Genomic insights and metabolism of indole by *Rubrivivax benzoatilyticus* JA2

Thesis submitted for the award of

Doctor of Philosophy

by

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Dedicated
To
My Parents,
Wife &
Friends

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DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Genomic insights and metabolism of indole by *Rubrivivax benzoatilyticus* JA2"** has been carried out by me under the supervision of Dr. Ch. V. Ramana and this work has not been submitted for any degree or diploma of any other university or Institute earlier. All the assistance and help received during the course of investigation have been duly acknowledged.

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CERTIFICATE

This is to certify that **Mr. Arvind Isukapatla** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis entitled **"Genomic insights and metabolism of indole by *Rubrivivax benzoatilyticus* JA2"** for submission for the degree of Doctor of Philosophy of this University.

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

Dr. Ch. V. Ramana
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List of Abbreviations

ABC	ATP binding cassette
ACN	Acetonitrile
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bch	Bacteriochlorophyll
BLAST	Basic Local Alignment Search Tool
bp	base pairs
C	Carbon
CoA	Coenzyme-A
Da	Dalton
DAD	Diode array detector
DDH	DNA-DNA hybridization
DEAE	Diethylaminoethyl cellulose
DHAP	Dihydroxyacetone phosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EDTA	Ethylenediamine tetra-acetic acid
FDR	False detection rate
GC	Guanine and Cytosine
h	hour
HPLC	High Pressure Liquid Chromatography
IAA	Indole-3-Acetic Acid
IS	Insertion elements
KBr	Potassium Bromide
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS	Liquid chromatography mass spectroscopy
LH	Light harvesting
M	Molar
m/z	Mass-to-charge ratio

MALDI	Matrix assisted laser desorption/ionization
Mb	Mega base pairs
MEP	2-*C*-methyl-D-erythritol 4-phosphate
mg	milligrams
min	minute
ml	milliliter
mM	milli molar
NADH	Nicotinamide adenine dinucleotide phosphate (reduced)
NCBI	National Center for Biotechnology information
NGS	Next Generation Sequencing
nm	nanometer
PAGE	Polyacrylamide gel electrophoresis
PCG	Photosynthetic Gene Cluster
PCR	Polymerase Chain Reaction
PDA	Photodiode array
PEP	Phosphoenol pyruvate
PLP	Pyridoxal phosphate
ppm	Parts per million
RC	Reaction Center
RND	Resistance nodulation cell division
SDS	Sodium dodecyl sulphate
TFA	Trifluoroacetic acid
TLC	Thin Layer Chromatography
t_R	Retention time
trp	Tryptophan
UV	Ultra Violet
V	Volts
v	Volume

Abstract

A metabolically versatile phototrophic *Betaproteobacteria*, *Rubrivivax benzoatilyticus* strain JA2 was used in this study to understand the genomic insights of various metabolic processes and the influence of exogenous indole on these processes. Strain JA2 has 99% 16S rRNA gene sequence similarity with *Rubrivivax gelatinosus* ATCC 17011^T, however a new genomo species was previously described for strain JA2 based on DNA-DNA hybridization (DDH) with a reassociation value of about 54.8 % with added differences in the phenotypic traits. The reassociation value was also confirmed in this study through *in silico* analysis done by the genome to genome distance calculator based on the regression DDH estimates and a value of 47.2 % is in agreement and justifies the separation of strain JA2 from *Rubrivivax gelatinosus* ATCC 17011^T. Pyrosequencing of the *Rubrivivax benzoatilyticus* JA2 whole genome has revealed 98% of its genome length comprising 4.13 mega base pairs and consisting of 3,762 open reading frames (ORFs). Out of the total ORFs 3,210 were functionally annotated, 382 hypothetical and 170 are of unknown function with the protein coding bases of 3,566,505 which is 86.3 % of the total genome. It consists of 46 tRNAs, an rRNA operon and 17 insertion elements. The 81.2% of the genome codes for protein coding genes involved in the metabolic, biosynthetic and other cellular processes, followed by transport, signal transduction, transcription, translation and the minimum are involved in replication and repair. The remaining 18.8 % are related to hypothetical, prediction general function and unknown function coding sequences.

For the phototrophic growth the genome has complete set of homologous gene coding sequences for Calvin cycle, bacteriochlorophyll biosynthesis, light harvesting antenna. Genome also codes for carbohydrate metabolism for glycolysis, Kreb's cycle, pentose phosphate pathway

and Entner-Doudoroff pathway. For the terpenoid biosynthesis methyl erythritol phosphate pathway genes are present along with genes related for both spheroidene and spirilloxanthin type of carotenoid biosynthesis. In the aromatic biosynthesis, complete set of genes for shikimate, chorismate and anthranilate biosynthesis along with genes involved in the indole 3-acetic acid and 4-aminobenzoate biosynthesis are present. The homologous gene coding sequences for prephanate/aromatic dehydratase were not found in the draft genome involved in the phenylalanine biosynthesis. In the nitrogen metabolism genes coding for two types of nitrogenases and other accessory proteins are present. In the polyhydroxyalkanoate biosynthesis polyhydroxyalkanoate synthase, depolymerase and regulatory protein coding genes along with glutamine synthetase for utilization of ammonia and different types of dehalogenases, dioxygenases, amidases and carboxyesterase useful in different degradation processes are present. Different gene sequences responsible for the transport, regulation and signaling which includes extrusion proteins, starvation and stress response proteins, efflux carrier proteins, antibiotics, solvent and camphor resistance proteins and signaling proteins like histidine kinases, response regulators, chemotaxis response proteins, entericidin, nitrogen regulatory and serine/threonine kinases are present in the genome.

Exogenous indole has effected *Rubrivivax benzoatilyticus* JA2 at different levels of the cellular organization. Its influence was directly seen on the growth rate, yield and was concentration dependent. A change in the color of the culture from purple to green above 2 mM indole was due to the inhibition of chlorophyll's and carotenoids (spheroidene, didehydrolycopene and neurosporene) and stimulated a novel unidentified carotenoid. Tryptophan and two novel indole dimers were observed in indole induced cultures and data suggests the IAA production was tryptophan dependent.

Exogenous indole had influenced the protein profile of strain JA2 and the iTRAQ proteome analysis indicated its influence on membrane, nitrogen metabolism, photosynthesis and respiration. Proteins involved in the function for immediate survival and adaptation of the cell to the environment, like proteins of nitrogen metabolism, energy metabolism, glycogen synthesis, membrane integrity maintenance, flagellar movement, proton pumping and import of organic molecules as nutrients were all up regulated. While proteins that are not necessary for immediate survival of the cell like citric acid cycle, reaction centre, transport and DNA metabolism were down-regulated. Matrix-assisted laser desorption/ionization (MALDI) based analysis of the colored indole-protein complex indicated the role of an outer membrane auto-transporter barrel protein involved in indole detoxification by *R. benzoatilyticus* JA2. Genomic details explicates the metabolic capability of strain JA2 and its large inventory of mono and dioxygenases, transport and chemotaxis genes implies that *Rubrivivax benzoatilyticus* JA2 had adopted for sensing and acquiring diverse compounds from its environment and the proteome profile explains the strategic adaptation steps for the survival against the impact of indole.

Introduction

1. Introduction

1.1 About phototrophic bacteria

Photosynthesis is the only significant solar energy storage process on earth and the source of all the food and energy resources on earth (Blankenship, 2010). Phototrophic bacteria through photosynthetic electron transport use light as the energy source to produce phosphate bond energy and reductants (Tang, 2011). It was proposed that the earliest phototrophs were photoheterotrophic instead of being photoautotrophic. Evidences from the studies suggest that the earliest photosynthetic organisms were anoxygenic, all photosynthetic reaction centers have been derived from a single source, and the antenna systems and carbon fixation pathways have been modified multiple times (Blankenship, 2010). Evolution of the purple photosynthetic bacteria, the non-photosynthetic proteobacteria and the aerobic anoxygenic photosynthetic bacteria have diverged from a single progenitor (Beatty, 2005). Many photosynthetic bacteria play essential roles in global carbon, nitrogen and sulfur cycles by photoautotrophic and photoheterotrophic growth. Phototrophic bacteria were categorized into oxygenic and anoxygenic depending on the capability in liberating and not liberating oxygen during the process of photosynthesis (Fig. 1), respectively. The oxygenic phototrophic bacteria (cyanobacteria) share similar electron transport pathways with other oxygenic phototrophs (Tang, 2011). Except cyanobacteria all other photosynthetic bacteria carry out only anoxygenic photosynthesis (Tang, 2011) and contain a single reaction center (RC) (Gupta, 2005), which include well studied strains like *Rhodopseudomonas palustris, Rhodobacter sphaeroides, Rubrivivax gelatinosus* and *Rhodobacter capsulatus*.

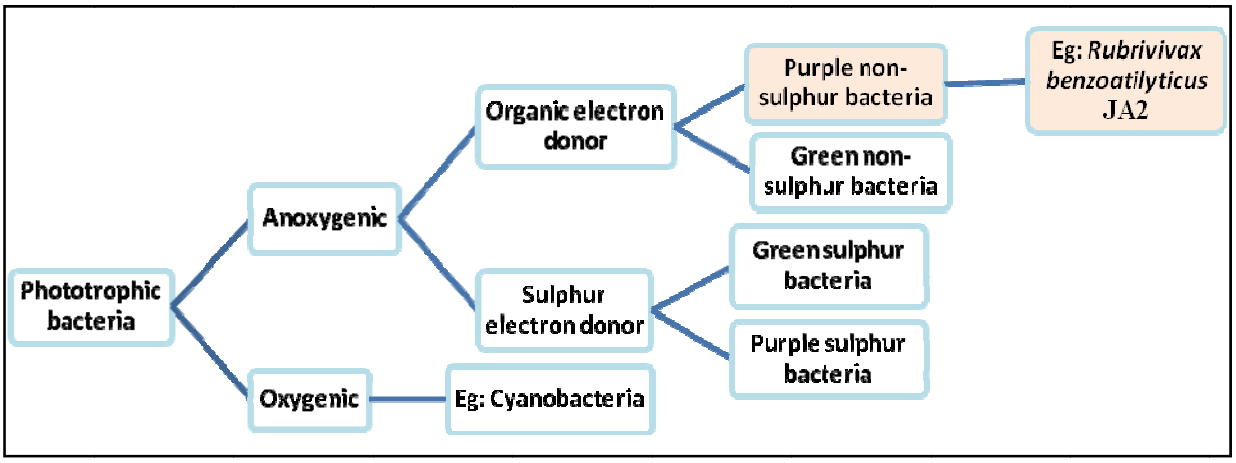


Fig.1 General overview of phototrophic bacteria

General categorization of phototrophic bacteria based on oxygen evolution and the source of electron donor during photosynthesis, and the position of *Rubrivivax benzoatilyticus* JA2

Anoxygenic phototrophic purple bacteria are a major group of photosynthetic microorganisms widely distributed in nature, primarily in aquatic habitats (Madigan and Jung, 2009). The studies of purple and other anoxygenic phototrophs have contributed in major ways in understanding the evolution of photosynthesis (Raymond et al., 2003; Swingley et al., 2009). Five out of six phyla of photosynthetic bacteria are anoxygenic phototrophs, Proteobacteria (anaerobic and aerobic anoxygenic phototrophs), green sulphur bacteria, filamentous anoxygenic phototrophs, heliobacteria and chloroacidobacteria (Tang, 2011). All phototrophic green and purple bacteria are gram negative and the main component of the outer membrane of gram negative cell envelops are the lipopolysaccharide carrying the type specific O-antigenicity (Luderitz et al., 1971). Analysis of different RCs suggests that all RCs have evolved from a single common ancestor and have a similar protein and cofactor structure (Sadekar et al., 2006). Earliest RCs were different from the present two RCs and multiple gene duplications have given rise to the heterodimeric complexes (Blankenship, 2010). Similarities in different RCs and the overall charge transfer mechanisms indicate that they all originated from a common ancestor (Blankenship 1994; Golbeck 1993; Nitschke and Rutherford 1991; Vermaas 1994; Sadekar et al., 2006).

The anoxygenic photosynthesis is a simpler form of photosynthesis than the oxygenic process and purple bacteria have emerged as ideal model systems for dissecting the physiology, biochemistry and molecular biology of photosynthesis (Raymond et al., 2003; Swingley et al., 2009). Species of *Rhodobacter* and *Rhodopseudomonas* are workhorses for laboratory studies of anoxygenic photosynthesis. Purple bacteria participate in the anoxic cycling of carbon both as primary producers (CO_2 fixation, photoautotrophy) and as light stimulated consumers of reduced organic compounds (photoheterotrophy) (Madigan and Jung, 2009). For photosynthetic electron donor, they depend on substances of a lower redox potential than water, such as reduced sulfur compounds, sulfur, molecular hydrogen or simple organic compounds (Pfennig, 1977). Photosynthetic bacteria have simplified photosynthetic apparatus with fewer subunits and unique photosystem, but their carbon metabolism pathways are rather complex (Gennis, 1993; Blankenship, 2002). For the growth of purple bacteria the media should have either sulfide plus bicarbonate (photoautotrophic growth) or an organic compound (photoheterotrophic growth)

(Raymond et al., 2003; Swingley et al. 2009). Yeast extract used as a source of B-vitamins as one or more of which are required by the majority of the purple non-sulfur bacteria and also stimulates the growth because of its assortment of organic compounds that can fuel photoheterotrophic growth. Several individual organic compounds like organic acids, amino acids, fatty acids, alcohols, carbohydrates and even C-1 compounds support photoheterotrophic growth of different species. With minor exceptions, the citric acid cycle intermediates malate, succinate and fumarate are universally used as well as pyruvate and acetate for growth. Ethanol, lactate and propionate are also utilized by many species (Sojka, 1978; Truper and Pfenning, 1981). Few purple non-sulfur bacteria photoassimilate aromatic compounds such as benzoate, hydroxyl derivatives of benzoate and cyclohexane carboxylate (Gibson and Harwood, 1995).

Most of the organic compounds that are photoassimilated by purple non-sulfur bacteria can also be used as electron donors and carbon source for dark respiratory growth. Certain purple non-sulfur bacteria can grow under anoxic dark conditions by either fermentation or anaerobic respiration on pyruvate (Uffen and Wolfe, 1970; Gurgen et al., 1976) and some sugars (Madigan and Gest, 1978; Scultz and Weaver, 1982). Dark chemolithotrophic growth of certain species of purple non-sulfur bacteria could possible using hydrogen or sulphite as electron donors (Madigan and Gest, 1979). With exceptions purple non-sulfur bacteria are excellent nitrogen fixing bacteria, the diazotrophy confers a significant competitive advantage on them in anoxic environments that are limited in fixed nitrogen (Madigan et al., 1984 & 1995). The difference between purple sulfur and non-sulfur bacteria is the ability or inability to form globules of elemental sulfur inside the cells (Molisch, 1907; Niel, 1957). 16S rRNA sequencing studies have shown that purple sulfur bacteria are species of *Gammaproteobacteria* while non-sulfur bacteria are either *Alphaproteobacteria* or *Betaproteobacteria* (Imhoff et al., 2005). Till 2012 no known purple sulfur bacteria are reported of hydrolyzing major polymeric substances such as cellulose, so they depend on heterotrophs for generation of low molecular weight compounds for their photoassimilation (Pfennig, 1978). The aerobic anoxygenic photosynthetic bacteria diverged from a purple photosynthetic like bacterial ancestor by becoming dependent on respiration of organic compounds for survival, losing oxygen repression of the photosynthetic apparatus and by producing large amounts of carotenoids (Beatty, 2005).

Purple non-sulphur bacteria

Purple non-sulfur bacteria are physiologically versatile group (Sojka, 1978). All species grow best as anaerobic photoorganotrophs and have the capacity to grow also as facultatively microaerophilic to aerobic chemoorganotrophs (Pfennig, 1974). Purple non-sulfur bacteria are specialized in photoheterotrophy instead of photoautotrophy, as they do not need to conserve energy from the carbon source, instead they photoassimilate, so quantitatively all the carbon goes into the cell material (Madigan and Jung, 2009). Evidence of similar pigments and photocomplexes in different species of purple non-sulfur bacteria suggests that the acquisition of phototrophic capacity in purple non-sulfur bacteria has occurred by lateral gene transfer (Nagashima et al., 1997). Evidences (less correlation between RC) indicates that phototrophic bacteria containing the same type of RC often have distinct central carbon metabolism pathways, suggesting horizontal/lateral gene transfers and late adaptation during the evolution of photosynthesis (Madigan and Jung, 2009). Anoxygenic phototrophic bacteria (purple non-sulfur bacteria) with a type II RC are facultative phototrophs, either photoautotrophs or photoheterotrophs and are metabolically versatile. Some anaerobic anoxygenic phototrophs either operate an active glyoxylate cycle (McKinlay and Harwood, 2011) or have genes (Oda et al., 2008; Lu et al., 2010; Strnad et al., 2010) or lack of genes (Lim et al., 2009).

Diversity of anoxygenic phototrophic bacteria

Anoxygenic photosynthetic bacteria have always attracted attention because of their coloration and ability to perform photosynthesis in the absence of oxygen and without producing oxygen (Zeng et al., 2007). Anoxygenic phototrophic bacteria have been isolated from soil, sewage, freshwater and brackish waters, sediments, hot springs and thermal vents in the sea floor and Antarctic ice covers, almost from every corner of Earth where light radiation can reach (Overmann and Garcia Pichel, 2003; Beatty et al., 2005). Extremophilic phototrophs have provided new insights into the evolution of photosynthesis and play ecological roles as primary producers in their unusual habitats (Madigan, 2003). Anoxygenic phototrophic extremophiles includes *Thermochromatium tepidum, Chlorobium tepidum, Choloflexus aurantiacus, Rhodovulum, Rhodovibrio, Rhodothalassium, Rhodobium, Roseospira* (Imoff, 2001), *Rhodobaca* (Milford et al., 2000), *Rhodoblastus acidophilus* (Pfennig, 1969), *Rhodoblastus sphagnolica* (Kulichevskaya et al., 2006) and *Rhodopila globiformis* (Pfennig, 1974).

Photosynthetic Gene Clusters in anoxygenic phototrophic bacteria

The structure of antenna complexes and the pigments present in them varied in different types of photosynthetic organisms (Blankenship, 2010). In anoxygenic species the apparatus consists of a reaction center surrounded by one to three types of antenna complexes (Fotiadis, 2004). The photosynthetic apparatus is encoded by a number of genes organized in a so-called photosynthetic gene cluster (PGC). PGCs are a feature of gene organization in several photosynthetic bacteria (Zheng, 2011). The occurrence of these clusters could facilitate lateral transfer of a carotenoid biosynthesis pathway among different micro-organisms (Naylor, 1999). This type of gene organization could be crucial for environmental adaptation (Liotenberg et al., 2008). PGC contain five main sets of genes: *bch, puf, puh* (for RC assembly), *crt* and various regulatory genes. The complex operon recombination in PGC occurred after phylogenetic divergence of anoxygenic phototrophic bacterial genera. Four structural types of *puf* gene organization are present in the PGCs *puf QBALMC, puf QBALM, puf BALM* and *puf LMCBA* (Zheng et al., 2011). In case of *R. sphaerodes* the PGC was reported as 40.7 kb in length and consists of 38 open reading frames encoding the reaction center H, L and M subunits, the alpha and beta polypeptides of the light- harvesting I (B875) complex, and the enzymes of bacteriochlorophyll and carotenoid biosynthesis (Naylor, 1999). The LH II polypeptides were found to be encoded by a region of DNA lying outside the PGC known as the *puc* operon (Youvan & Ismail, 1985).

Anoxygenic photosynthesis is tightly regulated, typically occurring under low oxygen tension due to the production of destructive singlet oxygen by the photosynthetic machinery when oxygen concentrations are high (Purcell and Crosson, 2008). Expression of the photosynthetic machinery of these species is thus highly regulated, occurring under low oxygen conditions (Gregor & Klug, 1999). Light quality and quantity can also serve as regulatory cues in the expression of photosynthetic genes (Purcell and Crosson, 2008). Two regulators (*ppsR* and *ppaA*) are observed having sensitivity to light intensity and oxygen concentration (Komiya, 1998). In the studies it was found that illumination with dim light (<1000 lux) stimulates expression of a light-harvesting apparatus, whereas high light intensities (>10,000 lux) cause a repression of pigment synthesis (Gemerden, 1980). This type of regulation could be due to increase the amount of absorbed photons under non-saturating conditions for photosynthesis and

to avoid photooxidative damage by strong light in presence of oxygen (Spring, 2009). Oxygen is the major external factor affecting the expression of photosynthesis genes in facultatively photosynthetic bacteria (Jutta and Klug, 2002). RegB/RegA two component system involved in oxygen-regulated expression of genes for nitrogen fixation, carbon dioxide assimilation and hydrogenase synthesis, and also plays a significant role in photosystem gene regulation, acting as an oxygen and light dependent regulator of the formation of photosynthetic complexes (Joshi and Tabita, 1996; Elsen et al., 2000).

The genes for the pigment binding proteins of the reaction center and the light harvesting I complex are organized in the polycistronic *puf* operon (Jutta and Klug, 2002). The *puf* operon was reported to be localized within the coding region of the *bchZ* gene. A low level of *puf* transcription occurs from promoters of upstream bacteriochlorophyll and carotenoid genes (Wellington et al., 1991). It was proposed that this type of gene organization guarantees low level of *puf* transcription at high oxygen tension and allows a faster adaption to changes in growth conditions (Jutta and Klug, 2002). *bchE* gene is expressed irrespective of the oxygen growth conditions, the BchE Mg-protoporphyrin monomethyl ester cyclase became inactive under high oxygenation (Ouchane et al., 2004). A drop in the oxygen tension in the environment leads to an increase in the levels of *puf* and *puc* expression. And so during the process of aerobic respiration, low amounts of photosynthetic complex genes are expressed (Zeilstra et al., 1998).

Applications of photosynthetic bacteria

Anoxygenic photosynthetic bacteria grow in the polluted environments and are extensively used in the waste remediation and treatment. These bacteria are also know for the production of hydrogen production, polyhyroxyalkanoic acids and carotenoids useful in numerous applications (Zeng et al., 2007). The main drawback in these bacteria is their production rate is considerably low, so the studies are in progress for overcoming this (Chen et al, 2006). These photosynthetic bacteria also serve as excellent model systems for the study of photosynthesis, the knowledge of the complete genome sequences of these bacteria makes it possible to apply DNA chip technologies and proteomics which may be the tools to better understand the regulatory network and to assign the functions for individual components in the future (Jutta and Klug, 2002).

1.2 Genome sequencing

Genome sequencing increases the understanding of the genetic diversity, distribution and metabolic capability of the organism. In whole genome sequencing the most important observation is of genetic diversity, there is a level of large-scale variation among the genomes of the same species, is far greater than was thought. Difference in the diversity with genome size of main chromosome ranging from 5.5 to 4.6 within the same species. The amount of genomic data available in the data base has provided significant advance in understanding a number of important observations including bacterial diversity, population characteristics, operon structure, mobile genetic elements and horizontal gene transfer, also in understanding ecology of, as yet, undiscovered bacterial worlds. When comparing bacterial genomes, two features are frequently analysed, gene presence and gene order (Binnewies et al., 2006).

Most of the whole genome sequencing was carried out using shortgun approach, involving large number of clones chosen randomly from a whole-genome library. Random sequences are then aligned and assembled together into contigs to reconstruct the structure of the genome (Tettelin and Feldblyum, 2009). Resulting assemblies typically consist of a number of contigs separated by gaps in which sequence information could be missing due to complex repeats that are hard to assemble and DNA regions that are difficult to sequence or fragments missing from genomic library. Gap closure is usually laborious because it inevitably tackles the most difficult regions of the genome to sequence and it involves a variety of molecular biology techniques, depending on the case at hand (Frangeul et al., 1999).

Development of chain termination method by Sanger established the bases for sequencing technologies (Sanger et al., 1977). These technologies offer dramatic increase in cost-effective sequence through-put, albeit at the expense of read lengths. Also for analysis of epigenetic modification of histones and DNA, gene expression profiling, genome annotation, small ncRNA discovery and profiling, and detection of aberrant transcription. Methods like fluorescence dyes and their detection, highly efficient emulsion PCR and capillary gel electrophoresis with arrays enhanced the separation of the sequencing efficiency. Till 2012, 2,577 complete bacterial genomes were published and another 12,874 ongoing bacterial genome projects, most of them are human pathogens, other pathogens and bacteria of environmental and industrial relevance. In these about 150 anoxygenic phototrophic bacterial genomes were listed

in the NCBI genome database including different genus and their species which accounts for 6 % of total bacterial genomes sequenced, and the number is still increasing with the advancement in the sequencing technologies which are more accurate and less time consuming (NCBI; http://www.genomesonline.org/cgi-bin /GOLD/index.cgi).

Next generation sequencing (NGS) have demonstrated the capacity to sequence DNA at unprecedented speed, thereby enabling previously unimaginable scientific achievements and novel biological applications (Zhang et al., 2010). The impact of NGS technologies on genomics will be far reaching and likely to change the field for years to come. NGS are called parallel sequencing as they are done by breaking the entire genome into small pieces, then ligating those small pieces of DNA to designated adapters for random read during DNA synthesis (sequencing by synthesis). In de novo sequencing a new genome or other genetic material are sequenced and assembled without direct comparison against a known sequence, due to this these sequencing technologies are suitable for new genetic material and genetic material that differs markedly from a previous sequenced strain (Bhattacharyya et al., 2002; Goo et al., 2004).

Next generation genome sequencing help to expand the utility of sequencing in drug discovery and development. Sequencing technologies have been applied in a variety of contexts like whole-genome sequencing, discovery of transcription factor binding sites and non coding RNA expression profiling. Sequencing larger and larger fractions of human genomes at an ever decreasing cost, an effort that will elucidate phenotype variants, extending the comprehension of disease susceptibility and pharmacogenomics, permitting personalized medicine. Other applications with pharmaceutical relevance are sequencing of multiple strains of pathogenic bacteria to monitor drug resistance and pathogenicity in bacteria, resequencing selected regions to search for human variation in populations (Hinds et al., 2005; Hardenbol et al., 2005) monitoring the onset of drug resistance in HIV or HCV (Gerhardt et al., 2005; Kapoor et al, 2004) profiling tumors to guide cancer therapies and discerning the mechanism of action of antibiotics (Andreis et al, 2005). Utility of sequencing can be applied in applications such as disease associated exons within a population, investigation of viral quasi-species present within a patient as a function of time and drug response, deep sequencing of microRNAs, querying somatic mutations in tumor samples, and monitoring pathogens for changes in their genome as a function of drug resistance or changing virulence. Till 2012 four types of next generation

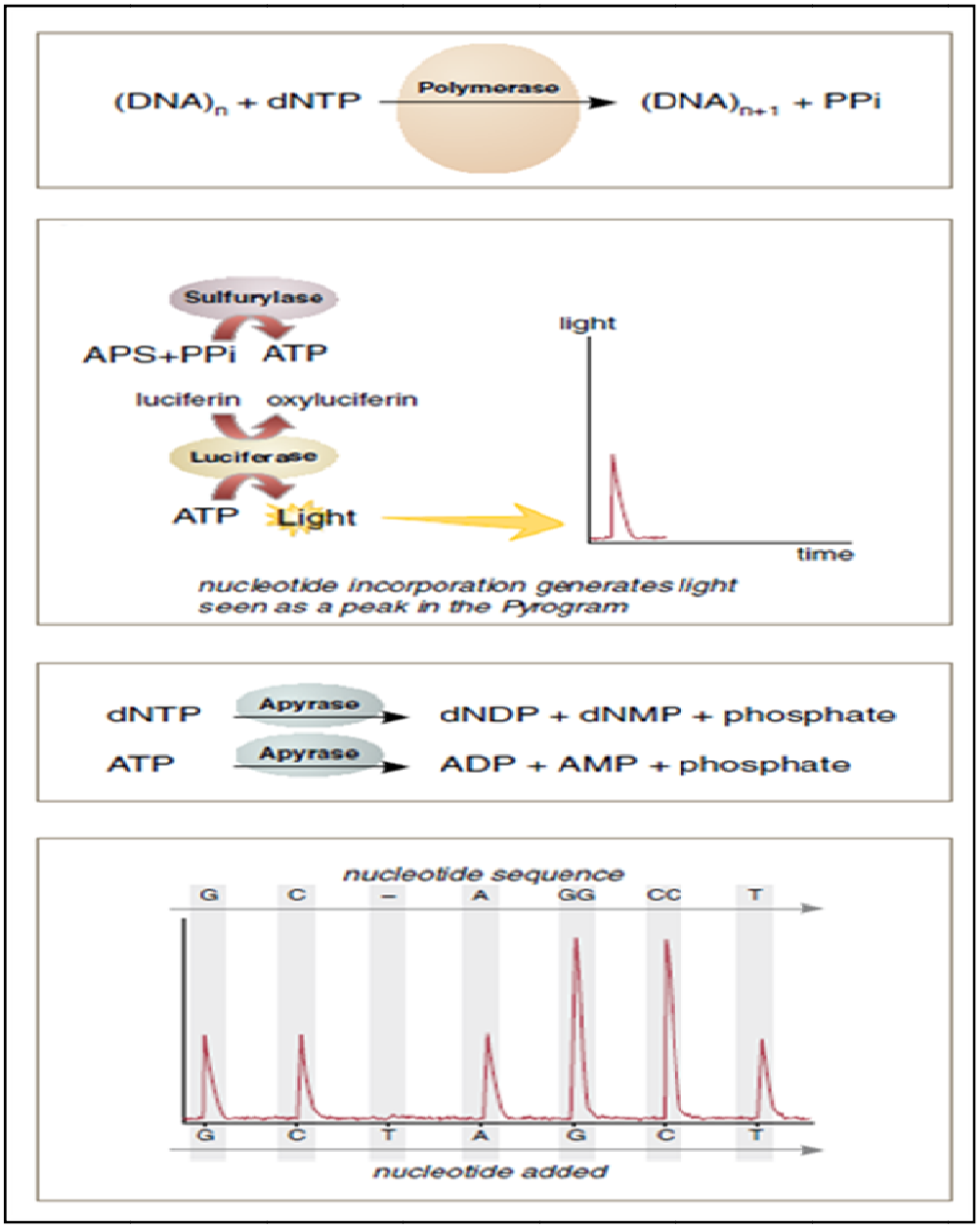
genome sequencing methods are employed they are 454 sequencing, Illumina-solexa, ABI SOLiD and Helicose (Tettelin and Feldblyum, 2009).

454 sequencing technology

454 sequencing technology was used to detect and map for more than 1,000 structural variations, 3kb or larger, between the two human genome. 454 sequencer was able to identify point mutations in the evolved strain. The sequence of DNA template was determined from a "pyrogram" based on the chemiluminescent signal proportional to the amount of pyrophosphate released corresponding to the correct nucleotide incorporated. 454 has been the most widely published next generation technology till 2012. Only 300 bp read lengths are only achievable by this method (Torres et al., 2008). Several studies found that 454 sequencing correlated well with the established gene expression profiling technologies such as microarray results and moderately with SAGE data. The quantity of reads, lengths and read accuracy becomes critical for mapping the reads to reference sequence and for detecting sequence polymorphisms. Pyrosequencing is a sequence by synthesis method where a successful nucleotide incorporation event occurs and detected as emitted photons (Ronaghi et al., 1998). The washing procedure for the removal of by products permits read lengths of over 400 bp. (250 bp in the GS FLX system and over 400 bp in the recently upgraded instrument of GS FLX Titanium). This limitation was due to negative frame shifts (incorporation of nucleotides in each cycle is not 100 % complete) and positive frame shifts (the population of nucleotides that is not fully degraded by the apyrase and can therefore be incorporated after the next nucleotide) that eventually will generate high levels of noise (Pettersson et al., 2008).

454 was the first commercial platform introduced in 2004, and uses pyrosequencing technology for the synthesis based sequencing, and the synthesis includes the emulsion based polymerase chain reaction (PCR) to the single stranded DNA binding beads bound in pico-liter wells in PicoTiter plate. With 600 bp the 454 sequencer has the longest short reads among all the NGS platforms and generates ~ 400-600 Mb of sequence reads per run (Zhang et al., 2010). In pyrosequencing the purified PCR samples are directly analyzed by real time monitoring of the DNA synthesis. Pyro can distinguish closely related bacterial strains by longer read lengths are necessary. Pryo can have broad range of applications like SNP genotyping, de novo mutation detection, gene identification and microbial genotyping (Ahmadian et al., 2006). Genome

sequencing will elucidate different metabolic pathways of the organism, in photosynthetic bacteria the key metabolic pathways include terpenoid, carotenoid and aromatic metabolism biosynthetic pathways for different growth modes of these organisms.



Flow chart.1 Pyrosequencing method overview

In the process of pyrosequencing a primer is used against the single stranded genomic DNA contigs and were PCR amplified and with incorporation of each deoxyribonucleotide an pyrophosphate was generated which results in the formation of ATP which drives the luciferase mediated conversion of luciferin to oxyluciferin that generates visible light in proportion to the amount of ATP are read by the detector. Apyrase is used in this method as to control the unused ATP and unincorporated dNTP's in order to switch off the light and also regenerates the reaction solution for the addition of the next dNTP.

1.3 Carotenoids

Terpenoid are the largest group of natural products with over 30,000 compounds, with different biological and physiological functions. Terpenoid biosynthesis occurs via two different pathways, classified as mevalonate and new 2-*C*-methyl-D-erythritol 4-phosphate (MEP or non-mevalonate) pathway leading to isopentenyl diphosphate. In higher plants the mevalonate pathway operates mainly in the cytoplasm and mitochondria, and the MEP pathway operates in the plastids with one common metabolite. And MEP pathway was first discovered in the biosynthesis of bacterial hopanoids. Tetra terpenoids units are the precursors for carotenoids (Sayed and Verpoorte, 2007).

Carotenoids are a major class of lipophilic isoprenoids. All photosynthetic organisms contain carotenoids which are essential for photoprotection and also functions as accessory pigments. Carotenoids are relatively hydrophobic molecules and are associated with membranes in noncovalent bond with specific proteins, as they display substantial bathochromic shifts. More than 600 different carotenoids produced by plants, algae, bacteria and fungi are described till 2011 (Kaur and Gill, 2011). Photosynthetic bacteria produces about 100 different types of carotenoids and around 50 different carotenoids are found in anaerobic purple photosynthetic bacteria which are unique from their eukaryotic counterparts (Takaichi, 2009). In general carotenoids are made up of C40 backbone and the carotenoids with longer C45 or C50 or shorter C30 backbones occur among nonphotosynthetic bacteria. The double bond in the polyene of naturally occurring carotenoids exist as all *trans* isomers only (Armstrong, 1997). In purple photosynthetic bacteria the two main pathways are proposed for carotenoid biosynthesis, they are the spirilloxanthin and okenone pathways (Takaichi, 1999), *Alphaproteobacteria* and *Betaproteobacteria* have spirilloxanthin pathway and *Gammaproteobacteria* either have spirilloxanthin or okenone pathway in different genus or species. Apart from these, carotenoid glucosides and carotenoid glucoside fatty acid esters were reported in some species and also some unusual non-photosynthetic carotenoids like carotenoid sulfates, carotenoid acids and hydroxy derivatives of β-carotene are present mainly in the aerobic anoxygenic photosynthetic bacteria whose function at present is unknown (Takaichi, 2009). The variation of carotenoid production was observed depending on the culture conditions, such as light intensity (Gardiner et al., 1993), oxygen concentration (Goodwin, 1956), growth phases (Schwerzmann and Bachofen, 1989) and also the strain specific in some species (Gardiner et al., 1993).

In the purple photosynthetic bacteria carotenoid biosynthesis occurs by three or four consecutive desaturations of phytoene to lengthen the polyene chromophore and yield neurosporene or lycopene, these carotenoid desaturation and cyclization are the main targets for many known carotenoid biosynthesis inhibitors. Most carotenoids accumulated in anoxygenic photosynthetic bacteria and cyanobacteria are associated with the membrane bound bacteriochlorophyll (*Bchl*) or chlorophyll (*Chl*) binding polypeptides of the photosynthetic apparatus. In nonphotosynthetic bacteria and some photosynthetic bacteria carotenoids and their glycosides can be found in the cytoplasmic and cell wall membranes and might have influence on the membrane fluidity (Armstrong, 1997).

The paramount function of carotenoids in all the photosynthetic bacteria is to provide photooxdative protection and in some useful for light harvest at higher wavelengths, except the unusual carotenoids. Carotenoids protect against the potentially damaging combination of oxygen, light and photosensitizing *Bchl* or *Chl* molecules by quenching both the triplet excited states of the photosensitizers and the singlet excited state of oxygen (Armstrong, 1997). And in non-photosynthetic bacteria and other organisms carotenoids are useful for protection against the reactive oxygen species by quenching them.

Gene organization of carotenoid biosynthesis genes in photosynthetic bacteria

The carotenoid biosynthesis genes mingled with other photosynthetic genes are arranged in the form of clusters called photosynthetic gene clusters (Zheng et al., 2011). The operons containing carotenoid genes *crtA, crtE* and *crtF* are embedded within superoperons that allow their cotranscription with *bch* genes and with the *puf* genes that encode the B870 light harvesting and reaction center polypeptides. *puc* genes that encode the B800-850 peripheral light harvesting antenna polypeptides are coregulated with *crt* and *bch* genes by separate mechanisms. The expression of most photosynthetic bacteria the *crt* genes was several fold higher under anaerobic photosynthetic conditions than in semiaerobic or aerobic conditions. Regulatory genes *ppsR* and *tspO* have been demonstrated to encode products that normally repress not only carotenoid and *Bchl* pigment levels, but also *crt, bch* and *puc* gene expression to several fold under aerobic growth conditions. *tspO* also serves as an environmental sensor present abundantly in anaerobic photosynthetic cells than in aerobic cells. The functionally related membrane bound carotene

biosynthetic enzymes of *Bchl* containing anoxygenic photosynthetic bacteria and oxygenic photosynthetic bacteria are structurally dissimilar (Armstrong, 1997).

The metabolically versatile, purple non-sulfur bacteria normally accumulate a mixture of two specialized acyclic xanthophylls-spheroidene and spheroidenone (Blankenship et al., 1995). Their abundance and distribution, as a function of the amount of dissolved oxygen in the growth medium, strongly influence the colour of the bacterial culture (Schmidt, 1978). The conversion of spheroidene to spheroidenone catalyzed by CrtA not only requires molecular oxygen as a source for the oxo-group but also stimulated at high light intensities. Interestingly, at a given oxygen tension and light intensity, the ratio of these two carotenoids correlates with the ratio of the peripheral spheroidene binding B800-850 light harvesting complex to the inner core spheroidenone binding B875 light harvesting complex (Yeliseev et al., 1996).

Applications of carotenoids

Carotenoids are valuable bioactive compounds with nutraceutical properties (Kaur and Gill, 2011). One of the most exciting prospects on the horizon in the field of carotenoid biosynthesis is the development of novel strategies for the production of carotenoids of nutritional, medical or aesthetic interest. Naturally occurring carotenoids of commercial interest as coloring agents for food, pharmaceuticals, cosmetics and animal feed include lycopene, β-carotene, lutein, zeaxanthin, canthaxanthin, astaxanthin and rhodoxanthin (Nelis and Leenheer, 1991). Microorganism offer economical biotechnological production of carotenoids and provide an alternative to chemical synthesis and there is a world wide growing market for microbial carotenoids and a comprehensive screening of the microbial carotenoid spectrum could help to identify novel compounds providing beneficial effects. Demand of carotenoids as safe and suitable colouring agents is on rise. Of all the carotenoids only few like β -carotene, lycopene, astaxanthin, canthaxanthin and lutein are commercially available. The polyene chain is also the feature mainly responsible for the chemically reactivity of carotenoids towards oxidizing agents and free radicals and hence has the antioxidant role. Various carotenoids of microbial origin such as astaxanthin, lycopene, β -carotene and canthaxanthin are being commercialized to some extent and finding applications in beverages, dairy foods, cereal products, meats, cosmetics, pharmaceutical, aquaculture and others. The growing demand of carotenoids has triggered the research to be more focused on the commercial production of carotenoids. Biotechnological

development of new strains that can withstand robust industrial conditions and utilization of industrial waste as substrate would help to bring down the economics of the whole process. A vast biodiversity of microorganisms has to be still explored from various habitats for exploitation as potential carotenoid factories (Kaur and Gill, 2011).

1.4 Occurrence of Aromatic compounds and their degradation

Aromatic compounds are the second most abundant class of organic compounds in nature and are ubiquitous growth substrates for microorganisms. Aromatic compounds and hydrocarbons have in common a great stability due to resonance energy and inertness of C-H and C-C bonds (Matthias et al., 2002). They form a large group of diverse compounds including lignin monomers, amino acids, quinones, flavonoids and others (Harwood et al., 1999). Microorganisms contribute to a large extent to the mineralization of the aromatic compounds. Aerobic aromatic metabolism is characterized by the extensive use of molecular oxygen. Monoxygenases and dioxygenases are essential for the hydroxylation and cleavage of aromatic ring structures (Heider and Fuchs, 1997a). Many of the aromatic nucleus-containing materials that are released into the environment by humans or by the degradation of plant material find their way into anaerobic sediments. Concerns about environmental pollution have resulted in many attempts to define conditions under which aromatic compounds degradation can occur, as this would help in the development of strategies for accelerating rates of biodegradation (Gibson and Harwood, 2002).

The capacity of some bacteria to metabolize hydrocarbons in the absence of molecular oxygen was first recognized only in early 1990s (Heider et al., 1999). Complete mineralization of aromatic molecules under anoxic conditions appears to be a quality of prokaryotes (Harwood and Gibson, 1997). Several alkylbenzenes, alkanes or alkenes are anaerobically utilized as substrate by several species of denitrifying, ferric iron-reducing, sulfate reducing bacteria and methanogens (Heider et al., 1999). Anaerobic bacterial degradation was observed in and around the oil fields also (Spormann and Widdel, 2000). Benzoate is the most common aromatic compound degraded by anaerobic bacteria (Gibson and Harwood, 2002). Anaerobic aromatic metabolism was also observed in sulfate reducing (Bellar et al., 1996), iron reducing bacteria (Gorny and Schink, 1994; Bellar et al., 1996) and in fermentative bacteria (Auburger and Winter, 1996). Anaerobic toluene, phenol and 3-chlorobenzoate degradation was reported in different

species of purple non-sulfur bacteria (Harwood, 2009). Anaerobic oxidation of aromatic amino acids via aryl pyruvate intermediates to the corresponding aryl acetates as final products is exerted by some fermentative microorganisms. Essential to anaerobic aromatic metabolism is the replacement of all the oxygen-dependent steps by an alternative set of novel reactions and the formation of different central intermediates for breaking the aromaticity and cleaving the ring, notably in aerobic pathways, the anaerobic pathways use a reductive biochemistry, so the aromatic ring is reduced rather than oxidised. Photosynthetic purple bacteria require anaerobiosis and the presence of light to grow under phototrophic conditions. Under these conditions they do not need to generate energy from oxidation or organic substrates (Heider and Fuchs, 1997b). In purple non-sulfur bacteria degradation of the aromatic compounds depends on the type of compound and the species of the bacteria and occurs aerobically under chemoheterotrophic conditions or anaerobically under photoheterotrophic conditions. Two different biochemical strategies are employed either by involving oxygenases or involving aromatic ring reduction, depending on the availability of oxygen in these bacteria (Harwood, 2009). Photosynthetic bacteria avoid many of the constraints, since all their energy is derived from light and aromatic compounds are degraded to form intermediary metabolites such as acetyl-CoA. These bacteria require organic substrates as a source of carbon and reducing equivalents, while energy is gained from light. This requirement can be satisfied by the degradation of aromatic compounds like acetyl-CoA serves as carbon source (Heider and Fuchs, 1997b; Gibson and Harwood, 2002).

In diverse pathways the aromatic hydrocarbons are degraded by anaerobic bacteria, chlorinated, phenolic and phenylalkanecarboxylates aromatic compounds form benzoyl-CoA as a common intermediate (Gibson and Harwood, 2002). The benzoate degradation involves benzoyl-CoA reductase (Mobitz and Boll, 2002), phenylacetate involves phenylacetate CoA ligase (Schneider et al., 1997), 3-cholorobenzoyl-CoA involves 3-cholorobenzoyl-CoA ligase (Egland et al., 2001), cyclohexanecarboxylate involves cyclohexanecarboxylate CoA ligase and cyclohexanecarboxylate CoA dehydrogenase (Kuver et al., 1995) and 4-Hydroxybenzoate is degraded by 4-hydroxybenzoate CoA ligase (Gibson et al., 1994).

Aromatic metabolism by anaerobic bacteria

Purple non-sulfur phototrophic bacteria and denitrifying bacteria are facultative aerobes that can oxidize many aromatic compounds both anaerobically and aerobically via two

completely different pathways, with the aerobic pathways requiring oxygen. The two outlined parts of anaerobic metabolism are the peripheral and central parts, in which peripheral pathways channel different aromatic substrates into a few central aromatic intermediates (Harwood et al., 1999). In general anaerobic metabolism the aromatic hydrocarbons are initially broken by reduction and the ring is subsequently opened by hydrolysis, different central aromatic intermediates can be reduced and the most common is benzoyl-CoA. Further catabolism of benzoyl-CoA appears to occur via reactions of a modified beta-oxidation pathway (Ziergler et al., 1989; Heider et al, 1999; Harwood et al., 1999). And the first step in the anaerobic degradation of most aromatic acids is conversion into CoA thioesters by the coenzyme A ligases. In most of the anaerobic aromatic metabolic pathways reduction of benzoyl-CoA to cyclic diene involves benzoyl-CoA reductase, after nitrogenase this is the second enzyme known which overcomes the high activation energy required for the reduction of a chemically stable bond by coupling electron transfer to the hydrolysis of ATP (Heider and Fuchs, 1997a). Two bacteria have been studied in some detail regarding anaerobic metabolism, they are *Rhodopseudomonas palustris* uses benzoate as the sole sources of carbon during anoxic photosynthetic growth (Harwood and Gibson, 1988) and *Thauera aromatic* a denitrifying bacteria also utilizes benzoate as sole carbon and energy source, benzoate degradation gene clusters were observed in these organisms (Springer et al., 1998). Comparative genomics can be expected to yield valuable insights of these metabolic pathways (Gibson and Harwood, 2002).

Many microorganisms which are unable to completely degrade aromatic compounds, but catalyse oxygen independent biotransformation reactions of the aromatics. Biotransformation reactions contribute significantly to aromate degradation in nature as parts of food webs with other microorganisms. Complex aromatic structures are reduced to a few simple products, which are preferred substrates for the organisms capable of aerobic or anaerobic aromate degradation. The reactions involved are oxidation, reduction of substituents, C-C cleavage, decarboxylations, removal of O-methyl groups and other sulfur or nitrogen containing substituents to serve as source for the respective element. Reductive anaerobic biotransformation of aromatic substrates takes place to dispose of redox equivalents generated in oxidative reactions. The known reductive biotransformation reactions are either involved in anaerobic respiratory chains or are part of fermentative pathways (Heider and Fuchs, 1997b). Reductive dehalogenation processes occurs in the anaerobic metabolism by halorespiring microorganisms (Holliger et al., 2003) and

the reductive dehalogenases are the key catalysts in the respiratory chain of halorespiring organisms (Smidt and Vos, 2004). Other reducible aromatic substituents include nitro groups and azo groups which are readily reduced to amino groups under anaerobic conditions (Field et al., 1995). Anaerobic dehalogenating microorganisms are of significant importance for the cleanup of contaminated ecosystems, including soils, sediments and groundwater (Smidt and Vos, 2004). Nitrogen containing substituent of aromatic compounds may also be utilized as nitrogen source by anaerobic bacteria (Preuss et al., 1993), as all three aromatic amino acids are readily degraded under anaerobic conditions. Many obligate anaerobic bacteria utilize them as substrates for amino acid fermentation and transform them to simple aromatic products (Heider and Fuchs, 1997b).

Anaerobic toluene catabolism has been shown to proceed via beta-oxidation of benzylsuccincate to benzoyl-CoA and succinate. Other pathways of anaerobic hydrocarbon catabolism involves a periplasmic molybdenum, iron-sulfur and heme-beta-containing enzyme ethylbenzene dehydrogenase (Matthias et al, 2002). Many microbes utilize aromatic ring substituents to their advantage while leaving the benzene ring untouched. Such modification include acyl side chain removal demethoxylation and ester hydrolysis (Gibson and Harwood, 2002). The side chains of phenylalkanoates like cinnamate are readily degraded by beta-oxidation, yielding acetyl groups for biosynthesis or for energy (Elder et al., 1992). Anthranilate supports anaerobic growth of denitrifying bacteria as sole carbon and nitrogen source. It is first thioesterified and subsequently reductively deaminated to benzoyl-CoA (Lochmeyer et al., 1992). Aniline serves as carbon and nitrogen source for both sulfate-reducing (Schnell et al., 1989) and denitrifying strains, it is first carboxylated to anthranilate and further metabolized (Kahng et al., 2000). Aromatic compounds are also utilized by some microorganisms as they can serve as electron shuttles (Lovely et al., 1999).

1.5 Occurrence of Indole

Indole and other N-heterocyclic aromatic compounds are produced by industries including cosmetics, agrochemicals, pharmaceuticals, pesticides, disinfectants, dyes and others (Novotny et al., 1981). Indole and its derivatives form a class of toxic recalcitrant environmental pollutants (Aldemir et al., 2003). Large majority of the indole and azo dyes are released into the

environment without preliminary treatments and thus represent a serious pollution problems (Beydilli et al., 1998). Indole had been reported to be growth inhibitory for microorganisms (Kamath and Vaidyanathan, 1990; Doukyu and Aono, 1997), a dynamic signaling in between multispecies communities (Lee and Lee, 2010) and acts as a general biologic oxidant (Garbe et al., 2000). Due to its toxicity, recalcitrance abundantly present in sewage (Doukyu and Aono, 1997), on the contrary indole and its structurally related compounds are present in most of the living organisms in the form of isatin, indoxyl sulfate an indole 3-propionic acid in enteric bacteria, while indole 3-acetic acid, 3-indolylacetonitrile and 3,3-diindolymethane in plants, epinephrine, serotonin and melatonin in animals performing different vital functions (Lee and Lee, 2010).

One of the key function of indole molecule in the photosynthetic bacteria is the production of amino acid tryptophan from indole, present in the active site of the carotenoid binding protein and have the significant role in the organization of the reaction centre. The hydrophilic methoxy head of spheroidenone is involved in a single carotenoid protein hydrogen bonding interaction formed between the methoxy oxygen of carotenoid and the hydrogen on the indole group nitrogen atom of Tryptophan M75 (Aleksander et al., 2004).

Bacterial metabolism of indole

Indole production was observed during the stationary phase of cell growth (Smith, 1897) by tryptophanase (Newton and Snell, 1965) in *E. coli*.. The expression of tryptophanase, TnaA and TnaB and indole production are repressed when the level of tryptophan is low. Extracellular tryptophan and other amino acids directly influence indole production (Gong and Yanofsky, 2002). The heterocyclic indole fulfills essential biochemical functions in the amino acid tryptophan, in plant growth regulators, hormones and neurotransmitters (Garbe et al., 2000). Environmental conditions and composition of media like carbon source, amino acids and growth status critically influences the level of extracellular indole (Wang et al., 2001). Temperature (Li et al., 2003) and pH (Blamkenhorn et al., 1999) are also important environmental factors that affect indole biosynthesis. Glucose repressed indole biosynthesis was observed due to catabolic repression of *tnaA* (John and Wyeth, 1919). High-affinity tryptophan permease Mtr is often cited as the main conduit for indole import and also on contrary reports of Indole transport is independent of Mtr or AcrEF under normal growth conditions as the indole rapidly crosses *E.*

coli membranes without the involvement of transport proteins (Fernandez et al., 2011). AcrEF plays a significant role in indole efflux. Indole also acts on sensor kinases (Hirakawa et al., 2005) of signaling pathways in bacteria.

Under anaerobic conditions indole could be metabolized to form methane (Wang et al., 1984). Indole degradation was also reported via oxindole and isatin by bacterial consortia under anaerobic and denitrifying conditions by the denitrifying bacteria (Gu and Berry, 1992). Most azo dyes are reduced anaerobically to the corresponding amines with cleavage of azo bonds by bacterial azoreductases. Anaerobic degradation of azo dyes results only in azo reduction and decolourization, further mineralization occurs by aerobically only (Manu and Chaudhari, 2002).

Beneficial effects of indole and its derivatives

Indole controls phenotypes of both types bacteria which can and cannot produce indole, as signal molecules coordinate the behaviour of the organisms to survive in dynamic multispecies communities (Nikaido et al., 2008). It appears indole plays an important role in bacterial physiology and ecological balance. As an intercellular signal molecule indole controls diverse aspects of bacterial physiology, such as spore formation, plasmid stability, drug resistance, biofilm formation and virulence in indole-producing bacteria (Lee and Lee, 2010). Indole 3-acetic acid (IAA) an derivative of indole is the first plant hormone identified and plays a central role in plant growth and development as a regulator of numerous biological processes, from cell division, elongation and differentiation to tropic responses, fruit development and senescence. Tryptophan aminotransferase catalyzes the conversion of tryptophan into indole pyruvic acid and it is found in many species of plants and bacteria. Indole 3-pyruvic acid is converted into indole 3-acetaldehyde by the action of indole pyruvate decarboxylase enzyme. Low levels of tryptophan can be efficiently converted into IAA by the presence of indolepyruvate decarboxylase with its high affinity for indole 3-pyruvic acid (Koga, 1995). Indole influencing multiple aspects of bacterial physiology and has proved to be an important factor in the transition to stationary phase, activating the global regulator RpoS (Lacour and Landini, 2004).

Indole might be an extracellular signal acting in the stationary phase to induce drug exporter genes and intrinsic xenobiotics exporter genes (Han et al., 2011). It has been reported

that indole regulates biofilm formation by *E. coli* associated with the virulence of *H. influenza* (Maritno et al., 2003). Indole also promotes resistance to a range of drugs and toxins through the induction of xenobiotic exporters (Lee et al., 2010) and is involved in preventing plasmid instability associated with the accumulation of plasmid multimers (Chant and Summers, 2007). Reports of intestinal epithelial cells response to gastrointestinal tract pathogens because indole beneficially affected gene expression of human epithelial cells and proposed to be involved in interkingdom signaling (Bansal et al., 2007). Production of indole 3-propionic acid in animal blood completely depended on indole producing enteric bacteria (Wikoff et al., 2009). It suggests that the intestinal microbial flora may protect itself using indole against pathogenic bacteria (Han et al., 2011). Different applications of indole molecules is used in various therapeutics (Sharma et al., 2010).

Harmful affects of indole

High concentration of indole (>2 mM) apparently decreases cell growth in *E. coli* most likely due to the blocking of cell division (Lee et al., 2009) or disruption of the bacterial envelope (Raffa and Raivio, 2002) and/or oxidant toxicity (Garbe et al., 2000). Oxidant toxicity of indole was demonstrated by the induction of alkylhydroperoxide reductase subunit C in *E. coli* (Garbe et al., 2000). The main target for the toxicity of organic compounds to bacterial and eukaryotic cells is the cell membrane (Sikkema et al., 1995). The effect of indole is by causing membrane changes is due to the generation of superoxides (Wang et al., 2001). A direct indication of membrane derangement in *E. coli* may be the indole inducible overproduction of spheroplast protein y and also causing the reduction of oxygen by semiquinones, resulting in lipid peroxidation and ultimately in the enrichment of the AhpC overproduction (Garbe et al., 2000). Indole activates the *astD* and *tnaB* genes in a concentration dependent manner, indicating its affect is on specific signaling pathway (Wang et al., 2001). Plant tissues exposed to indole showed low pigmentation, presumably due to the inhibition of anthraquinone biosynthesis (El-Shagi et al., 1984). IAA can be a signaling molecule in bacteria and have direct effect on bacterial physiology. IAA biosynthesis in diverse bacteria is related to environmental stress, including acidic pH, osmotic and matrix stress and carbon limitation (Spaepen et al., 2007).

1.6 Definition of the problem

Anoxygenic phototrophic bacteria (APB) are metabolically versatile and are able to survive in diverse environmental conditions by utilizing wide variety of organic compounds. Some of the APB are capable of utilizing aromatic compounds as sole source of carbon and/or nitrogen. On the other hand some of the APB have remarkable ability of biotransformation involving oxidation, reduction, C-C cleavage, decarboxylations and removal of methyl, sulfur and other nitrogen containing substituents for respective elements (Heider and Fuchs, 1997). One such anoxygenic photosynthetic bacterium is *Rubrivivax benzoatilyticus* JA2 grows under different growth modes such as chemo-organoheterotrophy, photo-organoheterotrophy and photolithoautotrophy. *Rubrivivax benzoatilyticus* JA2 was able to biotransform aromatic compounds like tryptophan (Ranjith et al., 2010), aniline (Mujahid et al., 2011) and phenylalanine (Prasuna et al., 2012) into novel metabolites of indole and phenols. *Rubrivivax benzoatilyticus* JA2 can also grow in the presence of aromatic compounds like benzoate, 4-hydroxybenzoate, cinnamate and phenylacetate (Ramana et al., 2006). It is producing polyhydroxyalkanoate and secondary metabolites of novel phenolic and indole derivatives when grown in presence of pyruvate and the aromatic amino acids like phenylalanine (Prasuna et al., 2012), tyrosine and tryptophan (Ranjith et al., 2010) respectively. As indole and its derivatives are known for their function as important heterocyclic signaling molecules in many bacteria, they play key role in bacterial physiology and ecological balance, such as spore formation, plasmid stability, drug resistance, biofilm formation and as a virulence enhancing factor in pathogenic bacteria (Lee and Lee, 2010).

However there are few reports of indole metabolism by anoxygenic photosynthetic bacteria in general (Rajashekar et al., 1998; Nanda devi et al., 2000) and *Rubrivivax benzoatilyticus* JA2 in particular (Sunayana et al., 2005; Ranjith et al., 2006), their metabolic pathways and the role in *Rubrivivax benzoatilyticus* JA2 is still unexplored. Gaining insights into metabolic and molecular adaptations require a functional analysis of the genome as gene expression enables survival of the cell and only about 6 % of the total bacterial genomes sequenced belong to photosynthetic bacteria so far. Comparative genomics will provide valuable insights and improvement in understanding the molecular mechanisms of the metabolic pathways employed by the organism for the adaptations and survival strategies acquired by

convergent evolution or horizontal gene transfer. Production of value added novel phenolic and indole molecules from the aromatic compounds made to look into the insights of the genome of *Rubrivivax benzoatilyticus* JA2 with the following objectives:

1.7 Objectives

1. Genome sequencing and metabolic pathway insights of *Rubrivivax benzoatilyticus* JA2
2. Affect of exogenous indole on the metabolic processes of *Rubrivivax benzoatilyticus* JA2

Materials
&
Methods

2. Materials and Methods

2.1 Glassware and chemicals

2.1.1 Glassware and cleaning: All the glassware used for the experiment which includes all the test tubes, pipettes, measuring cylinders, culturing flasks, reagent bottles, Petri dishes, screw cap test tubes were of Borosil or Duran brand. The glassware used in the experiments were soaked in the diluted chromic acid solution, made of potassium dichromate and sulphuric acid, for 24 h prior to the usage and cleaned with tap water and teepol detergent. After washing with detergent the glassware were rinsed with deionized water and kept for drying in the oven.

2.1.2 Deionized water: Deionized water was obtained from deionizer plant of the school (equipped with reverse osmosis plant attached to filtration assembly), routinely used for rinsing the glassware after washing and for media preparation. Distilled and double distilled water were obtained from the distillation plant, Millipore make, was used to make stock solutions and chemical reagents. Milli-Q water was used for HPLC analysis and protein assay preparations for *invitro* experiments.

2.1.3 Chemicals All the chemicals used in this study were of analytical grade obtained from Sigma-Aldrich, Qualigens, Merck solvents for HPLC and Himedia.

2.1.4 P^H determination P^H of the culture media and the chemical stocks were determined by using a digital P^H meter (Digisun make, model DI-707).

2.1.5 Sterilization Sterilization of the culture media, glassware and other heat stable stock solutions were done by autoclaving at 15 lbs for 15 min. Remaining heat labile stocks or standards are filtered through 0.22 μm membrane filters.

2.2 Organism and growth conditions

2.2.1 Organism growth conditions, stock culture and purity check: *Rubrivivax benzoatilyticus* JA2 was grown photoheterotrophically in light with 2400 lux at $30^oC \pm 2$ for 48 to 96 h maintaining micro-anaerobic conditions in screw cap test tubes (8ml, 10x100mm) or stop cock bottles (60ml, 250ml, 500ml, 1ltr & 2ltr). The culture was grown on Biebl and Pfennig's mineral medium with malate as carbon source and ammonium chloride or indole as nitrogen source and other macro nutrients include ($g.l^{-1}$) KH_2PO_4 -0.5, $MgSO_4.7\ H_2O$ -0.2, NaCl -0.4,NH_4Cl -1.2,$CaCl_2.2\ H_2O$ -0.05, 3.0, Yeast extract -0.12,

Ferric citrate (0.1 % w/v)- 5 ml and trace elements SL_7-1.0 ml ($mg.ml^{-1}$): HCl (25 % v/v)-1ml; $ZnCl_2$-7; $MnCl_2.4H_2O$-100; H_3BO_3-60; $CoCl_2.6H2O$-200; $CuCl_2.H_2O$-20; $NiCl_2.6H_20$-20; $NaMoO_4.6H_2O$-40. Stock cultures of *Rubrivivax benzoatilyticus* JA2 were maintained as agar stabs grown heterotrophically in ¾ volume filled screw cap test tubes. The cultures were stabbed with a sterile needle into the agar and incubated in presence of light (2,400 lux) at 30 $\pm$ 2°C. After 2-3 days of growth the stab cultures were preserved under refrigeration at 4°C up to a period of 2 months. For long time preservation sterilized 30 % glycerol stock inoculated with culture were maintained up to a period of 6 months. Purity of the culture was regularly checked before and after assay by streaking on nutrient agar plates ($g.l^{-1}$) peptone- 10, yeast extract- 3, NaCl- 5 and agar 20 and incubated aerobically under light at 30 $\pm$ 2°C.

2.2.2 Growth and Biomass Growth of bacterial culture was recorded in terms of optical density at 660nm colorimetrically against the uninnoculated media blank. Biomass yield was estimated by standard method (standardized in the lab i.e., 0.1 O.D ~ 0.15 mg dry weight ml^{-1}).

2.2.3 Bulk cultivation of *Rubrivivax benzoatilyticus* JA2 was carried out in 250 to 2000 ml screw cap reagent bottles containing the photoheterotrophic media with 20 % log phase inoculums culture was used and incubated under light (2,400 lux), anaerobic conditions at 30 $\pm$ 2°C. Culture was harvest after 96 h of growth by centrifugation at 8 k g for 10 min and the supernatant was used for extraction of metabolites and the cell pellet was used for isolation of the protein.

2.2.4 Preparation of cell free extracts and resting cells: Late log phase culture, about 48 hour grown culture was centrifuged (10000 g for 10 min), washed with buffer (0.02 M K-phosphate, P^H-7) and the pellet was sonicated (6-8 cycles, probe MS 72 in Bandelin sonoplus sonicator), protease inhibitor 0.2 M EDTA was added. The cell lysate was centrifuged (16000 g for 10 min) and the supernatant was used as enzyme source for cell free extract and debris for further studies. Late log phase culture, about 48 hour malate grown culture (control culture) was centrifuged, the pellet was washed with buffer (phosphate buffer P^H-7) once or twice and the final pellet was used for the assay. Equal

biomass cells are taken in screw cap test tubes with different aromatic compounds as supplement and incubated for 48 to 96 h and assayed for utilization by HPLC.

2.3 Extraction methods

2.3.1 Genomic DNA

Genomic DNA was isolated by using kit method from qiagen QIAamp DNA mini kit and followed the instructions given in the manual. The late log phase culture after pelleting the bacterial cells by centrifugation at 8000 g, the pellet was suspended in appropriate enzyme solution (20 $mg.ml^{-1}$ lysozyme or 200 $\mu g.ml^{-1}$ lysostaphin; 20 mM Tris-Hcl, P^H 8.0; 2 mM EDTA; 1.2 % Triton) and incubated for at least 30 min at 37^oC, after which proteinase K and 200 µl of buffer was added and mixed well by vortexing and centrifuged for few seconds. This mixture was loaded on to the column QIAamp spin column and centrifuged at 6000 g for 1 min placed in a 2 ml collection tube, care was taken to completely remove the solution. Further to the column AW 1 buffer was added and centrifuged at same speed as mentioned early along with a collection tube and followed by AW 2 buffer and centrifuged at 20,000 g for 3 min, the filtrate was discarded. Then the column was incubated with buffer AE at room temperature for 1-3 min and centrifuged at 6000 g for 1 min and the sample was stored at -20^oC and sent for analysis in a dry ice packing. The whole genome was sequenced by shotgun sequencing method on a Roche 454 sequencer by the Xcelris lab limited.

2.3.2 Proteins

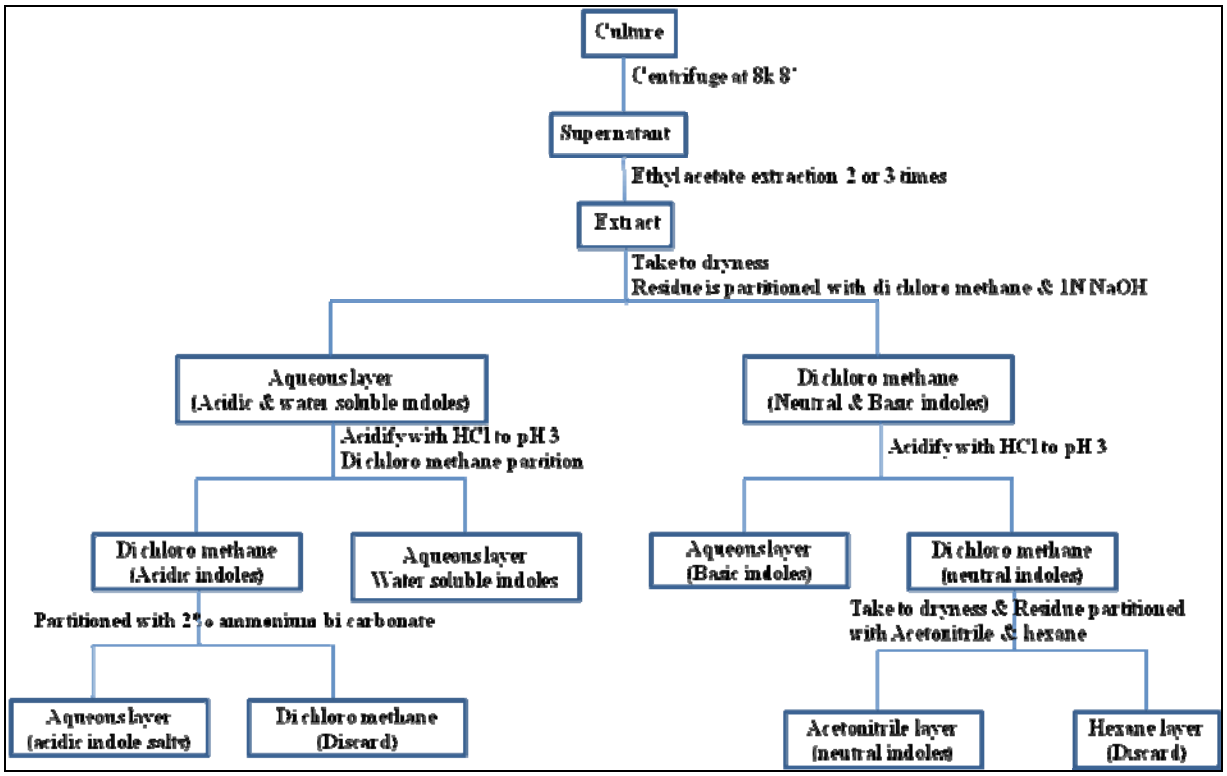
Proteins were extracted by sonicating the cell pellet after centrifugation and membrane proteins with the help of triton X 100 detergent for solubilizing the membrane fragments. For iTRAQ analysis of protein separate method was employed as given in the further methods.

2.3.2.1 Extraction of carotenoid proteins: Carotenoid proteins were isolated from centrifuged culture and dissolved in phosphate buffer (P^H-7.5) and sonicated at 4 ^oC. The cell lysate was incubated with 1 % Triton-X 100 for 400 $\mu g\ ml^{-1}$ of protein for 1-2 h at 4 ^oC, then the sample was centrifuged (at 15000 g for 12 min at 4 ^oC) and the supernatant fraction was taken for the coloured protein studies.

2.3.3 Pigments extraction: Pigments were extracted from the bacterial cell pellet by using solvent mixture of acetone: methanol in the ratio 7:2 (Cohen-Bazire et al., 1957).

2.3.4 Indoles

2.3.4.1 Fractionation of supernatant for indole derivatives:



Flow chart. 2 Extraction of indoles and other small hydrophobic molecules separation for HPLC and LC-MS analysis (Powell, 1964).

The supernatant was extracted thrice with ethyl acetate and taken to dryness by rota-vapourator. Dried concentrated residue was fractionated by the standard method. (POWELL-preparation of indole extracts). The extracted indoles were analyzed by TLC and HPLC methods as described in the flow chart. 2.

2.4 Analytical Techniques

2.4.1 Spectrophotometry

2.4.1.1 Pigment analysis The pigment analysis of the extracts were done from wavelength 300 to 900 nm on Shimadzu spectrophotometer with that of the respective blank solution. The quantification was one by following Cohen-Bazire *et al*., (1957) standard method.

2.4.1.2 Indoles quantification: the extracted indoles were estimated by following the methods

2.4.1.2.1 Salkowski reagent (Salper's method): To 1ml of sample 1 ml ethyl acetate and 2 ml salper's reagent (1ml of $Fecl_3$ was added to 50 ml of 35 % (v/v) percholric acid) and after incubation absorbance was read at 535 nm in Biochrom spectrophotometer (Libra S11/S12) (Gorden & Paleg, 1957).

2.4.1.2.2 Ehrlich's reagent: To 1 ml sample 1 ml of PDAB reagent (60 mg of p-dimethyl amino benzaldehyde was added per 1 ml of 3 N H_2SO_4) and the absorbance was read at 550 nm.

2.4.1.3 Proteins

Protein estimation was done by standard Bradford and Lowry methods. When Triton was used Lowry method was done only after precipitating the sample with ice cold acetone in equal volumes (1:1), as Triton-X 100 interferes in the assays (due to presence of aromatic ring in the Triton-X 100).

2.4.1.3.1 Bradford method: In Bradford method 100 mg Coomassie Brillant Blue G-250 dissolved in 100 ml of absolute / distilled ethanol or methanol was added and mixed well to this 88 % of O-phosphoric acid was added and mixed thoroughly and volume made up to 500 ml with distilled water. Finally filtered through whatman No. 1 filter paper and added to the sample in the ratio 1:1 to read absorbance at 550 nm against reagent blank. For standard graph known concentrations of BSA were taken and plotted in the graph.

2.4.1.3.2 Lowry method: Lowry *et al.,* (1951). To the 100 µl of sample 900 µl of water and 5 ml of alkaline solution (2 % Na_2CO_3 and 0.5 % copper sulphate in 50:1 ratio) was added and incubated for 10 min and 0.5 ml of Folin-Ciocalteau reagent (diluted with distilled water in 1:1 ratio before use) was added and thoroughly mixed before allowing them to stand for 30 min at room temperature. After incubation blue color developed was measured at 750 nm. A solution containing 5 ml of alkaline solution and 0.5 ml of Folin-Ciocalteau reagent served as blank with known concentrations of bovine serum albumin.

2.4.2 Infrared spectroscopy: Extracted carotenoid pigments by methanol: acetone in ratio 9:1 were dried and mixed with KBr powder to make pellets for analyzing by Perkin-Elmer infrared spectrophotometer against KBr pellet as blank**.**

2.4.3 Thin layer chromatography

2.4.3.1.1 TLC of the extracted pigments were done on ready made silica gel aluminum plates from Merck with the solvents petroleum ether: Acetone in the ratio 7:2 in a saturated chamber.

2.4.3.1.2 TLC of the indoles was carried on ready made silica gel coated aluminum plates from Merck with solvents Chloroform: Methanol in the ration 9:1 in a saturated chamber.

2.4.4 Ion-exchange chromatography: DEAE-cellulose matrix was used for cation exchange chromatography. Proteins are fixed by repeated loading on the column and then washed with plain tris buffer of P^H 7.8 till no further protein elution. Final elution was done with increasing concentration of NaCl starting from 0.05 M to 0.4 M in tris buffer.

2.4.5 High pressure liquid chromatography

2.4.5.1.1 HPLC analysis of the extracted pigments was done on Shimadzu SPD-M20A isocratic system with methanol, acetonitrile and ethyl acetate in a ratio of 5:4:1 as solvent at 1ml/min for 20 min, Luna 5µ C18 100 ^{o}A column (250 x 4.6 mm) and the compounds were detected in PDA detector at wavelength ranging from 200 to 700 nm.

2.4.5.1.2 Indoles identification and quantification was done on Shimadzu SPD-M20A system using a gradient method consisting of solvent A-0.6 % acetic acid and solvent B-100 % acetonitrile at a flow rate of 1.5 ml mi^{n-1}, with a gradient increase of B solvent from 0 to 100 % in 30 min with the same column described above in aromatic compound detection. PDA detection was done from 200 to 700 nm wavelength range.

2.4.5.1.3 Aromatic compounds utilization was estimated by HPLC at the absorbance maxima wavelengths of respective standards. Initial (immediately after inoculation) and final (after incubation with culture for 48 h) samples of the culture media are drawn, centrifuged to remove the pellet and the supernatant was analyzed by HPLC, the difference in the concentrations were measured in absorbing units with that of the known concentration of authentic standards. HPLC analysis of the samples was performed at room temperature using Shimadzu SPD-M20A isocratic system with methanol and water (1:1) mixture as solvent at 1ml/min flow rate, Luna 5µ C18 100 ^{o}A column (250 x 4.6 mm) and the compounds were detected in UV detector at respective wavelengths. Reduction (i.e., increase in peak height) was observed for some compounds.

2.4.6 Liquid chromatography-mass spectroscopy

Fractionated indoles were analyzed on shimadzu high pressure liquid chromatography attached to a agillant mass spectroscopy by using the modified HPLC program with solvent-A 1 % acetic acid and solvent-B 100 % acetonitrile. Gradual increase of solvent-B from 0 to 100 % in 60 min at a flow rate of 0.75 ml min^{-1}. Time-of-flight MS were acquired from *m/z* 50 to 800 on an electron spray ionizing probe. Their *m/z* values were obtained in both positive and negative modes.

2.4.7 Native Polyacrylamide gel electrophoresis (PAGE) : Quantified protein in equal quantities were loaded in the native polyacrylamide gel and electrophoresed at 60 V for four hours. PAGE was done with different concentrations of the acrylamide (5 %, 8 % and 10 %) for obtaining distinct separate protein bands, predominantly 6 % was used as to avoid smear of the protein.

2.4.7.1 Coomassie Staining of PAGE gel: Standard coomassie staining method was followed for staining the protein gels. (Pepper, 1992).

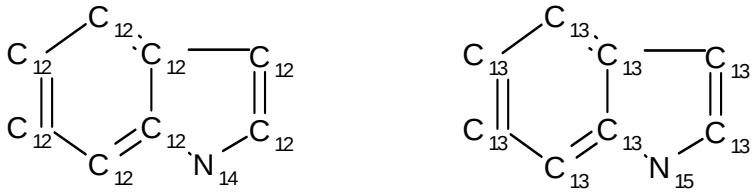
2.4.8 Indole analogues studies: Indole acetic acid is produced via two different pathways, they are tryptophan dependent and independent pathways. 5-fluoroindole is known inhibitor for the enzyme tryptophan synthase beta subunit, key enzyme in tryptophan dependent pathway by which tryptophan is produced from indole. As 5-fluoroindole metabolism leads to the production of toxic compounds like 5-fluoro-tryptophan, which inhibits the growth of the organism. Different concentrations were tested varying from 0.4, 0.2, 0.1, 0.05 and 0.025 mM. Other indole analogues like 2-methyltryptophan and indolecarboxylic acid were used.

2.4.9 Isotope tagged random and absolute quantification of proteins: Control and induced culture samples were pellet down, the culture pellet were lysed by sonication and the unlysed cells were removed by centrifuging at low speed. The lysed pellet samples were further solubilised in tris HCl P^H 7.5 with SDS 0.1 % (w/v) M and 0.1 % Triton-X 100. Finally the proteome was acetone precipitated and lyophilized after complete removal of acetone. 150 µg of lyophilized sample was dissolved in TNE [50 mM Tris (P^H 8.0), 100 mM NaCl, 1 mM EDTA]. Final concentration of samples were made to 0.1% by adding RapiGest SF reagent (Waters). Then samples were heated to 95 ^oC. Proteins were then reduced with 1mM TCEP (Pierce

chemical) and carboxymethylated with 0.5 mg/ml of iodoacetamide final concentration for 30 minutes at 37 ^{o}C. The iodoacetamide was then neutralized with addition of 1mM TCEP. Proteins were trypsin digested at a concentration of 1:100 (trypsin: protein) for overnight at 37 ^{o}C. The samples were treated with 50 mM HCl at 37 ^{o}C for 1 hour followed by centrifuging at max speed for 30 min at 4 ^{o}C for degrading sample and removal of RapiGest. The soluble fraction was then transferred to a new tube with solution of P^{H} 3 using NH_4OH. The sample was desalted using desalting tips (Thermo Scientific, Aspire RP 30). Peptides were re-quantified using BCA assay. 100 µg of each sample was labelled with a unique iTRAQ tag (with 114, 115, 116, 117) as prescribed by the manufacturer's protocol (ABSCIEX). After combining all the four samples they are dried in speed vacuum for removal of ethanol. Finally the samples were re-suspended in 100 µl of Buffer A (98 % H_2O, 2% ACN, 0.2 % formic acid and 0.005 % TFA) and 5 µl was injected for the Mudpit analysis.

Peptides were separated using nano-flow HPLC coupled with tandem mass spectroscopy (LC-MS/MS, QSTAR-Elite hybrid mass spectrometer [AB/MDS Sciex]) using nano spray ionization source. The column used was a 10 cm-180 micron ID glass capillary packed with 5 µm C18 Zorbax beads (agilent technologies). For SCX BioX-SCX (5 µm particle size, 500 µm inner diameter x15 mm) trap column was used. Sample was loaded onto the SCX column using 7.5 µl min^{-1} flow rate for 10 minutes. The SCX salt steps were 5 %, 7.5 %, 10 %, 15 %, 20 %, 25 %, 30 %, 40 %, 50 %, 75 % and 100 % buffer C (5 % CAN, 0.2 % formic acid and 0.5 M ammonium acetate) 7.5 µl min^{-1} flow rate for 10 min followed by reverse phase separation. For reverse phase the peptides were eluted from C18 column into the mass spectrometer using a linear gradient of 5-80 % buffer B (100 %, 0.2 % formic acid and 0.005 % TFA) over 60 min at 400 µl min^{-1} flow rate. LC-MS/MS data were acquired in a data-dependent fashion by selecting the 6 most intense peaks with charge state of plus 2 to 4 that exceeds 35 counts, with exclusion of former target ions set to "60 seconds" and the mass tolerance for exclusion set to 100 ppm. Time of flight MS were acquired at *m/z* 400 to 2000 Da for 0.75 s with 12 time bins to sum. MS/MS data were acquired for *m/z* 50 to 2000 Da by using "enhance all" and 24 time bins to sum, dynamic background subtract, automatic collision energy and automatic MS/MS accumulation with the fragment intensity multiplier set to 6 and maximum accumulation set to 2 s before rerunning to the survey using Protein Pilot 2.0 (Life Technologies Inc, Carlsbad, CA).

2.4.10 Stable isotope probing studies:



N-117 L – 126 (+9)

Fig. 1 N- normal (C^{14}) and L- labelled indole.

Indole has an aromatic benzene ring forming a pyrrole ring on the side containing 8 carbon (C^{12}) molecules and a nitrogen molecule summing up to a mass of 117, the stable isotope of indole was having 8 C^{13} and a N^{15}nitrogen molecule having a m/z value of 126 (117+8+1).

In the study stable isotope C^{13} and N^{15} labelled indole was used. Having eight C^{13} and one N^{15} of value 126 *m/z* (+9) with that of normal indole of value 117 *m/z* (from cambridge isotope laboratories, USA) (figure. 1). 60ml culture with stable and normal indole were incubated for 96h and harvested by centrifugation at 8,000 rpm for 10 min. The supernatant was fractionated and extracted by standard method (1), extraction was done with equal volumes of ethyl acetate (thrice) and taken to dryness in rotor evaporator under vacuum. To the dried sample dichloro methane and 1 M sodium chloride were added according to the procedure and two fractions of indole derivatives were obtained, acidic-water soluble and neutral-basic indole derivatives.

2.4.11 Matrix Assisted Laser Desorption/Ionization (MALDI) analysis: The native polyacrylamide gel electrophoresis coloured band was excised from the gel and thoroughly washed with HPLC grade water and the sample was analyzed by MALDI. The sample was digested with trypsin and loaded in the agillant fast beam III MALDI instrument. The amino acid sequences obtained was analyzed by using PUBMED protein BLAST analysis for identification of the protein.

Results

3. Results:

3.1. General overview of *Rubrivivax benzoatilyticus* JA2 genome

Pyrosequencing of *Rubrivivax benzoatilyticus* JA2 genome sequence has revealed 98 % of its genome length, comprising 4,130,131 base pairs (4.13 Mb) with an average GC content of 72.77 % and consists of 3,762 open reading frames (ORFs). Out of the total ORFs 3,210 (85.32 %) are functionally annotated, 382 (10.15 %) hypothetical and 170 (4.51 %) are unknown. Recently *Rubrivivax gelatinosus* IL144 has been completely genome sequenced from which R*ubrivivax benzoatilyticus* JA2 was separated as genomo species having a 16S rRNA sequence similarity of 99 % and based on the DNA-DNA hybridization similarity of 54.8 % which is similar with that 47.2 % of the insilico analysis done by the genome to genome distance calculator based on the regression DDH estimates.

Rubrivivax benzoatilyticus JA2 has an average gene length of 961 bases, with as least as 75 bases to as much as 9003 bases per gene. The protein coding bases were 3,566,505 which is 86.3 % of the total bases. It consists of 46 tRNAs, an rRNA operon and 17 insertion elements (Table. 1). With most of the protein coding sequences as much as 54.4 % were involved in the metabolic, biosynthetic and other cellular processes, 12.7 % are involved in the transport, 5.4 % involved in signal transduction, 4.8 % involved in transcription, 2.8 % involved in translation and 1.4 % involved in replication and repair. The remaining 18.8 % were related to hypothetical 10.2 %, prediction general function are 5.5 % and remaining 2.8 % are having unknown function (Details were presented in table. 2).

3.1.1. Genomic insights of *Rubrivivax benzoatilyticus* JA2 for phototrophic growth

Photosynthesis is one of the key processes including carbon fixation by Calvin cycle and by different dehydrogenases along with terpenoid biosynthesis, carotenoid biosynthesis and assembly of light harvesting antenna and other structural polypeptides biosynthesis for the photosystem assembly. The products of the photosynthesis are converted into energy currency by respiration which include the pathways like glycolysis, Krebs cycle and Entner-Doudoroff pathway.

Rubrivivax benzoatilyticus JA2 shows phototrophic growth and it has the complete set of homologous gene sequences responsible for Calvin cycle, for bacteriochlorophyll biosynthesis, 2 sets of light-harvesting complexes, a set of *puf* L, M and C probably present overlapping in an operon as observed in the draft circular genomic map and also various substrate dehydrogenases and formate dehydrogenases responsible for fixing the inorganic carbon. In the calvin cycle 4 ribulose bisphosphate carboxylase (RubisCO) gene sequences were observed in which 2 were similar with *Rubrivivax gelatinosus* IL144 in 94 to 97 % and the other 2 are having matching sequences of 87 % with *Rhodobacter sphaeroides* ATCC 17025 and 76 % with *Rhodobacter capsulatus* SB 1003. One ribulose activation protein was observed. 2 sets of light harvesting complex gene sequences were observed with 2 alpha and 2 beta genes which is having a similar sequence with *Roseateles depolymerans* DSM11814 67 % and *Rhodobacter sphaeroides* ATCC 17025 about 48 %. The *puf* L, M, C and H are having similarity of 99, 96, 94 and 93 % with the genes of *Rubrivivax gelatinosus* IL144 species respectively are present in near vicinity of each other. The genome consists of the cytochrome oxidoreductase complex gene sequences and also encodes for various substrate dehydrogenases and 4 formate dehydrogenases which can have potential function to supply reductant substrate for carbon dioxide fixation during anaerobic phototrophic growth or for both energy production and carbon dioxide fixation under aerobic chemoautotrophic growth conditions.

The terpenoid biosynthesis pathway present in *Rubrivivax benzoatilyticus* JA2 is the non-mevalnate or methyl erythritol phosphate pathway consisting of homologous genes for the *dxs, dxr, cms, cmk, mcs, ispG* and *ispH* having a similarity with the *Rubrivivax gelatinosus* IL144 of 98, 95, 94, 96, 94, 97 and 94 % (Fig. 3 and Table. 3). In the carotenoid biosynthetic pathway, the homologous gene sequences for *crtA*, *B*, *C*, *D*, *E*, *F*, *I* and *P* are found in the genome which are involved in the synthesis of two types of carotenoids, spheroidene and spirilloxanthin type of carotenoids. *crtA* is similar with *Rubrivivax gelatinosus* IL144 of about 91 %, two forms of *crtB/I/P* are present having similarity of 94 and 90 %, a putative *crtC* is present showing similarity with *Rubrivivax gelatinosus* IL144 of 98 %, *crtE* having a similarity of 94 %, two *crtF* are present and *crtI* gene is the key enzyme involved in the production of spirilloxanthin series of carotenoids having similarity of 97 % with *Rubrivivax gelatinosus* IL144 (Fig. 4 and Table. 4).

Complete set of homologous gene sequences involved in the processes of glycolysis, Krebs cycle, pentose phosphate pathway and also the alternate pathway for the catabolism of glucose to pyruvate by Entner-Doudoroff are present in the genome. In these metabolic pathways the gene sequences were showing more than 90 % similarity with the *Rubrivivax gelatinosus* IL144.

3.1.2 Genomic insights of aromatic biosynthesis in *Rubrivivax benzoatilyticus* JA2

In the aromatic biosynthesis the key processes of aromatic amino acid biosynthesis and the secondary metabolites biosynthesis are involved. In these processes the homologous gene sequences starting from the synthesis of DHAP in the shikimate biosynthesis to the production of the aromatic amino acids, some of the gene sequences for the indole acetic acid biosynthesis and the synthesis of aminobenzoate are present. Two DHAP synthases homologous gene sequences are present having a similarity with *Rubrivivax gelatinosus* IL144 of 98 and 96 %, a shikimate dehydrogenase gene is present having similarity with *Rubrivivax gelatinosus* IL144 of 92 %, *aroA*, *B*, *C*, *D*, *H* and *K* genes are present having similarity with *Rubrivivax gelatinosus* IL144 of 97, 95, 97, 94, 98 and 92 % respectively and are sparsely distributed in the genome as observed in the draft circular genome map. Aromatic amino acid synthesis gene sequences of *trpC, D, E* and *F* are present having a similarity of 96, 98, 99 and 98 % with of *Rubrivivax gelatinosus* IL144 respectively. Two gene sequences of *trpF* are present in the genome. The key genes involved in indole and tryptophan biosynthesis are the tryptophan synthase subunits, the genome is found to have an alpha and two beta subunits having a similarity of 96 and 98 % with that of the *Rubrivivax gelatinosus* IL144. *TrpA, B* and *F* are present within a range of thousand base pairs as observed in the genome map and *trp* operon type of gene arrangement was not observed. Nitrilase gene sequence is 90 % similar with that of the *Rubrivivax gelatinosus* IL144 and indole pyruvic acid decarboxylase are the two gene sequences involved in the indole 3-acetic acid biosynthesis *via* tryptophan dependent pathway further discussed in detail in next chapters. Auxin efflux carrier proteins useful for the transport of indole acetic acid like auxins across the cell are present.

A *tyrA* and two *tyrB* gene sequences useful in the biosynthesis of tyrosine amino acid are present in the genome having a similarity of 98 and 97 % with *Rubrivivax gelatinosus* IL144

respectively. Phrephanate dehydratase gene sequence involved in the phenylalanine was not found in the draft genome sequence of strain JA2. Genes responsible for the biosynthesis of 4-aminobenzoate from chorismate *pabB* and *C* coding for amiondeoxychorismate synthase and lyase respectively are found (Fig. 5 and Table. 5).

3.1.3 Genomic insights of nitrogen metabolism and other processes in *Rubrivivax benzoatilyticus* JA2

Other metabolic processes like nitrogen fixation, polyhydroxyalkanoate biosynthesis and also other biodegradation processes genomic details are looked in. In the nitrogen fixation two types of nitrogenase dimers are present with all the related cofactors and accessory genes for the nitrogenases. The nitrogenases present are molybdenum-dependent nitrogenase, found in all the nitrogen fixing bacteria and an iron dependent nitrogenase. In the polyhydroxyalkanoate biosynthesis polyhydroxyalkanoate synthase, depolymerase and a regulator are present having a similarity of 98 and 92 % with that of the *Rubrivivax gelatinosus* IL144 respectively. The genome also include the presence of glutamine synthetase and glutamine: oxo-glutarate aminotransferase helps in the incorporation of ammonia with the help of ammonium transporters.

Rubrivivax benzoatilyticus JA2 degrades nitrogen containing compounds like amino acids and heterocyclic aromatic compounds and it may also dehalogenate the compounds with the help of the different monooxygenases, dioxygenases, peroxidases and dehalogenase.

3.1.4 Genomic insights of signaling, regulation and transport in *Rubrivivax benzoatilyticus* JA2

Transport, regulation and signaling processes are about 18 % of the genome. These include the gene sequences for the synthesis of antimicrobial extrusion proteins, starvation and stress response proteins, efflux carrier proteins, antibiotic, solvent and camphor resistance proteins and signaling proteins include histidine kinases, response regulators, chemotaxis response proteins of A, B, W, Y and entericidin, nitrogen regulatory and serine/threonine protein kinases, etc. About 5 % of the genome is allocated for the regulation and signal transduction. 23 signal transduction histidine kinases indicates their involvement in regulating different signaling

processes. Genome consists of 10 RNA polymerase sigma factors and consists of 47 bacterial chemotaxis sensory transducer genes and have 8 GGDEF domain, 30 PAS domain, 5 GAF domain containing signal transduction gene sequences.

15 % of the genome consists of genes involved in transport which includes ATP-binding cassettes (ABC transporters) 291, 10 p-type, 3 type-II and type-III secretary systems. In this P-type ATPases likely to confer heavy metal resistance, type-II secretion systems are used for the biogenesis of pili and other general protein secretions, and type-III system for flagella biosynthesis. Other secondary transport systems include 7 major facilitator superfamily (MFS), 12 resistance-nodulation-cell division pumps (RND), 9 tripartate ATP-dependent periplasmic (TRAP) and 3 organic solvent resistance transporters. 11 multi drug efflux pumps (RND) gives the drug resistance to the organism against different drugs. The heavy metal efflux transporters and organic solvent resistance transporters present may allow the organism to survive in high concentrations of metals and other solvents. 9 *ilv* family proteins are present in this system which help in the transport of amino acids isoleucine, leucine and valine. 24 TonB systems are indicative of importance of iron acquisition by the organism.

25 response regulator receiver gene sequences and 13 different transcriptional regulatory proteins are present in the genome are having vital role in the two-component signal transduction systems making the organism to sense, respond and to adapt to the changes in the surroundings and also to stress and growth conditions. These are helpful in various cellular activities like adaption to different stimuli response which include nutrient, cellular redox, osmolarity, quorum sensing, antibiotics and other signals during the drug resistance, motility, phosphate uptake, osmoregulation and nitrogen fixation. The genome consists of 15 mono and dioxygenases, 13 amidases, 8 glutathoine S-transferase and a carboxyesterase which may be useful in different degradation processes including β-aryl ether bonds.

Some of the unique homologous sequences are bacteriophytochrome like protein, rhamnosidase proteins and multi drug resistance proteins are present. Other gene sequences for silent information regulator useful in gene silencing, phenazine biosynthesis protein, PhoH like protein useful in phosphate starvation, heat-inducible transcription repressor for transcription regulation, rubredoxin, benzoate transporter, phage shock proteins and stress response barrel.

Total bases	4,130,131 bp (4.13 Mb)
Average gene length	961 bases/gene
Protein coding features	3,618
Protein coding bases	3,566,505 (86.3 %)
tRNA	46
rRNA operons	1
GC percentage	72.77
IS elements	17

Table.1 Genomic details incurred from sequencing

The sequencing of the *Rubrivivax benzoatilyticus* JA2 genome has revealed the details inferred in the table. The sequencing was carried out on a pyrosequencing platform on a Roche 454 GS instrument (FLX titanium) has established the genome sequence of about 98 % and 2 % of gaps are left. By the BLAST tool from (NCBI) the protein coding features and with tRNA scan -SE, RNAmerr number of tRNA and rRNA were established

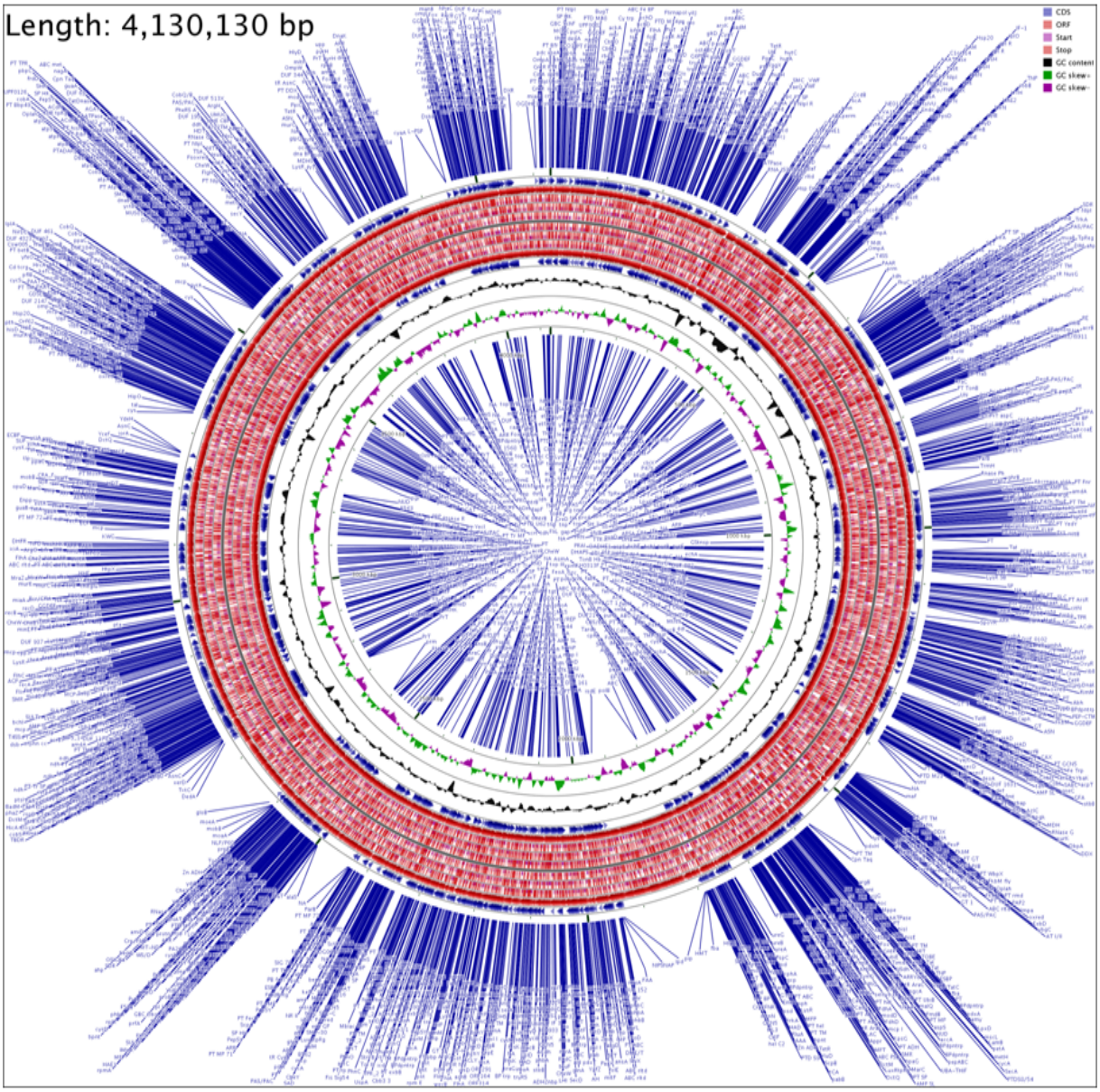


Figure.2 Circular genomic mapping of *Rubrivivax benzoatilyticus* JA2

The draft genome sequence of the *Rubrivivax benzoatilyticus* JA2 containing 3,762 gene coding sequences were mapped in circular form using GCView Server V 1.0 (2007), Jason Grant and Paul Stothard.

(http://stothard.afns.ualberta.ca /cgview_server/index.html)

Gene categories	Number of genes	Percentage of genome
Metabolic and cellular processes	1,970	54.4
Transport	458	12.7
Signal transduction	195	5.4
Transcription	175	4.8
Translation	100	2.8
Replication and repair	52	1.4
General function (predicted only)	200	5.5
Unknown function	100	2.8
Hypothetical	368	10.2

Table.2 Distribution of genes based on their functional characterization

After the genome sequencing as described in the Flow chart.1, the genome was functionally annotated by using BLAST tool from NCBI. Based on their function they are categorized in the table

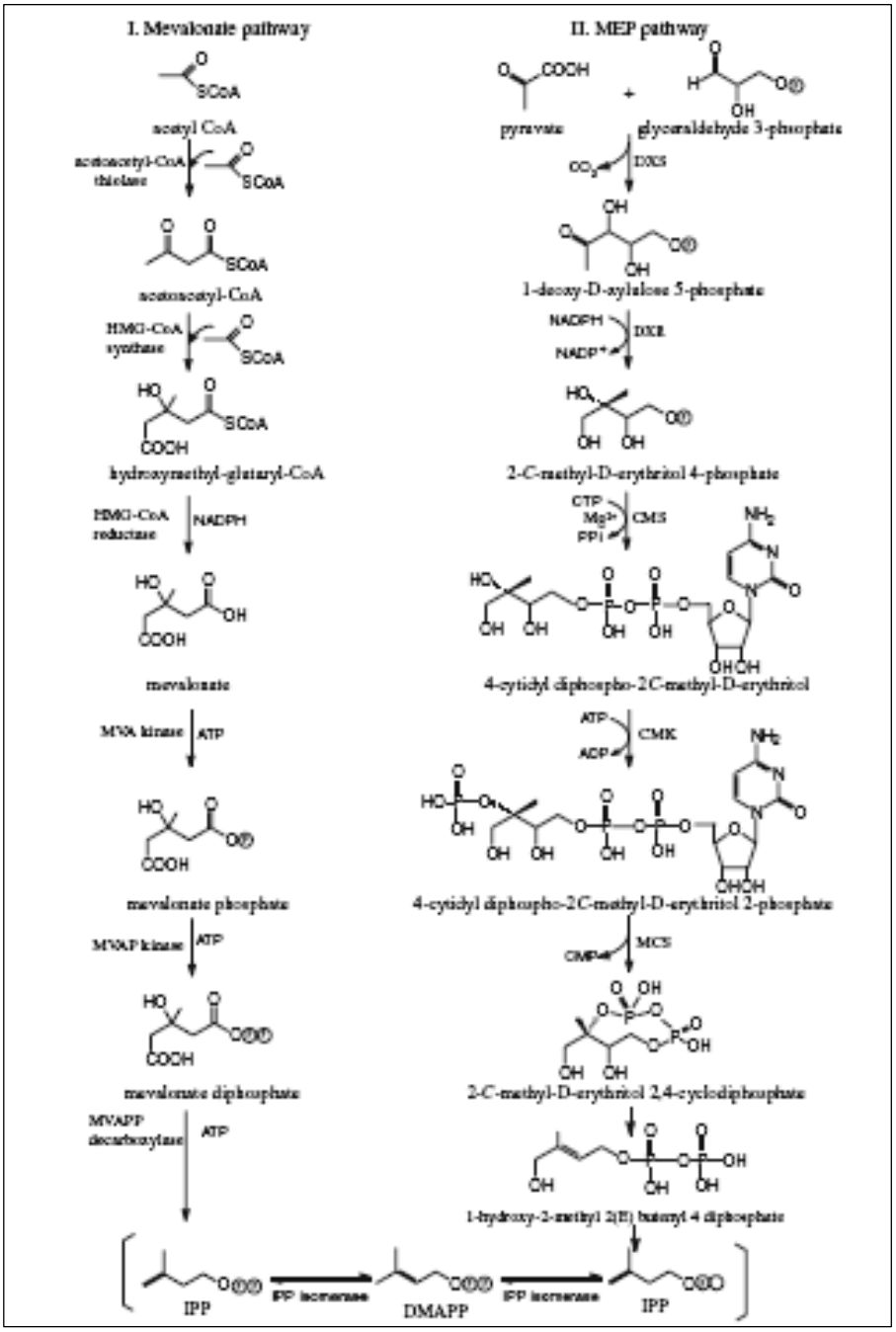


Figure.3 General terpenoid biosynthesis pathways (Sayed & Verpoorte, 2007)

Terpenoid biosynthesis occurs in the organisms by two known pathways till date as represented in the figure, which are I. Mevalnate pathway and II. Methyl erythritol phosphate pathway or non-mevalnate pathway. Methyl erythritol phosphate pathway enzymes are present in the *Rubrivivax benzoatilyticus* JA2

Gene (enzyme)	**Gene code**
Deoxyxylulose-5-phosphate synthase	*DXS*
Deoxyxylulose-5-phosphate reducto isomerase	*DXR*
4-Cytidyl diphospho-2 *C*-methyl-$_D$-erythritol synthase	*CMS*
4-Cytidyldiphospho-2-*C*-methyl-$_D$-erythritol kinase	*CMK*
2-C-Methyl-$_D$-erythritol 2,4-cyclodiphosphate synthase	*MCS*
4hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	*ispG*
hydroxymethyl butenyl pyrophosphate reductase	*ispH*

Table.3 Complete list of enzymes involved in the terpenoid biosynthesis and their codes

List of homologous gene sequences (with codes, as denoted in the circular genome) found in the *Rubrivivax benzoatilyticus* JA2 for the terpenoid biosynthesis *via* methyl erythritol phosphate pathway inferred from the genome sequence

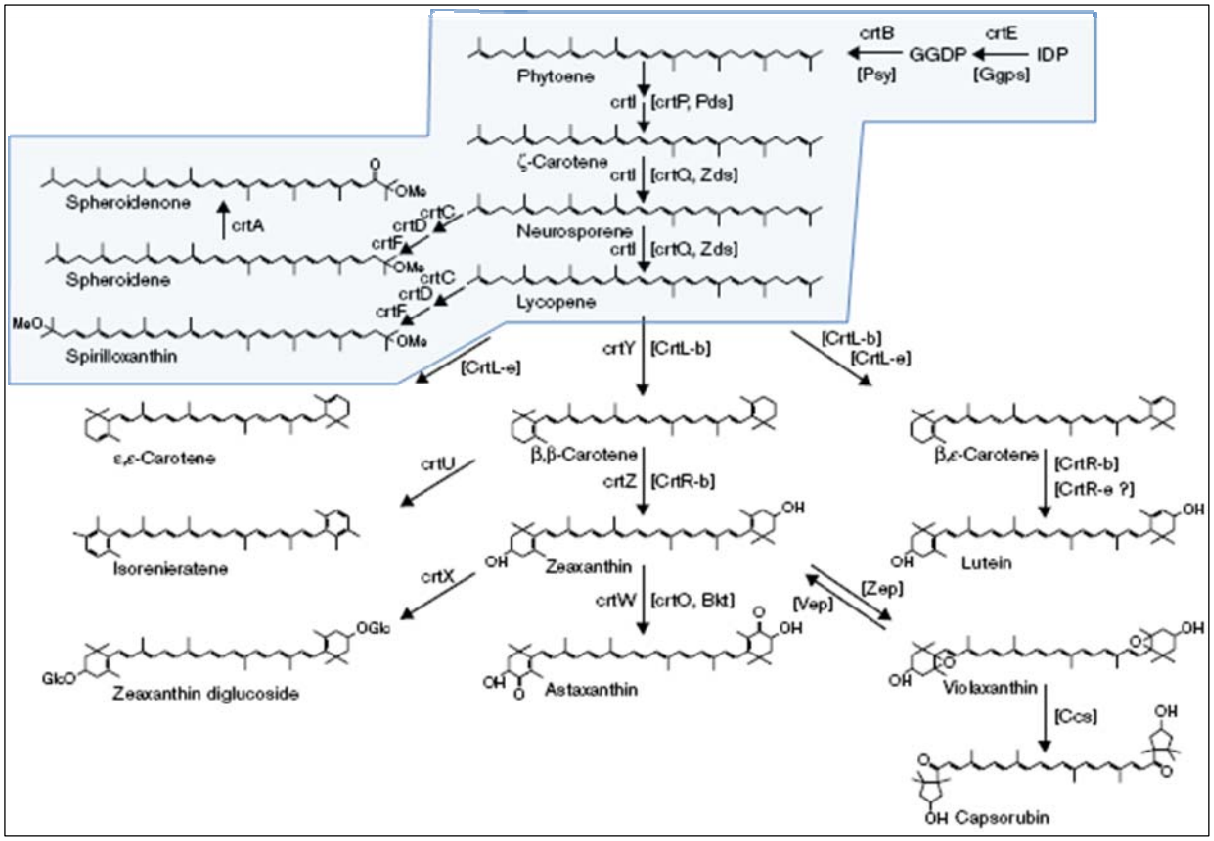


Figure.4 Carotenoid biosynthesis pathways (Dannert, 2000)

Carotenoid biosynthesis pathways in microorganisms and plants, the terminal products of biosynthesis routes are shown. The highlighted area denotes the pathway functional in *Rubrivivax benzoatilyticus* JA2

Gene (enzyme)	Gene code
Homologous gene sequences present	
Hydroxyneurosporene methytransferase/ dimethylspheroiden and didehydrorhodopin O-methyltransferase	*CrtF*
Methoxyneurosporene dehydrogenase	*CrtD*
Hydroxyneurosporene synthase	*CrtC*
Spheroidene/ spirilloxanthin monooxygenase	*CrtA*
Phytoene dehydrogenase/ Squalene/ Phytoene synthase	*CrtI/P/B*
Geranylgenranyl pyrophosphotase synthetase	*CrtE*
Homologous gene sequences not found	
Beta-end group ketolase	*CrtO*
Carotene desaturase	*CrtQ*
Beta-ionone desaturase	*CrtU*
Lycopene cyclase	*CrtY*
Beta-carotene ketolase	*CrtW*
Diapolycopene oxidase	*CrtN*
Diapolycopene synthase	*CrtM*
Carotene isomerase	*CrtH*

Table.4 Complete list of enzymes involved in the carotenoid biosynthesis and their codes

List of homologous gene sequences (with codes, as denoted in the circular genome) present and not found in the draft genome of *Rubrivivax benzoatilyticus* JA2 for the carotenoid biosynthesis inferred from the genome sequence.

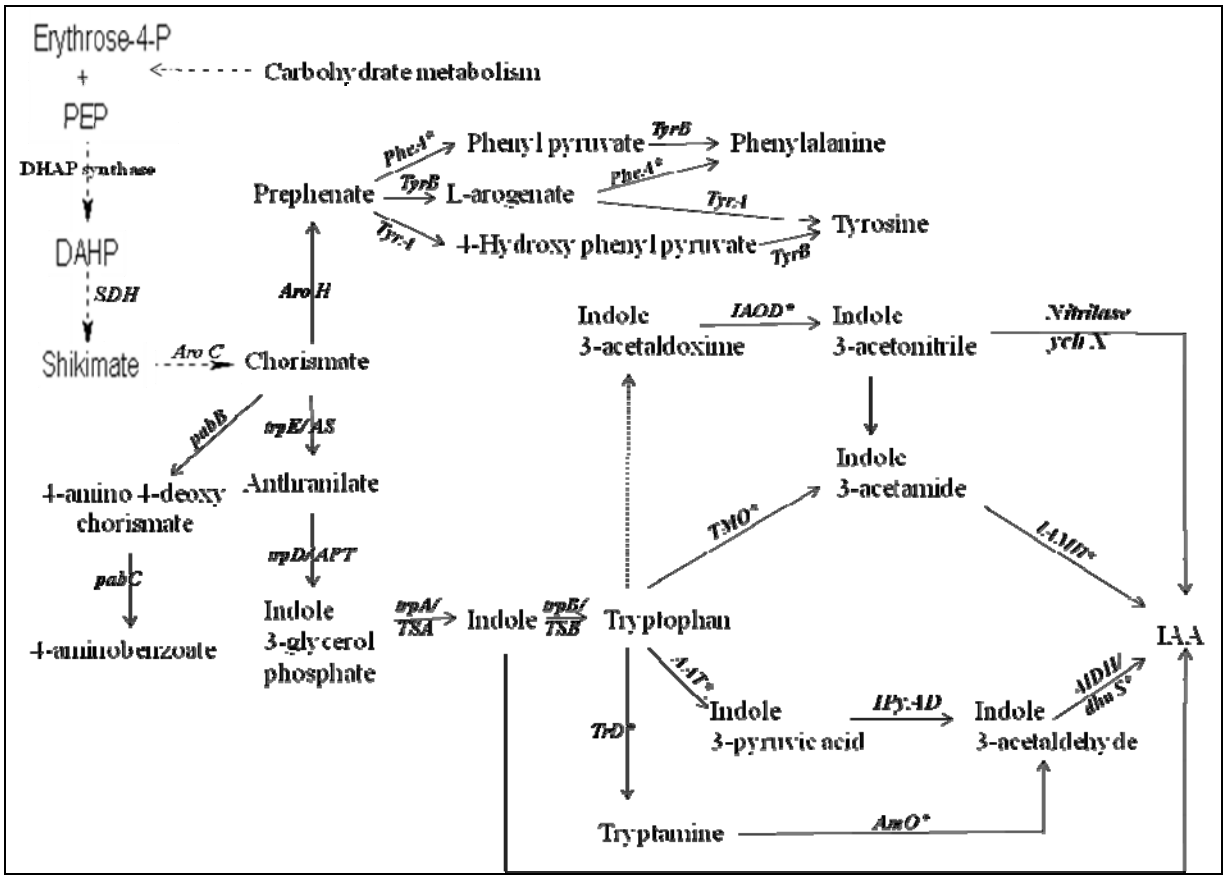


Figure.5 Aromatic biosynthetic pathway (Idris, *et al.*, 2007) * homologous gene coding sequences are not present in *Rubrivivax benzoatilyticus* JA2

In the aromatic biosynthetic pathway all the homologous gene sequences till the biosynthesis of chorismate are present, in the trifurcation from there to 4-aminobenzoate and to the aromatic amino acid biosynthesis prephenate dehydratase was not found and in the indole 3-acetic acid biosynthesis from tryptophan nitrilase and indole pyruvic acid decarboxylase were present as listed in the table.5

Gene (enzyme)	**Gene code**
2-dehydro-3-deoxyphosphoheptonate aldolase	*DHAPs*
Chorismate mutase	*AroH*
3-dehydroquinate synthase	*AroB*
3-dehydroquinate dehydratase	*AroD*
Shikimate 5-dehydrogenase	*SDH*
Shikimate kinase	*AroK*
Chorismate synthase	*AroC*
2-deoxyisochorismate synthase	*ADIC*
Anthranilate synthase	*AS/ trpE*
Anthranilate phosphoribosyl transferase	*APT/ trpD*
N-5-phosphoribosyl anthranilate isomerase	*trpF*
Indole 3-glycerophosphate synthase	*trpC*
Tryptophan synthase alpha	*TSA*
Tryptophan synthase beta	*TSB*
Indole 3-pyruvate decarboxylase	*IPyAD*
Nitrilase	*NIT/ychX*
Aminodeoxychorismate synthase	*PabB*
Aminodeoxychorismate lyase	*PabC*
Prephenate dehydrogenase	*TyrA*
Aromatic aminotransferase	*TyrB*

Table.5 List of enzymes and their codes involved in aromatic biosynthesis

List of homologous gene sequences (with codes, as denoted in the draft circular genome) found in the *Rubrivivax benzoatilyticus* JA2 for the aromatic biosynthesis inferred from the genome sequence.

3.2. Affect of indole on *Rubrivivax benzoatilyticus* JA2

Rubrivivax benzoatilyticus JA2 upon induction with indole as described in the materials and methods, affects were observed at different levels of the cellular organization. These affects observed at the physiological, metabolic and proteome levels were discussed in detail in the following results.

3.2.1. Physiological effects

3.2.1.1. Growth and utilization on various N-heterocyclic aromatics by *Rubrivivax benzoatilyticus* JA2

Rubrivivax benzoatilyticus JA2 culture grows photoheterotrophically on Biebl and Pfennig's medium additionally supplemented with indole and other aromatic hydrocarbons enlisted in the table. 6, for 96 h and assayed for utilization by HPLC analysis and spectroscopic analysis. Media Supplemented either with 4-nitrobenzene, 2-nitrotoluene, phenylalanine and 4-hydroxybenzene has inhibited the growth and resulted in no utilization of the compound. In presence of salicylate, 4-nitroaniline, 4-nitrotoluene and benzene growth was observed without utilization. In presence of 2-aminobenzene and trans-cinnamate with resting cells has resulted in the utilization of the compound. In presence of indole both growth and utilization were observed. Maximum growth yields were observed with salicylate and indole followed by 4-nitroaniline and 4-nitrotoluene (Table. 6).

3.2.1.2. Effect of light and oxygen on growth and indole consumption by *Rubrivivax benzoatilyticus* JA2

Rubrivivax benzoatilyticus JA2 with indole (1 mM) as additional supplement in the Biebl and Pfennig's media has shown only photoheterotrophic growth both anaerobically and aerobically, and no growth in absence of light. In presence of light equal biomass yield was observed in aerobic and anaerobic conditions with variation in indole consumption of 0.15 mM (15 %) and 0.6 mM (60 %) respectively (Table.7). Similar biomass yield of *Rubrivivax benzoatilyticus* JA2 is observed when indole (1 mM) is used as additional supplement or

complemented for NH_4Cl as nitrogen source. Upon increasing the concentration of indole in the media from 1 to 8 mM as nitrogen source growth of the culture has decreased (Fig. 6).

3.2.1.3. Effect of indole on pigments of *Rubrivivax benzoatilyticus* JA2

Rubrivivax benzoatilyticus JA2 grown on media with increasing concentration of indole from zero to 10 mM, the colour of the culture remains purple red with the total pigment content is almost similar till 2 mM, above 2 mM indole concentration the culture turns to green (Fig. 7 A). Till 2 mM concentration the total pigment concentration was similar and above this concentration both the carotenoid and chlorophyll content had shown a drastically down trend (Fig. 7 B). The concentration of the total pigment is same with variation in the concentrations of individual pigments like red, yellow and orange carotenoids and chlorophyll (Fig. 7 C).

In the spectrophotometer studies of extracted pigments from *Rubrivivax benzoatilyticus* JA2 in the visible region range of 400 to 850 nm against the reagent blank (Acetone: Methanol in ratio 7:2), there is a gradual loss of carotenoid pigment peaks in the range of 400 to 600 nm and chlorophyll peak at the 775 nm and increase of absorbance at the 400 nm with increase of indole concentration from 1 to 8 mM was observed (Fig. 8).

3.2.1.4. Effect of indole on carotenoid profile of *Rubrivivax benzoatilyticus* JA2

HPLC analysis of the solvent pigment extract has shown the major carotenoids as spheroidene at t_R 7.5 min, didehydrolycopene at t_R 6.1 min, neurosporene at t_R 4.2 min and a minor unidentified carotenoid at t_R 2.7 min in the control culture. When the *Rubrivivax benzoatilyticus* JA2 was induced with 1 mM of indole the spheroidene concentration was decreased and the unidentified and didehydrolycopene concentration was enhanced. With increasing concentration of indole the minor pigment at t_R 2.7 min concentration has gradually increased to higher concentrations (about 20 times) and is the prominent pigment peak from 2 mM and above concentrations of indole (Fig. 9).

3.2.1.5. Analysis of unidentified carotenoid in *Rubrivivax benzoatilyticus* JA2

The HPLC analysis of the extracted pigments of culture induced with 4 mM indole had a major chromatogram peak at t_R 56.3 min. it has absorbance at 440 nm visible region (as coloured

carotenoid molecules have) and in ultra violet region. It has absorbance maxima at λ 217 332 372 413 419 nm and a fragment value of 363 *m/z* in mass analysis (Fig. 10), not matching with the carotenoids in the data base.

Compound (1 mM) supplemented in the media	Biomass yield (mg dry wt. ml^{-1})	Substrate utilization (%)
Salicylate	0.6	-
4-nitroaniline	0.45	-
4-nitrobenzene	Nil	-
4-nitrotoluene	0.3	-
2-nitrotoluene	Nil	-
Benzene	0.045	-
4-hydroxybenzene	Nil	-
Phenylalanine	Nil	-
`Indole	0.69	+ (60 %)
*2-aminobenzene	NA	+ (5 %)
*Cinnamate	NA	+ (85 %)
None	0.75	NA

Table.6 Growth of *Rubrivivax benzoatilyticus* JA2 and utilization of the substrate (–,unutilized; +, utilized; NA- not applicable ` utilization measured by calorimetric method remaining measured by HPLC unless mentioned and * with resting cells only utilization is measured after 48 h)

Rubrivivax benzoatilyticus JA2 culture was grown photoheterotrophically in Biebl and Pfennig's medium (1981) with malate (22 mM) as sole carbon source and NH_4Cl (7 mM) as nitrogen source and additionally supplemented with aromatic hydrocarbons (1 mM) enlisted in the table for 96 h incubation and assayed for utilization. Growth was estimated by photometrically as mentioned in the materials and methods and HPLC analysis for utilization was accordingly carried out as described in the materials.

Culture condition		Biomass yield (mg dry weight ml^{-1})	Indole utilization (mM)
Anaerobic	Light	0.69	0.6
	Dark	Nil	Nil
Aerobic	Light	0.525	0.15
	Dark	Nil	Nil

Table.7 Growth of the *Rubrivivax benzoatilyticus* JA2 and indole utilization under variation in light and oxygen availability conditions.

Rubrivivax benzoatilyticus JA2 culture was grown on Biebl and Pfennig's medium (1981) containing 1 mM of indole as additional supplement with malate (22 mM) as carbon source and NH_4Cl (7 mM) as nitrogen source and incubated under 2,400 lux for phototrophic growth and kept in dark cupboard for anaerobic conditions. Aerobic conditions were provided in orbital shakers with and without light at 180 rpm at 30 $\pm$ 2 ^{o}C temperature were maintained. results expressed are average values of independent experiments in duplicates.

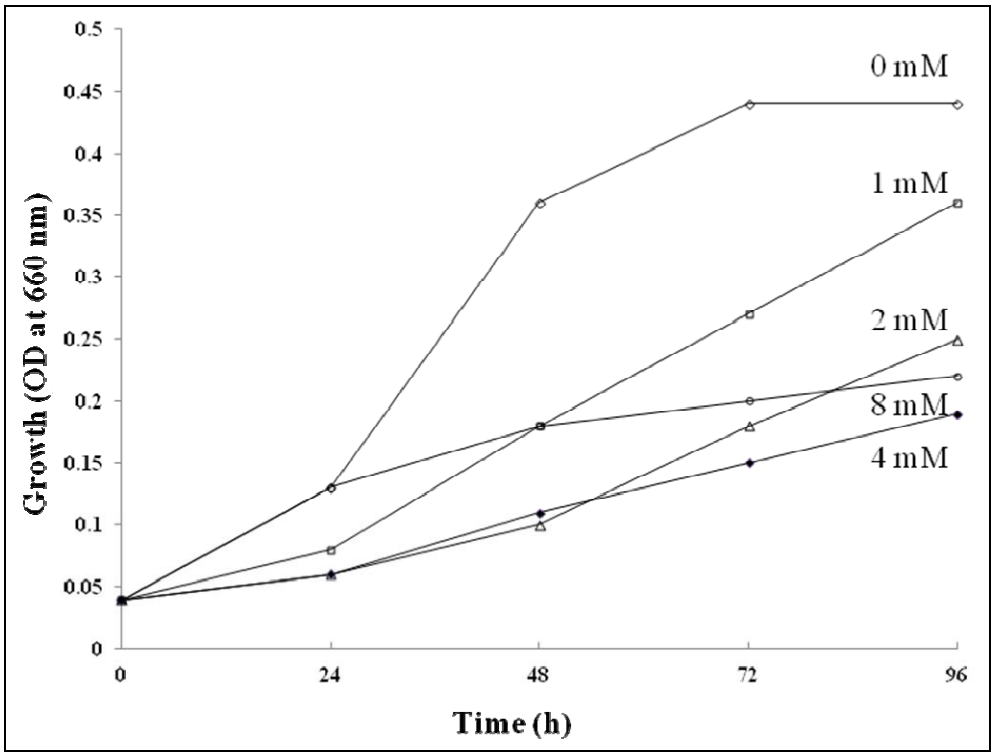


Figure.6 Affect of indole on growth of *Rubrivivax benzoatilyticus* JA2 at different concentrations with time.

Rubrivivax benzoatilyticus JA2 was grown on Biebl and Pfennig's medium with malate as carbon source and NH_4Cl as nitrogen source in control (0 mM) and indole was substituted in the media as nitrogen source at concentrations of 1, 2, 4 and 8 mM and growth was monitored spectrometrically.

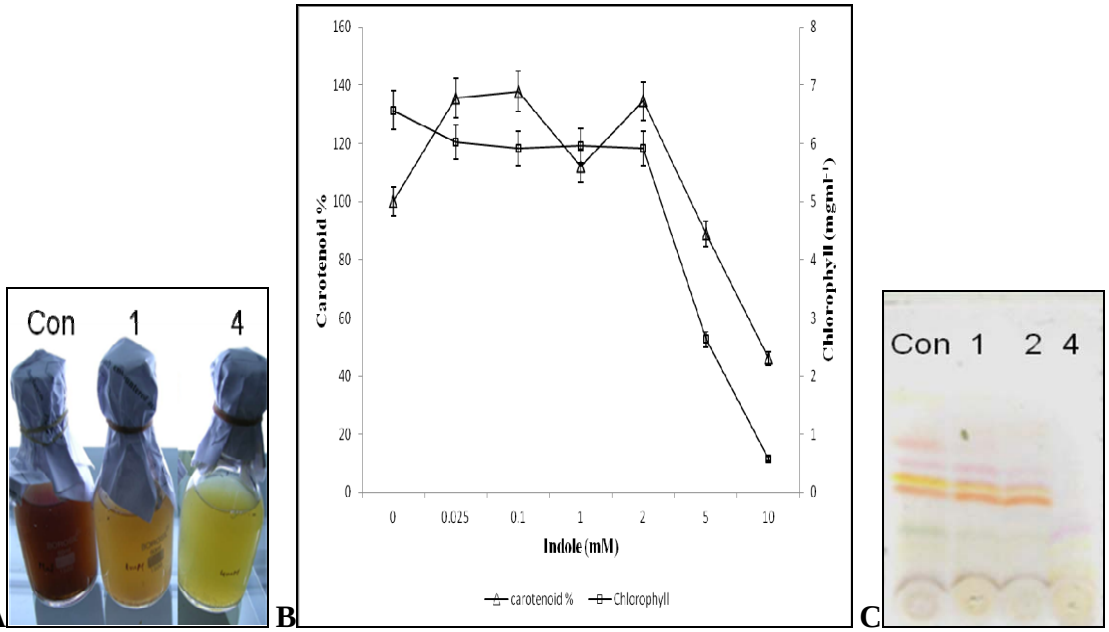


Figure.7 Affect of indole on culture colour and pigmentation of *Rubrivivax benzoatilyticus* JA2.

A. Affect of indole on culture colour of *Rubrivivax benzoatilyticus* JA2 without indole (Control), 1 and 4 are culture with 1 and 4 mM indole concentration, media and growth conditions are same as mentioned in the figure. 6.

B. *Rubrivivax benzoatilyticus* JA2 when grown in increasing concentration of indole from zero to 10 mM. Pigments were extracted using solvent Acetone: Methanol in the ratio 7:2 and total pigment content is calculated using the method of Cohen-Bazire *et al*., (1957) by reading absorbance at 450, 500 and 750 nm.

C. TLC of the extracted pigments from the culture grown at different concentrations of indole from 0 to 4 mM on silica coated aluminum plates with solvent petroleum ether: acetone in ratio 9:2; Con- control culture without indole (0 mM), 1, 2 and 4 indicate the concentration of indole in 1 mM, 2 mM and 4 mM respectively.

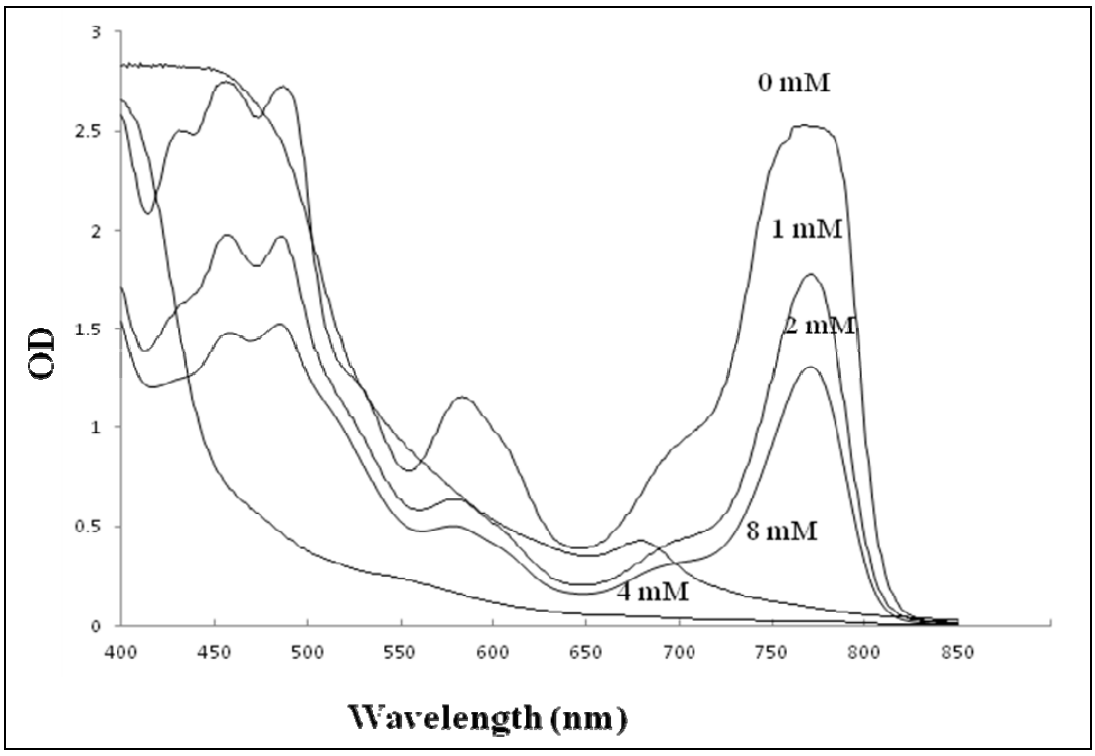


Figure.8 Influence of indole on pigments of the *Rubrivivax benzoatilyticus* JA2.

Extracted pigments of *Rubrivivax benzoatilyticus* JA2 cultured at 0 (control), 1, 2, 4 and 8 mM concentrations of indole, as nitrogen source, with solvent Acetone: Methanol in the ratio 7:2 were analyzed in spectrophotometer in the visible region ranging from 400 to 850 nm.

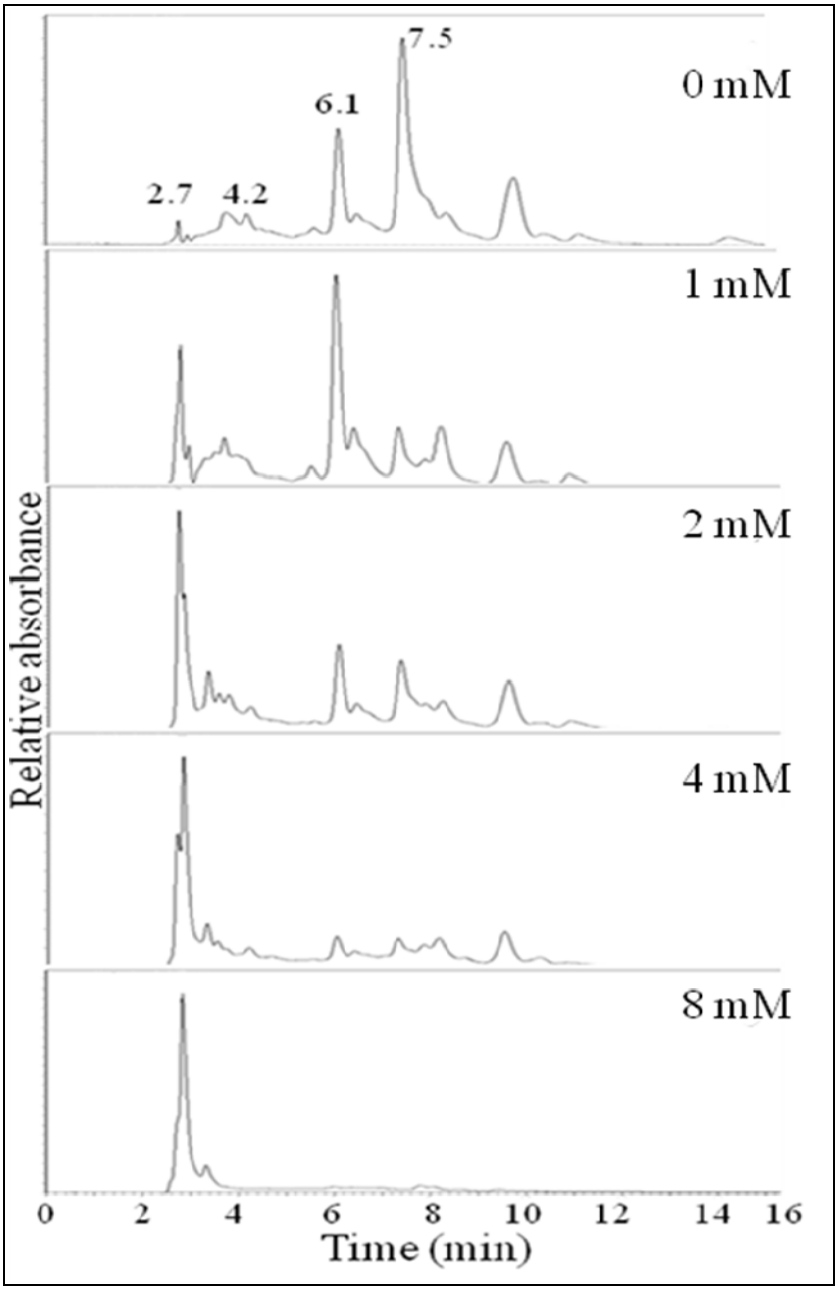


Figure.9 HPLC chromatogram profile of the extracted pigments of *Rubrivivax benzoatilyticus* JA2 with increase in concentration of indole from 0 to 8 mM.

HPLC analysis of the pigments from *Rubrivivax benzoatilyticus* JA2 was carried after extracting the pigments, as mentioned in figure. 7, in isocratic method with C18 column and solvent consisting of Acetonitrile: Methanol: Ethyl acetate in the ratio 5:4:1 at 440 nm with a photo diode array detector having a 15 min run time.

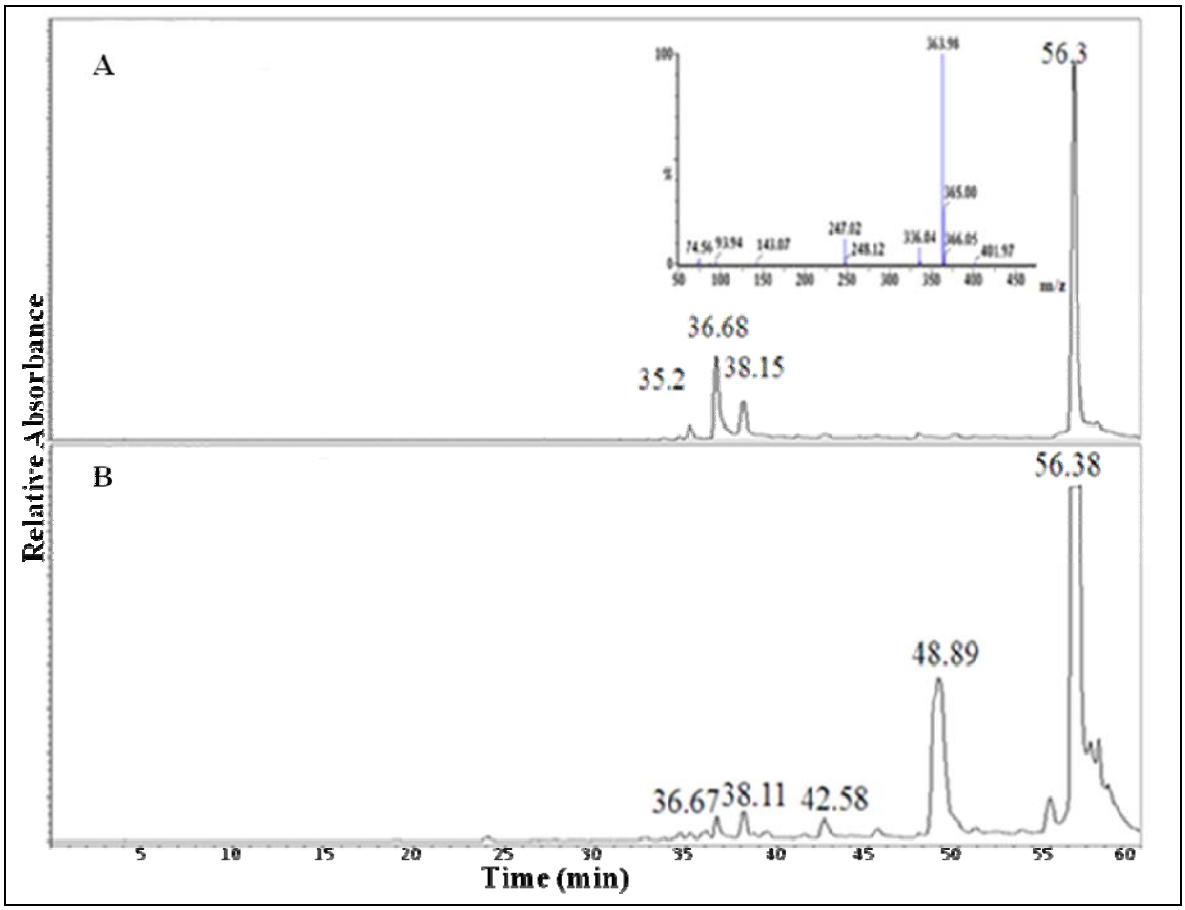


Figure.10 HPLC chromatogram of extracted pigments of *Rubrivivax benzoatilyticus* JA2 culture induced with 4 mM concentration of indole in visible region at 440 nm, A and in ultra-violet region at 280 nm, B; Inset is the *m/z* mass fragmentation of the pigment at t_R 56.3 min.

The HPLC analysis was carried out following an gradient method using a C18 column, solvent consisting of 0.1 % acetic acid as solvent A and 100 % acetonitrile as solvent B, increases from 0 to 100 % in 30 min run time including a after wash in the run, detected using a photo diode array detector at 440 nm and 380 nm as mentioned in the materials and methods. The mass analysis was carried out with the modified method of the same with extended run for 60 min and the mass detector was set between 50 to 800 *m/z*.

3.2.2 Indole metabolism by *Rubrivivax benzoatilyticus* JA2

The metabolites extracted from the culture supernatant of *Rubrivivax benzoatilyticus* JA2 induced with 1 mM indole were isolated (Powell, 1964), identified and characterized by using the HPLC and LC-MS.

3.2.2.1 Metabolites identified

Profiling of the culture supernatant for aromatic (indole ring) metabolites was done with the help of high pressure liquid chromatography. Control and induced cultures had shown production of indole acetic acid, and tryptophan production was observed in induced culture only. Maximum indole consumption and tryptophan production was observed in the culture supernatant supplemented with indole as nitrogen source than additional source. After removing the cell pellet by centrifugation, using ethyl acetate the metabolites in the supernatant were fractionated, extracted (flow chart. 1), concentrated and identified with the help of standards retention times and absorbance maxima. Standard indole, IAA and tryptophan was observed at t_R 24.3 m in, 18.2 min and 9.4 min respectively with that of the standards (Fig. 11).

Tryptophan is observed only in the indole induced culture supernatant after 96 h of incubation. In presence of indole growth of culture had a prolonged lag phase and enters log phase after 48 h, against the 24 h for the control, It reaches maximum growth at 72 h and remains almost constant till 96 h (Fig. 12). Maximum indole consumption of 0.6 mM (60 %) and tryptophan production of 0.1 mM (10 %) (Fig. 12) and 0.01 mM of indole acetic acid production was observed in the culture supernatant in presence of indole after incubation. Upon increase in indole induction concentration tryptophan production was scaled down.

3.2.2.2 Stable isotope probing studies: In the supernatant fraction of stable indole isotope four products were detected with a mass difference of +9 and +18 *m/z* at t_R 15, t_R 32, t_R 35 and t_R 44 minutes. Two of them were identified with the help of fragmentation pattern from mass bank data base, one *m/z* value is similar to the indole dimer, indigo and another *m/z* value is not matching with the standards mass in the data base.

Mass peak at t_R 15min shows the mass spectrum fragmentation same as the standard tryptophan having a mass of 205 *m/z* in positive mode along with another fragment of mass 186 *m/z* in normal indole, and in the stable isotope sample the +9 masses 214 *m/z* and 197 *m/z* were observed (Fig. 13).

Mass peak at t_R 32 min shows the mass fragmentation spectrum same as the standard indole acetic acid having a mass of 176 *m/z* in positive mode along with other fragments of mass 160 and 130 *m/z*, and in the stable isotope sample the +9 masses 185, 169 and 139 *m/z* were observed (Fig. 14).

Mass peak at t_R 35 min shows the mass fragmentation spectrum having a mass of 247 *m/z* in positive mode and in the stable isotope sample the +18 mass of 265 *m/z* were observed. None of the standard *m/z* values match with the data base in the mass bank (Fig. 15).

Mass peak at t_R 44 min shows the mass fragmentation spectrum pattern having a mass of 261 *m/z* in positive mode, similar to the mass of indigo molecule of *m/z* value of 262, other values of 185 and 144 *m/z* were also observed. In the stable isotope sample the +18 and +9 masses of 279, 194, and 153 *m/z* were observed (Fig. 16).

3.2.2.3 Metabolism of indole analogues: After products identification study of indole metabolism and the pathway leading to the production of indole acetic acid was done by using the competitive inhibitor for tryptophan synthase 5-fluoroindole, an indole analogue. Concentrations above 0.35 μM has resulted in complete growth inhibition of *Rubrivivax benzoatilyticus* JA2, without any biomass yield and the intermediate products of 5-fluoroindole metabolism are not detected. Concentration below 0.35 μM has not inhibited the growth and none of the intermediates are detected.

Further two of the indole analogues 2-methylindole and indole-carboxylic acid were used with functional group variation. The culture supernatant were analyzed with the help of HPLC, with 2-methyl indole major product identified is 2-methyl tryptophan and with indole carboxylic acid there is no growth yield (Table. 8).

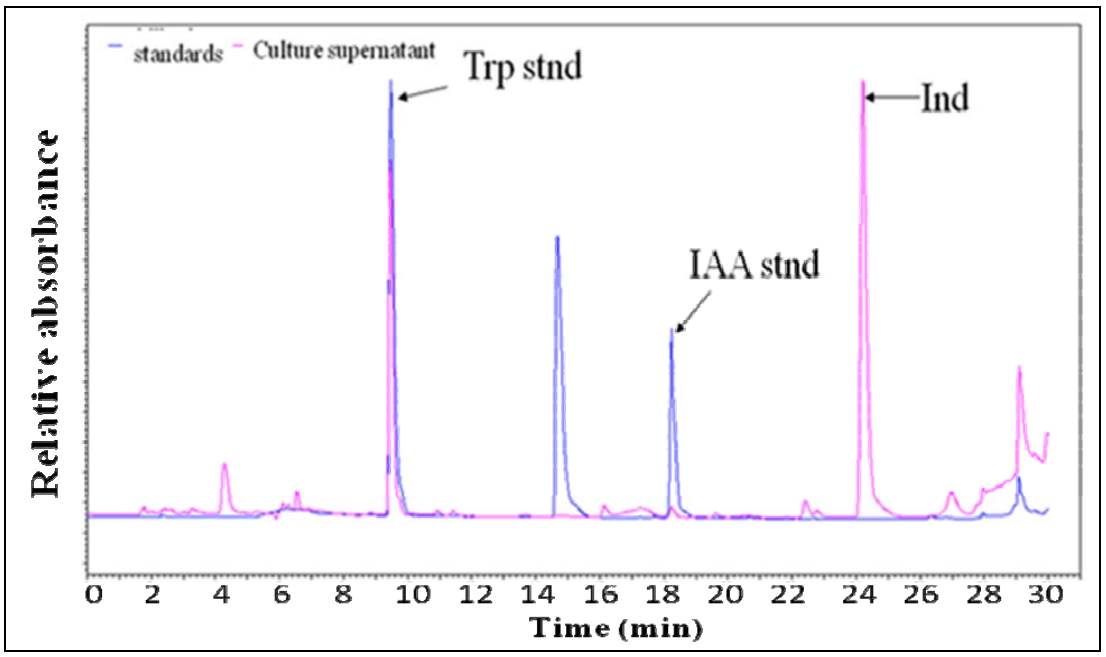


Figure.11 HPLC chromatogram of metabolites identified in the indole induced *Rubrivivax benzoatilyticus* JA2 culture supernatant (1 mM) and the mixed standards of tryptophan and indole acetic acid.

HPLC analysis of the culture supernatant was done in a gradient method consisting of 0.1 % acetic acid as solution A and 100 % acetonitrile as solution B, with a gradual increase from 0 to 100 % of solution B for a 30 min run time as mentioned in the materials and methods.

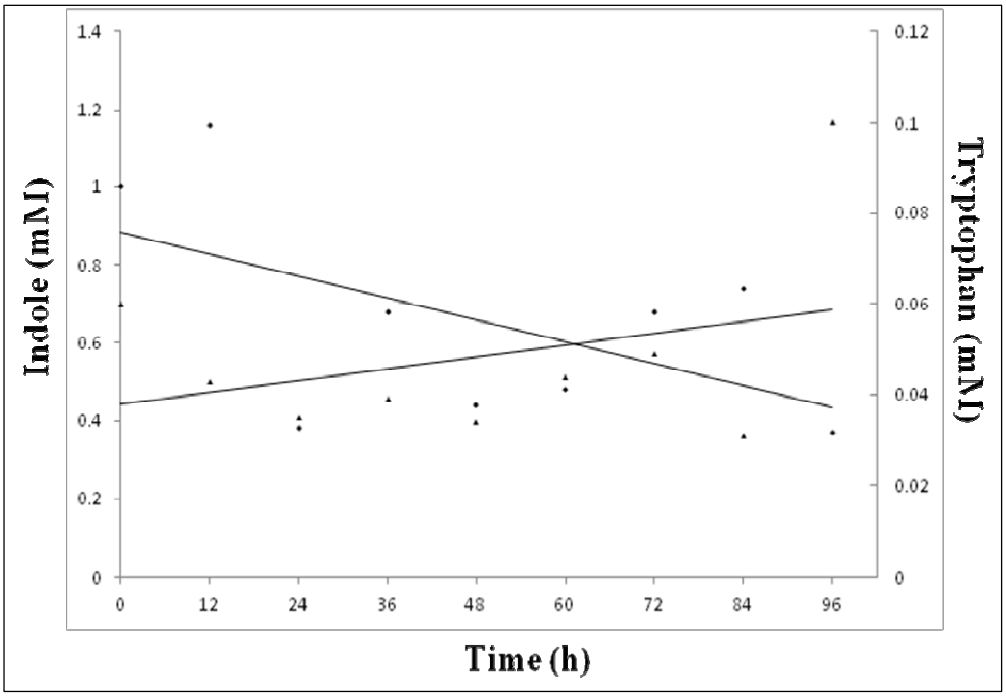


Figure.12 Indole consumption and tryptophan production (mM) with time (h) by *Rubrivivax benzoatilyticus* JA2.

Indole (1 mM) supplemented in the media as sole nitrogen source is incubated for 96 h under phototrophic (2,400 lux) conditions at 30 $\pm$ 2 ^{o}C and consumption was estimated either by Ehrlich's reagent or HPLC analysis as mentioned in the methods; tryptophan production is quantified by HPLC analysis. Results expressed are average values of two independent experiments done in triplicates.

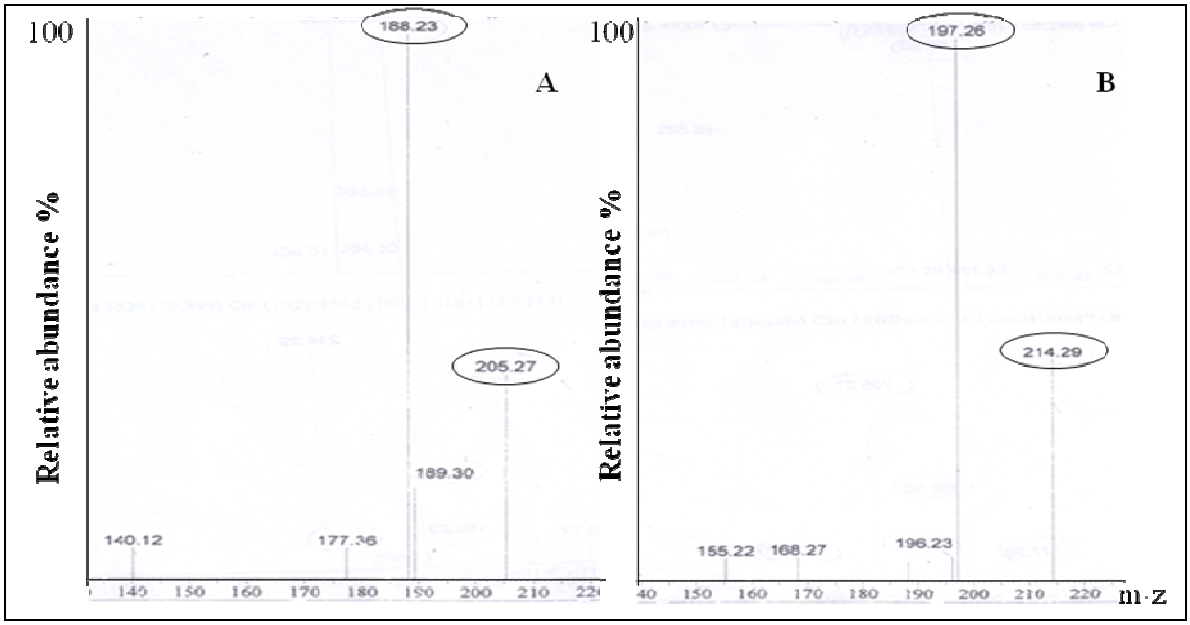


Figure.13 LC-MS chromatogram with mass fragmentation of the molecule with t_R 15 min in the culture supernatant extract.

m/z value observed at t_R 15 min from normal indole, A and its stable isotope culture supernatant, B observed with a mass difference of +9 and is matching with standard *m/z* value of tryptophan.

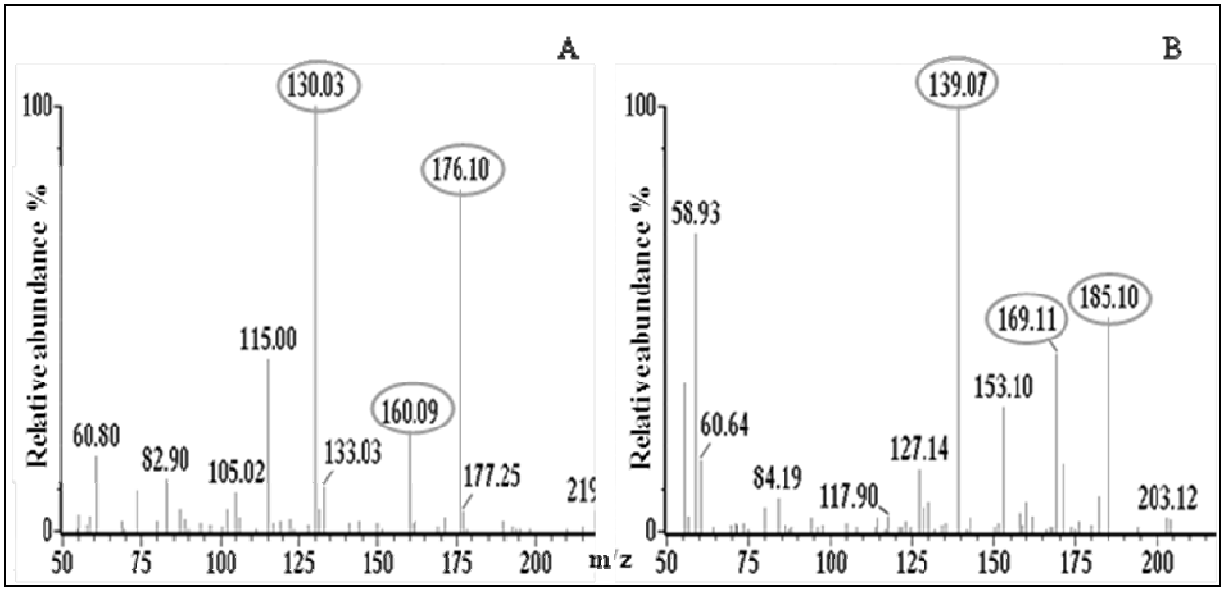


Figure.14 LC-MS chromatogram with mass fragmentation of the molecule with t_R 32 min in the culture supernatant extract.

m/z value observed at t_R 32 min from normal indole, A and its stable isotope culture supernatant, B observed with a mass difference of +9 and is matching with standard *m/z* value of indole acetic acid.

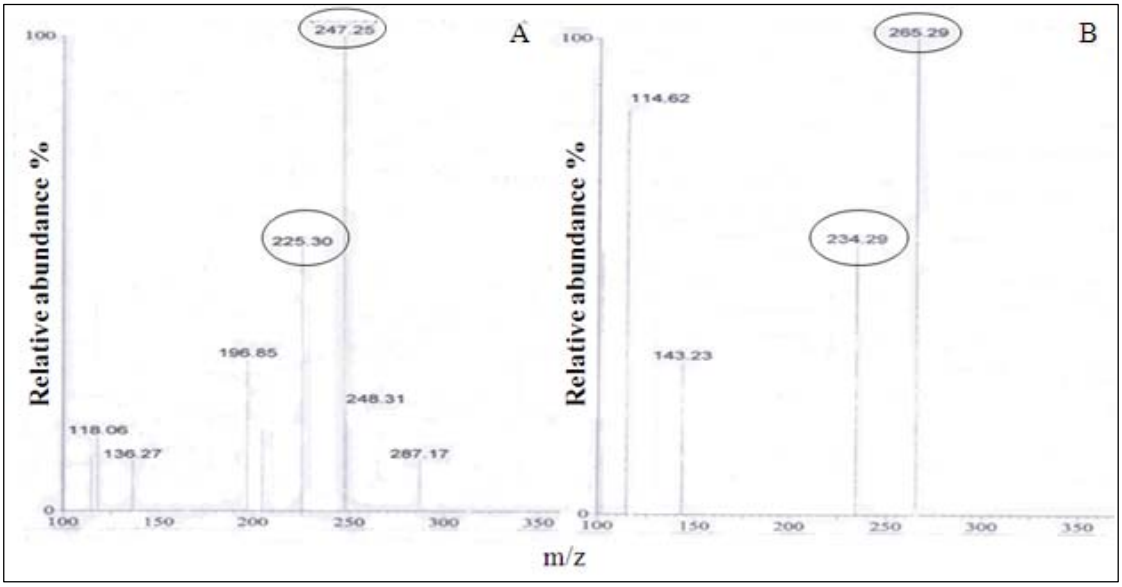


Figure.15 LC-MS chromatogram with mass fragmentation of the molecule with t_R 35 min in the culture supernatant extract.

m/z value observed at t_R 35 min from normal indole, A and its stable isotope culture supernatant, B observed with a mass difference of +9 and is not matching with any of the standards *m/z* value in the data bank.

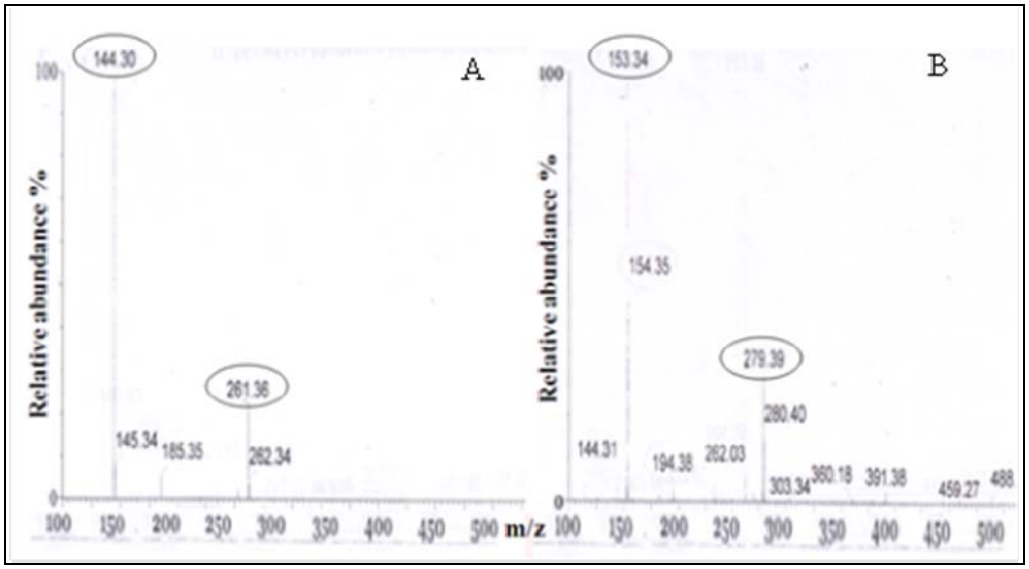


Figure.16 LC-MS chromatogram with mass fragmentation of the molecule with t_R 44 min in the culture supernatant extract.

m/z value observed at t_R 44 min from normal indole, A and its stable isotope culture supernatant, B observed with a mass difference of +9 and is similar with the standard *m/z* value of indigoid molecule

Indole and its substitutes	**Biomass yield (mg dry wt. ml^{-1})**	**Metabolites identified**
Indole	0.375	Tryptophan & Indole acetic acid
2-Methylindole	0.3	2-Methyltryptophan
5-fluoroindole	Nil (>0.35μM)	Not applicable
Indolecarboxylic acid	Nil	Not applicable

Table.8 Growth and identified metabolites in presence of indole and its derivatives by *Rubrivivax benzoatilyticus* JA2.

Rubrivivax benzoatilyticus JA2 is grown on media containing indole molecules substituted with different functional groups, 2-Methyl indole and indole carboxylic acid are supplemented at 1 mM concentration and 5-fluoro indole different concentrations were tested. The metabolites were identified with the help of HPLC analysis.

3.2.3 Proteomic responses of *Rubrivivax benzoatilyticus* JA2 to indole

3.2.3.1 Native Gel protein profile: The membrane proteins isolated from the cell pellet using the detergent Triton-X 100. When the isolated proteins were run on 6 % native gel have the colour pigment bands representing the pigment bound protein complexes along with indole when observed under ultra violet light. With increase in the indole concentration the pigment colour has diminished and the fluorescence has increased, with similar protein concentration (Fig. 17). From the MALDI analysis the outer membrane auto-transporter barrel protein was detected in the colour protein complex.

3.2.3.2 Proteome analysis of indole induced *Rubrivivax benzoatilyticus* JA2 by iTRAQ Proteome is isolated after centrifuging the pellet and lysed by sonication by using mixture of detergents SDS and Triton X-100 and centrifuged to remove the unlysed cells as pellet and the supernatant is lyophilized and iTRAQ analysis was done. The protein sample was enzyme digested and labeled with isobaric tagging and the proteins are identified and quantified by LC-MS analysis. False positive identification is reduced and the quality of obtained data was assessed, the false discovery rate (FDR) analysis was performed in addition to Protein Pilot software. FDR analysis including the yields of proteins and peptides with error rates of 1 %, 5 % and 10 % for both local and global FRDs were done. 924 distinct proteins were identified which is 24 % of the *Rubrivivax benzoatilyticus* proteome, the value lies within the range of 13-27 % obtained in large-scale proteome studies of bacteria.

Number of proteins with Pval <0.05 for two or more biological replicates are 108, out of which 27 remained unchanged, 36 suppressed and 45 up regulated (Fig. 18). The protein identification based on the NCBI genome blast search were further categorized based on their function from KEGG pathway and other means. Highly affected categories are the photosynthesis and respiration and membrane with maximum number of affected proteins, then is the protein translation mechanism, carbohydrate and also putative or hypothetical proteins are influenced due to the indole induction (Fig. 19).

3.2.3.2.1 Down regulated protiens Highly down regulated include proteins involved in the function of photosynthesis and respiration with maximum percentage of 22 %, next membrane

bound of 19 %, next carbohydrate metabolism of 17 %, putative proteins of 14 %, transport proteins of 11 %, purine and pyramidine biosynthesis of 8 %, proteins of transcription, translation and stress are down regulated of 3 % and other categories are not down regulated (Fig. 20).

A noticable amount of hypothetical proteins (about 14 %) have been down regulated. Their role in indole induced condition has to be elucidated.

3.2.3.2.2 Up regulate protiens Highly up regulated include proteins involved in the function of cellular processes of 16 %, followed by transcription and membrane proteins of 13 %, then proteins involved in photosynthesis and respiration are of 11 %, then are the translation proteins of 9 %, followed by putative, carbohyrate metabolism, purine and pyramidine metabolism and energy metabolism are of 7 %, stress and fatty acid biosynthesis are of 4 % and the least affected transport of 2 % (Fig. 21).

3.2.3.2.3 Ratios of up and down regulated proteins: Only up regulation is observed in cellular processes, energy metabolism and fatty acid biosynthesis. The ratio of up to down regulated is observed high in translation and transcription and then seen in stress proteins. Almost similar up to down regulation is observed in the purine and pyramidine biosynthesis. The ratio of down to up regulation is high as observed in transport proteins, carbohydrate metabolism, photosynthesis and respiration, putative proteins and the membrane proteins (Fig. 22).

3.2.3.2.4 Affect of indole on the expression of identified proteins in detail: In the iTRAQ analysis of the 108 proteins identified, proteins under different functional categories varied in their expression.

a) Membrane

Membrane proteins are the highly affected proteins having 15 % of the proteome, in which 19 % are down regulated and 13 % are up regulated. Down regulated proteins include extracellular solute binding protein family does not have any function related to the transport process but serve as receptors to trigger or initiate translocation of the solute through out the membrane by binding to the external sites of the integral membrane proteins of the efflux

system, in addition some proteins function in the initiation of sensory transduction pathways. Porin protein which form ion selective channel for small hydrophobic molecules (up to ~600 D) and the acetyl-CoA-binding protein which may function as an intracellular carrier of acetyl-CoA esters.

Up regulated membrane proteins includes the preprotein translocase subunit SecA is part of the export system involved in the extrusion of noxious substances. Most of the proteins of gram-negative type outer membrane porin protein are porin-like integral membrane proteins, but some are small lipid-anchored proteins and serves two functions in general, attaching to the cell wall to form the stator of the flagellar motor and also in the flow of ions across the cell membrane for the movement of the rotor. Membrane-bound proton-translocating pyrophosphatase have been characterized for soluble and membrane-bound proton-pumping pyrophosphatases. It is associated with proton translocation and ATP formation. The Peptidoglycan associated lipoprotein system appears to be involved both in the outer membrane integrity maintenance and in the import of certain organic molecules as nutrients. In majority of the ice cold region organisms Type I antifreeze protein HlyD family secretion proteins are useful in prevention of growth of ice crystals or retarded, and the freezing point is depressed. The high lysine content of these peptides may serve to promote the solubility of these proteins.

b) Photosynthesis and Respiration

Photosynthetic and respiration associated proteins are the second most affected ones with 15 % of the proteome in which 22 % are down regulated and 11 % are up regulated. Down regulated proteins include the 2-methylisocitrate dehydratase or bifunctional aconitate hydratase 2 is involved in the third step enzymatic reaction of methylcitrate cycle of propionate catabolism. Zn-dependent alcohol dehydrogenase involved in the reversible oxidation of ethanol to acetaldehyde involving NAD reduction. Succinate dehydrogenase subunits A and B catalyze oxidation of succinate in the citric acid cycle and transfers the electrons to the respiratory quinones present in the membrane. The external subunit A with the active site for succinate and a membrane subunit B has site for the quinone. The hydrophilic sequence present in the cytoplasmic space subunit A consists of a flavoprotein with the active site for succinate and an iron-sulphur protein subunit B carrying three iron-sulphur clusters which are involved in the

electron transfer from A to the membrane subunits B. Phosphate glycerate kinase is involved in the formation of ATP to ADP and vice versa during glycolysis where 1,3-diphosphoglycerate is converted to 3-phsophoglycerate. This step is essential for generation of ATP and for fermentation in aerobes. Aldehyde dehydrogenases enzymes catalyzing wide variety of aliphatic and aromatic aldehydes to their oxides using NADP as a cofactor. Glyceraldehydes-3-phosphate dehydrogenase transforms 1,3-diphosphoglycerate to glyceraldehydes-3-phosphate and vice versa which is an important step in glycolysis and gluconeogenesis.

In the photosynthesis apparatus of anoxygenic bacteria light harvesting (LH) complexes (I and II) have protein pigment assembly, the pigments carotenoid and bacteriochlorophyll act as primary donors. The LHs around acts as the energy collection hub, by temporarily storing the electrons and transferring them to the central photosynthetic reaction centre (RC), in some species additional subunits may be there along with LHI. Reaction centers uses the excitation energy to shuffle electrons across the membrane, *via* ubiquinol to cytochrome bc1 complex to establish the proton gradient across the membrane, useful for ATP synthesis by ATP synthetase. Reaction centers consists of three subunits L (light), M (medium) and H (heavy). Subunits L and M provide the support for the chromophore, while subunit H contains a cytoplasmic domain.

The up regulated include phosphoribulokinase catalyzing the ATP dependent phosphorylation of ribulose-3-phosphate to ribulose-1,5-phosphate of the pentose phosphate pathway where carbon is assimilated by the autotrophs, while photosynthetic bacteria are regulated by a system that has an absolute requirement for NADH. Two types of transketolases are present in this organism with small changes in the sequence useful for reversible transfer of a two carbon ketol from xylulose 5-phosphate to aldose receptor, like ribose 5-phosphate, resulting in the formation of sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate, with thiamine pyrophosphate as cofactor. Tranketolase along with transladolase form the enzymatic link between glycolysis and pentose-phosphate pathways. Tartrate dehydrogenase is involved in the oxidation of both meso and (+)-tartrate and also D-malate which are NAD dependent. It is involved in the oxidation of (+)-tartrate to form oxaloglycolate and oxidative decarboxylation of D-malate to form pyruvate and carbon dioxide, and decarboxylation of meso-tartrate forming D-glycerate and carbon dioxide without any net oxidation or reduction. Electron-transferring-

flavoprotein dehydrogenase are the electron transferring flavoprotein which serve as specific electron acceptors for primary dehydrogenases, transferring the electrons to the terminal respiratory systems. They are formed of two domains and are classified into two groups, group I mainly involved in the oxidation of fatty acids and group II produced by some prokaryotes under specific growth conditions, receiving electrons only from the oxidation of specific substrates.

c) Translation

The third most affected proteins up to 11 % belong to the protein biosynthesis translation machinery, most of them are unaffected, up regulated are about 9 % and only 3 % of proteins are down regulated. 2-isopropylmalate synthase is the protein responsible for conversion of alpha-ketoisovalerate to alpha-isopropylmalate the first step in leucine biosynthetic pathway.

The up regulated proteins involved in protein synthesis are aspartate-semialdehyde dehydrogenase which is the second enzyme in the aspartate pathway converting aspartyl phosphate to aspartate-semialdehyde where bifurcation of lysine and threonine-methionine pathways occur with NADP to NADPH conversion. Aspartate pathway is important for production of four amino acids lysine, threonine, methionine and isoleucine and also metabolic intermediates like diaminopimelic and dipicolinic acid required for bacterial cell wall biosynthesis and sporulation in gram positive bacteria respectively. Dihydroxy acid dehydratase enzyme involved in the biosynthesis of isoleucine and valine where dehydration of 2,3-dihydroxy isovaleic acid leads to alpha ketoisovalerica acid. 3-isopropylmalate dehydratase or isopropylmalate isomerase is the enzyme catalyzing the stereo specific isomerisation of 3-isopropylmalate and 3-isopropylmalate with an intermediate of 2-isopropylmaleate, in the biosynthesis of leucine. Some prokaryotic isopropylmalate dedhydrogenases can function as homoaconitase converting cis-homoaconitate to homoisocitric acid in lysine biosynthesis.

a) Carbohydrate metabolism

In the carbohydrates metabolism 10 % of protein of the proteome are affected in which most of the proteins about 17 % are down regulated includes isocitrate lyase catalyzing the isocitrate into succinate and glyoxylate conversion in the glyoxylate bypass an alternative to the tricarboxylic acid cycle in bacteria, fungi and plants. Malate dehydrogenase helps in the

reversible oxidation of malate to oxaloacetate in the tricarboxylic acid cycle. Type II citrate synthase catalyzes the first step in Kreb's cycle that is carbon-carbon bond formation between oxaloacetic acid and acetyl-coenzyme A to form citrate and coenzyme A without any cofactors requirement. It is a significant step in both energy generation and in carbon assimilation. Phosphoenolpyruvate carboxykinase or carboxylase useful for formation of oxaloacetate from phosphoenolpyruvate (PEP) and hydrocarbonate ion, useful in seizure and assimilation of carbon dioxide. PEPcarboxylase also have a key role as an antipleurotic supplying oxaloacetate to the tricarboxylic acid cycle, for the continuous supply of C4 molecules to replenish the intermediates removed for amino acid biosynthesis. S-adenosyl L-homocysteine hydrolase or Adenosylhomocysteinase has role in the activated methyl cycle, responsible for the reversible breakdown by hydration of S-adenosyl L-homocysteine into adenosine and homocysteine, it has a requirement of cofactor NAD and has a glycine rich central region for the NAD binding. It plays important role in maintenance of adenosylhomocysteine intracellular concentration. Fructose 1,6-bisphosphate aldolase catalyzes the reversible aldol cleavage or condensation of fructose 1,6-bisphophate into dihydroxyacetone-phosphate and glyceraldehydes 3-phosphate in the glycolysis. There are two classes in these enzymes depending on the requirement of metal ion as class I does not require metal ion and forms Schiff base intermediate between active site lysine and carbonyl group of substrate, and class II requires divalent metal ion in the active site.

7 % proteins are up regulated involved in carbohydrate metabolism includes phosphoenlopyruvate synthase and glycosyl transferase group I protein or glycogen/ starch/alpha-glucan phosphorylase. Phosphoenolpyruvate synthase converts pyruvate into phosphoenolpyruvate (PEP) an key step in gluconeogenesis using pyruvate or lactate as carbon source. PEP also utilized as an energy source for the phosphotransferase system uptake. Glycosyl transferase group I protein and glycogen/starch/alpha-glucan phosphorylase comes under glycosyltransferases and catalyzes the transfer of sugar moieties from activated donor molecules to the specific acceptors forming glycosidic bonds in the biosynthesis of disaccharides, oligosaccharides and polysaccharides. Regarding glycogen/starch/alpha-glucan phosphorylase is glycosyltransferase along with phosphorylase activity as these use phosphate to break alpha 1,4 linkages between pairs of glucose residues at the long glucose polymers end, releasing alpha-D-glucose 1-phosphate. The terminology is to preface the name according to the substrate, as in

glycogen phosphorylase, starch phosphorylase, etc. key function of glycogen phosphorylase is to provide phosphorylated glucose and regulated by allosteric affect mostly by phosphate molecule with the requirement of pyridoxal phosphate (PLP). Most of the phosphorylases role is still unclear, but thought of endogenous glycogen metabolism in periods of starvation, sporulation, stress response or quick adaptation to changing environments are possible.

c) Putative proteins

Putative proteins are the next most effected proteins about 10 % of the proteome, with 14 % down regulation and 7 % up regulation. The hypothetical RBXJA2T three forms were highly down regulated, 2-deacetyl-2-hydroxyethyl bacterochlorophyllide A dehydrogenase probably involved in the bacterial chlorophyll biosynthesis was down as observed physiologically also. Nitrite/sulfite reductase could be involved in the reduction of nitrite or sulfite useful in the process of oxidoreductase. The hypothetical proteins RBXJA2T two forms are up regulated whose function is not yet known. The putative outer membrane signal peptide protein hypothetically useful in the signaling process were also up regulated.

f) Transcription

In the protein synthesis machinery after the proteins involved in the translational process, proteins involved in the transcription are affected up to 7 % of the proteome. In this only 3 % are down regulated and 13 % of the up regulated proteins are observed. The down regulated protein is the two component LuxR family transcriptional regulator protein has a DNA-binding helix-turn-helix domain on the C terminal region and the N terminal region containing an auto-inducer binding domain or a response regulatory domain. This family proteins act as transcription activators, but some can be repressor or have dual role for different sites and control a wide variety of activities in biological processes. Some get activated by phosphorylation or binding to N-acyl homoserine lactones, which act as quorum sensing molecules in variety of gram negative bacteria.

The up regulated proteins include DNA-directed RNA polymerase subunit beta and beta prime, DNA-directed RNA polymerase subunit beta also known as DNA-dependent RNA polymerase catalyzes the polymerization of ribonucleotides into a sequence complementary to

the DNA template. Most RNA polymerases are multimeric having variable number of subunits but majorly five subunits are common they are two alpha, one each of beta, beta prime and omega sufficient for transcription, elongation and termination but sigma factor is required for initiation. Ribonucleases E is the enzyme related to RNA cleaving enzymes, along with ribonuclease G. mainly useful in maturation of 5' end of 16S RNA. It is the major subunit in degradosome of eukaryotes with a large multiprotein, multienzyme complex involved in RNA processing and degradation. Nitrogen regulatory protein P-II is a tetrameric protein involved in adenylation cascade in regulation activity and concentration of glutamine synthetase in response to the nitrogen source availability. In nitrogen limiting conditions when glutamine to 2-ketoglutamate decreases, P-II gets uridylylated to form P-II-UMP and allows the adenylation of glutamine synthetase by deuridylating it and also by controlling the transcription of glutamine synthetase by preventing phosphorylation of NR-I.

Osmolarity response regulator Protein involved in a system responding to environmental changes characterized usually by a sensor kinase in the cell membrane that phosphorylates itself in response to a signal and a response regulator to which the phosphoryl group is transferred. The responder is typically a DNA-binding protein that regulates transcription. Several of these systems are quite complex, involving many proteins in a signaling cascade or contributing to several responses simultaneously. They are involved in a variety processes such as chemotaxis, osmoregulation, magnesium transport, pH tolerance, sporulation, or response of virulent species to host cell's environments. TetR family transcriptional regulator are mainly involved in the tetracycline resistance by regulating the transcriptional control of the three types tetracycline resistance genes which are tetracycline efflux, ribosomal protection and tetracycline modification, by controlling the multidrug efflux pumps, control of catabolic pathways, differentiation processes and pathogenicity.

g) Purines and Pyramidines

Next are the proteins involved in the synthesis of purines and pyramidines with 7 % of the proteome with equal number of down and up regulated proteins 8 and 7 % respectively. Down regulated include phosphoribosyl-aminoimidazole succinocarboxamide synthase family members are involved in catalyses ATP dependent seventh step in de novo purine biosynthetic

pathway by converting 5'-phosphoribosyl-5-aminoimidazole-4-carboxylic acid and aspartic acid into phosphoribosyl-aminoimidazole-succinocarboxamide. Adenylate kinases catalyze the inter conversion of adenine nucleotide. Exodeoxyribonuclease or Exonuclease VII has two subunits one large and four small, useful in catalyzing exonucleolytic cleavage in either 5'-3' or 3'-5' direction to yield 5'-phosphomononucleotides.

Up regulated proteins include polynucleotide phosphorylase/polyadenylase has phosphorolytic domain with 3'-5' exoribonuclease activity with usage of phosphate as a nucleophile, adding it across the phosphodiester bond between the end two nucleotides in order to release ribonucleoside 5'-diphosphate from the 3' end of the RNA substrate. CTP synthetase catalyzes synthesis of CTP from UTP by amination of the pyramidine ring at the 4-position in pyrimidine ribonucleotide/ribonucleoside metabolism. Peptidase S9 prolyl oligopeptidase or post proline cleaving enzyme comes under proteolytic enzymes and grouped by their cleavage active site, it cleaves peptide bonds on the C-terminal side of prolyl residues.

h) Cellular processes

The cellular proteins affected due to indole are up to 7 % of the proteome, all of them are up regulated only, about 16 %. These include N-formlyglutamate amidohydrolase catalyzes the terminal reaction in the process of histidine utilization, where N-formyl L-glutamate is hydrolyzed to produce L-glutamate and formate. Glutamate 1-semialdehyde aminotransferase catalyses transamination of glutamate 1-semialdehyde to produce 5-aminoaevulinic acid during the tetrapyrrole biosynthesis by C5 pathway. Glutamine synthetase plays a essential role in nitrogen metabolism in catalyzing the condensation of glutamate and ammonia to form glutamine. PrkA family serine protein kinase helps in the reversible phosphorylation of proteins needed for the cellular activities along with phosphoprotein phosphatases. Protein kinases catalyze phosphorylation of protein substrates with gamma phosphate from nucleotide triphosphate (ATP) depending on the substrate specificity classified into three classes like serine/threonine, tyrosine or dual (both Thr and Tyr) specificity protein kinases.

In pyridoxal phosphate (PLP) dependent Tryptophanase/L-cysteine desulphohydrase, L-cysteine desulphohydrase is present most exclusively in nitrogen fixing bacteria belonging

epsilon proteobacteria and tryptophanase is common to most of the bacteria. Tryptophanase acts in reversible hydrolytic cleavage of L-tryptophan to indole and ammonium pyruvate, balancing the concentrations of both indole and tryptophan in the medium. Gamma glutamytransferase has a predicted role in assembly of spore coat proteins by breaking epsilon-(gamma-glutamyl) lysine cross links. Leucyl aminopeptidase comes under aminopeptidases family involved in processing and regular turnover of intracellular proteins, function is still unclear, leucine aminopeptidases cleave leucine residues from the N-terminal of polypeptide chains, substantial rates are evident for all amino acids. Phasin family proteins or phasins or granule associated proteins cover the surface of the polyhydroxyalkanoate storage granules in bacteria. Polyhydroxyalkanoates are linear polyesters produced by bacterial fermentation of sugars or lipids for carbon storage and energy purposes during the unfavorable conditions and for the stress resistance. Phasins stabilizes the granules and prevents concentration in the cytoplasm and nonspecific binding of other proteins to the hydrophobic surfaces of the granules.

i) Transport

Transport proteins constitute to 6 % of the proteome affected by indole, with 11 % down regulated and 2 % up regulated proteins. Down regulated proteins include polar amino acid transporter substrate binding protein or Amino acid permeases are integral membrane proteins involved in the transport of amino acids into the cell. TRAP dicarboxylate transporter subunit family have a three component system and are less characterized. These proteins involved in binding extracellular solutes for transport across the bacterial cytoplasmic membrane, includes sialic acid binding, periplasmic dicarboxylate binding protein, etc., proteins. Polyamine ABC transporter and maltose ABC transporter periplasmic protein comes under the ABC transporter system which is an ATP binding cassette super family. This family proteins function by binding to the ATP and in dimers consisting of two modules ATP binding and transmembrane. These proteins are involved in several physiological functions which include export of noxious substances and targeting of membrane components, which confer the organism with resistance to several fungal compounds by actively transporting out of the cell. Polyamine ABC transporter system have substrate binding proteins function as transporters and were not functionally fully characterized but some proteins with function of efflux pumps for molecules like indigoidine

pigment, amino acid metabolite, etc., were observed. Maltose ABC transporter periplasmic protein are the bacterial importers that transport the ligands to the extracellular of the transmembrane domain. These proteins have high specificity and affinity to the substrates. TonB dependent siderophore receptor are the class of proteins with "Ton-box" conserved sequence and have high specificity for carrying out active transport of iron, cobalamin and other molecules across the outer membrane by utilizing energy, as these small molecules are permeable through outer membrane. NMT1/THI5-like domain containing protein belong to the transport system with less functional characterization is the only up regulated protein.

J) Stress

Stress proteins affected are up to 5 % of the proteome, with 3 % down and 4 % up regulated, which are catalase/hydroperoxidase, heat shock protein Hsp20, chaperonin GroEL, endopeptidase La and UTP glucose 1-phosphate uridylyltransferase. The down regulated catalase/hydroperoxidase is haem containing bifunctional antioxidant enzyme exhibiting both catalase and peroxidase activities predominantly in bacterial species. These enzymes are for protection against oxidative stress by dismutating hydrogen peroxide to oxygen and water, present ubiquitously in aerobic bacteria.

The up regulated includes chaperonin GroEL or 60 belong to the subfamily chaperonins which help in the proper folding and subsequent assembly into oligomers by stabilizing and disassembly of polypeptides during the stress induced conditions. The chaperonin 60 have stacked rings structures assembled by self stimulation in presence of magnesium and ATP. It has probable role in response to hydrogen peroxide and inducing immune protection against unrelated bacterial infections. Endopeptidase La belong to the proteolytic enzymes with catalytic triad aspartate (electrophile), histidine (base) and serine (nucleophile). This signature defines the bacterial proteases which are ATP dependent and belong to the peptidase family S16. UTP glucose 1-phosphate uridylyltransferase or uridine diphosphoglucose pyrophosphorylase is responsible for the synthesis of UDP-glucose, an useful compound in the polysaccharide biosynthesis. It acts in the conversion of glucose 1-phosphate to UDP-glucose.

k) Fatty acid biosynthesis

The less number of affected proteins are the fatty acid biosynthesis proteins with 4 % of the proteome, in which mostly either not affected or up regulated with 4 %. The proteins include 3-oxoacid CoA transferase subunit B, 4-hydroxy 3-methylbut 2-en 1-yl diphosphate synthase and acetyl CoA carboxylase or carboxyl transferase subunits alpha and beta. 3-oxoacid CoA transferase subunit B catalyses the reversible transfer of coenzyme A from CoA-thioesters to free acids. 4-hydroxy 3-methylbut-2-en-1-yl diphosphate synthase or 1-hydroxy 2-methyl 2-(E)-butenyl 4-diphosphate catalyzes the conversion of 2C-methyl D-erythritol 2,4-cyclodiphosphate (ME-2,4CPP) into 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate in the non-mevalonate terpenoid biosynthesis, found mainly in bacteria. Acetyl CoA carboxylase or carboxyl transferase subunits alpha and beta catalyzing two step irreversible process of carboxylation of the carrier protein to form an intermediate and transfer of the carboxyl group from the intermediate to acetyl CoA forming malonyl CoA.

l) Energy metabolism

The least number of affected proteins belong to the energy metabolism with 3 % of the proteome all of them are up regulated only, with 7 %. These include the magnesium chelatase ATPase subunit I and F0F1 ATP synthase subunits beta and delta. Magnesium chelatase ATPase subunit I is one of the two ATPase subunits of the trimeric magnesium chelatase responsible for insertion of magnesium ion into protoporphyrin IX, an essential step in biosynthesis of chlorophyll and bacteriochlorophyll. F-ATPases are formed of two complexes F1 and F0 ATPases, F1 is the catalytic core with 5 subunits alpha, beta, gamma, delta and epsilon and F0 is the membrane embedded protein with 3 subunits. These enzymes use ion gradients to synthesize ATP, epsilon subunit appears to act as an inhibitor of ATPase activity.

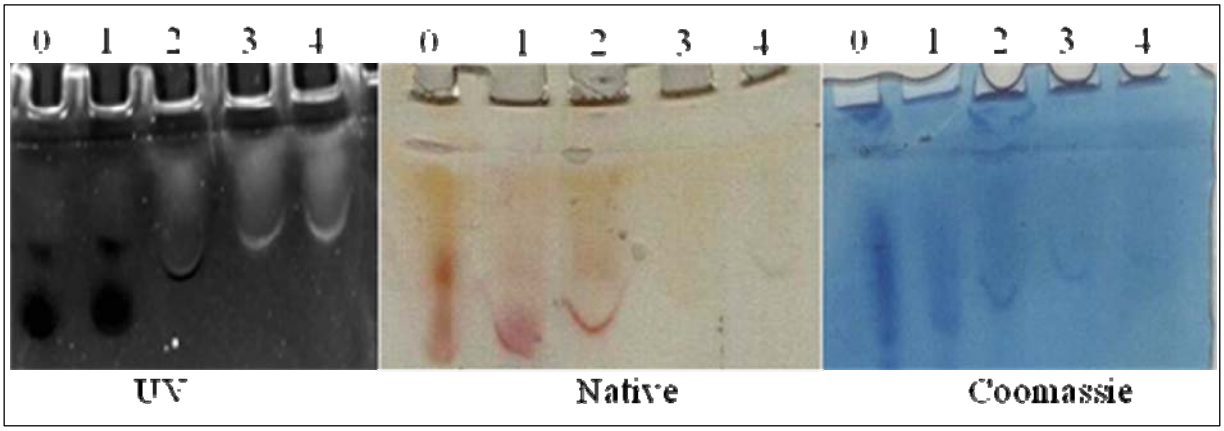


Figure. 17 6 % native gel picture of the membrane proteins.

Unstained 6 % native gel picture at the centre, same gel when observed under ultra violet light on left and on right after coomassie staining. Labels 0, 1, 2, 3 and 4 indicate the 0 (Malate and NH4Cl control), 1, 2, 3 and 4 mM concentration of indole induced cultures.

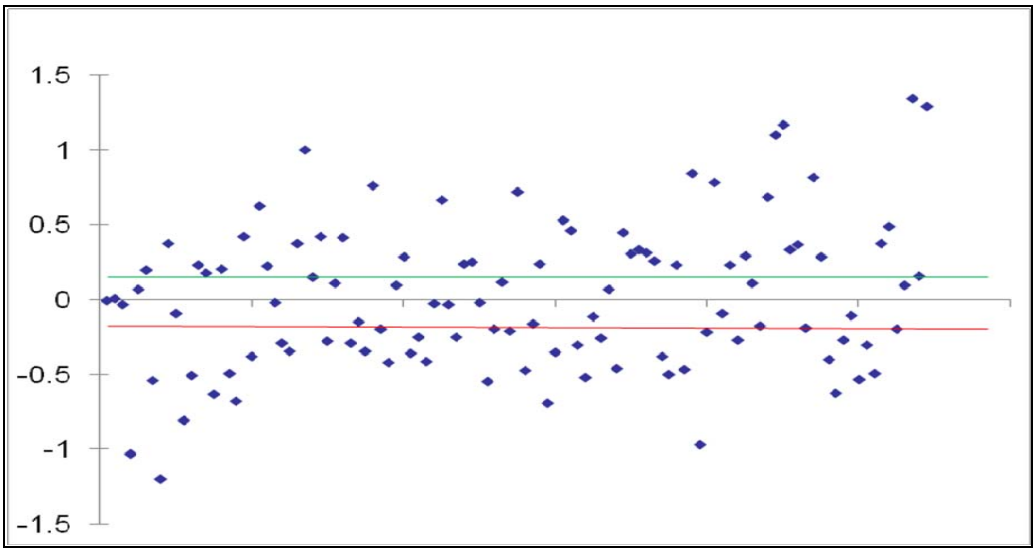


Figure.18 Differential expression of iTRAQ labeled proteins on incubation with indole (1 mM) by *Rubrivivax benzoatilyticus* JA2.

The $\log_{10}$ values of the P values with <0.05 were calculated and are plotted in a scattered plot with values between + and – 0.15 at the centre between the red and green lines which are unaffected proteins, with values above + 0.15 are above the green line which are up regulated and the values below -0.15 are below the red line are down regulated.

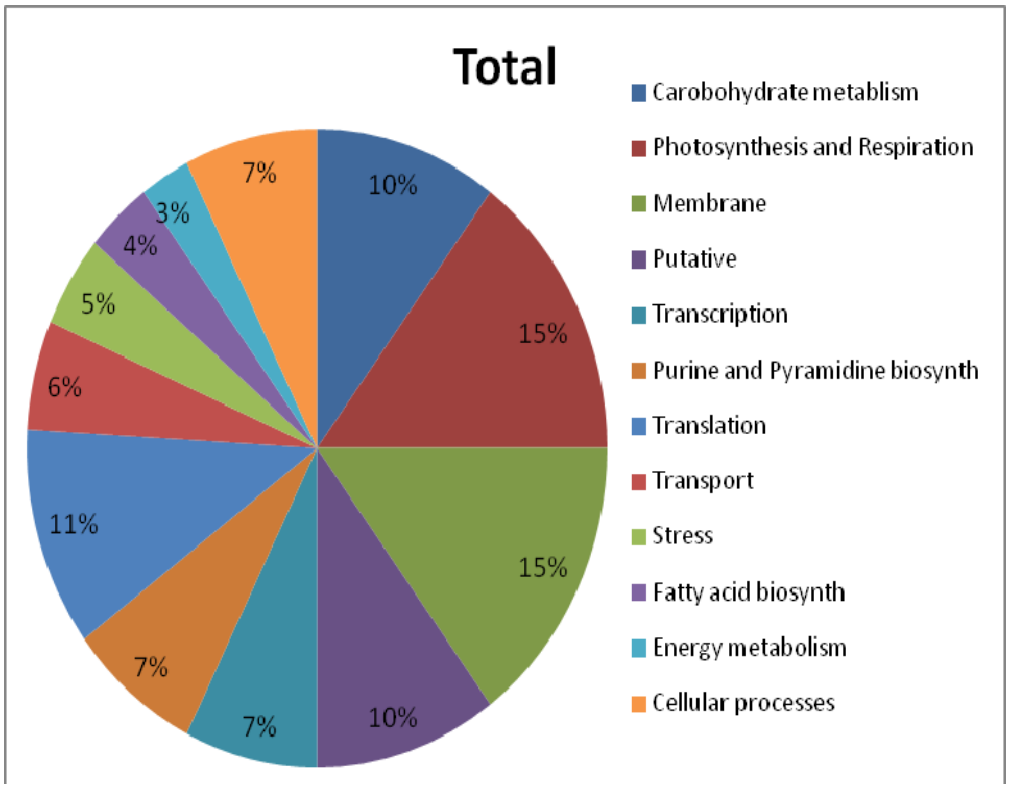


Figure 19 Distribution of affected proteins of indole (1 mM) induced culture by their function in accordance with the KEGG database.

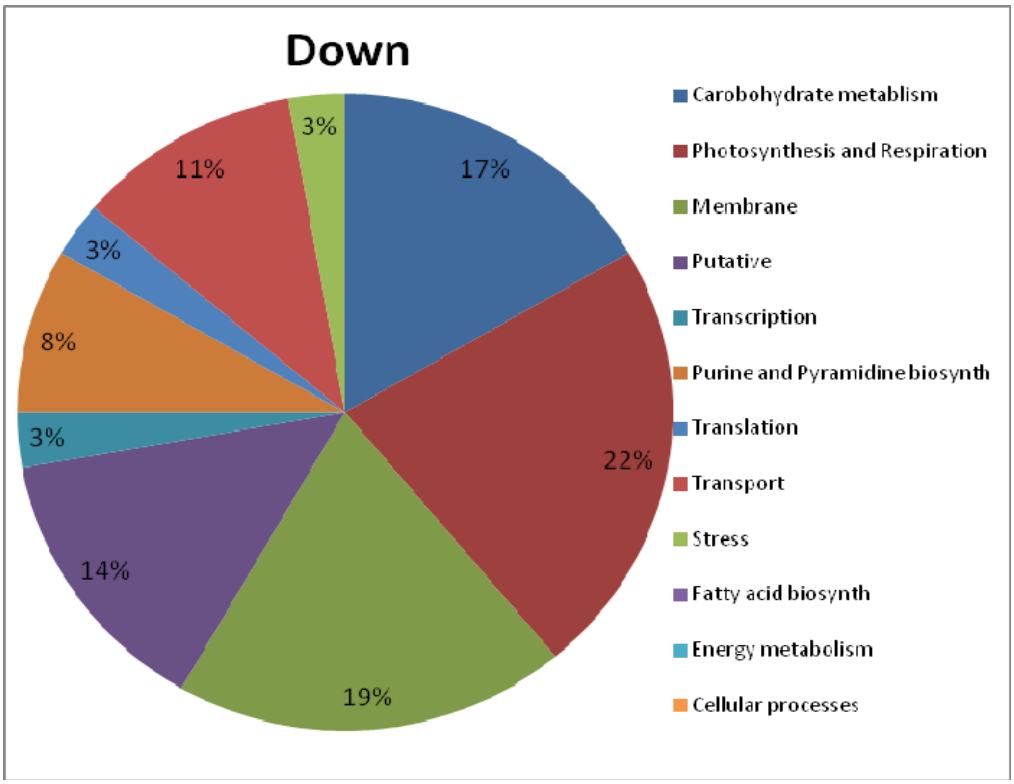


Figure.20 Distribution of down regulated proteins after isobaric labelling into different functional categories according to the KEGG database in presence of indole.

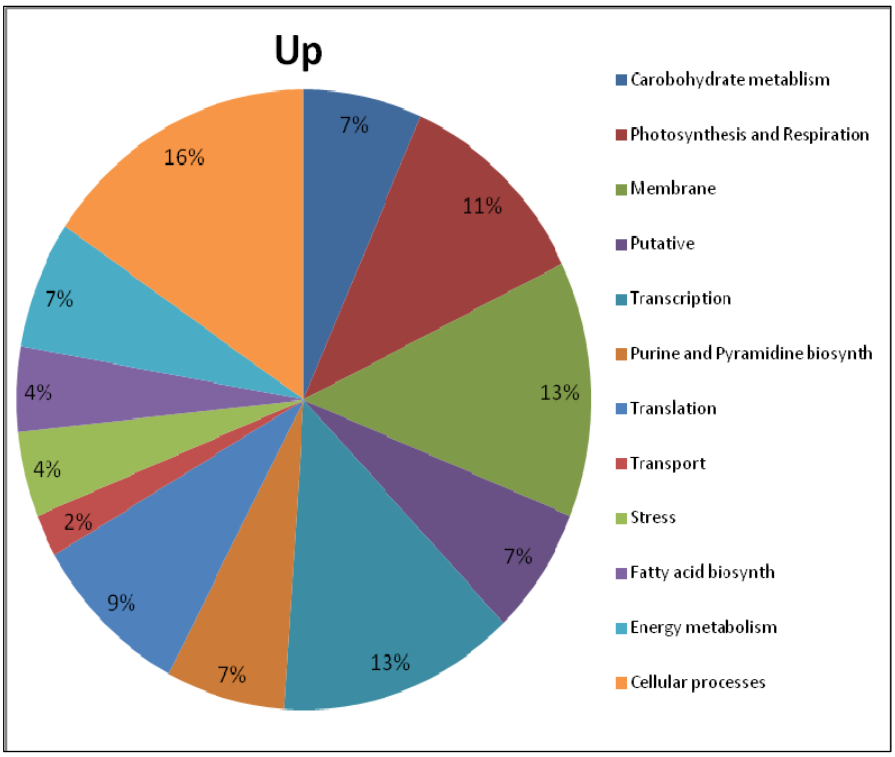


Figure.21 Distribution of up regulated proteins after isobaric labelling into different functional categories according to the KEGG database in presence of indole (1 mM).

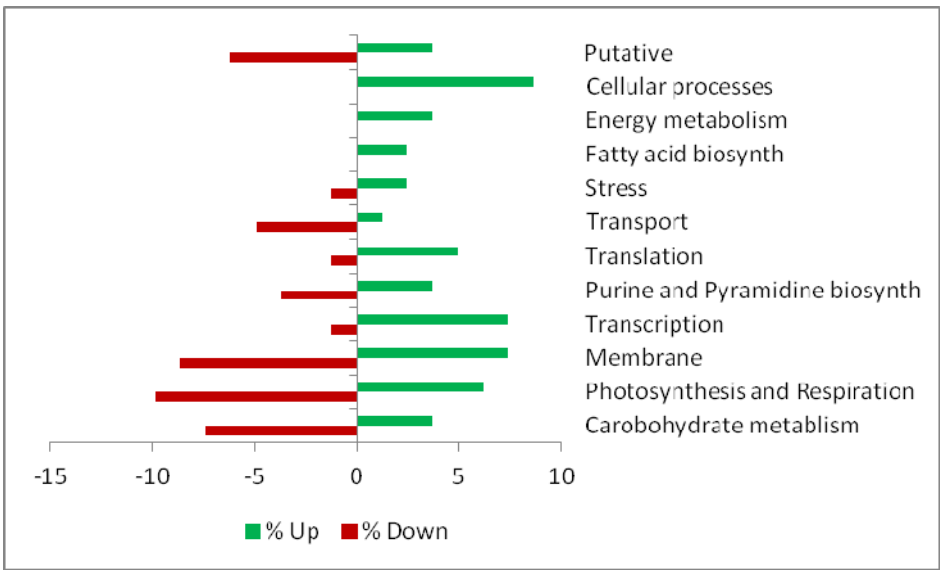


Figure.22 Differential expression of identified functional proteins in presence of indole.

Discussion

4. Discussion

The photosynthetic purple bacteria are the simplest photosynthetic organisms on earth. These bacteria are metabolically versatile organisms, having different growth modes along with the production of useful by-products like hydrogen, different nitrates and nitrites by atmospheric nitrogen fixation and also utilization of different toxic aromatic compounds either by degradation and/or biotransformation into useful products. All these characters are attributed to the protein coding sequences in their genome. The genomic sequencing reveals the details of the metabolic capability and the proteins involved in these pathways and the strategies and adaptations of the organism in response to the environmental conditions. Understanding the metabolic pathways will help in harnessing the by-products and also utilization of the organism in different applications like biotransformations, degradations and other processes. Only about 6 % of the genome sequenced bacteria belong to the photosynthetic bacteria, which gives much scope and advantage in understanding the photosynthetic bacteria by genome sequencing.

4.1 Insights of *Rubrivivax benzoatilyticus* JA2 genome

More than half of the coding sequences in the genome codes for different metabolic processes intimates the metabolic capability of the organism to adapt and respond to the variation in the environmental conditions for the survival. The key homologous gene sequences are involved in the photosynthesis, respiration, carbon and nitrogen fixation, aromatic metabolism, polyhydroxyalkanoate metabolism, signaling, transport, biodegradation and other processes. *Rubrivivax benzoatilyticus* JA2 was the first organism to be genome sequenced with accession number AEWG00000000 (Mujahid et al., 2011), in the genus *Rubrivivax* and the gene coding sequences were homologous matched with the known phototrophic bacteria. Key enzyme coding sequences BLAST search had shown maximum gene coverage and similarity (>95 %) with the *Rubrivivax gelatinosus* IL144 strain with accession number AP012320 (Nagashima et al., 2012), the gene coding sequences absent in the strain *Rubrivivax gelatinosus* are having maximum similarity with other phototrophic organisms like *Rhodobacter sphaeroides* strain KD131 (Lim et al., 2009) with accession number PRJNA31111 and *Rhodospirillum rubrum* strain ATCC 11170 (Munk et al., 2011) with accession number PRJNA57655. One of the major difference

between the *Rubrivivax benzoatilyticus* JA2 and *Rubrivivax gelatinosus* is the gelatin liquefaction ascribed to the presence of genes that possibly code for extracellular serine proteases and oligopeptidase (Nagashima et al., 2012), presence of these coding genes does not contribute the character to the *Rubrivivax benzoatilyticus* JA2.

In the draft genome the photosynthetic genes are arranged as the photosynthetic gene cluster (PGC) in *Rubrivivax benzoatilyticus* JA2 and the arrangement is found to be *crtF-bchCXYZ-puf* type with *puf* gene organization as *pufBLMC* (Zheng et al., 2011) and BChl *a* gene arrangement is *bchCXYZ* having a length of about 25.44 kb. Regulator of photosynthetic gene cluster like *ppsR* is present in the draft genome having sensitivity towards light intensity and oxygen concentrations (Komiya, 1998) as observed in most of other photosynthetic bacteria. While the precursor for carotenoids the terpenoid units are biosynthesized by the erythitol phosphate pathway as observed in the plastids of plants and most of the bacteria (Rohmer 1999; Rodriguez- Concepcion and Boronat 2002; Dubey et al., 2003) with all the genes present scattered in the genomes

Different gene coding sequences for the carbon dioxide fixation and photophosphorylation function along with the dehydrogenases, oxidoreductases and carbon degradation pathways support the organism in different growth modes like chemo-organoheterotrophy, photo-organoheterotrophy and photolithoautotrophy. With the help of the bacteriophytochrome like sensory protein might be helping the bacteria in detection of light intensity and switch between the growth modes, which is restricted to few photosynthetic bacteria (Larimer et al., 2004).

The gene coding sequences in the tryptophan operon are in the form of two broken clusters in the genome separated by a distance of about 500 kb. The broken clustered type of tryptophan operon usually found in archeal genomes (Merkl, 2007).. The clusters have the sequence of *trpCDE* and *trpABF* gene coding sequences.

Two different nitrogenases and different oxidoreductases presence were found only after genome sequencing of strain JA2 useful in fixing atmospheric nitrogen. The nitrogenases include the general and molybdenum-iron containing nitrogenases and does not contain the vanadium

containing nitrogenase as observed in the *Rhodopseudomonas palustris*. The genes coding for these nitrogenases are present in the form of two gene clusters in the sequence of *Rubrivivax benzoatilyticus* JA2 along with all the accessory proteins for the cofactors and also the regulators with a gap of about 1.4 kb, in contrast to the four gene clusters as observed in *Rhodobacter capsulatus* (Masepohl et al., 2004). The genome consists of the gene coding sequences for the denitrification which include the reductases, one nitrate reductase (Nar), nitrite reductase (Nir), one putative nitric oxide reductase (Nor) and without nitrous oxide reductase (Nos), as denitrification helps to dispose excess reducing equivalents, respiratory function, redox balancing or to reduce the toxicity of certain nitrogen oxide intermediates (Shapleigh, 2009).

Genome consists of the gene coding sequences for the production of polyhydroxy-alkanoate synthesis through the butyrate biosynthesis pathway, which include the poly-hydroxyalkanoate synthase, polyhydroxyalkanoate depolymerase and a repressor, and possibly be a potential organism for the biosynthesis.

Different enzymes like monooxygenases, dioxygenases, peroxidases, dimethylaniline monooxygenase and 2-haloacid dehydrogenase enzymes are useful in degradation or biocatalysis or dehalogenation of wide variety of molecules explains the photoheterotrophic growth of the organism on different aromatic compounds (Larimer et al., 2004). The phenylacetate CoA ligase might be involved in the growth of *Rubrivivax benzoatilyticus* JA2 on the phenylacetate media (Hirsch et al., 1998). Presence of 4-hydroxybenzoyl-CoA thioesterase and benzoyl-CoA oxygenase component A and benzoate transport protein coding sequence in the genome could be the possible reason for the survival of *Rubrivivax benzoatilyticus* JA2 in benzene containing media (Nishikawa et al., 2008), as the key enzyme for benzene metabolism like benzyl-CoA reductase were not found in the draft genome may not be utilizing benzene and could possibly an acquired character through horizontal gene transfer.

The gene coding sequences for more than 300 signaling proteins, above 30 types of response and transcription regulators and different transport proteins present in the genome are responsible for the organism to sense, respond and to adapt to the changes in the surroundings

and also during different stress and growth conditions. This explains the survival ability and could be reason for the resistance to various aromatic and/or toxic compounds.

4.2 Effect of indole on *Rubrivivax benzoatilyticus* JA2

Colour variation of the *Rubrivivax benzoatilyticus* JA2 observed was due to the change in the pigment concentration with increase in indole concentration above 2 mM (Fig. 8). Spectroscopic observations denoted the loss of chlorophyll at 775 nm and also decrease or loss of carotenoids having an absorbance between 400 to 600 nm, and increase at 350 to 400 nm representing the carotenoid precursors and other molecules (Fig. 3). The native carotenoids like spheroidene and didehydrolycopene present in major concentrations in normal conditions were surpassed by a novel minor carotenoid present in native or uninduced condition, a 20 fold increase in concentration was observed in induced condition (Fig. 4). From the elution patterns the enhanced carotenoid was much polar than other carotenoids and could be an oxidized form having a ketone functional group, with a 363 *m/z* value (Fig. 5). As reports suggest that inhibition of expression of photosynthetic genes in oxygenic conditions by the anoxygenic photosynthetic bacteria (Joshi and Tabita, 1996; Gregor and Klug, 1999; Elsen et al., 2000; Jutta and Klug, 2002; Purcell and Crosson, 2008; Spring, 2009) and production of reactive oxygen species by indole, the photosynthetic gene cluster which includes the carotenoids, bacteriochlorophyll and other accessory protein coding sequences could be inhibited in *Rubrivivax benzoatilyticus* JA2 when exposed to indole.

a) Metabolism of indole by *Rubrivivax benzoatilyticus* JA2

As observed in many of the anoxygenic phototrophic bacteria, *Rubrivivax benzoatilyticus* JA2 shows photoheterotrophy by utilizing some of the aromatic compounds as nitrogen and/or additional source only, for the heterotrophic growth carbon source like malate or pyruvate are a must (Ramana et al., 2006). In presence of aromatic compounds like benzene and others it has shown resistance than utilization (Table. 6), this could be due to the presence of different transporter proteins like the benzoate transporter as represented in the draft genome. In the strain JA2 indole utilization was a light dependent process as well as its production in this bacterium (Ranjith et al., 2010), and the consumption of indole was four folds higher in anaerobic

conditions than in aerobic conditions as mentioned in the results (Table. 7). Indole utilization as nitrogen, when replaced with NH_4Cl, was higher up to four folds than indole as additional supplement in the media.

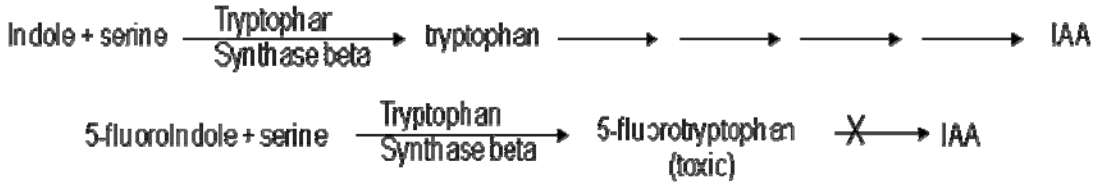
Indole 3-acetic acid (IAA) production was reported in many bacteria and over 80 % of the bacteria isolated from the rhizosphere are capable of IAA synthesis (Patten and Glick, 1996; Glick et al., 1999; Khalid et al., 2004), most requiring tryptophan as a precursor (Ryu and Patten, 2008). From indole supplemented in the media *Rubrivivax benzoatilyticus* JA2 had produced four molecules, by HPLC and LC-MS analysis were identified as tryptophan (205 *m/z*), indole 3-acetic acid (176 *m/z*) and two indole dimmer like molecules (261 and 247 *m/z*). Tryptophan and IAA were identified with the help of standards having the same retention times (t_R) and absorbance maxima with the help of photodiode array detector enabled HPLC system. The tryptophan and IAA mass fragmentation pattern analyzed on LC-MS (Fig. 8 and 9) matched with the online database (http:www.massbank.jp). Production of tryptophan, and due to increased availability of the tryptophan as biosynthetic precursor and/or as a requirement for induction of the genes in the IAA biosynthetic pathway, might have resulted in IAA production (Ryu and Patten, 2008). Multiple tryptophan-dependent biosynthetic routes to IAA are reported in microorganisms (Patten and Glick, 1996; Glick et al., 1999). Presence of the coding sequences for the enzymes like indole 3-pyruvate decarboxylase (Zimmer et al., 1998) and nitrilase (Carreno-Lopez et al., 2000) in the genome explains the production of IAA by the strain JA2. Absence of indole 2,3-dioxygenase, indole hydroxylase, indole monooxygenase, indole oxidase and indole oxygenase in the genome explains the inability of the strain JA2 for aromatic ring breakage and are in acceptance with the HPCL and LC-MS observations.

b) Tryptophan dependent IAA biosynthesis by *Rubrivivax benzoatilyticus* JA2

Indole acetic acid is synthesized mainly by two different pathways, they are tryptophan dependent and independent. These pathways are investigated by using indole analogues which are competitive inhibitors for the tryptophan synthase enzyme, they are 5-fluoroindole, 2-fluoroindole, 2,3-dihydro-L-tryptophan, oxindolyl-L-alanine, and others. 2-fluoroindole and 5-fluoroindole are utilized by tryptophan synthase and converted into respective 2 or 5-

fluorotryptophan, which are toxic to cell and inhibits the growth by interference in all the vital activities of the cell like protein synthesis, aromatic amino acid synthesis and also other aromatic compound synthesis (Ilic et al., 1999).

5-fluoroindole (>0.35 μM) has inhibited the *Rubrivivax benzoatilyticus* JA2 growth (Table. 3), the growth inhibition could be due to the formation of 5-fluorotryptophan as observed in the bacteria *Pseudomonas putida* and *Pseudomonas aeruginosa* (Widholm, 1981) and in fungi *Saccharomyces cerevisiae* and *Coprinus cinereus* (Miozzari et al., 1977; Tilby et al., 1978) and in higher plants and their cell cultures (Widholm, 1972; Singh and Widholm, 1975), no biomass yield and intermediates were detected as growth was inhibited. Concentrations <0.35 μM had not inhibited the growth, as 5-fluorotryptophan was reported as a competitive inhibitor of the tryptophan for the tryptophan synthase (Ilic et al., 1999), none of the intermediate products were detected at that low quantities.



With other indole substituents like 2-methylindole, methyltryptophan production has been indicating the activity of tryptophan synthase (Barlati and Ciferri, 1970). Therefore with similar type of molecules like indole, 5-fluoroindole and 2-methylindole substituted in the media, products of tryptophan, 5-fluorotryptophan and methyltryptophan were produced respectively with the activity of the enzyme tryptophan synthase. From these studies indole utilization by *Rubrivivax benzoatilyticus* JA2 in the production of IAA was indicative of tryptophan dependent pathway only.

For confirming the indole metabolism pathways and to identify the products formed from indole, stable isotope labeling studies were carried out. It is a non toxic and radiation free method for probing studies and can be carried out only with help of mass fragmentation analysis or/and nuclear magnetic resonance (NMR) studies by detecting the stable isotope atoms like $C^{13}$and N^{15} with the difference in mass and difference in the orientation of the electron spin. For the study

C^{13} and N^{15} atoms stable isotopes in the indole moiety are used, total of eight carbon atoms and one nitrogen with a final mass difference of +9 with that of the normal indole. Stable isotope method is very accurate method as the natural occurrence of C^{13} molecules is 1.1 % only and N^{15} isotope is very rare. The isotopic enrichment of any given precursor should be reflected in the product (Normanly et al., 1993). Once incorporated into a ring structure, the stable isotopes were non-exchangeable (Cohen et al., 1986). A total of 10 standard compound peaks have eluted in the HPLC, in which five elution peaks have variation of mass +9 and +18, +9 difference as explained and +18 *m/z* was due to the formation of indole dimerization. In the five compounds indole was the one supplemented in the media and in the remaining four eluted compound masses, two are identified as the products tryptophan and indole acetic acid, and two unidentified indole dimers.

The mass peak at t_R 15 min (Fig. 8) having a value of 205 *m/z* and 214 *m/z* in normal and stable indole samples are matching with the standard tryptophan mass fragmentation in the mass bank. The standard tryptophan has the mass fragments of 205 and 188 *m/z* are identical as observed in the normal indole sample and mass fragments of 214 and 197 *m/z* are observed with +9 difference in the indole stable isotope sample.

The mass peak at t_R 32 min (Fig. 9) having a value of 176 *m/z* and 185 *m/z* in normal and stable indole samples are matching with the standard tryptophan mass fragmentation in the mass bank. The standard IAA has the mass fragments of 176, 144 and 130 *m/z* are identical as observed in the normal indole sample and mass fragments of 185, 153 and 139 *m/z* are observed with +9 difference in the indole stable isotope sample.

Two indole dimer molecules were identified in the LC-MS analysis. The mass peak at t_R 44 min having a value of 261 *m/z* and 279 *m/z* (Fig. 11) in normal and stable indole samples has similarity with the standard indigo *m/z* value of the mass bank. The standard indigo has the mass fragments of 262 *m/z* similarly in the normal indole the dimmer molecule having 261 and 144 *m/z* and mass fragments of 279 and 153 *m/z* are observed with +18 and +9 difference in the indole stable isotope sample. Its an indigoid like molecule having a different fragmentation pattern but have the same indigo *m/z* value. The mass peak at t_R 35 min having a value of 247 and 265 *m/z* (Fig. 10) in normal and stable indole samples did not match with any of the standard

mass fragmentation in the mass bank. There was a difference of +18 between the normal and stable isotope referring to the indole dimer, the molecule characterization has to be done. The mass fragmentation pattern support the data of the HPLC of production of tryptophan and indole acetic acid from the indole molecule, confirms the tryptophan dependent indole acetic acid pathway in correlation with the inhibitor studies. The external supplemented indole in the media was converted into useful product of tryptophan and further utilized in the metabolic activities like indole 3-acetic acid biosynthesis, production of dimmers of indole like indigo and others, and also might be channelized into other functions like protein synthesis and secondary aromatic metabolite production. The monooxygenases and peroxidases (Burd et al., 2001) present in the genome and also electron-rich nature such as hydrogen peroxide and others easily oxidizes indole (Kanaoka et al., 1971), could explain the formation of the indole dimmers by the strain JA2.

c) Proteomic response of *Rubrivivax benzoatilyticus* JA2 to indole induction

The response of *Rubrivivax benzoatilyticus* JA2 on indole induction was studied by the native PAGE and iTRAQ studies. The whole proteome of *Rubrivivax benzoatilyticus* JA2 along with the membrane proteins were isolated as mentioned in the methods by the use of detergent Triton x-100. The isolated membrane proteins are electrophoresed on a 6 % native polyacrylamide gel for separation of membrane protein complexes. The unstained electrophoresis gel has red coloured bands, when the same gel on staining with PDBA reagent the coloured bands had given positive colouration for indole and on observation under UV light the bands were fluorescing, due to the presence of fluorescent indole molecule. On staining the gel with comessie the coloured and fluorescing bands had stained for proteins, indicating the presence of coloured pigments bound to the proteins along with indole. The indole binding to the complex could be due to the weak bonding of indole to the carotenoid binding proteins, as the indole group nitrogen atom of tryptophan in the carotenoid binding protein forms hydrogen bond interaction with the methoxy oxygen of carotenoid spheroidenon (Aleksander et al., 2004). These proteins might be having similar function as orange carotenoid proteins reported in the photosynthetic cyanobacteria useful in quenching the photo-oxidative species (Kerfeld C. A., 2004; Kirilovsky, 2007). Upon the MALDI analysis of the complex from the native PAGE, one

of the protein was identified as outer membrane autotransporter barrel protein, might be having a key role in the transport of proteins to the surface of the cell for tackling the indole, as it helps in the transport of the protein to the cell wall or to the extracellular. Key feature of an auto-transporter is that it contains all the information for secretion in the precursor of the secreted protein itself (Henderson et al., 1998; Koebnik et al., 2000). Auto-transporters comprise three functional domains:

a) an N-terminal targeting domain (amino-terminal leader sequence) that functions as a signal peptide to mediate targeting to and translocation across the inner membrane,

b) a C-terminal translocation domain (carboxy-terminal) that forms a beta-barrel pore to allow the secretion of and

c) the passenger domain, the secreted mature protein.

Global proteomes of control, without any indole and 1mM indole cultures were compared in the late log phase of their growth to look into details of survival and metabolic strategies of *R. benzoatilyticus* JA2 when induced with 1 mM indole. After the acclimatization process in the lag phase for 48 h, till the 96 h a log phase was observed, at this phase cultures were isolated and complete proteome samples were taken as described in the methods. Membrane proteins (15 %) were the most effected proteins in the iTRAQ analysis followed by photosynthesis and respiration (15 %), translation (11 %), carbohydrate metabolism (10 %), putative (10 %), transcription (7 %), purine and pyramidine biosynthesis (7 %), cellular processes (7 %), transport (6 %), stress (5 %), fatty acid biosynthesis (4 %) and the less effected were the energy metabolism (3 %) proteins of the identified proteins.

Membrane proteins are the most affected due to indole as they are the barriers between the extracellular media and the cell (Sikkema et al., 1995; Kawamura et al., 1999; Garbe et al., 2000), it includes the proteins of membrane transport and triggering the signaling system for the survivability of the cell, along with the suppression of the unwanted extracellular solute binding proteins. The photosynthetic and respiratory system proteins are also highly affected as they are involved in the carbon assimilation and power generating systems of the cell along with the biosynthesis of the carotenoids useful in the scavenging of the reactive oxygen species and photosynthesis, are located in the light harvesting complexes when induced with indole. The

inhibition of the reaction centre proteins could be due to the oxygen sensitive suppression of the PGC (Gregor and Klug, 1999; Purcell and Crosson, 2008), as indole induces reactive oxygen species (Kawamura et al., 1999; Garbe et al., 2000; Hwang et al., 2011). In the protein synthesis the aliphatic amino acid biosynthesis has been enhanced as the aromatic amino acid and nitrogen requirements are met from the tryptophan synthesized from the indole supplied. In case of translation the regulatory proteins were affected including RNA polymerases, nitrogen regulatory protein and other regulatory proteins useful in the stress conditions are up regulated, as nitrogen biosynthesis is up regulated in bacteria during low concentrations of ammonium and oxygen present in the environment (Schuddekopf et al., 1993; Masepohl et al., 2002).

IAA up regulates the genes involved in the central metabolic pathways such as the tricarboxylic acid cycle (TCA), glyoxylate shunt and amino acid biosynthesis (leucine, isoleucine, valine and proline) (Bianco et al., 2006b), in contrast the carbohydrate metabolism proteins of the TCA were down regulated affecting both carbon assimilation and energy production in the strain JA2. And at the same time proteins useful for the glycogen/starch synthesis were enhanced, helping the strain JA2 for the survival in the starvation conditions. Putative proteins were also significantly affected due to indole induction, there functions are not yet known. In the purine and pyramidine biosynthesis the *de novo* biosynthesis was affected along with the enzymes useful in the phosphorylation and polyadenylation representing the inhibition of the cell division. In the cellular processes protein kinases, tetrapyrrole biosynthesis, nitrogen metabolism, polyhydroxyalkanoates and phasins are affected. In case of the proteins involved in the transport system mainly the ABC type amino acid transporters, membrane permeases, polyamine and maltose transporters are affected. At 1 mM concentration of indole only few heat shock and the chaperonin GroEL peptidases useful in the polysaccharide biosynthesis were affected, representing that the cell was not facing stress conditions, as the reported inhibitory concentration is above 2 mM of indole (Lee et al., 2009) in some bacteria. The fatty acid biosynthesis were up regulated indicating the need of stabilization of the membrane and also the synthesis of the terpenoid. Energy metabolism proteins are the least affected and mainly the ATPases are affected are enhanced to meet the energy requirements of the cell.

Conclusions

Conclusions

- ✓ The versatile growth modes (photo-organoheterotrophy, chemo-organoheterotrophy and photolithoautotrophy) of *Rubrivivax benzoatilyticus* JA2 might be due to the gene coding sequences in the genome for different signaling and metabolic processes for sensing and adapting to the fluctuating supplies (carbon, nitrogen, light and oxygen) and other environmental conditions.
- ✓ *Rubrivivax benzoatilyticus* JA2 genome consists of indole 3-acetic acid biosynthesis gene coding sequences and the production of IAA with tryptophan proposes a tryptophan dependent pathway.
- ✓ *Rubrivivax benzoatilyticus* JA2 genome has the coding sequences for the biosynthesis of spheroidene and spirilloxanthin carotenoids. A gradient increase of novel carotenoid with increase of exogenous indole may have a protective role against indole toxicity, as carotenoids are known for their reactive oxygen species scavenging activities.
- ✓ The outermembrane autotransporter barrel protein of the indole-pigment protein complex of *Rubrivivax benzoatilyticus* JA2 might be involved in the indole detoxification process by acting as platform for the interaction of the toxic exogenous indole and the proteins.
- ✓ *Rubrivivax benzoatilyticus* JA2 adapts to the indole toxicity possibly by altering the membrane structure by the expression of membrane integrity proteins, enhancing the proteins involved in indole utilization (nitrogen source) and the proteins for energy production.

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