CITRIC ACID PRODUCTION USING *ASPERGILLUS NIGER* BY SOLID SUBSTRATE FERMENTATION

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To My Mother

Abstract

The aim of this work was to investigate solid substrate fermentation for citric acid production using *Aspergillus niger*, in an attempt to provide systematic information and an understanding of the process. Initial experiments were performed to select an appropriate substrate and organism. Thus, kumara and a strain of *Aspergillus niger*, Yang No.2 were found favourable for citric acid production, while potato was a poor substrate due to its excessive nitrogen content. The fermentations were carried out under various conditions, i.e. inoculum size, moisture content and particle size of the substrate to optimize these parameters. Inoculum sizes between 10^4 and 10^6 spores/40 g kumara, moisture contents between 65 and 71% and particle sizes between 4 and 6 mm were optimal for citric acid production. It was found that the organism takes up nutrient by penetrating into the substrate, thus the fermentation had a direct relationship with the available surface area. The solid substrate was found to have the potential ability to overcome the adverse effect of high concentrations of metal ions. Addition of 150 mg/kg substrate of Fe^{2+}, 25 mg/kg substrate of Cu^{2+}, 75 mg/kg substrate of Zn^{2+} and 150 mg/kg substrate of Mn^{2+} had slightly stimulatory effects on citric acid production rather than inhibitory effects. Based on the optimized conditions, the kinetics of the solid substrate fermentation in flasks were studied. The maximum observed gravimetric rate, maximum observed specific rate and overall productivity of citric acid production were 1.5 g/kg.h, 122 mg/g.biomass.h and 0.48 g/kg.h, respectively.

To develop the solid substrate fermentation process, experiments were performed in different types of reactors, including a gas-solid fluidized bed, a gas-liquid-solid fluidized bed, a rotating drum and a packed bed. Except for the packed bed reactor, these systems were found to be unsuitable for the fermentation, due to harsh conditions of abrasion, friction, low moisture supply, or combinations of these factors. The fermentation in the packed bed reactor was optimized with respect to air flow rate, bed loading and particle size. Based on these optimized conditions, the kinetics were studied,

and it was determined that the fermentation allowed much higher rates of citric acid production than were observed in flasks, i.e. a maximum observed gravimetric rate of 1.9 g/kg.h and an overall productivity of 0.82 g/kg.h. In an attempt to understand mass and heat transfer in the solid substrate fermentation, experiments were conducted in a multi-layer packed bed reactor. However, because of the complexity of mass transfer in solid substrate fermentation, the understanding of this aspect in this process was rather limited. Nevertheless, the multi-layer packed bed reactor improved the mass transfer considerably compared with the single layer packed bed with the same bed loading, and allowed precise measurement of the gradients for gases, citric acid, biomass and starch. The results suggest that the multi-layer packed bed reactor is a suitable reactor for further investigations, and has the possibility of being used for large scale production of citric acid in solid substrate fermentation.

This systematic investigation of solid substrate fermentation for citric acid production, which is the first reported, provides detailed information and understanding of this fermentation technology.

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Nomenclature

ATM	atmospheric pressure, 1 kg/cm^2
bar	pressure, 1.02 kg/cm^2
BM	Biomass concentration, in g/kg initial wet substrate
CA	Citric acid concentration, in g/kg initial wet substrate
cm	Centimetre(s)
°C	Degree Celsius
g	Gram(s)
h	Hour(s)
kcal	1000 Caloric
kg	kilogram
kj	1000 joule
l	Litre(s)
M	Mole concentration
mg	Milligram(s)
min	Minute(s)
ml	Millitre(s)
mm	Millimetre(s)
NAD^+, NADH	Nicotinamide adenine dinucleotide, and its reduced form
ppm	Parts per million
q	Specific rate, in g/g biomass.h
r	Rate of production or utilization, in g/kg.h
rpm	Revolutions per minute
RS	Resident starch concentration, in g/kg initial wet substrate
t	time, in hour or day
μ	Specific growth rate, in h^{-1}
μl	Microlitre(s)
%(w/w)	Percentage weight by weight
%(w/v)	Percentage weight by volume
%(v/v)	Percentage volume by volume

Chapter 1

Introduction

Since the introduction of submerged fermentation technology, this technique has dominated commercial production of citric acid and, consequently, research activities in this area. Our understanding of this fermentation technology is much more comprehensive than that of other technologies, such as liquid surface and solid substrate fermentations. However, with increasingly strict environmental requirements, the dominant position of submerged fermentation has been challenged, due mainly to the large amount of waste water released from the process, the limited range of substrates which can be used, and the high energy requirement. Solid substrate fermentation, because of its environmental friendliness, the wide range of substrates which can be used, its potentially higher productivity and smaller energy requirement, is becoming of interest worldwide. For citric acid production using solid substrate fermentation, Yamada (1977) reported that large scale production using starch residues was used in Japan, but no detailed description was provided. There are also several other reports where solid substrate fermentation has been described for citric acid production, and potential advantages, including the use of cheap substrates and production of high concentrations of citric acid, have been claimed. However, understanding of this fermentation technology appears very limited.

The work described in this thesis was undertaken to provide systematic information on a solid substrate fermentation process using a selected substrate which allowed easy biomass measurement. The investigation included attempts to optimize the process, and to understand the kinetics of the solid substrate fermentation both in flasks and in a packed bed reactor. The latter was chosen as an appropriate technology for the process, and preliminary studies on heat and mass transfer were conducted to provide some basic knowledge for future investigations.

Chapter 2
Literature Review

2.1 Introduction

At present, citric acid is the largest tonnage organic acid produced by commercial fermentation. Since the invention of production by fermentation late last century, development of citric acid production has progressed greatly. A prerequisite of this development has been the collaborative contributions from physiology, which provides knowledge of the fermentation mechanism, from genetics, which provides better strains of organisms, and from biochemical engineering, which provides fermentation technologies, including fermentation processes and reactors. In the past 30 years, an extensive body of literature has accumulated, including more than 500 reports published worldwide. Amongst these reports are some comprehensive reviews written from different aspects (Prescott and Dunn, 1959; Lockwood and Schweiger, 1967; Miall, 1978; Kapoor *et al*, 1982; Röhr *et al*, 1983; Milson and Meers, 1986; Dawson, 1986). However, these reviews have placed little emphasis on the development of fermentation technologies for citric acid production, despite the fact that since the first isolation of citric acid from lemon juice, its production has had a very close relationship with the development of these technologies. Every improvement in the basic technology has spurred citric acid production, particularly the development of submerged fermentation.

In this chapter, an attempt has been made to overview fermentation technologies for citric acid production, associated with an understanding of the organism and its biochemistry.

2.2 History of Citric Acid Production

The history of fermentation technologies for citric acid production can be divided into three phases. The first phase began in 1893 when Wehmer found that citric acid was

excreted by some strains of fungi, and a new era of citric acid production was commenced. However, Wehmer's efforts did not achieve successful commercial production due to problems of contamination and the long duration of the fermentation process. Currie solved these problems and optimized the fermentation medium, and brought citric acid production by fermentation into commercial use in 1923. Liquid surface fermentation was the technology used, and large amounts of citric acid were produced using this technology, until submerged fermentation was introduced. Although there were some early reports describing submerged fermentation for citric acid production (Shu and Johnson, 1948a, 1948b), application of this technology on an industrial scale, the second phase, did not come into operation until the early 1950s. Since it was introduced, however, submerged fermentation technology has dominated citric acid production. Many investigations have been carried out in submerged fermentation, and this technology has been improved considerably, with a corresponding improvement in citric acid productivity. Although the development of submerged fermentation is continuing, particularly in the development of new reactors, the third phase of development of fermentation technology may now have begun with the emergence of solid substrate fermentation. More and more research attention is being paid to solid substrate fermentation technology, because it is friendly to the environment and has other potential advantages. During this third phase, however, the submerged fermentation process will continue its dominant position in citric acid production, but it will coexist with surface and solid substrate fermentation.

2.3 Organisms

Many different groups of fungi have been found to accumulate citric acid, particularly the genera *Aspergillus* and *Penicillium*. Many strains of these two genera have been used for studies of citric acid production; Loesecke (1945), Foster (1949), and Kapoor *et al* (1982) have provided extensive reviews about them. However, only selected strains of *Aspergillus niger*, mainly mutants, have been reported to be used in commercial fermentation. Strain improvement programs have used a variety of techniques, such as induced mutation

ultraviolet light, but recently some more novel approaches have been reported. Kirimura *et al* (1988a, b) reported on diploid strains of *A. niger* obtained by protoplast fusion. Later, this group obtained 2-deoxyglucose-resistant mutant strains (Kirimura *et al*, 1992) and autodiploid strains for citric acid production from starch (Sarangbin *et al*, 1994). Islam *et al* (1986) isolated mutants of *A. niger* using gamma-ray induced mutagenesis for citric acid production from molasses. These strains were reported to have high tolerance to metal ions. No doubt, continuous efforts will be made to obtain mutant strains for use on different substrates and in different conditions.

Various yeasts, particularly those of the genera *Candida* and *Saccharomycopsis*, have been shown to accumulate citric acid, and some bacteria, such as *Bacillus licheniformis*, *Bacillus subtilis* and *Brevibacterium flavum* have been found to possess this property. Kapoor *et al* (1982) and Milson and Meers (1986) have provided comprehensive reviews of this subject. As stated above, yeasts of the genera *Candida* and *Saccharomycopsis* have been used in most investigations, e.g. Souw *et al* (1976, 1977) used a strain of *Candida*, with n-alkane as the substrate, while Tani *et al* (1990) used a strain of *Candida*, with methanol as the substrate. A strain of *S.lipolytica* was used by Maddox and Kingston (1983), while a strain of *Yarrow lipolytica* was used by Kautola *et al* (1991). Yeasts have the advantage over *A. niger* of producing citric acid at higher rate but are unable to use such a wide spectrum of substrates. Apparently, only a few commercial plants produce citric acid using yeasts, most preferring to use *A. niger*.

2.4 Nutritional Status of the Medium

Growth of organism in an appropriate medium, in association with optimum levels of several environmental factors, is a prerequisite for citric acid accumulation. In the medium, there is, firstly, carbohydrate, which is the carbon source to meet the needs of organism growth and citric acid production. Also, nitrogen and phosphate, and a range of metal ions and mineral ions are required. The amount of each individual component will affect both the organism growth and citric acid production.

2.4.1 Carbohydrate

It is generally accepted that any polysaccharide used for citric acid production by fungi, must be converted into monosaccharide by the organism. Therefore, the organism must possess the necessary enzymes to hydrolyse the polysaccharide. Many sugars can be used by *Aspergillus niger* for citric acid production. Among these, sucrose has been reported to be the most favoured carbon source (Hossain *et al*, 1984), while other sugars were favoured in the order of glucose, fructose and lactose. Galactose was found to be a poor substrate for citric acid production except in the presence of 1-3% (v/v) methanol (Maddox *et al*, 1986) and has been reported to be an inhibitor of citric acid production from glucose (Hossain *et al*, 1985). Xu *et al* (1989a) reported a similar order of preference, i.e. maltose, sucrose, glucose, mannose and fructose, but little citric acid production was observed from starch. Begum *et al* (1990) concluded that a combination of sugars, at 50% each, e.g. sucrose/glucose, glucose/sorbitol, glucose/xylose and xylose/sorbitol, could improve the citric acid yield over that obtained when using single sugars.

In present commercial practice, the raw materials which are generally used are cane or beet molasses and starch hydrolysate/glucose syrup. Röhr *et al* (1983) sorted these carbohydrates as follows:

> a) materials with low ash content, such as types of cane sugar, beet sugar and corn sugar. These materials are mainly used in submerged fermentation.
>
> b) materials with high ash content, such as types of cane molasses, beet molasses and starch hydrolysate. These materials are usually used in surface fermentation after appropriate treatment to remove contaminating metal ions.

Due to the high cost and limited supply of substrate, more and more new carbohydrates have been investigated for citric acid production, most of them in solid substrate fermentation. Starch residues have been used in Japan for large scale production of citric acid by solid substrate fermentation (Yamada, 1977). Many agricultural and food industrial solid wastes, such as apple pomace (Hang and Woodams, 1984, 1987; Hang,

1988), grape pomace (Hang and Woodams, 1985), cellulose hydrolysate from sugar cane bagasse (Manonmani and Sreekantiah, 1987) and wheatbran (Shankaranand and Lonsane, 1994), have also been used for citric acid production in solid substrate fermentation, where the process can be viewed as a waste treatment operation.

The optimum concentration of carbohydrate in the fermentation medium has been reported to be between 100-200 g/l, while 160 g/l is used in current commercial practice in submerged fermentation (Berry *et al.*, 1977).

2.4.2 Nitrogen and phosphate

Nitrogen and phosphate are generally used as the growth-limiting nutrients during citric acid production so that the fermentation operates under nitrogen and/or phosphate exhaustion during the production phase. Kristiansen and Sinclair (1978, 1979) reported that nitrogen or phosphate exhaustion was a prerequisite for citric acid accumulation in both batch and continuous fermentation. Dawson *et al* (1988, 1989) reported that nitrogen or phosphate limitation were necessary to obtain higher citric acid productivity values during fed-batch and batch fermentations. These reports suggested that nitrogen limitation was more favourable than phosphate limitation for citric acid production. However, Honecker *et al* (1989) reported that phosphate limitation had more pronounced effects on citric acid production using immobilized cells than did nitrogen limitation. There are no generally accepted data showing the exact relationship between exhaustion of either nitrogen or phosphate and the initiation of citric acid production. Röhr *et al* (1983) attempted to reveal the role of nitrogen metabolism in citric acid accumulation. However, it is likely that the effects of nitrogen and phosphate may be related to not only the physiology but also to the morphology of the organism. Furthermore, the actual limiting concentrations may vary among different strains of organism, and different fermentation conditions.

In practical terms, the limiting concentrations of nutrients to maximize citric acid production, with minimum biomass production, probably require more attention.

2.4.3 Metal ions

It is well established that the metal ion requirements for citric acid production must be investigated whenever a new strain or substrate is used (Berry *et al*, 1977; Jennings, 1988). Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} are the most sensitive metal ions with regard to citric acid production. They have received much research attention. Clark *et al* (1966) studied the effects of various metal ions on citric acid production, and concluded that very low levels of Fe^{2+}, Cu^{2+} and Zn^{2+}, and total absence of Mn^{2+} were required for high yields of citric acid. Kubicek, Röhr and their coworkers concluded that the physiological and metabolic changes of *A. niger* during citric acid production under Mn^{2+} deficiency are as follows:

a) lower levels of pentose phosphate pathway and tricarboxylic acid cycle enzymes (Kubicek and Röhr, 1977).
b) higher levels of pyruvate and oxaloacetate (Kubicek and Röhr, 1978);
c) elevated levels of amino acids during idiophase (Kubicek *et al*, 1979).
d) elevation of cell wall chitin and decreased β-1,3-glucan (Kisser *et al*, 1980).
e) increased monosome portion of ribosomes and elevated proteinase activity as well as rate of protein degradation (Ma *et al*, 1985).
f) no influence on cyclic AMP, which has been realised to have a direct influence on citric acid production (Xu *et al*, 1989c).

Jernejc *et al* (1989) found that in the presence of Mn^{2+}, the amount of total lipids in mycelia was up to two times higher than in conditions favouring citric acid production.

The metal ions, Fe^{2+}, Cu^{2+} and Zn^{2+} have been reported to have a critical influence on citric acid production. Several reports indicate that some or all of these metal ions should be absent from the medium or present in very low concentration (Shu and Johnson , 1948a, 1948b; Prescott and Dunn, 1959; La Nauze, 1966; Kapoor *et al*, 1982). Jernejc *et al* (1982) reported that no more than 40 mg/l of $CuSO_4.5H_2O$ in the medium resulted in high yields of citric acid. It is generally accepted that a high concentration of Cu^{2+} can eliminate the negative effect of Fe^{2+} (Röhr *et al*, 1983). Zn^{2+} was reported

to be critical for initiation of citric acid accumulation by Wold and Suzuki (1976).

In current practice, due to the complex composition of the commercial substrates used, particularly molasses, the presence of individual metal ions is always in excess rather than in deficit. Therefore, many investigations are related to methods for removing metal ions and to the organisms' tolerance to excessive amounts. The most used removal agent is ferrocyanide which can precipitate or complex ferrous and other ions. For example, it has been used to treat beet molasses (Horitsu and Clark, 1966), black strap molasses (Sánchez-Marroquín *et al* (1970) and tamarind seed powder hydrolysate (Purohit and Daginawaha, 1986), with a subsequent improvement in citric acid yield. However, excessferrocyanidehas a toxic effect on citric acid accumulation. Thus other agents, such as sodium dithionite, potassium pyrosulphate and sodium bisulphite have been added to eliminate the toxic action offerrocyanide(Ilczuk, 1983). Other chelating agents, such as tricalcium phosphate, hydrochloric acid (Kundu *et al*, 1984), sodium malonate, iodoacetate, sodium azide, sodium arsenate and sodium fluoride (Agrawal *et al*, 1983) have been described. Ion exchange resins have been widely used to remove the excess metal ions even in commercial practice (Kapoor *et al*, 1982), but the application is limited by the large requirement of the substrate used.

All these investigations were performed either in submerged or surface fermentations. Very few investigations have been related to solid substrate fermentations, where effects of these metal ions could be different, and the removal of excess ions would be very difficult. Shankaranand and Lonsane (1994) demonstrated that citric acid production in a solid substrate fermentation using wheat bran was resistant to high concentrations of metal ions, but the understanding of the effects appeared to be limited.

2.4.4 Other additives

In addition to metal ions, other additives have been reported to improve citric acid production. The addition of methanol to whey substrate (Hossain *et al*, 1983), apple pomace (Hang and Woodams, 1987), beet molasses (Roukas *et al*, 1987), and galactose (Maddox *et al*, 1986) have been reported to increase citric acid production. Ethanol and

isopropyl-alcohol (Moyer, 1953a, b) and acetate (Nowakawska-Waszczuk *et al*, 1984) have been reported to have similar effects.

2.5 Environmental Factors

The environmental factors required for successful citric acid production vary for different strains of organism, different media, and possibly for different fermentation technologies. There have been some comprehensive reviews of the environmental factors, e.g. Kapoor *et al* (1982), Röhr *et al* (1983) and Dawson (1986). However, the difference among fermentation technologies has not been taken account, e.g. moisture content of substrate and relative humidity (RH) of air in solid substrate fermentation. To focus on these, the environmental factors which are applicable to only one fermentation technology, have not been discussed in this section.

2.5.1 pH

The influence of the culture pH value was originally seen to be contradictory. Wehmer tried to optimize the fermentation medium for citric acid production at the beginning of this century, because low pH values inhibited citric acid production (partly due to the limitation of the strains used), while high pH values led to oxalic acid production and increased the risk of microbial contamination. With the development of mutant strains, however, the contradiction has been solved. A pH value between 2.0 to 3.0 was suggested by Berry *et al*(1977) to favour citric acid production. However, the optimum initial pH of the medium varies depending on the substrate used; a pH value of 2.5 to 4.0 was reported to be optimum for chemically defined media (Jernejc *et al*, 1982) while an initial pH value of 6.0 to 7.5 was required in molasses medium (Berry *et al*, 1977). Hossain *et al* (1983) observed that an initial pH of 4.5 was optimal in whey permeate for citric acid production, while Roukas and Alichanidis (1991) suggested an initial pH value of 3.0 in beet molasses. Control of the pH value during different fermentation phases was suggested by Kapoor *et al* (1982), where a higher pH is maintained in

growth phase, thereafter the pH is permitted to drop during citric acid production. The low pH value, on the other hand, can prevent bacteria contamination.

The pH value in submerged and surface fermentation media can be adjusted according to the particular situation, or even controlled during the period of fermentation process. However, in solid substrate fermentation, this is very difficult, and normally the pH value is allowed to change naturally with the progress of the fermentation.

2.5.2 Temperature

The incubation temperature is very important for citric acid production. The generally accepted optimum temperature ranges from 25 to 30°C to achieve high yields and rapid rates of citric acid production (Prescott and Dunn, 1959; Kapoor *et al*, 1982). Temperatures above 30°C could decrease the citric acid yield and increase oxalic acid production, while temperature below 25°C slow the organism growth and fermentation rates (Kapoor *et al*, 1982). In commercial practice using liquid media, heat exchange facilities, such as snake tubes and water jackets, are used to control the temperature. In the initial stage, warm water is supplied to maintain the required temperature, while in later stages, cool water is supplied to remove fermentation heat, thus keeping the temperature in the required range. However, the control of temperature in solid substrate fermentation becomes very difficult, due to the nature of the substrate and lack of effective reactors.

2.5.3 Aeration

Citric acid is an oxidation product of hexose sugar, and therefore, during the fermentation process, it is essential to ensure a supply of oxygen exceeding that required for the organism growth alone. Kubicek *et al* (1980) suggested that the minimum dissolved oxygen tension (DOT) in submerged fermentation for citric acid accumulation was about 25 mbar. Production increased steadily when the DOT was raised from 40 to 150 mbar, and the interruption of aeration or the lowering of DOT caused an irreversible

alteration in both citric acid production and respiration of the organism. In contrast, Dawson *et al* (1987) showed that above a DOT value of 20% of saturation there was no gross effect on citric acid production, and even if the oxygen supply was interrupted for as long as 85 minutes, during which time the culture DOT value was at zero, citric acid production recommenced after restoration of the oxygen supply. Roukas (1991) observed that increasing the DOT of the culture by raising the speed of the impeller did not lead to increased citric acid yields.

In surface fermentation, it is interesting that Sakurai and Imai (1992) concluded that the citric acid yield was constant when the dissolved oxygen tension was less than 74 % of saturation, but that it decreased in the range of 74 to 100 % (v/v). For a solid substrate fermentation, Hang (1988) reported that a high air flow rate through a packed bed reactor could improve citric acid production from apple pomace.

2.5.4 Other factors

The effects of some other factors on citric acid production have been also studied, e.g. Berovič *et al* (1982) suggested that the redox potential of submerged fermentation cultures could be related to citric acid production. Later, this group found that there was a relationship between the rheological characteristics of the culture and citric acid production (Berovič *et al*, 1991).

2.6 Fundamental Biochemistry of Citric Acid Accumulation

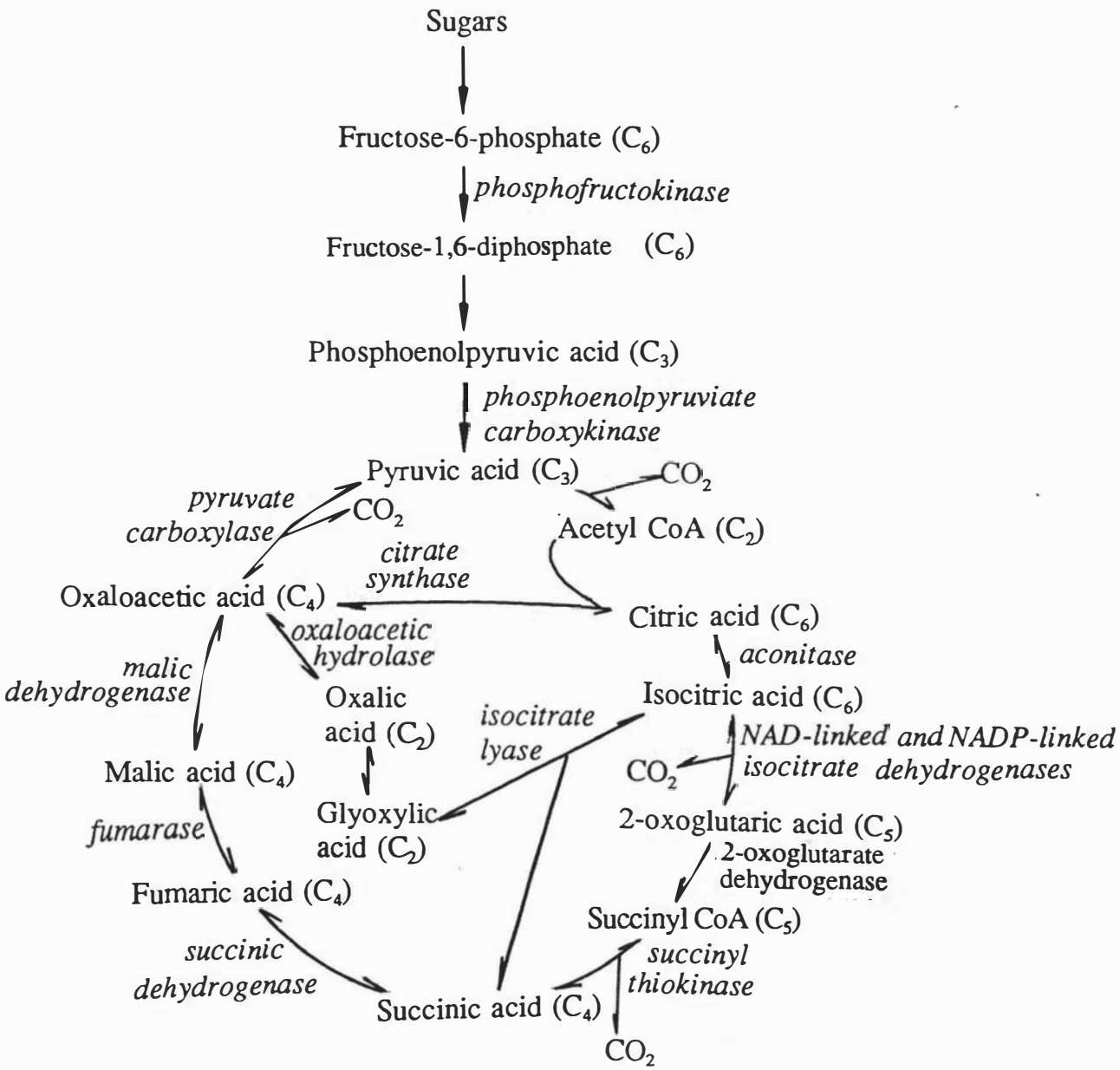
Biochemistry, or the mechanism of citric acid accumulation, has received much research attention since the beginning of citric acid production by fermentation, and there are many extensive reviews on this subject (e.g. Prescott and Dunn, 1959; Kapoor *et al* , 1982; Röhr *et al*, 1983; Dawson, 1986). However, due to the complex nature of the mechanism, there still may be a considerable gap in our knowledge. The purpose of this section is to summarize the recent reports on this subject.

It is generally accepted that the final step in the synthesis of citric acid is the condensation of acetyl CoA and oxaloacetate, as this is the major route of citric acid synthesis (Kapoor *et al*, 1982), as shown in Fig 2.1. A number of reports have been published to explain the operation of TCA cycle, and much effort has been spent to reveal the operating functions of the enzymes. Röhr *et al* (1983) and Dawson (1986) have made extensive reviews of these systems. They concluded that citric acid accumulation commences due to the incomplete operation of the TCA-cycle, when the activities of 2-oxoglutarate dehydrogenase, isocitrate dehydrogenase and succinic dehydrogenase were severely inhibited.

Recently, Legiša and Mattey (1986a) suggested that glycerol accumulation, present in the early stage of the fermentation, is capable of inhibiting mitochondrial $NADP^+$-dependent isocitrate dehydrogenase, which subsequently leads to citric acid accumulation. Further, these authors reported that glycerol accumulation in the early stages may trigger the change of carbohydrate metabolism from the pentose phosphate pathway to the glycolysis pathway (Legiša and Mattey, 1986b). Later, however, they found that the decrease of the pentose phosphate pathway was due to inhibition of 6-phosphogluconate dehydrogenase by intracellular citrate. This decrease occurred simultaneously with an increase in the activity of 6-phosphofructokinase, which regulates the glycolysis pathway (Legiša and Mattey, 1988). Legiša and Kidrič (1989) reported that there was a relationship between glycerol and the change in pH value during the fermentation.

From the technology viewpoint, understanding of the biochemistry of citric acid production is very meaningful to investigations of the kinetics, and subsequently to reactor designs. However, there are insufficient data to support this approach; only Szczodrak (1981) reported that the activities of some citrate accumulation related enzymes were much higher in submerged fermentation than in surface fermentation. However, the implications of these are unclear.

Figure 2.1 The tricarboxylic acid cycle with glyoxylic acid cycle and carbohydrate input (Dawson, 1986)



2.7 Fermentation Technologies for Citric Acid Production

2.7.1 Introduction

As generally described, there are three different fermentation technologies: a) liquid surface fermentation; b) submerged fermentation; c) solid substrate fermentation, the so called "koji" process. There are many differences among the three fermentation technologies, including the organism morphological characteristics and the substrates used. These differences can lead to different mass transfer characteristics, nutrient requirement and environmental conditions, which can subsequently lead to different reactor designs. However, although there are some extensive reviews describing the different fermentation technologies (Kapoor *et al*, 1982; Röhr *et al*, 1983; Dawson, 1986), detailed analyses associated with these differences have been rarely found. The purpose of this section is to analyze the fermentation technologies in the aspects of: substrate, morphological characteristics, mass transfer, kinetics and reactors, but to neglect the inoculation, sterilization and citric acid recovery processes.

2.7.2 Liquid surface fermentation

Liquid surface fermentation was the first used fermentation technology for citric acid production. The fermentation is generally carried out in shallow pans where the fungal mycelium develops on the surface of the medium. The system consists of, in general, fermentation rooms, in which a number of pans are mounted one over the other in stable racks. The pans, as reactors, are made of aluminium or of stainless steel, with sizes ranging from 2m×2.5m×0.15m to 2.5m×4m×0.30m, filled with medium depths of 0.08 to 0.25 m (Röhr *et al*, 1983).

After inoculation with spores, the medium is covered with a mycelial film after 2 to 3 days. During the fermentation, moist air is introduced into the room, and evenly distributed at all levels of the room. The organism is well aerated because it is in direct contact with the air. The organism takes up nutrients from the medium, and accumulated citric acid is secreted and dissolves in the medium. In this way, any product inhibition

is reduced considerably. The temperature is controlled by adjusting the air flow temperature, while the heat released during fermentation is removed by means of water evaporation, with the help of air circulation. During the intensive fermentation period, gentle mixing of the medium is necessary to reduce the temperature gradient in the medium. Therefore, in surface fermentation, the organism grows in conditions free of shear stress and product inhibition, with good aeration and nutrients supply although the contact between the organism and the medium is limited.

Beet or cane molasses, diluted to a sugar concentration of 15 to 20% (w/v), is used as a raw material for surface fermentation. Due to the presence of impurities, particularly of excessive metal ions content, the molasses are normally treated with potassium hexacyanoferrate (Röhr *et al*, 1983), or other chelating agents, including ferrocyanide salts (Clark *et al*, 1966; Ilczuk, 1983; Kundu *et al*, 1984), to precipitate or complex the metal ions. Recently, other substrates have been investigated for surface fermentation for citric acid production. Roukas and Kotzckidou (1987) used brewery wastes, and showed that the addition of metal ions (Mn^{2+}, Fe^{2+}, and Zn^{2+}) could improve citric acid production. Soluble starch (Sakurai and Imai, 1991) and cotton waste (Kiel *et al*, 1981) have also been tested as the carbon source, at least on a laboratory scale.

The pans, as the reactors for surface fermentation, have undergone no major changes since their first use. However, an innovation has been to incorporate the pans with appropriate overflow devices, so that the operation can be carried out in fed-batch and continuous culture (Röhr *et al* , 1983). A similar process was developed by Roukas and Alichanidis (1991) to retain the mycelium while replacing the fermented medium with fresh materials up to 7 times. Sakurai *et al* (1991) studied the kinetics of citric acid production, and concluded that a lower citric acid production rate is attained in surface fermentation than in all other fermentation technologies.

Due to intensive labour requirements, the high risk of contamination and the limitation of large scale production, the practical use of surface fermentation for commercial citric acid production is declining.

2.7.3 Submerged Fermentation

Since its introduction, submerged fermentation has dominated commercial citric acid production. Fermentation is carried out in an enclosed vessel, and the organism grows throughout the medium. Therefore, there are many differences from the surface fermentation, including morphological characteristics, mass transfer and substrates used.

The conventional process of submerged fermentation consists of a stirred vessel with an air supply system, incorporating a heat exchange facility, such as snake tubes and water jacket. A typical process is described thus: the organism, after 1 to 2 days, grows as pellets of approximately 0.5 cm diameter. These pellets are suspended freely in the medium, thus the organism has a huge contact surface area with the medium to take up the nutrients and oxygen if the dissolved oxygen tension (DOT) is high enough. The air flow is introduced into the vessel at high speed, and the agitation equipment mixes and breaks the air bubbles, to increase DOT levels. The heat exchange system is used to control the medium temperature at 28 to 30°C by supplying warm water or cool water depending on the fermentation requirement.

Substrates used in submerged fermentation include cane sugar, beet sugar and corn sugar. Starch hydrolysate from sweet potato and cassava are also used. The sugar concentration of the medium ranges from 15 to 27 % (w/v) (Röhr *et al*, 1983), while other nutrients, such as nitrogen, phosphate and metal ions, and even the pH value of the medium, can be controlled easily by addition or removal.

The conventional reactors used in submerged fermentation are the stirred vessel and tower fermenter, and their commercial capacity can be up to 1,000 m^3 (Brown, 1988). To increase the DOT value of the medium, the ratio of the reactor's height to diameter is up to 6:1 in the stirred vessel, and the reactor is sometimes operated under pressure to ensure higher DOT values. Another way to increase the DOT value is to raise the stirrer speed. However, this not only increases the huge energy input, but is also harmful to the organism pellets (Brown, 1988). Roukas (1991) observed that increasing

the DOT by raising the impeller speed led to decreased citric acid production. The air-lift tower reactors were introduced for citric acid production after the 1970s. Here, the higher ratio of height to diameter, up to 10:1, can increase the DOT in the medium, and its relatively large surface area for heat exchange makes the control of temperature more precise. It is possible to practise continuous operation by incorporating the vessels with feeding and draining systems.

In submerged fermentation, the filamentous organism becomes pellets which are suspended in the medium. Although the organism can take up nutrients easily, its morphological characteristics are very different from those in its natural environment. There are some published reports about these changes, e.g. Clark *et al* (1966) and Heinrich and Rehm (1982). However, the relationship between these changes and citric acid production is unknown. Nevertheless the morphological characteristics of the pellets have been reported to have a relationship with metal ions, including Mn^{2+} (Clark *et al*, 1966; Heinrich and Rehm, 1982) and/or Fe^{2+}, Zn^{2+}, Cu^{2+} (Honecker *et al*, 1992), and the nitrogen nutrient (Eikmeier *et al*, 1984).

One disadvantage of these free pellets is that it is difficult to practice continuous operation, e.g. in the air lift reactor, since the pellets are removed by the medium drainage. Recently, much research effort has been spent on cell immobilization. Anderson *et al* (1980) fixed the filamentous organism on a disc, while Vaija *et al* (1982) entrapped *A. niger* in calcium alginate beads and used them in an air-lift stirred reactor. In the latter, a 5 times higher productivity of citric acid than in batch fermentation was reported. Heinrich and Rehm (1982) immobilized *A. niger* in a glass-carrier, and used it in a fixed bed reactor for citric acid production. Eikmeier and Rehm (1984, 1987) studied the morphological characteristics of *A. niger* immobilized on calcium alginate, and developed an immobilization apparatus for use on a laboratory scale. Recently, this group has studied the influence of metal ions on the immobilized cells (Honecker *et al*, 1992). Lee *et al* (1989) developed a method for immobilizing *A. niger* on polyurethane foam, while Chung *et al* (1988) reported immobilization in hollow-fibres, and achieved continuous operation for up to 40 days, with high productivity of citric acid.

Other submerged fermentation technologies which have been developed using free pellets include repeated batch with repeated use of mycelium. On a laboratory scale, Dawson *et al* (1988) conducted fed-batch fermentation in a stirred tank reactor, while Kristiansen and Sinclair (1979) and Dawson *et al* (1988) have reported a continuous fermentation in a stirred tank reactor, but it is unclear if these techniques are used commercially.

For the kinetics of citric acid production, Kristiansen and Sinclair studied the kinetics of batch (1978) and continuous (1979) processes for citric acid production, while Roehr *et al* (1981) investigated the kinetics of a batch process on a pilot plant scale. Dawson (1986) systematically studied the kinetics of batch, fed-batch and continuous processes citric acid production. Briffaud and Engasser (1979) studied the kinetics of citric acid production in a trickle flow reactor.

2.7.4 Solid substrate fermentation

Traditionally, solid substrate fermentation has received little attention in western countries. But its use in oriental countries can be traced back more than 2000 years. For citric acid production, using starch residues from sweet potato and cassava, this solid substrate technology has been used in China, Japan and other oriental countries. It has been reported that a total of 5,400 tons/year of citric acid is produced by using solid substrate fermentation in Japan, mainly using starch waste and rice bran as substrate (Yamada, 1977). Unfortunately, no description of the process or reactor was provided by the author. The conventional solid substrate fermentation is described as follows: conidia, which are produced from rice bran or wheat bran by mixing with a spore suspension and cultivating for 3 to 4 days, are usually used for inoculation, by mixing with the substrate. The moisture content of the substrate is adjusted to 50-70%, and this inoculated substrate is conducted into trays in layer depths of 5 to 15 cm. These trays are the reactors, and have screen bottoms, allowing aeration by air diffusion across the substrate layers. They are incubated in incubation rooms similar to these used for surface fermentation. The room's temperature and atmosphere is controlled by air circulation,

and the fermentation is completed after 4-6 days. The fermented substrate is treated by water extraction for the recovery of citric acid. The extracted residues, with a rich microbiological protein content, are used as stockfeed. In this way, the solid substrate fermentation process releases little waste water during citric acid production.

One of the remarkable characteristics of solid substrate fermentation is that it is a process with no free water. Therefore, the filamentous organism is close to its natural situation, and takes in oxygen from the surrounding atmosphere and nutrients from the substrate.

The trays, as the reactors, have changed little during their history. Their temperature and aeration control is poor, so that the depth of the substrate is limited. Aidoo *et al* (1982) reported that heat generation from solid substrate fermentation for citric acid production using molasses, starch cake or rice bran ranged from 540 to 570 kcal/kg substrate. Based on these data, steep temperature gradients could exist in the substrate layer. However, according to an indirect report (Röhr *et al*, 1983), the substrate layer could be up to 1 m depth in the solid substrate fermentation process for citric acid in Japan.

A few investigations of solid substrate fermentation for citric acid production have been reported. However, the reactors used were mostly flasks, although Lakshminarayana *et al* (1975) used a conventional tray, while Hang (1987) used a packed bed reactor. Most of the investigations emphasized the development of new substrates for citric acid production, with the substrates used being: cane bagasse impregnated with sucrose (Lakshminarayana *et al*, 1975), cellulose hydrolysate (Manormani and Sreekantiah, 1987), or cellobiose (Sarangbin *et al*, 1993), apple pomace (Hang and Woodams, 1984), grape pomace (Hang and Woodams, 1985), kiwifruit peel (Hang *et al.* 1987) and wheat bran (Shankaranand and Lonsane, 1994). Hang and Woodams (1987) suggested that a moisture content of apple pomace between 65 and 75% (w/w) was optimal for citric acid production. Skankaranand and Lonsane (1994) claimed a high tolerance of the solid substrate fermentation process to the adverse effects of metal ions on citric acid production. There are no published reports about the effects of other nutrients, e.g. nitrogen or phosphate, on citric acid production in solid substrate fermentation, possibly

due to the complexity of the substrate compositions.

In summary, the present situation relating to solid substrate fermentation for citric acid production, although some potential advantages have been claimed, e.g. high yields and concentrations of citric acid, indicates that it is just the beginning of the application of this fermentation technology for this product. There has been no systematic investigation of the process, including the kinetics, the development of reactors, the effects of various environmental or nutrients requirements.

2.8 The General Solid Substrate Fermentation

2.8.1 Introduction

Solid substrate fermentation is generally defined as that in which microbial growth and product formation take place on a solid substrate with no free water. Solid substrate fermentation is present in natural environments e.g. wood rotting, plants decaying, and is widely used in the manufacture of western foods, e.g. bread and cheeses, and in eastern dairy ingredients, e.g. soya sauce, vinegar. There are extensive reviews of solid substrate fermentation, e.g. Hesseltine (1972, 1977a, b, 1981), Aidoo *et al* (1982), Mudgett (1986), Lonsane and Ramesh (1990). Doelle *et al* (1992) gave a systematical introduction from various aspects of solid substrate fermentation. This section will focus on the introduction of kinetics, mass transfer and reactor design, and some recent developments of solid substrate fermentation, related only to filamentous fungi.

2.8.2 Filamentous fungi in solid substrate fermentation

Various filamentous fungi are used in solid substrate fermentation and they have a common growth mode on the substrate. Mitchell (1992) has extensively reviewed the physiological capabilities of filamentous fungi. In summary, the basic growth mode of filamentous fungi on a solid substrate is a combination of apical extension of hyphal tips

through branching, the hyphal uptake of materials from the substrate, and the uptake of oxygen from the surrounding atmosphere to support the growth of the organism (Oliver and Trinci, 1985). Fungal hyphae extend branches to cover the surface area of the solid substrate, or extend between substrate particles in response to substrate concentration and other conditions, such as nutrients, aeration, pH and available surface area. In a low nutrients concentration, the colony is much less dense and it branches to its maximum extent (Bull and Trinci, 1977), allowing the organism to utilize the available substrate efficiently.

Another remarkable characteristic of filamentous fungi on solid substrate fermentation is the powerful penetrating ability into the substrate. With the production of hydrolytic enzymes at the hyphal tip, the organism is able to penetrate into the substrate. Therefore, the porosity of the substrate is an important advantage for the organism penetration, and thus nutrient uptake. Moo-Young *et al* (1983) stated that the physiological and morphological characteristics of the organism may change with the micro-porosity of the substrate.

Therefore, it appears that the most important feature of solid substrate fermentation is that the hyphal branches on the surface of the substrate, penetrate into the substrate, and utilize the nutrients efficiently, while the apical tips which extend outside the substrate, take in the oxygen. In this way, the growth mode of filamentous fungi in solid substrate fermentation is very efficient. However, a corollary of this mode of growth is that it is very difficult to separate fungal biomass from the substrate, as may be required when determining the biomass concentration.

2.8.3. Substrate

A very wide range of substrates can be used in solid substrate fermentation. In general, they can be divided into two groups: products and residues of agriculture, e.g. potato, cassava, wheat bran; And by-products or wastes of the food industry and human daily living, e.g. fruit pomace, trash. All these substrates have one feature in common: low

cost and the possibility of environmental pollution if no treatment. These substrates, after treatment by fermentation, may be converted to organic acids, enzymes or be upgraded by conversion of cellulosic and non-protein nitrogen contents to fungal protein (Mudgett, 1986). Reviews of the substrates used in solid substrate fermentation have been provided by Hesseltine (1972, 1981), Aidoo *et al* (1982), Lonsane and Ramesh (1990), and Mitchell *et al* (1992). From the carbon sources used by fungi, the substrates can be divided into two groups: lignocellulose and starch. Lignocellulose, as a major by product of agriculture, has the advantage of being cheap, but only a limited number of organisms can degrade it. It is represented in solid substrate fermentations by: wheat straw (Yadav, 1987; Vandevoorde and Verstraete, 1987; Valmaseda *et al*, 1991), oat straw (Levonen-Munoz *et al*, 1983), wheatbran (Thakur *et al*, 1990; Ramesh and Lonsane, 1991; Gowthanman *et al*, 1993, 1995) and apple pomace (Hang and Woodams, 1985; Hours *et al*, 1985; Ngadi and Corrcia, 1992). Starchy substrates, found mainly in different root crops, with sufficient nutrients, are easily utilized by many organisms after gelatinization. Most fungi are capable of excreting glucoamylase and amylase to hydrolyse starchy substrates. Therefore, these substrates are often used in solid substrate fermentations involving fungi. Substrates represented in the recent literature include cassava (Raimbault and Alazard, 1980; Carrizalez *et al*, 1981; Oriol *et al*, 1988; Soccol *et al*, 1994), sago-beads (Gumbira-Sa'id *et al*, 1993), rice (Sakurai *et al*, 1977; Sugama and Okazaki, 1979; Ito *et al*, 1991), and some other starch-containing substrates, such as banana wastes (Baldensperger *et al*, 1985), chickpeas (Paredes-López *et al*, 1991), buck wheat seeds (Larroche *et al*, 1988), and sweet potato residues (Yamada, 1977).

Solid substrates used in the fermentation are generally in particle form. The particle size, shape, porosity and strength are all strongly related to the surface area available to the organism and mass transfer, and subsequently to reactor performance and design. The particle size, in particular, which is readily controlled by appropriate processing while the other factors are generally described by the natural characteristics of the substrate, has attracted much attention. The particle size used varies for different substrates, e.g. for cassava meal, a size ranging from 0.8-2.0 mm was used by Soccol *et al* (1994) in a tray reactor, while an average size of 1.65 mm was used by Raimbault and Alazard

(1981). For wheatbran, 2 mm size was used by Ghildyal *et al* (1994) and Gowthaman *et al* (1993), while for sago-beads, a 2-4 mm size was used by Gumbira-Sa'id *et al* (1992, 1993). In addition, the heterogeneity of the particle substrate is a significant problem which makes study difficult and leads to poor reproducibility of the fermentation process (Daubresse *et al*, 1987: Smits *et al*, 1994). There are few systematic investigations found in the literature concerning the substrate characteristics, possibly due to the complexity of the particle substrate, and its strong relationship with the complicated mass transfer process (Mitchell *et al*, 1992; Murthy *et al*, 1993).

2.8.4 Environmental factors

In submerged fermentation, the environmental conditions can be controlled easily by agitation, aeration and the addition or removal of some nutrients. In solid substrate fermentation, however, the control of moisture content, pH, temperature and gaseous levels, and the removal of excess nutrients content are very difficult. This limits the industrial potential of the solid substrate fermentation (Kim *et al*, 1985). The Following provides a brief summary of recent investigations on these environmental factors.

2.8.4.1 Moisture content

As stated in the definition of solid substrate fermentation, the organism grows on the substrate without any free water. The moisture content required for optimal microbial growth varies for different organisms and substrates used. Prior *et al* (1992) gave a summary of the moisture content used in recent investigations, and showed that values as low as 25-35% and as high as 75% were used. This wide range of moisture contents was related to microbial growth and product formation.

For citric acid production, a moisture content of 45% was used by Shankaranand and Lonsane (1994) using wheat bran, while 65% was reported to be optimal using apple pomace by Hang and Woodams (1987). However, due to lack of biomass data, the effect of moisture content on fungal growth has not been analyzed in the literature. The reported moisture content, in general, is the value at the initial stage, and the value

declines due to evaporation of water during the fermentation. This evaporation of water, on the other hand, can remove a great deal of fermentation heat. However, the control of moisture content can be carried out by spraying water or by circulation of moist air (Carrizalez *et al*, 1981; Sato *et al*, 1982; Gumbira-Sa'id *et al*, 1993), and/or with appropriate agitation. Despite this, the control of moisture content is very difficult in practical production, especially in fermentations involving filamentous fungi, since agitation can be harmful to the organism.

Recently, another expression of moisture content, the water activity, which is based on the ratio of the vapour pressure of substrate to the vapour pressure of pure water, was introduced by Grajek and Gervais (1993), Acuña-Argüelles *et al* (1994) and Dorta *et al* (1994).

2.8.4.2 pH

The pH value of natural substrates is usually between 6.0-8.0. However, due to microbial growth, the pH value may change as nutrients are hydrolysed and products are formed. The optimum pH value for filamentous fungal growth and for formation of different products varies enormously, as summarized by Prior *et al* (1992). However, control of the pH value of the fermentation process seems impractical, for similar reasons to those described in the preceding section for control of moisture content. Few reports have been found in the literature. For organic acid production, including citric acid and gibberellic acid, the pH value is generally left to change naturally with acid accumulation. The lowest pH value of the fermentation process could be 2.0-3.0 (Röhr *et al*, 1983).

The low pH value in the substrate can prevent contamination from bacteria (Raimbault and Alazard, 1980; Gibbons *et al*, 1986;), and could aid substrate hydrolysis, especially for starchy substrates.

2.8.4.3 Temperature

Temperature is an extremely important parameter in solid substrate fermentation, due

to the low heat transfer capacity and high substrate and biomass concentration per unit volume, while the fermentation releases considerable metabolic heat. Aidoo *et al* (1982) reported that heat ranging from 419 to 2,617 KJ/kg fermenting solids was generated in koji processes for biomass, enzyme or organic acid production. Further, temperature gradients have been reported as high as 3°C/cm in soya bean fermentation (Rathbun and Shuler, 1983), and temperatures can reach as high as 60 to 70°C (Biddlestone and Graves, 1985). Thus, the steep temperature gradients, associated with the natural characteristics of the substrates, make it very difficult to remove the fermentation heat and control the temperature, particularly in large scale solid substrate fermentation.

To remove the fermentation heat, two methods are used, i.e. heat exchange and water evaporation with forced aeration. Representative heat exchange equipment include the snake tube and water jacket, which, normally, are inefficient, due to the poor conductivity of the solid substrate, and are expensive. Water evaporation with forced aeration has attracted much attention because of its capacity to remove large amounts of heat, and because it is easily controlled by adjusting the air flow rate and its relative humidity (Saucedo-Casteñeda *et al*, 1990). However, this may conflict with the control of the moisture content of the substrate. Recently, a combination of two methods has been used. Durand *et al* (1993) developed a pilot-plant reactor with a temperature and moisture content control system , in which the temperature was controlled by aeration, heat exchange and interval agitation, and the system was controlled by a computer program.

Despite considerable effort, information concerning heat transfer in solid substrate fermentation remains insufficient, partly due to the complexity of the heat transfer mechanism in the solid substrate, and heat generation during the fermentation. However, Sangsurasak and Mitchell (1993) have modelled heat transfer in a packed bed reactor, while Szewezyk and Myszka (1994) investigated the relationship between heat generation and the progress of fungal growth.

2.8.5 Kinetics

Because of the heterogeneity of many solid substrates, the complexity of mass transfer both inter-particular and intra-particular, and the difficulty of obtaining biomass data, studies on the kinetics of solid substrate fermentation have made little progress. This lack of understanding of the kinetics is a significant barrier to further application of solid substrate fermentation, especially for the development of the reactors.

Recent studies on the kinetics have been extensively reviewed by Mitchell (1992) and by Murthy *et al* (1993). Nielsen (1993) attempted the modelling of filamentous fungal growth based on the organism's morphological characteristics. To do this, the biomass was divided into different cell types, and structural models were used. To simplify the situation, some researchers have studied fungal growth on agar plates, e.g. Georgiou and Shuler (1986) used a computer model to simulate the fungal colony, while Laszlo and Silman (1994) used "cellular automata" model. These studies, although they gave some understanding of fungal colony expansion, are very difficult to apply for a particle substrate. To avoid involving the particle substrate, Sugama and Okazaki (1979) and Carrizalez *et al* (1981) used carbon dioxide data to model the growth of *A. oryzae* on rice grains and of *A. niger* on cassava flour meal, respectively. Okazaki *et al* (1980) modelled *A. oryzae* growth on rice grains based on the oxygen consumption data, and reasonable accuracy of the prediction was claimed. The gaseous data, however, may not necessarily express the production of mycelia. Mitchell and co-workers initially used an empirical model to simulate *Rhizopus oligosporus* growth on cassava flour medium, based on glucoamylase activity data (Mitchell *et al*, 1991a), and then developed a semi-mechanistic model to simulate the same system (Mitchell *et al,* 1991b). In this case, mass transfer between biomass and the substrate was taken account.

Ito *et al* (1989) have simulated distribution of *A. oryzae* on rice grains, while Nandakumar *et al* (1994) modelled degradation of wheatbran particles by *A. niger*. These cases gave an understanding of fungal growth on a particle substrate. To deal with the complexity of the substrate, Smits *et al* (1994) used a fuzzy logic model to simulate mycelial glucosinolate content during *A. clavatus* growth on rapeseed meal.

Despite the fact that much effort has been spent on the study of kinetics, a satisfactory model which is able to describe the solid substrate fermentation has not been achieved.

2.8.6 Reactors for solid substrate fermentation

Solid substrate fermentation reactors aim to provide appropriate environmental conditions for the fermentation. Although there are numerous designs of reactors found in literature, development on an industrial scale is still limited. Mudgett (1986) has provided an introduction to some industrial scale reactors used in Japan, but there is lack of sufficient description. Pandey (1991) classified the reactors as: fermentation without agitation, and with occasional or continuous agitation, while other researchers (Mitchell *et al*, 1992; Lonsane *et al*, 1985) divided them into five groups, as shown in Table 2.1. Table 2.2 gives a comparison of some basic characteristic of these reactors. The following gives a brief description of the operating process and mass transfer analysis of each type of reactor.

The tray reactor is the most popular for current commercial production, particularly in oriental countries for Koji or food fermentations (Aidoo *et al*, 1982; Mudgett, 1986). Its commercial scale is often in multi-tray. These trays, mounted one above another, are incubated in a room with air circulation. The oxygen supply, moisture content and temperature of the fermentation are controlled by the air flow. As the air is blown in a horizontal direction, the aeration and temperature of each tray are dependent on diffusion and/or water evaporation. Thus the mass transfer limits the thickness of substrate to just a few centimetres (Rathbun and Shuler, 1983). In this way, a large scale operation of this process may consist of a large room and a number of trays, where the temperature control and air distribution could be uneven, and the operation is probably labour-intensive.

The packed bed reactor has attracted much attention (Lonsane *et al*, 1985, Pandey, 1991), and has been used in several commercial applications (Mudgett, 1986), due to its potential advantages, as shown in Table 2.2. The typical design is a column with a

Table 2.1 Application of reactors in solid substrate fermentation

Type	Substrate	Capacity	Process	Organism	Reference
Tray	wheatbran	2 kg	fungal rennet	*Mucor miehei*	Thakur *et al*, 1990
Tray	bagasse+sucrose	12inx15inx2in	citric acid	*A. niger*	Lakshminarayana *et al*, 1975
Multi-tray	raw cassava	100 g	protein enrichment	*Rhizopus oligosporus*	Soccol *et al*, 1994
Packed bed	citrus peel	100 g	protein enrichment	*A. niger*	Rodriguez *et al*, 1985
Packed bed	cassava meal	450 g	protein enrichment	*A.niger*	Sauced-Castañeda *et al*, 1990
Packed bed	sago-beads	up to 350 g	protein enrichment	*Rhizopus oligosporus*	Gumbira-Sa'id *et al*, 1993
Packed bed	apple pomace	50 g	citric acid	*A. niger*	Hang, 1987
Packed bed	sugar beet pulp	1000 kg	protein enrichment	*Trichoderma viride*	Durand and Chereau, 1988
Rotating drum	wheatbran	150 g	hydrolase	*A. niger*	Nishio *et al*, 1979
Rotating drum	soya beans	NA	foods	*Rhizopus oligosporus*	Reu *et al*, 1993
G-S fluidized bed	wheatbran	400 g	enzymes	*A. niger*	Tanaka *et al*, 1986
G-S fluidized bed	sucrose	40 pound	ethanol	yeast	Röttenbacher *et al*, 1987
L-S-G fluidized bed	corncob	NA	mycelium	*Trichoderma reesi*	Adisasmito *et al*, 1987
L-S-G fluidized bed	liquid medium	1.2l	Antibiotics	*Penicillin Unticae*	Behie and Gaucher, 1988

Note:NA, not available; G-S, gas-solid; L-S-G; liquid-solid-gas.

Table 2.2 Characteristics of reactors for solid substrate fermentation

Type	Moisture control	Temperature control	Aeration control	Abrasion or Shear stress	Capacity	Mechanical complexity	Energy requirement	Developing expectation
Tray	natural	good	poor	no	limited	simple	little	large scale
Packed bed	good	fair	good	little	excellent	simple	fair	large scale
Rotating drum	good	good	good	large	limited	complicated	high	large scale
G-S Fluidized bed	poor	excellent	excellent	large	limited	simple	high	medium scale
L-G-S Fluidized bed	excellent	excellent	limited	medium	limited	simple	medium	small scale

Note:G-S, gas-solid; L-G-S, liquid-gas-solid.

perforated bottom, associated with forced aeration. The adjustable forced air flow makes it very flexible to control the temperature, oxygen supply and moisture content of the substrate. However, due to the complexity of bioreactions, and the nature of the substrate, there is insufficient understanding of mass transfer in the packed bed reactor (Mudgett, 1980). André *et al* (1981) have studied the measurement of mass transfer coefficient in a packed bed reactor, while Ghildyal *et al* (1994) and Gowthaman (1993) reported that steep gas and temperature gradients existed in this type of reactor. Gowthaman *et al* (1995) attempted to calculate the mass transfer coefficient, but unfortunately, they used an inappropriate calculation. Recently, more and more modifications have been made on packed bed reactors. Durand *et al* (1993) developed a large scale (1.6 m^3) packed bed reactor with agitation, while Xue *et al* (1992) used a 25 tons capacity packed bed reactor equipped with a movable agitation device. The addition of an agitation device to a packed bed reactor may be a great improvement for heat removal and mass transfer, however, it may also break the filamentous organism. Nonetheless, a packed bed reactor with forced aeration, an agitation device and even a heat exchange device may be the developing way for large scale solid substrate fermentation. The rotating drum reactor has many types (Murthy *et al*, 1993), and has been used for many solid substrate fermentations (Mudgett, 1986, Mitchell *et al*, 1992). In general, this reactor consists of a rotating drum and aeration system. The rotation provides agitation to the substrate, associated with aeration, so that the mass transfer and heat exchange are much improved compared to the packed bed reactor (Reu *et al*, 1993). Recently, many modifications have been made to this type of reactor, including the addition of baffles (Murthy *et al*, 1993), a heat exchange and water spray system (Mudgett, 1986; Ryoo *et al*, 1991), and a computer-controlled rocking motion (Ryoo *et al*, 1991). However, these modifications, although they may improve the reactor's performance, also complicate the reactor and increase the manufacturing cost.

With solid particles in suspension in an appropriate air flow, excellent mass and heat transfer can be provided by a gas-solid fluidized bed reactor. However, the operating procedure may be complicated, and the parameters may not be stable. In particular, the low moisture content of the air flow may restrict growth of the organism (Shügerl,

1989). Adisasmito (1987) reported that an air flow with low relative humidity made growth of *Trichoderma reesei* on corncob impossible, while it has also been reported that high humidity caused agglomeration of a particle substrate (Shügerl, 1989). Although, several reports are available using gas-solid fluidized bed reactors, most of them involve yeast, which is able to grow in low moisture conditions, and some additional equipment e.g. an impeller (Moebus and Teuber, 1982, 1985; Tanaka *et al*, 1986), was incorporated, or uneven fluidization was obtained (Hong *et al*, 1989). The only report of filamentous fungal growth in this reactor type was by Tanaka *et al* (1986), but the growth was limited. Due to the necessity for low humidity, and the high energy requirement, the application on a large scale of this type of reactor may be limited to a few organisms, and only for production of a few valuable enzymes (Shügerl, 1989; Bauer, 1986).

A liquid-gas-solid fluidized bed reactor has been widely used for waste water treatment (Cooper, 1983; Rao Bhamidimarri and Greenfield, 1990) and also for production of some valuable materials using, e.g. plant cells of *coffee arabica* (Bramble *et al*, 1990), and mammalian cells of mouse hybridoma (Dean *et al*, 1988), because of its excellent mass and heat transfer properties, and low shear stress. In solid substrate fermentation, Adisasmito *et al* (1987) used a liquid-gas-solid fluidized bed reactor for *Trichoderma reesei* growth on corncob, and improved biomass production and activity were claimed. However, because of its high operating cost and the high risk of contamination, this type of reactor has only limited application in solid substrate fermentation.

In addition to the types of reactors described above, other types, e.g. stirred tank reactors (Levonen-Munoz *et al*, 1983; Baldensperger *et al*, 1985), and liquid-solid fluidized bed reactors (Tzeng *et al*, 1991), have also been used in solid substrate fermentation. However, they are unlikely to be used on a large scale with solid substrates because of their high operating costs.

2.9 Summary

The main objective of this review has been to provide some informative background on citric acid production by solid substrate fermentation. A brief introduction to the history of citric acid production, including a description of nutritional and environmental factors and the biochemistry is provided in order to understand the process.

The comparison among liquid surface, submerged and solid substrate fermentation technologies for citric acid production indicates potential advantages and our limited understanding of the solid substrate fermentation. Referring to current investigations and applications of solid substrate fermentation, our understanding of heat and mass transfer, and subsequent reactor designs, is also limited.

The ultimate aim of this thesis is to investigate solid substrate fermentation for citric acid production, including nutritional requirements and environmental factors, and to investigate the kinetic characteristics, so that a reactor may be selected. Based on the selected reactor, and optimized conditions, attempts will be made to understand the kinetic and mass transfer characteristics.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Microbiological media

Two media were used for the sporulation of *Aspergillus niger*, sucrose-beef extract medium for strain MH15-15, as shown in Table 3.1; and malt extract agar medium, as supplied by Unipath Ltd. (Basingstoke, England), for strain Yang No.2.

Table 3.1 Sucrose-beef extract medium (Sánchez-Marroquín *et al*, 1970; Dawson, 1986, Dawson *et al*, 1987, 1988)

Component	Concentration (g/l)
Sucrose	2.5
Beef extract	10.0
Sodium chloride	5.0
Agar	15.0
other	distilled water

3.1.2 Chemicals

Chemicals used for fermentation and analytical work are listed in Table 3.2.

3.1.3 Raw substrates

Potato, kumara and taro, which were purchased fresh at a local supermarket, were used in the experiments. The fresh substrates were packed in plastic bags to prevent serious moisture loss, and stored at 4°C for long term use (less than 2 months)

3.1.4 Gases

The following gases were used in the experiments, and were supplied and certified by New Zealand Gases Ltd., Palmerston North, New Zealand:

Dry air, oxygen-free nitrogen, 4% (v/v) carbon dioxide in oxygen-free nitrogen

Table 3.2 Chemicals and suppliers

Name	Formula	Grade	Manufacturer	Supplier
Ammonium sulphate	$(NH_4)_2SO_4$	A.R.	BDH Chemicals Ltd, England	
Amylase	Termamyl-120L		NOVO, Nordisk, Bagsaerd, Denmark	CHEMICOLOUR INDUSTRIES NZ. LTD.
Amylose		A.R.	BDH Chemicals Ltd, England	
Citric acid	C_6HO_7	A.R.	Sigma Chemicals Co. St. Louis, U.S.A.	
Copper sulphate	$CuSO_4.5H_2O$	A.R.	BDH Chemicals Ltd, England	
Ferrous sulphate	$FeSO_4.7H_2O$	A.R.	BDH Chemicals Ltd, England	
Gluco-amylase	San-240L		NOVO, Nordisk Bagsaerd, Denmark	CHEMICOLOUR INDUSTRIES NZ. LTD.
Hydrochloric acid	HCl	A.R.	Ajax Chemicals Co., Australia	
Manganese sulphate	$MnSO_4.5H_2O$	A.R.	BDH Chemicals Ltd, England	
Nitric acid	HNO_3	A.R.	Ajax Chemicals Co., Australia	
Orthophosphoric acid	H_3PO_4	A.R.	Ajax Chemicals Co., Australia	
Potassium di-hydrogen phosphate	KH_2PO_4	A.R.	BDH Chemicals Ltd, England	
Sodium Chloride	NaCl	A.R.	BDH Chemicals Ltd, England	
Sodium Hydroxide	NaOH	A.R.	Ajax Chemicals Co., Australia	
Soda Lime 'Corbosorb'			Ajax Chemicals Co., Australia	
Soluble starch			Ajax Chemicals Co., Australia	
Zinc sulphate	$ZnSO_4.3H_2O$	A.R.	BDH Chemicals Ltd, England	

Note:Except otherwise stated, all other chemicals were supplied by BDH Chemicals N.Z. Co., Palmerston North, New Zealand.

3.1.5 Organism

Two strains of *Aspergillus niger* were used in the experiments. Strain MH15-15, was isolated in this laboratory by Hossain *et al* (1983) as a mutant strain of *A. niger* IMI41874, and was used by Dawson (1986) and Dawson *et al* (1987, 1988) for citric acid production in submerged fermentation. It was cultivated on slopes of sucrose-beef extract agar (Table 3.1) at 30°C for 8-9 days. Another strain, Yang No.2, supplied by Professor H. Imai, Waseda University, Tokyo, Japan, has been used in liquid surface fermentation for citric acid production (Sakurai and Imai, 1991; Sakurai *et al*, 1991, 1992). It was cultivated on slopes of malt extract agar at 30°C for 5-6 days to obtain spores.

For long term preservation of the organisms, the techniques described by Hossain (1983) and Dawson (1986), were used:

a) The spores of the organism were harvested from agar slopes with sterilized distilled water. Then the spore suspension was shaken for 1 hour to break the spore chains and clumps. The concentration of the suspension was adjusted to 1-2x10^8 spores/ml using sterilized distilled water.

b) Two ml spore suspension was dispensed aseptically into 3 ml of corresponding medium (no agar), containing 30% (v/v) glycerol, contained in a 10 ml capacity screw cap bottle. The inoculated bottles were then stored at –20°C.

When spores were required for sub-culturing, a loopful from a thawed bottle was transferred to a slope of the corresponding medium, and incubated at 30°C for a different period dependent on different strains of the organism. A further subculture from this initial slope, onto a fresh slope, was carried out to obtain improved sporulation. This second slope was used for up to 2 months as the stock culture for inoculum preparation.

3.2 Media Sterilization

Microbiological media were sterilized by autoclaving at 121°C for 15 minutes. Fermentation media, unless otherwise stated, were sterilized at 121°C for 20 minutes.

3.3 Cleaning of Glassware

All glassware, including glass reactors and flasks, was washed in hot Pyroneg$^{(R)}$ solution, rinsed in tap water, then in distilled water, and hot air dried. Glassware used in experiments involving metal ions, was treated with 10% (v/v) nitric acid after the detergent wash, then rinsed thoroughly with deionized water, and hot air dried.

3.4 Avoidance of Wall Growth in Flasks and Reactors

All glass flasks and reactors used in fermentation were treated with Sigmacote (Sigma Chemical Co., St Louis, Missouri, U.S.A.), which coated the inner surface of the glass with a microscopically thin film of silicone, thus preventing wall growth of mycelium. After coating, the glassware was dried in hot air and then washed in Milli-Q deionised water and hot air dried before use.

3.5 Analytical Methods

3.5.1 pH measurement

pH measurement was performed using a pH meter (PHM82, RADIOMETER, COPENHAGEN, Denmark), which was calibrated prior to use using pH 4.0 and pH 7.0 buffers.

3.5.2 Spore count

Spore counts were performed using a standard haemocytometer under 400x magnification.

3.5.3 Treatment of samples

The process which was adopted for treatment of fermentation samples and other starch-containing samples aimed to separate the starch from the fungal biomass and/or non-hydrolysable material, thus allowing the analysis of starch, citric acid, fungal biomass and non-hydrolysable materials.

3.5.3.1 Treatment process for raw substrate

Raw substrate (potato, kumara and taro) after preparation for fermentation (as described in Section 3.6.2), was sampled, in duplicate, for determination of initial starch and non-hydrolysable material (NHM). The process was as follows:

a) Sample (5-8 g) was mixed well with 100 ml of distilled water in a 250 ml flask using a glass rod. Three drops (approximately 0.04 ml per drop) each of amylase and gluco-amylase were added into the flask (If HCl was used as the hydrolysing agent, 50 ml of A.R. grade HCl was added instead of the enzymes, and the pH value was adjusted to 6.5-7.0 using 1 M NaOH solution after hydrolysis.). The flask was held in a reciprocal shaking incubator at a speed of 140 rpm at 70°C for 4 hours.

b) The separation of liquid and solid was accomplished using a centrifuge (Model RC5C, Sorvall^{R} Instruments, DU PONT, Wilmington, Delaware, USA) at a speed of 9,000 rpm for 15 minutes. The solid was washed 3 times using distilled water. The supernatant fluid and washings were collected and made up to 1,000 ml in a standard flask. A 5 ml sample was withdrawn and stored in a 10 ml screw cap bottle at -20°C for determination of starch (as glucose).

c) The solid was placed in a 105°C oven for 48 hours, and the weight of dry solid was expressed as the non-hydrolysable matter (NHM) of the substrate. For samples which required analysis for total carbon content, the dry solid was ground to a powder using a mortar and pestle, then placed in a 10 ml capacity screw cap bottle and stored in a 60°C oven.

3.5.3.2 Treatment process for fermentation samples

a) Fermentation samples (entire contents) were broken into small pieces using a glass rod, and mixed with 100 ml distilled water in a 250 ml flask. If a sample was more than 40 g, two or more flasks were used, with no more than 30 g in each flask; for samples taken within 3 days of inoculation, no more than 15 g was placed in each flask. NaOH solution (1 M or 4 M) was added, depending on the particular fermentation sample, to adjust the pH value to 6.5-7.0. Three drops (approximately 0.04 ml per drop) each of amylase and gluco-amylase were added into the flask, and the flask was held in a reciprocal shaking incubator, at a speed of 140 rpm and temperature of 70°C for 4 hours.

b) The separation of liquid and solid was achieved as described above. The supernatants were collected and made up to volume in a 2,000 or 3,000 ml standard flask, depending on the volume of supernatant and washings of the sample. The well-mixed solution from the standard flask was filtered through a membrane (pore size 0.45 μm) using a Swinney Filter kit (Millipore Corporation, Bedford, Massachusetts, U.S.A.) and 5 ml of this filtrate was placed in a 10 ml screw cap bottle and stored at -20°C for subsequent citric acid and residual starch analysis.

c) The solid was placed in a 105°C oven for 48 hours. The dry weight value obtained was used to calculate the fungal biomass weight associated with NHM weight. Any samples which were to be analysed for carbon content were treated as described in the preceding section.

3.5.4 Determination of fungal biomass

For each run of experiments, solid dry weights of non-hydrolysed residues were obtained. The fungal biomass was determined as:

Dry fungal biomass=Dry fermented solid – dry NHM

3.5.5 Analysis of starch

The starch content of samples was analysed by measuring the glucose released by

hydrolysis using a YSI glucose analyser (Model 27, Yellow Springs Instrument CO. Inc., Yellow Springs, Ohio, U.S.A.). The analyser can detect glucose, fructose and other monosaccharides . Therefore, the "glucose" determined by this analyser was a total concentration of these sugars, and the "starch" included the free monosaccharides in the substrate. Samples, if their sugar concentration was over 5.0 g/l, were diluted with distilled water. Prior to each operation, the analyser was calibrated using glucose standards of 2.0 g/l and 5.0 g/l. The starch was calculated as below:

$$Starch\ (g/l) = 0.9 \times Glucose\ (g/l)$$

3.5.6 Determination of glucoamylase activity and free glucose

When glucoamylase activity and the free glucose concentration was required, samples were treated as follows:

a) **Original glucose**

Uninoculated and cooked paste or particular substrate (5-8 g) was placed in a 250 ml flask using 100 ml of distilled water. This flask was placed on a shaking incubator at a speed of 140 rpm and at room temperature for 1 hour, after which the mixture was made up to 250 ml. Two samples were withdrawn and analysed using the YSI glucose analyser. The concentration of glucose, based on wet weight of the substrate, was recorded the "original" glucose of the substrate.

b) **Initial glucose**

The fermentation sample was conducted into a flask(s), as described in Section 3.5.3.2 and made up to 150 ml with distilled water. The flask(s) was placed on a shaking incubator at 30°C at a speed of 250 rpm for 5 minutes. A 1 ml sample was filtered using filter paper (Whatman No.1), and the filtrate was immediately analysed using YSI glucose analyser. The concentration of glucose, based on the wet weight of the sample, was the "initial" glucose (IG). The "original" glucose was subtracted from the "initial" glucose, to give the net glucose released in that fermentation period.

c) **Glucoamylase activity**

After the first sampling, the flask(s) was kept on the shaking incubator for 1 hour, after which a 1 ml sample was taken and analysed using a similar procedure to that described in b) above. The concentration of glucose, based on wet weight of the substrate, after subtraction of the "initial" glucose concentration and divided by 1 hour, represented the glucoamylase activity of the fungal biomass.

3.5.7 Determination of citric acid

Analysis of citric acid content of samples was carried out by HPLC (high performance liquid chromatograph). Prior to the analysis, the pH value of the samples was adjusted by adding 0.1 ml orthophosphoric acid into 1.0 ml of sample. The sample was shaken vigorously and then allowed to stand for 10 minutes. The tube was centrifuged (Model Biofuge 13, Heraeus, SEPATECH, Germany) at a speed of 12,000 rpm for 5 minutes, and 50 µml of the clear solution was injected into HPLC for the analysis of citric acid.

The HPLC consisted of a liquid chromatograph (Model ALC/GPC 244, Water Associates Inc., Milford, Masschusetts, U.S.A.), a solvent delivery system (Model 6000A), a septumless injector (U06K, Water Associates), a detector (Model 401 Differential Refractometer, Water Associates), and an integrating recorder (Waters 740 Data Module, Millipore Corporation, Bedford, Massachusetts, U.S.A.). A µ-Bondapak C18 reverse-phase column (4.0 mm IDx250 mm, Bio-sil, ODS-10, Bio-Rad Laborataries, Richmond, California, U.S.A.) was used. The response was recorded and calculated by the integrating recorder which determined the amount of citric acid by measuring the peak area with reference to the peak area of two standard injections, 1.0 g/l and 10.0 g/l.

The solvent system was 2% (w/v) potassium di-hydrogen orthophosphate solution prepared using Milli-Q deionised water, and adjusted to pH 2.45 using orthophosphoric acid (Coppola *et al*, 1978; Dawson, 1986). The solvent was degassed by passing through a membrane (pore size 0.4 µm, Millipore Corporation), and was used at flow rate of

2.0 ml/min during the analysis.

Thorough cleaning of the column was necessary following the use of the phosphate-based solvent. This was achieved by using freshly degassed Milli-Q deionised water at a flow rate of 1.5 ml/min for 1 hour. This was then followed by a methanol/water mixture (ratio 50:50) and pure methanol at a flow rate of 0.5 ml/min for 30 minutes and 1.0 ml/min for 20 minutes, respectively.

3.5.8 Determination of carbon

Solid samples were weighed to 0.03 to 0.05 g using an electronic balance (Model AT261, METTLER, DeltaRange, Switzerland), and packed in a special silver container (Type F, FISONS Scientific Equipment, Loughborough, Lecestershire, UK). The containers were conducted into an autosampler (Model, AS-200, FISONS), and the carbon analysis was performed using high temperature chromatograph (Model NA 1500/AS 200, FISONS). This highly automatic gas chromatograph consists of combustion and reduction furnaces, a detector, a pneumatic control system, an electronic programmer, an electronic peak sensing feature and a built-in potentiometer electronic recorder. Helium (g.c. grade) and oxygen (99.995% purity) were conducted through the analyser at flow rates of 100 ml/min and 50 ml/min, respectively. The responses were recorded and calculated by the built-in potentiometer recorder which determined the amount of carbon content by measuring the peak area with reference to the peak area of standard sulphanilamide. The analyser can analyse the nitrogen, carbon and sulphur contents of the sample, and provides results as a percentage (w/w), based on the weight of the sample.

3.5.9 Determination of carbon dioxide

Two methods to determine carbon dioxide were used in the experiments; an Infra-red carbon dioxide analyser, and the use of 0.2 M NaOH solution to absorb carbon dioxide when the Infra-red analyser was not available.

a) Infra-red carbon dioxide analyser for carbon dioxide analysis

Gas samples, at flow rates ranging from 0.2-1.4 l/min were dried by using a silica gel column before they were introduced into the Infra-red carbon dioxide analyser (The Analytical Development Co. Ltd., Hoddesdon, England). The response could be either read directly on the panel meter or recorded continuously by a twin-pen flat chart recorder (Model CR652S, JJ Instruments, Southampton, England). The analyser measured carbon dioxide concentration in % (v/v) of the sample air flow rate. Therefore, total air flow rate was corrected using a manometer connecting with the exiting of a reactor used in the manners shown in Figs 3.7 and 3.10 in a manner described in Section 3.5.12.

The analyser was calibrated using dry air (the carbon dioxide was stripped by passage through a 20% NaOH solution) as the zero concentration standard and 4% (v/v) carbon dioxide in oxygen-free nitrogen, prior to each analysis. Deviation of the measurement is under ±2%, according to the manufacture's manual. The analysis was conducted at ambient temperature 28-30°C.

b) NaOH solution to absorb carbon dioxide

NaOH solution (0.2 M, 10 ml or 20 ml) and two drops of indicator (0.1 g phenolphthalein in 100 ml 50:50 ethanol water solution) were added to a 250 ml flask, together with 150 ml distilled water. This flask replaced the collector which is shown in Fig 3.4 during the operation of the reactor, and prior to disappearance of the purple colour, a fresh 10 or 20 ml 0.2 M NaOH solution was added to the flask. If necessary, 0.1 M HCl was used to titrate the remaining NaOH in the flask. The total volume of consumed 0.2 M NaOH solution was used to represent the carbon dioxide produced by the fermentation.

3.5.10 Determination of oxygen

An oxygen analyser (Model 540A, Servomex, SYBRON Taylor, Taylor Instrument Ltd., England) was used to determine the oxygen concentration of the air flow. The oxygen

concentration in % (v/v) could be either read directly on the panel meter or recorded continuously by a twin-pen flat chart recorder (Model CR652S, JJ Instrument, Southampton, England). Prior to each analysis, the analyser was calibrated using oxygen-free nitrogen gas and dry air.

3.5.11 Measurement of temperature

Temperature within reactors was measured using thermocouples, and recorded continuously using a multi-point chart recorder (Honeywell Varsaprint, Amiens, France).

3.5.12 Measurement of air flow rate

Air flow rate was monitored by using rotameter. However, in the packed bed reactor, because of varying back-pressure caused by biomass growth, the air pressure was also measured, using a manometer (with 400ml volume), at intervals of 4-6 hours. The average value of the measurements during the fermentation was used to provide the corrected air flow rate.

3.5.13 Measurement of air pressure

A 1000 mm high U tube (8 mm diameter) filled with mercury, was used to measure the air pressure by connection with appropriate ports of the reactor or other units. The unit of measured pressure was mm Hg.

3.6 Culture Conditions

3.6.1 Preparation of substrate

Raw substrate was processed into three forms, i.e. paste, cubic particle and dry particle, for different fermentation experiments.

a) Paste substrate

The peeled substrate was cut into pieces (2-4 cm), and put into a container. This container was wrapped with two layers of aluminium foil and the material was cooked in an autoclave at 121°C for 25 minutes. This cooked substrate was blended into a paste using a blender (AUTO MIX, MSE LTD., England). If any additions where required, e.g. metal ions, the cooked substrate, was weighed prior to being placed in the blender, and the additions made on the basis of 100 g substrate.

The paste was transferred into flasks or other containers using a glue-gun (modified in this Department) to assist handling.

b) Cubic particle substrate

The peeled substrate was cut into flat pieces, using a chip-cutter (DiTo-SAMA, F23200, AUBUSSON, France), to the required thickness. The flat pieces were then cut into cubic particles using a knife and were placed in a beaker of volume of 200-1,000 ml, depending on the weight of the substrate. This container was wrapped with two layers of aluminium foil and sterilized at 110°C for 25 minutes.

c) Dry particle substrate

When making the paste substrate in a blender, 20 ml of deionised water was added for every 100 g substrate. The paste so obtained was spread onto plastic plates, and dried in an oven at 60°C for 4 days. The dry and fragile substrate films, of 2-3 mm thickness, were crushed into small pieces, and blended to break up the small pieces. The blended material was then conducted into a set of sieves (Endecott, ENDECOTTS Ltd., London, England). Particle sizes ranging from 105-150 μm, 150-210 μm, 210-300 μm, 300-420 μm and 450-500 μm, or bigger, were obtained and stored in a 60°C oven until use.

3.6.2 Preparation of inoculum for fermentations

A spore suspension (approximately 1-2x10^8 spores/ml) (Section 3.1.5) was adjusted to the required concentration using sterile distilled water. For a paste substrate, the spore suspension was added to the surface of the paste, and the flask was tilted to ensure even spreading. For the particle substrate, the flasks were inverted several times after inoculation to ensure even inoculation.

For the dry particle substrate, 10 g was used in each experiment. About 2 g of the substrate was placed in a 100 ml bottle containing a 6 day old slope of sporulated culture. The bottles was shaken until the yellow substrate particles turned black due to attachment of spores. The particles were then poured out of the bottle, and mixed with the remaining 8 g of the substrate. Any agglomerations of particles were separated using a glass rod. Finally, before use, the particles were examined under the microscope to ensure that each had at least 10-20 spores attached to it. All of the above processes were carried out aseptically.

3.6.3 Flask culture

250 ml Erlenmeyer flasks (250 ml) containing 40 or 50 g of paste or particle substrate, with cotton wool in the mouth, wrapped with two layers of aluminium foil, were sterilized in an autoclave at 121°C for 15 minutes. After cooling, the flasks were inoculated with the required inoculum size. These flasks were incubated at 30°C for a given period. For sampling, the entire contents of a flask were taken.

3.6.4 Tube culture

Tubes, of diameter ranging from 10 mm to 50 mm, were packed carefully with paste substrate using a glue gun and a rubber rod. It was important that there was no air entrapped inside the paste, otherwise the substrate was blown out and the uniform surface was destroyed during autoclaving.

After inoculation with required inoculum size, these tubes were incubated at 30°C. For sampling, the entire contents of a tube were taken.

3.6.5 Gas-solid fluidized bed reactor culture

The gas-solid fluidized bed reactor system was constructed in the Department of Process and Environmental Technology, Massey University. The construction and dimensions of the acrylic reactor are shown in Fig 3.1, while a schematic diagram of the system is shown in Fig 3.2.

Compressed air, from the University compressed air line, at a flow rate ranging from 5 to 20 l/min, was filtered and passed through a heater to raise its temperature to 30-35°C. To prevent serious heat loss, all connecting tubes were well wrapped with insulation material. The air was then passed through a humidifier, at the outlet of which the air RH was about 80% and the temperature 24-26°C. The auxiliary humidifier then adjusted the RH to 100% and the temperature to 28°C. Inside the reactor, as shown in Fig 3.1, the temperature of the glass beads was controlled by the water jacket. Thus, the temperature of the air could be increased by up to 6°C, with a concomitant decrease in RH to, say, 70-90% (It should be noted that the RH of the air was not measured directly. Rather, its value was taken from Psychometric charts, based on the temperature. Because there was no direct control of the RH, its value varied inversely with the temperature). The presence of these glass beads in the reactor was crucial to the control of temperature, and thus RH, and also to prevent any condensed water from reaching the particle substrate. The water jacket surrounding the reactor, and the extension zone, were used to keep the walls of the reactor at a temperature slightly higher than that of the air. thus preventing any water condensation. The distributor at the base of the reactor, above the glass beads, consisted of two layers of meshes. The upper mesh (pore size less than 104 μm) prevented substrate particles from exiting downwards, while the lower mesh simply provided support. Finally, the air exiting from the reactor was passed through a collector, to prevent dispersion of any spores or particles from the reactor. The measurement of temperature was by means of methods described in Section 3.5.11.

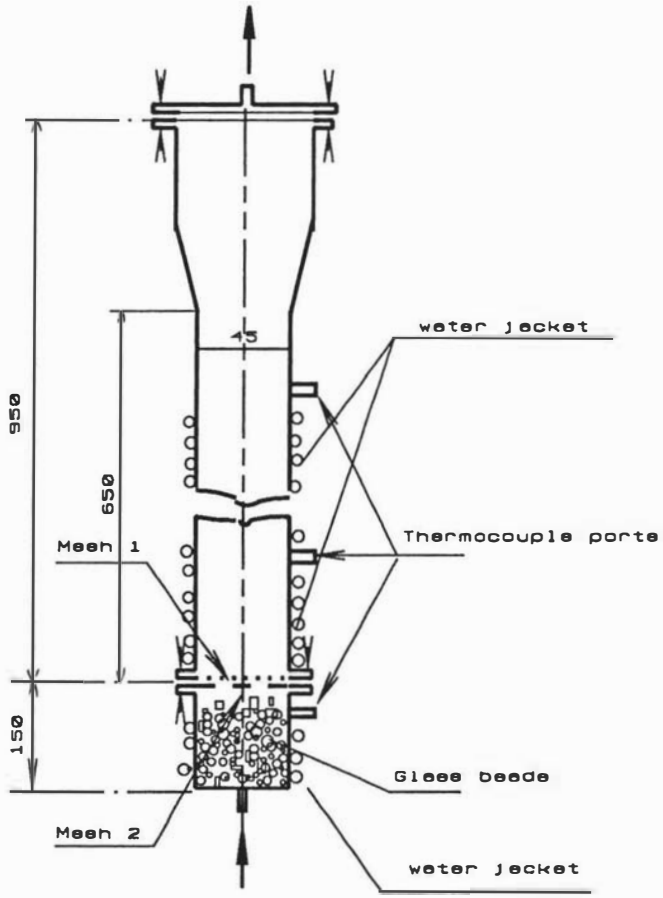


Figure 3.1 Diagram of gas-solid fluidized bed reactor (unit: mm)

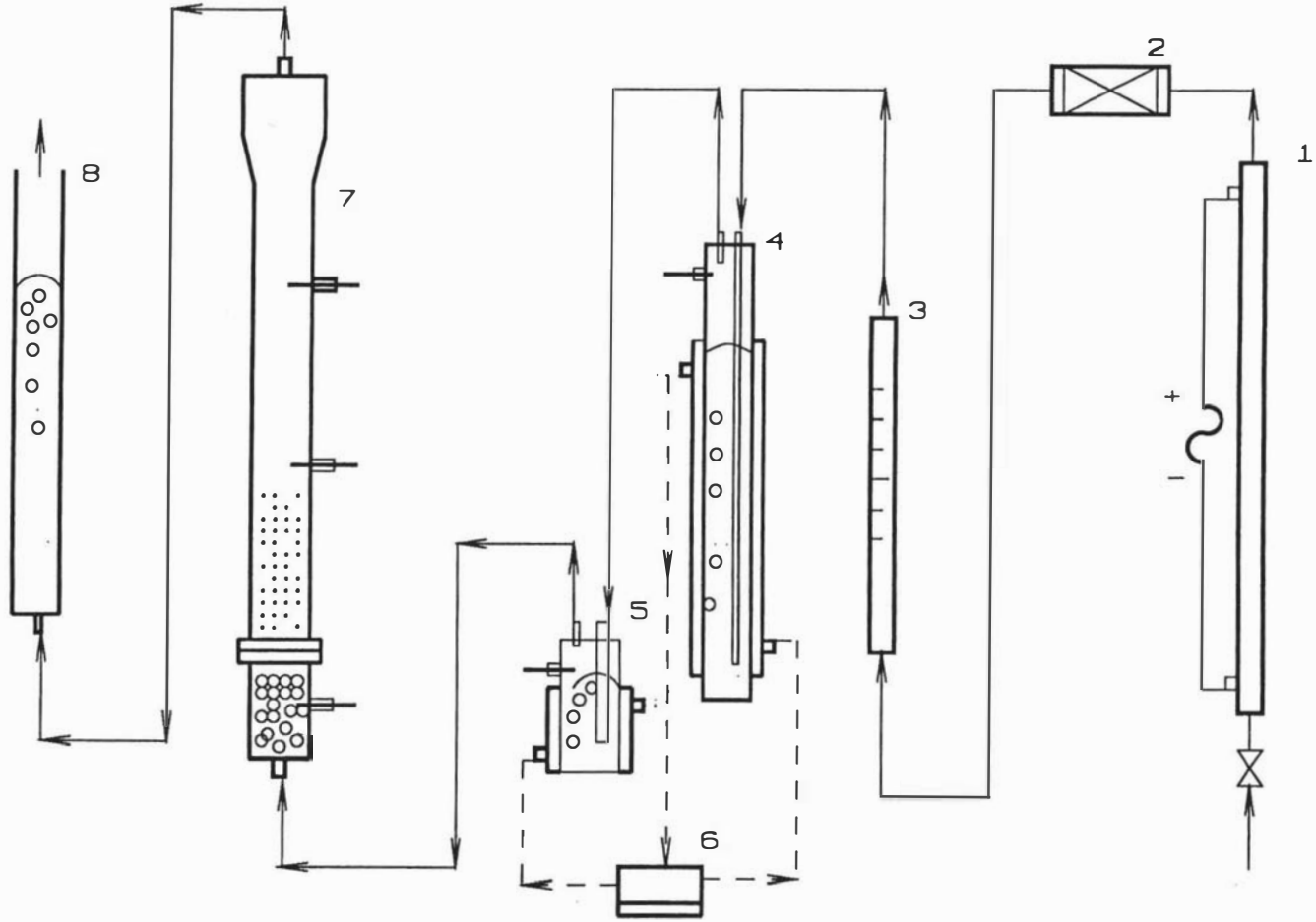


Figure 3.2 Schematic diagram of gas-solid fluidized bed reactor system
1. heater 2. filter 3. rotameter 4. humidifier 5. auxiliary humidifier
6. warm water supplier 7. reactor 8. collector

3.6.6 Liquid-solid-gas fluidized bed reactor culture

The liquid-solid-gas fluidized bed reactor system used was constructed in the Department of Process and Environmental Technology, Massey University. The reactor was made of glass by the Glass Workshop, Chemistry and Biochemistry Department, Massey University. Its construction and dimensions are shown in Fig 3.3, while a schematic diagram of the system is shown in Fig 3.4. The system was operated at room temperature. The system was connected to the University compressed air line, supplied with air at flow rates ranging from 1 to 5 l/min. The air passed through the cotton wool filter for sterilization. The remainder of the system was sterilized by running 50% ethanol through all lines for 2 hours.

The cubic particle substrate was used in this system. After loading of the inoculated substrate, air was supplied for 24 hours, but without supplying sterile water from the reservoir. This allowed the spores to germinate. When white filamentous hyphae appeared on the substrate, sterile water was pumped into the reactor to give a ratio of liquid to substrate of 2:1 (w/w), using a peristaltic pump (Model 7015, MASTERFLEX, Cole Parmer Instrument Co., Chicago, U.S.A.). The control valve was then turned to circulation, and air flow rate and liquid flow were adjusted carefully to achieve the fluidization. The temperature control of the system was carried out by controlling the temperature of the circulation solution, which was conducted through a heater as shown in Fig 3.2.

3.6.7 Rotating drum reactor culture

The rotating drum reactor system consisted of 4 1-litre capacity Agee jars and a rotating driver, as depicted in Fig 3.5. The construction and dimensions of the Agee jar are depicted in Fig 3.6. The inner surface of the jars was coated with a microscopically thin film using Sigmacote (Sigma Chemicals Co. Ltd.). A chimney (stainless steel), with cotton wool inside, which allowed natural aeration by the diffusion of air, was fixed on the top of the jar by a screw ring. Each jar's loading varied from 100 to 200 g of substrate. The substrate used was either paste or particles. The jars, after loading with

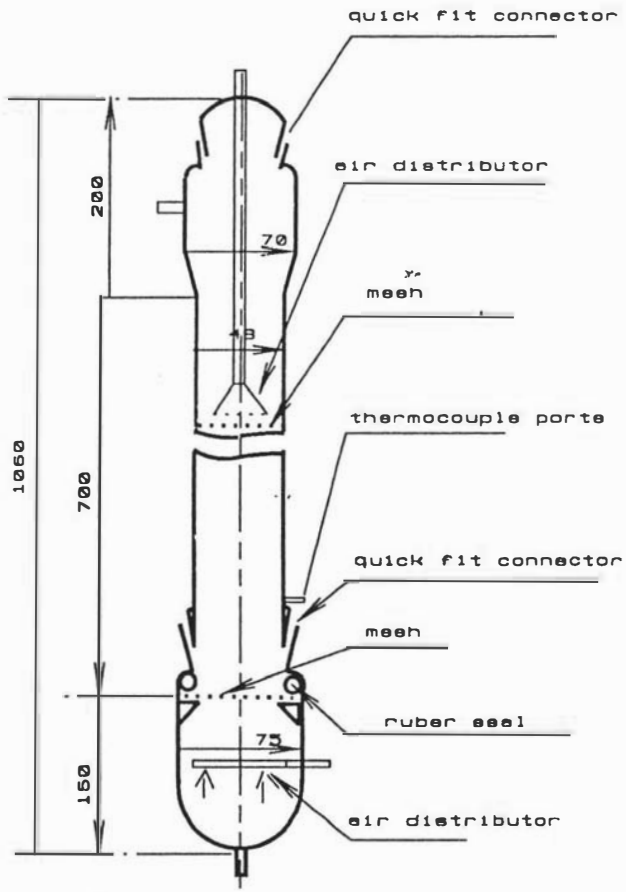


Figure 3.3 Diagram of liquid-solid-gas fluidized bed reactor (unit: mm)

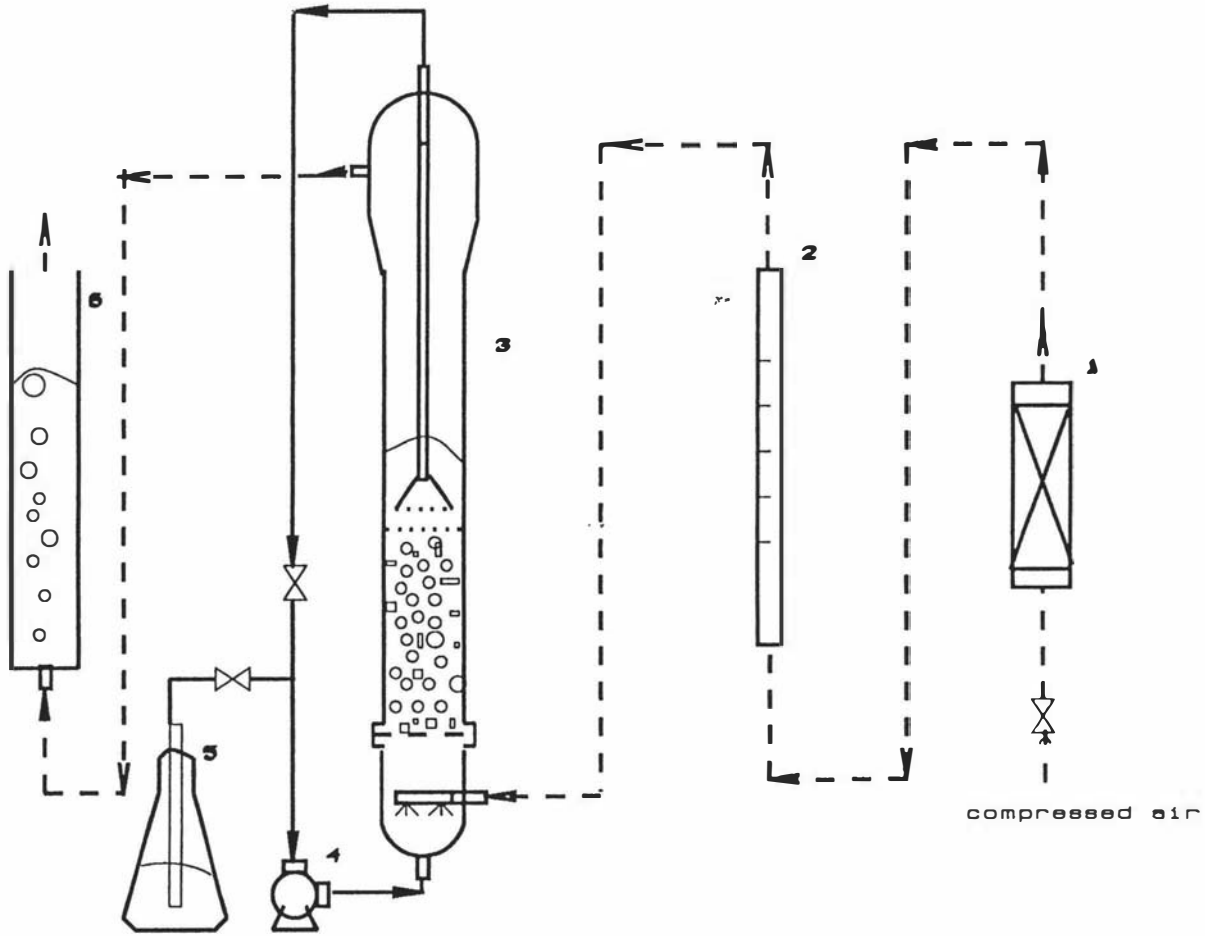


Figure 3.4 Schematic diagram of the liquid-solid-gas fluidized bed reactor
1. filter 2. rotameter 3. reactor 4. pump 5. reservoir 6. collector

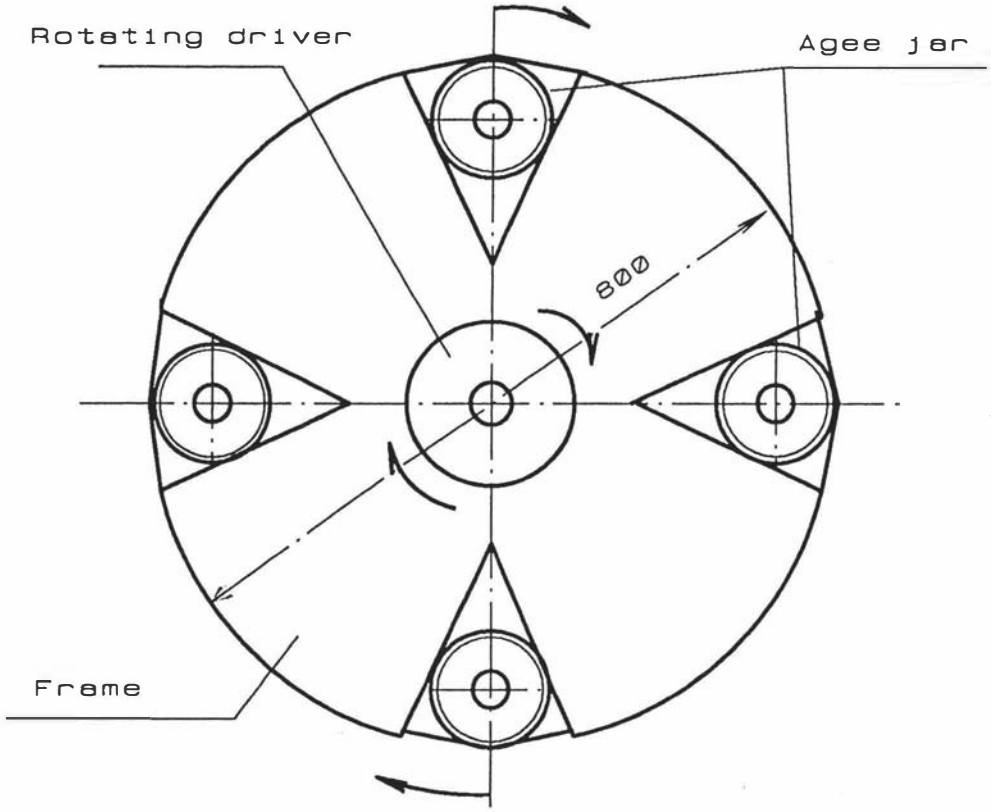


Figure 3.5 Schematic diagram of rotating drum reactor system

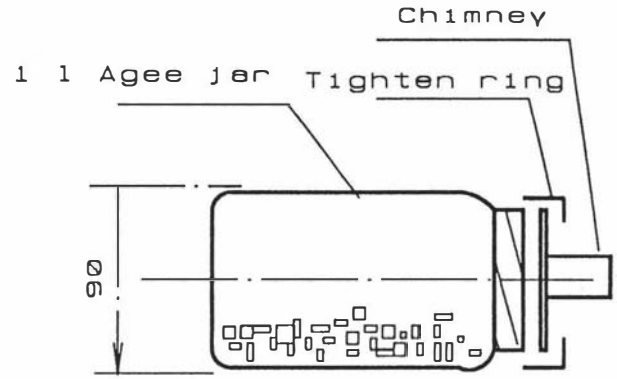


Figure 3.6 diagram of Agee jar (reactor)

(unit: mm)

substrate, were sterilized by autoclaving. The system was operated in a room at a temperature of 30°C. When sampling, the entire contents of the jar were taken.

3.6.8 Packed bed reactor culture

The packed bed reactor system, as depicted in Fig 3.7, was constructed in the Department of Process and Environmental Technology, Massey University. The reactor, of which construction and dimensions are depicted in Fig 3.8, was made of glass by the Glass Workshop, Chemistry and Biochemistry Department, Massey University. The system was operated in a room at a temperature of 30°C.

Dry air from a cylinder, of which the flow rate was monitored by a rotameter, was sterilized by passing through a cotton wool filter (sterilized at 160°C for 4 hours). Carbon dioxide was stripped as the air passed through a 20% (w/v) NaOH solution column, then the air was raised in temperature to 29-30°C and in RH to 95-100% while passing through the humidifier. The moist air entered the reactor at the top, to prevent pushing up the substrate which could cause serious blockage in the reactor during the later period of operation. After exiting, the air passed through a condenser (0°C), then a water trap to strip its moisture. The air passed through a silica gel column before entering the carbon dioxide analyser followed by the oxygen analyser. Between the water trap and the silica gel column, there was approximately 2 meters length of tube, which allowed an increase of the air temperature, and subsequently reduced the duty of the silica gel. The silica gel was replaced before its colour turned pink. The air went to vent after exiting from the oxygen analyser.

When 0.2 M NaOH solution was used to analyse carbon dioxide, a collector followed the air exiting from the reactor, to prevent interference by spores to the NaOH solution. Then the 0.2 M NaOH solution column followed the collector. To measure the temperature during the fermentation, thermocouples were inserted into the substrate through different ports at different positions, and reached the diameter centre point. The system was examined for leakage before each operation.

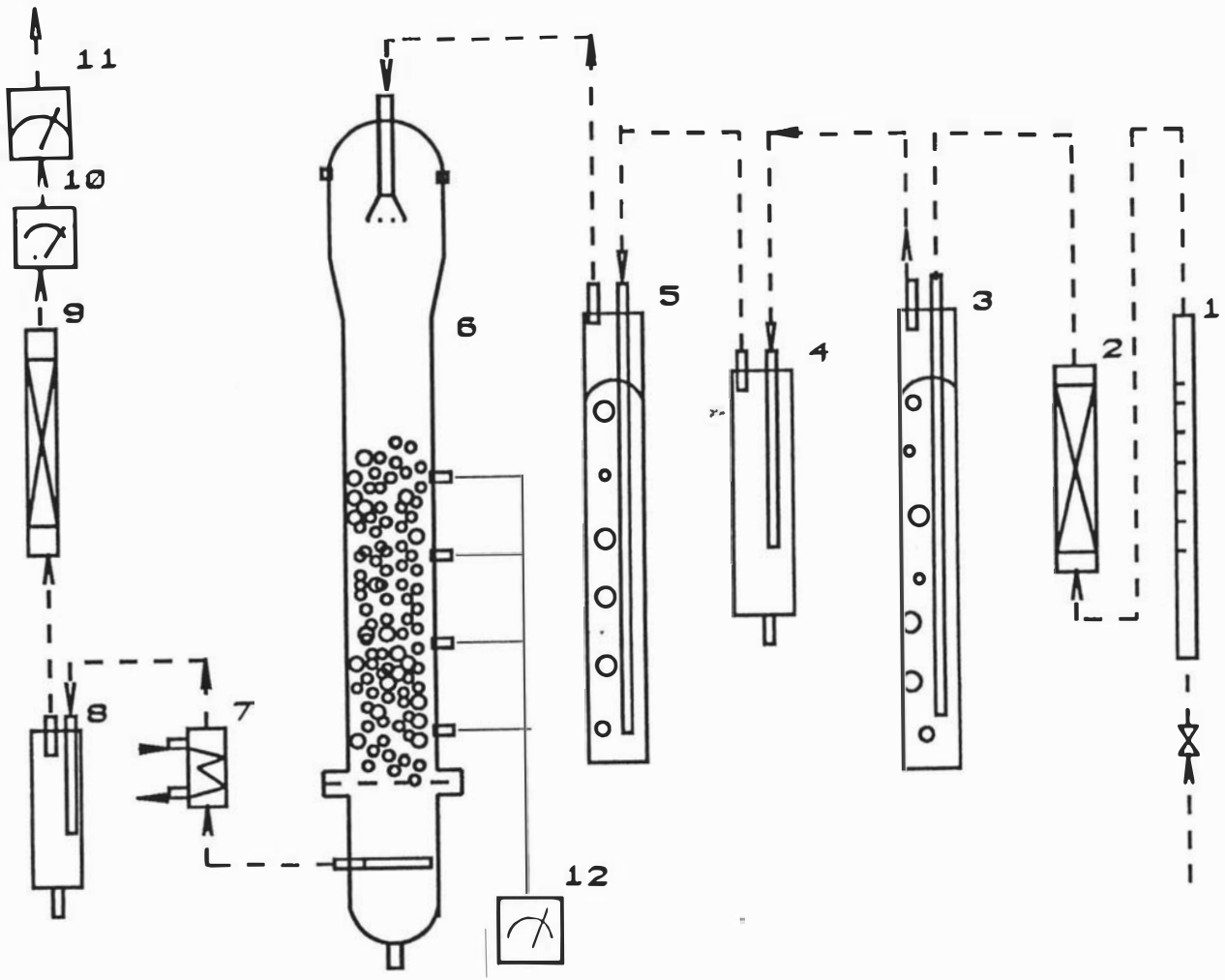


Figure 3.7 Schematic diagram of packed bed reactor system
1. rotameter 2. filter 3. 20% NaOH solution column 4. water trap
5. humidifier 6. reactor 7. condenser 8. water trap 9. silica gel column
10. carbon dioxide analyser 11. oxygen analyser 12. temperature recorder

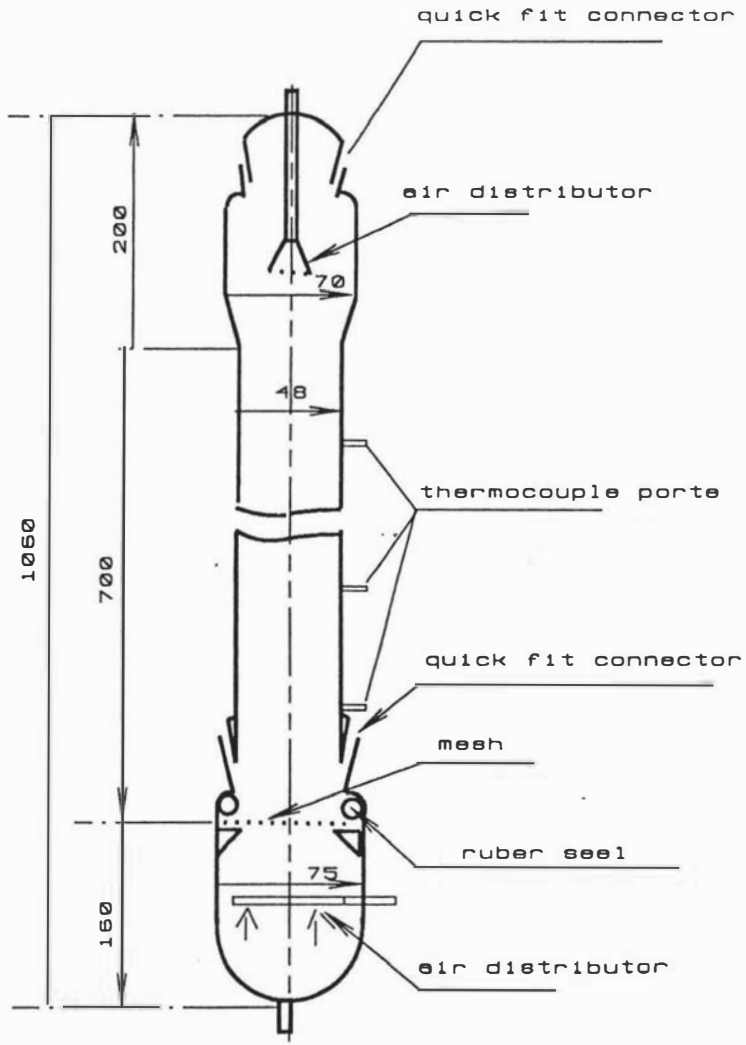


Figure 3.8 Diagram of packed bed reactor

3.6.9 Multi-layer packed bed reactor culture

The multi-layer packed bed reactor, which was a modification of the packed bed reactor (Fig 3.8), is depicted in Fig 3.9, while a schematic diagram of the system is shown in Fig 3.10. The system was operated in a room at a temperature of 30°C.

Except for air sampling and analysis, the operation of the system was the same as described for the packed bed reactor system (preceding section). There were 4 pairs of magnetic valves (Model 35A-ACA-DXXJ-1KD, MAC, WIXOM, Miami, U.S.A) with 4 sampling and 4 feeding back ports on the reactor. The magnetic valves were controlled by a timer (Model 88 646 2, CROUZET, France) with a 15-minute interval. A pair of valves was opened, allowing 0.2-0.3 l/min of air sample to be withdrawn. The air sample then passed through a condenser, a water trap and a silica gel column. After exiting the carbon dioxide analyser, the air passed through a humidifier to compensate for moisture loss during the carbon dioxide analysis process. The sample was then fed back to the reactor through the feeding back valve. In this way, the air after each substrate layer was analysed and recorded continuously by a recorder.

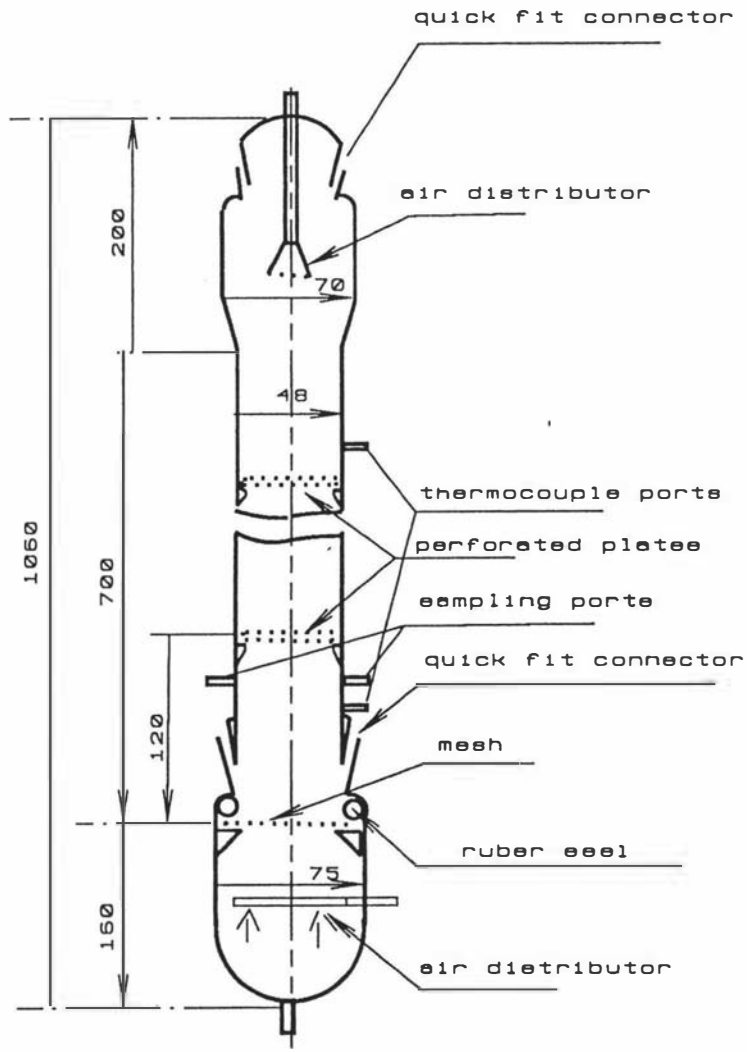


Figure 3.9 Diagram of multi-layer packed bed reactor (unit: mm)
(Layer order:from top to bottom, 1, 2, 3 and 4)

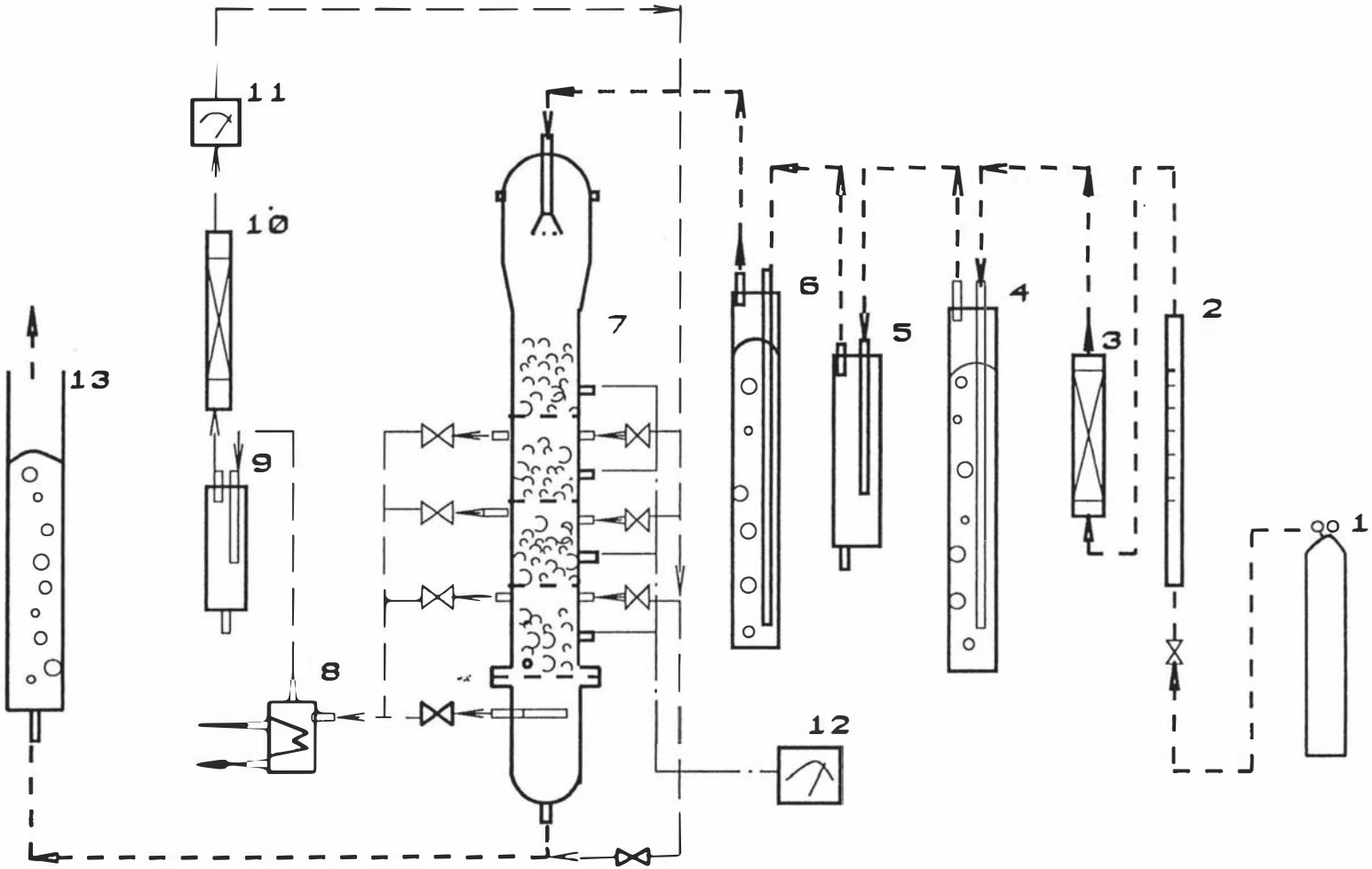


Figure 3.10 Schematic diagram of multi-layer packed bed reactor system
1. compressed air cylinder 2. rotameter 3. filter 4. 20% NaOH solution column 5. water trap
6. humidifier 7. reactor 8. condenser 9. water trap 10. silica gel column 11. carbon dioxide analyser
12. temperature recorder 13. collector

3.7 Discussion of Methods

3.7.1 Organism

The two strains of *Aspergillus niger*, MH15-15 and Yang No.2, had been used in reported works for submerged and liquid surface fermentations, respectively. In this project, they were used for the solid substrate fermentation. Their morphological characteristics were different from those in either submerged or liquid surface fermentations. However, the two strains of *A. niger* displayed excellent citric acid production in solid substrate fermentation, and demonstrated high stability during this project.

3.7.2 Raw substrate

For the raw substrate, the starch content and NHM contents may be different for each tuber of the substrate, and this may affect the evaluation of fermentation parameters. However, after selection depending on the appearance of the tubers, their starch content varied within only ±3% (w/w), and for NHM ±0.4%. These differences were reduced considerably by blender mixing (paste substrate) and vessel shaking (particles substrate).

3.7.3 Treatment of samples

It was important to limit the loading of each flask, to ensure complete hydrolysis during starch determination. It was experimentally proved that a starch concentration of 30 g of cooked substrate in 100 ml distilled water gave a hydrolysis value 10% less than that of 15 g. Therefore, for obtaining accurate starch data, the controlled loading of each flask as described in Section 3.5.3 was necessary.

3.7.4 Determination of fungal biomass

There were two problems in the determination of fungal biomass; the loss of spores during solid and liquid separation (Section 3.5.3), and the stability of the NHM value during fermentation. It was impossible to collect all spores using a centrifuge. However,

it was proved by verification experiments, which were carried out by filtering using a Buchner funnel with pre-weighed Whatman No.54 filter paper which had been dried at 105°C and stored in a desiccator, that no more than 3% of the total dry weight of the fermentation solids was lost during the centrifuge separation. Regarding the stability of NHM, it was shown in the results in Section 4.2, in which hydrolysis was conducted with 10% HCl at 100°C for 3 hours, that the NHM was identical with that obtained using the enzymes. Therefore, the NHM is assumed to be stable during the fermentation process.

3.7.5 Determination of carbon dioxide using 0.2 M NaOH solution

In this carbon dioxide analysis method, there were two problems which affected the absorption of carbon dioxide by the NaOH solution; air bubbles and the approach of absorption equilibrium. Large air bubbles emerged when the air was passed through the 0.2 M NaOH solution column, and so some carbon dioxide had no contact with the NaOH solution. When close to the absorption equilibrium, the ability of carbon dioxide absorption decreases considerably. Hence, even though the pH of the phenolphthalein indicator's colour change was at a value of 7.8, some errors may have occurred. However, this method of analysis was used only in experiments of optimization of the packed bed reactor, where the relative amounts of carbon dioxide could satisfy the experimental requirements.

3.7.6 Gas-solid fluidized bed reactor culture

The gas-solid fluidized bed reactor system was very sensitive, and this was mainly expressed as difficulties in controlling the RH of the air in the reactor. Control of RH was by means of temperature control of the air. However, the response of air temperature to the flow rate of water in the jacket was often delayed. Therefore, as the room temperature changed during the night time, the RH of the air drifted in a range of ±20% or more, causing condensed water to form in the reactor. During daytime, if the RH fell below 50%, there was insufficient moisture for the organism growth. Therefore, very close and frequent examinations were necessary to maintain stable operation.

3.7.7 Liquid-solid-gas fluidized bed reactor culture

It was found important that the system ran for the first 24 hours without liquid to allow the germination of the organism. Without this, there was considerable loss of material, and the circulation of solution was easily blocked.

3.7.8 Packed bed reactor culture

It was important to have the air entering at the top of the reactor to prevent the substrate from being pushed up. It is considered surprising that this has not been mentioned in the literature related to solid fermentation in a packed bed reactor, unless such serious channelling happened in the packed bed reactor that this phenomenon was not observed. This pushing up of the substrate by the air was observed after 3 days fermentation, and this would, at least, affect the temperature measurement, or even push the substrate out of the reactor, causing termination of the experiment. Gumbira-Sa'id *et al* (1993) reported the shrinkage of the substrate during the fermentation which was also observed in the present experiments.

In addition, a clear solution was found to accumulate at the bottom of the reactor after 2 days fermentation, and the organism grew on its surface. This solution was collected and mixed with the fermentation substrate when analysis was carried out.

3.7.9 Multi-layer packed bed reactor culture

The loading of substrate layer by layer was difficult. However, this was overcame by the use of a flexible pick-up tool (modified length of 80 cm). The multi-layer packed bed reactor allowed collection of representative air samples, compared to other reported work, in which air samples were withdrawn from the substrate (Gumbira-Sa'id *et al*, 1993, Gowthaman *et al*, 1993; Ghidyal *et al*, 1994). In the latter technique of sample withdrawal, firstly, because of the extra vacuum exerted at the sample area, the air distribution inside the substrate could be affected, and, secondly, it could cause

channelling. In addition, samples taken in this express only the air distribution at the sample port. Therefore, the multi-layer packed bed reactor was a successful piece of equipment to allow appropriate research on solid substrate fermentation.

3.7.10 Expression of Concentrations and Yields

Unless otherwise stated, all concentration are expressed on the basis of original wet weight of substrate, while biomass and citric acid yields are expressed on the basis of starch utilized.

Chapter 4

Selection of Substrate and Organism for Citric Acid Production

4.1 Introduction

Submerged fermentation processes for citric acid production by *A. niger* generally use sucrose- or starch-based media (Dawson, 1986, Röhr *et al.* 1983). Because the necessary nutrients are in solution, and mixing is provided by various agitation devices, these processes are reasonably homogeneous, and it is relatively easy to adjust the medium composition by addition or removal of nutrients. In addition, at the end of the process, separation of biomass from the fermented solution is easy to achieve by means of filtration. In solid substrate fermentation, there may be a heterogeneous distribution of the nutrient constituents and non-fermentable solid matter may be present, causing uneven growth of organism on the substrate. In addition, successful separation of fungal biomass from the substrate may be more difficult. The latter presents a particular problem since accurate measurements are required for any kinetic analysis which is basic to the understanding of solid substrate fermentation. As reported in the literature, apple pomace (Hang, 1988), wheatbran (Shankaranand and Lonsane, 1994), and even kiwi fruit peel (Hang *et al.* 1987) can be used as substrates for citric acid production in solid substrate fermentation, but the authors indicated that it was almost impossible to achieve separation of fungal biomass for its accurate estimation.

For the purpose of biomass estimation, many methods have been proposed, such as estimation based on glucosamine (Sakurai *et al.* 1977; Aidoo *et al*, 1982; Desgranes *et al.* 1991), on the combination of ergosterol and total sugar (Seitz *et al*, 1978; Desgranes *et al.* 1991), and on glucosinolate (Smits *et al*, 1994). In addition, carbon dioxide evaluation has been used for biomass estimation by Sugama and Okazaki (1979), Okazaki *et al.* (1980) and Carrizalez *et al.* (1981). Recently, mathematical models have been developed to explore the possibility of estimation (Mitchell *et al.* 1988, 1990; Laszlo and Silman, 1994), and even a fuzzy logic mathematical method has been used

by Smits *et al.* (1994) for this purpose. However, a reliable method for the direct measurement of biomass has not been reported in the literature.

Therefore, for the purpose of the present work, an appropriate substrate for citric acid production in solid substrate fermentation would focus not only on citric acid production but also on a successful procedure for biomass separation and measurement.

4.2 The Basic Parameters of the Substrates

Since the substrates which were being considered for this work contained starch as their main carbon source, it was likely that during the fermentation process, the fungal biomass would be an admixture with the starch and also with some other material. Hence, for the starch measurement and fungal biomass estimation in later experiments, it was decided to separate the starch from the other material by hydrolysis to glucose, followed by its determination as the monosaccharide. The remaining solid material, containing mainly fungal biomass, could then be estimated reasonably accurately by determination of its dry weight. To hydrolyse the starch, two techniques were investigated. The first used a combination of enzymes (Termamyl 120L, an amylase, and Super San 200L, a glucoamylase), while the second used hydrochloric acid. The methodology used for the enzymatic hydrolysis is described in Section 2.2.3, while for hydrochloric acid, the substrate was mixed with 10% HCl and held at 100°C for 30 minutes. The results for the starch contents of three substrates are shown in Table 4.1. Also shown are the contents of the residual, i.e. non-hydrolysable material (NHM). For potato, starch determination after acid hydrolysis gave a higher value than after enzymatic hydrolysis, with a correspondingly lower value of NHM. For kumara, the starch values from the two techniques were virtually identical, but the NHM value was much lower after acid hydrolysis. Consequently, since enzymatic hydrolysis gave a better figure for the starch content, and acid hydrolysis may hydrolyse some non-starch material (including, possibly, fungal biomass if present), enzymatic hydrolysis was selected for all further experiments.

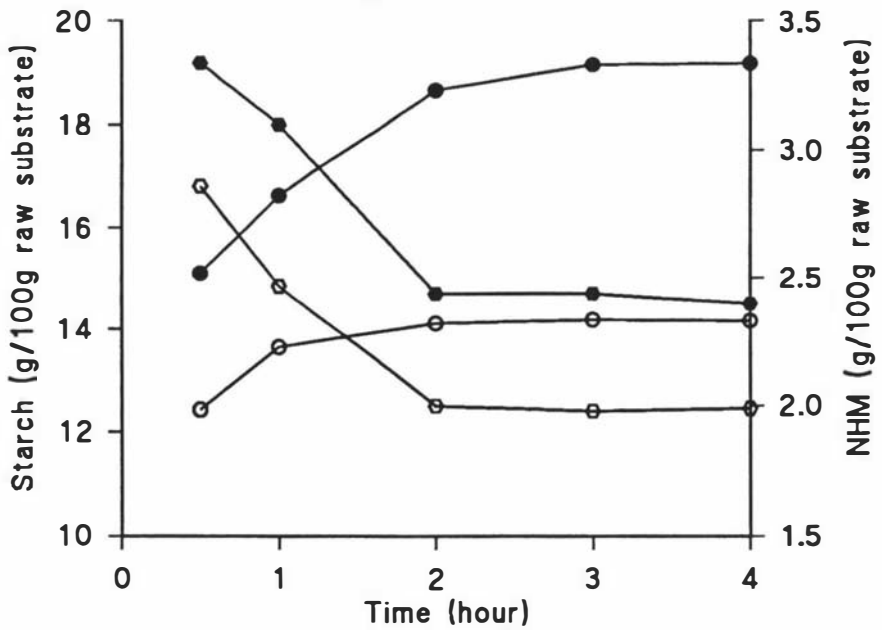
Table 4.1 Starch contents of substrates as determined following acid or enzymatic hydrolysis
(100 g raw substrate)

Substrate	hydrolysis method	Starch (g)	NHM(g)	Total dry matter(g)
Potato	enzymatic	14.1	2.0	21.3
Potato	acid	16.1	0.9	
Kumara	enzymatic	20.1	2.4	27.9
Kumara	acid	20.2	1.9	
Taro	enzymatic	24.5	3.0	34.8

Table 4.2 Starch contents of substrates based on wet and dry weight of the substrate

Substrate	Based on dry weight(%)		Based on wet weight(%)	
	starch	NHM	starch	NHM
Kumara	71.7	8.6	20.1	2.4
Potato	65.7	9.4	14.1	2.0
Taro	68.9	8.6	24.5	3.0

Figure 4.1 Time profiles of substrates hydrolysed using enzymes



Symbols:● starch, ● NHM of kumara; o starch, o NHM of potato

Fig 4.1 shows the relationship between hydrolysis time and the determined starch and NHM contents of potato and kumara. From this figure, the hydrolysis of both kumara and potato were almost complete after 2 hours. Therefore, 4 hours of hydrolysis time was used to guarantee the completion of hydrolysis and a stable NHM content of both potato and kumara, and was adopted for starch and NHM determination in all future experiments.

Sum up, the starch and NHM contents of substrates estimated following enzymatic hydrolysis are shown in Table 4.2. However, these parameters were measured in each experiment conducted during this work.

4.3 Comparison of Citric Acid Production among Potato, Kumara and Taro Using *A. niger* Yang No.2 and MH15-15

Potato, kumara and taro were tested for their effectiveness as substrates for citric acid production using *A. niger* Yang No.2 and MH15-15. All substrates were used as pastes, containing their original moisture content with an inoculum size of 10^8 spores per 50 g substrate. Experiments were conducted in flasks as described in Section 2.3.4, and the results are shown Table 4.3. Kumara and taro were excellent substrates for citric acid production, while potato was poor. However, the amounts of fungal biomass produced on all substrates were quite similar. There appeared be no major difference in citric acid production between the two strains of organism, but strain MH15-15 may be slightly superior to Yang No.2 in citric acid yield. However, Yang No.2 was selected for all future experiments because of the shorter culture time required for spore production, and because of the ready availability of its culture medium which could eliminate the handling of the synthetic sucrose-beef extract medium for MH15-15.

Table 4.3 Citric acid production from kumara, taro and potato using *A.niger* strain Yang No.2 and MH15-15

Organism	Substrate	Time (day)	initial starch (g/kg)	Fungal biomass (g/kg)	Citric acid (g/kg)	Yield of citric acid (%)	Yield of biomass (%)	Percentage of Starch utilised (%)
Yang No.2	Potato	6	141	31.7	3.4	3.3	30.4	74.0
	Potato	9	141	30.1	1.2	1.0	24.6	86.7
	Kumara	6	201	32.3	68.7	57.9	27.2	59.0
	Kumara	9	201	36.2	71.6	47.4	23.9	75.2
	Taro	6	245	30.9	66.3	78.2	36.5	34.6
	Taro	9	245	31.7	68.9	57.5	26.5	48.9
MH15-15	Potato	9	141	29.6	7.10	5.4	22.4	93.6
	Kumara	9	202	30.2	83.6	61.1	22.1	67.7
	Taro	9	245	30.7	76.5	55.5	22.3	56.3

Note:all concentrations are based on the initial wet weight of the substrate.

Table 4.4 Nutrients compositions of potato, kumara and taro (per 100 g)

nutrient	CBH (g)	Nitrogen (mg)	Phosphate (mg)	Iron (mg)	Copper (mg)	Zinc (mg)	Manganese (mg)
Potato[a]	17.01	280	33	0.57	0.14	0.30	0.12
Kumara[a]	27.50	200	21	0.53	0.11	0.20	0.75
Taro[b]	28.00	176	--	0.5	--	0.5	--

Note: a, data from FOODINFO, New Zealand Institute for Crop and Food Research, pp.1151, and 1203.
b, data from The Concise South Pacific Commission Food Composition Tables, 1993.
CHB, carbohydrate.

Table 4.5 Comparison of ratio of carbohydrate to other nutrients for potato, kumara, taro and a liquid medium

Ratio	CBH/N (g/g)	CBH/P (g/g)	CBH/Fe (g/mg)	CBH/Cu (g/mg)	CBH/Zn (g/mg)	CBH/Mn (g/mg)
Potato	61	515	30	122	57	142
Kumara	138	1309	52	250	138	37
Taro	159	--	56	--	56	--
[a]liquid medium	660	614	1400	2333	1400	--

Note:[a] a liquid medium based on sucrose (Dawson, 1986)
CBH, carbohydrate.

To attempt to understand the reason(s) for the superiority of taro and kumara over potato as substrate for citric acid production, the literature was consulted for their compositions, and a summary is shown in Table 4.4. To compare more easily these compositions, the ratios of carbohydrate to other nutrients were calculated, and the results are shown in Table 4.5, along with similar ratios from a liquid medium which supports strong citric acid production by *A. niger* MH15-15 (Dawson, 1986). Since it is known that citric acid production usually occurs under nitrogen-limited and/or phosphate-limited conditions (Kristiansen and Sinclair, 1978; Dawson *et al*, 1989), it is possible that potato is a poor substrate because of its relatively high nitrogen and/or phosphate content, which is indicated more clearly in Table 4.5. Experiments to investigate this hypothesis would be performed, at least with regard to the nitrogen content, as described below.

Nonetheless, kumara was selected as substrate for all further detailed work on citric acid production in solid substrate fermentation. Taro was abandoned because of its sticky consistency which made handling difficult, and because of its higher NHM content which would interfere with biomass estimation. In addition, although taro was the preferred substrate on the basis of the citric acid yield, the starch utilization was relatively low, and it was considered that this could complicate the analytical procedures of biomass separation.

4.4 Reason(s) for poor Citric Acid Production from Potato

Potato was originally selected as a potential substrate for citric acid production in solid substrate fermentation because it was readily available in local supermarkets. However, as described above, it proved to be a poor substrate. The following experiments were undertaken to ascertain the reason(s) for this and, if possible, to enhance citric acid production. *A. niger* strain MH15-15 was used as the strain of organism, potato paste was the medium, and an inoculum size of 10^8 spores per 50 g of substrate was used. All experiments were conducted in flasks.

4.4.1 Time course of citric acid production in solid substrate fermentation

The initial experiment to assess the potential of potato for citric acid production in solid substrate fermentation was performed as described above, and the full results are shown in Fig.4.2. Clearly the organism was able to use starch readily and to grow very well, but citric acid production was very poor. On day 6, 115 g/kg substrate of starch had been consumed, representing 83% of that present initially, while 34.1 g/kg substrate of biomass was produced. Biomass accumulation stopped and declined on day 9, coincidental with the depletion of starch. Citric acid production was very low during the process and even declined after day 9. This has been observed by other workers when the carbon substrate is exhausted.

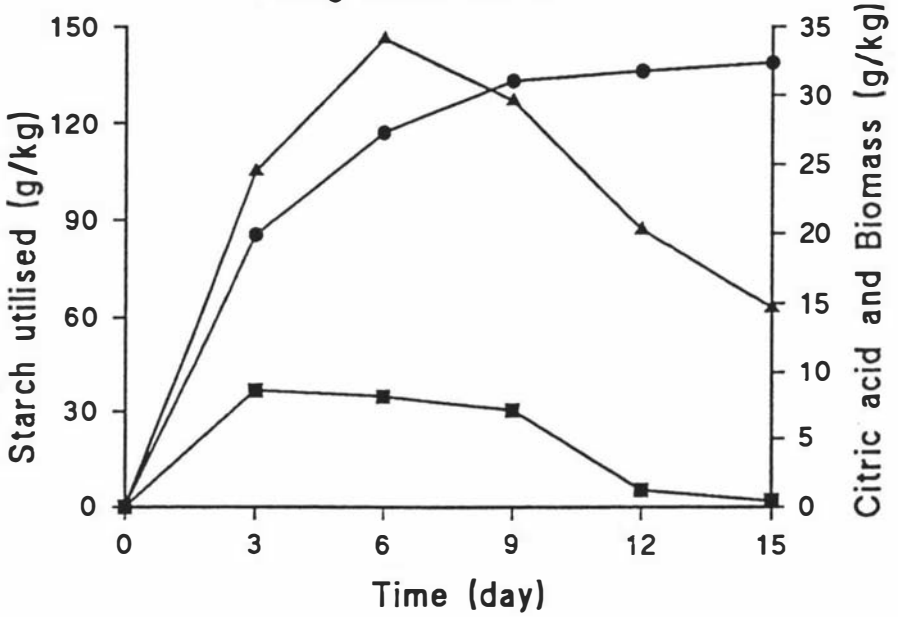
According to the generally accepted theory of citric acid accumulation, many factors might significantly affect citric acid production, including such things as nutrients excess or deficiency, or both carbon source and inoculum size. The following experiments were designed to investigate some of these factors.

4.4.2 Effect of nitrogen and phosphate addition on citric acid production

Among the various nutrients required by the organism, nitrogen and phosphate have a critical effect on citric acid production. Owing to the difficulty of removal of excess nitrogen or phosphate from the substrate, the experiments described here were conducted to assess the effect of supplementation with these nutrients. Nitrogen supplementation was achieved by the addition of the required amount of $(NH_4)_2SO_4$ to the potato, while phosphate supplementation was achieved using KH_2PO_4.

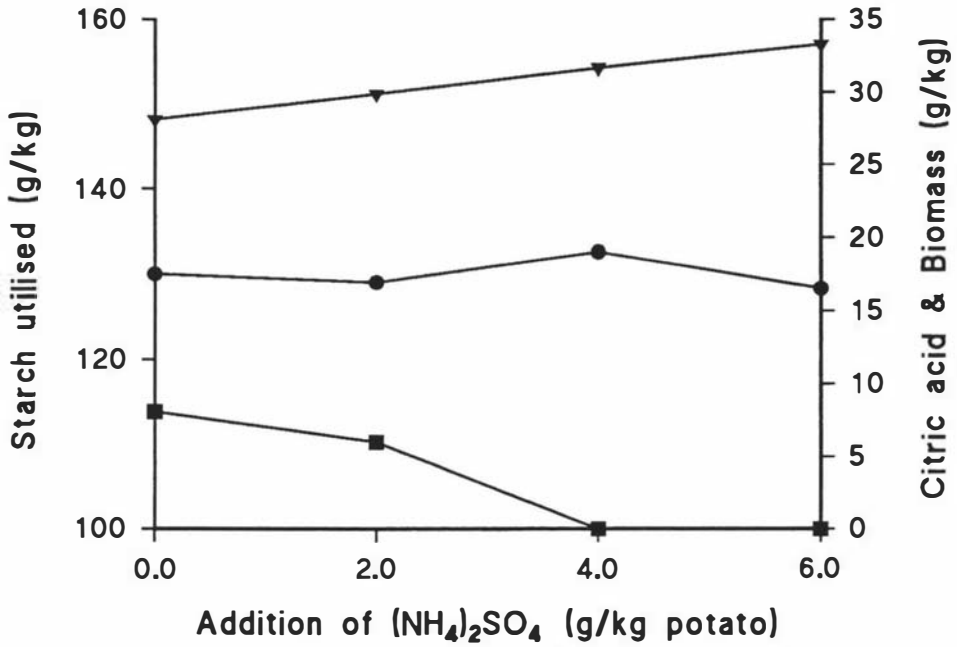
With increasing supplementation with either nitrogen or phosphate, citric acid production barely improved, or even declined, while biomass increased slightly, as shown in Fig.4.3 and 4.4 respectively. These results indicate that potato is not deficient in either nitrogen

Figure 4.2 Time course of citric acid production from potato using strain MH15–15



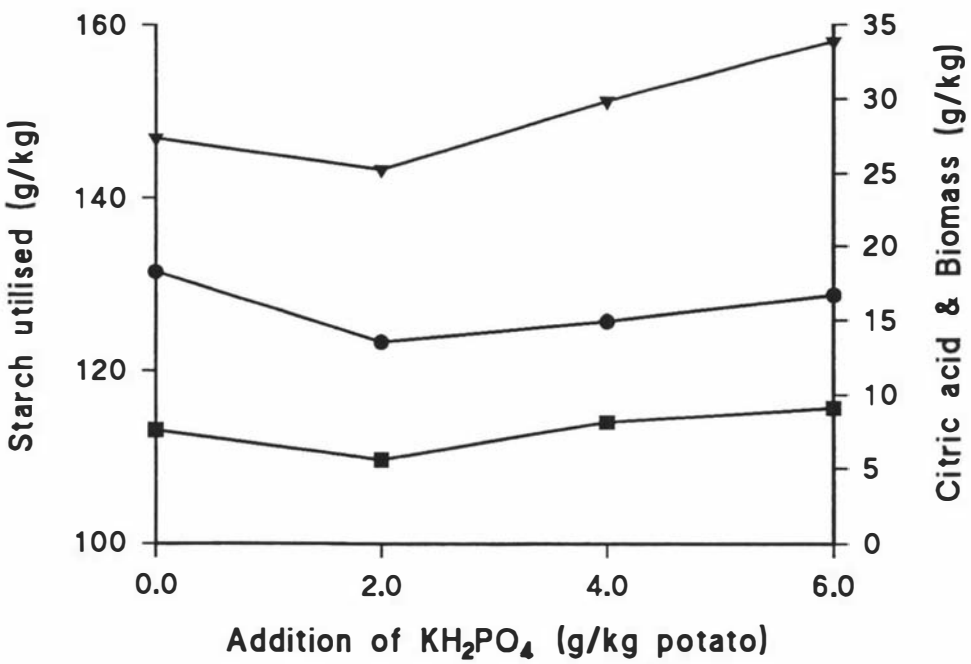
Symbols:● starch, ■ citric acid, ▲ biomass

Figure 4.3 Effect of addition of nitrogen on citric acid production from potato using strain MH15–15 (6 days fermentation)



Symbols:● starch, ■ citric acid, ▼ biomass

Figure 4.4 Effect of addition of phosphate on citric acid production from potato using strain MH15–15 (6 days fermentation)



Symbols:● starch, ■ citric acid, ▼ biomass

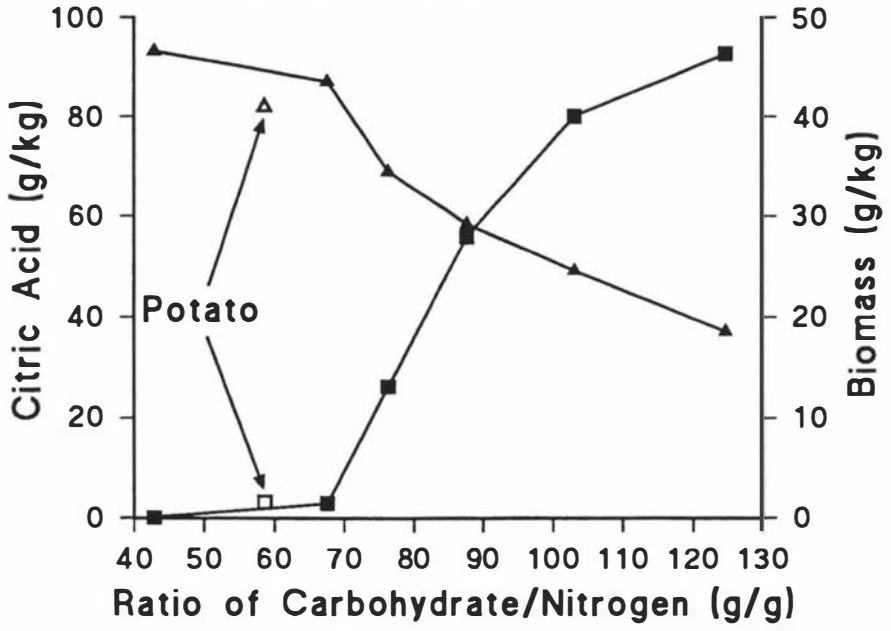
or phosphate so far as citric acid production is concerned. However, there is an indication that the nitrogen content of potato may be excessively high, with a resulting detrimental effect on citric acid production.

4.4.3 Effect of nitrogen addition on citric acid production from kumara

As discussed above, the poor production of citric acid from potato may be due to an excess of nitrogen content. Unfortunately, it would be difficult experimentally to remove nitrogen from potato. However, there are two other ways of testing the hypothesis. First, experiments could be conducted in which the potato was supplemented with additional starch, hence increasing the ratio of carbohydrate to nitrogen. However, the results of such experiments may be ambiguous due to the decreasing water activity of the substrate that occurs with increasing carbohydrate content. Secondly, experiments could be conducted with kumara (which has a higher ratio of carbohydrate to nitrogen and is an excellent substrate for citric acid production), supplemented with additional nitrogen. In this way, the ratio of carbohydrate to nitrogen could be adjusted to simulate that of potato. To perform this experiment, additions of $(NH_4)_2SO_4$ to kumara were carefully prepared to obtain a homogenous distribution of nitrogen as described in Section 3.6.1. Data for the original nitrogen content of kumara were obtained from Table 4.4 while the carbohydrate content was determined experimentally. Strain Yang No.2 was used for this experiment as it was easier to grow on the medium used for routine culture transfer.

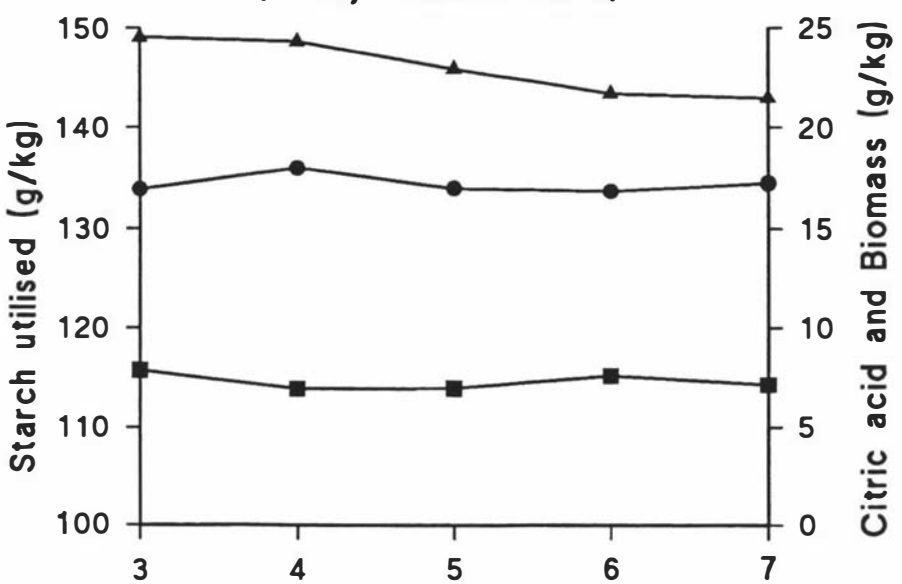
The effect on citric acid production of additions of $(NH_4)_2SO_4$ to kumara paste is shown in Fig.4.5. With increasing additions of nitrogen, citric acid production began to decline. When the ratio of carbohydrate to nitrogen reached a point similar to that of potato, citric acid production was very little, i.e. similar to that from potato itself. In contrast biomass production increased with the increasing addition of nitrogen.

Figure 4.5 Effect of addition of nitrogen on citric acid production from kumara using strain Yang No.2 (6 days fermentation)



Symbols: ■ citric acid, ▲ biomass.

Figure 4.6 Effect of inoculum size on citric acid production from potato using strain MH15–15 (9 days fermentation)



Symbols:● starch, ■ citric acid, ▲ biomass.

4.4.4 Effect of inoculum size on citric acid production

To test the effect of the inoculum size on citric acid production from potato, different concentrations of spore suspensions were carefully prepared by counting as described in Section 2.2.3. The experimental results observed after 9 days of fermentation are shown in Fig.4.6. The variation of inoculum size from 10^3-10^8 spores per 50 g potato paste had no obvious effect on citric acid or biomass production, or starch utilization. The inoculum size was therefore excluded as being a critical factor for citric acid production from potato.

4.5 Discussion

The enzymatic hydrolysis of starch and measurement of resulting monosaccharide appeared to be an effective technique for starch determination, and, in addition allowed successful separation of fungal biomass from the fermented material. Because the enzymes have no reaction which may dissolve fungal biomass, the loss of such biomass into solution during the analysis procedure is probably very little. However, some non-hydrolysable material (NHM) did remain after starch hydrolysis, and this would interfere with the estimation of fungal biomass. Consequently, for all determinations of biomass, the original value of NHM in the substrate was subtracted from the total dry matter to allow the estimation of fungal biomass.

The effect of the nitrogen content of the substrate has proved to be substantial for citric acid production in solid substrate fermentation. Potato, which had the highest nitrogen content of the substrates tested supported excellent fungal growth, but poor citric acid production. Taro and kumara, with lower nitrogen contents, supported equally good fungal growth, but citric acid production was markedly superior. The conclusion to be drawn is that citric acid production in solid substrate fermentation resembles that in submerged fermentation in that a nitrogen-deficient medium is required.

4.6 Conclusions

The main conclusions that can be drawn from this series of experiments are that potato is an unsuitable substrate for citric acid production because of its high nitrogen content. In contrast, kumara and taro are excellent substrates for citric acid production in solid substrate fermentation. However, taro was abandoned because of its very sticky consistency and high content of non-hydrolysable material. Thus, kumara and strain Yang No.2 were used for all further experiments.

Chapter 5

Optimization of Citric Acid Production from Kumara

5.1 Introduction

In general solid substrate fermentation, the initial moisture content of the substrate, the substrate particle size, its metal ion content and the inoculum size are critical to the fermentation in microbiological (organism growth and product formation) and biochemical (reactor design) aspects, and have been investigated to a greater or lesser extent. Because of their possible importance, it was considered imperative to investigate these factors and, if possible, to optimize them, prior to conducting a kinetic study of the process.

For citric acid production in solid substrate fermentation, Hang *et al.*(1987) and Hang (1988) reported that the initial moisture content had a substantial effect on citric acid production from apple pomace and grape pomace, respectively. Shankaranand and Lonsane (1994) recently studied the effects of multiple metal ions on citric acid production from wheatbran. However, as mentioned previously, these studies did not include any biomass data due to the difficulty of biomass measurement, so that the analysis and understanding of the processes were limited, particularly the effects of these factors on aspects of morphology, substrate uptake and mass transfer. In addition, some basic characteristics of solid substrate fermentation, such as the ability of the fungus to penetrate into the substrate, have been frequently described in relation to the uptake of nutrients from the solid substrate, but the relationship of this ability to substrate uptake, biomass and product formation has rarely been found in the literature. For these reasons, the experiments described in this chapter were conducted as a prelude to the kinetic study.

5.2 Effect of the Initial Moisture Content of the Substrate on Citric Acid Production

Generally, the initial moisture content of cooked kumara was approximately 71%. To set up the experiments, dry kumara (particle size between 1-2 mm, moisture content 5%) was obtained as described in Section 2.2.3. To adjust the moisture content to the appropriate value, distilled water was added to the dry kumara before sterilization of the substrate. The mouth of the flasks was wrapped firmly with two layers of aluminium foil to prevent the loss of moisture or invasion of steam during sterilization. The estimation of the initial moisture content included the dry kumara moisture content, any added distilled water and the spore inoculum (1 ml per flask). Each flask was loaded with 15 g dry kumara, and after the adjustment of moisture content and sterilization, each flask was inoculated with an inoculum size of 10^6-10^8 spores.

The results are shown in Figs 5.1 and 5.2. In Fig 5.1, the citric acid and biomass concentrations are expressed on the basis of the dry weight of kumara to provide an accurate comparison. Since the data are all taken after 6 days of fermentation, the citric acid and biomass concentrations may be interpreted as rates of production rather than the total amount achievable. On this basis, a moisture content of 65% or higher is necessary to achieve the maximum production rates of biomass and citric acid. When the initial moisture content was below 55%, the growth of the organism was seriously inhibited, and citric acid production and starch utilization were low. At a moisture content of 65% or higher, the biomass yield was approximately 12% based on the starch utilised, while the citric acid yield was approximately 50% based on starch utilised. It is noticeable, however, that at moisture contents below 60%, the biomass and citric acid yields, based on starch utilised were both substantially lower than that at the higher moisture contents. This may reflect the fact that at these low moisture contents, the fermentation is still in its early stages, and citric acid production has not yet reached its maximum rate. However, the fact that both the biomass and citric acid yields are low indicates that much of the starch is unaccounted for. Further, at these low moisture

Figure 5.1 Effect of initial moisture content on citric acid production (6 days fermentation)

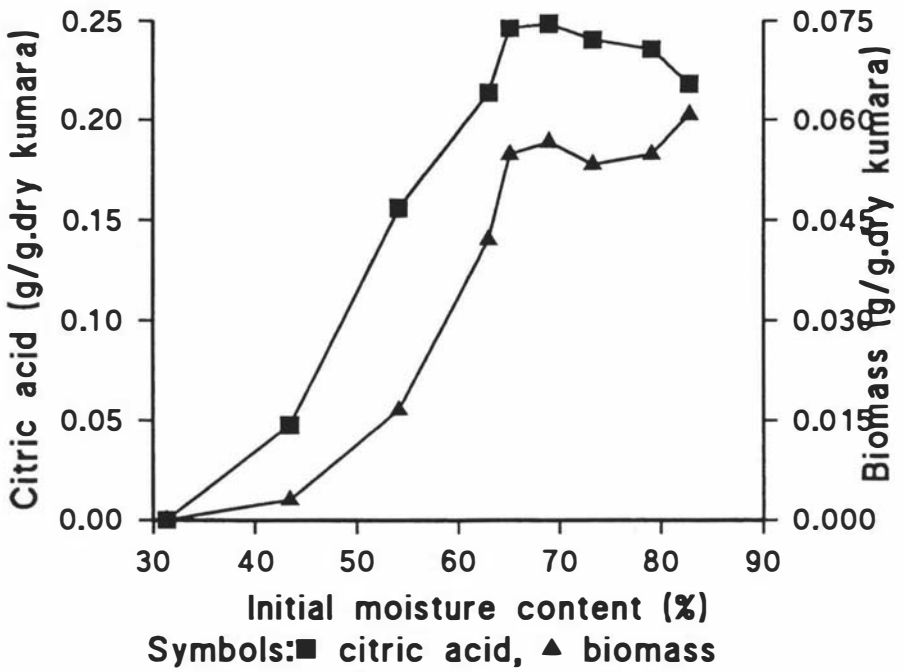


Figure 5.2 Effect of initial moisture content on yields citric acid and biomass (6 days fermentation)

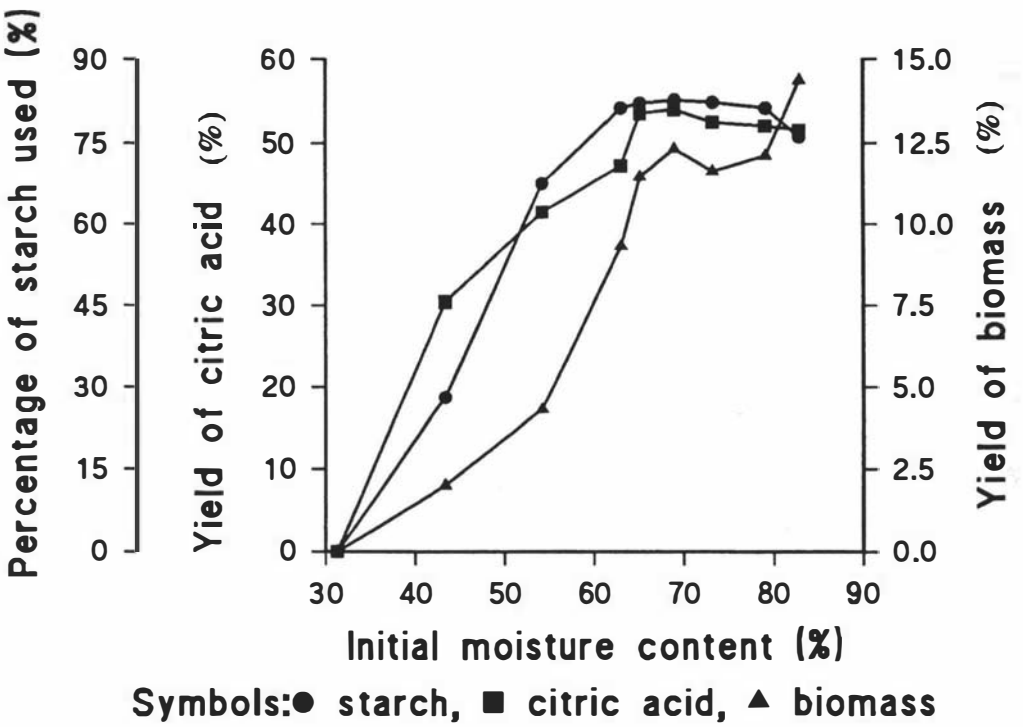
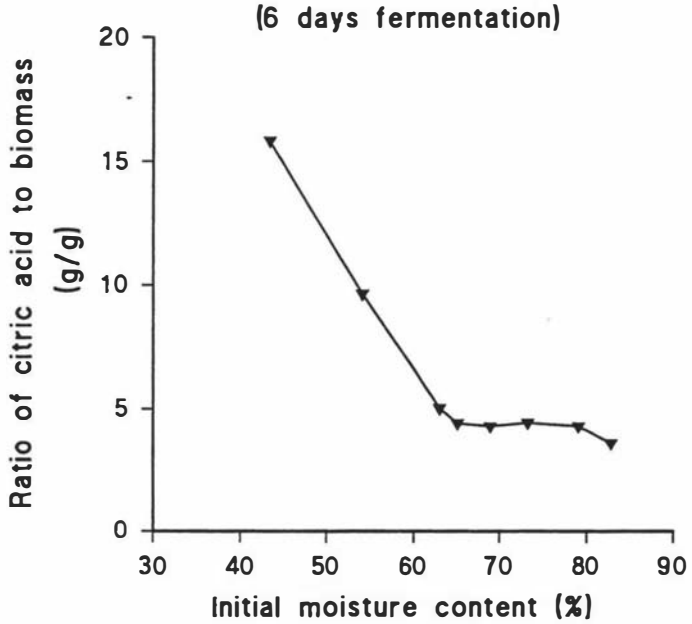


Figure 5.3 Effect of initial moisture content on the ratio of citric acid to biomass production (6 days fermentation)



contents, the biomass yield is more markedly depressed than is the citric acid yield (Fig 5.2), indicating that some factors other than the stage of the fermentation process are having an effect. Assuming that carbon dioxide is the only other major metabolic product, these results indicate that at low substrate moisture contents, *A. niger* converts relatively more starch to carbon dioxide than to citric acid production and/or biomass, perhaps reflecting an increased maintenance energy requirement at low values of substrate water activity.

At initial moisture content values higher than 65%, neither the biomass nor the citric acid production showed any further increase, and starch utilization stayed relatively constant. Hence the optimum moisture content appears to reach a plateau where the ratio of citric acid to biomass is stable at a value of 4.3 g citric acid/g biomass.

This was, perhaps, surprising, since, with the initial moisture content increasing, the nutrients concentration becomes diluted, e.g. at an initial moisture content of 82%, the nutrients concentration was diluted about to 1.5 times lower than that at 71%. But such dilution of nutrients had no gross effect on citric acid or biomass production, although there might have been a slight effect on starch utilization at initial moisture contents of 82% or higher, as shown in Fig 5.2.

To sum up, the initial moisture content of kumara substantially affected the production of biomass and citric acid, with an optimum value being between 65% and 71%. Similarly, for citric acid production in solid substrate fermentation, Hang (1987) and Hang *et al.*(1988) reported an optimum initial moisture content of approximately 65% for apple pomace and grape pomace, but Shankaranand and Lonsane (1994) used an initial moisture content of 40% of wheatbran. However, the latter authors provided no biomass data.

5.3 Effect of Inoculum Size on Citric Acid Production

To determine if there is an optimum inoculum size for citric acid production in solid substrate fermentation, 40 g kumara paste with an initial moisture content of 71% was used in each flask, and a range of different spore numbers from 10^1-10^8 was used as the inoculum.

Figs 5.4, 5.5 and 5.6 show the results after 4, 6 and 8 days respectively, while Fig 5.7 shows the ratio of citric acid to biomass after 6 days fermentation. From Figs 5.4 and 5.5, it is evident that there was very little or no growth at an inoculum size of 10^1. Even after 8 days, flasks with inoculum sizes lower than 10^3 had less growth than that at higher inoculum size, which shows that a longer lag time is needed to achieve the appropriate growth at the lower inoculum sizes. Consequently, citric acid production and starch utilization were low. As the inoculum size was progressively increased, higher values of starch utilization and biomass production were achieved. However, at an inoculum size of 10^6 or higher, a slightly negative effect on citric acid production was observed. Fig 5.7, which shows the ratio of citric acid to biomass production, gives a much clearer indication of this effect. The fermentation conducted with an inoculum size of 10^5 spores achieved the highest ratio of citric acid to biomass production, indicating that at this value starch was converted to citric acid rather than to fungal biomass.

Focusing on achieving high citric acid production in a short period, an optimum inoculum size of 10^4-10^6 spores per 40 g kumara paste, that is $2.5\text{x}10^2$-$2.5\text{x}10^4$ spores/g, was selected as being appropriate. For citric acid production using other substrates, Hang (1987) stated that the inoculum size had no gross effect on citric acid production when using an inoculum size range of $1\text{x}10^5$ to $4\text{x}10^5$ spores/g apple pomace. This value is rather higher than that observed in the present work, perhaps because apple pomace is a less nutritious substrate.

Figure 5.4 Effect of inoculum size on citric acid production (4 days fermentation)

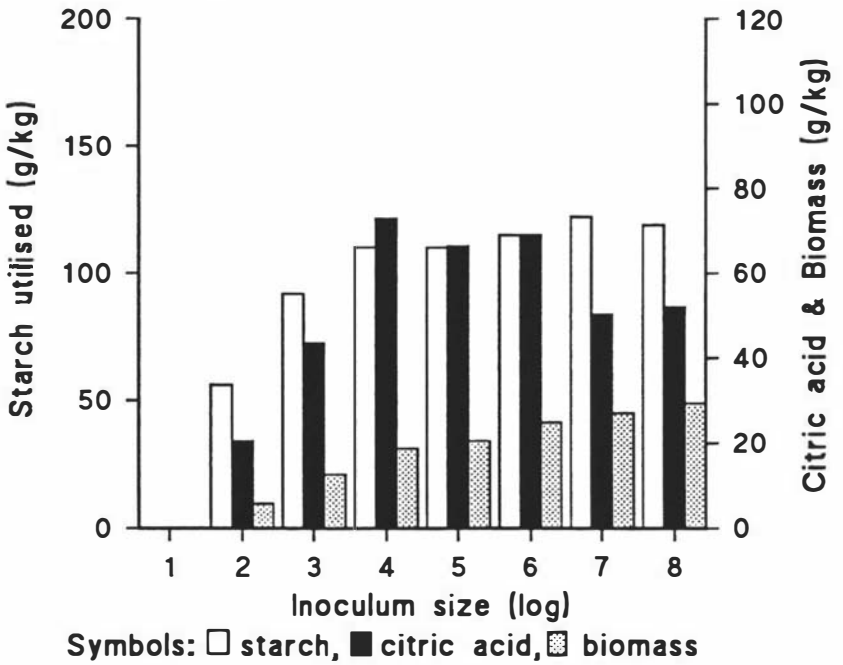


Figure 5.5 Effect of inoculum size on citric acid production (6 days fermentation)

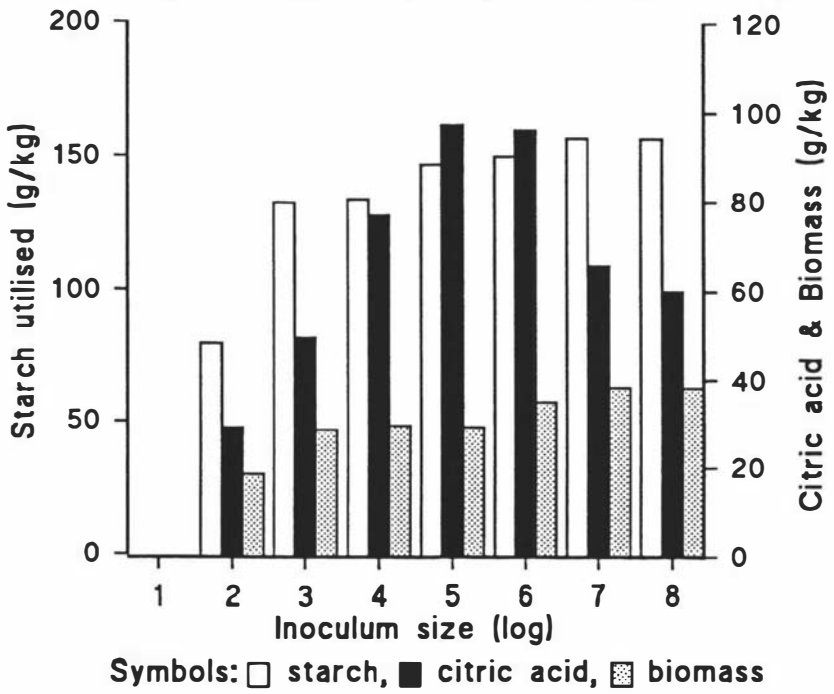


Figure 5.6 Effect of inoculum size on citric acid production (8 days fermentation)

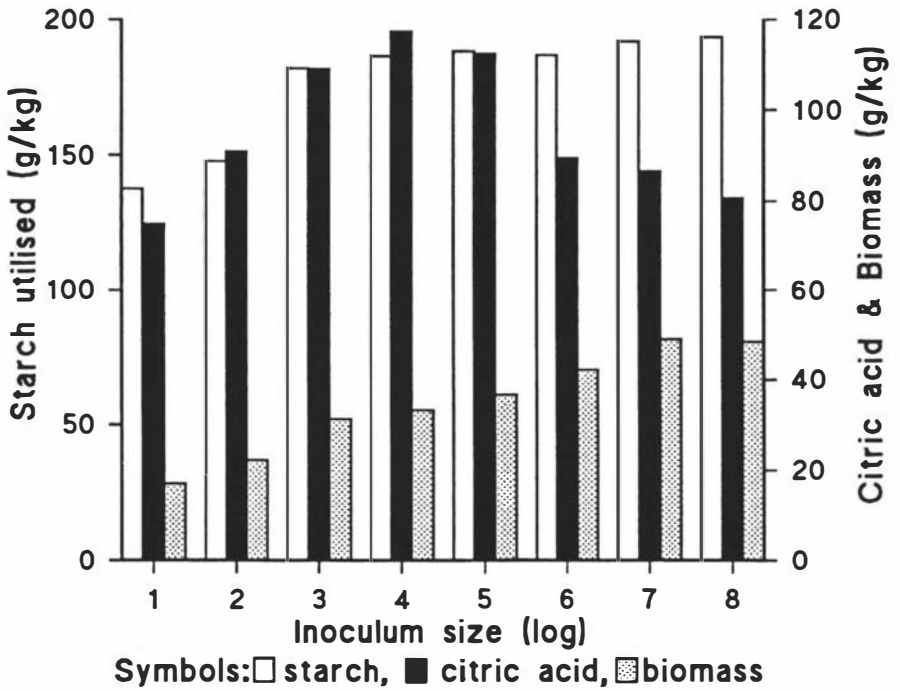
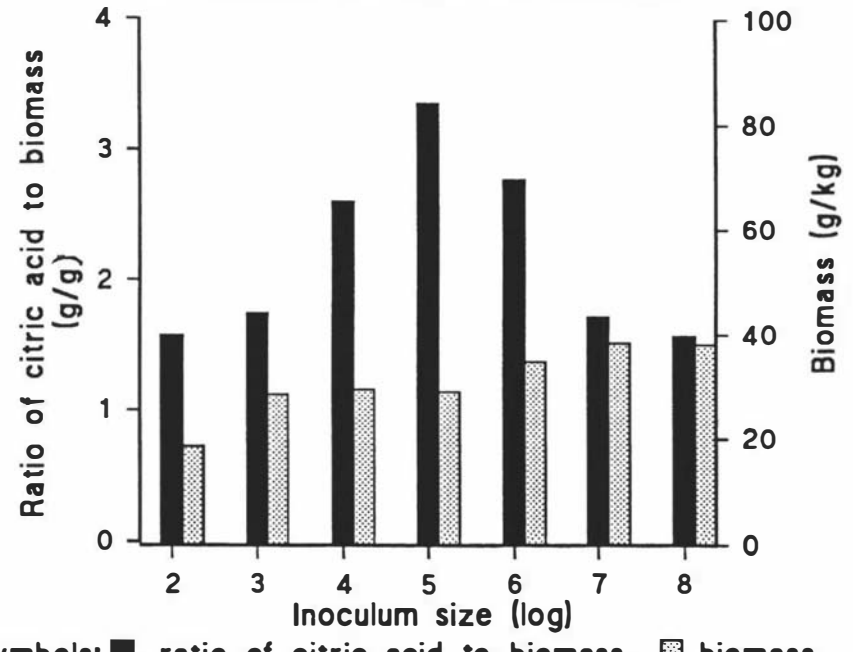


Figure 5.7 Effect of inoculum size on ratio of citric acid to biomass (6 days fermentation)



5.4 Effect of Particle Size of the Substrate on Citric Acid Production

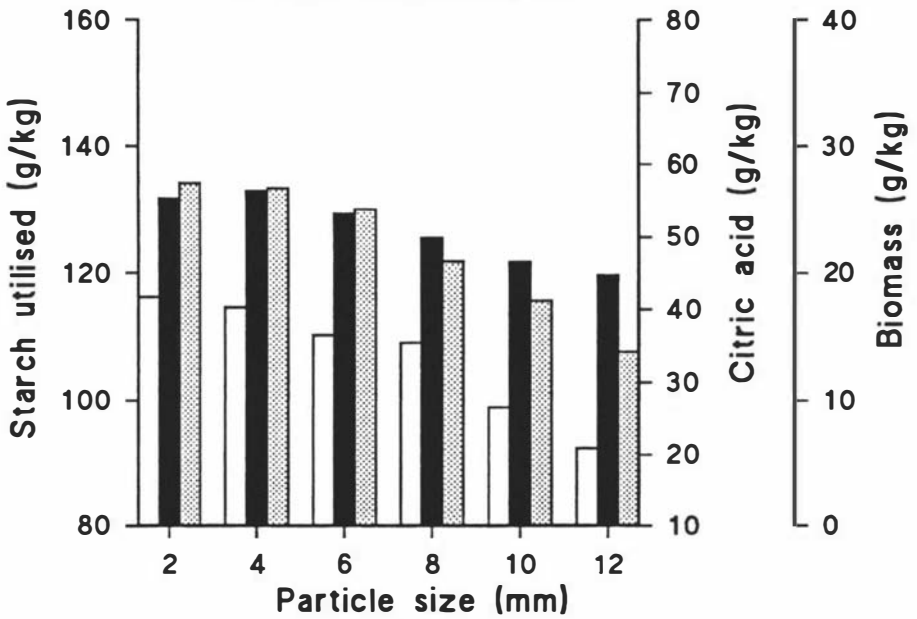
Substrate particles, of cubic shape, were obtained by cutting fresh, peeled kumara (moisture content of approximately 70%), and sterilizing as described in Section 2.3.1. Experiments were conducted in flasks, each flask containing 40 g of particular substrate. The flasks were inoculated with an inoculum size of 10^4 to 10^6 spores.

For a given amount of substrate, i.e. 40 g kumara, a different particle size will mean that a different amount of surface area is exposed to the organism. However, it is difficult to estimate the true exposed surface areas of particles of different sizes due to the way the particles pack within the flasks. Hence, an accurate assessment of exposed surface area cannot be made. Figs 5.8, 5.9 and 5.10 show the results of the experiments after 4, 5 and 6 days of fermentation for various particle sizes from 2 mm to 12 mm. Biomass production and starch utilization was favoured by the smaller particle sizes, probably reflecting the larger surface area available for fungal growth. Citric acid production was also favoured by the smaller particle size after 4 days fermentation, but after 6 days there was little difference in the concentrations achieved. The different effects of particle size on citric acid and biomass production are shown in Fig 5.11 where the ratio of citric acid to biomass production is plotted against the size. Clearly, higher production rates are favoured by smaller sizes. In terms of reasonable citric acid production, a compromise particle size may be 4 to 6 mm.

5.5 Effect of Ratio of Surface Area to Weight of the Substrate on Citric Acid Production

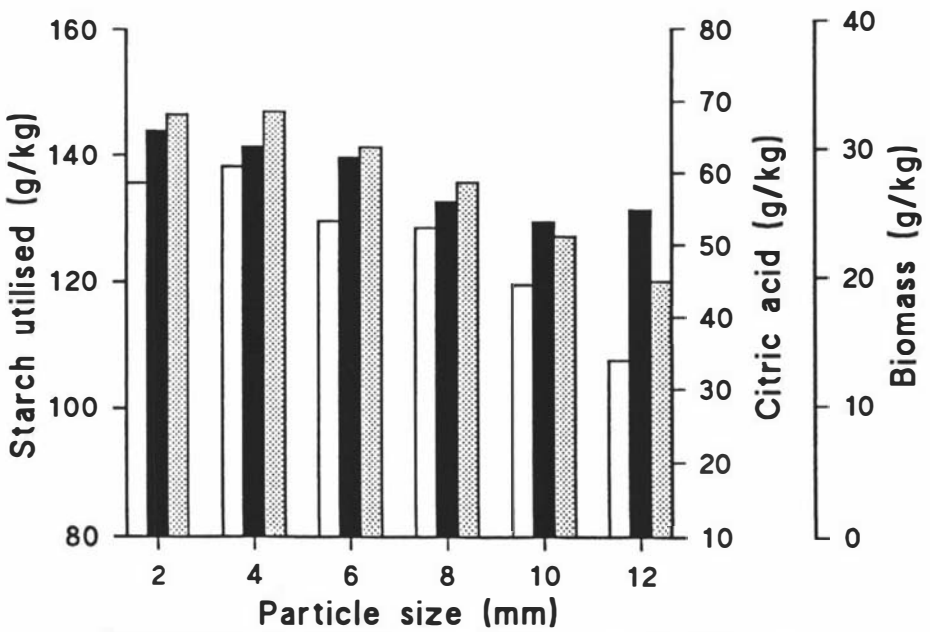
As described in Section 5.4, different particle sizes of substrate can provide different exposed surface areas for the organism. However it was difficult to estimate accurately the relationship between the surface area and citric acid production because of the way the particles packed inside the flasks. In order to obtain accurate surface area

Figure 5.8 Effect of particle size on citric acid production (4 days fermentation)



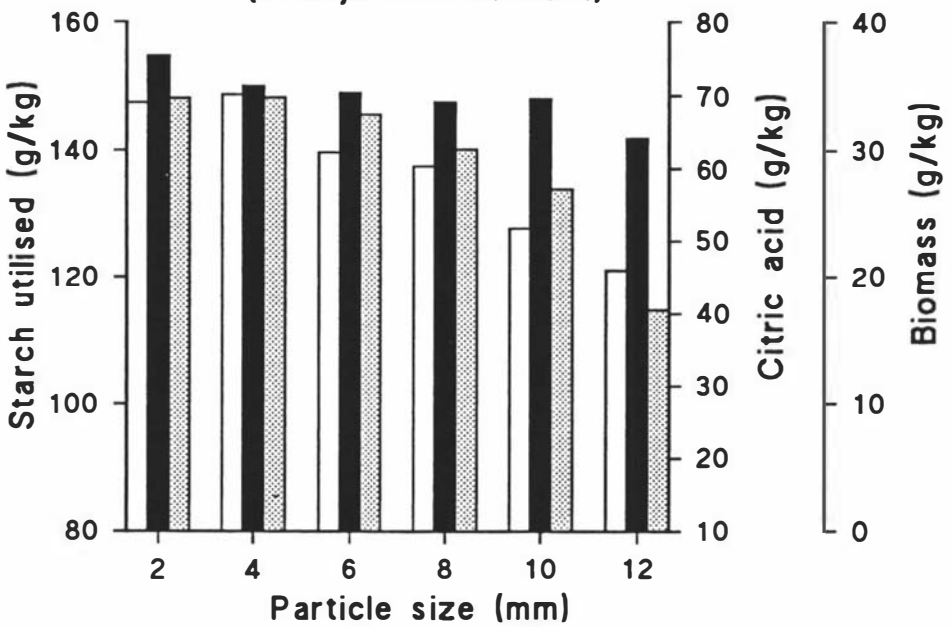
Symbols: □ starch, ■ citric acid, ▨ biomass

Figure 5.9 Effect of particle size on citric acid production (5 days fermentation)



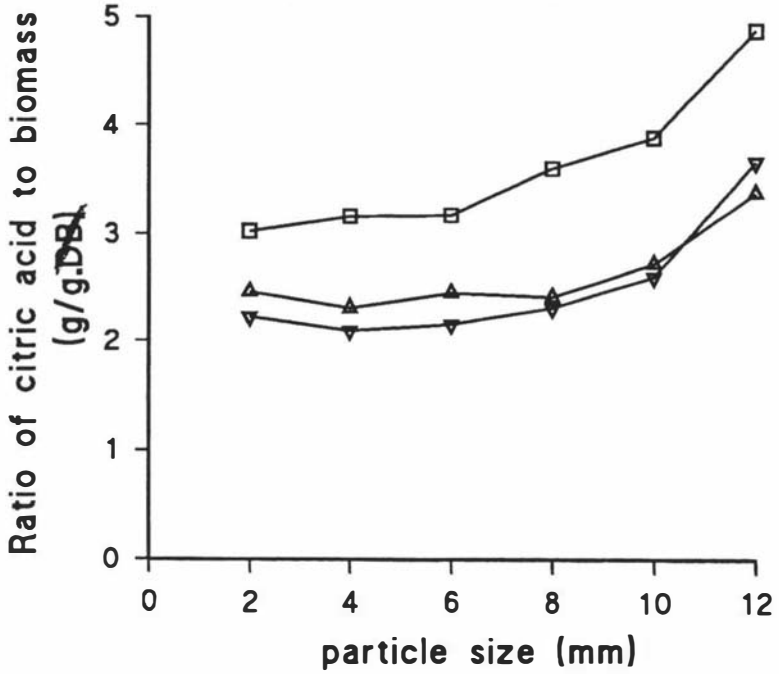
Symbols: □ starch, ■ citric acid, ▨ biomass

Figure 5.10 Effect of particle size on citric acid production (6 days fermentation)



Symbols: □ starch, ■ citric acid, ▨ biomass

Figure 5.11 Ratio of citric acid to biomass production varying on substrate particle size



Symbols: □ 4 days, △ 5 days, ▽ 6 days

measurements, experiments were performed in different diameter tubes, using homogenized kumara paste (moisture content approximately 70%) as substrate. Paste (5-20 g) was carefully conducted into the tubes, and an inoculum size of 10^4 to 10^6 spores was inoculated onto the surface of each tube. In this way, the inner radial cross sectional area of the tube represented the substrate surface area exposed to the organism when these tubes were loaded with the substrate. Since a constant inoculum size was used for a range of different surface areas, it is possible that the different inoculum sizes per cm^2 may have affected the results, but this was considered unlikely for the range of surface areas used.

The results are presented in Fig 5.12. As expected, citric acid and biomass production both increased with an increased ratio of surface area to weight of substrate. However, citric acid and biomass production, expressed per unit area, were rather similar at the ratios tested, as shown in Fig 5.13. This indicates that total biomass and citric acid production are limited directly by the surface area exposed to the organism rather than to the weight or volume of substrate.

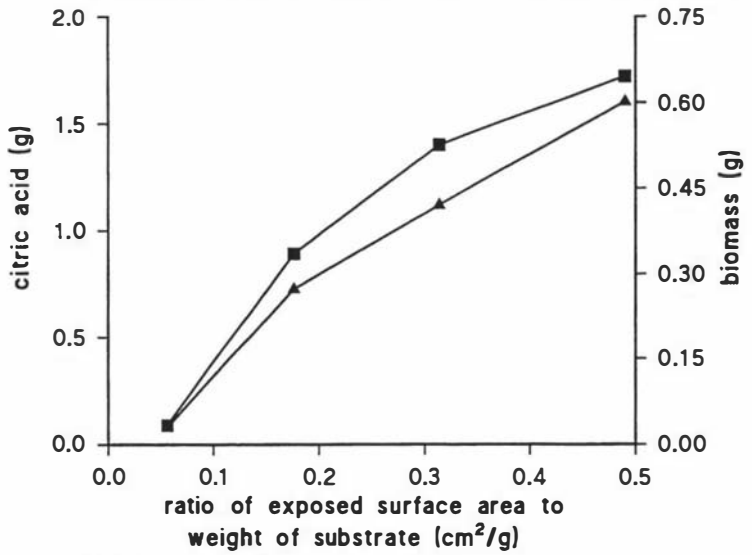
To focus on citric acid production, this result implies that for subsequent reactor design, the exposed surface area of the substrate should be as large as possible. The average citric acid and biomass production per unit area were 0.090 g/cm^2 and 0.035 g/cm^2 , respectively.

5.6 Measurement of Ability of Organism to Penetrate into Substrate

In solid substrate fermentation, fungi take up nutrients by means of penetration into the substrate. However, there is little published literature describing the measurement of this ability. Further, measurement of this ability is very important for the understanding of mass transfer in solid substrate fermentation. Experiments were conducted in tubes with a diameter of 30 mm, and with a load of 40 g kumara paste and an inoculum size of 10^4 to 10^6 spores per tube. The penetrating ability of the organism was expressed by the

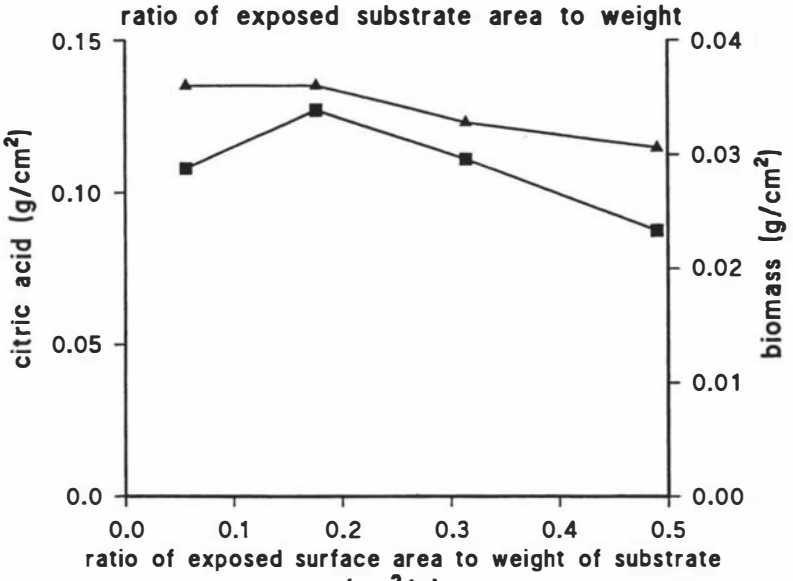
depth of substrate invaded by the organism, as judged by eye. The results are presented in Fig 5.14, based on the gram per unit area. On the first day, the organism still remained in the germination stage and there was no sign of penetration into the substrate. Starting from the second day, penetration was observed and coincided with commencement of biomass and citric acid production, and starch utilization. In the later period, after 4 days, possibly due to moisture loss, the substrate shrank and a gap emerged between it and the tube wall. Consequently, the organism penetrated into this gap, with the result that measurement of penetration depth became inaccurate. Despite this, the data provided give an initial indication of the ability of the organism to penetrate into the kumara substrate. Thus, it is clear that there is a close relationship between fungal growth and product formation, and the depth to which its hyphae penetrate the substrate. In the present experiments, penetration was observed to a depth of 15 mm after 6 days fermentation, compared to 1.2 mm depth in flour medium by *Rhizopus oligosporus* in a period of 48 hour (Mitchell *et al*, 1992) and 0.4 mm depth in rice grains by *A. oryzae* within 36 hours (Ito *et al*, 1989).

Figure 5.12 Effect on citric acid production of the ratio of exposed surface area to weight of substrate (6 days fermentation)



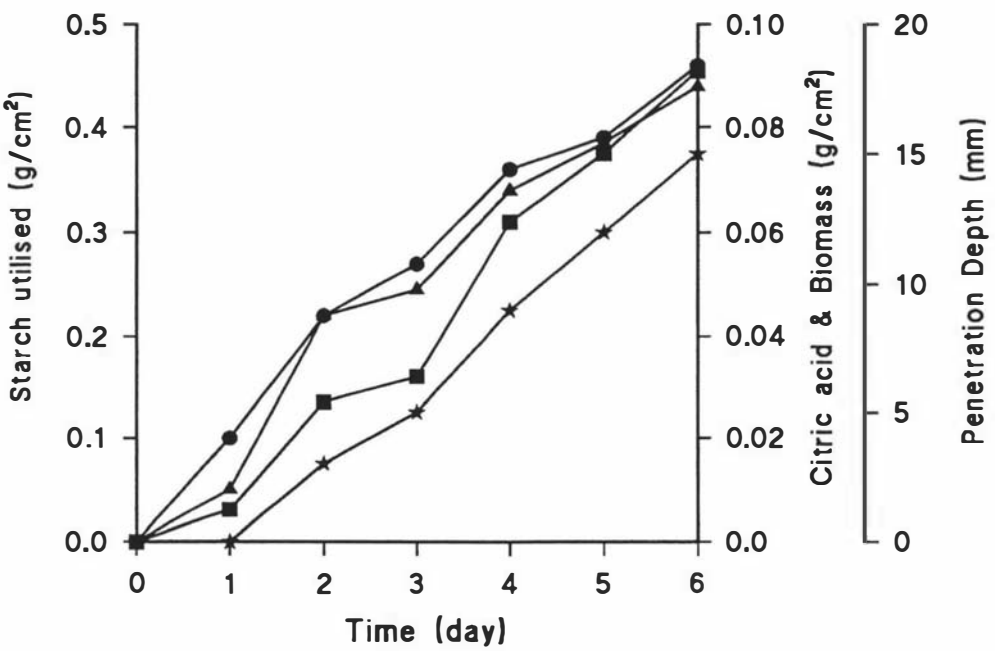
Symbols:■ citric acid, ▲ biomass

Figure 5.13 Effect on citric acid production per unit area of the ratio of exposed substrate area to weight



Symbols:■ citric acid, ▲ biomass

Figure 5.14 Measurement of penetrating ability of organism in SSF



Symbols:● starch, ■ citric acid, ▲ biomass and ★ depth.

5.7 Effect of Metal Ions on Citric Acid Production

As previously described in Chapter 2, the effects of metal ions on citric acid production in submerged and surface fermentation have been widely investigated. Generally, iron, copper, zinc and manganese are considered to be the major metal ions of importance. However, the inhibitory or stimulatory effects of these metal ions have been reported to vary in different fermentation states, with different strains of organism (Sanchez-Marroquin *et al.* 1970; Kubicek and Röhr, 1977; Roukas and Kotzekidou, 1987; Shankaranand and Lonsane, 1994) and, perhaps, with different substrates used. In citric acid production by solid substrate fermentation, Shankaranand and Lonsane (1994) recently studied the effect of some metal ions using a substrate of wheatbran. However, this detailed investigation lacked the support of biomass data. Furthermore, although a considerable number of experiments were conducted, the authors used the traditional method of experimental design, i.e. "one factor at time". Thus, the effects of interactions among the metal ions were analyzed separately from the main effects of the fermentations.

Many effective mathematical methods, such as fractional factorial design and Placket-Burman design, have been developed, which can overcome these disadvantages of traditional experimental method (Haaland, 1989). This could be particularly useful in solid substrate fermentation where there are many 'noise' factors which could interfere with the experimental results. However, a successful application of these mathematical methods has not been found in solid substrate fermentation for citric acid production. Although Srinivas *et al.* (1994) used a Placket-Burman design in SSF, their efforts simply screened multiple factors, while interactions were still ignored.

In the present experiments, a fractional factorial design was adopted to investigate the effect of iron, copper, zinc and manganese ions and their interactions on citric acid and biomass production from kumara.

5.7.1 The fractional factorial design for estimation of effects of metal ions on citric acid production

A half fractional factorial of a 2^4 design was adopted. Although the main effects are confounded with three-way and four-way interactions, the latter are considered to be negligible The design included three replicates of the centre points to test for non-linear characteristics of the main effects, and to provide a better estimation of experimental error. Table 5.1 shows the design matrix, from which the coded values would be used in the design analysis. The Minitab package (Copyright Pennsylvania State University, 1984) was used for the solution of the design.

The conditions used in the experiments were selected from those tested previously as being the optimum, i.e. initial moisture content of substrate of 71%, inoculum size of 10^4 to 10^6 spores/40 g kumara paste. To add the metal ions, a concentrated solution was used to minimize any effect on the initial moisture content of the substrate. To prevent the uneven distribution of added metal ions, kumara paste was blended in a blender for 10 minutes after the additions had been made. To prevent time trend effects, the experiments were carried out simultaneously. The original contents of metal ions in the substrate, which are shown in Table 4.5, were not included in the design analysis, while added concentrations were selected on the basis of being similar to those used by Shankaranand and Lonsane (1994) in their study using wheatbran. These added concentrations of metal ions are shown in Table 5.2 and the various responses of citric acid and biomass production to their addition, after 6 days fermentation, are presented in Table 5.3.

Table 5.1 The matrix of the fractional factorial design

Run number of experiments	Matrix of the design			
	Fe^{2+}	Cu^{2+}	Zn^{2+}	Mn^{2+}
1	-1	-1	-1	-1
2	-1	-1	+1	+1
3	-1	+1	-1	+1
4	-1	+1	+1	-1
5	+1	-1	-1	+1
6	+1	-1	+1	-1
7	+1	+1	-1	-1
8	+1	+1	+1	+1
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0

Table 5.2 The added concentrations of metal ions

Coded value	Added metal ions (mg/kg) (decoded value)			
	Fe^{2+}	Cu^{2+}	Zn^{2+}	Mn^{2+}
-1	0	0	0	0
0	75	12.5	37.5	75
+1	150	25	75	150

Metal ions sources:
Fe^{2+}---$FeSO_4.7H_2O$, Cu^{2+}---$CuSO_4.5H_2O$,
Zn^{2+}---$ZnSO_4.7H_2O$, Mn^{2+}---$MnSO_4.H_2O$

Table 5.3 Responses of citric acid production affected by the added metal ions after 6 days fermentation

Run	Response observations						
	citric acid (g/kg)	yield of citric acid (%)	biomass (g/kg)	yield of biomass (%)	starch used (g/kg)	percentage of starch used (%)	ratio of citric acid to biomass (g/g)
1	63.4	43.4	17.4	13.6	152	77.6	3.6
2	73.8	47.4	17.8	11.5	153	78.3	4.1
3	68.1	43.3	12.3	7.8	174	88.8	5.5
4	70.3	43.3	14.7	9.1	168	85.6	4.7
5	78.7	48.3	14.9	9.1	175	89.1	5.2
6	74.2	41.9	16.2	9.2	179	91.4	4.5
7	75.2	41.7	18.4	10.1	184	93.7	4.0
8	86.2	50.7	18.1	10.6	174	88.8	4.7
9	78.8	44.8	15.0	8.5	176	89.7	5.2
10	82.4	46.0	15.5	8.6	182	92.9	5.3
11	77.0	43.2	13.8	7.7	182	92.6	5.5

5.7.2 Estimated effects of metal ions on citric acid concentration and yield

The results of the statistical analysis for citric acid concentration, expressed in g/kg initial substrate, are presented in Table 5.4. Only addition of Fe^{2+} showed significant effect on citric acid production. None of the other metal ions has significant effect on citric acid production. Also, there were no significant interactions detected.

These results indicate that addition of metal ions to the substrate had no inhibitory effect on citric acid production. The concentrations of the added metal ions, 150 mg/kg of Fe^{2+}, 25 mg/kg of Cu^{2+}, 75 mg/kg of Zn^{2+} and 150 mg/kg of Mn^{2+}, were much higher than the values which were reported to have seriously inhibited citric acid production using wheatbran (Shankanranand and Lonsane, 1994).

The results of the statistical analysis for citric acid yield, based on the starch utilised, are shown in Table 5.5. Mn^{2+}, and the interaction between Fe^{2+} and Mn^{2+}, with p-values of 0.011 and 0.043, respectively, showed significant stimulatory effects on the citric acid yield. However, no other main effects or interactions showed any significant effects. Consequently, it appears that addition of Mn^{2+} and Fe^{2+} together might improve the yield of citric acid.

5.7.3 Estimated effects of metal ions on biomass concentration and yield

The results for the statistical analysis of biomass concentration are given in Table 5.6, and the biomass yield, based on starch utilised, in Table 5.7. For the biomass yield, no significant stimulatory or inhibitory effects were observed, while for the biomass production, the only significant effect was a stimulatory one due to the interaction between Fe^{2+} and Cu^{2+}. In contrast, less black spores than that without any addition of metal ions were observed during early period in the experiments. This suggests that metal ions may negatively affect on spores formation in the early period, but not necessary on the final biomass production.

Table 5.4 Estimated effects and coefficients for citric acid concentration (based on weight of initial substrate)

term	effect	coefficient	t-value	p-value
constant		75.33	46.58	0.000
Fe^{2+}	9.71	4.85	2.56	0.083
Cu^{2+}	2.46	1.23	0.65	0.563
Zn^{2+}	4.77	2.39	1.26	0.297
Mn^{2+}	5.94	2.97	1.57	0.215
$Fe^{2+}*Cu^{2+}$	1.83	0.91	0.48	0.664
$Fe^{2+}*Zn^{2+}$	-1.51	-0.76	-0.40	0.72
$Fe^{2+}*Mn^{2+}$	1.83	0.92	0.48	0.663
curvature				0.092

Table 5.5 Estimated effects and coefficients for yield of citric acid (based on weight of utilised starch)

term	effect	coefficient	t-value	p-value
constant		44.97	124.63	0.000
Fe^{2+}	1.30	0.65	1.53	0.223
Cu^{2+}	-0.47	-0.24	-0.56	0.616
Zn^{2+}	1.67	0.84	1.97	0.143
Mn^{2+}	4.83	2.41	5.70	0.011
$Fe^{2+}*Cu^{2+}$	1.59	0.80	1.88	0.156
$Fe^{2+}*Zn^{2+}$	-0.32	-0.16	-0.38	0.723
$Fe^{2+}*Mn^{2+}$	2.86	1.43	3.38	0.043
Curvature				0.762

Table 5.6 Estimated effects and coefficients for biomass concentration (based on weight of initial substrate)

term	effect	coefficient	t-value	p-value
constant		15.87	36.02	0.000
Fe^{2+}	1.33	0.66	1.28	0.293
Zn^{2+}	0.94	0.47	0.91	0.430
Mn^{2+}	-0.93	-0.46	-0.90	0.437
Cu^{2+}	-0.68	-0.34	-0.66	0.558
$Fe^{2+}*Cu^{2+}$	3.42	1.71	3.31	0.045
$Fe^{2+}*Zn^{2+}$	-0.47	-0.24	-0.45	0.680
$Fe^{2+}*Mn^{2+}$	0.11	0.05	0.10	0.925
curvature				0.127

Table 5.7 Estimated effects and coefficients for yield of biomass

term	effect	coefficient	t-value	p-value
constant		9.66	19.76	0.000
Fe^{2+}	-0.73	-0.36	-0.63	0.572
Cu^{2+}	-1.42	-0.71	-1.24	0.303
Mn^{2+}	-0.74	-0.37	-0.65	0.565
Zn^{2+}	-0.06	-0.03	-0.05	0.965
$Fe^{2+}*Cu^{2+}$	2.68	1.34	2.33	0.102
$Fe^{2+}*Zn^{2+}$	0.35	0.18	0.31	0.780
$Fe^{2+}*Mn^{2+}$	0.96	0.48	0.83	0.466
curvature				0.032

5.7.4 Estimated effects of metal ions on starch utilization

The results for the statistical analysis of starch utilization, expressed in terms of concentration and percentage of starch utilised, are given in Tables 5.8. and 5.9, respectively. Fe^{2+} ion presented the only significant effect on starch utilization, similar to its effect on citric acid production as discussed previously in Section 5.7.2. This possibly indicates that the effect of Fe^{2+} in this solid substrate fermentation may be both to stimulate starch utilization and to direct its metabolism towards citric acid rather than biomass production.

5.7.5 Estimated effects of metal ions on the ratio of citric acid to biomass production

The ratio of citric acid to biomass production could reflect the ability of the organism to direct the sugar metabolism in various ways. From the results of the statistical analysis of the ratio of citric acid to biomass concentrations, which are shown in Table 5.10, no significant stimulatory or inhibitory effects were observed.

5.7.6 Discussion

Some metal ions, such as Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} have been reported to be inhibitory to citric acid production using *A. niger*. However, studies on the effects of individual metal ions on citric acid production are difficult to interpret because of the interactions with other metal ions and even with other nutrients. Further, the many variations with different strains of *A. niger* used, the different substrates used and fermentation states, taken together make it extremely difficult to make conclusions regarding the effects of metal ions on citric acid production.

In the present experiments, addition of Fe^{2+} to a concentration of 150 mg/kg, was shown to stimulate significantly citric acid production and starch utilization, but for biomass

Table 5.8 Estimated effects and coefficients for starch utilised

term	effect	coefficient	t-value	p-value
constant		172.64	63.02	0.000
Fe^{2+}	16.25	8.13	2.53	0.085
Cu^{2+}	10.25	5.13	1.60	0.209
Zn^{2+}	-2.75	-1.38	-0.43	0.697
Mn^{2+}	-1.75	-0.88	-0.27	0.803
$Fe^{2+}*Cu^{2+}$	-8.25	-4.13	-1.28	0.289
$Fe^{2+}*Zn^{2+}$	-0.25	-0.13	-0.04	0.971
$Fe^{2+}*Mn^{2+}$	-5.25	-2.63	-0.82	0.474
curvature				0.032

Table 5.9 Estimated effects and coefficients for percentage of starch utilised

term	effect	coefficient	t-value	p-value
constant		88.05	64.05	0.000
Fe^{2+}	8.18	4.09	2.54	0.085
Cu^{2+}	5.13	2.56	1.59	0.210
Zn^{2+}	-1.28	-0.64	-0.40	0.719
Mn^{2+}	-0.83	-0.41	-0.26	0.815
$Fe^{2+}*Cu^{2+}$	-4.13	-2.06	-1.28	0.291
$Fe^{2+}*Zn^{2+}$	-0.02	-0.01	-0.01	0.994
$Fe^{2+}*Mn^{2+}$	-2.78	-1.39	-0.86	0.453
curvature				0.032

Table 5.10 Estimated effects and coefficients for ratio of citric acid to biomass

term	effect	coefficient	t-value	p-value
constant		4.81	23.10	0.000
Fe^{2+}	0.16	0.08	0.33	0.761
Cu^{2+}	0.37	0.19	0.76	0.501
Mn^{2+}	0.66	0.33	1.36	0.268
Zn^{2+}	-0.07	-0.03	-0.14	0.899
$Fe^{2+}*Cu^{2+}$	-0.88	-0.44	-1.81	0.168
$Fe^{2+}*Zn^{2+}$	0.07	0.03	0.14	0.899
$Fe^{2+}*Mn^{2+}$	0.03	0.01	0.06	0.959
curvature				0.022

production, the simulatory effect is observed only together with Cu^{2+}. Shankaranand and Lonsane (1994) reported that addition of 100 mg/kg of Fe^{2+} to wheatbran in solid substrate fermentation resulted in little citric acid production. In other fermentation systems, much lower additions of Fe^{2+} have been reported to be inhibitory, e.g. addition of 50 mg/kg of $FeCl_3$ to a filter paper surface fermentation severely inhibited citric acid production (Xu *et al.* 1989a) and addition of 50 ppb of Fe^{2+} to a brewery wastes medium reduced citric acid production by 25% (Roukas and Kotzekidou, 1987). In submerged fermentation, the inhibitory concentration of Fe^{2+} varies in different reports. Horitsu and Clark (1966) reported that an added concentration of 100 mg/l of Fe^{2+} into beet molasses had no gross effects on either the morphological characteristics or the citric acid production, while others reported that a tight control of Fe^{2+} was required for citric acid production, e.g. 0.01 mg/l of Fe^{2+} reported by Dawson (1986), 2 mg/l by La Nauze (1966). However, since the presence of Fe^{2+} in any fermentation substrate usually is of excess rather than of deficiency, the current study on the effect of Fe^{2+} on citric acid production is focused on the tolerance ability of the organism. In the case of solid substrate fermentation using kumara for citric acid production, the results show the high ability of the system to tolerate any adverse effect of higher concentrations of Fe^{2+}.

Cu^{2+} has been recognised to be an antagonist of Fe^{2+} (Berry, 1988) and also to balance effectively the inhibitory effect of manganese (Röhr *et al.* 1983). However, in the present experiments, neither Cu^{2+} nor the interaction between Cu^{2+} and Fe^{2+} showed any significant effect on citric acid production, though the interaction between Cu^{2+} and Fe^{2+} had a significant effect on biomass production. Shankaranand and Lonsane (1994) reported severe inhibition on addition of 1 mg/kg of Cu^{2+} to wheatbran which originally contained 16.99 mg/kg of Cu^{2+} based on dry weight of the substrate, while an addition of 1 ppm of Cu^{2+} reduced citric acid production from brewery wastes by 20% (Roukas and Kotzekidou, 1987). In submerged fermentation, addition of 0.06 ppm Cu^{2+} significantly reduced citric acid production (Shu and Johnson, 1948a, b), but addition of 30 mg/l $CuSO_4.5H_2O$ was reported to be necessary for citric acid production in a chemically defined medium (Jernejc *et al.* 1982). Therefore, the effect varies

considerably with the system under study. In the present work, the addition of 25 mg/kg of Cu^{2+} had no inhibitory effect on citric acid production, in which case the solid substrate fermentation using kumara shows high tolerance ability.

It has been well established that Zn^{2+} is an important metal ion which is necessary for the production of citric acid by *A. niger* (Kapoor *et al*, 1982). The onset of the citric acid accumulation phase has been reported to be controlled by the concentration of Zn^{2+} as growth becomes limited, and citric acid production occurs only in the presence of low concentrations of Zn^{2+} (Shu and Johnson, 1948; Wold and Suzuki, 1976). The addition of 170 ppb of Zn^{2+} reduced citric acid production by 35% in submerged fermentation (Roukas and Kotzekidou, 1987), while addition of 2 mg/l of $ZnSO_4.7H_2O$ in filter paper surface fermentation severely inhibited citric acid production (Xu *et al*, 1987). Shankaranand and Lonsane (1994) reported that citric acid production decreased on addition of 1.0 mg/kg of Zn^{2+} to wheatbran which contained 59.74 mg/kg of Zn^{2+} based on dry weight of the substrate. However, in the present experiment, addition of 75 mg/kg of Zn^{2+} showed no significant effect on either citric acid or biomass production.

Mn^{2+} has been studied in more detailed than others. Horitsu and Clark (1966) stated that Mn^{2+} had a most pronounced effect on citric acid production and the morphological characteristics of fungus. Kubicek and co-workers have spent considerable effort on the investigation of the role of Mn^{2+} in citric acid production in submerged fermentation. To sum up, their investigation concluded that a condition of Mn^{2+} deficiency (10 μM) in *A. niger* is a pre-condition for the accumulation of citric acid. In surface fermentation, Roukas and Kotzekidou (1987) reported that 146 ppb of Mn^{2+} reduced citric acid production from brewery wastes by 17.5%, and Xu *et al,* (1989c) reported that 30 ppb of $MnCl_2.H_2O$ severely inhibited citric acid production in a filter paper surface fermentation. In solid substrate fermentation, addition of 15 mg/kg wheatbran, which originally contained 124.9 ppm of Mn^{2+} based on dry weight of the substrate, allowed no citric acid production during 5 days fermentation. In the present work, however, Mn^{2+} has been shown to be stimulatory to citric acid yield, as has the interaction between

Mn^{2+} and Fe^{2+}. In addition, the addition of 150 mg/kg of Mn^{2+} indicates the ability of the organism to tolerate high concentrations of Mn^{2+}.

5.7.7 Conclusion of effects of metal ions on citric acid production

From the preceding discussion, it is clear that the effects of metal ions on citric acid production are complex. In the present experiments, this solid substrate fermentation system for citric acid production has a high ability to tolerate any adverse effects of metal ions. The significant effects are stimulatory rather than inhibitory on citric acid production.

5.8 Conclusions

The conclusions that can be drawn from this chapter may be summarized as follows:

1. The initial moisture content of the substrate is an important parameter for organism growth, and citric acid production. A value in the range of 65 to 71% was found to be optimum.
2. An inoculum size of 10^4 to 10^6 spores/40g kumara was optimum for citric acid production, with appropriate growth of the organism.
3. A cubic particle size between 4 and 6 mm was most favourable for organism growth and citric acid production.
4. Biomass and citric acid production were related directly to the surface area provided but not to the weight or volume of the substrate. Average values for citric acid and biomass production under the condition of limited surface area were 0.090 g/cm^2 and 0.035 g/cm^2, respectively. It was shown that the organism takes up nutrients by penetrating through the substrate. However, the attempt to measure accurately this ability was unsuccessful.

5. The solid substrate fermentation exhibited no adverse effect of added metal ions on citric acid production. Any significant effects were stimulatory rather than inhibitory.

Chapter 6

Kinetics of Citric Acid Production from Kumara in Flasks

6.1 Introduction

The kinetics of solid substrate fermentation involve mass transfer and bioreactions with oxygen and nutrients in the solid substrate, which, in turn, are affected by environmental conditions such as temperature, pH and the surrounding atmosphere. Therefore the kinetics in solid substrate fermentation are very complicated. However the kinetics are so vital for an understanding of solid substrate fermentation and for subsequent design of reactors that it cannot be ignored. In solid substrate fermentation involving fungi, some attempts to investigate the kinetics have been reported. Sugama and Okazaki (1979) and Okazaki *et al.* (1980) modelled *Aspergillus niger* growth on rice grains using carbon dioxide evolution. Mitchell *et al.*(1991a, b) used an empirical model and later a semi-empirical model to predict *Rhizopus oligosporus* growth on a starch medium based on data for glucoamylase activity, and consensus was reached with the experimental data. Laszlo and Silman (1993) used a "cellular automata model" to simulate filamentous fungal growth on a flat medium. Citric acid is a major metabolic product, the presence of which causes a decrease in pH value during the process, and may have an inhibitory effect on certain process parameters. So far, reports describing the kinetics of citric acid production in solid substrate fermentation have not been found in the literature, though the kinetics in submerged fermentation have been widely studied (Kristiansen and Sinclair, 1978, 1979; Roehr *et al*, 1981; Dawson *et al*, 1987).

Another characteristic of solid substrate fermentation involving fungi is that the organism takes up nutrients by penetrating into the substrate (refer to Section 5.6). Therefore, the volume and weight of substrate changes during the process of fermentation, although the concentrations of some of the nutrients may stay relatively constant and any inhibition from metabolic products will increase with continuing production. In contrast, in submerged fermentation, the volume can be kept constant,

the nutrient concentrations become progressively diluted and any inhibition due to metabolic products can be reduced considerably by dispersal into the medium. These critical differences between solid substrate fermentation and submerged fermentation seem to be acknowledged, but their adequate expression has not been developed. Okazaki *et al.* (1980) expressed their data using similar units to those used for submerged fermentation, i.e. based on initial dry matter, while Mitchell *et al.* (1991a, b) expressed their data based on surface area of substrate (g/cm^2). These units are unable to express the changing starch content of the substrate, the constant nutrient concentrations or the increased inhibition caused by metabolic product, if present.

The purpose of these experiments was to investigate the kinetic characteristics of the solid substrate fermentation for citric acid production, in an attempt to provide an initial understanding of the process. In addition, an attempt would be made to develop an adequate unit for the expression of this solid substrate fermentation.

6.2 Results and Discussion

In these experiments, 40 g homogenized kumara paste was used as fermentation medium with an initial moisture content of 71% and with an inoculum size of 10^4-10^6 spores per flask. For sampling, two flasks were sacrificed for duplicate analysis, the results are expressed as the average values.

6.2.1 Time course and kinetic characteristics

The time course of the process, expressed on the basis of the initial wet weight of the substrate, is presented in Fig 6.1. Citric acid production commenced on day 1, and increased continuously till day 8, after which its concentration decreased, presumably due to depletion of starch. Thus, the citric acid was used as carbon source after 95% of the starch had been utilized. The maximum citric acid concentration was achieved on

day 8, at a value of 91 g/kg, but the highest yield, based on starch utilized, of 65% was achieved on day 3. Fungal biomass kept increasing during the process of fermentation, even after the time when starch approached depletion and citric acid utilization commenced. The highest yield of biomass, based on starch used, was 26% on day 4.

The morphological observations are displayed in Figs 6.2 to 6.6, from day 1 to day 5. On day 1, just a little white filamentous mycelial growth could be observed. However, in the following 24 hours, between day 1 and day 2, a great deal of mycelial growth occurred and covered the surface of the substrate completely. After day 2, the mycelium became denser, the colour changed from white to yellow, and a few black spores could be observed on the surface. After day 3, the mycelium was completely covered with black spores.

The free glucose in the substrate and the activity of glucoamylase are shown in Fig 6.7. Both of these parameters showed peaks around day 2 to 3, after which they gradually declined. The significance of the timing of these peaks will be discussed in conjunction with the rate data below.

From the data presented in Fig.6.1 rates of citric acid and biomass production and starch utilization, based on the original wet weight of the substrate, were calculated, as follows:

For gravimetric rates:

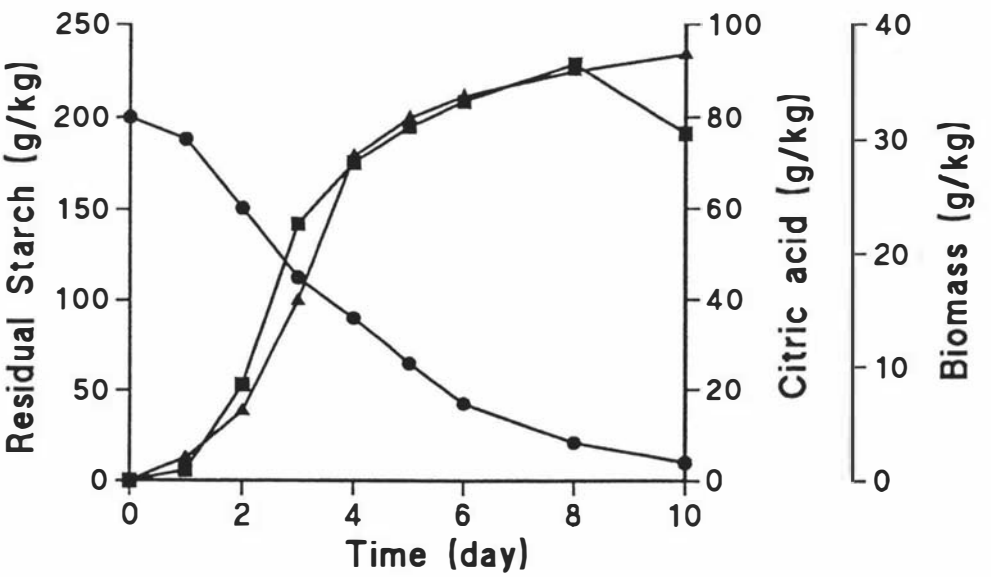
rate of citric acid production

$$r_{CA}(t)=\frac{CA(t)-CA(t-\Delta t)}{\Delta t} \qquad g/kg.h$$

rate of starch utilization

$$r_{S}(t)=\frac{RS(t-\Delta t)-RS(t)}{\Delta t} \qquad g/kg.h$$

Figure 6.1 Time course of the solid substrate fermentation for citric acid production



Symbols:● starch, ■ citric acid, ▲ biomass

Figure 6.2 The morphological observation of *A. niger* growth on kumara paste (day 1)

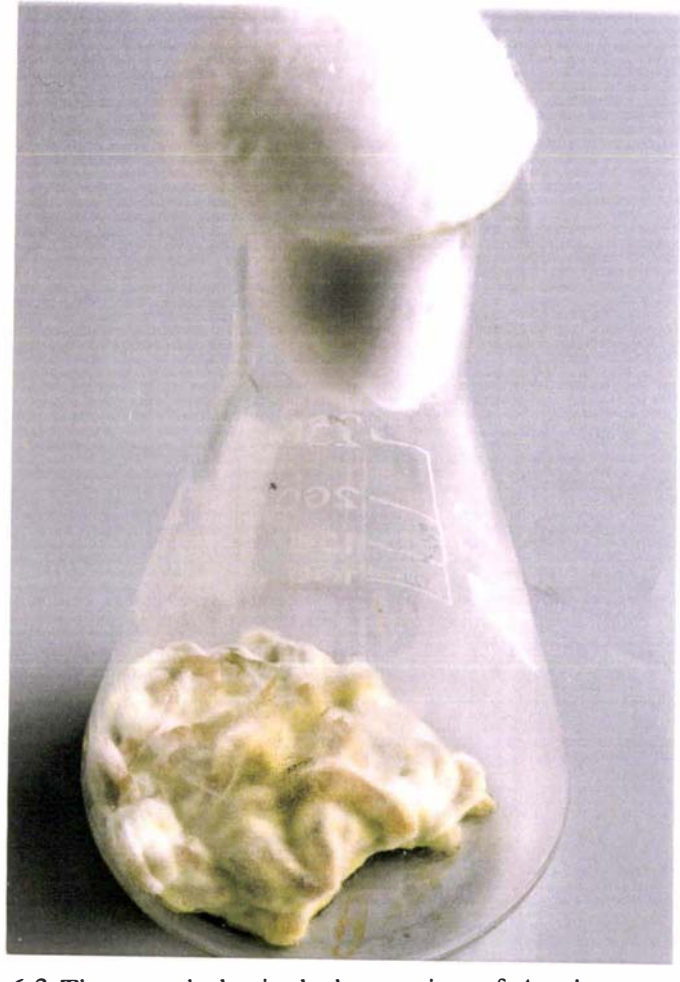


Figure 6.3 The morphological observation of *A. niger* growth on kumara paste (day 2)

Figure 6.4 The morphological observation of *A. niger* growth on kumara paste (day 3)

Figure 6.5 The morphological observation of *A. niger* growth on kumara paste (day 4)

Figure 6.6 The morphological observation of *A. niger* growth on kumara paste (day 5)

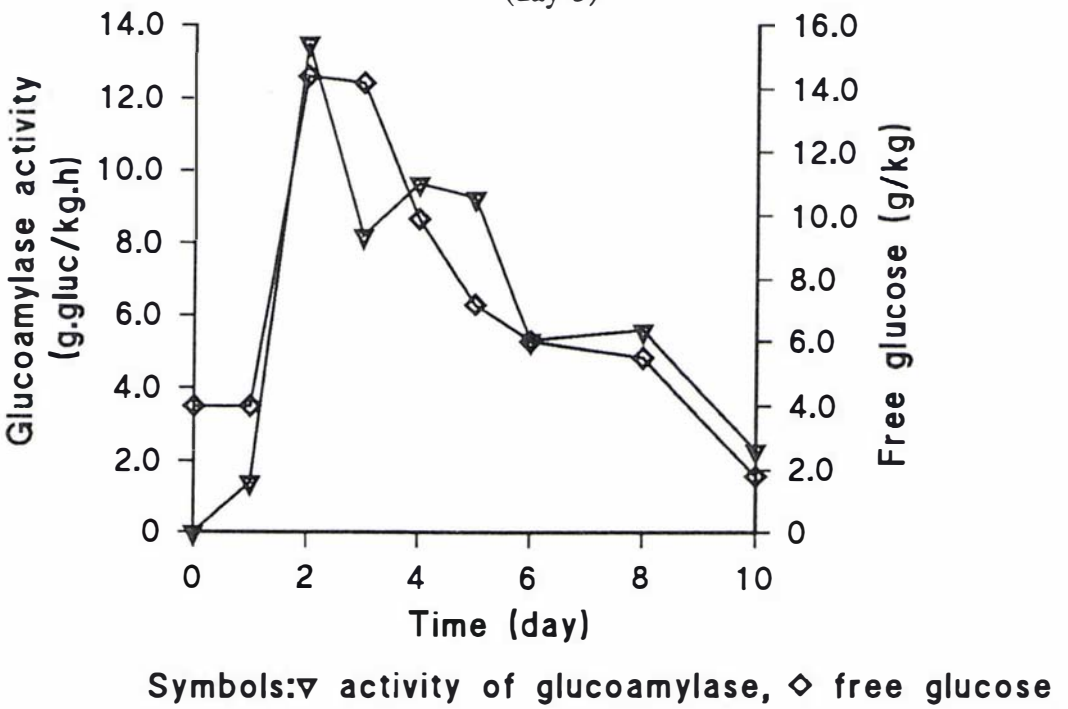


Figure 6.7 Time course of free glucose and glucoamylase activity

rate of fungal biomass production

$$r_{BM}(t)=\frac{BM(t)-BM(t-\Delta t)}{\Delta t} \qquad g/kg.h$$

For specific rates, i.e. rates based on fungal biomass:

rate of citric acid production,

$$q_{CA}(t)=\frac{r_{CA}(t)}{BM(t)} \qquad g/g \text{ biomass.h}$$

specific growth rate,

$$\mu(t)=\frac{r_{BM}(t)}{BM(t)} \qquad g/g \text{ biomass.h}$$

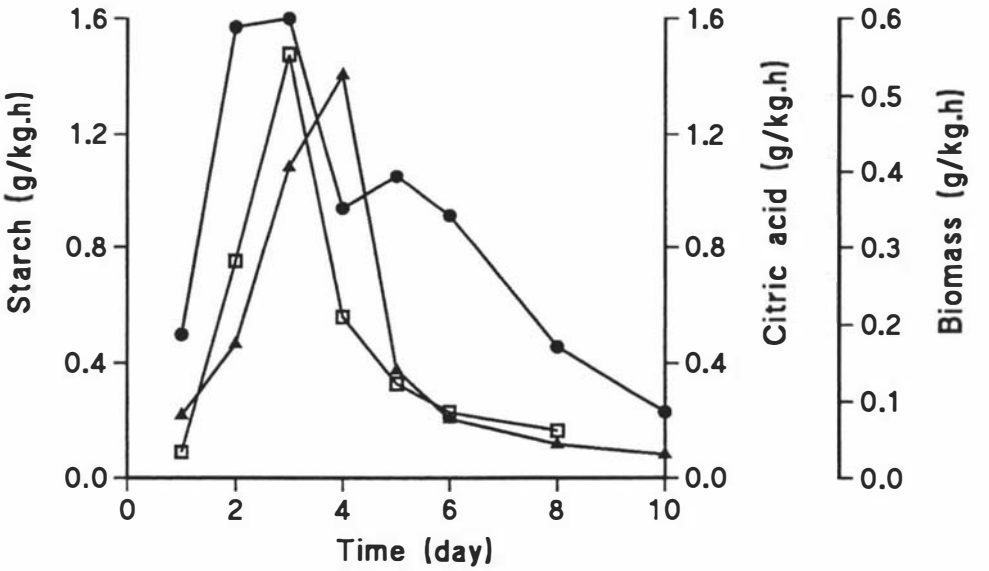
rate of starch utilization,

$$q_S(t)=\frac{r_S(t)}{BM(t)} \qquad g/g \text{ biomass.h}$$

where expressions and units were explained in Nomenclature.

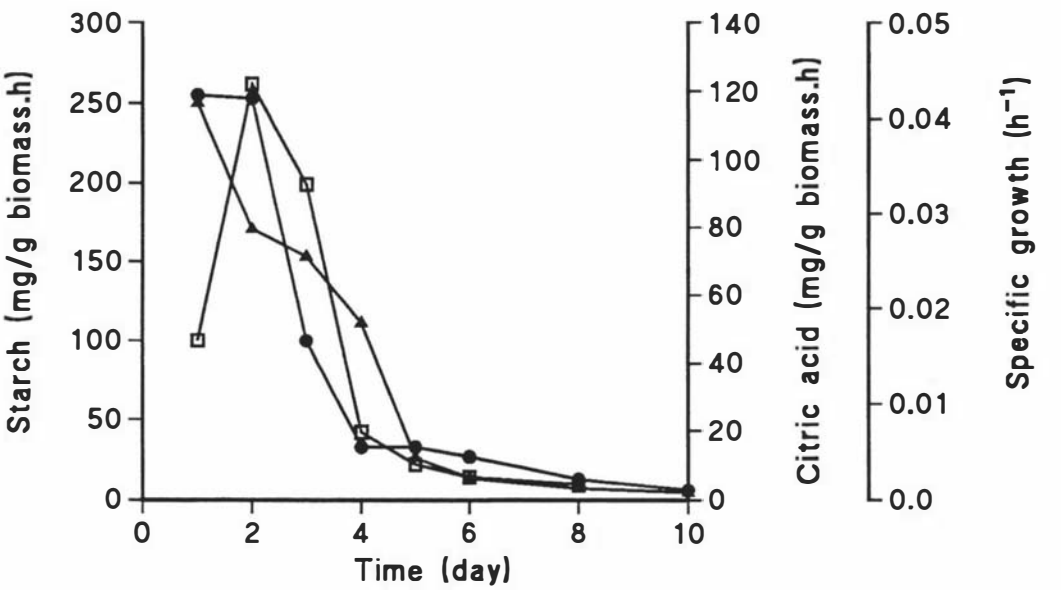
The gravimetric and specific rates of citric acid production, biomass formation and starch utilization are presented in Figs 6.8 and 6.9, respectively. The maximum citric acid production rates, both gravimetric and specific, were observed at values of 1.5 g/kg.h on day 3 and 122 mg/g biomass.h, on day 2, respectively. The maximum gravimetric rate of biomass production was observed on day 4 with a value of 0.48 g/kg.h, but the specific rate declined throughout the process from a value of approximately 0.04 h^{-1} which was observed on day 1. The rates of starch utilization, both gravimetric and specific, were observed at their highest values of 1.6 g/kg.h, on days 2 and 3, and 0.25 g/g biomass.h, on days 1 and day 2, respectively. The specific rates all declined sharply during the later period of fermentation.

Figure 6.8 Gravimetric rates during solid substrate fermentation process



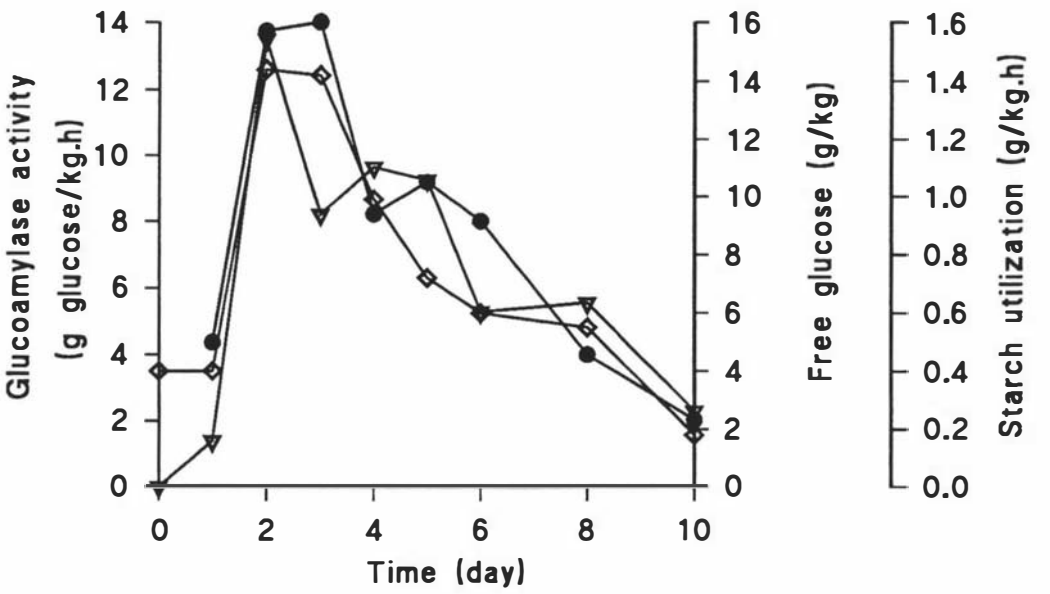
Symbols:● starch, □ citric acid, ▲ biomass

Figure 6.9 Specific rates during solid substrate fermentation process



Symbols:● starch, □ citric acid, ▲ biomass

Figure 6.10 Relationship between free glucose, glucoamylase activity and gravimetric rate of starch utilization



Symbols:▽ activity of glucoamylase, ◇ free glucose, ● starch

Fig 6.10, in which the data for glucoamylase and free glucose from Fig 6.7 are combined with the gravimetric rate of starch utilization from Fig 6.8, shows a clear relationship among these parameters. Thus, for starch utilization to occur, starch substrate must be converted to glucose by the glucoamylase which is released by the organism. The glucose is then transferred into the cell for intracellular metabolism. The free glucose and activity of glucoamylase occurred concomitantly during the whole process of the fermentation, and both of them coincided with the varying gravimetric rate of starch utilization. The maximum activity of glucoamylase was obtained on day 2 with values of 13.5 g/kg.h and 2.2 g/g biomass.h, respectively, based on the initial substrate and fungal biomass values, when the gravimetric rate of starch utilization was at its highest value. Subsequently, the activity of glucoamylase decreased along with the gravimetric rate of starch utilization.

The free glucose, the concentration changes of which coincided with the gravimetric rate of starch utilization during the progress of the fermentation, is a valuable parameter to express starch utilization and the activity of glucoamylase because its analysis is very rapid and simple.

6.2.2 An alternative unit for the expression of the reaction kinetics

As mentioned previously, the description of a solid substrate fermentation process based on the weight of the initial substrate may not accurately reflect the situation because of the changing starch content during the process. Hence, it may be useful to base the kinetic parameters on the starch content at any given time. The following calculations, from the data plotted in Fig 6.1, are based on the residual starch content of the substrate.

Corresponding to the gravimetric rates based on the initial substrate, the gravimetric rates based on residual starch are:

for starch utilization,

$$r_S'(t)=\frac{r_S(t)}{RS(t)} \qquad \text{g starch/kg residual starch.h}$$

for citric acid production,

$$r_{CA}'(t)=\frac{r_{CA}(t)}{RS(t)} \qquad \text{g citric acid/kg residual starch.h}$$

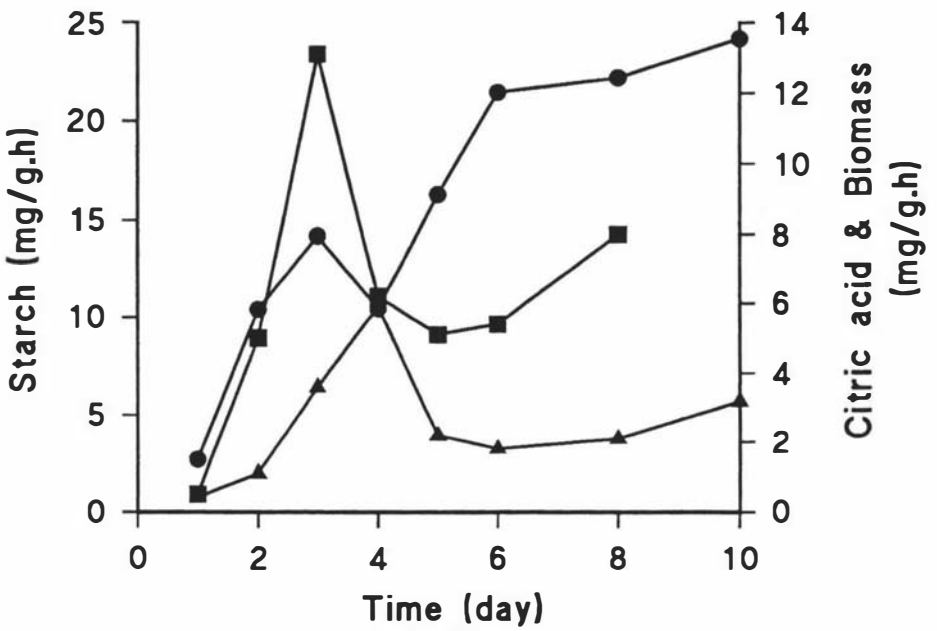
for fungal biomass production,

$$r_{BM}'(t)=\frac{r_{BM}(t)}{RS(t)} \qquad \text{g biomass/kg residual starch.h}$$

These calculated rate data are plotted in Fig 6.11. To interpret these data, it is necessary to recall the expressions which were based on the initial wet weight of the substrate, where the rate data are based on an unchanging base, i.e. the initial weight of the substrate. However, for the actual fermentation, the weight of substrate, biomass and citric acid are all changed during the process. Therefore, the rate data, as calculated above, based on residual starch, may reflect characteristics of the changing situation during the fermentation.

As shown in Fig 6.11, during the first 6 days of the fermentation, the starch utilization rate increased with time. This indicates that as the biomass increased, so the starch utilization rate increased, rather than decreased after day 3 as it did using the expression based on initial weight of substrate. The latter reflects the starch shortage rather than the ability of starch utilization by the fungal biomass. The rates of both biomass and citric acid production showed peaks similar to those based on the initial wet weight of the substrate, i.e. on days 3 and 4 for citric acid and biomass, respectively. However, for citric acid, the subsequent decline was less marked in Fig 6.11 than in Fig 6.8. This possibly implies that if sufficient starch were available, the citric acid production rate would be maintained at a high value. According to this, the fermentation could be

Figure 6.11 Gravimetric rates of the solid substrate fermentation represented by basing on residual starch



Symbols:● starch, ■ citric acid, ▲ biomass

characterized by a production phase which follows the initial acceleration period.

In Fig 6.11, the data after 6 days fermentation must be treated with caution since 85% of starch had been utilized at that time. In summary, the rates based on residual starch, which could eliminate any influence caused by the changing substrate concentration, are able to express the fermentation in a more accurate way than that based on the unchanged initial wet weight of the substrate.

6.3 Discussion

Basically, the solid substrate fermentation for citric acid production can be divided into three phases. Firstly, there is a lag period, for about one day, during which time the spores of the organism are germinating. Secondly, there is a growth period, from day 2 to day 6, during which time the biomass is accumulating, and this coincides with citric acid accumulation. The last period, after day 6, corresponds to the depletion of starch, and the fermentation slows down. According to the generally accepted theory of citric acid production in submerged fermentation, citric acid production occurs only under the condition of nitrogen- and/or phosphate-limitation (Dawson, 1986), and citric acid accumulation is commenced when nitrogen and/or phosphate become exhausted from the medium, i.e. when growth is ceasing (Dawson *et al*, 1986; Kristiansen and Sinclair, 1978). For the solid substrate fermentation, as described previously in Section 4.4, kumara is able to satisfy the requirement of nitrogen-limitation, though the content is higher than that of media used in submerged fermentation. However, the situation in solid substrate fermentation is not straightforward. As described at the beginning of this chapter, the nutrient concentrations in the substrate, including those of nitrogen and phosphate, would stay constant in areas not yet reached by the organism. Therefore, the total content of nitrogen or phosphate would not be exhausted until all substrate had been reached by the organism. But locally, there would be depletions. This explains why, in the present work, citric acid production in the solid substrate fermentation

and ignoring diffussion

commenced and reached a high rate by day 2, coinciding with the biomass accumulation. In other words, it is because of the time-space differences that citric acid and biomass accumulation appear to coincide, rather than biomass production preceding citric acid production, as occurs in submerged fermentation. A plot of the specific rate of citric acid production versus specific growth rate, which is presented in Fig 6.12, displays that citric acid production in solid substrate fermentation is basically a growth-associated process. The value of the specific citric acid production rate at the early stage, point (0.041, 50) in Fig 6.12, was lower than expected, probably because of the inclusion of the lag time for the spores germination, and so can be disregarded.

With the preceding kinetic analysis of this solid substrate fermentation, a comparison with data from submerged and liquid surface fermentations is possible, as shown in Table 6.1. Two major points arise from this comparison. Firstly, the overall productivity of citric acid from solid substrate fermentation, based on the initial wet weight of substrate, is comparable with the volumetric productivity from submerged fermentation processes. Secondly, the specific production rate of citric acid is markedly superior in solid substrate fermentation. This superiority may result from the difference in fermentation mode, carbon source or other factors but not the strain of organism, since it has been shown that there is no obvious difference between strains Yang No.2 and MH15-15 which was used in the submerged fermentation work, and since strain Yang No.2 was used in the liquid surface fermentation process.

Figure 6.12 Plot of specific citric acid production rate against specific growth rate in solid substrate fermentation

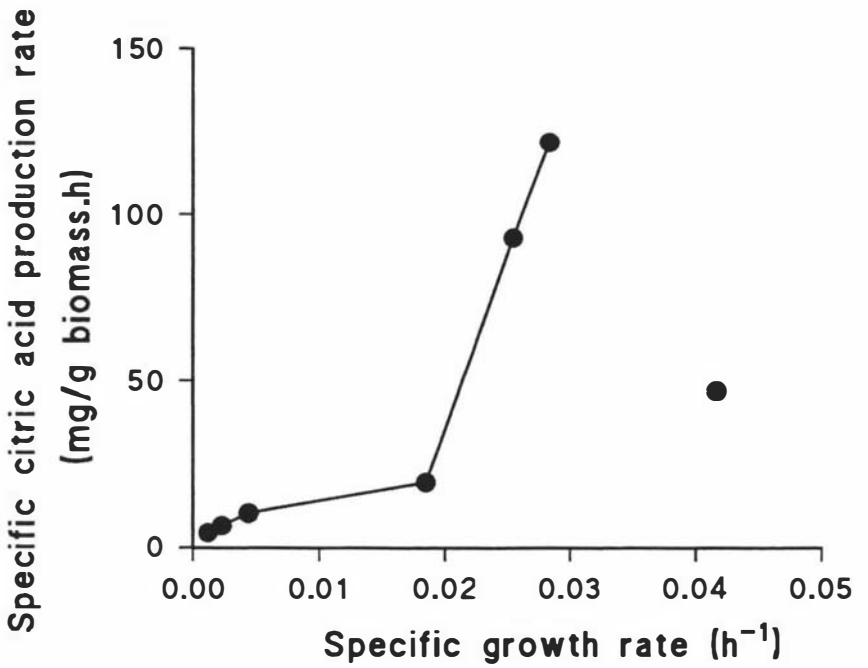


Table 6.1 Comparison of kinetic characters among different fermentation technologies

Technology	Substrate	Organism	Overall productivity of citric acid (g/kg.h or g/l.h)	Maximum specific rate of citric acid production (mg/g biomass.h)	Reference
Submerged					
-batch	sucrose	B60/B3	0.39	--	Roehr *et al*, 1981
-batch	sucrose	MH15-15	0.27	35	Dawson *et al*, 1988
-continuous	sucrose	MH15-15	0.40	79	Dawson *et al*, 1988
-fed-batch	sucrose	MH15-15	0.54	51	Dawson *et al*, 1988
Liquid surface					
	soluble starch	Yang No.2	0.13	20	Sakurai *et al*, 1991
Solid Substrate					
	apple pomace		1.08*		Hang, 1988
	kiwifruit peel		0.86*		Hang *et al*, 1987
	Wheatbran		0.47*		Shankaranand and Lonsane, 1994
	kumara	Yang No.2	0.48[b]	122	This work

Note:* calculated from the reference; [b] 8 days fermentation.

6.4 Conclusions

The major conclusions which can be drawn from this chapter are:

1. Based on the kinetic analysis, this solid substrate fermentation for citric acid production is superior to conventional submerged and liquid surface fermentation.
2. Citric acid production in the solid substrate fermentation is a growth-associated process.
3. In fermentations where the substrate concentration is changing, the expression of rates based on the residual starch can reflect the fermentation characteristics more accurately than calculations based on the initial wet weight of the substrate. However, for keeping the analysis uniform and to be comparable with other work, this expression will not be used for further kinetic analysis.

Chapter 7

Selection of Reactors for Citric Acid Production by Solid Substrate Fermentation

7.1 Introduction

Solid substrate fermentation is currently receiving increased attention. This is because of its advantages over submerged fermentation processes, including its low energy requirement and its environmental friendliness (Mudgett, 1986; Panday, 1991; Murthy *et al*, 1993). However, there has long been a need for improved solid substrate fermentation reactors to provide more favourable fermentation conditions including more effective mass and heat transfer. With the development of solid substrate fermentation, much effort has been spent on the study of reactors. A summary of reactors used in solid substrate fermentation and their basic characteristics is given in Table 2.1 and 2.2.

For solid substrate fermentation for citric acid production, Hang (1988) used a fixed (packed) bed reactor using apple pomace as substrate, and Lakshminarayana *et al* (1975) used a tray reactor, while most investigations have been carried out in flasks (Shankaranand and Lonsane, 1994; Hang and Woodams, 1984, 1985; Hang *et al*, 987). No detailed performance of these reactors has be described and other reactors have not been adopted.

The purpose of the experiments described in this chapter was to investigate the possibility of using different reactors for citric acid production in solid substrate fermentation. In addition, a comparison would be made among these reactors, and the most promising would be selected for further study.

7.2 Gas-solid Fluidized Bed Reactor

7.2.1 Introduction

The gas-solid fluidized bed reactor has been widely used in chemical engineering. This is because, compared with the packed bed and other reactors, it has several advantages including superior heat and mass transfer and it is easy to achieve continuous operation. However, in fermentation technology, only a few publications have referred to the gas-solid fluidized bed reactor, while other types of reactors have been mentioned frequently. Tanaka *et al.* (1986) reported on the use of an air-solid fluidized bed reactor with *Eupenicillium javanicum*, *Saccharomyces cerevisiae* and *Aspergillus niger*. They worked with wheat bran and concluded that a moisture content of at least 40% was required for microbial growth on this substrate. To prevent particle agglomeration at high values of moisture content, a blower was installed in the system. Recently, the same group has described an air-solid vibration fluidized bed reactor for the cultivation of *Saccharomyces cerevisiae*. In this process, the reactor was kept vibrating to prevent particle agglomeration, while a water-spraying nozzle was installed to adjust the moisture content of the particles with control by a computer (Matsuno *et al*, 1992). Bauer (1986) used a gas-solid fluidized bed reactor for the production of glutathione using *Saccharomyces cerevisiae*, and the same system has also been used for ethanol production (Röttenbacher *et al*, 1987). Similarly, Moebus and Teuber (1982) used a gas-solid fluidized bed, using CO_2 recirculation, for ethanol production using *Saccharomyces cerevisiae*. A gas-solid fluidized bed reactor was used for the cultivation of *Trichoderma reesei* on corncob (Adisasmito, 1987), but it failed owing to the inability of the system to provide the substrate with a sufficiently high moisture content for microbial growth. Despite these difficulties, most workers have achieved higher reactor productivity, or enzyme activity, in a fluidized bed reactor than in traditional solid substrate fermentation.

So far, there has not been any application of gas-solid fluidized bed reactors for citric acid production in solid substrate fermentation. Hence, the purpose of the present work was to investigate the possibility of application of a gas-solid fluidized bed reactor for citric acid production from kumara using *A. niger*.

7.2.2 Preparation of experiments

In these experiments, a gas-solid fluidized bed reactor system, as depicted in Fig 3.2, was used. Dry kumara particle substrates (5% moisture content) with size ranges of 105-150 μm, 150-300 μm and 300-420 μm, were obtained from the procedure described in Section 2.4.1. Prior to inoculation of an experiment, the particulate substrate was held at 60°C for 3 hours for sterilization. For each experiment, 10 g of the substrate was used. About 2 g of the substrate was placed in a 100-ml bottle containing an 6-day old sporulating culture. The bottle was shaken until the yellow kumara particles turned black owing to attachment of spores. The substrate was then poured out of the bottle, and mixed with the remaining 8 g of the substrate under aseptic conditions. Any agglomerations of particles were separated using a glass rod. Finally, before use, a sample of particles was examined under the microscope to ensure that each particle had at least 10-20 spores attached to it.

7.2.3 Results and discussion

Previous experiments have demonstrated that a minimum moisture content is necessary for growth of *A. niger* on kumara. The minimum content for satisfactory growth is 55%, while the optimum value is about 65-71%. Hence in a gas-solid fluidized bed reactor it is necessary for the initial moisture content of the substrate to be sufficiently high to allow spore germination. Unfortunately, it is not possible to commence operation of the reactor if the moisture content of the particles is too high, since particle agglomeration occurs. Hence during start-up, it was intended that the particles, of initial moisture

content 5%, would absorb sufficient water from the humidified air to initiate fungal growth while not causing particle agglomeration. Experiments were performed using air of different relative humidity, to determine suitable conditions for start-up of the reactor. The RH values were 60%, 70%, 80% and 90%, while air flow rates were in the range of 5-20 l/min depending on the particle size used. Samples were withdrawn from the reactor at intervals and examined under the microscope for fungal growth. For the experiment using air at a RH value of 60%, the reactor was operated for 6 days, but no fungal growth was observed on the particles. At 70% RH, the operation lasted 5 days, but again, no fungal growth was observed. At 80% RH, the reactor was operated for 5 days and microscopic observation revealed that the spores had begun to germinate. However, particle agglomeration occurred at this point and the operation was ceased. At 90% RH, after 3 days operation, the spores had commenced germination, but again, particle agglomeration occurred, causing reactor shut-down.

These results confirmed the importance of moisture content in the establishment of a gas-solid fluidized bed reactor of this type. As the moisture content of the particles became sufficiently high to allow spore germination, the particles agglomerated. Indeed, the problem appeared to be exacerbated by the spore germination in that this initiated liquefaction of the starch, thus promoting agglomeration. To confirm this effect, a simple experiment was performed in a petri dish in which a piece of aluminium foil was supported above sterile water. In this system, dried (5% moisture) kumara particles were spread out on the foil, such that they could readily absorb moisture from the surroundings, and were inoculated with the spores. Spore germination was observed within 2 days, and simultaneously, the particles agglomerated so that it became very difficult to break up the masses.

A noticeable factor during the fluidized bed experiments was the long lag time observed before spore germination commenced. In addition to the moisture content of the kumara particles, other factors which may have contributed to this include the high air flow rate

inside the reactor and friction or abrasion among the particles. *A. niger* is a filamentous fungus and growth occurs by hyphal extension. Since the air flow rates were in the range of 0.05-0.2 m/sec, the friction and/or abrasion could physically damage hyphae and had an inhibitory effect on germination. Microscopic examination, however, revealed that the spores remained attached to the particles throughout the experiments. The friction and/or abrasion among particles as they collided with each other within the reactor may have inhibited germination and/or dislodged filamentous growth as it was formed.

7.2.4 Conclusion

Despite several attempts, it proved very difficult to start up a gas-solid fluidized bed reactor using the filamentous fungus *A.niger* growing on kumara particles. The major reason for this was the conflicting moisture content requirements for fungal growth and particle fluidization. At the moisture contents required for spore germination, the kumara particles agglomerated and could no longer be fluidized. This problem was exacerbated by starch liquefaction within the particles. Minor reasons for the poor spore germination are thought to be the high air flow rate within the reactor and collisions between particles, which may have broken up any filamentous growth which emanated from spore germination.

7.3 Liquid-solid-gas Fluidized Bed Reactor

7.3.1 Introduction

A liquid-solid-gas fluidized bed reactor system has many potential advantages including improved mass and heat transfer. Its most marked advantages over the gas-solid fluidized bed reactor are, firstly, the substrate can have a sufficiently high moisture

content, and, secondly, there is low shear stress. For these reasons, liquid fluidized bed reactors have been widely adopted in microbiological waste treatment systems and fermentation reactors. However, most of the applications have been related to the use of cell immobilization for production of high value products, e.g. monoclonal antibodies (Dean *et al*, 1988), Penicillin-G (Jones *et al.* 1986), and for waste water treatment (Cooper, 1983). Nevertheless, Adisasmito *et al* (1987) used a liquid-solid-gas fluidized bed reactor for the production of culture starter of *Trichoderma reesei* on corncob, and achieved high mycelial biomass concentration within 24 hours.

Following the studies of citric acid production in solid substrate fermentation in flasks, and the experiences using the gas-solid fluidized bed reactor which was unable to supply sufficient moisture for the organism growth under fluidization fashion, the present experiments were designed to investigate the possibility of application of a liquid fluidized bed reactor for citric acid production in solid substrate fermentation.

7.3.2 Preparation of experiments

A liquid-solid-gas fluidized bed reactor system, as depicted in Fig 3.4, was used in these experiments, while fresh kumara substrate with a particle size range of 4-6 cm, which was obtained from the procedure described in Section 3.6.6, was used. The substrate was sterilized at 108°C for 20 minutes prior to inoculation. For inoculation, 2 ml of spore suspension (approximately 10^8 spores/ml) was well mixed with 100 g of sterilized substrate, after which the mixture was conducted into the reactor.

Because of the lag period required for the spores to germinate, and the softness of the particle substrate, for the first 24 hours the loaded reactor was kept under an air flow rate of 1.0 l/min, but without supplying sterile water. During this time, the organism germinated and white filamentous hyphae appeared on the substrate particles. Sterile water was then pumped into the reactor from the reservoir. Once the ratio of liquid to

particles reached 2:1 (v/w), the supply was stopped and the valve was turned to recirculation. Proper adjustment of the liquid flow rate (range from 200-400 ml/min) and air flow rate (range from 1-2 l/min) allowed fluidization to be achieved.

7.3.3 Results and discussion

The liquid fluidized bed reactor was run in a turbulent fluidized fashion, because of the heterogeneous density created by the air bubbles. It was operated for 3 days, after which time it became contaminated. During the process, the particles were broken into small pieces, or were abraded into round small balls. No biomass grew on the particles. Meanwhile, the original clear liquid became increasingly turbid, showing that a serious loss of material from the kumara particles and growth of organism in liquid rather than on particles was happening during the process. The shear stress created by the fluid and air bubbles and the friction among the particles were the major factors contributing to this breakage. In addition, because of the disintegration of the particles, blockage occurred at the top separation mesh and the bottom support mesh, creating difficulties with recirculation during the process. The large mass of liquid also increased the risk of contamination, thus eliminating one of the important advantages of solid substrate fermentation.

7.3.4 Conclusion

Because of the softness of the kumara particles, disintegration occurred during operation of the system. This was due mainly to the vigorous abrasion among the particles and shear stress from aeration. No fungal growth was seen on the particles despite the fact that hyphal growth had commenced prior to liquid being introduced into the reactor. Hence, the mycelium was probably dislodged during the fluidization process. For these reasons, this type of reactor was not considered suitable for the present work.

7.4 Rotating Drum Reactor

7.4.1 Introduction

With the development of solid state fermentation, various types of reactors have been investigated. The rotating drum reactor is one of the types that has attracted much research attention. It has been used in solid substrate fermentation for the production of soy sauce koji (Aidoo *et al*,1982) and ethanol by yeast (Kargi and Curme, 1985). Many modifications to rotating drum reactors, including the addition of baffles (Ryoo *et al*, 1991), the installation of a mixer (Laukevics *et al*, 1984) and even the application of a computer control system with rocking action (Ryoo *et al*, 1991), have been developed. Although using a rotating drum reactor could have its limitations, including the disruption of mycelial biomass and low packing capacity (Reu *et al*, 1993), its potential advantages cannot be neglected. It is relatively economic and provides efficient agitation; it is easy to achieve large scale production and it is easy to practise continuous operation.

Thus, following the previous experiments in flasks and the experiences with the gas fluidized bed reactor and liquid fluidized bed reactor, an investigation was conducted into the rotating drum reactor for citric acid production from kumara.

7.4.2 Preparation of experiments

Experiments were carried out in the rotating drum reactor system, which is depicted in Fig 3.5. The 1-litre Agee jars had a silicone coating inside to prevent adherence of material to the sides. A chimney with cotton wool inside, on the top of each jar provided natural aeration by the diffusion of air. The substrate loading in each jar was 100, 150 or 200 g and was used in two forms, one as a cubic particle (size of 4 mm), another as a paste. The paste and particle substrates were obtained by the same procedure as described previously. The inoculum size was 10^4-10^6 spores/g substrate and the whole system was kept at an ambient temperature of 30 °C and at a rotation speed of 15 rpm.

7.4.3 Results and discussion

The rotating drum reactor system was operated for 3 days. The cubic particles lost their shape during the first few hours and gradually became spherical after 10 hours. During this period, the particles showed movement relative to the jar's wall and to other particles with time. The particles became sticky and agglomeration occurred, forming a paste which attached onto the jar's wall. This phenomenon happened due to abrasion and friction between or among the wall of the jar and the substrate particles. Furthermore, the hydrolysis of the starch by the enzymes released by the organism during germination made the particles softer. After about 36 hours, mycelia emerged which made the paste attach even more firmly to the wall. However, these phenomena occurred only where the substrate loading was low (100 g per jar).

In the jars containing the higher loads of substrate, while the particles still became a paste, the paste still had a movement relative to the walls of the jars. This was essentially due to the force of gravity on the large mass of paste. Thus, the paste became a rolling form of material during the process. However, there was no growth of organism observed even after 3-days of culture, suggesting that the abrasion or friction between the paste and jars' walls seriously inhibited the organism's growth. For the original paste substrate, similar phenomena were observed, and there was no growth of organism. Although no quantitative measurements of this effect have been made, it is clear that such abrasion and friction between particles and solid surfaces must be considered during the development of such a reactor system involving filamentous fungi.

7.4.4 Conclusion

The rotating drum reactor system was not feasible for the solid substrate fermentation for citric acid production from kumara, due to abrasion and friction between or among the substrate particles and reactor walls which seriously inhibited the growth of the filamentous organism, *A. niger*.

7.5 Packed Bed Reactor

7.5.1 Introduction

The packed bed reactor has been used frequently in solid substrate fermentation because of its wide range of applications (Mitchell *et al*, 1992; Murthy *et al*, 1993). Compared with other reactors, the packed bed reactor is able to provide more favourable fermentation conditions, including the presence of sufficient moisture, and freedom from friction and abrasion on the substrate and organism. Recently, packed bed reactors have been developed on a large scale, e.g. Durand *et al* (1993) used a 1 m deep substrate layer in a packed bed reactor for protein enrichment of sugar beet pulp using *Rhizopus oligosporus*, while Xue *et al* (1992) developed a packed bed reactor 17.6 m in length, 3.6 m in breadth and 2.0 m in height for the protein enrichment of sugar beet using *Aspergillus oryzae*. However, in solid substrate fermentation for citric acid production, application of the packed bed reactor is just beginning.

The purpose of the experiments described here was to investigate the possibility of applying a packed bed reactor to citric acid production in solid substrate fermentation from kumara.

7.5.2 Preparation of experiments

The packed bed reactor system, as depicted in Fig 3.7, was used in these experiments. The particle substrate, its sterilization, inoculum size and inoculation procedure, were all as described in Section 7.3.2. The reactor was loaded with 150 g of substrate particles, with a bed depth of approximately 130 mm, and operated at temperature of 30 °C, with an air flow rate of 0.5 l/min.

7.5.3 Results and discussion

The results of two experiments are shown in Table 7.1, and morphological observations after 6 days of fermentation are provided in Figs 7.1 and 7.2. From Table 7.1, it is apparent that the solid substrate fermentation in a packed bed reactor achieved excellent citric acid and biomass production. It is also apparent that the progress of the fermentation was faster in the packed bed reactor than in flasks, i.e. 95.2% of starch was utilised in the former, after 6 days fermentation, compared with 78.7% in flasks (Section 6.2). Furthermore, the peak citric acid production occurred earlier in the packed bed reactor i.e. the concentration on day 4 was higher than that on day 6. However, the biomass concentration was lower in the packed bed reactor. The citric acid yield was also lower, with a value of 37% on day 4 and 31% on day 6, respectively, based on starch utilized.

The morphological observations of the fermentations showed that spore germination occurred earlier in the packed bed reactor than in flasks, and the substrate was covered completely by the white and yellow hyphae within 24 hours. Furthermore, from the observations shown in Figs 7.1 and 7.2, although considerable mycelial mass was present on the particles throughout the packed bed reactor, black spores were present only at the top and bottom, not in the middle of the reactor. These morphological differences are almost certainly related to the different aeration systems used. It was decided that the parameters of the packed bed reactor would be optimized in further experiments.

7.5.4 Conclusion

The packed bed reactor proved to be suitable for citric acid production from the substrate. Fungal growth occurred rapidly, and a high concentration of citric acid was achieved. The system was technically simple to operate and appeared robust.

Table 7.1 Fermentation parameters for citric acid production from kumara in a packed bed reactor

Fermentation Time (day)	Bed loading (g)	Initial starch (g/kg)	Citric acid (g/kg)	Biomass (g/kg)	Starch utilization (g/kg)	Yield citric acid (%)	yield of biomass (%)
4	150	200	62.7	13.6	170.6	36.8	8.0
6	150	200	59.7	24.8	190.4	31.3	13.0

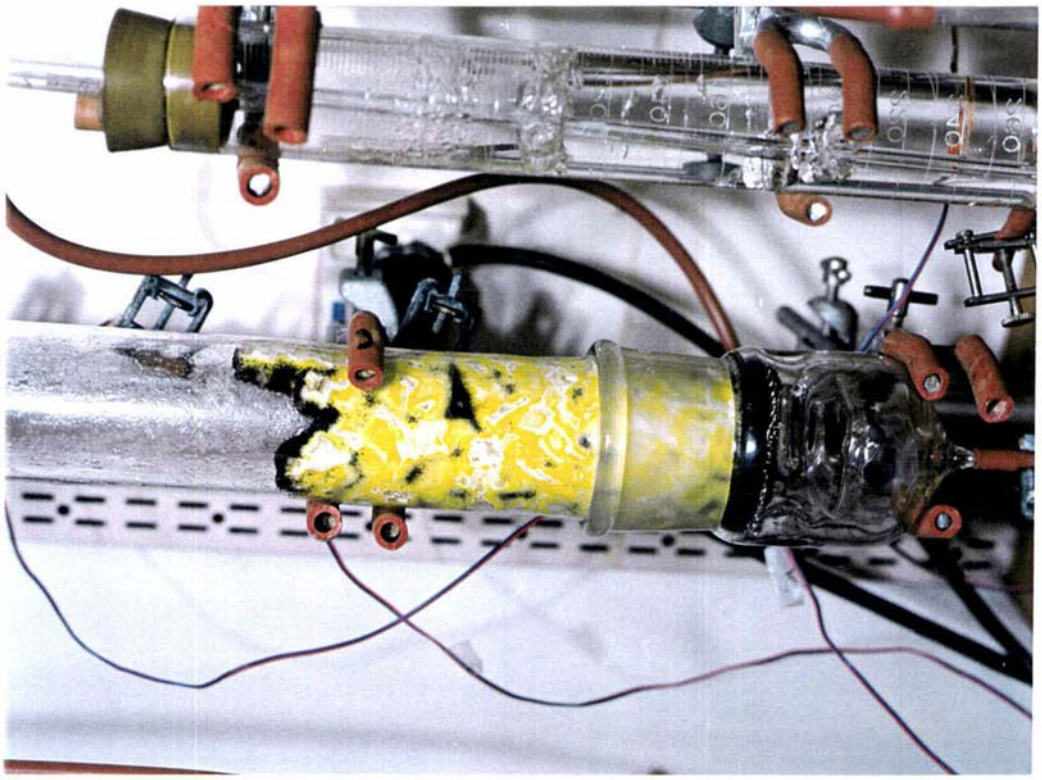


Figure 7.1 Morphological observation of fungal growth in packed bed reactor (6 days fermentation)

Figure 7.2 Morphological observation of fungal growth in packed bed reactor (a) in packed bed and, (b) a radius section view (6 days fermentation)

7.6 Overall Conclusions

Although considerable effort was spent on the application of various reactors to citric acid production in solid substrate fermentation, only the packed bed reactor proved to be suitable. The other reactors were not suitable because of their inability to allow adequate fungal growth under the necessary operating conditions. Hence, the packed bed reactor was selected for further study with a view to developing a superior system to the traditional flask technique.

Chapter 8

Optimization of Packed Bed Reactor for Citric Acid Production

8.1 Introduction

As shown in the previous chapter and as reported in the literature (Silman *et al*, 1977; Raimbault and Alazard, 1980; Durand and Chereau, 1988), the packed bed reactor has a wide range of applications in solid substrate fermentation. Recently, the studies on packed bed reactors have been extended into the details. Gowthaman *et al* (1993) investigated the effect of air flow rate on enzyme activity in a packed bed reactor using *A. niger* growing on wheatbran. Later, this group described the relationship between air flow rate and the temperature gradient of the substrate layer (Ghildyal *et al*, 1994). Gumbira-Sa'id *et al* (1993) studied the effects of interaction between the particle size and bed loading of sago-bead on the growth of *Rhizopus oligosporus*. For citric acid production, Hang (1987) reported that the air flow rate had a substantial effect on citric acid production using *A. niger* growing on apple pomace. Apparently, therefore, air flow rate, substrate particle size and bed loading are all critical factors for solid substrate fermentation in a packed bed reactor. However, although these variables have been investigated individually, their interactions have usually been ignored, despite the fact that such interactions may have profound effects on the fermentation process.

To optimize a packed bed reactor system, the conventional experimental method is to vary one of the parameters while keeping the others at a constant level. This method is not only laborious and time consuming, but is also unable to estimate the interactions to any accurate degree. Recently, with the development of statistical-based experimental designs and corresponding software packages, more experimental design techniques have been adopted in biotechnological research. Thus, Rodriguez *et al* (1985) used a 2^2 factorial design to test the effects of two types of nitrogen source on the growth of *A.*

niger on dried citrus peel in a packed bed reactor. Polakovič *et al* (1993) used a second-order central composite design to optimize a packed bed reactor utilising immobilized *Saccharomyces cerevisiae* to hydrolyse sucrose to glucose. Ramakrishna *et al* (1991) used a full factorial design to model a *Saccharomyces cerevisiae* immobilized cell reactor for production of alcohol. The advantage of the full factorial design is that, with a reasonably small number of experiments, simultaneous changes of all variables can be achieved in conjunction with a quantitative estimation not only of the main effects, but also of their interactions (Haaland, 1989).

In the present experiments, a full factorial design was adopted to optimize the packed bed reactor system with respect to the air flow rate, the bed loading and the particle size of the substrate. In this way, optimal conditions would be provided for further kinetic study.

8.2 Experimental Design

Based on previous experiments, an inoculum size of 10^4-10^6 spores/g substrate was used, while the moisture content of the substrate was left at the natural value, i.e. approximately 70%. The cubic particle substrate was obtained using the procedure described in Section 3.6.1. The range of particle sizes was selected to be 4 to 8 mm, based on the results described in Section 5.6. The ranges of air flow rate and bed loading were selected to be 0.5 to 1.5 l/min, and 100 to 300 g, respectively.

The full factorial design, its matrix and coded values are presented in Table 8.1 along with the decoded variable values. This would allow estimation of all main effects and all two-way and three-way interactions. The three replicates of the centre points were designed to estimate the experimental errors and to test the linear characteristics of the variables. The Minitab package (Copyright, 1991, Pennsylvannia State University) was used to develop the statistical analysis and regression equations for the experimental

data. The general model used has the form:

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{123}x_1x_2x_3$$

where: Y=the value of the variable being regressed

a_0=constant

a_{ij}=coefficients, i and j=0, 1, 2 or 3

x_1=coded value of air flow rate

x_2=coded value of bed loading

x_3=coded value of particle size

Statistical analysis will provide a test for lack of fit for a regression equation where a 90% confidence is introduced for arbitrary judgement, which is used a p-value to judge at a value less than 0.10. If there is no significant lack of fit, this equation can be fitted to obtain an optimized expression. However, an important point is that this regression gives an empirical model. The terms and coefficients are empirically related to, but do not necessarily determine, the values of the experimental variables.

Table 8.2 presents the experimental data obtained from the various runs, which were carried out in random order. During the experiments, 0.2 M NaOH solution was used to absorb the CO_2 at the exit of the packed bed reactor (as described in Section 3.5.9). The unit "ml 0.2 M NaOH/100 g substrate" was used to express the amount of CO_2 produced during the fermentations, and "ml 0.2 M NaOH/100 g starch utilised" to express the yield of CO_2, based on starch utilised.

Table 8.1 Matrix of full factorial design and decoded values

Run No.	Order of run	Coded value			Decoded value		
		air flow rate	bed loading	particle size	air flow rate (l/min)	bed loading (g)	particle size (mm)
1	1	-1	-1	-1	0.5	100	4
2	4	-1	-1	+1	0.5	100	8
3	10	-1	+1	-1	0.5	300	4
4	5	-1	+1	+1	0.5	300	8
5	2	+1	-1	-1	1.5	100	4
6	6	+1	-1	+1	1.5	100	8
7	3	+1	+1	-1	1.5	300	4
8	7	+1	+1	+1	1.5	300	8
9	8	0	0	0	1.0	200	6
10	9	0	0	0	1.0	200	6
11	11	0	0	0	1.0	200	6

Table 8.2 Responses of experiments for the full factorial design (after 4 days fermentation)

Run No.	Order of run	Citric acid (g/kg)	Yield of citric acid (%)	Biomass (g/kg)	Yield of biomass (%)	Carbon dioxide (ml 0.2M NaOH/ 100 g substrate)	Yield of carbon dioxide (ml 0.2M NaOH/ 100g starch used)	Percentage of starch utilised (%)
1	1	53.0	31.3	37.4	22.1	100	114.0	87.8
2	4	103.8	70.0	31.5	21.3	113	140.5	80.5
3	10	47.8	30.6	35.6	22.8	97	109.8	88.3
4	5	45.9	33.4	34.2	24.6	93	105.9	87.8
5	2	94.6	53.3	31.4	17.9	75	88.9	84.3
6	6	68.7	50.8	14.0	10.4	50	63.5	78.8
7	3	66.3	49.2	21.9	16.3	28	33.4	83.7
8	7	68.1	53.6	32.9	25.9	67	77.9	86.0
9	8	83.3	57.0	30.5	20.9	78	91.1	85.6
10	9	75.5	50.9	27.6	18.6	100	91.4	87.5
11	11	78.7	53.6	28.9	19.7	80	93.3	85.7

8.3 Statistical Analysis of Responses in the Full Factorial Design

To obtain an optimal model, the full regression equations for the responses were, firstly, tested for lack of fit, and 90% confidence level was introduced, i.e. a p-value less than 0.10 would suggest significant lack of fit. Then, each of the statistical coefficients was examined for its significance by using the t-test associated with the p-value. Any term(s) which was tested and showed significant of lack of fit, is not shown in the corresponding table. Any regression equations which exhibited significant lack of fit, will not be discussed further. For the significance of the regression, although a 90% confidence level was used, the judgement emphasized the significance of the total main effects, and the 2- and 3-way interactions, rather than the individual coefficients. This is because, in some cases, e.g. for the effects on citric acid concentration in Table 8.3, the main effects or interactions, individually, were not significant, but attempts to further fit the model by dropping them actually caused significant lack of fit.

8.3.1 Estimated effects for citric acid production

The statistical analysis of citric acid production, both of concentration and yield based on starch utilized, are shown in Tables 8.3 and 8.4, respectively. The regression for the citric acid yield showed significant lack of fit, with a p-value of 0.079 (Table 8.4). None of the three main effects showed any significant effect on the citric acid yield. The reason for the lack of fit is not clear, but it is possible that complex interactions among the 3 operating parameters are important. Unfortunately, the simple interaction terms used in the model may not represent the complexity of the situation.

In contrast, the regression for citric acid concentration showed no significant lack of fit (Table 8.3). Among the parameters, the bed loading, the 2-way interaction between air flow rate and particle size, and the 3-way interaction all showed significant effects. Neither the air flow rate nor its interaction with bed loading were significant. Nevertheless, if either the air flow rate or the interaction between air flow rate and bed

loading was dropped from the model, the regression would show significant lack of fit. These two effects, therefore, were kept in the regression. The regression equation is as follows:

$$Y_{CA} = 71.4 + 5.9x_1 - 11.5x_2 + 3.1x_1x_2 - 9.1x_1x_3 + 10x_1x_2x_3 \quad ---(2)$$

In an attempt to determine the conditions for maximum citric acid production, this regression equation was used to predict response surfaces. To accomplish this, one of the three variables must be kept constant, to allow the creation of a three dimensional response surface, e.g. while keeping the particle size at values of -1, 0, or +1, three corresponding equations were produced as follows:

$$x_3 = -1, \quad Y_{CA} = 71.4 + 15.0x_1 - 11.5x_2 - 6.9x_1x_2 \quad ---(3)$$

$$x_3 = 0, \quad Y_{CA} = 71.4 + 5.9x_1 - 11.5x_2 + 3.1x_1x_2 \quad ---(4)$$

$$x_3 = +1, \quad Y_{CA} = 71.4 - 3.2x_1 - 11.5x_2 + 13.1x_1x_2 \quad ---(5)$$

Then, equations 3, 4 and 5 were used to create three dimensional response surfaces using a software package of Stanford Graphics (Copyright of 3-D Visions Corporation, 1993, USA). In this way, three response surfaces were created for each of the three variables being kept constant. Thus, nine response surfaces were created, as shown in Figs 8.1, 8.2 and 8.3, which allow detailed analysis of the parameters.

As the aim of the experimental design was to identify the optimal conditions for high citric acid production, the analysis presented here will focus on these parameters and, as a consequence, on the lowest biomass and carbon dioxide production. Therefore, the intermediate situations described in the response surface will not be discussed.

Table 8.3 Estimated effects for citric acid concentration

Term	effect	coefficient	t-value	p
constant		71.4	24.7	0.000
AFR	11.8	5.9	1.7	0.155
BL	-23.0	-11.5	-3.4	0.023
AFR*BL	6.2	3.1	0.9	0.420
AFR*PS	-18.3	-9.1	-2.6	0.049
AFR*BL*PS	20.1	10.0	2.8	0.036

Analysis of variance

Effects	DF	Seq SS	F	P
Main effects	2	1338	6.7	0.038
2-way interactions	2	745	3.7	0.100
3-way interactions	1	806	8.1	0.036
Residual error	5	499		
curvature	1	245	3.9	0.120
lack of fit	2	223	7.3	0.120
pure error	2	30		
Total	10	3390		

Table 8.4 Estimated effects for yield of citric acid yield

Term	effect	coefficient	t-value	p
constant		48.5	20.7	0.000
AFR	10.4	5.2	1.9	0.131
BL	-9.6	-4.9	-1.8	0.155
PS	10.8	5.4	2.0	0.120
AFR*BL	9.0	4.5	1.6	0.177
AFR*PS	-9.9	-4.9	-1.8	0.145
AFR*BL*PS	10.7	5.4	2.0	0.123

Analysis of variance

Effects	DF	Seq SS	F	P
Main effects	3	637	3.5	0.128
2-way interactions	2	359	3.0	0.162
3-way interactions	1	230	3.8	0.123
Residual error	4	241		
curvature	1	118	2.9	0.189
lack of fit	1	104	11.2	0.079
pure error	2	30		
Total	10	3390		

Note: AFR, air flow rate; BL, bed loading; PS, particle size.

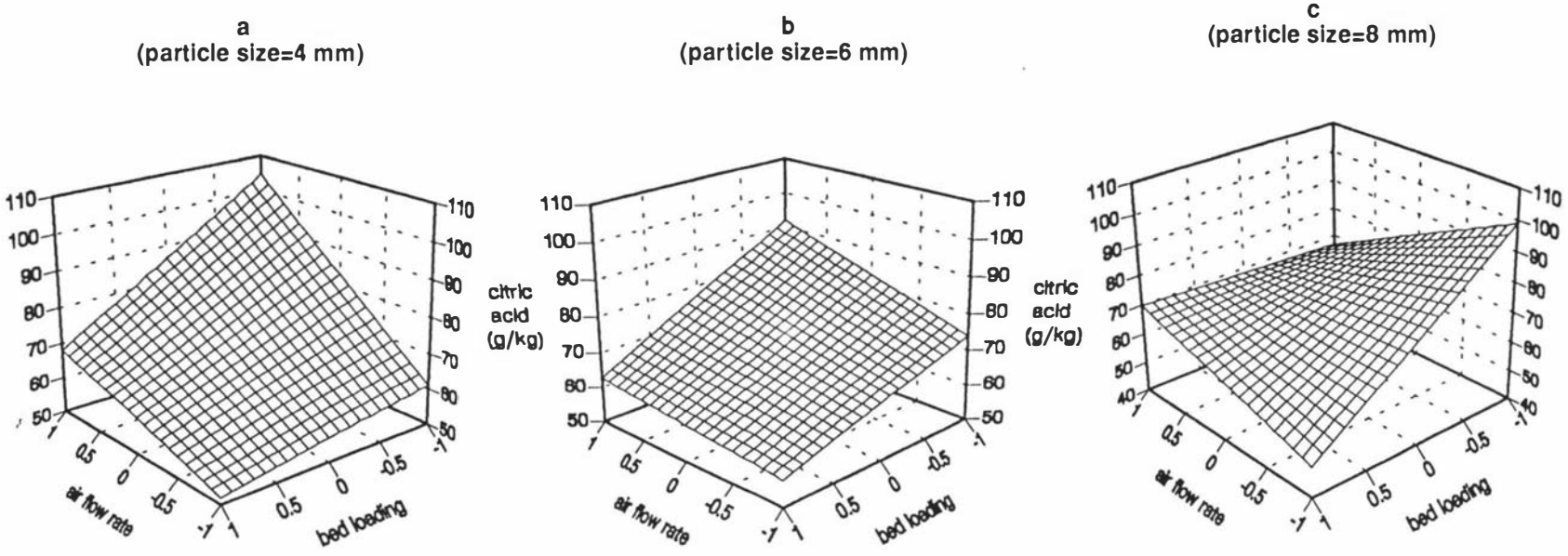


Figure 8.1 Response surfaces of citric acid concentration in packed bed reactor (keeping particle size constant)

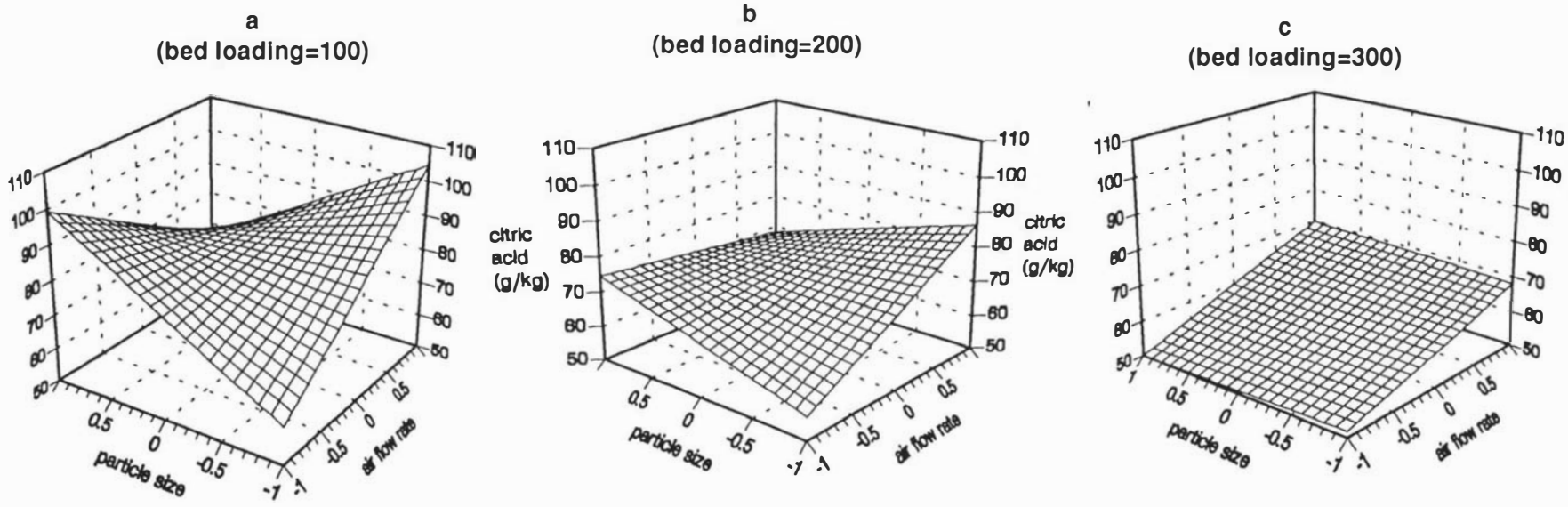


Figure 8.2 Response surfaces of citric acid concentration in packed bed reactor (Keeping bed loading constant)

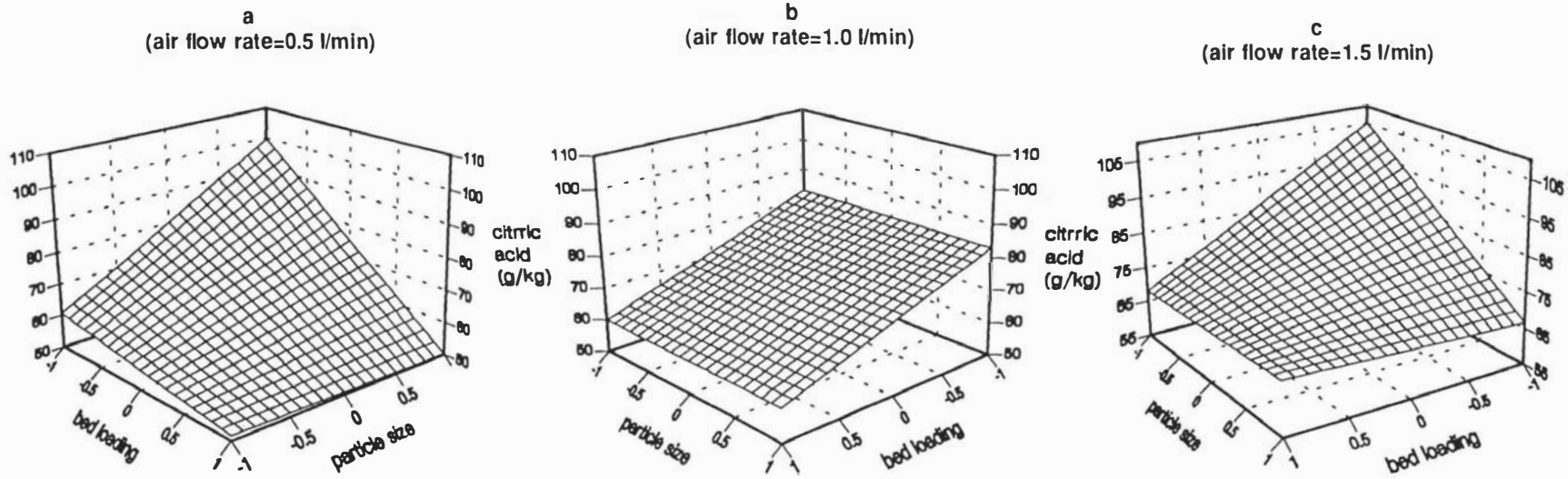


Figure 8.3 Response surfaces of citric acid concentration in packed bed reactor (keeping air flow rate constant)

From Fig 8.1, a high citric acid concentration can be obtained using a small particle size with a low bed loading and a high air flow rate (Fig 8.1a) and using a large particle size with a low bed loading and a low air flow rate (Fig 8.1c). In contrast, with a high bed loading and low air flow rate, low citric acid concentrations are observed within the whole range of particle sizes. From Fig 8.2, as shown in Fig 8.2a, a low bed loading favours citric acid production with either a high air flow rate and small particle size or a low air flow rate and a large particle size. In contrast, with a high bed loading, as shown in Fig 8.2c, low citric acid concentrations are observed with whatever levels of air flow rate and particle size. These results suggest that bed loading has the most important effect on citric acid production in the packed bed reactor. Another interesting situation observed in Fig 8.2a is that low citric acid concentration is obtained using a low bed loading with a high air flow rate and a large particle size. Favoured conditions for citric acid production shown in Figs 8.1 and 8.2 are confirmed in Fig 8.3, i.e. a high air flow rate with a small particle size and a low bed loading or a low air flow rate with a large particle size and a low bed loading. It is also shown that a high air flow rate is not a prerequisite for high citric acid production in the packed bed reactor. To sum up, under the conditions of low bed loading, with a high air flow rate and small particle size, or with a low air flow rate and large particle size, a citric acid concentration at a value of approximately 105 g/kg could be obtained.

8.3.2 Estimated effects for biomass production

The statistical analysis for biomass production, in terms of both concentration and yield based on starch utilized, is presented in Table 8.5 and 8.6, respectively. Neither of the regression equations showed a significant lack of fit. For biomass concentration, all of the effects were significant at very high confidence levels, while for the yield, most were significant.

For biomass concentration, the three main effects, the interaction between bed loading and particle size, and the interaction among air flow rate, bed loading and particle size were all significant. These significant factors were expressed in an equation, using

similar processes to those described in the preceding section (the equations analysis processes are shown in Appendix I), while the three dimensional response surfaces are displayed in Figs 8.4, 8.5 and 8.6. Similarly, for the biomass yield based on starch utilized, the significant factors were the air flow rate, the bed loading, the interactions between air flow rate and bed loading, and bed loading and particle size, and the interaction among the three parameters. The three dimensional response surfaces are shown in Figs 8.7, 8.8 and 8.9 respectively, while the equations analysis processes are given in Appendix I.

From Figs 8.4, 8.5 and 8.6, it is evident that a high biomass concentration, in the range of 30 to 35 g/kg, may be obtained under multiple combinations of conditions, such as low air flow rate with low bed loading and small particle size (Fig 8.4a), or high air flow rate with small particle size and high bed loading (Fig 8.5c). A low biomass concentration can be observed in Figs 8.4a, 8.4c, 8.5a, 8.5c and 8.6c, and was affected strongly by the interactions among the three parameters. It is difficult to identify the most important factor from the parameters. However, a high air flow rate did not always favour biomass production, e.g. a low biomass concentration was observed at high air flow rate and large particle size with low bed loading (Fig 8.4c), and at a small particle size with high bed loading and high air flow rate (Fig.8.6c). This may imply that, in a packed bed reactor, conditions other than aeration per se may be important, such as the shear stress of air flow, the specific surface area, or the porosity of the packed layer. During selection of optimized conditions it should be borne in mind that for citric acid production, a medium biomass production may be favoured rather than either extremely high or low, since production of sufficient biomass is necessary to catalyse the process of citric acid production, but not too much to waste some of the starch substrate. As shown in the above figures, a sufficient biomass concentration can be achieved under a wide range of operating conditions, allowing convenient selection of optimum conditions for citric acid production. In addition, this also shows the wide range of possible operating conditions of a packed bed reactor from the view of fungal biomass production.

Table 8.5 Estimated effects for biomass concentration

Term	effect	coefficient	t-value	p
constant		29.6	56.3	0.000
AFR	-9.6	-4.8	-7.8	0.001
BL	2.6	1.3	2.1	0.092
PS	-3.4	-1.7	-2.8	0.039
BL*PS	8.2	4.1	6.7	0.001
AFR*BL*PS	6.0	3.0	4.8	0.005

Analysis of variance

Effects	DF	Seq SS	F	P
Main effects	3	221	24.3	0.002
2-way interactions	1	135	44.3	0.001
3-way interactions	1	71	23.4	0.005
Residual error	5	15		
curvature	1	1.7	0.5	0.515
lack of fit	2	9.3	2.2	0.317
pure error	2	4.3		
Total	10	443		

Table 8.6 Estimated effects for biomass yield

Term	effect	coefficient	t-value	p
constant		20.0	74.4	0.000
AFR	-5.1	-2.5	-8.1	0.001
BL	4.5	2.2	7.1	0.002
PS	0.8	0.4	1.3	0.278
AFR*BL	2.5	1.2	3.9	0.017
BL*PS	4.9	2.5	7.8	0.001
AFR*BL*PS	3.7	1.8	5.8	0.004

Analysis of variance

Effects	DF	Seq SS	F	P
Main effects	3	93	38.9	0.002
2-way interactions	2	61	38.2	0.002
3-way interactions	1	27	33.9	0.004
Residual error	4	3.2		
curvature	1	0.4	0.5	0.551
lack of fit	1	0.2	0.1	0.765
pure error	2	2.6		
Total	10	184		

Note:AFR, air flow rate; BL, bed loading; PS, particle size.

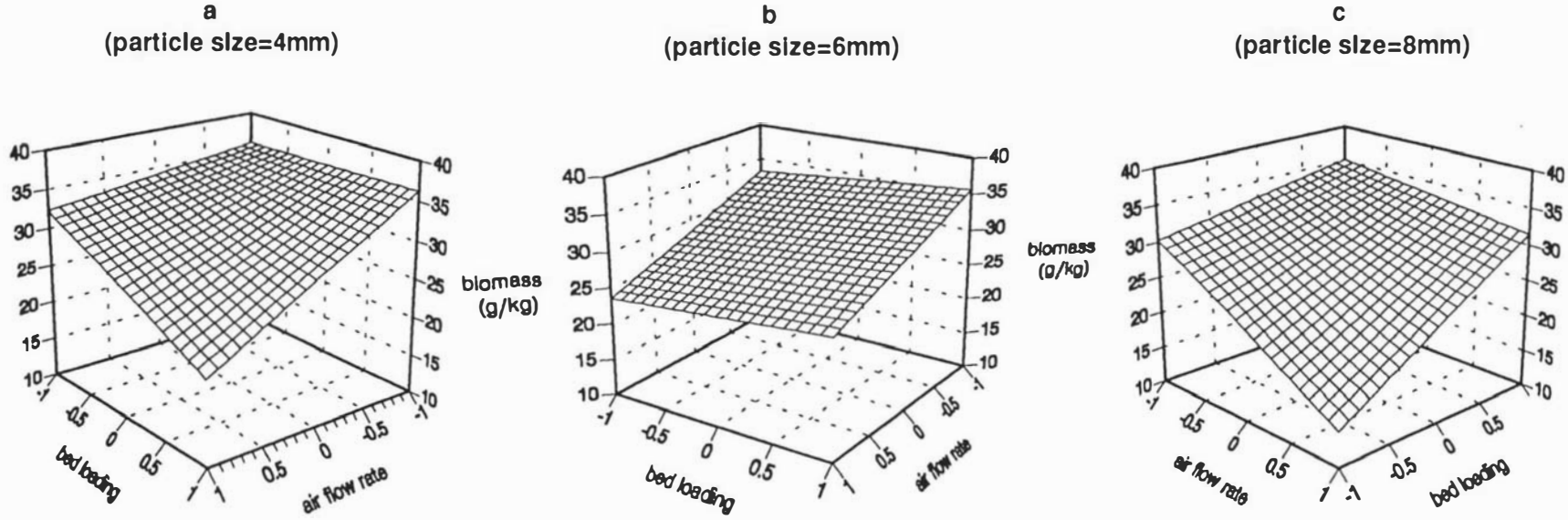


Figure 8.4 Response surfaces of biomass concentration in packed bed reactor (keeping particle size constant)

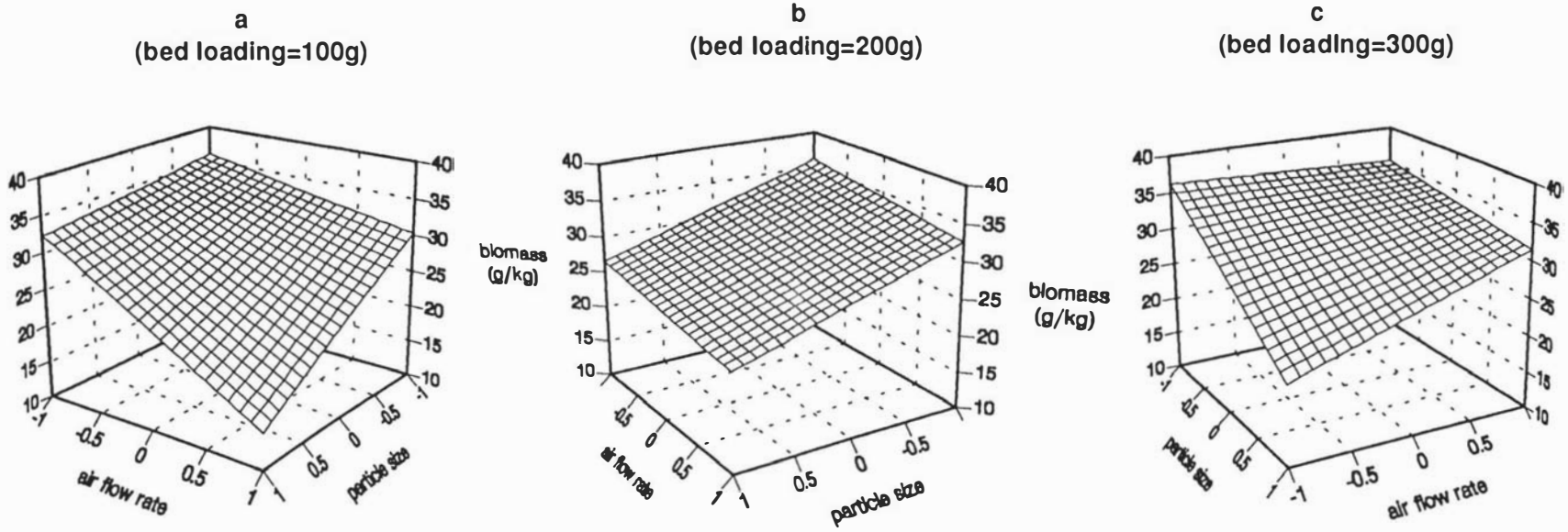


Figure 8.5 Response surfaces of biomass concentration in packed bed reactor (keeping bed loading constant)

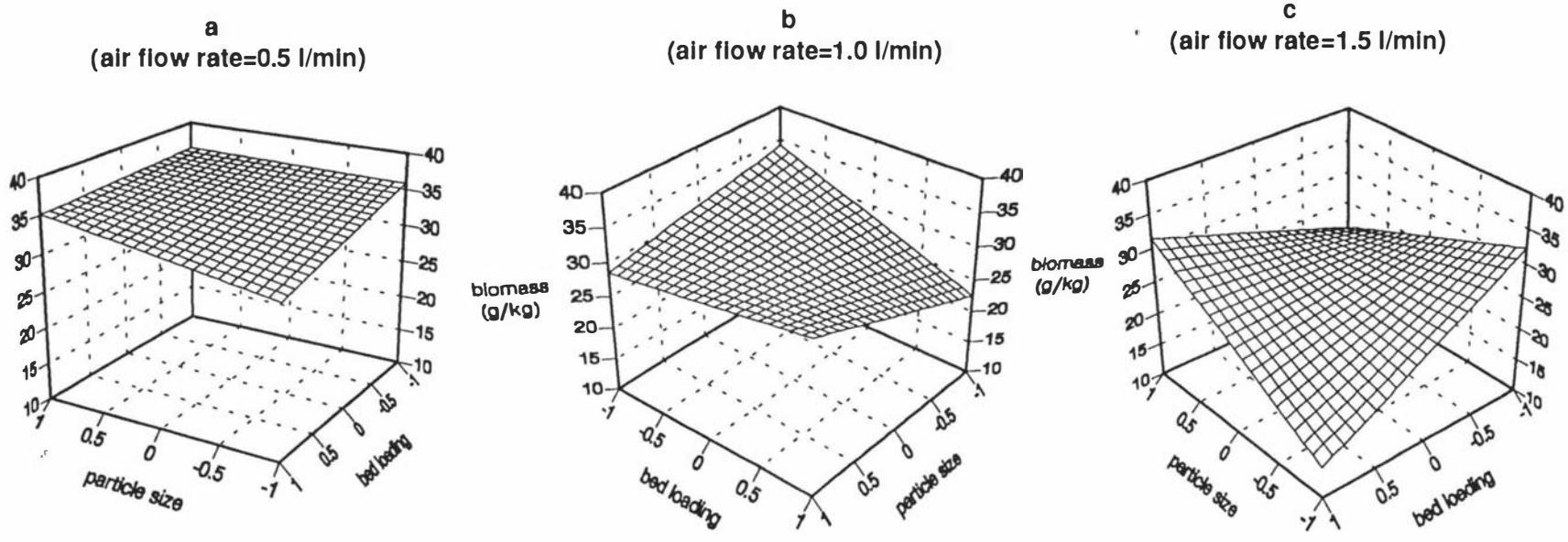


Figure 8.6 Response surfaces of biomass concentration in packed bed reactor (keeping air flow rate constant)

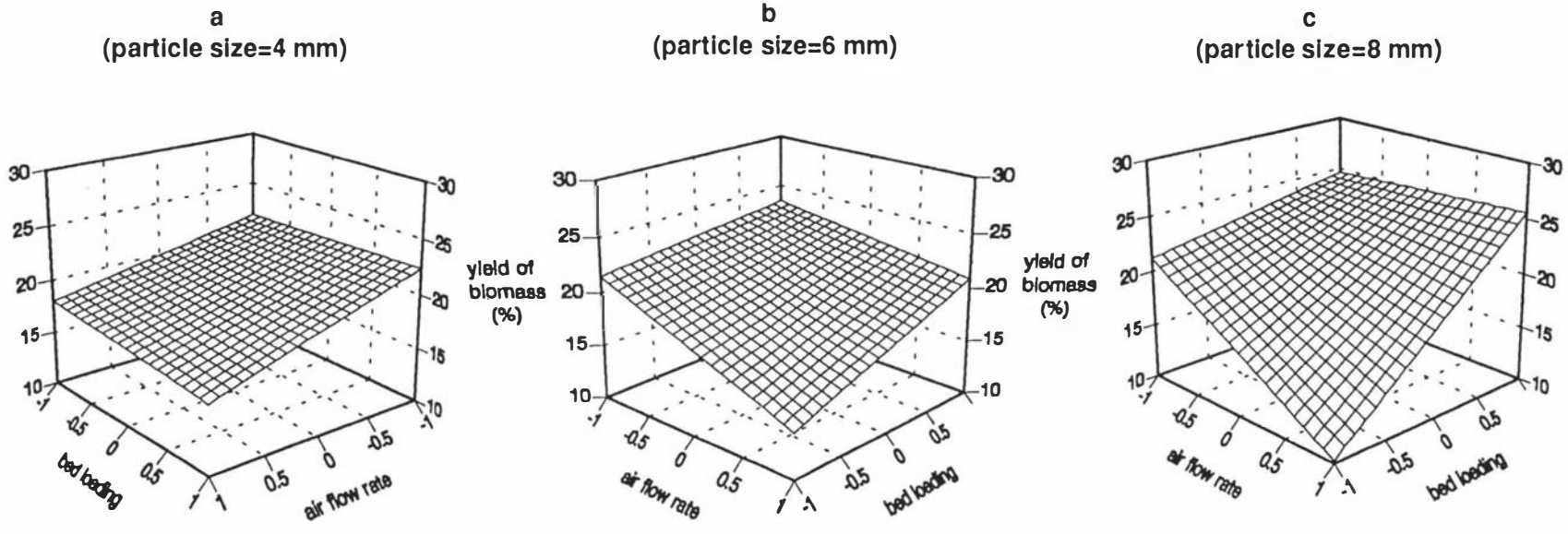


Figure 8.7 Response surfaces of biomass yield in packed bed reactor (keeping particle size constant)

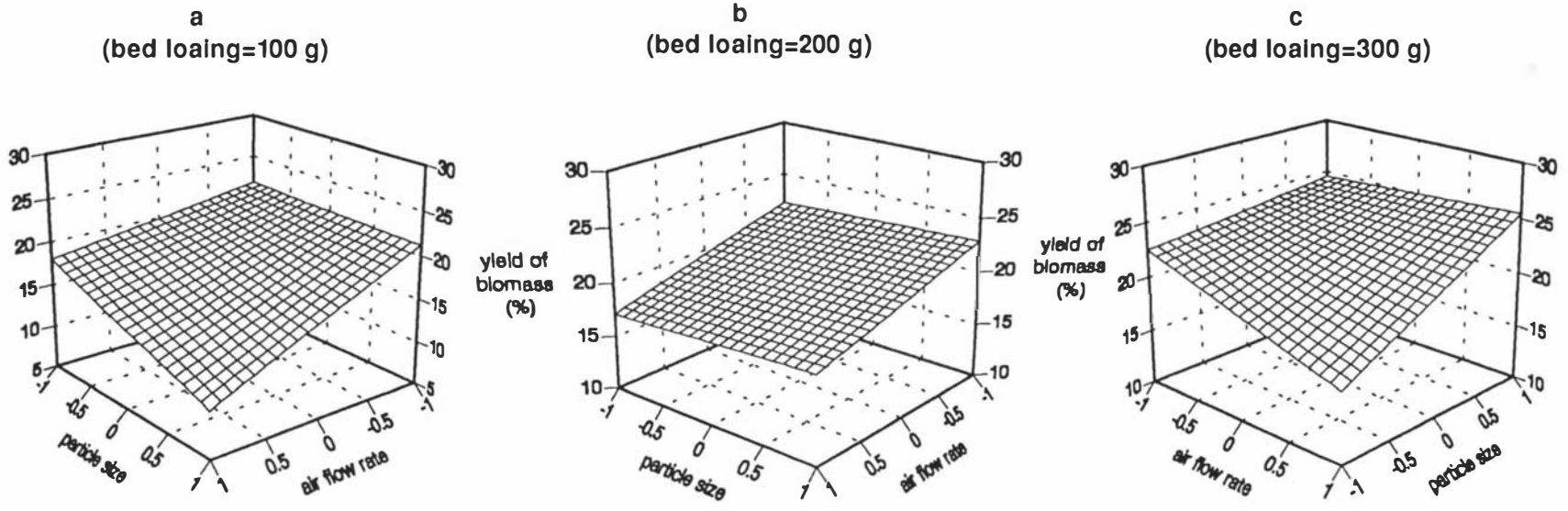


Figure 8.8 Response surfaces of biomass yield in packed bed reactor (keeping bed loading constant)

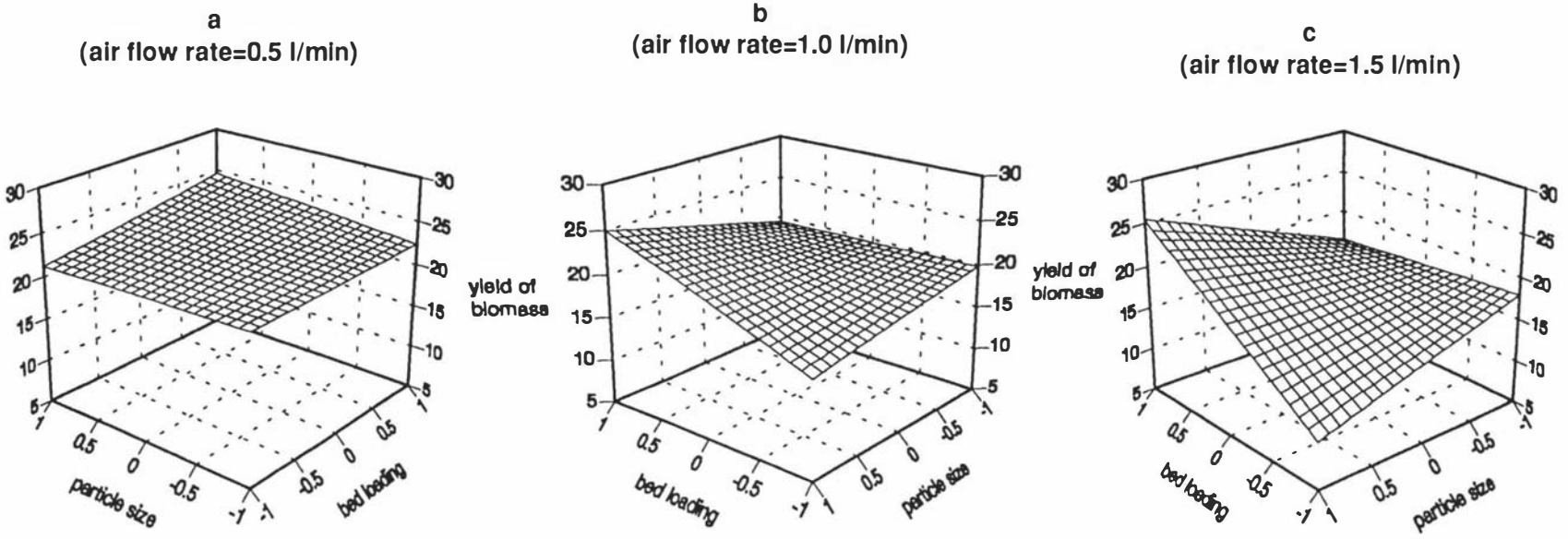


Figure 8.9 Response surfaces of biomass yield in packed bed reactor (keeping air flow rate constant)

The response surfaces for the biomass yield are shown in Figs 8.7, 8.8 and 8.9. Except for a few conditions, such as shown in Figs 8.7b, 8.7c, 8.8a and 8.9c, biomass yields ranging from 15 to 25% based on starch utilized, can be obtained. It appears that interactions among the three parameters strongly affected the biomass yield, and there is no dominant main effect among the three parameters. It confirms the interesting phenomenon in Section 8.3.1 that the combination of the conditions of large particle size with low bed loading and high air flow rate (Figs 8.7c, 8.8a, and 8.9c) favours a low biomass yield. However, conditions which favour a high biomass yield, such as high bed loading with high air flow rate and large particle size (Fig 8.9c) should also be avoided as they are likely to be detrimental to citric acid production because of diversion of starch (e.g. Fig 8.1c). Therefore, like the biomass concentration analysis above, a medium biomass yield will allow convenient selection of optimal conditions for citric acid production.

8.3.3 Estimated effects for carbon dioxide production

The role of CO_2 in the fermentation process for citric acid production has received little attention, and it is normally known as a respiratory product which accompanies sugar metabolism. In the context of the present study, CO_2 may be viewed as a product which takes a portion of the carbon source which may otherwise be converted to citric acid production. In addition, its presence in the substrate layer of the packed bed reactor can reduce the O_2 partial pressure and may adversely affect the O_2 distribution. Thus the purpose in the optimization process is to minimize CO_2 production, but not at the expense of citric acid yield.

The statistical analyses of CO_2 production both in amount, and yield based on starch utilized are shown in Tables 8.7 and 8.8, respectively. In Table 8.7, the regression for the amount of CO_2 is shown, but, although there is no significant lack of fit, the regression could not be fitted with only one factor, for reasons that are unclear However, including the bed loading, the regression was still significant at a 99.5% confidence level. The equation of the regression was used to create a response surface (as shown in Appendix I), which is presented in Fig 8.10. From this figure, the air flow rate had dominant effects on the amount of carbon dioxide produced, the amount decreasing considerably with increasing air flow rate. With reference to Section 8.3.1, this suggests that a good aeration can improve the conversion of starch from CO_2 to citric acid accumulation.

Table 8.7 Estimated effects for amount of carbon dioxide produced

Term	effect	coefficient	t-value	p
constant		80.1	25.2	0.000
AFR	-45.8	-22.9	-6.2	0.004
BL	-13.3	-6.6	-1.8	0.150

Analysis of variance

Main effects	DF	Seq SS	F	P
Main effects	2	4357	13.9	0.005
Residual error	6	1612		
curvature	1	163	0.8	0.404
lack of fit	5	1153	1.56	0.435
pure error	2	296		
Total	10	6149		

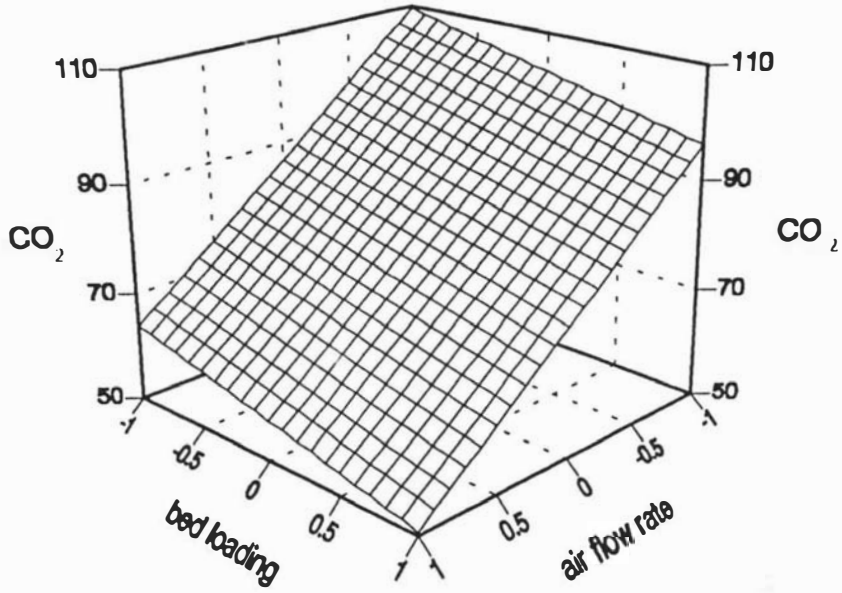
Table 8.8 Estimated effects for yield of carbon dioxide

Term	effect	coefficient	t-value	p
constant		91.8	295.7	0.000
AFR	-51.6	-25.8	-70.9	0.000
BL	-19.9	-10.0	-27.4	0.000
PS	10.4	5.2	14.3	0.000
BL*PS	9.9	4.9	13.6	0.000
AFR*BL*PS	25.7	12.5	34.4	0.000

Analysis of variance

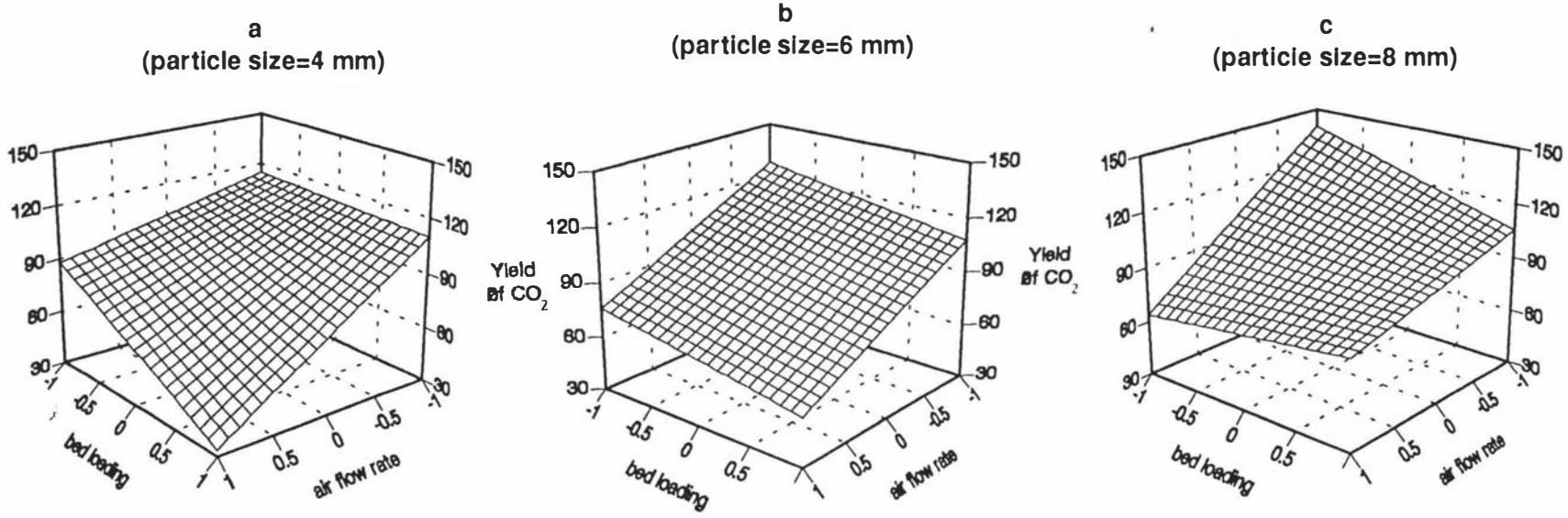
Main effects	DF	Seq SS	F	P
Main effects	3	6337	2000	0.000
2-way interactions	1	194	183.7	0.000
3-way interactions	1	1257	1000	0.000
Residual error	4	5.3		
curvature	1	0.1	0.1	0.790
lack of fit	2	2.4	0.9	0.542
pure error	2	2.8		
Total	10	7794		

Note: AFR, air flow rate; BL, bed loading; PS, particle size.



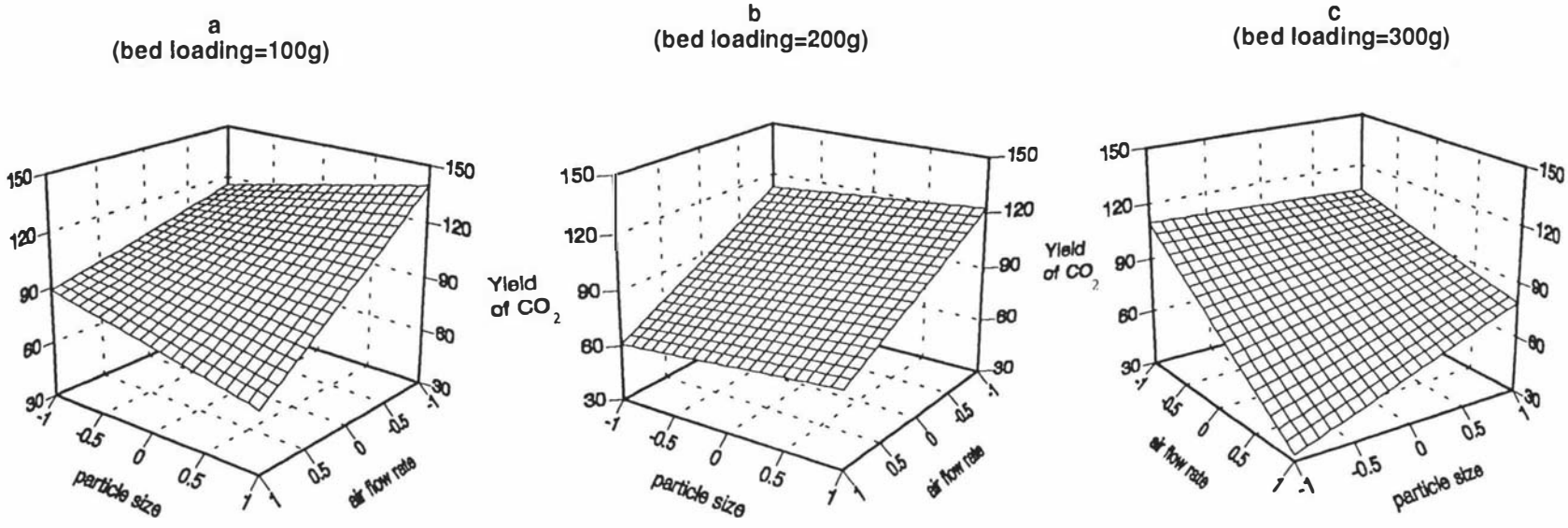
CO_2 Unit: ml 0.2 M NaOH/100 g substrate

Figure 8.10 Response surface of amount of carbon dioxide production in packed bed reactor



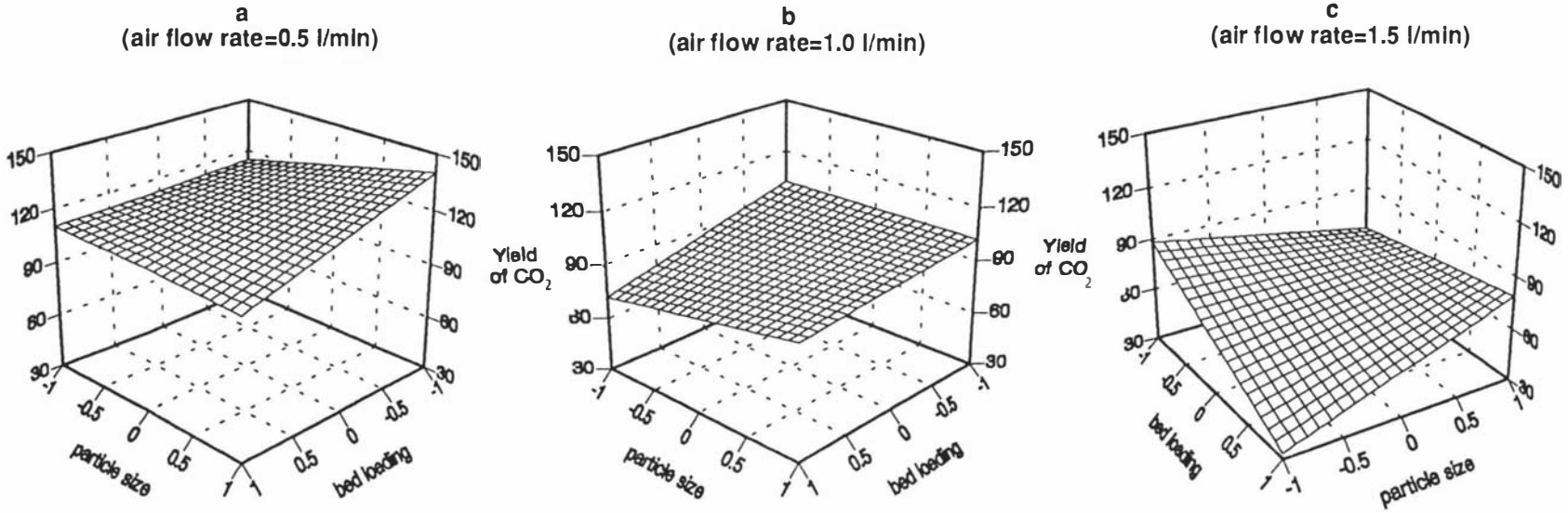
unit of the yield: ml 0.2 M NaOH/100 g starch utilised

Fgiure 8.11 Response surfaces of carbon dioxide yield in packed bed reactor (keeping particle size constant)



Unit of the yield : ml 0.2 M NaOH/100 g starch utilised.

Figure 8.12 Response surfaces of carbon dioxide yield in packed bed reactor (keeping bed loading constant)



unit of the yield: ml 0.2 M NaOH/100 g starch utilised.

Figure 8.13 Response surfaces of carbon dioxide yield in packed bed reactor (keeping air flow rate constant)

For the CO_2 yield, all of the coefficients listed in Table 8.8 were significant at the 100% level. All three main effects were significant, as well as the interactions between bed loading and particle size, and the interaction among the three main effects. The response surfaces are presented in Figs 8.11, 8.12 and 8.13.

From these figures, the lowest yields of CO_2 would be obtained under conditions of high air flow rate, with small particle size and high bed loading. Among the parameters, the air flow rate had the most pronounced effect on the yield of CO_2, as shown in Fig.8.13, the value obviously decreasing with increasing air flow rate. This effect is similar to that on the amount of CO_2 production.

8.3.4 Estimated effects for starch utilization

For each run of experiments in the packed bed reactor, different batches of kumara, which may have had different initial starch contents, were used. Thus, it was very difficult to maintain constant the initial starch content of the substrate. However, it has been shown previously (Section 5.2) that slight differences of initial starch content of the substrate have little effect on the fermentation. Thus, to investigate the conditions controlling starch utilization, only the percentage of starch utilized was analyzed. The statistical analysis is presented in Table 8.9. The regression equation showed no significant lack of fit. All of the coefficients in the equation were significant at the 97% or higher confidence level. The significant effects included all three main effects, and the interaction between bed loading and particle size. The equation is shown in Appendix I, while its response surfaces are presented in Figs 8.14, 8.15 and 8.16.

Under most conditions, the percentage of starch utilization ranged from 80% to 90%, and there was no dominant factor among the three parameters. The highest percentage of starch utilization, with a value of 92%, was observed under the conditions of small particle size, low air flow rate and low bed loading (Fig 8.14a). The lowest utilization, at a value of 78% was observed under conditions of high air flow rate, small particle size and high bed loading (Fig 8.16c). It is interesting that bed loading had little effect

Table 8.9 Estimated effects for percentage of starch utilised

Term	effect	coefficient	t-value	p
constant		85.1	210	0.000
AFR	-2.9	-1.4	-3.0	0.023
BL	-3.6	-1.8	3.8	0.009
PS	-2.8	-1.4	-2.9	0.027
BL*PS	3.6	1.8	3.8	0.009

Analysis of variance

Main effects	DF	Seq SS	F	P
Main effects	3	58.5	10.7	0.008
2-way interactions	1	26.8	14.8	0.009
Residual error	6	10.9		
curvature	1	5.8	5.6	0.064
lack of fit	3	2.9	0.9	0.571
pure error	2	2.2		
Total	10	96.2		

Note: AFR, air flow rate; BL, bed loading; PS, particle size

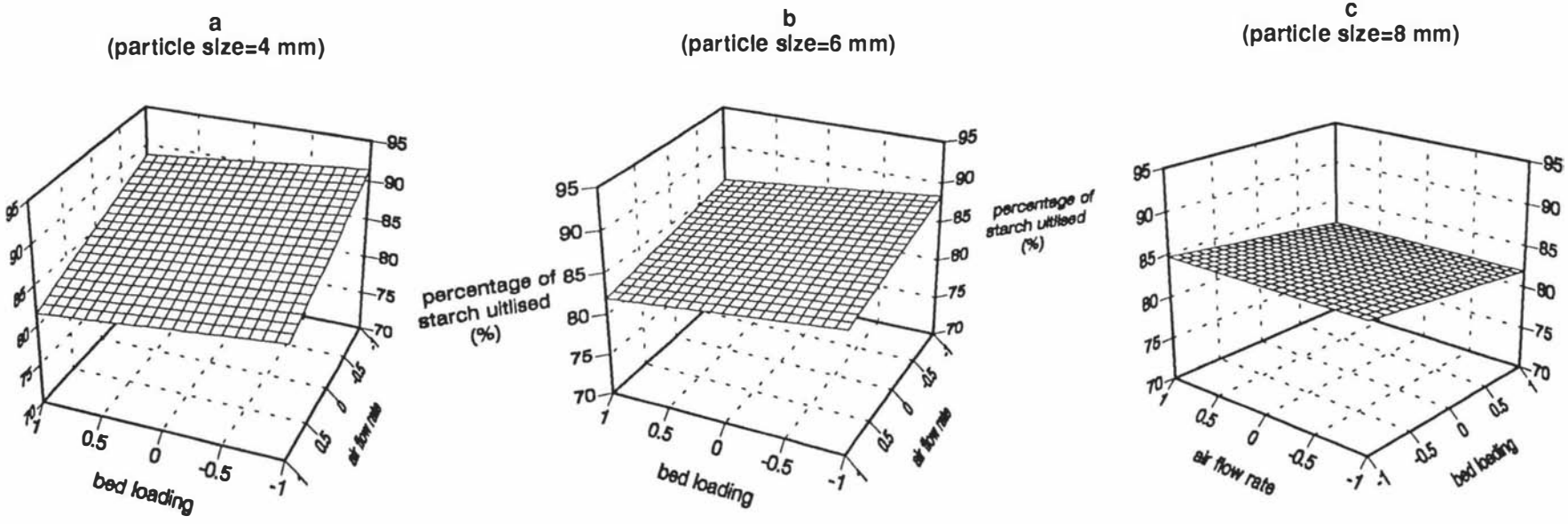


Figure 8.14 Response surfaces of percentage of starch utilized in packed bed reactor (keeping particle size constant)

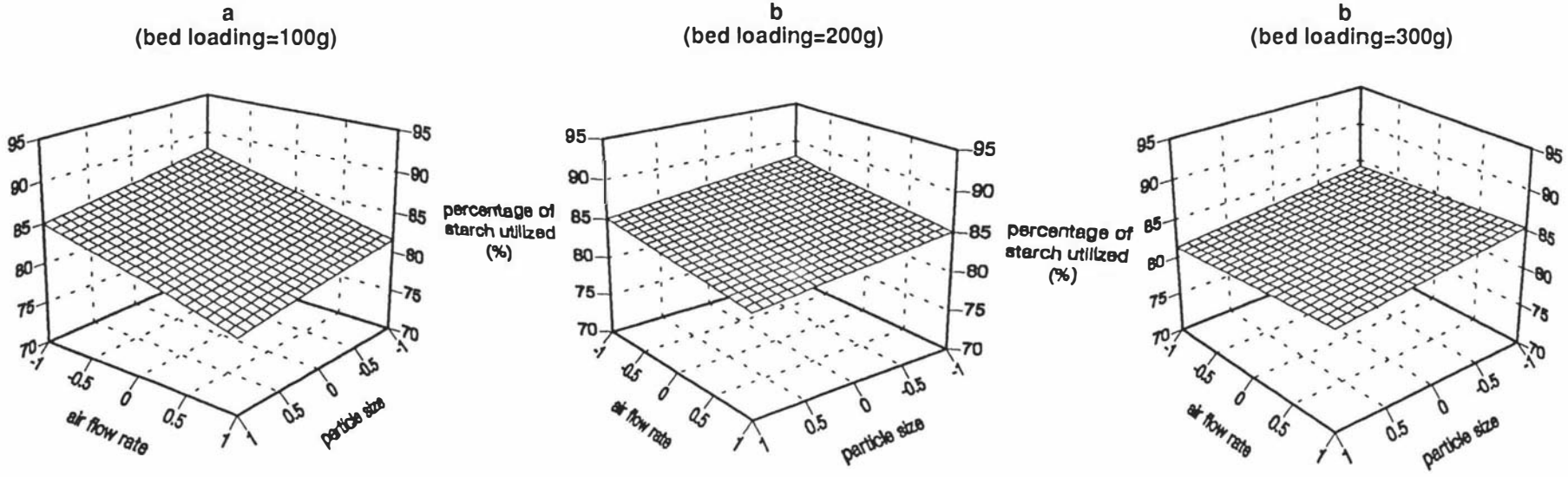


Figure 8.15 Response surfaces of carbon dioxide yield in packed bed reactor (keeping bed loading constant)

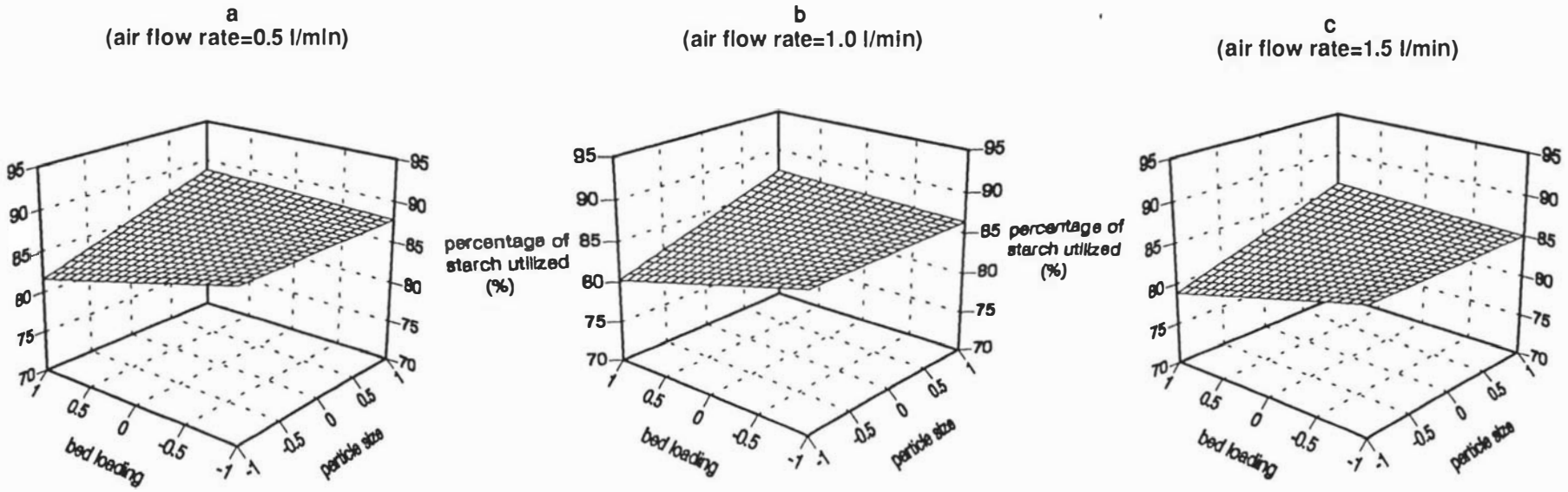


Figure 8.16 Response surfaces of percentage of starch utilized in packed bed reactor (keeping air flow rate constant)

on the starch utilization, as shown in Figs 8.15a, b and c, where the starch utilization percentage showed no obvious change when the bed loading increased from 100 g to 300 g. However, there were some changes caused by variations in air flow rate and particle size. This indicates that starch utilization can occur under different conditions, but it is not necessarily being converted to citric acid or biomass production. Possibly, it is being converted to carbon dioxide if conditions favour it.

8.4 Discussion and Conclusions

The fermentation can be affected by the air flow rate, the bed loading and the particle size of the substrate. Further, different particle sizes could provide different surface areas and porosity, which could subsequently lead to changes of morphology and metabolism of the organism. However, because of the restricted nature of the present investigation, it was very difficult to analyze the morphological and metabolic characteristics in a packed bed reactor, and to relate them directly to the air flow rate, bed loading and particle size. Thus, the aim was to focus empirically on higher citric acid production, and to minimize other products of starch utilization, such as biomass and CO_2 production. However, as pointed out in Section 6.4, the solid substrate fermentation process for citric acid production using kumara is a growth associated process, thus a reasonable biomass production is essential for citric acid production.

From the statistical analysis of citric acid production, the yield based on starch utilized showed significant lack of fit, which may imply that there are some other significant high order interactions among the parameters, which are not considered in this experimental design, as proposed by Maddox and Richert (1971) for the optimization of microbiological media. Alternatively, there may be other factors not included in the model. However, the maximum concentration could be obtained under two combinations of conditions, i.e. high air flow rate, low bed loading and small particle size; and low air flow rate, low bed loading and large particle size. Hence, it appears that conditions which favour the provision of air to the fungus can stimulate citric acid production,

which is similar to the high dissolved oxygen tension which is required in submerged fermentation (Dawson, 1986). The provision of low bed loading, which simply provides good aeration, was essential at all times. The particle size could affect citric acid production by providing good aeration rather than by providing a bigger surface area. Interestingly, the high air flow rate, although it could provide better aeration for the fermentation, had a negative effect on citric acid production under conditions of large particle size and low bed loading as described in Section 8.3.1 and 8.3.2. This is possibly due to the high air flow shear stress having a harmful effect on the filamentous organism, since under this condition, the lowest biomass production, both of concentration and yield based on starch utilized, was observed. Thus, the combination of high air flow rate and large particle size should be avoided in this packed bed reactor. When using the high air flow rate, the small particle size should be adopted, since this high air flow rate could improve the gaseous distribution in the packed substrate layer, and also result in less CO_2 production. The high bed loading, as discussed in Section 8.3.1, has a detrimental effect on citric acid production, and may act by adversely affecting the axial gaseous distribution. But with too low a bed loading, the advantage of the large capacity of the packed bed reactor will be lost. With reference to Tables 8.1 and 8.2, Run 2 (with conditions of low air flow rate, low bed loading and large particle size), resulted in the highest citric acid production both in concentration, at 104 g/kg, and in yield, at 70%. However, under this regime, the advantage of the packed bed reactor would be lost. Thus, this operating condition was not considered for further experiments.

Therefore, considering the above analysis of the three operating parameters, and the limitations of the equipment, an air flow rate of 1.5 l/min, a bed loading of 180 g and a particle size of 4 mm were selected for future experiments in a packed bed reactor. These conditions should allow reasonable fungal growth, starch utilization and citric acid production while minimizing carbon dioxide production. At that same time, the perceived advantages of the packed bed reactor, i.e. space saving and reactor productivity, can be exploited.

Chapter 9
Citric Acid Production in a Packed Bed Reactor

9.1 Introduction

In Chapter 8, it was demonstrated that the packed bed reactor was robust and suitable for solid substrate fermentation and its major operating parameters, including air flow rate, bed load and particle size, were characterized and optimized for citric acid production. Taking into account all previous experiments, including those in flasks and in the packed bed reactor, it is evident that this system could be used for an investigation of the kinetics and other characteristics, including gaseous concentrations and temperature gradients, and carbon balance, of solid substrate fermentation for citric acid production in a packed bed reactor. Data of this type are not currently available in the literature.

9.2 Kinetics of Solid Substrate Fermentation for Citric Acid Production in a Packed Bed Reactor

9.2.1 Introduction

Although the packed bed reactor has found widespread use in solid substrate fermentation, few descriptions of its kinetics have been found in the literature. Generally, this is because of the conflict between sample removal and continuous monitoring of the progress of fermentation. To allow continuous monitoring of the fermentation process, Carrizalez *et al* (1981) estimated the growth of *A. niger* in a packed bed reactor based on the CO_2 production data. Auria *et al* (1992) used air flow pressure drop through the substrate layer to estimate the growth of *A. niger* on an ion exchange resin impregnated with nutrients. This same technique was adopted by Gumbira-Sa'id *et al* (1993) to estimate the growth of *Rhizopus oligosporus* on sago-

beads. However, although it was able to monitor the progress of fermentation without interruption, this technique did not provide the true fermentation data, such as substrate utilization and biomass production. Particularly with regard to citric acid production, CO_2 production may somehow reflect the organism's respiration, but it may not have direct relationship with citric acid or fungal biomass production. Hang (1987) reported a study on citric acid production from apple pomace in a packed bed reactor, but neither mentioned the sampling procedure nor discussed the kinetics. Furthermore, the above mentioned investigations did not present any biomass data on the fermentations, possibly due to the difficulty of separation of biomass from substrate. However, the biomass data are necessary for the kinetic analysis of the fermentation.

Based on the previous investigation of the effect of initial moisture content of kumara on citric acid production (Section 5.6), which demonstrated that within the initial moisture content range from 65 to 71% there was little effect of moisture content on citric acid production, it was decided to use the "stop and sample" procedure to follow the progress of the fermentation in a packed bed reactor. By careful selection of kumara substrate for individual reactors, any variations could be minimized. In this way, the entire reactor/substrate was used as the sample for the analysis, and the effect of fermentation interruption could be eliminated, thus allowing the investigation of the kinetics of the solid substrate fermentation in a packed bed reactor. In addition, with reference to Table 8.2, the three replicates show that experiments in the packed bed reactor are reproducible. Therefore, it was considered unnecessary to duplicable each individual experimental data point.

9.2.2 Results and discussion

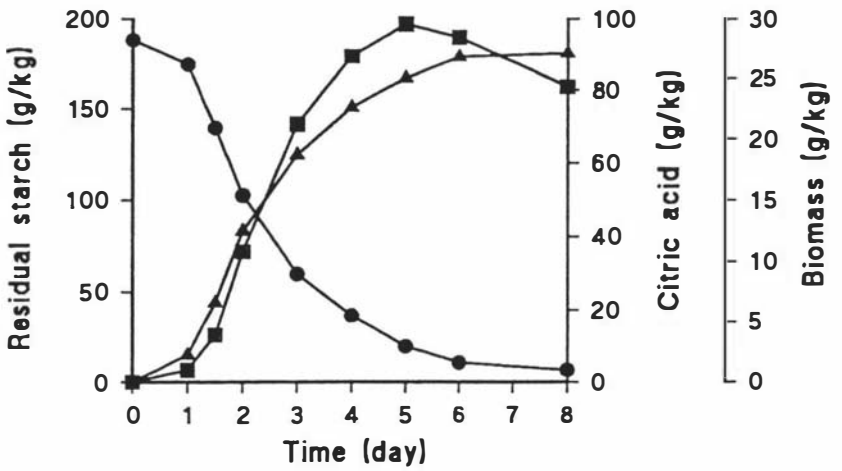
Experiments were conducted in the packed bed reactor system, which is depicted in Fig 3.7. As suggested in Chapter 8, operating conditions of 1.5 l/min of air flow rate, 180 g of bed load and 4 mm of particle size were used. An inoculum size of 10^4 to 10^6 spores per 40 g substrate was adopted, while the initial moisture content of the substrate

used ranged from 69 to 71%. To sample, each run was stopped at the end of a given time and the entire contents of the reactor were treated for the various analyses.

The time course of the process is shown in Fig 9.1. Citric acid accumulation commenced after day 1 and increased until day 5, at which time the concentration was 99 g/kg. As was observed in flasks (Section 6.2), but which now occurred earlier, the citric acid concentration declined after 90% of the starch had been utilized. Biomass production initially increased more rapidly than it had in the flask experiments (Section 6.2), possibly due to the considerably larger surface area and better aeration condition in the packed bed reactor. However, although biomass production continued increasing throughout the 8 day fermentation period, its final concentration of 27 g/kg, representing a 15% yield based on starch utilized, was lower than the 38 g/kg with a yield of 20%, observed in a period of 10 days fermentation in flasks. This suggests that in the packed bed reactor the limited space inhibited the production of the biomass and the grown biomass severely deteriorated the aeration, although the early good aeration condition allowed good initial growth rates.

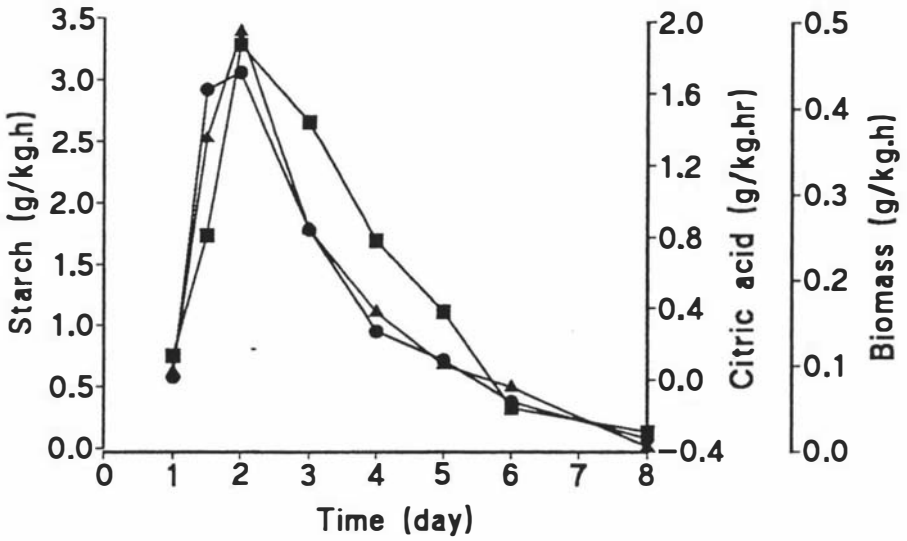
Figs 9.2 and 9.3 show gravimetric and specific rate data, respectively, which are calculated from the data plotted in Fig 9.1 using the same methods as described in Section 6.2. The rate of citric acid production increased rapidly during the early fermentation period, but after day 2 it decreased quickly, and had a negative value after day 5, indicating that citric acid was utilized by the organism to maintain its energy requirement. The maximum citric acid production rates, both gravimetric and specific, were achieved on day 2, with values of 1.9 g/kg.h and 150 mg/g biomass.h, respectively. The maximum biomass production rates, both gravimetric and specific, were also observed on day 2, with values of 0.49 g/kg.h and 0.055 h^{-1}, respectively. For starch utilization, similar to that in flasks, the maximum gravimetric rate was maintained for a period, but in the packed bed reactor, this maximum occurred earlier, beginning on day 1.5, and lasted for a shorter time (only 0.5 day). In flasks, the maximum rate began on day 2, and lasted for 1 day.

Figure 9.1 Time course of citric acid fermentation in packed bed reactor



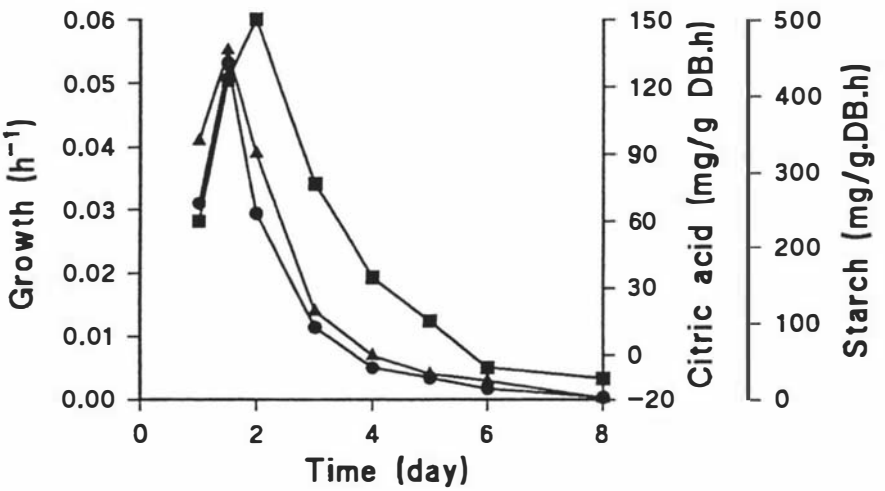
Symbols:● starch, ■ citric acid, ▲ biomass; Unit:g/kg wet kumara

Figure 9.2 Gravimetric rates of citric acid fermentation in packed bed reactor



Symbols:● starch, ■ citric acid, ▲ biomass

Figure 9.3 Specific rates of citric acid fermentation in packed bed reactor



Symbols:● starch, ■ citric acid, ▲ biomass

It is interesting to compare the kinetics of the fermentation in the packed bed reactor with those in flasks, as shown in Table 9.1. For the concentrations, fungal biomass production was much lower in the packed bed reactor than in flasks, while citric acid production was slightly higher. This indicates that the physical conditions in the packed bed reactor served to retard fungal growth, thus allowing more starch to be converted to citric acid. However, since the yields, based on starch utilized, of both fungal biomass and citric acid were lower in the packed bed reactor than in flasks, it appears that carbon dioxide production may be favoured in this reactor. Since little quantity of metabolic products other than citric acid were detected by the HPLC, it is assumed that carbon dioxide is the only other product of metabolism. Another major difference between the packed bed reactor and the flasks is seen in the initial reaction rates. Thus, starch utilization, fungal biomass production and citric acid production rates generally reached higher values earlier in the fermentation, with the result that the overall reactor productivity at the point of maximum citric acid concentration was effectively double in the packed bed reactor compared with the flasks. This phenomenon is probably due to the fact that in the packed bed reactor, the forced aeration system allowed rapid initial fungal growth, starch utilization, and citric acid production. Subsequently, however, due to limited space within the substrate/reactor, fungal growth slowed, and the starch was preferentially converted to citric acid and carbon dioxide. Observation of the packed bed reactor during its operation, particularly during the later period, revealed that the spaces among the substrate particles became filled with mycelium, causing some blockage. This blockage is reflected in Fig 9.4, which shows the pressure drop (ΔP) of air flowing through the substrate layer, represented by mm Hg. The ΔP increased quickly during the early period of fermentation and, after day 2, reached a value of 65 mm Hg which lasted until the end of fermentation. Thus, beginning at that time, the substrate layer became blocked and so there was less space in which further biomass growth could occur. Corresponding to the blockage, there was an increased possibility that channelling of the air flow might increase, leading to deterioration of aeration inside the substrate layer.

Table 9.1 Comparison of fermentations in packed bed reactor and flasks

Term	Unit	In packed bed reactor	In flasks
Highest citric acid concentration	g/kg	99	91
Time of occurrence	day	5	8
Highest yield of citric acid	%	60	64
Time of occurrence	day	4	3
Highest biomass concentration	g/kg	27	37
Time of occurrence	day	8	10
Highest yield of biomass	%	16	26
Time of occurrence	day	1	4
Overall starch utilization rate Period	g/kg.h day	0.94 8	0.79 10
Overall citric acid productivity Period	g/kg.h day	0.82 5	0.42 8
Overall biomass productivity Period	g/kg.h day	0.14 8	0.15 10
Maximum observed gravimetric rate of starch utilization	g/kg.h	3.1	1.6
Time of occurrence	day	2	3
Maximum observed gravimetric rate of citric acid production	g/kg.h	1.9	1.5
Time of occurrence	day	2	3
Maximum observed gravimetric rate of biomass production	g/kg.h	0.49	0.53
Time of occurrence	day	2	4
Maximum observed specific rate of starch utilization	mg/g biomass.h	443	255
Time of occurrence	day	1.5	1
Maximum specific biomass growth rate	h^{-1}	0.055	0.04
Time of occurrence	day	1.5	1
Maximum specific rate of citric acid production	mg/g biomass.h	150	122
Time of occurrence	day	2	2

Figure 9.4 Air flow pressure drop along the substrate layer in packed bed reactor

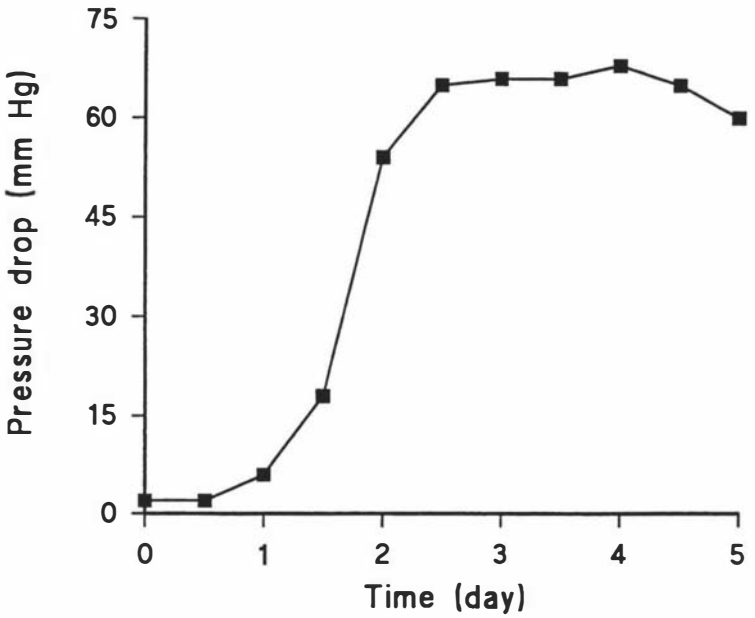
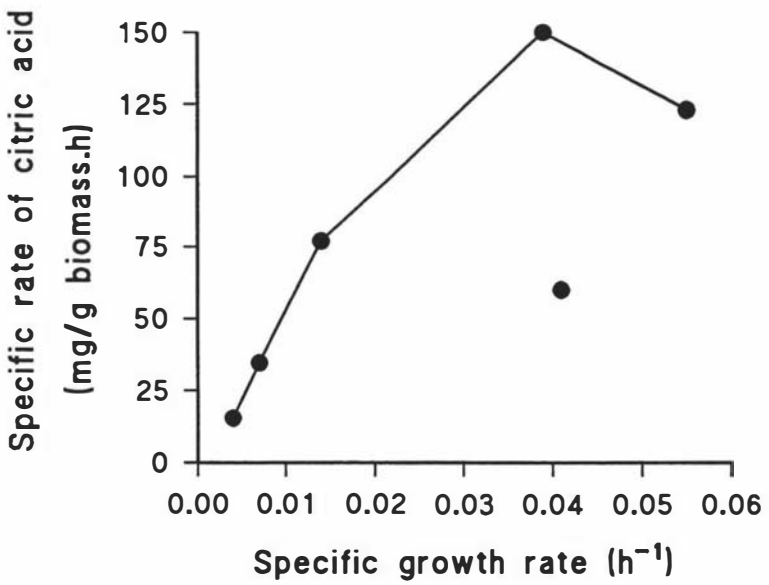


Figure 9.5 Plot of specific rate of citric acid production versus specific growth rate of fermentation in packed bed reactor



As pointed out in Chapter 6, the solid substrate fermentation in flasks was a growth-associated process. For the packed bed reactor, Fig 9.5 shows the plot of the specific rate of citric acid production (before citric acid production decreased) versus specific growth rate. Similar to the situation in flasks, citric acid production in the packed bed reactor was a growth-associated process. However, as described in Section 6.3, the value of the point(0.041, 60) on day 1 in Fig 9.5 was lower than it should be, probably due to the inclusion of the lag time for the organism germination.

9.3 Axial Concentration Gradients within the Substrate Layer in the Packed Bed Reactor

9.3.1 Introduction

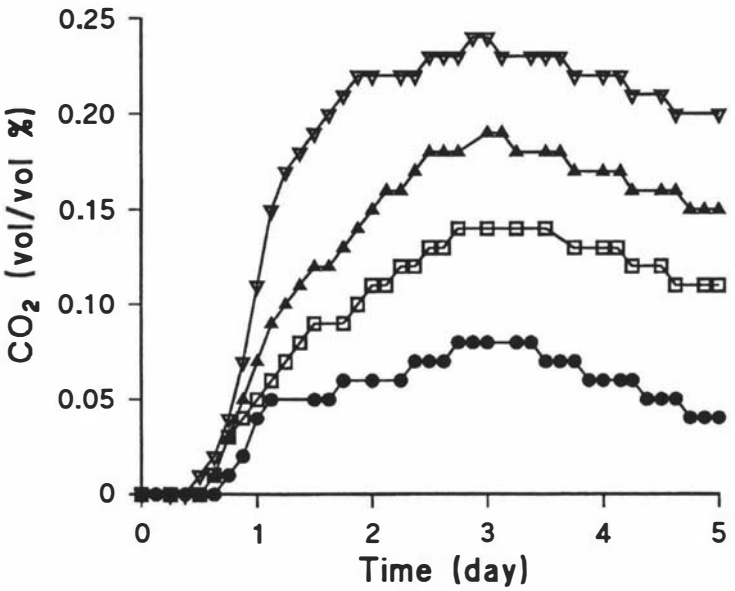
The packed bed reactor, as generally known, has many potential advantages, the most pronounced one of which is its large capacity of bed load. However, with bed load increasing, the axial concentration gradients, including those of O_2, CO_2 and temperature, developed rigorously, could significantly affect the fermentation process. Many reports are available concerning these gradients in solid substrate fermentations (Finger *et al*, 1976; Mudgett, 1980; Auria *et al*, 1992). Gowthaman *et al* (1993) studying the growth of *Rhizopus oligosporus* on wheatbran, reported that steep O_2 and CO_2 gradients existed in the wheatbran layer of a packed bed reactor and, associated with steep temperature gradients (Ghidyal *et al*, 1994), these subsequently adversely affected the progress of the fermentation. Rathbun and Schuler (1983) reported a temperature gradient of 3°C per cm in the axial direction during a soya bean fermentation, while in a composting solid substrate fermentation, the centre temperature reached as high as 60 to 70°C (Mitchell *et al*, 1992). Temperature gradients, if sufficiently steep, could severely inhibit the progress of a fermentation and effective and expensive methods, such as cool water jackets, must be used to remove the released heat. However, these methods may not be feasible because most substrates used in solid substrate fermentation have a poor conductivity.

Therefore, an understanding of gaseous and temperature gradients in the substrate layer, and their effects on the solid substrate fermentation process for citric acid production in a packed bed reactor, are very meaningful. These data have not been reported previously in the literature. In addition, the carbon balance for a fermentation is very meaningful for examining the carbon consumption, and it has been applied widely to submerged fermentation processes. In solid substrate fermentation, probably due to the complicated composition of the substrate and the difficulty of biomass measurement, no such carbon balance has been made. In the present experiments, equipped with an appropriate reactor and analysis equipment, it was possible to measure the carbon balance of the solid substrate fermentation process for citric acid production.

9.3.2 Results and discussion

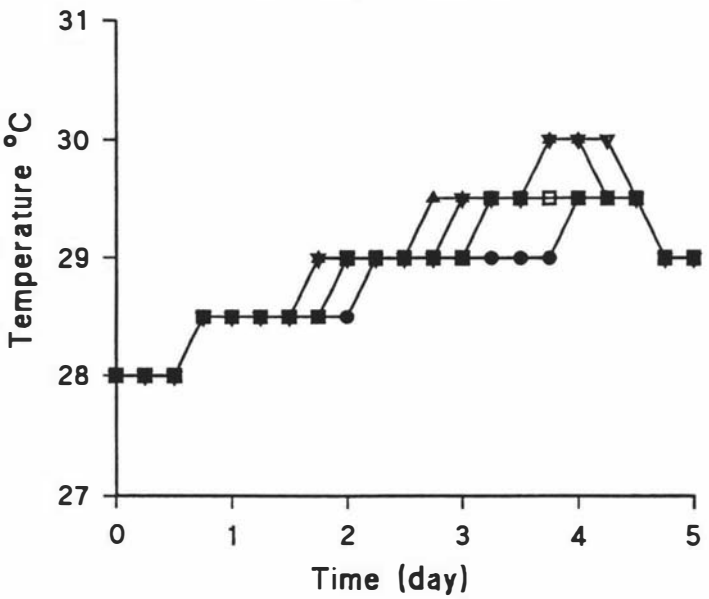
Experiments were carried out in a four-layer packed bed reactor system, as shown in Fig 3.10, of which the reactor (Fig 3.9) was modified based on the packed bed reactor shown in Fig 3.8, and which allowed the measurement of CO_2 above each layer. In each layer, there were 100 g substrate, with an initial moisture content range of 69 to 71% , a particle size of 4 mm and an inoculum size of 10^4 to 10^6 spores per 40 g substrate. To compensate for the total substrate increasing from 180 g to 400 g, the air flow rate was increased from 1.5 l/min to 1.8 l/min. A gas sample (0.2 l/min) was withdrawn from the sampling port of each layer, and after the analysis, the gas sample was re-humidified and fed back to the reactor. In this way, the air flow rate to the next layer of substrate was not decreased. Because the O_2 analyzer within a short response time requires a large gas sample, which may interfere the packed bed aeration, thus O_2 concentration of each layer was not be analyzed. At the end of each run, each layer of substrate was withdrawn from the reactor, and was analyzed separately. Thus the axial concentration gradients of CO_2, temperature, starch, biomass and citric acid were obtained, and their time profiles are presented in Figs 9.6, 9.7, 9.8, 9.9 and 9.10, respectively. Fig 9.11 shows a morphological observation after 5 days fermentation in the multi-layer packed bed reactor.

Figure 9.6 Time profiles of CO_2 production in multi–layer packed bed reactor



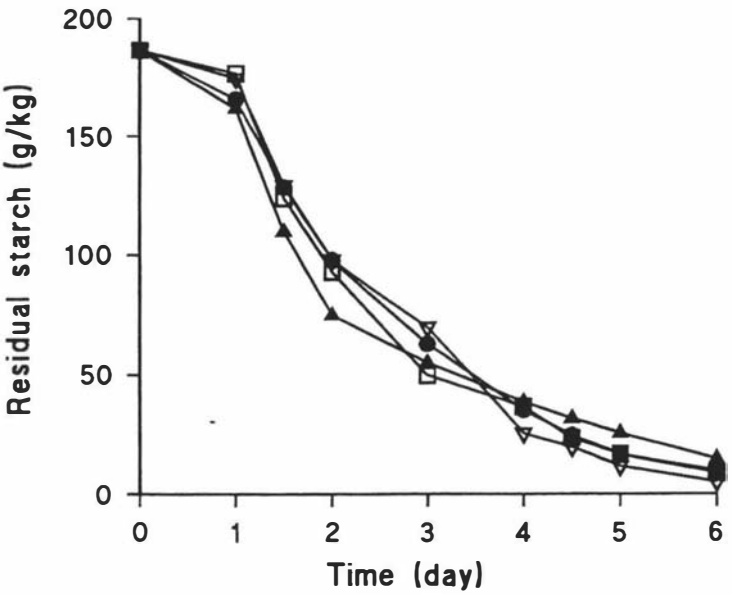
Symbols:● 1st layer, □ 2nd layer, ▲ 3rd layer, ▽ 4th layer; 4x100 g substrate

Figure 9.7 Time profiles of temperature in multi–layer packed bed reactor



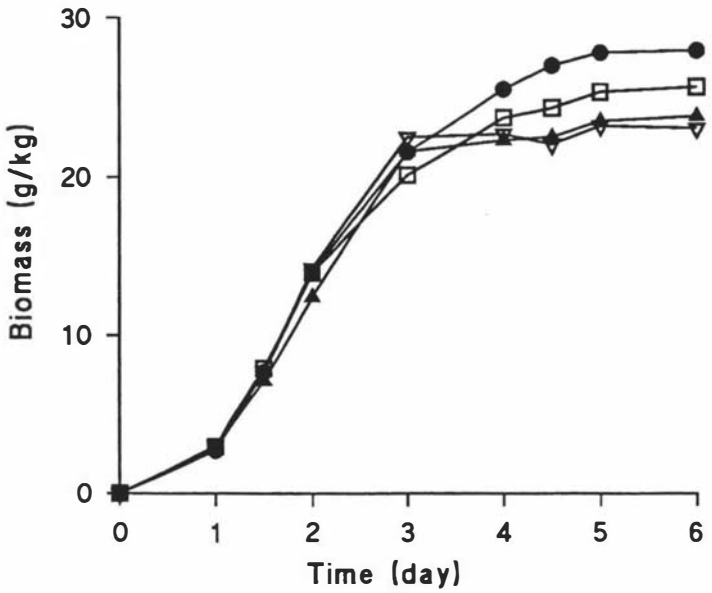
Symbols:● 1st layer, □ 2nd layer, ▲ 3rd layer, ▽ 4th layer; 4x100 g substrate

Figure 9.8 Time profiles of starch utilization in multi-layer packed bed reactor



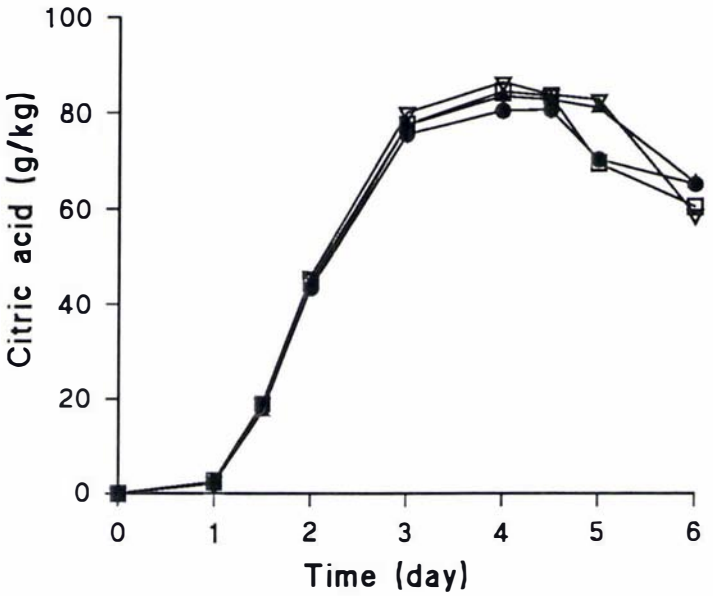
Symbols:● 1st layer, □ 2nd layer, ▲ 3rd layer, ▽ 4th layer; 4x100 g substrate

Figure 9.9 Time profiles of biomass production in multi-layer packed bed reactor



Symbols:● 1st layer, □ 2nd layer, ▲ 3rd layer, ▽ 4th layer; 4x100 g sustrate

Figure 9.10 Time profiles of citric acid production in multi–layer packed bed reactor



Symbols:● 1st layer, □ 2nd layer, ▲ 3rd layer, ▽ 4th layer; 4x100 g substrate

Figure 9.11 Morphological observation of the fermentation in a multi-layer packed bed reactor (5 days fermentation)

From Fig 9.6, CO_2 was detected after 18 to 20 hours fermentation and its production increased quickly during following 2 days. The highest CO_2 concentration in all layers was observed on day 3, at which point there was an average gradient between each layer of 0.05%. Generally, in microorganism reactions, CO_2 production directly correlates the O_2 consumption for respiration of the organism. Thus, in response to the CO_2 gradients, there could be O_2 gradients between the substrate layers.

Fig 9.7 shows the temperature profiles of each layer, which displayed no major temperature difference among all the layers. This is possibly due to the heat loss which occurred during withdrawal and analysis of gas samples and during air flow along the unpacked region above each layer, as can be seen in Fig 9.11.

From Fig 9.8, there was no obvious difference observed in starch utilization among the layers. For fungal biomass production, as shown in Fig 9.9, 3rd and 4th layer showed little further increase after day 3, while 1st and 2nd layer showed increases throughout the entire period. Thus, a fungal biomass gradient was observed among the top three substrate layers, but the gradient disappeared between the 3rd and 4th layer. For citric acid production, slight gradients were observed among the layers after 4 days of fermentation, but the differences were only small (Fig 9.10).

From Fig 9.11, there was no obvious morphological differences among the layers.

In summary, carbon dioxide production by the fungus during its growth led to the development of gradients within the reactor. However, the carbon dioxide gradient (and, presumably, the oxygen gradient) had no significant effect on either starch utilization or citric acid production throughout the different layers of the reactor. Biomass production, however, was slightly less in the bottom layers of the reactor.

Based on the data shown in Figs 9.6, 9.8 9.9 and 9.10, the rate data of carbon dioxide production, starch utilization, citric acid production and biomass production were

calculated. The results for each layer are shown in Figs 9.12, 9.13, 9.14 and 9.15. The rates, other than for carbon dioxide, are all similar to those reported in Section 9.2. The rates of carbon dioxide production, although they showed some irregularities, most of them showed the highest values between days 1 and 2, which corresponds to the maximum rates for the other parameters.

From the data of the multi-layer packed bed reactor, the carbon balance of each layer was calculated, using the following formulation:

$$\Delta C_{citric\ acid} + \Delta C_{biomass} + \Delta C_{CO_2} = \Delta C_{starch}$$

The assumption of this formulation and calculation of each item are shown in Appendix II. The data for the calculations were taken, after 4 days of fermentation, from Figs 9.6, 9.8, 9.9 and 9.10 for carbon dioxide, starch, biomass and citric acid, respectively. Assumptions and calculations are shown in Appendix II. The carbon balance for each layer is shown in Table 9.2. The percentage of the carbon utilized which was recovered in the products, including carbon dioxide, citric acid and biomass, was 87.8% in 1st layer , 84.0% in 2nd layer, 78.5% in 3rd layer and 85.7% in 4th layer. The average value for the whole reactor was 84.0%. Of the carbon accounted for, citric acid represented 36.6% in layer 1, 39.5% in layer 2, 39.2 in layer 3 and 38.1% in layer 4. Carbon dioxide represented the second largest carbon utilization, while fungal biomass took the least carbon utilization. With reference to the fact that only 84.0% of the carbon utilized was recovered, the following reasons may be suggested:

a) dissolved carbon dioxide in liquid falling to bottom of the column.

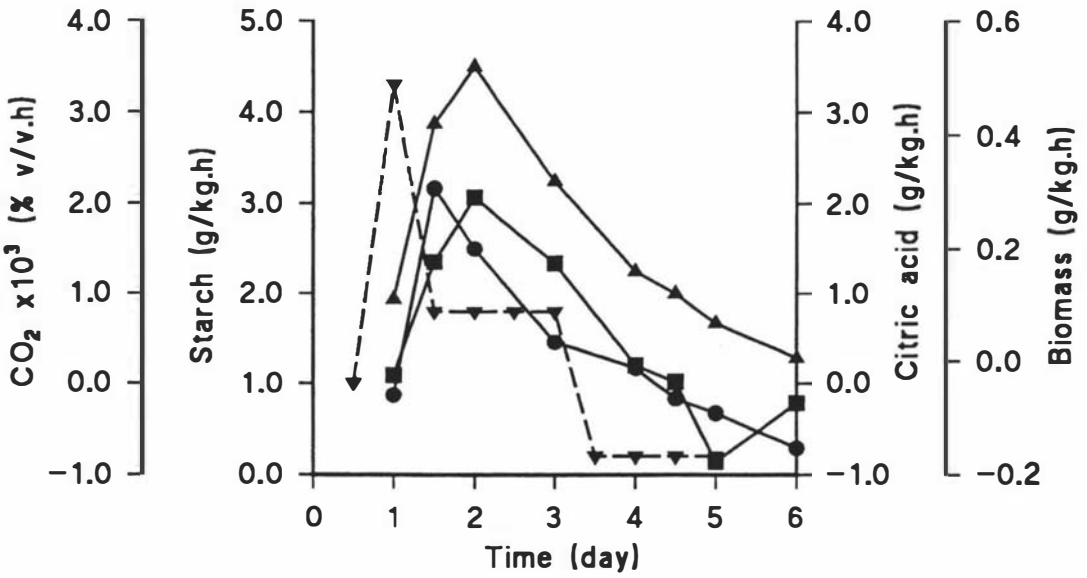
b) loss of spores in the air flow, since some spores were observed in the collector (Fig 3.10), particularly during the later fermentation period.

c) loss of spores during the fungal biomass analysis procedure (As described in Section 3.5.5).

d) other unaccounted carbon containing products.

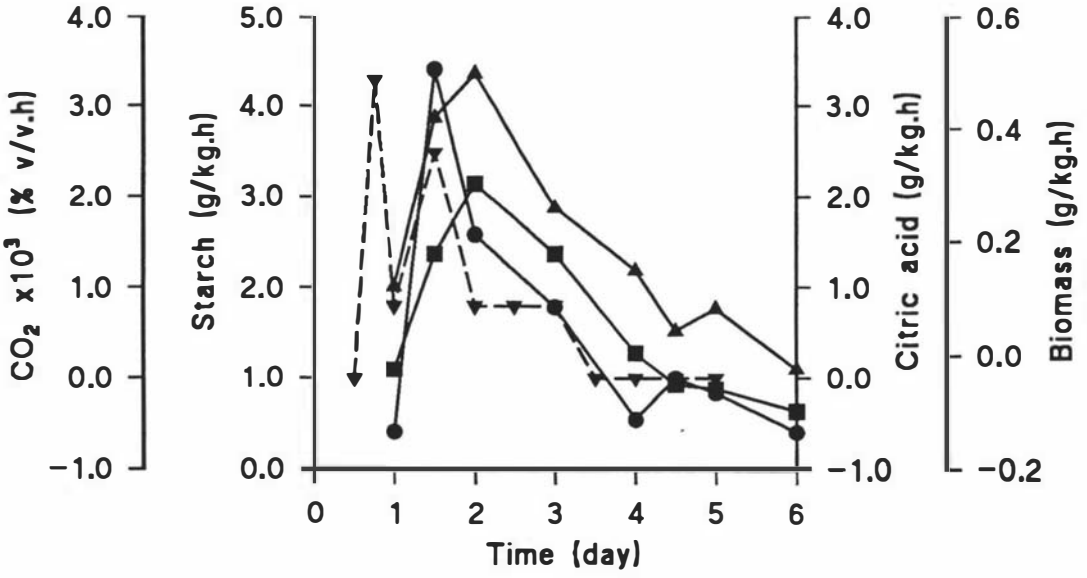
However, considering the complexity and initial stage of understanding of the solid substrate fermentation, an 84% carbon balance may be considered acceptable.

Figure 9.12 Gravimetric rates of Layer 1 in multi-layer packed bed reactor



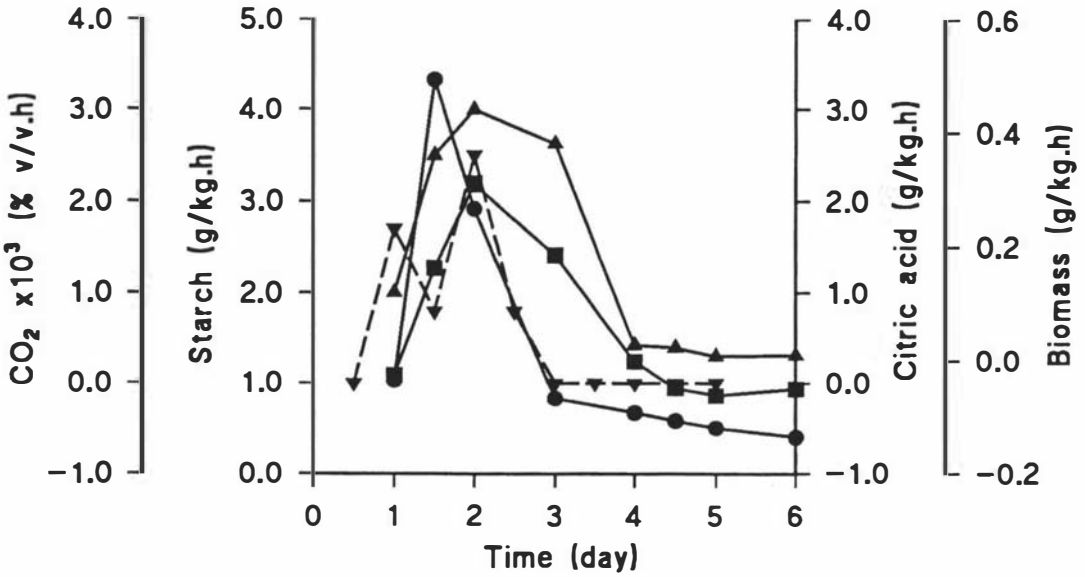
Symbols:● starch, ■ citric acid, ▲ biomass, ▼ CO_2

Figure 9.13 Gravimetric rates of Layer 2 in multi-layer packed bed reactor



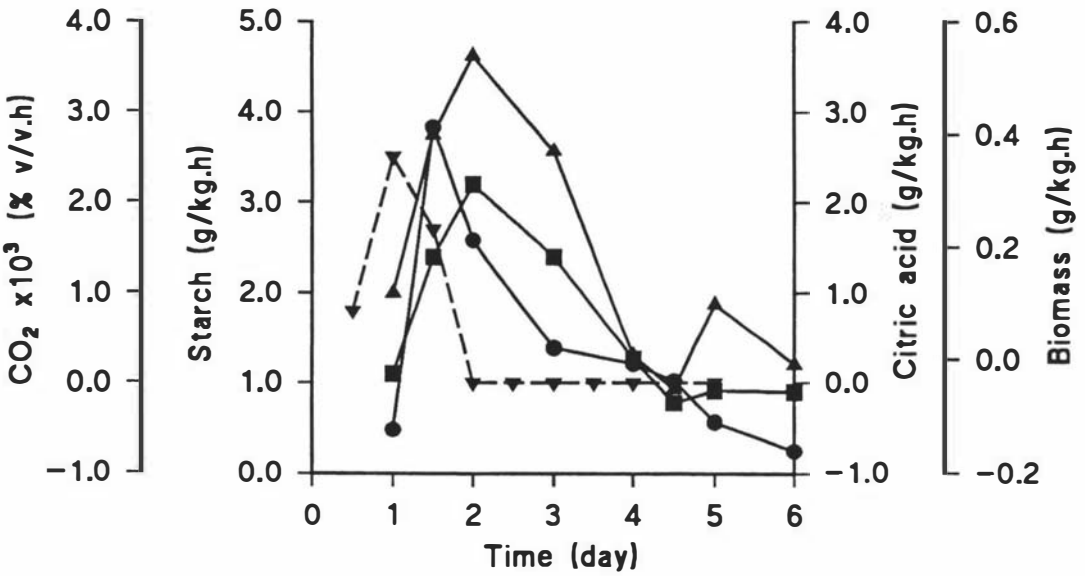
Symbols:● starch, ■ citric acid, ▲ biomass, ▼ CO_2

Figure 9.14 Gravimetric rates of Layer 3 in multi-layer packed bed reactor



Symbols:● starch, ■ citric acid, ▲ biomass, ▼ CO_2

Figure 9.15 Gravimetric rates of Layer 4 in multi-layer packed bed reactor



Symbols:● starch, ■ citric acid, ▲ biomass, ▼ CO_2

Table 9.2 Carbon balance of the fermentation in a multi-layer packed bed reactor (4 days fermentation)

Term		1st L	2nd L	3rd L	4th L
Starch utilized	g	15.2	15.0	14.8	15.7
Carbon content	%, g/g	53.7	53.7	53.7	53.7
Carbon utilized	**g**	**8.2**	**8.1**	**7.9**	**8.4**
Biomass production	g	2.6	2.4	2.2	2.3
Carbon content	%, g/g	64.7	64.4	67.3	65.8
Carbon in biomass	**g**	**1.7**	**1.5**	**1.5**	**1.5**
Citric acid production	g	8.0	8.4	8.3	8.6
Carbon content	%, g/g	37.5	37.5	37.5	37.5
Carbon in citric acid	**g**	**3.0**	**3.2**	**3.1**	**3.2**
CO_2 production	g	9.2	7.8	5.8	9.0
Carbon content	%, g/g	27.3	27.3	27.3	27.3
Carbon in CO_2	**g**	**2.5**	**2.1**	**1.6**	**2.5**
Sum of carbon except starch	g	7.2	6.8	6.2	7.1
$\frac{\text{Sum of carbon}}{\text{Carbon utilized}}$ x100	%	**87.8**	**84.0**	**78.5**	**85.7**

Note:L, layer.

9.4 Experiments Comparing the Multi-layer Packed Bed Reactor with Single Packed Bed Reactor

As pointed out in Fig 9.7, the empty regions in the multi-layer packed bed reactor and the sampling procedure for gas analysis could affect the temperature within the various substrate layers. To examine these effects, experiments were conducted in a single layer packed bed reactor with a bed loading of 400 g. This was comparable to the multi-layer packed bed reactor, but without the empty regions or the between-layer gas analysis system. All the other operating parameters were the same as in the multi-layer packed bed reactor (as described in Section 9.3), while gas analysis was reformed at the exit of the reactor and used same equipment as in the multi-layer packed bed reactor. For sampling, the entire reactor was sacrificed. It should be noted that according to the results obtained in Chapter 8, a reactor with this high bed loading should not favour citric acid production, so the experiment was also used to confirm the greater effectiveness of the multi-layer reactor for the same mass of substrate. Duplicate experiments were performed, and the temperature was monitored at selected points within the bed.

Figs 9.16 and 9.17 show the gas and temperature profiles during the fermentations, while Table 9.3 shows the other results. The O_2 consumption, as expected, had an opposite trend to CO_2 production, the lowest O_2 concentration being observed on day 3 at a value of 20.7%, corresponding to the highest CO_2 production. According to the O_2 profiles, with a value of not less than 20.6% at the exit, the aeration condition in the packed bed reactor should satisfy the fermentation requirements. However, citric acid production in these experiments, at an average concentration of 42 g/kg and a yield of 23%, based on starch utilized, was much lower than that in the multi-layer packed bed reactor, with an average concentration of 75 g/kg and yield of 54%, at the same fermentation time, indicating the advantages of the multi-layer packed bed reactor over the single packed bed reactor (as predicted in Chapter 8). To explain this, it is necessary to understand the gas transfer in a packed bed reactor for solid substrate fermentation. Mitchell *et al* (1992) pointed out that the O_2 transfer process consists of several steps:

a) Transfer of O_2 into interparticles space.

b) Diffusion of O_2 across stagnant gas films at the substrate.

c) Transfer of O_2 across the interface into the liquid film at the substrate surface.

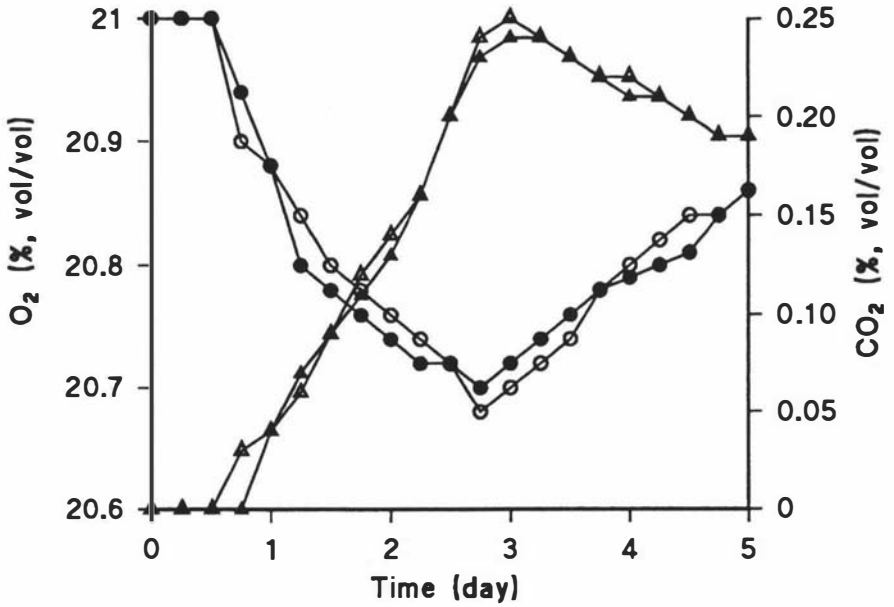
d) Uptake of O_2 by organism.

e) Diffusion of O_2 into the substrate particle itself.

An increased aeration rate can improve only the first two steps, i.e. in the present experiments, it could improve the axial and radial gas transfer in the packed bed reactor. On the other hand, the improvement of aeration condition in axial and radial directions of the substrate may not efficiently improve the oxygen transfer to the organism. As pointed out by Mudgett (1986) that oxygen transfer in solid substrate fermentation is controlled by the diffusion across the liquid film on the substrate surface, the c) step, and this diffusion can be affected by the interfacial surface area and oxygen partial pressures. Auria *et al* (1992) found that mass diffusion in a packed bed reactor deteriorated as the biomass concentration increased. The estimation of oxygen transfer across the liquid film is not yet achievable in solid substrate fermentation due to the complexity of this measurement (Andre, *et al*, 1981; Durand *et al*, 1988). Furthermore, the channelling which existed within the packed substrate may considerably offset the gaseous transfer. This can be demonstrated by the comparison of the fermentations performed in the single layer packed bed reactor and in the multi-layer packed bed reactor. In the multi-layer packed bed reactor, the smaller packed layer could considerably reduce the risk of channelling. Because, after each layer, the gas was re-distributed, therefore, even though channelling existed in some of the packed layers, it would not affect the following layers. This analysis is based only on oxygen transfer; if carbon dioxide transfer was taken into account, the gas transfer would deteriorate further owning to lower partial oxygen pressure inside the packed substrate. Therefore, the multi-layer packed bed reactor can reduce the gas flow channelling which otherwise then improve the gaseous transfer.

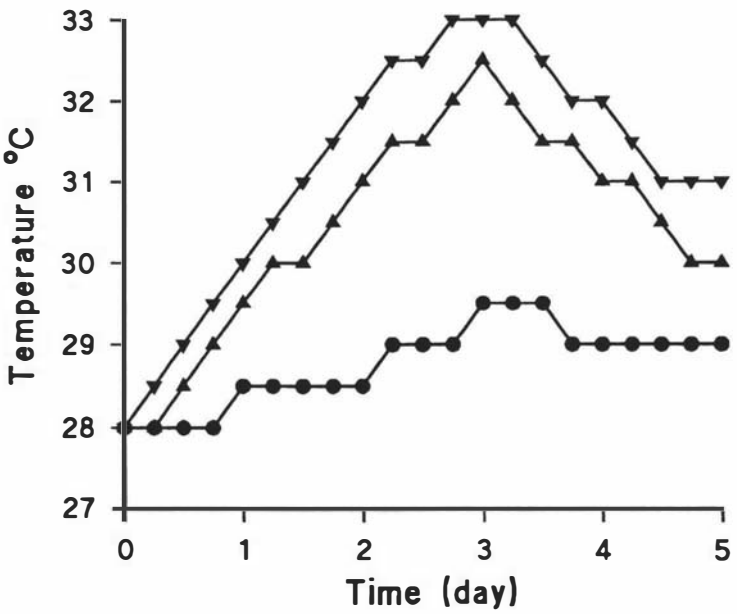
In addition, from the results of the multi-layer fermenter experiments, as described in Section 9.3, where there were no obvious citric acid or biomass gradients observed among

Figure 9.16 Time profiles of O_2 and CO_2 in a single packed bed reactor with 400 g bed loading



Symbols:● O_2 of Run 1, ○ O_2 of Run 2; ▲ CO_2 of Run 1, △ CO_2 of Run 2

Figure 9.17 Time profiles of temperature in a single packed bed reactor with 400 g bed loading



Symbos:● 15 mm depth, ▲ 60 mm depth, ▼ 105 mm depth

Table 9.3 Fermentations in a single layer packed bed reactor with 400 g bed loading (5 days fermentation)

Term	Unit	Run 1	Run 2
Initial starch	(g/kg)	211.8	211.8
Residual starch	(g/kg)	19.0	33.0
Percentage of starch utilized	(%)	91.0	84.4
Citric acid	(g/kg)	41.8	43.5
Yield of citric acid based on starch utilized	(%)	21.7	24.3
Biomass	(g/kg)	27.0	23.5
Yield of biomass based on starch utilized	(%)	14.0	13.1

the substrate layers, and where sufficiently high oxygen and very low carbon dioxide concentrations were present, it could prove that the solid substrate fermentation process is controlled by gas transfer through interfacial and intra-particles rather than through axial and radial transfer. However, a complete understanding of the details of gas transfer inside the substrate layers in a packed bed reactor is too huge a task for the present work.

As shown in Fig 9.17, the temperature profiles changed as the fermentation progressed. The maximum temperatures occurred on day 3, corresponding to the lowest oxygen and the highest carbon dioxide concentrations. The temperature gradients were not evenly distributed along the axial direction; the highest gradient, with a value of 2°C per 45 mm, was achieved in bottom part of the reactor, but only 0.5°C per 45 mm was observed in the top part. The highest temperature was detected at 105 mm depth of the packed substrate, at a value of 33°C, which is much lower than that of other solid substrate fermentations (Rathbun and Schuler, 1985; Mitchell *et al*, 1992). This implies that this particular solid substrate fermentation is a low heat-releasing process, and therefore the temperature increase is not a significant factor in this scale of packed bed reactor. However, Aidoo *et al* (1982) reported that citric acid production in solid substrate fermentations could generated 540-570 kcal/kg substrate heat which imply that high temperature may exist in the packed substrate. The maximum temperature before any adverse effects on citric acid production would be observed is about 35°C (Kapoor *et al*, 1982)

9.5 Conclusions

The conclusions that can be drawn from this chapter are:

1. Citric acid production in the packed bed reactor was superior to that in flasks, mainly in the much higher rates observed, and in the doubling of the overall productivity of citric acid. However, blockage of the system by biomass production could be a problem.
2. There were carbon dioxide and oxygen concentration and temperature gradients

within the substrate layer. The high risk of channelling in the packed bed reactor could have vital effects on gas transfer, leading to inhibition of citric acid production.

3. The multi-layer packed bed reactor, with a high bed loading, could considerably reduce the risk of channelling, and consequently gave improved citric acid production.

4. The carbon balance of the solid substrate fermentation for citric acid production in the multi-layer packed bed reactor was approximately 84.0% on average.

5. The solid substrate fermentation for citric acid production was a low heat-releasing fermentation. Thus temperature in this scale of the packed reactor is not a significant factor.

Chapter 10

Final Discussion and Conclusions

The major aim of this work was to investigate solid substrate fermentation for citric acid production using *Aspergillus niger*, to provide understanding of this process and to develop an appropriate reactor for the fermentation. Discussion and conclusions were made in each chapter, so the detailed information contained in those chapters will not repeated here.

Experiments were commenced using flasks to select an appropriate substrate and organism for citric acid production in solid substrate fermentation. Kumara was found to be an excellent substrate, using *A. niger* strain Yang No.2. Compared with other solid substrates, e.g. apple pomace (Hang and Woodams, 1984) and wheatbran (Shankaranand and Lonsane, 1994), kumara has many advantages, such as the achievement of a relative homogeneous condition, the ease of processing, and more importantly, the ease of separation of biomass from the substrate. The latter paved the way for further investigations with the support of biomass data, a condition that has been lacking in past solid substrate fermentations for citric acid production, and is still an obstacle in general solid substrate fermentations (Desgranges *et al*, 1991). Experiments were performed on the effects of some nutrients on the fermentation process and the nitrogen content of the substrate was found to be critical for citric acid production. As in submerged fermentation, nitrogen limitation appears to be a prerequisite for product accumulation. Optimal conditions, such as inoculum size, moisture content and particle size, were determined for citric acid production, and it was ascertained that the process had a high tolerance to any adverse effect of metal ions. This is in contrast to submerged fermentation processes. An initial study on the kinetics of the process provided a comparable base with other fermentation technologies, although the understanding of the kinetics was still rudimentary.

Subsequent experiments were performed to select an appropriate reactor for the solid substrate fermentation. A packed bed reactor proved to be more suitable than other types, such as a gas-solid fluidized bed, a gas-liquid-solid fluidized bed and a rotating drum reactor. A factorial design experiment was adopted to optimize the packed bed reactor with regard to the parameters of air flow rate, bed loading and substrate particle size. This experimental technique was found useful and convenient, particularly as it could provide analysis data for interactions among the parameters. The kinetic study in the packed bed reactor indicated that rates of both citric acid production and starch utilization were much higher than in flasks, due to the forced aeration and larger available surface area. Although heat transfer may be a problem in solid substrate fermentations, it was found that the process for citric acid production presented few problem in this regard. Mass transfer in solid substrate fermentation is very complicated, and our present understanding of this aspect is limited (André *et al*, 1981; Mudgett, 1986; Murthy *et al*, 1993). Thus, detailed analysis or measurement of mass transfer was not conducted in this work. However, the multi-layer packed bed reactor which was developed allowed accurate measurement of gas, citric acid, biomass, and starch gradients, and mass transfer was improved over that of a conventional packed bed reactor with the same bed loading. In addition, a corresponding improvement in citric acid production was observed. Thus, the multi-layer packed bed reactor may provide an excellent environment for further investigations of mass transfer and has the possibility for large scale solid substrate fermentation.

With comparison to other technologies, higher yields and concentrations of citric acid have been claimed by using solid substrate fermentations, e.g. Lakshminarayana *et al* (1975) using a tray reactor and Hang (1988) using a packed bed reactor. However, a comparison based on kinetic data is still not available in the literature. The present work allowed a comparison with other technologies based on kinetic data. Besides the above advantages, particularly when using the packed bed reactor, considerably higher productivity, gravimetric and specific rates were observed compared with other fermentation technologies.

In conclusion, solid substrate fermentation for citric acid production has many potential advantages over other fermentation technologies, and application of this fermentation in a packed bed reactor, particularly in the multi-layer packed bed reactor, was successfully achieved. These systematic investigations of the solid substrate fermentation provide a solid base and direction for further investigations, and have not been found in the literature.

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Appendix 1

Processing of Regression Equations to Create Response Surfaces

The general model of the regression is:

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{123}x_1x_2x_3$$

Where: Y=the value of the variable being regressed

a_0=constant

a_{ij}=coefficients, i and j=0, 1, 2 and 3

x_1=coded value of air flow rate

x_2=coded value of bed loading

x_3=coded value of particle size

1) Equation Processing for Citric Acid Production

a) For citric acid concentration, regression equation is:

$$Y = 71.4 + 5.9x_1 - 11.5x_2 + 3.1x_1x_2 - 9.1x_1x_3 + 10x_1x_2x_3 \quad ---(1)$$

Keeping x_3 constant,

When

$$x_3 = -1, \quad Y = 71.4 + 15x_1 - 11.5x_2 - 6.9x_1x_2 \quad ---(2)$$

$$x_3 = 0, \quad Y = 71.4 + 5.9x_1 - 11.5x_2 + 3.1x_1x_2 \quad ---(3)$$

$$x_3 = +1, \quad Y = 71.4 - 3.2x_1 - 11.5x_2 + 13.1x_1x_2 \quad ---(4)$$

Equations 2, 3 and 4 were used to create response surfaces as shown in Fig 8.1.

Keeping x_2 constant,

When

$$x_2 = -1, \qquad Y = 82.9 + 2.8x_1 - 19.1x_1x_3 \qquad ---(5)$$

$$x_2 = 0, \qquad Y = 71.4 + 5.9x_1 - 9.1x_1x_3 \qquad ---(6)$$

$$x_2 = +1, \qquad Y = 59.9 - 9.0x_1 - 0.9x_1x_3 \qquad ---(7)$$

Equations 5, 6 and 7 were used to create response surfaces as shown in Fig 8.2.

Keeping x_1 constant,

When

$$x_1 = -1, \qquad Y = 65.5 - 14.6x_2 + 9.1x_3 - 10x_2x_3 \qquad ---(8)$$

$$x_1 = 0, \qquad Y = 71.4 - 11.5x_2 \qquad ---(9)$$

$$x_1 = +1, \qquad Y = 77.3 - 8.4x_2 - 9.1x_3 + 10x_2x_3 \qquad ---(10)$$

Equations 8, 9 and 10 were used to create response surfaces as shown in Fig 8.3.

2) Equation Processing for Biomass Production

a) For biomass concentration

its regression equation is:

$$Y = 29.6 - 4.8x_1 + 1.3x_2 - 1.7x_3 + 4.1x_2x_3 + 3.0x_1x_2x_3 \qquad ---(11)$$

Keeping x_3 constant,

When

$$x_3 = -1, \qquad Y = 31.3 - 4.8x_1 - 2.8x_2 - 3.0x_1x_2 \qquad ---(12)$$

$$x_3 = 0, \qquad Y = 29.6 - 4.8x_1 + 1.3x_2 \qquad ---(13)$$

$$x_3 = +1, \qquad Y = 27.9 - 4.8x_1 + 5.4x_2 + 3x_1x_2 \qquad ---(14)$$

Equations 12, 13 and 14 were used to create response surfaces as shown in Fig 8.4.

Keeping x_2 constant,

When

$$x_2 = -1, \qquad Y = 28.3 - 4.8x_1 - 5.8x_3 - 3.0x_1x_3 \qquad ---(15)$$

$$x_2 = 0, \qquad Y = 29.6 - 4.8x_1 - 1.7x_3 \qquad ---(16)$$

$$x_2 = +1, \qquad Y = 30.9 - 4.8x_1 + 2.4x_3 + 3.0x_1x_3 \qquad ---(17)$$

Equations 15, 16 and 17 were used to create response surfaces as shown in Fig 8.5.

Keeping x_1 constant,

When

$$x_1 = -1, \qquad Y = 34.4 + 1.3x_2 - 1.7x_3 + 1.1x_2x_3 \qquad ---(18)$$

$$x_1 = 0, \qquad Y = 29.6 + 1.3x_2 - 1.7x_3 + 4.1x_2x_3 \qquad ---(19)$$

$$x_1 = +1, \qquad Y = 24.8 + 1.3x_2 - 1.7x_3 + 7.1x_2x_3 \qquad ---(20)$$

Equations 18, 19 and 20 were used to create response surfaces as shown in Fig 8.6.

b) For biomass yield

its regression equation is:

$$Y = 20 - 2.5x_1 + 2.2x_2 + 0.4x_3 + 1.2x_1x_2 + 2.5x_2x_3 + 1.8x_1x_2x_3 \qquad ---(21)$$

Keeping x_3 constant,

When

$$x_3 = -1, \qquad Y = 19.6 - 2.5x_1 - 0.3x_2 - 0.6x_1x_2 \qquad ---(22)$$

$$x_3 = 0, \qquad Y = 20 - 2.5x_1 + 0.4x_2 \qquad ---(23)$$

$$x_3 = +1, \qquad Y = 22.2 - 1.3x_1 + 2.9x_2 + 1.8x_1x_2 \qquad ---(24)$$

Equations 22, 23 and 24 were used to create response surfaces as shown in Fig 8.7.

Keeping x_2 constant,

When

$$x_2 = -1, \qquad Y = 17.8 - 3.7x_1 - 2.1x_3 - 1.8x_1x_3 \qquad ---(25)$$

$$x_2 = 0, \qquad Y = 20 - 2.5x_1 + 0.4x_3 \qquad ---(26)$$

$$x_2 = +1, \qquad Y = 22.2 - 1.3x_1 + 2.9x_3 + 1.8x_1x_3 \qquad ---(27)$$

Equations 25, 26 and 27 were used to create response surfaces as shown in Fig 8.8.

Keeping x_1 constant,

When

$$x_1 = -1, \qquad Y = 22.5 + x_2 + 0.4x_3 + 0.7x_2x_3 \qquad ---(28)$$

$$x_1 = 0, \qquad Y = 20 + 2.2x_2 + 0.4x_3 + 2.5x_2x_3 \qquad ---(29)$$

$$x_1 = +1, \qquad Y = 17.5 + 3.4x_2 + 0.4x_3 + 4.3x_2x_3 \qquad ---(30)$$

Equations 28, 29 and 30 were used to create response surfaces as shown in Fig 8.9.

3) Equation Processing for Carbon Dioxide Production

a) For amount of carbon dioxide production

its regression equation is:

$$Y = 80.1 - 22.9x_1 - 6.6x_2 \qquad (31)$$

Equation 31 was used directly to create Fig 8.10.

b) For carbon dioxide yield

Its equation is:

$$Y = 91.8 - 25.8x_1 - 10x_2 + 5.2x_3 + 4.9x_2x_3 + 12.5x_1x_2x_3 \qquad ---(32)$$

Keeping x_3 constant,

When

$$x_3 = -1, \qquad Y = 86.6 - 25.8x_1 - 14.9x_2 - 12.5x_1x_2 \qquad ---(33)$$

$$x_3 = 0, \qquad Y = 91.8 - 25.8x_1 - 10x_2 \qquad ---(34)$$

$$x_3 = +1, \qquad Y = 97 - 25.8x_1 - 5.1x_2 + 12.5x_1x_2 \qquad ---(35)$$

Equations 33, 34 and 35 were used to create response surfaces as shown in Fig 8.11.

Keeping x_2 constant,

When

$$x_2 = -1, \qquad Y = 101.8 - 25.8x_1 - 0.3x_3 - 12.5x_1x_3 \qquad ---(36)$$

$$x_2 = 0, \qquad Y = 91.8 - 25.8x_1 + 5.2x_3 \qquad ---(37)$$

$$x_2 = +1, \qquad Y = 81.8 - 25.8x_1 + 10.1x_3 + 12.5x_1x_3 \qquad ---(38)$$

Equations 36, 37 and 38 were used to create response surfaces as shown in Fig 8.12.

Keeping x_1 constant,

When

$$x_1 = -1, \qquad Y = 117.6 - 10x_2 + 5.2x_3 - 7.6x_2x_3 \qquad ---(39)$$

$$x_1 = 0, \qquad Y = 91.8 - 10x_2 + 5.2x_3 - 7.6x_2x_3 \qquad ---(40)$$

$$x_1 = +1, \qquad Y = 66 - 10x_2 + 5.2x_3 + 17.4x_2x_3 \qquad ---(41)$$

Equations 39, 40 and 41 were used to create response surfaces as shown in Fig 8.13.

4) Equation processing for percentage of starch utilization

The regression equation is:

$$Y = 85.1 - 1.4x_1 - 1.8x_2 - 1.4x_3 + 1.8x_2x_3 \quad ---(42)$$

Keeping x_3 constant,

When

$$x_3 = -1, \quad Y = 86.5 - 1.4x_1 - 3.6x_2 \quad ---(43)$$

$$x_3 = 0, \quad Y = 85.1 - 1.4x_1 - 1.8x_2 \quad ---(44)$$

$$x_3 = +1, \quad Y = 83.7 - 1.4x_1 \quad ---(45)$$

Equations 43, 44 and 45 were used to create response surfaces as shown in Fig 8.14.

Keeping x_2 constant,

When

$$x_2 = -1, \quad Y = 86.9 - 1.4x_1 - 3.2x_3 \quad ---(46)$$

$$x_2 = 0, \quad Y = 85.1 - 1.4x_1 - 1.4x_3 \quad ---(47)$$

$$x_2 = +1, \quad Y = 83.3 - 1.4x_1 + 0.6x_3 \quad ---(48)$$

Equations 46, 47 and 48 were used to create response surfaces as shown in Fig 8.15.

Keeping x_1 constant,

When

$$x_1 = -1, \qquad Y = 86.5 - 1.8x_2 - 1.4x_3 + 1.8x_2x_3 \qquad ---(49)$$

$$x_1 = 0, \qquad Y = 85.1 - 1.8x_2 - 1.4x_3 + 1{,}8x_2x_3 \qquad ---(50)$$

$$x_1 = 0, \qquad Y = 83.7 - 1.8x_2 - 1.4x_3 + 1{,}8x_2x_3 \qquad ---(51)$$

Equations 49, 50 and 51 were used to create response surfaces as shown in Fig 8.16.

Appendix 2

Calculation of Carbon Balance

Table 1 Original data of kumara and fermented solids in multi-layer packed bed reactor (after 4 days fermentation)

Kumara basic parameters		
Dry matter	(%)	27.6
Its carbon content	(%)	53.6
NHM	(%)	2.1
Its carbon content	(%)	52.6

Fermentation original data					
Item		1st L	2nd L	3rd L	4th L
Dry fermented solids	(g)	4.7	4.4	4.3	4.4
Its carbon content	(%)	59.3	57.6	60.1	59.4
Biomass concentration	(g/kg)	25.5	23.7	22.3	22.7
Amount of biomass	(g)	2.6	2.4	2.2	2.3
Initial starch concentration	(g/kg)	186.8	186.8	186.8	186.8
Residual starch concentration	(g/kg)	35.0	37.0	39.0	30.0
Amount of starch utilized	(g)	15.2	15.0	14.8	15.7
Citric acid concentration	(g/kg)	80.4	84.3	83.0	86.2
Amount of citric acid	(g)	8.0	8.4	8.3	8.6
Average air flow rate	(l/min)	1.8	1.8	1.8	1.8

Note: The dry fermented solids were produced from 100 g wet kumara during 4 days fermentation.
L, layer.

The original data of the fermentation are shown in Table 1, which will be used for the further calculations.

Calculations are based on the formula:

$$\Delta C_{citric\ acid} + \Delta C_{biomass} + \Delta C_{co_2} = \Delta C_{starch}$$

The following assumptions are made:

1. Starch is the only carbon source used by the organism.
2. Citric acid, biomass and CO_2 are only the products produced during the fermentation.

1. Calculation of ΔC_{starch}

Defining:

Starch as S	(g)
its carbon content as C_s	(%)
Solid of kumara as S_k, S_k=27.6%×100=27.6	(g)
its carbon content as C_k, C_k=53.6	(%)
NHM=2.1%×100=2.1.	(g)
its carbon content as C_{NHM}, C_{NHM}=52.6	(%)

Under assumptions:

1. The dry matter of kumara consists of starch and NHM only, S_k=S + NHM
2. Starch is the only fermented carbon source utilized
3. NHM is not changed during the fermentation process

Therefore, carbon balance of dry matter of kumara is:

$$S_k \times C_k = NHM \times C_{NHM} + C_s \times S$$

Then

$$C_s = \frac{S_k \times C_k - NHM \times C_{NHM}}{S_k - NHM} = \frac{27.6 \times 0.536 - 2.1 \times 0.526}{27.6 - 2.1} = 53.7\%$$

Referring to Table 1,

Starch utilized in Layer 1, ΔS_1=15.2	(g)
Starch utilized in Layer 2, ΔS_2=15.0	(g)
Starch utilized in Layer 3, ΔS_3=14.8	(g)
Starch utilized in Layer 4, ΔS_4=15.7	(g)

Therefore,

Layer 1, carbon utilized, $\Delta C_{starch}=\Delta S_1 \times C_s=15.2 \times 53.7\%=8.2$ (g)

Layer 2, carbon utilized, $\Delta C_{starch}=\Delta S_2 \times C_s=15.0 \times 53.7\%=8.1$ (g)

Layer 3, carbon utilized, $\Delta C_{starch}=\Delta S_3 \times C_s=14.8 \times 53.7\%=7.9$ (g)

Layer 4, carbon utilized, $\Delta C_{starch}=\Delta S_4 \times C_s=15.7 \times 53.7\%=8.4$ (g)

2. Calculation of $\Delta C_{biomass}$

Assumption: The dry fermented solid consists of NHM and biomass only

Defining:

Dry fermented solid as FS, its carbon content as C_{FS}

Biomass as BM, its carbon content as C_{BM}

Therefore,

$$FS = NHM + BM$$

And

$$FS \times C_{FS} = BM \times C_{BM} + NHM \times C_{NHM}$$

Then, for Layer 1,

$$C_{BM_1} = \frac{FS_1 \times C_{FS_1} - NHM \times C_{NHM}}{BM_1} = \frac{FS_1 \times C_{FS_1} - NHM \times C_{NHM}}{FS_1 - NHM}$$

Referring to Table 1 for FS_1, FS_2, C_{FS1}, C_{FS2}, NHM and C_{NHM}, then

$$C_{BM_1} = \frac{4.7 \times 0.593 - 2.1 \times 0.526}{4.7 - 2.1} = 64.7\%$$

Then for Layer 2, Layer 3 and Layer 4, respectively,

$$C_{BM_2} = \frac{4.4 \times 0.576 - 2.1 \times 0.526}{4.4 - 2.1} = 64.4\%$$

$$C_{BM_3} = \frac{4.3 \times 0.601 - 2.1 \times 0.526}{4.3 - 2.1} = 67.3\%$$

$$C_{BM_4} = \frac{4.4 \times 0.594 - 2.1 \times 0.526}{4.4 - 2.1} = 65.8\%$$

Therefore,

Carbon in biomass of Layer 1, $\Delta C_{biomass}=BM_1 \times C_{BM1} = 2.6 \times 0.647 = 1.7(g)$

Carbon in biomass of Layer 2, $\Delta C_{biomass}=BM_2 \times C_{BM2} = 2.4 \times 0.644 = 1.5(g)$

Carbon in biomass of Layer 3, $\Delta C_{biomass}=BM_3 \times C_{BM3} = 2.2 \times 0.673 = 1.5(g)$

Carbon in biomass of Layer 4, $\Delta C_{biomass}=BM_4 \times C_{BM4} = 2.3 \times 0.658 = 1.5(g)$

3. Calculation of $\Delta C_{citric\ acid}$

Carbon content of citric acid (C_{CA}) is based on its formula, $C_6O_7H_8$,

$$C_{CA} = \frac{6 \times C}{6 \times C + 7 \times O + 8 \times H} = \frac{6 \times 12}{6 \times 12 + 7 \times 16 + 8 \times 1} = 37.5\%$$

Referring to Table 1 for CA_1, CA_2, CA_3 and CA_4 in Layer 1, Layer 2, Layer 3 and Layer 4, respectively.

Therefore,

Carbon in citric acid of Layer 1, $\Delta C_{citric\ acid}=CA_1 \times C_{CA}=8.0 \times 0.375=3.0$ (g)

Carbon in citric acid of Layer 2, $\Delta C_{citric\ acid}=CA_2 \times C_{CA}=8.4 \times 0.375=3.2$ (g)

Carbon in citric acid of Layer 1, $\Delta C_{citric\ acid}=CA_3 \times C_{CA}=8.3 \times 0.375=3.1$ (g)

Carbon in citric acid of Layer 2, $\Delta C_{citric\ acid}=CA_4 \times C_{CA}=8.6 \times 0.375=3.2$ (g)

4. Calculation of ΔC_{CO2}

Carbon content of CO_2 is based on its formula, then

$$C_{CO_2} = \frac{1 \times C}{1 \times C + 2 \times O} = \frac{1 \times 12}{1 \times 12 + 2 \times 16} = 27.3\%$$

Defining:

Average air flow rate in the packed reactor as r, r=1.8 l/min, at 30°C and 1 atm absolute

Concentration of CO_2 after Layer i at given time (t) as $w_i(t)$, %, v/v, at 30°C and 1 atm absolute.

Volume of CO_2 production of Run 2 as V_i, l, at 30°C, its weight as W_i, g.

Here, unit of t is minute, and i=1, 2, 3 and 4, respectively.

Therefore, total CO_2 production during 4 days fermentation of Layer i,

$$V_i = 24 \times 60 \times r_i \times \sum_{t=0}^{t=4} \Delta t \times [w_i(t) - w_{i-1}(t)] = 1440\, r_i \int_0^4 [w_i(t) - w_{i-1}]\, dt =$$

$$1440\, r_i \left[\int_0^4 w_i(t)\, dt - \int_0^4 w_{i-}(t)\, dt\right]$$

To obtain the

$$\int_0^4 w_i(t)\, dt$$

The area under the time profile curve of CO_2 production during 4 days Fig 9.6 can be calculated by using the Trapezoidal method,

$$\int_0^4 w_1(t)\,dt = 0.201 \quad \text{min, \% } (v/v)$$

$$\int_0^4 w_2(t)\,dt = 0.369 \quad \text{min, \% } (v/v)$$

$$\int_0^4 w_3(t)\,dt = 0.498 \quad \text{min, \% } (v/v)$$

$$\int_0^4 w_4(t)\,dt = 0.697 \quad \text{min, \% } (v/v)$$

Therefore, for CO_2 production at each Layer,

V_1=1440 × r × 0.201%=1440 × 1.8 × 0.00201 = 5.2 l, at 30°C and 1 atm absolute.

V_2=1440×r×(0.369% - 0.201%)=1440×1.8×0.00168=4.4 l, at 30°C and 1 atm absolute.

V_3=1440×r×(0.498% - 0.369%)=1440×1.8×0.00129=3.3 l, at 30°C and 1 atm absolute.

V_4=1440×r×(0.695% - 0.498%)=1440×1.8×0.00197=5.1 l, at 30°C and 1 atm absolute.

Then based on the ideal gas formulation, for CO_2:

$$P \times V = M \times R \times T = \frac{W}{44} \times R \times T$$

Therefore

$$W = \frac{44 \times P \times V}{R \times T} \qquad (g)$$

Here 44 is CO_2 molecular weight, P=1, R=0.082, T=273+30=303 K

CO_2 production in Layer 1,

$$W_i = \frac{44 \times P \times V_i}{R \times T} \qquad (g)$$

Then

$$W_1 = \frac{44 \times 1 \times 5.2}{0.082 \times 303} = 9.2 \qquad (g)$$

$$W_1 = \frac{44 \times 1 \times 4.4}{0.082 \times 303} = 7.8 \qquad (g)$$

$$W_1 = \frac{44 \times 1 \times 3.3}{0.082 \times 303} = 5.8 \qquad (g)$$

$$W_1 = \frac{44 \times 1 \times 5.1}{0.082 \times 303} = 9.0 \qquad (g)$$

Finally,

Carbon of CO_2 production in Layer 1, $\Delta C_{CO2}=W_1 \times C_{CO2}=9.2 \times 27.3\%=2.5(g)$

Carbon of CO_2 production in Layer 2, $\Delta C_{CO2}=W_2 \times C_{CO2}=7.8 \times 27.3\%=2.1(g)$

Carbon of CO_2 production in Layer 3, $\Delta C_{CO2}=W_3 \times C_{CO2}=5.8 \times 27.3\%=1.6(g)$

Carbon of CO_2 production in Layer 4, $\Delta C_{CO2}=W_4 \times C_{CO2}=9.0 \times 27.3\%=2.5(g)$

Appendix 3

Publications

Note:The bottom page numbers in following pages are original.