University of Strathclyde

Metabolic Analysis of Genetically Engineered Micro-Organisms

By

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Master of Philosophy

2012

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed *Date*

Acknowledgements:

I wish to acknowledge and thank the following individuals for their contribution to the production of this thesis: My Supervisor, Dr M. Mattey; Dr D. M. Mousdale, and especially my parents James and Helen Dowdells for all their love and support.

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ABSTRACT

The Aspergilli are important industrial microorganisms used for the production of organic acids and enzymes. In this project the main objective was to increase productivity in industrial micro-organisms grown on glucose as the sole carbon source by expressing *A. niger* genes coding for the enzyme 6-phosphofructo-1-kinase (PFK). *A. terreus* was chosen as the recipient organism as it is closely related to *A. niger* and would hopefully be receptive to expression of *A. niger* genes.

The project aimed to engineer the metabolism of *A. terreus* to use features of *A. niger* such as its high productivity and yield which, along with optimisation of production and fermentation media has resulted in up to 80% of substrate (sucrose) being converted into the product, citric acid. Using this knowledge it is anticipated that the rates of glucose utilisation and glycolysis will be increased so that the precursors for the production of industrially important products are produced in higher yields by *A. terreus*.

When glucose is utilised during a fermentation, *A. terreus* is known to produce itaconic acid at low pH and lovastatin and other secondary metabolities at higher pH. However, when a starting pH of 6.5 was used that would favour lovastatin production, a novel pathway of glucose catabolism was demonstrated in this study by both wild type *A. terreus* (without any strain development) and by transformants containing the *A. niger* genes encoding PFK. Gluconic acid was produced by *A. terreus* at high yields (up to 0.7 mole per mole of glucose consumed). Although *A. niger* is used on an industrial scale to biomanufacture gluconic acid, this is the first demonstration of gluconic acid production by *A. terreus*. The implications for fermentation control with *A. terreus* when used for the production of secondary metabolites at high pH are explored.

Although glucose utilisation was not clearly different between the parental and modified strains, the modified strain produced less gluconic acid than the parental strain. This was found to be glucose concentration-dependent because accumulated gluconic acid could be utilised when glucose was exhausted in the fermentation medium. This knowledge furnishes new strategies for metabolite production with *A. terreus* .

The major significance of study is the realisation of *A. terreus* as a potential novel producer organism for gluconic acid which has many and varied uses as a bulk chemical. Employing the new understandings of glucose catabolism by *A. terreus*, new strategies for secondary metabolite production could be anticipated with glucose feeding employing pH as a feedback regulation.

This research work was funded by the European Commission under the international project ANTICO (Aspergillus Niger Transformed Into Commercial Organisms.

1. Literature Review

1.1 Introduction - History of Fermentation Technology

Fermentation technology is very old in human history; with the fermentation of sugars to ethanol (alcohol), archaeology has shown that this use of "biotechnology" dates back to the ancient Egyptians, at least as far back as 1500BC (El Mansi *et al*., 1999). The modern science of microbiology and, in particular, fermentation microbiology, came to fruition with the pioneering work of Pasteur in the 1850s showing how fermentation was associated with living cells.

Before the end of the nineteenth century, the concept that the biochemical actions of cells (later described as enzymes) were responsible for the fermentations involved in alcohol production and bread making had been developed (El Mansi *et al*., 1999**)**. In the twentieth century, microbial production of amino acids (Nakayama and Soda, 1998; Ericson and Kurz, 1962) and antibiotics, (Lam, 1998; Trepanier *et al*., 2002; Chater and Chandra, 2008) became the foundations of industrial scale bioprocesses. Since the work carried out with penicillins by Howard Florey in the 1930s, a considerable number of antibiotics and related secondary products of medical value have been produced and subsequently been commercialised (Lam, 1998).

Recent evidence of increased microbial resistance to antibiotics by microbes such as penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant Enterococci, methicillin-resistant *Staphylococcus aureus*, multidrug-resistant Salmonellae and *Mycobacterium tuberculosis* causing diarrhoeal diseases, respiratory tract infections, meningitis, sexually transmitted infections and hospital acquired infections (WHO, 2002; WHO 2007) are giving great cause for concern among health professionals.
It is therefore of great importance for scientists to look into other possible ways of eradicating or controlling the spread of these resistant strains. Developing more

efficient fermentation technologies to manufacture known, but little used antibiotics, is one strategy to diversify the range of antibiotics for clinical use. In addition, the ability to explore exotic places once thought of as inaccessible, such as tropical rain forests, volcanic spa regions and the great oceans, have thrown up new and diverse environments containing as yet unidentified microbial fauna which could address these concerns with their novel antibiotic and pharmaceutically valuable properties; once discovered, however, fermentation technologies for such microbes must be available or easily developed.

1.2 The ANTICO Project – Fundamentals

The main objective of the research borne out of the ANTICO project (*Aspergillus Niger* Transformed Into Commercial Organisms) was to increase productivity in industrial micro-organisms grown on glucose as sole carbon source by expressing *A. niger* genes coding for the enzymes 6-phosphofructo-1-kinase (PFK) and alternative oxidase (AOX), into another filamentous fungus (*A. terreus*), a yeast (*P. pastoris*) and into two distinct, industrially important, Streptomycetes: *S. rimosus S. clavuligerus.* During the project some rather unexpected results were obtained and additional avenues of research were explored, namely, discovery of a novel pathway for gluconic acid in *A. terreus*. The research reported here developed out of the ANTICO project; it was not part of the original intention and the work reported was additional to the Antico objectives.

Citric acid-producing strains of the fungus *A. niger* are highly efficient at metabolising glucose to the commercially important acid product. At the enzymatic level, this extraordinary productivity depends on the properties of two enzymes: phosphofructokinase (PFK) and a non-energy conserving alternative oxidase (AOX). ANTICO, a collaborative project funded under the European Commission Fifth Framework, genetically engineered the metabolism of other microbial species to use

these features and modify their use of glucose so that the production of a wide spectrum of other industrially important products was enhanced; this was hypothesised to be achieved by the following steps.

1. Glucose uptake rate was increased by having a higher initial glucose concentration in the medium, which in turn resulted in a more pronounced driving force that stimulated simple diffusion as a consequence of higher differences in concentration gradients.

2. Normally a high intracellular glucose concentration would prove toxic for cells as the uncontrolled phosphorylation of glucose would result in a build-up of the phospho-intermediates and ultimately a reduction in the amount of inorganic phosphate required for ATP synthesis, this coupled with the restricted availability of NAD^+ during the later stages of glycolysis would ultimately result in death of the cells, attributable to the significantly increased glucose concentrated media. However, if the incoming glucose were to be rapidly metabolised by up-regulated glycolysis, this would allow the cells to grow and make products in a glucose rich media without hitting any of the aforementioned problems.

3. To encourage metabolic flux through glycolysis, the gene encoding 6-phospho-1-fructokinase from the NRRL 2270 strain of *A. niger* was added to the original set of recipient genes. The known sequence of *pfkA* gene (EMBL Accs. No. Z79690) from the wild type of *A. niger* enabled the cloning and isolation of the gene from the NRRL 2270 strain gene library. A truncated gene coding for an enzyme shortened at the C-terminal part of the protein molecule was prepared and initially tested in *A. niger* cells however the enzyme still needed phosphorylation for activation. There were two options for inducing phosphorylation: either spontaneously inducing environmental conditions that trigger cAMP synthesis or by introducing *pkaC* gene coding for the catalytic subunit of cAMP-dependent protein kinase. Transformants were prepared in

which *pkaC* was under the control of inducible promoters, as well as expressed constitutively at low levels.

4. There was good evidence that the transfer of the *A. niger* alternative oxidase gene (*aox1*) into recipient micro-organisms should contribute to an increase in citrate cycle intermediates by uncoupling the oxidation of protons from the synthesis of ATP. The gene was cloned from an *A. niger* gene library, modified appropriately and transformed into recipient micro-organisms. Although *aox*1 is a mitochondrial enzyme, there was enough information available from the literature to suggest how the gene should be modified to be fully functional in bacterial cells.

My role within the project was to carry out any analysis to gauge the yield and productivity of each organism from all consortium member's after all genetic modifications and fermentations had taken place; however the main body of my research became focussed entirely on the novel pathway for gluconic acid production found in *A. terreus* discussed below and further in Chapter 3

1.1 .1 The ANTICO Project - *Aspergillus niger*

1.2.2 Nutrient inputs to fermentations

Media employed in the cultivation of micro-organisms must have an ample supply of all elements imperative for the propagation of the cell substance and the eventual production of metabolic products. In small-scale research using micro-organisms, pure defined chemical media consisting of a sugar carbon source, an ammonium salt (or amino acids) and inorganic compounds (such as a "trace elements" mix) are employed to facilitate the growth of the culture media. However, on an industrial manufacturing scale the use of chemically defined media is very expensive and growth restrictions are prevalent. Therefore, the use of "complex" media, with carbon and

nitrogen inputs that are themselves products or by-products of other industrial processes is more economic. For optimum product yield sometimes more than one carbon and nitrogen source is required (Nagodawithana and Wasileski, 1998; Lesniak, 1999).

Typical "complex" forms of glucose include starch hydrolysates and (together with sucrose, fructose and other disaccharides and oligosaccharides) cane and beet sugar molasses. These carbohydrate sources present great challenges if their use by microbial cells is to be accurately monitored in industrial fermentations (Mousdale *et al.*, 1999). The ability to use glucose is, in contrast, very easily followed using well established analytical techniques and can demonstrate when high efficiencies of glucose utilisation are achieved.

A. niger is a commercially important micro-organism involved in the production of citric acid and various enzymes including proteases, amylases, cellulases, xylanases and phytases. Commercially, citric acid is required in the food industry where it acts as a natural acidifying agent, taste enhancer and chelating agent (Kubicek, 1998). *A. niger* is most commonly the micro-organism of choice for citric acid production, where it has been employed for over 80 years. Worldwide approximately 1.6×10^6 tonnes is currently being produced annually ((Berovic and Legisa, 2007). *A. niger* has unrivalled productivity and yield and optimisation of production media and fermentation methods have resulted in up to 80% of substrate (sucrose) being converted into the product (Wolshek and Kubicek, 1999). The metabolic physiology of citric acid-producing strains of *A. niger* is well understood (Karaffa and Kubicek, 2003).

1.2.3 Carbon Sources

Industrial fermentations can employ polysaccharides (for example, purified corn starch) but cruder sources such as beet molasses, sugar cane molasses and fruit pulp can be used if they are locally economically viable. However, these cheaper carbon sources produce problems: as they are waste products they are usually contaminated by high levels of cations produced by earlier processes, which usually require precipitation out with potassium ferrocyanide or removed using cation exchange resins. Other methods proposed involve activated carbon, bone char and bauxite (Prescott and Dunn, 1959). Due to these problems and the complexity of pre-treatment, researchers usually use refined sugars such as sucrose or glucose.

In *A. niger* the carbon source for citric acid fermentation has been shown to be of considerable importance; this is because only sugars which are able to be swiftly taken up give a high yield of citric acid (Mattey, 1992). Sugar concentrations in the range of 14-22% have been found to give maximal yields, dependant on strain and conditions employed (Papagianni, 2007). Many studies have focussed on polysaccharide as the main carbon source but, unless already hydrolysed, polysaccharides create logistical problems in that they are broken down too slowly to match the high rate of sugar catabolism required for citric acid production; this is due to the low pH required for citric acid production being irreconcilable with maximum activities of the hydrolytic enzymes required (Papagianni, 2007).

Various researchers (Kubicek and Rohr 1989) have suggested that sucrose is favourable to glucose because *A. niger* has a highly active extracellular mycelium-bound invertase that is active at the low pH necessary for citric acid production and can rapidly hydrolyze sucrose to glucose and fructose. Sucrose's supremacy over glucose and fructose has been demonstrated by Gupta *et al*. (1976), Hossain *et al*. (1984) and Xu *et al*. (1989).

Monosaccharides (including fructose and glucose) and simple disaccharides such as sucrose and lactose can be determined by high performance liquid chromatography (HPLC) – (Hounsell, 1986; Dionex, 1998) – or by both chemical and enzymatic assays that are or have been commercially available (Table 1.1). However, more complex starch hydrolysates and polysaccharides are more difficult to determine quantitatively. The overall carbohydrate use can be monitored using the anthrone chemical assay (Yemm and Willis, 1954). Different manufacturing processes create different mixtures of oligosaccharides (Krzyzaniak *et al*., 2003). This is important, as different micro-organisms utilize different oligomers at different rates, so a specific micro-organism should be matched with the best mix for optimum productive growth.

Compound (s) Detected	Absorbance Wavelength	Enzymic method	Boehringer Mannheim Catalogue No	Sigma Catalogue No
Glucose	610	+	124036	
D-Glucos/D-fructose	340	+	139106	
D-Glucos/D-fructose/Sucrose	340	+	716260	
D-Sorbitol/Xylitol	340	+	670057	
Glycerol	340	+	148270	
Citric acid	340	+	139076	
l-Malic acid	340	+	139068	
Ammonia	340	+	1112732	171-A
Urea	530	-	542946	535-B
Urea	340	+		66.20,67.10
Uric acid	520	+		685.1
α-Amylase	405			577.3
Alkaline phosphatase	420			104.IS
Lipase	(titrimetric)			800.B
Acetic acid	340	+	148261	
Ethanol	340	+	176290	333-A
Gluconic acid	340	+	428191	
Succinic acid	340	+	176281	
Triglycerides	540	+		339.1

Table 1.1: Assay kits for fermentation substrates, metabolites and enzymes (from Mousdale, 1996)

1.2.4 Nitrogen sources

Proteins, peptides and amino acids are the organic forms of nitrogen commonly used as nitrogen inputs to fermentations (Nagodawithana and Wasileski, 1998).

For industrial fermentations, plant seed proteins are major fermentation inputs and are supplied by one of four ways:

- Whole (macromolecular) in the medium
- Treated with a protease to digest it in the medium
- Whole in a single or combined feed
- Treated proteolytically in a single or combined feed.

Digestion by enzymes usually only goes as far as the stage of peptides with varying molecular weights. Yeast extracts and corn steep liquors contain peptides, macromolecular protein and small amounts of free amino acids (Champagne et al., 2003); Nagarjun *et al*., 2005) whereas casein digests are mainly peptides and free amino acids with no macromolecular protein (Hall and Herejk, 2001). Although free amino acids can be used as nitrogen sources (Albers *et al*., 1996; Torija *et al*., 2003), economic considerations prefer the more cost-effective use of yeast extracts and corn steep liquors.

However, which amino acids are included in these feeds is particularly important as individual amino acids are employed in quite distinct roles. For example proline and glutamate are involved in osmoprotection (Csonka, 1989; Glassker *et al*., 1996) whereas glutamate and glutamine are required for biosynthetic amination reactions (Ertan, 1992).

In secondary product formation ammonia is the most important nitrogen source, as most of the nitrogen incorporated into organic molecules occurs by amination

reactions using glutamine and glutamate as amino donors **(**Ertan, 1992**).** Ammonium salts (for example, ammonium sulphate) or complex nitrogen inputs (for example, corn steep liquors) may be the preferred mode of inclusion of ammonia in the starting medium; pH regulation also plays a role in controlling ammonia concentration as buffering salts are added to counter the accumulation of acids or uptake of ammonium ion from starting medium (Gupta *et al*. 1976, Mousdale *et al*., 1999).

1.2.4.1. Nitrogen sources for *Aspergillus niger*

A study on nitrogen sources and the enhancement of β-galactosidase production in *A niger* and its mutant strain found that all 5 different nitrogen sources ammonium sulphate, corn steep liquor, diammonium phosphate, fish meal and urea produced significant results. Enzyme activity was highest with the corn steep liquor 168.0 and 371.15 IU/I/h, in the parent and mutant respectively compared to 73.1 and 176.3 IU/I/h in the parent and control (Awan *et al*., 2010). Although the nitrogen source was found to be significant when comparing wild type and mutant, the mutant was found to be more productive (2.2 fold). These results tell us that both the nitrogen source and the micro-organism (wild type or mutant organism) can have a marked response on production and for optimum production both nutrients and micro-organism used have to be complementary.

1.2.5 Inorganic inputs

The inorganic cations, sodium, potassium, magnesium and calcium play differing roles during a fermentation process. Sodium (usually as sodium hydroxide) can be added to medium initially to adjust pH and to control pH during a fermentation process; this can add to the osmotic potential of the medium. Both potassium and magnesium salts are used as sources of sulphate and chloride. Potassium also plays a role as an

osmoprotectant in certain microbes (Csonka, 1989). Calcium, as calcium carbonate (chalk) is used as a pH regulator.

Inorganic phosphate is very important in microbial fermentations due to its role in biomass formation (DNA, RNA, phospholipids, etc.). Phosphate levels have, however, been shown to be inhibitory to secondary product formation with Streptomycetes (Aharonowitz and Demain, 1978; McDowall *et al.*, 1999; Bibb, 2005); this is thought to be related indirectly to growth, as high phosphate levels stimulate excessive growth. Secondary product formation and excessive growth are generally incompatible; for example in *S. rimosus*, only during phosphate starvation is oxytetracycline production high (McDowall *et al*., 1999).

Some trace elements such as iron, zinc, copper, cobalt, and molybdenum are required for growth or product formation (Kubicek, 1998). The trace element manganese (in as little as one part per billion), if included in the citric acid fermentation with *A. niger*, will however have a detrimental effect on citric acid production (Clark *et al*., 1966).

1.2.6 Effect of manganese deficiency on citric acid production

One of the requirements for optimum citric acid production by *A. niger* is manganese deficiency. Its principal mode of action is by impairment of macromolecular protein synthesis (Kubicek *et al*., 1979; Hockertz *et al*., 1987), which in turn causes increased protein degradation (Kubicek *et al*., 1979; Ma *et al*., 1987); as a result, mycelia accumulate elevated concentrations of NH_4^+ (Kubicek *et al*., 1987) which causes a feedback inhibition on PFK. High glucose and ammonium concentrations repress the formation of 2-oxoglutarate dehydrogenatese, thus inhibiting citric acid catabolism.

It has become apparent that for increased yield the morphology of the hyphae is primary. Ideally they should be abnormally short, bulbous and heavily branched which manganese deficiency provides (Mattey and Kristiansen, 1999)

1.2.7 Mode of citric acid transport

A. niger strains have over the last two decades been studied in detail. Understanding overproduction of citric acid by *A. niger* is linked to the transport of sugars and ammonia into the cell and the export of citrate from the cell. There has been much conjecture among different researchers about biochemical mechanism underlying citric acid overproduction. Initial substrate availability and subsequent product removal during glycolytic reactions of *A. niger* play an important rate limiting role during citric acid production. This was shown using mathematical modelling (Torres, 1994 a and b), which concluded that the major process controlling citric acid production must occur either at the stage of hexose uptake or subsequent phosphorylation. Therefore, if glucose uptake increases then the rate of citrate production would also increase; the pathways involved are shown in Figure: 1.1

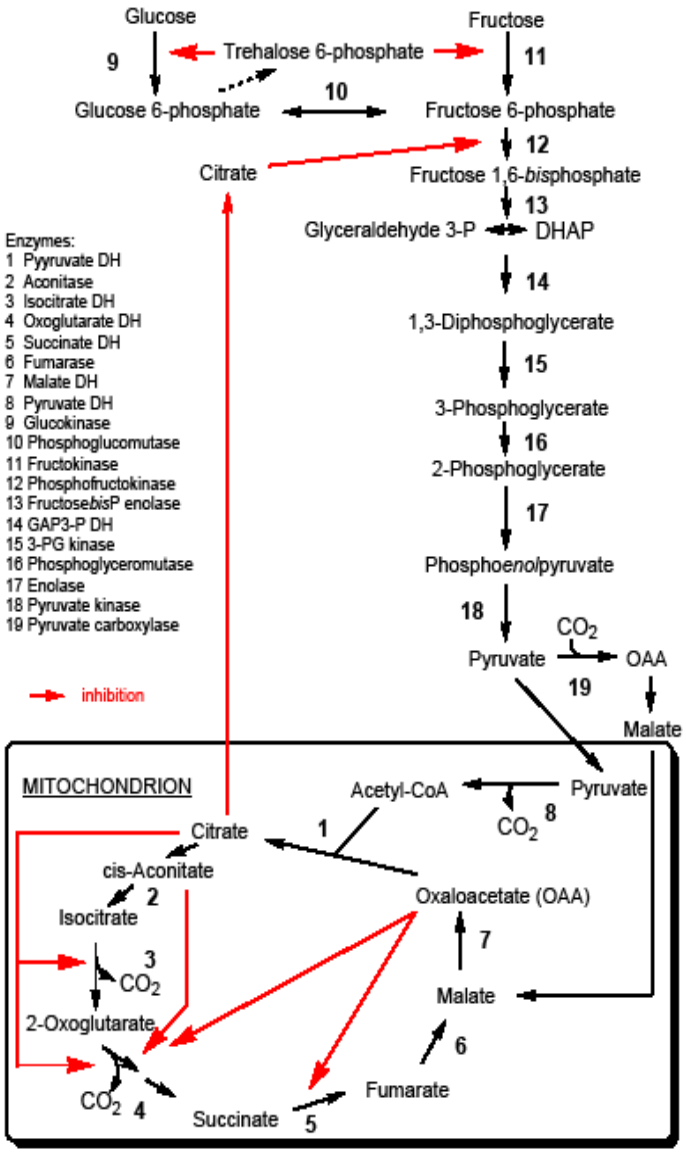


Figure 1.1: Summary of interrelations of the citrate cycle with glycolysis

Two glucose carriers have been identified. One is a high affinity carrier, important when glucose concentrations are below 50g/l but which is activated at all times; the second is a low affinity carrier, which is only expressed at elevated concentrations of glucose. Both have been shown to be inhibited by citric acid during production (Torres *et al*., 1996; Mischak *et al*., 1984); this runs contrary to the belief that uptake of glucose is mainly through the glucose carriers, as a pronounced effect with high citric acid concentrations on glucose uptake rates under carrier-saturated conditions might have been expected (but is not observed) and therefore implies that another major mode of glucose uptake operates at high glucose concentrations. It should not be ignored that with high levels of sugars in the medium, these molecules can diffuse through the membrane at significant rates giving rise to the simple diffusion model

1.2.8 The Simple Diffusion model

A simple diffusion model has been postulated (Wayman and Mattey, 2000; Wayman, 2001) which can account for the over production of citric acid observed. Simple diffusion is an inevitable physical process where regulation is not possible; this is corroborated by the similarity displayed by glucose uptake relationships between different strains and under different growing conditions.

The correlation between the glucose concentration and the specific glucose uptake rate has been explored using three sets of previously published batch fermentations data from shake flask cultures, an air-lift bioreactor and a stirred tank reactor employing two glucose uptake models (Wayman and Mattey, 2000). The Simple Diffusion Model accurately reflects the observed relationship between specific uptake rate and glucose concentration under carrier-saturated conditions (Torres, 1996; Wayman and Mattey, 2000).

1.2.9 Factors affecting citric acid production

To obtain substantially increased citric acid production under industrially favourable conditions several researchers have shown the importance of primary metabolism and the significance of the glycolytic flux control (Karaffa and Kubicek, 2003; Legisa and Mattey, 2007).

Extensive studies have shown that there are three main metabolic events that will predispose the cells to replenish tricarboxylic acid cycle and increased product formation (Mattey, 1992; Torres *et al*., 1996):

- Glucose utilization by simple diffusion
- Unrestricted metabolic flow through glycolysis, to provide precursors for the synthesis of TCA cycle intermediates
- Uncoupled NADH re-oxidation resulting in lower levels of ATP resulting in decreased anabolic reactions

1.2.10 Effect of genetic modification of the glycolytic pathway

Working on the basis that unregulated metabolic flux through glycolysis is a crucial prerequisite for rapid synthesis of citric acid, there have been two attempts to influence the efficiency of this pathway through genetically modified means. Firstly, trehalose-6-phosphate (a potent inhibitor of glycolysis) was eliminated by disrupting the trehalose-6-phosphate synthase gene (*ggs A*); as a consequence, synthesis of trehalose-6-phosphate was prevented and this resulted in a small increase in citric acid accumulation (Arisan-Atac *et al*., 1996). Next, the key regulatory enzymes 6-phosphofructo-1-kinase and pyruvate kinase were moderately over-expressed; citric acid production in the wild strain was, however, not affected (Ruijter *et al*., 1997).

1.2.11 Hypo-osmotic shock

The sudden change in the solute concentration around a cell is referred to as osmotic shock or osmotic stress, and results in a rapid change in the movement of water across its cell membrane. Conditions of high concentrations of either salts, substrates or any solute in the supernatant would result in water being drawn out of the cells through osmosis. As a consequence of this the transport of substrates and cofactors into the cell is inhibited thus "shocking" the cell. On the other-hand low concentrations of solutes, result in water entering the cell in large amounts, causing it to swell and either burst or undergo apoptosis. (Lang et al., 2005)

1.2.11.1 Recovery and tolerance mechanisms for hypo-osmotic stress

All organisms have mechanisms in place to respond to osmotic shock, with the osmolarity of its surroundings being conveyed to the cell by sensors and signal transduction networks (Kültz and Burg 1998). Single-celled organisms are the most vulnerable to osmotic shock due to their direct proximity to their environment; however, cells in large animals such as mammals are still susceptible to these stresses under certain conditions.

When hypo-osmotic conditions prevail cells respond to osmotic swelling by increasing membrane permeability to specific intracellular osmolytes. As a consequence of the efflux of these osmolytes, as well as water, a partial recovery of cell volume is obtained. Generally there are two types of osmolytes involved, inorganic ions such as K^+, Ca^{2+} and Cl^- and small molecular weight organic compounds, including certain quaternary ammonium compounds and specific amino acids (Pierce, 1982).

Depending on which osmotic concentration cells exist, dictates the kind of osmolytes which are found. At osmotic concentrations up to 300-400 milliosmoles where many types of freshwater animals and the majority of terrestrial vertebrate cells are found, mostly inorganic ions are present. Cells found at higher osmotic pressures, e.g. cells

from marine and brackish water organisms and dehydration-tolerant species, are dependant on organic osmolytes to maintain cell volume. Some of these organic osmolytes exhibit dual roles where they provide both osmotic volume regulation and also serve to stabilize proteins (Pierce, 2001).

1.2.11.2 Effect of hypo-osmotic shock on citric acid production

Hypo-osmotic shock was shown to increase the rate of accumulation of citric acid and decrease fermentation time in one strain; this was found to be a consequence of activation of cAMP signal transduction pathway which results in phosphorylation of 6-phosphofructo-1-kinase (PFK) by cAMP-dependant protein kinase (PKA - Legisa and Bencina, 1994). PKA contains a catalytic and regulatory subunit and the sequence of encoded genes for both units have been determined (Bencina *et al*., 1997) although no other key regulatory enzymes were found to be affected.

1.2.12 Effect of aeration on citric acid production

Another requirement for citric acid formation is strong aeration requiring higher dissolved oxygen tensions than for normal vegetative growth (Kubicek *et al*., 1980). The biochemical basis behind this observation is thought to involve the alternative cyanide-resistant respiratory pathway, where an alternative oxidase (AOX) enzyme catalyses the reduction of oxygen to water, functioning as a non-energy conserving member of the respiratory electron chain due to its ability to reduce oxygen to water without proton translocation across the inner mitochondrial membrane (Sluse and Jarmuszkiewicz, 1998). AOX is synthesised in the cytosol and translocated into the mitochondria (Kirimura *et al*., 1996). The alternative respiration pathway is found routinely in citric acid producing strains (Kirimura *et al*., 1986).

In my project, to promote the productivity of primary and secondary metabolite formation by the selected micro-organisms *A. terreus,* glycolysis would be

upregulated; a gene coding for 6-phosphofructo-1-kinase (PFK) which had been shown to be enhanced significantly by phosphorylation by cAMP-dependent protein kinase (PKA) was taken along with a gene coding *pkaC* gene (which codes for the catalytic sub-unit of PKA) from a wild strain of *A. niger* and expressed in the commercial micro-organisms.

The transfer of the *A. niger* alternative oxidase gene (*aox1*) into *Aspergillus terreus* was undertaken which should in theory result in an increase in TCA intermediates by uncoupling the oxidation glycolytically produced NADH from energy conservation (Kirimura *et al*., 1986, 1996, 2000).

1.2.13 Central metabolic pathways in *A. niger*

The central pathway of carbohydrate metabolism found in almost all cells (Fothergill-Gilmore and Michels, 1993) is glycolysis or the Embden-Meyerhof-Parnas pathway (EMP) (Figure 1.1). Evolutionary, this is one of the earlier pathways for sugar degradation whose enzymes or modified one's have been found in Archaea such as *Thermoproteus tenax (*Siebers and Hensel, 1993) and in the hyperthermophilic anaerobic bacterium *Thermotoga maritime* (Schroeder *et al*., 1994).

Even the simple and basic pathway of glycolysis, however, has a high plasticity and versatility both in a single species and across multi-genome comparisons (Dandekar *et al*., 1999).

1.2.14 Phosphofructo-1-kinase (*PFK*)

Phosphofructokinase catalyses the first irreversible step in the glycolytic pathway the phosphorylation of fructose-6-phospate to form fructose-1,6-*bis*phosphate using ATP as the phosphoryl donor and releasing ADP as a by-product.

Two forms of *A. niger* 6-phosphofructo-1-kinase (*PFK1*) have been identified: the 85-kDa native enzyme and a 49-kDa shorter fragment which is formed from the former as a consequence of posttranslational modification (Mesojednik and Legisa, 2005). To date no kinetic characteristics are known of the smaller enzyme purified to near homogeneity.

1.2.15 Alternative oxidase (*AOX1*)

The nuclear-encoded mitochondrial proteins termed "alternative oxidases" have two considered roles: they can function in parallel with the classic cytochrome oxidative pathway to produce ATP and they may counter oxidative stress within the mitochondria (Kirimura *et al*., 1986, 1996, 2000). In eukaryotic organisms, energy for growth, development, reproduction and response to external stresses is derived mainly through ATP production during mitochondrial respiration.

The formation of citric acid is dependent on strong aeration; dissolved oxygen tensions higher than those required for the vegetative growth of *A. niger* stimulate citric acid fermentation (Dawson *et al*., 1986; Kubicek *et al*., 1980). The biochemical basis of this observation is related to the presence of an alternative, cyanide-resistant respiratory pathway, which is required for the re-oxidation of glycolytically produced NADH when a high oxygen tension is maintained. The enzyme responsible for the additional respiratory pathway is an alternative oxidase, which catalyses reduction of oxygen to water without the translocation of protons across the inner mitochondrial

membrane, and thus functions as a non-energy-conserving member of the respiratory electron chain. The alternative respiration seems to be constitutively present in citric acid producing strains (Kirimura *et al*., 2000). The alternative oxidase (AOX) is synthesised in the cytosol and translocated into mitochondria (Kirimura *et al*., 1996).

1.3 *Aspergillus terreus – Introduction*

A. terreus produces the secondary metabolite lovastatin (Hendrickson *et al.,* 1999). Cholesterol can be absorbed from the diet from foods such as eggs and dairy products but most is synthesised in the liver (82%) or intestines (11%). If the diet is adequate then synthesis will all but stop; however too much cholesterol in the blood leaves the individual susceptible to a whole host of serious health problems including heart diseases and strokes (Faggiotto and Paoletti, 1999). Various cholesterol-lowering agents were discovered in the 1950s and the 1960s; unfortunately most displayed unpleasant side effects. In 1971 Japanese scientists endeavoured to produce a more acceptable drug to treat hyperchlesterolemia with as little of the side effects as possible; the secondary metabolite lovastatin (and related compounds) is now proving to be a great aid in the treatment of hypercholesterolemia. Lovastatin belongs to the family of cholesterol reducing agents called statins due to its strong inhibitory effects on HMG CoA reductase, the enzyme that catalyses the reduction of mevalonate during the synthesis of cholesterol (Figure 1.2).

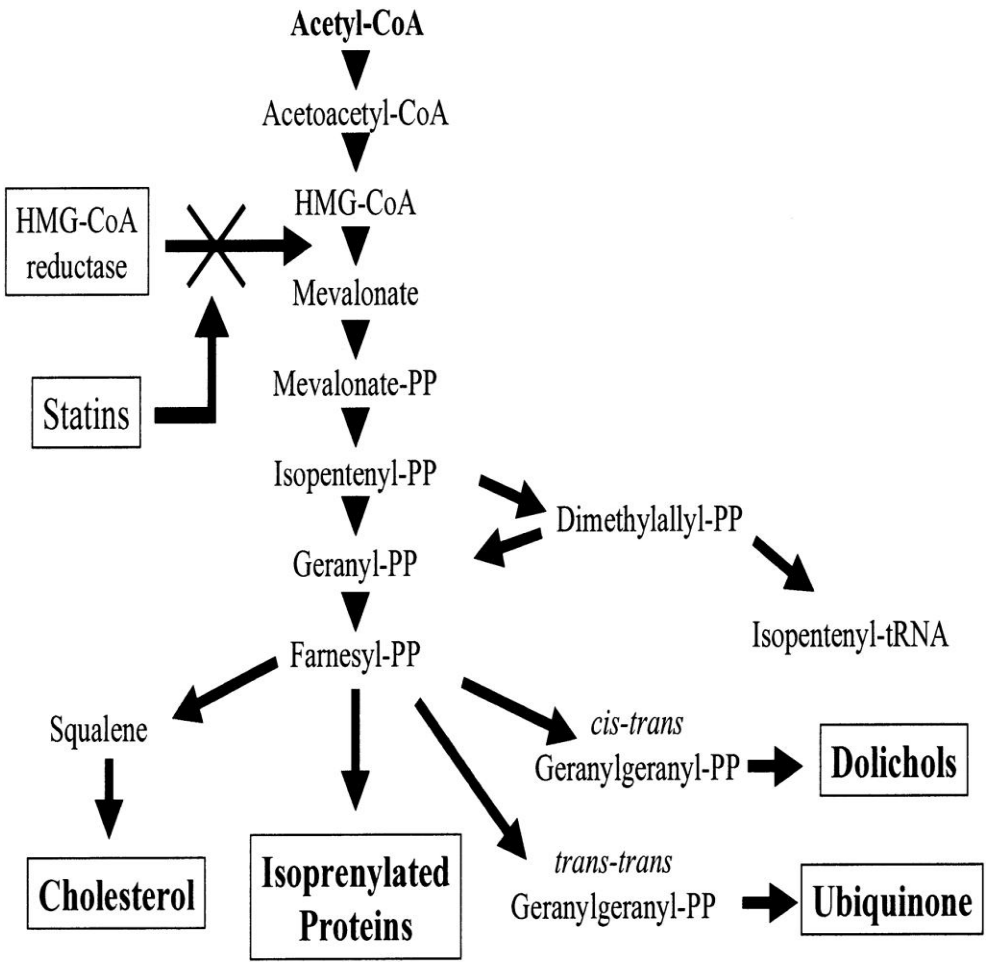


Figure 1.2: The mechanism of action of statins on cholesterol biosynthesis (taken from Faggiotto and Paoletti, 1999)

Lovastatin consists of two distinct polyketide chains joined through an ester linkage (Figure 1.3); proof for this structure was provided through cloning and partial characterisation of the lovastatin biosynthetic gene cluster from *A. terreus* (Hendrickson *et al*., 1999; Kennedy *et al*., 1999).

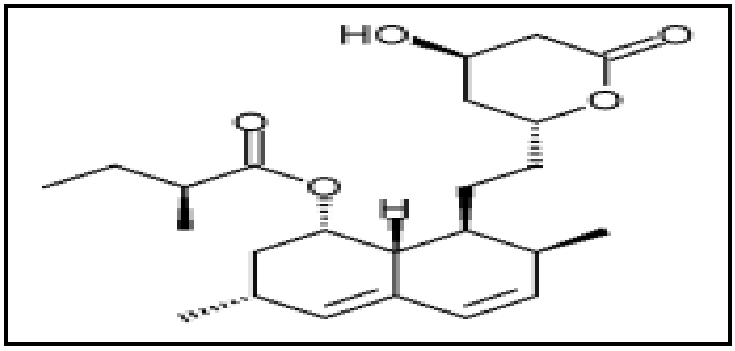


Figure 1.3: Lovastatin Structure: **8-[2-(4-hydroxy-6-oxo-oxan-2-yl)ethyl] -3,7-dimethyl-1,2,3,7,8,8a- hexahydronaphthalen- 1-yl] 2-methylbutanoate**

Although knowledge of the genes and enzymes involved in lovastatin biosynthesis exists, the regulation and physiology of the pathway are much less well understood.

It has been reported that biochemically the biosynthesis of lovastatin (mevinolinic acid) is performed in two stages. The first stage is catalysed by the nonaketide synthase (EC 2.3.1.161), which belongs to type I polyketide synthases, and catalyses the nine-step formation of the polyketide (Shen, 2003). This PKS stage leads to the lovastatin precursor 4a, 5-dihydromonacoline L (Sutherland *et al*., 2001).

It is thought that complex regulation of gene expression and enzyme activity for polyketide synthesis are controlled directly by its carbon and nitrogen sources. It was anticipated in the ANTICO project that increased levels of TCA intermediates as a consequence of modifying the *A. terreus* with the *A. niger* genes would result in an increase in both itaconic acid and lovastatin.

1.3.1 *Aspergillus terreus* - Gluconic acid production and feedback regulation by pH

Historically *A. terreus* is known to produce itaconic acid at low pH (Calam *et al*., 1937, Bentley and Thiessen 1957) and lovastatin and other secondary metabolites at

higher pH in the fermentation (Hajjaj *et al*. 2001). During investigations on glucose utilisation for secondary metabolite production (lovastatin) by *A. terreus* a novel pathway of glucose catabolism was demonstrated. It was found that the wild type of *A. terreus* without any strain development at pH 6.5 was able to produce gluconic acid as a bulk chemical at high molar conversion (up to 0.7mol mol^{-1} glucose consumed). These exciting findings have led to a manuscript being submitted to *Letters in Applied Microbiology* and which has been published (Dowdells *et al*., 2010)

It was also found that transformants harbouring *A. niger* genes encoding PFK exhibited superior kinetic and regulatory properties for the bio-production of metabolites from glucose without being shunted towards the novel gluconic acid pathway to the same extent as the wild type whereby showing an exploitable trait that could bestow a further genetic advantage (Berovic and Legisa, 2007) . These findings envisaged a novel gluconic acid pathway where pH could be used as a regulatory element. Results and evidence for these findings are presented in chapter 3.

1.3.2 Gluconic Acid Properties

Gluconic acid is an organic compound with molecular formula $C_6H_{12}O_7$ and condensed structural formula $HOCH_2$ $(CHOH)_4COOH$. It is one of the 16 stereoisomers of 2, 3, 4, 5, 6-pentahydroxyhexanoic acid. Gluconic acid was first discovered by Hlasiwetz and Habermann in 1870 (Röhr *et al.*, 1983) and acetic acid bacteria were first shown to produce gluconic acid in 1880. Forty two years later, *A. niger* was identified as a producer of gluconic acid by Molliard (1922) and later still gluconic acid was associated with the bacterial species *Pseudomonas*, *Gluconobacter* and *Acetobacter* and various fungal species such as *Penicillium* (Ramachandran *et al.*, 2006).

Gluconic acid (pentahydroxycaproic acid, (Fig. 1.4) is produced from glucose through a simple dehydrogenation reaction catalysed by glucose oxidase (fungi) and glucose dehydrogenase (bacteria). The preferred method is by microbial production and the most commonly studied fermentation process is with *A. niger*.

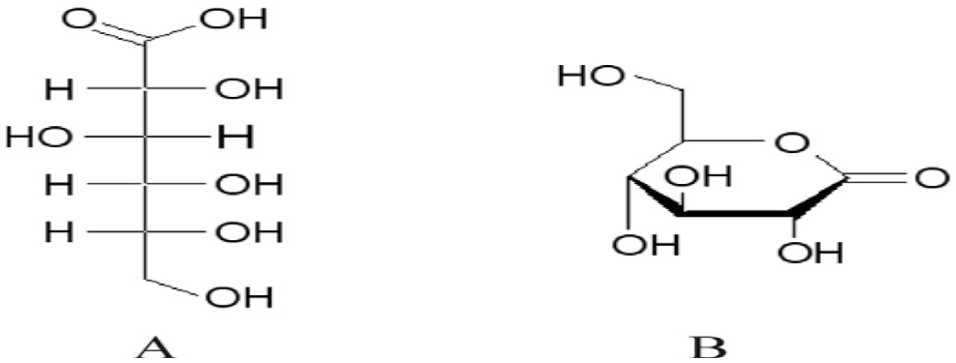


Figure 1.4: Formula of gluconic acid (A) and glucono- β--lactone (B)

Oxidation of the aldehyde group on the C-1 of β--D-glucose to a carboxyl group results in the production of glucono-δ-lactone ($C_6H_{10}O_6$,) and hydrogen peroxide. glucono- β--lactone is further hydrolysed to gluconic acid either spontaneously or by lactone hydrolysing enzyme, while hydrogen peroxide is decomposed to water and oxygen by peroxidase. The gluconic acid pathway is shown in Figure 1.5.

The conversion process also could be a biotransformation, taking place in the absence of cells with glucose oxidase and catalase derived from *A. niger*; nearly 100 % of the glucose is converted to gluconic acid under the appropriate conditions. This method is an FDA approved process. Production of gluconic acid using the enzyme has the potential advantage that no product purification steps are required.

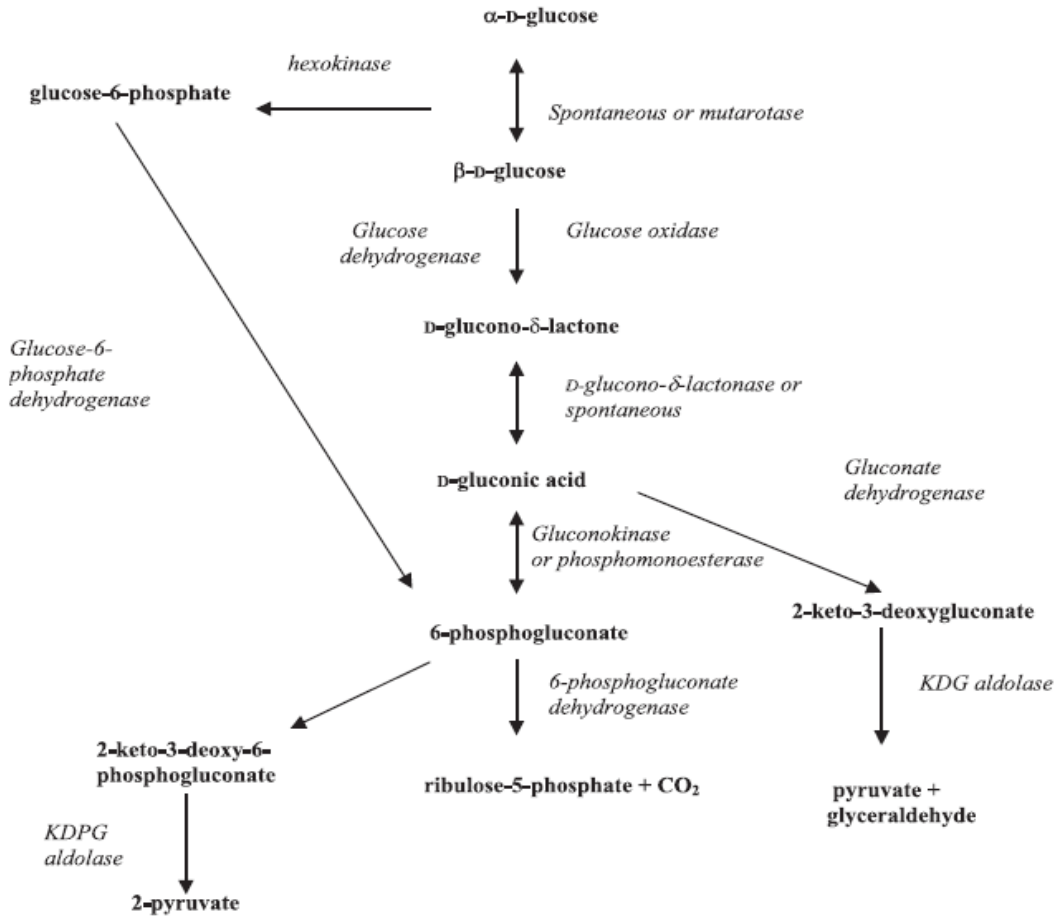


Figure 1.5 Gluconate pathways reproduced from Ramachandran *et al*. (2006)

1.3.3 Common uses of Gluconic Acid

Gluconic acid can be found naturally in fruit, honey, kombucha tea, and wine. As a food additive gluconic acid (E574) is approved by the Food Standards Agency of the United Kingdom; it is also used as an acidity regulator. (FSA, 2010). Cleaning products make use of its ability to dissolve mineral deposits especially in alkaline solution. The gluconate anion chelates Ca^{2+}, Fe^{2+}, Al^{3+} and other metal cations.

Calcium gluconate, in the form of a gel, is used to treat burns from hydrofluoric acid; (Saadi *et al*., 1989; Roblin *et al*., 2006) calcium gluconate injections may be used for

more severe cases to avoid necrosis of deep tissues. (Thomas *et al*., 2009). Quinine gluconate is a salt between gluconic acid and quinine, which is used for intramuscular injection in the treatment of malaria. Zinc gluconate injections are used to neuter male dogs. (Levy *et al*., 2008). Iron gluconate injections have been proposed in the past to treat anemia (Reznikoff and Goebel, 1937).

1.4 Analytical Chemistry and the Improvement of Fermentation Processes

Fermentation processes have been used and improved without any real understanding or knowledge of the biochemical processes behind them. However in the last 50 years, with the aid of modern analytical methods and techniques using a random selection of genetically modified strains, improvements to the media nutrient content, feeding strategies, fermentor design and process operation have been made (Allman, 1999; O'Kennedy *et al*., 2000). Analytical biochemistry has advanced greatly; now on-site or research laboratories have access to powerful analytical tools not available when industrial fermentations were first developed in the first half of the twentieth century (Mousdale, 1996; Mousdale *et al*., 1999).

1.4.1 High performance liquid chromatography (HPLC)

The Russian botanist M. S. Tswett (1872-1919) developed chromatography as a technique for the separation of coloured plant pigments, today HPLC (high performance or high pressure liquid chromatography) is the most widely used of all analytical separation techniques due to its ability to provide accurate results with adequate sensitivity. HPLC can give qualitative and quantitative separation of non-volatile or thermally labile compounds. The five most common modes of HPLC are Size Exclusion Chromatography, Ion Exchange Chromatography, Reversed Phase Chromatography, Hydrophobic Interaction Chromatography and Affinity Chromatography; these modes can be individually used for the analysis of

fermentation nutrients and metabolites, including carbohydrates, anions, cations, amino acids, peptides, lipids, vitamins, antibiotics, nucleotides, nucleosides and bases (Lim, 1987; Robards *et al*., 1994).

1.4.2 Capillary gas-liquid chromatography (GLC)

Capillary gas-liquid chromatography is a chromatographic technique that can be used to separate volatile organic compounds. The organic compounds are separated due to differences in their partitioning between the mobile gas phase and the stationary phase in the column. Organic acids, sugars and sugar alcohols can all be detected by GLC but, as they are not volatile compounds, they have to be converted to volatile chemical derivatives prior to GLC analysis (Chen *et al*., 1998; Adams *et al*., 1999).

1.4.3 Ion-selective electrodes

Ion selective electrodes measure the potential of a specific ion in solution the potential is measured against a stable reference electrode of constant potential. The potential difference between the two electrodes will depend on the specific activity of the ion that is related to concentration. Many ions in fermentation media are suitable for this method of analysis, including ammonium, sodium, potassium, calcium and chloride ions (Frant, 1994).

1.4.4 Capillary electrophoresis

Capillary electrophoresis is a useful tool employed both academically and industrially within pharmaceutical and biotechnological sectors, for the separation of charged and uncharged species ranging in size from metal ions to proteins and polynucleotides (Pena-Mendes *et al*., 2003).

1.4.5 Colorimetric and enzymatic assays for carbohydrates and related compounds

Some of the many commercially available kits to detect fermentation substrates and products are shown in Table 1.1. Included in these are specific methods for D-glucose, D-fructose and D-sorbitol, which can be determined enzymatically (Beutler, 1984). Total carbohydrate in the medium can be determined by the anthrone method (Yemm and Willis, 1954).

1.5 Fermentation Improvement by Metabolic Analysis

By employing the above (and other) analytical tools, metabolic analysis can be carried out to:

- Allow growth and product formation to be assessed accurately and quantitatively relating to the important chemical and biochemical events taking place.
- Identify areas for process improvement.
- Evaluate practical ways to improve these processes.
- Understand at the metabolic level the consequences of these changes to the fermentation process as a whole.

This is summarised in Figure 1.6 (taken from Mousdale *et al*., 1999).

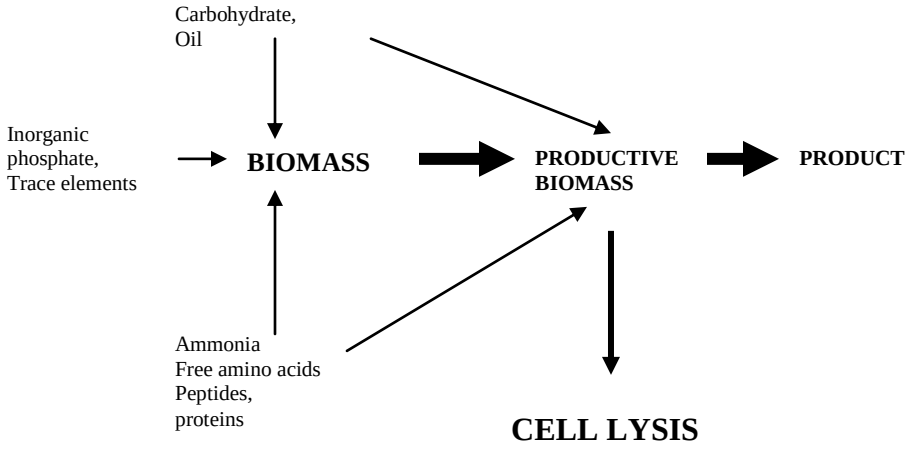


Figure 1.6: Analytical targets in fermentation process improvement

Many, if not all, of the major biosynthetic pathways are known. This provides an invaluable tool for learning what actually happens during fermentation processes and can give information of where to intervene and specify which part of the biochemical "map" requires further investigation (Michal, 1999).

1.5.1 Metabolic analysis

From the 1970's onwards several different approaches have been devised to understand metabolism in fermentations at the quantitative biochemical level using the modern and growing knowledge of biochemical pathways and computer models with which to organise data sets from analytical studies of microbial metabolism.

The earliest systematic methodologies were those of Metabolic Control Analysis (Kacser and Burns, 1973; Heinrich and Rapaport, 1974). These were later followed by Biochemical Systems Theory (Savageau et al., 1987), flux analysis (El-Mansi and Holms, 1989; Holms, 1996) and more sophisticated methods of flux distributions

derived from ^{13}C-glucose and other methods (Stephanopoulos and Vallino, 1991; Fell, 1997). Essentially, all these methods attempt to model metabolism at the cellular level using assumptions about the operation of biochemical pathways and the partition of substrates between rival pathways.

For commercial work with industrial scale fermentations (with fermentation vessels of 100,000 l or greater volume), only one method (flux analysis) has been successfully applied to complex media. The work of Bioflux Ltd. (later beòcarta Ltd.) in Glasgow developed analytical and mathematical techniques and applied them to individual bioprocesses that comprised:

- A strain (usually highly evolved by classical mutagenesis from the wild type).
- A medium (usually containing complex nitrogen sources such as corn steep liquors, yeast extracts and plant proteins).
- A feeding strategy for fed-batch fermentations using separately controlled carbon and nitrogen inputs.

Because of the idiosyncratic and proprietary nature of these commercial fermentations, the results ("rate models" or "metabolic kinetic models") were not published but were used to improve fermentation yields and process economics for example, the efficiency of production of a fermentation product from a glucose or vegetable oil feed.

This approach also anticipated the conclusion that metabolic fluxes are dominated by relatively few reactions (Almaas *et al*., 2004) and was used in the ANTICO project work. The principles of this simplified but highly practical method of fermentation analysis are easily summarised (Holms, 1996; Mousdale *et al*., 1999):

- Rates of metabolic utilisation of fermentation substrates (for example, glucose, ammonia, etc.) and all fermentation products and side-products are measured by direct analysis.
- The rates of utilisation and accumulation – expressed on a whole-fermentor basis – are then computed and compared using a simplified metabolic diagram.
- Measurable differences in the kinetic parameters between fermentations of differing productivities or (as used in the work for the ANTICO project) between fermentations with parent and genetically modified strains are then interpreted to identify means of optimising the process, i.e. increasing the rate of product formation, minimising wasted inputs, reducing the running costs of the process, etc.

In fermentation manufacturing as a worldwide industry, many production processes (and their scaled-down forms in pilot plants and laboratories) are limited by simple parameters: the rate of growth, the timing of net cell death (or culture lysis), rate of a crucial carbon or nitrogen feed or the exhaustion of a key nutrient. Novel strains evolved by classical mutagenesis or by rational genetic engineering often fail to express higher productivities in large fermentors and the reasons can frequently recognised from quantitative kinetic measurements.

It is, however, very important to distinguish between intracellular fluxes and whole-fermentor parameters. Although usually held to be useful factors in fermentation analysis, intracellular concentrations of metabolites are minute and change on a time scale of seconds (Oldiges *et al.*, 2004). Few laboratories have the sampling hardware to "capture" accurately such fleeting phenomena (using, in effect, modifications of rapid reaction technique apparatus) and with industrial vessels with volumes in the range 10,000 – 1,000,000 litres they are entirely inapplicable

1.5.2 Metabolic analysis in the ANTICO project

Primarily, my research within the ANTICO project was to analyse the fermentations samples produced after all the genetic manipulations of the recipient micro-organisms had been done, and compare the wild and the modified strain metabolically to gauge if these changes had resulted in an improved yield and productivity.

One of the most relevant and significant results to come out of the ANTICO project is the novel pathway for gluconic acid and is documented in chapter 3. Therefore, my work has resulted in some novel and unexpected results, which lead to significant contributions to knowledge within the field of microbial strains for industrial fermentations.

1.5.3 Motivation and Aims

The fungus *A. niger* is an important industrial microorganism used for the production of organic acids and enzymes. In this project the main aim is to increase productivity of specific products in *A. terreus*, itaconic acid and the secondary product lovastatin with glucose as sole carbon source, by expressing *A. niger* genes coding for the enzymes 6-phosphofructo-1-kinase (PFK). *A. terreus* was chosen as a recipient as it is closely related to *A. niger* and would be expected to express *A. niger* genes while also being an industrially used micro-organism capable of synthesising a range of products not biomanufactured by *A. niger*.

At low pH, *A. terreus* produces itaconic acid but lovastatin and other secondary metabolites are produced at higher pH in the fermentation (Osman *et al*., 2011). The utilization of glucose as a carbon substrate will be investigated and secondary metabolite production monitored in *A. terreus*.

Glucose catabolism will be investigated in *A. terreus*, using a wild type without any previous strain development. In addition, *A. terreus* strains which have been genetically modified to express the native *A. niger pfkA* gene and a shorter form of the gene will be investigated to determine if increased glucose utilization rates result in the increased production of both primary and secondary metabolites.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 MICRO-ORGANISMS

With the parental and modified strains, New Brunswick Bioflo 110 vessel fermentor (figure 2.1) and/or shake flask trials were performed by Dr. R. I. Jones and sampled for metabolic analysis in the Department of Bioscience, University of Strathclyde with *Aspergillus terreus*. In addition, some shake flask cultures with *A. niger* were grown and sampled in Glasgow. Samples from fermentors run with *A. terreus* in the National Institute of Chemistry, Ljubljana were shipped on dry ice to Glasgow

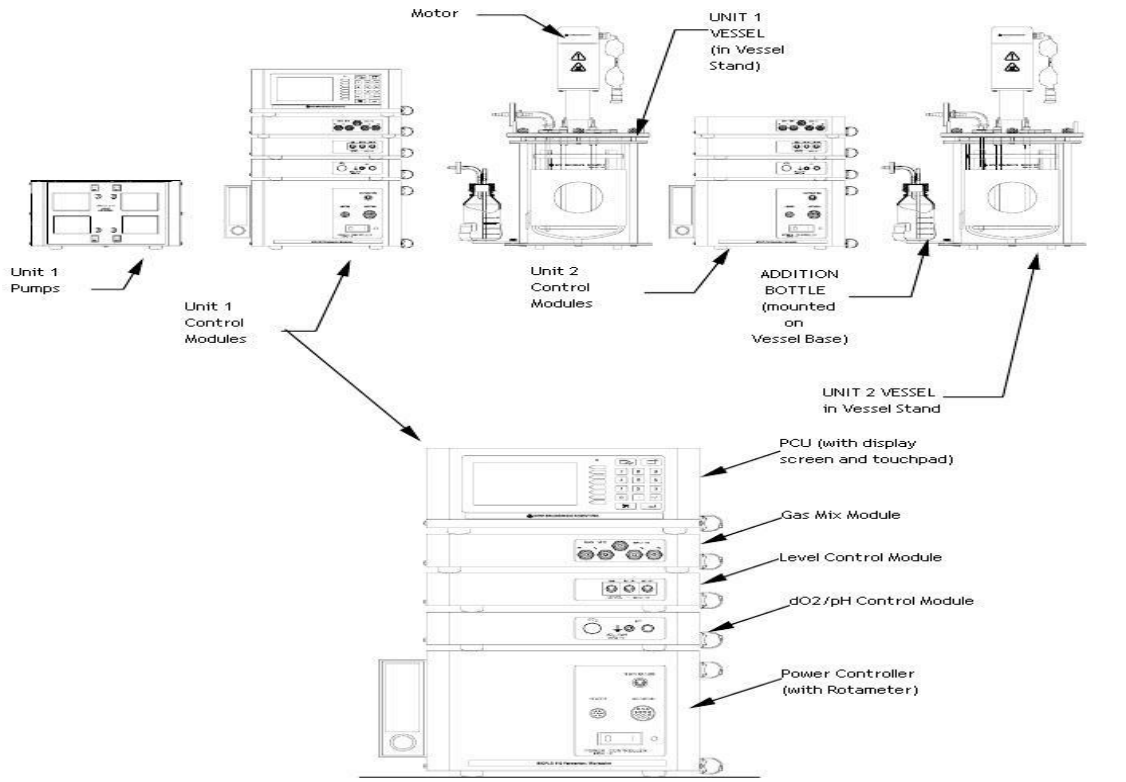


Figure 2.1 - New Brunswick Bioflo 110 vessel

2.2 Metabolic Analysis Of Genetically Modified Micro-organisms

Transformants of *A. terreus*, *S. rimosus*, *S. clavuligerus* and *P. pastoris* which had been constructed to express native or intron-less forms of the *A. niger* genes *pfkA*, a truncated form of *pfkA* (t-*pfk*), *pkaC* and *aox1* were analysed in laboratory fermentors and/or shake flasks to enable their performance characteristics under physical conditions similar to industrial processes be assessed and the finer points of how they differed metabolically from the respective parental strains be assessed.

The chemical fractionation procedures used were based on those described by Herbert *et al.* (1971) and Mousdale (1996) to separate low molecular weight components from nucleic acids, protein and other highly insoluble macromolecular material.

2.2.1 Preparation of Broth Samples

The method used is based on that described by Herbert *et al.* (1971) and Mousdale (1996)

For all strains other than *P. pastoris*, broth samples withdrawn from shake flasks and fermentors were treated with equal weights of ice-cold 0.4 M perchloric acid (PCA), incubated for 16 hr at 4-6^{o}C and then centrifuged (14000 x *g*, 20 min, 6^{o}C) to separate cold PCA-soluble phases (representing the sum of intra- and extra-cellular metabolites) and insoluble components (cellular macromolecules and extra-cellular proteins precipitated by PCA).

Broth samples from *P. pastoris* fermentations were separated into cells and supernatant phase by centrifugation (10000 x *g*, 20 min, 6^{o}C) before freezing at –70^{o}C; supernatant phases were subsequently treated with an equal volume of 0.4 M PCA,

incubated for 16 hr at 4-6^{o}C and then centrifuged (14000 x *g*, 20 min, 6^{o}C) prior to analysis.

Broth filtrates for soluble protein and other analysis were prepared by filtration through disposable 0.45 μm filters.

2.2.2 Preparation of Nucleic acid fractions (hot PCA-soluble)

The method used is based on that described by Herbert *et al.* (1971) and Mousdale (1996)

The pellets prepared in Section 2.2.1 were re-suspended in 0.5M PCA (10ml) and incubated for 2hr at 70^{o}C. The soluble material was separated by centrifugation (8500rpm in 6 x 250ml rotor with inserts for 15ml centrifuge tubes) and the extract volume was recorded.

2.2.3 Preparation of Alkali-soluble fractions

The method used is based on that described by Herbert *et al.* (1971) and Mousdale (1996)

Alkali-soluble samples for total protein measurements were prepared by digesting material insoluble in hot 0.5M PCA (Section 2.2.2.) in 10ml 0.5M NaOH for 16hr at 37^{o}C; the soluble material was separated by centrifugation (8500rpm /20min/20^{o}C) and the extract volume was recorded.

2.2.4 Determination of DNA by Burton Assay of Deoxyribose

The method was based on that described by Richards (1974).

The reagent was prepared by dissolving 4g diphenylamine in 100ml glacial acetic and then adding 10µl paraldehyde.

Procedure:

(1) A standard solution was made from 2-deoxyribose (25mg/l) in 0.5M perchloric acid (PCA) incubated at 70^{o}C for 2hr.

(2) 900µl reagent was added to 50-450µl of sample plus up to 400µl 0.5M PCA or 500µl standard in 1.5ml Eppendorf micro-centrifuge tubes.

(3) For each sample a "sample" blank was prepared by incubating 450µl sample with 900µl glacial acetic acid containing 100µl.l^{-1} paraldehyde; the blank was incubated and processed in parallel with the full assays.

(4) After incubation at 30^{o}C for 16hr, the assay mixtures were centrifuged in an Eppendorf micro-centrifuge tube for 2min at 14000rpm.

(5) The absorbance of the supernatant phases was measured at 600nm against acetic acid containing 100µl.l^{-1} paraldehyde.

(6) The absorbance of the sample blank was subtracted from the absorbance of the sample in the full assay with diphenylamine.

(7) The absorbance of the "reagent" blank (450µl 0.5M PCA incubated with the full diphenylamine reagent) was subtracted from the corrected absorbance from step (6).

(8) The assay was calibrated with 2-deoxyribose; the absorbance of the standard was corrected for any absorbance in the reagent blank.

2.2.5 Determination of RNA by the Orcinol Assay for Ribose

The method is based on that described in Herbert *et al.* (1971):

(1) The reagent was prepared by dissolving 20g orcinol in 100ml absolute ethanol.

(2) A standard solution was made from 25mg/l D-ribose.

(3) Assays were performed in glass test tubes and 0.1ml reagent was added to sample or standard in a final volume of 1.5ml 0.5M PCA.

(4) 1.5ml 0.03% ferric chloride in concentrated HCl was then added and the mixture placed in a heating block at 100^{o}C for 20min.

(5) After cooling to room temperature, the absorbance was measured at 665nm against a "blank" in which 1.5ml 0.5M PCA replaced the sample.

2.2.6 Determination of Total Nucleic Acid Phosphate

As described in method in section 2.2.11 but using hot PCA fractions prepared in Section 2.2.2

2.2.7 Determination of Total Protein

As described in method in section 2.2.20 but used with alkali-soluble fractions prepared in Section 2.2.3

2.2.8 Determination of Total Insoluble Carbohydrate

As described in method in section 2.2.24 but used with fractions of *Pichia pastoris* after alkali extraction section 2.2.3; the resulting pellets were re-suspended in deionised water before analysis.

2.2.9 Determination of Melanoid Pigment

Melanoid pigments were extracted in 0.5 M NaOH step for total protein (Section 2.2.3) and quantified by spectrophotometry (Nakano *et al*., 2000). From *S. rimosus* cultures, the melanoid pigments were purified by prolonged dialysis of alkaline

extracts against deionised water and lyophilisation of the resulting fraction; a 100mg/l solution of the purified pigment in 0.5M NaOH had an absorbance at 475nm of 0.497.

2.2.10 Determination of Inorganic Phosphate

Inorganic phosphate was measured with a colorimetric procedure using Malachite Green modified from the method described by Lanzetta *et al.* (1979).

Reagents: 0.045% (w/v) Malachite Green hydrochloride;
4.2% (w/v) ammonium molybdate in 5N hydrochloric acid;
34% (w/v) citric acid.

Standard: 0.2mM mono-potassium dihydrogen orthophosphate.

Procedure:

(1) 3:1 (v/v) Malachite Green molybdate reagent was prepared and left for 30min at room temperature.

(2) 50µl sample, standard or water (blank) added to 5ml plastic test tubes.

(3) 850µl Malachite Green reagent added and mixture vortexed.

(4) After 60sec100µl 34% citric acid added and mixture vortexed.

(5) Assays incubated at room temperature for 60min.

(6) The absorbance at 645nm was measured against the assay blank.

2.2.11 Determination of Total Phosphate

Reagents: concentrated sulphuric acid, 8.3% (w/v) ammonium molybdate, 1% (w/v) amidol (2,4-diaminophenol hydrochloride) in 20% (w/v) sodium metabisulphite (prepared fresh just before use).

Standard: 10mM mono-potassium dihydrogen orthophosphate (1.36g/L).

Samples: 0.2M PCA-soluble fractions or filtrates.

Procedure:

1) To 100µl sample was added 100µl concentrated sulphuric acid in Pyrex tubes and digested at 180^{o}C in a heating block for 60min. Standards (in triplicate) and blank (100µl water) were prepared and processed in parallel to samples.

2) After 60 min the tubes were removed and cooled to room temperature.

3) 0.5ml hydrogen peroxide ("100 volume") was added, the tubes returned to the heating block and digestion continued digestion for 30min.

4) After cooling the tubes, 4.2ml water and 0.2ml ammonium molybdate were added and the mixtures were vortexed.

5) 0.4ml amidol reagent was added, the tubes were vortexed and incubated for 60 min at room temperature.

6) The absorbance at 620nm was measured against the assay blank

2.2.12 Determination of Free Amino Acids by HPLC

For primary amino acids and amines a gradient HPLC separation of o-phthaldialdehyde (OPA) derivatives was used. The methodology is based on that in Jarrett *et al.* (1986).

Reagent: 50mg/ml OPA in 1ml methanol was added to 10ml 0.2M potassium tetraborate, pH 9.5, to which 40µl 2-mercaptoethanol (MESH) was then added. This reagent was stored in a dark bottle and 4µl MESH added every 2-3 days.

HPLC Conditions:

Column: Adsorbosphere OPA HR 5µ (150 x 4.6mm) – Alltech Part No. 28072., Applied Science Ltd., 6-7 Kellet Road Industrial Estate, Kellet Road, Carnforth, Lancashire LA5 9XP, United Kingdom

Apparatus: Jasco Gradient mixer
Jasco HPLC pump: PU – 1580
Jasco Sampler: AS – 155510

Detector: Jasco 1520-FP Spectrofluorometer, λ_{ex} 230nm, λ_{em} 455nm

Solvents: A: 20mM sodium acetate, pH5.9/4.5% 1,4-dioxan / 3% IPA

B: methanol, 1.5% 1,4-dioxan, 1.5% IPA

Gradient Method:		A%	B%
initial		100	0
segment 1	12min	60	40
segment 2	23min	45	55
segment 3	30min	25	75
segment 4	32min	15	85
segment 5	37min	100	0

Flow Rate: 1.5ml/min at room temperature

Injection volume: 20µl

Preparation of standard mix:

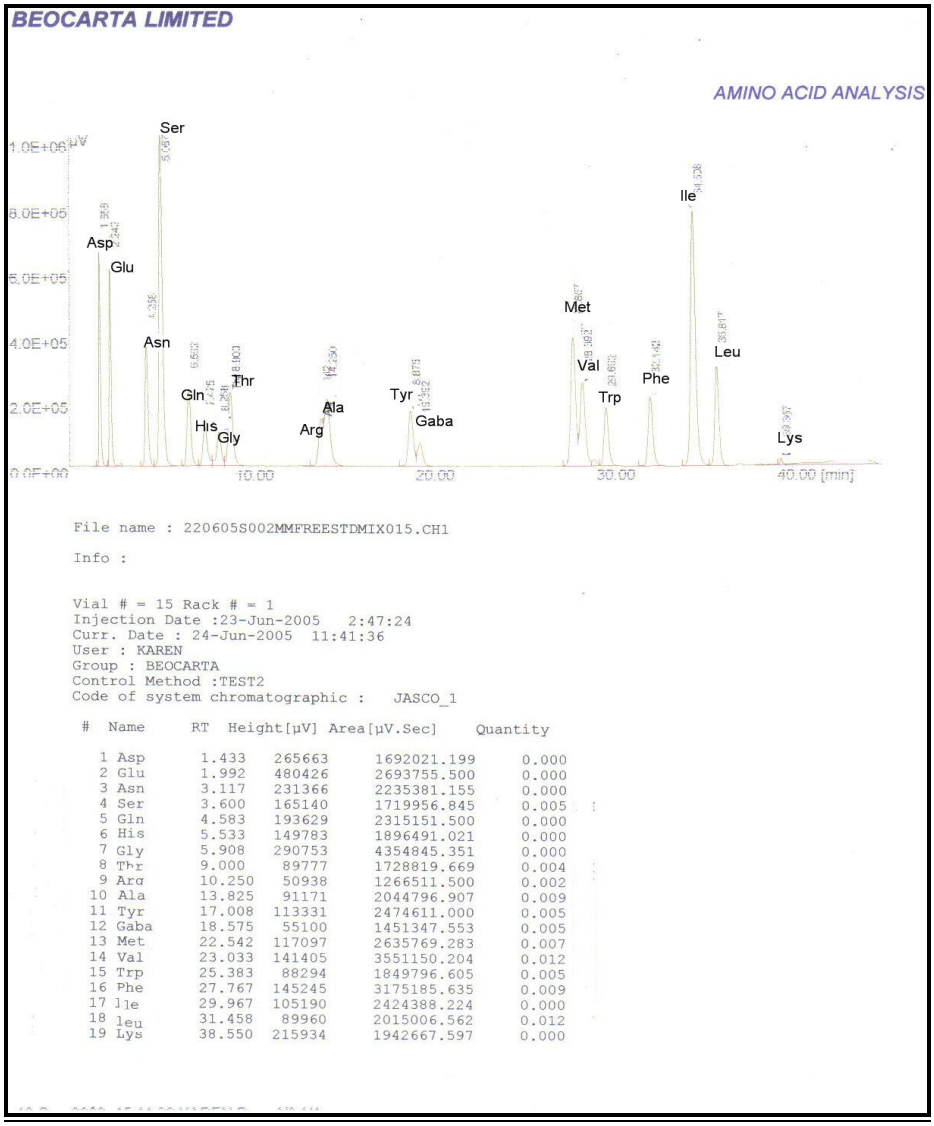
(1) All standards had prepared stock solutions of 10mM.

(2) 200µl of each standard was added to a 50ml volumetric flask.

(3) The solution was made up to volume with deionised water.

Amino acid standard mix:

All standards in the mixture had a concentration of 0.02mM. The mixture contained 19 amino acids integrated in the order of their elution: Asp-Glu-Asn-Ser-Gln-His-Gly-Thr-Arg-Ala-Tyr-4ABA-Met-Val-Trp-Phe-Ile-Leu-Lys (Figure 2.2).



File name : 220605S002MMFREESTDMIX015.CH1

Info :

Vial # = 15 Rack # = 1
Injection Date :23-Jun-2005 2:47:24
Curr. Date : 24-Jun-2005 11:41:36
User : KAREN
Group : BEOCARTA
Control Method :TEST2
Code of system chromatographic : JASCO_1

#	Name	RT	Height[µV]	Area[µV.Sec]	Quantity
1	Asp	1.433	265663	1692021.199	0.000
2	Glu	1.992	480426	2693755.500	0.000
3	Asn	3.117	231366	2235381.155	0.000
4	Ser	3.600	165140	1719956.845	0.005
5	Gln	4.583	193629	2315151.500	0.000
6	His	5.533	149783	1896491.021	0.000
7	Gly	5.908	290753	4354845.351	0.000
8	Thr	9.000	89777	1728819.669	0.004
9	Arg	10.250	50938	1266511.500	0.002
10	Ala	13.825	91171	2044796.907	0.009
11	Tyr	17.008	113331	2474611.000	0.005
12	Gaba	18.575	55100	1451347.553	0.005
13	Met	22.542	117097	2635769.283	0.007
14	Val	23.033	141405	3551150.204	0.012
15	Trp	25.383	88294	1849796.605	0.005
16	Phe	27.767	145245	3175185.635	0.009
17	Ile	29.967	105190	2424388.224	0.000
18	Leu	31.458	89960	2015006.562	0.012
19	Lys	38.550	215934	1942667.597	0.000

Figure 2.2: Amino Acid Standard

2.2.13 Determination of Inorganic Anions and Organic Acids by HPLC

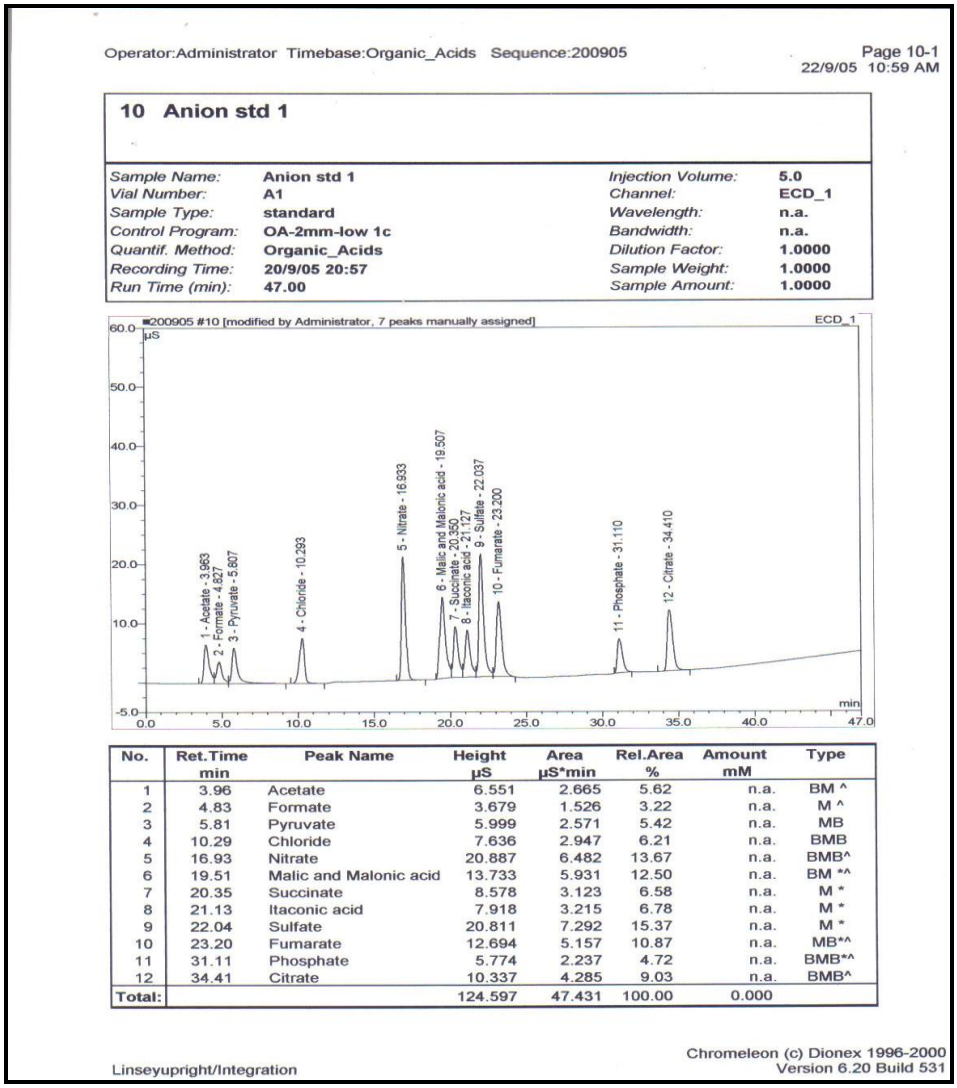
The methodology is based on that in Application note 123 "The Determination of Inorganic Anions and Organic Acids in Fermentation Broths" Dionex Corporation (UK Headquarters, 4 Albany Court, Camberley, Surrey, GU16 7QL).

Apparatus:	Dionex ED40 Electrochemical detector.
	Dionex HPLC pump: GP50
	Spectra System Autosampler AS – 3500
	DS3 Detection Stabiliser
	ASRS 4mm Suppressor
Column:	Dionex Ion Pac AS11-HC
Guard:	Dionex Ion Pac AG11-HC
Eluants:	E1 Water
	E2 5mM NaOH
	E3 100mM NaOH
Flow rate:	$0.38 ml.min^{-1}$
Temperature:	$30^{o}C$
Injection:	5µl
Run time:	47min
Detection:	Dionex ED40 Conductivity Mode
Software:	Dionex Chameleon

Gradient:

Time (min)	%E1	%E2	%E3
-10	80	20	0
0	80	20	0
8	80	20	0
20	90	0	10
33	75	0	25
47	40	0	60

All standards in the mixture had a concentration of 0.2mM; the standard mixture contained 13 organic and inorganic anions and their sequence of integration was: acetate, lactate, formate, pyruvate, chloride, nitrate, succinate, malate, sulphate, itaconic acid, fumarate, phosphate and citrate (Figure 2.3).



No.	Ret.Time min	Peak Name	Height µS	Area µS*min	Rel.Area %	Amount mM	Type
1	3.96	Acetate	6.551	2.665	5.62	n.a.	BM ^
2	4.83	Formate	3.679	1.526	3.22	n.a.	M ^
3	5.81	Pyruvate	5.999	2.571	5.42	n.a.	MB
4	10.29	Chloride	7.636	2.947	6.21	n.a.	BMB
5	16.93	Nitrate	20.887	6.482	13.67	n.a.	BMB^
6	19.51	Malic and Malonic acid	13.733	5.931	12.50	n.a.	BM *^
7	20.35	Succinate	8.578	3.123	6.58	n.a.	M *
8	21.13	Itaconic acid	7.918	3.215	6.78	n.a.	M *
9	22.04	Sulfate	20.811	7.292	15.37	n.a.	M *
10	23.20	Fumarate	12.694	5.157	10.87	n.a.	MB*^
11	31.11	Phosphate	5.774	2.237	4.72	n.a.	BMB*^
12	34.41	Citrate	10.337	4.285	9.03	n.a.	BMB^
Total:			124.597	47.431	100.00	0.000	

Figure 2.3: Anion Standard Mix

2.2.14 Determination of Cations by HPLC

The methodology is based on that in Chong *et al.* (2003).

Detector: Waters 431 Conductivity detector

Apparatus: Waters Action Analyzer HPLC System
Varian 9100 Autosampler:

Column: Dionex IonPac CS12A column (150 x 4.6mm)
Dionex guard column CG12A

Eluant: 10mM sulphuric acid (18Ω deionised water)

Flow rate: 1ml/min

Temperature: 40^{o}C

Injection volume: 25µl

Run time: 14min

Detection: Waters Conductivity

Range: 0.002µS sensitivity

Software: AL-450 Chromatography Software

Working standards (1mM) were prepared from standard stock solution (20mM) of sodium, ammonium, potassium, magnesium and calcium salts (Figure 2.4).

Data Reprocessed On 05/17/1958 06:32:10

Sample Name: CAT1	Date: 05/12/2028 03:37:10
Data File : C:\DX\DATA\WATERS\BYR\300\CAT22601.D01	
Method : C:\DX\METHOD\WATERSIC\CATIONS\nh4mg.MET	
ACI Address: 1 System: 1 Inject#: 1	Detector:COND
Analyst : AW	Column: Ionpac CS12A (4 * 250mm)

Calibration	Volume	Dilution	Points	Rate	Start	Stop	Area Reject
External	25	1	4500	5Hz	0.00	15.00	1000

********** Peak Report: All Peaks **********

Pk. Num	Ret Time	Component Name	Height	Area	Bl. Code	%Delta
1	4.37	Na	16563	175756		
2	4.90	NH4	12610	126498		
3	5.93	K	12062	159071		
4	9.43	Mg	20622	523505		
5	11.03	Ca	13305	388066		
			71550	1372896		

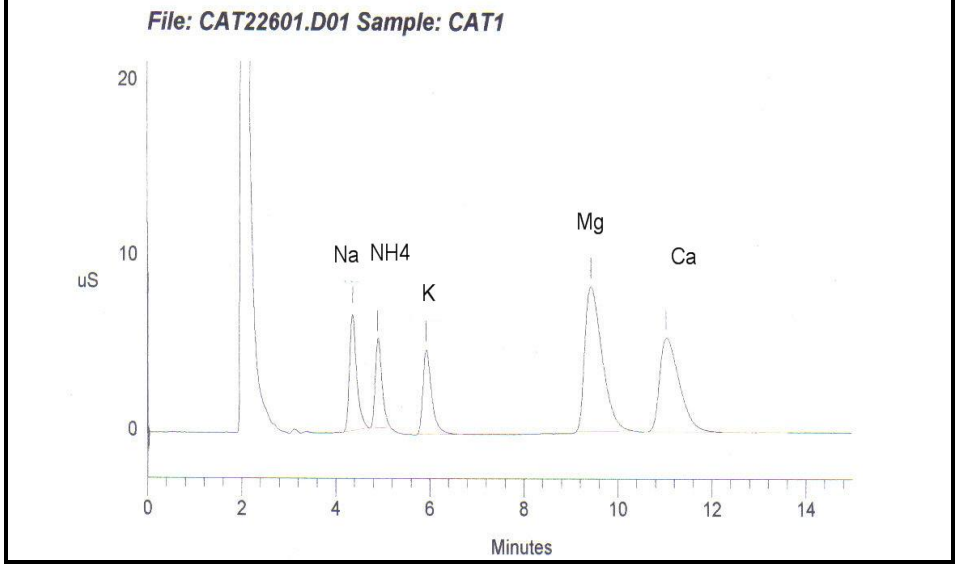


Figure 2.4: Cation Standard Mix

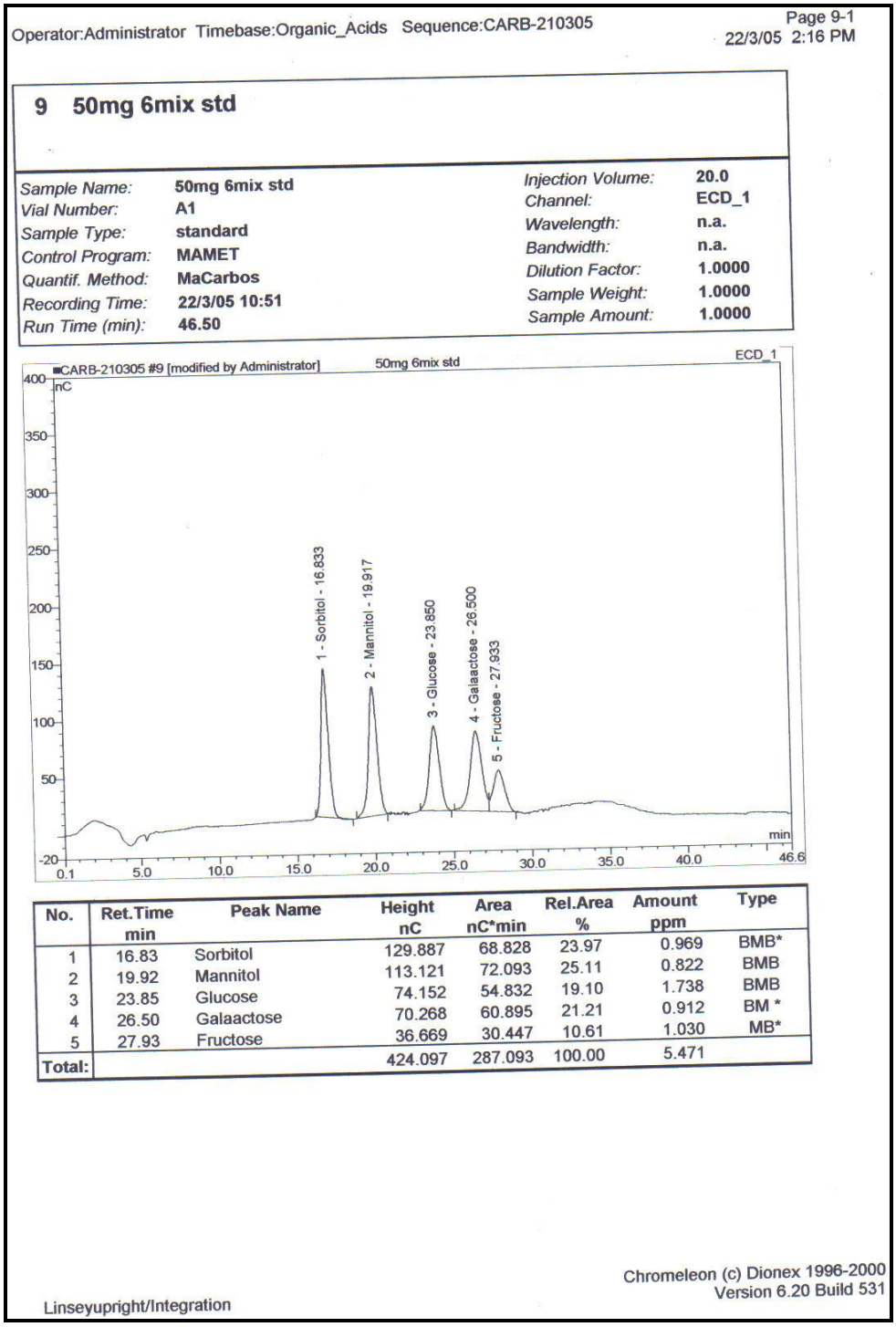
2.2.15 Determination of Carbohydrates by Gradient HPLC

The methodology is based on that in Application note 122 Dionex Corporation "The Determination of Carbohydrates, Alcohols, and Glycols in Fermentation Broth"

Apparatus: Dionex ED40 Electrochemical detector
Dionex: HPLC pump
Spectra System Autosampler AS – 3500
Column: Dionex CarboPac PA-1 Analytical, (4 x 250mm)
Guard column: Dionex CarboPac PA-1 (4 x 50mm).
Solvent: A: 16mM sodium hydroxide for electrochemical use
B: 200mM sodium hydroxide for electrochemical use
Flow rate: 1ml/min at room temperature
Injection: 20µl
Run Time: 47 min
Detection: Pulsed amperometric, gold electrode
Gradient:

Time (min)	A (%)	B (%)
Initial	99	1
0	100	0
22	100	0
25	0	100
35	0	100
37	100	0
47	100	0

A carbohydrate and polyol standard was prepared from sorbitol, mannitol, mannose, glucose, galactose and fructose each at 50mg.l^{-1} in deionised water. (Shown in figure 2.5)



No.	Ret.Time min	Peak Name	Height nC	Area nC*min	Rel.Area %	Amount ppm	Type
1	16.83	Sorbitol	129.887	68.828	23.97	0.969	BMB*
2	19.92	Mannitol	113.121	72.093	25.11	0.822	BMB
3	23.85	Glucose	74.152	54.832	19.10	1.738	BMB
4	26.50	Galaactose	70.268	60.895	21.21	0.912	BM *
5	27.93	Fructose	36.669	30.447	10.61	1.030	MB*
Total:			424.097	287.093	100.00	5.471	

Figure 2.5: Carbohydrate Standards

2.2.16 Determination of D-Glucose by Enzyme Kit

Glucose was measured by a commercial hexokinase-glucose 6-phosphate dehydrogenase assay kit method (catalogue number 10716251035, R-Biopharm AG, Darmstadt, Germany), following appropriate dilution of the sample.

2.2.17 Determination of D-Gluconic acid by Enzyme Kit

Gluconic acid was measured by a commercial gluconokinase/6-phosphogluconate dehydrogenase assay kit method (catalogue number 10428191035, R-Biopharm AG, Darmstadt, Germany) after appropriate dilution of the sample.

2.2.18 Determination of Soluble Protein

The colorimetric assay using Coomassie G250 dye was based on the method described by Bradford (1976).

Reagent: 100mg Coomassie Blue (Brilliant Blue) G250 was dissolved in 50ml 95% (v/v) ethanol; 100 ml ortho-phosphoric acid was added with constant stirring; 850ml water was then added with constant stirring; the reagent was stored in a brown glass bottle at room temperature.

Standard: bovine serum albumin (BSA), 1mg.ml^{-1}
Samples: filtered broths

Procedure:

(1) A standard curve was prepared before each set of assays with 12 plastic test tubes (5ml volume) with 0, 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200µl BSA and water (where necessary) to make a final volume of 200µl.

(2) 4.2ml dye solution was added and the assays and mix by gentle inversion using Parafilm.

(3) The absorbance at 595nm was measured using the tube with 200µl water as the assay blank.

(4) The absorbance at 595nm was plotted in an Excel worksheet against the amount of BSA added per assay and a trinomial fit was calculated to the results.

(5) Samples were assayed using sample and water to a final volume of 200µl.

(6) The protein content of the samples was computed from the BSA calibration curve.

2.2.19 Determination of Lactic acid by Enzyme Kit

Lactic acid was measured by a commercial assay kit method (catalogue number 10139084035, R-Biopharm AG, Darmstadt, Germany) after appropriate dilution of the sample.

2.2.20 Determination of Acetic acid by Enzyme Kit

Acetic acid was measured by a commercial assay kit method (catalogue number 10148261035, R-Biopharm AG, Darmstadt, Germany) after appropriate dilution of the sample.

2.2.21 Determination of Urea

Urea was measured by a commercial assay kit method (catalogue number 10542946035, R-Biopharm AG, Darmstadt, Germany) after appropriate dilution of the sample.

2.2.22 Determination of Total Carbohydrate

Total carbohydrate was measured by the anthrone method.
This method is based on that in Shields and Burnett (1960).

Reagent

1. Place 100ml water in a glass beaker in an ice-water mixture and cautiously add 250ml concentrated sulphuric acid with constant stirring.
2. When the acid solvent is cool, add 0.5g anthrone and stir until dissolved.

Standard: 1g/l D-glucose in deionised water

Procedure:

(1) Add 100↘l of sample and standards with up to 0.1mg glucose (with sufficient water to make the volume up to 0.1ml) in triplicate to 15ml Pyrex glass test tubes. Prepare parallel blanks with 0.1ml water.

(2) Add 5ml anthrone reagent.

(3) Place tubes immediately in boiling water bath and leave for 10min.

(4) Remove test tubes and place in water bath at room temperature.

(5) After 10min read the absorbance at 620nm in 3ml glass cuvettes against the blank as reference.

2.2.23 Measurement Units

Measurements on samples were treated with PCA were calculated on a weight basis since the samples were prepared using weights rather than volumes; in practice mmole/kg or g/kg were most common calculation bases. The calculations are expressed on a whole broth basis, i.e. including any insoluble material (this is essentially a means of manipulating data so that whole-fermentor data can be used after multiplying by the weight of the fermentation broth – frequently the only reliable data on fermentor contents from industrial sites).

Measurements on filtered samples are expressed as mM or g l^{-1}.

Chapter 3. Production of Lovastatin and Other Metabolites by *Aspergillus terreus*

3.1 Aims of genetic transformation of *Aspergillus terreus*

The central hypothesis of my project was that the productivities and yields of metabolites produced by *Aspergillus terreus* would be increased as a direct consequence of reinforcing anaplerotic reactions by the transfer of specific *A. niger* genes:

1. To stimulate metabolic flux through glycolysis, the gene encoding 6-phospho-1-fructokinase (PFK) of *A. niger* was added to the host recipient genes.
2. In addition, the transfer of the *A. niger* alternative oxidase gene (*aox1*) into the recipients was carried out to enable an increase in citrate cycle intermediates by uncoupling the oxidation of protons from the synthesis of ATP.

Itaconic acid (methylenesuccinic acid) is biosynthesized by *A. terreus* at low pH and is a product of industrial importance, its worldwide production exceeding $8x10^4$ tonne per year (Okabe *et al*., 2009). Itaconic has a market price approximately the same as for citric acid and its primary use is as a copolymer with synthetic resins (Miall, 1978). In addition, as its hexyl derivative, itaconic acid is used in agronomy a cheap and potent plant growth regulator that accelerates root growth (Suzuki *et al.*, 1986). While itaconic acid is a high-volume, low-value product, the same micro-organism when grown at a higher operating pH elaborates higher value secondary metabolites, including lovastatin, the first cholesterol-lowering agent to be approved for clinical use (Alberts, 1990; Bizukojc and Ledakowicz, 2009). Slowly metabolized carbon sources (for example, lactose) are considered to give the highest production of lovastatin (Casas López *et al*. 2003). The possibility that genetically transformed *A.*

terreus might product lovastatin from a more readily utilized carbon source such as glucose was, therefore, an attractive hypothesis to explore.

3.2 Experimental Results

3.2.1 Metabolic Analysis of Parental and *pfkA*-Modified Strains

The molecular genetic transformations were undertaken by ANTICO colleagues in the laboratories of the National Institute of Chemistry, Department for Biotechnology and Industrial Mycology, Ljubljana, Slovenia; transformed organisms where sent to Strathclyde university, where Dr. Robin Jones carried out fermentations. Initially the fermentations where carried out under conditions that favoured lovastatin (Hajjaj *et al.*, 2001). While no lovastatin could be detected, examination of HPLC analyses for carboxylic acids and anions indicated the accumulation of an unexpected metabolite, gluconic acid, and the project was refocused to investigate the production of this novel fermentation product for *A. terreus*.

3.2.2 *Aspergillus terreus* and gluconic acid production – fermentations at Strathclyde University

Two parallel 10-L trials with the parent (AT156) and *pfkA*-modified (AT10) strains were performed in Glasgow in a defined medium with pH control to pH 6.8. These conditions were expected to favour lovastatin formation; however, no lovastatin was detected at a detection limit of 10 mg/L.

The absence of lovastatin was not due simply to a failure of the cultures to either grow or utilise glucose from the medium (Figure 3.1).

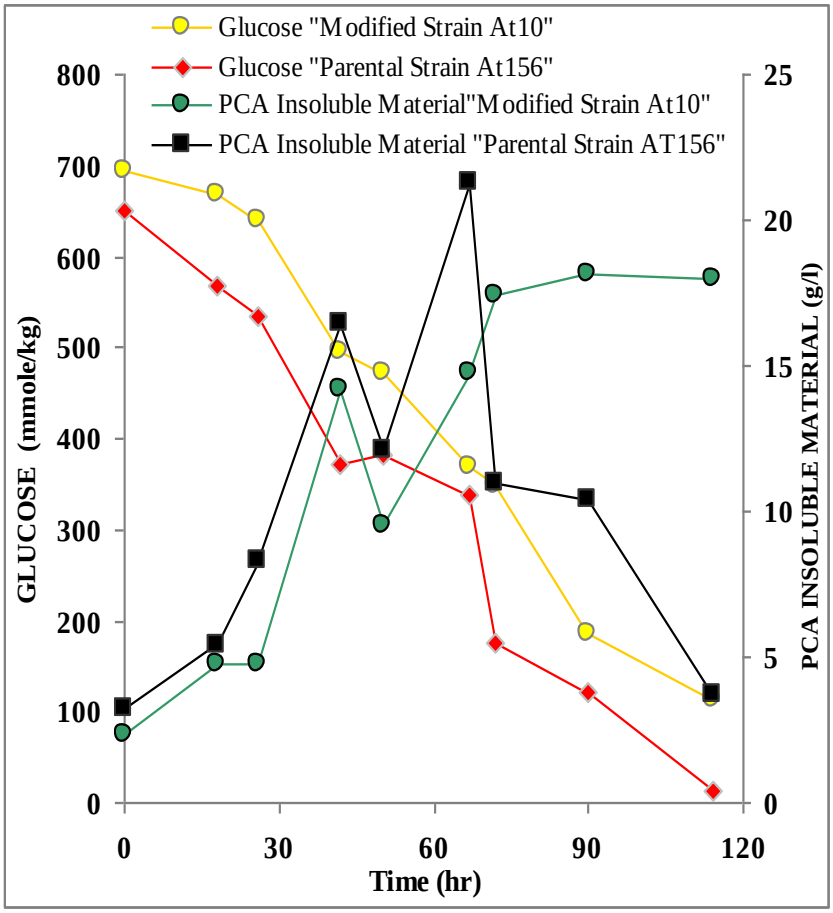


Figure 3.1: Glucose utilisation by modified (●) and parental (♦) strains of *A. terreus* and PCA-insoluble material with modified (●) and parental (■) strains of *A. terreus i*n 10-litre stirred tank fermentations. One sample at timed intervals was taken for analysis.

The accumulation of gluconic acid also indicated clearly that primary metabolism was active with over 30% of the utilised glucose being oxidised into gluoconic acid by the parental strain (Figure 3.2).

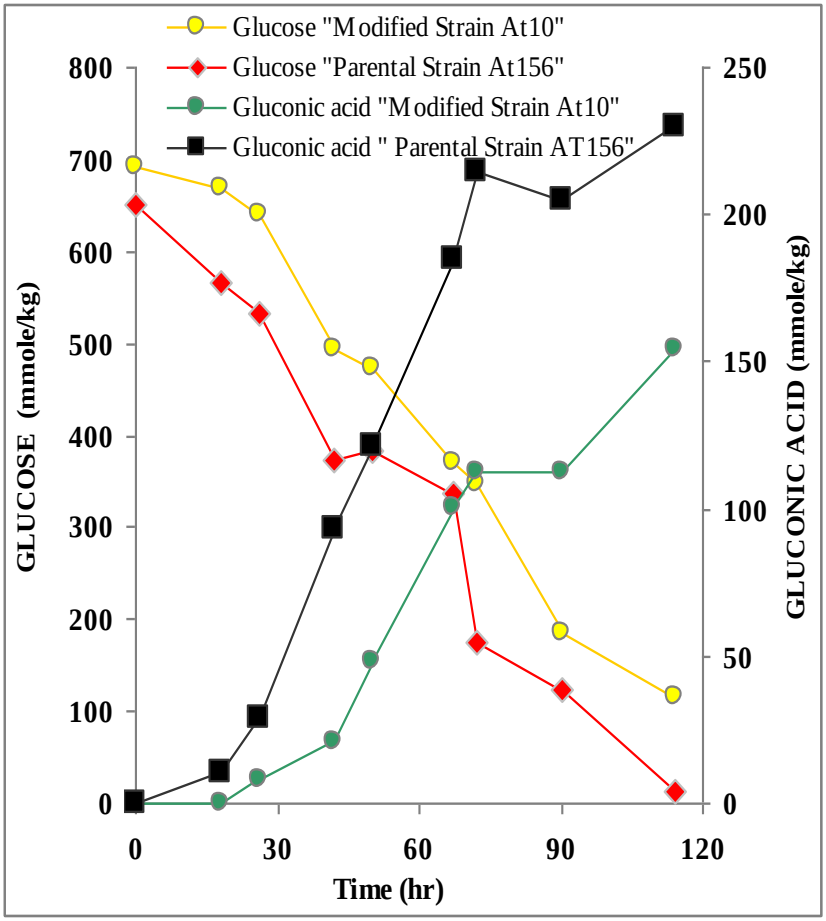
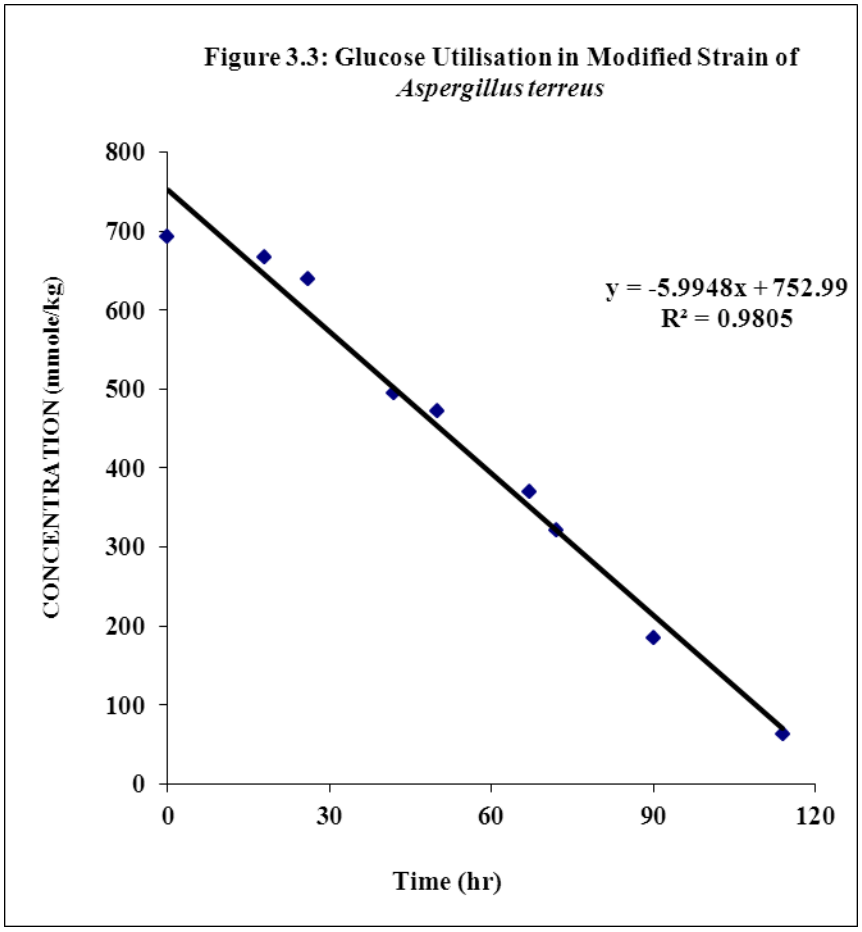
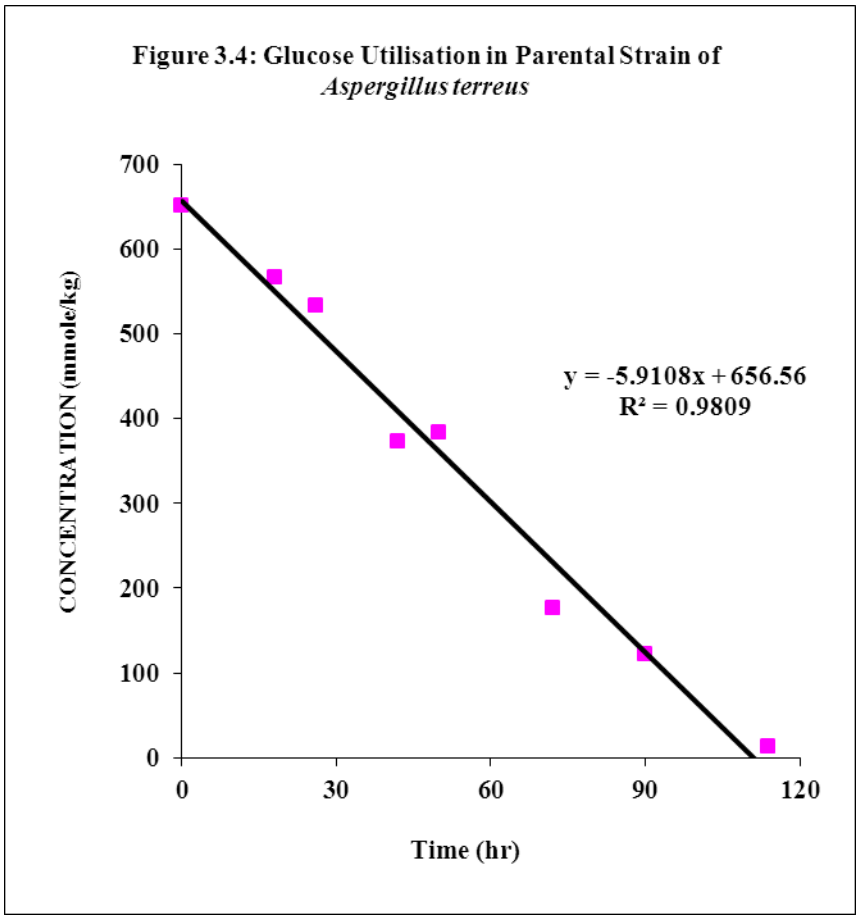


Figure 3.2: Glucose utilisation by modified AT10 (*A. niger pfk*A gene (●) and parental (♦) strains of *A. terreus* and gluconic acid production by modified AT10 (*A. niger pfk*A gene) (●) and parental (■) strains of *A. terreus* in 10-litre stirred tank fermentations. One sample at timed intervals was taken for analysis

The genetically transformed strain accumulated less gluconic acid than did the parent, the final gluconic acid titre being approximately 67% that of the parent (Figure 3.2). This interesting finding could not, however, be further investigated because of a lack of fermentation time available. The maximum rates of glucose use by the strains were

very similar, suggesting that the glucose utilising ability of the transformed strain had not been increased by the insertion of the *A. niger* gene (Figures 3.3-3.4).

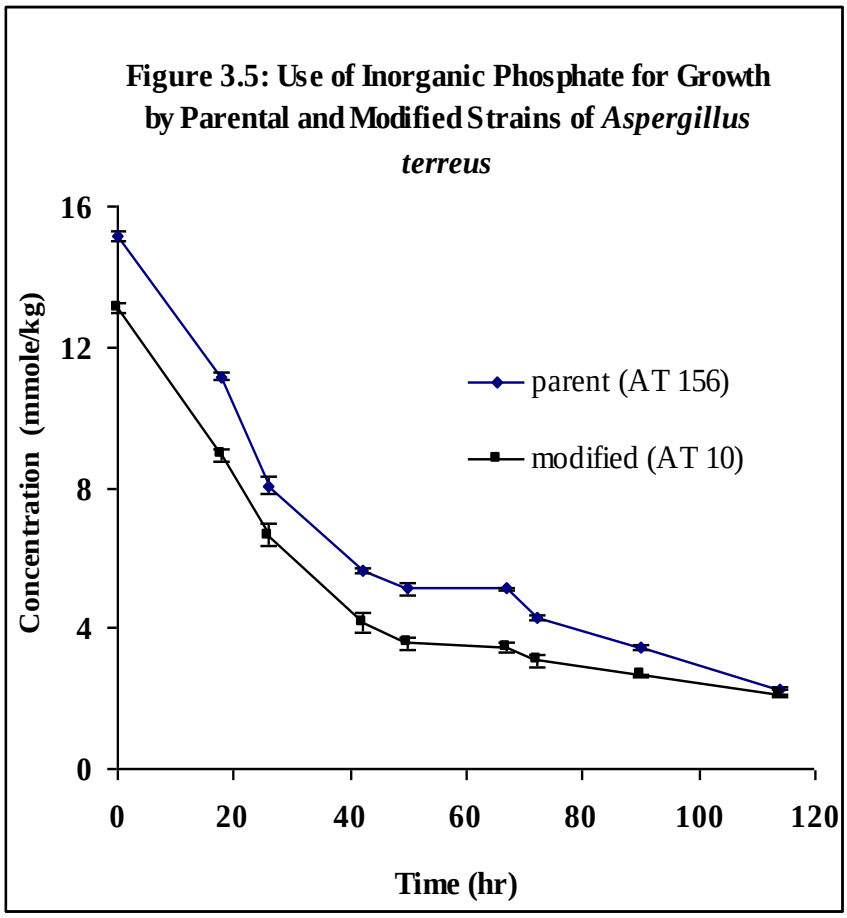




It could be deduced from results (Figures 3.3-3.4), that the rate of glucose use was very similar by the two strains.

Growth was difficult to accurately quantify by chemical measurement of cellular constituents in the perchloric acid (PCA)-insoluble material collectable by centrifugation because both cultures grew with large-pellet morphologies; after PCA treatment, samples gave irreproducible patterns of the weights of the insoluble material (Figure 3.1). Due to these technical problems, the uptake of inorganic phosphate from the media was investigated as a more accurate guide to growth kinetics. The fastest growth rates occurred in the first 40-50 hr in both fermentations;

after thus time, growths were very slow but neither strain fully exhausted the inorganic phosphate supply (Figure 3.5).



These results indicated that the genetic transformation had not impaired the ability of the strain to grow under the chosen fermentation conditions. Similar growth kinetics with reduced gluconic acid formation by the transformed strain was a potentially interesting combination of characteristics (Figures 3.5-3.7).

Figure 3.6: Gluconate Production by Modified Strain of *Aspergillus terreus*

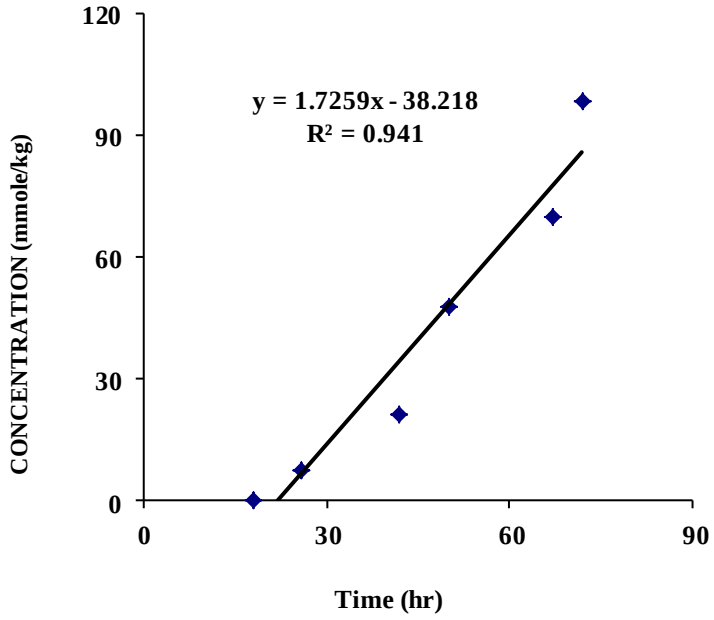
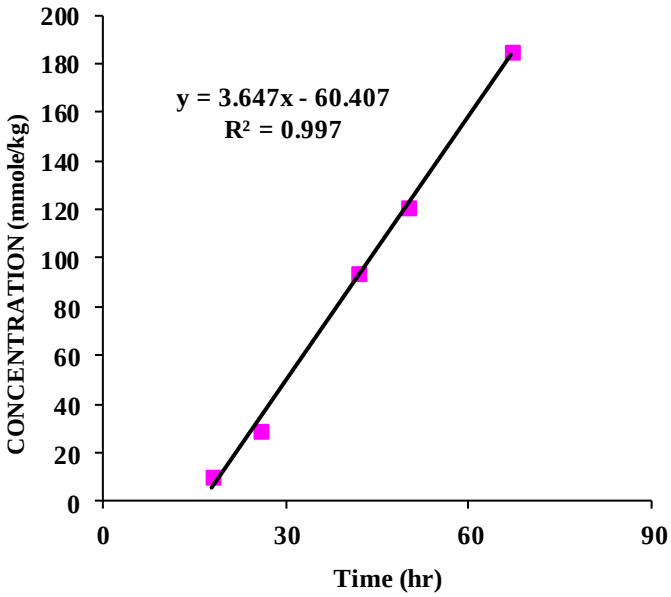


Figure 3.7: Gluconate Production by Parental Strain of *Aspergillus terreus*



If the introduced gene product in the transformed strain were functional, this phenotype can be interpreted as one in which the balance of fluxes has been altered, with more glucose directed towards glycolysis and the TCA cycle and less being "diverted" to gluconic acid (Figure 3.8-3.9 and tables 3.1-3.2)

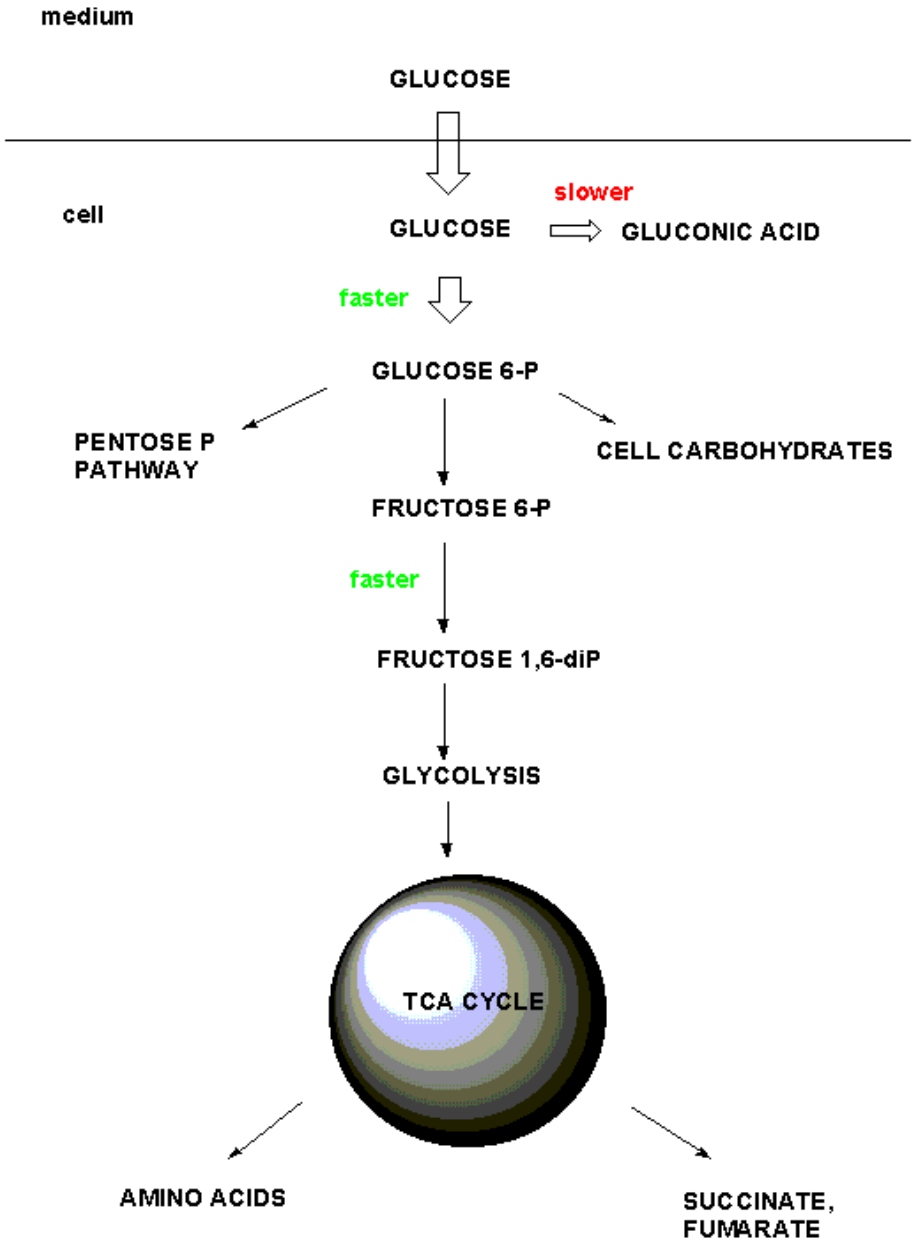
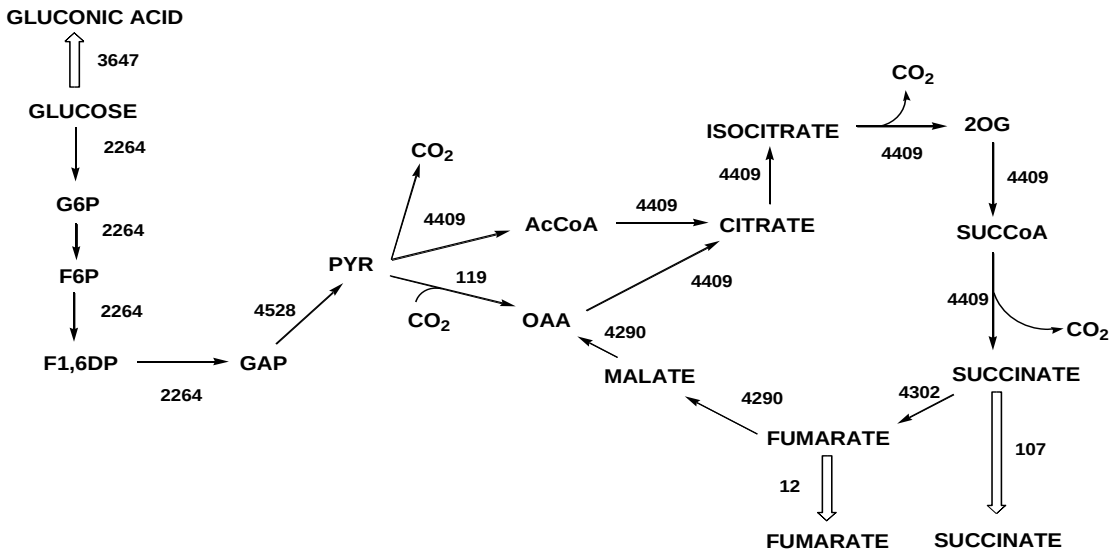


Figure 3.8: Glucose Utilisation by *pfkA*-transformant of *A. terreus*

AT156 0-67hr:



AT10 18-67hr:

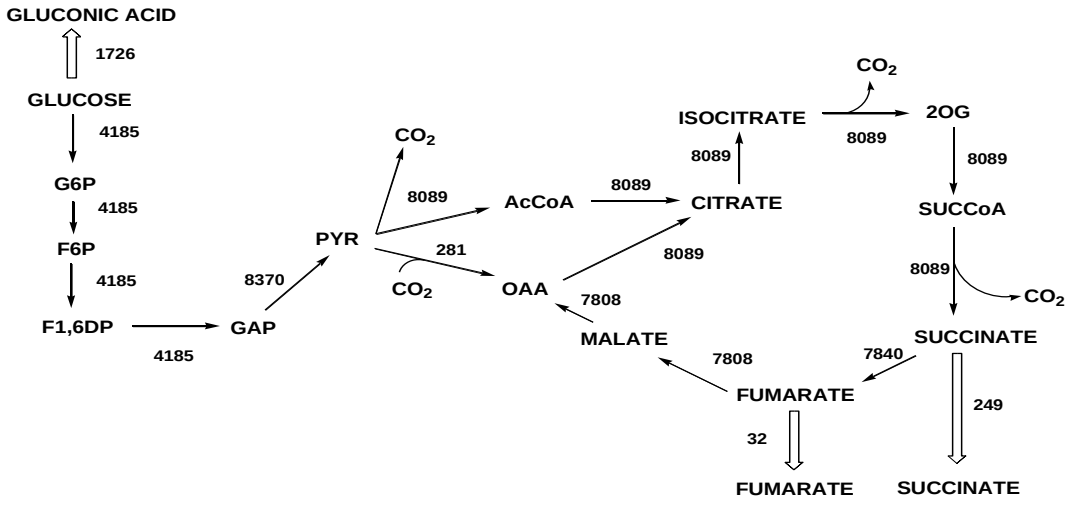


Figure 3.9 – Metabolic Rate Model For *Aspergillus terreus* Glucose Use IN 10-L Fermentors

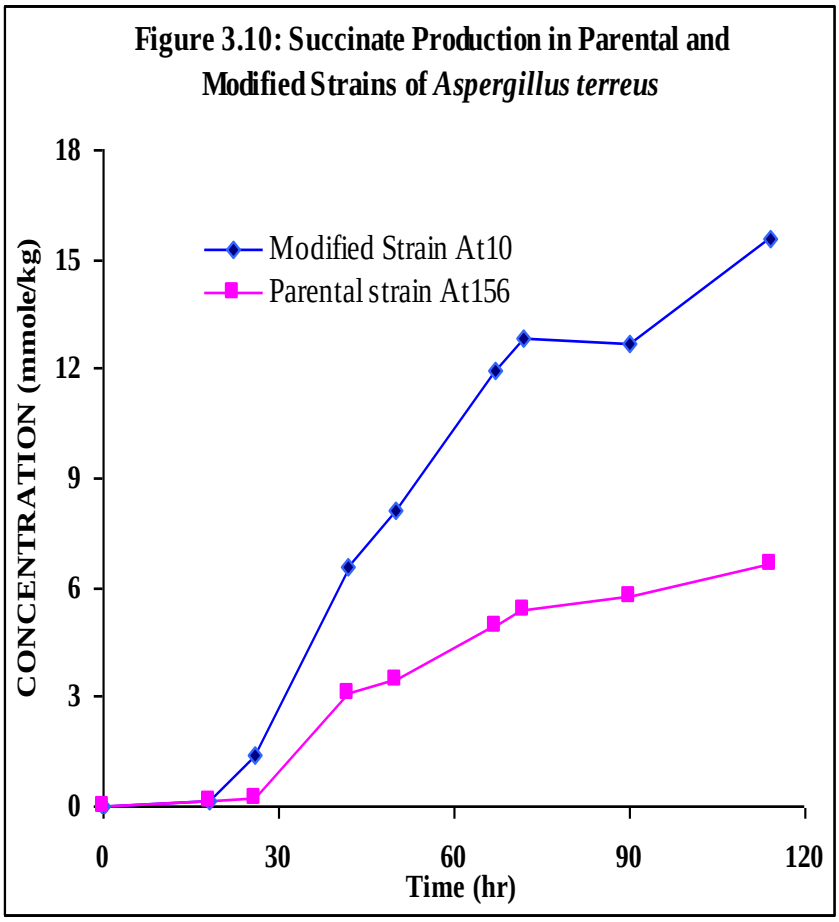
Strain	**AT10**						
Time (hr):	**18-67**						
Input	Use (mmole/kg/hr)	Use (umole/kg/hr)	Output	Rate (mmole/kg/hr)	Rate (umole/kg/hr)	Step	Rate (umole/kg/hr)
glucose	5.99	5990	gluconate	1.726	1726	glucose-glc6P	4185
			succinate	0.2487	249	glc6P-F6P	4185
			fumarate	0.323	32	F6P-F1,6-diP	4185
						F1,6diP-GAP	4185
						GAP-pyr	8370
						pyr-AcCoA	8089
						AcCoA-acetate	1726
						AcCoA-citrate	8089
						citrate-isocitrate	8089
						iscitrate-2OG	8089
						2OG-succinylCoA	8089
						succinylCoA-succinate	8089
						succinate-succinate	249
						succinate-fumarate	7840
						fumarate-fumarate	32
						fumarate-malate	7808
						malate-OAA	7808
						pyr-OAA	281
						OAA-citrate	8089

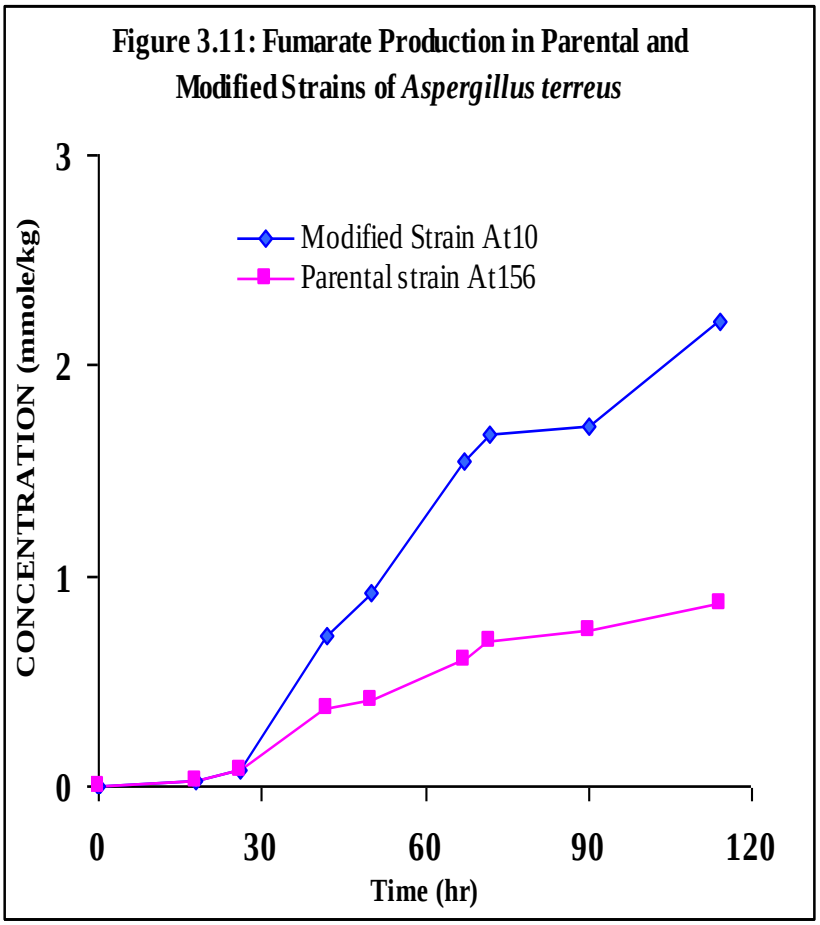
Table 3.1: Metabolic Rate Model for Modified *Aspergillus terreus*

Strain	**AT156**						
Time (hr):	**0-67**						
Input	Use (mmole/kg/hr)	Use (umole/kg/hr)	Output	Rate (mmole/kg/hr)	Rate (umole/kg/hr)	Step	Rate (umole/kg/hr)
glucose	5.911	5911	gluconate	3.647	3647	glucose-glc6P	2264
			succinate	0.107	107	glc6P-F6P	2264
			fumarate	0.012	12	F6P-F1,6-diP	2264
						F1,6diP-GAP	2264
						GAP-pyr	4528
						pyr-AcCoA	4409
						AcCoA-acetate	3647
						AcCoA-citrate	4409
						citrate-isocitrate	4409
						iscitrate-2OG	4409
						2OG-succinylCoA	4409
						succinylCoA-succinate	4409
						succinate-succinate	107
						succinate-fumarate	4303
						fumarate-fumarate	12
						fumarate-malate	4290
						malate-OAA	4290
						pyr-OAA	119
						OAA-citrate	4409

Table 3.2: Metabolic Rate Model for Parental *Aspergillus terreus*

The hypothesis that the modified strain was being "steered" towards the goal of increased glycolysis and TCA cycle intermediates was supported by the increasing amounts of the TCA intermediates fumaric and succinic acids being produced by the *pfkA*-modified strain (Figures 3.10 and 3.11).





The choice of nitrogen source is known to be important for the yield of gluocnic acid when *A. niger* is used as the producer organism (Ray and Banik, 1999). When the concentrations of the available nitrogen sources in the fermentation medium was investigated, ammonium was found to be the preferential nitrogen source (Figure 3.12-3.13). When the ammonium source was fully utilised, both strains used some of the nitrate but most of the free amino acids (Figures 3.13-3.15).

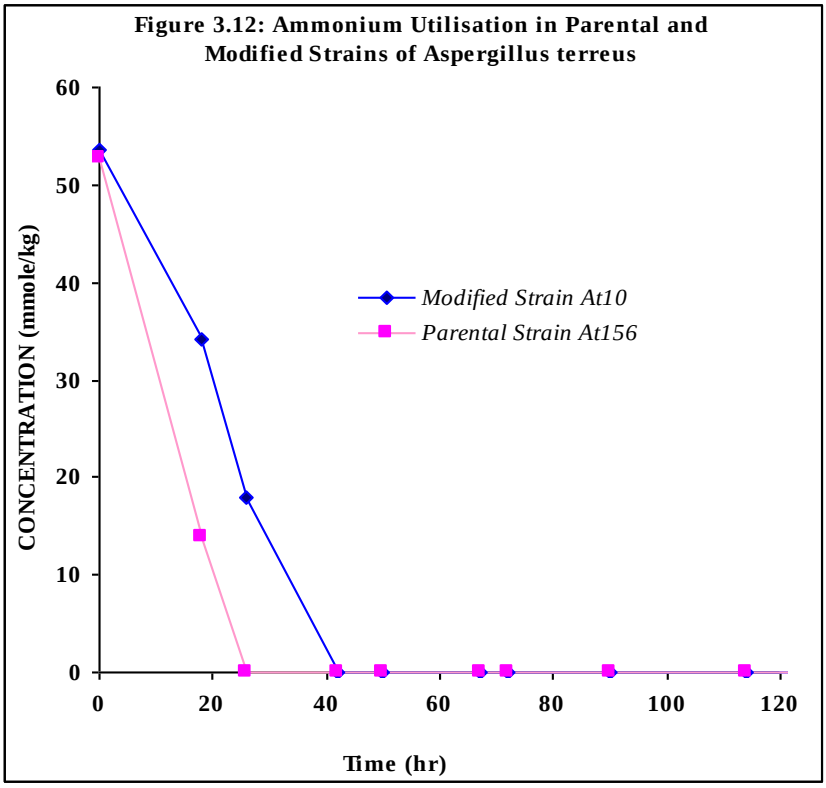


Figure 3.12: Ammonium Utilisation in Parental and Modified Strains of Aspergillus terreus
CONCENTRATION (mmole/kg)
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Time (hr)
Modified Strain At10
Parental Strain At156

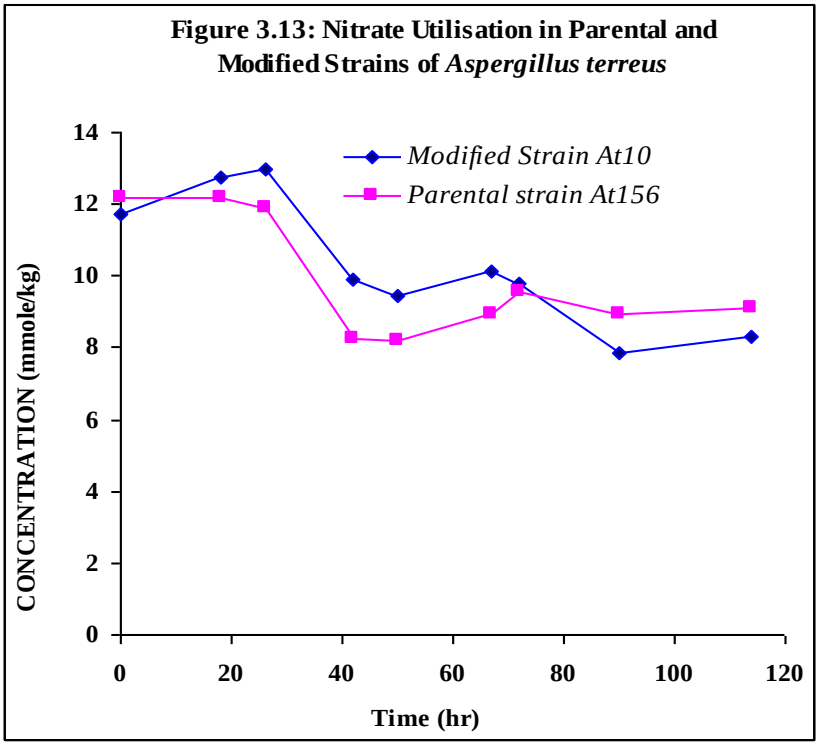


Figure 3.13: Nitrate Utilisation in Parental and Modified Strains of Aspergillus terreus
CONCENTRATION (mmole/kg)
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
Time (hr)
Modified Strain At10
Parental strain At156

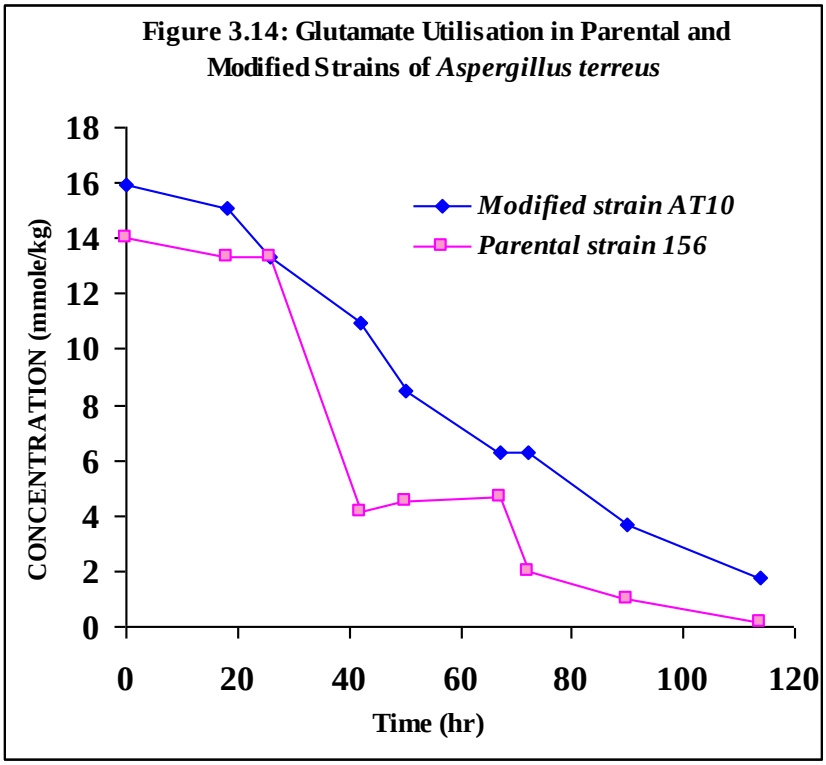
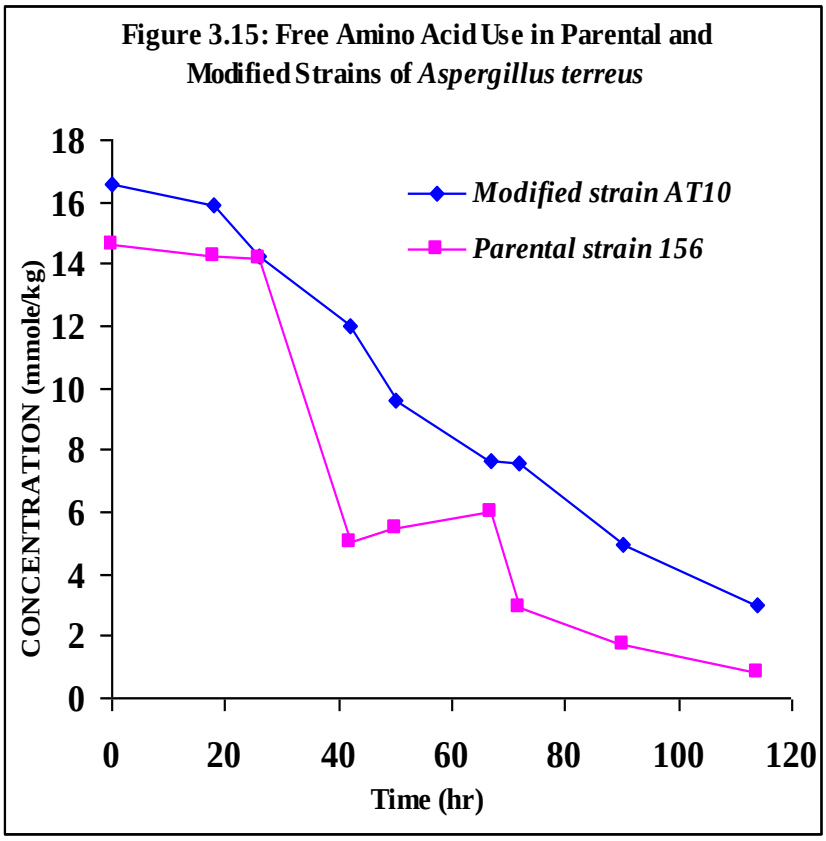


Figure 3.14: Glutamate Utilisation in Parental and Modified Strains of *Aspergillus terreus*
CONCENTRATION (mmole/kg)
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
Time (hr)
Modified strain AT10
Parental strain 156



3.2.3 *Aspergillus terreus* and gluconic acid production – fermentations at the National Institute for Chemistry, Ljubljana

ANTICO colleagues in Slovenia performed a group of fermentations with a lower glucose concentration than used in stirred tank fermentations in Glasgow. The initial medium contained 40 g l^{-1} glucose as the sole carbon source; all three strains tested (the parent and two transformants) showed rapid glucose use and gluconic acid accumulation (Figure 3.16). Once the limited amount of glucose in the medium was exhausted, gluconic acid production ceased and the metabolite was itself used a carbon source (Figure 3.17).

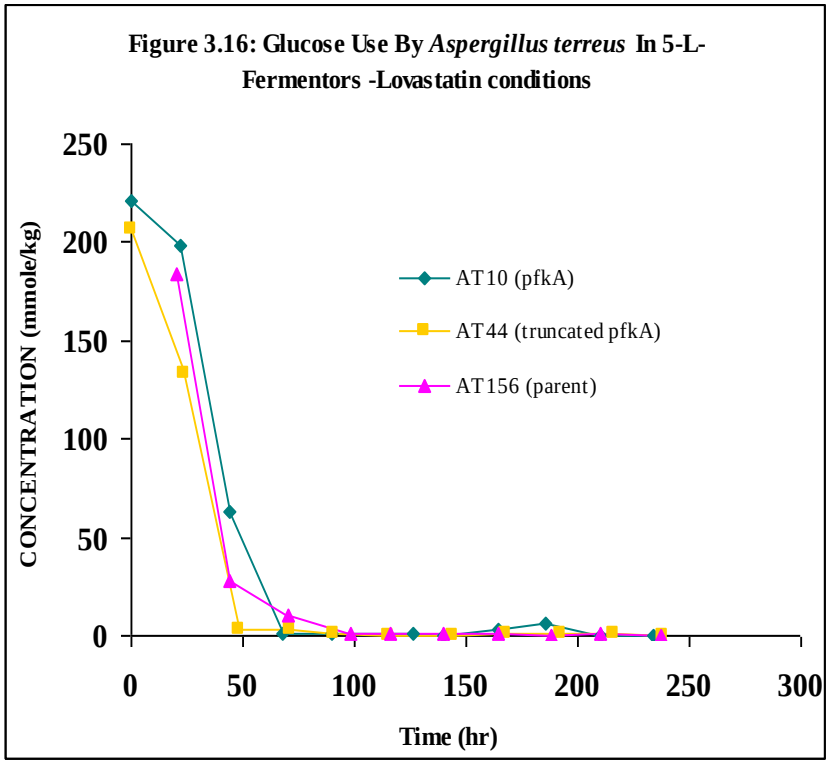


Figure 3.16: Glucose Use By *Aspergillus terreus* In 5-L-Fermentors -Lovastatin conditions
CONCENTRATION (mmole/kg)
250
200
150
100
50
0
0
50
100
150
200
250
300
Time (hr)
AT10 (pfkA)
AT44 (truncated pfkA)
AT156 (parent)

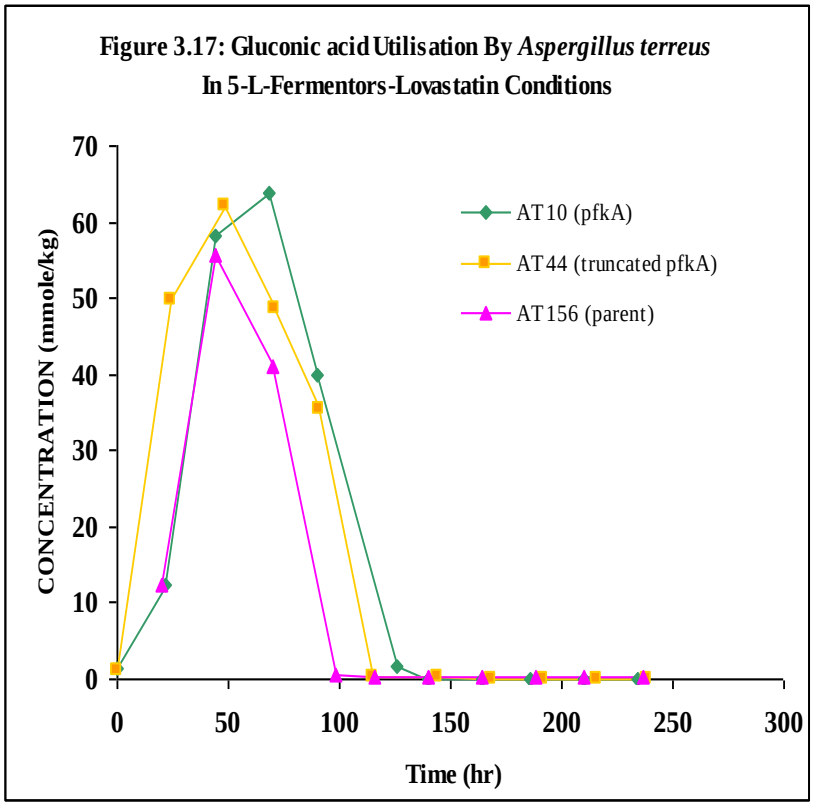
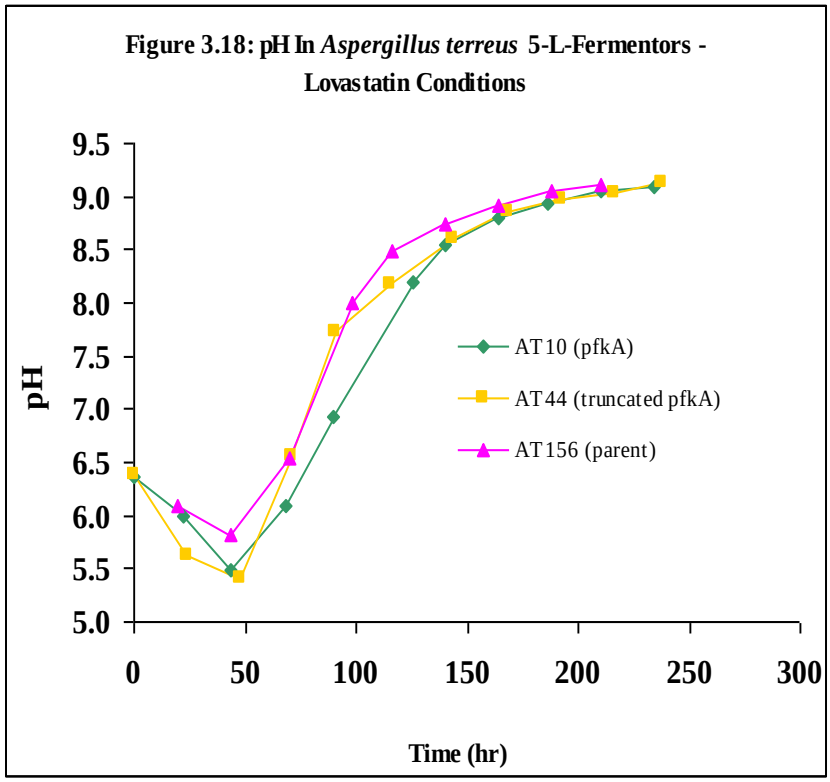
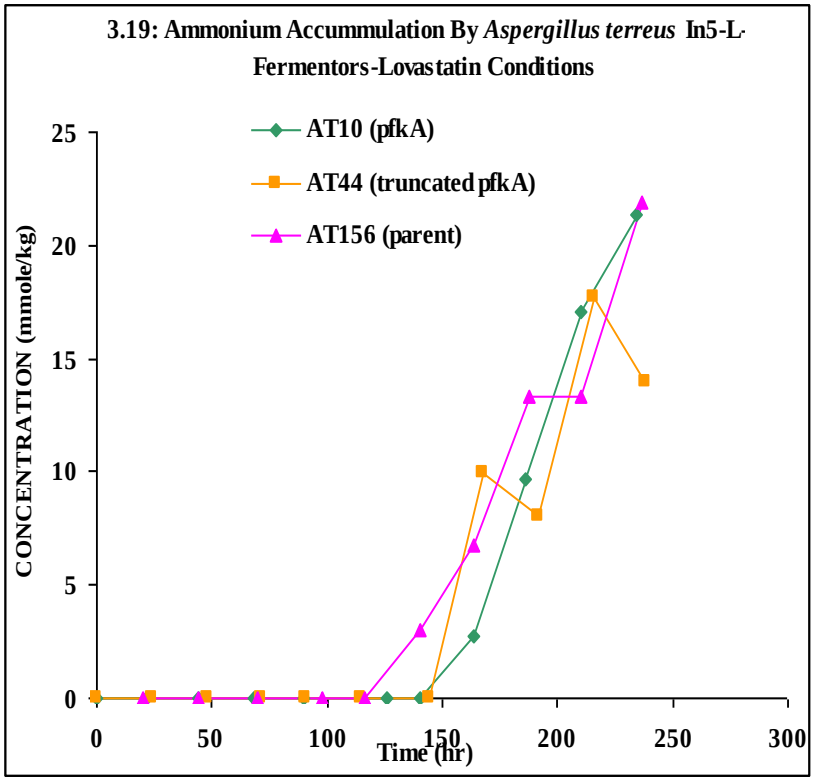


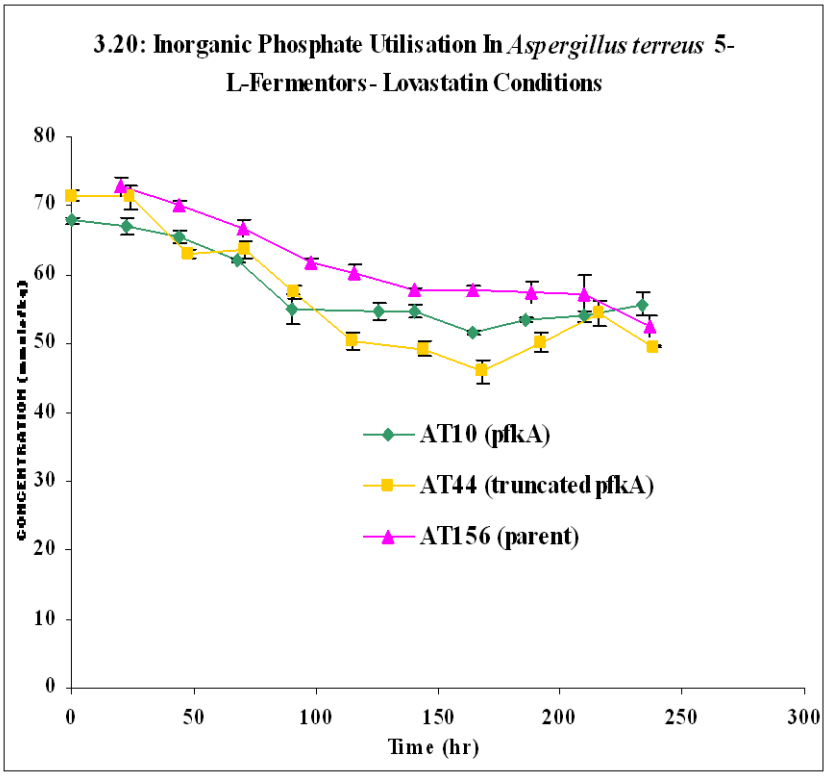
Figure 3.17: Gluconic acid Utilisation By *Aspergillus terreus* In 5-L-Fermentors-Lovastatin Conditions
CONCENTRATION (mmole/kg)
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
Time (hr)
AT10 (pfkA)
AT44 (truncated pfkA)
AT156 (parent)

No pH regulation was described for this defined medium fermentation in the original publication (Hajjaj *et al*., 2001); in the sampled fermentations, the pH increased rapidly after 50 hr when the accumulated metabolic acids were used (Figure 3.18). This severe increase in pH was caused by the metabolic utilisation of the accumulated gluconic acid and then by ammonia accumulating after 100hr (Figure 3.19).





Since the medium contained the amino acids, the likely source of the ammonia accumulated during the fermentation was deamination of amino acids when glucose and gluconic acid had been exhausted. This version of the medium contained abundant inorganic phosphate, which was only partly utilised for growth (Figures 3:20-3:22).


3.20: Inorganic Phosphate Utilisation In Aspergillus terreus 5-L-Fermentors- Lovastatin Conditions
CONCENTRATION (mmole/kg)
Time (hr)
AT10 (pfkA)
AT44 (truncated pfkA)
AT156 (parent)

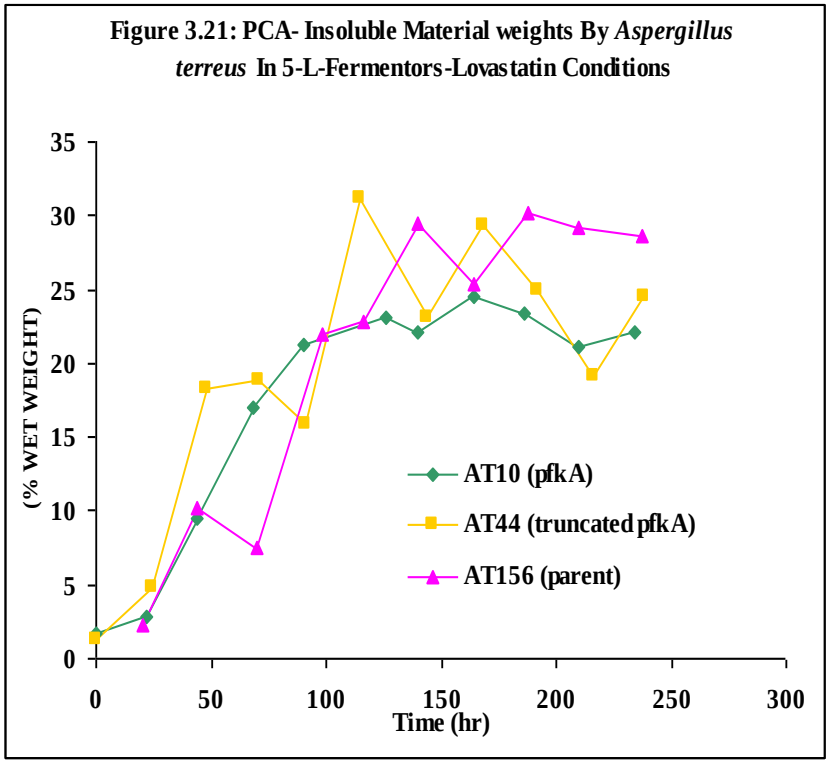
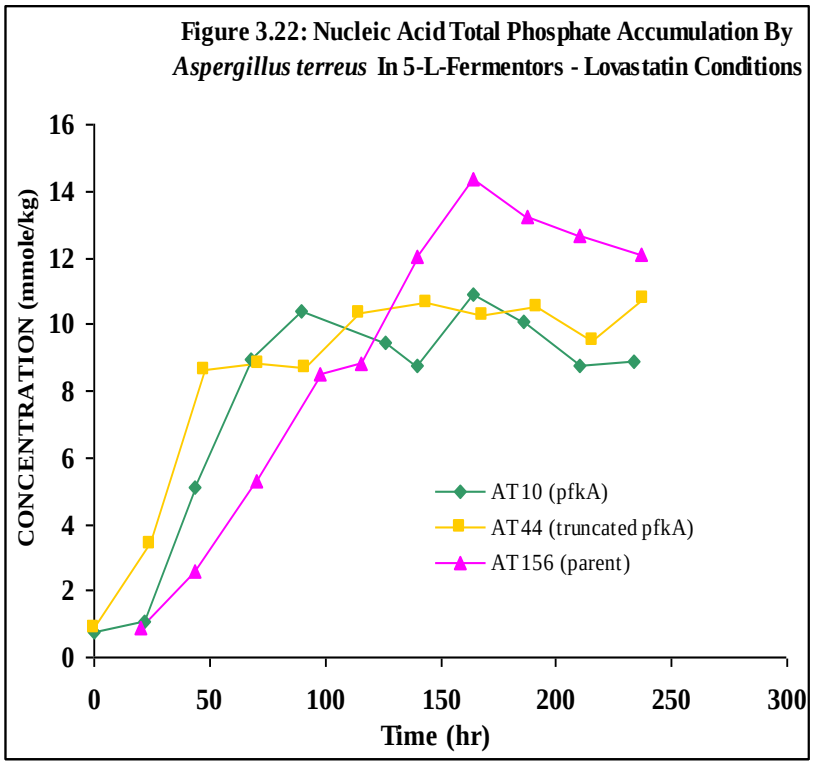


Figure 3.21: PCA- Insoluble Material weights By Aspergillus terreus In 5-L-Fermentors-Lovastatin Conditions
(% WET WEIGHT)
Time (hr)
AT10 (pfkA)
AT44 (truncated pfkA)
AT156 (parent)



While processing the samples for growth analysis by PCA-insoluble material and total phosphate in nucleic acids, it was apparent that a red pigment had accumulated in the fermentation. This red coloration was partially soluble but also present in the cellular material precipitated by perchloric acid. Both the transformed strains accumulated markedly less red pigment than did the parent (Figures 3.23-3:25).

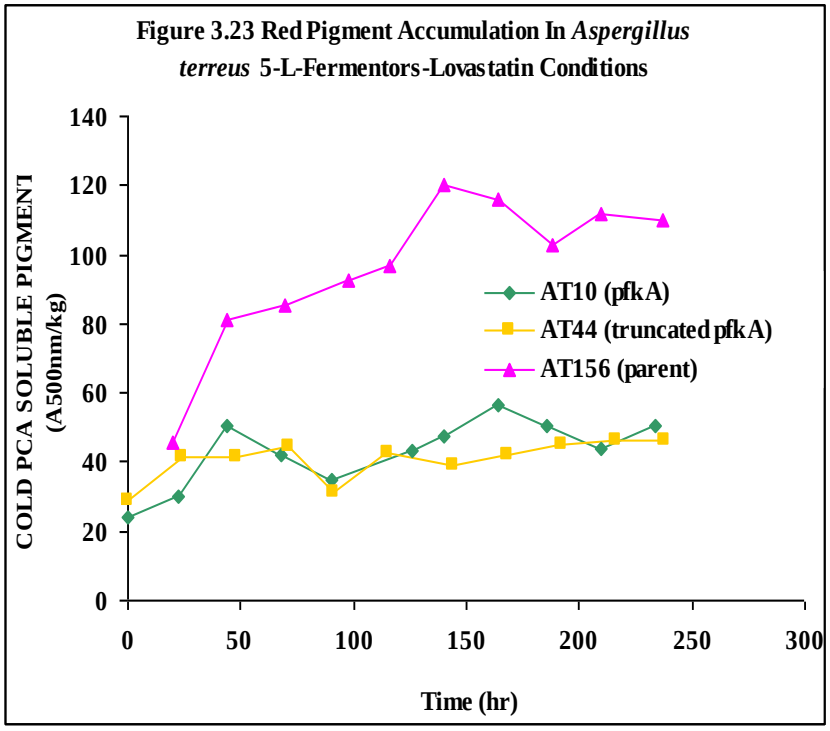
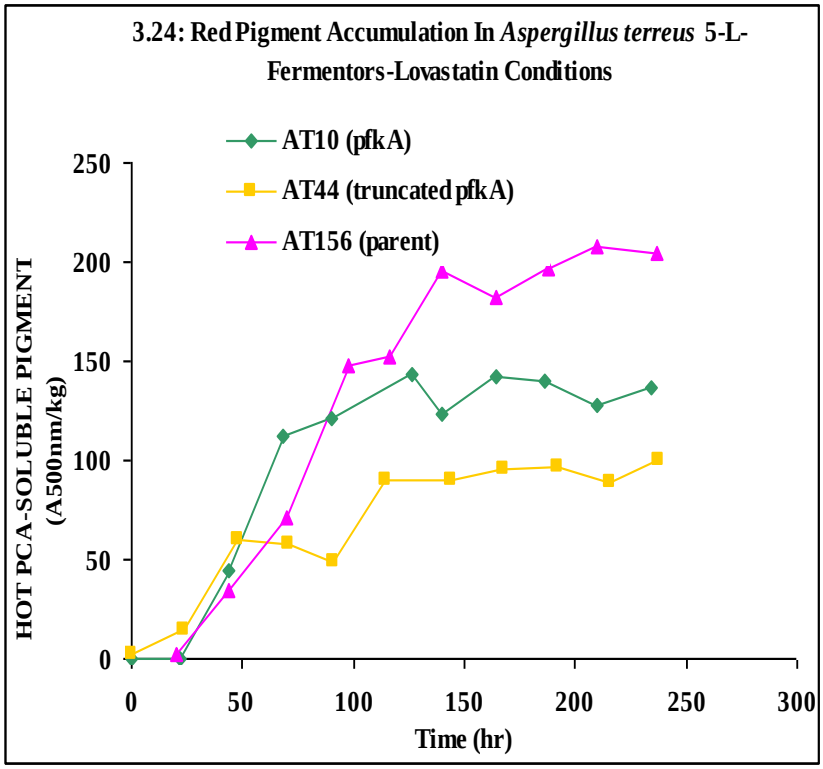
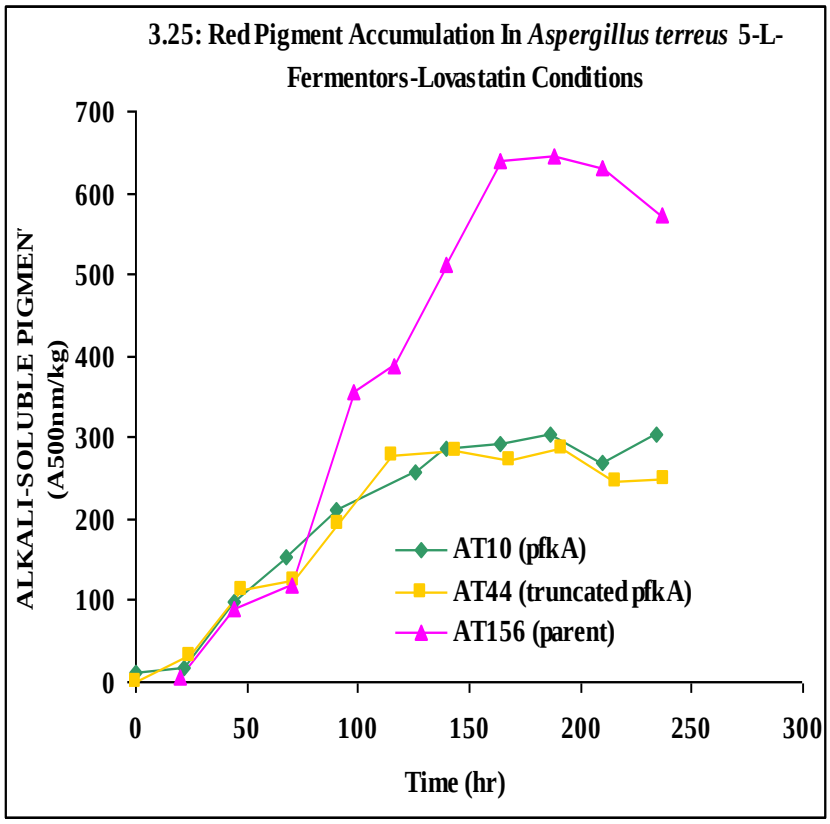


Figure 3.23 Red Pigment Accumulation In *Aspergillus terreus* 5-L-Fermentors-Lovastatin Conditions
COLD PCA SOLUBLE PIGMENT (A500nm/kg)
140
120
100
80
60
40
20
0
0
50
100
150
200
250
300
Time (hr)
AT10 (pfkA)
AT44 (truncated pfkA)
AT156 (parent)


3.24: Red Pigment Accumulation In *Aspergillus terreus* 5-L-Fermentors-Lovastatin Conditions
HOT PCA-SOLUBLE PIGMENT (A500nm/kg)
250
200
150
100
50
0
0
50
100
150
200
250
300
Time (hr)
AT10 (pfkA)
AT44 (truncated pfkA)
AT156 (parent)



The chemical identity of the red pigmentation was not further investigated. The integration of the *A. niger* genes into the *A. terreus* chromosome may have disrupted endogenous genes not essential for growth or cell viability.

3.2.4 Shake flask investigations of gluconic acid production by *A. terreus*

Since stirred tank fermenters became unavailable for further investigation of gluconic acid production by *A. terreus*, shake flasks were set up using glucose concentrations of 50, 100 and 150 g l^{-1} (Figures 3.26-3:31).

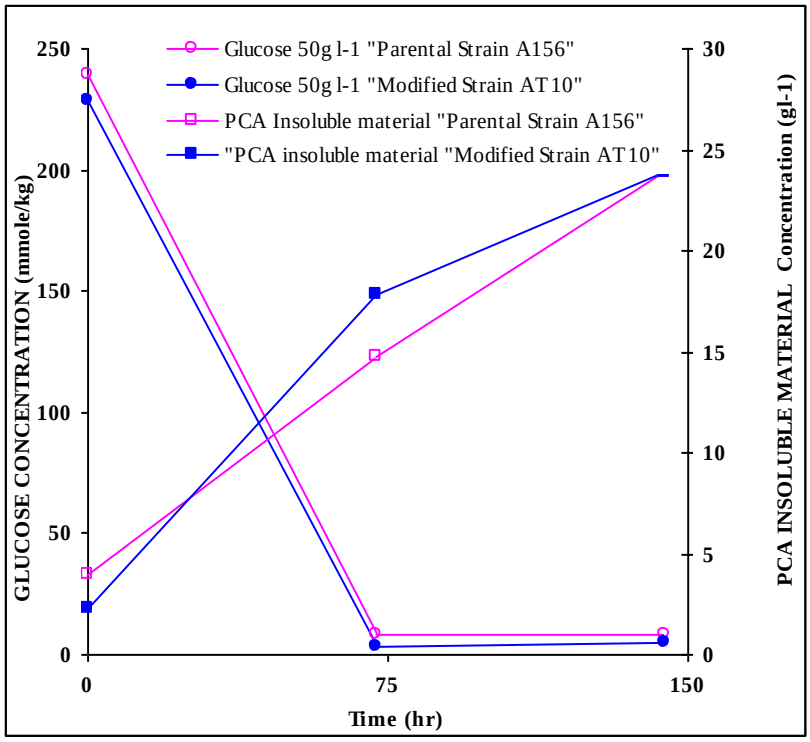


Figure 3.26: Glucose utilisation by parental AT156 (○) and modified AT10 (●) strains of *A. terreus* and PCA insoluble material with parental AT156 (□) and modified AT10 (■) strains of *A. terreus i*n shake flask fermentations. One sample at timed intervals was taken for analysis.

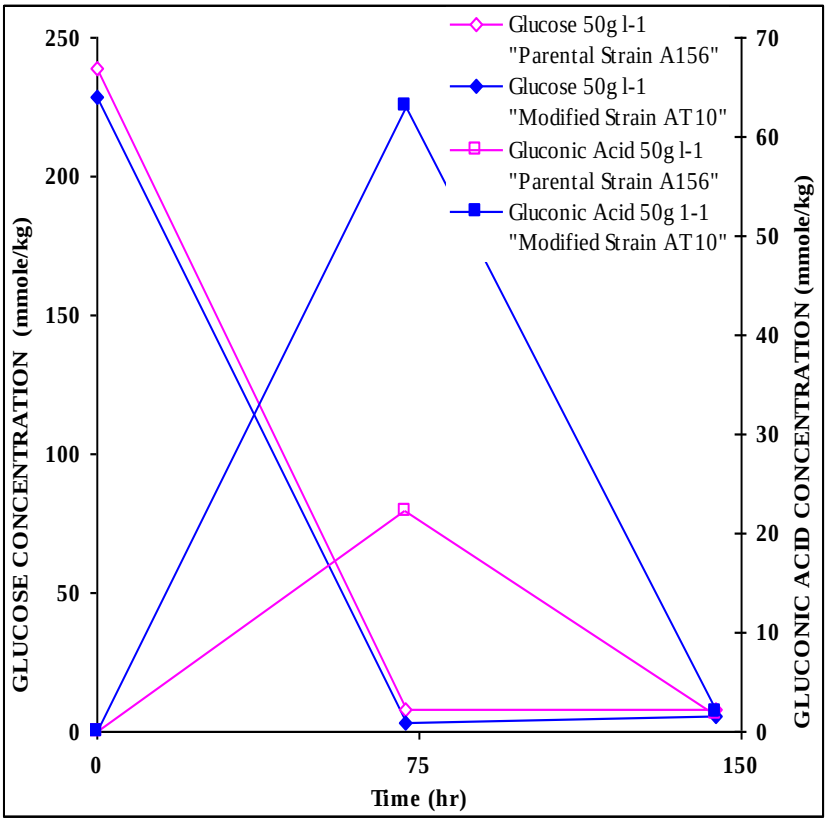


Figure 3.27: Glucose utilisation by parental AT156 (○) and modified AT10 (●) strains of *A. terreus* and gluconic acid production with parental AT156 (□) and modified AT10 (■) strains of *A. terreus* in shake flask fermentations. One sample at timed intervals was taken for analysis.

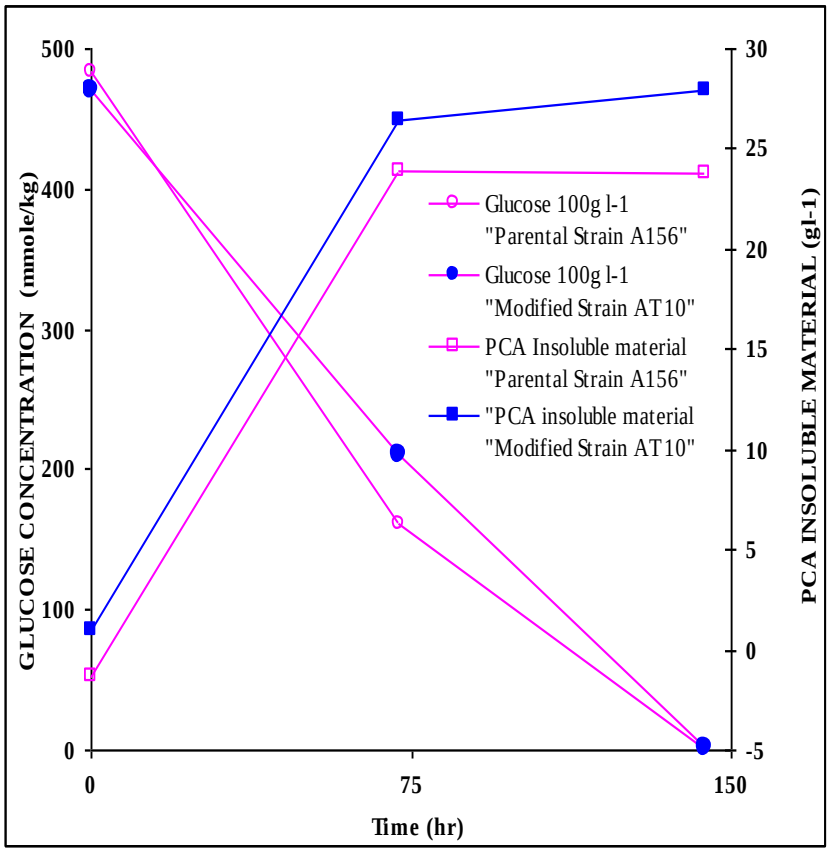


Figure 3.28: Glucose utilisation by parental AT156 (○) and modified AT10 (●) strains of *A. terreus* and PCA-insoluble material with parental AT156 (□) and modified AT10 (■) strains of *A. terreus i*n shake flask fermentations. One sample at timed intervals was taken for analysis.

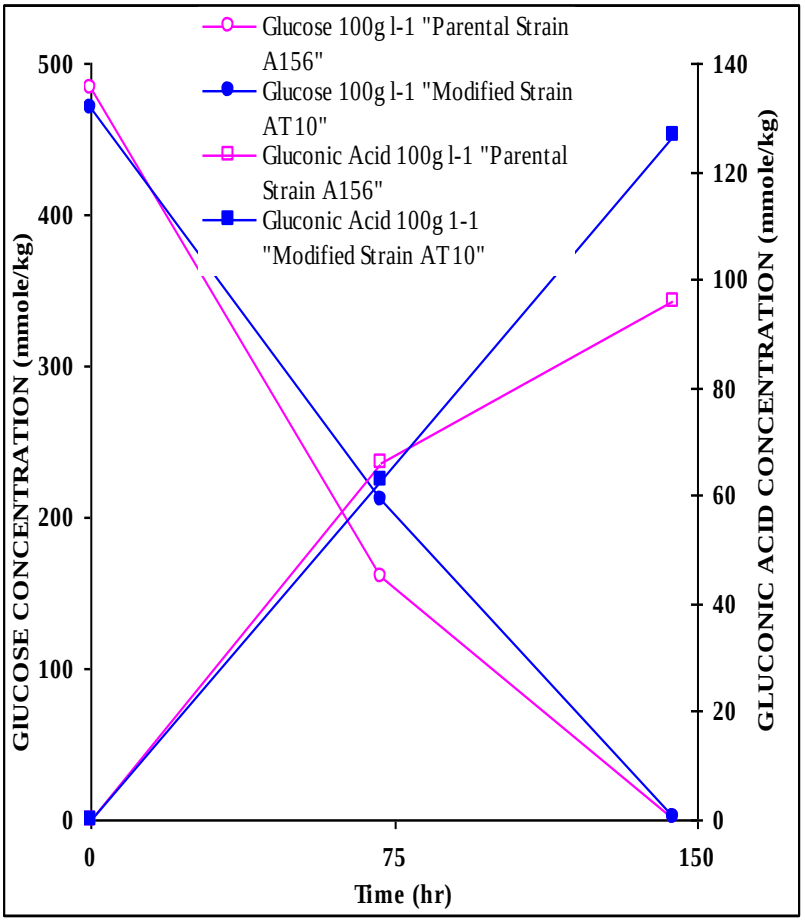


Figure 3.29: Glucose utilisation by parental AT156 (○) and modified AT10 (●) strains of *A. terreus* and gluconic acid production with parental AT156 (□) and modified AT10 (■) strains of *A. terreus* in shake flask fermentations. One sample at timed intervals was taken for analysis.

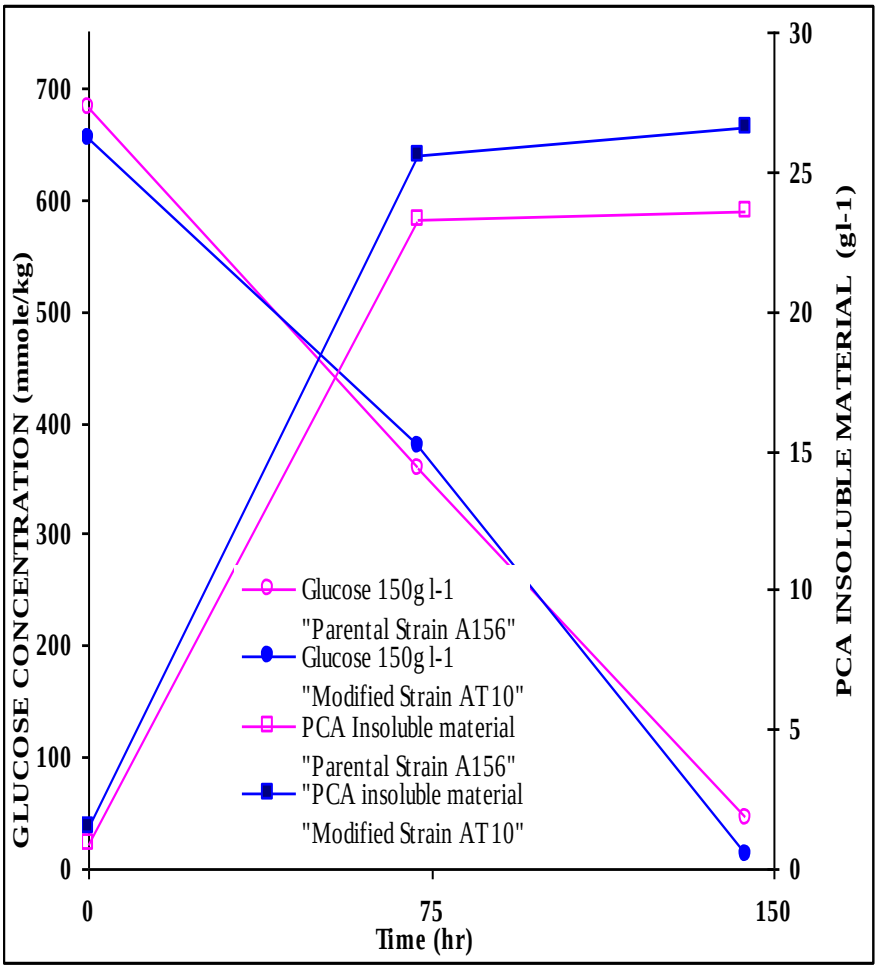


Figure 3.30: Glucose utilisation by parental AT156 (○) and modified AT10 (●) strains of *A. terreus* and PCA-insoluble material with parental AT156 (□) and modified AT10 (■) strains of *A. terreus* in shake flask fermentations. One sample at timed intervals was taken for analysis.

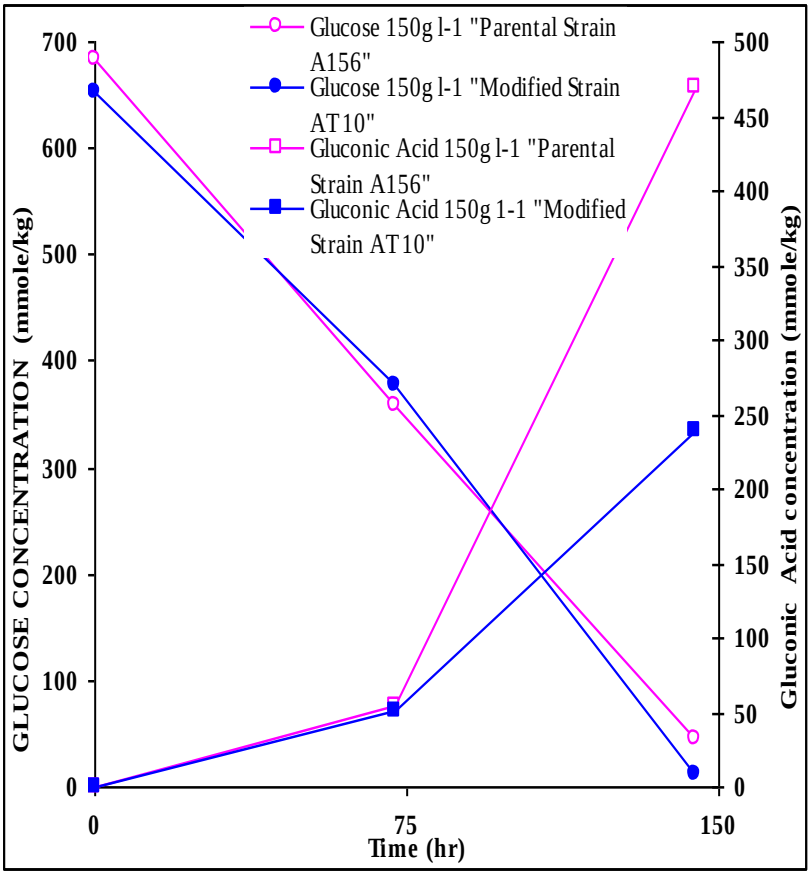


Figure 3.31: Glucose utilisation by parental AT156 (○) and modified AT10 (●) strains of *A. terreus* and gluconic acid production with parental AT156 (□) and modified AT10 (■) strains of *A. terreus* in shake flask fermentations. One sample at timed intervals was taken for analysis.

It was demonstrated that at the lowest glucose concentration used (50g 1^{-1}), gluconic acid had accumulated by 72hr but was then utilised as the supply of glucose became low (Figure 3.27). However, at concentrations of 100g 1^{-1} and 150g 1^{-1} where the glucose was not utilised fully before 100 hr, gluconic acid could remain at high concentrations (Figures 3.29 and 3.31). These results confirmed the fermentor data that gluconic acid was a primary metabolite of glucose but could serve as a carbon source once the glucose had been used.

3.3 Discussion

The failure of the either the parental or transformed strains to produce lovastatin was disappointing. Enquires to ANTICO colleagues in Slovenia later revealed that the parental strain had never been demonstrated in the laboratory to produce lovastatin. Nevertheless, the accumulation of gluconic acid gave a fortuitous new outlook on *A. terreus* metabolism.

Gluconic acid is produced commercially in fermentations using *A. niger* and glucose is the usual carbon source (Singh and Kumar, 2007). Highly mutated and selected strains of *A. niger* can biosynthesize gluconic acid at more than 95% of the theoretical yield from glucose (Ramachandran *et al.*, 2006). The biosynthetic pathway is simple and involveds only two enzymesg glucose oxidase (EC 1.1.3.4) is a glycoprotein with two bound FAD co-enzymes and catalyses the oxidation of D-glucose to D-gluconolactone (and hydrogen peroxide as a coproduct); D-gluconolactone may spontaneously hydrolyze to gluconic acid but this reaction is catalyzed by lactonase (Wong *et al*., 2008). Gluconate formation by *A. niger* is thought to occur extracellularly because immunocytochemical studies have shown that glucose oxidase is localized in the cell wall together with catalase and lactonase (Witteveen *et al*., 1992).

Gluconic acid has been identified as one of the top 30 "building block" compounds that can be produced in "biorefineries" from pant biomass sources (Werpy and Peterson, 2004). However, relatively high production costs presently limit the use of gluconic acid as a chemical on a large scale (Singh and Kumar, 2007). Research is continuing, therefore, into improving the efficiency and process economics of fermentation production processes. Recent studies have explored novel producer organisms (Anastassiadis *et al*., 2003), fermentation optimization strategies (Cheema *et al*., 2002), use of cheaper carbohydrate sources (Singh and Singh, 2006), solid-state

fermentation (Sharma *et al*., 2008) and using fungal spores (Ramachandran *et al*., 2007).

Figures 3.1 and 3.4 show that there was no difference in glucose utilisation between the parental and modified strains; however, when we look at gluconic acid production we see a very different picture as the *pfkA* genetically transformed strain (Figure 3.2) accumulated less gluconic acid (approximately 67%) than that of the parent.

Due to technical problems encountered, namely irreproducible patterns of weights (Figure 3.1), inorganic phosphate utilisation was employed as a more accurate guide to growth kinetics; this showed that the first 40-50hr was the most productive time, when both inorganic phosphate utilisation (Figure 3.5) and gluconic acid production were fastest (Figures 3.6-3.7)

The genetic modifications may have resulted in the balance of fluxes being altered so that more glucose was diverted towards glycolysis and the TCA cycle and less being diverted to gluconic acid (Figure 3.8). Gluconic acid production was slower in the *pfkA*- transformant of *A. terreus* compared to the parent in one fermentor trial carried out for strain comparison (Figures 3.6 and 3.7)

This hypothesis is illustrated in the metabolic rate model (Figure 3.9 and Table 3.1-3.2) where the rate of gluconic acid production by the transformant is seen to be lower, 1.9 mmole/kg/hr compared to 3.6 mmole/kg/hr, almost a 2 fold decrease. Support again is provided by the increasing rates of the TCA intermediates fumaric and succinic acid produced by the *pfkA*-modified strain: with succinate 0.25 mmole/kg/hr compared to 0.11 mmole/kg/hr, more than a two fold increase (Figures 3.10 and 3.11, Tables 3-1 and 3.2). Similar results (more than a two fold increase) were observed with fumarate: 0.032 mmole/kg/hr compared to 0.012 mmole/kg/hr.

Another important piece of information is that the choice of nitrogen source is crucial to the yield of gluconic acid with *A. Terreus*. The preferred nitrogen source may be ammonium (Figure 3.12) as this was completely utilised quickly, before 30hr with the parental strain and 45hr with the modified strain; however, this was not the case with nitrate (Figure 3.13) as nitrate was not utilised until after the ammonium was exhausted. In addition, amino acids were utilised at steady rate throughout the fermentation but were not fully utilised by either strain (Figure 3.14 – 3.15).

The results obtained with an initial medium of 40g l^{-1} glucose showed that the glucose concentration was crucial to sustaining the gluconic acid production. When all three strains where tested, the *pfkA*-transformed (AT10), the truncated *pfkA* (AT44) and the parent (AT156) it was found that when glucose in the medium was utilised fully (Figure 3.16) gluconic acid production also ceased (Figure 3.17). When the gluocose was fully used up, gluconic acid became itself a carbon source for growth, which was substantiated by the increase in pH (Figure 3.18) caused by the metabolic utilisation of the accumulated gluconic acid and then when ammonia accumulated after 100hr (Figure 3.19). The likely source of ammonia was the deamination of amino acids when glucose and gluconic acid became depleted.

Growth indicators inorganic phosphate (Figure 3.20), PCA-insoluble material weight (Figure 3.21) and total nucleic phosphate (Figure 3.22) show no marked differences between the three strains. However, during growth analysis an accumulation of red pigment became apparent in the fermentation; the red pigmentation which was both partially soluble and present in the cellular material precipitated by perchloric acid was accumulated by parental strain markedly more than the other two transformants (Figures 3.23-3.25). In this respect, the transformants both exhibited promising metabolic features for industrial use in that they were capable of diverting less of the carbon and other nutrients from the medium to the production of macromolecular

components (red pigmentation) that would inevitably be discarded during downstream processing.

The shake flask trials into gluconic acid production reinforced some interesting finding already reported (Figures 3.16 - 3.17). At low glucose concentrations (Figures 3.26-3.27) when glucose became depleted, gluconic acid was utilized for continued growth of micro-organism. These results confirmed the fermentor data that gluconic acid was a primary metabolite of glucose and when the glucose is used gluconic acid would serve as a carbon source. However, at elevated glucose concentrations (> 100g l^{-1}) the three strains continued to produce gluconic acid although at 100g l^{-1} the modified strain produced more gluconic acid than the parent (Figure 3.9) which was the opposite of the findings earlier in the fermentor trials (Figures 3.6-3.7) where the parental produced more gluconic acid. At glucose concentration of 150g $l^{-}1$ the parental strain again produced more gluconic acid (Figure 3.31).

These contradictory findings could be as a consequence of the *A niger pfkA* genes having two independent but contrary effects on gluconic acid production in *A. terreus* whereby a more efficient flux of glucose to amino acids for extracellular enzyme formation would increase the rate of gluconic acid formation but with a higher rate of glycolysis, gluconic acid production would be reduced consequently as intracellular glycolysis competed with extracellular gluconate formation for glucose. The earlier effect may go some way to explaining why the genetically engineered strains at low glucose concentrations produced more gluconic acid and it has been demonstrated with the *A. niger* transformant carrying *t-pfkA* gene, increased extracellular α-amylase production was observed by ANTICO colleagues in Slovenia.

The work presented in this thesis demonstrates that *A. terreus* has the potential for becoming a producer of gluconic acid as well as its established role in the production of itaconic acid. The most straightforward assumption in that the biochemistry of *A.*

terreus for carboxylic acid production resembles that in *A. niger*, whose ability to produce citric, gluconic or oxalic acid at different pH values has been the subject of academic and industrial research for over 30 years (Ramamchandran *et al.*, 2006). Even the wild type *A. terreus* can produce gluconic acid in high yields, up to 0.7 mol mol^{-1} glucose consumed (Dowdells *et al.*, 2010). The wild-type *A. terreus* is unlikely, however, to compete with the best industrial production strains of *A. niger* used for gluconic acid production

However, a target for rational strain improvement has been identified by the ANTICO project work. Galactonic acid is structurally very similar to gluconic acid and is known to be metabolized by *A. terreus* by a nonphosphorolytic pathway (Elshafeia and Abdel-Fataha, 1991). If gluconic acid metabolism in *A. terreus* requires a gluconate dehydratase activity to degrade gluconic acid into 2-keto-3-deoxy-gluconate followed by a 2-keto-3-deoxy-gluconate aldolase to form pyruvate and glyceraldehyde, the deletion of any genes for gluconate dehydratase would be predicted to produce strains with reduced gluconate catabolism; this would provide a rational strain improvement for industrially useful strains.

Another target for strain development would be reduction in secondary product formation, not only for lovastatin but also the elimination of the biosynthesis of the dangerous mycotoxin citreoviridin (Franck and Gehrken, 1980). The effect of expressing the *A. niger pfk*A gene(s) in *A. terreus* producing gluconic acid is not fully predictable because, while a more efficient flux of glucose to amino acids for extracellular enzyme formation could increase the rate of gluconic acid formation, a consistently higher rate of glycolysis could reduce gluconic acid production as intracellular glycolysis competed with extracellular gluconate formation for glucose. The first of these contrary effects might explain the higher rates of gluconic acid production observed with the *A. niger* transformant carrying t-*pfk*A gene increased extracellular α-amylase production (Legiša *et al*., 2007).

The realization of gluconic acid production from glucose by *A. terreus* at relatively high pH in the fermentation also suggests new ways of improving the production of lovastatin and other secondary metabolites. The nonphosphorolytic pathway of gluconate catabolism (Elshafeia and Abdel-Fataha, 1991) is bioenergetically inferior to the glycolytic pathway of glucose and the production of high concentrations of gluconic acid can disrupt pH regulation in fermentations and introduce high concentrations of osmotically active solutes (by base addition). If gluconic acid is accumulated and then metabolized as a carbon source, this would cause an acid demand to maintain a set pH.

To *minimize* gluconate formation in secondary product processes, therefore, high concentrations of glucose must be avoided. A fed-batch strategy can be envisaged in which the glucose feed rate is regulated by feedback control using fermentation broth pH as the operating parameter to maintain the pH against decreases caused by gluconic acid accumulation.

These novel findings lead to a manuscript being submitted to letters in Applied Microbiology and which has subsequently been accepted for publication. The final manuscript is presented below.

Letters in
Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Gluconic acid production by *Aspergillus terreus*

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Keywords
gluconic acid, fermentation, *Aspergillus terreus*, glucose oxidase.

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2010/0125: received 24 January 2010; revised 18 May 2010; accepted 9 June 2010

doi:10.1111/j.1472-765X.2010.02890.x

Abstract

Aim: *Aspergillus terreus* produces itaconic acid at low pH but lovastatin and other secondary metabolites at higher pH in the fermentation. The utilization of glucose as a carbon substrate was investigated for secondary metabolite production by *A. terreus*.

Methods and Results: With a starting pH of 6·5, glucose was rapidly metabolized to gluconic acid by the wild-type strain and by transformants harbouring *Aspergillus niger* genes encoding 6-phosphofructo-1-kinases with superior kinetic and regulatory properties for bioproduction of metabolites from glucose. On exhaustion of the glucose in batch fermentations, the accumulated gluconic acid was utilized as a carbon source.

Conclusions: A novel pathway of glucose catabolism was demonstrated in *A. terreus*, a species whose wild type is, without any strain development, capable of producing gluconic acid at high molar conversion efficiency (up to 0·7 mol mol^{-1} glucose consumed).

Significance and Impact of the Study: *Aspergillus terreus* is a potential novel producer organism for gluconic acid, a compound with many uses as a bulk chemical. With a new knowledge of glucose catabolism by *A. terreus*, fermentation strategies for secondary metabolite production can be devised with glucose feeding using feedback regulation by pH.

Introduction

Gluconic acid is a bulk chemical with many uses in the food, beverage and other industries and is produced commercially by submerged fermentation using *Aspergillus niger* and glucose as the carbon source (Singh and Kumar 2007). Strains of *A. niger* can biosynthesize gluconic acid at more than 95% of the theoretical conversion efficiency from glucose (Ramachandran *et al.* 2006). Glucose oxidase (EC 1·1·3·4) is a well-characterized glycoprotein with two bound FAD co-enzymes and catalyses the oxidation of D-glucose to D-gluconolactone and hydrogen peroxide; the D-gluconolactone product may spontaneously hydrolyse to gluconic acid, although this transformation is catalysed by lactonase (Wong *et al.* 2008). Gluconate formation by *A. niger* occurs extracellularly; immunocytochemical methods show that glucose oxidase is localized in the cell wall together with catalase and lactonase activities (Witteveen *et al.* 1992).

Another carboxylic acid, itaconic acid (methylenesuccinic acid), is biosynthesized by *A.* terreus at low pH, worldwide production exceeding $8{\cdot}0 \times 10^4$ tons per year (Okabe *et al.* 2009). The same organism under different fermentation conditions and with a higher operating pH elaborates secondary metabolites, including lovastatin (Bizukojc and Ledakowicz 2009). Lovastatin was the first cholesterol-lowering agent to be approved for clinical use (Alberts 1990). Slowly metabolized carbon sources (for example lactose) are considered to give the highest production of lovastatin (Casas López *et al.* 2003).

Citric acid production by *A. niger* is similar to the itaconic acid fermentation by *A. terreus*; one of the key features of glucose metabolism in citric acid-producing *A. niger* is a high rate of glycolysis (Legiša and Mattey 2007). There is good evidence that deregulated glycolytic flux in *A. niger* is caused by post-translational modification of 6-phosphofructo-1-kinase (PFK1), a key allosteric regulatory enzyme of glycolysis. After proteolytic cleavage

of the C-terminal part of the PFK1 enzyme, an initially inactive fragment is formed, which after phosphorylation regains activity and is resistant to citrate and ATP inhibition (Mesojednik and Legiša 2005; Mlakar and Legiša 2006). Recently, it has been shown that post-translational modification of PFK1 enzyme can be avoided by the insertion of a modified *pfk*A gene into a target microorganism that synthesizes the shorter PFK1 fragment (Capuder *et al.* 2009). In *A. niger* transformants carrying modified *pfk*A genes, increased production of citric acid was recorded as well as higher activities of α-amylases, which are typical extracellular enzymes (Legiša *et al.* 2007).

Aspergillus terreus strains genetically modified to express the native *A. niger pfk*A gene and a shorter form of the gene were investigated to determine whether increased glucose utilization rates could result in the increased production of both primary and secondary metabolites. Under fermentation conditions designed to produce lovastatin, however, a rapid metabolism of glucose by a previously unrecognized biochemical route in *A. terreus* was observed in both the wild type and transformants (with *A. niger* genes) of this industrially useful microbe.

Materials and methods

Aspergillus terreus strain A 156 from the cultural collection of the National Institute of Chemistry (Ljubljana) that is identical to ATCC 20542 was the wild-type strain and was used as the parent for genetic transformations.

Aspergillus terreus protoplasts were transformed with pRCR-PFK1 expression plasmid, carrying the native *A. niger pfk*A gene, and pRCR-PFK10 plasmid with truncated A. *niger* (t-*pfk*A10) gene that were constructed as reported previously (Capuder *et al.* 2009).

For protoplast formation, the mycelium was obtained by growing *A. terreus* cells for 16–18 h in a liquid culture on complete medium. Cell wall lysis, release of protoplasts and subsequent transformation of *A. terreus* protoplasts were performed essentially as described by Bagar *et al.* (2009) with the lytic enzyme Novozym234 replaced by Caylase-4 (10 mg ml^{-1}) (Cayla, 31400 Toulouse, France). For the co-transformantion of *A. terreus* cells, hygromycin resistance (*hph*) on pAN7-1 vector was taken as a dominant selection marker. One μg of selection marker vector and 15 μg of the co-transforming plasmid pRCR (carrying specific *A. niger* genes) were added to 1×10^8 protoplasts. Hygromycin as a selection factor was added to the regeneration medium in the concentration of 1 mg ml^{-1}. The transformants were selected and purified by replating at low spore densities on adequate selective medium. One transformant encoding the native *A. niger* PFK1 enzyme (A10) and one transformant encoding the shorter PFK1 fragment (A44) were further tested for gluconate production.

Shake flask fermentations were set up using the inoculation procedure and chemically defined medium used as described in Hajjaj *et al.* (2001) except that the sodium glutamate monohydrate concentration was to 7·0 g l^{-1}.

Stirred tank fermentors used in Glasgow were New Brunswick Bioflo 110 vessels (New Brunswick Scientific (UK) Ltd., St. Albans, AL4 0JJ, UK); the nominal volumes were 10 l, and the working volumes were 7 l; vessels were operated at 28°C; the pH was regulated to 6·5 with 1 mol l^{-1} H_2SO_4 and 2 mol l^{-1} NaOH; the initial aeration rate was 1 vvm; the initial agitation speed was 300 rev min^{-1}; dissolved oxygen levels were controlled to a minimum of 40% saturation by increases in aeration and agitation rates as required. The growth of inocula and the inoculation procedure were as described in Hajjaj *et al.* (2001); the chemically defined medium was based on that described for itaconic acid production by Riscaldati *et al.* (2000) supplemented with sodium glutamate (2·5 g l^{-1}) and NH_4NO_3 (1·0 g l^{-1}) and with increased KH_2PO_4 (2·0 g l^{-1}).

Stirred tank fermentations in Ljubljana were performed in a 5-l bioreactor (IS-100; Infors, Bottmingen, CH-4103, Switzerland) using the media described by Riscaldati *et al.* (2000). The vegetative phase was conducted in two 500-ml Erlenmeyer flasks with baffles; 100 ml of vegetative medium in each flask was inoculated by 1×10^7 spores and incubated on a rotary shaker at 100 rev min^{-1} and 30°C. After 24 h, the broth was transferred into a laboratory fermentor containing 4·5 l of production medium. The growth temperature was set at 30°C, the medium was aerated at 5 vvm, and the stirring rate was adjusted to 500 rev min^{-1}; no pH control was applied. Samples of the fermentation broth for analyses were collected in 10-ml polypropylene tubes, sealed, frozen under the liquid nitrogen and stored at -80°C. Dry cell weight (DCW) measurements were made with washed mycelia dried to constant weight at 105°C.

Glucose was measured by the hexokinase-glucose 6-phosphate dehydrogenase assay kit method (product number 10 716 251 035; R-Biopharm AG, 64293 Darmstadt, Germany) after appropriate dilution of the sample. Initial screening for acidic metabolites was by high-performance anion-exchange HPLC analysis with an Ion Pac AS11-HC column (Dionex, Sunnyvale, CA 94085-4074, USA) and conductimetric analysis using an ED40 detector (Dionex); the flow rate was 0·38 ml min^{-1}, and the gradient was generated from deionized water (E1), 5 mmol l^{-1} NaOH (E2) and 100 mmol l^{-1} NaOH (E3) developing a gradient from 1 to 60 mol l^{-1} NaOH over 47 min: 1 mol l^{-1} at 0–10 min, 10 mol l^{-1} at 20 min, 25 mol l^{-1}

at 33 min and 60 mol l^{-1} at 47 min. The injection volume was 0·005 ml. The standard analyte mixture (0·2 mmol l^{-1}) used for calibration included the following: L-lactate, acetate, formate, pyruvate, chloride, nitrate, malate, succinate, itaconate, sulfate, fumarate, phosphate and citrate; gluconic acid was poorly resolved from acetic acid in this chromatographic system and was quantified by the gluconokinase/6-phosphogluconate dehydrogenase assay kit method (product number 10 428 191 035; R-Biopharm AG, Darmstadt, Germany) after appropriate dilution of the sample. Inorganic cations (sodium, ammonium and potassium) were determined by ion chromatography (Mousdale 1996).

Results

Acid formation by *A. terreus* was indicated by a demand for base (NaOH) pH regulation during fermentations where glucose was consumed; the increase in extracellular sodium ion concentration was measured offline (Figs 1 and 2). Part of this base demand represented the substitution of ammonium ions present in the inoculated production medium at 53–54 mmol l^{-1}. Gluconic acid was produced at up to 0·45 mol mol^{-1} glucose consumed in the wild-type strain and up to 0·3 mol mol^{-1} glucose consumed in the genetically modified strain expressing the native *A. niger pfk*A gene. No major difference in the specific productivities and in the final yields of gluconate of both strains could be observed. Itaconic acid was below the limit of detection (0·5 mol l^{-1}); other acids present in minor amounts (<20 mmol l^{-1}) co-chromatographed with succinic and fumaric acids.

Galactonic acid is metabolized by *A. terreus* by a non-phosphorolytic pathway (Elshafeia and Abdel-Fataha 1991). Shake flask cultures were, therefore, used to investigate if gluconic acid is an end-point metabolite of glucose (Table 1). At the lowest glucose concentration used (50 g l^{-1}), gluconic acid had accumulated by 72 h but, with glucose depletion in the medium by that time, was subsequently metabolized. At concentrations of 100 g l^{-1} or higher, the glucose was not fully consumed and gluconic acid remained present in high concentration at 144 h (Table 1).

Stirred tank fermentations with no pH control and using the wild-type and two genetically modified strains (A10 with the complete native *pfk*A gene and A44 with the truncated variant) at a lower glucose concentration (40 mmol l^{-1}) were used to investigate if *A. terreus* would adapt to depletion of glucose as a carbon source and (as observed in some shake flask experiments – Table 1) metabolize gluconic acid. Glucose consumption was rapid in the initial 48 h; gluconic acid accumulated within that time but was subsequently metabolized (Figs 3–5). The pH (measured offline) exhibited a biphasic pattern of decrease to approximately 50 h followed by an increase after gluconic acid concentrations decreased. Growth kinetics as measured by DCW indicated the cessation of active growth after 100–120 h in the absence of any remaining major carbon sources (glucose or gluconic acid).

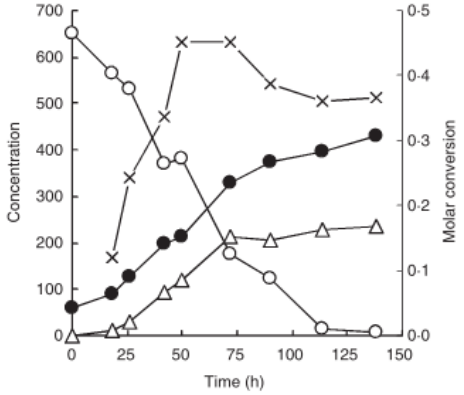

Figure 1 Concentrations (mol l^{-1}) of glucose (–○–), Na⁺ ion accumulated for pH regulation by NaOH at 6·5 (–●–) and gluconic acid (–△–) and the molar conversion (mol gluconate mol^{-1} glucose) of glucose to gluconic acid (–×–) in 10-l stirred tank fermentations with *Aspergillus terreus* wild-type strain (A 156).

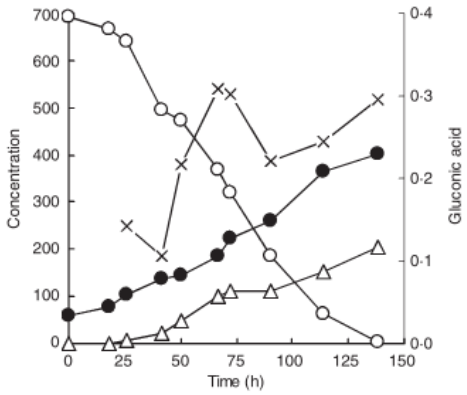

Figure 2 Concentrations (mol l^{-1}) of glucose (–○–), Na⁺ ion accumulated for pH regulation by NaOH at 6·5 (–●–) and gluconic acid (–△–) and the molar conversion (mol gluconate mol^{-1} glucose) of glucose to gluconic acid (–×–) in 10-l stirred tank fermentations with *Aspergillus terreus* strain (A 10) containing the *Aspergillus niger pfk*A gene.

Table 1 Glucose and gluconic acid concentrations in shake flasks

Time (h)	Glucose (mmol l^{-1})	Gluconic acid (mmol l^{-1})	Gluconic acid production (mol mol l^{-1} glucose used)
Strain A156 (50 g l^{-1} glucose)			
0	238·9	<0·1	
72	8·2	22·2	0·10
144	8·2	1·7	0·01
Strain A156 (100 g l^{-1} glucose)			
0	483·6	<0·1	
72	161·2	66·2	0·21
144	1·0	96·0	0·20
Strain A156 (150 g l^{-1} glucose)			
0	682·3	<0·1	
72	359·4	53·4	0·17
144	44·1	469·5	0·74
Strain A10 (50 g l^{-1} glucose)			
0	228·4	<0·1	
72	2·9	63·2	0·28
144	5·3	2·0	0·01
Strain A10 (100 g l^{-1} glucose)			
0	471·2	<0·1	
72	211·1	63·0	0·24
144	1·0	126·6	0·27
Strain A10 (150 g l^{-1} glucose)			
0	653·5	<0·1	
72	378·1	51·0	0·19
144	12·5	238·3	0·37

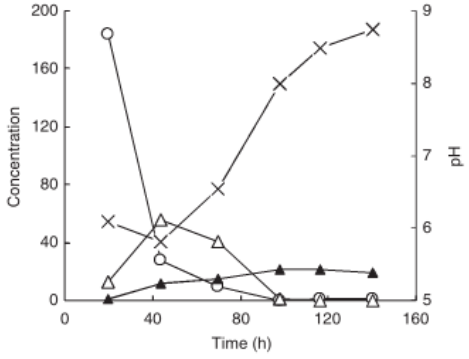

Figure 3 Concentrations (mol l^{-1}) of glucose (–○–) and gluconic acid (–△–), dry cell weight (g l^{-1}) (–▲–) and pH (–×–) in 5-l stirred tank fermentations with *Aspergillus terreus* wild-type strain (A 156).

In stirred tank fermentations where the pH was initially 3·0 and was allowed to decrease to below 2·0, itaconic acid was the only carboxylic acid produced by the wild type or either of the genetically transformed strains, and no gluconic acid production could be detected.

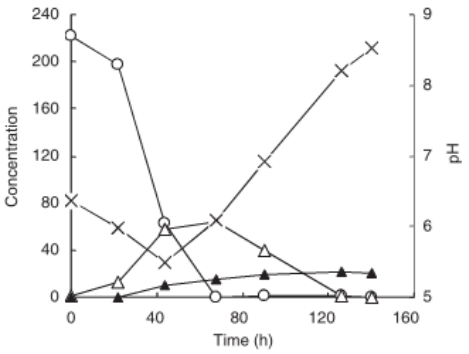

Figure 4 Concentrations (mol l^{-1}) of glucose (–○–) and gluconic acid (–△–), dry cell weight (g l^{-1}) (–▲–) and pH (–×–) in 5-l stirred tank fermentations with *Aspergillus terreus* strain (A 10) containing the *Aspergillus niger pfk*A gene.

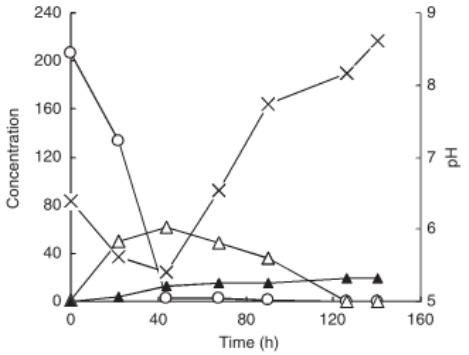

Figure 5 Concentrations (mol l^{-1}) of glucose (–○–) and gluconic acid (–△–), dry cell weight (g l^{-1}) (–▲–) and pH (–×–) in 5-l stirred tank fermentations with *Aspergillus terreus* strain (A 44) containing the *Aspergillus niger* t-*pfk*A gene.

Discussion

Gluconic acid has been identified as one of the top 30 'building block' compounds that can be produced from biomass sources in future biorefineries (Werpy and Peterson 2004). High production costs presently limit the use of gluconic acid as a bulk chemical (Singh and Kumar 2007). Research approaches to improve fermentation production processes have included the exploration of novel producer organisms (Anastassiadis *et al.* 2003), fermentation optimization strategies (Cheema *et al.* 2002), use of cheaper carbohydrate sources (Singh and Singh 2006),

solid-state fermentation (Sharma *et al.* 2008) and the use of fungal spores as biocatalysts (Ramachandran *et al.* 2007).

The present work demonstrates that *A. terreus* has the potential to become a producer of gluconic acid with the particular advantages that the extensive experience of this species as an industrial micro-organism brings. Defining the influences of pH, temperature, aeration rate and the choice of carbon source will, however, be essential if a fermentation capable of rivalling gluconic acid yields already achieved with *A. niger* is to be established. The biochemistry of *A. terreus* for carboxylic acid production resembles that in *A. niger*, whose ability to produce citric, gluconic or oxalic acid at different pH values has been recognized for over 30 years (Ramachandran *et al.* 2006). Even the wild type can produce gluconic acid at high bioconversion efficiencies (up to 0·7 mol mol^{-1} glucose consumed), and targets for rational strain improvement can be identified. If, by analogy with galactonic acid, gluconic acid metabolism in *A. terreus* requires a gluconate dehydratase activity to degrade gluconic acid into 2-keto-3-deoxy-gluconate followed by a 2-keto-3-deoxy-gluconate aldolase to form pyruvate and glyceraldehyde (Elshafeia and Abdel-Fataha 1991), gluconate dehydratase gene knockout could be efficient in producing strains for gluconic acid with reduced gluconate catabolism. While wild-type *A. terreus* is highly likely to compare poorly with intensively evolved strains of *A. niger* for gluconic acid production, rational strain improvement could result in a candidate strains for industrial production of gluconate. A key component of such strain development would include reduction in secondary product formation, in particular elimination of the biosynthesis of the mycotoxin citreoviridin (Franck and Gehrken 1980).

Expressing the *A. niger pfk*A genes might be anticipated to have two independent but contrary effects on gluconic acid production in *A. terreus*: a more efficient flux of glucose to amino acids for extracellular enzyme formation would increase the rate of gluconic acid formation, but a consistently higher rate of glycolysis could reduce gluconic acid production as intracellular glycolysis competed with extracellular gluconate formation for glucose. The first effect might explain the higher rates of gluconic acid production observed with genetically engineered strains at low glucose concentrations; with the *A. niger* transformant carrying t-*pfk*A gene, increased extracellular α-amylase production was observed (Legiša *et al.* 2007). The expression of *A. niger* genes inserted into *A. terreus* is under the control of *A. niger pfk*A promoter. Although the expression level of the genes under *pfk*A promoter was not yet determined at various environmental conditions, it might be anticipated that the shorter form of PFK1 enzyme was more intensely synthesized at low glucose conditions. To maximize gluconic acid production, therefore, detailed investigation of such genetically strains could be useful.

The realization of gluconic acid production from glucose by *A. terreus* at relatively high pH in the fermentation indicates innovative routes to glucose use for the production of lovastatin and other secondary metabolites without further genetic manipulation of industrial strains. The nonphosphorolytic pathway of gluconate catabolism is not only bioenergetically inferior to the glycolytic pathway of glucose, but the production of high concentrations of gluconic acid would disrupt pH regulation in the fermentation and introduce high concentrations of osmotically active solutes especially if the accumulated acid (causing base addition) was later utilized as a carbon source (resulting in acid demand).

To *minimize* gluconate formation in secondary product processes, therefore, high concentrations of glucose must be avoided; this implies a fed-batch strategy with another carbon source to support the growth of producing cells. Such a glucose feed could be easily regulated by feedback control using fermentation broth pH as the operating parameter and maintaining the pH at a set point against decreases caused by gluconic acid formation.

Acknowledgement

This work was supported by the European Commission Framework Five programme under contract number QLK3-CT-2002-02038 (''Demonstration of increased yields and productivity in selected commercial organisms by strategic transformation of key genes from *Aspergillus niger*'').

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Chapter 4: Conclusions

4.1 Metabolic engineering of the primary metabolic pathways of industrial micro-organisms

The ANTICO project aimed to alter the metabolic properties of industrial microbes by introducing "foreign" genes from *Aspergillus niger*. The project's specific target was in increasing the rate of glucose utilisation and consequent flux through glycolysis to provide more carbon for pathways of primary or secondary metabolism. That primary metabolism could be successfully altered by this approach had been established well before the project started and in 2007 colleagues from ANTICO published an in-depth review of this field of research (Kern *et al*., 2007).

This 2007 review covered published accounts of work with yeast, fungi and bacteria and identified several "success stories" and offered three different but overlapping definitions of metabolic engineering:

1. "Improvement of cellular activities by the manipulation of enzymatic, transport and regulatory functions of the cell with use of recombinant DNA technology" (Bailey, 1991).
2. The "purposeful modification of the intermediary metabolism using recombinant DNA technology" (Cameron and Tong, 1993).
3. The "genetic modification of cellular biochemistry to introduce new properties or to modify existing ones" (Jacobsen and Khosla, 1998).

Ethanol production by yeasts has been a major focus of metabolic engineering for the past 20 years because of its industrial importance with the rise of ethanol as a biofuel produced on a massive global scale. Among the approaches and strategies used have been:

- Introducing genes for pentose metabolism into strains of brewer's yeast, *Saccharomyces cerevisiae* (Eliasson *et al*., 2000; Toivari *et al*., 2001)
- Rebalancing carbon fluxes between glycolysis and the pentose phosphate pathway to improve the supply of oxidised and reduced forms of NAD and NADP when pentose are metabolised to ethanol by yeasts (Jeppsson *et al*. 2002)
- Reducing acetic acid formation by ethanol-producing yeasts (Eglinton *et al*., 2002).

As discussed by Kern *et al*., not every attempt to improve product synthesis by rational genetic and metabolic engineering yields the desired results. Particularly with bacteria such as *Escherichia coli*, attempting to reduce acetic acid production (a well characterised and much studied metabolic process) leads only to the excretion of other carboxylic acids (El-Mansi, 2004). As the other note:

"*E. coli* has shown a remarkable tolerance to drastic changes in metabolic flux implying a considerable elasticity in permitted pool size for key intermediates…"

There is not only a "metabolic robustness" as a consequence of gene duplication but previously silent genes become activated or alternative pathways function to minimise the effects of disruptions to metabolic fluxes (Wagner, 2000). Primary pathways of metabolism are highly interconnected and most of the enzymic activies may be present in vast excess and be only fully saturated when glucose and other substrates are present in very high concentrations. Indeed, *A. niger* synthesising citric acid at high rates may be a rare and well-defined example of this nearly full utilisation of a small group of primary pathway enzymes (Legiša and Mattey, 2007).

With this theoretical background of understanding how difficult it may be to accurately redirect primary fluxes to increased product formation, the results from the ANTICO project can be reviewed with some advantage of hindsight.

4.2 Results with Aspergillus terreus and Lactobacillus lactis.

While the genetic engineering work was successful and the expression of introduced *A. niger* genes into all the microbial species included in the project was demonstrated, the detailed metabolic comparison of parent and transformed strains proved to be very much restricted by the availability of fermentor time. As a consequence, replication of test results under exactly similar conditions was generally not possible. Nevertheless, fermentor trials with *A. terreus* and other industrially relevant micro-organisms gave sufficient evidence that the genetic transformations had indeed altered metabolic properties. After the ANTICO project itself had ended, continued work by colleagues in Slovenia eventually produced results that were incorporated into published articles. In addition, Intellectual Property Rights were pursued for some of the project's findings in the form of patent applications.

Following on from preliminary results in Glasgow, the increased production rate of itaconic acid by *A. terreus* fermented at low pH was demonstrated (Tevž et al., 2010). When a wider range of transformants carrying native or truncated forms of the *A. niger pfkA* gene were tested in replicated shake flasks, increases in itaconic acid titre of up to 213% while some strains could also produce itaconic acid at up to a 5.5-fold faster rate. Moreover, the growth of the transformants was impaired when compared to the parent and this gave the transformants higher specific productivities (g acid/g dry cell mass/hr). The earlier results obtained during the ANTICO had shown fermentor trials with transformed strains that showed increases in itaconic acid titres and specific productivities of more than 100% (results not included in this thesis).

Similarly, analyses of stirred tank fermentations with parental and transformed strains of *Lactobacillus lactis* showed that strains carrying *A. niger* genes had higher lactic acid productivities and specific productivities. This has recently been confirmed by the ANTICO project team in Greece (Papagianni and Avramidis, 2011). Additionally, these newly published results show not only increased lactic acid production (52% increase in g lactic acid/g dry cell mass/hr) but also in glucose uptake (213% increase in ↘mol/g dry cell mass/hr) in a strain carrying a modified *A. niger pfkA* gene. The transformed strain could also tolerate higher glucose concentrations in the medium.

4.3 Future research following from the ANTICO project

Taken together, the results from the recent publication from the Greek ANTICO research group (Papagianni and Avramidis, 2011), fit very well with the predictions of the project, i.e. that expressing *A. niger* genes in other microbial species would increase glucose uptake and metabolism via glycolysis and lead to increased production of metabolites as fermentation products. Subsequently, the same authors have shown that expression of the *A. niger aox* gene in *L. lactis* greatly increases the production of the antimicrobial peptide nisin (Papagianni and Avramidis, 2012).

The central hypothesis of the ANTICO project deserves, therefore, further attention and experimental testing. Results obtained during the time of project suggested that A. niger genes in *Streptomyces rimosus* could increase the production of tetracycline. With the yeast *Pichia pastoris*, no reliable effects of genetic transformation could be demonstrated but this organism already had very high native rates of glucose use. With the clavulanic acid producer *Streptomyces clavuligerus*, although a range of transformants were produced, they were not tested in with glucose as the sole carbon source in media that did support high rates of clavulanic acid formation. These micro-organisms could now be tested again in a more detailed manner with the knowledge from positive results with such very different species as *A. terreus* and *L. lactis*.

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