NOTE TO USERS

OPTIMIZATION OF CITRIC ACID PRODUCTION BY *ASPERGILLUS NIGER* NRRL 567 IN VARIOUS FERMENTATION SYSTEMS

by

JIN-WOO KIM

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Department of Biosystems Engineering

Macdonald Campus of McGill University

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ABSTRACT

Among the various fungal strains screened for citric acid production, *Aspergillus niger* is known to produce considerable amounts of citric acid and other organic acids when cultivated in carbohydrate-rich medium in solid substrate fermentation (SSF). Since *A. niger* on a solid substrate grows under conditions similar to the natural habitat, SSF is ideal to cultivate *A. niger* for the purpose of producing citric acid.

An initial optimization (study 1) was conducted in batch type fermentation experiments using peat moss supplemented with glucose to simulate an organic waste. The effects of various nutrients (glucose, $(NH_4)_2SO_4$, KH_2PO_4 and NaCl), fermentation parameters (moisture content, temperature, inoculum density, composition of solid substrate and particle size) and of initial level of potential stimulators (ethanol, methanol, phytate and surfactant) were evaluated with respect to citric acid production by *A. niger* grown on damp peat moss. In these experiments, optimization using a traditional 'one-factor-at-a-time' method was applied to determine key factor ranges for the production of citric acid. When the fermentation was carried out using the final optimal conditions. This allowed for a 50-fold increase in citric acid production compared to the production of citric acid by *A. niger* grown on peat moss supplemented with 100 g glucose/kg DPM.

A second set of experiments (study 2) was conducted to optimize fermentation conditions for citric acid production in a column bioreactor. *A. niger* NRRL 567 grown on damp peat moss was held within a column bioreactor and periodically irrigated with a glucose-rich solution simulating field conditions. Three variables including aeration, thickness of solid substrate bed and incubation temperature were optimized using a 2^3 full factorial design (FFD). Under optimum, the total citric acid production and yield were

120.6 g/kg DPM and 18.5% respectively.

A third experiment (study 3) compared the production of citric acid by *A. niger* in submerged fermentation using cheese whey, as opposed to batch and semi-continuous fermentation using peat moss. Various fermentation conditions such as nutrients (glucose, $(NH_4)_2SO_4$ and KH_2PO_4), stimulators (methanol, olive oil and phytate) and fermentation parameters (pH, fermentation time and inoculum density) were optimized using a central composite design (CCD). Citric acid production improved citric acid production by a factor of 13.3 when compared to the production of citric acid by *A. niger* NRRL 567 using whey-based medium (50 g/l) alone.

As compared to submerged and semi-continuous fermentation, Batch type SSF could take higher levels of initial glucose and produce the high concentration of citric acid within a shorter period of time. Thus, SSF may be considered to be better technique than submerged fermentation, if main disadvantage like non-homogeneous fermentation conditions could be overcome.

RÉSUMÉ

Parmi les souches fongiques qui produisent de l'acide citrique, l'*Aspergillus niger* est reconnu pour en produire de grandes quantités, en plus d'autres acides organiques, lorsqu'il est cultivé dans un milieu riche en hydrates de carbone et dans des conditions de fermentation en milieu solide (SSF). Étant donné que les conditions de croissance de l'*Aspergillus niger* en milieu solide sont similaires à celles de son milieu naturel, la fermentation en milieu solide est idéale pour produire de l'acide citrique.

Une première optimisation (phase 1) a été réalisée grâce à des expériences de fermentation en discontinue, à partir d'un substrat de mousse de tourbe auquel avait été ajouté du glucose, afin de simuler un polluant organique. Les effets des divers nutriments (glucose, $(NH_4)_2SO_4$, KH_2PO_4 et NaCl), des paramètres de fermentation (teneur en eau, température, grosseur de l'inoculum, composition du milieu solide et grosseur des particules) et du niveau initial des stimulateurs potentiels (éthanol, méthanol, phytates et agents de surface) ont été mesurés en tenant compte de la production d'acide nitrique d'une souche d'*Aspergillus niger* sur un substrat de mousse de tourbe humide. Au cours de ces expériences, c'est la méthode d'optimisation traditionnelle « un paramètre à la fois » qui fut utilisée pour déterminer la gamme approximative des facteurs clés qui interviennent dans la production d'acide citrique. La gamme a par la suite été précisée davantage à l'aide d'un procédé d'optimisation fondé sur les statistiques. Ceci augmentait de 50 fois, la production d'acide citrique par rapport à celle produite à partir d'un substrat de mousse de tourbe auquel a été ajouté 100 g de glucose/kg MTS.

Une deuxième série d'expériences (phase 2) a été réalisée, afin d'optimiser les conditions de fermentation nécessaires à la production d'acide citrique, en utilisant un

réacteur à colonne, dans des conditions de fermentation semi-continues. L'*Aspergillus niger* NRRL 567, produite à partir de mousse de tourbe humide, a été déposé dans un réacteur à colonne, puis irrigué de façon périodique d'une solution riche en glucose, afin de simuler les conditions du milieu réel. Les trois variables suivantes ont été optimisées en utilisant un plan entièrement factoriel de 2^3: aération, épaisseur de la couche du support solide et température d'incubation. Dans ces conditions, la production moyenne d'acide citrique était de 40,2 g/kg de mousse de tourbe humide et le rendement de 18,5%.

Une troisième expérience (phase 3) comparait la quantité d'acide citrique produite par la fermentation de l'*Aspergillus niger* immergé dans du lactosérum et celle produite au cours d'un procédé de fermentation semi-continu sur de la mousse de tourbe. Plusieurs conditions de culture, telles que les nutriments (glucose, $(NH_4)_2SO_4$ et KH_2PO_4), les stimulateurs (méthanol, huile d'olive et phytates) et les paramètres de fermentation (pH, temps de fermentation et taille de l'inoculum), ont été optimisées à l'aide d'un plan composé central. Les résultats démontrent que l'usage de niveau optimum pour tous les paramètres a permis d'augmenter la production d'acide citrique jusqu'à 74,6 g/l et son rendement jusqu'à 80.6%. Ce taux de production représente une amélioration de 13,3 fois comparativement au taux obtenu avec une solution de lactosérum (50 g/l) sans suppléments.

Comparativement aux essais en conditions submergées et semi continues, les essais en discontinues du type SSF pouvait accommoder de plus hauts niveaux initiaux de glucose et donc produire plus d'acide citrique en moins de temps. Donc, la méthode SSF est supérieure à la méthode submergée mais requière une attention particulière pour éviter toute situation hétérogène.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

CHAPTER 9

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

CHAPTER 9

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CHAPTER 1

INTRODUCTION

One of the most important fungi used in industrial microbiology, *Aspergillus niger* has been employed for many years for the commercial production of citric acid (Schuster *et al.*, 2002). Citric acid is produced commercially from the fermentation of bulk hydrated materials and by-product of sugar production by *A. niger* (Wang, 1998; Lesniak *et al.*, 2002). However, the worldwide demand for citric acid is increasing faster than its production and more economical processes are required (Tran *et al.*, 1998; Alvarez-Vasquez *et al.*, 2000). It was well known fact that growth and production of *A. niger* are strongly affected by the medium composition, fermentation parameters and stimulators. Thus, citric acid productivity by *A. niger* can be improved by optimizing the fermentation conditions.

Citric acid (2-hydroxy-1, 2,3-propanetricarboxylic acid) is an intermediate in the TCA cycle and its accumulation is strongly influenced by the balance of nutrients. The type and concentration of the carbon source, especially glucose and sucrose, has a significant effect on citric acid production. In general, the final concentration of citric acid increases as the initial concentration of the carbon source is increased (Papassiopi *et al.*, 1999). Also, citric acid production by *A. niger* also depends on presence of other nutrients such as nitrogen, phosphorous, potassium and other salts (Jianlong and Ping, 1998; Wen and Chen, 2001). The limitation or starvation of nitrogen, phosphorus or other trace elements during the fermentation resulted in the limited growth of *A. niger* and to the enhancement of citric acid production (Mirminachi *et al.*, 2002). In addition to the basal nutrients, to improve citric acid production, stimulators such as organic solvents, phytate and lipids can be

applied (Jianlong and Ping, 1998).

The important physico-chemical fermentation parameters influencing the growth of *A. niger* on a solid substrate and its production of citric acid are solid substrate composition, moisture content, particle size distribution, fermentation temperature, pH, fermentation time and inoculum density (Xu *et al.*, 1989; Jianlong and Ping, 1998). Most filamentous fungi are known for better citric acid production under acidic pHs ranging between 3 and 6, but some fungi are able to grow at pHs below 2 to better compete against bacteria (Fawole and Odunfa, 2003). Citric acid production is also known to be affected by inoculum density and fermentation time (Lee and Yun, 1999). Up to a specific limit, metabolite production generally increases with inoculum density (Kota and Sridhar, 1999; Nampoothiri *et al.*, 2003).

Soil flushing technique using weak organic acids can effectively remediate soils contaminated with heavy metals (Wasay *et al.*, 1998; Sayer and Gadd, 2001). Organic acids including citric acid, gluconic, oxalic and tartaric acid produced by *A. niger* have several carboxyl and hydroxyl groups, which compete against soil particles for heavy metal absorption. Among weak organic acids, citric acid produced by *A. niger* has advantages over chemical chelating agents and strong acids such as EDTA (ethylenediaminetetraacetic acid), DTPA (diethylenetriaminepentaacetic acid), HCl, HNO_3 and H_2SO_4 (Elliott and Shastri, 1999; Peter, 1999). The concept of using citric acid produced by *A. niger* NRRL 567 for such a purpose requires further research, including the optimization of medium, fermentation parameters and initial level of stimulating compounds.

OBJECTIVES

The objective of this study is to identify the optimal citric acid production condition (nutrients, fermentation parameters and stimulators) for the fungal strain *A. niger* NRRL 567, using two sets of fermentation methodologies: (1) laboratory scale solid substrate and (2) submerged fermentation.

The main objectives focused on the optimization of citric acid production by *A. niger* using inert solid substrate. First, using a simulated support material such as peat moss (PM) supplemented with glucose in a batch process compared to submerged fermentation. Second, as in the first but using a column bioreactor where *A. niger* is fed semi-continuously. Third, with whey as an alternative nutrient source for citric acid production, using batch experiments in submerged fermentation.

Thus the specific objectives of study 1, using batch experiments in solid substrate fermentataion, were:

A. To optimize the basal nutrient levels (glucose, $(NH_4)_2SO_4$, KH_2PO_4 and NaCl), fermentation parameters (moisture content, temperature, pH, inoculum density, composition of the solid substrate and particle size), stimulators (methanol, ethanol, phytate, surfactant and natural oil) and buffer solutions (acetate, phosphate and carbonate buffer) for citric acid production by *A. niger*, an initial empirical optimization to approximate the range of variables and then a statistically-based optimization method was used to predict optimum fermentation conditions.

B. To compare citric acid production by *A. niger* in solid substrate fermentation, as opposed to that obtained during batch experiments using Czapek-Dox medium in

submerged fermentation.

C. To evaluate the effect of the initial pH of the nutrient solution and buffer solutions on citric acid production by *A. niger* NRRL 567.

The specific objectives of study 2, using column semi-continuous fermentation, were:

A. To optimize the fermentation parameters (aeration, thickness of solid substrate and temperature) for the production of citric acid from *A. niger* grown on PM in semi-continuous fermentation, simulating a field test. Again, a statistically-based optimization method, namely full factorial design (FFD), was used to analyze the results and predict optimum fermentation condition.

B. To compare citric acid production by *A. niger* grown on PM under semi-continuous conditions, as opposed to that obtained during the previous batch experiments using solid substrate fermentation.

The specific objectives of study 3, using a whey-based medium in submerged fermentation, were:

A. To optimize the basal nutrients (Glucose, $(NH_4)_2SO_4$ and KH_2PO_4), fermentation parameters (pH, inoculum density and fermentation time) and stimulators (methanol, olive oil and phytate) for citric acid production by *A. niger* using whey as a nutrient-rich byproduct. The optimal conditions were identified via a statistically-based optimization of a central composite design (CCD).

B. To compare citric acid production by *A. niger* in submerged fermentation, as opposed to batch and semi-continuous fermentation that used solid substrate fermentation.

SCOPE OF THE STUDY

The study was performed at the laboratory scale. Study 1, solid substrate fermentation in flasks, made use of the 250-ml Erlenmeyer flasks containing 7 g dry peat moss (DPM) and was used to optimize citric acid production by *A. niger*. Study 2, termed semi-continuous fermentation, was conducted in an 890-ml column bioreactor to simulate a field test. The column bioreactor contained 80 g DPM in order to test citric acid production by *A. niger*. For both studies 1 and 2, as the solid substrate for the growth of *A. niger* and citric acid production, PM supplemented glucose was used to simulate sugar rich byproduct. Study 3 was performed in submerged fermentation using 50-ml of whey-based medium in the 250-ml Erlenmeyer flasks

CHAPTER 2

LITERATURE REVIEW

2.1 Citric acid production by *Aspergillus niger*

An intermediate in the tricarboxylic acid (TCA) cycle, citric acid is an important commercial product with global production reaching 736,000 tons/yr. Furthermore, it is produced almost through the submerged fermentation of the white rot fungus (Jianlong 2000; Vandenberghe *et al.*, 2000). Citric acid is widely used in the food, beverage, pharmaceutical and cosmetic industries and it has other applications including textiles, electroplating and bioremediation (Tran *et al.*, 1998; Wang, 1998; Ates *et al.*, 2002). Because of its numerous applications, the volume of citric acid production by fermentation is continually on the increase (Jianlong and Ping, 1998). The most popular white rot fungus for large-scale production of citric acid is *A. niger* due to its high citric productivity at low pH without the secretion of toxic byproducts. Besides citric acid, some strains of *A, niger* also accumulate other organic acids as well such as oxalic, malic, tartaric, fumaric and pyruvic acids under specific fermentation conditions (Sassi *et al.*, 1991).

Regarding the process of citric acid accumulation in *A. niger*, two main metabolic pathways have involved a major role: (1) the catabolic pathway of hexoses to pyruvate and Acetyl-Coenzyme A (Acetyl-CoA) by glycolysis and (2) citric acid formation by TCA cycle (Alvares-Vasquez *et al.*, 2000). As glucose is the starting carbohydrate in glycolysis for citric acid production, glucose plays an important role in citric acid production. According to Wasay in 1998a, over 90% of glucose can be converted into citric acid mainly and other organic acids under optimal fermentation conditions. The stoichiometry for microbial production of citric acid is described as follows:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6\,H_2O + 38\,ATP \quad (2.1)$$

$$C_6H_{12}O_6 + 1.5O_2 \rightarrow C_6H_8O_7 + 2H_2O \quad (2.2)$$

The TCA cycle plays an important role in generating high-energy fuel (ATP) or producing intermediates such as organic acids which can be used for various purposes in the cell through catabolic or metabolic process (Figure 2.1). The production of high-energy fuel is depicted in Equation (2.1) which represents the complete oxidation of glucose to carbon dioxide and water (Jianlong, 2000). When the cell needs high energy for cell propagation or maintenance, it initiates cellular respiration processes including glycolysis, the TCA cycle and electron transport/oxidative phosphorylation harnesses all three processes for more energy generation. During the complete oxidation of glucose to CO_2 and H_2O, cellular respiration process produces high-energy fuel molecules (38 ATPs).

Citric acid is produced instead of energy when glucose is oxidized (Equation 2.2). When the cell produces citric acid, the full respiration process stops during the TCA cycle and yields citric acid rather than energy. The fungus decides whether the process goes to energy or instead to an intermediate based upon growth conditions and concentration of end products. The reason for production of citric acid by native strains of *A. niger* is not clear, however several theories have been proposed. Most of these are related to biological competition against enemies and the increase of ionic mobility. Insoluble forms of trace elements or nutrients, such as copper, zinc, calcium and phosphate, can be solubilized by fungal citric acid and their mobility is thus increased. Also, citric acid can form stable complexes with various metallic ions and such formation resulted in high heavy metal tolerance for *A. niger*. Since many fungi are capable of survival and growth under very acidic conditions, citric acid production and acidification of their microenvironment are

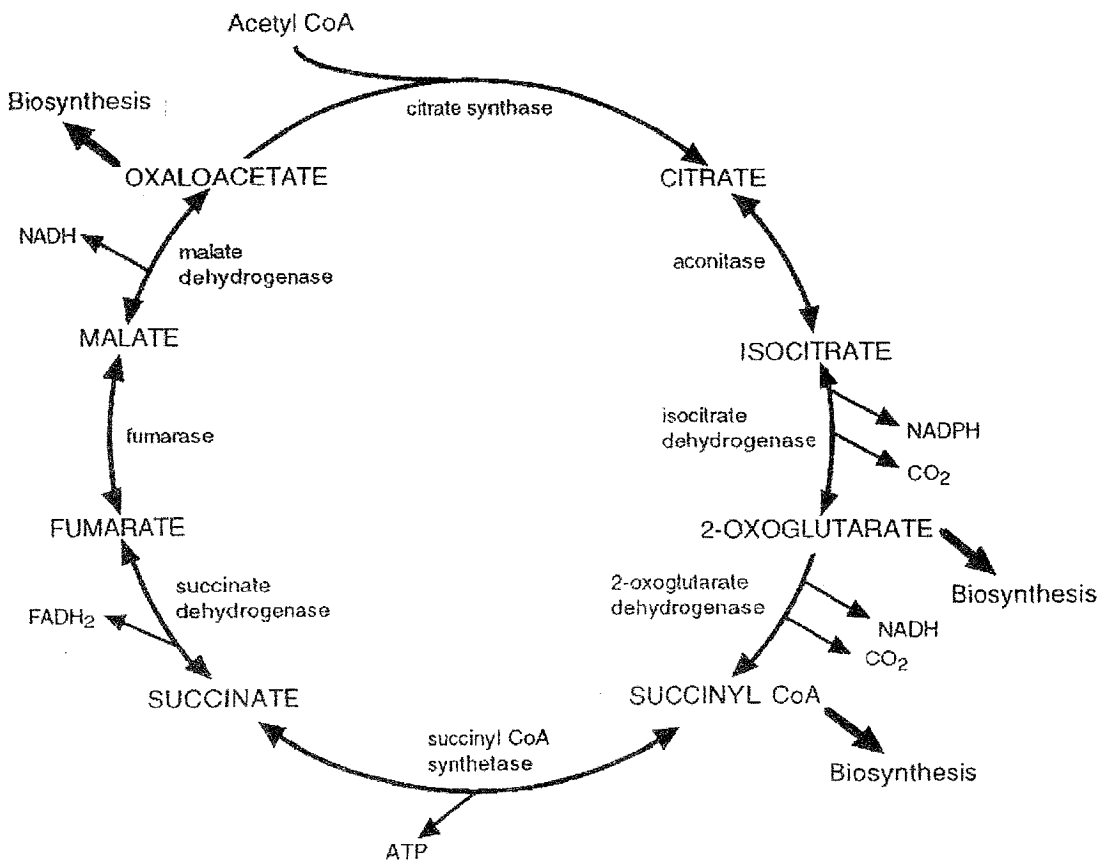

Figure 2.1 Major intermediates of the citric acid (Krebs) cycle (Dunn, 1998).

very effective strategies for biological competition (Sayer and Gadd, 2001).

2.2 Solid substrate fermentation

The most common process for the commercial scale production of citric acid involves submerged fermentation using the filamentous fungus, *A. niger*, growing on media containing glucose or sucrose (Leangon *et al.*, 2000; Kumar *et al.*, 2003). In recent years, the use of solid substrate fermentation has shown some potential as an alternative for the production of this organic acid (Romero-Gomez *et al.*, 2000). Solid substrate fermentation involves "the growth of microorganisms on moist solid substrates in the absence or near-absence of free flowing water" (Pandey, 1992; Robinson *et al.*, 2001; Ellaiah *et al.*, 2004). The solid substrate acts as a source of carbon, nitrogen, minerals and a growth surface which absorbs the water necessary for microbial growth (Chahal, 1985; Nandakumar *et al.*, 1994). In addition, the solid substrate provides anchor points for the growth and propagation of microorganisms (Larroche *et al.*, 1998). As microorganisms on a solid substrate are growing under conditions similar to their natural habitat, they may be able to produce certain enzymes, metabolites, proteins and spores more efficiently than in submerged fermentation (Goes, 1999).

Solid substrate fermentation has been widely used especially in East Asia for traditional food fermentations, enzyme production by the Koji process, mould-ripened cheese and composting of agricultural residues (Fujio *et al.*, 1985; Tengerdy, 1985; Omori *et al.*, 1994). In recent years, solid substrate fermentation has regained interest based on its technological and economic advantages over conventional submerged fermentation (Ellaiah *et al.*, 2004). The absence of a liquid phase and low substrate humidity level allow (1) facilitated aeration through the pore spaces between substrate particles; (2) reduction of

the fermentation and the liquid effluent volumes; (3) reduced risk of bacterial contamination because of low moisture level; (4) use of the non-sterile solid substrate in some cases; (5) reduction in water usage and wastewater management; (6) simplified media and (7) utilization of agro-industrial sugar rich wastes or byproducts (Szakacs and Tengerdy, 1996; Gutierrez-Correa and Tengerdy, 1997; Gutierrez-Correa *et al.*, 1999).

Solid substrate fermentation is used on a commercial scale for the production of different types of fermented foods, fungal metabolites and for the bioconversion of sugar rich wastes into useful products. Various agro-industrial residues, such as apple pomace, coffee husks, wheat straw, pineapple waste, cassava baggasse, banana peel, sugar beet and kiwi fruit peel have been used as solid substrates (Hang *et al.*, 1987, Tran *et al.*, 1998). Huge amounts of agricultural waste are produced daily and there is an urgent need to find suitable applications and disposal techniques for those. One economic alternative is to use such wastes as fermentation substrates in the production of value-added products such as enzymes, fine chemicals, metabolites and microbial biomass.

2.3 Solid substrate (peat moss)

According to the definition above, a solid material having a high carbon or energy content and a potential high water retention capacity may be a good candidate as a solid substrate. Peat moss (PM) contains high levels of polysaccharides but a low level of lignin (Table 2.1). Since polysaccharides have much higher moisture absorption potential than lignin, PM is able to retain higher moisture levels. Particles of PM can attract and retain water mainly because hydrophilic functional groups in its organic matter are able to form hydrogen bonds with water molecules. Examples of such groups are phenolic and alcoholic hydroxyls and carboxyls, or nitrogenous compounds like amines and N-bearing

heterocycles. PM is found all over the world in areas called peat lands. It is rich in organic matter formed from the accumulation of dead plants which are decomposed under water in land formations such as bogs, swamps and marches (Garcia-Moreno, 1993).

2.4 Whey-based medium for citric acid production

Whey is the chief by-product from the dairy industry, representing 80 - 90% by volume of the total milk utilized. The manufacture of cheese and casein releases large quantities of cheese whey, the annual global whey production reaching approximately 4.0 x 10^7 tons, which in turn contain approximately 4.5 - 5.0% (w/v) lactose, 0.8% (w/v) whey protein, 1.0% (w/v) salts, 0.1% (w/v) lactic acid and vitamins (Table 2.2). Because of the high concentration of organic species, the resulting biological demand (BOD) and chemical oxygen demands (COD) represent 30 – 60 g/l and 60 – 70 g/l respectively and disposal without proper treatment can cause considerable environmental problems (Wang and Lee, 1998; Rech *et al.,* 1999; Kosseva *et al.,* 2001). The production of value-added compounds using whey provides advantages from both the standpoint of waste material management and of lower capital costs for carbon substrates. Bioconversion of whey to value-added products such as organic acids, ethanol, enzymes, PHB (poly-3-hydroxybutyrate), xanthan gum and biomass has been extensively studied by many teams in the past (Lee and Yun, 1999; Rech *et al.,* 1999; Kawahara and Obata, 1998; Lee *et al.,* 2000).

2.5 Fermentation conditions affecting citric acid production

Citric acid production by *A. niger* is influenced by number of fermentation parameters. There are significant variations in fermentation environments reported in the previous

Table 2.1 Physico-chemical characteristics of the sphagnum PM

Property	Units	Value
Bulk density	kg/m^3	276.4
Moisture content	%	52.7
Carbon	% DW	53.1
Nitrogen	% DW	2.1
Phosphorus	% DW	0.01
Potassium	% DW	0.15
Ash	% DW	2.8
Volatile solids	% DW	97.1
NH_4-N absorption capacity	mg/kg	27.5
pH	-	4.38
Coarse particles (> 2.00 mm)	% DW	14.3
Medium particles (1.00 - 2.00 mm)	% DW	18.4
Fine particles (0.5 – 1.00 mm)	% DW	20.5
Very fine particles (< 0.5 mm)	% DW	46.8

Note:

All analysis are reported on a dry weight basis; the carbon content was calculated from {(100% - ash%)/183} (Barrington *et al.*, 2002);

DW = dry weight.

Table 2.2 Composition of Cheese whey

	Unit	Value
Moisture	wt %	3.5
Protein	wt %	9.0 - 11.0
Lactose	wt %	65
Fat	wt %	1.2
Ash	wt %	8.0
pH (10% Solution)		7.0

studies for citric acid production by *A. niger*. For achieving high production of citric acid, it is essential that the study of influence of physical and chemical environments on citric acid production (Jianlong and Ping *et al.*, 1998).

2.5.1 Medium composition

Growth and production of microorganism are strongly affected by the medium composition such as concentrations of carbon, nitrogen, phosphorous, potassium, trace elements and stimulators. Thus, citric acid productivity by *A. niger* can be improved by optimizing the medium composition.

2.5.1.1 Glucose

The commercial scale production of citric acid using *A. niger* grown on a solid substrate is strongly affected by the type of solid substrate and its level of available carbon (Wayman and Mattey, 2000). For the production of citric acid, sucrose, fructose and glucose are the carbon substrates for *A. niger* (Sassi *et al.*, 1991). Among them, as glucose doesn't need any future modification for the metabolites, it is readily used by fungus. Also, apple peels and pomace, grape pomace, banana extract, sugar cane baggasse and sugar beet molasses have been used to produce citric acid (Ngadi and Correia, 1992; Wang, 1998; Gutierrez-Correa *et al.*, 1999). Some strains of *A. niger* can convert up to 90% of the glucose into organic acids (Wasay *et al.*, 1998a). A substrate initiating glycolysis followed by the TCA cycle, glucose is the crucial factor affecting acid production. According to Leangon *et al.* (2000), an increase glucose flux through glycolysis may be the reason for over-production of citric acid in solid substrate fermentation. However, low glucose concentration in the solid substrate may cause oxalic acid accumulation (Alvarez-Vasquez

et al., 2000).

2.5.1.2 Nitrogen sources

The effect of nitrogen source on citric acid producion has been intensively studied in solid substrate and submerged fermentation. Ammonium chloride, ammonium sulphate, ammonium nitrate, pepton and yeast extract were the most suitable nitrogen source for production of citric acid by fungus (Abou-Zeid and Ashy, 1984). The limitation or starvation of nitrogen during the fermentation resulted in the limited growth of *A. niger* and to the enhancement of citric acid production (Mirminachi *et al.*, 2002).

2.5.1.3 Phosphorus sources

The concentration of exogenous phosphorus in medium had significant effect on cell multiplication and metabolite production. Abou-Zeid and Ashy (1984) reported KH_2PO_4 and K_2HPO_4 proved to be the best phosphorus source. Mirminachi *et al.* (2002) reported that phosphorus limitation induced higher citric acid production and yield, while Shu and Johnson (1984) reported higher citric acid production and yield with phosphorus in the range of 0.06 to 0.32 g /l.

2.5.2 Stimulators

Several reports have shown the stimulatory effects of additives on fungal citric acid accumulation and secretion (Pardo, 1996; Goes, 1999). To improve citric acid production, stimulators have been used, such as organic solvents, phytate and lipids (Jianlong and Ping, 1998).

2.5.2.1 Organic solvent

Higher citric acid production levels can be obtained by applying stimulators, such as methanol, ethanol, phytate, vegetable oil, oximes, *n*-dodecane, fluoroacetate and chelating agents (Navaratnam *et al.*, 1998; Jianlong, 2000).

Organic solvents including ethanol and methanol stimulate the production of citric acid by increasing the permeability of the cell membrane, decreasing cell growth or changing the activity of key enzymes. Ethanol supplementation is known to change the activity of key enzymes associated with the TCA cycle; it increases the activity of citrate synthetase and decreases the activity of aconitase. In addition, ethanol can improve citric acid production by being converted to acetyl-CoA and assimilated by *A. niger* as an alternative carbon source (Jianlong and Ping, 1998). The stimulating effect of methanol is not clear. However, it is proposed that the addition of methanol increases the permeability of the cell membrane and increases the transfer of nutrients, which in turn increases the excretion of citric acid across the cell membrane (Hang *et al.*, 1987; Jianlong and Ping, 1998; Navaratnam *et al.*, 1998).

2.5.2.2 Phytate

Citric acid production using *A. niger* is very sensitive to the concentration of trace element. A presence of trace elements in media can cause considerable negative effect on citric acid production (Adham, 2002). Phytate is also known to offer 12 replaceable protons providing the binding potential for positively charged molecules. Phytic acid acts as a metal-chelating agent, chelating free trace elements (Wang, 1998; Haq *et al.*, 2002; Mirminachi *et al.*, 2002). Phytate may also improve citric acid production by *A. niger*, by controlling key enzymes involved in the TCA cycle (Jianlong and Ping, 1998)

2.5.2.3 Fatty acid

Vegetable oils with high levels of unsaturated fatty acids, such as olive, maize and sunflower oil, are stimulators for citric acid production, when using both submerged and solid substrate fermentation by *A. niger*. The stimulating mechanism of natural oil was investigated by Adham (2002) who suggested that unsaturated lipids play a role of alternative hydrogen acceptors to oxygen. Vegetable oil can be converted to acetyl-CoA and assimilated by *A. niger* as an alternative carbon sources (Jianlong and Ping, 1998).

2.5.3 Fermentation parameters

Citric acid production by *A. niger* is influenced by number of culture parameters. There are considerable variations in fermentation environments reported in the previous studies for citric acid production by *A. niger*. For achieving high production of citric acid, it is essential that the study of influence of physical and chemical environments on citric acid production (Jianlong and Ping, 1998).

2.5.3.1 Moisture content

Even though solid substrate fermentation is conducted in the absence of free flowing water, a small variation in moisture content can produce changes in fungal growth and metabolite production (Miller, 1999). The initial moisture content influences nutrient solubility, heat and gas transfers and substrate swelling (Kamini *et al.*, 1998; Ellaiah *et al.*, 2003). For *A. niger* DS 1 grown on fruit waste, moisture contents ranging from 65 to 85% resulted in the best citric acid production (Kumar *et al.*, 2003). Moisture content between 70 and 80% increased citric acid and lipase production by *A. niger* species grown on semi-dried figs and wheat bran (Roukas, 2000; Mahadik *et al.*, 2002). However, a

moisture content under 60% was determinated to be found optimal for the growth of *A. niger* MTCC 2594 and *Bacillus* sp. using gingelly oil cake and agro-byproducts (Kamini *et al.*, 1998; Uyar and Baysal, 2003).

2.5.3.2 Fermentation temperature

Optimal fermentation temperature must be maintained despite the large amount of heat (14 MJ/kg of substrate dry matter) generated by the metabolic activity of microorganisms (Miller, 1999). A solid substrate with a poor heat transfer coefficient results in localized temperature build-up and non-homogeneous fermentation conditions, especially for large-scale fermentation (Nampoothiri *et al.*, 1003). Cultivated under temperatures other than ideal, cells show signs of adverse growth and metabolic production (Ellaiah *et al.*, 2003). Higher than optimal temperatures result in enzyme denaturation and inhibition, excess moisture losses and growth arrest while lower temperatures lead to lower metabolic activity (Adinarayana *et al.*, 2003). Although most filamentous fungi are mesophilic requiring optimal temperatures between 25 and 35°C, some species thrive at 50°C (Reid, 1998; Suresh *et al.*, 1999). A temperature of 40°C was identified as optimum for metabolite production and sugar utilization by *A. niger* ATCC 10577 and *A. niger* V. Tiegham (Roukas, 2000; Fawole *et al.*, 2003).

2.5.3.3 pH

The metabolic activity of fungus is very sensitive to pH level of media. The initial pH of the solid substrate was found to have a impact on citric acid production by *A. niger* grown on peat moss (PM) (Kim *et al.*, 2004). In addition, the type of buffer used in the nutrient solution is a key factor in governing citric acid production by the fungus *A. niger*

(Roukas, 2000; Uyar and Baysal, 2003).

Most filamentous fungi are observed to grow well under slightly acidic conditions, ranging from 3 to 6, but some fungi are able to growth at a pH below 2 to better compete against bacteria (Fawole and Odunfa, 2003). For the production of citric acid by *A. niger*, the initial pH range from 2 to 6 is commonly used in solid substrate and submerged fermentation (Watanabe *et al.*, 1998; Adham, 2002; Lesniak *et al.*, 2002).

2.5.3.4 Buffer solution

As citric acid production is impacted by the initial pH of the solid substrate and *A. niger* produced a high concentration of citric acid within the narrow optimal pH range during solid substrate fermentation, finding the optimal initial pH of the solid substrate, as well as pH control during the fermentation, are equally important to improving citric acid production (Kim *et al.*, 2004). The difficulty of control of pH during solid substrate fermentation is one of the drawbacks of the method due to low the MC, lack of mixing and the heterogeneous growth characteristic of fungus. Supplementation with a buffer solution can stabilize sudden pH fluctuations and may result in a stimulating effect on citric acid production. Buffer solutions such as phosphate, acetate, carbonate, citric acid and lactic acid are widely used in metabolite production from fungus and are considered to have no adverse effect on cell growth (Uyar and Baysal, 2003).

2.5.3.5 Inoculum density

A high inoculum density leads to population over-crowding, higher nutrient competition and rapid exhaustion of nutrients (Uyar and Baysal, 2003). Up to a specific limit, metabolite production generally increases with inoculum density (Kota and Sridhar,

1999; Nampoothiri *et al.*, 2003). At the lower inoculum density, metabolite production drops and contamination risks increase due to an insufficient cell population. According to the literature, an inoculum density between 1 x 10^4 to 1 x 10^9 spores/ml was found to be suitable for citric acid production by *A. niger* (Favela-Torres *et al.*, 1998; Ruijter *et al.*, 2000; Adham, 2002).

2.5.3.6 Particle size distribution

Decreasing the particle size distribution of the solid substrate increases its surface area, gas diffusion and improving growth of hyphae. However, too small a particle size results in limited inter-particle pores, increased agglomeration and lower gas and heat transfers (Ellaiah, 2003). Larger particles benefit heat and gas transfer but reduce surface area for fungal attachment. The best cellulase production from banana fruit stalks was obtained by *Bacillus subtilis* with a particle size of 0.4 mm (Krishna and Chandrasekaran, 1996). A particle size of 0.4 to 1.0 mm led to maximum α-amylase production by *Bacillus subtilis* grown on wheat bran while larger and smaller particles led to substantially lower production rates (Krishna, 1996).

2.6. Optimization procedures

Optimization of the fermentation parameters for citric acid production by *A. niger* NRRL 567 can be carried out using two methods, among others: empirical- and statistically- based optimization.

2.6.1. Empirical optimization

The empirical technique is a traditional optimization method employing one-at-a-time

strategies (Ooijkaas *et al.*, 1999). This involves varying one factor while keeping the other factors unchanged under a specific set of conditions. It is useful only when one or a small number of variables is applied. With a small number of factors, this method is convenient and simple to handle and allows for the interpretation of results without statistical analysis (Wen and Chen, 2001). However, the technique becomes more complex when a large number of factors need to be optimized. Moreover, when statistical interactions exist among factors, the empirical method gives limited information on the interactive effects (Ooijkaas *et al.*, 1999). Preliminary optimizations can be conducted using the empirical method. This method identifies the input variables that can have a significant effect on the response. Thus the empirical method can reduce the number of experiments to be carried out later when using a statistically-based method (Li *et al.*, 2001).

2.6.2. Statistically-based method

Statistically-based optimization is a proven tool for overcoming the limitations of the "one-factor-at-a-time" method. It is a more efficient technique since it can provide statistical data with a relatively small number of experiments. Moreover, it is a valuable tool for measuring interactions among factors and for the prediction of optimal fermentation conditions (Wen and Chen, 2001). Another strong point of using statistically-based optimization is that no complex calculations are required to analyze the resulting data (Berthouex and Brown, 1994). Within one class of experimental designs, two types were used in this work: the so-called central composite design (CCD) and full factorial design (FFD). Optimization through experimental design is a general method used in biotechnology and several researchers have employed this for the optimization of media (Sircar *et al.*, 1998; Ooijkaas *et al.*, 1999; Li *et al.*, 2001; Ramirez *et al.*, 2001).

The CCD requires five levels of each variable. These levels and the center point are determined by setting the range to be tested, based on conclusions from previous "one-factor-at-a-time" runs. From this range, CCD sets the real values (Z_i) tested for each variable parameter and codes them as χ_i ($-\alpha$, - 1, 0, +1 and $+\alpha$) according to the following equation.

$$\chi_i = (Z_i - Z_{cp})/\Delta Z_i \qquad (2.1)$$

where χ_i = the dimensionless value of an independent variable, Z_i = the real value of an independent variable in original units, Z_{cp} = the value of Z_i at the center point level in the original units, ΔZ_i = a step change in original units (Ooijkaas *et al.*, 1998; Ambati and Ayyanna, 2001).

The statistical software package Design-Expert® 6.0 (Stat Ease, Inc, Minneapolis, USA) was used to generate a regression model in order to predict the effect of combined parameters on responses such as citric acid production and yield. To construct the response surface model, a second-order polynomial equation was fitted to the data using multiple regressions. The response of tested variables can be predicted by following quadratic polynomial equation (e.g. a four-variable model):

$$Y = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \beta_3\chi_3 + \beta_4\chi_4 + \beta_{11}\chi_1^2 + \beta_{22}\chi_2^2 + \beta_{33}\chi_3^2 + \beta_{44}\chi_4^2 + \beta_{12}\chi_1\chi_2 + \beta_{13}\chi_1\chi_3 + \beta_{14}\chi_1\chi_4 + \beta_{23}\chi_2\chi_3 + \beta_{24}\chi_2\chi_4 + \beta_{34}\chi_3\chi_4 \qquad (2.2)$$

where Y = the predicted response; β_0 = the intercept; β_1, β_2, β_3, β_4 = linear coefficients; β_{11},

β_{22}, β_{33}, β_{44} = squared coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} = interaction coefficients; χ_i and χ_j = the coded level of variable X_i and X_j (Chowdary *et al.*, 2002; Vohra and Satyanarayana, 2002).

2.7 Remediation using fungal citric acid

In nature, many strains of fungi are able to produce organic acids and therefore dissolve macronutrient or trace elements for increased mobility and cellular uptake. As well, acid-producing fungi in acidic soils inhibit the growth of other microorganisms and often comprise the dominant biomass (Gadd, 1993; Sayer and Gadd, 2001).

Fungal organic acids can act as chelating agents and dissolve heavy metals. *A. niger* has attracted considerable interest because it can produce large amounts of organic acids such as citric, gluconic, oxalic and tartaric acids (Gupta and Sharma, 2002; Lesniak *et al.*, 2002). The organic acids produced by *A. niger* possess several carboxyl groups which tend to donate protons (H^+), resulting in negatively charged carboxyl groups capable of competing against soil particles for heavy metal binding. Citric acid anions have been shown to produce stable complexes with various metallic cations, such as copper and zinc (Sayer and Gadd, 2001). The number of carboxyl and hydroxyl groups determines the order of effectiveness in chelating heavy metals (citric acid > tartaric acid > other organic acid anions). Metal-citric acid complexes exhibit stronger chelating potential and can form more stable complexes than other acid anions, not surprisingly because citric acid carries three negative charges (Wasay, 1998)

The partitioning of heavy metals between their atoms on soil surface groups and the organic acid can be depicted using the following reactions.

$$S{:}HM + OA^{-} \leftrightarrow HM{:}OA + S^{-}$$

Where S, HM and OA stand respectively for soil, heavy metal and organic acid; S:HM represents heavy metal contaminated soil and HM:OA represent the soluble form of the heavy metal/organic acid complex.

Organic acids with carboxylic functional groups are most often proposed as potential metal extracting agents, replacing chemical chelating agents and strong acids. Fungal organic acids with one or more carboxylic functional groups such as citric acid, oxalic acid and tartaric acid have chelating properties and can effectively remove Cr, Ni and Zn from soil (Elliott and Shastri, 1999; Wasay *et al.*, 2001). Organic acids produced by *A. niger* are beneficial in remediation techniques because they are capable of improving soil properties by enhancing the formation of stable soil aggregates, do absorb heavy metals in a more specific way, are less likely to leach out soil macronutrients and are less costly and more biodegradable (Wasay *et al.*, 1998b).

CONNECTING STATEMENT

A comprehensive review of literature demonstrated the need for developing optimum fermentation conditions for citric acid production. Solid substrate fermentation has been recongnized to be relatively low-cost appropriate fermentation system for the production of citric acid when it used agro-industial byproducts. This following experiment employed an inert solid substrate, peat moss, to simulate agro-industrial byproducts for the production of citric acid using *Aspergillus niger*. Citric acid production by *A. niger* is strongly influenced by the composition of the medium including carbon, nitrogen, phosphate and other salts. Therefore, studies were conducted to evaluate the effect of the initial nutrient concentration on citric acid production. In the first optimization, the effects of nutrients on citric acid production were optimized by a traditional strategy, to approximate their optimal value for initial concentration of nutrients. And then, the second optimization, the application of a statistically-based procedure, used to find optimum concentration of nutrients. One class of statistically-based optimization, the central composite design was applied to evaluate the interactions between variables. Finally, the results obtained from statistically-based optimization were compared to those obtained using a traditional "one-factor-at-a-time" method.

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CHAPTER 3

Optimization of Nutrients for Citric Acid Production by *Aspergillus niger* NRRL 567 Grown on Peat Moss

3.1 Abstract

The efficient production of citric acid by *Aspergillus niger* grown on a sugar rich byproduct requires the supplementation of the optimized quantities of nutrients. The central composite design (CCD) was used to statistically compare 19 combinations of four different nutrients, namely glucose, $(NH_4)_2SO_4$, KH_2PO_4 and NaCl, where glucose and peat moss (PM) simulated the sugar rich byproduct on which *A. niger* NRRL 567 was grown. The CCD method was used to generate a nutrient optimization model and it predicted an optimal nutrient levels were tested in the laboratory. For the production of citric acid after 72 h of fermentation, the initial concentration of glucose and KH_2PO_4 were found to exert positive effects, while $(NH_4)_2SO_4$ was found to show a negative effect in the tested range. The optimization using the CCD predicted the composition of the optimal nutrients (967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl/kg DPM) predicting 73.4 g of citric acid/kg DPM. Using the optimal nutrient concentrations, *A. niger* produced the maximum citric acid level of 82.0 g/kg DPM, as compared to 73.4 g/kg DPM as predicted by the CCD method and 53.6 g/kg DPM as obtained with a control test with 1000 g glucose/kg DPM along.

Keywords: *Aspergillus niger*, optimization, solid substrate fermentation, peat moss, citric acid.

3.2 Introduction

A variety of fungi are reported to produce organic acids such as citric, oxalic, succinic and malic acid. Among them, citric acid production using the filamentous fungus *Aspergillus niger* is well known and widely used by industries producing food, beverages, chemicals and pharmaceutical products (Haq *et al.*, 2001). Presently, citric acid production by *A. niger* is economically produced using submerged fermentation. However, the global demand for citric acid is growing faster than its production, implying that more economical processes are required to supplement or replace the present processes (Hang *et al.*, 1987; Alvarez-Vasquez *et al.*, 2000; Pazouki *et al.*, 2000; Vandenberghe *et al.*, 2000). Furthermore, submerged fermentation cannot be adapted to specific conditions such as the use of sugar-rich byproducts (Wasay *et al.*, 2001).

The most important physico-chemical fermentation parameters influencing the growth of *A. niger* on a solid substrate and its production of citric acid are: nutrient balance, solid substrate composition, moisture content (MC) and particle size distribution, fermentation temperature, pH and inoculum density (Xu *et al.*, 1989; Jianlong and Ping, 1998). A substrate initiating glycolysis followed by the TCA cycle, glucose is the crucial factor affecting acid production (Alexander, 1977; Wasay, 1998). According to Leangon *et al.* (2000), an increase glucose flux through glycolysis may be the reason for over-production of citric acid in solid substrate fermentation. However, low glucose concentration in the solid substrate may cause oxalic acid accumulation (Alvarez-Vasquez *et al.*, 2000). The limitation or starvation of nitrogen or phosphorus during the fermentation resulted in the limited growth of *A. niger* and to the enhancement of citric acid production (Mirminachi *et al.*, 2002).

The fungus *A. niger* can be grown on sugar rich byproducts using solid substrate

fermentation to produce citric acid for various purposes, including bioremediation. Nevertheless, this process requires optimization, as *A. niger* needs salts besides sugars to grow and produce citric acid. Therefore, this work tested the response of *A. niger* to different level combinations of four different nutrients (glucose, $(NH_4)_2SO_4$, KH_2PO_4 and NaCl) to maximize citric acid production using the central composite design (CCD).

3.3 Methods

3.3.1 Microorganism and preparation of inoculum

Aspergillus niger NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was stored in a tube containing glycerol (30% v/v) at –76°C. *A. niger* spores were produced on potato dextrose agar (PDA, Sigma, St. Louis, MO, USA) plates at 30°C and were sub-cultured at biweekly intervals. After seven to ten days of incubation on PDA plates, 10 ml of 0.1% Tween 80 (sigma, Sigma, St. Louis, MO, USA) solution were add to each plate. Diluted spore suspensions of 1.0×10^7 spores/ml were counted using hemocytometer and this spore solution was used to inoculate the solid substrate fermentation.

3.3.2 Solid substrate

PM can be an interesting solid substrate for *A. niger*. When compared with other types of solid substrates, which contain high levels of lignin such as cereal straw, corn stover and sawdust, PM has much higher polysaccharide content (Ezeonu and Okaka, 1996). PM can retain 15 to 20 times its own weight in water and has a low bulk density even when wet, allowing for a high porosity and good air diffusion even under moist conditions (Garcia-Moreno, 1993).

Sphagnum PM (Schultz Company, Mississauga, Ontario, Canada) supplemented with glucose was used to simulate a sugar rich byproduct and tested as the solid substrate. PM was wetted to the moisture content (MC) of 80% wet weight with deionized water supplemented nutrients such as salts and glucose (Considine *et al.*, 1987).

3.3.3 Salt solution for preliminary studies

The following basal salt solution was used to optimize the production of enzymes using *Trichoderma reesei* RUT C30 (Miller, 1999; Kim, 2000). The basal salt solution used to wet the PM and to provide basic nutrients to the fungal culture contained the following salts: 5 g/l $(NH_4)_2SO_4$, 5 g/l KH_2PO_4, 1 g/l NaCl, 1 g/l $MgSO_4 \cdot 7H_20$ and 1 g/l $FeSO_4 \cdot 7H_2O$. Once used to wet the PM, this solution gave the following salt concentration in terms of kg DPM: 20 g $(NH_4)_2SO_4$, 20 g KH_2PO_4, 4 g NaCl, 4 g $MgSO_4 \cdot 7H_20$ and 4 g $FeSO_4 \cdot 7H_2O$/kg of DPM.

3.3.4 Salt solution for central composite design

In the laboratory, 17 different nutrient solutions were prepared, using various levels of glucose, $(NH_4)_2SO_4$, KH_2PO_4 and NaCl (Tables 3.1 and 3.2). As a preliminary experiment, the 'one-factor-at-the-time' method was used to approximate the optimal initial nutrient concentration for citric acid production for *A. niger* grown on PM (Kim *et al.*, 2004): 1000 g glucose 20 g $(NH_4)_2SO_4$, 20 g KH_2PO_4, 4 g NaCl, 4 g $MgSO_4 \cdot 7H_2O$ and 4 g $FeSO_4 \cdot 7H_2O$/kg dry peat moss (DPM). This medium composition was used as the basal nutrient solution for the optimization step using CCD method.

3.3.5 Czapek-Dox medium for submerged fermentation

For the production of citric acid in submerged fermentation by *A. niger* NRRL 567, the Czapek-Dox medium was supplemented with glucose at a level of 50 g/l. This basic medium contained the following salts : 1.0 g KH_2PO_4; 0.5 g KCl; 2.0 g $NaNO_3$; 0.5 g $MgSO_4 \cdot 7H_20$ and 0.01 g $FeSO_4 \cdot 7H_2O$ per liter. The Czapek-Dox medium was already balanced in terms of nutrients for submerged fermentation of *Aspergillus niger* NCIM 548 and *Candida lipolytica* for citric acid production (Pazouki *et al.*, 2000). The pH of the medium was adjusted to 4.0 before autoclaving, to correspond to conditions found with solid substrate fermentation using PM.

3.3.6 Fermentation conditions for solid substrate fermentation

The experiment consisted of small-scale batch fermentation in 250-ml Erlenmeyer flasks holding 7 g of DPM wetted with 28 ml of solution containing salts and the various amount of glucose. After autoclaving, the solid substrate was inoculated with 1 ml (1.0 x 10^6 spores/ml) of *A. niger* NRRL 567 inoculum. The 250-ml Erlenmeyer flasks and their contents were then incubated for 6 days in a closed chamber maintained at 30°C.

3.3.7 Fermentation conditions for submerged fermentation

The submerged fermentation was performed in the 250-ml Erlenmeyer flasks containing 60 ml of Czapek-Dox medium with 50 g/l glucose. The Erlenmeyer flasks were incubated at 30 °C for 264 h on an orbital shaker at 150 rpm. At 48 h intervals, 2 ml of sample was aseptically withdrawn from the each flask and stored at –20 °C for glucose and citric acid analysis (Lee and Yun, 1999).

3.3.8 Analytical procedure

For each sampling session, the contents of the duplicate 250-ml Erlenmeyer flasks were withdrawn and 10 g of wet sample was harvested from each flask; 5 g was used to measure the MC of the PM and the other 5 g of wet sample was placed in 50 ml of distilled water and shaken for 60 min at 150 rpm and 22°C. The supernatant was tested for the pH and stored at –20 °C for citric acid quantification (Xu *et al.*, 1989). As ten times higher amount of water was applied for extraction of citric acid from DPM, the obtained final concentrations of glucose and citric acid were ten times concentrated compared to those of submerged fermentation.

For the solid substrate fermentation experiments, all citric acid and residual glucose concentrations were expressed per units of PM dry mass. The MC of PM was determined by placing the samples in aluminum drying pans, which were then dried in an oven at 60°C for 48 h. Citric acid levels were determined by spectrophotometry at 420 nm after adding pyridine and acetic anhydride. For each 3 ml of sample, 1.7 ml of pyridine (Sigma, St. Louis, MO, USA) and 5.6 ml of acetic anhydrid (Sigma, St. Louis, MO, USA) were added to develop color. Glucose was analyzed by the 3,5-denitrosalicylic acid (DNS) method (Marier and Boulet, 1958; Miller, 1959). The MC, yield of citric acid (CA), glucose consumption rate were calculated as follows:

Moisture content (%) = 100 x [water in wet PM (g)/wet PM (g)]

CA yield (%) = 100 x [produced citric acid (g/kg DPM)/utilized glucose (g/kg DPM)]

Glucose consumption rate (%) = 100 x [utilized glucose (g/kg DPM)/initial glucose (g/kg DPM)]

3.4 Results and discussion

3.4.1 Citric acid production

The effect of the initial glucose level on citric acid production by *A. niger* grown on PM is illustrated in Figure 3.1. The time course change of citric acid production with various initial glucose levels for 144 h of fermentation indicates that initial glucose level had a significant effect on citric acid production.

With 100 g glucose/kg DPM, citric acid production started after 24 h of fermentation and maximized at 11.2 g/kg DPM at 48 h. The maximum citric acid production of 53.7 g/kg DPM was reached at 96 h with an initial glucose level of 1000 g/kg DPM. For 100 and 250 g glucose/kg DPM, extending the fermentation time beyond the maximum citric acid production resulted in the oxidation of citric acid due to the depletion or the low level of fermentable glucose (Hang and Woodams, 1998). The patterns for citric acid production were similar for initial glucose levels of 250, 500 and 750 g/kg DPM. Citric acid production began after 24 h, maximized at 72 h and declined thereafter.

For glucose levels of 1000 g/kg DPM, citric acid production maximized after 96 h of fermentation, indicating that a high initial level of glucose may have negative effects on the growth and production of citric acid during lag phase by creating high osmotic pressures or by catabolic repression. During 144 h of fermentation, very little if any citric acid was produced when no glucose was added, indicating that a high initial concentration of carbon substrate is required for *A. niger* to produce citric acid.

3.4.2 Citric acid yield

Citric acid yield is an important parameter in selecting the optimum initial glucose level. For each level of initial glucose tested, the profiles for citric acid yield indicates that

initial glucose level had an effect on citric acid yield (Figure 3.2). Maximum citric acid yield increased with higher initial glucose level employed. The 250 and 500 g/kg DPM treatments maximized at 72 h as compared to 100, 750 and 1000 g/kg DPM treatments which maximized at 48 h. The treatment of 1000 g/kg DPM produced a maximum citric acid yield of 47.0% after 48 h, which exceeded that of 14.5% obtained with 100 g glucose/kg DPM.

3.4.3 Glucose consumption

Figure 3.3 illustrates the consumption of glucose by *A. niger* NRRL 567 over time in solid substrate fermentation. The complete utilization of glucose from the solid substrate was detected at the lowest initial concentration added (100 and 250 g/kg DPM). For the treatments using more than 500 g/kg DPM, percent glucose consumption rate was less effective than that of the lowest initial glucose concentration. At higher initial glucose levels of 500, 750 and 1000g/kg DPM, the percent glucose consumptions rate dropped to 71.0, 54.4 and 47.8%, respectively. The profiles for residual glucose concentration were similar for 500, 750 and 1000 g/kg DPM. Despite of high concentration of residual glucose concentration after 72 h of fermentatin, citric acid productions for 500, 700 and 1000 g/kg DPM were dropped. The result from residual glucose concentration indicated that the decreases in citric acid production for high initial glucose treatments may not be resulted from depletion of glucose The same was reported by Falvela-Torres *et al.* (1998) who cultivated *A. niger* 10 and *A. niger* CH4 on sugar cane bagasse for the production of pectinases: they found that complete utilization of initial glucose was obtained only when provided at concentrations up to 450 g/l in solid substrate fermentation.

3.4.4 Change in PM pH

The initial PM pH of 4.42 dropped during the fermentation (Figure 3.4), proportionally with the amount of initial glucose and therefore, the amount of citric acid produced. Although the treatments of 0 and 100 g/kg DPM produced a maximum pH drop of 0.05 and 0.33, the 250, 500, 750 and 1000 g/kg DPM treatments produced higher drops in the pH, falling respectively to 1.04, 1.01, 1.27 and 1.36.

Citric acid concentrations were plotted versus the pH of PM obtained during fermentation to obtained correlation. Citric acid production was correlated well with the pH of PM and citric acid production can be predicted by using given equation in Figure 3.5 ($R^2 = 0.94$). The pH drop resulted from the production of citric and most likely. The protonated forms of organic acids inhibit growth of microorganisms. In low pH environment, the concentration of free citric acid increased and enhanced toxicity in comparison to a higher pH (Patel *et al.*, 2004). Thus, the pH evolution can be an indirect indicator of citric acid production during the fermentation. The pH of all glucose levels started to increase after 72 h, except that of the 1000 g/kg DPM treatment which started to increase after 96 h and this occurred in parallel with the drop in citric acid concentrations.

3.4.5 Modeling the effect of nutrient level on citric acid production

Table 3.2 presents the actual and predicted citric acid production values, obtained experimentally using the CCD after 48 and 72 h. The CCD produced second-order polynomial equations for predicting citric acid production (Y) as a function of coded value of variables at both 48 and 72 h:

$$Y_{48\,h} = 15.87 + 3.41\chi_1 - 5.20\chi_2 + 0.77\chi_3 - 0.68\chi_4 - 3.42\chi_1^2 + 0.077\chi_2^2 - 0.12\chi_3 - 1.02\chi_4^2 + 1.15\chi_1\chi_2 - 1.10\chi_1\chi_3 - 0.096\chi_1\chi_4 - 0.18\chi_2\chi_3 + 5.01\chi_2\chi_4 + 0.40\chi_3\chi_4 \quad (3.1)$$

$$Y_{72\,h} = 29.47 + 18.90\chi_1 - 11.58\chi_2 + 5.89\chi_3 + 0.17\chi_4 + 2.04\chi_1^2 - 1.01\chi_2^2 - 2.92\chi_3^2 + 0.058\chi_4^2 - 3.52_1\chi_2 + 1.37\chi_1\chi_3 + 3.96\chi_1\chi_4 + 0.11\chi_2\chi_3 + 11.78\chi_2\chi_4 - 0.86\chi_3\chi_4 \quad (3.2)$$

The goodness of fit of equations were determining by computing predicted citric acid production values and comparing them to those measured. At 48 and 72 h of fermentation, R^2 for citric acid production was 0.99 and 0.97, which implies a good agreement between the observed and predicted response (Panda *et al.*, 1999; Ambati and Ayyanna, 2001; Vohra and Satyanarayana, 2002).

The significance and adequacy of the second-order equations are measured using analysis of variance. ANOVA (analysis of variance) can be definded as "a method for estimating the amount of variation within all treatment and comparing it to the variables between treatments" (Berthouex and Brown, 1994). The results of the ANOVA are presented in Table 3.3. For citric acid production at 48 h, the levels of glucose and nitrogen, X_1 and X_2, were observed to be significant, while the levels of KH_2PO_4 and NaCl, X_3 and X_4, showed no significant effect. For citric acid production at 72 h, three variables, X_1, X_2 and X_3, showed a significant effect. Thus, glucose and $(NH_4)_2SO_4$ had the most significant impact on citric acid production by *A. niger* at 48 and 72 h.

The coefficients of the second-order regression equations (3.1) and (3.2) measured the effect of the nutrient on the response (Y). The coefficients for glucose (X_1) and potassium phosphate (X_3) were positive indicating that higher levels of such nutrients result in a higher citric acid production at 48 and 72 h of fermentation. All coefficients for nitrogen

(X_2) were negative, indicating a negative effect on citric acid production.

3.4.6 Response surface curves

Three-dimensional response surface curves show the interaction between the levels of two variables, while fixing that of the others. Figures 3.6a and 3.6b showed the interactive effect of glucose and $(NH_4)_2SO_4$ on citric acid production at 48 and 72 h of fermentation. In both plots, citric acid production was increased with increased levels of glucose and the decreased with increased levels of $(NH_4)_2SO_4$. A slight decrease in citric acid production was observed for an initial glucose concentration above 875 g/kg DPM. When $(NH_4)_2SO_4$ was increased from 15 to 45 g/kg DPM, citric acid concentration was decreased for all initial glucose concentrations. A maximum citric acid concentration of 64.5 g/kg DPM was achieved with initial levels of 1000 g glucose/kg DPM and 15 g $(NH_4)_2SO_4$/kg DPM. Similarly, Kristiansen and Sinclair (1979) and Mirminachi *et al.* (2002) reported that low levels of nutrients such as nitrogen, limit the growth of *A. niger* in continuous fermentation, which then fosters higher citric acid production and yield.

The interactive effects of glucose and KH_2PO_4 on citric acid production at 48 and 72 h with fixed level of $(NH_4)_2SO_4$ and NaCl are shown at Figures 3.7a and 3.7b. The surface curve showed curvature and maximum citric acid concentration of 16.9 g/kg DPM at 48 h with an initial glucose concentration of 900 g/kg DPM and 45 g KH_2PO_4/kg DPM. A marginal increase in citric acid production was only found at low glucose levels with higher KH_2PO_4 levels after 48 h, while at 72 h, this marginal increase was found for all levels of glucose. Glucose concentration was found to linearly affect citric acid production at all concentrations of KH_2PO_4 (Figure 3.7b). The effect of phosphorus concentration on citric acid production is still unclear, as Mirminachi *et al.* (2002) reported that phosphorus

limitation induced higher citric acid production and yield, while Shu and Johnson (1984) and Chen (1994) reported higher citric acid production and yield with phosphorus in the range of 0.06 to 0.32 g/l.

Figures 3.10a and 3.10b represents citric acid production as a function of glucose and NaCl at fixed levels of $(NH_4)_2SO_4$ and KH_2PO_4 at 48 and 72 h, respectively. Citric acid production was maximized with 850 g glucose and 7 g NaCl/kg DPM, while not significantly affected by variation of NaCl. At 72 h, maximum citric acid production was achieved with the highest levels of glucose and NaCl, as an increase in NaCl resulted in a slight increase in citric acid production only for initial glucose levels exceeding 750 g/kg DPM. Thus, initial glucose concentration strongly affects citric acid production by *A. niger*, at any concentration of the other tested nutrients.

At 48 and 72 h of fermentation, Figures 3.9a and 3.9b present the surface response curves of citric acid production as a function of $(NH_4)_2SO_4$ and KH_2PO_4. A maximum citric acid production of 22.0 g/kg DPM was predicted using the lowest $(NH_4)_2SO_4$ level and the highest KH_2PO_4 level at 48 h. A similar response surface curve was obtained at 72 h. A maximum citric acid production of 42.9 g/kg DPM was predicted when using 15 g $(NH_4)_2SO_4$ and 45 g KH_2PO_4/kg DPM. Thus, citric acid production increases slightly with KH_2PO_4, when all other nutrients are fixed in both 48 and 72 h of fermentation. These results showed that glucose and $(NH_4)_2SO_4$ are proved to be the two most critical positive and negative nutrients in the solid substrate fermentation of *A. niger* NRRL 567 using PM for citric acid production.

3.4.7 Optimum composition of nutrients

The numerical optimization of CCD method was used to determine the optimum

composition of nutrients, within the range of coded levels from −1 to 1 at 72 h based on the constraints listed on Table 3.4. Although the numerical optimization process suggested 10 possible optimal media (Table 3.5), the second solution was found to be the most economical, producing the highest citric acid for the lowest initial glucose concentration. This initial nutrients showing citric acid production of 73.4 g/kg DPM at 72 h was found to be composed of: 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl.

For validation, this optimal medium was used to grow *A. niger* in the laboratory on PM for 144 h. Figure 3.10 compares the level of citric acid produced in the optimum and basal medium. The control experiment with basal medium produced 53.6 g citric acid as compared to that of 82.0 g/kg DPM, obtained with the optimized medium. Moreover, as compared with the control, the optimized medium reduced the fermentation time from 96 to 72 h. The 1.5-fold increase in citric acid production, along with a reduction of fermentation time by 25% both will contribute to reducing the production cost using PM enriched with glucose.

3.4.8 Citric acid production in submerged fermentation

To compare the result from solid substrate fermentation using PM, a submerged fermentation was employed. Citric acid production and yield of *A. niger* NRRL 567 and the pH profiles are represented in Figures 3.11 and 3.12. Citric acid production and yield maximized at 10.6 g/l and 21.3%, after 144 h. Citric acid yield of 1000 g initial glucose/kg DPM for solid substrate fermentation (47%) was considerably higher than the citric acid concentration obtained from submerged fermentation (21.3%). As compared to the standard submerged fermentation, solid substrate fermentation leads to better overall citric acid production with short fermentation time.

3.5 Conclusions

Moist PM with a high level of initial glucose was found to be a suitable solid substrate for the production of citric acid by *A. niger* NRRL 567. Citric acid production and yield were strongly affected by initial glucose concentration. PM was a good support material only when supplemented with a relatively high level of readily available carbon substrate more than 250 g/kg DPM. However, as high cencentation of initial sugar will add costs to the process, it is necessary to find cheap source of fermentable carbon for citric acid production. In general, for the agro-industrial byproducts such as fruit peels, corn stover, rice straw, wheat straw and other crop residues have low concentration of fermentable sugars, containing up to 35 wt% residual sugars, to supply high concentration of initial sugar, addition of sugar rich byproducts like molasses (72 wt% sugar) is necessary to provide enough fermentable sugar to achieve high citric acid production.

A statistically-based optimization using the CCD method was proved to be useful in optimizing the nutrient compositions for citric acid production by *A. niger* NRRL 567 grown on PM. For citric acid production, glucose and KH_2PO_4 showed a positive impact. Nitrogen, as $(NH_4)_2SO_4$, had a negative effect most likely because it enhances cell growth as opposed to citric acid production, by depressing enzyme synthesis for citric acid production in the TCA cycle, resulting in more energy being spent for biomass production rather than for enzyme synthesis and citric acid production. The second-order polynomial model obtained using CCD method was able to predicted citric acid production given specific levels of nutrients and limited within the tested range.

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Table 3.1 Optimization of medium components (g/kg DPM) for the production of citric acid: Independent variables in central composition design

Variables	Parameter	Coded and actual level				
		-1.68	-1	0	+1	+1.68
X_1	Glucose (g/kg DPM)	330	500	750	1000	1170
X_2	$(NH_4)_2SO_4$ (g/kg DPM)	4.8	15	30	45	55.2
X_3	KH_2PO_4 (g/kg DPM)	4.8	15	30	45	55.2
X_4	NaCl (g/kg DPM)	1.3	4	8	12	14.7

Table 3.2 Experimental and predicted concentration of citric acid for nutrients optimization at 48 and 72 h

Run	X_1	X_2	X_3	X_4	Responses at 48 h			Responses at 72 h		
No					pH	Observed	Predicted	pH	Observed	Predicted
1	1000	45	45	4	3.16	5.14	5.60	2.75	20.00	23.76
2	1000	45	15	4	2.79	6.75	7.44	2.71	8.87	7.30
3	1000	15	45	12	2.71	12.85	13.32	2.84	56.52	60.28
4	500	45	15	12	3.37	3.27	3.96	2.97	6.49	4.92
5	1000	15	15	12	2.74	12.12	12.82	2.84	49.27	47.70
6	500	15	45	4	3.30	21.10	21.56	2.90	33.89	37.65
7	500	45	45	12	3.26	7.69	8.15	2.78	8.69	12.45
8	500	15	15	4	2.81	17.57	18.27	2.90	28.68	27.11
9	330	30	30	8	3.22	1.26	0.45	3.13	5.01	3.46
10	1170	30	30	8	3.10	12.73	11.92	2.78	68.57	67.02
11	750	5	30	8	3.03	25.65	24.83	2.90	47.64	46.09
12	750	55	30	8	3.44	8.17	7.35	2.69	8.68	7.14
13	750	30	5	8	2.60	15.34	14.25	2.96	6.53	11.32
14	750	30	55	8	3.00	17.39	16.84	2.58	39.01	31.13
15	750	30	30	1	3.02	14.95	14.14	2.90	30.90	29.35
16	750	30	30	15	3.00	12.67	11.85	2.86	31.46	29.92
17	750	30	30	8	3.04	15.49	15.87	2.82	28.74	29.47
18	750	30	30	8	2.96	16.43	15.87	2.83	30.52	29.47
19	750	30	30	8	2.90	15.03	15.87	2.82	29.17	29.47

X_1: Glucose; $(NH_4)_2SO_4$; X_2: KH_2PO_4; X_3: KH_2PO_4; X_4: NaCl; all units are g/kg DPM.

Table 3.3 ANOVA and regression analysis for the production of citric acid at 48 and 72 h fermentation

	48 h			72 h		
	Sum of Squares	*F* value	*P* level	Sum of Squares	*F* value	*P* level
Model	682.54	32.47	0.0002*	5934.28	15.11	0.0016*
X_1	65.78	43.81	0.0006*	2019.84	72.01	0.0001*
X_2	152.78	101.75	<0.0001*	758.68	27.05	0.002*
X_3	8.07	5.38	0.0595	473.44	16.88	0.0063*
X_4	2.60	1.73	0.2360	0.16	0.0006	0.9424
X_1^2	175.30	116.75	<0.0001*	62.22	2.22	0.1870
X_2^2	0.089	0.060	0.8153	15.25	0.54	0.4888
X_3^2	0.20	0.13	0.7290	127.03	4.53	0.0774
X_4^2	15.42	10.27	0.0185*	0.051	0.0002	0.9674
X_1X_2	4.37	2.91	0.1388	41.13	1.47	0.2715
X_1X_3	9.71	6.47	0.0439*	15.03	0.54	0.4917
X_1X_4	0.031	0.021	0.8908	51.87	1.85	0.2227
X_2X_3	0.26	0.18	0.3601	0.093	0.0003	0.9560
X_2X_4	83.04	55.30	0.0003*	460.07	16.40	0.0067*
X_3X_4	1.31	0.87	0.3868	5.92	0.21	0.6621

*Significant at the 95% level.

R^2 (coefficient of determination) = 0.99 (48 h) and 0.97 (72 h).

Table 3.4 The constraints for optimization of the medium composition using CCD at 72 h

Variables	Goal	Lower Limit (g/kg DPM)	Upper Limit (g/kg DPM)
Glucose	-	500	1000
$(NH_4)_2SO_4$	-	15	45
KH_2PO_4	-	15	45
NaCl	-	4	12
Citric acid conc. at 72 h	maximize	5.00	68.57

Table 3.5 Optimum levels of medium components (g/kg DPM) and predicted optimum citric acid concentration by numerical optimization using CCD at 72 h

Solutions Number	Glucose (X_1)	$(NH_4)_2SO_4$ (X_2)	KH_2PO_4 (X_3)	NaCl (X_4)	Citric acid (g/kg DPM)
1	993.77	15.03	44.57	7.29	69.5
2*	**967.88***	**15.35***	**43.90***	**4.04***	**73.4***
3	995.11	15.36	44.19	7.36	69.1
4	999.51	17.81	44.78	5.77	69.7
5	997.91	16.27	34.81	5.12	70.2
6	991.01	16.76	44.53	5.88	70.0
7	999.56	15.66	34.53	6.33	68.9
8	990.34	15.85	42.02	5.80	70.9
9	947.83	16.18	38.85	4.09	69.1
10	1000.00	35.57	41.50	12.00	56.4

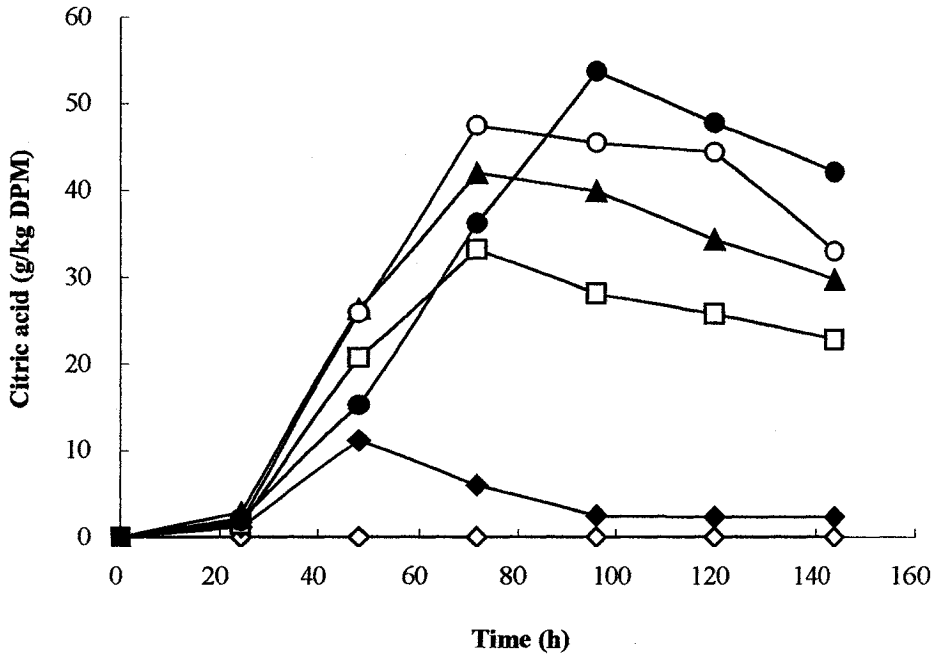

Figure 3.1 Effect of initial glucose concentration on citric acid production from PM by *A. niger* NRRL 567 grown at 30°C; basal salt solution containing 20 g $(NH_4)_2SO_4$, 20 g KH_2PO_4, 4 g NaCl, 4 g $MgSO_4 \cdot 7H_2O$ and 4 g $FeSO_4 \cdot 7H_2O$/kg of DPM; (◇: 0 g/kg DPM; ◆: 100 g/kg DPM; □: 250 g/kg DPM; ▲: 500 g/kg DPM; ○: 750 g/kg DPM; ●: 1000 g/kg DPM).

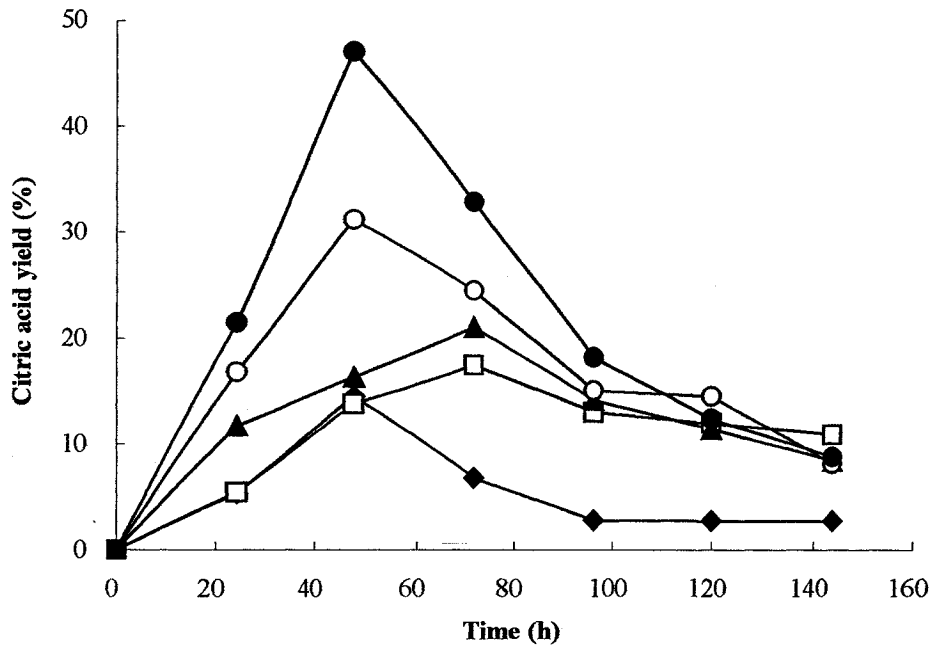

Figure 3.2 Effect of initial glucose concentration on the percent citric acid yield from PM by *A. niger* NRRL 567. The culture was grown at 30°C in a basal salt solution of 20 g $(NH_4)_2SO_4$, 20 g KH_2PO_4, 4 g NaCl, 4 g $MgSO_4 \cdot 7H_20$ and 4 g $FeSO_4 \cdot 7H_2O$/kg of DPM; (◆: 100 g/kg DPM; □: 250 g/kg DPM; ▲: 500 g/kg DPM; ○: 750 g/kg DPM; ●: 1000 g/kg DPM).

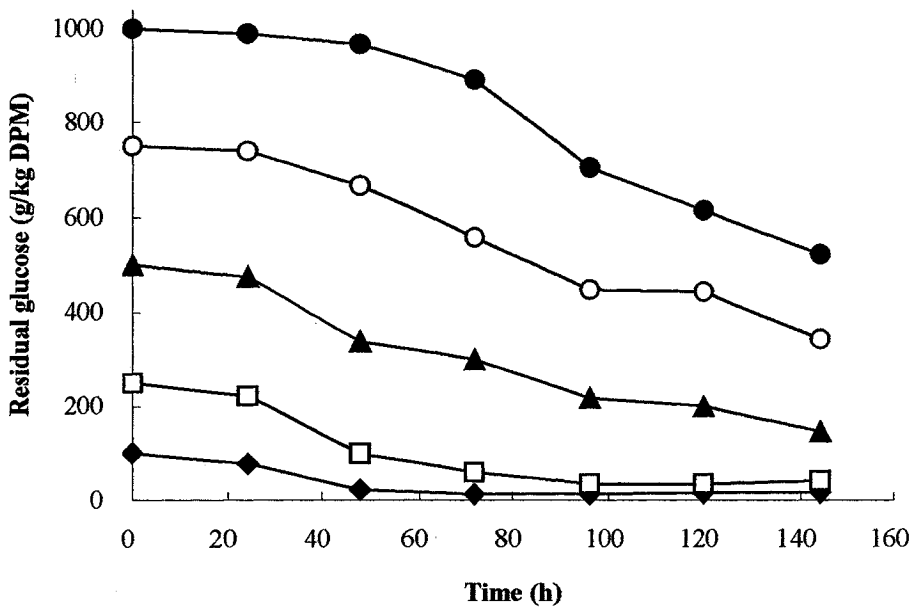

Figure 3.3 Time course of residual glucose concentration from PM by *A. niger* NRRL 567 with various initial glucose levels. The culture was grown at 30°C in a basal salt solution of 20 g $(NH_4)_2SO_4$, 20 g KH_2PO_4, 4 g NaCl, 4 g $MgSO_4 \cdot 7H_20$ and 4 g $FeSO_4 \cdot 7H_2O$/kg of DPM; (◆: 100 g/kg DPM; □: 250 g/kg DPM; ▲: 500 g/kg DPM; ○: 750 g/kg DPM; ●: 1000 g/kg DPM).

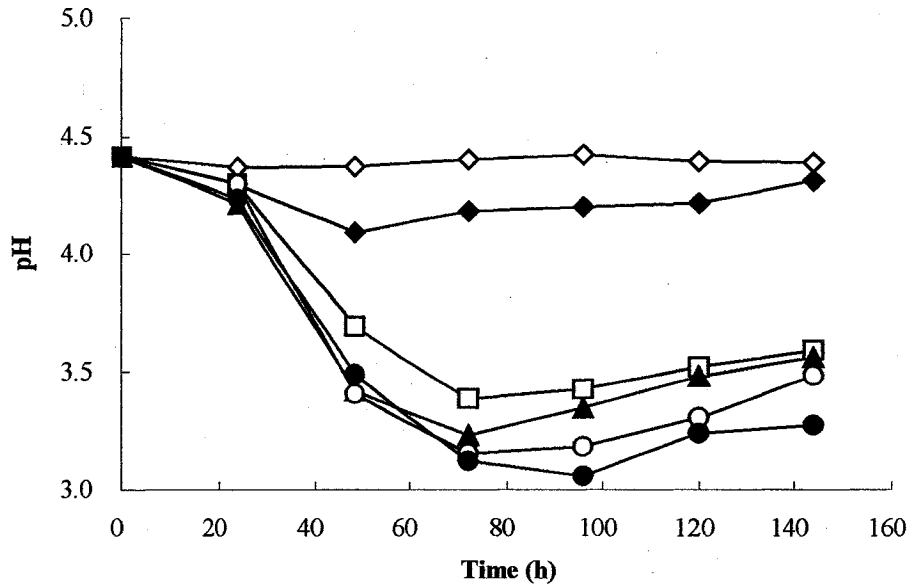

Figure 3.4 Effect of initial glucose concentration on the evolution of the pH from PM by *A. niger* NRRL 567. The culture was grown at 30°C using a basal salt solution of 20 g $(NH_4)_2SO_4$, 20 g KH_2PO_4, 4 g NaCl, 4 g $MgSO_4 \cdot 7H_20$ and 4 g $FeSO_4 \cdot 7H_2O$/kg of DPM; (◇: 0 g/kg DPM; ◆: 100 g/kg DPM; □: 250 g/kg DPM; ▲: 500 g/kg DPM; ○: 750 g/kg DPM; ●: 1000 g/kg DPM).

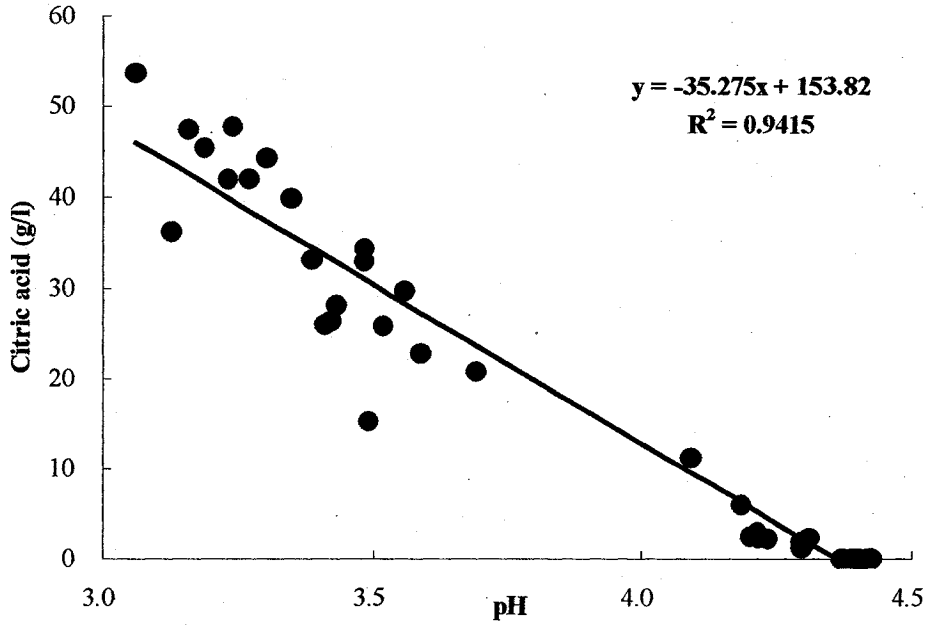

Figure 3.5 The relationship between citric acid concentration and the pH during the fermentation with various concentrations of initial glucose concentration from 0 to 1000 g/kg DPM.

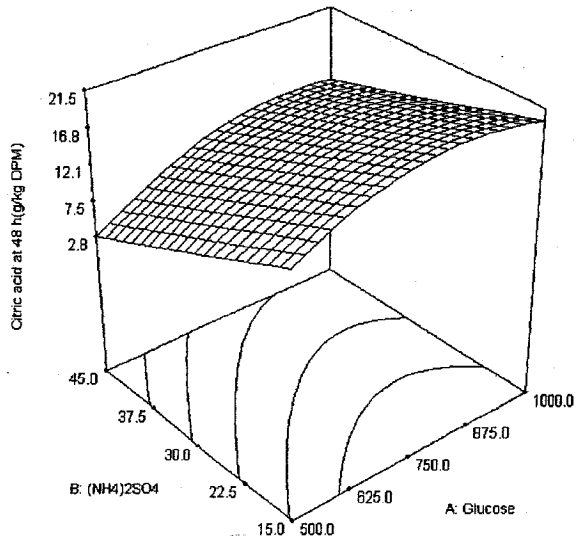

Figure 3.6a Response surface curve for citric acid production representing the interaction between the initial concentration of glucose and $(NH_4)_2SO_4$ at 48 h. Other variables are fixed at 30 g KH_2PO_4 and 8 g NaCl/kg DPM.

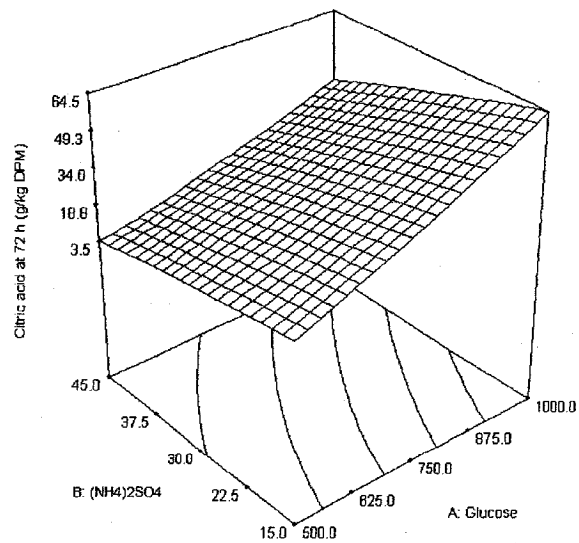

Figure 3.6b Response surface curve for citric acid production representing the interaction between the initial concentration of glucose and $(NH_4)_2SO_4$ at 72 h. Other variables are fixed at 30 g KH_2PO_4 and 8 g NaCl/kg DPM.

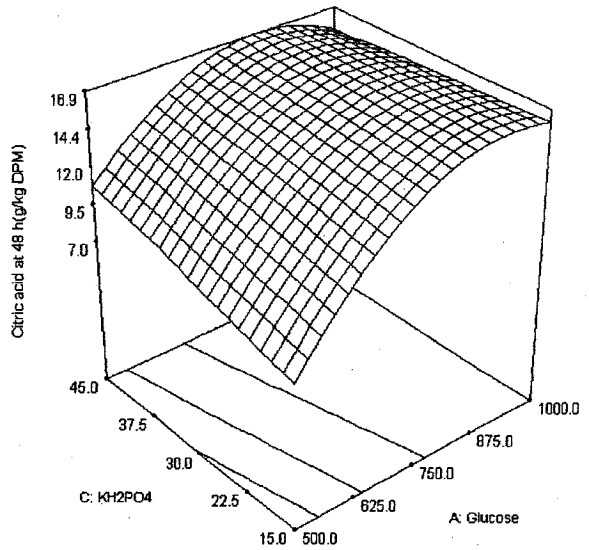

Figure 3.7a Response surface curve for citric acid production representing the interaction between initial concentration of glucose and KH_2PO_4 at 48 h. Other variables are fixed at 30 g $(NH_4)_2SO_4$ and 8 g NaCl/kg DPM.

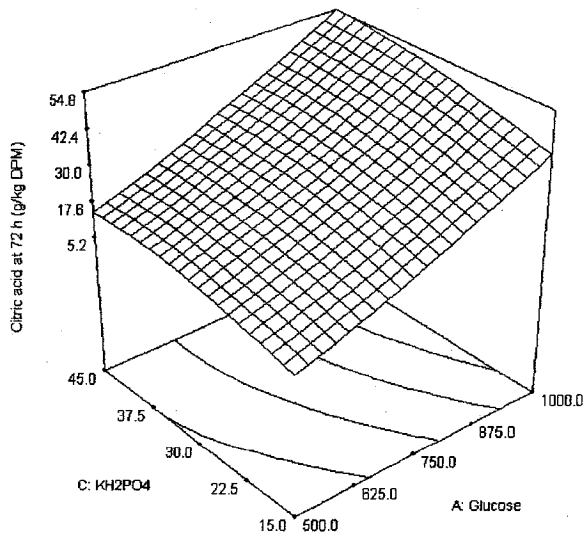

Figure 3.7b Response surface curve for citric acid production representing the interaction between the initial concentration of glucose and KH_2PO_4 at 72 h. Other variables are fixed at 30 g $(NH_4)_2SO_4$ and 8 g NaCl/kg DPM.

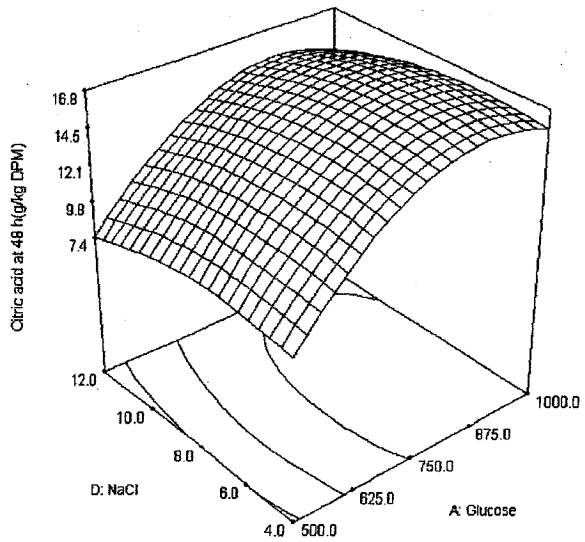

Figure 3.8a Response surface curve for citric acid production representing the interaction between the initial concentration of glucose and NaCl at 48 h. Other variables are fixed at 30 g $(NH_4)_2SO_4$ and 30 g KH_2PO_4/kg DPM.

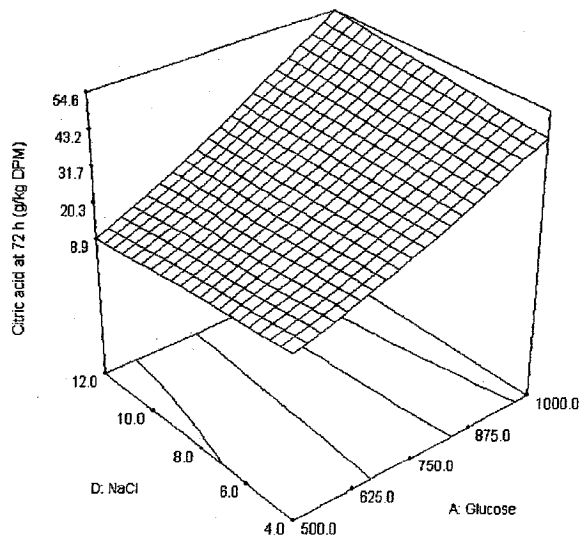

Figure 3.8b Response surface curve for citric acid production representing the interaction between the initial concentration of glucose and NaCl at 48 h. Other variables are fixed at 30 g $(NH_4)_2SO_4$ and 30 g KH_2PO_4/kg DPM.

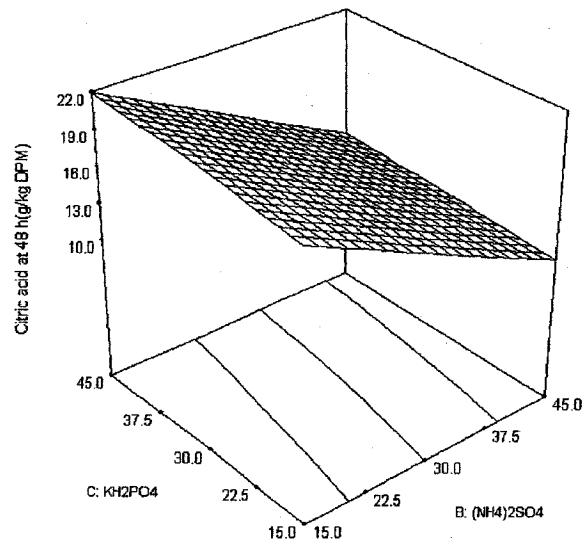

Figure 3.9a Response surface curve for citric acid production representing the interaction between the initial concentration of $(NH_4)_2SO4$ and NaCl at 48 h. Other variables are fixed at 750 g glucose and 8g NaCl/kg DPM.

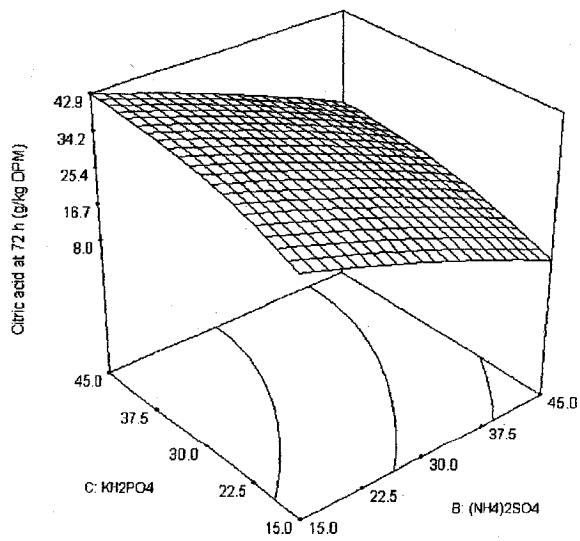

Figure 3.9b Response surface curve for citric acid production representing the interaction between the initial concentration of $(NH_4)_2SO_4$ and KH_2PO_4 at 72 h. Other variables are fixed at 750 g glucose and 8g NaCl/kg DPM.

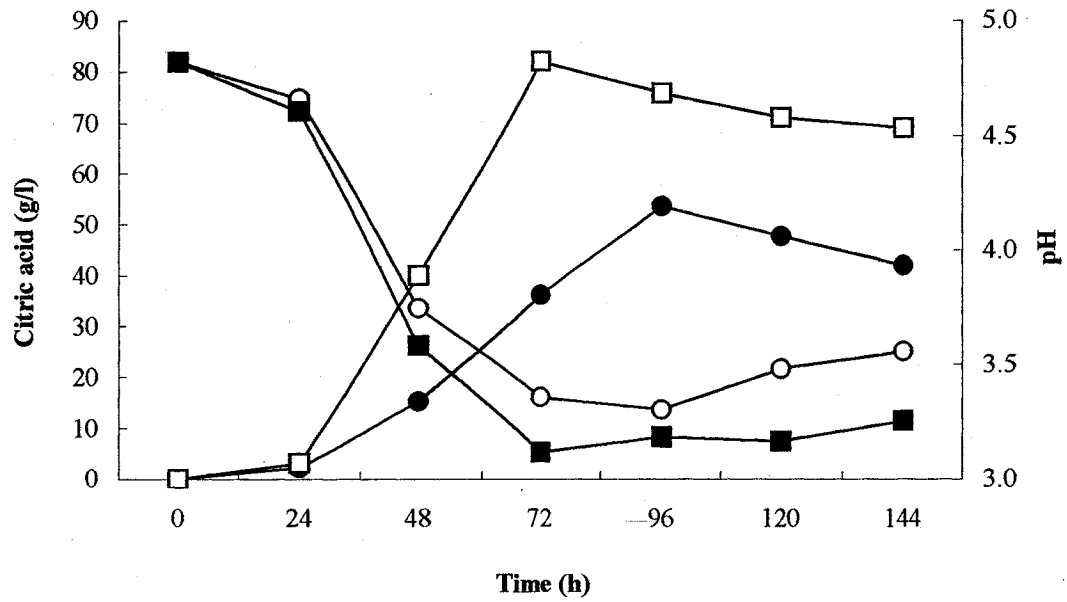

Figure 3.10 Time course behaviors of citric acid production and pH by *A. niger* NRRL 567 in solid substrate fermentation with different media compositions. The control contained used a basal salt solution with 1000 g glucose, 20 g $(NH_4)_2SO_4$, 20 g KH_2PO_4, 4 g NaCl, 4 g $MgSO_4 \cdot 7H_2O$ and 4 g $FeSO_4 \cdot 7H_2O$/kg DPM, while the optimized medium contained 967.9g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0g NaCl/kg DPM; ●:control (citric acid production); □:optimized medium (citric acid production); ○:control (pH); ■:optimized medium(pH).

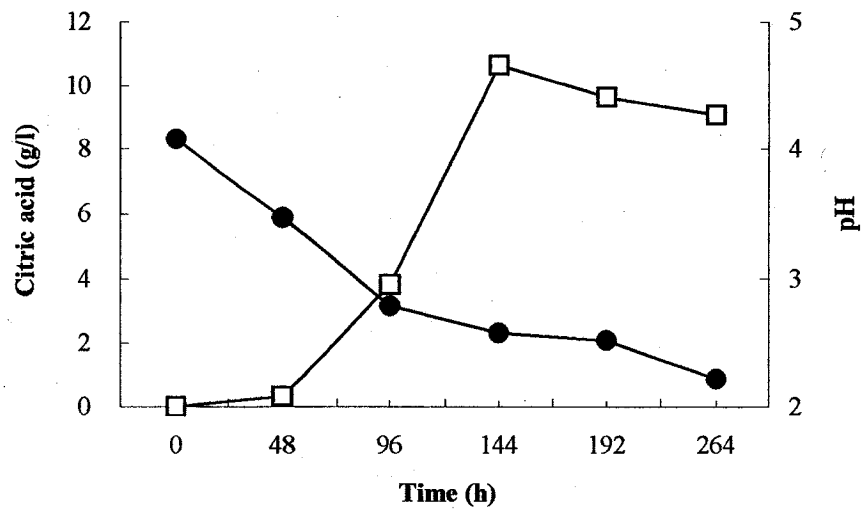

Figure 3.11 Time courses of citric acid production and the pH evolution obtained with *A. niger* NRRL 567 in submerged fermentation grown at 30°C using Czapek-Dox medium; (□: Citric acid; ●: pH).

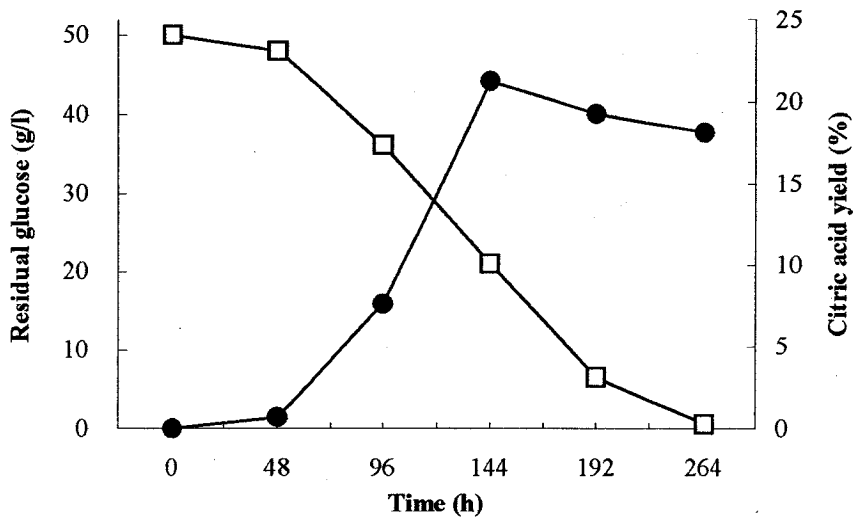

Figure 3.12 Time course of residual glucose concentration, percent yield of citric acid and biomass obtained with *A. niger* NRRL 567 in submerged fermentation at 30°C using Czapek-Dox medium; (□: Residual glucose; ●: Citric acid yield).

CONNECTING STATEMENT

Besides glucose and salt levels, physico-chemical fermentation parameters significantly affect citric acid production by *A. niger*. The next chapter studies the optimization of the physico-chemical fermentation parameters in solid substrate fermentation, using two consecutive steps: first step, "one-factor-at-a-time" was applied while keeping all other constant, to approximate the optimum value of each fermentation parameter. Second step, several fermentation parameters were changed simultaneously, while still keeping them within the range of the optimum value found in the first step.

The chapter 4 deals with optimization of the fermentation parameters for citric acid production by *A. niger* grown on peat moss. Various initial parameters such as solid substrate composition, moisture content, particle size distribution, fermentation temperature, pH and inoculum density were studied for finding optimum fermentation condition. To predict the optimum physico-chemical fermentation condition, this study describes conducting an experiment where a central composite design tested the interactive effects between parameters to optimize them as a group rather than individually. The results from this study were compared with the output of the previous experiment.

This paper has been presented in **Process Biochemistry in 2004 for review. Authors: Jin-Woo Kim, Suzelle Barrington, John Sheppard and Mari Shin.** The contribution of authors are: 1) First author carried out entire experiment work and writing of manuscripts, 2) second author supervised and technical correction of the work and manuscripts, 3) third author provided fermentation facilities and manuscript correction, and 4) fourth author provided her analytical advice and manuscript correction.

CHAPTER 4

Effect of Fermentation Parameters on Citric Acid Production by *Aspergillus niger* 567 Grown on Peat Moss: Statistically-based Optimization

4.1 Abstract

Solid substrate fermentation is ideal to grow *Aspergillus niger* on a sugar rich byproduct to produce citric acid for various purposes. Such technology requires the optimization of the physico-chemical fermentation parameters, the objective of the project. An initial optimization study was conducted using simulated sugar-rich byproduct wetted with an optimized glucose and mineral solution inoculated with *A. niger* NRRL 567 using "one-factor-at-a-time" method. Duplicate samples were fermented under a given set of fermentation conditions: initial moisture content of solid substrate (MC), composition of various ratios of peat moss (PM) and bark-less pine shaving (PS) and particle size distribution; incubation temperature; initial nutrient solution pH; and inoculum density. And then, statistically-based optimization was employed to investigate the inter-related effects of variables using a central composite design (CCD). This model was used to identify the optimum fermentation conditions leading to a maximum citric acid production: a fermentation temperature of 35°C, an initial substrate pH of 8, an inoculum density of 4 x 10^6 spores/ml and a PM initial MC of 80%. Growing *A. niger* on under the conditions optimized by the the CCD method produced a maximum citric acid level of 133.0 g/kg dry peat moss (DPM) at 72 h, compared to the predicted by of 122.0 g/kg DPM at 72 h and to that of 82.0 g/kg DPM obtained with the previously optimized conditions.

Keywords: *A niger*, solid substrate fermentation, citric acid, optimization.

4.2 Introduction

A variety of fungi are reported to produce organic acids such as citric, oxalic, succinic and malic acid. Citric acid production using the filamentous fungus *Aspergillus niger* is well known and widely used by industries producing food, beverages, chemicals and pharmaceutical products (Haq *et al.*, 2001). Presently, citric acid production by *A. niger* is economically produced using submerged fermentation (Pazouki *et al.*, 2000; Vandenberghe *et al.*, 2000). However, the global demand for citric acid is growing faster than its production, implying that more economical processes are required to supplement or replace the present processes (Hang *et al.*, 1987; Alvarez-Vasquez *et al.*, 2000).

The most important physico-chemical fermentation parameters influencing the growth of *A. niger* on a solid substrate and its production of citric acid are: nutrient balance, solid substrate composition, MC and particle size distribution, fermentation temperature, pH and inoculum density (Xu *et al.*, 1989; Jianlong and Ping, 1998). Kim *et al.* (2004a,b) simulated such a system by growing *A. niger* on PM wetted with a glucose and mineral solution optimized to produce a citric acid level of 82.0 g/kg DSS after 72 h of fermentation. Besides sugar and mineral levels, the most important physico-chemical fermentation parameters listed above also need optimization (Battaglin *et al.*, 1991; Wen and Chen, 2001).

The objective of the research was therefore to optimize the physico-chemical fermentation parameters, using two consecutive steps: the first step, where one condition at a time was changed while keeping all other constant, to approximate the optimum value of each fermentation condition and; the second step, a statistical procedure was then used to identify the optimal set of interactive fermentation parameters where several fermentation parameters were changed at the same time, while still keeping them within

the range of the optimum value found in the first step.

4.3 Materials and Methods

4.3.1 Microorganism

Aspergillus niger NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was stored in a tube containing glycerol (30% v/v) at –76°C. *A. niger* spores were produced on a potato dextrose agar (PDA, Sigma, St. Louis, MO, USA) plates at 30°C and were sub-cultured at biweekly intervals. After ten days of incubation on PDA plates, 10 ml of 0.1% Tween 80 were added to each plate to harvest spores. Diluted spore suspensions of 1.0×10^7 spores/ml were counted using hemocytometer and prepared as inoculum for the experimental media.

4.3.2 Solid substrate

Sphagnum PM (Schultz Company, Mississauga, Ontario, Canada) and bark-less PS were used as the solid substrates. Both were characterized for density, total carbon, total nitrogen, total phosphorus, pH and NH_4-N absorption (Table 4.1). After drying at 60°C for 48 h and sieved to obtain desired particle sizes, both were rewetted with the nutrient solution before growing *A. niger*.

4.3.3 Fermentation condition for "one-factor-at-a-time" method

Small-scale experiments were conducted using the 250-ml Erlenmeyer flasks containing 7 g of DSS wetted with the required volume of solution containing glucose and minerals adjusted to obtain the following optimum levels. In a previous study, Kim *et al.*, (2004a,b) produced citric acid in batch experiments using *A. niger* grown on PM, using an

optimized initial glucose and mineral solution composed per kg dry solid substrate (DSS) of: 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl (Kim, 2004a,b). After autoclaving, each flask was inoculated with a 1.0 ml spore solution of *A. niger* NRRL 567 and incubated in a closed chamber for 48 and 72 h.

To evaluate the effect of each physico-chemical parameter on citric acid production, the "one-condition-at-a-time method" was applied. The effect of initial MC was varied by adding more deionized water to the solid substrate to obtain initial levels of 60, 70, 75, 80 and 85% (Miller, 1999). After addition of nutrients to the wet solid substrate, its resulting MC corresponded to either 40.6, 53.9, 61.0 67.3 and 74.7%, respectively (Table 4.2). To evaluate an effect of fermentation temperature on citric acid production, the fermentation temperature was varied from 13 to 42°C. The initial nutrient solution pH was varied from 2.08 to 7.10 by adding 1 N HCl or 1 N NaOH. Similarly, 1.0 ml of inoculum containing either 0.25, 0.5, 1.0, 2.0 or 4.0 x 10^6 spores/ml was added to the each flask after autoclaving. The solid substrate composition was varied using PM and PS at a ratio ranging from 0:1 to 1:0 based on dry weight. Further, the PM particle size was varied by sieving to various particle size distributions.

Finally, citric acid was produced using the conditions optimized previously and using the initial control conditions consisting of: as the solid substrate of 100% PM sieved to remove all particles over 2.0 mm and initially wetted to a MC of 80% using an nutrient solution with an initial pH of 4.28, incubated at 30°C with 1.0 ml of inoculum containing 1.0 x 10^6 spores/ml.

4.3.4 Fermentation condition for central composite desing (CCD)

The initial pH of the nutrient solution was adjusted to either 2, 4, 6, 8 or 10 by adding

1 N NaOH or 1 N HCl. Because of the high buffering capacity of the PM, its resulting initial pH corresponded to either 3.92, 4.35, 4.71, 5.47 or 5.87, respectively. The effect of initial MC was investigated by supplementing with deionized water, to obtain levels of 65, 70, 75, 80 and 85%. In preparation for solid substrate fermentation, the PM was wetted with an optimized nutrient solution containing glucose and minerals and was autoclaved for 15 minutes at 121°C. After autoclaving, the solid substrate was inoculated with 1.0 ml of inoculum with either 0.5, 2.0, 3.5, 5.0 or 6.5 x 10^6 spores/ml. The effect of incubation temperature was measured by incubating the 250-ml Erlenmeyer flasks and their contents for 3 days in a closed chamber maintained at either 20, 25, 30 35 or 40°C.

4.3.5 Analytical methods

For each sampling session, the contents of the duplicate 250-ml Erlenmeyer flasks were withdrawn and 10 g of wet sample was harvested from each flask; 5 g was used to measure the MC of the PM and the other 5 g of wet sample was placed in 50 ml of distilled water and shaken for 60 min at 150 rpm and 22°C. The supernatant was tested for the pH and stored at –20 °C for citric acid quantification (Xu *et al.*, 1989).

After adding pyridine and acetic anhydride to develop color, citric acid was determined by spectrophotometry at 420 nm (Marier and Boulet, 1958; Miller, 1958). The substrate MC was determined by drying at 60°C for 48 h.

4.4 Results and discussion

4.4.1 Effect of initial moisture content (MC)

Initial PM MC had a considerable effect on citric acid production by *A. niger*, after 48 and 72 h of fermentation, as illustrated in Figure 4.1. A maximum citric acid production of

78.4 g/kg DSS was obtained with 75% and 80% MC after 72 h, where both levels produced similar results. An initial MC dropping from 70% to 60% resulted in a drop in citric acid production from 54.6 to 5.7 g/kg DSS, after 72 h of fermentation. Also, an initial PM MC over 80%, such as 85%, dropped citric acid production after 48 and 72 h. The MCs of PM at 48 and 72 h are listed in Table 4.3. In all treatments, MCs were not varied during the fermentation. If the MC of the substrate is too low, this generally results in a low nutrient solubility and poor substrate swelling reducing citric acid production (Nampoothiri *et al.*, 2003). If the MC exceeds the substrate's retention capacity, this results in insufficient aeration and less mycelium growth (Battaglin *et al.*, 1991).

4.4.2 Effect of temperature

After 48 h of fermentation, *A. niger* produced a higher level of citric acid at 35°C, while after 72 h, its production ranged between 75 and 78 g of citric acid/kg DSS at 25, 30 and 35°C (Figure 4.2). Modifying the fermentation temperature from its optimum range reduced citric acid production. For temperatures of 13 and 42°C, the results obtained indicate that *A. niger* NRRL 567 would produce only 6 g of citric acid/kg DSS. In a low or high incubation temperature, Nampoothiri *et al.*, (2003) found that citric acid production could be affected by a retarded germination of fungi, slow metabolic activity, enzyme denaturation and low cell viability.

4.4.3 Effect of pH

The initial pH of the nutrient solution was adjusted to 2.08, 3.00, 4.28 and 7.10, but due to the substrate's high buffering capacity, its initial pH once wetted was 4.06, 4.26, 4.37 and 5.12, respectively (Figure 4.3). The results showed that nutrient solution pH had

to have an important effect on citric acid production. The best citric acid production was obtained at a pH 7.1. However, the highest tested initial pH of 7.1 did not lead to a maximum in citric acid production after 72 h of fermentation. Thus, higher pH conditions must be tested to optimize growth conditions. The time course changes of the pH during the fermentation are shown in Figure 4.4. All initial PM pHs decreased during the fermentation and pH drops were directly proportional to citric acid production. The treatments of a pH 7.1 showed a maximum pH drop from a pH 5.12 at 0 h to 2.86 at 72 h. The initial pH of 3.0 and pH 4.28 showed similar tendencies for pH, to drop respectively to pH 3.85 and pH 3.84 at 72 h. Therefore, it was assumed that the decrease in pH during the fermentation was caused by the production of citric acid (R^2 = 0.84). Thus, pH variation during the solid substrate fermentation can be on indirect indicator of citric acid production.

4.4.4 Effect of inoculum density

As illustrated in Figure 4.5, increasing the inoculum density had an effect on increasing citric acid production after 48 and 72 h of fermentation. A maximum in citric acid production had not yet been reached with the highest inoculum density of 4.0 x 10^6/ml, where *A. niger* produced 95.8 g of citric acid/kg DSS corresponding to almost a 22.2% increase as compared to the control using an inoculum density of 1.0 x 10^6/ml. Thus, inoculum density above 4.0 x 10^6/ml must be tested to optimize citric acid production.

4.4.5 Effect of PM to PS ratio

Maximizing the level of PM, against that of PS, increased citric acid production

(Figure 4.6) after 48 and 72 h of fermentation. A solid substrate composed of 100% PM produced a maximum citric acid production of 78.4 g/kg DSS after 72 h. Due to its high lignin content, lower moisture absorption capacity and, most probably, its tannin content, PS proved to be a poor substrate for *A. niger*, as compared to PM.

4.4.6 Effect of particle size

The particle size distribution of the solid substrate influences fungal growth and citric acid production by affecting air diffusion, particle surface area for microbe adhesion, heat exchange and water retention capacity. Used as a solid substrate, only the PM with the finest particles reduced citric acid production (Figure 4.7). The highest citric acid production was obtained with a mixed range of particle size under 2.0 mm. The negative effect of the finest particles is most likely due to smaller pores clogging more easily under moist conditions, resulting in less aeration. The mixed particle size distribution under 2.0 mm most likely helped retain a better porosity under the same moisture conditions, offering better surface aeration, mycelium attachment and cell propagation.

4.4.7 Modeling citric acid production

Table 4.5 presents the 19 combinations of input variables and their citric acid production. This data produced two second-order polynomial equations where citric acid production was correlated with all four physico-chemical parameters after 48 ($Y_{48\,h}$) and 72 h ($Y_{72\,h}$) of fermentation, respectively:

$$Y_{48\,h} = 18.69 + 0.94\chi_1 + 13.86\chi_2 + 2.73\chi_3 + 7.15\chi_4 - 2.86\chi_1^2 + 3.46\chi_2^2 - 1.55\chi_3 + 0.33\chi_4^2 - 6.66\chi_1\chi_2 + 1.49\chi_1\chi_3 + 21.53\chi_1\chi_4 + 0.23\chi_2\chi_3 - 11.93\chi_2\chi_4 + 1.27\chi_3\chi_4 \quad (4.1)$$

$$Y_{72\,h} = 78.19 + 4.60\chi_1 + 23.0\chi_2 + 6.24\chi_3 + 25.51\chi_4 - 15.94\chi_1^2 - 0.81\chi_2^2 - 1.05\chi_3^2 - 5.62\chi_4^2 - 10.36\chi_1\chi_2 - 1.93\chi_1\chi_3 + 18.22\chi_1\chi_4 + 2.36\chi_2\chi_3 + 4.40\chi_2\chi_4 - 3.77\chi_3\chi_4 \quad (4.2)$$

where χ_1, χ_2, χ_3 and χ_4 correspond to the coded values of physico-chemical parameters (temperature, a nutrient solution initial pH, inoculum density and initial PM MC).

The quality of fit for this second-order regression equation was checked using the coefficitent of determination (R^2), found to be 0.981 and 0.987 for 48 and 72 h of fermentation, respectively. Such higher coefficients indicate good agreement between the observed and predicted response, as only 1.9 and 1.3% of the total response variation was not explained by the model (Pazouki *et al.*, 2000).

The significance of the second-order equations are measured using analysis of variance (ANOVA). It can be definded as "a method for estimating the amount of variation within all treatment and comparing it to the variables between treatments" (Berthouex and Brown, 1994). The model *F* values of 14.41 and 21.30 for citric acid production at 48 and 72 h (Table 4.6). These high *F* values imply that the two models (48 and 72 h) are significant and can accurately predict the experimental results. For each parameter tested (X_1, X_2, X_3 or X_4), the significance in variation in citric acid production can be evaluated. For citric acid production after 48 and 72 h, produced *P* levels less than 0.05 for X_2 (initial pH) and X_4 (initial MC), indicating a significant effect (Ellaiah *et al.*, 2003).

The coefficients of each first term of the second-order regression equation provided a measure of the effect level of the independent variable on the response. A large positive value for the coefficient indicates a significant positive response (Kumar *et al.*, 2003). Thus, a nutrient solution initial pH and initial MC of PM had a significant effect on citric

acid production.

4.4.8 Modeling the effect of physico-chemical conditions on citric acid production

Using the model presented in equations (4.1) and (4.2), Figure 4.8a and 4.8b predict the interactive effect of fermentation temperature and a nutrient solution initial pH on citric acid production after 48 and 72 h, respectively. Both plots indicate that citric acid production increased with the nutrient solution initial pH, but not with temperature. Citric acid production showed a steep increase as initial pH increased from 4.0 to 8.0. As for temperature, the surface plot shows a slight curvature peaking near 30°C. Thus, a maximum citric acid production of 101.0 g/kg DPM was obtained with the initial pH and temperature combination of 8.0 and 30°C, after 72 h of fermentation.

Figure 4.9a and 4.9b predict the interactive effects of temperature and inoculum density on citric acid production after 48 and 72 h of fermentation. A fermentation temperature of 30 and 32.5°C combined with the highest inoculum density of 5.0 x 10^6 spores/ml produced the most citric acid after 48 and 72 h, respectively. Inoculum density linearly affected citric acid production at all ranges of fermentation temperature, which maximized at 83.5 g/kg of DPM, after 72 h of fermentation.

Since the MC of PM during the fermentation a had strong correlation with the fermentation temperature (Roukas, 2000), the interactive effect of these two conditions was examined (Figures 4.10a and 4.10b). The highest citric acid production level was obtained using the lowest fermentation temperature and MC and the highest fermentation temperature and MC after 48 and 72 h, respectively. Because of water evaporation, prolonged fermentation at higher temperatures led to low PM MC and a drop in citric acid production. Only a higher initial MC can compensate for this. Thus, a high fermentation

temperature accompanied by a high initial MC resulted in a predicted maximum citric acid production of 106.2 g/kg DPM. This observation also led to the conclusion that the solid substrate must have a high water retention capability, or else, it must be regularly watered.

Figure 4.11 and 4.11b predict the interactive effects of the nutrient solution initial pH and MC of the PM on citric acid production after 48 and 72 h of fermentation at 30°C. High citric acid production levels were predicted using a combination of high pH with all ranges of MC after 48 h while after 72 h, the effect of initial MC was more pronounced and a maximum citric acid production of 124.7 g/kg DPM was predicted for a nutrient solution with an initial pH of 8.0 and an initial PM MC of 80%.

4.4.9 Predicting optimum conditions for citric acid production

To predict optimum fermentation conditions, considering all physico-chemical parameters, the numerical optimization method of CCD was employed (Table 4.7). The first five series of physico-chemical conditions show similar maximum citric acid production levels after 72 h. However, if economic aspects are considered, the fifth series predicts the best results, since it uses a lower inoculum density (3.94×10^6 spores/ml) for a relatively high citric acid production of 46.1 and 122.0 g/kg DPM after 48 and 72 h of fermentation using a temperature of 35°C, a nutrient solution initial pH of 8.0 and an initial MC of 80%.

The control, one-parameter-at-a-time, and CCD method optimized fermentation conditions were used to grow *A. niger* NRRL 567 for 144 h, to compare their citric acid productions (Figure 4.12). After 80 h of fermentation, the optimized set of conditions produced maximum citric acid concentrations of 133.0 g/kg DPM, as compared to the control and traditional optimization peaking at 82.0 and 104.2 g/kg DPM. The physico-

chemical parameters optimized traditional had produced a maximum citric acid concentration of 102.7 and 104.2 g/kg DPM after 72 and 96 h of fermentation. Thus, CCD method further optimized physico-chemical fermentation parameters by increasing its level of citric acid production. Those conditions found optimal using the CCD method were: 100% PM as solid substrate, wetted initially to 80% MC using a nutrient solution with an initial pH of 8.0, fermented at 30°C using an inoculum density of 4.0 x 10^6 spores/ml.

4.5 Conclusions

The traditional "one-condition-at-a-time" strategy produced a set of optimal fermentation conditions which were tested simultaneously against a control. Using a combination of all conditions at their optimal level, citric acid production reached 102.7 and 104.2 g/kg DSS after 72 and 96 h of fermentation, as compared to 82 g/kg DSS with the initial control conditions, representing a 26.8% increase. (Figure4.12). For both fermentation conditions, extending the fermentation time beyond the peaks, the depletion or the low level of fermentable glucose in the solid substrate may result in oxidation of citric acid as an alternative carbon source (Hang and Woodams, 1998).

In this study, the fermentation conditions were varied one at a time. Nevertheless, each physico-chemical parameter may exert an interaction on the others. Thus, the values obtained are a first optimization step. As opposed to traditional optimization method, the CCD method further optimized citric acid production by growing *A. niger* on PM. The physico-chemical parameters having the most impact on citric acid production were: a nutrient solution initial pH and initial MC of PM. Temperature (25 – 35°C) and inoculum density (2.0 – 5.0 x 10^6 spores/ml) had no effect on citric acid production for the range of

values tested.

4.6 Acknowledgements

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Table 4.1 Physico-chemical characteristics of PM and PS

	Unit	Peat moss (PM)	Pine shavings (PS)
Bulk density	kg/m^3	276.4	780
Carbon	% DW	53.1	54.4
Nitrogen	% DW	2.1	0.064
Phosphorus	% DW	0.01	-
Potassium	% DW	0.15	-
Ash	% DW	2.8	0.38
Volatile solids	% DW	97.1	-
NH_4-N absorption capacity	mg/kg	27.5	-
pH	-	4.38	4.4
Coarse particles (> 2.0 mm)	% DW	14.3	100
Medium particles (1.0 – 2.0 mm)	% DW	18.4	-
Fine particles (0.5 – 1.0 mm)	% DW	20.5	-
Very fine particles (< 0.5 mm)	% DW	46.8	-

Note:

All analysis are reported on a dry weight basis; the carbon content was calculated from {(100% - ash%)/183} (Barrington et al., 2002);

DW = dry weight.

Table 4.2 Levels of various physico-chemical conditions tested

Parameters	Unit	Level 1	Level 2	Level 3	Level 4	Level 5
Initial MC	%	60[a]	70[a]	75[a]	80[a]	85[a]
		(40.6[b])	(53.9[b])	(61.0[b])	(67.3[b])	(74.7[b])
Solution pH*		2.08[c]	3.00[c]	4.28[c]	7.10[c]	-
(PM pH)		(4.06[d])	(4.26[d])	(4.37[d])	(5.12[d])	
Temperature	°C	13	25	30	35	42
Inoculum density	spores/ml	0.25×10^6	0.5×10^6	1.0×10^6	2.0×10^6	4.0×10^6
PM MC	%	0	25	50	75	100
Particle size	mm	Mixed[e]	0.15–0.25	0.25–0.5	0.5–1.0	1.0–2.0

Note :

[a]Initial MC of wet PM before adding nutrients,

[b]MC after adding nutrient,

[c]Nutrient solution initial pH,

[d]pH of solid substrate after autoclaving,

[e]Mixed particle smaller than 2.0 mm.

Table 4.3 Effect of initial MC (%) of PM on citric acid production

Initial MC	MC + nutrient [a]	MC at 0 h	MC at 48 h	MC at 72 h
60	40.6	40.6	41.3	39.7
70	53.9	53.9	56.1	57.3
75	61.0	61.0	62.6	62.8
80	67.3	67.3	63.0	64.7
85	74.7	74.7	76.3	73.7

Note:

[a]MC + nutrient: MC after adding nutrient (967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl.)

Table 4.4 Coded values used in CCD to optimize the physico-chemical fermentation conditions for citric acid production

Variables	Parameter	Unit	Coded and actual level				
			-2	-1	0	+1	+2
X_1	Temperature	°C	20	25	30	35	40
X_2	pH	-	2	4	6	8	9
X_3	Inoculum density	10^6 spores/ml	0.5	2.0	3.5	5.0	6.5
X_4	Moisture content	%	65	70	75	8	85

Table 4.5 Experimental and predicted citric acid production optimization after 48 and 72 h

Run	Temp	pH	ID	MC	Response at 48 h		Response at 72 h	
	X_1	X_2	X_3	X_4	Observed	Predicted	Observed	Predicted
No	°C		10^6spore/ml	%				
1	35	8	5.0	70	15.70	12.63	28.27	34.39
2	35	8	2.0	70	8.68	6.29	15.49	13.50
3	35	4	5.0	80	60.75	57.68	78.05	84.17
4	25	8	2.0	80	10.98	8.59	90.50	88.51
5	35	4	2.0	80	49.55	47.16	89.80	87.81
6	25	4	5.0	70	1.86	-1.20	17.31	23.43
7	25	8	5.0	80	17.13	14.07	95.93	102.04
8	25	4	2.0	70	1.70	-0.69	6.24	4.25
9	20	6	3.5	75	2.63	5.36	7.29	5.23
10	40	6	3.5	75	6.41	9.14	25.70	23.64
11	30	2	3.5	75	2.07	4.79	30.86	28.80
12	30	10	3.5	75	57.50	60.23	123.15	121.09
13	30	6	0.5	75	5.00	7.04	55.45	61.49
14	30	6	6.5	75	14.55	17.95	96.64	86.47
15	30	6	3.5	65	2.98	5.71	6.75	4.68
16	30	6	3.5	85	31.58	34.31	108.78	106.71
17	30	6	3.5	75	17.84	18.69	77.41	78.19
18	30	6	3.5	75	19.27	18.69	82.58	78.19
19	30	6	3.5	75	18.96	18.69	74.58	78.19

Note: ID – inoculum density (spores/ml); MC – initial moisture content (%).

Table 4.6 Regression analyses for the model and ANOVA results after 48 and 72 h of fermentation

	Citric acid production					
	48 h			72 h		
	Sum of Squares	*F* value	*P* level	Sum of Squares	*F* value	*P* level
Model	6147.42	14.41	0.0098*	27122.21	21.30	0.0047*
X_1	7.14	0.23	0.6536	169.48	1.86	0.2440
X_2	1536.76	50.42	0.0021*	4258.45	46.82	0.0024*
X_3	119.03	3.91	0.1193	623.85	6.86	0.0589
X_4	408.93	13.42	0.0215*	5204.89	57.22	0.0016*
X_1^2	169.82	5.57	0.0776	5273.76	57.98	0.0016*
X_2^2	247.86	8.13	0.0463*	13.68	0.15	0.7179
X_3^2	49.73	1.63	0.2706	23.02	0.25	0.6413
X_4^2	2.26	0.074	0.7988	656.43	7.22	0.0549
X_1X_2	177.38	5.82	0.0734	429.72	4.72	0.0954
X_1X_3	17.69	0.58	0.4886	29.89	0.33	0.5972
X_1X_4	1854.40	60.85	0.0015*	1328.43	14.60	0.0188*
X_2X_3	0.41	0.013	0.9132	44.59	0.49	0.5224
X_2X_4	569.39	18.68	0.0124	77.37	0.85	0.4086
X_3X_4	12.94	0.42	0.5502	113.82	1.25	0.3259

*significant levels at a 95% confidence level.

X_1 – fermentation temperature; X_2 – initial nutrient solution pH; X_3 – inoculum density; X_4 – initial MC.

Table 4.7 Predicted maximum citric acid production for optimal fermentation conditions using CCD after 48 and 72 h of fermentation. The values underlined were those found to be the most feasible

	Temp.	pH	Inoculum density	MC	Predicted citric acid	
	°C	-	10^6 spores/ml	%	g/kg DPM	
	X_1	X_2	X_3	X_4	48 h	72 h
1	34.96	8.00	4.81	80.00	48.24	123.15
2	35.00	8.00	4.45	80.00	47.50	122.66
3	35.00	7.54	5.00	80.00	48.31	118.99
4	35.00	8.00	3.98	80.00	46.17	122.06
5	**35.00***	**8.00***	**3.94***	**80.00***	**46.06***	**122.01***
6	25.31	8.00	5.00	70.44	60.75	88.27
7	25.31	8.00	5.00	70.88	58.60	89.94
8	25.00	8.00	5.00	71.13	58.34	90.28
9	35.00	5.71	4.18	80.00	48.68	102.36
10	25.71	8.00	3.06	70.28	60.75	71.09

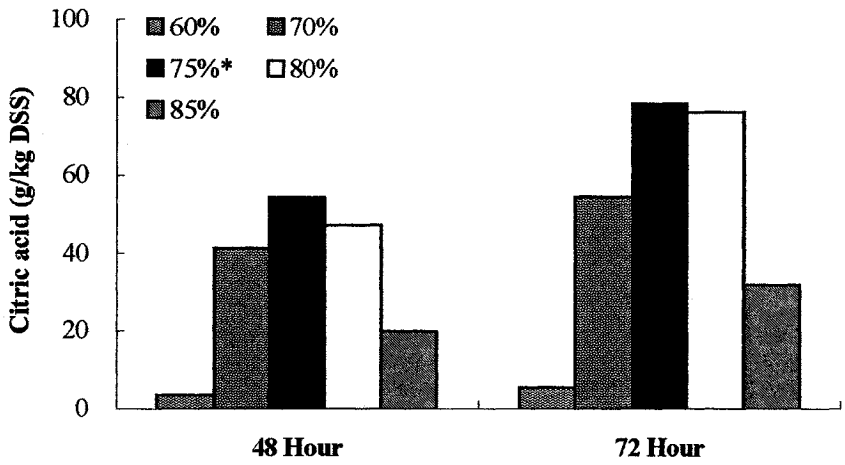

Figure 4.1 Citric acid production using various initial MC after 48 and 72 h. The other variables were fixed at: 30°C, a nutrient solution initial pH of 4.28, inoculum density of 1.0 x 10^6 spores/ml, 100% PM with particles smaller than 2.0 mm. All values were averaged from duplicate tests and an asterisk (*) identifies the control experiment.

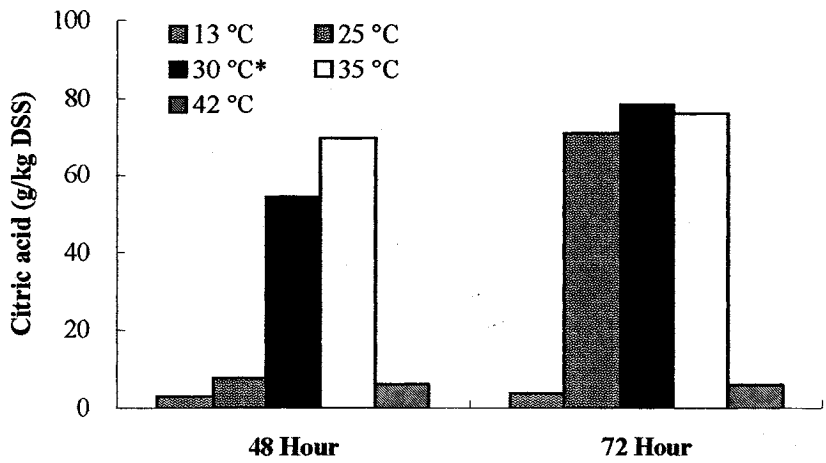

Figure 4.2 Citric acid production under various temperatures after 48 and 72 h. The other variables were fixed at: initial 80% MC, a nutrient solution initial pH of 4.28, inoculum density of 1.0 x 10^6 spores/ml, 100% PM with particles smaller than 2.0 mm. All values were averaged from duplicate tests and an asterisk (*) identifies the control experiment.

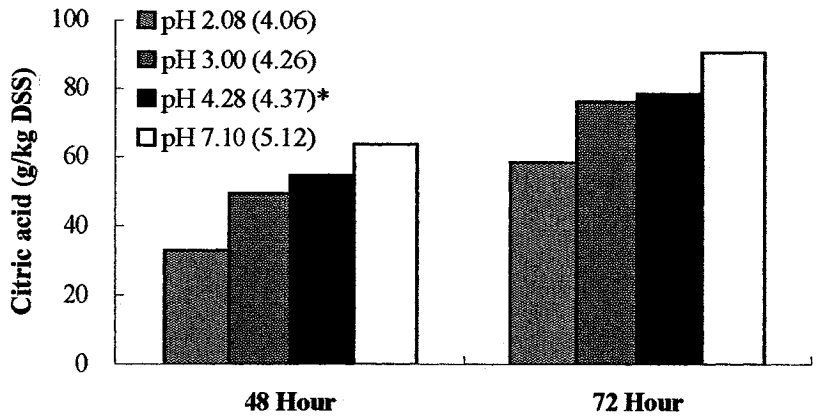

Figure 4.3 Citric acid production at various initial pH levels after 48 and 72 h. The other variables were fixed at: initial 80% MC, 30°C, inoculum density of 1.0 x 10^6 spores/ml, 100% PM with particles smaller than 2.0 mm. All values were averaged from duplicate tests and an asterisk (*) identifies the control experiment. Values in the parenthesis were the initial substrate pH once wetted with the nutrient solution.

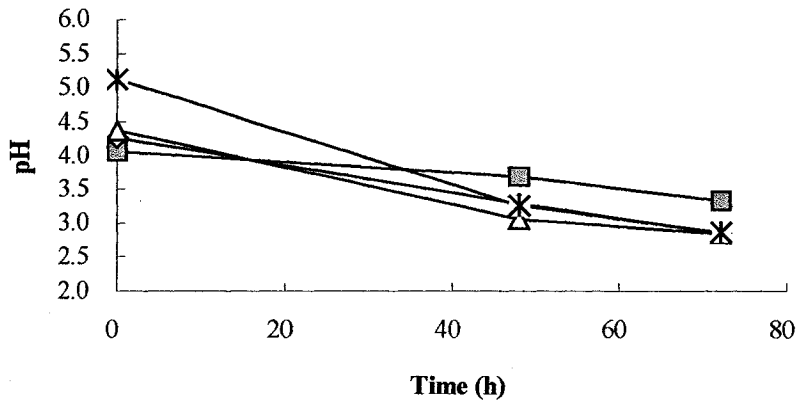

Figure 4.4 Profile of pH changes in PM by *A. niger* at the different initial pH of nutrient solution. The other variables were fixed at: initial 80% MC, 30°C, inoculum density of 1.0 x 10^6 spores/ml, 100% PM with particles smaller than 2.0 mm. [■: pH 2.08 (4.06); ◇: pH 3.00 (4.26); △: pH 4.28 (4.37); *: pH 7.10 (5.12)].

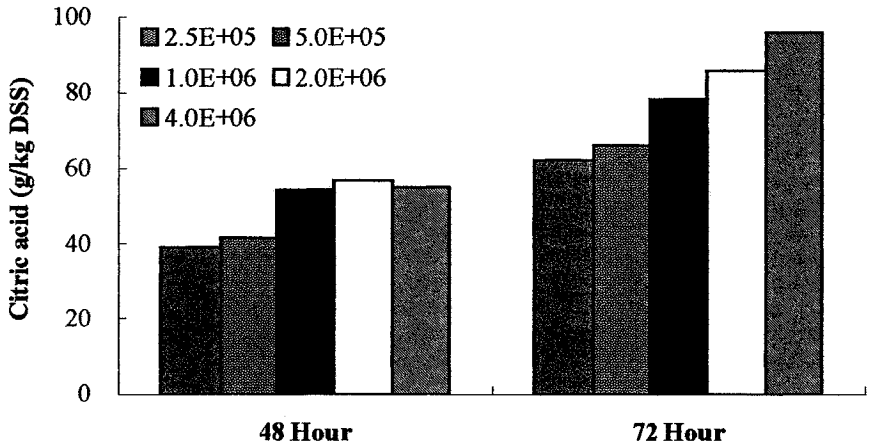

Figure 4.5 Citric acid production using various inoculum density after 48 and 72 h. The other variables were fixed at: initial 80% MC, 30°C, a nutrient solution initial pH of 4.28, 100% PM with particles smaller than 2.0 mm. The control experiment used an inoculum density of 1.0×10^6 spores/ml. All values were averaged from duplicate tests.

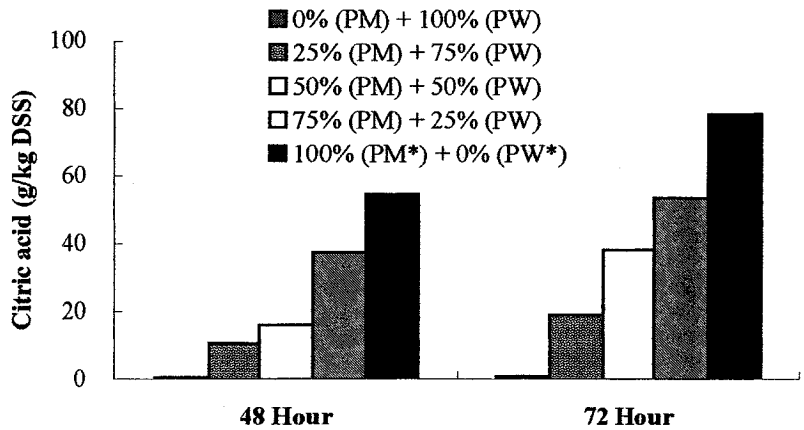

Figure 4.6 Citric acid production using various ratios of PM and PS at 48 and 72 h. The other variables were fixed at: 80% MC, 30°C, the initial pH of 4.28, inoculum density of 1.0×10^6 spores/ml and PM and PS with particles smaller than 2.0 mm. All values were averaged from duplicate tests and * identifies the control experiment.

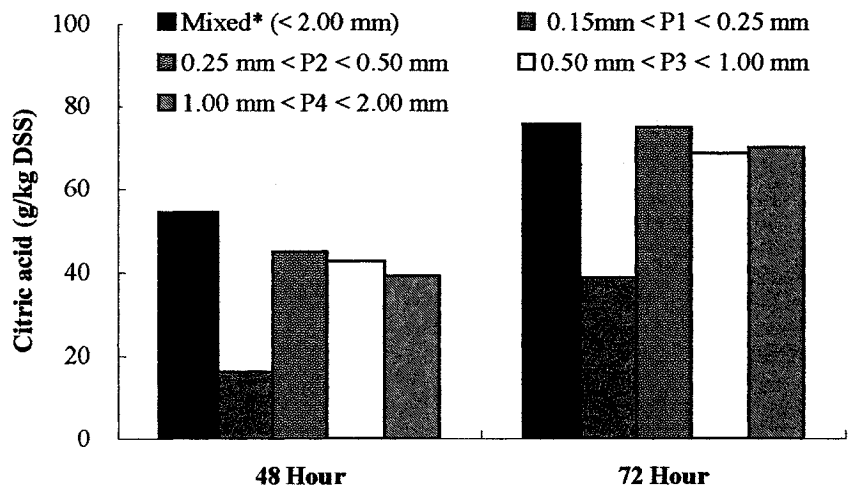

Figure 4.7 Citric acid production using various particle size ranges for PM after 48 and 72 h. The other variables were fixed at: initial 80% MC, 30°C, a nutrient solution initial pH of 4.28, inoculum density of 1.0 x 10^6 spores/ml and 100% PM. All values were averaged from duplicate tests and * identifies the control experiment.

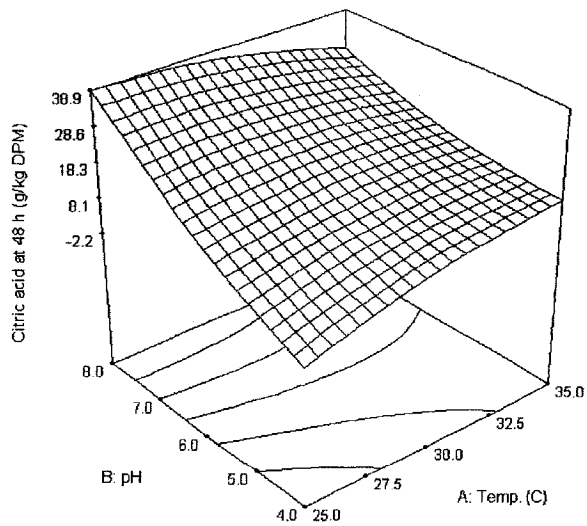

Figure 4.8a Response surface curve representing the interactive effect of fermentation temperature and a nutrient solution initial pH on citric acid production at 48 h, using an inoculum density of 3.5 x 10^6 spores/ml and an initial PM MC of 75%.

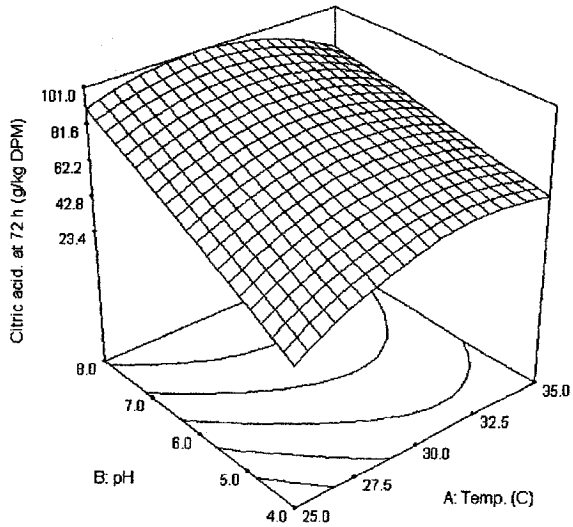

Figure 4.8b Response surface curve representing the interactive effect of fermentation temperature and a nutrient solution initial pH on citric acid production at 72 h, using an inoculum density of 3.5 x 10^6 spores/ml and an initial PM MC of 75%.

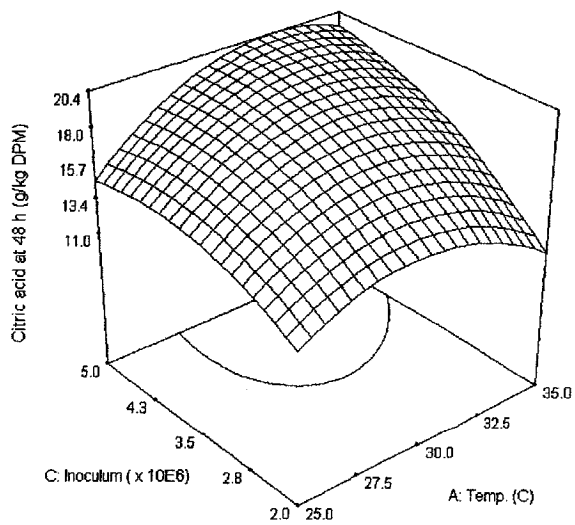

Figure 4.9a Response surface curve representing the interactive effect of fermentation temperature and inoculum density on citric acid production at 48 h, using a nutrient solution initial pH of 6.0 and an initial PM MC of 75%.

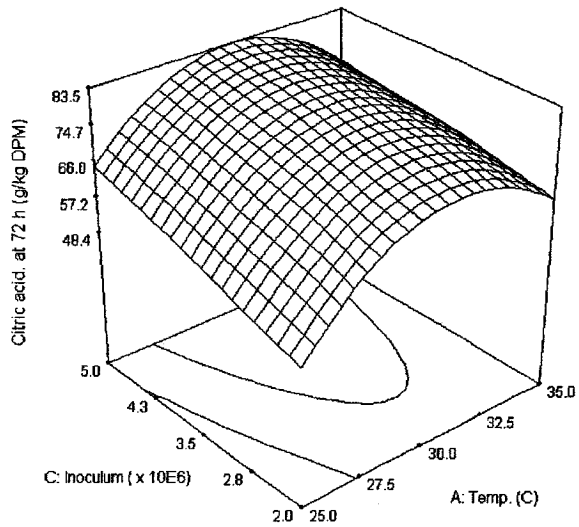

Figure 4.9b Response surface curve representing the interactive effect of fermentation temperature and inoculum density on citric acid production at 72 h, using a nutrient solution initial pH of 6.0 and an initial PM MC of 75%.

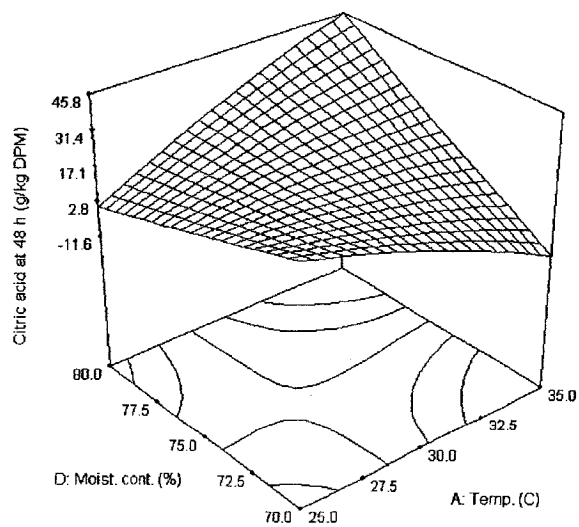

Figure 4.10a Response surface curve representing the interactive effect of fermentation temperature and initial PM MC on citric acid production at 48 h, using a nutrient solution initial pH of 6.0 and an inoculum density of 3.5 x 10^6 spores/ml.

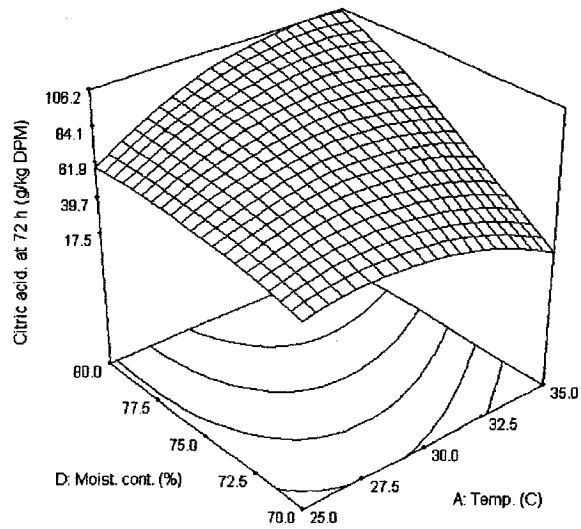

Figure 4.10b Response surface curve representing the interactive effect of fermentation temperature and initial PM MC on citric acid production at 72 h, using a nutrient solution initial pH of 6.0 and an inoculum density of 3.5×10^6 spores/ml.

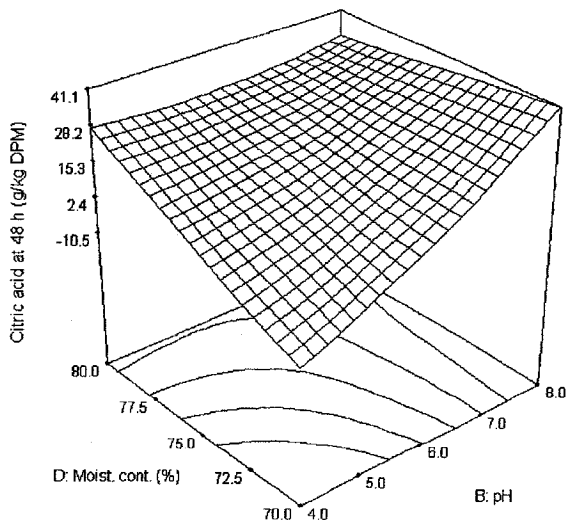

Figure 4.11a Response surface curve representing the interactive effect of a nutrient solution initial pH and initial PM MC on citric acid production at 48 h, using a fermentation temperature of 30°C and an inoculum density of 3.5×10^6 spores/ml.

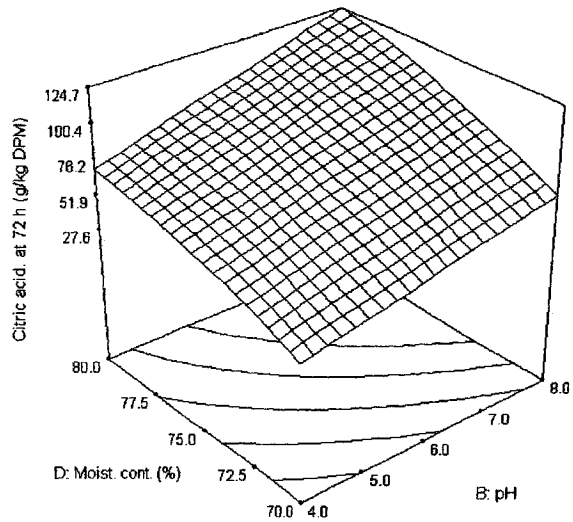

Figure 4.11b Response surface curve representing the interactive effect of a nutrient solution initial pH and initial PM MC on citric acid production at 72 h, using a fermentation temperature of 30°C and an inoculum density of 3.5×10^6 spores/ml.

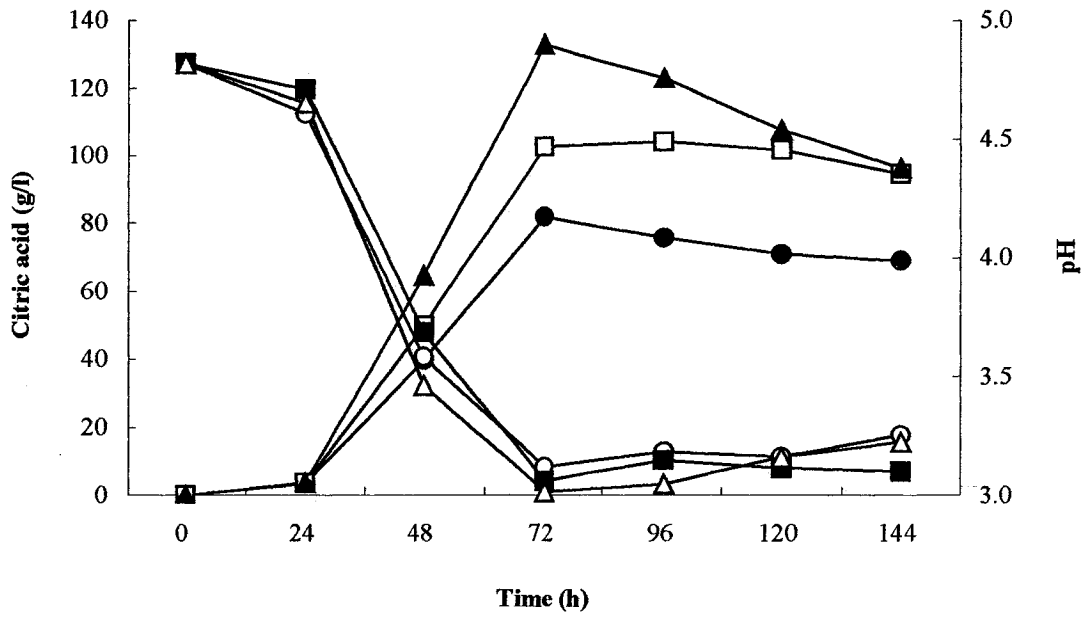

Figure 4.12 Time course behavior of citric acid production and pH by *A. niger* grown on PM using the control fermentation conditions (100% PM wetted initially to 80% MC using a nutrient solution with an initial pH of 4.28 (initial PM pH of 4.37), fermented at 30°C and inoculated with 1.0 ml of 1.0 x 10^6 spores/ml) and optimization condition (100% PM wetted initially to 80% MC using a nutrient solution with an initial pH of 8.0 (initial PM pH of 5.47), fermented at 35°C and inoculated with 1.0 ml of 4 x 10^6 spores/ml), ●:control (citric acid production); □: Optimized by traditional optimization (citric acid production); ▲: Optimized by CCD (citric acid production); ○:control (pH); ■: Optimized by traditional optimization (pH); △: Optimized by CCD (pH).

CONNECTING STATEMENTS

In a previous study, citric acid in batch experiments using *A. niger* grown on peat moss wetted to an initial MC of 80%, using an optimized initial glucose and mineral solution composed of per kg DPM: 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl. Such optimal nutrient levels produced 133 g of citric acid/kg DPM after 72 h of fermentation at a fermentation temperature of 35°C, a nutrient solution initial pH of 8.0 and an inoculum density of 4 x 10^6 spores/ml. Nevertheless, to improve citric acid production, the initial concentration of stimulators must also be optimized along with the nutrient concentration and fermentation parameters.

Chapter 5 deals with the effect of stimulators on citric acid production by *A. niger* in solid substrate fermentation. This study was conducted to determine the potential of stimulators including ethanol, methanol, phytate and Tween 80 for citric acid production by *A. niger* grown on peat moss. To evaluate the effect of individual factors, the "one-factor-at-a-time" strategy was used for the first step optimization and the results were then employed for second step optimization using central composite design. In a study using "one-factor-at-a-time" method, the effects of four stimulators were optimized by a traditional strategy, to approximate their optimal value for citric acid production. As the second optimization, a statistically-based optimization procedure was employed to optimize the concentration of stimulators for the production of citric acid by *A. niger* grown on peat moss. One class of statistically-based optimization, central composite design was used to find optimum levels of stimulators including phytate, olive oil and methanol and to evaluate the interactions between variables. Finally, the results obtained from this study were compared to those obtained using a traditional "one-factor-at-a-time"

method.

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CHAPTER 5

Effect of Stimulators on Citric Acid Production by *Aspergillus niger* NRRL 567 in Solid Substrate Fermentation

5.1 Abstract

The potential stimulating effect of five additives including ethanol, methanol, phytate, olive oil and surfactant (Tween 80) were evaluated on citric acid production by *Aspergillus niger* NRRL 567 grown on peat moss (PM). The results from "one-factor-at-a-time" optimization showed ethanol, methanol and phytate had an effect on citric acid production. The addition of methanol between 15 and 30 g/kg dry peat moss (DPM) at the beginning of fermentation enhanced citric acid production whereas a reduction in citric acid production was observed with a higher concentration of methanol. The production of citric acid was increased by adding 15 g ethanol/kg DPM while higher levels than 30 g/kg DPM of ethanol had an inhibitory effect on citric acid production. Supplementation with a high concentration of phytate decreased citric acid production, however, a low level of phytate had no adverse or stimulating effect. The second optimization employed a central composite design (CCD), where the variables selected were phytate (2.6 – 87.4 g/kg DPM), vegetable oil (olive oil, 2.6 – 87.4 g/kg DPM) and methanol (2.6 – 87.4 g/kg DPM). Using three-factor and five-level CCD, the two independent variables, phytate and methanol, were identified to have effects on citric acid production at 48 and 72 h. The predicted optimum concentration of stimulators for citric acid production was phytate 19.6 g, olive oil 28.9 g and methanol 40.9 g/kg DPM. After optimization, citric acid production reached a maximum (354.8 g/kg DPM) at 120 h when fermentation was carried out with the optimized concentration of stimulators and allowed a 2.7-fold increase compared to citric

acid production of previous optimzation.

Keywords: *Aspergillus niger*, citric acid, solid substrate fermentation, stimulators, statistically-based optimization.

5.2 Introduction

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is mainly produced by fermentation using *Aspergillus niger* (Wang, 1998; Pera and Callieri, 1999; Crolla and Kennedy, 2001). Citric acid accumulation by *A. niger* is achieved by two main metabolic pathways: (1) the catabolic pathway where hexose is transformed into pyruvate and acetyl-Coenzyme A (Acetyl-CoA) by glycolysis, and; (2) citric acid formation by the TCA cycle (Alvares-Vasquez *et al.*, 2000). In citric acid production, glucose plays an important role as the initial carbohydrate source for glycolysis. According to Wasay *et al.* (1998), *A. niger* can convert over 90% of glucose into citric acid and other organic acids in optimum fermentation conditions.

Besides the optimization of casal nutrient concentration, higher citric acid production levels can be obtained by applying stimulators, such as methanol, ethanol, phytate, vegetable oil, oximes, *n*-dodecane, fluoroacetate and chelating agents (Navaratnam *et al.*, 1998; Jianlong, 2000). Organic solvents including ethanol and methanol stimulate the production of citric acid by increasing the permeability of the cell membrane, decreasing cell growth or changing the activity of key enzymes. As compared to methanol, which is not assimilated, *A. niger* assimilates ethanol to convert it into acethyl-CoA, a key precursor of the TCA cycle (Jana and Ghosh *et al.*, 1995). Also, ethanol supplementation is known to change the activity of key enzymes associated with the TCA cycle; it increases

the activity of citrate synthetase and decreases the activity of aconitase (Jianlong and Ping, 1998). Phytate is also known to offer 12 replaceable protons providing the binding potential for positively charged molecules. It acts as a metal-chelating agent, chelating free trace elements and removes heavy metals providing a negative effect on citric acid production (Wang, 1998; Haq *et al.*, 2001; Mirminachi *et al.*, 2002). Vegetable oils with high levels of unsaturated fatty acids, such as olive, maize and sunflower oil, are stimulators for citric acid production, when using both submerged and solid substrate fermentation by *A. niger*. The stimulating mechanism of natural oil was investigated by Adham (2002) who suggested that unsaturated lipids play a role of alternative hydrogen acceptors to oxygen. Also, vegetable oil can be converted to acetyl-CoA and assimilated by *A. niger* as an alternative carbon sources (Jianlong and Ping, 1998).

The main objective of the present project was to determine the potential increase in citric acid production by *A. niger* NRRL 567 in the presence of stimulators such as of ethanol, methanol, phytate, olive oil and Tween 80. *A. niger* was grown on a simulated sugar rich byproduct, namely peat moss (PM) supplemented with glucose and a nutrient solution. The experiment tested each stimulator based on the traditional "one-parameter-at-a-time" method, to approximate an optimal range. And then, the CCD method was used to find optimum levels of stimulators for citric acid production by *A. niger* in solid substrate fermentation. The CCD method is capable of evaluating the interactions between each stimulator. The production level obtained from CCD method was also compared to that obtained using a traditional "one-factor-at-a-time" method, as tested in a previous experiment (Kim *et al.*, 2004).

5.3 Materials And Methods

5.3.1 Microorganism

The white rot fungus *Aspergillus niger* NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was stored in tubes containing glycerol (30% v/v) at –76°C. *A. niger* spores were produced on potato dextrose agar (PDA, Sigma, St. Louis, MO, USA) plates at 30°C. After ten days of incubation on PDA plates, 10 ml of 0.1% Tween 80 were added to each plate to harvest spores. Diluted spore suspensions of 4.0 x 10^7 spores/ml were counted using hemocytometer and prepared as inoculum for the experimental media.

5.3.2 Fermentation conditions for "one-factor-at-a-time" optimization

To simulate a sugar rich byproduct, sphagnum PM (Schultz Company, Mississauga, Ontario, Canada) supplemented a high concentration of glucose was used as solid substrates for *A. niger*. Initially, it was dried at 60°C for 48 h to 3 ± 1% moisture contents (MC) and then screened to remove all particles over 2.0 mm. Before being characterized for density, total carbon, total nitrogen, total phosphorus, pH and NH_4-N absorption.

Small scale experiments were carried out in 250-ml Erlenmeyer flasks holding 7 g of dry peat moss (DPM) re-wetted with a nutrient solution (Considine *et al.*, 1987). The glucose and salt content of the nutrient solution was adjusted to obtain the following content per kg DPM: 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl. The initial pH of nutrient solution was adjusted to 8.0 by adding 1 N NaOH. The final MC of PM was adjusted to 80% using the required volume of deionized water and used as the control treatment.

The potential stimulators, Tween 80 (Sigma Chemical Co., U.S.A.) and phytate (40

wt%, Aldrich Chemical Co, U.S.A.), were added to the PM before autoclaving while the stimulators ethanol (99.9%, Sigma Chemical Co, U.S.A.) and methanol (99+%, Sigma Chemical Co, U.S.A.) were added after autoclaving, before inoculating with 1 ml of spore solution (4 x 10^6 spores/ml) was inoculated. To investigate the influence of the initial concentration of ethanol, methanol and phytate, the "one-factor-at-a-time" optimization was carried out with various stimulator concentrations (15, 30, 90 and 150 g/kg DPM), keeping other conditions at their basal level. Three initial concentrations of Tween 80 (3.3, 16.5 and 82.2 g/kg DPM) were tested to evaluate the in effect on citric acid production. The 250-ml Erlenmeyer flasks and their contents were then incubated for 3 days in a closed chamber maintained at 35°C. All treatments were duplicated.

5.3.3 Fermentation conditions for statistically-based optimization

The quantities of stimulators added to the PM were based on those prescribed by the CCD method (Table 5.1). A final experiment was carried out to measure the citric acid produced when using those stimulator levels optimized by the CCD method.

5.3.4 Analysis of citric acid

For each sampling session, the contents of two 250-ml Erlenmeyer flasks were withdrawn. The wet sample was split into a 10 g portion to verify the final MC of the PM and into another 5 g portion for citric acid determination. This last portion was soaked in 50 ml of distilled water and shaken for 60 min at 150 rpm at 22°C (Xu *et al.*, 1989). Once collected, the supernatant was separated by centrifugation at 11,000 x g for 10 min and tested for pH and stored at -20°C for citric acid quantification. After adding pyridine and acetic anhydride to develop color, citric acid was determined by spectrophotometry at 420

nm (Marier and Boulet, 1958).

5.4 Results and Discussion

5.4.1 Optimization using "one-factor-at-a-time" method

5.4.1.1 Effect of ethanol

Ethanol was added at a concentration varying between 0 to 150 g/kg DPM. After 48 and 72 h of fermentation, ethanol had an effect on citric acid production. Without ethanol, the control produced to 102.3 g of citric acid/kg DPM after 72 h (Figure 5.1). The addition of 15 and 30 g ethanol/kg DPM, increased citric acid production to 126.6 g/kg DPM, after 72 h. Thus, ethanol had no inhibitory effect on citric acid production after 48 and 72 h of fermentation, at a concentration equal to and below 30 g/kg DPM. High concentrations of ethanol adversely affect citric acid production or cell growth while low concentrations potentially stimulate the production of citric acid. Several researchers have reported the stimulating effect of ethanol on citric acid production and suggested that ethanol could be used as a carbon source to be converted to citric acid via the TCA cycle of *A. niger* (Moyer, 1953; Navarantnam *et al.*, 1998). In addition to its assimilation, ethanol can also increase the permeability of the cell membrane and, thus, increase the secretion of citric acid (Pazouki *et al.*, 2000).

5.4.1.2 Effect of methanol

Figure 5.1 shows the effect of methanol on citric acid production. With no methanol, *A. niger* produced 102.3 g citric acid/kg DPM after 72 h. Up to 30 g/kg DPM, the addition of methanol improved citric acid production up to 2.3-fold, but any further increase in methanol concentration had an inhibitory effect. There are several hypothesis related to the

stimulating effect of methanol on citric acid production. Hang and Woodams (1998) reported that the increase in citric acid production resulted from a decrease in mycelial growth, glucose was used for citric acid production rather than for cell growth. Also, organic solvents such as alcohol and methanol are known to increase the permeability of the cell membrane and, thus, improve the secretion of citric acid across this membrane (Hang *et al.*, 1987; Navaratnam *et al.*, 1998). However, methanol concentration above 30 g/kg DPM considerably decreased citric acid production, because of its inhibitory effect on spore germination and cell growth (Navaratnam *et al.*, 1998).

5.4.1.3 Effect of Phytate

The effect of phytate on citric acid production is presented in Figure 5.1. Phytate showed an adverse effect on citric acid production. Some stimulating effect occurred with a phytate concentration of 15 g/kg DPM, but the effect was negative at a phytate level equal to and exceeding 30 g/kg DPM. Many researchers observed the stimulating effect of phytate on citric acid production. During the submerged fermentation of *A. niger*, 10 g/l of phytate stimulated citric acid production (Wang, 1998; Jinglong and Ping, 1998). Jianlong (1998) also observed higher citric acid production with the addition of low concentrations of phytate. The initial pH of the solid substrate was noticeably affected by the initial concentration of phytate (Figure 5.2, $R^2 = 0.97$). It may be concluded that a high level of phytate decreased the initial pH of the solid substrate and provide adverse fermentation conditions for citric acid production.

5.5.1.4 Effect of Tween 80

The nonionic synthetic surfactant, Tween 80, was tested as a potential stimulator

(Figure 5.3). The addition of Tween 80 up to 82.2 g/kg DPM, had an insignificant effect on citric acid production after 48 and 72 h of fermentation. A maximum citric acid production of 119.0 g/kg DPM was obtained with 82.2 g/kg DPM of Tween 80 after 72 h, and only slightly better than that obtained with the control of 102.3g/kg of DPM.

Various nonionic surfactants, including Tween 80, Tween 20 and Triton X-100 have been applied in the submerged and solid substrate fermentation by microorganisms (*Aspergillus, Trichoderma* or *Nectria*) to improve production of cellulosic enzyme such as endoglucanase and exoglucanase and the stimulating effect of surfactants was reported (Reese and Maguire, 1969; Pardo, 1996; Goes and Sheppard, 1999). In our study, the results revealed that Tween 80 had no stimulating effect on citric acid production using solid substrate fermentation by *A. niger* NRRL 567 grown on PM.

5.4.1.5 Citric acid production using optimized levels of stimulators

Using the results from the traditional "one-parameter-at-a-time" method, the time course behavior of citric acid production was investigated using: 15 g ethanol, 30 g methanol and 15 g phytate per kg DPM. This combination of stimulators produced a maximum citric acid production of 240.2 g citric acid/kg DPM after 120 h of fermentation (Figure 5.4). This value exceeded that of the control by 1.8-fold, which maximized at 72 h of fermentation. For the control test without stimulators, extending the fermentation time beyond the point of maximum citric acid production resulted in a drop in citric acid production thereafter, because of the microbial oxidation of citric acid (Hang and Woodams, 1998). However, the stimulators seem to have prevented the oxidation of citric acid for 144 h of fermentation. Jinglong and Ping (1998) reported that, in the TCA cycle, supplementation with ethanol changed the activity of key enzymes such as citrate

synthetase and aconitase. When the activity of aconitase is decreased while that of citrate synthetase is increased, citric acid degradation is retarded. Thus, the stimulators acted to suppress the oxidation of citric acid in the latter half of the fermentation.

5.4.2 Optimization using statistically-based optimization

As an initial concentration of Tween 80 had insignificant effect on citric acid production and it was out of consideration for the further optimization. The function and effect of organic solvents, methanol and ethanol, on citric acid production were identical. As, both organic solvents showed an antagonic effect, less effective stimulator, ethanol, was excluded from the further experiments. After 48 and 72 h of fermentation, the level of citric acid production obtained is summarized in Table 5.2. The 11 combinations of stimulators produced citric acid levels varying between 0.2 and 239.4 g/kg of DPM. Varying the level of stimulators therefore had a marked effect on citric acid production.

The measured levels of citric acid production were used to compute the coefficients of equations capable of predicting citric acid production (Y) as a function of the levels of each three variables or stimulators (χ_1, χ_2 and χ_3), after 48 and 72 h of fermentation respectively:

$$Y_{48\,h} = 31.54 - 22.27\chi_1 + 4.98\chi_2 - 19.66\chi_3 + 4.80\chi_1^2 - 2.63\chi_2^2 - 1.31\chi_3 + 6.69\chi_1\chi_2 + 15.32\chi_1\chi_3 - 7.51\chi_2\chi_3 \quad (5.1)$$

$$Y_{72\,h} = 223.13 - 38.81\chi_1 + 13.67\chi_2 - 20.15\chi_3 - 21.50\chi_1^2 - 18.17\chi_2^2 - 63.74\chi_3^2 + 12.54\chi_1\chi_2 + 22.31\chi_1\chi_3 - 10.55\chi_2\chi_3 \quad (5.2)$$

Equations (5.1) and (5.2) yielded high R^2 coefficients of 0.994 and 0.997 after 48 and

72 h fermentation. Thus, these equations model most of the effect of each three stimulators on the production of citric acid (Panda *et al.*, 1999; Pham *et al.*, 1998; Coello *et al.*, 2002).

The ANOVA results are presented in Table 5.3. The effect on citric acid production of varying all three stimulators was highly significant, both after 48 and 72 h of fermentation. After 48 and 72 h of fermentation, phytate and methanol also had a significant effect on citric acid production ($P < 0.05$) as opposed to olive oil, which had a less significant effect ($P = 0.09$). However, at a 90% confidence level, all tested three variables had a significant effect.

Being able to accurately predict citric acid production, equations (5.1) and (5.2) were used to obtained response surface curves relating citric acid production to various levels of stimulators. Figures 5.5a and 5.5b show the interactive effect of phytate and olive oil on citric acid production after 48 and 72 h fermentation, respectively. Both plots show that citric acid production is not affected by varying the initial concentration of olive oil but is affected by varying the initial concentration of phytate. After 72 h of fermentation, a maximum citric acid concentration of 240.7 g/kg DPM was obtained with 45 g olive oil, 15 g phytate and 45 g methanol/kg DPM. Although several studies have indicated the stimulating effect of phytate on citric acid production, for and solid substrate fermentation using *A. niger*, this study shows a depressing effect. As, the initial pH of solid substrate was correlated with the initial concentration of phytate (Figure 5.6, $R^2 = 0.96$), it is presumed that a high level of phytate may decrease the initial pH of solid substrate and provide unfavorable fermentation conditions.

Figures 5.7a and 5.7b show the response surface curve of citric acid production from *A. niger* NRRL 567 as a function of initial phytate and methanol concentration after 48 and 72 h fermentation. The predicted citric acid production decreased with phytate and

increased methanol concentrations. A maximum citric acid concentration of 92.3 g/kg DPM was predicted after 48 h of fermentation with respective concentrations of methanol and phytate of 15 and 15 g/kg DPM. After 72 h of fermentation, citric acid production increased up to 45 g methanol/kg DPM and fell thereafter for methanol concentrations of up to 75 g/kg DPM. After 72 h of fermentation, a maximum citric acid production of 247.4 g/kg DPM was predicted with 45 g methanol and 15 g phytate/kg DPM.

Figures 5.8a and 5.8b predict citric acid production as a function of olive oil and methanol concentrations, and at a fixed phytate concentration after 48 and 72 h of fermentation respectively. After 48 h of fermentation, citric acid production maximized using the highest and lowest concentrations of olive oil and methanol, respectively, indicating that methanol had an inhibitory effect on citric acid production during the early stage of fermentation. After 72 h of fermentation, citric acid production increased with methanol concentrations increasing up to 45 g/kg DPM, but fell thereafter, at a fixed concentration of olive oil. A maximum citric acid production of 228.0 g/kg DPM was predicted for the highest level of olive oil of 75 g and 45 g methanol/kg DPM, after 72 h of fermentation.

The combined effects of varying all three stimulators after 72 h of fermentation is illustrated in Figure 5.9. Each cube corner represents eight different experimental conditions, where the plus (+) and minus (-) signs represent the coded levels (-1 and +1) for each variable. A maximum citric acid production of 212.7 g/kg DPM was predicted when combining a low concentration of phytate (A-), with a high concentration of olive oil (B+) and a low concentration of methanol (C-). The cube graph shows a dominant surface on the left side with a higher citric acid production at a low phytate concentration (A-).

The numerical optimization method of CCD was employed to predict the optimum

level of phytate, olive oil and methanol using equations (5.1) and (5.2) and after 72 h of fermentation (Tables 5.4 and 5.5). Ten possible optimal combinations were predicted. However, the fifth solution is the most economical using the lowest concentration of the three combined stimulators. Thus, a maximum citric acid production of 237.1 g/kg DPM is predicted when using 19.6 g phytate, 28.9 g olive oil and 40.9 g methanol/kg DPM.

These optimal levels of stimulators were appliedfor SSF once more to test its citric acid production in the laboratory (Figure 5.10). The effect of using the levels of stimulators predicted using "one-factor-at-a-time" (control) was compared to that where all three stimulators were optimized using CCD method. The CCD method optimized levels increased maximum citric acid production by 1.5-fold. Using 15 g phytate, 15 g olive oil and 30 g methanol/kg DPM, citric acid production maximized at 240.2 g/kg DPM after 120 h of fermentation. The CCD method optimized test produced a maximum citric acid production of 354.8 g/kg DPM after 120 h.

5.5 Concludions

The influence of stimulators on citric acid production was studied using the traditional "one-parameter-at-a-time" method. It was demonstrated that the addition of 15 g of ethanol, 30 g of methanol and 15 g of phytate per kg DPM increased citric acid production by 1.8-fold, compared to using no stimulators. Using solid substrate fermentation, the addition of low concentrations of ethanol and methanol stimulated citric acid production, whereas, phytate and Tween 80 had no stimulating effect for the range tested after 48 and 72 h of fermentation. The optimal range of stimulator concentration identified by the present experiment can provid basic information for further testing the synergistic effect of stimulators on each other, using a statistically-based optimization method. Such a method

can investigate the interactive effect of various stimulators including ethanol, methanol, phytate and vegetable oil. Olive oil and methanol were found to have a positive significant effect on citric acid production after 72 h of fermentation, whereas phytate was found to have a negative significant effect. A maximum citric acid production of 354.8 g/kg DPM was obtained after 120 h of fermentation.

5.6 Acknowledgements

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Table 5.1 Experimental plan for optimization of level of stimulators: Independent variables in CCD

Variables	Parameter	Unit	Coded and actual level				
			-1.68	-1	0	+1	+1.68
X_1	Phytate	g/kg DPM	2.6	15	45	75	87.4
X_2	Olive oil	g/kg DPM	2.6	15	45	75	87.4
X_3	Methanol	g/kg DPM	2.6	15	45	75	87.4

Table 5.2 Effect of stimulator constituents: the observed and predicted values

No	Phytate	Olive oil	Methanol	Responses at 48 h		Responses at 72 h	
	(g/kg DPM)	(g/kg DPM)	(g/kg DPM)	Observed	Predicted	Observed	Predicted
1	75.00	75.00	15.00	32.24	33.65	113.55	115.50
2	75.00	15.00	75.00	0.22	1.63	65.48	67.43
3	15.00	75.00	75.00	9.05	10.46	104.69	106.65
4	15.00	15.00	15.00	82.46	83.87	187.36	189.31
5	2.57	45.00	45.00	74.06	72.65	236.97	235.02
6	87.43	45.00	45.00	11.06	9.65	127.21	125.26
7	45.00	2.57	45.00	20.65	19.24	169.41	167.46
8	45.00	87.43	45.00	34.72	33.31	208.06	206.11
9	45.00	45.00	2.57	58.14	56.73	126.10	124.15
10	45.00	45.00	87.43	2.53	1.12	69.11	67.16
11	45.00	45.00	45.00	33.36	31.54	228.23	223.13
12	45.00	45.00	45.00	26.90	31.54	214.14	223.13
13	45.00	45.00	45.00	28.93	31.54	239.37	223.13

R^2 (coefficient of determination) = 0.994 (48 h) and 0.997(72 h)

Table 5.3 ANOVA: influence of stimulators on citric acid production at 48 and 72 h

	Citric acid production					
	48 h			72 h		
	Sum of Squares	*F* value	*P* level	Sum of Squares	*F* value	*P* level
Model	7960.82	39.52	0.0249*	40743.47	62.42	0.0159*
X_1	1984.58	88.68	0.0111*	6023.74	83.06	0.0118*
X_2	99.07	4.43	0.1701	746.94	10.30	0.0849
X_3	1546.20	69.09	0.0142*	1623.71	22.39	0.0419*
X_1^2	138.50	6.19	0.1307	2773.36	38.24	0.0252*
X_2^2	41.61	1.86	0.3059	1981.86	27.33	0.0347*
X_3^2	10.26	0.46	0.5682	24376.46	336.12	0.0030*
X_1X_2	89.60	4.00	0.1834	314.28	4.33	0.1728
X_2X_3	469.66	20.99	0.0445*	995.82	13.73	0.0657
X_1X_3	112.86	5.04	0.1538	222.55	3.07	0.2219

* *P* level less than 0.05 indicate the model terms are significant.

X_1 = phytate; X_2 = olive oil; X_3 = methanol

Table 5.4 Constraint for the optimization of initial stimulator (g/kg DPM) after 72 h

Variables	Goal	Lower Limit	Upper Limit
Phytate		15	75
Olive oil		15	75
Methanol		15	75
Citric acid at 72 h	maximize	65.5	237.0

Table 5.5 Predicted citric acid concentration after 72 h of fermentation

	Phytate (g/kg DPM)	Phytate (g/kg DPM)	Phytate (g/kg DPM)	Citric acid at 72 h (g/kg DPM)
1	28.8	42.9	34.7	240.5
2	32.0	43.4	40.6	238.4
3	32.5	47.4	33.5	238.4
4	21.5	39.1	41.4	242.2
5*	**19.6***	**28.9***	**40.9***	**237.1***
6	30.4	64.2	36.9	239.9
7	21.6	33.6	42.8	238.4
8	22.9	57.4	30.3	243.6
9	24.9	37.1	38.1	240.9
10	23.5	56.2	38.9	244.6

Phytate: X_1; Phytate: X_2; Phytate: X_3

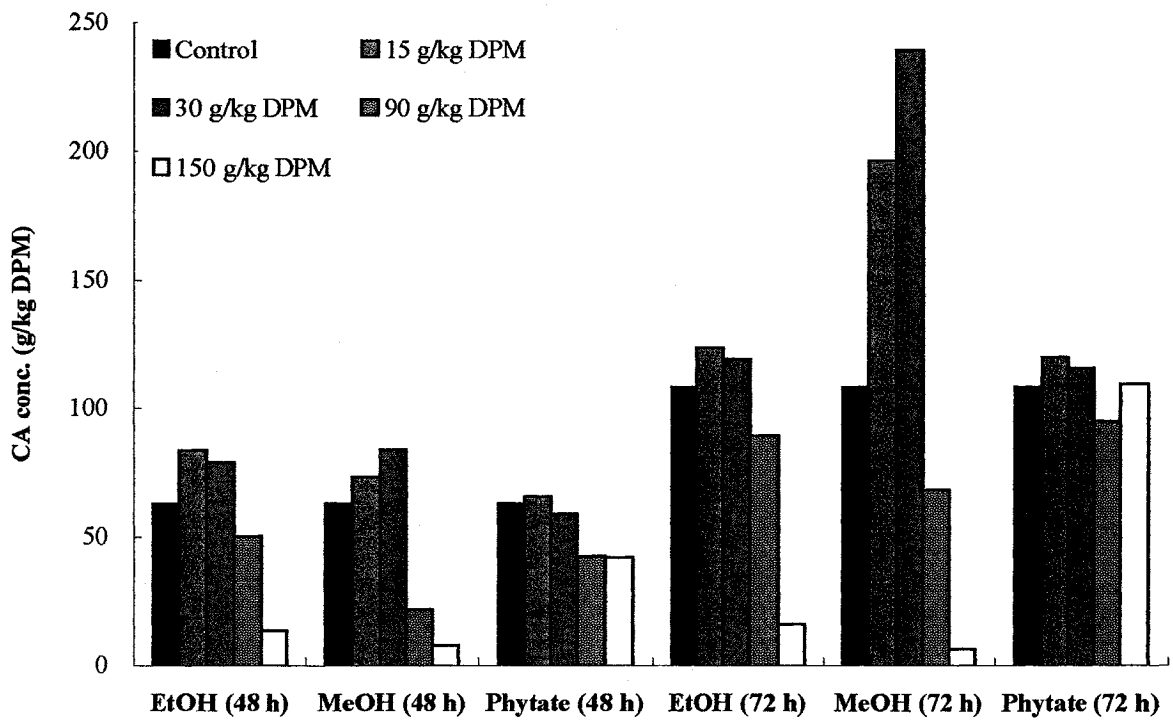

Figure 5.1 Citric acid production with various concentrations of stimulators at 48 and 72 h. All other variables were fixed per kg DPM: 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl; 80% water content; 4 x 10^6 spores/ml of inoculum and a fermentation temperature of 35°C.

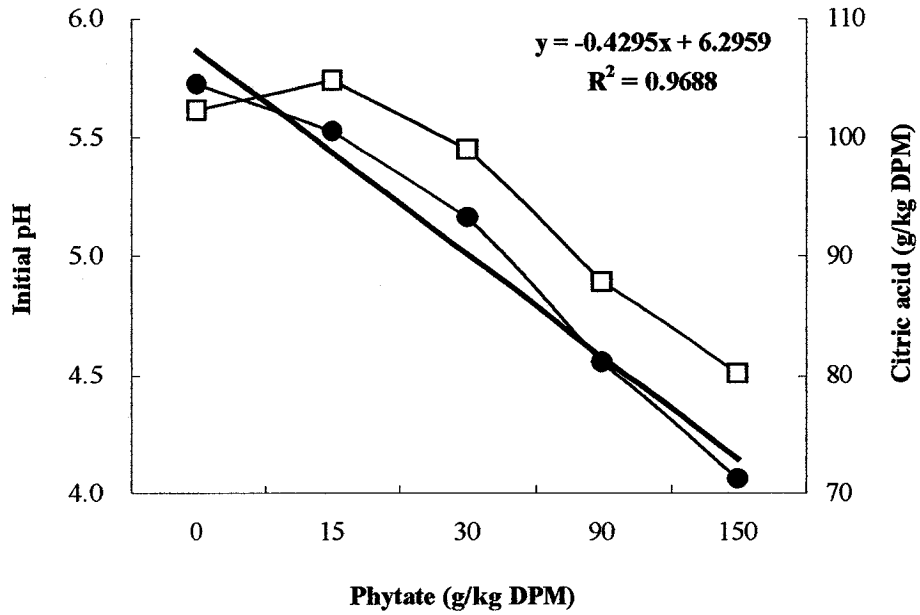

Figure 5.2 Effect of initial phytate concentration on citric acid production after 72 h of fermentation and linear relation between the initial concentration of phytate and the initial pH of solid substrate (●: initial pH of solid substrate; □: citric acid concentration)

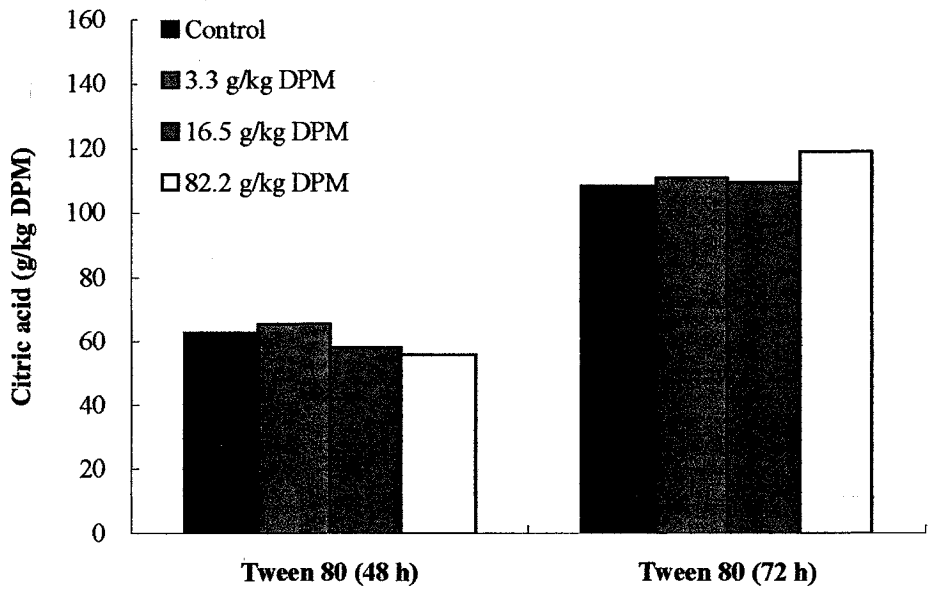

Figure 5.3 Citric acid production with various concentrations of Tween 80 at 48 and 72 h. The other fixed variables were, per kg DPM: 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4, 4.0 g NaCl; 80% water content; 4 x 10^6 spores of inoculum and a fermentation temperature of 35°C.

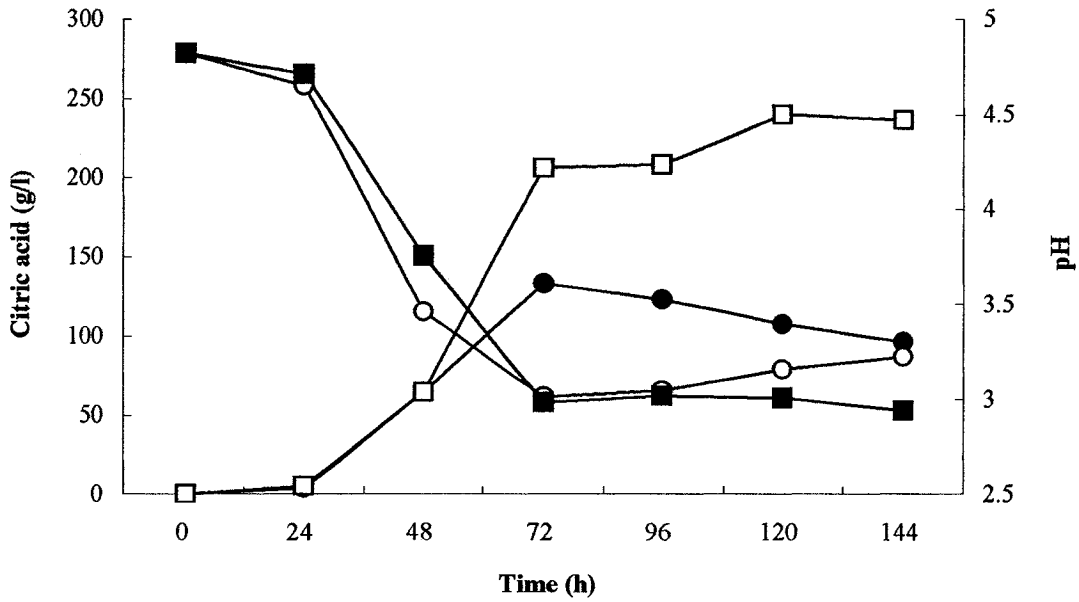

Figure 5.4 Time course behavior of citric acid production and pH by *A. niger* NRRL 567 in solid substrate fermentation. The control treatments used no stimulator. The optimized treatment was conducted by adding to the PM 15 g of ethanol, 30 g of methanol and 15 g of phytate/kg DPM. Both the control and optimized experiments containing, per kg DPM: 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4, 4.0 g NaCl. At an initial pH of 8 and wetted to 80% water content, the medium was inoculated with 4 x 10^6 spores and incubated at 35°C. ●: control (citric acid production); □: optimized condition (citric acid production); ○: control (pH); ■: optimized condition (pH).

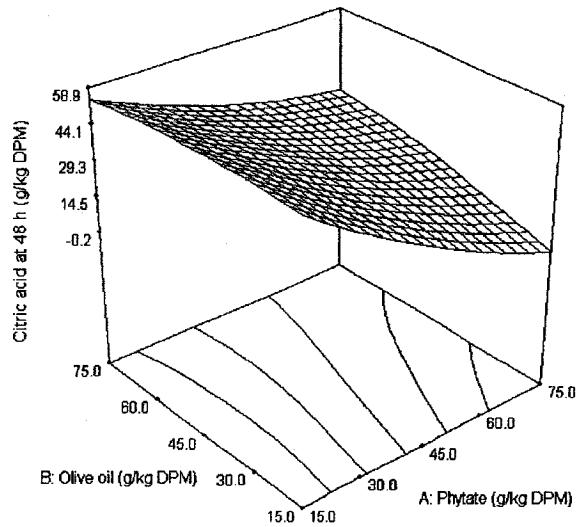

Figure 5.5a Predicted citric acid production by *A. niger* NRRL 567 as a function of initial concentration of olive oil and phytate after 48 h of fermentation with a fixed methanol concentration of 45 g/kg DPM.

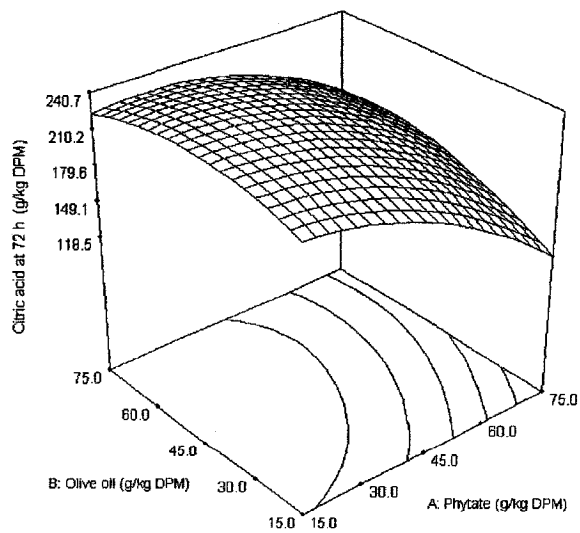

Figure 5.5b Predicted citric acid production from *A. niger* NRRL 567 as a function of initial concentration of olive oil and phytate with a fixed methanol concentration of 45 g/kg DPM after 72 h of fermentation.

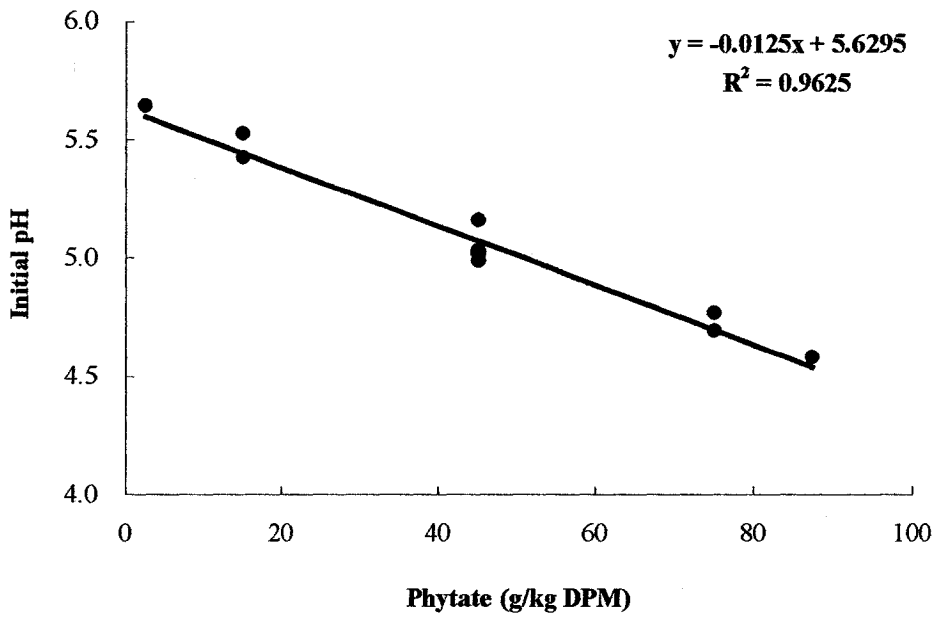

Figure 5.6 Linear relations between the initial concentration of phytate and the initial pH of solid substrate.

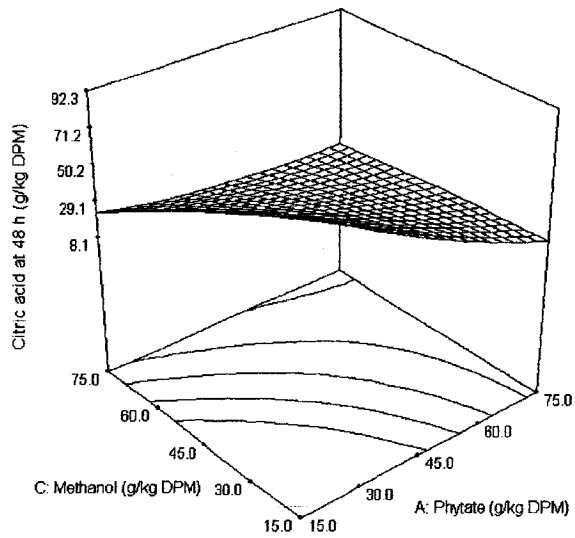

Figure 5.7a Predicted citric acid production from *A. niger* NRRL 567 as a function of initial concentration of phytate and methanol with a fixed olive oil concentration of 45 g/kg DPM after 48 h of fermentation.

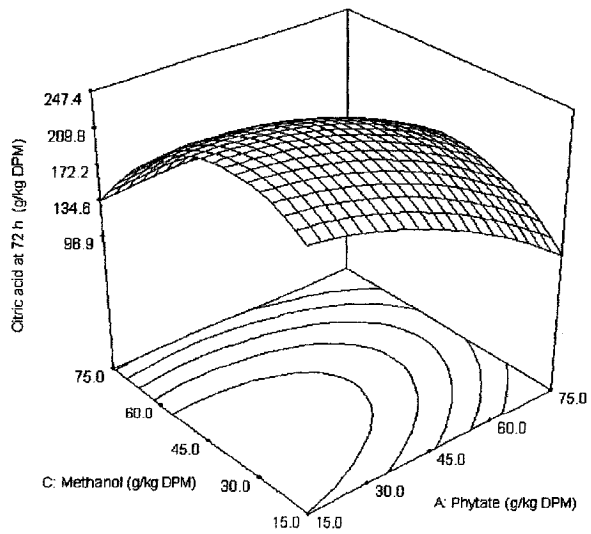

Figure 5.7b Predicted citric acid production from *A. niger* NRRL 567 as a function of initial concentration of phytate and methanol with a fixed olive oil concentration of 45 g/kg DPM after 72 h of fermentation.

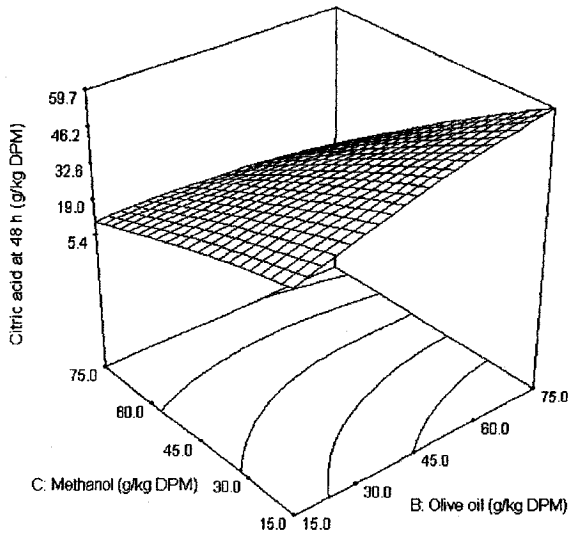

Figure 5.8a Predicted citric acid production from *A. niger* NRRL 567 as a function of initial concentration of olive oil and methanol with a fixed phytate concentration of 45 g/kg DPM after 48 h of fermentation.

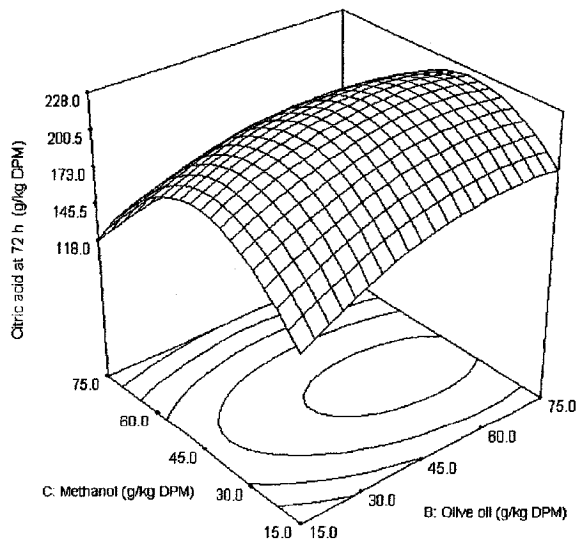

Figure 5.8b Predicted citric acid production from *A. niger* NRRL 567 as a function of initial concentration of olive oil and methanol with a fixed phytate concentration of 45 g/kg DPM after 72 h of fermentation.

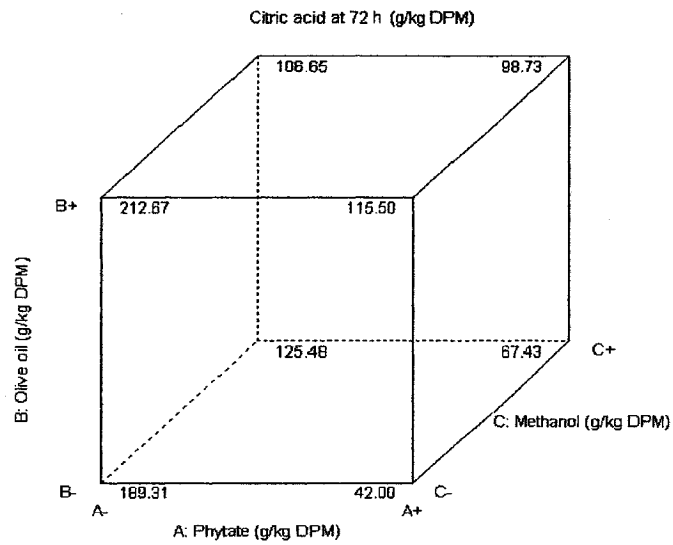

Figure 5.9 Cube plot predicting citric acid production after 72 h of fermentation, as a function of each stimulator level. The values of each cube corners expresses the value of citric acid production computed by equation (5.2).

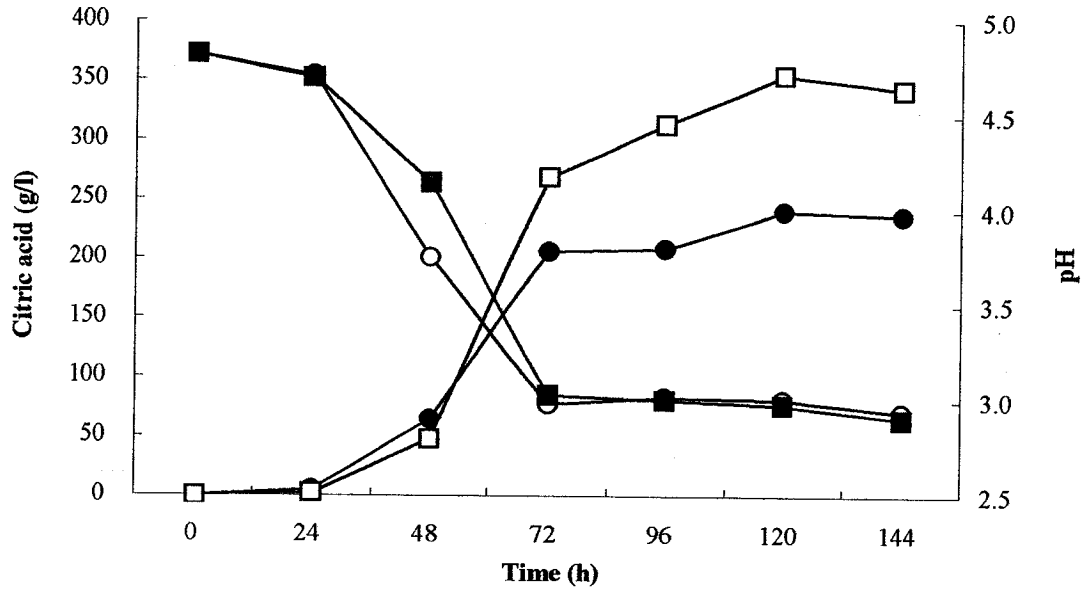

Figure 5.10 Time course behavior of citric acid production and pH by *A. niger* NRRL 567 grown on PM with levels of stimulators optimized one at a time (15 g phytate, 15 g olive oil and 15 g methanol/kg DPM) and using CCD method (19.6 g phytate, 28.9 g olive oil and 40.9 g methanol/kg DPM). For both treatments, the PM was supplemented with: 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl/kg DPM. The PM was wetted to 80% water content, inoculated with 4.0 x 10^6 spores, fermented at 35°C of fermentation temperature and adjusted initially to a pH 8. ●: control (citric acid production); □: optimized condition (citric acid production); ○: control (pH); ■: optimized condition (pH).

CONNECTING STATEMENTS

It was reported that the concentration of hydrogen ion is a significant factor for citric acid production in submerged as well as solid substrate fermentation. In previous study (Chapters 4), the effect of the initial pH of nutrient solution on citric acid production was evaluated and concluded that the initial pH had a considerable effect on the physiology and growth of *A. niger* and production of citric acid grown on peat moss. In the static solid substrate fermentation, the control and montoring of pH during fermentation is very difficult due to uneven distribution of produced organic acid and supplemented acid or base solution for controlling pH. Even though, fungi have a broad pH range for growth and organic acid production, due to an end product inhibition, a high concentration of hydrogen ion at the end of fermentation may suppress organic acid production. To prevent an end product inbibition by produced hydrogen ion, buffer solution was used to improve citric acid production. As the effect of various buffer solutions and the initial pH of the solid substrate were not investigated in previous studies, in this chapter, to study the effect of buffer solutions, various buffer solutions were used for determining the effect of the initial pH of different buffer solution on citric acid production by *A. niger* grown on peat moss. The results from the experiments were compared with previous studies.

This paper will be presented in **Biotechnology Letter in 2004. Authors: Jin-Woo Kim, Suzelle Barrington and John Sheppard.** The contribution of authors are: 1) First author carried out entire experiment work and writing of manuscripts, 2) second author supervised and technical correction of the work and manuscripts and 3) third author provided the analytical advice and manuscript correction

CHAPTER 6

Effect of Buffer Solutions on Citric Acid Production by *Aspergillus niger* NRRL 567 Grown on Peat Moss

6.1 Abstract

In the solid substrate fermentation of *Aspergillus niger* NRRL 567, it was known that pH has an effect on metabolite production and it has been observed that a high level of pH enhances citric acid production. Various growth conditions were simulated with different buffer solutions at various initial pH levels to evaluate the effects on citric acid production by *A. niger* NRRL 567 grown on peat moss (PM). A phosphate buffer and a carbonate buffer with pH 8.6 and pH 10.0 respectively were identified as suitable buffer solutions for citric acid production. The maximum citric acid production of 564.3 g/l was achieved employing the carbonate buffer at a pH of 10.0.

Keywords: *Aspergillus niger*, solid substrate fermentation, citric acid, buffer solution

6.2 Introduction

Solid substrate fermentation is defined as the growth of microorganisms on a moist solid substrate, in the absence of free flowing water (Robinson *et al.*, 2001). It has been widely used for thousands of years, especially in East Asia, for the fermentation of traditional foods such as tempeh, shoyu and miso, the production of enzymes (Koji process), mushrooms and mould-ripened cheese (Fujio *et al.*, 1985; Tengerdy, 1985; Omori *et al.*, 1994, Bellon-Maurel *et al.*, 2003). Recently, solid substrate fermentation has been recognized for its advantages over submerged fermentation (SmF): the absence of a liquid phase and a low substrate humidity level; simpler and smaller fermentation processors; lower risks of bacterial contamination; reduction of waste material and of liquid effluent volumes, and less energy consumption. Moreover, the non-sterile operating condition, the simplification of the media, the utilization of sugar-rich agro-industrial byproducts, and the higher productivity of solid substrate fermentation have provided other incentives for process cost cutting (Szakacs and Tengerdy, 1996; Gutierrez-Correa *et al.*, 1999, Ellaiah, 2004). Accordingly, solid substrate fermentation has a lot of potential and can be an alternative of SmF, for the more efficient production of fine chemicals, enzymes, antibiotics, proteins, immuno-suppressants and spores (Goes and Sheppard, 1999; Ellaiah *et al.*, 2004).

The accumulation of citric acid by *Aspergillus niger* grown on solid substrate is strongly influenced by a number of factors such as nutrient balance, pH, temperature, moisture content (MC), aeration, particle size and level of stimulators (Hang *et al.*, 1987; Jinglong, 1998; Kumar *et al.*, 2003). The initial pH of the solid substrate was found to have an impact on citric acid production by *A. niger* grown on peat moss (PM) (Kim *et al.*, 2004). In addition, the type of buffer used in the nutrient solution is a key factor in

governing citric acid production by the fungus *A. niger* (Roukas, 2000; Uyar and Baysal, 2003).

Most filamentous fungi are observed to grow well under slightly acidic conditions, ranging from 3 to 6, but some fungi are able to growth at a pH below 2 to better compete against bacteria (Fawole and Odunfa, 2003). For the production of citric acid by *A. niger*, the initial pH range from 2 to 6 is commonly used in solid substrate and submerged fermentation (Watanabe *et al.*, 1998; Adham, 2002; Lesniak *et al.*, 2002).

As citric acid production is impacted by the initial pH of the solid substrate and *A. niger* produced a high concentration of citric acid within the narrow optimal pH range during solid substrate fermentation, finding the optimal initial pH of the solid substrate, as well as pH control during the fermentation, are equally important to improving citric acid production (Kim *et al.*, 2004). The the difficulty of control of pH during solid substrate fermentation is one of the drawbacks of the method due to low the MC, lack of mixing and the heterogeneous growth characteristic of fungus. Supplementation with a buffer solution can stabilize sudden pH fluctuations and may result in a stimulating effect on citric acid production (Nagel *et al.*, 1999). Buffer solutions such as phosphate, acetate, carbonate, citric acid and lactic acid are widely used in metabolite production from fungus and are considered to have no adverse effect on cell growth (Nagel *et al.*, 1999; Uyar and Baysal, 2003).

Intensively used by industries, *A. niger* is known as a high producer of citric acid. The technique of solid substrate fermentation allows for the production of citric acid using sugar-rich agro-industrial by-products. Furthermore, citric acid is well known as a bioremediation agent for soils contaminated with heavy metals. The development of an *in-site* solid substrate fermentation system for the production of citric acid requires the

knowledge of optimal citric acid production conditions, including that of medium pH.

The objective of this study was to evaluate the effect of the initial pH of the nutrient solution and buffering on citric acid production by *A. niger* NRRL 567. The fungus was grown on PM supplemented with glucose and a nutrient solution to simulate a sugar rich byproduct.

6.3 Materials and Methods

6.3.1 Microorganism

Aspergillus niger NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was stored in a tube containing glycerol (30% v/v) at –76°C. *A. niger* spores were produced on a potato dextrose agar (PDA, Sigma, St. Louis, MO, USA) plates at 30°C and were sub-cultured at biweekly intervals. After seven to ten days of incubation on PDA plates, 10 ml of 0.1% Tween 80 (Sigma, St. Louis, MO, USA) solution were add to each plate. The surface of PDA was scraped to correct spores and counted using hemocytometer and dilute again to obtain a solution containing 4 x 10^6 spores/ml. This spore solution was used to inoculate the solid substrate fermentation.

6.3.2 Solid substrate preparation and fermentation

Sphagnum PM (Schultz Company, Mississauga, Ontario, Canada) was used as solid substrate. It was initially dried at 60°C for 48 h and screened to remove all particles over 2.0 mm.

Three buffers were selected for this experiment, namely, phosphate, acetate and carbonate. Phosphate tends to control the solution pH at around 6, while acetate and carbonate maintain the solution at acidic or alkaline conditions, respectively. Three types

of buffer solution were prepared and their pH was adjusted to various levels (Table 6.1). For the phosphate buffer solution, the pH was adjusted to 4.7, 6.1 and 8.6 by adding various concentrations of KH_2PO_4 and Na_2HPO_4. More KH_2PO_4 was added to obtain a lower pH and while more Na_2HPO_4 was added to obtain a higher pH. The pH of the acetate buffer was adjusted to between 4.9 and 6.1, by adding different concentrations of CH_3COONa and CH_3COOH. More CH_3COOH was added to obtain a lower pH and more CH_3COONa was added to obtain a higher pH. The pHs of the carbonate were adjusted to between 10.0 and 11.0 by adding Na_2CO_3 and $NaHCO_3$.

Once prepared, all 7 buffer solutions were supplemented with glucose and nutrients, to obtain on the basis of 1.0 kg of dry peat moss (DPM): 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl. Then, the pH of each buffer in the medium was measured (S+N). After adding the same amount of glucose and nutrients as buffered medium, the pH of the control nutrient solution (no buffer) was adjusted to 4.4 or 10.0, using 1 N HCl or 1 N NaOH respectively.

The buffered media and nutrient solutions were added at a rate of 28 ml per duplicate 7 g sample of DPM, held in individual 250-ml Erlenmeyer flasks. Thus, the initial MC of PM was 80%. Finally, each wetted PM samples was supplemented with a stimulator, namely olive oil, on the basis of 28.9 g/kg DPM (Kim *et al.*, 2004b). All PM samples were autoclaved at 121°C for 15 minutes. Once cooled to room temperature, all samples received a second stimulator, methanol, under aseptic conditions at a rate of 40.9 g/kg DPM (99+%, Sigma Chemical Co, U.S.A.). Then, after adding all nutrients and stimulators, the entire contents of the Erlenmeyer flasks were harvested and the pH measured PM (S+N+PM). Finally, 1 ml of spore suspension (4×10^6 spores/ml) was used to inoculate under aseptic conditions. Duplicate PM samples were then incubated for 8

days in a closed chamber maintained at 35°C. After 48, 96, 144 and 192 h, duplicated PM samples were withdrawn for citric acid and glucose quantification.

6.3.3 Analytical methods

Each sampling session, each wet PM sample was split into two fractions: one of 5 g to verify the final MC of PM, to express citric acid production on a DPM basis, and; the second of 5 g for citric acid and glucose determination. Citric acid and glucose extraction was carried out by placing 5 g of wet PM sample in 50 ml of distilled water and shaking for 60 min at 20°C (Jianling and Ping, 1998). The supernatant removed by centrifugation at 11,000 x g for 10 min was tested for pH using a pH meter (Corning, NY, USA) and stored at –20°C for citric acid and residual glucose quantification.

Citric acid quantification was carried out by adding pyridine and acetic anhydride to develop color. The intensity of color, directly related to citric acid concentration, was determined by spectrophotometry at 420 nm (Marier and Boulet, 1958). Glucose was analyzed by the 3,5-denitrosalicylic acid (DNSA) method (Miller, 1959). All citric acid concentrations were expressed on a DPM basis. PM dry matter was determined by drying at 60°C for 48 h. Citric acid yield and glucose consumption were calculated by the following equations:

Citric acid yield (%) = 100 x [produced citric acid (g/l)/utilized glucose (g/l)] (6.1)

Glucose consumption (%) = 100 x [utilized glucose (g/l)/initial glucose (g/l)] (6.2)

6.4 Results

6.4.1 Effect of the initial pH of nutrient and buffer solutions on the pH of PM

The pH of the PM samples after autoclaving (S+N+PM) did not vary among treatments compared to the pH of the buffer solution (S) and that of the buffer medium after adding nutrients (S+N). For example, the PM samples treated with a nutrient solution at a pH of 4.4, without buffer, had a final pH of 5.01 (S+N+PM), while that treated with the buffer solution of pH of 10.0 had a final pH of 5.99 (S+N+PM). Thus, the PM had a major effect on the alkalinity or acidity of the final medium (Table 6.1).

6.4.2 Effect of the initial pH of buffer solution on citric acid production

After 48, 96, 144 and 192 h of fermentation, patterns in citric acid concentration were affected by the type of buffer used and the initial pH of the solution (Figure6.1). Furthermore, the results where highly correlated with the initial pH of the buffer solution, rather than the PM pH after autoclaving. Up to 144 h of fermentation, the control treatments (no buffer) produced citric acid concentrations increasing with the initial pH of nutrient solution; after 144 h of fermentation, citric acid concentrations decreased for both initial nutrient pH levels (pH 4.4 and pH 10.0).

The citric acid concentration obtained using both acetate buffers (AB 4.9 and AB 6.1) were not different. During fermentation, the maximum citric acid productions for both acetate media never exceeded 30 g/l. Although the initial pH of both the acetate buffers and the initial pH of PM were similar to those of the control experiment (pH 4.4), citric acid production when using the acetate buffers were considerably lower than obtained with the control media.

When using the phosphate buffer, citric acid concentration increased up to 144 h of

fermentation but did not drop thereafter; citric acid concentration increased with the initial pH of the phosphate buffers.

Also, when using the carbonate buffer, citric acid concentration increased up to 144 h of fermentation and did not decrease thereafter, but decreased as the initial buffer solution pH increased from 10.0 to 11.0. The stimulating effect of calcium carbonate on citric acid production by C. *lipolytica* PC 711 and *Candida* strains was also reported by Abou-Zeid and Ashy (1984). According to Roukas (2000) using solid substrate fermentation, the highest level of *A. niger* biomass production and, the best citric and gluconic acid yields were obtained using a neutral initial pH.

6.4.3 Effect of the initial pH of buffer solution on glucose consumption

The residual glucose concentration obtained from the fermentation of *A. niger* grown on PM over a various range of initial pH is shown in Figure 6.2. The glucose consumption profiles were similar except for the acetate buffer. For the solid substrate fermentation with the phosphate and carbonate buffers, glucose consumption started within 48 h of fermentation and glucose depletion was observed after 144 h. Glucose consumption reached 96%.

With the acetate buffers, a very lower glucose consumption (41%) was found. The low glucose utilization may indicate a low biomass production and/or a low metabolites production. When the acetate buffer was used, *A. niger* mycelia growth was not observed during the fermentation, also indicating low biomass production.

6.4.4 Effect of the initial pH of nutrient and buffer solution on citric acid yield

For each fermentation period, citric acid yield is reported in Fig 6.3. As observed before, the initial nutrient solution pH (no buffer) had an effect on citric acid yield. Maximum citric acid yield increased with the phosphate and the carbonate buffer employed. When, using no buffer, maximum citric acid yields of 33.6 and 48.4% were achieved after 144 h at the respective initial nutrient solution pH levels of 4.4 and 10.0.

For the PM treated with the phosphate buffer, citric acid yield increased from 51.3 to 57.7%, as the initial buffer solution pH increased from 4.7 to 8.6. For the PM treated with the acetate buffer, citric acid yield dropped from 9.6 to 7.6%, as the initial buffer solution pH increased from 4.9 to 6.0. Finally, for the PM treated with the carbonate buffer, citric acid yield dropped from 59.8 to 51.4%, as the initial buffer solution pH increased from 10 to 10.9.

The carbonate (CB 10.0) and phosphate buffers (PB 8.6) produced a maximum citric acid yield of almost 60% after 144 h of fermentation, which represents a 1.8 fold improvement over the control treatment (pH 4.4). The increase in citric acid production and yield using the buffer solution were associated with the neutralization of acids produced by *A. niger*, as a highly acidic environmental may inhibit fungi functions (Manohar and Divakar, 2002).

6.4.5 Effect of pH of nutrient and buffer solution on pH of PM

The change in PM pH with fermentation time varied among treatments (Figure 6.4). Up to 96 h of fermentation, the pH of all PM samples, exept for those treated with the acetate buffer, decreased proportionally with the amount of citric acid produced along with, most likely, other organic acids. The pH profiles of the acetate buffer treatment did not

vary during fermentation. Citric acid and orther organic acids were likely not producted by *A. niger* grown using the acetate buffer.

Up to 144 h, the pH profiles did not change after 96 h of fermentation despite the increase in citric acid production up to 144 h (Figures 6.1 and 6.4). Citric acid production decreased the pH of PM proportionally up to a certain level (pH 2.8), however, after that minimum, the pH of PM did not drop with an increase in citric acid production. Thus, pH evolution can indirectly indicate citric acid concentration, especially during the initial phase of fermentation, however, the pH profiles during the middle and end of the fermentation period, cannot be correlated with citric acid production.

6.5 Discussion

From the results of our work, it was concluded that citric acid production and yield were strongly affected by initial pH level and buffers. The high initial pH of buffer supported high citric acid production. Also the result suggested pH 10 of carbonated buffer enhanced citric acid production and yield. The result from this study is in agreement with the research of Roukas (2000). According to the conclusion of Roukas, the highest level of *A. niger* biomass production, citric acid production and yield, and gluconic acid production and yield were obtained at the neutral initial pH range of solid substrate. The increase in citric acid production and yield at near neutral pH of solid substrate and presenting of buffering agent may be due to a neutralization of produced organic acids by *A, niger*, especially citric acid and preventing product inhibition during the fermentation (Manohar and Divakar, 2002).

6.6 Acknowledgements

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6.7 References

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Table 6.1 Experimental buffer solutions and their effect on medium initial pH

Buffer	Treatment notation	Buffer strength	pH (S)	pH (S+N)	pH (S+N+PM)
None*	pH 4.4	-	-	4.42	5.01
	pH 10.0	-	-	9.97	5.99
Phosphate (PB)	PB 4.7	0.2 M	4.68	4.52	4.66
	PB 6.1	0.2 M	6.09	5.67	4.90
	PB 8.6	0.2 M	8.60	6.43	5.34
Acetate (AB)	AB 4.9	0.037 M	4.92	4.80	4.89
	AB 6.1	0.1 M	6.05	5.48	5.05
Carbonate (CB)	CB 10.0	0.2 M	10.00	6.76	5.65
	CB 11.0	0.2 M	10.92	6.94	5.85

Note:

*None – control,

(S)- pH of buffer solution before being added to nutrients,

(S+N) - pH of buffer medium (buffer solution after being added to nutrient,

(S+N+PM) - pH of PM with buffer medium or nutrient solution after autoclaving.

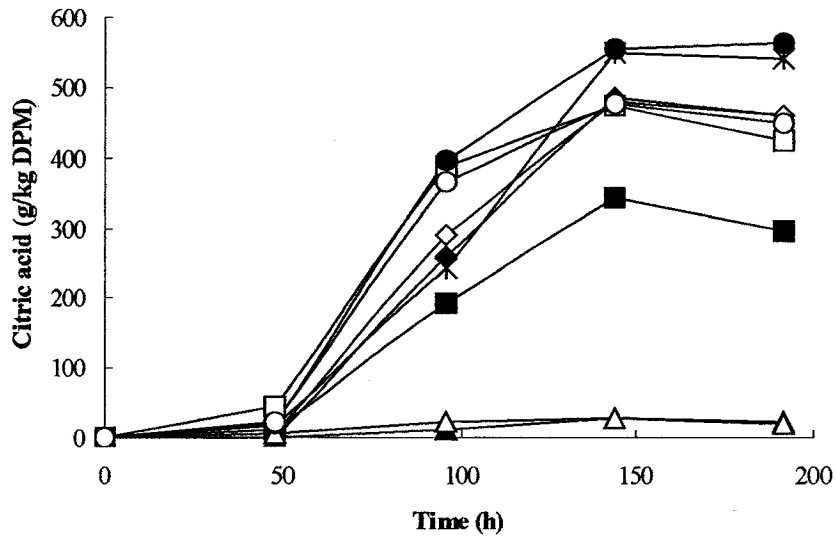

Figure 6.1 Effects of buffer solutions on citric acid production by *A. niger*; (■) pH 4.4; (□) pH 10.0; (◆) P.B. 4.7; (◇) P.B. 6.1; (*), P.B. 8.6; (▲) A.B. 4.9; (△) A.B. 6.1; (●) C.B. 10.0; (○) C.B. 11.0.

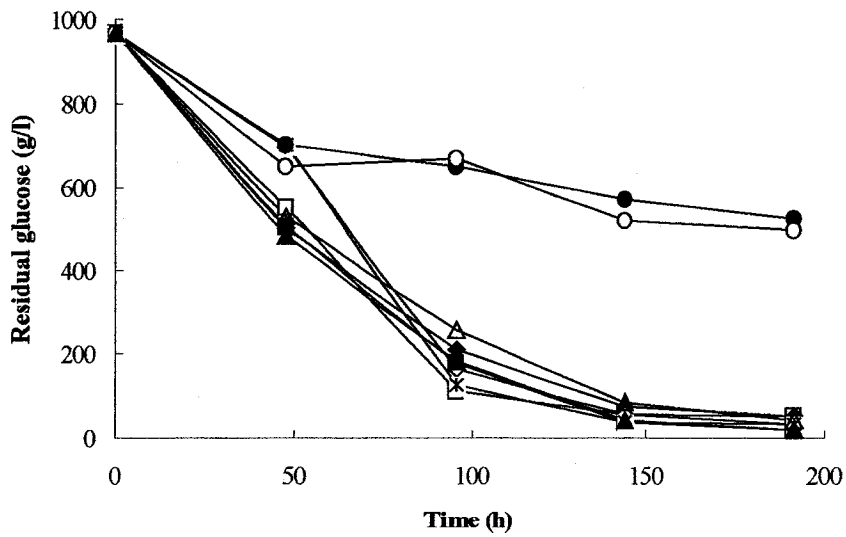

Figure 6.2 Effect of various buffer solutions on residual glucose concentration; (■) pH 4.4; (□) pH 10.0; (◆) P.B. 4.7; (◇) P.B. 6.1; (*); P.B. 8.6; (▲) A.B. 4.9; (△) A.B. 6.1; (●) C.B. 10.0; (○) C.B. 11.0.

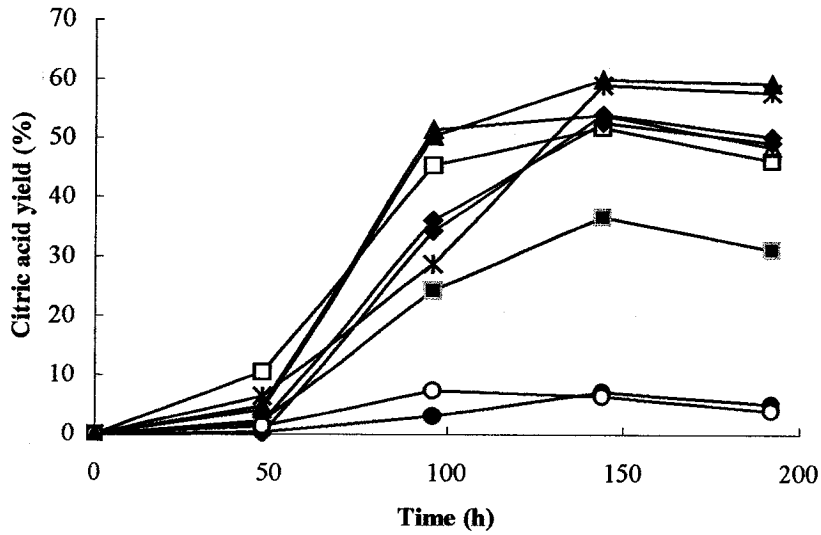

Figure 6.3 Effect of various buffer solutions on citric acid yield; (■) pH 4.4; (□) pH 10.0; (◆) P.B. 4.7; (◇) P.B. 6.1; (*); P.B. 8.6; (▲) A.B. 4.9; (△) A.B. 6.1; (●) C.B. 10.0; (○) C.B. 11.0.

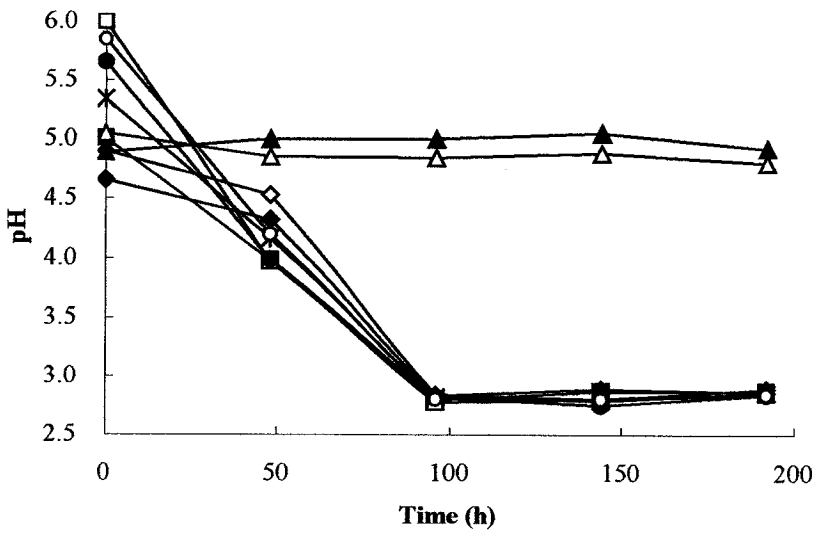

Figure. 6.4 Variation in pH with the fermentation period; (■) pH 4.4; (□) pH 10.0; (◆) P.B. 4.7; (◇) P.B. 6.1; (*); P.B. 8.6; (▲) A.B. 4.9; (△) A.B. 6.1; (●) C.B. 10.0; (○) C.B. 11.0.

CONNECTING STATEMENTS

Soil flushing paratice for remediation of heavy metal contaminated soil generally employs acids, bases, chelating agents, surfactants, alcohols or reducing agents as extractants. Among the chelating agents, synthetic chemicals and strong acids are the most popular extractants for heavy metals. However their toxicity and high costs have limited their use in soil flushing. By being absorbed on the surface of soil particles, strong acids dissolve useful soil minerals and macronutrients. Moreover, using strong acids as extractants acidifies the soil, destroys soil structure and damages the soil ecosystem.

Fungal citric acid with carboxylic functional groups has chelating properties and is most often proposed as potential metal extracting agents for effective remediation of Cr, Ni and Zn from soil. Citric acid produced by *A. niger* are beneficial in remediation techniques because they are capable of improving soil properties by enhancing the formation of stable soil aggregates, do absorb heavy metals in a more specific way, are less likely to leach out soil macronutrients and are less costly and more biodegradable.

For the future application of fugal citric acid for the remediation of heavy metal contaminated soil uing soil flushing, this chapter evaluates the citric acid production potential in a semi-continuous fementation system using column reactor. For optimization of citric acid production in the column reactor, a full factorial design was used to find optimum levels of physical parameters including aeration, thickness of solid substrate and incubation temperature. The results from this experiment were then compared with the results of batch type solid substrate fermentation to evaluate its efficiency in citric acid production and potential for a future remdiaition practice.

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CHAPTER 7

Optimization of Citric Acid Production by *Aspergillus niger* 567 Using Peat Moss in a Column Bioreactor

7.1 Abstract

Citric acid production using *Aspergillus niger* NRRL 567 grown on peat moss (PM) has been optimized in a column bioreactor using a statistically-based method. The 2^3 full factorial design having eight different fermentation conditions was applied to evaluate their significance on total citric acid production and yield, where the three independent variables evaluated were aeration, thickness of solid substrate bed and fermentation temperature. Aeration and temperature were identified to be important variables and had a positive effect on responses, however, the thickness of solid substrate had an insignificant effect on citric acid production and yield within the tested range. The optimum fermentation condition for citric acid production and yield consisted of high aeration (0.84 vvm), deeper thickness of bed (22 cm) and high fermentation temperature (32°C), respectively. Under these conditions total citric acid production and yield were 123.9 g/kg dry peat moss (DPM) and 17.8%, respectively.

7.2 Introduction

Solid substrate fermentation involves the growth of microorganisms on moist solid substrates in the absence of free flowing water (Chahal, 1985; Nandakumar *et al.,* 1994). In general, the solid substrate acts as physical support, provides nutrients and holds water meeting the requirements of the microorganism (Ghildyal *et al.,* 1994; Pintado *et al.,* 1998, Goes and Sheppard, 1999). Solid substrate fermentation has been widely used for traditional food fermentation, enzyme production by the Koji process, mushroom production, mould-ripening of cheese and partial composting of agricultural residues (Omori, *et al.,* 1994; Jianlong and Ping, 1997). As solid substrate fermentation provides growth conditions simulating natural habitats, such as layers of agro-industrial byproducts, it is presently gaining interest for the manufacturing of bulk chemicals and highly purified enzymes at a lower cost than submerged fermentation (Kokitkar and Tanner, 1990; Smits *et al.,* 1998; Goes and Sheppard, 1999).

The potential use of agro-industrial byproducts with solid substrate fermentation for citric acid production has been intensively studied over the past five decades (Favera-Torres *et al.,* 1998; Romero-Gomez *et al.,* 2000). The white rot fungus *A. niger* can be grown on sugar rich agro-industrial byproducts using solid substrate fermentation to produce organic acids for various purposes, including bioremediation. Weak organic acids such as citric, tartaric, oxalic and formic acids have been used as chelating agents for the bioremediation of soils and sediments contaminated with heavy metals (Wasay *et al.,* 1998). The organic acids produced by *A. niger* have carboxyl groups which tend to donate protons (H^+), resulting in negatively charged carboxyl groups capable of competing against soil particles for heavy metal adsorption. A prerequisite for the development of such an *in-site* bioremediation technique, using the fungus *A. niger*, is the optimization of

a semi-continuous production technique using sugar-rich byproducts.

The objective of the present study was, therefore, to evaluate citric acid production by *A. niger* when grown using a semi-continuous system consisting of a column bioreactor holding the sugar rich byproduct periodically supplemented with nutrients. This column reactor also requires the flushing of its solid substrate to recover the citric acid produced for the bioremediation of heavy metals from contaminated soils. The sugar rich byproduct was simulated using peat moss (PM) wetted on a periodic basis, with a glucose solution. The physical fermentation parameters optimized were: aeration, thickness of solid substrate and temperature. The present study used a 2^3 full factorial design (FFD) to identify, for each variable or physical fermentation parameter, the level optimizing citric acid production and to evaluate the interactions between the variables.

7.3 Materials and Methods

7.3.1. Microorganism

Aspergillus niger NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was stored at –76°C, in tubes containing glycerol (30% v/v). *A. niger* spores were produced on a potato dextrose agar (PDA, Sigma, St. Louis, MO, USA) plates kept at 30°C. After ten days of incubation on PDA plates, 10 ml of 0.1% Tween 80 (a non ionic surfactant) was added to each plate to harvest the spores. Diluted spore suspensions of 4.0×10^6 spores/ml were counted using hemocytometer and prepared as inoculum.

7.3.2 Solid substrate

Sphagnum PM (Schultz company, Mississauga, Ontario, Canada) was used as the

solid substrate. It was dried at 60°C for 48 h and screened to remove all particles over 2.0 mm, before being rewetted and inoculated with *A. niger*.

7.3.3 Experimental columns

Figure 7.1 shows the fermentation device used in semi-continuous fermentation by *A. niger* grown on PM. The semi-continuous fermentation with 135 and 270 g wet PM was carried out in a column bioreactor with the following dimensions: internal diameter 7 cm, height 23 cm and internal volume 0.89 l. The cylindrical bioreactor consisted of a 3 mm thickness Plexiglas with acrylic material of top and bottom. Non-sterile air was saturated with water by passing through humidifiers before entering the bioreactor from the bottom. Outlets on the top and bottom allowed for air exhaustion and excess water drainage. After inoculation, the bioreactor was placed in an incubator for 12 days under non-sterile conditions.

7.3.4 Fermentation condition

Dry PM samples of 40 and 80 g were placed in 0.5 l autoclave bags (Sigma Chemical Co, U.S.A) and wetted using a nutrient solution with an initial pH adjusted to 8, by adding 1 N NaOH. The nutrient solution provided the following glucose and salt levels per kg DPM: 250 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl (Kim *et al.*, 2004a,b). The moisture content (MC) of the DPM was further raised to 80% by adding an additional amount of distilled water. Each wetted PM sample was supplemented with stimulators, namely olive oil (Bertolli, Laval, Qc, Canada) and phytate (40 %wt, Aldrich, St. Louis, MO, USA), on the basis of 28.9 and 19.6 g/kg DPM (Kim *et al.*, 2004b). Once supplemented with nutrients and stimulators, the wet PM samples were autoclaved for 20

minutes at 121°C.

Once cooled to room temperature, all samples received a third stimulator, methanol (99+% wt, Sigma Chemical Co, U.S.A.), at a rate of 40.9 g/kg DPM, and 1.0 ml of inoculum containing 4 x 10^6 spores/ml for every 20 g DPM. The initial pH of the inoculated PM was determined by thoroughly mixing the content of each inoculation bag, removing a 5 g of wet sample, soaking it in distilled water at a ratio of 1:10 and measuring its pH using a pH probe. This initial pH value corresponded to 5.47. The prepared wet PM samples were removed from the autoclave bags before being placed in the column bioreactors.

The procedure was designed to evaluate the effect of the following fermentation parameters on citric acid production and yield: aeration level, thickness of PM bed and fermentation temperature. These parameters were varied simultaneously according to factorial design matrix (Table 7.1). Each column could hold up to 300 g of wet PM. The two wet PM depths tested corresponded to 22 ± 0.5 cm (270 g of wet PM) and 11 ± 0.5 cm (135 g of wet PM). The effect of temperature was tested by incubating the column bioreactors for 12 days, in a closed chamber maintained at either 22 or 32°C. The effect of aeration was tested by bubbling humidified non-sterile air at the base of the reactors. The aeration rates tested were 0 and 0.84 vvm (volumes of air per minute per working volume of reactor).

At four-day intervals under non-sterile condition, the PM was fed and washed as follows. The PM contained in each column bioreactor was soaked twice for one hour, using non-sterile de-ionized water. After each soaking, the PM was drained and collected to determine its citric acid and glucose content. Once soaked and drained twice, each column bioreactor was placed in an incubator for 24 h to remove excess free flowing water.

After completion of all washing processes, the 40 and 80 g DPM samples each received 20 ml and 40 ml of concentrated solutions with 500 g glucose /l and at the first and second washing cycles (days 4 and 8), each cycle total 250 g glucose/kg DPM was supplemented (Roukas, 1991; Petruccioli *et al.*, 1996). On day 12, the entire content of each column bioreactor was harvested for glucose and citric acid quantification, by soaking in distilled water for 60 min at 150 rpm at room temperature for citric acid and glucose extraction (Xu *et al.*, 1989).

7.3.5 Analytical methods

For each washing session, the washing effluent was collected, centrifuged at 11,000 x g for 10 min. to remove all PM particles, and tested for pH, residual glucose and citric acid. After adding pyridine and acetic anhydride to develop color, citric acid was determined by spectrophotometry at 420 nm (Marier and Boulet, 1958). Glucose was analyzed by the 3,5-denitrosalicylic acid (DNSA) method (Miller, 1959).

All citric acid and residual glucose concentrations were calculated based on the mass of kg DPM by drying at 60°C for 48 h. After measuring citric acid production at each sampling session, total citric acid concentration was calculated by summing the glucose production level of each sampling session. Citric acid yield and glucose consumption were calculated as follows:

Citric acid yield (%) = 100 x [produced citric acid (g/l)/utilized glucose (g/l)] (7.1)

Glucose consumption (%) = 100 x [utilized glucose (g/l)/initial glucose (g/l)] (7.2)

7.3.6 Statistical Procedure

A 2^3 FFD method was applied to determine the effect of fermentation parameters on citric acid production by *A. niger* grown in column reactors under semi-continuous fermentation. The 2^3 FFD method requires that two levels of each parameter, where each high and low levels are coded +1 and –1 (Table 7.1). Thus, 8 combinations of fermentation conditions were tested and their input variables are reported in Table 7.2.

The performance of the 8 different conditions can be used to produce a behavior model expressed using the following equation, which includes all linear and interaction terms:

$$Y = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \beta_3\chi_3 + \beta_{12}\chi_1\chi_2 + \beta_{23}\chi_2\chi_3 + \beta_{13}\chi_1\chi_3 + \beta_{123}\chi_1\chi_2\chi_3 \qquad (7.3)$$

where Y = predicted response; β_0 = intercept; β_1, β_2, β_3 = linear coefficients; β_{11}, β_{22}, β_{33} = squared coefficients; β_{12}, β_{13}, β_{23}, β_{123}= interaction coefficients;

The significance and adequacy of the second-order equation (7.3) was measured using analysis of variance (ANOVA), definded as "a method for estimating the amount of variation within all treatment and comparing it to the variables between treatments" (Berthouex and Brown, 1994). The *F*-test was applied to evaluate the statistical significance of the model. The *F* value represents "the ratio of the mean square due to regression to the mean square due to error" (Panda *et al.,* 1999). High *F* values imply that models are significant and can accurately predict the experimental results. The importance of the *F* values was interpreted as a level of provability (*P),* where a level under 0.05 implies a level of confidence of over 95%. The coefficients of all linear terms of the model

equation provided a measure of the effect of the level of the independent variable on the response (Medeiros *et al.*, 2002).

7.4 Results and Discussion

7.4.1. Experimental design: 2^3 full factorial design (FFD)

The measured citric acid production level and yield obtained with the 8 different variable combinations of parameters are reported in Table 7.2. Citric acid production ranged from 30 to 124 g/kg DPM while the yield ranged from 4.4 to 17.8%. The highest level of production was obtained using the aeration rate of 0.84 vvm, the PM thickness of 22 cm and a temperature of 32°C. Citric acid production level and yields measured at 32°C were four times as high as those measured at 22°C. Aeration was another parameter having a major impact, as an air flow rate of 0.84 vvm increased citric acid production and yield by a factor of 3, as compared to no aeration. Also PM thickness was the parameter which had the effect, as doubling its thickness increased citric acid production and yield by a 33%.

The measured levels were used to generate a second-order equation for predicting citric acid production and yield, by computing the significant linear, squared and interaction coefficients. Neglecting the terms, χ_1 and $\chi_1\chi_2\chi_3$, which were insignificant, the result of 2^3 FFD produced the following equation which describes the effects of the variables on the response. The equation shows that total citric acid production ($Y_{CA\ total}$) and yield ($Y_{CA\ yield}$) are a function of the coded levels of the three tested input variables. The second-order equation used to predict those optimal fermentation conditions for best citric acid production (Abdel-fattah and Olama, 2002):

$$Y_{CA\ total} = 54.47 + 16.01\chi_1 + 21.46\chi_3 + 6.75\chi_1\chi_2 + 17.38\chi_1\chi_3 + 4.54\chi_2\chi_3 \quad (7.4)$$

$$Y_{CA\ yield} = 9.41 + 1.82\chi_1 + 3.37\chi_3 + 0.45\chi_1\chi_2 + 2.60\chi_1\chi_3 + 0.81\chi_2\chi_3 \quad (7.5)$$

where the coefficients χ_1, χ_2 and χ_3 correspond to the coded value of fermentation parameters such as aeration, thickness of solid substrate bed and incubation temperature.

The goodness of fit of the second-order regression equation was checked by determining its coefficient of determination (R^2), where R^2 measures the correlation between the measured and predicted citric acid production values. A value of 1.0 indicates perfect correspondance or goodness of fit between the two values. The R^2 of equations (7.4) and (7.5) were found to be 0.988 and 0.986 for citric acid production and yield, respectively, which indicates good agreement between the quadratic model of FFD and the measured data. These values indicate that 98.8 and 98.6% of the variability in the response can be explained by equations (7.4) and (7.5).

The ANOVA procedure produced *F* values of 31.9 and 28.8 for citric acid production and yield, indicating a good fit between the equations and the actual values (Table 7.3). For the total citric acid production and its yield, ANOVA produced *P* levels under 0.05 for χ_1 (aeration) and χ_3 (temperature), indicating that these two variables have a highly significant effect, for the range tested (Panda *et al.*, 1999; Li *et al.*, 2001; Vohra and Satyanarayana, 2002).

7.4.2 Modeling the effects of the independent variables

The effect of the three independent variables on citric acid production and yield during semi-continuous fermentation were studied using equations (7.4) and (7.5) to plot response

surface curves.

The interaction between aeration and thickness of solid substrate was plotted for an incubation temperature fixed at 27°C. Figure 7.2a shows a surface curve where citric acid production is mainly influenced by aeration. At a bed thickness of 22 cm, citric acid production increased noticeably with increased aeration rate. However, at constant aeration rate, citric acid production did not changed significantly with a deeper solid substrate bed. As compared to a bed thickness of 22 cm, a fixed bed thickness of 11 cm demonstrated a less aeration effect. Under optimum levels, a maximum citric acid production of 77.2 g/l was predicted. A similar response surface curve was obtained for citric acid yield (Figure 7.2b), which also maximized at 11.7%, but varied little between bed thicknesses. Thus, thick beds of solid substrates need a forced aeration to support high citric acid production rates. The effect of aeration has been intensively investigated by many researchers who reported that the growth of fungi is aerobic, that forced aeration is indispensable for the over-production of citric acid (Abou-Zeid and Ash, 1984).

The interactive effect of aeration and incubation temperature on citric acid production and yield are plotted in Figures 7.3a and 7.3b. At a high incubation temperature, aeration had a significant effect on increasing citric acid production, at a constant solid substrate thickness of 16.5 cm, while at a low incubation temperature, aeration level had no significant effect. Thus, high citric acid production rates obtained under the optimal incubation temperatures require forced aeration. Considering the effect of an incubation temperature on citric acid production by *A. niger* NRRL 567 grown on PM, Kim *et al.* (2004a,b) reported that citric acid concentration increased with the incubation temperature up to 35°C. As the cylindrical column bioreactor has a relatively small surface area compare to its bed volume, surface aeration leads to insufficient oxygen supply within the

center of the solid substrate mass. At low incubation temperatures, cell growth and citric acid production are lower and surface aeration suffices in supplying oxygen to the fungi.

Figures 7.4a and 7.4b plot the response surface curves for citric acid production and yield, respectively, as a function of the solid substrate thickness and incubation temperature. The two plots show the dominant effect of the incubation temperature on citric acid production and yield and the limited effect of thickness. Optimum citric acid production was achieved at an incubation temperature of 32°C and a bed thickness of 22 cm.

A cube plot was developed (Figures 7.5a and 7.5b) to evaluate the combined effect of the three tested factors. This plot has eight corners representing eight different experimental conditions. The plus (+) and minus (-) signs represent the coded level (-1 and +1) of each variable. The maximum citric acid concentration and yield were predicted under a high level of aeration (A+), high thickness of solid substrate (C+) and high temperature (C+) (Berthouex and Brown, 1994). Thus, the combined influence of temperature and aeration rate had a dominant effect on stimulating citric acid production and yield.

The results also indicate that the statistically-based optimization procedure using a FFD proved to be an effective method in optimizing fermentation conditions. In the experiment using the column bioreactor, total citric acid production and yield from three washing sessions were 123.9 g/kg DPM and 17.8%.

Citric acid production and yield using a semi-continuous fermentation was much lower than that obtained with batch fermentation tests using the same simulated substrate and *A. niger*. While the semi continuous process produced a citric acid level and yield of 123.9 g/kg DPM and 17.8%, respectively, batch tests produced 561.5 g/kg DPM of citric

acid and a yield of 58%. The low productivity of citric acid in the column bioreactor may result from low surface aeration and heterogeneous fermentation condition such as temperature gradient, variation of MC and restricted gas exchange.

7.5 Conclusions

The results demonstrated that for citric acid production by *A. niger* NRRL 567, the semi-continuous fermentation process is less productivity than a batch process. As the semi-continuous fermentation provides citric acid production and the flushing of its solid substrate to recover the citric acid produced for the bioremediation of heavy metals simultaneously, in spite of disadvantages of semi-continuous fermentation system for citric acid production, it still has the potential for using *in-situ* remediation system for heavy metal contaminated soil. In addition, *in-situ* soil flushing using fungal organic acids, especially citric acid, represents a biodegradable approach and effective remediation technique compared to using chemical chelators (Elliott and Shastri, 1999; Sayer and Gadd, 2001).

Thus, to obtain this purpose, an appropriate bioreactor type and fermentation conditions must be used. The values obtained from semi-continuous fermentation and other fermentation parameters need to be optimized by using sequential optimization for further improvement in citric acid production and yield.

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Table 7.1 Coded values used in 2^3 factorial design to optimize the fermentation parameters for citric acid production in column reactor

Variables	Parameter	Unit	Coded and actual level	
			-1	1
X_1	Aeration	vvm	0	0.84
X_2	Thickness	cm	11	22
X_3	Temperature	°C	22	32

vvm (min^{-1}): volume of aeration (ml)/working volume of reactor (ml)/minute (min).

Table 7.2 Experiment and predicted values of citric acid production and yield

Run	X_1	X_2	X_3	pH		Total citric acid (g/kg DPM)		Citric acid yield (%)	
No	vvm	cm	°C	Day 3	Day 12	Observed	Predicted	Observed	Predicted
1	0	11	22	3.822	3.350	42.35	45.67	8.71	8.08
2	0.84	11	22	3.529	3.275	33.46	29.43	6.10	5.62
3	0	22	22	4.255	3.950	26.40	23.09	4.93	5.56
4	0.84	22	22	3.608	3.094	29.82	33.85	4.42	4.89
5	0	11	32	3.910	3.452	48.79	44.76	8.48	8.01
6	0.84	11	32	3.979	2.960	94.71	98.02	16.57	15.94
7	0	22	32	3.789	3.663	36.30	40.34	8.26	8.73
8	0.84	22	32	4.008	3.085	123.91	120.60	17.83	18.46

R^2 = 0.988 (total citric acid production) and 0.986 (citric acid yield),

X_1 = aeration ; X_2 = thickness of solid substrate bed; X_3 = incubation temperature.

Table 7.3 ANOVA table: influence of fermentation parameters on citric acid production and yield

	Citric acid production and yield					
	Total citric acid (g/kg DPM)			Citric acid yield (%)		
	Sum of Squares	*F* value	*P* level	Sum of Squares	*F* value	*P* level
Model	8678.58	31.87	0.0307*	178.26	28.81	0.0339*
X_1	2049.41	37.63	0.0256*	26.41	21.34	0.0438*
X_3	3684.46	67.65	0.0145*	91.00	73.54	0.0133*
X_1X_2	364.51	6.69	0.1225	1.60	1.29	0.3738
X_1X_3	2415.42	44.35	0.0218*	53.98	43.62	0.0222*
X_2X_3	164.79	3.03	0.2241	5.26	4.25	0.1752

* *P* level less than 0.05 indicate model terms are significant

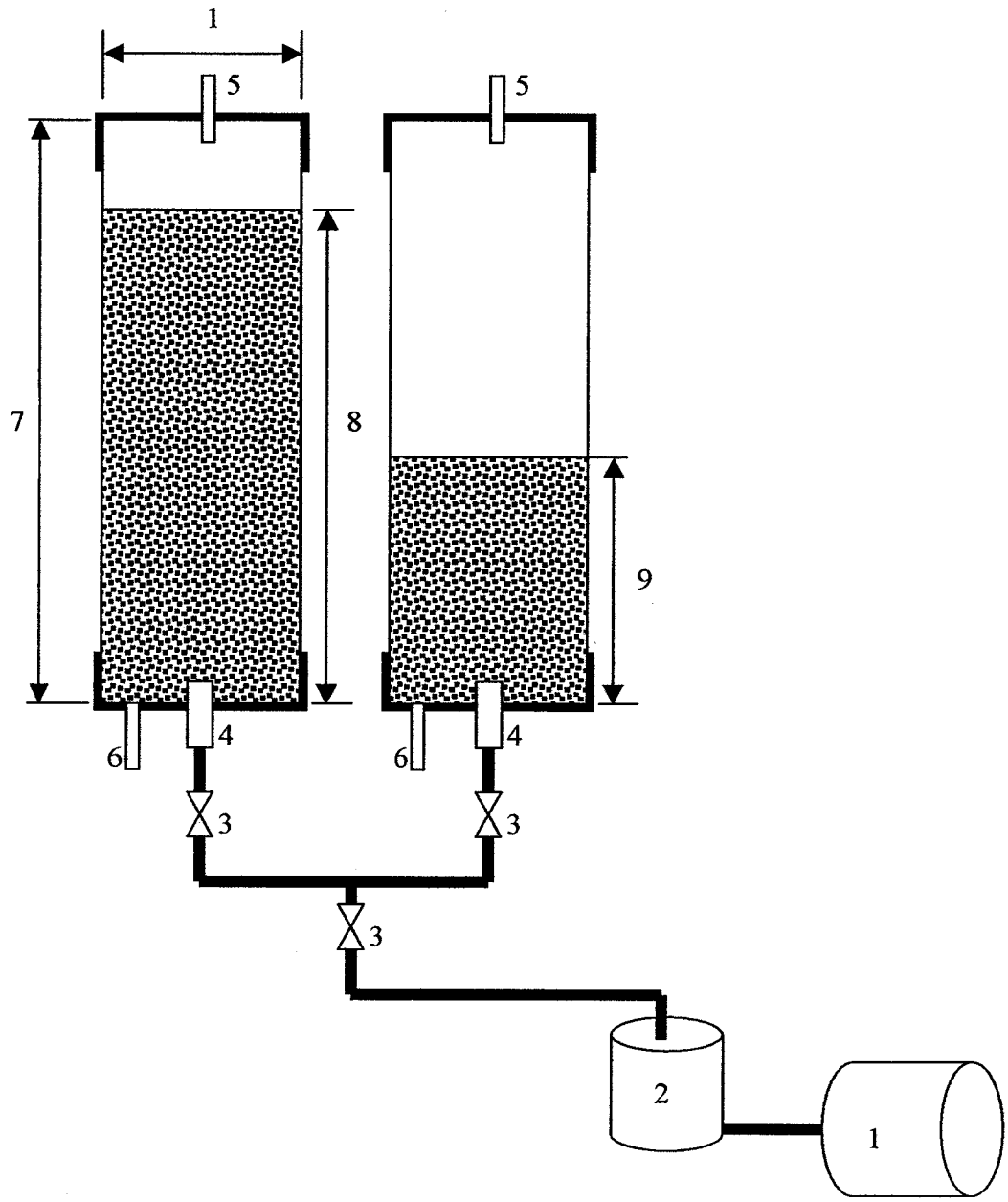

Figure 7.1 Schematic diagram of the system.

(1) air compressor, (2) humidifier, (3) pressure regulating valve, (4) air inlet, (5) air outlet, (6) water outlet, (7) hight of reactor (23 cm), (8) thickness of solid substrate bed (22 cm), (9) thichness of solid substrate bed (11 cm), (9) diameter of reactor (7 cm).

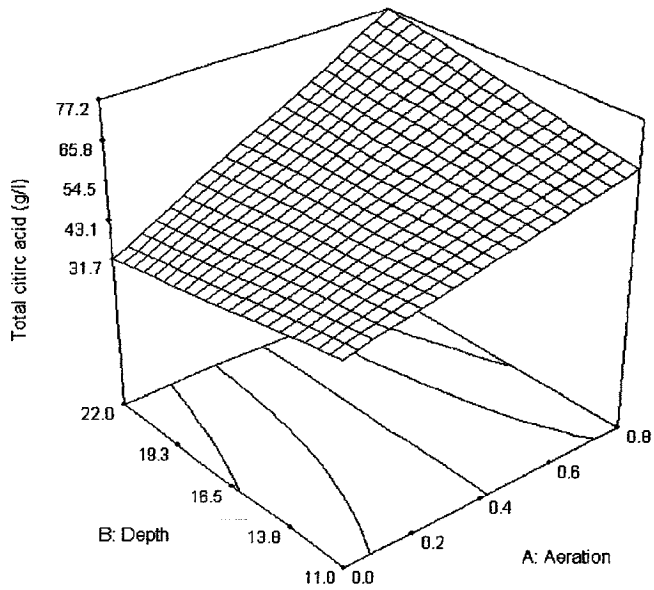

Figure 7.2a Predicted total citric acid production from *A. niger* as a function of aeration and thickness of solid substrate at the constant incubation temperature (27°C).

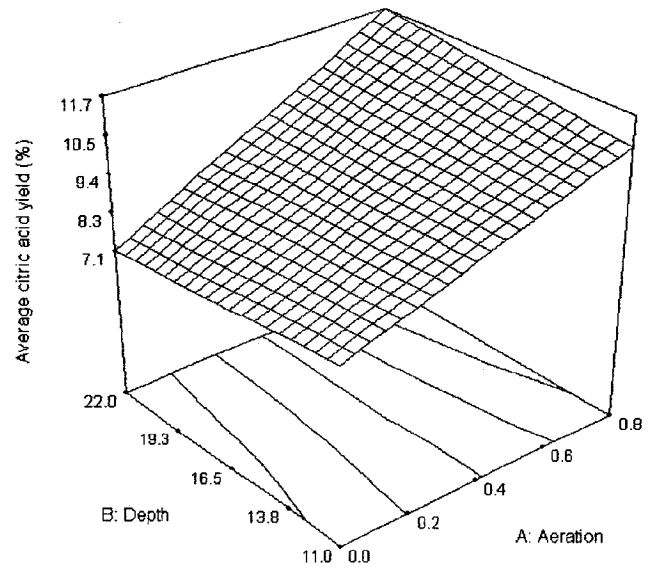

Figure 7.2b Predicted citric acid yield from *A. niger* as a function of aeration and thickness of solid substrate at the constant incubation temperature (27°C).

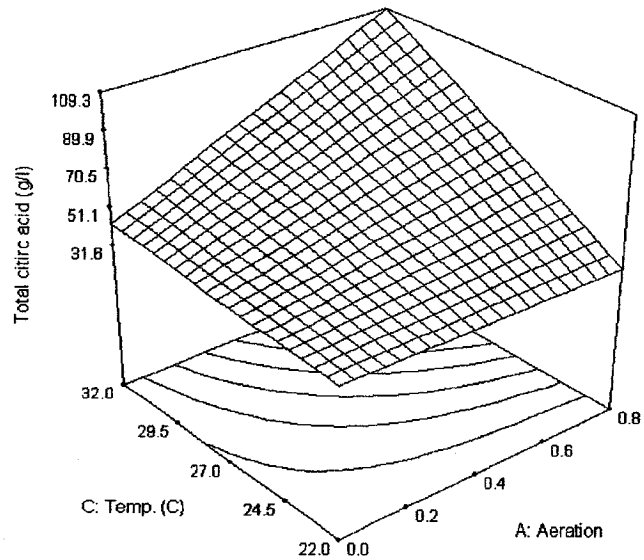

Figure 7.3a Predicted total citric acid production from *A. niger* as a function of aeration and incubation temperature at constant thickness of solid substrate (16.5 cm).

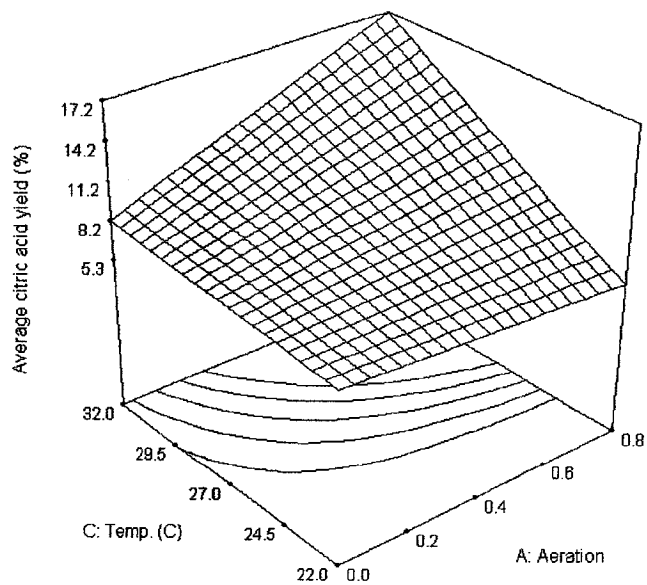

Figure 7.3b Predicted citric acid yield from *A. niger* as a function of aeration and incubation temperature at constant thickness of solid substrate (16.5 cm).

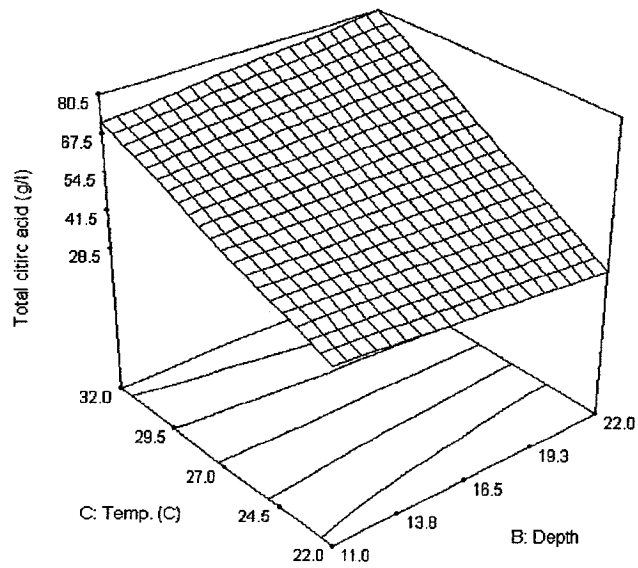

Figure 7.4a Predicted total citric acid production from *A. niger* as a function of thickness of solid substrate and temperature at constant level of aeration (0.42 vvm).

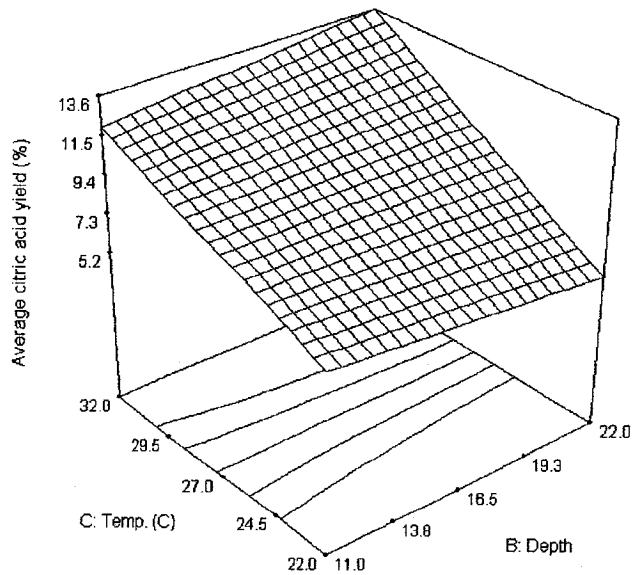

Figure 7.4b Predicted citric acid yield from *A. niger* as a function of thickness of solid substrate and incubation temperature at constant level of aeration (0.42 vvm).

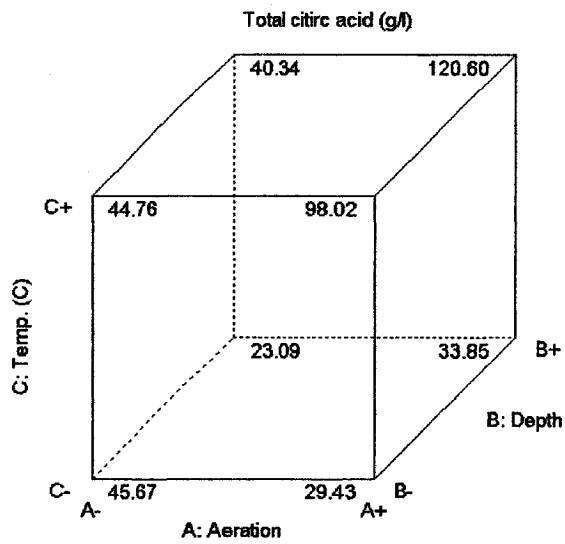

Figure 7.5a Cube plot showing the interactive effect of aeration, thickness of solid substrate and incubation temperature on total citric acid production. The cube corner values are the level of citric acid production predicted using the second-order polynomial equation (7.4).

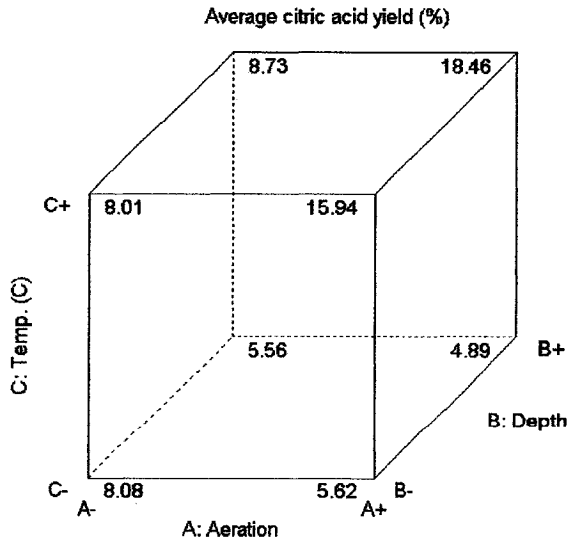

Figure 7.5b Cube plot showing the interactive effect of aeration, thickness of solid substrate and temperature on citric acid yield. The cube corner values are the level of citric acid production predicted using the second-order polynomial equation (7.5).

CONNECTING STATEMENTS

Whey, a byproduct of cheese making, is a nutrient rich substrate containing substantial amounts of lactose, proteins, minerals and vitamins. Due to its high lactose content, cheese whey could be an economical source of carbohydrate for the production of value-added products, especially citric acid, from the fermentation of the fungus *Aspergillus niger*. Moreover, the production of citric acid using a byproduct provides considerable advantages of waste material management as well as decrease of production cost.

Citric acid production by *A. niger* is significantly affected by the composition of medium including carbon, nitrogen, phosphate and stimulators. This chapter deals with optimization of composition of whey-based medium for citric acid production in submerged fermentation by *A. niger* using statistically-based optimization. In this study, cheese whey (50 g/l) was used as basal nutrients and the effect of additional important nutrients and stimulators including glucose, $(NH_4)_2SO_4$, KH_2PO_4, methanol, phytate, olive oil on citric acid production was evaluated using central composite design. Then, the results obtained from this study were compared to those obtained from solid substrate fementation system.

This paper will be presented in **Appl. Microbiol. Biotechnol. in 2004. Authors: Jin-Woo Kim, Suzelle Barrington, John Sheppard and Byong H Lee.** The contribution of authors are: 1) First author carried out entire experiment work and writing of manuscripts, 2) second author supervised and technical correction of the work and manuscripts, 3) third author provided fermentation facilities and manuscript correction, and 4) fourth author provided advice for the design of experiment and manuscript correction.

CHAPTER 8

Optimization of Medium Composition for Citric Acid Production using Cheese Whey by *Aspergillus niger* NRRL 567

8.1 Abstract

The effect of supplementing nutrients to a cheese whey medium was investigated using *Aspergillus niger* NRRL 567 in submerged fermentation to produce citric acid. In batch experiments, 50 g/l whey media were prepared and supplemented with different levels of glucose, $(NH_4)_2SO_4$ and KH_2PO_4. Also, effects of three additives including methanol (1.25 – 93.75 g/l), olive oil (1.25 – 93.75 g/l) and phytate (0.12 – 9.38 g/l) in whey-based medium were investigated after optimization of basal nutrients using central composite design (CCD). Glucose supplementation influenced citric acid production, while $(NH_4)_2SO_4$ and KH_2PO_4 had insignificant effects in tested ranges. Citric acid production was identified to correlate with the initial concentration of methanol, olive oil and phytate, as described by the second-order polynomial model with high confidence levels. The predicted maximum citric acid production was obtained with 48.3 g methanol, 47.7 g olive oil and 3.76 g phytate at 168 and 240 h of fermentation. Under the conditions studied, the predicted citric acid concentrations were 16.4 and 36.9 g/l. These matched well with the values from the validation experiment of 14.7 and 32.8 g/l at 168 and 240 h. The application of the statistical optimization method using CCD resulted in an improvement of maximum citric acid production from 5.6 to 41.8 g/l.

8.2 Introduction

Whey is a byproduct of the dairy industry representing 80 to 90% of the total milk

volume processed. The world production of cheese and casein yields annually 4.0 x 10^7 tons of whey, containing 4.5 to 5.0 % (w/v) lactose, 0.8 % (w/v) whey protein, 1.0 % (w/v) salts and 0.1 % (w/v) lactic acid along with vitamins (Wong and Lee, 1998; Rech et al., 1999). Accordingly, whey has a high biological (BOD) and chemical oxygen demand (COD), which exerts a significant oxygen demand on any wastewater treatment processes. It is a disposal challenge and the discarge of waste without treatment can cause considerable environmental problems (Kosseva *et al.*, 2001). Any process utilizing some of the whey constituents which brings revenue can help offset the treatment cost for the remaining portion.

The bioconversion of whey to value-added products such as organic acids, ethanol, enzymes, PHB (poly-3-hydroxybutyrate), xanthan gum and biomass has been intensively studied (Lee *et al.*, 1997; Kawahara and Obata, 1998; Rech *et al.*, 1999). Whey is an interesting source of organic substrates because of its relatively high concentration of nutrients. Because of its high lactose content, whey can be a good source of carbohydrates for the production of citric acid by the fungus *Aspergillus niger* (Lee and Yun, 1999; Lee *et al.*, 2000)

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is a common fungal metabolite and is widely used by various food, beverage, chemical, pharmaceutical and cosmetic industries. Commercial citric acid is produced by submerged fermentation using fungi such as *A. niger,* known for its high citric acid production (Pazouki *et al.*, 2000; Ambati and Ayyanna, 2001; Crolla and Kennedy, 2001; Schuster *et al.*, 2002). Nevertheless, the worldwide demand for citric acid is increasing faster than its production, implying that new production facilities and more efficient processes are required (Tran *et al.*, 1998; Alvarez-Vasquez *et al.*, 2000).

Citric acid is an intermediate in the TCA cycle and its accumulation by *A. niger* is strongly influenced by the nutrient balance, especially the level of a readily available carbon, nitrogen and phosphorus (Jianlong and Ping, 1998; Favela-Torres *et al.*, 1998). Under optimal fermentation conditions, over 90% citric acid yield can be obtained with monosaccharides or disaccharides when fermenting *A. niger* in submerged and solid substrate conditions (Xu *et al.*, 1989; Wasay *et al.*, 1998; Gupta and Sharma, 2002). Beet and cane molasses are good examples of processing byproducts rich enough in crude carbohydrates and salts for the production of citric acid by *A. niger* (Wang, 1998; Lesniak *et al.*, 2002). However, such sugar rich byproducts may offer challenges because of their more concentrated heavy metal content and the increasing sugar extraction efficiency leading to waste of lower sugar levels (Pazouki *et al.*, 2000; Mirminachi *et al.*, 2002).

The objective of this project was to investigate an alternate strategy for the utilization of cheese whey. This strategy consists in using cheese whey as a source of nutrients for the production of citric acid by *A. niger*, in submerged fermentation. The development of this strategy requires the optimization of nutrient levels. The project aimed at optimizing the supplementation of glucose, $(NH_4)_2SO_4$, KH_2PO_4 and stimulators for the efficient production of citric acid by *A. niger* fermented cheese whey. The CCD was used to simultaneously optimize the levels of these interactive nutrients first. Based on previously optimized nutrient supplementation, the effect of stimulators including methanol, olive oil and phytate on the production of citric acid by *A. niger* fermenting whey medium were optimized using CCD.

8.3 Materials and Methods

8.3.1 Microorganism

The white rot fungus *Aspergillus niger* NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was stored in a tube containing glycerol (30% v/v) at –76°C. *A. niger* spores were produced on a potato dextrose agar (PDA, Sigma, St. Louis, MO, USA) plates kept at 30°C. After ten days of incubation on PDA plates, 10 ml of 0.1 % (v/v) Tween 80 were added to each plate to harvest spores. Diluted spore suspensions of 1.0 x 10^6 spores/ml were counted using hemocytometer and prepared as inoculum for the experimental media.

8.3.2 Medium composition and fermentation condition for nutrient optimization

Spray dried bovine whey powder (Sigma, St. Louis, MO, USA) containing at least 65% lactose (w/w), 11% protein (w/w) and 2% lactic acid was used as the basal nutrient. The whey medium was prepared by dissolving 50 g of whey powder in 1.0 l of distilled water at 50°C (Lee and Yun, 1999). To avoid protein precipitation during autoclaving, the whey was heated at 90°C for 20 min and the proteins were removed by centrifugation at 11,000 x g for 20 min (Lee *et al.*, 1997). Then, the supernatant was collected and used to prepare 17 different supplemented media, where the level of glucose, $(NH_4)_2SO_4$ and KH_2PO_4 varied from 6.36 to 73.64, 1.59 to 18.41 and 0.16 to 1.84 g/l, respectively (Table 8.1). The pH of the whey-based media before autoclaving ranged from 5.14 to 5.56, depending on the level of nutrients supplemented (Figure 8.1). Each of the 17 experimental media was placed in a 250 ml Erlenmeyer flask and then autoclaved at 121°C for 15 min. Once autoclaved, each medium (50 ml) was inoculated with 0.5 ml spore suspension of 1.0 x 10^6 spores/ml and allowed to ferment at 30°C while being

shaken at 150 rpm.

Citric acid production was also measured using a standard fermentation medium, namely the Czapek-Dox medium. This basic medium already contained balanced levels of nutrients for the production of citric acid by *A. niger* NCIM 548: 50 g glucose, 1.0 g KH_2PO_4; 0.5 g KCl; 2.0 g $NaNO_3$; 0.5 g $MgSO_4 \cdot 7H_20$ and 0.01 g/l of $FeSO_4 \cdot 7H_2O$ (Pazouki *et al.*, 2000).

8.3.3 Medium composition and fermentation condition for optimization

The supernatant was collected, reformed by adding supplements (60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g/l KH_2PO_4) and autoclaved at 121°C for 15 min (Lee *et al.*, 2000). The stimulators tested were phytate, (40% solution, Aldrich, St. Louis, MO, USA), olive oil (Bertolli, Laval, Qc, Canada) and methanol (99+%, Sigma, St. Louis, MO, USA). While phytate and olive oil were added to the whey-based medium before autoclaving, methanol was added after (Table 8.2).

8.3.4 Analytical methods

As the preliminary experiments showed the maximum citric acid production after 240 h of fermentation, 1.7 ml of cultured medium was harvested from each flask under aseptic conditions after 196 and 288 h of fermentation and centrifuged at 11,000 x g for 2 min (Lee and Yun, 1999). Pyridine and acetic anhydride were added to the supernatant to develop color for citric acid quantification by spectrophotometry at 420 nm (Marier and Boulet, 1958). The diluted supernatant was used for the estimation of residual glucose concentration using glucose assay kit (Sigma, St. Louis, MO, USA).

8.3.5 Statistical analysis

According to the CCD table, the experiment needed to test 17 experimental media representing the combination of 5 different levels of nutrient supplements (independent variable) calculated based on 5 coded values (Tables 8.1 and 8.3). For statistical purposes, a triplicate center point was tested. The nutrient supplement level X_i associated with each coded level, –1.68, -1.0, 0, 1.0 and 1.68, was calculated as:

$$X_i = \chi_i \bullet \Delta X_i + X_{cp} \qquad (8.1)$$

where i = 1, 2 and 3 and corresponds to each one of the three nutrient supplement; χ_i = dimensionless coded level for X_i, namely –1.68, -1, 0, 1 and 1.68; X_i = real concentration of the independent variable for the code used; X_{cp} = concentration of independent variable at the coded value 0; ΔX_i = step change in concentration calculated as 20 g/l for glucose, 0.5 g/l for $(NH_4)_2SO_4$ and 5 g/l of KH_2PO_4. The X_{cp} and ΔX_i value were determined from preliminarily unreported experimentation (Ambati and Auuuanna, 2001; Abalos *et al.*, 2002).

These citric acid production levels obtained by fermenting each of the 17 experimental media were used to develop a second-order equation capable of predicting citric acid production as a function of nutrient supplement levels, after 196 and 288 h of fermentation. This equation could also predict nutrient supplement levels optimizing citric acid production (Ooijkaas *et al.*, 1999; Vohra and Satyanarayana, 2002):

$$Y = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \beta_3\chi_3 + \beta_{11}\chi_1^2 + \beta_{22}\chi_2^2 + \beta_{33}\chi_3^2 + \beta_{12}\chi_1\chi_2 + \beta_{23}\chi_2\chi_3 + \beta_{13}\chi_1\chi_3 \qquad (8.2)$$

where Y = predicted response (citric acid); β_0 = intercept; β_1, β_2, β_3 = linear coefficients; β_{11}, β_{22}, β_{33} = squared coefficients; β_{12}, β_{13}, β_{23} = interaction coefficients.

8.4 Results

8.4.1 Optimization of nutrients

After 196 and 288 h of fermentation, the batch experiments produced citric acid levels varying between 1.21 to 12.70 g/l, depending on the level of nutrient supplementation (Table 8.2). From these results, a second-order polynomial equation was developed to predict citric acid production (Y, g/l) based on the level of nutrient supplementation, after 196 and 288 h of fermentation, respectively:

$$Y_{196\,h} = 7.18 + 2.82\chi_1 + 0.19\chi_2 - 1.35\chi_3 + 0.28\chi_1^2 + 0.66\chi_2^2 + 0.042\chi_3^2 + 0.68\chi_1\chi_2 + 1.26\chi_1\chi_3 + 0.25\chi_2\chi_3 \quad (8.3)$$

$$Y_{288h} = 7.89 + 3.95\chi_1 + 0.12\chi_2 - 0.76\chi_3 - 0.39\chi_1^2 - 0.23\chi_2^2 - 1.41\chi_3^2 + 0.41\chi_1\chi_2 - 0.17\chi_1\chi_3 - 0.17\chi_2\chi_3 \quad (8.4)$$

The R^2 of these equations (8.3) and (8.4), comparing the predicted to the measured values, shows a high degree of correlation, as it ranges between 0.965 and 0.939 (Table 8.3). Accordingly, they implied that only 3.5 and 4.1% of the overall variation is not explained by the equations (Ramirez *et al.*, 2001; Chowdary *et al.*, 2002).

The ANOVA indicated that the combined over-all effect was significant, in terms of citric acid production (Table 8.4). For the combined effect of changing the level of all

three experimental nutrients, after 196 and 288 h of fermentation, the respective F values were 21.56 and 14.43, indicating a highly significant impact on citric acid production. Similarly, glucose (X_1) had a significant effect on citric acid production, after 196 and 288 h of fermentation while KH_2PO_4 and $(NH_4)_2SO_4$ (X_2) had no significant effect.

8.4.1.1 Interactive effect of nutrients on citric acid production

Proven accurate to predict citric acid production, second-order equations were used to analyze the interactive effects of the three experimental nutrients, within their individually tested range. The results of equations (8.3) and (8.4) including their linear, quadratic and cross product terms, were applied to plot a three-dimensional curve predicting citric acid production (Al-Zenki, 2000).

The predicted interaction between glucose and $(NH_4)_2SO_4$ on citric acid production after 196 and 288 h of fermentation is presented in Figures 8.2a and 8.2b, respectively. Both plots depict an increase in citric acid production with increasing initial glucose concentration within all ranges of $(NH_4)_2SO_4$ tested. Increasing the initial $(NH_4)_2SO_4$ concentration did not significantly increased citric acid production. Thus, maximum citric acid production was predicted using the highest levels of supplemented glucose and but not necessarily the highest level of $(NH_4)_2SO_4$.

The predicted interaction between glucose and KH_2PO_4, on citric acid production at a constant concentration using a constant $(NH_4)_2SO_4$ level, after 196 and 288 h of fermentation, is illustrated in Figures 8.3a and 8.3b, respectively. Citric acid production significantly increased with higher levels of supplemented glucose but not with higher levels of supplemented KH_2PO_4, both after 196 and 288 h of fermentation. Citric acid production maximized using the highest level of glucose and the lowest level of KH_2PO_4.

The predicted interaction between $(NH_4)_2SO_4$ and KH_2PO_4, at a constant glucose level of 40 g/l, is shown in Figures 8.4a and 8.4b, after 196 and 288 h of fermentation, respectively. Citric acid production was found to decrease with increasing levels of supplemented KH_2PO_4, after 196 h of fermentation, while after 288 h a maximum citric acid production was obtained with intermediate levels of KH_2PO_4. Citric acid production was not significantly affected by levels of $(NH_4)_2SO_4$.

The interaction analysis therefore predicted a linear effect between initial glucose concentration and citric acid production, irrespective of $(NH_4)_2SO_4$ and KH_2PO_4 levels. Thus, higher citric acid levels are achievable with a whey-based medium supplemented with levels of glucose higher than those tested. Similar observations for citric acid production were reported by Xu *et al.* (1989) and Favela-Torres *et al.* (1998) who found that optimal levels of sugar supplementation ranged between 10 to 15% (w/v) and that citric acid production increased with higher sugar levels.

Cube graphs were used to predict the simultaneous interaction between all three nutrient supplements, after 196 and 288 h of fermentation (Figures 8.5a and 8.5b). Each cube has eight corners representing eight different experimental conditions. The plus (+) and minus (-) signs represent the coded levels (-1 and +1) for each nutrient (Berthouex and Brown, 1994; Anderson *et al.*, 2000). The cube's upper right side gives a maximum citric acid production when using the highest concentration of glucose (A+) along with the highest concentration of $(NH_4)_2SO_4$ (B+), both after 196 and 288 h of fermentation. Also, citric acid production did not increase from 196 to 288 h of fermentation, indicating that 196 h is adequate.

8.4.1.2 Predicting optimum nutrient supplementation

Optimum levels of nutrient supplementation after 196 and 288 h of fermentation were established using second-order equations (8.3) and (8.4) and the concentration ranges or constraints indicated in Table 8.5 Table 8.6 lists ten possible optimal combinations of nutrient supplementation. As all combinations give similar results, the following medium composition was selected as the optimized combination producing 11.7 g/l of citric acid at 196 h: glucose at 60 g/l, $(NH_4)_2SO_4$ at 1.5 g/l and KH_2PO_4 at 8.1 g/l. These nutrient levels, used under batch laboratory conditions, produced 12.8 g/l of citric acid after 240 h of fermentation (Figure 8.6). This production level represents a 2.2 times increase, as compared to the whey medium alone producing 5.6 g/l of citric acid.

The measured citric acid production agreed with that predicted by the second-order equations, of 11.9 g/l after 288 h of fermentation. Citric acid production obtained with the supplemented whey based medium, was compared to that obtained with the Czapek-Dox medium. This standard submerged fermentation medium produced a maximum citric acid production of 10.6 g/l, after 144 h, with 50 g/l of supplemented glucose. For 60.0 g/l or 20% more glucose supplementation, the whey-based medium produced 20% more citric acid after 288 h of fermentation, as compared to the Czapek-Dox medium after 144 h of fermentation.

8.4.2 Optimization of stimulators

The levels of citric acid production (Y in g/l) obtained from the batch experiments produced the following second-order equations, for 168 and 240 h of fermentation, respectively.

$$Y_{168\,h} = 16.24 + 0.18\chi_1 + 0.21\chi_2 - 1.83\chi_3 - 3.50\chi_1^2 - 4.03\chi_2^2 - 3.61\chi_3^2 + 0.092\chi_1\chi_2 - 0.082\chi_1\chi_3 + 0.027\chi_2\chi_3 \quad (8.5)$$

$$Y_{240\,h} = 33.71 + 0.72\chi_1 - 0.54\chi_2 - 10.97\chi_3 - 4.27\chi_1^2 - 1.68\chi_2^2 - 6.34\chi_3^2 + 0.16\chi_1\chi_2 - 0.37\chi_1\chi_3 + 1.60\chi_2\chi_3 \quad (8.6)$$

The quality of fit of both equations (8.5) and (8.6) was high, as the determination coefficient (R^2) between the measured and predicted values were 0.938 and 0.939 (Table 8.7).

The coefficients of equations (8.5) and (8.6) indicate the effect of each stimulator concentration on the response, namely citric acid production. The coefficient of methanol (X_1) was positive while that for phytate (X_3) was negative, indicating a positive and negative effect on citric acid production after 168 and 240 h of fermentation, respectively. As for olive oil, the coefficient was positive after 168 h, but negative after 240 h of fermentation. ANOVA evaluated the significance of the effect on citric acid production, of the over-all change in all three stimulators and that of each individual stimulator (Table 8.8). Only phytate had a significant effect on citric acid production in tested range.

8.4.2.1 Response surface curves

Using both equations (8.5) and (8.6), predicted citric acid production values were computed and plot using a three-dimensional response surface curve to evaluate their interactive effects. For a constant phytate level of 4.75 g/l, the effect of the initial concentration of methanol and olive oil is plotted in Figures 8.8a and 8.8b, for 168 and 240 h of fermentation, respectively. For both fermentation periods, convex surfaces

maximized near the center point of the tested range. Citric acid production maximized at 16.2 and 33.8 g/l, respectively, as methanol and olive oil level increased from 20 to 48 g/l. For higher values of methanol and olive oils, citric acid production decreased.

For a constant olive oil concentration of 47.5 g/l, second-order equations predicted citric acid productions as a function of methanol and phytate, after 168 and 240 h of fermentation (Figures 8.9a and 8.9b). Again a convex response curves were obtained, after 168 h of fermentation. Citric acid production maximized at 16.5 g/l as methanol increased from 20 to 50 g/l and as phytate increase from 2 to 4 g/l. After 240 h of fermentation, a different response surface curve was obtained, where citric acid production significantly decreased with increasing phytate concentration and increased only slightly with increasing methanol concentration. Thus, citric acid production maximized with a methanol and phytate concentration of 47.5 and 4 g/l, respectively.

The interactive effect of phytate and olive oil was predicted for a constant methanol level of 47.5 g/l, after 168 and 240 h of fermentation (Figures 8.10a and 8.10b). After 168 h of fermentation, citric acid production maximized with 4 g phytate and 48 g/l of olive oil. After 240 h of fermentation, citric acid production dropped rapidly with increasing phytate concentration while not being affected by olive oil concentration. Although many researchers reported the stimulating effect of phytate on citric acid production, the supplementation of phytate in our experiment had a negative effect. In experimental media 5, 6, 7, 8 and 14 (Table 8.7), higher concentration of phytate (phytic acid) than 15 g/l resulted in a significant initial pH decrease, which had an adverse affect on citric acid production at 240 h (Figure 8.11). The final citric acid production at 240 h was correlated with the initial pH of the medium ($R^2 = 0.78$) and high medium pH increased in citric acid production. The highest level of citric acid production of 36.9 g/l was obtained from the

experimental medium with the highest initial pH of 4.8, while lower medium initial pH of 2.68 led to a significant decrease in citric acid production.

8.4.2.2 Cube graphs

Using CCD to predict citric acid production, cube graphs were drawn to demonstrate the effect between all three stimulators, after 168 and 240 h of fermentation (Figures 8.12a and 8.12b).

The cube plot indicated two dominant surfaces, the front side with a low phytate concentration and the right side with a high methanol concentration, both increasing citric acid production. However, citric acid production values were not significantly different, for the top and the bottom sides. The cube plot predicted a maximum citric acid concentration of 31.5 g/l, for the highest methanol and olive oil concentrations (A+ and B+, respectively) and the lowest phytate concentration (C-), after 240 h of fermentation.

8.4.2.3 Optimal stimulator level

Equations (8.5) and (8.6) predict a maximum citric acid production of 16.4 and 36.9 g/l, after 168 and 240 h of fermentation, when using initial methanol, olive oil and phytate concentrations of 48.3 g, 47.7 g and 3.8 g/l, respectively (Tables 8.9 and 8.10).

A final batch experiment was conducted to validate each predicted value (Figure 8.13). Using optimal levels of stimulators, citric acid productions of 32.8 and 41.8 g/l were achieved after 240 and 312 h of fermentation, respectively. This represents a 3.3-fold increase in citric acid production as compared to the control experiment without stimulators which produced 12.8 g/l of citric acid. The measured citric acid production, after 240 h of fermentation, was in good agreement with the predicted value of 36.9 g/l.

8.5 Discussion

The first statistical procedure was a valid and efficient tool in optimizing the supplementation of a whey-based growth medium for citric acid production by *A. niger* NRRL 567 using whey-based medium. Although nutrient supplementation of the whey-based solution increased citric acid production, the final levels were neither significantly different nor faster from those obtained with a Czapek-Dox medium. To further improve citric acid production using a whey-based medium, additional testing is required to optimize the initial concentration of stimulators. While a 12 g/l of citric acid production was obtained with a standard whey-based medium supplemented with 60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g/l KH_2PO_4, the same medium supplemented with 48.3 g, 47.7 g and 3.8 g/l of methanol, olive oil and phytate, respectively, produced a maximum citric acid production of 32.8 and 41.8 g/l, after 240 and 312 h of fermentation.

The second CCD proved to be an efficient method in optimizing the level of stimulators. The supplementation of methanol showed a positive effect on citric acid. The restarted spore germination or cell growth was observed during the fermentation with the supplementation of methanol, indicating low biomass production. It is suspected that depressing cell growth or spore germination might result in high citric acid production (Jianlong, 1998). The production of citric acid decreased with the increase in the concentrations of phytate. The reason for the negative effect of phytate on citric acid production may be resulted from the decrease in pH. For a future study to evaluate the stimulating effect of phytate, the initial pH should be controlled to avoid the pH effect. As revealed by citric acid production profiles on Figure 8.13, whey-based medium with glucose and stimulator is a better substrate for citric acid production. Citric acid production from the optimized medium using CCD was 3.9-fold higher than that achieved

achieved from Czapek-Dox medium alone.

During the cofermentation of glucose and whey with adding stimulators, the profiles of residual glucose present in the medium were similar to the control test. After depletion of glucose, cell mass of optimized medium was slightly decreased, while citric acid production was increased. In general, *A. niger* can utilize both of sugars, glucose and lactose, as carbon sources for citric acid production, but when glucose is added in lactose containing medium, glucose inhibits the synthesis of ß-galactosidase and is preferentially consumed than whey lactose during fermentation (Shuler and Kargi, 2002). Before being accessible to *A. niger*, lactose must be hydrolyzed to glucose and galactose by the enzyme ß-galactosidase. In the experiment with 50 g/l cheese whey alone, 5.6 g/l of citric acid and 3.8 g/l of dry cell mass were obtained thus it may be concluded that *A. niger* NRRL 567 is a strain able to produce ß-galactosidase and can utilize lactose as a carbon source for citric acid production and cell growth (Figure 8.7). However, as the conversion of lactose to glucose and galactose was not sufficient, the residual glucose concentration during the fermentation was very low and the slow fermentation of whey may be resulted from limited levels of available glucose, despite its high lactose content. When glucose and whey were fermented together (optimized medium), glucose was utilized first and almost depleted (Shuler and Kargi, 2002). After the glucose depleted, citric acid production and cell growth at a similar level or decreased. This result means that limited level of available sugar for cell growth and citric acid production remained in the medium after 312 h. As shown in Figures 8.13 and 8.13, it was presumed that utilization of whey lactose is controlled by catabolic repression when glucose is present in the medium. However, after depletion of glucose, the activity of ß-galactosidase is increased and lactose can be utilized for citric acid.

As whey powder contains 65% (w/w) of lactose, the initial sugar concentration, lactose (32.5 g/l) and glucose (60 g/l), can be calculated as 92.5 g/l. Even though adding stimulators increased citric acid production up to 41.8 g/l, the increase in citric acid yield (45.2%) by adding stimulators were not considerable. To further improve citric acid production in the experiment with whey-based media with supplementation of stimulators, additional study is needed to optimize other fermentation parameters including pH of medium, fermentation time and inoculum density.

8.6 Acknowledgments

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8.7 References

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Table 8.1 Values of independent variables and their coded level of nutrient optimization

Variables	Components	Coded and actual level				
		-1.68	-1	0	+1	+1.68
X_1	Glucose (g/l)	6.36	20.00	40.00	60.00	73.64
X_2	$(NH_4)_2SO_4$ (g/l)	0.16	0.50	1.00	1.50	1.84
X_3	KH_2PO_4 (g/l)	1.59	5.00	10.00	15.00	18.41

Table 8.2 Actual values of the coded level of variables of stimulator optimization

Variables	Components	Coded and actual level				
		-1.68	-1	0	+1	+1.68
X_1	Methanol (g/l)	1.25	20.00	47.50	75.00	93.75
X_2	Olive oil (g/l)	1.25	20.00	47.50	75.00	93.75
X_3	Phytate (g/l)	0.12	2.00	4.75	7.50	9.38

Table 8.3 Experimental plan for optimization of medium constituents along with experimental and predicted concentration of citric acid at 196 and 288 h

Run	Glucose	$(NH_4)_2SO_4$	KH_2PO_4	Responses at 196 h (g/l)		Responses at 288 h (g/l)	
No	(g/l)	(g/l)	(g/l)	Observed	Predicted	Observed	Predicted
1	20.00	0.50	5.00	9.03	8.69	1.21	2.61
2	60.00	0.50	5.00	10.67	10.44	9.46	10.04
3	20.00	1.50	5.00	7.60	7.21	1.74	2.38
4	60.00	1.50	5.00	12.70	11.67	11.68	11.44
5	20.00	0.50	15.00	2.76	2.96	1.51	1.79
6	60.00	1.50	15.00	10.21	9.77	9.12	8.52
7	20.00	1.50	15.00	3.09	2.50	1.40	0.87
8	6.36	1.00	15.00	12.51	12.01	10.59	9.23
9	73.64	1.00	10.00	2.96	3.23	1.19	0.15
10	40.00	1.00	10.00	11.79	12.70	12.45	13.43
11	40.00	0.16	10.00	8.64	8.72	8.01	7.04
12	40.00	1.84	10.00	8.28	9.37	6.53	7.44
13	40.00	1.00	1.59	8.78	9.56	6.57	5.18
14	40.00	1.00	18.41	4.64	5.03	1.30	2.63
15	40.00	1.00	10.00	6.92	7.18	8.21	7.89
16	40.00	1.00	10.00	6.96	7.18	8.04	7.89
17	40.00	1.00	10.00	7.85	7.18	7.42	7.89

R^2 (coefficient of determination) = 0.9652 (196 h) and 0.9393 (288 h)

Table 8.4 Analysis of variance (ANOVA) for the second-order model for citric acid production at 196 and 288 h

	196 h			288 h		
	Sum of Squares	*F* value	*P* level	Sum of Squares	*F* value	*P* level
Model	155.85	21.56	0.0003*	253.47	14.43	0.0017*
X_1	108.22	134.75	< 0.0001*	220.83	94.43	< 0.0001*
X_2	0.51	0.63	0.4534	0.51	0.22	0.6536
X_3	24.77	30.84	0.0009*	6.39	2.73	0.1423
X_1^2	0.87	1.08	0.3333	1.97	0.84	0.3895
X_2^2	4.91	6.12	0.0426*	0.75	0.32	0.5887
X_3^2	0.020	0.024	0.8804	23.31	9.97	0.0160*
X_1X_2	3.68	4.58	0.0695	0.64	0.28	0.6159
X_1X_3	12.78	15.91	0.0053*	0.71	0.30	0.5981
X_2X_3	0.51	0.63	0.4518	0.71	0.30	0.5981

X_1 = Glucose; $X_2 = (NH_4)_2SO_4$; $X_3 = KH_2PO_4$

*Significant at the 95% level

Table 8.5 CCD medium optimization constraints (g/l) after 196 and 288 h of fermentation

Variables	Goal	Lower Limit	Upper Limit
Glucose	-	20	60
$(NH_4)_2SO_4$	-	0.5	1.5
KH_2PO_4	-	5	15
Citric acid at 196 h	maximize	2.76	12.7
Citric acid at 288 h	maximize	1.19	12.45

Table 8.6 Predicted citric acid production (g/l) using ten combinations of medium supplements

	Glucose	$(NH_4)_2SO_4$	KH_2PO_4	Predicted Citric acid	
	X_1	X_2	X_3	196 h	288 h
1	60.00	1.50	8.08	11.74	12.00
2	60.00	1.50	8.19	11.74	12.00
3	60.00	1.50	8.42	11.75	11.98
4	60.00	1.50	8.13	11.74	12.00
5	60.00	1.50	7.45	11.72	12.00
6	60.00	1.50	6.58	11.70	11.93
7	59.59	1.50	7.47	11.66	11.93
8	60.00	1.50	5.55	11.68	11.73
9	60.00	1.50	12.40	11.89	10.78
10	60.00	1.33	12.03	11.18	10.99

Table 8.7 Experimental plan on optimization of stimulator constituents for the experimental and predicted concentration of citric acid at 168 and 240 h

Run	X_1	X_2	X_3	Initial	Responses at 168 h (g/l)		Responses at 240 h (g/l)	
No	(g/l)	(g/l)	(g/l)	pH	Observed	Predicted	Observed	Predicted
1	20.0	5.0	0.5	4.61	5.94	6.58	33.09	33.61
2	60.0	5.0	0.5	4.58	5.27	6.91	32.31	35.47
3	20.0	5.0	1.5	4.59	5.90	6.76	26.41	29.00
4	60.0	5.0	1.5	4.60	6.14	7.46	29.49	31.51
5	20.0	15.0	0.5	2.99	2.48	3.03	6.45	9.22
6	60.0	15.0	1.5	3.08	2.02	3.04	7.38	9.58
7	20.0	15.0	1.5	3.03	3.09	3.32	9.37	11.01
8	6.4	15.0	1.0	3.08	2.45	3.69	7.77	12.03
9	73.6	10.0	1.0	4.06	6.48	6.03	22.58	20.42
10	40.0	10.0	1.0	4.04	8.83	6.62	27.46	22.84
11	40.0	10.0	0.16	3.88	5.88	4.49	32.72	29.88
12	40.0	10.0	1.84	3.94	6.47	5.20	32.00	28.06
13	40.0	1.6	1.0	4.81	10.87	9.12	36.86	34.24
14	40.0	18.4	1.0	2.68	3.87	2.97	1.50	-2.66
15	40.0	10.0	1.0	4.00	16.74	16.24	30.42	33.71
16	40.0	10.0	1.0	4.00	16.41	16.24	36.14	33.71
17	40.0	10.0	1.0	4.00	15.11	16.24	32.42	33.71

R^2 (coefficient of determination) = 0.938 (168 h) and 0.939 (240 h)

X_1: methanol; X_2: olive oil; X_3: phytate

Table 8.8 Regression statistics of the model and ANOVA at 168 and 240 h

	168 h			240 h		
	Sum of Squares	*F* value	*P* level	Sum of Squares	*F* value	*P* level
Model	342.36	11.78	0.0019*	2207.14	11.84	0.0018*
X_1	0.42	0.13	0.7293	7.07	0.34	0.5775
X_2	0.60	0.19	0.6791	4.00	0.19	0.6734
X_3	45.68	14.15	0.0071*	1643.27	79.36	< 0.0001*
X_1^2	138.43	42.87	0.0003*	205.68	9.93	0.0161*
X_2^2	183.00	56.68	0.0001*	31.66	1.53	0.2561
X_3^2	146.51	45.38	0.0003*	452.68	21.86	0.0023*
X_1X_2	0.068	0.021	0.8891	0.22	0.010	0.9215
X_1X_3	0.054	0.017	0.9007	1.11	0.054	0.8236
X_2X_3	0.0057	0.0018	0.9678	20.50	0.99	0.3529

*Significant at the 95% level

X_1 = methanol

X_2 = olive oil

X_3 = phytate

Table 8.9 The constraints for optimization of level of additives (g/l) using CCD at 168 and 240 h

Variables	Goal	Lower Limit	Upper Limit
Methanol		20	75
Olive oil		20	75
Phytate		2	7.5
Citric acid at 168 h	maximize	2.02	16.74
Citric acid at 240 h	maximize	1.50	36.86

Table 8.10 The results of combinations of medium components (g/l) and predicted optimum citric acid concentration (g/l) by numerical optimization

	Methanol (X_1)	Olive oil (X_2)	Phytate (X_3)	Predicted citric acid 168 h	Predicted citric acid 240 h
1	48.56	47.73	3.76	16.4	36.9
2	48.30	47.74	3.76	16.4	36.9

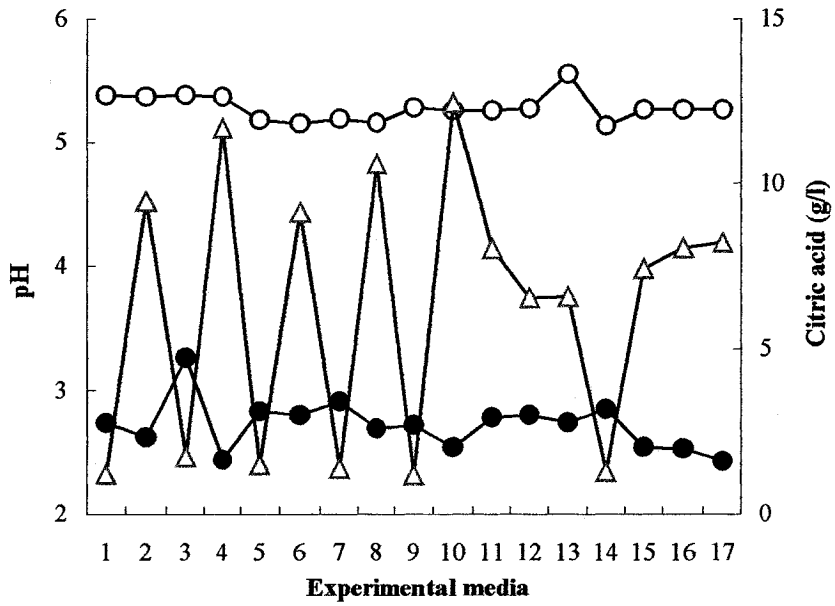

Figure. 8.1 Change in pH for 17 experimental media before and after fermentation (○: initial pH; ●: Final pH at 288 h; △: citric acid production at 288 h)

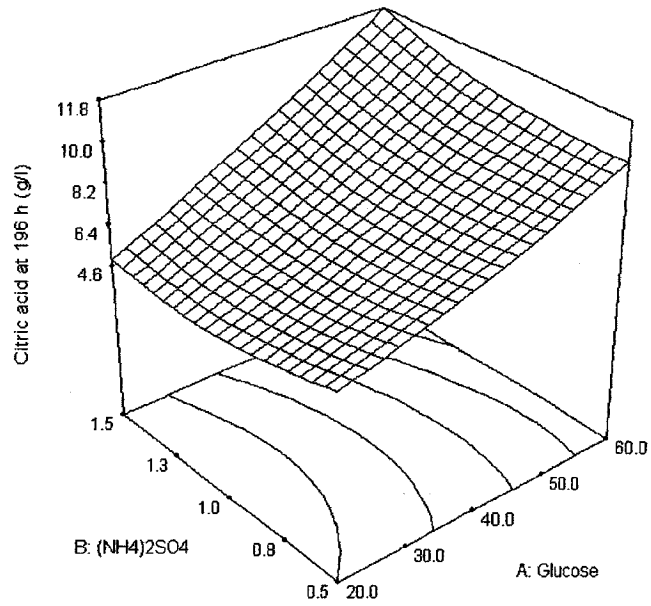

Figure. 8.2a Predicted citric acid production as a function of glucose and $(NH_4)_2SO_4$ with constant levels of KH_2PO_4 (10 g/l) and whey (50 g/l) after 196 h of fermentation.

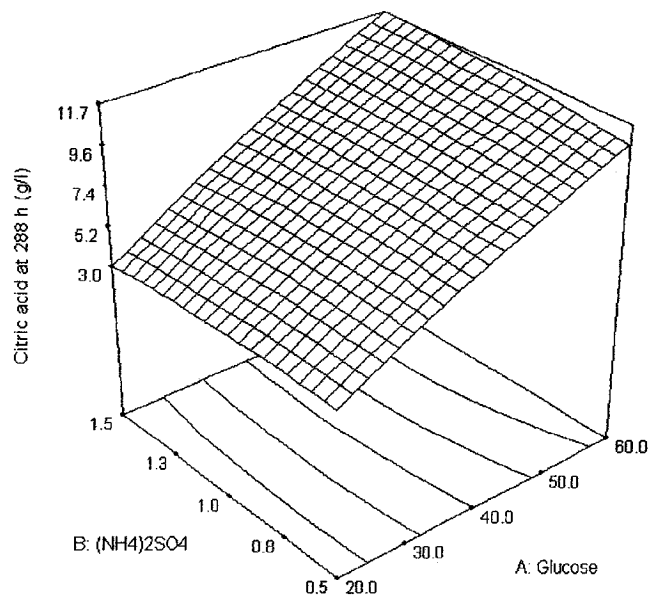

Figure. 8.2b Predicted citric acid production as a function of glucose and $(NH_4)_2SO_4$ with constant levels of KH_2PO_4 (10 g/l) and whey (50 g/l) after 288 h of fermentation.

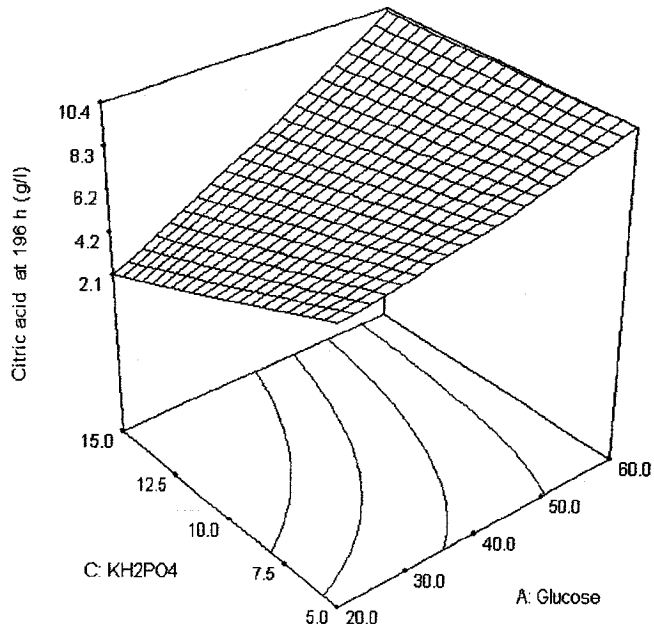

Figure 8.3a Predicted citric acid production as a function of glucose and KH_2PO_4 with constant levels of $(NH_4)_2SO_4$ (1 g/l) and whey (50 g/l) after 196 h of fermentation.

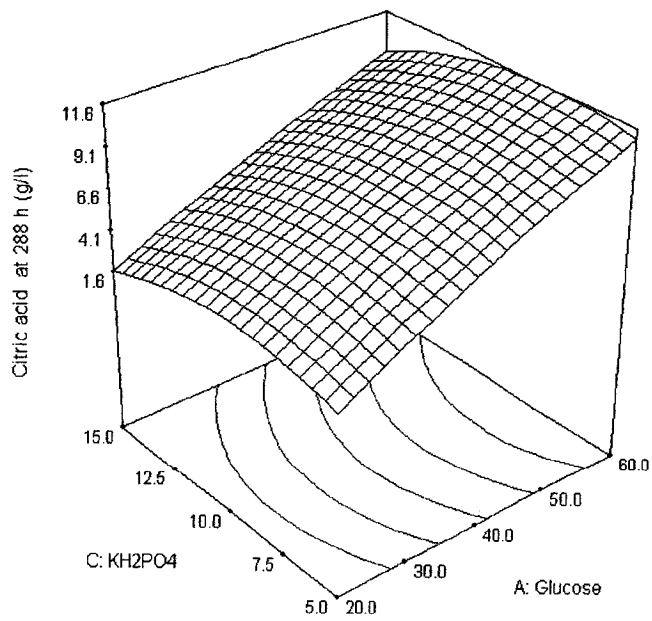

Figure. 8.3b Predicted citric acid production as a function of glucose and KH_2PO_4 with constant levels of $(NH_4)_2SO_4$ (1 g/l) and whey (50 g/l) after 288 h of fermentation.

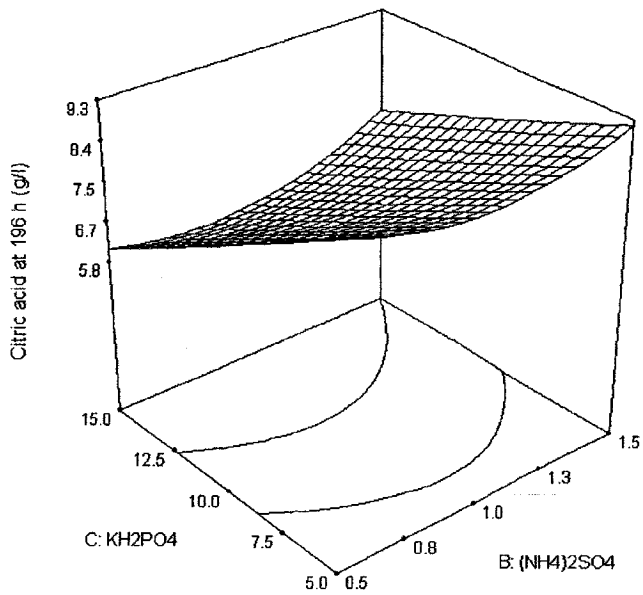

Figure. 8.4a Predicted citric acid production as a function of $(NH4)_2SO_4$ and KH_2PO_4 with constant levels of glucose (40 g/l) and whey (50 g/l) after 196 h of fermentation.

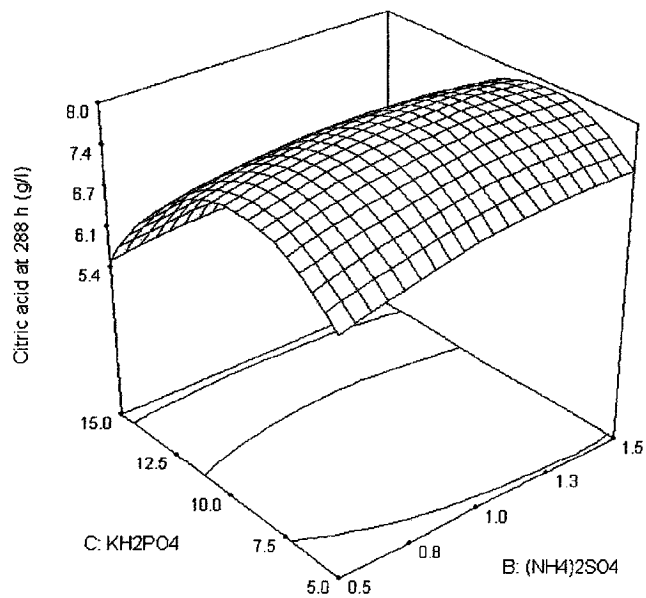

Figure. 8.4b Predicted citric acid production as a function of $(NH4)_2SO_4$ and KH_2PO_4 with constant levels of glucose (40 g/l) and whey (50 g/l) after 288 h of fermentation.

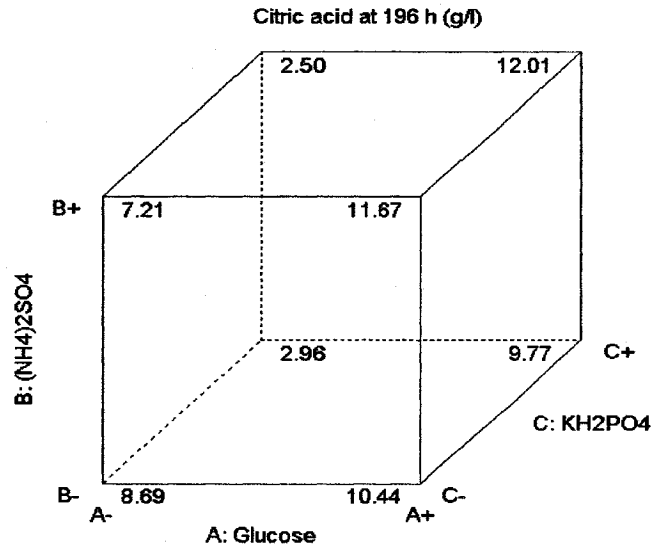

Figure. 8.5a Cube plot of CCD for 196 h of fermentation. The cube corner values are the levels of citric acid production predicted using the second-order polynomial equation (8.3) applied to a whey concentration of 50 g/l.

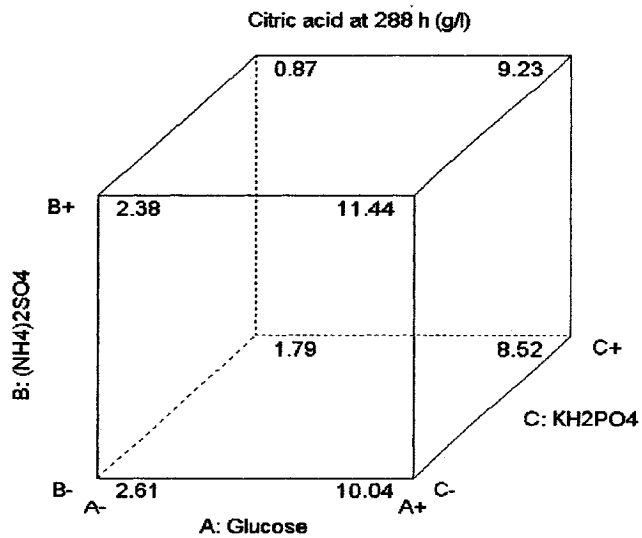

Figure. 8.5b Cube plot of CCD for 288 h of fermentation. The cube corner values are the levels of citric acid production predicted using the second-order polynomial equation (8.4) applied to a whey concentration of 50 g/l.

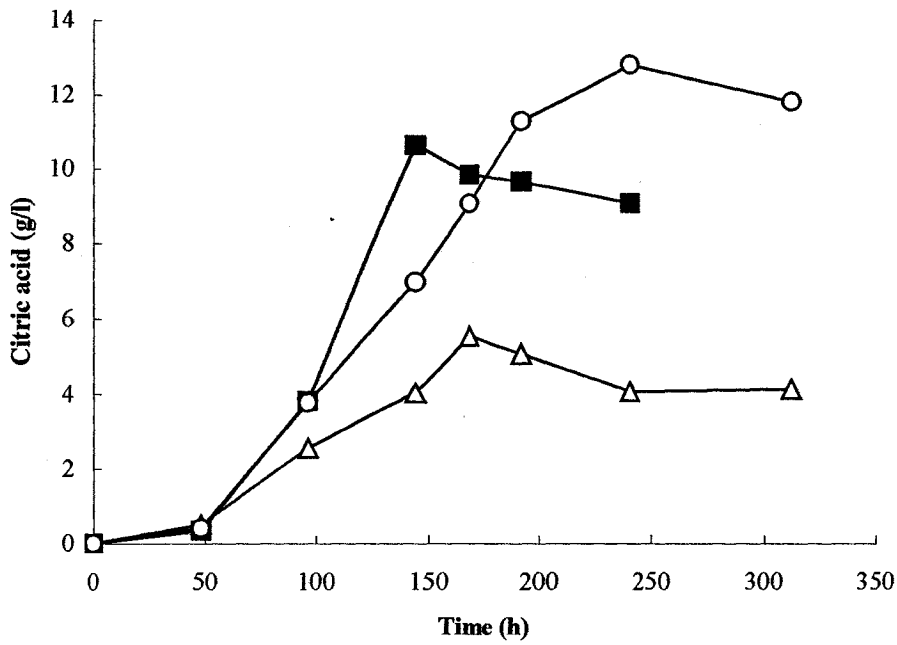

Figure. 8.6 Citric acid production by *A. niger* NRRL 567 in submerged fermentation using a control whey-based medium (50 g/l) alone, a whey-based medium supplemented with 60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g/l KH_2PO_4 and a standard Czapek-Dox medium containing 50 g/l glucose and other nutrient. All media were inoculated with 0.5 ml of 1.0 x 10^6 spores/ml and fermented at 30°C with agitation at 150 rpm (△: whey-based medium; ■: Czapek-Dox medium; ○: optimized medium).

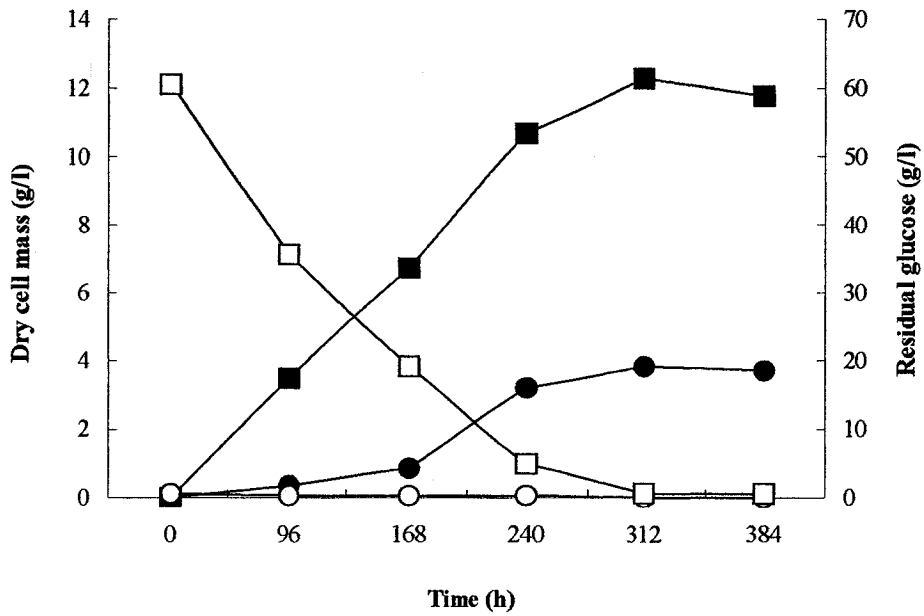

Figure. 8.7 Time course of cell mass and residual glucose concentration in submerged fermentation using a control whey-based medium (50 g/l) alone and a whey medium supplemented with 60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g/l KH_2PO_4. All media were inoculated with 1 ml of 1.0 x 10^6 spores/ml and fermented at 30°C with agitation at whey-based medium. ●: cell mass (whey-based medium); ■: cell mass (optimized medium); ○ : residual glucose concentration (whey-based medium); □: residual glucose concentration (optimized medium).

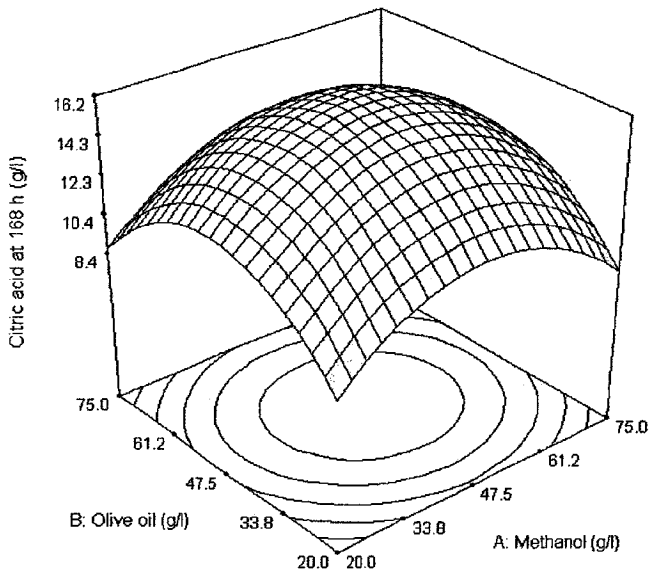

Figure. 8.8a Predicted citric acid production as a function of methanol and olive oil with constant phytate level of 4.75 g/l after 168 h of fermentation.

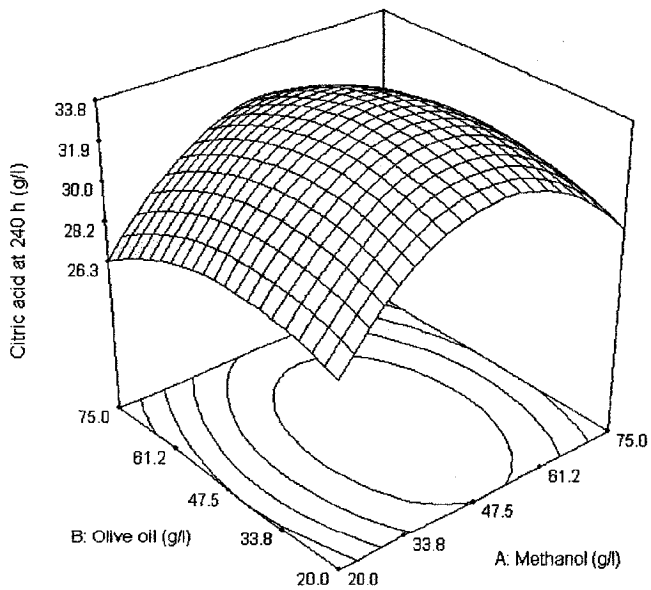

Figure 8.8b Predicted citric acid production as a function of methanol and olive oil with constant phytate level of 4.75 g/l after 240 h of fermentation.

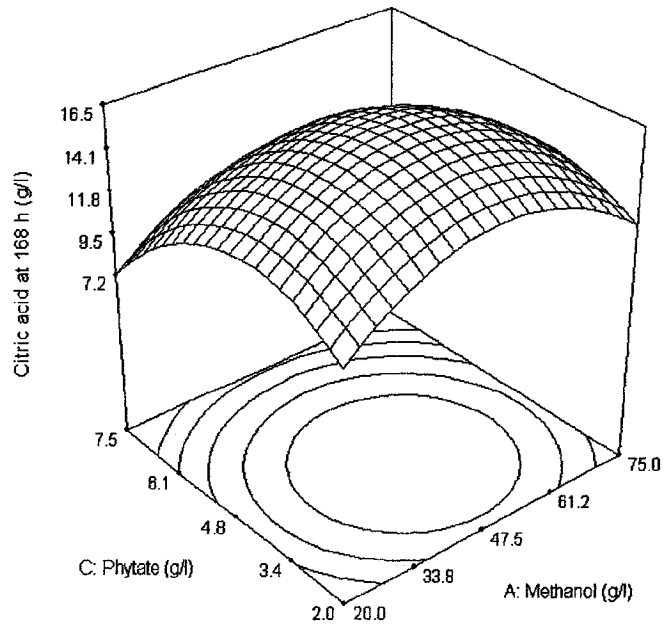

Figure 8.9a Predicted citric acid production as a function of methanol and phytate with constant olive oil of 47.5 g/l after 168 h of fermentation.

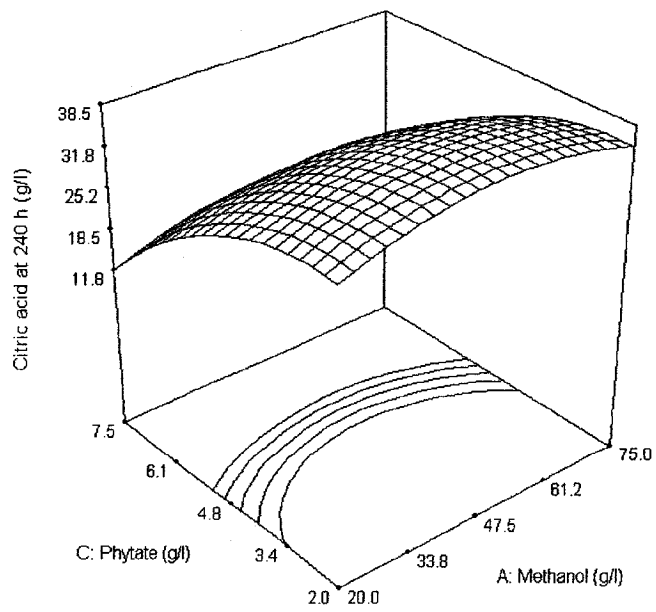

Figure 8.9b Predicted citric acid production as a function of methanol and phytate with constant olive oil of 47.5 g/l after 240 h of fermentation.

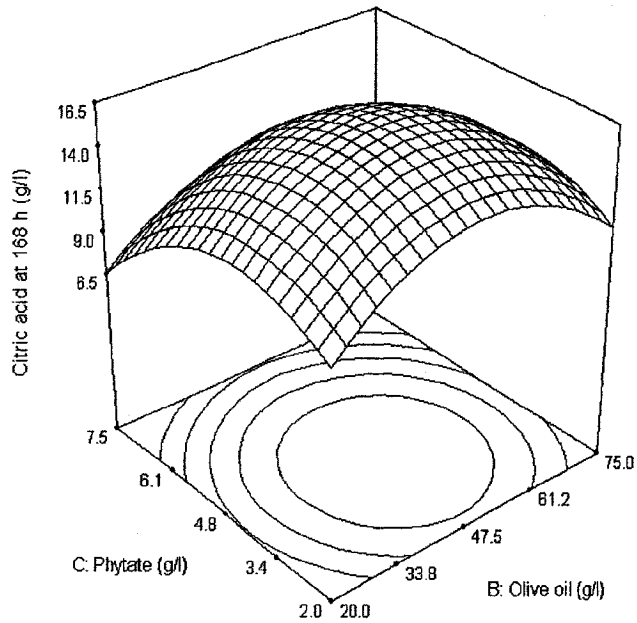

Figure 8.10a Predicted citric acid production as a function of olive oil and phytate with constant methanol level of 47.5 g/l after 168 h of fermentation.

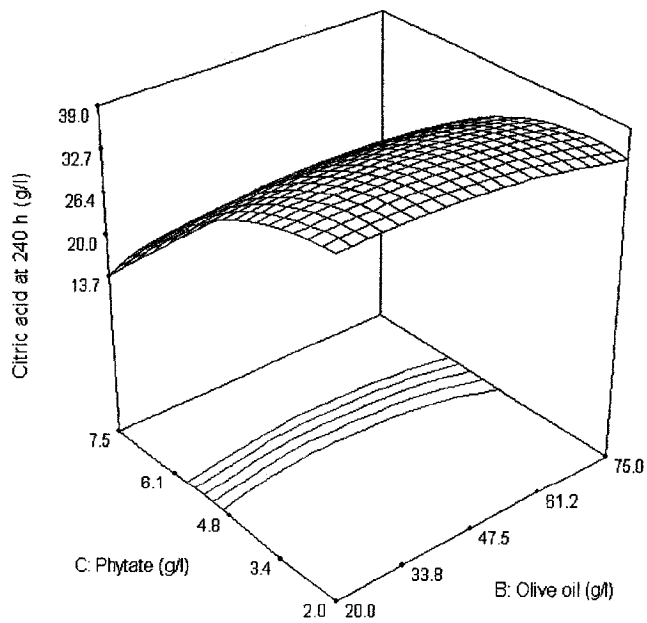

Figure 8.10b Predicted citric acid production as a function of olive oil and phytate with constant methanol level of 47.5 g/l after 240 h of fermentation.

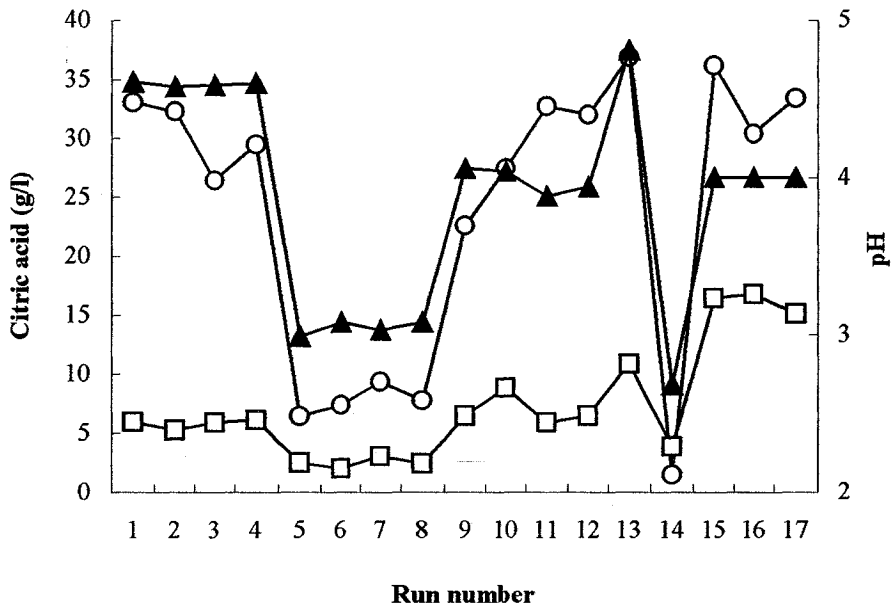

Figure 8.11 Change in pH for 17 experimental media before and after fermentation (▲: initial pH; □: citric acid production at 168 h; ○: citric acid production at 240 h).

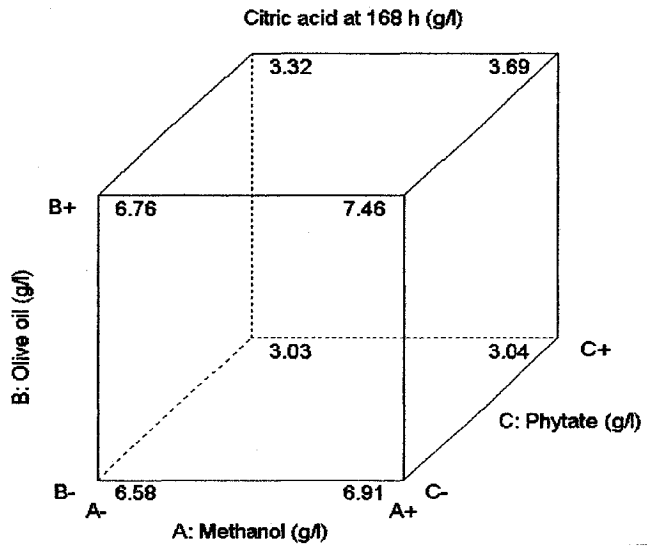

Figure 8.12a CCD cube plot predicting citric acid production after 168 h. The corner values are those predicted using the second-order polynomial model and stimulators were coded values of −1 and +1.

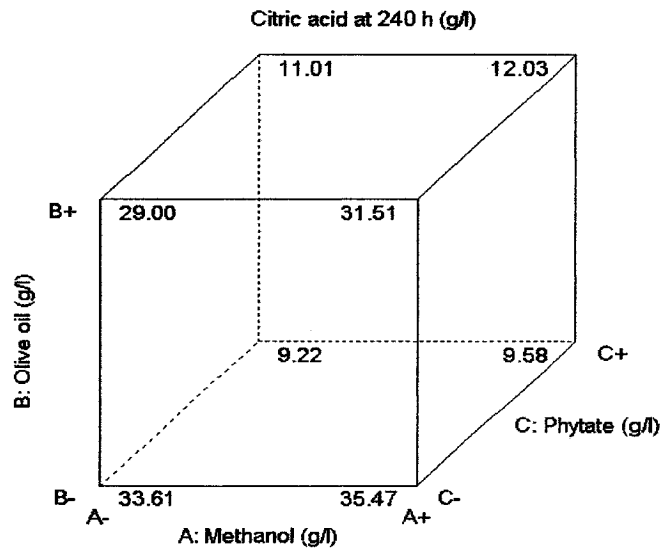

Figure 8.12b CCD cube plot predicting citric acid production after 240 h. The corner values are those predicted using the second-order polynomial model and stimulators were coded values of −1 and +1

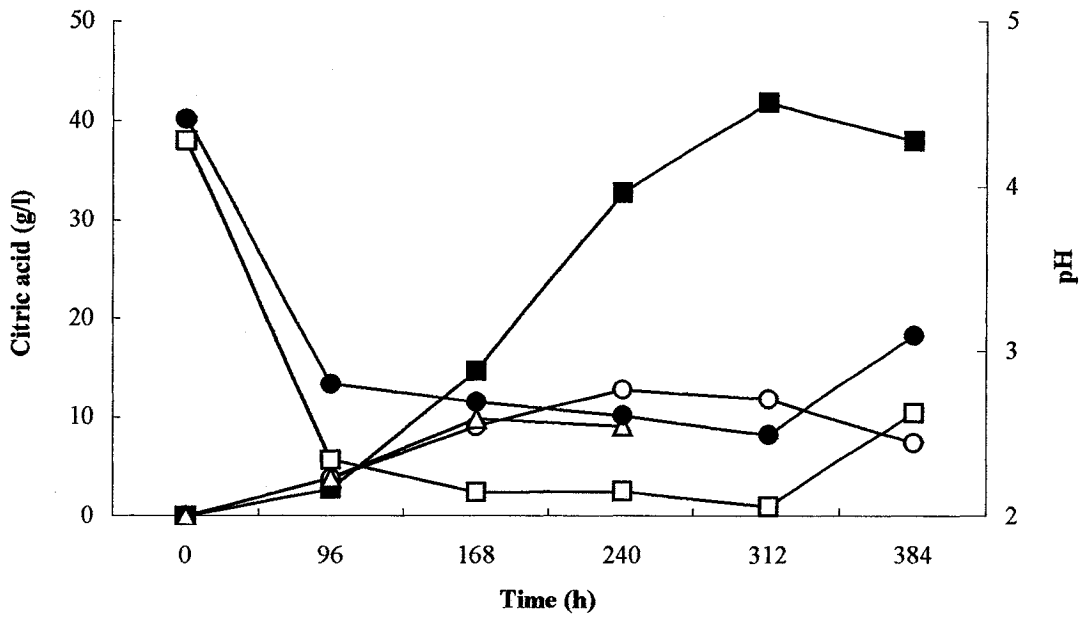

Figure 8.13 Time course of citric acid production and pH by *A. niger* NRRL 567 in submerged fermentation using a control whey-based medium without stimulators (50 g whey, 60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g/l KH_2PO_4); an optimized medium containing 50 g whey, 60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g KH_2PO_4, 48.3 g methanol, 47.7 g olive oil and 3.8 g/l phytate; and a standard Czapek-Dox medium containing 50 g/l glucose and other nutrient. All media were inoculated with 0.5 ml of 1.0 x 10^6 spores/ml and fermented at 30°C with agitation at 150 rpm. ○: control whey-based medium (citric acid); ■: optimized medium (citric acid); △: Czapek-Dox medium(citric acid); ●: control whey-based medium (pH); □: optimized medium (pH).

CONNECTING STATEMENTS

In a previous study, citric acid production in batch experiments using whey-based medium with optimum medium composition (60 g glucose, 1.5 g $(NH_4)_2SO_4$, 8.1 g/l KH_2PO_4, 48.3 g methanol, 47.7 g , olive oil and 3.8 g/l phytate) produced a maximum citric acid production at 312 h of fermentation. Even though adding high concentration of glucose and stimulators increased citric acid production up to 41.8 g/l, the increase in citric acid yield (45.2%) were not considerable. To achieve a high concentration of citric acid, the initial level of fementation parameters must also be optimized along with the initial concentration of nutrient and stimulators.

Next chapter deals with optimization of fermentation parameters for citric acid production by *A. niger* using whey-based medium. For the development of economical process using whey-based medium for citric acid production in submerged fermentation, statistically-based optimization was used to investigate and optimize fermentation parameters such as pH, fermentation time and inoculum density.

This paper will be presented in **Applied Microbiology and Biotechnology in 2004. Authors: Jin-Woo Kim, Suzelle Barrington, John Sheppard and Byong H Lee.** The contribution of authors are: 1) First author carried out entire experiment work and writing of manuscripts, 2) second author supervised and technical correction of the work and manuscripts, 3) third author provided fermentation facilities and manuscript correction and 4) forth author provided fermentation facilities and manuscript correction.

CHAPTER 9

Optimization of Fermentation Parameters for Citric Acid Production by *Aspergillus niger* NRRL 567 in Submerged Fermentation

9.1 Abstract

For the production of citric acid by *Aspergillus niger* NRRL 567 fermented using a cheese whey in submerged fermentation, the effect of three fermentation parameters including initial pH, time and inoculum density was investigated using the central composite design (CCD). For each variable, five combinations of levels were tested using batch experiment: initial pH ranging from 1.6 to 8.4; fermentation time of 103 to 426 h and inoculum density of 0.32 – 3.68 x 10^6 spores/ml added to 50 ml of whey-based medium. From CCD, a second-order regression equation was produced to predict citric acid production as a function of fermentation parameters. The batch results indicated that initial pH and inoculum density had a significant effect on citric acid production, while fermentation time was insignificant in tested range of fermentation. The second-order equation predicted optimum citric acid production of 61.5 g/l, when using an initial pH 5.37 and an inoculum density of 2.35 x 10^6 spores/ml after 252 h of fermentation. Testing these optimal conditions using a validation experiment, a maximum citric acid concentration of 74.6 g/l was obtained after 312 h of fermentation. The predicted citric acid production of 61.5 g/l after 252 h matched that measured citric acid production of the validation experiment, indicating the effectiveness of the CCD.

9.2 Introduction

Whey is a byproduct of the dairy industry, representing 80 to 90% of the total milk

volume utilized (Wang and Lee, 1998; Rech *et al.*, 1999). As it is rich in lactose, fat, proteins, lactic acid and has trace amounts of vitamins, cheese whey can be used as an inexpensive substrate for the fermentation of organic acids, ethanol, enzymes, PHB (poly-3-hydroxybutyrate,), xanthan gums and biomass (Lee *et al.*, 1997; Lee and Yun, 1999; Rech *et al.*, 1999; Kawahara and Obata, 1998).

Our previous experiment indicated that 50 g/l of cheese whey-based medium, used in submerged fermentation, was relatively ineffective as a medium for the production of citric acid by *A. niger* NRRL 567 (Kim *et al.*, 2004). Nevertheless, the supplementation of additional nutrients (60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g/l KH_2PO_4) and stimulators (48.3 g methanol, 47.7 g olive oil and 3.76 g/l of phytate) increased citric acid production by 7.2-fold, in which 41.8 g/l was obtained after 312 h. Although this is a significant improvement, citric acid yields (45.2%) are still too low to be economical.

The pH of a liquid medium strongly influences the growth of *A. niger* and the production of citric acid (Goes and Sheppard, 1999). Different pH optima have been report by several researchers. Most filamentous fungi are known for better citric acid production under acidic pHs ranging between 3 and 6, but some fungi are able to grow at pHs below 2 to better compete against bacteria (Fawole and Odunfa, 2003). However, Roukas (2000) reported that *A. niger* produced large amounts of citric and gluconic acid at an initial pH of 7, while Kamini *et al.* (1998) reported an ideal pH of 6 to 8, for the production of citric acid and lipase by *A. niger* ATCC 10577 and *A. niger* MTCC 259. In our previous study (Kim *et al.*, 2004), where whey media pHs were adjusted from 2.68 to 4.80, maximum citric acid production in submerged fermentation was obtained at a pH 4.80, while a pH of 2.68 led to a dramatic decrease in citric acid production.

Citric acid production is also known to be affected by inoculum density and

fermentation time (Lee and Yun, 1999, Kim *et al.*, 2003). Up to a specific limit, metabolite production generally increases with inoculum density (Kota and Sridhar, 1999; Nampoothiri *et al.*, 2003). At the lower inoculum density, metabolite production drops and contamination risks increase due to an insufficient cell population. A high inoculum density leads to population over-crowing, higher nutrient competition and rapid exhaustion of nutrients (Adinarayana *et al.*, 2003; Ellaiah *et al.*, 2003; Uyar and Baysal, 2003). According to the literature, an inoculum density between 1×10^4 to 1×10^9 spores/ml was found to be suitable for citric acid production by *A. niger* in submerged fermentation (Favela-Torres *et al.*, 1998; Ruijter *et al.*, 2000; Adham, 2002). The production of metabolites varies depending on the medium, fermentation parameters, products and fungus species employed. In general, optimal citric acid production is obtained between 120 to 360 h in submerged fermentation (Abou-Zeid and Ashy, 1984; Hang *et al.*, 1987; Favela-Torres *et al.*, 1998; Gupta and Sharma, 2002).

The objective of this study was therefore to test the effect of fermentation parameters on the production of citric acid by *A. niger* NRRL 567 on fermented cheese whey medium. Temperature will not be optimized as 30°C is known to produce maximum yields from *A. niger* (Abou-Zeid and Ashy, 1984; Haq *et al.*, 2001). The statistically-based optimization procedure CCD was used to select the best combinations of fermentation parameters from the results.

9.3 Materials and Methods

9.3.1 Microorganism

The white rot fungus *Aspergillus niger* NRRL 567 was obtained from the American Type Culture Collection (ATCC) and was stored in a tube containing glycerol (30% v/v) at

–76 °C. *A. niger* spores were produced on a potato dextrose agar (Sigma, St. Louis, MO, USA) plate kept at 30°C. After ten days of incubation on PDA plates, 10 ml of 0.1% Tween 80 were added to each plate to harvest spores. Diluted spore suspensions of 1.0 x 10^7 spores/ml were counted using hemocytometer and prepared as inoculum for the experimental media.

9.3.2 Method

Spray dried whey powder (Sigma, St. Louis, MO, USA) containing at least 65% lactose (w/w), 11% protein (w/w) and 2% lactic acid was used as a basal medium. The whey-based medium was prepared by dissolving 50 g of whey powder in 1.0 l of distilled water at 50°C (Lee and Yun, 1999). To avoid protein precipitation during autoclaving, the whey was heated at 90°C for 20 min and the proteins were removed by centrifugation at 11,000 x g for 20 min (Lee *et al.*, 1997). Then, the supernatant was collected and reformed by adding supplements (60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g KH_2PO_4). The fermentation medium was also supplemented with previously optimized additives, 3.76 g phytate (40% (w/v), Aldrich, St. Louis, MO, USA.), 47.7 g olive oil (Bertolli, Laval, Qc, Canada) added before autoclaving and 48.3 g/l of methanol (99+%, Sigma, St. Louis, MO, USA) added after autoclaving.

The effects of fermentation parameters were investigated by adjusting the initial pH to either 1.6, 3.0, 5.0, 7.0 or 8.4 by adding 1.0 N NaOH or 1.0 N HCl before autoclaving (Table 9.1). Then, 50 ml samples of each of the 17 experimental media were placed in 250 ml Erlenmeyer flasks and autoclaved at 121°C for 15 min. Once autoclaved, the 17 media were assigned to one of the treatments presented in Table 9.2. The inoculum density was varied from 0.32 to 3.68 x 10^6 spore/ml and the fermentation time was varied from 102 to 426 h. All media were incubated in a rotary shaker (150 rpm) at 30°C.

At each sampling session, 1.7 ml of cultured medium was sampled from each flask under aseptic conditions and centrifuged at 11,000 x g for 2 min (Lee and Yun, 1999). The citric acid level in the supernatant was determined by adding pyridine and acetic anhydride to develop color that was measured by spectrophotometry at 420 nm (Marier and Boulet, 1958). The diluted supernatant was used for the estimation of residual glucose concentration using a glucose assay kit (Sigma, St. Louis, MO, USA).

9.3.3 Statistical procedure

The CCD was shown to effectively produce an equation predicting citric acid production as a function of variables (Kim *et al.*, 2004). Thus, this method was used to optimize the combination of fermentation parameters in this study. Table 9.1 represents the coded level for the three independent variables and Table 9.2 presents the 17 combinations tested. These levels were coded –1.68, -1.0, 0, 1.0 and 1.68. For the statistical calculations, the concentration of the variables (X_i) were calculated by a following equation (Ambati and Auuuanna, 2001; Abalos *et al.*, 2002):

$$X_i = \chi_i \cdot \Delta X_i + X_{cp} \qquad (9.1)$$

where i = 1, 2 and 3 and corresponds to each one of the three variables; χ_i = dimensionless coded level for X_i, namely 0, +/-1 and +/- 1.68; X_i = real level of the independent variable for the code used; X_{cp} = level of independent variable at the coded value 0; ΔX_i = step change in concentration calculated as 2.0 for the initial pH, 96 h for fermentation time and 1 x 10^6 of inoculum density. The actual levels of the initial pH, fermentation time and inoculum density were determined as follows: $X_1 = \chi_1 \cdot 2 + 5$; X_2 (h) = $\chi_2 \cdot 96$ (h) + 264 (h); X_3 (10^6 spores/ml) =

χ_3•1 (10^6 spores/ml) + 2 (10^6 spores/ml). The high, low and center point concentration for each nutrient were selected by preliminarily unreported experimentation.

The results obtained from experiment are used to produce a quadratic equation including all linear and interactive terms predicting citric acid production (Y) as:

$$Y = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \beta_3\chi_3 + \beta_{11}\chi_1^2 + \beta_{22}\chi_2^2 + \beta_{33}\chi_3^2 + \beta_{12}\chi_1\chi_2 + \beta_{23}\chi_2\chi_3 + \beta_{13}\chi_1\chi_3 \quad (9.2)$$

where Y = predicted response; β_0 = intercept; β_1, β_2, β_3 = linear coefficients; β_{11}, β_{22}, β_{33} = squared coefficients; β_{12}, β_{13}, β_{23} = interaction coefficients (Panda *et al.*, 1999; Vohra and Satyanarayana, 2002).

9.4 Results

Seventeen experimental fermentation conditions showed the different production levels between 12 to 66 g/l (Table 9.2). These production levels (Y, g/l) produced the following second-order polynomial equation (Panda *et al.*, 1999):

$$Y = 60.39 + 4.35\chi_1 - 2.52\chi_2 + 3.05\chi_3 - 14.62\chi_1^2 - 14.81\chi_2^2 - 5.79\chi_3^2 + 0.098\chi_1\chi_2 + 3.09\chi_1\chi_3 - 3.54\chi_2\chi_3 \quad (9.3)$$

The goodness of fit was confirmed by a coefficient of determination (R^2) of 0.971. Thus, equation (9.3) predicted citric acid production within accuracy of 97% (Pham *et al.*, 1998; Ramirez *et al.*, 2001).

Using equation (9.3), three-dimensional response surface curves were generated to

evaluate the interactive effect between the tested variables for citric acid production. For a constant inoculum density, Figure 9.1 illustrates the interaction of an initial pH and fermentation time. The convex surface curve indicated maximum citric acid production near an intermediate initial pH level of 5.5 and a fermentation time of 264 h. Under such conditions, citric acid production maximized at 60.8 g/l.

The response surface curve predicting citric acid production, as a function of the initial pH and inoculum density for a constant fermentation time of 264 h was plotted in Figure 9.2. Citric acid production increased with an initial pH level up to 5.5 but decreased thereafter, while for inoculum density, it increased from 1.0 to 2.0 x 10^6 spores/ml but did not change thereafter. A maximum production of 60 g/l was reached under optimal combinations of the initial pH and inoculum density, after 264 h of fermentation.

Figure 9.3 predicted the interactive effect of fermentation time and inoculum density for a fixed initial pH of 5.0. Citric acid production maximized between 200 and 250 h of fermentation time. An inoculum density near 2.0 x 10^6 spores/ml led to a maximum production of 61 g/l. Thus, for an initial pH level of 5, citric acid production increased up to 264 h.

Figure 9.4 is a cube graph predicting the interaction between each of the three independent variables, fermentation time, an initial pH and inoculum density. Each cube corner represented a different experimental condition. The plus (+) and minus (-) signs represent the coded levels -1 and +1 (Berthouex and Brown, 1994). The cube plots indicated one dominant surface, that of the right side with a high initial pH level resulting in the most citric acid production. A maximum production of 41.8 g/l was therefore predicted with a high initial pH level (A+), a short fermentation time (B-) and a high inoculum density (C+). The optimum initial pH for the whey-based medium was found to

be 5.4, while the optimal fermentation time and inoculum density were found to be 252 h and 2.35 x 10^6 spores, respectively. (Figure 9.5).

The final fermentation conditions for citric acid production after optimization using CCD is as follows: pH 5.4, 252 h fermentation time and 2.35 x 10^6 spores/ml. Under the above fermentation conditions, *A. niger* NRRL 567 produced 74.6 g/l citric acid at 312 h in the validation study which is a 78.5% increase as compared to the maximum citric acid concentration from previous studies (Figure 9.6).

When glucose and lactose are cofermented for citric acid production by *A. niger,* glucose inhibits the synthesis of ß-galactosidase and is preferentially consumed than whey lactose (Shuler and Kargi, 2002). Thus, hydrolysis of lactose by ß-galactosidase is detected after depletion of glucose and then lactose can be used as a carbon source. During the fermentation of whey-based medium supplemented with glucose with optimized conditions, residual glucose concentration achieved was close to that obtained in the control experiment (Figures 9.6 and 9.7). In both experiments, residual glucose concentrations were almost depleted after 240 h of fermentation. After the depletion of the glucose in the medium, dry cell weight of *A. niger* and citric acid production at 312 h were increased to 7.2 and 74.6 g/l, respectively. Thus, *A. niger* NRRL 567 is a strain able to produce the enzyme ß-galactosidase and it is economical to use a whey-based medium for citric acid production as a nutrient for citric acid production.

9.5 Discussion

The CCD was instrumental in reaching this maximum production level. Considering the three variables tested, the initial pH of the whey-based medium and the inoculum density had a significant positive effect on citric acid production. Whereas, fermentation

time had an insignificant effect, for the ranges tested.

The initial sugar concentration based on the content of lactose (65% w/w) in whey can be calculated; the theoretical initial sugar concentration of 92.5 g/l and citric acid yield of 80.6% can be obtained. These results are comparable to citric acid production and yield of previously reported results by Lee and Yun (1999) using whey-based medium.

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Table 9.1 Coded and actual levels tested using CCD

Variables	Components	Unit	Coded and actual level				
			-1.68	-1	0	+1	+1.68
X_1	pH	-	1.6	3.0	5.0	7.00	8.4
X_2	Fermentation time	Hour	102.5	168	264	360	425.5
X_3	Inoculum density	10^6 spores/ml	0.32	1	2	3	3.68

Table 9.2 Experimental plan and measured versus predicted citric acid production

Run No	pH	Fermentation time (h)	Inoculum density ($\times 10^6$)	Citric acid (g/l)	
				Observed	Predicted
1	3.0	264.0	2.0	20.96	19.73
2	7.0	264.0	2.0	20.12	22.46
3	3.0	264.0	2.0	18.95	21.97
4	7.0	168.0	1.0	27.06	24.31
5	3.0	168.0	1.0	22.36	26.74
6	7.0	360.0	1.0	43.21	41.81
7	3.0	360.0	1.0	15.54	14.82
8	7.0	168.0	3.0	26.66	29.51
9	1.6	168.0	3.0	14.19	11.72
10	8.4	360.0	3.0	26.19	26.36
11	5.0	360.0	3.0	24.37	22.72
12	5.0	264.0	2.0	14.90	14.26
13	5.0	264.0	2.0	38.93	38.89
14	5.0	102.5	2.0	51.41	49.16
15	5.0	425.5	2.0	55.14	60.39
16	5.0	264.0	0.3	59.12	60.39
17	5.0	264.0	3.7	66.51	60.39

R^2 (coefficient of determination) = 0.971

X_1 = initial pH; X_2 = fermentation time; X_3 = inoculum density

Table 9.3 Analysis of variance (ANOVA) for the second-order model for citric acid production

	Sum of Squares	*F* value	*P* level
Model	4430.29	25.65	0.0002*
X_1	258.84	13.49	0.0079*
X_2	86.48	4.51	0.0714
X_3	127.16	6.62	0.0368*
X_1^2	2409.47	125.53	< 0.0001*
X_2^2	2474.24	128.90	< 0.0001*
X_3^2	377.45	19.66	0.0030*
X_1X_2	0.076	0.0039	0.9514
X_1X_3	76.30	3.98	0.0864
X_2X_3	100.10	5.22	0.0563

*Significant at the 95% level; X_1 = initial pH; X_2 = fermentation time; X_3 = inoculum density

Table 9.4 Optimization constraints at 168 and 240 h of fermentation

Variables	Goal	Unit	Lower Limit (-1)	Upper Limit (+1)
pH (X_1)			3	7
Fermentation time (X_2)		h	168	360
Inoculum density (X_3)		10^6	1	3
Predicted citric acid (*Y*)	maximize	g/l	14.2	66.5

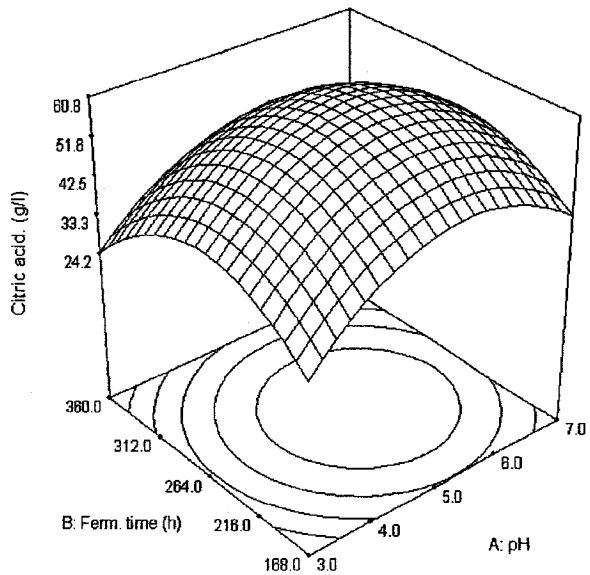

Figure 9.1 Predicted citric acid production as a function of pH and fermentation time, for a fixed inoculum density of 2.0×10^6 spores/ml; Ferm. time = fermentation time.

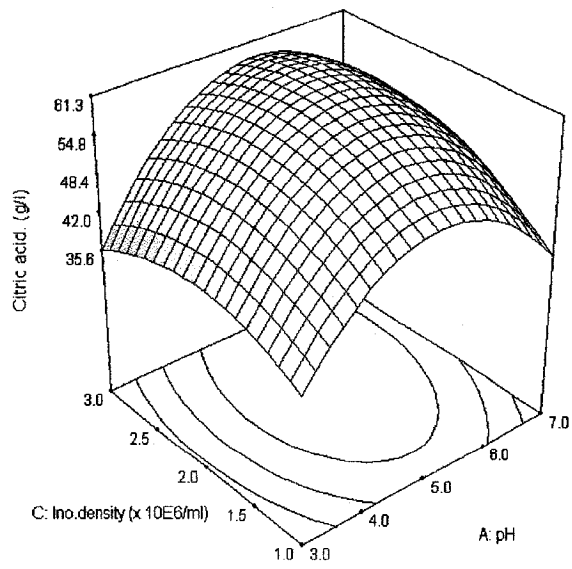

Figure 9.2 Predicted citric acid production as a function of pH and inoculum density, for a fixed fermentation time of 264 h; Ino. density = inoculum density.

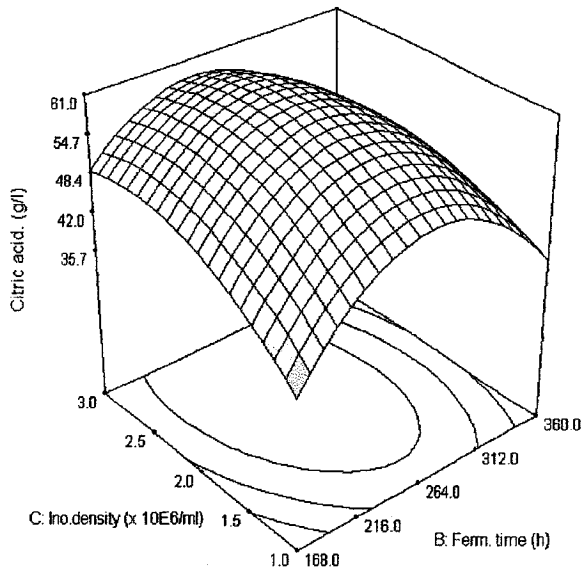

Figure 9.3 Predicted citric acid production as a function of ferm. time and ino. density, for a pH fixed at 5; Ino. density = inoculum density; Ferm. time = fermentation time.

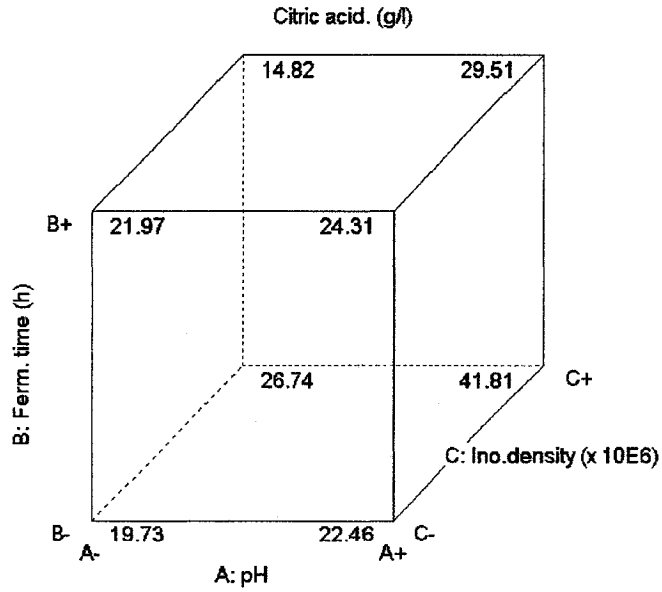

Figure 9.4 CCD cube plot predicting citric acid production. The corner values correspond to the coded vales of −1 and +1, computed using the second-order polynomial equation; Ino. density = inoculum density; Ferm. time = fermentation time.

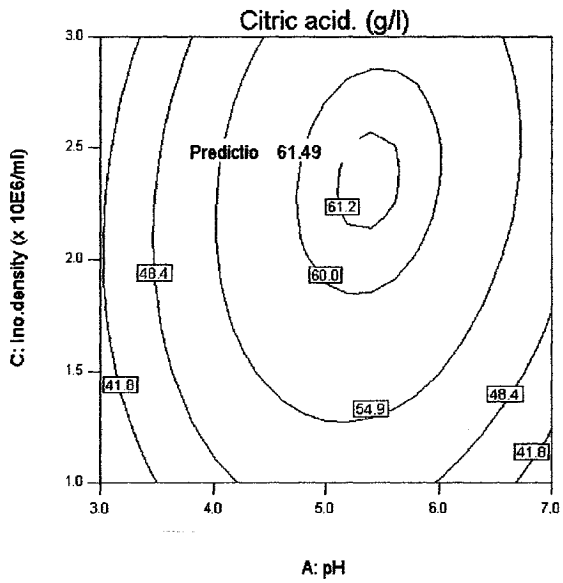

Figure 9.5 Two-dimensional contour plots showing the predicted optimum level of citric acid production as a function of pH (X_1) and inoculum density (X_3), at a fixed fermentation time (X_3) of 252 h; Ino. density = inoculum density.

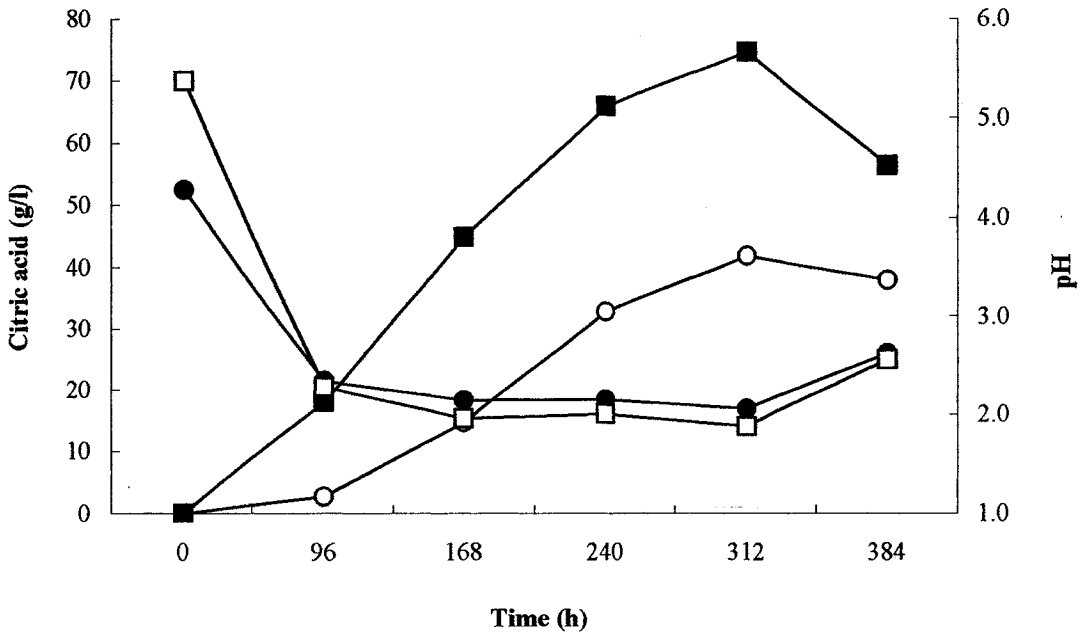

Figure 9.6 Time course of citric acid production and pH by *A. niger* NRRL 567 in submerged fermentation using the control and optimized parameters. Both media contained 50 g whey, 60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g KH_2PO_4, 48.3 g methanol, 47.7 g olive oil and 3.8 g/l phytate and were fermented at 30°C while shaking at 150 rpm. The initial pH and inoculum density of the control experiment were pH 4.28 and 1 x 10^6 spores/ml. The initial pH and inoculum density of optimized parameters were pH 5.37 and 2.35 x 10^6 spores/ml. ○: control experiment (citric acid); ■: optimized parameters (citric acid); ●: control experiment (pH); □: optimized parameters (pH).

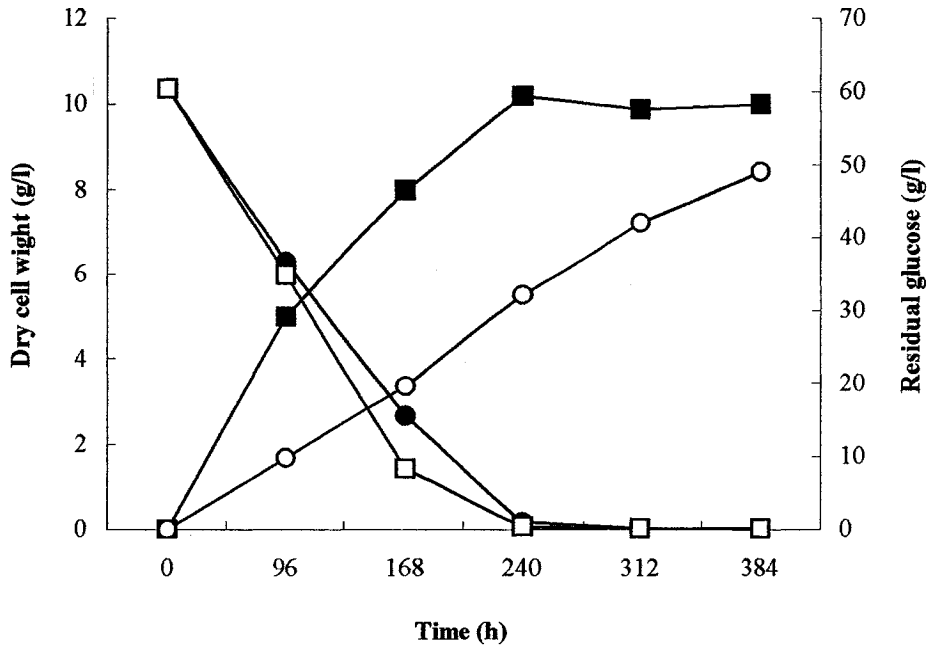

Figure 9.7 Time course of cell mass and residual glucose concentration in submerged fermentation using the control and optimized parameters. Both media contained 50 g whey, 60 g glucose, 1.5 g $(NH_4)_2SO_4$ and 8.1 g KH_2PO_4, 48.3 g methanol, 47.7 g olive oil and 3.8 g/l phytate and were fermented at 30°C while shaking at 150 rpm. The initial pH and inoculum density of the control experiment were pH 4.28 and 1 x 10^6 spores/ml. The initial pH and inoculum density of optimized parameters were pH 5.37 and 2.35 x 10^6 spores/ml. ○: control experiment (dry cell weight); ■: optimized parameters (dry cell weight); ●: control experiment (residual glucose); □: optimized parameters (residual glucose).

CHAPTER 10

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

10.1 Cost analysis for citric acid production in solid substrate fermentation

In recent years, the advantages of solid substrate fermentation have been recognized, because it offers various rewards in comparison with submerged culture and fits practical requirements for bulk production. For that reason, solid substrate fermentation for citric acid has been proposed for the application in remediation of heavy metal contaminated soil and anticipated to offer a potentially low cost and environmentally friendly solution. However, as the price of commercial citric acid is cheap less than one US dollar per kg, to apply fungal citric acid for heavy metal remediation practice, the economics of solid substrate fermentation for the production of fungal citric acid using solid substrate fermentation need to be discussed.

It is clear that the economic competitiveness of citric acid production by solid substrate fermentation depends mainly on the investment and production costs of the process. It would be difficult to estimate an accurate operating cost of solid substrate fermentation. However, based on the common concerns, approximate costs of solid substrate fermentation in comparison to conventional submerged fermentation can be estimated (Table 10.1). Even though, solid substrate fermentation can use a cheap substrate, agro-industrial byproducts, they can be used for a feeding matrial for animals without any futher treatment. Therefore, as compared to animal feed, solid substrate fermentation for citric acid production using agro-industral byproducts need to generate more profits.

As solid substrate fermentation allows very low moisture content of support matrials even without free flowing water, it can provide many cost effective advantages in substrate and fermentation preparation. For the cost analysis for the investment and plant operation,

solid substrate fermentation system is not required to construct large fermentation facilities due to a space saving and simplified fermentor. However, despite of a cost advantage in the initial setup for fermentation facilities, due to the difficulties in on-line monitoring and control of fermentation parameters, labor and maintenance may be higher than the expenditure of submerged fermentation (Goes, 1999).

The agro-industrial byproduct can be used as solid substrate and it may bring revenue can help offset the initial cost for substrate preparation and the treatment cost for the remaining waste from agro-industrial processes. However, for fungal citric acid production using agro-industrial byproducts, it is required to collect, convey and pretreat of large amount of byproduct and would cause major complexity and expenditure for whole process. Also, to obtain high concentration of citric acid, high concentration of sugar, nutrient and stimulators were required to be added and it will add costs to the process.

In view of drawbacks of solid substrate fermentation, a more practical approach with low cost of sugar sources, simplified fermentation equipments and cost-effective purification process would be to decrease production cost and prove an economical efficiency of solid substrate fermentation. To achieve more efficient process, the utilization of agro-industrial sugar rich byproducts containing high concentration of sugar will be required. In general, for the byproducts such as fruit peels and and crop residues have low concentration of fermentable sugars, containing up to 35 wt%, addition of sugar-rich byproduct like molasses (72 wt% sugar) is necessary to provide enough fermentable sugar. The facts obtained in this study point out the production of citric acid by solid substrate fermentation of agro-industrial byproducts containing high sugar concentration may have economic potential in solving waste treatment and in the production of citric acid. For the recovery process, as citric acid and other organic acid such as oxalic, tartarlic and malic

acid produced by *A. niger* have a potential for heavy metal remediation, washing effluent containing various organic acids, mainly citric acid, can be directly used for heavy metal remediation practice without purification of a target product, citric acid, using conventional precipitaton process. Therefore, the simplified recovery process with removal of only biomass could reduce the cost. Also, alternatives to overcome the heterogeneous fermentation environmental of solid substrate fermentation are being considered, to decrease the cost for labor and maintanace using Ko-ji and rotated drum solid substrate fermentors. Attempts are also being undertaken to develop a simple, large-scale and well-controlled solid substrate fermentation systems that would be allowed for future achievement of application of fungal citric acid for remediation practice.

Once we achieve the efficient citric acid fermentation and purification process, citric acid can be industrially produced much cheaper by use of sugar-rich byproducts and may fit practical requirements for use in *in-situ* soil flushing for soil remediation. The proposed *in-situ* soil flushing technique using fungal citric acid provides a potentially low cost and environmentally friendly solution.

10.2. Summary and Conclusions

This study was focused on the evaluation of the use of an solid substrate fermentation system for the production of citric acid using simulated solid substrate. Citric acid production by *A. niger* NRRL 567 grown on peat moss was optimized using a sequential optimization using a traditional and a statistically-based optimization in various fermentation system. The production of citric acid from batch type solid substrate fermentation was compared to submerged and semi-continuous fermentation.

Based on the results of study 1 (optimization of citric acid production by *A. niger* grown on PM in batch type solid substrate fermentation), the following general conclusions were obtained:

1. PM was a good support material only when supplemented with a relatively high level of readily available carbon substrate.
2. For citric acid production, glucose and KH_2PO_4 showed a positive significant impact. Nitrogen, as $(NH_4)_2SO_4$, had a negative effect most likely because it enhances cell growth as opposed to citric acid production.
3. As produced citric acid provided an end product inhibition, citric acid production was decreased in low level of pH. Among the physico-chemical fermentation parameters having the most impact on citric acid production were the initial pH of the nutrient solution and the initial MC of PM.
4. Stimulators such as olive oil and methanol were found to have a positive significant effect on citric acid production, whereas phytate was found to have a negative significant effect.
5. The buffer used to control the pH of solid substrate significantly influenced citric acid production. The phosphate and carbonate buffers, with pH 8.6 and pH 10.0 respectively, were identified as suitable buffer solutions for citric acid production. The maximum citric acid production of 564.3 g/kg DPM was achieved with the carbonate buffer at an initial pH 10.0
6. The sequential optimization using the traditional and CCD effectively optimized fermentation condition for citric acid production by *A. niger* NRRL 567. The sequential optimization allowed for a 50-fold increase in citric acid production compared to the production of citric acid by *A. niger* grown on PM supplemented

with 100 g glucose/kg DPM alone.

7. As compared to standard submerged fermentation, solid substrate fermentation could produce a high concentration of citric acid within a shorter period of time. However, the control of fermentation parameters such as temperature, pH, water content and mixing are more of a challenge.

Based on the results of study 2 (optimization of citric acid production by *Aspergillus niger* 567 using PM in columns bioreactor), the following general conclusions were obtained:

1. Citric acid production and yield using a semi-continuous fermentation system was much lower than that obtained with batch fermentation tests in solid substrate fermentation. This may result from inefficient aeration and gas exchange and heterogeneous fermentation conditions, especially for temperature and MC.
2. The improvement of citric acid production in the semi-continuous system requires the further development of the bioreactor and its fermentation conditions.

Based on the results of study 3 (cheese whey for citric acid production by *A. niger* in submerged fermentation), the following general conclusions were obtained:

1. Glucose supplementation significantly influenced citric acid production. Thus, higher citric acid levels are achievable with a whey-based medium supplemented with a high level of glucose.
2. The supplementation of methanol had a positive effect on citric acid production, while that of phytate decreased citric acid production.
3. The initial pH of the whey-based medium and the inoculum density had a significant positive effect on citric acid production, whereas, fermentation time had an

insignificant effect, for the tested ranges.

4. In all experiments, the statistically-based optimization methods, namely CCD and FFD, were instrumental in generating regression equations that accurately predicted optimal parameter values. These statistically-based optimization methods proved to be efficient tools in achieving optimal fermentation condition with a minimum number of treatment combinations.
5. Sequential optimization using CCD and FFD allowed for a 13.3-fold increase in citric acid production compared to the production of citric acid by *A. niger* NRRL 567 using a whey-based medium (50 g/l) alone.
6. Cheese whey, as a nutrient medium, offered fermentable sugar during the fermentation. Whey lactose was utilized for citric acid and cell mass production. Thus, *A. niger* NRRL 567 is a strain able to produce the enzyme ß-galactosidase and whey can be an interesting nutrient for citric acid production.
7. The facilitation of monitoring and controlling fermentation parameters during the fermentation is one of advantages of submerged fermentation. In addition, a high citric acid yield (80.6%) can be obtained using a whey-based medium.

10.3 Contributions to Knowledge

This project offered the following major contributions to knowledge:

1. Establish optimal fermentation conditions including glucose and salts, fermentation parameters and stimulators for solid substrate fermentation of *A. niger* to produce citric acid in batch fermentation grown on a glucose-rich byproduct.
2. Establish optimal physico-chemical fermentation conditions for citric acid

production in semi-continuous fermentation grown on a glucose-rich byproduct.

3. Evaluate the effects of the initial pH of various buffer solutions on citric acid production and optimize the initial pH of PM.
4. Demonstrate that PM can provide an adequate support material and moisture reservoir for growth and citric acid production by *A. niger* in batch fermentation.
5. Demonstrate that cheese whey can be used as carbon source for citric acid production in submerged fermentation.
6. Demonstrate that the statistically-based optimization method using FFD and CCD is an effective tool in defining interactions between variables and in optimizing fermentation conditions for citric acid production grown on PM and on a whey-based medium.

10.4. Recommendation for Future Research

Three fermentation types including solid substrate, submerged and semi-continuous fermentation were used to evaluate the potential for citric acid production. The enhancement of citric acid production from various fermentation systems and the application of fungal citric acid to bioremediation can be achieved from the following future work:

1. An alternative cheap carbon sources is needed to decrease the operational costs. Agro-industrial byproducts such as cane molasses, pineapple waste, apple pomace, kiwi peel, grape pomace or mixture of byproducts can be used as the bulk carbon substrate, because they offer high levels of readily available carbon sources.
2. Cheese whey is a nutrient rich substrate and its high lactose content can be utilized by *A. niger* NRRL 567. A strain of *A. niger* which overproduces ß-galactosidase and lactose permease could utilized cheese whey more efficiently for citric acid

production.

3. Further improvements and optimization are required to develop the appropriate bioreactor and the fermentation parameters for citric acid production in semi-continuous fermentation.
4. For applying to the field test, a large-scale experiment should be conducted on a site contaminated by heavy metals.

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