MECHANISM OF CITRIC ACID ACCUMULATION BY *ASPERGILLUS NIGER* IN SOLID STATE FERMENTATION

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Abstract

The main purpose of this work was to study the mechanism of citric acid accumulation in solid state fermentation of *Aspergillus niger*. Two strains, Yang No.2 and MH 15-15, represented the high-accumulating organisms from which the low-accumulating mutants, SL-1 and SL-2, were generated by ultraviolet treatment. Comparative solid state fermentations, with a starch-containing material as the substrate, were conducted in petri-dishes, a technique which conferred a major advantage in allowing recovery of metabolically active mycelia for biochemical assays. Apart from the decreased citric acid accumulation, the selected mutants displayed lower starch consumption and enhanced production of oxalic acid, while their growth were generally equal to that of their respective parents. Evidence on elevated levels of free glucose in the cultures of the mutants, despite there being no alteration of α-amylase and glucoamylase from their parents, has prompted a hypothesis that the mutants were defective in the rates of glucose uptake.

The biochemical work started with the primary steps of carbon assimilation, *viz* measurement of glucose uptake and activity assay of hexokinase. The results confirmed the reduced glucose uptake rates by the mutants and a hypothesis that this is caused by some defects in certain components of the glucose transport mechanism, but not at membrane ATPase, has been proposed. In addition, hexokinase showed higher *in vitro* activities in the parents and, presumably, their glycolytic fluxes were greater than those of their mutants.

Investigation of activities of some selected TCA cycle enzymes and other metabolic steps *in vitro* strongly indicated the decreased activity of 2-oxoglutarate dehydrogenase and, possibly, NAD-and NADP-specific isocitrate dehydrogenases in the parents. Although most other enzymes decreased their activities during the later phase of cultivation, there was no definite difference between each parent and its mutant. However, oxaloacetate hydrolase, for oxalate formation, was at higher activity in the mutants than in the parents.

Measurements of intracellular concentrations of products of certain enzymes and adenine nucleotides were conducted in order to assess the *in vivo* catalytic function of the enzymes of interest. It was concluded that internal accumulation of citrate or oxalate is an immediate cause of its excretion. Supplemented by evidence from the ratio of ATP/AMP in the cells, a complete hypothesis describing citrate accumulation in *A.niger* Yang No.2 and MH 15-15 is proposed. Hence, the rate by which glucose is taken up into the cells is the primary trigger determining the capacity of glycolytic metabolism and it is proposed that the primary cause of citric acid accumulation in the high-accumulating strains is the deregulation of glucose uptake. When the glucose supply exceeds the requirement of the cells, i.e. when growth is slow, the TCA cycle is balanced by allosteric deactivation of isocitrate dehydrogenases by ATP which is excessively generated *via* the active glycolysis. The observed low level of activity of 2-oxoglutarate dehydrogenase is, therefore, a result of this metabolic block, rather than a cause. Because of the equilibria of the reactions, citrate is accumulated and then excreted out of the cells. In contrast, when the glucose supply is below such level, this enzyme regulation does not occur and oxalate, instead of citrate, acts as the drain of excess carbon going around the fully operative TCA cycle.

In conclusion, the current hypothesis for citric acid accumulation is basically similar to that proposed for submerged fermentation conditions. However, the rationale for carbon sink *via* oxalate is novel. Finally, it has been shown that two *A.niger* strains of different origins displayed a similar mechanism, although the fine control may be different.

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Abbreviations

Abbreviation of Units

^0C	degree Celcius
cm	centimetre
g	gram
h	hour
ID	inner diameter
kg	kilogram
kJ	kilojoule
l	litre
µg	microgram
µl	microlitre
µm	micrometre
µmol	micromole
m	metre
mbar	millibar
min	minute
mg	milligram
ml	millilitre
mm	millimetre
mm^3	cubic millimetre
mM	millimolar
M	molar
nm	nanometre
rpm	revolutions per minute
%	percentage
% (v/v)	percentage, volume related to volume
% (w/v)	percentage, weight related to volume

Other Major Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
C	carbon
CoA	coenzyme A
DB	dry biomass
EDTA	ethylenediamine tetraacetic acid
HPLC	high performance liquid chromatography
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
TCA	tricarboxylic acid

CHAPTER 1

Introduction

As the second largest product, in terms of volume, obtained from microorganisms, the prominence of citric acid is granted. Since the development of the industrial production process for this acid in the last century, there has been enormous success in improving the fermentation technology. The process using submerged fermentation technology has dominated in commercial-scale production because of numerous production advantages. In the meantime, production using the solid state process, which has also a long history for the production of citric acid, has recently been investigated more systematically. Although advantages of cultivation using this type of process are well known, the development of the process has been neglected in the past, mainly because of the non-homogeneous character of the system which causes problems in determining the necessary fermentation parameters. Nevertheless, there has been at least one report which systematically investigated the kinetics of citric acid production in the solid state process, that of Lu (1995). In that study, a starch-containing solid substrate was used as the carbohydrate source for the producing organism without addition of any other nutrients. The organism selected for the study was *A.niger* Yang No.2 which showed high ability to produce and accumulate citric acid within a relatively short period (4 to 6 days). All the methods for the measurement of essential fermentation parameters have also been developed, particularly that for biomass measurement.

Although considerable knowledge was achieved from that study, it provided very little information on the biochemical aspects of the producing organism during the fermentation. As a consequence, there remains the necessity to research into this area since this has a tremendous influence on the fermentation performance. Moreover, although the biochemical pathway and the mechanism by which citric acid is accumulated in high concentration by certain yeast and fungal strains have been

elucidated to an acceptable extent, they typically represent the situation for the submerged conditions. Since environmental factors, such as nutrition and other cultivation conditions, have a tremendous influence on growth and metabolism of an organism, there is no guarantee that the mechanisms which operate during the fermentation using the submerged and the solid state conditions will be identical. For these reasons, it is important that research into the biochemical aspects be conducted in the solid state culture for citric acid production.

This thesis, therefore, intends to fulfil the concept of fermentation using the solid state system initiated by Lu (1995). Based on this purpose, additional strains of the fermenting organisms will be investigated since this assists in elucidating the biochemical mechanism by means of comparative studies of strains which vary in their ability to accumulate citric acid. Hence, the primary work will be to isolate and select some useful mutant strains from certain high-accumulating strains of *A.niger*, after which a general biological study of all the strains will be conducted. Then, comparative studies on enzymatic and some essential metabolic events will be carried out. The information obtained will be a useful tool to illustrate the mechanism and regulation of citric acid accumulation in solid state cultivation.

CHAPTER 2

Literature Review

2.1 Introduction : History of Citric Acid Production

Citric acid is an organic acid with numerous industrial applications. The original commercial source of this tricarboxylic acid was citrus fruits such as lime and lemon. However, at present the supply is provided entirely by microbial sources. The annual worldwide production of about 700,000 tonnes (Roehr *et al.*, 1996) has seen it become a major industrial compound produced by microorganisms. Because of the value of this acid, a great number of reviews describing its production have been published. These articles are sources of knowledge on all aspects including types of production process, producing organisms and mechanism and regulation of citric acid production. Examples of recent reviews are those by Röhr *et al.*(1983), Milsom (1987), Bigelis and Arora (1992), Roehr *et al.* (1992), Grewal and Kalra (1995), and Roehr *et al.* (1996).

The discovery of the microbial source of citric acid can be traced back to the late nineteenth century when the production of citric acid during oxalic acid production by some species of the fungus *Penicillium* was observed (Roehr *et al.*, 1992). However, industrial application began following the work of Currie (1917) who optimized the conditions to achieve a high citric acid yield from the fungus *Aspergillus niger* using the so-called surface cultivation method. Since then, this process has been developed for the commercial production of citric acid from a variety of carbohydrates, mainly sucrose and molasses, using selected strains of *A.niger*. Subsequently, following progress in the development of new fermentation technologies, the submerged fermentation process was introduced for commercial citric acid production but the main producing organisms remained strains of *A.niger*. Intensive investigations on many aspects of this technology have led to tremendous

improvements and have seen the submerged process emerge as the main commercial route for citric acid production (Röhr *et al.*, 1983). Meanwhile, new organisms, including various yeasts, have been investigated for their production proficiency, while in other practice, genetic manipulation techniques have been studied to improve the productivity of natural strains. Recently, due to certain limitations of the conventional raw materials, particularly their cost, new, cheaper sources have been investigated, often for application in the solid state process which has been used successfully for production of many other microbial products (Moo-Young *et al.*, 1983).

Although our knowledge on the microbial production of citric acid has shown great advances, there are still some aspects that require further clarification. It is apparent that the understanding of the biochemical mechanism of citric acid accumulation is still incomplete. At present, there is no single comprehensive theory to explain the mechanism and regulation of citric acid accumulation in *A.niger*. Furthermore, although a great deal of investigation on this subject has been conveyed for the submerged fermentation process, there are very few reports describing the biochemistry in other fermentation modes, such as solid state fermentation. There is no guarantee that an organism will perform an identical metabolism in different fermentation modes.

This chapter will discuss the general knowledge of citric acid production by fungal fermentation. The emphasis will be on growth description, as well as on the biochemistry and regulation of citric acid accumulation by *A.niger*.

2.2 Uses and Properties of Citric Acid and Its Salts

Citric acid is widely used because of its properties of solubility, nontoxicity and biodegradability (McGraw-Hill Encyclopedia of Science, 7th ed). Basically, citric acid and its salts are used for acidification or chelation purposes. The first major use of these compounds is in the food and beverage industries because it is classified as "GRAS" (generally regarded as safe) (Roehr *et al.*, 1996). Mainly they serve as pH adjustment and flavour improvement agents for these products (Meyrath, 1967; Kapoor

et al., 1982). The second main use of citric acid and citrate salts is in pharmaceutical and cosmetic products, for acidification and metal ion chelation. In addition, citric acid and its derivatives also have some minor applications, such as to substitute for phosphate in detergents, to control pH and remove heavy metals in textiles, in the synthesis of plastic, and to replace many strong mineral acids for various purposes (Grewal and Kalra, 1995; Roehr *et al.*, 1996).

2.3 Citric Acid-Accumulating Organisms

A large number of moulds, yeasts and bacteria are able to produce and excrete citric acid into their culture media. Besides the well-known fungus *Aspergillus niger*, other species of *Aspergillus*, as well as members of the genera *Penicillium*, *Trichoderma* and *Mucor* also show this property (Kapoor *et al.*, 1982; Röhr *et al.*, 1983). However, most industrial processes employ *A.niger*, usually genetically improved strains, as the producing organism (Bigelis and Arora, 1992; Grewal and Kalra, 1995). The advantages of fermentation with these particular strains include high yield, genetic stability, and the possibility for shifting to new raw materials (Grewal and Kalra, 1995).

Yeasts of several genera have also been described for their citric acid-producing capabilities. Among these, some species of the genus *Candida* and a few other genera accumulate considerable amounts of citric acid from carbohydrates and hydrocarbons (Kapoor *et al.*, 1982). *Yarrowia lipolytica* (syn. *Saccharomycopsis lipolytica*, *C.lipolytica*) and *C.guilliermondii* are the most widely studied yeasts, and one major world producer of citric acid is known to have used a yeast on a commercial scale for 15 years (Maddox, I.S. personal communication). Basically, fermentation with yeasts offers potential in performing a continuous process, a technique which is more difficult for the fungal fermentation. However, a considerable amount of isocitric acid is commonly produced by yeasts as an impurity (Kapoor *et al.*, 1982; Röhr *et al.*, 1983). The review article of Roehr *et al.* (1996) is an excellent source on the use of yeast as a citrate producing organism. Besides fungi and yeasts, bacteria of several genera including *Bacillus* and *Brevibacterium* can

produce citric acid (Kapoor *et al.*, 1982). However, little investigation has been carried out on this group of microorganisms.

In general practice, selected strains of *A.niger* are still the major organisms for large-scale citric acid production followed by certain yeast strains. Unfortunately, the procedures for strain selection have been kept confidential by the manufacturers. However, usually there are two techniques for strain selection (Roehr *et al.*, 1996). The first is selecting through the single spore technique. In this regard, a great number of single spores from a spore suspension are checked for their citrate producing ability using various methods and is, thus, tedious and time-consuming. The second strain selection technique is the passage method in which certain measures are set as the selection criteria. Some examples are selection based on resistance to low pH (Currie, 1917), deoxyglucose (Kirimura *et al.*, 1992; Sarangbin *et al.*, 1993), high sugar concentrations (Schreferl-Kunar *et al.*, 1989, Xu *et al.*, 1989a), or sensitivity to cycloheximide (Rugsaseel *et al.*, 1996). The applicable strains should also display a stable acid production capability.

Mainly, the natural strains have to be improved for better characteristics including their acid production ability. The subject of strain improvement has been described in the reviews of Perlman and Sih (1960), Bigelis (1985), Roehr *et al.* (1992), and Roehr *et al.* (1996). Usually the mutation technique is used, by inducing a random mutation to a population of spores with various mutagenic agents, such as ultraviolet (UV) light, X-rays, and certain chemicals. In many cases, a multi-step mutation provides a more favourable mutant than a single-step mutation(Gardner *et al.*, 1956; Das and Nandi, 1969). Other techniques such as heterokaryosis and polyploidy based on the parasexuality property of *A.niger* have also been attempted to generate the desired strains, as reported in some laboratories. However, instability of the improved strains is a major problem for these methods (Roehr *et al.*, 1996). The protoplast fusion technique is also used as a tool by many groups, for example, some fusants of *A.niger* with superiority in citric acid production have been isolated (Kirimura *et al.*, 1986; 1988a,b). Apart from a high acid productivity, however, the desired mutants may be selected on other criteria such as the ability to utilize cheap raw materials or

to tolerate some detrimental environmental factors such as metal ions (Bigelis, 1985; Roehr *et al.*, 1992).

It is obvious that more intensive work has to be accomplished on this subject. Although knowledge of the genetic component of *A.niger* is being advanced markedly (Roehr *et al.*, 1996), this has yet to be integrated with the genetic control of citric acid biosynthesis and accumulation. Such knowledge at the genetic level will lead to a greater understanding of the mechanism of citric acid accumulation, and thereby to a better control of the process.

2.4 A Brief Description of *A.niger*

Since *A.niger* is the fungal species which is the most used for commercial citric acid production, and is also used as the organism for the current study, a brief description of its classification and morphological characteristics will be presented (Smith, 1969; Alexopoulos and Mins, 1979).

Aspergillus niger is a filamentous fungus of the genus *Aspergillus*. It is often called "black mould" because of the colour of its spore or conidial heads. The fungi in this group are commonly distributed in the environment and, because of their great enzymatic activities, have been employed in many industrial processes. *A.niger* is a member of the Class Deuteromycetes or Fungi Imperfecti because it lacks a perfect or sexual reproduction process. The hyphae are well-developed, profusely branched, hyaline and septate with multinucleate cells. Asexual reproduction of *A.niger* is by formation of conidia or conidiospores at the end of the conidiophores which originate from the "foot cells" of the hyphae, forming the conidial heads. Typically, conidia are spherical, coarse, hyaline and black to brown in colour and produced abundantly. The colony on agar medium of *A.niger* is usually compact to faintly loose with yellow pigmentation.

2.5 Fermentation Conditions

In addition to a productive strain of organism, a profitable citric acid fermentation process requires the development of optimum environmental conditions. For a producing strain to produce and accumulate a high concentration of citric acid, the composition of the fermentation medium as well as other environmental conditions must be appropriate. In general, the production medium should contain a high sugar concentration while other nutrients such as nitrogen and/or phosphate are at growth-limiting concentrations. In addition, certain metal ions should be present at minimum levels. Under these conditions, the mycelial growth phase is restricted and most of the excess carbon is converted to citric acid (Roehr *et al.*, 1992). The following is a description of the major fermentation components and their influence on citric acid production.

2.5.1 Carbohydrates

Because citric acid is an intermediate product of carbohydrate metabolism, the type of the carbon source has a profound influence on its production and yield. As a heterotrophic organism, *A.niger* can utilize a variety of carbohydrates. In practice, however, the mono-or di-saccharides which are readily consumed by the organism are not all as favourable as each other in terms of citrate production. Normally, sucrose is the most favourable among simple sugars for allowing high yields of citrate (Hossain *et al.*, 1984). Xu and coworkers (Xu *et al.*, 1989b) reported that maltose and sucrose were superior to glucose and fructose in promoting high yields and rates of citric acid production. Among these simple sugars, galactose has been shown to not only be a poor source, but also to have an inhibitory effect on citric acid production from glucose (Hossain *et al.*, 1985). In addition to the type of sugar, its concentration in the medium has a major influence on citrate production. The importance of sugar concentration over other nutritional parameters when the organism is exposed to different fermentation systems has been implied (Xu *et al.*, 1989a). Usually, sugar should be present at as high as 14 to 22 % (w/v) (Röhr *et al.*, 1983).

Xu *et al.* (1989b) reported that the optimum sugar concentration for the process was about 10 % (w/v) while at less than 2.5 % (w/v) sugar, no citrate was produced. These authors also proposed that the high sugar concentration can induce an imbalance in the fungal metabolism which then leads to accumulation of citric acid. An increase in sugar uptake by *A.niger* grown in high sugar concentration has been reported (Xu *et al.* (1989c). Further, the influence of sugar type and concentration has been related to regulation of glycolysis of the fungal cells, starting from the rate of sugar uptake. The detail on this subject is provided in Section 2.7.2. As a consequence, it is understandable that sugars of the "poor" type and low concentrations of sugars of the "preferred" type would fail to activate the necessary glycolytic operation, and thus provide only low yields and rates of citric acid production. In addition, there are reports that lower sugar concentrations tend to promote excess growth as well as increase production of undesirable by-products such as oxalic acid (Kovats, 1960, in Kapoor *et al.*, 1982). In contrast, however, a report by Honecker *et al.* (1989) stated that a high sucrose concentration may reduce citrate yields and increase polyol formation when *A.niger* is cultured in an immobilised form.

Other crude carbohydrates which have been investigated include low-grade beet and cane molasses, cane juice, corn and wheat starch (Perlman and Sih, 1960; Röhr *et al.*, 1983). These raw materials usually require some special treatments to remove excess metal ions which can have negative effects on the fermentation performance. This can usually be done by precipitation with chelating compounds such as ferrocyanide or with other precipitants (Perlman and Sih, 1960), or by passing the raw substrate through an ion-exchange resin (Kapoor *et al.*, 1982). Another approach is to use an organism which has been selected to tolerate these metals (Trumpy and Millis, 1963; Sanchez-Marroquin *et al.*, 1970).

Recently, a variety of other crude carbohydrates have been investigated for their potential use as cheap raw materials for citric acid production using the solid state fermentation process. Examples of these carbohydrates are apple pomace (Hang and Woodams, 1984), grape pomace (Hang and Woodams, 1985), kiwifruit peel (Hang *et al.*, 1987), wheat bran (Shankaranand and Lonsane, 1994), and pineapple waste (Tran

and Mitchell, 1995). These substrates are mostly residues or wastes from agriculture and food-processing, therefore conversion to citric acid can increase their values.

2.5.2 Nitrogen

Conventionally, nitrogen in the fermenting medium is added as ammonium or nitrate ions (Röhr *et al.*, 1983). Both the type and concentration of the nitrogen nutrient can influence the growth and citric acid productivity of the organism. Ammonium sulphate has been reported to prolong vegetative growth while ammonium nitrate can reduce the growth period (Kapoor *et al.*, 1982). Excess ammonium nitrate stimulates oxalic acid production (Berry *et al.*, 1977), promotes fungal growth and sugar consumption and reduces citric acid productivity (Prescott and Dunn, 1959). Kristiansen and Sinclair (1978) investigated the effect of some environmental factors on citric acid production by *A.niger* in batch culture and reported citric acid accumulation following exhaustion of nitrogen from the medium. In some circumstances, the effect of nitrogen is related to other nutrients. Shu and Johnson (1948a) showed that under conditions of excess nitrogen, citric acid can accumulate if phosphate in the medium is limited. This can explain the possible use of beet molasses, rich in nitrogen, for citric acid production.

It is now generally accepted that accumulation of citrate by *A.niger* occurs after growth has ceased or slowed. This correlation is due to a nutrient limitation, and nitrogen limitation is a commonly accepted way of achieving this. Thus, there must be sufficient nitrogen nutrient present to allow reasonable production of fungal biomass, but not so much that too much carbohydrate is diverted away from citrate production.

2.5.3 Phosphate

As phosphate is an integral component of many cellular macromolecules, coenzymes and nucleotides, it has a tremendous influence on the cellular metabolism (Garraway and Evans, 1984). The influence of phosphate on citric acid production has been rather controversial, but it is now clear that most of the reports can be readily

interpreted on the basis of whether or not phosphate was the limiting nutrient. The fact that phosphate can act as the limiting nutrient is generally accepted, in which case sufficient must be present in the medium to allow reasonable fungal growth, but not so much that growth is excessive, preventing conversion of sugar to citrate. Shu and Johnson (1948a) reported that the requirement for phosphate by *A.niger* varied in a wide range (0.5 to 5.0 g/l) for a chemically defined medium. Martin and Steel (1955) showed that addition of phosphate could lower the ratio of citric acid to total acids by increasing the ratio of minor acids such as 5-ketogluconic and malic acid. In chemostat culture, Kristiansen *et al.* (1982) reported the superiority of nitrogen over phosphate limitation for citric acid accumulation, and this concept was supported by the work of Dawson *et al.* (1989) in a fed-batch fermentation. In contrast, Honecker *et al.* (1989) have found that a growth restriction of immobilized cells of *A.niger* due to phosphate limitation was preferable to nitrogen limitation with regard to citric acid accumulation.

2.5.4 Metal ions

A number of divalent metal ions such as copper (Cu^{2+}), iron (Fe^{2+}), manganese (Mn^{2+}), and zinc (Zn^{2+}) have profound influence on growth and citric acid production by *A.niger* (Kapoor *et al.*, 1982, Röhr *et al.*, 1983). These metal ions function as activators for many cellular enzymes (Garraway and Evans, 1984). It is generally agreed that in order for citric acid to be accumulated, these metallic ions should be present but in limited concentrations. The minimum tolerance to each trace element depends on the individual organism as well as on the fermentation conditions. Tomlinson *et al.* (1950, 1951) showed that these metal ions were necessary for high yields of citric acid but their concentrations should be less than those required for growth. Wold and Suzuki (1976) studied the effect of Zn^{2+} and reported that its deficiency (at a concentration of about 0.2 μM) is a prerequisite for the fungal cell to pass into the acidogenesis phase. In the case of Cu^{2+}, it was used as a growth limiting agent by Jernejc *et al.* (1982) who reported that a concentration of 40 mg/l improved citric acid accumulation.

The significance of Mn^{2+} to citric acid accumulation has been mentioned by many authors. Shu and Johnson (1948b) reported that the presence of only 20 μg/l of Mn^{2+} could significantly reduce citric acid yields. Tomlinson *et al.* (1951) showed an effect of Mn^{2+} addition on the growth morphology of *A.niger* in a static surface culture, in that its concentration should be about 3 to 4 μg/l to obtain the desirable mycelial growth form. The detrimental effect of Mn^{2+} on citric acid accumulation has also been demonstrated by Clark *et al.* (1966). The physiological roles of Mn^{2+} deficiency in *A.niger* during citric acid production have been described in the reports of Kubicek and Röhr and other workers, and can be summarized as follows :

- it decreases the activity of the enzymes of the pentose phosphate and the tricarboxylic acid pathways (Kubicek and Röhr, 1977);
- it increases the levels of pyruvate and oxaloacetate, and thereby increases the citrate formation rate (Kubicek and Röhr, 1978);
- it strongly reduces triglyceride and phospholipid levels of the cells (Orthofer *et al.*, 1979);
- it increases cellular levels of NH_4^+ by increasing the rates of protein degradation (Kubicek *et al.*, 1979);
- it induces the morphology required for high yield of citric acid and alters the composition of the cell wall (Kisser *et al.*, 1980);
- it increases the protein degradation rate, as well as decreases the contents of polysomes and 80 S ribosomes (Ma *et al.*, 1985);
- it alters the membrane lipid composition (Meixner *et al.*, 1985) and total mycelial lipid content and composition (Jernejc *et al.*, 1989).

These influences of Mn^{2+} deficiency are believed to contribute to citric acid accumulation, particularly in relation to the effect of NH_4^+. It has been postulated that an elevated level of NH^+_4 in the fungal cell caused by Mn^{2+} deficiency can relieve the enzyme phosphofructokinase of the glycolytic pathway from feedback inhibition by citrate (Kubicek *et al.*, 1979; Habison *et al.*, 1983). The importance of this deregulation of glycolysis will be mentioned in more detail later in the chapter.

The negative effect of these metal ions on citric acid accumulation in the solid state cultivation process appears to be to a lesser extent than in the submerged fermentation conditions. There is a report on the resistance of the organism to these metal ions and, in some conditions, the yield of citric acid was stimulated (Shankaranand and Lonsane, 1994). Lu *et al.* (1995), however, reported that there was no significant change of citric acid yield as a result of addition of these metal ions in the solid state fermentation process.

2.5.5 pH and temperature

The initial pH of the culture medium has been reported to be important for the performance of the citric acid fermentation. Generally, the starting pH should be low but not so low that spore germination is retarded. The optimal starting pH varies with the nature of the substrate. Thus, whereas a pH range of 2.4 to 4.0 is optimal for a chemically defined medium, a range of pH 6.0 to 7.5 is required for a molasses based medium (Berry *et al.*, 1977). During the progress of the fermentation, the pH should be controlled to be less than 3.0 to prevent the formation of undesirable organic acids such as gluconic and oxalic acids (Röhr *et al.*, 1983). The enzymes which catalyse the formation of these two by-products are known to be pH dependent: glucose oxidase for gluconic acid synthesis, which is a cell wall-bound enzyme, is sensitive to low pH (Witteveen *et al.*, 1992), while oxaloacetate hydrolase for oxalic acid synthesis, which is a cellular enzyme, is induced by neutral pH (Kubicek *et al.*, 1988). In contrast, Heinrich and Rehm (1982) compared different types of fermentation and reported that gluconic acid could be formed at a fermenting pH of as low as 2.5 in a fixed bed reactor, but not in surface or shake-flask cultures.

The optimum temperature for citric acid fermentation by the fungus is generally in the range of 25 to 30^{0}C (Prescott and Dunn, 1959; Kapoor *et al.*, 1982). At below 25^{0}C, growth of the fungus is delayed whereas at above 30^{0}C the yield of citric acid is decreased while more oxalic acid is produced (Kapoor *et al.*, 1982).

2.5.6 Aeration

Biochemically, citric acid biosynthesis is an oxidative pathway, therefore, sufficient oxygen must be provided to the process. It is generally accepted that a high oxygen concentration, expressed as dissolved oxygen tension (DOT), is necessary for citric acid production. Kubicek *et al.* (1980) reported a minimum DOT of 25 mbar for a submerged process. Dawson *et al.* (1988) also demonstrated a high DOT of the medium as having great influence on the rate of citric acid production in chemostat and fed-batch cultures, while Gomez *et al.* (1987) reported an agitation speed of 1,000 rpm in submerged culture to be the optimum value for high citric acid productivity.

In addition, interruption of aeration has been reported to cause an irreversible negative effect on citric acid production as well as on respiration of *A.niger* (Kubicek *et al.*, 1980). However, restoration of product formation has been reported after an interruption of as long as 85 min (Dawson *et al.* 1987). An adverse effect of aeration on citrate production has been reported in a surface culture. Thus, Sakurai and Imai (1992) reported that the citric acid yield from this system decreased when the DOT value was high (74 to 100 % of saturation). In contrast, very little work has been reported on the oxygen requirement in solid state culture. Nevertheless, Hang (1988) has reported the use of a high air flow rate to improve the yield of citric acid in a packed-bed reactor.

2.5.7 Other additives

A number of compounds can be added to the culture medium to improve the yield of citric acid. Among these, methanol has been widely investigated since its stimulatory effect was reported by Moyer (1953a,b). The addition of 3 to 4 % (v/v) methanol to the fermenting medium could retard fungal growth and sporulation while the citric acid yield is improved. The exact function of methanol is still unknown but it is believed that this alcohol is a source of acetyl CoA or can alter the cell permeability to citrate (Kapoor *et al.*, 1982; Maddox *et al.*, 1985).

The effects of lipid additives have also been investigated. Millis *et al.*(1963) have shown that oleic acid and other natural oils with a high unsaturated fatty acid content could improve citric acid yield when present at a concentration of 2 % (v/v). These authors proposed a function of lipid as being an alternative hydrogen acceptor. In addition, starch and other viscous substances have been added into shake-flask cultures to improve the yield of citric acid by acting as a cell protectant to the physiological stress cause by shaking (Rugsaseel *et al.*, 1995).

2.6 Production Processes

2.6.1 Introduction

As mentioned previously, the microbial processes for citric acid production can be categorized into three main modes: (i) liquid surface process, (ii) submerged process, and (iii) solid state process. These processes are clearly different in their physical aspects, but it is not known whether or not they differ in their biochemical aspects. A description of these processes can be found in most reviews (for example, Röhr *et al.*, 1983; Dawson, 1986, and Lu, 1995). In this section, an overview of these fermentation technologies will be provided.

2.6.2 The liquid surface process

This is the classical process for citric acid production in which the fungus grows on the surface of a liquid medium of a limited depth (Roehr *et al.*, 1992). This process may be referred to as the "shallow pan process", as the fermentation is performed in shallow pans containing a sterile liquid medium (Lockwood, 1975). These pans, or trays, are made of high grade-aluminium or stainless steel to avoid trace metal contamination due to corrosion of the containers. The width and the length of these containers may vary from 2 m x 2.5 m to 2.5 m x 4 m, with a depth of about 0.25 m (Roehr *et al.*, 1996). They are, indeed, bioreactors and are arranged on racks which are placed in a fermentation room. Usually, metal-removed beet molasses, diluted to a sugar concentration of 14 to 20 % (w/v) and treated with hexacyanoferrate, is

used as the carbon source and is placed in the trays to the medium depth of 0.1 to 0.18 m. Other substrates may be raw sugar, high test syrup, or high-grade molasses (Lockwood, 1975). Kiel *et al.* (1981) attempted to use cotton waste as the substrate for surface cultivation and reported that sucrose had to be included as an additional carbon source. Roukas and Kotzekidou (1987) cultured *A.niger* in brewery waste to produce citric acid using the liquid surface process.

Inoculation is usually done by spraying spores, either in suspension or in dried form, over the medium surface (Roehr *et al.*, 1992). Alternatively, pregrown pellicles containing spores may be used after being mechanically dispersed (Lockwood, 1975). The temperature in the incubation room is controlled to about 30^{0}C by air circulation which, at the same time, helps provide aeration (Röhr *et al.*, 1983). After a day or two, the fungus develops a wrinkled mycelial pellicle on the surface of the liquid medium. By this form of growth, the fungus takes up oxygen from the atmosphere and utilizes nutrients dissolved in the liquid medium to an efficient extent because of the large contact area of the mycelial mat. As the cultivation continues, the mycelial mat becomes thicker with differentiated layers. Citric acid excretion appears to be associated with the mycelial layer in contact with the liquid medium (Berry *et al.*, 1977). As more citric acid is excreted into the medium the pH drops from the initial value of 6.0 to 6.5 to below 2.0 (Röhr *et al.*, 1983). Because it is a static condition, heat and concentration gradients occur along the liquid depth and a gentle mixing of the medium may be beneficial to the process. By other means, however, the heat may be removed by the supplied air (Roehr *et al.*, 1996). After the fermentation is complete citric acid is recovered from the fermented liquid. In some cases, the grown mycelium is reused as the inoculum for the next operation (Berry *et al.*, 1977).

Although this process requires much labour and space, the power needed for the operation is fairly low. For this reason, it is still currently used by some manufacturers to produce citric acid, though only on a small scale.

2.6.3 The submerged process

Since its first introduction for microbial production of citric acid, the submerged cultivation process has undergone substantial development. Unlike the static surface culture process, the submerged fungus develops in a pelletal or filamentous form, dispersing in the liquid medium due to vigorous mixing (Whitaker and Long, 1973). In such a situation, the whole organism can effectively accomplish the uptake of nutrients as well as oxygen, supplied in dissolved form.

At present this process accounts for the majority of the industrial-scale citric acid production because of its high yield and for other economic reasons (Roehr *et al.*, 1992). The commonly used reactors are the stirred tank and tower fermenters which are specially coated to prevent corrosion (Röhr *et al.*, 1983; Milsom and Meers, 1985). The process can be operated in batch, continuous, or fed-batch mode, but generally the first is preferable. The raw materials for the process are mainly glucose, cane syrup, or molasses diluted to a sugar concentration of 15 to 27 % (w/v) (Röhr *et al.*, 1983). The initial pH is usually adjusted to 2.0 to 4.0, with sucrose- or glucose-based media. Generally, the medium is inoculated with a suspension of spores, although sometimes, pregrown pellets prepared in a seed fermenter may be used to accelerate the fermentation (Röhr *et al.*, 1983). Gomez *et al.* (1987) demonstrated the advantage of inoculation with the pregrown pellets over inoculation with spores. Aeration is supplied through an air sparger and the air bubbles are further mixed and disintegrated by an agitator to increase the dissolved oxygen tension in the liquid medium. However, if agitation is too vigorous, damage to the pellets results.

The form of mycelial growth has been implicated as having a great influence on the citric acid fermentation performance in this type of process (Berry *et al.*, 1977). The preferable pellet morphology of *A.niger* in the citric acid-accumulating phase is described as small (0.2 to 0.5 mm diameter) with a dense, smooth surface (Clark, 1962; Gomez *et al.*, 1987). This form of pellet consists largely of extremely short, forked-like, bulbous, and swollen hyphae (Röhr *et al.*, 1983). Papagianni *et al.* (1994)

measured the morphological parameters of *A.niger* growing in a tubular loop and a stirred tank fermenter and reported a correlation between the hyphal length and citric acid production in that a shorter length is preferred. The physiological conditions for the development of these hyphae have been studied; for example, a deficiency in Mn^{2+} (Kisser *et al.*, 1980; Röhr *et al.*, 1983) or the presence of cyclic AMP (Obaidi and Berry, 1980) have been suggested. In addition, the organism grown in submerged culture is extremely sensitive to many environmental factors, particularly to metal ion concentrations. As a consequence, the medium for the process should be selected or treated properly. Moreover, sufficient oxygen should be supplied constantly to the system.

Being a homogeneous system, the kinetics of citric acid production in the submerged culture process have been intensively studied for most types of operation, for example, batch (Kristiansen and Sinclair, 1978), continuous (Kristiansen and Sinclair, 1979) and fed-batch (Dawson, 1986). At present, this type of process accounts for the main part of industrial scale production of citric acid.

2.6.4 The solid state process

This type of citric acid fermentation originated from the traditional "koji" process developed in Japan (Lockwood, 1979; Röhr *et al.*, 1983). The system consists of solid raw materials, such as sweet potato residues or wheat bran, spread on trays of about 0.6 m x 0.4 m x 0.05 m in size, with screen bottoms. The depth of the substrate layer is limited to improve heat transfer and to reduce any temperature gradient. The moisture content of the substrate is adjusted to between 65 to 70 % and the starting pH is about 5.5 (Röhr *et al.*, 1983). Inoculation can be done by mixing conidia of a selected strain of *A.niger*, usually an amylolytic enzyme-producing strain, with the substrate. These conidia may be prepared in rice or wheat bran, in the same method as in koji preparation. Since it is impractical to remove excess trace metal ions contained in the solid substrates, the organism used should be able to resist the negative effects caused by these ions (Röhr *et al.*, 1983). The fermentation is performed in a room, without continuous mixing of the fermented mass, and the

temperature is controlled to about 30^{0}C by circulated aeration. After completion of the process, the fermented materials, containing concentrated citric acid, are extracted with water. At present, this process is still operated in, mainly, Japan (Roehr *et al.*, 1996). In some conditions, the solid substrates for the process are inert solids impregnated with a sugar-based liquid medium (Lakshiminarayana *et al.*, 1975; Lee *et al.*, 1989).

Because of its nonhomogeneity, the method to expand citric acid production using the solid state process is a major concern due to the lack of necessary kinetic information. Moreover, the process is described as a non-automated type, thus, huge space is required which results in limitation of the operation. However, this process can be considered as a simple means to convert cheap solid organic materials or solid wastes into useful products. In addition, other advantages of the solid state over the submerged process have also been cited (Hesseltine, 1977; Mitchell and Lonsane, 1992).

There is only a little information on environmental factors influencing the production of citric acid using the solid state process. Hang and Woodams (1987) optimized the moisture content of apple pomace for use in citric acid production while, recently, Shankaranand and Lonsane (1994) reported less sensitivity to metal ions of *A.niger* during citric acid production from wheat bran when compared to submerged fermentation. Some fermentation kinetics of this process has been studied by Lu (1995) during cultivation in flasks and in a packed-bed reactor. So far, however, no morphological descriptions of the fungus are available during growth on a solid substrate for citric acid production and there appears to be no information regarding the biochemistry of the organism in this mode of growth.

2.7 Biochemistry of Citric Acid Accumulation by *A.niger*

2.7.1 Introduction

The metabolic pathways leading to citric acid overproduction in *A.niger* have received

as much attention as progress on the technology of the process. However, although many metabolic schemes have been proposed by different groups of researchers, there remain several controversial points on the mechanism and regulation of citric acid accumulation and thus a full description has not been established. This topic has always formed a major part of most reviews on citric acid production (see Röhr *et al.*, 1983; Milsom and Meers, 1985; Dawson, 1986; and Roehr *et al.*, 1996).

This part of the literature review will provide a description of the biosynthesis and regulation of citric acid production by the fungus *A.niger*, by considering each stage of the metabolic scheme from glucose to citrate. In this regard, a general metabolic scheme for the conversion of glucose to citric acid is proposed in Figure 2.1. However, it should be stressed that virtually all of this information originates from studies in submerged or liquid surface cultures, and may or may not hold true for a solid state fermentation process.

2.7.2 The role of glycolysis

After glucose is transported into *A.niger* cells, it can be assimilated *via* the Embden-Meyerhof-Parnas (EMP) glycolytic pathway or the pentose phosphate (PP) pathway, and pyruvate is formed as the terminal metabolic intermediate. The contribution of glycolysis during citric acid production has been demonstrated. Thus, Cleland and Johnson (1954) used a radiolabelled technique to prove that glucose is split to two C_3 molecules (pyruvate). Further, Kubicek and Röhr (1977) measured the activities of selected enzymes from both pathways from *A.niger* cultured in the presence or absence of Mn^{2+} which has been known to strongly influence citric acid accumulation. They concluded that glycolysis is favoured during the production phase of the Mn^{2+}-deficient culture, i.e. under conditions which promote citric acid accumulation. In order that citrate is accumulated at high rates, the flow of carbon through glycolysis and subsequently to the citrate precursors, acetyl CoA and oxaloacetate, should be stimulated (Röhr *et al.*, 1983). As a consequence, the regulation of certain glycolytic enzymes has been investigated. Among these, phosphofructokinase (PFK) has been described as the most likely regulatory enzyme. This enzyme, later named as 6-

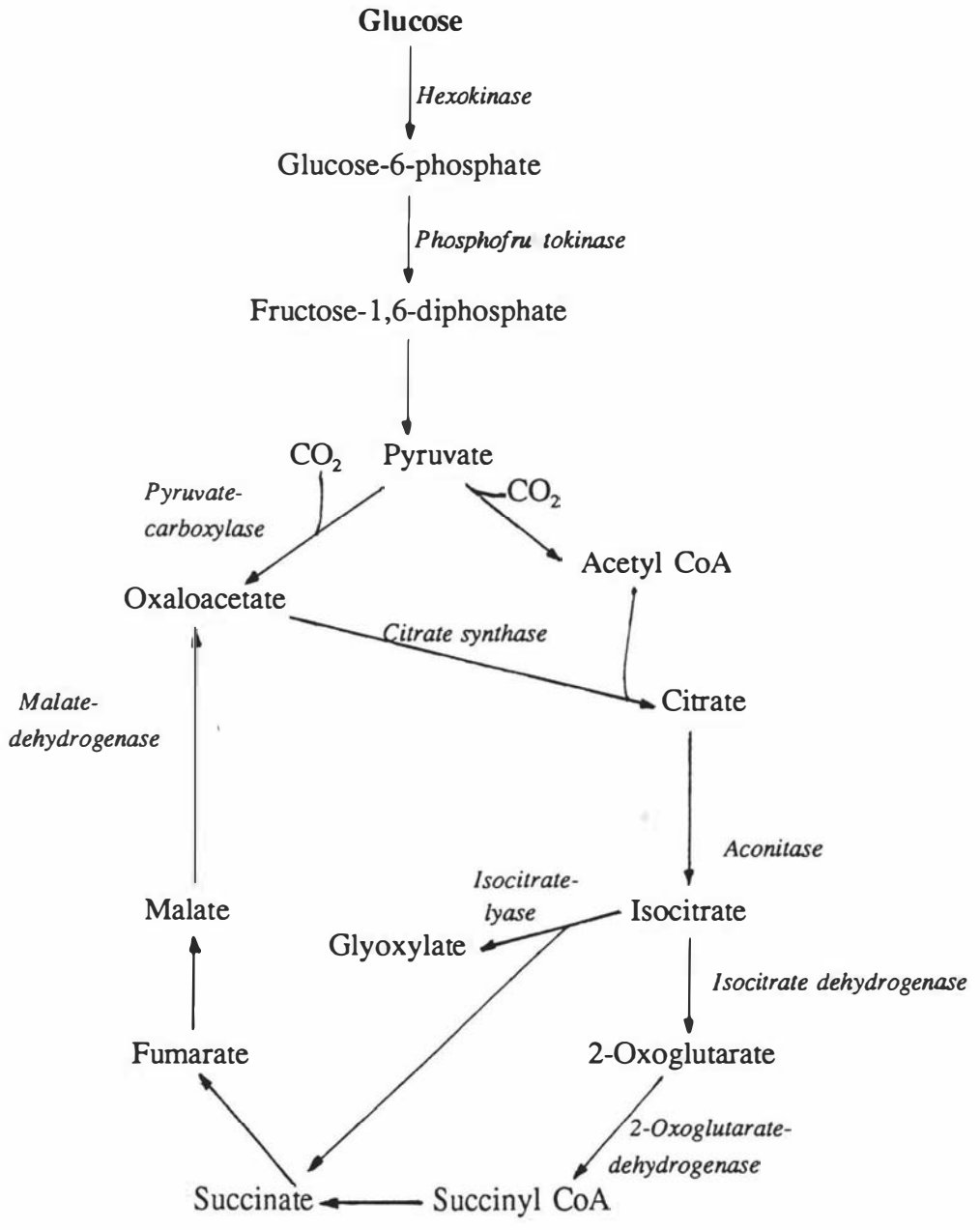


Figure 2.1 A simplified metabolic scheme for citric acid synthesis by *A.niger* (modified from Dawson (1986) and Legisa *et al.* (1995)).

phophofructokinase or PFK 1, has been shown to be inhibited by citrate at cellular concentrations (Mattey, 1977; Kubicek and Röhr, 1978). This inhibition was confirmed by Habison *et al.* (1979) who demonstrated that a crude extract containing PFK was inhibited by citrate at concentrations of 2 to 10 mM. In addition, by measuring various metabolite concentrations, this group also proposed the possibility of releasing this feedback inhibition by elevated levels of NH_4^+ ions and AMP. Later, the enzyme was purified and its kinetic properties were studied by Habison *et al.* (1983). These workers demonstrated that purified PFK 1 is inhibited by citrate, phosphoenolpyruvate and ATP, and that the feedback inhibition by citrate is neutralised by NH_4^+, AMP, and phosphate. The concept of NH_4^+ deactivation can be referred to the physiological roles of Mn^{2+} deficiency of the culture medium (see Section 2.5.4). In addition, Arts *et al.* (1987) postulated that PFK 1 can be stimulated by fructose 2,6-biphosphate (Fru-2,6-P_2) by increasing the affinity of the enzyme for its substrate, fructose 6-phosphate (Fru-6-P), and by neutralizing the inhibitory effect of ATP on the enzyme.

The concept of high sucrose concentration and increased citric acid accumulation has been previously reported (Xu *et al.*, 1989a,b). Further, Kubicek-Pranz *et al.* (1990) explained that this condition induced an elevated level of Fru-2,6-P_2 which then increased the rates of glycolysis and citric acid accumulation. The enzyme catalysing the formation of Fru-2,6-P_2, phosphofructokinase 2 (PFK 2), hence, has been studied in a citric acid-accumulating strain of *A.niger* (Harmsen *et al.*, 1992). Its kinetic properties suggest a major control by the presence of its substrate, Fru-6-P, which is also effected by the concentration of glucose or sucrose in the medium. Therefore, in the presence of high concentrations of these sugars, Fru-6-P accumulates in the cells and this can lead to an increase in Fru-2,6-P_2 levels from the action of activated PFK 2. These glycolytic regulations can relate to the effect of type and concentration of sugar on citric acid accumulation, in that either should allow these situations to occur. In addition, the regulatory role of Fru-6-P has led to a hypothesis on the existence of controls at steps prior to its formation, either at glucose transport or glucose phosphorylation. This assumption is supported by the steady-state sensitivity analysis proposed by Torres (Torres, 1994a,b). Thus, hexokinase, which functions in the

phosphorylation of hexose sugar, has been investigated in *A.niger*. The enzyme from a citrate-accumulating strain of *A.niger* was purified and was found to be present in only a single form (Steinböck *et al.*, 1994). Kinetic studies of the enzyme by this group revealed inhibition by citrate and this could be counteracted by increasing the concentration of the enzyme. In addition, a correlation was proposed by this group between reduced hexokinase activity and decreased citrate production rate. This relationship was also acknowledged previously in some strains of *A.niger* cultured under a high sucrose concentration (Schreferl-Kunar *et al.*, 1989). Mutants resistant to 2-deoxyglucose, showing reduced citric acid accumulation rates, have also been reported to contain reduced hexokinase activity (Kirimura *et al.*, 1992). However, it has been further postulated that the reduction of hexokinase may be a "result" rather than a "cause" of the low carbon flux through glycolysis (Roehr *et al.*, 1996).

The mechanism of glucose transport in citric acid-accumulating *A.niger* has been investigated only recently by Torres *et al.* (1996). It has been shown that in conditions of citric acid accumulation, the fungus contains a low-affinity glucose transport protein in addition to the high-affinity type. This low-affinity transporter is less sensitive to inactivation by low pH and citrate in the medium, and thus, is responsible for the glucose transport in relation to the high glycolysis fluxes necessary for high rates of citric acid accumulation.

Evidence for the relative contributions of the EMP and the PP pathways with regard to citric acid accumulation is also provided by Legisa and coworkers. Thus, it was reported that during the early stage of growth, the PP pathway predominated and glycerol is produced, resulting in the "bulbous" characteristic of the hyphae. Glycerol then enters the mitochondrion and inhibits the NADP-specific isocitrate dehydrogenase causing initial accumulation of citrate (Legisa and Mattey, 1986a,b, Legisa and Kidric, 1989). Citrate then, in turn, regulates glycerol synthesis by inhibiting 6-phosphogluconate dehydrogenase which results in a shift from the PP pathway to glycolysis, and citrate accumulation then continues (Legisa, 1988). Overall, however, this information has also led to the conclusion that a high flux through glycolysis is important to citric acid accumulation.

2.7.3 Metabolism of pyruvate

As citric acid is formed from the condensation of oxaloacetate and acetyl CoA (Figure 2.1), there has been considerable research into the metabolic routes leading to the formation of these two precursors. The evidence that the source of both citrate precursors is the metabolism of pyruvate, the terminal product of glycolysis, was first provided by Cleland and Johnson (1954). Using a tracer technique, these authors demonstrated that one molecule of pyruvate is decarboxylated to form acetyl CoA while another is carboxylated to form oxaloacetate. Verhoff and Spradlin (1976), in contrast, examined the mass balance in which the input is pyruvate and proposed a metabolic scheme relating the biosynthesis of citrate, oxalate, and CO_2. In this approach, one molecule of oxaloacetate, which originated from pyruvate, is split to oxalate and acetate while another oxaloacetate molecule is condensed with acetate to form citrate. Thus, this metabolic scheme explains the presence of oxalic acid as a by-product of citric acid production.

Following the evidence of carboxylation of pyruvate in *A.niger* reported by Cleland and Johnson (1954), the enzyme that catalyses this reaction, i.e.pyruvate carboxylase, has been investigated by many workers. Bloom and Johnson (1962) reported that the enzyme is active during citric acid formation and this was later supported by Hossain *et al.* (1984) and Dawson (1986). The kinetics of the catalytic function of this enzyme have been studied by Feir and Suzuki (1969). However, its regulation and location in the fungal cells are still controversial. Röhr *et al.* (1983) noted that pyruvate carboxylase of *Aspergillus* is produced constitutively when growing on glucose. In contrast to most other fungi, the enzyme from *Aspergillus,* such as *A.nidulans* (Osmani and Scrutton, 1983) and *A.terreus* (Jaklitsch *et al.*, 1991a) is located in the cytoplasm. This leads to a hypothesis that citrate can be transported from the mitochondrion in an exchange for cytosolic malate formed from cytosolic oxaloacetate which is supplied by the action of cytosolic pyruvate carboxylase (Roehr *et al.*, 1996). In this regard, citrate may be excreted and accumulated without the requirement for inhibition of the TCA cycle. However, this concept is still uncertain

since there are also reports of mitochondrial pyruvate carboxylase, as by Purohit and Ratledge (1988). Furthermore, Bercovitz *et al.* (1990) detected both cytoplasmic and mitochondrion enzyme in various species of *Aspergillus* including *A.niger* and concluded that there is no correlation between location of pyruvate carboxylase and citric acid accumulation. However, Jaklitsch *et al.* (1991b) provided evidence for cytosolic pyruvate carboxylase in *A.niger* during the acidogenesis phase but reported that the enzyme is also present in the mitochondrion of the growth phase mycelium. It was, therefore, suggested by this group that more investigation should be carried out on the regulation of this enzyme.

2.7.4 The role of the tricarboxylic acid cycle

The operation and regulation of the tricarboxylic acid (TCA) cycle in connection with citric acid accumulation has been the centre of controversy. By using labelled glucose, Shu *et al.* (1954) demonstrated that about 40 % of citrate was formed from recycled C_4 carboxylic acid of the cycle. In contrast, Bomstein and Johnson (1952) and Cleland and Johnson (1954) reported only little recycle of this dicarboxylic acid during the citrate accumulation phase. Earlier information from the measurement of TCA cycle enzymes led to the conclusion that citrate accumulated because of interruptions at certain steps of the cycle. The most disputatious publication is that of Ramakrishnan *et al.* (1955), who claimed that citric acid accumulated in *A.niger* because aconitase and isocitrate dehydrogenase disappeared from the TCA cycle. However, the presence of these two enzymes has subsequently been demonstrated, though at reduced levels, during the citric acid production phase (La Nauze, 1966; Kubicek and Röhr, 1977; Szczodrak, 1981; Hossain *et al.*, 1984; Dawson, 1986). The lability of these enzymes to acid conditions during the extraction procedure may explain the activity loss reported by Ramakrishnan *et al.* (1955) and thus strict cautions are advised during the preparation of enzymes for the assay (Röhr *et al.*, 1983; Kubicek, 1988).

Other evidence which disproves the theory of complete blockage of the TCA cycle is the allosteric and isoenzyme feature of isocitrate dehydrogenase (Kubicek, 1988).

This enzyme is present in two forms: the NADP-specific enzyme that is located in both the mitochondrion and the cytosol, the mitochondrial one being inhibited by citrate at a physiological concentration (Mattey, 1977; Mattey and Bowes, 1978), and the NAD-specific enzyme which is located exclusively in the mitochondrion, and is not inhibited by citrate. Meixner-Monori *et al.* (1986) purified the mitochondrial NADP-specific enzyme for a kinetic study and the inhibitory effects of citrate and ATP have been confirmed. In contrast, the NAD-specific enzyme which is very labile (and therefore, may lose activity during the preparation) is activated by citrate and AMP, whereas ATP is a negative effector (Kubicek, 1988). Both forms of isocitrate dehydrogenase are also subject to regulation by the presence of their cofactors, NAD or NADP. Since most previous publications did not consider the location or even the allosteric nature of isocitrate dehydrogenase, it may cause difficulties in interpretation of the results. It is, hence, suggested that a compartmentation rather than whole cell should be studied (Kubicek, 1988).

Another interruption in the TCA cycle has been proposed at the step of 2-oxoglutarate dehydrogenase. This enzyme could not be detected in *A.niger* during citric acid production (Kubicek and Röhr, 1977; Hossain *et al.*, 1984). Kubicek and Röhr (1978) demonstrated this blockage by measuring the intracellular levels of certain metabolic intermediates of the TCA cycle. Meixner-Monori *et al.* (1985), however, could detect activity of this enzyme by stabilising with its substrate, 2-oxoglutarate, when the enzyme was in the labile form because of dilution in the *in vitro* assay conditions. This is probably the main reason for the failure to detect the enzyme by other workers. Kinetic studies of the crude enzyme revealed inhibition by several metabolites but the most probable inhibitors are oxaloacetate and NADH, and this inhibition may cause citrate to accumulate due to the equilibria of the reactions. This activity "inhibition" hypothesis proposed by Meixner-Monori and coworkers, however, disputes the concept of "repression" of the enzyme synthesis by glucose proposed by Röhr and Kubicek (1981). Later, using this improved assay technique, the enzyme could be detected in *A.niger*, and which, during the citrate accumulation phase, decreased in activity (Dawson 1986).

2.7.5 The involvement of the glyoxylate cycle and other metabolic steps

Knowledge on the role of the glyoxylate cycle in citric acid production and accumulation is still very limited. The enzyme of this pathway which is of most interest is isocitrate lyase, since it catalyses the breakdown of isocitrate to glyoxylate and succinate (Figure 2.1). This enzyme has been reported to be active in *A.niger* during the citrate production phase (Ahmed *et al.*, 1972; Ng *et al.*, 1973). Further, Verhoff and Spradlin (1976) proposed a metabolic scheme of *A.niger* based on a mass balance involving lipid accumulation, in which the lipid produced after the active growth phase is converted back to sugar and since sugar in the medium is still in excess, the pathway shifts to oxalate which is then converted to glyoxylate. The enzyme isocitrate lyase is then activated to convert glyoxylate to isocitrate, and thus citrate is accumulated because of the reaction equilibrium. However, because the biochemical pathway of oxalate formation is still mainly unknown, this proposed scheme is questionable. Kubicek and Röhr (1977), in contrast, could not detect isocitrate lyase at all in a citric acid-producing *A.niger*. More recently, however, the enzyme has been reported to be active during the acidogenesis phase (Hossain *et al.*, 1984; Dawson, 1986). It is clear that there is more need to investigate the actual role of this enzyme, and probably other enzymes of the glyoxylate cycle.

One morphological change of *A.niger* during the citric acid accumulation phase is the increase in lipid content of the mycelium (Orthofer *et al.*, 1979; Jernejc *et al.*, 1982). Since biosynthesis of lipid, which takes place in the cytoplasm, requires acetyl CoA as a precursor, the source of this intermediate has been studied. A cytosolic enzyme, ATP:citrate lyase, has been studied in some detail because it requires citrate as the substrate for this purpose. A study by Pfitzer *et al.* (1987) has shown that this enzyme is constitutive and its activity appears not to be a prerequisite for citric acid accumulation. In addition, Jernejc *et al.* (1991) reported inhibition of the enzyme activity during the acidogenesis phase and proposed an inverse correlation between the enzyme activity and the concentration of excreted citric acid.

2.7.6 Summary

It is apparent that citric acid biosynthesis and accumulation by *A.niger* involve the collaboration of many regulatory events. In summary, it is now believed that the complete inhibition of certain steps in the TCA cycle is not necessary, while evidence for a high carbon flux through glycolysis is increasing. This condition is established by specific physiological conditions in the growth medium, i.e. high sugar concentration and deficiency of Mn^{2+}. Oxaloacetate is considered as a central metabolic intermediate and is supplied constitutively by the poorly-regulated pyruvate carboxylase. When the cellular concentrations of oxaloacetate reach certain levels, inhibition of 2-oxoglutarate catabolism may occur and cause the operation of the so-called "horseshoe cycle" in which more citrate is produced. The rise in intracellular levels of citrate can further reduce its own breakdown by inhibiting NADP-specific isocitrate dehydrogenase. However, because of lack of studies on the compartmentation of various enzymes there is still insufficient support for these hypotheses. Moreover, it is uncertain whether or not the situation will be identical when the organism is grown under the solid state environment.

2.8 General Principles of Solid State Cultivation

2.8.1 Introduction

Solid state cultivation generally refers to any microbial process in which the substrate consists of solid particles without free water (Aidoo *et al.*, 1982; Moo-Young *et al.*, 1983). This type of process has wide potential application in the production of many microbial products and a number of reviews on the topic are available, for example, Cannel and Moo-Young, 1980a,b; Aidoo *et al.*, 1982, Mudgett, 1986. Recently, Doelle and his colleagues have published a book on this subject (Doelle *et al.*, 1992) which is a comprehensive source of knowledge.

The classical products of solid state cultivation include bread, mould-ripened cheese, soy sauce and microbial biomass. In addition, certain natural microbial activities such as microbial growth in soil, composting and wood rotting may also be included (Mudgett, 1986). Solid state processes have also been studied for the production of a variety of microbial metabolites. Some of the products from the recent reports are lactic acid (Soccol *et al.*, 1994), enzymes (Padmamabhan *et al.*, 1993; Hours and Sakai, 1994), pigments (Johns and Stuart, 1991), and alcohol (Roukas, 1994). Citric acid is another organic acid which can be produced by this process (see Section 2.6.4). The cultivation can be carried out statically, i.e. without agitation, or with various degrees of mixing (Hesseltine, 1972). The group of microorganisms most commonly involved in the process is fungi since they require, by nature, only a low amount of water for growth and product formation (Cannel and Moo-Young, 1980a). The advantages of cultivation using solid state over submerged process include high volumetric productivity, reduced product recovery and operation cost, fewer pollution problems, and possible use of unrefined substrates.

This following section will describe the general features of solid state cultivation based on those review articles already mentioned. The growth morphology of the fungi on solid substrates and the characteristics of the solid substrate will be emphasized.

2.8.2 Solid Substrates

The sources of the solid raw materials for solid state processes are mainly agriculture and the food industry. Ralph (1976) classified solid substrates according to their nutritional properties as those which are a major source of nutrients for the microorganisms and those which are not. For the latter group, they act only as inert supports and growth of the organism is associated with the nutrients which are absorbed to these solids.

The solid substrate normally consists of complex polymeric molecules, therefore, an appropriate pretreatment is often required in order that the substrate becomes more

accessible and susceptible to microbial action (Mudgett, 1986). Most substrates used in the system contain complex carbohydrates such as lignocellulose or starch. The organisms capable of utilizing these polymers, therefore, should be able to hydrolyse these macromolecules into simple units, using the extracellular enzymes excreted from the cells. This step is considered as a rate-limiting step in solid state cultivation (Mitchell *et al.*, 1992b). For lignocellulosic materials, only a limited number of microorganisms, mainly fungi, can degrade these complicated structures, and in most cases, these substrates are converted to fungal biomass. Some examples of the lignocellulosic substrates used for this purpose are beet pulp (Considine *et al.*, 1987), wheat straw (Abdullah *et al.*, 1985) and sugar cane bagasse (Poonam-Nigam and Nigam, 1990). In the case of starch-containing raw materials, the structure of this complex carbohydrate is more readily available for microbial degradation than is that of the former group, therefore, they are widely used for solid state cultivation. Starchy materials may be fermented to improve their protein contents, such as the cassava fermentation (Raimbault *et al.*, 1985; Daubresse *et al.*, 1987), or they may be used as a substrate for other microbial products (see Mitchell *et al.*, 1992b).

Besides the nutritional properties, the physical factors of the solid substrate are also important. The most significant characters are the size and shape of the substrate particle, as well as the porosity of the substrate mass. These are associated with the surface area to volume ratio of the substrate particle and to the packing density within the substrate mass (Mudgett, 1986; Mitchell *et al.*, 1992b). Essentially, substrates with a smaller particle size provide a larger access area for microbial activities and for better heat transfer as well as gas exchange. Nevertheless, the complex, non-uniform character of the solid particle itself, or which arises after undergoing fermentation, can result in heterogeneity of the system (Mitchell *et al.*, 1992b). This leads to gradients of nutrients, exoenzymes, products, pH and temperature in the substrate layer and which are the major disadvantages for large-scale process development of the solid state fermentation.

2.8.3 Fungal growth characteristics on solid substrates

Filamentous fungi grow differently in the solid state environment than in the liquid state condition (Moo-Young *et al.*, 1983). Furthermore, the growth pattern of fungi on a solid substrate particle is more complicated than the growth on a solid agar medium. Generally, on the agar surface growth is indicated by radial extension of the hyphae in response to nutrient concentration gradients (Trinci, 1969). The kinetics of hyphal extension and branching of filamentous fungi on agar surfaces has received considerable attention, for example, as reported by Trinci (1971, 1974).

In solid state culture, the fungus also grows by extending its apical hyphae along the surface of the solid particles, but the direction and the growth rate rely on both nutritional and geometric properties of the substrate matrix (Moo-Young *et al.*, 1983). The oxygen required for fungal growth in the solid state system is supplied mainly from the atmosphere in the form of gas, though, to a minor extent, the organism may use oxygen dissolved in the water associated with the solid particles (Ramana Murthy *et al.*, 1993). Because of the complicated nature of the system, the growth description and kinetics are presented in sophisticated mathematical formulae. For example, Sugama and Okazaki (1982) studied the growth kinetics of *Rhizopus oryzae* on rice grains and developed a logistic growth model, while Ito *et al.* (1989) described the distribution of *A.oryzae* mycelia in rice grains using a mathematical model. Mitchell and coworkers used a model solid substrate to develop an empirical model (Mitchell *et al.*, 1991a) and a semimechanistic mathematical model (Mitchell *et al.*, 1991b) to depict the kinetics of glucoamylase activity and starch and glucose consumption during growth of *R.oligosporus.* Recently, Nandakumar *et al.* (1994) proposed a mathematical model to describe wheat bran particle degradation by the fungus *A.niger.*

As the fungus is growing on the solid substrate surface, the nutrients become limited so the fungus penetrates into the solid particle. Hence, the process of penetration has received some attention. For example, Jerus and Sundberg (1976) studied the frequency and depth of penetration into soybean cotyledons of *R.oligosporus* during

tempeh production and reported a rapid change in both physical and chemical properties of the soybeans due to the mechanical force during the hyphal penetration in addition to the action of fungal exoenzymes. Mudgett *et al.* (1982) observed the growth characteristics of *R.oryzae* on rice solids and reported a penetration depth of about 0.15 mm after 2 days incubation. A model solid substrate was used by Mitchell *et al.* (1990) for a penetration study of *R.oligosporus*. This fungus developed two forms of penetrative hyphae : the one found at the superficial depth was dense and parallel to the substrate surface whereas that which penetrated deeper was less compact and perpendicular to the surface. Recently, Lu (1995) measured the ability of *A.niger* to penetrate into the layer of the starch-containing solid substrate prepared from sweet potato (kumara) by observing the reduction of the depth of the substrate layer caused by the fungal growth. However, this author appeared to measure the total change of the solid substrate mass rather than the penetration into the particles of the solid substrate.

A major problem with the study of the growth kinetics associated with the intricate fungal growth characteristics in the solid state system is the direct measurement of biomass. Most of the methods which are used in submerged cultivation are inapplicable to solid state systems. Hence, various indirect methods based on measurement of cellular components have been used to estimate the growth. For example, Desgranges *et al.* (1991) estimated the fungal biomass from agar medium and clay granules by measuring glucosamine and ergosterol contents of the mycelia. Cordova-Lopez *et al.* (1996) used the soluble protein content as the growth index for *A.niger* cultured on a model substrate and on cane bagasse. In addition, when growing in a condition of limited water, the fungi usually perform sporulation which is a crucial step of the morphological development (Moo-Young *et al.*, 1983). In filamentous fungi including *A.niger,* sporulation is stimulated by certain environmental changes (Smith and Gabaith, 1971; Smith, 1978). This incidence is often enhanced in solid state cultivation due to the difficulty of removing such stimulators (Moo-Young *et al.*, 1983), thus making estimation of fungal growth even more difficult.

2.8.4 Environmental factors

The same environmental conditions which effect growth and product formation of microorganisms in submerged cultivation also influence the performance in solid state systems. However, control of these factors in the latter is more difficult, mainly because of the nonhomogeneous character of the systems.

2.8.4.1 Moisture content and water activity

Ideally, there is no water in the free form in the system for solid state cultivation. Instead, water exists as moisture either adsorbed to the substrate surface or in a complex form within the solid matrix (Cannel and Moo-Young, 1980a).

The critical value of adsorption depends on the water binding capacity of each substrate. Whereas moisture levels of 50 to 55 % were reported as maximum for cassava (Oriol *et al.*, 1988), a lower level of 40 % moisture was maximum for maple bark (Moo-Young *et al.*, 1983). The requirement for water of microorganisms also varies with the organism. This water requirement may be expressed as water activity, which refers to the ratio of the vapour pressure of the substrate to that of pure water (Scott, 1957). In general, at low water activity the lag phase of the fungus is extended and the growth rate is decreased (Prior *et al.*, 1992). The initial moisture content of a solid state process can vary within the range of 30 to 85 %, depending upon the substrate type (Laukevics *et al.*, 1984; Oriol *et al.*, 1988). Excess moisture leads to agglomeration of the substrate, and thus limits gas exchange as well as increases the potential for bacterial contamination (Moo-Young *et al.*, 1983; Lonsane *et al.*, 1985; Considine *et al.*, 1987). During the course of the fermentation, the content of water is changed due to microbial activities. Usually, the substrate moisture is decreased by evaporation caused by heat build-up. The control of the moisture content in the system, though difficult, may be achieved, for example, by aerating with humidified air or by spraying with water (Sato *et al.*, 1982; Mitchell *et al.*, 1992b). This would result in non-absorbed, i.e. free water, in the system.

2.8.4.2 Temperature and heat removal

The heat generated per unit volume is much greater in solid state than in liquid state systems because of the high substrate density (Smith and Aidoo, 1988). For example, in composting, a total heat generation of more than 13,000 kJ/kg dry matter occurs (Finger *et al.*, 1976). This excess heat must be removed or it may be detrimental to the system. Moreover, the solid nature of the substrate and the mode of operation also cause a temperature gradient in the system. As reported by Rathbun and Shuler (1983), a temperature gradient of 3^0C/cm was observed during a soya bean fermentation.

The control of temperature, or the removal of heat, in solid state cultivation is very difficult because of the low moisture content together with the poor thermal conductivity of the organic solid substrate. This situation becomes increasingly severe for a large-scale process (Tengerdy, 1985). Removal of the generated heat can be achieved by forced aeration with moist or dry air, or by water-cooling (Mitchell *et al.*, 1992a). However, evaporation with dry air may lead to the problem of water loss from the system (Trevelyan, 1974).

2.8.5 Reactors

The simplest and most practical bioreactor for solid state fermentation is the tray. There has been little success in the development of other types of reactor because of the lack of accurate and reliable measurements for the necessary process parameters (Aidoo *et al.*, 1982; Moo-Young *et al.*, 1983). Nevertheless, a few other types of bioreactor have been developed in the laboratory or small pilot scale such as rotating drums (Lindenfelser and Ciegler, 1975) and packed columns (Raimbault and Alazard, 1980; Yang, 1988). Examples of these bioreactors have been reviewed by Pandey (1991) and Mitchell *et al.* (1992a). For the production of citric acid using a solid state process, Lu (1995) developed a packed-bed column for use as the fermentation reactor.

The majority of reactors used for solid state cultivation are operated in the batch mode, mostly without vigorous mixing or agitation, although other modes of operation have been investigated on the laboratory scale (Mitchell *et al.*, 1992a). A major problem associated with this static condition is the temperature gradient, therefore, the depth of the substrate in the reactor must be limited.

2.9 Summary

This literature review may be considered in two main parts. The first part provided fundamental knowledge on citric acid production by microbial processes, beginning with a short history on citric acid production and the types of producing microorganisms. Then the emphasis was placed on the production processes and on the environmental factors influencing the process performance. Other subjects which have been emphasised are the biochemistry and regulation of citric acid production by the fungus *A.niger*, in which almost all the information had been obtained from submerged liquid fermentation, and very little from surface liquid or solid state fermentation.

The second part was the review on solid state cultivation which provided some general knowledge on this type of the process, although the focus was on the biological fundamentals of the fungi associated with solid substrates. A combination from both parts of the review will lead to a more comprehensive knowledge of citric acid production by the solid state fermentation process.

The aim of this thesis is to study the fundamental biology of *A.niger* strains during citric acid production in solid state fermentation. Appropriate mutants will be developed and isolated allowing comparative studies of these strains at the biochemical level. It is hoped that this study will lead to an understanding of the mechanism of citric acid accumulation by *A.niger* in solid state fermentation.

CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Microbiological media

The medium used for cultivation and spore propagation of all fungal strains was Malt Extract (ME) agar (Oxoid, England). For screening of mutants, ME agar was supplemented with glucose (20 g/l), calcium carbonate (10 g/l) and bile salts No.3 (Oxoid, 2.0 g/l).

3.1.2 Substrates

The substrate used for solid state fermentation experiments was prepared from fresh tubers of sweet potato, as described in Section 3.2.4.

For cultivation in submerged conditions, the SL medium according to Kirimura *et al.* (1992) was used. The composition of this liquid medium is given in Table 3.1. This liquid medium was prepared without glucose and adjusted to pH 3.0 before sterilizing at 121^0 C for 15 min. Glucose was sterilized separately using the same method, then added to this basal medium to the required final concentration.

Table 3.1 Composition of SL medium for submerged cultivation of *A.niger* (Kirimura *et al.*, 1992).

Component	Concentration (g/l)
Glucose	120.0
$(NH_4)_2SO_4$	3.0
K_2HPO_4	2.0
KH_2PO_4	2.0
$MgSO_4.7H_2O$	0.5
$FeCl_3.6H_2O$	1.0×10^{-2}
$MnSO_4$	1.4×10^{-2}

3.1.3 Chemicals

The chemicals used for the fermentation and analyses were of analytical or HPLC grade. Their names and sources are :

BDH Chemicals Ltd. (England)

- Antimony potassium tartrate; ammonium molybdate; ammonium sulphate; L-ascorbic acid; calcium carbonate; calcium chloride; citric acid; copper sulphate; ethylenediamine tetraacetic acid, disodium salt; dipotassium hydrogen phosphate; ethyl alcohol; ferrous sulphate; glacial acetic acid; glucose; glycerol; hydrochloric acid; iodine; magnesium chloride; magnesium sulphate; manganese chloride; manganese sulphate; mercaptoethanol; methyl alcohol; orthophosphoric acid; oxalic acid; perchloric acid; phenylhydrazine hydrochloride; potassium carbonate; potassium chloride; potassium citrate; potassium cyanide; potassium diphosphate; potassium hydrogen phosphate; potassium hydroxide; potassium iodide; potassium permanganate; potassium pyrophosphate; sodium azide; sodium bicarbonate; sodium citrate; sodium hydroxide; sulphuric acid; trichloroacetic acid;

triethanolamine; tris (hydroxymethyl) aminomethane; zinc chloride, and zinc sulphate.

Sigma Chemicals Co., (St Louis, Missouri, U.S.A.)

- *cis*-Aconitic acid; adenosine 5'-monophosphoric acid (AMP, muscle adenylic acid); adenosine 5'-diphosphoric acid (ADP, sodium salt); adenosine 5'-triphosphoric acid (ATP, sodium salt); bovine serum albumin; citric acid; coenzyme A, sodium salt; L-cysteine hydrochloride; dithiothreitol; DL-isocitric acid, tetrasodium salt; glycylglycine; glyoxylic acid; lyolecithin; 2-(*N*-morpholino) ethanesulfonic acid; ß-nicotinamide adenine dinucleotide (NAD); ß-nicotinamide adenine dinucleotide, reduced form (NADH, disodium salt); ß-nicotinamide adenine dinucleotide phosphate (NADP, monosodium salt); ß-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, tetrasodium salt); 2-oxoglutarate, monosodium salt; phosphoenol pyruvate; pyruvic acid, sodium salt; oxaloacetic acid; succinate; thiamine pyrophosphate chloride, and tricine.

3.1.4 Enzymes

The types and sources of enzymes used in the study are:

NOVO, Nordisk (Bagsaerd, Denmark)

- α-Amylase, from *Bacillus licheniformis* and glucoamylase, from *A.niger*.

Sigma Chemicals Co., (St Louis, Missouri, U.S.A.)

- Aldehyde dehydrogenase, from bakers' yeast; catalase, from *A.niger*; citrate lyase, from *Enterobacter aerogenes*; glucose-6-phosphate dehydrogenase, type III, from bakers' yeast; glutamate dehydrogenase, type I, from bovine liver; hexokinase, type C-300, from bakers yeast; lactate dehydrogenase, type II, from rabbit muscle; malic dehydrogenase, from bovine heart; myokinase, from rabbit muscle; oxalate oxidase,

from barley seedling, and pyruvate kinase, type II, from rabbit muscle.

3.1.5 Organisms

Four strains of citric acid-producing fungus *A.niger* were used in this study. The first two strains were the high-yielding strains, *A.niger* Yang No.2 and *A.niger* MH 15-15 (ATCC 64065). The former strain was obtained from Waseda University, Tokyo, Japan, while the latter was an improved strain originating from *A.niger* IMI 41874, and isolated by Hossain (1983) in this laboratory.

The other two strains were mutants which had been selected after an ultraviolet treatment of those two high-yielding strains in this laboratory. They were characterized by their decreased citric acid yields and were designated as strains SL-1 and SL-2. The procedures for the isolation and the selection of these mutants are described in Section 3.2.6. All these strains were maintained and cultured for spore production using the conditions given in Table 3.2.

Table 3.2 Cultural conditions for growth and spore production of *A.niger* strains used in the study.

Strain	Culture Medium	Incubation Condition
Yang No.2	ME agar	30^{0}C, 5 days
SL-1	ME agar	20^{0}C, 10 days
MH 15-15	ME agar	30^{0}C, 5-7 days
SL-2	ME agar	30^{0}C, 10-15 days

To prepare stock cultures of each fungal strain, the spores were harvested and were suspended in ME broth containing 30 % (v/v) glycerol to a concentration of about 10^7 spores/ml. Small portions of the suspension were dispensed in Eppendorf tubes and were then kept at -20^{0}C. When subcultivation was required, a tube of the stock culture was defrosted and then inoculated on ME agar. Usually, there had been no

more than 10 passages of subcultivation of each stock culture.

3.2 Methods

3.2.1 Cleaning procedure for glassware

All glassware such as flasks and petri-dishes were cleaned by washing with hot detergent solution. After rinsing with tap water and followed by distilled water, they were dried in hot air. Glassware used in the enzyme assays, metabolite and adenylate determinations was specially treated as described by Dawson (1986) which included treatments with 2.0 M NaOH and 50 % (v/v) HCl solutions.

3.2.2 Avoidance of wall growth in flasks

To avoid wall growth of the fungi, all flasks used in the submerged cultivation experiment were treated with Sigmacote (Sigma Chemical Co., St Louis, Missouri, U.S.A.). In the method, the inner surface of the dried clean flasks was rinsed thoroughly with this coating solution. After drying under hot air, the flasks were washed with distilled water and redried in hot air before use.

3.2.3 Preparation of spore suspension

Spores were harvested from the surface of agar slopes by shaking with sterile water. The suspension was then shaken vigorously to break spore chains and clumps and the spore number was counted with a standard haemocytometer slide (0.1 mm depth, 1/400 mm^2, Weber Scientific International Ltd.), then diluted to desired concentrations.

3.2.4 Preparation of solid substrate

The solid substrate used for the solid state fermentation work was prepared from sweet potato (kumara, *Ipomoea batatas*) by blending the peeled, cooked (121^0C, 15 min) sweet potato in a blender (AUTO MIX, MSE Ltd., England) until homogeneity. To

reduce variation in the substrate composition, this substrate was prepared in an excess amount and was frozen at -20^{0}C until required. The general composition of nutrient contained in sweet potato is given in Table 3.3.

Table 3.3 Nutritional composition of sweet potato (kumara, *Ipomoea batatus*).

Composition	Concentration/100 g
Carbohydrate	27.50 g
Nitrogen	200 mg
Phosphate	21 mg
Copper	0.11 mg
Iron	0.53 mg
Manganese	0.75 mg
Zinc	0.20 mg

Note : from FOODINFO, New Zealand Institute for Crop and Food Research, pp. 1151, 1203.

For a fermentation study, a 40 g (wet weight) amount of this solid substrate was transferred into each glass petri-dish (1.5 cm depth x 9.0 cm diameter). The paste was packed to a uniform thickness then sterilised at 121^{0}C for 15 min.

3.2.5 Inoculation and cultivation

Each of the dishes containing substrate was inoculated with 1 ml of 5 x 10^5 spores/ml suspension, by uniformly distributing the suspension through a pipette tip. Cultures were then incubated at 30^{0}C. For submerged cultivation, each 250 ml flask containing 50 ml of SL medium was inoculated with the same amount of spores and was incubated at 30^{0}C on a rotary shaker (Gallenkamp Orbital Incubator) operating at a speed of 200 rpm.

3.2.6 Isolation and selection of mutants

3.2.6.1 Mutagenic treatment

Spore suspension (10 ml, containing 10^7 spores/ml) was transferred into a sterile glass petri-dish and was exposed to ultraviolet (UV) light at 254 nm generated from a universal UV lamp (CAMAG, Muttenz-Schweiz Ultra-Violet products, Inc., San Gabriel, California, U.S.A.). The distance between the dish and the source of light was about 15 cm. The irradiation was continued for 45 min with periodic gentle swirling to distribute the spores. This treatment was performed in a dark room to avoid any photoreactivation effects to the mutants.

3.2.6.2 Screening of mutants

After UV-irradiation, appropriate dilutions of the spore suspension were spread onto the surface of the "screening" medium (ME agar containing glucose, calcium carbonate and bile salts, Section 3.1.1) and were incubated at 30°C for 5 days. The untreated spore suspension was also plated as a control.

For screening mutants of *A.niger* Yang No.2, any colonies showing marked reduction in sporulation ability, as compared to their original strain, were collected. These conidial mutants were further subcultured onto the "selection" medium (the screening medium without bile salts) and were incubated at both 30°C and 20°C, for 5 days. Colonies which showed superior sporulation at 20°C than at 30°C incubation, or the so-called "temperature-sensitive conidial" mutants, were then collected.

For screening mutants of *A.niger* MH 15-15, any colonies showing marked reduction in acid production on the screening medium after incubation at 30°C for 5 days were collected regardless of their sporulation abilities.

3.2.6.3 Selection of mutants

To select the desired mutants, the ability to produce acid, assuming citric acid, of the collected mutants were rechecked. This was done by inoculating an agar cube (2 mm^3) from a 48 h colony of each fungal strain onto a plate of the selection medium. After incubation at 30^0C for 4 days, the diameters of the colony and the surrounding clear zone were measured. The ratio of the latter to the former was used as a measure of the citric acid producing ability of that colony.

3.2.7 Extraction and treatment of fermented solid culture

To extract a fermented sample, the whole contents from each petri-dish were transferred into a 250 ml flask and 150 ml of water was added. The solid part (fungal mycelium plus residual substrate) was disintegrated using a glass rod and the flask was then shaken at 200 rpm for 5 min. A volume of 2 ml was taken from the mixture for measurement of free glucose (Section 3.2.9). The remaining sample was adjusted to pH 6.0 with 10 % (w/v) NaOH solution and 2 drops of crude solution of α-amylase (TERMAMYL, NOVO, Denmark) were added. The liquefaction process was carried out for 1 h at 90^0C in a shaker-bath. After cooling to 70^0C, the mixture was adjusted to pH 4.5 with 10 % (v/v) HCl solution and 2 drops of crude glucoamylase (SAN, NOVO, Denmark) were then added. After saccharification for about 4 to 5 h at 70^0C in a shaker-bath, the non-hydrolysed materials were removed by centrifugation at 5,000 rpm for 15 min using a bench-top centrifuge (BHG HERMLE Z320, Germany). This solid residue was reextracted three times, each time with water of about twice the volume of the solid mass. The supernatant collected from all centrifugations was combined and the final volume was recorded. This liquid extract was used for determination of residual starch (in form of glucose), citric acid and oxalic acid.

3.2.8 Determination of fungal biomass

A gravimetric method was used to determine the fungal biomass of a fermented

sample. To harvest the mycelium, the solid residue from the final centrifugation (Section 3.2.7) was dispersed in water, and the biomass, which appeared as broken pieces of mycelial mat, was collected. This was then dried to a constant weight at $103 \pm 0.2^{0}C$.

3.2.9 Measurement of glucose

Glucose was measured using a glucose analyzer (YSI Model 27, Yellow Springs Instrument Co.Inc., Yellow Springs, Ohio, U.S.A.), which was based on the enzyme electrode method. Before each analysis, the insoluble part of the sample, if present, was removed by centrifugation and the liquid portion was passed through a 0.45 μm membrane (Millipore Corporation, Bedford, Massachusetts, U.S.A.). A standard glucose solution of 200 mg/100 ml was used for calibration. If necessary, the sample was diluted with distilled water before the glucose measurement.

3.2.10 Determination of starch

The amount of starch was determined indirectly, by measuring the glucose released after hydrolysis with amylase enzymes. To determine the starch contained in a fermented sample, the total glucose concentration (measured in the final supernatant obtained after NOVO amylases hydrolysis, Section 3.2.7) less the free glucose concentration (glucose existing before the amylase treatment, Section 3.2.7) was determined. That is :

$$\textit{glucose in starch (g/l)} = \textit{total glucose (g/l)} - \textit{free glucose (g/l)}$$

The glucose value obtained from the above equation was then converted to starch content by multiplying with a factor of 0.9 (Lu, 1995), as below :

$$0.9 \times \textit{glucose (g/l)} = \textit{starch (g/l)}$$

3.2.11 Measurement of glucose-releasing enzymes

This measurement was based on the method of Lu (1995) and measured the release of glucose from the starch contained in the solid substrate by actions of the fungal enzymes. In the method, the whole fermenting culture was transferred into a 250 ml flask and 100 ml of water was added. The solid content (residual substrate and fungal biomass) was disintegrated using a glass rod and was mixed by shaking vigorously for about 5 min. A 2 ml portion of the mixture was then taken for analysis of "original" glucose using the glucose analyzer (Section 3.2.9), while the rest of the content was incubated at 30^{0}C while shaking at a speed of 200 rpm. After incubating for 1 h, another 2 ml sample was taken for analysis of glucose (Section 3.2.9). The enzyme activity was based on the initial wet weight of substrate, i.e. g glucose released/kg.h.

3.2.12 Measurement of starch-hydrolysing enzymes

3.2.12.1 Preparation of enzyme sample

To extract the amylolytic enzymes from the fermenting medium, a whole dish of fermented culture was transferred into a 250 ml flask and 100 ml of water was added. The solid content was disintegrated using a glass rod and the flask was shaken at 200 rpm for 5 min, as described in Section 3.2.7. The mixture was centrifuged at 5,000 rpm for 15 min. The solid residue was reextracted once with a small volume of water. The supernatant from both centrifugations was combined and was used as the crude extract for the activity assays of α-amylase and glucoamylase.

3.2.12.2 Assay of α-amylase

The assay method for α-amylase was based on that of Wilson and Ingledew (1982), with slight modifications.

The assay mixture contained in a test tube : 2.0 ml of 0.2 % (w/v) soluble starch in 0.05 M potassium phosphate buffer (pH 6.0) and 1.0 ml of diluted enzyme sample. The reaction mixture was incubated at 40^{0}C in a shaker-bath for 10 to 15 min, and the reaction was stopped by immersing the tubes in boiling water for 5 min. To measure the amount of starch remaining after α-amylase action, 0.2 ml of this mixture was reacted with 5.0 ml of iodine solution (0.1 % (w/v) iodine in 1.0 % (w/v) potassium iodide containing 0.05 M HCl). The starch-iodine complex formed was measured at 620 nm against a reagent blank.

One unit of α*-amylase* is defined as the amount of enzyme which hydrolysed 0.1 mg of starch in 10 min at 40^{0}C when 4.0 mg of starch was present.

3.2.12.3 Assay of glucoamylase

The activity of glucoamylase in the enzyme sample was determined according to the method of Rugsaseel *et al.* (1993) with slight modifications.

The reaction mixture in a tube contained : 1.6 ml of 2 % (w/v) soluble starch in 0.05 M sodium acetate buffer (pH 5.5) and 0.4 ml of diluted enzyme sample. The mixture was incubated at 45^{0}C in a shaker-bath for 30 to 60 min, after which the tubes were immersed in boiling water to end the reaction. The amount of glucose released from glucoamylase action was measured using the glucose analyzer (Section 3.2.9).

One unit of glucoamylase is defined as the amount of enzyme which catalysed the release of 1.0 μmole of glucose per min under the assay conditions used.

3.2.13 Measurement of glucose uptake by intact mycelium

The uptake of glucose was measured using a modification of the exhaustion method described by Kirimura *et al.* (1992), which was modified from that of Mischak *et al.* (1984). Mycelium used for the measurement was pregrown for 48 h in SL medium containing 12 % (w/v) glucose (Table 3.1) in a shake-flask condition, and was

harvested by rapid filtration through Whatman No.54 filter paper. The mycelium was then suspended in SL medium without carbon or nitrogen source, and incubated on the shaker at 30°C for 60 min to allow glucose exhaustion. This starvation procedure was repeated twice in fresh SL medium. After the final filtration, the exhausted mycelia were resuspended in fresh SL medium (without glucose or nitrogen source) to a concentration of 4 to 10 mg dry weight/ml. At zero time, a glucose solution in SL medium was added to the suspension to give a final concentration of 1.8 mg glucose/ml and the mixture was shaken at 30°C. At appropriate intervals, a 1 ml suspension was taken for glucose measurement using the YSI glucose analyzer (Section 3.2.9).

3.2.14 Determination of extracellular citric acid

The concentration of citric acid in the fermented medium was determined using the HPLC method according to Dawson (1986) and Lu (1995). The citric acid sample was prepared according to the extraction procedure described in Section 3.2.7.

The HPLC system consisted of a Model ALC/GPC 244 liquid chromatograph , a Model 6000A solvent delivery system, a Model U06K septumless injector, and a Model 401 Differential Refractometer (all were from Waters Associates Inc., Milford, Massachusetts, U.S.A.), and a Data Module integrating recorder (Millipore Corporation). The column used was a μ-Bondapak reverse-phase column (4.0 mm ID x 250 mm, Bio-sil ODS-10, Bio-Rad Laboratories, Richmond, California, U.S.A.). The mobile phase was 2 % (w/v) potassium dihydrogen phosphate adjusted to pH 2.45 with orthophosphoric acid and was passed through a membrane (0.45 μm pore size, Millipore Corporation) before use. The flow-rate was set to 2.0 ml/min and 50 μl of sample was used for injection. Before the analysis, the system was calibrated with standard citric acid solutions (1.0 and 10.0 g/l). The samples to be analysed were adjusted to pH 2.45 with orthophosphoric acid then passed through the 0.45 μm Millipore membrane before injection.

3.2.15 Determination of extracellular oxalic acid

The concentration of oxalic acid in the fermented sample was determined using a titration method according to Baseman and Beer (1965). Oxalic acid was extracted from the sample as described in Section 3.2.7.

In the determination procedure, the sample was added to a mixture of glacial acetic acid and 10 % (w/v) calcium chloride solution of 5:3 (v/v) to precipitate oxalic acid as the calcium salt. The precipitate was recovered by centrifugation at 5,000 rpm for 15 min and was washed twice with water. The calcium oxalate was then dissolved in 4 M H_2SO_4 and titrated at 90^0C with 0.3 M $KMnO_4$ solution. The titration end-point was indicated by the formation of permanent faint-pink colour. The concentration of oxalic acid in the sample was calculated from a standard curve prepared from oxalic acid.

3.2.16 Extraction of enzymes and other cellular components from mycelium

3.2.16.1 Preparation of mycelium for extraction

The mycelium used for the extraction was harvested from the fermented medium, by peeling off the sheet of mycelium from the solid substrate surface. It was then sprayed with several volumes of ice-cold water to wash out residual substrate and citric acid. This was followed by washing with 0.1 M potassium phosphate buffer (pH 7.4 to 7.8) until neutralized. To minimize any physiological changes of the mycelium sample, these steps were completed as quickly as possible and, at the same time, the temperature was controlled to not exceed 7^0C. Extraction of enzymes and other metabolites from this clean mycelium was conducted immediately.

3.2.16.2 Extraction of enzymes (other than ATPase)

To extract the cellular enzymes, the mycelium prepared as above was pre-disrupted

by hand-grinding in a pre-cooled porcelain mortar for about 2 to 3 min. The extracting solution (0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA) was added in a ratio of 4 ml per g mycelium (wet weight). The pre-homogenate sample was then transferred into a pre-chilled homogenizer container (a stainless steel bottle of 75 ml capacity) and glass beads of 0.3 to 0.5 mm diameter (Glasperlen, Kt. Nr.54180, B. Braun Melsungen AG., Germany) were added in a ratio of 2.5 g per g mycelium (wet weight). The container was shaken using a mechanical homogenizer (Cell Homogenizer MSK, Type 853034, B. Braun Melsungen Ag, Germany) at a speed of 4,000 rpm for a total time of 60 to 90 sec. During the operation, the system was cooled by passage of liquid carbon dioxide. After centrifugation at 2^0C for 15 min at a speed of 12,000 rpm using a refrigerated centrifuge (Sorvall Superspeed RC 2-B Automatic Refrigerated Centrifuge), the cell debris was discarded, while the supernatant was used for the enzyme assays.

3.2.16.3 Extraction of ATPase

The extraction procedure for the membrane ATPase was according to the method of Gutierrez and Maddox (1993) which was based on Viegas and Sa-Correia (1991), with some appropriate modifications.

The enzyme was extracted from the mycelium (prepared as in Section 3.2.16.1) according to the procedure described in Section 3.2.16.2, except that a solution containing 5 mM EDTA, 100 mM Tris (pH 8.0 adjusted with diluted HCl solution) and 2 mM dithiothreitol was used as the extracting solution. The final mycelial homogenate was centrifuged at 3,000 rpm and 4^0C for 3 min and the supernatant was retained (supernatant fraction). Meanwhile, the pellet fraction was resuspended in a solution containing 20 % (v/v) glycerol, 10 mM Tricine (pH 7.5, adjusted with NaOH solution), 0.1 mM EDTA and 0.1 mM dithiothreitol (pellet fraction). The activity of ATPase was determined in both fractions and the results were reported as sums of the data from both fractions.

3.2.16.4 Extraction of metabolites and adenylates

The cellular metabolites as well as the adenine nucleotides were extracted using the acid extraction method described by Kubicek and Röhr (1978), which was modified from that of Ng *et al.*(1973), as follows :

The mycelium sample (prepared as in Section 3.2.16.1) was added to 8 % (v/v) $HClO_4$ at a ratio of 1 g (wet weight) per 10 ml acid solution. After pre-disruption by manual grinding, followed by shaking homogenization (Section 3.2.16.2), the homogenate was centrifuged at 12,000 rpm for 15 min. The pellet was reextracted with 6 % (v/v) $HClO_4$ and recentrifuged. The supernatant from all centrifugations was pooled and was neutralized to pH 6.0 to 7.0 with 3 M K_2CO_3 in 0.5 M triethylamine. The $KClO_4$ precipitate formed was removed by centrifugation at 12,000 rpm for 15 min. The final supernatant was used for the determination of metabolites and adenine nucleotides.

3.2.17 Measurement of protein

The soluble protein in the mycelial extract prepared for enzyme assays (Sections 3.2.16.2 and 3.2.16.3), was measured using the microassay method of Bio-Rad (Bio-Rad Laboratories, Hercules, CA, U.S.A.) which was based on the dye-binding method of Bradford (1976), as follows :

Diluted sample (800 µl) containing 10 to 100 µg protein was transferred into a test tube to which was added 200 µl of concentrated protein reagent (Bio-Rad, U.S.A.). The absorbance was measured against a reagent blank at 595 nm after standing for 5 min. The concentration of protein was calculated from a standard curve prepared with bovine serum albumin.

3.2.18 Assay of intracellular enzymes

All the enzyme assays were carried out at 25^{0}C by monitoring the changes in absorbance using a double-beam spectrophotometer (Hitachi U 2000, Hitachi Ltd., Tokyo, Japan). This spectrophotometer was equipped with a temperature control unit, and could continually record the absorbance. Triplicate assays were performed for each enzyme and the average value was reported.

Enzyme activity was expressed as specific activity (unit/mg protein). *One unit of enzyme* is defined as the amount of enzyme which catalyses the formation of 1 µmole of product per min under the assay conditions. The amount of product formed by the action of a certain enzyme was calculated from a standard curve of that product. The assays were carried out by monitoring changes in absorbance due to the catalytic activity of a particular enzyme. If not otherwise specified, the final volume of the reaction mixture was 3.0 ml and the monitoring time was 5 min. If necessary, the enzyme sample was diluted to give an absorbance change of 0.02 to 0.2 per min.

3.2.18.1 Hexokinase (E.C. 2.7.1.1)

Hexokinase activity was determined by measuring the rate of NADPH formation from NADP, according to the method of Bergmeyer (1974).

The reaction mixture contained in 2.61 ml : 50 mM triethanolamine buffer, pH 7.6, 1.0 ml; 100 mg/ml glucose (in buffer), 1.0 ml; 100 mM $MgCl_2$, 0.2 ml; 10 mg/ml NADP, 0.2 ml; 10 mg/ml ATP, 0.1 ml; 0.55 unit/ml glucose 6-phosphate dehydrogenase, 0.01 ml; and enzyme solution, 0.1 ml. The blank contained all the components except glucose. The reaction was started by adding the enzyme solution and was monitored for increased absorbance at 340 nm.

3.2.18.2 Aconitase (E.C. 4.2.1.3)

Aconitase activity was determined by measuring the rate of *cis*-aconitate formation from isocitrate, according to the method of La Nauze (1966).

The reaction mixture contained : 100 mM DL-isocitrate in 500 mM potassium phosphate buffer (pH 7.4), 0.4 ml; enzyme solution, 0.1 ml; and 50 mM phosphate buffer (pH 7.4), 2.5 ml. The blank contained all the components except isocitrate. The reaction was started by adding the enzyme solution and was monitored for increased absorbance at 240 nm.

3.2.18.3 NAD-specific isocitrate dehydrogenase (E.C. 1.1.1.41)

Activity of this NAD-specific enzyme was determined by measuring the rate of NADH formation from NAD, according to the method of La Nauze (1966).

The reaction mixture contained : 500 mM potassium phosphate buffer (pH 7.4), 0.2 ml; 5 mM NAD, 0.3 ml; 2.5 mM AMP, 0.3 ml; 100 mM $MgCl_2$, 0.1 ml; 300 mM KCN (freshly neutralized), 0.1 ml; 100 mM DL-isocitrate in 500 mM potassium phosphate buffer (pH 7.4), 0.1 ml; enzyme solution; 0.1 ml; and distilled water, 1.8 ml. The blank contained all the components except isocitrate. The reaction was started by adding the enzyme sample and was monitored for increased absorbance at 340 nm.

3.2.18.4 NADP-specific isocitrate dehydrogenase (E.C.1.1.1.42)

Activity of this NADP-specific enzyme was determined by measuring the rate of NADPH formation from NADP, according to the method of La Nauze (1966).

The reaction mixture contained : 500 mM potassium phosphate buffer (pH 7.4), 0.2 ml; 2.5 mM NADP, 0.3 ml; 100 mM $MgCl_2$, 0.1 ml; 300 mM KCN (freshly

neutralized), 0.1 ml; 100 mM DL-isocitrate in 500 mM potassium phosphate buffer (pH 7.4), 0.1 ml; enzyme solution, 0.1 ml; and distilled water 2.1 ml. The blank contained all the components except isocitrate. The reaction was started by adding the enzyme solution and was monitored for increased absorbance at 340 nm.

3.2.18.5 2-Oxoglutarate dehydrogenase (E.C.1.2.4.2)

Activity of 2-oxoglutarate dehydrogenase was determined by measuring the rate of NADH formation from NAD, according to the method of Reed and Mukherjee (1969).

The reaction mixture contained : 500 mM potassium phosphate buffer (pH 8.0), 0.3 ml; 10 mM $MgCl_2$, 0.3 ml; 10 mM NAD, 0.3 ml; 30 mM cysteine hydrochloride (neutralized), 0.3 ml; 20 mM thiamine pyrophosphate, 0.03 ml; 3 mM coenzyme A (freshly prepared), 0.06 ml; 100 mM 2-oxoglutarate, 0.03 ml; enzyme solution, 0.1 ml; and distilled water, 1.58 ml. The blank contained all the components except isocitrate. The reaction was started by adding coenzyme A and was monitored for increased absorbance at 340 nm.

3.2.18.6 Pyruvate carboxylase (E.C. 6.4.1.1)

Activity of pyruvate carboxylase was determined by measuring the rate of oxidation of NADH to NAD, according to the method of Feir and Suzuki (1969).

The reaction mixture contained : 50 mM tris hydrochloride buffer (pH 7.9), 1.5 ml; solution containing 5 mM sodium pyruvate, 5 mM sodium bicarbonate, 3 mM $MgCl_2$, 1.2 mM ATP, and 33 mM KCl, 1.0 ml; 0.01 mM NADH, 0.3 ml; malic dehydrogenase, 1 unit (0.1 ml); and enzyme solution, 0.1 ml. The blank contained all the components except pyruvate. The reaction was started by adding the enzyme solution and was monitored for decreased absorbance at 340 nm.

3.2.18.7 Isocitrate lyase (E.C. 4.1.3.1)

Activity of isocitrate lyase was determined by measuring the rate of glyoxylic acid phenylhydrazone formation, according to the method of Dixon and Kornberg (1959).

The reaction mixture contained : 500 mM potassium phosphate buffer (pH 6.85), 0.4 ml; 100 mM DL-isocitrate in 50 mM potassium phosphate buffer (pH 6.85), 0.5 ml; 100 mM $MgCl_2$, 0.1 ml; 100 mM phenylhydrazine hydrochloride, 0.02 ml; enzyme solution, 0.1 ml; and distilled water, 1.88 ml. The blank contained all the components except isocitrate. The reaction was started by adding the enzyme solution and was monitored for increased absorbance at 324 nm for 10 min.

3.2.18.8 ATP:citrate lyase (E.C. 4.1.3.8)

Activity of ATP:citrate lyase was determined by measuring the rate of oxidation of NADH to NAD, according to the method of Takeda *et al.*(1969) with small modifications.

The reaction mixture contained in 2.0 ml : 100 mM Tris buffer, pH 8.4, 0.8 ml; 200 mM $MgCl_2$, 0.1 ml; 200 mM potassium citrate, 0.2 ml; 200 mM mercaptoethanol, 0.1 ml; 100 mM ATP, 0.2 ml; 2 mM coenzyme A, 0.2 ml; 10 mM NADH, 0.04 ml; 20 units/ml malate dehydrogenase, 0.02 ml; enzyme solution, 0.1 ml; and distilled water, 0.24 ml. The blank contained all the components except ATP. The reaction was started by adding coenzyme A and was monitored for decreased absorbance at 340 nm.

3.2.18.9 Oxaloacetate hydrolase (E.C. 3.7.1.1)

Activity of oxaloacetate hydrolase was determined by measuring the rate of disappearance of oxaloacetate according to the method of Lenz *et al.* (1976).

The reaction mixture in 2.6 ml contained : 100 mM Tris buffer, pH 7.6, 1.0 ml; 20 mM oxaloacetic acid, 0.5 ml; 0.3 mM $MnCl_2$, 0.1 ml; and enzyme solution, 1.0 ml. The blank contained all the components except the enzyme solution. The decrease in absorbance at 255 nm was monitored for 10 min.

3.2.18.10 ATPase

ATPase was determined as total membrane activity, by measuring the rate of inorganic phosphate released from ATP, according to the method of Viegas and Sa-Correia (1991), with slight modifications.

The reaction mixture contained : enzyme sample, 2.5 ml and assay medium, 2.4 ml. The assay medium consisted of 50 mM 2-(*N*-morpholino) ethanesulfonic acid, pH adjusted to 5.5 to 6.5 with Tris; 5 mM sodium azide; 0.2 mM ammonium molybdate; 10 mM $MgSO_4$; 50 mM KCl; 0.1 mg/ml egg yolk lyolecithin (Serrano, 1983). The mixture was incubated at 30^0C for 5 min in a shaker bath. Then, the ATPase reaction was started by adding 0.1 ml of 0.1 M ATP (to a final concentration of 2 mM ATP). After incubating in the same condition for 15 min, the reaction was stopped by adding 0.5 ml of 10 % (w/v) trichloroacetic acid. The precipitate was removed by centrifugation at 12,000 rpm for 5 min and inorganic phosphate was measured in the supernatant fraction using the phosphomolybdate method of John (1970).

3.2.19 Measurement of intracellular metabolites

The mycelial extract containing cellular metabolites was prepared as previously described in Section 3.2.16.4. Determination of each metabolite was based on the enzymatic analysis methods described in Bergmeyer (1985) which measured the absorbance change caused by reaction of a given metabolite and the particular enzyme(s). Triplicate measurements were conducted for each metabolite and the mean value was reported. Concentration of each metabolite was calculated from the standard curve of the product of the respective enzymatic reaction(s).

3.2.19.1 Citrate

The concentration of citrate in an extract sample was determined by measuring the enzymatic disappearance of NADH, according to the method of Möllering (1985), with appropriate modifications.

The reaction mixture contained in 3.05 ml : 500 mM glycylglycine buffer (pH 7.8) containing 0.6 mM $ZnCl_2$, 1.00 ml; 10 mM NADH, 0.01 ml; distilled water, 2.0 ml; extract sample, 0.20 ml; a mixture of 600 units/ml malic dehydrogenase and 1,375 units/ml lactic dehydrogenase, 0.02 ml; 40 units/ml citrate lyase, 0.02 ml. The blank contained all the components except the extract sample. The reaction was started by adding the enzyme solutions. The decrease in absorbance at 340 nm was monitored for 10 min.

3.2.19.2 2-Oxoglutarate

The concentration of 2-oxoglutarate in an extract sample was determined by measuring the disappearance of NADH, according to the method of Burlina (1985).

The reaction mixture contained in 2.04 ml : extract sample, 2.00 ml; 8.5 mM NADH in 1 % (w/v) $NaHCO_3$ solution, 0.03 ml; 360 units/ml glutamate dehydrogenase, 0.01 ml. The blank contained all the components except the extract sample. The reaction was started by adding the enzyme solution. The decrease in absorbance at 340 nm was monitored for 12 min.

3.2.19.3 Oxaloacetate

The concentration of oxaloacetate in an extract sample was determined by measuring the enzymatic disappearance of NADH, according to the method of Rej (1985) with appropriate modifications.

The reaction mixture contained in 3.005 ml : extract sample, 2.00 ml; 6.0 mg NADH in 10.0 ml of 300 mM Tris buffer pH 7.8 containing 20 mM EDTA, 1.0 ml; 230 units/ml malic dehydrogenase containing 0.5 volume glycerol, 0.005 ml. The blank contained all the components except the extract sample. The reaction was started by adding the enzyme solution. The decrease in absorbance at 340 nm was monitored for 10 min.

3.2.19.4 Oxalate

The concentration of oxalate in an extract sample was determined by measuring the enzymatic formation of NADPH, according to the method of Heinz and Kohlbecker (1985) with appropriate modifications.

The reaction mixture contained in 1.84 ml : 8.58 mM succinate buffer pH 3.8 containing 4.29 mM EDTA, 2.35 M ethanol and 5,550 units/ml catalase, 0.45 ml; extract sample, 0.45 ml; 2.5 units/ml oxalate oxidase, 0.02 ml; 200 mM potassium diphosphate buffer containing 1.27 mM NADP, 0.9 ml; aldehyde dehydrogenase, 10 units/ml, 0.02 ml. The blank contained all the components except the extract sample. The reaction was started by adding the enzyme solutions. The increase in absorbance at 340 nm was monitored for 15 min.

3.2.20 Determination of adenylates

Adenylates were extracted from the mycelium using the acid extraction method described in Section 3.2.16.4, and their concentrations were determined based on the enzymatic analysis methods of Bergmeyer (1985). For each adenylate, triplicate assays were conducted and the mean of absorbance change was converted to concentration data using an appropriate calibration curve, as described for the case of metabolite determination.

3.2.20.1 Adenosine triphosphate (ATP)

The concentration of ATP was determined by measuring the rate of enzymatic formation of NADPH, according to the method of Trautschold *et al.* (1985).

The reaction mixture contained in 3.0 ml : 50 mM triethanolamine buffer (pH 7.5), 2.27 ml; 10 mM NADP, 0.1 ml; 100 mM $MgCl_2$, 0.2 ml; sample, 0.1 ml; 140 units/ml glucose-6-phosphate dehydrogenase, 0.01 ml; and 500 mM glucose solution, 0.3 ml. The blank contained all the components except the extract sample. The reaction was started by adding 0.02 ml of 280 units/ml hexokinase. The increase in absorbance at 340 nm was read after 15 min.

3.2.20.2 Adenosine diphosphate (ADP) and adenosine monophosphate (AMP)

The concentrations of both ADP and AMP were determined in a single assay system, by measuring the enzymatic oxidation of NADH to NAD, according to the method of Jaworek and Welsch (1985).

The reaction mixture contained in 2.21 ml : extract sample, 2.0 ml; 0.15 ml of a solution containing 14 mM phosphoenol pyruvate, 500 mM $MgSO_4$, and 1.8 M KCl; 2,750 units/ml lactate dehydrogenase, 0.02 ml. The blank contained all the components except the extract sample. The reaction for ADP was initiated by adding 0.02 ml of 2,000 units/ml pyruvate kinase and the decrease in absorbance at 340 nm was measured after 5 min. Further decrease in absorbance at 340 nm caused by reaction with AMP was started by adding 0.02 ml of 1,800 units/ml myokinase and was monitored for 15 min.

3.3 Discussion

3.3.1 Organisms

Of the two high citrate-yielding strains used in this study, *A.niger* Yang No.2 was originally isolated using a mutation technique, and was used for citric acid production from sugar cane bagasse and concentrated liquor of pineapple waste in semi-solid state fermentation (Usami and Fukutomi, 1977), and from soluble starch both in surface culture fermentation (Sakurai and Imai, 1991; Sakurai *et al.*,1991) and in shake-flask fermentation (Rugsaseel *et al.*, 1995). Recently, this strain has been reported to accumulate high yields of citric acid from sweet potato by the solid state process (Lu, 1995).

The other high-producer, *A.niger* MH 15-15, was an improved mutant of *A.niger* IMI 41847 (ATCC 9142). Its parent strain accumulated high yields of citric acid from various substrates, such as cotton waste (Kiel *et al.*, 1981) and molasses (Roukas and Alichanidis, 1991). The strain MH 15-15 showed enhanced citric acid yields from glucose (Hossain, 1984) and sucrose (Dawson, 1986), by the submerged fermentation process and was also found to accumulate high citric acid yields from sweet potato by the solid state process (Lu, 1995). Notably, it was discovered in the current study that this strain produced spores more profusely on Malt Extract agar than on the Beef Extract Sucrose medium used in those previous reports.

The two mutant strains of *A.niger*, SL-1 and SL-2, were selected on the basis of reduced citric acid yields. However, strain SL-1, which originated from strain Yang No.2, did not sporulate at 30^{0}C incubation but this property was recovered at 20^{0}C incubation, while strain SL-2 also had a reduced sporulation ability compared to its parent strain, MH 15-15.

3.3.2 Determination of extracellular oxalic acid

Before a decision was made on the selection of method for oxalic acid determination in the fermented medium sample, there had been some attempts with HPLC methods. The two reverse-phase columns, Protesil 300 octyl 25 and Polypore PPH-224 were found unsatisfactory because of superimposition of oxalic acid and glucose peaks. Thus, the titration method, based on the property of oxalic acid to reduce potassium permanganate was selected. This method was quite simple and provided a high recovery (95 to 98 %) without interference by citric acid.

3.3.3 Extraction of cellular enzymes from solid state cultures

The mycelium harvested from a fermentation dish was in the form of a single sheet, and thus differed from that of the pellet form of submerged cultures. Therefore, the method for mycelial disruption and enzyme extraction from the solid state mycelium had to be modified. Usually, shaking with materials such as glass beads is satisfactory for the mycelial pellets. However, in the case of the mycelial sheet, it was discovered that before undergoing this step the mycelium should be pre-disrupted by hand-grinding. This pre-homogenisation step improved the efficiency of the subsequent mechanical homogenization, as a preliminary study had shown greater amounts of protein released from the samples with pre-disruption than those without (data not shown).

3.3.4 Determination of ATPase activity

From the extraction procedure employed in this study (Section 3.2.16.3), it was not possible to fractionate the plasma membrane from the homogenate sample by centrifugation. This was evidenced by a preliminary study that considerable activity of ATPase remained in the pellet fraction (detail not shown). Thus, is was decided to measure ATPase activity as "total" membrane activity present in both the supernatant and the pellet fractions. The data were, therefore, reported as sum values

of the activity measured from both fractions.

3.3.5 Measurement of intracellular metabolites

It is certain that some extracellular metabolites of interest, particularly citrate and oxalate, can interfere with the measurement of their intracellular concentrations. This is because it was not possible to thoroughly wash all their extracellular amounts from the mycelium sample. For this reason, it was necessary to determine the interfering concentrations of citrate and oxalate. Thus, the mycelium sample was shaken vigorously in a volume of distilled water for about 5 min. This washing procedure was repeated twice and the combined supernatant from all the centrifugations was measured for citrate and oxalate. Thus, the citrate or oxalate content within the cells was obtained by subtracting this "background" concentration from the "total" concentration in the mycelial extract sample. In the case of 2-oxoglutarate and oxaloacetate measurement, the background concentrations were assumed not to be significant compared to their intracellular concentrations.

3.3.6 Reproducibility of experiments

All results were the average of at least duplicate determinations. Duplicates were accepted provided that they were within 10 % of each other.

CHAPTER 4

Isolation and Selection of Mutants

4.1 Introduction

It is well accepted that the ability of *A.niger* to produce and accumulate citric acid from carbohydrates varies among strains. To accumulate a considerable amount of citric acid, a strain of *A.niger* should be able to perform certain metabolic pathways under a given set of fermentation conditions. In addition to the genetic component of the producing organism, the environmental factors, particularly nutrition, also assert a significant influence on the fermentation performance.

Knowledge of the biochemistry of citric acid accumulation has become an area of interest along with advances in process technology. There are several means to achieve information on the mechanism of citric acid accumulation. A common technique is to measure changes in activity of certain enzymes involving citric acid synthesis of one or more strains of the organism cultured under a condition of citric acid accumulation, as employed in the studies of, for example, Ahmed, *et al.* (1972), Kubicek and Röhr (1978), Hossain (1983), and Dawson (1986). By another means, such as reported by Jernejc *et al.* (1991), the enzymatic difference of a given strain was compared when the fermentation was performed under conditions promoting and non-promoting citric acid accumulation. In other approaches, comparative studies have been conducted among various strains of *A.niger* which differ in ability to accumulate citric acid under a given set of fermentation conditions. For example, a wild-type has been compared with its modified strains, as reported by La Nauze (1966), Schreferl *et al.* (1986), and Wallrath *et al.* (1991).

Although much progress on this biochemical aspect has been achieved for submerged fermentation processes, very little work has been done for other types of process.

One exception is the work of Szczodrak (1981) that compared a high-yielding strain of *A.niger* under conditions of submerged and surface cultivation. However, no similar investigation for a solid state fermentation process has been reported in the literature.

To achieve the information on the biochemical aspects of *A.niger* during citric acid production in the solid state process, some appropriate mutants are required for further comparative studies. This chapter, thus, describes the isolation and selection of mutants from the representative strains of *A.niger* showing good yields of citric acid in solid state fermentation. The fundamental biology of both the selected mutants and their respective parents is described, accompanied by their preliminary solid state fermentation for citric acid production. The acquired information will serve as a general biological background of the fungal strains for the subsequent work on mechanism of citric acid accumulation in the solid state fermentation condition.

4.2 Results and Discussion

4.2.1 Mutation and selection of mutants

Two strains of *A.niger*, Yang No.2 and MH 15-15, represented the parent strains for induction of the desired mutants. These two strains were employed for the study because they had shown ability to accumulate high concentrations of citric acid from sweet potato in solid state fermentation (Lu, 1995).

To conduct the mutagenesis, spore suspension prepared from each parent culture was exposed to ultraviolet light as described in Section 3.2.6.1. Screening of acid-producing colonies was performed according to the procedure described in Section 3.2.6.2). The medium used for the screening work was the Malt Extract (ME) agar supplemented with glucose, calcium carbonate and bile salts (Section 3.1.1). The colonies developed on this medium after incubation at 30^{0}C for 5 days were compact and discrete due to the growth retarding effect of bile salts, as illustrated in Figure 4.1. The presence of bile salts, thus, assists in the screening performance by reducing

merging of the fungal colonies.

Figure 4.1 Formation of compact colonies by *A.niger* Yang No.2 on the medium used for screening of acid-producing mutants

Production of acid by a fungal colony on this screening medium was visualized by a clear zone, developed from calcium carbonate dissolution by the diffused acid. To determine the ability to produce acid of each colony, it was subcultured onto the "selection" medium (the screening medium without bile salts) and the ratio of the diameter of the clear zone and that of the colony, or "acid unitage" (James *et al.*, 1956), was estimated, as described in Section 3.2.6.3.

The indication of acidity using calcium carbonate as employed in this study is undoubtedly a practical method but suffers from the disadvantage of not being specific for citric acid production. The same is true of acid/base indicators which detect acidity by a change in colour of the medium, for example, indication with bromcresol green as used by James *et al.* (1956). Röhr *et al.* (1979) improved the specificity of citric acid indication by using paradimethylaminobenzaldehyde which reacts specifically with citric acid, causing a visible change of the medium.

Nevertheless, despite its poor specificity for citric acid, calcium carbonate offers a benefit besides its acid indication. It was discovered that some mutated colonies of *A.niger* produced a white, cloudy precipitate on the selection medium. This substance was later shown to be the insoluble calcium salt of oxalic acid formed from the

reaction between oxalic acid produced by the fungus and calcium ions present in the medium.

4.2.2 Selection of mutant from *A.niger* Yang No.2

The first objective for the mutagenesis of *A.niger* Yang No.2 was to select for a mutant with reduced or lost sporulation ability under normal cultivation conditions. This idea originated from the knowledge that sporulation, or conidiation, in filamentous fungi, such as *A. niger*, is triggered by particular environmental changes in which certain cellular activities are expressed in favour of this developmental process (Smith *et al.*, 1977). It was thus proposed that repression of sporulation in a citric acid-accumulating *A.niger* would be beneficial to its acid productivity and thus some superior mutants should be procured. Based on this hypothesis, mutants with marked reduction in sporulation, i.e. sporogenous or conidial mutants, were sought among the survivors from the mutagenesis of this parental strain (Section 3.2.6.2). However, since spores would be needed as inoculum for the fermentation work, these mutants should lose their sporulation ability only temporarily, i.e. they would be conditional mutants.

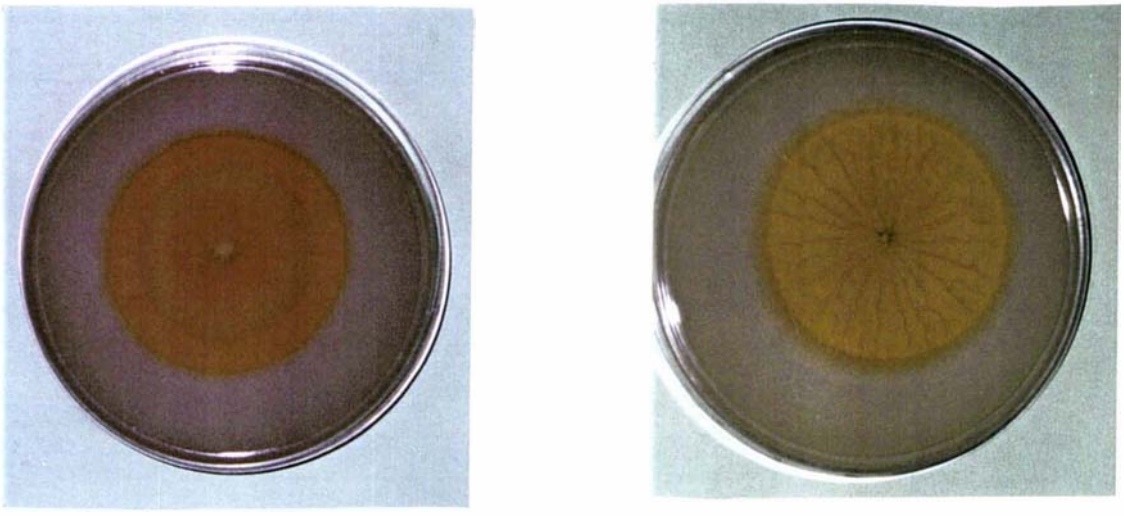
The use of temperature-shift as a trigger to induce sporulation in *A.niger* was first reported by Glenn (1986). This author found that by changing the incubation temperature from the normal (37^0C) to the lower (30^0C), certain asporogenous mutant strains of *A.niger* could regain their sporulation abilities. In the current study, such a "permissive" temperature was set to be 20^0C in which some sporulation-defective mutants of *A.niger* Yang No.2 were checked for the recovery of this property.

Of the total of about 45,000 colonies screened, 44 were found to have markedly altered sporulation at 30^0C incubation and were then tested for their sporulation ability at 20^0C. Among these strains, only 5 colonies were designated as temperature-sensitive mutants, i.e. these strains could produce spores abundantly at 20^0C, but not or very little at the normal fermentation temperature of 30^0C. These potentially useful fungal isolates were subsequently rechecked for their acid-producing ability, by

determining the acid unitage on the selection medium (Section 3.2.6.3) after incubation at 30^{0}C for 4 days. Thus, it was found that all of these five temperature-induced sporulation mutants produced *less* acid than did their parent, strain Yang No.2. The acid unitage obtained from these strains varied from 0.12 to 0.51 (detail not given), whereas that of strain Yang No.2 was 0.75. The mutant designated as strain SL-1, with an acid unitage of 0.43, was finally selected because it sporulated well at 20^{0}C. Additionally, it was observed that this selected mutant strain produced oxalic acid, as observed from the formation of the white cloudy halo around the colony on the selection medium.

4.2.3 Selection of mutant from *A.niger* MH 15-15

Since it was clear from the above results that the proposed relationship between asporogenesis and enhanced citric acid accumulation was unlikely, mutants of *A.niger* MH 15-15 were selected directly using the criterion of reduced acid production. Primary screening from about 27,000 colonies of the survivors of strain MH 15-15 after the UV treatment yielded 15 isolates with clearly reduced acid production on the screening medium. When confirming on the selection medium, the acid unitage of these colonies varied in the range of 0.21 to 0.71 (detail not given), compared with the value of 0.79 of strain MH 15-15. Subsequently, mutant SL-2, with an acid unitage of 0.56, was selected since the remainder of the isolates had much reduced sporulation abilities. It was also observed during the mutant selection programme that a large halo of white precipitate was formed by this strain of mutant. Figure 4.2 illustrates the formation of white precipitate by mutant SL-2, in comparison with the clear zone around the colony of strain MH 15-15.



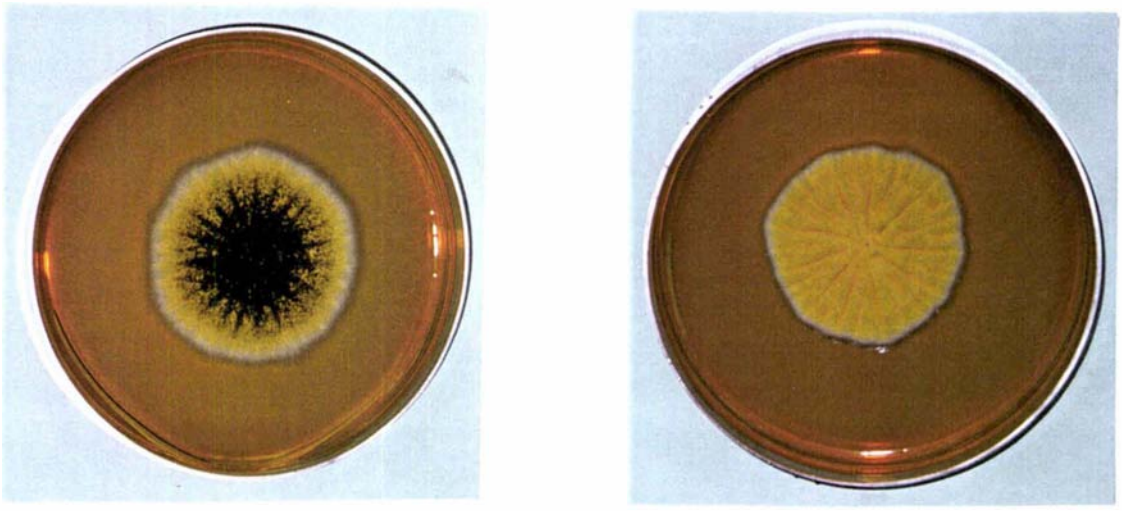
a. b.

Figure 4.2 Formation of clear zone and white precipitate on the selection medium after incubation at 30°C for 4 days
a. clear zone with white precipitate of *A.niger* SL-2
b. clear zone of *A.niger* MH 15-15

Although it was later discovered that mutant SL-1 produced more oxalic acid in the solid state fermentation than did mutant SL-2 (Chapter 5, Figure 5.8 and 5.14), this was not the case on the selection medium since mutant SL-2 appeared to produce more of this by-product, as observed from the formation of a larger area of the white precipitate. It is, therefore, suggested that nutritional properties exert some influence on this ability of the two mutant strains.

4.2.4 Cultural characteristics on agar medium

The growth morphology of each fungal strain was studied on ME agar by observing the colony after incubation at 30°C for 4 days, and, in addition, at 20°C for 4 days for Yang No.2 and SL-1. It was found that both *A.niger* Yang No.2 and mutant SL-1 developed yellow colonies, but with some differences. The aerial hyphae of the mutant were aberrantly short and were not developed into spore heads. In contrast, strain Yang No.2 produced an abundance of aerial hyphae bearing black spores. These observations are displayed in Figure 4.3.

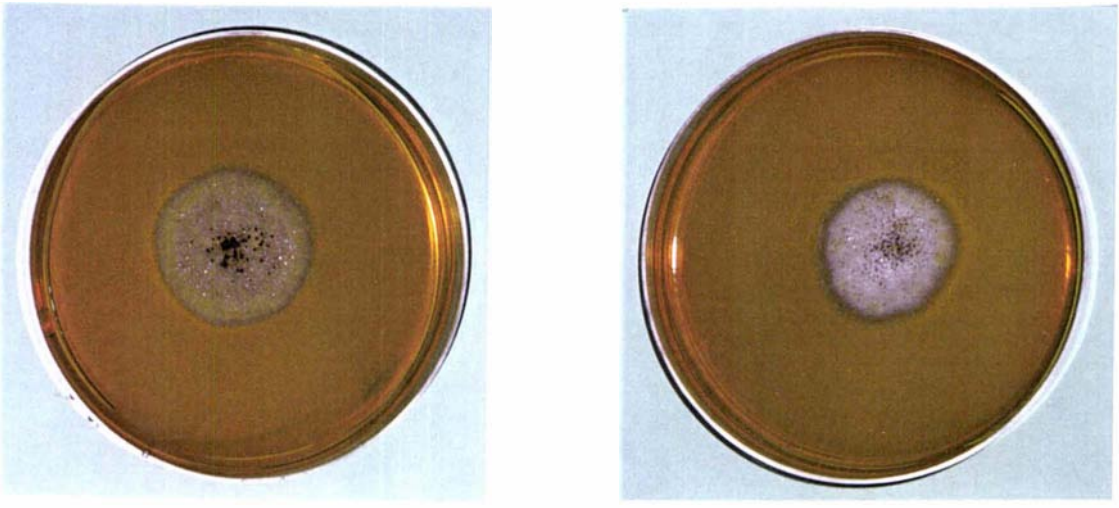


a. b.

Figure 4.3 Growth morphology of *A.niger* Yang No.2 and *A.niger* SL-1 on ME agar after incubation at 30°C for 4 days
a. strain Yang No.2
b. strain SL-1

It was also observed that at 20°C incubation, which is the permissive temperature for sporulation of mutant SL-1, growth of both Yang No.2 and SL-1 was slower than at the normal incubation temperature of 30°C. Spore production was observed in both strains, though delayed and less massive than observed at 30°C (figures not shown).

In the case of the growth morphology on ME agar of *A.niger* MH 15-15 and its selected mutant, strain SL-2, the colonies after 4 days incubation at 30^{0}C were similar, i.e. light yellow in colour with moderate sporulation. In addition, the spores produced by these two fungal strains were dark-brown but were less abundant in the mutant than in the parent, as displayed in Figure 4.4.



a. b.

Figure 4.4 Growth morphology of *A.niger* MH 15-15 and *A.niger* SL-2 on ME agar after incubation at 30^{0}C for 4 days
a. strain MH 15-15
b. strain SL-2

4.2.5 Preliminary solid state fermentation

The aim of this experiment was to ascertain citric acid production by the four strains of *A.niger* used for this study in a solid state fermentation condition. The cultivation was conducted in petri-dishes, with sweet potato as the solid substrate (Section 3.2.4 and 3.2.5). After fermentation at 30^{0}C for 5 days, the gross morphology of each fungal strain was recorded. The fermented material was then analyzed for citric acid, biomass, and residual starch, and the yields based on utilized starch were determined. The results are summarised in Table 4.1 along with the growth morphology of each fungal strain. In the case of strains Yang No.2 and SL-1, another set of solid state cultures was prepared but the fermentation was performed at 20^{0}C, rather than at 30^0 C, for a period of 5 days.

Table 4.1 Yields of citric acid and biomass and morphology of *A.niger* strains in the preliminary solid state fermentation at 30^{0}C for 5 days.

Strain	Yield (% w/w)		Morphology
	Citric acid	Biomass	
Yang No.2	38.8	23.6	A thick mycelial sheet with massive amounts of black conidia
SL-1	17.4	32.5	A thick mycelial sheet with very short aerial hypha. No spore production was observed.
MH 15-15	54.7	32.2	A thick mycelial sheet with white fluffy hyphae. A few dark-brown spores were produced.
SL-2	33.7	32.1	A thick mycelial sheet with white fluffy hyphae. Very few dark-brown spores were produced.

The data shown in Table 4.1 confirmed the reduction of citric acid yields of the two selected mutants, as compared to their respective parent strains. As shown in Table 4.1, mutant SL-1 accumulated only 17.4 % yield of citric acid in this solid state condition compared to a value of 38.8 % citric acid yield of its parent, Yang No.2. Similarly, the yield of citric acid at 33.7 % of mutant SL-2 was markedly lower than the value of 54.7 % yield of its parent, MH 15-15. Growth yield under the solid state fermentation condition of mutant SL-1 was slightly higher than that of parent Yang No.2 while this was generally equal for mutant SL-2 and parent MH 15-15. In

addition, the growth morphology of each fungal strain in this solid state fermentation closely resembled its colonial growth on the agar medium. Moreover, spore production was still not observed in mutant SL-1. From the results, it can be stated that mutant SL-1 exhibited a strong modification from its parent strain. In contrast, the morphological variation was only slight in the case of mutant SL-2 and parent MH 15-15.

It was observed that at 20^0C incubation, growth of Yang No.2 and SL-1 in the solid state condition was slower than at 30^0C incubation but sporulation was expressed in both strains. After fermentation for 5 days, yields of citric acid and biomass from the former were 20.2 % and 14.4 %, respectively, whereas those of the latter were 8.8 % % and 16.4 %, respectively. Therefore, mutant SL-1 was still able to accumulate less citric acid than was its parent at this incubation temperature.

4.3 Summary

From the UV treatment of the two high-citric acid-accumulating strains of *A.niger*, Yang No.2 and MH 15-15, the acid-alteration mutants were sought on an agar medium containing glucose and calcium carbonate. Two mutants markedly reduced in this ability were selected, one from each of the parent strains. Mutant SL-1, originating from Yang No.2, was characterized by its temperature-dependent sporulation whereas mutant SL-2, originating from MH 15-15, displayed no distinctively different growth morphology from its parent. In addition, the production of oxalic acid by these selected mutants was indicated. The growth and acid-accumulation characteristics of each organism in the solid state condition supported the agar plate results, and ensured the reliability of the strains for the following solid state fermentation work.

CHAPTER 5

Production of Citric Acid Using *A.niger* in Solid State Fermentation

5.1 Introduction

The first intensive work on citric acid production by the fungus using the solid state process was that of Lu (1995). A medium prepared from sweet potato (kumara, *Ipomoea batatus*) was used as the solid substrate for the process which employed *A.niger* Yang No.2 as the producing organism. Initially the fermentation was conducted in flasks, after which the process was scaled up in various types of reactor.

Although representing a systematic investigation, this report provided little detail on the biological aspects of the process. As emphasized earlier, the interaction between the fungus and the solid substrate particles is very complicated. This leads to difficulty in obtaining pure active fungal biomass from the fermented sample and may have contributed to the lack of a biochemical study of the process. Although Lu (1995) was able to determine growth of the organism by measuring the mycelial dry weight, the data were achieved indirectly and the procedure was inapplicable for obtaining viable biomass. In the present study, petri-dishes, rather than flasks, were employed as the reactor for performing the fermentation in which the solid substrate was arranged as a layer resembling an agar medium. In this manner, the fungus grew covering the surface of the substrate layer and formed a single piece of mycelial sheet which could subsequently be easily removed for the biochemical assays.

This chapter will describe the solid state fermentation for citric acid production using the two high-citric acid-accumulating strains of *A.niger*, Yang No.2 and MH 15-15, and the two low-accumulating mutants, SL-1 and SL-2, which had been selected through the mutation programme reported in Chapter 4. All the fermentations were performed in petri-dishes with sweet potato being used as the solid substrate. The

progress of the fermentation, the kinetic parameters, and the growth morphology of each fungal strain will be described. Furthermore, a comparison between the petri-dish and the flask systems for the fermentation using *A.niger* Yang No.2 will also be made. Thus, this investigation provided necessary background knowledge of all the tested strains for further biochemical work.

5.2 Physical Properties of The Solid Substrate

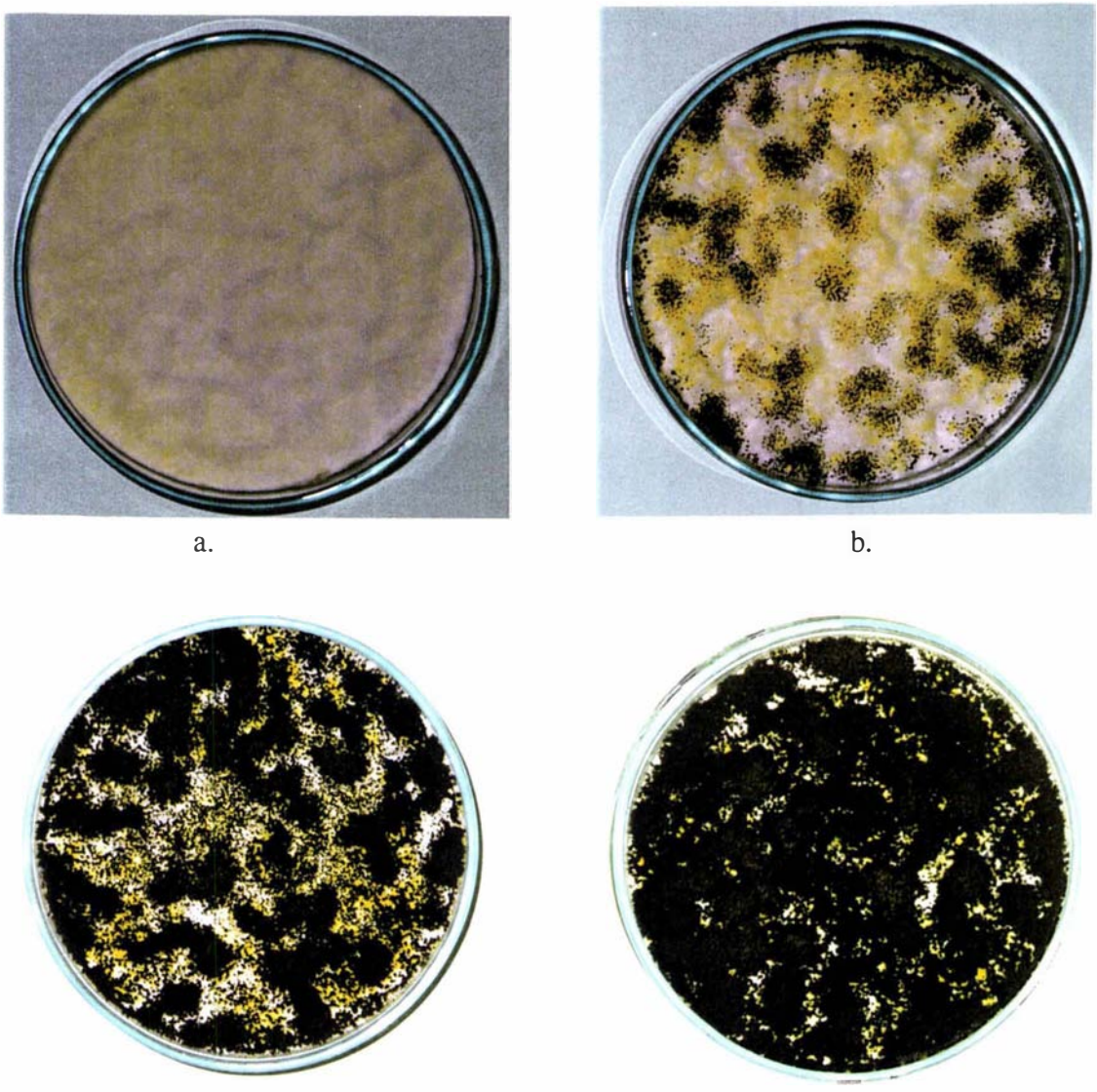
The solid substrate used for the fermentation was prepared from sweet potato, as described in Section 3.2.4. It was packed in a petri-dish as a layer of about 0.4 cm depth. In this manner, the upper surface was the only side exposed directly to the atmosphere. The surface area of the substrate layer was approximately equal to the circular area of the dish (diameter of 9.0 cm), which was 63.6 cm^2. The density of spores inoculated on this surface was about 790 spores/cm^2, given that 50,000 inoculated spores were uniformly distributed over the surface.

Since this substrate was prepared from fresh tubers of sweet potato, it would normally vary in water content and nutrient composition. Thus, to diminish this problem and to ensure the reproducibility of the fermentation the substrate was prepared in a large quantity, sufficient for the entire experiment. Before conducting any fermentation, the major physical properties of the substrate were usually recorded. It was found that the substrate usually contained an initial moisture content of about 70 to 72 % (w/w). The initial starch content varied in the range of 200 to 230 g/kg substrate on wet weight basis, as determined by the enzymatic method (Section 3.2.10), with a small amount of free glucose, at 6.0 to 6.4 g/kg wet weight of the substrate. The pH before starting the fermentation, measured after diluting 40 g of the substrate with 150 ml of water, was between 6.0 to 6.4. These physical parameters of the substrate were in the optimum ranges for initiation of citric acid production, as recommended by Lu (1995).

5.3 Growth Morphology on Solid Substrate in Petri-dishes

The morphological development of each fungal strain during the progress of the fermentation were observed over a period of 10 days. The parameters examined were intensity of growth, pigmentation and sporulation.

Figures 5.1a to 5.1d display the morphology of *A.niger* Yang No.2 during the course of the fermentation. Spore germination was observed after about 18 h incubation, followed by a rapid surface extension of the fungal hyphae creating patches of thin mycelium after about 24 h. By 48 h incubation, the entire surface of the substrate was covered with yellow mycelium and conidiophore development could be observed (Figure 5.1a). Upon further incubation, the mycelial sheet became thicker with patches of black spores expanding with the incubation time (Figures 5.1b,c). By day 8, the entire surface of the thick mycelium sheet was covered with black spores (Figure 5.1d).



a. b.

c. d.

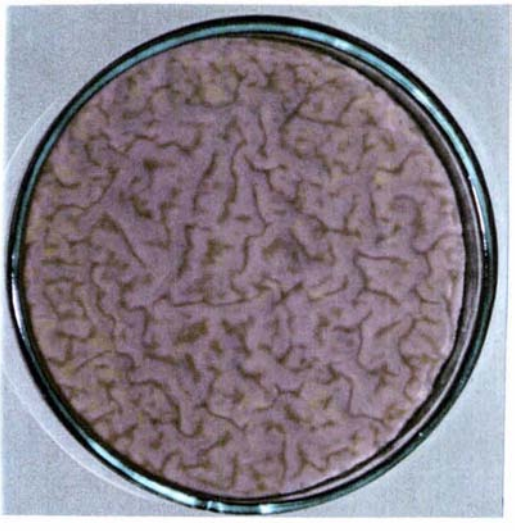
Figure 5.1 Growth morphology of *A.niger* Yang No.2 during solid state fermentation for citric acid production

a. day 2 b. day 4
c. day 6 d. day 8

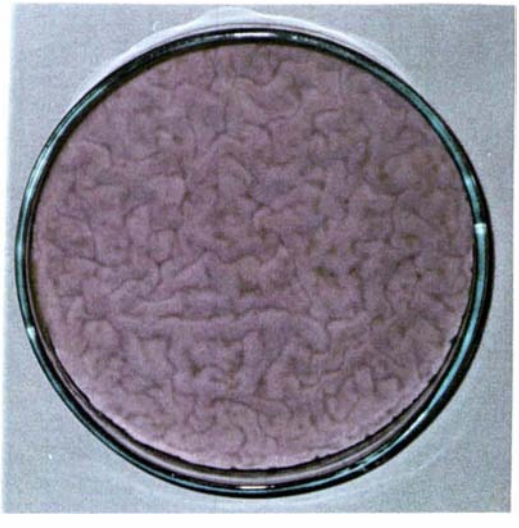
For the morphological appearance of strain SL-1, there were some variations from that of its parent, strain Yang No.2. In the early phase of growth, the mutant developed a yellow mycelial sheet over the substrate layer. Upon further incubation, the mycelium became thicker, however, no sporulation was observed. By the end of the incubation, this mutant strain had developed a thick, cream-coloured mycelial sheet, with very short aerial hyphae, indicating incomplete development into spores. These morphological descriptions are illustrated in Figures 5.2a to 5.2d.

a.

b.



c.

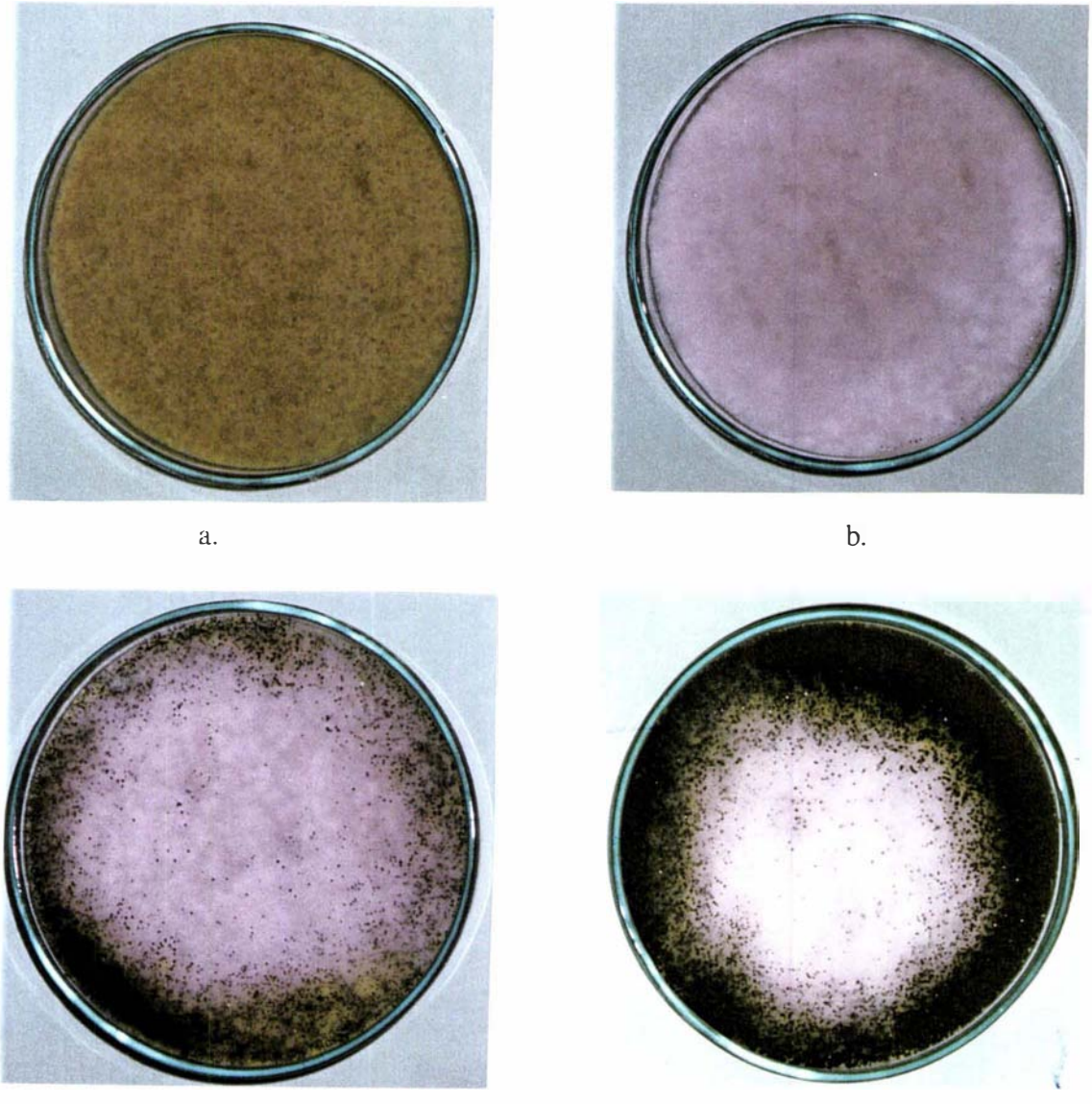


d.

Figure 5.2 Growth morphology of *A.niger* SL-1 during the solid state fermentation for citric acid production

a. day 2 b. day 4
c. day 6 d. day 8

For the morphological development of ***A.niger*** MH 15-15 during the solid state fermentation, a wrinkly, yellow-brown mycelial sheet was formed after the germination of spores. As the cultivation proceeded, there was an increase in the formation of white aerial hyphae creating a fluffy mycelium. Sporulation, which commenced on day 2, was dark-brown in colour and less profuse than that of strain Yang No.2 upon further incubation. These growth characteristics are shown in Figures 5.3a to 5.3d.



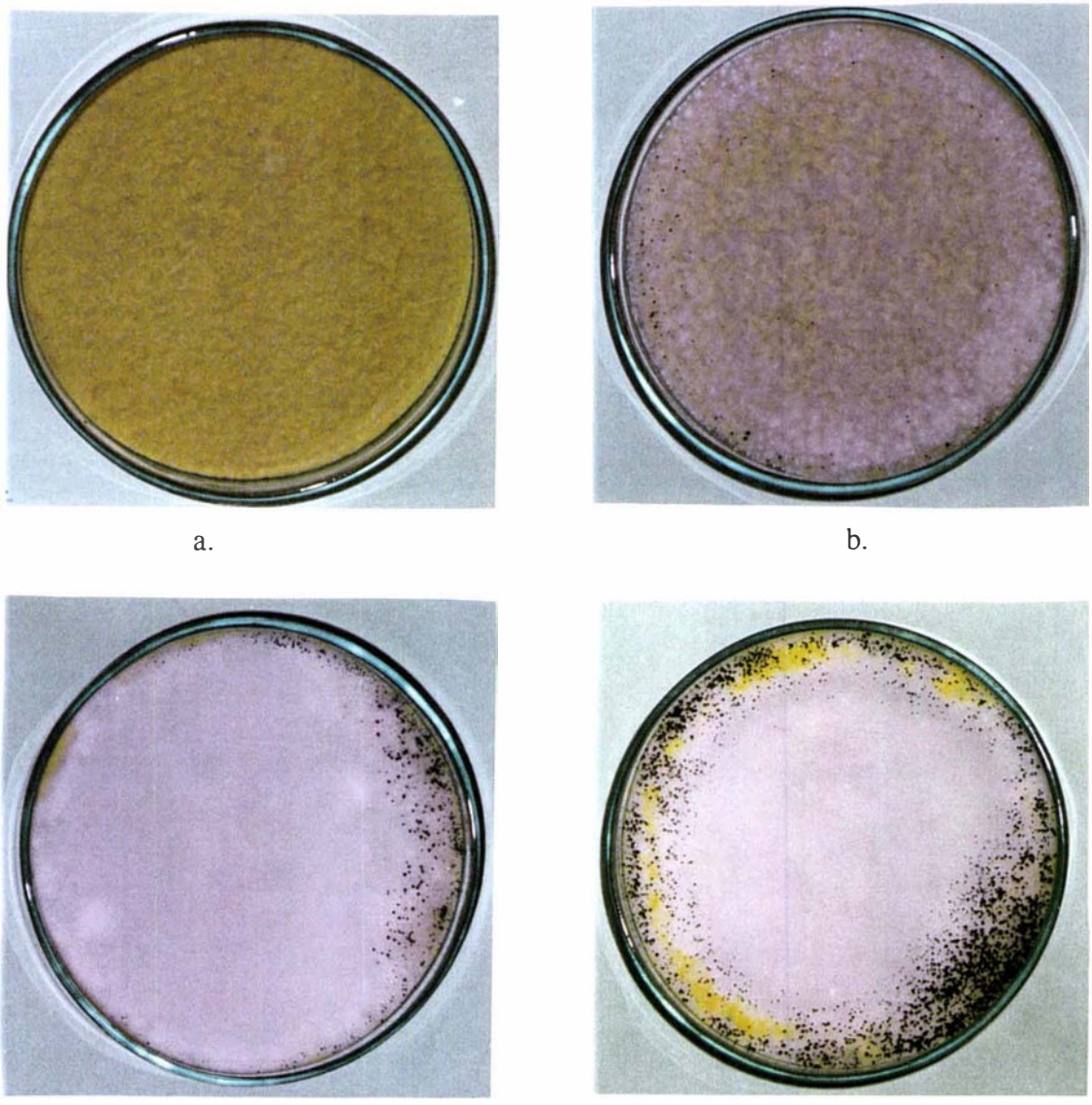
a. b.

c. d.

Figure 5.3 Growth morphology of ***A.niger*** MH 15-15 during solid state fermentation for citric acid production

a. day 2 b. day 4
c. day 6 d. day 8

In the case of the growth development of strain SL-2, there was no major difference to that of its parent, strain MH 15-15, except a small variation in colour of the mycelium. In addition, fewer spores were produced by the mutant even at later stage of growth. These growth characteristics are displayed in Figures 5.4a to 5.4d.



a. b.

c. d.

Figure 5.4 Growth morphology of *A.niger* SL-2 during solid state fermentation for citric acid production

a. day 2 b. day 4
c. day 6 d. day 8

The mycelial growth characteristics of the solid state cultures, as described above, resemble fungal growth on the surface of a liquid medium. Thus, it has been described than when growing on the static liquid surface, the fungus developed a thick wrinkled mycelial sheet without a submerged mycelium (Roehr, *et al.*, 1992). The mycelium formed on the solid substrate layer, however, comprised two main structures. The sub-aerial mycelium was formed when the hyphae grew towards the nutrient source and became thicker with prolonged incubation. The aerial hyphae, on the other hand, grew towards the surrounding atmosphere and, in some cases, developed further into spores.

The growth morphology which developed in the petri-dish, as reported in the present study, conferred some benefits to the methodology of biomass separation and determination. When using the flask fermentation system (Lu, 1995), the biomass was determined indirectly as the non-hydrolysed material remaining after the fermented sample had been treated with crude amylase enzymes (NOVO, Denmark). The biomass from the current study, however, could be measured directly by the gravimetric method (Section 3.2.8). This is because it was practical to recover the mycelial mat, in the form of broken pieces, from the residue of the centrifugation while the recovery of mycelium from the flask system was more difficult due to inconsistency of the fungal growth form. As a consequence, the petri-dish system allowed a more accurate measurement of this parameter. Another advantage of cultivating in petri-dish is that it granted a method to obtain a pure mycelium which was still metabolically active for the biochemical study since the fungus developed into a single piece of mycelium on the substrate surface thus allowing a simply detachment of the mycelium.

5.4 Fermentation Time Course and Kinetic Descriptions

The changes in the concentration of following parameters of the four *A.niger* strains were determined in the solid state fermentation: starch and free glucose, biomass, citric acid and oxalic acid. These parameters were monitored over a period of 10 days in which at each 48 h interval the whole fermented cultures were taken and treated

with the procedure described in Section 3.2.7. Moreover, the activity of glucose-releasing enzymes excreted by the fungal strains was also measured after the enzymes were extracted from the fermenting medium, as described in Section 3.2.11. It is noted that the unit of each parameter was based on the initial wet weight of the substrate, i.e. g/kg, or g glucose released/kg.h for the glucose-releasing enzyme activity. The data reported were averaged from two separate fermentation experiments.

5.4.1 Fermentation using *A.niger* Yang No.2 and *A.niger* SL-1

In this section, the progress of solid state fermentation using *A.niger* Yang No.2 and its mutant, strain SL-1, is reported.

Figure 5.5 presents the changes in starch, free glucose, biomass and citric acid of strain Yang No.2 over the period of 10 days. The data indicate a decrease in the starch content as the fermentation proceeded. This coincided with an increase in free glucose from its initial level, though this soon decreased, presumably due to utilization by the organism. Citric acid was accumulated concomitantly with biomass production and reached a peak value of 70.5 g/kg by day 8, after which it decreased slightly, possibly due to utilization by the fungus following depletion of the starch. Meanwhile, the biomass continued increasing to a maximum concentration of 52.0 g/kg at the completion of the process, in which about 93 % of the original starch had been utilized.

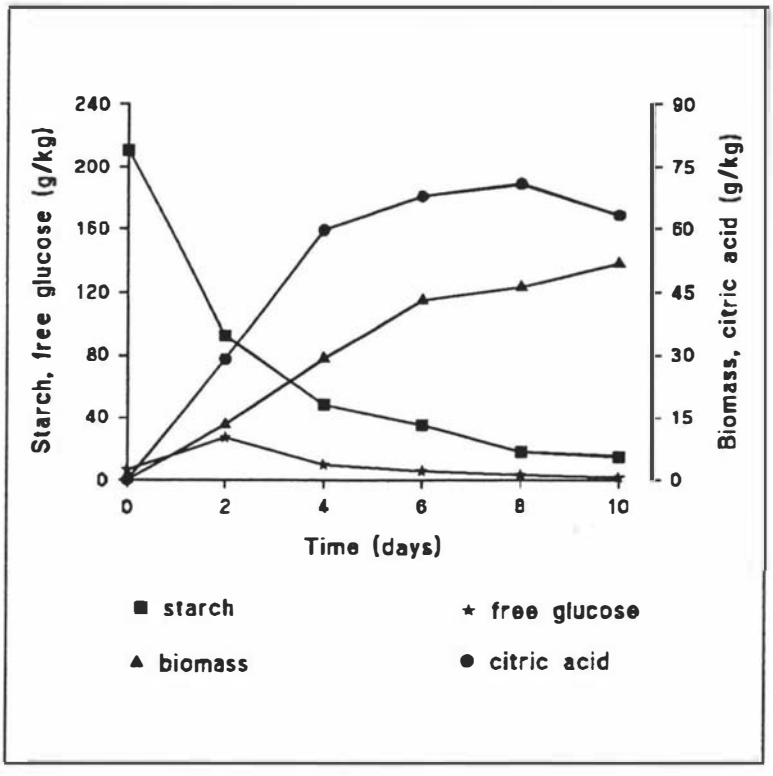


Figure 5.5 Time course of solid state fermentation for citric acid production using *A.niger* Yang No.2

Considering the work of Lu (1995), who used a flask rather than a petri-dish culture, a maximum citric acid concentration of 91.0 g/kg was obtained using this strain of *A.niger*, corresponding to a yield of citric acid of 65.0 % based on consumed starch. In comparison, the yield obtained in the present work was 40.0 %. However, by the end of the process a comparable amount of starch had been utilized under the two conditions. This observed difference is considered to be the result of different cultivation systems rather than properties of the solid substrate. Thus, the flask culture appeared to be more favourable to citric acid production than did the petri-dish culture, possibly because it allowed a more rapid starch utilization. Table 5.1 is a summary of some kinetic parameters obtained from these two fermentation systems.

Table 5.1 Some maximum kinetic parameters from solid state fermentation using *A.niger* Yang No.2 in the petri-dish (present work) and in the flask (Lu, 1995) systems.

Parameter	Present work		Lu (1995)	
	Value	Day	Value	Day
Starch utilization, %	93.0	10	95.0	10
Biomass, g/kg	52.0	10	37.0	10
Biomass, % yield	27.7	10	26.0	4
Citric acid, g/kg	70.5	8	91.0	8
Citric acid, % yield	40.0	6	65.0	3

The progress of fermentation using mutant SL-1 under the same conditions is shown in Figure 5.6. The results clearly reveal that much less citric acid was produced by this strain, as compared to its parent, Yang No.2. Thus, the maximum concentration achieved by day 4 was only 23.3 g/kg, about one-third of that of its parent. Thereafter, there was little change of the concentration until day 8, after which there was a slight drop in the concentration, suggesting its reabsorption by the fungus. The decrease in starch content appeared slower than that of the parent and by the end of the fermentation only 74.4 % of the initial content had been utilized. The maximum yield of citric acid based on the consumed starch was observed on day 2, at 20.9 %, which was about half that of its parent. However, a comparable concentration of biomass was produced by the mutant after 10 days incubation, at 53.8 g/kg.

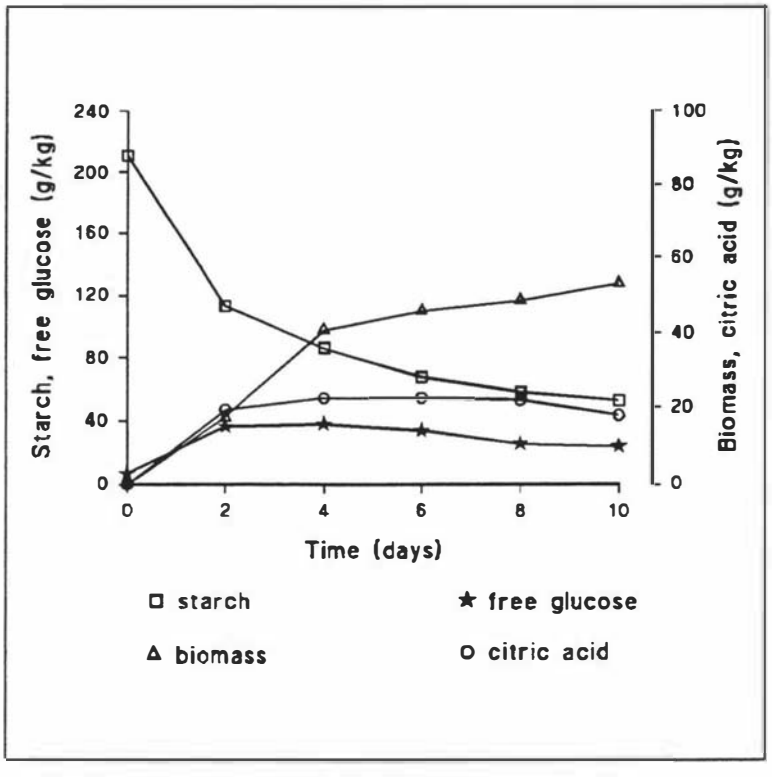


Figure 5.6 Time course of solid state fermentation for citric acid production using *A.niger* SL-1

An interesting feature noticed during the fermentation using mutant SL-1 was that there were relatively high levels of free glucose in the fermenting culture throughout the course of the process. These glucose molecules are the hydrolysis product of starch contained in the sweet potato medium, formed by the action of amylolytic enzymes known to be produced by *A.niger* and many other fungi (Blain, 1975). By the function of these extracellular amylolytic enzymes, particularly glucoamylase, the polymeric molecule of starch is hydrolysed into single glucose units. It was thus possible that the high free glucose levels detected in the cultures of mutant SL-1 resulted from high production of these amylases. However, the observed rate of starch consumption was actually lower in the mutant than in its parent, Yang No.2, so it is probable that the greater accumulation of free glucose by mutant SL-1 is a reflection of the glucose uptake rate rather than its formation rate. To confirm this, the total activity of glucose-releasing enzymes was measured in the fermenting medium of both strains Yang No.2 and SL-1. The assay method employed was based on the release of glucose after a given incubation period (Section 3.2.11). The data, averaged from two separate experiments, are expressed as g glucose released/kg.h and presented in Figure 5.7.

The measurement of this enzyme activity was conducted only on day 2 and day 4 of the time course. The results reveal high activity levels on day 2 for both of the fungal strains, with a slightly higher value observed for mutant SL-1. By day 4 there was a decrease in the activity, though it was still higher for the mutant. The activity variation between these two strains may possibly be considered as an experimental error in the assay procedure. In the method, a whole fermented culture was taken as the enzyme sample without removal of the active mycelium. The fungus could, therefore, utilize the released glucose molecules during the incubation, leading to unpredictable changes in the net concentrations of free glucose which were subsequently used for the activity calculation. It is therefore impossible to state that mutant SL-1 is significantly different from its parent, Yang No.2, in the production of amylolytic enzymes. Hence, other explanations must be considered for the decreased starch utilization and increased glucose accumulation of the mutant when

compared with its parent. The most likely hypothesis which can be proposed is that mutant SL-1 may consume the released glucose at a lower rate than does its parent. The investigation into this subject will be reported in a later chapter.

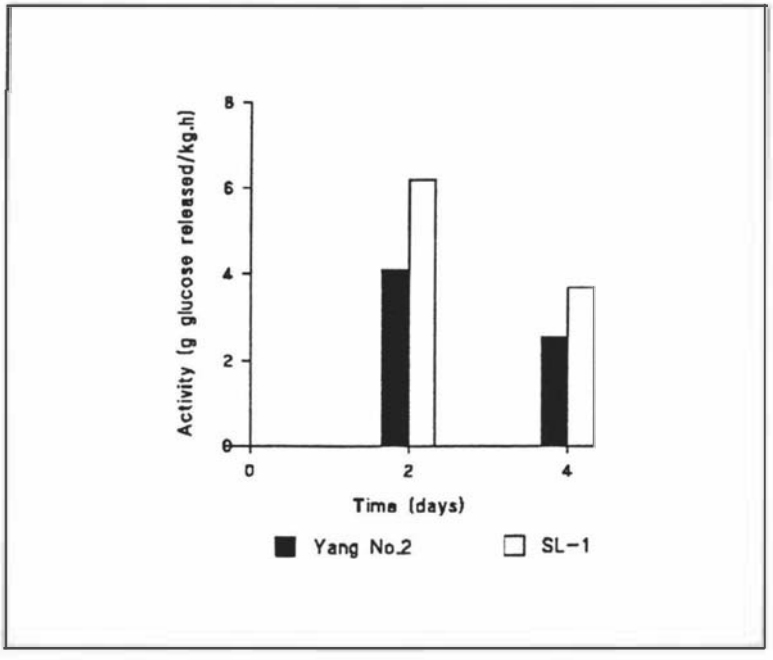


Figure 5.7 Glucose-releasing enzyme activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

Considering the production of glucose-releasing enzymes by *A.niger* Yang No.2 under different cultivation systems, the observed activities were markedly different. Thus, with the flask cultivation (Lu, 1995), the fungus produced a maximum activity of 14.3 g glucose released/kg.h, about twice higher than that with the current petri-dish cultivation. This difference indicates the influence of reactor types on the production of amylases by this fungus. In addition, the greater activities with the flask cultivation may result in a higher rate of starch hydrolysis, and thus may be an additional explanation for a more rapid utilization of starch by this fungal strain with the flask than with the petri-dish systems.

In Chapter 4 it was mentioned that mutants SL-1 and SL-2 appeared to produce oxalic acid as a by-product, as evidenced by the formation of the white precipitate on the medium containing calcium ions. This character has also been reported in other *A.niger* strains under certain conditions during citric acid production (Röhr, *et al.*, 1983). As a result, the production of this acid during the time course of citric acid production in the solid state process was determined. Oxalic acid was extracted from the fermented medium, accompanied by citric acid (Section 3.2.7), and its concentration was determined by a titration method (Section 3.2.14).

Figure 5.8 clearly reveals that oxalic acid was produced by both strains Yang No.2 and SL-1, but at a greater amount by the latter. Moreover, the production by the mutant was observed until day 6, as shown by the increased concentrations, while this ceased soon after day 2 in the case of its parent. The maximum concentration of this minor acid produced by the mutant was 26.4 g/kg, representing a yield of 19.8 %, whereas only 6.2 g/kg and 5.7 % maximum yield were observed for the parent strain.

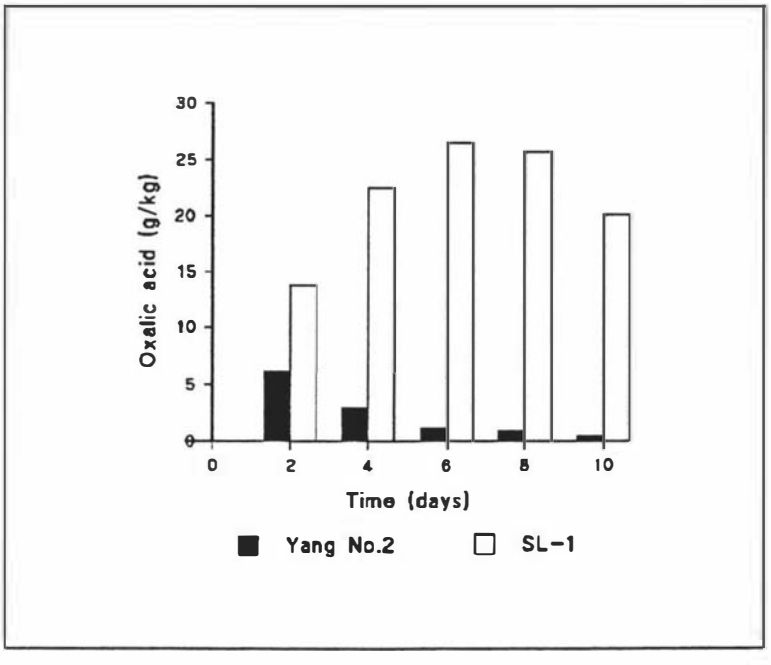


Figure 5.8 Production of oxalic acid by *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid-state process

The physiology of oxalic acid production by *A.niger* during citric acid accumulation is mainly unknown, although there have been a few reports on the favourable effect of neutral pH values in the medium on its biosynthesis (Cleland and Johnson, 1955; Kubicek and Röhr, 1986). In the present work, for mutant SL-1, the pH value of the culture never dropped to below pH 3.5 during the fermentation, whereas for the parent it decreased to below pH 2.5 within 48 h of incubation (details not given). For some reason, therefore, the situation in the mutant culture may become more favourable to oxalic acid synthesis. However, the relationship between this property of mutant SL-1 and reduced citric acid production is unknown at this stage.

From the concentration data of all parameters, i.e. starch consumption, biomass and citric acid production, the gravimetric and the specific rates over the fermentation time course were determined. For oxalic acid, it was possible to determine the production rate in mutant SL-1 over a period of 10 days cultivation, whereas this could be obtained only on day 2 in parent Yang No.2 due to the observed concentration reduction (day 2 onwards). The gravimetric rate is defined as the change in concentration per unit time, i.e. g/kg.h. For the specific rate, this change is based on the dry weight of biomass, i.e. g/g DB.h. A full description of these units has been provided by Lu (1995). It is remarked that in the case of the citric acid and oxalic acid rates, only the positive data are plotted on the graphs.

Figure 5.9 compares the gravimetric rates from the fermentation of *A.niger* Yang No.2 and mutant SL-1. The results reveal that the maximum rates of all the parameters were observed on day 2, after which they declined with the progress of the fermentation. The maximum gravimetric rate of citric acid production by the parent was 0.651 g/kg.h, whereas that of the mutant was only 0.146 g/kg.h. A significant difference was also observed for the starch consumption rates in that it was markedly lower in the mutant, particularly at the early growth phase. Thus, this maximum starch utilization rate for the parent was 1.310 g/kg.h, whereas that of the mutant was only 0.568 g/kg.h. However, the maximum biomass production rates for both strains were approximately equal.

The oxalic acid production rate of strain SL-1 was the highest on day 2, at 0.208 g/kg.h compared to the value of 0.036 g/kg.h of strain Yang No.2.

For the specific rates of the fermentation using strain Yang No.2 and SL-1, the most marked difference between the two strains for all the parameters was also observed on day 2. As shown in Figure 5.10, the maximum specific rate of citric acid production for the parent, based on the biomass present, was 0.048 g/g DB.h, whereas a much lower value was obtained for the mutant, at 0.008 g/g DB.h. In addition, the data indicate the impaired starch utilization by mutant SL-1 since the maximum specific rate observed from the mutant was about 2.6 times lower than that of its parent. For oxalic acid production, the specific rates resembled those of the gravimetric rates as the observed value on day 2 of the mutant was higher than that of its parent.

For a clearer comparison, the maximum values of these parameters obtained from the fermentation of *A.niger* Yang No.2 and SL-1 are summarised in Table 5.2 together with the days on which they were observed.

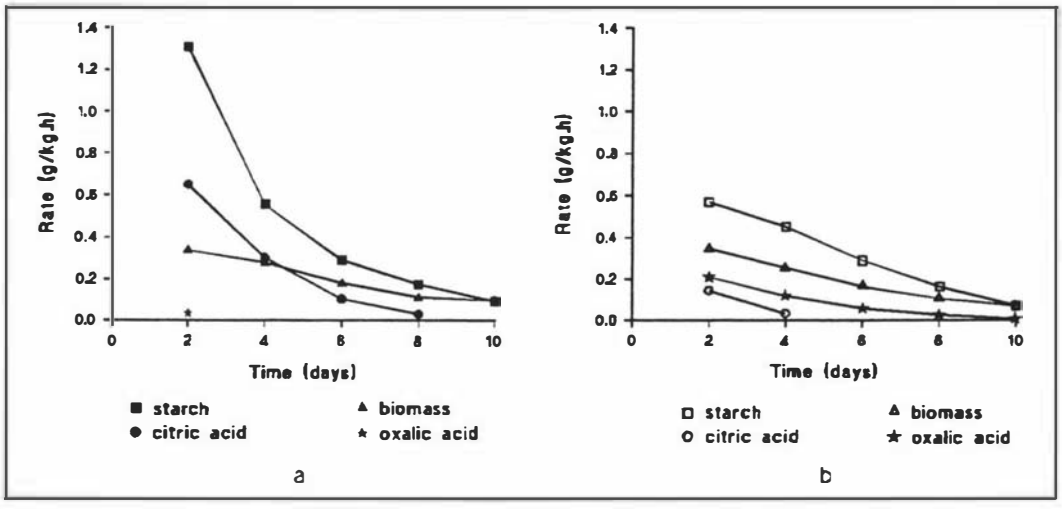


Figure 5.9 Gravimetric rates from the solid state fermentation for citric acid-production using *A.niger* Yang No.2 and *A.niger* SL-1
a. Yang No.2 b. SL-1

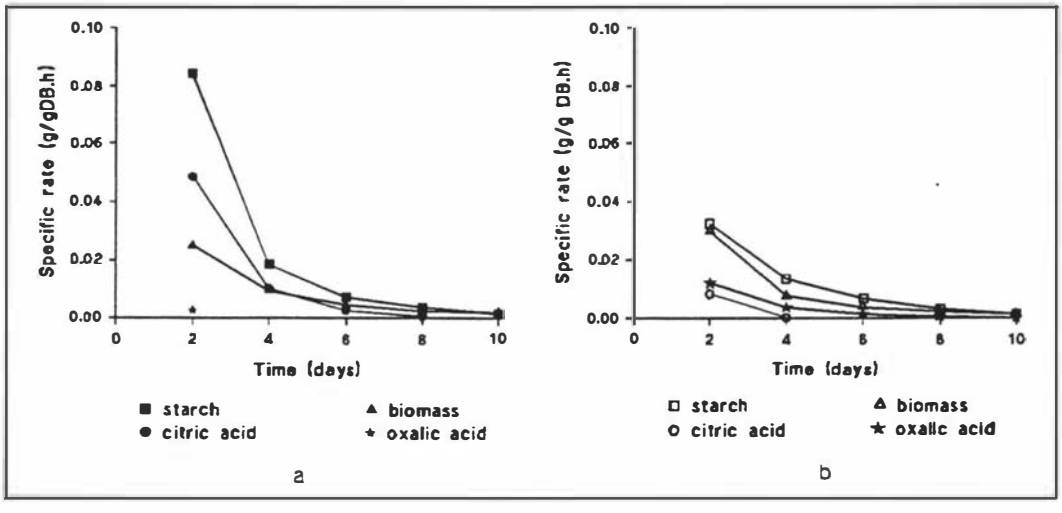


Figure 5.10 Specific rates from the solid state fermentation for citric acid production using *A.niger* Yang No.2 and *A.niger* SL-1
a. Yang No.2 b. SL-1

Table 5.2 Summary of maximum values of kinetic parameters during fermentation using *A.niger* Yang No.2 and *A.niger* SL-1 in the solid state process.

Parameters	*A.niger* Yang No.2		*A.niger* SL-1	
	Value	Day	Value	Day
Starch				
- utilization, %	93.0	10	74.4	10
- gravimetric rate, g/kg.h	1.310	2	0.568	2
- specific rate, g/g DB.h	0.084	2	0.032	2
Biomass				
- concentration, g/kg	52.0	10	53.8	10
- yield, %	27.7	10	35.9	10
- gravimetric rate, g/kg.h	0.338	2	0.347	2
- specific rate, g/g DB.h	0.025	2	0.020	2
Citric acid				
- concentration, g/kg	70.5	8	23.3	6
- yield, %	40.0	6	20.9	2
- gravimetric rate, g/kg.h	0.651	2	0.146	2
- specific rate, g/g DB.h	0.048	2	0.008	2
Oxalic acid				
- concentration, g/kg	6.2	2	26.4	6
- yield, %	5.7	2	19.8	2
- gravimetric rate, g/kg.h	0.036	2	0.208	2
- specific rate, g/g DB.h	0.003	2	0.013	2

5.4.2 Fermentation using *A.niger* MH 15-15 and *A.niger* SL-2

The progress of the fermentation using *A.niger* MH 15-15 and SL-2 was also studied during citric acid production by the solid state process. The parameters determined and their analytical methods were as those reported for Yang No.2 and SL-1 strains.

Figure 5.11 shows the progress of starch utilization, free glucose, biomass and citric acid production from the fermentation using strain MH 15-15. As another hyper-producing strain, *A.niger* MH 15-15 was able to accumulate considerable amounts of citric acid under these conditions. It was found that up to 104 g/kg of this acid were accumulated by day 8, a considerably higher value than that of strain Yang No.2. This represented a citric acid yield of 57.2 % based on starch used. The consumption of starch by this strain of parent was slightly faster than strain Yang No.2, with only about 1.5 % of the original content remaining at completion of the process. The free glucose concentration in the fermented medium increased by day 2 but soon dropped to low levels, indicating utilization by the fungus. Biomass production increased concomitantly with citric acid production and reached a maximum concentration of 57.0 g/kg by day 10.

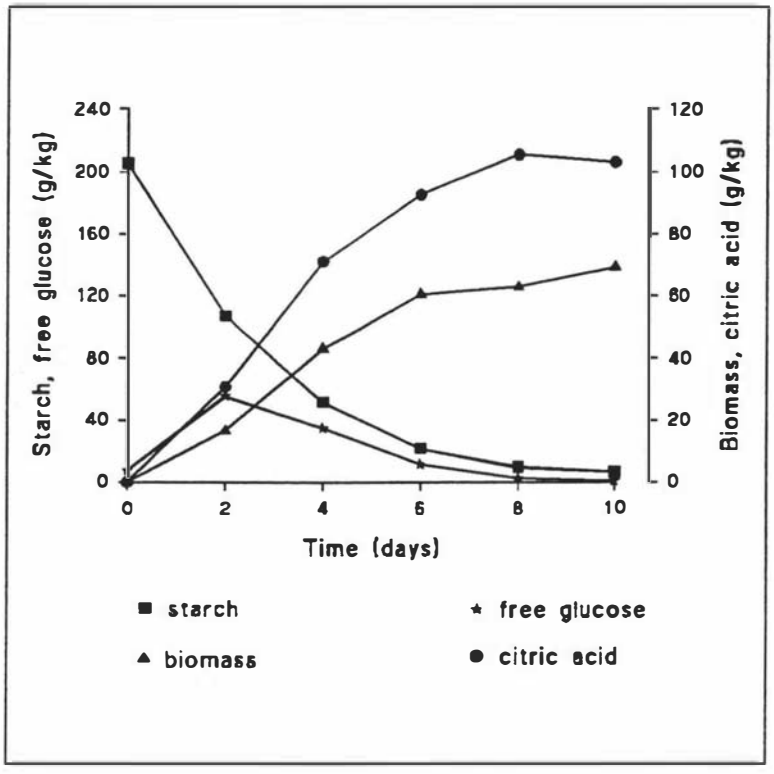


Figure 5.11 Time course of solid state fermentation for citric acid production using *A.niger* MH 15-15

For the fermentation using mutant SL-2, the graph presented in Figure 5.12 reveals that this mutant accumulated a maximum citric acid concentration of 51.0 g/kg by day 8, which was about half that of its parent, MH 15-15. Starch utilization, although slower during the early phase of growth, was almost complete, with only 4.7 % remaining after completion of the process. Biomass production by mutant SL-2 was comparable to that of its parent, with a concentration of 56.8 g/kg obtained by day 10. The maximum yield of citric acid based on starch used was only 30.7 %, markedly lower than that of its parent. The level of free glucose increased substantially from its original concentration, then remained high during the early growth phase, after which it declined through to the end of the fermentation.

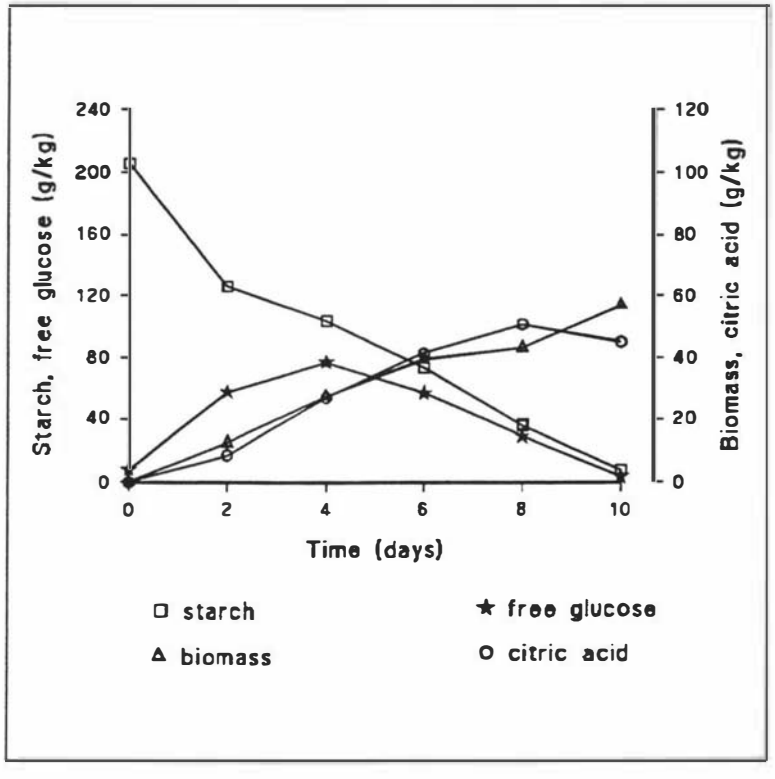


Figure 5.12 Time course solid state fermentation for citric acid production using *A.niger* SL-2

For the reason described earlier, the glucose-releasing enzymes excreted by strains MH 15-15 and SL-2 were also examined, by measuring the activity in the fermented samples. The data show that the enzymes were more active on day 2 than on day 4 (Figure 5.13). However, there was no major difference between the two strains, except that the level was slightly higher in the mutant strain. The methodology of the enzyme assay technique may be applied as an explanation for this. Thus, the accumulation of free glucose in the culture medium of mutant SL-2 may also be an indicator of an impaired glucose consumption rate, as hypothesised for mutant SL-1.

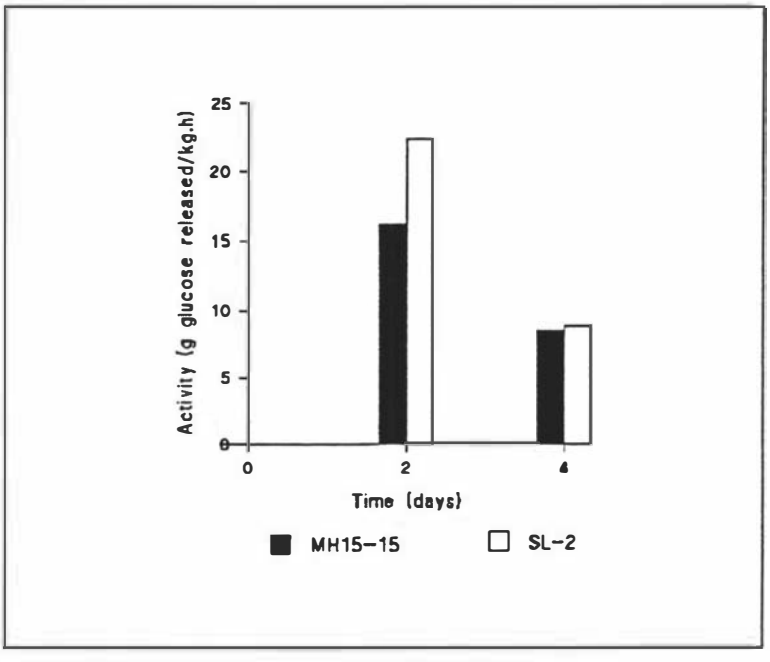


Figure 5.13 Glucose-releasing enzyme activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

It is noticeable that the levels of starch-hydrolysing enzymes observed with the cultures of MH 15-15 and SL-2 were about 2.3 to 3.3 times higher than those reported for Yang No.2 and SL-1. One possible explanation for this lies in the growth morphology in petri-dishes of these fungal strains. As described in Section 5.3, both MH 15-15 and SL-2 formed wrinkled mycelia on the solid substrate surface. This mycelial structure would increase its surface area where secretion of many enzymes,

including amylases, occurs. The secretion of exoenzymes and other proteins by *A.niger* takes place at the growing hyphal tips (Wosten *et al.*, 1991). Hence, the mycelial structures of MH 15-15 and SL-2 appeared to comprise a larger number of these hyphal structures, and so increased amylase excretion per unit of mycelium dry weight could be expected.

The concentration of oxalic acid was determined from both the cultures of MH 15-15 and SL-2. The results demonstrate that oxalic acid was produced by both organisms, but at a greater extent by the mutant than by the parent, as shown in Figure 5.14. Thus, an amount of 26.1 g/kg was detected from the day 2 culture of the mutant while only 16.6 g/kg was detected from the parent culture. As the fermentation proceeded, oxalic acid concentrations decreased in both cultures

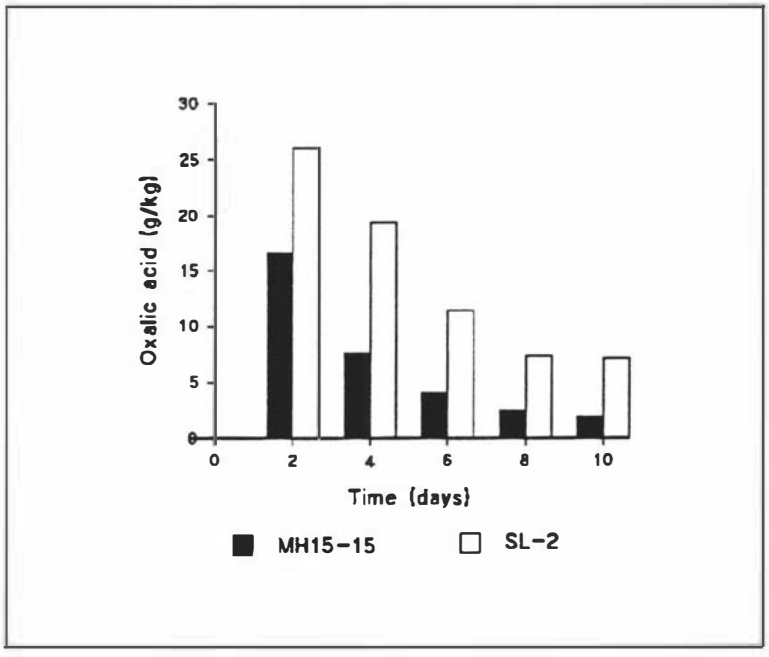


Figure 5.14 Production of oxalic acid by *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid-state process

The gravimetric rates for the fermentation using strains MH 15-15 and SL-2, calculated from the concentration data, are compared in Figure 5.15. In general, starch was utilized at a higher rate by the parent, particularly in the early phase of the incubation. The mutant, in comparison, consumed starch at lower, almost constant, rate throughout the course of the fermentation. The rates of biomass and citric acid production were similar to those of starch utilization, i.e. higher for the parent strain. Notably, the biomass production rate for the parent rapidly fells and became similar to that of the mutant by day 6, while the citric acid production rate was similar for both strains from day 4. The maximum rate of citric acid production for parent MH 15-15, which was observed on day 2, was 0.787 g/kg.h, while that of mutant SL-2 was about twice lower, at 0.387 g/kg.h. Oxalic acid production rate, which could be obtained only on day 2 for both strains, was higher in the mutant than in the parent.

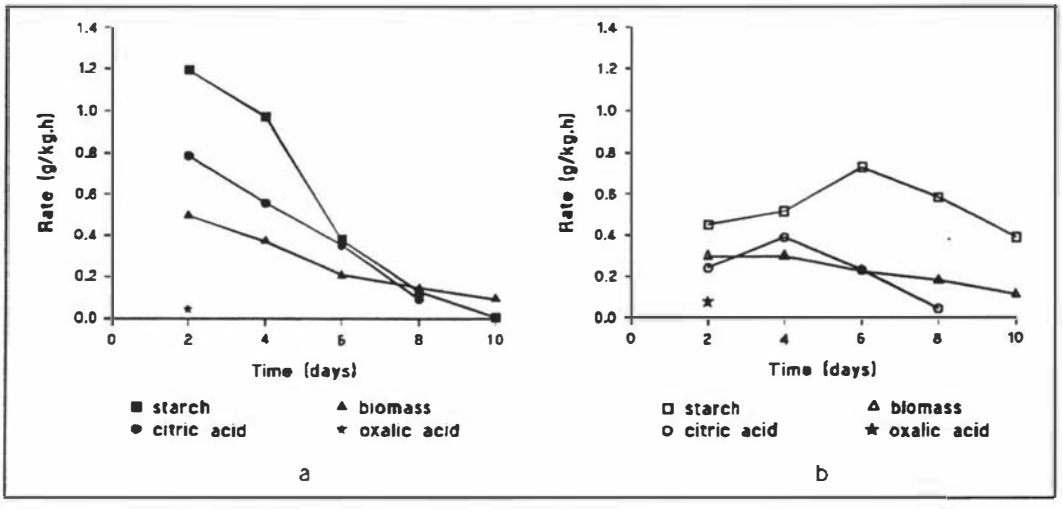


Figure 5.15 Gravimetric rates from the solid state fermentation for citric acid production using *A.niger* MH 15-15 and *A.niger* SL-2
a. MH 15-15 b. SL-2

The specific rates, based on the mycelial dry weight, from the fermentation of *A.niger* MH 15-15 and SL-2 reveal similarities to those of the gravimetric rates. As shown in Figure 5.16, all the measured parameters showed maximum values on day 2. Starch was utilized at higher specific rates by the parent than by the mutant. In addition, the maximum specific rate for citric acid production for this parent was 0.048 g/g DB.h, about 2.5 times higher than that of its mutant. However, all corresponding rates were similar in the two strains by day 4. For oxalic acid production, the specific rate was higher in the mutant than in the parent. These kinetic parameters are summarised in Table 5.3.

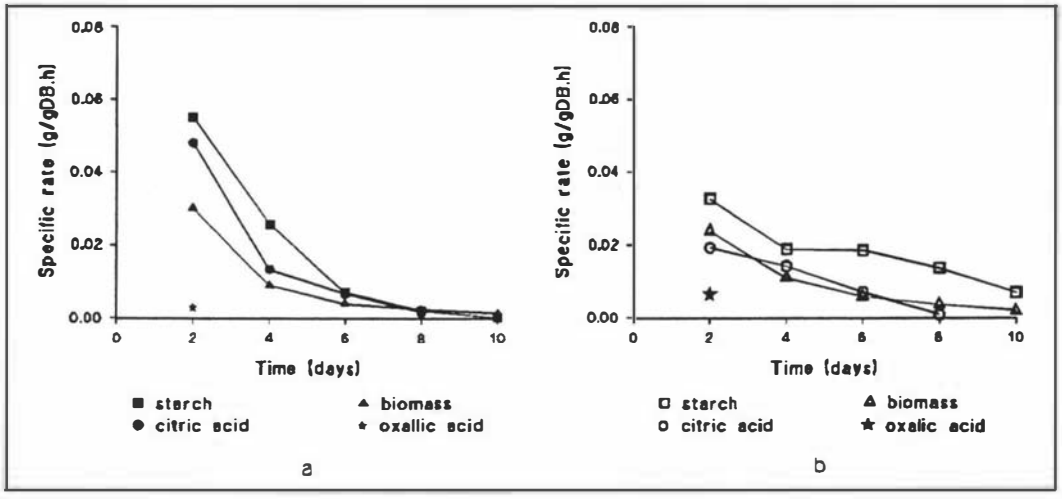


Figure 5.16 Specific rates from the solid state fermentation for citric acid production using *A.niger* MH 15-15 and *A.niger* SL-2
a. MH 15-15 b. SL-2

Table 5.3 Summary of maximum values of kinetic parameters during fermentation using *A.niger* MH 15-15 and *A.niger* SL-2 in the solid state process.

Parameters	*A.niger* MH 15-15		*A.niger* SL-2	
	Value	Day	Value	Day
Starch				
- utilization, %	98.5	10	95.3	10
- gravimetric rate, g/kg.h	1.071	2	0.729	4
- specific rate, g/g DB.h	0.055	2	0.036	2
Biomass				
- concentration, g/kg	57.0	10	56.8	10
- yield, %	35.6	10	32.1	6
- gravimetric rate, g/kg.h	0.495	2	0.295	2
- specific rate , g/g DB.h	0.030	2	0.024	2
Citric acid				
- concentration, g/kg	104.0	8	50.9	8
- yield, %	57.2	8	30.7	6
- gravimetric rate, g/kg.h	0.787	2	0.387	2
- specific rate , g/g DB.h	0.048	2	0.019	2
Oxalic acid				
- concentration, g/kg	16.6	2	26.1	2
- yield, %	13.9	2	30.0	2
- gravimetric rate, g/kg.h	0.044	2	0.077	2
- specific rate, g/g DB.h	0.027	2	0.062	2

5.5 Conclusions

A major conclusion from this chapter is that the petri-dish system of solid state fermentation allows comparable results to those obtained in flask culture. In addition, it should have the advantage of allowing more accurate biomass measurements and practical recovery of viable biomass for biochemical study.

In comparison with their parent strains, the mutant strains exhibited :

- lower rates of starch utilization,
- lower glucose consumption rates
- lower concentrations, yields and production rates of citric acid,
- higher concentrations, yields and production rates of oxalic acid.

Despite these differences, the glucose-releasing enzyme activities and the biomass concentrations were relatively similar between each parent and its respective mutant. The specific growth rates, however, were slightly lower in the mutant than in the parent strains. Given these observed physiological differences between parents and mutants, attempts were now made to understand the biochemical basis of the observations.

CHAPTER 6

Primary Metabolism of Carbon and Citric Acid Accumulation in Solid State Fermentation

6.1 Introduction

From a biochemical viewpoint, citric acid is a product of carbohydrate metabolism of particular strains of microorganisms. The carbohydrates which promote high accumulation of this tricarboxylic acid are sucrose and glucose, either of which must be present in the fermenting medium at a high concentration (Röhr *et al.*, 1983; Xu *et al.*, 1989a,b). In the method of solid state cultivation, as employed in the current study, a starch-containing substrate was used as the source of carbon for the producing organisms. Generally, strains of *A.niger* can utilize starch due to their ability to produce and excrete amylolytic enzymes, such as α-amylase and glucoamylase (Berka *et al.*, 1992; Fogarty, 1994). This property of the organism is considered to be a rate-limiting step for sugar consumption and subsequently to the metabolic flux which favours citric acid overproduction (Röhr *et al.*, 1983). Due to the actions of these amylases the polymeric structure of starch is hydrolysed to more simple units and finally to glucose which is then transported into the fungal cells for metabolism.

Most filamentous fungi usually operate two metabolic schemes for the utilization of glucose, namely the Embden-Meyerhof-Parnas (glycolysis) pathway and the pentose phosphate pathway (Bull and Trinci, 1977). During citric acid accumulation by *A.niger*, the participation of these pathways is shifted in favour of the former (Cleland and Johnson, 1954; Kubicek and Röhr, 1977; Legisa and Mattey, 1988). This implies the importance of the glycolytic pathway to the overproduction of citric acid.

There have been a number of investigations into regulation of glycolysis in relation

to the accumulation of citric acid by *A.niger*. In addition to a major regulatory enzyme of this pathway, phosphofructokinase (Habison *et al.*, 1979; 1983; Arts *et al.*, 1987), hexokinase has been proposed to be another regulatory enzyme (Torres, 1994a,b). Its characterization, as well as its regulation, has been studied recently (Steinböck *et al.*, 1994). Moreover, the mechanism by which glucose is transported into the fungal cells has also been proposed to be an important step for initiation of the glycolytic overflow which is necessary for a high rate of citric acid production and has been studied by Torres *et al.* (1996).

The main purpose of the investigation reported in this chapter was to study the primary steps of carbon utilization, i.e., the hydrolysis of starch, the uptake of glucose and the activity of hexokinase during citric acid production in solid state fermentation. It was believed that the results would indicate differences between the high-and the low-citric acid-accumulating strains with respect to initiation of citric acid accumulation.

6.2 Enzymatic Hydrolysis of Starch

From the preliminary study in Chapter 5, the two high-citric acid-accumulating *A.niger* strains, Yang No.2 and MH 15-15, displayed different abilities to produce the enzymes acting in the liberation of glucose molecules from the starch contained in sweet potato substrate. Since α-amylase and glucoamylase, which are commonly produced by *A.niger* (Berka *et al.*, 1992), are the enzymes responsible for the hydrolysis of starch to glucose, they were investigated during the course of citric acid production. The enzymes were extracted from the fermenting cultures with water, according to the procedure in Section 3.2.12.1. α-Amylase activity was determined using the assay method described in Section 3.2.12.2 while glucoamylase was assayed according to the method described in Section 3.2.12.3. Briefly, determination of α-amylase activity was based on the ability to reduce the formation of the starch-iodine complex by the enzyme sample (Wilson and Ingledew, 1982). For glucoamylase, the activity was estimated from the release of glucose residues from soluble starch by the enzyme

sample (Rugsaseel *et al.*, 1993). Two experiments were conducted and the means, expressed as unit/g DB, are reported.

6.2.1 Measurement of α-amylase activity

α-Amylase (α-1,4-D-glucan 4-glucanohydrolase, E.C. 3.2.1.1) is an endo-acting enzyme catalysing the hydrolysis of α-1,4 glucosidic linkages in amylose, amylopectin and related structures, with maltooligosaccharides of varying chain lengths as the product. Many groups of microorganisms, including *Aspergillus spp.*, can produce and excrete this enzyme in the presence of its substrate (Forgarty and Kelly, 1979, Forgarty, 1994). The external activities of α-amylase from all the four *A.niger* strains were monitored over a period of 10 days and are reported in this section.

The activity profiles of α-amylase during the fermentations of *A.niger* Yang No.2 and SL-1 are displayed in Figure 6.1. The results reveal a strong similarity between the two strains in that the peak levels were observed at 30 h, with a slightly lower value in the parent. By day 2, however, the activity had decreased sharply and remained at consistently low levels through to the end of the fermentation.

For α-amylase measurement of *A.niger* MH 15-15 and its mutant, similarity of the profiles between the two strains was also observed. The activity, which was also at a maximum level after 30 h incubation and was at a slightly lower level in the parent, gradually decreased through to the end of the process. It is also observed that these maximum activity values observed with MH 15-15 and SL-2 strains were rather lower than those reported with the previous strains. Thus, the maximum observed activity for strain MH 15-15 was only 380 units/g DB compared to the level of 1,300 units/g DB for strain Yang No.2.

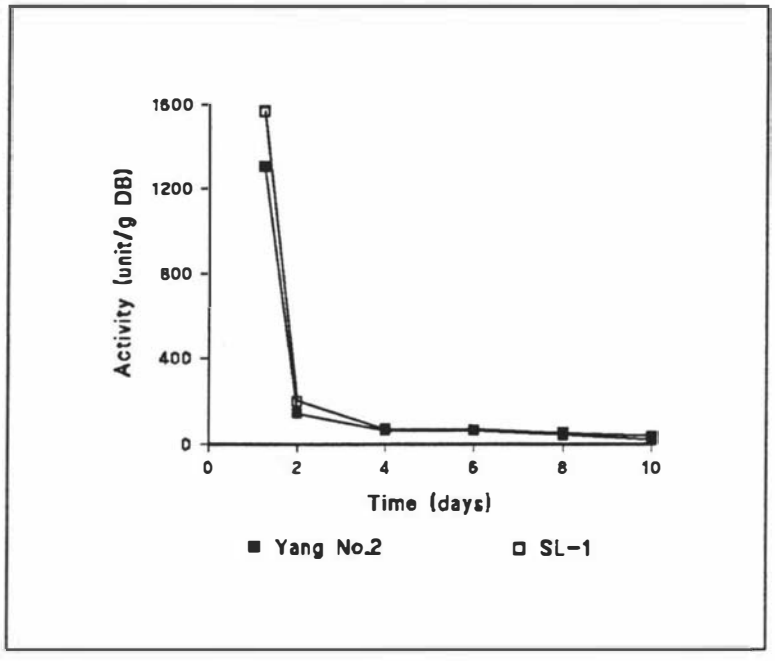


Figure 6.1 α-Amylase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid-state process

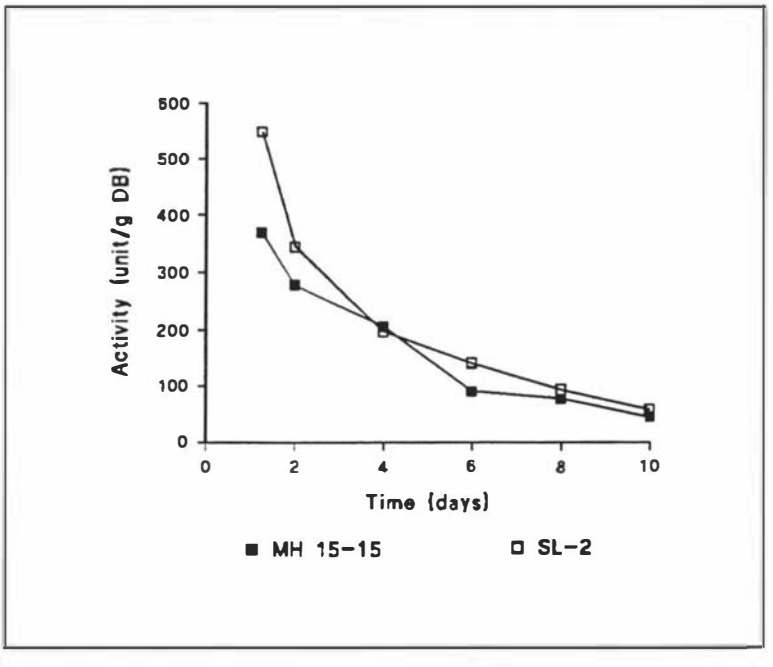


Figure 6.2 α-Amylase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid-state process

6.2.2 Measurement of glucoamylase activity

Glucoamylase (amyloglucosidase, α-1,4-D- glucan maltohydrolase, E.C. 3.2.1.3) is an exo-acting enzyme capable of releasing ß-D-glucose by consecutive hydrolysis of, mainly, α-1,4 linkages from the non-reducing ends of amylose and amylopectin. The most crucial source of this enzyme from microorganisms is fungi, in which *A.niger* is one of the major species (Forgarty and Kelly, 1979; Forgarty, 1994).

The results from glucoamylase measurements of strains Yang No.2 and SL-1 are compared in Figure 6.3. It is clearly shown that these two activity profiles are almost identical and the activity reached the highest level on day 2, followed by a sharp drop by day 4 and then a decreasing level through to the end of the fermentation.

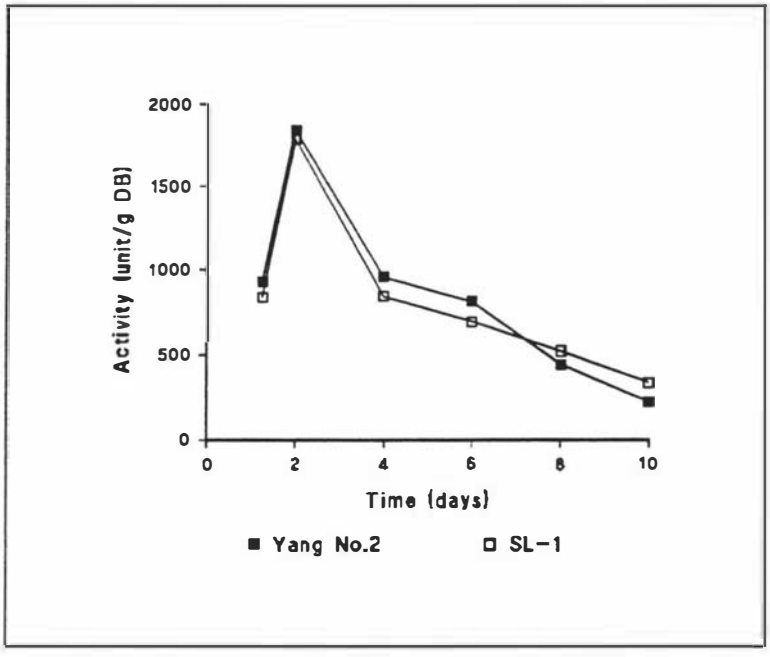


Figure 6.3 Glucoamylase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid-state process

Similarly, the activity profile of glucoamylase from strain MH 15-15 is, in general, comparable to that of its mutant, strain SL-2 (Figure 6.4). The levels measured at 30 h increased and reached the peak values by day 4, after which they decreased through to the end of the time course.

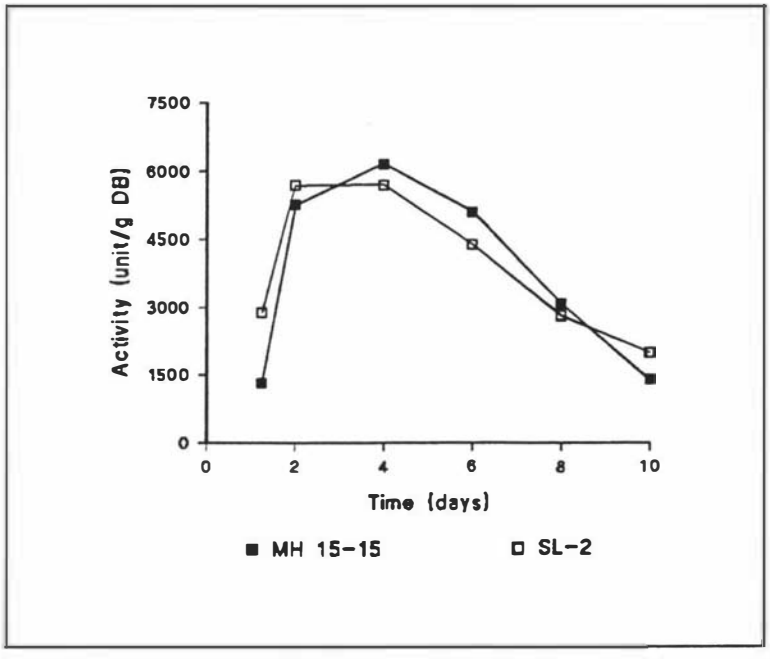


Figure 6.4 Glucoamylase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid-state process

There are two additional points to be mentioned regarding these glucoamylase measurements. Firstly, the activities observed with strains MH 15-15 and SL-2 were markedly higher than those with strains Yang No.2 and SL-1. According to the data, the maximum level for glucoamylase for strain MH 15-15 was as high as 6,150 units/g DB, compared to the value of 1,840 units/g DB for strain Yang No.2. Secondly, the peak activities of strains MH 15-15 and SL-2 were observed on day 4, whereas they appeared on day 2, in the case of strains Yang No.2 and SL-1.

6.2.3 Discussion

In this section, the production of extracellular α-amylase and glucoamylase by the four *A.niger* strains was investigated. The results reveal that these fungal strains could produce and excrete these two amylases for hydrolysis of starch present in the substrate.

With respect to the catalytic activity of α-amylase, it is presumed that there was active liquefaction of starch during the early growth phase of the fungal strains, as the observed activity levels were very high at this stage. This can be explained on the basis that; at the beginning of the fermentation the starch contained in the medium remained available and accessible to the action of this enzyme excreted by the fungus. As the hydrolysis and consumption of starch continued, less α-amylase was produced, probably due to exhaustion of the substrate and, possibly due to expression of certain regulatory mechanisms.

The similarity of the α-amylase profile between each parent and mutant strain suggests no modification of this property as a consequence of mutagenic treatment. The slight differences in the levels at 30 h incubation may not be too significant because of the rapid change in the metabolic events at this stage. This view is supported by the relatively equal α-amylase levels observed between each parent and its respective mutant strains during the later growth phase (day 2 onwards).

Examination of glucoamylase reveals that the enzyme production by each fungal strain reached its peak later than that of α-amylase activity. This can be explained on the basis that the action of α-amylase is necessary to supply the molecules required for induction of glucoamylase production (Barton *et al.*, 1972). However, as more glucose units are produced by the action of glucoamylase a regulatory mechanism, such as catabolite repression (Ghosh *et al.*, 1990) may operate, thus causing the observed activity reduction during the later growth phase.

The similarity between the glucoamylase profiles of each parent and the appropriate strain of mutant also suggests that this property is not the mutation target. The markedly higher levels of glucoamylase observed with strains MH 15-15 and SL-2, compared to those with strains Yang No.2 and SL-1 confirm the preliminary examination in Chapter 5 although different techniques were used for assessment of the activity.

The relationship between the production of these two amylolytic enzymes, the consumption of starch and the presence of external free glucose in these fungal strains is considered. Comparing the two parental strains, the observed starch utilization rate was slightly higher with Yang No.2 than with MH 15-15 (Chapter5, Tables 5.2 and 5.3). A similar trend was observed for the α-amylase activity which was also higher with Yang No.2 than with MH 15-15 (Figures 6.1 and 6.2). In contrast, the glucoamylase activity of MH 15-15 was higher than that of Yang No.2 (Figures 6.3 and 6.4). Thus, glucoamylase activity agrees well with the levels of free glucose in the culture medium which were higher with MH 15-15 than with Yang No.2 (Chapter 5, Figures 5.5 and 5.11). In contrast, the levels of α-amylase activity may reflect the primary hydrolysis of starch rather than the starch consumption rate *per se*. Notably, when comparing each strain of parent with its respective mutant the rates of starch consumption were much lower and the levels of free glucose were higher with the mutant despite the fact that the amylolytic properties had not been changed. Thus, a hypothesis based on the lower rates of glucose consumption by the mutants, previously proposed in Chapter 5, has been supported.

6.3 Uptake of glucose

Following starch saccharification, the released glucose can be readily used as the carbon source for fungal metabolism. Usually there are two components of the transport systems responsible for the uptake of solutes, including glucose (Garrill, 1995). A constitutive low-affinity type allows a facilitated diffusion of glucose when

this solute is present at a high external concentration. At a lower glucose concentration, a high-affinity type becomes derepressed and additionally takes part in the uptake mechanism. In filamentous fungi, the mechanism by which glucose is transported into the cells has been studied extensively in *Neurospora crassa* (Scarborough, 1970; Schneider and Wiley, 1970a,b), and *A.nidulans* (Mark and Romano, 1971). In *A.niger*, specifically the citric acid-accumulating strains, this has recently been investigated by Torres *et al.* (1996). This group demonstrated two types of glucose transport protein varying in affinity and specificity for their substrate, similar to those in other fungi.

As previously reported in Chapter 5, mutants SL-1 and SL-2 accumulated relatively high levels of free glucose in their culture media, and a hypothesis based on defective glucose consumption rates of these strains has been proposed (Section 6.2.3). Hence, the measurement of glucose uptake of these fungal strains would assist in clarification of this concept.

6.3.1 Measurement of glucose uptake

Since external citrate has been reported to inhibit uptake of glucose by *A.niger* (Mischak *et al.*, 1984), it was decided to conduct the glucose uptake measurement in a condition freed from this interference, such as using the exhaustion technique according to Kirimura *et al.* (1992). According to the procedure described in Section 3.2.13, the intact pelletal mycelia (obtained by growing the fungus in SL medium containing 12 % (w/v) glucose in a shake-flask condition) was subject to a series of glucose depletion and starvation regimes. These exhausted mycelia were then incubated in a glucose solution and the glucose remaining after various incubation time intervals was determined. The experiment was repeated twice and averaged data are reported.

Figure 6.5 shows the graphs of glucose uptake by the exhausted mycelia of *A.niger* Yang No.2 and mutant SL-1. The data reveal a linear decrease in the amount of glucose due to consumption by each of the fungal strains through to about 90 min of incubation. However, mutant SL-1 appeared to take up glucose more slowly than did its parent, based on the higher amount of glucose remaining at each time interval.

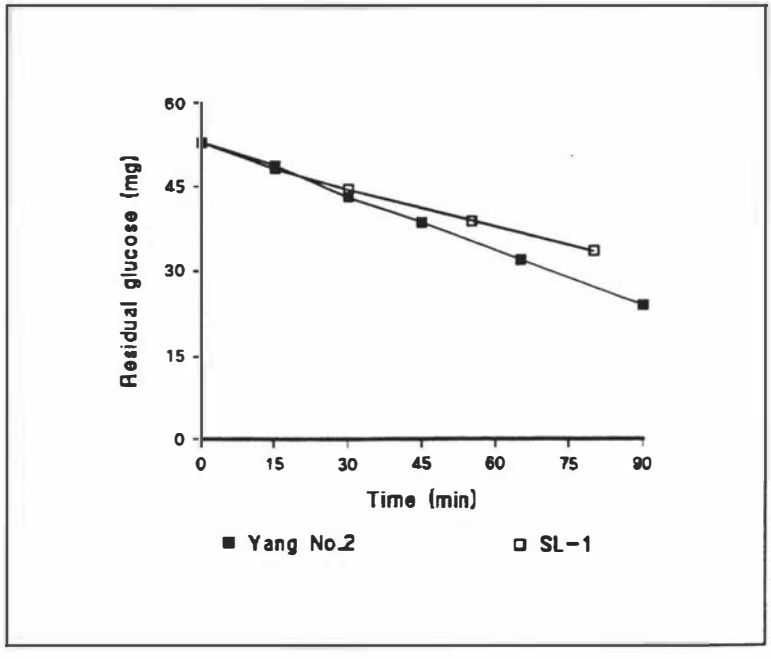


Figure 6.5 Time course of glucose uptake by exhausted-mycelia of *A.niger* Yang No.2 and *A.niger* SL-1

When plotting the amounts of glucose taken up by an equal amount of mycelium against the time, the slope of the graph depicts the specific glucose uptake rate of the fungus. As shown in Figure 6.6, mutant SL-1 exhibited a defective glucose uptake rate as the slope of the displayed graph was lower than that of Yang No.2. Hence, the specific glucose uptake rate at a linear range for the mutant was 0.0033 mg/mg DB.min, compared to the value of 0.0069 mg/mg DB.min of the parent.

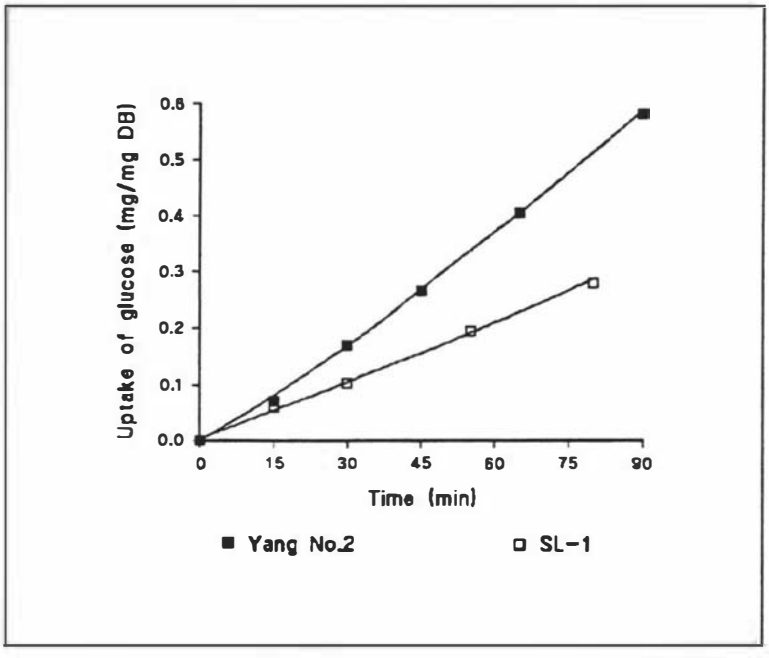


Figure 6.6 Time course of glucose uptake rate by the exhausted mycelia of *A.niger* Yang No.2 and *A.niger* SL-1

As for *A.niger* MH 15-15 and SL-2, the glucose uptake curves obtained by the same method, are presented in Figure 6.7. Similarly, this mutant also consumed glucose more slowly than did its parent, particularly during the later stage of incubation.

The specific glucose uptake rates of mutant SL-2 were also lower than those of strain MH 15-15, estimating from the plots between mg glucose consumed per mg dry biomass, and time (Figure 6.8). At a linear range, therefore, the specific rate for the mutant was obtained at 0.0031 mg/mg DB.min, compared to the value of 0.0078 mg/g DB.min for the parent.

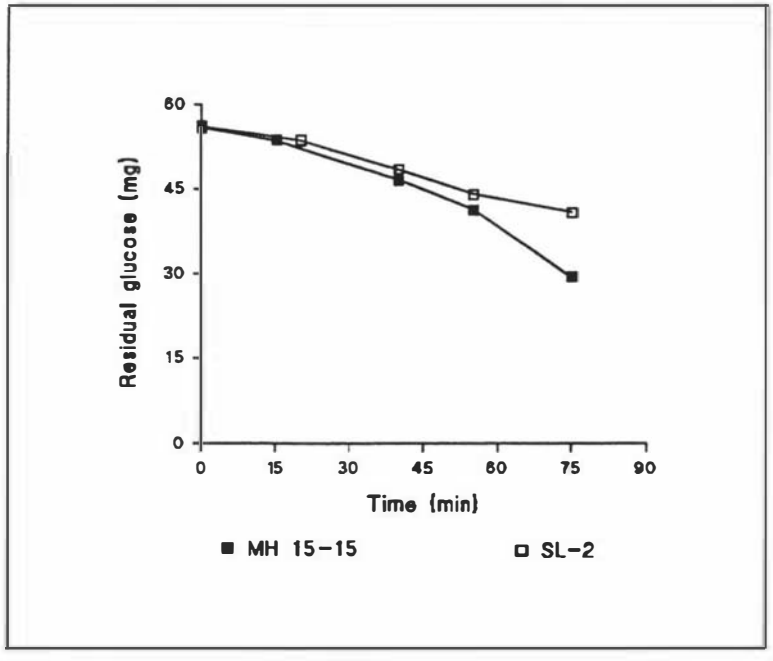


Figure 6.7 Time course of glucose uptake by exhausted-mycelia of *A.niger* MH 15-15 and *A.niger* SL-2

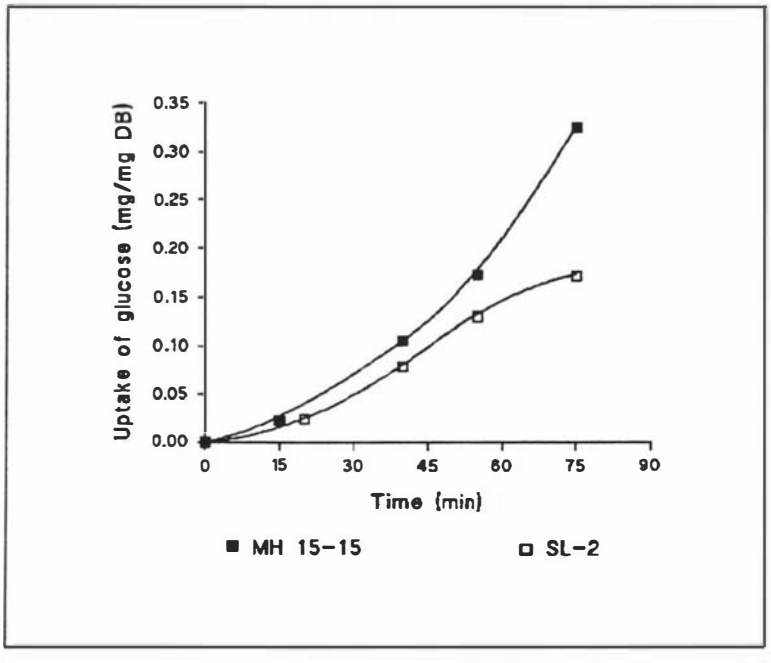


Figure 6.8 Time course of glucose uptake rate by the exhausted mycelia of *A.niger* MH 15-15 and SL-2

6.3.2 Discussion

The role of glucose uptake as a crucial initial step causing the operation of glycolysis necessary for the overproduction of citric acid by *A.niger* has been mentioned (Chapter 2, Section 2.7.2). Generally for citric acid to be actively synthesized and excreted to accumulate in a high concentration in the culture medium, the rate by which glucose is taken up into the fungal cells should be sufficiently high to allow the subsequent flow of carbon through the glycolytic pathway, and thus generate an abundant supply of the precursors for citrate production.

In the current section, the rates by which glucose was consumed by each strain of *A.niger* were determined using the exhaustion technique. The results show that mutants SL-1 and SL-2 exhibited defective rates of glucose uptake as the observed specific rates were lower than those reported for their parents. These findings agree well with the observation on the elevated levels of free (unconsumed) glucose in the culture medium of the mutants during the process of solid state fermentation. It also confirms that this impaired glucose uptake is not caused by the effect of external citric acid, but rather is due to the glucose transport mechanism *per se*, presumably of the glucose transport proteins. It has been reported that in a medium containing a high glucose concentration (15 %, w/v), *A.niger* derepressed its low-affinity protein for glucose transport in addition to the normal high-affinity type (Torres *et al.*, 1996). In the current work on glucose uptake measurement, the fungal strains were pregrown in a liquid medium containing 12 % (w/v) glucose, which has been reported to be the optimum concentration for high yield of citric acid from *A.niger* Yang No.2 (Kirimura *et al.*, 1992). Therefore, under a normal circumstance, these fungal strains should operate both carrier systems to transport glucose. However, it is hypothesised that there are some alterations in mutants SL-1 and SL-2, regarding these transport proteins, leading to the abnormal glucose uptake rates. As some mutated strains of *A.niger* which showed reduced citric acid production rates have been reported to have lost their ability to form the low-affinity glucose transporter in a condition of high

glucose concentration (Torres *et al.*, 1996), it is likely that a similar defect occurs in mutants SL-1 and SL-2 and is responsible for their decreased citric acid accumulation.

The discovery that these two mutant strains, which showed reduced ability for citric acid production, displayed decreased glucose uptake rates from those of their parents is considered very important. This also supports the significance of glucose transport to citrate overproduction. Additionally, another major regulatory step of primary carbon metabolism in relation to citric acid formation which has been proposed is at the phosphorylation of glucose, catalysed by the enzyme hexokinase (Torres, 1994a,b). Investigation into this subject will be reported later in the chapter.

6.4 Measurement of ATPase Activity

ATPase generally refers to a large group of enzymes which hydrolyse the phosphate bond in ATP (adenosine triphosphate). The energy derived from the ATP hydrolysis by certain ATPases may be used by the cells for various types of energy-requiring activity, including solute uptake. Transport of nutrients across the membrane, in some circumstances, requires an electrochemical proton gradient generated by the plasma membrane ATPase as a driving force (Serrano, 1984). It is, thus, presumed that any modifications to this energy-generating system would affect this function of the cells, and it is possible that the abnormal glucose uptake of mutants SL-1 and SL-2 may relate to certain alterations of the plasma membrane ATPase.

For this reason, the measurement of the membrane-bound ATPase activity would help in defining the energy transforming capability of the cells in relation to the transport of glucose. In the present work, however, the total membrane rather than the plasma membrane ATPase activity was measured because of some difficulties in the fractionation of cellular components. Nevertheless, this is thought not to affect the conclusions that can be drawn from the results. The methods for extraction and activity measurement of the total membrane ATPase are explained in Section 3.2.16.3 and 3.2.18.10, respectively. The data were obtained only at day 2 and day 4 of the

time course and were expressed as specific activity (unit/mg protein). Two individual experiments were conducted and averaged results are reported.

6.4.1 Results

The activities of the total membrane ATPase of strains Yang No.2 and SL-1 are presented in Figure 6.9. The results imply that the two fungal strains did not alter in this property as the observed activities were approximately equal.

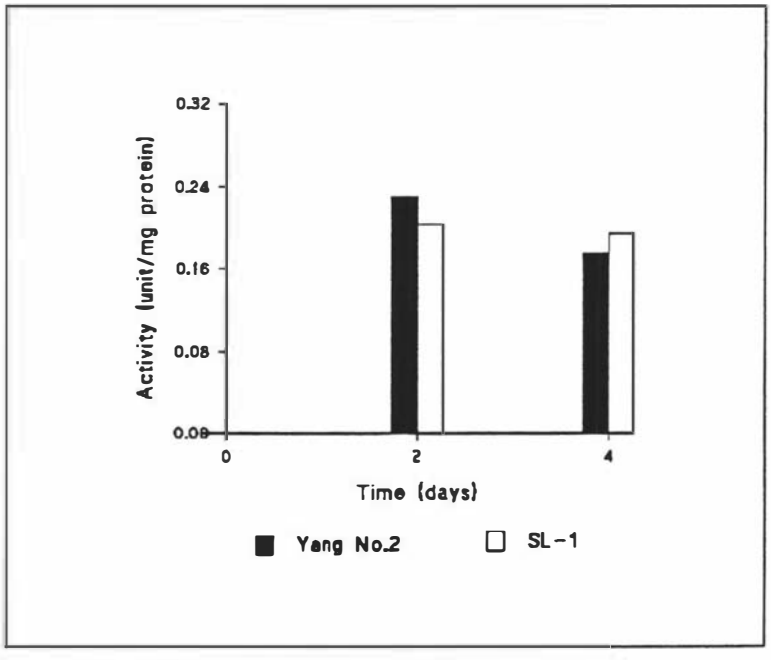


Figure 6.9 Total membrane ATPase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

A similar result was observed for the total membrane ATPase measurement of strains MH 15-15 and SL-2. Thus, there was no major difference between the activity observed in the two strains, as shown in Figure 6.10.

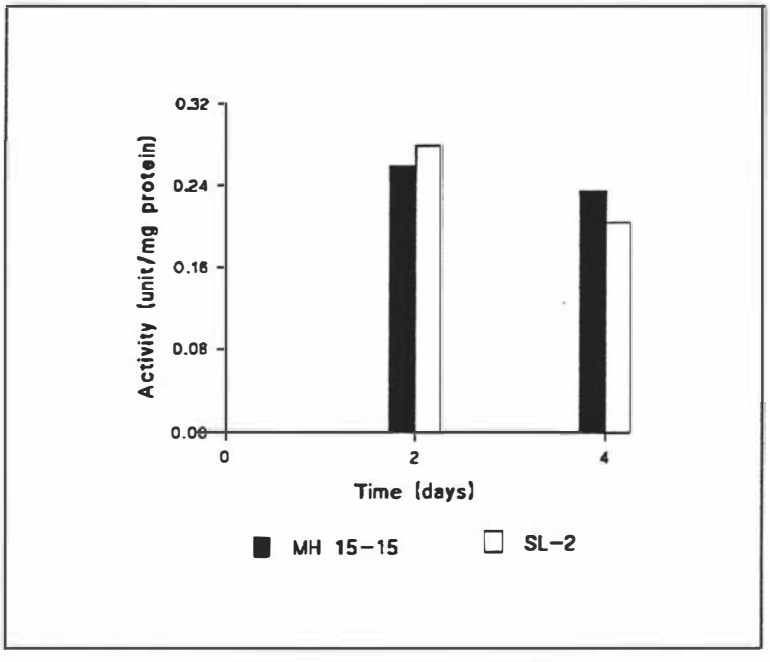


Figure 6.10 Total membrane ATPase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

6.4.2 Discussion

The activities of total membrane ATPase were investigated in the solid state cultures of all *A.niger* strains in order to relate them to the glucose transport. To our knowledge, this measurement has never been previously reported for this species of fungus during cultivation for citric acid production but it has been studied in a citric acid-accumulating strain of yeast in relation to citrate transport (Gutierrez and Maddox, 1993). The results obtained from the present study suggest that neither mutant SL-1 nor mutant SL-2 had been modified in this property from that of its respective parent. It is, therefore, suggested that the reduction in glucose uptake rates of the two mutants is not a result of alterations of this energy-generating system, but

rather is a defect in a component in the glucose transport system, as previously proposed.

6.5 Measurement of hexokinase activity

Hexokinase is the first enzyme of glucose assimilation *via* the Embden-Meyerhof-Parnas (glycolysis) pathway after it enters the fungal cells. This enzyme functions in the phosphorylation of glucose, yielding glucose 6-phosphate which is subsequently metabolised within the cells. The importance of hexokinase as a potential control step of the glycolytic pathway, and then of citric acid accumulation, has been described in several recent reports, therefore, its activity was investigated in the current study. The enzyme was extracted from the mycelium using the procedure described in Section 3.2.16.2 and was measured for its activity using the *in vitro* assay method (Section 3.2.18.1). The assays were conducted over a period of 10 incubation days and the data, reported as specific activity, are the means from three separate experiments.

6.5.1 Results

The changes in hexokinase activity of *A.niger* Yang No.2 and SL-1 are displayed in Figure 6.11. The results clearly demonstrate a difference between these two strains, though they show a similar pattern of changes. The activity from the parent strain was high until day 4, but dropped sharply by day 6 then remained at low levels through to the end of the process. The mutant also displayed a similar activity profile, but the activity reduced to generally half that of its parent.

Considering *A.niger* strains MH 15-15 and SL-2, there were also differences in the levels of hexokinase activity between these two strains. It is revealed that the activities in the parent were slightly higher than those of the mutant over the entire period of the fermentation, although their patterns of change were similar (Figure 6.12). Thus, in both cultures, hexokinase activity increased and reached a peak by day 4, after which it declined to low levels by the end of the process.

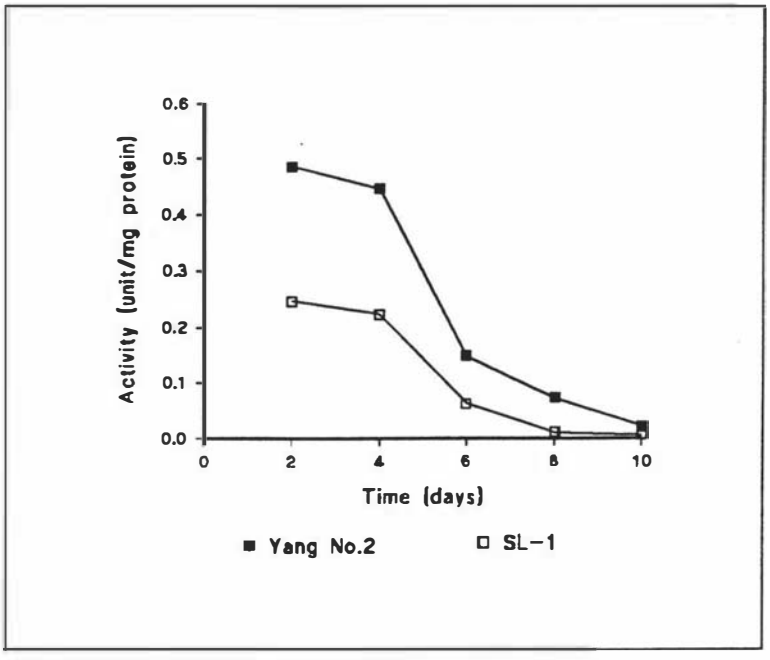


Figure 6.11 Hexokinase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid-state process

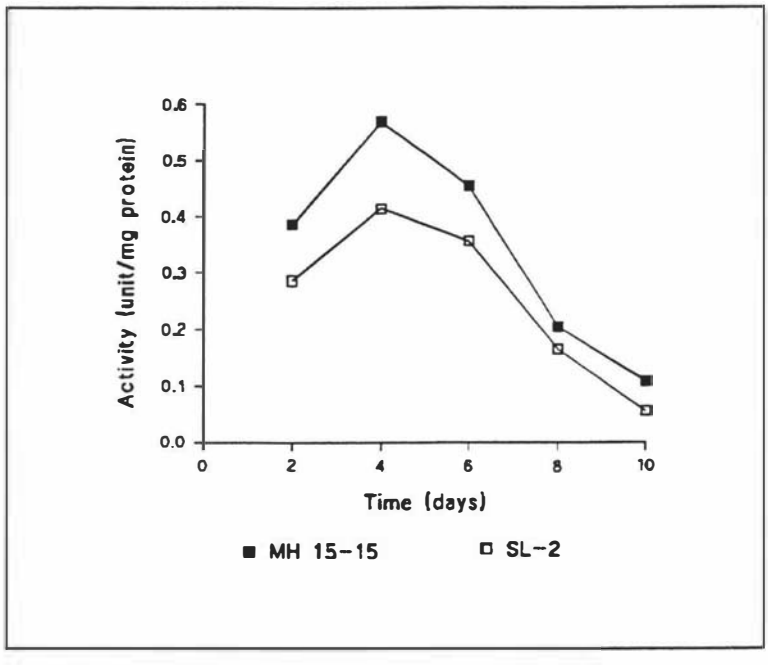


Figure 6.12 Hexokinase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid-state process

6.5.2 Discussion

The importance of an active glycolysis as a prerequisite for citric acid overproduction has been documented (Röhr *et al.*, 1983, Roehr, *et al.*, 1992). Besides the control at phosphofructokinase of the glycolytic pathway, hexokinase also plays a significant role in the regulation of this pathway in relation to accumulation of citric acid by *A.niger* which is described in Section 2.7.2. Hexokinase has been purified and characterised and its increased activity by high concentrations of glucose or sucrose has been shown (Steinböck *et al.*,1994). It has also been reported that some *A.niger* mutants which improved citrate yields displayed increased hexokinase activities (Schreferl-Kunar *et al.*, 1989), whereas the defective mutants exhibited significantly reduced activity of this enzyme (Kirimura *et al.*, 1992; Steinböck *et al.*, 1994).

The current findings on hexokinase activities, therefore, agree well with the above literature. Thus, mutants SL-1 and SL-2, which showed decreased yields and rates of citric acid production also displayed reduced hexokinase activities. Moreover, there has been a report on the relationship between reduced hexokinase activity and impaired glucose transport (Torres *et al.*, 1996). As a consequence, both the impaired glucose uptake rate and the reduced hexokinase activity of these two strains of mutant may be responsible, at least partly, for their decreased citric acid production rates and yields and, at the same time, the results affirm the importance of these two characters to the hyperproduction of citric acid by *A.niger* Yang No.2 and MH 15-15. However, it remains unclear as to which character is the more important, and whether the decreased activity of hexokinase is a result or a cause of the decreased glucose uptake rate.

6.6 Conclusions

There are at least two amylolytic enzymes, α-amylase and glucoamylase, contributing to the saccharification of starch by *A.niger* Yang No.2 and MH 15-15. However, mutants SL-1 and SL-2 displayed no observable differences in these properties to their respective parents. In contrast, reduced glucose uptake rates of the mutants have been demonstrated, but this is not due to alterations of ATPase activity. Compared to their respective parents, the mutants also showed decreased hexokinase activities during the fermentation. It is hypothesised that these two defective characters of the mutants are responsible for the impairment of the high glycolytic capacities necessary for the overproduction of citric acid.

CHAPTER 7

Selected TCA Cycle and Other Enzymes and Citric Acid-Accumulation in Solid State Fermentation

7.1 Introduction

As citric acid is a metabolic intermediate of the tricarboxylic acid (TCA) cycle, a great deal of work has been conducted to investigate the regulation of this metabolic scheme in relation to accumulation of this acid. The most controversial point is whether an interruption at certain steps of the TCA cycle is established during the acidogenic phase. The enzymes that have been studied in this regard are those catalysing direct conversion of citrate, i.e. aconitase; or those enhancing breakdown of citrate, i.e. isocitrate dehydrogenases (NAD-and NADP-specific) and 2-oxoglutarate dehydrogenase (see reviews of Kubicek, 1987; 1988). In addition, the involvement of metabolic steps following the glycolytic pathway, yet prior to the TCA cycle, has also received some interest. Particular enzymes investigated include pyruvate carboxylase (Feir and Suzuki, 1969; Hossain *et al.*, 1984; Dawson, 1986), pyruvate kinase (Meixner-Monori *et al.*, 1984) and citrate synthase (Kubicek and Röhr, 1980). Recently, there have been studies on cytosolic routes of citrate metabolism after it has been transported from its biosynthetic location in the mitochondrion. A cytosolic enzyme, ATP:citrate lyase, catalysing the breakdown of citrate to acetyl CoA and oxaloacetate, has thus become an additional subject of research towards a better understanding of the mechanism of citric acid accumulation by the fungus *A.niger*.

The main purpose of this chapter was to provide primary information on the operation of the TCA cycle and other relevant metabolic steps during fermentation in the solid state system. This was achieved by monitoring changes in activity of the corresponding enzymes in the high- and the low-citric acid-accumulating strains over the course of the fermentation.

7.2 Measurement of Selected TCA Cycle Enzymes

The following TCA cycle enzymes were determined : aconitase, NAD-specific and NADP-specific isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase. The high-accumulating strains, Yang No.2 and MH 15-15, and their low-accumulating mutants, SL-1 and SL-2, were cultivated for citric acid production using the solid state fermentation method described in Chapter 5. At various stages of the incubation, the enzymes were extracted from the mycelium (Section 3.2.16.2) and their activities were assayed *in vitro* using the method specified for each enzyme given in Section 3.2.18. Protein in the enzyme samples were estimated using the dye-binding method (Section 3.2.17). Triplicate assays were conducted for each enzyme and the activity, expressed as specific activity (unit/mg protein), was averaged. The data, reported as the means from three separate experiments, are presented in comparison between each parent and the appropriate strain of mutant.

7.2.1 Results

7.2.1.1 Aconitase

Aconitase is the enzyme which catalyses equilibrium reactions of citrate, *cis*-aconitate, and isocitrate in the TCA cycle. Because it is the first enzyme of citrate catabolism, its putative inhibition during the acidogenesis phase has often been referred to in early publications.

Figure 7.1 compares the changes in aconitase activity of *A.niger* Yang No.2 and its mutant, strain SL-1. The data show a marked difference between the two strains. Thus, the peak value for the parent culture was observed on day 2 followed by a sharp drop by day 4. The activity continued declining to a very low level at the completion of the process. In contrast, aconitase activity of the mutant was low on day 2, and it then rose to reach a maximum by day 6.

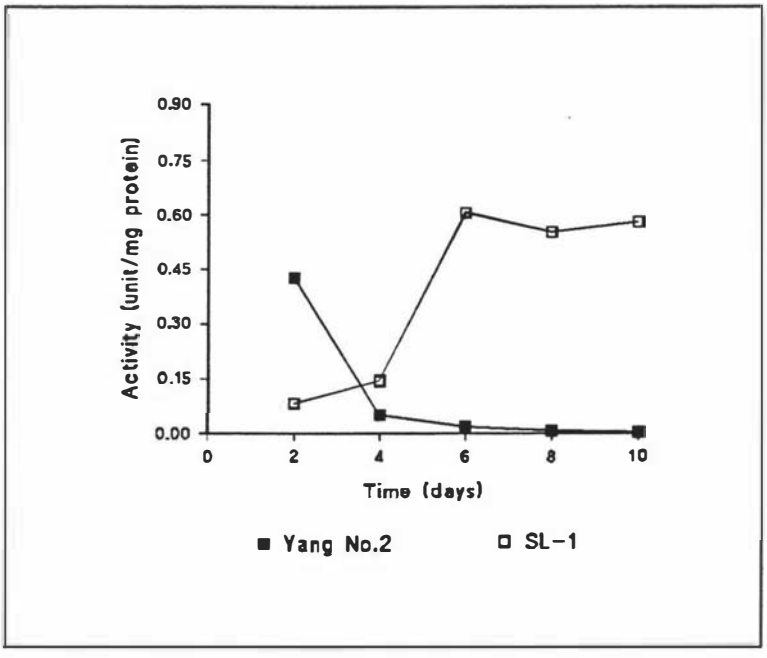


Figure 7.1 Aconitase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid-state process

There were similarities to the above results from the measurement of aconitase activity of strains MH 15-15 and SL-2. As shown in Figure 7.2, the activity pattern of this parent strain resembles that of strain Yang No.2. Similarly, there was also a resemblance between the aconitase profile of mutant SL-2 and mutant SL-1.

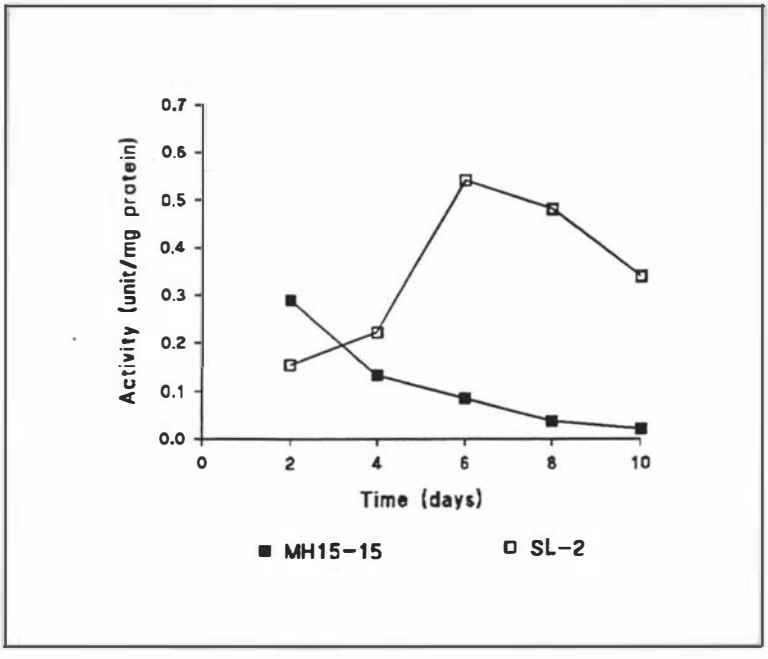


Figure 7.2 Aconitase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid-state process

7.2.1.2 NAD-specific isocitrate dehydrogenase

Filamentous fungi, including *A.niger*, usually contain two forms of isocitrate dehydrogenase which differ in cofactor requirements. The NAD-requiring enzyme is normally located in the mitochondrion, while the NADP-requiring enzyme is generally located in the cytoplasm, but also in the mitochondrion, of the fungal cells (Osmani and Scrutton, 1983). Both types of isocitrate dehydrogenase have usually been investigated along with aconitase enzyme.

Figure 7.3 compares NAD-specific isocitrate dehydrogenase of strains Yang No.2 and SL-1. The results show that the observed activity of strain Yang No.2 was at the highest level on day 2 but this then decreased sharply by day 4. Thereafter, the activity continued to decrease to a very low level at the end of the time course. A similar activity profile was reported for mutant SL-1 but it appeared that the activity reduction was less pronounced than for its parent.

As for strains MH 15-15 and SL-2, the activity profiles of this enzyme were comparable to those of strains Yang No.2 and SL-1. As revealed in Figure 7.4, the activity was generally rather higher in the mutant than in the parent.

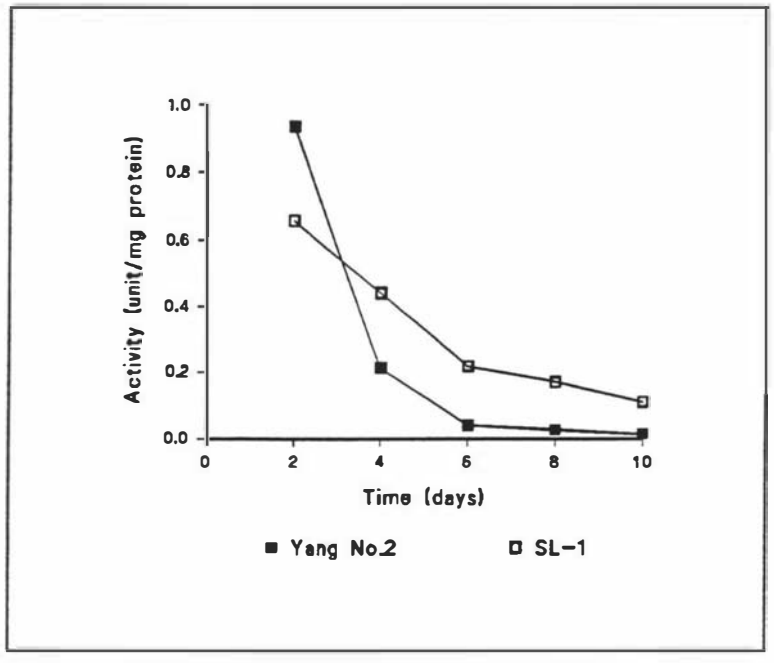


Figure 7.3 NAD-specific isocitrate dehydrogenase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid-production by the solid state process

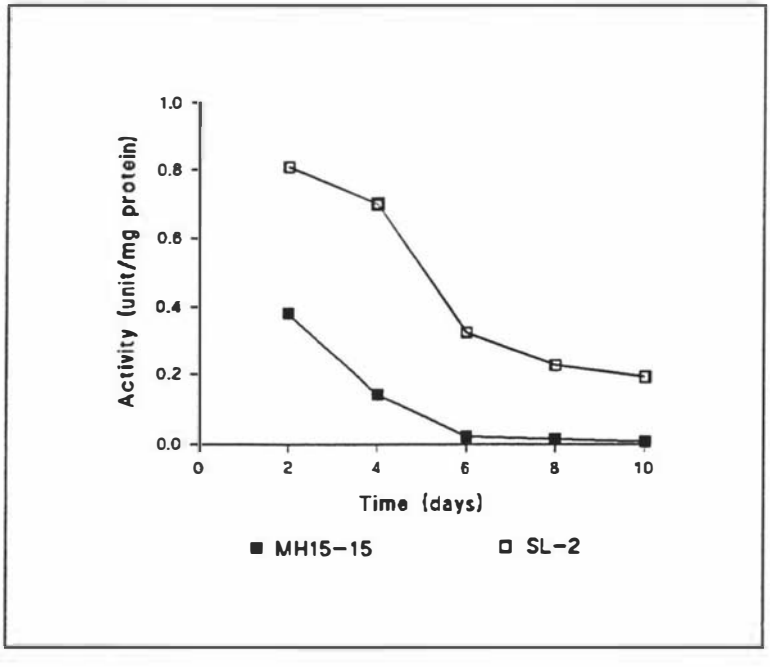


Figure 7.4 NAD-specific isocitrate dehydrogenase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid-production by the solid state process

7.2.1.3 NADP-specific isocitrate dehydrogenase

The importance of NADP-specific isocitrate dehydrogenase in citric acid accumulation has been suggested (Mattey, 1977; Meixner-Monori *et al.*, 1986).

The changes in activity of this enzyme observed from strain Yang No.2 are compared with those from strain SL-1 in Figure 7.5. It appeared that the activity profile of the NADP-specific enzyme followed a similar pattern to that of the NAD-specific enzyme as the maximum value was detected on day 2, after which there was a substantial reduction of the activity level. Moreover, the mutant displayed an almost identical activity profile to that of its parent, except that rather higher values were observed.

Figure 7.6 displays the activity profile of this enzyme detected from strains MH 15-15 and SL-2. The results generally resemble those reported for strains Yang No.2 and SL-1 except for some variations in the rate of activity decrease and the level of activity observed.

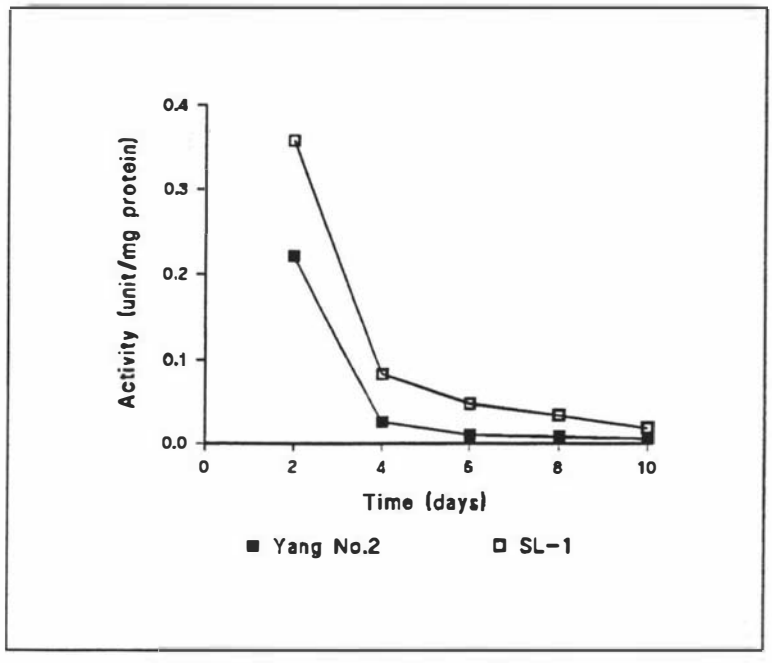


Figure 7.5 NADP-specific isocitrate dehydrogenase activities of *A.niger* Yang and *A.niger* SL-1 during citric acid production by the solid state process

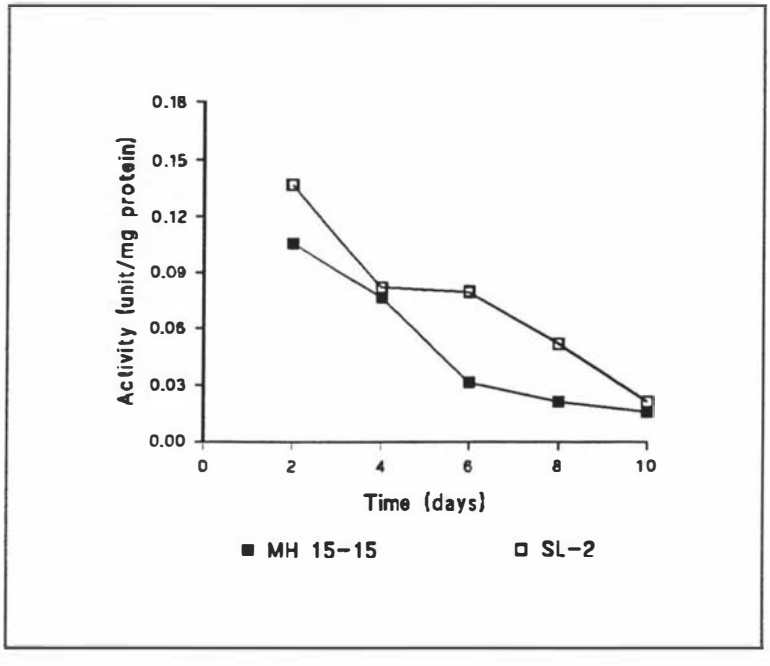


Figure 7.6 NADP-specific isocitrate dehydrogenase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

7.2.1.4 2-Oxoglutarate dehydrogenase

The enzyme 2-oxoglutarate dehydrogenase functions at the only irreversible step of citrate catabolism in the TCA cycle, therefore, any alteration at this step would influence the performance of the whole cycle. The decrease in activity of this enzyme during the citric acid accumulation phase of *A.niger* has been reported (Ng *et al.*, 1973; Kubicek and Röhr, 1977; Hossain *et al.*, 1984, Meixner-Monori *et al.*, 1985; Dawson, 1986).

The results clearly show that there was a marked difference in the 2-oxoglutarate dehydrogenase activity between each parent and its mutant. As shown in Figure 7.7, the enzyme detected from strain Yang No.2 was at very low levels through to the end of the fermentation. In contrast, it was present at much higher levels in strain SL-1 and the observed values were about 3 to 15 times higher than those of its parent.

In the case of 2-oxoglutarate dehydrogenase of strains MH 15-15 and SL-2, the data were similar to those observed for the previous strains. As shown in Figure 7.8, the enzyme activity detected from the mutant was at much greater levels than that observed from its parent.

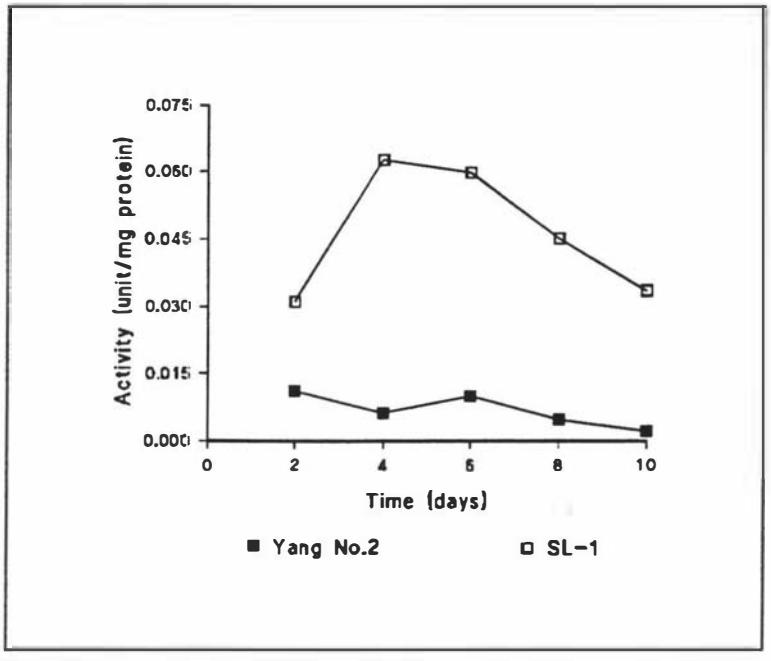


Figure 7.7 2-Oxoglutarate dehydrogenase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid-production by the solid state process

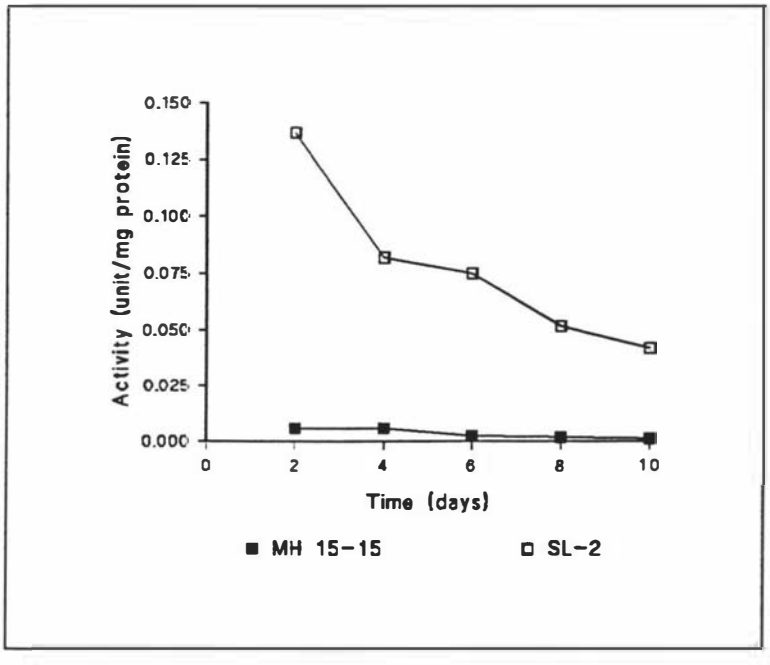


Figure 7.8 2-Oxoglutarate dehydrogenase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid-production by the solid state process

7.2.2 Discussion

This part of the enzyme measurements in the solid state cultures was aimed to describe the operation of the TCA cycle and to relate it to accumulation of citric acid by *A.niger*. By considering activity profiles of some selected enzymes of this metabolic pathway from each high-citric acid-accumulating strain and its respective low-accumulating mutant when all organisms were grown under the same condition, it was hoped that some explanations could be proposed.

Before any further discussion, two things must be borne in mind on the interpretation of the results of the enzyme activity measurements. Firstly, the enzyme assays were performed *in vitro* and may not accurately reflect the *in vivo* situation. For example, the catalytic function of a particular enzyme generally requires one or more cofactors. Usually they are added in excess in the *in vitro* assay, but their concentrations in the cell are unknown. In addition, the enzymes may be modified or controlled by certain effector(s) within the metabolically active cells which will affect their *in vivo* activity. Secondly, it may be difficult to distinguish between "cause" and "effect" with regard to the activity of any given enzyme.

Theoretically for citrate to accumulate, there should be some prevention of its catabolism in the TCA cycle. In this solid state fermentation condition, the most likely enzyme could be 2-oxoglutarate dehydrogenase, as each parent and its respective mutant displayed a marked activity difference. Thus, the very low activities observed in both Yang No.2 and MH 15-15 strains (Figure 7.7 and 7.8) suggest reduced fluxes around their TCA cycle, either by a repression (Röhr and Kubicek, 1981) or an inhibition (Meixner-Monori *et al.*, 1985) mechanism, thus allowing citrate to accumulate because of the equilibria of the enzymes acting between citrate and 2-oxoglutarate. The regulation of this enzyme activity by the mechanism of inhibition, as proposed by Meixner-Monori *et al.*, (1985), has been demonstrated to occur by several metabolites, the most likely, by oxaloacetate and NADH. However, it is not

possible at this stage to specify whether such a control mechanism is operating and so is responsible for this *in vitro* activity reduction. Additionally, the current results verify that the fungal TCA cycle is not completely blocked during the acidogenesis phase since 2-oxoglutarate dehydrogenase was detected from both Yang No.2 and MH 15-15 throughout the entire fermentation, although the flux around their TCA cycle may be greatly reduced.

It must also be borne in mind that during fermentation using this solid state condition, the first possible determination in the time course from which the recovery of active mycelium for the enzyme assays was conceivable was on day 2. However, at this growth stage the organisms had already entered their acidogenic phase, judging from their maximum rates of citric acid production which occurred on day 2 (Chapter 5). This situation means that the transition into this phase, or the onset of citric acid accumulation, had commenced prior to this first observation. Therefore, in the case of 2-oxoglutarate dehydrogenase, and possibly of other enzymes being measured, it is not clear whether this activity reduction is a prerequisite for, or a result of, citric acid accumulation.

The higher levels of 2-oxoglutarate dehydrogenase observed in mutants SL-1 and SL-2 indicate active breakdown of citrate *via* the TCA cycle, and so may account for the reduced external citrate accumulation by these strains. An explanation for this non-interrupted cycle, therefore, should be sought and this will be discussed later in the section.

Another approach which can be discussed regarding the TCA cycle enzymes is that of the enzymes accelerating the catabolism of citrate, *viz* aconitase and isocitrate dehydrogenase, both the NAD-and the NADP-specific types. There were some differences in the activity profiles observed with each parent and its mutant in the sense that the activities in the latter were generally higher. These findings conflict with the report of La Nauze (1966) who, using a condition promoting citric acid accumulation, compared these enzymes from a pair of parent and mutant of *A.niger*

varying in the ability to accumulate citric acid. According to that report, there was no significant activity difference between aconitase and isocitrate dehydrogenases of the low-and the high-yielding strains. However, no definitive conclusions were proposed to explain this.

Although there has remained controversy over the role of citrate-catalysing enzymes in citric acid accumulation, the concept of aconitase interruption is now believed to be invalidated, whereas the role of both types of isocitrate dehydrogenase has remained inconclusive (Kubicek, 1988). The progressive activity reduction of both types of isocitrate dehydrogenase in Yang No.2 and MH 15-15 strains during the later growth phase seems to agree well with most previous publications (Kubicek and Röhr, 1977; Szczodrak, 1981; Hossain *et al.*, 1984; Dawson, 1986; Roukas, 1991). However, the properties and locations in the fungal cells of these enzymes should also be taken into account. Interestingly, the mitochondrial located NADP-specific isocitrate dehydrogenase has been demonstrated to be specifically inhibited by citrate at cellular levels (Mattey, 1977; Meixner-Monori *et al.*,1986). In the current findings, a more marked decrease in the *in vitro* activity of this enzyme was revealed with parents Yang No.2 and MH 15-15 than with mutants SL-1 and SL-2. Possibly, this activity reduction may be caused by external citrate remaining in the enzyme samples (Kubicek, 1988), but this mechanism may also occur *in vivo* by intracellular citrate. As a consequence, a further investigation, such as the measurement of metabolic products of appropriate enzymes, would be a useful depiction of the *in vivo* enzyme operation.

In the situation in which aconitase and isocitrate dehydrogenases are more active, as with mutants SL-1 and SL-2, a relationship to their lower citric acid production rates could also be postulated. Thus, under such circumstances, more citrate is being channelled through the TCA cycle rather than being excreted out of the cells. A hypothesis explaining the apparent cause of such TCA cycle operation with regard to the control of these citrate-degrading enzymes will be discussed in more detail in Chapter 8.

From the preceding information, however, a general hypothesis depicting a possible primary cause of the observed TCA cycle operation can be attempted. Thus, the glucose uptake rate, which has been characterised to be strongly defective in both strains of mutant (Chapter 6), should be taken into account as this character may be considered as the most primary step of glucose assimilation by the fungal cells. Also, the significance of this step as having a crucial role in controlling citric acid accumulation in *A.niger* grown under submerged fermentation conditions has been proposed (Torres, 1994a,b). Thus, it is hypothesised that when glucose is supplied to the cells of the mutants at rates lower than those required for initiation and maintenance of the necessary fluxes through glycolysis which operate in strain Yang No.2 or MH 15-15, citric acid formation rates decrease because of the fully operative TCA cycle which allows catabolism of citrate. A more conclusive hypothesis suggesting a possible cause of this non-interrupted TCA cycle will be put forward in Chapter 8.

7.3 Measurement of Other Selected Enzymes

The changes in activity of pyruvate carboxylase, isocitrate lyase, ATP:citrate lyase, and oxaloacetate hydrolase, which have been proposed to be involved in the accumulation of citric acid were studied in all these *A.niger* strains. The enzymes were extracted from the mycelia using the same procedure as for the TCA cycle enzymes and were assayed for their activities according to the method specific for each enzyme, as provided in Section 3.2.18. The data obtained over the course of the fermentation are presented in comparison between the parent and its respective mutant.

7.3.1 Results

7.3.1.1 Pyruvate carboxylase

The occurrence of carbon dioxide fixation in *A.niger* by the function of pyruvate carboxylase has been reported (Cleland and Johnson, 1954, Woronick and Johnson, 1960; Bloom and Johnson, 1962; Feir and Suzuki, 1969). The importance of this enzyme to citric acid accumulation bears on its role in providing oxaloacetate, a direct precursor for citrate synthesis, to the citric acid cycle.

Figure 7.9 shows pyruvate carboxylase activity profiles of *A.niger* Yang No.2 and its mutant. The results reveal a similarity between the two strains as the activity was reasonably high through to day 6 of the fermentation, after which it decreased. However, the values for the parent were consistently higher than those of the mutant.

Similar results were obtained for pyruvate carboxylase measurement of *A.niger* MH 15-15 and its mutant. As shown in Figure 7.10, the enzyme of this parent was consistently higher than that of its mutant, particularly during the early growth phase.

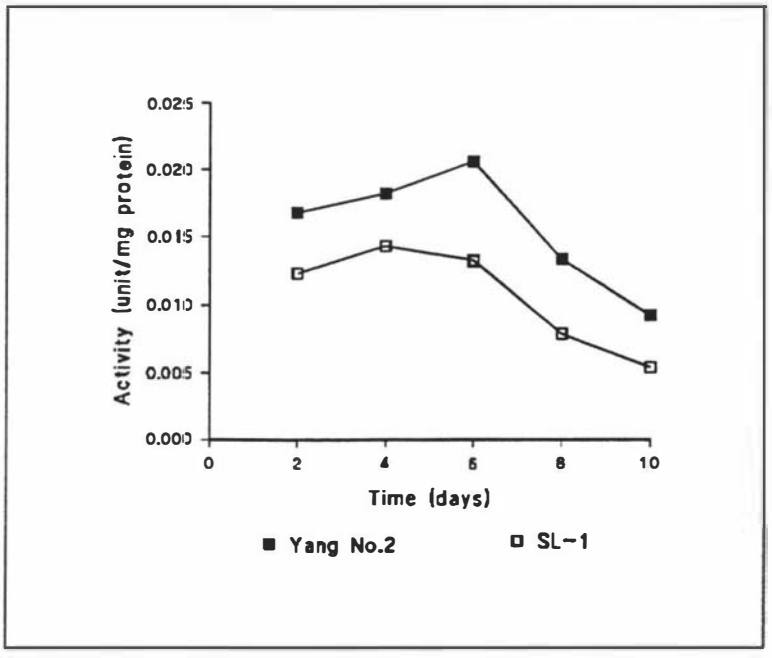


Figure 7.9 Pyruvate carboxylase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

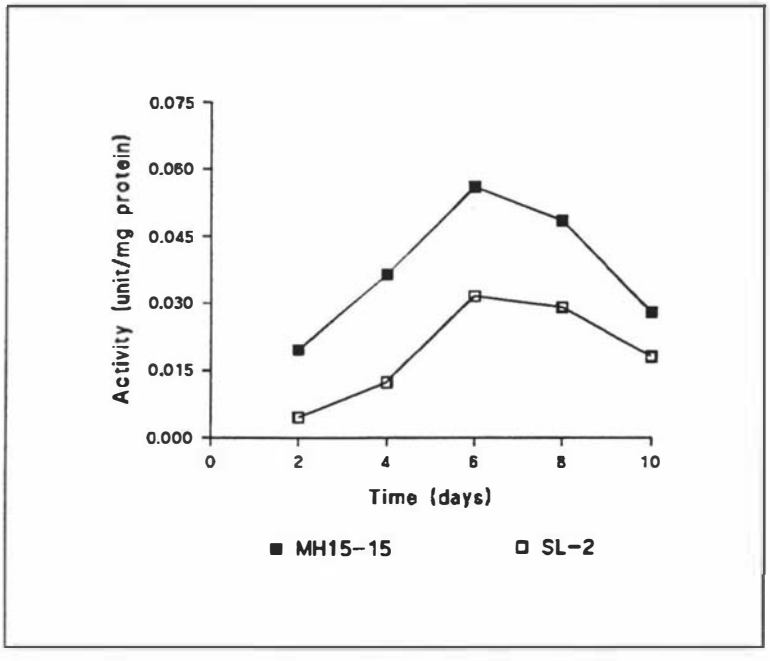


Figure 7.10 Pyruvate carboxylase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

7.3.1.2 Isocitrate lyase

The catalytic function of isocitrate lyase is in the breakdown of isocitrate *via* the glyoxylate pathway. There have been a few studies on the role of this enzyme in relation to citric acid accumulation (Verhoff and Spradlin, 1976; Ahmed *et al.*, 1972; Dawson, 1986).

Figure 7.11 shows activities of isocitrate lyase measured from strains Yang No.2 and SL-1. In general, the enzyme was more active during the early growth phase, particularly in the parent. During the later stage, there were no consistent differences between the parent and the mutant.

Similarly, there was no major difference between isocitrate lyase profiles of strains MH 15-15 and SL-2 except that the enzyme of the mutant appeared to be more active on day 2, as displayed in Figure 7.12.

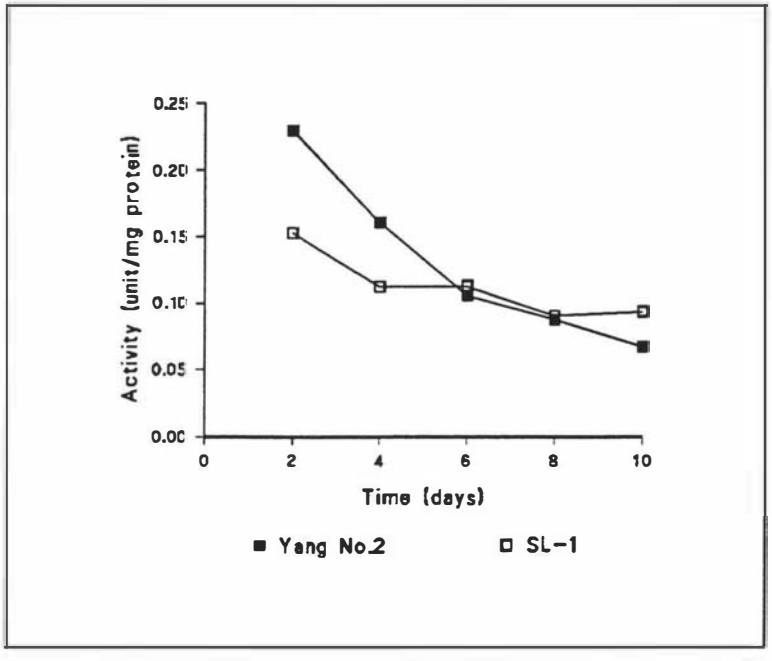


Figure 7.11 Isocitrate lyase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid-state process

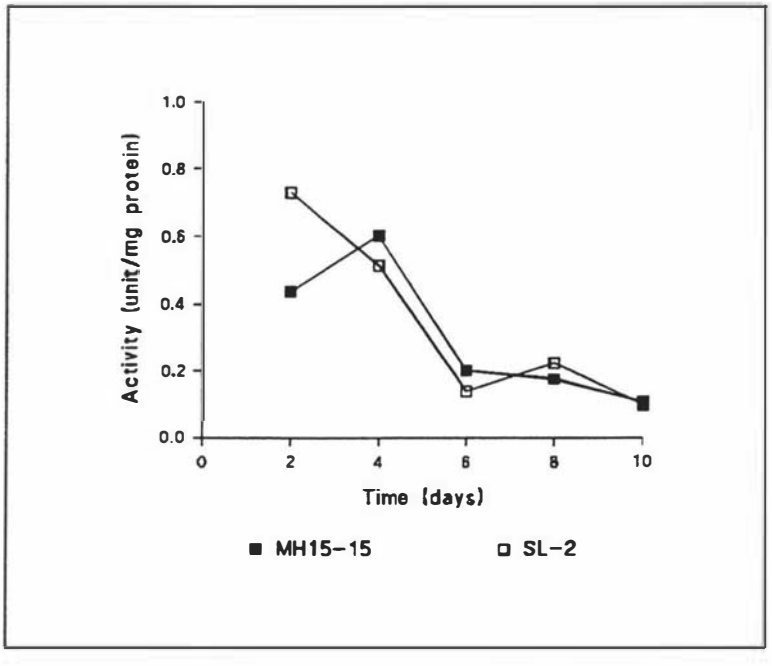


Figure 7.12 Isocitrate lyase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid-state process

7.3.1.3 ATP:citrate lyase

The role of ATP:citrate lyase in the biosynthesis and accumulation of lipid by certain strains of yeast is well-established (Boulton and Ratledge, 1981; Evans and Ratledge, 1985). In addition, its role in relation to the accumulation of citric acid has been investigated in the fungus *A.niger*. This cytosolic enzyme has been reported to be absent or inhibited under conditions of high citric acid accumulation (Pfitzner *et al.*, 1987; Jernejc *et al.*, 1991).

The graphs shown in Figure 7.13 clearly demonstrate a major difference in ATP:citrate lyase activity between strains Yang No.2 and SL-1. Thus, the activities detected on day 2 were comparably high for both strains. However, the level detected for the parent dropped markedly by day 4 and was then maintained at very low levels through to the end of the process. In contrast, strain SL-1 maintained the high activity of this enzyme although the activity decreased later in the process.

A similar result was also observed between parent MH 15-15 and its mutant. Thus, ATP:citrate lyase activity was lower in this parent than in its mutant. However, this difference was much less marked than was observed in strains Yang No.2 and SL-1 except for the value at day 2. These results are presented in Figure 7.14.

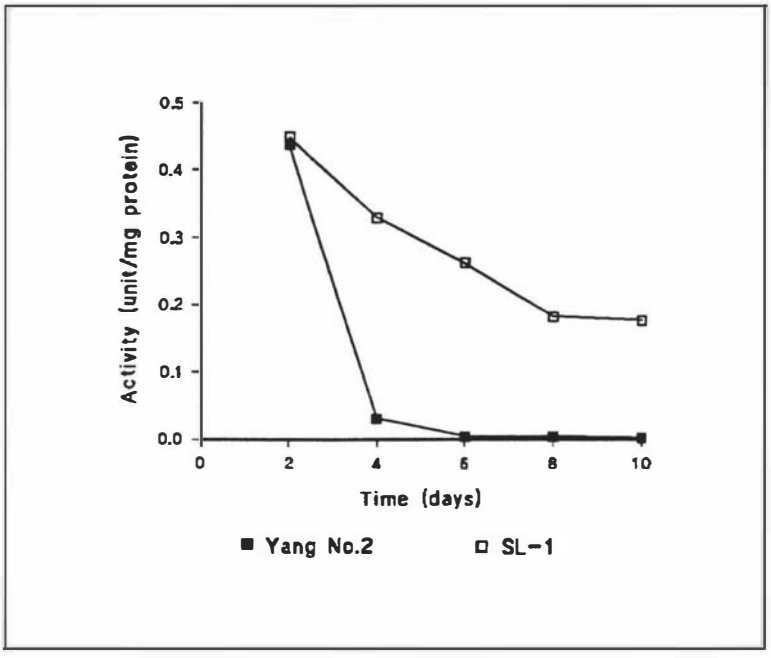


Figure 7.13 ATP:citrate lyase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid-state process

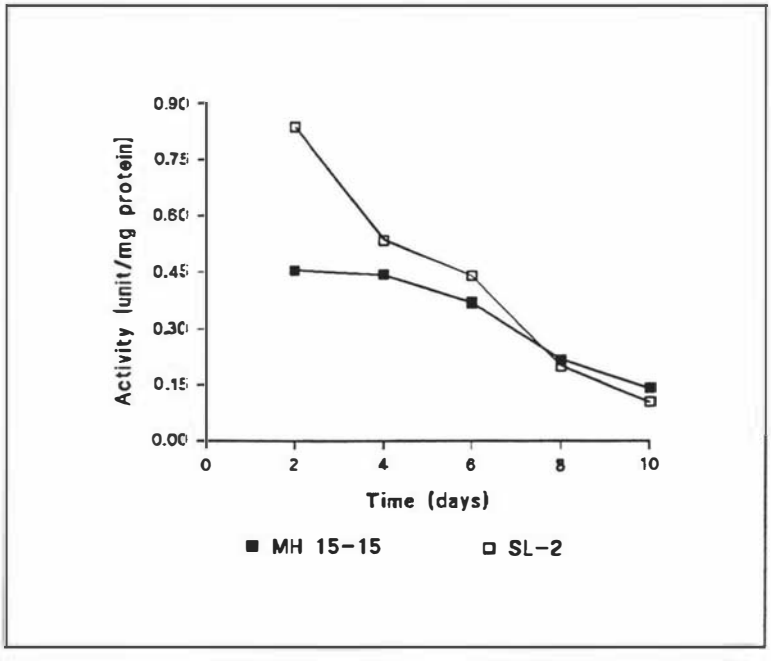


Figure 7.14 ATP:citrate lyase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid-state process

7.3.1.4 Oxaloacetate hydrolase

The presence of oxalic acid as a major by-product of citric acid production by *A.niger* has been known since early this century, although the physiology and the biochemistry of its biosynthesis remain largely unresolved (Kubicek, 1987; Kubicek *et al.*, 1988). The enzyme responsible for oxalate formation by *A.niger* has been believed to be oxaloacetate hydrolase (Cleland and Johnson, 1955; Kubicek *et al.*, 1988). As reported previously (Chapters 4 and 5), *A.niger* strains selected for this study, particularly the two strains of mutant, displayed the ability to produce and accumulate oxalic acid during citric acid production in the solid state process. As a consequence, the activity of this enzyme was investigated in all the tested strains. It is noted that the measurement was carried out only on day 2 and day 4 of the cultivation.

Figure 7.15 represents oxaloacetate hydrolase measurement of strains Yang No.2 and SI-1. The results show that the activity was markedly higher in the mutant than in its respective parent, particularly on day 2. By day 4, the activity in the mutant reduced sharply while it was completely absent in the parent.

Considering strains MH 15-15 and SL-2, oxaloacetate hydrolase was also more active in the mutant than in the parent, but the difference was less in magnitude than that observed for strains Yang No.2 and SL-1.

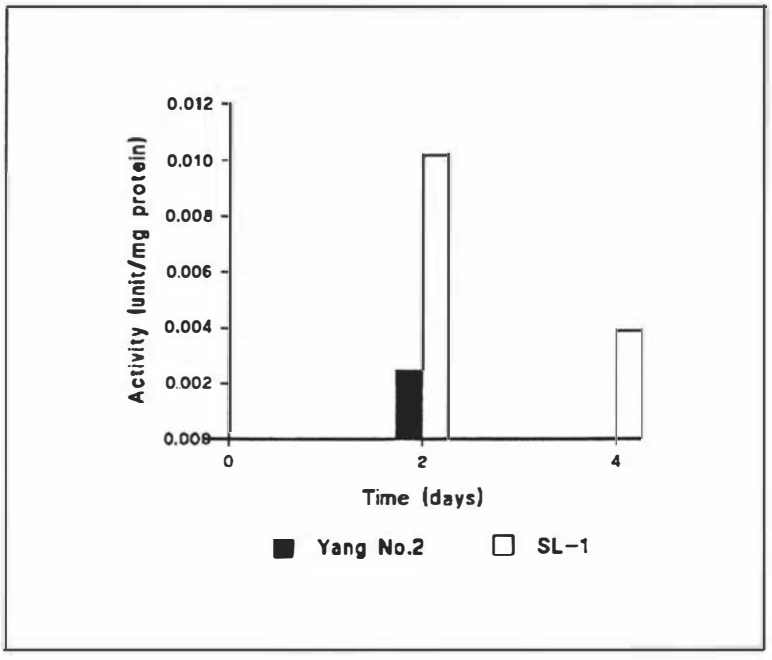


Figure 7.15 Oxaloacetate hydrolase activities of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

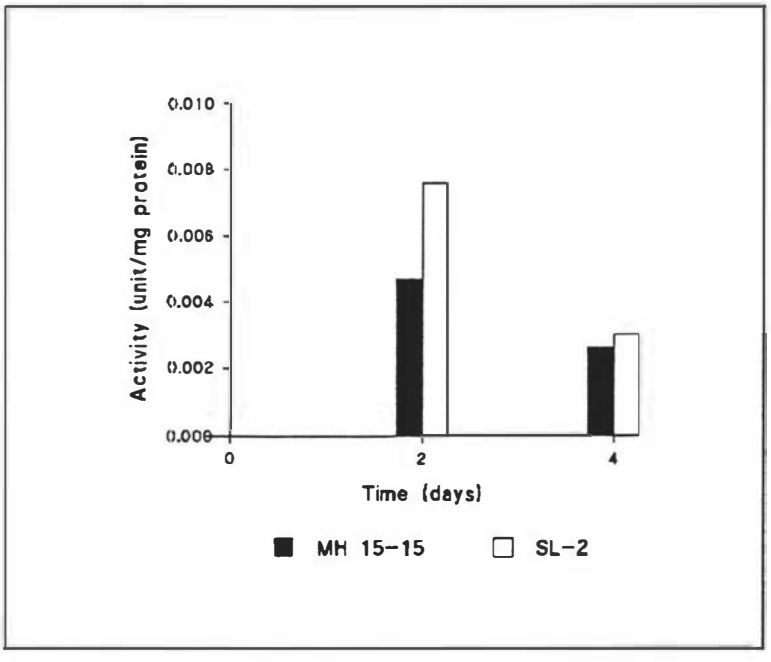


Figure 7.16 Oxaloacetate hydrolase activities of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

7.3.2 Discussion

This part of the *in vitro* enzyme measurements concerns the enzymes of some metabolic schemes other than those of the TCA cycle, which may also relate to citric acid and/or oxalic acid accumulation. The enzyme pyruvate carboxylase was investigated because of its crucial role in supplying oxaloacetate, a key metabolite, to the TCA cycle. There have been reports that pyruvate carboxylase is active when the oxaloacetate supply is reduced due to interruptions in the TCA cycle (Hossain *et al.*, 1984; Dawson, 1986). The current findings (Figures 7.9 and 7.10) affirm that the enzyme is generally more active during citric acid accumulation in both Yang No.2 and MH 15-15. It is thus proposed that, in these hyperproducing *A.niger* strains, oxaloacetate can be supplied at rates which allow citrate accumulation to continue. On the other hand, the lower pyruvate carboxylase activities observed in mutants SL-1 and SL-2 are possibly a result of the non-interrupted (active) 2-oxoglutarate dehydrogenase, thus reducing the need for this additional source of oxaloacetate. These results, therefore, do not support the constitutively produced property of the enzyme (Röhr and Kubicek, 1981; Röhr *et al.*, 1983), but rather the enzyme may be inducible, at least in part.

Regarding the role of the glyoxylate by-pass in citric acid catabolism, there has been some interest but only the enzyme isocitrate lyase, catalysing the breakdown of isocitrate to glyoxylate and succinate, has been investigated in some detail. Nevertheless, the contribution of this enzyme to citric acid accumulation in *A.niger* has remained largely unclarified. The formation of citrate and oxalate from oxaloacetate involving the glyoxylate cycle has been proposed (Müller and Frosch, 1975; Verhoff and Spradlin, 1976), and the presence of isocitrate lyase has been demonstrated in citric-acid accumulating strains of *A.niger* (Ahmed *et al.*, 1972; Dawson, 1986). In contrast, however, Kubicek and Röhr (1977) could not detect this enzyme under conditions which promoted citric acid accumulation. In the present work, the enzyme was detected in all of the strains tested, and the observed differences between each parent and its mutant were only slight (Figures 7.11 and

7.12). Based on these remarks, it has remained difficult to specify the role of isocitrate lyase to citric acid or oxalic acid accumulation by these *A.niger* strains.

Another enzyme investigated is ATP:citrate lyase which converts cytoplasmic citrate to acetyl CoA, a precursor of lipid biosynthesis, and oxaloacetate. It was found that the enzyme activity of strain Yang No.2 reduced markedly after a massive accumulation of citric acid (Figure 7.13). A similar reduction was also observed in some high-accumulating strains of *A.niger* (Pfitzner *et al.*, 1987; Jernejc *et al.*, 1991). In contrast, the enzyme of mutant SL-1 remained at relatively high levels during the entire fermentation. According to the catabolic function of this enzyme, it can be proposed that after the active growth phase, lipid synthesis in strain Yang No.2 may decline due to a deficit in its precursor while this metabolic route may remain operative in its mutant strain. Although not a prerequisite (Pfitzer *et al.*, 1987), the reduced ATP:citrate lyase activity should confer some advantages to citrate accumulation by reducing the flow of citrate to lipid.

Decreased ATP:citrate lyase activities were also demonstrated in the accumulating strain MH 15-15 during the acidogenic phase (Figure 7.14). However, the observed decrease was much less marked than in strain Yang No.2, and much less difference was observed between strain MH 15-15 and its mutant, strain SL-2. Based on these results, it is more likely that the observed activities of ATP:citrate lyase indicate the availability of cytosolic citrate as a substrate, rather than citrate accumulation being the result of decreased activity of this enzyme.

Finally, the enzyme oxaloacetate hydrolase is considered. This enzyme has been proposed to be responsible for the formation of oxalic acid, a known by-product during citric acid production, and acetate (Cleland and Johnson, 1955; Hayaishi *et al.*, 1956; Müller, 1975), and which was more active in the low- than in the high-citrate-accumulating strains in the present study. However, there are still discrepancies on the role and the location within the cells of this enzyme. The enzyme has been proposed to be located in the mitochondrion and its catalytic function involves the

TCA cycle (Müller, 1975). More recently, however, a cytosolic location of the enzyme has been demonstrated (Kubicek *et al.*, 1988). The current study verifies the presence of this enzyme in all *A.niger* strains but it was more active in the mutants than in their respective parents (Figures 7.15 and 7.16), and these findings coincide with the observed concentrations of external oxalic acid reported in Chapter 5. As a consequence, it is likely that oxaloacetate hydrolase is responsible for the formation of oxalic acid by these fungal strains.

Based on these *in vitro* enzyme activities, the following hypothesis in which the glucose uptake rate is considered as the primary drive may now be proposed to explain citric acid accumulation in *A.niger* Yang No.2 and MH 15-15, while oxalic acid is accumulated in mutants SL-1 and SL-2. In this regard, a metabolic scheme representing the metabolism of starch to citrate and oxalate, and all the necessary enzymes, is illustrated in Figure 7.17. The proposed hypothesis is :

(1) Citric and/or oxalic acid are produced when glucose is being metabolised at a greater rate, and to a greater extent than is required for growth.

(2) If the glucose uptake rate, and thus the glycolytic flux, is sufficiently high, 2-oxoglutarate dehydrogenase and, possibly, isocitrate dehydrogenases, are prevented from a normal function. Thus, citrate formation and then excretion is promoted.

(3) If the glucose uptake rate, and thus the glycolytic flux, is rather lower than the critical values in (2) above, 2-oxoglutarate dehydrogenase and isocitrate dehydrogenases continue fully functioning. Thus, there is a high carbon flux around the TCA cycle and oxaloacetate is accumulated. If the enzyme responsible for oxalate formation is active, oxaloacetate is then hydrolysed to oxalate, which acts as a 'carbon sink' and is excreted out of the cells.

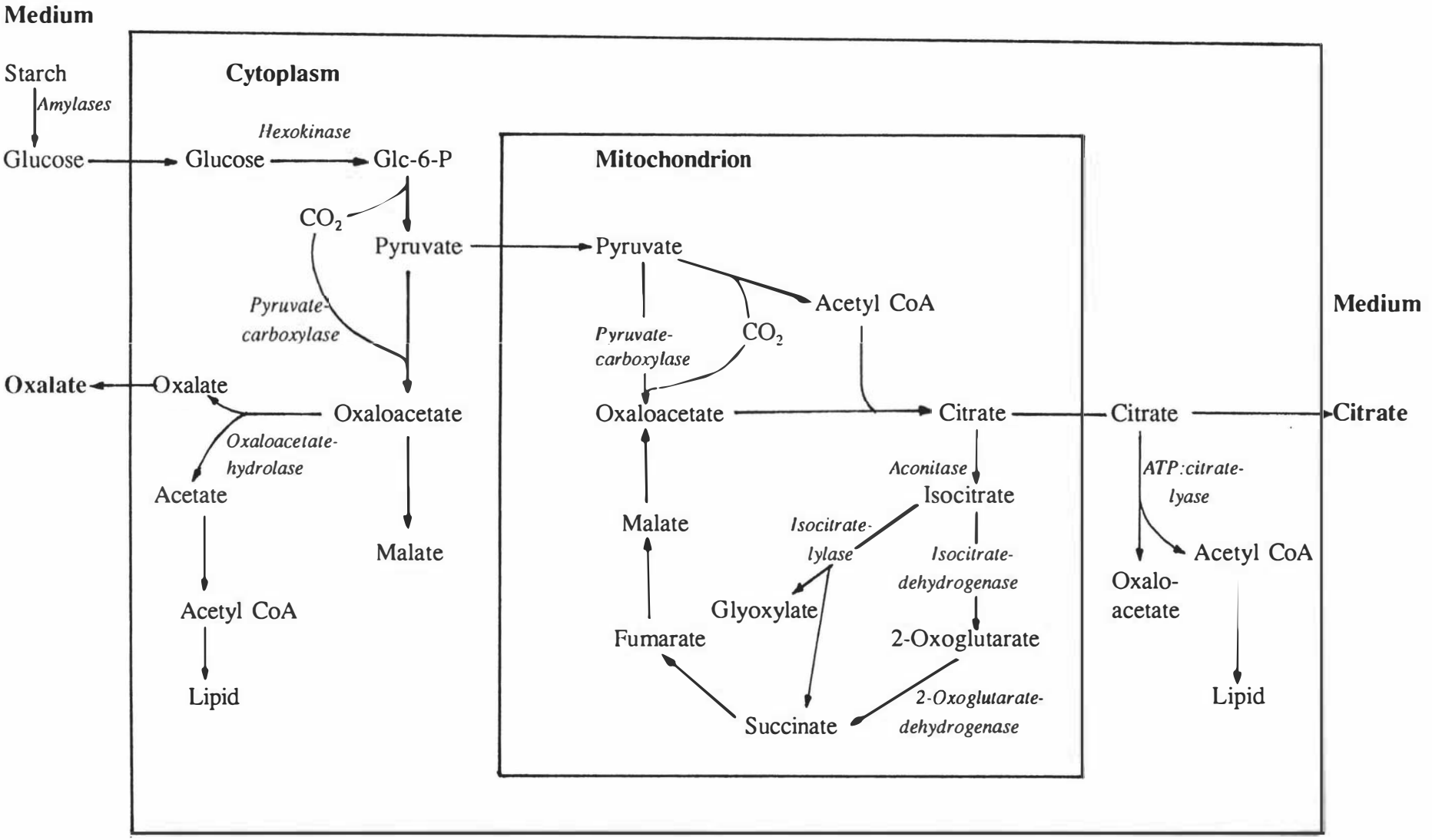


Figure 7.17 A metabolic scheme for the conversion of starch to citric acid and oxalic acid by *A.niger* (modified from Kubicek (1988) and Kubicek *et al.*, (1988)).

The concept of a high glucose uptake rate and glycolytic flux being a trigger for citrate accumulation has been proposed previously (Roehr *et al.*, 1992) but, the proposed mechanism for oxalate accumulation has never been reported before.

7.5 Conclusions

The major conclusion which can be drawn from these *in vitro* enzyme measurements relating to the high citric acid accumulation by *A.niger* Yang No.2 and *A.niger* MH 15-15 is that there are some interruptions of citrate breakdown *via* the TCA cycle. The most likely step is at 2-oxoglutarate dehydrogenase, while it remains inconclusive for isocitrate dehydrogenases. Moreover, pyruvate carboxylase is active when such interruptions occur, while the roles of other enzymes are not so direct. Thus, a hypothesis describing the primary cause of these enzyme interruptions with respect to citric acid and oxalic acid accumulation has been proposed. There remains the question, however, on the mechanism by which such enzyme interruptions occur in these *A.niger* strains, and this will be clarified to some degree in the following chapter.

CHAPTER 8

Intracellular Metabolite and Adenine Nucleotide Concentrations and Citric Acid Accumulation in Solid State Fermentation

8.1 Introduction

Although measurement of enzyme activities using the *in vitro* assay method can provide some information relating to the mechanism of citric acid accumulation, the data obtained may not represent the situation *in vivo*. For example, enzymes often require cofactors for their catalytic activity, and while these are provided in *in vitro* assays, they may or may not be present in the required concentration *in vivo*. Also, enzyme effectors may be present *in vivo*, which influence the enzyme catalytic function, but this may not be reflected in an *in vitro* assay. Therefore, examination of the concentration of a metabolic product of an enzyme in the cells may provide a more accurate depiction of the *in vivo* catalytic activity of that enzyme, and so may allow speculation of the carbon flux through that enzymatic step.

There are only a few publications dealing with intracellular concentrations of certain metabolic intermediates of the fungus *A.niger*. Smith and Valenzuela-Perez (1971), for example, studied intermediates of the Embden-Meyerhof-Parnas pathway during the growth cycle of a strain of *A.niger*. With respect to citric acid accumulation, the metabolites of the TCA cycle have been investigated in the cells of a citrate-accumulating strain of *A.niger* (Kubicek and Röhr, 1978).

In addition to the enzymatic intermediates, other important molecules which are circulated through cellular metabolism are adenine nucleotides. These phosphate-containing molecules play a crucial role in controlling many cellular activities (Chapman and Atkinson, 1977). In addition to being the direct substrates for energy

generation for many cellular functions, they also behave as allosteric effectors for many enzymatic reactions and metabolic sequences, by mechanisms of either stimulation or inhibition (Chapman and Atkinson, 1977; Krämer and Sprenger, 1993). The control of certain allosteric enzymes by certain adenylates has been studied in some citric acid-accumulating yeasts (Marchal *et al.*,1977; Mitsushima *et al.*, 1978), and has also been reported to function in *A.niger* (Kubicek, 1988). An example is the activation of NAD-isocitrate dehydrogenase by adenosine monophosphate (AMP), while its triphosphate derivative, ATP, acts as an inhibitor of the enzyme. Hence, the absolute capacity of a given enzyme should relate to the relative concentrations of these adenine nucleotides. In addition, regulation by these adenylates has also been reported for other enzymes, such as NADP-specific isocitrate dehydrogenase (Meixner-Monori *et al.*, 1985) and phosphofructokinase (Habison *et al.*, 1983).

This chapter will present and discuss the cellular concentrations of intermediary products of some selected enzymes of the four *A.niger* strains during cultivation for citric acid production. In addition, the concentrations of adenine nucleotides in the cells will be reported in order to provide a more complete depiction of cellular metabolism in relation to the mechanism of citric acid accumulation by these fungal strains.

8.2 Measurement of Intracellular Metabolites

The following metabolites were measured for their intracellular concentrations in *A.niger* strains: citrate, 2-oxoglutarate, oxaloacetate, and oxalate. All, with the exception of oxalate, are generated as the catalytic products of certain TCA cycle enzymes. These metabolites were extracted from the mycelia of *A.niger* strains cultured for citric acid production in the solid state condition, using the acid extraction method described in Section 3.2.16.4. The methods based on the enzymatic analysis (Bergmeyer, 1985), given in Section 3.2.19, were employed for the metabolite determination. The measurement was conducted on day 2 and day 4 of the time course of two separate experiments. The results, expressed as concentration per unit

biomass dry weight (μmol/g DB), are presented in comparison between each parent and its respective mutant strain.

8.2.1 Results

8.2.1.1 Citrate

As the product of interest, citrate concentrations within the fungal cells were measured since the data may indicate a relationship with the external concentrations.

Compared to the other enzymatic intermediates being measured, citrate was present in the cells at extremely high concentrations, ranging from about 33 to 205 μmol/g DB. Comparison between the high- and the low-citric acid accumulating strains revealed higher internal citrate concentrations in the former. Considering strains Yang No.2 and SL-1 (Figure 8.1), citrate was observed on day 2 at 155.2 μmol/g DB in the former, compared to only 45.3 μmol/g DB in the latter. Similar results were obtained with strains MH 15-15 and SL-2 (Figure 8.2), as this parent retained its internal citrate at as high as 205 μmol/g DB on day 2, while that of its mutant was lower, at 124.2 μmol/g DB. In addition, by day 4, the citrate concentrations of all strains decreased but remained lower in the mutants than in the parents. It is notable that a slightly higher cellular citrate concentration was present in parent MH 15-15 than in parent Yang No.2, and the difference from its mutant was not so large as observed with the latter.

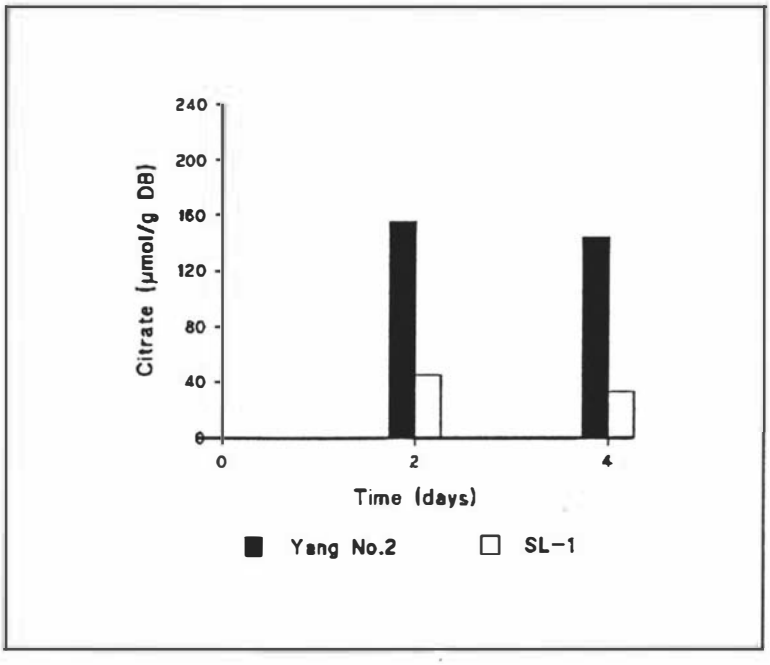


Figure 8.1 Intracellular citrate concentrations of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

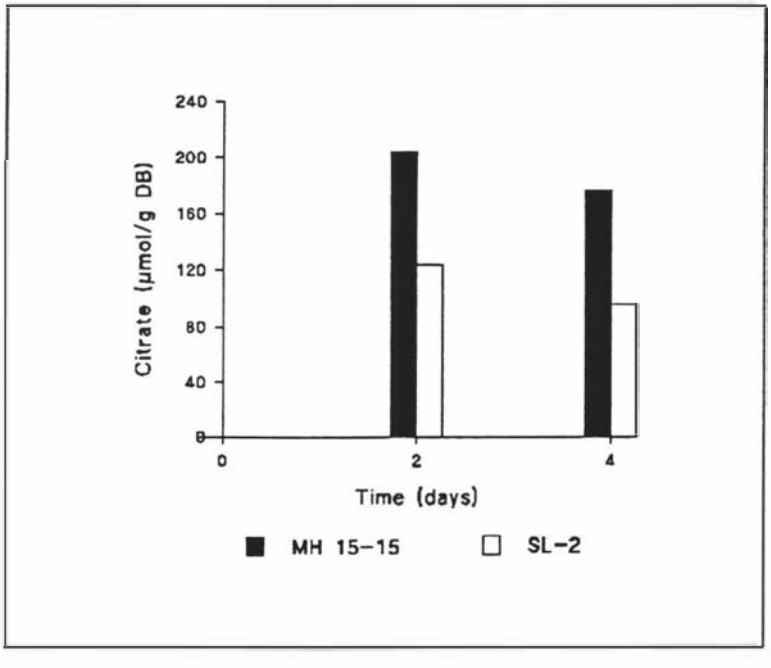


Figure 8.2 Intracellular citrate concentrations of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

8.2.1.2 2-Oxoglutarate

This metabolite is the 2-oxo-acid product of the catalytic activity of isocitrate dehydrogenase, and which will subsequently be the substrate for 2-oxoglutarate dehydrogenase. Determination of this metabolic product, therefore, could possibly refer to the operation of these two enzymes in the fungal cells.

It is noteworthy that the 2-oxoglutarate concentrations detected in the cells were considerably less, by approximately 200-fold, than the cellular citrate concentrations. Thus, the observed concentrations in all these fungal strains ranged from 0.144 to 0.760 µmol/g DB. The results show that 2-oxoglutarate was higher with day 2 than with day 4 cultures. In addition, while there appeared to be differences between the concentration in each parent and its respective mutant, these were only slight. In general, the concentrations were slightly higher with the mutants than with the parents, particularly on day 2. Hence, the concentrations were detected at 0.678 and 0.539 µmol/g DB for SL-1 and Yang No.2 strains, respectively (Figure 8.3). Similar results were obtained with strains MH 15-15 and SL-2, as revealed in Figure 8.4.

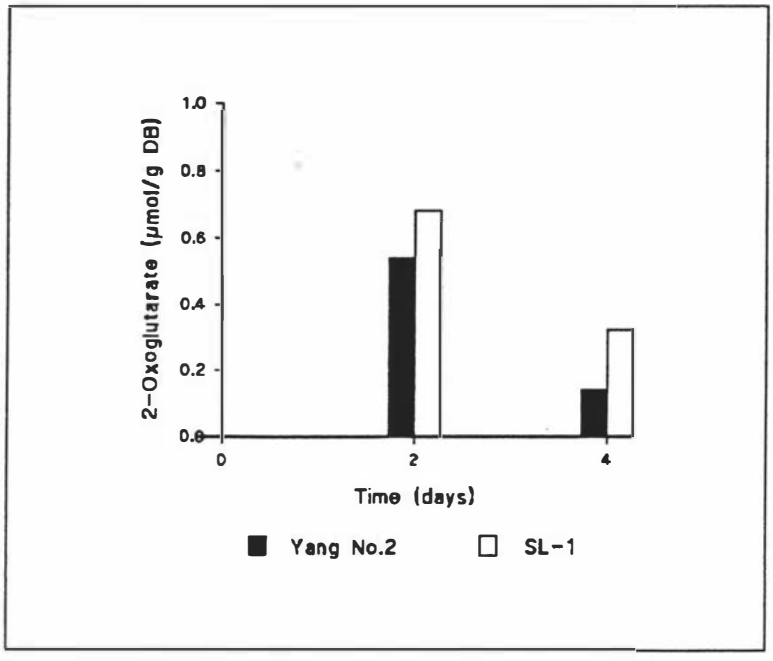


Figure 8.3 Intracellular 2-oxoglutarate concentrations of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid-production by the solid state process

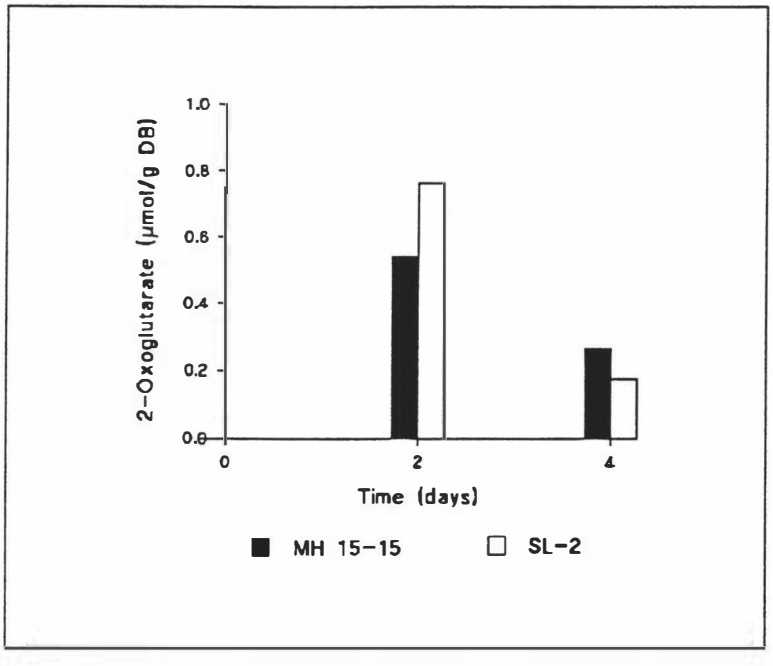


Figure 8.4 Intracellular 2-oxoglutarate concentrations of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid-production by the solid state process

8.2.1.3 Oxaloacetate

The significance of oxaloacetate to citric acid accumulation has already been emphasized (Chapter 2 and Chapter 7). This metabolite can be generated either from malate *via* the TCA cycle, or by carboxylation of pyruvate catalysed by pyruvate carboxylase. Besides being a direct precursor for citrate and oxalate formation, this molecule has been reported to act as a negative effector of 2-oxoglutarate dehydrogenase (Meixner-Monori *et al.*, 1985). As a consequence, its cellular concentrations were investigated.

It is apparent that oxaloacetate was present in the fungal cells at very low levels compared to the cellular citrate concentrations. In addition, *A.niger* Yang No.2 clearly showed a difference from its mutant in the sense that the concentration detected on day 2 of the mutant was rather higher than that of the parent (Figure 8.5). By day 4, however, both concentrations had decreased yet remained slightly higher in the mutant. In contrast, there was little concentration difference observed between strains MH 15-15 and SL-2 at each determination. In addition, oxaloacetate existing in this strain of parent was maintained at a high concentration on day 4, while it had decreased in the case of its mutant, as shown in Figure 8.6.

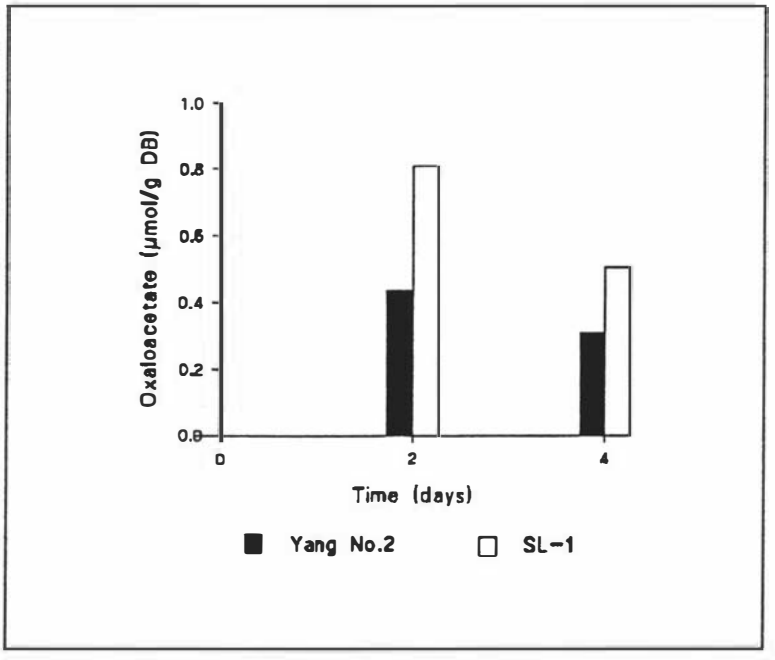


Figure 8.5 Intracellular oxaloacetate concentrations of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid-production by the solid state process

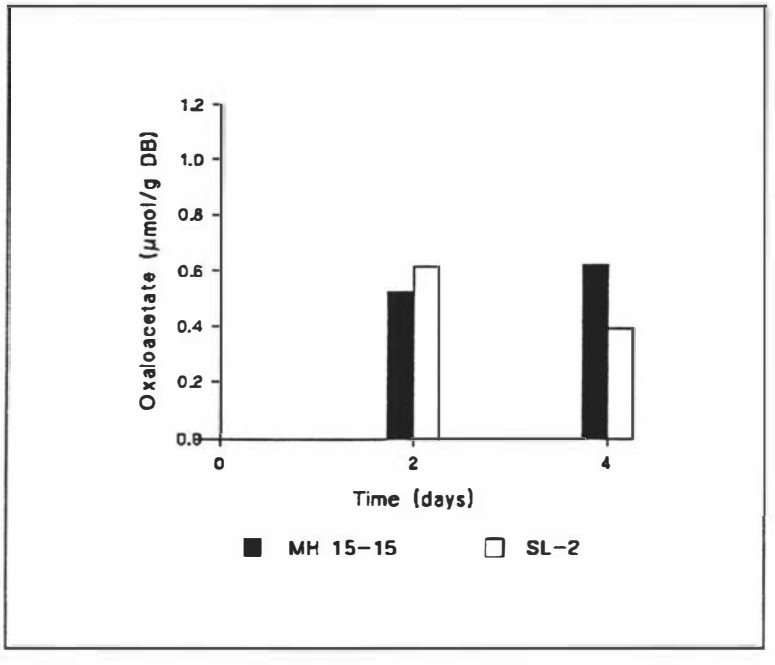


Figure 8.6 Intracellular oxaloacetate concentrations of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid-production by the solid state process

8.2.1.4 Oxalate

The occurrence of oxalic acid as a minor acid product in the culture medium of *A.niger* during citric acid production strongly suggested that its intracellular concentration should be measured. The biosynthetic pathway of oxalate is still largely unverified and there are only a few reports dealing with this subject. However, the pathway involving oxaloacetate hydrolysis by the action of the enzyme oxaloacetate hydrolase has been demonstrated in *A.niger* (Hayaishi *et al.*, 1956; Müller, 1975; Kubicek *et al.*, 1988), although there has remained controversy over the precise location of this enzyme. Since the *in vitro* activity of oxaloacetate hydrolase had been investigated (Chapter 7), measurement of cellular oxalate, a catalytic product of this enzyme, would assist in revealing the cellular function of this enzyme.

Oxalate was present at high concentrations in the fungal cells compared to cellular 2-oxoglutarate and oxaloacetate, though still much lower than cellular citrate concentrations. The data clearly reveal the presence of internal oxalate at higher concentrations with the mutants than with their parents. This situation is more pronounced in the case of Yang No.2 and SL-1 strains since the oxalate concentration on day 2 in the mutant was as high as 22.7 μmol/g DB, while only 8.7 μmol/g DB was detected in the parent (Figure 8.7). Moreover, the elevated oxalate level was retained in SL-1 mycelia through to day 4 while, in Yang No.2, it decreased to a very low level.

Figure 8.8 shows cellular oxalate concentrations of strains MH 15-15 and SL-2. Although more oxalate was accumulated in this mutant than in its parent, this was not so obvious as for strains SL-1 and Yang No.2. Moreover, by day 4 the concentrations from both MH 15-15 and SL-2 strains decreased to be relatively equal. This correlates with the observed extracellular levels.

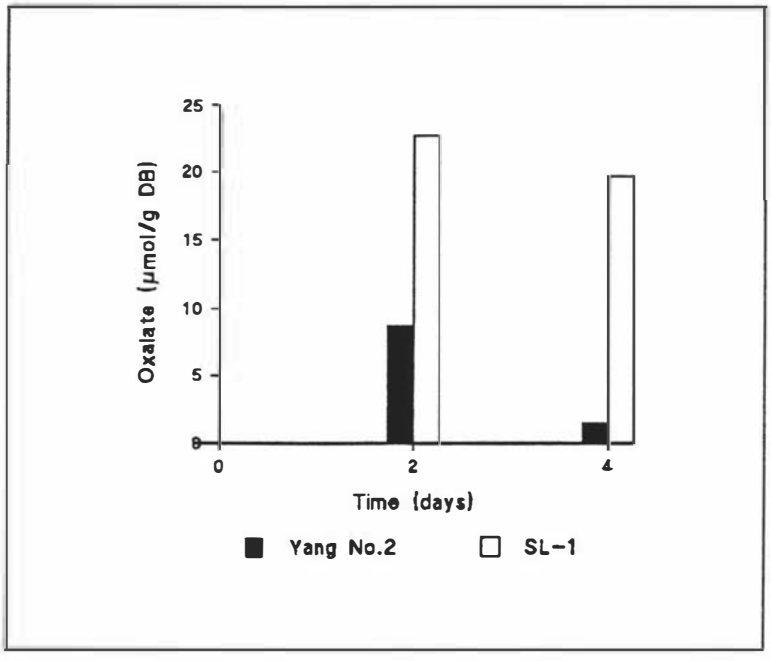


Figure 8.7 Intracellular oxalate concentrations of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

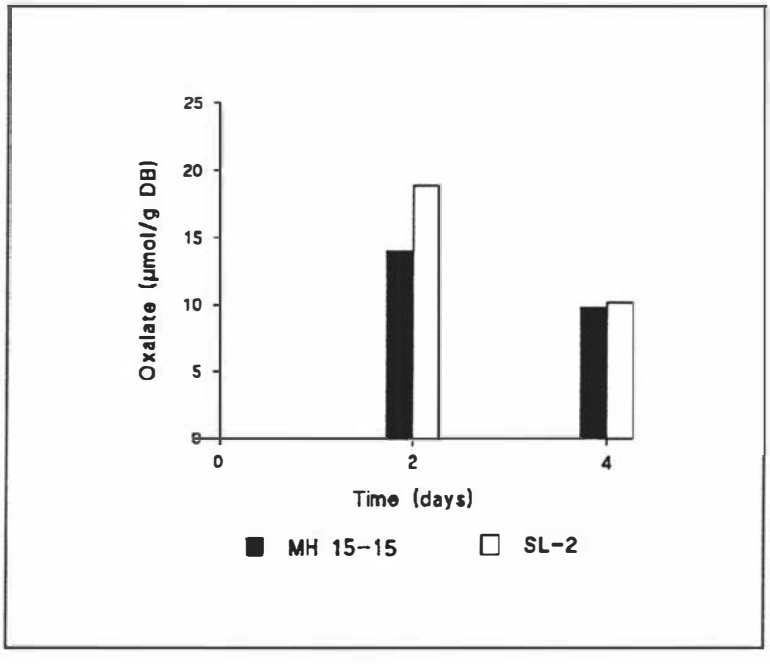


Figure 8.8 Intracellular oxalate concentrations of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

8.2.2 Discussion

In this section of the work on the mechanism of citric acid accumulation in solid state fermentation, the intracellular concentrations of citrate, 2-oxoglutarate, oxaloacetate, and oxalate have been reported. The metabolite with the highest concentration was citrate followed by oxalate, and this observation corresponds well to these acids being excreted from the mycelia and accumulating to high concentrations in the culture medium. Hence, it may be postulated that an elevated intracellular concentration of the metabolite is an immediate cause of its excretion and accumulation.

The point, however, is to determine the reason for the rise in intracellular concentration of a metabolite. In the present work, two different strains of *A.niger* have been investigated. The major reason for using two strains was to determine if the same mechanism of citrate accumulation could be applied to both. Because of the somewhat different results obtained in the present part, it is proposed to deal with each strain on its own.

With *A.niger* Yang No.2, and its mutant, SL-1, both accumulated high concentrations of citrate (Figure 8.1) and oxalate (Figure 8.7) in the cells. However, whereas in the parent, citrate predominated, in the mutant the relative concentrations shifted in favour of oxalate. Thus, it is postulated that there is a correlation between enhanced oxalate formation and reduced citrate synthesis of the mutant. Before any explanation can be sought, however, one must realise that there exists a controversy on the location of the biosynthetic pathway for oxalate, i.e. whether or not the TCA cycle is involved.

Considering the possible involvement of the TCA cycle, as proposed by Müller (1975) and Müller and Frosch (1975), a simplified diagram, as presented in Figure 8.9, may be presented.

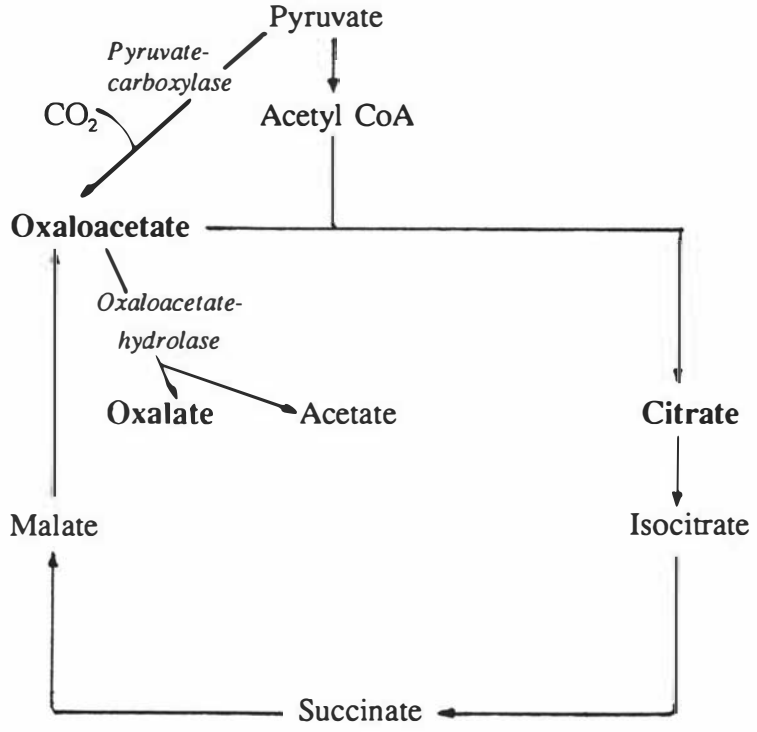


Figure 8.9 A metabolic scheme for oxalate biosynthesis involving the tricarboxylic acid cycle (modified from Dawson (1986))

Based on this metabolic scheme, oxalate may be viewed as a natural "sink" for excess carbon going around the TCA cycle. If both the TCA cycle and the enzyme pyruvate carboxylase are operating, and there is no "drain" of the TCA cycle intermediates for biosynthetic purposes (i.e. when growth is slow), then the oxaloacetate concentrations within the cells would continually increase. Hence, oxalate may behave as a natural "sink" to remove excess carbon *via* oxaloacetate which is generated through the TCA cycle. Indeed, the rise in intracellular oxaloacetate concentrations observed with mutant SL-1 (Figure 8.5) corresponds well with the elevated internal and external concentrations of oxalate which have been reported.

Citrate, on the other hand, is not usually considered to be a natural carbon sink, but rather, is an intermediate in a cycle which operates to supply a range of metabolites for biosynthesis. Hence, an explanation must be sought as to why citrate, rather than,

or in addition to, oxalate accumulates. The occurrence of high intracellular citrate concentrations in *A.niger* Yang No.2 agrees well with the hypothesis put forward previously (Chapter 7, Section 7.3.2), in which high fluxes of glycolysis arising by high rates of glucose uptake are responsible. Based on the proposed concept, under the situation of strong formation of citrate, 2-oxoglutarate dehydrogenase, and perhaps, isocitrate dehydrogenases, are less active because of certain negative control mechanisms. However, the fact that 2-oxoglutarate, a catabolic intermediate of these metabolic steps, did not accumulate to any great extent (Figures 8.3) is, perhaps, not too surprising, since both isocitrate dehydrogenases and aconitase are reversible, and the equilibrium lies towards citrate. Hence, citrate acts as the carbon "sink" when a high glucose uptake rate, together with certain defects in the TCA cycle, prevents the "oxalate sink" from removing the excess carbon from the TCA cycle. In fact, citrate may be viewed as the major "sink" if the TCA cycle becomes interrupted.

Considering strains MH 15-15 and SL-2, the situation is not so clear, although it may only be a matter of degree. Compared to its parent, mutant SL-2 accumulated relatively lower citrate in the cells (Figure 8.2), corresponding to a lower external concentration. The rise in cellular oxalate concentration in this mutant (at least on day 2, Figure 8.8), again corresponds to its higher external concentration. However, no clear difference was observed between the intracellular oxaloacetate concentrations of these two strains (Figure 8.6). Therefore, it is not so clear as to what is providing the "drive" for enhanced accumulation of oxalate in mutant SL-2.

If, on the contrary, the oxalate biosynthetic pathway is independent of the TCA cycle, but rather is exclusively cytosolic, as proposed by Kubicek *et al.* (1988), the above postulations should be modified as shown in Figure 8.2.

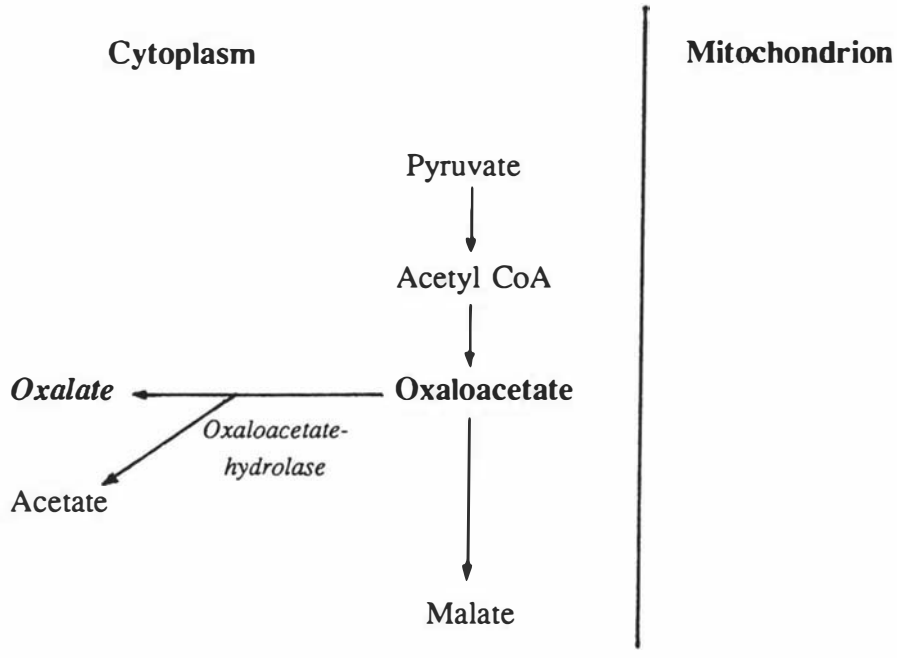


Figure 8.10 A metabolic scheme for cytoplasmic oxalate biosynthesis (modified from Kubicek *et al.*, 1988)

Based on the above metabolic scheme, oxalate remains the hydrolysis product of oxaloacetate. Hence, a rise in oxalate precursor, oxaloacetate, in mutant SL-1, may also correspond to the high internal and then, the high external oxalate concentrations. The cause of the elevated level of cellular oxaloacetate in this mutant, hence, is considered. One possibility is that mutant SL-1 may be defective at dehydrogenation of oxaloacetate, catalysed by cytosolic malate dehydrogenase. However, considering the fact that this enzyme was not determined and that the absolute concentrations of metabolites in individual cellular compartments are unknown, this concept remains unverified.

In addition, if oxalate synthesis is conclusively cytoplasm-located, its formation may be irrelevant to citrate being formed *via* the mitochondrion-located TCA cycle, since their common precursor, oxaloacetate, can not interchange between the cytoplasm and the mitochondrion (Roehr *et al.*, 1996). However, oxaloacetate has its origin from pyruvate which is the terminal product of glycolysis. Hence, with *A.niger* Yang No.2, which has been shown to have active glycolysis, oxaloacetate may be formed at a high

rate from pyruvate which enters the mitochondrion and so allow an increase rate of citrate formation in the mitochondrion. Meanwhile, the mitochondrial oxaloacetate may assert a negative effect on the catalytic function of 2-oxoglutarate dehydrogenase (Meixner-Monori *et al.*, 1985). However, given the higher cellular oxaloacetate concentration (Figure 8.5) and the higher activity of this dehydrogenase (Chapter 7) with mutant SL-1 than with parent Yang No.2, this negative control mechanism is questionable.

Alternatively, the defective glycolytic capacity of mutant SL-1, in cooperation with its activated oxaloacetate hydrolase, may, somehow, allow the cytosolic oxalate synthesis from oxaloacetate at a high rate independently of the TCA cycle, and oxalate is then is excreted into the culture medium.

In the case of *A.niger* MH 15-15 and SL-2, these postulations based on the cytosolic pathway of oxalate formation can also be applied to explain oxalate accumulation in the mutant while citrate accumulated in the parent, although the situation is not so conclusive as for the previous strains.

8.3 Measurement of Adenine Nucleotides

The role of adenine nucleotides in cellular functions and, possibly, in regulation of citric acid accumulation has been mentioned. In this section, the mycelial contents of the three derivatives of adenine nucleotides: ATP, ADP, and AMP, are reported. These adenylates were extracted from the fungal cells using the acid extraction method as for metabolite extraction (Section 3.2.16.4). Determination of these molecules was based on the enzymatic analysis technique (Bergmeyer, 1985), as described in Section 3.2.19. The measurement was conducted over a period of 10 days and the results, which were averaged from two separate experiments, are expressed as concentration per unit dry weight of fungal biomass (μmol/g DB). The data are presented in comparison between each high-and its low-citrate accumulating strains.

8.3.1 Results

8.3.1.1 Adenine 5'-triphosphate (ATP)

ATP is a high energy biological molecule which actively participates in a range of cellular processes and reactions which involve conversion or expenditure of energy. In filamentous fungi, this nucleotide is formed by oxidative or substrate-level phosphorylation of adenosine dinucleotide (ADP). It also plays a role in the control of certain allosteric enzymes.

According to the data from the present study, ATP was present at generally high concentrations during the early growth phase of all the cultures, after which it decreased through to the end of the process, though at different degrees among these strains.

Comparing each parent with its respective mutant, the ATP concentrations of strains Yang No.2 and SL-1 displayed little difference, though perhaps those of the latter were slightly higher towards the end of the fermentation (Figure 8.11). In contrast, strain MH 15-15 showed consistently higher levels of ATP than did strain SL-2 (Figure 8.12). In addition, this adenylate remained at high levels in the cells of MH 15-15 after day 2, while it dropped sharply with strain Yang No.2.

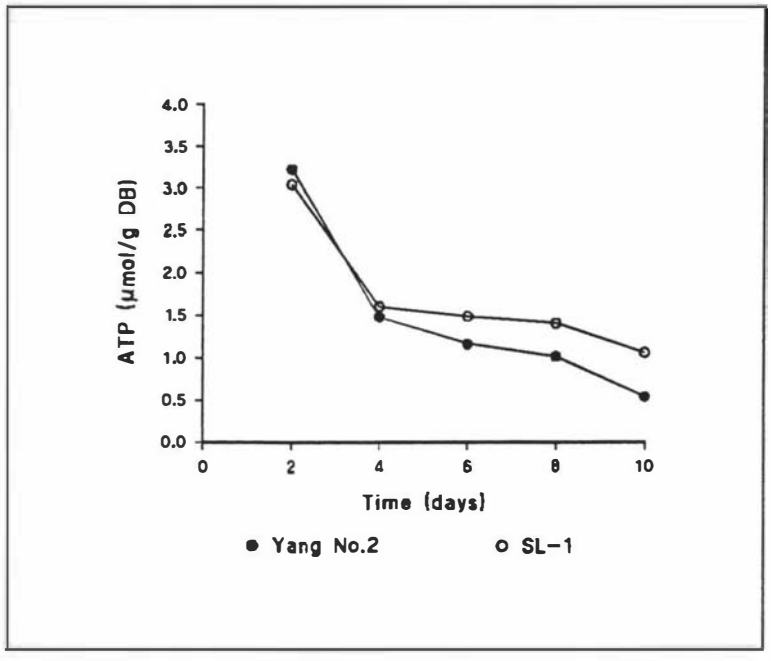


Figure 8.11 Intracellular ATP concentrations of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

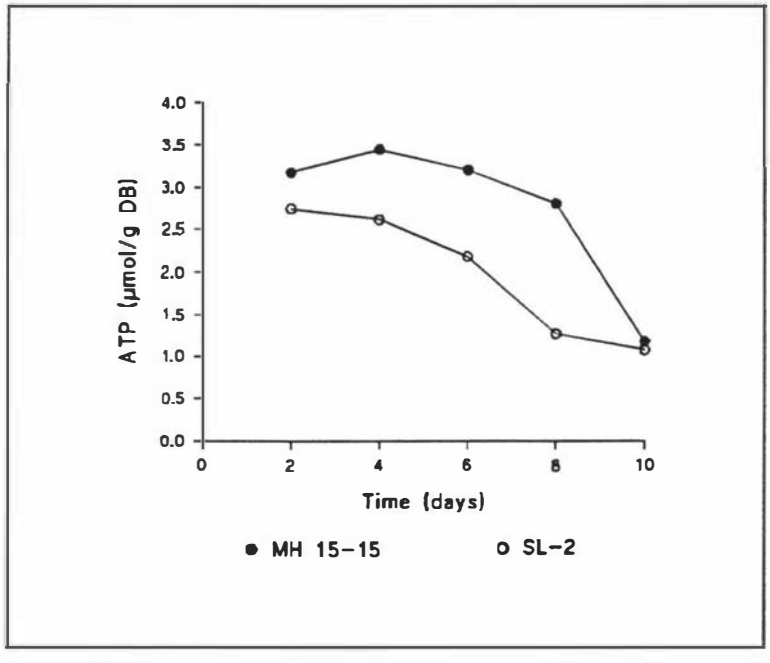


Figure 8.12 Intracellular ATP concentrations of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

8.3.1.2 Adenosine 5'-diphosphate (ADP)

As an intermediary product of ATP, as well as being its precursor, ADP also plays an important role in the energy conversion of the cells. The concentrations in these *A.niger* strains during the course of citric acid fermentation, hence, were investigated.

This adenylate, although present in the fungal cells at very low levels compared to those of ATP, was at relatively high concentrations during the early growth phase, after which the concentrations decreased through to the end of the fermentation. The data displayed in Figure 8.13 show a marked difference between the cellular ADP contents observed with *A.niger* Yang No.2 and SL-1 on day 2 in such a manner that the observed concentration in the mutant was lower, about half that of its parent. As the fermentation progressed, however, this adenylate remained reasonably constant and equal between the two strains.

With *A.niger* MH 15-15 and SL-2, the observed ADP concentrations were generally slightly higher in the parent than in the mutant (Figure 8.14).

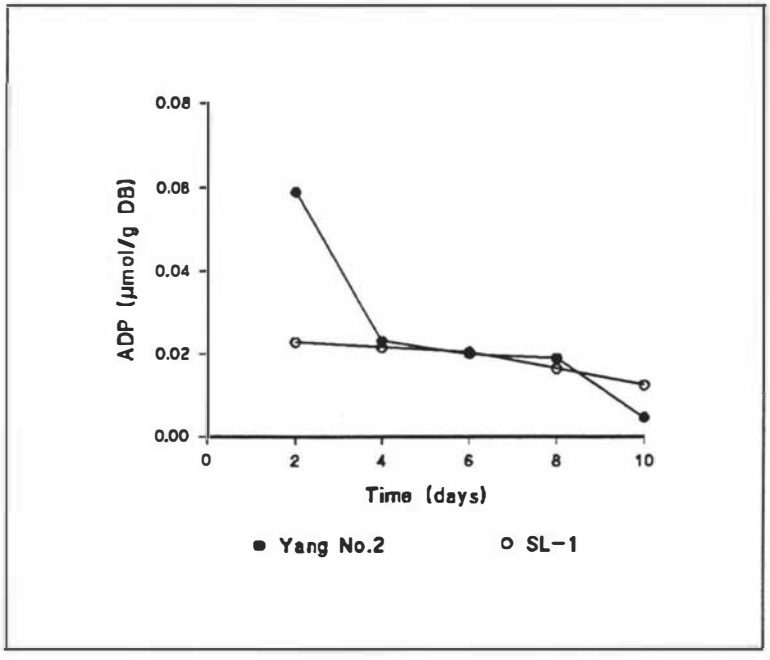


Figure 8.13 Intracellular ADP concentrations of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

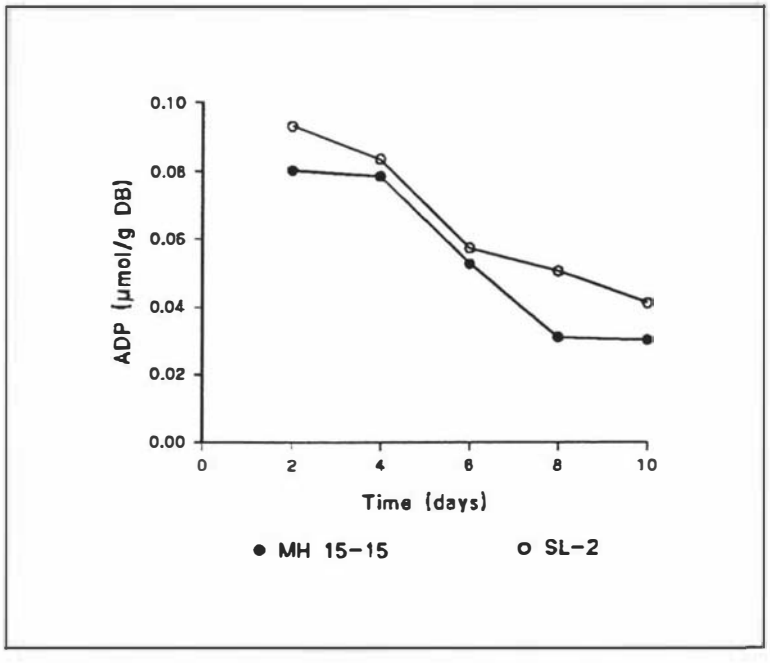


Figure 8.14 Intracellular ADP concentrations of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

8.3.1.3 Adenosine 5'-monophosphate (AMP)

The contribution of this monophosphate adenylate in allosterically controlling the activity of certain enzymes involving accumulation of citric acid has been proposed. Enzymes which have been reported to be activated by AMP are phosphofructokinase and isocitrate dehydrogenase, both the NAD-and the NADP-specific types.

The results show that AMP concentrations, although much lower than cellular ATP, were relatively high during the early growth phase, after which they decreased as the fermentation proceeded. A marked difference was observed between strains Yang No.2 and SL-1 during the later growth phase. Thus, the concentrations were comparably high on day 2 but these soon dropped sharply in the parent while only a small change was observed in the mutant (Figure 8.15). On day 10, the concentration remained higher in the mutant than in the parent.

The cellular AMP concentrations observed in strains MH 15-15 and SL-2, in contrast, were similar. In addition, the concentrations in both cultures decreased simultaneously during the time course. These results are presented in Figure 8.16.

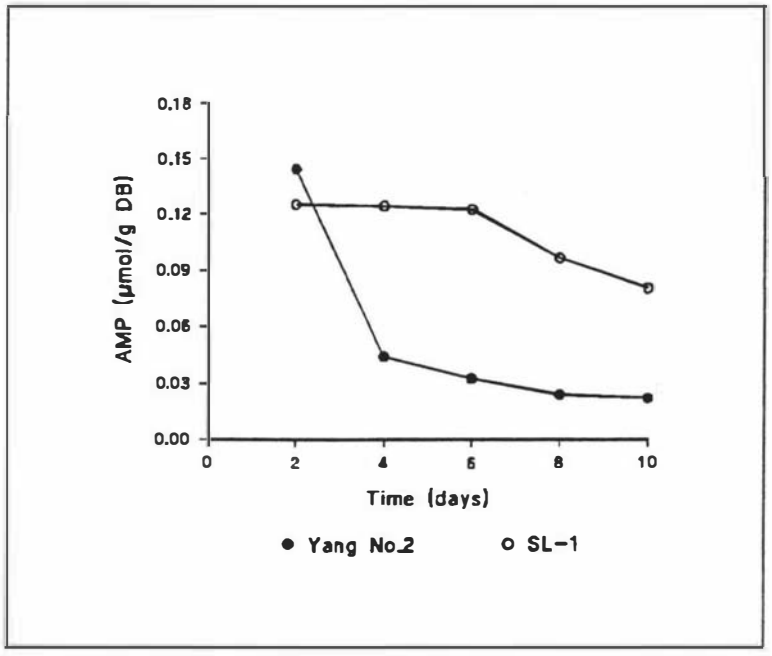


Figure 8.15 Intracellular AMP concentrations of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

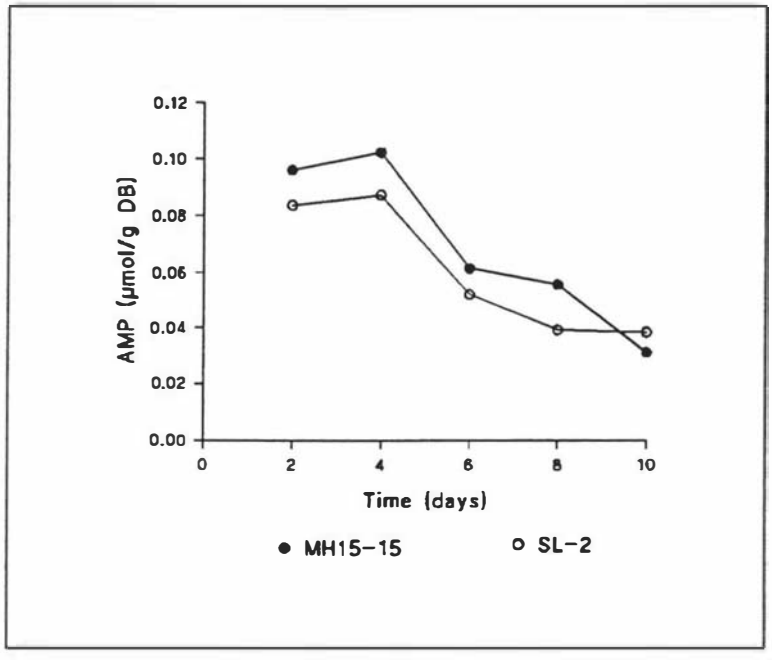


Figure 8.16 Intracellular AMP concentrations of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by the solid state process

8.3.2 Discussion

The concentrations of adenine nucleotides in the cells of the four strains of *A.niger* were investigated. ATP was present at considerably higher levels compared to the others two adenylates, ADP and AMP. Generally, the concentrations of all these molecules were high during the early growth phase and declined as the fermentation progressed. However, there are notable differences between the two high-citrate accumulating strains, and also between each parent and its respective mutant, with respect to adenylate concentrations. Hence, for the same reason as for the discussion on metabolite concentrations, it is relevant to consider the adenylate results through each strain of the parent.

In the case of *A.niger* Yang No.2 and its mutant, the presence of high ATP levels on day 2 corresponds with the work of Kubicek *et al.* (1980) who reported a high ATP content in a citrate-accumulating strain of *A.niger* during the active growth phase in submerged fermentation. The major source of ATP during the condition promoting accumulation of citric acid, i.e. when growth is restricted and the requirement for energy of the cells is declining, has been postulated to be *via* substrate level phosphorylation of glycolysis which is active during citric acid accumulation phase.

Comparison of the adenylate patterns between the Yang No.2 and SL-1 strains reveals a significant difference between the AMP concentrations (Figure 8.15), and the implication of this should be considered. As stated earlier, ATP and AMP act as regulators of certain allosteric enzymes. However, because of the interchangeable property of these adenylates, the relative, rather than the absolute, concentration of the individual adenylate is more useful as a parameter as to whether an enzyme is activated or inhibited. Based on this concept, ATP/AMP ratios were used to evaluate the allosteric control of NAD-isocitrate dehydrogenase in a citrate-accumulating yeast, *Candida lipolytica* (Mitsushima *et al.*, 1978). From the ATP and AMP concentrations measured in the present work, the ATP/AMP ratios were determined.

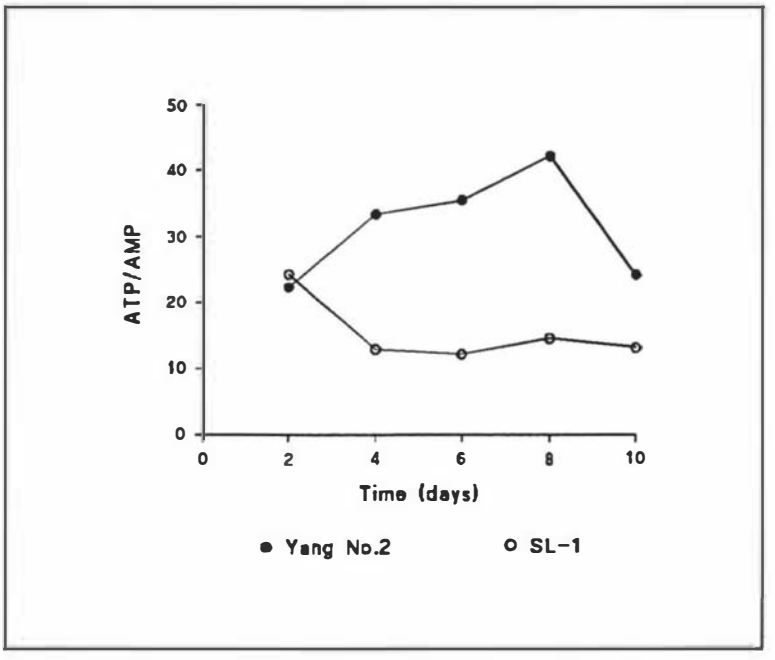


Figure 8.17 Molar ratios of ATP to AMP of *A.niger* Yang No.2 and *A.niger* SL-1 during citric acid production by the solid state process

As shown in Figure 8.17, considerably greater ratios of ATP/AMP were observed with strain Yang No.2 than with strain SL-1, particularly during the later growth phase. Since AMP acts as an allosteric activator of the NAD-specific and also of the mitochondrial NADP-specific isocitrate dehydrogenases of *A.niger* (Meixner-Monori *et al.*, 1986; Kucicek, 1988), while ATP is a negative effector of these enzymes, it may be postulated that these enzymes are less active in parent Yang No.2 than in mutant SL-1. The fact that such differences were not so pronounced during the *in vitro* enzyme activity measurements (Chapter 7, Sections 7.2.1.2 and 7.2.1.3) can be explained by the condition under which the enzymes were assayed, in which their cofactors were provided in excess. If this postulate is true, then it implies that a second blockage, in addition to, or instead of, that at the 2-oxoglutarate dehydrogenase step, may exist in the TCA cycle to allow accumulation of citrate. Thus, the hypothesis proposed in Chapter 7 can be expanded in that a high rate of glucose uptake and glycolytic flux cause a rise in the relative concentration of ATP to AMP within the cells of parent Yang No.2, leading to an inhibitory effect on these isocitrate

dehydrogenases. Since aconitase is reversible, with an equilibrium strongly favouring citrate, citrate then accumulates in the cells and, subsequently, is excreted into the culture medium.

Similarly to the previous strains, these adenine nucleotides were also present in high levels during the active growth phase of *A.niger* MH 15-15 and SL-2, after which the concentrations fell. However, a marked difference was observed for the ATP, rather than the AMP, concentrations which were lower in the mutant than in the parent (Figure 8.12). In order to relate this with the control of an enzyme, the ATP/AMP ratios were determined from the absolute concentrations of each nucleotide, and are shown in Figure 8.18.

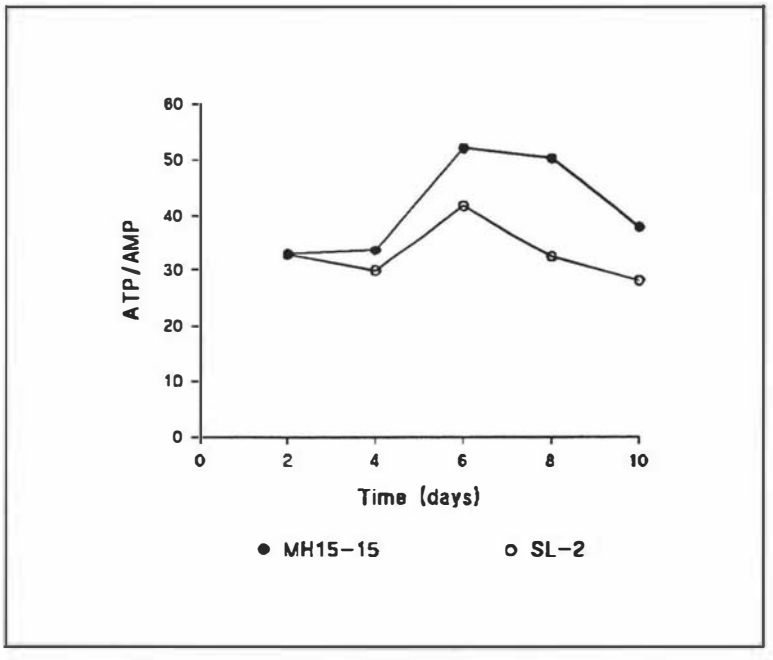


Figure 8.18 Molar ratios of ATP to AMP of *A.niger* MH 15-15 and *A.niger* SL-2 during citric acid production by solid state process

Although the ratios were generally higher with the parent than with the mutant, the differences are not as great as those between Yang No.2 and SL-1. However, it remains possible that the differing ratio allowed a greater *in vivo* activity of isocitrate dehydrogenases in SL-2 than in MH 15-15. In other words, the enzymes of the latter

were less operative. As a consequence, a similar hypothesis, as proposed for Yang No.2 and SL-1, is applicable to the mechanism by which more citric acid is accumulated in *A.niger* MH 15-15 than in mutant SL-2.

8.4 Conclusions

Evidence provided in this chapter, both on metabolites and adenine nucleotides, could indicate reduction of the carbon flow through isocitrate dehydrogenases in addition to, or instead of, through the 2-oxoglutarate dehydrogenase step of *A.niger* Yang No.2 and MH 15-15. A hypothesis explaining the regulation of isocitrate dehydrogenases by ATP/AMP ratios has thus been proposed. The role of oxaloacetate in citric and oxalic acids accumulation, though not completely clear at this stage, has also been attempted. It is possible that the mechanism by which high concentration of citric acid is accumulated is somewhat different in each of the two *A.niger* strains, although this may be a matter of degree rather than of mechanism *per se*.

CHAPTER 9

Final Discussion and Conclusions

The mechanism by which *A.niger* accumulates high concentrations of citric acid in a solid state fermentation condition has been studied. The two high-citric acid-yielding strains, Yang No.2 and MH 15-15, were chosen to represent the fermenting organisms. Mutants SL-1 and SL-2, with decreased citric acid yields were used for appropriate comparative studies, including fermentation kinetics and certain physiological and biochemical properties.

It is postulated that there is a common trigger causing a set of metabolic events leading to citric acid accumulation in *A.niger* Yang No.2 and MH 15-15. The most likely trigger is the rate of glucose uptake, which was shown to be markedly lower in the mutants, compared to their respective parents. Hence it is postulated that deregulation of glucose uptake is the primary cause of accumulation of high concentrations of citric acid. Other supportive findings were that the parents displayed strong *in vitro* hexokinase activity while 2-oxoglutarate dehydrogenase and, possibly, isocitrate dehydrogenase activities were weaker than in the mutant strains. Supplemented with the evidence on the cellular concentrations of metabolic intermediates and adenine nucleotides, a full hypothesis can now be proposed to describe the overall mechanism of citric acid accumulation by the two parental strains.

The hypothesis is that in *A.niger* Yang No.2 and MH 15-15 the vigorous formation and then excretion of citric acid is a means to dispose of excess carbon which is entering the cells at a rate over the requirement for growth, i.e. when growth is slow or restricted. The mechanism operating for this disposal is allosteric deactivation of NAD- and NADP-specific isocitrate dehydrogenases by cellular adenylates, and citrate, rather than isocitrate or *cis*-aconitate, is accumulated because of the equilibria of the reactions. The allosteric negative regulation of these dehydrogenases occurs because

there is excess ATP generated *via* substrate level phosphorylation during active glycolysis and thus its relative concentration to AMP, i.e. the ATP/AMP ratio, results in inhibition of these enzymes. Under this situation, 2-oxoglutarate dehydrogenase then becomes less active as a consequence of lack of its substrate. With this means, the organisms can restrict the formation of excess energy and metabolic intermediates *via* the TCA cycle while the requirement of the cells is declining.

Considering mutants SL-1 and SL-2, the lower rate of glucose uptake can be related to their decreased citric acid accumulation. Hence, when the supply of glucose is lower than that of their parents, glycolysis functions in "subexcess" capacity and the ATP/AMP ratios do not prevent isocitrate dehydrogenases from functioning. As a result, oxalate, rather than citrate, acts as a drain for the excess carbon generated through the active TCA cycle to prevent overproduction of energy and metabolic intermediates. The fact that the formation of oxalate from oxaloacetate does not require or generate energy or reducing power supports the concept of metabolic conservation of the cells through oxalate formation.

It can be stated that the deregulated glucose transport of the parents is the true primary cause determining the subsequent pathways of carbon dissimilation in the most economic way to the cells. However, since starch is the carbon source in the solid state fermentation process, it is believed that its conversion to glucose may be another important step. Further research, therefore, should be focused on kinetic properties and regulation of starch hydrolysis. In addition, the mechanism and regulation of glucose transport and glycolysis should be characterized in more detail. Other additional work may include study of the relationship between sporulation and citric acid accumulation, as this property is more pronounced in the solid state than in the submerged fermentation conditions.

Lastly, this work has provided a strong biochemical background to the mechanism of citric acid accumulation by *A.niger* in a solid state fermentation system and is the first report on this subject. Basically, the mechanism is similar to the situation in submerged fermentation conditions but the concept of the oxalate "sink" is original.

Also, it has been shown that the mechanism operating in the two strains which have no recorded genetic relationship is similar.

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