performance liquid chromatography (HPLC) and liquid chromatography–mass spectroscopy (LC–MS) analysis. The conversion of L-tryptophan into indole 3-pyruvic acid and glutamate by an enzyme aminotransferase was confirmed and the catabolism of indole-3-pyruvic acid via side chain oxidation followed by ring oxidation, gallic acid and pyrogallol were confirmed as metabolites. In addition, the proposed pathway sequential conversion of indole-3-pyruvic acid to the end product of pyrogallol was identified, including an enzymatic step that would convert isatin to benzaldehyde by an enzyme yet to be identified. At this stage of the study, the enzyme tryptophan aminotransferase in *R. benzoatilyticus* JA2 was demonstrated.

Keywords L-Tryptophan · Indole 3-pyruvic acid · Indole 3-acetic acid · Tryptophan aminotransferase · *Rubrivivax benzoatilyticus* JA2

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Introduction

The metabolism of aromatic compounds by a few purple non-sulfur anoxygenic phototrophic bacteria has been reviewed (Sasikala and Ramana 1998). A total genome analysis of *Rhodopseudomonas palustris* has helped in predicting at least five different aromatic ring cleavage pathways in this organism (Larimer et al. 2004). In contrast to this aromatic ring cleavage capability in *R. palustris*, benzene ring cleavage has not been reported thus far within the genus *Rhodobacter*; however, *Rhodobacter capsulatus* can transform nitro aromatic compounds in a light dependent process (Blasco and Castillo 1992). On the other hand, in *Rubrivivax benzoatilyticus* JA2, a few incubation experiments have resulted in the transformation of aromatic compounds such as aniline (Vijay et al. 2006), anthranilate (Nanda et al. 2000; Sunayana et al. 2005a, b), L-tryptophan (Ranjith et al. 2007a) and

L-phenylalanine (Ranjith et al. 2007b; Sáez et al. 1999) into indole esters, ethers, phenol esters or hydroxylated benzenes. Recent studies (Ranjith et al. 2007a) have also indicated a novel pathway of L-phenylalanine/L-tyrosine catabolism in *R. benzoatilyticus* OU5, which occurs through the intermediate 3,4-dihydroxyphenylalanine (DOPA). In the presence of 2-oxoglutarate as an amino acceptor, the downstream of DOPA proceeds through the intermediate 3,4-dihydroxyphenyl pyruvate (DOPP), catalyzed by DOPA-aminotransferase. 3,4-Dihydroxyphenylpropionic acid (DPPA) was the intermediary in the absence of 2-oxoglutarate catalyzed by DOPA-reductive deaminase. This is in contrast to our understanding of L-phenylalanine and L-tyrosine catabolism by *Rhodobacter sphaeroides* OU5 (Ranjith et al. 2007b) and *R. capsulatus* (Sáez et al. 1999), with the exception of the production of indole 3-acetic acid (Rajasekhar et al. 1999a) and rhodethrin (Ranjith et al. 2007a) from L-tryptophan. The catabolism of L-tryptophan has not been subject of detailed study thus far in purple anoxygenic bacteria. This report provides evidence for the existence of a pathway of L-tryptophan catabolism in *R. benzoatilyticus* JA2 with intermediate indole 3-pyruvic acid that involves a key enzyme, L-tryptophan aminotransferase (an aromatic aminotransferase) (Lee et al. 2004; Zuther et al. 2008) and a novel step isatin to the benzaldehyde conversion enzyme (which needs to be established).

Materials and methods

Organism and growth conditions

The research group responsible for this study (Rajasekhar et al. 1998; 1999a, b, c; 2000; Nanda et al. 2000; Sunayana et al. 2005a, b; Vijay et al. 2006; Usha et al. 2007; Ranjith et al. 2007a, b, 2008, 2010) has worked extensively with a phototrophic purple nonsulfur bacterium, *R. sphaeroides* OU5; however, molecular characterization of the OU5 strain had not been conducted earlier. Using the 16S rRNA gene sequence analysis (Anil Kumar et al. 2007), the OU5 strain is clustered within the *Betaproteobacteria* with its closest relative (99.6% sequence similarity), *R. benzoatilyticus* JA2^T (ATCC BAA-35^T = JCM 13220^T = MTCC 8097^T) (Ramana et al. 2006). Based on polyphasic characterization, the OU5 strain is confirmed as *R. benzoatilyticus*. *R. benzoatilyticus* JA2 was grown photoheterotrophically (anaerobic/light) (2,400 lux) in fully filled screw cap test tubes (10 × 100 mm) or in reagent bottles (250/2000 ml) on a mineral medium (Biebl and Pfennig 1981) with malate (22 mM) and ammonium chloride (7 mM) as the carbon and nitrogen sources, respectively, at 30 ± 2°C. For growth on L-tryptophan, ammonium chloride was replaced with the L-tryptophan (1 mM) when used as the nitrogen source, or malate was replaced when used as the carbon source, or both malate and ammonium chloride were replaced when used as the sole source of carbon and nitrogen. The amount of time required for generation derived from the slope of the log phase of growth was measured by bacterial cells versus time as the bacterial cell numbers at 660 nm. All the metabolites were purchased from Sigma-Aldrich HPLC grade, and the data provided in this study was reproducible or consistent with the best of our knowledge.

Preparation of cell-free extracts

Cells grown on L-tryptophan (2 L) for 6 h were harvested by centrifugation (16,000*g* for 15 min at 4°C), the pellet was washed twice with 50 ml of a wash buffer [with 0.05 M potassium phosphate buffer (pH 7.5)], and resuspended in the same buffer. Cells were lysed by sonication, using a MS-72 probe at 4°C (Bandelin, Germany make, model-UW 2070) to complete the cell lyses after eight to nine cycles. The cell homogenate was centrifuged (16,000*g* for 30 min at 4°C) and the clear supernatant obtained was used as a source of the enzymes.

Enzyme activities

Tryptophan aminotransferase (EC. 2.6.1.27)

This was determined by measuring the loss of L-tryptophan and the formation of indole 3-pyruvic acid and glutamate using HPLC. At a final volume of 1.0 ml (0.05 M potassium phosphate buffer pH 7.8), the reaction mixture contained 25 µmole/ml of 2-oxoglutarate, 100 µmole/ml of L-tryptophan, 25 µmole/ml of pyridoxal-5-phosphate (PLP) (as a co-factor) and an appropriate amount of partially purified cell extract. The reaction was carried out in

eppendorf tubes (1.5 ml) and incubated at 30°C. The reaction was terminated after 5 min (unless otherwise mentioned) and proteins were denatured by acidification with 10% (w/v; 100 mg ml^{-1}) trichloroacetic acid (TCA). The sample was centrifuged (12,000*g* for 5 min at 4°C) and analyzed by injecting 20 µl of clear supernatant in HPLC. A unit (U) of enzymatic activity is defined as the amount of the enzyme that catalyzed the formation of 1 µmole of indole 3-pyruvic acid, mg $protein^{-1}$ min^{-1}.

Tryptophanase (EC 4.1.99.1)

Tryptophanase enzyme assay was conducted according to the procedure described by Snell, 1975. In a final volume of 1 ml (0.05 M potassium phosphate buffer pH 7.8), the assay mixture contained 100 µmole/ml of L-tryptophan and 20 µmole/ml of pyridoxal-5-phosphate (PLP), with an appropriate volume of cell free extract added. The reaction was carried out at 37°C for five to 6 min. The assay mixture was chilled in an ice bath and extracted into 2.0 ml of ethyl acetate. A 1.0 ml sample of the ethyl acetate was assayed for indole using kovacs reagent (paradimethylamino benzaldehyde).

Extraction of metabolites

After the enzyme assay was conducted, the metabolites were extracted with 3 ml of ethyl acetate and further processing was done, as described earlier (Ranjith et al. 2007b). Metabolites were identified based on their molecular masses.

Analytical methods

Growth was measured turbidometrically (O.D) at 660 nm. L-tryptophan and total indoles (Kupfer and Atkinson 1994), proteins (Bradford 1976), pyruvate (Umbreit et al. 1964), amino acids (0.2% (w/v) ninhydrin in acetone) and ammonia (Solórzano 1969) were analyzed using standard protocols.

HPLC analysis was performed at room temperature using a Shimadzu SPD-10AVP (Shimadzu LC 20AT Japan) isocratic liquid chromatography. Methanol plus water (1:1) was used as a solvent at 0.5 ml min^{-1}, Luna 5 µm RP-C_{18} (2) 100A (250 × 4.6 mm), and the compounds were detected using UV–vis at 200 nm for glutamate and 2-oxoglutarate or 254 nm for indoles and other aromatic compounds.

LC–MS analysis was done using a Shimadzu LC-MS-2010A analysis, performed at 40°C for the LC column oven and at 85°C for the MS ionization chamber. Methanol plus water (1:1) was used as a solvent at 0.5 ml min^{-1}, Luna 5 µm C_8 (2) 100A was the column (250 × 4.6 mm), and the compounds were detected (LC) at 254 nm. The column effluent from the LC was nebulized into an electro spray ionization (ESI) or atmospheric pressure chemical ionization (APCI) region under N_2 gas in order to generate the molecular masses in both positive and negative modes.

Results and discussion

Growth, simultaneous utilization of L-tryptophan, production and identification of indoles

Rubrivivax benzoatilyticus JA2 could grow on L-tryptophan only under phototrophic conditions and when used as the sole source of nitrogen. The compound could not support growth as a source of carbon or of carbon and nitrogen. The organism had a doubling time of ~11 h (compared to 8 h with ammonium chloride) during the growth of the culture, with 100% consumption of L-tryptophan observed within 6 h (Fig. 1a). Meanwhile, the concentration of indoles increased stoichiometrically within 6 h and later decreased. This indicates that the identified metabolite products include the indole derivatives. When using the cell free extracts, L-tryptophan consumption and indoles production (Fig. 1b) was observed at various time intervals.

Cell free extracts

Presence of 2-oxoglutarate as an amino acceptor

With the cell free extract of *R. benzoatilyticus* JA2, nearly 90% of L-tryptophan consumption was observed within 1 min, while the formation of compounds which were collectively called indoles was simultaneously increased (Fig. 1b). The metabolite products identified include the indole derivatives, Indole 3-pyruvic acid (m/z 202), indole 3-acetaldehyde (m/z 159), indole

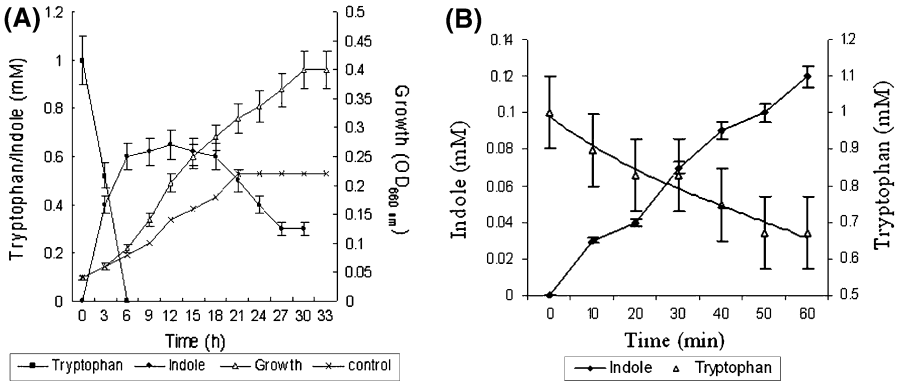

Fig. 1 Growth, L-tryptophan utilization and the production of indoles by whole cells (**a**), and L-tryptophan consumption and production of total indoles by cell free extract enzyme assay (**b**) of *Rubrivivax benzoatilyticus* JA2. L-tryptophan (1 mM) was used as the sole source of nitrogen in the presence of malate (22 mM) as the carbon source and incubated under phototrophic (2,400 lux) conditions at 30 ± 2°C. The data represents the means of triplicate experiments, and the *error bars* indicate 95% confidence intervals

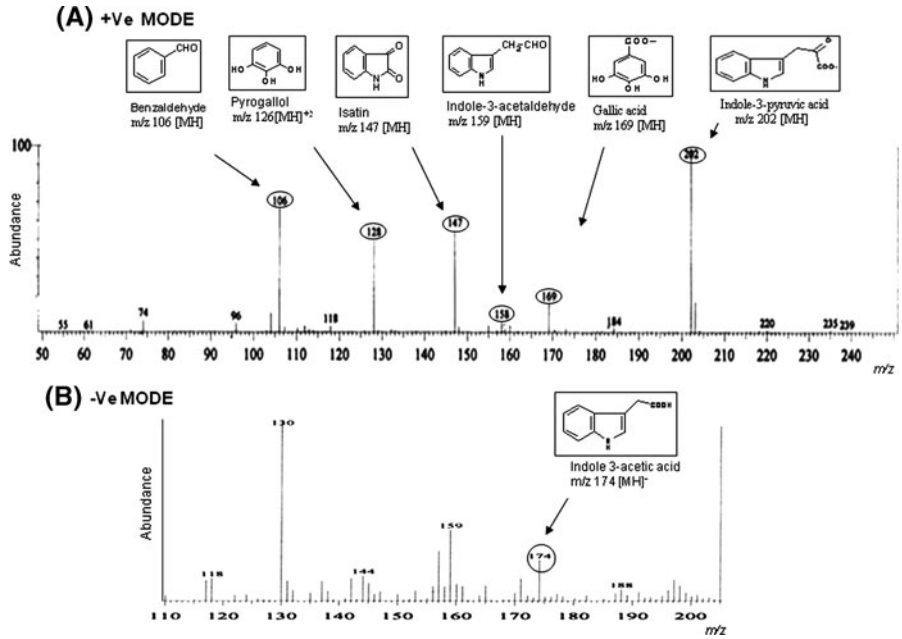

Fig. 2 Liquid chromatography–mass spectroscopy (LC–MS) profiling of the partially purified cell free extract enzyme assay supernatant performed in the presence of L-tryptophan and 2-oxoglutarate (amino acceptor). **a** Metabolites were identified by ESI–MS positive mode. **b** Metabolite was identified by ESI–MS negative mode. The molecular masses of the encircled metabolites identified were, namely, indole-3-pyruvic acid, indole-3-acetaldehyde, indole-3-acetic acid, isatin, benzaldehyde, gallic acid and pyrogallol, respectively

3-acetic acid (m/z 175) and isatin (m/z 147), as well as the benzene derivatives, benzaldehyde (m/z 106), gallic acid (m/z 170) and pyrogallol (m/z 126). These were identified from the assay supernatant of the cell free extracts, based on their molecular masses (Fig. 2a and b). Indole-3-pyruvic acid ($R_t = 16.9$), isatin ($R_t = 5.8$), benzaldehyde ($R_t = 8.9$), gallic acid ($R_t = 4.4$) and pyrogallol ($R_t = 5.0$) were confirmed using their respective standards. However, indole-3-acetaldehyde could not be detected in the HPLC analysis, due to the low concentration of metabolite, we are able to identified using LC–MS analysis.The metabolites are sequential intermediates of a single pathway, as proposed in the studies that used cell free extracts where L-tryptophan (Fig. 3a1), IAA (Fig. 3b1), isatin, gallic acid or benzaldehyde (Fig. 3c1) were used as substrates and the absence of substrates was used as the control measure (Fig. 3a, b, c). The above products were further identified in this study by LC–MS analysis using enzymatic assays (Fig. 2a, b). The results indicate that the catabolism of L-tryptophan in the presence of 2-oxoglutarate by

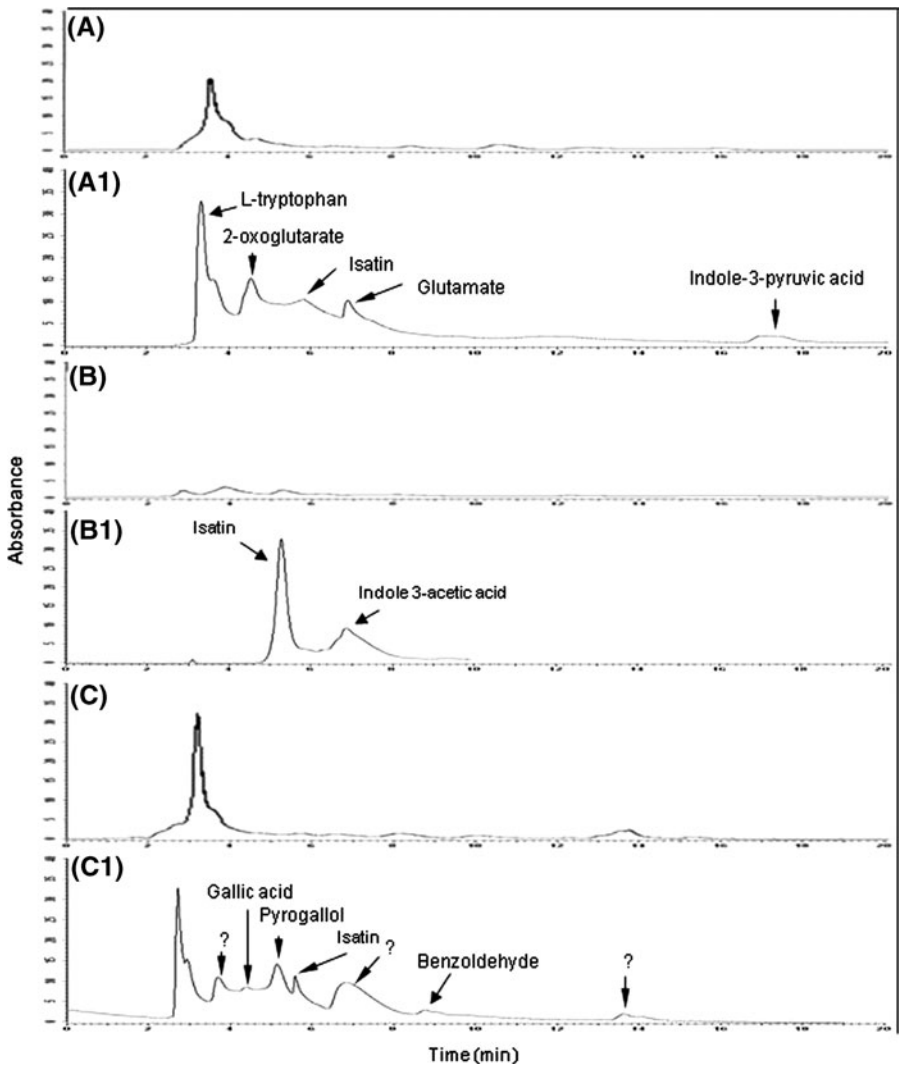

Fig. 3 HPLC chromatograms showing the conversion of L-tryptophan to indole derivatives and benzene derivatives by cell free extracts of *Rubrivivax benzoatilyticus* JA2. Assay performed in the presence and absence of 2-oxoglutarate, where L-tryptophan (**a1**), Indole-3-acetic acid (**a1**) and isatin (**a1**) were used as precursors and control chromatograms (**a**, **b** and **c**) without substrates

R. benzoatilyticus JA2 proceeds through the indole-3-pyruvate pathway, leading to the synthesis of indole-3-acetic acid with the intermediate indole-3-acetaldehyde (Truelsen 1972; Lee et al. 2004; Ryu and Patten 2008; Reineke et al. 2008). The putative proposed pathway of L-tryptophan catabolism in the presence of 2-oxoglutarate by *R. benzoatilyticus* JA2, which was hypothesized based on the sequential enzymatic products, is shown in Fig. 4. However, the proposed pathway confirmation requires an isotopic labeling analysis, and this work is currently in progress in our laboratory. The L-tryptophan aminotransferase (EC 2.6.1.27) activity was measured in the presence and absence of 2-oxoglutarate using HPLC and LC–MS analysis, and the enzyme products indole-3-pyruvic acid and glutamate was confirmed (Figs. 2a, 3a1, 5). The tryptophan aminotransferase is well documented in plant, animal and microbial systems (Stanley et al. 1984; Koga et al. 1992; 1994; Zuther et al. 2008). The enzyme specific activities was done by using spectroscopy analysis with the substrate; L-tryptophan, L-phenylalanine, L-tyrosine, L-histidine, L-glutamate and DL-alanine/L-glycine were 1.0, 0.06, 0.2, 0.05, 0.6 and 0.2 μmol product.mg protein^{-1} min^{-1}, respectively.

In conclusion, many aerobic/anaerobic bacteria commonly produce and degrade a number of indoles (Gu and Berry 1991; Chang et al. 2003). *R. benzoatilyticus* JA2 produced indole derivatives in this study, indole and DL-alanine could not be found from

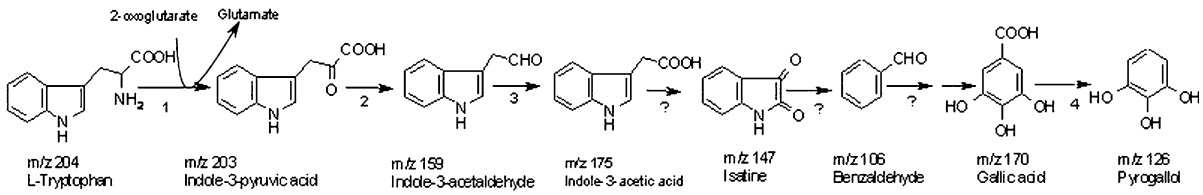

Fig. 4 Proposed intracellular catabolism of L-tryptophan by *Rubrivivax benzoatilyticus* JA2. The proposed pathway for L-tryptophan catabolism is based on experimental evidence observed in the present study and the evidence for some of the enzymes was also obtained from the literature. *1* = Tryptophan aminotransferase (EC 2.6.1.27; (Koga et al. 1994; Stanley et al. 1984); *2* = Indole-3-pyruvicacid decarboxylase (EC 4.1.1.74; (Koga et al. 1992; Ryu and Patten 2008), *3* = Aldehyde dehydrogenase (EC 1.2.3.1; Basse et al. 1996)/aldehyde oxidase (EC 1.2.3.1; Basse et al. 1996); *4* = Gallate decarboxylase (EC 4.1.1.59; Zeida et al. 1998); *?* = enzymes not identified

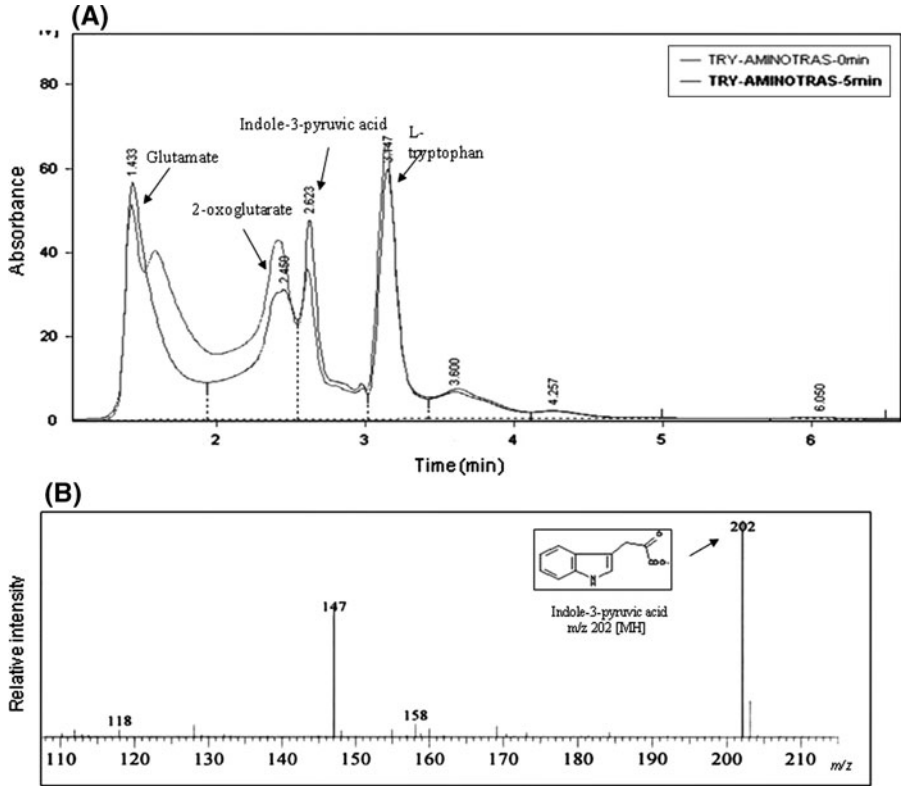

Fig. 5 HPLC chromatogram showing the conversion of L-tryptophan to indole-3-pyruvic acid and glutamate by *Rubrivivax benzoatilyticus* JA2. HPLC analysis of the substrates/products was performed at room temperature using the Shimadzu SPD-10AVP isocratic system. Methanol plus water (1:1) was used as a solvent at 1.5 ml.min^{-1}, Luna 5 μC 8 (2) 100A column (250 × 4.6 mm) and the compounds were detected using UV–VIS detector at 200 nm. The reaction times (R_t; min) for L-tryptophan (R_t = 3.1 min), 2-oxoglutarate (R_t = 2.4 min), indole-3-pyruvic acid (R_t = 2.6 min) and glutamate (R_t = 1.4 min) are shown. The compounds were identified by conducting a comparison of the peak for the corresponding standard

L-tryptophan, thus probably lack the enzyme tryptophanase (EC 4.1.99.1), which is the key enzyme for L-tryptophan catabolism. In addition, the study indicates the presence of L-tryptophan catabolism through indole-3-pyruvic acid, and isatin to benzaldehyde conversions were not reported in any organisms to the best of our knowledge. More experimental evidence is required to prove the existence of the hypothetical pathway. This may contribute significantly to scientific knowledge of aromatic amino acids metabolism

in photosynthetic purple nonsulfur bacterium *R. benzoatilyticus* JA2.

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