CITRIC ACID PRODUCTION FROM YEASTS:

COMPARISON OF A PARENT AND A MUTANT STRAIN OF *CANDIDA GUILLIERMONDII*, AND SUBSEQENT REVERSION OF THE MUTANT

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ABSTRACT

Citric acid production from yeasts has been studied widely owing to the short duration of fermentation, the broad choice of carbon source and the better yields obtained when compared to the currently used submerged or surface fermentation with *Aspergillus niger*.

In this work two strains of *Candida guilliermondii* were compared for their citric acid-producing capabilities, these being parent strain *Candida guilliermondii* NRRL Y-448, and mutant strain *Candida guilliermondii* IMK1. The mutant was previously selected for its ability to produce much higher concentrations of citric acid than the parent. These strains were grown under various nutrient limitations to determine if nutrient limitation had an effect on the amount of citric acid produced.

Several differences were observed between the non-citric acid-producing parent and the citric acid-producing mutant. The mutant generally consumed less glucose ($g.g^{-1}$), produced less biomass ($g.L^{-1}$) and produced much higher levels of citric acid – the best production (7.34 $g.g^{-1}$) seen from the culture grown under phosphorus-limited (0.15 mM) conditions. Upon assessment of enzyme activities it was found that the mutant also exhibited reduced activity of the enzyme NAD-ICDH (NAD-dependent isocitrate dehydrogenase), a recognised control point for the over-production of citric acid. NAD-ICDH is inhibited by increased concentrations of ATP - these are associated with the accumulation of citric acid in the cell in the stationary phase of growth. This reduction in NAD-ICDH activity correlated with a dramatic increase in the activity of NADP-ICDH (NADP-specific isocitrate dehydrogenase), the activity of which was thought to compensate for the loss of activity of NAD-ICDH. However, in a subsequent experiment, the mutant was found to have reverted - losing its ability to produce citric acid. This loss of productivity occurred before the levels of adenine

nucleotides in the cell could be assessed, meaning that the suggested inhibition of NAD-ICDH by elevated levels of ATP could not be confirmed.

Upon analysis of the revertant, it was found that glucose consumption (grams per gram of cells) had increased, as had the production of biomass ($g.L^{-1}$). Even though the revertant failed to consume as much glucose as the parent, in many instances it produced higher levels of biomass. Upon analysis of enzyme activity, it was found that the activity of NAD-ICDH had increased, so reducing the accumulation of citric and isocitric acids. The activity of NADP-ICDH had decreased somewhat, but activity of this enzyme remained at significant levels. It is proposed that the activity of NADP-ICDH in the revertant was responsible for the increased efficiency of biomass production.

In conclusion, it is suggested that overproduction of citric acid in *Candida guilliermondii* IMK1 was due to the consumption of lowered levels of glucose combined with the reduced activity of the enzyme NAD-ICDH, which it is speculated was due to elevated concentrations of ATP in the cell.

ACKNOWLEDGEMENTS

When I started on this journey of discovery, who would have known where the next few years were to take me. However, this particular little journey has finally been completed. I would like to thank my Mum and Dad for all of their love and support, which helped to keep me going when all was not going as planned. I would also like to thank John Brooks - his support and encouragement was greatly appreciated. I can't forget the other technical staff in the Food Technology department, most of whom at some time have offered advice and encouragement. Lastly I want to acknowledge myself – I finally made it!

A cloud does not know why it moves in such a direction and at such a speed
It feels an impulsion……this is the place to go now
But the sky knows the reasons and patterns behind all clouds
And you will know too – when you lift yourself high enough to see beyond horizons

Richard Bach
Illusions

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CHAPTER 1

INTRODUCTION

Citric acid is currently produced industrially using the submerged or surface fermentation with *Aspergillus niger*. Citric acid has a wide range of uses, but the majority of citric acid produced is used in the food and pharmaceutical industries.

Recently attention has been focused on the use of yeasts for the production of citric acid. Yeasts have certain advantages over fungi, some of these being the shorter duration of fermentation, broad choice of carbon source and better yields.

Much work has been performed on the mechanism of accumulation of citric acid in yeasts. The organisms most commonly used are those of the genus *Candida, C. lipolytica* (Synonym: *Yarrowia lipolytica, Saccharomycopsis lipolytica*) being the most favoured. It has been found that the optimum production of citric acid can vary depending on the type of carbon source, the medium pH, aeration and also the presence or absence of trace elements.

It is generally accepted that for citric acid production to occur, the culture must be under conditions of nutrient limitation - usually nitrogen limitation. However, other nutrient limitations have been assessed successfully for the production of citric acid.

The object of this research was to compare two strains of *Candida guilliermondii* - the parent (NRRL Y-448) and a mutant strain (IMK1). The mutant was chosen for its increased production of citric acid. Comparisons were made between the parent and the mutant to attempt to identify the differences that were responsible

for the increased production of citric acid from the mutant. Comparisons were made of glucose consumption, rates of production of citric acid, levels of intermediates and enzymes of the tricarboxylic acid (TCA) cycle.

Unfortunately, the mutant proved to be unstable and citric acid producing activity was lost after a period of time. Attempts were made to revive this mutant and to isolate a new citric acid producing mutant, but this proved to be unsuccessful.

CHAPTER 2

LITERATURE REVIEW

2.1 Citric Acid - General

2.1.1 History

Citric acid was first isolated from lemon juice by Scheele in 1784. It was first produced commercially from 1826 onwards by John & Edmund Sturge in England, using calcium citrate from Italy.

At the beginning of this century Italy had a monopoly on citric acid production, producing about 10 000 tons of citric acid a year. This was obtained by pressing citrus fruits, which contain about 7 - 9% citric acid, and subsequent precipitation of the calcium salt. This process required the processing of about 30 - 40 tons of lemons to obtain 1 ton of citric acid. This citric acid produced from fruits is known as 'natural citric acid', as opposed to 'fermented citric acid'.

Wehmer, in 1893, was the first to observe the accumulation of citric acid in a fermentation medium by a culture of *Penicillium glaucum*, with sugar as a source of carbon. He was well aware of the commercial potential of a fermentation process based on the ability of these strains to produce considerable amounts of citric acid.

However, attempts to transfer it into an industrial process were unsuccessful, mainly due to problems with contamination and periods of fermentation lasting several weeks.

The first patent for citric acid production using *Sterigmatocystis nigra*, a synonym for *Aspergillus niger*, was obtained by Zahorsky in 1913.

In 1917, Currie opened the way for industrial production of citric acid using Aspergilli. The most important finding was that *Aspergillus niger* could grow well at pH values of around 2.5 - 3.5, and that citric acid was abundantly produced at pH values even lower than 2.0. Yields of over 60% were achieved in as little as 1 - 2 weeks. Through this work it was also found that high sugar concentrations were most favourable for optimal production and highest yields were attained when growth of mycelium was restricted.

The first plant to successfully produce citric acid on an industrial scale was opened in Belgium in 1919. This was followed by plants erected in New York by Chas. Pfizer & Co in 1923, J & E Sturge in England in 1927, and in 1928 a factory in Czechoslovakia which used beet molasses as a source of sugar. Further plants were later erected in the USSR and in Germany.

By 1933, the world production of citric acid was 10 400 tons. Of this 1 800 tons was produced in Italy from lemons, the rest came from fermentation.

Different carbon sources were also experimented with, such as *n*-alkanes. Paraffin hydrocarbons were used for a time, but this is now economically unviable.

In 1966/67, it was found that bacterial mutants such as *Corynebacterium, Arthrobacter* and *Brevibacterium* produced citric acid from *n*-alkanes. In 1969, (Röhr and Kubicek 1983) it was discovered that the *Candida* yeasts produce citric acid when grown on glucose and on hydrocarbon.

The production of citric acid by yeasts is now more widely studied and more is known and understood about the processes and conditions under which citric acid is produced in excess.

2.1.2 Uses and Properties of Citric Acid

Citric acid is the most versatile and widely used food acidulant, with 75% of the total citric acid produced used in the food industry. It is accepted worldwide as a safe food ingredient i.e. approved by the joint FAE/WHO Expert Committee on Food Additives without limitation. The most extensive application of citric acid is in food, sugar confectionary and beverages (Röhr and Kubicek 1985; Milsom and Meers 1985).

Another important area of application is the pharmaceutical industry (10%). Purity for these purposes and several others is regulated by the respective Food Codices and Pharmacopoeia. The sequestering action of citric acid is used in the stabilization of ascorbic acid. Another large pharmaceutical use is based on the effervescent effect it produces when combined with carbonates and bicarbonates e.g. in antacid and soluble aspirin preparations. Citric acid and its salts are also used extensively for their buffering capacity. Trisodium citrate is widely used as a blood preservative, where it prevents clotting by complexing calcium.

Industry accounts for the remaining 15% of use of citric acid. Here citric acid is used in varying degrees of purity. It is used as a raw material for the manufacture of derivatives such as citric acid esters, which may be used in the plastics industry e.g. as plasticizers etc.

Citric acid is able to complex heavy metals such as iron and copper. This property has led to its increasing use as a stabilizer of oils and fats where it greatly reduces oxidation catalysed by these metals. The ability to complex metals combined with its low degree of attack on special steels allows the use of solutions of citric acid in the cleaning of power station boilers and similar installations. The main application of the chelating property is probably that of providing a readily biodegradable ingredient of detergents, thus being a favourable substitute for phosphates in various kinds of laundry detergents and household cleaners.

Citric acid forms a wide range of metallic salts, many of which are used in commerce. In terms of volume, trisodium and tripotassium citrates are probably the most important. Citric acid esters of a wide range of alcohols are known. In particular, the triethyl, tributyl and acetyltributyl esters are employed as non-toxic plasticizers in plastic films used to protect foodstuffs. Monostearyl citrate can be used instead of citric acid as an antioxidant in oils and fats. It is more easily incorporated than the free acid, and an improved method of preparation has been developed.

2.1.3 Biochemistry of the Citric Acid Cycle

For a summary of the reactions and the enzymes involved see Table 2.1.

Table 2.1: The Citric Acid Cycle
(From: Stryer 1981)

Step	Reaction	Enzyme	Cofactor
1	Acetyl CoA + oxaloacetate + H_2O → citrate + CoA + H^+	Citrate synthase	CoA
2	Citrate ↔ *cis*-aconitate + H_2O	Aconitase	Fe^{2+}
3	*cis*-Aconitate + H_2O ↔ isocitrate	Aconitase	Fe^{2+}
4	Isocitrate + NAD^+ ↔ 2-oxoglutarate + CO_2 + NADH	Isocitrate dehydrogenase	NAD^+
5	2-Oxoglutarate + NAD^+ CoA ↔ succinyl CoA + CO_2 + NADH	2-oxoglutarate dehydrogenase complex	CoA; TPP; Lipoic acid FAD
6	Succinyl CoA + P_i + GDP ↔ succinate + GTP + CoA	Succinyl CoA synthase	CoA
7	Succinate + FAD (enzyme-bound) ↔ fumarate + $FADH_2$ (enzyme-bound)	Succinate dehydrogenase	FAD
8	Fumarate + H_2O ↔ malate	Fumarase	None
9	*L*-Malate + NAD^+ ↔ oxaloacetate + NADH + H^+	Malate dehydrogenase	NAD^+

2.1.4 Regulation of the Citric Acid Cycle - General

The citric acid cycle is largely regulated by substrate availability, product inhibition, and inhibition by other cycle intermediates (See Figure 2.1).

It is proposed that possibly the most important regulators of the citric acid cycle are its substrates, acetyl-CoA and oxaloacetate, and its product NADH (Voet and Voet 1995). Both acetyl-CoA and oxaloacetate are present in mitochondria in concentrations that do not saturate citrate synthase. The metabolic flux through the enzyme therefore varies with substrate concentration and is subject to control by substrate availability.

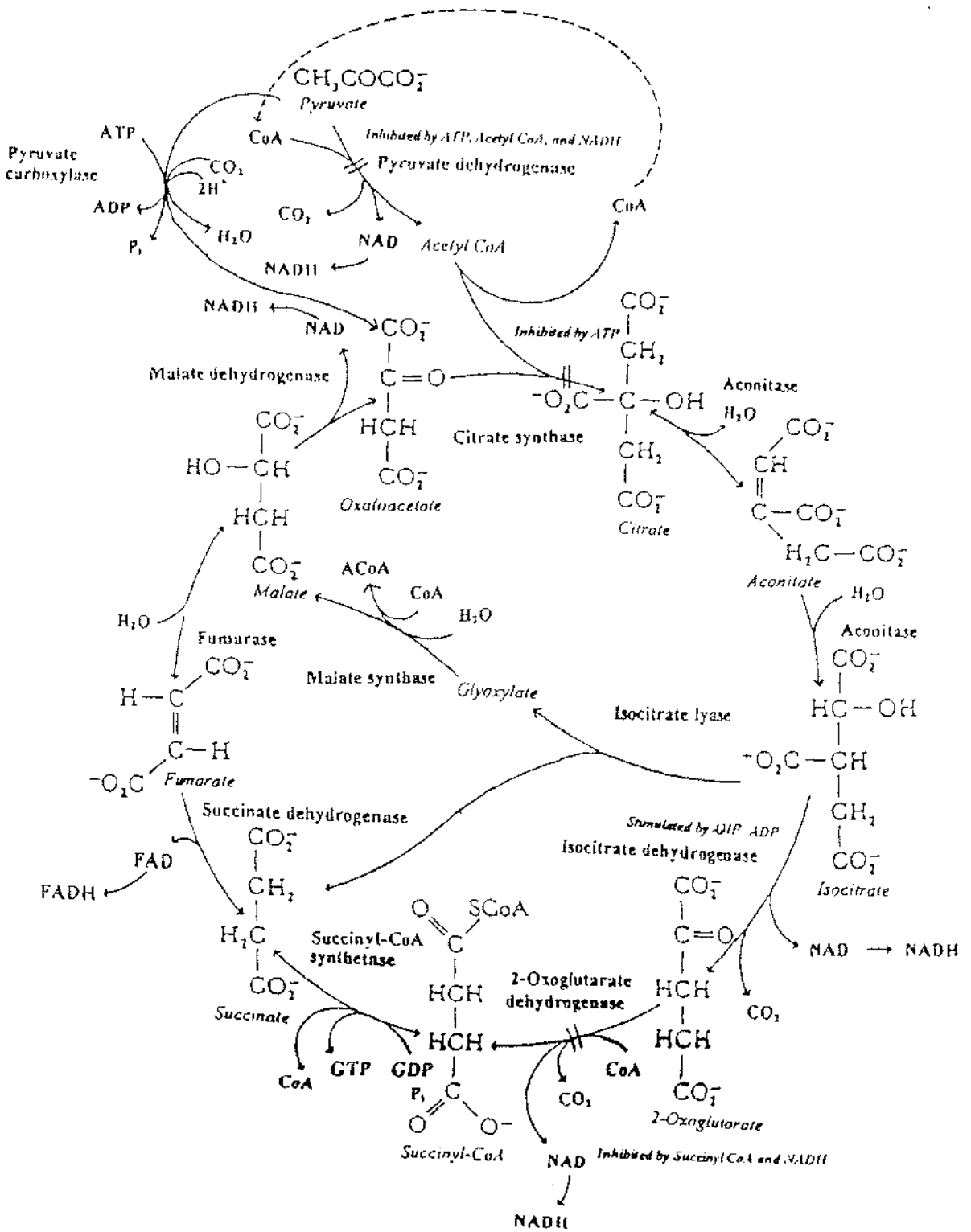


Figure 2.1: **The Citric Acid Cycle.**
Regulation of the citric acid cycle and anaplerotic pathways.
(From: Stryer 1981)

Under normal conditions, the rate of glycolysis and the rate of the citric acid cycle are integrated with each other so that only as much glucose is broken down to form pyruvate as is needed to supply the citric acid cycle with acetyl-CoA (Lehninger 1982). This is achieved by inhibition of glycolysis by high levels of ATP and NADH, but also by the concentration of citrate. Citrate serves as an important allosteric inhibitor of the phosphorylation of fructose-6-phosphate by phosphofructokinase.

Another important factor for controlling the activity of the citric acid cycle is the intramitochondrial ratio of [NAD^+] to [NADH] (Mathews and Holde 1996). NAD^+ is a substrate for three cycle enzymes as well as for pyruvate dehydrogenase. Key sites for allosteric regulation are the reactions catalysed by isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase. In many cells, isocitrate dehydrogenase is activated by ADP and is inhibited by NADH and ATP. The [NADH]/[NAD^+] ratio also causes fluctuations in the concentration of oxaloacetate, which is in equilibrium with malate.

In order to maintain flux through the cycle, citric acid cycle intermediates are replenished by means of anaplerotic pathways. The glyoxylate cycle is one such pathway. It involves the breakdown of isocitrate by isocitrate lyase into succinate and glyoxylate, the latter of which is converted by malate synthase with acetyl-CoA to form malate. The other important anaplerotic pathway is the conversion of pyruvate to oxaloacetate which is catalysed by pyruvate carboxylase.

2.1.5 Strains Used in the Production of Citric Acid

Only a few classes or even genera of microorganisms have been reported to excrete sustantial amounts of citric acid into the fermentation medium under certain conditions.

In 1916, Thom and Currie documented the use of *Penicillium*. This was followed in 1961 by a patent granted to Kinoshita *et al.*, who used *Penicillium janthinellum Biourge var. kuensanii* and *P. restrictum Gilman and Abbot var kuensanii*, with appreciable yields and productivities of citric acid being claimed (Röhr and Kubicek 1983).

Apart from *Aspergillus niger*, the following strains were also found to produce citric acid: *A. awamori, A. clavatus, A. fenicus, A. fonsecaeus, A. fumaricus, A. luchensis, A. saitoi, A. usumii* and *A. wentii*. Of these only *A. wentii* has been applied in a well-documented process (Röhr and Kubicek 1983).

Other moulds recorded as producing relatively large amounts of citric acid are *Botrytis cinerea, Mucor piriformis* and *Trichoderma viride*.

Citric acid is produced in substantial amounts by some coryneform bacteria e.g. *Arthrobacter*, and related organisms.

With regard to yeasts, the genus *Candida* has been almost exclusively utilised. This includes: *C. lipolytica, C. tropicalis, C guilliermondii, C. intermedia, C. parapsilosis, C. zeylanoides, C. fibriae, C. subtropicalis* and *C. oleophila*. Some of these strains are mutant isolates developed in the course of studies of citric acid.

2.1.6 Citric Acid Production by *Aspergillus niger*

A. niger is an imperfect filamentous fungus, which is capable of converting as much as 90% (w/w) of the carbohydrate supplied (usually sucrose or molasses) to citric acid (Röhr and Kubicek 1981).

Production of citric acid by *Aspergillus niger* is currently carried out by the following processess: (a) Surface fermentation using beet molasses

(b) Submerged fermentation using beet or cane molasses or glucose syrup

The surface fermentation method is still extensively used. It is labour intensive, but the power requirements are less than the submerged fermentation.

The submerged fermentation differs depending on the substrate. This method of fermentation has its drawbacks as the fungus is more sensitive to trace metals as well as other deviations of environmental conditions. Recovery of the mycelial mass is also more difficult and often requires the use of a filtering aid (Röhr and Kubicek 1983).

It is generally agreed that in order to achieve an abundant excretion of citric acid, growth of the production strain must be restricted (Röhr and Kubicek 1983; Milsom and Meers 1985). The precondition for sufficient citric acid production is a medium deficient in one or more essential elements which can be realised by limiting the concentration of one of the nutrition elements e.g. nitrogen or phosphorus.

Other growth conditions shown to have an effect on the production of citric acid from *A. niger* include the carbon source, pH and oxygen. Only sugars which are rapidly taken up by the fungus are useful carbon sources for citric acid production. These include sources such as sucrose, molasses, glucose and fructose. The

concentration of the carbon source is also important. Maximum production of citric acid is usually achieved at concentrations of 14 - 22% (w/v). Yields have been obtained of 70 - 90% of citric acid monohydrate per 100 kg of carbohydrate supplied. The theoretical yield is 123% - assuming that no carbon is diverted to biomass, CO_2 or other byproducts (Milsom and Meers 1985).

A pH of 1.7 - 2.0 has been shown to be optimum for citric acid production (Röhr and Kubicek 1983). A pH of less than 3 has been shown to be preferable as at this pH citric acid is the major fermentation product. At higher pH values substantial amounts of oxalic and gluconic acid may be produced.

A high oxygen tension is required - especially in submerged fermentations. Citric acid by *A. niger* has been shown to be stimulated by increased aeration (Röhr and Kubicek 1983).

It is concluded by Röhr and Kubicek (1981), that an excessive production of oxaloacetate is the key event for citric acid accumulation. Due to the poor regulatory role of pyruvate carboxylase and citrate synthase, and to the non-cyclic operation of the TCA cycle, this leads to an accumulation of cycle metabolites, which because of the equilibria of the enzymes, is most pronounced with citrate. After exceeding a certain cellular level, citrate can enhance its own formation by inhibiting isocitrate dehydrogenase.

Phosphofructokinase has been identified as a regulatory enzyme in a citric acid producing strain of *A. niger* (Röhr and Kubicek 1981). Phosphofructokinase is inhibited by citrate and ATP, but is activated by AMP, inorganic phosphate and ammonium ions (NH_4^+). The presence of NH_4^+ ions can effectively release phosphofructokinase from inhibition by citrate and ATP.

It has been shown that there is a close correlation between the rate of citric acid production and the concentration of NH_4^+ ions (Röhr and Kubicek 1981). It is believed that an elevated NH_4^+ pool is responsible for the apparent insensitivity of phosphofructokinase towards intracellular citrate accumulation.

In *A. niger*, it has been shown that an unusually high NH_4^+ pool occurs particularly during cultivation on manganese deficient media with a high sugar concentration (Röhr and Kubicek 1981). This leads to accumulation and excretion of amino acids derived from 2-oxoglutarate which eventually act as detoxifiers of the cell from possible NH_4^+ poisoning. Accumulation of amino acids was also seen to coincide with a decrease in cellular proteins and nucleic acids.

In conclusion, the complex regulatory events in the industrial production of citric acid by *A. niger* can be expressed as follows:

Unbalanced growth conditions, caused by severe limitations of manganese ions and/or nitrogen or phosphate, which manifest themselves as an impaired protein synthesis, lead to the build-up of an unusually high intracellular NH_4^+ pool. This intracellular pool is able to counteract feedback inhibition of phosphofructokinase by citric acid and ATP, thus leading to an unlimited flux through the glycolytic sequence and thus leading to the formation of citric acid without any further regulation. The above mentioned high glucose and NH_4^+ concentrations on the other hand, strongly repress the formation of 2-oxoglutarate dehydrogenase and thus inhibit the catabolism of citric acid within the TCA cycle, producing the enormous overflow and excretion of citric acid which characterises the industrial process.

2.2 Citric Acid Production From Yeasts

2.2.1 Introduction

The production of citric acid from yeasts has been studied widely with a view to using yeast as a possible alternative to the currently used submerged fermentation involving *Aspergillus niger*. Citric acid production from a variety of substrates has been studied, such as: carbohydrates, hydrocarbons and agricultural wastes (See Appendix 1: Table 1). A variety of fermentation methods have been used, from shake flask to continuous fermentations, with a wide variation in the resulting yields of citric acid produced.

It is generally agreed that there are certain advantages to using yeasts over *A. niger* for the production of citric acid (Rane and Sims 1993), these include:

- Broad choice of carbon source
- Greater tolerance of the yeast to high substrate concentration.
- Higher conversion rates - in many cases yields are over 100% (w/w)
- Insensitivity to metal ions - which permits the use of lower-grade or less refined molasses
- Better process control due to the unicellular nature of yeast.

Candida lipolytica strains in particular are considered to be suitable for citric acid production, as they are capable of producing citrate with yields and productivity similar to those produced by *A. niger* in batch culture (Rane and Sims 1993). However, the main disadvantage that has been encountered with citric acid production from yeast is the simultaneous production of citric and isocitric acids. Ratios can vary from 1:1 to 20:1 depending on the organism, the carbon source, and the micronutrient concentration (Rane and Sims 1993).

2.2.2 Yeast Biology

The following is an introduction to the yeast cell, its growth requirements and general cell structure. This is intended to provide some understanding as to the effects that nutrient limitation has on the citric acid-producing yeast cell.

It has been shown that the composition of a yeast cell can vary enormously according to how it is grown i.e. aerobically or anaerobically, or with a surfeit or deficit of carbon or other nutrients. Changes in temperature and pH can also cause variations in composition (Ratledge 1991). Thus a cell responds to its environment by controlling the expression of its genetic information as well as controlling the activity of the enzymes within the cell.

2.2.2.1 Growth and Multiplication of Yeasts

The basic requirements for the growth of yeasts are summarised below. Those factors that may affect citric acid production are covered in more detail.

Water is required at a level of at least 30% for yeast-like forms. It is involved in the transport of nutrients into the cell and also in the draining off of surplus heat produced by the cell. Yeasts can only take up oxygen when in the form of a molecular solution in water, i.e. in the form of dissolved oxygen. The amount of oxygen depleted from the medium is replenished by diffusion from the atmosphere.

The sugars are the form of carbon that are most readily utilized by yeasts. Basic sugars utilised include D-glucose, D-fructose, and D-mannose. However, sucrose

is the disaccharide found to be most easily utilised by yeast cells. These sugars are usually added to fermentation media in concentrations of 1 - 10%. Other carbon sources utilised by yeasts include: D-xylose, L-arabinose and polyols such as arabitol and glucitol. Polysaccharides such as soluble starch, are also utilised as are alcohols e.g. ethanol and methanol, and hydrocarbons such as *n*-alkanes.

Nitrogen is usually provided by organic compounds. Ammonium salts (sulphate, phosphate, nitrate) are also utilized by cells. Ammonium salts of organic acids are better utilized than salts of inorganic acids, since their decomposition gives rise to weak acids that can serve as an additional carbon source. Inorganic salts produce strong inorganic acids that can change pH and have an inhibitory effect. The exception being ammonium phosphate.

It has been shown, that often under nitrogen-limited conditions, when a cell becomes depleted of nitrogen for further growth, proteases may be activated to degrade surplus copies of enzymes so that the amino acids therein can be scavenged and used for the biosynthesis of enzymes in short supply (Ratledge 1991).

Phosphorus is an important element. It plays a central role in both energy metabolism and in the biosynthesis of membrane phospholipids (Jones and Gadd 1990).

Phosphorus is transported into the cell in the form of free H_2PO_4. Intracellular concentrations usually range from 3 - 10 mM. This uptake is via three different tranport systems, all of which are highly sensitive to intracellular pH - more so than extracellular pH.

Phosphorus is present in "compartments" in the cell. i.e. in the form of phospholipids, RNA, DNA and nucleotides, two distinct forms of metaphosphate

granules and free phosphate. The amount in each compartment varies with growth conditions and growth history. Vacuolar reserves of polyphosphate and free phosphate act as a mobilization store of phospate and metabolic energy without alteration of the cytoplasmic environment (Jones and Gadd 1990).

The level of free phosphate is also linked to glycolysis and other metabolic pathways due to its role in reactions involving ADP and ATP. Limitation of phosphorus can therefore affect the yeast cell in various ways. Alteration in the structure of the plasma membrane may occur due to imbalanced phospholipid metabolism which can lead to changes in permeability of the cell. Phosphorus starvation also causes reduction of vacuolar polyphosphate materials and the vacuoles associated store of ionic species. Phosphorus-starved cells should therefore be more sensitive to the deleterious effects of the external environment.

Phosphate is added to media in the form of potassium, ammonium or sodium phosphates. The most common salt KH_2PO_4, is often used as it buffers the media at a suitable pH. Depending on the pH, the salt used is sometimes K_2HPO_4, or the two may be combined.

The magnesium ion (Mg^{2+}), acts to stimulate the specific growth rate and cell division in the yeast cell. It is involved in many enzyme catalyzed processes. In some cases it can readily be replaced by manganese or cobalt. It is commonly added as $MgSO_4$ to artificial media.

Potassium plays a central role in the regulation of yeast growth and fermentation under both aerobic and fermentative conditions. e.g. under aerobic conditions, a medium which is deficient in K^+ (i.e. levels of around 500 μM) promotes the production of ethanol due to the resulting energetic demands for its uptake against a very high concentration gradient (Jones and Gadd 1990).

It has also been found that there is an interrelationship between K^+ and Na^+, where the requirement for K^+ increases with Na^+ concentration. There is a critical intracellular Na^+:K^+ ratio above which Na^+ becomes toxic. However, in K^+ deficient conditions it has been shown that Na^+ may enhance growth and reduce the minimum concentration of K^+ required to prevent cell death (Jones and Gadd 1990).

Growth substances such as vitamins are also required for the growth of yeasts. It is mainly the B-group vitamins that are of importantance e.g. m-Inositol, pantothenate, niacin, thiamine, pyridoxine and biotin.

2.2.2.2 Yeast Cell Structure

The plasma membrane (cytoplasmic membrane) makes up 13 - 20% of biomass dry weight. It contains several hydrolytic enzymes and enzymes that are associated with the synthesis of cell wall components. It is the site of strong ATPase (EC 3.6.1.3) activity which requires Mg^{2+} (optimum concentration 3mM), and hence is sometimes called Mg-ATPase. This enzyme is different to the ATPase found in mitochondria.

The plasma membrane ATPase (and vacuolar ATPase) are associated with the transport of ions and other nutrients, intracellular compartmentalization and also the regulation of cell pH. Suggested regulators of activity include internal levels of ATP and ADP, external and intracellular pH as well as concentrations of Mg^{2+} (cofactor) and Ca^{2+} (inhibitor) inside the cell.

Vacuoles are highly conspicuous and important organelles in yeast cells, and may occupy as much as 25 - 95% of cell volume. Cells usually contain one large vacuole which is mostly spherical in shape, and several smaller ones.

Vacuoles contain hydrolases. i.e. proteases, ribonucleases, and esterases. They also contain amino acids – 80 - 90% of dissolved amino acids out of the total free amino acid pool in yeast cells has been discovered to be localized in vacuoles.

It is indicated that the vacuoles represent the central storage space for deposition of intermediates, and also many ions such as inorganic phosphate, and monovalent and divalent cations, which are preferentially located in vacuoles in cells (Jones and Gadd 1990). These play the role of reserves which are prepared for utilization when the external conditions change. Therefore, the growth rate and ion uptake rates of yeasts may reflect the precultivation conditions. If the yeast cell is precultured in unlimited media, the vacuoles will serve as a store of ionic species should the yeast cell encounter any form of nutrient limitation. This means that it is only when the cell has exhausted its intracellular stores of nutrients that effective nutrient limitation occurs.

Mitochondria are built up by two kinds of membranes: outer and inner membranes. The mitochondrial membrane system contains all mitochondrial proteins and also contains ribosomes.

Mitochondria are responsible for the final oxidation of pyruvate arising from glucose metabolism, to yield intermediates of the tricarboxylic (TCA) acid cycle. These intermediates are then used for the manufacture of new cell material, as well as for the provision of energy via oxidative phosphorylation, where oxygen is consumed and energy is produced at the expense of pyruvate (See Figure 2.2).

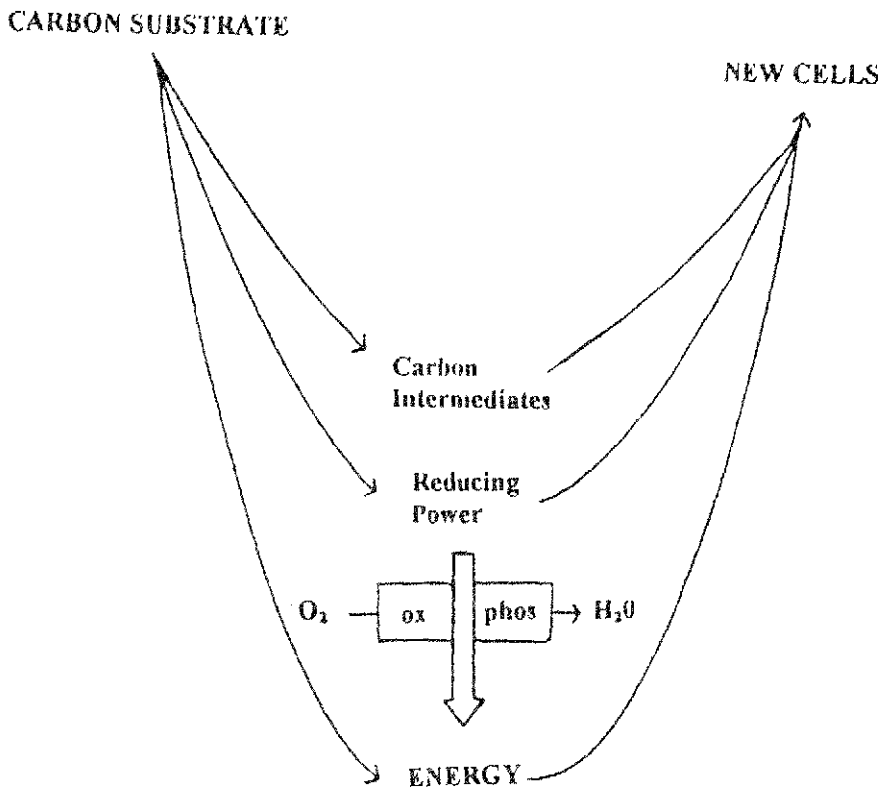


Figure 2.2: **Processes of catabolism (degradation) and anabolism (biosynthesis) linked to energy production and provision of reducing power - Aerobic metabolism**
(Ox - Phos = oxidative phosphorylation)
(From: Ratledge 1991)

Mitochondrial morphogenesis and structure can be affected by factors such as lowered partial pressure of oxygen. Under anaerobic conditions, yeast cells contain fewer mitochondria with a reduced number of cristae. Changes in the number and shape of mitochondria can also be induced by different carbon sources and different concentrations of these sources.

Mitochondria represent a system handling the cell energy. Electron flow within them is associated with ATP synthesis and the active transport of soluble molecules or ions. The two membrane systems fulfil different roles, which is indicated by the location of enzymes.

A number of factors are proposed for regulating the rate at which the TCA cycle operates within mitochondria:

1. Availability of NAD^+ for the dehydrogenation steps of the cycle
2. The rate at which acetyl CoA is synthesized, and intermediates of the cycle are drained for biosynthesis.
3. Intramitochondrial concentrations of AMP, ADP and ATP.

2.2.3 Media Used in the Production of Citric Acid

2.2.3.1 Carbon source

A variety of carbon sources have been assessed for the production of citric acid from yeasts, under varying conditions of growth (See Appendix 1: Table 1 for details and references).

The assessment of citric acid production from yeasts has been carried out using a variety of methods (Hattori *et al.* 1974; Marchal *et al.* 1977b; Finogenova *et al.*

1986; Tani *et al.* 1990; Finogenova *et al.* 1991; Abou-Zeid and Khoja 1993; McKay *et* al. 1994; Rane and Sims 1995). Small-scale batch fermentations in shake flasks have been used, with a fermentation medium volume of (on average) 100 mL. Larger-scale fermentors under more controlled conditions, with volumes ranging from 1 L to 10 L, have also been used along with a variety of fermentation methods. These methods include: batch, trickle-flow, cell-recycle, continuous and immobilized fermentations.

Work done on a smaller scale using batch culture conditions in shake flasks predominantly deals with the use of glucose as a carbon source, with concentrations in the fermentation medium ranging from 20 $g.L^{-1}$ (Mandeva *et al.* 1977; Finogenova *et al.* 1986; Shah *et al.* 1989; Olama *et al.* 1990), to 30 $g.L^{-1}$ (Gutierrez *et al.* 1992; Gutierrez and Maddox 1993). 50 $g.L^{-1}$ (Evans *et al.* 1985a; Mitsushima *et al.* 1976), up to 72 $g.L^{-1}$ (McKay *et al.* 1994). Much work has also been done using large-scale fermentations with glucose as a carbon source. Concentrations used ranged from 20 $g.L^{-1}$ to 240 $g.L^{-1}$ (Treton *et al.* 1978; Briffaud and Engasser 1979a, b; Marchal *et al.* 1980; Evans *et al.* 1983; Ermakova *et al.* 1986; Kautola *et al.* 1991; Klasson *et al.* 1991; Rane and Sims 1993, 1994, 1995; Rymowicz *et al.* 1993; Kulakovskaya *et al.* 1993).

The use of *n*-alkanes as a carbon source has been studied widely. The development of methods using *n*-alkanes however, came to a sudden stop due to the oil crisis of 1973/74. There were also reservations expressed (Good *et al.* 1985) that citric acid produced from *n*-alkanes could potentially contain impurities, such as carcinogenic polycyclic hydrocarbons, thus making it unsuitable for use in food. The *n*-alkanes utilised for citric acid production were generally a mixture of C_{10} - C_{18} (Hattori *et al.* 1974; Treton *et al.* 1978). The best mixture was found to be C_{13} - C_{18} (Nakanishi *et al.* 1972). Concentrations used varied from 50 $g.L^{-1}$ to 100 $g.L^{-1}$ for large-scale fermentations (Nakanashi *et al.*

1972; Hattori and Suzuki 1974; Hattori *et al.* 1974; Marchal *et al.* 1977a, b; Aiba and Matsuoka 1978) and 45 to 80 $g.L^{-1}$ (Aiba *et al.* 1978; Furukawa *et al.* 1982) for shake flask fermentations.

Other media used include ethanol (Marchal *et al.* 1977b; Finogenova *et al.* 1986, 1991), hexadecane (Marchal *et al.* 1980; Mandeva *et al.* 1981; Ermakova *et al.* 1986; Finogenova *et al.* 1986), glycerol (Treton *et al.* 1978), sucrose (Martin and Denton 1970), potassium acetate (Marchal *et al.* 1977a), acetic acid (Finogenova *et al.* 1986), and canola (rapeseed) oil (Good *et al.* 1985).

2.2.3.2 Nutrient limitation

It is generally accepted that in order for overproduction of citric acid to occur, the culture requires the carbon source to be in excess and nitrogen to be the limiting nutrient (Marchal *et al.* 1977a; Mitsushima *et al.* 1978; Evans and Ratledge 1985b; Kubicek and Röhr 1986; Klasson *et al.* 1991; Rane and Sims 1994). It is also accepted that accumulation of citric acid and consequent excretion into the medium by yeasts does not usually begin until all nitrogen in the medium has been utilised and intracellular levels of nitrogen have also dropped i.e. when the culture has made the transition from unlimited exponential growth to that of growth retardation (Mitsushima *et al.* 1976; Briffaud and Engasser 1979a; Finogenova *et al.* 1986). This has been shown to coincide with an intracellular rise in ATP, which in combination with other factors, leads to increased citric acid production and excretion.

The majority of published data deals with nitrogen as the limiting nutrient, but some work has also been done looking at the effects of limiting phosphorus,

sulphur, magnesium and potassium concentrations in the production medium. (Mandeva *et al.* 1981; McKay *et al.* 1994)

Nitrogen

The levels of nitrogen required to ensure maximum levels of citric acid production have been studied by some authors. Levels of 3.0 - 4.0 $g.L^{-1}$ of NH_4Cl or $(NH_4)_2SO_4$ are commonly used as the limiting nitrogen source (Hattori *et al.* 1974). Röhr and Kubicek (1983), also reported that optimum concentrations of total acid and the ratio of citric to isocitric acid were obtained at 0.3% (w/v) NH_4Cl. Levels used in both batch fermentations in shake flasks, and large scale fermentations, vary from 0.1 to 5.0 $g.L^{-1}$, depending on the carbon source used.

Phosphorus

Phosphorus has been stated as necessary for the growth of yeasts and for fermentation. Uptake of phosphorus is related to cell multiplication. When assessing optimal conditions for the growth of *S. lipolytica* on *n*-alkanes, Furukawa *et al.* (1982) showed that a low phosphorus:carbon (w/w) ratio was important for high citric acid productivity. A ratio of P:C (w/w) of 0.6 - 1.0 x 10^{-3} gave the highest amount of citric acid. Phosphorus-limited cells have been shown to produce increased levels of citric acid (McKay *et al.* 1994). The increased level of citric acid production with phosphorus-limited cultures may be related to changes in the plasma membrane due to imbalanced phospholipid metabolism. This may also have some effect on the membrane ATPase(s) (See Section 2.2.2).

KH_2PO_4 has been stated as being the most favourable source of phosphorus for citric acid production. Levels of 0.3 $g.L^{-1}$ KH_2PO_4 were shown by Hattori *et al.*

(1974b) to be optimal for citric acid production from *n*-alkane, but not for cell growth. Abou-Zeid and Khoja (1993), while assessing the use of date-coat sugar extracts as a carbon source, found that the best concentration of phosphorus was 1.0 mg KH_2PO_4 mL^{-1}. Rymowicz *et al.* (1993) used levels of 0.1 $g.L^{-1}$ for citric acid production from *Y. lipolytica* immobilized in calcium-alginate.

Vitamins and Minerals

Work has also been done on the effects of the addition of vitamins and minerals to the growth media and their effect on the production of citric acid (Akiyama *et al.* 1973; Good *et al.* 1985; Rane and Sims 1993). It was found that the vitamins thiamine, nicotinic acid and nicotinamide, as well as the addition of ferrous ion increased production (Rane and Sims 1993). Yeasts assimilating *n*-alkane are known to require thiamine in the form of thiamine pyrophosphate, as it functions as a coenzyme in the oxidative decarboxylation of *n*-alkane (Röhr and Kubicek 1983).

Hattori *et al.* (1974), Mandeva *et al.* (1981) and Good *et al.* (1985) found that the presence or absence of various trace metals such as iron, zinc or manganese affected the ratio of citric acid to isocitric acid. The yield of citric acid was also affected. It was shown by Good *et al.* (1985), that iron and zinc had negative effects of the yield of citric acid from canola oil, while manganese stimulated citrate synthesis. Marchal *et al.* (1977b) demonstrated that the addition of 400 mg of $FeSO_4.7H_20$ per litre, reduced citric acid productivity from *S. lipolytica* by 30% when this yeast was grown on *n*-alkanes.

Copper ions are known to inhibit aconitase activity. Furukawa *et al.* (1982) showed while growing *S. lipolytica* on *n*-alkanes that the activity of aconitase was inhibited by the addition of 0.1 $mg.mL^{-1}$ $CuSO_4$. This concentration also resulted

in optimal production of citric acid. Rymowicz *et al.* (1993) used a concentration of 21 $mg.L^{-1}$ of copper sulphate (5.2 $mg.L^{-1}$ copper) to increase citric acid production from *Y. lipolytica* grown on glucose. It was also found by Furukawa *et al.* (1982), that the addition of sodium borate (0.1 $mg.mL^{-1}$ $Na_2B_4O_7.10H_2O$), decreased by half the amount of isocitrate produced in the acid mix when *S. lipolytica* was grown on *n*-alkanes. Although this observation was not addressed further by the authors, it is assumed that the reduction in isocitrate levels was due to some form of inhibition of aconitase activity.

It is known that ferrous ion is an essential factor for aconitase activity. High levels of ferrous ions result in high activities of aconitase and hence reduces the amount of citric acid produced. Therefore, the level of ferrous ion plays an important role in deciding the ratio of citric acid to isocitric acid (Akiyama *et al.* 1973). Levels of ferrous ion quoted range from 0.001 g L^{-1} to 10 g L^{-1} of $FeSO_4.7H_2O$ (Aiba and Matsuoka 1978; Briffaud and Engasser 1979a; Moresi *et al.* 1980; Ermakova *et al.* 1986; Abou-Zeid and Khoja 1993). This is a very wide range of levels - the latter perhaps being a misprint or error, as it has been stated by Akiyama *et al.* (1973) that extremely low levels of < 1 ppm of ferrous ion (as heptahydrous ferrous sulphate), are preferred for a low production ratio of citric acid to isocitric acid. But, they also conceded that maintenance of this low level of ion in the fermentation medium was almost impossible.

The requirement for ferrous ions has been shown to vary depending on the species being studied. Nakanishi *et al.* (1972), reported that *Candida guilliermondii* when grown on a chemically-defined medium with *n*-alkanes as the carbon source, requires the presence of ferrous ions ($FeSO_4.7H_2O$ - 0.002% (w/v)) for the production of citric acid. However, for citric acid production by *Candida zeylanoides*, using *n*-alkanes as a carbon source, ferrous ions were removed from the medium to increase the ratio of citric acid to isocitric acid. Activity of

aconitase was reduced in this instance to only 60% of that in the medium in which sufficient ferrous ions were provided.

Ferrocyanide is known to inhibit aconitase activity, and hence increase citric acid production. Ferrocyanide acts as a chelating agent and so reduces the content of free iron in the medium.

But, in contrast, addition of potassium ferrocyanide (200 μM) was shown by Nakanishi *et al.* (1972) to decrease the accumulative ratio of citric acid to total citric acid in *C. zeylanoides* and *C. guilliermondii.* This also resulted in a reduction of NAD-ICDH activity, but did not greatly affect aconitase activity. A change in activity of aconitase might be expected due to the increased levels of isocitrate in the acid mix. In moulds, the addition of potassium ferrocyanide has also been shown to decrease the accumulative ratio of citric acid to total acid. It was seen to cause more inhibition of NAD-ICDH than of NADP-ICDH (Abou-Zeid *et al.* 1984).

Marchal *et al.* (1977b), showed that the addition of iron-complexing agents such as quinaldinic acid were effective in increasing the citric acid production rate. For example, the addition of 100 $mg.mL^{-1}$ of quinaldinic acid to medium containing 10 $mg.mL^{-1}$ $FeSO_4.7H_20$, increased the production rate by 71% when compared with medium that contained only 10 $mg.mL^{-1}$ $FeSO_4.7H_20$. This also resulted in an increase in the ratio of citric acid to isocitric acid in the acid mix, from 60 to 70%.

2.2.3.3 Growth conditions

Growth conditions used for the fermentations varied between authors. The temperatures used ranged from 27°C to 30°C. The pH values also differed considerably, the range being 5.5 to 6.5. pH has been found to have an effect on the yield of citric acid. The most suitable medium pH for citric acid production was 5.5 when grown on glucose (Nakanishi *et al.* 1972), or 5.0 (Marchal *et al.* 1977b) for growth on alkanes. Hattori and Suzuki (1974) showed that for *C. zeylanoides* grown on *n*-alkanes, activity of citrate synthase (and citric acid production), was optimal at an intracellular pH of 6.5, and that an intracellular pH of 5.5 was associated with the increased production of polyols. Shifting of the medium pH from 5.5 to 3.5 led to an immediate change in the intracellular pH from 6.5 to 5.5, thus indicating that the medium pH does affect the intracellular pH and as a result, enzyme activity. In contrast, Kim and Roberts (1990) used a medium pH of 3.4 - 3.5 for the production of citric acid by *S. lipolytica* grown on glucose in a continuous system. Good *et al.* (1985) also maintained a medium pH of 3.5 during the citric acid production phase. The use of such acidic medium pH would surely have some effect on the activity of citrate synthase if the data presented by Hattori and Suzuki (1974) is correct.

The aeration rate in the fermentors and dissolved oxygen content of the medium also affected citric acid production from yeasts - these have been studied frequently by various authors (Moresi *et al.* 1980; Finogenova *et al.* 1991; Rane and Sims 1994). The best level of aeration was found by (Marchal *et al.* 1977b) to be 0.2 vvm, which is low in comparison with later studies. Sufficient aeration was found to be more important when *n*-alkanes were used as the carbon source. There is much variation between authors with regard to the speed of agitation and aeration of the culture (Nakanishi *et al.* 1972; Treton *et al.* 1978; Rymowicz *et al.* 1993; Moresi 1994). For example, Rane and Sims (1993) achieved optimum citric acid production at aeration of 1.5 vvm (volume of air per volume of medium

per minute) and agitation of 1000 rpm, whereas Hattori *et al.* (1974) found that aeration of 2.0 vvm and agitation of 700 rpm gave the best levels of citric acid production, but with higher levels of isocitric acid in the acid mix. The dissolved oxygen content of the medium that has been deemed to be optimal by Rane and Sims (1990), for the production of citric acid is 80% of saturation - the dissolved oxygen content of the medium being more important with regards to measuring the uptake of oxygen by yeasts, rather than the rate of agitation (See Section 2.2.2). Levels of aeration in the shake flasks are harder to assess but there is great variation between the speed of agitation of flasks, these ranging from 120 - 260 rpm. Generally, most authors used a small volume of liquid in a larger flask, so giving greater surface area and hence better aeration.

Time of fermentation also seems to vary between authors and the yeast used. This was due to the rate at which the carbon source was utilised and exhausted. The production phase is usually considered to be the stationary phase of growth, i.e. when cell growth has stopped. Citric acid production then begins and continues until the exhaustion (or near-exhaustion) of the carbon source. However, it has been found that with some yeasts accumulation of citric acid begins in the exponential phase of growth (Gutierrez *et al.* 1992).

It would be reasonable to conclude that growth conditions in the shake flask cultures might be less than optimum and perhaps production of citric acid could be increased further in fermentors, in which it is possible to elicit tighter control on both the pH and level of aeration with a greater resultant yield of citric acid.

Generally, the best growth conditions for the production of citric acid from yeasts appear to be:

- Excess carbon source - glucose 20 - 240 $g.L^{-1}$ (Depending on method of fermentation)
- Growth temperature of 28 - 30°C

- pH controlled at 6.5
- Nitrogen source: NH_4Cl at 3.0 - 4.0 $g.L^{-1}$
- Trace elements: Low levels of ferrous ion, also presence of thiamine, nicotinic acid and nicotinamide.
- Aeration to give 80% of saturation of dissolved oxygen.
- Time of production - yeast dependent

Many of these factors can change depending on the strain of yeast and also the carbon source used. It would be advisable to determine for each yeast species optimum conditions for citric acid production under the method of fermentation chosen.

2.2.4 Yeast Strains Used in the Production of Citric Acid

Appendix 1: Table 1 summarises many of the yeast strains that have been assessed for their ability to produce citric acid and the carbon sources used. Many of these strains are mutants that have been developed in the course of finding the most effective producers. For levels of citric acid produced by the parent and mutant strains of *Candida guilliermondii* in this work see Figures 5.4 and 5.5 and Appendix 3: Table 6.

2.2.5 Control of Citric Acid Production From Citric Acid Producing Yeasts

2.2.5.1 Introduction

Although much work has been done on the development of yeast mutants and obtaining optimum conditions for the production of citric acid, little is known about the exact mechanism of accumulation and excretion of citric acid from the cell into the fermentation medium.

It was proposed by Marchal *et al.* (1977b) and Finogenova *et al.* (1986) that there are certain events that are of crucial importance to any citrate accumulation metabolism:

(a) An increased supply of precursors for citrate (Acetyl-CoA and oxaloacetate).

(b) A relatively low catabolism of citrate.

(c) Blockage of anaplerotic reactions requiring TCA cycle intermediates i.e. isocitrate and oxaloacetate

Work has been carried out assessing the mechanisms of citric acid accumulation in citric acid-producing yeasts, and also oleaginous (lipid-producing) yeasts. Evans and Ratledge (1985d), found that there are striking metabolic similarities between oleaginous yeasts and citric acid-accumulating micro-organisms. The pathways for the utilisation of carbon are the same until citrate is excreted into the cytosol from the mitochondria. Oleaginous yeasts, unlike citric acid-producing yeasts (Boulton and Ratledge 1981a), have the enzyme ATP-citrate lyase, which further breaks down citrate in the cytosol, into the lipid precursors acetyl-CoA and oxaloacetate.

It is the work on oleaginous yeasts by Botham and Ratledge (1979), Boulton and Ratledge (1979) and Evans and Ratledge (1985b, c) and work on the production of

citric acid from *n*-alkanes (Mitsushima *et al.* 1977; Marchal *et al.*1977a; Botham and Ratledge 1979) and glucose (Bartels and Jensen 1979), which has led to the currently accepted hypothesis for the overproduction and excretion of citric acid:

When growth stops, due to nitrogen limitation, the intracellular (intramitochondrial) concentration of AMP and ADP decreases, whereas the concentration of ATP rises sharply. This causes inhibition of NAD-specific (AMP-requiring) isocitrate dehydrogenase. Due to inhibition of this enzyme, flux through the citric acid cycle is reduced, leading to the accumulation of citric and isocitric acids. The ratio of citric and isocitric acids obtained is dependent on the activity of aconitase, the carbon source, and the activity of anaplerotic pathways.

2.2.5.2 Nutrient Limitation and Citric Acid Production

It is generally agreed that for the overproduction and accumulation of citric acid, or any metabolite to occur, there must be a limiting nutrient. In the vast majority of cases nitrogen has been used as the limiting nutrient, and has been stated as necessary for the production of citric acid. It has been shown by McKay *et al.* (1994) and Mandeva *et al.* (1981), that over-production of citric acid also occurs under other nutrient limitations and not exclusively under nitrogen limitation as was previously thought.

Mandeva *et al.* (1981) found that the type of nutrient limitation affected the metabolic products. For example, *C. guilliermondii* was found to excrete polyols under nitrogen, phosphorus, sulphur and magnesium limitation and citric acid under phosphorus and nitrogen limitation.

It is widely accepted that citric acid production begins at the end of the exponential growth phase, when nitrogen has been exhausted from the fermentation medium (Mitsushima *et al.* 1976; Marchal *et al.* 1977a; Rane and Sims 1993). Production and excretion of citric acid continues through the stationary phase for a limited period of time, depending on factors such as the carbon source, and growth conditions. Finogenova *et al.* (1986) have found that although there is no increase in numbers of cells during the stationary phase, there may be an increase in biomass. It was also found that citric acid is produced only by non-propagating cells of the stationary phase, with more than 70% of these cells remaining viable and capable of propagating.

It has been assumed by Treton *et al.* (1978), that as citric acid can be produced in continuous culture, it is not incompatible with growth. It has also been seen in this laboratory on work with *C. guilliermondii* IMK1, that low levels of citric acid production occur in the exponential phase of growth - before nutrient limitation is reached (Gutierrez *et al.* 1992). It was found by Moresi *et al.* (1980) while assessing citric acid production from *C. lipolytica* Cooper, that citric acid production commenced with cell growth. The capability of a yeast to produce citric acid during the exponential phase of growth would surely be advantageous with regard to yields and possible reduction of fermentation time.

It has been suggested by Finogenova *et al.* (1991), that during adaptation of a yeast to a medium that is deficient in a particular nutrient, protein molecules whose functions are lost, such as enzymes, disintegrate. This implies that, if the limitation is reversed, the cell will be unable to continue normal functioning in unlimited conditions. There appears to be little evidence to support this claim. It was common practice in this laboratory to prove nutrient limitations at the end of a fermentation, by addition of the limiting nutrient in excess which resulted in a significant increase in cell numbers as measured by spectrophotometric methods.

As there is little increase in cell biomass during citric acid production, it would suggest that the enzymes involved in reproduction etc. are inactive at this stage.

However, on removal of the limitation, the resultant increase in cell numbers indicates that the enzymes involved in reproduction (at least) are still functional.

2.2.5.3 Enzyme Activity

The activity of the TCA cycle enzymes, particularly citrate synthase, aconitase and NAD-specific isocitrate dehydrogenase (NAD-ICDH), are thought to have an important role in the overproduction of citric acid.

It has been suggested by Finogenova *et al.* (1986), that the ability of a strain of *C. lipolytica*, to produce citric acid and isocitric acid is associated in part with very high activity of citrate synthase compared with the relatively low activity of other enzymes of the cycle. Reported levels of citrate synthase activity vary greatly between authors, from 0.5 to 14.8 U/mg protein. (See Appendix 1: Table 2a) It has been suggested by Abou-Zeid *et al.* (1984), that citrate synthase is a key regulatory site of aerobic metabolism. But in work done on oleaginous yeasts by Boulton and Ratledge (1980) it was found that citrate synthase was not susceptible to rigorous or severe regulatory controls.

Aconitase activity has been studied widely. Low aconitase activity is seen as preferable as this will reduce the amount of isocitrate in the total acid mixture. Methods used to reduce the activity of aconitase involve the development of aconitase-defective mutants or the addition of aconitase inhibitors to the medium, as will be discussed shortly.

Botham and Ratledge (1979) stated that as aconitase catalyses the reversible conversion of citrate to isocitrate, it is also responsible for the conversion of isocitrate to citrate following any build up of isocitrate in the mitochondria.

Finogenova *et al.* (1990) developed a glucose-grown mutant (*C. lipolytica* Mutant 1), which was found to synthesise citric acid almost exclusively. This mutant was found to have low aconitase activity compared with relatively high citrate synthase activity and a high total activity of NAD- and NADP-ICDH. A second glucose-grown mutant (*C. lipolytica* Mutant 2), was shown to have higher aconitase activity and so synthesised citric acid and isocitric acid simultaneously.

Furukawa *et al.* (1982), developed *n*-alkane-grown mutants which produced higher levels of citric acid. These mutants showed reduced aconitase activity and higher citrate synthase activity - one mutant exhibiting activity of citrate synthase that was 40% higher than the parent.

Aconitase inhibitors such as monofluoroacetate and 2-picolinic acid (Good *et al.* 1985), have been investigated as a means to increase the ratio of citric acid to the total acid produced. Monofluoroacetate is metabolised to form monofluorocitrate which is a competitive inhibitor of aconitase (Akiyama *et al.* 1973a, b).

Akiyama *et al.* (1973a, b) developed monofluoroacetate-sensitive mutants of *C. lipolytica* for improved production of citric acid from *n*-alkanes. Under normal conditions a ratio of 60:40 (CA:ICA) was obtained, while the presence of 0.1% monofluoroacetae in the culture medium improved the ratio to 85:15. It was also shown that the aconitase activities of the mutants had reduced to $^{1}/_{10}$ and $^{1}/_{100}$ of that of the parent. While assessing the production of citric acid from methanol Tani *et al.* (1990), developed a fluoroacetate-resistant mutant (*Candida* sp Y-1), which produced levels of isocitrate so low as to be undetectable. It was suggested that the mutation had caused structural changes in the aconitase gene, resulting in

lowered activity and resistance to fluoroacetate. Nakanishi *et al.* (1972), also showed that aconitase inhibitors caused a large increase in the ratio of citric acid to total acid. However, the use of monofluoroacetate and 2-picolinic acid is not appropriate as they have toxic effects, and so are unacceptable for the production of food-grade citric acid. For this reason it is suggested that development of mutants with reduced aconitase activity would be a preferable option. Reduction of aconitase activity would also be expected to result in lower growth yields due to the lower flux of carbon through the TCA cycle.

Enzyme activity has also been shown to vary between exponential and stationary phases of growth (Akiyama *et al.* 1973; Mitsushima *et al.* 1976). Mitsushima *et al.* (1976), showed that the activities of aconitase, ICDH and 2-oxoglutarate dehydrogenase were seriously reduced in the citric acid-accumulating (stationary) phase. However, Hattori *et al.* (1974) showed (using *C. zeylanoides* grown on *n*-alkane) that although activity of aconitase changed during the course of the fermentation - decreasing in the stationary phase, the activity of NADP-ICDH remained constant throughout the fermentation. Finogenova *et al.* (1991) while growing *C. lipolytica* on ethanol, found that the activities of NAD-ICDH and aconitase were highest in the stationary (citric acid-producing) phase, whereas the activities of NADP-ICDH and isocitrate lyase were at their lowest during this phase of growth. It would be expected that as the stationary phase is the main period of citric acid accumulation, that activities of aconitase and NAD-ICDH would be at their lowest during this time. The fact that Finogenova *et al.* (1991) found increases in activity is surprising, although it may suggest that due to lowered flux through the cycle, enzyme activity has increased in order to utilise any carbon that may become available, or the increase is a result of the increased levels of precursor - citric acid.

The control of the activity of NAD-dependent ICDH, is probably the most important control point. This enzyme is located in the mitochondria in yeasts and

is involved in the conversion of isocitrate to 2-oxoglutarate. Under conditions of nitrogen limitation the levels of AMP in the cell decrease, with a correlating increase of ATP. This causes inhibition of the enzyme, decreasing the net flux through the 2-oxoglutarate dehydrogenase complex, resulting in accumulation of citric acid in the mitochondria which is then transported into the cytosol and out of the cell (Mitsushima *et al.* 1976; Finogenova *et al.* 1991).

Evans and Ratledge (1985c) showed that NAD-ICDH in *Y. lipolytica* was weakly inhibited by glutamate, 2-oxoglutarate and citrate. They also stated that it would be expected that a rapid inactivation of NAD-ICDH at the onset of nitrogen limitation would occur, as there is a dramatic decrease in intracellular AMP and an increase in ATP. Ermakova *et al.* (1986) found that NAD-ICDH was more active in the oxidation of glucose than hexadecane. It was suggested that this was due to the fact that during growth on *n*-alkanes, some of the isocitrate is metabolized via the glyoxylate cycle.

See Appendix 1: Tables 2a - 2e for a summary of enzyme activities for citric acid producing yeasts.

3.2.5.4 Adenine Nucleotides

The regulatory effect of adenine nucleotides on the production of citric acid has already been mentioned with regard to the effects of ATP on NAD-dependent ICDH.

The addition of 5.0 mM ATP has been shown experimentally to increase the efflux of citric acid from the cell. This level of ATP has been shown (Marchal *et al.* 1977a) to cause 40% inhibition of citrate synthase activity, but this is thought

to depend on the intracellular concentration of the precursor acetyl-CoA. ATP inhibition has been found to be competitive with respect to the concentration of acetyl-CoA.

Mitsushima *et al.* (1978) showed that addition of 0.1 mM ADP to the mitochondria of the citric acid-producing yeast *C. lipolytica*, caused a decrease in citric acid production and a four-fold increase in the concentration of 2-oxoglutarate, thus indicating that ADP has a role in the activation of NAD-ICDH activity. Addition of 0.3 mM ATP to the mitochondria increased citric acid production by 50%. This showed that the reduced activity of NAD-ICDH that is commonly seen during the citric acid phase may be due in part to inactivation/inhibition of activity by ATP.

2.2.5.5 Transport of Glucose and Citric Acid in Citric Acid-Producing Yeasts

Glucose Transport

It has been shown by various authors that under conditions of increased citric acid accumulation the rate of uptake of glucose decreases (Aiba and Matsuoka 1978; McKay *et al.* 1994). This might be expected due to the feedback control of glycolysis by citric acid causing inhibition of the enzyme phosphofructokinase.

The rate of glucose utilisation that can be maintained by the yeast whilst under limitation affects citrate production. This was shown by McKay *et al.* (1994) by the differing rates of uptake observed under citric acid-producing and non-citric acid-producing limitations such as the potassium and magnesium-limited cultures. It is recognised that potassium, magnesium and phosphorus ions play an essential role in both glucose catabolism and substrate transport by yeasts, implying that

limitation of these nutrients may affect the ability of the cell to transport nutrients, i.e. glucose, into the cell.

Citric Acid Transport

The exact mechanism of transport of citric acid out of the mitochondria and subsequently into the medium is as yet unknown.

Evans *et al.* (1983) showed evidence of a carrier system in mitochondria, for dicarboxylic and tricarboxylic anions, phosphate and pyruvate. It was also concluded that in fully functional yeast mitochondria, citrate efflux is limited by the amount of intramitochondrial citrate made available to the citrate translocator for exchange.

Evans *et al.* (1983), stated that the continuous supply of citrate to the cytosol from mitochondria will depend on the action of several metabolic events:

(a) The rate and extent of citrate efflux from the mitochondria to the cytosol.

(b) The rate of citrate synthesis, which in turn depends on

(c) The regeneration of intramitochondrial acetyl CoA and oxaloacetate.

Acetyl-CoA is provided by pyruvate, which can enter mitochondria by a specific carrier. Mitochondria are thought to be relatively impermeable to oxaloacetate, but this intermediate may enter on a dicarboxylate carrier system, or indirectly via malate and pyruvate. The addition of malate was shown to increase citrate efflux from the mitochondria of *S. lipolytica NCYC 153* (Evans *et al.* 1983). Mitsushima *et al.* (1978) showed that in *Y. lipolytica* there is an active citrate-malate translocase to remove accumulating citrate.

Evans and Ratledge (1985d), showed that the malate-citrate exchange is stimulated by phosphate and pyruvate. This stimulation is attributed to the operation of the citrate translocase, as a proton-compensated electroneutral carrier, which transports a proton in the same direction as the citrate during exchange, i.e. the exchange is malate $^{2-}$ for citrate^{3-} + H^+.

Kulakovskaya *et al.*(1993) investigated the possibility that vacuoles were involved in the transport of citric acid out of the cell. It was found that this was not the case and it was proposed that excretion into the medium was probably by passive diffusion.

2.2.5.6 Growth Conditions Affecting Citric Acid Production

Carbon Source

The carbon source used for the production of citric acid can affect the yield obtained. It has been found that the quantity of isocitrate in the total acid mix can be lowered by the use of glucose as a carbon source (Hattori *et al.* 1974; Boulton and Ratledge 1981b; Aiba and Matsuoka 1982).

When yeasts are grown on glucose, pyruvate carboxylation has been found to be the anaplerotic pathway, whereas on alkanes, the anaplerotic pathway is the glyoxylate cycle, and so higher levels of isocitrate are required as precursors (Aiba and Matsuoka 1978; Marchal *et al.* 1980; Ermakova *et al.* 1986).

Ermakova *et al.* (1986), showed that for yeasts grown on *n*-alkanes, the assimilation of substrate was accompanied by the formation of a large number of peroxisomes in which the enzymes of the glyoxylate cycle are localised. It was also found that yeasts without isocitrate lyase were unable to grow and produce

citric acid in media containing hydrocarbons, thus providing evidence for the operation of the glyoxylate pathway in yeasts grown on *n*-alkanes.

Aiba and Matsuoka (1978), demonstrated that when yeasts were grown on glucose, pyruvate carboxylase provided the excess oxaloacetate needed for the spilling-over of citrate. Due to repression of the glyoxylate cycle, less isocitrate is required outside of the mitochondria and so the ratio of citrate to isocitrate is higher than by cultivation on *n*-alkanes.

Some work has been done on the ability of citrate-producing yeasts to reutilise citrate as a carbon source. This, if it occurs, is undesirable as it will affect the final yields of citrate obtained. This phenomenon seems to depend on the original carbon source used for growth. Treton *et al.* (1978) found that when *S. lipolytica* was grown on glucose or glycerol, neither citric or isocitric acid was reconsumed after being excreted. When grown on *n*-alkanes, only citric acid was reconsumed, but at a lower rate than excretion, so isocitric acid accumulated in the medium faster than citric acid. This provides another explanation for the higher levels of isocitrate found in cultures grown on *n*-alkanes, other than the activity of aconitase which has been mentioned previously.

Treton *et al.* (1978) attempted to explain the reason for the non-consumption of isocitrate in the presence of citric acid (when grown on glucose or glycerol):

(a) If the same permease mediates the uptake of both isocitric acid and citric acid, one could expect competitive inhibition to occur at the expense of isocitric acid uptake. (There is always an excess of citric acid in the total acid mixture.)

(b) It is possible that the carriers might be different. Citric acid could act as a repressor/inhibitor of the transport system specific for isocitric acid.

However, there has been little investigation into the exact mechanism of excretion of citric acid out of the cell, so there is little evidence to either confirm or deny these theories.

Marchal *et al.* (1980), attempted to find the reasons for the accumulation of isocitrate. The ratio of isocitrate found in the majority of cases is far too low when compared with the thermodynamic equilibrium of aconitase. (The theoretical percentage of isocitrate in the intracellular pool is 7% (Treton *et al.* 1978)). It was suggested that this results from the localisation of these acids in different compartments. Citric acid is strictly mitochondrial, while isocitric acid occurs in the mitochondria, cytosol and peroxisomes. As aconitase is absent from the cytosol, isocitric acid can accumulate there in high concentrations. Thus, a higher proportion can be excreted than would be predicted from the equilibrium of the aconitase reaction.

Marchal *et al.* (1980), suggested that this compartmentalisation of citric and isocitric acid probably involves a selective transport of a portion of isocitric acid from the mitochondria to the cytoplasm. It is suggested from the results that acid excretion from the cytoplasm to the external medium takes place by passive diffusion. This was explained by the observation that external and internal concentrations of citric and isocitric acids were of similar levels during the phase of acid excretion. It was assumed that the concentration of isocitric acid was greater than that of the entire cell and was at least as high as the external concentration. Again, this differs from the proposed active carrier system proposed by Treton *et al.* (1978).

The accumulation of isocitric acid was further explained by Marchal *et al.* (1980) as follows: a portion of isocitric acid is transported to the cytoplasm where it is either converted to glutamate or metabolized through the glutamate cycle. This metabolism takes place in 'microbodies' (Aiba and Matsuoka 1978). This

metabolism suggests the existence of a selective system for transporting of isocitric acid from mitochondria to the cytoplasm where it can then accumulate in concentrations greater than expected. Internal concentrations increase until biosynthesis stops, then excretion occurs. Therefore, transport activity of isocitric acid from mitochondria to the cytoplasm will be much lower during growth on a carbon source that does not require the glyoxylate cycle to be active. e.g. glucose. This results in a lower level of isocitrate in the mix of acids excreted.

Kim and Roberts (1990) assessed glucose-grown *S. lipolytica*. Using a medium pH of 3.4 - 3.5 in a continuous-stirred-tank-membrane-reactor system, they showed that the citrate production rate increased at high glucose concentrations, whereas the glucose consumption rate remained almost constant. This was thought to indicate that the citrate yield is a function of glucose concentration in the fermentation broth. Extracellular citric acid was found to inhibit the glucose consumption rate and the citric acid production rate. The product inhibition constant was found to be 235 $g.l^{-1}$. This concentration of citrate was found to inhibit non-competitively the glucose consumption rate. It is assumed that this is due to the effect of citric acid on phosphofructokinase although it must be noted that the levels shown to inhibit glucose consumption are far above levels of citric acid obtained by the majority of authors (See Appendix 1: Table 1). The reduced rate of glucose consumption associated with citric acid production is therefore probably associated with something other than inhibition of glucose uptake by citric acid.

pH Levels

pH levels have been shown to affect the ratio of citric acid to isocitric acid. Hattori and Suzuki (1974) found that during growth of *C. zeylanoides* on *n*-alkane, a shift of pH of the medium from 5.5 to 3.5 resulted in an immediate increase in

the production of rate of erythritol and a decrease in citric acid production. This change in pH was found to affect the activity of citrate synthase. Citrate synthase activity was found to be highest at an intracellular pH of 6.5. Enzyme activites of *C. zeylanoides* were compared at pH 6.5 (citric acid producing) and pH 5.5 (polyol producing). Significant differences in activity were found in NAD-ICDH (1:3, pH 5.5:pH 6.5), citrate synthase (1:13) and isocitrate lyase (1.62:1). This would have quite a significant effect on the amounts of citric acid produced. However, it is not known if this effect of pH on intracellular enzymes is also manifest on other carbon sources such as glucose.

Hattori *et al.* (1974b) showed that the type of medium pH-control agent affected the ratio of citric acid to total acid while growing *C. zeylanoides* on *n*-alkane. For example, under conditions where the pH was controlled by ammonia, the acid mix was 50% citric acid, whereas when the pH was controlled by NaOH solution, the acid mix was 90% citric acid. Enzyme activities of both types of pH control were assessed, and it was found that of the ammonia-controlled cultures, the enzymes aconitase and NADP-ICDH had activities that were $^1/_3$ and $^1/_4$, respectively, of those controlled by NaOH.

Finogenova *et al.* (1991), while assessing isocitric acid production by *C. lipolytica* grown on ethanol, also found that the pH of the medium affected citric acid ratios. For example, at pH 4.5, the ratio was 1.0:1.7 (ICA:CA), at pH 6.0 the ratio was 2.0:1.7. This has similarities with the results seen earlier, in that total citric acid was found to be highest at a pH of 6.0, which is close to the pH found to be optimal for citrate synthase activity. This fermentation was carried out with ethanol as a carbon source, suggesting that the effect of pH on the activity of citrate synthase may be the same no matter what the source of carbon.

Therefore, it appears that control of the medium pH is an important factor in optimising the production of citric acid. In work done on shake-flask cultures, as

opposed to the more controlled fermentor systems, the pH was in many cases only adjusted every 24 hours - this could have a significant effect on production. Indeed, citric acid production in a buffered medium (15 mM phosphate buffer) has been seen to cause changes in pH from 6.5 to 2.4 over a 24 hour period (McKay *et al.* 1994). Given the evidence for the effects of pH on the activity of citrate synthase, this would surely have some effect on citric acid production.

McKay *et al.* (1994) observed the production of mannitol during citric acid production. It is thought that this may be associated with internal pH regulation. It is known that the plasma membrane ATPase is involved in the regulation of internal pH. It has been shown that when yeast is grown in a medium in which a final external pH of less than 4 is attained, there is a 2- to 3-fold increase in plasma membrane ATPase activity (Jones and Gadd 1990). This acid-mediated activity is only found in late logarithmic or stationary phase cells, and only cells that are able to activate the membrane ATPase involved are able to maintain a constant internal pH in acidic conditions.

Therefore, it appears that the results of work performed on the effects of medium pH on enzymes of the citric acid cycle may be subject to question, as they may not take into account the ability of the yeast cell *in vivo* to control internal pH.

Oxygen Levels

It has been shown that highest levels of citric acid production were associated with a high rate of oxygen consumption (Moresi *et al.* 1980; Tani *et al.* 1990; Finogenova *et al.* 1991).

Moresi *et al.* (1980) reported that an air dilution rate of 0.4425 dm^3 (of air) $(kg.min)^{-1}$ (kg of fermentation medium per minute), in combination with pH

control, was optimal for citric acid production from *C. lipolytica* Cooper grown on *n*-alkanes. Rane and Sims (1994), obtained optimum citric acid production from *C. lipolytica* at a dissolved oxygen concentration of 80% of saturation. This was also found to be the case by Finogenova *et al.* (1991), who found that decreasing the dissolved oxygen content of the medium to 28 - 30% of saturation, caused a cessation in citric acid production.

Abou-Zeid *et al.* (1984) stated that about 1 g of oxygen is required for the production of 1 g of yeast dry matter. Under controlled conditions it is necessary to provide 0.75 vol. air/min to maximise the production of citric acid. If insufficient oxygen is provided, the rate of citric acid formation is low, while the biomass increases markedly. The oxygen level is critical when yeasts are grown on hydrocarbons. Briffaud and Engasser (1979a) found that during growth of *S. lipolytica* on glucose, respiration during citric acid excretion is a very demanding process that accounts for 60% of consumed oxygen and 35% of consumed glucose. Rane and Sims (1994) showed that oxygen uptake is highest in the logarithmic phase of growth and hence is associated with maximum cell growth. Uptake rates of oxygen were lower in the stationary or production phase.

This high oxygen consumption associated with citric acid production may be explained by the fact that production of citric acid takes place in mitochondria, which is also the site of oxidative phosphorylation, an oxygen-consuming energy production process. Ermakova *et al.* (1986) demonstrated that the production of citric acid was associated with increased levels of mitochondria in the yeast cell. This may also be related to the decrease in activity of NAD-ICDH, also associated with increased production of citric acid, in that the cell is compensating for the loss of energy production via the TCA cycle, by obtaining its energy by other means.

2.2.6 Productivity of Citric Acid Producing Yeasts

The amounts of citric acid obtained by authors varies considerably. This is due to a variety of factors which have been discussed earlier, such as the yeast strain, carbon source and pH. Many authors have reported levels of citric acid produced in terms of $g.L^{-1}$ (grams per litre of medium). These levels range from 5.8 - 200 $g.L^{-1}$ (For a summary, see Appendix 1: Table 1). This is not the ideal means of expressing the levels of citric acid produced as it does not take into account the biomass, nor the concentration of other variables such as carbon source.

Many authors, however, have taken into account the cell biomass when reporting their results. These are summarised in Table 2.1. Quite significant yields per gram of cells have been obtained, but as demonstrated in this work, much higher yields of citric acid are possible.

Table 2.2: Citric acid and isocitric acid production from yeasts (grams per gram of cells)

Organism	Substrate	Citric Acid $g.g^{-1}$	Isocitric Acid $g.g^{-1}$	Reference
S. lipolytica	Glucose	1.87	0.35	Finogenova *et al.* 1986
	Hexadecane	1.27	1.67	
S. lipolytica (Mutant 1)	Glucose	2.41	0.07	
	Hexadecane	3.33	0.19	
S. lipolytica (Mutant 2)	Glucose	2.15	1.19	
	Hexadecane	0.95	3.38	
C. zeylanoides	Glucose	3.19	0.41	Hattori *et al.* 1974
	n-alkane	4.01	3.61	
C. guilliermondii IMK1	Glucose	0.38	NA	Gutierrez *et al.* 1992
C. lipolytica	Glucose	0.71	0.05	Rane and Sims 1995
Y. lipolytica	Date-Coat sugar	0.48	NA	Abou-Zeid and Khoja 1993
C. tropicalis		0.46	NA	
C. utilis		0.38	NA	
C. guilliermondii		0.34	NA	
C. lipolytica	Glucose	2.1	0.4	Rane and Sims 1993
Y. lipolytica	Glucose	1.2	0.6	

Only a few authors have calculated specific rates of citric acid production and glucose consumption. Briffaud and Engasser (1979a, b), showed specific rates of citric acid production of 2.5 and 4.1 ($g.g^{-1}.h^{-1}$) and specific rates of glucose consumption of 3.4 and 5.5 ($g.g^{-1}.h^{-1}$). Marchal *et al.* (1977b) showed a rate of citric acid production of 0.058.

2.3 Yeast Mutation and Repair

Mutation of yeasts has been performed by various authors in an attempt to produce organisms that will give higher yields of citric acid.

The method of mutation used to select for better citric acid producers is generally one of the following :

(a) Ultraviolet light at 253 nm

(b) Chemical mutagens such as 4-nitroguinoline 1-oxide, *N* -methyl *N*-nitrosoguanidine, nitrous acid, ethylmethanesulphonate.

Little is known about the actual site of the lesion(s) which cause increased production of citric acid. Indeed, these may vary depending on the strain of yeast. In this study, the reversion or apparent repair of the mutant strain proved to be a major problem, one that perhaps could have been overcome if more were known about the mechanism and site of mutation.

Following is a review of information that is currently available regarding mutation and repair in yeasts: (From: Parry and Cox 1968; Haynes and Kunz 1981; Lawrence 1982; Game 1983; James and Nasim 1987)

2.3.1 Introduction

It is known that the primary lesions induced in DNA by radiation, or by other agents, are removed through the actions of naturally occurring repair mechanisms in yeasts. However, it has become evident that the molecular pathways of repair are exceedingly complex, and that these repair systems are not identical in different species of yeasts.

Much research has performed relating to the effects of radiation on yeasts and the elucidation of these repair systems. It has become evident that the repair processes do not only eliminate damage, but are also instrumental in the production of genetic modifications. Therefore, repair is sometimes responsible for the effects caused by radiation.

Previously, radiation effects such as cell inactivation, recombination and mutation have been considered to be independent subjects, and have been treated as such. However, it is now evident that such effects are often caused by the same initial DNA lesions, and that the end-point or fate of an irradiated cell depends on the course of subsequent events in the presence of repair processes. This means that the various effects of irradiation are inseparable, except at the level of ultimate expression - in this case, citric acid production.

Saccharomyces cerevisiae, as well as some pathogenic strains of *Candida* have been widely studied with regard to the effects of mutagens. Different species of yeast will act differently in response to UV radiation - enough so that even differentiation of species is possible. So, much of what is currently known about *Saccharomyces cerevisiae*, may not in fact apply to citric acid producers such as *Candida lipolytica* and *Candida guilliermondii*.

2.3.2 Repair

The existence of repair mechanisms became evident with the discovery that treatment with visible light could reactivate cells that had been treated with UV irradiation i.e. photoreactivation.

Photoreactivation is mediated by a single enzyme which specifically binds to the pyrimidine dimers in DNA and splits these *in situ* in the presence of visible light (310-400 nm), to restore DNA to its normal condition. A pyrimidine dimer occurs where adjacent pyrimidine residues on a DNA strand become covalently linked after UV irradiation. Replication and gene expression is then blocked unless the lesion is removed.

The results of incubating UV irradiated cells in the dark in non-growth conditions before plating, and the effects of visible light after such dark incubation suggested that a major repair process that operates without light in yeast acts on the same UV-induced DNA damage as the photoreactivation system. It was found that a gradual increase in survival occurs in wild-type yeast cells held in the dark for periods as long as several days before plating. So, the major dark repair system in yeast appears to operate on potentially photoreactivable lesions.

Pyrimidine dimers are the major souce of UV-induced lethality in yeast. One or two unrepaired dimers are lethal to a yeast cell, and all dimer repair in wild-type yeast in the dark can be accounted for by the three identified repair systems: excision, error-prone and recombinational repair. Excision repair appears to account for most dimer damage in the wild-type, since excision defective mutants tend on the whole to be more sensitive than those in the error-prone group, which in turn are more sensitive than those defective in the recombinational repair of UV-induced damage.

Pyrimidine dimers are of major importance with regard to the primary ultraviolet-induced lesions responsible for mutation. Mutant frequency declines with removal of dimers by post-treatment exposure to visible light. However, a fraction of mutations are not photoreversible and these may be attributable not only to dimers that escape the action of the photoreactivating enzyme, but also to lesions other than dimers.

The molecular changes responsible for UV-induced mutations do not always occur at the site of the primary lesion. i.e. are untargeted.

2.3.3 Inactivation

The most obvious immediate consequence of irradiation of yeast cells is a disturbance in the timing of cell division. It is also apparent that in many instances cell death is a long-delayed consequence of the inability of cells to reproduce.

Disruption of the cell-division process is just one example that can cause disturbances to cell function. Other transient morphological and physiological effects have been described, including cell enlargement, malformation, enzymic changes, cell-wall changes that cause a delay in the physical separation of a mother cell from its bud, and alterations in permeability. (Changes in permeability have been observed that result in a loss of potassium ions.) In particular, morphological changes, among other metabolic changes, have been observed with the mutant *Candida guilliermondii* IMK1 (Gutierrez *et al.* 1992).

The most dramatic consequence of irradiation is complete inactivation of cells. This can be exhibited in various ways:

- Some cells lyse almost immediately
- More frequently (at lower doses), cells divide once to produce a doublet
- On occasion, cell division is not halted until several progeny cells have been produced
- Lethal damage may be expressed as lethal sectoring, in which case inactivation is confined to a fraction of the mitotic progeny of an irradiated cell

The relative frequencies of these different expressions of death are dose-dependent.

With non-ionizing radiation, it is found that most mutations are base-pair substitutions, (small deletions and additions being less frequent). UV irradiation is apparently non-specific with regard to the base pair altered.

It is evident that the molecular mechanism(s) responsible for mutations in *S. cerevisiae* are not yet clear. The important finding that many mutations are untargeted suggests that cellular processes that normally insure fidelity of DNA replication are saturated in cells irradiated by UV radiation.

2.3.4 Modification of Effects of Radiation

Circumstances under which the response of an organism is modified can clarify the site of the lesion(s).

2.3.4.1 Post-irradiation Treatments

The most commonly used post-irradiation treatment that can modify radiation effects in yeasts, apart from treatment with visible light, is liquid holding. This treatment, which can alleviate radiation damage, consists of holding irradiated cells in a non-nutritive medium for an interval before plating. There is a progressive loss in photoreversibility during liquid holding after ultraviolet irradiation, an indication that dimers are being removed during this period.

This could have implications in the mutation selection process used to isolate a new citric acid-producing mutant in that the irradiated cells were held in sterile peptone water, a non-nutritive medium, in the dark, for a period of 20 minutes before plating.

2.3.4.2 Cell phase

Radiosensitivity of yeasts varies with cell stage. Studies carried out on *Saccharomyces cerevisiae* has shown that budding cells in a culture of a haploid strain are more resistant to ionizing radiation than are non-budding cells. This is also apparent after ultraviolet radiation.

2.3.4.3 Mitochondria

The mitochondrial system of *S. cerevisiae* has been studied extensively with regards to the effects of radiation. Mutations are mainly of two classes, one leading to drug resistance, the other to respiratory-deficient "petites" (designated σ^-). The former are a consequence of point mutations, and can be induced by ultraviolet radiation. Induction of σ^- mutants are a consequence of gross deletions and re-arrangements. These are also induced with a high frequency by ultraviolet radiation.

It has been suggested that there is a role for dimers in the induction of the σ^- mutation as there is evidence for photoreversibility. Liquid holding also results in a decrease in the frequency of σ^- mutants, which suggests the involvement of dark-repair processes.

2.3.5 Mutation and Citric Acid Producing Yeasts

Mutation techniques used by researchers to develop better producers of citric acid usually involve UV irradiation or chemical mutagens. These methods have resulted in a variety of effects, from changes in enzyme activity, to changes in transport across the plasma membrane of the cell.

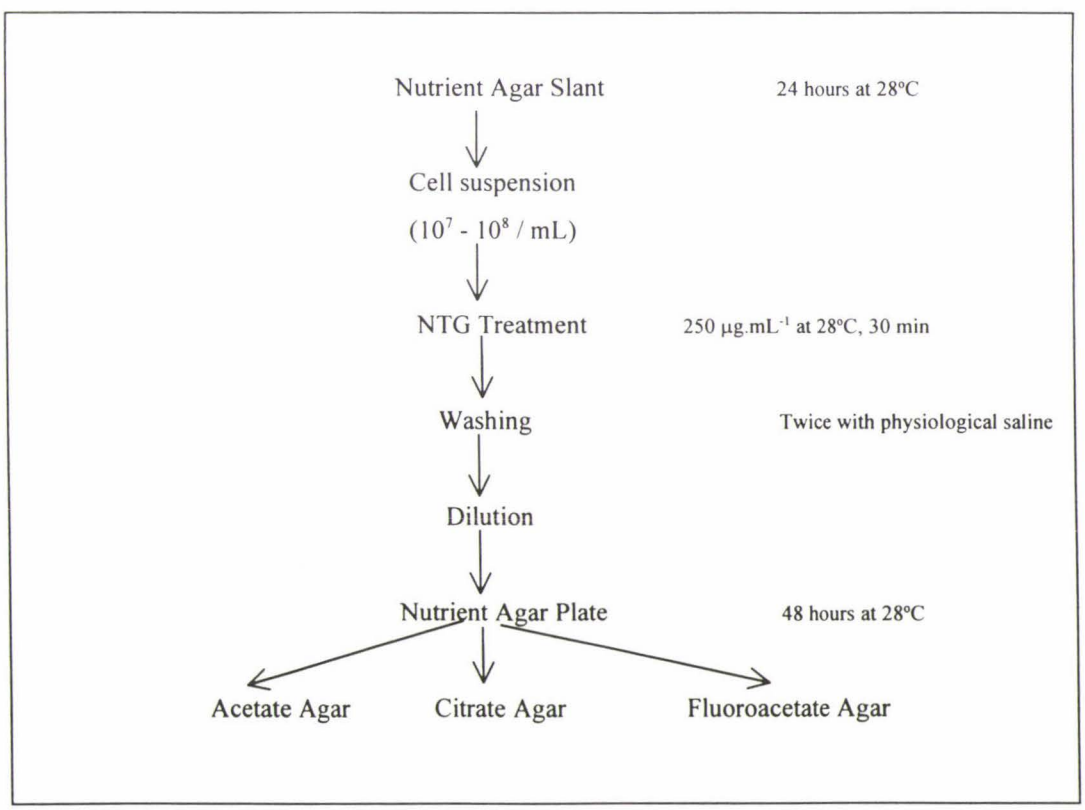
Most authors are in agreement that a decrease in aconitase activity is necessary to increase the ratio of citric acid to isocitric acid (Akiyama *et al.* 1973a, b; Furukawa *et al* 1982; Good *et al.* 1985; Ermakova *et al.* 1986; Finogenova *et al.* 1986; Tani *et al.* 1990). Agents such as the expensive chemical 2-picolinic acid and fluoroacetate (which is toxic), have been proven to reduce aconitase activity, but the addition of such chemicals is unacceptable for the production of food-grade citric acid (Good *et al.* 1985). It is therefore apparent that mutagenesis is the preferable option to induce metabolic controls that will provide the desired results.

In an attempt to produce a mutant that would show improved levels of citric acid production, Akiyama *et al.* (1973a) used the method shown in Figure 2.4.1 for the isolation of a fluoroacetate-sensitive citric acid-producing mutant which had low isocitrate production (due to low aconitase activity), and also showed poor growth with citric acid provided as the sole source of carbon. Conversely, Tani *et al.* (1990) developed a fluoroacetate-resistant mutant to give enhanced production of citric acid from methanol by *Candida* sp. Y-1. This mutant produced three more times citric acid than the parent, and also had lower aconitase activity.

In addition to changes in aconitase activity Ermakova *et al.* (1986) reported increases in the activity of citrate synthase in a citric acid-producing mutant. Activity of citrate synthase was found to be higher than the other enzymes of the

TCA cycle. This higher activity was explained by the fact that citric acid in mitochondria acts as a substrate for TCA cycle reactions and also as a substrate for transport of acetyl groups to the cytoplasm via the mitochondrial membrane. The mitochondrial membrane is impenetrable by acetyl-CoA and so higher activity of citrate synthase is required to supply these requirements. The observation was also made that the mitochondria of the cells involved in citric acid production had well-developed mitochondria.

Figure 2.3: Method for isolation of a fluoroacetate-sensitive, non-citrate metabolizing mutant.
(Akiyama *et al.* 1973a)



It is also considered desirable for a mutant to be unable to use citric acid as a sole source of carbon (Gutierrez *et al.* 1992; Akiyama *et al.* 1973b). This will result in an increase in the final yield of citric acid obtained, as citric acid is unable to be reutilised when the original carbon source has been depleted.

Gutierrez *et al.* (1992) found that mutant *C. guilliermondii* IMK1 was unable to use citric acid as the sole source of carbon. Although the mutant was able to utilise citric acid after depletion of glucose, it differed from the parent strain in that the parent was able to utilise both glucose and citric acid simultaneously. Two possible explanations were offered: (a) The lesion in the mutant was at the site of the enzyme(s) aconitase and/or ICDH, meaning citric acid is unable to be further metabolised and so is excreted from the cell, and (b) The lesion is at the level of citric acid transport into the cell, and so only the parent strain can reassimilate citric acid.

It was further implied that the lesion in IMK1 could be at the site of the plasma membrane ATPase, as the activity of this enzyme was found to be lower in the mutant. As the plasma membranae ATPase is involved in nutrient uptake into the cell, decreased activity of this enzyme would also help account for the lower growth rate and impaired citric acid uptake in IMK1.

In summary, it would appear that yeast mutations that result in increased citric acid production are likely to be the result of one or more of the following:

- Increased activity of citrate synthase
- Decreased activity of aconitase, so lowering the amount of isocitric acid in the acid mix
- Inability of the mutant to utilise citric acid as a sole source of carbon
- Inhibition of the enzyme NAD-dependent ICDH

So, for improved levels of citric acid production from yeasts, mutagenesis and development of the medium, as well as growth conditions are necessary.

CHAPTER 3

NUTRIENT LIMITATION EXPERIMENT MATERIALS AND METHODS

3.1 Introduction

In this experiment the rates of glucose utilisation were compared with the rates of citrate accumulation under different limitations to determine whether there was a link between the rates of glucose uptake and citrate accumulation. Other aspects of citrate acid production such as enzyme activity and levels of intermediate metabolites were also taken into consideration. The results might give an indication as to why citric acid accumulation was occurring and show optimum conditions for this accumulation.

3.2 Cultures - Origin and Maintenance

3.2.1 Yeast Strains

The organisms used were parent strain *Candida guilliermondii* NRRL Y-448, and mutant strain *Candida guilliermondii* IMK1. The mutant had been previously selected on the basis of increased levels of citric acid production following treatment with ultraviolet (UV) light (McKay *et al.* 1994; Gutierrez *et al.* 1992).

However, this mutant was later lost apparently due to reversion. Attempts to reisolate were not successful (See Chapters 4 and 7).

3.2.2 Maintenance

Cultures were maintained on Potato Dextrose Agar slants (Oxoid, Basingstoke England). However, it was discovered that the levels of citric acid produced by the mutant decreased steadily when the organism was subjected to repeated subculturing. Therefore, it was necessary to reisolate the organism on plates of Potato Dextrose Agar, with calcium carbonate added at 25 $g.L^{-1}$, and D-Glucose at 50 mM before each fermentation.

These plates were poured thinly, and an appropriate dilution of *C. guilliermondii* IMK1 was spread-plated onto the surface. These plates were then incubated at 30°C for up to 144 hours, after which colonies that produced the largest zones of clearing in the media were selected i.e. those that were the best acid producers.

3.3 Nutrient Limitation Experiment

3.3.1 Media Composition and Concentration

Following preliminary work by I.A. McKay using this organism (McKay *et al.* 1994), it was decided to use the limitations listed below:

No limitation	- as a control
Nitrogen limitation	- 10 mM NH_4Cl
Sulphur limitation	- 10 μM $MgSO_4$
Phosphorus limitation	- 0.15 mM KH_2PO_4
	- 0.23 mM KH_2PO_4
	- 0.30 mM KH_2PO_4

Potassium limitation - 0.04 mM KCl

- 0.10 mM KCl

Magnesium limitation - 15 μM $MgCl_2$

- 50 μM $MgCl_2$

Appendix 2: Table 1 gives the concentrations of stock solutions and amounts used. It also shows additional substrates used.

All solutions were made from chemicals of AnalR Grade and were obtained from BDH Chemicals (Poole England) and Sigma Chemical Co (St Louis, Missouri USA).

Flasks were prepared containing only glucose and distilled water and were autoclaved at 121°C for 15 minutes. The sterile solutions listed in Appendix 2: Table 1, were added at the quoted volumes prior to inoculation with the yeast culture. Those solutions unable to be autoclaved, such as the antibiotics and the Yeast Nitrogen Base (YNB), were filter-sterilised using 0.45 μm filters (Millipore).

3.3.2 Inoculation of Flasks

Yeast cells were pre-cultured in unlimited media. See Appendix 2: Table 1 for the medium composition. Cells in exponential phase were harvested and washed twice in 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES)-NaOH. 1 mL of resuspended cells was used to inoculate each flask. The final volume of each flask was 100 mL.

The flasks were then incubated at 30°C on an orbital shaker at approximately 160 rpm.

3.4 Analysis of Samples

3.4.1 Preparation of Sample for Further Analysis

Samples were taken every 24 hours, at which time the optical density (OD) and the pH were recorded and samples taken for further analysis.

The flask was well mixed before taking a sample of approximately 10 mL. This was then centrifuged at 180 rpm for 15 minutes. The supernatant fluid was then decanted into a suitably labelled bottle and frozen until required.

3.4.2 Optical Density

Optical density (OD) was read every 24 hours at 600 nm as a measure of cell growth, using a Hitachi U2000 Double Beam Spectrophotometer (Hitachi Ltd, Tokyo, Japan). The sample was diluted appropriately in water to obtain a value equal to, or below, an optical density of 0.30. This value was then multiplied by the dilution factor to give the correct value.

3.4.3 pH

The pH of each of the flasks was monitored, and adjusted if required, every 24 hours. This was to ensure that the pH was returned to approximately pH 6.0, to allow for further acid production. 10 M NaOH (AnalR BDH) was used to make these adjustments.

3.4.4 Dry Weight Determination

Dry weight or biomass determinations were made at the end of the fermentation i.e. at 144 hours. 10 mL of yeast suspension was dispensed into clean, pre-dried and weighed, 15 mL Corex centrifuge tubes (Corning Glass Works). This was then centrifuged at 180 rpm for 15 minutes, after which the supernatant fluid was decanted off. The pellet was resuspended using 10 mL of distilled water and then recentrifuged at 180 rpm for a further 15 minutes. The supernatant fluid was again decanted off, and the tubes were placed in a hot air oven at 120°C for 18 hours. After this time, the tubes were allowed to cool in a desiccator and then weighed. The difference in weight after drying was calculated and results were given in grams per litre of medium ($g.L^{-1}$).

At the same time as the sample for biomass determination was taken, another sample was taken, diluted serially and the OD read at 600 nm. By dividing the biomass by the same value as the sample was diluted for the OD, a series of results was obtained, enabling the development of a calibration curve for dry weight. This enabled the estimation of the dry weight at any part of subsequent fermentations by measuring the OD of the sample.

e.g.	At time *x*:	Optical density	= 16
		Biomass	= 20 $g.L^{-1}$
	Therefore:	If OD	= 16 ÷ 2 = 8
		Biomass	= 20 ÷ 2 = 10 $g.L^{-1}$

3.5 Analysis of Concentrations of Glucose and Citric Acid Cycle Metabolites in the Fermentation Medium

All enzymatic analyses were carried out using a Hitachi U2000 Double Beam Spectrophotometer (Hitachi Ltd, Tokyo, Japan), fitted with a six-cell positioner.

3.5.1 Glucose

Glucose was measured enzymatically using the method of Trinder (1969). The principle of this reaction is the oxidation of β-D-Glucose by glucose oxidase to D-glucono-δ lactone. The hydrogen peroxide produced in the glucose oxidase reaction is determined by means of phenol and 4-aminoantipyrine in the presence of peroxidase, yielding a coloured product. The amount of dye formed is a measure of the glucose concentration in the sample. The absorption of the dye is measured at 510 nm. The measured absorbance of the sample is compared with that of a glucose standard.

Concentration in the reaction mixture:

Glucose	Up to	0.18 mM
Phosphate		90.91 mM
NaN_3		1.36 mM
4-aminophenazone		0.70 mM
Phenol		10.0 mM
Glucose oxidase		19.1 $kU.L^{-1}$
Peroxidase		1.4 $kU.L^{-1}$

3.5.2 Citric Acid

Citric acid concentration was measured enzymatically according to the method of Mollering (1985). This assay operates on the principle that oxaloacetate and its decarboxylation product pyruvate are reduced in the presence of the enzymes malic dehydrogenase (MDH) (EC 1.1.1.37), and L-lactic dehydrogenase (LDH) (EC 1.1.1.27), by NADH to produce L-malate and L-lactate. The amounts of NADH oxidised are measured by the decrease in absorbance at 339 nm, and this is proportional and stoichiometric to the amount of citrate in the sample.

(a) Citrate $\leftrightarrow$ Oxaloacetate + Acetate *(Citrate lyase)*

(b) Oxaloacetate + NADH + H^+ $\rightarrow$ L-malate + NAD^+ *(MDH)*

(c) Oxaloacetate $\rightarrow$ Pyruvate + CO_2 *(Decarboxylase)*

(d) Pyruvate + NADH + H^+ $\rightarrow$ L-lactate + NAD^+ *(LDH)*

Concentration in the reaction mixture:

Citric Acid	Up to	130 µM
Glycylglycine		0.16 M
Zn^{2+}		0.19 mM
NADH		0.32 mM
Malate dehydrogenase		3.8 $kU.L^{-1}$
Lactic dehydrogenase		8.8 $kU.L^{-1}$

3.5.3 Isocitric Acid

Isocitric acid was measured enzymatically using the method of Beutler (1985). In this assay, D-isocitrate is oxidatively decarboxylated to 2-oxoglutarate by NADP in the presence of isocitrate dehydrogenase (ICDH) (EC 1.1.1.42). The amount of NADP formed is stoichiometric with the amount of D-isocitric acid. The increase of NADP is measured by a change in absorbance at 339 nm.

D-Isocitrate + $NADP^+$ $\rightarrow$ 2-Oxoglutarate + NADPH + CO_2 + H^+

(Isocitrate dehydrogenase)

Concentration in the reaction mixture:

Isocitric acid	Up to	170 µM
Imidazol		125 mM
EDTA		125 µM
Mn^{2+}		2.3 mM
NADP		358 µM
Isocitrate dehydrogenase		62 $U.L^{-1}$

3.5.4 Pyruvate

Pyruvate was measured enzymatically using the method of Lamprecht and Heinz (1985). In this reaction catalysed by LDH, the decrease in NADH concentration, measured by the change in absorbance at 339 nm, is proportional to the amount of pyruvate required.

Pyruvate + NADH + H^+ ↔ L-lactate + NAD^+

Concentration in the reaction mixture:

Pyruvate	Up to	0.2 mM
NADH		0.33 mM
Lactic dehydrogenase		2.12 $kU.L^{-1}$

3.5.5 2-Oxoglutarate

2-Oxoglutarate was measured enzymatically using the method of Burlina (1985a). In this reaction catalysed by glutamic dehydrogenase (EC 1.4.1.3) one mole of NADH is oxidised for each mole of 2-oxoglutarate. The decrease in NADH concentration, measured by a change in absorbance at 339 nm, is proportional to the amount of 2-oxoglutarate in the reaction mixture.

2-Oxoglutarate + NADH + NH_4^+ ↔ Glutamate + NAD^+ + H_2O

(Glutamate dehydrogenase)

Concentration in the reaction mixture:

2-Oxoglutarate	Up to	60 μM
NADH		0.1 mM
Glutamate dehydrogenase		1.8 $kU.L^{-1}$

3.5.6 Fumarate

Fumarate was measured enzymatically based on the method of Burlina (1985b). This reaction, catalysed by fumarase (EC 4.2.1.2) and MDH, gives an increase in concentration of reduced acetyl-pyridine-adenine nucleotide (NADPH), which is measured by an increase in absorbance at 339 nm. This increase is proportional to the amount of fumarate present.

(a) Fumarate + H_2O ↔ Malate *(Fumarase)*

(b) Malate + NAD + Hydrazine ↔ Oxaloacetate-hydrazone + NADH + H^+ *(Malate dehydrogenase)*

Concentration in the reaction mixture:

Fumarate	Up to	0.30 mM
Hydrazine buffer		0.084 M
EDTA		0.17 mM
NAD		6 mg
Fumarase		1 $U.mL^{-1}$
Malate dehydrogenase		1 $U.mL^{-1}$

3.5.7 Malate

Malate was measured enzymatically using the method of McKay *et al.* (1988). This reaction catalysed by MDH and aspartate aminotransferase (EC 2.6.1.1) measures the formation of NADH at 339 nm.

(a) L-malate + NAD^+ → Oxaloacetate + NADH + H^+

(Malate dehydrogenase)

(b) Oxaloacetate + L-glutamate → L-aspartate + 2-oxoglutarate

(Aspartate aminotransferase)

Concentration in the reaction mixture:

Malate	Up to 0.30 mM
L-glutamate	0.04 M
NAD	2 mg
Aspartate aminotransferase	20 $U.mL^{-1}$
Malate dehydrogenase	60 $U.mL^{-1}$

3.6 Analysis of Enzyme Activities of the Citric Acid Cycle

3.6.1 Fermentation and Sampling

It has been found by some authors that TCA cycle enzymes can show variation in activity between logarithmic and stationary phases of growth (Ermakova *et al.* 1986; Finogenova *et al.* 1986, 1991). Therefore it was decided to investigate enzyme activity for the parent and mutant strains in both phases of growth, to determine if there was a difference in activity which might elucidate the reasons for the accumulation of citric acid in *C. guilliermondii* IMK1. Table 3.6.1 shows the limitations selected for their citric acid producing ability (P-lim 0.15 mM, P-lim 0.30 mM and N-lim 10 mM) and times of sampling for enzymatic analysis. A non-citrate accumulating limitation (Mg-lim 15 μM) was also chosen as a comparison.

Table 3.1: Limitations Chosen for Enzymatic Analysis

Limitation	Strain	Logarithmic Phase	Stationary Phase
		Time (hours)	Time (hours)
P-lim 0.15 mM	Parent	48	120
	Mutant	72	120
P-lim 0.30 mM	Parent	48	120
	Mutant	72	120
N-lim 10 mM	Parent	48	120
	Mutant	72	120
Mg-lim 15 μM	Parent	48	120
	Mutant	72	120

The time of analysis was chosen according to the growth data obtained in the first experiment. The mutant was sampled later in the logarithmic phase of growth as

it was slower to grow, and generally had a shorter logarithmic phase of growth when compared to the parent.

3.6.2 Disruption of Cells and Sample Preparation

At the chosen time of sampling, 15 mL of the yeast suspension was centrifuged at 4°C and 6000 rpm, for 20 minutes. This first supernate was retained to determine the concentration of metabolites excreted into the medium. The pellet was resuspended in an extraction medium containing EDTA 5 mM (BDH), Tris 100 mM (Sigma) and Dithiothreitol 2 mM (Serva) at pH 8.0. This yeast suspension was then passed twice through a French Press pressure cell at 12 000 psi. This suspension was again centrifuged as above and the supernatant was used for the subsequent enzyme assays. As far as possible the temperature was kept at 4°C until assayed. Some of the supernatant was retained to be later analysed for levels of metabolites within the yeast cell.

3.6.3 Protein Analysis

Protein was analysed using the method of Lowry *et al.* (1951). This was performed using the cell-free extract as prepared above. After addition of the reagents, the samples were left at room temperature for 30 minutes to allow for colour development. The absorbance was then read at 720 nm. A standard curve was produced using Bovine Serum Albumin (Sigma) at 1.0 $mg.mL^{-1}$.

3.6.4. Citrate Synthase (EC 4.1.3.7)

This assay was based on that of Srere (1969). The assay measures the increase of absorbance of the reaction mixture by measuring the formation of CoA-SH using

Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic) acid or DNTB), to measure the appearance of thiol groups. The initial rate of increase of absorbance at 412 nm is proportional to citrate synthase concentration. One unit is the amount of enzyme catalysing the formation of 1 μM of the 2-nitrobenzoate anion per minute. Specific activity is expressed in Units (U) per mg of protein.

Acetyl-SCoA + Oxaloacetate ↔ Citric Acid + CoASH

3.6.5 Aconitase (EC 4.2.1.3)

This assay was based on that of Fansler and Lowenstein (1969). The reaction is started by the addition of isocitrate. One unit is the amount of enzyme which catalyses an increase in absorbance of 0.001 per minute at initial rates. Each 0.001 change in OD is equivalent to 2.82×10^{-4} μM of cis-aconitate per mL.

Citrate ↔ *Cis*-aconitate ↔ Isocitrate

3.6.6 NAD-Dependent Isocitrate Dehydrogenase (ICDH) (EC 1.1.1.41)

This assay was based on that of Plaut (1969). The reaction was started by the addition of isocitrate. The resultant increase in absorbance at 340 nm due to the formation of NADH was measured, and the initial slope used to calculate the enzyme activity using the equation below.

$$\text{L-isocitrate} + NAD^+ \rightarrow \text{2-oxoglutarate} + CO_2 + NADH + H^+$$

$$\text{Specific Activity} = \frac{\Delta A340 \times V}{t \times \Sigma \times d \times v \times \text{protein}} \qquad (\text{mol.mg}^{-1}.\text{min}^{-1})$$

Δ 340 nm / t	=	absorbance change per minute at 340 nm
V	=	total volume of cuvette
Σ	=	extinction coefficient of NADH at 340 nm ($0.631\ \text{mL}.\text{mol}^{-1}.\text{min}^{-1}$)
d	=	cuvette length (light path = 10 mm)
v	=	sample volume (mL)
protein	=	protein content of sample (mg.mL)

3.6.7 NADP-Specific Isocitrate Dehydrogenase (EC 1.1.1.42)

This assay was based on that of Cleland *et al.* (1969). This reaction measures activity based on the formation of NADPH, causing an increase in absorbance at 340 nm. Calculation of the specific activity uses the same equation as above.

L-isocitrate + $NADP^+$ → 2-oxoglutarate + CO_2 + NADPH + H^+

3.6.8 Pyruvate Carboxylase (EC 6.4.1.1)

This assay was based on that of Sumegi and Alkonyi (1983). The reaction is started by the addition of pyruvate. Activity is measured by the formation of NADH per minute, measured as an increase in the absorbance at 340 nm.

Pyruvate + NAD^+ + CoA-SH → Acetyl-S-CoA + CO_2 + NADH

CHAPTER 4

MUTANT REISOLATION

4.1 Introduction

Owing to the gradual loss of citric acid-producing activity of IMK1 over a period of time of approximately five months in both working and stock cultures, it was decided to perform mutagenesis in order to isolate another, if not the same, citric acid-producing yeast. If a different mutant was isolated i.e. that was still a citric acid producer but showed different characteristics to IMK1, it may help further elucidate the mechanism of citric acid accumulation and excretion in *Candida guilliermondii*.

4.1.2 U.V. Mutagenesis

Mutagenesis was carried out on the parent strain and the IMK1 revertant strain in an attempt to isolate another citric acid-producing yeast.

This was carried out as follows: The yeast culture to be irradiated was grown overnight in 10 mL of nutrient broth. A plate count was carried out on this culture to determine total numbers.

The overnight culture was centrifuged at 180 rpm and then washed three times using 0.05 M phosphate buffer at pH 5.5. The pellet was then resuspended in 10 mL of phosphate buffer prior to irradiation.

The resuspended cells were placed into a petri dish and irradiated by UV light at 254 nm, the light being at a distance of 30 cm from the plate. Samples were taken from the petri dish at 0, 1, 2, 4, and 6 minutes, appropriate dilutions were made and the sample was spread-plated onto PDA agar.

From this, a death curve was obtained and a time of irradiation was selected that gave a 2 - 3 log reduction in cell numbers. This was found to be between 2 - 4 minutes.

The above procedure was repeated for the sample. The cells were exposed to UV for 2 - 4 minutes. Samples were taken at 0.5 minute intervals and were then kept in the dark for at least 20 minutes to prevent photoreactivation. However, as it is apparent that there are also dark repair mechanisms that can operate in yeast cells (See Section 2.3.2), it is presumed that a period of 20 minutes dark-holding is insufficient for these repair mechanisms to manifest themselves. Although it has also been shown that damage to the cell resulting in mutation may result in cell death in subsequent generations.

Samples were then diluted appropriately and spread-plated onto plates of PDA + $CaCO_3$. These were incubated at 30 °C and monitored daily for isolated colonies that produced zones of clearing the fastest and also the largest zones.

These isolated colonies were restreaked onto PDA (+ $CaCO_3$) agar before each experiment.

However, problems were experienced in attempting to isolate another citric acid-producing mutant. Colonies were obtained that produced acid, causing zones of clearing in the agar, but upon assessment in shake flasks containing unlimited media which previously produced levels of citric acid of the region of 2.0 $g.L^{-1}$ this

was found not to be citric acid. It is speculated that this acid may have in fact been isocitric acid, but this was not tested for.

It is not known why this attempt at the reisolation of a citric acid-producing mutant failed. The mutation process was repeated many times, and hundreds of plates used in an attempt to find a significant acid producer - each time unsuccessful.

CHAPTER 5

NUTRIENT LIMITATION EXPERIMENT RESULTS AND DISCUSSION

5.1 Introduction

The parent and mutant strains were grown under various nutrient limitations for a period of 144 hours as stated in Chapter 3: Materials and Methods. Samples (10 mL), were taken and the pH was measured and adjusted, if necessary, every 24 hours. These samples were then assessed to measure glucose consumption and citric acid production by each strain under the various nutrient limitations. Biomass production under each nutrient limitation was ascertained at the completion of the fermentation. At the same time, determinations of levels of selected intermediates of the citric acid cycle excreted into the fermentation medium were made.

Generally, the results obtained were as follows: The mutant produced higher levels of citric acid than the parent but also consumed less glucose, and hence produced less biomass (See Table 5.1). Of the nutrient limitations assessed in this experiment, it was the phosphorus-limited mutant cultures that produced the highest levels of citric acid (See Appendix 3: Table 6). Citric acid production from the magnesium- and potassium-limited cultures was very low in comparison.

Levels of isocitric acid excreted into the medium by the mutant also increased when compared with the parent, as did pyruvate. Quantities of other intermediates excreted into the medium varied between the parent and mutant strains, but generally were at low concentrations in both.

5.2 Optical Density (OD)

Optical densities were recorded as a measure of cell growth over a period of 144 hours (See Chapter 3: Materials and Methods). The results can be seen in Appendix 3: Tables 1 and 2, and in Figures 5.1 and 5.2.

Generally, the optical densities for the parent strain were higher than for the mutant. When related to glucose consumption (See Appendix 3: Table 6), this suggested that the parent used much of the glucose consumed for cell biomass production and not for the production of citric acid. This was also reflected in the data obtained from the biomass estimations taken at the end of the fermentation (See Table 5.1).

While assessing the growth of the parent strain when grown under the different nutrient limitations, it was observed that the nitrogen- and sulphur-limited cultures produced a higher optical density after 144 hours than the unlimited strain. These results were also reflected in the biomass results (See Table 5.1). This was unusual, in that it would be expected that a culture grown under conditions where all nutrients were provided in sufficient quantities would produce a higher optical density than a culture where nutrients were limited.

The phosphorus-limited cultures also showed an interesting trend in that the least-limited culture (0.30 mM) showed the lowest optical density after 144 hours when compared with the more limited 0.15 mM and 0.23 mM cultures. Again, this was an unexpected result. i.e. the less limited a culture, the more nutrients are available for growth, and so it would be expected to have a higher optical density than a more limited culture.

The magnesium-limited cultures showed the lowest gain in optical density after 144 hours. It was apparent that the absence of adequate levels of magnesium has

Figure 5.1: Optical Density at A600 nm of parent strain *Candida guilliermondii* NRRL Y-448 at 24 hour intervals

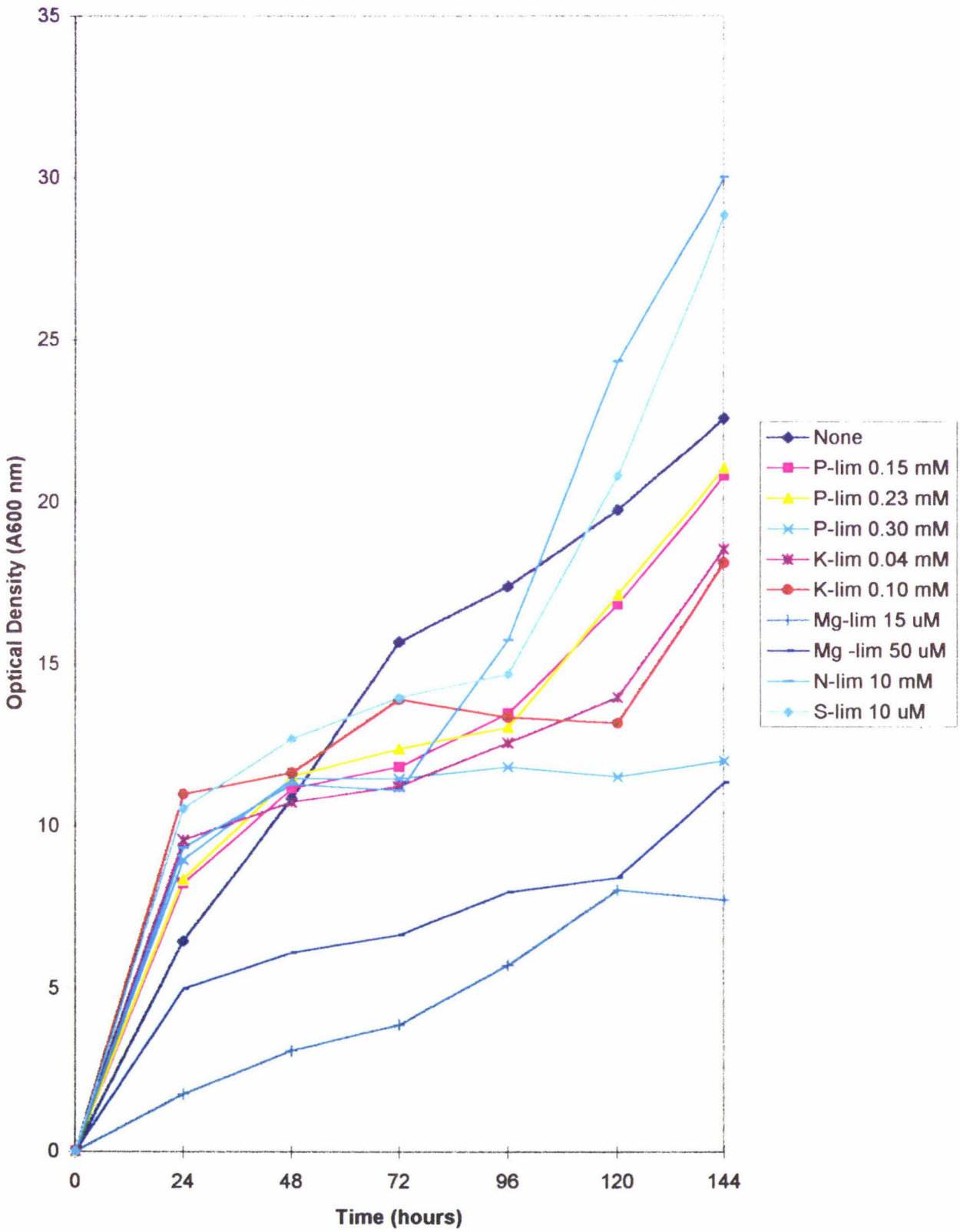
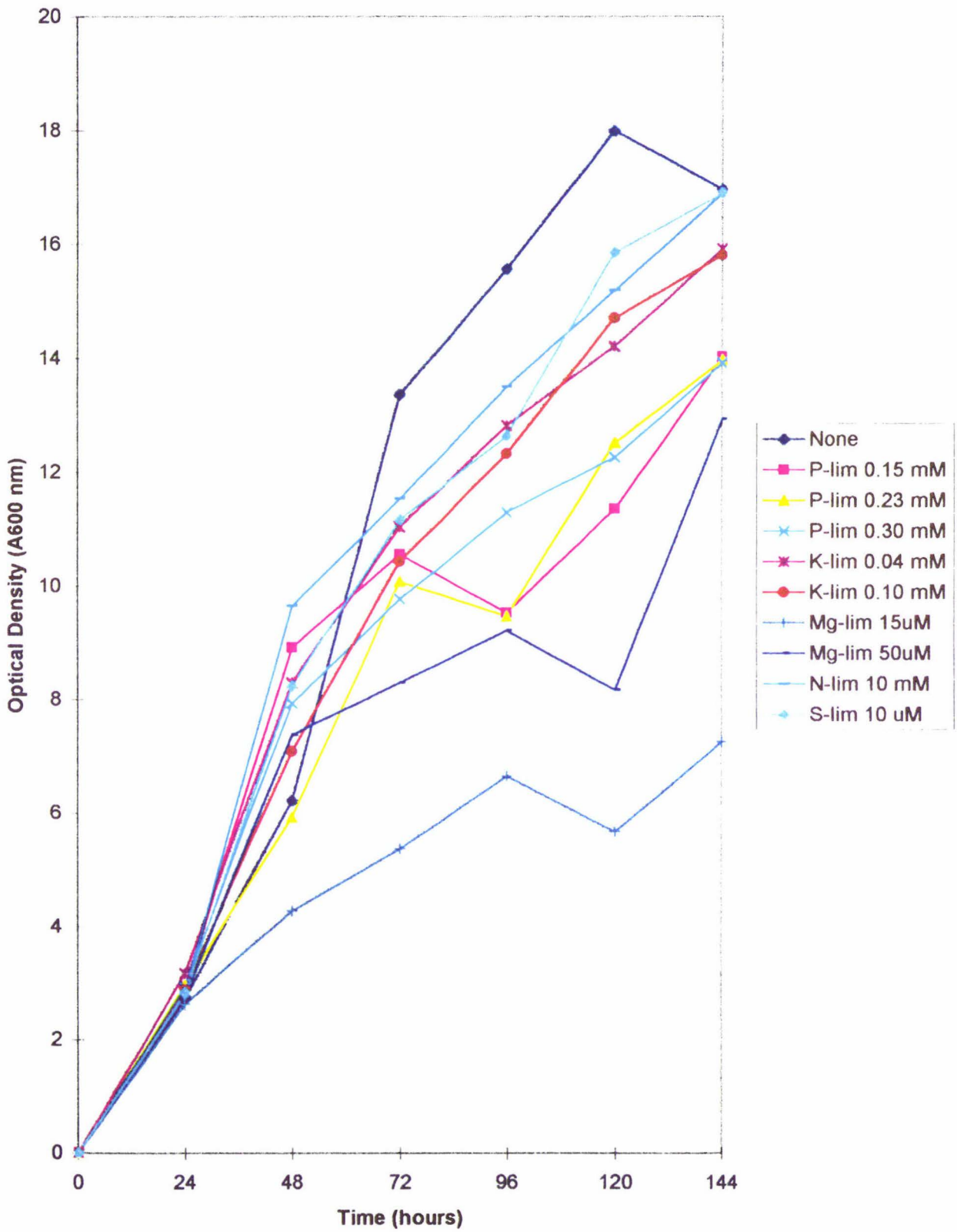


Figure 5.2: Optical Density at A600 nm of mutant strain *Candida guilliermondii* IMK1



for some reason severely impaired growth. This may be related to the requirement of the plasma membrane ATPase for Mg^{2+} as discussed later (See Section 5.5.1).

The initial increase in optical density (over the first 24 hours) in the mutant strain was slower, and the optical densities obtained after 144 hours were lower than the parent strain. Most limited cultures achieved optical densities that were only approximately half that of the parent.

Limited cultures that did not produce citric acid achieved a higher optical density than the citric acid-producing phosphorus limited cultures. This observation was also reflected in the biomass results (See Section 5.3).

Again, the magnesium-limited cultures showed little increase in optical density, levels in the mutant being very similar to those obtained in the parent.

5.3 Changes in pH

The pH of the cultures was adjusted every 24 hours with 10M NaOH (See Chapter 3: Materials and Methods). These values can be seen in Appendix 3: Tables 3 and 4.

The parent strain in many cases showed increases in pH rather than decreases (decreases being associated with the production of citric acid), over the period of fermentation. This indicated that very little citric acid, if any was being produced, and indeed the excretion of some other undetected metabolite is suggested by the slight increase in alkalinity of the culture.

The mutant strain showed significant reductions in pH over a 24 hour period under the citric acid-producing phosphorus limited cultures (See Appendix 3:Tables 3 and 4). The greatest change in pH was seen over the first 48 hours, indicating that acid production commenced in the logarithmic phase of growth, and not at the onset of nutrient limitation as has been stated to be the case by many researchers (Rane and Sims 1993; Marchal *et al.* 1977a; Mitsushima *et al.* 1978). These large reductions in pH have been seen previously by McKay *et al.* (1994) with *Candida lipolytica* IMK2 grown in media containing 15 mM phosphate buffer.

It has been shown by Hattori *et al.* (1974), that the optimum pH for the activity of citrate synthase is 6.4, also that the medium pH can affect the intracellular pH. Therefore it is possible that in the mutant strain the activity of citrate synthase, and hence the levels of citric acid produced may have been affected by the large fluctuations in pH over each 24 hour period. This would occur only if the yeast cell were incapable of maintaining its intracellular pH (Jones and Gadd 1990), which may be the case under conditions of nutrient limitation. It would require the more controlled conditions of a chemostat system to determine whether or not this is the case.

5.4 Biomass Production

Biomass levels were measured as stated in Chapter 3 - Materials and Methods. Results can be seen in Table 5.1.

For the parent strain, the biomass measurements obtained reflect those previously observed in the measurement of optical density. The nitrogen- and sulphur-limited cultures produced the highest levels of biomass, followed by the unlimited

culture. Again, as seen with optical density, the magnesium-limited culture was unable to produce as much biomass as the other limitations, the more limited 15 μM culture being the worst affected.

As seen with the optical density measurements, there appears to be a movement against the trend with the phosphorus-limited cultures. The less-limited 0.30 mM culture showed lower levels of biomass when compared with the more limited cultures, even though it consumed more glucose (See Appendix 3: Table 6). This particular limitation was also shown to produce high levels of citric acid compared with the other nutrient limitations (See Appendix 3: Table 6). This may account for the lower levels of biomass produced, in that glucose is being channelled into the production of citric acid, rather than through the remainder of the TCA cycle that ultimately results in the production of cell biomass.

For the mutant strain, biomass levels for the nitrogen-, sulphur-limited and unlimited cultures were shown to be at a similar level, although these were much lower per litre of medium than for the parent strain (See Table 5.1).

The magnesium-limited cultures again showed low levels of biomass production, especially the 15 μM culture. The less-limited 50 μM culture produced levels that were comparable to those obtained by other limitations.

Table 5.1: Comparison of Biomass (Dry Weights) of parent strain *Candida guilliermondii* NRRL Y-448 and mutant strain *Candida guilliermondii* IMK1 after 144 hours

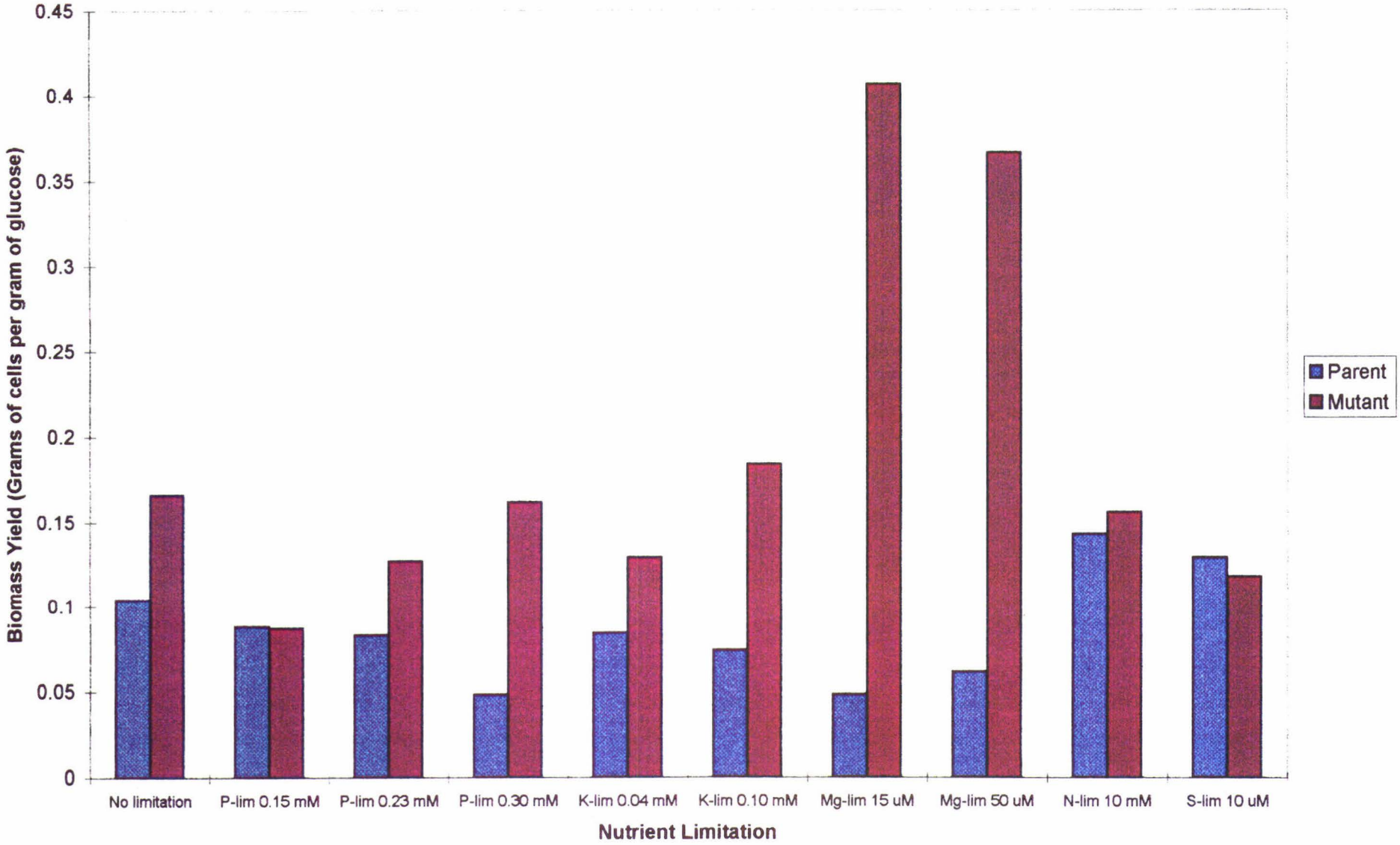
Limitation	Mutant ($g.L^{-1}$)	Parent ($g.L^{-1}$)
No limitation	3.41	6.40
P- limitation: 0.15 mM	2.80	5.96
P-limitation: 0.23 mM	3.11	6.31
P-limitation: 0.30 mM	2.82	3.81
K-limitation: 0.04 mM	3.49	5.69
K-limitation: 0.10 mM	3.39	5.47
Mg-limitation: 15 µM	1.04	2.75
Mg-limitation: 50 µM	3.00	4.60
N-limitation: 10 mM	3.83	9.55
S-limitation: 10 µM	3.82	9.82

5.5 Biomass Yield

The biomass yield was also assessed for each nutrient limitation. This shows the amount of cell biomass (grams) produced from each gram of glucose utilised by the yeast. Results can be seen in Figure 5.3 and in Appendix 3: Table 7.

The parent strain exhibited similar trends to those seen with the results obtained for optical density. The nitrogen- and sulphur-limited cultures showed the highest yields, followed by the unlimited culture. This again is unusual, in that it would be expected that the unlimited culture would produce the higher biomass. However, the unlimited culture was seen to produce low levels of citric acid, which may account for the loss of carbon. The phosphorus-limited cultures showed an interesting trend in that the less limited the culture, the less biomass per gram of glucose was produced - although these differences were not great.

Figure 5.3: Biomass Yield (Grams of Cells per Gram of Glucose) of Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1



Again, the magnesium 15 μM culture showed the lowest cell biomass, but when the amount of glucose that was consumed by this limitation was taken into account, it became apparent that this particular limitation was somewhat more efficient in the conversion of glucose into biomass than was expected. The biomass yield for this limitation being very similar to those obtained for the other nutrient limitations.

In the mutant strain, when glucose consumption was related to biomass production (Biomass Yield - grams of cells per gram of glucose. See Figure 5.3 and Appendix 3: Table 7), some notable differences to the observations that were made previously on the parent strain became apparent:

The magnesium-limited cultures proved to be the most efficient limitation for the conversion of glucose to cell biomass, even though the amount of glucose consumed was very low.

The phosphorus-limited cultures showed a trend in that the less-limited the culture, the better the biomass yield. The 0.15 mM culture also had the lowest yield of all mutant cultures grown under limitation. This was interesting in that it was this limitation which was later shown to produce the highest levels of citric acid (See Section 5.5.2), showing that glucose consumed was being channelled into citric acid production under this limitation, rather than the production of cell biomass. The phosphorus-limited (0.15 mM) culture was also shown to consume the highest levels of glucose when compared with growth under the other nutrient limitations.

5.6 Glucose Consumption and Citric Acid Production

5.6.1 Glucose Consumption

Glucose consumption was measured enzymatically as stated in Chapter 3: Materials and Methods. Results can be seen in Appendix 3: Table 6.

Average utilisation of glucose by all parent strain limitations was 95%. The parent strain was found to utilise 99% of available glucose in the phosphorus-limited cultures, whereas the magnesium-limited 50 μM culture showed levels of utilisation of around 86% of available glucose. The 15 μM magnesium-limited culture showed even lower levels of utilisation.

In the limitations involving the mutant, the citric acid-producing phosphorus-limited 0.15 mM culture showed greatest utilisation of glucose. However, this was still only at a level of 45% of available glucose - much lower than that of the parent.

The phosphorus-limited cultures also showed a trend in that the less-limited the culture, the lower the level of glucose utilisation. This again corresponds to the optical density and biomass results, but is unexpected.

5.6.2 Citric Acid Production

Citric acid production was assessed enzymatically as stated in Materials and Methods. Results can be seen in Figure 5.4 and Appendix 3: Tables 6 and 8.

In the parent strain the citrate yield (grams of citrate produced per gram of cells), was highest in the phosphorus-limited 0.23 and 0.30 mM cultures respectively, followed by the unlimited culture. The phosphorus-limited 0.15 mM culture, which in the mutant produced the highest levels of citric acid, in the parent produced levels so low as to be negligible (See Figure 5.4).

In the mutant strain, the phosphorus-limited cultures showed the highest levels of production per gram of cells, followed by the unlimited and nitrogen-limited cultures. The phosphorus-limited cultures showed a trend in that the most limited culture (0.15 mM) showed the highest production, and the least-limited (0.30mM) showed the lowest levels of production of citric acid.

All limited mutant cultures showed an increase in the amount of citric acid produced when compared with the parent strain. The lowest increase of 3.5-fold was seen with the sulphur-limited culture. The low production of citric acid under this limitation was unexpected as sulphur-limitation was previously shown by McKay *et al.* (1994) to produce significant levels of citric acid in a different mutant, *Y. lipolytica* IMK2.

It must be mentioned that the levels of citric acid produced by IMK1 are much higher than those achieved by many authors (See Appendix 1: Table 1). Even the unlimited parent culture produced levels of citric acid that are comparable to those produced by other workers under nitrogen-limitation. It was hoped that the comparison of IMK1 with the parent culture would identify the factors that led to the increased production of citric acid from the mutant, and might help to clarify further the controls involved in the overproduction of citric acid in yeasts.

Figure 5.4: Citrate yield (Grams of Citrate per Gram of Cells) for Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1

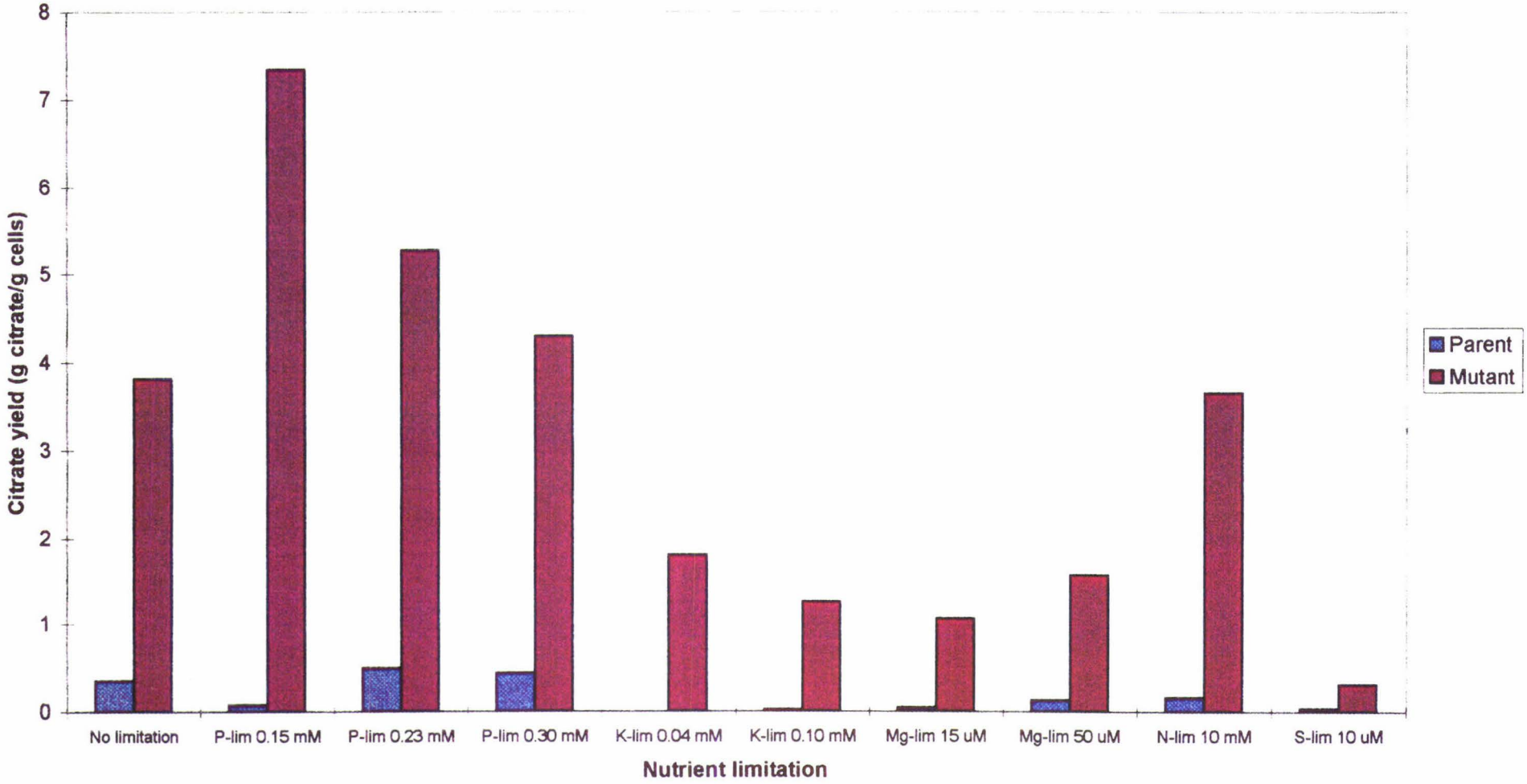


Table 5.2: Comparison of levels of glucose consumed and citric acid produced for parent strain *Candida guilliermondii* NRRL Y-448 and mutant strain *Candida guilliermondii* IMK1 after 144 hours.

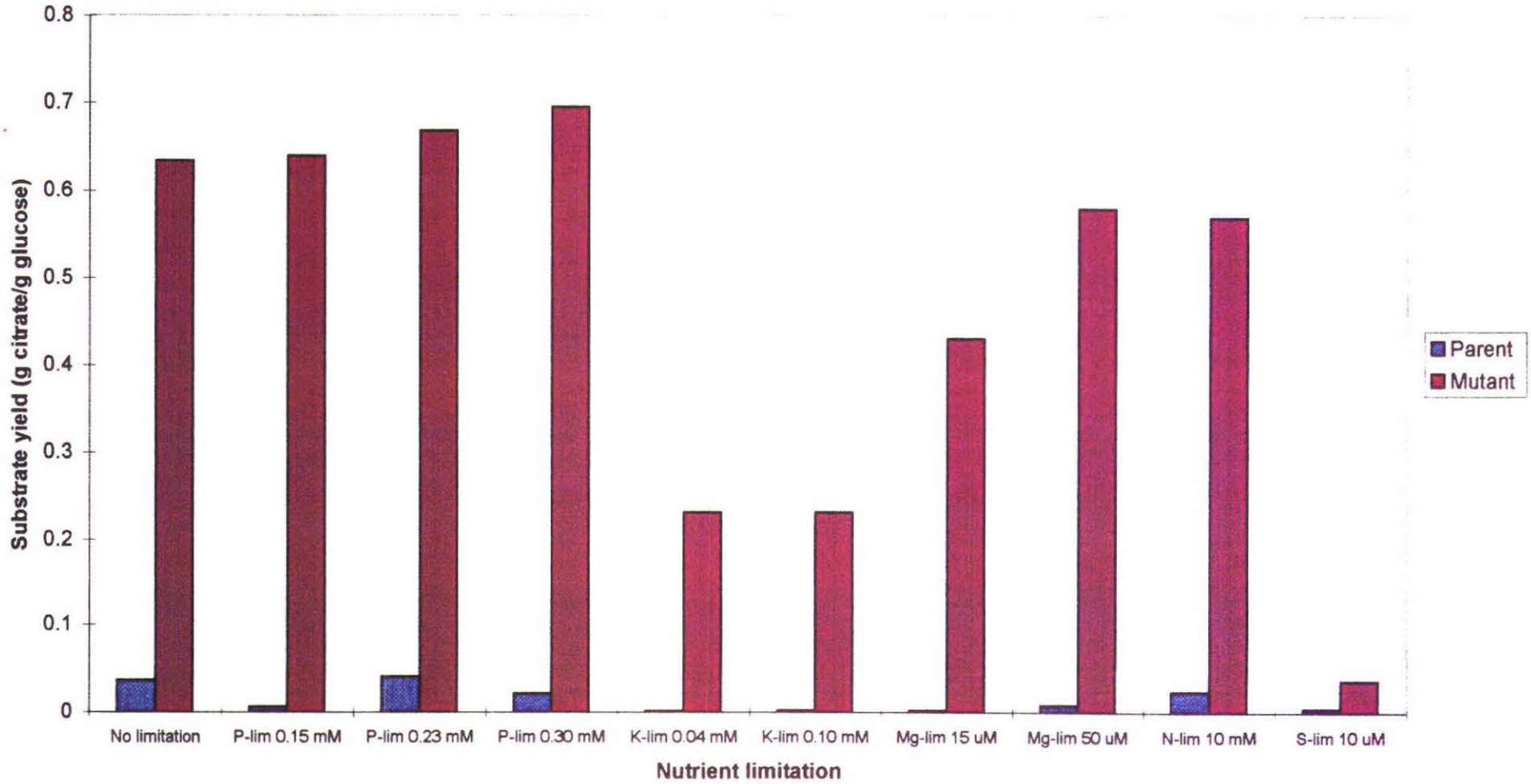
Limitation	Glucose consumption ($g.L^{-1}$)		Citric acid production ($g.L^{-1}$)	
	Mutant	Parent	Mutant	Parent
No limitation	20.49	61.28	12.98	2.30
P-lim 0.15 mM	32.15	67.63	20.55	0.42
P-lim 0.23 mM	24.58	75.88	16.41	3.07
P-lim 0.30 mM	17.42	78.62	12.11	1.69
K-lim 0.04 mM	27.14	67.79	6.30	0.007
K-lim 0.10 mM	18.44	73.57	4.28	0.13
Mg-lim 15 μM	2.56	57.87	1.10	0.11
Mg-lim 50 μM	8.19	75.33	4.75	0.59
N-lim 10 mM	24.58	66.99	13.99	1.56
S-lim 10 μM	32.27	76.36	1.17	0.33

5.6.3 Substrate Yield

It is the substrate yield (grams of citrate per gram of glucose consumed), that provides more precise information as to the most efficient producers of citric acid (See Figure 5.5 and Appendix 3: Table 9).

As expected, the substrate yields obtained from the parent were very low due to the high level of glucose consumption. In the mutant cultures, a trend previously observed with the citrate yields (grams of citrate produced per gram of cells), obtained from the phosphorus limitations was reversed. Previously, the 0.15 mM culture was shown to have the better yield of citric acid per gram of cells. However the 0.30 mM culture was shown to be the most efficient (marginally) at

Figure 5.5: Substrate yield (Grams of Citrate per Gram of glucose) for Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1



the conversion of glucose to citric acid, as well as the conversion of glucose into cell biomass.

Surprisingly, the magnesium cultures also show a high substrate yield, producing significant amounts of citric acid per gram of glucose consumed. But this limitation also produced the best biomass yield (grams of cells per gram of glucose). It appears that it is the low levels of glucose consumed that render this limitation unable to produce the levels of citric acid that are seen with the phosphorus-limited cultures. Therefore, it appears that the amount of glucose taken up is a critical factor in the production of citric acid in the magnesium-limited cultures, and possibly in the other nutrient limitations.

5.7 Specific Rates of Glucose Consumption and Citric Acid Production

Maximum specific rates of glucose consumption and citric acid production (grams per gram of cells per hour), were calculated to determine if the rate of glucose consumption was a controlling factor in the production of citric acid. The results can be seen in Figures 5.6 and 5.7, and Appendix 3: Tables 10 and 11.

5.7.1 Glucose Consumption

In the parent strain, the maximum specific rates of glucose utilisation were found to be highest in the magnesium-limited cultures at 72 hours, followed by the phosphorus-limited 0.15 mM and 0.23 mM cultures.

Figure 5.6: Maximum Specific Rates of Glucose Utilisation for Parent *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1

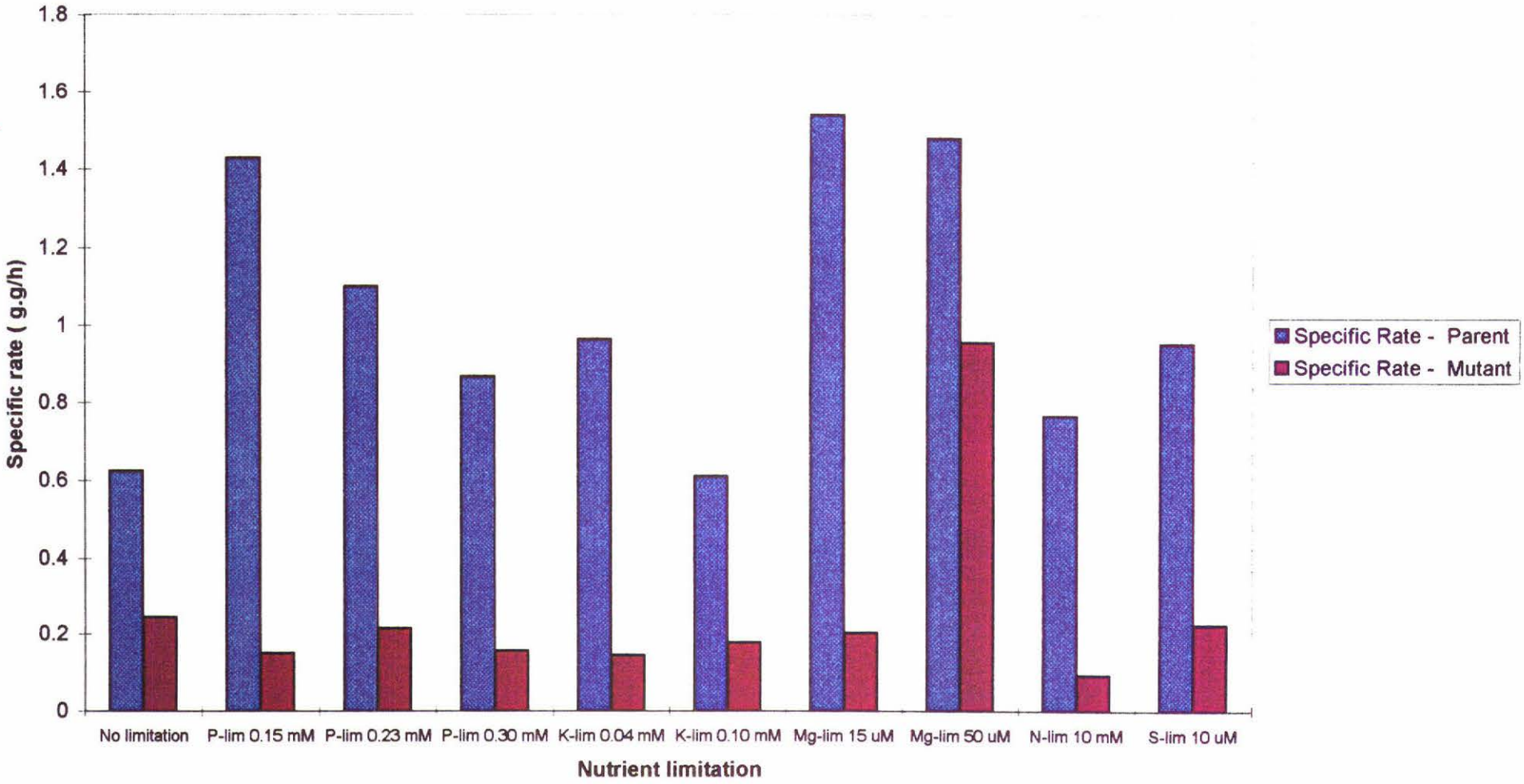
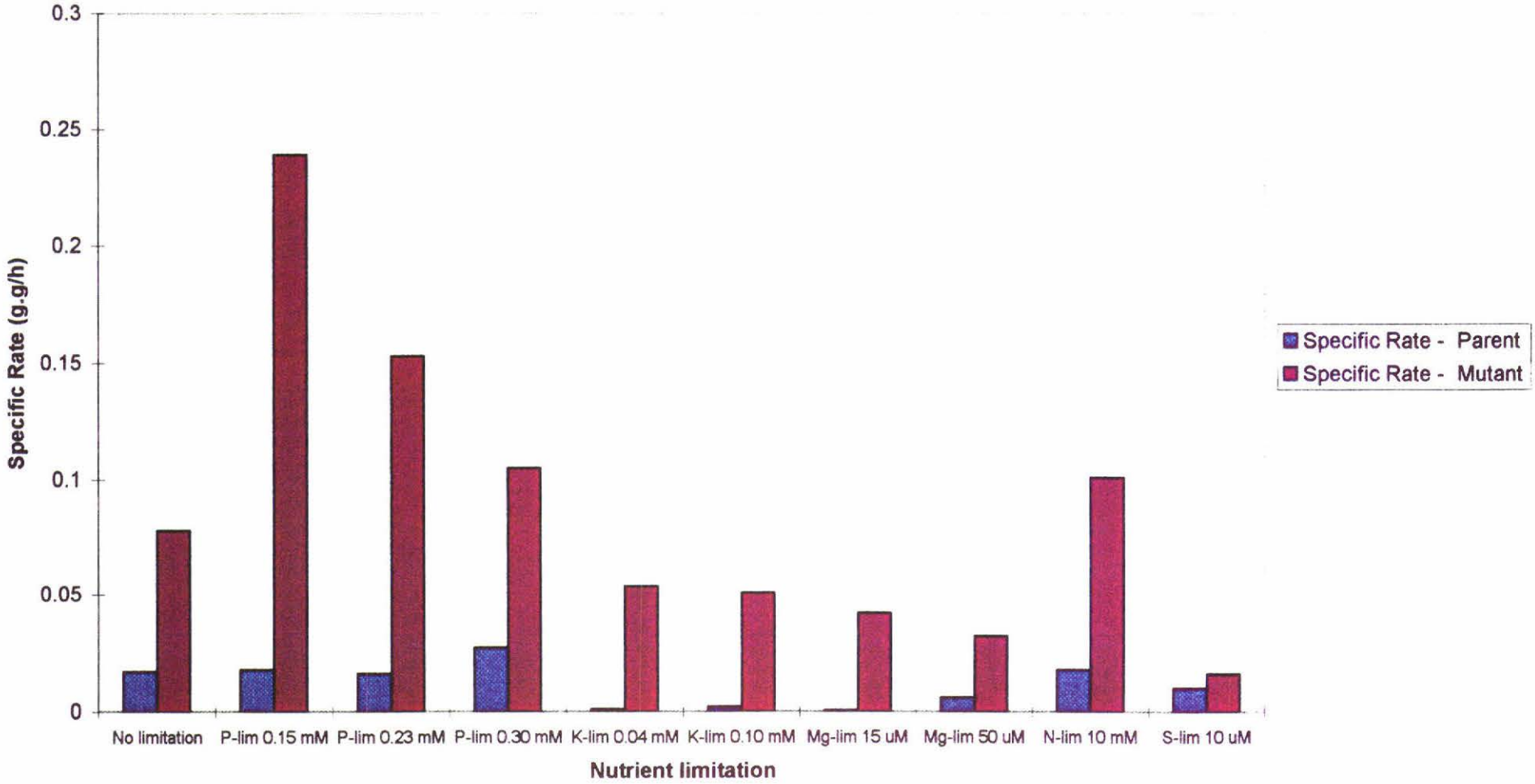


Figure 5.7: Maximum Specific Rates of Citric Acid Production for Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1



The mutant strain showed maximum specific rates of glucose utilisation that were noticeably lower than those observed for the parent, except in the magnesium-limited (50 μM) culture.

Although the magnesium-limited cultures showed high rates of utilisation early in the fermentation, it appears that it was the amount (grams) of glucose consumed that was the limiting factor for citric acid production from these cultures.

5.7.2 Citric Acid Production

Overall, the maximum specific rates of citric acid production were much higher in the mutant strain - especially in the phosphorus-limited cultures. The phosphorus-limited 0.15 mM culture showed the highest rate of production followed by the other less-limited phosphorus limitations. This trend with the more-limited cultures also showing the highest rates of production was also seen with the potassium- and magnesium-limited cultures, although not to the same extent. Again the unlimited culture showed rates of production similar to the nitrogen-limited culture.

Overall, the 0.15 mM phosphorus mutant culture was the best citric acid-producing system. This limitation showed the highest rate of citric acid production, the highest level of glucose consumption, but produced less biomass. The extra glucose taken up by this limitation has been converted into citric acid, as seen by the rates of citric acid production and the levels of citric acid produced. The mutant, however, seemed to be incapable of utilising all of the carbon available in the medium – a problem that was not encountered by the parent strain.

5.8 Ratio of Citric Acid to Isocitric Acid

The ratio of citric acid to isocitric acid was calculated using data obtained at the completion of the fermentation. Quantities of isocitric acid excreted into the medium after 144 hours can be seen in Figure 5.8 and Appendix 3: Table 12. Ratios can be seen in Table 5.3.

In the parent strain, the most desirable ratio - being that with a higher proportion of citric acid and low levels of isocitric acid - was seen in the phosphorus-limited 0.23 mM culture (See Table 5.3). The unlimited and nitrogen-limited cultures also produced favourable ratios.

High levels of isocitric acid in the total acid mix were seen in the potassium-limited 0.04 mM culture giving a ratio of 0.05:1. The potassium-limited 0.10 mM and magnesium-limited 15 μM cultures showed ratios that were close to 1:1.

In the mutant strain most ratios changed considerably, in that much more isocitric acid was produced than in the parent. The best ratio was seen in the phosphorus-limited 0.15 mM culture (3:1). This limitation also showed the highest level of combined citric and isocitric acids.

Some notable changes in ratio were observed when the parent and mutant strains were compared. In the parent, the potassium-limited (0.04 mM) culture showed a ratio of 0.05:1 (CA:ICA), whereas in the mutant the ratio had improved to 1.3:1. In the sulphur-limited culture the ratio moved in favour of isocitric acid, from 3.4:1 to 0.3:1.

Although the levels of citric acid produced by the mutant increased when compared to the parent, so did the levels of isocitric acid. This suggests that

further improvement of the citric acid yield may be obtained through use of an aconitase inhibitor, to prevent or reduce isocitric acid formation.

It is suggested that the increase in concentration of citric and isocitric acid in the mutant was due to inhibition of the enzyme NAD-ICDH. This inhibition is speculated to be a result of increased concentrations of ATP in the cell causing the resultant accumulation of the citric and isocitric acid.

Table 5.3: Comparison of Ratios of Citric Acid to Isocitric Acid (Grams per gram of cells) for parent strain *Candida guilliermondii* NRRL Y-448 and mutant strain *Candida guilliermondii* IMK1.

Limitation	Parent		Mutant	
	Total acid $g.g^{-1}$	Ratio CA:ICA	Total Acid $g.g^{-1}$	Ratio CA:ICA
No limitation	0.38	11.5:1	5.75	1.9:1
P-lim 0.15 mM	0.087	4:1	9.77	3:1
P-lim 0.23 mM	0.506	32:1	7.53	2.3:1
P-lim 0.30 mM	0.758	1.4:1	6.67	1.8:1
K-lim 0.04 mM	0.019	0.05:1	3.23	1.3:1
K-lim 0.10 mM	0.038	1.1:1	2.33	1.2:1
Mg-lim 15 µM	0.076	1.1:1	1.82	1.4:1
Mg-lim 50 µM	0.152	6.1:1	2.08	3.2:1
N-lim 10 mM	0.170	15.7:1	5.49	2.0:1
S-lim 10 µM	0.044	3.4:1	1.45	0.3:1

Ratio calculated on a basis of 1 g of isocitric acid to *x* grams of citric acid per gram of cells.
Total acid = Citric acid + Isocitric acid

5.9 Levels of Intermediates Excreted Into the Medium

Levels of some intermediates of the citric acid cycle excreted into the medium were assessed enzymatically as stated in Chapter 3: Materials and Methods. Data correspond to the amount of metabolite excreted into the medium after 144 hours. Results can be seen in Figures 5.8 - 5.12 and in Appendix 3: Table 12.

5.9.1 Isocitric Acid

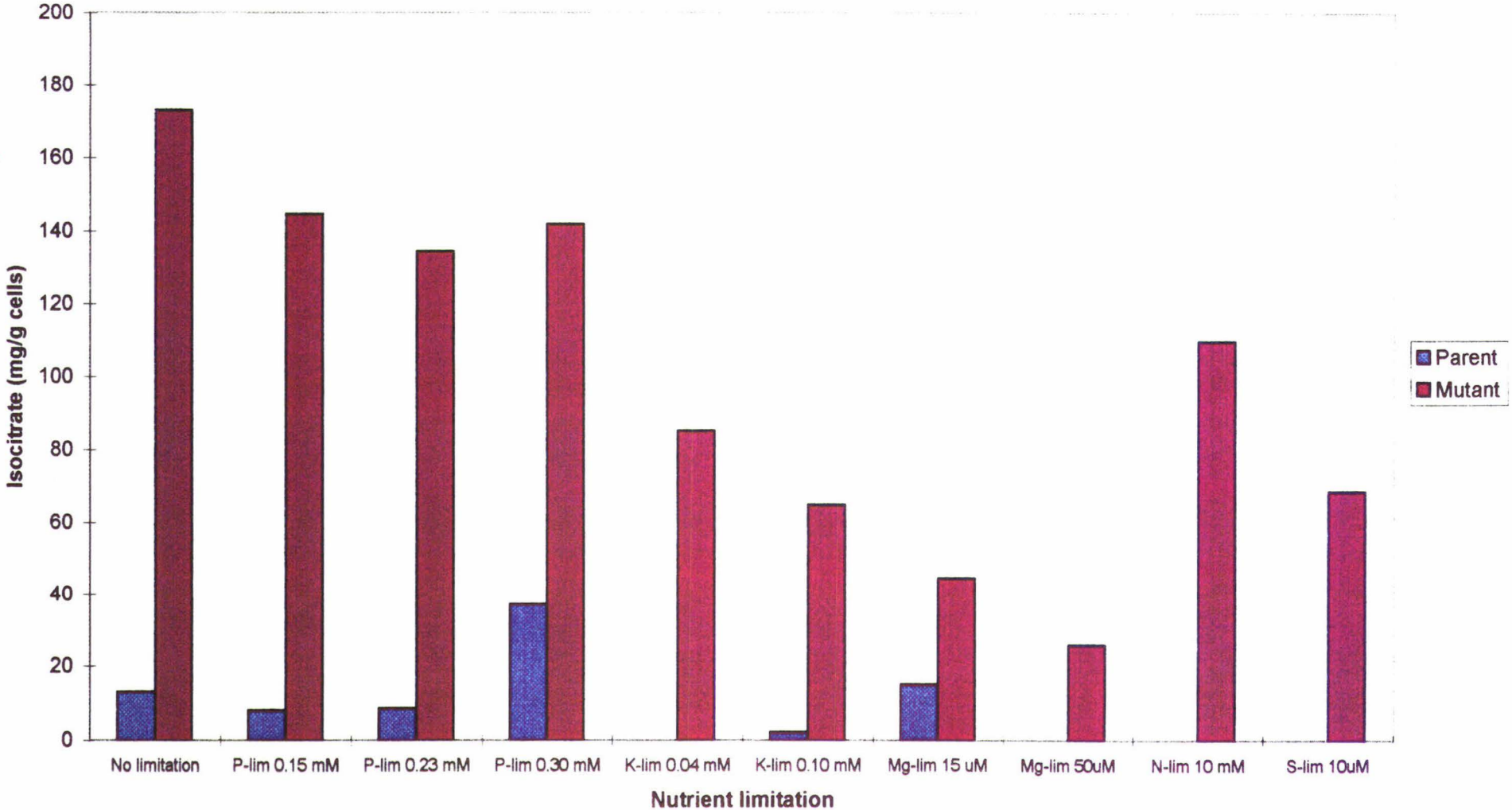
Levels of isocitric acid produced by the mutant were much higher than those produced by the parent strain (See Figure 5.8 and Appendix 3: Table 12).

The most significant producer of isocitric acid from the limited parent strain cultures was the phosphorus-limited 0.30 mM culture. This limitation was previously shown to produce the highest levels of citric acid (See Section 5.7), and also produced the highest levels of total acid per gram of cells for the parent strain cultures.

The mutant strain showed dramatic increases in the amount of isocitric acid produced when compared to the parent. Highest production was seen in the unlimited culture, followed by the citric acid-producing phosphorus-limited cultures.

As observed previously with citric acid, the most limited potassium- and magnesium-limited cultures showed the highest levels of isocitric acid production when compared to the less-limited cultures.

Figure 5.8: Comparison of Isocitrate Production (Milligrams per gram of Cells) from Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1



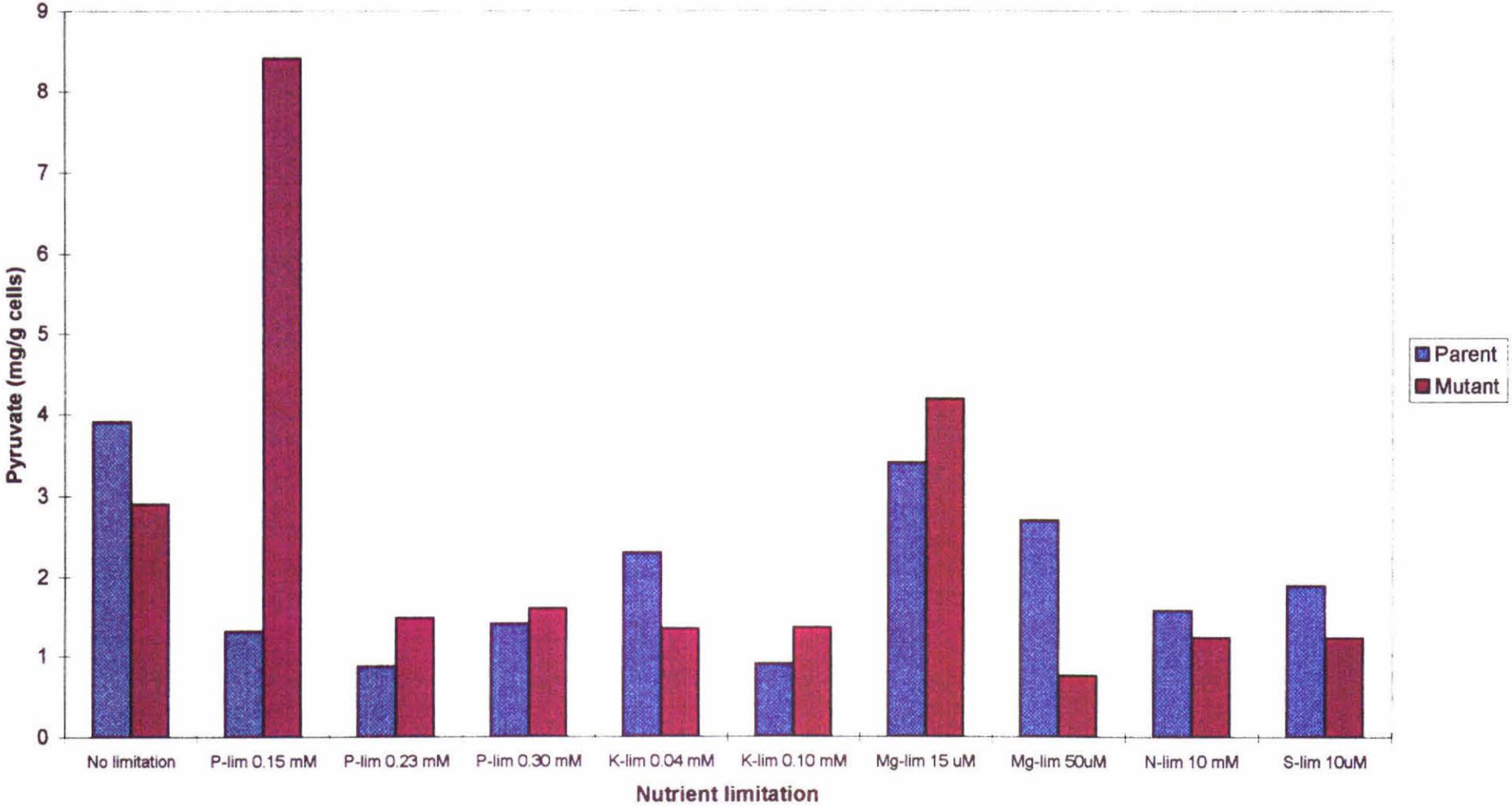
5.9.2 Pyruvate

Pyruvate production from both parent and mutant strains was generally similar, with the exception of the phosphorus-limited cultures (See Figure 5.9 and Appendix 3: Table 12).

In the parent strain, the highest level of excretion of pyruvate into the medium was seen by the unlimited culture and the magnesium-limited cultures. The high level excreted by the culture under magnesium limitation may be attributed to the inability of the magnesium-limited cell to take pyruvate into the mitochondrion. It is suggested that the limitation of magnesium (15μM), had a detrimental effect on the activity of the mitochondrial membrane ATPase and its ability to transport pyruvate into the mitochondrion, resulting in the excretion of excess pyruvate into the medium. This accumulation was also apparent in the 50 μM magnesium-limited culture, but not to the same extent. However, the unlimited culture takes more glucose into the cell - this being accounted for by the production of more biomass and the accumulation of small amounts of citric acid and other cycle intermediates. Any pyruvate that is excess to the requirements of the cell is excreted.

In the mutant strain, the highest level of pyruvate excreted was seen in the citric acid-producing phosphorus 0.15 mM culture. This culture was also observed to consume the highest amount of glucose of the mutant cultures, but produced less biomass. It is suggested that the levels of pyruvate excreted by this particular limitation may account for the extra carbon that was assimilated but not converted into cell biomass or citric acid. In the other cultures grown under phosphorus limitation, a higher biomass yield was obtained (See Figure 5.3) along with a lower citrate yield (See Figure 5.4). These cultures were observed to excrete much lower levels of pyruvate into the medium. It is suggested that the low levels of phosphorus provided in the 0.15 mM limitation may have affected the ability of

Figure 5.9: Comparison of Pyruvate Production (Milligrams per Gram of Cells) from Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1



the mitochondrion to utilise pyruvate, resulting in the excretion of excess pyruvate from the cell. It is known that the conversion of pyruvate to the precursor acetyl-CoA by pyruvate dehydrogenase is inhibited by high levels of ATP in the cell. As elevated levels of ATP are associated with the production of citric acid, it is quite likely that some inhibition of this enzyme occurred in the 0.15 mM culture. However, as high ATP in the cell has also been shown to cause inhibition of the enzymes NAD-ICDH, 2-oxoglutarate dehydrogenase and citrate synthase (Stryer 1981), the inability of the cell to utilise the available pyruvate may be a result of a combination of factors such as enzyme activity and transport of nutrients into and out of the cell.

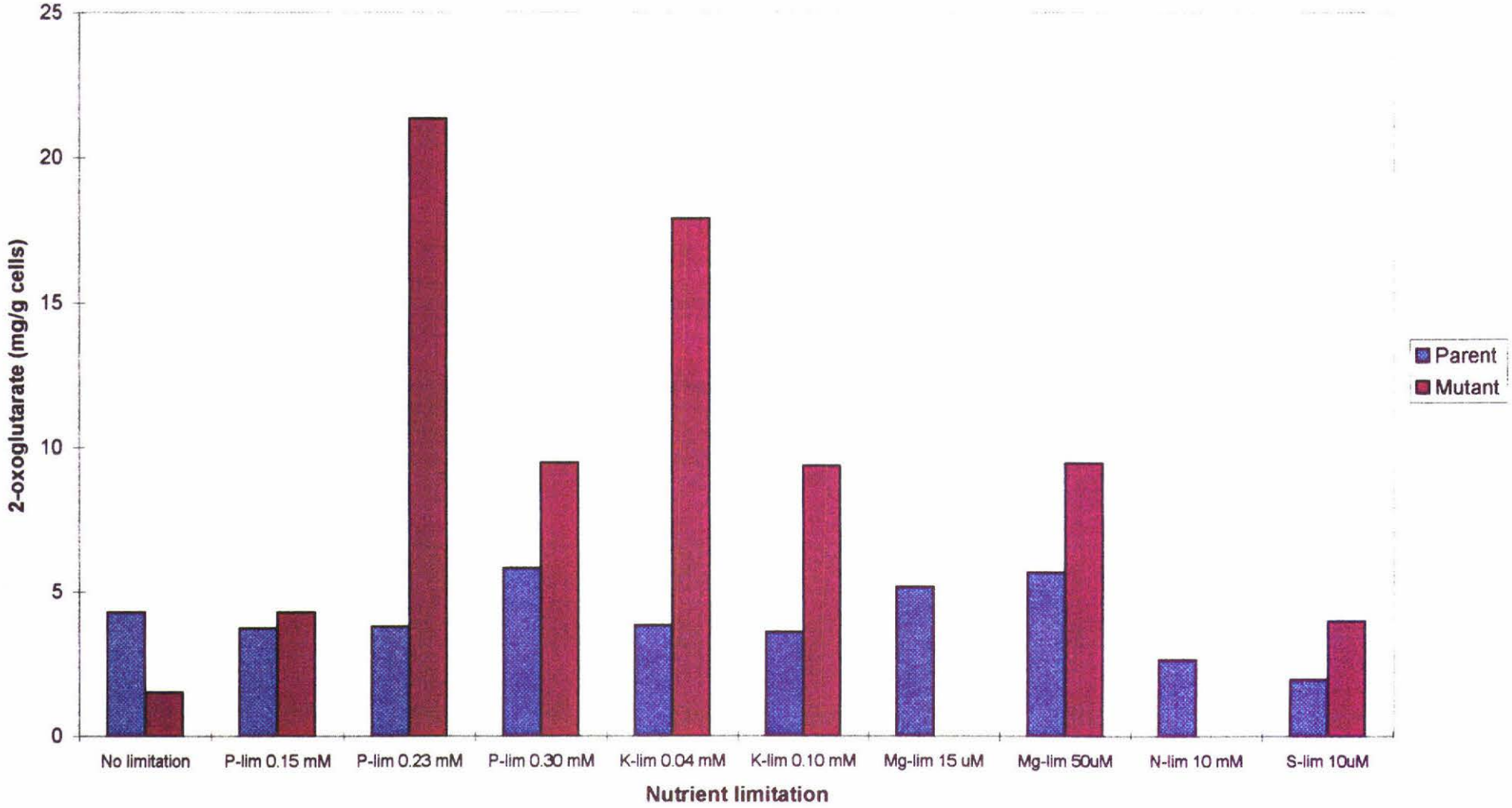
5.9.3 2-Oxoglutarate

Overall, levels in both the parent and mutant strains were very low. This was to be expected as is has been proposed that the enzyme NAD-ICDH is a likely control point for the production of citric acid. However, noticeable differences were found when the two strains were compared (See Figure 5.10 and Appendix 3: Table 12).

Overall, the parent showed higher levels of production. This would be expected, as in the parent strain the cell consumed more carbon and produced higher biomass than that observed for the mutant. This was also reflected by the fact that the parent accumulated lower concentrations of citric and isocitric acid.

The parent showed highest production of 2-oxoglutarate in the phosphorus-limited 0.30 mM culture, followed by the magnesium-limited 50 µM culture. The 0.30 mM culture also produced the most citric acid in the parent strain limitations. The higher level of 2-oxoglutarate in this limitation could be explained by the

Figure 5.10: Comparison of 2-Oxoglutarate Production (Milligrams per Gram of Cells) from Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1



observation that because this limitation produced more citric acid (2.3 $g.L^{-1}$), more substrate was available for utilisation in subsequent reactions of the citric acid cycle. The phosphorus-limited parent cultures showed a trend that related increased 2-oxoglutarate production with a decrease in limitation. This also mirrored the trend for glucose consumption observed for these limitations.

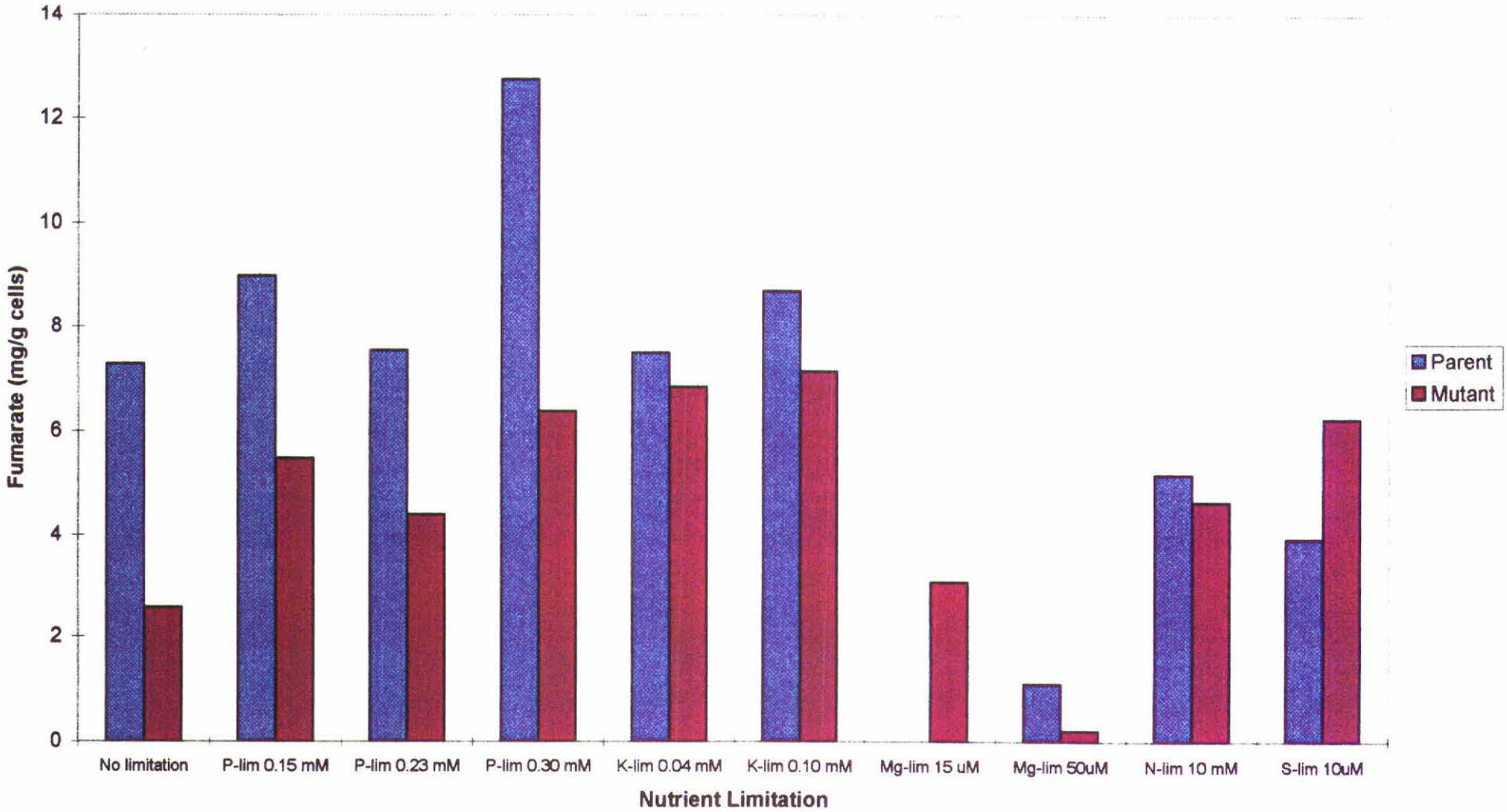
In the mutant, levels were lower. This was as expected due to the observed accumulation of citric acid, thought to be a result of decrease in activity of the enzyme NAD-ICDH. The phosphorus-limited cultures exhibited a similar trend in 2-oxoglutarate production that had previously been seen with citric acid production. The amount of 2-oxoglutarate produced by the mutant decreased with increasing limitation. This was a reversal of the trends observed with the parent strain.

It is suggested that in the mutant, the high limitation of the phosphorus 0.15 mM culture resulted in the cell being unable to metabolise the acid further through the cycle. It was also possible that some of the 2-oxoglutarate produced was converted into glutamate. As this accumulation of 2-oxoglutarate occurred in the limitation that produced the highest levels of citric acid, it is conceivable that the activity of 2-oxoglutarate dehydrogenase was inhibited by the high ATP content of the cell. There was no detectable production of 2-oxoglutarate by the mutant in the 15 μM magnesium-limited and the nitrogen-limited cultures.

5.9.4 Fumarate

The parent strain showed highest production from the phosphorus-limited 0.30 mM culture, and again there was a general trend (with exception of the 0.23 mM culture), of increase in production with decreasing limitation (See Figure5.11 and

Figure 5.11: Comparison of Fumarate Production (Milligrams per Gram of Cells) from Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1



Appendix 3: Table 12). Again, this can be associated with the consumption of glucose.

The magnesium-limited cultures produced very low levels of fumarate in comparison with the other limited parent cultures. These low levels are unusual when the levels of 2-oxglutarate produced by this limitation are taken into consideration. It appears that due to the relatively high levels of 2-oxoglutarate excreted by the parent into the medium, very little was converted to fumarate within the cell.

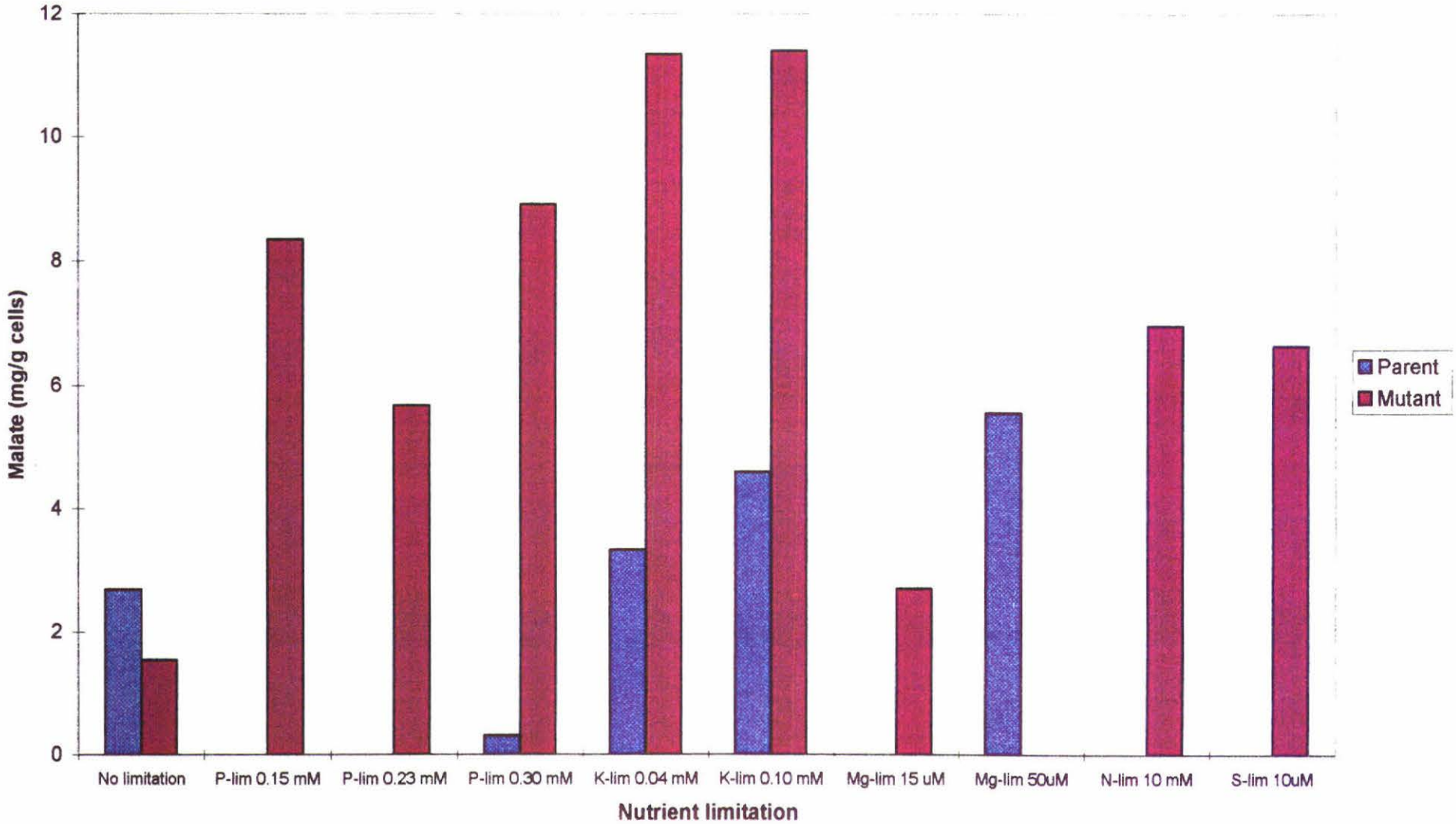
5.9.5 Malate

Overall, levels of malate detected were not high, but differences were observed between the parent and mutant strains.

In the parent, the only significant production was seen in the unlimited, the potassium- and magnesium-limited (50 μM) cultures. This may be attributed to functioning of the glyoxylate pathway under these limitations that acts to convert isocitric acid to malate via glyoxylate.

The mutant showed noticeable increases in the production of malate in most limitations when compared to the parent. The highest levels of malate production were seen in the non-citric acid-producing potassium-limited cultures, followed by the citric acid-producing 0.30 mM phosphorus culture. These levels suggested activity of the glyoxylate pathway, especially in the potassium limitations that produced little 2-oxoglutarate and only low concentrations of fumarate. Two possible reasons for the accumulation of malate are: (1) The enzyme pyruvate dehydrogenase was inhibited by increased levels of ATP, resulting in insufficient

Figure 5.12: Comparison of Malate Production (Milligrams per Gram of Cells) from Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1



levels of the precursor acetyl-CoA. (2) The enzyme citrate synthase was inhibited by the elevated levels of ATP, meaning that flux of substrate through this enzyme slowed, resulting in the accumulation of precursor i.e. malate.

The phosphorus-limited cultures also showed higher levels of malate production when compared to the parent. This may have other connotations in a citric acid-producing culture, as malate has been shown by various authors (Evans and Ratledge 1985d; Evans *et al.* 1983; Mitsushima *et al.* 1978), to be involved in the transport of citric acid out of mitochondria, and it is possible that the increase in levels in the mutant strain was due to this phenomenon.

5.10 Experiment 1: Summary & Discussion

In comparing the data obtained thus far for the parent and mutant strains, a number of differences have become apparent.

The parent strain took up much more glucose than the mutant strain - practically exhausting all reserves of glucose in the medium. The parent produced little citric acid and produced higher levels of biomass. This suggests that the citric acid cycle in the parent was fully operational and was performing its primary function - the conversion of pyruvate into energy in the form of ATP, and the formation of intermediates required for the synthesis of cell components.

Both parent and mutant have shown that the best production of citric acid was in the phosphorus-limited cultures; this was also related to the consumption of glucose i.e. the consumption of high levels of glucose was associated with the production of higher levels of citric acid. These strains also produced the lowest cell biomass.

Some work has been performed examining the effects of biomass concentration on citric acid production. Rane and Sims (1994) looked at the effect of biomass concentration on the production of citric acid by *C. lipolytica* in fermenters. Concentrations of 30 and 50 $g.L^{-1}$ biomass were assessed. It was found that better citric acid production was obtained at a biomass of 30 $g.L^{-1}$. This however, is much higher than the final biomass obtained from the mutant under the various nutrient limitations in this work. The biomass for the citric acid-producing strains was around 3.0 $g.L^{-1}$ at the end of the fermentation. However, as this experiment was carried out in shake flasks, control of biomass levels was not possible.

Gutierrez *et al.* (1992) obtained a biomass of 5.5 $g.L^{-1}$ and a biomass yield of 0.15 $g.g^{-1}$ while evaluating citric acid production from glucose by *C. guilliermondii* IMK1. The biomass yield was comparable to that obtained in this work (See Figure 5.3 and Appendix 3: Table 7). Abou-Zeid and Khoja (1983), obtained a biomass from *C. guilliermondii* of 4.4 $g.L^{-1}$ and although this is slightly lower than that obtained for the parent strain in this experiment, it is still comparable.

It was suggested by McKay *et al.* (1994), that high specific rates of glucose utilisation were necessary for citric acid production in *Yarrowia lipolytica* mutant IMK2. However, in the citric acid-producing mutant IMK1, the phosphorus limitation with the highest maximum specific rate of citric acid production showed the lowest maximum specific rate of glucose consumption. It appears that the high specific rate of glucose uptake is not necessary for citric acid production in IMK1.

The mutant generally, over all limitations, utilised less glucose than the parent, and consequently produced less biomass, but the mutant also produced much higher concentrations of citric acid. High levels of citric acid in the citric acid-producing yeast are known to correlate with high levels of ATP (Marchal *et al.* 1977a; Mitsushima *et al.* 1978). NAD-ICDH is known to be inhibited by high levels of ATP, as are the enzymes pyruvate dehydrogenase, citrate synthase and 2-oxoglutarate dehydrogenase (Stryer 1981). It is proposed that there is a block or reduction of activity in the cycle, probably at the level of the enzyme NAD-ICDH as has been found by other researchers (Mitsushima *et al.* 1976; Finogenova *et al.* 1991). However, it is likely that inhibition of the other enzymes affected by elevated ATP would also play a role. Unfortunately, it was not possible to assess levels of ATP in the mutant owing to the loss of citric-acid producing ability during the course of this work. Other indications that activity of this key enzyme was lower in the mutant, apart from the increased levels of citric and isocitric

acids, were the reduced levels of 2-oxoglutarate and other intermediates that are produced subsequent to this enzyme.

There also appears to be a lesion or some alteration at the level of the plasma membrane ATPase, which is associated with nutrient uptake in the cell. This ATPase has a requirement for Mg^{2+}, which became apparent in both the parent and mutant magnesium-limited cultures. These cultures showed the lowest levels of glucose uptake and biomass production, suggesting that levels of Mg^{2+} provided in these limitations were insufficient for optimum activity of this enzyme. This provides a possible explanation for the lower levels of glucose consumed by the parent and mutant under magnesium-limitation. The magnesium-limited mutant cultures were surprisingly efficient at the conversion of glucose to citric acid - as much so as the best citric acid-producing phosphorus limitation (See Figure 5.5). However, it appears that due to the magnesium-limited cell being unable to take up sufficient glucose, the culture was hindered in the production of citric acid. Upon analysis of intermediates excreted into the medium, it also became apparent that transport across the mitochondrial membrane was a limiting factor in the magnesium-limited cell, leading to the subsequent excretion of unutilised pyruvate into the fermentation medium.

A trend was observed with the phosphorus-limited mutant cultures, and to a lesser extent with other limitations, in that the lower the concentration of growth-limiting nutrient, the more glucose was consumed, the less biomass was produced, the more citric acid produced per gram of cell and also more isocitrate and 2-oxoglutarate produced. An opposite trend was seen in the parent in that it was the less-limited cultures - as expected - that consumed more glucose and produced more citric acid.

It appears that for the mutant strain, in the more-limited (0.15 mM) phosphorus culture, the amount of glucose taken up is an important factor. Phosphorus is used

in the cell for synthesis of components such as phospholipids and nucleic acids etc, and also has a role in energy production (See Section 2.2.2). The 0.15 mM culture consumed the highest level of glucose of all the phosphorus limitations, but also produced the lowest biomass. Therefore, it is suggested that the higher levels of glucose taken up by the 0.15 mM limitation were an attempt by the cell to provide energy and/or substrate for biosynthesis by means of the citric acid cycle. As the cell was under conditions of phosphorus limitation, insufficient phosphorus was available for biosynthesis of cell components and so biomass was reduced. Only limited amounts of the citric acid produced can be utilised further through the cycle owing to the reduced activity of the enzyme NAD-ICDH. It must be noted that the phosphorus limitation of the cell must be quite severe, as it has been stated (See Section 2.2.2) that before effects of limitation are seen, the cytoplasmic stores of phosphorus must be mobilised - it is possible that the increased consumption of glucose was associated in part with this mobilisation. In the less-limited cultures, more phosphorus was available - with the result that more biomass was produced. These cultures also consumed less glucose and produced less citric acid.

The trend of higher levels of citric acid production with the more-limited cultures was seen with other nutrient limitations. It is possible that there was some preferential channelling of nutrients through the citric acid cycle in order to produce energy when the cell was under conditions of nutrient limitation. The reasons for the lower limitations showing these effects may also be due to the mobilisation of nutrient stores held by the cell, and subsequent exhaustion of these stores. Jones and Gadd (1990) stated that it was not until the stores of phosphorus within the cytoplasm are reduced that the true effects of limitation would become apparent.

The most noticeable differences in intermediates excreted into the fermentation medium by the mutant when compared to the parent strain were seen in the levels

of isocitric acid and pyruvate. Levels of isocitric acid increased markedly in the mutant strain suggesting that the enzyme aconitase - which catalyses the conversion of citric acid to isocitric acid - was fully functional. The mutant appears to be subject to some restriction of the enzyme NAD-dependent isocitrate dehydrogenase, which resulted in accumulation of citric and isocitric acids. In the parent strain, aconitase was presumed to be functioning normally, but as there was no inhibition of the enzyme NAD-ICDH, any citric or isocitric acid that was produced was further processed through the cycle.

Reduction of aconitase activity, either by mutation or by means of chemical inhibition, has been found by researchers to increase the amount of citric acid in the total acid mix. Some form of inhibition of this enzyme is likely to cause a reduction of isocitrate in the total acid mix.

Of the levels of pyruvate produced by the mutant strain limitations, the phosphorus-limited 15 mM culture produced levels that were around double that produced by any other limitation. It is speculated that the reason for this accumulation may be due to some restriction or inhibition of the transport of pyruvate across the mitochondrial membrane by the inhibition of the enzyme pyruvate dehydrogenase due to increased levels of ATP (See Figure 2.1). Pyruvate dehydrogenase catalyses the conversion of pyruvate to acetyl-CoA. Increased levels of ATP are associated with the phase of citric acid accumulation in the cell and are known to inhibit the enzyme NAD-ICDH. However, it is not known what concentrations of ATP will result in the inhibition of pyruvate dehydrogenase in citric acid-accumulating yeasts. This has not been addressed in the literature.

The magnesium-limited 15 μM culture also excreted relatively high levels of pyruvate into the fermentation medium. This may be associated with the mitochondrial membrane ATPase, and the effects of magnesium limitation.

Inhibition of the mitochondrial ATPase would partly account for the low levels of citric acid produced by this limitation and the low biomass due to the reduced flux of carbon through the citric acid cycle.

Unfortunately, levels of oxaloacetate were not assessed. It has been stated (Voet and Voet 1995), that the availability of oxaloacetate as a substrate for the formation of citric acid is one of the most important regulators of the citric acid cycle (See Section 2.1.4). Knowledge of the levels of oxaloacetate would also help to further clarify the mechanism of accumulation of citric acid in the cell.

The aim of the next experiment was to examine the activities of some enzymes involved in the citric acid cycle and compare the parent and mutant to try to determine whether there are any major differences that would elucidate further the mechanisms of citric acid production in *Candida guilliermondii* IMK1. It was also decided to examine the relative concentrations of the metabolites of the citric acid cycle both inside and outside the cell at the same time as enzyme activities were being analysed, as this is possible while separating the mitochondrial fraction.

CHAPTER 6

NUTRIENT LIMITATION EXPERIMENT - PART 2 RESULTS AND DISCUSSION

6.1 Introduction

The aim of this experiment was to determine activities of selected enzymes of the TCA cycle to elucidate the reasons for increased citric acid production by the mutant strain when compared with the parent. Enzyme activity was determined at both logarithmic and stationary phases of growth. It has been shown by other researchers (Finogenova *et al.* 1986, 1991), that enzyme activity can vary in the different phases of growth and that these differences in enzyme activity can be related to the major phase of citric acid accumulation which occurs in the stationary phase of growth.

Concentrations of TCA cycle intermediates excreted into the medium and those within the cell were also determined at this time. This was carried out in an attempt to provide more information with respect to differences in cell function and patterns of citric acid accumulation between parent and mutant.

The cultures assessed for enzyme activity were the phosphorus-limited 0.15 mM, 0.30 mM cultures, the nitrogen-limited 10mM culture and the magnesium-limited 15 μM culture. The phosphorus-limited cultures were to provide data from citric acid-producing cells, the nitrogen-limited culture an example of a mediocre citric

acid producer, and the magnesium-limited as an example of a low citric acid-producer.

Unfortunately, it was discovered when comparing data with those obtained in Experiment 1, that the mutant strain had started to lose its ability to produce citric acid. This also affected levels of intermediates produced. However, it was still deemed worthwhile to treat this fermentation separately and compare data obtained from each limitation for the parent and mutant strains.

6.2 Comparison of Internal and External Levels of Intermediates

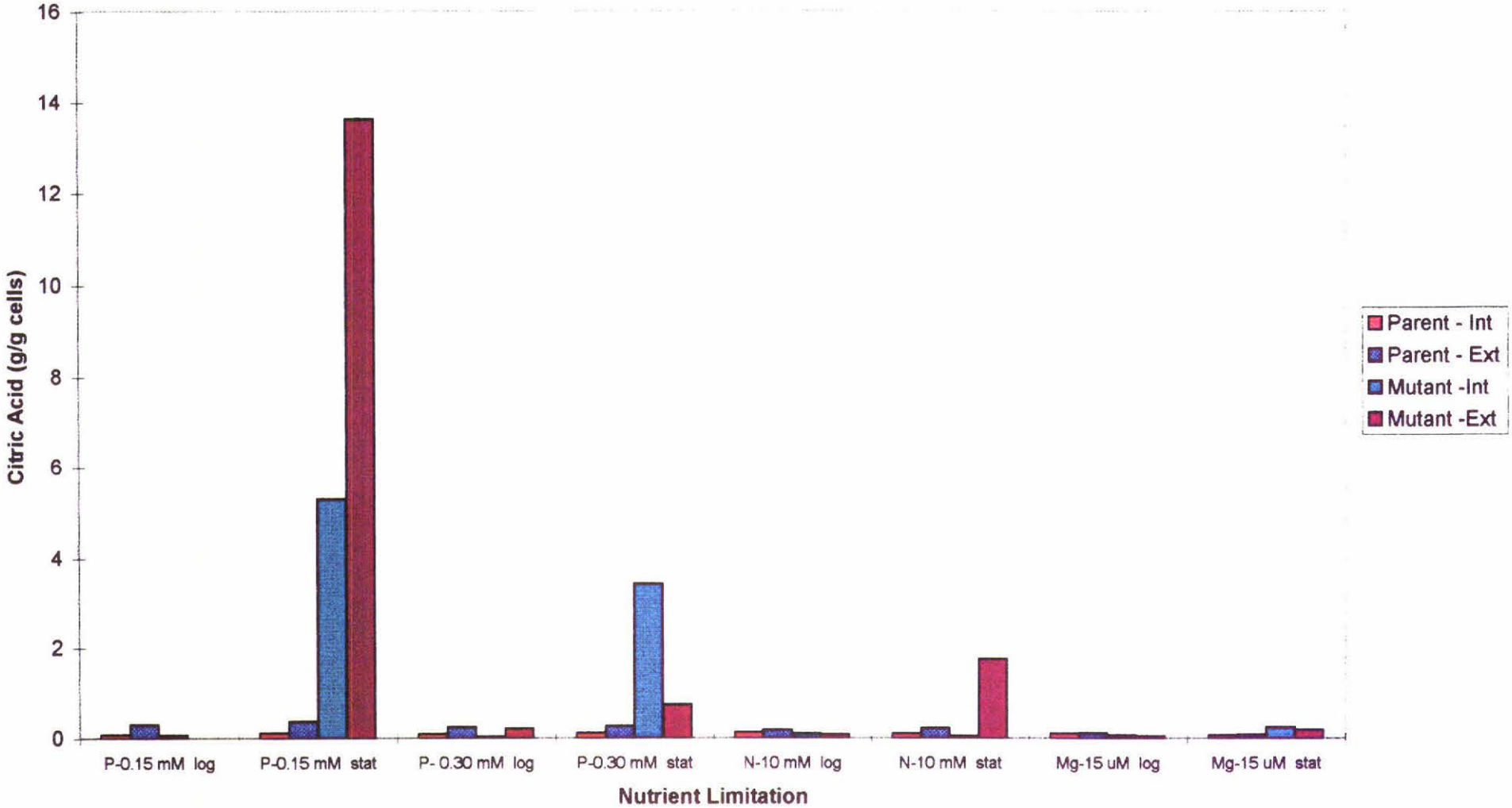
6.2.1 Citric Acid

Concentrations of citric acid in the internal and external pools were assessed as stated in Chapter 3: Materials and Methods. Results can be seen in Figure 6.1 and in Appendix 4: Tables 1 and 2.

In the parent strain, levels of citric acid increased in the phosphorus-limited cultures in the stationary phase of growth. This observation agrees with the theory of citric acid accumulation, in that major accumulation occurs in the stationary phase of growth. The nitrogen- and magnesium-limited cultures showed slight decreases in the amount of citric acid detected in the stationary phase - indicating some possible reassimilation of citric acid.

Externally, levels of citric acid increased in the stationary phase in the parent cultures, with the exception of the magnesium-limited culture. This reduction in the level of citric acid in the magnesium-limited culture indicated that this culture

Figure 6.1: Comparison of Internal and External Levels of Citric Acid Produced by Parent *Candida guilliermondii* NRRL Y-448 and Mutant *Candida guilliermondii* IMK1



may have been using citric acid as a source of carbon. It is likely that this consumption was taking place because of the inability of the cell to consume further glucose owing to the depletion of magnesium from the medium, which would cause inactivation of the magnesium-requiring plasma membrane ATPase. The fact that the cell was able to take up citric and isocitric acids (See Appendix 4: Tables 1 and 2), suggests that the carrier(s), or site(s), where glucose is transported into the cell and citric acid into (and out) of the cell are separate.

In the mutant strain, the levels of citric acid inside the cell increased substantially in the stationary phase of growth in most limitations – the magnesium-limited culture being the exception. External levels of citric acid also increased in the stationary phase as was expected. Highest levels of citric acid production were again seen from the phosphorus-limited 0.15 mM culture.

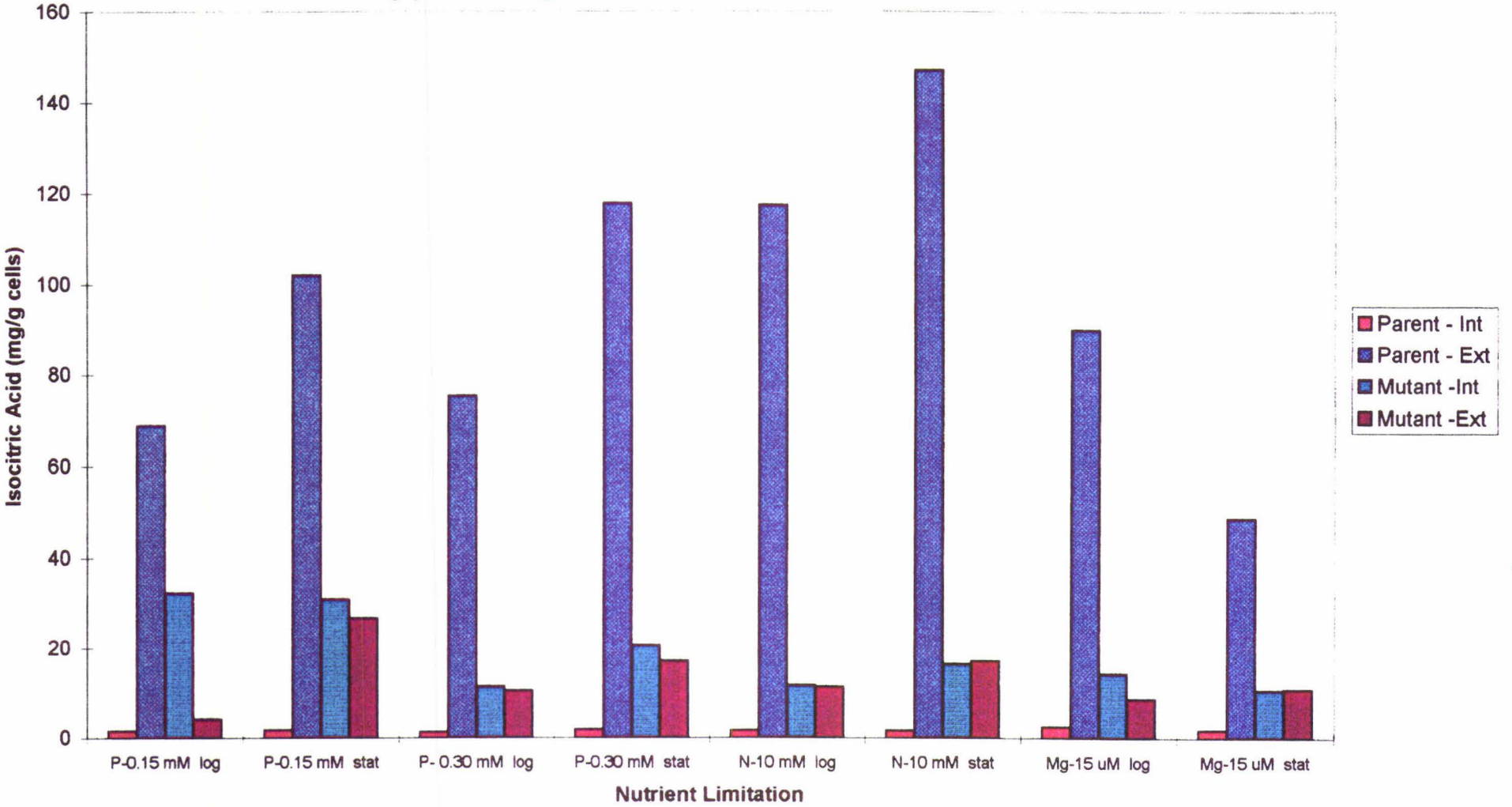
6.2.2 Isocitric Acid

Isocitric acid was assessed as in Chapter 3: Materials and Methods. Results can be seen in Figure 6.2 and in Appendix 4: Tables 1 and 2.

In the parent strain, internal concentrations of isocitric acid increased slightly in the stationary phase of growth. The magnesium-limited culture showed evidence of reassimilation of isocitric acid in that the external concentration of isocitric acid decreased in the stationary phase.

External levels of isocitric acid produced by the parent increased in the stationary phase. It was also observed that levels of isocitric acid excreted by the parent were around four times higher than the citric acid excreted into the medium. This may indicate that the reversible reaction catalysed by aconitase was working in

Figure 6.2: Comparison of Internal and External Levels of Isocitric Acid from parent *Candida guilliermondii* NRRL Y-448 and mutant *Candida guilliermondii* IMK1 at Logarithmic and Stationary phases of growth



favour of isocitrate. The nitrogen-limited culture was seen to produce ten times more isocitric acid than citric acid. A possible explanation may be that limitation of nitrogen had an effect on the activity of the aconitase enzyme, resulting in the production of isocitric acid in preference to citric acid. Nitrogen is required for the conversion of 2-oxoglutarate to glutamate. It is suggested that limitation of nitrogen may have caused some accumulation of isocitric acid, due to the inability of the cell to utilise the 2-oxoglutarate produced.

Levels of isocitric acid inside the cell were similar to the levels of citric acid. Citric acid levels in the stationary phase were generally of the order of 0.1 g/g cells, as were levels of isocitric acid.

In the mutant strain, levels of isocitric acid found internally were variable between the different limitations, but there were no notable increases or decreases observed between the logarithmic and stationary phases of growth.

Overall, levels of isocitric acid in the mutant were found to be much lower than those found in the parent strain. This is a complete reversal to what was discovered earlier in Experiment one. This change correlated to the loss of citric acid production by the mutant. It is unclear as to whether the difference in results obtained here are solely a result of difference in activity of the aconitase enzyme, or whether these differences may be a reflection of the reduced levels of citric acid produced. It is also possible that reduced production of isocitric acid is a result of a combination of enzyme activity and substrate utilisation. The mutant takes up less carbon in the form of glucose and so there is less substrate passing through the TCA cycle. However, in the reverting strain, less citric acid is accumulating and so more substrate is passing through the cycle. In addition, the enzymes of the TCA cycle showed reduced activity due to reduced substrate availability.

6.2.3 Pyruvate

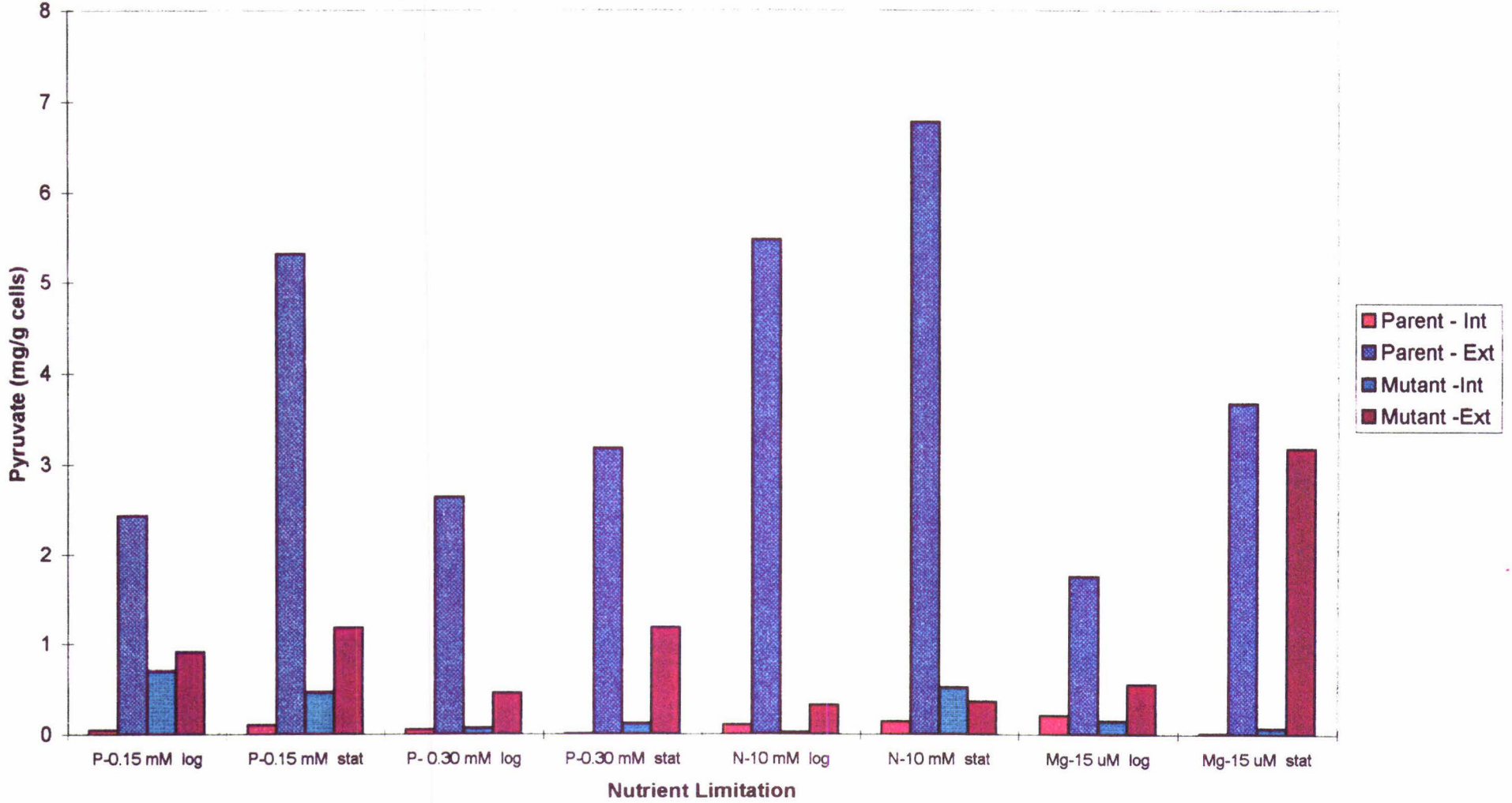
Levels of pyruvate were assessed as in Chapter 3: Materials and Methods. Results can be seen in Figure 6.3 and in Appendix 4: Tables 1 and 2.

The internal concentration of pyruvate in the parent cell was so low as to be practically undetectable. This may be because pyruvate was utilised as soon as it was produced, and so accumulation did not occur. This suggested that only pyruvate that was required by the cell was taken into the mitochondrion. Excess was excreted, resulting in minimal accumulation in the cell. Externally, levels increased substantially in the stationary phase of growth.

In the mutant strain, internal concentrations again were low. The most notable level of excretion was from the magnesium-limited culture in the stationary phase of growth. High levels of pyruvate production in magnesium-limited cultures were also seen in the first experiment. (See Section 5.8.4) Internally, the mutant generally also showed higher levels of accumulation than the parent.

Again, levels of pyruvate produced in this experiment were lower than seen in Experiment one. Levels produced by the mutant decreased considerably, while the parent produced levels of pyruvate that were slightly lower, but still comparable to those seen before. This decreased level of production also correlated to the lower levels of isocitric and citric acid produced, suggesting that some repair had taken place. During this experiment, glucose levels were not assessed. Therefore, it was impossible to determine whether these changes in production in the mutant were a result of changes in the utilisation of glucose, or changes in enzyme activity. However, biomass estimations were made which indicated a substantial increase in cell biomass produced by the mutant. The increase in biomass could be explained by the decrease in excretion of pyruvate and other intermediates such as citric and isocitric acids, indicating that some

Figure 6.3: Comparison of Internal and External Levels of Pyruvate from Parent *Candida guilliermondii* NRRL Y-448 and Mutant *Candida guilliermondii* IMK1 at Logarithmic and Stationary Phases of Growth



change has taken place – which may be enzymatic – allowing the cell to take more pyruvate into the mitochondrion, and hence produce more biomass.

6.2.4 2-Oxoglutarate

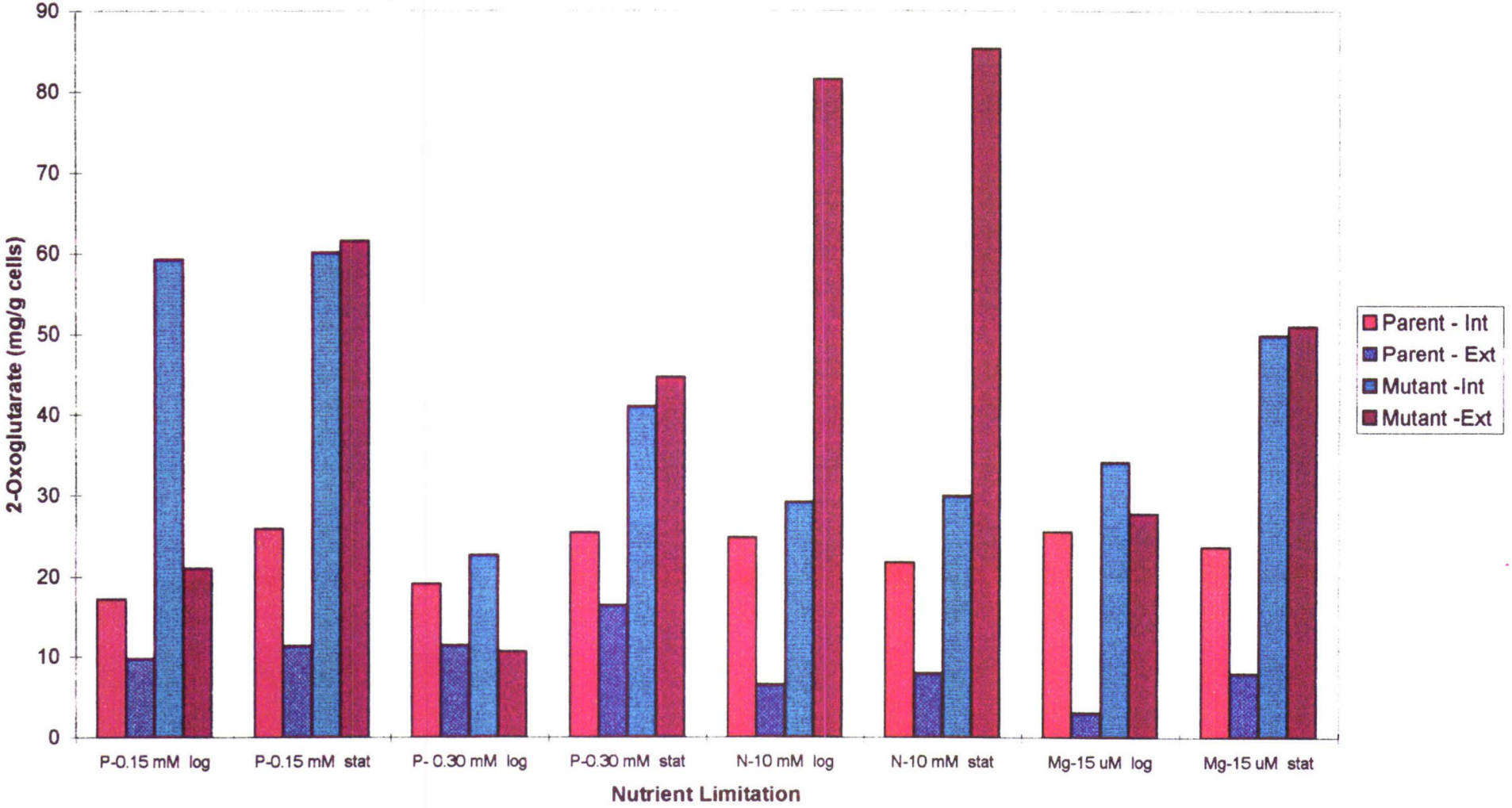
Levels of 2-oxoglutarate were assessed as stated in Chapter 3: Materials and Methods. Results can be seen in Figure 6.4 and in Appendix 4: Tables 1 and 2.

In the parent strain, internal levels of 2-oxoglutarate produced by the phosphorus-limited cultures increased slightly in the stationary phase. In both phases of growth internal concentrations were higher than those excreted, suggesting that most of the 2-oxoglutarate produced by the parent cell was being further utilised.

The mutant – with the exception of the 0.30 mM phosphorus culture - showed higher levels of production than the parent internally and externally, the highest levels of excretion being seen in the nitrogen-limited culture in both phases of growth. This indicated that there might be some inhibition of the enzyme 2-oxoglutarate dehydrogenase, causing accumulation of 2-oxoglutarate and its subsequent excretion into the medium. Nitrogen is required for the conversion of 2-oxoglutarate into glutamate. It is speculated that as there is insufficient nitrogen for this reaction to take place, 2-oxoglutarate is excreted into the medium.

Levels of 2-oxoglutarate observed in the mutant increased when compared with Experiment 1. This suggested that the increase in concentration of 2-oxoglutarate might be due in part to the loss of production of citric acid. Increases in concentration of 2-oxoglutarate were observed to be 10 times that in the original experiment. The mutant continued to take up low levels of glucose (monitored with OD readings), but processed more carbon through the citric acid cycle, and

Figure 6.4: Comparison of Internal and External Levels of 2-Oxoglutarate from Parent *Candida guilliermondii* NRRL Y-448 and Mutant *Candida guilliermondii* IMK1 at Logarithmic and Stationary Phases of Growth



consequently produced more biomass. The accumulation of 2-oxoglutarate indicated some form of inhibition or a "slow" enzyme at a subsequent step in the cycle. It is suggested that the enzyme 2-oxoglutarate dehydrogenase was inhibited, possibly by high levels of ATP in the cell.

6.2.5 Malate

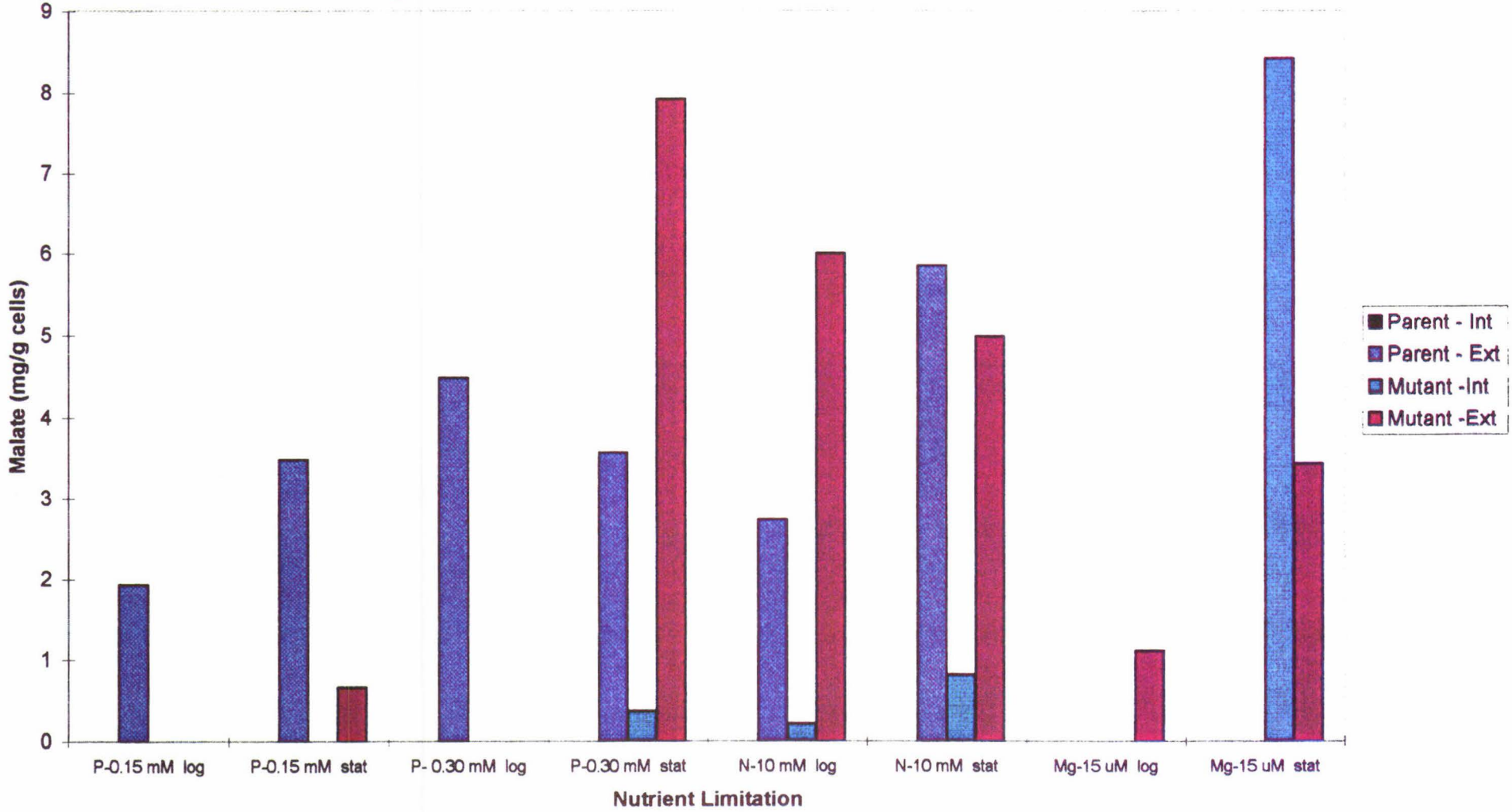
Malate was assessed as stated in Chapter 3: Materials and Methods. Results can be seen in Figure 6.5 and in Appendix 4: Tables 1 and 2.

Levels of malate found internally in the parent strain were in all cases so low as to be undetectable. Externally, levels of malate were relatively low, but these increased in the stationary phase compared with all except the magnesium-limited culture - low levels explained here by the apparent inability of the magnesium-limited cell to transport pyruvate into the mitochondrion.

Generally, the mutant strain produced very low levels of malate internally, these generally increasing slightly in the stationary phase of growth. The magnesium-limited culture was the only one to accumulate notable amounts of malate internally in the stationary phase of growth.

Malate has been stated to be involved in the excretion of citric acid and possibly isocitric acid out of the mitochondrion (Mitsushima *et al.* 1978; Evans *et al.* 1983; Evans and Ratledge 1985d). It is possible that in IMK1 mitochondria, malate is exchanged for citrate, owing to the similar levels observed for each acid under different nutrient limitations. However, due to the high levels of isocitric acid excreted it is probable that isocitric acid is excreted on another carrier. If malate were involved in transport of citrate, higher concentrations of malate would be

Figure 6.5: Comparison of Internal and External Levels of Malate from Parent *Candida guilliermondii* NRRL Y-448 and Mutant *Candida guilliermondii* IMK1 at Logarithmic and Stationary Phases of Growth



expected internally. The higher levels of malate observed in the mutant may be due to inhibition by ATP of citrate synthase, causing the accumulation of oxaloacetate and hence malate. Malate may also accumulate due to inhibition of the enzyme pyruvate dehydrogenase also by ATP, resulting in a lack of precursor in the form of acetyl-CoA. When compared with the low levels of fumarate produced, it is suggested that operation of the glyoxylate cycle played a role in the increased concentrations of malate

Externally, levels of malate were more significant, higher levels being seen in the phosphorus 0.30 mM and nitrogen-limited cultures. The nitrogen-limited culture produced significant levels of malate in both phases of growth, unlike any other limitation. The high levels of malate produced by the nitrogen-limited culture are in contrast to those seen previously in Experiment one.

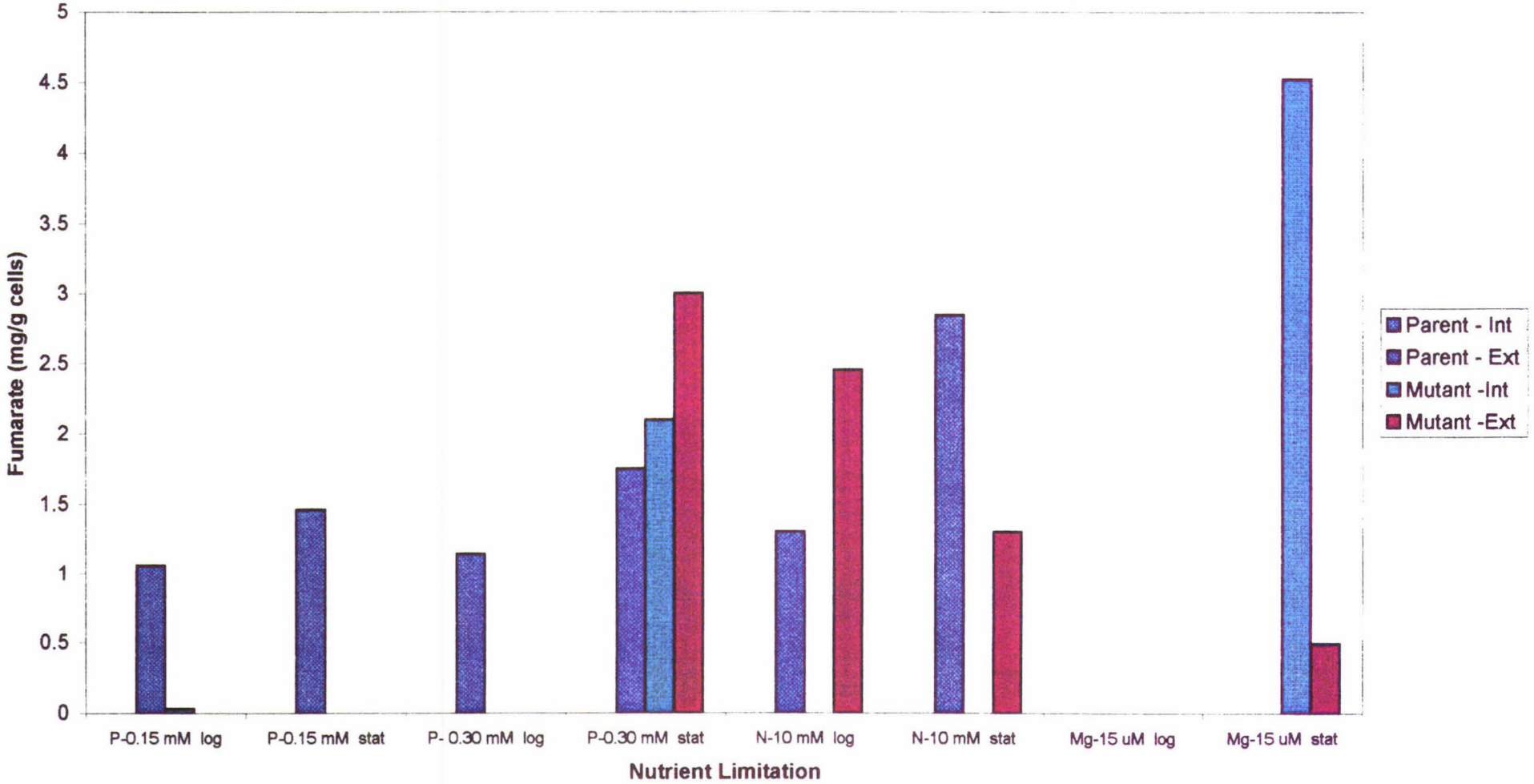
6.2.6 Fumarate

Levels of fumarate in both the parent and the mutant, internally and externally, were relatively low. Results can be seen in Figure 6.6 and in Appendix 4: Tables 1 and 2.

In the parent, internal levels were so low as to be undetectable. Externally, levels were higher. It is suggested that any fumarate that was excess to requirements was excreted by the cell.

In the mutant 0.30 mM phosphorus culture, accumulation of fumarate was observed internally and externally in the stationary phase of growth. It is suggested that this was due to the level of limitation of the culture, in that more phosphorus was available for the cell resulting in more substrate passing through

Figure 6.6: Comparison of Internal and External Levels of Fumarate Produced by Parent *Candida guilliermondii* NRRL Y-448 and Mutant *Candida guilliermondii* IMK1 at Logarithmic and Stationary Phases of Growth



the cycle. The stationary-phase magnesium culture showed the highest levels of fumarate to accumulate internally. It is suggested that fumarate accumulated due to the low levels of pyruvate transported into the mitochondrion, ultimately resulting in a lack of precursor for further production of citric acid. It is also suggested that some of the organic acids that normally accumulate in the mitochondrion may be excreted in exchange for pyruvate, with the aid of the membrane Mg^{2+}- ATPase. As pyruvate is not taken into the mitochondrion under this limitation, there is no exchange for fumarate and hence it accumulates.

6.3 Enzyme Activities

Enzyme activities were assessed at logarithmic and stationary phases of growth as stated in Chapter 3: Materials and Methods. Only four limitations were investigated, the citric acid-producing phosphorus 0.15 mM and 0.30 mM limitations, and the non-citric acid-producing nitrogen (10 mM) and magnesium (15 μM) limitations.

When the parent and mutant cultures were compared, only one major difference in enzyme activity was observed, this being the activity of the enzyme NADP-ICDH. Activities of other enzymes were either similar or varied only slightly.

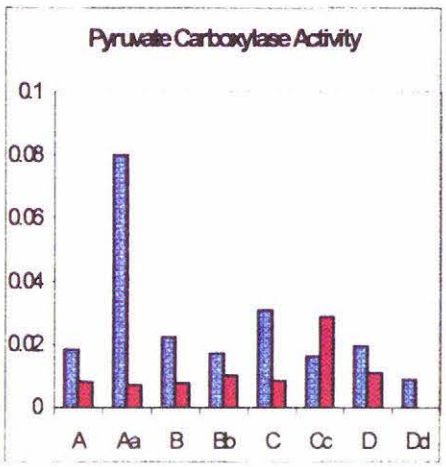
It should be noted that activities determined in this experiment are only a measure of *in vitro* activity of the enzyme (Units per mg of protein), not a determination of the *in vivo* activity, as enzyme may be present in the cell but inactive, due to insufficient substrate or cofactors.

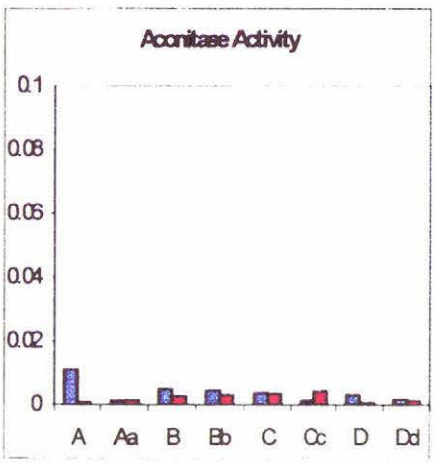
6.3.1 Citrate Synthase (EC 4.1.3.7)

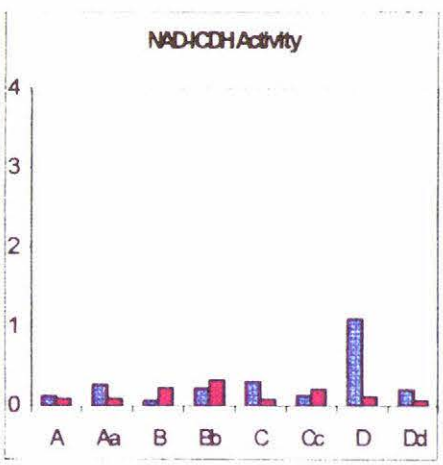
Activities of citrate synthase were determined as stated in Chapter 3: Materials and Methods. Results can be seen in Figure 6.6 and in Appendix 4: Table 3.

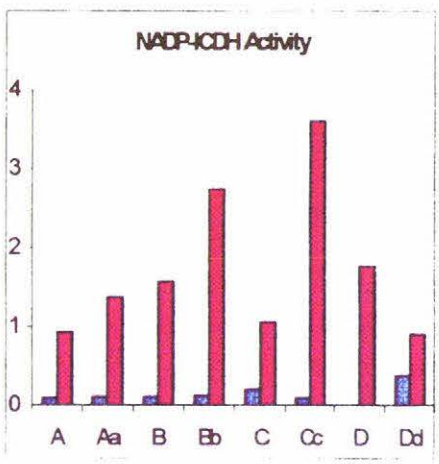
Overall, levels of citrate synthase activity were low in both the parent and mutant strains, especially when compared with those found in the literature (See Appendix 1: Table 2a).

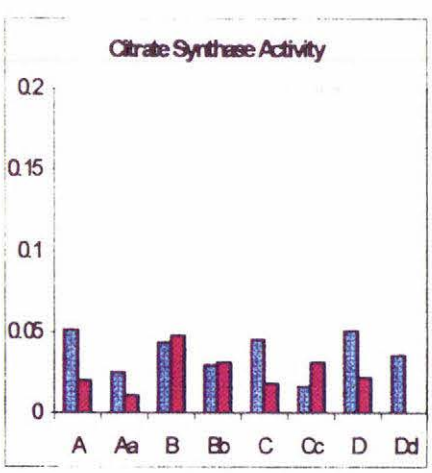
Figure 6.7: Comparison of Enzyme Activities of Parent Strain *Candida guilliermondii* NRRL Y-448 and Mutant Strain *Candida guilliermondii* IMK1











Legend:		
	A	P-lim 0.15 mM Log
	Aa	P-lim 0.15 mM Stat
	B	P-lim 0.30 mM Log
	Bb	P-lim 0.30 mM Stat
	C	N-lim 10 mM Log
	Cc	N-lim 10 mM Stat
	D	Mg-lim 15 uM Log
	Dd	Mg-lim 15 uM Stat

Parent
Mutant

X-axis: Nutrient limitation

Y-axis: Enzyme Activity (U/mg Protein)

Note: Different scales have been used for each pair of enzymes

When the parent and mutant were compared, it was found that in the citric acid-producing phosphorus-limited 0.15 mM culture, levels of citrate synthase activity were lower than in the parent. This is surprising, in that it would be expected that activity of this enzyme would be higher in the mutant to account for increased production of citric acid. It is suggested that due to the slight accumulation of pyruvate, the citrate synthase enzyme may be saturated and unable to process the pyruvate as it becomes available. This may a result of increased levels of ATP in the cell, or a lack of acetyl-CoA

When activities in the logarithmic and stationary phase of growth were compared, it was found that the activity of citrate synthase decreased in the stationary phase of growth. This may be because during the stationary phase of growth the rate of growth has stabilized and less substrate is passing through the TCA cycle.

It is assumed that because differences have been observed in the concentrations of intermediates excreted into the medium, enzyme activities may also have changed from Experiment one to Experiment two. This suggested that activities of citrate synthase detected in this experiment may not be a true reflection of activity in the citric acid-producing mutant. This would provide a likely explanation for the reduction of citric acid produced by the mutant in this experiment. However, the mutant was consuming more carbon, and more pyruvate was being utilised by the cell. This would be expected to result in an increase in activity of citrate synthase. Therefore, it appears that citrate synthase activity is not an important control point in the citric acid-producing cell.

6.3.2 Aconitase (EC 4.2.1.3)

Activity of the enzyme aconitase was determined as stated in Chapter 3: Materials and Methods. Results can be seen in Figure 6.6 and in Appendix 4: Table 3.

Aconitase activity in both the parent and mutant strain was low when compared to the activities of other enzymes assessed. When activities of the parent and mutant were compared these were found to be similar. Highest activity of aconitase was seen in the parent, in the phosphorus-limited 0.15 mM logarithmic phase culture.

When activities in the logarithmic and stationary phases of growth were compared, differences were seen between the parent and mutant. In the parent strain, the activity of aconitase was highest in the logarithmic phase of growth, whereas in the mutant higher activity was seen in the stationary phase of growth.

The higher activity in the mutant in the stationary phase of growth may be a result of there being more substrate available in the form of citric acid. It is suggested that aconitase activity in the mutant was a reflection of the amount of pyruvate taken into the mitochondrion i.e. activity of the enzyme is dependent on the amount of substrate available.

Activity of aconitase was lower in the mutant when compared with the activity of citrate synthase. The mutant also exhibits a high total activity of NAD- and NADP-ICDH (see 6.3.3 and 6.3.4). This agrees with Finogenova *et al.* (1986), who stated that these characteristics were necessary for high levels of citric acid production.

However, there was some doubt as to the validity or consistency of the enzyme activities measured in this experiment. Isocitric acid levels that were assessed at the same time as enzyme activity had decreased to levels significantly lower than

those previously observed with this mutant. It is possible that aconitase activity here was lower than in the previous fermentation, so accounting for the lower levels of isocitric acid measured. However, it is also possible that in *Candida guilliermondii*, measurable activity of aconitase is dependent on the amount of substrate available, and that the changes caused by mutation have not affected the activity of this enzyme.

6.3.3 NAD-dependent Isocitrate Dehydrogenase (EC 1.1.1.41)

Activity of the enzyme NAD-dependent isocitrate dehydrogenase (NAD-ICDH) was determined as stated in Chapter 3: Materials and Methods. Results can be seen in Figure 6.6 and in Appendix 4: Table 3.

Reduced activity of NAD-ICDH has been proposed as an important control in the accumulation of citric acid. In this experiment, it was not possible to determine if this enzyme was a control, owing to there being no significant increase or decrease in activity. The reduced production of citric acid in this experiment may have affected enzyme activity, therefore making it difficult to make any assumptions.

The magnesium-limited culture showed higher activity in both strains during the logarithmic phase of growth. This was probably due to there being more substrate available prior to the inhibition of uptake of substrate caused by magnesium limitation. There was sufficient carbon available for the production of 2-oxoglutarate, but it is speculated that much of this was utilised for the production of glutamate – the concentrations of which were not assessed. It is suggested that the proposed inhibition by ATP actually inhibits 2-oxoglutarate dehydrogenase

more than NAD-ICDH, so preventing the conversion of 2-oxoglutarate to succinyl-CoA.

Activity of NAD-ICDH was generally lower in the mutant strain than in the parent. This reduced activity suggested that less isocitrate was converted into 2-oxoglutarate. However, there was sufficient activity to provide a flux of carbon through the enzyme to produce the low levels of 2-oxoglutarate observed. As has been found with other citric acid-producing yeasts, it is suggested that a reduction in NAD-ICDH activity has a role in the accumulation of citric and isocitric acids in the mutant *Candida guilliermondii* IMK1.

6.3.4 NADP-specific Isocitrate Dehydrogenase (EC 1.1.1.42)

Activity of NADP-specific isocitrate dehydrogenase (NADP-ICDH) was determined as stated in Chapter 3: Materials and Methods. Results can be seen in Figure 6.6 and in Appendix 4: Table 3.

The enzyme NADP-ICDH showed the most notable differences in activity when comparisons were made between the parent and mutant strains.

Very large increases in activity in the mutant were apparent - in most cases this increase was in the region of ten-fold. The largest increase in activity was seen in the nitrogen-limited culture in the stationary phase of growth. The reasons for this huge increase in activity are not clear. NADP-ICDH is used in the cell to provide reducing power that is needed in addition to ATP for biosynthesis. It is found both in mitochondria and in the cytoplasm. It is suggested that this increase in activity was an attempt by the cell to compensate for the reduction in activity of NAD-ICDH. It is also known that NADP-ICDH is not subject to control by ATP,

and so the inhibition of NAD-ICDH by ATP would not affect NADP-ICDH activity. NADP is required for the formation of glutamate from 2-oxoglutarate, which in turn is used for the synthesis of amino acids. This may offer a partial explanation for the large increase in activity, and would help explain the reduced levels of fumarate and malate produced. The high activity of NADP-ICDH in the mutant grown under nitrogen-limited conditions correlated to the quantities of 2-oxoglutarate produced in this culture.

The levels of activity detected in this mutant conflict with work reported by Hattori *et al.* (1974) with *C. zeylanoides*, who found that activity of NADP-ICDH remained constant between the logarithmic and stationary phases of growth. Mitsushima *et al.* (1978), found that total ICDH activity decreased in the citric acid-accumulating phase; they also found that levels of ATP were higher in the stationary phase of growth.

6.3.5 Pyruvate Carboxylase (EC 6.4.1.1)

Activity of pyruvate carboxylase was determined as stated in Chapter 3: Materials and Methods. Results can be seen in Figure 6.6 and in Appendix 4: Table 3.

This enzyme forms part of the anaplerotic pathway that is involved in the provision of oxaloacetate for the citric acid cycle, therefore if this pathway were in use we would expect to see higher activities of this enzyme.

Overall, activities of pyruvate carboxylase were low in the parent and mutant strain, although when compared to the activity of other enzymes determined in this experiment, levels of activity detected were comparable to those of citrate synthase and aconitase. The parent in the majority of cases showed higher activity

of pyruvate carboxylase than the mutant. This suggested that the anaplerotic pathway was in operation to some extent in the parent strain, providing extra precursor for the citric acid cycle. In the mutant this pathway appeared to have been functioning – although to a limited extent - owing to the higher levels of malate produced.

6.4 Experiment 2: Summary and Discussion

Generally, in both parent and mutant strains, citric acid accumulated internally in the logarithmic phase of growth and reached significant levels in the stationary phase where excretion of citric acid began.

In the parent, isocitric acid was excreted into the medium at much higher concentrations than seen for the mutant. These levels also exceeded the citric acid excreted into the medium. The parent culture under magnesium limitation showed a decrease in the concentration of isocitric acid excreted into the medium from the logarithmic to the stationary phase. This suggested that the magnesium-limited culture was using isocitric acid (and to a lesser extent citric acid) as a source of carbon. As this was in the stationary phase, it corresponded with the time when the magnesium-limited cultures ceased to take up glucose. This apparent utilisation of isocitric acid suggested that the uptake of this acid was associated with a different transport system than was used for the uptake of glucose. It appears that this phenomenon was associated with the limitation of magnesium, the exact mechanisms unknown.

The most significant difference in enzyme activity was seen with the activity of NADP-ICDH. This increase in activity was also associated with a slight decrease in the activity of NAD-ICDH. It is known that during the phase of citric acid accumulation in yeast cells, the level of ATP increases in the cell (Marchal *et al.* 1977a; Mitsushima *et al.* 1978). This increase is sufficient to cause reduction in activity of NAD-ICDH. It is also known that ATP causes inhibition of the enzymes pyruvate dehydrogenase, citrate synthase and 2-oxoglutarate dehydrogenase (Stryer 1981). NADPH, a by-product of the reaction catalysed by NADP-ICDH, is required for the reaction that converts 2-oxoglutarate to glutamate. Therefore, this increase in NADPH produced may also explain the

utilisation of 2-oxoglutarate. It is proposed that as NADP-ICDH is not subject to control by ATP, activity increased in order to provide reducing power for biosynthesis, compensating for the loss of NAD-ICDH. However, it was not clear if the reduction in activity of NAD-ICDH in this mutant was the result of inhibition by ATP, or whether the change in activity was due to damage to the enzyme caused by mutation.

Assessment of adenine nucleotide levels in the cell would have helped to further elucidate the reasons for the change in activity in this enzyme, and the role of other enzymes in the citric acid cycle.

The accumulation of 2-oxoglutarate in the mutant in this experiment was proposed to be because of the increase in carbon passing through the cycle – a consequence of the loss of citric acid production in the cell. It is speculated that the enzyme 2-oxoglutarate dehydrogenase was also inhibited, either due to the effects of ATP, or as a result of damage caused by the mutation.

Activity of citrate synthase was found to be lower in the mutant than in the parent. This was unexpected, as it was thought that increased production of citric acid would also be reflected in an increase in citrate synthase activity. It is suggested that as the parent consumed more glucose, it would require higher activity of citrate synthase to process the higher levels of carbon taken up by the yeast, through the citric acid cycle. It has been stated by other authors (Boulton and Ratledge 1980), that citrate synthase is not an important control point in the citric acid cycle. It is speculated that activity of citrate synthase in both parent and mutant is dependent on the amount of substrate available, and is not subject to significant inhibition by ATP.

Aconitase activity was seen to decrease slightly in the mutant strain, resulting in decreased amounts of isocitric acid. It is suggested that again, as with citrate

synthase, the parent was consuming more glucose, and so had a higher activity of aconitase to cope with the high levels of substrate.

Pyruvate carboxylase, which catalyses the anaplerotic pathway involved in the formation of oxaloacetate, was found to be functioning in the parent, and in the mutant, but to a limited extent. In the parent, this enzyme ensures that sufficient precursor in the form of oxaloacetate is available for the formation of citric acid. In the mutant strain, the contribution of this pathway was observed in the higher concentrations of malate when compared to fumarate.

In the mutant magnesium-limited cultures, a number of factors have become apparent that appear to influence the ability of this particular culture to produce citric acid. These may also help elucidate the mechanism of citric acid accumulation in *Candida guilliermondii* IMK1.

The 15 µM culture showed the lowest levels of glucose uptake. It is proposed that this was due to the effect of the limitation of magnesium on the cell membrane ATPase. The magnesium-limited cells were seen to take up less glucose in the stationary phase, apparently due to the depletion of magnesium from the medium. At this time, a decrease in the concentrations of isocitric and citric acids in the medium was observed, suggesting that the cell was using isocitric and citric acids as an additional source of carbon. This reutilisation of acids was only apparent in the magnesium-limited cultures.

The magnesium-limited cultures also seem to have impaired transport of pyruvate into the mitochondrion. It is suggested that this was due to inhibition of the mitochondrial membrane ATPase by magnesium limitation. The excess pyruvate was subsequently excreted from the cell. This inability to take up pyruvate was also reflected by the low concentrations of intermediates produced and low biomass production.

The mutant culture grown under magnesium limitation was observed to accumulate low levels of some intermediates of the citric acid cycle – particularly fumarate and malate. It is suggested that this accumulation was due to the lack of pyruvate being taken into the mitochondrion, resulting in a reduction of availability of precursor at later stages in the fermentation.

In conclusion, it appears that the following factors were important in the accumulation of citric acid in IMK1. The amount of glucose transported into the cell was shown to be a limiting factor in the magnesium-limited cell, but was also of importance in the more limited phosphorus culture. The 0.15 mM phosphorus culture was observed to consume more glucose, to produce more citric acid and to produce less biomass. This showed that glucose consumed was being channelled into the production of citric acid instead of biomass. This was thought to be due to the level of limitation of the culture, the exact mechanisms being unclear.

As was also shown by the magnesium-limited cell, the transport of pyruvate into the mitochondrion was important for citric acid production. From results obtained from the mutant limitations, it appeared that only pyruvate required by the cell was taken into the mitochondrion, the excess being excreted. The mutant, for some reason, was able to accumulate higher levels of pyruvate within the cell than the parent. This may have had some effect on the ability of the cell to produce and accumulate citric acid.

It also appeared that control of the cycle by ATP was important. Unfortunately, concentrations of ATP within the cell were unable to be measured. It is speculated that ATP concentrations within the cell will also affect the activity of other enzymes such as 2-oxoglutarate dehydrogenase, as well as the activity of NAD-ICDH, a known control point in citric acid production in other yeasts.

Therefore, to provide a clearer picture of the reasons for the accumulation of citric acid in *Candida guilliermondii* IMK1, further analyses were required. Estimations of the concentrations of ATP within the cell, and the effects of ATP on the enzymes of the citric acid cycle would be particularly useful. Because ATP has been shown to inhibit the enzymes pyruvate dehydrogenase, citrate synthase, 2-oxoglutarate dehydrogenase as well as NAD-ICDH in other organisms, it would have been useful to determine if this was the case in IMK1. In addition, estimations of the concentrations of the intermediates that were not assessed in this experiment would provide a more complete picture as to the mechanism(s) of citric acid accumulation in IMK1.

CHAPTER 7

REVERSION AND REISOLATION OF MUTANT STRAIN *CANDIDA GUILLIERMONDII* IMK1

7.1 Introduction

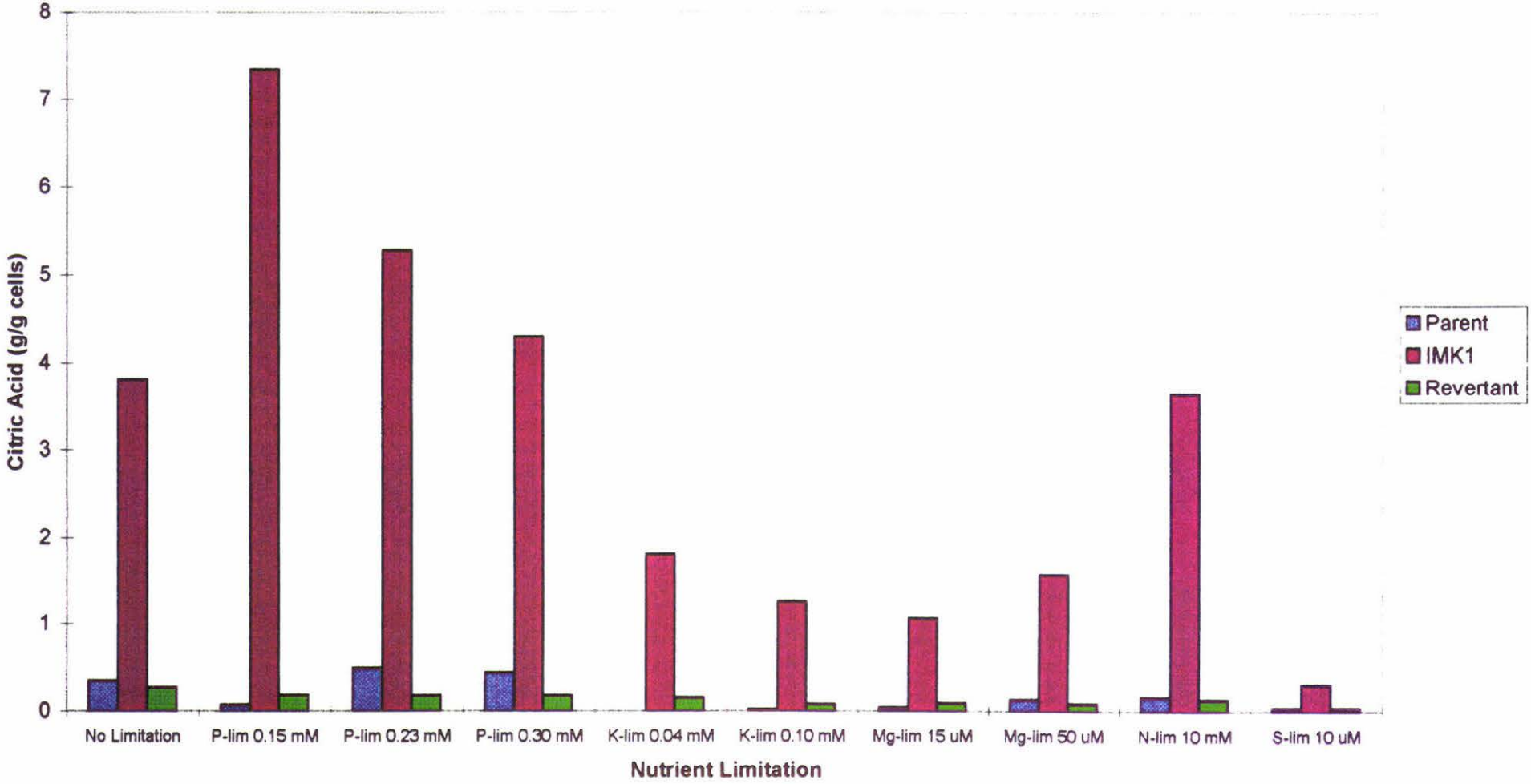
In the course of enzyme analysis and measurement of levels of citric acid production, it was discovered that the mutant strain was undergoing some form of reversion or repair. This happened over a reasonably short period of time. This was initially made apparent by the loss of production of citric acid.

Following is a comparison of levels of metabolites produced by the mutant at the commencement of the work and subsequent levels determined over a period of several months. The revertant culture was compared with both the parent and the citric acid-producing mutant to try to elucidate where repair had taken place. This may also shed some light on the site of the initial lesions, if in fact the changes were due to repair and not to the cell by-passing the damage in some way.

7.2 Citric Acid Production

Changes in the amount of citric acid produced from the parent, mutant and revertant cultures can be seen in Figure 7.1 and in Appendix 5: Table 1.

Figure 7.1: Comparison of Citric Acid Production (grams of citrate per gram of cells) from *Candida guilliermondii* - Parent, Mutant and Revertant strains



Citric acid production decreased considerably over a period of approximately one month. This was the time between the initial citric acid data being collected and the time of the second experiment where enzyme activity was assessed. For example, levels of citric acid produced by the culture grown under phosphorus limitation (0.15 mM), decreased from 7.2 g/g to 1.8 g/g. All limitations showed large reductions in levels of production. In many cases, the yield of citric acid (grams per gram of cells), produced by the revertant strain were less than those produced by the parent. The revertant also showed changes with regards to the trends that were observed previously for the various nutrient limitations and citric acid production: in the revertant, the highest production of citric acid was from the unlimited culture, the lowest from the culture grown under sulphur limitation. The sulphur-limited culture was also the lowest producer in the IMK1 cultures.

This reduction in citric acid production in the revertant suggested that repair of some form had occurred in the mutant strain, leading to low levels of citric acid excretion into the medium. This repair may have been due to one or more factors. The site(s) of mutation or inhibition in IMK1 that led to the accumulation of citric acid have been previously suggested to be associated with the transport of glucose into the mutant cell, and the subsequent transport of pyruvate into the mitochondrion. The revertant showed increased levels of glucose uptake and increased biomass production. Another proposed control point was high concentrations of ATP in the cell causing inhibition of some enzymes of the citric acid cycle – particularly NAD-ICDH. It was also possible that enzymes such as pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and to a lesser extent citrate synthase may have been affected. It was likely that in the revertant strain, concentrations of ATP within the cell had decreased (as evidenced by accumulation of citric acid), so reversing some of this inhibition.

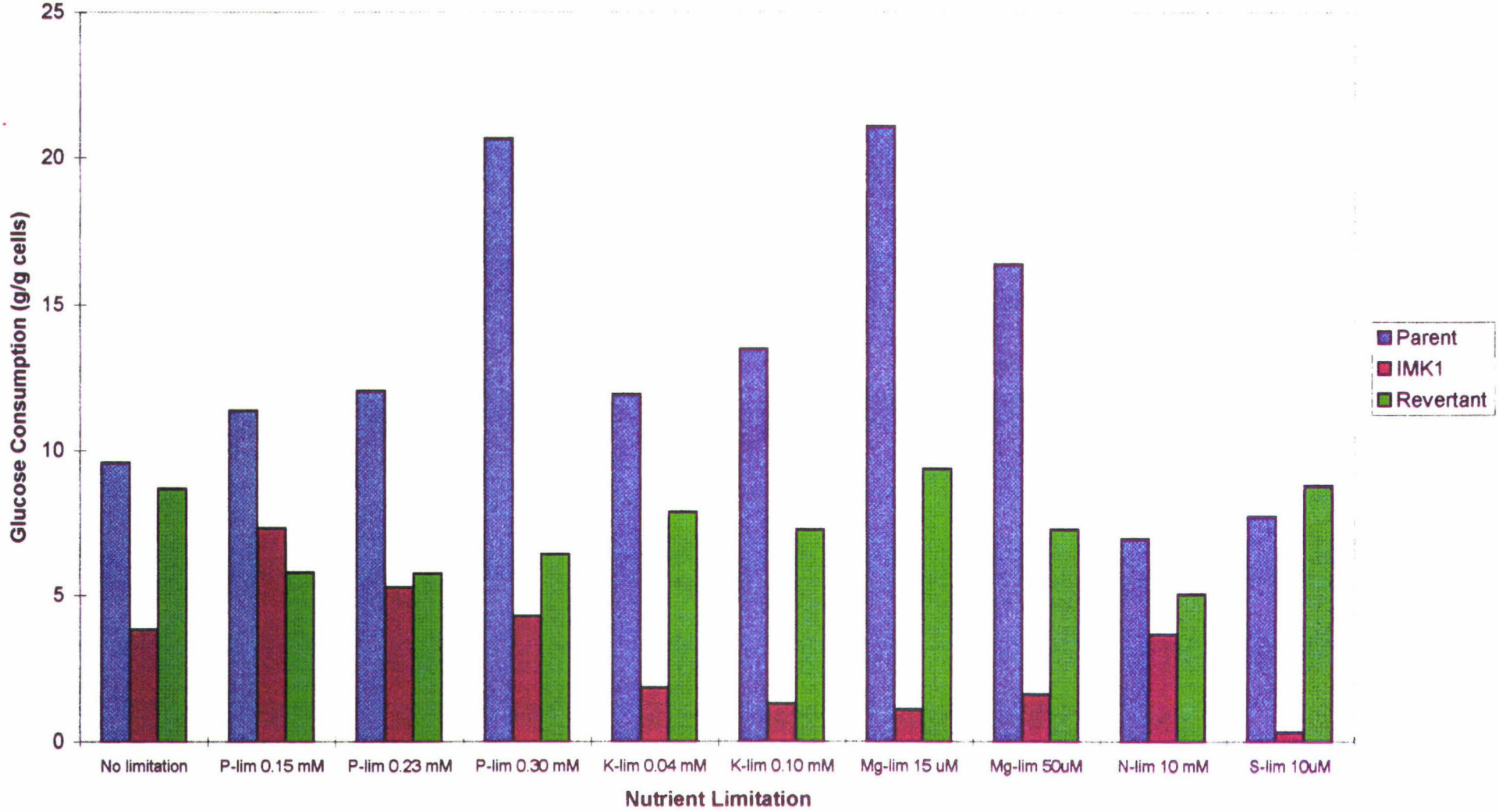
7.3 Glucose Consumption

Glucose consumption results can be seen in Figure 7.2 and in Appendix 5: Table 1.

Glucose consumption by the revertant strain increased when compared to IMK1, however levels of glucose consumed by the revertant did not reach the high levels attained by the parent strain. Previously in IMK1, the highest consumption of glucose was seen in the citric acid-producing 0.15 mM phosphorus-limited culture and lowest consumption was observed in the culture grown under magnesium limitation (15 μM). Therefore, it was suggested that magnesium limitation might have resulted in the inability of this particular mutant limitation to utilise high levels of glucose, due to inhibition of the magnesium-requiring ATPase. Surprisingly, the revertant showed the highest level of glucose consumption under magnesium limitation, but was unable to convert all of this glucose into biomass. This carbon may be accounted for in the high levels of pyruvate and 2-oxoglutarate excreted when compared to the mutant strain.

The ability of the revertant to utilise more glucose was a significant difference when compared to the citric acid-producing mutant. It is likely that this increased utilisation was related to the loss of citric acid production from the mutant. It is suggested that the damage or inhibition, that resulted in a reduction in the amount of glucose transported into the mutant cell, had been reversed or by-passed. It was also found that much of this glucose consumed by the revertant was converted to biomass. This was probably the most significant difference between the mutant and the revertant, with the exception of citric acid production. The magnesium-limited culture showed considerable signs of repair. The 15 μM culture, previously the lowest consumer of glucose, took up more glucose than any other revertant culture. These results cast doubt on the theory that magnesium

Figure 7.2: Comparison of Glucose Consumption from *Candida guilliermondii* - Parent, Mutant and Revertant strains



limitation was responsible for reduction of glucose consumption. The effect of magnesium limitation on the transport of pyruvate across the mitochondrial membrane may also be minimal. Therefore, in the citric acid-producing mutant, it was likely that ATP-induced inhibition of pyruvate dehydrogenase led to a reduction in the amount of pyruvate that was transported across the mitochondrial membrane and converted to acetyl-CoA. It was assumed that the revertant did not have as high an energy charge (in the form of ATP), within the cell and so inhibition by ATP was not as apparent, meaning that more substrate could be converted through the cycle.

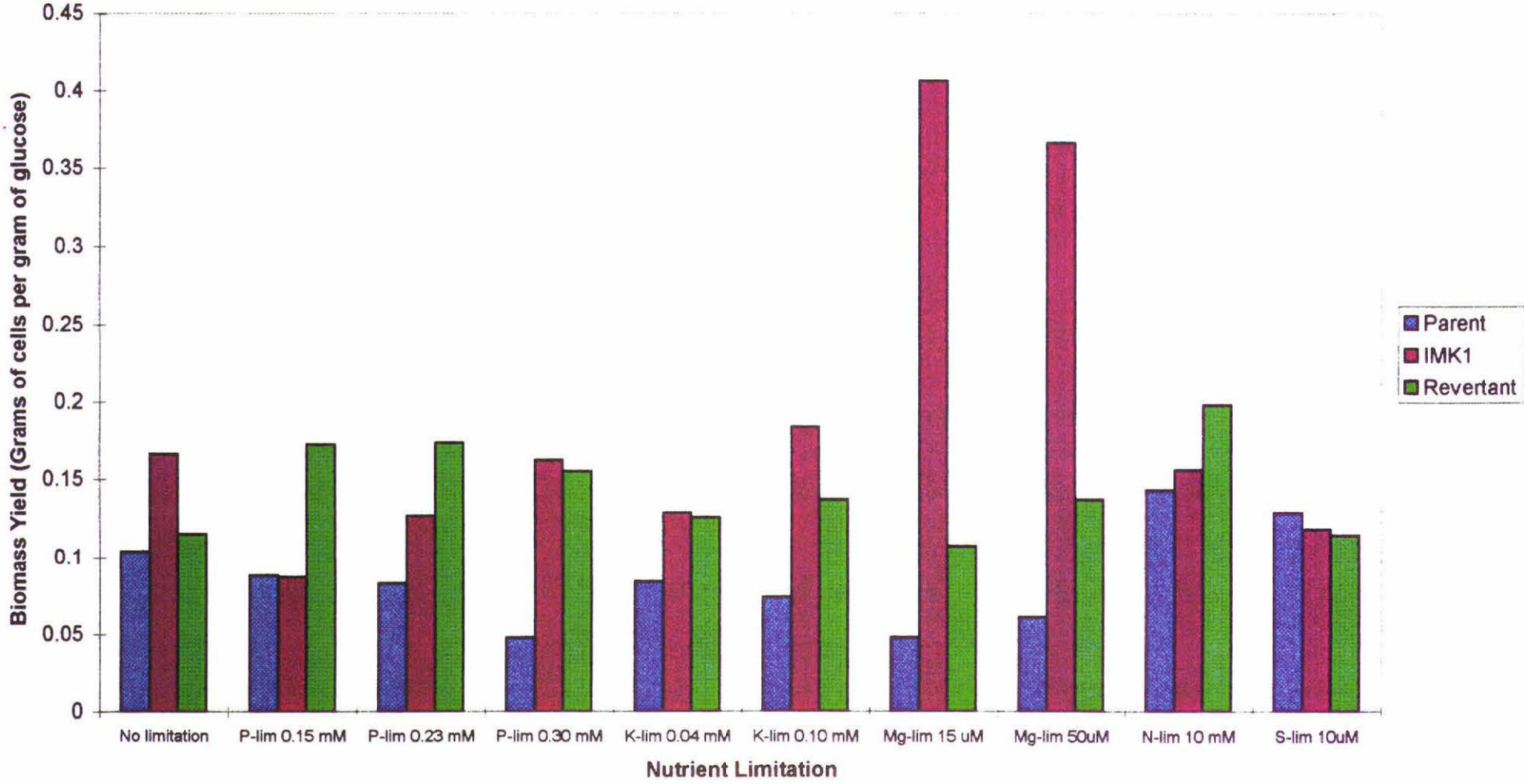
7.4 Biomass Production and Biomass Yield

Comparisons of biomass production and yields can be seen in Table 7.1, Figure 7.3 and in Appendix 5: Table 1.

The mutant cultures originally produced lower levels of biomass than the parent (See Section 5.3). However in the revertant strain, levels of biomass approached that of the parent, and in most cases biomass production per litre of medium exceeded that of the parent by a large margin. The mutant had previously shown some signs of reversion in Experiment two when enzyme activity was assessed. This was observed as a decrease in the production of citric acid, which in turn was associated with an increase in biomass.

This increase in biomass production by the revertant strain demonstrated that the lesions that had previously inhibited growth and production of cell biomass had been repaired or bypassed. In some cases, particularly the cultures grown under phosphorus limitation, the repair was exceedingly efficient, perhaps using

Figure 7.3: Comparison of Biomass Yield (Grams of Cells per Gram of Glucose) for *Candida guilliermondii* - Parent, Mutant and Revertant Strains



different pathways to process metabolites. It is unclear as to which pathways might be involved without extensive analysis for other cycle intermediates.

Table 7.1: Comparison of Biomass (Dry Weights) of parent, mutant and revertant strains after 144 hours.

Limitation	Mutant ($g.L^{-1}$)	Parent ($g.L^{-1}$)	Revertant ($g.L^{-1}$)
No limitation	3.41	6.40	7.10
P- limitation: 0.15 mM	2.80	5.96	9.6
P-limitation: 0.23 mM	3.11	6.31	9.6
P-limitation: 0.30 mM	2.82	3.81	10.6
K-limitation: 0.04 mM	3.49	5.69	9.10
K-limitation: 0.10 mM	3.39	5.47	8.6
Mg-limitation: 15 μM	1.04	2.75	6.2
Mg-limitation: 50 μM	3.00	4.60	10.0
N-limitation: 10 mM	3.83	9.55	10.8
S-limitation: 10 μM	3.82	9.82	8.3

The efficiency of conversion of glucose into biomass became less apparent with comparison of the biomass yields (grams of cells per gram of glucose). The largest increase in yield was seen in the citric acid-producing 0.15 mM phosphorus-limited culture and to a lesser extent the 0.23 mM culture. The culture grown under nitrogen limitation also showed an increase in yield. It was apparent that this increase in biomass yield was a consequence of the loss of ability by the revertant to produce citric acid. All other limitations showed decreases in yields – the magnesium cultures showed the most significant reduction. This reduction in yield in the magnesium cultures can also be accounted for by the increased levels of intermediates and increased enzyme activities observed in the revertant strain.

7.5 Levels of Intermediates Excreted Into the Medium

7.5.1 Isocitric Acid

Levels of isocitric acid produced by the revertant decreased markedly. In many cases, these were less than the levels produced by the parent strain. These results can be seen in Figure 7.4 and in Appendix 5: Table 2

These results suggested that most isocitric acid produced (as with citric acid), was metabolised through the citric acid cycle, and eventually converted into biomass. This reduction of isocitric acid in the mutant coincided with the increase in activity of NAD-ICDH (See Figure 7.8), so showing that there was increased flux of substrate through this enzyme ultimately resulting in the production of cell biomass.

7.5.2 Pyruvate

Levels of pyruvate produced by the revertant decreased significantly, and in many cases again were lower than those produced by the parent strain. Results can be seen in Figure 7.5 and in Appendix 5: Table 2.

This reduction in levels of pyruvate excreted suggested that pyruvate was being channelled through the citric acid cycle for the production of energy and biomass. The high levels of pyruvate that were previously accumulated in the magnesium-limited (15 μM) culture were no longer apparent. It was previously suggested that this accumulation of pyruvate in the magnesium- limited culture was a result of some form of inhibition of transport across the mitochondrial membrane. This was speculated to have been due to inhibition of the membrane ATPase, or to

Figure 7.4: Comparison of Isocitrate production (milligrams per gram of cells) from *Candida guilliermondii* - Parent, Mutant and Revertant strains

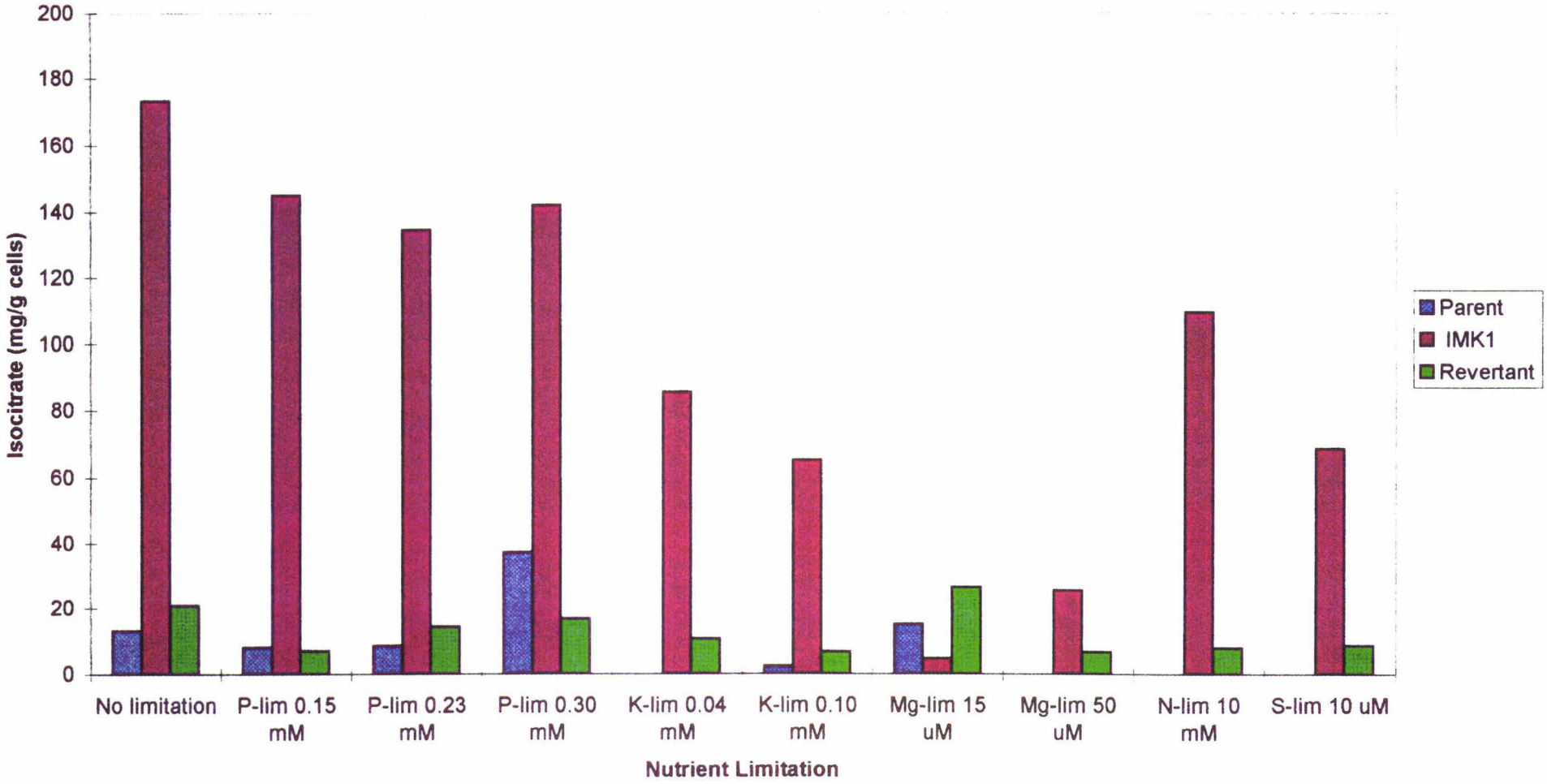
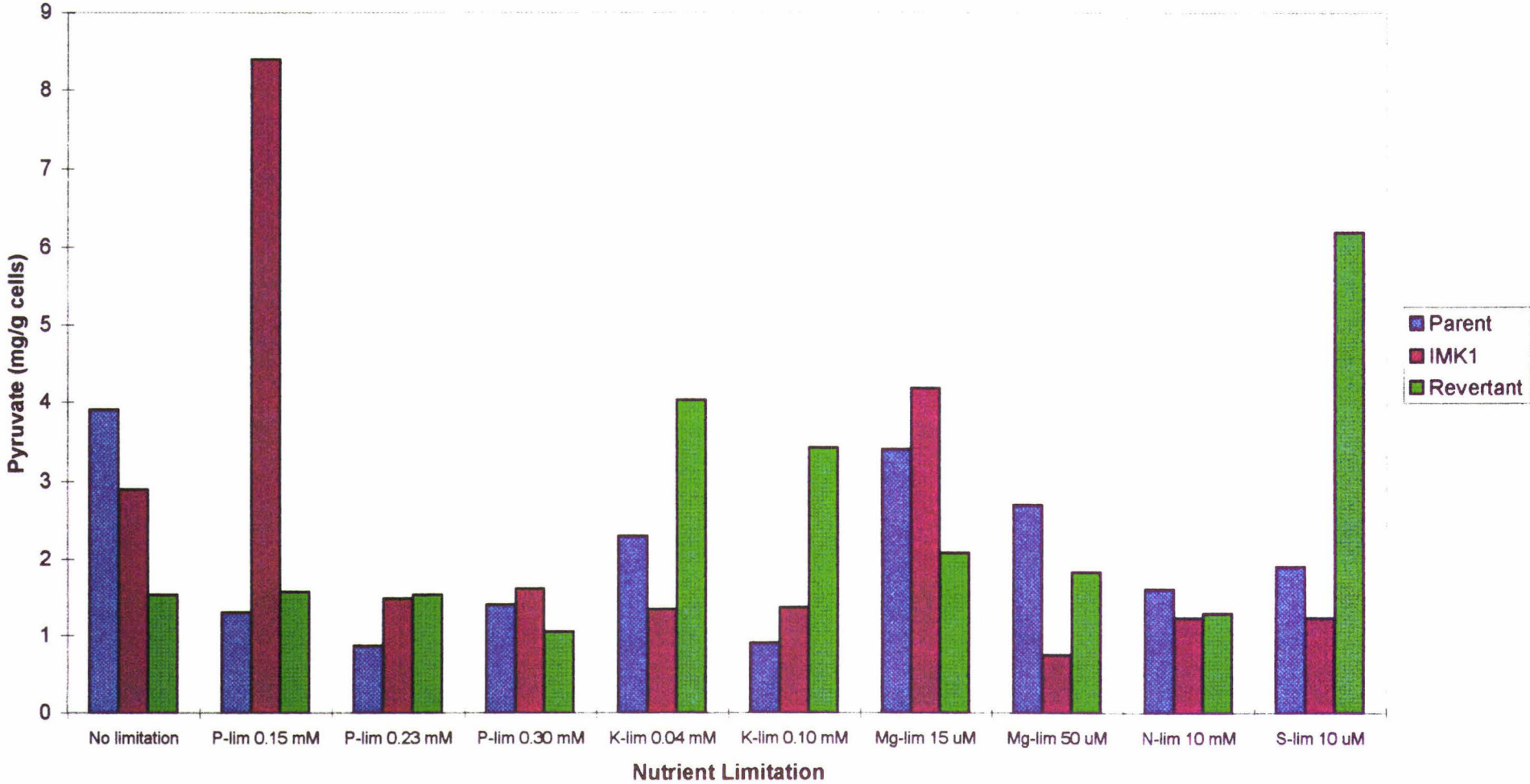


Figure 7.5: Comparison of Pyruvate production (milligrams per gram of cells) from *Candida guilliermondii* - Parent, Mutant and Revertant strains



inhibition of pyruvate dehydrogenase by ATP. It is now suggested that it was the latter (inhibition of pyruvate dehydrogenase), as the limitation of magnesium in the revertant has had little effect on the ability of this particular culture to transport pyruvate into the mitochondrion. However, it was impossible to confirm inhibition of pyruvate dehydrogenase by ATP without data on the concentration of adenine nucleotides within the cell.

The mutant culture grown under phosphorus limitation (0.15 mM) excreted high quantities of pyruvate, but in the revertant levels excreted decreased significantly. It is suggested that this decrease in levels of pyruvate excreted by the revertant was a result of the loss of citric acid-producing ability. Inhibition of the enzyme NAD-ICDH has been reversed, meaning that pyruvate could be utilised as soon as it was produced, eliminating the need for the excretion of excess pyruvate from the cell. It is also possible that the revertant had improved or repaired the mechanism for transport of pyruvate into the mitochondrion – resulting in increased uptake.

The revertant cultures grown under potassium- and sulphur-limited conditions showed significant increases in the levels of pyruvate produced when compared to both the parent and mutant. This again was thought to be a direct consequence of the increased amount of glucose taken up by the revertant strain. These particular limitations showed a slight decrease in the levels of biomass produced, indicating that the extra carbon taken up by these limitations was not used for the production of biomass - as was seen in the phosphorus limitations that previously produced large amounts of citric acid.

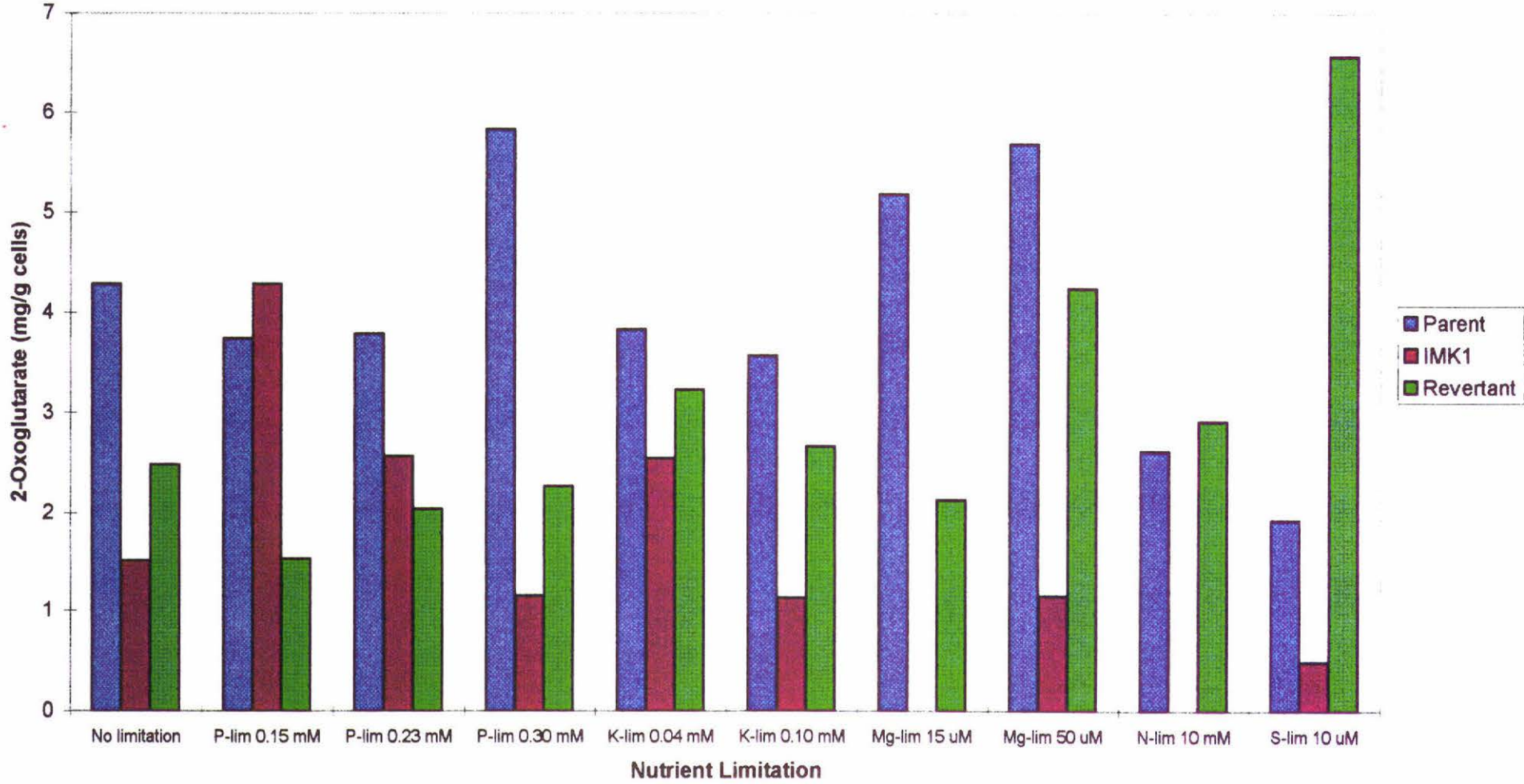
7.5.3 2-Oxoglutarate

Generally, levels of 2-oxoglutarate produced by the revertant strain decreased under the limitations that previously produced citric acid. Results can be seen in Figure 7.6 and in Appendix 5: Table 2.

Increases in the concentration of 2-oxoglutarate excreted by the revertant strain were observed in some limitations, although it must be noted that the overall levels of 2-oxoglutarate detected were low. The most significant increase was seen in the nitrogen- and sulphur-limited cultures. The culture grown under sulphur limitation showed levels of production much higher than those seen previously for both mutant and parent strains. This culture also excreted high levels of pyruvate and produced reduced levels of biomass per gram of glucose consumed. The low levels of fumarate obtained may explain the reason for this large increase (See Figure 7.8). The sulphur-limited revertant culture produced levels of fumarate much lower than the mutant and parent cultures. This suggested that under this particular limitation there might have been a block – or "slow point" - in the cycle, which was preventing the immediate utilisation of 2-oxoglutarate. This may have been due to inhibition of the enzyme 2-oxoglutarate dehydrogenase, which converts 2-oxoglutarate to succinyl-CoA. This enzyme is inhibited by ATP, NADH and succinyl-CoA (See Figure 2.1). It may be that the products of this reaction were accumulating to some extent to cause inhibition. Alternatively, this enzyme may have been inhibited owing to the lack of sulphur, which is required for the formation of succinyl-CoA and so resulting in accumulation.

The levels of 2-oxoglutarate produced by the revertant culture grown under phosphorus limitation (0.15 mm) dropped considerably, indicating increased flux of substrate through the cycle, ultimately resulting in the production of biomass.

Figure 7.6: Comparison of 2-Oxoglutarate production (milligrams per gram of cells) from *Candida guilliermondii* - Parent, Mutant and Revertant strains



The nitrogen-limited culture showed a dramatic increase in 2-oxoglutarate produced: from undetectable levels in the mutant, to levels in the revertant that exceeded those of the parent. This may be accounted for by the increased consumption of glucose, which was also associated with the production of increased levels of biomass - which in this limitation exceeded that of the parent.

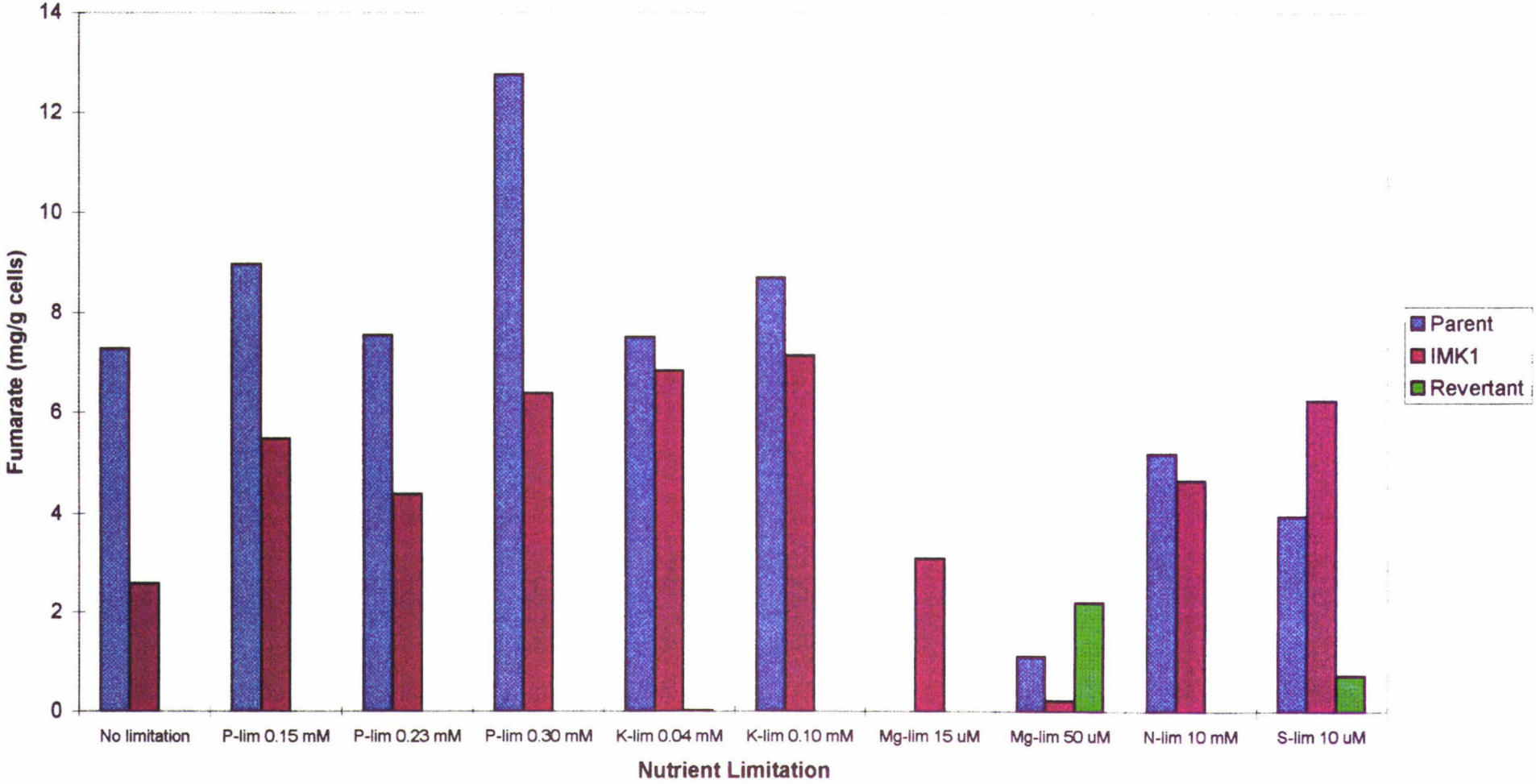
7.5.4 Fumarate

Levels of fumarate in the parent and in the citric-acid producing mutant were high, but in the revertant strain had reduced to levels that were negligible. Results can be seen in Figure 7.7 and in Appendix 5: Table 2.

This reduction in levels indicated that a significant change had occurred in the revertant cultures. It is suggested that any fumarate produced by the revertant strain was utilised immediately for the subsequent steps of the citric acid cycle, preventing any accumulation. It is also speculated that the high levels of intermediates (2-oxoglutarate and pyruvate) noted previously in the nitrogen and sulphur limitations were utilised for the production of biomass, having been removed from the cycle for example, at 2-oxoglutarate - in order to be utilised for the formation of glutamate and amino acids.

Low levels of fumarate were detected in the sulphur- and magnesium-limited revertant cultures. Previously these cultures had shown accumulation of significant levels of pyruvate and 2-oxoglutarate. It is speculated that this accumulation of fumarate occurred as a result of the high levels of 2-oxoglutarate produced, and that these exceeded some "threshold level" of fumarate for the revertant.

Figure 7.7: Comparison of Fumarate production (milligrams per gram of cells) from *Candida guilliermondii* - Parent, Mutant and Revertant strains



7.6 Enzyme Activity

Enzyme activities for the parent, mutant and revertant strains can be seen in Figure 7.8 and in Appendix 5: Table 3.

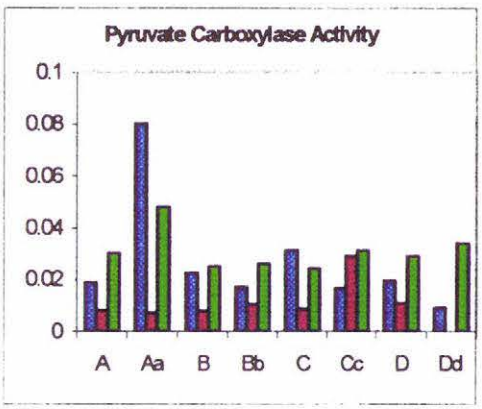
The information provided by the comparison of enzyme activities of the mutant and revertant would help to confirm whether enzyme repair, or reversal of inhibition, has occurred at any of the enzymes of the cycle. It was proposed that it was the reduced activity of NAD-ICDH in the mutant that was largely responsible for the increased levels of accumulation of citric acid. Therefore, significant changes at the site of this, or any other enzyme of the TCA cycle, may provide some indication as to the type of change that occurred resulting in reduced production of citric acid.

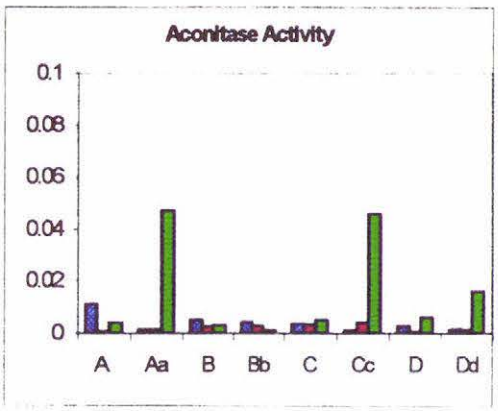
7.6.1 Citrate synthase

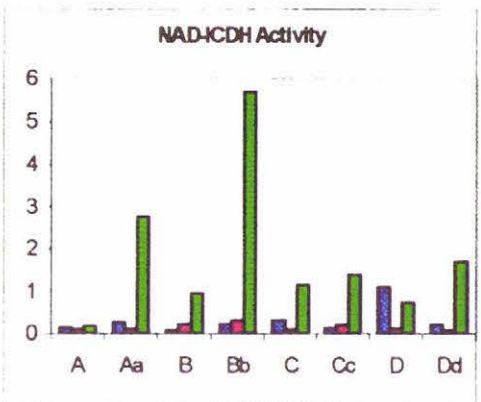
Citrate synthase is responsible for the conversion of acetyl-CoA and oxaloacetate into citric acid. Generally, activities of citrate synthase in the revertant strain were higher than those of the parent and mutant strains. Surprisingly, the magnesium-limited culture showed the highest activity of all limitations in the stationary phase of growth. This was unusual in that this limitation previously was a low producer of both citric acid and biomass. However, this increase in activity in the revertant correlated with the "repair" of glucose uptake into the magnesium-limited cell, in addition to the production of increased levels of intermediates.

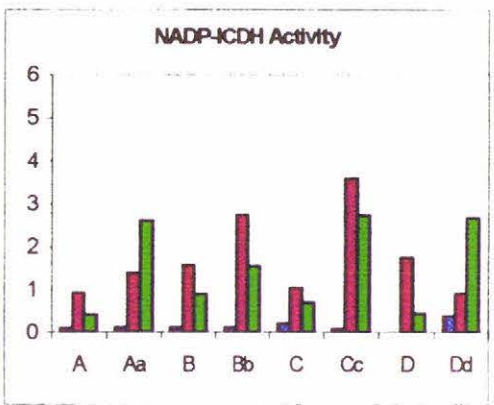
It was previously determined that activity of this enzyme was not an important control point in the citric acid cycle, but that citrate synthase activity was controlled by the availability of substrate. This appeared to be the case for the

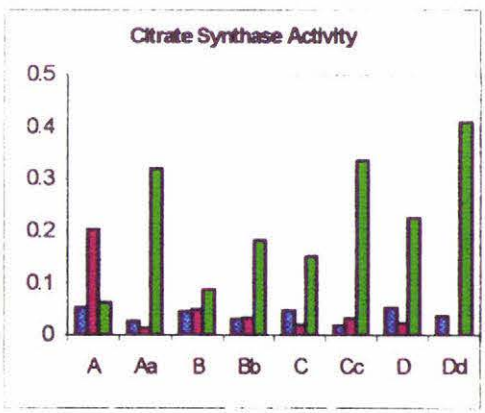
Figure 7.8: Comparison of Enzyme Activities of *Candida guilliermondii* - Parent, Mutant and Revertant Strains











Legend:

A	P-lim 0.15 mM Log
Aa	P-lim 0.15 mM Stat
B	P-lim 0.30 mM Log
Bb	P-lim 0.30 mM Stat
C	N-lim 10 uM Log
Cc	N-lim 10 uM Stat
D	Mg-lim 15 uM Log
Dd	Mg-lim 15 um Stat

Parent
Mutant
Revertant

X-axis: Nutrient Limitation
Y-axis: Enzyme Activity (U/mg Protein)

N.B. Please note the different scales used for each pair of enzymes

revertant strain i.e. increased activity was associated with the increase in glucose consumption. However, activities were observed for the revertant that were much higher than in the phosphorus-limited cultures that previously produced citric acid, even though the revertant consumed less glucose. It was also possible that citrate synthase in the mutant was inhibited to some extent by ATP. If this were the case it would be expected that this inhibition would be reversed in the revertant where limited accumulation of citric acid was taking place.

7.6.2 Aconitase

Aconitase catalyses the reversible reaction between citric acid and isocitrate. Inhibition of this enzyme has been shown to increase the amount of citric acid excreted by citric acid-producing yeasts.

Aconitase activity in all strains was low. Generally, activity of aconitase increased in the revertant in the stationary phase of growth. The largest increases in activity were observed in the 0.15 mM phosphorus culture as well as the magnesium- and nitrogen-limited cultures. This increase in activity of aconitase was probably a result of the increased availability of substrate, combined with the reversal of inhibition of NAD-ICDH. This in turn led to the accumulation of lower levels of citric and isocitric acids, with more substrate then being processed through the cycle.

7.6.3 NAD-dependent Isocitrate Dehydrogenase

When comparisons were made between the mutant and revertant cultures, notable changes in activity of NAD-ICDH became apparent. Activity of the revertant strain under all limitations was found to be higher in the stationary phase of growth when compared to IMK1. This increased activity of NAD-ICDH in the stationary phase of growth suggested that the inhibition of this enzyme seen previously had been reversed and that the enzyme NAD-ICDH was processing more carbon. This was also reflected in the increased biomass production as well as the lowered levels of accumulation of citric and isocitric acids and the increases in levels of 2-oxoglutarate in some limitations.

This reversal in inhibition also suggested that the levels of ATP in the cell had decreased. Decreases in ATP have been associated with increases in ADP and AMP, that in turn cause activation of NAD-ICDH. Unfortunately levels of adenine nucleotides in the cell were not tested after it was discovered that the culture had lost the ability to produce citric acid.

7.6.4 NADP-specific Isocitrate Dehydrogenase

Previously, levels of this enzyme in the mutant were found to be exceptionally high when compared to the activities of other enzymes assessed. Levels in the revertant generally decreased, but were still higher than activity in the parent strain.

It was suggested that in the mutant, the increase in activity of this enzyme was a response to the decrease in activity of NAD-ICDH. It was then proposed that the

increased activity of NADP-ICDH occurred to compensate for the reduced activity of NAD-ICDH, and hence lowered production of reducing power. As the activity of NAD-ICDH had now increased, the higher levels of NADP-ICDH were no longer required.

However, the levels of activity of NADP-ICDH remained at relatively high levels. This may have been a result of the increased activity of citrate synthase observed in the revertant, so providing more substrate for subsequent reactions of the TCA cycle, ultimately resulting in higher biomass.

7.6.5 Pyruvate carboxylase

Levels of pyruvate carboxylase assessed in the revertant culture increased when compared to the mutant. Pyruvate carboxylase is involved in the anaplerotic reaction to provide precursor in the form of oxaloacetate for the citric acid cycle. As the revertant was taking up more carbon in the form of glucose it was possible that this pathway would be in utilisation in order to provide sufficient precursor for the cycle.

With the exception of the phosphorus-limited culture, all revertant limitations assessed showed increased activity of pyruvate carboxylase in the stationary phase of growth. These activities also exceeded that of the parent, the magnesium-limited culture exhibited the most notable change. This increase in activity of pyruvate carboxylase may also help to account for the increased utilisation of glucose and pyruvate in the revertant, in addition to the increase biomass production.

7.7 Possible Site(s) of Repair of the Mutant Strain

From the data obtained from the mutant and revertant it was apparent that changes had occurred in the cell with regard to the metabolism of glucose, and the subsequent production of citric acid:

7.7.1 Glucose Consumption

The most apparent and possibly the most significant change in the revertant strain was the increase in total glucose consumption. The citric acid-producing mutant had previously exhibited lower levels of glucose consumption (when compared to the parent), which was thought to be of significance with regard to the accumulation of citric acid. As the amount of glucose taken up by the revertant strain had increased when compared with mutant IMK1, it was assumed that the inhibition or damage that may have affected the efficiency of the glucose transporting system had been reversed or by-passed to some extent. This increase in glucose consumption when compared to the mutant was also reflected in increased biomass production by the revertant strain.

However, although the revertant culture had shown an increase in glucose consumption, it did not consume as much glucose as the parent. But even so, in some limitations the revertant was able to produce levels of biomass that exceeded those produced by the parent. This increased efficiency of biomass production may be related to the activity of the enzyme NADP-ICDH, which in the revertant was still seen to have increased activity than seen previously in the parent (See Section 7.6.4).

7.7.2 Enzyme Activity

Enzyme activities also showed a significant change especially NAD-ICDH, citrate synthase, and to a lesser extent NADP-ICDH. The reductions in activity of NADP-ICDH along with the rise of NAD-ICDH activity in the revertant culture suggested that NADP-ICDH had a role in the accumulation of citric acid in the mutant strain. It was previously suggested that the increased activity of NADP-ICDH in the mutant was to compensate for the loss of activity NAD-ICDH and the resulting loss of production of reducing power. NADP-ICDH has been shown to have a role in biosynthesis, in that NADPH is required for the formation of glutamate from 2-oxoglutarate. The revertant showed increased levels of activity of NAD-ICDH in addition to levels of activity of NADP-ICDH that were still higher than those in the parent – indicating that the activity of this enzyme may have been significant in the high levels of biomass produced by the revertant.

It was originally intended to assess the levels of ATP in the citric acid-producing mutant strain which would have provided information on the suspected inhibition of NAD-ICDH by ATP, but owing to the rapid reversion of the culture this was not possible. Comparison of levels of ATP in parent, mutant and revertant would help to clarify the effects of these nucleotides on enzyme activity and citric acid production. Assessment of levels of NAD^+ and NADH would also help to confirm their role in biomass production.

7.8 Efficiency of Mutation Method

Activity was lost from the mutant strain both with repeated subculturing, and from stock cultures which were stored under conditions (which with any other yeast strain) had proved to be satisfactory practice in the past.

Many attempts were made to reisolate a citric acid producing mutant from the parent. Mutants were obtained that were shown to produce acid - demonstrated by zones of clearing on the PDA + $CaCO_3$ agar plates within 144 hours.

However, when the isolates were transferred to fermentation media, acid was produced was found not to be citric acid. It is possible that isocitric acid was being produced in large quantities, but no determinations were made. It is not known why it was found to be impossible to isolate another citric acid producing mutant. The mutation and selection process was the same as used previously to obtain IMK1, and so reasons for failure in this instance are unknown.

Therefore, in conclusion, it was apparent that the mutant studied here became unstable, and apart from displaying morphological effects of mutation – as also seen by Gutierrez *et al.* (1992), the cell was also showing some form of inactivation, (See 3.4.3) in that cell division may have been affected. In this case it was not until many generations later that the cell was unable to continue cell division. This suggested that the mutations that resulted in the increased production of citric acid in IMK1 were in fact lethal, but it was not until several hundred generations had passed that these lethal effects of mutation became evident. Although this mutant was able to repair some of the damage caused by UV-irradiation over a period of time – as seen in the revertant, the ultimate consequence was death of the culture. This was also demonstrated by the near complete inability of a mutant stock culture, which was assessed for growth and citric acid production towards the end of this work, to consume glucose and produce significant levels of biomass.

CHAPTER 8

CONCLUSION

8.1 Conclusion

Increased citric acid production in mutant *Candida guilliermondii* IMK1 appeared to be due to a combination of factors, rather than a single effect.

In Experiment one, it was found that the mutant strain, over all limitations, utilised less glucose than the parent and consequently produced less biomass, but produced much higher concentrations of citric acid, particularly in the cultures grown under phosphorus limitation. Accumulations of high concentrations of citric acid are known to correlate with high internal concentrations of ATP within the cell (Mitsushima *et al.* 1977; Marchal *et al.* 1977a; Bartels and Jensen 1979; Botham and Ratledge 1979). The enzyme NAD-ICDH which has been proposed to be an important control point in the production of citric acid, is known to be inhibited by high levels of ATP, as are the enzymes pyruvate dehydrogenase, citrate synthase and 2-oxoglutarate dehydrogenase. It was proposed that increased production of citric acid in the mutant was due primarily to reduced activity of NAD-ICDH caused by ATP, (although it was likely that some inhibition of other enzymes of the cycle had occurred – such as 2-oxoglutarate dehydrogenase).

The magnesium-limited mutant cultures showed low levels of glucose uptake and biomass production, suggesting that limitation of magnesium had affected the activity of the magnesium-requiring plasma membrane ATPase, so resulting in reduced utilisation of glucose. Magnesium limitation was also thought to affect the transport of pyruvate across the mitochondrial membrane, as the magnesium-

limited cultures were shown to excrete relatively high concentrations of pyruvate into the medium. This decreased flux of carbon through the TCA cycle was also reflected in the lower levels of citric acid cycle intermediates accumulated and in the production of low levels of biomass.

The mutant culture grown under phosphorus limitation (0.15 mM), was the best producer of citric acid, and also excreted significant quantities of pyruvate. This suggested that the ability or inability of the mutant to transport pyruvate across the mitochondrial membrane was an important factor in the production of citric acid in this particular limitation. Although the phosphorus-limited cell was taking up more glucose, once this carbon was converted to pyruvate much of it was unable to be utilised through the cycle for the production of biomass. It may be due to this impaired utilisation of pyruvate – possibly due to inhibition of pyruvate dehydrogenase by ATP – that citric acid accumulation occurred in this limitation.

The mutant also showed a tendency to produce more citric acid from the more-limited cultures, this was also associated with higher glucose uptake but lower biomass production. It is speculated that the more-limited cell consumes more glucose in an attempt to produce biomass, but due to limitation of nutrients biomass production is not possible.

In Experiment two, where enzyme activities were assessed, it was found that there had been some loss of citric acid-producing ability from the mutant – in that the total citric acid excreted by the mutant had decreased considerably. However, it was observed that the mutant began to produce citric acid in the logarithmic phase of growth with more significant accumulation occurring within the cell in the stationary phase, with subsequent excretion of acids into the medium. It was likely that this accumulation of citric acid in the stationary phase of growth caused an accumulation of ATP, which subsequently caused inhibition of the enzyme NAD-ICDH. The activity of NAD-ICDH in the mutant had decreased

considerably when compared with the non-citric acid-producing parent. This reduction in activity was expected, owing to the proposed inhibition by increased levels of ATP within the cell that are associated with citric acid production.

NADP-ICDH activity increased considerably in the mutant. NADP-ICDH is not subject to control by ATP, and so it was speculated that increased activity of this enzyme was to compensate for the loss of activity of NAD-ICDH. NADP-ICDH provides NADPH, which is used in the conversion of 2-oxoglutarate to glutamate and the subseqent production of amino acids. It was also suggested that there was some inhibition of the enzyme 2-oxoglutarate dehydrogenase, as evidenced by the accumulation of 2-oxoglutarate.

Activity of citrate synthase was found to be lower in the mutant. It was stated by Boulton and Ratledge (1980) that citrate synthase is not subject to rigorous control by ATP. Therefore it was likely that this lowered activity in the mutant was a reflection on the decreased availability of substrate. This was confirmed with the increase in citrate synthase activity in the revertant, which consumed higher levels of glucose.

The activity of pyruvate carboxylase was seen to play a role in the mutant in topping up levels of oxaloacetate – this was important especially in the mutant as in the latter part of the cycle (from 2-oxoglutarate onwards), the amount of substrate passing through had decreased dramatically.

The revertant culture it was hoped, would provide some clarification as to the reasons for the loss of citric acid production in the mutant - the sites of repair/change in the revertant perhaps indicating the site(s) in the mutant which were responsible for the production of citric acid.

The most significant difference between the revertant and the original mutant was the utilisation of glucose. The loss of citric acid production in the mutant was

accompanied by increased glucose consumption (although not as high as in the parent), and increased biomass production that in most instances exceeded that of the parent strain. This indicated that the revertant had succeeded to some extent in achieving its requirement to produce sufficient levels of cell biomass from lowered levels of glucose consumption.

Generally, levels of citric acid cycle intermediates excreted by the revertant decreased. This indicated that increased utilisation of carbon had occurred throughout the cycle, resulting in the increased production of biomass.

Enzyme activity in the revertant increased when compared to the mutant. Much of this increase was thought to be a result of increased availability of substrate e.g. the activity of the enzymes citrate synthase, aconitase and pyruvate carboxylase all showed increases in activity in the revertant. In particular, the activity of NAD-ICDH increased. This resulted in the reduction of accumulation of citric and isocitric acids. Interestingly, the activity of NADP-ICDH which was very high in the citric acid-producing mutant, showed a decrease of activity in the revertant, but activity was not as low as observed in the parent. The significant activity that was exhibited by this enzyme in the revertant would also help to explain the higher biomass production by this culture.

The different nutrient limitations were also affected differently with regard to the ability to consume glucose and produce citric acid. The mechanisms involved in each nutrient limitation and utilisation of nutrients is beyond the scope of this work.

In conclusion, it would appear that citric acid accumulation in *Candida guilliermondii* IMK1 was due to the following: The consumption of lower levels of glucose (when compared to the parent) combined with some damage or inhibition to the enzyme NAD-ICDH, resulted in the accumulation of citric acid.

Glucose taken up by the cell was converted to pyruvate, which was transported across the mitochondrial membrane to be converted to citric and isocitric acids. These acids began to accumulate in the logarithmic phase of growth, reaching levels in the stationary phase which were speculated to correlate with an increase in concentrations of ATP within the cell. These increases in the concentration of ATP in turn caused inhibition of NAD-ICDH resulting in further accumulation and excretion of acids. It was also proposed that these increased levels of ATP in the cell also caused partial inhibition of pyruvate dehydrogenase, leading to the accumulation and excretion of excess pyruvate from the cell. This was more apparent in the more-limited cultures, which also consumed more glucose, perhaps in an attempt to provide the reducing power needed for biosynthesis. However, owing to the inhibition of critical enzymes of the TCA cycle i.e. NAD-ICDH, production of adequate levels of biomass was not possible. It was also possible that increased concentrations of ATP caused inhibition of glucose consumption i.e. at the level of the enzyme phosphofructokinase, but it was not possible to ascertian this without knowledge of ATP levels within the cell. However, it is possible that the change(s) to the glucose uptake mechanisms of the mutant may have been a result of mutation as may the transport of pyruvate into the cell and may not have been subject to control by ATP.

8.2 Further Work

Although the mutant in this case was lost, apparently due to reversion, a number of factors have been shown to need more explanation.

The effects of each particular type of nutrient limitation on the cell deserve more investigation, especially phosphorus-limited cultures. The stores of phosphorus in the citric acid-producing cell and the subsequent mechanism of mobilization of

these stores may help to explain the production of citric acid from phosphorus-limited cultures.

It would have been advantageous to have been able to assess concentrations of ATP within the cell during both phases of growth, as this would have been the key to understanding many of the activities occurring within the cell. The effects of ATP on the activities of the enzymes of the citric acid cycle would provide useful information with regard to the control of citric acid production, particularly the enzymes NAD-ICDH, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. Unfortunately, due to the instability of the mutant, this was not possible. In addition to determining inhibitory levels of ATP it would be useful to investigate the possible inhibition of glucose consumption and inhibition of TCA cycle reactions by citric and isocitric acids and other cycle intermediates i.e. pyruvate.

In addition, the development of a more stable mutant, and/or development of means of maintenance of the mutant would be advantageous. Owing to the problems encountered in attempting to reisolate the mutant, it is also suggested that development of mutation methods for citric acid producers would be of help.

There is a gap in the literature with regard to the mechanism of excretion of citric acid from citric acid-producing yeasts. More information is required on the mechanism of transport of pyruvate into the mitochondrion, and the subsequent excretion of citric acid from the mitochondrion and into the external medium. Further work in this area would provide more understanding as to the controls involved in the transport of citric acid, and ways of optimising excretion.

APPENDIX 1

LITERATURE

Table A1.1: Summary of Citric Acid Production from Yeasts: Substrates, Methods and Yields.

ORGANISM	SUBSTRATE	CONC	METHOD	CITRIC ACID ($g.L^{-1}$)	ISOCITRIC ACID ($g.L^{-1}$)	RATIO CA:ICA	CA YIELD (g/g)	REFERENCE
Y. lipolytica IMK2*	Glucose	72 g L^{-1}	Shake flask					McKay *et al.* 1994
C. guilliermondii IMK1*	Glucose	72 g L^{-1}	Shake flask					
Y. lipolytica YB 423	Glucose	72 g L^{-1}	Shake flask					
Y. lipolytica A101	Glucose	100 g L^{-1}	Immob. shake	14.95	2.25			Kautola *et al.*1991
Y. lipolytica A101	Glucose	100 g L^{-1}	Air-lift bioreactor	16.4				
C. guilliermondii IMK1*	Glucose	36 g L^{-1}	Shake flask	13.5			0.38	Gutierrez & Maddox 1993
C. guilliermondii NRRL Y448	Glucose	36 g L^{-1}	Shake flask	2.0			0.06	
Y. lipolytica NRRL Y1095	Glucose	20 g L^{-1}	Shake flask	2.4				Abou-Zeid & Khoja 1993
C. tropicalis	Glucose	20 g L^{-1}	Shake flask	2.0				
C. utilis NRRL Y423	Glucose	20 g L^{-1}	Shake flask	1.9				
C. utilis NRRL Y2001	Glucose	20 g L^{-1}	Shake flask	1.8				
C. guilliermondii	Glucose	20 g L^{-1}	Shake flask	1.5				
C. guilliermondii NRRL Y448	Glucose	36 g L^{-1}	Shake flask	2.0			0.06	Gutierrez *et al.*1992
C. guilliermondii IMK1*	Glucose	36 g L^{-1}	Shake flask	13.5			0.38	
Candida sp Y-1	Glucose	30 g L^{-1}	Shake flask	1.65				Tani *et al.* 1990
Candida MA92*	Glucose	30 g L^{-1}	Shake flask	7.2				
S. lipolytica IFP29#*	Glucose	240 g L^{-1}	Batch fermentor	96.1	9.4			Treton *et al.* 1978
Y. lipolytica DS-1	Glucose	200 g L^{-1}	Shake flask	115	60			Shah *et al.* 1989
C. zeylanoides KY6161	Glucose	78 g L^{-1}	Continuous batch	37	4.8			Hattorri *et al.* 1974
S. lipolytica 704 VKM Y2372	Glucose	20 g L^{-1}	Shake flask	5.8	1.1	5.3:1		Finogenova *et al.* 1986
S. lipoytica Mutant 1*	Glucose	20 g L^{-1}	Shake flask	6.5	0.2	32:1		
S. lipolytica Mutant 2*	Glucose	20 g L^{-1}	Shake flask	5.6	3.1	1.8:1		
C. lipolytica Y1095	Glucose	100 g L^{-1}	Batch	0.70		12		Rane & Sims 1995
	Glucose	100 g L^{-1}	Cell recycle	0.65		9		
	Glucose	100 g L^{-1}	Fed Batch	0.56		10		
C. lipolytica Y1095	Glucose	100 g L^{-1}	Batch					Rane & Sims 1994
C. lipolytica Y1095	Glucose	100 g L^{-1}	Batch					
C. lipolytica	Hexadecane	8 g L^{-1}	Shake flask	3.8	5.0	1:1.3		Finogenova *et al.* 1986
C. lipolytica Mutant 1*	Hexadecane	8 g L^{-1}	Shake flask	7.0	0.4	17.5:1		
C. lipolytica Mutant 2*	Hexadecane	8 g L^{-1}	Shake flask	2.0	7.1	1:3.6		
S. lipolytica 102	*n*-alkane	50 g L^{-1}	Continuous Batch					Marchal *et al.* 1977b
C. zeylanoides KY 6161	*n*-alkane	78 g L^{-1}	Batch	56.2	50.5			Hattori *et al.* 1974b

ORGANISM	SUBSTRATE	CONC	METHOD	CITRIC ACID ($g.L^{-1}$)	ISOCITRIC ACID ($g.L^{-1}$)	RATIO CA:ICA	CA YIELD (g/g)	REFERENCE
	n-alkane	78 g L^{-1}	Continuous	65.3	51.7			
C. lipolytica ATCC 20114*	*n*-alkane	80 g L^{-1}	Shake Flask			60:40	(130%)	Akiyama *et al.* 1973
C. lipolytica #	*n*-alkane	50 mL L^{-1}	Continuous	5.84	5.02			Aiba & Matsuoka 1982
C. guilliermondii KY 5822	*n*-alkane	50 g L^{-1}	Shake flask	18	1.7	90:10		Nakanishi *et al.* 1972
C. zeylanoides KY 5802	*n*-alkane	50 g L^{-1}	Shake flask	23.7	14.4	50:50	(150%)	
Torulopsis famata KY 5801	*n*-alkane	50 g L^{-1}	Shake flask	27	15			
C. lipolytica KY 5809	*n*-alkane	50 g L^{-1}	Shake flask	12	8.2			
S. lipolytica MT1002	*n*-alkane	45 g L^{-1}	Shake flask	53.8	4.7		(119.6%)	Furukawa *et al.* 1982
S. lipolytica ON4-9*	*n*-alkane	45 gL^{-1}	Shake flask	45.2	9.9			
S. lipolytica NT1-33*	*n*-alkane	45 g L^{-1}	Shake flask	57.4	9.4			
S. lipolytica MN3-1	*n*-alkane	45 g L^{-1}	Shake flask	67.5	8.3			
S. lipolytica MO6-8	*n*-alkane	45 gL^{-1}	Shake flask	45.4	22.8			
S. lipolytica MU3-28	*n*-alkane	45 g L^{-1}	Shake flask	49.1	11.5			
S. lipolytica IFO 129#*	*n*-alkane	2 mL hr^{-1}	Continuous batch	75.5	37.4			Treton *et al.* 1978
S. lipolytica ATCC 20228	Canola oil	100 g L^{-1}	Batch	109.3	75.3	1.5	(87.9%)	Good *et al.* 1985
S. lipolytica NTG9*	Canola oil	100 g L^{-1}	Batch	137.5	49.2	2.8	(102%)	
S. lipolytica JF2	Canola oil	100 g L^{-1}	Batch	152.3	38.5	3.96	(113.4%)	
C. guilliermondii NRRL Y448	Galactose	36 g L^{-1}	Shake flask	1.2			0.04	Gutierrez *et al.* 1992
C. guilliermondii IMK1*	Galactose	37 g L^{-1}	Shake flask	12.6			0.37	
S. lipolytica	Glycerol	20 g L^{-1}	Shake flask	4.2	0.6	7:1		Finogenova *et al.*1986
S. lipolytica Mutant 1*	Glycerol	20 g L^{-1}	Shake flask	7.4	0.1	74:1		
S. lipolytica Mutant 2*	Glycerol	20 g L^{-1}	Shake flask	5.1	2.4	2.1:1		
S. lipolytica	Acetic acid	20 g L^{-1}	Shake flask	1.8	1.4	1.3:1		
S. lipolytica Mutant 1*	Acetic acid	20 g L^{-1}	Shake flask	4.1	0.3	13.6:1		
S. lipolytica Mutant 2*	Acetic acid	20 g L^{-1}	Shake flask	2.0	2.0	1:1		
S. lipolytica	Ethanol	20 g L^{-1}	Shake flask	1.2	4.0	1:3.3		
S. lipolytica Mutant 1*	Ethanol	20 g L^{-1}	Shake flask	4.7	0.5	9.4:1		
S. lipolytica Mutant 2*	Ethanon	20 gL^{-1}	Shake flask	0.4	0	-		
Candida sp Y-1	Methanol	30 g L^{-1}	Shake flask	1.11				Tani *et al.* 1990
Candida MA92*	Methanol	30 g L^{-1}	Shake flask	2.8				
Candida Y-1	Ethanol	30 g L^{-1}	Shake flask	2.18				
Candida MA92*	Ethanol	30 g L^{-1}	Shake flask	1.8				
S. lipolytica	Glycerol	240 gL^{-1}	Batch	125.5	11.4			Treton *et al.* 1978
C. inconspicua VKM Y740	Ethanol	15 g L^{-1}	Shake flask	1.5	0			Finogenova *et al.* 1991
Y. lipolytica (C.lipolytica 9b)	Ethanol	15 g L^{-1}	Shake flask	2.0	3.7			
C. lipolytica 704 VKM Y2373	Ethanol	15 g L^{-1}	Shake flask	6.4	12.2			

ORGANISM	SUBSTRATE	CONC	METHOD	CITRIC ACID ($g.L^{-1}$)	ISOCITRIC ACID ($g.L^{-1}$)	RATIO CA:ICA	CA YIELD (g/g)	REFERENCE
C. lipolytica 716 VKM Y2376	Ethanol	15 g L^{-1}	Shake flask	1.65	4.2			
P. canadensis VKM Y50	Ethanol	15 g L^{-1}	Shake flask	2.2	0.02			
Sacch. crataegensis VKM Y 2210	Ethanol	15 g L^{-1}	Shake flask	1.45	0.18			
S. fibuliger VKM Y1067	Ethanol	15 g L^{-1}	Shake flask	1.4	0.04			
Y. lipolytica NRRL Y1095	Date-coat sugar extract	25-30 mg mL^{-1}	Shake flask	3.0				Abou-Zeid & Khoja 1993
C. zeylanoides KY 6161	Glycerol	78 g L^{-1}	Continuous	39.0	4.1			Hattori *et al.* 1974b
C. zeylanoides KY 6161	Sucrose	78 g L^{-1}	Continuous	23.0	5.4			
C. zeylanoides KY 6161	Sorbitol	78 g L^{-1}	Continuous	17.0	3.2			
C. zeylanoides KY 6161	Acetic acid	78 g L^{-1}	Continuous	13.0	12.0			
C. zeylanoides KY 6161	Soy Bean oil	78 g L^{-1}	Continuous	51.0	48.0			

* denotes mutant

\# denotes average value of several runs

Table A1.2a: Summary of Citrate Synthase Activity from Citric Acid Producing Yeasts

YEAST	CARBON SOURCE	GROWTH PHASE	ACTIVITY (U/mg protein)	REFERENCE
C. zeylanoides KY 6161	*n*-alkane	Stationary	14.8	Hattori *et al.* 1974
C. lipolytica 704	Ethanol	Exponential	2.3	Finogenova *et al.* 1991
		Retardation	2.0	
		Stationary	2.6	
C. lipolytica	*n*-alkane	Stationary	1.38*	Aiba & Matsuoka 1982
Candida Y-1 (Wild strain)	Methanol	Stationary	33.2	Tani *et al.* 1990
Candida Y-1 (Mutant - MA92)	Methanol	Stationary	42.6	
C. zeylanoides KY 6166	*n*-alkane	Stationary	13.2	Hattori & Suzuki 1974
C. lipolytica (Wild strain)	Glucose	Exponential	1.4	Finogenova *et al.* 1986
		Retardation	0.61	
		Stationary	0.60	
C. lipolytica (Mutant M1-1)	Glucose	Exponential	2.0	Finogenova *et al.* 1986
		Retardation	0.72	
		Stationary	0.66	
C. lipolytica (Mutant M2-1)	Glucose	Exponential	1.14	Finogenova *et al.* 1986
		Retardation	0.52	
		Stationary	0.47	
C. lipolytica (Wild strain)	Hexadecane	Exponential	2.6	Finogenova *et al.* 1986
		Retardation	1.3	
		Stationary	1.0	
C. lipolytica (Mutant M1-1)	Hexadecane	Exponential	3.0	Finogenova *et al.* 1986
		Retardation	2.0	
		Stationary	2.2	
C. lipolytica (Mutant M2-1)	Hexadecane	Exponential	2.12	Finogenova *et al.* 1986
		Retardation	1.93	
		Stationary	1.79	
C. lipolytica (Wild strain)	Glucose	Stationary	0.60	Ermakova *et al.* 1986
C. lipolytica (Mutant 1)	Glucose	Stationary	1.0	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Glucose	Stationary	0.70	Ermakova *et al.* 1986
C. lipolytica (Wild strain)	Hexadecane	Stationary	2.4	Ermakova *et al.* 1986
C. lipolytica (Mutant 1)	Hexadecane	Stationary	2.25	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Hexadecane	Stationary	2.45	Ermakova *et al.* 1986

Table A1.2b: Summary of Aconitase Activity of Citric Acid Producing Yeasts

YEAST	CARBON SOURCE	GROWTH PHASE	ACTIVITY (U/mg protein)	REFERENCE
C. zeylanoides KY 6161	*n*-alkane	Stationary	0.0034	Hattori *et al.* 1974
C. lipolytica 704	Ethanol	Exponential	0.60	Finogenova *et al.* 1991
		Retardation	0.48	
		Stationary	0.90	
Candida Y-1 (Wild strain)	Methanol	Stationary	16.5	Tani *et al.* 1990
Candida Y-1 (Mutant MA92)	Methanol	Stationary	4.50	
C. zeylanoides	*n*-alkane	Stationary	347*	Nakanishi *et al.* 1972
C. guilliermondii	*n*-alkane	Stationary	363.3*	
C. zeylanoides KY 6166	*n*-alkane	Stationary	4.7	Hattori & Suzuki 1974
C. lipolytica (Wild strain)	Glucose	Exponential	0.48	Finogenova *et al.* 1986
		Retardation	0.27	
		Stationary	0.28	
C. lipolytica (Mutant M1-1)	Glucose	Exponential	0.46	Finogenova *et al.* 1986
		Retardation	0.23	
		Stationary	0.22	
C. lipolytica (Mutant M2-1)	Glucose	Exponential	0.57	Finogenova *et al.* 1986
		Retardation	0.42	
		Stationary	0.38	
C. lipolytica (Wild strain)	Hexadecane	Exponential	0.63	Finogenova *et al.* 1986
		Retardation	0.61	
		Stationary	0.61	
C. lipolytica (Mutant M1-1)	Hexadecane	Exponential	0.46	Finogenova *et al.* 1986
		Retardation	0.29	
		Stationary	0.22	
C. lipolytica (Mutant M2-1)	Hexadecane	Exponential	0.98	Finogenova *et al.* 1986
		Retardation	0.82	
		Stationary	0.73	
C. lipolytica (Wild strain)	Glucose	Stationary	0.34	Ermakova *et al.* 1986
C. lipolytica (Mutant 1)	Glucose	Stationary	0.38	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Glucose	Stationary	0.34	Ermakova *et al.* 1986
C. lipolytica (Wild strain)	Hexadecane	Stationary	0.63	Ermakova *et al.* 1986
C. lipolytica (Mutant 1)	Hexadecane	Stationary	0.67	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Hexadecane	Stationary	1.42	Ermakova *et al.* 1986

Table A1.2c: Summary of NAD-Dependent Isocitrate Dehydrogenase Activity of Citric Acid Producing Yeasts

YEAST	CARBON SOURCE	GROWTH PHASE	ACTIVITY	REFERENCE
C. zeylanoides KY 6161	*n*-alkane	Stationary	0.017	Hattori *et al.* 1974
C. lipolytica 704	Ethanol	Exponential	0.11	Finogenova *et al.* 1991
		Retardation	0.08	
		Stationary	0.18	
C. lipolytica	*n*-alkane	Stationary	0.089*	Aiba & Matsuoka 1982
Candida Y-1 (Wild strain)	Methanol	Stationary	3.80	Tani *et al.* 1990
Candida Y-1 (Mutant MA92)	Methanol	Stationary	4.31	
C. zeylanoides	*n*-alkane	Stationary	25.7*	Nakanishi *et al.*1972
C. guilliermondii	*n*-alkane	Stationary	48.23*	
S. lipolytica	n-alkane	Stationary	0.028	Marchal *et al.* 1977a
S. lipolytica	Ethanol	Stationary	0.260	
S. lipolytica	Acetate	Stationary	0.337	
S. lipolytica	Glucose	Stationary	0.110	
C. zeylanoides KY 6166	*n*-alkane	Stationary	50.2	
C. lipolytica (Wild strain)	Glucose	Exponential	0.17	Finogenova *et al.* 1986
		Retardation	0.15	
		Stationary	0.17	
C. lipolytica (Mutant M1-1)	Glucose	Exponential	0.37	Finogenova *et al.* 1986
		Retardation	0.22	
		Stationary	0.21	
C. lipolytica (Mutant M2-1)	Glucose	Exponential	0.11	Finogenova *et al.* 1986
		Retardation	0.12	
		Stationary	0.11	
C. lipolytica (Wild strain)	Hexadecane	Exponential	0.10	Finogenova *et al.* 1986
		Retardation	0.13	
		Stationary	0.15	
C. lipolytica (Mutant M1-1)	Hexadecane	Exponential	0.11	Finogenova *et al.* 1986
		Retardation	0.10	
		Stationary	0.10	
C. lipolytica (Mutant M2-1)	Hexadecane	Exponential	0.04	Finogenova *et al.* 1986
		Retardation	0.05	
		Stationary	0.12	
C. lipolytica (Wild strain)	Glucose	Stationary	0.08	Ermakova *et al.* 1986
C. lipolytica (Mutant 1)	Glucose	Stationary	0.18	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Glucose	Stationary	0.11	Ermakova *et al.* 1986
C. lipolytica (Wild strain)	Hexadecane	Stationary	0.06	Ermakova *et al.* 1986
C. lipolytica (Mutant 1)	Hexadecane	Stationary	0.085	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Hexadecane	Stationary	0.015	Ermakova *et al.* 1986

Table A1.2d: Summary of NADP-Specific Isocitrate Dehydrogenase Activity of Citric Acid Producing Yeasts

YEAST	CARBON SOURCE	GROWTH PHASE	ACTIVITY (U/mg protein)	REFERENCE
C. zeylanoides KY 6161	*n*-alkane	Stationary	0.121	Hattori *et al.* 1974
C. lipolytica	Ethanol	Exponential	1.20	Finogenova *et al.*1991
		Retardation	0.60	
		Stationary	0.50	
Candida Y-1 (Wild strain)	Methanol	Stationary	86.9	Tani *et al.* 1990
Candida Y-1 (Mutant MA 92)	Methanol	Stationary	90.2	
C. zeylanoides	*n*-alkane	Stationary	1096.67*	Nakanishi *et al.* 1972
C. guilliermondii	*n*-alkane	Stationary	236.67*	
S. lipolytica	n-alkane	Stationary	0.175	Marchal *et al.* 1977a
S. lipolytica	Ethanol	Stationary	0.458	
S. lipolytica	Acetate	Stationary	0.278	
S. lipolytica	Glucose	Stationary	0.084	
C. zeylanoides KY 6166	*n*-alkane	Stationary	38.7	Hattori & Suzuki 1974
C. lipolytica (Wild strain)	Glucose	Exponential	0.28	Finogenova *et al.* 1986
		Retardation	0.24	
		Stationary	0.20	
C. lipolytica (Mutant M1-1)	Glucose	Exponential	0.63	Finogenova *et al.* 1986
		Retardation	0.31	
		Stationary	0.21	
C. lipolytica (Mutant M2-1)	Glucose	Exponential	0.34	Finogenova *et al.* 1986
		Retardation	0.27	
		Stationary	0.27	
C. lipolytica (Wild strain)	Hexadecane	Exponential	0.28	Finogenova *et al.* 1986
		Retardation	0.29	
		Stationary	0.26	
C. lipolytica (Mutant M1-1)	Hexadecane	Exponential	0.42	Finogenova *et al.* 1986
		Retardation	0.31	
		Stationary	0.28	
C. lipolytica (Mutant M2-1)	Hexadecane	Exponential	0.17	Finogenova *et al.* 1986
		Retardation	0.14	
		Stationary	0.14	
C. lipolytica (Wild strain)	Glucose	Stationary	0.23	Ermakova *et al.* 1986
C. lipolytica (Mutant 1)	Glucose	Stationary	0.64	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Glucose	Stationary	0.32	Ermakova *et al.* 1986
C. lipolytica (Wild strain)	Hexadecane	Stationary	0.18	Ermakova *et al.* 1986
C lipolytica (Mutant 1)	Hexadecane	Stationary	0.30	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Hexadecane	Stationary	0.14	Ermakova *et al.* 1986

Table A1.2e: Summary of Isocitrate Lyase Activity of Citric Acid Producing Yeasts

YEAST	CARBON SOURCE	GROWTH PHASE	ACTIVITY (U/mg protein)	REFERENCE
C. lipolytica	Ethanol	Exponential	0.26	Finogenova *et al.* 1991
		Retardation	0.08	
		Stationary	0.05	
C. lipolytica	*n*-alkane	Stationary	0.122	Aiba & Matsuoka 1982
Candida Y-1 (Wild strain)	Methanol	Stationary	1.92	Tani *et al.* 1990
CandidaY-1 (Mutant MA92)	Methanol	Stationary	1.00	
S. lipolytica	n-alkane	Stationary	0.178	Marchal *et al.* 1977a
S. lipolytica	Ethanol	Stationary	0.065	
S. lipolytica	Acetate	Stationary	0.050	
S. lipolytica	Glucose	Stationary	0.004	
C. zeylanoides KY 6166	*n*-alkane	Stationary	1.89	
C. lipolytica (Wild strain)	Glucose	Exponential	0.04	Finogenova *et al.* 1986
		Retardation	0.02	
		Stationary	0.02	
C. lipolytica (Mutant M1-1)	Glucose	Exponential	0.16	Finogenova *et al.* 1986
		Retardation	0.04	
		Stationary	0.05	
C. lipolytica (Mutant M2-1)	Glucose	Exponential	0.009	Finogenova *et al.* 1986
		Retardation	0.003	
		Stationary	0.002	
C. lipolytica (Wild strain)	Hexadecane	Exponential	0.47	Finogenova *et al.* 1986
		Retardation	0.08	
		Stationary	0.13	
C. lipolytica (Mutant M1-1)	Hexadecane	Exponential	0.70	Finogenova *et al.* 1986
		Retardation	0.32	
		Stationary	0.35	
C. lipolytica (Mutant M2-1)	Hexadecane	Exponential	0.04	Finogenova *et al.* 1986
		Retardation	0.02	
		Stationary	0.01	
C. lipolytica (Wild strain)	Glucose	Stationary	0.02	Ermakova *et al.* 1986
C. lipolytica (Mutant 1)	Glucose	Stationary	0.07	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Glucose	Stationary	0.006	Ermakova *et al.* 1986
C. lipolytica (Wild strain)	Hexadecane	Stationary	0.40	Ermakova *et al.* 1986
C. lipolytica (Mutant 1)	Hexadecane	Stationary	0.60	Ermakova *et al.* 1986
C. lipolytica (Mutant 2)	Hexadecane	Stationary	0.06	Ermakova *et al.* 1986

APPENDIX 2

MATERIALS AND METHODS

Table A2.1: Nutrient Limitation Experiment - Media and Composition

Medium Component	Limitation									
	Unlimited	Phosphorus			Magnesium		Potassium		Nitrogen	Sulphur
		0.15mM	0.23mM	0.30 mM	15 μM	50 μM	0.10 mM	0.40 mM	10 mM	10 μM
Glucose (10M)	4.0 mL	4.0 mL	4.0 mL	4.0 mL	4.0 mL	4.0 mL	4.0 mL	4.0 mL	4.0 mL	4.0 mL
Antibiotics	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL
Vitamins/Trace	-	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL
Amino Acids	0.25	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL
Fe^{2+}	-	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL
NaCl	-	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL
$CaCl_2$	-	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL
10% Yeast Extract	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL
Yeast Nitrogen Base	10 mL	-	-	-	-	-	-	-	-	-
Na_2SO_4 (0.01M)	-	-	-	-	0.25 mL	0.25 mL	-	-	-	-
KPi (1.5 M)	-	-	-	-	1.0 mL	1.0 mL	-	-	1.0 mL	1.0 mL
NaH_2PO_4 (1.5 M)	-	-	-	-	-	-	1.0 mL	1.0 mL	-	-
Mes-NaOH (15 mM)	-	1.0 mL	1.0 mL	1.0 mL	-	-	-	-	-	-
NH_4Cl (3.0 M)	-	5.0 mL	5.0 mL	5.0 mL	5.0 mL	5.0 mL	5.0 mL	5.0 mL	0.33 mL	5.0 mL
$MgSO_4$ (0.01 M)	-	0.25 mL	0.25 mL	0.25 mL	-	-	0.25 mL	0.25 mL	0.25 mL	0.10 mL
KH_2PO_4 (0.01 M)	-	1.5 mL	2.3 mL	3.0 mL	-	-	-	-	-	-
KCl (0.01 M)	-	-	-	-	-	-	0.40 mL	1.0 mL	-	-
$MgCl_2$ (0.01 M)	-	-	-	-	0.15 mL	0.50 mL	-	-	-	-

Table A2.2: Concentrations of Amino Acids, Vitamins and Trace Elements

Amino Acids	L-Histidine monohydrochloride	10 mg
	LD-Methionine	20 mg
	LD-Tryptophan	20 mg
Vitamins	Biotin	2 μg
	Calcium pantothenate	400 μg
	Folic acid	2 μg
	Inositol	2000 μg
	Niacin	400 μg
	p-Aminobenzoic acid	200 μg
	Pyridoxine hydrochloride	400 μg
	Riboflavin	200 μg
	Thiamine hydrochloride	400 μg
Trace Elements	Boric acid	500 μg
	Copper sulphate	40 μg
	Potassium iodide	100 μg
	Ferric chloride	200 μg
	Manganese sulphate	400 μg
	Sodium molybdate	200 μg
	Zinc sulphate	400 μg

APPENDIX 3

NUTRIENT LIMITATION EXPERIMENT PART 1

Table A.3.1: Optical density at A600 nm of parent strain *Candida guilliermondii NRRL Y-448* at 24 hour intervals.

Limitation	Time (hours)						
	0	24	48	72	96	120	144
None	0	6.47	10.86	15.68	17.39	19.76	22.57
P-lim 0.15 mM	0	8.24	11.16	11.83	13.48	16.84	20.8
P-lim 0.23 mM	0	8.36	11.53	12.38	13.05	17.14	21.05
P-lim 0.30 mM	0	8.97	11.47	11.47	11.83	11.53	12.02
K-lim 0.04 mM	0	9.58	10.74	11.22	12.57	13.97	18.54
K-lim 0.10 mM	0	10.98	11.65	13.91	13.36	13.18	18.12
Mg-lim 15 μM	0	1.77	3.11	3.90	5.73	8.05	7.75
Mg -lim 50 μM	0	5.00	6.10	6.65	7.99	8.42	11.35
N-lim 10 mM	0	9.33	11.29	11.10	15.74	24.34	30.01
S-lim 10 μM	0	10.55	12.69	13.97	14.7	20.8	28.85

Table A3.2: Optical density at A600 nm of mutant strain *Candida guilliermondii IMK1* at 24 hour intervals

Limitation	Time (hours)						
	0	24	48	72	96	120	144
None	0	2.71	6.22	13.36	15.56	17.99	16.96
P-lim 0.15 mM	0	2.91	8.91	10.55	9.52	11.35	14.03
P-lim 0.23 mM	0	2.95	5.92	10.07	9.46	12.51	13.97
P-lim 0.30 mM	0	2.91	7.93	9.76	11.29	12.26	13.91
K-lim 0.04 mM	0	3.16	8.30	11.04	12.81	14.21	15.92
K-lim 0.10 mM	0	2.87	7.08	10.43	12.32	14.7	15.8
Mg-lim 15 μM	0	2.62	4.27	5.37	6.65	5.67	7.26
Mg-lim 50 μM	0	2.75	7.38	8.30	9.21	8.17	12.93
N-lim 10 mM	0	2.83	9.64	11.53	13.50	15.19	16.90
S-lim 10 μM	0	2.83	8.24	11.17	12.63	15.86	16.90

Table A3.3: pH changes of parent strain *Candida guilliermondii NRRL Y-448* over period of fermentation.

Limitation	Sampling Time											
	24 Hours		48 Hours		72 Hours		96 Hours		120 Hours		144 Hours	
	A	B	A	B	A	B	A	B	A	B	A	B
No limitation	2.9	6.1	4.5	6.7	5.2	6.4	4.5	6.9	4.9	6.0	4.8	*
P-lim 0.15 mM	2.4	5.7	3.6	6.0	6.6	#	6.2	#	7.1	6.2	4.4	*
P-lim 0.23 mM	2.4	6.3	3.8	6.8	6.7	#	6.5	#	6.5	#	4.5	*
P-lim 0.30 mM	2.3	6.3	3.7	5.9	6.0	#	6.3	#	7.2	#	7.8	*
K-lim 0.04 mM	2.4	6.0	4.2	6.3	5.9	#	5.9	#	6.4	#	7.4	*
K-lim 0.10 mM	2.4	6.2	4.2	6.1	5.5	6.0	5.8	6.5	6.4	#	6.6	*
Mg-lim 15 µM	5.4	6.8	6.4	#	6.4	#	6.2	#	5.9	#	5.3	*
Mg-lim 50 µM	3.2	6.5	4.5	6.4	5.3	6.6	6.1	#	6.7	#	6.2	*
N-lim 10 mM	2.8	6.2	4.4	6.1	5.6	6.7	7.2	6.5	7.7	6.5	5.3	*
S-lim 10 µM	2.6	6.3	4.1	6.1	5.8	6.6	6.0	#	7.1	6.4	6.2	*

\# No adjustment made to pH

* Cells harvested

A = pH after 24 hours of fermentation

B = pH after adjustment with 10M NaOH

Table A3.4: pH changes of mutant strain *Candida guilliermondii IMK1* over the period of fermentation.

Limitation	Sampling Time											
	24 Hours		48 Hours		72 Hours		96 Hours		120 Hours		144 Hours	
	A	B	A	B	A	B	A	B	A	B	A	B
No limitation	4.2	6.7	6.0	#	3.4	7.3	4.5	6.0	4.5	6.0	6.0	*
P-lim 0.15 mM	4.2	6.1	2.1	6.0	3.8	7.4	4.2	7.1	4.4	6.8	6.8	*
P-lim 0.23 mM	4.3	7.2	3.5	6.8	2.8	6.1	3.6	6.5	3.9	6.2	6.2	*
P-lim 0.30 mM	4.3	7.1	2.7	6.7	3.3	7.0	3.8	7.5	4.3	6.3	6.3	*
K-lim 0.04 mM	4.3	6.1	3.3	6.0	3.9	6.6	3.9	6.5	4.2	6.5	6.5	*
K-lim 0.10 mM	4.2	6.1	3.1	6.1	4.2	6.3	4.2	6.1	4.2	5.9	5.9	*
Mg-lim 15 µM	5.7	#	5.4	#	3.8	5.9	4.3	6.1	5.5	#	5.5	*
Mg-lim 50 µM	5.6	#	2.8	5.9	4.2	6.3	4.6	7.2	6.4	#	6.4	*
N-lim 10 mM	5.7	#	2.5	6.0	4.3	6.5	4.0	6.0	4.1	5.9	5.9	*
S-lim 10 µM	5.6	#	2.5	6.4	4.5	6.4	4.4	6.0	4.5	6.0	6.0	*

\# No adjustment made to pH

* Cells harvested

A = pH after 24 hours of fermentation

B = pH after adjustment with 10M NaOH

Table A3.6: Citric Acid Production and Glucose Consumption from Parent strain *Candida guilliermondii* NRRL Y-448 and Mutant strain *Candida guilliermondii* IMK1 over a fermentation period of 144 hours.

Limitation	Time (hours)	Parent		Mutant	
		Glucose Consumption ($g.L^{-1}$)	Citric Acid Production ($g.L^{-1}$)	Glucose Consumption ($g.L^{-1}$)	Citric Acid Production ($g.L^{-1}$)
Unlimited	0	0	0.00	0.00	0.00
	24	35.12	0.00	5.12	0.007
	48	35.12	0.00	8.19	0.007
	72	73.93	0.00	11.27	3.66
	96	74.13	0.00	18.95	6.18
	120	75.89	0.00	20.49	12.98
	144	75.89	0.00	20.49	12.98
P-lim 0.15 mM	0	0	0.00	0.00	0.00
	24	20.65	0.103	10.57	0.033
	48	71.04	2.13	10.57	0.70
	72	71.04	2.13	17.17	5.41
	96	78.89	7.74	21.91	12.39
	120	78.89	10.02	28.56	14.92
	144	79.46	2.19	32.15	20.55
P-lim 0.23 mM	0	0.0	0.00	0.00	0.00
	24	25.19	0.00	0.00	0.00
	48	78.06	0.00	0.00	0.00
	72	78.06	0.56	8.19	1.72
	96	80.13	11.78	11.78	5.23
	120	80.13	13.08	14.85	16.41
	144	81.12	5.26	24.58	16.41
P-lim 0.30 mM	0	0.00	0.00	0.00	0.00
	24	21.06	0.83	0.00	0.00
	48	64.43	1.11	2.56	0.00
	72	64.43	1.11	8.7	0.92
	96	75.99	1.85	13.31	5.01
	120	76.42	8.12	16.39	12.17
	144	76.42	8.16	17.42	12.17
K-lim 0.04 mM	0	0.00	0.00	0.00	0.00
	24	30.15	0.00	0.51	0.00
	48	72.28	0.37	2.05	0.00
	72	72.28	0.00	11.78	0.30
	96	72.28	0.43	9.73	1.83
	120	75.33	0.43	23.05	6.29
	144	76.67	0.43	27.14	6.30
K-lim 0.10 mM	0	0.00	0.00	0.00	0.00
	24	0.00	0.00	0.51	0.00
	48	5.77	0.94	1.53	0.00

	72	37.80	1.8	9.22	0.00
	96	48.12	1.09	13.31	0.00
	120	45.46	1.23	16.33	4.28
	144	46.39	1.71	18.44	4.28
Mg-lim 15 µM	0	0.00	0.00	0.00	0.00
	24	0.00	0.02	0.00	0.014
	48	2.63	0.05	2.56	0.014
	72	15.78	0.06	2.56	0.014
	96	19.28	0.04	2.56	0.005
	120	16.61	0.08	2.05	1.10
	144	19.81	0.15	2.56	1.10
Mg-lim 50 µM	0	0.00	0.00	0.00	0.00
	24	7.89	0.00	0.00	0.00
	48	19.07	0.00	2.05	0.66
	72	24.99	0.04	2.05	2.42
	96	40.02	0.02	5.63	2.42
	120	47.21	0.09	5.63	4.75
	144	45.34	0.13	8.19	4.75
N-lim 10 mM	0	0.00	0.00	0.00	0.00
	24	16.44	0.69	0.00	0.00
	48	57.86	0.00	0.51	0.00
	72	57.86	0.00	6.14	0.00
	96	57.86	5.9	11.78	0.00
	120	76.87	7.8	15.88	4.75
	144	80.60	9.44	24.58	13.99
S-lim 10 µM	0	0.00	0.00	0.00	0.00
	24	7.89	0.101	0.00	0.00
	48	44.74	0.101	0.00	0.00
	72	44.71	0.052	11.78	0.08
	96	57.36	0.052	21.51	0.19
	120	76.25	5.42	27.66	1.17
	144	76.25	6.23	32.27	1.17

Table A3.7: Biomass Yield (Grams of cells per gram of glucose) for Parent strain *Candida guilliermondii NRRL Y-448* and Mutant strain *Candida guilliermondii IMK1.*

Limitation	Parent	Mutant
No limitation	0.104	0.166
P-lim 0.15 mM	0.088	0.087
P-lim 0.23 mM	0.083	0.127
P-lim 0.30 mM	0.048	0.162
K-lim 0.04 mM	0.084	0.129
K-lim 0.10 mM	0.074	0.184
Mg-lim 15 μM	0.048	0.406
Mg-lim 50 μM	0.061	0.366
N-lim 10 mM	0.143	0.156
S-lim 10 μM	0.129	0.118

Table A3.8: Citrate yield (Grams of citrate per gram of cells) for Parent strain *Candida guilliermondii NRRL Y-448* and Mutant strain *Candida guilliermondii IMK1.*

Limitation	Parent	Mutant
No limitation	0.35	3.81
P-lim 0.15 mM	0.07	7.34
P-lim 0.23 mM	0.49	5.28
P-lim 0.30 mM	0.44	4.29
K-lim 0.04 mM	0.001	1.81
K-lim 0.10 mM	0.02	1.26
Mg-lim 15 μM	0.04	1.06
Mg-lim 50 μM	0.13	1.58
N-lim 10 mM	0.16	3.65
S-lim 10 μM	0.034	0.306

Table A3.9: Substrate yield (grams of citrate per gram of glucose) for Parent strain *Candida guilliermondii NRRL Y-448* and Mutant strain *Candida guilliermondii IMK1.*

Limitation	Parent	Mutant
N$_0$ limitation	0.036	0.633
P-lim 0.15 mM	0.006	0.639
P-lim 0.23 mM	0.040	0.668
P-lim 0.30 mM	0.021	0.695
K-lim 0.04 mM	0.001	0.232
K-lim 0.10 mM	0.002	0.232
Mg-lim 15 μM	0.002	0.430
Mg-lim 50 μM	0.008	0.580
N-lim 10 mM	0.023	0.570
S-lim 10 μM	0.004	0.036

Table A3.10: Maximum specific rates of glucose utilisation for parent strain *Candida guilliermondii NRRL Y-448*, and mutant strain *Candida guilliermondii IMK1*.

Nutrient Limitation	Time (Hours)	Specific Rate: Parent ($g\ g^{-1}\ h^{-1}$)	Time (Hours)	Specific Rate: Mutant ($g\ g^{-1}\ h^{-1}$)
No limitation	48	0.625	120	0.244
P-lim 0.15 mM	48	1.43	96	0.149
P-lim 0.23 mM	48	1.1	96	0.215
P-lim 0.30 mM	48	0.869	96	0.155
K-lim 0.04 mM	48	0.963	144	0.143
K-lim 0.10 mM	48	0.611	96	0.177
Mg-lim 15 μM	72	1.54	72	0.202
Mg-lim 50 μM	72	1.48	120	0.956
N-lim 10 mM	24	0.767	144	0.091
S-lim 10 μM	24	0.953	96	0.224

Table A3.11: Maximum specific rates of citric acid production for parent strain *Candida guilliermondii NRRL Y-448*, and mutant strain *Candida guilliermondii IMK1*.

Nutrient Limitation	Time (Hours)	Specific Rate: Parent ($g\ g^{-1}\ h^{-1}$)	Time (Hours)	Specific Rate: Mutant ($g\ g^{-1}\ h^{-1}$)
No limitation	72	0.017	144	0.078
P-lim 0.15 mM	120	0.018	120	0.239
P-lim 0.23 mM	120	0.016	144	0.153
P-lim 0.30 mM	48	0.027	144	0.105
K-lim 0.04 mM	24	0.001	144	0.054
K-lim 0.10 mM	48	0.002	144	0.051
Mg-lim 15 μM	144	0.0004	144	0.042
Mg-lim 50 μM	144	0.006	144	0.032
N-lim 10 mM	120	0.018	144	0.101
S-lim 10 μM	48	0.01	120	0.016

Table A3.12: Levels of Intermediates (Milligrams per gram of cells) excreted into the fermentation medium by parent strain *Candida guilliermondii NRRL Y-448* and mutant strain *Candida guilliermondii IMK1* after 144 hours

Limitation	Glucose Consumed		Citrate Produced		Isocitrate		Pyruvate		2-Oxoglutarate		Fumarate		Malate	
	Parent	Mutant	Parent	Mutant	Parent	Mutant	Parent	Mutant	Parent	Mutant	Parent	Mutant	Parent	Mutant
No Limitation	9.58	6.01	0.35	3.81	12.97	173.03	3.90	2.90	4.28	1.51	7.28	2.57	2.70	1.53
P-lim 0.15mM	11.35	11.48	0.07	7.34	7.92	144.81	1.30	8.40	3.74	4.28	8.98	5.47	0.00	8.34
P-lim 0.23 mM	12.03	7.90	0.49	5.28	8.43	134.36	0.87	1.48	3.79	21.34	7.54	4.38	0.00	5.67
P-lim 0.30 mM	20.64	6.18	0.44	4.29	37.19	141.86	1.40	1.61	5.84	9.48	12.75	6.38	0.30	8.89
K-lim 0.04 mM	11.91	7.78	0.001	1.81	0.00	85.27	2.30	1.34	3.83	17.90	7.50	6.84	3.34	11.34
K-lim 0.10 mM	13.45	5.44	0.02	1.26	2.17	64.94	0.90	1.36	3.57	9.36	8.70	7.14	4.58	11.39
Mg-lim 15 μM	21.04	2.46	0.04	1.06	15.02	44.42	3.40	4.18	5.18	0.00	0.00	3.08	0.00	2.70
Mg-lim 50 μM	16.38	2.73	0.13	1.58	0.00	25.72	2.70	0.75	5.69	9.48	1.10	0.21	5.56	0.00
N-lim 10 mM	7.01	6.42	0.16	3.65	0.00	109.57	1.60	1.23	2.62	0.00	5.19	4.65	0.00	6.97
S-lim 10 μM	7.78	8.45	0.034	0.306	0.00	68.58	1.90	1.23	1.93	3.97	3.94	6.25	0.00	9.64

APPENDIX 4

NUTRIENT LIMITATION EXPERIMENT PART 2

Table A4.1: Comparison of Internal and External Metabolites (Milligrams per gram of cells) of Parent *Candida guilliermondii* NRRL Y-448 in Logarithmic and Stationary Phases of Growth.

Limitation	Time (Hours)	Citric Acid		Isocitric Acid		Pyruvate		Malate		2-Oxoglutarate		Fumarate	
		Internal	External	Internal	External	Internal	External	Internal	External	Internal	External	Internal	External
P-lim 0.15 mM	48	0.079	0.309	1.47	68.71	0.047	2.42	0.00	1.93	17.19	9.72	0.00	1.06
	120	0.114	0.376	1.65	101.88	0.103	5.31	0.00	3.48	25.85	11.30	0.00	1.46
P-lim 0.30 mM	48	0.088	0.250	1.29	75.31	0.050	2.63	0.00	4.49	19.07	11.32	0.00	1.14
	120	0.114	0.266	1.83	117.79	0.007	3.17	0.00	3.57	25.34	16.41	0.00	1.75
N-lim 10 mM	48	0.120	0.177	1.55	117.33	0.100	5.47	0.00	2.73	24.69	6.49	0.00	1.30
	120	0.100	0.229	1.62	147.05	0.143	6.77	0.00	5.85	21.71	7.94	0.00	2.84
Mg-lim 15 μM	48	0.107	0.110	2.45	89.94	0.207	1.76	0.00	0.00	25.49	3.10	0.00	0.00
	120	0.071	0.082	1.66	48.52	0.010	3.67	0.00	0.00	23.67	7.95	0.00	0.00

Table A4.2: Comparison of Internal and External Metabolites (Milligrams per gram of cells) of Mutant *Candida guilliermondii* IMK1 in Logarithmic and Stationary Phases of Growth.

Limitation	Time (Hours)	Citric Acid		Isocitric Acid		Pyruvate		Malate		2-Oxoglutarate		Fumarate	
		Internal	External	Internal	External	Internal	External	Internal	External	Internal	External	Internal	External
P-lim 0.15 mM	48	0.070	0.000	32.27	4.09	0.70	0.90	0.00	0.00	59.25	21.03	0.03	0.00
	120	5.291	13.636	30.85	26.75	0.46	1.18	0.00	0.66	60.09	61.51	0.00	0.00
P-lim 0.30 mM	48	0.0313	0.212	11.23	10.40	0.07	0.45	0.00	0.00	22.64	10.59	0.00	0.00
	120	3.444	0.746	20.40	16.94	0.12	1.18	0.37	7.92	40.96	44.58	2.10	3.00
N-lim 10 mM	48	0.0967	0.077	11.61	11.29	0.02	0.32	0.21	6.00	29.09	81.47	0.00	2.45
	120	3.448	1.746	16.30	16.99	0.52	0.36	0.81	4.98	29.86	85.31	0.00	1.30
Mg-lim 15 μM	48	0.065	0.050	14.19	8.55	0.15	0.55	0.00	1.11	34.10	27.67	0.00	0.00
	120	0.2483	0.193	10.51	10.72	0.07	3.17	8.42	3.43	49.85	50.93	4.52	0.49

Table A4.3: Enzyme Activities (U/mg protein) of parent strain *Candida guilliermondii NRRL Y-448* and mutant strain *Candida guilliermondii* IMK1 in logarithimic and stationary growth phases.

Limitation	Time (hours)		Protein (mg/mL)		Citrate synthase		Aconitase		NAD-ICDH		NADP-ICDH		Pyruvate carboxylase	
	Parent	Mutant	Parent	Mutant	Parent	Mutant	Parent	Mutant	Parent	Mutant	Parent	Mutant	Parent	Mutant
P-lim 0.15 mM	48	72	3.32	8.44	0.0514	0.0202	0.0108	0.0006	0.1225	0.0878	0.0897	0.9190	0.0187	0.00810
	120	120	3.67	6.94	0.0254	0.0113	0.0012	0.0014	0.2600	0.0885	0.1057	1.386	0.0801	0.00712
P-lim 0.30 mM	48	72	3.67	5.11	0.0435	0.0479	0.0048	0.0027	0.0628	0.2260	0.1022	1.563	0.0225	0.0078
	120	120	3.29	3.90	0.0295	0.0315	0.0043	0.0029	0.2156	0.3197	0.1116	2.739	0.0172	0.0103
N-lim 10 mM	48	72	3.16	6.33	0.0457	0.0182	0.0036	0.0033	0.3102	0.0771	0.2050	1.051	0.0311	0.0086
	120	120	3.43	3.33	0.0163	0.0313	0.0011	0.0042	0.1276	0.2117	0.0849	3.603	0.0165	0.0290
Mg-lim 15 µM	48	72	3.37	4.71	0.0508	0.0221	0.0028	0.0004	1.0965	0.1151	0.0000	1.753	0.0195	0.0110
	120	144	3.02	10.66	0.0357	0.0000	0.0016	0.0012	0.2002	0.05894	0.3769	0.900	0.0091	0.0000

APPENDIX 5

REVERSION OF *CANDIDA GUILLIERMONDII* IMK1

Table A5.1: Comparison of Citric Acid Production, Glucose Consumption and Biomass Yield from *Candida guilliermondii* - Parent, Mutant and Revertant strains

Limitation	Citric Acid Production (grams/gram cells)			Glucose Consumption (grams/gram cells)			Biomass Yield (grams/gram glucose)		
	Parent	IMK1	Rev	Parent	IMK1	Rev	Parent	IMK1	Rev
No Limitation	0.35	3.81	0.27	9.58	6.01	8.71	0.104	0.166	0.115
P-limitation 0.15 mM	0.07	7.34	0.181	11.35	11.48	5.80	0.088	0.087	0.172
P-limitation 0.23 mM	0.49	5.28	0.175	12.03	7.90	5.77	0.083	0.127	0.173
P-limitation 0.30 mM	0.44	4.29	0.175	20.64	6.18	6.46	0.048	0.162	0.155
K-limitation 0.04 mM	0.001	1.81	0.153	11.91	7.78	7.92	0.084	0.129	0.126
K-limitation 0.10mM	0.02	1.26	0.079	13.45	5.44	7.30	0.074	0.184	0.137
Mg-limitation 15μM	0.04	1.06	0.087	21.04	2.46	9.38	0.048	0.406	0.107
Mg-limitation 50μM	0.13	1.58	0.085	16.38	2.73	7.32	0.061	0.366	0.137
N-limitation 10 mM	0.16	3.65	0.131	7.01	6.42	5.06	0.143	0.156	0.198
S-limitation 10 μM	0.034	0.306	0.037	7.78	8.45	8.81	0.129	0.118	0.114

Table A5.2: Comparison of Levels of Intermediates (mg/g cells) Excreted by *Candida guilliermondii* - Parent, Mutant and Revertant strains after 144 hours

Limitation	Isocitrate			Pyruvate			2-Oxoglurarate			Fumarate		
	Parent	IMK1	Rev	Parent	IMK1	Rev	Parent	IMK1	Rev	Parent	IMK1	Rev
No Limitation	12.97	173.03	20.77	3.90	2.90	1.53	4.28	1.51	2.48	7.28	2.57	0.00
P-lim 0.15 mM	7.92	144.81	6.76	1.30	8.40	1.57	3.74	4.28	1.53	8.98	5.47	0.00
P-lim 0.23 mM	8.43	134.36	14.13	0.87	1.48	1.53	3.79	2.57	2.04	7.54	4.38	0.00
P-lim 0.30 mM	37.19	141.86	16.70	1.40	1.61	1.05	5.84	1.15	2.26	12.75	6.38	0.00
K-lim 0.04 mM	0.00	85.27	10.37	2.30	1.34	4.03	3.83	2.55	3.23	7.5	6.84	0.01
K-lim 0.10 mM	2.17	64.94	6.48	0.90	1.36	3.42	3.57	1.13	2.66	8.7	7.14	0.00
Mg-lim 15 µM	15.02	44.42	26.64	3.40	4.18	2.08	5.18	0.00	2.13	0.00	3.08	0.00
Mg-lim 50 µM	0.00	25.72	6.49	2.70	0.75	1.83	5.69	1.15	4.24	1.10	0.21	2.19
N-lim 10 mM	0.00	109.57	7.65	1.60	1.23	1.29	2.62	0.00	2.92	5.19	4.65	0.00
S-lim 10 µM	0.00	68.58	8.53	1.90	1.23	6.19	1.93	0.49	6.57	3.94	6.25	0.73

Table A5.3: Comparison of Enzyme Activities (U/mg protein) of *Candida guilliermondii* - Parent, Mutant and Revertant strains

Limitation	Time (hours)	Citrate synthase			Aconitase			NAD-ICDH			NADP-ICDH			Pyruvate Carboxylase		
		Parent	IMK1	Rev	Parent	IMK1	Rev	Parent	IMK1	Rev	Parent	IMK1	Rev	Parent	IMK1	Rev
P-lim 0.15 mM	72	0.0514	0.2020	0.061	0.0108	0.0006	0.004	0.1225	0.0878	0.174	0.0897	0.9190	0.400	0.0187	0.0081	0.030
	120	0.0254	0.0113	0.319	0.0012	0.0014	0.047	0.2600	0.0885	2.753	0.1057	1.3860	2.597	0.0801	0.0071	0.048
P-lim 0.30 mM	72	0.0435	0.0479	0.087	0.0048	0.0027	0.003	0.0628	0.2260	0.940	0.1022	1.5630	0.883	0.0225	0.0078	0.025
	120	0.0295	0.0315	0.182	0.0043	0.0029	0.001	0.2156	0.3197	5.692	0.1116	2.7390	1.543	0.0172	0.0103	0.026
N-lim 10 mM	72	0.0457	0.0182	0.151	0.0036	0.0033	0.005	0.3102	0.0771	1.134	0.2050	1.0510	0.703	0.0311	0.0086	0.024
	120	0.0163	0.0313	0.335	0.0011	0.0042	0.046	0.1276	0.2117	1.383	0.0849	3.6030	2.727	0.0165	0.0290	0.031
Mg-lim 15µM	72	0.0508	0.0221	0.224	0.0028	0.0004	0.006	1.0965	0.1151	0.746	0.000	1.7530	0.438	0.0195	0.0110	0.029
	144	0.0357	0.0000	0.408	0.0016	0.0012	0.016	0.2002	0.0589	1.701	0.3769	0.9000	2.663	0.0091	0.0000	0.034

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