Genetic modification of *Citrobacter* DHRSS and *Pseudomonas fluorescens* 13525 for enhanced citric acid and oxalic acid secretion

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STATEMENT UNDER O. Ph.D. 8/(iii) OF

THE M. S. UNIVERSITY OF BARODA, VADODARA

This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of **Dr. G. Naresh Kumar.**

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This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph. D. degree of The M. S. University of Baroda by Ms. Kavita Yadav is her original work. The entire research work and the thesis have been built up under my supervision.

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Dedicated to my

Beloved Mother

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Kavita Yadav

List of Abbreviations used

ATCC	American type culture collection
MTCC	Microbial type culture collection
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D ga*lac*topyranoside
IPTG	Isopropyl β-D thio ga*lac*topyranoside
LB	Luria broth
SDS	Sodium Dodecyl Sulphate
Ori	Origin of replication
WT	Wild type
PPC	Phosphoenolpyruvate carboxylase
CS	Citrate synthase
OAH	Oxaloacetate acetyl hydrolase
ICL	Isocitrate lyase
ICDH	Isocitrate dehydrogenase
G-6-PDH	Glucose-6-phosphate dehydrogenase
PEPCk	Phosphoenolpyruvate carboxykinase
PYC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
PTS	Phosphotransferase system
G-6-p	Glucose-6-phosphate
ATP	Adenosine triphosphate
PEP	Phosphoenolpyruvate
OAA	Oxaloacetate
DNA	Deoxyribonucleic acid
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
DTT	1, 4-Dithiothreitol
EMP	Embden–Meyerhof–Parnas pathway
TCA	Tricarboxylic acid
PPP	Pentose phosphate pathway

ETC	Electron transport chain
ED	Entner-Doudoroff
PSM	Phosphate solubilizing microorganisms
PSB	Phosphate solubilizing bacteria
P/Pi	Phosphate / inorganic phosphate
HPLC	High performance liquid chromatography
BLAST	Basic local alignment search tool
PCR	Polymerase Chain Reaction
PAGE	Polyacrylamide Gel Electrophoresis
EDTA	Ethylene diamine tetra acetic acid
Tris	Tris (hydroxymethyl) aminomethane buffer
CTAB	Cetyl- trimethylammonium bromide
RE	Restriction Endonuclease
nm	Nanometer
ng	Nanogram
kb	kilobase (s)
bp	base pair (s)
kDa	Kilodalton
rpm	Revolutions per minute
OD	Optical Density
DCW	Dry cell weight
mM/M	Millimolar/Molar
µl/ml	Microlitres/milliliters
Ap	Ampicillin
Str	Streptomycin
Km	Kanamycin
Gm	Gentamycin

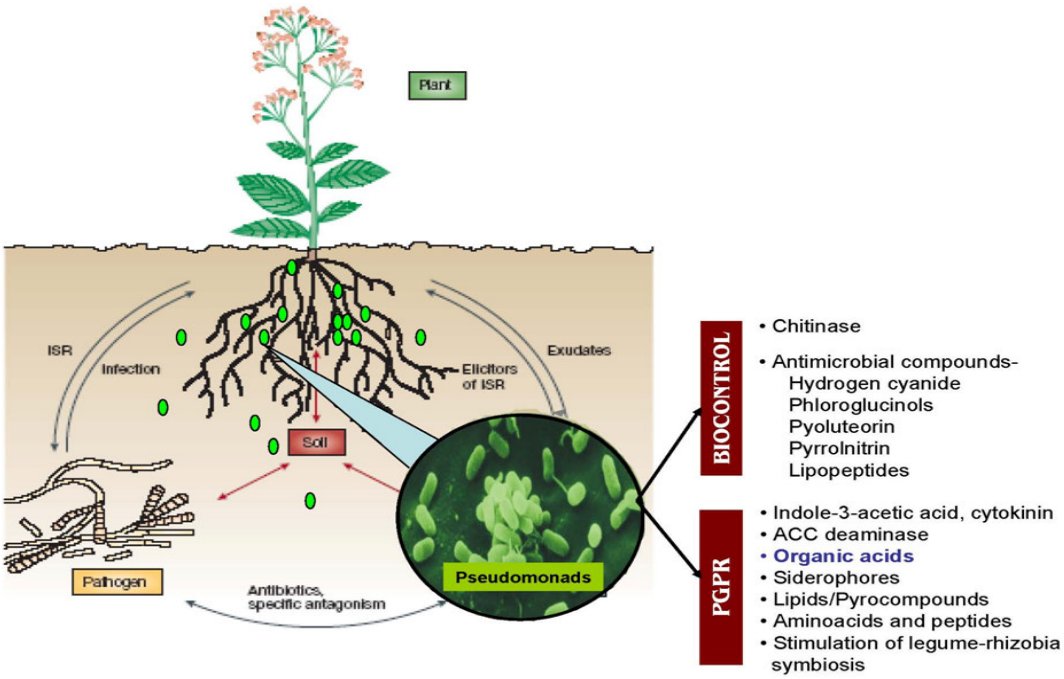
Note: The full forms of several rarely used abbreviations have been described within the text.

TABLE OF CONTENTS

Genetic modification of *Citrobacter* DHRSS and *Pseudomonas fluorescens* 13525 for enhanced citric acid and oxalic acid secretion

LIST OF FIGURES

LIST OF TABLES



CHAPTER 1

Review of Literature and Introduction

1.1 Metabolic Engineering

Metabolic engineering is a process of optimizing genetic architecture and regulatory process within the cell to achieve desired phenotype or production of certain substances using the tool for genetic engineering (Bailey, 1991; Stephanopaulous et al., 1998; Nielsen, 2001; 2003). Process of developing desired recombinant strain required several round of modifications (Nielsen, 2001). This cyclic process is required to integrate several discipline including mathematics, chemical engineering, biochemistry, microbiology, bioinformatics and the most emerging field of recombinant DNA technology (Fig. 1.1). The technical manifestation of metabolic engineering involves manipulations of enzymatic, transport and regulatory functions of cell using r-DNA technology (Bailey, 1991; Stephanopaulos and Vallino, 1991; Koffas et al., 1999). Hence, metabolic engineering has two main stages (i) carefully analyzing cellular networking to suggest possible targets, and (ii) introducing directed genetic modification using r-DNA technology (Ostergaard et al., 2000). Metabolic engineering is a direct improvement process of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology. An essential element of this definition is the specificity of the biochemical reaction(s) targeted for modification or to be newly introduced. "The directed improvement of product formation or cellular properties through the modification of specific modification like amplify, inhibit or delete, transfer, or deregulate the corresponding gene(s) or enzyme(s) of specific biochemical reaction(s) with the use of recombinant DNA technology" was coined as **Metabolic engineering** (Stephanopoulos., 1998).

In 1973, Cohen and coworkers reported the first successful genetic modification of *E. coli*. Which open the new avenue in the field of genetic engineering confer organisms with novel

configuration resulting in beneficial properties. Classical approach of genetic modification involved extensive use of chemical mutagens leading to random mutagenesis and screening for strains with superior properties of product formation or growth. This method was well exploited for the production of antibiotics, solvents and vitamin (Cameron and Tong, 1993; Koffas et al., 1999; Nielson, 2001). Today genetic engineering allows directed modification in the enzymatic reaction of the metabolic network leading to better product formation or redirection of cellular metabolism thus leading to enhanced product formation.

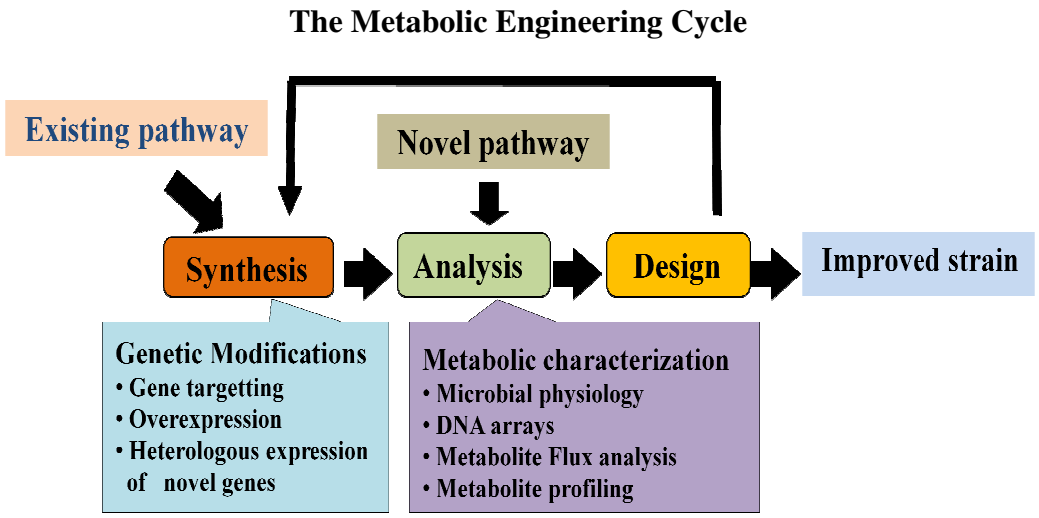


Fig. 1.1: The cycle of metabolic engineering (Thykaer and Nielsen, 2003).

1.1.1 Concepts of Metabolic Engineering

Metabolic engineering emphasis on integrated metabolic pathway compared to an individual reaction so it includes complete biochemical reaction network, pathway synthesis output, thermodynamic feasibility, pathway flux and control. So there are paradigm shift away from individual enzymatic reactions and toward interactions of biochemical reactions participating in a metabolic network. Main focus on metabolic network in the sense that an enhanced perspective of metabolism and cellular function can be obtained by considering reactions in their entirety rather than in isolation from one another. Metabolic engineering

depends on the whole system instead of its constituent parts. Metabolic engineering thus seeks to synthesize and design using techniques and information developed from extensive reductionist research. The basic steps involving metabolic engineering are: identifying the rate limiting steps in the biochemical network based on the carbon-flux distributions and using this information for further applications or construction of recombinant strain for efficient product formation or lesser by-product outflow and based on the results targeting the second modification. Gathering the information from both the strategies further modification employed are overexpression(s) of the rate limiting enzyme or down regulation of the inefficient pathway contributing to high by-product formation (Stephanopaulous and Sinskey, 1993; Nielson, 2001; Vemuri and Aristodou, 2005). Thus metabolic engineering involves analysis, synthesis and designing of a strain with the help of molecular biology tools and information acquired of the cellular network.

One of the most important aspects of metabolic rigidity includes identifying and removing the rigid nodes in the metabolic network (Stephanopoulos and Vallino, 1991). Rigidity of the metabolic network or its resistance to manipulations has been attributed to the control architecture of the cellular systems that ensure proper growth.

This has often led to difficulties in bringing about the desired changes to improve cellular systems. Hence, it is important to understand the host system to determine the kind of genetic modifications needed and its probable effects on the physiological properties of the cell like growth and unrelated systems. Overexpression of phosphoenol pyruvate forming enzymes led to inhibit heat shock response and nitrogen regulation (Liao et al., 1996). Metabolic engineering thus involves a holistic approach of understanding cellular networking and cellular physiology.

1.1.2 Strategies of Metabolic Engineering.

In recent years various reviews have been described advancement in metabolic engineering to a more detailed, defined and rational approach, application in various fields and upcoming fields (Bailey, 1991; Cameron and Tong, 1993; Stephanolpoulos and Vallino, 1993; Ostergaard et al., 2000). Metabolic engineering can be successfully implemented by designing strategies based on the host metabolism and nature of the product.

(i) Constructive metabolic engineering

Constructive metabolic engineering based upon the host metabolic framework and a series of genetic modification to achieve desired product. This approach has been divided into seven categories (Cameron and Tong, 1993; Nielsen, 2001). Although this approach was successful in many cases, it fails to address the engineering problems leading to multi component systems of the cells that strive to maintain homeostasis making it difficult to engineer these systems for maximum product yield.

(ii) Inverse metabolic engineering (IME)

IME account for an iterative cycle which includes, identification, construction or calculation of desired phenotype, determinations of factors contributing to desired phenotype and creating the phenotype in another strain through genetic engineering or environmental manipulation (Bailey et al., 2002) (Fig.1.2).

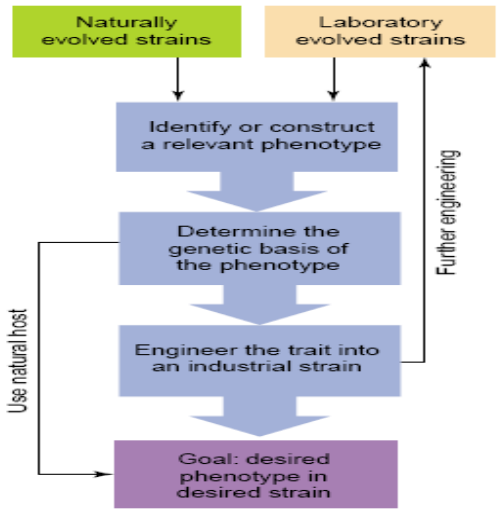


Fig. 1.2: The inverse metabolic engineering approach (Gill, 2003).

Inverse metabolic engineering has developed as a cycle where previous successful recombinant host can be further characterized to elucidate its characteristic in order to detect new targets for improved strategies (Otero and Neilsen 2010). Advantage of inverse metabolic engineering is its access to the less understood cell subsystems that are necessary to bring out the desire change in phenotype.

1.1.3 Metabolic engineering in the -Omics era

Recent development in Omics technologies, genome sequencing, transcriptome, proteome and metabolic profiling has not fulfilled the potential due to lack of knowledge how these things work in unison to produce the desired trait in the cell. Genome sequencing and transcriptome data has paved a new direction to metabolic engineering. This generated the need to assign function to various unknown genes in order to generate potential targets for drug therapy and to develop biomarkers of diseases. Flux control nodes begin with transcription (induction-repression mechanism mRNA degradation) followed by translation (protein activation and proteolysis) and enzyme activity (allostery) or sometimes involves signaling cascade

(Vemuri and Aristodou, 2005). Integrated approach of these technologies successfully employed in improving metabolic engineering of many biochemicals. On the other hand, the emerging field of the three *omics* led to the development of various high throughput analytical techniques and a large amount of data that needed to be integrated in useful information.

1.1.4: The metabolic engineering toolbox

Rapid growth in the field of metabolic engineering can be attributed to the development and availability of high throughput techniques for successful genetic modifications and numbers of programs developed for the analysis of cellular functions.

1.1.3.1 Metabolic flux analysis

Metabolic flux analysis (MFA) in the cell represents the final outcome of cellular regulation in a certain condition. Due to this reason analysis of how metabolic flux distributed in the cell give insight of its metabolic state, bridging the gap to what is observed at the transcription level and level of gross phenotype. MFA summarizes series of metabolic events involving conversion of a feedstock to biomass and by-product which requires precise values of feedstock input, generation of biomass and excretion of by-product (Holms, 2001). The concept of **Metabolite balancing** for quantification of metabolic fluxes has been demonstrated for various microbial systems (Vallino et al., 1993; Sauer et al., 1999; Pramanik and Keasling, 1997). The flux depends on the co-factor balances e.g. NADH, NADPH or ATP so it is obvious that all the reactions involving these cofactors are included. It's very difficult to identify all co-factors of the reactions, the metabolic fluxes may be under-estimated. Distribution of intracellular flux in cell can't analyze directly but by providing labeled substrate, it is possible to observed distribution of substrate in metabolic network and then calculates the flux. Hence, an alternative approach was the use of ^{13}C labeled glucose which measured intracellular metabolites

by the monitoring the labeling pattern deduced using NMR, LC-MS or GC-MS (Zupke and Stephanopoulos, 1994; Kelleher, 2001; Rohlin et al., 2001; Antoniewicz et al., 2007). This technique enabled to apply balance of individual carbon atoms in addition to the metabolite balances and does not involve balances of co-factors.

Metabolic fluxes quantification of different branches in the network does not suggest the flux control at all these nodes. Hence, it is mandatory to quantify the flux distribution throughout the network for designing suitable methods. Kinetic, thermodynamic, stoichiometry, flux capacity, and regulatory restraints play major role in flux control under which reactions operate in the metabolic network (Varma and Palsson, 1994; Bonarius et al., 1997; Edwards et al., 2002; Price et al., 2003). However, material balance concept used to calculate fluxes does not provide information on the flux control nodes. Flux control can be decoded from the regulation of enzymes of the branch points in the metabolic network. Further, the metabolite levels along with the affinities of the enzymes can provide valuable information on the *in vivo* regulation. **Fig. 1.3** shows the various methodologies employed for metabolome analysis.

Concept of metabolic flux control is used for Metabolic Flux analysis. This includes the flux control coefficient (FCCs) which quantifies the relative increase in the network based on the enzyme activities. FCCs have been derived for the glycolytic pathway in yeast (Galazzo and Bailey, 1990) from the enzyme activities of certain pathways and the metabolite concentrations. Due to advent of many advanced sequencing technologies like NGS (Next generation sequencing) platform focus shifted towards system biology which required many genome based models which can evaluate and study intrinsic biological properties that operate in the cell. Constraint based models are the first step to such a study. Many reviews have put forward

various models like METAFoR analysis, Mixed integer linear program (MILP), Minimization of metabolic adjustment (MOMA) for flux control calculations (Reed and Palsson, 2003).

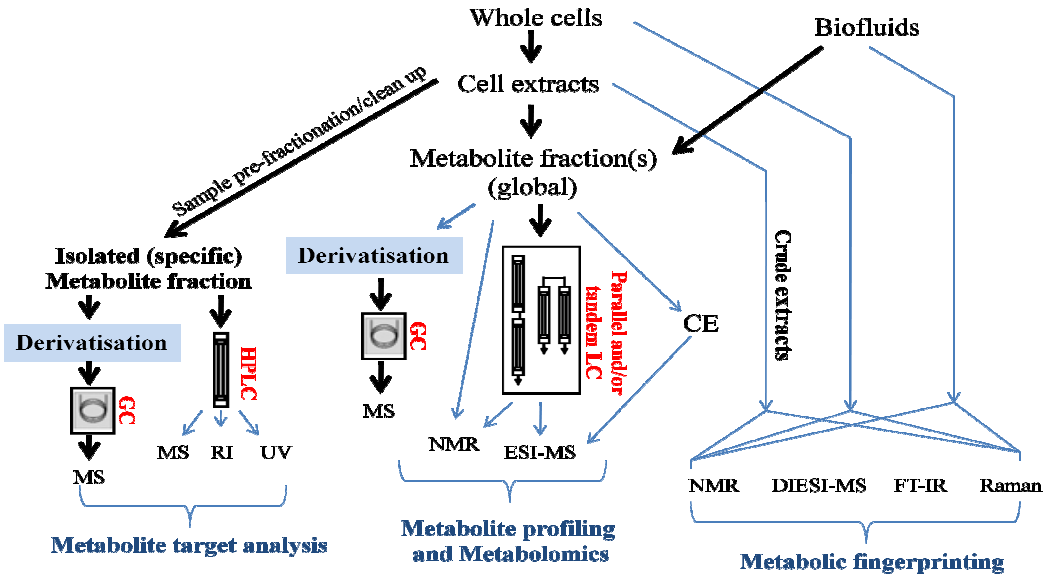


Fig. 1.3 General strategies of metabolome analysis (Goodacre et al., 2004) CE-capillary electrophoresis, DIESI-direct infusion ESI, FT-ICR –MS -Fourier transform ion cyclotron resonance mass spectrometry, NMR- nuclear magnetic resonance; RI- refractive index detection; UV-ultraviolet detection.

1.1.4.2: Current approach of metabolic engineering

Emergence of new technology to manipulate the living organisms can serve to address important and current problem in agriculture, environment, energy and health. However, with all complexity and interconnectivity of cell pathways, biology has for so many years trying hard to engineering manipulations. Recent advances in synthesis, modeling and analysis finally provided the platform to do necessary manipulation living cell in meaningful way, which leads to coin a new filled called **synthetic biology**. Synthetic biology is complicated technology, encompassing many branch of science and provides endless applications. New DNA synthesis and assembly techniques made routinely customization of very large DNA which allows incorporation of many genes or whole pathways. Using these tools now scientist creating completely novel biological

pathways. Field of synthetic biology lies at the interface of many different biological research areas such as, functional genomics, protein engineering, chemical biology, metabolic engineering, systems biology and bioinformatics. Exact meaning varies for different people so it was defined by Liang et al, (2011) as "deliberate design of improved or novel biological systems that draws on principles elucidated by biologists, chemists, physicists, and engineers." Synthetic biology has varieties of applications in medical, chemical, food and agricultural industries. In addition to practical applications, synthetic biology also aims to increase our understanding of basic life sciences.

1.1.4.2.1: Methods used in synthetic biology (Fig. 1.4)

Tools: Its comprises of synthesizing and modifying a broad range of biological entities like DNA, proteins, pathways, organelles, viruses and genome in cost effective manner. Recently many tools have been developed to generate large size of DNA, pathway component, proteins and genomes.

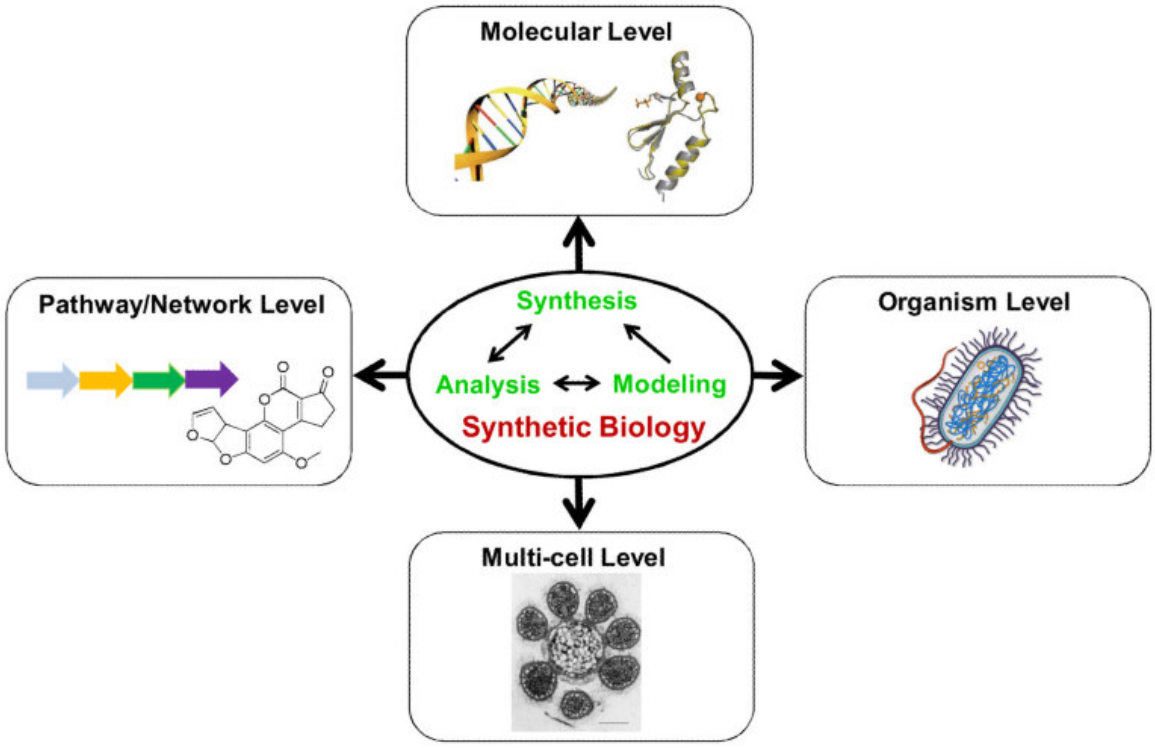


Fig. 1.4: Synthetic biology overview. Figure describes the relationship among the various methods used in synthetic biology and different applications of synthetic biology on different levels (Liang et al., 2011).

(i) DNA synthesis: Unlike traditional recombinant DNA technology using cloning, chemical synthesis of DNA revolutionaries the generation of rationally designs new DNA sequence. Commercially up to 10kb DNA can be synthesized (May, 2009).This approach allow rapid generation of genes, remove restriction enzyme site constraints, undesired RNA secondary structure or codon optimization.

J. Craig Venter Institute (JCVI) synthetically assembled 583 kb *Mycoplasma genitalium* genome which was constructed from 101 pieces of DNA fragments, each approximately 5–6 kb in length (Gibson et al., 2008a), which was done in two step in first step multiple *in vitro* enzymatic recombination to create six large pieces of DNA, while in second step

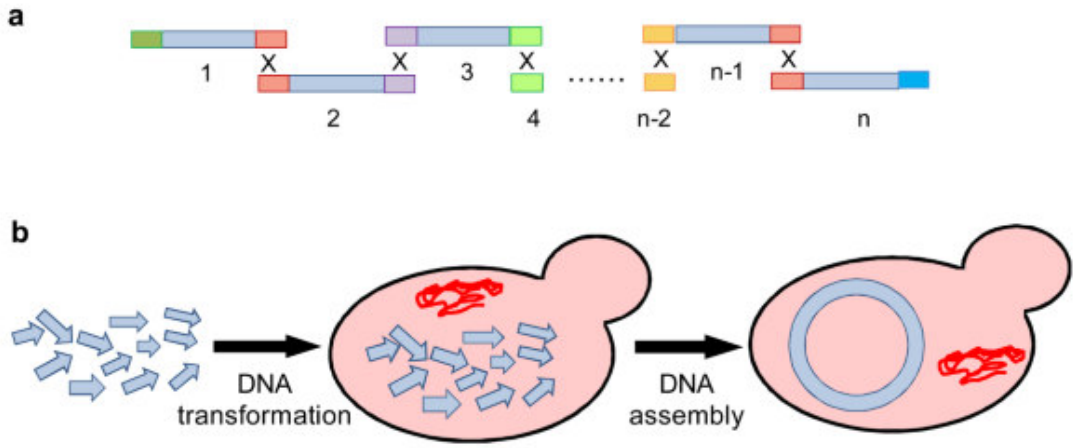


Fig. 1.5: Synthesis of large DNA molecules in yeast. (**a**) Yeast homologous recombination mechanism. (**b**) Construction of a synthetic *M. genitalium* genome. 25 different overlapping DNA segments (blue arrows, 17~35 kb each) composing the genome were co-transformed into yeast followed by assembly of the entire genome in a single step.

in vivo yeast recombination mechanism was used to produce the final synthetic genome (Fig. 1.5). Finally entire genome assembly process was simplified by directly assembling 25 overlapping DNA fragments in a single step in yeast (Gibson et al., 2008b). DNA assembler a similar method was developed by Shao and co-workers (2009) to assemble a biochemical pathway. A biochemical pathway consisting of 8 genes (a total of ~19 kb) was assembled in a single step in yeast and the resulting recombinant strain could utilize D-xylose to produce a nutraceutical zeaxanthin. This method offers unmatched versatility and flexibility in pathway engineering.

(ii) Protein engineering: Generally two approaches are used in this case- rational and directed evolution. Rational designs are often difficult to achieve due to incomplete knowledge of protein structure, function and dynamics while directed evolution is more suited to preexisting protein

function. It is based on Darwinian evolution process in test tube involve repeated cycle of selection. It has been successfully used to improve variety of protein properties such as activity, stability and selectivity (Schimid-Dannert et al., 2001, Zhao et al., 2002). However this method has its own limitations. To overcome these limitation many novel methods have been designed like, Iterative Saturation Mutagenesis (ISM), Incorporating Synthetic Oligonucleotides *via* Gene Reassembly (ISOR), One-pot Simple methodology for CAssette Randomization and Recombination (OSCARR), Overlap Primer-Walk Polymerase Chain Reaction (OPW-PCR) and rapid Site Directed Domain Scanning Mutagenesis (SDDSM) (Chockalingam et al., 2005; Reetz and Carballeira, 2007; Herman and Tawfik, 2007; Hidalgo et al., 2008; Kumar and Rajagopal, 2008; Deng et al., 2008).

1.1.4.2.2: Pathway engineering

Most of the effort put on the construction and optimization of an existing biochemical pathway either in a native or a heterologous host which can be achieved by *de novo* DNA synthesis tool. Pathway engineering can be improved by rationally designed well known modular part by mixing and matching and modulating gene expression through various control mechanisms. However designing of pathway includes necessary biological part including, promoters, genes and proteins but it also require optimal function of those parts. Failure in balance of flux in designed synthetic pathway will result in bottleneck and accumulation of intermediate which are non essential (Pitera et al., 2007). Balance can be done by optimization of transcription of the various genes in the pathway. Pitera and coworkers (2007) have managed to engineer the flux balance pathway with improved yield and reduced metabolic burden by fusing a library of promoter to various enzymes in isoprenoid production pathway. Well characterized transcriptional regulator emerges as powerful tool in metabolic engineering because they allow

rational coordination and control of multigene expression, so decoupling pathway design from construction (Bennett and Hasty, 2009). Protein co-localization can also be used for Flux balance. Dueber and coworker (2009) developed a method where protein scaffold with modular interaction domain physically linked with the pathways enzymes together result in limits the loss of intermediates to competing pathways. This enables the direct control of metabolic flux by varying the number of interaction domains on the scaffold, thereby adjusting the composition of enzyme complex. Regulatory parts such ribosome binding sites (RBS), riboswitches and operator-regulator pair can be redesigned and engineered to overcome problems involved in synthetic metabolic pathways (Dueber et al., 2009; Landrain et al., 2009)

1.1.4.2.3: Genome Engineering

Invention of methods to generate and manipulate large DNA has opened the new era of genome engineering (Fig. 1.6). According to requirement, editing of genomes can be carried out by variety of approaches, such as constructing a genome *de novo* (Gibson et al., 2008a), removal of unstable DNA elements (Pósfai et al., 2006), combining one genome to other genome (Itaya et al., 2005), reorganizing genome component (Chan et al., 2005), whole genome shuffling. Multiplex Automated Genome Engineering (MAGE) a very powerful approach was developed for large scale programming and evolution of cell. In this method a pool of degenerate oligonucleotides is cyclically introduce into various cells within the population to generate sequence diversity across the chromosome by allelic replacement.

1.1.4.2.4: Genomic Integration

In many conditions like biomedical, agriculture and recombinant product productions plasmid based modification are not recommended due to the presence of antibiotic markers,

metabolic burden on cells, chance of horizontal gene transfer or loss of plasmid in due course of time. To overcome these problems it's necessary to do the modification in genome itself.

Bacterial genome can be manipulated by gene integration or excision. Chromosomal DNA manipulation can be done by three ways *viz* (i) Homologous recombination; (2) site-specific recombination and (3) transposon mediated gene integration (Fig. 1.7). Homologous recombination is mediated by recombinase RecA catalytic activity between two long homologous sequences (Baitin et al., 2003). Site-specific recombination includes enzymes belonging to two major families: (1) the resolvase-invertase family and (2) Int family (FLP-FRT, Cre-*lox*P). Transposon mediated gene integration involves phage integration and excision via site-specific recombination between the phage attachment site, *att*P, and the bacterial attachments site, *att*B. These events lead to either gene insertion or excision in the chromosome. Transposition is mediated *via* two mechanisms either cut-and-paste transposition (e.g., Tn5 and Tn10) (Reznikoff, 2008) or replicative transposition (e.g., Tn3, Mu, and many IS) (Shapiro, 1979) by one or both enzymes of transposase and resolvase, leaving one copy on the target DNA or two copies on both donor and target DNA, respectively.

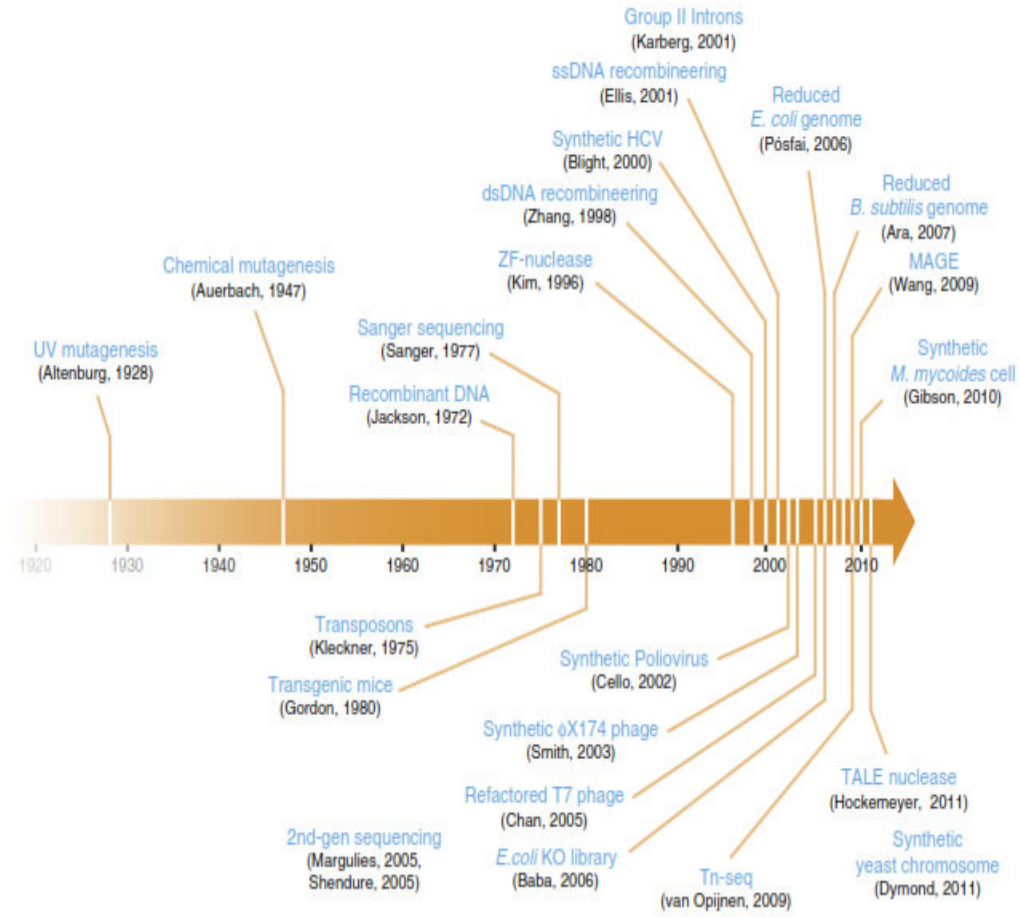


Fig. 1.6: Historical timeline of genome scale engineering (Esvelt and Wang, 2012).

In transposon-based gene integration homologous sequences are not required for transfer to the chromosome, unlike recombination-associated chromosomal manipulations. However, transposons are extensively used for random mutagenesis.

Small number of transposons can be integrated at a preferred, neutral, naturally evolved and defined target site without any deleterious effects or preparatory genetic modification, which paves the way to construct genetically engineered bacterial strain harboring a chromosomal insertion of genes of interest.

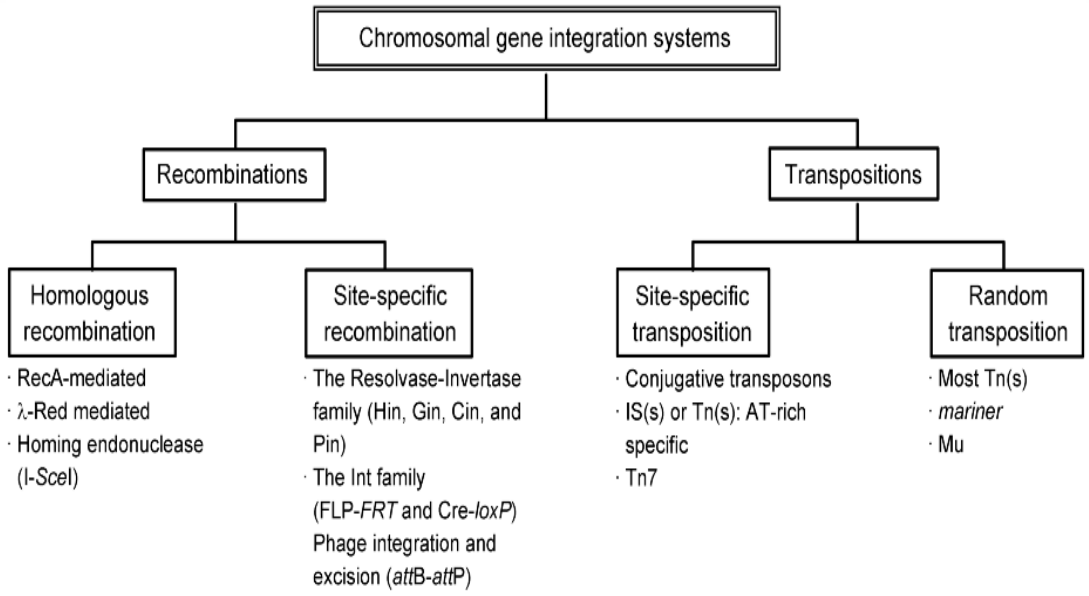


Fig. 1.7: Chromosomal gene integration system used in bacteria (Choi and Kang-Ju, 2009**).**

Our focus is mainly to discuss Tn7 based site specific integration and its mechanism. Tn7 does not share sequence identity between the target insertion site and the Tn7 ends. Site-specific integration of Tn7 is governed by the TnsD-targeting protein (Bainton, 1993). The unique chromosomal attachment Tn7 site, referred to as *att*Tn7, resides downstream of the essential *glmS* gene, which encodes for the glucosamine-6-phosphate synthetase is an essential gene involved in cell wall biosynthesis (Craig, 1989). The site-specific Tn7 insertion site has been discovered in many Gram-negative bacteria, including *Desulfovibrio desulfuricans*, *E. coli*, *Serratia marcescens*, *Sphingomonas yanoikuyae*, *Vibrio anguillarum* and *Vibrio fischeri*. The mini-Tn7 transposon system is used as tool for a single copy tagging of bacteria in a site-specific manner at a unique and neutral site without any deleterious effects. Many fluorescent genes have been successfully integrated in bacterial chromosome under constitutive promoters or its own promoters like GFP and luciferase genes integrated into chromosomes of *Erwinia chrysanthemi*, *P. syringae* (Shen et al., 1992), *Pseudomonas fluorescens* (Koch et al., 1991) and *P. putida*

(Lambertsen et al., 2004) by the Tn7-based system. Recently, mini-Tn7-based gene integration system has been improved for gene complementation, gene expression analysis, strain construction and reporter gene-tagging of *P. aeruginosa* and *Yersinia pestis*, particularly in biofilm and animal models (Choi et al., 2005). Moreover, modified gene delivery system was applied to other organisms, such as *Burkholderia* spp. and *Proteus mirabilis*, which were determined to have multiple *glmS*-linked *att*Tn7 sites and secondary, non-*glmS*-linked *att*Tn7 site, respectively (Choi et al., 2006; Choi and Schweizer, 2006).

Analogs of the *glmS* gene are found universally in all organisms, including bacteria, yeast, and even humans. Hence, the Tn7 transposition could be used as a universal tool for genetic manipulation. Presence of two analogs of *glmS*, glutamine-fructose-6osphate-transaminase-1 and -2 (*gfpt*-1 and *gfpt*-2) were identified in the human chromosome, and their adjacent positions are a potential target of Tn7 transposition with a higher frequency of insertion neighboring to *gfpt*-1 than *gfpt*-2, can be useful tool for site-specific DNA delivery in a gene therapy (Kuduvalli et al., 2005). *Saccharomyces cerevisiae* harbors the glmS analog, *gfa*-1, at its chromosome XI, does not transpose adjacent to the possible target *gfa*-1 sequence (Kuduvalli et al., 2005). However a Tn7-like transposon, Tn5468, was found at the intergenic region between *glmU* encoding for N-acetylglucosamine1-uridyltransferase and the *glmS* gene in *Thiobacillus ferrooxidans* (Oppon et al., 1998).

1.2: Global phosphorus status

Green revolution especially in developing countries like India had led to extensive use of chemical fertilizers and other chemicals which have resulted in reduction in soil fertility and to environmental degradation (Gyaneshwar et al., 2002). Use of chemical fertilizers reached to the theoretical maximum level beyond that no further improvement in grain production is possible

(Ahmad, 1995). Current studies show that by the year 2050, population will touch the 9 billion mark. To provide food for this population, agriculture will have to grow more while soil erosion poses a challenge even to sustain. Phosphorus is chief macronutrient after nitrogen required for plant growth and grain yield (Bieleski, 1973; Vance et al., 2000). Nucleic acids, phospholipids, ATP and several other biologically active compounds require phosphorus for their synthesis. It participates directly in generating the biochemical energy necessary to drive virtually every anabolic process within the cell and is a prerequisite in every phase of cellular metabolism (Goldstein, 1995).

Crop production needs to be improved to meet the global population demand which requires efficient utilization of phosphate reserves of earth. Green revolution created one way overflow of phosphorus from rock to lakes and oceans which dramatically impaired costal and marine ecosystem. In current scenario, supply of phosphorus for fertilizers is obtained from the rock phosphates.

Recent IFDC survey produces an assessment of global phosphorus reserves. New survey increased the estimated phosphorus reserve of Moracco and Western Sahara territory from 15 billion ton to 65 billion ton (van Kauwenbergh, 2010). Overall, three countries have more than 85% of known phosphorus reserves (Fig. 1.8).

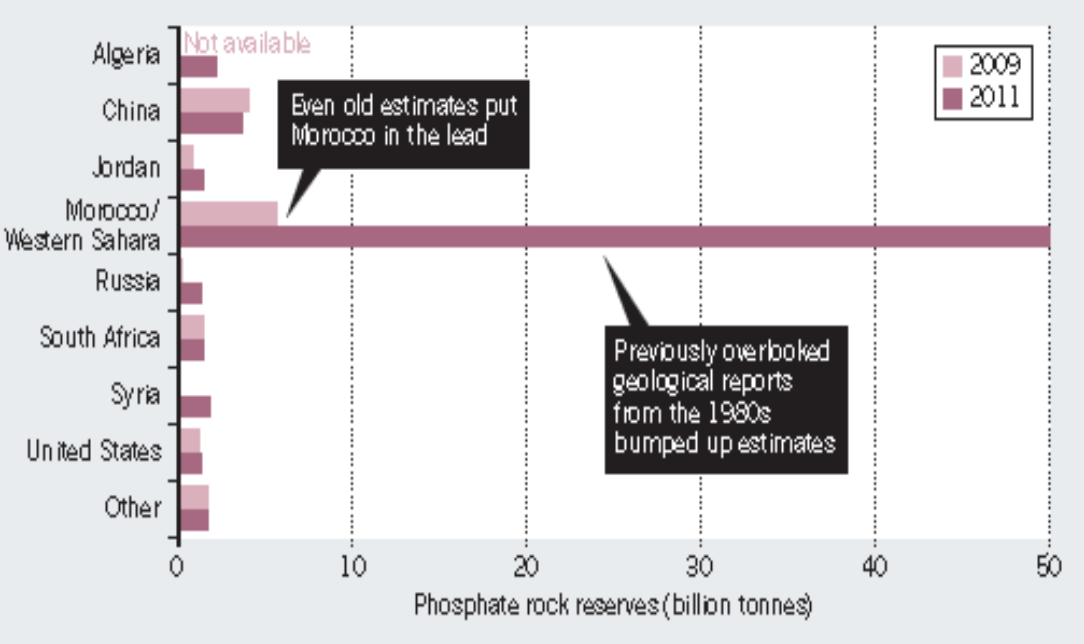


Fig. 1.8: Global distribution of phosphorus reserve (Elser and Bennett, 2011).

P fertilizers are essential for maintaining and increasing the world food production (Koning et al., 2008). Phosphorus is completely produced from non-renewable resources, phosphate rocks, and the current extraction rates would lead to a rapid depletion of these resources.

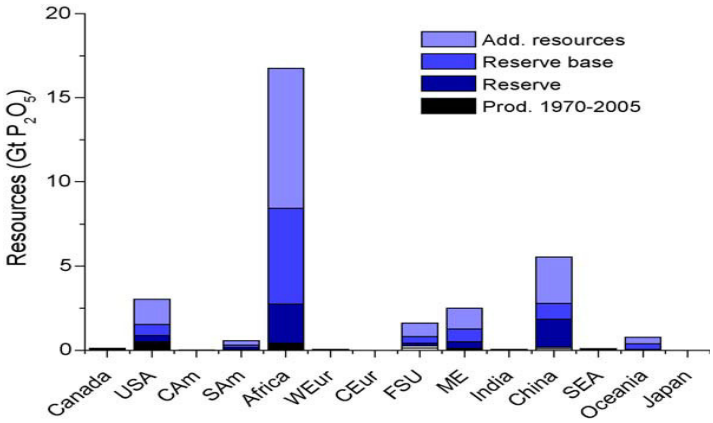


Fig. 1.9: Available P resources per region (Van Vuuren et al., 2010).

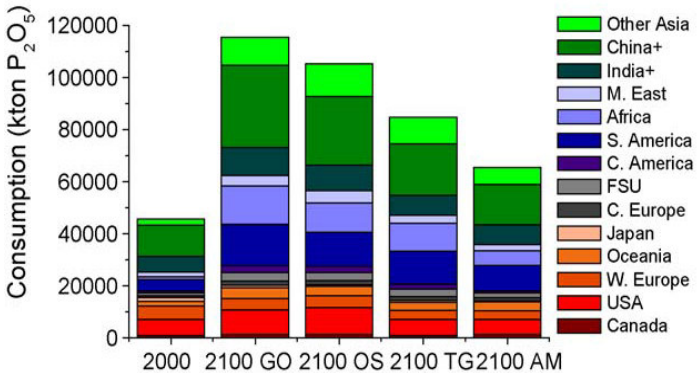


Fig. 1.10: Total global P consumption (Van Vuuren et al., 2010).

The main producers are Africa, China, the United States, the Middle East and Russia (Fig. 1.9) and according to the model results, by 2050 Africa would totally dominate the market, with more than half the global production. In contrast, production in United States would decrease. Most studies expect a further increase in the use of P fertilizers in the coming decades (Fig. 1.10) (USGS, 2008; Cordell et al., 2009). In developing countries, P fertilizer use for the production of food crops is increasing while in industrialized regions the P fertilizer use is declining. For 2100, the resulting range in P use would be 65–115Mt P_2O_5. P production is dominated by the United States, Africa and China (Fig. 1.11).

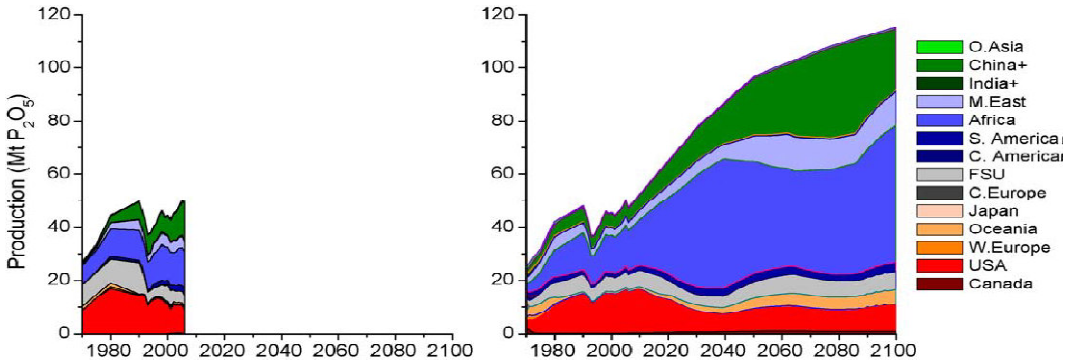


Fig. 1.11: P production for the 1970–2006 period and for the 2000–2100 period (Van Vuuren et al., 2010).

Under the Geological orchestration scenarios (medium resource estimates), about half of the current reserves is projected to be depleted by 2050, and the other half before the end of the century (Fig. 1.12). As a result of the associated increase in prices, at the same time a larger part of the reserve base would become economic for exploitation and by the end of the century about 40% of the reserve base would also have been exploited.

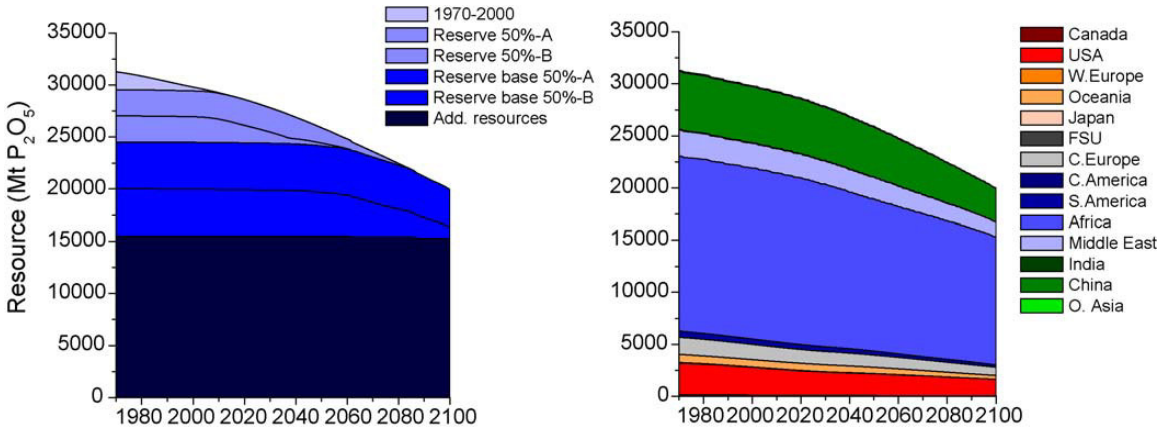


Fig. 1.12: Depletion of resource base of phosphate rock under the Global Orchestration (GO) scenario (Van Vuuren et al., 2010**).**

For the total resource base, this implies a depletion of 35% by the end of the century. Thus, scarcity of P resources would become a more important issue by the end of the century, with only 65% of the resource base being left. Hence, current reserves or resources with a very high probability of being available would be totally depleted by the end of the century.

1.3: Status of phosphorus in soil

After N, phosphorus (P) is the major plant growth limiting nutrient despite being abundant in soils in both inorganic and organic forms. However, many soils throughout the world are P-deficient and the free phosphorus concentration (the form available to plants) even in fertile soils is generally not higher than 10 μM even at pH 6.5 where it is most soluble (Arnou,

1953). On an average, most mineral nutrients in soil solution are present in millimolar amounts; however, phosphorus is present only in micromolar or lesser quantities (Ozanne, 1980). These low levels of P are due to high reactivity of soluble P with Calcium (Ca), iron (Fe) or aluminum (Al) that lead to P precipitation. Inorganic P in acidic soils is associated with Al and Fe compounds (Sharpley et al., 1984) whereas calcium phosphates are the predominant form of inorganic phosphates in calcareous soils. Organic P contributes to a large fraction of soluble P, as much as 50% in soils with high organic matter content (Barber, 1984). Depending upon the weathering of rock and other environmental factors, the amount of P in soil varies from 0.02 to 0.5% (Kucey et al., 1989).

1.3.1: Alkaline Vertisol

Alkaline vertisols, also called as calcareous soils, have high buffering capacity and mainly contains P in bound form with Ca (Ca-P complexes) which could be solubilized by acidification of soil. Thus, organic acids which lower the pH of the medium could efficiently release P form vertisols. However, the amount of organic acid required could vary from 10-100 mM depending on the nature of organic acid (Gyaneshwar et al., 1998b).

1.3.2: Acidic Alfisols:

Acid soils such as alfisols, aridisols, entisols and ultisols occupy about 30% of world's ice free land area (von Uexull et al., 1995). Deficiency of nutrients such as P and toxicity of metals such as aluminium (Al) chiefly mark the crop growth in acid soils. Of the total land area of the world, about 24.2% is considered potentially arable land and most of this belongs to acid soils. In alfisols, phosphate is predominantly present in iron (Fe) and Al bound forms which are difficult to be solubilized by even strong organic acids (Ae et al., 1991; Jones and Darrah., 1994; Srivastava et al., 2006). Low molecular weight organic acids effectively chelate Fe and Al from

ferric phosphate and aluminium phosphate, thereby solubilize P (Kpomblekou and Tabatabai, 1994; Gadd, 1999). However, addition of organic acids at high concentrations (10 mM-50 mM) did not result in the release of detectable amount of P from alfisol although pH of the soil suspension dropped below 4.0 (Srivastava et al., 2006). When alfisol was supplemented with 40 mg RP per g of soil, addition of organic acids resulted in drop in pH to less than 4.0 as well as release of P in the supernatant. Among different organic acids tested, their effectiveness in solubilizing P from alfisol is in the order oxalic> citric> tartaric> gluconic> succinic> acetic acid. On the other hand, 5mM oxalic acid could solubilize P in the range of 217-440 µM alfisol amended with 10-30 mg RP /g RP soil (Table 1.1).

Hence, to enhance P nutrition to plants grown in acidic soils, RP was directly applied as P fertilizer (Rajan et al., 1996). RP is cheaper than inorganic P-fertilizer, creates less environmental pollution as it requires minimum processing and its dissolution results in slow release of P in the soil. RP are rich in CaP complexes and hence are suggested to be soluble in acidic soils.

Table 1.1: Efficacy of different organic acids at P-solubilization from alfisol supplemented with RP (40 mg / g soil) (Srivastava et al., 2006).

Organic acid	Concentration (mM)	0 h		24 h	
		pH	Solution P (µM)	pH	Solution P (µM)
None	-	6.73±0.21	U.D.	6.69±0.02	U.D.
Oxalic	10	3.42±0.05	1560±4.0	3.14±0.01	1092±2.4
Citric	10	3.45±0.04	409±8.0	3.22±0.02	387±3.0
Citric	20	2.80±0.034	850±6.5	3.18±0.431	646±2.9
Tartaric	20	3.50±0.02	550±6.0	3.60±0.23	502±2.1
Gluconic	50	3.40±0.05	800±2.0	3.71±0.01	300±5.0
Lactic	50	3.25±0.015	920±7.0	3.36±0.02	200±3.0

Succinic	50	3.64±0.04	332±7.0	3.66±0.07	359±5.3
Acetic	50	3.80±0.23	102±2.0	4.45±0.3	80±2.0

U. D.: Undetectable. Values expressed as mean ± S. D. for minimum of three independent experiments.

Various reports show the usage of partially acidulated rock phosphate (PARP) in acid soil to provide P nutrition (Marwah, 1983; Bolland, 1992; Butegwa, 1996; Abekoe, 1998). However, the use of RP is limited due to variations in the dissolution of RP and refixation of soluble P in soils. Thus, the effectiveness of RP differs with soil properties, climatic conditions and nature of the crops (Nahas, 1996; Rajan et al., 1996). In a study of 228 soil samples from South Western Australia, only 29 of the soils dissolved more than 40% of North Carolina RP (Huges, 1998).

One of the attractive approaches to solubilize RP would be to use microorganisms that are able to secrete organic acids (Vassilev et al., 1996).Application of RP along with P-solubilizing bacteria and vesicular arbuscular mycorrhiza (VAM) had shown to increase the P nutrition of plants in alkaline soils (Piccini and Azcon, 1987; Omar, 1998; Vassileva et al., 1998).

Secretion of piscidic acid, which is a derivative of tartaric acid, has been implicated to solubilize FeP and provide P for effective growth of pigeon pea in P deficient acid soil (Ae et al., 1990; 1991). Optimization of concentration of oxalic acid and RP with alfisols suggests that PSMs secreting 5-10 mM of oxalic acid could be act as phosphate biofertilizers in alfisols supplemented with RP. *Penicillium bilaii* strain has been reported to secrete oxalic and citric acids at about 10 mM concentrations (Cunningham and Kuiack, 1992) and various *Bacillus* and *Aspergillus* strains secrete oxalic acid (Gupta et al., 1994). However, their effectiveness in alfisols has not been studied.

1.4: Phosphate solubilization

Phosphate mineralization in rhizosphere is achieved by different mechanisms – acidification and chelation.

1.4.1: Acidification

MPS ability of PSMs have been attributed to the acidification of the surrounding by secretion of organic acids (Gyaneshwar et al., 1999; Whitelaw, 2000; Sharma et al 2005; Patel et al., 2008). Protons of the organic acid are the major reason for the release of inorganic P from the bound P (Lin et al., 2006). Secreted organic acid can dissolve mineral phosphate by anion exchange of PO_4^{-2} by acid anion or chelation of Ca, Fe and Al ions complexed with phosphate or competes with phosphate for adsorption in soil site (Gyaneshwar et al., 2002; Khan et al., 2006; Zaidi et al., 2009). PSMs generally secrete gluconic, 2-ketogluconic, citric, malic, acetic acid etc. when grown on simple carbohydrates as carbon source. In case of *E. asburiae* PSI3, gluconic acid secretion is induced by phosphate starvation (Gyaneshwar et al., 1999). PSM ability of *Rhizobium/Bradyrhizobium* was demonstrated due to the presence of 2-ketogluconic acid (Halder and Chakrabarty, 1993). Biochemical and molecular mechanism for MPS ability of these nodulating bacteria is not clear.

1.4.2: Chelation

Acidification is not effective in releasing P from alfisol soils (Illmer and Schinner, 1995). Chelation ability has been shown to contribute for the MPS ability along with acidification. P release was observed when 0.05M EDTA was added to the medium in *Penicillium bilaii* inoculation (Kucey, 1988). Complex formation of cations (Al^{+3}, Fe^{+3} and Ca^{+2}) depends upon the number and kind of functional groups present on chelator. Organic acid having good chelating properties could be most effective as P solubilizer in alfisols, which was demonstrated by the

amount of P released when 1 mM of citric and 1 mM of oxalic acid was added to RP (Kpomblekou and Tabatabai, 1994; Xu et al., 2004). Citrate form complex with Ca^{+2} more easily than dicarboxylic acids, like malic and tartaric (Whitelaw, 2000). Dicarboxylic acids like oxalic, citric and tartaric acid could help in efficient P release from soil. In acidic alfisols, oxalic acid is most effective, much better than that of citric acid, in releasing P from RP (Srivastava et al., 2006).

1.4.3: Phosphate Solubilizing Microorganisms

PSMs are integral component of the soil which controls the biofertility of the soil. Numerous microorganisms reported to have MPS ability (Gyaneshwar et al., 1998a; Hameeda et al., 2008; Zaidi et al., 2009). Important genera reported as PSMs include *Bacillus, Enterobactor* and *Pseudomonas* (Illmer and Schinner, 1992; Wani et al., 2007a; Archana et al., 2012) while *Penicilium, Aspergillus* and *Rhizopus* are the important fungal genera (Pandey et al., 2008). Many other genera also show MPS ability including *Rhodococcus, Arthrobacter, Chryseobacterium, Gordonia, Phyllobacterium, Arthrobacter, Delftia* sp. (Chen et al., 2006), *Azotobacter* (Kumar et al., 2001), *Xanthomonas* (de Freitas et al., 1997), *Pantoea*, and *Klebsiella* (Chung et al., 2005). Some symbiotic nitrogen fixers also showed MPS ability. *Rhizobium leguminosarum* bv. *trifolii* (Abril et al., 2007), *R. leguminosarum* bv. *viciae* (Alikhani et al., 2007) and *Rhizobium* species nodulating *Crotalaria* sp. (Sridevi et al., 2007) showed improvement in plant P content by mobilizing inorganic and organic P from the soil.

1.4.4: Factors influencing the efficacy of PSMs in field conditions

Many factors influence the efficacy of PSMs in field conditions. Many successful field studies reported with consortium of microorganism which improved plant growth due to P availability or other plant growth promoting activities like nitrogen fixation, efficient nutrient

uptake, alleviation of metal toxicity and PGPR traits including siderophores production, antibiotics production, phytohormones etc. (Archana et al., 2012).

1.4.4.1: Root colonization ability

Competitiveness of the PSMs depends upon the nutrient availability, survival and colonization ability of the organism in rhizosphere (van Veen et al., 1997). Effectiveness of the inoculated microorganism depends upon the ecological condition. Reports for understanding the factors influencing the survival and efficacy of PSMs in field conditions are scanty. In case of *Rhizobia,* 300 cells per seed are sufficient for optimal nodulation (Giddens et al., 1982) and efficiency of *Rhizobia* nodulation can also be improved by amending soil with specific substrate.

Generally microbial population size decreases rapidly in soil after inoculation (Ho and Ko, 1985). Survival of microorganisms in soil depends upon number of factors including soil composition (Heijnen et al., 1993; 1995; Bashan et al., 1995), temperature, moisture, carbon status (Richardson and Simpson, 2011) and presence of recombinant plasmid (van Veen et al., 1997). Biotic factors also play an important role in the survival of the inoculated strains which could be explained by decline of microorganisms in non sterile soil which is minimal in sterile soils (Heijnen et al., 1988; Heijnen and van Veen, 1991). Increased population of inoculated microorganisms was observed in sterile soil (Postma et al., 1988).

Root colonization is a major factor which determines the successfulness of inoculants. Majority of the microbe population found in the soil are associated with the plant roots where their population can reach up to 10^9 to 10^{12} per gram of soil leading to biomass equivalent to 500 kg ha^{-1} (Whipps, 1990; Metting, 1992). Abundance of the microbes in rhizosphere is due to secretion of high amount of root exudates (Brimecombe, 2001). Root associated bacterial diversity; growth and activity vary in response to the biotic and abiotic rhizospheric environment

of the particular host plant (Berg and Smalla, 2009). Interaction of rhizobacteria with plant roots is mediated by secreted compounds (signals and nutrients) that vary in abundance and diversity depends upon the characteristics of the particular host (Bais et al., 2006; Lugtenberg and Kamilova, 2009).

1.4.4.2: Soil Properties

Soil properties may vary in term of texture, particle and pore size of soil. Pore size distribution determines the efficacy of the microorganism in soil and different behavioral pattern in bacteria when released in different texture soil can be correlated by protective pore spaces present in these soils (van Veen et al., 1997). *P. fluorescens* inoculation in loamy sand and silt loam soils was observed for three years and it showed better survival in finer-texture soil i.e. silt loam soil than in the sandy soil (van Elsas et al., 1986). P solubilization varies with composition of the medium and type of mineral phosphate provided. Nature of the organic acid secreted by PSB varies depending on the nature of rock phosphate and thus affects the P solubilization ability of the organism (Vyas and Gulati, 2009). Efficacy of PSMs reduced drastically in soil due to high buffering capacity of alkaline vertisol and very low amount of available P, high refixation and Al toxicity in acidic alfisols (Gyaneshwar et al., 1998a; Srivastava et al., 2006).

1.4.4.3: Nutrient Availability

Substrate availability often limits the performance of the inoculated organisms. In natural environment nutrients are mostly present in very limited amount (Paul and Clark, 1988; Gottschal et al., 1992; Koch et al., 2001). Physiological status of the inoculated microorganism also determines the efficacy in field conditions. In rhizosphere, the abundance and activity of microorganisms is significantly correlated with the increased exudation of photosynthetic carbon from root. 5-20% photosynthetic carbon generally secreted into the rhizosphere as simple hexose

sugar, high molecular weight mucilage and organic anions along with complex carbon derived from root turn over (Jones et al., 2009). These available carbon sources are utilized by microorganisms and which leads to significant increase in microbial biomass (C and P) within the rhizosphere as observed in perennial ryegrass and radiate pine by using the rhizobox system (Chen et al., 2002).

Scarcity of C in bulk soil compared to rhizospheric soil was observed by reporter gene technology using carbon limitation dependent gene expression system fused with *lacZ* in *P. fluorescens* (Koch et al., 2001). Different root portions like root hair zone, sub apical zone and emerging site of secondary ramification release specific exudates (Curl and Truelove, 1986; Bais et al., 2006). These exudates are used as convenient source of carbon, energy and likely to favor fast growth of metabolically versatile microorganism. Rhizobacteria utilizing broad substrate (carbon source) for organic acid production appear to be better PSB (Sharma et al., 2005, Patel et al., 2008).

Amount of carbon used in the laboratory studies (several mg per gram soil) are very high compared to the carbon present in the rhizosphere (Kozdroj and van Elsas, 2001; Schutter and Dick, 2001; Griffiths et al., 2004). Daily carbon input estimated around 50-100 mg per gram of rhizospheric soil (Trofymow et al., 1987; Iijima et al., 2000). Plant roots secrete complex mixture of organic compounds like organic acids, amino acids and sugars. Different sugars are secreted in root exudates including glucose, fructose, maltose, ribose, sucrose, arabinose, mannose, galactose and glucuronic acid (Jaeger III et al., 1999; Lugtenberg et al., 1999; 2001; Kupier et al., 2002; Bacilio- Jimenez et al., 2003; Bais et al., 2006; Kamilova et al., 2006). Organic acids secreted by root exudates in the rhizosphere are malate, citrate, oxalate, succinate, formate, etc. (Jones, 1998).

1.4.5: Genetic modifications for developing P solubilizing ability

In gram negative bacteria, direct oxidation of glucose is major pathway for glucose utilization, in which glucose gets converted into gluconic and 2-ketogluconic acid (Goldstein, 1995). These acids play a major role in MPS ability of microrganisms and their secretion has been incorporated to achieve and improve the MPS ability of non- or weak-PSMs. Gene responsible for MPS ability were cloned and expressed in different hosts which imparts or improves the MPS phenotype of genetically modified organisms (Table 1.2).

Mostly genetic modifications were done for enzymes or cofactors of direct oxidation pathway including the genes responsible for gluconic, 2-ketogluconic acid and cofactor for glucose dehydrogenase. However, previous studies shows that gluconic acid is required in very high amount (50 mM) for phosphate solubilization while citric and oxalic acid are required in very low amount (5-10 mM) and shows efficient P release (Gyaneshwar et al., 1998a). Thus, rhizobacteria secreting citric or oxalic acid could be most efficient in P release from both alkaline vertisol and acidic alfisols. Genetic modification in the central carbon metabolism of rhizobacteria for secretion of citric and oxalic acid could be a great approach for converting rhizobacteria into potential phosphate solubilizing bacteria (PSB). Overexpression of *E. coli* citrate synthase gene (*glt*A) in *P. fluorescens* ATCC 13525 results in secretion of citric acid however the secreted citrate was not sufficient to release P (Buch et al., 2009).

MPS ability of PSMs depends upon the secretion of organic acids but amount of P release in the medium does not always correlate with the amount of organic acid (Thomas, 1985; Asea et al., 1988). Therefore, nature and strength of organic acid seems to be effective in P solubilization. Oxalic acid is more effective as compared to citric acid in acidic alfisols

supplemented with RP but in alkaline vertisol both the acids efficiently release P from RP (Gyaneshwar et al., 1998a; Srivastava et al., 2006).

Phosphorus biofertilizers in the form of micro-organisms can help in increasing the availability of accumulated phosphates for plant growth (Goldstein 1986; Kucey et al., 1989; Richardson, 1994; Subba Rao, 1982; Tandon, 1987). In addition, the micro-

Table 1.2: Genetic modifications of microorganisms for phosphate solubilization (Kumar et al., 2013)

Gene/Function	Source	Host	Mineral P solubilized	Organic acid	References
PQQ biosynthesis	*Serratia marcescens*	*E. coli*	TCP	GA	Krishnaraj and Goldstein (2001)
pqqE	*Erwinia herbicola*	*Azospirillum sp.*	TCP	GA ?	Vikram et al. (2007)
pqqED genes	*Rahnella aquatilis*	*E. coli*	HAP	GA	Kim et al. (1998)
Unknown	*Enterobacter Agglomerans*	*E. coli*		GA ?	Kim et al., 1997
pqqABCDEF genes	*Enterobacter intermedium*	*E. coli* DH5α	HAP	GA	Kim et al., 2003
Ppts-gcd, P gnlA-gcd	*E. coli*	*Azotobacter vinelandii*	TCP	GA	Sashidhara and Podille, 2009
gabY Putative PQQ transporter	*Pseudomonas Cepacia*	*E. coli* HB101		GA	Babu-Khan et al., 1995
Unknown	*Erwinia herbicola*	*E. coli* HB101	TCP	GA	Goldstein and Liu, 1987
gltA/ citrate synthase	*E. coli* K12	*Pseudomonas fluorescens* ATCC 13525	DCP	Citric acid	Buch et al., 2009
Unknown	*Synechocystis* PCC 6803	*E. coli* DH5α	RP	Unknown	Gyaneshwar et al., 1998b
gad / Gluconate dehydrogenase	*P. putida* KT2440	*E. asburiae* PSI3	RP	GA and 2KG	Kumar et al., 2013

GA- Gluconic acid, 2KG- 2 ketogluconic acid, DCP- Dicalcium phosphate, TCP- Tricalcium phosphate, RP- Senegal rock phosphate, HAP- Hydroxyapatite

organisms involved in P solubilization as well as better scavenging of soluble P (P biofertilizers) can enhance plant growth by increasing the efficiency of biological nitrogen fixation, enhancing the availability of other trace elements such as Fe, zinc (Zn), etc. and by production of plant growth promoting substances (Kucey et al., 1989).

The involvement of micro-organisms in solubilization of inorganic phosphates was known as early as 1903 (Kucey et al., 1989). Since then, there have been extensive studies on the solubilization of mineral phosphates by micro-organisms. These have been reviewed by Subba Rao (1982), Goldstein (1986), Tandon (1987) and Kucey et al. (1989). Phosphate solubilizing microorganisms (PSMs) are ubiquitous, and their numbers vary from soil to soil. In soil, P-solubilizing bacteria constitute 1–50% and fungi 0.5%–0.1% of the total respective population. In general, P solubilizing bacteria generally out number P solubilizing fungi by 2–150 fold (Banik and Dey, 1982; Kucey, 1983; Kucey et al., 1989). The majority of the PSMs solubilize CaP complexes and only few are effective in solubilizing FeP and AlP (Banik and Dey, 1982; Kucey et al., 1989). Hence, these PSMs could be effective in calcareous soils in which CaP complexes are present, but not in other soils such as alfisols in which phosphates are complexed with Fe and Al ions.

1.5: Citric acid secretion in microorganisms

Citric acid secretion is reported in large number of microorganisms including bacteria, fungus and yeast (Khan et al., 2006). Plants are also known to secrete high amount of citrate in root exudates. However, bacteria secrete citrate in very low amounts (~ 1 mM) (Gyaneshwar et al., 1998a). *A. niger* is known to produce citrate in molar amounts and thus the organism is used to produce citrate at large scale (Pappagiani, 2007).

1.5.1: Citrate synthase

Citrate synthase is a ubiquitous enzyme found in all eukaryotes and in most microorganisms. Its role as a catalyst of the entry point reaction for entry of two-carbon units into the citric acid cycle indicates its importance for organisms with a complete cycle, but even where the complete cycle does not function; it is an essential step in the biosynthesis of amino acids related to glutamate. Some organisms have a single CS, while a few, such as baker's yeast and *Bacillus subtilis*, have as many as three. *E. coli* citrate synthase contain 427 amino acid encoded by *gltA* gene. Most CS molecules are homodimers with a polypeptide chain between 380 and 440 residues in length. *E. coli* citrate synthase contain 427 amino acid encoded by *gltA* gene. Monomeric hexamer has molecular weight 48 KD. Type I CS is found in eukaryotes, archaea and Gram-positive bacteria. Type II CS, found in gram negative bacteria especially in members of *Enterobacteriaceae* family, was first recognized by Weitzman in 1966. Type II CS is a hexameric molecule, found only in Gram-negative bacteria. Its most striking functional property, which clearly distinguishes it from type I enzymes, is that type II CS is strongly and specifically inhibited by NADH via an allosteric mechanism (Fig. 1.13) (Weitzman, 1966).

E. coli CS is a mixture of dimers and hexamers of identical subunits, 426 amino acids in length. The dimer–hexamer equilibrium is affected by many factors as pH, ionic strength, and the presence of the allosteric inhibitor, NADH (Tong and Duckworth, 1975; Ayed et al., 1998). Citrate synthase, the ubiquitous enzyme which catalyzes entry of acetyl-coA carbon into the Krebs cycle, is potentially a key control point for energy production. In eukaryotic and Gram-positive bacteria citrate synthase, is dimer of identical subunits. The more complex citrate synthases of Gram-negative bacteria are allosteric hexamers, displaying specific inhibition by

NADH and a sigmoid saturation curve for substrate, acetyl-coA (Weitzman and Danson, 1976; Donald et al., 1991).

Acetyl-coA is enolized by simultaneous deprotonation of the methyl carbon by an aspartic acid and protonation of the carbonyl oxygen by a histidine. The neutral enol then attacks the carbonyl carbon of OAA to form the product, citryl-CoA which is subsequently hydrolyzed to citrate and coenzyme A. OAA is bound in the active site by 5 amino acids including 3 arginines and 2 histidines (Karpusas et al., 1990).

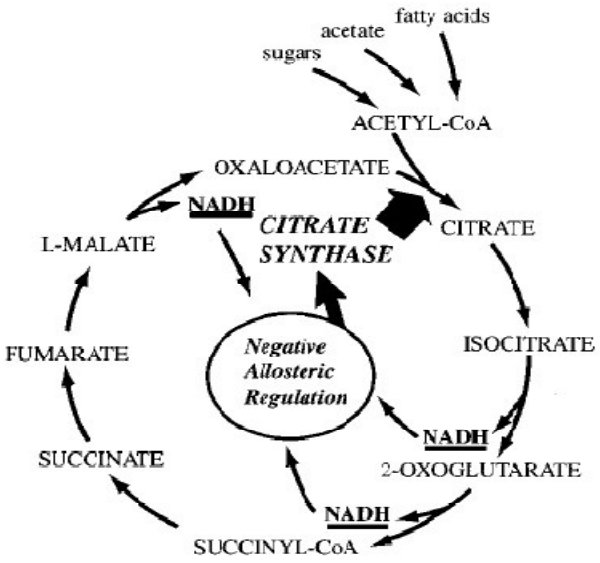


Fig. 1.13: Schematic representation of NADH inhibition of *E. coli* Type II CS (Nguyen et al., 2001).

E. coli citrate synthase loses most of its ability to discriminate between OAA and the inhibitor, α-KG, when the functional group of Arg^{407} was substituted to leucine by mutation (Pereira et al., 1993). *E. coli* citrate synthase is example of type II hexameric CS allosterically inhibited by NADH. Type I and type II subunit have overall similar fold. Type II hexamer is formed by three dimers each one folded in two domain arrangement containing 18 α helices. Presence of a 53 residue novel N-terminal domain, rich in β sheet is the major structural

difference which is absent in type I CS. Also, the length of interhelical loops is different in type I and type II CS.

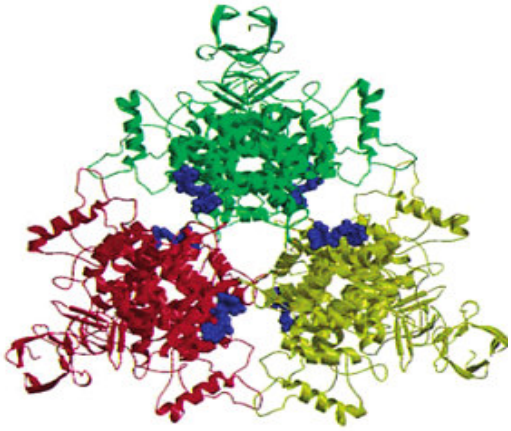


Fig. 1.14: Ribbon structure of NADH bound hexameric *E. coli* F383A type II CS. Green, red and yellow ribbons shows three equivalent dimers of this complex having central 3- fold axis while the location of the six bound NADH molecule (one per subunit) is shown in blue (Maurus et al., 2003).

Kinetic properties of CS variant, in which active site phenylalanine (Phe 383) is replaced by alanine, suggest that the equilibrium between the R (active) and T (Inactive) confirmation strongly shifted to T state. NADH acts as allosteric inhibitor and selectively binds to T state. In CS dimer state NADH occupies approximately six sites making close contact (Fig. 1.14). One face of the hexamer having three identical sites arrange with 3-fold symmetry and three more identical site slightly different from first three, on the other face. Pocket of the binding site involved are cationic and bind NADH in an unusual, roughly horseshoe confirmation.

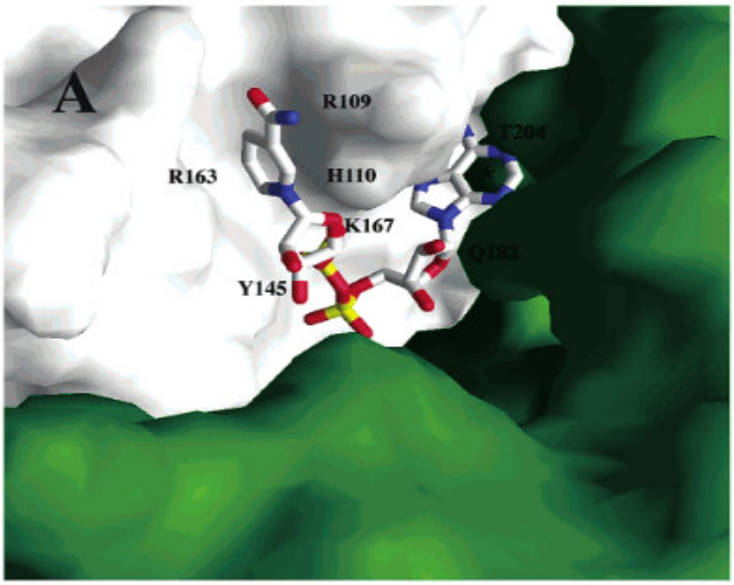


Fig. 1.15: Close-up view of the contour of the enzyme surface in the vicinity of one NADH binding site in hexameric *E. coli* CS (Maurus et al., 2003).

This is significantly different from the Rossman fold where NADH binds to the most common nucleotide binding protein motif and showed extended confirmation. Thus, NADH sites can only formed in hexameric form, by interaction of two different dimer subunits. Most of the binding pocket, including positive charge of pyrophosphate-binding, is provided by one subunit and residue from adjacent dimer required to complete formation of the adenine subsite. Arg 163, Arg 109, Tyr 145, Lys 167, His 110, Tyr 204 and Gln 182 are identified as key residues that interact with the NADH. The binding site is made up of structural component having two different subunit which are part of two different enzyme dimers in overall hexameric confirmation of *E. coli* CS (Fig. 1.15) (Maurus et al., 2003). Binding site of NADH is distinct from active sites. Thus, NADH inhibition can be weakened by different conditions like salt or alkaline pH, which favors enzyme activity of CS. Treatment with sulfydryl reagent and Ellman's reagent, affects the activity but abolish the NADH inhibition. *E. coli* CS exits in two

conformational state i.e. R state which binds acetyl-CoA selectively whereas T state binds with NADH.

In R109L variant protein of *E. coli* CS, residue 262-298 refold into a confirmation towards functional acetyl-CoA binding site. In wild type and R109L variant CS structure, residue 316-342 form helix turn helix which act as helical linker. Hexameric CS shows helical linker region (residue 316-341), structurly linkes refolded active site polypeptide chain segment region (262-298) to an NADH binding site. However, the NADH binding site involved is not from the same subunit, instead being located across the dimer-dimer interface on an adjacent subunit (Fig. 1.16).

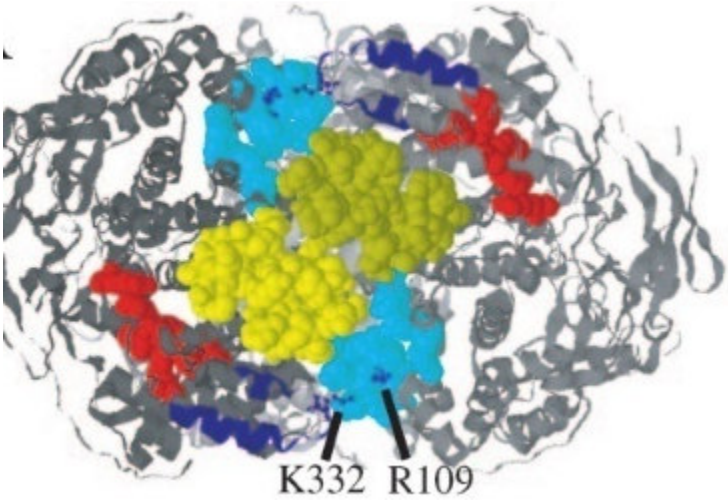


Fig. 1.16: RasMol model of *E. coli* citrate synthase (hexamer). Figure shows perpendicular 3 fold axis that relate dimer subunit and face onto one of the three relatively flat sides. Running through the middle of each side face is a 2-fold axis that relates two NADH binding sites and two active sites (Stokell et al., 2003).

In T (inactive) state in wild type CS, Arg-109 positioned so as to cause high thermal motion in helical linker region. This leads to refolding of polypeptide chain in active site so enzyme unable to bind substrate acetyl CoA. In this condition NADH stabilizes hexameric

structure of enzyme in this T state confirmation. This indicates the presence of NADH has negligible effect on the segmental mobility and overall folding of the R109L CS protein (Fig. 1.17).

In case of wild type CS, switching of R (active) state involves the release of NADH and movement of Arg to alleviate the charge- charge conflict with adjacent residue present in helical linker region. Rigidity of this linker region promote refolding of residue 262-298 form acetyl CoA binding site to catalytic reaction to be happen. This way helical linker region play a role in cross talk between NADH and active site which is in close proximity across the dimer-dimer interface in hexameric form of enzyme.

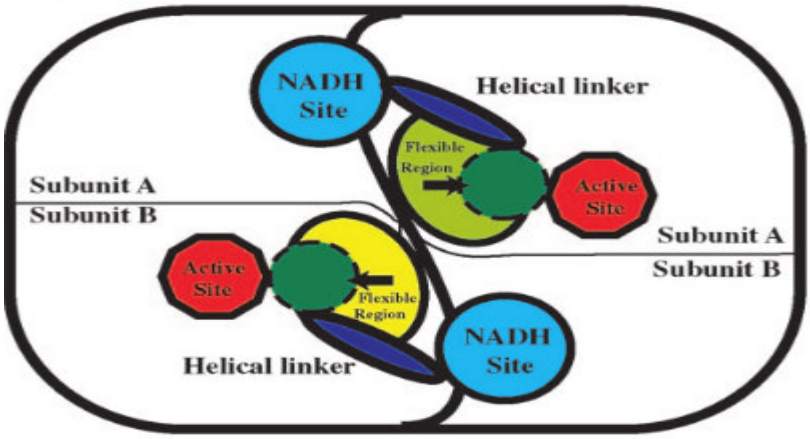


Fig. 1.17: Diagram showing proposed route of communication between NADH binding sites and active sites in the Type II CS of *E. coli* (Stokell et al., 2003).

Replacement of Arg 109 (in the NADH binding site) by Leu is accompanied by substantial changes in position and mobility of two other regions: the "helical linker" (residues 316–342, dark blue) and the "flexible region" (residues 262–298, bright yellow for one dimer, greenish yellow for the other). A regulatory signal, originating at the NADH binding sites (cyan circles), is transmitted to the 316–342 linker of the other dimer and hence via the flexible 262–

298 region to the active site (red decagons; the entry points for the two visible active sites are from the lower left and upper right, respectively). The initial step in this process may be modulated by a charge interaction between Arg and Lys 332. Changes in position and mobility of the 316–342 linker provide a more suitable surface upon which the flexible 262–298 region can pack. This packing occurs partially in R109L structure. It induces refolding of part of the flexible region, to form a binding site (green circle) for the substrate acetyl-CoA. This mechanism suggests how the six NADH binding sites and six active sites in the Type II CS hexamer might communicate so as to induce either the T (inactive) or R (catalytically active) states that are required for the control exerted by NADH on this system.

Side chains of nine amino acids are proposed to make hydrogen bonds with the NADH. Functional properties of nine sequence variants, in which these amino acids have been replaced by nonbonding residues, were checked. All of the variants show some changes in NADH binding and inhibition and small but significant changes in kinetic parameters for catalysis. NADH inhibition in three mutants (Y145A, R163L, and K167A) has become extremely weak (Maurus et al., 2003) Replacement of Arg163 by Leu is accompanied by substantial changes in position and mobility of two other regions: the “helical linker” (residues 316–342, dark blue) and the “flexible region” (residues 262–298, bright yellow for one dimer, greenish yellow for the other) they propose that a regulatory signal, originating at the NADH binding sites (cyan circles), is transmitted to the 316–342 linker of them other dimer and thence via the flexible 262–298 region to the active site (red decagons; the entry points for the two visible active sites are from the lower left and upper right, respectively). Changes in position and mobility of the 316–342 linker provide a more suitable surface upon which the flexible 262–298 region can pack which induces refolding of part of the flexible region, to form a binding site (green circle) for the substrate

acetyl-CoA (Fig. 1.17). This mechanism suggests how the six NADH binding sites and six active sites in the Type II CS hexamer might communicate so as to induce either the T (inactive) or R (catalytically active) states that are required for the control exerted by NADH on CS activity.

1.5.2: Citrate transporter

Citrate is very abundant in nature and most bacteria have transport proteins in the cytoplasmic membrane for uptake of citrate. The carriers belong to the class of secondary transporters which use the free energy stored in transmembrane electrochemical gradients of ions to drive the uptake of the substrates (Poolman and Konings, 1993). The citrate transporter CitH of *Klebsiella pneumoniae* is driven by the proton motive force and the transporters CitS and CitC of *K. pneumoniae* and *Salmonella* serovars are driven by both the proton motive force and sodium ion motive force (Lolkema, 2006). Mechanistically these transporters catalyze coupled translocation of citrate and H^+ and/or Na^+ (symport). However, citrate carriers of lactic acid bacteria take up citrate by an electrogenic uniport mechanism or by exchange with lactate (Lolkema, 2006). These citrate transporters are involved in generation of metabolic energy. A number of structural genes coding for citrate transporters have been cloned and the primary sequences have been deduced from the base sequences (Table 1.3).

The 2-hydroxycarboxylate transporter (2HCT) family is a family of bacterial secondary transporters for substrates like citrate, malate and lactate. The 2HCT family of secondary transporters contains 54 unique members that are all found in the bacterial kingdom. The characterized members of the family are transporters for citrate, malate and lactate which are substrates that contain the 2-hydroxycarboxylate motif. The members of the 2HCT family represent different modes of energy coupling like other families of secondary transporters. The transporters are H^+ or Na^+ symporters or they catalyze exchange between two substrates. Mostly

under anaerobic conditions, the transporters of the 2HCT family function in citrate and malate breakdown pathways. Na^+ -coupled citrate transporters like CitS of *Klebsiella pneumonia* and CitC of *Salmonella enterica* found in the γ subdivision of the phylum Proteobacteria which are involved in the fermentative degradation of citrate to acetate and carbon dioxide yielding ATP (Lolkema, 2006; Dobrowolski et al., 2010).

Table 1.3: Characterized members of 2HCT family (Lolkema, 2006).

Transporter	Bacteria	Substrates	Transport mode
CitS	*K. pneumoniae*	citrate	Na^+ symport
CitC	*Salmonella typhimurium*	citrate	Na^+ symport
CitW	*K. pneumoniae*	citrate, acetate	Exchange
MleP	*Lactococcus lactis*	malate, lactate	exchange
CitP	*Leuconostoc mesenteroides*	Citrate, lactate	exchange
CimH	*Bacillus subtilis*	Citrate, malate	H^+ symport
MalP	*Streptococcus bovis*	malate	H^+ symport
MaeN	*Bacillus subtilis*	malate	Na^+ symport

The pathway that consists of only exchanger and the decarboxylase results in generatation of proton motive force in the form of both a membrane potential and a pH gradient across the cytoplasmic membrane. Similarly, CitP generates membrane potential by exchange of divalent citrate for monovalent lactate. Secondary transporters are widely distributed in nature and they come in a great genetic and structural diversity which probably reflects many different translocation mechanisms (Sobczak and Lolkema, 2005). The transporters in the 2HCT family

are integral membrane proteins consisting of about 440 amino acid residues. The core of the structure is formed by two homologous domains that are connected by a large hydrophilic loop which resides in the cytoplasm. The domains contain 5 transmembrane segments (TMSs) each and they have opposite orientations in the membrane (Lolkema, 2006; Dobrowolski et al., 2010).

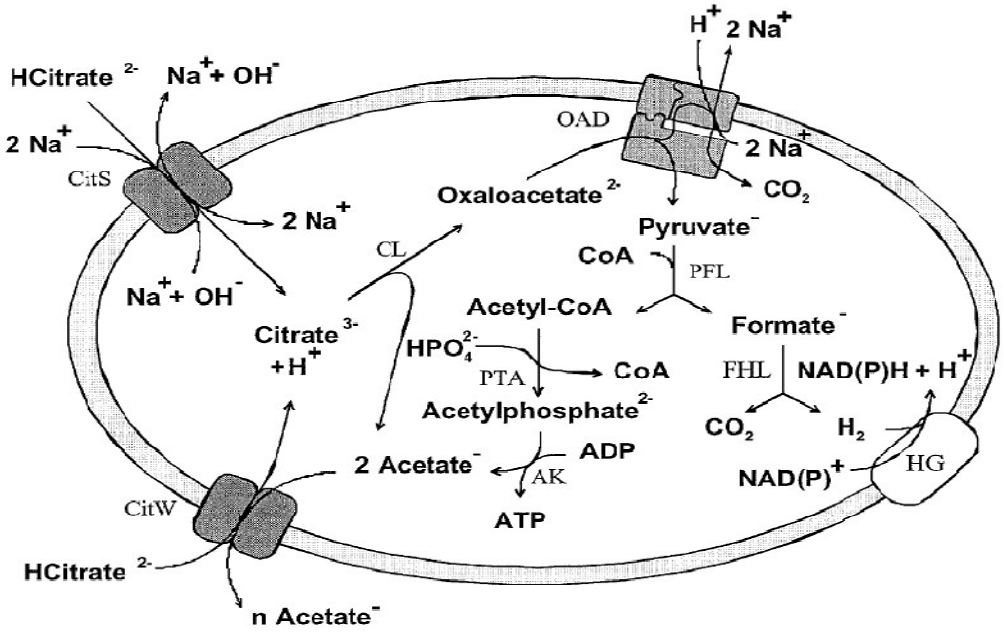


Fig. 1.18: Citrate fermentation pathway in *Klebsiella pneumonia* (K□stner et al., 2002).

In *K. pneumoniae* citrate is transported into the cell by the Na^+-dependent citrate carrier CitS and the citrate/acetate exchanger CitW. Internal citrate is cleaved by the citrate lyase (CL) to oxaloacetate and acetate. Oxaloacetate is decarboxylated to pyruvate by the oxaloacetate decarboxylase Na^+ pump (OAD). Pyruvate formate lyase (PFL) converts pyruvate to formate and acetyl-CoA. Acetyl-CoA is converted to acetyl-phosphate by phosphotransacetylase (*PTA*) and further to acetate by the acetate kinase (*AK*) with ATP generation. Formate is converted to CO_2 and H_2 by formate hydrogen lyase (*FHL*) and the H is utilized for NAD (P) reduction by membrane-bound hydrogenase (HG) (Steuber et al., 1999). The end product acetate is expected

to leave the cell via the citrate/acetate antiporter CitW in exchange for citrate (Fig. 1.18) (Kustner et al., 2002).

1.6: Microorganisms secreting oxalic acid

Plants are also known to secrete oxalic acid in root exudates for Al tolerance and phosphate mineralization. Although oxalic acid secretion has been reported in bacteria and fungi, only few bacteria are known to secrete oxalate up to few millimolar amount (2 mM) including *Arthrobacter* sp., *Micrococcus* sp. and *Bacillus* sp. (Khan et al., 2006).

1.6.1: Oxalic acid biosynthesis in bacteria

In bacteria, oxalic acid biosynthesis is mediated by glyoxylate dehydrogenase which breaks down glyoxalate to oxalate when flux towards glyoxalate pathway is more instead of TCA cycle. *P. fluorescens* ATCC 13525 is also known to secrete oxalic acid under Al stress when Al-citrate was given as carbon source (Apanna et al., 2003). Thus, the organism secretes oxalic acid to alleviate Al toxicity by diverting the flux towards glyoxylate pathway involving increase in isocitrate lyase activity under Al toxic conditions.

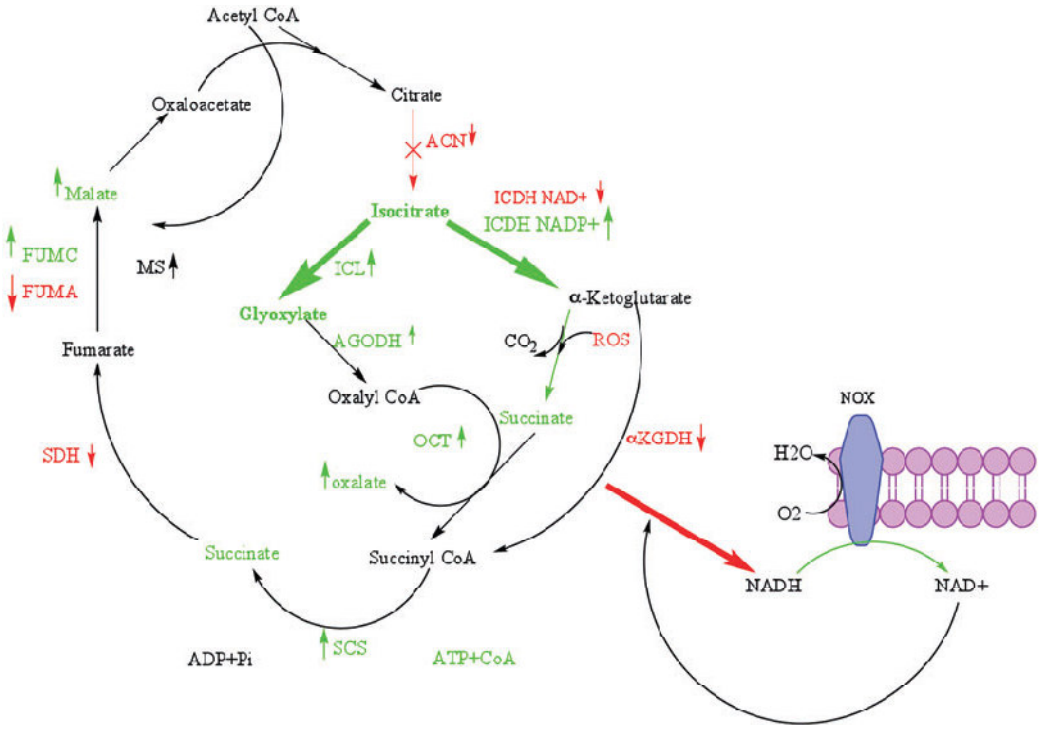


Fig. 1.19: Al-induced metabolic adaptation to counter Fe deprivation in *P. fluorescens*. ↑ increase in activity, ↓ decrease in activity. OCT, oxalyl-CoA transferase; AGODH, acylating glyoxylate dehydrogenase; SCS, succinyl-CoA synthase (Mailloux et al., 2007; Chenier et al., 2008; Singh et al., 2009).

Al is also called as pro-oxidant due to its ability to increase the free Fe-burden of the cell which leads to increased ROS production (Exley, 2004; Beriault et al., 2007). During aerobic respiration, the majority of ROS is formed when electrons from NADH and $FADH_2$ are transported via the ETC to the O_2. ROS generation is decreased by two key enzymes of the TCA cycle (ICDH-NAD and KGDH) involved in the production of NADH which were down regulated under Al-stress conditions (Mailloux et al., 2007). Furthermore, formation of ATP promoted by the ETC is not favoured during Al stress due to the diminished bioavailability of Fe. Thus, the production of ATP via oxidative phosphorylation is inefficient under Al stress. To circumvent this problem, *P. fluorescens* possesses an intricate system involving succinyl-CoA

synthetase (SCS) and oxalate-CoA transferase (OCT) to produce ATP via substrate level phosphorylation (Singh et al., 2009) (Fig. 1.6). This metabolic alteration not only fulfils the energy requirement, but also enables the microbe to synthesize oxalate which efficiently sequesters Al^{+3}. All though ACN, a key critical enzyme of the TCA cycle is severely impeded, the microbe increases the ICL activity to facilitate the degradation of citrate. This metabolic shift provides ATP, NADPH and oxalate; ingredients that help mediate the survival of *P. fluorescens* challenged by Al toxicity (Fig. 1.19).

1.6.2: Oxalic acid biosynthesis in fungi

Numerous filamentous fungi, including the food biotechnology fungus *Aspergillus niger*, the opportunistic human pathogen *Aspergillus fumigatus*, the phytopathogenic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum*, *Penicillium* sp. as well as many brown-rot and white-rot basidiomycetes, are able to efficiently produce large quantities of oxalate (Dutton et al., 1996; Gadd, 1999). Oxalic acid biosynthesis in fungi is a toxic by-product of citric acid production and its biosynthesis depend on whether glucose or citric acid is used as the carbon source (Wolschek and Kubicek, 1999). Oxalate secretion is associated with fungal pathogenesis (Munir et al., 2001). In the wood-rotting fungus *Fomitopsis palustris* oxalate is formed chiefly by glyoxalate pathway (Munir et al., 2001). Production of oxalate in fungi occurs by three pathways - oxidation of glyoxylate (Maxwell and Bateman., 1968; Balmforth and Thomson 1984), oxidation of glycolaldehyde (Hammel et al., 1994), and hydrolysis of oxaloacetate (Lenz et al., 1976). The results of studies of [^{14}C] CO_2 incorporation into the metabolite pools of *A. niger* demonstrated that oxalate is derived from oxaloacetate (Kubicek et al., 1988). Oxaloacetate acetyl hydrolase (OAH) catalyzes the hydrolytic cleavage of oxaloacetate to form acetate and oxalate (Lenz et al., 1976).

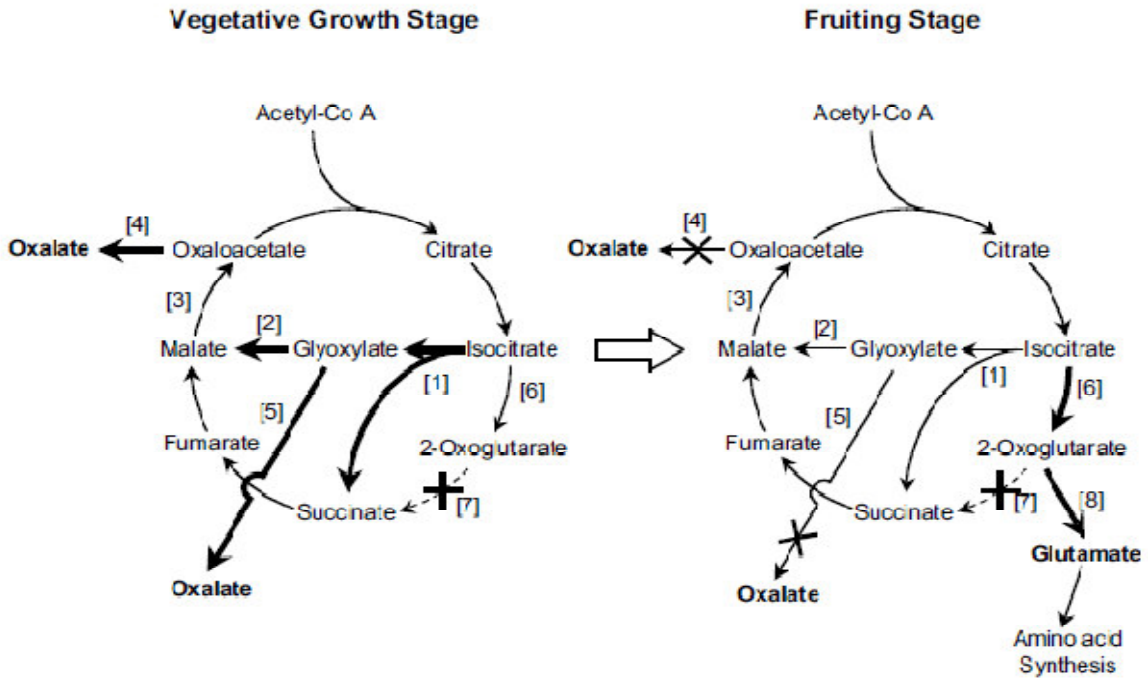


Fig. 1.20: Proposed carbon metabolism in the vegetative growth and fruiting stages of the wood-rotting basidiomycete *F. palustris* during fruit body development. 1- ICL; 2- MS; 3- MDH; 4- OXA; 5- GLOXDH; 6- IDH; 7- ODH; 8- GLTDH (Yoon et al., 2002).

In a subsequent study, a mutant *A. niger* strain NW228 was found to be deficient in both oxalate production and in the synthesis of active OAH (Hombergh et al., 1995; Ruijter et al., 1999). These observations suggest that oxalate is produced only by the OAH catalyzed process in *A. niger*.

TCA cycle enzymes play a more important role than the GLOX cycle enzymes in mycelium during fruit body formation (Fig. 1.20) (Yoon et al., 2002). Activities of the two key enzymes of the GLOX cycle and the two oxalate-producing enzymes in mycelium increased during vegetative growth of the fungus, whereas they decreased during fruit body formation.

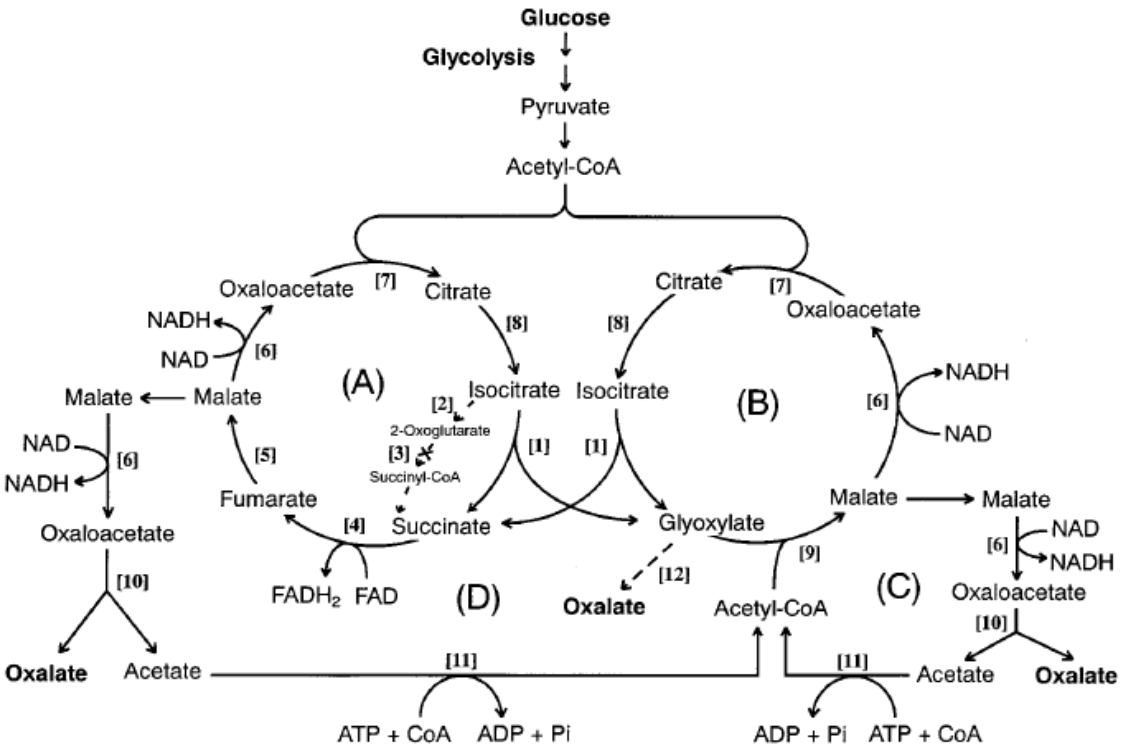


Fig. 1.21: A proposed metabolic mechanism for the oxalate biosynthesis in the wood-rotting basidiomycete *F. palustris*. (A) TCA cycle; (B) GLOX cycle; (C and D) acetate-recycling routes. 1. Isocitrate lyase (ICL); 2. Isocitrate dehydrogenase (IDH); 3. 2-Oxoglutarate dehydrogenase (ODH); 4. Succinate dehydrogenase; 5. Fumarase; 6. Malate dehydrogenase (MDH); 7. Citrate synthase; 8. Aconitase; 9. Malate synthase (MS); 10. Oxaloacetase (OXA); 11. Acetyl-CoA synthase;12. Glyoxylate dehydrogenase (GLOXDH) (Munir et al., 2001).

IDH activity, which was much lower than the ICL activity at the stage of vegetative growth, predominated over ICL activity during fruit body formation which indicates that the carbon flow of isocitrate directed from oxalate synthesis to glutamate synthesis mediated by increase in glutamate dehydrogenase (GLTDH) activity (Fig. 1.20) (Yoon et al., 2002). However, ICL and OXA predominate over IDH and GLOXDH, respectively, regardless of the culture media. Furthermore, acetyl-CoA synthase was detected in a significant amount which

indicates that acetate, liberated from oxaloacetate hydrolyzed by OXA, was recycled by routes C and D involving acetyl-CoA synthase (Fig. 1.21) (Munir et al., 2001).

Oxalate formation in fungi is known to be associated with plant pathogenesis as oxalate and polygalacturonase act synergistically in depolymerization of plant tissue (Maxwell and Bateman 1968; Balmforth and Thomson 1984; Amadioha 1994). Several pathways leading to oxalate production have been elucidated in fungi. One involves the cleavage of oxaloacetate by the enzyme oxaloacetate hydrolase to form oxalate and acetate (Kubicek et al. 1988); in another, glyoxylate is oxidized to oxalate by the enzyme NAD-glyoxylate dehydrogenase (Balmforth and Thomson, 1984). An NADP-glyoxylate dehydrogenase that produces oxalyl-CoA as the initial product has also been found (Quayle and Taylor, 1961).

In *A. niger*, oxalate is produced solely by oxaloacetate hydrolase (Kubicek et al., 1988). OAH was induced by growth on acetate with nitrate as the nitrogen source at pH 8. The enzyme oxaloacetate hydrolase has been partially purified from *A. niger* (Lenz et al., 1976).

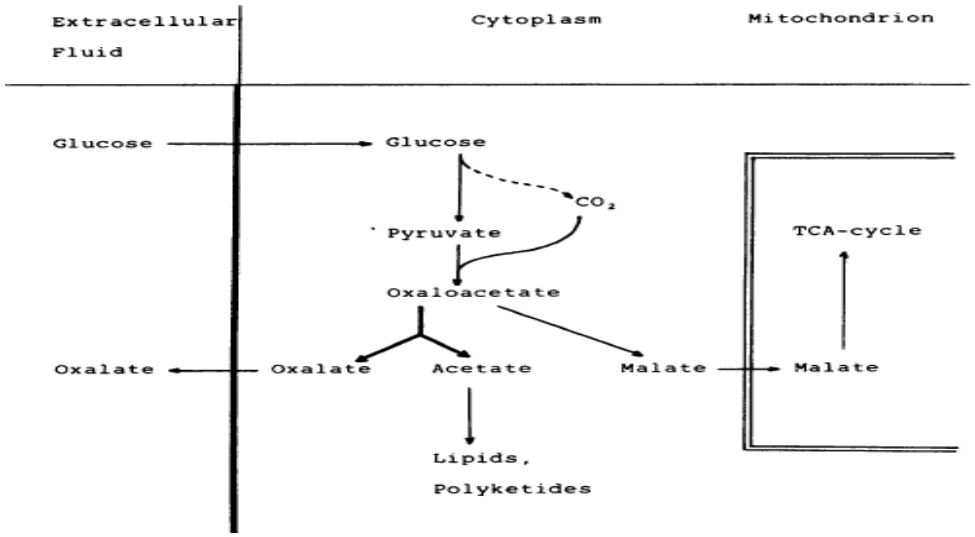


Fig. 1.22: Oxalate biosynthesis in *A. niger* (Kubicek et al., 1988).

The biosynthetic pathways of organic anions were first studied in saprotrophic fungi such as *A. niger*, as this ascomycete species is able to produce huge amounts of oxalate and/or citrate from glucose supplied in the culture medium. Oxalate may be formed by splitting of oxaloacetate or oxaloasuccinate, but not by direct oxidation of acetate, and that citrate is probably mainly produced by condensation of carbon dioxide with a 3-carbon compound (Bomstein and Johnson, 1952).

A combination of labelling studies, subcellular fractionation methods and inhibition of mitochondrial citrate metabolism, demonstrated that oxalate was formed by a cytoplasmic pathway which does not involve the tricarboxylic acid cycle (Kubicek et al., 1988). Initial steps involve fixation of carbon dioxide onto a molecule of pyruvate by the cytoplasmic pyruvate carboxylase to produce oxaloacetate. Inhibition of mitochondrial citrate metabolism by fluorocitrate did not significantly decrease the oxalate yield, suggesting that oxalate formation does not involve the tricarboxylic acid cycle (Fig 1.22).

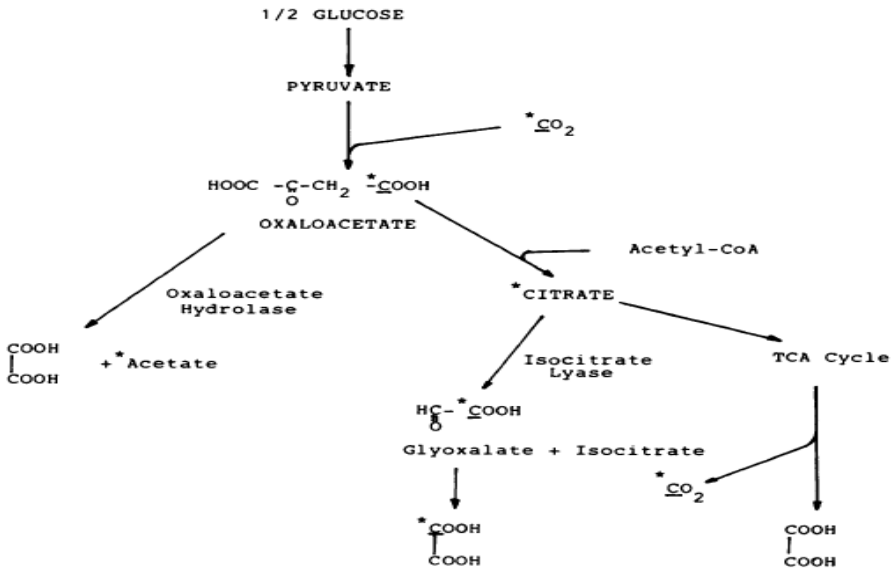


Fig. 1.23: Pathways of oxalate biosynthesis in *A. niger* (Kubicek et al., 1988).

Exogenously added $^{14}CO_2$ was not incorporated into oxalate, but was incorporated into acetate and malate, indicating the biosynthesis of oxalate from the cleavage of oxaloacetate which suggested cytoplasmic OAH responsible for this cleavage (Fig. 1.23) (Kubicek et al., 1988). ^{14}C-acetate produced from oxaloacetate hydrolysis never accumulated in the culture medium but a significant ^{14}C label in ethyl acetate extract of *A. niger* was measured, presumably containing polyketides and lipids, indicating that acetate is converted to acetyl coenzyme A under these conditions. This pathway and the main role of OAH were confirmed by the discovery of a mutant of *A. niger* lacking OAH that did not produce any oxalate, implying that OAH is the only enzyme involved in oxalate production in this fungal species (Ruijter et al., 1999). This pathway for oxalate production also demonstrated in the phytopathogenic fungal species *Botrytis cinerea* as the disruption of *oah*A gene coding OAH resulted in the loss of oxalate formation (Han et al., 2007).

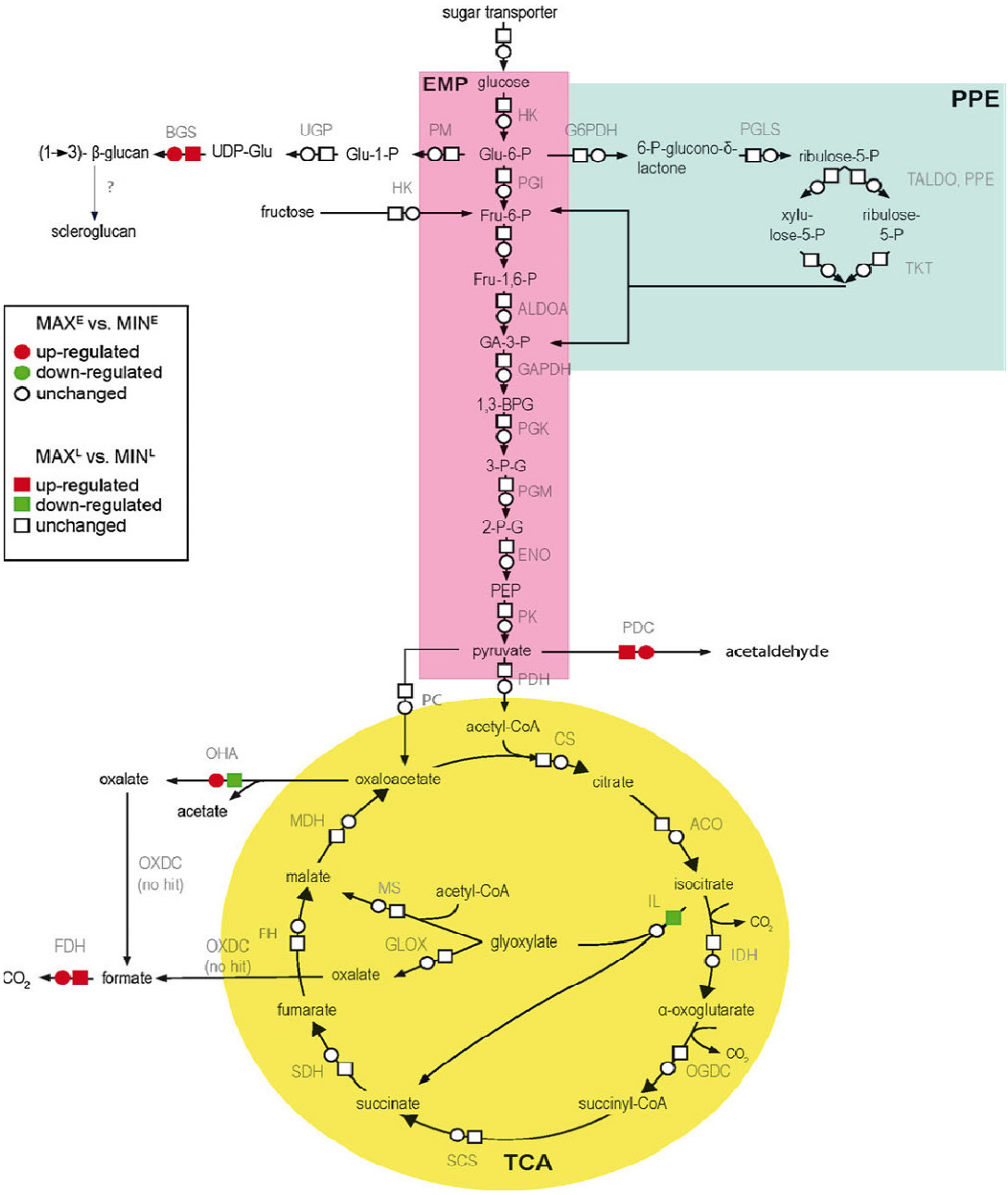
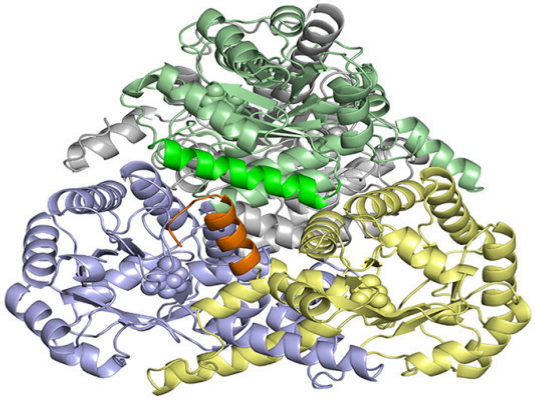


Fig. 1.24: Changes in transcript levels of genes associated with scleroglucan and oxalate metabolism, glycolysis, pentose-phosphate pathway and TCA (Schmid et al., 2011). MAX represents scleroglucan synthesis in high-level producing media and MIN represents scleroglucan synthesis in low-level producing media, respectively. E and L represent the early (E, 37 h) and the late (L, 61 h) stage of scleroglucan synthesis.

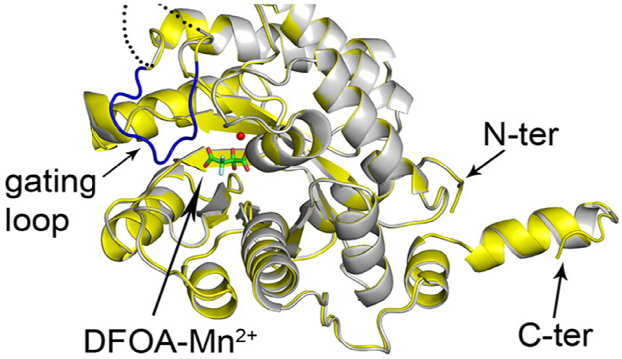
1.6.3 Structure of oxaloacetate acetylhydrolase

Oxalacetate acetylhydrolase (OAH) catalyzes the hydrolysis of oxalacetate to oxalic acid and acetate. OAH is a member of the phosphoenolpyruvate mutase (PEPM)/ ICL superfamily. Members of this superfamily act on α-oxycarboxylate substrates and cleave/form C–C or P–C bonds. Memebers of PEPM/ICL superfamily are oligomeric proteins, mostly tetramers, and each subunit adopts an $(\alpha/\beta)_8$ barrel fold. The tetramer is composed of a dimer of dimers arranged in a manner that the two molecules within a dimer swap the 8th helix of the α/β barrel. The active site is located at the C-terminal side of the β-barrel. In most PEPM/ICL superfamily members a mobile loop controls interaction with the active site. Gating loop closes to sequester the catalytic site from bulk solvent when substrate binds. All PEPM/ICL superfamily members require Mg^{2+} or Mn^{2+} ions for activity as the metal mediates the interactions between the protein and the substrate (Chen et al., 2010). The overall oligomeric structure of OAH shows that it forms a dimer of dimers consisting of four $(\alpha/\beta)_8$ barrels (Fig. 1.25 A and B), with two subunits swapping their 8th helices (Fig. 1.25C). The bottom of each $(\alpha/\beta)_8$ barrel is flanked by an additional α -helix. In a barrel 8th helix, the polypeptide chain meanders to form two more α -helices and traverses the top of the $(\alpha/\beta)_8$ barrel of the partner above its active site entrance which is indicated as “partner subunit cap” (Fig. 1.25 C). Interaction between the orange-colored C terminus of the yellow subunit with the molecule colored green shows that the C terminus reaches a third subunit (Fig. 1.25 A). Much of the active site gating loop in the ligand-free OAH and OAH/oxalate structures is disordered.

(A)



(B)



(C)

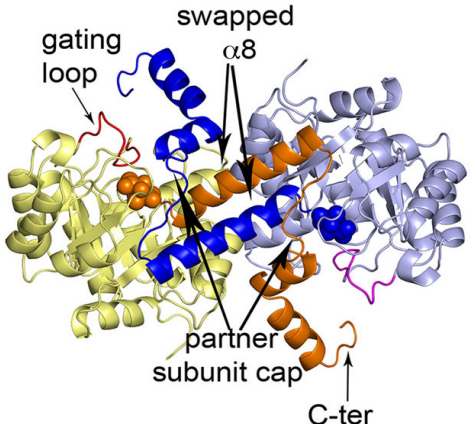


Fig. 1.25: Ribbon representation of the overall fold and oligomeric association of OAH (Chen et al., 2010).

The DFOA-bound OAH exhibits a well ordered gating loop in the closed conformation (Fig. 1.25 B). As with all members of the PEPM/ICL superfamily, the OAH active site is located in the center of the α/β barrel at the C-terminal side of the β-strands. Residues that flank the substrate-binding cavity are located on loops connecting the β-strands with the α-helices and the gating loop is the longest of them. When bound with DFOA the protein undergoes localized conformational transition that organizes the gating loop in closed conformation (Fig. 1.25 B).

In figure part A shows dimer of dimers association that define the physiologically relevant tetramer and bound DFOA molecules are shown as space-filling models. The helix colored dark orange depicts the C terminus of the yellow subunit which interacts with the green subunit. Part B shows overall fold of a monomer and the ordered gating loop corresponding to the ligand-bound state is shown in blue color.

The disordered gating loop in the ligand-free state is indicated by dashed line. The bound inhibitor (DFOA) is shown in stick model and the Mn^{2+} cofactor is shown as a sphere. Part C shows dimer formed by swapping α-helices. The swapped 8th α-helices are colored orange. The inhibitor is shown as space-filling model and ter shows the terminal.

1.6.4 Oxalic acid transporters in microorganisms

Oxalic acid secretion in bacteria is not very clear as the oxalate efflux system is not known. However, in obligate anaerobe *Oxalobacter formigens* an oxalate antiporter (OxlT) is involved in uptake of oxalate, which was converted to formate by oxalate decarboxylase (Ye et al., 2001). This would result in generation of a pH gradient leading to ATP synthesis by using proton motive force. In some organisms, the dicarboxylate transporters (DctA and DctB) may help in efflux of oxalate in the extracellular medium. Wood rotting fungus *Fomitopsis plaustris*

possess an oxalate transporter encoded by the gene *FpOAR*, which results in secretion of very high amount of oxalate (up to 78 mM) in the extracellular medium (Watanabe et al., 2010).

1.7: *Vitreoscilla* heamoglobin

Oxygen is very important for life as it is required in the energy metabolism as an electron acceptor molecule. Additionally, oxygen is required for regulation of various cellular functions and byproduct accumulated by stress response (Frey and Kallio, 2003). Oxygen and cognate oxygen radicals can affect microbial cell either by altering the function or biosynthesis of specific proteins mediated by global transcription regulation.

Globins are present in all five kingdoms of life and divided into vertebrate and non-vertebrate groups which include plants, fungi and protozoans, Common characteristics of all hemoglobins (Hbs) is that it binds reversibly to oxygen. Obligate anaerobic bacterium *Vitreoscilla* synthesizes homodimeric hemoglobin (VHb) which is the best characterized member of bacterial Hb family (Webster and Hacket, 1966). *vgb* gene encodes 15.7 kDa protein and is expressed under the control of oxygen dependent promoter (*Pvhb*) which induced under oxygen limiting condition. *Pvhb* has been characterized in *E. coli* and shown to be functional in many heterologous hosts *viz Pseudomonas*, *Azatobactor*, *Rhizobium etli*, *Streptomyces* sp., *Burkhulderia* sp., *Enterobactor aerogens*, yeast etc. Pvhb expression gets induced when dissolved oxygen level is less than 2% air saturation in both *Vitreoscilla* and *E. coli* (Khosla and Bailey, 1988). Pvhb activity in *E. coli* is modulated by CRP and FNR and in the absence of CRP or cAMP, expression of *Pvhb* decreased substantially. Maximal Pvhb induction increased recombinant protein production to about 10% of total cellular protein (Khosala et al., 1990).

High expression level of *Pvhb* and its regulatory mechanism could be interesting to study for biotechnological process applications. Kallio et al. (1994) had proposed that VHb acts as an additional oxygen source and increases intracellular dissolved oxygen level under microaerobic conditions which result in increased activities of cytochrome o and cytochrome d. Increase in terminal oxidase activity facilitates proton pumping for generation of ATP when proton reenters the cytoplasm via ATPase. Increased proton pumping results increased amount of ATP production subsequently (Fig. 1.26). This model was further supported by study on energy metabolism in *E. coli* and *Saccharomyces cerevisiae* (Chen et al., 1994; Kallio et al., 1994; Tsai et al., 2002).

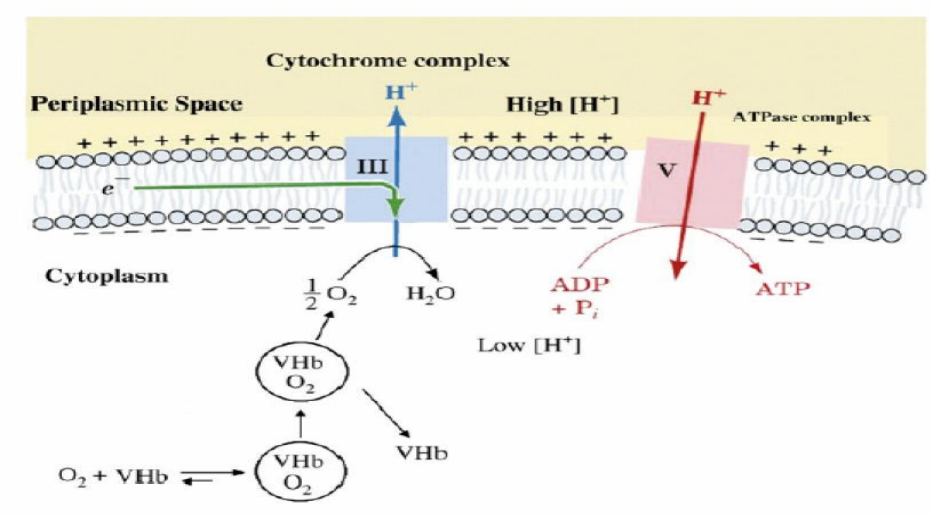


Fig. 1.26: Proposed Energy generation model in presence of VHb (Zhang et al., 2007).

Investigation of tRNA and ribosome content revealed that cells expressing VHb showed higher level of tRNA and ribosome as compared to control cells, which help in enhancing translational component by VHb (Roos et al., 2002). VHb provides oxygen to respiration apparatus of host organism under limited oxygen condition (Webster, 1988; Chi et al., 2009). This hypothesis is also supported by localization and concentration of VHb near

periphery of cytosolic face of cell membrane in *Vitreoscilla* and *E. coli* (Ramandeep et al., 2001) which could improve the uptake of oxygen (Erenler et al., 2004), and specifically interacts with lipids of cell membrane (Rinaldi et al., 2006) and cytochrome O (Ramandeep et al., 2001; Park et al., 2002).

VHb stimulates oxygenase activity and acts as terminal oxidase (Dikshit et al., 1992), modulates redox status of cells (Tsai et al., 1995), detoxifies nitric oxide (Frey et al., 2002; Kaur et al., 2002) and give protection against oxidative stress (Geckil et al., 2003; Kvist et al., 2007). Presence of VHb in cells altered antioxidant enzymes status to protect cells from oxidative damage. *vgb* expressing *Enterobactor aeruginosa* showed elevated level of catalase and become more tolerant to oxidative stress (Geckil et al., 2003). In another study, *vgb* expressing *Streptomyces lividens* exhibits significant upregulation of *katA* (catalase peroxidase A) and *sodF* (superoxide dismutase F) which are antioxidant enzymes (Kim et al., 2007). In oxidative stress condition VHb expression significantly minimizes H_2O_2 effect on cells growth rate and survival (Anand et al., 2010). Protective effects of VHb are reported in many heterologous hosts where vhb not expressed from native promoter could get induced from an alternate promoter by OxyR transcriptional activator (Abrams et al., 1990; Anand et al., 2010) (**Fig. 1.27**).

VHb has been extensively used for metabolic engineering and improved in fermentation processes. Industrial production of metabolites requires modification in complex metabolic pathway. Limited aeration condition during the cultivation of various microbial strains result in decreased activity of cytochrome P-450 monooxygenase which is required for the processing of pathway intermediates into final form leading to the overflow of intermediate metabolic

products instead of final product.

To minimize this problem, *vgb* was expressed in *E. coli* and results showed enhanced growth and protein production under microaerobic condition (Khosla and Bailey, 1988a,b; Khosla et al., 1990). Oxygen is required at several steps in degradation pathway of hazardous contaminants of soil and ground water. But low levels of dissolved oxygen (DO) seem to be limiting factor for the bioremediation mediated by microorganisms (Zhang et al., 2007). To overcome this problem, several attempts had been made to develop organism which could show better growth and bioremediation property under hypoxic condition (Liu et al., 1996; Patel et al., 2000; Nasr et al., 2001; Urgun-Demirtas et al., 2003; 2004; So et al., 2004).

vgb expressing *Burkholderia* sp. enhanced 2,4-dinitrotoluen (DNT) degradation in continuous flow sand column bioreactor. Degradation of benzoic acid by *Xanthomonas mathophillia* (Liu et al., 1996) and 2-chlorobenzoic acid by *Burkholderia cepacia* (Urgun-Demirtas et al., 2003; 2004) also result in similar type of effects. VHb incorporation in *Rhodococcus erythropolis* LSSE8-1 improved growth and biodesulfurization under lower oxygen conditions (Xiong et al., 2007).

Under oxygen limited condition, *R. etli* expressing VHb increased respiratory activity, chemical energy content and expression of *nifHC* gene which result in increased efficiency of ATP production, nitrogenase activity and total nitrogen content in bean plants inoculated with recombinant *R. etli* (Ramirez et al., 1999).

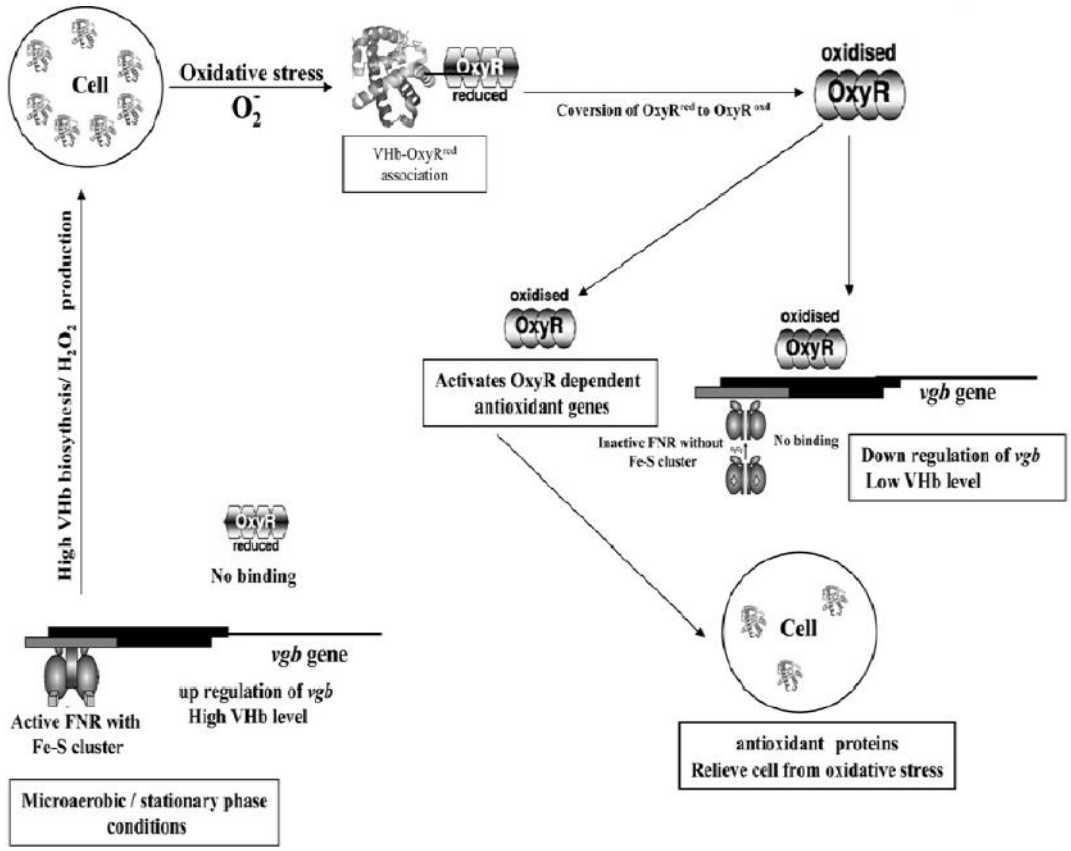


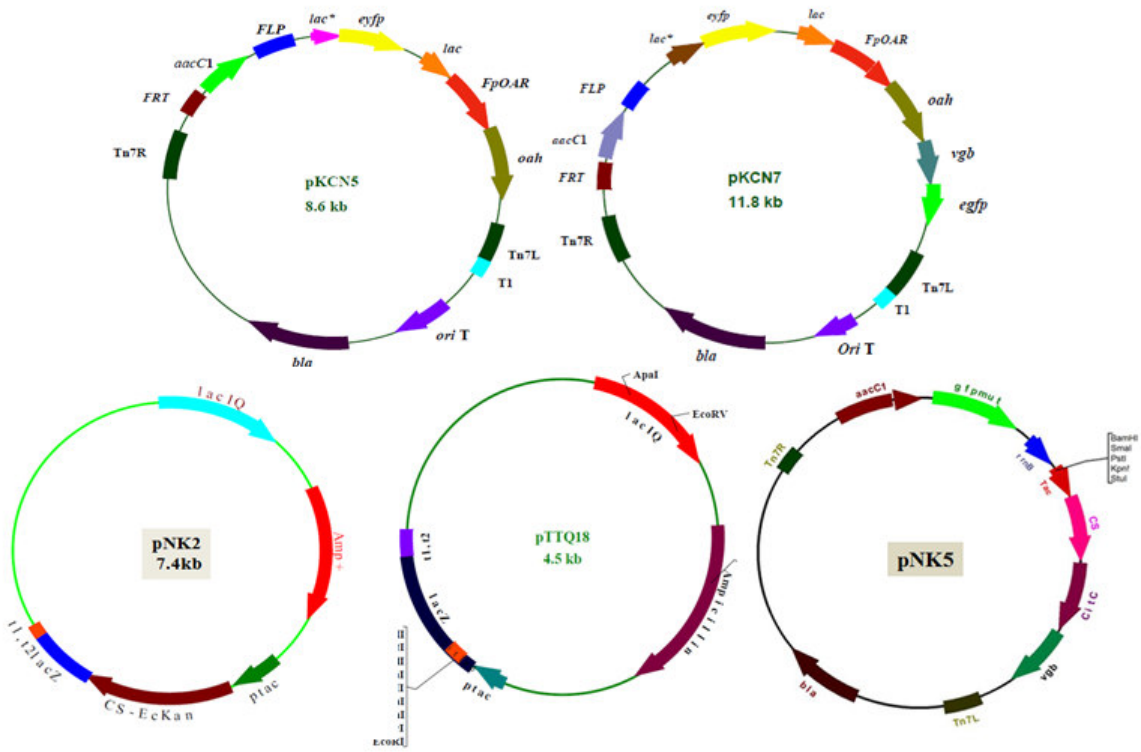
Fig. 1.27: Proposed role of *vgb* during oxidative stress (Anand et al., 2010).

1.8: OBJECTIVES OF THE PRESENT STUDY

The objectives of the present study were

1. Effect of overexpression of NADH insensitive *E. coli* CS (*cs**) gene in *Citrobacter* DHRSS.
2. Effect of coexpression of *E. coli cs** and Sodium dependent transporter (*citC*) of *Salmonella typhimurium* genes on citric acid efflux in *Citrobacter* DHRSS.
3. Effect of coexpression of NADH insensitive *E. coli* CS, Sodium dependent transporter (*citC*) of *Salmonella typhimurium* and *Vitreoscilla* haemoglobin (*vgb*) (*tac*cs*-citC-vgb* gene cluster) on MPS ability and citric acid secretion in *Citrobacter* DHRSS.
4. Effect of heterologous overexpression of *A. niger oah* gene in *Pseudomonas fluorescens* 13525**.**

5. Effect of coexpression of *A. niger oah* gene and oxalate transporter (*FpOAR*) of *Fomitopsis plaustris* in *Pseudomonas fluorescens* 13525.
6. Effect of *oah-oxlT-vgb-gfp* gene cluster integration in *Pseudomonas fluorescens* 13525 on MPS ability.



CHAPTER 2

Materials and Methods

2.1: Bacterial strains and Plasmids

All the wild type and genetically modified *E. coli*, *Citrobacter* sp. DHRSS, *Pseudomonas fluorescens* and fungus strains are listed in **Table 2.1**. The plasmids used in the present study and their restriction maps are given in **Table 2.2** and **Fig. 2.1**. *E. coli* DH10B was used for all the standard molecular biology experiments wherever required. The *glt*A (citrate synthase gene) mutant of *E. coli* was obtained from *E. coli* Genetic Stock Center (CGSC), Yale University, U.S.A. The NADH insensitive *E. coli cs* mutant (Y145F) was a generous gift from Dr. H. W. Duckworth, University of Manitoba, Canada. pUC8:16 containing *Vitreoscilla* hemoglobin (*vgb*) gene was generously gifted by Dr. B. C. Stark, Illinois Institute of Technology, USA.

Bacterial Strains	**Genotype**	**Reference**
E. coli DH10B	F^- *endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ (ara, leu) 7697 mcrA Δ(mrr-hsdRMS-mcrBC)* $^{\lambda-}$	Invitrogen, USA
E. coli W620	CGSC 4278 - *glnV44* ***gltA6*** *galK30 LAM- pyrD36 relA1 rpsL129 thi-1*; Str^r	*E. coli* Genetic Stock Center
W620 (pTTQ18K)	*E. coli* W620 with pTTQ18K plasmid; Str^r, Km^r	Ch. 3
W620 (pNK2)	*E. coli* W620 with pNK2 plasmid; Str^r, Km^r	Ch. 3
W620 (pNK3)	*E. coli* W620 with pNK3 plasmid; Str^r, Km^r	Ch. 3
W620 (pNK5)	*E. coli* W620 with pNK5 plasmid; Str^r, Gm^r	Ch. 5
Citrobacter sp. DHRSS	Sugarcane rhizosphere isolate	Patel et al., 2008
C. DHRSS (pTTQ18K)	*C.* DHRSS with pTTQ18K plasmid; Ap^r, Km^r	Ch. 4
C. DHRSS (pNK2)	*C.* DHRSS with pNK2 plasmid; Ap^r, Km^r	Ch. 4
C. DHRSS (pNK3)	*C.* DHRSS with pNK3 plasmid; Ap^r, Km^r	Ch. 4

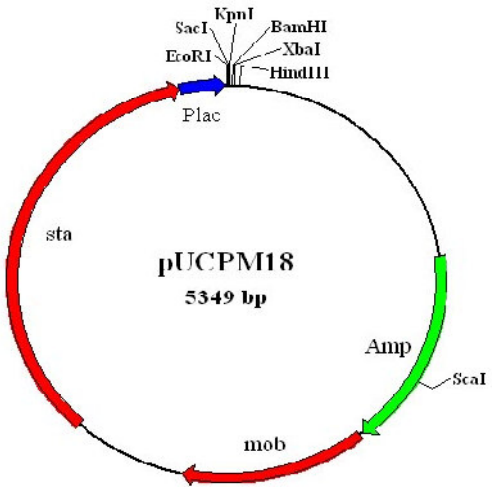
C. DHRSS (pNK5)	*C.* DHRSS with pNK5 plasmid; Ap^r, Gm^r	Ch. 5
DH10B (pKCN2)	*E. coli* DH10B with plasmid pKCN2; Ap^r, Gm^r	Ch. 6
DH10B (pKCN4)	*E. coli* DH10B with plasmid pKCN4; Ap^r, Gm^r	Ch. 6
DH10B (pKCN6)	*E. coli* DH10B with plasmid pKCN6; Ap^r, Gm^r	Ch. 6
DH10B (pKCN5)	*E. coli* DH10B with plasmid pKCN5; Ap^r, Gm^r	Ch. 7
DH10B (pKCN7)	*E. coli* DH10B with plasmid pKCN7; Ap^r, Gm^r	Ch. 7
P. fluorescens ATCC 13525	Wild Type	ATCC
Pf (pUCPM18G)	*P. fluorescens* 13525 with plasmid pUCPM18G; Ap^r, Gm^r	Ch. 6
Pf (pKCN2)	*P. fluorescens* 13525 with plasmid pKCN2; Ap^r, Gm^r	Ch. 6
Pf (pKCN4)	*P. fluorescens* 13525 with plasmid pKCN4; Ap^r, Gm^r	Ch. 6
Pf int1	*P. fluorescens* 13525 genomic integrant containing *oah, FpOAR*; Ap^r	Ch. 7
Pf int2	*P. fluorescens* 13525 genomic integrant containing *oah, FpOAR, vgb, gfpmut*3; Ap^r	Ch. 7
Pf int3	*P. fluorescens* 13525 genomic integrant containing *gfpmut*3; Ap^r	Ch. 7
Aspergillus niger K85	Soil isolate	Ch. 6
Fomitopsis plaustris	Wood rotting fungus	MTCC

Table 2.1: List of Bacterial and Fungul strains used in the present study. Ap= Ampicillin; Km= Kanamycin; Gm= Gentamycin; Str =Streptomycin; r = resistant.

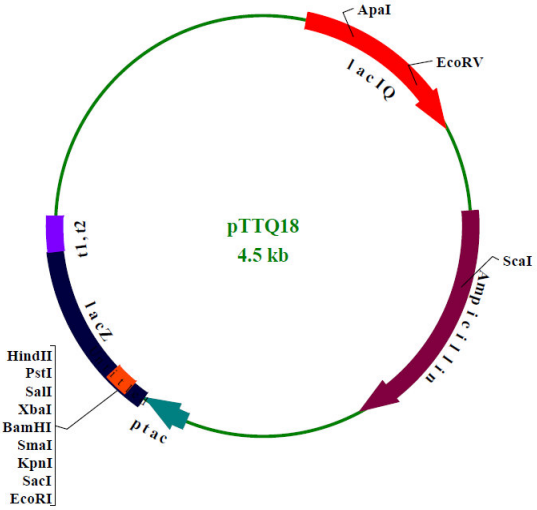
Plasmids	Features	Reference
pTTQ18	Cloning vector- ColE1 origin, P *tac*, Ap^r	Stark et al., 1987
pTTQ18K	pTTQ18 containing *nptII* gene; Ap^r, Km^r	Ch. 3
pTTQ18Gm	pTTQ18 containing *aacC1* gene, Ap^r, Gm^r	Ch. 5
pUCPM18Gm	pUC18 derived Broad-Host-Range vector; Ap^r, Gm^r	Ch. 6
pUC18T-mini Tn7 T-Gm-*gfpmut*3	Mini-Tn7 delivery vector, gfpmut3; Ap^r, Gm^r	Choi et al., 2010
pUC18T-mini-Tn7T-Gm-*eyfp*	Mini- Tn7 delivery vector, P *lac*, eyfp; Ap^r, Gm^r	Choi et al., 2010
pNK2	pTTQ18 with *E. coli cs** gene of *E. coli*; Ap^r, Km^r	Ch. 3
pNK3	pTTQ18 with *E. coli cs** gene of *E. coli* and *S. typhimurium citC* gene; Ap^r, Km^r	Ch. 3
pNK5	pUC18T-mini Tn7T with constitutive *tac-cs-citC* operon and *vgb* gene of *Vitreoscilla*; Ap^r, Gm^r	Ch. 5
pNK6	pTTQ18Gm with *vgb* gene of *Vitreoscilla*; Ap^r, Gm^r	Ch. 5
pKCN2	pUCPM18G with *oah* gene of *A. niger* K85; Ap^r, Gm^r	Ch. 6
pKCN6	pUCPM18G with *FpOAR* gene of *F. plaustris*; Ap^r, Gm^r	Ch. 6
pKCN4	pUCPM18G with *oah* gene of *A. niger* K85 and *FpOAR* gene of *F. plaustris*; Ap^r, Gm^r	Ch. 6
pKCN5	pUC18T-mini-Tn7T with *oah* gene of *A. niger* K85 and *FpOAR* gene of *F. plaustris*; Ap^r, Gm^r	Ch. 7
pKCN7	pUC18T-mini-Tn7T with *oah* gene of *A. niger* K85, *FpOAR* gene of *F. plaustris*, *vgb* gene of *Vitreoscilla* and *egfp*; Ap^r, Gm^r	Ch. 7
pUCVHb-egfp	pUC16 with *vgb* gene of *Vitreoscilla*, *egfp*; Ap^r	Ch. 7

Table 2.2: List of plasmids used in the present study. All the plasmids were stored as stocks at -20ºC in the form of ethanol precipitates.

(a) **(b)**

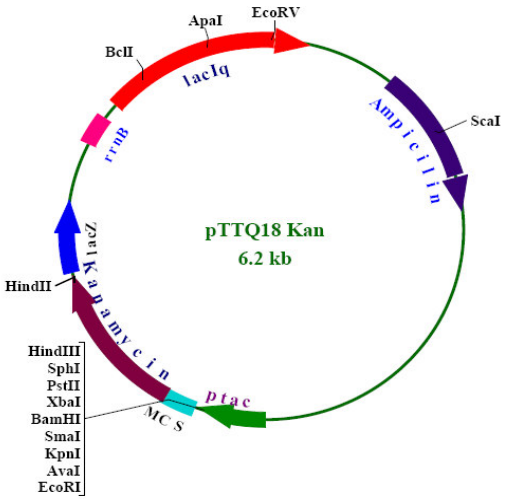


Generous gift from **Dr. J. R. Sokatch**, University of Oklahoma Health Sciences



Generous gift from **Dr. Michael J.R. Stark**, University of Leicester

(c)



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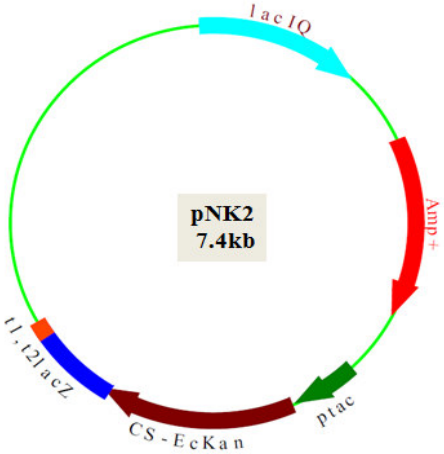

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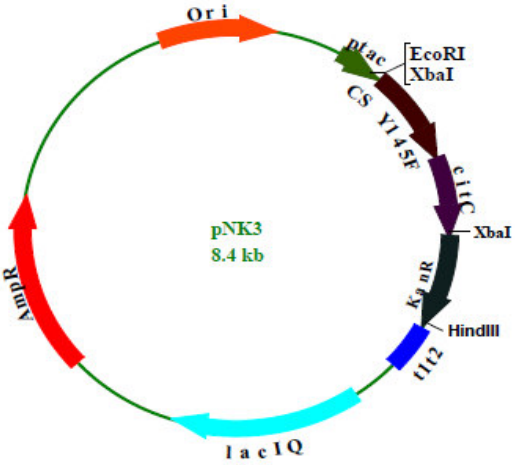

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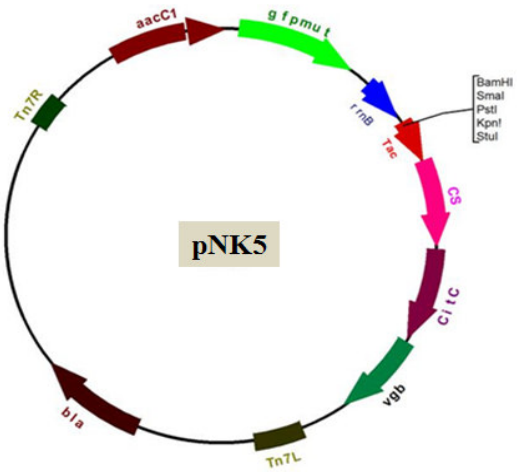

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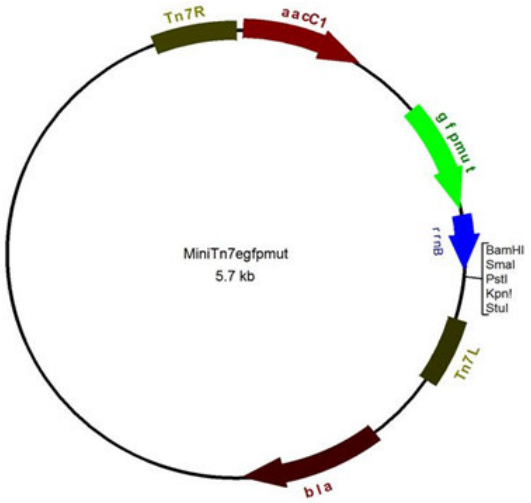

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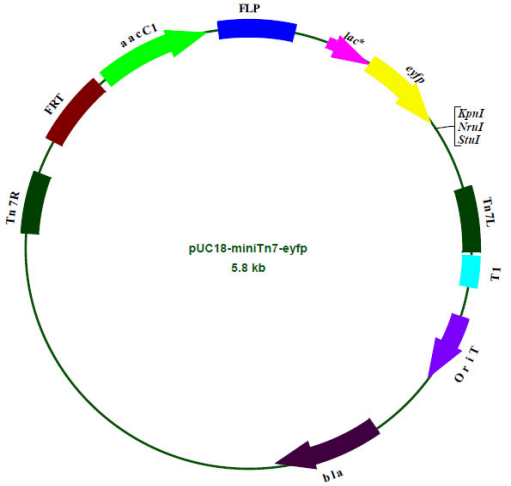
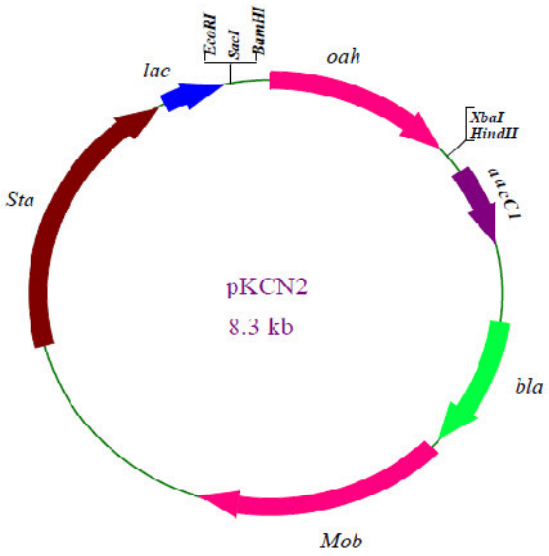


Generous gift from **Dr. H. P Schweizer**, Colorado State University, USA.

(i)

(j)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oah
XbaI
HindII
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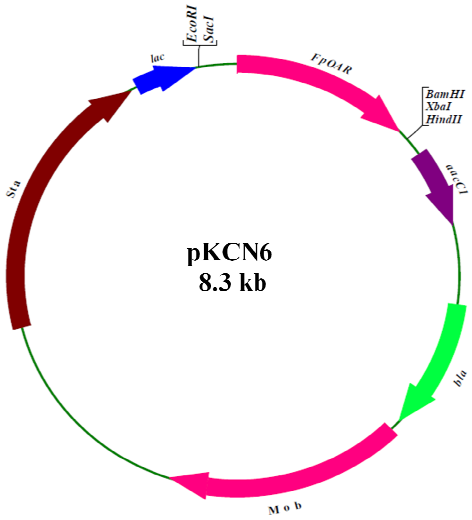
bla
Mob

(k)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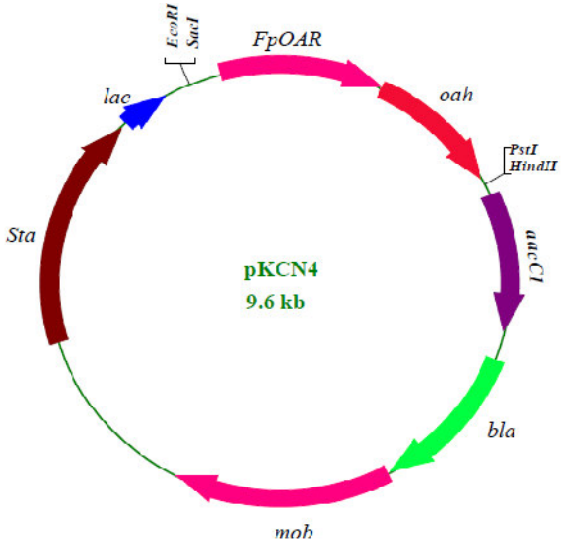
(l)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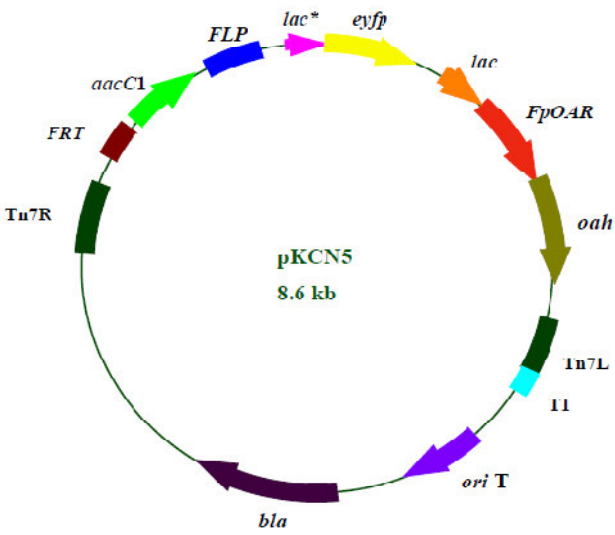
(m)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eyfp
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pKCN5
8.6 kb
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(n)

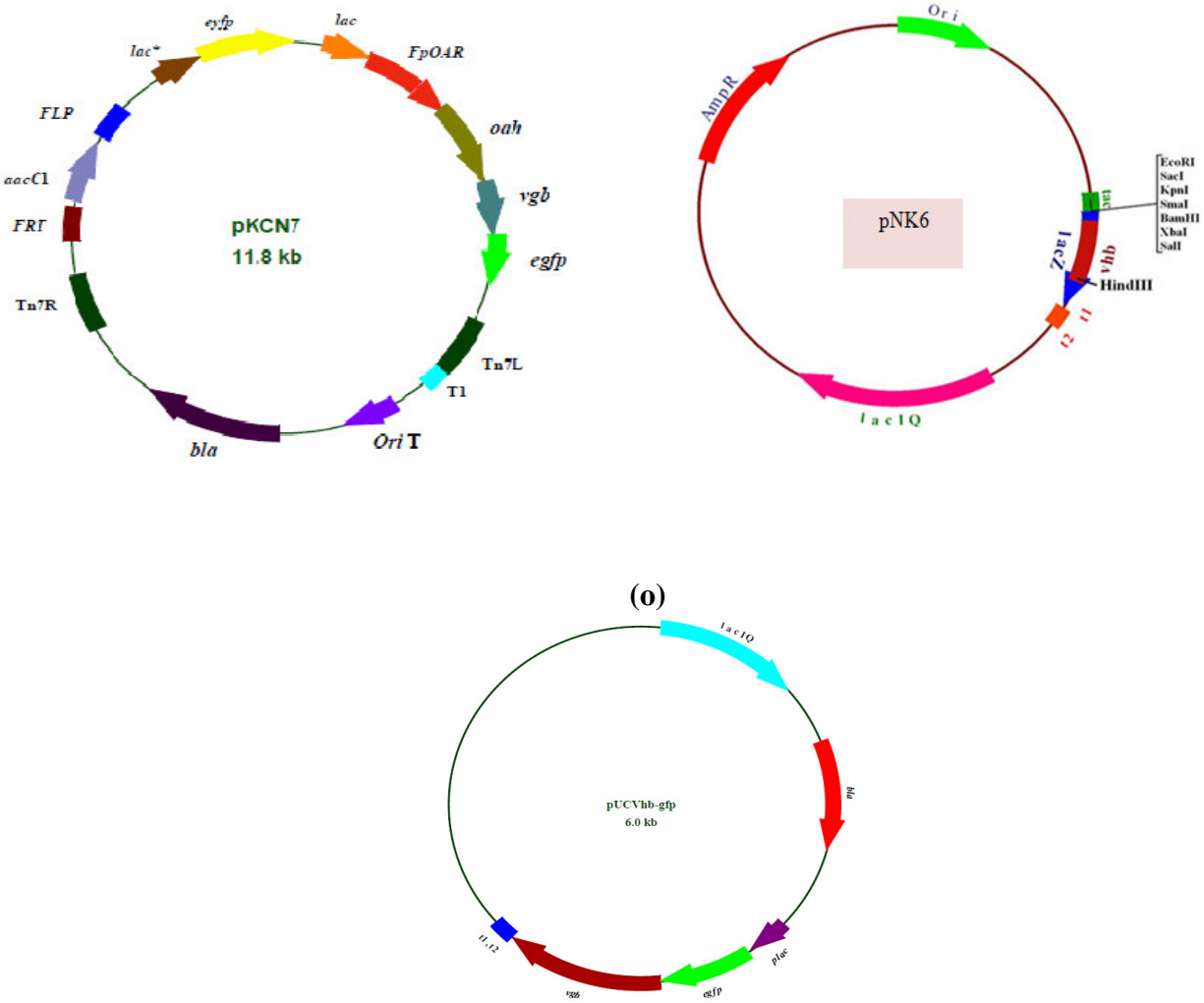


Fig. 2.1: Restriction maps of the plasmids used in this study. (a) is the broad host- range vectors used for expression of heterologous genes under *lac* promoter in *P. fluorescens* 13525. (b) and (c) are used for expression of heterologous genes under *tac* promoter in enterobacteriacae family. (d), (e), (f) and (n) are the final constructs of plasmids used for physiological studies in *Citrobacter* DHRSS, (g) and (h) are integration vectors used for cloning and genomic integration in *P. fluorescens* 13525. (i), (j) and (k) are final constructs of plasmids used for physiological studies in *P. fluorescens* 13525. (l) and (m) are final constructs of plasmids used for integration in genome of *P. fluorescens* 13525. (o) is the plasmid used to obtain *vgb*, *egfp* gene.

Broad host range, multicopy plasmid vector pUCPM18 was selected for expression in *P. fluorescens* 13525 due to their small size and versatile multiple cloning sites. pUCPM18 is derived from a pUC-derivative, pUCP18 plasmid which is stably maintained in both *E. coli* and

Pseudomonas species (Schweizer, 1991). pUCPM18 was developed from pUCP18 (GenBank accession number: U07164) by incorporating a 750 bp mob fragment from pLAFRI (broad host range vector used for genetic analysis of gram-negative bacteria; Vanbleu et al., 2004), in order to enable convenient mobilization within *Pseudomonas* species (Hester et al., 2000). It replicates in *E. coli* using ColE1 origin of replication (*ori*) while in *Pseudomonas* it replicates owing to a pRO1614 derived DNA fragment encoding a putative *ori* and a replication-controlling protein (West et al., 1994).

pTTQ18 is a pUC18 based plasmid containing unique multiple cloning site and provides direct selection of recombinant plasmids based on blue-white selection strategy. *Lac*Zα gene in pTTQ18 is under *tac* promoter which is strong and tightly regulated in *Enterobactericeae* family (Stark, 1987). pTTQ18Km and pTTQ18Gm are the plasmids derivatives of pTTQ18 containing antiobiotic resistance genes kanamycin and gentamycin, respectively.

pUC18Tn7T-Gm-*gfpmut*3 is a pUC18- based suicide delivery plasmid containing mini-Tn7 base element. This plasmid is a mobilizable derivative of pUC18-mini-Tn7 and has ColE1 origin of replication, transcriptional terminators from bacteriophage λ, *E. coli rrnB* promoter and *gfpmut*3. It contains *bla* and *aacC1* genes as selection markers. The selection marker *aacC1* present in mini-Tn7 is flanked by *FRT* sites and can be excised from the chromosome with *FLP* recombinase after mini-Tn7 insertion. pUC18T-mini-Tn7T-Gm-*eyfp* is also a pUC18-based suicide delivery plasmid same as pUC18Tn7T-Gm-*gfpmut*3 plasmid, having constitutive *lac* promoter and *eyfp* as fluorescent tag.

2.2: Media and Culture conditions

The *E. coli*, *Citrobacter* DHRSS and *Pseudomonas fluorescens* were cultured and maintained on Luria Agar (LA) and Pseudomonas Agar respectively (Hi-Media Laboratories,

India). *Aspergillus niger* and *Fomitopsis plaustris* were cultured and maintained on Potato Dextrose Agar (PDA) (Hi-Media Laboratories, India). Fungus cultures were grown at 27°C, *E. coli* and *Citrobacter* DHRSS cultures were grown at 37ºC while *Pseudomonas* cultures were grown at 30ºC. For growth in liquid medium, shaking was provided at the speed of 200 rpm. The plasmid transformants of *E. coli*, *Citrobacter* DHRSS and *Pseudomonas fluorescens* were maintained using respective antibiotics at the final concentrations as mentioned in **Table 2.3** as and when applicable. *Citrobacter* DHRSS, *E. coli* and *Pseudomonas fluorescens* wild type strains and plasmid transformants grown in 3ml Luria broth (LB) containing appropriate antibiotics were used to prepare glycerol stocks which were stored at -20ºC. *Aspergillus niger* and *Fomitopsis plaustris* were maintained on PDA slants.

Antibiotic	Rich Medium	Minimal Medium
Ampicillin	50µg/ ml	12.5µg/ ml
Kanamycin	50µg/ ml	12.5µg/ ml
Gentamycin	20µg/ ml	5µg/ ml
Streptomycin	20µg/ ml	_

Table.2.3: Recommended dozes of antibiotics used in this study (Sambrook and Russell, 2001). All the antibiotics were prepared in sterile distilled water at the stock concentrations of 1000x and were used accordingly to have the desired final concentrations.

The compositions of different minimal media used in this study are as described below. Antibiotic concentrations in all the following minimal media were reduced to 1/4th of that used in the above mentioned rich media (**Table 2.3**).

2.2.1: Koser's Citrate medium

Composition of the Koser's Citrate medium included magnesium sulphate, 0.2g/L; monopotassium phosphate, 1.0g/L; sodium ammonium phosphate, 1.5g/L; sodium citrate, 3.0g/L and agar, 15g/L. The readymade media was obtained from Hi- Media Laboratories, India, and was used according to manufacturer's instructions.

2.2.2: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including Na_2HPO_4 $7H_2O$, 34g/L; KH_2PO_4, 15g/L; NH_4Cl, 5g/L; NaCl, 2.5g/L; 2mM $MgSO_4$; 0.1mM $CaCl_2$ and micronutrient cocktail. The micronutrient cocktail was constituted of $FeSO_4.7H_2O$, 3.5 mg/L; $ZnSO_4.7H_2O$, 0.16 mg/L; $CuSO_4.5H_2O$, 0.08 mg/L; H_3BO_3, 0.5 mg/L; $CaCl_2.2H_2O$, 0.03 mg/L and $MnSO_4.4H_2O$, 0.4 mg/L. Carbon sources used was glucose. For solid media, 15g/L agar was added in addition to above constituents.

5X M9 salts, micronutrients (prepared at 1000X stock concentration) and carbon source (2M stock) were autoclaved separately. Fixed volumes of these were added aseptically into pre-autoclaved flasks containing distilled water to constitute the complete media with the desired final concentrations. Volume of water to be autoclaved per flask was calculated by subtracting the required volumes of each ingredient from the total volume of the media to be used.

2.2.3: Tris buffered medium

The media composition included Tris-HCl (pH-8.0), 100 mM; NH_4Cl, 10 mM; KCl, 10 mM; $MgSO_4$, 2 mM; $CaCl_2$, 0.1 mM; micronutrient cocktail; Glucose, 50 mM. 20 mM $MnCl_2$ was used as and when required (Gyaneshwar et al., 1998a). 1mg/ml Senegal Rock phosphate (RP) was used as P source. Each ingredient was separately autoclaved at a particular stock concentration and a fixed volume of each was added to pre-autoclaved flasks containing sterile distilled water (prepared as in Section 2.2.2) to constitute complete media.

2.2.4: Acidic Alfisol Soil Medium

Alfisol was obtained from agricultural soil near Hyderabad, India. The pH of the soil solution was 7.4 and total P content was 17.9 Kg/ha of alfisol. Alfisol was suspended at 0.5 g/ml in sterile medium containing 100 mM glucose and 10 mM KNO_3. RP was added at 30 mg/g of soil.

2.2.5: Murashige-Skoog's Medium

Murashige-Skoog's (MS) medium was composed of macro elements and micro elements. The macro elements included $CaCl_2.2H_2O$, 0.440g/L; KH_2PO_4, 0.17g/L; KNO_3, 1.9g/L; $MgSO_4.7H_2O$, 0.37g/L; NH_4NO_3, 1.65g/L and 10g/L agar. The micro elements included the essential trace elements. Micronutrients (autoclaved separately) were added aseptically to reconstitute the complete media. The readymade micronutrient was obtained from Hi-Media Laboratories, India, and was used (half strength concentration) according to manufacturer's instructions while the macronutrients were reconstituted as and when required.

2.2.6: Fungus Minimal Medium

The media composition included $(NH_4)_2SO_4$, 7.3g/L; KH_2PO_4, 1.5 g/L; $MgSO_4.7H_2O$, 1 g/L; NaCl, 1 g/L; $CaCl_2.2H_2O$, 0.1 g/L and 0.5 ml of trace metal solution. The trace metal solution contained: $ZnSO_4.7H_2O$, 14.3 g/L; $CuSO_4.5H_2O$, 2.5 g/L; $NiCl_2.6H_2O$, 0.5 g/L; $FeSO_4.7H_2O$, 13.8 g/L and $MnCl_2$, 6 g/L. The carbon source was sucrose (20 g/L) unless otherwise indicated (Pedersen et al., 2000). Salts, micronutrients (prepared at 1000X stock concentration) and carbon source (2M stock) were autoclaved separately and a fixed volume of each was added to pre-autoclaved flasks containing sterile distilled water (prepared as in Section 2.2.2) to constitute complete media.

2.3: Molecular biology tools and techniques

2.3.1: Isolation of plasmid, genomic DNA and RNA

2.3.1.1: Plasmid DNA isolation from *Citrobacter* DHRSS, *E. coli* and *P. fluorescens*

The plasmid DNA from *Citrobacter* DHRSS, *E. coli* and *P. fluorescens* 13525 was isolated using standard alkaline lysis method (Sambrook and Russell, 2001).

2.3.1.2 Genomic DNA isolation from *Aspergillus niger*

Loop full of fungus spores were inoculated aseptically in Fungus Minimal medium (described in section **2.2.7**) and incubated at 27°C and shaking was done at 200 rpm. Mycelium was harvested on 4^{th} day, washed twice with phosphate buffer, dried completely and crushed to fine powder in liquid N_2. 60- 100 mg of fine powder, crushed in liquid N_2, was taken in 1.5 ml microfuge tube then 750µL of lysis buffer (50 mM Tris-HCl; 50 mM EDTA; 3% SDS) and 10µL of 2- mercaptoethanol were added. To ensure good mixing of solution, it was kept at 65°C for 1 hr. After incubation solution was centrifuged at 3400 rpm for 5 min, to remove cell debris. Aqueous phase was transferred to new eppendorf, 700µL of chloroform: isoamyl alcohol (24:1) was added and then vortexed. Solution was centrifuged at 12,000g for 10 min, aqueous phase was transferred to new eppendorf. 600µL of isopropanol and 20µL of 3M NaOAc were added to supernatant and then microcentrifuged for 2 min. Then the supernatant was discarded and microfuge tube was inverted for 1 min, pellet was washed with 70% ethanol and then air dried or kept at 65°C for 15 min. Pellet was resuspended in 50µL of double distilled water.

2.3.2 RNA isolation and cDNA synthesis

2.3.2.1 RNA isolation from Fungus

Both the fungus *Aspergillus niger* and *Fomitopsis plaustris* used in this study were harvested, washed twice with phosphate buffer and dried completely. Then the dried mycelium was crushed

to fine powder in liquid N_2. This fine powder was proceed for RNA isolation by TRizole reagent (Sigma Aldrich, India) as described by Chomczynski and Sacchi (2006). Integrity of isolated RNA was checked by 2% agarose gel electrophoresis.

2.3.2.2 cDNA synthesis

The total RNA isolated, was used for first strand cDNA synthesis using First strand cDNA synthesis kit (Thermo Scientific, USA) by following the manufacturer's instructions. The second strand cDNA was prepared by using gene specific primers by PCR amplification.

2.3.3: Transformation of plasmid DNA

2.3.3.1: Transformation of plasmid DNA in *E. coli* and *Citrobacter* DHRSS

The transformation of plasmids in *E. coli* and *Citrobacter* DHRSS using $MgCl_2$-$CaCl_2$ method was carried out according to Sambrook and Russell (2001).

2.3.3.2: Transformation of plasmid DNA in *P. fluorescens* 13525

Plasmid transformation in *P. fluorescens* 13525 was done using the NaCl-$CaCl_2$ method (Cohen et al., 1972) with slight modifications which are as follows. *P. fluorescens* 13525 was grown at 30ºC in LB broth to an O.D. $_{600}$ of 0.6-0.8. At this point, the cells were chilled for about 10 minutes, centrifuged at 5000 rpm for 5 minutes and washed once in 0.5 volume 10mM NaCl (chilled). After centrifugation (5000 rpm for 5 minutes), bacteria were re-suspended in half the original volume of chilled 0.1M $CaC1_2$, incubated on ice-bath for 1hr, centrifuged (5000rpm for 5 minutes) and then resuspended in 1/10th of the original culture volume of chilled 0.1M $CaC1_2$. 0.2ml of competent cells treated with $CaCl_2$ was used per vial (microcentrifuge tube) to add DNA samples (minimum 0.8-1.0µg is required) and were further incubated on ice-bath for 1hr. Competent cells were then subjected to a heat pulse at 42°C for 2 min to enable DNA uptake, immediately chilled for 5 minutes and then were supplemented with 0.8ml of sterile LB broth

followed by incubation for 1hour at 30°C under shake conditions. These cells were then centrifuged and plated on Pseudomonas Agar plates containing appropriate antibiotics. The colonies obtained after overnight incubation of the plates at 30°C were then subjected to fluorescence check and plasmid DNA isolation.

2.3.4 Integration of genes in chromosome of *P. fluorescens* 13525

The desired operon or gene cluster was cloned in broad host range mini-Tn7 suicide delivery vector. The insertion of genes in chromosome of *P. fluorescens* 13525 was done as described by Choi and Schweizer (2006). Integrants were selected on Koser's Citrate medium containing appropriate antibiotic and checked for fluorescence. Confirmation of integration was done by polymerase chain reaction amplification of inserted genes, functional confirmation and enzyme assays as and when required. Confirmed cultures were processed for *Flp* recombinase mediated excision of gentamycin resistant marker as described by Hoang et al., (1998).

2.3.5: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose (containing 1µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator.

2.3.6: Restriction enzyme digestion analysis

0.5-1.0µg DNA sample was used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10µl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by Ethidium bromide staining after electrophoresis on 0.8% agarose gels and were subsequently photographed. In case of double digestion, a

compatible buffer for the two REs was essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

2.3.7: Gel elution and purification

The DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band. The purification was done by using kit method (Invitrogen, USA) and following the instructions described by manufacturer. The purification efficiency was checked by subjecting 1μL DNA solution to gel electrophoresis and visualizing the sharp DNA band of desired size. The purified DNA was used for ligation experiments.

2.3.7: Ligation

The ligation reaction was usually done in 20 μL volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 2μl; T4 DNA ligase (MBI Fermentas), 0.5- 1.0U and sterile double distilled water to make up the volume. The cohesive end and blunt ligation reaction was carried out at 22°C for 1h and 4h, respectively. The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:3 was maintained, with a total of 50-150 ng of DNA in each ligation system.

$$\text{pmoles of DNA} = \frac{\text{Amount of DNA (μg) x 1, 515}}{\text{Size of the DNA fragment (no. of base pairs)}}$$

2.3.8: SDS-PAGE

(A) Monomer solution (30%) (Store at 4° C in dark)		**(B) Resolving gel buffer-1.5M Tris (pH 8.8)** Adjust pH with HCl		**(C) Stacking gel buffer 1.0M Tris (pH 6.8)** Adjust pH with HCl	
Acrylamide	14.6 gm	Tris base	9.1 gm	Tris base	3.02 gm
Bisacrylamide	0.4 gm	SDS	0.2 gm	SDS	0.20 gm
D. H20 Till	50 ml	D. H20 Till	50 ml	D. H20 Till	50ml

(D) Tank Buffer (pH 8.3)		**(E) Sample Loading buffer (2X)**		**(F) Other reagents**	
Tris base	6.0 gm	SDS	4%	APS (fresh)	10% (w/v)
Glycine	28.8 gm	Glycerol	20%	TEMED	2-3 µl
SDS	2.0 gm	Tris-Cl (pH6.8)	0.125M	Isopropanol	
D. H_20 Till	2 L	Bromophenolblue	0.05%w/v	Bangalore Genie Mid range Protein mol. wt. marker (15 Kd-97 Kd)	
Adjust the pH with HCl		β-mercaptoethanol	10mM		
		D. H_20 Till	10ml		

(G) Separating Gel (8%)		**(H) Stacking Gel (3.9%)**		**(I) Staining Solution**
30% Monomer	2.7 ml	30% Monomer	0.65 ml	0.025% Commassie Blue R-250 in 40% Methanol and 7% Acetic acid
Separating gel buffer (pH 8.8)	2.5 ml	Stacking gel buffer (pH 6.8)	1.25 ml	
D. H_20	2.3 ml	D. H_20	3.05 ml	**(J) De-staining solution**
10% APS	50 µl	10% APS	25 µl	(10% methanol and 10% Acetic acid)
TEMED	2 µl	TEMED	3 µl	

Table 2.4: Composition of SDS-PAGE reagents (Sambrook and Russell, 2001) SDS-PAGE gel electrophoresis was carried out using 8% - 15% acrylamide gel, as per requirement, by following the procedures described by Sambrook and Russell (2001).

After electrophoresis the gel was stained using the staining solution (**Table 2.4**) for about 1h and then de-stained with de-staining solution (**Table 2.4**) by incubating overnight under mild shaking conditions. Result was recorded by direct scanning of gel.

2.3.9: Polymerase Chain Reaction (PCR)

The PCR reaction set up was based on the guidelines given in Roche Laboratory Manual. The assay system and the temperature profile used are described in **Table 2.5.**

Assay system used		**Temperature Profile**	
Sterile DDW	39.3µl	Initial denaturation	94°C - 1 min
dNTP (10mM with 2.5mM each)	3.µl	Denaturation	94°C - 30 sec
Forward Primer 20pmoles	1.5µl	Annealing	52-58°C - 45sec*
Reverse Primer 20pmoles	1.5µl	Elongation	72°C –1 min- 3 min
Template DNA (100ng/µl)	0.2µl	Final Elongation	72°C - 5 min-7 min
Buffer (10X)	5µl	No. of cycles	25- 30
DNA Polymerase (1 unit/µl)#	0.5µl		
Total System	50µl		

Table 2.5: PCR conditions used in the present study PCR amplifications were performed in DNA Engine thermal cycler (Bio Rad, USA) and Veriti thermal cycler (Applied Biosystem). *Exact primer annealing temperature and primer extension time varied with primers (designed with respect to different templates) and has been specified in the text as and when applicable. # Different DNA polymerases were used as per requirement. *Taq* DNA polymerase and *Pfu* polymerase, XT-20 polymerase, dNTPs and primers were obtained from Thermo Scientific, USA, New England Biolabs, USA, Bangalore Genei Pvt. Ltd., India, Sigma Chemicals Pvt. Ltd.

and Integrated DNA Technologies, USA, respectively and were used according to manufacturer's instructions.

The theoretical validation of the primers with respect to absence of intermolecular and intramolecular complementarities to avoid primer-primer annealing and hairpin structures and the appropriate %G-C was carried out with the help of online primer designing software Integrated DNA Technology. The sequence, length and % G-C content of primers are subject to variation depending on the purpose of PCR and will be given as and when applicable in the following chapters. The PCR products were analyzed on 1.0% agarose gel along with appropriate molecular weight markers (Section 2.3.5).

2.3.10: Circular Polymerase Extension Cloning

Artificial operons were constructed using circular polymerase extension cloning method as described by Quan and Tian, 2011. Overlapping primers were also designed accordingly containing 20 bp sequence of each gene.

2.3.11: DNA sequencing

The DNA sequencing service was obtained from Chromous Biotech. Pvt. Ltd. (India) for 18SrDNA sequence for fungal culture identification. Complete sequences of genes were obtained from First Base, Malaysia (details in Chapter 6.)

2.3.12: BLAST Search

The online computational tool "BLAST" for the DNA sequence alignments and homology search was used for identification of fungus culture and for gene confirmation (details in Chapter 6). Integrated DNA technology and other online software's were used for primer designing and other bioinformatics studies as and when required.

2.4: P-solubilization phenotype

P-solubilizing ability of the *Citrobacter* DHRSS and *P. fluorescens* 13525 transformants was tested on Tris buffered RP-Methyl red (TRP) agar plates which represents a much more stringent condition of screening for PSMs (Gyaneshwar et al, 1998a). Liquid medium for RP solubilization is described in Section 2.2.3 with an addition of methyl Red as pH indicator dye and 1.5% agar for all the plate experiments. *Citrobacter* DHRSS and *Pseudomonas fluorescens* cell suspension for these experiments was prepared as described in Section 2.6.1 and 5µl of it was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 37°C and 30°C, respectively for 4-7 days. P solubilization was determined by monitoring the red zone on the TRP agar plates. Media acidification from pH 8.0 to pH<5 on TRP broth was used as an indicator for P-solubilization and organic acid secretion.

2.5: Mutant Complementation Phenotype

E. coli cs mutant (W620) was used to confirm the functionality of the cloned *cs* gene. The recombinant plasmid containing *cs* gene under *tac* or constitutive *tac* promoter was transformed into mutant strain. The *cs* transformants were selected on streptomycin and kanamycin or gentamycin as per requirement. These transformants were grown on M9 minimal medium with 0.2% (11.11mM) glucose as carbon source in presence and absence of 340µg/ml sodium glutamate, under shaking conditions at 37°C. *cs* mutants exhibited glutamate auxotrophy (Underwood et al., 2002) as they could not grow on glucose as the sole carbon source in the absence of glutamate supplementation.

2.6: Physiological experiments

The physiological experiments were carried out using wild type *Citrobacter* DHRSS, *Pseudomonas fluorescens* and their transformants, which included growth, pH profile and enzyme assays.

2.6.1: Inoculum preparation

The inoculum for M9, Tris minimal media and acidic alfisol soil medium was prepared by growing the *Citrobacter* DHRSS and *Pseudomonas* cultures overnight at 37°C and 30°C, respectively, in 3ml LB broth. Cells were harvested aseptically, washed thrice by normal saline and finally re-suspended in 1ml normal saline under sterile conditions. Freshly prepared inoculum was used for all the experiments.

2.6.2: Growth characteristics and pH profile

Growth parameters and pH profile of the wild type well as transformants were determined using three different media conditions including **(i)** TRP medium with RP as P source (Gyaneshwar et al, 1998a). The media composition in this case was same as mentioned in Section 2.2.3; **(ii)** Acidic alfisol soil medium- the media composition was same as mentioned in section 2.2.4 and **(iii)** M9 minimal medium (Section 2.2.2). 50 mM glucose was used as the carbon source for all the experiments unless and until stated categorically.

In 150 ml conical flasks, 30 ml of relevant minimal broth was inoculated with cell suspensions to have 0.01-0.03 $O.D._{600nm}$ initially. The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker (Scigenics, India) maintained at the required temperature with agitation speed at 75 and 200 rpm as and when required. 1ml samples were aseptically harvested at regular intervals and were subjected to various analytical techniques.

2.6.3: Analytical techniques

The cell density determinations were done at 600 nm as monitored spectrophotometrically. Change in absorbance was considered as the measure of growth and drop in pH of the media was taken as the measure of acid production. In all the cases, the observations were continued till the media pH reduced to less than 5. 1ml aliquots withdrawn aseptically at regular time intervals were immediately frozen at -20°C until further used for biochemical estimations. The stored samples were centrifuged at 9, 200x *g* for 1 min at 4°C and the culture supernatants derived were used to estimate organic acid analysis using HPLC. For HPLC analysis, the culture supernatant was passed through 0.2µm nylon membranes (Pall Life Sciences, India) and the secreted metabolites were quantified using RP-18 column. The column was operated at room temperature using mobile phase of 0.01M H_2SO_4 at a flow rate of 1.0 ml min^{-1} and the column effluents were monitored using a UV detector at 210 nm. For citric acid estimation the same column was operated at room temperature using mobile phase of 0.2% orthophosphoric acid at a flow rate of 1.0 ml min^{-1}. Standards of organic acids were prepared in double distilled water, filtered using 0.2µm membranes and were subjected to chromatography for determining the individual retention time. Measurements of area under peak with an external standard were used for quantification.

2.7: Enzyme assays

2.7.1: Preparation of cells and cell free extracts

Glucose grown cells under above mentioned minimal media conditions were harvested in appropriate growth phase from 30 ml of cell culture by centrifugation 9,200x *g* for 2 minutes at 4°C. Citrate synthase (CS) was assayed in the stationary phase and oxaloacetate acetyl hydrolase (OAH) was assayed when medium pH was above 6. The preparation of cell free extracts for CS assay was carried out according to Kodaki et al, (1985). The cell pellet was washed once with 80

mM phosphate buffer (pH 7.5) followed by resuspension in same buffer containing 20% glycerol and 1mM DTT. In OAH assay 2mM $MnCl_2$ was also added to the sonication buffer. The cells were then subjected to lysis by sonicating for maximum 1-1.5 minute in an ice bath, followed by centrifugation at 9,200x *g* at 4°C for 30 minutes to remove the cell debris. The supernatant was then used as cell-free extract for the enzyme assays.

2.7.2: Enzyme Assay Protocols

2.7.2.1: CS assay

CS activity was estimated by following the absorbance of 5,5- dithiobis(2-nitrobenzoic acid) (DTNB) at 412nm which would change due to its reaction with sulfhydryl group of CoA (Serre, 1969). The assay mixture per cuvette contained following ingredients in 1.0ml: Tris-HCl (pH=8.0), 93mM; acetyl CoA, 0.16mM; OAA, 0.2mM; DTNB, 0.1mM and cell lysate. The reaction was started by addition of OAA. Molar absorbance coefficient was taken as 13.6 mM-1cm-1 at 412nm. The rate of increase in absorbance was used to calculate CS activity.

2.7.2.2: OAH assay

OAH activity was estimated by direct optical determination of oxaloacetate (OAA) as described by Lenz et al, 1976. Enolic OAA absorbs in the range of 220- 300 nm. At 255 nm absorption is maximum, and intensity of OAA strongly dependent on the solvent composition and the presence of metal ions (influence on the keto-enol equilibrium). The reaction mixture, which was kept at 25°C in a total volume of 1.0 ml, contained: 0.1 M imidazol buffer, pH 7.6, 1.0 mM oxaloacetate, 0.3 mM $MnCl_2$. Reaction was started by addition of oxaloacetate acetyl hydrolase after a 3-min preincubation to ensure constant absorbance; d= 1 cm; A= 255 nm. The change of absorbance in the complete system was determined in a Shimadzu UV- Vis 1700 double beam, spectrophotometer *versus* the same solution without enzyme.

CS and OAH enzyme activities were determined at 30°C and 25°C, respectively and were expressed per mg total protein. Total protein concentration of the crude extract was measured by modified Lowry's method (Peterson, 1979) using bovine serum albumin as standard. Enzyme activities were calculated using following formula

$$\text{Specific enzyme activity (U)} = \frac{\Delta A_y \text{ nm/min}}{€ \times \text{enzyme (sample) aliquot (ml)} \times \text{Total protein (mg/ml)}}$$

where, ΔA_y nm is the difference in the absorbance at any given wavelengths (y nm) and € is the millimolar extinction coefficient at *y* nm.

One unit of enzyme activity was defined as the amount of protein required to convert 1 nmole of substrate per minute.

2.8: Estimation of organic acids

Organic acids levels determined by HPLC were confirmed by using enzymatic analysis and spectrophotometric analysis, as per the requirement. Culture supernatants were filtered through 0.2µm nitrocellulose membrane and different aliquots were used to estimate oxalic and citric acids.

2.8.1: Extracellular citric acid

Citrobacter transformant cultures grown on TRP buffered media were harvested when pH of medium goes below 5 and were pelleted by centrifuging at 9, 200x *g* for 2 minutes at 4°C. The supernatant was filtered through 0.2 µm nitrocellulose membrane and filtrate was used to estimate the amount of extracellular citric acid using the method described by Petrarulo et al (1995) and Buch et al (2009).

According to this method citrate lyase converts citrate to OAA and acetate and the resultant OAA can rapidly react with phenyl hydrazine at slightly acidic pH to form the corresponding phenyl hydrazone which can absorb ultraviolet (UV) light. Increase in the absorbance at 330 nm would be an expression of the citrate concentration. The assay system per cuvette contained- 50 mM phosphate buffer mix, 1.0 ml; 246 mM phenyl hydrazine, 0.02 ml; citrate lyase, 0.27U (0.02 ml of 13.3 units/ml stock); citric acid standard or test sample. Buffer mix contained 50 mM phosphate buffer (pH 6.5), 0.1 mM $ZnSO_4.7H_2O$ and 0.2 g/L sodium azide. Citric acid standards of 5µM, 7.5µM, 10µM, 15µM and 20µM concentrations were used to generate standard curve. Difference in $O.D._{330nm}$ after 3 minutes of addition of citrate lyase was used for the calculations.

Extracellular concentration (mM) = Xnmoles/ml where,

$$\text{Xnmoles/ml} = \frac{\Delta\ O.D._{330nm}/3\text{min (test)} \times \text{Std (nmoles)}}{\text{Test aliquot (ml)} \times \Delta\ O.D._{330nm}/3\text{min (Std)}}$$

2.8.2: Oxalic acid Estmation

Oxalic acid estimation of samples was also done by catalytic kinetic spectrophotometric method by quantitative determination of oxalic acid using Victoria Blue B based (Yan et al., 2004).

2.9: Inoculation of mung beans (*Vigna radiata*)

Mung bean seeds were surface sterilized by treating with 0.1% (w/v) mercuric chloride for ~3-5 minutes followed by 5 rounds of thorough washing with 100 ml freshly sterilized distilled water (Ramakrishna et al., 1991). These seeds were placed in autoclaved petri-dishes

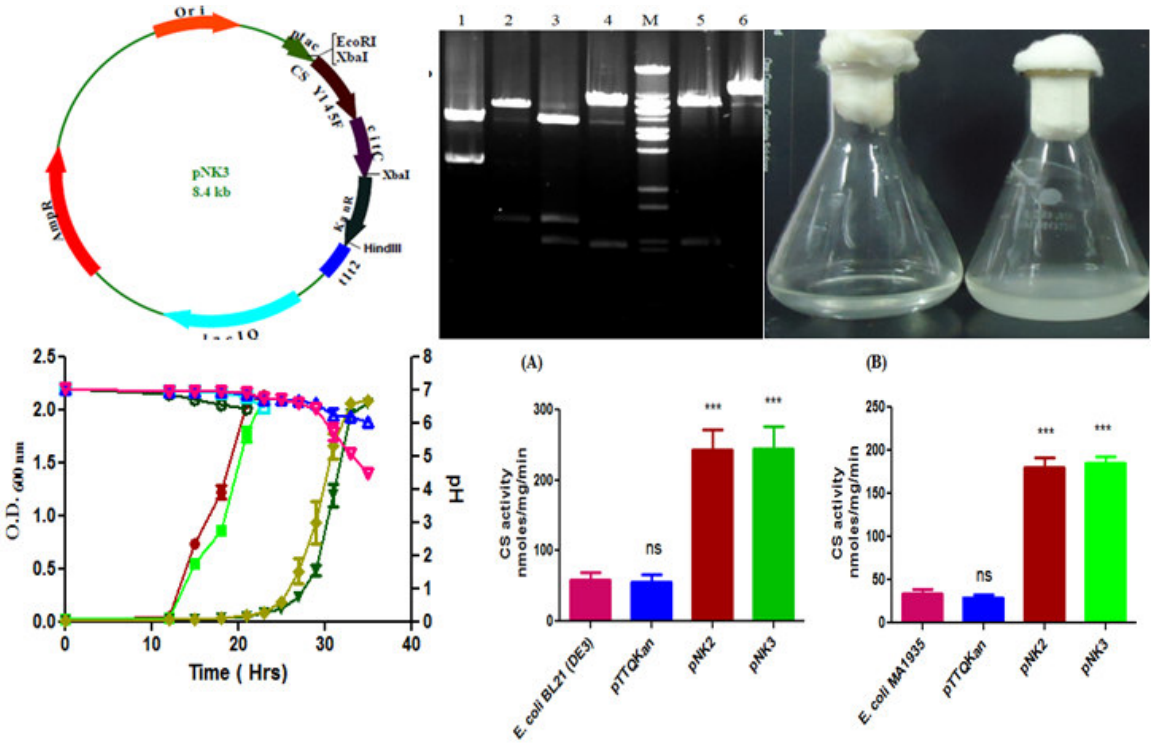
containing moist filter paper and were allowed to germinate in dark for 24 h at room temperature. *Pseudomonas fluorescens* wild type, plasmid transformants and chromosome integrants were inoculated in 10 ml sterile LB broth and were allowed to grow at 30°C with overnight shaking. Fresh culture was harvested by centrifuging at 9, 200x *g* for 2 minutes; the pellet was aseptically washed twice with sterile normal saline and finally was re-suspended in 4 ml of normal saline (Ramos et al., 2000). Healthy germinated seeds were incubated with this cell suspension in a sterile Petri-dish under aseptic conditions for 10-15 minutes. Root tips of hence obtained *Pseudomonas fluorescens* coated mung bean seeds were implanted with the help of forceps in non sterile acidic alfisol soil with added RP (10 mg/g of soil) as P source. Plants were allowed to grow at room temperature with sufficient light conditions for 15- 20 days after which effect of *Pseudomonas fluorescens* transformant and integrant inoculation was monitored in terms of overall plant growth. In case of MS medium, inoculation was done in aseptic condition.

Note:

(i) All the centrifugations were carried out using Heraeus Biofuge or Multifuge. The spectrophotometric measurements of $O.D._{600nm}$, oxalic acid estimation and enzymatic determinations were carried out using UV- Vis 1700 double beam, spectrophotometer, Shimadzu (path length of 1cm) while the citric acid estimation was done using JASCO V-530 spectrophotometer. The cell-lysis by sonication was achieved using Branson Sonifier Model 450. HPLC equipment from Shimadzu, India was used for organic acid analysis.

(ii) The fine chemicals acetyl-CoA, pure enzymes like citrate lyase were obtained from Roche diagnostics Switzerland. While other routine chemicals and solvents were procured from Sigma Aldrich, Hi-media Laboratories, Qualigens, Sisco Research Laboratories and Merck, India.

(iii) All molecular biology grade reagents and kits were procured from Hi-Media Laboratories, India; Thermo Scientific, USA; Sisco Research Lab (SRL), India; Bangalore Genei Pvt. Ltd., India and Sigma Chemical Pvt. Ltd. until and unless specified.



CHAPTER 3

Effect of genetic modification of *E. coli* MA1935 (*icd* mutant) and *E. coli* BL21 (λDE3) strains on citric acid secretion by incorporation of artificial citrate operon

3.1: INTRODUCTION

Citric acid secretion is well known in large number of fungus and yeast species (Papagianni, 2007). Some bacterial species are also known to secrete or accumulate citrate including *Corynebacterium*, *Arthrobacter*, *Brevibacterium*, *Bacillus sp*., *Bradyrhizobium japonicum* and *Citrobacter koseri* (Carson et al., 1992; Rohr et al., 1996; Gyaneshwar et al., 1998a; Khan et al., 2006). Genetic modifications for accumulation of higher amount of citrate in fungus and yeast has been achieved by overexpression of phosphofructokinase (*pfk*), pyruvate kinase (*pk*) and *cs* genes in *Aspergillus niger*, *icl* and invertase gene overexpression in *Yarrowia lipolytica* (Ruijter et al., 1997; 2000; Forster et al., 2007a; b). In bacteria the genetic modifications for citrate secretion include mutation in isocitrate dehydrogenase (*icd*) gene of *Escherichia coli* (K and B strains) and *Bacillus subtilis*, aconitase mutation in *Streptomyces coelicolor* (Lakshmi and Helling, 1976; Matsuno et al., 1999; Viollier et al., 2001; Aoshima et al., 2003; Kabir and Shimizu, 2004), *E. coli cs* overexpression in *P. fluorescens* ATCC 13525 and heterologous overexpression of *Synechococcus elongatus* phosphoenolpyruvate carboxylase (*ppc*) gene in fluorescent pseudomonads (Buch et al., 2009; 2010). Overexpression of *cs*, *pk* and *pfk* genes in *A. niger* does not increase citrate production. On the other hand, overexpression of *icl* and invertase genes in *Y. lipolytica* resulted in production of higher amount of citrate.

Many fungi are known to secrete citric acid in molar amounts while bacteria secrete to only a few millimolar (Gyaneshwar et al., 1998a; Papagianni, 2007). However,
~10 mM citric acid is required for P release from soils by rhizobacteria for plant growth promotion (Gyaneshwar et al., 1998a). *E. coli* and *Bacillus subtilis icd* mutants resulted in accumulation of high amount of citrate in rich medium and *E. coli icd* mutants shows 2 fold increase in CS activity (Matsuno et al., 1999; Aoshima et al., 2003; Kabir and Shimizu, 2004). *E.*

coli MA1935 is an *icd* gene mutant of *E. coli* BL21 (λDE3) and showed increased accumulation and secretion of citric acid in rich medium. But the mechanism of citric acid is not clear as *E. coli* does not possess a functional citrate transporter (Hall, 1982). *P. fluorescens* ATCC 13525 overexpressing *cs* gene of *E. coli* resulted in accumulation of citrate upto 20 mM but secreted only ~1.2 mM which indicates that the native H^+ dependent citrate transporter is not sufficient to efflux higher amount of citrate (Buch et al., 2009).

Type II CS of *E. coli* is a hexamer of identical subunits which is strongly and allosterically inhibited by NADH (Weitzman, 1966). Three mutants of *E. coli* CS protein show extremely weak inhibition by NADH (Stokell et al., 2003). On the other hand, citrate uptake in bacteria is mediated by different citrate transporters *viz* 2- hydroxycarboxylate transporter (2HCT) family in *Klebsiella pneumoniae*, *Bacillus subtilis*, *Streptococcus bovis*, *Lactococcus lactis* and *Salmonella typhimurium* (Lolkema , 2006). Additionally, CitC of *Salmonella typhimurium* and CitS of *Klebsiella pneumoniae*, are citrate specific Na^+ coupled symporter belonging to 2HCT family (Lolkema, 2006; Ishiguro et al., 1992).

Rationale of Present Study

Present study deals with the genetic manipulation of *E. coli* BL21 wild type and *icd* mutant strains for citric acid secretion by overexpression of NADH insensitive *E. coli cs* gene alone and in combination with sodium dependent citrate transporter (*citC*) gene of *Salmonella typhimurium* in an artificial citrate operon in minimal medium containing 50 mM glucose as carbon source.

3.2: EXPERIMENTAL DESIGN

3.2.1: Bacterial Strains used in this study

Bacterial Strains	Relevant characteristics	Source/Reference

E. coli DH10B	Host strain for routine DNA manipulations	Invitrogen, USA
E. coli W620	CGSC 4278 - *glnV44 gltA6 galK30 LAM-pyrD36 relA1 rpsL129 thi-1*; Str^r	*E. coli* Genetic stock center
E. coli MA1935	*icd* mutant of *E. coli* BL21 (λDE3)	((Aoshima et al., 2003)
icd (pNK2)	*E. coli* MA1935 containing pNK2, Ap^r, Km^r	This study
icd (pNK3)	*E. coli* MA1935 containing pNK3, Ap^r, Km^r	This study
icd (pTTQK)	*E. coli* MA1935 containing pTTQKan, Km^r	This study
E. coli BL21 (λDE3)	F^-, *ompT*, *hsd*S_B (r_{B-}, m^{B+}), *dcm*, *gal*, (DE3)	(Sambrook and Russell, 2001)
E. coli (pNK2)	*E. coli* BL21 (λDE3) containing pNK2, Ap^r, Km^r	This study
E. coli (pNK3)	*E. coli* BL21 (λDE3) containing pNK3, Ap^r, Km^r	This study
E. coli (pTTQK)	*E. coli* BL21 (λDE3) containing pTTQKan, Ap^r, Km^r	This study

Table 3.1: List of bacterial strains used in this study. Ap= Ampicillin; Km= Kanamycin; Str= streptomycin; r= resistance

3.2.2: Cloning of NADH insensitive *gltA* gene under *tac* promoter

NADH insensitive Y145F *gltA* gene (*cs**) of *E. coli* was sub cloned from pHA3 in pTTQ18, a pUC based high copy number plasmid with *Lac*Zα gene, under *tac* promoter. Both the plasmids were digested with *Eco*RI/*Hind*III enzymes. pHA3 gave fragments of 5.1 kb (vector backbone) and 2.9 kb corresponding to NADH insensitive citrate synthase Y145F (*cs**) gene along with kanamycin marker (*npt*) gene, while pTTQ18 was linearized. Both the vector and

insert were gel purified by using Gel extraction Kit (Invitrogen, USA) method and were ligated **(Fig. 3.1)**.

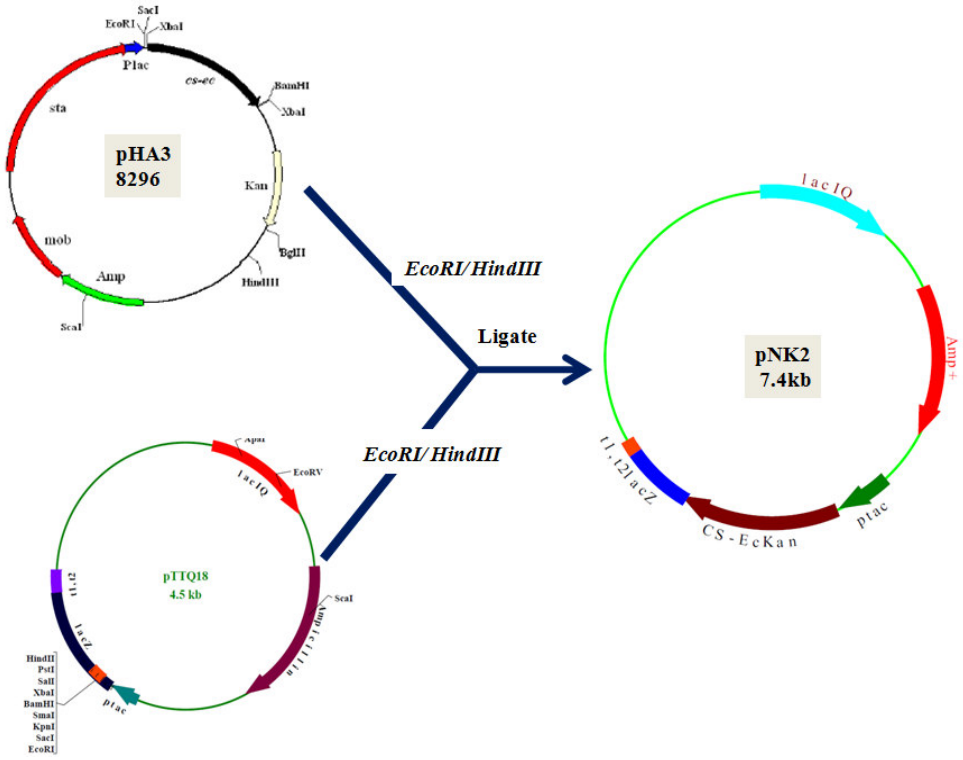


Fig. 3.1: Schematic representation of construction of vector containing NADH insensitive *gltA* gene (*cs) of *E. coli* under *tac* promoter.**

Ligation mixture was transformed in *E. coli* DH10B and transformants were selected on kanamycin containing Luria Agar (LA) plates. The plasmid containing NAHD insensitive citrate synthase (Y145F) gene was designated as pNK2.

3.2.3: Construction of artificial citrate operon under *tac* promoter

Citrate operon was constructed by cloning of Sodium dependent citrate transporter (*citC*) gene of *Salmonella typhimurium* in pNK2 plasmid. *S. typhimurium citC* gene was obtained by polymerase chain reaction amplification using gene specific primer pair: FP- 5' GGGGGCCCCGGGATCCCGCCAGGAGGAATCAACTTATGACCAACATGACC CAGC 3' and RP- 5' GGGGTACCCCGCTCTAGAGCTTACACCATCATGCTGAACACGATGC 3'. Underline sequence shows

*BamH*I restriction enzyme sites used for cloning. PCR amplification was done using *Pfu* polymerase (Thermo Scientific, USA) from plasmid pBKS-*citC*.

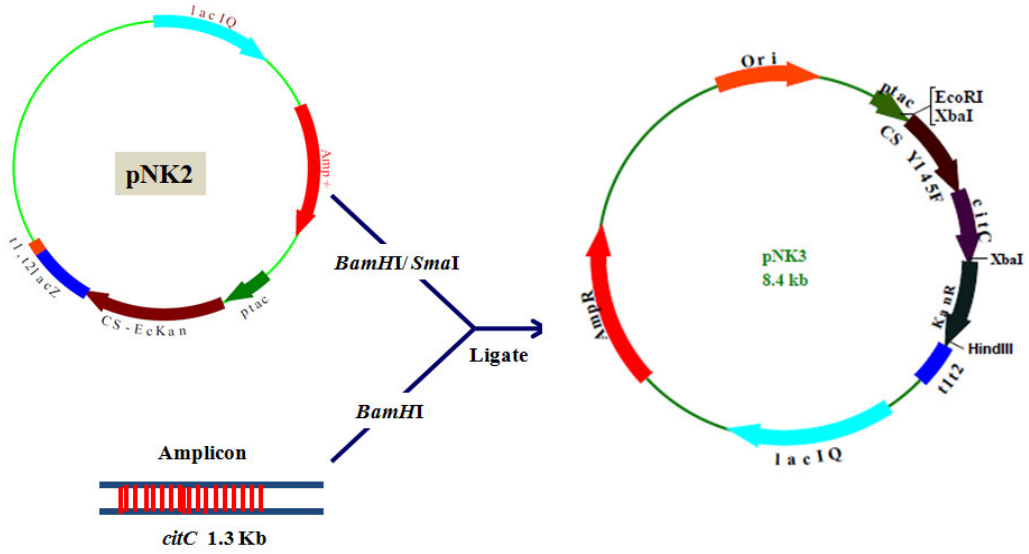


Fig. 3.2: Schematic representation of construction of pNK3 plasmid.

Amplicon of 1.3 Kb, containing *citC* gene, was digested with *Bam*HI, gel eluted, purified and ligated in *BamH*I / *Sma*I digested, gel purified plasmid pNK2 **(Fig. 3.2)**. The resultant construct containing citrate operon (*cs** and *citC*) was designated as pNK3.

All molecular biology techniques like plasmid DNA isolation and transformation, restriction digestion, gel elution, ligation and gel electrophoresis were performed as described in Section 2.3.

3.2.4: Functional confirmation of *E. coli cs** expressed from pNK2 and pNK3

E. coli W620 *gltA* mutant required glutamate supplementation when grown on minimal medium containing glucose. pTTQKm, pNK2 and pNK3 plasmids were transformed in *E. coli* W620 and the transformants were selected on streptomycin and kanamycin plates (Table 2.3). Plasmids were isolated and confirmed by restriction digestion. Confirmed transformants were subjected to complementation studies as described in Section 2.5. Functional confirmation of

citrate transporter was checked by growth of *E. coli* pNK3 transformant on Koser's citrate broth containing IPTG as described in Section 2.2.1.

3.2.5: Physiological Experiments

Bacterial cells were grown overnight in 3 ml Luria-Bertani broth and agitated at 200 rpm at 37°C. Overnight grown culture was harvested aseptically and washed thrice with 0.9 % (w/v) normal saline and finally dissolved in 1 ml saline under aseptic conditions. The resultant inoculum was used to inoculate M9 minimal medium. The composition of minimal medium used for the physiological studies was according to Sambrook and Russell, (2001). Carbon source used was glucose. Batch culture studies were done in 150 ml conical flask containing 30 ml of inoculated medium on an Orbitek rotary shaker at 200 rpm and 37°C.

3.2.6: Measurement of CS, G-6-PDH and ICL activities

Cells grown in M9 minimal medium were harvested aseptically at stationary phase by centrifugation at 9200 g for 2 min at 4°C. Cell free extract was prepared by sonication (Kodaki et al., 1985). CS activity in *E. coli* BL21 transformants was estimated by method described by Serre (1969). CS activity in *E. coli* MA1935 was estimated by DNPH method (Else et al., 1998). One unit of specific enzyme activity was defined as the amount of protein required to convert 1 nmol substrate per min per mg total protein. Total protein was estimated using a modified Lowry's method (Peterson, 1979). G-6-PDH activity was determined spectrophotometrically at room temperature by following the reduction of NADP at 340 nm as a function of time (Eisenberg and Dobrogosz, 1967). ICL activity was measured by a modified method of Dixon and Kornberg (Dixon and Kornberg, 1959).

3.2.7: Analytical methods and estimation of citric acid

Growth was monitored as increase in the absorbance at 600 nm and pH of the medium was monitored at different time intervals until the $O.D._{600nm}$ reaches 2.0. Culture was harvested and supernatant was used to estimate extracellular citric acid. Cell free extract was prepared and was used for intracellular citrate estimation. Intracellular and extracellular citrate estimation was done spectrophotometrically as described by Petrarulo et al, (1995) and Buch et al, (2009).

3.2.8: Data analysis

Physiological experiments were done in three – four independent experiments. Data are expressed in mean and standard deviation. The statistical analysis of all the parameters has been done using Graph Pad Prism (version 5.0) software.

3.3: Results

3.3.1: Construction of *E. coli cs** gene under *tac* promoter

Based on the above mentioned strategies (**Fig. 3.1**; section 3.2.2) NADH insensitive citrate synthase gene (*cs**) of *E. coli* was cloned under inducible *tac* promoter in high copy number pTTQ18 plasmid with kanamycin resistance (*npt*) gene. Plasmid pNK2 was confirmed by restriction digestion pattern **(Fig. 3.3)**.

4.3.2: Construction of artificial citrate operon (*cs*-citC*) under *tac* promoter

Based on the above mentioned strategies (**Fig. 3.2**; section 3.2.3) sodium dependent citrate transporter *citC* gene of *S. typhimurium* was cloned under inducible *tac* promoter in pNK2 plasmid containing *E. coli cs** gene. Plasmid pNK3 containing citrate operon was confirmed by restriction digestion pattern and polymerase chain reaction amplification **(Fig. 3.4; 3.5)**.

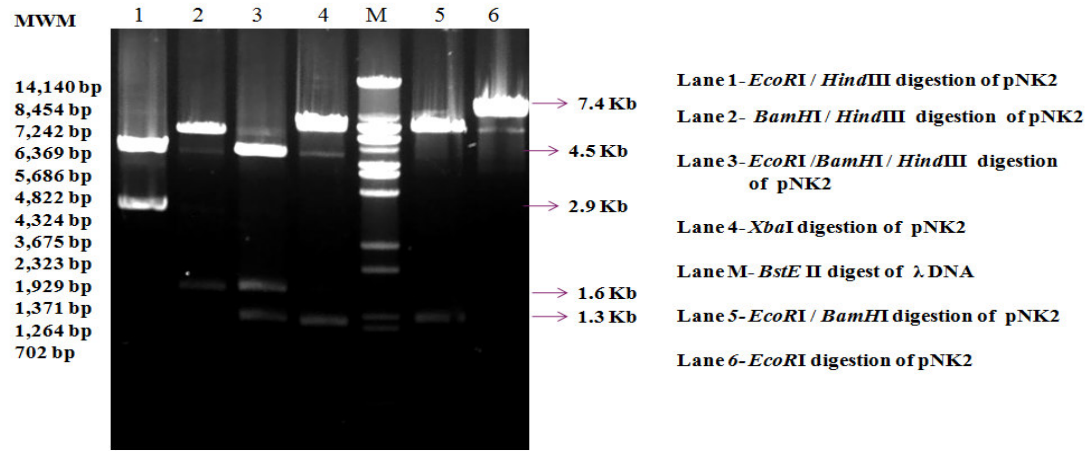


Fig. 3.3: Restriction digestion pattern of pNK2.

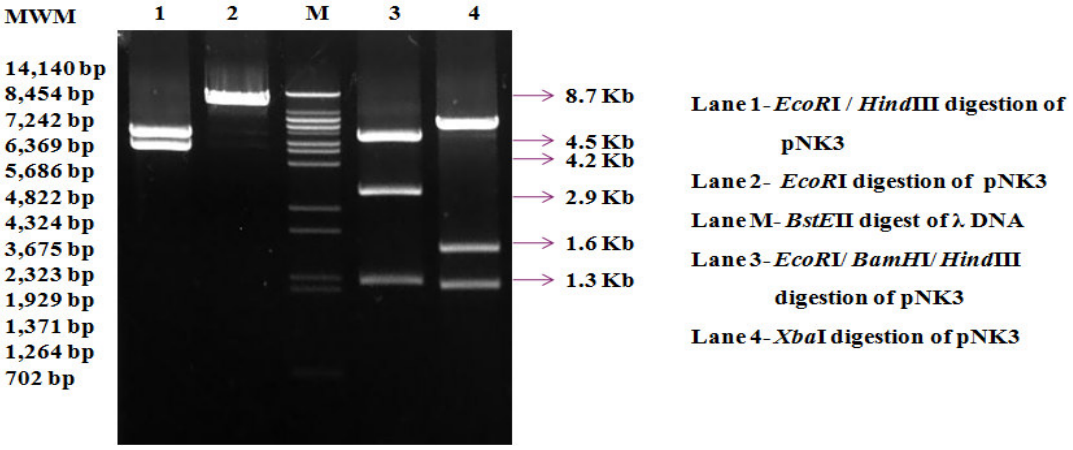


Fig. 3.4: Restriction digestion pattern of pNK3.

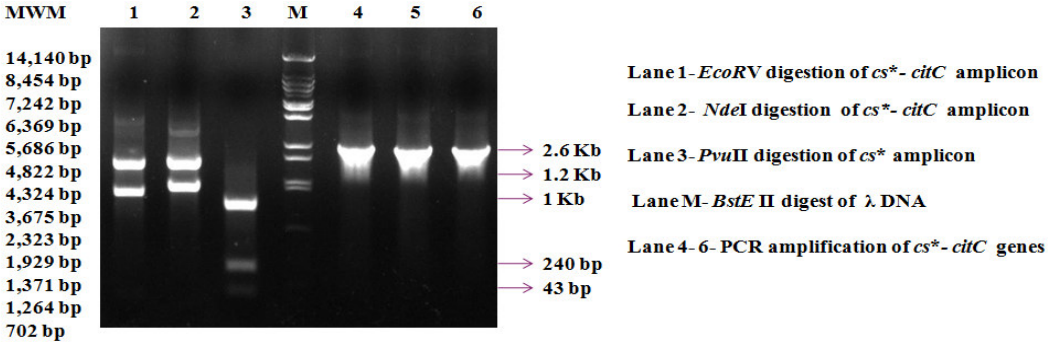


Fig. 3.5: PCR amplification and restriction digestion confirmation of pNK3.

3.3.3: Functional characterization of *cs** and *citC* genes

The functional confirmation of *E. coli cs** gene in pNK2 and pNK3 plasmids was done by mutant complementation. *E. coli* W620 *gltA* mutant strain harboring pTTQKm, pNK2 and

pNK3 were grown on M9 minimal medium with glucose as carbon source in presence and absence of glutamate.

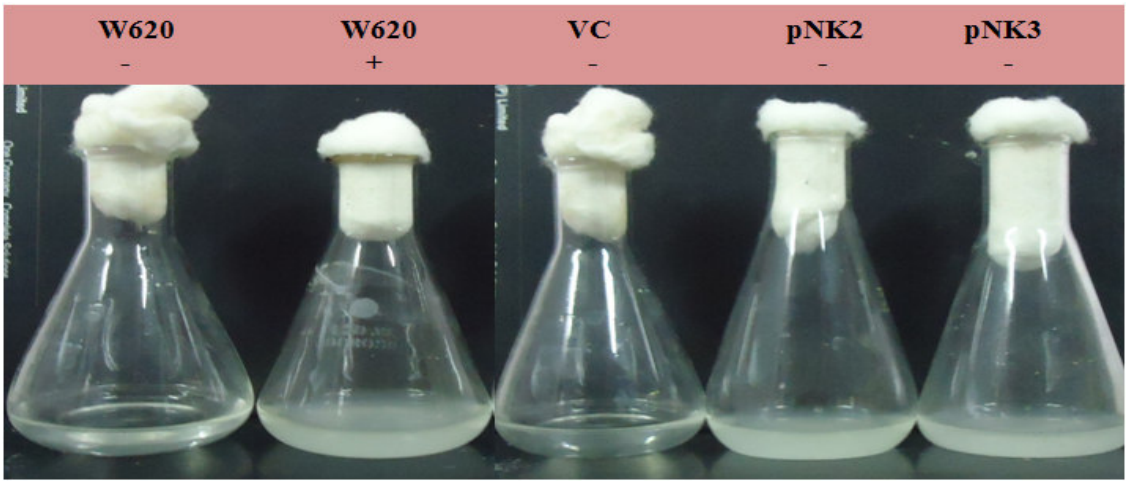


Fig. 3.6: Complementation study of *E. coli* W620 mutant phenotype by pNK2 and pNK3 constructs in M9 minimal medium containing 0.2% glucose as carbon source. –/+ at the corners of each image indicates absence and presence of glutamate (340µg/ml) in the medium, respectively.

E. coli *E. coli* (pNK2) *E. coli* (pNK3)

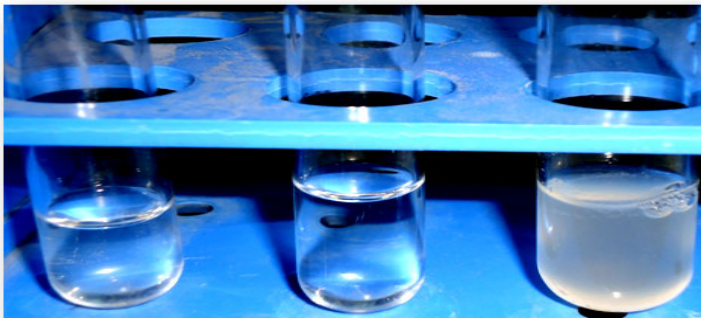


Fig. 3.7: Functional confirmation of citrate transporter in Koser's citrate broth.

E. coli W620 harboring pNK2 and pNK3 grew on M9 minimal medium containing IPTG in the absence of glutamate unlike the vector control harboring pTTQKm **(Fig. 3.6)**. The functional confirmation of sodium dependent citrate transporter (*citC*) gene of pNK3 plasmid was checked on Koser's citrate broth. *E. coli* DH10B harboring pNK2 and pNK3 plasmids were grown on Koser's citrate broth which contains citrate as sole carbon source (section 2.2) with

added IPTG. *E. coli* harboring pNK3 grew on Koser's citrate broth while the vector control was not able to grow **(Fig. 3.7)**.

3.3.4: Effect of *E. coli* NADH insensitive *cs* gene and artificial citrate operon on growth and citric acid levels in *E. coli* BL21 and *E. coli* MA1935

E. coli BL21 harboring pNK2 and pNK3 plasmid grew up to 2.0 $O.D._{600nm}$ in about 32 h and acidified the medium to pH 6.0 and pH 4.2, respectively **(Fig. 3.8)**. pNK2 and pNK3 transformants of *E. coli* MA1935 could only grow till 0.5 and 0.7 $O.D._{600nm}$ and acidified the medium till pH 6.6 and 4.4, respectively **(Fig. 3.9)**.

Incorporation of pNK2 plasmid in *E. coli* BL21 and *E. coli* MA1935 resulted in accumulation of 15.83 mM and 17.25 mM of intracellular citric acid **(Table 3.2)**, respectively, in M9 minimal medium containing 50 mM glucose as carbon source. However, extracellular citric acid was not detected in both the strains harboring pNK2 plasmid. *E. coli* BL21 and *E. coli* MA1935 harboring pNK3 plasmid showed 9.06 mM and 10.23 mM of intracellular citric acid and secreted 5.6 mM and 6.3 mM of citric acid, respectively, in M9 minimal medium with 50 mM glucose as carbon source **(Table 3.2)**.

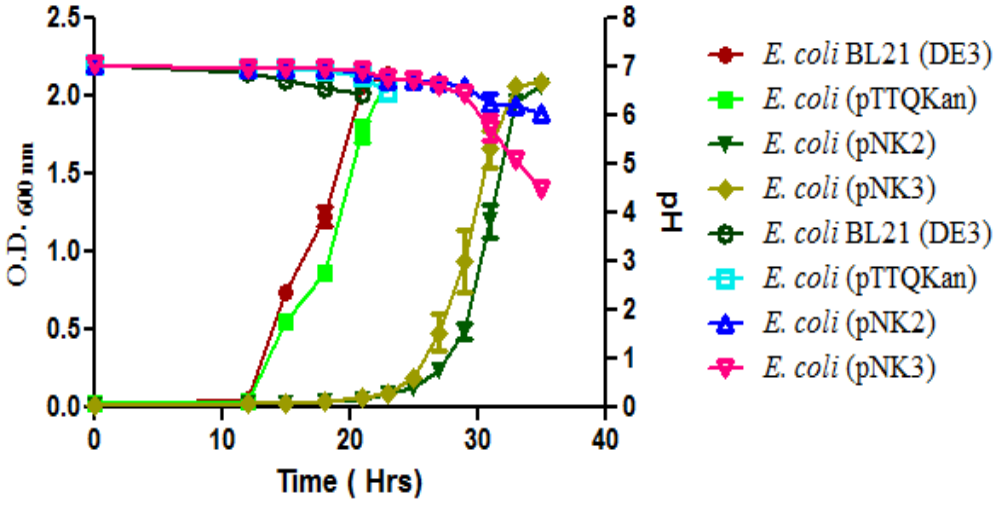


Fig. 3.8: Growth and pH profile of *E. coil* BL21 transformants in M9 minimal medium containing 50 mM glucose as carbon source. $O.D._{600}$ and pH values at each time point are represented as the mean ± SD of four to six independent observations.

Strain	Plasmid	Citric acid (mM)	
		Intracellular	Extracellular
E. coli MA1935	pNK2	17.25 ± 3.01	UD
	pNK3	10.23 ± 1.83	6.35 ± 1.52
E. coli BL21 (λDE3)	pNK2	15.83 ± 2.95	UD
	pNK3	9.06 ± 2.11	5.6 ± 1.68

Fig. 3.9: Growth and pH profile of *E. coil* MA1935 transformants in M9 minimal medium containing 50 mM glucose as carbon source. $O.D._{600}$ and pH values at each time point are represented as the mean ± SD of four to six independent observations.

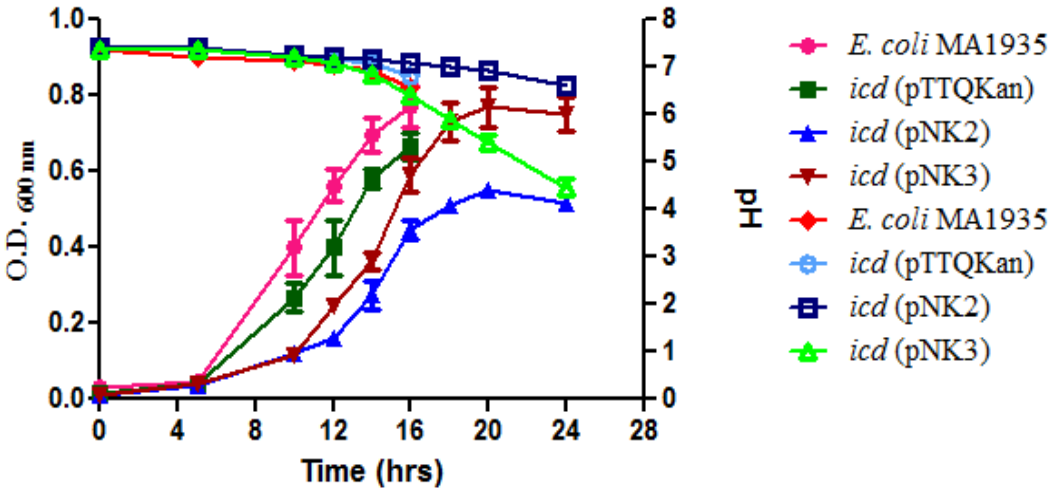


Table 3.2: Effect of citrate operon overexpression on amount of intracellular and extracellular citrate levels. The results are the mean ± SD of four to six independent observations, UD- Undetectable.

3.3.5: Effect of *cs* gene and artificial citrate operon overexpression on CS, G-6-PDH and ICL activities of *E. coli* BL21 and *E. coli* MA1935

E. coli BL21 and *E. coli* MA1935 vector controls showed CS activities of 55 ± 10.82 and 29.40 ± 3.6 U, respectively while pNK3 transformants showed 244.2 ± 28.9 and 184.6 ± 8.2 U of CS activities **(Fig. 3.10)**, respectively, in M9 minimal medium containing 50 mM glucose as carbon source which is 4.2 fold and 5.4 fold higher than vector controls. *E. coli* BL21 harboring pNK2 plasmid showed 73.80 ± 3.8 U of G-6-PDH activity which similar to the vector control. On the other hand, *E. coli* BL21 harboring pNK3 plasmid showed increased G-6-PDH activity of 125.6 ± 3.2 U which is 1.7 fold as compared to pNK2 transformants **(Fig. 3.11a)**.

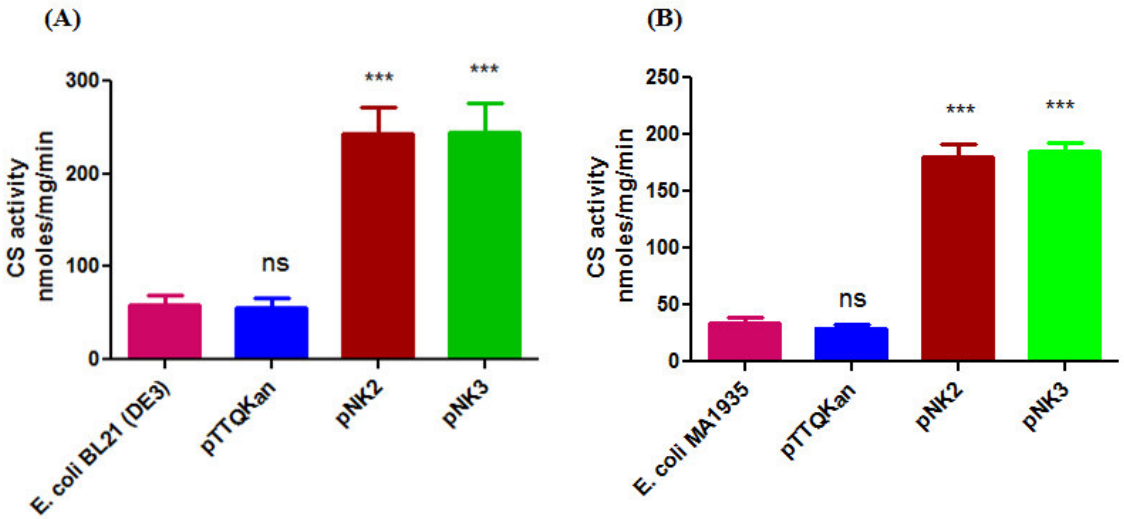


Fig. 3.10: Citrate synthase activity of *E. coli* BL21 and *E. coli* MA1935 transformants in M9 minimal medium. The results are the mean ± SD of three independent observations. ***, P < 0.0001; ns, non-significant.

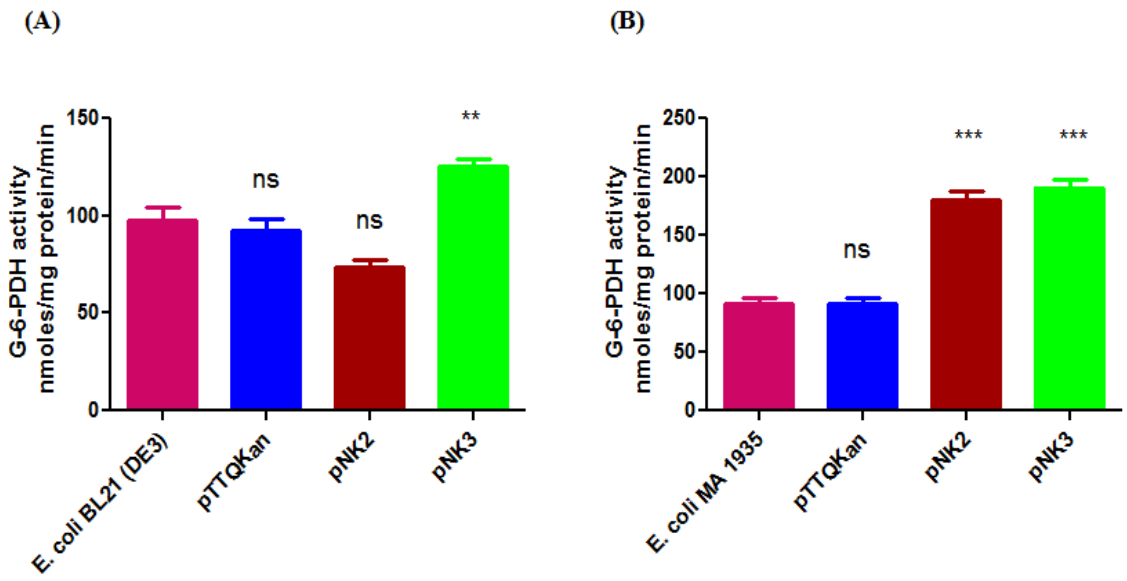


Fig. 3.11: Effect of *cs and citrate operon overexpression on G-6-PDH activity.** The results are the mean ± SD of three independent observations. **, P<0.0001; ***, P<0.0001; ns, non-significant.

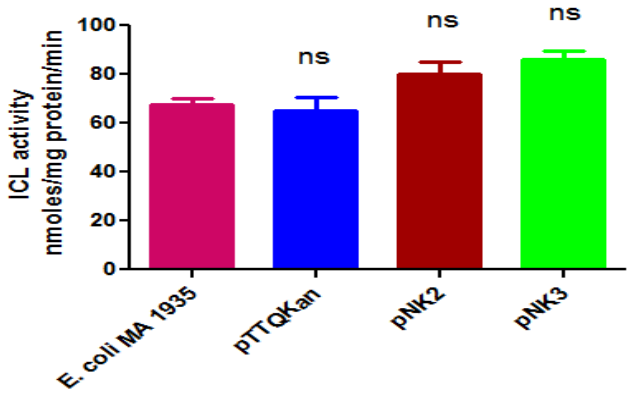


Fig. 3.12: Effect of *cs and citrate operon overexpression on ICL activity.** The results are the mean ± SD of three independent observations. ns, non- significant.

Similarly, *E. coli* MA1935 harboring pNK2 and pNK3 plasmids showed 2 fold increase in G-6-PDH activity as compared to vector control. G-6-PDH activity in *E. coli* MA1935 transformants was higher as compared to *E. coli* BL21 trasnformants (**Fig. 3.11b**). ICL activity in *E. coli* MA1935 transformants **(Fig. 3.12)** was much higher as compared to *E. coli* BL21 and its transformants which showed undetectable amount of activity.

3.4: Discussion

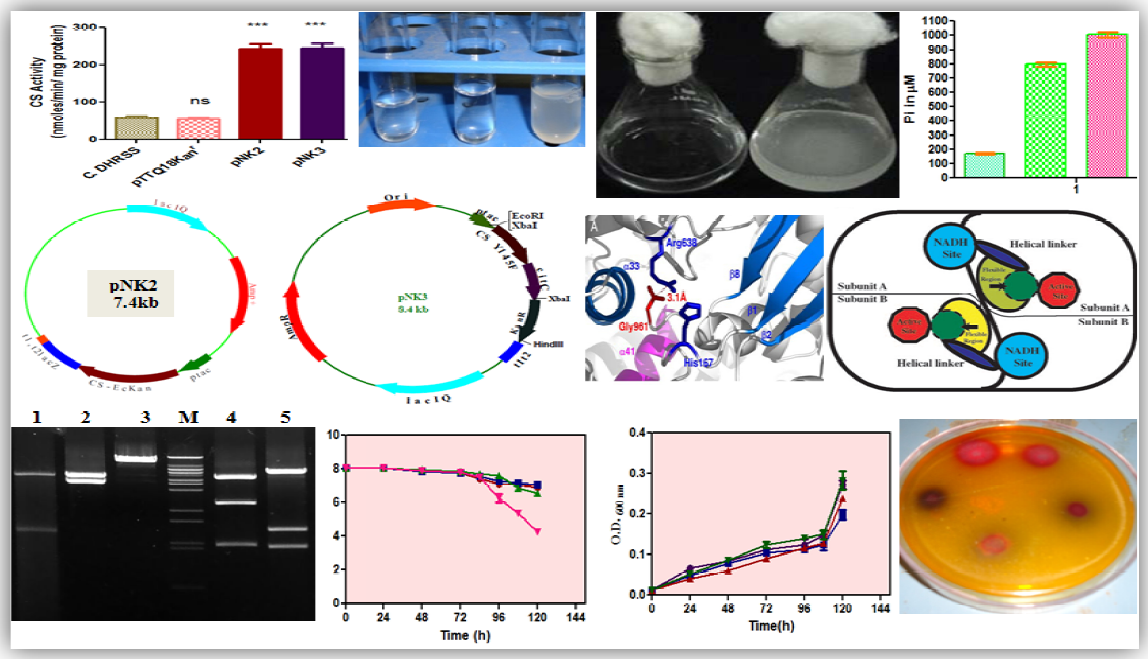
Genetic manipulation for citric acid secretion in fungus, involving overexpression of *cs*, *pk* and *pfk* genes in *A. niger* could not increase citrate production while overexpression of *icl* and invertase genes in *Y. lipolytica* resulted in production of higher amount of citrate (Ruijter et al., 1997; 2000; Forster et al., 2007a; 2007b). Fungi secrete citric acid in very high amounts (upto 1M) which is achieved by alterations of glycolytic pathway, tricarboxylic acid cycle in mitochondria, transport of citric acid from mitochondria to cytoplasm and in turn to extracellular medium (Papagianni, 2007). On the other hand, *Citrobacter koseri* and *Bacillus coagulans* are known to secrete low levels of citric acid (~1 mM) (Gyaneshwar et al., 1998a). *Bacillus subtilis* mutation in *icd* gene resulted in accumulation of 20 mM citrate but secreted only 0.9 mM citrate (Matsuno et al., 1999). Deletion of aconitase (*acn*) gene in *Corynebacterium glutamicum* resulted in secretion of 2 mM citrate while *Streptomyces coelicolor acn* mutant accumulated citrate up to 14 mM (Viollier et al., 2001; Meike et al., 2011). Thus, all these studies demonstrate that mutation in genes of TCA cycle downwards of *cs* resulted in accumulation of higher amount of citrate. Similar results of citrate accumulation up to 20 mM and 1.2 mM secretion was achieved when *E. coli cs* gene was overexpressed in *P. fluorescens* (Buch et al., 2009). Citric acid secretion in *P. fluorescens* could be mediated by the presence of H^+ dependent citrate transporter. Thus metabolic engineering of the central carbon metabolism (CCM) of the industrially important bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Corynobacterium glutamicum*, *Streptomyces spp*., *Lactococcus lactis* and other lactic acid bacteria could be a great strategy for the improvement of cells by engineering of selected metabolic steps to reroute carbon fluxes toward precursors for important metabolites (Papagianni, 2012). The extensive

knowledge gained on *E. coli* CCM offers key advantages in metabolic engineering of strain to achieve increased metabolite production.

E. coli BL21 *icd* mutants are reported to accumulate citric acid upto 11 mM and secrete 3.4 mM in rich medium (Aoshima et al., 2003). However, the mechanism of citric acid secretion in *E. coli* MA1935 is unclear as it does not possess a functional citrate transporter (Hall, 1982). Overexpression of *E. coli* NADH insensitive *cs* gene in both *E. coli* BL21 and *E. coli* MA1935 strains accumulated citrate to similar levels suggesting that citric acid is diverted towards glyoxylate pathway in *icd* mutant. This is supported by the observation of increased level of ICL activity in *icd* mutant as compared to wild type. Thus, citric acid accumulation does not require deletion of either *icd* or *acn* genes.

Most bacteria utilize citrate as the sole carbon source and contain H^+ dependent citrate transporters. Additionally, some bacteria possess sodium and magnesium dependent transporters belonging to 2-hydroxycarboxylate transporter family (Lolkema, 2006). *E. coli* strains possess cryptic citrate transporter and cannot utilize citrate as the carbon source (Hall, 1982). Secretion of 6.35 mM and 5.6 mM of citric acid in *E. coli* BL21 and *E. coli* MA1935 harboring artificial citrate operon, demonstrates that the sodium dependent citrate transporter can also function in citrate efflux. On the other hand, the citrate efflux system in *A. niger* is mediated by H^+ coupled ATP transport system (Papagianni, 2007). The efficient efflux of citrate in fungi results in low intracellular citrate levels (~ 10 mM) as compared to high extracellular citric acid (up to 1M) (Papagianni, 2007). However, incorporation of citrate operon in *E. coli* resulted in intracellular and extracellular citrate gradient of 2:1 is achieved which is much higher than *acn* and *icd* mutations (10:1) in bacteria.

The present study shows that incorporation of citrate operon secrete high amount of citric acid efficiently in both- wild type and *icd* mutant strains. However, *E. coli* BL21 harboring citrate operon has good growth in minimal medium while the *icd* mutant is growth compromised. The amount of citric acid secreted in *E. coli* harboring citrate operon is more than 5 mM. 5-10 mM citric acid levels could help in transforming natural rhizobacteria into novel phosphate solubilizing bacteria as these levels of citric acids are sufficient to release P from mineral phosphates (Gyaneshwar et al., 1998a).



CHAPTER 4

Effect of coexpression of NADH insensitive *E. coli cs** and *Salmonella typhimurium* citrate transporter (*citC*) genes on citric acid efflux in *Citrobacter* DHRSS

4.1: INTRODUCTION

MPS ability of rhizobacteria is mainly due to secretion of low molecular weight organic acids such as citric, oxalic, gluconic, 2-ketogluconic, lactic, malic, succinic, tartaric and acetic (Archana et al., 2012). Depending on the nature of the acid, concentrations ranging from 10 to 100 mM of these organic acids is required for solubilization of mineral P from soils (Gyaneshwar et al., 1998a). Among the different organic acids, citric and oxalic acid are most effective in the solubilization of different mineral phosphate rocks by good chelating and strong acid properties (Kpomblekou and Tabatabai, 1994; Liu et al., 2012). Our earlier reports showed that 5-10 mM citric acid is sufficient for releasing P from alkaline vertisols (Gyaneshwar et al., 1998a). Very few bacterial strains are known to secrete citrate including *Citrobacter* sp. and *Bacillus coagulans* which secrete this acid in very low amounts (~ 1 mM). *Citrobacter* DHRSS has been isolated from the rhizosphere of sugar cane which demonstrated MPS ability on a

variety of aldosugars and ketosugars like glucose, fructose and sucrose by secreting organic acids such as gluconic, acetic and pyruvic acids (Patel et al., 2008).

Organic acid secretion in rhizobacteria has been incorporated by genetic modification of pathway leading to biosynthesis of gluconic acid including cloning and overexpression of glucose *dehydrogenase gene (gcd) in Azotobacter vinelandii.* Genes responsible for biosynthesis and transport of pyrroloquinoline quinone (PQQ), an essential cofactor for GDH, has been cloned and overexpressed in *Escherichia coli* and *Erwinia herbicola*. Heterologous overexpression of GAD operon for 2-ketogluconic acid secretion has been done in *E. asburiae* PSI3 (Goldstein, 1995; Khan et al., 1995; Krishnaraj and Goldstein., 2001; Kim et al., 2003; Shashidhar and Podile, 2009; Kumar et al., 2013). All these genetic modification were done for converting the rhizobacteria in to efficient phosphate solubilizing bacteria.

Citric acid secretion in bacteria has been achieved by mutations including gene-disruption of *icd* gene of *Escherichia coli* (K and B strains) and *Bacillus subtilis* (Aoshima et al., 2003; Kabir and Shimizu, 2004). Also, aconitase mutation in *Streptomyces coelicolor* has been reported for accumulation of higher amount of citrate (Matsuno et al., 1999; Viollier et al., 2001). Mutation in *icd* gene of *E. coli* increased citric acid secretion up to 3.4 mM (Aoshima et al., 2003). Although, the mutation achieved enhancement in citric acid levels, the strain was growth compromised and showed glutamate auxotrophy. Hence, these modifications in rhizobacteria are not suitable for field conditions. Earlier reports from our laboratory demonstrated that overexpression of *gene encoding citrate synthase (gltA) in Pseudomonas fluorescens* increased citric acid secretion up to 1.2 mM (Buch et al., 2009). However, the levels were insufficient for releasing P from soil (Gyaneshwer et al., 1998a). With a view to increase the flux through the *anaplerotic node* for increasing oxaloacetate levels, *ppc* gene of

Synechococcus elongatus was over-expressed in fluorescent pseudomonads. *ppc* overexpression enhanced cellular biomass, glucose catabolism through intracellular phosphorylative pathway and resulted in increased gluconic, pyruvic and acetic acids but citric acid was not detected (Buch et al., 2010). When *ppc* and *gltA* were overexpressed, the biomass yield was increased however citric acid secretion was not altered as compared to *cs* overexpression alone (Buch et al., 2009). *E. coli* has Type II CS which is a hexamer of identical subunits, is strongly and specifically inhibited by NADH (Weitzman, 1966). This allosteric regulation of CS may result in tight homeostasis of citrate levels *in vivo* and this might explain why *cs* overexpression did not result in high accumulation of citrate (Buch et al., 2009). Gram positive bacteria, on the other hand, possess Type I CS which is insensitive to NADH inhibition, but it is inhibited by ATP (Stokell et al., 2003). Several specific point mutations in the NADH binding pocket of *E. coli* CS are reported which show extremely weak inhibition by NADH. Presence of NADH insensitive *cs* gene on citrate accumulation in gram negative host is not known.

Citrate transport in *Enterobacteriaceae* family is mainly mediated by cation dependent or ATP dependent transporters (Lolkema, 2006). The Na^+-dependent citrate carriers CitS of *Klebsiella pneumoniae* and CitC of *Salmonella typhimurium serovars*, are citrate specific Na^+ coupled symporter, belonging to 2-hydroxycarboxylate family, and are driven by both the proton motive force and sodium ion motive force (Ishiguro et al., 1992; Lolkema, 2006). The citrate transporter CitH of *K. pneumoniae* is driven by the proton motive force (Lolkema., 2006). In other bacteria such as *Bacillus subtilis*, *Streptococcus bovis*, *Lactococcus lactis* the citrate transporters are Mg^{+2} or proton dependent. Hence, intracellularly accumulated citrate could be secreted by incorporation of an efficient citrate transporter.

Rationale for the present study

Objective of the present study was to enhance the MPS ability of *Citrobacter* DHRSS in field conditions, which is hindered by low availability of carbon sources, high buffering capacity of soils. The improvement in the MPS ability was investigated by targeted genetic manipulation of the central carbon metabolic pathway towards citrate secretion by overexpression of NADH insensitive citrate synthase (*cs**) of *E. coli* and *S. typhimurium* Na^+ dependent citrate transporter (*citC*) gene.

4.2: EXPERIMENTAL DESIGN

4.2.1: Bacterial Strains used in this study

Bacterial Strains	Relevant Characteristics	Source/Reference
Citrobacter DHRSS	Sugarcane rhizosphere isolate	Patel et al., 2008
C. DHRSS (pTTQKm)	*Citrobacter* DHRSS with pTTQKm plasmid; Amp^r, Km^r	This study
C. DHRSS (pNK2)	*Citrobacter* DHRSS with pNK2 plasmid; Amp^r, Km^r	This study
C. DHRSS (pNK3)	*Citrobacter* DHRSS with pNK3 plasmid; Amp^r, Km^r	This study

Table 4.1: List of bacterial strains used in this study. The details of the plasmids and the concentration of the antibiotics used are given in the table 2.2 and 2.3. All the strains were grown at 37°C.

4.2.2: Development of *Citrobacter* DHRSS harboring *E. coli cs** and artificial citrate operon

The recombinant plasmids pNK2, pNK3 and pTTQKm were transformed in *Citrobacter* DHRSS (Section 2.3.3). The transformants were selected on kanamycin plates and were confirmed by plasmid isolation and restriction digestion (Section 2.3).

4.2.3: Effect of heterologous overexpression of *cs** gene and citrate operon on MPS ability of *Citrobacter* DHRSS

P-solubilizing ability of the *Citrobacter* DHRSS transformants was checked on Tris buffered RP-Methyl red (TRP) agar plates (Section 2.4). Cell suspension of *Citrobacter* DHRSS transformants was prepared (Section 2.6.1) and 5µl of it was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 37°C for 3-5 days. P solubilization was determined by monitoring the red zone on the TRP agar plates.

4.2.4: Effect of *cs** gene and citrate operon overexpression on physiology and organic acid secretion of *Citrobacter* DHRSS

Citrobacter DHRSS transformants were grown on Tris- HCl medium containing 50 mM glucose as carbon source and subjected for physiological experiments. IPTG (0.1 mM) was used for induction. The samples withdrawn at 24 h intervals were analyzed for $O.D._{600nm}$ and pH of the medium (Section 2.6). Stationary phase cultures harvested, when pH of medium was below 5, were subjected for Pi and organic acid estimation (Ames, 1964; Section 2.6.3). CS assay was done in late-log phase in M9 minimal medium containing 50 mM glucose (2.7.2; 2.2) as carbon source.

4.3: Results

4.3.1: Heterologous overexpression of *E. coli cs** gene in *Citrobacter* DHRSS

In M9 minimal medium containing 50 mM glucose *Citrobacter* DHRSS harboring pNK2 and pNK3 showed 243.6 ± 28.09 U and 244.2 ± 29.8 U of CS activities, respectively, this is ~5 fold higher as compared to vector control which showed 55.0 ± 10.82 U of CS activity (**Fig. 4.1a**).

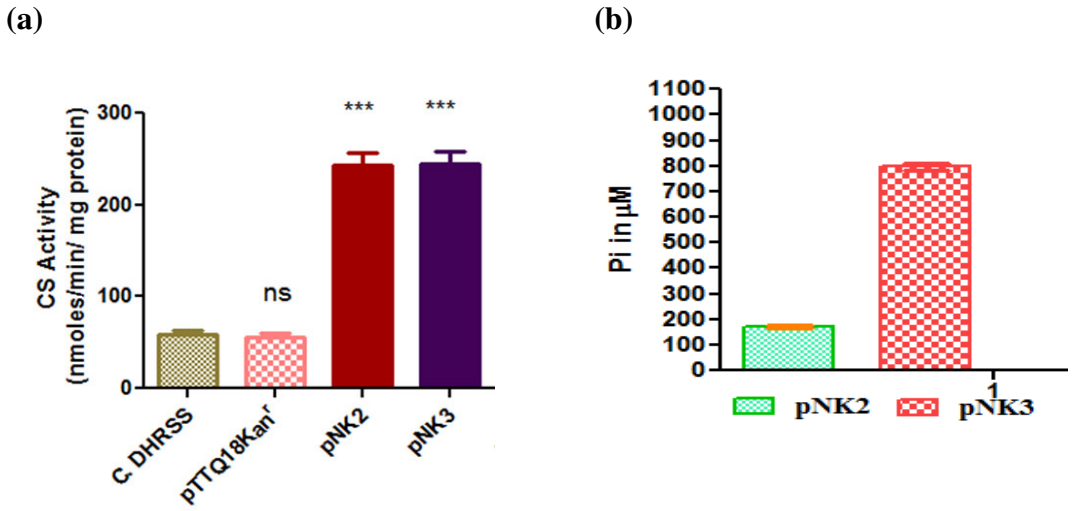


Fig. 4.1: CS activity and Pi estimation of *Citrobacter* DHRSS transformants. The results are the mean ± SD of three independent observations. ***,P < 0.0001; ns, non-significant.

4.3.2: Effect of heterologous overexpression of *E. coli cs and artificial citrate operon on MPS ability of *Citrobacter* DHRSS**

Citrobacter DHRSS pNK3 transformants showed red zone of acidification on TRP plates containing 75 mM, 100 mM Tris HCl (pH-8.0) and 50 mM glucose while wild type, vector control and pNK2 transformants were not able to show zone of acidification **(Fig. 4.2)**. In liquid TRP medium *Citrobacter* DHRSS (pNK3) showed acidification of medium till pH 4.2 in 120 h **(Fig. 4.3)**, released 0.82 mM Pi while there is very less amount of Pi release 0.17 mM, was observed in case of *Citrobacter* DHRSS (pNK2) **(Fig. 4.1b; Table 4.2)**.

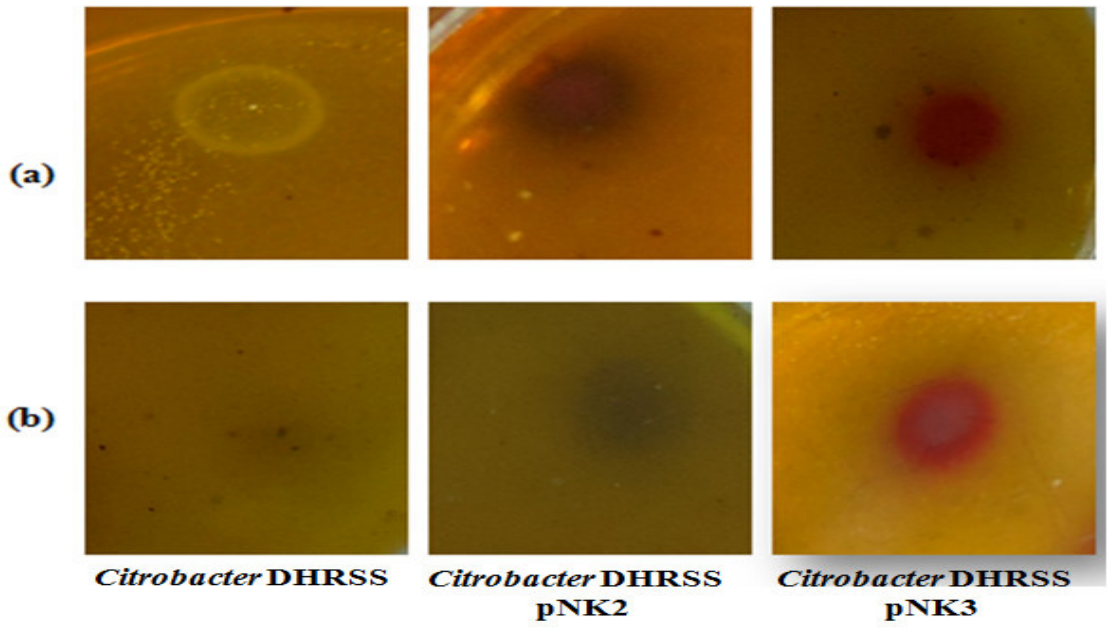


Fig. 4.2: MPS phenotype of *Citrobacter* DHRSS transformants on TRP plates. In TRP minimal medium containing 75 mM (a) and 100 mM (b) Tris HCl (pH-8.0) and 50 mM glucose as carbon source.

4.3.3: Effect of heterologous overexpression of *E. coli cs** and artificial citrate operon on physiology of *Citrobacter* DHRSS

Citrobacter DHRSS harboring pNK2 and pNK3 plasmids were grown on 75 mM and 100 mM Tris-HCl buffered media containing 50 mM glucose. *Citrobacter* DHRSS harboring pNK2 grew up to 0.28 and 0.23 O.D. and acidified the medium till pH 6.2 and 6.5 in Tris buffered medium containing 75 mM and100 mM Tris- HCl, respectively (**Fig. 4.3**). Incorporation of citrate operon, containing additional citrate transporter *citC* gene, in *Citrobacter* DHRSS shows growth up to 0.32 and 0.28 O.D. and acidified the medium til pH 4.2 in Tris buffered medium containing 75 mM and100 mM Tris- HCl, respectively, in 120 h (**Fig. 4.3**). *Citrobacter* DHRSS harboring pNK2 plasmid shows slow growth and low acidification of medium as compared to pNK3 transformants.

4.3.4: Effect of heterologous overexpression of *E. coli cs** and artificial citrate operon on organic acid secretion ability of *Citrobacter* DHRSS

Citrobacter DHRSS wild type secreted 20 mM gluconic acid and undetected amount of citric acid. *Citrobacter* DHRSS harboring pNK2 and pNK3 plasmids secreted 2.3 and 7.2 mM citric acid, and 18.9 and 20 mM gluconic acid, respectively **(Table 4.2)**.

(A)

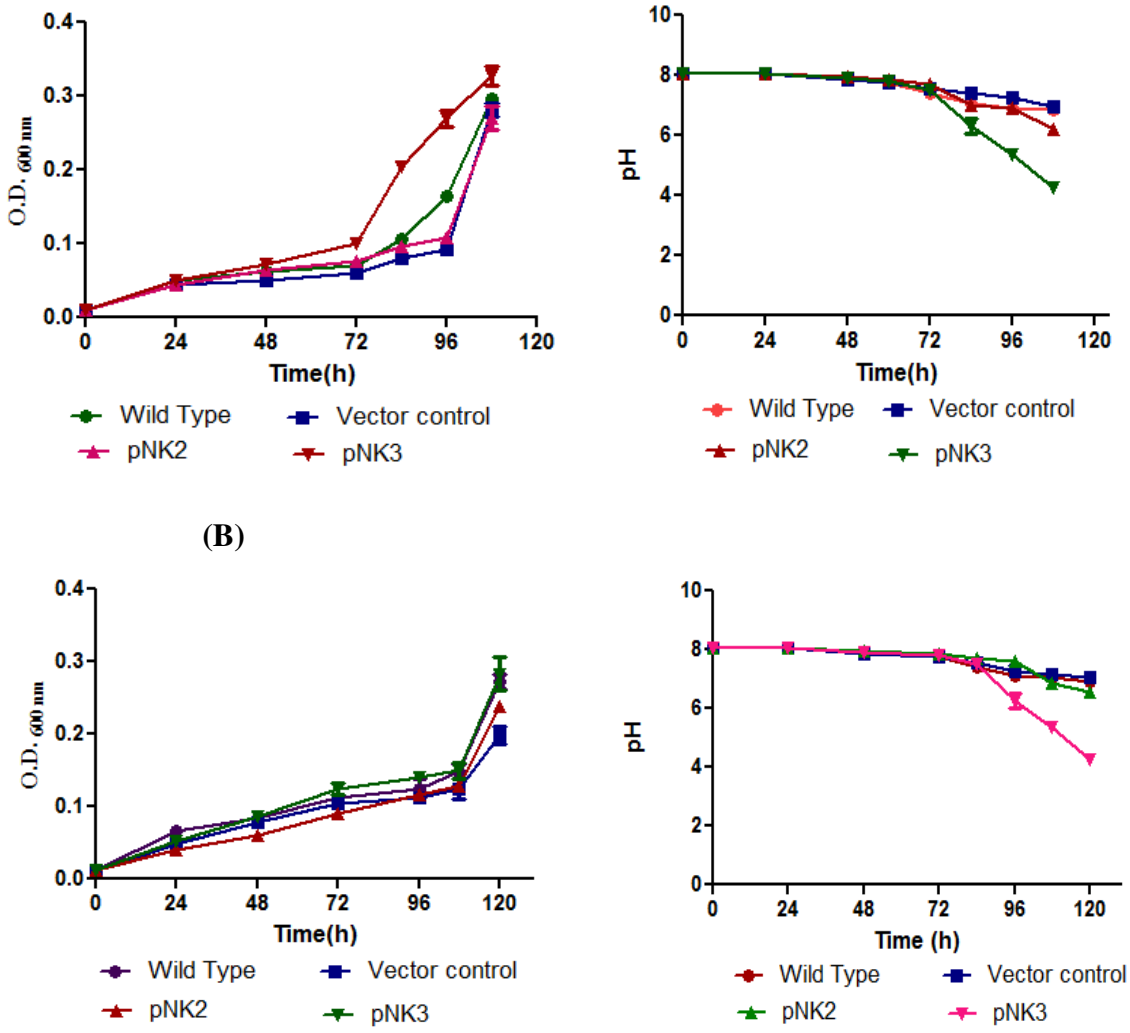


Fig. 4.3: Growth and pH profile of *Citrobacter* DHRSS wild type and transformants in Tris- HCl medium containing 50mM glucose. (A) 75mM Tris- HCl; **(B)** 100mM Tris- HCl. In TRP minimal medium containing 50 mM glucose as carbon source. OD_{600} and pH values at each time point are represented as the mean ± SD of three to four independent observations.

Table 4.2: Summary of results of *cs- *citC* overexpression in *Citrobacter* DHRSS.**

Parameters	*Citrobacter* DHRSS	pNK2	pNK3
O.D.$_{600nm}$	0.19 ± 0.03	0.23 ± 0.05	0.28 ± 0.08
pH	6.9	6.5	4.2
Pi	UD	0.169 ± 0.05	0.82 ± 0.11
CS activity	58.80 ± 10.99	243.6 ± 28.09	244.2 ± 32.22
Gluconic acid	19.48 ± 4.1	18.9 ± 3.8	20.02 ± 5.4
Citric acid	UD	2.3 ± 0.72	7.2 ± 1.9

4.4: Discussion

Manipulation of citric acid production by bacteria is important from a fundamental point of view to understand central carbon metabolism and it's over flow to different metabolic end-products. In this work, the citrate secretion was engineered for improving the MPS ability of the rhizospheric bacterium *Citrobacter* DHRSS. Citric acid secretion up to 3.4 mM has been reported in *E. coli icd* mutant (Aoshima et al., 2003). However, this strategy is not useful for field applications as the mutants were growth defective.

In our previous studies, overexpression of *cs* gene in *P. fluorescens* resulted in intracellular accumulation of 20 mM citric acid and secretion of 1.2 mM in the growth medium with no adverse effect on growth (Buch et al., 2009). The low amount of citric acid secretion could be attributed to the inefficiency of the native H^+ dependent citrate transporter in *P. fluorescens.* To further increase the citrate production and its efficient secretion, the present

study dealt with the over-expression of NADH insensitive CS and Na^+ dependent citrate transporter was carried out in *Citrobacter* DHRSS as it is proficient at secretion of other organic acids such as gluconate, pyruvate and acetate (Patel et al., 2008). Additionally, *Citrobacter koseri* is known to secrete about 1-2 mM citric acid (Gyaneshwar et al., 1998a). Intracellular accumulation of citrate upon overexpression of NADH insensitive *cs* gene in *Citrobacter* DHRSS was 26 mM, in 100 mM glucose as carbon source, but did not show any efflux of citrate although it can utilize citrate as sole carbon source.

Incorporation of artificially constructed citrate operon containing Na^+-dependent CitC transporter in addition to NADH insensitive CS in *Citrobacter* DHRSS resulted in the secretion of citric acid up to 7 mM in TRP medium indicating that the native transporter was the limiting factor for citrate secretion.

Citrobacter DHRSS containing artificial citrate operon showed improved MPS ability on TRP plates in presence of 50 mM glucose under 100 mM Tris buffered medium while wild type gives phenotype with higher glucose and lower buffer strength of the medium indicating a higher efficiency of MPS ability. In flask studies, artificial

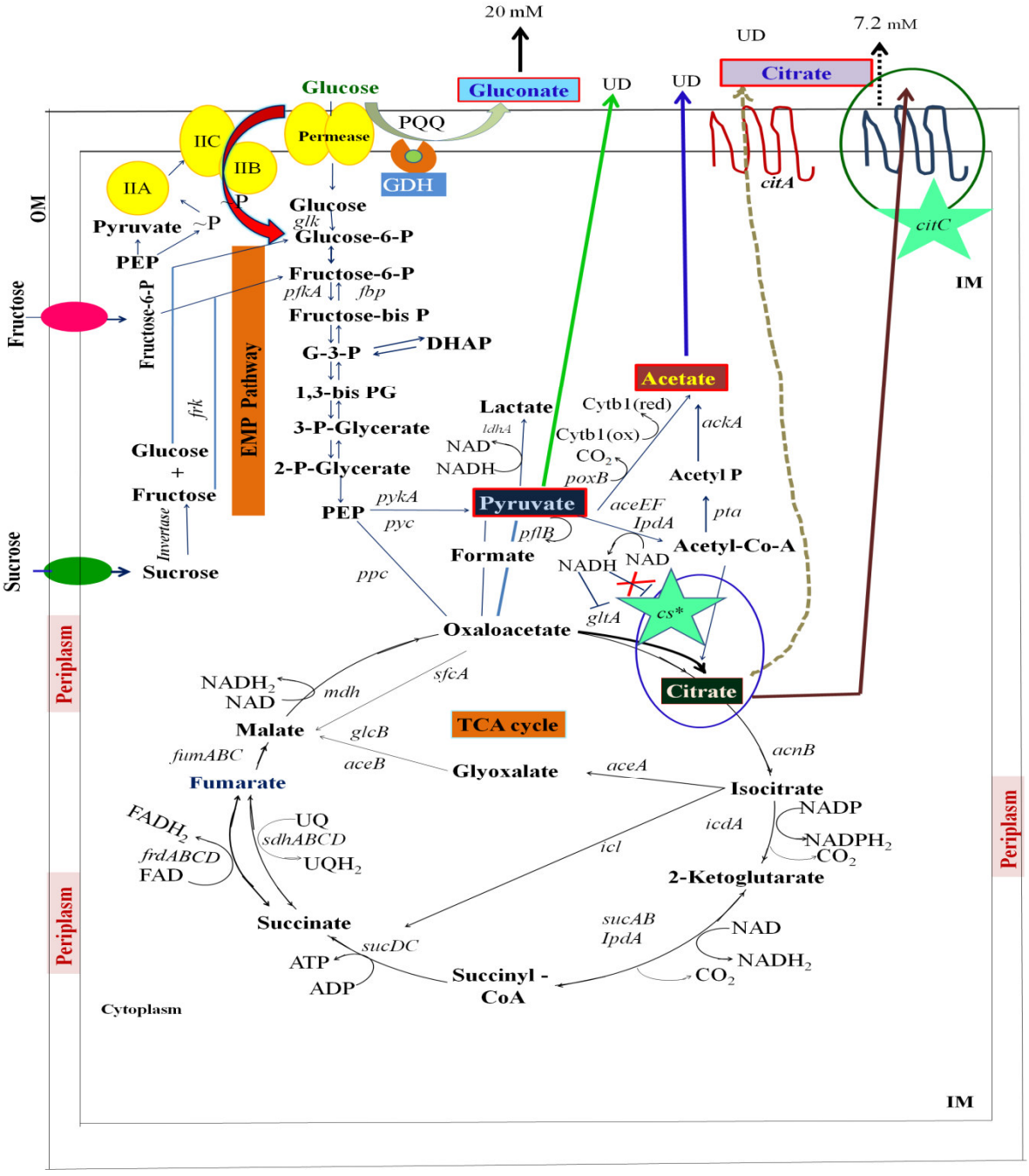


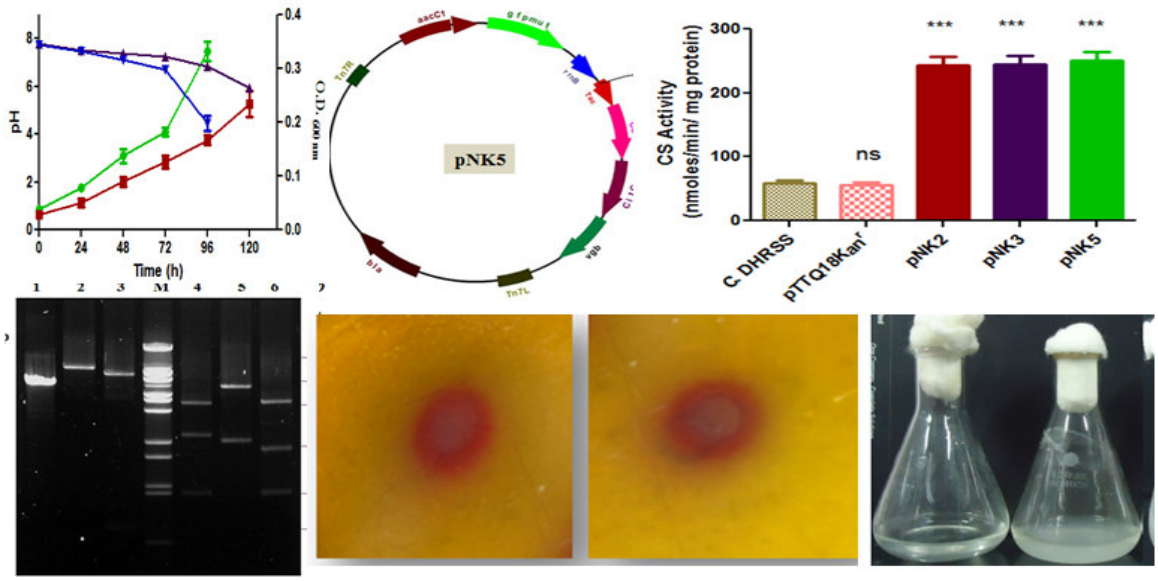
Figure 4.4: Schematic representation of citric acid secretion in *Citrobacter* DHRSS harboring artificial citrate operon.

citrate operon transformants secreted 7 mM citric acid, released 0.82 mM P in the medium and showed enhanced MPS ability. The high efficiency can be attributed to citrate production and is in agreement with the fact that 10 mM citric acid released around 0.7 mM P from alkaline

vertisols (Gyaneshwar et al., 1998a). The levels of gluconic acid (20 mM) produced by the transformant may also contribute to its MPS ability.

Citric acid biosynthesis in bacteria is governed by the nature of carbon source and the related coordination of catabolic and biosynthetic processes. *Citrobacter* DHRSS is a member of *Enterobacteriaceae* family of facultative anaerobes. The effect of the genetic modifications at the central carbon metabolism pathways of *Citrobacter* spp. are depicted in **Fig. 4.4**. In *Citrobacter* spp., glucose uptake is mediated by the phosphotransferase system (PTS) involving phosphoenol pyruvate (PEP) as phosphate donor which is a component of *anaplerotic node*. Glucose is mainly catabolized in *Citrobacter* spp. by the Embden-Meyerhof pathway resulting in pyruvate and is also subjected to direct oxidation resulting in gluconate. In contrast to *E. coli*, *Citrobacter* spp. possesses a functional GDH leading to gluconic acid secretion.

To summarize, in this study we report the genetic modification of *Citrobacter* DHRSS for secretion of higher amount of citric acid under aerobic conditions for improved mineral phosphate solubilization. *Citrobacter* DHRSS harboring NADH insensitive CS resulted in accumulation of higher amount of citric acid and incorporation of citrate transporter increased the efflux of citrate without affecting gluconic acid secretion which in turn improved phosphate solubilization under aerobic conditions.



CHAPTER 5

Effect of incorporation of artificial citrate operon and *Vitreoscilla* hemoglobin gene on MPS ability of *Citrobacter* DHRSS under microaerobic conditions

5.1: INTRODUCTION

Inoculation of PSMs resulted in variable efficacies in supplementation of phosphate to plants under field conditions (Richardson and Simpson, 2011). Soil properties, nature and amount of sugars in root exudates and catabolite repression influence the phosphate solubilization (van Veen et al., 1997; Jones et al., 2009; Patel et al., 2011). Colonization survival and functioning of PSMs is influenced by many abiotic stresses like temperature, pH and salinity. Thus, PSMs have been isolated possessing the P solubilizing ability under the stresses (Vassilev et al., 2012). Oxygen is present in limited amounts in the rhizosphere and bacterial metabolism gets altered under these conditions (Ramírez et al., 1999). Hence, the colonization and secretion of organic acids in the rhizosphere could be limited by the metabolic constraints imposed by microaerobic conditions. The obligate aerobic bacterium, *Vitreoscilla*, synthesizes elevated quantities of homodimeric hemoglobin (VHb) under hypoxic growth conditions and improves growth under microaerobic conditions when dissolved oxygen is less than 2% of air saturation (Khosla and Bailey, 1988a; b). Expression of *vgb* gene encoding VHb protein in heterologous hosts often enhances growth and metabolism by facilitating oxygen transfer to the respiratory membranes (Stark et al., 2011). Beneficial effect of *vgb* overexpression for improved bacterial growth has been demonstrated in plant associated bacteria (Ramírez et al., 1999).

VHb improves growth, metabolite and protein synthesis under oxygen limiting condition (Frey and Kallio, 2003). Incorporation of *vgb* gene in bacteria leads to improvement of recombinant protein and metabolite production. *vgb* gene has been expressed successfully in numerous organisms for growth improvement or metabolite production (Stark et al., 2011). Heterologous expression of *vgb* gene in *Streptomyces aureofaciens*, *Saccharopolyspora erythraea, Streptomyces cinnamonensis* and *Acremonium chrysogenum* increased yield of

antibiotic production under low oxygen conditions (DeModena et al., 1993; Brunker et al., 1998; Wen et al., 2001; Meng et al 2002).

Oxygen is essential in degradation of many soil or water contaminants by microorganisms. *vgb* gene has been extensively used, to overcome the problem of low availability of oxygen, by enabling the bacteria with better growth and improved degradation of organic contaminants under hypoxic condition. (Liu et al., 1996; Patel et al., 2000; Nasr et al., 2001; Urgun-Demirtas et al., 2003; 2004; So et al., 2004). 2, 4-Dinitrotoluene degradation has also been done successfully by *vgb* gene incorporation in *Burkholderia* sp. in continuous flow sand column reactor (So et al., 2004).

Extensive physiological studies in *E. coli* demonstrated that the heterologous expression of *vgb* gene increases natural resistance to nitrosative stress and oxidative stress (Frey et al., 2002; Anand et al., 2010). These stresses can damage nucleic acids, proteins and cellular membrane when level of free radical increased beyond capacity of cell's mechanism of elimination (Farr and Kogoma, 1991). Cells expressing *vgb* gene minimally affected by free radicals as compared to plasmid controls which clearly indicates the positive effect on cell survival (Frey et al., 2002; Anand et al., 2010).

Rationale of Present Study

Present study aims to determine the effect of incorporation of *vgb* gene along with artificial citrate operon, containing NADH insensitive *E. coli cs* and sodium dependent citrate transporter (*citC*) genes, on citric acid secretion and mineral phosphate solubilizing ability in *Citrobacter* DHRSS under microaerobic conditions.

5.2: EXPERIMENTAL DESIGN

5.2.1: Bacterial Strains used in this study

Bacterial Strains	Relevant Characteristics	Source/Reference
***E. coli* Strains**		
E. coli W620	*gltA* mutant strain exhibiting glutamate auxotrophy, Str^r	*E. coli* Genetic Stock Center
W620 (pNK5)	W620 with pNK5 plasmid; Str^r, Gm^r, Amp^r	This study
***Citrobacter* DHRSS Strains**		
Citrobacter DHRSS	Sugarcane rhizosphere isolate	Patel et al., 2008
C. DHRSS (pNK2)	*Citrobacter* DHRSS with pNK2 plasmid; Amp^r, Km^r	Chapter 4
C. DHRSS (pNK3)	*Citrobacter* DHRSS with pNK3 plasmid; Amp^r, Km^r	Chapter 4
C. DHRSS (pNK5)	*Citrobacter* DHRSS with pNK5 plasmid; Amp^r, Gm^r	This study

Table 5.1: List of bacterial strains used in this study. The details of the plasmids and the concentration of the antibiotics used are given in the table 2.2 and 2.3. All the strains were grown at 37°C.

5.2.2: Construction of artificial citrate gene cluster (*tac*-cs*-citC*)

Citrate gene cluster contains *E. coli cs** and *S. typhimurium citC* gene under constitutive *tac* (*tac**) promoter and *vgb* gene under its natural promoter in pUC18T-mini-Tn7T-Gm-*gfpmut*3 vector. Inserts, *tac*-cs*-citc* and *vgb*, were amplified from plasmid pNK3 **(Fig. 3.2)** and pNK6 (Section 2.1), respectively, using high fidelity Phusion DNA polymerase (New England Biolabs, USA) and gel eluted by Pure Link gel extraction kit according to manufacturer's instructions (Invitrogen, USA).Vector was digested with *BamH*I, eluted from agarose gel and proceed for cloning **(Fig. 5.1)** by circular polymerase extension cloning (CPEC) method (Quan and Tian,

2011) (Section 2.3.10). Gene specific primers used for cloning by CPEC method are listed in **Table 5.2**. Inducible *tac* promoter was mutated to constitutive *tac* (*tac**) promoter by PCR based mutagenesis in its operator region, primers for *tac** promoter are listed in **Table 5.2**. The resultant construct containing citrate gene cluster was designated as pNK5.

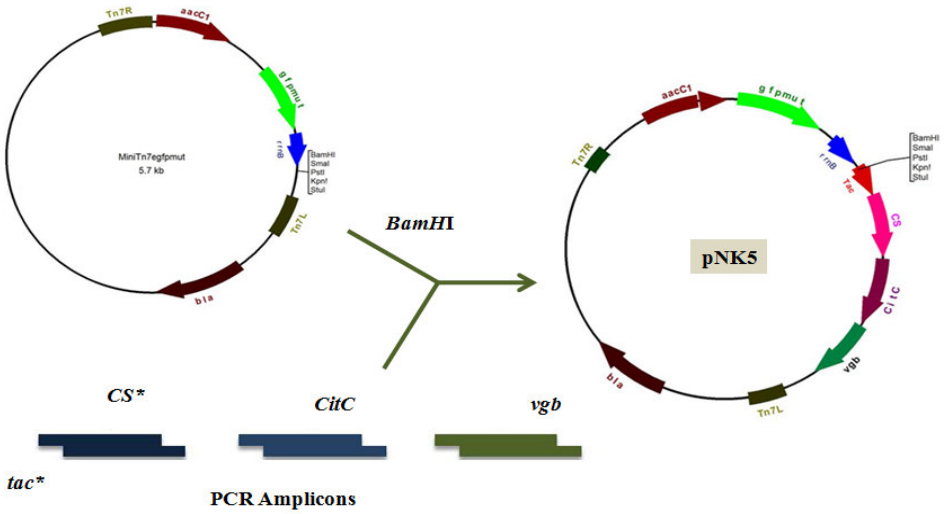


Fig. 5.1: Schematic representation of construction of artificial citrate gene cluster.

Primer	Sequence (5' – 3')
***tac* forward primer**	CGCGCAACGAGCTCACTAGTGGATCTTGACAATTAATCATCG GCTCGTATAATGGATCGAATTGTGAGCGGAATCGATTTTCAC ACAG
***tac* 2nd - *cs* forward primer**	CGGAATCGATTTTCACACAGGAAACAGACCATGGCTGATA CAAAAGCAAA
***cs* Reverse primer**	GCCTGGGTCATGTTGGTCATGGTCTGTTTCCTGTG TTAACGCTTGATATCGCTTT
***citC* forward primer**	AAAGCGATATCAAGCGTTAACACAGGAAACAGACC ATGACCAACATGACCCAGGC
***citC* reverse primer**	CCCCAGCGTCCTGTAAGCTTTTACACCATCATGCTGAACA
***vgb* forward primer**	TGTTCAGCATGATGGTGTAAAAGCTTACAG GACGCTGGGG
***vgb* reverse primer**	AGGAATTCCTGCAGCCCGGGGATGCCAAGGCACACCTGAAG

Table 5.2: List of primers used for construction of artificial citrate gene cluster by CPEC method.

All the molecular biology techniques like plasmid DNA isolation and transformation, restriction digestion, gel elution, ligation and gel electrophoresis were performed as described in Section 2.3.

5.2.3: Functional confirmation of *vgb* and *E. coli cs** gene in pNK5 plasmid

Expression of *vgb* gene in citrate cluster was checked on SDS-PAGE (Section 2.3.8) and by in-gel catalase activity (Zymographic analysis). *E. coli* W620 *gltA* mutant required glutamate supplementation when grown on minimal medium containing glucose. Plasmid pNK5 was transformed in *E. coli* W620 and the transformants were selected on streptomycin and gentamycin plates (Table 2.3). Plasmids were isolated and confirmed by restriction digestion. Confirmed transformants were subjected to complementation studies in absence of IPTG, as described in Section 2.5. The recombinant plasmid pNK5 was transformed in *Citrobacter*

DHRSS (Section 2.3.3). The transformants were selected on gentamycin plates and were confirmed by plasmid isolation and restriction digestion (Section 2.3).

5.2.4: Effect of overexpression of citrate gene cluster on MPS ability of *Citrobacter* DHRSS

P-solubilizing ability of the *Citrobacter* DHRSS transformants was checked on Tris buffered RP-Methyl red (TRP) agar plates (Section 2.4). Cell suspension was prepared (Section 2.6.1) and 5µl of it was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 37°C for 3-5 days. P solubilization was determined by monitoring the growth and red zone of acidification on the TRP agar plates.

5.2.5: Effect of artificial citrate operon and artificial citrate gene cluster overexpression on physiology and organic acid secretion of *Citrobacter* DHRSS under full aeration and microaerobic conditions in flask and fermenter studies

Citrobacter DHRSS transformants were grown on Tris- HCl medium containing 50 mM glucose as carbon source and subjected for physiological experiments. IPTG (0.1 mM) was used for induction in case of pNK3 transformants. In flask study shaking was provided at 200 rpm and 75 rpm for full aeration and limited aeration conditions, respectively. Fermenter studies were done in Biostat 1.5 L vessel (Sartorius, Germany) containing 1 L 100 mM TRP medium and 50 mM glucose as carbon source under full oxygen and low oxygen conditions (2-8%) at 800 rpm and 37°C. The samples withdrawn at 24h intervals were analyzed for $O.D._{600nm}$ and pH of the medium (Section 2.6). Stationary phase cultures harvested when pH of medium was below 5, were subjected for Pi and organic acid estimation (Ames, 1964; Section 2.6.3). CS assay was done in late-log phase in M9 minimal medium containing 50 mM glucose (Section 2.7.2; 2.2) as carbon source.

5.3: Results

5.3.1: Construction of artificial citrate gene cluster

Based on the above mentioned strategies (**Fig. 5.1**; section 5.2.2) citrate gene cluster, containing NADH insensitive citrate synthase gene (*cs**) of *E. coli*, *citC* gene of *S. typhimurium* under constitutive *tac* promoter and *vgb* gene of *Vitreoscilla* under its natural oxygen sensitive promoter, was cloned in pUC18T-mini-Tn7T-Gm-*gfpmut*3 vector. Construct containing citrate gene cluster was designated as pNK5. Plasmid pNK5 was confirmed by restriction digestion pattern **(Fig. 5.2)**.

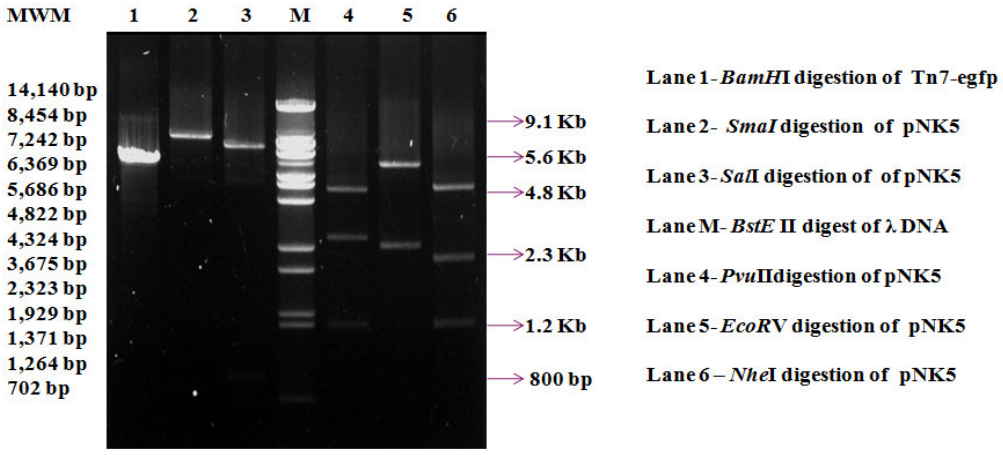
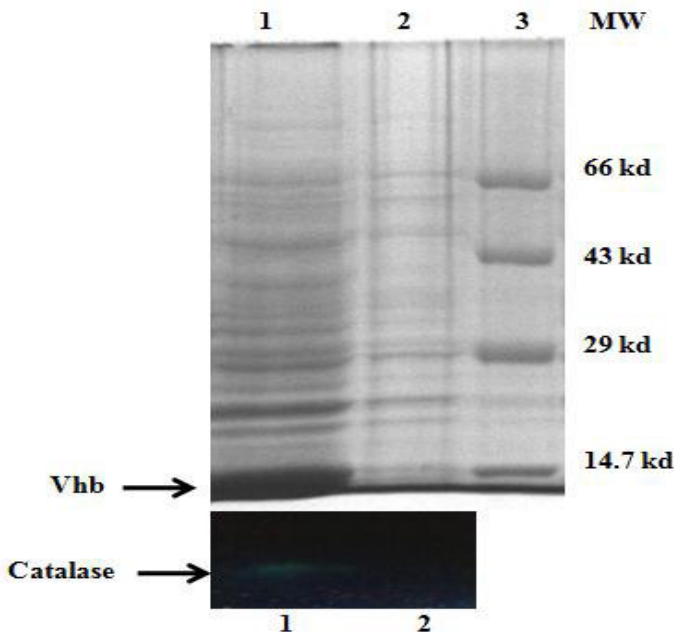


Fig. 5.2: Restriction digestion pattern of pNK5 plasmid containing artificial gene cluster.



(a) **SDS-PAGE analysis**

Lane 1- *C*. DHRSS (pNK5)

Lane 2- *C*. DHRSS (pNK3)

Lane 3- Mid-range Protein Molecular weight marker.

(b) **Zympgraphic analysis**

Lane 1- *C*. DHRSS (pNK5)

Lane 2- *C*. DHRSS (pNK3)

Fig. 5.3: *vgb* expression in *Citrobacter* DHRSS pNK5 (*ptac* cs*-citC, vgb*) transformants.

5.3.2: Expression of *vgb* gene in *Citrobacter* DHRSS containing pNK5 plsamid and functional characterization of NADH insensitive citrate synthase gene under constitutive *tac* promoter.

Expression of *vgb* gene form *Citrobacter* DHRSS harboring pNK5 plasmid (artificial citrate gene cluster) was confirmed by SDS-PAGE analysis and in-gel catalase assay. 14.7 kd band, corresponding to VHb, was detected in *Citrobacter* DHRSS (pNK5) transformants while in case of pNK3 transformants the band was absent **(Figure 5.3a)**. Further confirmation of *vgb* expression was done by in- gel catalase assay, where unstained band of catalase, due to H_2O_2 stress in presence of VHb, was detected in pNK5 transformants while it was absent in *Citrobacter* DHRSS pNK3 transformants **(Fig. 5.3b)**.

The functional confirmation of *E. coli cs** gene of pNK5 plasmids was done by mutant complementation. *E. coli* W620 *gltA* mutant strain harboring pNK5 and pNK3 plasmids were grown on M9 minimal media with glucose as carbon source in presence and absence of

glutamate. *E. coli* W620 harboring pNK5 grew on M9 minimal media in the absence of glutamate and IPTG unlike the vector control harboring pNK3 (**Fig. 5.4**), which grew in absence of glutamate when IPTG was added.

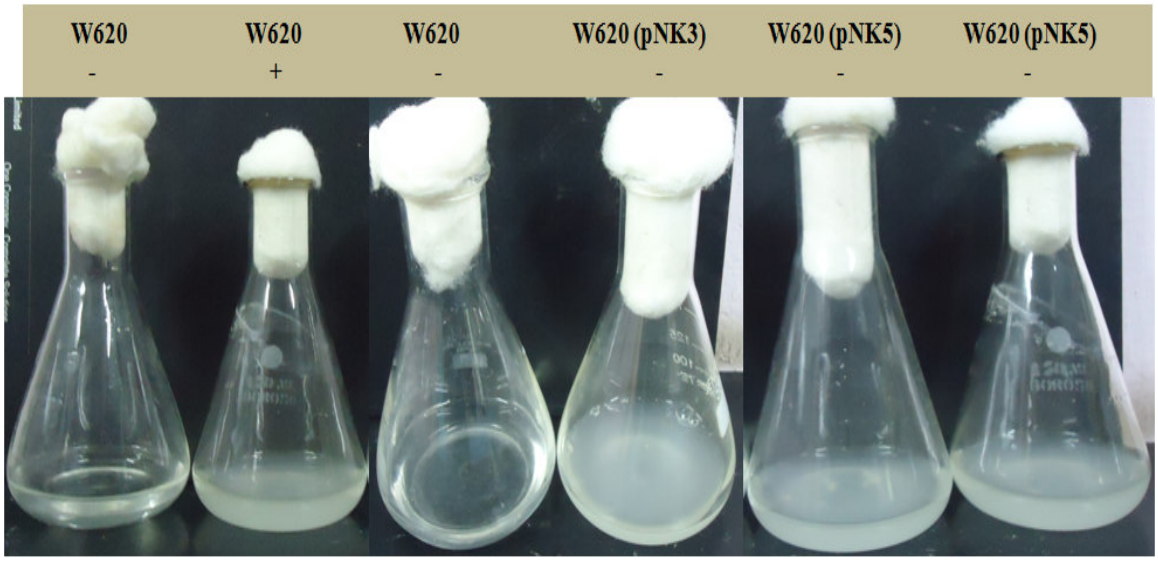


Fig. 5.4: Complementation study of *E. coli* W620 mutant phenotype by pNK3 and pNK5 constructs in M9 minimal medium containing 0.2% glucose as carbon source. –/+ at the corners of each image indicates absence and presence of 340µg/ml glutamate in the medium, respectively.

5.3.3: Effect of heterologous overexpression of artificial citrate gene cluster in *Citrobacter* DHRSS on CS activity

In M9 minimal medium without IPTG, containing 50 mM glucose, *Citrobacter* DHRSS harboring pNK5 showed 250.2 ± 30.2 U of CS activity **(Fig. 5.5; Table 5.3)** which is ~5 fold higher as compared to vector control which showed 55.0 ± 10.82 U of CS activity and same as found in pNK2 and pNK3 transformants.

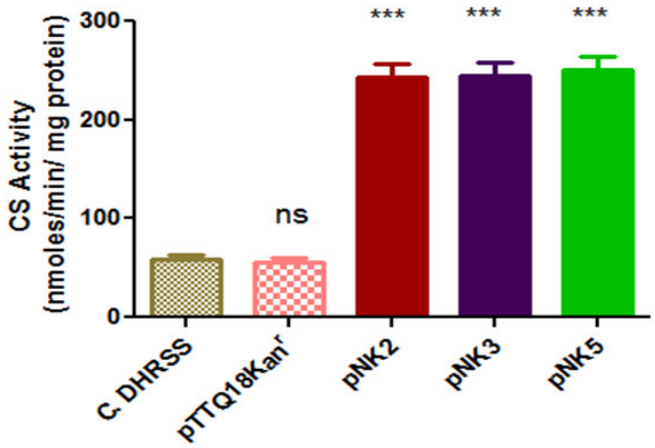


Fig. 5.5: CS activity of *Citrobacter* DHRSS transformants. The results are the mean ± SD of three independent observations. ***, $P < 0.0001$; ns, non-significant.

5.3.4: Effect of heterologous overexpression of citrate gene cluster on MPS ability and physiology of *Citrobacter* DHRSS

Citrobacter sp. DHRSS pNK5 and pNK3 transformants showed red zone of acidification on TRP plates containing 75 mM, 100 mM Tris HCl (pH-8.0) and 50 mM glucose while wild type, vector control and pNK2 transformants were not able to show zone of acidification **(Fig. 5.6)**.

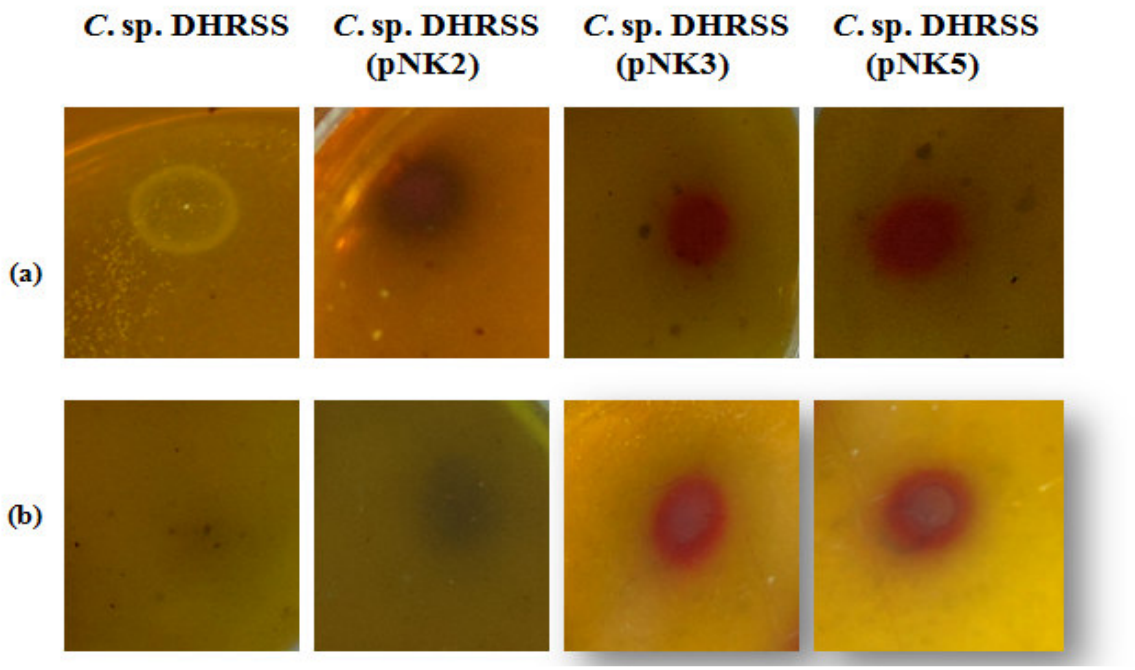


Fig. 5.6: MPS phenotype of *Citrobacter* DHRSS transformants on TRP plates. (a) 75 mM Tris- HCl and (b) 100 mM Tris- HCl.

Flask Experiments:

Growth studies of *Citrobacter* DHRSS pNK5 and pNK3 transformants harboring citrate gene cluster and citrate operon, respectively, were done under two different conditions- (i) O_2 sufficient or full aeration and (ii) O_2 deficient or low aeration conditions. In liquid TRP medium, *Citrobacter* DHRSS (pNK5) grew upto 0.26 $O.D._{600}$, showed acidification of medium till pH 4.2 in 120 h and released 0.96 mM Pi **(Fig. 5.7; Table 5.3)** from RP, under full aeration conditions. Under low aeration conditions, the growth was slow while pH drop and Pi release were similar **(Fig. 5.7; Table 5.3)** as in O_2 sufficient conditions.

Fermenter experiments:

Under full aeration conditions, both *Citrobacter* DHRSS pNK3 and pNK5 transformants grew up to $O.D._{600}$ 0.54 and 0.62, acidified the medium till pH 4.2 and released 0.84 mM and 1.1mM

Pi from RP **(Fig. 5.8; Table 5.3)**, respectively. Under low aeration conditions, growth in *Citrobacter* DHRSS (pNK3) was observed till $O.D._{600}$ 0.23 and pH drop of the medium was 5.9 while no Pi release was observed till 120 h **(Fig. 5.8; Table 5.3)**. In *Citrobacter* DHRSS (pNK5) under low aeration conditions growth was observed till O.D. 0.38, medium was acidified till pH 4.2 in 94 h and released 1.0 mM Pi **(Fig. 5.8; Table 5.3)**.

5.3.5: Effect of heterologous overexpression of citrate gene cluster and citrate operon on organic acid secretion ability of *Citrobacter* DHRSS

Citrobacter DHRSS wild type secreted 20 mM gluconic acid and undetected amount of citric acid. Under full aeration conditions in flask studies *Citrobacter* DHRSS harboring pNK2 and pNK3 plasmids secrete 2.3 and 7.2 mM citric acid, and 18.9 and 20 mM gluconic acid, respectively **(Table 5.3)**. In liquid TRP medium, *Citrobacter* DHRSS (pNK5) secreted 7.8 mM citric acid and 21 mM gluconic acid under full aeration conditions in flask studies **(Table 5.3)**. Under full aeration conditions in fermenter studies *Citrobacter* DHRSS harboring pNK3 and pNK5 secreted 7.4, 8.1 mM of citric acid, 23 mM and 25 mM of gluconic acid, respectively

(Table 5.3). Under low aeration

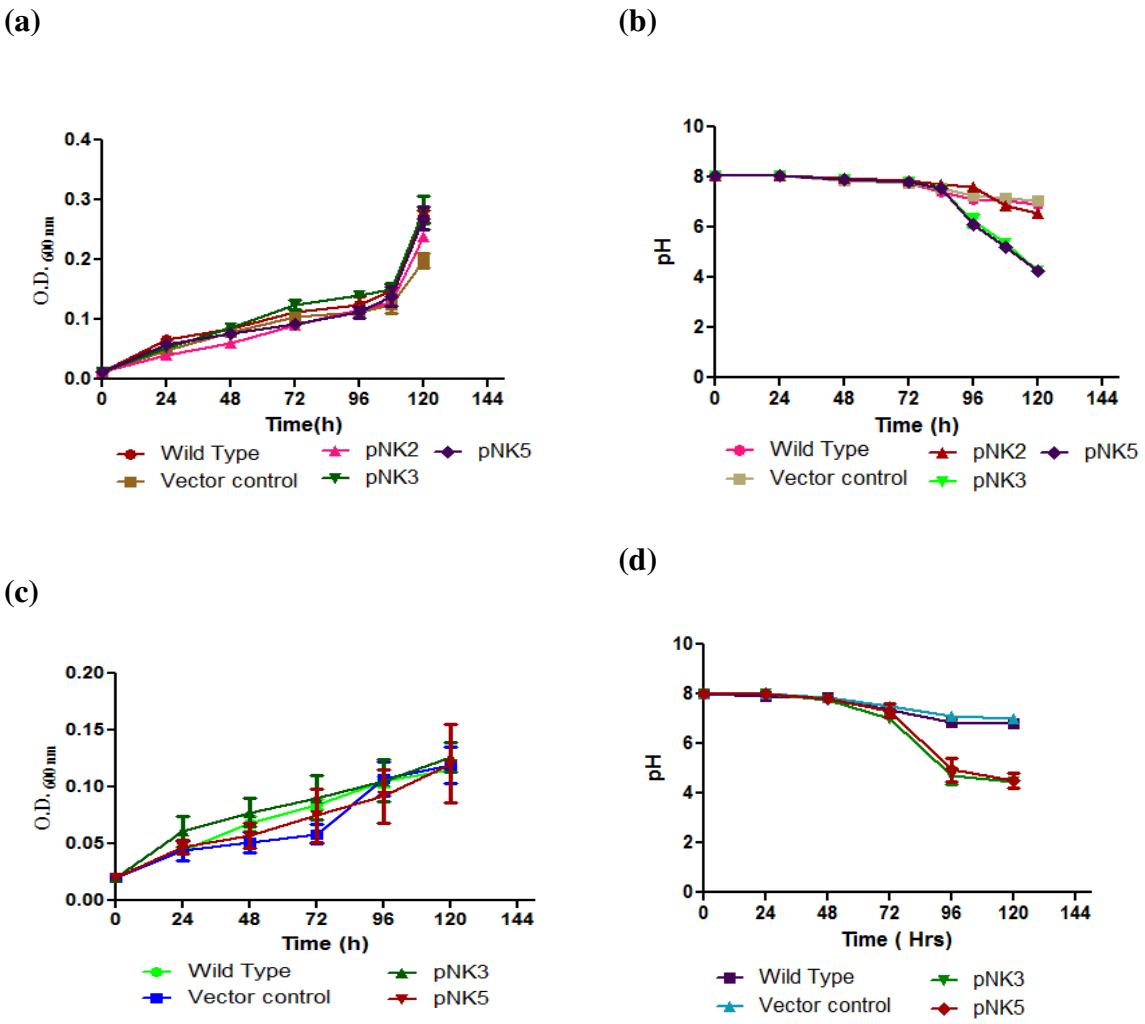
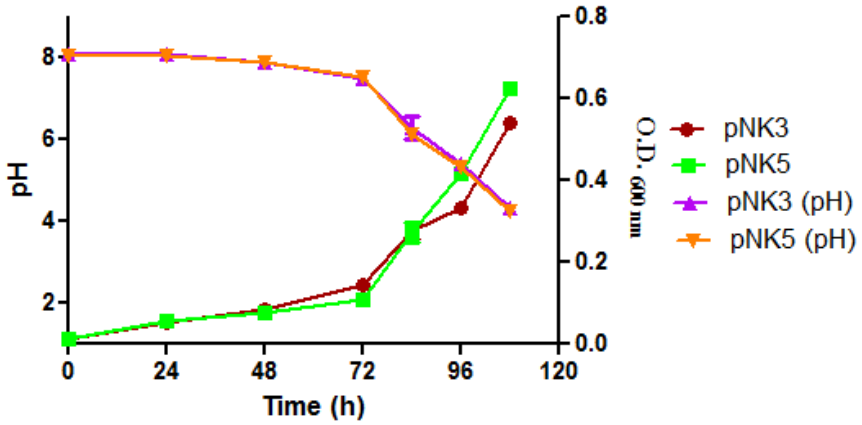


Fig. 5.7: Growth and pH profile of *Citrobacter* DHRSS transformants in flask study. Growth and pH profile under full aeration conditions (a) and (b); under limited aeration conditions (c) and (d), respectively, in TRP minimal medium containing 50 mM glucose and 100 mM Tris HCl (pH 8.0). OD_{600} and pH values at each time point are represented as the mean ± SD of three to four independent observations.

(a)



(b)

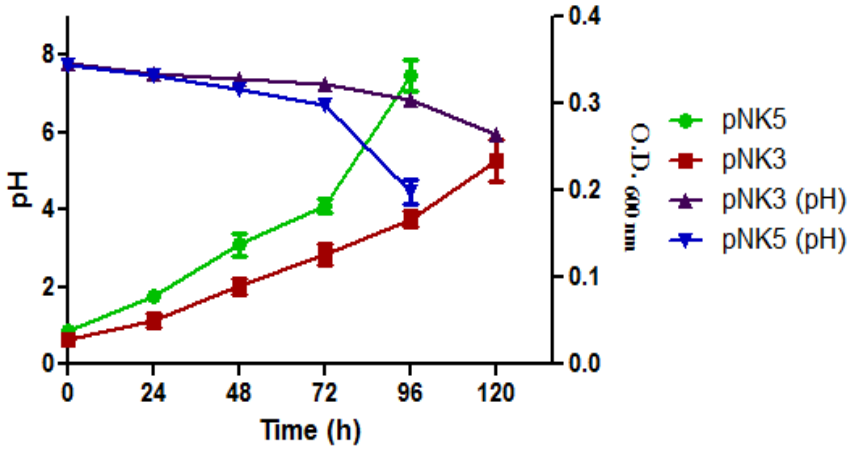


Fig. 5.8: Growth and pH profile of *Citrobacter* DHRSS transformants in fermenter study. Under full aeration (a) and low aeration (b) conditions in TRP minimal medium containing 50 mM glucose and 100 mM Tris HCl (pH 8.0). OD_{600} and pH values at each time point are represented as the mean ± SD of three observations.

conditions, in *Citrobacter* DHRSS pNK3 transformants both gluconic and citric acid were not detected while *Citrobacter* DHRSS pNK5 transformants secreted 9 mM citric acid and 17 mM of gluconic acid into the medium **(Table 5.3)**.

Table 5.3: Organic acid secretion, pH drop and Pi release by *Citrobacter* DHRSS transformants.

Condition	Plasmid	pH	Pi (mM)	Citric acid (mM)	Gluconic acid (mM)
Flask study under full aeration	*C*. sp. DHRSS	6.9	UD	ND	19.48 ± 4.1
	pNK2	6.5	0.169 ± 0.05	2.3 ± 0.72	18.9 ± 3.8
	pNK3	4.2	0.82 ± 0.11	7.2 ± 1.9	20.02 ± 5.4
	pNK5	4.2	0.96 ± 0.13	7.8 ± 1.6	21.48 ± 4.12
Flask study under low aeration	pNK3	4.5	0.79 ± 0.12	6.9 ± 2.1	17.3 ± 2.08
	pNK5	4.4	0.96 ± 0.13	7.6 ± 1.8	17.9 ± 2.11
Fermenter study under full aeration	pNK3	4.2	0.84 ± 0.39	7.4 ± 2.11	23.7 ± 2.19
	pNK5	4.2	1.1 ± 0.48	8.1 ± 2.8	25.31 ± 5.3
Fermenter study under limited aeration	pNK3	5.9	UD	ND	ND
	pNK5	4.4	1.0 ± 0.18	9.08 ± 3.8	17.33 ± 2.08

Table 5.3: Effect of heterologous overexpression of artificial citrate operon and citrate gene cluster in *Citrobacter* DHRSS. In TRP medium containing 50 mM glucose and 100 mM Tris HCl under full aeration and limited aeration conditions in fermenter and flask studies.

5.4: Discussion

In addition to the metabolic alterations in the central carbon pathway, this work also deals with the overexpression of *vgb* gene in *Citrobacter* DHRSS along with artificial citrate operon to

improve the recombinant protein production and better survival of host under microaerobic conditions (Frey and Kallio., 2003). It has been reported that the MPS ability was lost when grown without an air in case of *Enterobacter intermedium* which secreted 2-ketogluconic acid (Hwangbo et al., 2003). Present study showed similar loss of MPS ability under low aeration conditions in case of *Citrobacter* DHRSS containing citrate operon with a concomitant loss of citric and gluconic acid secretion. Presence of *vgb* gene restored the citric and gluconic acid secretion along with MPS ability under microaerobic conditions. This could be accounted by the fact that presence of VHb is known to improve growth, metabolite and protein synthesis under oxygen limiting condition. Presence of VHb in *Rhizobium etli* increased nitrogenase activity and N content in bean plants (Ramirez et al., 1999). This is of significance in the rhizosphere environment where the conditions are microaerobic and affect the metabolism and phenotype of microorganism drastically.

In this study we report the genetic modification of *Citrobacter* DHRSS for secretion of higher amount of citric acid under microaerobic conditions for improved mineral phosphate solubilization **(Fig. 5.9)**. *Citrobacter* sp. DHRSS harboring NADH insensitive CS resulted in accumulation of higher amount of citric acid and incorporation

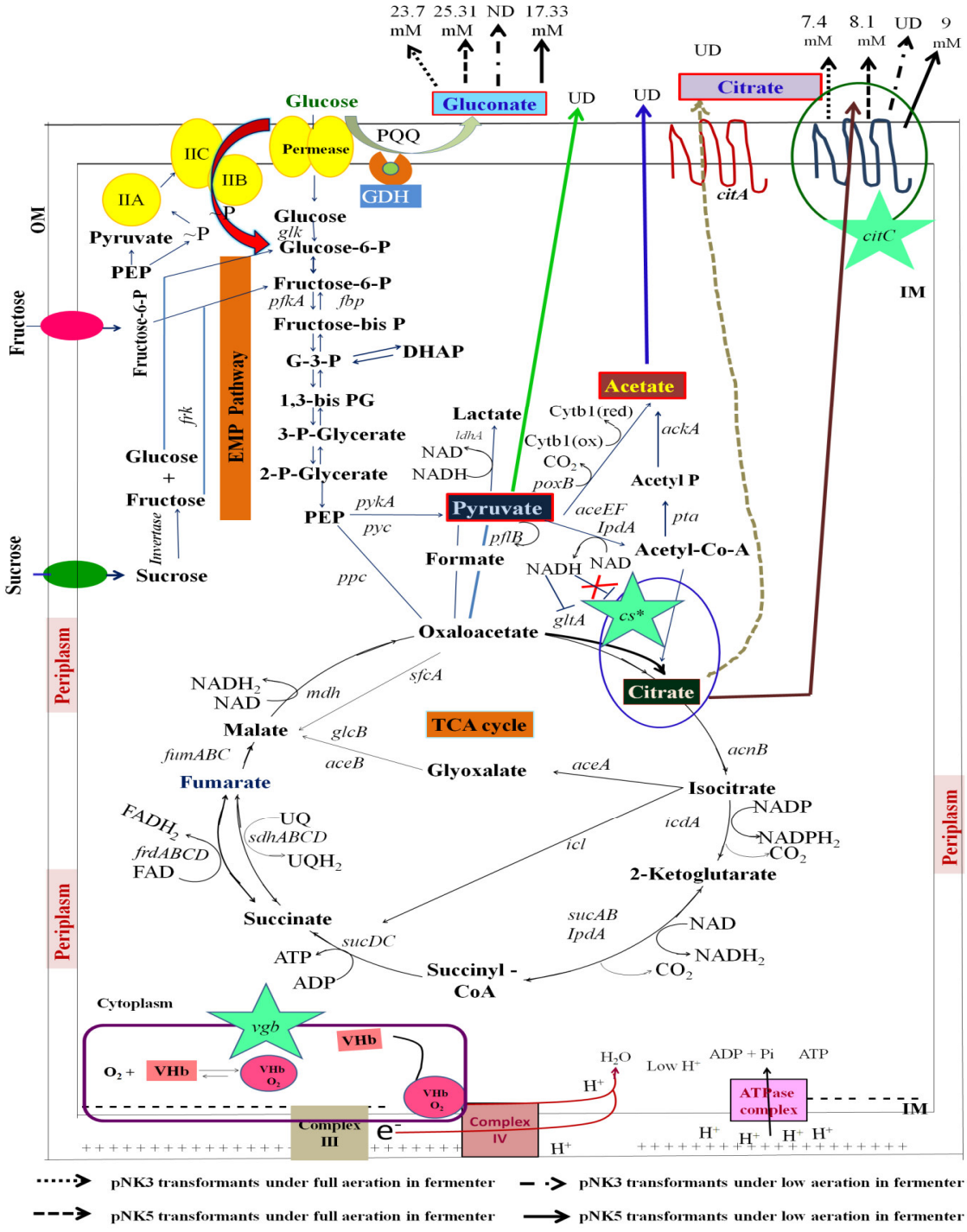


Fig. 5.9: Schematic representation of citric acid secretion in *Citrobacter* DHRSS containing artificial citrate gene cluster under microaerobic conditions.

of citrate transporter increased the efflux of citrate, without affecting gluconic acid secretion, which in turn improved phosphate solubilization under aerobic conditions.

However, under microaerobic conditions, citrate secretion and MPS ability of organism were lost and incorporation of *vgb* gene in artificial citrate operon containing strain restored these properties under microaerobic conditions.

Incorporation of citrate secretion in *Citrobacter* DHRSS improves the MPS phenotype of genetically modified organism while growth and physiological parameters remains unaltered. Under full aeration conditions *Citrobacter* DHRSS harboring pNK3 shows secretion of higher amount citric acid which is sufficient to release ~3 fold increase in Pi release from RP as compared to pNK2 transformants, and same as pNK5 transformants. In fermentor studies under low aeration conditions MPS ability of pNK3 transformants diminished drastically, due to secretion of undetectable amount of citric acid, which is in accordance with the results of previous studies of *Enterobacter intermedium* which lost its MPS ability and 2-KGA production when grown without an air supply (Hwangbo et al., 2003). Incorporation of *vgb* gene in artificial citrate operon restored the MPS ability of *Citrobacter* DHRSS by secreting higher amount of citric acid which leads to medium acidification and release of Pi, under 2% aeration conditions in fermenter, these results are in accordance of recent report where genomic integration of *vgb* gene in *Bacillus amyloliquefaciens* LL3-PVK showed an increase of 30% in γ-PGA production, while the biomass was increased by 7.9% under low aerobic condition (Zhang et al., 2013). Similar type of effect of VHb was observed in production of many biochemicals in either aerobic or microaerobic conditions **(Table 5.4)**.

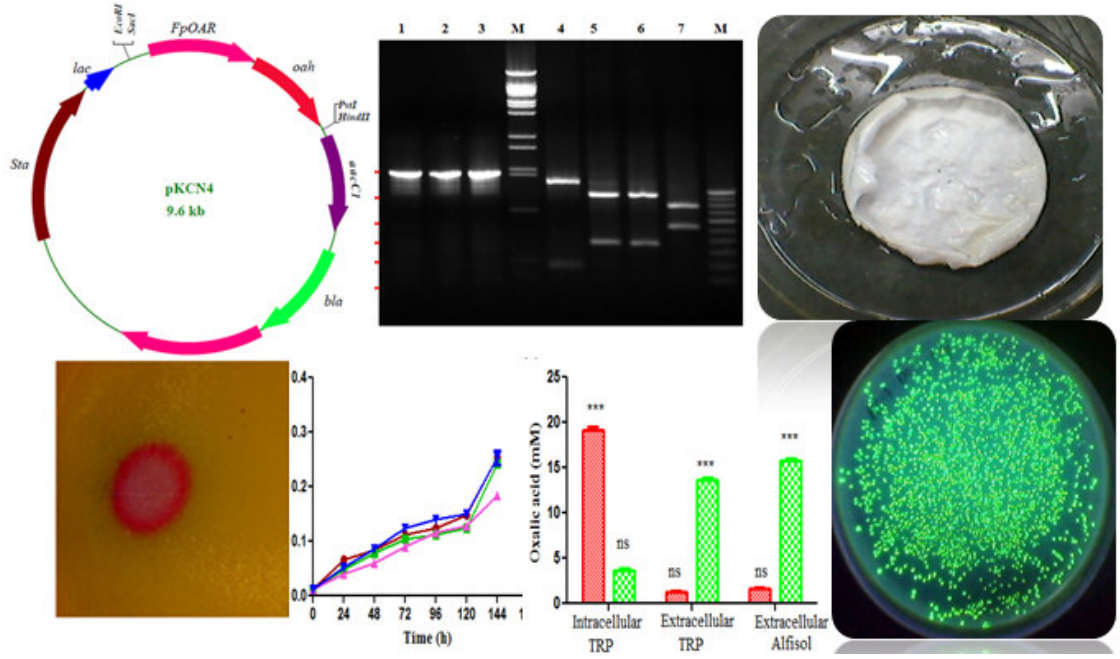
Biochemical	Organism(s)	Reference
α-amylase	*Schwanniomyces occidentalis*	Suthar and Chattoo, 2006
L-Asparaginase	*Pseudomonas aeruginosa*	Geckil et al., 2006
Insecticidal crystal proteins	*Bacillus thuringiensis*	Feng et al., 2007
Hyaluronic acid	*Bacillus subtilis*	Chien and Lee, 2007
S-adenosylmethionine	*Pichia pastoris*	Chen et al., 2007
Mussel adhesive protein	*Escherichia coli*	Kim et al., 2008
D-amino acid oxidase	*Escherichia coli*	Yu et al., 2008
L-glutamate, L-glutamine	*Corynebacterium glutamicum*	Liu et al., 2008
L-DOPA, dopamine	*Citrobacter freundii, Erwinia herbicola*	Kurt et al., 2009
Poly-γ-glutamic acid, *Bacillus amyloliquefaciens* LL3-PVK	*Bacillus subtilis*	Su et al., 2010 Zhang et at., 2013
Dihydroxyacetone	*Gluconobacter oxydans*	Li et al., 2010
Ethanol	*Escherichia coli*	Sanny et al., 2010
GDH, Gluconic acid	*E. asburiae* PSI3	Kumar, 2012
Citric acid	*Citrobacter* DHRSS	This study

Table 5.4: Effect of overexpression of *vgb* on host metabolism (Stark et. al., 2011 with new additions)

Co-over expression of Na^+ dependent citrate transporter of *S. typhimurium* in addition to the *cs** (as an artificial operon) resulted in secretion of 7 mM citrate along with gluconic acid and improves MPS ability of the strain under aerobic conditions. Additionally, heterologous

overexpression of *vgb* gene along with artificial citrate operon retained citric acid secretion and MPS ability of transformants under low O_2 conditions.

The genetically modified strain is expected to be an efficient phosphate solubilizing bacterium (PSB) in rhizospheric soil where the environment is microaerobic. This paves the way for genetic modification of other plant growth promoting rhizobacteria to enable them to secrete higher amount citric acid and converting into efficient PSB.



CHAPTER 6

Effect of coexpression of *A. niger oah* gene and *Fomitopsis plaustris FpOAR* gene on oxalic acid secretion in *Pseudomonas fluorescens* 13525

6.1: INTRODUCTION

Acid soils such as alfisols, aridisols, entisols and ultisols occupy about 30% of world's icefree land area (El Swaify et al., 1985; Von Uexull and Mutert, 1995). Of the total land area of the world, about 24.2% is considered potentially arable and most of this belongs to acidic soils. Thus, acid soils represent largest potential land area for future agricultural development. Plant growth and crop yield in alfisols is limited by many factors including, very low availability of P, high refixation of available P, Al toxicity and acidic properties of soil (Srivastava et al., 2006). In acidic alfisols P is mainly bound to Fe and Al oxides (Ae et al., 1990; 1991) which are difficult to dissolve by organic acid secretion mediated acidification due to strong complex formation. However, organic acids having good chelating properties are more effective in P release from alfisols (Srivastava et al., 2006). Phosphorus (P) is the major plant growth limiting nutrient in soil after nitrogen (N). Although, it is present in abundant amounts in soils as bound form, most soils are P deficient and contains very less (~ 10 µm) amount of free inorganic phosphate (Pi) (Arnou, 1953) due to high reactivity of soluble P with aluminum (Al), iron (Fe) and calcium (Ca). Al-P and Fe-P are the major bound form in acidic soils while Ca-P is the predominant form in alkaline vertisols (Sharpley et al., 1984). AlP and FeP could be solubilized by chelation but acidification does not result in P release from these complexes, however CaP complexes can be dissolved by acidification and release P. To enhance P nutrition to plants grown in acidic soils, direct application of rock phosphate (RP) as P fertilizer has been studied (Rajan et al., 1996). RP is rich in calcium phosphate (CaP) complexes and could be solubilized in acidic soils due to acidification. Earlier reports showed that use of partially acidulated rock phosphate

(PARP) in acid soils provides P nutrition (Marwah, 1983; Bolland., 1992; Butegwa, 1996; Abekoe, 1998). However, the amount of PR dissolved was very low due to many factors including dissolution of RP, its availability to plants and high refixation capacity of alfisols. Alternate approach to solubilize RP in alfisols is use of organic acid secreting microorganisms (Vassilev et al., 1996; Gadd, 1999). Low molecular weight organic acids are known to effectively chelate Fe and Al from ferric phosphate (FeP) and aluminium phosphate (AlP), thereby solubilising P (Bolan et al., 1994; Jones and Darrah, 1994; Kpomblekou and Tabatabai, 1994; Gilroy and Jones, 2000).

Chemical fertilizers are commonly used to supplement P for plants. However, very small amount of P is available for plants in alfisols due to low buffering capacity, acidic pH and efficient refixation (Kucey et al., 1989; Agbenin, 2003; Srivastava et al., 2006). Very few microorganisms are known to solubilize FeP and AlP (Kang et al., 2002; Srivastava et al., 2006). *E. asburiae* PSI3 known to solubilize P from alkaline vertisols and rock phosphate could not release free P from alfisols supplemented with rock phosphate (Srivastava et al., 2006). However, addition of 5-10 mM oxalic acid solubilizes P from rock phosphate in acidic alfisol which could be attributed to its excellent chelating property (Tabatbai et al., 1994; Srivastava et al., 2006; Liu et al., 2012). High amount of oxalic acid secretion is reported in fungi, including *Aspergillus niger*, *A. fumigatus*, *Botrytis cinerea*, *Fomitopsis plaustris* and *Penicillium* spp. (Khan et al., 2006). In fungus oxalic acid synthesis is governed by a cytoplasmic enzyme oxaloacetate acetyl hydrolase

(OAH), which breaks down oxaloacetate into oxalic and acetic acid (Lenz et al., 1976; Nielsen et al., 1999). In bacteria, oxalic acid biosynthesis is mediated by breakdown of glyoxalate, when flux toward glyoxalate pathway is high. Very few bacterial strains including *Bacillus subtilis*,

Arthrobacter sp. and *Micrococcus* spp. are known to secrete oxalic acid in very low amount (~ 2 mM) (Singh et al., 2009; Khan et al., 2006).

OAH of *A. niger* is a pH inducible enzyme belonging to PEP mutase/isocitrate lyase superfamily and requires divalent metal ions for catalysis (Lenz et al., 1976, Nielsen et al., 2000; Han et al., 2007). In bacteria such as *Oxalobacter formigens* oxalate specific transporter (OxlT) is responsible for oxalate uptake and helps in ATP generation (Fu and Maloney, 1997; Ye et al., 2001). On the other hand, high amount of oxalate secretion in fungi is mediated by efficient oxalate transporter (Watanabe et al., 2010). *Fomitopsis plaustris* is a wood rotting fungus and degradation of wood is mediated by oxalic acid secretion with the help of an oxalate transporter encoded by *FpOAR* gene (Watanabe et al., 2010).

Fluorescent pseudomonads are well-known plant-growth promoting rhizobacteria with root colonization and efficient biocontrol property (Rodrı´guez and Fraga, 1999; Haas and De´fago, 2005). Thus, incorporation of mineral phosphate solubilizing gene or operon in fluorescent pseudomonads could convert these organisms into efficient PSB.

Rationale of Present Study

Present study deals with the genetic modification of *Pseudomonas fluorescens* ATCC 13525 for oxalic acid secretion by incorporation of *A. niger oah* and *F. plaustris FpOAR* genes to study its effect on MPS ability in acidic alfisols.

6.2: EXPERIMENTAL DESIGN

6.2.1: Bacterial and fungal strains used in this study

Strains	Relevant Characteristics	Source/Reference
Bacterial Strains		

E. coli DH10B	Host strain for routine DNA manipulations	Invitrogen
P. fluorescens ATCC 13525	Wild type	MTCC, Chandigarh
Pf (pUCPM18G)	*P. fluorescens* 13525 with pUCPM18G plasmid; Amp^r, Gm^r	This study
Pf (pKCN2)	*P. fluorescens* 13525 with pKCN2 plasmid; Amp^r, Gm^r	This study
Pf (pKCN4)	*P. fluorescens* 13525 with pKCN4 plasmid; Amp^r, Gm^r	This study
Fungal Strains		
A. niger K85	Soil isolate	This study
Fomitopsis plaustris	Wood rotting fungi	MTCC, Chandigarh

Table 6.1: List of bacterial and fungal strains used in this study. The details of the plasmids and the concentration of the antibiotics used are given in the table 2.2 and 2.3. All the bacterial strains were grown at 30°C and fungal cultures were grown at 27°C.

6.2.2: Cloning of *A. niger oah* gene under *lac* promoter

oah expression in *A. niger* was induced by adding 2 M sodium carbonate to medium till the pH of medium goes to 8.0. After induction, when the medium pH was 6.5 (*oah* expression was maximum), total RNA was isolated from *A. niger* by TRizol method (Sigma Aldrich, India). First strand cDNA was synthesized by using First strand cDNA kit (Fermantas, India) by

following the instructions. *oah* gene was amplified from first strand cDNA using gene specific primers by *Taq* DNA polymerase (New England Biolabs, USA). Sequence of gene specific primers was: FP- 5'CAG GGATCC *CACGGA GGAATCAACTT*ATGAAAGTTGATACCCCCG3' and RP- 5'GTCGGGCCCCTGCAG TTAGACACCATTAGCAAACC 3'. Underline sequences shows *Bam*HI and *Pst*I

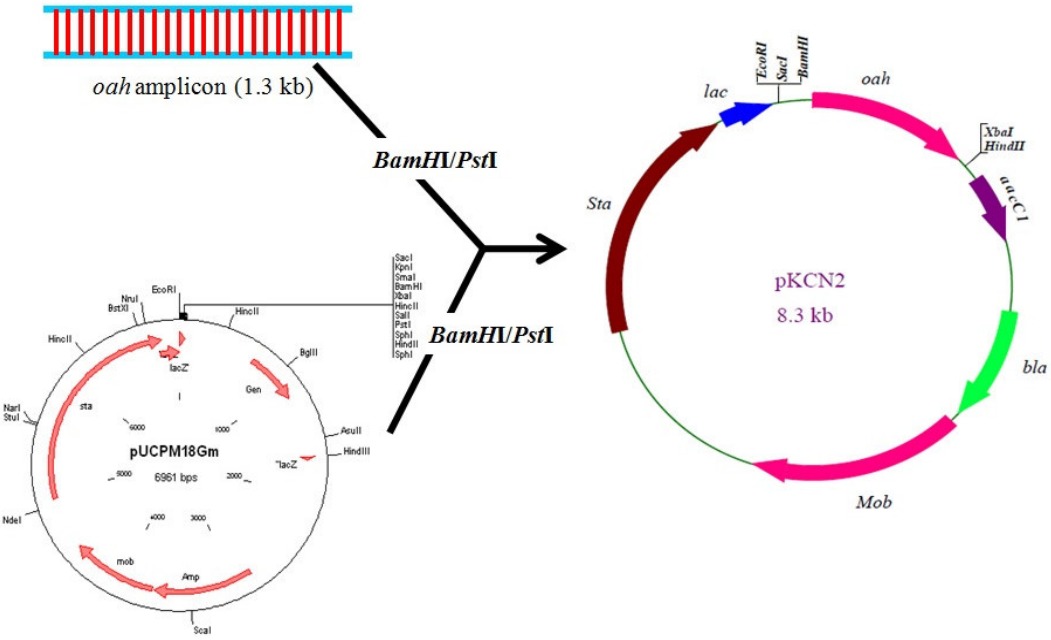


Fig. 6.1: Schematic representation of construction of pKCN2 plasmid containing *A. niger oah* gene under *lac* promoter.

restriction enzyme sites used for cloning and Ribosome binding sequence (RBS) is shown in italics. Amplicon of 1.3 kb size (*oah*) was digested with *Bam*HI/ *Pst*I, gel eluted, purified using Pure Link gel extraction kit (Invitrogen, USA) and ligated in *Bam*HI/ *Pst*I, gel eluted, purified plasmid pUCPM18Gen **(Fig. 6.1)**. Clone was confirmed by restriction digestion, PCR amplification and sequencing. Resultant construct of 8.2 kb, containing *oah* gene of *A. niger* under *lac* promoter in pUC based broad host range plasmid pUCPM18Gen, was designated as pKCN2.

6.2.3: Cloning of *F. plaustris FpOAR* and artificial oxalate operon

F. plaustris was grown in medium described by Watanabe et al, (2010) in dark conditions at room temperature without shaking. Total RNA was isolated from *F. plaustris* by TRizol method (Sigma Aldrich, India). First strand cDNA was synthesized by using First strand cDNA kit (Thermo Scientific, USA) by following the instructions. *FpOAR* gene was amplified from first strand cDNA by *Taq* DNA polymerase (NEB, USA) using gene specific primers. Sequence of gene specific primers was: FP- 5' CGAGCTCG*CACGGAGGAATCAACTT*ATGACCGACCTGCATCGA3' and RP- 5'CG GGATCCCGTCAGAGAAGATCTTCTTG3'. Underline sequences shows *Sac*I and *BamH*I restriction enzyme sites used for cloning and RBS sequence is shown in italics. Amplicon of 1.3 kb containing *FpOAR* gene was digested with *Sac*I/*BamH*I, gel eluted, purified and ligated in *Sac*I/*BamH*I digested, gel eluted, purified plasmid pUCPM18Gen **(Fig. 6.2)**. Clone was confirmed by restriction digestion, PCR amplification and sequencing. Resultant construct of 8.3 kb, containing *F. plaustris FpOAR* gene under *lac*

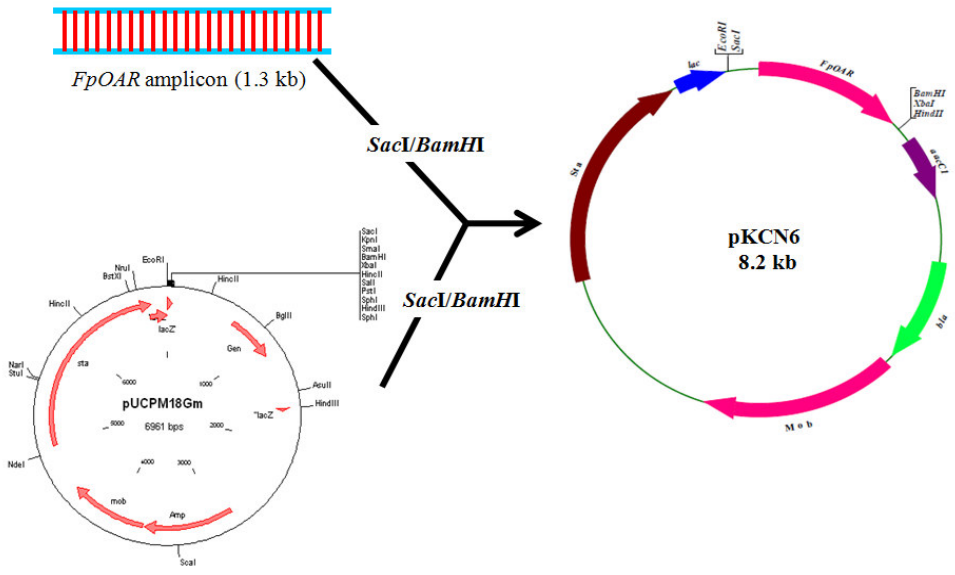


Fig. 6.2: Schematic representation of construction of pKCN6 plasmid containing *F. plaustris FpOAR* gene under *lac* promoter.

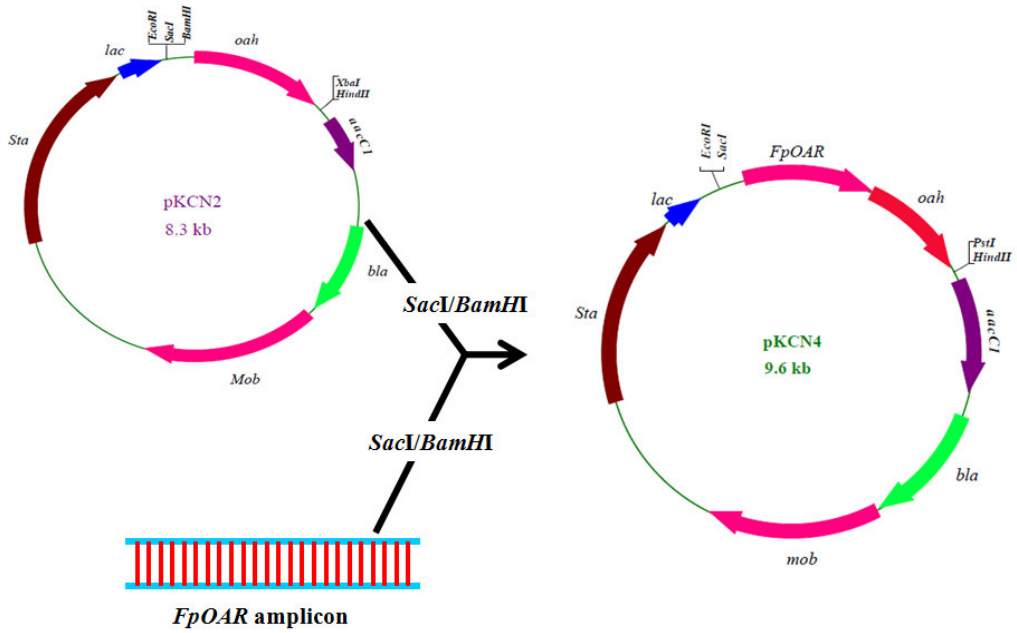


Fig. 6.3: Schematic representation of construction of pKCN4 plasmid.

promoter in broad host range plasmid pUCPM18G under *lac* promoter was designated as pKCN6. *FpOAR* gene amplicon digested with *Sac*I/*BamH*I, gel purified was ligated in *Sac*I/*BamH*I digested, gel eluted, purified plasmid pKCN2 **(Fig. 6.3)**. Resultant construct consisting of artificial oxalate operon, containing *F. plaustris FpOAR* and *A. niger oah* gene under *lac* promoter, was designated as pKCN4. Clone was confirmed by restriction digestion, PCR amplification and sequencing.

6.2.4: Identification of fungal culture and sequencing of *A. niger oah*

Aspergillus strain isolated from soil was identified by 18SrDNA partial sequencing (Chromous Biotech Pvt. Ltd., India). *A. niger oah* gene cloned in pKCN2 was confirmed by complete sequencing using forward (*lac*) and reverse (*oah* RP) primers (First Base Sequencing,

Malaysia) and this sequence was used to find homology with other genes in NCBI database by using blast.

6.2.5: Development of *P. fluorescens* ATCC 13525 harboring *A. niger oah* and artificial oxalate operon

The recombinant plasmids pKCN2, pKCN4 and pUCPM18G were transformed in *P. fluorescens* ATCC 13525 (Section 2.3.3.3). The transformants were selected on gentamycin plates and were confirmed by plasmid isolation and restriction digestion (Section 2.3).

6.2.6: Effect of heterologous overexpression of *A. niger oah* gene and artificial oxalate operon on MPS ability of *P. fluorescens* ATCC 13525

P-solubilizing ability of the *P. fluorescens* ATCC 13525 transformants was checked on Tris buffered RP-Methyl red (TRP) agar (Section 2.4). Cell suspension of *P. fluorescens* ATCC 13525 transformants was prepared (Section 2.6.1) and 5µl of it was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 30°C for 3-5 days. P solubilization was determined by monitoring the red zone of acidification on the TRP agar plates.

6.2.7: Effect of *oah* gene and artificial oxalate operon overexpression on physiology and organic acid secretion of *P. fluorescens* ATCC 13525

P. fluorescens transformants were grown on Tris-HCl medium and alfisol soil medium containing 100 mM glucose as carbon source (2.2.3; 2.2.4) and subjected for physiological experiments. The samples withdrawn at 24 h intervals were analyzed for $O.D._{600nm}$ and pH of the medium (Section 2.6). Stationary phase cultures harvested, when pH of medium was below 4.5, were subjected for Pi and organic acid estimation (Ames, 1964; Section 2.6.3). OAH assay was

done in late-log phase in M9 minimal medium containing 100 mM glucose (2.7.2.2; 2.2.2) as carbon source.

6.3: Results

6.3.1: Construction of *oah* gene and artificial oxalate operon under *lac* promoter

Total RNA was isolated from *A. niger* and integrity was checked on agarose gel **(Fig. 6.4)**. Based on the above mentioned strategies (**Fig. 6.1**; section 6.2.2) *A. niger oah* gene was cloned under *lac* promoter in pUC based broad host range plasmid pUCPM18G containing gentamycin resistance (*aacC1*) gene. Plasmid pKCN2 was confirmed by restriction digestion pattern **(Fig. 6.5a)**, PCR amplification **(Fig. 6.5b)** and sequencing.

F. paustris RNA was isolated and integrity was checked by agarose gel electrophoresis **(Fig. 6.6)**. *FpOAR* amplicon was confirmed by agarose gel electrophoresis and restriction

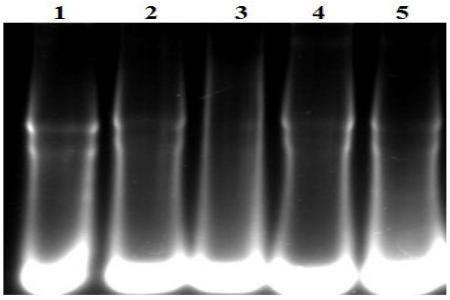
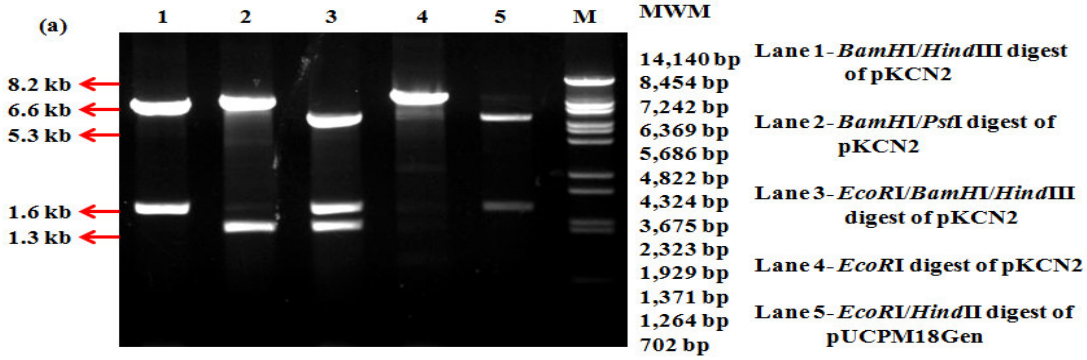


Fig. 6.4: Total RNA isolated from *A. niger*.



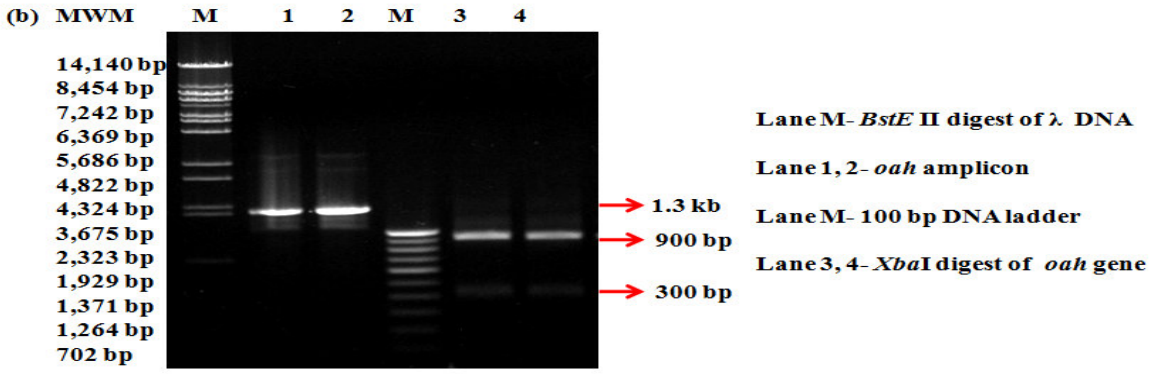


Fig. 6.5: (a) Restriction digestion pattern and (b) PCR confirmation of pKCN2.

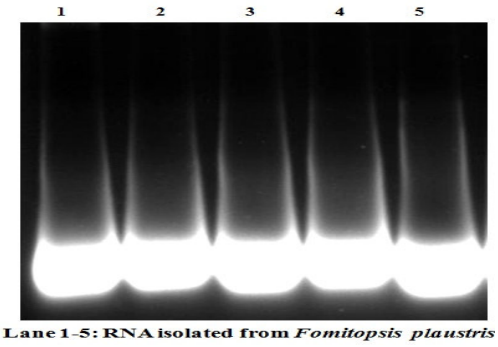


Fig. 6.6: Total RNA isolated from *F. palustris*.

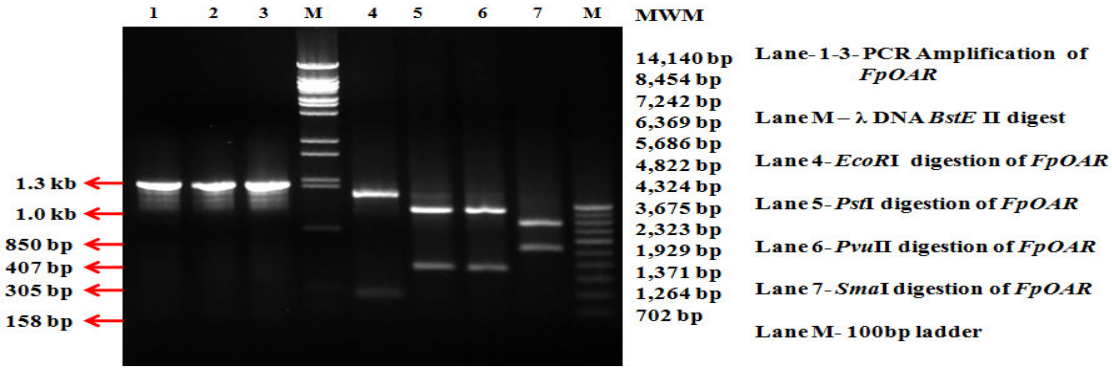


Fig. 6.7: PCR amplification and restriction digestion confirmation of *FpAOR*.

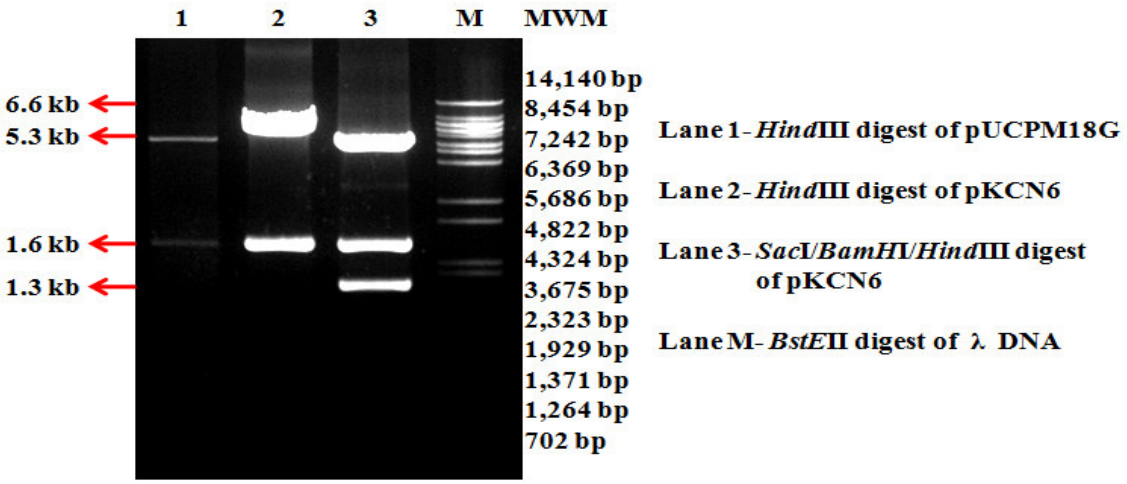


Fig. 6.8: Restriction digestion confirmation of pKCN6.

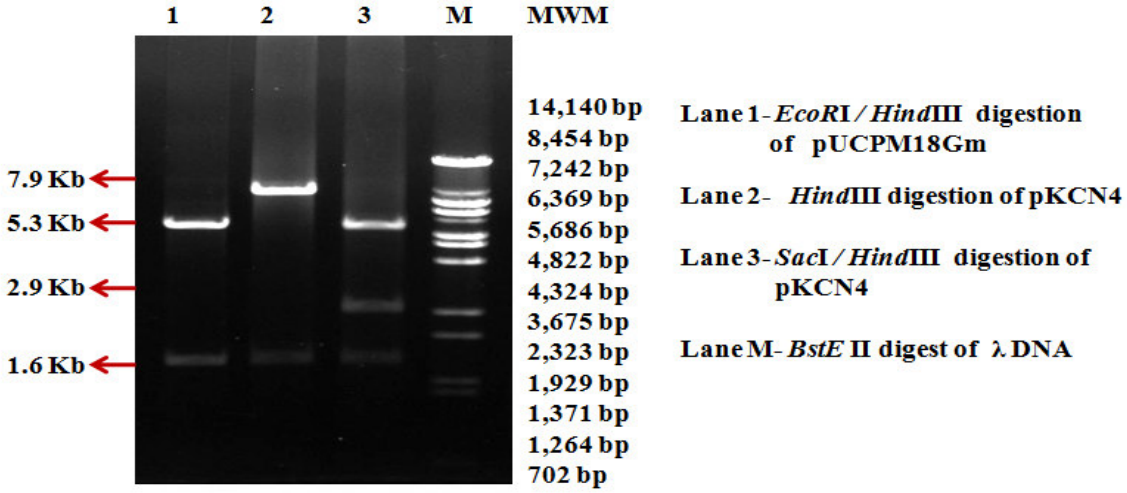


Fig. 6.9: Restriction digestion confirmation of pKCN4 plasmid.

enzyme digestion **(Fig. 6.7)**. *FpOAR* gene was cloned in pUCPM18G plasmid using the strategy mentioned in section 6.2.3 and Fig. 6.2. Plasmid pKCN6 was confirmed by restriction digestion pattern **(Fig. 6.8)**. Artificial oxalate operon was constructed, according to the strategy mentioned in **Fig. 6.3** (section 6.2.3), under *lac* promoter in pKCN2 plasmid. The resultant construct KCN4 was confirmed by restriction digestion **(Fig. 6.9)**, PCR amplification and sequencing.

6.3.2: Sequencing of *A. niger* and *oah* gene

Aspergillus strain was identified by 18S sequencing of culture. Genomic DNA was isolated from the sample and ~ 900 bp rDNA fragment was amplified using high –fidelity DNA polymerase. The PCR product was sequenced bi directionally. The sequence data was aligned and analyzed to identify the culture and its closest neighbors and the information is given in alignment view and distance matrix table **(Table 6.2)**. Alignment results show that the culture is closest to *A. niger* and *A. heteromorphus* and shows 98% homology.

Sequence of *oah* obtained from sequencing was used for nucleotide blast in NCBI for finding out the highly similar gene sequences and maximum identity (99%) was found with *A. niger oah* gene **(Table 6.3)**. Thus, the cloned *oah* gene was also confirmed by sequencing.

NCBI Accession No.	Organism Name	Score
JN048503.1	Aspergillus heteromorphus strain MTCC 5466	98%
JN938930.1	Aspergillus niger strain DAOM 221143	98%
JN938956.1	Aspergillus ochraceus strain DAOM 222007	96%
JF922028.1	Aspergillus restrictus strain CBS541.65	96%
JN938926.1	Aspergillus flavus strain DAOM 225949	95%
HQ825090.1	Aspergillus oryzae strain HK	95%
JN938934.1	Aspergillus parasiticus strain DAOM 225948	95%
JN938938.1	Aspergillus terreus strain DAOM 215379	95%
AY176752.1	Petromyces alliaceus strain ATCC 16891	95%
JF922041.1	Aspergillus candidus strain CBS567.65	95%

Table 6.2: Alignment view and distance matrix table of sequencing results. Score is sequence match score obtained based on nucleotide alignment.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB723988.1	Aspergillus niger oahA gene for oxaloacetate hydrolase, complete cds	2226	2226	94%	0.0	99%
AJ567910.1	Aspergillus niger oahA gene for oxaloacetate acetylhydrolase, exons 1-3	1520	1520	93%	0.0	89%
AM270216.1	Aspergillus niger contig An10c0050, genomic contig	1513	1513	93%	0.0	89%
DQ426539.1	Aspergillus niger strain CBS 513.88 oxaloacetate hydrolase (An10g00820), conserved hypothetical protein (An10g00830), and conserved hypothetical protein (An10g00840) genes, complete cds	1513	1513	93%	0.0	89%
XM_001402436.2	Aspergillus niger CBS 513.88 oxaloacetate acetylhydrolase, mRNA	870	1482	76%	0.0	95%
XM_744097.1	Aspergillus fumigatus Af293 carboxyphosphonoenolpyruvate phosphonomutase (AFUA_2G00540), partial mRNA	433	433	45%	2e-117	80%

Table 6.3: Alignment results of *oah* sequence for finding highly similar genes.

6.3.3: Effect of genetic modifications on OAH activity and growth of *Pf*13525

*Pf*13525 harboring pKCN2 and pKCN4 plasmids showed around 230 u/mg of OAH activity in M9 minimal medium containing 100 mM glucose **(Fig. 6.10)**.

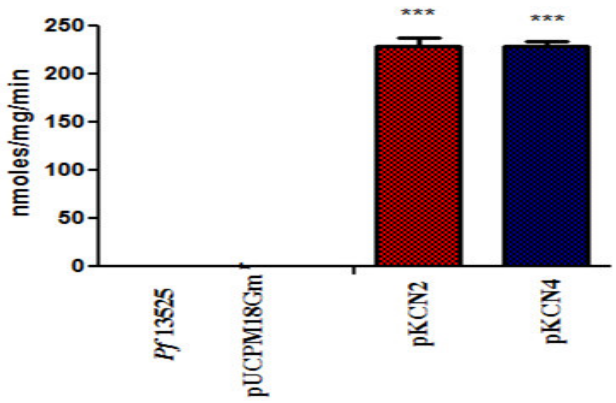
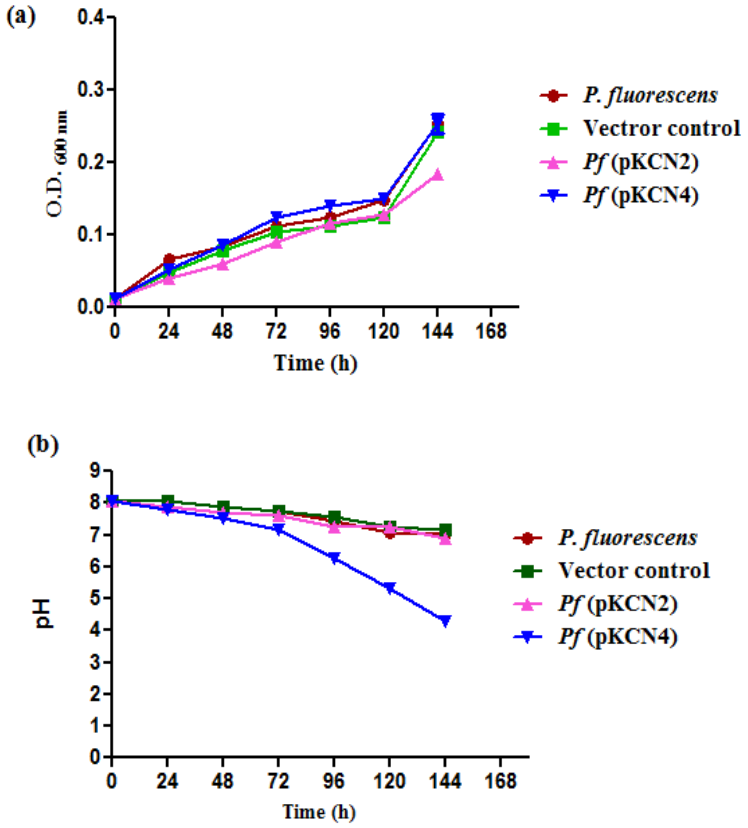


Fig. 6.10: *oah* activity of *Pf*13525 and transformants in M9 minimal medium containing 100 mM glucose as carbon source. The results are the mean ± SD of three independent observations. ***,$P < 0.0001$.

On the other hand, *Pf*13525 and plasmid control does not show *oah* activity due to the absence of *oah* gene. The growth of pKCN4 transformant of *Pf*13525 was not significantly different than the untransformed strain in 50 mM Tris-HCl (pH 8.0) medium containing 100 mM glucose as carbon source and RP as the sole P source **(Fig. 6.11a)**. However, the pKCN2 transformant grew somewhat slowly and reached 0.28 $O.D_{600nm}$ after 144 h. pKCN4 transformant was most effective in acidification of the medium from pH 8.0 to 4.2 while pKCN2 did not decrease the pH less than 7.0 **(Fig. 6.11b)**. Medium acidification by *Pf*13525 transfromants was also observed in alfisol.



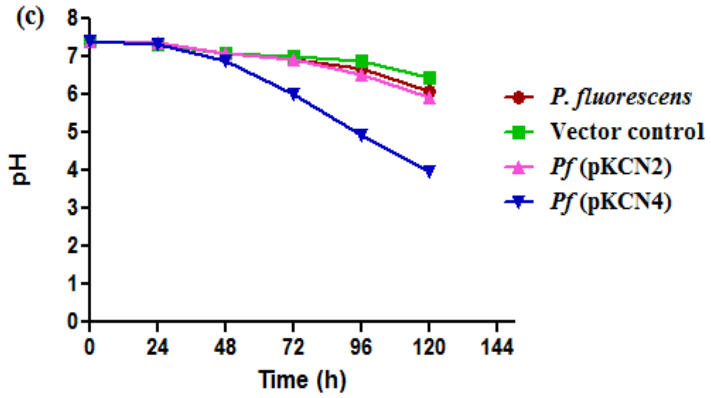


Fig. 6.11: Growth and pH profile of *Pf*13525 strains in TRP medium and alfisol soil containing 100 mM glucose as carbon source. (a) Growth profile **(b)** pH profile in TRP medium and **(c)** pH profile in alfisol soil medium. OD_{600} and pH values at each time point are represented as the mean ± SD of four to six independent observations.

In alfisol soil medium (Section 2.2.4) *Pf* (pKCN2) acidified the medium up to pH 5.9 in 120 h while *Pf* (pKCN4) transformant acidified the medium pH to 3.9. On the other hand, *Pf*13525 and vector control does not showed acidification below pH 6.0 in alfisol soil **(Fig. 6.11c)**.

6.3.4: Effect of genetic modifications on organic acid secretion by *Pf*13525

To study the effect of increased OAH activity on organic acid secretion in *Pf*13525 tranformants, the cell lysate and extracellular culture supernatant were analysed for oxalic acid levels using HPLC. pKCN2 transformant carrying the *oah* gene showed highest accumulation of oxalic acid intracellularly (up to 19.1 mM) and secreted relatively less amount (1.2 mM) in the medium **(Fig. 6.12a)**. On the other hand, pKCN4 transformant possessing artificial oxalate operon consisting of *oah* gene along with oxalate transporter accumulated only 3.6 mM oxalic acid and secreted 13.6 mM in the medium **(Fig. 6.12a)**. Oxalic acid was not detected in wild type *Pf*13525 and vector control transformant.

In order to study the organic acid secretion in soil conditions, alfisol soil supplemented with 100 mM glucose and 30 mg RP/g of soil was inoculated with genetically modified *Pf*13525 strains and the oxalate secreted in the soil solution was estimated. In agreement with the TRP medium studies, in alfisol pCNK4 secreted 15 mM of oxalate while pKCN2 transformant secreted 1.6 mM oxalate **(Fig. 6.12a)**. Wild type *Pf*13525 secreted 2.0 mM gluconic acid under similar growth conditions but does not show neither oxalate accumulation nor secretion. All genetic modifications resulted in a decrease in gluconic acid secretion **(Fig. 6.12b)**.

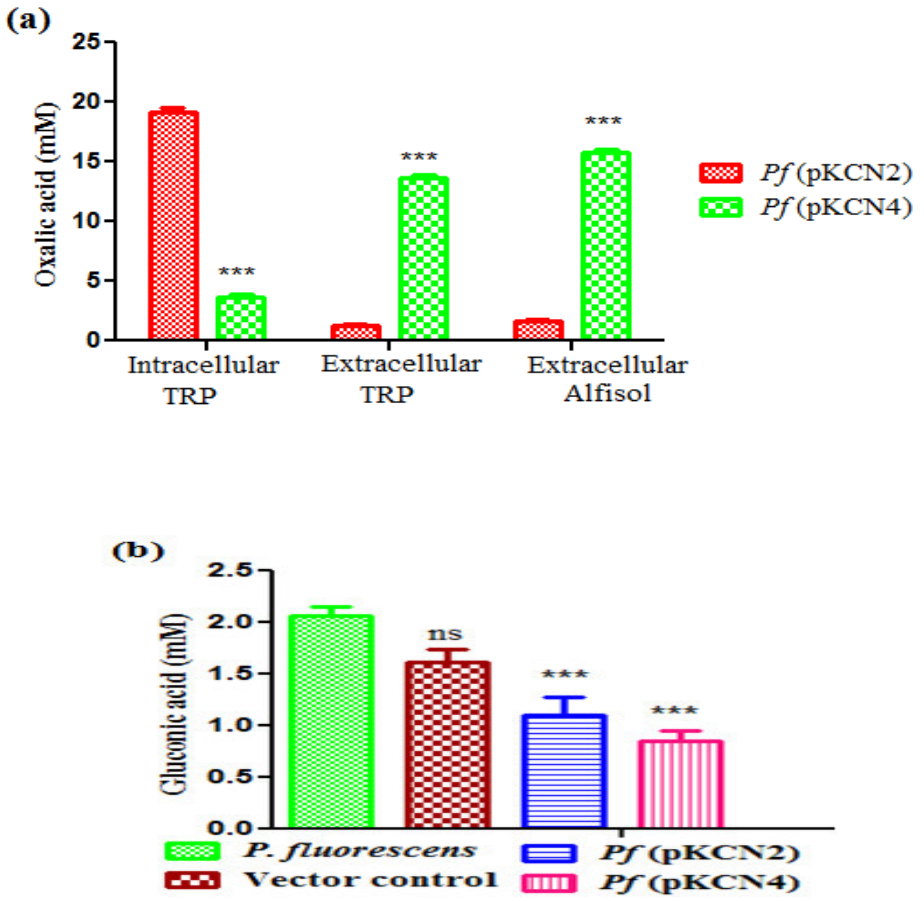


Fig. 6.12: Organic acid secretion by *Pf*13525 strains in alfisol soil and TRP minimal medium. The results are the mean ± SD of four to six independent observations. ***,P < 0.0001; ns, non-significant.

6.3.5: Effect of genetic modifications on the MPS ability of *Pf*13525

Further effect of organic acid mediated acidification of genetically modified *Pf*13525 strains was observed on TRP plates. *Pf*13525 and pKCN2 transformant did not show red zone of acidification while pKCN4 transformant acidified TRP agar plate containing 100 mM glucose as carbon source and 50 mM Tris HCl (pH 8.0) **(Fig. 6.13)**. Thus, after observing the medium acidification in flask studies and on TRP plates, P solubilization was checked in TRP and alfisol minimal medium. In TRP minimal medium, *Pf* (pKCN2) and *Pf* (pKCN4) released 62 µM and 217 µM of P from RP, respectively **(Fig. 6.14)**. In alfisol soil medium, containing 100 mM glucose as carbon source and supplemented with 30 mg RP/g of soil, *Pf* (pKCN2) and *Pf* (pKCN4) released 112 µM and 509 µM of P, respectively **(Fig. 6.14)**. P released was not observed in *Pf*13525 and vector control.

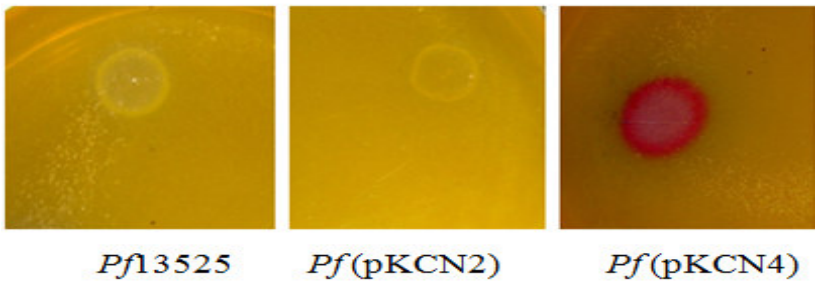


Fig. 6.13: MPS ability of *Pf*13525 strains on TRP agar plates containing 50 mM Tris-HCl and 100 mM glucose.

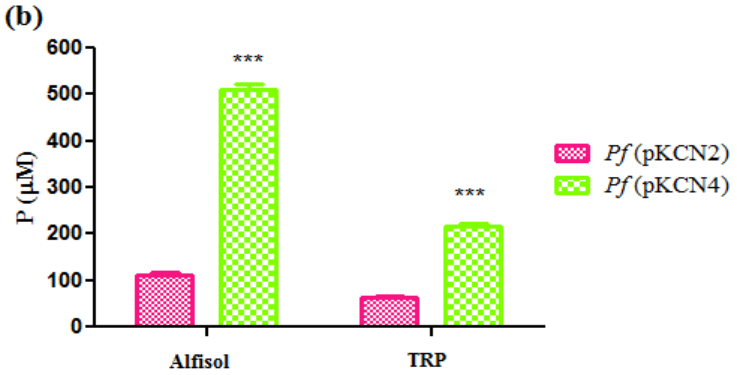


Fig. 6.14: P release by *Pf*13525 transformants in alfisol soil and TRP minimal medium containing 100 mM glucose as carbon source. The results are the mean ± SD of four to six independent observations. ***,P < 0.0001; ns, non-significant.

Discussion

Growth and yield of crops is low in alfisols due to low amount of available P, high P refixation capacity of soil, acidic pH and Al toxicity. Pi is strongly bound to Fe and Al oxides in alfisols which is difficult to solubilize even in the presence of strong organic acids. However, Pigeon pea shows improved growth as compared to other crops in alfisol due to piscidic acid mediated FeP solubilization (Ae et al., 1990). Only oxalic and citric acids solubilize RP in alfisol. Oxalic acid is most effective at 5 to 10 mM concentration plausibly hinders refixation of P by chelation of Fe and Al ions. Thus, addition of oxalate in different phosphate rocks and alfisol result in efficient mineral phosphate solubilization (Tabatbai et al., 1994; Srivastava et al., 2006; Liu et al., 2012). Fungus secretes oxalate in molar amounts while only few bacteria are known to secrete oxalate in very low amount. *Penicillium bilai* has efficient MPS ability and is known to secrete 10 mM of oxalic or citric acid under different conditions (Kuiack and Cunningham, 2003).

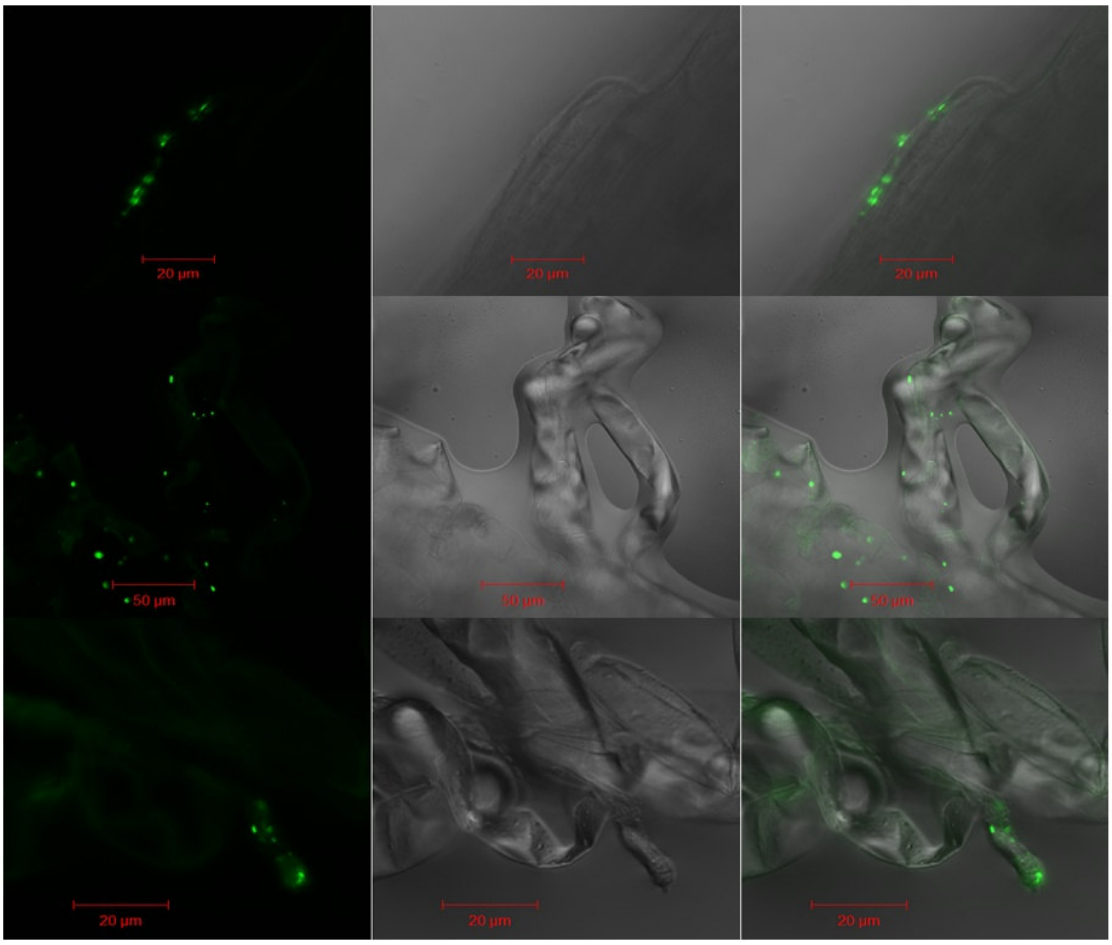
Oxalate biosynthesis in bacteria involves conversion of glyoxalate, the intermediate of glyoxylate shunt, to oxalate mediated by glyoxylate dehydrogenase (AGODH). Secretion of oxalic acid is not very clear but broad substrate range dicarboxylate transporter (DctA) could be responsible for the transport in bacteria. In *Oxaolobacter formigens,* oxalate uptake is mediated by oxalate-formate antiporter (Valentini et al., 2011). Thus, in bacteria oxalic acid biosynthesis is dependent on flux towards glyoxalate pathway. On the other hand, alternate pathway of *oah* mediated oxalate formation is expected to be preferred pathway in *Pf*13525, as flux towards

tricarboxylic acid is high as compared to glyoxalate shunt in *P. fluorescens* (Fuhrer et al., 2004). Oxalic acid conversion from glyoxalate involves multiple steps as against single step breakdown of oxaloacetate by OAH. Accordingly, observation of accumulation of high levels of oxalate upon incorporation of *oah* gene in *P. fluorescens* validates this perspective.

P. fluorescens ATCC 13525 transformant harboring *oah* gene secreted low amount of oxalic acid which could be mediated by dicarboxylate trasporter DctA. *P. fluorescens* strains contain two dicarboxylate transporters mainly implicated in the uptake of dicarboxylic acids (Valentini et al., 2011). DctA is a broad substrate range transporter while DctB is specific for succinate. Secretion of oxalic acid under Al toxicity conditions by *P. fluorescens* ATCC 13525 in presence of external Al-citrate (Appanna et al., 2003) suggests that these dicarboxylate transporters could export oxalic acid. However, *FpOAR* enhanced accumulated oxalate secretion in *P. fluorescens* ATCC 13525 transformant harboring artificial oxalate operon in the present work.

P. fluorescens ATCC 13525 transformant harboring oxalate operon exhibited MPS phenotype in TRP medium and in alfisol which is attributed to the oxalic acid secretion. This is supported by the fact that 5-10 mM oxalate is known to be sufficient to solubilize RP in alfisols (Srivastava et al., 2006). The oxalic acid secreted by the transformant is also expected to be effective in releasing P from alkaline vertisol which contain significant amount of CaP (Gyaneshwar et al., 1998a). Oxalic and citric acids have been shown to solubilize significant quantities of fixed P. Plants secreting piscidic, citric and oxalic acid in root exudates showed increased shoot P content (Bolan et al., 1994; Jones and Darrah, 1994; Khademi et al., 2010). Pigeon pea shows better growth and crop yields in alfisol as compared to other crops due to secretion of piscidic acid (Ae et al., 1990).

To summarize, in this study we reports genetic manipulation mediated secretion of oxalic acid in *Pf*13525 for mineral phosphate solubilization from acidic alfisol. *Pf*13525 harboring *oah* gene result in accumulation of higher amount of oxalic acid and incorporation of oxalate operon, containing additional oxalate transporter, secreted the intracellular oxalate in the medium which in turn incorporate MPS ability in the organism. Potential of mineral phosphate solubilization of oxalic acid by rhizobacteria could also help plants to overcome Al toxicity.



CHAPTER 7

Integration of artificial oxalate operon and artificial oxalate gene cluster in *Pseudomonas fluorescens* 13525 and its effect on MPS ability.

7.1: INTRODUCTION

Fluorescent pseudomonads are well-known plant-growth promoting rhizobacteria with high biocontrol efficacy as well as phosphate-solubilizing ability (Rodrı´guez and Fraga, 1999; Haas and De´fago, 2005). Pseudomonads utilize glucose by the periplasmic direct oxidation pathway mediated by pyrroloquinoline-quinone-dependent glucose dehydrogenase (PQQ-GDH) and intracellular phosphorylative oxidation mediated by glucokinase and glucose-6-phosphate dehydrogenase (G-6-PDH) (Lessie and Phibbs, 1984). Other metabolic advantages include high carbon flux through the TCA cycle resulting in lower acetate overflow and increased capacity to produce OAA due to the presence of pyruvate carboxylase (PYC) and/or phosphoenolpyruvate carboxylase (PPC) at the anaplerotic node and absence of glucose-mediated catabolite repression (Lessie and Phibbs, 1984; Stover et al., 2000; Nelson et al., 2002; Basu et al., 2006). Plant growth promoting rhizobacteria (PGPR) having MPS ability can help in better growth and crop improvement.

Incorporation of artificial oxalate operon mediated oxalic acid secretion imparts MPS ability in *Pf*13525 (Chapter 6). However, plasmids are not very stable in natural soil isolates and presence of antibiotic marker also impose problem in field conditions. Presence of plasmid affects the host metabolism adversely which result in different metabolic perturbations including alterations of several metabolic pathways in *E. coli* depending on the growth conditions due the presence of colE1 based plasmid (Wang et al. 2006), impaired growth, phosphate solubilization, nitrogen fixation, siderophore production and indole acetic acid biosynthesis, under various conditions in *E. asburiae* PSI3, *Azotobacter vinelandii*, *Azospirillum brasilense*, respectively, (Glick 1995; Sharma et al. 2010), variable stability in *P. fluorescens* WCS365 under rhizospheric conditions and reduction in competitiveness in *Pseudomonas putida* GR12-2 (Schmidt-Eisenlohr

et al., 2003; Simons et al. 1996). Previous reports from our laboratory shows that genetic engineering of bacteria by overexpression of gene in plasmids exerted greater metabolic alterations on host metabolism despite of low copy number (Buch et al., 2010). Thus to minimize the plasmid load, instability and antibiotic marker free genetic manipulation all the genes were constructed on integration vector as an artificial operon and artificial gene cluster. Artificial oxalate operon and artificial oxalate gene cluster were integrated into the genome of *P. fluorescens* ATCC 13525 by Tn7 based integration system at *att* site.

Oxygen is present in limited amounts in the rhizosphere which could limit the colonization and survival of rhizobacteria (Ramírez et al., 1999). The obligate aerobic bacterium, *Vitreoscilla*, synthesizes elevated quantities of homodimeric hemoglobin (VHb) under hypoxic growth conditions which allows improved growth under microaerobic conditions when dissolved oxygen is less than 2% of air saturation (Khosla and Bailey, 1988a; 1988b). Expression of *vgb* gene encoding VHb protein in heterologous hosts often enhances growth and metabolism by facilitating oxygen transfer to the respiratory membranes (Stark et al., 2011). Beneficial effect of *vgb* overexpression for improved bacterial growth has been demonstrated in plant associated bacteria (Ramírez et al., 1999). Thus, incorporation of *vgb* gene in genetically modified rhizobacteria harboring MPS ability could help the organism to survive in soil conditions and improvement in recombinant protein production.

Rationale of Present Study

Present study deals with the genetic modification of *P. fluorescens* ATCC 13525 for oxalic acid secretion by integration of *A. niger oah* and *F. plaustris FpOAR* genes to study its effect on MPS ability and growth promotion of *Vigna radiata* in acidic alfisols. Additionally,

incorporation of *vgb* gene along with artificial oxalate operon could help in better survival and colonization of organism in soil environment.

7.2: EXPERIMENTAL DESIGN

7.2.1: Bacterial strains used in this study

Strains	Relevant Characteristics	Source/Reference
Bacterial Strains		
P. fluorescens 13525	Wild type	MTCC, Chandigarh
Pf (pUCPM18G)	*P. fluorescens* 13525 with pUCPM18 plasmid; Amp^r, Gm^r	Chapter 6
Pf (pKCN2)	*P. fluorescens* 13525 with pKCN2 plasmid; Amp^r, Gm^r	Chapter 6
Pf (pKCN4)	*P. fluorescens* 13525 with pKCN4 plasmid; Amp^r, Gm^r	Chapter 6
Pf int 3	Genomic integrant of *P. fluorescens* containing *egfp*; Amp^r	This study
Pf int 1	Genomic integrant of *P. fluorescens* containing *lac-FpOAR-oah*; Amp^r	This study
Pf int 2	Genomic integrant of *P. fluorescens* containing *lac-FpOAR-oah, vgb, egfp*; Amp^r	This study

Table 7.1: List of bacterial strains used in this study. The details of the plasmids and the concentration of the antibiotics used are given in the table 2.2 and 2.3. All the bacterial strains were grown at 30°C.

7.2.2: Cloning of artificial oxalate operon in integration vector

Artificial oxalate operon containing *F. palustris FpOAR* and *A. niger oah* gene under *lac* promoter was PCR amplified using XT-20 polymerase (Bangalore Genei, India) from plasmid pKCN4. Forward (*lac*) primer: TTTACACTTTATGCTTCCGG CTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAG CG and reverse primer of *oah* gene (Chapter 6; section 6.2.2) was used to amplify artificial oxalate operon. Plasmid pUC18T-mini-Tn7T-Gm-*eyfp* was digested with *Sma*I, gel purified and ligated with 2.9 kb amplicon containing artificial oxalate operon **(Fig. 7.1)**.

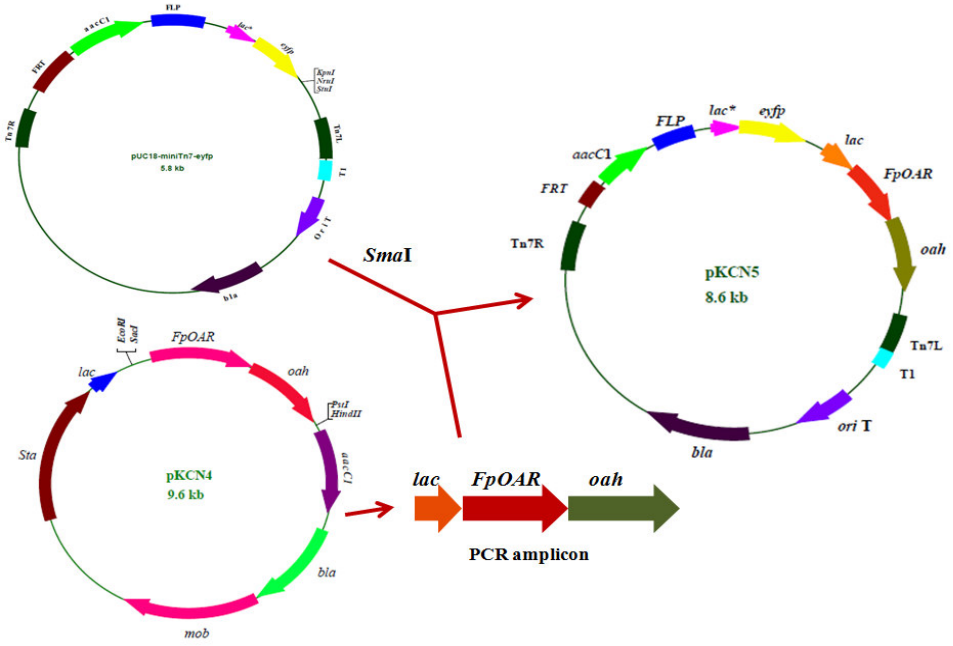


Fig. 7.1: Schematic representation of construction of pKCN5 plasmid containing *F. plaustris FpOAR* and *A. niger oah* gene under *lac* promoter.

Clone was confirmed by restriction digestion and PCR amplification. Resultant construct of 8.6 kb consisting of artificial oxalate operon, containing *F. plaustris FpOAR* and *A. niger oah* gene under *lac* promoter in Mini- Tn7 delivery vector, was designated as pKCN5.

7.2.3: Cloning of artificial oxalate gene cluster in integration vector

Plasmid pUCVHb-*egfp* was digested with *Pvu*II to obtain 3.2 kb insert containing *vgb* and *egfp* genes. Insert was cloned in pKCN5 digested with *Nhe*I and end filled using Klenow fragment (Thermo Scientific, USA).

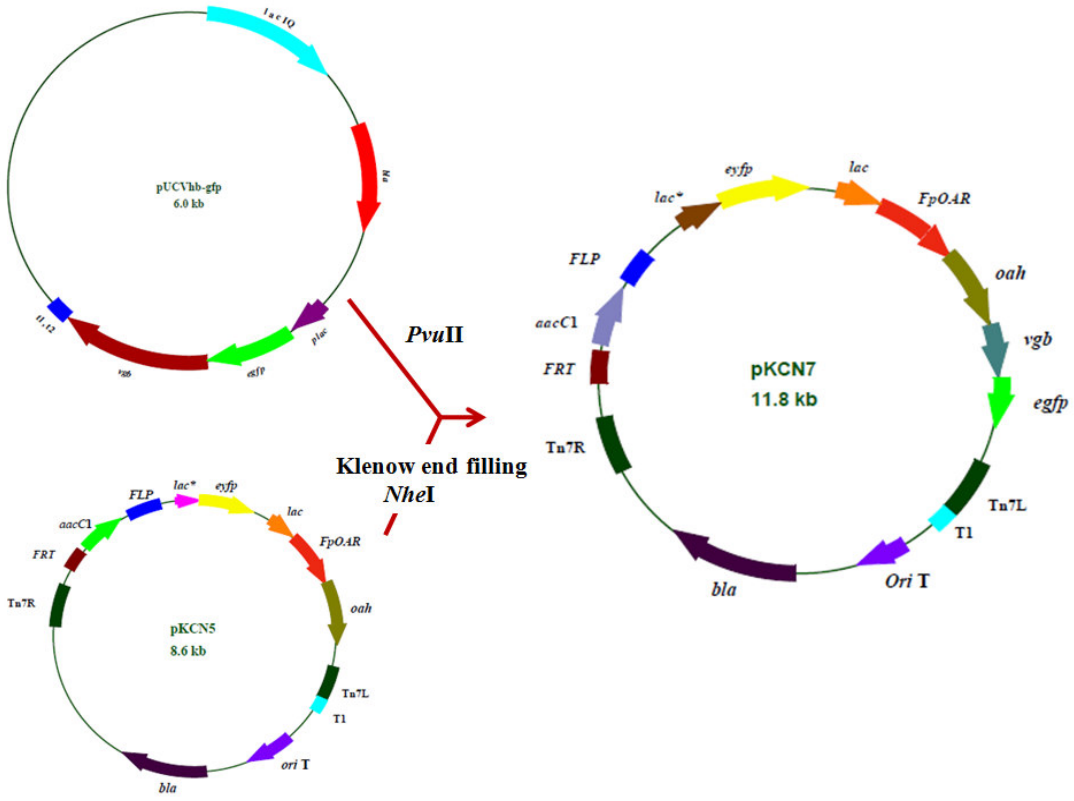


Fig. 7.2:

Schematic representation of construction of pKCN7 plasmid.

The resultant construct (artificial oxalate gene cluster) containing *F. plaustris FpOAR* and *A. niger oah* genes under *lac* promoter, *vgb* gene under its natural oxygen sensitive promoter and *egfp* under *rrnB* promoter in pUC18T-mini-Tn7T-Gm-eyfp vector, was designated as pKCN7 (**Fig. 7.2**). Clone was confirmed by restriction digestion, PCR amplification and fluorescence detection of GFP.

7.2.4: Integration of artificial oxalate operon and artificial oxalate gene cluster in genome of *P. fluorescens* ATCC 13525

Artificial oxalate operon and artificial gene cluster cloned in Mini- Tn7 delivery vector, pKCN5 and pKCN7, respectively, were integrated into the genome of *P. fluorescens* by transformation method (Choi and Schweizer, 2006). The integrants were selected on gentamycin containing Koser's citrate agar plates. Integration confirmation was done by PCR amplification of genes (section 2.3.4).

7.2.5: Effect of genomic integrants on MPS ability of *P. fluorescens* ATCC 13525

P-solubilizing ability of the *P. fluorescens* ATCC 13525 integrants was checked on Tris buffered RP-Methyl red (TRP) agar plates (Section 2.4). Cell suspension of *P. fluorescens* ATCC 13525 transformants was prepared (Section 2.6.1) and 5µl of it was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 30°C for 3-5 days. P solubilization was determined by monitoring the red zone of acidification on the TRP agar plates.

7.2.6: Effect of genomic integrants of artificial oxalate operon and artificial oxalate gene cluster on physiology and organic acid secretion of *P. fluorescens* ATCC 13525

P. fluorescens integrants were grown on Tris-HCl medium and alfisol soil medium containing 100 mM glucose as carbon source (Section 2.2.3; 2.2.4) and subjected for physiological experiments. The samples withdrawn at 24h intervals were analyzed for $O.D._{600nm}$ and pH of the medium (Section 2.6). Stationary phase cultures harvested, when pH of medium was below 4.5, were subjected for Pi and organic acid estimation (Ames, 1964; Section 2.6.3). OAH assay was done in late-log phase in M9 minimal medium containing 100 mM glucose (Section 2.7.2.2; 2.2.2) as carbon source.

7.2.7: Plant experiments

Plant studies were done in MS medium as well as in Alfisol soil (10 mg RP/g of soil). Soil analysis was done from Pulse Research Station (Anand Agriculture University, Vadodara) and was found to contain 0.085% organic carbon, 165.1 kg/ha total nitrogen, 262.4 kg/ha available K and 17.9 kg/ha available P. *Vigna radiata* (mung bean) seeds were germinated in aseptic conditions and plant inoculation studies were carried out in pots containing non-sterile acidic alfisol soil. Mung bean (*Vigna radiata*) seeds were surface sterilized with 0.1% $HgCl_2$ solution, followed by 70% ethanol wash and germinated in sterile distilled water under aseptic conditions. Overnight 50 ml LB grown cultures of *P. fluorescens* wild type and integrants were washed thrice with normal saline and dissolved in 5 ml saline. Mung bean seeds were soaked in bacterial culture for 30 min. Uninoculated control seeds were treated with sterile saline. 20 to 30 grown seeds were inoculated in a single pot. Three sets of (9 pots) each wild type, vector control and integrants were grown under natural light at ambient temperature for 15 d after which plants were uprooted, shoots and roots were dried at 65^{o}C up to constant dry weight measurement. Dried shoots and roots (n=6-8) were processed for ashing in muffle furnace at 550° C for 15 h and ashes were dissolved in 0.9 M H_2SO_4 at 10 mg dry weight per ml acid (Richardson et al., 2001). P contents were estimated by molybdate- blue method (Murphy and Reilay, 1962). Root colonization study of *P. fluorescens* integrant was done in MS medium (hydroponic study) and non sterile alfisol soil supplemented with 10 mg RP/g of soil. Plants were uprooted on 5th and 10th day after inoculation and different sections of root were observed for bacterial colonization by using Confocal laser scanning microscopy (LSM 700 Carl Zeiss, GmbH).

7.2.8: Data analysis

Physiological experiments were done in three – four independent experiments for batch culture studies and data are expressed in mean with standard deviation. In plant experiments,

three independent triplicate studies were performed. Differences in mean values were determined using general analysis of varience (ANOVA) and linear regression analysis was done using GraphPad Prism5.0.

7.3: Results

7.3.1: Construction of artificial oxalate operon in integration vector

Based on the above mentioned strategies (**Fig. 7.1**; section 7.2.2) artificial oxalate operon, containing *F. plaustris FpOAR* and *A. niger oah* gene under *lac* promoter, was cloned in Mini- Tn7 delivery vector (pUC18T-mini-Tn7T-Gm-*eyfp*) having gentamycin resistance (*aacC1*) gene. Plasmid pKCN5 was confirmed by restriction digestion pattern **(Fig. 7.3)**.

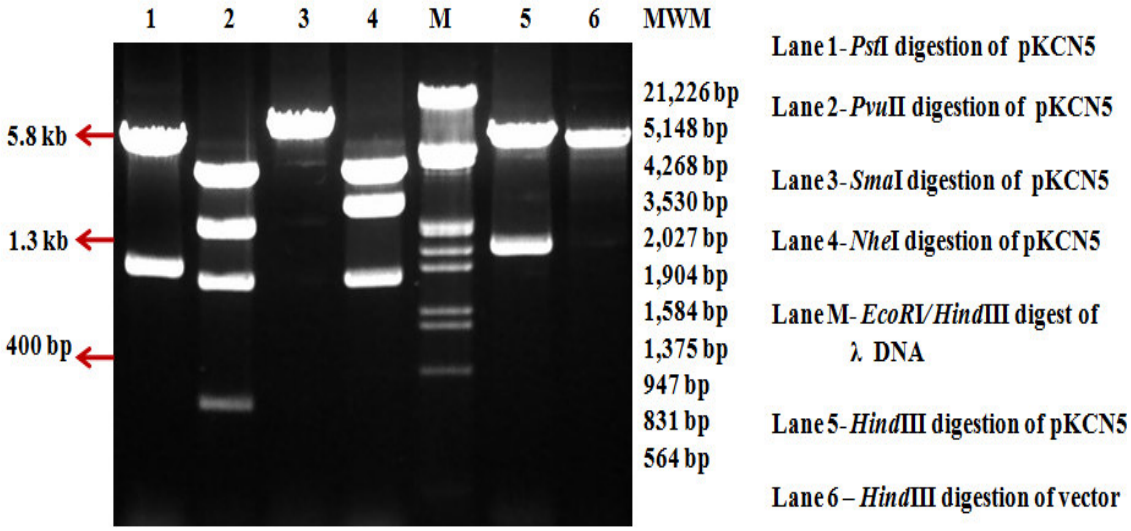


Fig. 7.3: Restriction digestion pattern of pKCN5.

7.3.2: Construction of artificial oxalate gene cluster in integration vector

Artificial oxalate gene cluster contains *lac-FpAOR-oah*, *vgb*, *egfp* genes. Based on the above mentioned strategies (**Fig. 7.2**; section 7.2.3) artificial oxalate gene cluster was cloned in Mini- Tn7 delivery vector (pUC18T-mini-Tn7T-Gm-*eyfp*) having

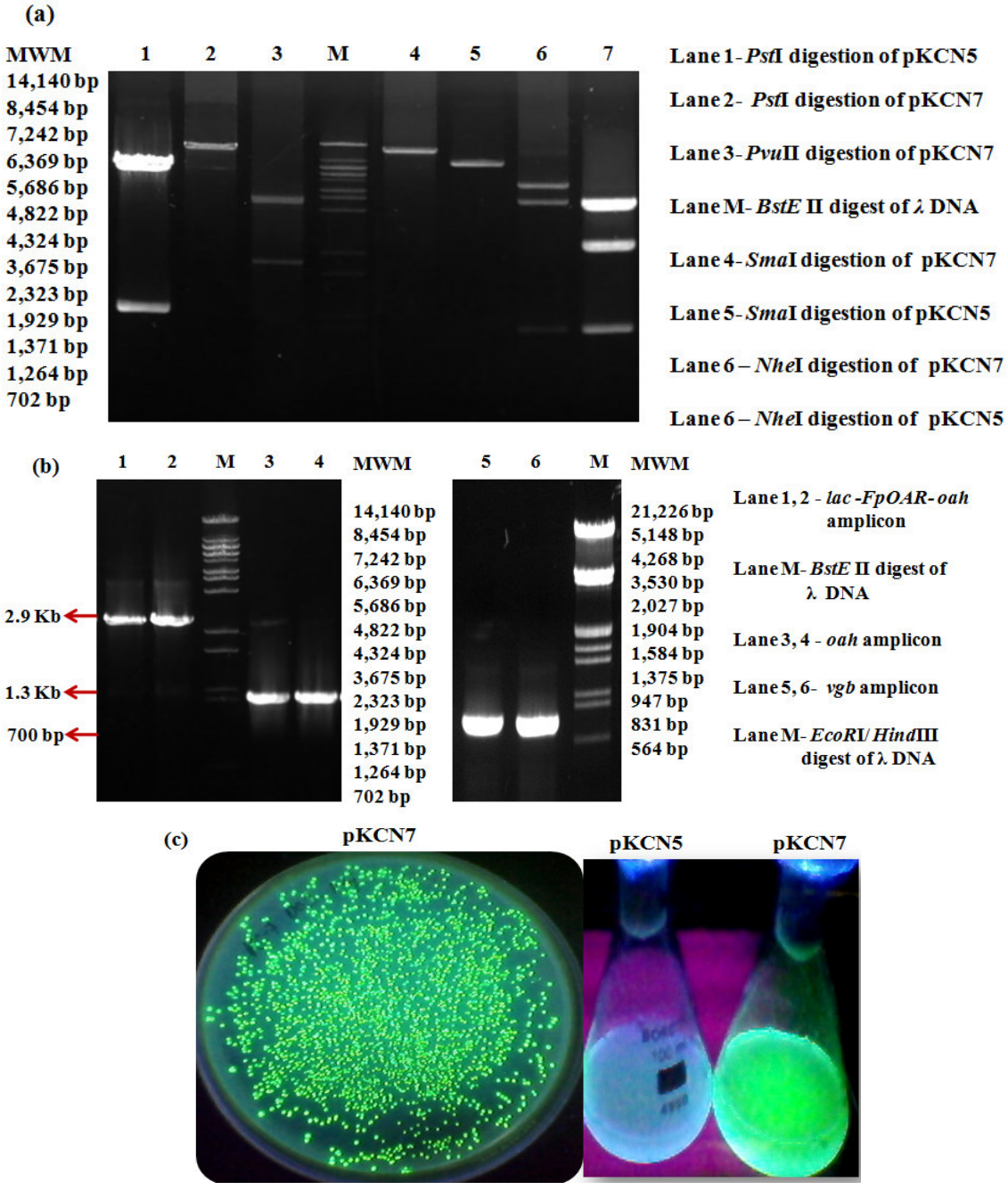


Fig. 7.4: (a) Restriction digestion pattern, (b) PCR amplification and (c)

fluorescence confirmation of pKCN7.

gentamycin resistance (*aacC1*) gene. Plasmid pKCN7 was confirmed by restriction digestion pattern, PCR amplification and fluorescence of GFP **(Fig. 7.4 a; b and c)**.

7.3.3: Effect of genetic modifications on OAH activity, growth and medium acidification of *Pf*13525

The integrants *Pf* int1 and *Pf* int2 showed about 165 U/mg of OAH activity while *Pf*13525 harboring pKCN2 and pKCN4 plasmids showed around 230 u/mg of OAH activity in M9 minimal medium containing 100 mM glucose **(Fig. 7.5)**. On the other hand, *Pf*13525 and plasmid control does not show detectable amount of *oah* activity as the gene is absent in the organism.

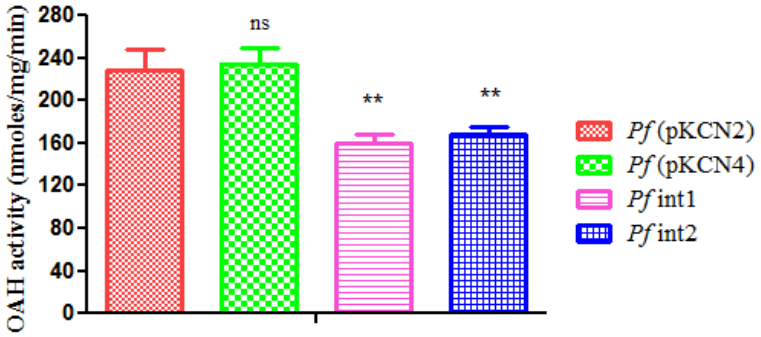


Fig. 7.5: OAH activity of *Pf*13525 transformants and integrants in M9 minimal medium. The results are the mean ± SD of three independent observations. **,P < 0.0067; ns, non-significant.

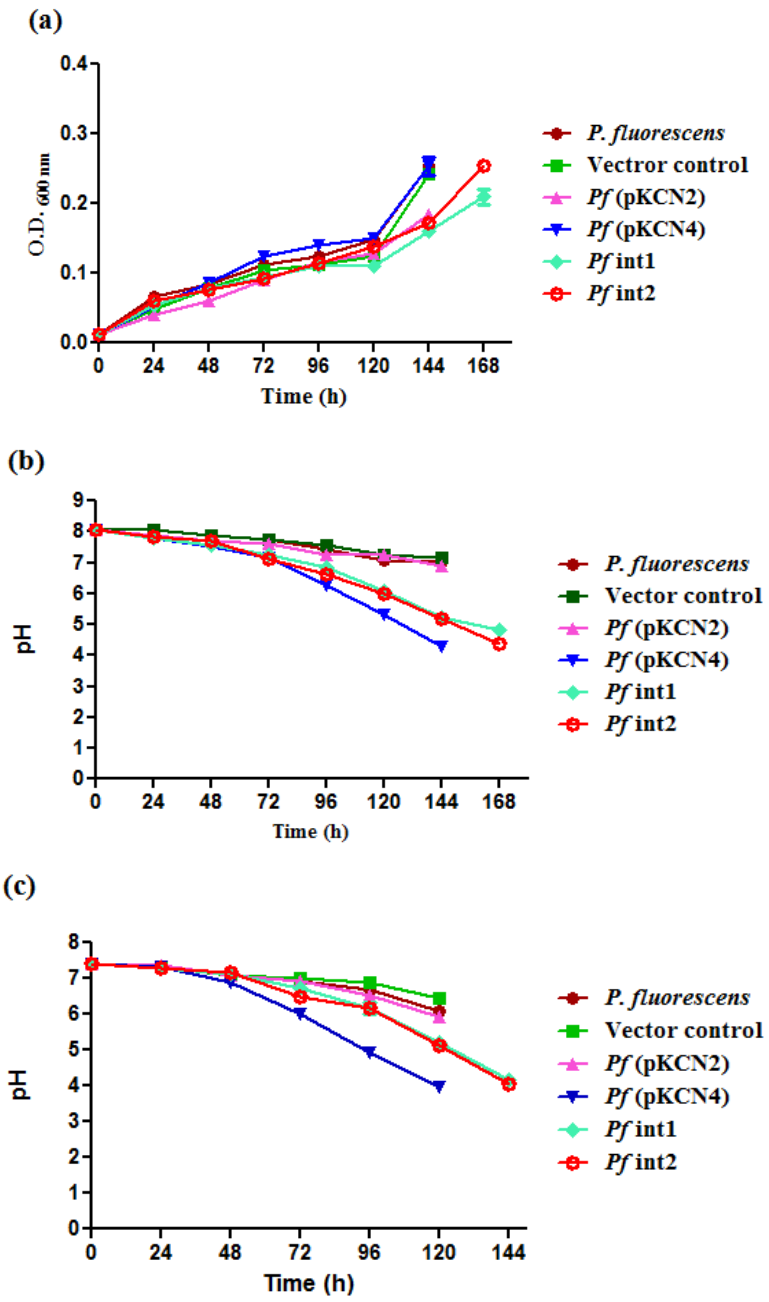


Fig. 7.6: Growth and pH profile of *Pf*13525 strains in TRP medium and alfisol soil containing 100 mM glucose as carbon source (a) Growth profile and (b) pH profile in TRP medium (c) pH profile in alfisol soil medium. OD_{600} and pH values at each time point are represented as the mean ± SD of four to six independent observations.

The integrants and pKCN2 transformant grew somewhat slowly and reached 0.28 $O.D_{600nm}$ after 168 h in 50 mM Tris-HCl (pH 8.0) medium containing 100 mM glucose as carbon source and RP as the sole P source **(Fig. 7.6a)**. The growth of pKCN4 transformant of *Pf*13525 was not significantly different than the untransformed strain. Although, integrants growth was slower as compared to the pKCN4 transformant still they were effective at reducing the pH of the medium till 4.3 **(Fig. 7.6b)** indicating organic acid secretion. pKCN4 transformant was most effective in acidification of the medium from pH 8.0 to 4.2 while pKCN2 transformant did not decrease the pH less than 7.0 **(Fig. 7.6b)**.

Medium acidification by *Pf*13525 integrants and transformants was also observed in alfisol. In alfisol soil medium (Section 2.2.4) *Pf* int1 and *Pf* int2 acidified the medium pH to 4.0 in 144 h. *Pf* (pKCN2) acidified the medium up to pH 5.9 in 120 h while *Pf* (pKCN4) transformant acidified the medium pH to 3.9. On the other hand, *Pf*13525 and vector control does not showed acidification below pH 6.0 in alfisol soil **(Fig. 7.6c)**.

7.3.4: Effect of genetic modifications on organic acid secretion by *Pf*13525

To study the effect of increased OAH activity on organic acid secretion in *Pf*13525 tranformants, the cell lysate and extracellular culture supernatant were analysed for oxalic acid levels using HPLC. Genomic integrants of *Pf*13525, int1 and int2 secreted 4.1 and 4.7 mM of oxalic acid, respectively, while intracellular levels were 2.6 and 3.1 mM, respectively **(Fig. 7.7a)**. pKCN2 transformant carrying the *oah* gene showed highest accumulation of oxalic acid intracellularly (up to 19.1 mM) and secreted relatively less amount (1.2 mM) in the medium **(Fig. 7.7a)**. On the other hand, pKCN4 transformant accumulated only 3.6 mM oxalic acid and secreted 13.6 mM in the medium.

(a)

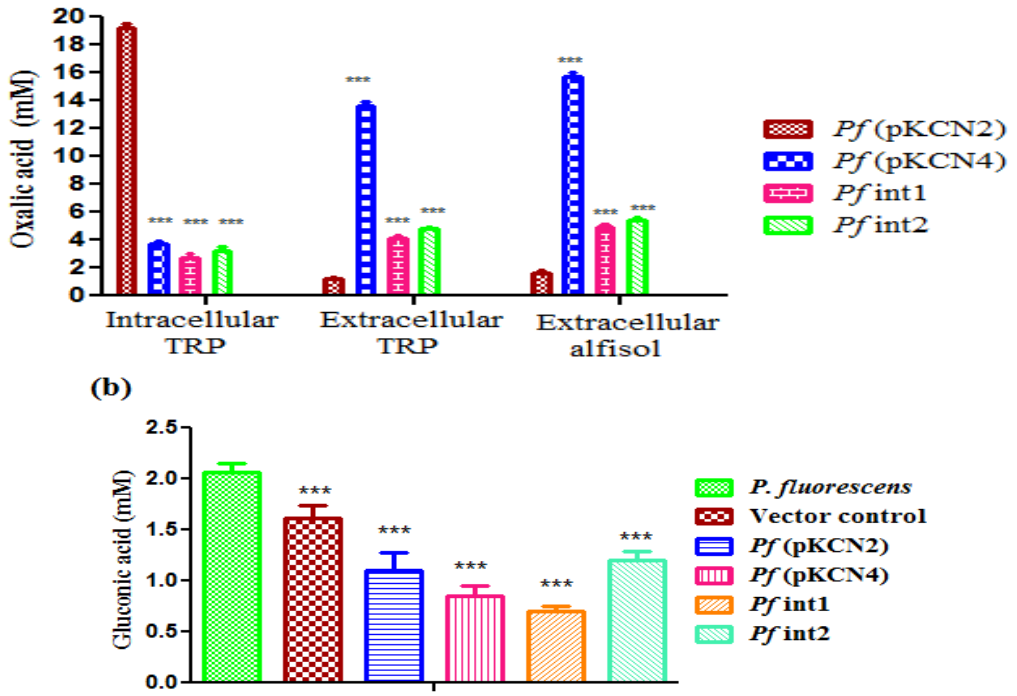


Fig. 7.7: Organic acid secreted by *Pf*13525 strains in TRP and alfisol medium- Effect of genetic modification on organic acid secretion by *Pf*13525 integrants and transformants in TRP medium (50 mM Tris- HCl) and alfisol soil containing 100 mM glucose as carbon source. The results are the mean ± SD of four to six independent observations. ***, P< 0.0001; ns, non-significant.

In order to study the organic acid secretion in soil conditions, alfisol soil supplemented with 100 mM glucose and 30 mg RP/g of soil was inoculated with different genetically modified *Pf*13525 strains and the oxalate secreted in the soil solution was estimated. In alfisol soil *Pf* int1 and *Pf* int2 secreted 4.8 and 5.4 mM of oxalate while pCNK4 secreted 15 mM of oxalate **(Fig. 7.7a)**. Thus oxalate secretion by integrants in alfisol was comparable to that on TRP medium. Wild type *Pf*13525 secreted 2.0 mM gluconic acid under similar growth conditions but does not

show neither oxalate accumulation nor secretion. All genetic modifications resulted in a decrease in gluconic acid secretion **(Fig. 7.7b)**.

7.3.4: Effect of genetic modifications on the MPS ability of *Pf*13525

Further effect of organic acid mediated acidification by genetically modified *Pf*13525 strains was observed on TRP plates. *Pf* int1, *Pf* int 2 and pKCN4 transformant

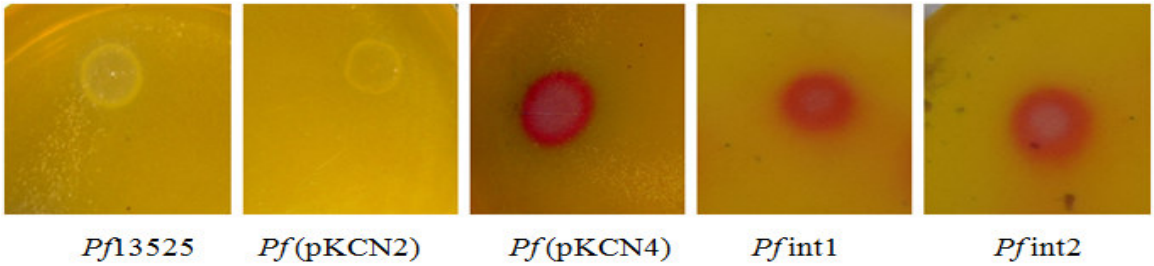


Fig. 7.8: MPS ability of *Pf*13525 strains on TRP agar plates containing 50 mM Tris-HCl and 100 mM glucose.

acidified TRP agar plates containing 100 mM glucose as carbon source and 50 mM Tris HCl (pH 8.0) while *Pf*13525 and pKCN2 transformant did not show red zone of acidification **(Fig.7.8)**.

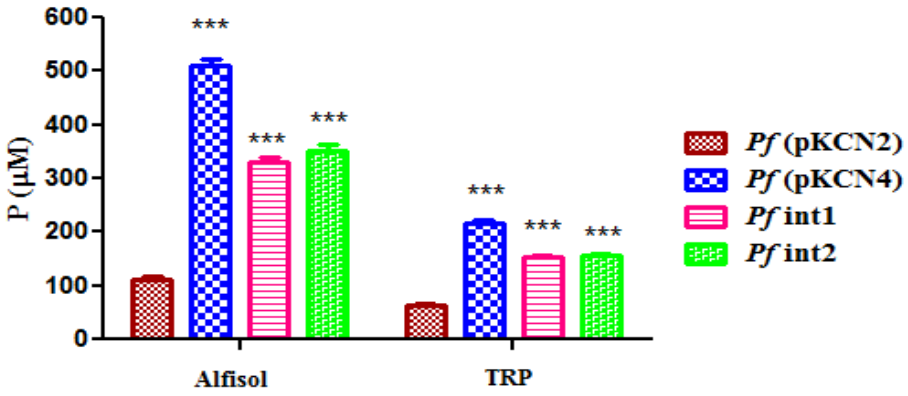


Fig. 7.9: P released from genetically modified *Pf*13525 strains in TRP and alfisol minimal medium containing 100 mM glucose as carbon source. The results are the mean ± SD of four to six independent observations. ***, P<0.0001.

In TRP minimal medium, *Pf* int1 and *Pf* int2 released 152 µM and 155 µM of P from RP, respectively. On the other hand, *Pf (*pKCN2) and *Pf (*pKCN4) released 62 µM and 217 µM of P, respectively. In alfisol soil medium containing 100 mM glucose as carbon source and supplemented with 30 mg RP/ g of soil *Pf (*pKCN4), *Pf* int1 and *Pf* int2 released 509 µM, 329 µM and 352 µM of P, respectively **(Fig. 7.9)**.

7.3.5: Root colonization study

Root colonization ability of *Pf* int 2 was observed in *Vigna radiata* (mung bean) in Murashige-Skoog's medium and alfisol soil. On 5^{th} day, in both media the abundant colonization was observed on the root surfaces, root tips, at the branching points. However, on 10^{th} day decreased colonization was observed **(Fig. 7.10; 7.11)**.

7.3.6: Effect of inoculation of genetically modified *Pf*13525 strains on growth parameters of *Vigna radiata*

Inoculation of *Vigna radiata* with *Pf* int1 and *Pf* int2 in pot experiments with non-sterilized alfisol soil supplemented with RP showed better growth (root and shoot length), increased root and shoot dry weight. *Pf* int1 and *Pf* int2 inoculations shows 1.7 and 1.9 fold increase in root length, 1.3 and 1.4 fold increase in shoot length of V*igna radiata*, respectively, as compared to wild type inoculations **(Fig. 7.12)**.

On the other hand, *Pf* int1 and *Pf* int2 inoculations result in 1.5 and 2 fold increase in root dry weight and 1.3 and 1.4 fold increase in shoot dry weight, respectively **(Fig. 7.13)**. The P content in *Pf* int1 and *Pf* int2 inoculations increased in shoots and roots by 1.8 and 2.1 fold, respectively, as compared to plants inoculated with wild type strain (Fig. 7.14). The

improvement in plant parameters is correlated with amount of oxalic acid secreted by *Pf* int1 and *Pf* int2.

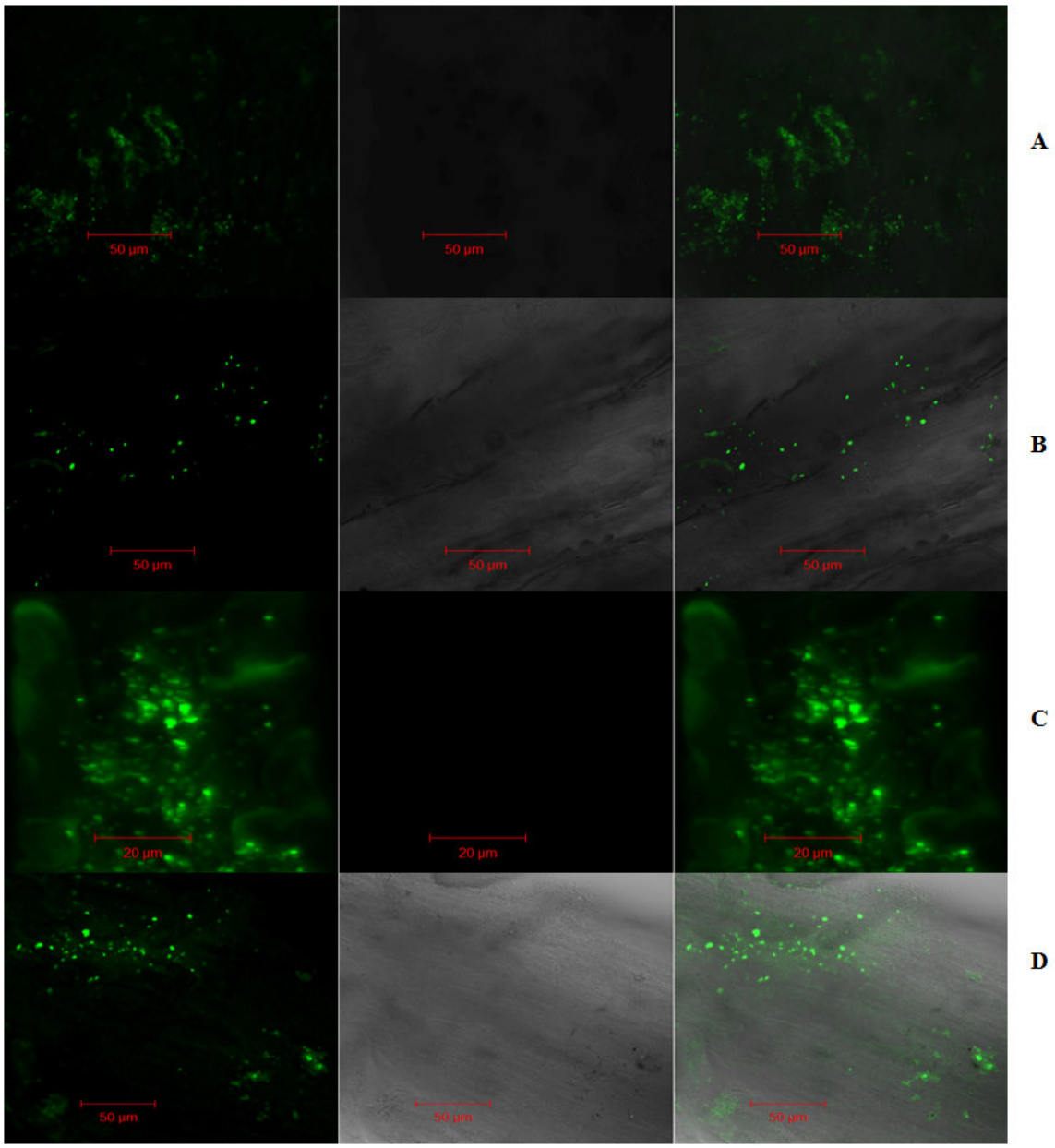


Fig. 7.10: Root colonization study of *Vigna radiata* inoculated with *Pf* int2: A- MS medium (hydroponics) on 5th day; B- MS medium (hydroponics) on 10th day; C- alfisol soil (pot experiment) on 5th day and D- alfisol soil (pot experiment) on 10th day by CLSM.

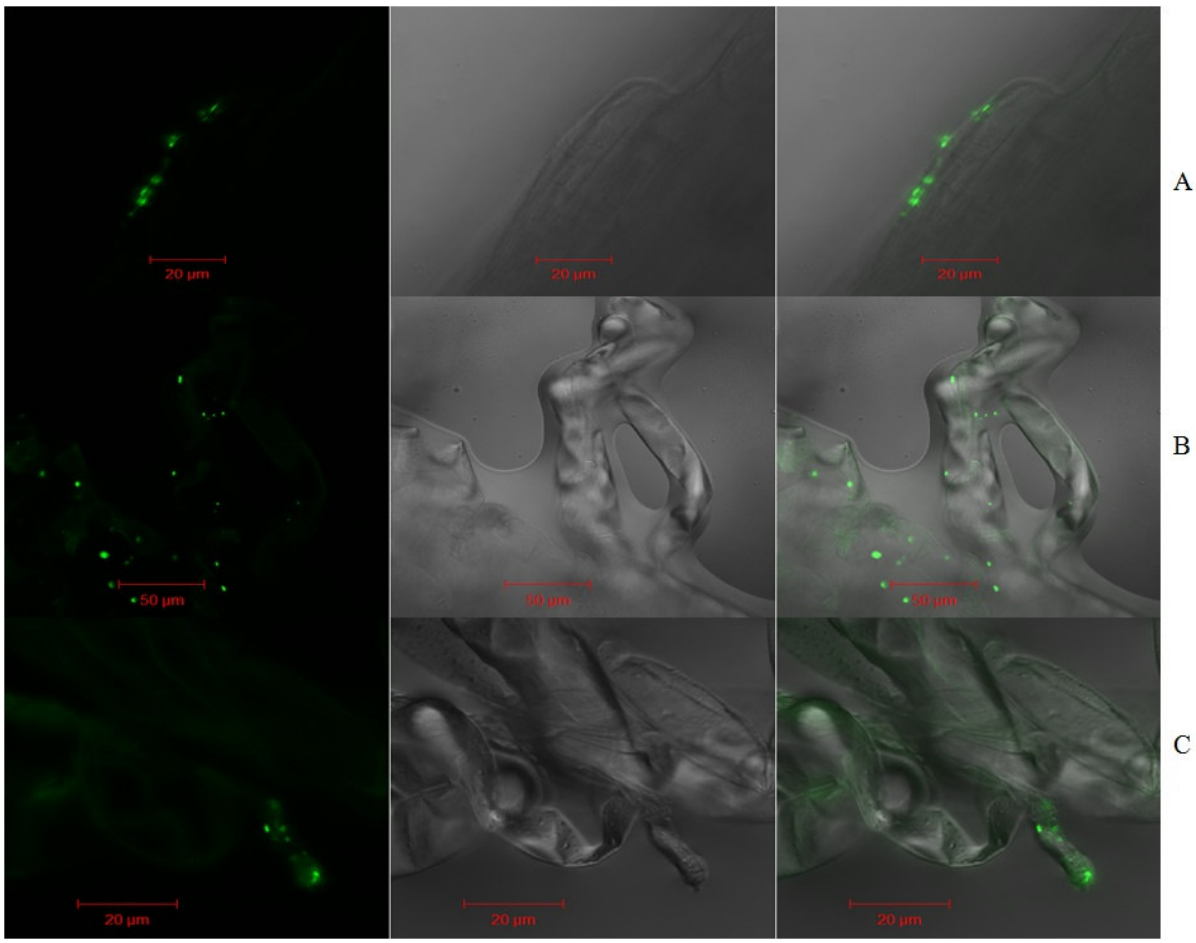


Fig. 7.11: Colonization study of *Vigna radiata* inoculated with *Pf* int2 on different parts of root by confocal microscopy. A- Lateral side of root; **B-** branching point and **C-** the root tip.

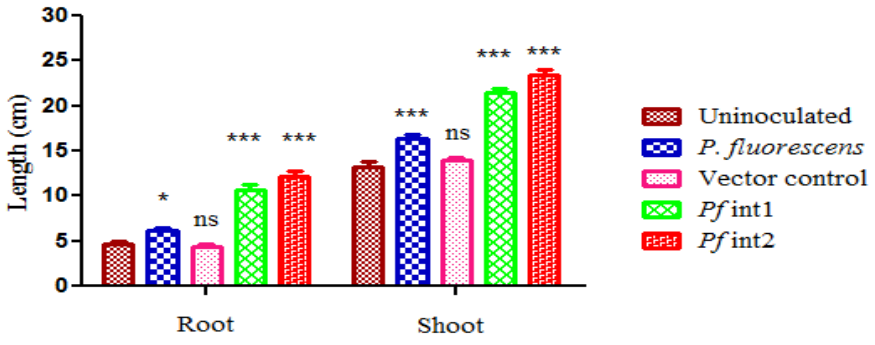


Fig. 7.12: Effect of *Pf*13525 integrants on growth of *Vigna radiata*. All the values are represented as Mean ± SD of n=9 observations, *,P<0.05; *** P<0.001; ns, non-significant.

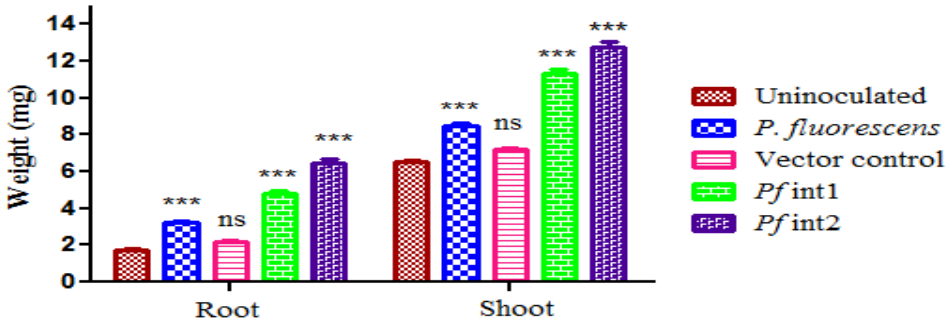


Fig. 7.13: Effect of *Pf*13525 integrants on dry weight of *Vigna radiata*. All the values are represented as Mean ± SD of n=9 observations, ***, P<0.001; ns, non-significant.

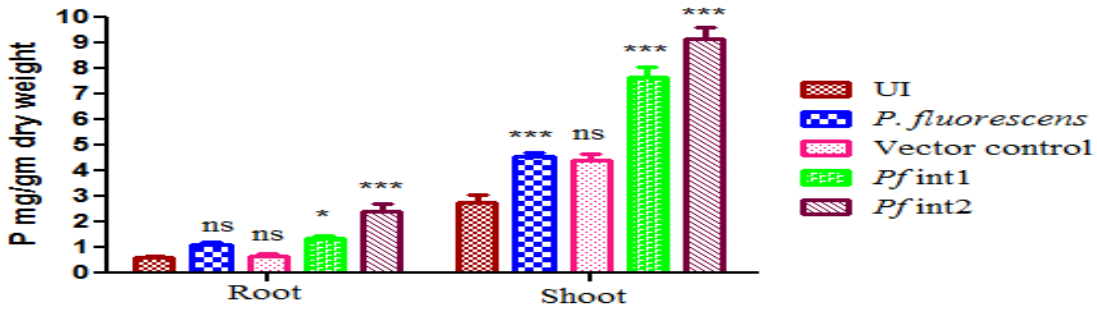


Fig. 7.14: Effect of *Pf*13525 integrants on P content of *Vigna radiata*. All the values are represented as Mean ± SD of n=4-8 observations, ***, P<0.001; ns, non-significant.

7.4: Discussion

Acidic alfisols have high refixation capcity, Al toxicity, acidic properties of soil and extremely low amount of available P (Yarzábal, 2010). The use of phosphate solubilizing rhizobacteria for biofertilizers is the most attractive way for increasing crop productivity in acidic soils, as rhizobacteria have good colonization ability and efficiently mobilize P from the soil by secreting organic acid. Organic acid mediated acidification does not release free P in alfisols, as most of the P is fixed in bound form to Fe and Al. *Enterobacter asburie* PSI3 is known to solubilize P from alkaline vertisol, due to secretion of high amount of gluconic acid, is not effective in P release from acidic alfisol (Gyaneshwar et al., 1998a; Srivastava et al., 2006). Low molecular weight organic acids with efficient chelating properties can effectively chelate

the Fe and Al from FeP and AlP, thus result in P release from alfisols (Kpomblekou and Tabatabai., 1994; Bolan et al., 1994). Few microorganisms are known to release P from alfisols by forming complexes with Al and Fe, including *A. niger* and *P. regulosum* (Nahas et al., 2002). Pigeon pea solubilizes FeP from alfisols by secretion of piscidic acid (Ae et al., 1990). Root exudates of plants contain organic acids, which help to improve the P nutrition of plants and provides resistance against heavy metal toxicity in the rhizosphere (Hocking, 2001). *P. fluorescens* ATCC 13525 secreted oxalic acid when Al- citrate was used as carbon source, to alleviate toxic effect of Al (Appanna et al., 2003). Nature and amount of organic acid determines the amount of P release from alfisols and alkaline vertisols (Srivastava et al., 2006).

Integration of genes is preferred as against plasmid transformants due to increased stability of the integrant, absence of antibiotic resistance genes and metabolic load (Buch et al., 2010). However, the integrants have the limitation of single copy expression leading to weak phenotype. This is reflected in the decrease in levels of oxalate accumulation and secretion in the genomic integrants as compared to the plasmid transformants. However, the amount of oxalate secreted by integrants was sufficient to solubilize RP in buffered medium as well as in alfisols.

Pseudomonads are known to be efficient in root colonization ability and promote plant growth by various mechanisms (Simons et al., 1996; Hass and Defago, 2005). Hydroponic and alfisol pot experiments of *Vigna radiata* inoculation with genomic integrants demonstrated enhanced root colonization as compared to the vector control suggesting that genetic manipulation did not adversely affect colonization and survival in the rhizosphere. On the other hand, *E. asburie* PSI3, which is efficient in releasing P from alkaline vertisols mediated by high levels of gluconic acid, did not improve the growth of *Vigna radiata* in alfisol (Srivastava et al., 2006).

Significant enhance in plant growth parameters and P content in *Pf* int2 inoculations as compared to *Pf* int1 could be attributed to the presence of VHb, which is known to improve the metabolism of bacteria under microaerobic conditions.

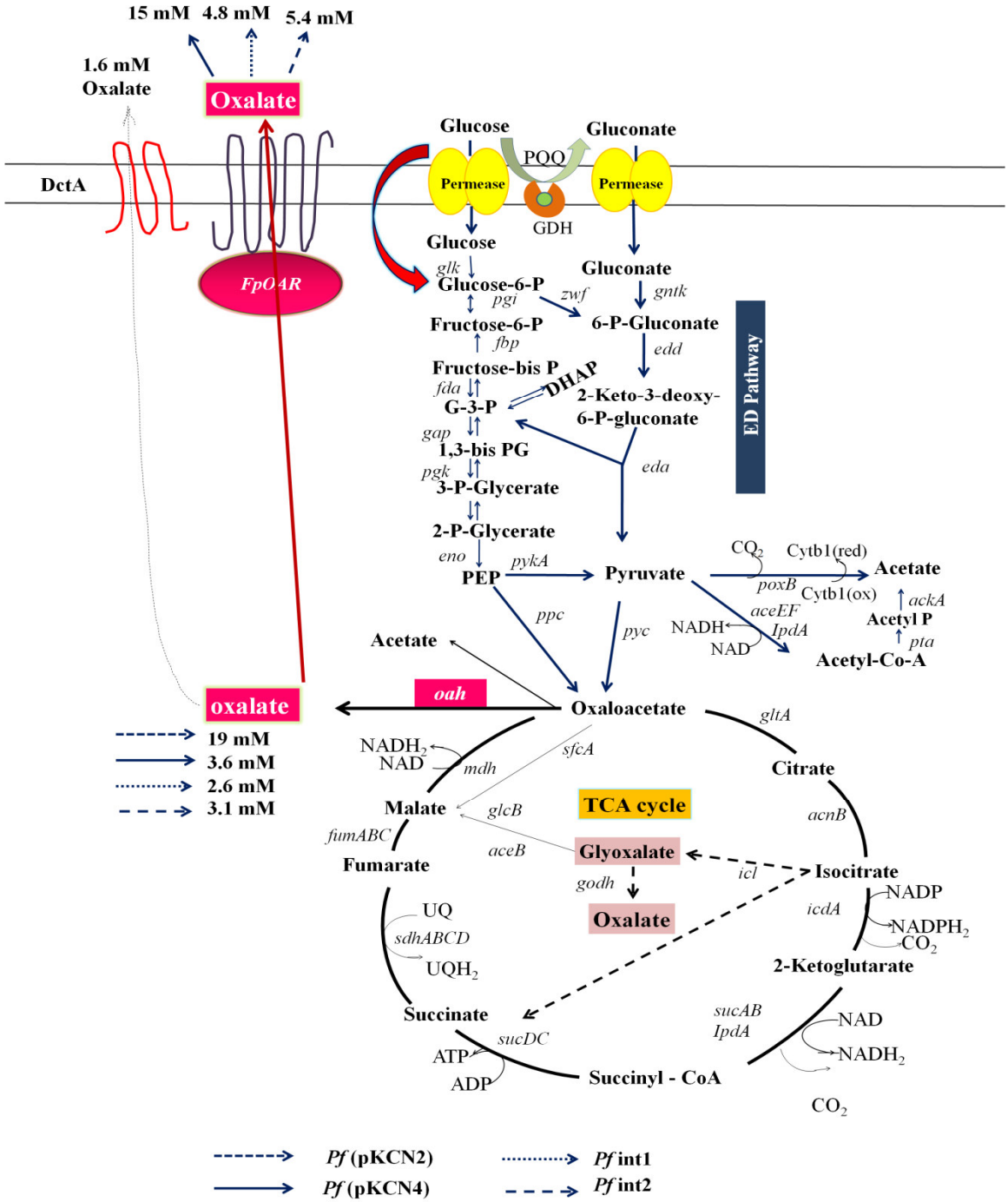
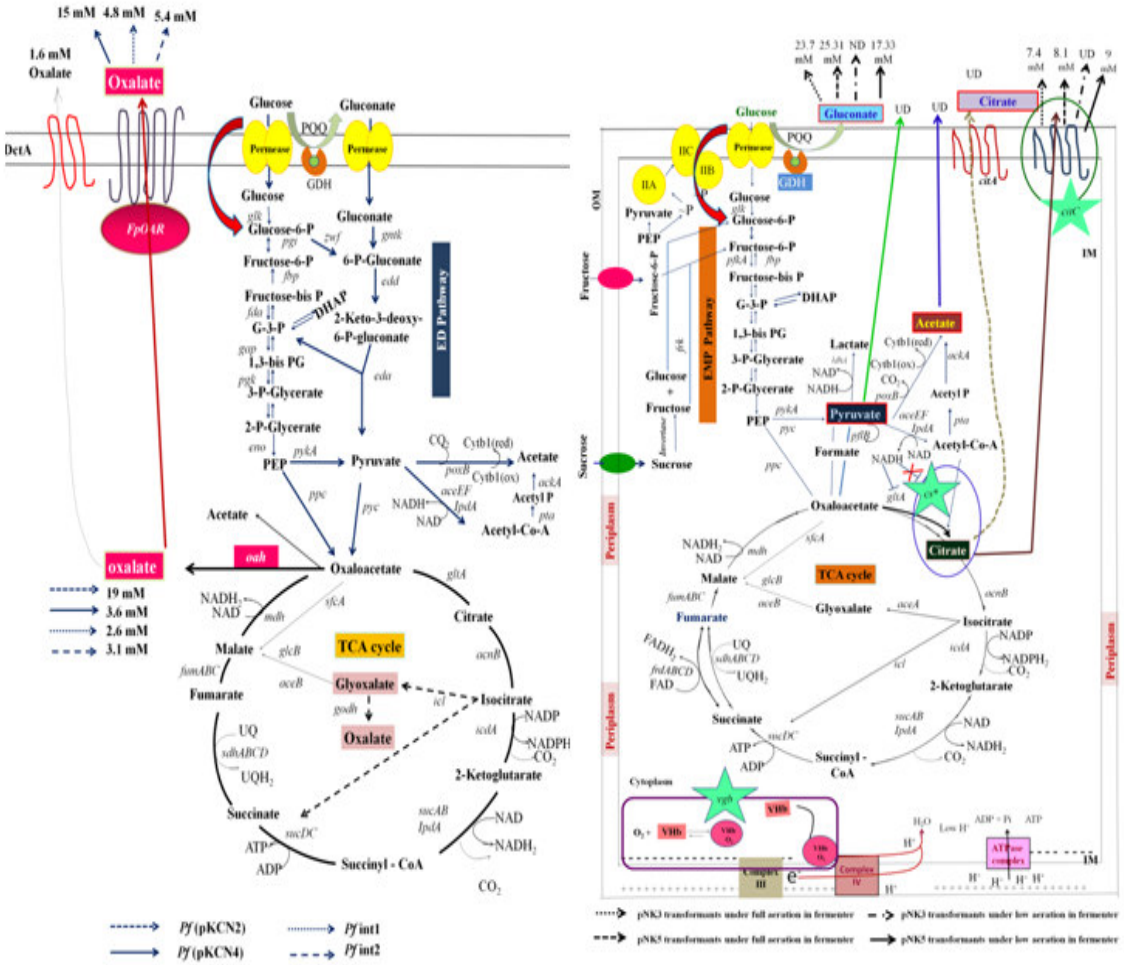


Fig. 7.15: Schematic representation of oxalic acid secretion in *Pf*13525 containing artificial oxalate operon and artificial oxalate gene cluster.

This suggests that metabolism of bacteria in the rhizosphere corresponds to microoxic conditions. Similarly, presence of VHb in *Rhizobium etli* increased nitrogenase activity and N content in bean plants (Ramirez et al., 1999).

Pigeon pea shows better growth and crop yields in alfisol as compared to other crops due to secretion of piscidic acid (Ae et al., 1990). To summarize, in this study we reports genetic manipulation mediated secretion of oxalic acid in *Pf*13525 for mineral phosphate solubilization from acidic alfisol. *Pf*13525 harboring *oah* gene result in accumulation of higher amount of oxalic acid and incorporation of oxalate operon, containing additional oxalate transporter, secreted the intracellular oxalate in the medium **(Fig. 7.15)** which in turn incorporate MPS ability in the organism while genomic integrant *Pf* int1 and *Pf* int2 shows lower amount of oxalate accumulation and secretion as compared to transformants **(Fig. 7.15)**.

However, the amount of oxalate secreted by both the integrants is sufficient to solubilize P from RP in alfisols. Genomic integrant of oxalate operon showed improved growth and increased P content of *Vigna radiata* in alfisol soil. Further, presence of VHb helps in colonization and better survival of *Pf*13525 integrant in soil, thus, improve plant growth and P content. Potential of mineral phosphate solubilization of oxalic acid by rhizobacteria could also help plants to overcome Al toxicity and in turn better survival in alfisol soils.


15 mM 4.8 mM 5.4 mM
1.6 mM Oxalate
Oxalate
DetA
FpOAR
Glucose
Gluconate
PQQ
Permease
GDH
Glucose-6-P
Fructose-6-P
6-P-Gluconate
Fructose-bis P
DHAP
2-Keto-3-deoxy-6-P-gluconate
ED Pathway
G-3-P
1,3-bis PG
3-P-Glycerate
2-P-Glycerate
PEP
Pyruvate
Acetate
Acetyl P
Acetyl-Co-A
oah
Oxaloacetate
Citrate
Malate
TCA cycle
Fumarate
Glyoxalate
Isocitrate
Oxalate
2-Ketoglutarate
Succinate
Succinyl - CoA
UQ
UQH2
NADP
NADPH
NAD
NADH2
CO2
ATP
ADP
oxalate
19 mM
3.6 mM
2.6 mM
3.1 mM
Pf (pKCN2)
Pf (pKCN4)
Pf int1
Pf int2
23.7 mM 25.31 mM ND 17.33 mM
Gluconate
UD
Citrate
7.4 mM 8.1 mM UD 9 mM
IIC
IIB
IIA
P
Pyruvate
PEP
Fructose
Sucrose
EMP Pathway
Lactate
Formate
Glyoxalate
Periplasm
OM
IM
Cytoplasm
VHb
Complex III
Complex IV
ATPase complex
H2O
Less H+
ADP + Pi
pNK3 transformants under full aeration in fermenter
pNK3 transformants under low aeration in fermenter
pNK5 transformants under full aeration in fermenter
pNK5 transformants under low aeration in fermenter

Summary

Phosphorus deficiency is a major problem for plant growth and crop yield. This problem persists in whole world over the century but very little progress has been done towards meeting the demand of food supply required into next 50 years. Multiple factors are responsible for this problem which includes depletion of natural rock phosphate (RP) ore reserve, high refixation of applied P in alfisols, pollution generated during the chemical fertilizers production and high cost of chemical fertilizers limit the use of chemical fertilizers. Thus, use of biofertilizer could be an effective approach. In alfisol soils, the crop yield is affected by many factors including very low amount of available P, Al toxicity and acidic properties of soli. In alfisols, P is fixed by Fe and Al ions while in vertisols the P is bound to Ca ions. CaP complexes can be dissolved by acidification and result in release of free P. On the other hand, FeP and AlP complexes in alfisols are difficult to dissolve due to very strong complex formation of P with Al and Fe. Organic acids which result in medium acidification are effective in alkaline vertisols for P solubilization. However, in alfisols the medium acidification does not result in P release which could be explained by the earlier studies where *E. asburie* PSI3, which is an efficient PSM in alkaline vertisol due to secretion of 50 mM gluconic acid, inoculation in alfisols does not show significant difference in P release and plant parameters. Thus, rhizobacteria secreting citric and/or oxalic acid could be a potential PSB in alkaline vertisol and alfisol soils. Also, secretion of oxalic acid by plants and microorganisms is reported to alleviate Al toxicity in alfisols.

Various phosphate solubilizing microorganisms (PSMs) have been isolated from different soil and environmental conditions. In soils, phosphate solubilizing bacteria (PSB) dominated over PS fungi in numbers due to good colonization property of of PSB. Variations were observed in the performance of PSMs under laboratory and field condition which could be due to the variations in amount of organic acid secretion. Nature and amount of organic acid secreted

determine the efficacy of PSM in rhizosphere. Organic acids secreted by PSMs play a major role in P solubilization by chelating mineral ions and acidification of surrounding medium. PSMs secrete gluconic, 2-ketogluconic, citric, succinic, malic, oxalic and acetic acid. However, the amount of organic acid secreted by these microorganisms is not sufficient for phosphate solubilization in soils. Thus, genetic modifications in central carbon metabolism of microorganisms for secretion of higher amount of strong organic acids could be an alternate approach for incorporation of MPS ability in rhizobacteria.

Recent advances in genetic engineering makes it is very easy to design new pathway and incorporation of genes in the genome of host bacteria for increasing production of desired product. Use of synthetic biology tools for construction of artificial operons and gene clusters could result in metabolic engineering of the central carbon metabolism (CCM) of rhizobacteria, could be an effective strategy for the improvement of cells by engineering of selected metabolic steps to reroute carbon fluxes toward precursors for important organic acid biosynthesis or secretion. Presence of plasmids affects the host metabolism drastically. Also, plasmids are not very stable in natural isolates, have antibiotic marker gene and leads to plasmid load. To alleviate all these factors integration of genes in bacterial genome could be an excellent strategy.

Availability of oxygen plays an important role in the colonization and survival of PSMs in rhizosphere, as oxygen levels modulate the energy metabolism of microorganisms. Oxygen and cognate oxygen radicals can affect bacterial cell either by altering the function or biosynthesis of specific proteins mediated by global transcription regulation. *Vitreoscilla* hemoglobin (VHb) encoded by *vgb* gene gets induced when dissolved oxygen is less than 2% air saturation. VHb protein known to significantly increase energy metabolism, protein biosynthesis, metabolite production and prevents cell for deleterious effect of oxidative and nitrasive stress

under hypoxic conditions. Thus, present study deals with the genetic modification of central carbon metabolism of *Citrobacter* DHRSS and *Pseudomonas fluorescens* for citric and oxalic acid secretion to convert them into potential PSB.

E. coli wild type and *icd* mutant were used as model organisms for studying citric acid secretion by incorporating artificial oxalate operon. High amount of citric acid secretion (up to 7 mM) was observed in both *E. coli* strains. The amount of citric acid secreted is sufficient for mineral phosphate solubilization (required 5-10 mM). Thus, now the operon can be incorporated in rhizobacteria to study its effect on MPS ability of host.

Citrobacter DHRSS is a good PSB and in presence of various carbon sources secretes gluconic, pyruvic and acetic acids leading to P solubilization. Incorporation of NADH insensitive CS gene in *Citrobacter* DHRSS resulted in accumulation of higher amount of citrate but the amount of citrate secreted outside was very low (2.3 mM). However, incorporation of artificial oxalate operon (pNK3, containing sodium dependent citrate transporter of *S. typhimurium*, and NADH insensitive *E. coli cs* genes) resulted in secretion of 7.2 mM citric acid in the TRP medium containing 50 mM glucose and 100 mM Tris-HCl (pH-8.0). Incorporation of artificial citrate operon improved the MPS ability of *Citrobacter* DHRSS. In liquid TRP medium, *Citrobacter* DHRSS harboring artificial citrate operon released 0.82 mM Pi.

Citrobacter DHRSS harboring artificial citrate gene cluster (pNK5, containing VHb in addition to citrate operon) and artificial citrate operon showed red zone of acidification on TRP plates containing 75 mM Tris HCl (pH-8.0) and 50 mM glucose while wild type, vector control and pNK2 transformants were not able to show zone of acidification. Under full aeration conditions in liquid TRP medium, *Citrobacter* DHRSS (pNK5) released 0.96 mM Pi from RP. Under low aeration conditions Pi release was similar as in O_2 sufficient conditions.

Under full aeration conditions in fermentor, both *Citrobacter* DHRSS pNK3 and pNK5 transformants grew up to $O.D._{600}$ 0.54 and 0.62, acidified the medium till pH 4.2 and released 0.84 mM and 1.1 mM Pi from RP, respectively. Under low aeration conditions in fermentor, growth in *Citrobacter* DHRSS (pNK3) was observed till $O.D._{600}$ 0.23 and pH drop of the medium was 5.9 while no Pi release was observed till 120 h. In *Citrobacter* DHRSS (pNK5) under low aeration conditions in fermentor, growth was observed till O.D. 0.38, medium was acidified till pH 4.2 in 94 h and released 1.0 mM Pi. Under low aeration conditions the MPS ability and citrate secretion was lost in *Citrobacter* DHRSS harboring artificial oxalate operon but incorporation of VHb in addition to artificial citrate operon retains the organic acid secretion and MPS ability. Thus, incorporation of *vgb* gene increased the citrate secretion and improves MPS ability under low oxygen conditions.

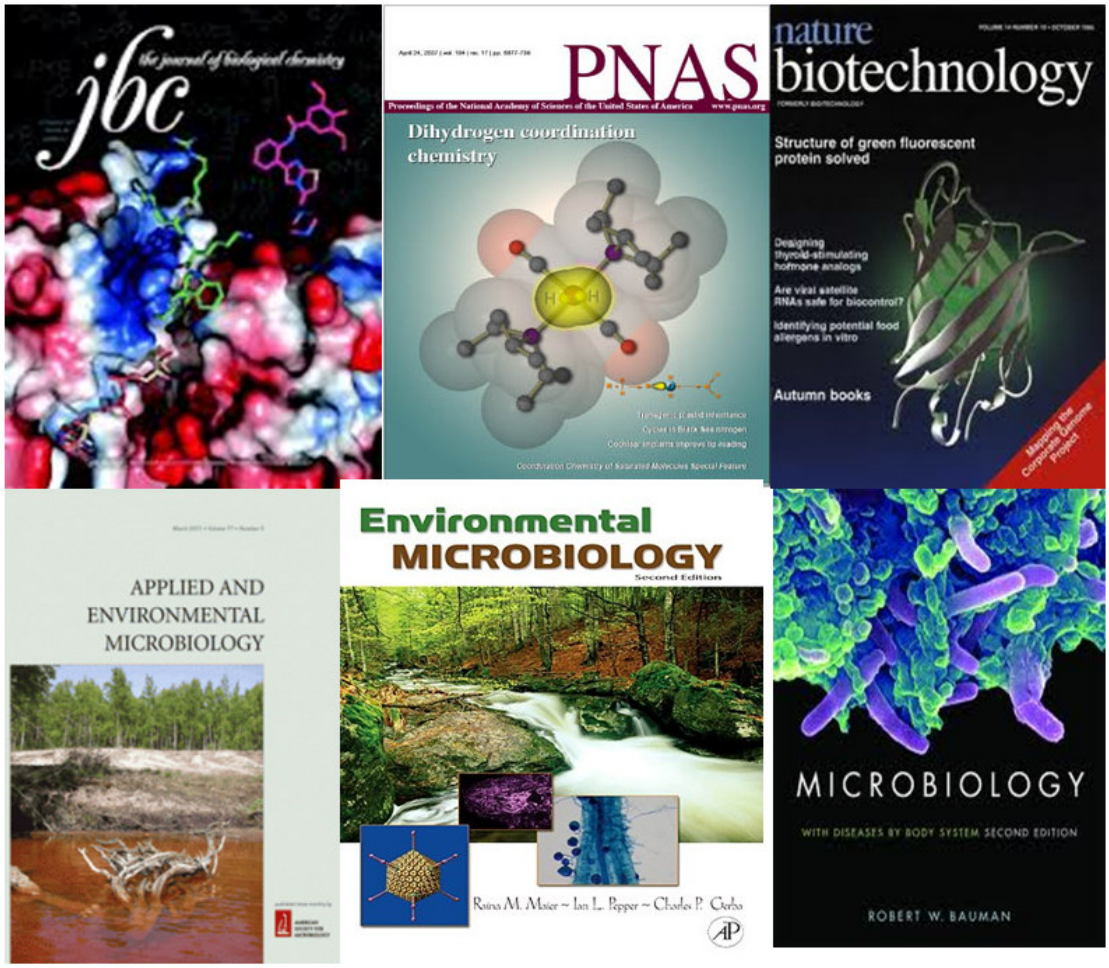
Fluorescent pseudomonads are well-known plant-growth promoting rhizobacteria with root colonization and efficient biocontrol property. Thus, incorporation of mineral phosphate solubilizing ability in fluorescent pseudomonads could convert these organisms into efficient PSB. Incorporation *of A. niger oah* gene in *Pf*13525 showed around 230 U/mg of OAH activity in M9 minimal medium containing 100 mM glucose while wild type and vector control does not show *oah* activity. *Pf* pKCN2 transformant did not decrease the pH of TRP medium less than 7.0 while incorporation of *F. plaustris* oxalate transporter (pKCN4 transformant) result in acidification of the medium from pH 8.0 to 4.2. Medium acidification by *Pf*13525 transfromants was also observed in alfisol. In alfisol soil medium *Pf* (pKCN2) acidified the medium up to pH 5.9 in 120 h while *Pf* (pKCN4) transformant acidified the medium pH to 3.9. On the other hand, *Pf*13525 and vector control does not showed acidification below pH 6.0 in alfisol soil. pKCN2 transformant carrying the *oah* gene showed highest accumulation of oxalic acid intracellularly

(up to 19.1 mM) and secreted relatively less amount (1.2 mM) in the medium. However, pKCN4 transformant possessing artificial oxalate operon consisting of *oah* gene along with oxalate transporter accumulated only 3.6 mM oxalic acid and secreted 13.6 mM in the medium. Oxalic acid was not detected in wild type *Pf*13525 and vector control transformant. In alfisol, pCNK4 secreted 15 mM of oxalate while pKCN2 transformant secreted 1.6 mM oxalate. In TRP minimal medium, *Pf (*pKCN2) and *Pf (*pKCN4) released 62 µM and 217 µM of P from RP, respectively and in alfisol soil medium containing 100 mM glucose as carbon source and supplemented with 30 mg RP/ g of soil *Pf (*pKCN2) and *Pf (*pKCN4) released 112 µM and 509 µM of P, respectively, while P released was not observed in *Pf*13525 and vector control. Thus, incorporation of *A. niger oah* gene resulted in oxalate accumulation but secretion was very low. Addition of *F. plaustris* oxalate transporter in addition to *oah* gene (artificial oxalate operon) improves the oxalic acid secretion and in turn MPS ability of organism. *Pf*13525 harboring oxalate operon showed mineral phosphate solubilization in both liquid medium and alfisol soil.

To minimize plasmid load on host metabolism, the artificial oxalate operon and artificial oxalate gene cluster was integrated into the genome of *Pf*13525. The integrants *Pf* int1 and *Pf* int2 showed less OAH activity (165 U/mg), secreted 4.1 and 4.7 mM of oxalic acid, respectively, while intracellular levels were 2.6 and 3.1 mM, respectively, which are less as compared to plasmid transformants. In alfisol soil *Pf* int1 and *Pf* int2 secreted 4.8 and 5.4 mM of oxalate. Thus oxalate secretion by integrants in alfisol was comparable to that on TRP medium. *Pf* int1, *Pf* int 2 and pKCN4 transformant acidified TRP agar plate containing 100 mM glucose as carbon source and 50 mM Tris HCl (pH 8.0) while *Pf*13525 and pKCN2 transformant did not show red zone of acidification. In TRP minimal medium, *Pf* int1 and *Pf* int2 released 152 µM and 155 µM of P from RP while in alfisol soil medium supplemented with 30 mg RP/ g of soil *Pf* int1 and *Pf*

int2 released 329 µM and 352 µM of P, respectively. Root colonization ability of *Pf* int2 was observed in *Vigna radiata* (mung bean) in Murashige-Skoog's medium and alfisol soil, which shows that colonization in case of *Pf* int2, was better as compared to vector control. In both media on 5^{th} day, abundant colonization was observed on the root surfaces, root tips and at the branching points but after 10^{th} day decreased colonization was observed. Inoculation of *Pf* int1 and *Pf* int2 to *Vigna radiata* in pot experiments with non-sterilized alfisol soil supplemented with RP showed 1.7 and 1.9 fold increase in root length, 1.3 and 1.4 fold increase in shoot length, 1.5 and 2 fold increase in root dry weight and 1.3 and 1.4 fold increase in shoot dry weight of V*igna radiata*, respectively, as compared to wild type inoculations. The P content increased in shoots and roots by 1.8 and 2.1 fold, respectively, as compared to plants inoculated with wild type strain. The improvement in plant parameters is correlated with amount of oxalic acid secreted by *Pf* int1 and *Pf* int2. Thus, genetic modification of *Pf*13525 for oxalic acid secretion imparts MPS ability to the organism. Albeit, plasmid transformants showed better phenotype as compared to integrants but the amount of oxalic acid secreted in integrants is sufficient for P release from alfisols and showed improved growth of mung bean plants.

In this study, genetic manipulation with a set of genes consisting of *cs*, citC, oah, FpOAR and vgb* resulted in improvement or incorporation of MPS ability in *Citrobacter* DHRSS and *Pf*13525. These genetic modifications significantly improved the efficacy of host strains in soil conditions. Similar genetic manipulations could be useful in incorporation of secretion of higher amounts citric and oxalic acid in transforming plant growth promoting rhizobacteria (PGPR) into efficient PSB.



BIBLIOGRAPHY

1. **Abekoe MK (1998)** Fertilizer P transformation and P availability in hill slope soils of Northern Ghana. Nutr. Cycl. Agroecosyst. 52: 45–54.
2. **Abrams JJ, Webster DA (1990)** Purification, partial characterization, and possible role of catalase in the bacterium *Vitreoscilla*. Arch. Biochem. Biophys. 279: 54–59.
3. **Abril A, Zurdo-Pineiro JL, Peis A, Rivas R, Velazquez E (2007)** Solubilization of phosphate by a strain of *Rhizobium leguminosarium* bv *trifolii* isolated from *Phaseolus vulgaris* in EI Chaco Arido soil (Argentina). In Velazquez, E., Rodriguez-Berrueco, C.,(Eds) Book Series: Developments in Plant and Soil Sciences. Springer, The Netherlands. pp 135-138.
4. **Ae N, Arihara J, Okada K (1991)** Phosphorus response of chick pea and evaluation of phosphorus availability in Indian alfisols and vertisols Phosphorus Nutrition of Grain Legumes. India: International Crop Research Institute for the Semi- Arid Tropics. ISBN 92–9066–200. 33P.
5. **Ae N, Arihara J, Okada K, Yoshihara T, Johansen C (1990)** Phosphorus uptake by pigeon pea and its role in cropping systems of the Indian subcontinent. Science. 248: 477-480.
6. **Agbenin JO (2003)** Extractable iron and aluminum effects on phosphate sorption in a savanna alfisol. Soil Sci. Soc. Am. J. 67: 589-595.
7. **Ahmed S (1995)** Agriculture–Fertilizer interface in Asia issues of growth and sustainability. Oxford and IBH publishing Co, New Delhi.
8. **Alikhani HA, Saleh-Rastin N, Antoun H (2007)** Phosphate solubilization activity of rhizobia native to Iranian soils. In Velazquez, E., Rodriguez-Berrueco, C.,(Eds) Book Series: Developments in Plant and Soil Sciences. Springer, The Netherlands. pp:135-138.
9. **Amadioha AC (1994)** Depolymerisation of potato tissue by oxalic acid, polygalacturonase and cellulase produced by *Aspergillus niger*. Food/Nahrung, 38: 341-342.
10. **Ames BN (1964)** Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol. 8: 115-118.

11. **Anand A, Duk BT, Singh S, Akbas MY, Webster DA, Stark BC, Dikshit KL(2010)** Redox-mediated interactions of VHb (*Vitreoscilla* haemoglobin) with OxyR: novel regulation of VHb biosynthesis under oxidative stress. Biochem. J. 426: 271-280.
12. **Antoniewicz MR, Kraynie DF, Laffend LA, González-Lergier J, Kelleher JK, Stephanopoulos G (2007)** Metabolic flux analysis in a nonstationary system: fed-batch fermentation of a high yielding strain of E. coli producing 1, 3-propanediol. Biotechnol. Bioeng. 9: 277.
13. **Aoshima M, Ishii M, Yamagishi A, Oshima T, Igarashi Y (2003)** Metabolic characteristics of an isocitrate dehydrogenase defective derivative of *Escherichia coli* BL21 (DE3). Biotechnol. Bioeng. 84: 732–737.
14. **Appanna VD, Hamel R, Mackenzie C, Kumar P, Kalyuzhnyi SV (2003)** Adaptation of *Pseudomonas fluorescens* to Al-citrate: involvement of tricarboxylic acid and glyoxylate cycle enzymes and the influence of phosphate. Curr. Microbiol. 47: 521-527.
15. **Archana G, Buch A, Naresh Kumar G (2012)** Pivotal role of organic acid secretion by rhizobacteria in plant growth promotion, In: Satayanarayan T, Johri BN, Prakash A, editors. Microorganisms in Sustainable Agriculture and Biotechnology. Springer, pp. 35-53.
16. **Arnou, DI (1953)** *In* Soil and Fertilizer Phosphorus in Crop Nutrition (IV). Ed. WH Pierre. Noramn, AG. Acad. Press NY.
17. **Asea PEA, Kucey RMN, Stewart JWB (1988)** Inorganic phosphate solubilization by two *Penicillium* species in solution culture and soil. Soil Biol. Biochem. 20: 459- 464.
18. **Ayed A, Krutchinsky AN, Ens W, Standing KG, Duckworth HW (1998)** Quantitative evaluation of proteinprotein and ligand-protein equilibria of a large allosteric enzyme by electrospray ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 12: 339-344.
19. **Babu-Khan S, Yeo C, Martin WL, Duron MR, Rogers RD, Goldstein AH (1995)** Cloning of a mineral phosphate-solubilizing gene from *Pseudomonas cepacia*. Appl. Environ. Microbiol. 61: 972-978.
20. **Bailey JE (1991)** Toward a science of metabolic engineering. Science 252:1668-1675.

21. **Bailey JE, Sburlati A, Hatzimanikatis V, Lee K, Renner WA, Tsai PS (2002)** Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. Biotechnol. Bioeng. 79: 568-579.

22. **Bainton RJ, Kubo KM, Feng JN, Craig NL (1993)** Tn7 transposition: Target DNA recognition is mediated by multiple Tn7-encoded proteins in a purified *in vitro* system. Cell 72: 931-943.

23. **Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006)** The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. 57: 233-266.

24. **Baitin DM, Zaitsev EN, Lanzov VA (2003)** Hyper recombinogenic RecA protein from *Pseudomonas aeruginosa* with enhanced activity of its primary DNA binding site. J. Mol. Biol. 328: 1 -7.

25. **Balmforth AJ, Thomson A (1984)** Isolation and characterization of glyoxylate dehydrogenase from the fungus *Sclerotium rolfsii*. Biochem. J. 218: 113.

26. **Banik S, Dey BK (1982)** Available phosphate content of an alluvial soil as influenced by inoculation of some isolated phosphate solubilizing microorganisms. Plant Soil 69: 353–364.

27. **Barber SA (1984**) Soil nutrient bioavailability. John Wily, New York, USA.

28. **Bashan Y, Puente ME, Rodriquea MN, Toledo G, Holguin G, Ferrera-Cerrato R, Pedrin S (1995)** Survival of *Azorhizobium brasilense* in the bulk soil and rhizosphere of 23 soil types. Appl. Environ. Microbiol. 61: 1938-1945.

29. **Bennett MR, Hasty J (2009)** Overpowering the component problem. Nat. Biotechnol. 27: 450– 451.

30. **Berg G, Smalla K (2009)** Plant species versus soil type: which factors influence the structure and function of the microbial communities in the rhizosphere? FEMS Microbiol. Ecol. 68: 1–13.

31. **Beriault R, Hamel R, Chenier D, Mailloux RJ, Joly H, Appanna VD (2007)** The overexpression of NADPH-producing enzymes counters the oxidative stress evoked by gallium, an iron mimetic. Biometals 20: 165-176.

32. **Bieleski RL (1973)** Phosphate pools, phosphate transport, and phosphate availability. Annu. Rev. Plant Physiol. 24: 225-252.

33. **Bolan NS, Naidu R, Mahimairaja S, Baskaran, S (1994)** Influence of low-molecular-weight organic acids on the solubilization of phosphates. Biol. Fert. Soils. 18: 311-319.
34. **Bolland MDA (1992)** Agronomic effectiveness of Partially Acidulated Rock Phosphate (PARP) and fused calcium-magnesium phosphate compared with superphosphate. Fertil. Res. 32: 169–183.
35. **Bomstein RA, Johnson MJ (1952)** The mechanism of formation of citrate and oxalate by *Aspergillus niger*. J. Biol. Chem. 198: 143-153.
36. **Bonarius HP, Schmid G, Tramper J (1997)** Flux analysis of underdetermined metabolic networks: the quest for the missing constraints. Trends Biotechnol. 15: 308-314.
37. **Brimecombe MJ, De Lelj FA, Lynch JM (2001)** The rhizosphere. The effect of root exudates on rhizosphere microbil populations. In: Pinton, R., Varanini, Z., Nannipieri, P., (Eds.). The rhizosphere, biochemistry and organic substances at the soil-plant interface. Marcel Dekker, New York, pp: 95-140.
38. **Brunker P, Minas W, Kallio PT, Bailey JE (1998)** Genetic engineering of an industrial strain of *Saccharopolyspora erythraea* for stable expression of the *Vitreoscilla* haemoglobin gene (*vgb*). Microbiol. 144: 2441–8.
39. **Buch A D, Archana G, Kumar G N (2010)** Broad-host-range plasmid-mediated metabolic perturbations in *Pseudomonas fluorescens* 13525. App. Microbiol. Biotechnol. 88: 209-218.
40. **Buch AD, Archana G, Naresh Kumar G (2008)** Metabolic channeling of glucose towards gluconate in phosphate solubilizing *Pseudomonas aeruginosa* P4 under phosphorus deficiency. Res. Microbiol. 159: 635-642.
41. **Buch AD, Archana G, Naresh Kumar G (2009)** Enhance citric acid biosynthesis in *Pseudomonas fluorescence* ATCC 13525 by overexpression of the *Escherichia coli* citrate synthase gene. Microbiol. 155: 2620-2629.
42. **Buch AD, Archana G, Naresh Kumar G (2010)** Heterologous expression of phosphoenolpyruvate carboxylase enhances the phosphate solubilizing ability of fluorescent pseudomonads by altering the glucose catabolism to improve biomass yield. Bioresour. Technol. 101: 679–687.

43. **Butegwa, CN (1996)** Agronomic evaluation of fertilizer products derived from Sukulu hills phosphate rock. Fertil. Res. 44: 113–122.
44. **Cameron DC, Chaplen FWR (1997)** Developments in metabolic engineering. Curr. Opin. Biotechnol. 8: 175-180.
45. **Carson KC, Holiday S, Glenn AR, Dilworth MJ (1992)** Siderophore and organic acid production in root nodule bacteria. Arch. Microbiol. 157: 264-271.
46. **Chan LY, Kosuri S, Endy D (2005)** Refactoring bacteriophage T7. Mol Syst Biol 1.
47. **Chanchal Kumar:** Thesis 2012.
48. **Chen C, Sun Q, Narayanan B, Nuss DL, Herzberg O (2010)** Structure of oxalacetate acetylhydrolase, a virulence factor of the chestnut blight fungus. J. Biol. Chem. 285: 26685-26696.
49. **Chen CR, Condron LM, Davis MR, Sherlock RR (2002)** Phosphorus 360 dynamics in the rhizosphere of perennial ryegrass (*Lolium perenne* L.) and radiata pine (*Pinus radiata* D. Don.). Soil Biol. Biochem. 34: 487-499.
50. **Chen W, Hughes DE, Bailey JE (1994)** Intracellular expression of Vitreoscilla hemoglobin alters the aerobic metabolism of *Saccharomyces cerevisiae*. Biotechnol. Prog. 10: 308–313.
51. **Chenier D, Beriault R, Mailloux R, Baquie M, Abramia G, Lemire J, Appanna V (2008)** Involvement of fumarase C and NADH oxidase in metabolic adaptation of *Pseudomonas fluorescens* cells evoked by aluminum and gallium toxicity. App. Env. Microbiol. 74: 3977-3984.
52. **Chi PY, Webster, DA, Stark BC (2009)** *Vitreoscilla* hemoglobin aids respiration under hypoxic conditions in its native host. Microbiol. Res. 164: 267-275.
53. **Chockalingam K, Chen ZL, Katzenellenbogen JA, Zhao HM (2005)** Directed evolution of specific receptor-ligand pairs for use in the creation of gene switches. P. Natl. Acad. Sci. USA 102: 5691–5696.
54. **Choi K-H, DeShazer D, Schweizer HP (2006)** MiniTn7 insertion in bacteria with multiple glmS-linked attTn7 sites: Example *Burkholderia mallei* ATCC 23344. Nat. Protocols 1: 162-169.

55. **Choi K-H, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP (2005)** A Tn7-based broad-range bacterial cloning and expression system. Nat. Methods 2: 443 -448.

56. **Choi K-H, Kim K-Ju (2009)** Applications of transposon-based gene delivery system in bacteria. J. Microbiol. Biotechnol. 19: 217-228.

57. **Choi KH, Schweizer HP (2006)** Mini-Tn7 insertion in bacteria with single *att*Tn7 sites: example *Pseudomonas aeruginosa*. Nat Protoc. 1: 153–161.

58. **Choi KH, Schweizer HP (2006)** Mini-Tn7 insertion in bacteria with secondary, non-glmS-linked attTn7 sites: Example *Proteus mirabilis* HI4320. Nat. Protocols 1: 170 - 178.

59. **Chomczynski P, Sacchi N (2006)** The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. Nature Protocol 1: 581-585.

60. **Chung H, Park M, Madhaiyan M, Seshadri S, Song J, Cho H, Sa T (2005)** Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biol. Biochem. 37:1970-1974.

61. **Cohen S, Chang ACY, Leslie HSU (1972)** Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA ($CaCl_2$ /extrachromosomal DNA/plasmid). Proc. Nat. Acad. Sci. USA 69: 2110-2114.

62. **Cordell D, Drangert JO, White S (2009)** The story of phosphorus: Global food security and food for thought. Glob. Env. Change 19: 292-305.

63. **Craig NL (1989)** Transposon Tn7. pp. 211-225. In D. E. Berg and M. M. Howe (eds.) Mobile DNA. American Society for Microbiology, Washington, DC.

64. **Cunningham JE, Kuiack C (1992)** Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaji*. Appl. Environ. Microbiol. 58: 1451-1458.

65. **Curl EA, Truelove B (1986)** The Rhizosphere. p. 1-8, Introduction; p. 140- 166, Microbial Interactions; p. 167-190, Rhizosphere in Relation to Plant Nutrition and Growth. Springer-Verlag, New York.

66. **De Freitas JR, Banerjee MR, Germida JJ (1997)** Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biol. Fertil. Soils 24: 358–364.

67. **DeModena JA, Gutierrez S, Velasco J, Fernandez FJ, Fachini RA, Galazzo JL (1993)** The production of cephalosporin C by *Acremonium chrysogenum* is improved by the intracellular expression of a bacterial hemoglobin. Biotechnol. 11: 926–9.

68. **Deng QW, Luo WS, Donnenberg MS (2007)** Rapid site site-directed domain scanning mutagenesis of enteropathogenic *Escherichia coli* espD. Biol. Proced. Online 18–26.

69. **Dikshit RP, Dikshit KL, Liu Y, Webster DA (1992)** The bacterial hemoglobin from Vitreoscilla can support the aerobic growth of *E. coli* lacking terminal oxidases. Arch. Biochem. Biophys. 293: 241–245.

70. **Dixon GH, Kornberg HL (1959)** Assay methods for key enzymes of the glyoxylate cycle. Proc. Biochem. Soc. 3P.

71. **Dobrowolski A, Fusetti F, Lolkema JS (2010)** Domain Interactions and Close Vicinity of trans Reentrant Loops in the Na+-Citrate Transporter CitS of *Klebsiella pneumoniae*. Biochem. 49(48).

72. **Donald LJ, Crane BR, Anderson DH, Duckworth HW (1991)** The role of cysteine 206 in allosteric inhibition of Escherichia coli citrate synthase. Studies by chemical modification, site-directed mutagenesis, and 19F NMR. J. Biol. Chem. 266: 20709-20713.

73. **Duckworth HW, Tong EK (1976)** The binding of reduced nicotinamide adenine dinucleotide to citrate synthase of *Escherichia coli* K12. Biochem. 15: 108-114.

74. **Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, et al. (2009)** Synthetic protein scaffolds provide modular control over metabolic flux. Nat. Biotechnol. 27: 753–759.

75. **Dutton MV, Evans CS (1996)** Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Can. J. Microbial. 42: 881-895.

76. **Edwards JS, Covert M, Palsson, BO (2002)** Metabolic modelling of microbes: the flux-balance approach. Env. Microbiol. 4: 133-140.

77. **Eisenberg RC, Dobrogosz WJ (1967)** Gluconate metabolism in *Escherichia coli*. J. Bacteriol. 93: 941-949.

78. **Else AJ, Barnes SJ, Danson MJ, Weitzman DJP (1998)** A new spectrophotometric assay for citrate synthase and its use to assess the inhibitory effects of palmitoyl thioesters. Biochem. J. 251: 803-807.

79. **El-Swaify, SA, Pathak P, Rego TJ, Singh S (1985)** Soil management for optimized productivity under rainfed conditions in the semi-arid tropics (pp. 1-64). Springer New York.

80. **Erenler SO, Gencer S, Geckil H, Stark BC, Webster DA (2004)** Cloning and expression of the *Vitreoscilla* hemoglobin gene in *Enterobacter aerogenes:* effect on cell growth and oxygen uptake. Appl. Biochem. Microbiol. 40: 241–8.

81. **Esler J, Bennett E (2011)** A broken geochemical cycle. Nature. 478: 29-31.

82. **Esvelt KM, Wang HH (2013)** Genome-scale engineering for systems and synthetic biology. Mol. Sys. Biol. 9:641

83. Exley C ***(2004)*** The pro-oxidant activity of aluminum. Free Rad. Biol. Med. 36*:* 380–387.

84. **Farr SB, Kogoma T (1991)** Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. 55: 561-585.

85. **Forster A, Aurich A, Mauersberger S, Barth G (2007)** Citric acid production from sucrose using a recombinant strain of the yeast *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 75: 1409-1417.

86. **Forster A, Jacobs K, Juretzek T, Mauersberger S, Barth G (2007)** Overexpression of the ICL1 gene changes the product ratio of citric acid production by *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 77: 861-869.

87. **Frey AD, Farres J, Bollinger CJ, Kallio PT (2002)** Bacterial hemoglobins and flavohemoglobins for alleviation of nitrosative stress in *Escherichia coli.* Appl. Environ. Microbiol. 68: 4835–40.

88. **Frey AD, Kallio PT (2003)** Bacterial hemoglobins and flavohemoglobins: versatile proteins and their impact on microbiology and biotechnology. FEMS Microbiol. Rev. 27: 525–545.

89. **Fu D, Maloney PC (1998)** Structure-function relationships in OxlT, the oxalate/formate transporter of *Oxalobacter formigenes* topological features of trans

membrane helix 11 as visualized by site-directed fluorescent labeling. J. Biol. Chem. 273: 17962-17967.

90. **Fuhrer T, Fischer E Sauer U (2005)** Experimental identification and quantification of glucose metabolism in seven bacterial species. J. Bacteriol. 187: 1581-1590.
91. **Gadd GM (1999)** Fungal production of citric and oxalic acid: Importance in metal speciation, physiology and biogeo-chemical processes. Adv. Microb. Physiol. 41: 48–92.
92. **Galazzo JL, Bailey JE (1990)** Fermentation pathway kinetics and metabolic flux control in suspended and immobilized *Saccharomyces cerevisiae*. Enz. Micro. Technol. 12: 162-172.
93. **Geckil H, Gencer S, Kahraman H, Erenler SO (2003)** Genetic engineering of *Enterobacter aerogenes* with the *Vitreoscilla* hemoglobin gene: cell growth, survival, and antioxidant enzyme status under oxidative stress. Res. Microbiol. 154: 425–431.
94. **Gibson DG, Benders GA, Andrews-Pfannkoch C, Denisova EA, Baden-Tillson H, et al. (2008a)** Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. Science 319: 1215–1220.
95. **Gibson DG, Benders GA, Axelrod KC, Zaveri J, Algire MA, et al. (2008b)** One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic *Mycoplasma genitalium* genome. P. Natl. Acad. Sci. USA 105: 20404–20409.
96. **Giddens JE, Dunigan EP, Weaver RW (1982)** Legume inoculation in the southeastern USA. South Coop. Ser. Bull. 283: 1-38.
97. **Gill, RT (2003)** Enabling inverse metabolic engineering through genomics. Curr. Opin. Biotechnol., 14: 484-490.
98. **Gilroy S, Jones DL (2000)** Through form to function: root hair development and nutrient uptake. Trends Plant Sci. 5: 56–60.
99. **Glick BR (1995)** The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. 41: 109-117.
100. **Goldstein AH (1995)** Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. Biol. Agric. Hor. 12: 185–193.

101. Goldstein AH, Liu ST (1987) Molecular cloning and regulation of a mineral phosphate solubilizing (*mps*) gene from *Erwinia herbicola*. *Nature Biotechnol* 5: 72-74.

102. Golstein AH (1986) Bacterial phosphate solubilization: Historical perspective and future prospects. Am. J. Alt. Agric. 1: 57–65.

103. Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB (2004) Metabolomics by numbers: acquiring and understanding global metabolite data. Trends Biotechnol. 22: 245-252.

104. Gottschal JC (1992) Substrate capturing and growth in various ecosystems. J. Appl. Bacteriol. Symp. Suppl. 73: 39-48.

105. Griffiths BS, Kuan HL, Ritz K, Glover LA, McCaig AE, Fenwick C (2004) The relationship between microbial community structure and functional stability, tested experimentally in an upland pasture soil. Microb. Ecol. 47: 104- 113.

106. Gupta RR, Singal R, Shanker A, Kuhad RC, Saxena RK (1994) A modified plate assay for screening phosphate solubilizing microorganisms. Gen. Appl. Microbiol. 40: 255–260.

107. Gyaneshwar P, Naresh Kumar G, Parekh LJ (1998a) Cloning of mineral phosphate solubilizing genes from *Synechocystis* PCC6803. Curr. Science 74: 1097-1099.

108. Gyaneshwar P, Naresh Kumar G, Parekh LJ (1998b) Effect of buffering on the phosphate solubilizing ability of microorganisms. World J. Microbiol. Biotechnol. 14: 669-673.

109. Gyaneshwar P, Naresh Kumar G, Parekh LJ, Poole PS (2002) Role of soil microorganisms in improving P nutrition of plants. Plant Soil 245: 83–93.

110. Gyaneshwar P, Parekh LJ, Archana G, Poole PS, Collins MD, Hutson RA, Naresh Kumar G, (**1999**) Involvement of a phosphate starvation inducible glucose dehydrogenase in soil phosphate solubilization by *Enterobacter asburiae*. FEMS Microbiol. Lett. 171: 223–229.

111. Halder AK, Chakrabartty PK (1993) Solubilization of inorganic phosphate by *Rhizobium*. Folia Microbiol. 38: 325-330.

112. Hall BG (1982) Chromosomal Mutation for Citrate Utilization by *Escherichia coli* K-12. J. Bacteriol. 151: 269-273.

113. **Hameeda B, Harini G, Rupela OP, Wani SP, Reddy G (2008)** Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. Microbiol. Res. 163: 234-242.

114. **Hammel KE, Mozuch MD, Jensen Jr KA, Kersten PJ (1994)** H_2O_2 recycling during oxidation of the arylglycerol. Beta.-aryl ether lignin structure by lignin peroxidase and glyoxal oxidase. Biochem. 33: 13349-13354.

115. **Han Y, Joosten HJ, Niu W, Zhao Z, Mariano PS, McCalman M, Dunaway-Mariano D (2007)** Oxaloacetate hydrolase, the C–C bond lyase of oxalate secreting fungi. J. Biol. Chem. 282: 9581-9590.

116. **Hass D, Défago G (2005)** Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Rev. Microbiol. 3: 307-319.

117. **Heijnen CE, Chenu C, Robert M (1993)** Micro-morphological studies on clay-amended and unamended loamy sand, relating survival of introduced bacteria and soil structure. Geoderma 56: 195-207.

118. **Heijnen CE, Page S, van Elsas JD (1995)** Metabolic activity of *Flavobacterium* strain P25 during starvation and after introduction into bulk soil and the rhizosphere of wheat. FEMS Microbiol. Ecol. 18: 129-138.

119. **Heijnen CE, van Veen JA (1991)** A determination of protective microhabitats for bacteria introduced into soil. FEMS Microbiol. Ecol. 85: 73-80.

120. **Herman A, Tawfik DS (2007)** Incorporating Synthetic Oligonucleotides via Gene Reassembiv (ISOR): a versatile tool for generating targeted libraries. Protein Eng. Des. Sel. 20: 219–226.

121. **Hester KL, Lehman J, Najar F, Song L, Roe BA, MacGregor CH, Hager P W, Phibbs PV Jr, Sokatch JR (2000)** Crc is involved in catabolite repression control of the bkd operons of *Pseudomonas putida* and *Pseudomonas aeruginosa*. J. Bacteriol. 182: 1144-1149.

122. **Hidalgo A, Schliessmann A, Molina R, Hermoso J, Bornscheuer UT (2008)** A one-pot, simple methodology for cassette randomization and recombination for focused directed evolution. Protein Eng. Des. Sel. 21: 567–576.

123. **Ho WC, Ko WH (1985**) Soil microbiostasis: Effects of environmental and edaphic factors. Soil Biol. Biochem. 17: 167–170.

124. **Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP (1998)** A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene 212: 77–86.

125. **Hocking P J (2001)** Organic acids exuded from roots in phosphorus uptake and aluminum tolerance of plants in acid soils. Adv. Agro. 74: 63-97.

126. **Holms H (2001)** Flux analysis: A basic tool of microbial physiology. Adv. Microbial. Physiol., 45: 271-340.

127. **Huges JC (1998)** The dissolution of North Carolina phosphate rock in some South-Western Australian soils. Fertil. Res. 38: 249–253.

128. **Hwangbo H, Park RD, Kim YW, Rim YS, Park KH, Kim TH, Suth JS, Kim KY (2003)** 2-ketogluconic acid production and phosphate solubilization by *Enterobacter intermedium.* Curr. Microbiol. 47: 87-92.

129. **Iijima M, Griffiths B, Bengough AG (2000)** Sloughing of cap cells and carbon exudation from maize seedling roots in compacted sand. New Phytol. 145: 477-482

130. **Illmer P, Schinner E (1995)** Solubilization of inorganie calcium phosphates solubilization mechanisms. Soil Biol. Biochem. 27: 257-263.

131. **Ishiguro N, Izawa H, Shinagawa M, Shimamoto T, Tsuchiya T (1992)** Cloning and nucleotide sequence of the gene (*citC*) encoding a citrate carrier from several *Salmonella* serovars. J. Biol. Chem. 267: 9559- 9564.

132. **Ishiguro N, Izawa H, Shinagawa M, Shimamoto T, Tsuchiya T** (1992) Cloning and nucleotide sequence of the gene (*citC*) encoding a citrate carrier from several *Salmonella* serovars. J. Biol. Chem. 267: 9559- 9564.

133. **Itaya M, Tsuge K, Koizumi M, Fujita K (2005)** Combining two genomes in one cell: Stable cloning of the *Synechocystis* PCC6803 genome in the *Bacillus subtilis* 168 genome. P. Natl. Acad. Sci. USA 102: 15971–15976.

134. **Jaeger III CH, Lindow SE, Miller W, Clark E, Firestone MK (1999)** Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. Appl. Environ. Microbiol. 65: 2685–2690.

135. **Jones DL (1998)** Organic acids in the rhizosphere - a critical review. Plant Soil 205: 25-44.

136. **Jones DL, Darrah PR (1994)** Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. Plant Soil 166: 247–257.
137. **Kabir MM, Shimizu K (2004)** Metabolic regulation analysis of *icd*-gene knockout *Escherichia coli* based on 2D electrophoresis with MALDI-TOF mass spectrometry and enzyme activity measurements. Appl. Microbiol. Biotechnol. 65: 84–96.
138. **Kallio PT, Kim DJ, Tsai PS, Bailey JE (1994)** Intracellular expression of *Vitreoscilla* hemoglobin alters *Escherichia coli* energy metabolism under oxygen-limited conditions. Eur. J. Biochem. 219:201–8.
139. **Kamilova F, Kravchenko LV, Shaposhnikov AI, Azarova T, Makarova N, Lugtenberg BJ (2006)** Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. Mol. Plant-Microbe Interact. 19: 250-256.
140. **Karpusas M, Branchaud B, Remington SJ (1990)** Proposed mechanism for the condensation reaction of citrate synthase: 1.9-Å structure of the ternary complex with oxaloacetate and carboxymethyl coenzyme A. Biochem 29: 2213-2219.
141. **Kästner CN, Schneider K, Dimroth P, Pos KM (2002)** Characterization of the citrate/acetate antiporter CitW of *Klebsiella pneumoniae.* Arch. Microbiol. 177: 500-506.
142. **Kaur R, Pathania R, Sharma V, Mande S, Dikshit KL (2002)** Chimeric *Vitreoscilla* hemoglobin (VHb) carrying a flavoreductase domain relieves nitrosative stress in Escherichia coli: new insight into the functional role of VHb. Appl. Environ. Microbiol. 68: 152–160.
143. **Kelleher JK (2001)** Flux estimation using isotopic tracers: common ground for metabolic physiology and metabolic engineering. Met. Eng. 3: 100-110.
144. **Khademi Z, Jones DL, Malakouti MJ, Asadi F (2010)** Organic acids differ in enhancing phosphorus uptake by *Triticum aestivum L.* effects of rhizosphere concentration and counterion. Plant and soil 334: 151-159.
145. **Khosla C, Bailey JE (1988a)** Heterologous expression of a bacterial hemoglobin improves the growth properties of recombinant *Escherichia coli.* Nature 331:633–635.

146. **Khosla C, Bailey JE (1988b)** The *Vitreoscilla* hemoglobin gene: molecular cloning, nucleotide sequence and genetic expression in *Escherichia coli*. Mol. Gen. Genet. 214: 158–61.

147. **Khosla C, Curtis JE, DeModena J, Rinas U, Bailey JE (1990)** Expression of intracellular hemoglobin improves protein synthesis in oxygen limited *Escherichia coli*. Biotechnol. 8: 849–53.

148. **Kim CH, Han SH, Kim KY, Cho BH, Kim YH, Koo BS, Kim YC (2003)** Cloning and expression of pyrroloquinoline quinine (PQQ) genes from a phosphate-solubilizing bacterium *Enterobacter intermedium.* Curr. Microbiol. 47: 457–461.

149. **Kim YJ, Sa SO, Chang YK, Hong SK, Hong YS (2007)** Overexpression of *Shinorhizobium meliloti* hemoprotein in *Streptomyces lividans* to enhance secondary metabolite production. J. Microbiol. Biotechnol. 12: 2066–2070.

150. **Koch B, Jensen LE, Nybroe O (2001)** A panel of Tn7based vectors for insertion of the gfp marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. J. Microbiol. Methods 45: 187195.

151. **Koch B, Worm J, Jensen LE, Hojberg O, Nybroe O (2001)** Carbon limitation induces σ**S**-dependent gene expression in *Pseudomonas fluorescens* in soil. Appl. Environ. Microbiol. 67: 3363-3370.

152. **Kodaki T, Katagiri F, Asano M, Izui K, Katsuki H (1985)** Cloning of phosphoenolpyruvate carboxylase gene from a cyanobacerium, *Anacystis nidulans*, in *Escherichia coli*. J. Biochem. **97:** 533-539.

153. **Koffas MA, Roberge CM, Lee K, Stephanopoulos G (1999)** Metabolic Engineering. Annu. Rev. Biomed. Eng. 1: 535-557.

154. **Koning NBJ, Van Ittersum MK, Becx GA, Van Boekel MAJS, Brandenburg WA, Van Den Broek JA, Smies M (2008)** Long-term global availability of food: continued abundance or new scarcity? NJAS-Wageningen J. Life Sci. 55: 229-292.

155. **Kpomblekou AK, Tabatabai MA (1994)** Effect of organic acids on release of phosphorus from phosphate rocks1. Soil Sci. 158: 442-453.

156. **Krishnaraj PU, Goldstein AH (2001)** Cloning of a *Serratia marcescens* DNA fragment that induces quinoprotein glucose dehydrogenase-mediated gluconic acid

production in *Escherichia coli* in the presence of stationary phase *Serratia marcescens*. FEMS Microbiol. Lett. 205: 215–220.

157. **Kubicek CP, Schreferl-Kunar G, Wöhrer, W, Röhr M (1988)** Evidence for a cytoplasmic pathway of oxalate biosynthesis in *Aspergillus niger*. App. Env. microbial. 54: 633-637.
158. **Kucey RMN (1983)** Phosphate solubilizing bacteria and fungi in various cultivated and virgin Alberta soils. Can. J. Soil Sci. 63: 671-678.
159. **Kucey RMN, Janzen HH, Leggett ME (1989)** Microbially mediated increases in plant-available phosphorus. Adv. Agron. 42: 198–228.
160. **Kuduvalli PN, Mitra R, Craig NL (2005)** Site-specific Tn7 transposition into the human genome. Nucleic Acids Res. 33: 857-863.
161. **Kuiper I, Kravchenko L, Bloemberg G V, Lugtenberg BJJ (2002)** Pseudomonas putida strain PCL1444, selected for efficient root colonization and naphthalene degradation, effectively utilizes root exudate components. Mol. Plant-Microbe Interact. 15:734-741.
162. **Kumar C, Yadav K, Archana G, Kumar GN (2013)** 2-Ketogluconic acid secretion by incorporation of *Pseudomonas putida* KT 2440 gluconate dehydrogenase (gad) operon in *Enterobacter asburiae* PSI3 improves mineral phosphate solubilization. Curr. microbial. 1-7.
163. **Kumar R, Rajagopal K (2008)** Single-step overlap-primer-walk polymerase chain reaction for multiple mutagenesis without overlap extension. Anal. Biochem. 377: 105–107.
164. **Kumar V, Behl RK, Narula N (2001)** Establishment of phosphate-solubilizing strains of *Azotobacter chroococcum* in the rhizosphere and their effect on wheat cultivars under greenhouse conditions. Microbiol. Res. 156: 87–93.
165. **Kvist M, Ryabova ES, Nordlander E, Bulow L (2007)** An investigation of the peroxidase activity of *Vitreoscilla* hemoglobin. J. Biol. Inorg. Chem. 12: 324–334.
166. **Laemmli UK (1970)** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
167. **Lakshmi TM, Helling RB (1976)** Selection for citrate synthase deficiency in *icd* mutants of *Escherichia coli*. J. Bacteriol. 127: 76-83.

168. **Lambertsen L, Sternberg C, Molin S (2004)** Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. Environ. Microbiol. 6: 726-732.

169. **Landrain TE, Carrera J, Kirov B, Rodrigo G, Jaramillo A (2009)** Modular model-based design for heterologous bioproduction in bacteria. Curr. Opin. Biotech. 20: 272–279.

170. **Lenz H, Wunderwald P, Eggerer H (1976)** Partial purification and some properties of oxalacetase from *Aspergillus niger*. Eur.J. Biochem. 65: 225-236.

171. **Lessie T, Phibb P (1984)** Alternative pathways of carbohydrate utilization in pseudomonads. Ann. Rev. Microbiol. 38: 359-388.

172. **Liao JC, Hou SY, Chao YP (1996)** Pathway analysis, engineering, and physiological considerations for redirecting central metabolism. Biotechnol. Bioeng. 52: 129-140.

173. **Lin TF, Huang HI, Shen FT, Young CC (2006)** The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by *Burkholderia cepacia* CC-Al74. Biores. Technol 97: 957-960.

174. **Liu SC, Liu YX, Webster DA, Stark BC (1994)** Sequence of the region downstream of the *Vitreoscilla* hemoglobin gene: *vgb* is not part of a multigene operon. Appl. Microbiol. Biotechnol. 42: 304-308.

175. **Liu SC, Liu YX, Webster DA, Wei ML, Stark BC (1996)** Genetic engineering to contain the *Vitreoscilla* hemoglobin gene enhances degradation of benzoic acid by *Xanthomonas mathophillia*. Biotechnol. Bioeng. 49:101–5.

176. **Liu Y, Feng L, Hu H, Jiang G, Cai Z, Deng Y (2012)** Phosphorus release from low-grade rock phosphates by low molecular weight organic acids. J. Food Agri. Environ. 10: 1001- 1007.

177. **Lolkema JS (2006)** Domain structure and pore loops in the 2-hydroxycarboxylate transporter family. J. Mol. Microbiol. Biotechnol. 11: 318-325.

178. **Lugtenberg BJ, Dekkers L, Bloemberg GV (2001)** Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu. Rev. Phytopathol. 39: 461-490.

179. **Lugtenberg BJJ, Dekkers LC (1999)** What makes Pseudomonas bacteria rhizosphere competent? Environ. Microbiol. 1: 9-13.

180. **Lugtenberg BJJ, Kamilova F (2009)** Plant growth promoting rhizobacteria. Ann. Rev. Microbiol. 63: 541-556.

181. **Mailloux RJ, Bériault R, Lemire J, Singh R, Chénier DR, Hamel RD, Appanna VD (2007)** The tricarboxylic acid cycle, an ancient metabolic network with a novel twist. PLoS One 2(8): e690.

182. **Marwah B C (1983)** Partially acidulated rock phosphate as a source of fertilizer phosphorus with special reference to high P-fixing acid soil - A review. Proc. Indian Nat. Sci. Acad. B49: 436–446.

183. **Matsuno K, Blais T, Serio AW, Conway T, Henkin TM, Sonenshein AL (1999)** Metabolic imbalance and sporulation in an isocitrate dehydrogenase mutant of *Bacillus subtilis*. J. Bacteriol. 181: 3382–3391.

184. **Maurus R, Nham T, Nguyen, Stokell DJ, Ayeda A, Hultin PG, Duckworth HW, Brayer GD (2003)** Insights into the Evolution of Allosteric Properties. The NADH Binding Site of Hexameric Type II Citrate Synthases, Biochem. 42: 5555-5565.

185. **Maxwell DP, Bateman DF (1968)** Oxalic acid biosynthesis by *Sclerotium rolfsii*. Phytopathol. 58: 1635-1642.

186. **May M (2009)** Engineering a new business. Nat. Biotechnol. 27: 1112–1120.

187. **Meike B, Nurije M, Andreas K and Michael B** (**2011**) Deletion of the aconitase gene in *Corynebacterium glutamicum* causes strong selection pressure for secondary mutations inactivating citrate synthase. J. Bacteriol. 193: 6864.

188. **Meng C, Ye Q, Shi X, Qiu L, Song S, Guo Y (2002)** Cloning and expression of *Vitreoscilla* hemoglobin in *Streptomyces aureofaciens*. Wei Sheng Wu Xue Bao 42:305–10 [in Chinese].

189. **Metting FB Jr (1992)** Structure and physiological ecology of soil microbial communities. In: FB Metting, Jr., (Eds.) Soil microbial ecology. Marcel Dekker, New York, pp: 3–25.

190. **Munir E, Yoon JJ, Tokimatsu T, Hattori T, Shimada M (2001)** A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete *Fomitopsis palustris*. Proc. Nat. Ac. Sci. 98: 11126-11130.

191. **Nahas E (1996)** Factors determining rock phosphate solubilization by microorganisms. World J. Microbiol. Biotechnol. 12: 562–572.

192. **Nasr MA, Hwang KW, Akbas M, Webster DA, Stark BC (2001)** Effects of culture conditions on enhancement of 2,4-dinitrotoluene degradation by *Burkholderia* engineered with the *Vitreoscilla* hemoglobin gene. Biotechnol. Prog. 17: 359–61.

193. **Nguyen NT, Maurus R, Stokell DJ, Ayed A, Duckworth HW, Brayer GD (2001)** Comparative analysis of folding and substrate binding sites between regulated hexameric type II citrate synthases and unregulated dimeric type I enzymes, Biochem. 40: 13177-13187.

194. **Nielsen J (2001)** Metabolic engineering. Appl. Microbiol. Biotechnol. 55: 263-283.

195. **Nielsen J (2003)** It is all about metabolic fluxes. J. Bacteriol. 185: 7031–7035.

196. **Omar SA (1998)** The role of rock-phosphate-solubilizing fungi and vesicular-arbuscular-mycorrhiza (VAM) in the growth of wheat plants fertilized with rock phosphate. World J. Microbiol. Biotechnol. 14: 211-218.

197. **Oppon JC, Sarnovsky RJ, Craig NL, Rawlings DE (1998)** A Tn7-like transposon is present in the glmUS region of the obligately chemoautolithotrophic bacterium *Thiobacillus ferrooxidans*. J. Bacteriol. 180: 3007-3012.

198. **Ostergaard S, Olsson L, Nielsen J (2000)** Metabolic Engineering of *Saccharomyces cerevisiae.* Microbiol. Mol. Biol. Rev. 64: 34-50.

199. **Otero JM, Nielsen J (2010)** Industrial systems biology. Biotechnol. Bioeng. 105: 439-460.

200. **Ozanne P G (1980)** Phosphate nutrition of plants: A general treatise. *In* The Role of Phosphorus in Agriculture. Eds. FE Khasawneh, EC Sample and EJ Kamprath. Soil Sci. Soc. Am. Madison WI.

201. **Pandey A, Das N, Kumar B, Rinku K, Trivedi P (2008)** Phosphate solubilization by Penicillium sps. isolated from soil sample of Indian Himalayan region. World J. Microbiol. Biotechnol. 24: 97-102.

202. **Papagianni M (2007)** Advances in citric acid fermentation by *Aspergillus niger*: Biochemical aspects, membrane transport and modeling. Biotechnol. Adv. 25: 244-263.

203. **Papagianni M (2012)** Recent advances in engineering the central carbon metabolism of industrially important Bacteria. Microb. Cell Fact. 11: 50.

204. Park KW, Kim KJ, Howard AJ, Stark BC, Webster DA (2002) *Vitreoscilla* hemoglobin binds to subunit I of cytochrome bo ubiquinol oxidases. J. Biol. Chem. 277: 33334–33337.

205. Patel DK, Archana G, Naresh Kumar G (2008) Variation in the nature of organic acid secretion and mineral phosphate solubilization by *Citrobacter* sp. in the presence of different sugars. Curr. Microbiol. 56: 168–174.

206. Patel DK, Murawala P, Archana G, Naresh Kumar G (2011) Repression of mineral phosphate solubilizing phenotype in the presence of weak organic acids in plant growth promoting fluorescent pseudomonads. Biores. Technol. 102: 3055-3061.

207. Patel SM, Stark BC, Hwang KW, Dikshit KL, Webster DA (2000) Cloning and expression of *Vitreoscilla* hemoglobin gene in Burkholderia sp. strain DNT for enhancement of 2,4-dinitrotoluene degradation. Biotechnol. Prog. 16: 26–30.

208. Paul EA, Clark FE (1988) Soil microbiology and biochemistry. Academic Press, Inc., San Diego, Calif.

209. Pedersen H, Hjort C, Nielsen, J (2000) Cloning and characterization of *oah*, the gene encoding oxaloacetate hydrolase in *Aspergillus niger*. Mol Gen Genetics 263: 281-286.

210. Pereira DS, Donald LJ, Hosfield DJ, Duckworth HW (1994) Active site mutants of Escherichia coli citrate synthase. Effects of mutations on catalytic and allosteric properties, J. Biol. Chem. 269: 412-417.

211. Peterson GL (1979) Review of the Folin phenol quantitation method of Lowry, Rosenberg, Farr and Randall. Anal. Biochem. 100: 201.

212. Petrarulo M, Facchini P, Cerelli E, Marangella M, Linari F (1995) Citrate in urine determined with a new citrate lyase method. Clin. Chem. 41: 1518–1521.

213. Piccini D, Azcon R (1987) Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizal fungi on the utilization of Bayovar rock phosphate by alfalfa plants using a sand-vermiculite medium. Plant and soil 101: 45-50.

214. Pitera DJ, Paddon CJ, Newman JD, Keasling JD (2007) Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. Metab. Eng. 9: 193–207.

215. Pósfai G, Plunkett G, Fehér T, Frisch D, Keil GM, et al. (2006) Emergent properties of reduced-genome *Escherichia coli*. Science 312: 1044–1046.

216. Postma J, van Elsas JD, Govaert JM, van Veen JA (1988) The dynamics of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil as determined by immunofluorescence and selecting plating techniques. FEMS Microbiol. Ecol. 53: 251–260.

217. Pramanik J, Keasling JD (1997) Stoichiometric model of Escherichia coli metabolism: Incorporation of growth-rate dependent biomass composition and mechanistic energy requirements. Biotechnol. Bioeng. 56: 398-421.

218. Price ND, Papin JA, Schilling CH, Palsson BO (2003) Genome-scale microbial in silico models: the constraints-based approach. Trends Biotechnol. 21: 162-169.

219. Quan J, Tian J (2011) Circular polymerase extension cloning for high-throughput cloning of complex and combinatorial DNA libraries. Nature Protocol. 6 (2): 242- 251.

220. Quayle JR, Taylor GA (1961) Carbon assimilation by *Pseudomonas oxalaticus* (OX1). 5. Purification and properties of glyoxylic dehydrogenase. Biochem. J. 78: 611.

221. Rajan SSS, Watkinson JH, Sinclair AG (1996) Phosphate rocks for direct application to soils. Adv. Agron. 77: 57–159.

222. Ramakrishna N, Lacey J, Smith JE (1991) Effect of surface sterilization, fumigation and gamma irradiation on the microflora and germination of barley seeds. Int. J. Food Microbiol. 13: 47-54.

223. Ramandeep D, Hwang KW, Raje M, Kim KJ, Stark BC, Dikshit KL, Webster DA (2001) Vitreoscilla hemoglobin: intracellular localization and binding to membranes. J Biol Chem 276: 24781–24789.

224. Ramírez M, Valderrama B, Arredondo-Peter R, Soberón M, Mora M, Hernández G (1999) *Rhizobium etli* genetically engineered for the heterologous expression of *Vitreoscilla* sp. hemoglobin: effects on free-living and symbiosis. Mol. Plant Microb. Interact. 12: 1008-1015.

225. Ramos C, Molbak L, Molin S (2000) Bacterial activity in the rhizosphere analyzed at the single-cell level by monitoring ribosome contents and synthesis rates. Appl. Environ. Microbiol. 66: 801-809.

226. Reed JL, Palsson BO (2003) Thirteen years of building constraint-based *in silico* models of *Escherichia coli*. J. Bacteriol. 185: 2692-2699.

227. Reetz MT, Carballeira JD (2007) Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes. Nat Protoc 2: 891–903.

228. Reznikoff WS (2008) Transposon Tn5. Annu. Rev. Genet. 42: 269-286.

229. Richardson AE (1994) Soil micro-organisms and phosphate availability. *In* Soil Biota Management in Sustainable Agriculture. Eds. CE Pankhurst, BM Doube, VVSR Gupts and PR Grace pp. 50–62. CSIRO, Melbourne, Australia.

230. Richardson AE, Simpson RJ (2011) Soil microorganism mediating phosphorus availability. Plant Physiol 156: 989-996.

231. Rinaldi AC, Bonamore A, Macone A, Boffi A, Bozzi A, Di Giulio A (2006) Interaction of *Vitreoscilla* hemoglobin with membrane lipids. Biochem. 45: 4069–4076.

232. Rodríguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion, Biotechnol Adv 17: 319-339.

233. Rohlin L, Oh MK, Liao JC (2001) Microbial pathway engineering for industrial processes: evolution, combinatorial biosynthesis and rational design. Curr. Opin. Microbiol., 4: 330-335.

234. Rohr M, Kubicek CP, Kominek J (1996) Citric acid. In: Rohr, M. ed. Biotechnology (Vol. 6). Wiley-VCH. Weinheim, New York, 307-366.

235. Roos V, Andersson CI, Arfvidsson C, Wahlund KG, Bülow L (2002) Expression of double *Vitreoscilla* hemoglobin enhances growth and alters ribosome and tRNA levels in *Escherichia coli*. Biotechnol. Prog. 18: 652–656.

236. Ruijter GJ, Panneman H, Visser J (1997) Overexpression of phosphofructokinase and pyruvate kinase in citric acid-producing *Aspergillus niger*. Biochim. Biophys. Acta. 1334: 317-326.

237. Ruijter GJ, Panneman H, Xu D, Visser J (2000) Properties of *Aspergillus niger* citrate synthase and effects of *citA* overexpression on citric acid production. FEMS Microbiol Lett 184: 35-40.

238. Ruijter GJ, van de Vondervoort PJ, Visser J (1999) Oxalic acid production by Aspergillus niger: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiol. 145: 2569-2576.

239. Sambrook J, Russell DW (2001) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

240. **Sauer U, Lasko DR, Fiaux J, Hochuli M, Glaser R, Szyperski T, Wüthrich K, Bailey JE (1999)** Metabolic flux ratio analysis of genetic and environmental modulations of *Escherichia coli* central carbon metabolism. J. Bacteriol., 181: 6679-6688.

241. **Schmid J, Meyer V, Sieber V (2011)** Scleroglucan: biosynthesis, production and application of a versatile hydrocolloid. App. Microbiol Biotechnol. 91: 937-947.

242. **Schmidt-Dannert C (2001)** Directed evolution of single proteins, metabolic pathways, and viruses. Biochem. 40: 13125–13136.

243. **Schmidt-Eisenlohr H, Gast A, Baron C (2003)** Inactivation of gacS does not affect the competitiveness of *Pseudomonas chlororaphis* in the *Arabidopsis thaliana* rhizosphere. App. Env. Microbiol. 69: 1817-1826.

244. **Schutter ME, Dick RP (2001)** Shifts in substrate utilization potential and microbial community structure in response to carbon substrates. Soil Biol. Biochem. 33: 1481-1491.

245. **Schweizer, H. P. (1991)** *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene 97:109-112.

246. **Serre PA (1969)** Citrate synthase. Methods Enzymol. 13: 3–11.

247. **Shao Z, Zhao H, Zhao H (2009)** DNA assembler, an *in vivo* genetic method for rapid construction of biochemical pathways. Nucleic Acids Res. 37: e16.

248. **Shapiro JA (1979)** Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. Proc. Natl. Acad. Sci. U.S.A. 76: 1933-1937.

249. **Sharma V, Archana G, Naresh Kumar G (2011)** Plasmid load adversely affects growth and gluconic acid secretion ability of mineral phosphate-solubilizing rhizospheric bacterium *Enterobacter asburiae* PSI3 under P limited conditions. Microbiol. Res. 166: 36-46.

250. **Sharma V, Kumar V, Archana G, Naresh Kumar G (2005)** Substrate specificity of glucose dehydrogenase (GDH) of *Enterobacter asburiae* PSI3 and rock phosphate solubilization with GDH substrates as C sources. Can. J. Microbiol. 51: 477–482.

251. Sharpley AN, Jones CA, Gray C, Cole CV (1984) A simplified soil and plant phosphorus model: II. Prediction of labile, organic, and sorbed phosphorus. Soil Sci. Soci. Am. J. 48: 805-809.

252. Shashidhar B, Podile AR (2009) Transgenic expression of glucose dehydrogenase in *Azotobacter vinelandii* enhances mineral phosphate solubilization and growth of sorghum seedlings. Microbiol. Biotechnol. 4: 521-529.

253. Shen H, Gold SE, Tamaki SJ, Keen NT (1992) Construction of a Tn7-lux system for gene expression studies in Gram-negative bacteria. Gene 122: 27 -34.

254. Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon muta-genesis in gram negative bacteria. Bio/Technology 1: 784–791.

255. Simons M, Van Der Bij AJ, Brand I, De Weger LA, Wijffelman CA, Lugtenberg BJ (1996) Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting Pseudomonas bacteria. Mol. Plant Micro. Int. 9: 600-607.

256. Singh R, Lemire J, Mailloux RJ, Chénier D, Hamel R, Appanna VD (2009) An ATP and oxalate generating variant tricarboxylic acid cycle counters aluminum toxicity in *Pseudomonas fluorescens*. PLoS One 4(10): e7344.

257. So J, Webster DA, Stark BC, Pagilla KR (2004) Enhancement of 2, 4- dinitrotoluene biodegradation by *Burkholderia* sp. in sand bioreactors using bacterial hemoglobin technology. Biodegradation **15:** 161–71.

258. Sobczak I, Lolkema JS (2005) The 2-hydroxycarboxylate transporter family: physiology, structure, and mechanism. Microbiol. Mol. Biol. Rev. 69: 665-695.

259. Sridevi M, Mallaiah KV, Yadav NCS (2007) Phosphate solubilization by *Rhizobium* isolates from *Crotalaria species*. J. Plant Sci. 2: 635-639.

260. Srivastava S, Kausalya MT, Archana G, Rupela OP, Naresh Kumar G (2006) Efficacy of organic acid secreting bacteria in solubilization of rock phosphate in acidic alfisols. In Velazquez E, Rodriguez-Barrueco C, editors. Developments in Plant and Soil Sciences, Springer, 102, pp. 117-124.

261. Stark BC, Dikshit KL, Pagilla KR (2011) Recent advances in understanding the structure, function and biotechnological usefulness of the hemoglobin from the bacterium *Vitreoscilla*. Biotechnol. Lett. 33: 1705-1714.

262. Stark BC, Dikshit KL, Pagilla KR (2012) The Biochemistry of Vitreoscilla hemoglobin. Comp. Str. Biotechnol. J. 3.

263. Stark MJR (1987) Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli.* Gene. 51: 255-267.

264. Stephanopoulos G (1999) Metabolic fluxes and metabolic engineering, Met. Eng. 1: 1-11.

265. Stephanopoulos G, Sinskey AJ (1993) Metabolic engineering-methodologies and future prospects. Trends Biotechnol. 11: 392-396.

266. Stephanopoulos G, Vallino JJ (1991) Network rigidity and metabolic engineering in metabolite overproduction. Science 252: 1675-1681.

267. Steuber J, Krebs W, Bott M, Dimroth P (1999) A membrane-bound NAD(P)+-reducing hydrogenase provides reduced pyridine nucleotides during citrate fermentation by *Klebsiella pneumoniae*. J Bacteriol. 181:241-245.

268. Stokell DJ, Lynda JD, Maurus R, Nguyen NT, Sadler G, Choudhary K, Hultin PG, Brayer GD, Duckworth HW (2003) Probing the roles of key residues in the unique regulatory NADH binding site of type II citrate synthase of *Escherichia coli*. J Biol Chem. 278: 35435–35443.

269. Stover CK, Pham, XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FSL et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406: 959-964.

270. Subba Rao NS (1982) *In* Advances in Agricultural Microbiology. Ed. NS Subba Rao. pp. 229–305. Oxford and IBH Publ. Co.

271. Suthar DH, Chattoo BB (2006) Expression of *Vitreoscilla* hemoglobin enhances growth and levels of alpha-amylase in *Schwanniomyces occidentalis*. Appl. Microbiol. Biotechnol. 72: 94–102.

272. Tandon HLS (1987) Phosphorus Research and Production in India. Fertilizer Development and Consultation Organization New Delhi. pp.160

273. Thomas GV (1895) Occurrence and ability of phosphate-solubilizing fungi from coconut plant soils. Plant Soil 87: 357-364.

274. Thykaer J, Nielsen J (2003) Metabolic engineering of β-lactam production. Met. Eng. 5: 56-69.

275. Tong EK, Duckworth HW (1975) The quaternary structure of citrate synthase from Escherichia coli K12. Biochem. 14: 235-241.

276. Trofymow JA, Coleman DC, Cambardella C (1987) Rates of rhizodeposition and ammonium depletion in the rhizosphere of axenic oat roots. Plant Soil 97: 334-344.

277. Tsai PS, Nageli M, Bailey JE (2002) Intracellular expression of *Vitreoscilla* hemoglobin modifies microaerobic Escherichia coli metabolism through elevated concentration and specific activity of cytochrome O. Biotechnol. Bioeng. 79: 558–67.

278. Underwood SA, Buszko ML, Shanmugam KT, Ingram LO (2002) Flux through citrate synthase limits the growth of ethanologenic *Escherichia coli* KO11 during xylose fermentation. Appl. Environ. Microbiol. 68: 1071–1081.

279. Urgun-Demirtas M, Pagilla KR, Stark BC (2004) Enhanced kinetics of genetically engineered *Burkholderia cepacia:* the role of vhb in the hypoxic metabolism of 2-CBA. Biotechnol. Bioeng. 87: 110–118.

280. Urgun-Demirtas M, Pagilla KR, Stark BC, Webster DA (2003) Biodegradation of 2-chlorobenzoate by recombinant *Burkholderia cepacia* expressing *Vitreoscilla* hemoglobin under variable levels of oxygen availability. Biodegradation 14: 357–365.

281. Valentini M, Storelli N, Lapouge K (2011) Identification of C4-Dicarboxylate Transport Systems in Pseudomonas aeruginosaPAO1. J. Bacteriol. 193: 4307-4316.

282. Vallino JJ, Stephanopoulos G (1993) Metabolic flux distributions in Corynebacterium glutamicum during growth and lysine overproduction. Biotechnol. Bioeng. 41: 633-646.

283. van Elsas JD, Dijkstra AF, Govaert JM, van Veen JA (1986) Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Microbiol. Ecol. 38: 151-160.

284. Van Kauwenbergh SJ (2010) World phosphate rock reserves and resources technical bulletin IFDC. 75.

285. Van Veen JA, Leonard S, Van Overbeek LS, Van Ellsas JD (1997) Fate and activity of microorganisms introduced into soil. Microbiol. Mol. Biol. Res. 61: 121-135

286. Van Vuuren DP, Bouwman AF, Beusen AHW (2010) Phosphorus demand for the 1970–2100 period: a scenario analysis of resource depletion. Glob. Env.change. 20: 428-439.

287. Vanbleu E, Marchal K, Vanderleyden J (2004) Genetic and physical map of the pLAFR1 vector. DNA Seq. 15: 225-227.

288. Vance CP, Graham, PH, Allan DL (2000) Biological nitrogen fixation: Phosphorus Ba critical future need? p. 509-518. *In*: F.O. Pederosa, M. Hungria, M.G. Yates, and W.E. Newton (ed.), Nitrogen fixation from molecules to crop productivity. Kluwer Academic Publishers, Dordrecht, Netherlands.

289. Varma A, Palsson BO (1994) Metabolic Flux Balancing: Basic Concepts, Scientific and Practical Use. Bio/technology 12.

290. Vassilev N, Irena F, Maria V, Azcon R (1996) Improved plant growth with rock phosphate solubilized by *Aspergillus niger* grown on sugar-beet waste. Biores. Technol. 55: 237– 241.

291. Vassileva M, Vassilev N, Azcon R (1998) Rock phosphate solubilisation by *Aspergillus niger* on olive cake-based medium and its further application in a soil-plant system. World J. Microbiol. Biotechnol. 14: 281–284.

292. Vassileva V, Demirevska K, Simova-Stoilova L, Petrova T, Tsenov N, Feller U (2012) Long-term field drought affects leaf protein pattern and chloroplast ultrastructure of winter wheat in a cultivar-specific manner. J. Agr. Crop Sci. 198: 104-117.

293. Vemuri, GN, Aristidou AA (2005) Metabolic engineering in the-omics era: elucidating and modulating regulatory networks. Microbiol. Mol. Bio. Rev. 69: 197-216.

294. Viollier PH, Nguyen KT, Minas W, Folcher M, Dale GE, Thompson CJ (2001) Roles of aconitase in growth, metabolism, and morphological differentiation of *Streptomyces coelicolor*. J. Bacteriol. 183: 3193–3203.

295. Von Uexküll HR, Mutert E (1995) Global extent, development and economic impact of acid soils. Plant and soil. 171: 1-15.

296. Vyas P, Gulati A (2009) Organic acid production *in vitro* and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. BMC Microbiol. 9: 174.

297. Wang Z, Xiang L, Shao J, Węgrzyn A, Węgrzyn G (2006) Effects of the presence of ColE1 plasmid DNA in Escherichia coli on the host cell metabolism. Micro. cell fact. 5: 34.

298. Wani PA, Khan MS, Zaidi A (2007a) Effect of metal tolerant plant growth promoting *Bradyrhizobium* sp. (vigna) on growth, symbiosis, seed yield and metal uptake by green gram plants. Chemosphere 70: 36-45.

299. Wani PA, Khan MS, Zaidi A (2007b) Cadmium, chromium and copper in greengram plants. Agron. Sustain. Dev. **27**: 14-153.

300. Watanabe T, Shitan N, Suzuki S, Umezawa T, Shimada M, Yazaki K, Hattori T (2010) Oxalate efflux transporter from the brown rot fungus *Fomitopsis palustris*. App. Env. Microbiol. 76: 7683-7690.

301. Webster DA, Hackett DP (1966) The purification and properties of cytochrome O from *Vitreoscilla*. J. Biol. Chem. 241: 3308–3315.

302. Weitzman PD (1966) Regulation of citrate synthase in *Escherichia coli.* Biochim. Biophys. Acta. 128 (1): 213-215.

303. Weitzman PD, Danson MJ (1976) Citrate synthase. Current topics in cellular regulation, 10: 161.

304. Wen Y, Song Y, Li JL (2001) The effects of Vitreoscilla hemoglobin expression on growth and antibiotic production in *Streptomyces cinnamonensis*. Sheng Wu Gong Cheng Xue Bao 17:24–8 [in Chinese]

305. West SE, Schweizer HP, Dall C, Sample AK, Runyen-Janecky LJ (1994) Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **148**: 81-86.

306. Whipps JM (1990) *Carbon economy. In: Lynch JM, (Eds).* The rhizosphere. *Chichester: Wiley,* 59*–97.*

307. Whitelaw MA (2000) Growth promotion of plants inoculated with phosphate solubilizing fungi. Adv. Agron. 69: 100-151.

308. Wolschek, MF, Kubicek CP (1999) Biochemistry of citric acid accumulation by *Aspergillus niger*. Citric acid biotechnology. Taylor & Francis, London, 11.

309. Xiong X, Xing J, Li X, Bai X, Li W, Li Y, Liu H (2007) Enhancement of biodesulfurization in two-liquid systems by heterogeneous expression of *Vitreoscilla* hemoglobin. Appl. Environ. Microbiol. 73: 2394–2397.

310. Xu RK, Zhu YG, Chittleborough D (2004) Phosphorus release from phosphate rock and an iron phosphate by low-molecular-weight organic acids. J. Environ. Sci. 16: 5- 8.

311. Yan ZY, Xing GM, Li ZX (2004) Quantitative determination of oxalic acid using victoria blue B based on a catalytic kinetic spectrophotometric method. Microchimica Acta, 144: 199-205.

312. Yarzábal LA (2010) Agricultural development in tropical acidic soils: potential and limits of phosphate-solubilizing bacteria. In Soil Biology and Agriculture in the Tropics pp. 209-233. Springer Berlin Heidelberg.

313. Ye L, Jia Z, Jung T, Maloney PC (2001) Topology of OxlT the oxalate transporter of *Oxalobacter formigenes*, determined by site-directed fluorescence labeling. J. Biol. Chem. 183: 2490-2496.

314. Yoon JJ, Hattori T, Shimada M (2002) A metabolic role of the glyoxylate and tricarboxylic acid cycles for development of the copper-tolerant brown-rot fungus Fomitopsis palustris. FEMS microbial. Lett. 217: 9-14.

315. Zaidi A, Khan MS, Ahemad M, Oves M, Wani PA (2009) Recent advances in plant growth promotion by phosphate-solubilizing microbes. In Khan, M. S., Zaidi, A., and Musarrat, J., (Eds.), Microbial Strategies for Crop Improvement, Springer, Berlin, pp: 23-50.

316. Zhang L, Li Y, Wang Z, Xia Y, Chen W, Tang K, (2007) Recent developments and future prospects of *Vitreoscilla* hemoglobin applications in metabolic engineering. Biotechnol. Adv. 25:123–136.

317. Zhang W, Xie H, He Y, Feng J, Gao W, Gu Y, Song C (2013) Chromosome integration of the *Vitreoscilla* hemoglobin gene (*vgb*) mediated by temperature-sensitive plasmid enhances γ-PGA production in *Bacillus amyloliquefaciens*. FEMS microbial. lett.

318. Zhao HM, Chockalingam K, Chen ZL (2002) Directed evolution of enzymes and pathways for industrial biocatalysis. Curr. Opin. Biotech. 13: 104–110.

319. Zupke C, Stephanopoulos G (1994) Modeling of isotope distributions and intracellular fluxes in metabolic networks using atom mapping matrices. Biotechnol. Prog. 10: 489-498.