QUANTIFICATION OF FUNGAL BIOMASS GROWTH DURING CITRIC ACID PRODUCTION BY *ASPERGILLUS NIGER* ON EXPANDED CLAY SOLID SUBSTRATE

By

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DEDICATED TO MY LATE GRAND PARENTS
AND
JABIR IBN HAYYAN

Abstract

The growth of fungi on sugar rich wastes can be an economical way of producing citric acid. Nevertheless, conditions for the optimum production of citric acid still need to be established. Using *Aspergillus niger*, the objective of the present study was to measure the effect on citric acid accumulation and fungal biomass, of sugar and nitrogen supplementation namely as glucose and ammonium, respectively. An inert solid substrate (Hydroton® or HSS) made of expanded clay and wetted with a nutrient solution was used to grow *A. niger* ATCC12846 and measure its fungal biomass with fermentation time. Citric acid accumulation and fungal biomass were measured during 168 h of fermentation with glucose concentrations ranging 0 to 475 g $(kg\ HSS)^{-1}$ and ammonium ranging from 2 to 16 g $(kg\ HSS)^{-1}$. Fungal biomass growth was monitored by measuring the change of total volatile solids (TVS) less residual glucose and citric acid, and; organic nitrogen accumulation. Glucose and ammonium had a significant effect ($P < 0.10$) on both fungal biomass and citric acid accumulation. For citric acid, the highest concentration of 52 g and yield of 14% were obtained with 475 and 250 g glucose $(kg\ HSS)^{-1}$ and 8 g of N $(kg\ HSS)^{-1}$. Nevertheless, only the glucose concentration of 475 g $(kg\ HSS)^{-1}$ resulted in citric acid accumulation continuing after reaching a peak in fungal biomass. This high glucose concentration could have yielded more citric acid by using a pH of 5.5 during spore germination and of 2.0 during fungal biomass growth. Because the C:N ratio of the fungal biomass was observed to vary with nitrogen supplementation, it is not recommended to use organic N to quantify fungal biomass.

Résumé

La fermentation de champignons sur des résidus riches en sucre pourrait être une façon économique de produire de l'acide citrique, à condition de bien maîtriser les paramètres de fermentation. La présente étude avait comme objectif d'évaluer l'effet de la charge de sucre, soit en glucose, et d'azote, soit en ammonium, sur la biomasse du champignon *Aspergillus niger* ATCC12846 et sur sa production d'acide citrique. De l'argile expansée (Hydrotron® ou HSS) fut utilisée comme substrat solide pour le champignon *A. niger* ATCC 12846. Le substrat fut humecté d'une solution offrant différents taux de glucose, de 0 à 475 g (kg HSS)$^{-1}$ et d'azote sous forme d'ammonium, de 2 à 16 g (kg HSS)$^{-1}$. La biomasse fongique fut obtenue en mesurant la masse volatile totale moins la masse résiduelle de glucose et la masse d'acide citrique, et; l'augmentation de la masse d'azote organique. Le taux de glucose et d'ammonium a eu un effet significatif sur la biomasse fongique et la production d'acide citrique pendant les 168 h de fermentation. Une concentration en glucose de 475 et 250 g (kg HSS)$^{-1}$ maximisaient la concentration de 52 g (kg HSS)$^{-1}$ et le rendement de 14% en acide citrique, respectivement, avec 8g d'azote (kg HSS)$^{-1}$. Par contre, seulement la concentration en glucose de 475 g (kg HSS)$^{-1}$ permettait d'accumuler de l'acide citrique après avoir atteint le plus de biomasse. Un rendement supérieur exigerait un meilleur contrôle du pH à 5.5 pendant le développement des spores et à 2.0 pendant la fermentation. Puisque le ratio C:N de la biomasse fluctuait avec la concentration d'azote dans la solution, il n'est pas recommandé d'utiliser l'azote organique pour suivre l'évolution de la biomasse.

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Authorship and Manuscript

This thesis is written in manuscript-based format. 1) First author carried out experiment and wrote the first draft of the manuscript. 2) Second author supervised the entire experiment and provided technical correction of the work and manuscript.

The authorship of the paper is as follows:

CHAPTER 3: Iqbal Q, Barrington S, Lyew D. This paper will be submitted to the journal of Applied Microbiology and Biotechnology

Abbreviations

1. *A. niger* = *Aspergillus niger*
2. ATP= adenosine triphosphate
3. ATCC= American Type Culture Collection
4. C= Carbon
5. C: N= Carbon to Nitrogen ratio
6. ^{o}C= degree Celsius
7. Ca^{2+}= Calcium
8. Cd= Cadmium
9. $Ca_{p=}$ Citric acid produced
10. Ca_{y}= Citric acid yield
11. CO_2= Carbon dioxide
12. Cu= Copper
13. DOT= Direct oxygen tension
14. Fe^{+2}= Iron, Ferrous
15. $FeSO_4.7H_2O$= Ferrous Sulphate heptahydrate
16. G_c= Glucose consumed
17. g/l, g L^{-1}= Gram per Liter
18. g (kg of Hydroton solid substrate)$^{-1}$= Gram per Kilogram of Hydroton Solid Substrate
19. g (kg HSS)$^{-1}$= Gram per Kilogram of Hydroton Solid Substrate
20. HCl= Hydrochloric acid
21. HSS= Hydroton Solid Substrate
22. H_2SO_4= Sulphuric acid
23. HPLC= High Performance Liquid Chromatography
24. KH_2PO_4= Potassium Phosphate
25. $MgSO_4.7H_2O$= magnesium sulphate
26. Mn= Manganese
27. N= Nitrogen
28. NaCl= Sodium Chloride
29. 0.02 N= 0.02 Normal
30. NAD= Nicotinamide adenine dinucleotide,
31. NADPH= Nicotinamide adenine dinucleotide phosphate-oxidase
32. $(NH_4)_2\ SO_4$= Ammonium Sulphate
33. O_2= Oxygen
34. PDA= Potato Dextrose Agar
35. Pb= Lead
36. rpm= Revolutions Per Minute
37. SSF= Solid state fermentation
38. TCA cycle= Tricarboxylic acid cycle
39. TKN= Total Kjeldahl nitrogen
40. TVS= Total volatile solids
41. Zn= Zinc

List of Equations

CHAPTER 1

INTRODUCTION

1.1 Problem statement

Produced using filamentous fungi, such as *Aspergillus niger*, citric acid is widely used by the food and pharmaceutical industries. In the 8th century, citric acid (2-hydro-1, 2, 3-propanetricarboxylic acid) was first isolated from lemon juice by a Muslim alchemist Jabir Ibn Hayyan better known as Geber. Many scholars reported on the acidic nature of lemon and lime juices, such as in the 13th century encyclopedia Speculum Majus (The Great Mirror) compiled by Vincent of Beauvais. Citric acid was first crystallized from lemon juice by the Swedish chemist, Carl Wilhelm Scheele, in 1784 (Papagianni, 2007).

Approximately 70% of all citric acid production is used by the food and beverage industry for various purposes such as an acidifying and flavor-enhancing agent (Yaykasli et al., 2005). About 12% is used by the pharmaceutical industry and 18% for miscellaneous purposes such as in soaps and laundry detergents. In the past few years, citric acid was demonstrated to chelate metals and therefore, it has been used in various industries such as photography, electroplating, copper plating, leather tanning, printing, reactivation of old oil wells, inks, floor cement, linoleum, silvering compounds, algicide formulations, dyeing of fabrics and removal of contamination caused by radioactive isotopes (Abou-Zeid et al., 1984).

Even more recently, citric acid has been used as a soil heavy metal remediation agent. Wasay et al. (2001) demonstrated that citric acid could leach Cd, Cu, Pb and Zn from loams and clay loams, in the range of 84 to 91, 73 to 84, 56 to 70 and 72 to 81%, respectively. Mulligan et al. (2004) reported that *Aspergillus niger* exhibits good potential to

produce a variety of organic acids, including citric acid, which can solubilize Cu and leach this heavy metal from soils.

Every year, about one million tons of citric acid is produced by *Aspergillus niger* world wide (Xie et al., 2006). Citric acid can also be isolated chemically from the juice of citrus fruits by adding lime (calcium oxide) to form the salt calcium citrate, which is then recovered by filtration and subsequently acidified by adding sulfuric acid. Most citric acid is now produced by fungal fermentation because the chemical synthesis of citric acid is more expensive than fungal fermentation (Prado et al., 2005).

The fungus *Aspergillus niger* is the most commonly used organism to produce citric acid because of its relatively high yield (Haq et al., 2001). Nevertheless, examples of other fungi which can also synthesize citric acid include: *A. cavatus, A. fumigatus, A. japonicus, A. wentii, Pencillium lutcum, P. citrinum, Paecilomyces divaricatum, Mucor Piriformis, Ustulina vulgaris, Trichoderma viride, Candida lipolytica,* and other *Candida* species (Harvey, et al., 1994).

Citric acid from *Aspergillus niger* has been produced industrially by the submerged or solution technique. Solid state fermentation (SSF) requires less energy, provides a higher yield, exposes the process to lower risks of bacterial contamination and generates less waste-water for a lower environmental impact (Kumar et al., 2003; Nandakumar, et al., 1994). Solid state fermentation also permits for the supplementation of more minerals which enhances citric acid fermentation (Shankaranand et al., 1994). Furthermore, some metal ions (Fe^{++}, Mn^{++}, Zn^{++} etc.) inhibit citric acid production by *Aspergillus niger* in submerged fermentation whereas with SSF, such metals have a lower inhibition effect (Gutiérrez-Rojas et al., 1995; Mulligan et al. 2004).

Accordingly, SSF could be the preferred process for the production of citric acid from sugar rich wastes, such as fruit and sugar cane residues. The economical and mass production of citric acid by *Aspergillus niger* on a solid bed of sugar-rich waste can generate industrial quantities of this remediation agent on sites that have been contaminated with heavy metals. Many sugar-rich wastes have demonstrated a high potential for the commercial production of citric acid. Submerged fermentation did produce citric acid economically from sucrose and molasses rich media. Other sugar rich wastes inculding coffee husk, apple pomace, wheat straw, pineapple waste, cassava baggasse, banana peel, sugar beet, kiwi fruit peel and corn cob, were also used to support the production of citric acid by *Aspergillus niger* (Shankaranand et al., 1994).

Citric acid accumulation is strongly influenced by the growth rate of the fungi. This growth rate is influenced by the availability of nutrients, the type and concentration of the carbon source, the fermentation method and the environmental conditions such as aeration rate, pH, and temperature (Xu et al., 1989; Jianlong et al., 1998). Citric acid production and the growth rate of *Aspergillus niger* can be limited by insufficient nitrogen, phosphorus or other bulk elements (Jianlong et al., 1998; Mirminachi, 2002). Some stimulators such as organic solvent, phytate and lipids have been found to increase citric acid yield (Jianlong and Ping, 1998). Ethanol is also known to change the enzyme activity (increases the activity of citrate synthetase and decreases the activity of aconitase) associated with the TCA cycle (tricarboxylic acid cycle). Finally, citric acid production is affected by fermentation time and inoculum density (Lee et al., 1999). Therefore, monitoring fungal growth rate during citric acid production can increase the understanding of the process and help improve yield.

As citric acid production reaches an optimum, the fungal biomass also grows rapidly. It is well-known that the control of fungal growth is a very important factor in the production of citric acid during solid state fermentation (Prado et al., 2005).

Solid state fermentation should control fungal mass growth rate to maximize sugar consumption and citric acid yield. In the past, the fungal mass growth rate has been monitored by CO_2 production and O_2 consumption (Prado et al., 2005), and measurement in relation to chitin (Narahara et al., 1982) and nucleic acid concentrations (Koliander et al., 1984). These methods are complicated and required the use of expensive instruments. A simple method is therefore required to monitor fungal mass growth during the SSF for citric acid production.

1.2 Objective

The general objective of the current study was therefore to monitor citric acid production by *Aspergillus niger* along with its mass growth rate, and as a function of available sugar and ammonium. More specifically, the present project had the following objectives, for the solid state production of citric acid by *Aspergillus niger* ATCC 1248 using a sugar rich substrate:

1) test a simple method to monitor the growth rate of the fungal mass;
2) investigate the effect of fungal growth rate on citric acid production;
3) measure the effect of sugar and ammonium ion concentration on citric acid yield.

1.3 Hypothesis

The first objective is based on the hypothesis that fungal mass has a specific carbon to nitrogen ratio and therefore, can be monitored by conducting an organic nitrogen and

carbon mass balance, parameters that are relatively easy to measure. The second objective is based on the hypothesis that citric acid production is directly related to fungal mass.

1.4 Scope

This experiment was limited to the laboratory and used a solid substrate consisting of an inert medium composed of Hydroton® clay particles. The organism used in this test was *Aspergillus niger* ATCC 1248. The range of sugar supplementation was limited to 125, 250, 350, and 475 g (Kg of Hydroton solid substrate)$^{-1}$. The range of $(NH_4)_2\ SO_4$ was to be limited to 2, 4, 8, 9, 12, 16 g (Kg of solid substrate)$^{-1}$.

CHAPTER 2

LITERATURE REVIEW

2.1 Citric Acid Production

Because of a high demand by the pharmaceutical, chemical, food, beverage and cosmetics industries, citric acid ($C_6H_8O_7$) is mass produced worldwide at the rate of 1,000,000 metric tons per annum (Prado et al., 2005). Recently, citric acid was also used in electroplating and for the bioremediation of soils contaminated with heavy metal (Wang, 1998; Ates et al., 2002). Although it can be produced through chemical processes, microbiological processes using *Aspergillus niger* are still preferred to commercially produce citric acid.

Historically, citric acid was first extracted from lemon juice, explaining the origin of its Latin name *citrus*. Citric acid was first isolated from lemon juice in 1784 by the Carl Wilhelm Scheele, a Swedish chemist. In 1860, the Italian citrus fruit industry started to market citric acid extracted from lemon juice and dominated the market for one century. In 1893, it was discovered that penicillin could produce citric acid from sugar, and in 1917, James Currie, an American, discovered that citric acid could be produced industrially by *Aspergillus niger*. Pfizer was the first group to produce citric acid industrially from the fermentation of sugars by *A. niger* two years later, and this technology was taken over by a Belgian group, namely “Citrique Belge” in 1929 (Wikipedia, 2008). During the 1960’s and 1970’s, when hydrocarbons were relatively cheap, citric acid was produced from n-alkanes (ethane, butane, propane) using strains of yeast (Grimoux et al., 1880; Mattey, 1999). Today, the most economical technology for the production of citric acid uses carbohydrates fermented by fungi and yeast, under submerged conditions.

Citric acid can be produced by various types of microorganisms. Among yeasts, several species have been evaluated for this purpose, such as *Candida*, *Torula*, *Saccharomyces* and *Pichia*. Although the specie *Candida* is used for the commercial production of citric acid, it also produces substantial amounts of unwanted isocitric acid. Among fungi, several species can produce citric acid, such as *Aspergillus*, *Eupenicillium*, *Acremonium*, and *Penicillium*. Of these, *Aspergillus niger* remains the main industrial producer of citric acid because of its high yield, exceeding 70% of the theoretical value calculated from the carbon source.

Although citric acid can be produced by submerged and solid state fermentation, industry still prefers submerged fermentation because of its continuous operation (Leangon et al., 2000; Kumar et al., 2003). Nevertheless, the yield obtained with submerged fermentation techniques is highly dependent on the composition of the fermentation medium.

In submerged fermentation, the most influential factors are the carbon source and concentration, the limited availability of nitrogen and phosphorous, a regulated aeration rate and the presence of trace elements (Papagianni, 2007). Citric acid is produced efficiently when sugar, acidity, and dissolved oxygen are present at high concentrations or nitrogen, phosphorous and trace elements are present at suboptimal concentrations (Papagianni, 2007). For a high citric acid yield, the sugar source must be highly available. This automatically excludes polysaccharides which are hydrolyzed slowly at low pH. Sucrose is the preferred source because *A. niger* has an extracellular enzyme bound to the hyphae (branches of fungal cells forming the mycelium) cell wall which is active at a low pH, namely invertase (Xu et al., 1989). For submerged fermentation, a sugar concentration

ranging from 14 to 22% was found to optimize citric acid production, although such high concentration results in a lag phase extended by 18 h and a rate of fungal growth reduced by 20%. Nitrogen and phosphorous must be provided in limited quantities. In the form of ammonia, the consumed nitrogen results in a pH drop even within the fungal cells, thus improving conditions for citric acid production (Jernejc and Legisa, 2004). For high citric acid yields using the submerged process, the solution pH is required to drop to 2.0 within 2h of initiating fermentation. Furthermore, a low pH reduces the risk of contamination by other microbes. A pH of 4.5 during the production phase can drop the yield by 80% according to Papagianni (1995). Nevertheless, a pH of 5.5 is required for spore development. Finally, aeration rate must be limited, because high air flow rates spurge the carbon dioxide necessary in the metabolic pathway of citric acid production.

Low citric acid prices ranging between $0.70 to $2.00 US/kg have forced manufacturers to look for more efficient production methods and cheaper sources of carbohydrates such as sugar rich wastes (Soccol et al., 2006). Nevertheless, the fermentation of sugar rich wastes require the development of efficient solid state fermentation techniques to produce citric acid using *A. niger* (Leangon et al., 2000; Kumar et al., 2003). Solid state fermentation involves the growth of microorganism in an environment of low water activity on an insoluble medium that functions as both support and nutrient source (Soccol et al., 2006). Solid state fermentation is gaining interest because of its technological and economical advantages over submerged fermentation (Ellaiah et al., 2004). These advantages include a decrease in both energy requirement and wastewater production (Prado, 2004). In addition, most fungi grown under submerged fermentation are highly sensitive to metal ions such as Fe^{2+}, Mn^{2+}, Zn^{2+}, as compared to solid state fermentation

conditions (Soccol et al., 2006). As a result, solid substrate fermentation has been the subject of further investigations (Romero-Gomez et al., 2000) focusing on optimizing the production of citric acid by *A. niger* grown on a sugar rich solid substrate.

2.2 Fungi as micro-organisms

Fungi are heterotrophic organisms belonging to the eukaryote kingdom. Occurring worldwide, there are some 70,000 species of fungi including mushrooms, molds, rust, smuts, truffles, puffballs, morels, and yeast (Alexopoulos et al., 1996).

Fungi are considered micro-organisms because they fall in the category of those smaller than 1.0mm, which is generally not distinguishable by the human eye (Stanier et al., 1970). Fungi are classified as protist organisms, along with protozoa, algae and bacteria, because of their relatively simple biological organization. Protist are unicellular (coenocytic) or, if they are multi-cellular, their cell type lacks internal differentiation such as those cells associated with tissue regions characteristics of plants and animals.

Besides the fact that they can be multi-cellular, fungi are differentiated from bacteria by their distinct nucleus and cytoplasmic organelles. Bacteria are prokaryotes and lack such features. Examples of membrane bound organelles in the fungi are: the Golgi apparatus or dictyosome which store various cellular products; a chloroplast and mitochondria where metabolic functions occur and where some genetic material (DNA) specifies the property of the organelle and provides translation information for the synthesis of proteins, and; the nucleus enclosing genetic material known as chromosomes.

The chromosomes of eukaryotic cells are generally replicated during cell division by a complex mechanism called mitosis. The genetic material of eukaryotic cells is more

evolved than that of prokaryotic cells, such as that of the bacteria, which have a nuclear region not enclosed by a membrane but which contains a single molecule of DNA.

Asexual reproduction is typical of fungi which produce a special structure called the sporangia, which develops and releases spores, each capable of forming new hyphae which will form a branched structure forming a mass called the mycelium.

2.3. The Filamentous Fungus *Aspergillus*

Among 200 genuses of molds, *Aspergillus* is the most important filamentous fungus. The most common species of this genus is *Aspergillus niger* which, like other fungi, requires an aerobic environment for growth. With carbon rich substrates such as glucose, *A. niger* produces colonies which are powdery in texture, with a black surface color and pale yellow underneath (Figure 2.1). The morphology of *Aspergillus niger* is complex because it exhibit different structural forms thorough out its growth cycle (Papagianni, 2004). The basic differences between *A. niger* and other *Aspergillus* species are colour, growth rate and thermo-tolerance.

As opposed to *A. niger*, *A. nidulans* produces blue green colonies with a white or brownish underneath. The underneath color of *Aspergillus* species ranges from uncolored to pale yellow and even to purple and olive in some strains of *A. nidulans*, and orange to purple in *A. versicolor* (Collier et al., 1998).

Observed under microscopy, *A. niger* is known to grow relatively rapid with its colony diameter increasing from 1 to 9 cm, over 7 days at 25°C on Czapek-Dox agar. This can be compared to the growth rate of *A. nidulans* and *A. glaucus* which can increase their colony diameter from 0.5 to 1 cm under the same incubation conditions (Larone, 1995).

A. niger is considered a mesophilic fungus, and can be distinguished from others on the basis of temperature tolerance range. On agar, *A. niger* tolerates temperatures in the range of 25 to 35^{o}C, with 30^{o}C being ideal (Havden and Maude, 1994). Other fungi, such as *A. fumigatus,* grows well at temperatures over 40°C (St-Germain et al., 1996).

In submerged conditions and during fermentation, the morphology of *A. niger* can range from pellets to fee filaments. Free filaments in the form of mycelia or single hyphae with conidiophores occur when the nutrient solution is mixed at a high speed, shearing the hyphae from one another while pellets are formed under mild mixing conditions (Pazouki et al., 2002). For citric acid production under submerged conditions, the pellet form is preferred while, for pectic enzyme production, filamentous growth is preferred (Steel et al., 1954; Kristiansen and Bullock, 1988). When the nutrient solution is not mixed, the fungi tend to growth at the liquid surface forming a mat of mycelium.

Figure 2.1 *Aspergillus niger* ATTC 12486 colonies grown on expanded clay after 96 h of fermentation; the fungal hyphae heads (Conidia) are black in colour and their underneath is white (Iqbal et al., 2007).

2.3.1 Growth Stages of the fungus *Aspergillus niger*

Fungi generally exhibit four basic growth stages: the spore which is a dormant phase, followed by the spore germination or lag phase, the growth or hyphae phase, and the spore formation phase. During the growth phase, the fungus develops tubular filaments known as hyphae, and these hyphae branch out repeatedly to form a mass know as the mycelium. During this phase, the rate of production of biomass is exponential (Figure 2.2). Citric acid production is known to occur when fungal growth is limited (Vaija and Linko, 1986).

New organisms originate from spores produced asexually by a mature *A. niger* mycelium. To reproduce, fungi develop special hyphae called sporangiophores which stand erect and develop numerous spores at their tip. These spores are released into the environment and will remain dormant until proper conditions allow for their development (Nason, 1968). The metabolism of spores will generally be activated by physical or chemical factors present in the environment, such as moisture, light, temperature, and substrates such as carbohydrates.

The time required for the spores to form hyphae and for the hyphae to start branching out is called the lag phase. Spore germination is generally initiated by a swelling and the division of the nucleus, the production of endoplasmic reticulum (a structure translating and transporting proteins), ribosomes (a structure assembling protein through translation from DNA) and mitochondria (a structure generating ATP and controlling cell growth). The introduction of any micro-organisms, including *A. niger*, to a new environment requires the synthesis of enzymes and intermediates to initiate nutrient ingestion for growth. The duration of the lag phase therefore depends on several factors, including environmental conditions and level of inoculum. For *A. niger* grown under submerged conditions, high

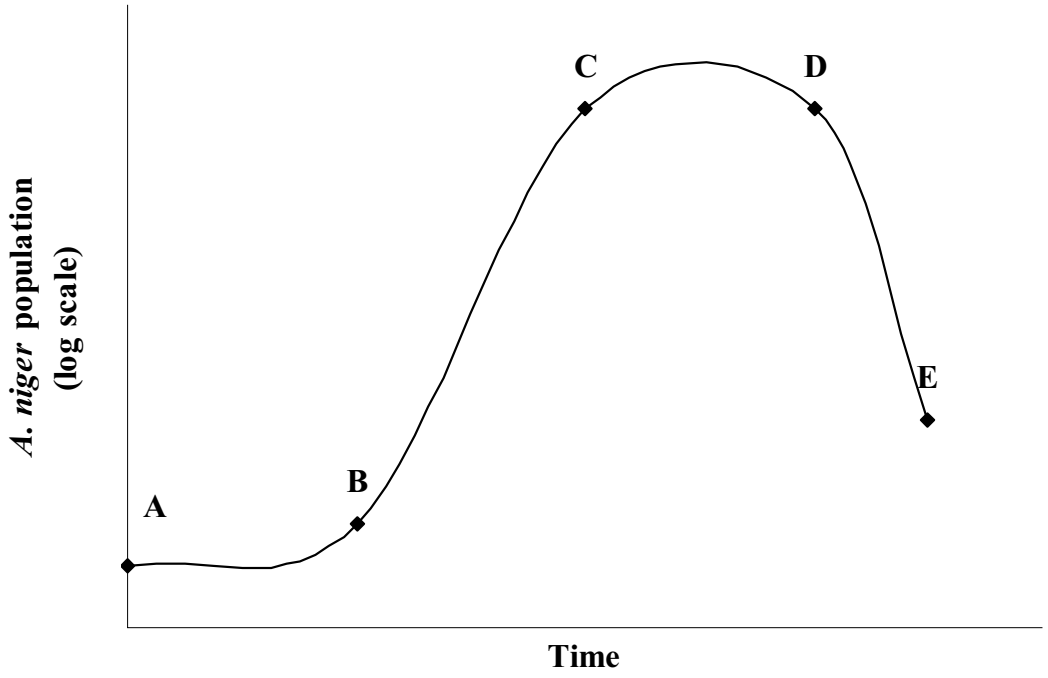

Figure 2.2 Growth stages of *Aspergillus niger*: A to B-initial lag phase; B to C-exponential growth phase; C to D-stationary phase, and; D to E-decline phase. The spores are formed during the exponential phase of *A. niger.*

levels of sugar have resulted in an extended lag phase, because such conditions repress the formation of the enzyme α–keto-glutarate dehydrogenase, an enzyme involved in the citric acid cycle (Papagianni, 2004; Hossain et al., 1984).

The lag phase is followed by the exponential growth of the fungus *A. niger*, when growth conditions are favorable and nutrients are available. During exponential growth, hyphae grow in length at their tips, branch out and extend outwards. The fungal hyphae responsible for the supply of nutrients will penetrate rich solid substances. For those grown under submerged condition and limited mixing, the hyphae tend to form pellets. The hyphae morphology and growth rate depend on environmental conditions and the presence of inhibitors such as Ca^{2+} and buffers (Jackson and Heath, 1993).

The exponential growth of the fungal mass may not be uniform within a colony. The older and central hyphae may exhaust their nutrient supply while the hyphae found at the surface may access new sources of nutrients and maintain their growth (Moore-Landecker, 1996). When nutrients become limited, especially for the older hyphae, a stationary phase is exhibited where growth is virtually stopped. According to Papagianni (2004), this phase establishes the balance between hyphal mass increase and decrease.

If nutrients are lacking over a prolonged period, the fungal hyphae undergoing a stationary growth phase may experience a loss of cell mass and even die as food reserves are deoleted. Death of fungal hyphae also occurs in the presence of toxins or unfavorable environmental conditions such as intolerable pH, and high levels of heavy metals and salts.

2.4 Biochemistry of Citric Acid Production by *Aspergillus niger*

Citric acid or citrate, the ionic form of citric acid, is a major intermediate compound produced by the tri-carboxylic acid (TCA) cycle. A product resulting from the oxidation of glucose, namely glycolysis, pyruvate ($HO(CO)_2CH_3$) is the compound required to initiate the TCA cycle (Figure 3.3). Pyruvate must first be converted to acetaldehyde (CH_3COH) and then Acetyl CoA (Figure 2.3) to activate the TCA. The first intermediate compound produced by the TCA cycle is citrate ($C_3H_5O(COO)_3^{-3}$) which is the ionic form of citric acid ($CH_2HOC(COOH)_3CH_2$). In *A. niger*, the TCA cycle occurs in the matrix of the mitochondrion (a structure generating ATP and controlling cell growth).

Once formed, citrate or citric acid can be excreted out of the cell, if conditions prohibit the second stage of the TCA cycle or can continues within the cycle to finally generate Oxaloacetic acid (Oxaloacetate) and several compounds used to store energy such as ATP and NADPH (Dunn, 1998).

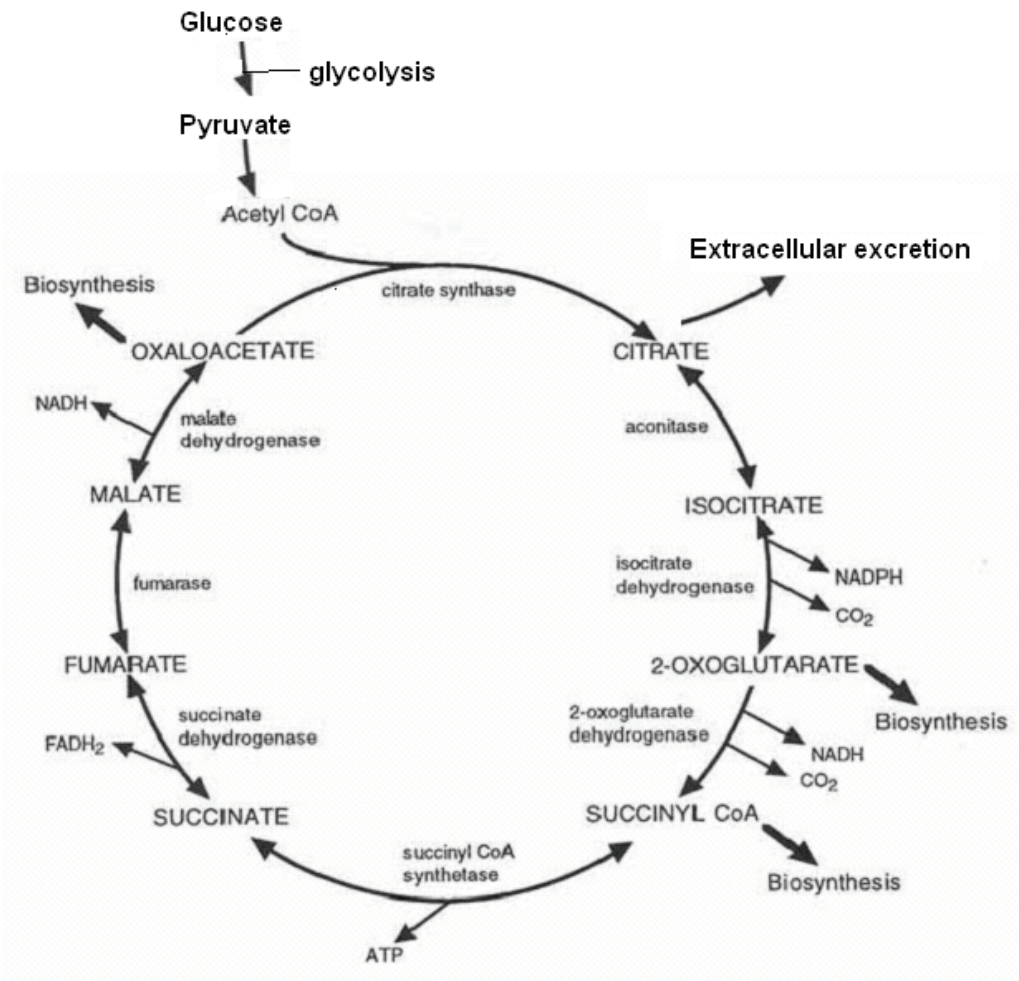

Figure 2.3 The Tri-carboxylic acid (TCA) cycle responsible for the production of citrate (the ionic form of citric acid). Citrate can be used as a major intermediate in the TCA cycle, or can be excreted outside the cell wall. If nutrients become limited, the citrate accumulated outside the cell wall can be reabsorbed and degraded within the TCA cycle by the enzyme aconitase with the eventual generation of Oxaloacetate and several compounds used to store energy such as ATP and NADPH (Dunn, 1998).

Several theories were proposed to explain the extracellular excretion of citric acid by *A. niger*. Citric acid excretion is said to drop the general pH of the environment in the vicinity of the hyphae to enable the fungus to biologically compete against its enemies and to enhance the mobility of compounds such as heavy metals and for phosphate (Sayer and Gadd, 2001).

According to Anastassiadis et al. (1993, 1994, 2001, 2002 and 2005), the intracellular accumulation of citric acid results from the inactivation of citrate degrading enzymes such as aconitase or isocitrate dehydrogenases. Ahmed et al. (1972) mentioned that the inactivation of these enzymes results from a low internal level of free amino acids. Nevertheless, a significant increase in cell protein level has been observed during citric acid accumulation (Jernejc et al., 1992). Accordingly, the TCA cycle produces major compounds required for biomass formation. In the TCA cycle, enzymes like mitochondrial isocitrate dehydrogenases, ά-ketoglutarate dehydrogenase, and succinate dehydrogenase are affected by NADH/NAD and NADPH/NADP ratios, cis-aconitate, oxaloacetate, and mitochondrial AMP. The in vivo effect of these factors is unknown (Chan et al., 1965; Meixner-Monori et al., 1985, 1986). The production of malate occurs prior to the accumulation of citrate (Ma et al., 1981; Röhr and Kubicek, 1981). The mitochondrial citrate carriers of *A. niger* accumulate citrate but the phenomenon still need more investigation (Papagianni, 2007).

2.5 Fermentation Technologies

2.5.1 Submerged fermentation

The commercial production of citric acid by filamentous fungi such as *A.niger* is mainly conducted using submerged fermentation techniques in which the growing solution contains sugars such as glucose or sucrose (Leangon et al., 2000; Kumar et al., 2003).

Submerged fermentation can be controlled to provide an optimal environment for the growth of filamentous and non-filamentous fungi. In this type of fermentation process, sterility of material can be controlled by maintaining the proper temperature, pH, dissolved oxygen, and mixing conditions such as speed of mixing, size of vessel, and fermentation volume. In the absence of mixing (static culture) for submerged fermentation, filamentous fungi form a mat of hyphae at the liquid surface (Carlile et al., 2001). When mixing is present, the culture is generally submerged in the solution and *A. niger* tends to develop pellets of hyphae, unless the mixing speed is too high and shears the hyphae away from each other. During the fermentation process, excessive mixing leads to cell structure damage, morphological changes, variations in growth rate and less efficient product formation (Papagianni, 2004).

2.5.2 Solid state fermentation

Solid substrate fermentation implies the use of a solid but porous matrix with a large surface area per unit volume. The solid substrate material can be biodegradable but must provide a suitable support for fungal growth and the exchange of gases. For citric acid production, oxygen acts as a direct regulator. In a study measuring the effect of oxygen tension (DOT)

on oxygen uptake and acid formation, DOT values of 18-21 and 23-26 mbar were found optimal for triphophase and idiophase production, respectively (Kubicek et al., 1980).

Typical examples of solid substrate fermentation techniques are: the Koji process which is the fermentation of steamed rice by *Aspergillus oryzae* to produce soy sauce; the Indonesian Tempeh and Indian ragi which is a meal of steamed legumes seeded with a non toxic mould; the composting of lignocellulosic fibers which are naturally contaminated by bacteria, moulds and *Streptomyces sp*., and; the famous French blue cheese which is fresh cheese perforated to allow for the growth of the fungus *Penicillium roquefortii*.

As compared to submerged fermentation, solid substrate fermentation offers many advantages:

1) limited consumption of water;

2) low heat transfer capacity with easy aeration;

3) high surface exchange air/substrate;

4) low energy consumption for the process;

5) lower water usage and wastewater treatment costs;

6) limited contamination risks because of a lower moisture level;

7) simplified nutrient media composition.

On the other hand, the disadvantages of solid state fermentation are:

1) difficulty in controlling process parameters such as pH, heat and nutrient conditions;

2) problems with heat build-up;

3) higher product impurity level;

4) solid waste to manage, including that of the fungal biomass;

5) higher product recovery costs.

In recent years, many different types of substrate have been used in solid state fermentation: coffee husk, apple pomace, wheat straw, pineapple waste, cassava baggasse, banana peel, sugar beet, kiwi fruit peel, and corn cob (Ngadi and Correia, 1992; Wang, 1998; Gutierrez-Correa et al., 1999). These substrates were also used to produce commercial compounds such as: enzymes, fine chemicals, microbial biomass, and secondary metabolites. As solid substrates are mainly agricultural wastes, solid substrate fermentation can transform a waste residue into value-added products.

2.6 Medium supplementation for citric acid production by *Aspergillus niger*

Solid substrate fermentation requires the addition of nutrients to maximize the yield of citric acid and the growth of fungi, such as *A. niger* (Krishna, 2005). Nutrients are generally classified as macronutrients (carbon, oxygen, hydrogen, nitrogen, phosphorus, potassium, sulfur, and magnesium) and micronutrients (manganese, iron, zinc, copper, and molybdenum).

2.6.1 *Glucose*

Filamentous fungi are heterotrophic organisms, meaning that they require as energy and carbon source, organic compounds such as glucose, fructose and sucrose. Other compounds such as polysaccharides, amino acids, lipids, organic acids, proteins, alcohols and hydrocarbons can also be used by fungi (Krishna, 2005). The preferred carbon source for *A. niger* is sugar because this substrate can be oxidized directly into pyruvate which is responsible for initiating the TCA cycle. The sugar was reported for citric acid production by *A. niger* include maltose, sucrose, glucose, mannose and fructose. Sucrose is preferred

over other sugars, because *A. niger* produces an extracellular mycelium bound invertase that is active at low pH and rapidly hydrolyzes sucrose (Kubicek and Röhr, 1989).

In recent years, grape pomace, banana extract, sugar cane baggasse, sugar beet molasses, and apple peels have been used to produce citric acid using *A. niger* because of their high sugar content (Wang, 1998; Wayman and Mattey, 2000). These wastes also provide essential micronutrients.

During glycolysis, an excess of glucose is known to lead to the over-production of citric acid (Leangon, et al., 2000), while a low glucose level may lead to the accumulation of oxalic acid. In the mitochondrion, citrate results from the condensation of oxaloacetate with the acetyl CoA enzyme. If the TCA cycle cannot be completed because of a lack of enzymes, citrate is excreted, and then oxaloacetate is produced from pyruvate as a result of pyruvate carboxylase. Also, limitation of carbon can cause cell aging and autolysis in fungal culture, which leads to fragmentation and heavily vacuolated hyphae (Papagianni, 2004) which ultimately affects citric acid production.

Initial glucose concentration is therefore a key factor in citric acid production when a solid substrate is used. Glucose was found to be unavailable at a concentration of 100-250 g/Kg of dry peat moss as solid substrate (Kim et al. 2006). Initial glucose level must be balanced with nitrogen ratio for fungal growth and a C:N ratio of 16 is found suitable for fungal biomass growth (Krishna, 2005). Shu and Johnson (1984) reported that high citric acid yields were obtained under submerged conditions with initial glucose concentrations of 14-22%, by far exceeding the C:N ratio of 16.

2.6.2 *Nitrogen*

In the nutrient medium for solid substrate fermentation, nitrogen is generally supplied as nitrate, ammonia or amino acids (Krishna, 2005). The most suitable sources of nitrogen are yeast extracts, peptone, ammonium sulphate, ammonium chloride and ammonium nitrate. Ammonium is known to lower the pH of the medium as well as that of the cell (Xu et al., 1989). The lack of nitrogen during fermentation is said to limits the growth of *A. niger* and enhance citric acid production (Mirminachi, et al. 2002).

2.6.3 *Phosphorous*

Phosphorous plays an important role in fungal morphology. It is the main component of ATP, a compounds used to accumulate and transport energy within cells. Phosphorous is also involved in cell multiplication and metabolite production. It may be supplied as KH_2PO_4 or K_2HPO_4 (Abou-Zeid and Ashy, 1984) and the range of 0.06 to 0.32 g/l was found to optimize citric acid yields (Shu and Johnson, 1984). The excess concentration of exogenous phosphorous can induce low yield of citric acid.

2.6.4 *Stimulators*

In recent years, stimulators such as organic solvents (ethanol, methanol), phytate, and vegetable oils were found to enhance the production of citric acid using *A. niger* grown on solid substrates. These stimulators were found to increase the permeability of cell walls, decrease cell growth and change enzyme activity. A key enzyme associated with the TCA cycle, ethanol is known to change the citrate synthetase activity, besides being assimilated by *A. niger* as an alternative carbon source (Jianlong and Ping, 1998). By increasing the

permeability of cell membranes, ethanol enhances the transfer of nutrients and compounds including citric acid excretion outside the cell wall (Hang et al., 1987).

2.7 Factors affecting solid substrate fermentation

Citric acid production by *A. niger* is greatly influenced by parameters such as moisture, pH, temperature, mechanical shaking, inoculum density and the solid substrate particle size. For high citric acid yields, all these parameters must be optimized (Jianlong, 1998).

2.7.1 *pH*

A pH ranging from 2 to 6 is considered adequate for solid substrate fermentation, as fungi grow well in slightly acidic conditions. Some investigators have reported that specific fungi can grow at a pH below 2 to compete against bacteria (Fawole and Odunfa, 2003). A low pH inhibits the production of unwanted organic acids such as gluconic acid and oxalic acid. Increasing the pH to 4.5 during the production phase reduces the final yield of citric acid by up to 80% (Papagianni, 1995).

2.7.2 *Temperature*

Most fungi produced a higher yield of citric acid at temperatures ranging between 25 and 35^{o}C. Higher temperatures lead to enzyme denaturation and moisture loss while lower temperatures lead to reduced metabolic activity (Adinarayana et al., 2003). Fawole et al. (2003) reported that a temperature of 30^{o}C was optimum for metabolite production by *A. niger*. The fungal cells showed signs of adverse growth and metabolic production if an ideal temperature was not provided during fermentation (Ellaiah et al., 2003).

In solid state fermentation *A. niger* grows under conditions which are closer to its natural environment. Considering this important fact, *A. niger* may be more capable of producing certain enzymes and metabolites which usually are produced at low yield in submerged cultures (Szewczyk and Myszka, 1994).

2.7.3 *Substrate medium particle size*

The medium used in solid substrate fermentation must provide a large surface area for surface fungal attachment and a suitable porosity for oxygen and carbon dioxide diffusion and heat transfer. A particle size ranging from 0.2 to 1.0 mm has been considered as optimal for gas diffusion and fungal hyphae growth (Ellaiah, 2003).

2.8 Bioremediation of heavy metal using citric acid

Many strains of fungi are able to produce organic acids which can be used as an efficient chelating agent for heavy metals. The organic acids that are produced by selected fungi such as *A. niger* contain a number of carboxyl groups which donate protons (H^+) and become negatively charged, binding heavy metals such as copper and zinc adsorbed on by soil particles (Sayer and Gadd, 2001). Being relatively milder than EDTA and sulphuric acid, citric acid produced by *A. niger*, can leach heavy metals while leaving behind soil macronutrients such as calcium and potassium (Wasay, et al., 2001).

2.9 Measurement of fungal biomass in solid state fermentation

Citric acid production is governed by fungal biomass growth and its measurement is fundamental in understanding the fermentation process. Mitchell et al. (1991) reported a

direct method to recover fungal biomass using a membrane filter which prevents the penetration of fungus hyphae into the liquid substrate. The whole fungal mycelium can be recovered and directly weighed. This measurement cannot be used with solid substrate fermentation SSF) because the hyphae must penetrate the medium in order to access nutrients (Raimbault 1998).

Accordingly, indirect measurement methods were developed such as dry mass monitoring, CO_2 production and O_2 consumption (Prado et al., 2005), chitin concentration (Narahara et al. 1982), nucleic acid concentration (Koliander et al. 1984), nutrient consumption (Matcham et al., 1984) and protein concentration. Other methods involve the measurement of biological activity such as that of enzymes (Barak and Chet, 1986) and ATP (Brezonik et al., 1975).

2.10 Conclusions

The comprehensive literature review presented here demonstrated that the C:N ratio offered by the solid substrate can have a marked effect on blocking the TCA cycle and the subsequent citric acid accumulation. Because the TCA cycle provides the energy required for fungal biomass growth, its inhibition can be monitored in terms of fungal biomass growth. Fungal biomass growth must therefore be monitored along with citric acid accumulation, to evaluate the impact of different nutrient levels on the fermentation process. In SSF, fungal biomass monitoring by simply keeping track of organic N and C is complicated by the substrate which is offers an increased amount of N and C. Therefore, there is a need to develop a fungal biomass monitoring technique for SSF. Although levels of organic C and N can provide an estimate of fungal biomass, their evaluation can be complicated by that offered by the solid substrate, in quantities exceeding by far the fungal biomass.

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CHAPTER 3

Effect of sugar and nitrogen on citric acid and biomass of *Aspergillus niger*

3.1 Abstract

Several applications require the optimization of citric acid produced by *Aspergillus niger* grown on a solid bed of sugar rich waste. This project measured fungal biomass and citric acid accumulation as influenced by available sugar and nitrogen. A. *niger* ATCC 12846 was cultured on expanded clay particles (HSS), wetted with 55 ml of a standard nutrient solution containing 0 to 475 g glucose (kg HSS) $^{-1}$ and 2 to 16 g NH_4^+-N (kg HSS)$^{-1}$. All samples were autoclaved, inoculated with *A. niger* except for the controls and incubated at 30°C for 168 h. Fungal biomass was equated to the total volatile solids (TVS) less residual glucose and accumulated organic acids. Fungal biomass and citric acid accumulation in the medium were significantly affected by the glucose and NH_4^+-N concentration ($P < 0.1$). The highest citric acid accumulation in the medium of 52 g (kg HSS)$^{-1}$ and yield of 14 % were obtained with 475 and 250 g of glucose (kg HSS)$^{-1}$ respectively, supplemented with 8 g of NH_4^+-N (kg HSS)$^{-1}$ after 144 and 96 h of incubation. Only 475 g glucose (kg HSS)$^{-1}$ resulted in citric acid being accumulated in the medium after reaching a peak fungal biomass. The high C:N ratio of the fungal biomass lead to the conclusion that medium water content restricted the diffusion of organic acids and its accumulation outside the fungal hyphae. Otherwise, a citric acid yield of 65% was likely with 250 g glucose (kg HSS)$^{-1}$ and 8 g NH_4^+-N (kg HSS)$^{-1}$. Future research should include a procedure for citric acid extraction from the hyphae.

Keywords *Aspergillus niger*, solid state fermentation, fungal biomass, citric acid, glucose, ammonium.

3.2 Introduction

Mass produced mainly by the filamentous fungus *Aspergillus niger*, citric acid is used by the food, beverage and pharmaceutical industries (Xie et al. 2006). Whereas submerged fermentation is the preferred industrial process for the production of citric acid using *A. niger*, recent interest in the valorization of sugar rich wastes shifted the focus towards solid state fermentation (Soccol et al. 2003). In solid state fermentation, the sugar rich substrate is non soluble and acts both as nutrient source and support medium for growth (Pandey et al. 2000).

Citric acid is an intermediate product of the Tri-carboxylic acid (TCA) cycle. This cycle is initiated in the presence of pyruvate produced from the glycolysis or oxidation of sugars such as sucrose, glucose and fructose. Within the fungal cell, the TCA cycle is the main process generating ATP (Adenosine Tri-phosphate) used to transport energy, and NAD (Nicotinamide adenine dinucleotide) an enzyme used in redox reactions such as the oxidation of sugars, and in the transport of electrons (Moore-Landecker 1996). Within the TCA cycle, citric acid is an intermediate metabolic compound unless accumulated and excreted outside the fungal cell wall (Papagianni 2007), when the TCA cycle is inhibited by: a lack of enzymes resulting from a shortage of nutrients such as nitrogen; by enzyme inactivation as a result of low pH conditions, and; by insufficient nutrient for growth in the presence of an abundant sugar supply. Jernecj et al. (1992) observed that optimal citric acid production occurred when the fungal mycelium stopped accumulating lipids and nucleic acid, resulting in large changes in intra- and extra-cellular proteins and amino acids, and intra-cellular TCA cycle acids.

Sugar type and concentration are known to have a marked effect on citric acid production. For submerged conditions, the initial sucrose or glucose concentration must be in the range of 10 and 7.5 %, respectively, and such high concentrations generally lead to a lag phase in fungal mass growth of 12 to 18 h, as well as a 20 % drop in growth rate (Xu et al. 1989). Optimal citric acid production is achieved under submerged conditions when the solution pH falls to 2.0, within 2 hours of spore germination. Nevertheless, spore germination is optimized at a pH of 5.5 (Papagianni 1995). In Japan, citric acid is commercially produced by growing *A. niger* on wheat bran with an initial pH adjusted between 4 and 5 (Smith 1994). Dawson et al. (2004) reported that citric acid production was inversely proportional to solution nitrogen concentration and fungal mass growth rate. Ammonium is the preferred form of nitrogen because its rapid depletion by the fungus within 24 to 36 h is required for pH drop and citric acid accumulation (Soccol et al. 2006; Papagianni 2005; Dawson et al. 2004).

Accordingly, fungal biomass growth must be investigated along with citric acid production, because citric acid is effectively produced in the presence of substantial amounts of sugar, when fungal biomass is slowed down as a result of TCA cycle inhibition. With solid substrate fermentation, monitoring fungal biomass growth is complicated by the presence of substances found in both the fungus and the medium. Several indirect methods were used in the past to determine fungal biomass growth during liquid state fermentation. Prado and Vandenberghe (2004) and Prado et al. (2005) used the online measurement of CO_2 production and O_2 consumption, which requires a correlation between gas production and fungal biomass growth and assumes that the relationship is constant (Koutinas et al. 2002). Narahara (1982) quantified chitin concentration and Koliander (1984) measured

nucleic acids concentration to determine fungal biomass, but this technique does not apply to solid state fermentation as many medium also contain these compounds.

The objective of the present study was therefore to measure the effect of sugar and nitrogen concentration on fungal biomass and citric acid accumulation, using solid state fermentation and *A. niger*. The solid medium was inert to measure without interference, fungal biomass growth in terms of changes in total volatile solids (TVS) and organic N.

3.3 Materials and methods

3.3.1 Experimental materials

In the present study, *Aspergillus niger* (ATCC 12846, NRRL 567, van Tieghem, anamorph) was obtained from the American Type Culture Collection *(ATCC, Manassas, VA, USA*) and stored at -76 ^{o}C, in test tubes containing 30 % glycerol on a volumetric basis. This strain of *A*. *niger* ATCC 12846 was previously used to produce citric acid, gluconic acid and L-malic acid from different substrates such as apple and grape pomace (Hang and Woodams 1985).

The fungus was grown on potato dextrose agar (PDA, Sigma, St-Louis, MO, USA) plates at 30^{o}C and sub-cultured at biweekly intervals. After 7 to 10 days of incubation on PDA plates, 10 ml of 0.1 % Tween 80 (Sigma, St Louis, MO, USA) solution was added to each plate, to collect the spores and dilute the suspension to 1.0×10^{7} spores ml^{-1}. The spore concentration of the inoculum was verified by microscopic count using a hemocytometer.

Expanded clay balls (Hydroton®, Hydromax, Montréal, Canada) were used as inert solid substrate. Produced by firing clay at 1200 ^{o}C, Hydroton® has a pH of 7.0 and is used world wide as plant hydroponics support medium. To provide a large surface area for fungal hyphae growth and water absorption, the expanded clay balls were crushed and sieved to

obtain a particle size of 0.4 mm. Because Hydroton® is a mineral, fungal biomass could be monitored as total volatile solids (TVS) with minimal interference from the solid substrate. In this paper, the dry mass of Hydroton® (HSS) will be used to express nutrient supplementation, fungal biomass and citric acid accumulation.

The basic nutrient solution used to grow *A. niger* ATCC 12846 was prepared according to Kim et al. (2006) and contained per L of distilled water: 68, 137, 190 or 260 g glucose; 1.1, 2.2, 4.4, 4.9, 6.5 or 8.7 g of N as $(NH_4)_2SO_4$; 22 g KH_2PO_4; 2 g NaCl; 2 g $MgSO_4.7H_2O$, and; 2 g $FeSO_4.7H_2O$. A volume of 55 ml of solution was added to 30 g samples of autoclaved HSS, to obtain glucose concentrations of: 0, 125, 250, 350, and 475 g (kg HSS) $^{-1}$, and NH_4^+-N concentrations of: 2, 4, 8, 9, 12 and 16 g (kg HSS) $^{-1}$.

3.3.2 Methodology

Samples of HSS weighing 30 g were wetted with 55 ml of nutrient solution to obtain the right nutrient level, autoclaved at 121 ^oC for 30 min and then inoculated with 1 ml of *A. niger* ATCC 12846 inoculum, except for the control samples. All inoculated and control samples were weighed again and incubated in a shaking incubator (110 rpm) at 30 ^oC for 168 h. After 0, 48, 96, 120, 144 and 168 h of incubation, triplicate inoculated and control samples were sacrificed, weighed and kept at -4^oC until analyzed for pH, TKN (total Kjeldahl nitrogen), NH_4^+-N, TVS, residual glucose and organic acids namely citric acid, L-malic acid, gluconic acid. The control HSS samples (samples without inoculants) were analyzed to check for possible contamination and extraction efficiencies.

A first set of inoculated HSS samples was tested with the full range of glucose and 8 g NH_4^+-N (kg HSS) $^{-1}$. Then, the test was repeated using in turn, 2, 4, 12 and 16 g NH_4^+-N (kg HSS) $^{-1}$ combined with 0, 125, 250, 350, 475 g glucose (kg HSS) $^{-1}$.

Citric acid yield (Ca_y) was calculated based on peak citric acid accumulation (Ca_a, g (kg HSS) $^{-1}$) and initial nutrient solution glucose (G_c, g (kg HSS) $^{-1}$):

$$Ca_y\ (\%) = 100 \times [Ca_a / G_c\ ] \quad (1)$$

During each test, fungal biomass was measured with time by measuring organic C, as TVS, less residual glucose and accumulated organic acids:

$$\textit{Fungal biomass (g) = TVS (g) - Residual glucose (g) – Accumulated citric acid (g)} \quad (2)$$

The change in organic N with fermentation time was also computed as organic N (Total Kjeldahl nitrogen or TKN) less NH_4^+-N:

$$\textit{Fungal biomass N (g) = TKN (g) - ammonium-N (g)} \quad (3)$$

Equations (2) and (3) were used to compute the C: N ratio of the fungal biomass, where the fungal biomass C was obtained by dividing TVS by 1.83 (Barrington et al. 2002).

3.3.3 Analytical procedures

All analyses were conducted according to standard methods (AOAC 2002). Moisture content was determined by drying at 70^{o}C for 24 h (Haq et al. 2001). Total volatile solids were determined after drying by incinerating at 500°C for 4 h (Haq et al. 2001).

Total Kjeldahl nitrogen was determined by digesting 0.5 g samples with sulfuric acid and hydrogen peroxide, at 500^{o}C for 10 minutes, and by quantification after adjusting the pH to 13, using an ammonia sensitive probe connected to a pH meter (Coroning pH/ion meter 450, coroning, NY, 14831, USA). The NH_4^+-N content of each sample was determined by soaking samples in distilled water, adjusting their pH to 13, and also quantifying using an ammonia- sensitive probe.

Organic acids (citric, gluconic, and L-malic acids) were determined after preserving the fermented samples at -4^{o}C with a mercuric chloride solution (10 mg L^{-1}) (Blake and Clarke 1987). After defrosting in a hot air bath at 30 ^{o}C, 1.0 g samples were soaked in 20 ml of 0.1 M HCl to extract the organic acids from HSS (Roser et al. 1994) and centrifuged at 3000 rpm for 30 minutes, after agitating for 60 min at ambient temperature. The supernatant was removed and filtered using initially a filter paper (Whatman No. 54) and then a 13 mm syringe with a 0.2 μm nylon filter. Quantification was conducted using an HPLC (Agilent 1100 chromatograph, Santa Clara, USA) with a diode array detectors equipped with a Phenomenex Resex ROA column 300x7.8 mm, 8 μm (Bio-Rad, Canada) and 0.02 N H_2SO_4 as mobile phase. A flow-rate of 0.5 ml min^{-1} and an injection volume of 10 μL were used. For calibration, HPLC-grade organic acids were purchased (Fisher Scientific, Montreal, Canada).

Glucose was measured according to Miller (1959), after homogenizing 2.0 g samples with distilled water (HPLC grade). The samples were heated for 25 min at 55 ^{o}C and centrifuged at 3000 rpm for 30 min, at room temperature. The supernatant fraction was cooled before filtering through a 13-mm syringe with a 0.2 μm nylon filter. All samples were refrigerated until analyzed using an HPLC (Agilent 1100 chromatograph, Santa Clara, USA) equipped with a 600E system controller, a differential refractometer R401, an auto sampler and a temperature control module. A Phenomenex Resex ROA column 300 x 7.8 mm, 8 μm (Bio-Rad, Canada) was used along with a 0.02 N H_2SO_4 solution mobile phase and a flow-rate of 0.8 ml min^{-1} with injection volume of 10 μL and a column temperature of 45 ^{o}C. For calibration, HPLC-grade glucose was used (Fisher Scientific, Canada).

3.3.4 Statistical analysis

Fungal biomass and citric acid accumulation for the different levels of glucose and nitrogen were compared using ANOVA through *SAS*® (SAS 9.1, SAS system, USA). To establish which level of glucose and nitrogen had a significance effect on fungal biomass and citric acid accumulation, Duncan's Multiple Range test was used.

3.4 Results

3.4.1 Glucose level and citric acid accumulation

With an initial nitrogen level of 8 g (kg HSS)$^{-1}$, citric acid accumulation was monitored over 168 h for initial glucose levels of 125, 250, 350 and 475 g (kg HSS)$^{-1}$ (Fig. 1). The non inoculated control samples produced no citric acid, maintained their initial glucose level, and maintained at time 0, their initial NH_4^+-N level, indicating no contamination or

absorption by HSS (results not shown). The control samples were observed to loose some of their initial NH_4^+-N because of volatilization. Finally, only citric acid was observed while gluconic acid and L-malic acid were not detected, during fermentation.

All initial glucose levels had a significant effect ($P<0.05$) on citric acid accumulation, except for treatments with 250 and 350 g glucose (kg HSS)$^{-1}$ where citric acid accumulation followed a similar concentration pattern. Peak citric acid concentration was delayed with higher glucose levels. With 125 g glucose (kg HSS)$^{-1}$, citric acid concentration peaked at 14 g (kg HSS)$^{-1}$ after 48 h of fermentation, while with 250 and 350 g glucose (kg HSS)$^{-1}$, citric acid concentration peaked at 35 g (kg HSS)$^{-1}$ after 96 h, and with 475 g glucose (kg HSS)$^{-1}$, citric acid concentration peaked at 52 g (kg HSS)$^{-1}$ after 144 h of fermentation. Only the treatment with 475 g glucose (kg HSS)$^{-1}$ demonstrated a 48 h lag phase before observing citric acid accumulation.

Glucose level therefore had a significant impact on the accumulation of citric acid in the medium and only the treatment with 475 g glucose (kg HSS)$^{-1}$ resulted in a lag phase, recognized in submerged fermentation as a prerequisite for high citric acid yields (Honecker et al. 1989). This is substantiated by the fact that as compared to 250 and 350 g glucose (kg HSS)$^{-1}$, the 475 g glucose (kg HSS)$^{-1}$ treatment produced a jump in citric acid accumulation in the medium, from 36 to 52 g (kg HSS)$^{-1}$.

All treatments lead to a drop in citric acid accumulation, once a peak value was reached, because of the batch nature of the experiment and as a resulted of glucose depletion. This phenomenon was also observed by several authors, such as Vaija and Linko (1986) while producing citric acid with *A. niger*, using a continuously fed reactor where nitrogen was supplied at a high rate of 0.3 g L^{-1}. This phenomenon was less obvious with a

nitrogen level of 0.05 g L^{-1} and absent with no nitrogen supplementation. Kontopidis et al. (1995) observed citrate to be taken up by fungal cells when the medium exhibited a pH rise.

Citric acid yield was also significantly influenced by glucose level ($P< 0.05$). The highest citric acid yield of 14 % was obtained with 250 g glucose (kg HSS)$^{-1}$. The citric acid yield with 125, 350 and 475 g glucose (kg HSS)$^{-1}$ reached 11, 10 and 11 %, respectively. Despite its much higher citric acid accumulation, the treatment with 475 g glucose (kg HSS)$^{-1}$ resulted in a lower yield because more sugar was supplemented initially. The same observation explains the large yield difference between the 250 and 350 g glucose (kg HSS)$^{-1}$, despite their similar citric acid accumulation in the medium.

3.4.2 Effect of nitrogen supplementation during fermentation

The effect of varying NH_4^+-N supplementation from 2 to 16 g (HSS)$^{-1}$, was tested for glucose levels varying from 125 to 475 g (HSS)$^{-1}$ (Figures 2 a, b, c, d and e). Nitrogen level had a significant effect on citric acid and fungal biomass accumulation ($P <0.10$), in combination with glucose level.

When using a nitrogen level of 2 g (kg HSS)$^{-1}$, maximum citric acid accumulation in the medium was achieved with a glucose level of 250 g (kg HSS)$^{-1}$, 48 h before reaching a peak fungal biomass of 125 g (kg HSS)$^{-1}$. In comparison, a glucose level of 125 g (kg HSS)$^{-1}$ gave almost as good a citric acid accumulation but with a low fungal biomass of 10 g (kg HSS)$^{-1}$. The treatments with 125 and 250 g glucose (kg HSS)$^{-1}$ yielded respectively, 9.5 and 4.8 % citric acid. The 350 g glucose (kg HSS)$^{-1}$ treatment was the least productive in terms of citric acid accumulation, but the most in terms of biomass. The treatment with 475 g glucose (kg HSS)$^{-1}$ produced a peak citric acid accumulation 48 h after reaching a peak in

fungal biomass, with a residual glucose concentration of 190 g (kg HSS)$^{-1}$ after 168 h of fermentation.

With 4 g nitrogen (kg HSS)$^{-1}$, the treatments using 350 and 475 g glucose (kg HSS)$^{-1}$ produced the most citric acid. Whereas the treatments with 250 and 350 g glucose (kg HSS)$^{-1}$ resulted in a simultaneous peak citric acid and biomass accumulation, that with 475 g glucose (kg HSS)$^{-1}$ resulted in a peak citric acid accumulation occurring 48 h after reaching a peak biomass level. Again, with 475 g glucose (HSS)$^{-1}$, 175 g glucose (kg HSS)$^{-1}$ remained after 168 h of fermentation.

As opposed to the treatments with 2 and 4 g nitrogen (kg HSS)$^{-1}$, that with 8 g resulted in much higher citric acid accumulation in the medium especially with an initial 475 g glucose (kg HSS)$^{-1}$ resulting this time in no residual glucose after 168 h of fermentation. Whereas glucose levels of 125, 250 and 350 g (kg HSS)$^{-1}$ produced peak citric acid accumulation and fungal biomass occurring simultaneously after 96h of fermentation, the treatment with 475 g glucose (kg HSS)$^{-1}$ accumulated a peak citric acid level 48 h after reaching a peak fungal biomass.

With 9, 12 and 16 g nitrogen (kg HSS)$^{-1}$, no citric acid accumulation and fungal biomass accumulation were observed for glucose levels of 125, 250 and 350 g (kg HSS)$^{-1}$. For the treatment with 475 g glucose supplemented with 9 and 12 g N (kg HSS)$^{-1}$, peak citric acid accumulation occurred simultaneously with peak biomass, a situation similar to that obtained with lower nitrogen and glucose levels. With 16 g nitrogen (kg HSS)$^{-1}$, an inhibitory effect was observed as no citric acid and biomass where measured during the entire 168 h of fermentation.

3.4.3 Medium pH and fungal biomass C: N ratio

Once wetted with the nutrient solution, the pH of the medium dropped from 7.0 to 4.5 (Fig. 3a). Then, during fermentation, the medium pH dropped even further to 2.8 - 3.5, and in direct relationship with the accumulation of citric acid (Fig. 3b). Despite the fact that the medium pH dropped with citric acid accumulation, this drop was not sufficient to achieve a level of 2.0 within 2 h of initiating fermentation, as suggested to optimize citric acid production with liquid state fermentation.

Computed using Equations (2) and (3), fungal biomass C:N ratio is illustrated in Fig. 4a and b for the various nitrogen and glucose combinations. Fermentation time and nutrient solution initial C:N ratio both had a significant effect the fungal biomass C:N ratio which peaked simultaneously with fungal biomass level. Also, all fungal C:N ratio were unusually high at ratios varying from 1:50 up to 1:180, except for the treatment with 475 g glucose (kg HSS)$^{-1}$ in the presence of 9 and 12 g N (kg HSS)$^{-1}$, where this ratio reached 200 and 250:1. Fungal biomass generally exhibits a C:N ratio in the range of 12 to 13 (Barbhuiya et al. 2004). These results indicate that citrate was accumulated inside the fungal biomass rather than being excreted outside the cell wall, despite its high diffusion rate (Papagianni 2007). Despite the fact that sample containers were covered, moisture was observed to evaporate during fermentation lowering the medium moisture content, likely resulting in pH differences and citrate accumulation outside the cell wall. This also explains the drop in citric acid accumulation in the medium after reaching peak levels of fungal mass, because local pH differences may have resulted in citrate diffusing back into the hyphae.

3.5 Discussion

This project investigated the effect on citric acid and fungal biomass, of glucose and NH_4^+-N supplementation during the fermentation of *A. niger* ATCC 12846 on an inert solid substrate wetted with a nutrient rich solution. Citric acid is known to be optimized in the presence of an abundant supply of sugar and a large biomass of *A. niger* where conditions are such that the fungal TCA cycle is inhibited immediately after the first step responsible for the formation of citric acid. Under such conditions, citric acid is excreted by the fungal biomass. TCA cycle inhibition occurs when a lack of nitrogen prevents the formation of enzymes and the enzymes are inactivated because of fungal cytoplasm acidification. When monitoring citric acid production, it is also recommended to check for L-malic acid which is an intermediate in citric acid biosynthesis (Goldberg et al. 2006).

In the present experiment, citric acid accumulation in the medium and *A. niger* growth were significantly affected by glucose while NH_4^+-N supplementation while no L-malic acid was detected. Higher glucose levels delayed peaks in citric acid accumulation in the medium and NH_4^+-N supplementation controlled the time interval between peak biomass and citric acid accumulation. With low nitrogen and glucose levels, citric acid peaked before biomass while, with an intermediate nitrogen level and the highest glucose level of 475 g $(kg\ HSS)^{-1}$, citric acid accumulation peaked after biomass. This last condition is preferred for high citric acid yields. The highest citric acid accumulation peak in the medium of 52 g $(kg\ HSS)^{-1}$ and yield of 14% were obtained using 8 g NH_4^+-N $(kg\ HSS)^{-1}$ and 475 and 125 g glucose $(kg\ HSS)^{-1}$, respectively. The quantity of citric acid accumulated in the medium did drop its pH between 2.8 and 3.5.

The high calculated C:N of the fungal biomass was likely an indication that citrate, produced in excess by TCA cycle inhibition, was accumulated inside the fungal hyphae rather than diffuses into the extra-cellular medium. This is substantiated by the fact that peak C:N ratios coincided with peak fungal biomass levels. Generally, citrate diffuses quickly through the fungal cell membrane when there is a large pH gradient between the cytosol and the external medium (Papagianni 2007).

Fungal biomass typically exhibits a C:N ratio of 10 to 15 (Barbhuiya et al. 2004). If the fungal biomass values obtained in this project are corrected using a typical C:N ratio of 15, then, the highest yield of 65 % is obtained with 250 g glucose and 8 g NH_4^+-N (kg HSS)$^{-1}$.With 475 g glucose and 9 or 12 g NH_4^+-N (kg HSS)$^{-1}$, yields of 36 and 40% were likely. This corrected yield of 65 % is more in line with other research reporting 70 to 90 % yields (Hang and Woodams 1985; Vandengerghe et al. 2000; Xie and West 2006; Papagianni 2007) for submerged fermentation. The treatment with 475 g glucose and 9 or 12 g NH_4^+-N (kg HSS)$^{-1}$ was negatively affected by the fermentation conditions, because the peak fungal mass never exceeded 150 g (kg HSS)$^{-1}$, whereas for the other treatment combination, peak values of 250 g (kg HSS)$^{-1}$were obtained. Furthermore, adding moisture to the medium during fermentation could have improved citrate diffusion, and corrected localized pH differences. Vaija and Linko (1986) observed citric acid yields of over 70 % at an optimum pH ranging from 2.2 to 2.3, again for submerged conditions. Jernejc and Legisa (2004) observed that *A. niger* species capable of high citric acid yields and biomass growth, are not as effective at eliminating intra-cellular H^+ produced from the absorption of ammonium as N source and the resulting cytoplasmic acidification stimulates citric acid production.

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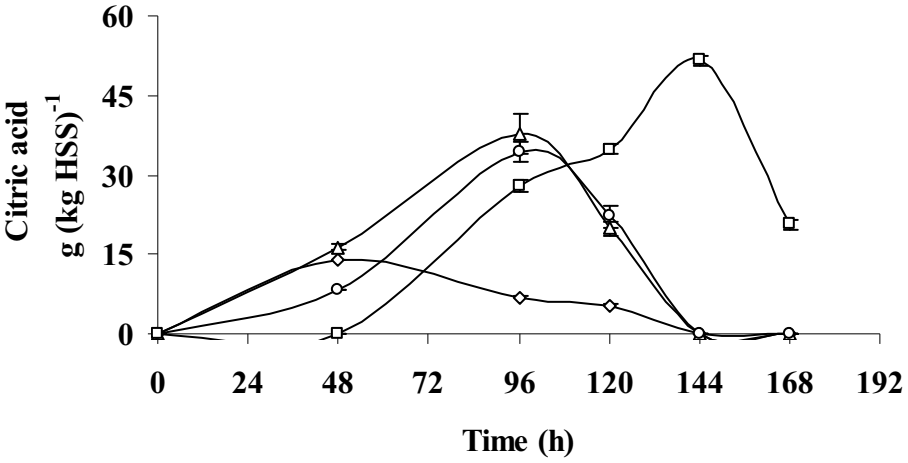
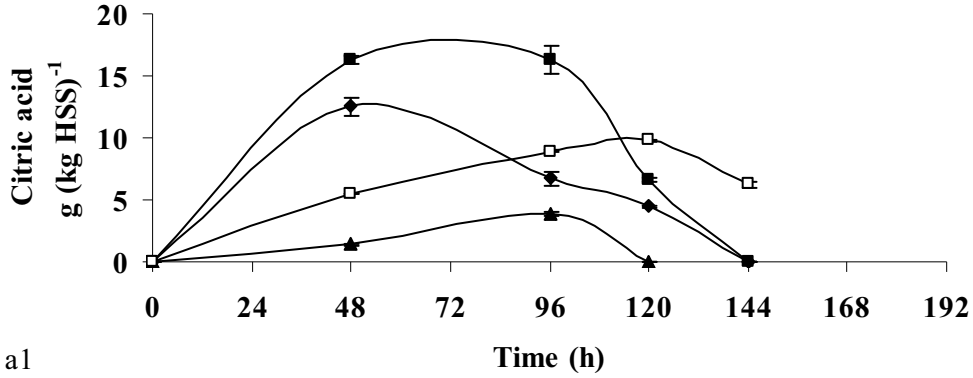


Fig.3.1. Effect on citric acid accumulation of glucose level at 125 (diamond), 250 (triangle), 350 (circle), and 475 (square) g (kg HSS)$^{-1}$.



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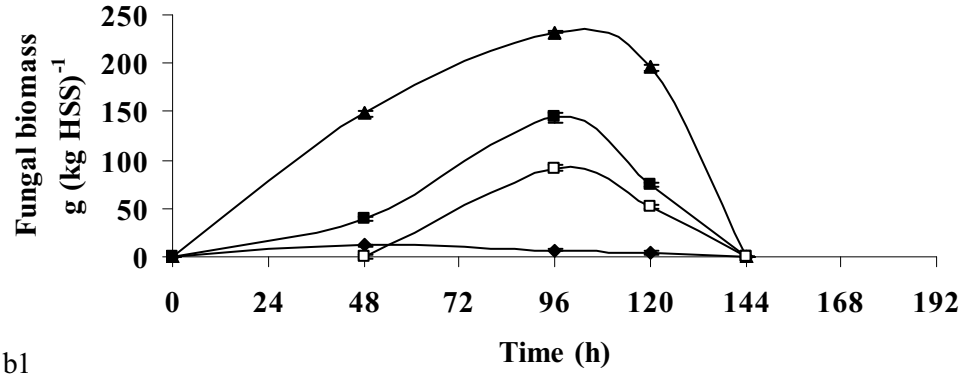

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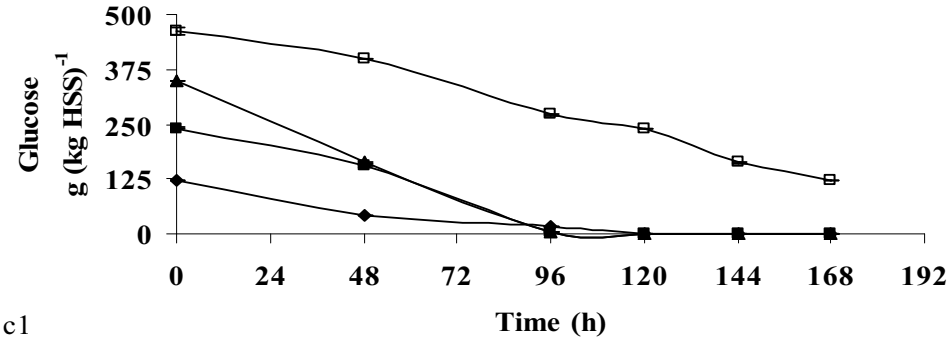
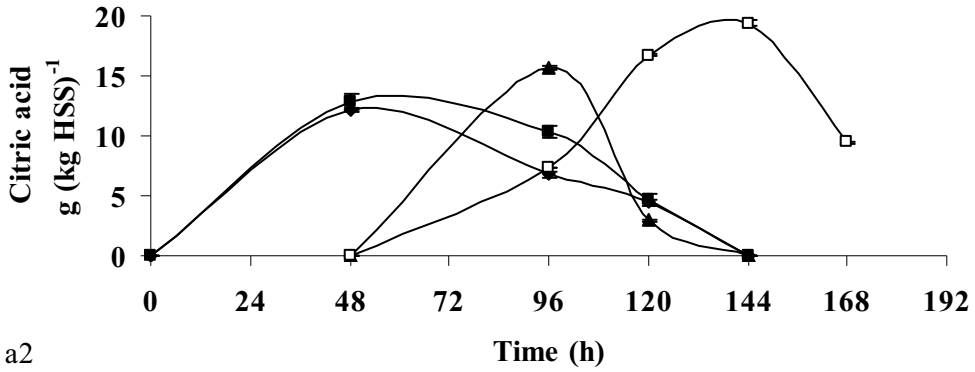


c1

Fig 3.2a. For 2 g of nitrogen (kg HSS)$^{-1}$ and glucose at 125 (Full diamond), 250 (Full square), 350 (Full triangle), and 475 g (kg HSS)$^{-1}$ (Open square): a1) citric acid; b1) fungal biomass, and; c1) residual glucose. The vertical bars indicate the standard deviation.



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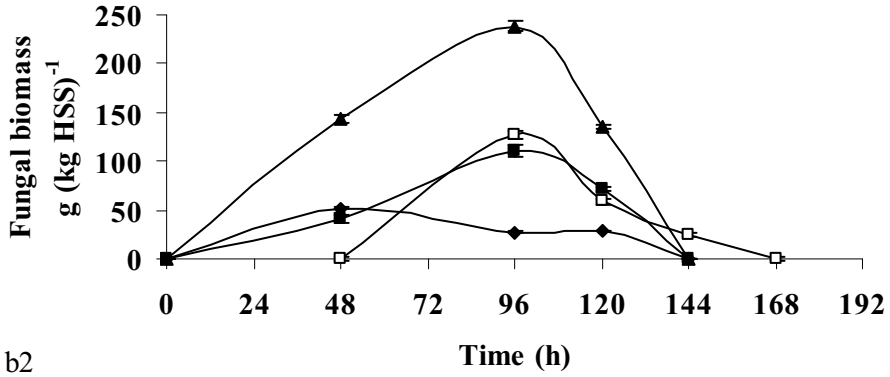

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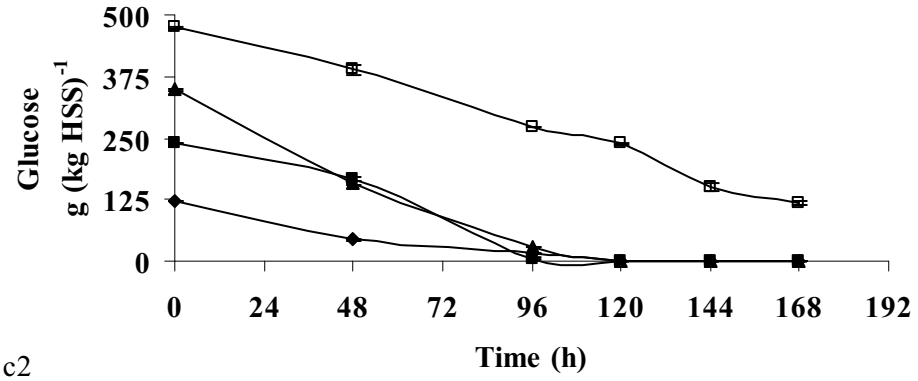
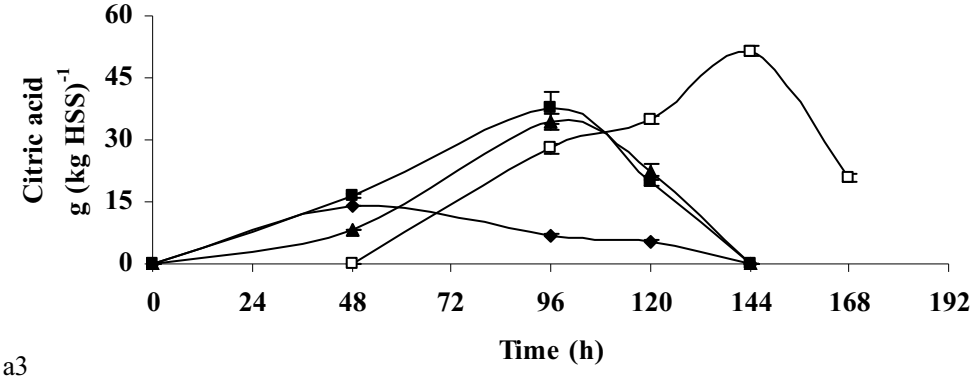


c2

Fig 3.2b. For 4 g of nitrogen (kg HSS)$^{-1}$ and glucose at 125 (Full diamond), 250 (Full square), 350 (Full triangle), and 475 g (kg HSS)$^{-1}$ (Open square): a1) citric acid; b1) fungal biomass, and; c1) residual glucose. The vertical bars indicate the standard deviation.



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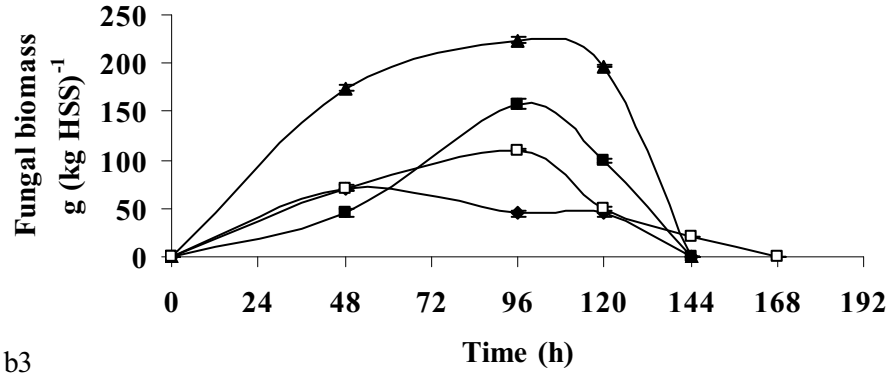

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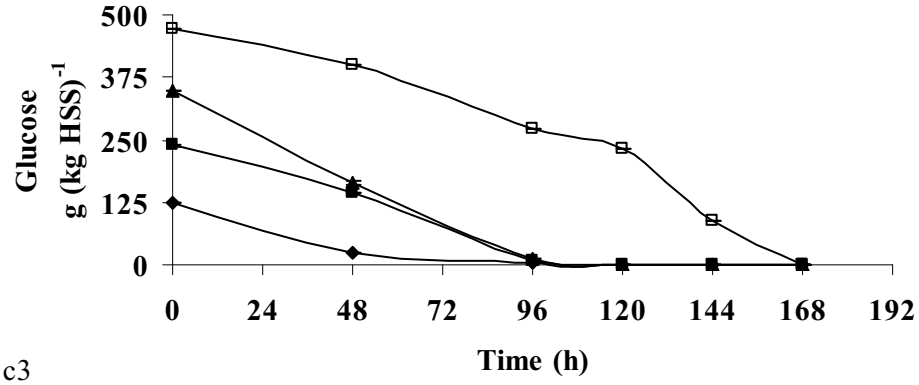
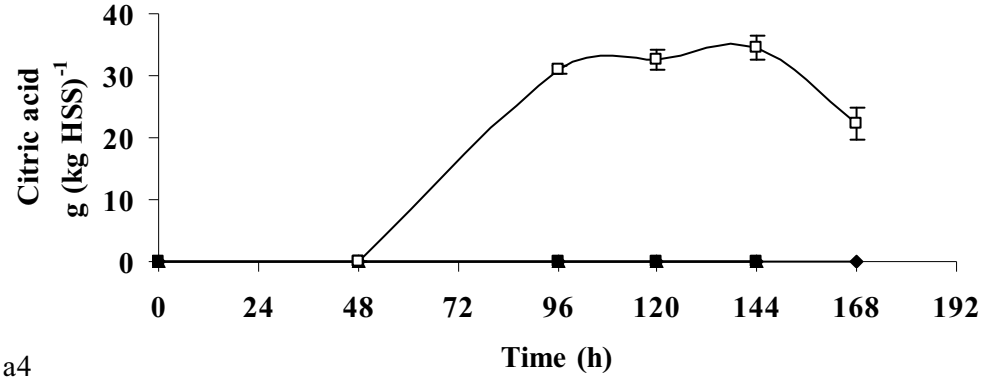


c3

Fig 3.2c. For 8 g of nitrogen (kg HSS)$^{-1}$ and glucose at 125 (Full diamond), 250 (Full square), 350 (Full triangle), and 475 g (kg HSS)$^{-1}$ (Open square): a1) citric acid; b1) fungal biomass, and; c1) residual glucose. The vertical bars indicate the standard deviation.



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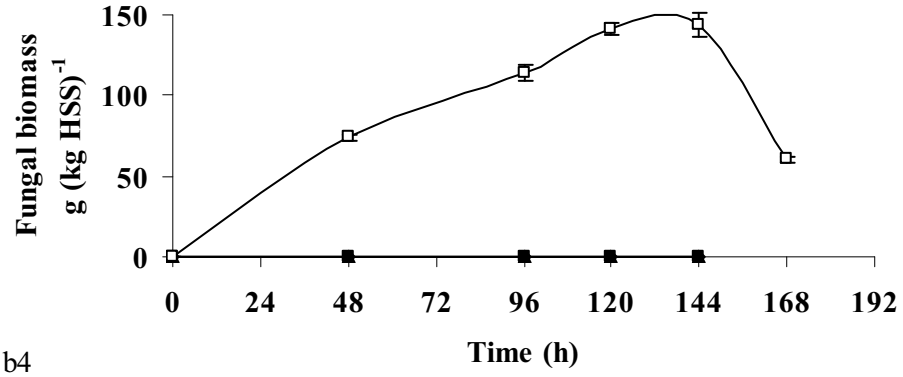

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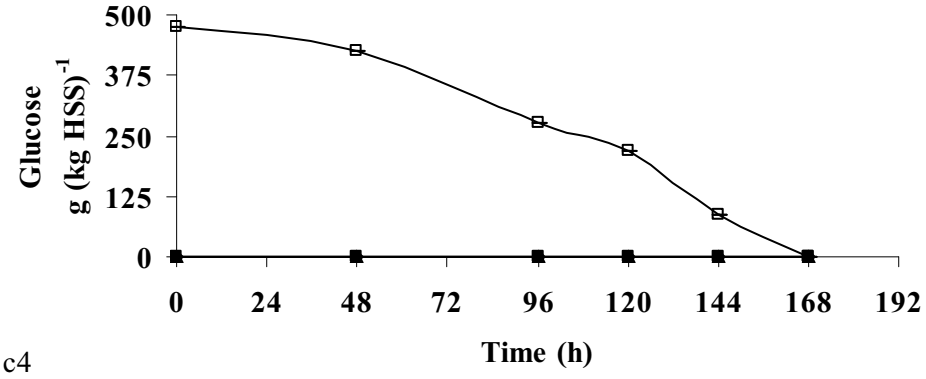
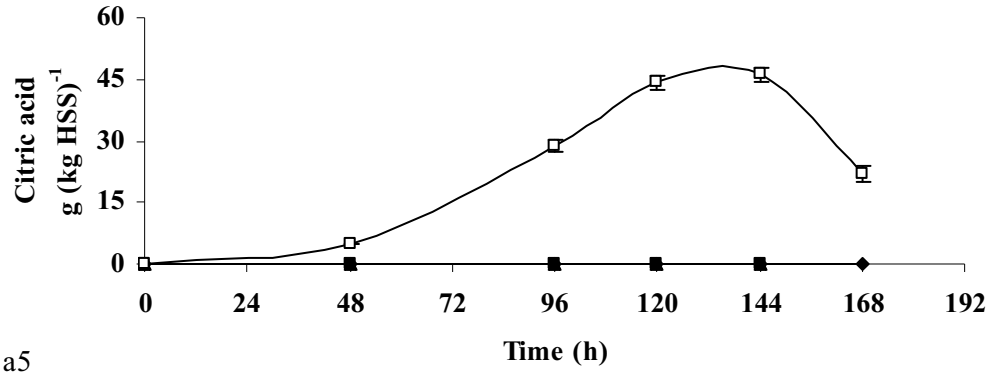
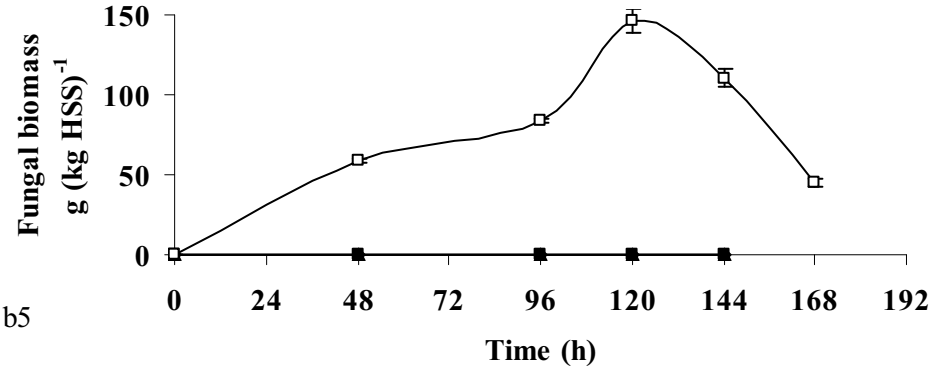


c4

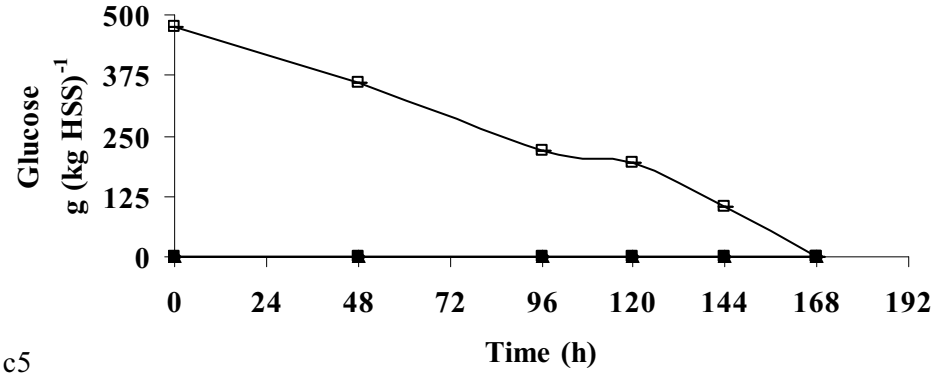
Fig 3.2d. For 9 g of nitrogen (kg HSS)$^{-1}$ and glucose at 125 (Full diamond), 250 (Full square), 350 (Full triangle), and 475 g (kg HSS)$^{-1}$ (Open square): a1) citric acid; b1) fungal biomass, and; c1) residual glucose. The vertical bars indicate the standard deviation.



a5

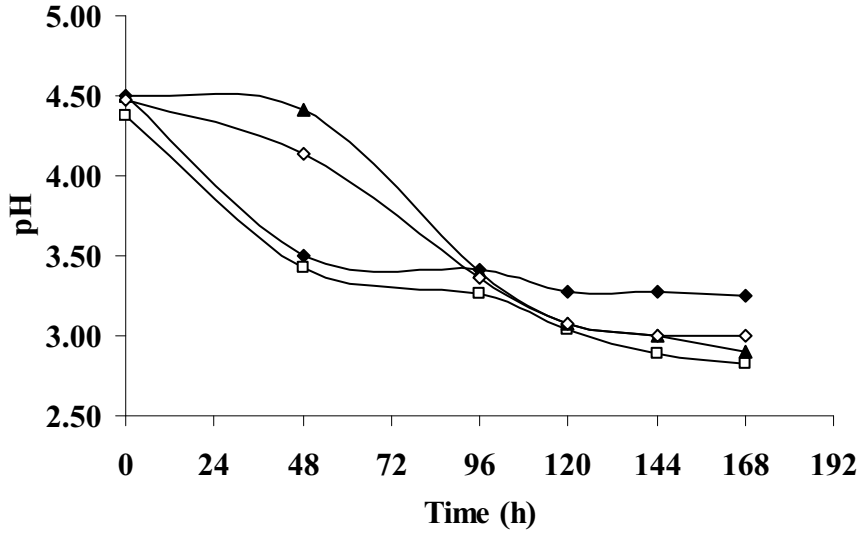


b5



c5

Fig 3.2e. For 12 g of nitrogen (kg HSS)$^{-1}$ and glucose at 125 (Full diamond), 250 (Full square), 350 (Full triangle), and 475 g (kg HSS)$^{-1}$ (Open square): a1) citric acid; b1) fungal biomass, and; c1) residual glucose. The vertical bars indicate the standard deviation.



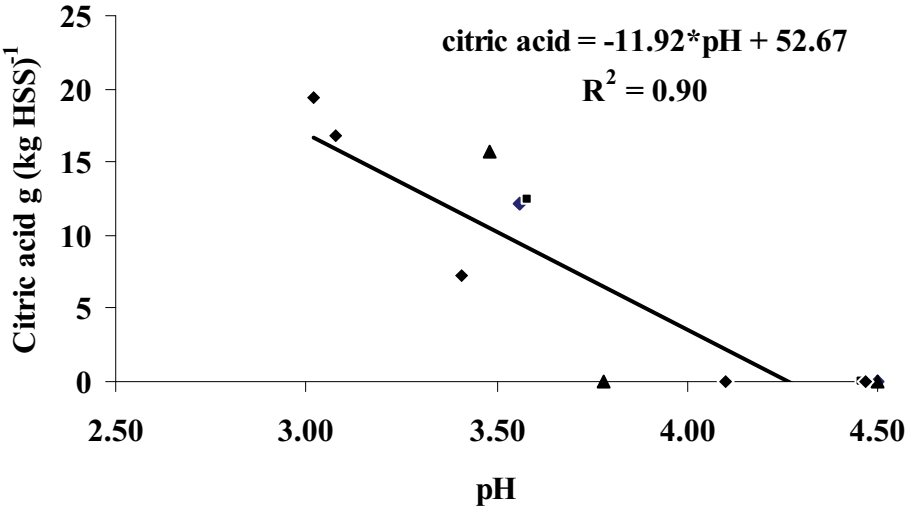
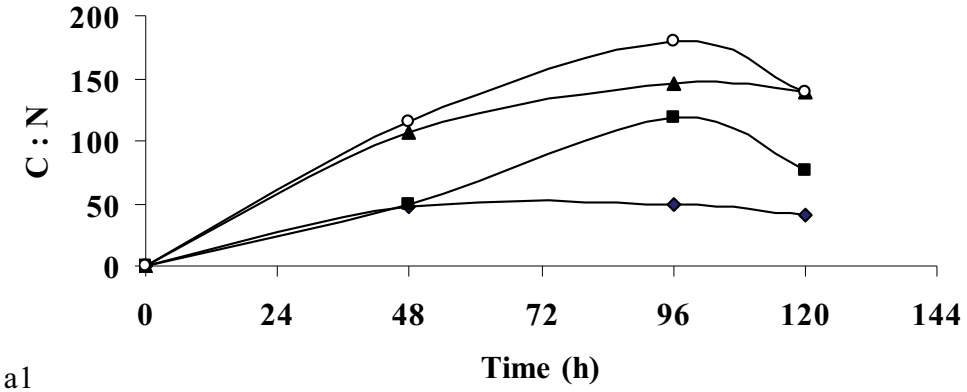
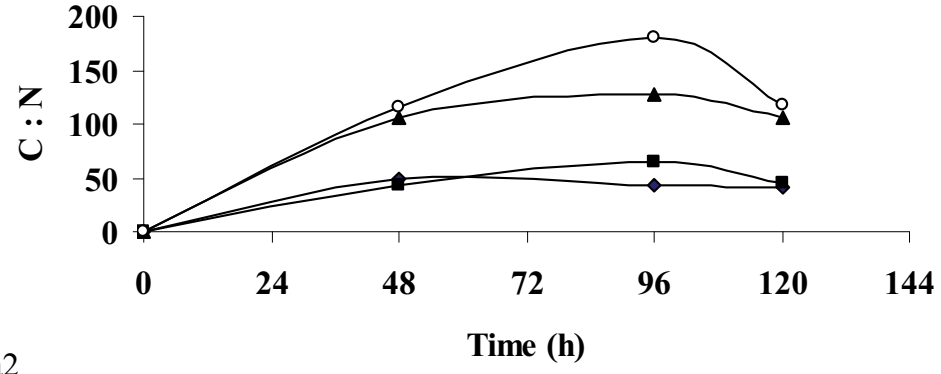


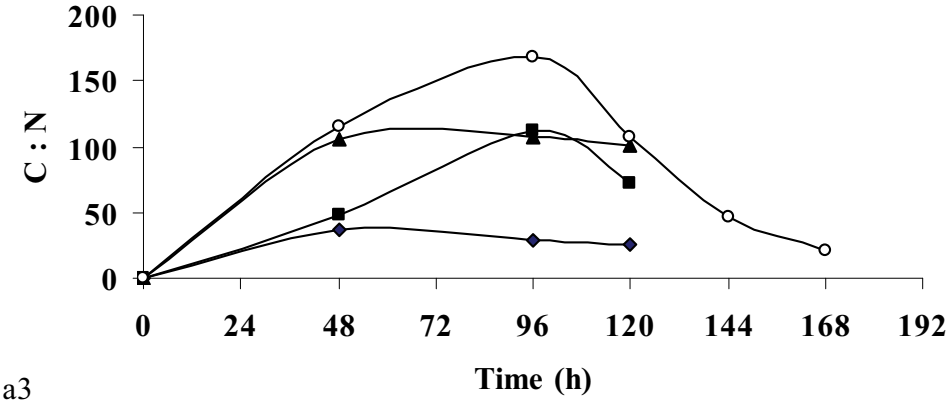
Fig 3.3a) Medium pH with fermentation time, using 8 g N (kg HSS)$^{-1}$ and glucose level of: 125 (full diamond), 250 (open square), 350 (full triangle), and 475 (open diamond) g (kg HSS)$^{-}$, and; **3 b**) pH during fermentation as a function of citric acid accumulation.



a1

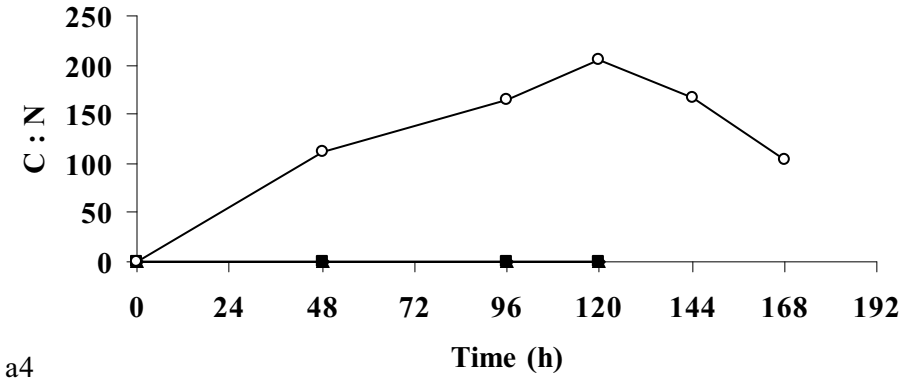


a2

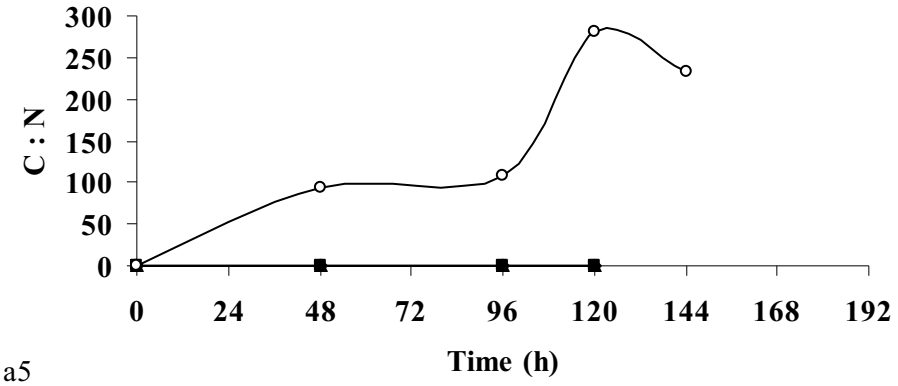


a3

Fig 3.4a. Fungal biomass C:N ratio for *A. niger (ATCC 12846)* for glucose levels of 125 (Full diamond), 250 (full square), 350 (Full triangle), and 475 (Open circle) g (kg HSS)$^{-1}$ with 2 g N (a1), 4 g N (a2), 8 g N (a3) (kg HSS)$^{-1}$.



a4



a5

Fig 3.4b. Fungal biomass C:N ratio for *A. niger (ATCC 12846)* for glucose levels of 125 (Full diamond), 250 (full square), 350 (Full triangle), and 475 (Open circle) g (kg HSS)$^{-1}$, with 9 g N (a4) and 12 g N (a5) (kg HSS)$^{-1}$.

CHAPTER 4

FINAL CONCLUSIONS

The present study focused on the effect of glucose and N supplementation on citric acid accumulation and fungal biomass of *A. niger* ATCC 12846, grown on an inert solid substrate wetted with a nutrient rich solution.

Citric acid accumulation and *A. niger* growth were significantly affected by glucose and ammonium supplementation. Higher glucose levels delayed citric acid accumulation peaks. Nitrogen supplementation controlled the time interval between peak biomass and citric acid accumulation; with low nitrogen and glucose levels, citric acid peaked before biomass while, with an intermediate nitrogen level and the highest glucose level of 475 g $(kg\ HSS)^{-1}$, citric acid accumulation peaked after biomass. This last condition is preferred for high citric acid yields.

The highest citric acid accumulation peak of 52 g $(kg\ HSS)^{-1}$ and yield of 14% were obtained using 8 g nitrogen $(kg\ HSS)^{-1}$ and 475 and 250 g glucose $(kg\ HSS)^{-1}$, respectively. Higher citric acid yields could be reached if the pH of the medium was adjusted to 5.5 during spore germination and then dropped to 2 during the exponential growth stage for the solution offering 475 g glucose and 8 g nitrogen $(kg\ HSS)^{-1}$.

Finally, fungal biomass cannot be monitored by measuring the changes in organic N then translated into total volatile solids representing biomass, because the C:N ratio of fungal biomass varied with fermentation time, and initial solution sugar and nitrogen content.

Future research should repeat the treatment with *475* g glucose and 8 g N $(kg\ HSS)^{-1}$ with the initial pH corrected at 5.5 and then allowed to drop and corrected to 2.0

when a peak fungal biomass is reached, after 96 h of fermentation. Also, the new trial should test increasing the amount of water added to each 30 g sample of expanded clay (HSS), because 55 ml may not have been enough.

Appendix

Table A1: ANOVA for Dependent Variable Fungal biomass growth for N 2, 4, and 8 g (kg HSS)$^{-1}$ with glucose 125, 250, 350, and 475 g (kg HSS)$^{-1}$

Source	D.F	Type III SS	Mean Square	F value	Pr>F
N	2	12593.73	6296.86	9352.56	<.0001
G	3	219447.58	73149.19	108646	<.0001
time	5	306996.58	61399.31	91194.7	<.0001
N*G	6	23498.74	3916.45	5817.01	<.0001
N*time	10	14263.84	1426.38	2118.57	<.0001
G*time	9	129036.94	14337.43	21295.0	<.0001
N*G*time	18	48692.03	2705.11	4017.83	<.0001

Table A2: ANOVA for Dependent Variable Fungal biomass growth for Glucose 475 g (kg HSS)$^{-1}$ with N 2, 4, 8, 9, and 12 g (kg HSS)$^{-1}$

Source	D.F	Type I SS	Mean Square	F value	Pr>F
N	4	37410.73	9352.68	1051.38	<.0001
time	5	116037.57	23207.51	2608.87	<.0001
N*time	20	43384.97	2169.24	243.86	<.0001

Table B1: ANOVA for Dependent Variable Citric acid production for N 2, 4, 8 g (kg HSS)$^{-1}$ with glucose 125, 250, 350, and 475 g (kg HSS)$^{-1}$

Source	DF	Type I SS	Mean Square	F Value	Pr > F
N	2	3879.15	1939.57	20987.3	<.0001
G	3	1168.05	389.35	4213.00	<.0001
time	5	6726.07	1345.21	14556.0	<.0001
N*G	6	1355.93	225.98	2445.33	<.0001
N*time	10	2963.11	296.31	3206.25	<.0001
G*time	9	2907.96	323.10	3496.20	<.0001
N*G*time	10	925.91	51.43	556.61	<.0001

Table B2: ANOVA Dependent Variable Citric acid production for Glucose 475 g (kg HSS)$^{-1}$ with N 2, 4, 8, 9, and 12 g (kg HSS)$^{-1}$

Source	DF	Type I SS	Mean Square	F Value	Pr > F
N	4	5741.12	1435.28	5584.11	<.0001
time	5	12173.43	2434.68	9472.40	<.0001
N*time	20	3738.86	186.94	727.32	<.0001

Table C1: Duncan's Multiple Range Test for the effect of N levels on Fungal biomass growth for Glucose 125, 250, 350, and 475 g (kg HSS)$^{-1}$ *

Duncan Grouping	Mean	N	N
A	72.87	54	8
B	66.21	54	2
C	53.82	54	4

Means with the same letter are not significantly different.

*Note: * For N 2, 4, 8 g (kg HSS)$^{-1}$*

Table C2: Duncan's Multiple Range Test for the effect of N levels on Fungal biomass growth for Glucose 475 g (kg HSS)$^{-1}$ *

Duncan Grouping	Mean	N	N
A	86.0817	18	9
A	85.6106	18	12
B	56.7989	18	4
C	40.8950	18	8
C	40.3544	18	2

Means with the same letter are not significantly different.

*Note: * For N 2, 4, 8, 9, and 12 g (kg HSS)$^{-1}$*

Table D1: Duncan's Multiple Range Test for the effect of N levels on Citric acid production for Glucose 125, 250, 350, and 475 g (kg HSS)$^{-1}$*

Duncan Grouping	Mean	N	N
A	**16.53**	**54**	**8**
B	**6.92**	**54**	**2**
C	**5.51**	**54**	**4**

Means with the same letter are not significantly different.

*Note: * For N 2, 4, 8 g (kg HSS)$^{-1}$*

Table D2: Duncan's Multiple Range Test for the effect of Nitrogen levels on Citric acid production for Glucose 475 (g kg HSS^{-1}) *

Duncan Grouping	Mean	N	N
A	24.1344	18	12
B	22.8099	18	8
C	22.4639	18	9
D	9.1550	18	4
E	5.0294	18	2

Means with the same letter are not significantly different.

*Note: * For N 2, 4, 8, 9, and 12 g (kg HSS)$^{-1}$*

Table E1: Duncan's Multiple Range Test for the effect of Glucose levels on Fungal biomass growth for Glucose 125, 250, 350, and 475 g (kg HSS)$^{-1}$ *

Duncan Grouping	Mean	N	G
A	129.83	36	350
B	65.99	36	250
C	46.01	54	475
D	24.51	36	125

Means with the same letter are not significantly different.

*Note: * For N 2, 4, 8 g (kg HSS)$^{-1}$*

Table E2: Duncan's Multiple Range Test for the effect of Glucose levels on Citric acid production *

Duncan Grouping	Mean	N	G
A	12.33	54	475
B	11.50	36	250
C	7.51	36	350
D	5.95	36	125

Means with the same letter are not significantly different.

*Note: * For N 2, 4, 8 (g kg HSS^{-1})*

Table F1: Correlation between Fungal biomass growth and Citric acid production for N 2, 4, 8 g (kg HSS)$^{-1}$with glucose 125, 250, 350, and 475 g (kg HSS)$^{-1}$

Pearson Correlation Coefficients, N = 162 Prob > |r| under H0: Rho=0		
	FM	**CA**
FM	1.00000	0.39252 <.0001
CA	0.39252 <.0001	1.00000

FM= Fungal biomass, CA= citric acid

Note: The correlation coefficients in table (3.7a) showed significant correlation with citric acid production and fungal biomass growth with glucose 125, 250, 350, 475 g (kg HSS)$^{-1}$.

Table F2: Correlation between Fungal biomass growth and Citric acid production for N 2, 4, 8, 9, and 12 g (kg HSS)$^{-1}$with glucose 125, 250, 350, and 475 g (kg HSS)$^{-1}$

Pearson Correlation Coefficients, N = 90 Prob > |r| under H0: Rho=0		
	FM	**CA**
FM	1.00000	0.68221 <.0001
CA	0.68221 <.0001	1.00000

FM= Fungal biomass, CA= citric acid

Note: The correlation coefficients in table (3.7b) showed a highly significant correlation with citric acid production and fungal biomass growth for glucose 475 g (kg HSS)$^{-1}$.