L-Carnitine Production in *Pseudomonas fluorescens* under phosphate starvation

By

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science (M.Sc.) in Chemistry

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Abstract

Glycerol is a by-product of the biodiesel industry and an important carbon source for bacterial growth. Phosphorus is an essential element in all living organisms and is utilized in numerous metabolic processes, such as the tricarboxylic acid (TCA) cycle and the electron transport chain. Without phosphorus, no organism can either grow or perform regular functions. In this study, we demonstrate that when the industrially-important microbe *Pseudomonas fluorescens* is deprived of phosphate, it elaborates a metabolic reconfiguration aimed at producing and secreting copious amounts of L-carnitine. To accomplish this biochemical adaptation, the organism bypassed the TCA cycle and utilized the glyoxylate shunt to generate a constant supply of L-carnitine through different metabolic networks. The upregulation of numerous enzymes including L-carnitine dehydrogenase (lcdH) and isocitrate lyase (aceA) mediated this process. The metabolic reprogramming triggered by phosphate may provide an effective means to transform an industrial waste into valuable L-carnitine.

Keywords: L-carnitine, phosphate starvation, glyoxylate shunt, metabolic networks

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List of Abbreviations

aceA	Isocitrate lyase gene
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
BN-PAGE	Blue-native polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
caiD	Carnityl-CoA dehydratase
caiT	L-carnitine/γ-butyrobetaine antiporter
CFE	Cell free extract
CDH	L-carnitine dehydrogenase
CSB	Cell storage buffer
DCPIP	Dichloroindophenol
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
$FADH_2$	Reduced flavin adenine dinucleotide
FRD	Fumarate reductase
FT-MS	Fourier transform mass spectrometry
GB	Gamma-butyrobetaine
HPLC	High performance liquid chromatography
ICDH	Isocitrate dehydrogenase
ICL	Isocitrate lyase
INT	Iodonitrotetrazolium

LP	Low phosphate
metK	S-adenosylmethionine synthase
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OP	Oxidative phosphorylation
PDH	Pyruvate dehydrogenase
P_i	Inorganic phosphate
PMS	Phenazine methosulfate
PMSF	Phenylmethylsulfonyl fluoride
psiF	Phosphate starvation inducible protein
RNA	Ribonucleic acid
SLP	Substrate level phosphorylation
TCA	Tricarboxylic acid
mM	Millimolar
mL	Milliliter
P_i	Inorganic phosphate
g	Gram
mg	Milligram
μL	Microliter
mg/L	Milligram per liter
nm	Nanometer
^oC	Celsius

min	Minute
µg	Microgram
rpm	Revolutions per minute
h	Hour
s	Second
g/L	Gram per liter

CHAPTER 1: Introduction and Objectives

1.1 Introduction

1.1.1 Cell Metabolism

Cellular metabolism is defined as a sequence of biochemical pathways occurring in all living organisms dedicated to the sustain life. These pathways are characterized by the participation of nutrients such as carbohydrates, fatty acids and amino acids that help maintain ATP homeostasis, the main energy currency (Deberardinis and Thompson, 2012, Auger et al., 2021). Cellular metabolism is regulated by enzyme activity that drive sophisticated reactions and specific inter-connected pathways (Rohmer et al., 2011, Auger and Appanna, 2015). These pathways are classified as catabolic where degradation occurs and may result in ATP production, or anabolic where products are synthesized. Anabolic reactions are characterized by the presence of smaller molecules that are involved in the synthesis into complex molecules with the input of energy. Common examples include synthesizing sugars from carbon dioxide, large proteins from amino acid building blocks and the DNA strands from nucleic acid building blocks. These processes require high energy molecules such as ATP, NADH and NADPH. In catabolic reactions, energy stored in the bonds of complex molecules are released and harvested in a way that it can be used to produce ATP. Starch, proteins, fats and lipids are also broken down to release energy and generate ATP (Alberts et al., 2015). Bacterial cells can modify these reactions to meet energy production or storage needs. For example, in the process of breaking down nutrients, bacteria produce enzymes with different affinity for the substrates in order to modulate the degradative process. Any organism must produce energy if it is to survive. Bacteria fulfill their energy needs by means of glycolysis, the TCA cycle, glyoxylate shunt, the electron transport chain and other inter-connected metabolic pathways (Fernie et al., 2004, Auger and Appanna, 2015).

These processes share a common molecule, known as adenosine 5’-triphosphate that stores free energy produced from the oxidation of carbohydrates, lipids and proteins. Its structure comprises

a pentose sugar ribose linked to an adenine nitrogenous base and three phosphodiester bonds (Fontecilla-Camps, 2021). When energy is needed inside the cell, an enzyme hydrolyses the bond between the second and third phosphate group in ATP to form ADP and an inorganic phosphate ion. This is known as substrate level phosphorylation. For this transfer to occur, the Pi group on the donor molecule must have a high group transfer potential so that the energy released (Gibbs free energy) is high enough to attach the Pi to ADP. Typically, the energy must be greater than -35 kJ/mol, which is the energy released from ATP hydrolysis (White et al., 2012). The phosphorylated metabolites participating in SLP are limited due to this requirement. For example, phosphoenolpyruvate (PEP) can participate in SLP whereas glucose-6-phosphate cannot because of its low transfer potential, making the phosphorylation of ADP infeasible (Berg et al., 2019). PEP is able to drive several phosphotransferase enzymes, such as pyruvate phosphate dikinase (PPDK), creatine kinase (CK) and adenylate kinase (AK) to generate maximum energy in the form of ATP (Dzeja et al., 2004, Bringaud et al., 2009, Radolf et al., 2016 Thomas et al., 2016). On the other hand, the conversion of succinyl-CoA to succinate in the TCA cycle generates ATP via SLP with the succinyl-Pi intermediate donor. Furthermore, the secretion of acetate is aided by the activity of acetate kinase, an enzyme that can generate ATP from acetyl-Pi and ADP (Table 1.1). Substrate level phosphorylation can occur as part of the TCA cycle and is implemented by organisms that are subjected to an anaerobic environment (Bailey-Serres et al., 2010). In fact, *Pseudomonas fluorescens* is known to utilize this alternative ATP-generating strategy when subjected to oxidative stress (Alhasawi et al., 2015b, Appanna et al., 2016).

Table 1.1: Enzyme-driven substrate-level phosphorylation reactions (Caspi et al., 2018)

Enzymes	Reaction	Gibbs free energy (ΔG^o; kJ/mol)
Acetate kinase	Acetyl-phosphate + ADP → Acetate + ATP	-29.2
Succinyl-CoA synthetase	Succinyl-CoA + Pi + ADP→ Succinate + CoA + ATP	-4.4
Pyruvate kinase	Phosphoenolpyruvate + ADP→ Pyruvate + ATP	-9.3

In oxidative phosphorylation, bacterial cells use enzymes to oxidize nutrients, a process that releases energy in the process to form ATP. This process takes place in the electron transport chain where the ATP is derived from the proton gradient generated upon the reoxidation of coenzymes NADH or $FADH_2$ (Krebs et al., 1952, Mailloux et al., 2007). The tricarboxylic acid cycle (TCA) cycle is programmed in such a way that three NADH and one $FADH_2$ are generated from acetyl-CoA upon the liberation of two CO_2. This cycle is driven by several enzymatic reactions that take place in the mitochondria of eukaryotic cells and in the cytoplasmic membrane of prokaryotes under aerobic conditions (Cavalcanti et al., 2013). Pyruvate from glycolysis is converted to acetyl-CoA, which in turn enters the TCA cycle. The TCA cycle undergoes several reactions to generate intermediates, one of which is succinate that is directly linked to complex II of the ETC. The electron transport chain is assembled of four complexes (I-IV), where electrons are passed along to molecular oxygen, which is reduced to water in the presence of protons (Figure 1.1). A portion of energy is released during these transfers that serve towards the generation of ATP, by ATP synthase (Complex IV) (Van Dongen et al., 2011). The coenzymes

are re-oxidized by energy-yielding redox reaction coupled to the phosphorylation of ADP. In the absence of oxygen, alternative compounds such as nitrate, nitrite, sulfate, trimethylammonium oxide and fumarate can serve as electron acceptors that function as proton pumps (Ju et al., 2013).

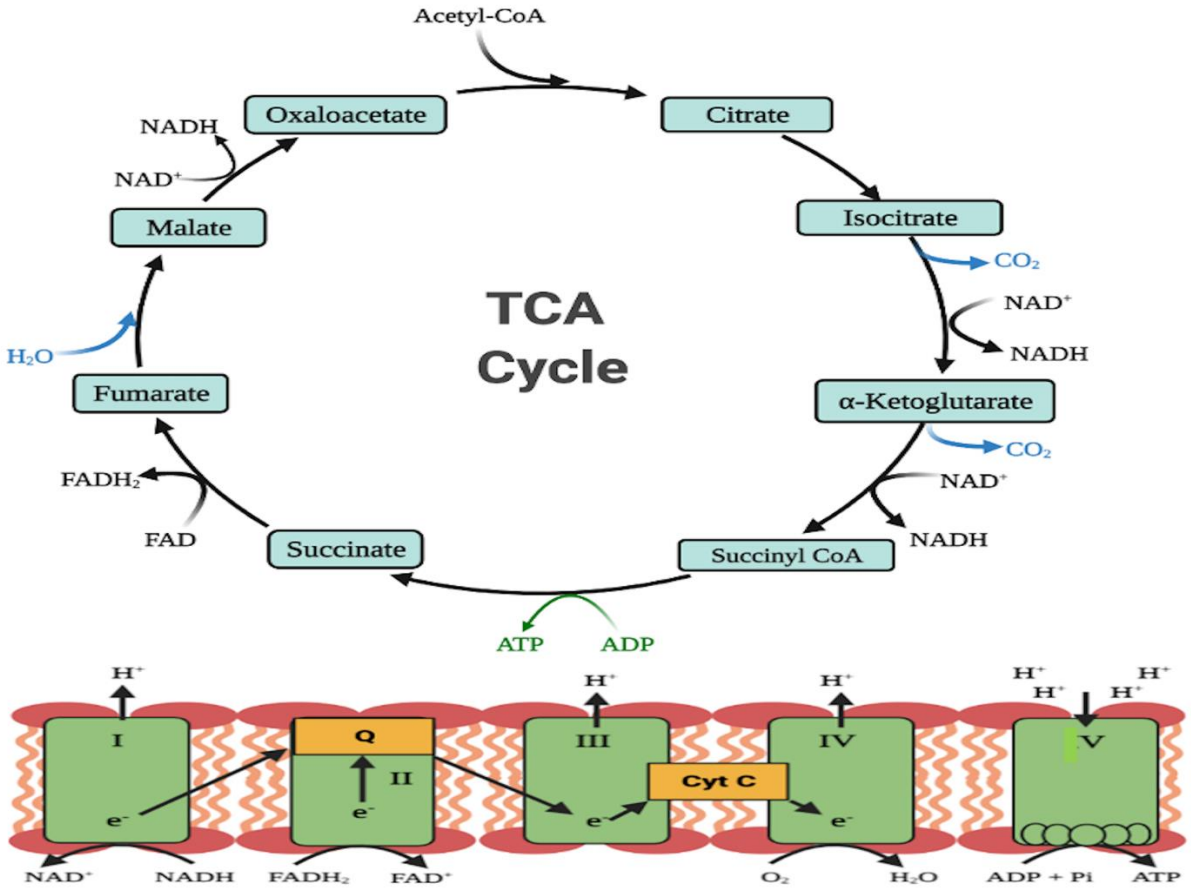

Figure 1.1: TCA Cycle and the electron transport chain. Q: Ubiquinone; Cyt C; Cytochrome c.

Despite ATP being the energy currency of the cell, energy can also be released by high energy phosphates compounds (Meis et al., 1997) (Table 1.2). These compounds are capable of yielding free energy equal to or greater than that of ATP (-30.5 kJ/mol). Some low energy phosphate compounds such as glucose-6-phosphate and glycerol-3-phosphate can produce energy in hydrolytic reactions (Poian and Castanho, 2015).

Table 1.2: Classification of high energy phosphate compounds (Fromm and Hargrove, 2012)

Type of compound	Example	(ΔG^o; kJ/mol)
Pyrophosphate	ATP	-30.5
	Pyrophosphate	-33.5
Enol phosphates	Phosphoenolpyruvate	-61.9
Acyl phosphates	1,3-biphosphoglycerate	-49.4
	Carbamoyl phosphate	-51.5
Thiol esters	Acetyl-CoA	-32.2
	Propionyl-CoA	-25.9
Guanido phosphates	Phosphocreatine	-43.1
	Phosphoargenine	-31.8

These cellular processes are driven by the presence of primary and secondary metabolites. Primary metabolites are compounds that are part of metabolic pathways assisting in the growth and cell proliferation of microorganisms. Carbohydrates, amino acid and lipids are products derived from primary metabolism. Secondary metabolites are termed as specialized metabolites or natural products that are not directly involved in the growth and reproduction of bacteria (Newmann et al., 2012). Common examples include steroids, oils, alkaloids, antibiotics, etc. (Figure 1.2). These compounds are often of value in industrial settings. Secondary metabolism is often the chosen route for bacteria that are subjected to stressors such as changes in nutrient levels and temperature. These metabolic networks dedicated to generate unique metabolites enable these microbes to adapt (Auger et al., 2013).

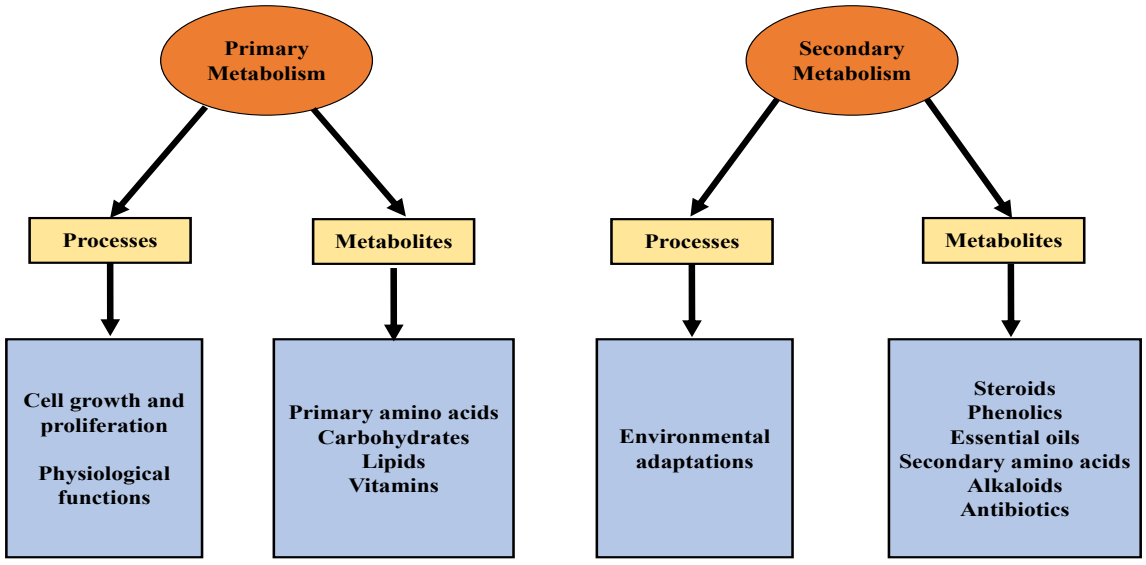

Figure 1.2: Primary and secondary metabolic processes and compounds

1.1.2 Glycerol Metabolism

Bacterial metabolism is often shaped around the carbon source present in the environment. Some bacteria are limited to growth from a single carbon source while others can grow from a variety of carbon sources. Glycerol is a three-carbon sugar alcohol with a hydroxyl group attached to each carbon. Its physical appearance is described as a clear, colourless, odourless, viscous and sweet- tasting liquid. This molecule was discovered by the chemist K. W. Scheele while heating a mixture of olive oil and litharge (lead monoxide). He called it the “sweet principle of fat” and was also able to reproduce this reaction with other metals and glycerides (Jungermann, 2018). Moreover, glycerol is a by-product in the production of biodiesel through transesterification, soap manufacturing and hydrolysis. Triglycerides and methanol are converted to biodiesel and waste glycerol (10%) as the main by-product in the presence of a catalyst such as sodium hydroxide (Figure 1.3).

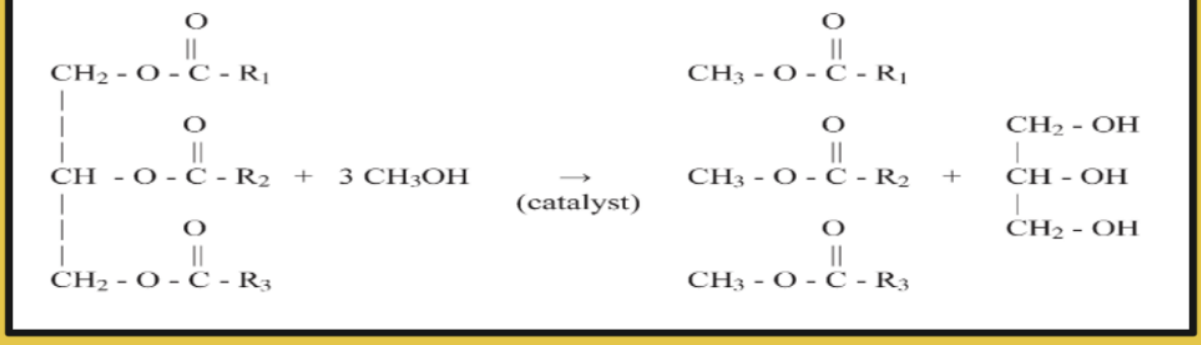

Figure 1.3: Transesterification of triglycerides with methanol, rendering biodiesel and glycerol (Taken from Gerpen, 2005)

Although his compound can be manufactured synthetically, its purity tend to be low (Tan et al., 2013). Several methods have been uncovered for the conversion of glycerol to its constituent valued products. These processes are either chemical, biochemical or bio-catalytic. Chemical catalysis is the major type of reaction but is often performed under harsh conditions such as high temperature, pressure and in the presence of toxic substrates. For this reason, bio-catalysis has emerged as one of the prominent techniques, mainly because it is sustainable and environmentally-neutral. This approach constitutes the programing of microorganisms on crude glycerol from biodiesel sources. The global production of crude glycerol and has increased significantly over the years as the prices have gone down (Figure 1.4).

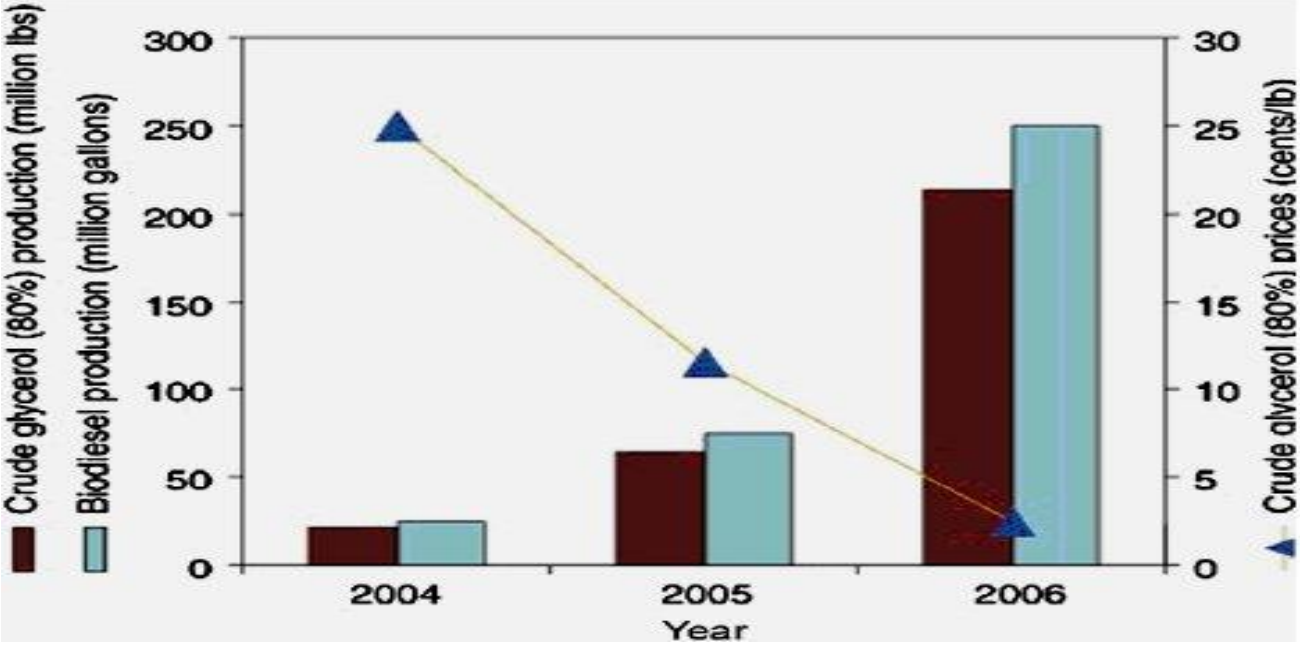

Figure 1.4: Global trends in crude glycerol, biodiesel production and prices in the early years. Taken from (Yazadani and Gonzalez, 2007)

Several studies have revealed different types of organisms (bacteria, fungi and algae) and their ability of utilizing this carbon source as a feedstock to generate valued products. Some of the products that can be synthesized by microorganisms subjected to glycerol include citric acid, polyhydroxyalkanoate, 1,3-propanediol and α-ketoglutarate (Figure 1.5) (Barbirato et al., 1997, Solaiman et al., 2006, Alhasawi et al., 2016). In fact, *Klebsiella pneumonia* and *Clostridium butyricum* are the two most widely utilized organisms in the production of 1,3-propanediol (Chatzifragkou et al., 2011). There are some drawbacks associated with this technique such as the presence of inhibitory compounds (by-products) from fermentation processes which affect the growth and cell proliferation of the organism, as well as the yield of the end product(s).

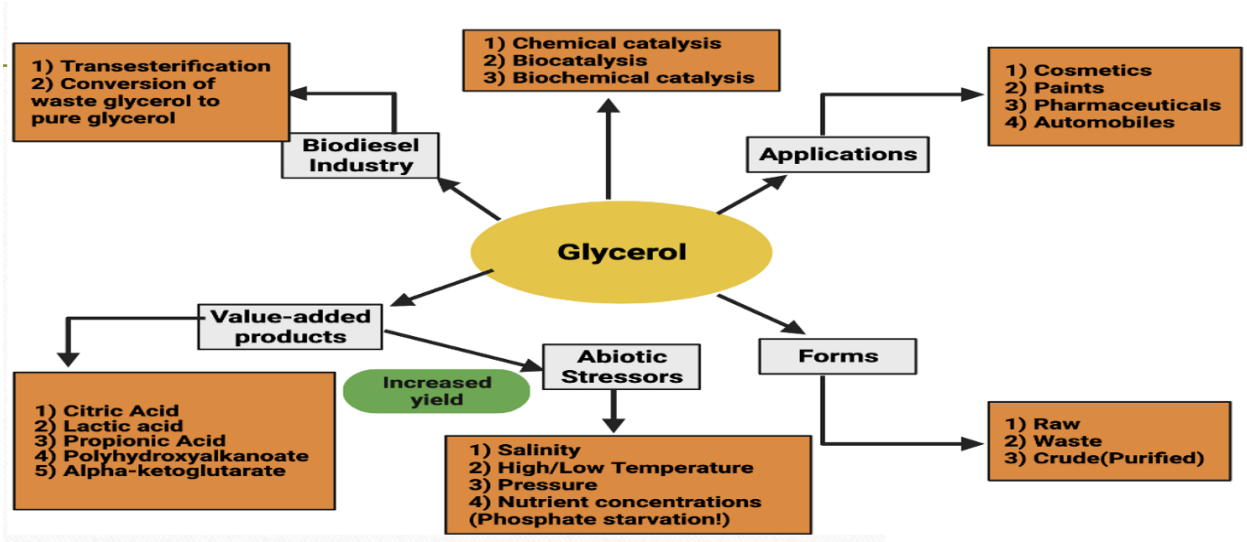

Figure 1.5: Biochemical significance of glycerol

To compensate for this shortcoming, the introduction of abiotic stressors and supplements are known to elevate the yield of the desired product. These stresses include increased salinity, change in temperature and pressure, and nutrient starvation. The ability of *Pseudomonas fluorescens* to increase the yield of α-ketoglutarate (10 x fold) in a glycerol-based medium supplemented with manganese (50 μM $MnCl_2$) (Alhasawi et al., 2016) has recently been demonstrated. Furthermore, when *Aspergillus niger* was supplemented with *Parkia biglobosa* fruit pulp, several parameters (pH, inoculum size, substrate concentration, incubation temperature and fermentation period) were modified to increase the yield of citric acid. High temperatures and acidic pH were the determining factors in the yield of this valued-product. (Auta et al., 2014). Another study demonstrated the ability of *Pseudomons putida* KT2440 to maximize the synthesis of polyhydroxyalkanoate from glycerol supplemented with lignin and glucose (Xu et al., 2021). These optimizations are extremely important because organisms such as *Pseudomonas fluorescens, Clostridium butyricum* and *Yarrowia lipolytica* can tolerate concentrations of glycerol up to 40% (w/v) (Dobson et al., 2012). However, when *Ruminococcus flavefaciens* and

Fibrobacter succinogenes subsp. are subjected to glycerol concentrations above 5%, their growth and metabolism are inhibited (Roger et al., 1992) and this could play a major factor in the manufacturing process of the commercial product. Furthermore, under aerobic conditions, *E. coli* can only grow and consume glycerol when global regulators such as cAMP receptor protein, catabolic repressor/activator protein (Cra) and other control proteins from gluconeogenesis and glycolysis pathways are present (Chiang et al., 2020).

The compatibility of glycerol with a wide range of other materials allows for the widespread utilization in food, medical, pharmaceutical and personal care industries (Wang et al., 2001). Furthermore, glycerol functions as a humectant, solvent, sweetener and preservative in foods and beverages. Glycerol may form other compounds and intermediates that play important roles in living systems. This polyol compound is converted to pyruvate and oxaloacetate before entering the TCA cycle, meanwhile it can participate in gluconeogenesis where it is metabolized to glucose. While glycerol may serve as a starting material for most organisms, others may synthesize it for their growth and survival. In fact, microbes, plants and numerous other organisms utilize this compound as a compatible solute in response to increased external osmotic pressure (Oren, 2016).

1.1.3 Phosphate as a modulator of cellular processes

Phosphorus is required by all living organisms to ensure cellular metabolism and energy transfer. The phosphate ion, also known as inorganic phosphate (P_i) is deemed to be a limiting factor in all living organisms and accounts for approximately 3% of the dry weight of all living organisms. (Sosa et al., 2017). It is a principal component of phospholipids, DNA and RNA. The latter nucleotides work in tandem as DNA provides the code for the cell's activities while RNA helps convert that code into proteins in order for the cell to function. P_i is also found in high energy

molecules such as ATP/ADP and cofactors (NADP and NADPH) from the TCA cycle. (Figure 1.6). Unicellular organism such as bacteria and yeast express specific P_i-binding proteins located on the membrane that respond to changes in P_i availability in order to regulate the expression of genes involved in P_i uptake by the transduction of intracellular signals (Kritmetapak and Kumar, 2019). Multicellular organisms like humans, on the other hand, have endocrine pathways which integrate signals from various organs to regulate the concentrations of P_i in the serum. Mammals depend on phosphate to modulate type III sodium-phosphate cotransporter activity on the plasma membrane. High levels of extracellular P_i induce activation of fibroblast growth factor receptor as well as several kinase pathways which control gene expression in various cell types (Michigami and Ozono, 2019).

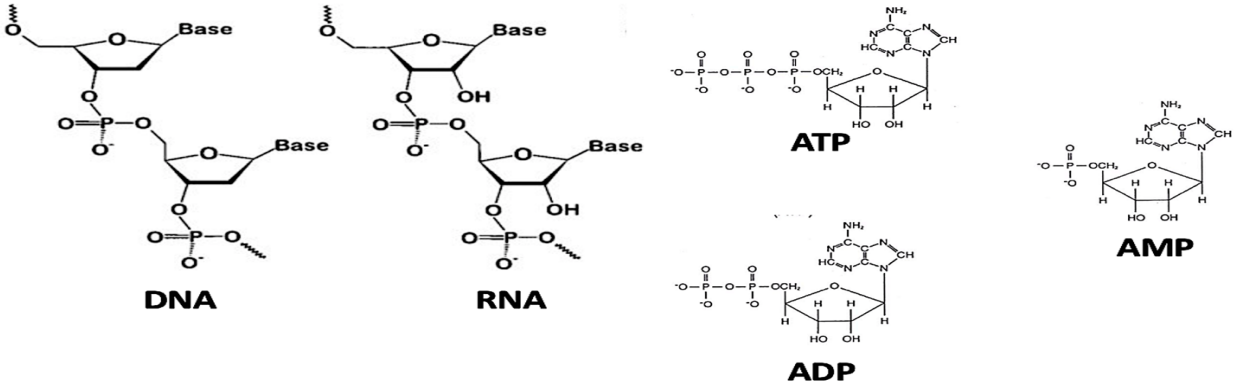

Figure 1.6: Chemical structures of DNA, RNA, ATP, ADP and AMP containing phosphate-linkages (Adapted from Noronha, 1998).

While bacteria mainly consume P_i for their growth, they may also convert it into polyphosphate for energy storage purposes. Phosphate maintains the shape and integrity of the cell, and is also the limiting nutrient in most environments, meaning that it has a substantial effect on the rate of growth and cell replication of an organism. Therefore, the levels of Pi supplemented in the respective media must be taken into account as this essential nutrient can affect the growth and

metabolism of the microbe. Given the diversity of P_i compounds, it is not surprising that many microorganisms are capable of using these ions or molecules as growth substrates. To summarize, phosphate is required for a number of cellular processes including replication, transcription and translation (Figure 1.7)

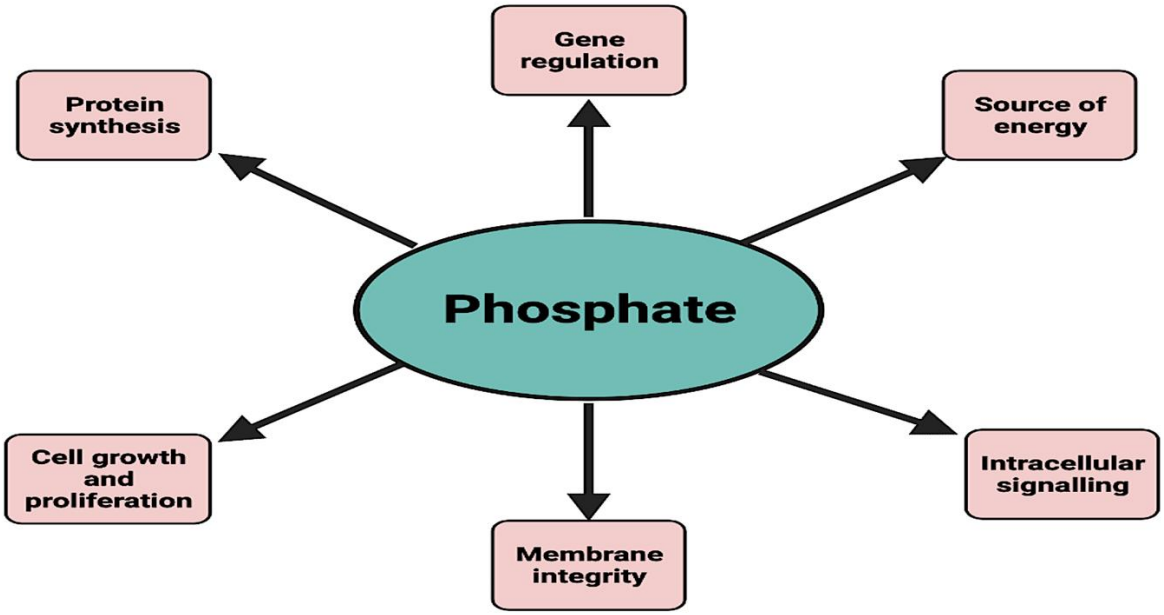

Figure 1.7: Importance of phosphate in living systems

1.1.4 Phosphate starvation and bacterial adaptation

Cellular activities vary according to the levels of phosphate introduced in the medium. For example, *Pseudomonas fluorescens* dictates the framework of the metabolic networks based on conditions with either low phosphate (LP) (<64 μM), normal phosphate (64 μM-0.64 mM) or high phosphate (HP) (>0.64 mM) (Appanna et al., 1994). There is no consensus for the minimum concentration of P_i required for growth of microorganisms. *E. coli* activates the low-affinity transport systems Pit and Pst, as well as the outer-membrane porin PhoE to transport P_i across the cells below a threshold as the organism cannot survive with external Pi levels lower than 20 μM

(Rao and Torriani, 1990). Low soluble salts are required for growth as bacteria reserve phosphate in complex forms such as polyphosphate for energy transduction and metabolism. The cultivation of different bacterial species at varying P_i concentrations is supported by a complex set of genes and mechanisms that mediate their survival and adaptation (Figure 1.8).

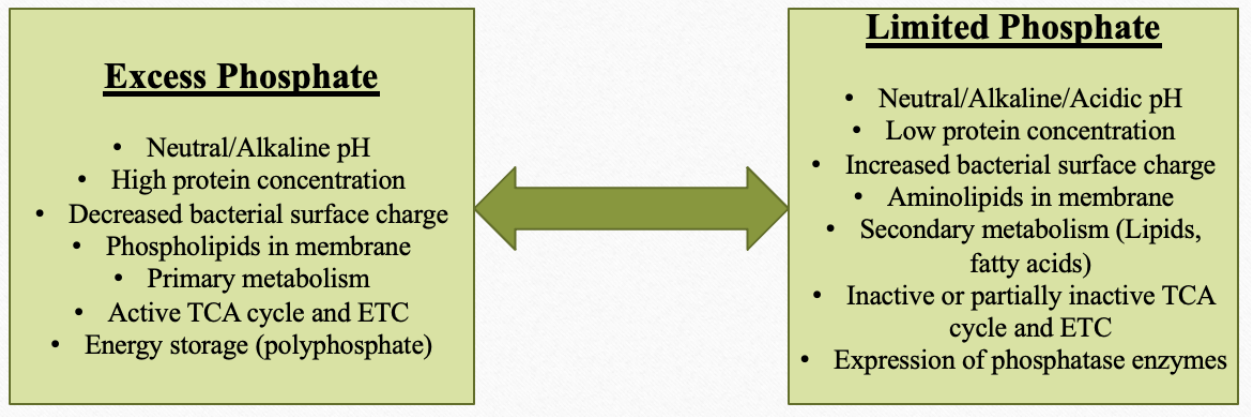

Figure 1.8: Characteristic traits of bacterial metabolism under low and excess phosphate

Phosphatase genes are expressed to combat a dearth of phosphate in environment. When P_i level is adequate these genes are supressed. Sufficient amounts of phosphate are normally required for bacteria to proliferate at a high rate and secrete high amounts of protein in the cell. In this setting, they utilize phospholipids in the membrane resulting in decreased bacterial surface charge. The presence of lipids and proteins are often correlated with neutral or alkaline pH at stationary phase of growth. The pH of spent fluid is generally associated with the types of compounds secreted. Primary metabolism occurs under these conditions with the activation of the TCA cycle and the ETC. Since microbes like *Pseudomonas fluorescens* only require a certain concentration of phosphate for proper growth, they may utilize the excess phosphate and convert it to polyphosphate for energy storage. When a microorganism is starved of phosphate, genes associated with the Pho regulon are expressed (White and Metcalf, 2007). These genes are so-

called P_i starvation-inducible genes. In addition, bacteria express high levels of phosphatase activity, an enzyme that is used to generate pools of phosphate for the organism's survival (Muller et al., 2019). In comparison to high phosphate conditions, P_i induces changes in bacterial physiology, protein expression and secondary metabolite production (Romano et al., 2015). Under phosphate deprivation, the organism's growth is characterized by a longer lag phase and lower protein concentration at stationary phase of growth, but this may vary depending on the level of phosphatase activity that could generate extracellular phosphate in a timely manner. Furthermore, their surface charge is increased due to the presence of aminolipids in the membrane, as well as the secretion of organic and amino acids. When a microbe is subjected to oxidative or nutrient stress, several ketoacids may act as ROS scavengers and the secretion of these dicarboxylic compounds may contribute to a decrease in pH (Lemire et al., 2017). Secondary metabolism is the favoured route as both the TCA cycle and ETC are severely depleted, forcing the organism to undergo an alternative metabolic pathway. Growing microorganisms under minimal levels of phosphate and studying their metabolism has become of interest to many scientists as the geochemical accessibility of phosphate on primordial Earth was limited. One such study uncovered a phosphate-independent metabolism comprised of a network enriched with enzymes requiring inorganic and iron-sulfur cofactors (Goldford et al., 2017). These findings would indicate that iron-sulfur proteins were primarily involved in ancient biological systems as they can carry out electron transfer, oxygen sensing and nitrogen fixation. Hence, as phosphate became more readily available, they became basic components of many biomolecules and essential in biochemical reactions but still more research needs to be done to unravel the roles of phosphate in the origin of life.

1.1.5 L-Carnitine: a potent valued product with numerous applications

L-Carnitine is a quaternary ammonium compound initially discovered in muscle extraction back in 1905 Its major physiological function, its biosynthetic pathway, transport mechanisms and metabolic role were elucidated in subsequent years. The biological significance of L-Carnitine was not unknown until it was found to be essential for the growth of the mealworm *Tenebrio molitor*. The growth of the organism was supported by the stimulation of fatty acids into the mitochondria for ß-oxidation (Ramsay, 1997). The overall process was described as the carnitine shuttle system, decrypting the role of this modified amino acid in energy production and fatty acid metabolism. L-Carnitine was promoted as a nutritional and pharmaceutical supplement in the 1960s to treat patients with carnitine deficiency and impaired fatty acid oxidation. L-Carnitine can be synthesized endogenously from lysine and methionine in the presence of cofactors such as ascorbic acid, ferrous ion, pyridoxine and NAD^+. Any dysregulations of these cofactors can lead to carnitine deficiency (Wolf, 2006). L-Carnitine is a natural compound obtained from diet via active and passive transport across intestinal cell, in red meats and dietary products. This compound can also be manufactured as a pharmaceutical supplement to improve exercise tolerance, burn fats and produce energy. The nutritional supplement of the biologically active form of carnitine can improve nerve conduction and immune function and ease neuropathic pain in patients diagnosed with diabetes. In fact, L-carnitine increases the production of antigen-specific immunoglobin G and downregulates inflammatory cytokines such as TNF-α and IL-6 (Mast et al., 2000, Winter et al., 1995). Acetyl-L-carnitine, an ester of L-carnitine is also recognized in the treatment of patients with cardiovascular disease, and may be beneficial in the treatment of obesity, ameliorating glucose intolerance and energy expenditure. Clinical trials have also highlighted a potential role of L-carnitine in Alzheimer's disease, hepatic encephalopathy and other neuropathic disorders (Flanagan et al., 2010) (Figure 1.9).

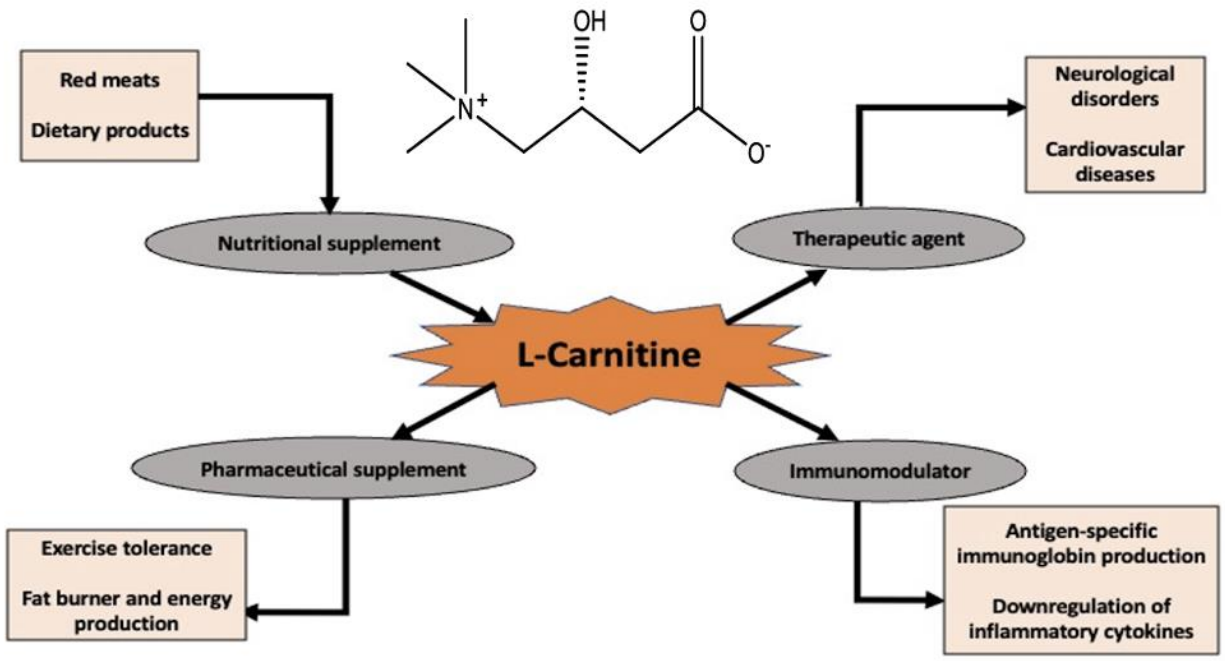

Figure 1.9: Commercial importance and applications of L-carnitine

While L-carnitine is mainly used as a feedstock for animals and microorganisms, its applications in medicine and manufacturing products are expected to grow in the upcoming years. Furthermore, the commercial value of this product has been increasing steadily over the years with increasing demand in numerous industries, thus increasing its value (Figure 1.10).

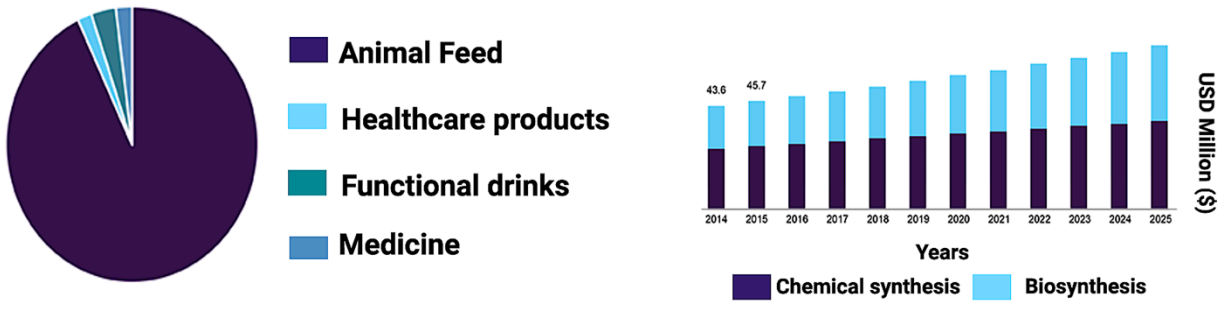

Figure 1.10: Global application share (%) and predicted market size of L-carnitine in the U.S. in upcoming years (Adapted from Grandview Research, 2019).

1.1.6 *Pseudomonas fluorescens*

Pseudomonas fluorescens is a common Gram-negative, rod-shaped bacterium that colonizes soils, plants and water surface environments. Its name is derived from the ability of the microbe to secrete a soluble fluorescent pigment called pyoverdine, which serves as a siderophore. This microbe possesses a number of interesting traits and requires simple nutrients that allows it to grow in mineral media (Stoimenova et al., 2009). It is classified as an obligate aerobe, except for some strains that can utilize NO_3^- during cellular respiration, meaning that they need oxygen present in order to survive. *P. fluorescens* is utilized in suppressing plant diseases by the production of secondary metabolites such as siderophores and antibiotics, and also used in bioremediation processes (Scales et al., 2014) (Figure 1.11).

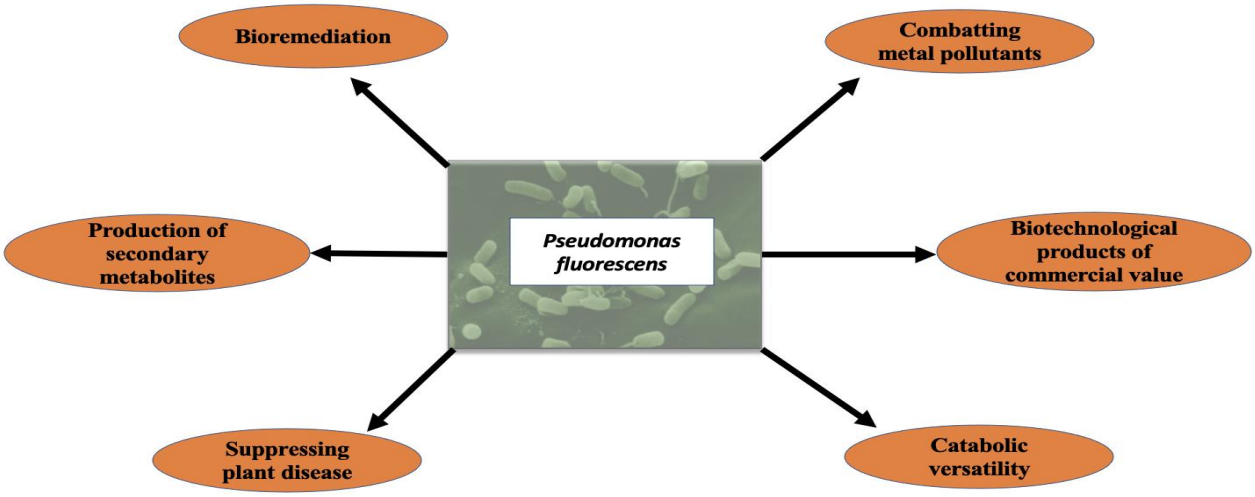

Figure 1.11: Biochemical characteristics of the nutritionally-versatile microbe *Pseudomonas fluorescens*

Pseudomonas fluorescens is mainly known for orchestrating metabolic reconfigurations when exposed to adverse conditions. For example, under oxidative stress, the TCA cycle and ETC are severely impeded as the microbe is forced to adapt and produce primary and secondary

metabolites aiding in its survival. When *P. fluorescens* is subjected to a citrate or glycine-based medium in the presence of hydrogen peroxide (H_2O_2), its metabolism is altered to produce ATP, NADPH and glyoxylate in order to neutralize the ROS (Alhasawi et al., 2015b). Formate is liberated upon the neutralization of ROS with glyoxylate, and is used as a reducing force to generate NADPH and succinate as an anti-oxidant defence mechanism (Thomas et al., 2015). Several ketoacids such as α-KG, oxaloacetate, pyruvate and glyoxylate were proven successful in combatting reactive oxygen species (Alhasawi et al., 2015a, Lemire et al., 2011, MacLean et al., 2020, Mailloux et al., 2010, Singh et al., 2008, Thomas et al., 2015). Hence, ICDH-$NADP^+$ and ICDH-NADPH are key mediators in the antioxidative defence mechanisms deployed by *P. fluorescens* (Figure 1.12). This organism can also modify its metabolic networks to increase the yield of oxalate to combat metal stress (Alhasawi et al., 2015b). Furthermore, nutrient changes are known to trigger biochemical reconfigurations that may lead to a product of commercial value. In fact, the addition of manganese to a high phosphate medium with glycerol elicits the formation of α-KG (Alhasawi and Appanna, 2016). Owing to its nutritional versatility and ability to survive in extreme environments, the soil microbe *P. fluorescens* is an excellent model system to study biochemical adaptation.

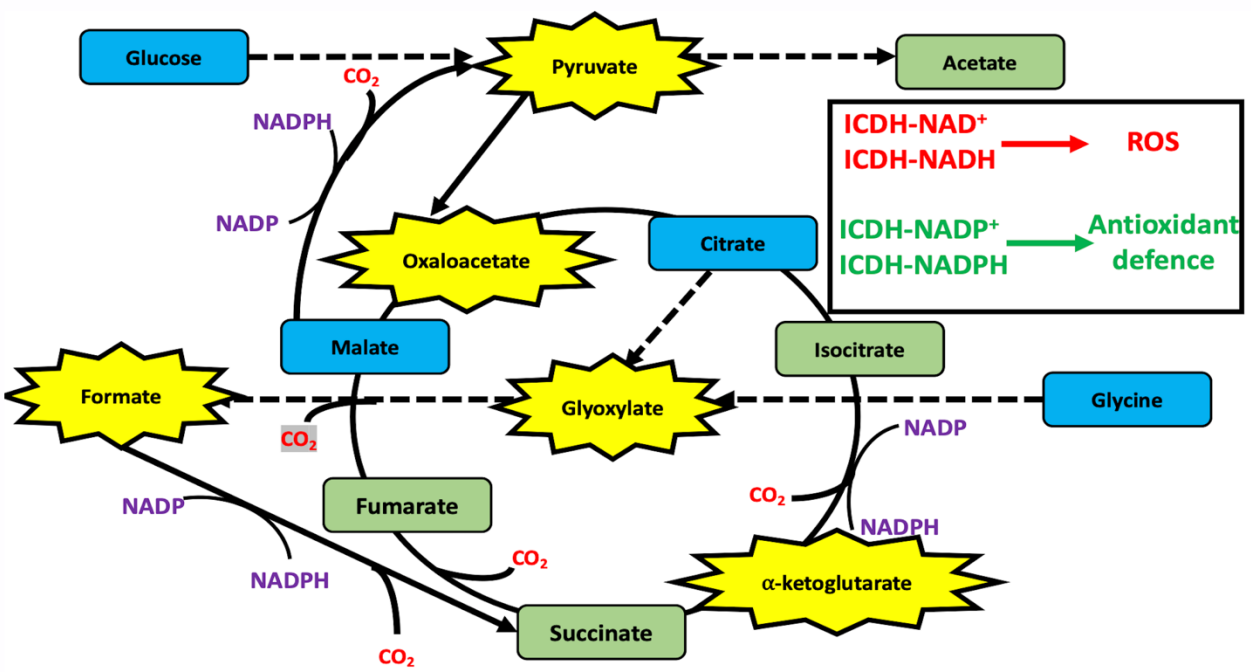

Figure 1.12: Metabolic networks deployed by *Pseudomonas fluorescens* to combat oxidative stress. [NADPH and ketoacids are the antioxidants].

1.2 Thesis Objectives

The main objective of this research is to elucidate the metabolic shift of *P. fluorescens* reconfigured under phosphate starvation compared to a condition with sufficient phosphate in a glycerol environment. *P. fluorescens* is an extremely versatile soil microbe and an excellent model to study metabolic reprogramming as a consequence of abiotic stress. It is characterized with a number of functional traits that enable it to grow in a mineral medium under diverse carbon sources. In this instance, the ability of the organism to survive and adapt in a mineral medium with glycerol as the sole carbon source and limited phosphate (25 μM) will be evaluated. Under phosphate starvation where P_i is consumed rapidly, bacteria deploy unique strategies for survival and may generate alternative high energy compounds such as polyphosphate or pyrophosphate upon expression of phosphate-inducible starvation enzymes. In addition, glycerol

is an excellent source of feedstock for bacteria to grow on and generate valued-products from reprogrammed metabolic pathways. Functional proteomics will be studied by means of high-performance liquid chromatography (HPLC) and blue native polyacrylamide gel electrophoresis (BN-PAGE). Furthermore, gene expression of metabolic enzymes will be characterized by real time quantitative polymerase chain reaction (RT-qPCR) (Figure 1.13). This template provides a systematic procedure how to obtain molecular insights into glycerol metabolism in *P. fluorescens*. The findings on these metabolic networks may reveal effective strategies to generate valuable secondary products under phosphate starvation and provide insights into how organisms manage their energy budget despite a dearth of phosphate.

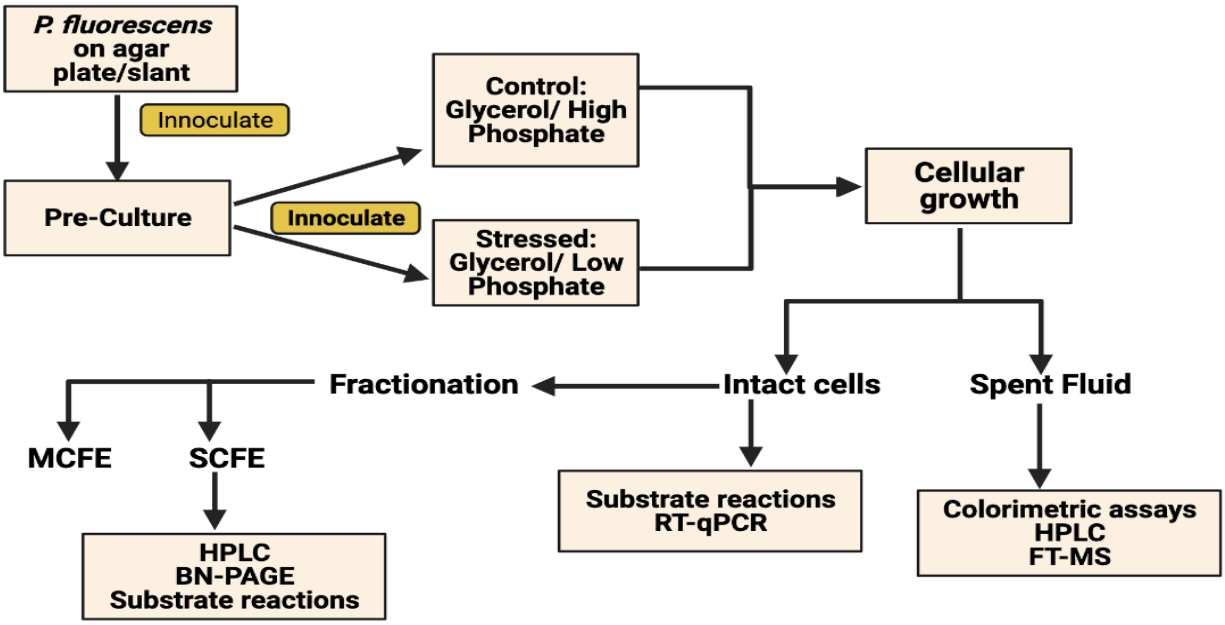

Figure 1.13: General experimental design of the project. MCFE; Membrane cell free extract; SCFE: soluble cell free extract; HPLC: High performance liquid chromatography; BN-PAGE: Blue-native polyacrylamide gel electrophoresis; RT-qPCR: Real-time polymerase chain reaction; FT-MS: Fourier transform mass spectrometry.

CHAPTER 2: Phosphate stress triggers the conversion of glycerol into L-carnitine in *Pseudomonas fluorescens*

Phosphate stress triggers the conversion of glycerol into L-carnitine in *Pseudomonas fluorescens*

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2.1 Abstract:

Glycerol, a by-product of the biofuel industry is transformed into L-carnitine when the soil microbe *Pseudomonas fluorescens* is cultured in a phosphate-limited mineral medium (LP). Although the biomass yield was similar to that recorded in phosphate-sufficient cultures (HP), the rate of growth was slower. Phosphate was completely consumed in the LP cultures while in the HP media, approximately 35 % of the initial phosphate was detected at stationary phase of growth. The enhanced production of α-ketoglutarate (KG) in HP cultures supplemented with manganese was recently reported (Alhasawi et al., 2017). L-carnitine appeared to be a prominent metabolite in the spent fluid while the soluble cellular-free extract was characterized with peaks attributable to lysine, γ-butyrobetaine (GB), acetate and succinate in the LP cultures. Upon incubation with glycerol and NH4Cl, the resting cells readily secreted L-carnitine and revealed the presence of such precursors like GB, lysine and methionine involved in the synthesis of this trimethylated moiety. Functional proteomic studies of select enzymes participating in tricarboxylic acid cycle (TCA), oxidative phosphorylation (OP), glyoxylate cycle and L- carnitine synthesis revealed a major metabolic reconfiguration evoked by phosphate stress. While isocitrate dehydrogenase-NAD^+ dependent (ICDH-NAD^+) and Complex I were markedly diminished, the activities of γ-butyrobetaine aldehyde dehydrogenase (GBADH) and L-carnitine dehydrogenase (CDH) were enhanced. Real- time quantitative polymerase chain reaction (RT-qPCR) analyses pointed to an increase in transcripts of the enzymes γ-butyrobetaine dioxygenase (bbox1), S-adenosylmethionine synthase (metK) and L-carnitine dehydrogenase (lcdH). The L-carnitine/γ-butyrobetaine antiporter (caiT) was enhanced more than 400-fold in the LP cultures compared to the HP controls. This metabolic reprogramming modulated by phosphate

deprivation may provide an effective technology to transform glycerol, an industrial waste into valuable L-carnitine.

Keywords: *Pseudomonas fluorescens*, Metabolic Networks, Glycerol, Phosphate starvation, L-carnitine

2.2. Introduction

Glycerol is a trihydroxy sugar alcohol, alternatively named glycerin, and has numerous applications in cosmetics, paints, foods, tobacco, pharmaceuticals and automobiles. This compound is a by-product resulting from soap manufacturing, microbial fermentation and bio-diesel production. It is an excellent source of carbon that can be readily utilized by microorganisms in order to generate high-value chemicals. Currently, citric acid, lactic acid, polyhydroxyalkanoate (PHA), hydrogen and ethanol can be synthesized from crude glycerol (Li et al., 2013). For example, microbes like *Yarrowia lipolytica* can yield up to 0.67 g/g of citric acid from glycerol in batch-regulated processes (Rywin´ska et al., 2011). *Pseudomonas fluorescens* ATCC 13525 is known to increase citric acid biosynthesis by over 20-fold due to the overexpression of the citrate synthase gene (Buch et al., 2009). Pro- duction of the biopolymer PHA from crude glycerol in concentrations ranging from 49 % to 63 % of the cell dry weight by *Pandorea* spp. has also been reported (Paula et al., 2017). Abiotic stress including salinity, change in temperature and nutrient levels can also help modulate the synthesis of metabolites. Indeed, nitrogen starvation has been shown to maximize PHA formation in *Novosphingobium* spp. (Teeka et al., 2012). Furthermore, the highest production of PHA by *Bacillus megaterium* was uncovered in media subjected to high temperatures and alkaline pH (Alvarado-Cordero et al., 2017). *Clostridium butyricum* grown under anaerobic conditions and high osmotic pressure with

glycerol as a car- bon source resulted in optimal synthesis of 1,3-propanediol (Szymanowska-Powalowska and Bialas., 2014). Recently, work in our laboratory revealed the ability of *P. fluorescens* to generate elevated amounts of alpha ketoglutarate (KG) in a glycerol medium enriched with manganese (Mn) (Alhasawi et al., 2017). This stimulation was not observed in media devoid of added Mn.

Phosphate (P_i) is an essential nutrient for all living systems. It is responsible for the functioning of a variety of biochemical pathways including energy transfer, protein activation, carbohydrate and amino acid metabolic processes. Without a sufficient amount of P_i, these pro- cesses are markedly altered (Appanna et al., 2016). Hence, to acquire this nutrient, numerous living systems have evolved intricate biochemical pathways to sense, transport and regulate P_i. P_i starvation triggers the expression of P_i starvation-inducible (psi) genes, which appear to be involved in multiple P_i-dependent metabolic networks (Romano et al., 2015). Most bacteria store this crucial moiety in the form of an inorganic polymer, polyphosphate (polyP_i), usually located in the cytoplasm (Müller et al., 2019). In an environment with a dearth of P_i, high-affinity transporters and phosphatases are overexpressed to in- crease the pool of Pi (Nussaume, 2011). Since P_i is a key ingredient propelling cellular respiration, a decrease in the concentration of this nutrient triggers metabolic reconfiguration aimed at limiting the use of this ion. The inclusion of aminolipids in cellular membranes and metabolic pathways not necessitating P_i are promoted. The synthesis of antibiotics and other secondary metabolites such as lipids and fatty acids evoked by P_i-deficiency have also been recorded in microbial systems (Yang et al., 2018). For instance, *Streptomyces* spp. can mediate the synthesis of antibiotics through the activity of the Pho regulon- phosphate regulator (PhoR-PhoP) system and overexpression of the alkaline phosphatase gene (Martiń,2004).This type of response is not only observed in bacterial systems

but also in plants. In *Arabidopsis* spp, the protein phosphate starvation response 1 transcription factor (PHR1) dictates metabolic configurations under limited phosphate and regulates the secretion of primary and secondary metabolites (Pant et al., 2014). Thus, P_i is an important modulator of metabolic processes in numerous living organisms (Dick et al., 2011).

In this report, P_i deficiency has been invoked to induce the conversion of glycerol into a value-added product by the metabolically- versatile soil microbe *P. fluorescens.* Herein, we demonstrate that this microbe produces L-carnitine from glycerol in a mineral medium with limited P_i. The metabolic reprogramming orchestrating the formation of L-carnitine is assessed. The significance of intact cells to secrete this quaternary ammonium compound when incubated with glycerol is explained. The modulation of the tricarboxylic acid cycle (TCA cycle), oxidative phosphorylation (OP), L-carnitine synthesizing enzymes and efflux system that contribute to the secretion of copious amounts of this non-essential amino acid is also discussed. These findings may provide an efficient route to a value-added chemical from glycerol, an industrial waste.

2.3 Materials and Methods

2.3.1 Microbial growth conditions and cellular fractionations

The bacterial strain *Pseudomonas fluorescens* (ATCC 13525) obtained from the American Type Culture Collection was grown in a defined medium of Na_2HPO_4 (0.0023 g), KH_2PO_4 (0.0013 g), NH_4Cl (0.8 g), $MgSO_4 \cdot 7H_2O$ (0.2 g) per liter of deionized water. Trace elements (1 mL) were also added to the medium (Anderson et al., 1992). Glycerol (10 % w/v): 1.1 M) from Fisher Scientific (Ottawa, Canada) was utilized and the pH of the medium was adjusted to 6.8 with 2 N NaOH. In the high phosphate medium, 1.2 g of Na_2HPO_4 and 0.6 g of KH_2PO_4 were utilized. Aliquots of the media (200 mL) were dispensed in 500 mL Erlenmeyer flask, followed by

inoculation of 1 mL of *P. fluorescens* at stationary phase of growth. The cultures were incubated in a gyratory water bath shaker [Model 76; New Brunswick Scientific] at 26 °C and a speed of 140 rpm. The bacterial cells were grown to stationary phase (96 h) and harvested by centrifugation at 11,000 x g for 20 min at 4 °C. The supernatant was removed and the pellet was resuspended in cell storage buffer (CSB) consisting of 50 mM Tris-HCl, 5 mM $MgCl_2$ and 1 mM phenylmethylsulfonylfluoride (PMSF) at pH 7.6. Following the resus- pension, the cells were disrupted by sonication with the aid of a sonicator [Brunswick] on ice at a power level of 4 [16 s, 5 times with 3 min wait intervals]. Intact cells were removed at 11,000 x g and the super- natant was then centrifuged at 30,000 x g for 90 min to afford the soluble fraction (sCFE) and the membrane fraction (mCFE) that was reconstituted in the storage buffer (Alhasawi et al., 2015). The Bradford assay was performed in triplicate to determine the protein concentration in both fractions. Bovine serum albumin (BSA) was used as the reference standard (Bradford, 1976). A modified 2,4-dinitrophenylhydrazine (DNPH) assay was used to detect the presence of compounds with carbonyl groups (Mesquita et al., 2014). The consumption of ammonium and phosphate were measured as described previously (Solorzano, 1969; Pradhan and Pokhrel, 2013). Phosphatase activity was measured by a spectrophotometric assay based on the ability of the enzyme to catalyze the hydrolysis of para-nitrophenylphosphate (pNPP) to *p*-nitrophenol (Ninfa et al., 2015).

2.3.2 Enzyme activity detection with blue-native polyacrylamide gel electrophoresis (BN-PAGE)

Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was performed to assess the activity of various enzymes upregulated or downregulated in L-carnitine biosynthesis. Linear gradient

gels (16 % and 4 %) were prepared to ensure optimal protein separation in the Biorad Mini-Protean TM system using1 mm comb spacers (Mailloux et al., 2008). Sixty micrograms of protein from the membrane and soluble cell free extract were loaded in every second well and the gels were electrophoresed under native conditions (50 mM ε-aminocaproic acid, 15 mM Bis-Tris, pH 7, 4 ˚C) at 85 V to ensure proper gel stacking for 1 h. The voltage was increased to 150 V for the second hour and 300 V for the last hour, allowing the proteins to migrate through the gel. Blue cathode buffer (50 mM Tricine, 15 mM Bis-Tris, 0.02 % (w/v) Coomassie Brilliant Blue G-250 (pH 7)) was poured between the gel plates to visualize the proteins migrating and then substituted with a colorless cathode buffer (50 mM Tricine, 15 mM Bis-Tris (pH 7) at 4 ˚C) when the proteins migrated halfway through the gel. In-gel visualization of enzyme activity was revealed via formazan precipitation. Reaction mixtures were prepared as follows: 5 mM substrate, 0.5 mM cofactor with 0.2 mg/mL of phenazine methosulfate (PMS) or 2,6-dichlorophenolindophenol (DCPIP), 0.4 mg/mL of iodonitrotetrazolium (INT) and reaction buffer based on the fraction being analyzed, and then poured onto the respective gel lanes (Han et al., 2012). Complex I activity was revealed using reaction buffer with NADH, ICDH-NAD^+ was visualized using isocitrate and NAD^+, while pyruvate dehydrogenase (PDH) was assessed with pyruvate, CoA (0.25 mM), and NAD^+. In the soluble fraction, ICDH-$NADP^+$ activity was visualized as described in (Legendre et al., 2019), while the L-carnitine dehydrogenase activity band was obtained using L-carnitine (5 mM) and NAD^+. The band was cut from the gel and incubated with the same substrate and cofactor to visualize the formation of 3-dehydrocarnitine and NADH. Lastly, γ-butyrobetaine aldehyde dehydrogenase (GBADH) activity was monitored as described in (Lemire et al., 2011). The activity band was excised from the gel and incubated with γ-butyrobetaine and NADH to monitor the formation of γ-butyrobetaine aldehyde. To stop

the reaction, an aqueous destaining solution (40 % methanol, 10 % glacial acetic acid) was used at the time of maximal intensity. The gels were visualized with CanoScan LiDE 220.

2.3.3 Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted using the TRIzol method as described previously (Alhasawi et al., 2019; Tharmalingam et al., 2020). RNA samples were treated with DNase (Sigma) and reverse transcribed to complementary DNA using random primers (Sigma), oligo dT (NEB), and M-MLV reverse transcriptase (Promega). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using Quant- Studio5 (ThermoFisher) as reported previously (Legendre et al., 2019). Primer sequences were designed (Primer-BLAST) and validated in-house (Table 1) (Davidson et al., 2020). Normalization was achieved using two independent control housekeeping genes (rpoB and groL). mRNA expression was reported using the ΔΔCT method as relative mRNA fold increase (Livak and Schmittgen, 2001).

2.3.4 Metabolite analysis: High Performance Liquid Chromatography and Fourier transform mass spectrometry

Metabolites and intermediate compounds were detected and quantified by means of high performance liquid chromatography (HPLC) in the spent fluid and soluble cell free extracts. Samples were injected in an Alliance HPLC with a C18 reverse-phase column (Synergi Hydro-RP; 4 μm; 250 × 4.6 mm, Phenomenex). A Waters Dual Absorbance Detector and Empower software were used as described in (Alhasawi et al., 2014). The mobile phase was composed of 20 mM KH_2PO_4, 5 % (v/v) acetonitrile at pH 2.9 and a flow rate of 0.7 mL/min at ambient temperature was used to separate the substrates and products. Compounds with carbonyl groups

were detected at 210 nm and biological samples were spiked with known standards to confirm the identity of the metabolite. *P. fluorescens* cells were harvested at stationary phase of growth by centrifugation at 11,000 x g for 20 min at 4 °C. An incubation mixture (1 mL) consisting of 200 µg of protein, glycerol and NH_4Cl (5 mM) in reaction buffer [50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4] was prepared. The reaction was performed at ambient temperature, and 300 µL aliquots were taken at 30 min, 1 h and 24 h and centrifuged at 11,000 x *g* for 10 min. The production of L-carnitine in the spent fluid over time was assessed by HPLC.

The detection of this compound was further confirmed by Fourier transform mass spectrometry (FT-MS). High-resolution measurements were obtained with a mass spectrometer (Thermo Scientific Q-Exactive) fitted with a heated electrospray ionization source (HESII) operating in the positive and negative ionization modes. The ionization spray was set to 3.5 kV. For LC–MS and tandem mass spectrometry experiments, the resolving power was set at 70 000 ΔM. Mass spectra were acquired over an *m/z* range from *m/z* 100 to *m/z* 1200, displaying the five most abundant ions. Analyses were conducted using a UHPLC (Thermo Sci- entific Ultimate 3000) equipped with a C18 reverse phase column (5.0 mm x 2.1 mm, 1.9 µm, Thermo Scientific Hypersil Gold). Elution was conducted at 40 °C using a mobile phase gradient with a flow rate of 0.3 mL/min. Phase A consisted of 5 mM acetate with pH adjusted to 6 in ddH_2O while phase B was methanol. The gradient started at 5 % for 1 min, followed by a linear gradient to 100 % for 6 min and remained for another 3 min at 100 %. The system returned to the initial solvent composition for 1 min and re-equilibrated under these conditions for 4 min. Samples were diluted accordingly before analysis and the auto- sampler was kept at 4 ° C. Detection limits were set with a mass tolerance of 10 ppm and a minimum peak intensity of 1×10^4. Databases were derived from Human Metabolome Database (HMDB), MetaCyc and KEGG.

2.3.5 Statistical analysis

Data was expressed as the means of one ± standard deviation (SD). Statistical significance of the data was assessed by the student τ test ($p \leq 0.05$) and all experiments were performed three times and in triplicate readings. Differential analysis and statistical calculations of FT-MS data were obtained from the Thermo Scientific Compound Discoverer 2.1 software.

2.4. Results

2.4.1 Phosphate as a modulator of carnitine production

When *P. fluorescens* was subjected to low phosphate (LP) medium with glycerol as the sole carbon source, a completely different biochemical profile was observed compared to the high phosphate (HP) culture. As expected, the rate of growth was slower in the LP medium, however the cellular yield at stationary phase of growth was similar to that observed in the HP medium. There were no significant differences in the consumption of glycerol and ammonium in these cultures as both nutrients were utilized for cellular proliferation. At stationary phase of growth, the nitrogen source was completely consumed, while only 70 % of the carbon source was utilized in the LP compared to 63 % in the HP condition. The LP cultures were characterized with a decrease in pH, a complete consumption of P_i and an increase in phosphatase activity. At stationary phase of growth, 35 % of the initial phosphate was detected in the spent fluid and no discernable phosphatase activity was evident in the HP cultures. As previously reported (Alhasawi and Appanna, 2017), copious amounts of KG were produced in a HP medium as revealed by the DNPH colorimetric assay (Table 2).

2.4.2 Identification of L-carnitine in the spent fluid from the LP cultures

These disparate physico-chemical parameters were coupled to marked variations in metabolic profiles observed in the spent fluids and the soluble cellular fractions from these cultures. While a major peak attributable to L-carnitine was detected in the LP culture, several prominent peaks indicative of PEP, pyruvate and acetate respectively were evident in the spent fluid of the HP medium at the stationary phase of growth (Fig. 2.7.1A). To identify the time-dependent secretion of L- carnitine, the presence of this metabolite was monitored at different time intervals and an increase in the production of this non-essential amino acid was observed over time. The relative production of L-carnitine was characterized by high performance liquid chromatography (AUC) (Fig. 2.7.1B). To further confirm that L-carnitine was being secreted in elevated amount, the spent fluid was analyzed by FT-MS. The mass to charge ratio of 162.1 [M+H] for the derived amino acid matches the spectrum obtained from the MassBank Record (Sawada et al., 2012) (Fig. 2.7.1C). The HPLC analyses of the soluble CFE (sCFE) revealed numerous metabolites such as lysine, GB, KG, isocitrate, methionine, acetate and succinate that may contribute to the elevated amounts of L-carnitine associated with the spent fluid from the LP cultures (Fig. 2.7.1D).

2.4.3 LP-treated cells and the biotransformation of glycerol into L-carnitine

As the spent fluid was characterized with copious amounts of L- carnitine and numerous precursors of this non-essential amino acid were evident in the soluble CFE, it was important to assess the ability of the intact cells to produce this value-added product from glycerol. The intact cells harvested at stationary phase of growth were incubated with 5 mM glycerol and NH_4Cl in the reaction buffer. Cells were centrifuged (11,000 x g) at different time intervals and the

secretion of L-carnitine in the spent fluid was monitored by means of HPLC (Fig. 2.7.2A). To determine which intermediates were involved in the synthesis of this derived amino acid, the cells were resuspended in cell storage buffer (CSB) and sonicated to break them apart. At this stage, several intermediates such as lysine, acetyl-CoA, GB, methionine and succinate were detected in the cells (Fig. 2.7.2B).

2.4.4 Assessment of metabolic pathways and enzymes contributing to L-carnitine biosynthesis

As the evidence pointed to L-carnitine being secreted in the spent fluid and to the ability of the intact cells to produce this compound from glycerol, the metabolic pathways prompting this biotransformation were deciphered by monitoring enzyme activities of the CFE. Since ac- etate and lysine were detected in the soluble cell free extract, these substrates were incubated with 200 µg equivalent of protein of sCFE to detect the formation of L-carnitine over time. The formation of the quaternary ammonium compound was concomitant with the utilization of lysine, acetate and GB (Fig. 2.7.3A). Enzymes related to the TCA cycle and the electron transport chain (ETC) were monitored in both the LP and HP (m & s) CFE to gain insight into the energy metabolism in both systems. Complex I and ICDH-NAD^+ were downregulated in the LP while bands indicative of ICDH-$NADP^+$ and PDH were prominent (Fig. 2.7.3B). The presence of CDH and GBADH provided further evidence how L-carnitine was being generated. To confirm the activity of these enzymes, the bands were excised from the gel and incubated with the substrate and cofactor. L-carnitine and NAD^+ produced 3-dehydrocarni- tine and NADH upon incubation with the excised CDH activity band. (Fig. 2.7.3C). The GBADH activity band converted GB to GB aldehyde as the end product (Fig. 2.7.3D).

3.5 Enhanced transcripts responsible for L-carnitine biosynthesis

After assessing the activity of several enzymes on gel, it was important to verify the mRNA expression of some of the prominent enzymes in these metabolic networks by RT-qPCR. The genetic information of *Pseudomonas fluorescens* ATCC 13525 on the ATCC genome portal was utilized. RT-qPCR primers were designed based on this sequence (Table 1). Detailed analysis revealed increased mRNA expression of several enzymes specific to L-carnitine biosynthesis in the LP cultures compared to the control cultures ($n = 3$; $p < 0.05$) (Fig. 2.7.4). These transcripts attributable to L-carnitine dehydrogenase (714.6-fold), γ-butyrobetaine dioxygenase (334.2-fold), L-carnitine/γ-butyrobetaine antiporter (455.8 fold), S-adenosylmethionine synthase (51.9-fold) and carnityl-CoA dehydratase (1.0-fold) were augmented. Additionally, the expression of isocitrate lyase (31.8-fold) and phosphate starvation- inducible protein (2.7-fold) were also notably higher in the stressed cultures.

2.5. Discussion

The data in this study demonstrate the ability of *P. fluorescens* to produce copious amounts of L-carnitine when deprived of phosphate in a mineral medium with glycerol as the sole carbon source. The metabolic reprogramming observed in this microbe under phosphate starvation affords a unique opportunity to generate a valuable commercial product. L-carnitine, a non-essential amino-acid was first isolated from muscle cells of the mealworm *Tenebrio molitor* in 1905. Its role in microbial metabolism and fatty acid degradation was not confirmed until later on. Several studies revealed the ability of bacteria to utilize this modified amino acid in aerobic or anaerobic environments for a variety of functions such as a carbon, nitrogen or energy source, an electron acceptor or an osmoprotectant. For instance, some *E. coli* strains can use L-carnitine

and crotonobetaine as final electron acceptors in the absence of oxygen or other common electron acceptors (Walt and Kahn, 2002). In addition to osmoprotection, compatible solutes can protect bacteria from additional sources of stress. In fact, L. *monocytogenes* and *Bacillus subtilis* use the OpuC carnitine transport system to protect against bile stress and cold shock (Watson et al., 2009; Hoffman and Bremer, 2011). Aside from its biological functions, L-carnitine has a wide range of applications in pharmaceuticals, food products, and feed additives and increasing demand for this product has become prominent (Naidu et al., 2000). Over the last few decades, the viability of producing L-carnitine from waste products of the chemical industry through the biotransformation mediated by microbial organisms has drawn significant interest from researchers (Bernal et al., 2007a).

The evidence suggests that the production of L-carnitine is evoked by a phosphate-limited medium as the presence of increased phosphate resulted in the secretion of other metabolites like PEP and pyruvate in the spent fluid. Various analytical techniques confirmed the nature of this non-essential amino acid that is known to be synthesized by two main pathways in bacteria (Meadows and Wargo, 2015). The first route is the γ-butyrobetaine (GB) pathway whereby *N*-trimethyllysine is formed and converted into subsequent intermediates including GB. The last step involves the conversion of GB into L-carnitine with the assistance of γ-butyrobetaine dioxygenase (GBD), a reaction necessitating $Fe^{3+}$and KG as cofactors (Lemire et al., 2011). The second pathway is the uptake of choline and betaine to yield 3-dehydrocarnitine. This precursor can yield L-carnitine by the activity of L-carnitine dehydrogenase (CDH). The presence of lysine, GB and 3-dehydrocarnitine associated with the cellular extracts and the various in-gel enzyme reactions would argue for the possibility that *P. fluorescens* might be invoking these two pathways to generate this metabolite. In fact, the ability of the soluble CFE to produce L-carnitine when

incubated with lysine and acetate would suggest an important contribution of this pathway in the enhanced production of L-carnitine observed under phosphate (Pi) starvation. Furthermore, the activation of CDH that contributes to the synthesis of L-carnitine may also help modulate the homeostasis of NADH, a pro-oxidant that may not be readily utilized in the formation of ATP via oxidative phosphorylation (Singh et al., 2007; Mailloux et al., 2010). This finding is consistent with our previous observations where *P. fluorescens* converts NADH into NADPH as an anti-oxidative defence strategy (Singh et al., 2008). Previous studies have shown the ability of genetically-modified *E. coli* strains to yield high levels of this compatible solute from precursors such as GB and crotonobetaine (Arense et al., 2013). L-carnitine is transported into the bacterial cytosol with the aid of a ATP-binding cassette- [ABC] trans- porter or an L-carnitine/γ-butyrobetaine antiporter (Ziegler et al., 2010). The antiporter allows for the rapid exchange of L-carnitine and its pre- cursors, an event that enables the acquisition of the modified amino acid required to combat osmotic stress. Crotonobetaine can also be converted into L-carnitine, a reaction mediated by carnityl-CoA dehydratase (caiD). In *E. coli*, caiT is highly specific and is able to work independently of cellular energy (Jung et al., 2002). In fact, the overexpression of caiT has been shown to yield a 3-fold increase in L-carnitine in both growing and resting cells (Bernal et al., 2007b). The over-expression of caiT and caiD mRNA that were 450- and 1-fold higher respectively in the LP cultures compared to the bacteria harvested from the HP media would provide an effective biosynthetic and export system that would further accelerate the release of this non-essential amino acid. This L-carnitine secretory pathway appeared to be supplemented by the increased expression of mRNA responsible for GBD, an enzyme dedicated to the synthesis of L-carnitine from GB. In fact, a more than 300-fold elevation of this transcript was observed.

It is quite evident that P_i is an important modulator of the metabolic network dedicated to the amplified secretion of L-carnitine. The syn- thesis of L-carnitine diminishes when the concentrations of P_i are increased (personal observation). It is well-established that under oxidative or nutrient stress, the energy budget has to be properly modulated. When organisms are deprived of P_i, they activate P_i acquisition proteins, like psiF, which in turn activate enzymes such as phosphatase to generate pools of P_i. The overexpression of the phosphate starvation inducible protein in the LP cultures and acid phosphatase enzyme in this study may help fulfill the need for this nutrient. ATP levels are known to decrease while AMP and ADP levels tend to increase (Mailloux et al., 2007; Appanna et al., 2016). To combat this situation, pyrophosphate (PP_i) can be utilized as an alternative form of energy for growth and survival. It can be synthesized by pyrophosphate synthase or the combined reactions of pyruvate phosphate dikinase (PPDK) and pyruvate kinase (PK). Recently, a PP_i-dependent kinase of the ribokinase family was discovered in *Entamoeba histolytica* (Nagata et al., 2018). In fact, the coupling of PPDK to the ubiquitous adenylate kinase (AK) which catalyzes the formation of ATP and AMP from 2 ADP allows the regeneration of AMP for PPDK activity. In fact, some organisms can substitute PP_i for ATP during glycolysis when subjected to a phosphate-deficient environment (Mustroph et al., 2013; Igamberdiev and Kleczkowski, 2021). When *Pseudomonas fluorescens* is exposed to nitrosative stress, the ETC complexes are inactivated and the aerobic microbe harnesses PP_i in the production of ATP from AMP in an O_2-in- dependent manner (Auger et al., 2011; Auger and Appanna, 2015). In *Syntrophus acidtrophicus*, acetyl-CoA, AMP and pyrophosphate are con- verted into ATP with the aid of acetyl-CoA synthetase (James et al., 2016). Similar alternative energy-generating machinery has been observed in *Pseudomonas fluorescens* subjected to oxidative and nutrient stress (Alhasawi et al., 2015; Aldarini et al., 2017). Therefore, it is not

surprising that enzymes involved in the TCA cycle (ICDH-NAD^+, PDH), and ETC (Complex I) are downregulated under phosphate starvation as the microbe seeks alternative metabolic networks to acquire energy. Even though Complex I is sharply reduced, it is within the realm of possibilities that concentrations of the other Complexes and ATP synthase may be enhanced to provide ATP for the P_i-starved microbe. The involvement of other metabolic networks including the glyoxylate shunt (ICL), as well as substrate-level phosphorylation may also assist in this adaptation (Hamel et al., 2004; Alhasawi et al., 2014; Thomas et al., 2015). The enhanced activity of ICDH- $NADP^+$ may be an important source of NADPH and KG. While the former is an important anti-oxidant and an anabolic reducing factor, the latter is a key metabolite in amino acid metabolism and a pivotal cofactor in the synthesis of L-carnitine, a reaction facilitated by GBD (Middaugh et al., 2005; Strijbis et al., 2010). The metabolic reprogramming aimed at countering P_i starvation may have compelled *P. fluorescens* to produce L-carnitine, a multi-functional metabolite. Although *P. fluorescens* has been shown to convert glycerol into various metabolites such as KG, pyruvate and PEP under disparate conditions, this study reveals L-carnitine is an important exocellular product in a P_i-deficient medium. This quaternary ammonium com- pound may serve as an end product of anaerobic respiration and help regenerate NAD^+, especially under conditions where OP appears to be ineffective. Such a situation would also limit β-oxidation of fatty acids, an event that may favor the accumulation of L-carnitine. As P_i starvation leads to the switch of aminolipids in the membrane to substitute the traditional phospholipids, the presence of elevated amounts of lysine and methionine in stressed microbes may both serve to synthesize amphipathic moieties and L-carnitine (Schubotz, 2019). It is also tempting to speculate that the trimethylammonium moiety with its positive charge can help scavenge the negatively charged P_i and alleviate this stress. The compatible solute characteristic

attribute of this metabolite that numerous living organisms have come to depend on may have propelled *P. fluorescens* to produce L-carnitine to combat P_i starvation as it can fulfill a range of diverse functions.

In conclusion, this report demonstrates the significance of abiotic modulators such as P_i in evoking a metabolic reconfiguration in *P. fluorescens* that results in the enhanced secretion of L-carnitine in a medium with glycerol as the sole carbon source (Fig. 2.7.5). As P_i influences a variety of metabolic pathways involved in energy homeostasis, membrane biosynthesis and redox regulation, L-carnitine appears to be the metabolite of choice that this microbe produces in elevated amounts. It is likely utilized to combat the dangers associated with P_i-deficiency since it participates in a range of biological processes including NADH homeostasis and as a compatible solute. Furthermore, the harnessing of the up-regulated genes responsible for the synthesis and export of L- carnitine will provide an easy access to this valuable product especially as it is being derived from glycerol, a by-product of the biodiesel industry. Optimization of these biochemical processes can potentially lead to nano-factories designed to make the biodiesel industry more sustainable, effective and profitable.

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2.7 Figures, legends and table

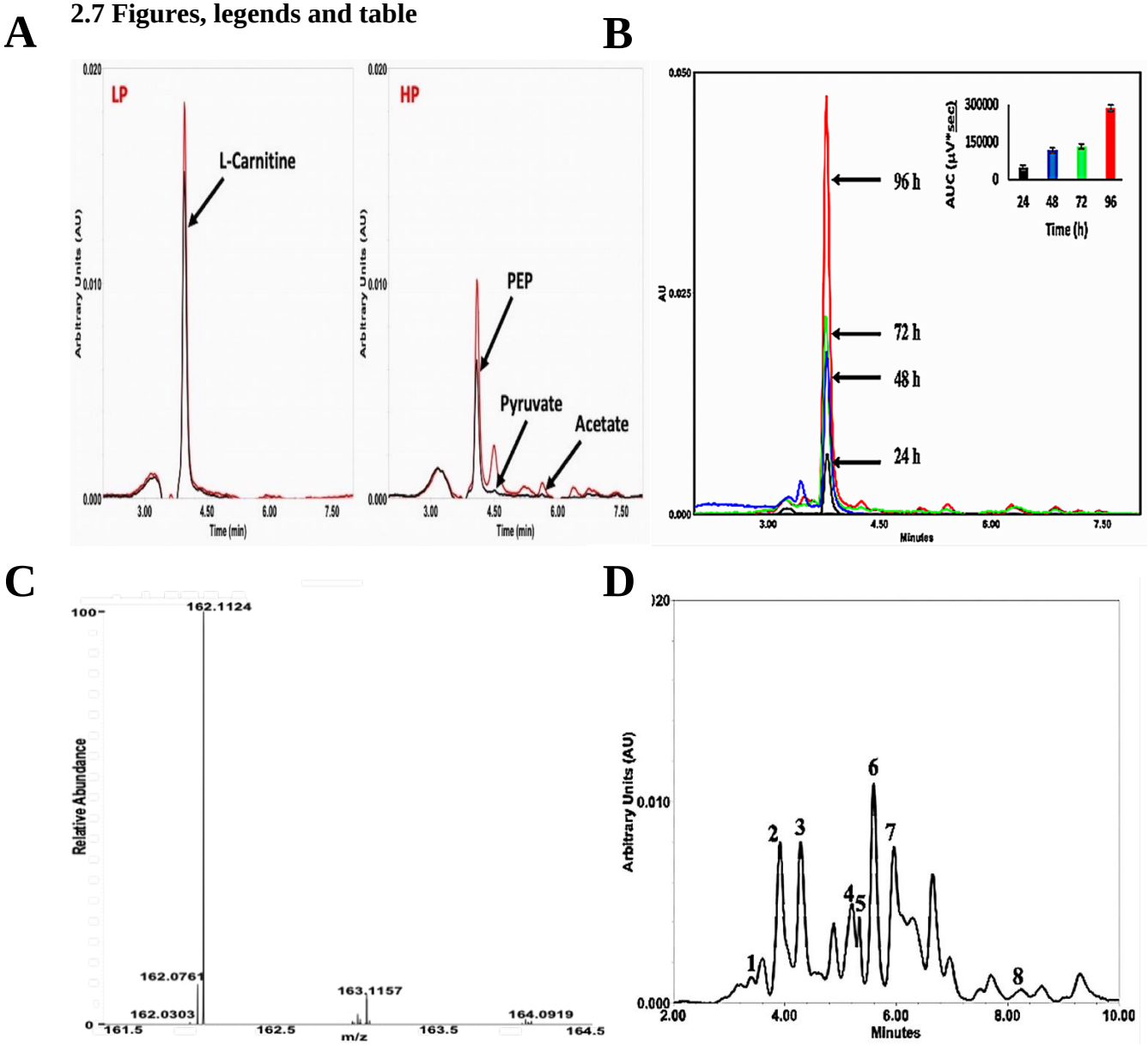


Figure 2.7.1. L-carnitine secretion in the spent fluid from low phosphate cultures. A) HPLC analysis of spent fluid in LP and HP cultures [**Black = Spent Fluid**, **Red = Spent Fluid spike with standard compounds**] (B) L-carnitine production at different time intervals (C) Mass Spectrum of L-carnitine ([M+H], m/z = 162.1) (D) Metabolites detected in the sCFE. 1, lysine; 2,

L-carnitine; 3, γ-butyrobetaine; 4, α-ketoglutarate; 5, isocitrate; 6, methionine; 7, acetate; 8, succinate.

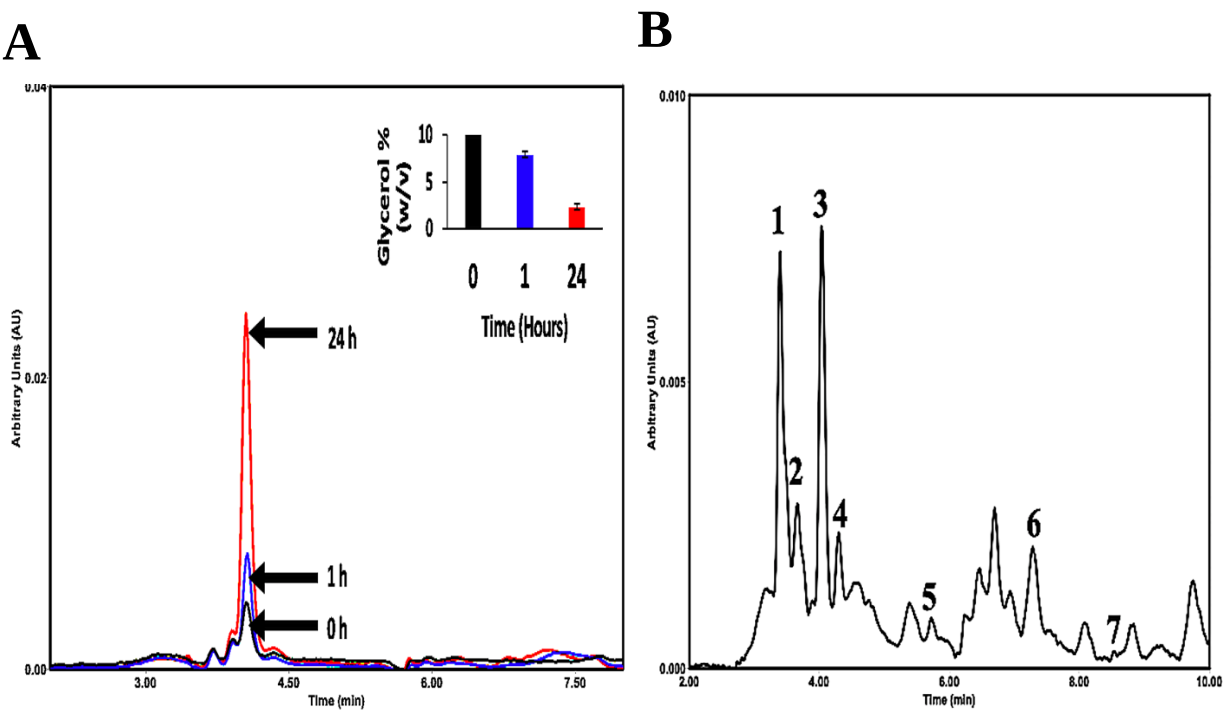


Figure 2.7.2. Synthesis of intermediates and L-carnitine in the spent fluid of the whole cells. A) L-Carnitine formed in whole cells incubated with glycerol and ammonium [Note: 80% of the glycerol was consumed in the reaction, ± SD, n = 3]. (B) Intermediates in the cell driving the conversion of glycerol to L-carnitine. 1, lysine; 2, glycine; 3, acetyl-CoA; 4, GB; 5, methionine; 6, trimethylamine; 7, succinate.

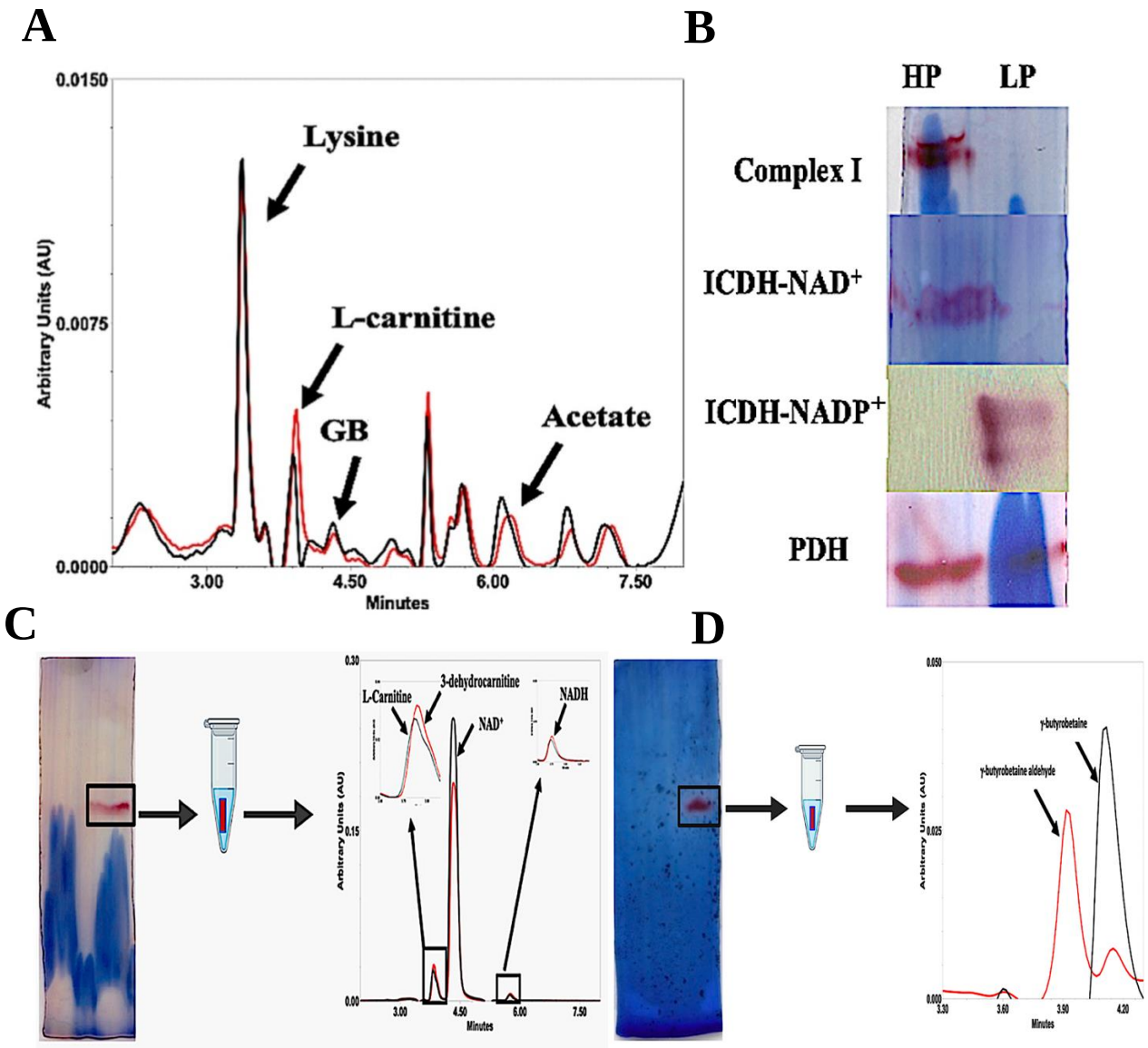
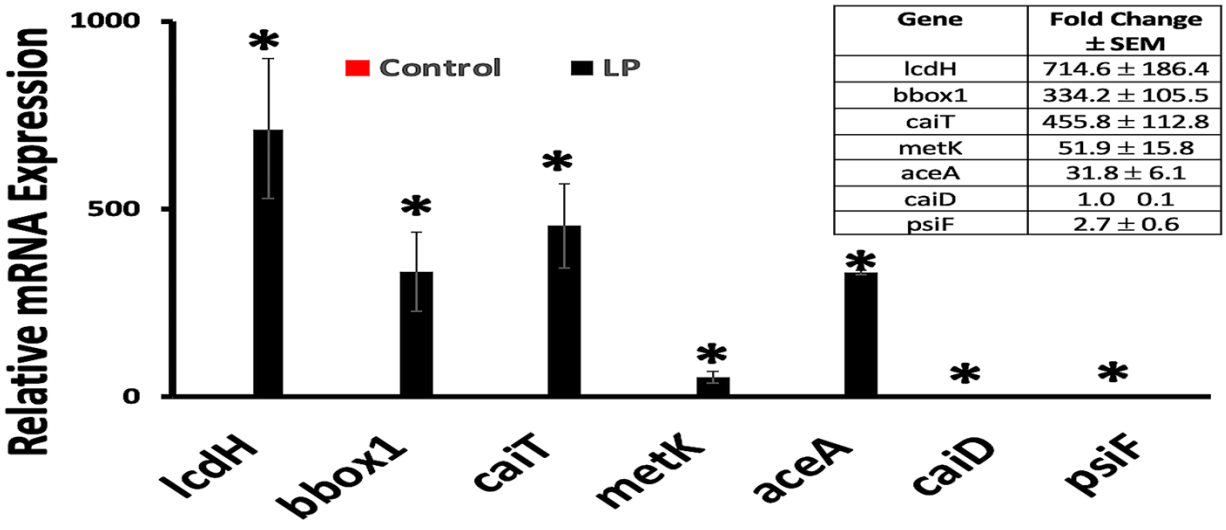


Figure 2.7.3. Metabolic and enzymatic activity in the soluble cell free extract. (A) sCFE incubated with lysine and acetate (B) Activity of TCA cycle and ETC-related enzymes (C) CDH excised band incubated with L-carnitine and NAD^+ (D) GBADH excised band incubated with GB and NADH. [**Black = 0 min**, **Red = 24** h]



Gene	Fold Change ± SEM
lcdH	714.6 ± 186.4
bbox1	334.2 ± 105.5
caiT	455.8 ± 112.8
metK	51.9 ± 15.8
aceA	31.8 ± 6.1
caiD	1.0 0.1
psiF	2.7 ± 0.6

Figure 2.7.4. mRNA expression analysis of metabolic enzymes in LP cultures by RT-qPCR. mRNA transcripts of L-carnitine dehydrogenase (lcdH), γ-butyrobetaine dioxygenase (bbox1), L-carnitine/γ-butyrobetaine antiporter (caiT), S-adenosylmethioninesynthase (metK), isocitrate lyase (aceA), carnityl-CoA dehydratase (caiD) and phosphate starvation inducible protein (psiF) were determined relative to DNA-directed RNA polymerase subunit beta (rpoB) and chaperonin 60 (groL) reference genes.Three independent experiments were performed and the statistical significance was assessed at $p<0.05$ (*) with the one-way ANOVA test and Turkey's post hoc analysis.

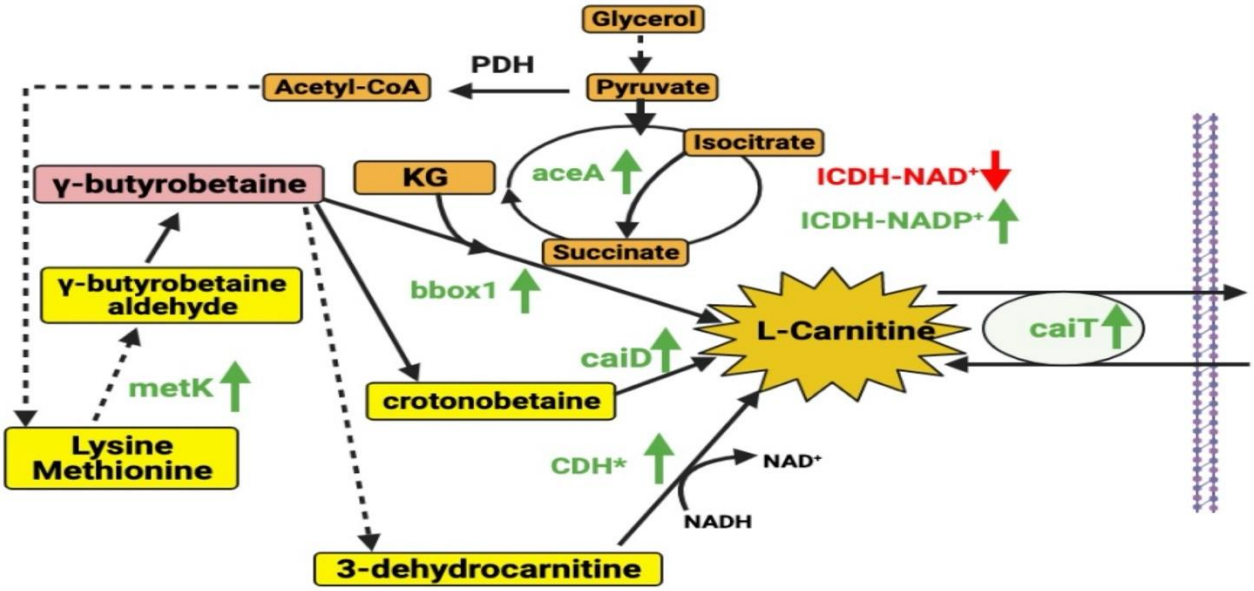


Figure 2.7.5. Metabolic scheme showing L-carnitine production from glycerol under phosphate starvation. PDH: Pyruvate dehydrogenase; ICDH: isocitrate dehydrogenase, aceA: isocitrate lyase; NAD: Nicotinamide adenine dinucleotide; NADH: reduced dicotinamide adenine dinucleotide; KG: alpha-ketoglutarate; metK: S-adenosylmethionine synthase; bbox1: γ-butyrobetaine dioxygenase; caiD: carnityl-CoA dehydratase; CDH: L-carnitine dehydrogenase; caiT: L-carnitine/γ-butyrobetaine antiporter. Increase;↑; decrease:↓ ; *:Both enzyme activity and transcripts were enhanced.

Table 1 RT-qPCR sequences utilized in the present study

Gene Name	Gene Location	Sequence (5'→3')	PCR product size	Annealing temp. (°C)
L-carnitine dehydrogenase (lcdH)	6,207,7481-6,208,446	Forward primer: TACCGGGCTTTATCGCTGAC Reverse primer: TCAGGAAGGTGCCCATGAC	150	57
γ-butyrobetaine dioxygenase (bbox1)	6,209,9062-6,210,222	Forward primer: GCATTGCCTGGTGAACGATG Reverse primer: GATCTCACACAGGGCACGA	82	57
L-carnitine/γ-butyrobetaine antiporter	6,217,7603-6,219,594	Forward primer: CTGTTCTCGTCGGGTATCGG Reverse primer: TGAAGTAGTGGTCCAACGGC	70	57
S-adenosylmethionine synthase (metK)	6,249,256-6,250,527	Forward primer: CCGTGACGTCATCACCAAGA Reverse primer: ATGTTCATCACGCCACAGGT	78	57
Isocitrate lyase (aceA)	4,254,795-4,256,120	Forward primer: GTCCTACAACTGCTCGCCTT Reverse primer: GCTGGAATTTGGCGATGGTC	71	57
Carnityl-coA dehydratase (caiD)	3,618,656-3,619,774	Forward primer: TCGGCTATTTCCCGGATGTG Reverse primer: CTGTCGATAGACCAGTCCGC	142	57
Phosphate starvation-inducible protein (psiF)	935,251-935,550	Forward primer: CGTTGTTGATGATGGGCCTG Reverse primer: TCAGCATTGCAGGTGGTCAT	85	57
DNA-directed RNA polymerase (rpoB)	6,050919-6,054,992	Forward primer: GTCGTCACGGTAACAAGGGT Reverse primer: TGACCAACGTTCATACGCGA	130	57
Chaperonin 60 (cpn60)	5,505,175-5,506,821	Forward primer: GCGACATGATCGAAATGGC Reverse primer: GCCAGTCGAGCCTTCTTTCT	140	57

Genes of interest were identified from the ATCC genome portal for Pseudomonas fluorescens ATCC 13525. Forward and reverse primer sequences were designed using Primer-BLAST.

Table 2. Analyses of various physico-chemical parameters in the spent fluid at stationary phase of growth.

Physico-chemical parameters[a]	Low Phosphate Medium (LP)	High Phosphate Medium (HP)
pH	2.2* ± 0.18	6.51 ± 0.08
Protein concentration (mg/mL)	0.50 ± 0.01	0.52 ± 0.01
Glycerol utilized (%)	70 ± 0.03	63 ± 0.02
Phosphate consumed (%)	100	65 ± 0.23
Phosphatase Activity[b]	17.17* ± 0.16	ND
Ammonia consumed (%)	100	100
Ninhydrin[c]	ND	ND
Ketoacid[d]	1*	4*

[a] monitored at stationary phase of growth,
[b] monitored by the PNPP assay and measured in nmol*min^{-1},
[c] not discernable [ND]
[d] fold change in absorbance by DNPH assay.
*Statistical significance assessed by student t-test, n = 3 ± S.D.

CHAPTER 3: Conclusions, Future Research and General Bibliography

3.1 Conclusions

Findings from this project indicate that *P. fluorescens* is capable of surviving and adapting under limited phosphate conditions with glycerol as the sole carbon source. It orchestrates a metabolic reconfiguration to overcome phosphate stress. In addition, the microbe was shown to express high activity of the acid phosphatase enzyme to generate pools of phosphate for its survival. Under phosphate starvation, it was expected that the bacteria would utilize a secondary metabolic pathway to generate a valued product, as the TCA cycle would be severely depleted, along with oxidative phosphorylation for ATP making. As a result, *P. fluorescens* secreted copious amounts of L-carnitine. The discovery of this amino acid prompted an investigation of the role of the TCA cycle in the synthesis of this modified amino acid as γ-butyrobetaine and KG are readily converted into succinate and L-carnitine. Succinate is generated from isocitrate by the activity of isocitrate lyase (aceA), which was highly expressed in the LP cells. Isocitrate lyase can also lead to the formation of glyoxylate which can be converted to glycine, an important ingredient in the synthesis of L-carnitine. Pyruvate, a main precursor of the TCA cycle can also act as a central metabolite in the synthesis of this moiety, as it can form glycine betaine, leading towards the synthesis of 3-dehydrocarnitine or it can go towards serine, which in turn is transformed to choline in the presence of several intermediates before generating glycine betaine. Glycerol, glucose or glycine can be used as starting material to synthesize L-carnitine with the concomitant utilization of the tricarboxylic acid cycle as a supplier of precursor metabolites (Figure 3.1).

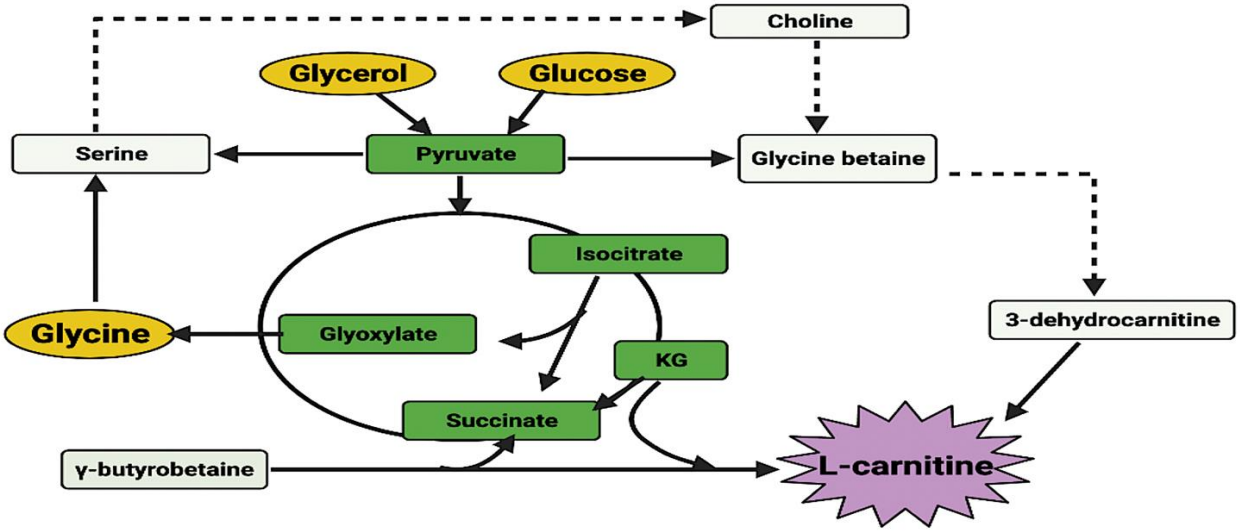


Figure 3.1: Precursor metabolites involved in L-carnitine biosynthesis

Aside from the interaction of L-carnitine with the TCA cycle, this quaternary ammonium compound is involved in the transport of fatty acids across the mitochondrial membrane so they can be oxidized to produce energy. L-Carnitine also functions as a scavenger in amino acid (AA) metabolism, binding acyl residues derived from AA in the process of removing abnormal organic acids (Housten and Wanders, 2010). Furthermore, L-carnitine can be metabolized to GB during anaerobic growth via crotonobetaine upon a two-step reaction with L-carnitine dehydratase and crotonobetaine reductase (Jung et al., 1987). L-Carnitine can potentially act as a scavenger of phosphate under phosphate starvation, when secreted in the cell. Moreover, the positive charge on the nitrogen atom can attract the negative charge on the phosphate ion intracellularly and may be involved in a membrane potential that could be a source of energy. One of the reasons why L-carnitine may interact with phosphate is because of its role as a compatible solute for stress protection (Figure 3.2). Minimal levels of phosphate in the bacterial medium may result in low osmolality and to compensate for this loss, *P. fluorescens* may be releasing L-carnitine in the

system to maintain cellular integrity. The solute can be accumulated by a transport system designed to maintain an optimal amount of the modified amino acid in the cells (Wood et al., 2001).

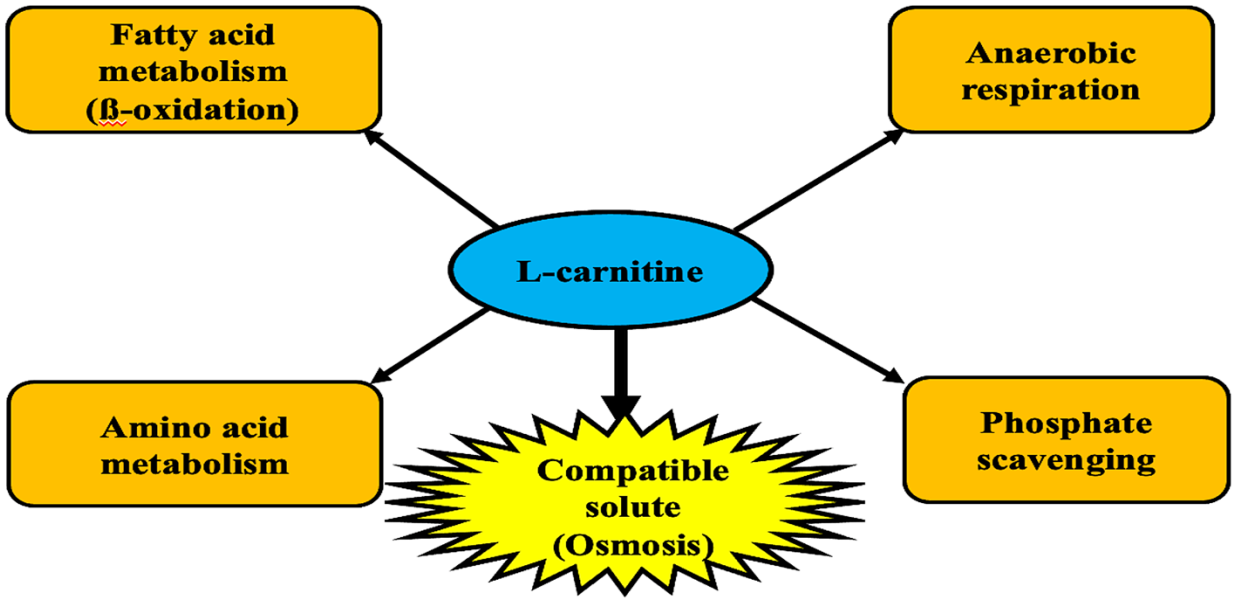


Figure 3.2: Biological significance of L-carnitine

The upregulation of the CaiT antiporter in the LP medium allowed the exchange of L-carnitine for γ-butyrobetaine in order to secrete the end product of the L-carnitine pathway. This amino acid derivative was constantly generated upon the expression of bbox1 (γ-butyrobetaine dioxygenase), lcdH (L-carnitine dehydrogenase) and caiD (carnityl-CoA dehydratase). ICDH-NAD^+ was found to be diminished in the LP cells but the activity of ICDH-$NADP^+$ was found to be augmented in these cells, which is consistent with previous findings where *P. fluorescens* was exposed to oxidative stress (Beriault et al., 2007). Upon the establishment of an antioxidative defence system, an antiport system for the movement of L-carnitine across the membrane systems and expression of a complex set of genes related to the biosynthesis of this valued product, *P. fluorescens* was able to survive and adapt in an environment with limited phosphate.

Future work

The discovery of L-carnitine as a compatible solute responsive to P_i starvation in a glycerol-supplemented medium provides leads for further investigation to verify if these results can be repeated with waste glycerol. This will help make the biodiesel industry be more cost-effective. Furthermore, the levels of phosphate and ammonium can also be optimized to potentially increase the yield of the valued product. Following these findings, the use of labelled glycerol (^{13}C) or phosphate (^{31}P) in subsequent experiments can unveil how the carbon and phosphate source from the growth medium are being utilized in the depicted metabolic networks. Additionally, CRISPR technology can be applied to this work by editing specific genes to increase the yield of L-carnitine. All in all, the discovery of this amino acid derivative secreted *by P. fluorescens* presents a unique opportunity to tailor this microbe and its nutrients for commercial application in the renewable biomass industry. A microbial bioreactor system could be designed in such a way where the glycerol medium is inoculated in a bioreactor tank containing microbial biomass and/ or immobilized enzymes agitated in batch processes before being processed downstream to purify L-carnitine for commercial use (Figure 3.3).

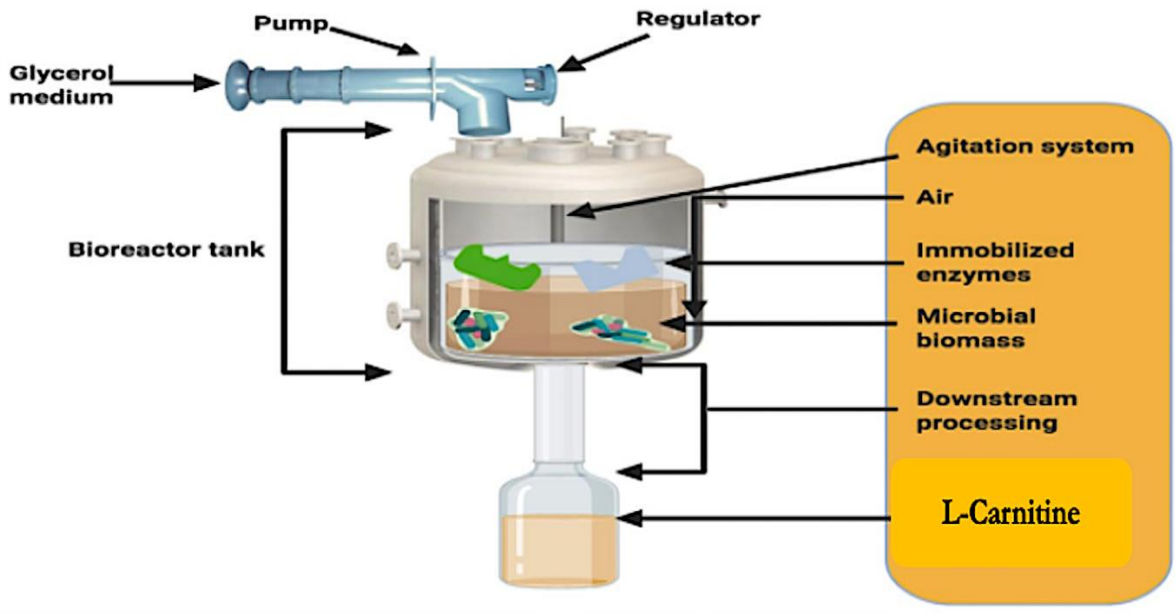


Figure 3.3: Microbial bioreactor system for the production of L-carnitine (Adapted from Legendre et al. 2020)

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