L-Phenylalanine catabolic multitasking and metabolic shift in *Rubrivivax benzoatilyticus* JA2

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

By

M. Lakshmi Prasuna

(Regd. No. 08LPPH04)

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

June 2014

University of Hyderabad
(A central university established in 1974 by Act of Parliament)
HYDERABAD-500046

DECLARATION

I **M. Lakshmi Prasuna**, hereby declare that this thesis entitled **"L-Phenylalanine catabolic multitasking and metabolic shift in *Rubrivivax benzoatilyticus* JA2"** submitted by me under the guidance and supervision of **Prof. Ch.V. Ramana** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

M. Lakshmi Prasuna
(Ph.D student)

Supervisor
Prof. Ch.V. Ramana

University of Hyderabad

(A central university established in 1974 by Act of Parliament)

HYDERABAD-500046

CERTIFICATE

This is to certify that the thesis entitled **"L-Phenylalanine catabolic multitasking and metabolic shift in *Rubrivivax benzoatilyticus* JA2"** is a record of bonafide work done by **Ms. M. Lakshmi Prasuna**, a research student for Ph.D programme in Department of Plant Sciences, School of Life Sciences, University of Hyderabad, under my guidance and supervision.

Prof. Ch. V. Ramana

Supervisor & Head

Department of Plant Sciences

DEAN

School of Life Sciences

Acknowledgements

I extend my gratitude to my supervisor Dr. Ch. V. Ramana for his constant guidance and support throughout my doctoral research.

I thank the present and former Heads of the Department of Plant Sciences, Prof. Ch. V. Ramana, Prof. A. R. Reddy for the Departmental facilities.

I thank Prof. A.S. Raghavendra, Dean, School of Life Sciences, and former Deans, Prof. M. Ramanadham, Prof. R.P. Sharma, Prof. Aparna Dutta Gupta for allowing me to use the general facilities of the school.

I thank my Doctoral Committee members Prof. Appa Rao Podile and Dr. Sarada Tetali for their valuable suggestions.

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

I would like to thank Prof. Appa Rao Podile and Dr. J. S. S. Prakash for extending their lab facilities.

I am grateful to all my teachers, who are instrumental in shaping up my life.

I am grateful to Dr Srinivas who taught me ABCs of biology.

I thank all the faculty of School of Life Sciences.

I would like to thank present and former labmates for their cooperation throughout my research.

I thank all the research scholars of School of Life Sciences for their cooperation.

I thank all my friends at University of Hyderabad for making my stay in the campus delightful.

The help and cooperation of the non-teaching staff is highly acknowledged.

I acknowledge the CSIR and UPE for financial aid. DST-FIST, UGC-SAP, DBT-CREEB are acknowledged for school and departmental facilities.

I am extremely grateful to my parents, my brothers and family members for their unwavering love, support and patience throughout my life.

Above the all, I vow my thanks to Almighty, for providing me everything.

I sincerely apologies to all whom I may have hurt knowingly or unknowingly.

Finally I thank one and all for their support.

M. Lakshmi Prasuna…


Dedicated
to my
Grandmother

Table of contents

ABSTRACT

Bacteria thrive under diverse environmental conditions, capable of metabolising wide range of organic compounds for their growth. Aromatic compounds are the most abundant class of organic substrates, among which aromatic amino acids serve as growth substrates for bacteria. Some bacteria can degrade/transform both aliphatic/aromatic molecules and can utilise as carbon/nitrogen source. Phototrophic *Proteobacteria* are metabolically versatile group of bacteria utilises organic hydrocarbons for growth as carbon/nitrogen source. *Rubrivivax benzoatilyticus* JA2 a metabolically versatile phototrophic betaproteobacterium, able to grow under oxic/anoxic conditions. *Rubrivivax benzoatilyticus* JA2 can utilise aromatic amino acids as sole source of nitrogen for growth and can transform into wide range of value added compounds. Transformation of L-phenylalanine into rubrivivaxin under anoxic conditions was reported in *R. benzoatilyticus* JA2. However, in depth study of L-phenylalanine metabolism under anoxic/oxic conditions was not done. Hence, an attempt was made to know the insights of L-phenylalanine metabolism under anoxic/oxic conditions in *R. benzoatilyticus* JA2 with metabolomics approach.

Rubrivivax benzoatilyticus JA2 utilised L-phenylalanine as sole source of nitrogen but not as carbon source for its growth and simultaneously produced phenolic compounds under anoxic/oxic conditions. Phenylpyruvic acid, phenyllactic acid, phenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylpyruvic acid, 4-hydroxyphenylacetic acid, benzoic acid, 4-hydroxybenzaldehyde were identified using HPLC, LC-MS/MS analysis. Volatile aryl metabolites like phenylacetaldehyde, 2-phenylethanol, benzaldehyde, benzylalcohol were identified based on GC-MS. Stable isotope studies and enzyme activities revealed the L-phenylalanine catabolism in *R. benzoatilyticus* JA2, which includes Ehrlich's pathway, N-acetyl-L-phenylalanine, tyrosine dependent melanin biosynthesis and some anthocyanin-like pigments production. Purified blue pigment was identified based on mass 537 [M^-] *m/z* obtained from mass spectral analysis and characteristic chemical shifts observed in Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared (FTIR) spectral analysis and it is identified as an anthocyanin-like pigment. The purified blue pigment with absorption maxima at 560 nm represent the anthocyanin-like compound which is similar to plant derived anthocyanin pigments. Pigments production from L-phenylalanine was observed only under oxic conditions but not in anoxic conditions. These results suggested oxygen dependent catabolism of L-phenylalanine and possible metabolic variations to oxic and anoxic conditions.

Further, metabolic responses of *R. benzoatilyticus* JA2 was studied through metabolomics approach.

Metabolome was studied with GC-MS based metabolomics for control (without L-phenylalanine), L-phenylalanine fed cultures under anoxic/oxic conditions. Multivariate statistical analysis such as hierarchical cluster analysis (HCA), principal component analysis (PCA), partial least square discriminant analysis (PLS-DA) explained the variations in the metabolome under anoxic/oxic conditions. Relative fold change analysis revealed that total 69 variables/metabolites significantly differentially regulated and they were responsible for the variations in the metabolome. The biosynthetic pathways of aryl metabolites, carbohydrates, fatty acids, hydrocarbons were significantly influenced under oxic/anoxic conditions. Relative fold change revealed, total 29 aryl metabolites were significantly regulated under anoxic/oxic conditions in presence of L-phenylalanine. Among which 12 metabolites were up-regulated under oxic conditions indicating that these are oxygen dependent metabolites. As the metabolome suggests phenylpyruvic acid, phenyllactic acid, phenylacetic acid, N-acetyl-L-phenylalanine were up-regulated under anoxic conditions and some hydroxy aryl metabolites were up-regulated under oxic conditions. Flux analysis of L-phenylalanine deciphered that ~70% of L-phenylalanine was channelized into Ehrlich's pathway under anoxic condition whereas only 30% channelized to Ehrlich's pathway and remaining was channelized into pigments production under oxic conditions. Shift in the metabolism was explained clearly through metabolomics study. 4-hydroxyphenylpyruvic acid was high under anoxic condition where it converted into pyomelanin when transferred to oxic conditions. *De novo* synthesis of 4-hydroxybenzoic acid was also influenced in presence of L-phenylalanine under anoxic/oxic conditions. Production of 4-hydroxybenzoic acid was inhibited in presence of glyphosate inhibitor. Fatty acid metabolism significantly influenced with saturated/unsaturated ratios and branched-chain fatty acids were high under anoxic condition. Metabolic responses were revealed the metabolic shift to changing conditions and the existence of multiple catabolic pathways explained the catabolic multitasking of L-phenylalanine under anoxic/oxic conditions.

LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µs	Microsecond
1D	One dimensional
2D	Two dimensional
4-HBAld	4-Hydroxybenzaldehyde
4-HPPY	4-Hydroxyphenylpyruvic acid
ACN	Acetonitrile
APB	Anoxygenic photosynthetic bacteria
ATCC	American Type Culture Collection
BLAST	Basic local alignment search tool
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
Blu P1	Blue pigment one
Blu P2	Blue pigment two
Brwn P	Brown pigment
CD	Circular dichroism spectroscopy
$CDCl_3$	Deuteriated chloroform
CID	Collision induced dissociation
COSY	Correlation spectroscopy
CP/TOSS	Cross polarization/total suppression of spinning sidebands
Da	Dalton
DHICA	5,6-Dihydroxyindole-2-carboxylic acid
DHI	5,6-Dihydroxyindole
DMSO	Dimethyl sulfoxide
DOPA	3,4-Dihydroxyphenylalanine
DOPAATS	3,4-Dihydroxyphenylalanine transaminase
DOPAPP	3,4-Dihydroxyphenylalanine phenylpyruvic acid
DOPARDA	3,4-Dihydroxyphenylalanine reductive deaminase

EPR/ESR	Electro paramagnetic resonance/Electron spin resonance
ESI	Electrospray ionization
eV	Electron volt
FAME	Fatty acid methyl esters
FTIR	Fourier transform infra-red spectroscopy
g	Gram
GC-MS	Gas chromatography-mass spectrometry
h	Hour
HAL	Histidine ammonia-lyase
HCA	Hierarchical cluster analysis
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HMG	Homogentisic acid
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantun coherence spectroscopy
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS	Liquid chromatography-mass spectrometry
L	Litre
M^-	Molecular ion mass in negative mode.
m/z	Mass-to-charge ratio
M^+	Molecular ion mass in positive mode
MEGA 5	Malocular evolutionary genetics analysis
MeOD	Deuteriated methanol
MeOH	Methanol
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
mT	Millitesla
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCBI	National center for botechnology information

NIST	National institute of standards and technology
nm	Nanometre
NMR	Nuclear magnetic resonance
Org P1	Orange pigment one
Org P2	Orange pigment two
PAA	Phenylacetic acid
PAL	Phenylalanine ammonia-lyase
PAld	Phenylacetaldehyde
PCA	Principal component analysis
PDA	Photodiode array
Phe	L-Phenylalanine
Pin P	Pink pigment
PLA	Phenyllactic acid
PLP	Pyridoxal-phosphate
PLS-DA	Partial least squares discriminant analysis
PPM	Parts per million
PPY	Phenylpyruvic acid
QTOF	Quadrupole time-of-flight
Rpm	Rotations per minute
R_t	Retention time
s	Second
SEM	Scanning electron microscopy
TAL	Tyrosine ammonia-lyase
TMCS	Trimethylchlorosilane
TOCSY	Total correlation spectroscopy
Tris	Tris (hydroxymethyl) aminomethane
Trp	Tryptophan
Tyr	Tyrosine
v/v	Volume per volume
VIP	Variable importance in projection
w/v	Weight per volume

INTRODUCTION

1.0 Introduction

1.1 Aromatic amino acid metabolism in bacteria

Bacteria are the most diverse group of microorganisms and they thrive in diverse habitats. Bacteria uses wide range of organic compounds as carbon source for their growth and can metabolise both aliphatic and aromatic hydrocarbons. Next to carbohydrates, aromatic hydrocarbons are the most widely distributed group of organic molecules (Teufel et al., 2010). Lignin polymer, monolignols, phenolic compounds and aromatic amino acids are a few abundantly found natural aromatic compounds in nature (Carmona et al., 2009). Aromatic amino acids are one such group of natural aromatic compounds (Carmona et al., 2009; Teufel et al., 2010). These are of plant and microbial origin, found in nature mainly due to plant and animal decay, and also by release of plant root exudates. Copious amounts of *meta*-tyrosine found in the root exudates of fine fescue cultivar *Festuca rubra* L. ssp. *commutata* (Tengfang et al., 2011). Concentrations of aromatic amino acids in nature varies greatly and their absolute concentrations in natural conditions is still unknown (Phillips et al., 2004). Derivatives of aromatic amino acids such as indolic (Ryu and Patten, 2008) and phenolic (Badri et al., 2013) compounds also present in root exudates.

Aromatic amino acids and their derivatives which are present in root exudates are utilised by bacteria for their growth (Ryu and Patten, 2008; Tengfang et al., 2011). Aromatic amino acids are excellent sources of carbon and nitrogen and some bacteria utilise aromatic amino acids for its growth as sole source of carbon and nitrogen or nitrogen sources (Tengfang et al., 2011). Bacteria are able to degrade/transform aromatic amino acids (L-phenylalanine, L-tyrosine and L-tryptophan) under oxic/anoxic conditions into an array of metabolites (Carmona et al., 2009; Ismail and Gescher, 2012). Anoxic aromatic metabolism *via* central benzoyl-CoA pathway was studied in denitrifying, phototrophic and fermentative bacteria (Carmona et al., 2009). Aromatic amino acids (L-tryptophan, L-phenylalanine, L-tyrosine) catabolism was also reported in *Lactobacillus casei* and *Lactobacillus helveticus via* transamination and dehydrogenation reactions resulting in the production of aromatic metabolites (Gummalla and Broadbent, 2001). *Lactobacillus casei* and *Lactobacillus helveticus* catabolised L-tryptophan to indole-3-pyruvic acid and indole-3-lactic acid (a chemically liable compound), L-phenylalanine to phenylpyruvic acid, phenyllactic acid and L-tyrosine to 4-hydroxyphenylpyruvic acid and 4-hydroxyphenyllactic acid (Gummalla and Broadbent, 2001). Bacteria are able to transform L-phenylalanine/L-tyrosine to phenolic compounds and L-tryptophan to indolic compounds under

oxic and anoxic conditions (Smith and Macfarlane, 1996; Diaz et al., 2001; Mechichi et al., 2002). Like other aromatic amino acid, L-phenylalanine is also used as carbon or nitrogen source by bacteria under oxic and anoxic conditions.

1.2 Anoxic L-phenylalanine catabolism in bacteria

1.2.1 L-phenylalanine catabolism as carbon source

Bacteria utilise L-phenylalanine as a sole source of carbon for its growth and catabolise L-phenylalanine to TCA cycle intermediates under anoxic conditions. L-Phenylalanine catabolism *via* phenylacetic acid was reported in *Escherichia coli and Pseudomonas putida* (Teufel et al., 2010). Complete anaerobic degradation of L-phenylalnine to TCA cycle intermediates *via* cinnamic acid, phenylpyruvic acid, phenylacetic acid, phenylacetyl-CoA and benzoyl-CoA was reported in *Desulfobacula toluolica* Tol2 (Wohlbrand et al., 2013). L-Phenylalanine was used as sole source of carbon by *Thauera* and *Azoarcus* strains under anoxic conditions and complete degradation of L-phenylalanine to TCA cycle intermediates *via* benzoyl-CoA was reported in denitrifying bacterium, *Thauera aromatica* (Schneider et al., 1997; Laempe et al., 2001; Fuchs, 2008; Carmona et al., 2009) (Fig. 1). In bacteria such as *Escherichia coli* (Diaz et al., 2001) and *Rhodococcus* sp. strain RHA1 (Navarro-Llorens et al., 2005) L-phenylalanine degraded *via* phenylacetic acid (PA) catabolic pathway and which further catabolised to acetoacetic acid and fumaric acid (Abe-Yoshizumi et al., 2004).

1.2.2 L-Phenylalanine catabolism as nitrogen source

Amino acids also serves as nitrogen source and bacteria utilizes aromatic amino acids as sole source of nitrogen. Utilisation of aromatic amino acids as sole source of nitrogen leads to the production of aromatic acids and alcohols (Wei et al., 2013).The catabolic pathways of amino acids involving chemical reactions to produce respective alcohols or carboxylic acids containing one carbon less than the starting amino acid is referred as Ehrlich's pathway (Hazelwood et al., 2008; Ravasio et al., 2014). In general branched-chain amino acids (valine, leucine and isoleucine) (Wei et al., 2013), aromatic amino acids (L-phenylalanine, L-tyrosine and L-tryptophan) and sulphur-containing amino acid (methionine) are catabolised *via* Ehrlich's pathway which led to the production of respective fusel acids and alcohols (Somers et al., 2005). The enzyme reactions involved in the Ehrlich's pathway are transamination, decarboxylation and reduction/oxidation reactions (Hazelwood et al., 2008). Transformation of aromatic amino acid, such as L-phenylalanine *via* Ehrlich's pathway was studied in bacteria

(Behrends et al., 2009; Afzal et al., 2013; Banerjee et al., 2014). Bacteria catabolise L-phenylalanine *via* phenylpyruvic acid and phenylacetaldehyde to phenylacetic acid and the enzymes involved in the transformation are aromatic aminotransferase, first enzyme that catalyses the conversion of L-phenylalanine to phenylpyruvic acid, pyruvate decarboxylase which convert phenylpyruvic acid to phenylacetaldehyde and aldehyde dehydrogenase converts phenylacetaldehyde to phenylacetic acid (Wei et al., 2013) (Fig. 1).

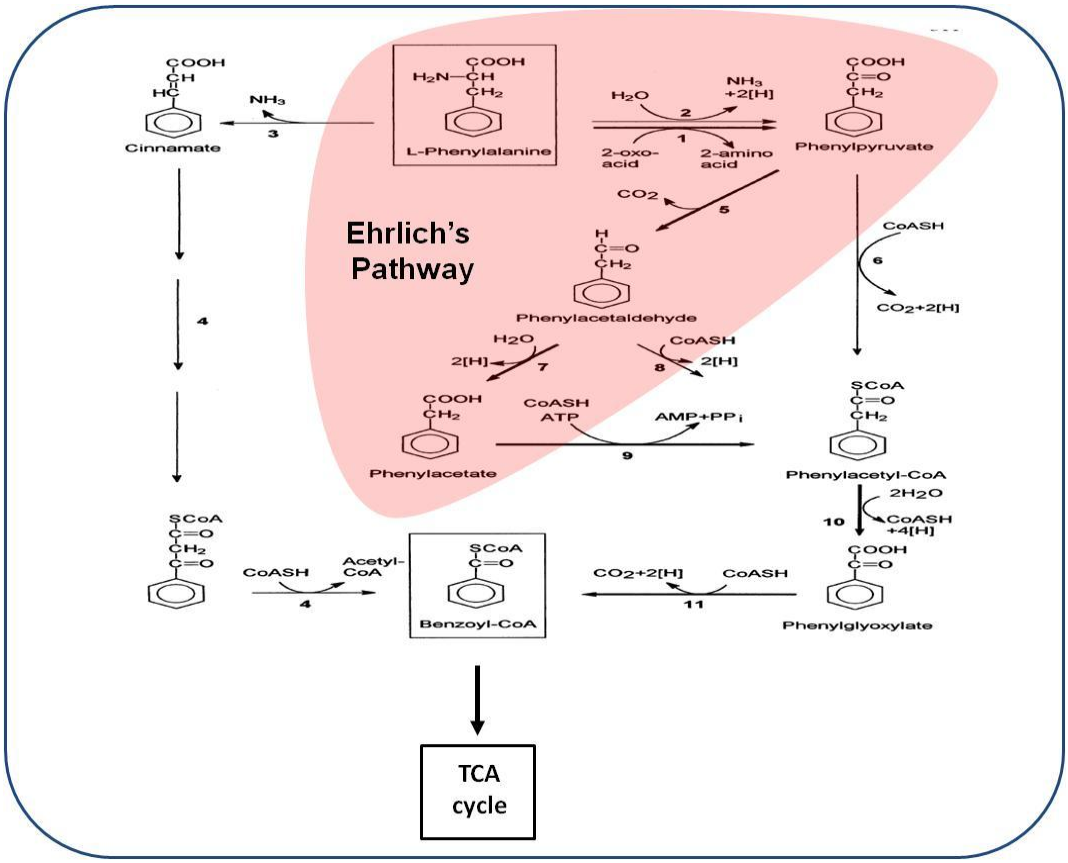


Fig. 1. Anoxic catabolism of L-phenylalanine *via* central intermediate benzoyl-CoA by denitrifying and phototrophic bacteria (Schneider et al., 1997).

Reaction numbers 1, L-phenylalanine:2-oxoglutarate aminotransferase; 2, L-phenylalanine NAD+ oxidoreductase; 3, L-phenylalanine ammonia-lyase; 4, β-oxidation pathway; 5, phenylpyruvate decarboxylase; 6, phenylpyruvate:acceptor oxidoreductase; 7, phenylacetaldehyde dehydrogenase; 8, phenylacetaldehyde dehydrogenase (CoA-acylating); 9, phenylacetate-CoA ligase; 10, phenylacetyl-CoA α-oxidizing enzyme; 11, phenylglyoxylate : acceptor oxidoreductase; TCA cycle, tricarboxylic acid cycle. Bold arrows indicate anoxic L-phenylalanine degradation in *Thauera aromatica* DSM698. Shaded region indicate the Ehrlich's pathway.

A branch point of Ehrlich's pathway leads to the conversion of phenylpyruvic acid to phenyllactic acid, catalysed by lactate dehydrogenase (EC 1.1.1.27). Phenyllactic acid production was reported in lactic acid bacteria (Li et al., 2007; Mu et al., 2009) and propionibacteria (Thierry and Maillard, 2002). L-Phenylalanine catabolism to phenylacetic acid *via* Ehrlich's pathway and phenyllactic acid production from phenylpyruvic acid was reported in lactic acid bacteria under anoxic condition (Svanstrom et al., 2013; Cortes-Zavaleta et al., 2014). L-Phenylalanine conversion to homogentisic acid *via* L-tyrosine, 4-hydroxyphenylpyruvic acid under anoxic conditions was reported in *Rhodobacter capsulatus* B10S (Saez et al., 1999). L-Phenylalanine conversion to 3,4-dihydroxybenzoic acid (protocatachuic acid) *via* L-tyrosine, 3,4-dihydroxy L-phenylalanine (DOPA) under anoxic conditions is reported in *Rhodobacter sphaeroides* (Ranjith et al., 2007b) (Fig. 2). Some of the intermediates like phenylacetic acid, mandelic acid and phenylglyoxylic acid were also observed in the L-phenylalanine transformation by lactic acid bacteria (Nierop Groot and de Bont, 1999). Apart from using L-phenylalanine under anoxic conditions, bacteria also catabolise L-phenylalanine for their growth as carbon/nitrogen source under oxic conditions (Arias-Barrau et al., 2004).

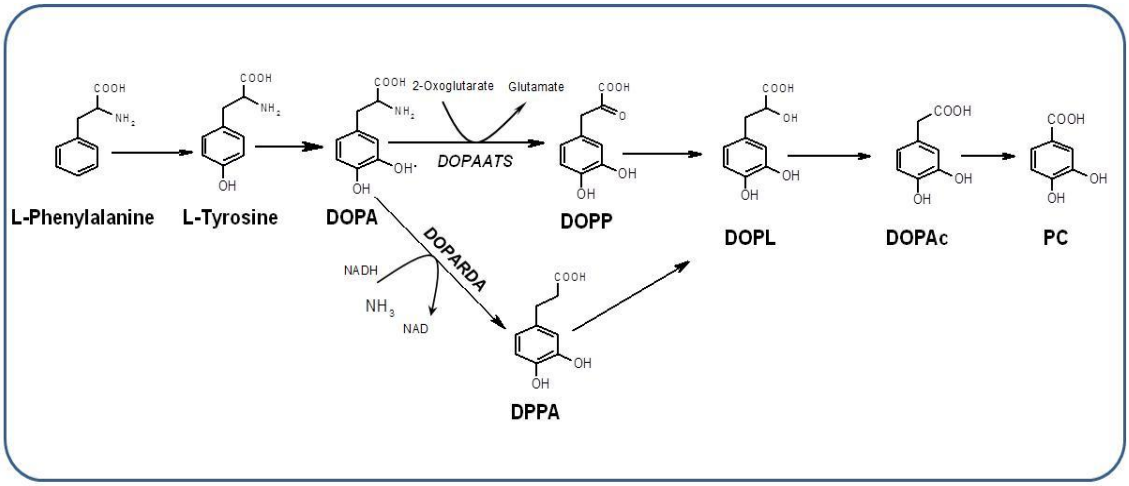


Fig. 2. Biotransformation of L-phenylalanine under anoxic conditions in *Rhodobacter sphaeroides* OU5 (Ranjith et al., 2007b)

DOPA, 3,4-Dihydroxy L-phenylalanine; DOPP, 3,4-dihydroxyphenylpyruvic acid; DOPL, 3,4-dihydroxyphenyllactic acid; DOPAc, 3,4-dihydroxyphenylacetic acid; PC, protocatachuic acid; DPPA, 3,4-dihydroxyphenylpropionic acid; DOPAATS, 3,4-dihydroxyphenylalanine aminotransferase; DOPARDA, 3,4-dihydroxyphenylalanine reductive deaminase.

1.3 Oxic L-phenylalanine metabolism in bacteria

1.3.1 Degradation of L-phenylalanine as carbon source

Bacteria utilise L-phenylalanine as sole source of carbon, carbon and nitrogen under oxic conditions. Oxidative degradation of aromatic amino acids by bacteria led to TCA cycle intermediates (Arias-Barrau et al., 2004; Camarero et al., 2008). Under oxic conditions L-phenylalanine is completely catabolised *via* two different pathways i.e homogetisic acid or phenylacetic acid pathway in bacteria (Arias-Barrau et al., 2004; Khan et al., 2013). In homgentisic acid dependent L-phenylalanine catabolism, L-phenylalanine was hydroxylated to L-tyrosine further converted to 4-hydroxyphenylpyruvic acid followed by hydroxylation to homogentisic acid which on ring cleavage with homogentisate dioxygenase leads to fumarate and acetoacetate (Arias-Barrau et al., 2004). Complete L-phenylalanine degradation into fumarate and acetoacetate *via* homogentisic acid was reported in different groups of bacteria. L-phenylalanine and L-tyrosine were completely catabolised *via* homogentisic acid in *Streptomyces setonii* 75Vi2 (Pometto and Crawford, 1985) and *Psuedomonas putida* U (Arias-Barrau et al., 2004).

Under oxic conditions, degradation of L-phenylalanine to TCA cycle intermediates through central phenylacetic acid pathway along with production of tropodithietic acid was reported in *Pheobacter gallaeciensis* (Berger et al., 2012) (Fig. 3). Some bacteria are capable of operating multiple catabolic pathways for the transformation of L-phenylalanine and L-tyrosine under oxic/anoxic conditions (Jimenez et al., 2002). A facultative methylotrophic bacterium, *Nocardia* sp. 239 capable of utilising L-phenylalanine and L-tyrosine as source of carbon, nitrogen and energy was reported (De Boer et al., 1988). Catabolism of both L-phenylalanine and L-tyrosine through homogentisic acid was reported in bacteria which further converts to TCA cycle intermediates (De Boer et al., 1988). Oxidative degradation of phenylacetic acid into phenylacetyl-CoA was reported in *Azoarcus evansii* in which L-phenylalanine was metabolised into phenylacetic acid (Mohamed et al., 2002).

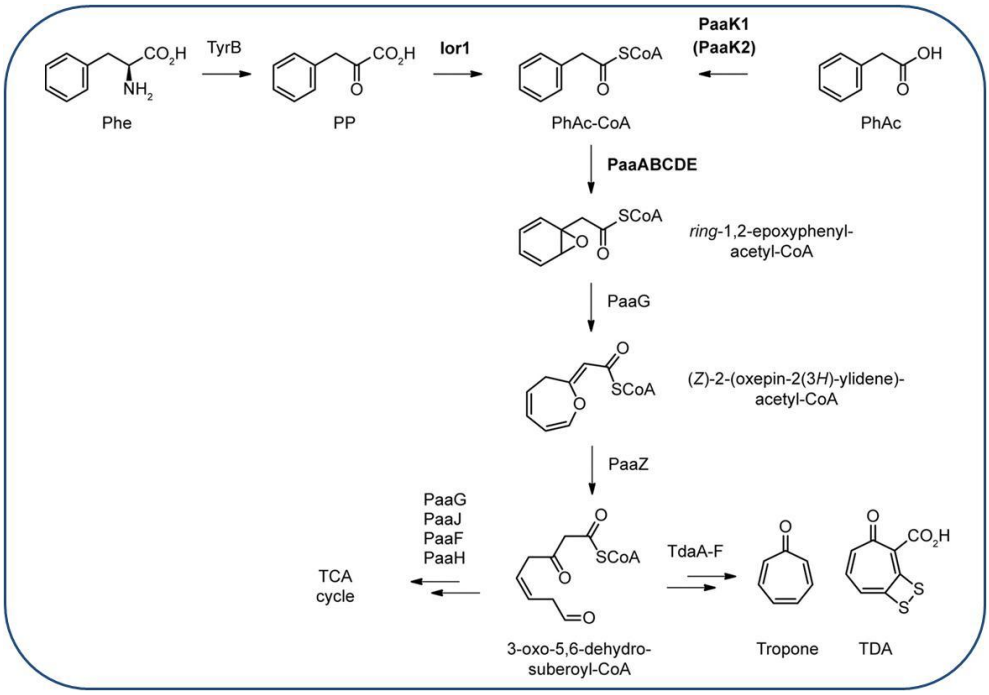


Fig. 3. Oxic degradation of L-phenylalanine *via* phenylacetyl-CoA in *Pheobacter gallaeciensis* (Berger et al., 2012).

Phe, L-phenylalanine; PP, phenylpyruvic acid; PhAc-CoA, phenylacetyl-CoA; PhAc, phenylacetic acid; TDA, tropodithietic acid; TCA cycle, tricarboxylic acid cycle; TyrB, aromatic amino acid transaminase; PaaK, phenylacetate-CoA ligase; PaaG, 2-(1,2-epoxy-1,2-dihydrophenyl)acetyl-CoA isomerase; PaaJ, 3-oxoadipyl-CoA/3-oxo-5,6-dehydrosuberyl-CoA thiolase; PaaF, 2,3-dehydroadipyl-CoA hydratase; PaaH, 3-hydroxyadipyl-CoA dehydrogenase; PaaZ, oxepin-CoA hydrolase/3-oxo-5,6-dehydrosuberyl-CoA semialdehyde dehydrogenase

1.3.2 L-phenylalanine as sole source of nitrogen

Under oxic conditions, many hydroxylases and oxygenases such as monooxygenases and dioxygenases catalyse the bioconversion reactions (Wang et al., 2011; Leiros et al., 2013) and play significant role in aromatic catabolism. Biotransformation of L-phenylalanine into different aromatic metabolites in the presence of oxygen were reported in bacteria (Berger et al., 2012). Bioconversion of L-phenylalanine into stilbene (4-isopropyl-3,5-dihydroxystilbene) under oxic conditions was reported in *Photorhabdus luminescens* (Joyce et al., 2008). L-phenylalanine transformed into *trans*-cinnamic acid and then to cinnamoyl-CoA which further conjugate with leucine derivative by an enzyme cyclase to form stilbene (Joyce et al., 2008). In the presence of oxygen some of the monooxygenases and dioxygenases transform L-phenylalanine into aromatic metabolites like L-tyrosine and 4-hydroxyphenylpyruvic acid, homogentisic acid in bacteria (Carreira et al., 2001; Arias-Barrau et al., 2004).

Oxic metabolism of L-phenylalanine *via* L-tyrosine, homogentisic acid leads to production of pigments such as melanin in bacteria. For example, *Psuedomonas sp* (Arias-Barrau et al., 2004; Hunter and Newman, 2010) (Fig. 4) and *Schewenella colweliana* (Turick et al., 2008) transformed L-tyrosine into pyomelanin *via* 4-hydroxyphenylpyruvic acid and homogentisic acid in presence of oxygen. Oxic L-phenylalanine and L-tyrosine biotransformation by a thermophilic *Geobacillus stearothermophilus* bacterium produced aromatic compounds like phenylpyruvic acid, phenylacetic acid, phenylacetaldehyde, phenylethylamine, *trans*-cinnamic acid, cinnamaldehyde and protocatachuic acid, benzaldehyde, homovanillic acid, homogentisic acid (Afzal et al., 2013). Under oxic conditions also L-phenylalanine catabolised *via* Ehrlich's pathway in bacteria (Hazelwood et al., 2008) when L-phenylalanine is used as sole source of nitrogen.

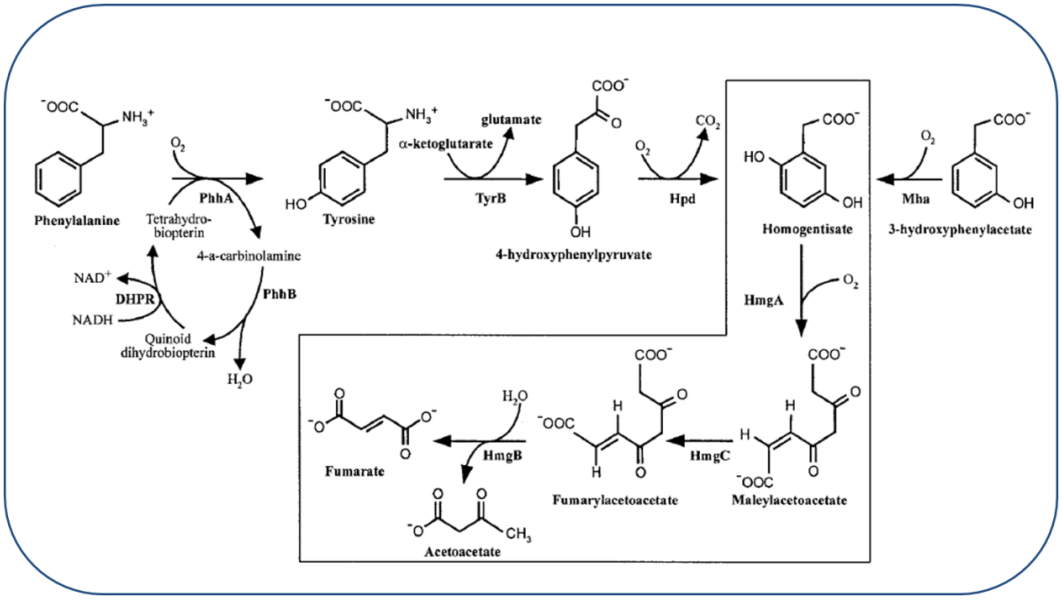


Fig. 4. Oxic L-phenylalanine degradation *via* homogentisic acid dependent pathway reported in *Psuedomonas putida* (Arias-Barrau et al., 2004).

PhhA, L-phenylalanine hydroxylase; TyrB, aromatic amino acid transaminase; Hpd, hydroxyphenylpyruvic acid dioxygenase; HmgA, homogentisic acid 1,2-dioxygenase; HmgC, maleylacetoacetic acid isomerase; HmgB, fumarylacetoacetase.

1.3.3 Production of pigments and antibiotics from L-phenylalanine by bacteria

Bacteria produce an array of aromatic compounds as a result of L-phenylalanine metabolism under oxic conditions, some of them are pigments such as melanins (Leiros et al., 2013; Banerjee et al., 2014; Coelho-Souza et al., 2014), anthroquinones (Li et al., 2013; Malak et al., 2013), stilbenes (Li et al., 1995; Joyce et al., 2008) and antibiotics such as tropodithietic acid (Berger et al., 2012), enterocin (Xiang and Moore, 2005), actinorhodin (Taguchi et al., 2012). A red pigment identified as anthraquinone derivative; 1,6-dihydroxy-4-methoxy-9,10-anthraquinone and an antibiotic identified as hydroxystilbene derivative; 3,5-dihydroxy-4-isopropylstilbene were reported in entomopathogenic bacterium, *Xenorhabdus luminescens* from L-phenylalanine (Richardson et al., 1988). *Streptomyces maritimus,* a marine bacterium, produces an antibiotic called enterocin (a polyketide) under anoxic condition where L-phenylalanine is converted to *trans*-cinnamic acid and β-oxidation of *trans*-cinnamic acid to benzoyl-CoA which on further converts to enterocin (Xiang and Moore, 2002). Production of antibiotic tropodithietic acid from L-phenylalanine through central phenylacetic acid pathway was reported in *Pheobacter gallaeciensis* (Berger et al., 2012) (Fig. 6).

Phenylpropanoid pathway is widely distributed among plant kingdom. Pigments such as anthocyanins and flavonoids were biosynthesised from L-phenylalanine through phenylpropanoid pathway (Fig. 7) (Ghasemzadeh et al., 2012). *Trans*-cinnamic acid, coumaric acid, chalcone are the key intermediates of phenylpropanoid pathway and the key enzymes of phenylpropanoid pathway are phenylalanine ammonia-lyase (PAL) (EC 4.3.1.24) and chalcone synthase (CHS) (EC 2.3.1.74). Phenylalanine ammonia-lyase convert L-phenylalanine to *trans*-cinnamic acid and chalcone synthase involves in the ring cyclisation and formation of a compound called chalcone, a precursor for the different flavonoids and anthocyanins (Maskeya et al., 2003). Phenylalanine ammonia-lyase (PAL) (Xiang and Moore, 2005) and chalcone synthase (CHS) are the rate limiting steps in the phenylpropanoid pathway (Fowler and Koffas, 2009). A novel bacterial L-phenylalanine ammonia-lyase (*EncP*) was identified in *Streptomyces maritimus* (Xiang and Moore, 2002) (Fig. 5). The PAL gene which is homologous to PAL gene sequence of plants was observed in the genome of *Rhodobacter capsulatus* B10S. The genome project of *Rhodobacter capsulatus* B10S (www.integratedgenomics.com) has an open reading frame (ORF) of PAL gene (1626 bp, contig 58-60, ORF 1844) located upstream of Photoactive Yellow Protein (PYP) gene (Kyndt et al., 2004).

Anthocyanins and flavonoids biosynthesis is widely distributed among plants. However, some bacteria and fungi transform flavonoids/anthocyanins by glycosidases. Some of the isoflavonoids were isolated from bacteria and fungi whereas their biosynthetic origin was not proven (Maskeya et al., 2003). A few actinomycetes were reported to transform isoflavonoids which most probably not reflect the *de novo* biosynthesis rather the presence of glycosidases can transform the flavonoid derivatives (Maskeya et al., 2003; Yuan et al., 2007). However, many bacteria have PAL and CHS genes of phenylpropanoid pathway in their genome whereas the functionality of those genes is not yet proved. Some photosynthetic bacteria such as *Rhodobacter sphaeroides* ATCC 17029 (gi|126102442), *Pheospirillum molischianum* DSM120, *Pheospirillum fulvum* MGU-K5, *Rhodomicrobium vannielli* also consists PAL and CHS genes in their genomes whereas functionality of these genes is unknown.

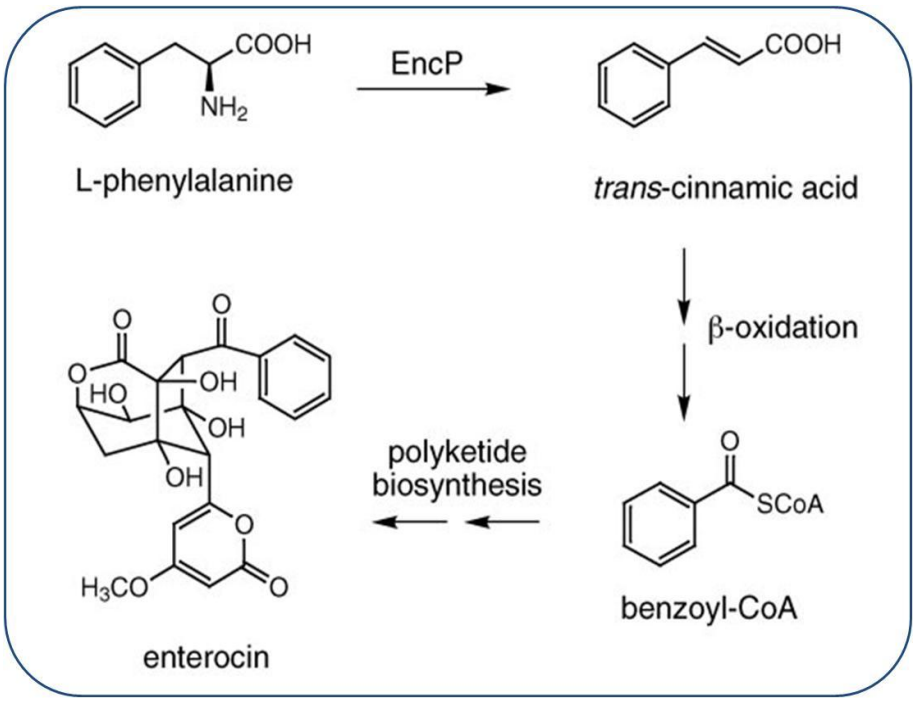


Fig. 5. Biotransformation of L-phenylalanine *via trans*-cinnamic acid and biosynthesis of benzoyl-CoA derived antibiotic, enterosin in *Streptomyces maritimus* (Xiang and Moore, 2002)

Double arrow reactions in the pathway indicate the multiple steps. EncP, phenylalanine ammonia-lyase gene associated to enterosin biosynthetic gene cluster.

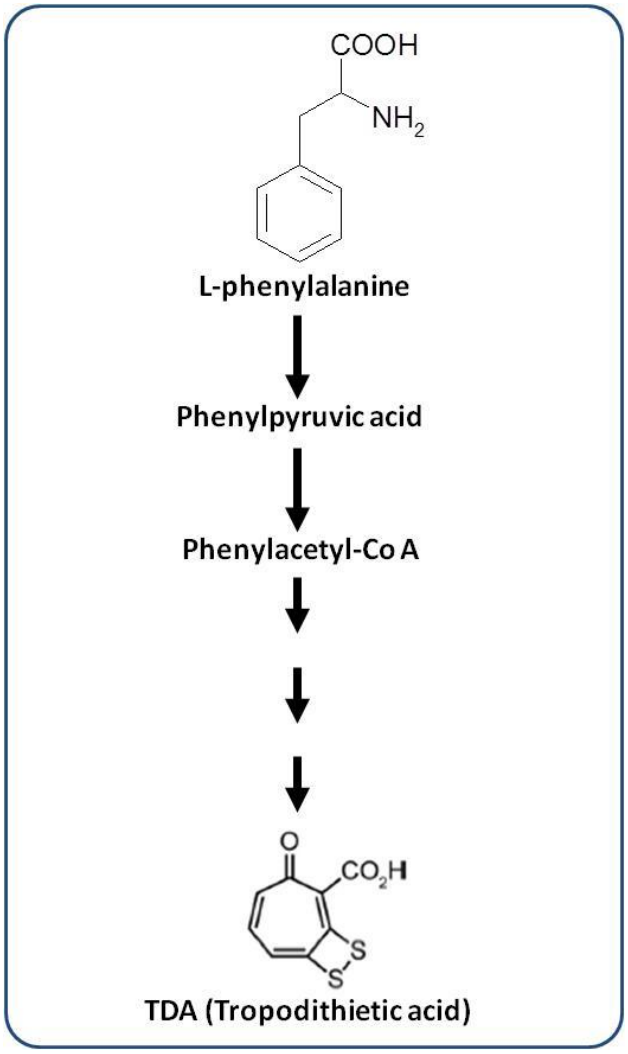


Fig. 6. Biosynthetic pathway of an antibiotic compound, Tropodithietic acid

Multiple arrows indicate the presence of multiple reactions in the pathway

TDA, tropodithietic acid, TCA cycle, tricarboxylic acid cycle.

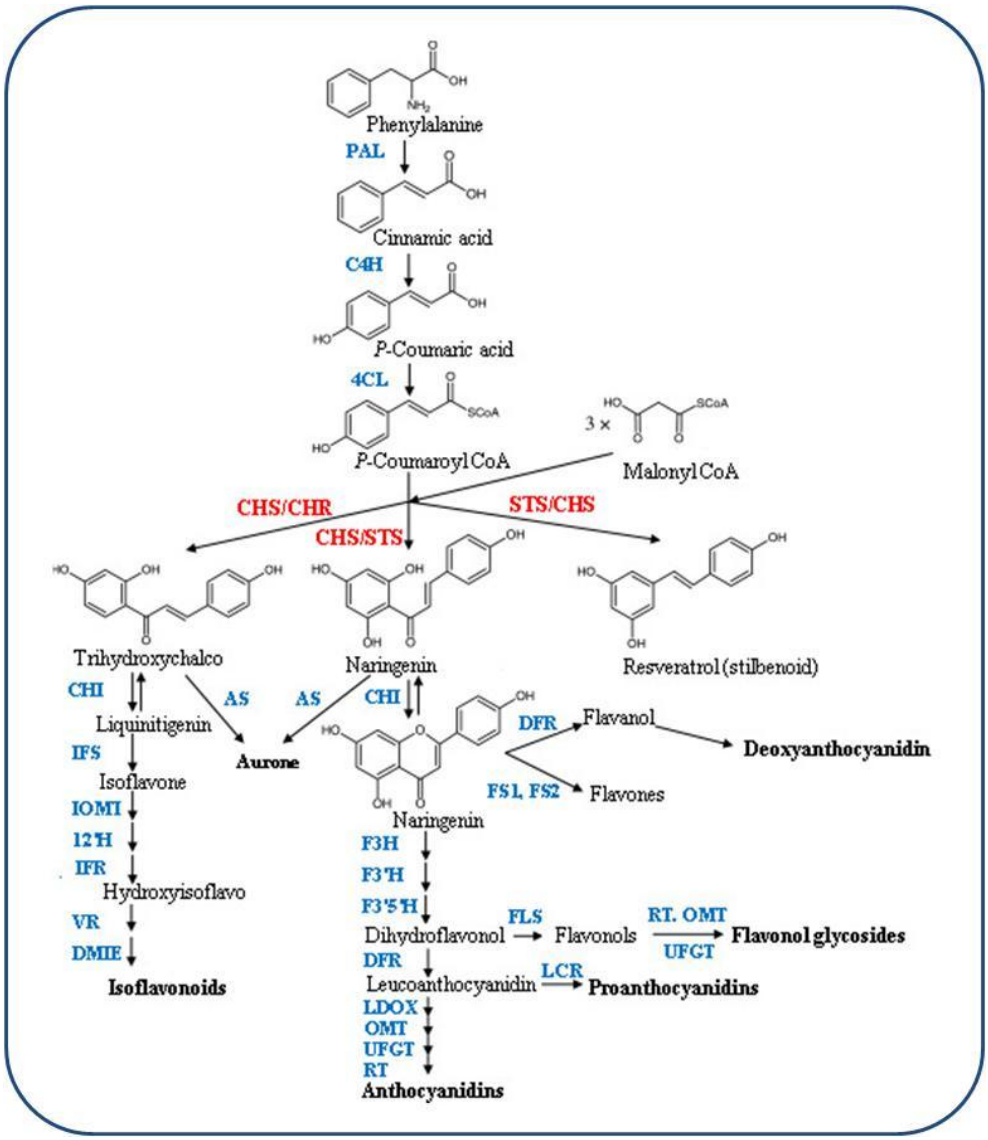


Fig. 7. Phenylpropanoid pathway observed in plants (Ghasemzadeh et al., 2012)

PAL/TAL, L-Phenylalanine/L-Tyrosine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-hydroxycinnamate lyase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; IFS, Isoflavone synthase; IOMT, Isoflavone *O*-methyltransferase; I2'H, isoflavone 2'-hydroxylase; IFR, isoflavone reductase; VR, vestitone reductase; DFR, dihydroflavonol 4-reductase; DMID, 7,2'-dihydroxy,4'-methoxyisoflavonol dehydratase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3' hydroxylase; F3'5'H, flavonoid 3'5' hydroxylase; FS1/FS2, flavone synthase; LDOX, leucoanthocyanidin dioxygenase OMT, *O*-methyltransferase; PAL, phenylalanine ammonia-lyase; RT, rhamnosyl transferase; UFGT, UDP flavonoid glucosyl transferase; STS, stilbene synthase; FLS, flavanol synthase.

1.4 Melanin biosynthesis in bacteria

Melanins are enigmatic pigments, important natural biopolymers with multiple functions, yet their structures are not well understood (Manivasagan et al., 2013). L-Tyrosine is the precursor for the melanin biosynthesis. Melanin is also produced from L-phenylalanine *via* L-tyrosine by some bacteria like *Rhizobium* species (Mercado-Blanco et al., 1993). Melanins have radioprotective and antioxidant properties (Dadachova et al., 2008; Khajo et al., 2011). Oxic L-phenylalanine/L-tyrosine catabolism lead to the production of melanin pigment by a thermophilic *Geobacillus stearothermophilus* (Afzal et al., 2013).

1.4.1 Occurrence

Melanin is a dark brown to black colored ochronotic pigment produced by all groups of biological kingdom. Melanins are widely distributed among plants, animals and microorganisms (Gessler et al., 2014). Melanin biosynthesis is L-tyrosine dependent in almost all the systems. Melanin exist in different forms like amorphous, crystalline. Melanin is predominantly found in the hair and skin of humans. Eumelanin and pheomelanin biosynthesis is abundant in vertebrates. Allomelanin is found in plants, fungi and bacteria (Singh et al., 2013).

1.4.2 Types of melanin and biosynthetic pathway of melanin

Different types of melanins were described in plants, animals, fungi and bacteria: eumelanin, pheomelanin, allomelanin, pyomelanin, neuromelanin. Eumelanins and pheomelanins were of animal origin. Eumelanin and pheomelanins are present in the human abundantly in hair and skin (Piletic et al., 2010). Eumelanins are black or brown colored pigments derived from L-tyrosine. Polymers of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) are called eumelanins (Glass et al., 2012). Pheomelanins are synthesised from the same precursor, L-tyrosine while cysteine is involved during the pheomelanin synthesis (Napolitano et al., 2008). Pheomelanins are polymers of benzothiazine units and has both nitrogen and sulfur in their structures (Napolitano et al., 2008). Allomelanins are most heterogenous group of polymers, synthesised from nitrogen free precursors (Tarangini and Mishra, 2013) and they are formed by oxidation or polymerisation of dihydroxynaphthalene [Di-(DHN)] or tetrahydroxynaphthalene [tetra-(DHN)]. Pyomelanins are dark brown colored pigment synthesised extracellularly by oxidative polymerisation of

homogentisic acid, an intermediate of L-tyrosine catabolism (Turick et al., 2008; Almeida-Paes et al., 2012; Zheng et al., 2013; Coelho-Souza et al., 2014).

Different types of melanins were biosynthesised through a common precursor L-tyrosine (Fig.8). L-tyrosine catabolism leads to the production of eumelanin, pheomelanin, pyomelanin, neuromelanin. L-tyrosine catabolism leads to the production of pyomelanin through 4-hydroxyphenylpyruvic acid and homogentisic acid. Homogentisic acid on autooxidation and copolymerisation produces pyomelanin (Leiros et al., 2013; Coelho-Souza et al., 2014). The key enzymes involved in pyomelanin biosynthesis are tyrosine aminotransferase which converts L-tyrosine to 4-hydroxyphenylpyruvic acid and 4-hydroxyphenylpyruvate dioxygenase converts 4-hydroxyphenylpyruvic acid to homogentisic acid (Chai et al., 2012). Biosynthesis of eumelanin is 3,4-dihydroxyphenylalanine (DOPA) dependent. L-tyrosine transforms into DOPA and dopaquinone and the reactions were catalysed by tyrosinase (Apte et al., 2013). Dopaquinone further cyclise into 5,6-dihydroxyindole (DHI) and 5,6 dihydroxyindole-2-carboxylic acid (DHICA). Eumelanins are polymers of DHI and DHICA (Glass et al., 2012). Dopaquinone with cysteine together form benzothiazine derivatives which lead to pheomelanins (Thureau et al., 2012). Dopaquinone is common for both eumelanin and pheomelanin biosynthesis. Neuromelanin was synthesised from L-tyrosine *via* tyramine, Dopamine and Dopaminochrome. Transformation of L-phenylalanine to L-tyrosine also leads to production of different types of melanin in bacteria (Singh et al., 2013) (Fig. 8).

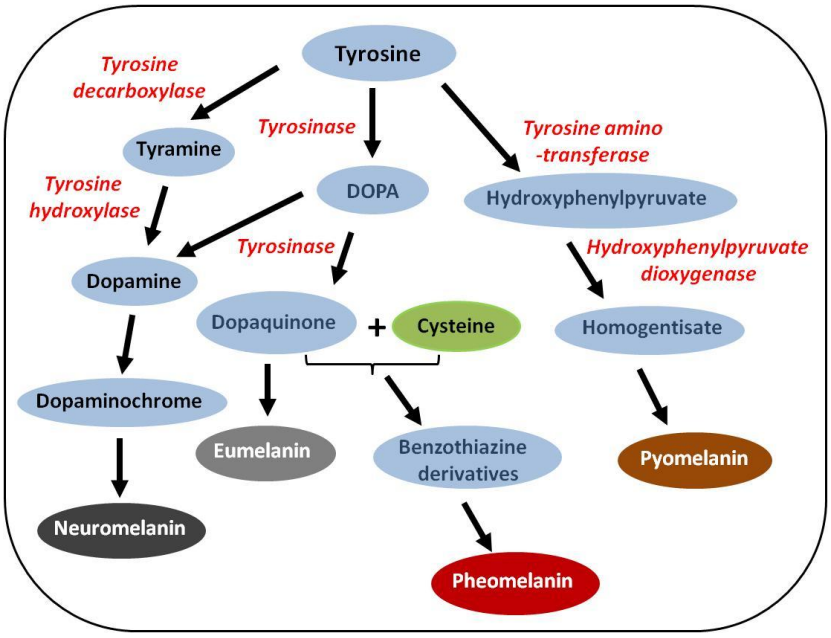


Fig. 8. An overview of melanin biosynthetic pathways present in human, plants, fungi and bacteria (Singh et al., 2013). DOPA, 3,4-dihydroxyphenylalanine.

1.5 Aromatic amino acid catabolism in purple bacteria

Anoxygenic phototrophic bacteria utilise aromatic amino acids as sole source of carbon/nitrogen. *Rhodopsuedomonas palustris* catabolise/degrade aromatic amino acids and heterocyclic aromatic compounds (Larimer et al., 2004; Oda et al., 2008). *Rhodobacter sphaeroides* utilises L-phenylalanine as sole source of nitrogen and produces phenolic compounds such as caffeic acid, protocatachuic acid, gallic acid and alkyl esters of gallic acid (Kumavath et al., 2010a). *Rhodobacter sphaeroides* OU5 utilised L-phenylalanine as sole source of nitrogen under anoxic conditions and produced aryl metabolites such as L-tyrosine, 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylpyruvic acid (DOPP), 3,4-dihydroxyphenyllactic acid (DOPLA), 3,4-dihydroxyphenylacetic acid (DOPAc), 3,4-dihydroxybenzoic acid (protocatachuic acid; PC). The catabolic pathway of L-phenylalanine was demonstrated with enzyme activies such as DOPA transaminase (DOPAATS) and DOPA reductive deaminase (DOPARDA). DOPA transminase convert DOPA into DOPAphenylpyruvic acid (DOPAPP) and DOPA reductive deaminase convert DOPA to 3,4-dihydroxypenylpropionic acid. A novel DOPA reductive deaminase enzyme was further purified and characterised (Ranjith et al., 2007b). Anoxic catabolism of L-phenylalanine to homogentisic acid *via* L-tyrosine and 4-hydroxyphenylpyruvic acid is reported in *Rhodobacter capsulatus* B10S. L-phenylalanine hydroxylated to L-tyrosine and transminated to 4-hydroxyphenylpyruvic acid and finally oxidised to homogentisic acid by *Rhodobacter capsulatus* B10S (Saez et al., 1999). A novel indole terpenoid ether called rhodethrin was biosynthesised by *Rhodobacter sphaeroides* OU5 when grown in the presence of L-tryptophan as sole source of nitrogen (Ranjith et al., 2007a).

Aromatic amino acids (L-tryptophan, L-phenylalanine and L-tyrosine) metabolism as sole source of nitrogen was also reported in *Rubrivivax benzoatilyticus* JA2 under oxic and anoxic conditions. L-Tryptophan catabolism *via* indole-3-pyruvic acid, indole-3-acetaldehyde, indole-3-acetic acid is reported in *R. benzoatilyticus* JA2 when grown in the presence of L-tryptophan as sole source of nitrogen (Kumavath et al., 2010b). Further multiple catabolic pathways of L-tryptophan were reported in this organism (Mujahid et al., 2011). *Rubrivivax benzoatilyticus* JA2 utilised L-phenylalanine as sole source of nitrogen but not as carbon source. *Rubrivivax benzoatilyticus* JA2 able to produce L-tryptophan in the presence of aniline (Mujahid et al., 2014). *Rubrivivax benzoatilyticus* JA2 transformed L-phenylalanine into rubrivivaxin under anoxic conditions (Kumavath et al., 2011). Genome sequence of *Rubrivivax*

benzoatilyticus JA2 revealed the diverse metabolic pathways of aromatic metabolism and also possible multiple catabolic pathways of L-phenylalanine (Mohammed et al., 2011).

1.6 Metabolic responses in bacteria under varied growth conditions

The changes/responses of bacteria to different physiological conditions at the metabolic level were not extensively studied. Metabolic responses varies with the surrounding environmental conditions (Frimmersdorf et al., 2010) such as pH, temperature (Azizan et al., 2012; Ye et al., 2012), salinity (Kol et al., 2010), and some physiological changes like oxic/anoxic growth modes (Frick and Wittmann, 2005; Chen et al., 2011; Schellenberger et al., 2012). Global view of metabolome revealed the metabolic variations in *Psuedomonas aeruginosa* when grown under different carbon sources and growth modes (Frimmersdorf et al., 2010). Metabolomics is a wholistic approach to know the metabolic responses of the system towards surrounding conditions. Metabolic changes were monitored over the course of time in batch cultures of well studied organisms, *Escherichia coli* and *Pseudomonas aeruginosa* using time-resolved metabolic footprinting (TReF) (Behrends et al., 2009). Metabolomics approach offered a promising avenue to fingerprint the intestinal microbiota and its interaction with the host (Marcobal et al., 2013). Metabolomics approach provided snapshot of bacterial metabolic adaptations to changing conditions (Chen et al., 2011).

1.7 Definition of the problem

Bacteria are well known for their metabolic diversity among groups of microorganisms and they thrive under diverse habitats. Bacteria are capable of degrading and transforming different aromatic compounds which are second abundant class of hydrocarbons (Carmona et al., 2009). Aromatic amino acids are abundantly found group of natural aromatic compounds. Anoxygenic phototrophic bacteria are capable of transforming aromatic amino acids like L-phenylalanine, L-tyrosine and L-tryptophan under different physiological conditions like oxic and anoxic conditions. *Rubrivivax benzoatilyticus* JA2, a betaproteobacterium transforms aromatic amino acids under oxic and anoxic conditions. *Rubrivivax benzoatilyticus* JA2 transforms L-phenylalanine to phenolic compounds (Kumavath et al., 2011) and L-tryptophan to indolic (Kumavath et al., 2010b; Mujahid et al., 2011) compounds under anoxic conditions. *R. benzoatilyticus* JA2 able to utilise aromatic amino acids as sole source of nitrogen but not as carbon source. Genomic insights also revealed *R. benzoatilyticus* JA2 lacks the gene coding for ring cleavage enzymes but genome sequence analysis of *R. benzoatilyticus* JA2 indicated the existence of multiple catabolic pathways of aromatic metabolism under oxic and anoxic

conditions. Reports on aromatic amino acids catabolism in *Rubrivivax benzoatilyticus* JA2 revealed the capability of producing aryl metabolites by utilising aromatic amino acids as sole source of nitrogen. L-tryptophan catabolised *via* indole-3-pyruvic acid, indole-3-acetaldehyde and indole-3-acetic acid in *Rubrivivax benzoatilyticus* JA2 when given as sole source of nitrogen (Kumavath et al., 2010b). A novel compound, rubrivivaxin was isolated from *Rubrivivax benzoatilyticus* JA2 when L-phenylalanine was used as sole source of nitrogen (Kumavath et al., 2011).

Previous reports of L-phenylalanine catabolism in *R. benzoatilyticus* JA2 was targeted to few aryl metabolites produced under anoxic conditions whereas insights of oxic L-phenylalanine catabolism was not studied yet. Nevertheless the comprehensive insights of the L-phenylalanine metabolism under oxic and anoxic conditions was not studied in this organism and moreover, how changing oxic/anoxic conditions will influence L-phenylalanine metabolism is of particular interest. Wholistic study of L-phenylalanine metabolism under oxic and anoxic conditions may provide catabolic potential of *R. benzoatilyticus* JA2. Hence, *Rubrivivax benzoatilyticus* JA2 was used as a model organism, and a detailed study was taken up to elucidate the insights of L-phenylalanine metabolism in *Rubrivivax benzoatilyticus* JA2 and its catabolic potential under varying conditions. Metabolomics approach was employed to decipher the multiple L-phenylalanine catabolic pathways and metabolic responses of bacteria to oxic/anoxic conditions. L-phenylalanine catabolism under oxic and anoxic conditions in *R. benzoatilyticus* JA2 was studied by using LC-MS based stable isotopic studies. An attempt was made to know the metabolic changes under oxic and anoxic conditions when L-phenylalanine was used as sole source of nitrogen. Untargeted GC-MS based metabolomics approach was employed to know the overall metabolism under oxic and anoxic conditions.

1.8 Objectives

- **Deciphering the insights of L-phenylalanine metabolism of *Rubrivivax benzoatilyticus* JA2**

- **Elucidating the metabolic responses of *Rubrivivax benzoatilyticus* JA2 to L-phenylalanine under oxic/anoxic conditions**

MATERIALS AND METHODS

2.0 Materials and methods

2.1 Materials, chemicals and devices

2.1.1 Glassware

The glassware used for the present work including conical flasks, beakers, measuring cylinders, pipettes, petri plates, culture bottles, screw capped culture tubes, test tubes, round bottom flasks, extraction funnel, reagent bottles were of Borosil or Duran company.

2.1.2 Deionized water

Deionized water was obtained from the Deionizer plant (Ion Exchnge India LTD. Model-CA 20/U). Single distilled water was used for autoclaving and also for rinsing the glassware. Double distilled water was used for preparing the media and also in the preparation of media stocks. Milli-Q water was used for the dissolution of standards and for HPLC analysis.

2.1.3 Chemicals

Chemicals used for the present work are of analytical grade from Sigma Aldrich, Fluka, Merck, HiMedia, Lancaster, Cambridge Stable Isotope Laboratories.

2.1.4 Filtration Devices

Membrane filters (0.2 µm and 0.45 µm) and filtration units used were from Millipore, PALL and filter papers from Whatmann.

2.1.5 Determination of pH

pH of the media and buffer solutions were determined by the digital pH meter (Digisun Electronics, India model DI-707). pH meter was often calibrated with standard buffer solutions of pH 4.2, 7 and 9.2.

2.1.6 Buffers and standard solutions

All the buffers used for the work were prepared by using the deionised double distilled water. Tris buffer: 100 mM Tris/HCl pH 7 was prepared according to the standard protocols and the pH was adjusted at room temperature unless otherwise stated.

2.1.6.1 Standard stock solutions

L-Phenylalanine (100 mM) stock was prepared using double distilled water and was sterilised by autoclaving / filter sterilisation. Stock solutions of glucose, fructose, galactose, mannose, maltose, lactose were prepared (100 mM) in distilled water and were sterilised by autoclaving.

2.1.7 Sterilisation

Sterilisation of media and glassware was done by autoclaving at 120°C, at 15 lb pressure for 15 min. All thermolabile solutions were filter sterilised by filtering through 0.22 µm membrane filters (Millipore).

2.1.8 Media preparation

2.1.8.1 Mineral media composition (g.l^{-1})

KH_2PO_4-0.5, $MgSO_4.7H_2O$-0.2, NaCl-0.4, NH_4Cl-0.37, $CaCl_2.2H_2O$-0.05, Yeast extract-0.2, Ferric citrate-0.1% (w/v), SL_7 (trace element solution)-1ml.l^{-1}.

SL_7 (mg.ml^{-1}): HCl 25% (v/v), $ZnCl_2$-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.

Malate (22 mM) was used as a carbon and electron donor, ammonium chloride (7 mM) as nitrogen source. The components were dissolved in distilled water, adjusted the pH to 6.8 with 5 N NaOH. Malate was replaced with other carbon sources wherever required.

2.1.8.2 Nutrient Agar (components g.l^{-1})

Peptone-10, yeast extract-3.5, NaCl-5, agar-20. The ingredients were weighed and dissolved in distilled water except agar. pH was adjusted to 7.0 and agar was added to media just before autoclaving.

2.2 Organisms and growth conditions

2.2.1 Organisms

Initially different purple bacteria were used for screening the phenolic compound production. *Rhodobacter* species and *Rubrivivax benzoatilyticus* JA2 were used for screening of

phenols production. *Rubrivivax benzoatilyticus* JA2^T (=ATCC BAA-35^T=JCM 13220^T=MTCC 7087^T) was used as model organism for all the experiments in the present work.

2.2.2 Growth conditions

Rubrivivax benzoatilyticus JA2 was grown photoheterotrophically (under anaerobic conditions, 30±2ºC; light 2,400 lux) on minimal media supplemented with malate (22 mM) as carbon and ammonium chloride (7 mM) as nitrogen sources in fully filled 7 ml screw cap test tubes (10x100 mm) / culture bottles (250 ml) at pH 6.8. The cultures were grown aerobically on minimal media in 250/500 ml conical flasks at 30º C, under agitation with 180 rpm. When L-phenylalanine was used as sole source of nitrogen, ammonium chloride was replaced with L-phenylalanine. Malate was replaced with glucose, maltose, fructose, mannose, galactose, fumarate and pyruvate when different carbon sources were used as sole source of carbon.

2.2.3 Maintenance of stock cultures

Stock cultures were maintained in the form of agar stabs. Agar stabs were prepared with 2% (w/v) agar in malate mineral media poured ¾ volume in 5 ml screw cap tubes. The culture was inoculated by stabbing into the agar with inoculation needle. The culture was incubated at 30º C under illumination (2,400 lux). After the growth was observed, the culture was stored at 4º C and used whenever required. The stock culture was inoculated and grown photoheterotrophically to reach 0.25 O.D at 660 nm. This active culture was used as 10% inoculum for further experiments.

2.2.4 Purity of cultures

Culture purity was checked by streaking the culture on nutrient agar plates. The plates were incubated under illumination (2400 lux) at 30±2º C. Purity of the culture was checked before and after the experiments.

2.2.5 Growth and biomass

Growth was measured turbidometrically and biomass was measured in the form of dry weight. The growth was monitored by measuring O.D at 660 nm. Dry weight was calculated based on the empirical formula generated by ploting the growth $O.D_{660\ nm}$ against dry weight. 0.1 O.D corresponds to 0.15 mg dry weight ml^{-1}

2.2.6 L-Phenylalanine feeding

Rubrivivax benzoatilyticus JA2 was grown photoheterotrophically under illumination (2400 lux) at 30±2º C for 24 h (early log phase 0.2 O.D) with malate (22 mM) as carbon source. L-phenylalanine (1 mM) was used as sole source of nitrogen instead of ammonium chloride. The culture was then incubated for 48 h or desired time period. *Rubrivivax benzoatilyticus* JA2 was also grown under aerobic condition at 30±2ºC for 24 h with agitation 180 rpm. L-phenylalanine (1 mM) was added as nitrogen source instead of ammonium chloride and incubated for 48 h.

2.2.7 Glyphosate treatment

To observe the effect of glyphosate on aryl metabolite production, glyphosate (1 mM) was added along with L-phenylalanine (1 mM) to the 24 h grown culture.

2.2.8 Sulcotrione treatment

Sulcotrione (0.5 mM) was added to the 24 h grown culture of *R. benzoatilyticus* JA2 along with L-phenylalanine (1 mM).

2.3 Extraction of metabolites

2.3.1 Extraction of aryl metabolites

The control and L-phenylalanine fed cultures were grown as described in the section 2.2.6 were used for the extraction of aryl metabolites. The cultures were harvested by centrifugation (10,000 x g, 10 min, 4° C) and the supernatant was taken for the ethyl acetate extraction. The supernatant was acidified to pH 2 with 5 N HCl and the acidified supernatant was extracted thrice with equal volumes of ethyl acetate. The ethyl acetate fraction was separated by using separation funnel and then evaporated to dryness under vacuum using rotary flash evaporator (Heidolph, Germany) at 35°C. Finally, re-suspended in 1 ml HPLC grade methanol. The extract was filtered through membrane filters (Supro, 0.22 µm) and the filtered extract was used for HPLC analysis (Fig. 9).

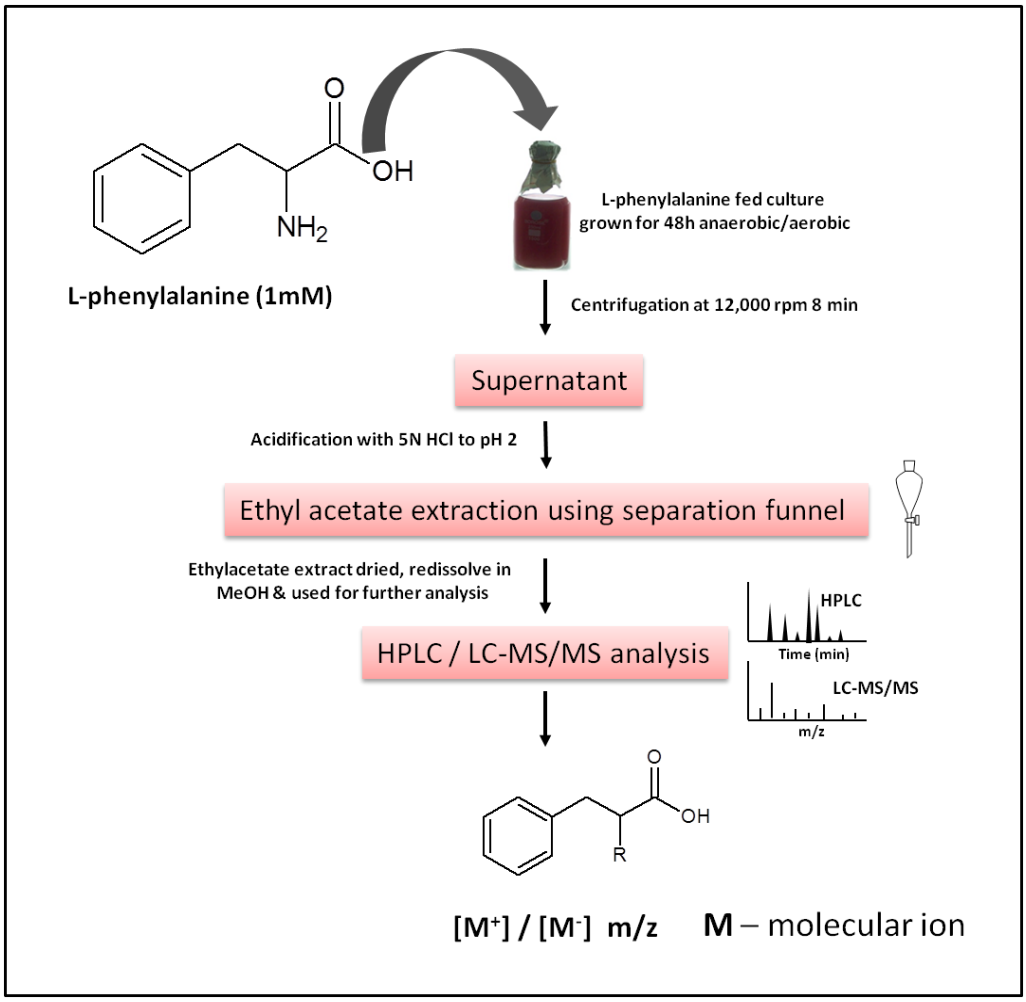


Fig. 9. Schematic representation of metabolite extraction and identification of aryl metabolites in *Rubrivivax benzoatilyticus* JA2.

Cultures were grown in the presence of L-phenylalanine for 48 h under oxic and anoxic conditions. The supernatants were acidified and extracted into ethyl acetate, the extract was concentrated to dryness and finally redissolved in HPLC grade methanol and subjected to HPLC and LC-MS analysis. M-molecular ion mass, *m/z*; mass/charge, M^+, Molecular ion mass ionised in positive mode, M^-, molecular ion mass ionised in negative mode.

2.3.2 Extraction of pigments

L-Phenylalanine fed cultures of *Rubrivivax benzoatilyticus* JA2 were grown under oxic condition as described in section 2.2.6 and the cultures were harvested by centrifugation at 12000 rpm for 10 min, 4° C, The supernatant was acidified to pH 2 with 5 N HCl and stored at 4°C for 48 h. Brown colored precipitate was settled at the bottom of the flask. Brown precipitate was collected by centrifugation 12000 rpm, 10 min, 4° C and the supernatant was discarded. The brown precipitate was suspended in methanol and fractionated. Fraction-II (methanol undissolved fraction) and Fraction-II (methanolic fraction) were taken for further purification of pigments (Flow chart. 1).

2.3.2.1 Purification of brown pigment (Brwn P)

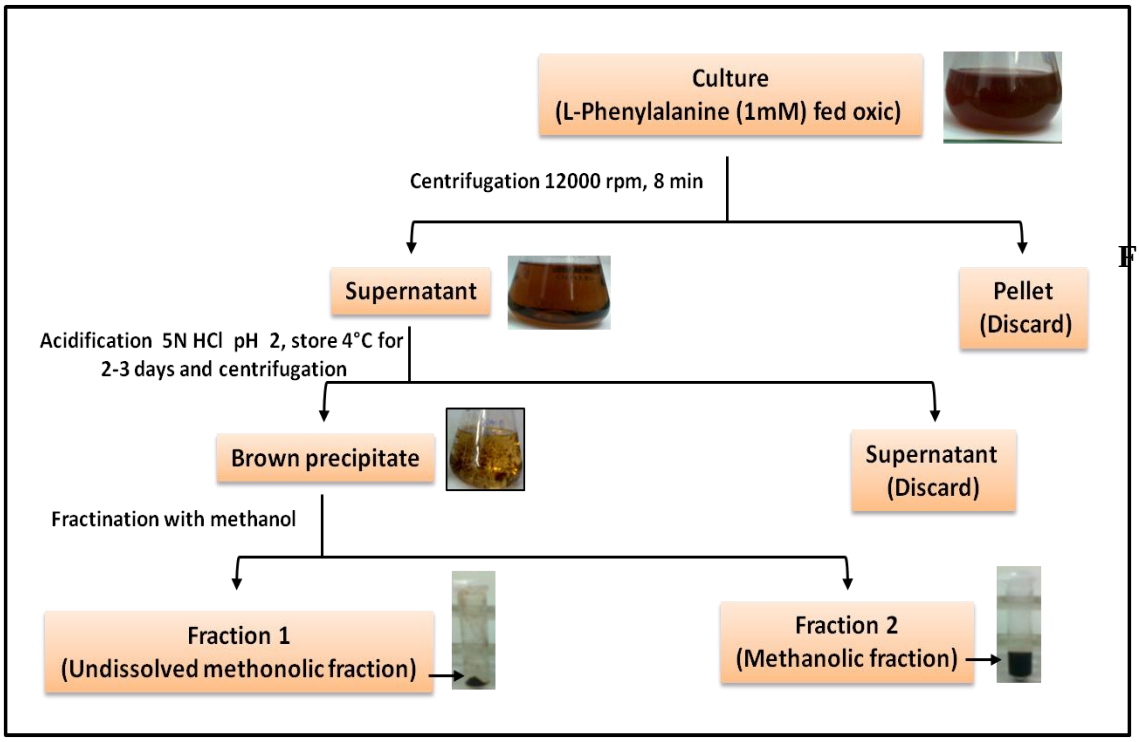
Fraction-I was further purified by serial washings with solvents (hexane, ethyl acetate, chloroform, acetone, methanol) and later dissolved in water. Finally the brown pigment was dissolved in 5 N NaOH and then precipitated with 5 N HCl. The brown precipitate formed with HCl precipitation was characterised based on NMR, FTIR, UV and ESR/EPR spectral analysis and the characteristic features of purified Brwn P was studied by physico-chemical properties (Flow chart. 2).

2.3.2.2 Purification of blue, pink and orange pigments

Fraction-II was subjected to TLC using mobile phase Chloroform:Methanol:Acetic acid (8:1.95:0.05 v/v). Blu P1 and P2 (blue pigments), Pin P (pink pigment), Org P (orange pigment) were partially purified by scrapping the colored bands along with silica from the TLC plates and suspended in HPLC grade methanol. Blu P1, P2 were purified by using HPLC as described in section 2.4.2. Brown pigment and blue pigments were characterised by LC-MS/MS, NMR, FTIR, UV spectral analysis (Flow chart. 3).

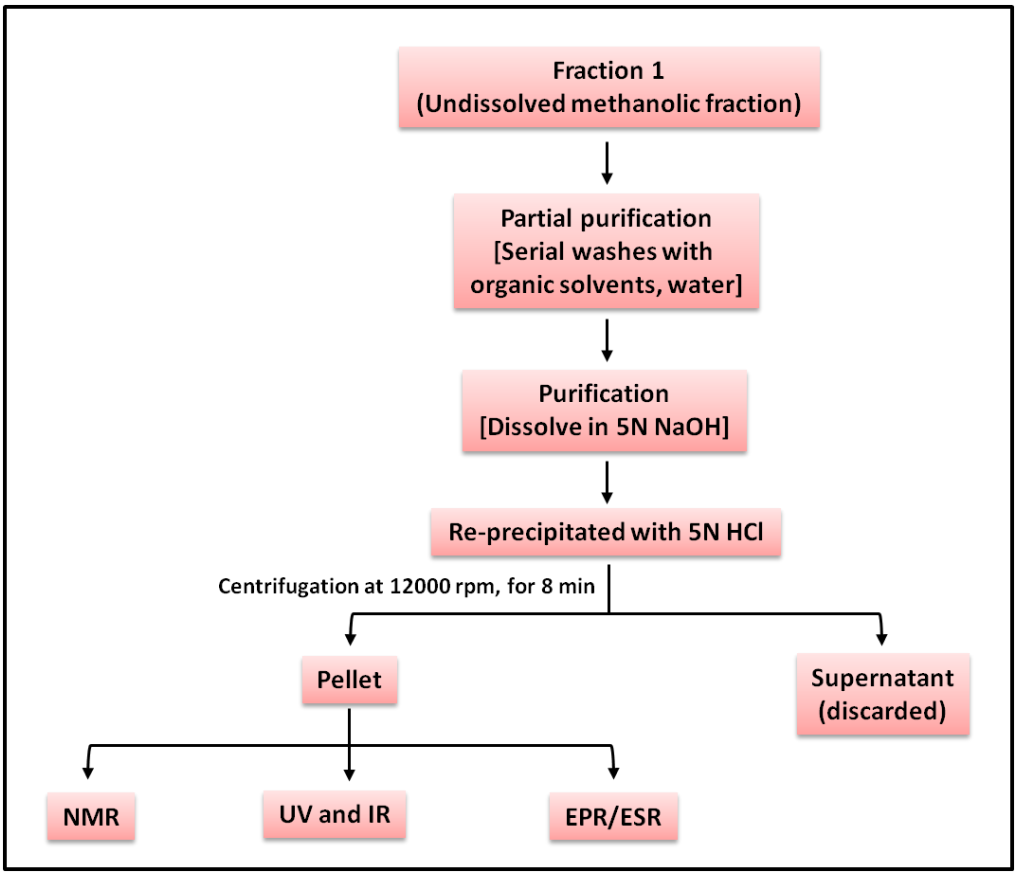
2.3.3 Extraction of extracellular metabolites (exometabolome)

L-Phenylalanine fed and control cultures were grown in 250 ml culture bottles under anoxic conditions and 100 ml cultures in conical flasks incubated with agitation (180 rpm) at 30°C under oxic conditions for 48 h. Growth O.D.s were noted to normalise biomass, the cultures were harvested by centrifugation (12,000 rpm, 10 min, 4°C) and the supernatants were stored in -80° C, later supernatants were freeze dried. The dried samples were stored at -20° C till the GC-MS analysis (Fig. 11)



Flow chart. 1. Extraction of pigments produced by *Rubrivivax benzoatilyticus* JA2 under oxic conditions in presence of L-phenylalanine.

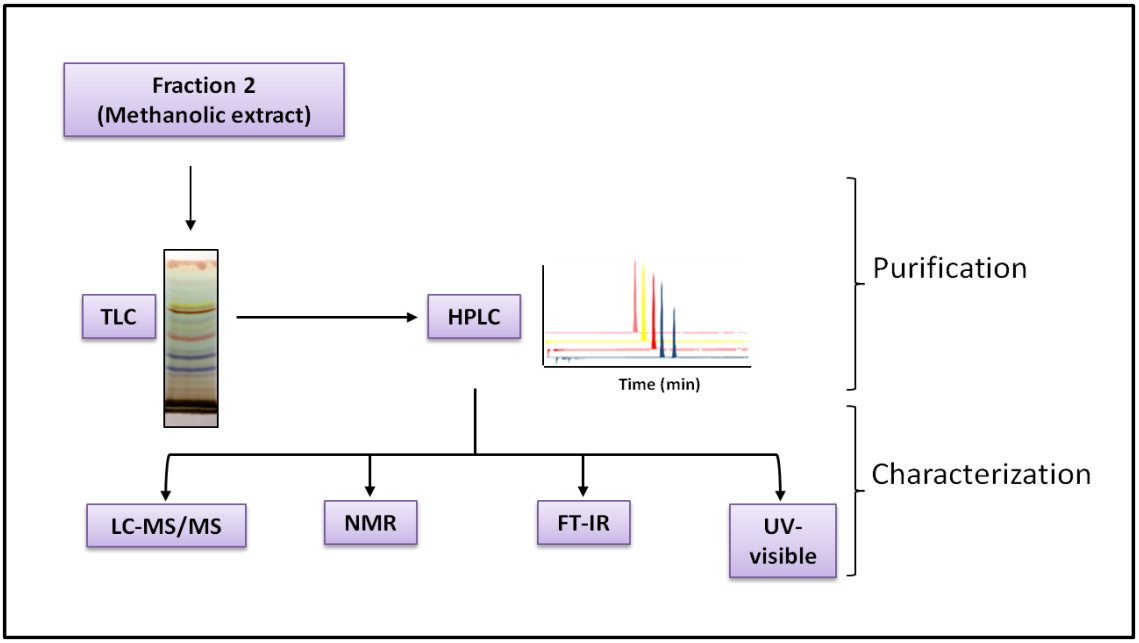
Culture was grown in presence of L-phenylalanine under oxic conditions for 48 h and the culture was harvested and the supernatants were acidified. The brown precipitate formed at the bottom was further fractionated into 2 fractions. Fraction-I (undissolved methanolic fraction) and Fraction-II (methanolic fraction).



Flow chart. 2. Extraction, purification and identification of brown pigment in *Rubrivivax benzoatilyticus* JA2

Fraction 1 obtained from the protocol mentioned in the Flow chart. 1, was further purified with serial washes using organic solvents, water and finally re-precipitated with 5 N HCl and collected on centrifugation. The purified brown pigment was further characterised based on polychem analysis.

NMR; Nuclear Magnetic Resonance spectroscopy, UV-IR; Ultra violet-visible-Infra-Red spectroscopy, EPR/ESR; Electron Paramagnetic Resonance/Electron Spin Resonance, min; minutes



Flow chart. 3. Extraction, purification and characterisation of colored pigments produced by *Rubrivivax benzoatilyticus* JA2.

Colored pigments were isolated and purified from the fraction 2 which was obtained from the brown precipitate according to the protocol given in Flow chart. 1 and the pigments were further characterised based on the polychem analysis.

2.4 Analytical methods

2.4.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was performed using the TLC plates (Silica Gel $60F_{254}$, Merck) to isolate and separate the pigments. The pigments were extracted into the methanol and the methanolic extract is subjected to TLC. Thin layer chromatographic analysis was performed using mobile phase, a mixture of chloroform:methanol:glacial acetic acid (8:1.95:0.05 v/v). The TLC chamber was kept for saturation before the analysis. The sample was loaded on to the TLC plate, distinct colored (blue, pink, yellow and orange) bands were observed. The pigments were partially purified by eluting from the TLC plate and further completely purified by using HPLC.

2.4.2 High Performance Liqud Chromatography (HPLC)

HPLC was done according to Mujahid et al. (2010). HPLC system consists of binary pump, photodiode array detector, fluorescence detector and Phenomenex C-18 column (Luna, 5 µm, 250 x 4.6 mm). Gradient programme was employed to identify the metabolites. Mobile phase used is 1% (v/v) acetic acid in water and acetonitrile (100%). Initially, the programme runs with 1-55% acetonitrile for 25 min and step gradient for 3 min with 100% acetonitrile and finally ends with 1% water wash. The absorption spectra were recorded with photodiode array detector and metabolites were quantified by the peak areas of the known concentrations of their reference authentic standards.

2.4.3 Liquid chromatography tandem mass spectrometry (LC-MS/MS QTOF)

Mass spectrometry analysis was done with QTOF (Agilent 6520) mass spectrometer coupled to HPLC (Agilent 1200 series) equipped with UV-visible detector and auto sampler. Metabolites separation was done on reverse phase column (Phenomenex), C-18 (Luna, 5 u, 150 x 4.6 mm) or C-18 (Luna, 5µm, 50 x 4.6 mm) followed with same gradient programme as mentioned in section 2.4.6 for HPLC analysis except the flow rate, 0.8 ml min^{-1}. The separated metabolites were entered into the electron spray ionisation (ESI) ion source (80^{o} C, cone voltage 15-25V) and detected by mass detector, the absorption spectra were recorded with UV-visible detector. Molecular masses were detected with ESI ion source and the mass fragmentation was done at collision energy 10-20 eV, which is dependent on the nature of the metabolite, mass range for metabolite identification was 50-1000 Da and nitrogen as collision gas. The

metabolites were identified based on mass spectra of the authentic standards and also by comparing the mass spectra of the compounds present in databases (http://metlin.scripps.edu/, http://www.massbank.jp).

2.4.4 Gas chromatography mass spectrometry (GC-MS) of exometabolome

The cultures of control (without L-phenylalanine) and L-phenylalanine fed were harvested and the supernatants were stored immediately at -80°C overnight. Supernatants were freeze dried and the samples were derivatised by adding 30 µl of methoxyamine (40 µg/ml pyridine) and incubated for 90 min at 30 °C under slight agitation, then 60 µl of BSTFA + TMCS (99:1 Sigma Aldrich), incubated at 37 °C for 1 h and subjected to GC-MS analysis. GC-MS analysis was done on the Pegasus HT TOF-MS (Leco, USA) system equipped with Agilent (7890 series) gas chromatography. One microlitre of derivatized sample was injected into HP-5 column (30 m, internal diameter 0.32 mm, thickness 0.25 µm) with helium as carrier gas at a constant flow of 1 ml min^{-1}. Initially temperature was taken to 80°C for 2 min, slowly temperature increased to 240°C with 8°C per min gradient and finally to 325°C with 20°C per min and temperature hold for 2 min. Inlet temperature 280°C, transfer line temperature 225°C, ion source temperature 250°C and ionisation energy -70eV. Mass spectra were recorded at 40-1000 *m/z* with acquisition rate 10 spectra /sec. Data processing was done with 50 as mass threshold, >700 as similarity match, library search matching with NIST, LECO-Fiehn library. Concentration of the metabolites was determined by peak areas. Multivariate statistical analysis was done using online software MetaboAnalyst (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp).

2.4.5 Nuclear magnetic Resonance (NMR) analysis

2.4.5.1 Solid state C-13 NMR

Brown pigment was purified as described in section 2.3.2.1 according to Flow chart. 2. The sample was used for solid state NMR analysis. Solid state ^{13}C-NMR spectra were recorded at room temperature on Avance III Bruker 500 MHz NMR at ^{13}C frequency of 100.62 MHz, ^{1}H 400 MHz, equipped with 5 mm double resonance probe head. Eighty milligrams of freeze dried melanin powder was packed into zirconia rotor. ^{13}C cross polarization/total sideband suppression (CP/TOSS) experiment was run at spinning speed of 7 kHz, 1H 90° pulse length was 2.83 µs and the ^{13}C 180° pulse length was 6.52 µs. The contact time was 2000 µs, acquisition time of 33 ms, the recycle delay was 8 s with total 11000 scans. Chemical shifts were refered to $CDCl_3$ (77.4 ppm).

2.4.5.2 1D, 2D ^{1}H and ^{13}C NMR analysis

The NMR analysis was outsourced at Central California 900 MHz NMR facility. Nuclear magnetic resonance spectra were recorded on Bruker Avance II 900 MHz NMR spectrometer equipped with a TXI cryoprobe at temperature of 298 K and a proton spectral width of 9.6 ppm. 1D ^{1}H NMR spectrum was recorded with 128 k points, 320 scans, and recycle delay of 3 seconds using pulse sequence zg. 1D ^{13}C NMR spectrum was recorded with 64 k points, 92,160 scans, with a recycle delay of 2 seconds using pulse sequence zgpg30. The carbon carrier was set to 102.76 ppm and the carbon spectral width was 245 ppm. 2D COSY and 2D TOCSY experiments were recorded with 4 k points in the direct dimension and 256 points in the indirect dimension, 48 scans per increment, and with a recycle delay of 2 seconds. The 2D COSY using pulse sequence cosydfetgp.2 and for 2D TOCSY, a mixing time of 100 ms, using pulse sequence dipsi2ph. 2D HSQC experiment was recorded using 2 k points in the direct dimension and 200 points in the indirect dimension whereas 2D HMBC was recorded with 4 k points in the direct dimension and 128 points in the indirect dimension. The scans per increment for 2D HSQC, 2DHMBC were 128 and 256 scans with a recycle delay of 1.8 seconds and 1.5 seconds, using pulse sequence hsqcetgpsi2 and hmbcgplpndqf. The carbon carrier was set to 80 ppm for 2DHSQC and 102.76 ppm for 2D HMBC. The carbon spectral width was set to 165 ppm for 2D HSQC and 250 ppm for 2D HMBC.

2.4.6 Fourier Transform Infrared (FTIR) analysis

The spectra was recorded on Bruker, ALPHA FTIR/ATR spectrometer. The purified Blu P1 was dissolved in methanol and the spectra were recorded. Purified Brwn P1 was made into KBR pellet by grinding the sample along with KBR. The pellet was kept in holder and the spectra were recorded in transmittance mode with wavelength ranging 200 to 800 nm

2.4.7 UV-Visible spectral analysis

UV spectral analysis was done in SCHIMADZU spectrophotometer. The spectra were recorded for the purified pigments, Brwn P (dissolved in 5 N NaOH) and Blu P1 (dissolved in methanol). Blu P1 was dissolved in methanol and the pH of the pigment was changed to acidic (pH 2-3) and basic (pH 8-9) with 5 N HCl and 5 N NaOH. The shift in the spectra with pH was recorded.

2.4.8 Electron Spin Resonance (ESR/EPR) analysis

The purified brown precipitate obtained from the L-phenylalanine/L-tyrosine added culture was used for the ESR/EPR and experiment was done on JEOL X-band ESR spectrometry. The dried brown powder was loaded into the ESR tube. The ESR spectra were recorded and analysed.

2.4.9 FAME analysis

Fatty acids of the samples were identified by FAME analysis done by Royal Life Science Pvt Ltd. Fatty acids were saponified, methylated, and extracted by using the protocol of the Sherlock microbial identification system (MIDI Inc.). FAMEs were analysed by gas chromatography.

2.5 Microscopic Techniques

2.5.1 Scanning electron microscopy (SEM)

Purified brown pigment powder was used for SEM analysis. Scanning electron microscopy was done according to the standardised protocol with slight modifications. The brown powder was mounted on glass pieces (0.5x0.5 cm) and dried by critical point dryer using standard protocol, dried samples were fixed to SEM stubs and coated with gold. The specimens were examined by using SEM (Philips XL30 series) at different magnification ranges.

2.6 Biochemical methods

2.6.1 Spectrophotometric assays

2.6.1.1 Estimation of total phenols

Total phenols estimation was done with Folin-Ciocalteau reagent (FC reagent), and 20% (w/v) sodium carbonate (Na_2CO_3). The supernatants (500 µl) of control and L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 were checked for the phenols production by using 500 µl of FC reagent and 2 ml of l20% (w/v) Na_2CO_3. The absorbance at 650 nm was measured and the total phenols were estimated by the standard gallic acid equivalents.

2.6.1.2 Total sugars

Total sugars were measured by phenol-sulfuric acid method (Jiao *et al*., 2010). Fifty microlitres of sample was mixed with 125 µl of the concentrated H_2SO_4 followed by 25 µl of the 10% phenol (w/v) and incubated at 100°C water bath for 10 min. Samples were cooled and absorbance was read at 490 nm. Glucose was used as standard.

2.6.1.3 Total Proteins

Protein content was measured by Bradford's method (Bradford, 1976) which involves the binding of Coomassie brilliant blue G-250 to proteins. Dissolve 100 mg of G-250 in 100 ml of absolute/distilled ethanol/methanol and kept on a shaker for ~ 60 min. Hundred millilitre of 88% O-phosphoric acid was added, mixed well, volume made up to 500 ml with distilled water. Filtered through Whattman No.1, diluted 1:1 with water and the absorption was read at 550 nm against water blank (OD ~ 1.1), reagent was stored in 4° C. Twenty microliter of the sample was mixed with 180 µl of the water and to this 800 µl of the Bradford's reagent was added, incubated for 5 min and absorbance was read at 595 nm against reagent blank. Bovine serum albumin (BSA) was used as standard.

2.7 Enzyme activities

2.7.1 Preparation of cell free extracts

The control and L-phenylalanine fed cultures were centrifuged at 4° C, 10,000 rpm for 8 min. The supernatants were discarded and the pellets were washed thrice with milliQ water and then with 50 mM Tris buffer (pH 7.5) and finally suspended in 4 ml of 50 mM Tris buffer (pH 7.5). Cells were lysed by sonication (BANDELIN, 8 cycles, 43% amplitude) and the cell lysate was centrifuged with 16,000 rpm, at 4° C for 20 min. The cell free extract (supernatant) was separated and collected into a vial and then used for assay as an enzyme source.

2.7.2 Aminotransferase /Transaminase activity

Aminotransferase/transaminase activity was carried in 3 ml volume, with L-phenylalanine (1 mM) as substrate, α-ketoglutarate (1 mM) and pyridoxal phosphate (PLP; 50 µM) as co factors. Reaction was started with addition of appropriate enzyme source to reaction mixture. The reaction was incubated for 1 h at 37° C and the reaction was stopped with 5 N HCl after 1 h incubation and the blank was pre-denatured with 5 N HCl. The reaction mixture was

centrifuged at 16000 rpm, at 4° C for 20 min, supernatant was taken and extracted thrice with 3 volumes of ethyl acetate. The ethyl acetate extracts were pooled and evaporated to dryness in rotary flash evaporator. Finally, the extract was dissolved in 100 µl of methanol (HPLC grade). L-phenylalanine consumption or phenylpyruvic acid formation was measured with HPLC. One unit (U) of enzyme activity was expressed as amount of enzyme required for the formation of 1 µmol of phenylpyruvic acid and specific activities were measured.

2.7.3 Dehydrogenase activity

Dehydrogenase activity was carried in 3 ml volume, with phenylpyruvic acid (2 mM) as precursor, NADH (0.5 mM) as co factor. Reaction was started with addition of appropriate enzyme source to reaction mixture. The reaction was incubated for 1 h at 37° C and the reaction was stopped with 5 N HCl after 1 h incubation and the blank was pre-denatured with 5 N HCl. The reaction mixture was centrifuged, supernatant was taken and extracted thrice with 3 volumes of ethyl acetate. The ethyl acetate extracts were pooled and evaporated to dryness in rotary flash evaporator. Finally, the extract was dissolved in 100 µl of methanol (HPLC grade). The phenylpyruvic acid consumption or phenyllactic acid formation was measured using HPLC. One unit (U) of enzyme activity was expressed as amount of enzyme required for the formation of 1 µmol of phenyllactic acid and specific activities were measured.

2.7.4 Pyruvate decarboxylase activity

Pyruvate decarboxylase activity was carried in 3 ml volume, with phenylpyruvic acid (2 mM) as precursor. Reaction was started with addition of appropriate enzyme source to reaction mixture. The reaction was incubated for 1h at 37° C and the reaction was stopped with 5 N HCl after 1 h incubation and the blank was pre-denatured with 5 N HCl. The reaction mixture was centrifuged, supernatant was taken and extracted thrice with 3 volumes of ethyl acetate. The ethyl acetate extracts were pooled and evaporated to dryness in rotary flash evaporator. Finally, the extract was dissolved in 100 µl of methanol. The product phenylacetaldehyde was detected in GC-MS.

2.7.5 Aldehyde Dehydrogenase activity

Aldehyde dehydrogenase activity was carried in 3 ml volume, with phenylacetaldehyde (2 mM) as precursor, NAD (0.5 mM) as co factor. Reaction was started with addition of appropriate enzyme source to reaction mixture. The reaction was incubated for 1h at 37° C and

the reaction was stopped with 5 N HCl after 1 h incubation and the blank was pre-denatured with 5 N HCl. The reaction mixture was centrifuged, supernatant was taken and extracted thrice with 3 volumes of ethyl acetate. The ethyl acetate extracts were pooled and evaporated to dryness in rotary flash evaporator. Finally, the extract was dissolved in 100 µl of methanol (HPLC grade). The phenylacetaldehyde consumption or phenylacetic acid formation was measured using HPLC.

2.8 Metabolomic methods

2.8.1 Stable isotope labeled L-phenylalanine feeding

Rubrivivax benzoatilyticus JA2 was grown photoheterotrophically for 24 h (0.2 OD_{660nm}) then culture was fed with C^{13} ring labeled L-phenylalanine (L-phenylalanine-^{13}C), control culture was fed with unlabeled L-phenylalanine and incubated for 48 h. Cells were harvested and the supernatant was extracted for phenols as described in 2.6.1.1. The ethyl acetate extracts were analysed by LC-MS/MS as described in section 2.4.3 (Fig. 10).

2.8.2 GC-MS data processing and statistical analysis

Data obtained from GC-MS analysis of exometabolome as described in section 2.4.4 was used for processing and subjected to statistical analysis. Relative abundance of metabolites were calculated from peak areas of the metabolites obtained from GC-MS analysis. Peak areas were normalised to dry weight of the sample and more than 50% of missing values excluded from the data. Data was log transformed (log_2) and subjected to quantile normalization using MetaboAnalyst and normalised data was used for multivariate statistical analysis. Principal component analysis (PCA), partial least-squares discrimination analysis (PLS-DA) and hierarchical cluster analysis (HCA) was performed using MetaboAnalyst software (*www.metaboanalyst.ca/MetaboAnalyst*). HCA was done using Pearson correlation as distance matrix. Data was subjected to *t*-test analysis to identify metabolites significantly regulated between control and L-phenylalanine fed cultures of *R. benzoatilyticus* JA2. Metabolites having fold change >2 and *p* value <0.05 were considered as statistically significant, finally significant metabolites were annotated to metabolic pathways according to KEGG (*Kyoto Encyclopedia of Genes and Genomes, www.genome.jp/kegg*) database to identify metabolic pathways influenced in control and L-phenylalanine fed culture of *R. benzoatilyticus* JA2 under oxic and anoxic conditions (Fig. 11).

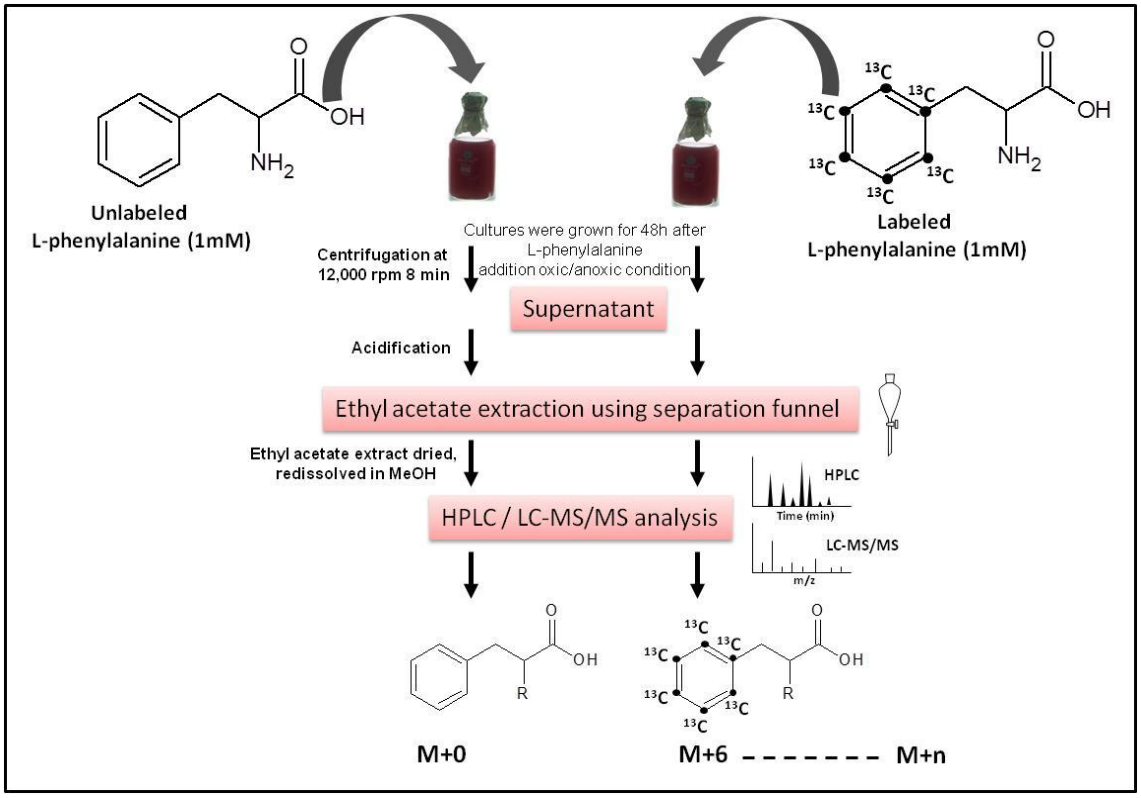


Fig. 10. Schematic representation of stable isotopic metabolite profiling in *Rubrivivax benzoatilyticus* JA2

Cultures were as mentioned in the Flow chart. 1, both with unlabeled and labeled L-phenylalanine (1 mM) and the further protocol followed as mentioned in the Fig. 9.

M+0, molecular ion mass without labeleing, M+6 to M+n, molecular ion mass with ring labelled 6 to n carbon atoms with increase in their masses.

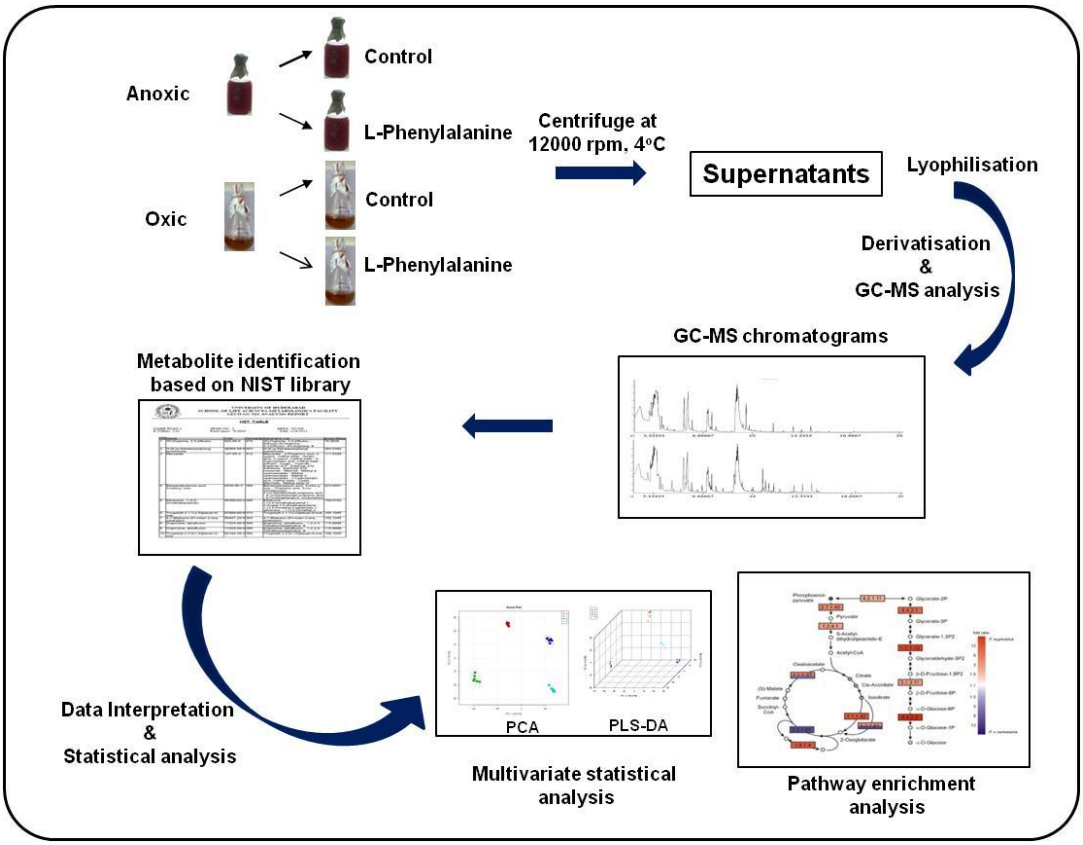


Fig. 11. Schematic representation of GC-MS based metabolic profiling of *Rubrivivax benzoatilyticus* JA2

GC-MS; gas chromatographic mass spectrometry, NIST; National Institute of Standards and Technology, PCA; principal component analysis, PLS-DA; partial lest square discriminant analysis,

RESULTS

3.0 RESULTS

3.1 Anoxic L-phenylalanine metabolism in Rubrivivax benzoatilyticus JA2

3.1.1 Phenols production by phototrophic Proteobacteria

Nine phototrophic *Proteobacteria* were screened for the production of phenols. All the bacteria were grown with/without L-phenylalanine as sole source of nitrogen, for 48 h and the supernatants were used for quantification of total phenols. All the bacteria tested were positive for phenols production (Table.1). Among the screened *Proteobacteria*, *Rubrivivax benzoatilyticus* JA2 and *Marichromatium indicum* JA100 produced significantly high phenolic compounds in the presence of L-phenylalanine compared to other *Proteobacteria*. *Rubrivivax benzoatilyticus* JA2, produced high quantity of phenols (8.8 µg. mg dry wt^{-1}) compared to *Marichromatium indicum* JA100 (3.3 µg. mg dry wt^{-1}) in presence of L-phenylalanine. Phenols production was significantly high in presence of L-phenylalanine compared to control by *R. benzoatilyticus* JA2 (Table.1). Phenols production also observed in the absence of L-phenylalanine by *R. benzoatilyticus* JA2 (2.8 µg. mg dry wt^{-1}) and *M. indicum* JA100 (2.3 µg. mg dry wt^{-1}). Significant difference in the production of phenolic compounds in the presence or absence of L-phenylalanine was not observed in *Marichromatium indicum* JA100 (Table.1).

3.1.2 Growth, utilisation of L-phenylalanine and phenols production by Rubrivivax benzoatilyticus JA2

Rubrivivax benzoatilyticus JA2 was able to grow on L-phenylalanine as sole source of nitrogen but not as carbon source under anoxic conditions. Growth of *R. benzoatilyticus* JA2 was observed in the presence of L-phenylalanine as sole source of nitrogen with simultaneous utilisation of L-phenylalanine and production of phenols (Fig. 12A). Within 24 h of growth, L-phenylalanine was completely consumed (0.9 – 0.92 mM) with 2.2 - 2.8 µg.mg dry wt^{-1} phenols production (Fig. 12A). Total phenols production was significantly increased by nearly 10 folds in the L-phenylalanine fed (72 µg.mg dry wt^{-1}) cultures of *R. benzoatilyticus* JA2 compared with control (7.5 µg.mg dry wt^{-1}) (Fig. 12B).

Table.1. Production of phenols by a few phototrophic *Proteobacteria* grown with/without L-phenylalanine under anoxic conditions.

Phototrophic *Proteobacteria*	Total phenols (µg. mg dry wt^{-1})	
	Control (without L-phenylalanine)	L-Phenylalanine (1mM)
Alphaproteobacteria		
Rhodobacter aestuarii JA296^T	0.1	0.2
Rhodobacter azotoformans KA25^T	0.1	0.1
Rhodobacter littoralis JA541^T	0.1	0.2
Rhodobacter maris JA276^T	0.2	0.2
Rhodobacter sphaeroides DSM158^T	0.2	0.7
Rhodobacter vinaykumarii JA123^T	0.1	0.1
Betaproteobacteria		
Rubrivivax benzoatilyticus JA2^T	2.3	8.8
Gammaproteobacteria		
Marichromatium purpuratum DSM1591^T	0.2	0.4
Marichromatium indicum JA100^T	2.8	3.3

Malate grown mid log phase (24 h grown) culture (O.D $_{660\ nm}$ 0.25) was used as inoculum (10%) for the experiment. Cultures were grown photoheterotrophically in nitrogen deficient (without NH_4Cl) malate (carbon source) medium for 24 h and then fed with L-phenylalanine (1 mM) as nitrogen source, incubated for 48 h. After 48 h of incubation the cultures were centrifuged and the supernatants of the cultures were used for the phenols estimation with Folin-Ciocalteau reagent.

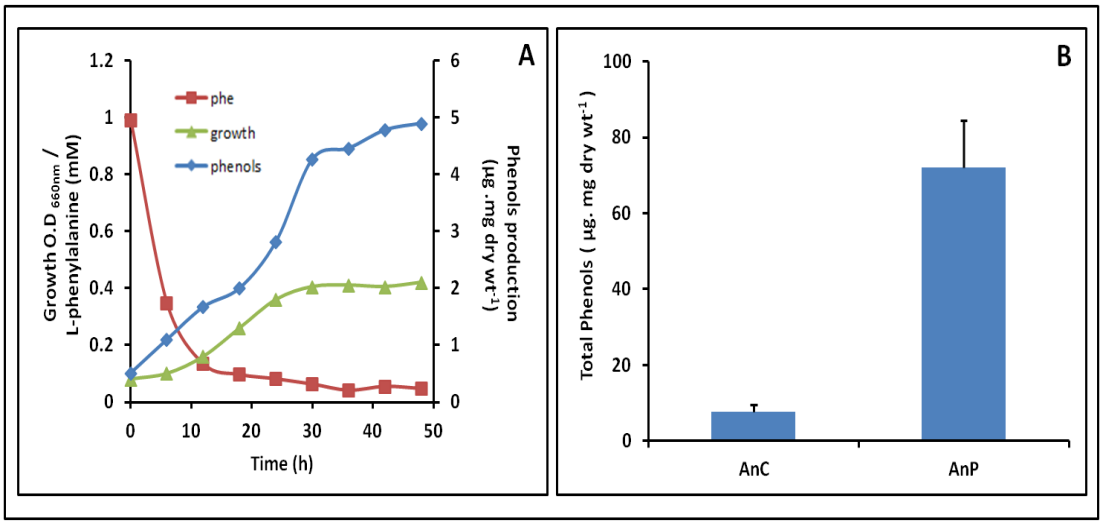


Fig. 12. Production of phenols with simultaneous utilisation of L-phenylalanine by *Rubrivivax benzoatilyticus* JA2 under anoxic condition.

Phenols production, L-phenylalanine consumption and growth of *R. benzoatilyticus* JA2 **(A)**. Total phenols production by *R. benzoatilyticus* JA2 fed with/without L-phenylalanine **(B).**

Growth conditions were same as mentioned in the Table. 1. Experiment was done by taking malate grown mid log phase (24 h) culture (O.D $_{660nm}$ 0.25) of *R. benzoatilyticus* JA2 as inoculum (10%). The supernatants of *R. benzoatilyticus* JA2 cultures grown with/without L-phenylalanine for 48 h was taken for the estimation of L-phenylalanine and total phenols. AnC, Anoxic control (without L-phenylalanine); AnP, Anoxic L-phenylalanine fed.

3.1.3 Comparitive metabolite profiling of control and L-phenylalanine fed cultures of Rubrivivax benzoatilyticus JA2

The supernatants of L-phenylalanine fed and control cultures of *R. benzoatilyticus* JA2 were acidified, extracted into ethyl acetate and analysed using HPLC. The comparative HPLC metabolite profiling of ethyl acetate fractions of both L-phenylalanine fed and control conditions was done at 260 nm. HPLC chromatograms of the ethyl acetate fractions were overlaid and the peaks specific to L-phenylalanine fed condition were indicated with asterisk marks. Eighteen peaks were observed particularly in L-phenylalanine fed condition compared to control (Fig. 13). The ethyl acetate fraction of L-phenylalanine fed culture supernatants of *R. benzoatilyticus* JA2 was taken further for the identification of metabolites through HPLC, LC and GC-MS analysis.

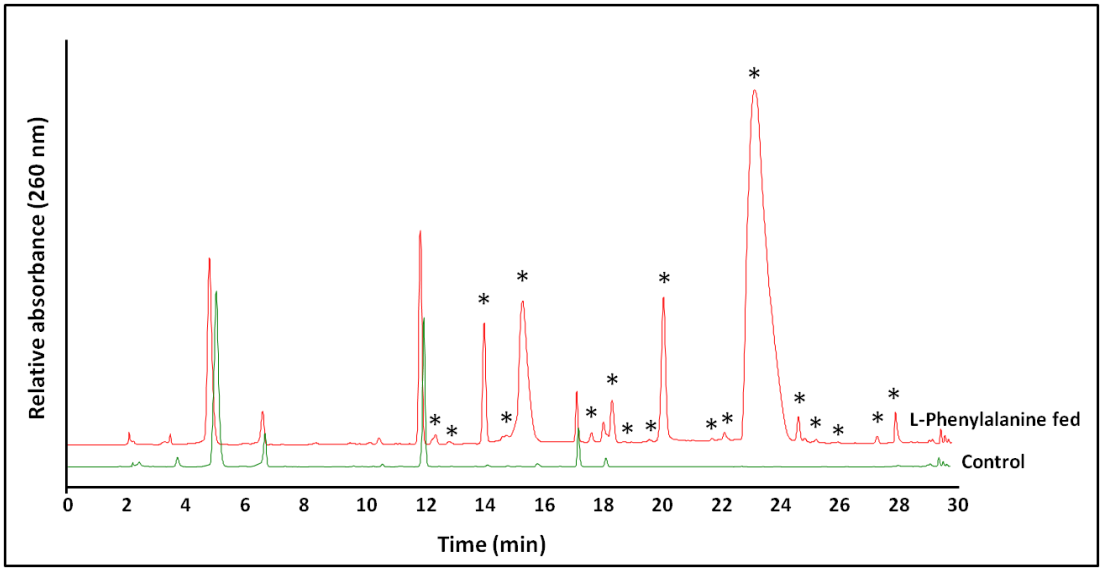


Fig. 13. Comparitive metabolite profiling of ethyl acetate fractions obtained from the supernatants of *Rubrivivax benzoatilyticus* JA2 grown with/without L-phenylalanine under anoxic condition.

Chromatograms represented in green color, control (without L-phenylalanine) and red, L-phenylalanine fed. Peaks labelled with '*' mark were present only in L-phenylalanine fed condition.

Experimental conditions were same as described in Fig. 12, except for extraction of acidified supernatants with ethyl acetate. The ethyl acetate extracts were subjected to HPLC analysis according to the standard protocol mentioned in analytical methods section 2.4.2 using ACN/water as mobile phase. The metabolites were analysed at 260 nm.

3.1.4 HPLC and LC-MS/MS based metabolite identification of ethyl acetate fractions of control and L-phenylalanine fed culture

The ethyl acetate fractions obtained from the L-phenylalanine fed culture of *R. benzoatilyticus* JA2 were subjected to HPLC and LC-MS/MS analysis to identify the metabolites. Metabolites were identified using HPLC, based on the retention times and absorption spectra. The LC-MS analysis was done to identify the metabolites based on the mass spectra and mass fragmentation in reference with the authentic standards obtained from the databases like Metlin (http://metlin.scripps.edu/), Mass Bank (http://www.massbank.jp/), Human Metabolome Database (http://www.hmdb.ca/). Identified metabolites were further confirmed by co-eluting with authentic standards.

The peak at retention time (R_t) 12 min having absorption of 254 nm (Fig. 14A) and molecular ion mass of 137[M^-] (Fig. 14B) was identified as 4- hydroxybenzoic acid (4-HBA). The peak at R_t 12.4 min having absorption maxima 273 nm (Fig. 14C) and mass 151[M^-] (Fig. 14D) was identified as 4-hydroxyphenylacetic acid (4-HPAA). Metabolite eluted at R_t 14 min with absorption 283 nm (Fig. 15A) and mass 123[M^-] (Fig. 15B) was identified as 4-hydroxybenzaldehyde (4-HBald); metabolite with R_t 15 min, absorption 303 nm (Fig. 15C) and mass 179[M^-] (Fig. 15D) was identified as 4-hydroxyphenylpyruvic acid (4-HPPY). The peak with R_t 17.8 min, had absorption maxima at 273 nm (Fig. 16A) and mass 121[M^-] (Fig. 16B) was identified as benzoic acid (BA); peak at 18.3 min, with absorption 257, 254, 263, 247 nm (Fig. 16C) having mass of 135[M^-] (Fig. 16D) was identified as phenylacetic acid (PAA). Peak with R_t 22.5 min, absorption maxima at 287 nm (Fig. 17A) and mass 163[M^-] (Fig. 17B) was identified as phenylpyruvic acid (PPY). The metabolites identified based on HPLC and LC-MS/MS analysis were further confirmed with co-elution of available authentic standards.

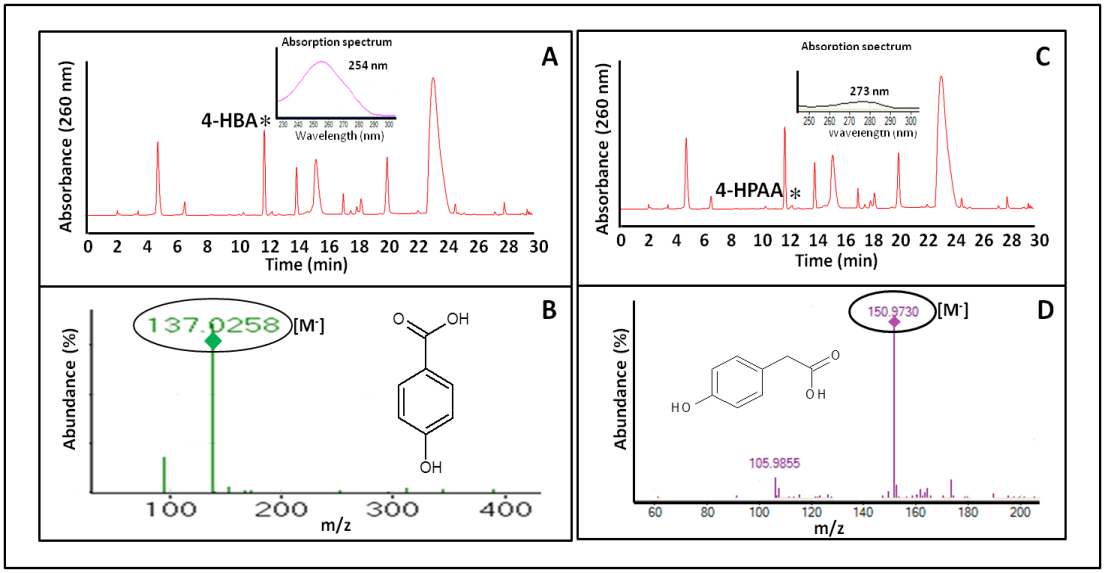


Fig. 14. HPLC and LC-MS metabolite profiling of ethyl acetate extracts obtained from the supernatants of *Rubrivivax benzoatilyticus* JA2 grown in presence of L-phenylalanine.

HPLC chromatograms with * marked peaks indicated the metabolites 4-HBA with R_t 12 min and its absorption at 254 nm **(A)** and mass spectrum, **(B)**; 4-HPAA at R_t 12.4 min with absorption at 273 nm **(C)** and mass spectrum **(D)**.

Experimental conditions were similar as mentioned in Fig. 13 except for mass analysis. The ethyl acetate extracts were subjected to LC-MS/MS analysis. LC-MS conditions were as described in analytical methods (2.4.3) under materials and methods section. Absorption spectra were represented as insert in HPLC chromatograms. 4-HBA, 4-Hydroxybenzoic acid; 4-HPAA, 4-Hydroxyphenylacetic acid; R_t, Retention time; [M^-], mass ionisation in negative mode, *m/z*, mass to charge ratio.

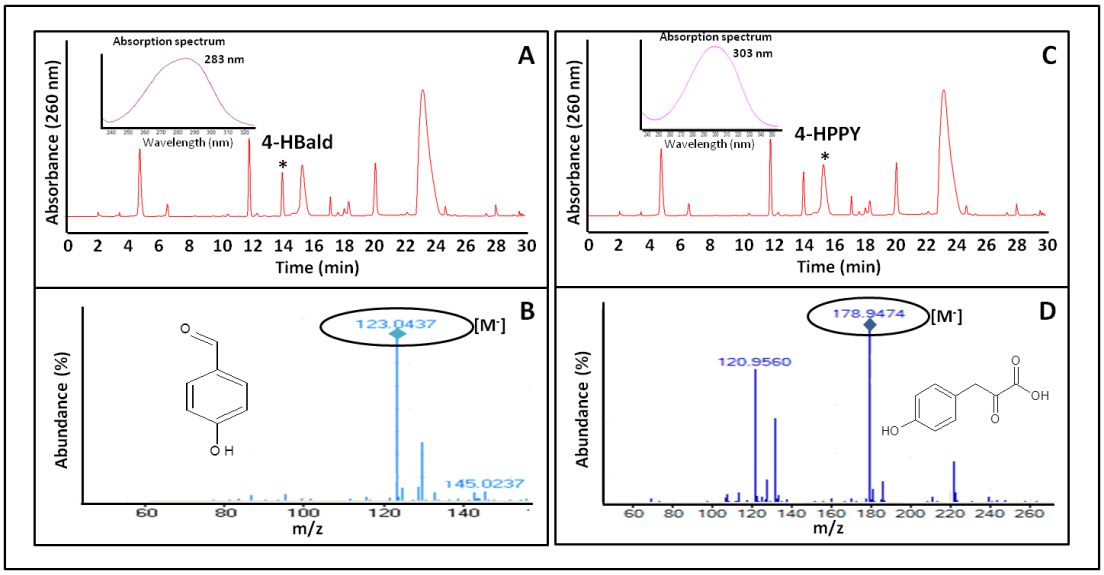


Fig. 15. HPLC and LC-MS metabolite profiling of ethyl acetate extracts obtained from the supernatant of *Rubrivivax benzoatilyticus* JA2 grown in presence of L-phenylalanine.

HPLC chromatograms with * marked peaks indicated the metabolites 4-HBald with R_t 14 min and its absorption at 283 nm **(A)** and mass spectrum **(B)**; 4-HPPY with R_t 15 min having absorption at 303 nm **(C)** and mass spectrum **(D)**.

Experimental conditions were similar as mentioned in Fig. 14. Absorption spectra were represented as insert in HPLC chromatograms. 4-HBald, 4-Hydroxybenzaldehyde; 4-HPPY, 4-Hydroxyphenylpyruvic acid; R_t, Retention time; [M^-], mass ionisation in negative mode, *m/z*, mass to charge ratio.

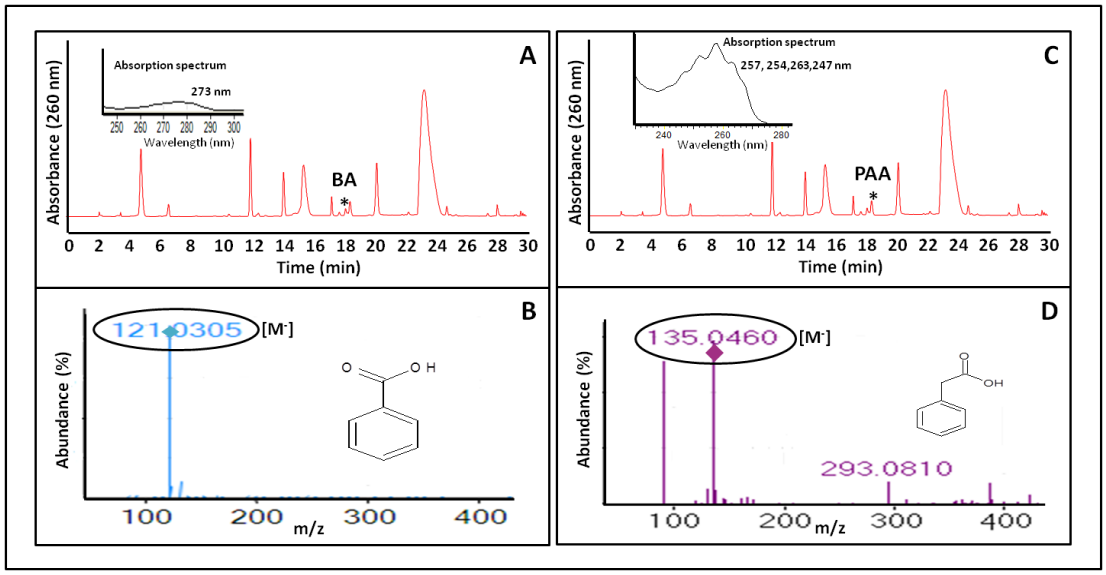


Fig. 16. HPLC and LC-MS metabolite profiling of ethyl acetate extracts obtained from the supernatant of *Rubrivivax benzoatilyticus* JA2 grown in presence of L-phenylalanine.

HPLC chromatograms with * marked peaks indicated the metabolites BA with R_t 18.3 min having absorption at 273 nm **(A)** and mass spectrum **(B)**; PAA with R_t 18.5 min having absorption at 257, 254, 263, 247 nm **(C)** and mass spectrum **(D)**.

Experimental conditions were similar as mentioned in Fig. 14. Absorption spectra were represented as insert in HPLC chromatograms. BA, Benzoic acid; PAA, Phenylacetic acid; R_t, Retention time; [M^-], mass ionisation in negative mode, *m/z*, mass to charge ratio.

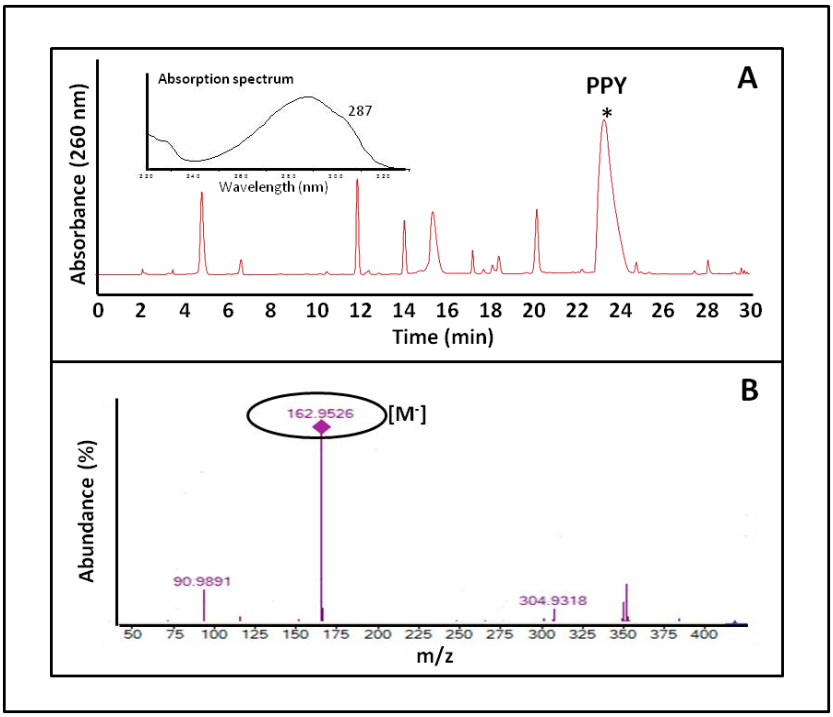


Fig. 17. HPLC and LC-MS metabolite profiling of ethyl acetate extracts obtained from the supernatant of *Rubrivivax benzoatilyticus* JA2 grown in presence of L-phenylalanine.

HPLC chromatogram with * marked peaks indicated the metabolite PPY with R_t 22.5 min having absorption at 287 nm **(A)** and mass spectrum **(B)**.

Experimental conditions were similar as mentioned in Fig. 14. Absorption spectrum was represented as an insert in HPLC chromatogram. PPY, Phenylpyruvic acid; R_t, Retention time; $[M^-]$, mass ionisation in negative mode, *m/z*, mass to charge ratio.

3.1.5 GC-MS analysis of the ethyl acetate extract of L-phenylalanine fed cultures of Rubrivivax benzoatilyticus JA2

The ethyl acetate extract obtained from the supernatant of L-phenylalanine fed *R. benzoatilyticus* JA2, was subjected to gas chromatography mass spectrometric analysis to identify the volatile aryl metabolites. Metabolites were identified by comparing the mass spectra of the authentic standards obtained from National Institute of Standards and Technology (NIST) library. GC chromatogram represented the peak at R_t 3.2 min (Fig. 18A), with mass of 120 m/z (Fig. 18B) was identified as phenylacetaldehyde (PAcld) and another peak at R_t 3.8 min (Fig. 18C), with mass of 108 m/z (Fig. 18D) identified as benzyl alcohol (BAlc). Peaks with R_t's 4.2 and 9.3 min (Fig. 19 A,C) had masses 106 m/z and 179 m/z (derivatized mass), respectively (Fig. 19B,D) and the metabolites were identified as benzaldehyde (Bald), 2-phenylethanol (PEtoh) respectively.

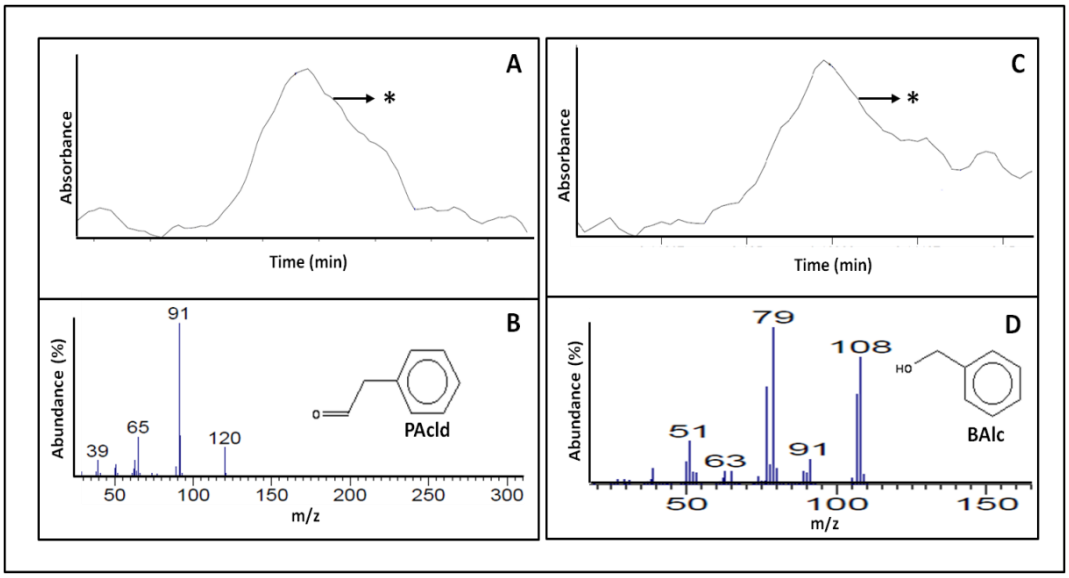


Fig. 18. GC-MS analysis of derivatised ethyl acetate extracts of L-phenylalanine fed culture supernatants of *Rubrivivax benzoatilyticus* JA2.

Phenylacetaldehyde **(**PAcld) with R_t 3.2 min **(A)** and its respective mass spectrum **(B)**; BAlc at R_t 3.8 min **(C)** and its mass spectrum **(D)**.

Experimental conditions were similar as mentioned in Fig. 13 except for the GC-MS analysis. Samples were derivatisation with BSTFA:TMCS and subjected to GC-MS analysis as mentioned under analytical methods (2.4.4) in materials and methods section. Metabolites were identified by comparing the spectra with NIST library. PAcld, Phenylacetaldehyde; BAlc, Benzyl alcohol; R_t, Retention time.

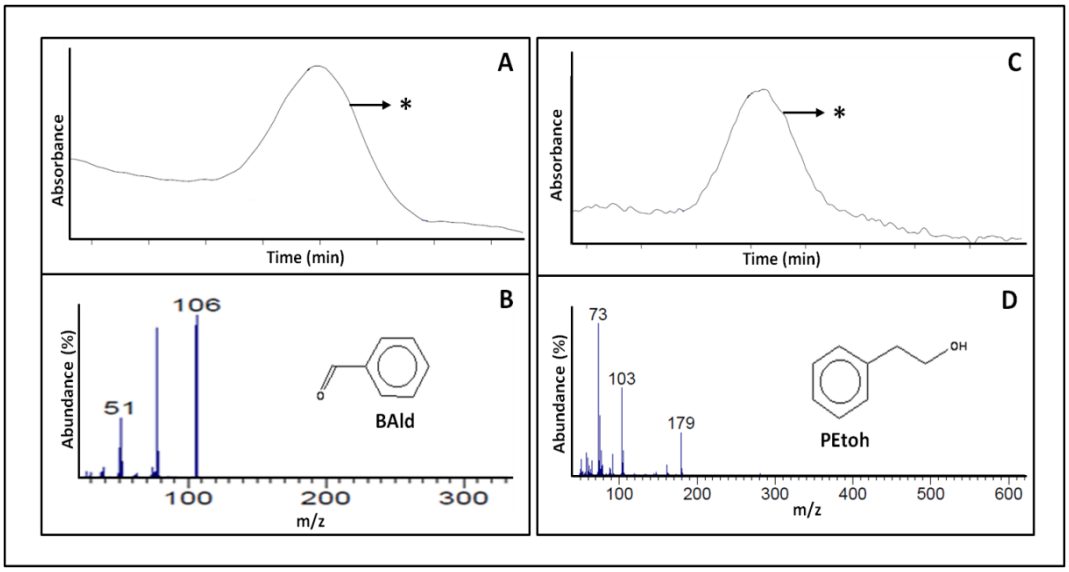


Fig. 19. GC-MS analysis of derivatised ethyl acetate extracts of *Rubrivivax benzoatilyticus* JA2 grown in presence of L-phenylalanine.

GC chromatograms with * marked peaks of metabolites, BAld with R_t 4.2 min **(A)** and its mass spectrum **(B)**; PEtoh at R_t 9.3 min **(C)** and its mass spectrum **(D)**.

Experimental conditions were similar as mentioned Fig. 18. BAld, Benzaldehyde; PEtoh, Phenylethanol; R_t, Retention time.

3.1.6 Characterisation and production of L-phenyllactic acid (PLA)

3.1.6.1 Polychem analysis of peak with R_t 15.5 min

Comparative metabolite profiling of L-phenylalanine and control HPLC chromatogram indicated that the metabolite at R_t 15.5 min was produced only in L-phenylalanine fed condition. Peak with R_t 15.5 min and absorption spectra 257, 254, 263, 247 nm indicated the metabolite as a phenyl derivative (Fig. 20). LC-MS/MS analysis revealed the mass spectra with molecular ion mass of 167[M^+] and its mass fragmentation with masses of 149,121,103 m/z (Fig. 21A). FTIR spectra with specific spectral shifts ranging 3400 cm^{-1} attributed to –OH group, 1700 cm^{-1} indicated the -C=O, 1000-1200 cm^{-1} represented the -C-O group and 600-800 cm^{-1} contribute to -C-H stretching. The specific spectral shifts are in accordance with standard phenyllactic acid (Fig. 21B). ^1H and ^{13}C NMR spectral analysis represented the characteristic chemical shifts similar to phenyllactic acid. The ^1H NMR analysis revealed the specific spectral shift indicated the aromatic group at 7.2 ppm. Spectral shifs at 2.7 and 2.9 ppm indicated the $–CH_2$ group; 4.1

ppm indicated the –C-H group indicated the spectral shifts similar to the phenyllactic acid (Fig. 21C). ^{13}C NMR spectral analysis revealed the specific spectral shifts at 126, 128, 129, 137 ppm attributed to the aromatic group; 39 ppm represented the $–CH_2$ group; 71 ppm, represented –CH group and 175 ppm, –COOH group ofthe phenyllactic acid (Fig. 21D). Chirality of the metabolite was confirmed based on Circular Dichroism spectroscopic analysis (CD spectroscopy). Polychem analysis validates the peak with R_t 15.5 min as L-phenyllactic acid.

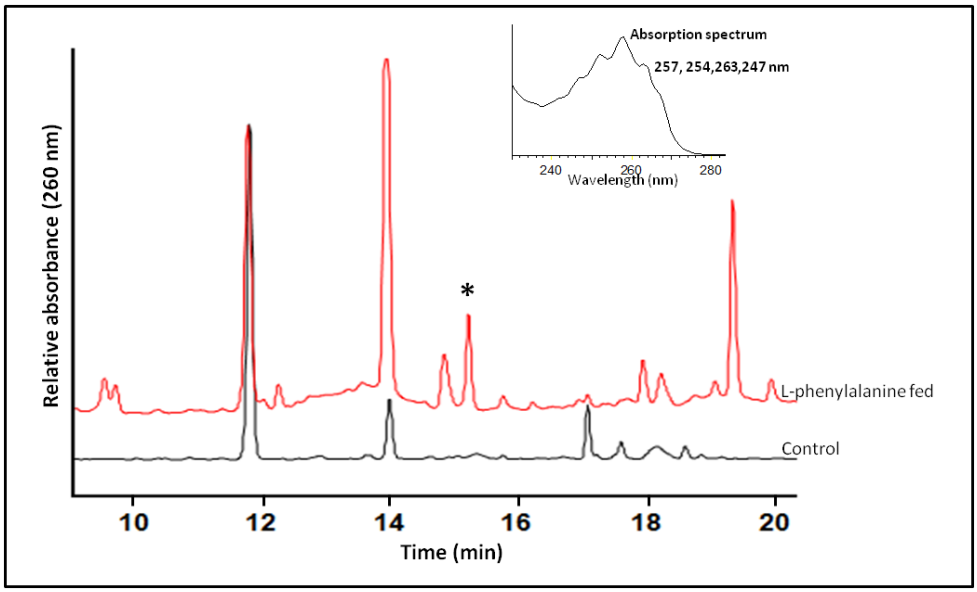


Fig. 20. HPLC comparative metabolite profiling of ethyl acetate extracts of *Rubrivivax benzoatilyticus* JA2 grown with/without L-phenylalanine under anoxic condition.

HPLC chromatograms with * marked peak with R_t 15.5 min present only in L-phenylalanine fed condition. Chromatograms represented in green color, control (without L-phenylalanine); red, L-phenylalanine fed.

Experimental conditions were similar as mentioned in Fig. 13. R_t, retention time.

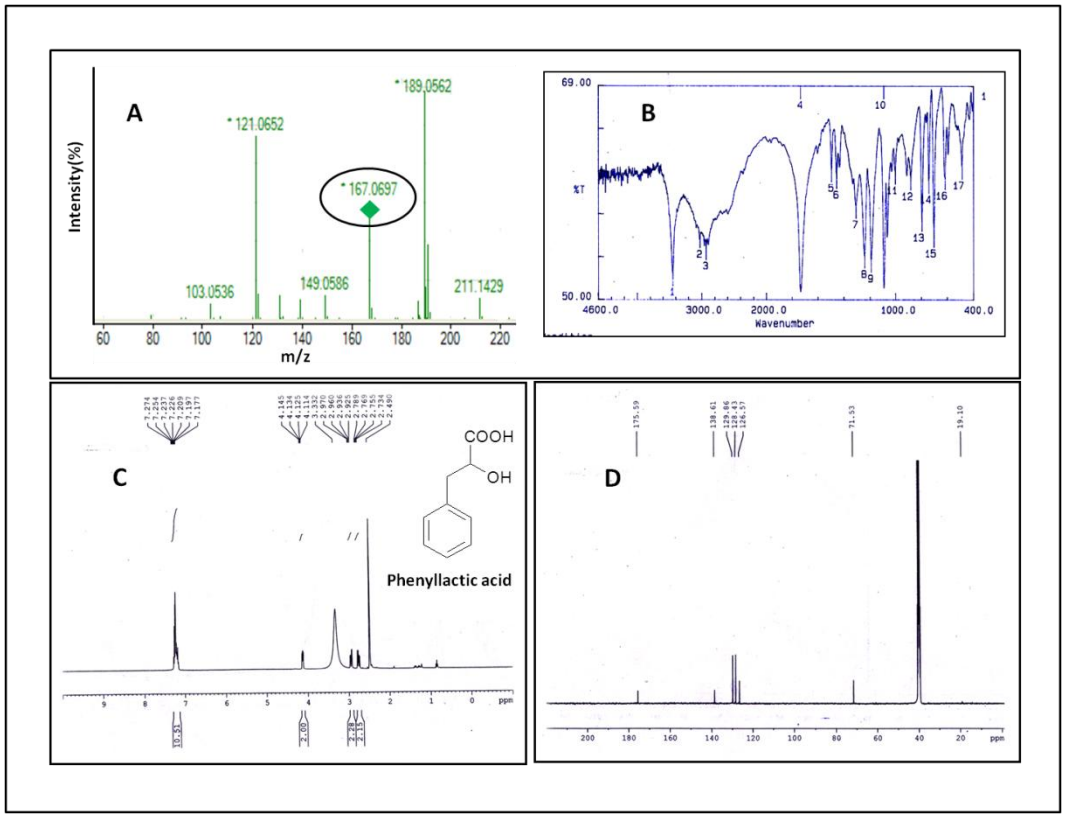


Fig. 21. Polychem analysis of purified metabolite (R_t 15.5 min).

The purified metabolite with R_t 15.5 was characterised based on LC-MS analysis, mass 167 $[M+H]^+$ ionized in positive mode **(A)**; FTIR spectrum **(B)**; NMR spectra, H^1NMR **(C)** and C^{13}NMR **(D)** indicated the metabolite as phenyllactic acid (PLA).

Experimental conditions were similar as mentioned in Fig. 13, except for the purification of compound through HPLC and characterisation was done based on polychem analysis which includes LC-MS, NMR and FTIR analysis as mentioned under analytical methods (2.4) in material and methods section. PLA, Phenyllactic acid; R_t, Retention time.

3.1.6.2 Production of L-phenyllactic acid by Rubrivivax benzoatilyticus JA2

3.1.6.2a L-phenylalanine utilisation and L-phenyllactic acid production

L-phenylalanine utilisation with simultaneous production of L-phenyllactic acid was observed in *R. benzoatilyticus* JA2. After 24 h of growth on L-phenylalanine (along the growth), 0.9 mM of L-phenylalanine was consumed and 0.4 mM of L-phenyllactic acid was produced with 45% conversion of L-phenylalanine into L-phenyllactic acid (Fig. 22A). Conversion efficiency of L-phenylalanine to L-phenyllactic acid was increased to 70% when L-phenylalanine was added to 24 h grown (mid log phase) culture of *R. benzoatilyticus* JA2.

3.1.6.2b Effect of carbon sources on L-phenyllactic acid production

Carbon sources influenced the production of L-phenyllactic acid in *R. benzoatilyticus* JA2. All the carbon sources supported the production of L-phenyllactic acid in presence of L-phenylalanine as nitrogen source (Fig. 22B). The yields of L-phenyllactic acid were high (0.8-0.92 mM) in presence of sugar carbon sources compared to non-sugar carbon sources (0.18-0.38 mM) (Fig. 22B).

3.1.6.2c Substrate dependent L-phenyllactic acid production

The yield of L-phenyllactic acid was influenced by different concentrations of L-phenylalanine and phenylpyruvic acid. Production of L-phenyllactic acid increased in a dose dependent manner upto 4 mM of L-phenylalanine and remained static at higher concentrations (Fig. 22C). In contrast, L-phenyllactic acid production increased with increase in concentration of phenylpyruvic acid, an immediate precursor of L-phenyllactic acid (Fig. 22D).

3.1.6.2d L-phenyllactic acid production under different growth conditions

Production of L-phenyllactic acid was influenced by different growth conditions such as anoxic light, anoxic dark, aerobic light, aerobic dark and with resting cells. L-phenyllactic acid production was significantly high (56.9 ± 2.35 mg. l^{-1}) when the culture fed with L-phenylalanine as sole source of nitrogen compared to additional source (25.2 ± 1.09 mg. l^{-1}). The yield of L-phenyllactic acid was high under phototrophic conditions (light anoxic) compared to chemotrophic (dark aerobic; 3.6 ± 1.908 mg. l^{-1}). The production of L-phenyllactic acid also varied with resting cells of *R. benzoatilyticus* JA2 (12.9 mg. l^{-1}) (Table. 2).

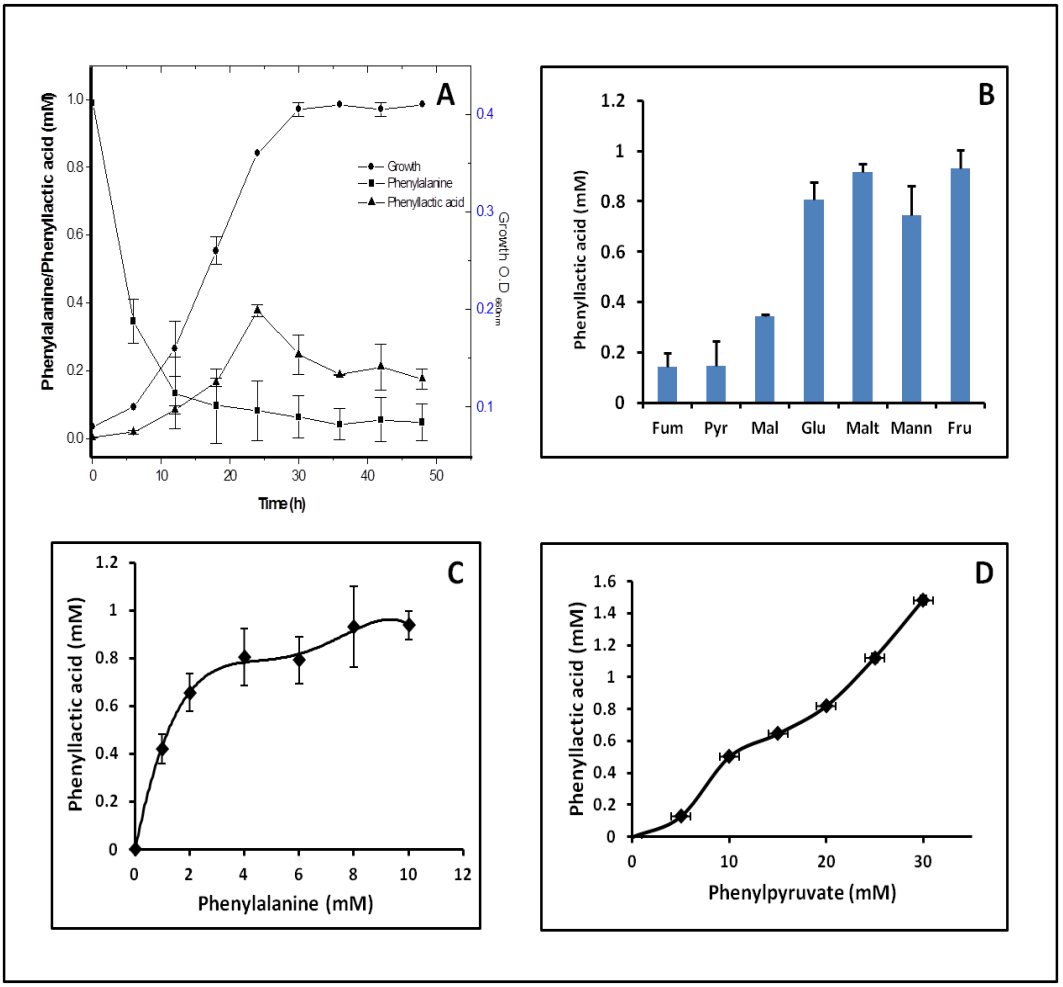


Fig. 22. Production of phenyllactic acid (PLA) by *Rubrivivax benzoatilyticus* JA2 fed with L-phenylalanine (1mM).

Utilization of L-phenylalanine (1 mM) for its growth with simultaneous production of Phenyllactic acid (PLA) **(A)**. Effect of carbon source on phenyllactic acid production **(B)**. Phenyllactic acid production under different concentrations of L-phenylalanine **(C)** and phenylpyruvic acid **(D)**.

Experiments were done as mentioned in Fig. 13 except for the growth conditions with different carbon sources, and different concentrations of L-phenylalanine and phenylpyruvic acid were added to media. Data expressed as mean ± standard deviation of three independent experiments. Fum, fumarate; Pyr, pyruvate; Mal, malate; Glu, glucose; Malt, maltose; Mann, mannose; Fru, fructose.

Table.2. Phenyllactic acid (PLA) production under different physiological conditions in L-phenylalanine fed culture of *Rubrivivax benzoatilyticus* JA2

Growth conditions	PLA production mg. lit^{-1}
Anaerobic Light	
Control (without L-phenylalanine)	ND
L-phenylalanine as additional source	25.2 ± 1.09
L-phenylalanine as nitrogen source	56.9 ± 2.35
Anaerobic dark	No growth
Aerobic Light	16.8 ± 4.22
Aerobic Dark	3.6 ± 1.908
Resting cells	12.9

Experimental conditions were same as mentioned in Fig. 20, except for the ethyl acetate extract of the cultures grown under different growth conditions in presence and absence of light, and also with resting cells.

Data expressed as mean ± standard deviation of three independent experiments. PLA, phenyllactic acid; ND, not detected.

3.1.7 Stable isotope studies of L-phenylalanine metabolism under anoxic condition

Rubrivivax benzoatilyticus JA2 was fed with ^{13}C labeled (stable isotope) and unlabeled L-phenylalanine. The ethyl acetate extracts obtained from the acidified supernatants of both labeled/unlabeled L-phenylalanine fed cultures were subjected to LC-MS/MS analysis to detect the L-phenylalanine catabolites. The metabolites derived from labeled L-phenylalanine were identified based on +6/+12 mass units increase in their molecular ion masses in comparison with those of unlabeled metabolites.

Peak with R_t 22.7 min had molecular ion mass of 163[M^-] in unlabeled fraction and 169[M^-] in labeled fraction which corresponds to phenylpyruvic aicd (Fig. 23A) with 6 mass units increase. Peak at R_t 15.5 min, molecular ion mass 167[M^+] in unlabeled fraction, having mass of 173[M^+] in labeled fraction with 6 units increase in the mass (Fig. 23B) represented the L-phenyllactic acid. Mass spectrum of the metabolite with molecular ion mass 206[M^-] in unlabeled fraction and its mass increased by 6 units to 212[M^-] in labeled fraction indicated the N-acetyl-L-phenylalanine (Fig. 24). Mass spectrum of unlabeled fraction had mass of 179[M^-], whose mass increased by 6 units to 185[M^-] was indicated as 4-hydroxyphenylpyruvic acid (Fig. 25). Unlabeled fraction with molecular ion mass 137[M^+] and labeled fraction with mass 143[M^+] represented the phenylacetic acid (Fig. 26). Molecular ion mass 151[M^-] in unlabeled fraction and mass 157[M^-] in labeled fraction indicated 4-hydroxyphenylacetic acid. Metabolites like 4-hydroxybenzaldehyde , phenylacetaldehyde with molecular ion masses 123[M^+], 121[M^+], increased by 6 units to 129[M^+], 127[M^+].

Incorporation of six ^{13}C labelled carbon atoms in the metabolites phenylpyruvic acid, L-phenyllactic acid, N-acetyl-L-phenylalanine, phenylacetic acid, 4-hydroxyphenylpyruvic acid, 4-hydroxyphenylacetic acid and 4-hydroxybenzaldehyde indicated that these were catabolites of L-phenylalanine. The metabolites in unlabeled fraction having masses 341[M^-], 203[M^+] and their mass increased by 6 units, to 347[M^-] and 209[M^+] respectively in labeled fractions, which were remain unidentified. Amongst the 10 metabolites with increase in mass by 6 units, eight metabolites were identified and two remain unidentified (Table. 3). Some aryl metabolites like 4-hydroxybenzoic acid, benzoic acid were not the L-phenylalanine catabolites as there is no increase of 6 mass units increase in their molecular ion masses.

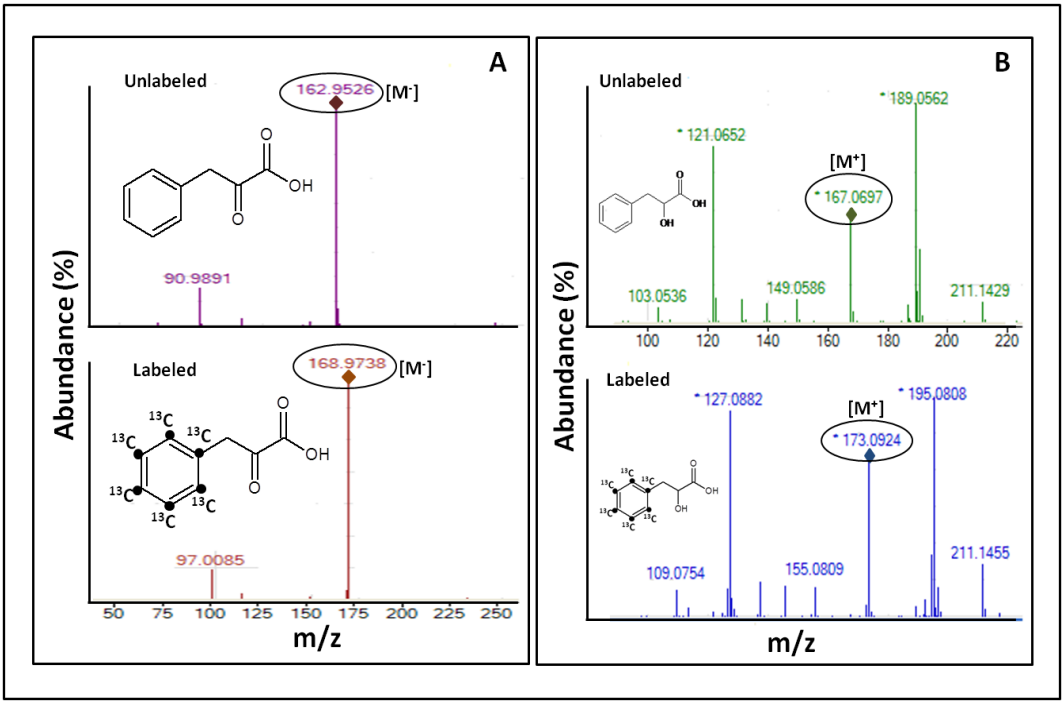


Fig. 23. Stable isotope probing of L-phenylalanine catabolism in *Rubrivivax benzoatilyticus* JA2 under anoxic condition.

Mass spectra of unlabeled and labeled phenylpyruvic acid (PPY) ionized in negative mode **(A)** and phenyllactic acid (PLA) ionized in positive mode **(B)**

Experimental conditions were similar as mentioned in Fig. 14 except for the growth of the cultures of *R. benzoatilyticus* JA2 fed separately with unlabeled or labeled L-phenylalanine. PLA, Phenyllactic acid; PPY, Phenylpyruvic acid; [M^-], mass ionisation in negative mode; [M^+], mass ionisation in positive mode. The structures with labelled and unlabelled carbons in the aromatic ring.

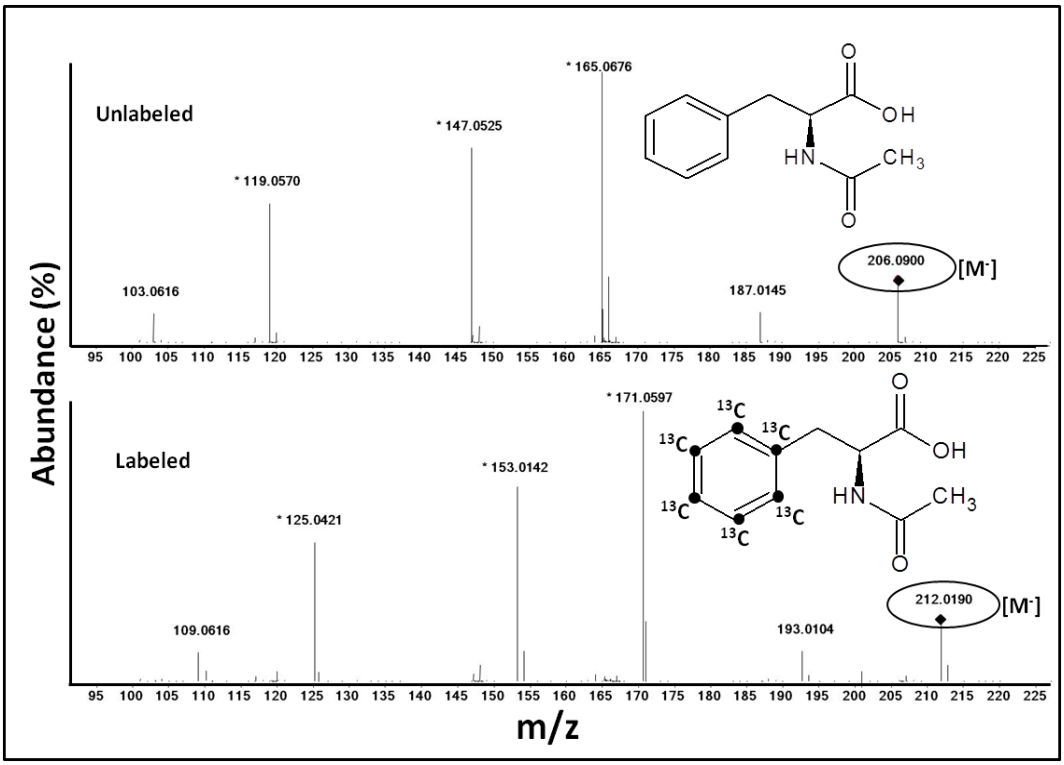


Fig. 24. Stable isotope probing of L-phenylalanine catabolism in *Rubrivivax benzoatilyticus* JA2 under anoxic condition.

Mass spectrum of unlabeled and labeled N-Acetyl-L-phenylalanine, ionized in negative mode.

Experimental conditions were similar as mentioned in Fig. 23. [M^-], mass ionisation in negative mode. The structures with labeled and unlabeled carbons in the aromatic ring, *m/z*, mass to charge ratio.

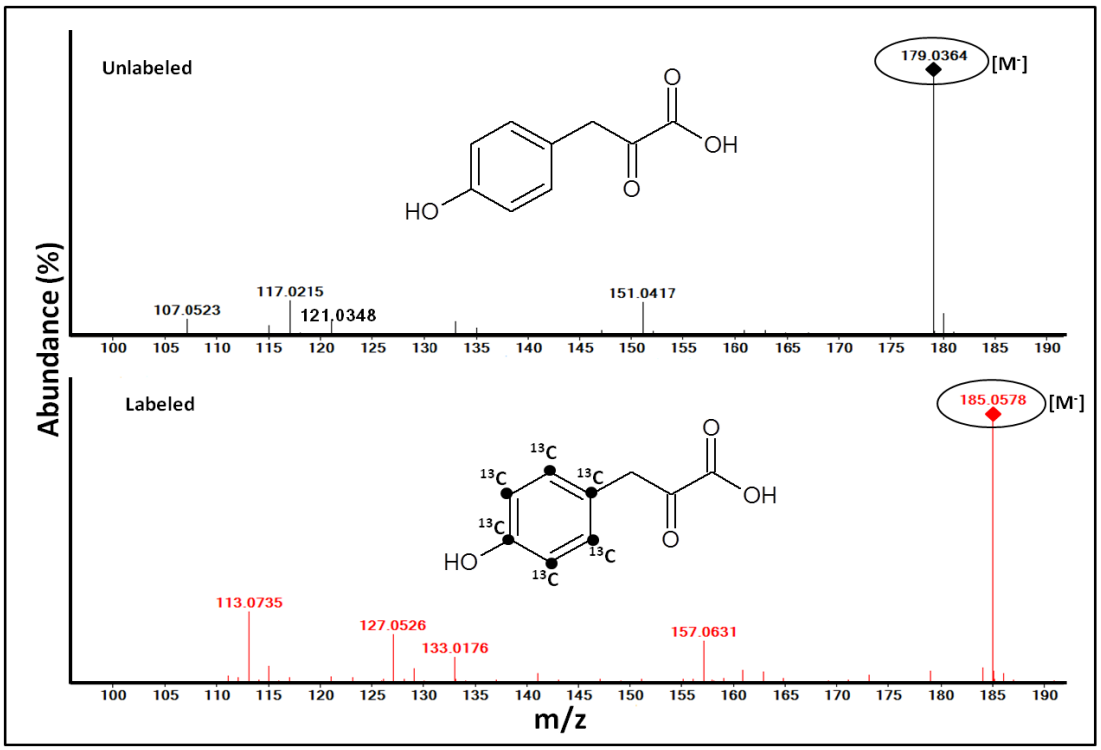


Fig. 25. Stable isotope probing of L-phenylalanine catabolism in *Rubrivivax benzoatilyticus* JA2 under anoxic condition.

Mass spectrum of unlabelled and labelled 4-Hydroxyphenylpyruvic acid (4-HPPY), ionized in negative mode.

Experimental conditions were similar as mentioned in Fig. 23. 4-HPPY, 4-Hydroxyphenylpyruvic acid; [M^-], ionisation in negative mode. The structures with labeled and unlabeled carbons in the aromatic ring. *m/z*, mass to charge ratio.

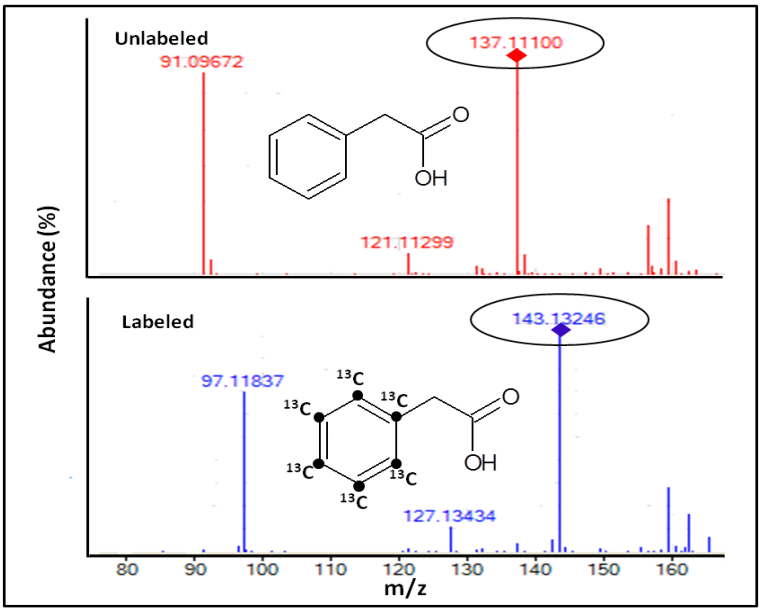


Fig. 26. Stable isotope probing of L-phenylalanine catabolism in *Rubrivivax benzoatilyticus* JA2 under anoxic condition.

Mass spectrum of unlabelled and labelled phenylacetic acid (PAA).

Experimental conditions were similar as mentioned in Fig. 23. PAA, phenylacetic acid; [M^+], ionisation in positive mode. The structures with labeled and unlabeled carbons in the aromatic ring. *m/z*, mass to charge ratio.

Table.3. An overview of the stable isotope analysis of L-phenylalanine catabolism in *Rubrivivax benzoatilyticus* JA2 under anoxic conditions.

Retention time (min)	Absorbance (nm)	Mass (m/z)		Compounds identified
		C12	C13	
5.16	275	151[M⁻]	156[M⁻]	α-Hydroxyphenylacetic acid
5.75	283	123[M⁺]	129[M⁺]	4-Hydroxybenzaldehyde
5.86	300	179[M⁻]	185[M⁻]	4-Hydroxyphenylpyruvate
6.34	257,263,252	167[M⁺]	173[M⁺]	L-Phenyllactic acid
7.7	260	209[M⁺]	215[M⁺]	N-Acetyl L-Phenylalanine
8.79	257,252,263	137[M⁺]	143[M⁺]	Phenylacetic acid
8.88	250	121 M⁺]	127 M⁺]	Phenylacetaldehyde
10.16	287	163[M⁻]	169[M-]	Phenylpyruvic acid
6.08	-	341[M⁻]	347[M⁻]	Un identified
8.9	260	203[M⁺]	209[M⁺]	Un identified

Metabolites were identified based on the absorption spectra and mass analysis. Molecular ion masses of unlabled and labelled masses with +6/+12 mass units (a.m.u) were represented. Experimental conditions were same as mentioned in Fig. 23.

3.1.8 Enzyme assays of L-phenylalanine catabolism under anoxic conditions

3.1.8.1 Aminotransferase/Transminase and Lactate/phenyllactate dehydrogenase activity

Aminotransferase/transaminase catalyses the conversion of L-phenylalanine to phenylpyruvic acid with co-factor 2-oxoglutarate (Fig. 27A) and lactate/phenyllactate dehydrogenase catalyses the conversion of phenylpyruvic acid to L-phenyllactic acid with NADH as co factor (Fig. 28A). Aminotransferase/transminase and lactate/phenyllactate dehydrogenase activities were demonstrated with cell free extracts of L-phenylalanine fed culture pellets of *R. benzoatilyticus* JA2. The substrate L-phenylalanine acts as amino donor and 2-oxoglutarate as amino acceptor in the presence of pyridoxal phosphate in aminotransferase/transminase reaction. Phenylpyruvic acid acts as substrate, NADH as co factor in lactate/phenyllactate dehydrogenase reaction (Fig. 27A, 28A). The products formed were analysed through HPLC. The products phenylpyruvic acid at R_t 22.7 min (Fig. 27B) and L-phenyllactic acid at 15.5 min (Fig. 28B) were observed only in enzyme reaction but not in pre-denatured blank. The specific activities of aminotransferase/transminase (0.2 U mg^{-1}protein) (Fig. 27C) and lactate/phenyllactate dehydrogenase (0.7 U mg^{-1}protein) (Fig. 28C) were measured.

3.1.8.2 Pyruvate/phenylpyruvate decarboxylase activity

Pyruvate/phenylpyruvate decarboxylase catalyses the phenylpyruvate conversion to phenylacetaldehyde with release of carbondioxide (Fig. 29A). Enzyme assay performed and the product phenylacetaldehyde was observed in the reaction but not in pre-denatured blank. The product phenylacetaldehyde was analysed through GC-MS analysis (Fig. 29B) and the product phenylacetaldehyde had R_t 3.2 min and its mass spectrum (120 m/z) (Fig. 29C).

3.1.8.3 Aldehyde/phenylacetaldehyde dehydrogenase activity

The enzyme catalyses the conversion of phenylacetaldehyde to phenylacetic acid with NAD^+ as co factor (Fig. 30A). Enzyme activity demonstrated the production of phenylacetic acid (PAA). The product phenylacetic acid (PAA) was observed only in reaction but not detected in pre-denatured blank (Fig. 30B). The product phenylacetic acid was detected in HPLC at R_t 18.5 min.

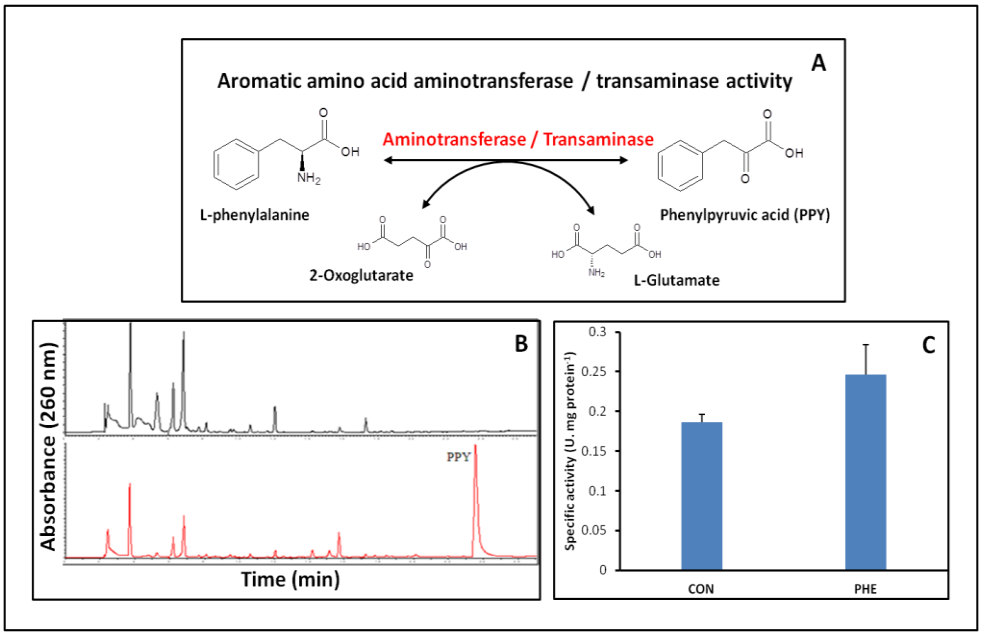


Fig. 27. Aminotransferase/Transaminase activity of *Rubrivivax benzoatilyticus* JA2 under anoxic condition.

Enzyme reaction of aminotransferase/transaminase, which converts L-phenylalanine to phenylpyruvic acid (PPY) **(A)**. HPLC analysis of aminotransferase /transaminase activity in pre-denatured blank (black chromatogram) in comparison to reaction mixture after 1 h incubation (red chromatogram) **(B)**. Product phenylpyruvic acid (PPY) observed at R_t 22.5 min. Specific activities of aminotransferase/transaminase in *R.benzoatilyticus* JA2 **(C)**.

Experimental conditions were similar as mentioned in Table.1 except for *R. benzoatilyticus* JA2 culture pellets of control and L-phenylalanine fed were taken for the experiment. Cell free extracts were prepared as mentioned in the materials and mthods section (2.7.1) and used as enzyme source for the enzyme activities. Enzyme assay was performed with cell lysate as enzyme source and it contained L-phenylalanine (1 mM) as substrate, co factor 2-oxoglutarate and tris buffer (pH-7.5) and incubation for 1 h at 37°C and analysed through HPLC. Reaction was stopped by adding 5 N HCl.

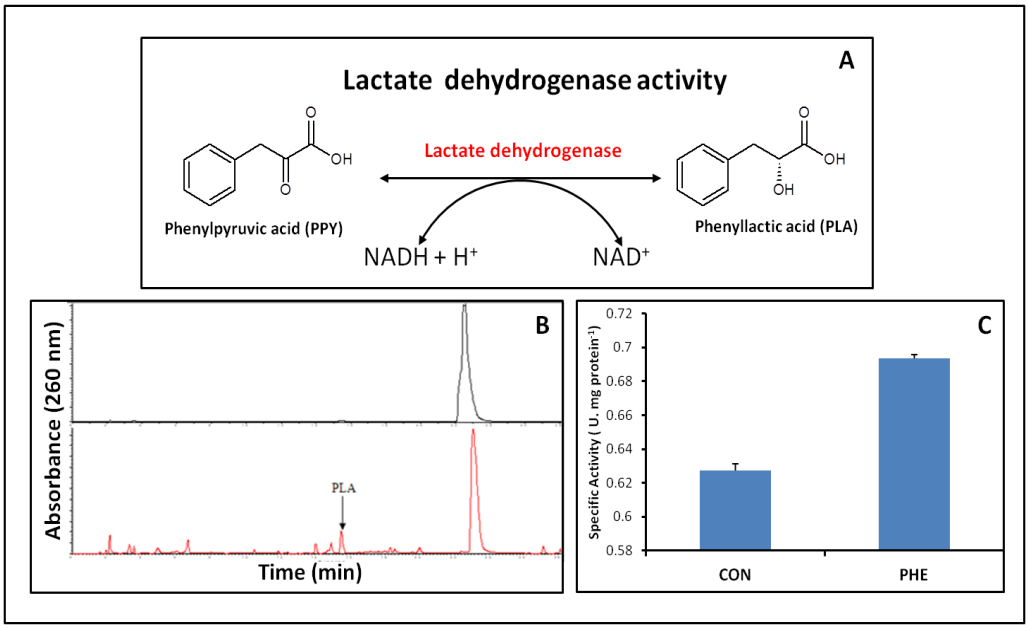


Fig. 28. Lactate/phenyllactate dehydrogenase activity of *Rubrivivax benzoatilyticus* JA2 under anoxic conditions.

Enzyme reaction of lactate dehydrogenase which converts phenylpyruvic acid (PPY) to phenyllactic acid (PLA) **(A)**. HPLC analysis of lactate dehydrogenase activity in pre-denatured blank (black chromatogram) in comparison to reaction mixture after 1 hour incubation (red chromatogram) **(B)**. Product phenyllactic acid (PLA) observed at R_t 15.5 min. Specific activities of lactate dehydrogenase in *R.benzoatilyticus* JA2 **(C)**.

Experimental conditions were similar as mentioned in Fig. 27, except for the precursors and co factors, phenylpyruvic acid (1 mM) as substrate, NADH as co factor.

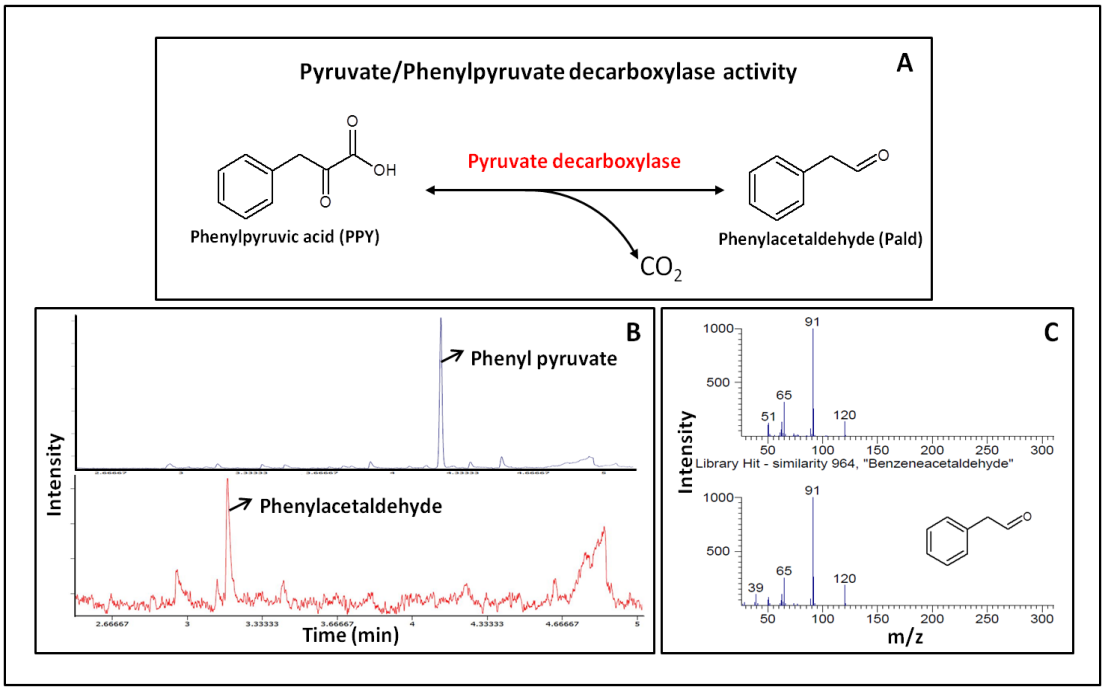


Fig. 29. Pyruvate/phenylpyruvate decarboxylase activity of *Rubrivivax benzoatilyticus* JA2 under anoxic condition.

Enzyme reaction of pyruvate decarboxylase which converts phenylpyruvic acid (PPY) to phenylacetaldehyde (PAcld) **(A)**. GC-MS analysis of pyruvate decarboxylase activity in pre-denatured blank (black chromatogram) in comparison to reaction mixture after 1 hour incubation (red chromatogram) **(B)** and the respective mass spectrum of the product phenylacetaldehyde (PAcld).

Experimental conditions were similar as mentioned in Fig. 27, except for precursor phenylpyruvic acid (1 mM) and GC-MS analysis to detect the product formed. The GC-MS conditions were similar as mentioned under analytical methods (2.4.4) in materials and methods section.

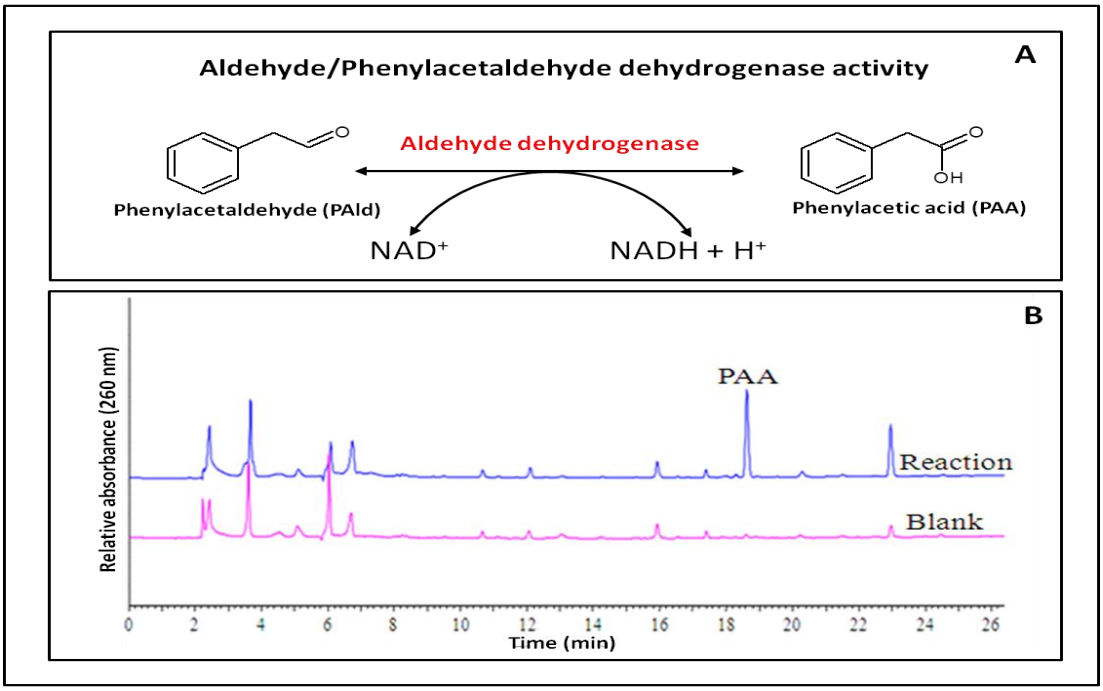


Fig. 30. Aldehyde/phenylacetaldehyde dehydrogenase activity of *Rubrivivax benzoatilyticus* JA2 under anoxic condition.

Enzyme reaction of aldehyde dehydrogenase which converts phenylacetaldehyde (PAcld) to phenylacetic acid (PAA) **(A)**. HPLC analysis of aldehyde dehydrogenase activity in pre-denatured blank (pink chromatogram) in comparison to reaction mixture after 1 h incubation (blue chromatogram) **(B)**. The product phenylacetic acid (PAA) at R_t 18.5 min.

Experimental conditions were similar as mentioned in Fig. 27, except for precursor phenylacetaldehyde (1 mM).

3.1.9 Elucidation of L-phenylalanine catabolic pathways in Rubrivivax benzoatilyticus JA2 *under anoxic condition*

Ehrlich's pathway of L-phenylalanine catabolism was observed in *R. benzoatilyticus* JA2 under anoxic conditions which was evident from the identification of metabolites, phenylpyruvic acid, L-phenyllactic acid, phenylacetic acid, phenylacetaldehyde, 2-phenylethanol. The pathway was confirmed with enzyme activities such as aminotransferase/transaminase, lactate dehydrogenase, pyruvate decarboxylase, and aldehyde dehydrogenase activities in cell free extracts of *R. benzoatilyticus* JA2. Apart from Ehrlich's pathway, other catabolic pathways were also observed under anoxic conditions which were evident from the identification of metabolites like N-acetyl-L-phenylalanine and 4-hydroxyphenylpyruvic acid, 4-hydroxyphenylacetic acid indicated L-phenylalanine catabolism probably via tyrosine. Enzyme activities such as aromatic amino acid aminotransferase, N-acetyltransferase confirm the catabolic pathways of L-phenylalanine under anoxic condition in *R. benzoatilyticus* JA2. The catabolites of L-phenylalanine were confirmed with ^{13}C labeled stable isotope studies. Based on the identification of metabolites, stable isotope studies and enzyme activities, L-phenylalanine catabolism was proposed in *R. benzoatilyticus* JA2. Stable isotope studies revealed some unidentified L-phenylalanine catabolites indicating the other unknown catabolic pathways of L-phenylalanine. The existence of multiple catabolic pathways of L-phenylalanine under anoxic conditions was proposed in *R. benzoatilyticus* JA2 (Fig. 31).

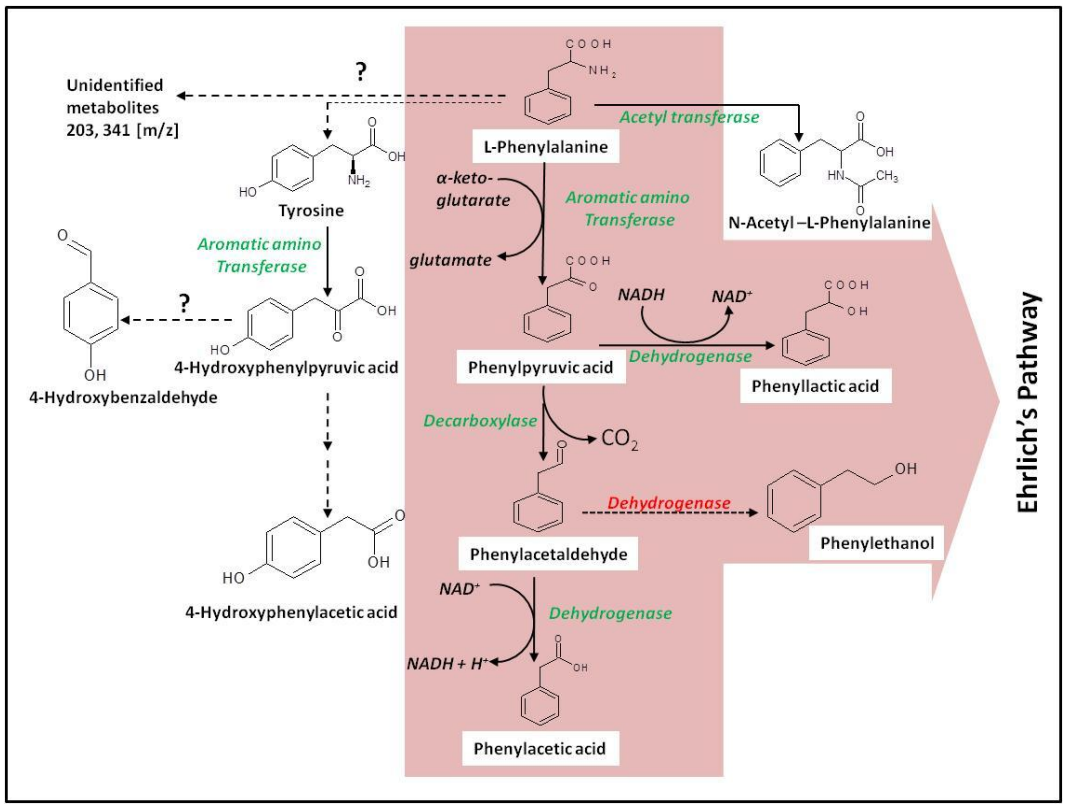


Fig. 31. Proposed pathway of L-phenylalanine catabolism in *Rubrivivax benzoatilyticus* JA2 under anoxic conditions.

Solid arrows indicate the reactions confirmed based on the enzyme activities as well as metabolite identification; dashed arrows indicate the possible reactions based on the metabolites identified; " ? " represent unknown reactions.

Enzyme activities confirmed in *R. benzoatilyticus* JA2 were represented in green color and activities which are not confirmed, represented in red color. Shaded region in the pathway indicated the Ehrlich's pathway of L-phenylalanine catabolism in *R. benzoatilyticus* JA2.

3.2 Oxic L-phenylalanine metabolism in *Rubrivivax benzoatilyticus* JA2

3.2.1 Growth, utilisation of L-phenylalanine and phenols production by Rubrivivax benzoatilyticus JA2

Rubrivivax benzoatilyticus JA2 produced phenols under oxic conditions with simultaneous growth and utilisation of L-phenylalanine as sole source of nitrogen. Within 24 h, L-phenylalanine (1 mM) was completely consumed. Maximum phenols production (18.8 µg. mg dry wt^{-1}) was observed at 12 h, with 87% consumption of L-phenylalanine (Fig. 32A). Total phenols production increased significantly in the presence of L-phenylalanine (168 µg. mg dry wt^{-1}) compared to control (32 µg. mg dry wt^{-1}) cultures of *R. benzoatilyticus* JA2 (Fig. 32B).

3.2.2 Comparitive metabolite profiling of control and L-phenylalanine fed cultures of Rubrivivax benzoatilyticus JA2

The spent media of L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 were acidified and extracted into ethyl acetate. The ethyl acetate fractions of L-phenylalanine fed and control conditions were analysed using HPLC. The comparative metabolite profiling of ethyl acetate fractions of both L-phenylalanine fed and control conditions indicated the aryl metabolites production. Twenty two peaks were observed only in the L-phenylalanine fed conditions compared to control (Fig. 33). The ethyl acetate fraction of L-phenylalanine fed culture of *R. benzoatilyticus* JA2 was taken further for the identification of metabolites through HPLC, LC-MS and GC-MS analysis.

3.2.3 HPLC, LC-MS and GC-MS analysis of ethyl acetate fraction of L-phenylalanine fed cultures of Rubrivivax benzoatilyticus JA2

Metabolites were identified using HPLC, LC-MS and GC-MS analysis based on the retention time, absorption spectra and mass spectra of the metabolites. 4-Hydroxybenzoic acid, phenyllactic acid, phenylpyruvic acid, phenylacetic acid, 4-hydroxyphenylpyruvic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzaldehyde were the metabolites identified based on HPLC and LC-MS analysis. Metabolites like 4-hydroxyphenyllactic acid (4-HPLA), 2-hydroxyphenylacetic acid (mandelic acid) (MA), 4-hydroxymandelic acid (4-HMA), α-oxo benzeneacetic acid (benzoylformic acid), 2,5-dihydroxyphenylacetic acid (Homogentisic acid) (HMG), benzaldehyde (Bald), 2-hydroxybenzoic acid (salicylic acid) (SA) were identified based on GC-MS analysis by comparing the mass spectra with, National Institute of Standards and

Technology (NIST) library based on the percentage similarity (Table. 4). Some of the metabolites were confirmed by co-elution with available standards.

3.2.4 Stable isotope probing of L-phenylalanine metabolism under oxic conditions

Stable isotope labeled/unlabeled fractions were analysed using LC-MS analysis. The metabolites of labeled fractions were identified based on the increase in masses by 6 units. Amongst the sixteen labeled metabolites, five metabolites were identified and eleven metabolites were unidentified. The metabolites with masses increased by 6 units were identified as phenylpyruvic acid (PPY), L-phenyllactic acid (PLA), phenylacetic acid (PAA), 4-hydroxyphenylpyruvic acid (4-HPPY), 4-hydroxybenzaldehyde (4-HBald). Within the unidentified metabolites, three metabolites had +6 units increase in their mass and eight metabolites had +12 increase in their masses. Stable isotope probing studies revealed metabolites with masses increased by +6/+12/+18/+24 units indicated that the metabolites are derived from L-phenylalanine. Labeled metabolites and their respective absorption spectra were represented in Table. 4. Some the unidentified metabolites having absorption spectra ranging 300-560 nm indicated that the metabolites are pigments.

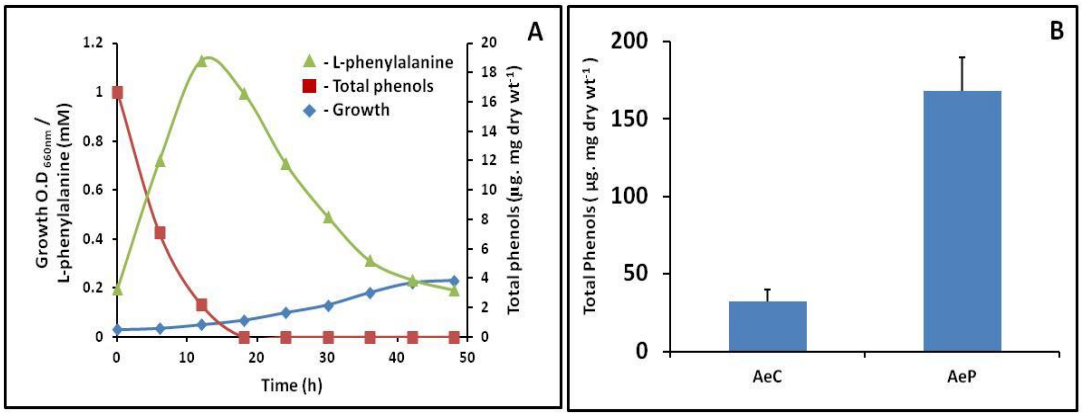


Fig. 32. Production of phenols with L-phenylalanine utilization by *Rubrivivax benzoatilyticus* JA2 under oxic conditions.

Phenols production with simultaneous consumption of L-phenylalanine (1 mM) for growth as sole source of nitrogen **(A)**. Total phenols production by *R. benzoatilyticus* JA2 grown in presence/absence of L-phenylalanine under oxic conditions **(B).**

Rubrivivax benzoatilyticus JA2 was grown under oxic conditions with agitation at 180-200 rpm for 24 h, L-phenylalanine (1 mM) was added and incubated for 48 h. Total phenols produced and L-phenylalanine consumed were estimated from the supernatants. AeC, Oxic control (without L-phenylalanine); AeP, Oxic L-phenylalanine fed .

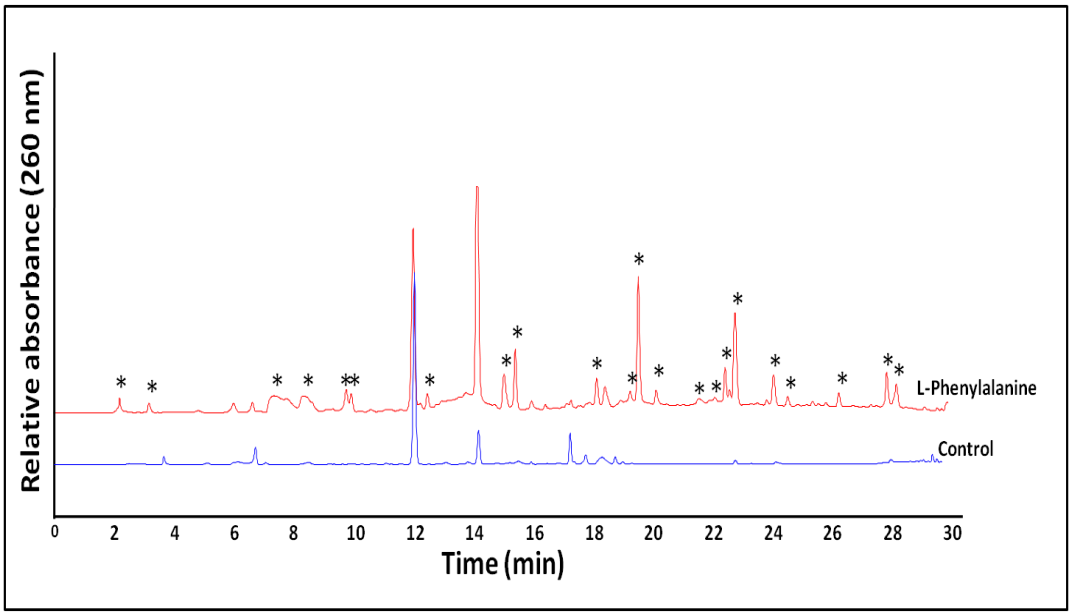


Fig. 33. HPLC metabolite profiling of ethyl acetate extracts obtained from supernatant of *Rubrivivax benzoatilyticus* JA2 grown with/without L-phenylalanine under oxic conditions.

HPLC chromatograms of ethyl acetate fractions extracted from acidified supernatants of control (without L-phenylalanine) and L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 grown under oxic condition. Chromatograms represented in blue color, control (without L-phenylalanine); red, L-phenylalanine fed. Peaks labelled with '*' mark were present only in L-phenylalanine fed condition.

Experimental conditions were similar as mentioned in the Fig. 32, except for the ethyl acetate extraction. The ethyl acetate extracts were used for the metabolite profiling. HPLC conditions were as mentioned in the Fig. 13.

Table.4. An overview of stable isotope labeled metabolites of L-phenylalanine fed cultures of *Rubrivivax benzoatilyticus* JA2 grown under oxic conditions.

R_t	Absorbance (nm)	m/z C12	m/z C13	Compounds
3.66	260,320	220[M^+]	226[M^+]	Unidentified
10.5	287	166[M^+]	172[M^+]	Unidentified
11.0	254,263,257, 340	148[M^-]	154[M^+]	Unidentified
8.8	254,263,257,355,460	316[M^+]	328[M^+]	Unidentified
12.1	ND	307[M^+]	319[M^+]	Unidentiified
13.3	280,460,563	503[M^+]	515[M^+]	Unidentified
13.8	254,263,257, 295,350	415[M^+]	427[M^+]	Unidentified
16.5	260,320	331[M^-]	343[M^-]	Unidentified
15.4	400-540	546[M^+]	558[M^+]	Unidentified
15.5	260,550	535[M^+]	559[M^+]	Unidentified
15.8	280,300,390	389[M^-]	407[M^+]	Unidentified
16.3	ND	413[M^+]	425[M^+]	Unidentified
16.6	260,295,380	331[M^+]	343[M^+]	Unidentified

Experimental conditions were same as mentioned in Fig. 33, except for the LC-MS/MS analysis, mass ionisation was done both in positive and negative modes. Metabolites represented in red color were having +6 mass units increase in their masses and metabolites represented in green color with +12, blue color with +18 and purple with +24 mass units increase in their masses. ND, not detected.

3.2.5 Pigments production in the presence of L-phenylalanine

Rubrivivax benzoatilyticus JA2 was grown 48 h under oxic conditions with/without L-phenylalanine for pigments production. The L-phenylalanine fed cultures turned dark brown over a period of 48 h. The spent media of L-phenylalanine fed cultures retained the dark brown color compared to control condition indicating the pigments are released into the media and are exogenous metabolites. Pigments produced in the presence of L-phenylalanine were precipitated on acidification followed by storage at 4°C for 2-3 days (Fig. 34). Thus precipitated brown pigment was taken for further purification and identification.

3.2.6 Screening Proteobacteria for pigment production

Seven *Proteobacteria* (*Rhodobacter sphaeroides* DSM158^T, *Rhodobacter blasticus* ATCC 33485^T, *Rhodobacter viridis* JA737^T, *Pheospirillum molischianum* DSM120^T, *Rhodobacter maris* JA276^T, *Rhodobacter azatoformans* KA25^T, *Rubrivivax benzoatilyticus* JA2^T**)** were tested for the pigments production. Among which, only *Rubrivivax benzoatilyticus* JA2 gave dark brown color in presence of L-phenylalanine indicating the pigments production under oxic conditions (Fig. 35).

3.2.7 Pigments production by viable culture of Rubrivivax benzoatilyticus JA2

Pigments production was studied with and without culture in presence of L-phenylalanine and also with viable and heat killed cells of *R. benzoatilyticus* JA2. The mineral media without culture, with L-phenylalanine (blank) (Fig. 36A) did not show any dark brown color, while mineral media with culture (in presence of L-phenylalanine) (Fig. 36C) gave dark brown color indicated that the pigment production is not a chemical synthesis. Pigments production was observed only in the presence of L-phenylalanine but not in control (Fig. 36B). Pigment production was observed only with the viable cells (Fig. 36E) and no pigment production was observed with the heat killed cells (Fig. 36D). Pigments production with viable cells indicated the biological synthesis of pigments in the presence of L-phenylalanine under oxic conditions.

3.2.8 Time dependent pigment production by Rubrivivax benzoatilyticus JA2

Pigment production was observed at different time intervals (0, 12, 24, 36, 48, 72, 96 h) in presence and absence of L-phenylalanine under oxic conditions. Pigment production was not observed in the absence of L-phenylalanine at any time interval (Fig. 37A) whereas, pigments

production started from at 24 h and continued till 96 h (Fig. 37B) in phenylalanine fed conditions.

3.2.9 Effect of carbon sources on pigment production

Pigment production was studied with different carbon sources (non-sugar/sugars) like malate (Mal), pyruvate (Pyr), fumarate (Fum), maltose (Malt), glucose (Glu) and fructose (Fru) under oxic conditions. Pigments production was observed in presence of non-sugar carbon sources (malate, pyruvate, fumarate) rather than sugar carbon sources (maltose, glucose, fructose) (Fig. 38). Amongst the sugar carbon sources, slight pigmentation was observed in presence of fructose whereas no pigmentation was observed in glucose and maltose (sugar carbon sources).

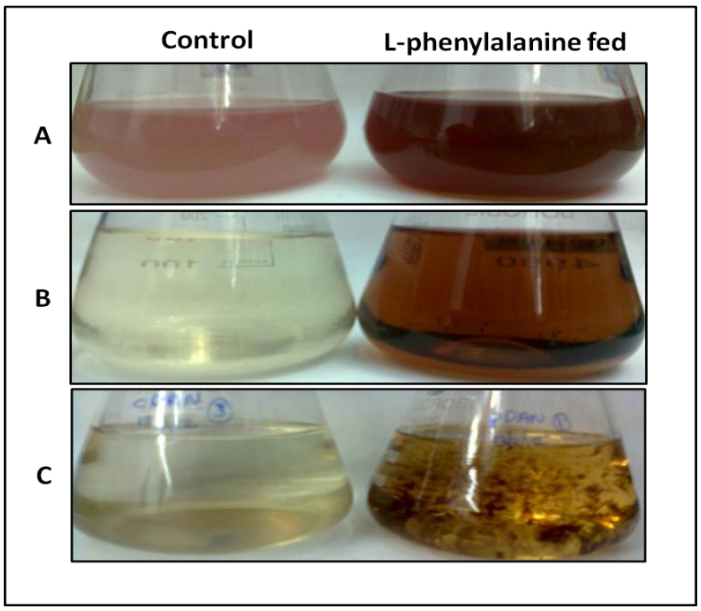


Fig. 34. Pigments production by L-phenylalanine fed culture of *Rubrivivax benzoatilyticus* JA2 grown under oxic conditions.

L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 turned dark brown compared to control, when grown under oxic condition **(A)**, L-phenylalanine fed culture supernatants retain the dark brown color **(B)**. Brown precipitate was observed on acidification only in L-phenylalanine fed culture supernatant **(C)**.

Experiment was done as mentioned in Fig. 32, except for the brown precipitate formed after acidification to pH 2 with 5 N HCl and storage at 4°C for 2-3 days.

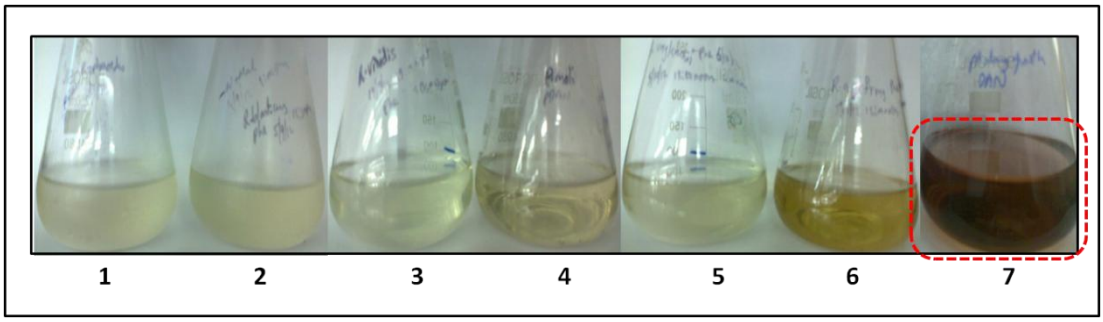


Fig. 35. Screening bacteria for pigment production in presence of L-phenylalanine under oxic conditions.

Different phototrophic *proteobacteria* - **(1)** *Rhodobacter sphaeroides* DSM158^T, **(2)** *Rhodobacter blasticus* ATCC 33485^T, **(3)** *Rhodobacter viridis* JA737^T, **(4)** *Pheospirillum molischianum* DSM120^T, **(5)** *Rhodobacter maris* JA276^T, **(6)** *Rhodobacter azatoformans* KA25^T, **(7)** *Rubrivivax benzoatilyticus* JA2^T**.**

Experiment was done same as mentioned in Fig. 34, except for different *proteobacteria*.

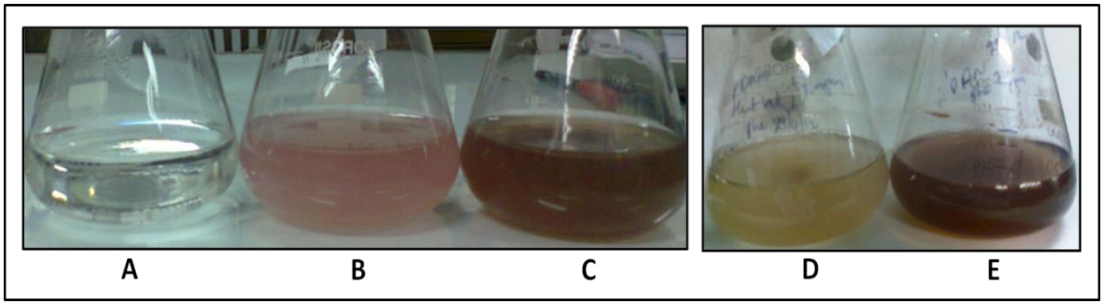


Fig. 36. Production of pigments by *Rubrivivax benzoatilyticus* JA2 under oxic conditions.

Mineral media with L-phenylalanine added as nitrogen source with no culture **(A);** control (culture without L-phenylalanine) **(B)**; culture with L-phenylalanine **(C)**. Heat killed cells **(D);** and viable cells **(E)**.

Experiment was done same as mentioned in Fig. 34, except for the heat killed and viable cells of *R. benzoatilyticus* JA2.

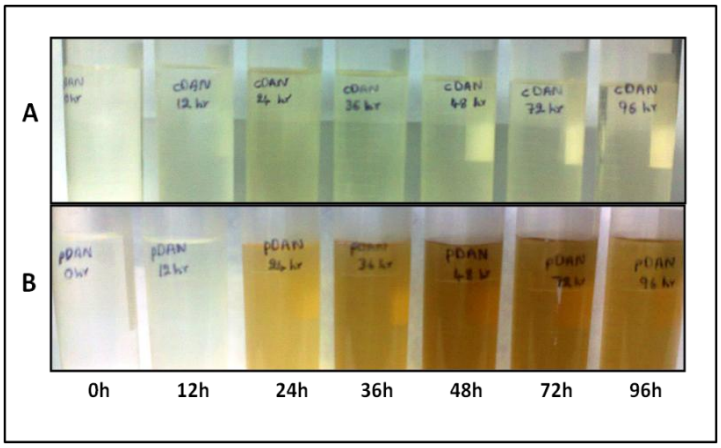


Fig. 37. Time dependent pigments production by *Rubrivivax benzoatilyticus* JA2 grown with/without L-phenylalanine under oxic condition.

Pigments production in control **(A)**, pigment production in L-phenylalanine fed culture over time period time **(B)**.

Experiment was done same as mentioned in Fig. 34, except for harvesting the cultures at different time intervals.

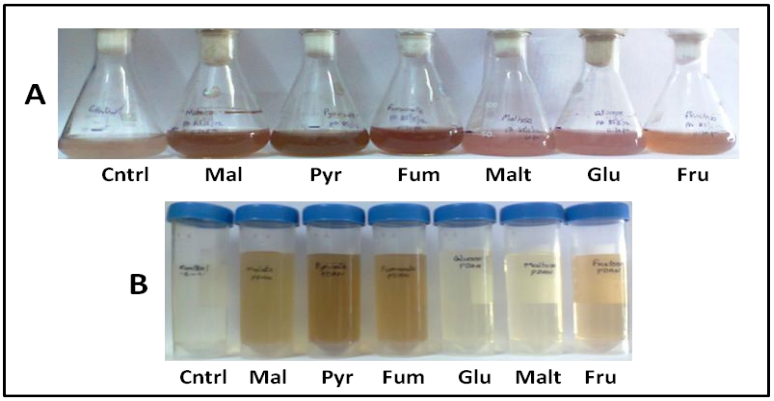


Fig. 38. Effect of carbon source on the pigments production

Cultures were grown in different carbon sources **(A)**, and their supernatants retained the pigments **(B)**.

Experiment was done as mentioned in Fig. 34, except for the culture grown on different carbon sources. Cntrl, control; Mal, malate; Pyr, pyruvate; Fum, fumarate; Malt, maltose; Glu, glucose, Fru, fructose

3.2.10 Identification and production of brown pigment from Fraction-I

3.2.10.1 Purification and characterisation of brown pigment

The brown pigment was isolated from the brown precipitate obtained from the acidified supernatant of L-phenylalanine fed culture of *R. benzoatilyticus* JA2 under oxic conditions. Purified brown pigment was characterised based on NMR, FTIR, UV and EPR/ESR analysis. Solid state carbon-13 NMR (^{13}C NMR) analysis indicated the chemical shifts at 174 ppm corresponding to C-O/C=O, 120-130 ppm to aromatic carbons, multiplet signals at 18-36 ppm represent the saturated aliphatic carbons. The signals observed with characteristic chemical shifts indicated the melanin pigment (Fig. 39A). FTIR spectrum with stretching vibration band at 3369cm^{-1} attributed to O-H stretch. The bands at 2854 and 2920 cm^{-1} due to the aliphatic C-H stretch. The C-H stretch (2920 cm^{-1}) is may be due to the presence of aromatic rings and bands at 1632 cm^{-1} also correspond to aromatic C=C appear. The vibration at 1445 cm^{-1} represents the aliphatic C-H stretch. The C-O stretch at 1074 cm^{-1} and broad band centered at 3370 cm^{-1} could be attributed to phenolic groups. The band at 1737cm^{-1} attributed to vibration stretch C=O possibly due to the presence of -COOH group (Fig. 39B).

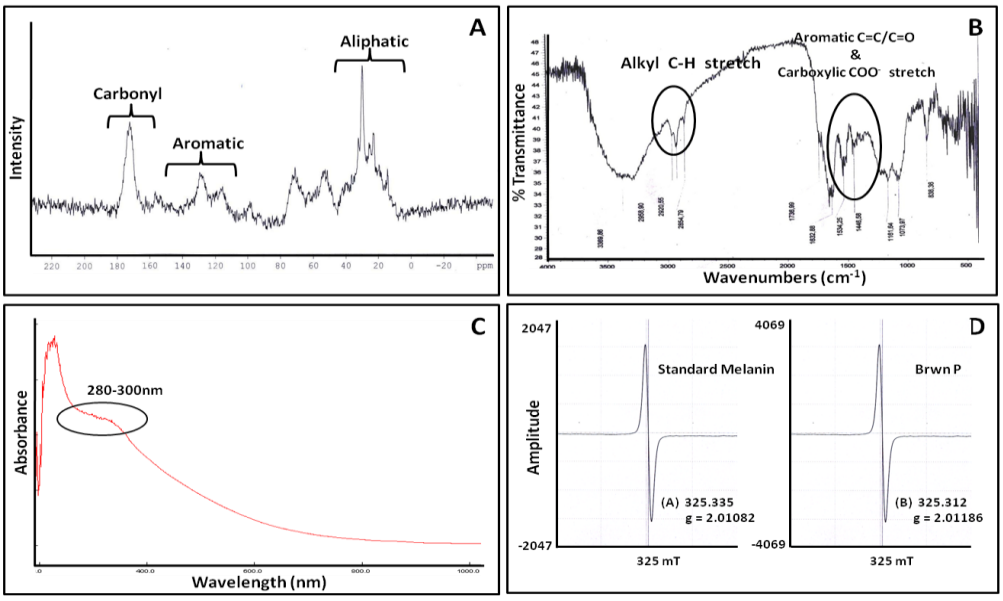
The UV-visible spectrum showed a broadband spectra with wavelength ranging 280-300 nm (Fig. 39C) with a continuous decrease in the absorbance from UV to visible wavelengths. Synthetic melanin also showed comparable absorption spectra which is most similar to absorption spectra of purified Brwn P. The electron paramagnetic resonance (EPR)/electron spin resonance (ESR) spectral analysis of purified brown pigment had signals at 325 mT representing the free radical concentration and the spectral features were similar to standard pyomelanin. Based on polychem analysis the purified brown pigment was identified as pyomelanin (Fig. 39D).

3.2.10.2 Scanning electron microscopic analysis of purified brown pigment

The purified brown pigment was observed for its form/shape/structure under scanning electron microscopic analysis. The amorphous powder of the dark brown pigment was observed as spherical ball-like structures (indicated with red colored arrows) (Fig. 40A) which were similar with the pure melanin (Fig. 40B).

3.2.10.3 Physico-chemical properties of purified brown pigment

The physical and chemical properties of the purified brown pigment was checked with several chemical tests which are characteristic to melanin. Physical properties like color, form, shape, solubility and chemical properties like reaction with oxidising agents, ferric chloride and acid precipitation were demonstrated with the purified brown pigment. The purified brown pigment was dark brown in color, amorphous in form and spherical in shape. The purified brown pigment was insoluble in solvents like hexane, chloroform, acetone, ethyl acetate, methanol and water but only soluble in 5 N NaOH/alkaline pH. The dissolved pigment precipitated at acidic pH with 5 N HCl. Reactions with oxidising agent like H_2O_2 decolorised the brown pigment. Reaction with ferric chloride (2% w/v) readily precipitated (Table. 5) and based on the physico-chemical properties, the brown pigment was identified as melanin.



Fig, 39. Characterisation of brown pigment (Brwn P) produced by *Rubrivivax benzoatilyticus* JA2 under oxic conditions.

Solid state C^{13}NMR spectrum **(A)**, FTIR spectrum **(B)**, UV spectrum **(C)**, EPR/ESR spectra of standard melanin and purified Brwn P **(D)**.

Experiment was done as mentioned in Fig. 23, except for the brown precipitate collected by centrifugation and then purified by suspending in water, different solvents. Finally Brwn p was purified by treating with 5 N NaOH and then the Brwn P was precipitated with 5 N HCl. Brwn P, brown pigment.

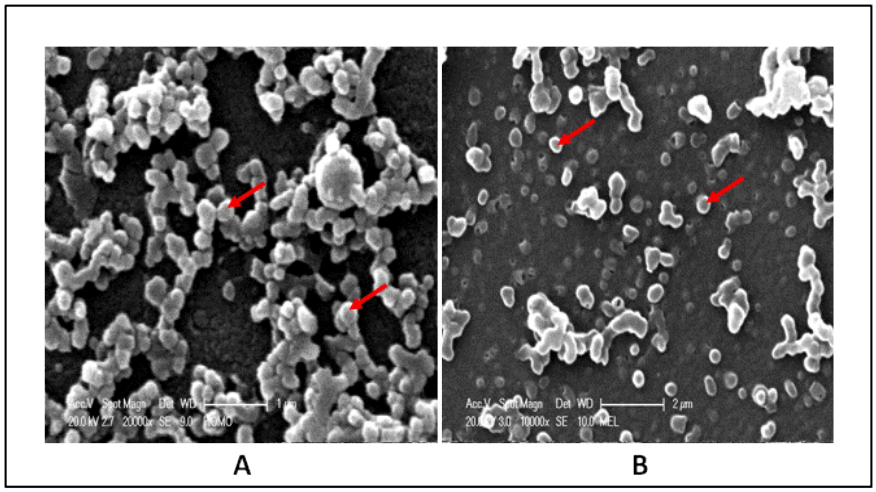


Fig. 40. Scanning Electron microscopic (SEM) analysis of purified Brwn P.

SEM image of standard melanin (A) and purified Brwn P (B).

Experiment was done same as mentioned in Flow chart-2, except for the SEM analysis. The brown pigment was dried and the powder was mounted on stab for gold sputtering before SEM analysis. Brwn P, brown pigment.

Table.5. Physico-chemical properties of the purified Brwn P (brown pigment)

Characteristic properties	Purified brown pigment
Color	Dark brown
State	Amorphous (powder)
Solubility	
(a) Water	Insoluble
(b) Organic solvents	Insoluble
(c) 5N NaOH	Soluble
Acid precipitation	Readily precipitated with 5N HCl
Reaction with oxidising agent	Decolorised with H_2O_2
Reaction with ferric chloride (2% w/v)	Brown precipitate

Experiment was done as mentioned in Fig. 34, and the physico-chemical properties were performed with brown pigment.

3.2.10.4 Substrate dependent melanin production by Rubrivivax benzoatilyticus JA2

Melanin production was measured at 300 nm and the production was checked with different precursors like L-phenylalanine, L-tyrosine, 4-hydroxyphenylpyruvic acid and homogentisic acid. *Rubrivivax benzoatilyticus* JA2 gave positive for the melanin production when grown with precursors like L-phenylalanine, L-tyrosine, 4-hydroxyphenylpyruvic acid and homogentisic acid. Highest melanin production was observed with precursor, homogentisic acid ($O.D_{300\ nm}$ 3.022) followed by L-tyrosine ($O.D_{300nm}$ 2.74), 4-hydroxyphenylpyruvic acid ($O.D_{300nm}$ 2.1) and L-phenylalanine ($O.D_{300\ nm}$ 1.84) in comparison to control ($O.D_{300\ nm}$ 0.22). Precursors feeding such as L-phenylalanine, L-tyrosine, 4-hydroxyphenylpyruvic acid, homogentisic acid to *R. benzoatilyticus* JA2 produced melanin under oxic conditions (Fig. 41). This confirmed that the melanin produced in *R. benzoatilyticus* JA2 is a pyomelanin.

3.2.10.5 Effect of sulcotrione on melanin biosynthesis in Rubrivivax benzoatilyticus JA2

Pyomelanin biosynthesis was further confirmed by enzyme inhibitor study. The sulcotrione inhibits the 4-hydroxyphenylpyruvic acid dioxygenase and their by the pyomelanin biosynthesis. Melanin production was observed only in L-phenylalanine fed culture (Fig. 42A), whereas pigment production was inhibited in presence of sulcotrione (Fig. 42B) and this confirmed the pyomelanin biosynthesis in *R. benzoatilyticus* JA2. Pyomelanin biosynthesis from L-phenylalaninein *R. benzoatilyticus* JA2 is a homogentisic acid dependent pyomelanin biosynthetic pathway (Fig. 42C).

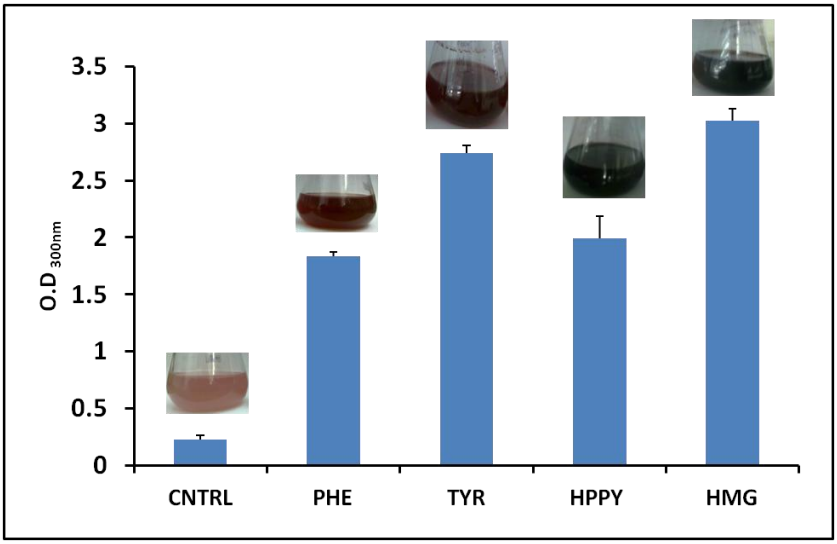


Fig. 41. Pyomelanin production in presence of different precursors in *Rubrivivax benzoatilyticus* JA2 grown under oxic conditions.

Experiment was done as mentioned in Fig. 34, except for the precursors L-tyrosine, 4-hydroxyphenylpyruvate, homogentisate and measuring the O.D. at 300 nm. CNTRL, control; PHE, L-phenylalanine; TYR, L-tyrosine; HPPY, 4-hydroxyphenylpyruvic acid; HMG, homogentisic acid.

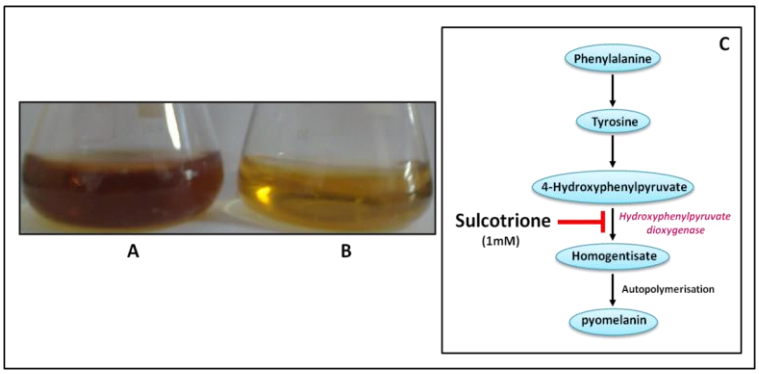


Fig. 42. Effect of sulcotrione on melanin production in *Rubrivivax benzoatilyticus* JA2

Melanin production in presence of L-phenylalanine (A), melanin production in presence of sulcotrione (1 mM) (B) and inhibition of 4-hydroxyphenylpyruvate dioxygenase by sulcotrione (C)

Experiment was done as mentioned in Fig. 34 except for the addition of sulcotrione (1mM) along with L-phenylalanine (1 mM).

3.2.11 Identification of pigments from the Fraction-II (methanolic fraction)

The pigments were purified from the fraction-2 as mentioned in the Flow chart-3. The TLC analysis of fraction 2 (methanolic fraction) showed different blue, pink, red, orange, yellow colored bands (Fig. 43A). Each colored band was eluted into methanol and further purified by HPLC. The HPLC chromatograms of the purified pigments were represented along with their absorption spectra as an insert in the chromatograms. Orange colored band was scrapped from the TLC and subjected to HPLC. Two peaks eluted at R_t 11.8 and 12.5 min with absorption spectra 344, 468, 465, 472 nm and 345, 463, 468, 472 nm, represented as orange pigment 1 (Org P1) and orange pigment 2 (Org P2) (Fig. 43B). Pink colored pigment (Pin P) eluted at R_t 8.5 min having absorption spectra 358,488,491 nm (Fig. 43C). Blue pigments (Blu P1 and Blu P2) eluted at R_t 9.8 and 10.2 min and absorption spectra 559, 353, 286, 399 nm and 563, 357, 289 nm (Fig. 43D).

3.2.12 Purification and characterisation of blue pigment 1 (Blu P1)

Blue pigments were partially purified on TLC and further purified through HPLC. The Blue pigment 1(Blu P1) at R_t 9.8 min (Fig. 44) was characterised based on LC-MS, NMR, FTIR and UV spectral analysis.

3.2.12.1 Mass spectral analysis of blue pigment 1 (Blu P1)

Mass spectral analysis of the purified blue pigments (Blu P1 and Blu P2) was done to identify the compounds. Stable isotopic profiling revealed the +24 units of mass increase i.e 535 m/z (M+0) to 559 m/z (M+24) (Fig. 45A) and 447 m/z (M+0) to 470 m/z (M+24) (Fig. 45B), in the molecular ion masses indicating that the two blue pigments are derivatives of L-phenylalanine. These two compounds were ionized under positive mode. The molecular ion mass of the blue pigment 1 was 537 m/z and the mass fragments 509, 456, 346, 302, 246, 202, 141 m/z were observed in the MS/MS analysis (Fig. 46A).

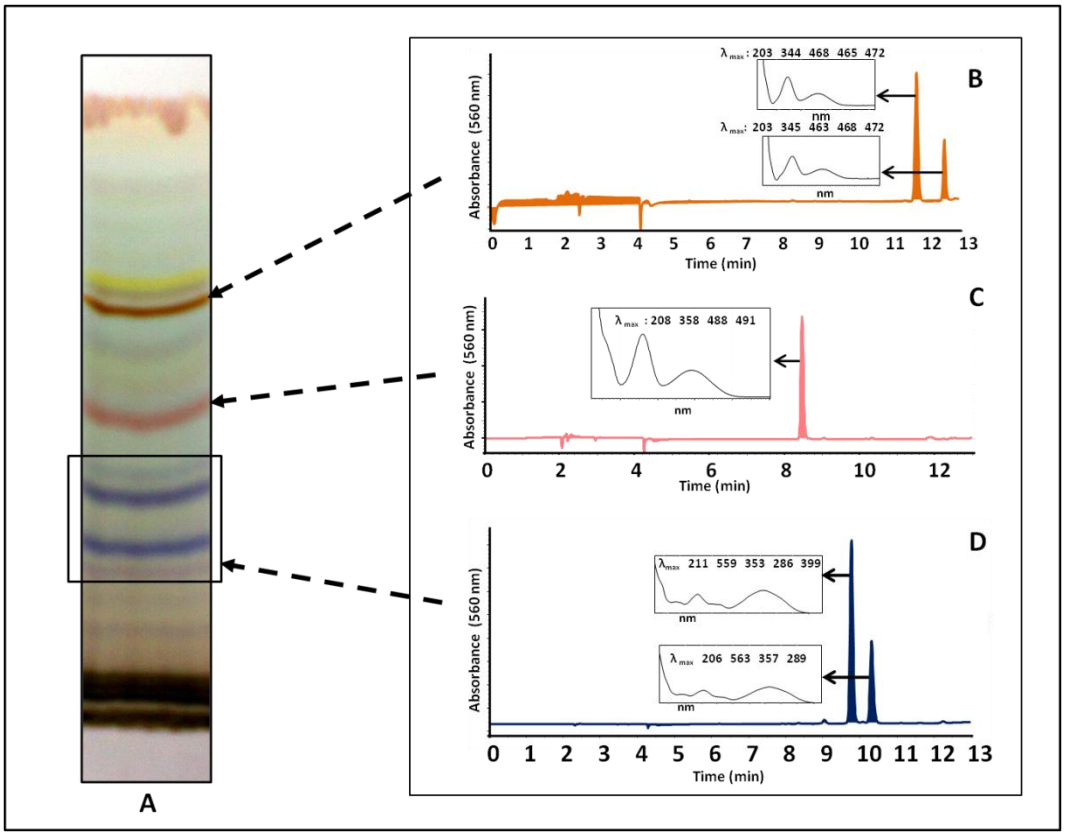


Fig. 43. TLC and HPLC analysis of pigments produced by *Rubrivivax benzoatilyticus* JA2 grown under oxic conditions.

TLC analysis of fraction II (methanolic fraction) of brown precipitate **(A)**, HPLC chromatograms of partially purified pigments, orange **(B)**, pink **(C)**, blue **(D)**.

Experiment was done same as mentioned in Fig. 34 and the fractionation was done as mentioned in flow chart 1 and purification and identification of pigments as mentioned in flow chart 3. Thin layer chromatography was performed by using mobile phase chloroform:methanol:acetic acid (8:1.95:0.05 v/v) to separate the pigments in fraction 2 (methanolic fraction). The pigments were scrapped from the TLC plate and suspended in methanol. Arrows indicated the pigments and their respective HPLC chromatograms. Absorption spectra of respective pigments were shown as insert in the HPLC chromatograms. Blue pigments encircled with a box in TLC plate were further taken up for characterisation.

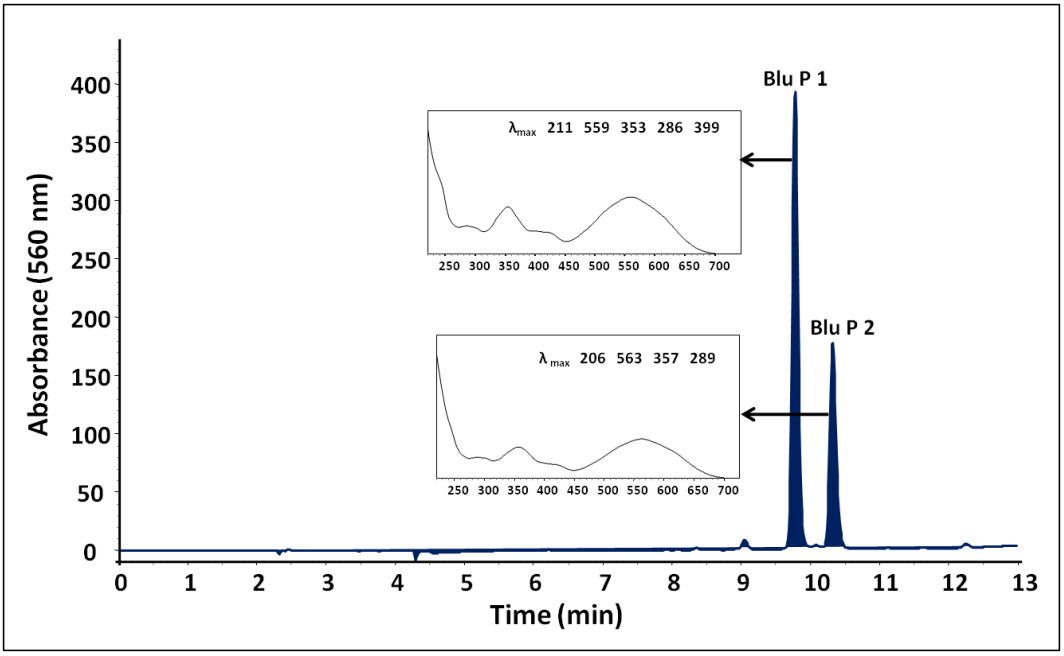


Fig. 44. HPLC chromatogram of blue pigment partially purified from TLC.

HPLC chromatogram indicated the blue pigments Blu P1 with R_t 9.8 min and absorption spectra 559, 353, 286, 399 nm and Blu P2 with R_t 10.2 min and absorption spectra 563, 357, 289 nm. Absorbance was measured at 560 nm.

Experiment was done same as mentioned in the Fig. 33. The blue pigment was purified using HPLC and pure blue pigment was used for characterisation. Absorption spectra were represented as insert in chromatogram. Blu P1, blue pigment 1; Blu P2, blue pigment 2.

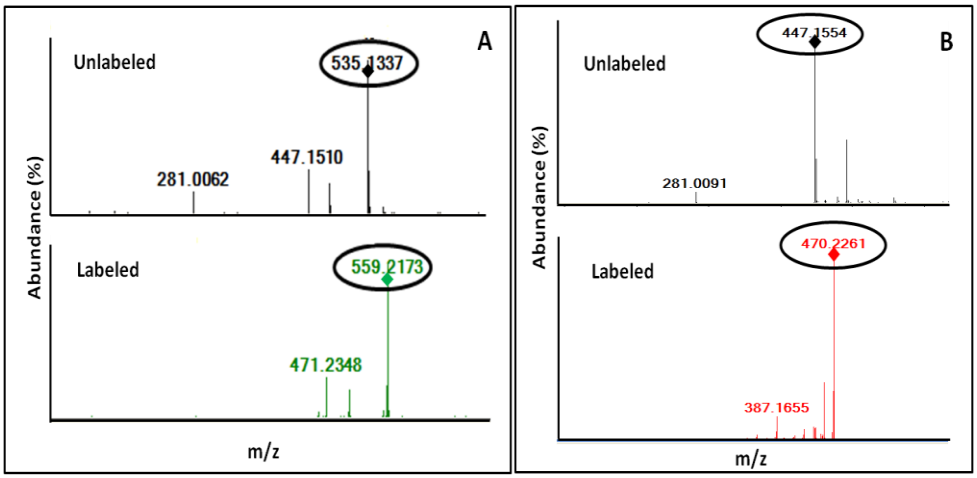


Fig. 45. Stable isotope studies of blue pigments produced by *Rubrivivax benzoatilyticus* JA2.

LC-MS analysis of the unlabeled and labeled purified blue pigments, Blu P1 (A), Blu P2 (B) indicating +24 mass units (amu) increase in their masses.

Experiment was done as mentioned in Fig. 44 except for growing cultures with unlabelled, stable isotope labelled L-phenylalanine and mass analysis as mentioned in Fig. 14. Encircled masses indicate the molecular ion masses having 24 mass units increase in their masses.

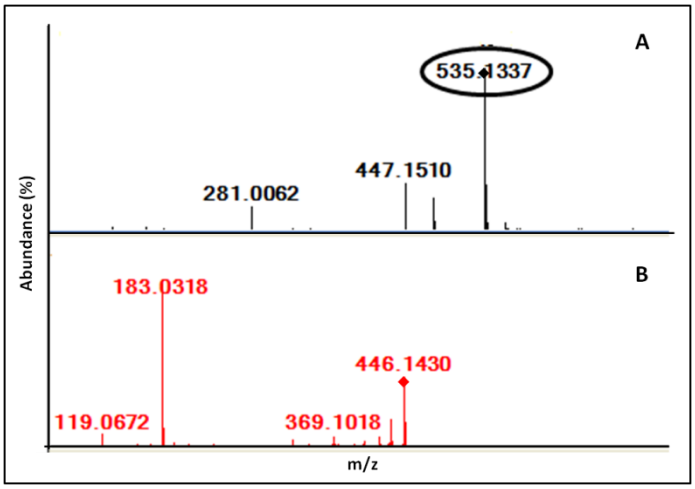


Fig. 46. LC-MS/MS analysis of purified Blu P1 produced by *Rurivivax benzoatilyticus* JA2

Mass spectra of pure Blu P1 (A), and mass fragmentation by MS/MS analysis (B).

Experiment was done as mentioned earlier in Fig. 45.

3.2.12.2 Nuclear magnetic resonance spectroscopic analysis

The purified blue pigment 1 (Blu P1) was subjected to one dimensional ^{1}H and ^{13}C NMR analysis initially and 2D NMR analysis like homonuclear correlation spectroscopy (COSY, TOCSY) and heteronuclear bond correlation spectroscopy (HSQC, HMBC),of the purified blue pigment was performed.

The chemical shifts (δ) obtained from the ^{1}H NMR and ^{13}C NMR analysis revealed that the purified blue pigment is nearly matching to the anthocyanins. The chemical shifts ranging δ 6.4 to 7.6 ppm clearly attributed to aromatic protons of aglycone molecule. The doublets at δ 5.34 ppm corresponded to anomeric sugar proton; δ 3.2-3.4 ppm attributed to the protons of glucose moiety and the δ 3.8 ppm represented the methoxy group (Fig. 47A). The ^{13}C NMR analysis with chemical shifts (δ) 112.58, 117.58, 124.61, 125.26, 131.36, 132.25, 134.02, 145.17, 147.21, 153.1, 153.43, 156.09, 160.06, 163.66, 168.76, 174.34 ppm attributed to the carbons of aromatic ring (aglycone/benzopyrilium ring). The chemical shifts 102.57, 103.14 ppm contributed to the anomeric carbon atom of sugar moiety (Fig. 47B). The 2D NMR;Total Correlation Spectroscopy (TOCSY) and Correlation Spectroscopy (COSY) indicated the signal on the diagonal at 5 ppm corresponded to the anomeric proton, and the rest of sugar signals at 1 to 2 ppm (Fig. 48A,C). The heteronuclear single quantum coherence spectroscopy (HSQC) chemicals shifts of ^{1}H-^{13}C bond signals ranging 125 to 175 ppm corresponded to the coupling constants of anthocyanins (Fig. 48B). The ^{1}H-^{13}C heteronuclear multiple bond correlation spectrum (HMBC) with cross peak at 5.2/158 ppm revealed the linkage between aglycone and sugar unit (Fig. 48D). The 2D-NMR analysis revealed the tentative structure of the compound which likely to be anthocyanin or anthocyanin like molecule.

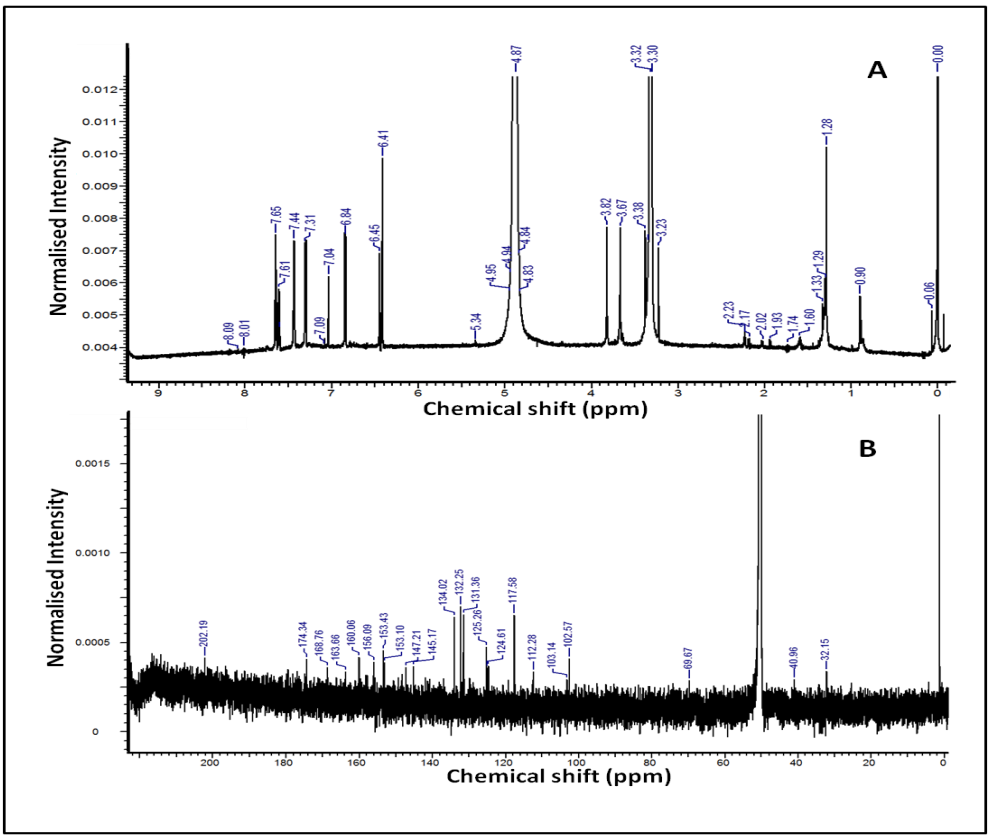


Fig. 47. 1D ^{1}H and ^{13}C NMR analysis of purified Blu P1 produced by *Rubrivivax benzoatilyticus* JA2

NMR analysis of pure Blu P1 was done in one dimension. H^{1} NMR spectrum (A) and C^{13} NMR (B).

Experiment was done as mentioned in Fig. 44, except for NMR analysis the pure Blu P1was completely dried and sent for analysis. NMR analysis was outsourced at University of California, Berkeley.

^{1}H NMR, Proton Nuclear Magnetic Resonance spectroscopy; ^{13}C NMR, Carbon-13 Nuclear Magnetic Resonance spectroscopy.

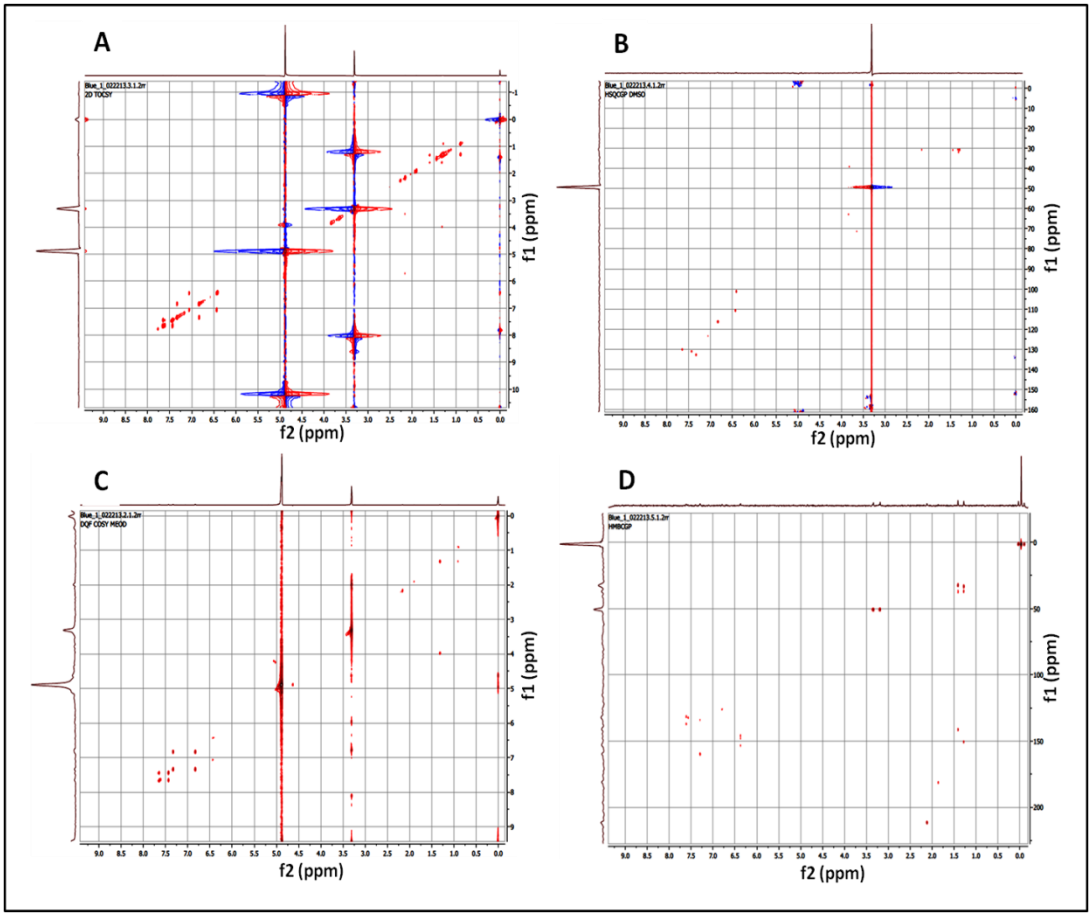


Fig. 48. 2D NMR analysis of purified Blu P1 produced by *Rubrivivax benzoatilyticus* JA2

Structural confirmation of Blu P1 was done with 2D NMR analysis. TOCSY **(A)**, HSQC **(B)**, COSY **(C)**, HMBC **(D)**.

Experiment was done as mentioned in Fig. 47. 2D NMR, Two Dimensional Nuclear Magnetic Resonance spectroscopy; TOCSY, Total Correlated Spectroscopy; HSQC, Heteronuclear Single Quantum Coherence spectroscopy; COSY, Correlated Spectroscopy; HMBC, Heteronuclear Multiple-Bond Correlation spectroscopy.

3.2.12.3 FTIR analysis

FTIR analysis of the purified blue pigment revealed the characteristic signals attributed to flavonoid/anthocyanins. Medium signal at 2160 cm^{-1} indicated the aromatic-O-CH_3, specific variable signals at 1409, 1450 cm^{-1} and a very strong signal at 1648 cm^{-1} attributed to the characteristic pyrylium cation. Strong signals at 1113, 1013 cm^{-1} contribute to -C-O stretching vibration of carbohydrates. Weak to medium signals at 2840, 2951 cm^{-1} explained the C-H symmetric stretching vibration represented the –O-CH_3 group. A broad signal at 3283 cm^{-1} of –OH vibrational stretch was identified (Fig. 49). Based on the above FTIR spectral analysis the compound showed the resemblance to the anthocyanin compound.

3.2.13 Detection of sugar moiety with phenol sulfuric acid test

Phenols sulfuric acid test is used in to detect the sugar. Purified blue pigment was tested with phenol sulfuric acid reagent. The purified blue pigment and also the standard glucose turned red when treated with phenol sulfuric acid. The color was not observed in the blank (Fig. 50B). The red color formation indicated the presence of sugar moiety in the purified blue pigment. The predicted structure nearly matches with malvidin glucoside, anthocyanin from plants (Fig. 50A,C).

3.2.14 pH dependent UV spectral shift

The UV spectra were recorded at different pH like acidic (pH 2), neutral (pH 6-7), basic (pH 11-12). When the pH of the purified blue pigment adjusted to acidic pH (pH 2) with 5N HCl, the color changed from blue to red and when pH adjusted to basic (pH 12) with 5N NaOH, the color changed to yellow (Fig. 51). As the color of the compound changed with pH, the shift in the absorption spectra was also observed indicating the blue pigment as pH indicator which is yet another anthocyanin character.

3.2.15 Predicted structure of the purified blue pigment

Based on the HPLC, LC-MS/MS the compound is near matching with flavones whereas NMR, UV & IR spectral analysis the blue pigment was tentatively identified as possible anthocyanin glycoside derivative/anthocyanin-like molecule and structure confirmation needs further analysis. Yet the data clearly indicate the presence of benzopyrilium ring, anomeric carbon that joins the sugar moiety with benzopyrene ring, positive to phenol sulfuric acid test and the spectral shift at acidic and basic pH which are characteristic features of anthocyanins.

observed with blue pigment produced by *R. benzoatilyticus* JA2 indicating that the blue pigment is possibly a anthocyanin/anthocyanin-like pigment (Fig. 50).

3.2.16 Phylogenetic studies of histidine ammonia-lyase (HAL)

Phenylalanine ammonia-lyase (PAL) is rate limiting enzyme in the flavonoid/anthocyanin biosynthesis in plants. Histidine ammonia lyase (HAL) is the enzyme which is homologous to phenylalanine ammonia-lyase present in bacteria. Histidine ammonia-lyase (HAL) is present in the genome of *R. benzoatilyticus* JA2 and the phylogenetic similarity studies were done with the sequence of the histidine ammonia-lyase gene. The BLAST analysis was done using the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the nucleotide sequence of HAL gene of *R. benzoatilyticus* JA2. The BLAST results were subjected to Molecular Evolutionary Genetics Analysis (MEGA5) (http://www.megasoftware.net) to construct the phylogenetic tree which showed the nearest related member with high sequence similarity. The phylogenetic tree showed *Rubrivivax gelatinosus* IL144 AP012320 with highest sequence similarity (100%) of HAL gene rather than PAL gene present in plants (Fig. 52).

3.2.17 Elucidation of L-phenylalanine catabolism under oxic conditions in Rubrivivax benzoatilyticus JA2

Ehrlich's pathway was also observed in *R. benzoatilyticus* JA2 under oxic conditions which was evident from the identification of metabolite such as phenylpyruvic acid, L-phenyllactic acid, phenylacetic acid, phenylacetaldehyde, 2-phenylethanol. In addition to the Ehrlich's pathway, other biosynthetic pathways of pigments like pyomelanin and flavonoid/anthocyanin-like pigments was also illustrated in *R. benzoatilyticus* JA2 under oxic conditions. Pyomelanin biosynthetic pathway was explained clearly by metabolites identification such as 4-hydroxyphenylpyruvic acid, homogentisic acid and stable isotope studies, enzyme activities under oxic conditions. Another group of compounds like flavonoids/anthocyanin-like pigments biosynthesis also demonstrated in *R. benzoatilyticus* JA2. Multiple catabolic pathways of L-phenylalanine such as Ehrlich's pathway, pyomelanin biosynthetic pathway and unknown pathways of pigments biosynthesis were elucidated in *R. benzoatilyticus* JA2 under oxic conditions (Fig. 53).

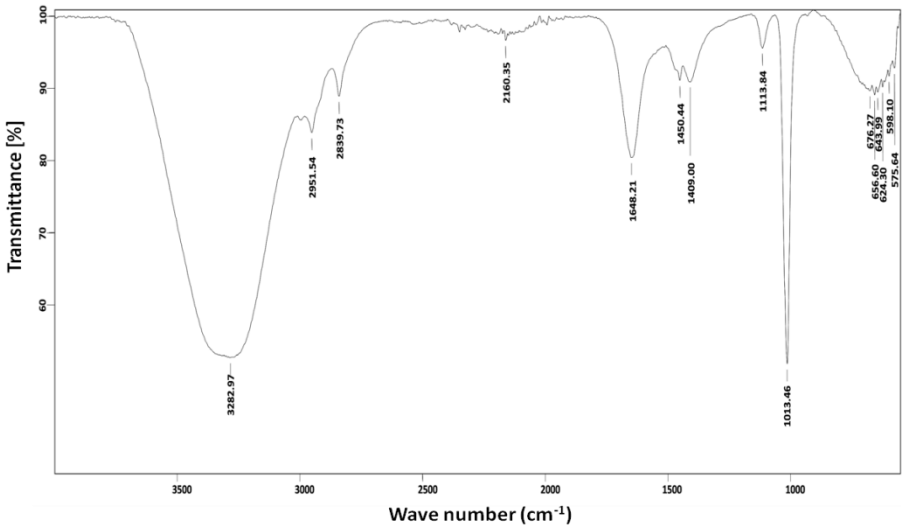


Fig. 49. FTIR analysis of purified Blue Pigment 1 (BluP1)

Experiment was done as mentioned in Fig. 44 except for the analysis of purified blue pigment for the FTIR analysis.

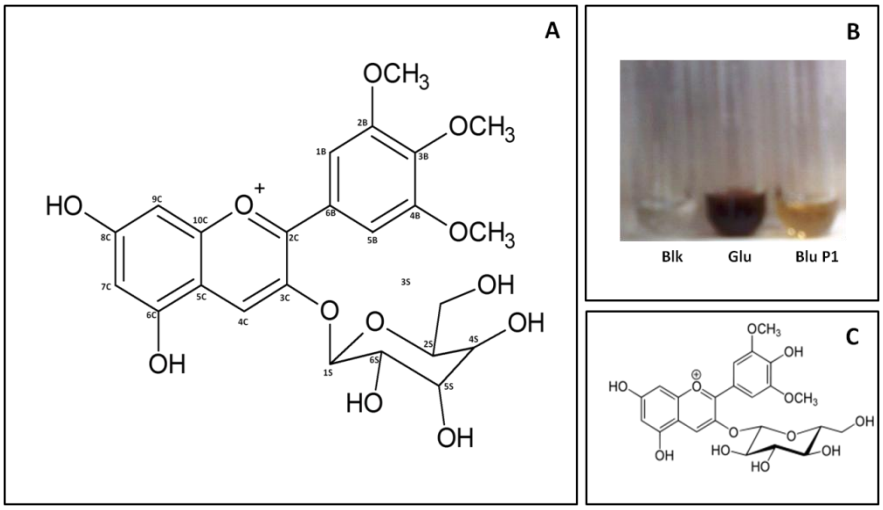


Fig. 50. Structural elucidation and phenol-sulfuric acid test of Blu P1

Based on polychem analysis predicted structure of pigment Blu pP1 **(A)**. The presence of glucose moiety was detected with phenol-sulfuric acid test using glucose as standard **(B)**. The nearest structure of predicted Blu p1 was malvidin 3-glucoside of plant systems **(C)**.

Experiment was done as mentioned in Fig. 44, except for phenol-sulfuric acid test for detection of glucose moiety. Blk, Blank; Glu, glucose; Blu P1, blue pigment 1.

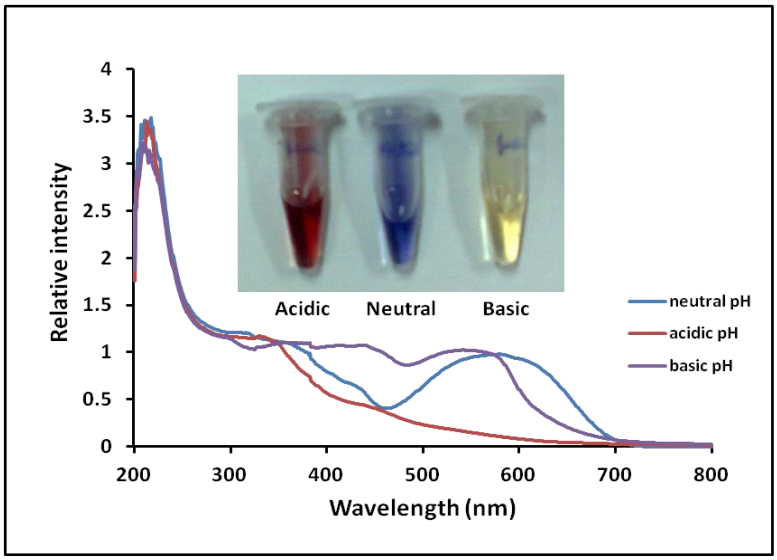


Fig. 51. UV spectral shift analysis of pure Blu P1

UV spectral shift at pH acidic (red), neutral pH (blue) and basic pH (yellow) was observed with pure Blu P1.

Experiment was done as mentioned in Fig. 44, except in recording the spectra by adjusting the pH to acidic with 5 N HCl and to basic pH with 5 N NaOH. The insert in the figure represent the color change with respect to change in pH of the Blu P1.

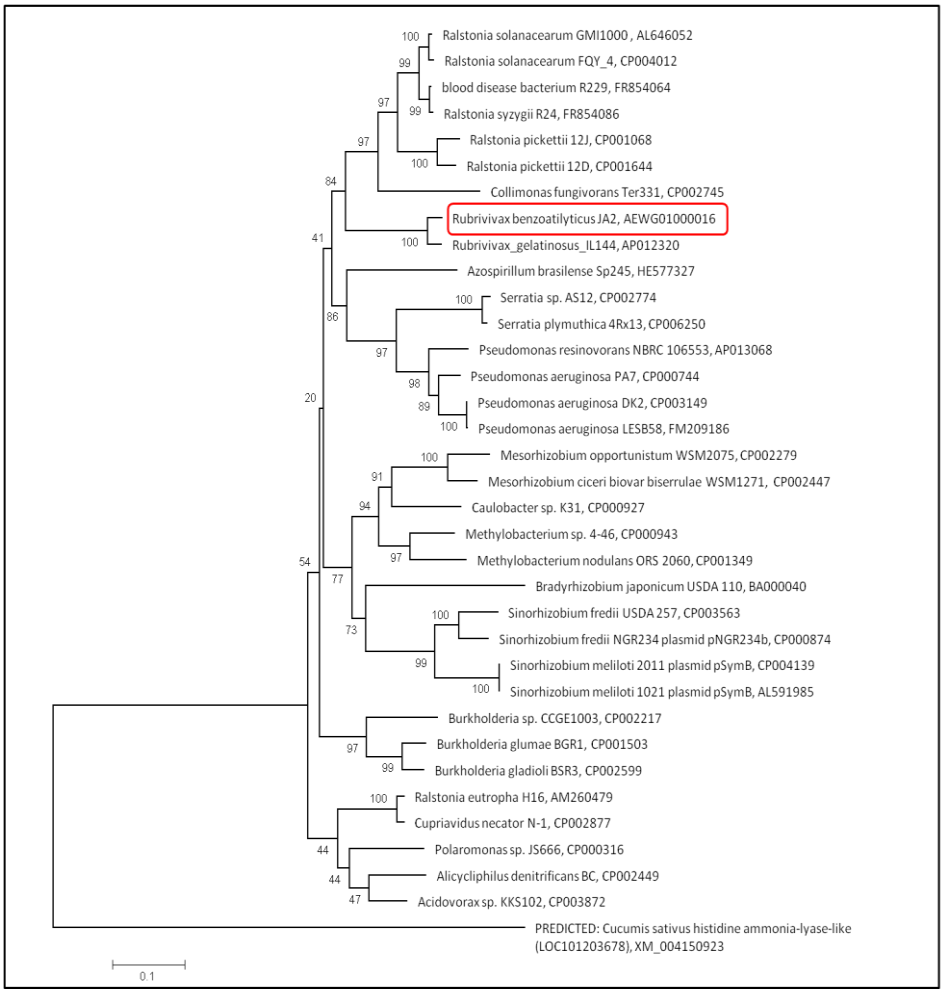


Fig. 52. Phylogenetic similarity of HAL (histidine ammonia-lyase) gene in the genome of *Rubrivivax benzoatilyticus* JA2

Experiment was done by taking the HAL gene nucleotide sequence and BLAST with other systems in NCBI. The BLAST results were subjected to MEGA5 software for constructing the phylogenetic tree. Red box represented the status of *R. benzoatilyticus* JA2 in the phylogenetic tree.

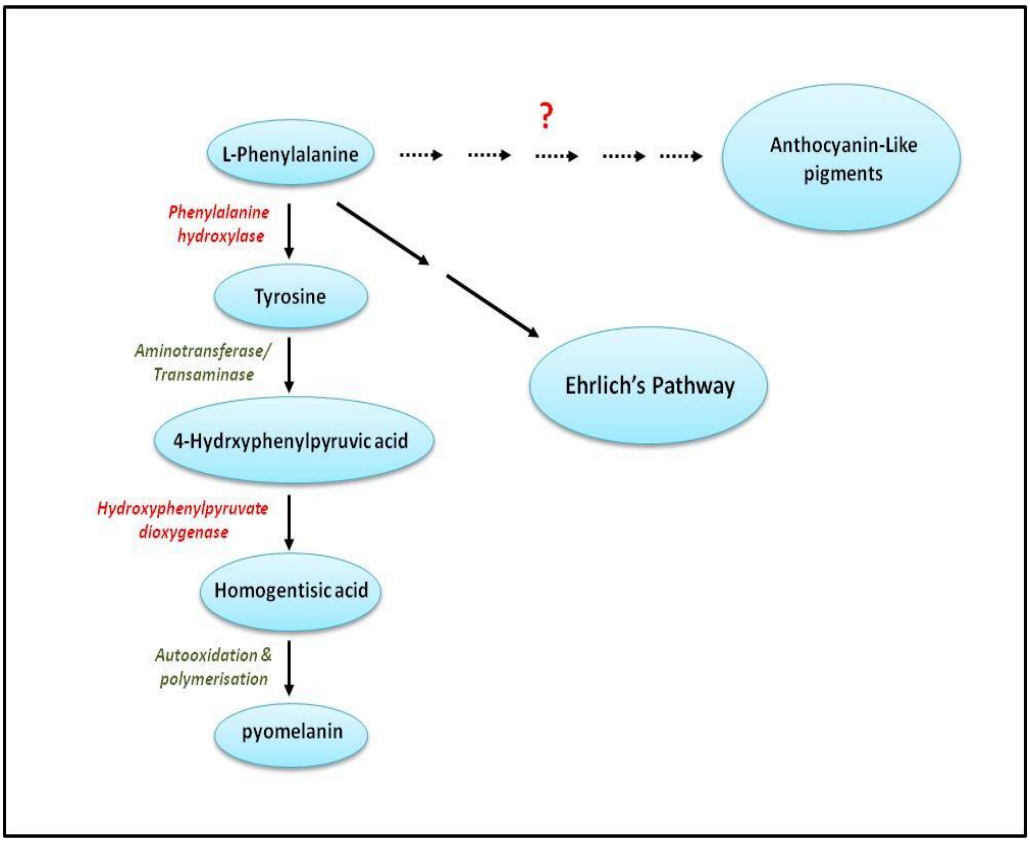


Fig. 53. Proposed pathway of L-phenylalanine catabolism in *Rubrivivax benzoatilyticus* JA2 grown under oxic condition.

Enzymes represented in red color indicate the activities not yet confirmed and enzymes represented in green color whose enzyme activities were confirmed. Solid arrows indicated the experimentally confirmed pathway based on metabolites identified. Dotted arrows with '?' indicated unknown pathway.

3.3 GC-MS based metabolic responses of *Rubrivivax benzoatilyticus* JA2 grown with/without L-phenylalanine under oxic and anoxic conditions.

3.3.1 Hierarchical cluster analysis (HCA)

Hierarchical cluster analysis is an unsupervised multivariate statistical method used to illustrate the metabolites concentration patterns in the metabolome of *R. benzoatilyticus* JA2 when grown with/without L-phenylalanine under oxic/anoxic conditions. Hierarchical cluster analysis was performed by taking the logarithmized mean values of metabolite areas using the Pearson correlation as distant matrix with the online software MetaboAnalyst (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp). The metabolome is differentiated into two major groups with metabolites clustered together. Few metabolites which were present only under oxic conditions with/without L-phenylalanine were clustered into Group I and metabolites pattern under anoxic condition as Group II (Fig. 54). Hierarchical cluster analysis revealed the two major groups such as oxic and anoxic clusters indicating that oxic metabolism varied with anoxic metabolism in *R. benzoatilyticus* JA2. Groups of control and L-phenylalanine conditions were separated within oxic and anoxic conditions indicating that the metabolome varied in the presence of L-phenylalanine under oxic and anoxic conditions (Fig. 54).

3.3.2 Principal component analysis (PCA)

Principal component analysis is a multivariate statistical analysis which explains the statistically significant metabolomic variations between the groups. Variations in the metabolome were clearly demonstrated with the well-separated groups of metabolome. The four groups with/without L-phenylalanine under oxic/anoxic conditions were clearly distinct and separate. The PCA score plot demonstrated nearly 60% of total variance was attributed to first two principal components, 39.5% variance explained by principal component 1 (PC1) and 18.9% by principal component 2 (PC 2) indicated metabolic differences under different growth conditions (Fig. 55A). Separation of 4 groups by PCA analysis suggested that the oxic and anoxic, with/without L-phenylalanine conditions are metabolically distinct.

3.3.3 Partial Least Square Discriminant Analysis (PLS-DA)

Partial least square discriminant analysis (PLS-DA) is employed to identify the statistically significant metabolites that contributed to group separation. Partial least square discriminant analysis is a supervised multivariate statistical method which enhanced the separation seen in PCA score plot. All the four groups were clearly separated in three principal components in PLS-DA (Fig. 55B). The R^2 value (0.9216) indicated the goodness of fit and Q^2 value (0.8613) indicted the goodness of predictability of PLS-DA model. High R^2 and Q^2 suggest that PLS-DA was based on the true metabolic variations.

3.3.4 Identification of key metabolic features in metabolome of Rubrivivax benzoatilyticus **JA2**

The key metabolic features which are responsible for the metabolic variations were identified based on the variable importance projection (VIP) scores obtained from the PLS-DA analysis. Total 50 metabolites with VIP scores >1.2 were considered as statistically significant that were responsible for the metabolic variations (Fig. 56). Aryl metabolites were significantly responsible for the variations in the metabolome of control and L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 grown under oxic and anoxic conditions. Differentially regulated metabolites under oxic and anoxic conditions in the presence and absence of L-phenylalanine were identified.

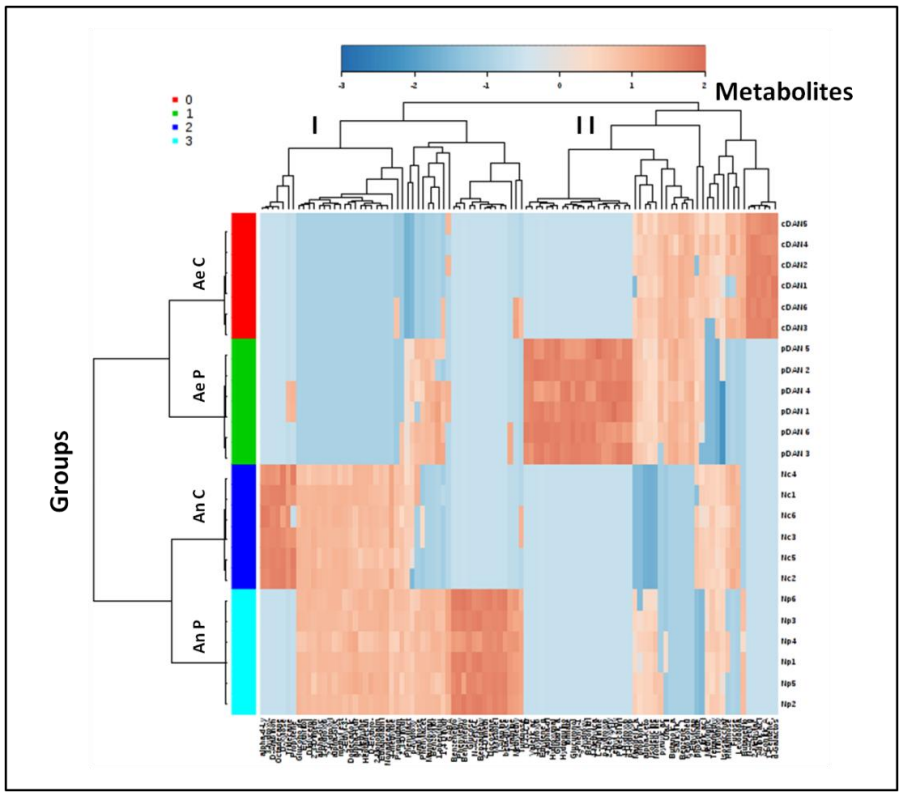


Fig. 54. Hierarchical cluster analysis of metabolome of *Rubrivivax benzoatilyticus* JA2 grown with/without L-phenylalanine under oxic/anoxic conditions.

Clustering was performed with parameters Pearson correlation as distance matrix using MetaboAnalyst (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp) online software. Metabolites in red indicated high concentration and blue indicated low concentrations.

Growth conditions were similar as mentioned in the Fig. 12 and Fig. 32, except for the extraction of the extracellular metabolites as described in section 2.3.3 and the GC-MS analysis was done as described in section 2.4.4. The data processing and statistical analysis was done as described in the section 2.8.2.

Data was normalised by taking the equal dry weights of lyophilized supernatants of culture grown under different conditions. Statistical normalisation of data was done with row-wise normalisation and log (log_2) transformed using MetaboAnalyst (http://www.metaboanalyst.ca/ MetaboAnalyst/faces/Home.jsp). Data represents mean of biological duplicates of three independent experiments.

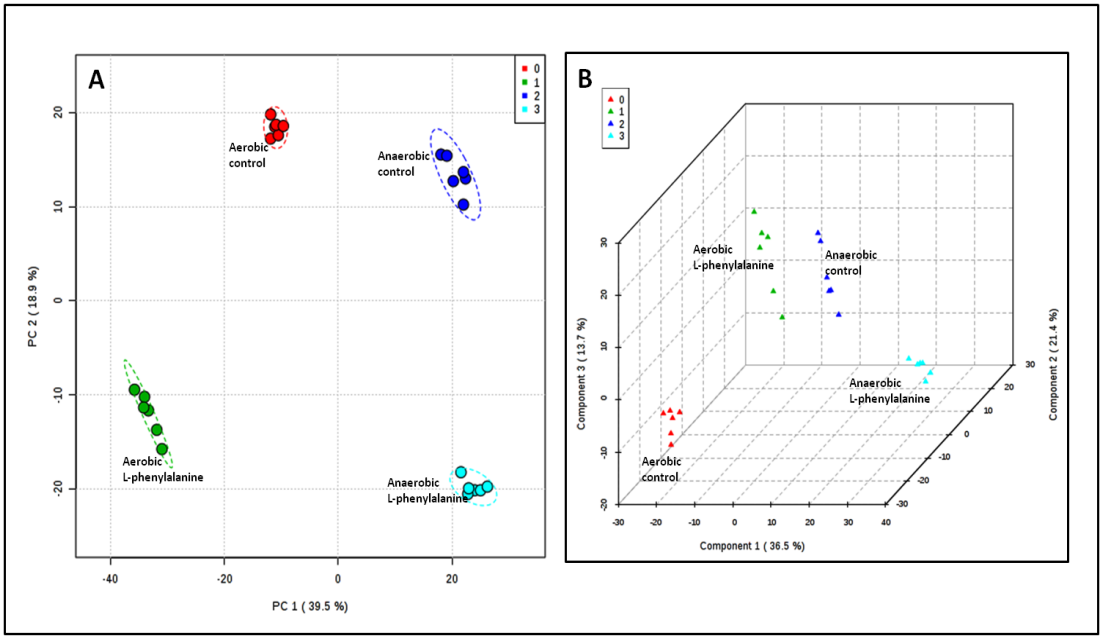


Fig. 55. Multivariate statistical analysis of exometabolome of *Rubrivivax benzoatilyticus* JA2

Principal Component Analysis (PCA) **(A)**, Partial Least Square Discriminant Analysis (PLS-DA) **(B),** score plot analysis between control and L-phenylalanine fed oxic and anoxic cultures of *Rubrivivax benzoatilyticus* JA2,

Data obtained from the GC-MS analysis as described in Fig. 54, used for statistical analysis. Normalised data obtained from GC-MS analysis was subjected to PCA, PLS-DA analysis using MetaboAnalyst.

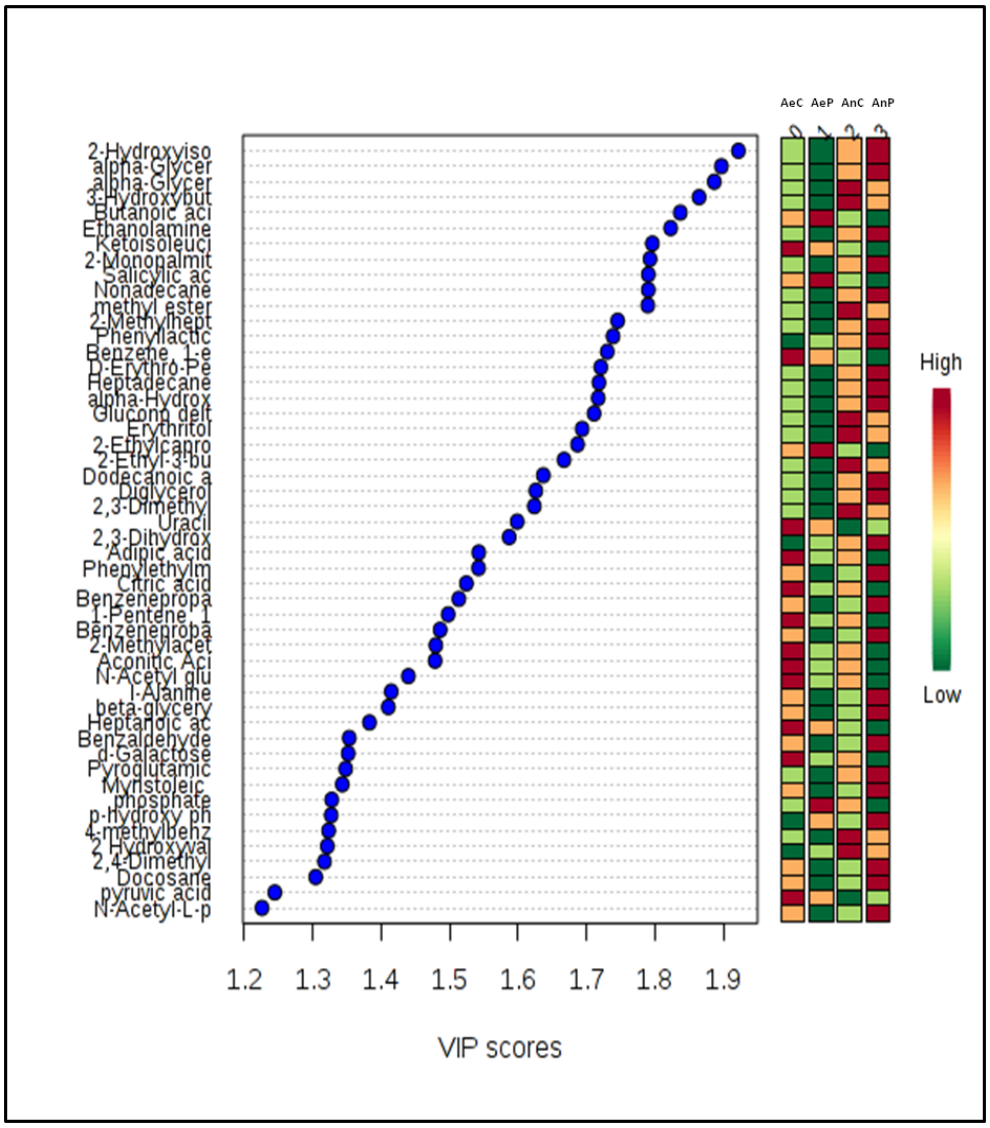


Fig. 56. Variable importance on projection (VIP) scores of the metabolites obtained from PLS-DA analysis of control and L-phenylalanine fed cultures grown under anoxic and oxic conditions.

Coloured boxes on right side indicate relative abundance of metabolites between control and L-phenylalanine fed conditions of oxic and anoxic cultures. Red colour indicates high concentration and green indicates low concentration of metabolites. Metabolites with VIP score >1.2 were considered to be statistically significant.

Data processing parameters were same as described in Fig. 54, and the same data was subjected to VIP analysis using MetaboAnalyst.

3.3.5 Relative fold change analysis of metabolome of Rubrivivax benzoatilyticus JA2

Relative fold change analysis is a univariate statistical analysis explains the differential regulation of the key metabolites that are responsible for the metabolic variations. Metabolites with fold change greater than 2 and the *p*-value less than 0.05 were considered as statistically significant. Fold change analysis revealed that total 51 metabolites were significantly differential regulated. Among which 34 metabolites were produced in high concentration and 17 metabolites were produced in low concentration under anoxic condition in presence of L-phenylalanine (Fig. 57). Aryl metabolites (phenyllactic acid, phenylacetic acid, N-acetyl-L-phenylalanine, tyramine, benzaldehyde, benzenepropanoic acid, mandelic acid, 1,4-dihydrobenzene, phenylethylmalonic acid, benzoyl formic acid, benzenepropanoic acid, benzenepropanoic acid alpha-oxo) followed by fatty acids (2-monopalmitin, beta-glyceryl caprinate, alpha-ketoisocaproic acid, myristoleic acid) were significantly regulated key metabolites that were responsible for the metabolomic variations. Carbohydrates, amino acids, butanoate metabolism, hydrocarbons, organic acids were also high under anoxic condition in presence of L-phenylalanine.

Under oxic conditions a total of 68 metabolites were differential regulated, among which thirty four metabolites were up-regulated and thirty four metabolites were down-regulated in L-phenylalanine fed conditions by *R. benzoatilyticus* JA2 (Fig. 58). Aryl metabolites (mandelic acid, 1-phenylbut-3-en-1-ol, salicylic acid, ethyl 2-hydroxymandelic acid, 4-hydroxyphenyllactic acid, 4-methyl catechol, 2-hydroxy 3(4-hydroxyphenyl) propionic acid, 4-hydroxybenzaldehyde, benzoylformic acid, 4-hydroxyphenyl-dihydroxy ethane, 2-phenyl 1,2-dihydroxypropane, phenylacetic acid, 4-hydroxyphenylacetic acid, benzonitrile, 4,alpha-dihydroxy cinnamic acid, phenyllactic acid, hydroquinone) were major group of metabolites that were high under oxic L-phenylalanine fed conditions.

3.3.6 Functional classification of differentially regulated metabolites

Functional annotation of differentially regulated metabolites was done to identify the significantly influenced metabolic pathways in L-phenylalanine in comparison to control cultures of *R. benzoatilyticus* JA2. The metabolic pathways that were influenced under oxic/anoxic conditions in L-phenylalanine fed cultures were categorised based on the KEGG database (http://www.genome.jp/kegg/pathway). Biosynthesis of aryl metabolites (29%) followed by carbohydrate metabolism (19%), fatty acid & lipid metabolism (16%) and

hydrocarbon metabolism (10%) were highly influenced under anoxic conditions in L-phenylalanine fed compared to control. Metabolic pathways of butanoate (6%), amino acids (6%), TCA cycle (6%), dicarboxylic acid & glyoxalate (4%), nucleic acid (2%), vitamins & co factors (2%) were also influenced under anoxic conditions. (Fig. 59).

The major metabolic pathways that were influenced under oxic conditions in the presence of L-phenylalanine compared to control cultures of *R. benzoatilyticus* JA2 were aryl metabolites biosynthesis (32%) and carbohydrate metabolism (22%). Butanoate metabolism (9%), dicarboxylic acid and glyoxalate (8%) amino acids metabolism (8%), fatty acid metabolism (6%), TCA cycle (5%), hydrocarbon metabolism (5%), nucleic acid metabolism (3%) and nicotinic acid metabolism (2%) were also influenced under oxic condition in the presence of L-phenylalanine compared with control cultures of *R. benzoatilyticus* JA2 (Fig. 60).

3.3.7 Relative fold change of aryl metabolites produced by Rubrivivax benzoatilyticus JA2

Aryl metabolites were the major group of key metabolic features that were influenced under both oxic and anoxic conditions in presence of L-phenylalanine. Functional annotation of key metabolites also revealed the aryl metabolites biosynthesis influenced in both oxic/anoxic conditions. Relative fold change analysis explained the differential regulation of aryl metabolites contributed to metabolomic variations of oxic condition in comparison with anoxic condition (Fig. 61A). Total 21 aryl metabolites were differentially regulated under oxic/anoxic condition, while 12 metabolites were produced in high concentration and 9 metabolites were produced in low concentration under oxic condition compared with anoxic conditions (Fig. 61A). Mandelic acid, ethyl 2-hydroxymandelic acid, 4-methyl catechol, 2-hydroxy 3(4-hydroxyphenyl) propionic acid, 4-hydroxybenzaldehyde, 2-phenyl 1,2-dihydroxypropane, 4-hydroxyphenyl-dihydroxy ethane, benzonitrile, alpha-dihydroxy cinnamic acid, 4-hydroxyphenylacetic acid, salicylic acid and hydroquinone were produced high concentrations in the presence of molecular oxygen. Tyramine, phenylpyruvic acid, 4-methyl benzoate, benzaldehyde, N-acetyl-L-phenylalanine, phenyllactic acid, phenylethylmalonic acid, benzenepropanoic acid, methyl ester of salicylic acid were produced high under anoxic condition (Fig. 61B).

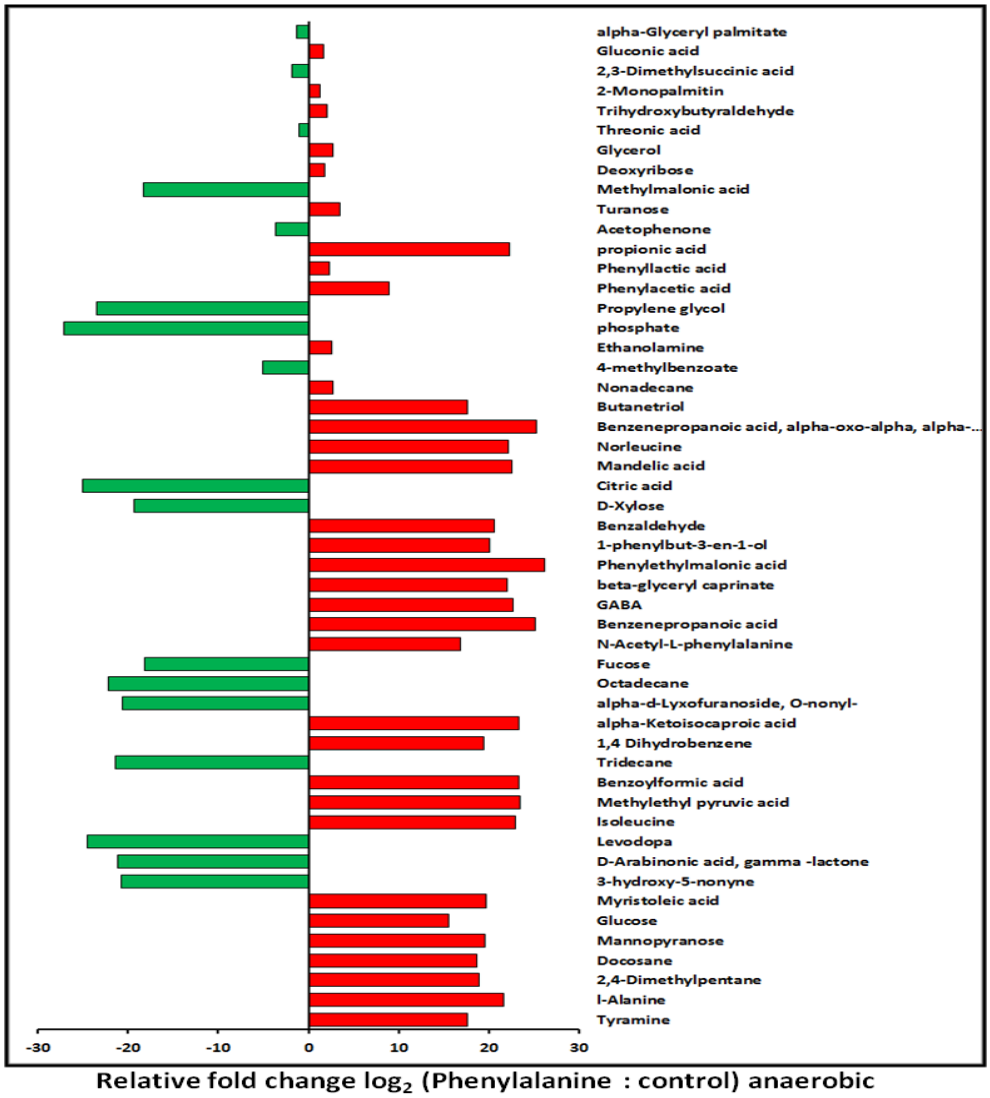


Fig. 57. Relative fold change of metabolites from L-phenylalanine fed cultures with that of control cultures of *Rubrivivax benzoatilyticus* JA2 grown anoxic conditions.

Metabolites with significant fold change of (L-phenylalanine fed /control) > 2.0 and *p*-value <0.05 were considered for data analysis. Relative fold change correspond to peak area of the metabolites of L-phenylalanine fed/control cultures. Fold change analysis was performed by using MetaboAnalyst.

Data obtained from GC-MS analysis as described in Fig. 56, was $\log_2$ transformed and subjected to Student's *t*-test by using Metaboanalyst.

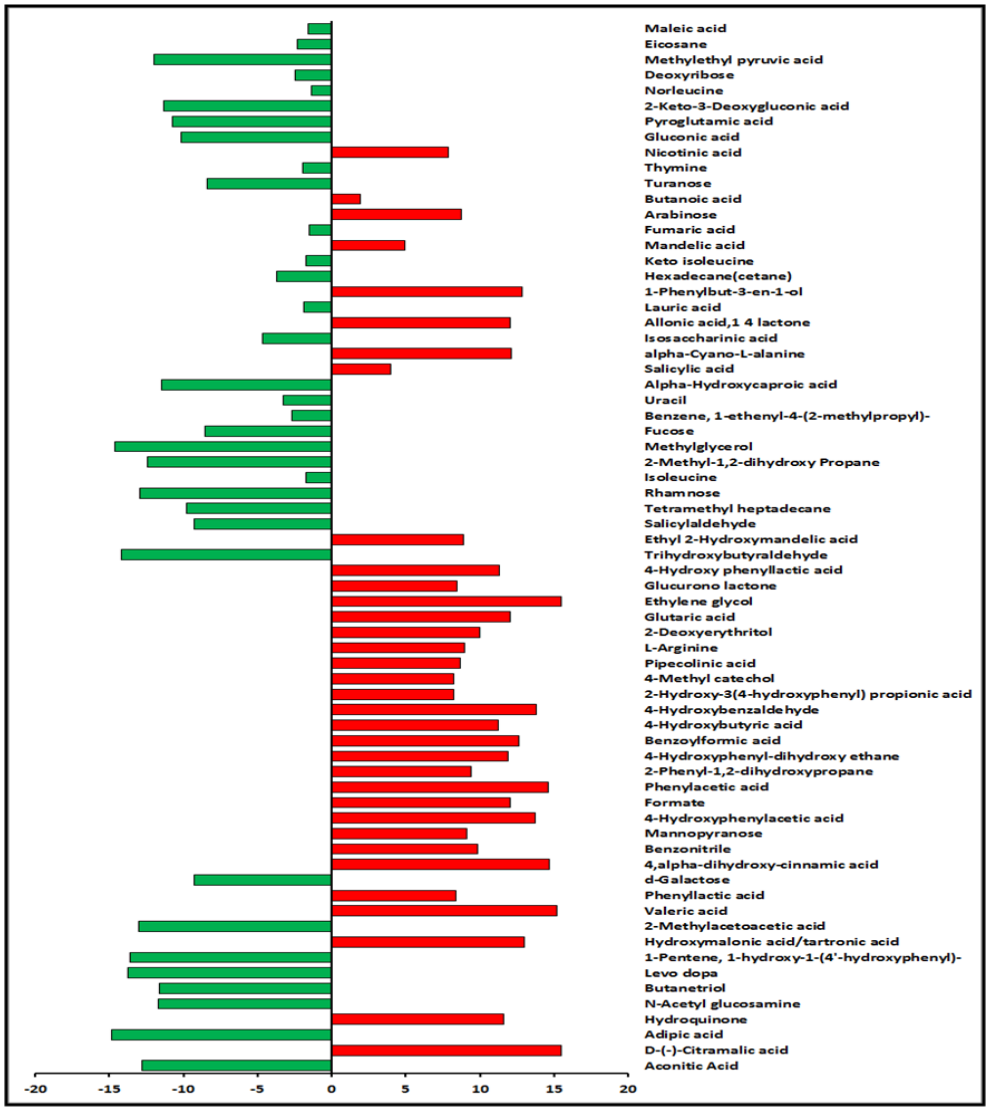


Fig. 58. Relative fold change of metabolites from L-phenylalanine fed cultures with that of control grown under oxic conditions of *Rubrivivax benzoatilyticus* JA2.

Metabolites with significant fold change (L-phenylalanine fed /control) > 2.0 and *p*-value <0.05 were considered for data analysis. Relative fold changes correspond to peak area of the metabolites of L-phenylalanine fed/control cultures. Fold change analysis was performed by using MetaboAnalyst.

Data obtained from GC-MS analysis as described in Fig. 56, was $\log_2$ transformed and subjected to Student's *t*-test by using MetaboAnalyst.

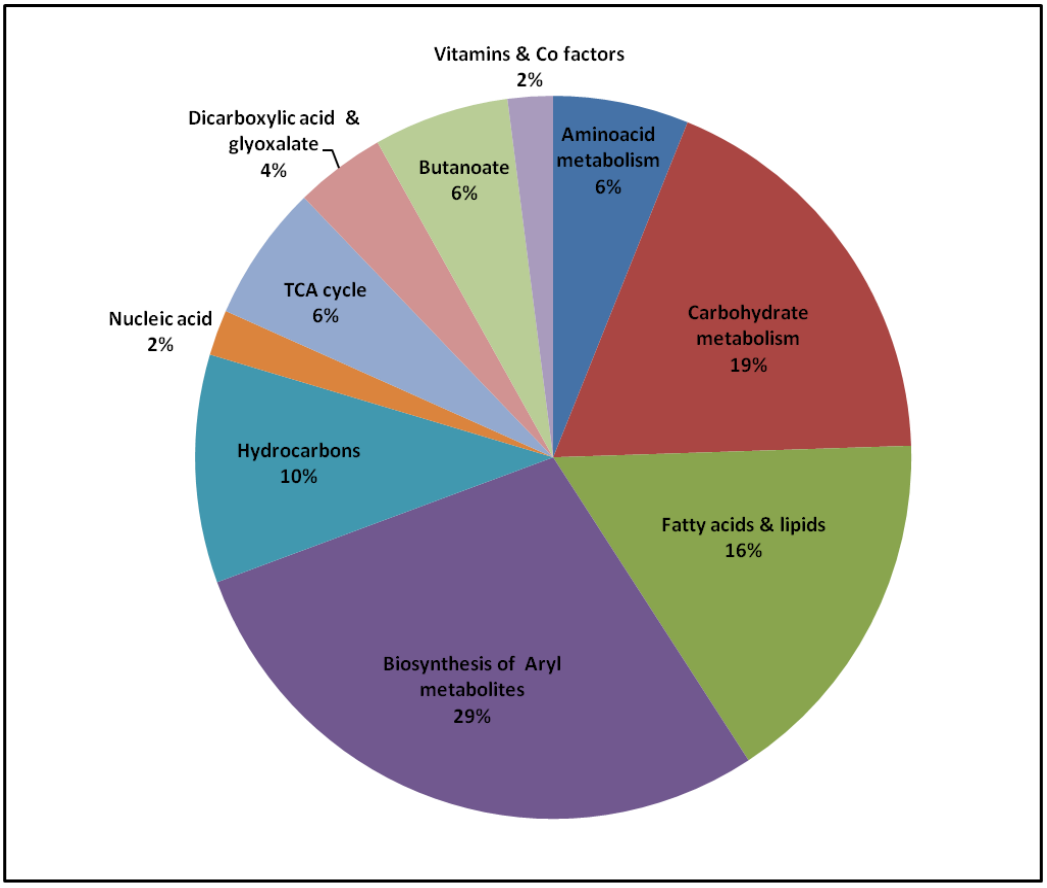


Fig. 59. Functional classification of differential regulated metabolites of L-phenylalanine fed cultures of *Rubrivivax benzoatilyticus* JA2 grown under anoxic conditions.

Metabolites with fold change (L-phenylalanine fed /control) >2.0 and *p*-value < 0.05 were considered for analysis. Functional classification was done according to KEGG database (http://www.genome.jp/kegg/pathway.html.)

Metabolites identified as significant by fold change analysis described in Fig. 57, were used for functional classification according to KEGG.

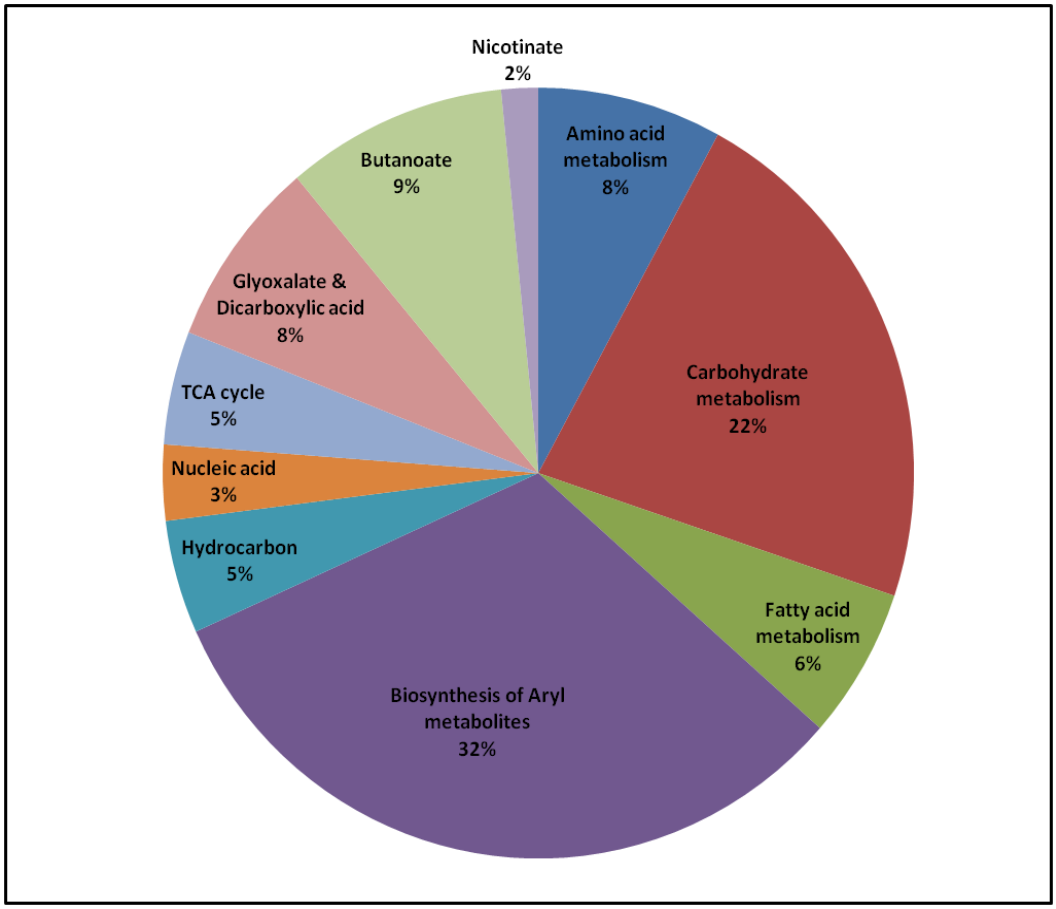


Fig. 60. Functional classification of differentially regulated metabolites of L-phenylalanine fed cultures of *Rubrivivax benzoatilyticus* JA2 grown under oxic conditions.

Metabolites with fold change (L-phenylalanine fed /control) >2.0 and *p*-value < 0.05 were considered for analysis. Functional classification was done according to KEGG database (http://www.genome.jp/kegg/pathway.html.)

Metabolites identified as significant by fold change analysis described in Fig. 59, were used for functional classification according to KEGG.

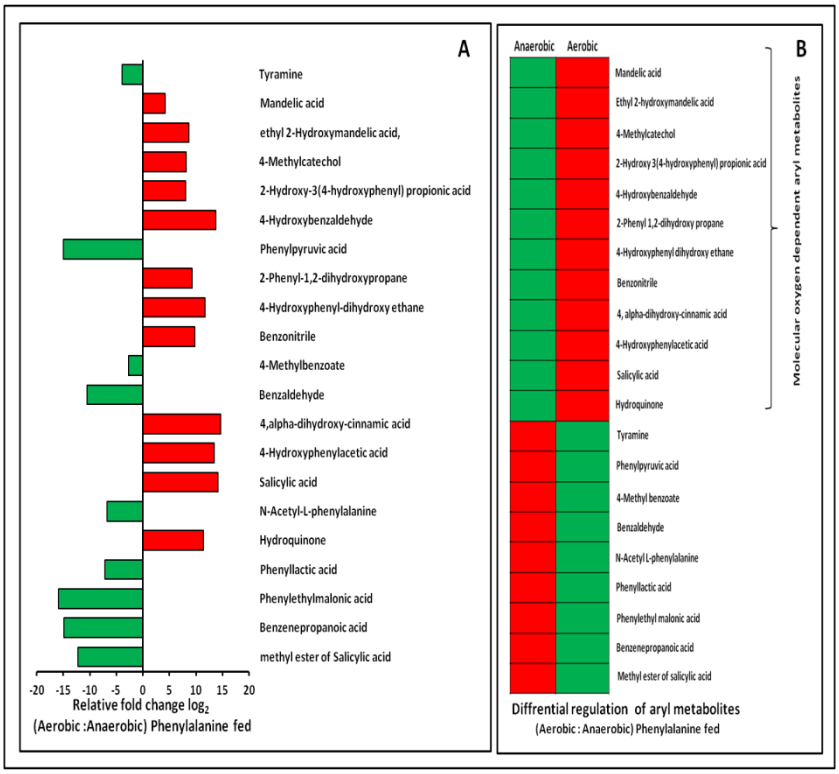


Fig. 61. Relative fold change of aryl metabolites from oxic with that of anoxically grown culture of *Rubrivivax benzoatilyticus* JA2 in presence of L-phenylalanine.

Aryl metabolites with significant fold change (oxic/anoxic) > 2.0 and *p*-value <0.05 were considered for data analysis **(A)**. Differential regulation of aryl metabolites compared between oxic and anoxic conditions **(B).** Relative fold change correspond to peak area of the metabolites of oxic and anoxic L-phenylalanine fed cultures. Fold change analysis was performed by using MetaboAnalyst.

Data obtained from GC-MS analysis as described Fig. 57, 58, aryl metabolites were log_2 transformed and subjected to Student's *t*-test by using MetaboAnalyst. Aryl metabolites represented in red were up-regulated, green color were down regulated.

3.3.8 Production of L-phenylalanine catabolites by Rubrivivax benzoatilyticus JA2

Metabolome suggested the aryl metabolites biosynthesis is the highly influenced metabolism under oxic/anoxic conditions. Quantification of aryl metabolites was done with known concentrations of authentic standards by using HPLC. L-Phenylalanine catabolites like phenyllactic acid (48 µg/ml), phenylpyruvic acid (0.9 µg/ml), 4-hydroxyphenylpyruvic acid (1.5 µg/ml) were produced in high concentrations under anoxic conditions in presence of L-phenylalanine compared to oxic condition while phenylacetic acid (19.3 µg/ml) produced in high concentrations under oxic condition (Fig. 62).

Production of 4-hydroxybenzaldehyde (4-HBAld), benzoic acid (BA) under oxic/anoxic conditions was significantly high in the presence of L-phenylalanine compared to control cultures of *R. benzoatilyticus* JA2. Under anoxic conditions, 4-hydroxybenzaldehyde and benzoic acid production in presence of L-phenylalanine (79.5 µg. mg dry wt^{-1} , 569 µg. mg dry wt^{-1}) is high compared to control (4.4 µg. mg dry wt^{-1} , 397 µg. mg dry wt^{-1}) while 4-hydroxybenzoic acid production was not significantly varied in the presence (175.5 µg. mg dry wt^{-1}) and absence(160.5 µg. mg dry wt^{-1}) of L-phenylalanine (Fig. 63A). Production of 4-HBald and BA were also significantly high in the presence of L-phenylalanine (11 µg. mg dry wt^{-1},23.3 µg. mg dry wt^{-1}) compared to control (0.9 µg. mg dry wt^{-1},1.3 µg. mg dry wt^{-1}) under oxic conditions while 4-HBA was not significantly varied in presence (3.55 µg. mg dry wt^{-1}) and absence (2.8 µg. mg dry wt^{-1}) of L-phenylalanine (Fig. 63B).

.

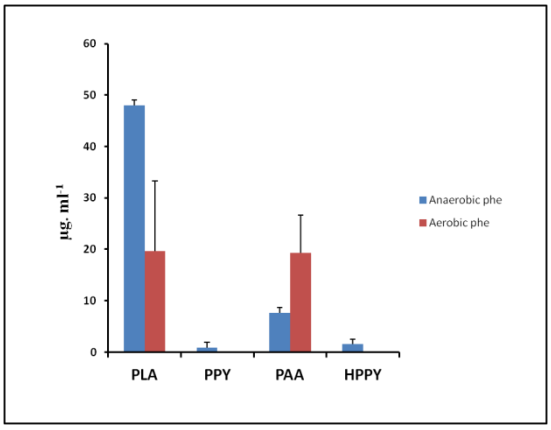


Fig. 62. Production of L-phenylalanine catabolites under anoxic and oxic conditions by *Rubrivivax benzoatilyticus* JA2

Experiment was done as described in Fig. 13, 33, except for quantification of metabolites from the oxic and anoxic cultures were done with authentic standards in HPLC. PLA, phenyllactic acid; PPY, phenylpyruvic acid; PAA, phenylacetic acid; HPPY, 4-hydroxyphenylpyruvic acid.

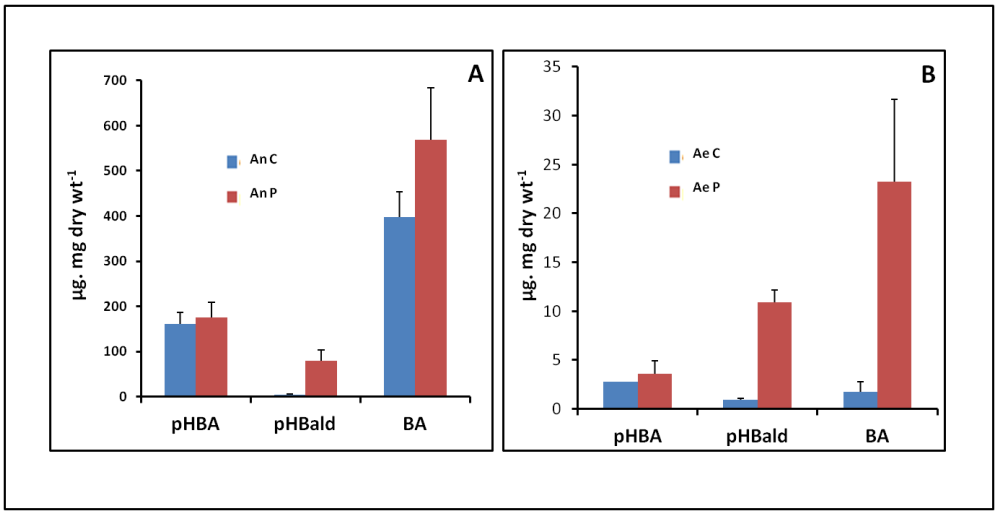


Fig. 63. Production of aryl metabolites by *Rubrivivax benzoatilyticus* JA2 under anoxic/oxic conditions

Aryl metabolites production by *Rubrivivax benzoatilyticus* JA2 with/without L-phenylalanine fed cultures under anoxic **(A)** and oxic condition **(B)**.

Experiment was done as referred in Fig. 62, pHBA, 4-hydroxybenzoic acid; pHBald, 4-hydroxybenzaldehyde; BA, benzoic acid.

3.3.9 Metabolic shift in presence of molecular oxygen by Rubrivivax benzoatilyticus JA2

Metabolome data suggested the shift in the metabolism of L-phenylalanine under oxic/anoxic conditions by *R. benzoatilyticus* JA2. Molecular oxygen induced the brown pigment production by *R. benzoatilyticus* JA2 only in presence of L-phenylalanine. The brown pigment production was not observed in presence/absence of L-phenylalanine when grown under anoxic conditions whereas the same anoxically grown cultures of *R. benzoatilyticus* JA2 shifted to oxic conditions, the brown pigment production was observed only in presence of L-phenylalanine (Fig. 64A). 4-Hydroxyphenylpurivic acid (4-HPPY) produced within the 24 h of addition of L-phenylalanine remained unused under anoxic conditions, but when the cultures were transferred to oxic conditions, 4-HPPY transformed into homogentisic acid (HMG) which further oxidised and polymerised to form melanin indicating the shift in the metabolism of L-phenylalanine (Fig. 64A). Production of 4-HPPY produced in presence of L-phenylalanine under anoxic conditions is 2 µg/250ml whereas no 4-HPPY was observed under oxic conditions (Fig. 64B).

3.3.10 Effect of glyphosate on de novo synthesis of 4-hydroxybenzoic acid

Production of 4-hydroxybenzoic acid (4-HBA) both in control as well as L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 was observed. 4-Hydroxybenzoic acid was produced from chorismate and the reaction was catalysed by the enzyme chorismate pyruvate lyase (CPY) (Fig. 65A). The enzyme activity of the chorismate pyruvate lyase was performed with cell free extracts of the *R. benzoatilyticus* JA2 as enzyme source and chorismate as precursor. The product 4-hydroxybenzoic acid formation was observed only in the reaction at R_t 12 min in HPLC chromatogram but absent in pre-denatured blank (Fig. 65B). Effect of glyphosate was observed on the production of 4-hydroxybenzoic acid. Production of 4-hydroxybenzoic acid was significantly low in presence of glyphosate (0.5 µg. mg dry wt^{-1}) (Fig. 65C) compared to malate (1.1 µg. mg dry wt^{-1}) grown cultures of *R. benzoatilyticus* JA2 under anoxic conditions indicating the *de novo* synthesis of 4-HBA from chorismate.

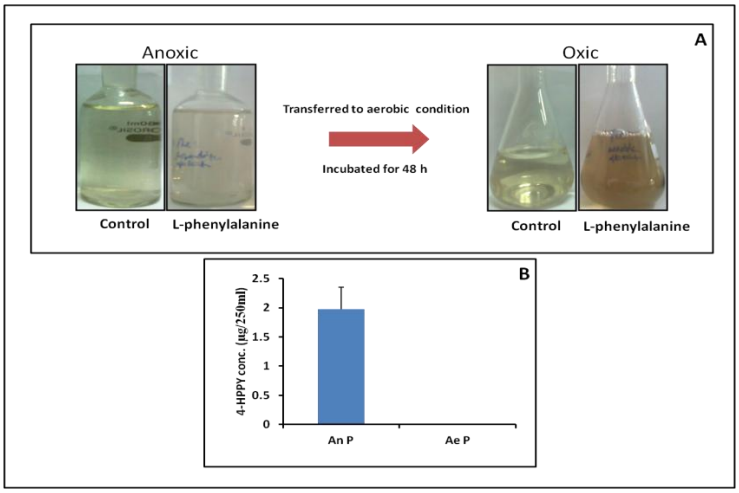


Fig. 64. Metabolic shift of L-phenylalanine catabolism in *Rubrivivax benzoatilyticus* JA2 under oxic/anoxic conditions.

Pigment production was observed when anoxically grown L-phenylalanine fed cultures transferred to oxic condition **(A)**. 4-hydroxyphenylpyruvic acid levels before and after transferring of L-phenylalanine fed cultures to oxic conditions **(B)**.

Experiment was done first same as mentioned in Fig. 32, 33, except for the 24 h grown L-phenylalanine fed cultures were transferred to oxic condition incubated for 48 h and harvested. An P, anoxic L-phenylalanine fed; Ae P, oxic L-phenylalanine fed.

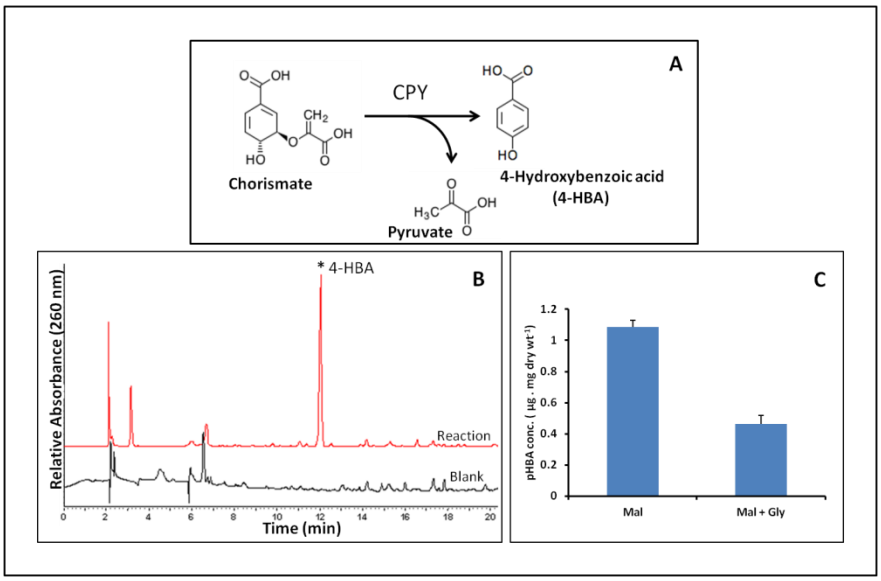


Fig. 65. *De novo* synthesis of 4-hydroxybenzoic acid from chorismate by *Rubrivivax benzoatilyticus* JA2

Reaction indicating the synthesis of 4-HBA from chorismate by chorismate pyruvate lyase enzyme (A), activity was detected in reaction mixture by using HPLC, where as no activity was observed in pre-denatured blank (B). Effect of glyphosate on synthesis of 4-HBA (C).

Experiment was done same as mentioned in Fig. 27, except for precursor chorismate. 4-HBA, 4-hydroxybenzoic acid; Mal, malate; Gly, glyphosate.

3.3.11 Fatty acid profiling under oxic and anoxic conditions of Rubrivivax benzoatilyticus JA2

Profiling of total cellular fatty acid was performed by fatty acid methyl ester analysis (FAME analysis). Total saturated fatty acid content increased in the presence of L-phenylalanine (42.3%) compared to control (39%) under oxic conditions while they are decreased in presence of L-phenylalanine (38.5%) compared to control (43.7%) under anoxic conditions. Total unsaturated fatty acid content was decreased in presence of L-phenylalanine (48.8%) compared to control (52.7%) under oxic conditions while they remain same in L-

phenylalanine (48.6%) and to control (48%) under anoxic condition. $C_{16:0}$ and $C_{16:1}$ ω7c/ ω6c are the major fatty acids observed both in control and L-phenylalanine conditions under oxic and anoxic conditions. Among saturated fatty acids, $C_{10:0}$ and $C_{12:0} - C_{17:0}$, $C_{15:0}$ 2OH, $C_{16:1}$ ω7c alcohol, $C_{16:1}$ ω7c/ω6c, $C_{17:1}$ ω8c/ ω6c, $C_{18:1}$ ω7c/ ω6c, $C_{19:1}$ ω6c/ω7c/19cy were the fatty acids observed under both oxic as well as anoxic conditions of *R. benzoatilyticus* JA2 in the presence and absence of L-phenylalanine. $C_{19:0}$ cyclo ω10c observed only in presence of L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 under both oxic/anoxic conditions. Hydroxy fatty acids content increased in presence of L-phenylalanine (7%) under oxic as well as anoxic conditions compared to control (5.2%). Branched chain fatty acids were also produced more under anoxic conditions in presence (5.6%) and absence (6.7%) of L-phenylalanine compared to oxic conditions in presence (1.7%) and absence (3.1%) of L-phenylalanine. Cyclo fatty acids are also produced high in presence of L-phenylalanine under oxic (1.4%) conditions compared to anoxic (0.3%) conditions.

Table.6. Fatty acid profiling of *Rubrivivax benzoatilyticus* JA2 grown with/without L-phenylalanine under oxic and anoxic conditions

Fatty acids	mol %			
	Anoxic		Oxic	
	Control	L-phenylalanine	Control	L-phenylalanine
Saturated	43.7	38.5	39.0	42.3
Unsaturated	48.6	48.6	52.7	48.8
Hydroxy	0.4	7.1	5.2	7.0
Cyclo propane	-	0.3	0.1	1.4
Branched chain	6.7	5.6	3.1	1.7
Polyunsaturated	0.8	0.3	0.2	0.1

Experiment was done as mentioned in Fig. 45, except for the lyophilised pellets were used for analysis. Fatty acids were analysed by gas chromatography.

DISCUSSION

4.0 Discussion

Anoxygenic phototrophic bacteria (APB) are unique physiological group of bacteria which can grow under anoxic and oxic zones by utilizing wide range of organic compounds (Larimer et al., 2004; Oda et al., 2008). Anoxic metabolism of aromatic amino acids was studied in APB whereas insights of oxic metabolism were not yet studied. Moreover, metabolic responses and metabolic shifts associated with aromatic amino acids metabolism under anoxic and oxic conditions was not elucidated so far though they thrive under anoxic and oxic zones. Gaining insights of aromatic metabolism of APB under oxic and anoxic conditions may shed light on their role in aromatic metabolism under different growth modes. *Rubrivivax benzoatilyticus* JA2 utilises L-phenylalanine as sole source of nitrogen and produced an array of value added L-phenylalanine derivatives under anoxic conditions (Kumavath et al., 2011). As *R. benzoatilyticus* JA2 utilises L-phenylalanine as sole source of nitrogen both under oxic and anoxic conditions, we selected this organism for the present study to elucidate the L-phenylalanine metabolism under anoxic and oxic conditions. Stable isotope metabolic profiling revealed the multiple catabolic pathways of L-phenylalanine and catabolic shift under anoxic and oxic conditions. GC-MS based global metabolomics deciphered metabolic responses of *R. benzoatilyticus* JA2 to anoxic and oxic conditions. The present study provided comprehensive understanding of L-phenylalanine catabolism under oxic and anoxic conditions and the same was discussed in greater detail.

L-Phenylalanine metabolism in Rubrivivax benzoatilyticus JA2 *under oxic and anoxic conditions*

Rubrivivax benzoatilyticus JA2 could grow at the expense of L-phenylalanine as sole source of nitrogen but not as carbon source both under oxic and anoxic conditions and this is due to lack of genes coding for ring cleavage enzymes such as aromatic dioxygenases (oxic) and benzoyl-CoA thioesterases (anoxic) in the genome. Similarly other proteobacteria, *Rhodobacter sphaeroides* OU5 (Ranjith et al., 2007b) and *Rhodobacter capsulatus* B10S (Saez et al., 1999) also utilised L-phenylalanine as sole source of nitrogen but not as carbon source. Bacteria utilise L-phenylalanine/L-tyrosine as nitrogen source and simultaneously produce phenols under different physiological conditions (Ranjith et al., 2007b; Kumavath et al., 2010a). Similarly, *Rubrivivax benzoatilyticus* JA2 produced phenols when grown under oxic and anoxic conditions with simultaneous utilisation of L-phenylalanine as sole source of nitrogen (Kumavath et al., 2011). *Rhodobacter sphaeroides* OU5 (Ranjith et al., 2007b), *Rhodobacter capsulatus* B10S

(Saez et al., 1999) produced phenols when grown in the presence of L-phenylalanine as sole source of nitrogen. *Rubrivivax benzoatilyticus* JA2 produced high phenols under oxic condition compared to anoxic conditions and this may be due to the presence of oxygen activated hydroxylases, mono and dioxygenases which catalyse the bioconversion of L-phenylalanine/L-tyrosine to phenolic compounds (Mohammed et al., 2011). Phenols are the group of aryl metabolites, which include aryl acids, aryl aldehydes and aryl alcohols which derive from aromatic amino acids such as L-phenylalanine and L-tyrosine (Lapadatescu et al., 2000; Ravasio et al., 2014).

HPLC comparative metabolic profiling indicated the production of different aryl metabolites such as phenyl derivatives (260-280 nm), benzyl derivatives (250-260 nm) and cinnamyl derivatives (280-320, 350-550 nm) under L-phenylalanine fed condition. Production of these metabolites only in the presence of L-phenylalanine under both oxic and anoxic conditions indicates that the metabolites are L-phenylalanine derivatives. Under oxic conditions also *R. benzoatilyticus* JA2 produced phenyl derivates, benzyl derivatives and in addition some pigments with absorption ranging from 300 to 550 nm which remained unidentified (Table. 4). However, stable isotope studies revealed that the molecular ion masses (M+0) of pigments increased by M+12, M+18, and M+24 units suggesting that these pigments are conjugated aromatic compounds (Table. 7) and are derived from L-phenylalanine. Production of pigments only under L-phenylalanine fed oxic conditions suggests that pigments production in *R. benzoatilyticus* JA2 is an oxygen dependent L-phenylalanine catabolism. Comparative metabolite profiling of L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 revealed wide range of aryl metabolites production under oxic conditions compared to anoxic condition indicating the diversity in L-phenylalanine catabolism under oxic conditions. Stable isotope profiling revealed more number of high molecular weight unidentified aryl metabolites (M+12, M+18, M+24) under oxic conditions indicating the conjugated aromatic metabolites formation by unknown pathway (Table.7). Stable isotope studies revealed that L-phenylalanine is catabolised into multiple pathways such as Ehrlich's pathway, 4-hydroxyphenylpyruvate pathway, N-acetyl-L-phenylalanine and pigments biosynthetic pathway under oxic and anoxic conditions.

L-Phenylalanine catabolism via Ehrlich's pathway

Ehrlich's pathway a common central catabolic pathway of amino acids lead to the production of alcohols or carboxylic acids which contain one carbon atom less than the

precursor amino acid (Ravasio et al., 2014). Few amino acids such as leucine, isoleucine (branched chain aminoacids), L-phenylalanine, L-tyrosine, L-tryptophan, and methionine are catabolised *via* Ehrich's pathway. Aromatic amino acids catabolism *via* Ehrlich's pathway results in production of aryl α-keto acids which on decarboxylation led to aryl aldehyde. Finally aryl aldehyde on oxidation led to aryl acid and reduction led to aryl alcohol (Hazelwood et al., 2008).

Detection of phenylpyruvic acid (PPY), phenylacetaldehyde (PAld), phenylacetic acid (PAA) based on LC-MS metabolite profiling indicated the L-phenylalanine catabolism *via* Ehrlich's pathway under anoxic conditions in *R. benzoatilyticus* JA2 (Fig. 31). Further Ehrlich's pathway was confirmed with stable isotope studies and the Ehrlich pathway specific enzyme activities (Fig. 31). Catabolism of L-phenylalanine *via* Ehrlich's pathway was reported in *Rhodobacter capsulatus* B10S (Saez et al., 1999), lactic acid bacteria (Li et al., 2007; Mu et al., 2009a) and *Bravibacterium* (Lavermicocca et al., 2000). The enzyme L-phenylalanine/aromatic amino acid aminotransferase converts L-phenylalanine to phenylpyruvic acid and the genome analysis revealed the presence of aromatic amino acid transaminase/aminotransferase genes which may convert the L-phenylalanine/L-tyrosine to phenylpyruvic acid/4-hydroxyphenylpyruvic acid (Mohammed et al., 2011). Furthermore, In the genome of *R. benzoatilyticus* JA2 putative decarboxylases and dehydrogenases protein coding genes were found, which may convert phenylpyruvic acid to phenylacetic acid indicating the existence of possible Ehrlich's pathway genes. Although Ehrlich's pathway metabolites were observed in both oxic and anoxic conditions, their production was high under anoxic conditions.

Stable isotope studies revealed nearly ~70% of L-phenylalanine has been channelized into Ehrlich's pathway under anoxic conditions whereas only 30% under oxic conditions. This explains the plausible differential flux of L-phenylalanine to Ehrlich's pathway (Fig. 64). Only 30% of L-phenylalanine flux to Ehrlich's pathway under oxic conditions, possibly due to the major flux of L-phenylalanine towards oxygen dependent catabolic pathways (pigments) of L-phenylalanine (Fig. 64). Taken together, L-phenylalanine was differentially fluxed into multiple catabolic pathways under oxic conditions (Weiner et al., 2014). Stable isotopic profiling revealed wide range of labeled aryl metabolites production, some of them are unidentified and this suggests that L-phenylalanine was channelized less towards Ehrlich's pathway and more towards pigments synthesis under oxic conditions (Fig. 64).

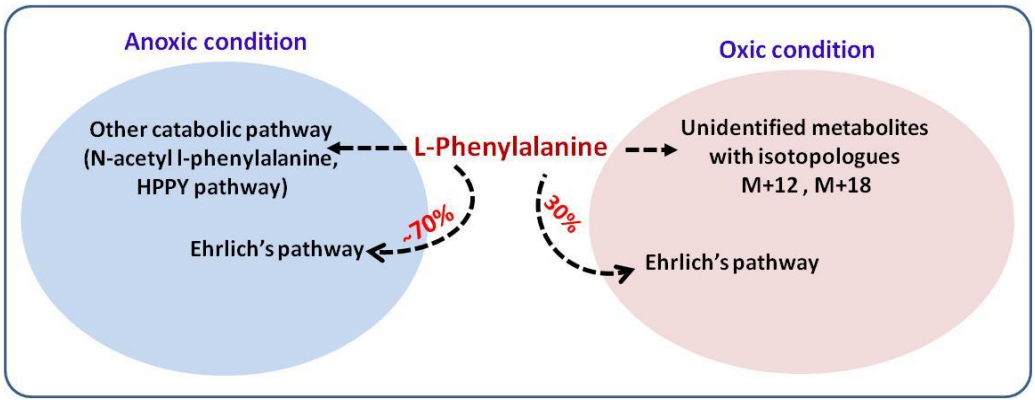


Fig. 64. Schematic representation of L-phenylalanine catabolism under oxic and anoxic conditions in *Rubrivivax benzoatilyticus* JA2

Dotted line represented the differential flux of L-phenylalanine under anoxic and oxic conditions.

Phenyllactic acid (PLA) was the major metabolite produced in the presence of L-phenylalanine under anoxic conditions and this was the first report of its production among APB. Phenyllactic acid was produced from phenylpyruvic acid, an intermediate of Ehrlich's pathway and the reaction was catalysed by dehydrogenase enzyme. Phenyllactic acid (PLA), is a biologically active chiral compound with antimicrobial property (Svanstrom et al., 2013). Phenyllactic acid production was restricted to a few groups of bacteria such as lactic acid bacteria (Li et al., 2007; Mu et al., 2009b), propionibacteria (Thierry and Maillard, 2002), *Geotrichum candidum* UCMA 4574 (Naz et al., 2013), *and Bravibacterium* (Mu et al., 2012a). Production of 3-phenyllactic acid (PLA), was reported in *Pediococcus acidilactici* DSM 20284 under anoxic conditions when supplemented with L-phenylalanine and tyrosine (Mu et al., 2012b). As the PLA is a biologically active compound its production and also the optimisation methods of PLA production were studied. The production of PLA in *R. benzoatilyticus* JA2 was influenced by different physiological factors such as precursor feeding, carbon sources and different conditions of culture i.e anoxic and oxic conditions (Fig. 22). Phenyllactic acid production increased with increase in the precursor, L-phenylalanine concentration ranging (1 mM – 10 mM). Similarly, production of PLA increased with increasing L-phenylalanine concentrations (0.6 – 30.2 mM) in *Geotrichum candidum* UCMA 4574 (Naz et al., 2013). High yields of PLA was observed in the presence of sugar carbon sources compared to non-sugar carbon sources in *R. benzoatilyticus* JA2 (Fig. 22B) and this may be due to availability of NADH cofactor for the enzyme lactate dehydrogenase which converts the phenylpyruvic acid

to phenyllactic acid as glucose readily generates the cofactor NADH under anoxic conditions (Li et al., 2007). Similarly, high yields of PLA was observed in *Bacillus coagulans* SDM, when grown on glucose along with PPY (Zhaojuan et al., 2011).

Phenyllactic acid production from phenylpyruvic acid was catalysed by phenyllactate dehydrogenase and the genome sequence of *R. benzoatilyticus* JA2 revealed the presence of lactate dehydrogenase gene which can also acts as a phenyllactate dehydrogenase. Earlier reports in *Bacillus coagulans* SDM also suggested, lactate dehydrogenase converted phenylpyruvic acid to phenyllactic acid (Zhaojuan et al., 2011). L-Phenylalanine was catabolised into multiple pathways by *R. benzoatilyticus* JA2 under anoxic conditions i.e Ehrlich's pathway, 4-hydroxyphenylpyruvic acid pathway, N-acetyl-L-phenylalanine pathway. L-Phenylalanine was channelized into N-acetyl-L-phenylalanine only under anoxic conditions in *R. benzoatilyticus* JA2 indicating that this pathway is specific for anoxic conditions and the conversion was catalysed by an enzyme phenylalanine-N-acetyltransferase. However, N-acetyl-L-phenylalanine production was not reported in bacteria and this was the first report of production of N-acetyl-L-phenylalanine. However, production of a bioactive metabolite N–acetyl-3,4-dihydroxy-L-phenylalanine was reported in *Streptomyces* sp.8812 (Solecka et al., 2012). The genome of *R. benzoatilyticus* JA2 consists of N-acetyltransferase which may act as phenylalanine N-acetyltransferase and may convert L-phenylalanine to N-acetyl-L-phenylalanine (Mohammed et al., 2011).

L-Phenylalanine catabolism via 4-hydroxyphenylpyruvic acid and pyomelanin biosynthesis

Rubrivivax benzoatilyticus JA2 under oxic and anoxic conditions produced 4-hydroxyphenylpyruvic acid (HPPY) from L-Phenylalanine. However, HPPY was further converted to homogentisic acid (HMG) under oxic conditions in *R. benzoatilycus* JA2 whereas, it remained as a dead-end molecule under anoxic conditions. Bacteria degrade/transform tyrosine into homogentisic acid *via* HPPY (Arias-Barrau et al., 2004) while in *R. benzoatilyticus* JA2, L-phenylalanine was transformed to HPPY, indicating the possible tyrosine dependent L-phenylalanine catabolism. However, tyrosine was not detected either in HPLC or LC-MS may be due to its trace amounts or due to its rapid conversion into 4-hydroxyphenylpyruvic acid. Nevertheless, genome sequence of *R. benzoatilyticus* JA2 revealed the presence of phenylalanine-4-monooxygenase gene which converts L-phenylalanine to L-tyrosine indicating plausible conversion of L-phenylalanine to tyrosine. Production of brown pigment was observed under oxic conditions which was identified as pyomelanin based on physico-chemical

properties (Table. 5) and polychem analysis (Fig. 39) in *R. benzoatilyticus* JA2. However, pyomelanin pigment production was not observed when *R. benzoatilyticus* JA2 was grown in presence of L-phenylalanine under anoxic conditions. In general, pyomelanin was produced from L-phenylalanine/L-tyrosine *via* 4-hydroxyphenylpyruvic acid and homogentisic acid which on autooxidation and copolymerisation led to pyomelanin and the enzymes involved in pyomelanin biosynthesis are phenylalanine-4-monoxygenase, aromatic aminotransferase, 4-hydroxyphenylpyruvate dioxygenase (Coelho-Souza et al., 2014). Pyomelanin, a brown colored extracellular pigment production reported in pathogenic bacterium *Legionella pneumophila* BAA75 by utilising L-phenylalanine/L-tyrosine as sole source of carbon and also as nitrogen source (Flydal et al., 2012). Similarly, production of L-tyrosine dependent DOPA-melanin *via* 4-hydroxyphenylacetic acid was reported in *Klebsiella sp.*GSK (Sajjan et al., 2010).

Pyomelanin production was observed only under oxic conditions. Though pyomelanin production was observed under oxic conditions, its precursor 4-hydroxyphenylpyruvic acid was not observed under oxic conditions. This may be due to its immediate conversion to homogentisic acid under oxic conditions. Even though high levels of 4-hydroxyphenylpyruvate was detected in anoxic conditions, pyomelanin production was not observed. When anoxic culture was transferred to oxic condition within 24 h, the pigment production was observed indicating the shift in the catabolism of L-phenylalanine towards melanin synthesis under oxic conditions in *R. benzoatilyticus* JA2 (Fig. 64). In support of this we found conversion of HPPY to HMG as soon as the culture was shifted from anoxic to oxic condition by the active oxygenases which lead to the production of pyomelanin pigment in *R. benzoatilyticus* JA2. The pyomelanin biosynthetic pathway was confirmed in *R. benzoatilyticus* JA2 by using different precursors feeding, inhibitor studies, enzyme activities. Sulcotrione, an inhibitor, of 4-hydroxyphenylpyruvate dioxygenase and blocks the the pyomelanin biosynthesis (Fig. 42). Inhibition of melanin synthesis by sulcotrione indicated the homogentisic acid dependent pyomelanin biosynthesis in *R. benzoatilyticus* JA2 (Fig. 42). Similarly, sulcotrione inhibited pyomelanin biosynthesis in *Sporothrix* spp.(Almeida-Paes et al., 2012), and *Acinetobacter baumonii* 456MDp (Coelho-Souza et al., 2014). Further, the pyomelanin biosynthesis was confirmed by feeding precursors such as L-tyrosine, 4-hydroxyphenylpyruvic acid, homogentisic acid which led to the production of melanin (Fig. 41). Genome of *R. benzoatilyticus* JA2 revealed the presence of genes involved in pyomelanin biosynthesis such as phenylalanine 4-monoxygenase, aromatic amino acid aminotransferase and 4-hydroxyphenylpyruvate dioxygenase and this confirms the biosynthesis of pyomelanin in *R.*

benzoatilyticus JA2. L-Phenylalanine/L-tyrosine catabolised into different types of melanin such as eumelanin, pheomelanin neuromelanin and pyomelanin. Except pyomelanin, all other types of melanins are DOPA dependent melanins, while pyomelanin was homogentisic acid dependent melanin (Singh et al., 2013). Pyomelanin production by *R. benzoatilyticus* JA2 is a homogentisate dependent melanin biosynthesis and oxic conditions specific L-phenylalanine catabolism (Fig. 53).

Stable isotope studies revealed formation of some aryl metabolites which are not labelled but produced in the presence of L-phenylalanine which are absent in control under oxic and anoxic conditions in *R. benzoatilyticus* JA2. Aryl metabolites such as benzaldehyde, benzyl alocohol were not labelled but produced only in presence of L-phenylalanine under anoxic and oxic conditions by *R. benzoatilyticus* JA2. This indicate the *de novo* pathways of aryl metabolites is influenced in the presence of L-phenylalanine under oxic and anoxic conditions. Aryl metabolites such as benzaldehyde, 4-hydroxybenzaldehyde were also identified under anoxic as well as oxic conditions in *R. benzoatilyticus* JA2. Among which 4-hydroxybenzaldehyde was labeled in stable isotopic profiling indicating that 4-hydroxybenzaldehyde is derivative of L-phenylalanine (Table. 3). L-Phenylalanine under anoxic conditions channelized into Ehrlich's pathway, L-tyrosine dependent 4-hydroxyphenylpyruvate pathway, N-acetyl-L-phenylalanine and some unidentified pathways (Fig. 31). *Rubrivivax benzoatilyticus* JA2 exhibits catabolic multitasking of L-phenylalanine and the metabolic shift to oxic and anoxic conditions.

***L-Phenylalanine dependent pigments biosynthesis in Rubrivivax benzoatilyticus* JA2**

Bacteria naturally produce pigments under different conditions (Louise et al., 2010). Bacteria also capable of producing different pigments such as melanins (Sajjan et al., 2010; Youngchim et al., 2013; Coelho-Souza et al., 2014), anthroquinones (Li et al., 1995; Li et al., 2013; Malak et al., 2013), actinorhodin (Bystrykh et al., 1996; Taguchi et al., 2012; Manikprabhu and Lingappa, 2013) as a result of biotransformation of aromatic amino acids under oxic and anoxic conditions. HPLC metabolite profiling indicated production of some aryl metabolites having absorption maxima 300 – 550 nm suggesting that they are pigments produced under oxic conditions. The stable isotopic studies revealed that the pigments produced under oxic condition are the L-phenylalanine derivatives. *Rubrivivax benzoatilyticus* JA2 did not show any pigment production under anoxic conditions while oxic conditions only led to pigments production indicating that the this property is an oxygen dependent mechanism. Pigments of different colores such as blue, pink, orange, red, yellow were observed in thin layer

chromatography. Among screened phototrophic bacteria, only *R. benzoatilyticus* JA2 produced pigments indicating that the pigment production is specific to *R. benzoatilyticus* JA2. The pigments production was observed only with viable cells but not with heat-killed cells of L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 indicating that the pigment production is a biological process rather than a chemical process (Fig. 36). Different carbon sources also affect the pigment production, non-sugar carbon sources such as malate, fumarate and pyruvate compared to sugar carbon sources such as glucose, fructose and maltose induced pigment production indicating that the influence of physiological conditions on pigment production (Fig. 38).

Rubrivivax benzoatilyticus JA2 also produced blue, red, yellow orange pink pigments under oxic conditions in presence of L-phenylalanine (Fig. 43). Similarly, a red pigment production was observed in entomopathogenic bacterium *Xenorhabdus luminescens* which was identified as anthraquinone derivative (1,6-dihydroxy-4-methoxy-9,10-anthraquinone) derived from L-phenylalanine (Richardson et al., 1988). NMR analysis of purified blue pigment revealed the chemical shifts characteristic to benzopyran (chromophoric) ring and sugar moiety of anthocyanin glycoside derivatives. Mass spectral analysis also revealed the mass with molecular ion mass 535 *m/z* in positive mode along with mass fragment 302 *m/z* which is a characteristic fragment of one of the anthocyanidin (peonidin) derivative (Fig. 50). The blue colored purified compound has given positive for phenol-sulfuric acid test indicating that the compound has sugar moiety (Fig. 50B).

The absorption spectra of these pigments ranged from 260-560 nm indicating the L-phenylalanine derived anthocyanin pigments as almost all anthocyanins have characteristic UV-Visible spectra at 260-280, 520-560 nm (Yoshida et al., 2003). The blue pigment also showed spectral shift with change in color acidic pH (red) and basic pH (yellow to green) which is one of the characteristic property of anthocyanins. In general anthocyanins at acidic pH turns red to purple and at basic pH turns yellow to green and at neutral pH they will be in blue color (Yoshida et al., 2003). Stable isotopic labeling suggested that that the blue pigment was derived from L-phenylalanine, as molecular ion mass increased by 12 and 24 units due to incorporation of aromatic rings of labelled L-phenylalanine. Based on the polychem analysis and other characteristic features the blue pigment was tentatively identified as anthocyanin-like compound (putative). Some of the characteristic signals which represent anthocyanins are very poor due to the low concentration of the sample and the structure of the pigment could not be confirmed. Hence the pigment was mentioned as putative or tentatively identified as anthocyanin-like

pigment (Fig. 50A). Bacteria produce some colored compounds such as a blue colored polyketide antibiotic, actinorhodin was reported in *Streptomyces coelicolor* A3(2) (Bystrykh et al., 1996; Bruheim et al., 2002). Another group of pigments, anthraquinones and hydroxystilbenes were also reported in an entamopathogenic bacterium *Xenorhabdus luminescens* (Richardson et al., 1988).

In general anthocyanins are produced from L-phenylalanine *via* phenylpropanoid pathway and this pathway is restricted to plants (Zeng et al., 2014). The rate limiting steps of phenylpropanoid pathway are the phenylalanine ammonia-lyase which is the first committed enzyme and the other one is chalcone synthase (CHS) (Ghasemzadeh et al., 2012). Genome sequence of *R. benzoatilyticus* JA2 revealed that there was no specific phenylalanine ammonia lyase gene but it has a histidine ammonia lyase gene. However, there are reports that bacterial histidine ammonia-lyase gene can act as phenylalanine ammonia lyase gene of plant systems (Xiang and Moore, 2005). Another rate limiting enzyme is chalcone synthase which is a ring cyclisation enzyme that synthesises chalcone.

Rubrivivax benzoatilyticus JA2 genome sequence neither have chalcone synthase gene nor the other genes of phenylpropanoid pathway. This gives a clue that *R. benzoatilyticus* JA2 may have an alternative route for anthocyanin-like pigments biosynthesis (Fig. 50C). However, phenylalanine ammonia lyase and chalcone synthase genes are present in some of the phototrophic members such as *Rhodobacter sphaeroides*, *Rhodopsuedomonas palustris* but none of these bacteria produce pigments when grown in presence of L-phenylalanine as sole source of nitrogen under oxic conditions indicating that the genes present in the genomes are may not be functional. The stable isotopic studies also revealed that the purified pink, orange, red, yellow colored pigments were also derived from L-phenylalanine with incorporated labeled L-phenylalanine aromatic ring. These pigments also have UV-Visible absorption ranges from 260-450 nm indicating the pigments may be flavonoid/anthocyanin derivatives.

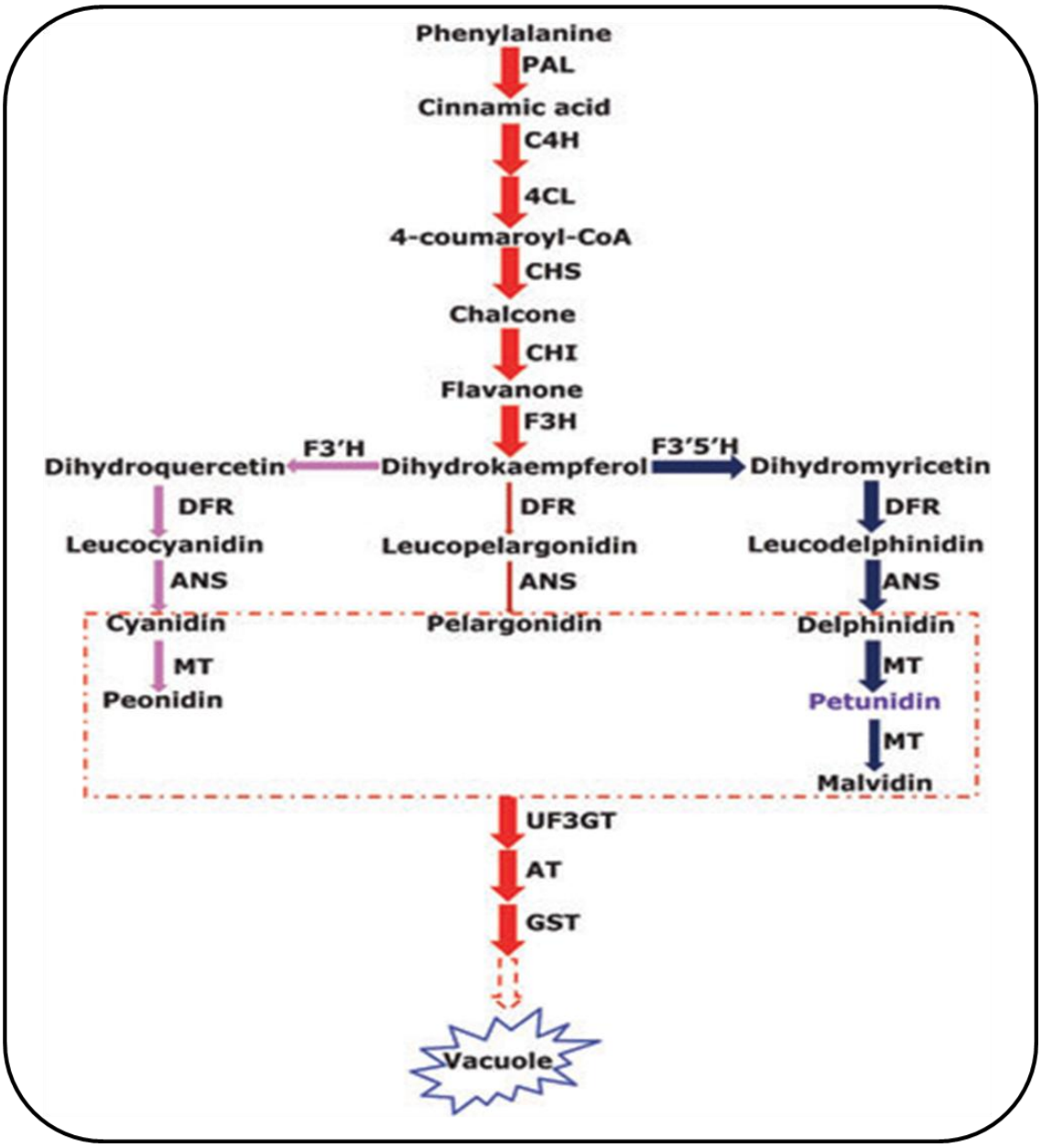


Fig. 65. Phenylpropanoid pathway in plant (*Lycium ruthenicum*) (Zeng et al., 2014).

PAL; phenylalanine ammonia-lyase, C4H; cinnamate 4-hydroxylase, 4CL; 4-coumaroyl: CoA-ligase, CHS; chalcone synthase, CHI; chalcone isomerise, F3H; flavanone 3-hydroxylase, F3'H; flavonoid 3'hydroxylase, F3'5'H; flavonoid 3'5'hydroxylase, DFR; dihydroflavonol 4-reductase, ANS; anthocyanidin synthase, MT; anthocyanin methyltransferase, UF3GT; flavonoid 3-glucosyl transferase, AT; anthocyanin acyltransferase, GST; glutathione *S*-transferase.

***Metabolic responses to L-phenylalanine under oxic and anoxic conditions by Rubrivivax benzoatilyticus* JA2**

GC-MS based metabolomics approach was employed to know the global metabolomic variations of *R. benzoatilyticus* JA2 when grown in the presence and absence of L-phenylalanine under oxic and anoxic conditions. Multivariate statistical analysis such as principal component analysis (PCA) (Wu and Li, 2013), partial least square discriminant analysis (PLS-DA) (Xu et al., 2013) and hierarchical cluster analysis (HCA) (Butler et al., 2012) deciphered the metabolomic variations of control and L-phenylalanine fed cultures of *R. benzoatilyticus* JA2 under oxic and anoxic conditions. The clustering of oxic and anoxic groups under different clusters in HCA represents that metabolism under oxic and anoxic conditions was different (Fig. 54).

Principal component analysis (PCA), partial least square discriminant analysis (PLSDA) explained the metabolic variations based on the groups separation (Fig. 55). The distinctly separated groups revealed the metabolic variations in the exometabolome of L-phenylalanine treated and control cultures of *R. benzoatilyticus* JA2 under oxic and anoxic conditions. Further, insights of metabolic variations were explained by identification of key metabolites from the variable importance projection (VIP) score plot (Xia and Wishart, 2011). The metabolic features with VIP scores >1 obtained from the PLS-DA plot are significantly responsible for the variations in metabolism. VIP score plot also explains the differential concentrations of the metabolic features of four different groups which represent control and L-phenylalanine fed *R. benzoatilyticus* JA2 under oxic and anoxic conditions (Fig. 56).

Relative fold change analysis revealed the differential regulation of metabolites in L-phenylalanine fed cultures compared to that of control under oxic and anoxic conditions (Fig. 57, 58). Functional annotation of these differentially regulated metabolites indicated the metabolic pathways influenced by L-phenylalanine (Fig. 59, 60). Aryl metabolism, fatty acid/lipid, and carbohydrate metabolisms were highly influenced. Relative fold change analysis of aryl metabolites explained the up-regulation of hydroxyderivatives of L-phenylalanine under oxic conditions (Fig. 61). Production of hydroxyphenyl derivatives only in oxic conditions indicates the oxygen dependent L-phenylalanine metabolism. Biosynthesis of aryl metabolites other than phenyl derivatives only in L-phenylalanine fed conditions indicated the influence of L-phenylalanine on *de novo* synthesis of aryl metabolites (Fig. 63).

The catabolic shift in oxic conditions and existence of multiple catabolic pathways under oxic and anoxic conditions is also validated by metabolome data indicating the multitasking nature of *R. benzoatilyticus* JA2 under oxic and anoxic conditions. Based on stable isotopic profiling, enzyme activities and metabolomic studies an overview of L-phenylalanine catabolism was proposed in *R. benzoatilyticus* JA2 (Fig. 66).

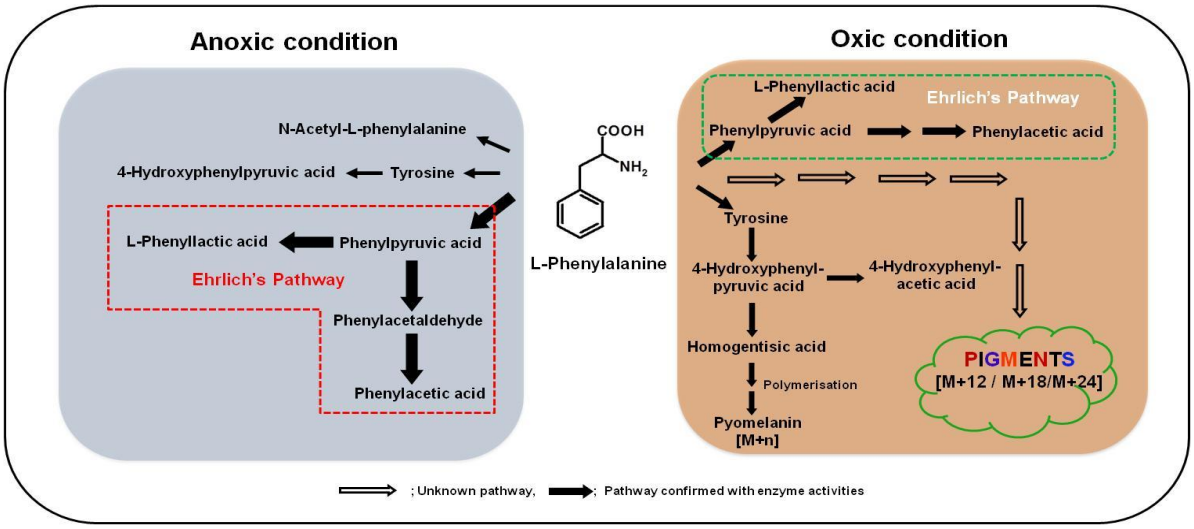


Fig. 66. Schematic representation of L-phenylalanine catabolism under oxic and anoxic conditions in *Rubrivivax benzoatilyticus* JA2.

M+12/M+18/M+24 represent the increased masses by 12/18/24 units.

CONCLUSIONS

5.0 Conclusions

Aromatic compounds are the most abundant group of compounds that exist in the environment. Bacteria utilise these aromatic compounds for growth under oxic and anoxic conditions. Aromatic amino acids are one such group of naturally occurring aromatic compounds found abundantly in nature. Bacteria utilise aromatic amino acids for its growth as sole source of carbon or nitrogen. Among bacteria, anoxygenic phototrophic bacteria are metabolically diverse group of bacteria that occupy diverse habitats and thrive on wide array of organic compounds. *Rubrivivax benzoatilyticus* JA2, one such anoxygenic phototrophic bacteria thrive under oxic and anoxic conditions by utilising different organic compounds. *Rubrivivax benzoatilyticus* JA2 utilise aromatic amino acids as sole source of nitrogen but not as carbon source. *Rubrivivax benzoatilyticus* JA2 has remarkable ability to transform aromatic amino acids into phenolic and indolic compounds. In the present work *R. benzoatilyticus* JA2 was used as model system to study the L-phenylalanine catabolism under oxic and anoxic conditions by using stable isotope based metabolomics approach.

Rubrivivax benzoatilyticus JA2 could not utilise L-phenylalanine as sole source of carbon neither under oxic nor in the anoxic conditions. However, *R. benzoatilyticus* JA2 utilise L-phenylalanine as sole source of nitrogen both under oxic and anoxic conditions and produce phenols (aryl metabolites) indicating the metabolic potential of *R. benzoatilyticus* JA2 to utilise L-phenylalanine under different growth modes. Comparative HPLC and LC-MS based metabolic profiling revealed the production of different aryl metabolites under oxic and anoxic conditions and this suggests catabolic diversity of L-phenylalanine by *R. benzoatilyticus* JA2 under oxic and anoxic conditions. Stable isotope studies and enzyme activities revealed the existence of multiple catabolic pathways such as Ehrlich's, N-acetyl L-phenylalanine pathway, 4-hydroxyphenylpyruvic acid pathway and some unknown pathways under anoxic conditions. However, under oxic conditions apart from Ehrlich's pathway, L-phenylalanine catabolised into pyomelanin and anthocyanin-like pigments. Existence of multiple catabolic pathways of L-phenylalanine under oxic and anoxic conditions indicated the catabolic multitasking of *R. benzoatilyticus* JA2. L-Phenylalanine is mainly fluxed towards Ehrlich's pathway under anoxic conditions whereas under oxic conditions towards pigments production indicating the differential flux of L-phenylalanine. Multiple pathways with differential flux of L-phenylalanine under oxic and anoxic conditions suggest plausible oxygen dependent regulation of L-phenylalanine in *R. benzoatilyticus* JA2.

L-Phenylalanine catabolised via 4-hydrxyphenylpyruvic acid followed by homogentisic acid only under oxic conditions by *R. benzoatilyticus* JA2 and this is the first report among anoxygenic phototrophic bacteria. However, 4-hydroxyphenylpyruvic acid under anoxic conditions remained as a dead-end molecule, when shifted to oxic conditions 4-hydroxyphenylpyruvic acid is further catabolised into homogentisic acid and finally to pyomelanin. This suggests that presence of oxygen evoked the pyomelanin biosynthesis by activating oxygen dependent enzymes and metabolic shift of L-phenylalanine under oxic conditions. In addition to pyomelanin, *R. benzoatilyticus* JA2 also produced different colored compounds under oxic conditions in presence of L-phenylalanine. Among those pigments, blue pigment tentatively identified as anthocyanin-like pigment derived from L-phenylalanine. However, genome sequence revealed the absence of homologous genes of anthocyanin biosynthetic pathway suggesting the possible alternative pathway of anthocyanin-like pigment biosynthesis which needs further investigation.

Metabolomics study based on the multivariate statistical analysis such as hierarchical cluster analysis (HCA), principal component analysis (PCA), and partial least squares discriminant analysis (PLS-DA) analysis revealed the metabolic variations of *R. benzoatilyticus* JA2 under oxic and anoxic conditions. The relative fold change analysis and functional classification explained the differential regulation of key metabolites and their metabolic pathways influenced under anoxic and oxic conditions. Metabolic variations under oxic and anoxic conditions suggests metabolic shift in *R. benzoatilyticus* JA2 to survive under changing conditions.

Finally metabolic shift and catabolic multitasking of L-phenylalanine in *R. benzoatilyticus* JA2 suggest a possible adaptive strategy to survive and utilize L-phenylalanine under oxic/anoxic transition zones. Diverse metabolic adaptations of *R. benzoatilyticus* JA2 to utilise L-phenylalanine under anoxic and oxic conditions was clearly demonstrated and similar kind of adaptations may exist in other anoxygenic phototrophic bacteria for metabolizing organic compounds which may help in carbon recycling in the oxic/ anoxic environments.

REFERENCES

6.0 References

Abe-Yoshizumi, Kamei U, Yamada A, Kimura M, and S, I. (2004) The evolution of the phenylacetic acid degradation pathway in bacteria. *Biosci Biotechnol Biochem.* **68**: 746-748.

Afzal , and, S.A.-A., and Oommen, S. (2013) L-Phenylalanine and L-tyrosine catabolism by thermophilic *Geobacillus stearothermophilus*. *British Biotechnology Journal* **3**: 581-591.

Almeida-Paes, R., Frases, S., Araujo Gde, S., de Oliveira, M.M., Gerfen, G.J., Nosanchuk, J.D., Zancope-Oliveira, R.M. (2012) Biosynthesis and functions of a melanoid pigment produced by species of the S*porothrix* complex in the presence of L-tyrosine. *Appl Environ Microbiol* **78**: 8623-8630.

Apte, M., Girme, G., Bankar, A., Ravikumar, A., and Zinjarde, S. (2013) 3, 4-Dihydroxy-L-phenylalanine-derived melanin from *Yarrowia lipolytica* mediates the synthesis of silver and gold nanostructures. *J Nanobiotechnology* **11**: 2.

Arias-Barrau, E., Olivera, E.R., Luengo, J.M., Fernandez, C., Galan, B., Garcia, J.L. et al. (2004) The homogentisate pathway: a central catabolic pathway involved in the degradation of L-phenylalanine, L-tyrosine, and 3-hydroxyphenylacetate in *Pseudomonas putida*. *J Bacteriol* **186**: 5062-5077.

Azizan, K.A., Baharum, S.N., and Mohd Noor, N. (2012) Metabolic profiling of *Lactococcus lactis* under different culture conditions. *Molecules* **17**: 8022-8036.

Badri, D.V., Chaparro, J.M., Zhang, R., Shen, Q., and Vivanco, J.M. (2013) Application of natural blends of phytochemicals derived from the root exudates of *Arabidopsis* to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *J Biol Chem* **288**: 4502-4512.

Banerjee, A., Supakar, S., and Banerjee, R. (2014) Melanin from the nitrogen-fixing bacterium *Azotobacter chroococcum*: a spectroscopic characterization. *PLoS One* **9**: e84574.

Behrends, V., Ebbels, T.M., Williams, H.D., and Bundy, J.G. (2009) Time-resolved metabolic footprinting for nonlinear modeling of bacterial substrate utilization. *Appl Environ Microbiol* **75**: 2453-2463.

Berger, M., Brock, N.L., Liesegang, H., Dogs, M., Preuth, I., Simon, M. et al. (2012) Genetic analysis of the upper phenylacetate catabolic pathway in the production of tropodithietic acid by *Phaeobacter gallaeciensis*. *Appl Environ Microbiol* **78**: 3539-3551.

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

Camarero, S., Canas, A.I., Nousiainen, P., Record, E., Lomascolo, A., Martinez, M.J., and Martinez, A.T. (2008) P-hydroxycinnamic acids as natural mediators for laccase oxidation of recalcitrant compounds. *Environ Sci Technol* **42**: 6703-6709.

Carmona, M., Zamarro, M.T., Blazquez, B., Durante-Rodriguez, G., Juarez, J.F., Valderrama, J.A. et al. (2009) Anaerobic catabolism of aromatic compounds: a genetic and genomic view. *Microbiol Mol Biol Rev* **73**: 71-133.

Carreira, A., Ferreira, L.M., and Loureiro, V. (2001) Brown pigments produced by *Yarrowia lipolytica* result from extracellular accumulation of homogentisic acid. *Appl Environ Microbiol* **67**: 3463-3468.

Chai, B., Wang, H., and Chen, X. (2012) Draft genome sequence of high-melanin-yielding *Aeromonas media* strain WS. *J Bacteriol* **194**: 6693-6694.

Chen, X., Alonso, A.P., Allen, D.K., Reed, J.L., and Shachar-Hill, Y. (2011) Synergy between (13)C-metabolic flux analysis and flux balance analysis for understanding metabolic adaptation to anaerobiosis in E. coli. *Metab Eng* **13**: 38-48.

Coelho-Souza, T., Martins, N., Maia, F., Frases, S., Bonelli, R.R., Riley, L.W., and Moreira, B.M. (2014) Pyomelanin production: a rare phenotype in *Acinetobacter baumannii*. *J Med Microbiol* **63**: 152-154.

Cortes-Zavaleta, O., Lopez-Malo, A., Hernandez-Mendoza, A., and Garcia, H.S. (2014) Antifungal activity of *Lactobacilli* and its relationship with 3-phenyllactic acid production. *Int J Food Microbiol* **173**: 30-35.

Dadachova, E., Bryan, R.A., Howell, R.C., Schweitzer, A.D., Aisen, P., Nosanchuk, J.D., and Casadevall, A. (2008) The radioprotective properties of fungal melanin are a function of its chemical composition, stable radical presence and spatial arrangement. *Pigment Cell Melanoma Res* **21**: 192-199.

De Boer, W. Harder, and Dijkhuizen, L. (1988) Phenylalanine and tyrosine metabolism in the facultative methylotroph *Nocardia* sp. 239. *Arch Microbiol* **149**: 459-465.

Diaz, E., Ferrandez, A., Prieto, M.A., and Garcia, J.L. (2001) Biodegradation of aromatic compounds by *Escherichia coli*. *Microbiol Mol Biol Rev* **65**: 523-569, table of contents.

Fowler, Z.L., and Koffas, M.A. (2009) Biosynthesis and biotechnological production of flavanones: current state and perspectives. *Appl Microbiol Biotechnol* **83**: 799-808.

Frick, O., and Wittmann, C. (2005) Characterization of the metabolic shift between oxidative and fermentative growth in *Saccharomyces cerevisiae* by comparative 13C flux analysis. *Microb Cell Fact* **4**: 30.

Frimmersdorf, E., Horatzek, S., Pelnikevich, A., Wiehlmann, L., and Schomburg, D. (2010) How *Pseudomonas aeruginosa* adapts to various environments: a metabolomic approach. *Environ Microbiol* **12**: 1734-1747.

Fuchs, G. (2008) Anaerobic metabolism of aromatic compounds. *Ann N Y Acad Sci* **1125**: 82-99.

Gessler, N.N., Egorovaa, A.S., and Belozerskaya, T.A. (2014) Melanin pigments of fungi under extreme environmental conditions (Review). *Applied Biochemistry and Microbiology* **50**: 105-113.

Ghasemzadeh, A., Jaafar, H.Z., and Karimi, E. (2012) Involvement of salicylic acid on antioxidant and anticancer properties, anthocyanin production and chalcone synthase activity in ginger (*Zingiber officinale* Roscoe) Varieties. *Int J Mol Sci* **13**: 14828-14844.

Glass, K., Ito, S., Wilby, P.R., Sota, T., Nakamura, A., Bowers, C.R. et al. (2012) Direct chemical evidence for eumelanin pigment from the Jurassic period. *Proc Natl Acad Sci U S A* **109**: 10218-10223.

Gummalla, S., and Broadbent, J.R. (2001) Tyrosine and phenylalanine catabolism by *Lactobacillus* cheese flavor adjuncts. *J Dairy Sci* **84**: 1011-1019.

Hazelwood, L.A., Daran, J.M., van Maris, A.J., Pronk, J.T., and Dickinson, J.R. (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl Environ Microbiol* **74**: 2259-2266.

Hunter, R.C., and Newman, D.K. (2010) A putative ABC transporter, hatABCDE, is among molecular determinants of pyomelanin production in *Pseudomonas aeruginosa*. *J Bacteriol* **192**: 5962-5971.

Ismail, W., and Gescher, J. (2012) Epoxy Coenzyme A Thioester pathways for degradation of aromatic compounds. *Appl Environ Microbiol* **78**: 5043-5051.

Jiao, Y., Cody, G.D., Harding, A.K., Wilmes, P., Schrenk, M., Wheeler, K.E. et al. (2010) Characterization of extracellular polymeric substances from acidophilic microbial biofilms. *Appl Environ Microbiol* **76**: 2916-2922.

Jimenez, J.I., Minambres, B., Garcia, J.L., and Diaz, E. (2002) Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ Microbiol* **4**: 824-841.

Joyce, S.A., Brachmann, A.O., Glazer, I., Lango, L., Schwar, G., Clarke, D.J., and Bode, H.B. (2008) Bacterial biosynthesis of a multipotent stilbene. *Angew Chem Int Ed Engl* **47**: 1942-1945.

Khajo, A., Bryan, R.A., Friedman, M., Burger, R.M., Levitsky, Y., Casadevall, A. et al. (2011) Protection of melanized *Cryptococcus neoformans* from lethal dose gamma irradiation involves changes in melanin's chemical structure and paramagnetism. *PLoS One* **6**: e25092.

Khan, F., Kumari, M., and Cameotra, S.S. (2013) Biodegradation of the allelopathic chemical m-tyrosine by *Bacillus aquimaris* SSC5 involves the homogentisate central pathway. *PLoS One* **8**: e75928.

Kol, S., Merlo, M.E., Scheltema, R.A., de Vries, M., Vonk, R.J., Kikkert, N.A. et al. (2010) Metabolomic characterization of the salt stress response in *Streptomyces coelicolor*. *Appl Environ Microbiol* **76**: 2574-2581.

Kumavath, R.N., Ramana Ch, V., and Sasikala, C. (2010a) Production of phenols and alkyl gallate esters by *Rhodobacter sphaeroides* OU5. *Curr Microbiol* **60**: 107-111.

Kumavath, R.N., Ramana Ch, V., and Sasikala, C. (2010b) L-Tryptophan catabolism by *Rubrivivax benzoatilyticus* JA2 occurs through indole 3-pyruvic acid pathway. *Biodegradation* **21**: 825-832.

Kumavath, R.N., Ramana, C.V., and Sasikala, C. (2011) Rubrivivaxin, a new cytotoxic and cyclooxygenase-I inhibitory metabolite from *Rubrivivax benzoatilyticus* JA2. *World journal of biotechnology* **27**: 11-16.

Kyndt, J.A., Hurley, J.K., Devreese, B., Meyer, T.E., Cusanovich, M.A., Tollin, G., and Van Beeumen, J.J. (2004) *Rhodobacter capsulatus* photoactive yellow protein: genetic context, spectral and kinetics characterization, and mutagenesis. *Biochemistry* **43**: 1809-1820.

Laempe, D., Jahn, M., Breese, K., Schagger, H., and Fuchs, G. (2001) Anaerobic metabolism of 3-hydroxybenzoate by the denitrifying bacterium *Thauera aromatica*. *J Bacteriol* **183**: 968-979.

Larimer, F.W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L. et al. (2004) Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. *Nat Biotechnol* **22**: 55-61.

Leiros, H.K., Flydal, M.I., and Martinez, A. (2013) Structural and thermodynamic insight into phenylalanine hydroxylase from the human pathogen *Legionella pneumophila*. *FEBS Open Bio* **3**: 370-378.

Li, H., Guo, J., Lian, J., Zhao, L., Xi, Z., Du, H., and Yang, J. (2013) Effective and characteristics of anthraquinone-2,6-disulfonate (AQDS) on denitrification by *Paracoccus versutus* sp.GW1. *Environ Technol* **34**: 2563-2570.

Li, J., Chen, G., Wu, H., and Webster, J.M. (1995) Identification of two pigments and a hydroxystilbene antibiotic from *Photorhabdus luminescens*. *Appl Environ Microbiol* **61**: 4329-4333.

Li, X., Jiang, B., and Pan, B. (2007) Biotransformation of phenylpyruvic acid to phenyllactic acid by growing and resting cells of a *Lactobacillus* sp. *Biotechnol Lett* **29**: 593-597.

Malak, L.G., Bishay, D.W., Abdel-Baky, A.M., Moharram, A.M., Cutler, S.J., and Ross, S.A. (2013) New anthraquinone derivatives from *Geosmithia lavendula*. *Nat Prod Commun* **8**: 191-194.

Manivasagan, P., Venkatesan, J., Sivakumar, K., and Kim, S.K. (2013) Actinobacterial melanins: current status and perspective for the future. *World J Microbiol Biotechnol* **29**: 1737-1750.

Marcobal, A., Kashyap, P.C., Nelson, T.A., Aronov, P.A., Donia, M.S., Spormann, A. et al. (2013) A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. *ISME J* **7**: 1933-1943.

Maskeya, R.P., Ratnakar N. Asolkara, Michael Speitlinga, Volker Hoffmanna, Iris Gr¨un-Wollnyb, Werner F. Fleckc, a., and Laatsch, H. (2003) Flavones and new isoflavone derivatives from Microorganisms: Isolation and structure elucidation. *Z. Naturforsch* **58b**: 686-691.

Mechichi, T., Stackebrandt, E., Gad'on, N., and Fuchs, G. (2002) Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of *Thauera phenylacetica* sp. nov., *Thauera aminoaromatica* sp. nov., and *Azoarcus buckelii* sp. nov. *Arch Microbiol* **178**: 26-35.

Mercado-Blanco, J., Garcia, F., Fernandez-Lopez, M., and Olivares, J. (1993) Melanin production by *Rhizobium meliloti* GR4 is linked to nonsymbiotic plasmid pRmeGR4b: cloning, sequencing, and expression of the tyrosinase gene mepA. *J Bacteriol* **175**: 5403-5410.

Mohamed, M.S., Ismail, W., Heider, J., and Fuchs, G. (2002) Aerobic metabolism of phenylacetic acids in *Azoarcus evansii*. *Arch Microbiol* **178**: 180-192.

Mohammed, M., Isukapatla, A., Mekala, L.P., Eedara Veera Venkata, R.P., Chintalapati, S., and Chintalapati, V.R. (2011) Genome sequence of the phototrophic betaproteobacterium *Rubrivivax benzoatilyticus* strain JA2T. *J Bacteriol* **193**: 2898-2899.

Mu, W., Chen, C., Li, X., Zhang, T., and Jiang, B. (2009) Optimization of culture medium for the production of phenyllactic acid by *Lactobacillus* sp. SK007. *Bioresour Technol* **100**: 1366-1370.

Mujahid, M., Sasikala, C., and Ramana Ch, V. (2011) Production of indole-3-acetic acid and related indole derivatives from L-tryptophan by *Rubrivivax benzoatilyticus* JA2. *Appl Microbiol Biotechnol* **89**: 1001-1008.

Mujahid, M., Sasikala, C., and Ramana Ch, V. (2014) Aniline is an inducer, and not a precursor, for indole derivatives in *Rubrivivax benzoatilyticus* JA2. *PLoS One* **9**: e87503.

Napolitano, A., De Lucia, M., Panzella, L., and d'Ischia, M. (2008) The "benzothiazine" chromophore of pheomelanins: a reassessment. *Photochem Photobiol* **84**: 593-599.

Navarro-Llorens, J.M., Patrauchan, M.A., Stewart, G.R., Davies, J.E., Eltis, L.D., and Mohn, W.W. (2005) Phenylacetate catabolism in *Rhodococcus* sp. strain RHA1: a central pathway for degradation of aromatic compounds. *J Bacteriol* **187**: 4497-4504.

Nierop Groot, M.N., and de Bont, J.A. (1999) Involvement of manganese in conversion of phenylalanine to benzaldehyde by lactic acid bacteria. *Appl Environ Microbiol* **65**: 5590-5593.

Oda, Y., Larimer, F.W., Chain, P.S., Malfatti, S., Shin, M.V., Vergez, L.M. et al. (2008) Multiple genome sequences reveal adaptations of a phototrophic bacterium to sediment microenvironments. *Proc Natl Acad Sci U S A* **105**: 18543-18548.

Phillips, D.A., Fox, T.C., King, M.D., Bhuvaneswari, T.V., and Teuber, L.R. (2004) Microbial products trigger amino acid exudation from plant roots. *Plant Physiol* **136**: 2887-2894.

Piletic, I.R., Matthews, T.E., and Warren, W.S. (2010) Probing near-infrared photorelaxation pathways in eumelanins and pheomelanins. *J Phys Chem A* **114**: 11483-11491.

Pometto, A.L., 3rd, and Crawford, D.L. (1985) L-Phenylalanine and L-tyrosine catabolism by selected *Streptomyces* species. *Appl Environ Microbiol* **49**: 727-729.

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

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

Ravasio, D., Walther, A., Trost, K., Vrhovsek, U., and Wendland, J. (2014) An indirect assay for volatile compound production in yeast strains. *Sci Rep* **4**: 3707.

Richardson, W.H., Schmidt, T.M., and Nealson, K.H. (1988) Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. *Appl Environ Microbiol* **54**: 1602-1605.

Ryu, R.J., and Patten, C.L. (2008) Aromatic amino acid-dependent expression of indole-3-pyruvate decarboxylase is regulated by TyrR in *Enterobacter cloacae* UW5. *J Bacteriol* **190**: 7200-7208.

Saez, L.P., Castillo, F., and Caballero, F.J. (1999) Metabolism of L-phenylalanine and L-tyrosine by the phototrophic bacterium *Rhodobacter capsulatus*. *Curr Microbiol* **38**: 51-56.

Schellenberger, S., Kolb, S., and Drake, H.L. (2012) Metabolic responses of novel cellulolytic and saccharolytic agricultural soil bacteria to oxygen. *Environ Microbiol* **12**: 845-861.

Schneider, S., Mohamed, M.E., and Fuchs, G. (1997) Anaerobic metabolism of L-phenylalanine via benzoyl-CoA in the denitrifying bacterium *Thauera aromatica*. *Arch Microbiol* **168**: 310-320.

Singh, S., Malhotra, A.G., Pandey, A., and Pandey, K.M. (2013) Computational model for pathway reconstruction to unravel the evolutionary significance of melanin synthesis. *Bioinformation* **9**: 94-100.

Smith, E.A., and Macfarlane, G.T. (1996) Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. *J Appl Bacteriol* **81**: 288-302.

Somers, E., Ptacek, D., Gysegom, P., Srinivasan, M., and Vanderleyden, J. (2005) *Azospirillum brasilense* produces the auxin-like phenylacetic acid by using the key enzyme for indole-3-acetic acid biosynthesis. *Appl Environ Microbiol* **71**: 1803-1810.

Svanstrom, A., Boveri, S., Bostrom, E., and Melin, P. (2013) The lactic acid bacteria metabolite phenyllactic acid inhibits both radial growth and sporulation of filamentous fungi. *BMC Res Notes* **6**: 464.

Taguchi, T., Ebihara, T., Furukawa, A., Hidaka, Y., Ariga, R., Okamoto, S., and Ichinose, K. (2012) Identification of the actinorhodin monomer and its related compound from a deletion mutant of the actVA-ORF4 gene of *Streptomyces coelicolor* A3(2). *Bioorg Med Chem Lett* **22**: 5041-5045.

Tarangini, K., and Mishra, S. (2013) Production, characterization and analysis of melanin from isolated marine *Pseudomonas* sp. using vegetable waste. *Res. J. Engineering Sci.* **2**: 40-46.

Tengfang, H., Ludmila, R., and Georg, J. (2011) meta-Tyrosine in *Festuca rubra* ssp. *commutata* (Chewings fescue) is synthesized by hydroxylation of phenylalanine. *Phytochemistry* **75**: 60-66.

Teufel, R., Mascaraque, V., Ismail, W., Voss, M., Perera, J., Eisenreich, W. et al. (2010) Bacterial phenylalanine and phenylacetate catabolic pathway revealed. *Proc Natl Acad Sci U S A* **107**: 14390-14395.

Thierry, A., and Maillard, M.-B. (2002) Production of cheese flavour compounds derived from amino acid catabolism by *Propionibacterium freudenreichii*. *Lait* **82**: 17-32.

Thureau, P., Ziarelli, F., Thevand, A., Martin, R.W., Farmer, P.J., Viel, S., and Mollica, G. (2012) Probing the motional behavior of eumelanin and pheomelanin with solid-state NMR spectroscopy: new insights into the pigment properties. *Chemistry* **18**: 10689-10700.

Turick, C.E., Caccavo, F., Jr., and Tisa, L.S. (2008) Pyomelanin is produced by *Shewanella algae* BrY and affected by exogenous iron. *Can J Microbiol* **54**: 334-339.

Wang, R., Wang, H., Zhou, H., Wang, Y., Yue, J., Diao, B., and Kan, B. (2011) Characters of homogentisate oxygenase gene mutation and high clonality of the natural pigment-producing *Vibrio cholerae* strains. *BMC Microbiol* **11**: 109.

Wei, J., Timler, J.G., Knutson, C.M., and Barney, B.M. (2013) Branched-chain 2-keto acid decarboxylases derived from *Psychrobacter*. *FEMS Microbiol Lett* **346**: 105-112.

Wohlbrand, L., Jacob, J.H., Kube, M., Mussmann, M., Jarling, R., Beck, A. et al. (2013) Complete genome, catabolic sub-proteomes and key-metabolites of *Desulfobacula toluolica* Tol2, a marine, aromatic compound-degrading, sulfate-reducing bacterium. *Environ Microbiol* **15**: 1334-1355.

Xiang, L., and Moore, B.S. (2002) Inactivation, complementation, and heterologous expression of encP, a novel bacterial phenylalanine ammonia-lyase gene. *J Biol Chem* **277**: 32505-33209.

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

Ye, Y., Zhang, L., Hao, F., Zhang, J., Wang, Y., and Tang, H. (2012) Global metabolomic responses of *Escherichia coli* to heat stress. *J Proteome Res* **11**: 2559-2566.

Yuan, W., Burleigh, S.H., and Dawson, J.O. (2007) Melanin biosynthesis by *Frankia* strain CeI5. *Physiol Plant* **131**: 180-190.

Zheng, H., Chatfield, C.H., Liles, M.R., and Cianciotto, N.P. (2013) Secreted pyomelanin of *Legionella pneumophila* promotes bacterial iron uptake and growth under iron-limiting conditions. *Infect Immun* **81**: 4182-4191.

PUBLICATIONS

JOURNAL OF BACTERIOLOGY, June 2011, p. 2898–2899 Vol. 193, No. 11
0021-9193/11/$12.00 doi:10.1128/JB.00379-11

Genome Sequence of the Phototrophic Betaproteobacterium *Rubrivivax benzoatilyticus* Strain JA2$^{T\nabla}$

Mujahid Mohammed,[1] Arvind Isukapatla,[1] Lakshmi Prasuna Mekala,[1] Rama Prasad Eedara Veera Venkata,[1] Sasikala Chintalapati,[2] and Venkata Ramana Chintalapati[1]*

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500 046, India,[1] and Bacterial Discovery Laboratory, Center for Environment, IST, JNT University Hyderabad, Kukatpally, Hyderabad 500 085, India[2]

Received 21 March 2011/Accepted 25 March 2011

Herein we report the draft genome sequence of a phototrophic bacterium, *Rubrivivax benzoatilyticus* strain JA2^T, which apparently is the first genome sequence report of a phototrophic member belonging to the class *Betaproteobacteria*. The unique feature of this strain is its capability to synthesize carotenoids through both spirilloxanthin and spheroidenone pathways. Strain JA2^T produces several novel secondary metabolites, and the genome insights help in understanding the unique machinery that the strain adapted.

Rubrivivax benzoatilyticus JA2^T was isolated from a flooded paddy field in South India and was found to be closely related to *Rubrivivax gelatinosus* ATCC 17011^T based on 16S rRNA gene sequence similarity (6). Extended growth modes (photo-/chemoheterotrophy and photoautotrophy) coupled with utilization of a variety of organic compounds make the strain highly metabolically flexible. *Rubrivivax benzoatilyticus* JA2^T can fix N_2 and also produces H_2. *Rubrivivax benzoatilyticus* JA2^T produces polyhydroxyalkanoates and secondary metabolites such as phenolic compounds (8) and indole derivatives (4, 5, 7). Here, we report the draft genome sequence of this bacterium, which may provide insights into the molecular aspects of its metabolic versatility.

Rubrivivax benzoatilyticus JA2^T (= ATCC BBA-35^T = JCM13220^T = MTTC7087^T) was grown photoheterotrophically on mineral medium (1) with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen sources, respectively. DNA was isolated with the QIAamp minikit (Qiagen), and the authenticity of the genome was confirmed by 16S rRNA gene sequencing. The genome of *Rubrivivax benzoatilyticus* JA2^T was sequenced by using the Roche 454 GS (FLX titanium) pyrosequencing platform. Sequencing resulted in a total of 262,346 high-quality reads with approximately 24× coverage of the entire genome. All of the reads were assembled by using a Newbler assembler (Roche Life Sciences), which generated 165 contigs. Functional annotation was carried out by using the BLAST tool (NCBI), identification of tRNA genes was carried out by using tRNAscan-SE (3), and identification of rRNA genes was carried out by using RNammer (2). GC content was estimated from the genome sequence.

The *Rubrivivax benzoatilyticus* JA2^T draft genome consists of a single circular chromosome of 4,130,132 bp with an average GC content of 72.77%. A total of 3,762 open reading frames (ORFs) were found in the genome where 3,210 (85.32%) are functionally annotated, 382 (10.15%) are hypothetical, and 170 (4.51%) are unknown. The numbers of genes transcribed were 1,926 from the positive strand and 1,836 from the negative strand. The coding density of the genome is 87.5%, with an average gene length of 961 bp, and the average intergenic length is 136 bp. The *Rubrivivax benzoatilyticus* JA2^T genome contains a single 16S-23S-5S operon, 46 tRNA genes, and 25 aminoacyl-tRNA synthetase genes for all 20 amino acids. The genome consisted of 56 predicted ABC transporters, 17 putative transposases, and 29 methyl-accepting chemosensory transducer genes. The *Rubrivivax benzoatilyticus* JA2^T genome has a complete set of genes for bacterial chlorophyll, carotenoid, quinone, and vitamin biosynthesis. Light-harvesting complex and reaction center-encoding genes along with Calvin-Benson cycle-dependent autotrophic CO_2 fixation genes were also located in the genome. Nitrogenase regulatory and nitrogen molybdenum-iron protein- and nitrogenase iron protein-encoding genes were predicted. Many multidrug-resistant transporter, organic solvent resistance, and aromatic compound metabolism genes were also revealed in the genome. A detailed analysis of the *R. benzoatilyticus* JA2^T genome and comparative genome analysis with other photosynthetic members will provide insights into the unique biochemical and molecular characteristics of this strain.

Nucleotide sequence accession number. The complete genome sequence was deposited in GenBank under accession number AEWG00000000.

The Department of Biotechnology, the Department of Science and Technology, the Ministry of Earth Sciences, the Council for Scientific and Industrial Research, and the Government of India are acknowledged for financial support. M.M. thanks the CSIR (India) for the Senior Research Fellowship.

We acknowledge the help of Xcelris Labs for genome sequence deposition. We thank Niyaz Ahmed, Department of Biotechnology, University of Hyderabad, for his critical suggestions.

* Corresponding author. Mailing address: Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500 046, India. Phone: 91 040 23134502. Fax: 91 040 23010120. E-mail: r449@sify.com.

▽ Published ahead of print on 8 April 2011.

REFERENCES

1. **Kalyan Chakravarthy, S., T. N. R. Srinivas, P. Anil Kumar, C. Sasikala, and C. V. Ramana.** 2007. *Roseospira visakhapatnamensis* sp. nov. and *Roseospira goensis* sp. nov. Int. J. Syst. Evol. Microbiol. **57:**2453–2457.
2. **Lagesen, K., et al.** 2007. RNammer: consistent annotation of rRNA genes in genomic sequences. Nucleic Acids Res. **35:**3100–3108.
3. **Lowe, T. M., and S. R. Eddy.** 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. **25:**955–964.
4. **Mujahid, M., C. Sasikala, and C. V. Ramana.** 2010. Aniline-induced tryptophan production and identification of indole derivatives from three purple bacteria. Curr. Microbiol. **61:**285–290.
5. **Mujahid, M., C. Sasikala, and C. V. Ramana.** 2011. Production of indole-3-acetic acid and related indole derivatives from L-tryptophan by *Rubrivivax benzoatilyticus* JA2. Appl. Microbiol. Biotechnol. **89:**1001–1008.
6. **Ramana, C. V., et al.** 2006. *Rubrivivax benzoatilyticus* sp. nov., an aromatic, hydrocarbon-degrading purple betaproteobacterium. Int. J. Syst. Evol. Microbiol. **56:**2157–2164.
7. **Ranjith, N. K., C. V. Ramana, and C. Sasikala.** 2010. L-tryptophan catabolism by *Rubrivivax benzoatilyticus* JA2 occurs through indole 3-pyruvic acid pathway. Biodegradation **21:**825–832.
8. **Ranjith, N. K., C. V. Ramana, and C. Sasikala.** 2010. Rubrivivaxin, a new cytotoxic and cyclooxygenase-I inhibitory metabolite from *Rubrivivax benzoatilyticus* JA2. World J. Microbiol. Biotechnol. doi:10.1007/s11274-010-0420-9.

Microbiological Research 167 (2012) 526–531

Contents lists available at SciVerse ScienceDirect

Microbiological Research

journal homepage: www.elsevier.de/micres

Research paper

L-Phenylalanine catabolism and L-phenyllactic acid production by a phototrophic bacterium, *Rubrivivax benzoatilyticus* JA2

M. Lakshmi Prasuna[a], Md. Mujahid[a], Ch. Sasikala[b], Ch.V. Ramana[a,*]

[a] Department of Plant Sciences, University of Hyderabad, Hyderabad 500046, India
[b] Bacterial Discovery Laboratory, Centre for Environment, IST, JNTUH, Hyderabad 500085, India

ARTICLE INFO

Article history:
Received 28 November 2011
Received in revised form 6 March 2012
Accepted 11 March 2012

Keywords:
L-Phenylalanine
L-Phenyllactate
Phenylpyruvic acid
Rubrivivax benzoatilyticus JA2

ABSTRACT

A phototrophic bacterium (*Rubrivivax benzoatilyticus* JA2) grows at the expense of L-phenylalanine as sole source of nitrogen but not as carbon source. Near stoichiometric yields of L-phenylpyruvic acid (0.4 mM) and L-phenyllactate (0.4 mM) were observed from L-phenylalanine (0.9 mM consumed). Aminotransfarase and dehydrogenase activities involved in the formation of L-phenylpyruvic acid and L-phenyllactate were demonstrated unequivocally in *Rubrivivax benzoatilyticus* JA2. Growth conditions and carbon sources had an influence on L-phenyllactate production. The process yielded a maximum of 0.92 mM L-phenyllactate from L-phenylalanine (1 mM) when fructose served as carbon source for *R. benzoatilyticus* JA2.

1. Introduction

Bacteria utilize wide variety of compounds as carbon and nitrogen sources for their growth including amino acids. Amino acids support growth of bacteria as an excellent source of nitrogen and this process of catabolism, many a times, leads to secondary metabolite production (Thierry and Maillard 2002; Kumavath et al. 2010). Utilization of aromatic amino acids as nitrogen source leads to the production of their corresponding aromatic acids and alcohols (Somers et al. 2005; Narayanan and Ramananda Rao 1974). Purple phototrophic bacteria are metabolically versatile, utilizes wide range of organic compounds as carbon and nitrogen source for their growth and few purple bacteria can also metabolize aromatic compounds (Sasikala and Ramana 1998). *Rubrivivax benzoatilyticus* JA2 is an anoxygenic photosynthetic purple nonsulfur bacterium which transforms aromatic compounds like phenylalanine, tryptophan and aniline to value added compounds (Mujahid et al. 2011; Ranjith et al. 2011). The genome of strain JA2 is now available (Mohammed et al. 2011) and a critical analysis indicated potential pathways of aromatic compound metabolism by this strain. In the present study, we made an attempt to understand the metabolism of L-phenylalanine by *R. benzoatilyticus* JA2.

2. Materials and methods

2.1. Organism and growth conditions

R. benzoatilyticus JA2 (=ATCCBAA-35) was grown photoheterotrophically on a mineral media (Kalyan et al. 2007) containing malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen source respectively, pH 6.8 at 30 ± 2 °C and light 2400 lux, under anaerobic condition in screw cap test tubes (10 mm × 100 mm)/250 ml culture bottles. Aerobic dark growth was performed in 250 ml conical flasks under continuous agitation (150 rpm). When L-phenylalanine was added as sole source of nitrogen, ammonium chloride was replaced with 1 mM of L-phenylalanine. Growth was measured turbidometrically at 660 nm.

2.2. Analytical methods

HPLC analysis was done according to Mujahid et al. (2011) on a SHIMADZU (LC-20AT) system equipped with photodiode array (PDA) detector and Phenomenex C18 column (Luna, 5 μm, 250 mm × 4.6 mm). A linear gradient method of 30 min was used with mobile phase 1% glacial acetic acid (solvent A) and 100% acetonitrile (solvent B) for the separation of metabolites. Flow rate was 1.5 ml min^{-1}, injection volume 20 μl at 260 nm. L-Phenylalanine, phenylpyruvic acid and L-phenyllactic acid were quantified by HPLC with reference to the peak areas of known concentrations of authentic standards. Mass analysis was performed on MicroTOF-Q (Brukers deltanoics) mass spectrometer with electro spray ionization (ESI) ion source (80 °C, cone voltage 15–25 V). ESI (−) ion mode

* Corresponding author. Tel.: +91 40 23134502; fax: +91 40 23010120/45.
E-mail addresses: r449@sify.com, chvrsl@uohyd.ernet.in (Ch.V. Ramana).

0944-5013/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2012.03.001

was used to detect molecular ion mass ([M]$^-$) and fragmentation was obtained by collision energy of 5–20 eV and mass spectrum was recorded from 50 to 1000 Da. Chirality of the compound was determined by CD spectroscopy on JASCO J-810 spectropolarimeter.

2.3. Extraction and purification

The supernatant of L-phenylalanine added *R. benzoatilyticus* JA2 culture was acidified with 5 N HCl and then extracted with ethylacetate. The ethyl acetate extract was evaporated to dryness under vaccum at 30 °C using rotary flash evaporator (Heidolph, Germany). The dried extract was finally dissolved in 1 ml of HPLC grade methanol. Purification of the compound was done with HPLC method. The method used for purification was similar to the HPLC method used for the L-phenyllactic acid analysis.

2.4. Enzyme assays

2.4.1. Aminotransferase/transaminase activity and dehydrogenase activity

Aminotransferase/transaminase and dehydrogenase activities were done with L-phenylalanine added culture pellets. The L-phenylalanine added culture was centrifuged at 10,000 rpm, 4 °C, for 8 min and the pellet was washed and suspended in 4 ml of 50 mM Tris buffer (pH 7.5). Cells were lysed by sonication (BANDELIN, 8 cycles, 43% amplitude) and the lysate was centrifuged at 16,000 rpm, 4 °C for 20 min. The cell free extract was taken for the assay as enzyme source. Aminotransferase activity was carried in a final volume of 3 ml, containing L-phenylalanine (1 mM), α-ketoglutarate (1 mM) and pyridoxal phosphate (PLP; 50 μM). Dehydrogenase enzyme activity was carried out with the substrates phenylpyruvic acid (2 mM) and NADH (0.5 mM). Reaction was started with the addition of substrates to appropriate amount of cell free extract, incubated for 1 h and then stopped by adding 5 N HCl, the reaction mixture was centrifuged and the clear supernatant was extracted thrice with equal volume of ethyl acetate. Ethylacatate fractions were pooled and concentrated to dryness using Rotary Flash evaporator. The dried extract was dissolved in 100 μl of HPLC grade methanol. Production of L-phenylpyruvic acid and L-phenyllactic acid was measured by HPLC (Mujahid et al. 2010). 1 unit (U) of enzyme activity was expressed as the amount of enzyme required for the formation 1 μmol of product and the results are expressed per unit protein, which was measured by Bradford method (1976).

3. Results and discussion

3.1. Growth and utilization of L-phenylalanine

Growth and utilization of L-phenylalanine was observed with *R. benzoatilyticus* JA2 when the substrate was used as sole source of nitrogen (Fig. 1) but not as carbon source. L-Phenylalanine utilization as sole source of nitrogen by *R. benzoatilyticus* JA2 was observed under both light-anaerobic and dark-aerobic conditions. L-Phenylalanine utilization was less (0.2 mM) in the presence of ammonium chloride compared to that of in its absence (0.85 mM) as also observed earlier with other bacteria (Sáez et al. 1999; Andreotti et al. 1994; Kumavath et al. 2010). During growth and utilization of L-phenylalanine, formation of free ammonia into the media was not observed which, indicates the L-phenylalanine metabolism in *R. benzoatilyticus* JA2 occurs through a transamination process.

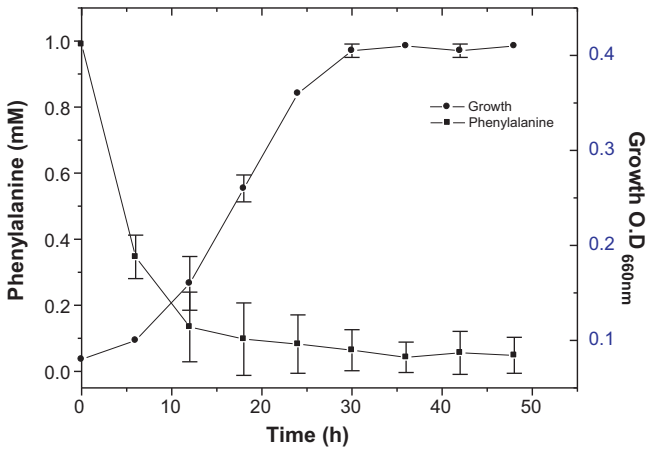


Fig. 1. Growth dependent L-phenylalanine utilization as nitrogen source by *R. benzoatilyticus* JA2.

3.2. Identification of metabolites

HPLC analysis of ethyl acetate extract of L-phenylalanine grown culture supernatant of *R. benzoatilyticus* JA2 indicated a few metabolites whose absorption maxima ranged between 240 and 290 nm depicting them as phenol derivatives (Gómez-Romero et al. 2010) and the same were absent in control condition (without supplementation of L-phenylalanine in the medium). Metabolites with retention times (R_t) 15.5, 23.8 were observed only in L-phenylalanine added culture supernatants (Fig. 2A and B). These two metabolites were identified as L-phenyllactic acid (UVmax 248, 254, 257, 263) and phenylpyruvic acid (UVmax 230, 287) based on their absorption spectra and retention times identical to that of authentic standards. The mass spectral analysis of the metabolites with R_t 15.5 and 23.8 indicated molecular ion peaks at 165[M$^-$] and 163[M$^-$], respectively (Fig. 2C and D) which confirms the metabolites as L-phenyllactic acid and phenylpyruvic acid. Phenylacetic acid (PAA), an intermediate of the L-phenylalanine catabolism was also identified based on the retention time (R_t 18.6 min) and absorbtion spectrum (UVmax 254, 257, 263; Fig. 3) identical to that of authentic standard. However, phenylethanol formation was not observed under anaerobic/microaerophilic conditions in *R. benzoatilyticus* JA2 rather PAA and PLA formation was seen. With the near complete utilization of the L-phenylalanine (0.9 mM), 0.33–0.38 mM of phenylpyruvic acid, 0.31–0.4 mM of L-phenyllactic acid and 0.09–0.1 mM of phenylacetic acid production was observed. CD spectroscopic analysis indicated that compound to be L-phenyllactic acid. Many bacteria utilize L-phenylalanine as nitrogen source and the catabolism leads to the production of phenyl derivates (Somers et al. 2005; Kumavath et al. 2010).

Production of L-phenylpyruvic acid during L-phenylalanine catabolism via transamination reaction was reported earlier in bacteria (Helinck et al. 2004). L-Phenylalanine catabolism by *R. benzoatilyticus* JA2 has lead to the formation of PAA/PLA and neither of the two metabolites was catabolized further. Genome insights also revealed that *R. benzoatilyticus* JA2 lacks PAA catabolic operon (genes) and L-phenylalanine could not support the growth of the organism as sole sources of carbon. These results suggest that L-phenylalanine catabolism occurs by Ehrlich pathway (Hazelwood et al. 2008) leading to the formation of PAA and not complete mineralization of L-phenylalanine in *R. benzoatilyticus* JA2. Production of L-phenyllactic acid is a restricted metabolic potential among bacteria and so far reported with a few lactic acid bacteria (Li et al. 2007; Mu et al. 2009), *Geotrichum candidum* (Dieuleveux

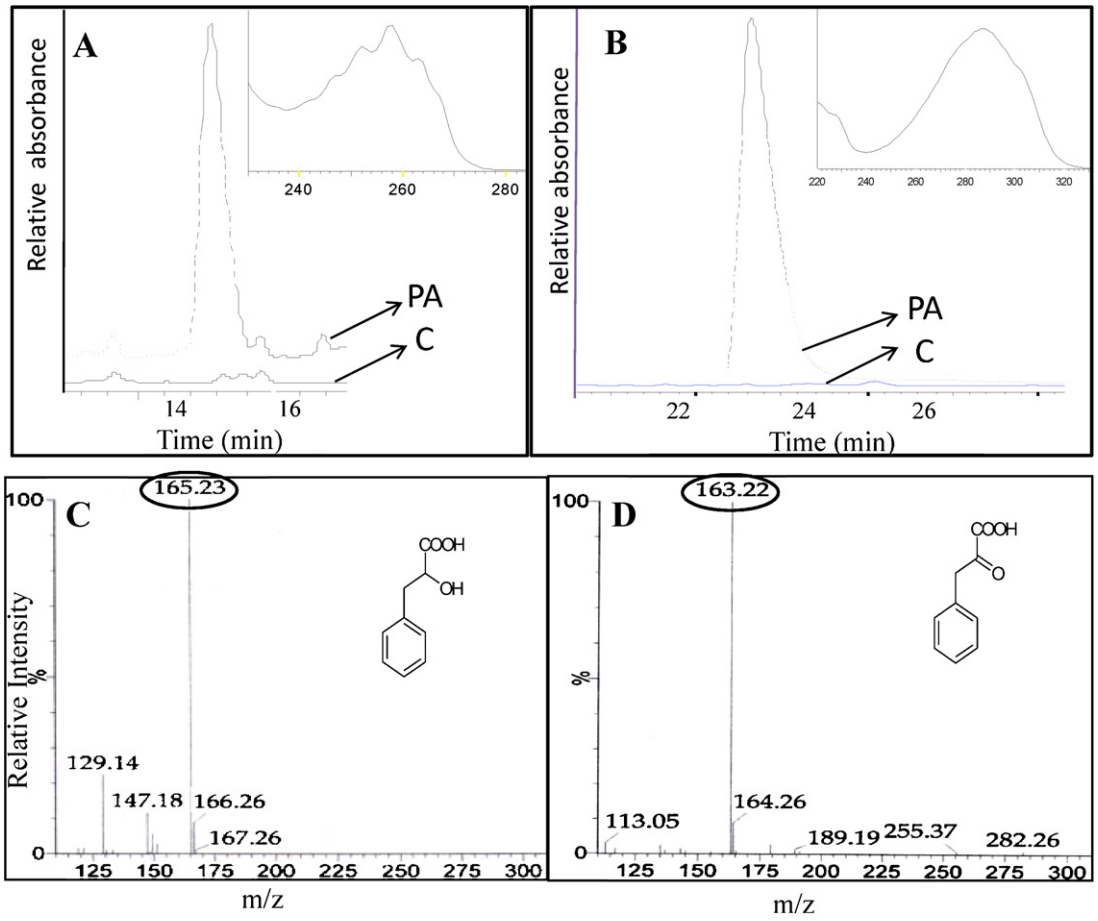


Fig. 2. HPLC chromatograms of ethyl acetate extract obtained from culture supernatants of *R. benzoatiltyicus* JA2. HPLC chromatograms of L-phenyllactic acid (A), phenylpyruvic acid (B), and their corresponding mass spectra (C and D). Chromatogram with solid line indicates control (without L-phenylalanine), dotted one for L-phenylalanine supplemented. Insert shows UV absorption spectra.

et al. 1998), *Bravibacterium* (Kamata et al. 1986) and *Propionibacteria* (Thierry and Maillard 2002). This is the first report of L-phenyllactic acid production by a purple bacterium, which also happens to be the major catabolite of L-phenayalanine. Production of L-phenylpyruvic acid in L-phenylalanine added cultures without ammonia release into the supernatant indicted an aminotransfarase dependent L-phenylalanine metabolism in *R. benzoatilyticus* JA2.

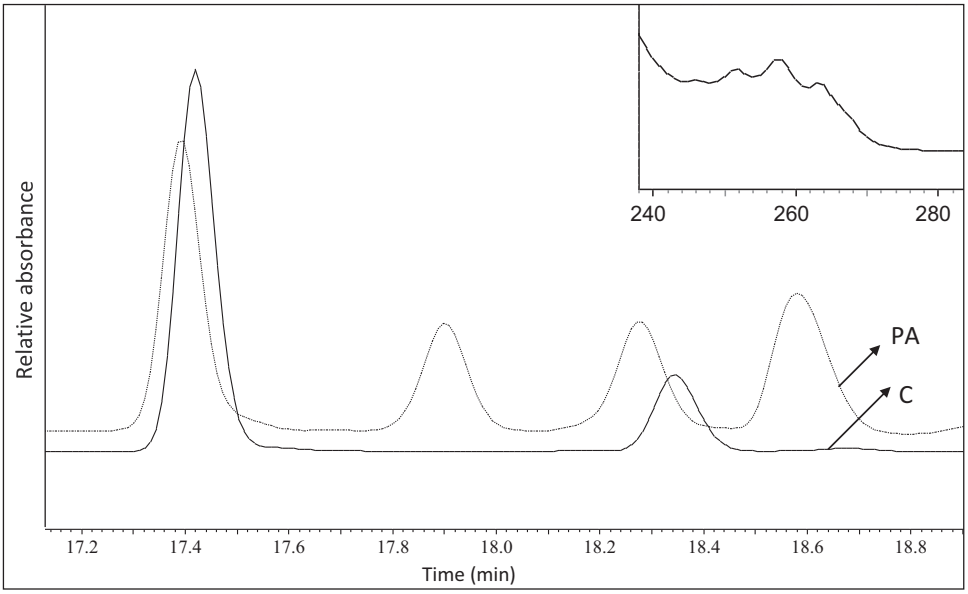


Fig. 3. HPLC chromatograms of L-phenylacetic acid with R_t 18.6 min (chromatogram with solid line indicates control (without L-phenylalanine), dotted one for L-phenylalanine supplemented). Insert shows UV absorption spectrum.

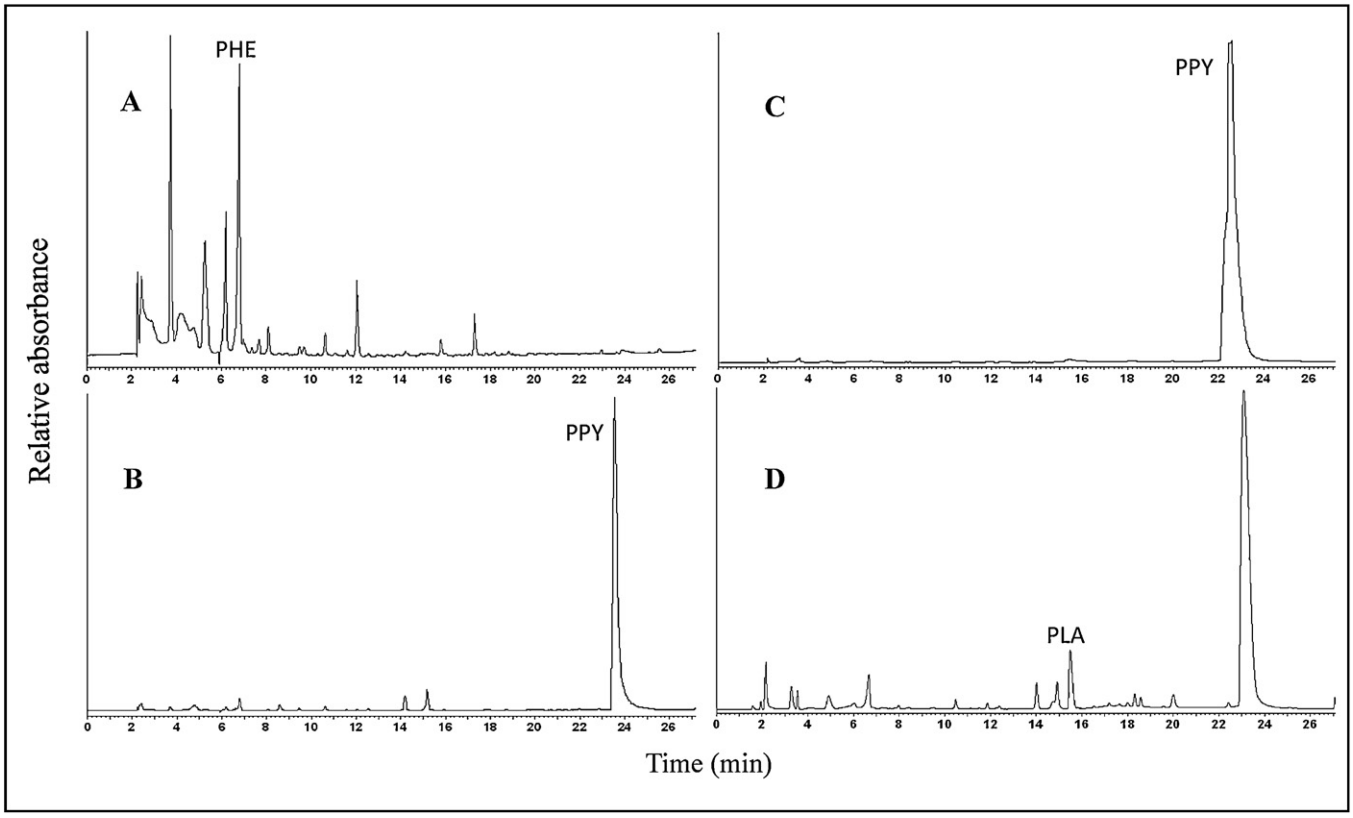


Fig. 4. Aminotransferase activity with pre-denatured sample (A) and reaction after 1 h of incubation (B) with cell free extracts of *R. benzoatilyticus* JA2. Dehydrogenase activity with pre-denatured sample (C) and reaction after 1 h of incubation (D) with cell free extracts of *R. benzoatilyticus* JA2.

3.3. Enzyme activities

Aminotransferase and dehydrogenase are the key enzymes involved in the conversion of L-phenylalanine to L-phenylpyruvic acid and L-phenyllactic acid (Li et al. 2008). Aminotransferase activity was demonstrated with cell free extracts of *R. benzoatilyticus* JA2, when L-phenylalanine acted as an amino donor, α-ketoglutarate as an acceptor in the presence of PLP. L-Phenylpyruvic acid was not observed in the pre-denatured sample (Fig. 4A) while its presence in 1 h incubated sample (Fig. 4B) indicates the presence of an aminotransferase (0.2 U mg^{-1}) in *R. benzoatilyticus* JA2. Dehydrogenase activity (0.7 U mg^{-1}) was also observed (Fig. 4C and D) in *R. benzoatilyticus* JA2 with the formation of L-phenyllactic acid from L-phenylpyruvic acid and the reaction was NADH dependent. Production of L-phenylpyruvic acid and L-phenyllactic acid and their corresponding enzyme activities confirms the L-phenylalanine catabolism via L-phenylpyruvic acid in *R. benzoatilyticus* JA2.

Identification of intermediates like L-phenylpyruvic acid, L-phenyllactic acid, L-phenylacetic acid indicates the L-phenylalanine catabolism via Ehrlich pathway (Fig. 5; Hazelwood et al. 2008). Genome analysis of *R. benzoatilyticus* JA2 revealed the presence of two aromatic aminotransferase genes and one lactate dehydrogenase gene possibly involved in L-phenylalanine catabolism. L-Phenylalanine/L-tryptophan grown cultures could show the transaminase activity for both the substrates L-phenylalanine and L-tryptophan this suggests that aromatic aminotransferase may be non-specific in *R. benzoatilyticus* JA2 (data not shown). Lactate dehydrogenase activity was also reported to be involved in L-phenylpyruvic acid to L-phenyllactic acid production by a *Lactobacillus* sp. (Li et al. 2008). L-Tryptophan catabolism in *R.*

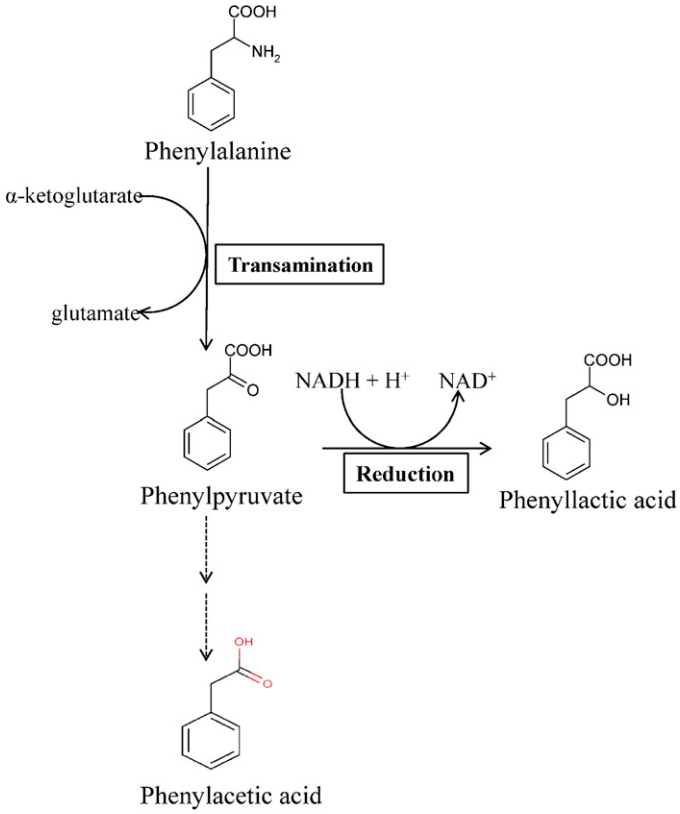


Fig. 5. Proposed pathway of L-phenylalanine metabolism in *R. benzoatilyticus* JA2.

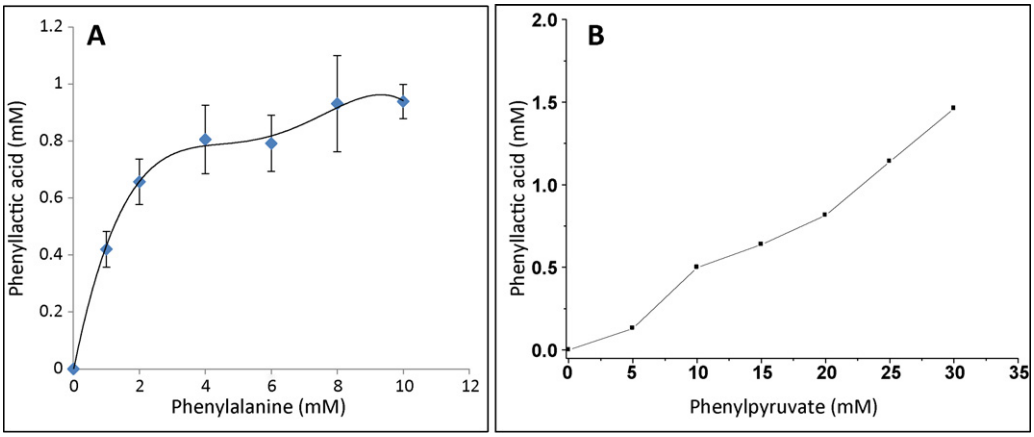


Fig. 6. Effect of L-phenylalanine (A) and phenylpyruvic acid (B) on L-phenyllactic acid production in *R. benzoatilyticus* JA2.

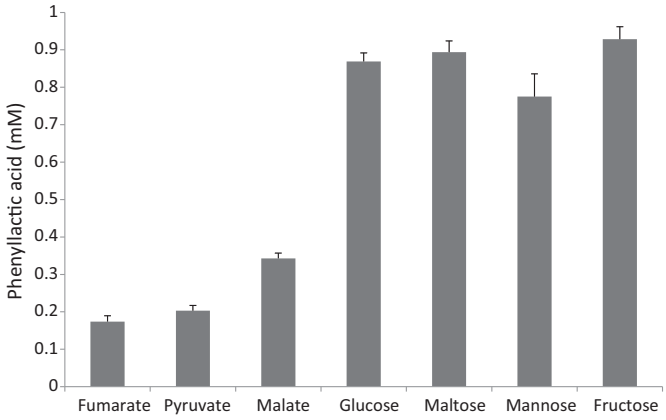


Fig. 7. Effect of carbon sources on L-phenyllactic acid production from L-phenylalanine in *R. benzoatilyticus* JA2. Malate (22 mM) was replaced with same concentration of other carbon sources.

benzoatilyticus JA2 via indole-3-pyruvate pathway was recently reported (Ranjith et al. 2010).

3.4. Production of L-phenyllactic acid

L-Phenyllactic acid was used as an antimicrobial compound (Dieuleveux and Gueguen 1998; Lavermicocca et al. 2003) and is of importance to pharmaceutical industries (Mu et al. 2009), hence its production by *R. benzoatilyticus* JA2 was monitored under different growth conditions. After 24 h of growth, 0.8–0.9 mM of L-phenylalanine was consumed with the production of 0.4 mM of L-phenyllactic acid, accounting to 45% conversion efficiency. The conversion efficiency could be increased to about 70%, when L-phenylalanine was supplemented to the 24 h grown (mid log phase) cultures of *R. benzoatilyticus* JA2.

Concentration of L-phenylalanine and growth conditions further influenced L-phenyllactic acid production by *R. benzoatilyticus* JA2. L-Phenyllactic acid production increases in a dose dependent manner up to 4 mM of L-phenylalanine and remains static at higher concentrations (Fig. 6A). In contrast, production of L-phenyllactic acid increased with increasing L-phenylpyruvic acid (Fig. 6B), an intermediate in the production of L-phenyllactic acid from L-phenylalanine (Li et al. 2007; Mu et al. 2009). L-Phenyllactic acid production was high (0.45–0.7 mM) when L-phenylalanine was used as sole source of nitrogen compared to that used as an additional source (0.15 mM). The yield of L-phenyllactic acid was high (0.45–0.7 mM) under phototrophic conditions (light anaerobic) than under chemotrophic (dark aerobic; 0.02 mM) conditions. Presence of carbon sources influenced L-phenyllactic acid yield in *R. benzoatilyticus* JA2 (Fig. 7). L-Phenyllactic acid yields were high (0.8–0.92 mM) in the presence of sugars. Genome sequence of *R. benzoatilyticus* JA2 indicated the presence of genes encoding decarboxylase and dehydrogenase enzymes which may have involved in the conversion of L-phenylpyruvic acid to L-phenylacetaldehyde and L-phenylacetaldehyde to L-phenylacetic acid (fusel acid).

Our study is the first report of L-phenyllactic acid production by a photosynthetic bacterium, particularly with 70% conversion efficiency of L-phenylalanine to L-phenyllactic acid which is the maximum known (to the best of our knowledge) from bacterial systems till now. We also report L-phenylalanine catabolism via Ehrlich pathway coupled to the production of L-phenyllactic acid in *R. benzoatilyticus* JA2.

Acknowledgements

LP and MM thanks for the CSIR, New Delhi for the award of Senior Research Fellowships.

References

Andreotti G, Cubellis MV, Nitti G, Sannia G, Mai X, Marino G, et al. Characterization of aromatic aminotransferases from the hyperthermophilic archaeon *Thermococcus litoralis*. Eur J Biochem 1994;220:543–9.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

Dieuleveux, Gueguen M. Antimicrobial effects of D-3-phenyllactic acid on *Listeria monocytogenes* in TSB-YE medium, milk, and cheese. J Food Prot 1998;61:1281–5.

Dieuleveux V, Van Der Pyl D, Chataud J, Gueguen M. Purification and characterization of anti-Listeria compounds produced by *Geotrichum candidum*. Appl Environ Microbiol 1998;64:800–3.

Gómez-Romero M, Segura-Carretero A, Fernández-Gutiérrez A. Metabolite profiling and quantification of phenolic compounds in methanol extracts of tomato fruit. Phytochemistry 2010;71:1848–64.

Hazelwood LA, Daran JM, van Maris AJ, Pronk JT, Dickinson JR. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol 2008;74:2259–66.

Helinck S, Le Bars D, Moreau D, Yvon M. Ability of thermophilic lactic acid bacteria to produce aroma compounds from amino acids. Appl Environ Microbiol 2004;70:3855–61.

Kalyan Chakravarthy S, Srinivas TNR, Anil Kumar P, Sasikala Ch, Ramana ChV. *Roseospira visakhapatnamensis* sp. nov. and *Roseospira goensis* sp. nov. Int J Syst Evol Microbiol 2007;57:2453–7.

Kamata M, Toyomasu R, Suzuki D, Tanaka T. D-Phenyllactic acid production by Brevibacterium or Corynebacterium; Brevet Ajinomoto Co Inc., Japan Patent JP 86,108,396; 1986.

Kumavath RN, Ramana ChV, Sasikala Ch. Production of phenols and alkyl gallate esters by *Rhodobacter sphaeroides* OU5. Curr Microbiol 2010;60:107–11.

Lavermicocca P, Valerio F, Visconti A. Antifungal activity of phenyllactic acid against molds isolated from bakery products. Appl Environ Microbiol 2003;69:634–40.

Li X, Jiang B, Pan B. Biotransformation of phenylpyruvic acid to phenyllactic acid by growing and resting cells of a *Lactobacillus* sp. Biotechnol Lett 2007;29: 593–7.

Li X, Jiang B, Pan B, Mu W, Zhang T. Purification and partial characterization of Lactobacillus species SK007 lactate dehydrogenase (LDH) catalyzing phenylpyruvic acid (PPA) conversion into phenyllactic acid (PLA). J Agric Food Chem 2008;56:2392–9.

Mohammed M, Isukapatla A, Mekala LP, Eedara Veera Venkata RP, Chintalapati S, Chintalapati VR. Genome sequence of the phototrophic betaproteobacterium *Rubrivivax benzoatilyticus* strain JA2^T. J Bacteriol 2011;193(11):2898–9.

Mu W, Chen C, Li X, Zhang T, Jiang B. Optimization of culture medium for the production of phenyllactic acid by *Lactobacillus* sp SK007. Bioresour Technol 2009;100:1366–70.

Mujahid M, Sasikala Ch., Ramana ChV. Aniline-induced tryptophan production and identification of indole derivatives from three purple bacteria. Curr Microbiol 2010;61:285–90.

Mujahid Md, Sasikala Ch, Ramana ChV. Production of indole-3-acetic acid and related indole derivatives from L-tryptophan by *Rubrivivax benzoatilyticus* JA2. Appl Microbiol Biotechnol 2011;89:1001–8.

Narayanan TK, Ramananda Rao G. Production of 2-phenethyl alcohol and 2-phenyllactic aid in *Candida* species. Biochem Biophys Res Commun 1974;58:728–35.

Ranjith NK, Ramana ChV, Sasikala Ch. Rubrivivaxin, a new cytotoxic and cyclooxygenase-I inhibitory metabolite from *Rubrivivax benzoatilyticus* JA2. World J Microbiol Biotechnol 2011;27:11–6.

Ranjith NK, Ramana ChV, Sasikala Ch. L-Tryptophan catabolism by *Rubrivivax benzoatilyticus* JA2 occurs through indole 3-pyruvic acid pathway. Biodegradation 2010;21:825–32.

Sáez LP, Castillo F, Caballero FJ. Metabolism of L-phenylalanine and L-tyrosine by the phototrophic bacterium *Rhodobacter capsulatus*. Curr Microbiol 1999;38:51–6.

Sasikala Ch, Ramana ChV. Biodegradation and metabolism of unusual carbon compounds by anoxygenic phototrophic bacteria. Adv Microbial Physiol 1998;39:339–77.

Somers E, Ptacek D, Gysegom P, Srinivasan M, Vanderleyden J. *Azospirillum brasilense* produces the auxin-like phenylacetic acid by using the key enzyme for indole-3-acetic acid biosynthesis. Appl Environ Microbiol 2005;71:1803–10.

Thierry A, Maillard MB. Production of cheese flavour compounds derived from amino acid catabolism by *Propionibacterium freudenreichii*. Lait 2002;82:17–32.