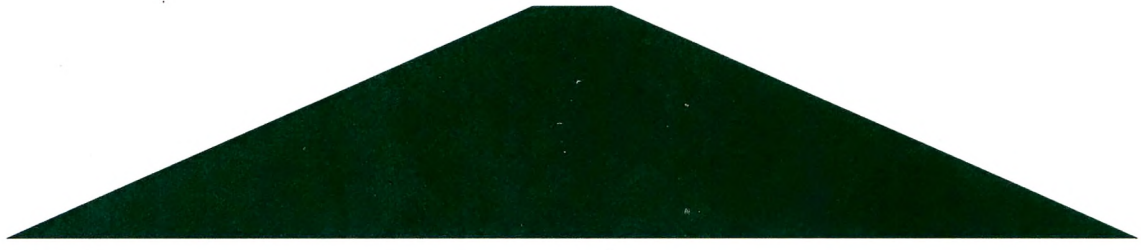
INTRODUCTION AND REVIEW OF LITERATURE



CHAPTER 1

1 INTRODUCTION

"Recall the face of the poorest and the weakest man whom you have seen, and ask yourself, If the steps you contemplate are going to be of any use to him. Will he gain anything by it? Will it restore to him control over his own life and destiny?"

Mahatma Gandhi

1.1 Metabolic engineering

Metabolic engineering can be defined as purposeful modification of cellular metabolism using recombinant DNA and other molecular biological techniques (Bailey 1991; Lee et al. 2009). Metabolic engineering considers metabolic and cellular system as an entirety and accordingly allows manipulation of the system with consideration of the efficiency of overall bioprocess, which distinguishes itself from simple genetic engineering (Stephanopoulos et al., 1998; Lee, S.Y. et al., 1999). Furthermore, metabolic engineering is advantageous in several aspects, compared to simple genetic engineering or random mutagenesis, since it allows defined and directed engineering of the cell, thus avoiding unnecessary changes to the cell and allowing further engineering if necessary.

1.1.1 Modern metabolic engineering: converting microbes to a chemical factory

Development of recombinant DNA technology in the post genomic era has accumulated staggering volume of gene, protein and metabolite data. The cost of oligonucleotide synthesis has exponentially declined and more precise techniques for studying cellular metabolism have been developed. Empowered by these developments the focus of metabolic engineering research has gradually been shifted from perturbing individual pathways to manipulating the entire cell itself giving rise to the concept of system metabolic engineering converting microbes to a chemical factory (Yadav et al., 2012).

Microbes have naturally evolved enzymes and pathways that can convert biomass into hundreds of unique chemical structures, a property that can be effectively exploited for

their engineering into Microbial Chemical Factories (MCFs). The first step in engineering novel or natural pathways for MCFs is to identify potential natural cell metabolites or biomass derived feedstock's that can serve as starting materials and the series of biochemical reactions required to convert these into the desired product. Martin et al., (2009) have reviewed some of the computational tools available for identifying and selecting from the multiple possible pathways connecting different starting materials to a product of interest. Once a pathway is selected, appropriate natural enzymes expected to catalyze pathway reactions need to be selected using enzyme information from various databases. *In silico* approaches such as protein BLAST searches and molecular docking may help in such enzyme selection. Further pathway optimization to enhance product titers relies on an integrated approach composed of (1) metabolic engineering to enhance precursor metabolite availability using gene knockouts and enzyme expression level manipulation, (2) protein engineering to enhance pathway enzyme specificity and activity and (3) cofactor balancing via effective cofactor (NADH/NADPH) recycling (**Fig. 1.1**).

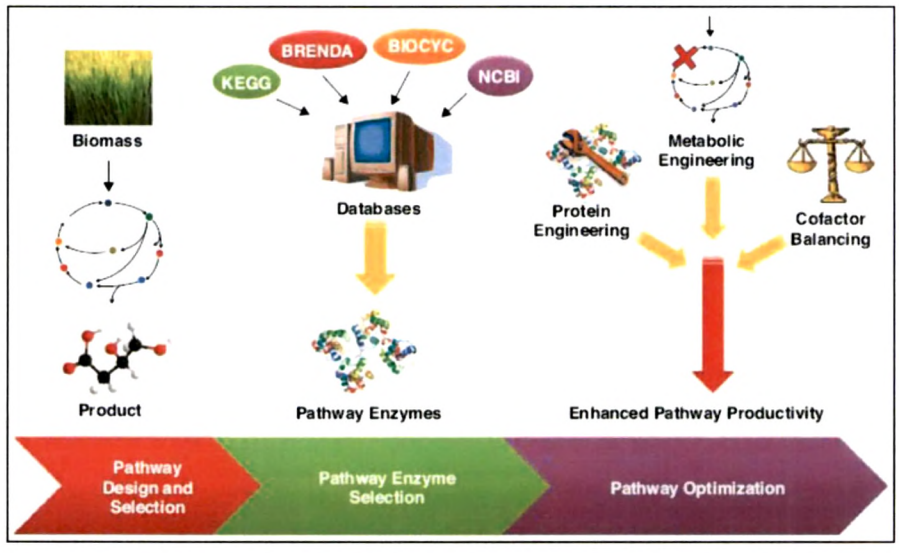


Figure 1.1: Design and engineering of pathways for microbial chemical factories (MCFs) (Dhamankar et al., 2011).

A number of research groups have employed combinations of these strategies towards developing novel pathways and enhancing productivities of already established pathways for the microbial synthesis of a number of value added biochemicals to name few

polymer and pharmaceutical building blocks putrescine, Cadaverine, succinic acid (Raab et al., 2010; Blankschien et al., 2010; Qian et al.,2009; 2011), 3-hydroxybutyric acid and 3-hydroxyvaleric acid (Tseng et al.,2009; 2010), native silk protein, the high value flavoring agent natural vanillin (Hansen et al., 2009; Lee et al., 2009; Xia et al., 2010), pharmaceutical drug precursors such as taxadiene, amorpha-4,11-diene and D-glucaric acid (Anthony et al., 2009; Ajikumar et al., 2010; Moon et al., 2010) and plant secondary metabolites such as flavanoids, stilbenoids and isoprenoids (Trantas et al., 2009; Asadollahi et al., 2010; Stephanopolous et al., 2011).

1.1.2 Strategies of Metabolic engineering for Strain improvement

Productivity of microbes isolated from nature is generally low. Genetic and metabolic engineering strategies have been used increasingly to modify or introduce new cellular or metabolic capabilities. Conventional metabolism-oriented engineering strategies often fail to obtain expected phenotypes owing to focusing narrowly on targeted metabolic capabilities while neglecting microbial physiological responses to environmental stresses. To meet the new challenges posed by the biotechnological production of fuels, chemicals and materials, microbes should exert strong physiological robustness and fitness, in addition to strong metabolic capabilities, to enable them to work efficiently in actual bioprocesses or in various environmental conditions.

Before 1970, microbes were used mainly in the traditional brewing and fermentation industries, such as the production of soy sauce, cheese, alcohol, antibiotics and other natural products. The production strains used were either selected from nature or mutated physically and chemically. Since the 1970s, with the development of recombinant DNA technology, scientists began to engineer microbes to meet desired requirements using genetic engineering, which enabled the introduction of novel microbial metabolic pathways. Among the products produced biotechnologically were recombinant pharmaceuticals and their precursors, antibiotics, amino acids and enzymes. Since the 1990s, the concept of metabolic engineering has enabled further improvements in microbial engineering and facilitated the broadening of substrates spectra, enabling improved titers and yields, heterologous protein production, engineering pathways for xenobiotic degradation, as well as the synthesis of

new bioproducts (Zhang et al., 2009). Metabolically engineered microbes have driven the rapid development of the biotechnological production of fine-chemicals, amino acids and biodegradable polymers. More recently, increasing food demands together with impending energy crises and environmental pollution, have driven the application of microbes for the production of biofuels, bulk-chemicals and biomaterials from renewable non-food biomass, which requires further improvements of the microbes used to increase product titer and productivity and to decrease the incurring production cost (Zhang et al., 2010). Since the 1990s, metabolically engineered microbes have been applied extensively in the production of organic acid, amino acids (Wendisch et al., 2006), sugar alcohols (Akinterinwa et al., 2008), biofuels (Atsumi et al., 2008; Lawrence et al., 2011; Zhang et al., 2011) and pharmaceuticals (Chartrain et al., 2000). Recent developments in '-omics' technologies have powered metabolic engineering strategies from the level of a local pathway to that of the global metabolic network (**Fig. 1.2**).

Advances in metabolic engineering combined with various '-omics' approaches (Park et al., 2008) have contributed to improving cellular metabolic activities to achieve a more efficient biotechnological production of target products (Lee et al., 2005; 2006; Park et al., 2007).

The term 'physiological engineering' was first used by Jens Nielsen in 1997 in relation to penicillin production by Penicillium chrysogenum (Nielsen et al., 1997). At that time, physiological engineering referred to understanding the function of important pathways in microorganisms by using an integrated approach of microbial physiology and bioreaction engineering. It involved metabolic flux analysis, metabolic control analysis and kinetic modeling to generate fundamental knowledge for metabolic engineering that was based on reproducible cultivation experiments and reliable measurements. During the past decade, metabolic engineering has become the central approach for strain improvement, mainly targeted at improving specific metabolic capabilities. However, the main physiological characteristics related to industrial and agriculture applications applications including microbial metabolic capability, insensitivity of pathway key enzymes to end-

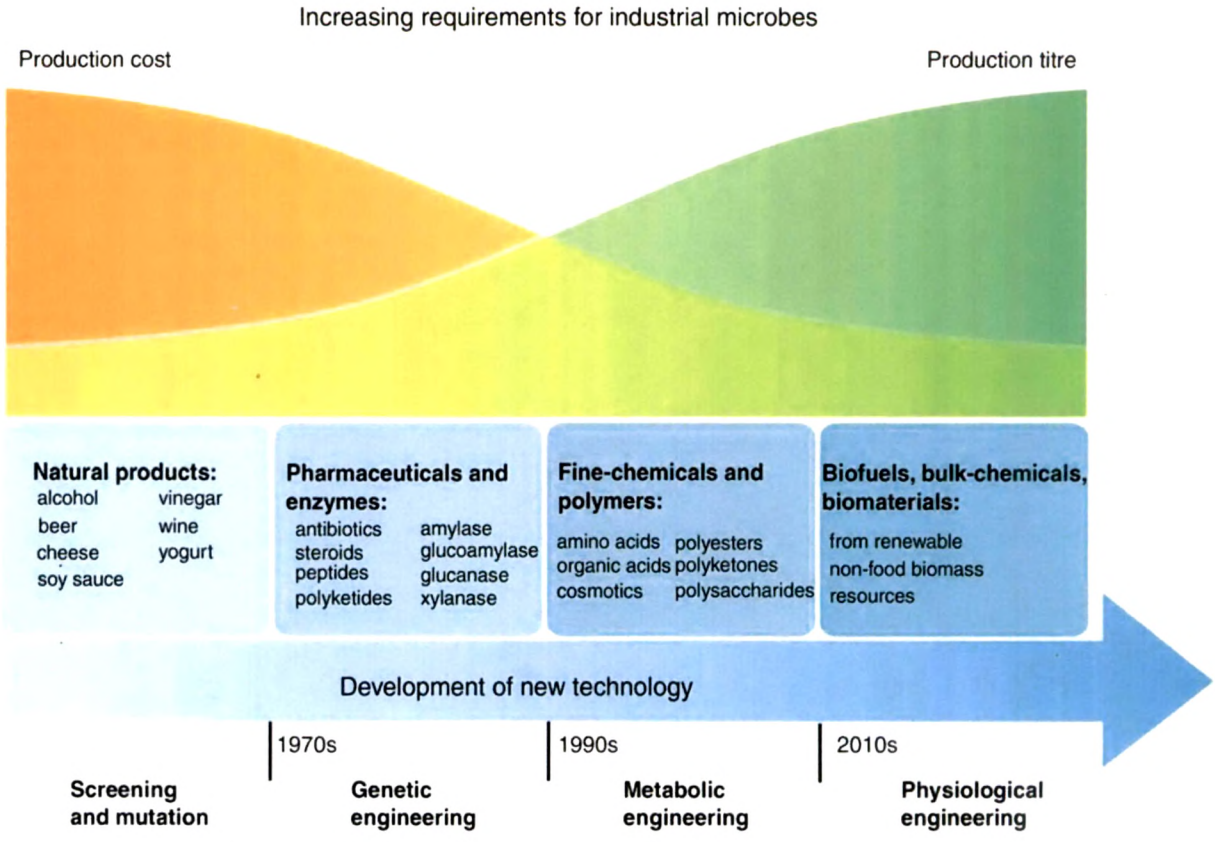


Figure 1.2: Recent advances in the engineering of microbes (Zhang et al., 2011).

1.1.3 Physiological engineering: New evolving technology for strain improvement

product inhibition or feedback repression, robustness under adverse environmental perturbations, tolerance of high concentration substrates or metabolites, and fitness throughout the entire biological cycle are also important for efficient biotechnological production of fuels, chemicals, materials and performance in ecohabitats. In this context, an evolved concept of physiological engineering comes into picture which refers to a strategy of strain improvement with the aim of either improving existing or engineering novel functionalities into microbes (Tyo et al., 2009). Different from the conventional metabolism-oriented engineering strategy, such a strategy focuses primarily on the physiological status of microbial cells and on the physiological functionality related to the actual biotechnological production processes. Therefore, this strategy aims not only to improve microbial metabolic activities at a specific physiological status, but also to further

investigate the molecular mechanisms underpinning the desired physiological characteristics.

1.1.4 Successful examples of metabolic engineering: Recent reports

Recent advances in system biology and synthetic biology approaches are providing support to metabolic engineering to become a growing tree of chemical diversity (**Fig. 1.3**) at the whole cell level particularly of microorganisms. Systems biology merged with metabolic engineering principles is quickly expanding the chemical palate of cells. Our capacity to engineer cells is becoming a competing force against traditional synthetic organic chemistry and heterogeneous catalysis. These new microbial factories have the capability to produce countless products, with new ones being added constantly. Synergies with the fields of synthetic biology have enabled new-found technologies for *de novo* design of enzymes, altering gene expression and creating novel network regulation completely unrelated to the native cellular regulatory network (Jiang et al., 2008; Young and Alper, 2010; Zastrow et al., 2012).

1.2 Engineering the central carbon metabolism

The term "central carbon metabolism" (CCM) describes the integration of pathways of transport and oxidation of main carbon sources inside the cell. In most bacteria, the main pathways of the CCM are those of the phosphotransferase system (PTS), glycolysis, gluconeogenesis, pentose phosphate (PP) pathway, and the tricarboxylic acid cycle (TCA) with the glyoxylate bypass. As a whole, the system has a complex structure and it is regulated by complex networks of reactions. The knowledge about regulation in CCM has great industrial relevance as it may allow the engineering of selected metabolic steps to reroute carbon fluxes toward precursors for industrially important metabolites (Nielsen, 2011). This kind of metabolic engineering however is a difficult task as the knowledge is incomplete regarding the regulation of central carbon metabolism flux for many industrially and agriculturally important bacteria.

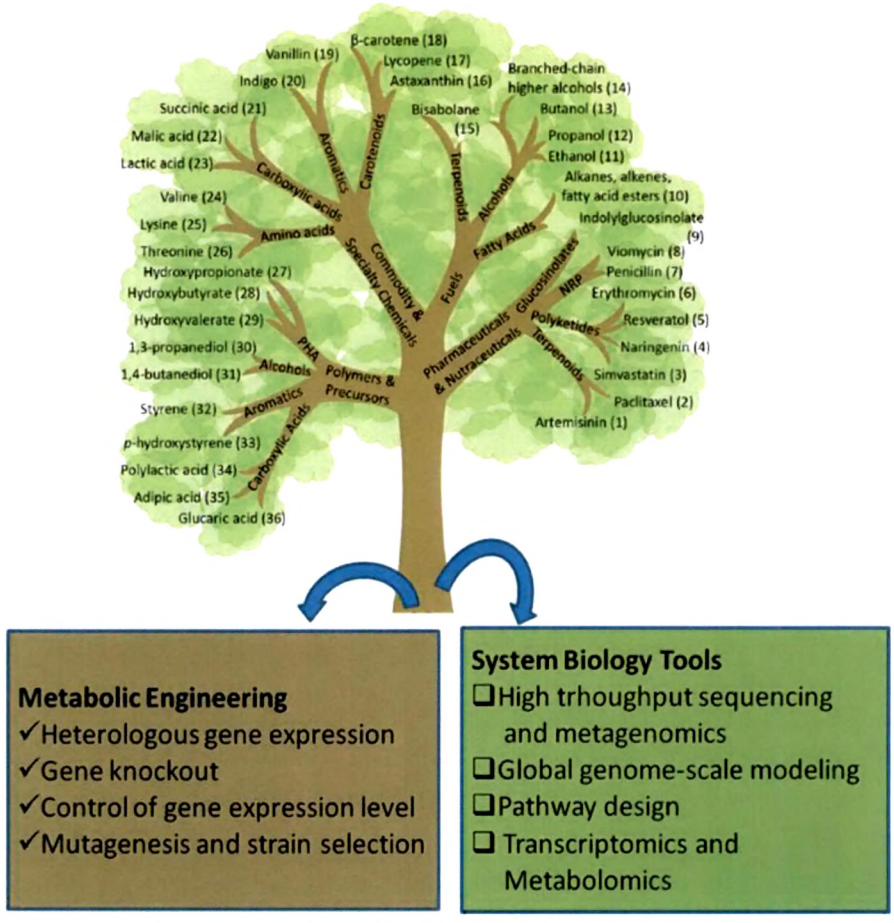


Figure 1.3: Metabolic engineering and system biology converting microbes as cell factory. Numbers correspond to the list of references. Abbreviations: NRP; nonribosomal peptide, PHA; polyhydroxyalkanoate (Modification of the figure from Curran et al., 2012).

1.2.1 *Escherichia coli*

E. coli, a Gram-negative bacterium, is being used widely today in a large number of biotechnological processes. The ease of cultivation as it grows quickly in minimal medium, as well as its ability to metabolize both pentoses and hexoses (Zaldivar et al., 2001), have made it the bacterium of choice for research and over the years the wealth of information in genomics, proteomics and metabolism have led it to be regarded as the prime prokaryotic model (Kadir et al., 2010). The CCM of *E. coli* and specifically the metabolism of glucose are intensively studied and well known topics (Sauer et al., 2005); Shiloach et al., 2009). Glucose metabolism starts with its uptake via the PTS and proceeds with several interconnected pathways with the major being: glycolysis, gluconeogenesis, the pentose-

monophosphate bypass with the Entner-Dudoroff pathway, the TCA cycle with the glyoxylate bypass, anaplerotic reactions and acetate production and assimilation and shikimic acid pathway (**Fig. 1.4**).

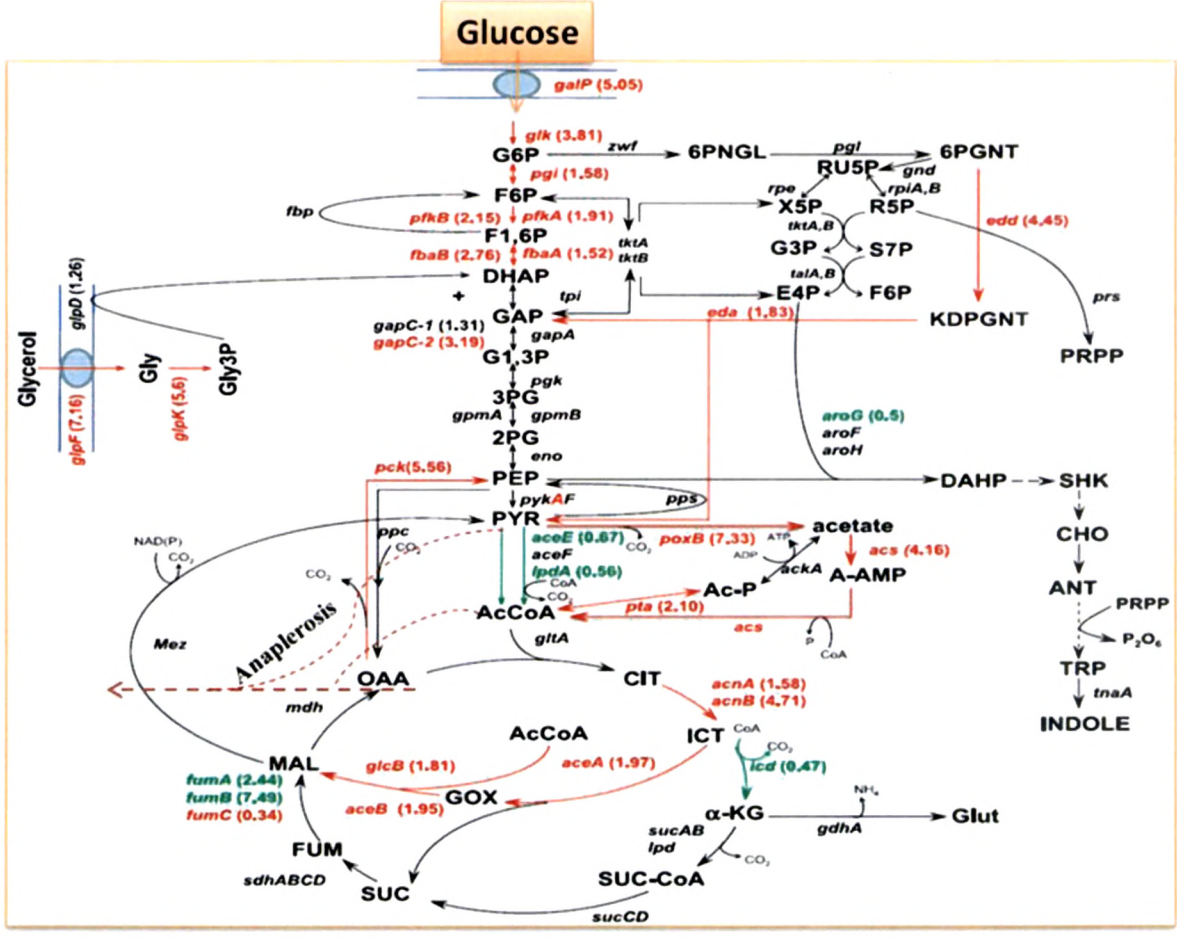


Figure 1.4: Central Carbon metabolism of *E. coli* JM101 grown on glucose and glycerol (Modification from Martínez-Gómez et al 2012). Relative transcription values are highlighted with colors when grown on glycerol as c source compared to glucose. **Genes in red: overexpressed. Genes in green: underexpressed. Genes in black: no change.** Metabolite abbreviations: Gly – glycerol; Gly3P - glycerol-3-phosphate; G - glucose; G6P - glucose-6-phosphate; F6P - fructose-6-phosphate; F1,6P - fructose-1,6-biphosphate; DHAP - dihydroxyacetone phosphate; G3P, glyceraldehydes 3-phosphate; G1,3P, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phophoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate;6PGLN, 6-phosphoglucono-δ-lactone; 6PGNT, 6-phophogluconate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; Xu5P, xylulose-5-phosphate;S7P, seudoheptulose-7-phosphate; E4P, erythrose-4-phosphate; Ac-CoA, acetyl coenzyme A; Ac-P, acetyl phosphate; A-AMP, acetyl-AMP; CIT, citrate; ICT,isocitrate; GOX, glyoxylate; α-KG, α-ketoglutarate; SUC-CoA, succinyl-coenzyme A; SUC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate;KDPGNT, 2-keto-3-deoxy-D-gluconate-6-phosphate; PRPP, 5-phospho-D-ribosyl-

α-1-pyrophosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; SHK, shikimate; CHO, chorismate; ANT, anthranilate; TRP, L-tryptophan (Papagianni, 2012).

The network controlling the carbon uptake integrates metabolism, signal transduction and gene expression (Baldazzi et al., 2010). In addition to the glycolytic metabolism, a gluconeogenic carbon recycling process that involves acetate is occurring simultaneously in *E. coli* JM101 when growing on glycerol. Glycerol, an energy-poor carbon source, has enhanced its biotechnology importance as carbon source since it is a byproduct of the biodiesel synthesis, whose production is expected to increase in the future (Dharmadi et al., 2006; Vasudevan et al., 2008; Bisen et al., 2010). *E. coli* growing aerobically on glycerol incorporates this molecule into central metabolism as dihydroxyacetone phosphate (DHAP), a metabolite which can participate in both gluconeogenic and glycolytic processes (Frankel et al., 1996; Martínez-Gómez et al., 2012). A balanced aerobic growth on glycerol depends on three global regulators: cAMP-CRP as the principal inducer of the glycerol catabolic regulon (including *glp*F, *glp*K *and glp*D); Cra(FruR) as regulator of some gluconeogenic genes, and ArcA as regulator of several central metabolic genes including the TCA cycle and others involved in respiration (Weissenborn et al., 1992; Iuch et al., 1995). Several genes are upregulated (*pyk*A, *pck*A, *glt*A, *fum*ABC, *sdh, mdh* and *acn*A) while *ackA* is downregulated when grown in glycerol as compared to glucose (Oh et al., 2000). It appears that when glycerol is used as the sole carbon source in addition to the glycolytic metabolism, a carbon stress response occurs that includes acetate scavenging and gluconeogenesis mediated by RpoS and Crl through indole. Interestingly, when JM101 was grown on a mixture of glycerol plus acetate, the μ of this strain was not enhanced but both compounds are utilized (**Table 1.1; Figure 1.5**).

Table 1.1: Specific growth rates (μ) and stoichiometric parameters of *E. coli* JM101 strain grown on single or mixtures of carbon sources (Martínez-Gómez et al., 2012).

Condition	μ(h-1)	Yx/s (g/mmolC)	Qs (mmolC /gdcw h)	mmolC of acetate produced(+) or consumed(-)
Glucose	0.69	0.013	51.8	+28.2
Glycerol	0.49	0.014	34.3	Not detected
Glucose+Glycerol	0.72(0.45)	0.017(0.006)	43.1	+4.1
Glycerol+acetate	0.43	0.011	39.5	-11.0
Glucose+acetate	0.72(0.1)	0.013(0.017)	55.4(6.55)	+6.0

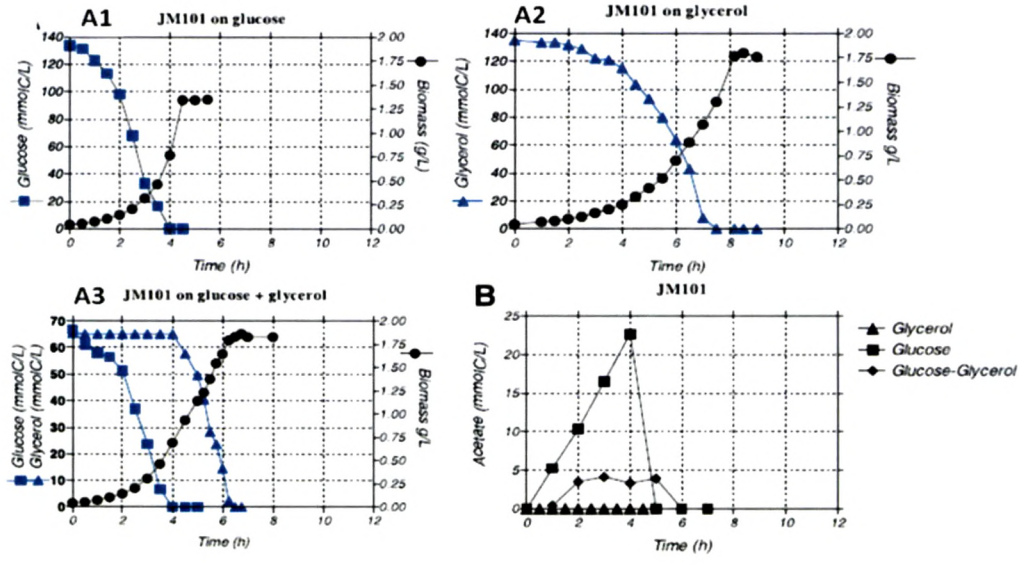


Figure 1.5: Growth profiles and substrate utilization (mmolC/L) of strain JM101 grown on glucose (A1) or glycerol (A2) and in the mixture glucose plus glycerol (A3). Acetate levels (mmolC/L) of strain JM101 grown on glucose, or glycerol and on a mixture of glucose plus glycerol (B) (Martínez-Gómez et al., 2012).

1.2.2 *Bacillus subtilis*

Bacillus subtilis is a gram positive spore forming bacterium and is the second most intensively studied bacteria after *E. coli.* Glucose is internalized via PTS *and* metabolizes a large proportion of it to pyruvate and acetyl CoA, and subsequently converts these compounds to lactate, acetate and acetoin as by-products of metabolism which are excreted into the extracellular environment . The enzymes of glycolysis depend on the cofactor NAD+ to take up electrons and hydrogen atoms that are released by substrate oxidation; the conversion of pyruvate to lactate has the advantage of regenerating NAD+ from its reduced form, NADH, which is a step that is essential for continued glycolysis. The conversion of acetyl CoA to acetate is coupled to the synthesis of ATP by the activities of the enzymes phosphotransacetylase and acetate kinase. Thus, these overflow pathways enable the cell to maintain redox balance and generate ATP without using the cytochrome system. When the glucose has been fully consumed, the cells reintroduce the by-products into central metabolism (using lactate dehydrogenase, acetoin dehydrogenase and acetyl CoA synthetase) and metabolize them further through the citric acid cycle, so generating additional ATP and reducing power. The genes that encode the enzymes that are involved in overflow metabolism are regulated by proteins that sense the nutritional status of the cell. In *B. subtilis*, CcpA activates the expression of the genes that are required for the synthesis of acetate, lactate and acetoin.The uptake of PTS sugar lead to an increase in FBP concentration in the cell which triggers ATP dependent HPr kinase/phosphatase-catalyzed phosphorylation o Hpr and Crh at Ser-46.The seryl phosphorylated complex with CcpA forming P-Ser-Hpr/CcpA and P-Ser-Crh/CcpA, now binds to cre to cause CCR or CCA depending upon the position of cre by transcriptional repression of the gene involved in the overflow metabolism.(Sonenshein 2007;Gorke and Stulke 2008; Fujita 2009) (**Fig. 1.6**).

The overall flux distribution done by ^{13}C metabolic flux analysis suggested glycolysis as the main catabolic pathway for glucose, acetate secretion, significant anaplerosis, and absent gluconeogenesis (**Fig. 1.7**) (Martin et al., 2011). The TCA cycle is one of the major routes of carbon catabolism in *B. subtilis* (Blenke et al., 2006).

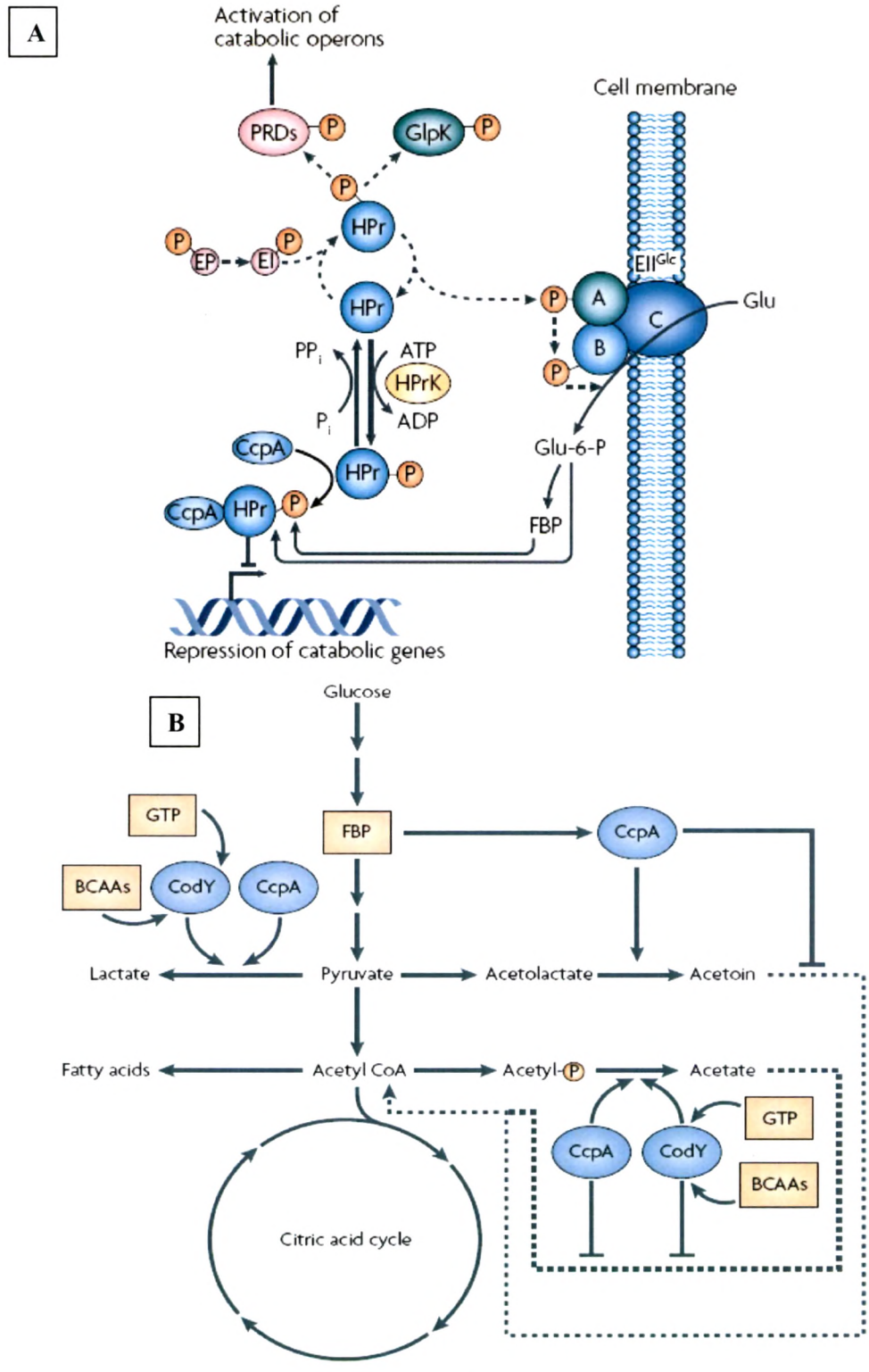


Figure 1.6: Glucose uptake and contribution of global CCR regulators for nutritional intersections in *B. subtilis* (A and B) (Sonenshein, 2007; Gurke and Stulke, 2008).

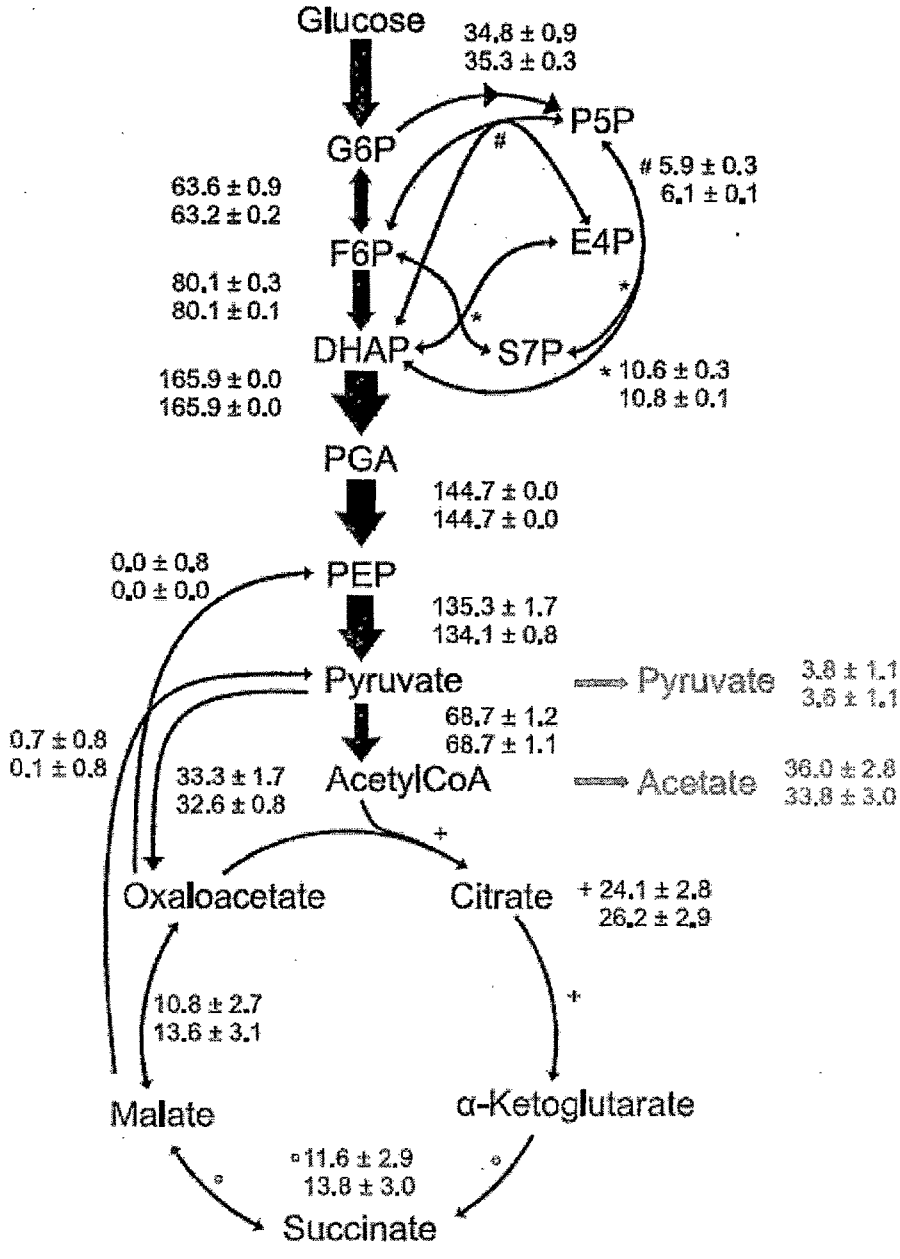


Figure 1.7: Intracellular flux distribution of *B. subtilis* wild-type during exponential growth on 20% (w/w) [U-13C] and 80% [1-13C] glucose derived from ^{13}C-patterns of solely intact metabolic intermediates (top values), or intact and fragmented carbon backbones of metabolic intermediates (bottom values) of the same culture. Shown are relative flux values normalized to the glucose uptake rate of 8.2mmol g^{-1} h^{-1}. Black arrows depict maximum and inner white arrows the minimum estimated flux value based on the Monte Carlo bootstrap error estimates with a confidence interval of 95%. (Martin et al., 2011).

Acetoin (3-hydroxy-2-butanone, AC) is an extensively used flavor compound as well as an important metabolic product produced by various microorganisms. It is classified as one of the 30 platform chemicals which were given priority to their development and utilization by the U.S. Department of Energy (Werpy and Petersen 2004). Production of acetoin can be achieved by chemical or biological synthesis. Compared to chemical synthetic and enzyme conversion methods, the microbial fermentation route has advantages of being more environmentally friendly and cost-effective. In a recent study, *B. subtilis* BSUW06 strain yields high levels of acetoin by disruption of *bdhA, acoA*, and *pta* genes involved in acetoin catabolic and competing pathways (Wang et al., 2012). The overexpression of *alsSD* increased pyruvate availability to acetoin biosynthesis, redirecting carbon flux towards the desired pathway (**Fig. 1.8**)

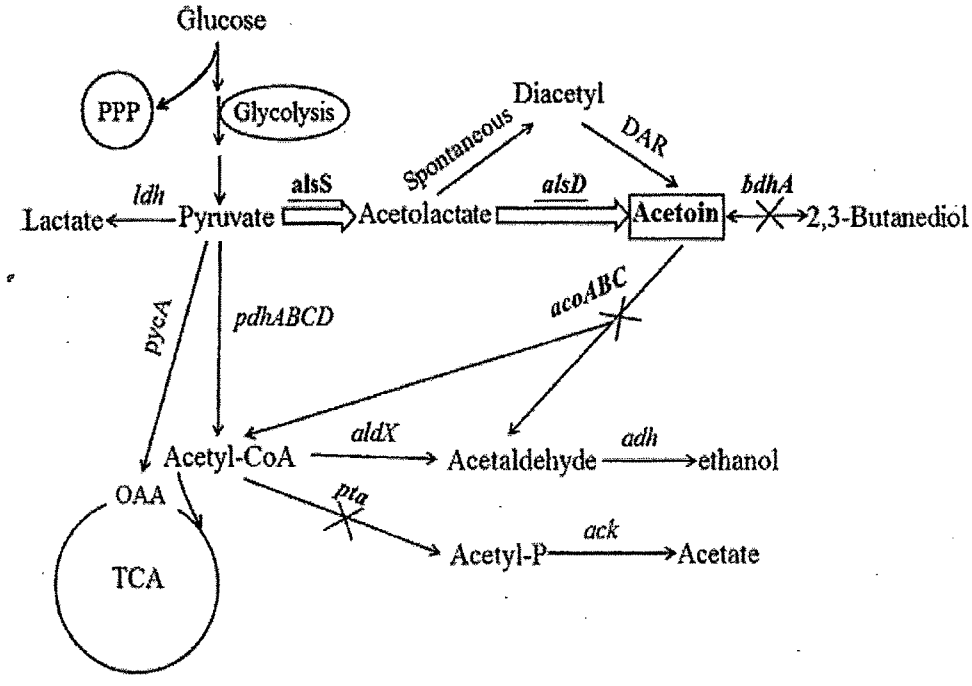


Figure 1.8: The acetoin biosynthetic pathway and other overflow metabolism pathways in *B. subtili* .Genes *alsS, alsD, bdhA, acoABC, ldh, pdhABCD, pycA, pta, ack, aldx* and *adh* encode acetolactate synthase, acetolactate decarboxylase, 2,3-butanediol dehydrogenase, acetoin dehydrogenase, lactate dehydrogenase, pyruvate dehydrogenase, pyruvate carboxylase, phosphotransacetylase, acetate kinase, aldehyde dehydrogenase and alcohol dehydrogenase. PPP pentose phosphate pathway, TCA tricarboxylic acid cycle, DAR diacetyl reductase. Overexpressed genes are underlined. Disrupted pathway steps are indicted by arrow breaks (Wang et al., 2012).

B. subtilis emerged an important model microorganism in the field of metabolic engineering for the production of riboflavin (Sauer et al., 1998). The PP pathway and the pyruvate shunt were identified as major pathways of glucose catabolism in a recombinant riboflavin-producing *B. subtilis* strain (Sauer et al., 1997). *B. subtilis* metabolism has an unusually high capacity for the reoxidation of NADPH. Riboflavin formation in *B. subtilis* is limited by the fluxes through the biosynthetic rather than the central carbon pathways. Therefore overexpression of enzymes (e.g. G6PDH and 6PGDH) that facilitate the route of carbon flow towards the PP pathway increased the pool of ribulose-5P which is a precursor for riboflavin biosynthesis and led to increased riboflavin yields (Zhu, Chen et al. 2007; Duan et al., 2010; Wang et al.,2011).

1.2.3 *Corynobacterium glutamicum*

A species of the class Actinobacteria *C. glutamicum* was discovered 50 years ago as a natural overproducer of glutamate. Today, it is used for the industrial production of more than 2 million tons of amino acids (glutamate, lysine and tryptophan) per year (Wittmann et al., 2010; Nešvera et al., 2011). Genetic manipulations in *C. glutamicum* were initiated in 1984, after isolation of small native plasmids. Progress in the genetic analysis of *C. glutamicum* accelerated after the determination of the complete genome sequences of two *C. glutamicum* strains (Yukawa et al., 2007).

The central metabolism of *C. glutamicum* involving glycolysis, pentose phosphate pathway (PPP), TCA cycle as well as anaplerotic and gluconeogenetic reactions (**Fig. 1.9**). Different enzymes are involved in the interconversion of carbon between TCA cycle (malate/oxaloacetate) and glycolysis (pyruvate/phosphoenolpyruvate). For anaplerotic replenishment of the TCA cycle, *C. glutamicum* exhibits pyruvate carboxylase and phosphoenolpyruvate (PEP) carboxylase as carboxylating enzymes. Malic enzyme and PEP carboxykinase catalyze decarboxylation reactions from the TCA cycle. Gluconeogenetic enzymes, oxaloacetate decarboxylase and PEP synthetase around the anaplerotic node is involved in the regeneration of excess ATP. The major enzymes, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the oxidative part of the PPP and the TCA cycle enzyme isocitrate dehydrogenase are linked to supply NADPH.

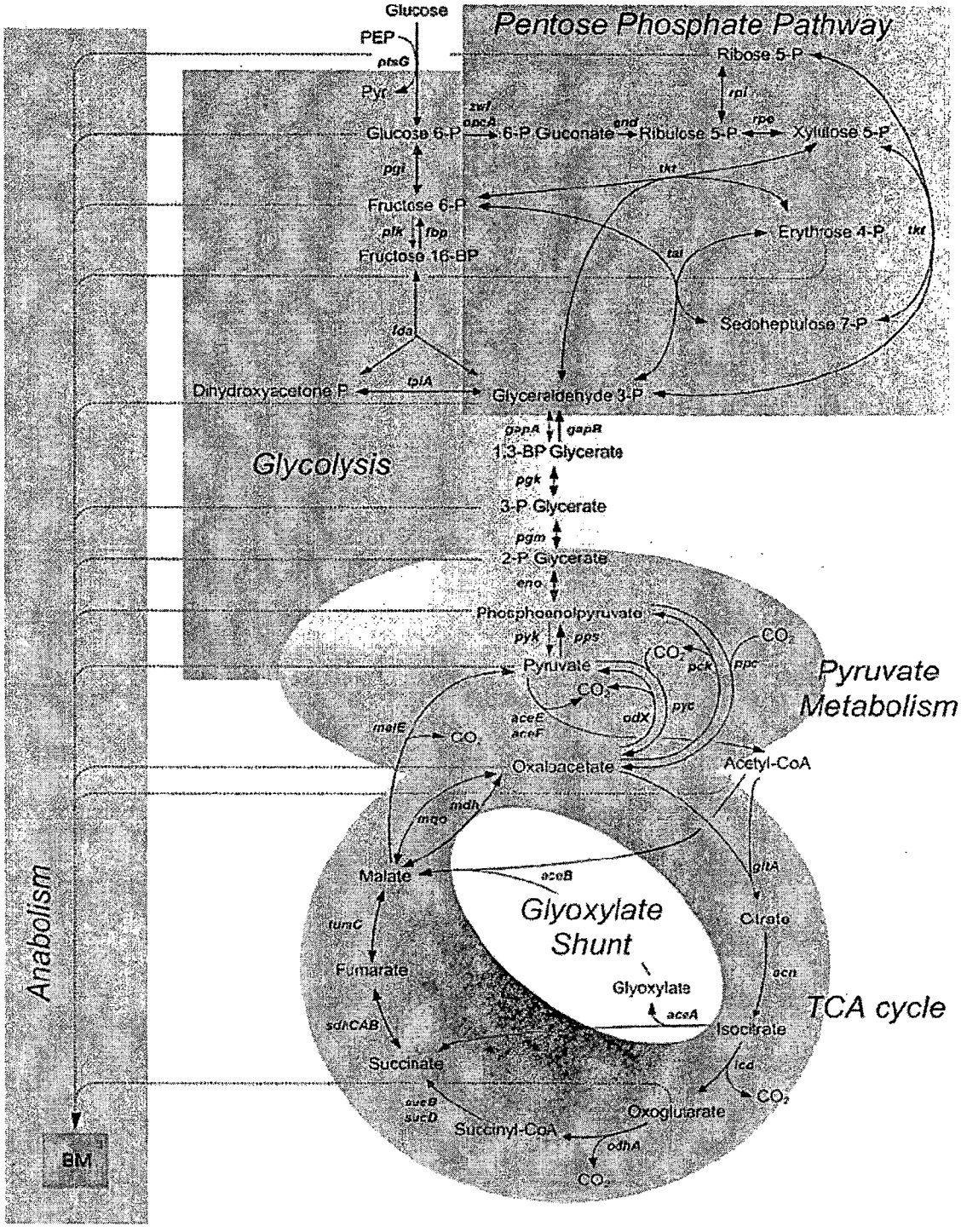


Figure 1.9: Central carbon metabolism in *C. glutamicum* (Wittmann, 2010).

High carbon loss through CO_2 formation by the TCA cycle is generally undesired with respect to carbon yield for the desired product. Hence, targeted down regulation of

TCA cycle is employed for improved lysine production (Becker et al., 2011). Upon replacement of the start ATG codon of *icdh* gene in *C. glutamicum* by GTG, the enzyme activity was reduced by 70% (Becker et al., 2009). This modification redirected the flux from the TCA cycle towards anaplerosis which enhanced lysine production by more than 40%.

1.2.4 *Streptomyces spp.*

Bacteria of the genus *Streptomyces* is very efficient producer of antibiotics. The Embden-Meyerhof-Parnas (EMP) pathway, the PP pathway and the TCA cycle are present in a number of *Streptomyces* species (Hodgson et al., 2000). Secondary metabolic pathways have been extensively studied in these bacteria for strain and yield improvement. The productivity of secondary metabolites is mainly determined by the availability of biosynthetic precursors (e.g. acetyl-CoA and malonyl-CoA). The carbon fluxes into the PPP or the glycogen synthetic pathway were reduced by deleting genes for G6PDH isozymes and 6PGDH, respectively. Since acetyl-CoA and/or malonyl-CoA is a precursor for the biosynthesis of actinorhodin (Act), a gene complex for acetyl-CoA carboxylase (ACCase) when overexpressed resulted in more rapid utilization of glucose and increased the efficiency of Act biosynthesis (**Fig. 1.10**) (Ryu et al., 2006).

Genetic engineering of glycolytic pathway in *Streptomyces clavuligerus* was carried out for improving clavulanic acid production (Li and Townsend, 2006). Clavulanic acid is a potent β-lactamase inhibitor used to combat resistance to penicillin and cephalosporin antibiotics. Clavulanic acid biosynthesis is initiated by the condensation of L-arginine and D-glyceraldehyde-3-phosphate (G3P). The limited G3P pool was overcome by targeted disruption of *gap1* genes which doubled production of clavulanic acid. Addition of arginine to the cultured mutant further improved clavulanic acid production.

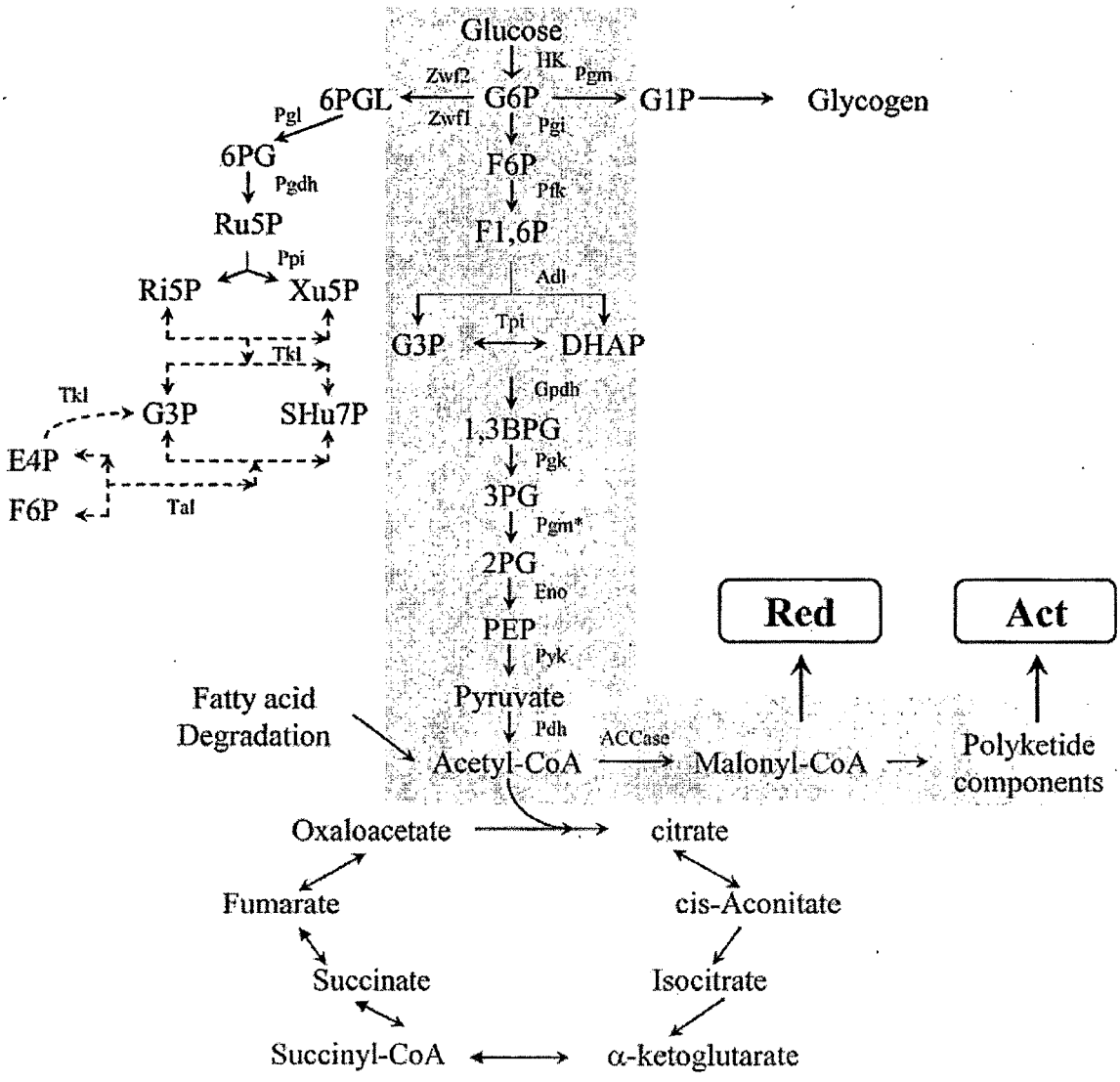


Figure 1.10: Central carbon metabolism and intermediates from primary metabolism for Act production in *S. coelicolor* (Ryu et al., 2006). G6P, glucose-6-phosphate;F6P, fructose-6-phosphate; F1,6DP, fructose-1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; 1,3 BPG, 1,3-diphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; 6PGL, 6-phosphoglucolactone; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; Ri5P, ribose-5-phosphate; Xu5P, xylulose-5-phosphate; SHu7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; HK, hexokinase;*Pfk*, phosphofructokinase; *Adl*, aldolase; *Tpi*, triose phosphate isomerase; *Gpdh*, glyceraldehyde-3-phosphate dehydrogenase; *Pgk*, phosphoglycerate kinase; *Pgm**, phosphoglycerate mutase; *Eno*, enolase; *Pyk*, Pyruvate kinase; *Pdh*, pyruvate dehydrogenase; *ACCase*, acetyl-CoA carboxylase; *Zwf*, glucose-6-phosphate dehydrogenase; *Pgm*, phosphoglucomutase; *Pgl*, phosphoglucolactonase; *Pgdh*, phosphogluconate dehydrogenase;*Ppi*, phosphopentose isomerase; *Tkl*, transketolase; *Tal*, transaldolase.

1.2.5 *Lactococcus lactis* and other lactic acid bacteria

Small genome size (~2-3 Mb) and simple energy and carbon metabolism that converts sugars to pyruvate via glycolytic pathway (**Fig. 1.11**) make lactic acid bacteria a promising targets of metabolic engineering strategies. *L. lactis* shows homolactic metabolism when growing in rapidly metabolized sugars with more than 90% of the metabolized sugar being converted to lactic acid. Deviation from homolactic fermentation is observed under aerobic conditions or during the metabolism of galactose or maltose. PFK was identified as the key regulatory enzyme of the glycolytic flux .The control of the glycolytic flux also resides to a large extend in processes outside the glycolytic pathway itself, like glucose transport and the ATP consuming reactions (Papagini et al., 2007; 2011, 2012).

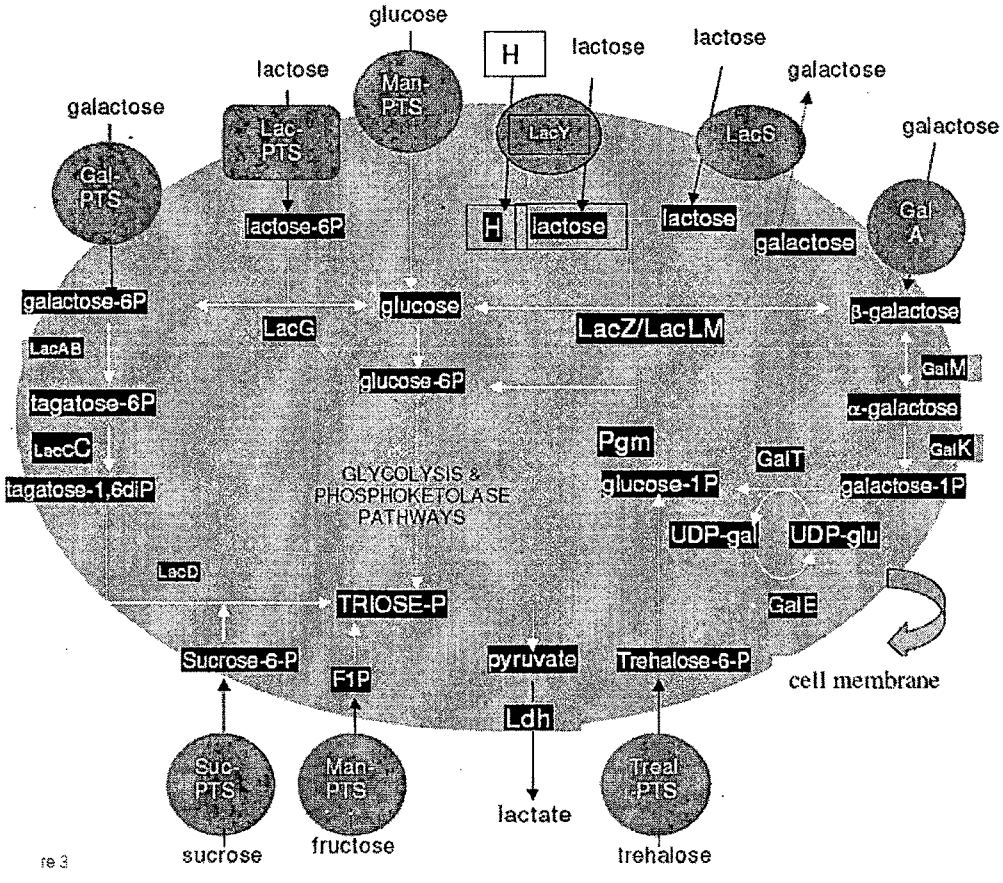


Figure 1.11: Central carbon metabolic pathway of *Lactobacillus lactis* (Papagini, 2012).

Cloning of *pfk*A gene from *Aspergillus niger* in *L. lactis* resulted in a two-fold increase in specific PFK activity (from 7.1 to 14.5 U/OD600) with a proportional increase of the maximum specific rates of glucose uptake (from 0.8 to 1.7 μMs^{-1} g CDW^{-1}) and lactate formation (from 15 to 22.8 g lactate (g $CDW)^{-1}$ h^{-1}) (Papagianni and Avramidis, 2009; 2011). Glycolytic flux in *L. lactis* is also affected by the carbon catabolite protein (CcpA) which besides its role in catabolite repression also regulates sugar metabolism through activation of the *las* operon encoding the glycolytic enzymes PFK and PYK, and LDH (Luesink et al., 1998).

1.2.6 *Pseudomonas spp.*

The genus *Pseudomonas* comprises of a large group of highly diverse Gram negative bacteria that are found abundantly as free-living organisms in soils, fresh water and marine environments, and in many other natural habitats. According to the microbial classification based on rRNA similarities, the largest group comprises of fluorescent species including *P. aeruginosa*, *P. fluorescens* (several biovars), *P. putida*, *P chlororaphis*, *P. syringae* (many pathovars), *P. cichorii*, *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. agarici*, etc., (predicted according to Palleroni et al.1973). The great catabolic versatility of pseudomonads has conferred an important ecological advantage which has allowed them (i) to colonize new habitats, including those toxic for most micro-organisms, (ii) to acquire and develop the specific mechanisms responsible for their natural resistance to harmful compounds and adaptations against metal stresses (Schleissner et al., 1997; Hamel et al, 2001), (iii) to promote plant growth and (iv) control plant pathogens by secretion of several antibiotics and antifungal molecules (Preston, 2004).

1.2.6.1 Glucose metabolism in *Pseudomonas* sp.

A common characteristic of Pseudomonas is their considerable metabolic versatility, being able to assimilate a wide range of compounds and environmental conditions (Palleroni and Moore, 2004). Some species, as for example *Pseudomonas aeruginosa*, can behave as severe opportunistic pathogens. Other species, such as *Pseudomonas fluorescens or*

Pseudomonas putida, can be beneficial for plants and thrive in the plant rhizosphere (Molina et al., 2000; Martins dos Santos et al., 2004).

Compared to facultative anaerobe *E. coli*, the glucose metabolism of *Pseudomonas* differs chiefly in the following aspects - (i) Absence of PTS mediated glucose uptake and Entner–Doudoroff (ED) pathway being almost exclusive catabolic route; (ii) Most Pseudomonas species (*P. aeruginosa, P. putida, P. fluorescens, Pseudomonas syringae, Pseudomonas mendocina* and *Pseudomonas entomophyla*, but not *Pseudomonas stutzeri*) lack phosphofructokinase which impedes the assimilation of glucose through the glycolytic pathway. (iii) PP pathway exhibiting completely biosynthetic functions; (iv) The respiratory mechanisms being highly efficient with very low overflow metabolism; (v) Significantly functional pyurvate bypass instead of the malate dehydrogenase (MDH) in TCA cycle; (vi) Glucose is extracellualrly converted to gluconate and 2-ketogluconate and simultaneously is also internalized by an active mechanism; and (vii) Absence of cAMP dependent glucose mediated catabolite repression (Mac Gregor et al., 1992; del Castillo and Ramos, 2007).

Glucose crosses the outer membrane into the periplasmic space through the OprB-1 porin (**Fig. 1.12**). Thereafter, glucose can be directly transported into the cell, or converted to gluconate or to 2-ketogluconate in the periplasmic space catalyzed by a membrane-bound PQQ-GDH and gluconate dehydrogenase (GADH), respectively, via direct oxidative pathway. Gluconate and 2-ketogluconate are then internalized using specific transporters into the cell and are acted upon by ATP dependent gluconokinase and 2-ketogluconokinase, respectively and 2-keto-6-phosphogluconate reductase finally producing 6-phosphogluconate (6-PG) which is further oxidized through the ED pathway to enter the central metabolism (Swanson et al., 2000; Fuhrer et al., 2005; Buch et al., 2009, 2008). 6-PG dehydratase (encoded by *edd*) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (encoded by *eda*) are the two enzymes comprising the ED pathway This complex mechanism for glucose uptake has been demonstrated earlier for *P. aeruginosa* PAO , *P. aeruginosa* M60 and *P. fluorescens* A3.12 (Williams et al., 1996). Generally, the genes responsible for direct oxidation of glucose and subsequent intracellular metabolism of gluconate and 2-KG occur variably among as evident from **Table 1.2** (Nelson et al., 2002; Joardar et al., 2005; Lee et al., 2006). Alternately, pseudomonads can also accumulate

glucose *via* an active transport mechanism (Midgley and Dawes, 1973; Eisenberg et al., 1974; Guymon and Eagon, 1974) which is induced by glucose and transports glucose in the form of free sugar; requiring a periplasmic glucose binding protein in *P. aeruginosa* (Lessie and Phibbs, 1984; Cuskey, 1985). Thus, direct oxidation is not the obligatory step for glucose metabolism. Intracellular glucose is rapidly phosphorylated by glucokinase (*glk*) followed by oxidation to 6-PG by glucose-6-phosphate dehydrogenase (*zwf*). These reactions comprise of the intracellular phosphorylative pathway.

Table 1.2: Distribution of the essential glucose catabolism genes across the partially and completely sequenced genomes of *Pseudomonas* spp. (Stover et al., 2000)

Psendomonas strain	GDH	GAD	GLK	6-PGDH	GNK	KGK	KGR
P. aeruginosa PAO1	+	+	+	-	+	-	-
P. aeruginosa UCBPP-PA14	+	+	+	-	+	-	+
P. putida KT2440	+*	putative	+	+	+	-	+
P. putida F1	+	+	+	+	-	-	-
P. putida GB-1	-	-	+	+	-	-	-
P. fluorescens Pf5	putative	putative	+	-	putative	-	putative
P. fluorescens Pf0-1	-	-	+	+	-	-	-
P. syringae pv. phaseolicola 1448A	+	-	+	putative	putative	-	-
P. stutzeri A1501	+	-	+	-	-	-	-
P. mendocina ymp	+	-	+	+	-	-	-
P. syringae pv. tomato DC3000	putative	-	+	+	+	-	-
P. entomophila L48	+	-	+	putative	+	-	-

GDH, glucose dehydrogenase;GAD, gluconate dehydrogenase; GLK, Glucokinase; GNK, gluconokinase; KGK, 2-ketogluconokinase; KGR, 2-ketogluconate-6-phosphate reductase; 6-PGDH, 6-phosphogluconate dehydrogenase. *GDH in *P. putida* KT2440 remains controversial as genome sequence reveals presence of *gcd* gene while Fuhrer et al (2005) demonstrated absence of the GDH mediated direct oxidation pathway in the same strain.

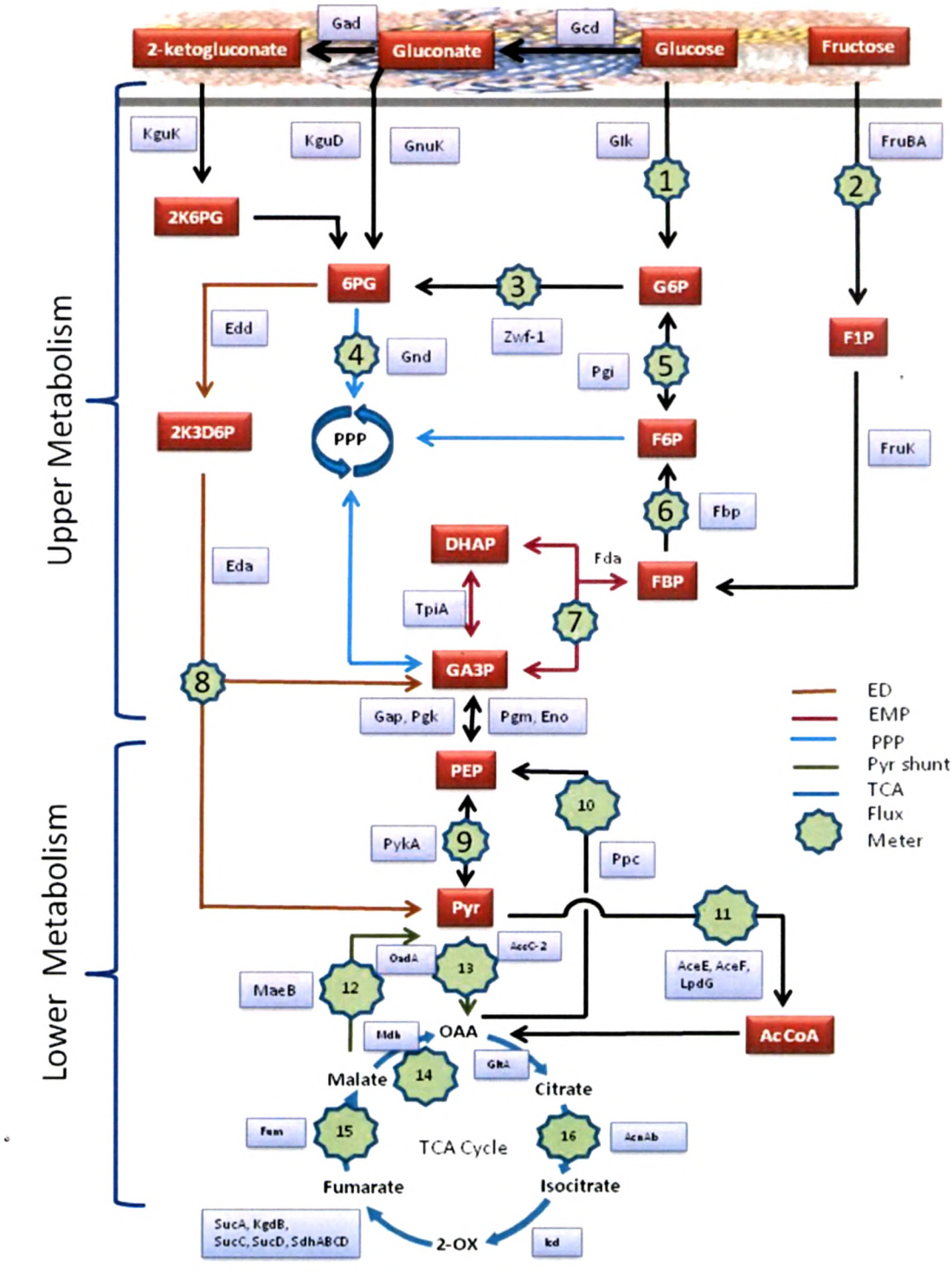


Figure 1.12: Glucose and fructose metabolism in *Pseudomonas* sp. (Modification from Chavarría et al., 2012).

In most pseudomonads, both the glucose oxidation pathways are operative with few exceptions. Contribution of periplasmic and intracellular pathways in the glucose catabolism varies in pseudomonads according to the physiological conditions and nature of substrate utilized. At least in *P. putida*, the direct import of gluconate into the cell normally accounts for only 10% of glucose metabolism, the remaining 90% occurring by direct uptake of glucose and of the 2-ketogluconate generated in the periplasmic space from glucose (del Castillo et al., 2007) (**Fig. 1.12**). *P. putida* C5V86 (Basu and Phale, 2006) and *P. citronellolis* (Fuhrer et al., 2005) exclusively catalyze glucose by intracellular phosphorylative oxidation as they lack the GDH and GADH activities whereas *P. acidovorans* lacks GLK as well as GDH thereby failing to assimilate glucose (Lessie and Phibbs, 1984). Glucose does not play central role in *Pseudomonas* as it does in *E. coli, B. subtilis* or lactic acid bacteria. In fact, the preferred carbon sources for *Pseudomonas* are some organic acids or amino acids, rather than glucose. For example, in the presence of succinate and glucose, the expression of enzymes of the *P. aeruginosa* central pathway for glucose catabolism such as G6PDH or 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase is repressed until succinate is consumed (Rojo, F., 2010). The expression of genes for the assimilation of other sugars such as gluconate, glycerol, fructose and mannitol is also inhibited by succinate or acetate. Glucose, however, has a repressing effect on the expression of several genes, for example on the *P. aeruginosa* regulons for mannitol or histidine utilization, on the *P. aeruginosa* amidase genes, on the *P. putida* pWW0 plasmid genes for the degradation of toluene (del Castillo and Ramos, 2007), on the *P. putida* genes involved in the assimilation of methylphenol) and phenylacetic acid or on the genes required to degrade styrene in *P. fluorescens* ST (Rampioni et al., 2008).

1.2.6.2 Growth of fluorescent pseudomonads on PTS versus non-PTS sugars

Fructose is the only carbohydrate which is uptaken in fluorescent pseudomonads *via* PEP:fructophosphotransferase system, except in *P. cepacia* and certain strains of *P. saccharophila* which accumulate fructose by active transport (Lessie and Phibbs, 1984). Fructose is imported through a typical PTS permease (FruA) consisting of a fusion of EIIBFru and EIICFru domain and is then converted to fructose 1-phosphate (F1P) and fructose 1,6-bisphosphate (F1,6BisP) (Nogales et al., 2008; Puchalka et al. 2008). F1,6BisP can enter the ED pathway and/or a standard glycolytic Embden-Meyerhof-Parnas (EMP)

route (**Fig. 1.12**). Despite these differences in the earlier stages of their metabolism, glucose and fructose ultimately converge into the production of phosphoenolpyruvate (PEP) and pyruvate as the point of entry into the TCA cycle. Interestingly the carbon flux of the cell is totally different when grown on glucose as compared to fructose. The central biochemical landscape following the uptake of carbohydrate as shown in the **Fig. 1.12** divided into an upper domain (i.e., the EMP, ED, and PP pathways) for the breakdown of the hexoses into C3 compounds (pyruvate and phosphoenolpyruvate) and a lower domain that encompasses the phosphoenolpyruvate-pyruvate-oxaloacetate (PEP-Pyr-OAA) checkpoint node and the TCA cycle. In a recent study, carbon fluxes of *P. putida* were high upper metabolic domain and get reduced significantly in the lower metabolism (Chavarría et al., 2012). Bulk (~96%) of glucose was metabolized via the ED pathway to pyruvate, while the other ~4% of the sugar was channeled into the PP route (**Fig. 1.13 & 1.14**). The fate of fructose was, however, altogether different in which channeling to ED pathway occurs at ~52% whereas ~34% and ~14% are diverted into EMP and PPP, respectively.

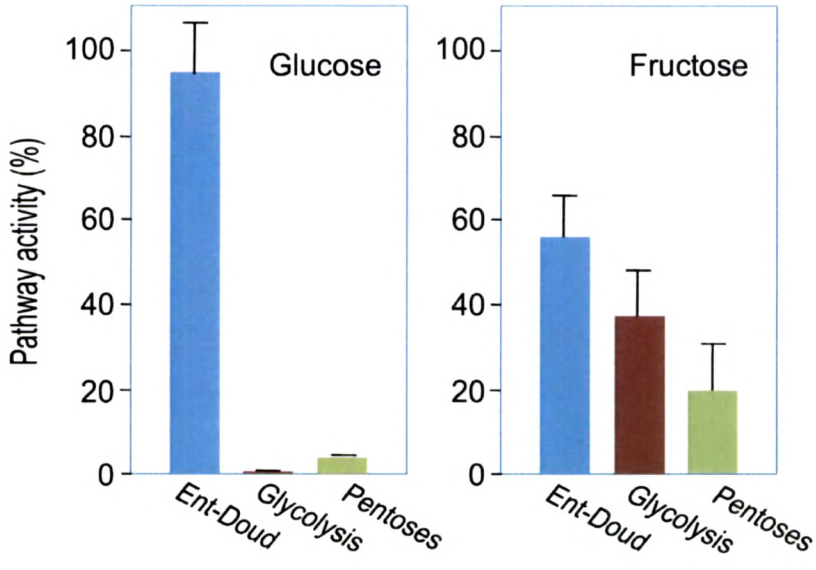


Figure 1.13: Channeling of glucose and fructose through each of the upstream sugar-catabolic pathways. The activities on the *y* axes represent the net fluxes of carbon through each of the routes calculated using metabolic flux analysis of *P. putida* MAD2 grown on the compound indicated in each case.Note that *P. putida* degrades glucose mainly through the Entner-Doudoroff (ED) pathway (~96%). Fructose is also catabolized mostly by the ED route (52%) but with an important contribution from standard glycolysis (the Embden-Meyerhof-Parnas (EMP) pathway), which accounts for ~34% of the corresponding flux (Chavarría et al., 2012).

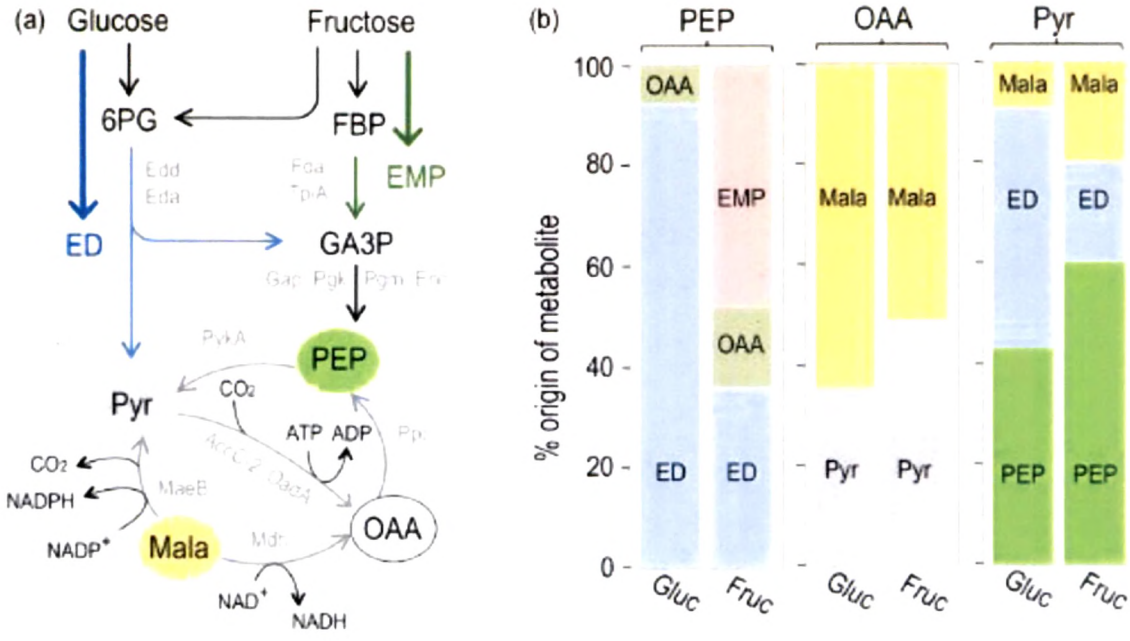


Figure 1.14: The origin of the components of the phosphoenolpyruvate-pyruvate-oxaloacetate (PEP-Pyr-OAA) node. (a) Major biochemical reactions that connect the ED and EMP routes with the malate and oxaloacetate components of the Krebs cycle (Chavarría et al., 2012). The routes necessary for the conversion of glucose and fructose into metabolic currency (Pyr and PEP) are illustrated on top along with the enzymes that belonging to the ED or the EMP pathways. The bottom highlights the reactions at the boundary between the upper and the lower metabolic domains, including the PEP-Pyr-OAA node and the Pyr shunt. (b) A breakdown of the route of key metabolites (PEP, OAA, and pyruvate) through each of the connecting reactions of the upper and lower metabolic boundaries for either glucose or fructose. The percentage of each precursor compound or pathway is indicated in every case.

1.2.6.3 Anaplerotic node in fluorescent pseudomonads

The central glycolytic/gluconeogenic pathways and the TCA cycle are metabolically linked by the crucial junction represented by the phosphoenolpyruvate–pyruvate–oxaloacetate node also referred to as *anaplerotic node* (**Fig. 1.15**) (Sauer and Eikmanns, 2005). This node comprises a set of reactions that direct the carbon flux into appropriate directions and thus, it acts as a highly relevant switch point for carbon flux distribution within the central metabolism. Under glycolytic conditions, the final products of glycolysis PEP and pyruvate enter the TCA cycle via acetyl-CoA (oxidative pyruvate decarboxylation and fueling of the cycle) and via formation of oxaloacetate by carboxylation (C3-

carboxylation). Under gluconeogenic conditions the TCA cycle intermediates oxaloacetate or malate are converted to pyruvate and PEP by decarboxylation (C4-decarboxylation) and thus, the PEP–pyruvate–oxaloacetate node provides the direct precursors for gluconeogenesis.

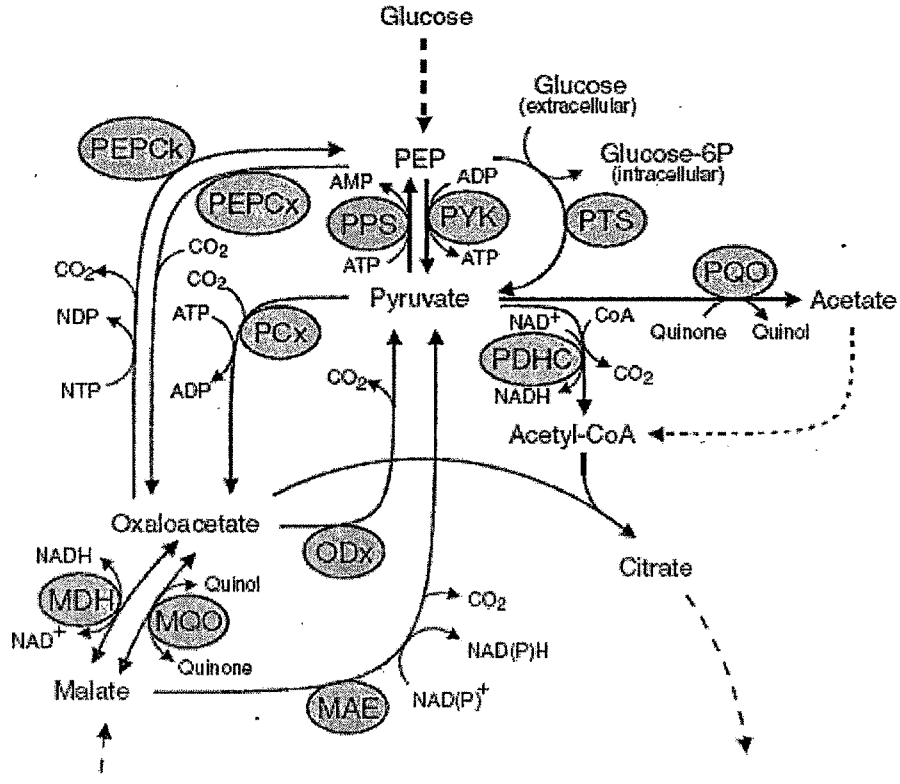


Figure 1.15 The enzymes at the PEP–pyruvate–oxaloacetate node in aerobic bacteria (Sauer and Eikmanns, 2005)). Abbreviations: MAE, malic enzyme; MDH, malate dehydrogenase; MQO, malate: quinone oxidoreductase; ODx, oxaloacetate decarboxylase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PEPCk, PEP carboxykinase; PEPCx, PEP carboxylase; PPS, PEP synthetase; PQO, pyruvate: quinone oxidoreductase; PTS, phosphotransferase system; PYK, pyruvate kinase.

The anaplerotic node in pseudomonads involves PYC for oxaloacetate biosynthesis in addition to phosphoenolpyruvate carboxylase (PPC); however, the distribution of these two enzymes in *Pseudomonas* sp. varies from strain to strain (Sauer and Eikmanns, 2005). Additionally, the metabolite balance at the anaplerotic node in fluorescent pseudomonads could also be influenced by malic enzyme of the TCA cycle as well as differential metabolic regulations attributed to the general lack of a PTS for glucose uptake and the key glycolytic

enzyme phosphofructokinase. Understanding the metabolic flexibility at the anaplerotic node in fluorescent pseudomonads could be significant since oxaloacetate at the node is not only a key anabolic precursor but also is intermediate in the biosynthesis of organic acids like citric, malic, succinic, pyruvic and acetic acids, which could be implicated in P-solubilization (Khan et al., 2006). Moreover, P-limitation has been shown to affect the anaplerotic node in *Pseudomonas fluorescens* ATCC 13525 by causing a decrease in PYC activity; thereby having an adverse effect on growth (Buch et al., 2008). Buch et al (2010) showed an enhancement in P solubilization ability by manipulating the enzymes at the anaplerotic node. Heterologous overexpression of *S. elongatus* PCC 6301 *ppc* gene in *P. fluorescens* ATCC 13525 lead to 12–14-fold higher PPC activity both in M9 and TRP minimal media as compared to the control without *ppc* gene. Improved biomass yield and unaltered growth rate of *ppc* overexpressing strain along with reduced glucose consumption (80% from 89%) and decreased yields of the metabolic by-products like pyruvate and acetate, indicated efficient carbon utilization and decreased carbon overflow probably due to diversion of the carbon units towards anabolic processes. Thus, a physiological level of PYC activity at the anaplerotic node of *P. fluorescens* ATCC 13525 was apparently not optimal for efficient carbon tilization and PPC served the anaplerotic function.The enhanced direct oxidation pathway counterbalanced the reduced glucose consumption under P-sufficient condition, as demonstrated by increased gluconic acid yield and GDH activity. On TRP medium, ppc transformants of Fp315 showed faster growth, media acidification and rock-phosphate solubilization as compared to its control. Elevating the flux through the anabolic pathways in *P. fluorescens* by enhancing the biosynthesis of precursor oxaloacetate, could benefit the cellular growth under P-limitation.

1.3 Global phosphate resources and the potential of fluorescent pseudomonads as P Biofertilizers

P fertilizers are essential for maintaining and increasing the world food production. Phosphorous is completely produced from large scale mining of rock phosphate which is non renewable resources. In ancient times P was applied to agricultural soil by recycling animal manure, crushed animal bones, human and bird excreta, city waste and ash. Industrial revolution replaced this by phosphate material from non renewable resources. According to

the International Fertilizer Industry Association (IFA), in 2008, close to 53.5 million tonnes (Mt) of P_2O_5 (i.e. 175 Mt of phosphate concentrates, averaging 30.7% P_2O_5 content) was mined (IFA, 2009a). Hence there is a gradual depletion of global high-grade P resources (USGS, 2008), (Vuuren et al., 2010; Childers et al., 2011).

1.3.1 Depleting global P resources

According to the reports, about 40–60% of the current resource base would be extracted by 2100. At the same time, production will concentrate in Asia, Africa and West Asia, and production costs will likely have increased.

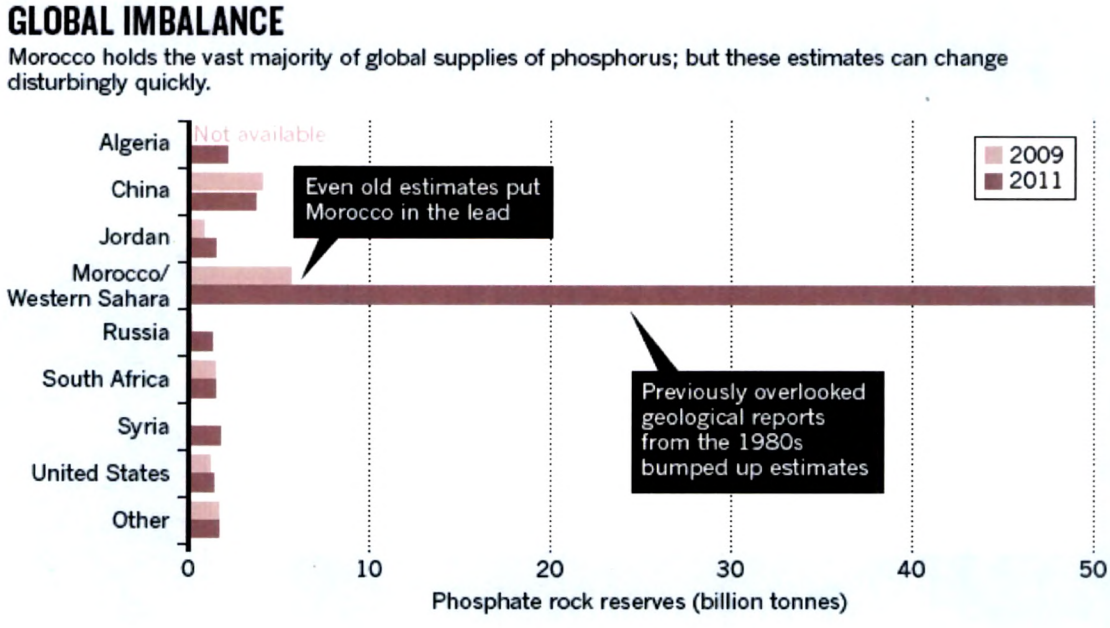


Figure 1.16: Global imbalance of P resources (Elser and Bennett, 2011).

Most of the current resource base is concentrated in Africa (Morocco) (**Fig. 1.16**). The depletion of resources outside Africa may lead to a high share of African production in world phosphate rock supply which is more than half the global production, unless new important resources are identified and exploited in other regions. Short-term focus on domestic resources may lead to higher prices and in the long run to even higher imports.

Depletion is also likely to increase phosphate production costs by about a factor of 3–5, during this century (**Fig. 1.17-1.19**).

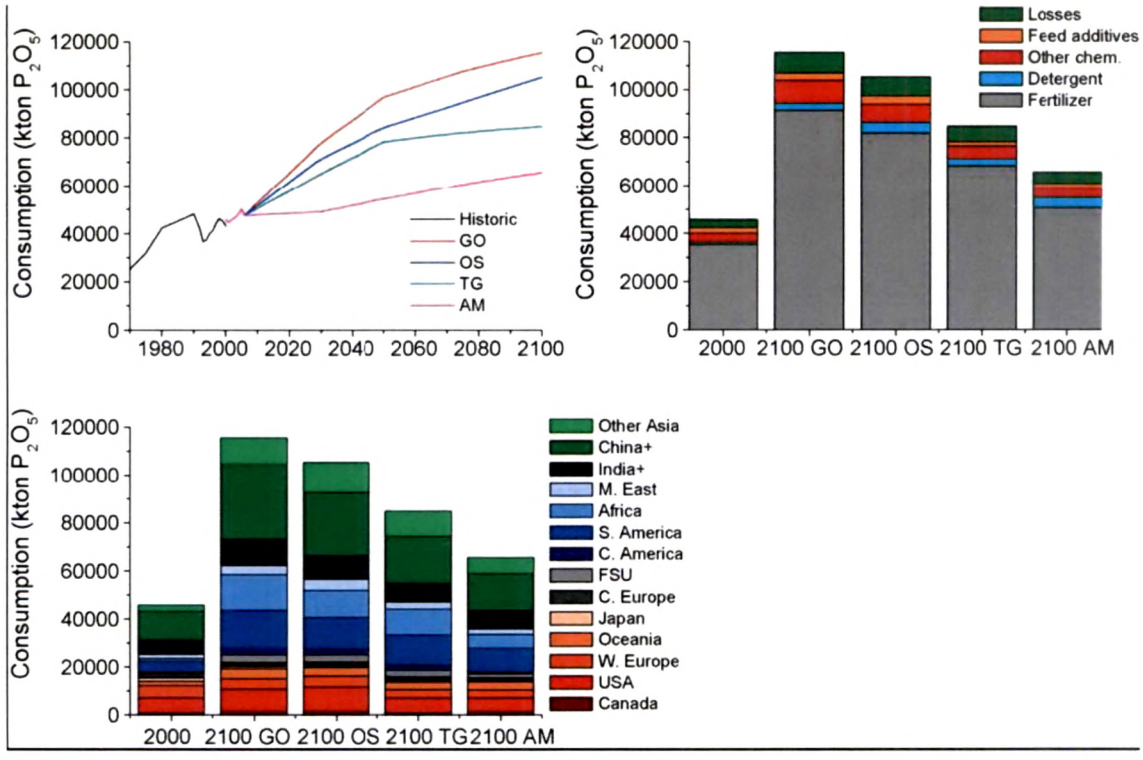


Figure 1.17: Total global P consumption (a), P use per use category (b), and P use per world region (c), for the four Millennium Ecosystem Assessment (MA) scenarios (Vuuren et al., 2010).

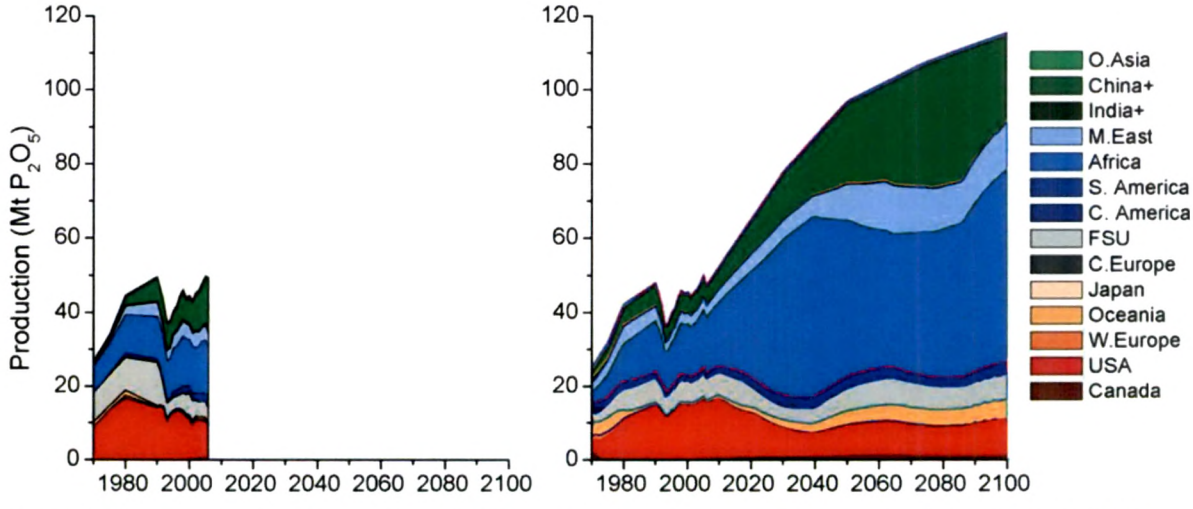


Figure 1.18: P production for the 1970–2006 period (historical data; left panel) and for the 2000–2100 period, in the Global Orchestration (GO) scenario (Vuuren et al., 2010).

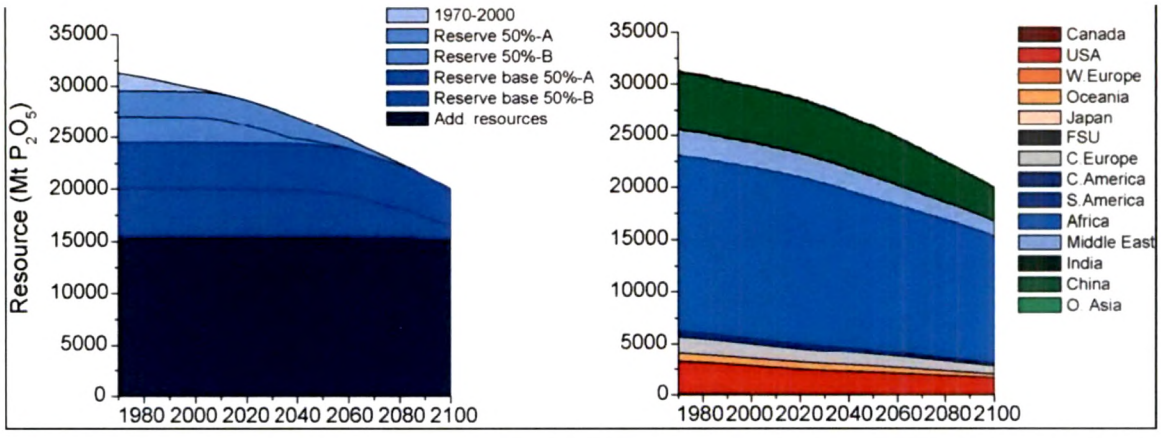


Figure 1.19: Depletion of resource base (reserves, reserve bases and additional resources) of phosphate rock under the GO scenario (default estimate) (Vuuren et al., 2010). The 1970–2000 category represents the cumulative production in this period (thus creating a best-guess resource e base estimate for 1970).

Increasing the efficiency of P application seems to be an urgent need to mitigate the increasing global P imbalance and fulfil the phosphorous requirement of crop and livestocks. In this context mineral phosphate solubilization by microbes like *P. fluorescens* holds tremendous importance. Exploitation of phosphate solubilizing bacteria through biofertilization has enormous potential for making use of ever increasing fixed P in the soil, and natural reserves of phosphate rocks. (Elser and Bennet, 2011).

1.3.2 Phosphate solubilization by fluorescent pseudomonads

Phosphorous is one of the major nutrient limiting plant growth.70% of the phosphate present in the soil is in the complexed form which is unavailable to plants. Soluble P-ion oncentration in most soils varies from 0.1 to 10µM while P required for optimal growth ranges from 1 to 60 µM. Thus, inspite of abundant phosphate in the soil the plants show phosphate deficient conditions. Phosphate solubilizing microorganisms (PSM) are the most promising bacteria among the plant growth promoting rhizobacteria (PGPR); which may be used as biofertilizers for plant growth and nutrient use efficiency in phosphate deficient soil. Rhizospheric microorganisms including bacteria like few *Pseudomonas* sp., *Serratia*,

Bacillus sp, *Rhizobium* sp., *Azotobacter*, *Azospirullum* and fungi like *Aspergillus*, *Penicillium*, etc. (Archana et al., 2012) are now known to act as powerful PSMs.

Fluorescent pseudomonads act as one of the promising plant growth promoting rhizobacteria. These PSMs dissolve the soil P through production of low molecular weight organic acids including the mono-, di- and tri-carboxylic acids like acetic, lactic, oxalic, tartaric, succinic, citric, gluconic, 2-ketogluconic, formic, malic, pyruvic and glyoxalic acid **(Table 1.3)**.

Table 1.3: Organic acid secreted by phosphate solubilizing pseudomonads (Reframmed from Archana et al 2012).

Fluorescent pseudomonads	**Organic acid secreted**
Pseudomonas cepacia	Gluconic and 2-ketogluconic
P. fluorescens	Gluconic, Citric, Formic, Lactic, Oxalic
P. fluorescens RAF15	Gluconic,2-ketogluconic, Tartaric
Pseudomonas corrugata NRRL B-30409	Gluconi, 2-ketogluconic
Pseudomonas sp	Lactic
P. trivialis	Gluconic,2-ketogluconic,lactic,formic, oxalic, citric
P. poae	Gluconic,formic,lactic,oxalic acid,Citric
P. fluorescens AF15	Formic
P. aeruginosa	Gluconic
P. corrugata	Gluconic
P. striata	Oxalic, succinic, Tartaric Fumaric, glyoxalic, isovaleric, isobutyric,Itaconic, ketobutyric, malonic, propionic
P. aerogenes	Fumaric, Lactic,acetic glyoxalic, isovaleric, isobutyric, Itaconic, ketobutyric, malonic, propionic

The hydroxyl and carboxyl groups of these acids chelate cations (Al, Fe, Ca) and make the P available for plants. in addition to lowering the pH of rhizosphere (Deubel *et al.*, 2000; Stevenson, 2005). The pH of rhizosphere is lowered through biotical production of proton/bicarbonate release (anion/cation balance) and gaseous (O_2/CO_2) exchanges.

Inorganic acids e.g. hydrochloric acid can also solubilize phosphate but they are less effective compared to organic acids at the same pH (Kim *et al.*, 1997). In certain cases phosphate solubilization is induced by phosphate starvation (Gyaneshwar *et al.*, 1999). A general sketch of P solubilisation and mechanisms in soil by bacteria is shown in **Fig. 1.20** and **Fig. 1.21**.

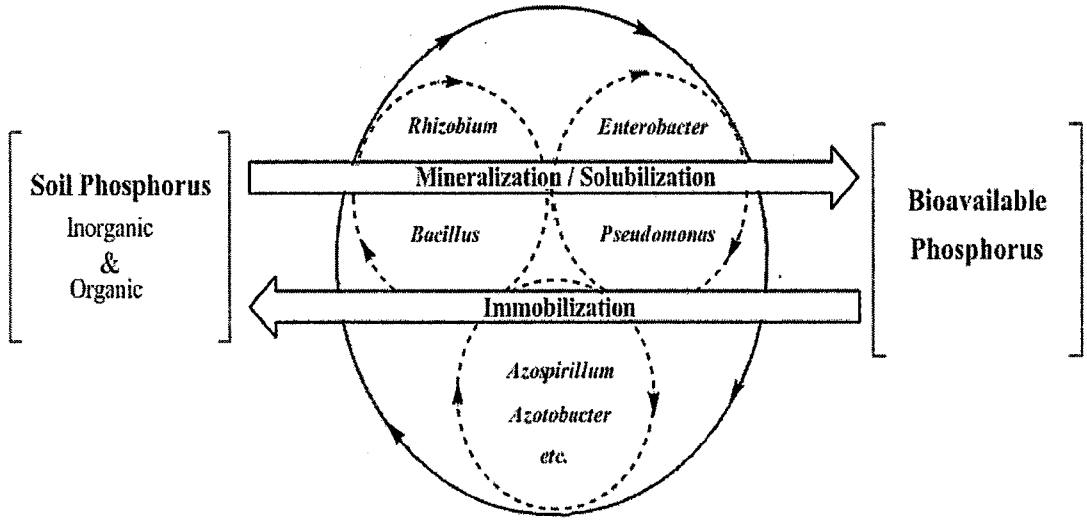


Figure 1.20: Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (Khan et al., 2009).

Plant inoculation experiments resulted in variable effects on P supply, plant growth and crop yields (Gyaneshwar et al., 2002). These varied effects are attributed to the nature of the soil and survival of inoculated microbes in the rhizosphere and their colonizing ability. Also, the nature and amount of organic acids limit the efficacy of PSM in soils and in field conditions (Gyaneshwar *et al.*, 2002; Khan *et al.*, 2007; Srivastava *et al.*, 2006). High buffering capacity of soil reduces the effectiveness of PSB in releasing P from bound phosphates.

Insertion of P-solubilizing genes in agriculturally important microorganisms lacking P-solubilizing ability or enhancing the microbial activity of weak PSMs has been an attractive approach to develop beneficial microbes with improved utility as soil inoculants (Rodriguez et al., 2006). Goldstein and Liu (1987) cloned a gene from *Erwinia herbicola*

that is involved in mineral phosphate solubilization by screening the antibiotic-resistant recombinants from a genomic library in a medium containing hydroxyapatite as the source of P. The expression of this gene allowed production of gluconic acid and mineral phosphate solubilization activity in *E. coli* HB101. Sequence analysis of this gene (Liu et al., 1992) suggested its probable involvement in the synthesis of the enzyme pyrroloquinoline quinone (PQQ) synthase, which directs the synthesis of PQQ. Several Gram-negative bacteria are capable of producing organic acids by such direct oxidation of aldehydes, which then get diffused in surroundings and help in the acidification of poorly soluble mineral phosphates (Goldstein 1986; Sashidhar and Podile, 2010). Glucose dehydrogenase (GDH) requires PQQ as a redox cofactor for direct oxidation of glucose to gluconic acid, which then diffuses in the soundings of bacterial niche and helps in acidic solubilization of insoluble phosphates in soil. Both membrane-bound and soluble forms of GDH, in spite of having different substrate specificity, use PQQ as a cofactor.

The role of PQQ as a redox coenzyme has been reported for several dehydrogenases, including methanol dehydrogenase, ethanol dehydrogenase and GDH. There are plant growth-promoting bacteria that use GDH-PQQ holoenzyme for solubilization of inorganic phosphates in soil (Han et al. 2008). These studies suggest that microbes producing PQQ can increase the phosphate availability in soil for the growth and development of crop plants, which in turn increase crop productivity (Mishra et al., 2012). Following a similar strategy, a mineral phosphate solubilization gene from *Pseudomonas cepacia* was isolated (Babu-Khan et al., 1995). This gene (*gabY*), whose expression also allowed the induction of the mineral phosphate solubilization phenotype via gluconic acid production in *Escherichia coli* JM109, showed no apparent homology with the previous cloned PQQ synthetase gene (Goosen et al., 1989), but it did with a permease system membrane protein. The *gabY* gene could play an alternative role in the expression and/or regulation of the direct oxidation pathway in *P. cepacia*, thus acting as a functional mineral phosphate solubilization gene *in vivo*.

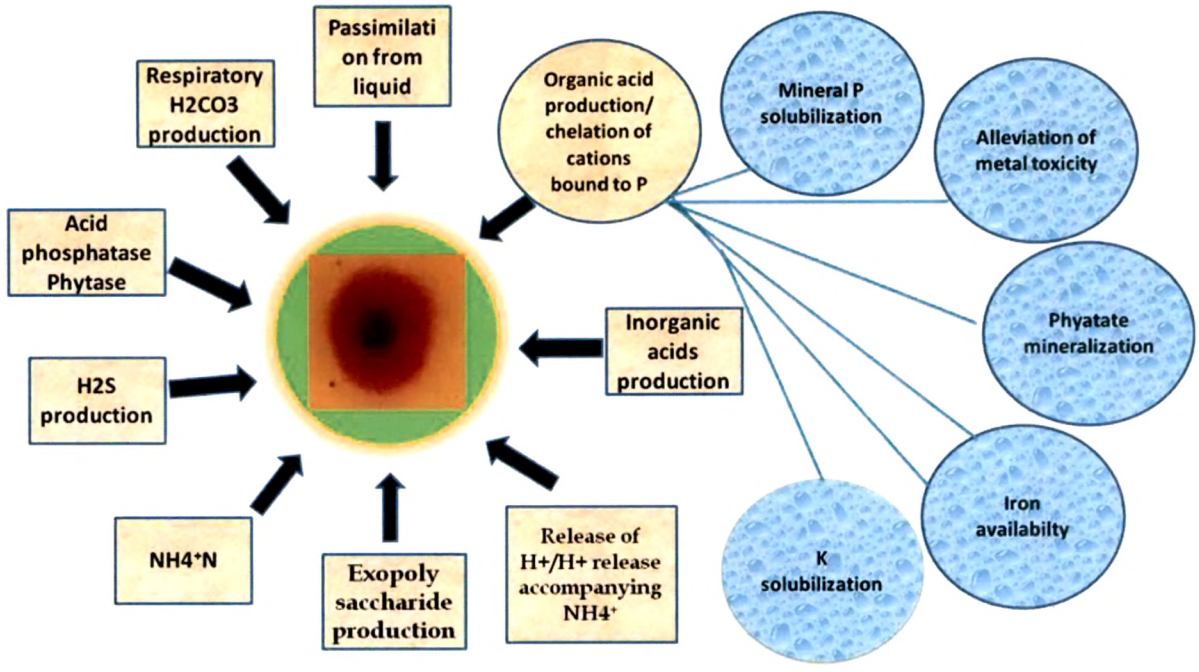


Figure 1.21: Mechanism of p solubilization by phosphate solubilizing bacteria and role of organic acid secretion in plant growth promotion (Redrawn from Archana et al., 2012).

Fluorescent pseudomonads are efficient biocontrol PGPR which also find application in bioremediation. Beneficial effects of the inoculation with *Pseudomonas* sp. to many crop plants have been well established (Brundrett, 2009; Gianinazzi *et al.*, 2010). Additional mineral phosphate solubilizing ability would help enhancing the efficacy of these pseudomonads as a phosphate biofertilizer.

1.3.3 Role of citric acid in P solubilization

The secretion of gluconic acid is the major mechanism of P-solubilization by gram negative bacteria (Goldstein 1995; Kim et al., 1998). The acidification of soil by organic acids depends on both the nature and quantity of the organic acid for e.g. acetic, lactic and succinic at 100 mM bring about a drop in pH of a soil solution from around 9.0 to about 6.0; a similar drop is brought about by only 20 mM of gluconic acid, 10 mM of oxalic acid and even lesser amount of citric and tartaric acids (Gyaneshwar et al. 1998; Srivastava *et al.*, 2006) (**Table 1.4**). Addition of organic acids decreases the pH of the alkaline vertisol soil solution in the order Acetic = Succinic = Lactic < <Gluconic < <Oxalic < Tartaric = Citric

and results in P release in a similar order. *Penicillium billai* secretes 10 mM each of citric and oxalic acids (Cunningham & Kuiack, 1992) and has been shown to be effective in releasing P in the field conditions (Asea et al., 1988). On the other hand, *C. koseri* and *B. coagulans* were found to secrete various organic acids in the range 1-5 mM whereas as the concentration of these acids required to reduce the pH of the soil was 20-50 times more.

Table 1.4: Organic acids for phosphate solubilisation in different soil types (Srivastava *et al.*,2006).

Organic acid	**Alkaline vertisol pH > 7**	**Acidic alfisol supplemented with RP pH < 7**
Citric acid	10 mM	10-20Mm
Oxalic acid	10 mM	5-10 mM
Gluconic acid	20mM	50mM
Tartaric acid	10mM	20mM

Citric acid has better chelation properties due to presence of its three –COOH group's having pKa values of 3.15, 4.77, and 6.40, respectively. Hence, fluorescent pseudomonads producing citric acid could be effective as P biofertilizers in alkaline soils.

1.3.4 Metabolic engineering strategies in fluorescent pseudomonads for P solubilization by citric acid.

Secretion of oxalic acid and citric acid is a well reported phenomena in *Pseudomonas fluorescens* exposed to Al toxicity.CS, an enzyme that condenses oxaloacetate and acetylCoA to citrate, experienced a two-fold increase activity in the Al-stressed cells, compared to the control cells. While a six fold increase in fumarase activity and five fold decrease malate synthase activity was found in the Al-stressed cells compared to the controls (Apanna et al., 2003). Excretion of citric acid by anamorphic fungi *viz.*, *Aspergillus* and *Penicillium* is a frequent phenomenon in natural habitats and in laboratory cultures (Wolfgang 2006). Naturally pseudomonads do not secrete any citric acid. The role of citrate synthase (CS) in citric acid biosynthesis and glucose catabolism in pseudomonads was investigated by overexpressing the *Escherichia coli gltA* gene in *Pseudomonas*

fluorescens ATCC 13525 (Buch et al., 2009.). Approximately, 2-fold increase in CS activity in the *gltA* overexpressing strain was observed with an enhanced intracellular and extracellular citric acid yields during the stationary phase, by about 2- and 26-fold, respectively, as compared to the control, without affecting the growth rate, glucose depletion rate or biomass yield. This is in contrast to the earlier reports from the known citric-acid producing bacteria, in which increase in CS activity is either the result of a TCA block in the form of *icd* mutation or is in response to aluminium toxicity (Barone et al., 2008). Increasing CS activity in *P. fluorescens* for citric acid overproduction from glucose is a better strategy than *icd* mutation in *E. coli*, which reduces biomass and growth (Aoshima et al., 2003).

1.4 Rationale of thesis

Fluorescent pseudomonads are well known for the biochemical and metabolic diversity. Genome sequence of several *Pseudomonas* sp. and metabolic data reveals that there are lot of interspecies diversity in terms of occurrence and regulation of enzymes at the central metabolism and PEP-Pyruvate-OAA node. This study is an effort to genetically engineer a stable system for phosphate biofertilizer and to examine its applicability amongst diverse fluorescent pseudomonads. Present study describes improvement in citric acid secretion in fluorescent pseudomonads to the required amount for P release from soils. Additionally, the genetic manipulations need be directed to the chromosomal integration as it would lead not only to increased stability but also decrease the metabolic load caused by the presence of the plasmids in the bacterial cell (Buch et al., 2010; Sharma et al., 2011).The overall strategy of the present study is depicted in **Fig. 1.22**.

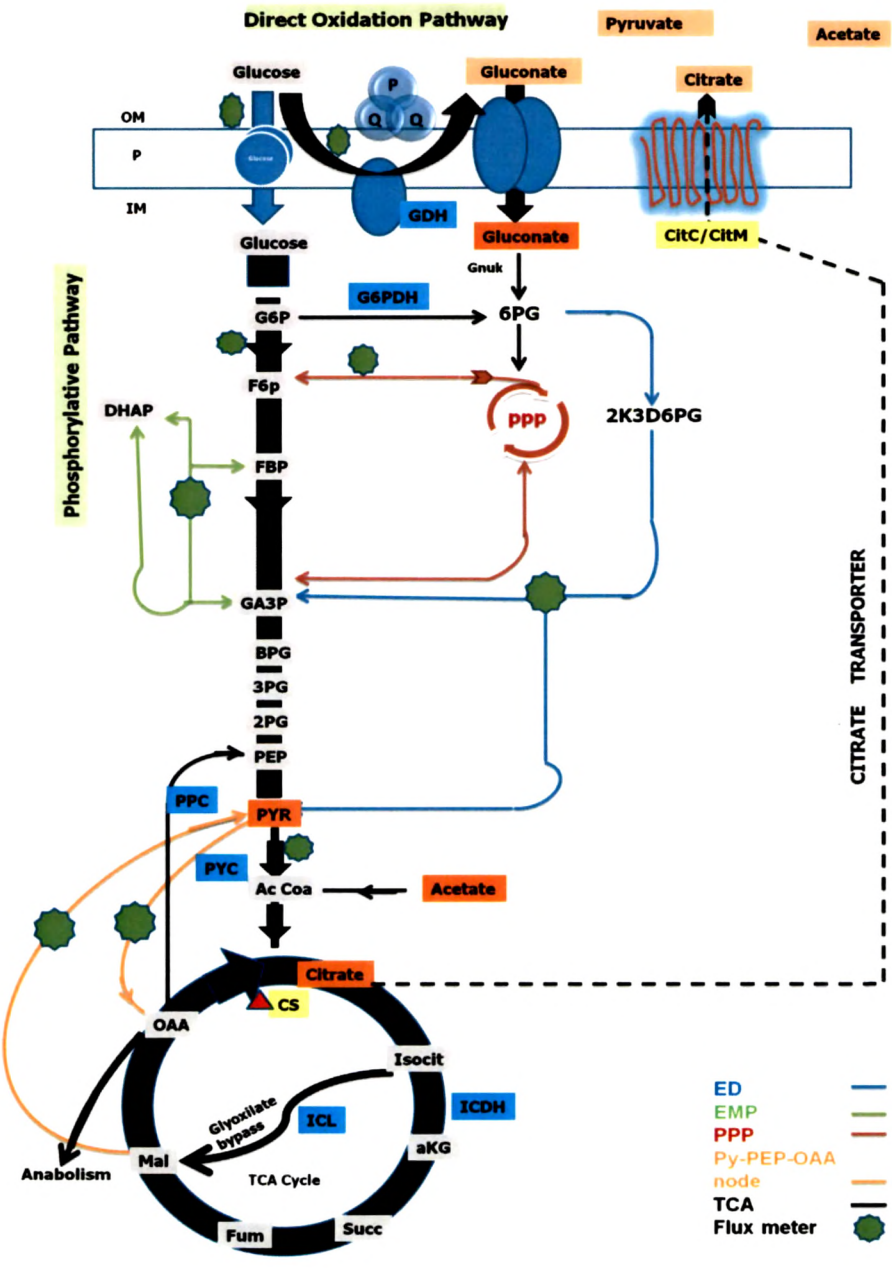


Figure 1.22: Design and analysis of genetic modification in fluorescent pseudomonads. Yellow boxes indicate the gene heterologously overexpressed, red triangle attached to the CS indicate NADH insensitivity.Blue bosex indicate those enzymes whose fluctuations are reported due to genetic modification. Orange boxes indicate the metabolite whose change is being monitored both intracellular and extracellular. Abbreviations: *ppc*, phosphoenolpyruvate carboxylase; *cs*, citrate synthase; *pyc*, pyruvate carboxylase; *pyk*, pyruvate kinase; *icl*, isocitrate lyase; *icd*, isocitrate dehydrogenase; *pdh*, pyruvate dehydrogenase complex, *glt*, glucose transporter; *glk*, glucokinase; *zwf*, glucose-6-phosphate dehydrogenase; *edd*, 6-phosphogluconate dehydratase and *eda*, 2-keto-6-phosphogluconate aldolase

1.5 Objectives

The objectives of the present study were defined as follows-

1. Effect of constitutive heterologous overexpression of *E. coli* NADH insensitive *cs* in *Pseudomonas fluorescens* Pfo-1

2. Metabolic characterization of engineered *Pseudomonas fluorescens* Pfo1coexpressing *E. coli* NADH insensitive CS and *Salmonella typhimurium* sodium citrate transporter or *Bacillus subtilis* magnesium dependent citrate transporter operon.

3. Evaluation of the effect of engineered genetic modifications on P-solubilizing ability of plant growth promoting rhizospheric fluorescent pseudomonads.

4. Genomic integration of *E. coli* NADH insensitive CS and *Salmonella typhimurium* Na^{+}/*Bacillus subtilis* Mg^{+2}–dependent citrate transporter operon in the genome using a Mini-Tn7 transposon and study their effects on P solubilisation and plant growth promotion.