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FEASIBILITY OF THE BIO-CONVERSION OF HYDROCARBON FEEDSTOCK TO CITRIC ACID

by

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ABSTRACT

Currently, the majority of worldwide microbial production of citric acid utilizes *Aspergillus niger* in a carbohydrate based submerged fermentation. An innovative alternative to utilizing carbohydrates are hydrocarbon feedstocks. Due to their high carbon content, hydrocarbons theoretically have the potential of producing high yields of citric acid. With the increasing prices in the sugars market, it is of economical interest to evaluate the feasibility of utilizing hydrocarbons in citric acid production. It is therefore the focus of this study to examine the feasibility of using hydrocarbon oils as the sole source of carbon for the production of citric acid and biomass.

Initial lab experiments were conducted using 1875 ml batch flask fermentations to determine the yeast strain of *Candida lipolytica* and hydrocarbon feedstock of choice. The two yeast strains investigated were *Candida lipolytica NRRL-Y-1094 and Candida lipolytica NRRL-Y-1095*. The two feedstocks evaluated were kerosene (Kero 1-K) and n-paraffin solvent (Norpar 15), both obtained from Imperial Oil Ltd. From these tests it was found that *Candida lipolytica NRRL-Y-1095* assimilated both feedstocks more effectively than *Candida lipolytica NRRL-Y-1094.* A citric acid productivity of 47 mg/L-h was obtained when *Candida lipolytica NRRL-Y-1095* was used with the n-paraffin solvent, while a citric acid productivity of 23 mg/L-h was obtained when using kerosene. Therefore all subsequent studies were conducted using *Candida lipolytica NRRL-Y-1095* and n-paraffin solvent.

To determine the optimum level of initial biomass concentration, n-paraffin concentration, iron concentration and temperature for the production of citric acid, a central composite design was developed using 200 ml batch flask fermentations. The design involved conducting 31 batch flask fermentations under various combinations of high and low values of these four parameters. From this investigation it was found that the optimum levels of each parameter for citric acid production were, 10-12 % volume for initial biomass inoculum, 10-15% volume for n-paraffin concentration, 10 mg/L for ferric nitrate concentration, and 26-30°C for temperature.

These optimum levels of the above operating parameters were implemented in larger scale (7litre) fermentations. These fermentations were conducted in a 14L Chemap fermentor. The fermentations investigated the effects of aeration, agitation (mechanical agitation from the fermentor and an external agitator), and batch versus fed-batch systems on biomass and citric acid production. It was determined that an aeration increase from 0.5 to 2.0 vvm in batch systems did not affect overall citric acid yield and productivity. An agitation rate increase from 400 to 800 rpm in batch systems increased average citric acid productivity by 39%. In fed-batch systems, an agitation increase from 400 to 1000 rpm resulted in a citric acid productivity increase of 125 %. The utilization of an external agitator running at 1400 rpm (while the fermentor agitator is running at 400 rpm) increased citric acid productivity by 75% from when an external agitator is absent from the system. However, an external agitation of 1600 rpm resulted in a 20-30 % increase in cell lyses. Citric acid yields were increased by 100% when moving from batch to fed-batch systems. When a three cycle fed-batch system was implemented, citric acid yields were increased by 100% from fed-batch systems and 200% from batch systems.

RÉSUMÉ

Actuellement, la majorité de la production microbienne mondiale d'acide citrique utilise le *Aspergillus niger* dans une fermentation submergée à base de glucide. L'utilisation de charges d'alimentation à base d'hydrocarbure plutôt que de glucides est une alternative innovatrice. Vu leur teneur élevée en carbone, les hydrocarbures ont, en théorie, le potentiel de produire de hauts rendements d'acide citrique. Face à l'augmentation du prix des sucres sur le marché, on peut avoir intérêt, sur le plan économique, à évaluer la faisabilité d'utiliser des hydrocarbures dans la production d'acide citrique. La présente étude a donc pour objet d'examiner la faisabilité d'utiliser des huiles d'hydrocarbures comme source exclusive de carbone pour la production d'acide citrique et la biomasse.

Les premières expériences en laboratoire ont été effectuées au moyen de fermentations par lots en ballon de 1875 mL pour déterminer la souche de levure de *Candida lipolytica* et la charge d'hydrocarbure optimale. Les deux souches de levure étudiées ont été la *Candida lipolytica NRRL-Y-1094* et la *Candida lipolytica NRRL-Y-1095.* Les deux charges d'alimentation évaluées ont été le kérosène (Kero 1-K) et le solvant n-paraffine(Norpar 15), tous deux obtenus de la Compagnie pétrolière impériale Ltée. Ces tests ont révélé que la *Candida lipolytica NRRL-Y-1095* assimilait les deux charges plus efficacement que la *Candida lipolytica NRRL-Y-1094.* On a obtenu une productivité d'acide citrique de 47 mg/l-h en utilisant la *Candida lipolytica NRRL-Y-1095* avec la n-paraffine, comparativement à une productivité d'acide citrique de 23 mg/l-H avec le kérosène. Toutes les études subséquentes ont par conséquent été effectuées avec la *Candida lipolytica NRRL-Y-1095* et la n-paraffine.

Pour déterminer le niveau optimum de concentration initiale de la biomasse, de concentration de la n-paraffine, de concentration du fer et de la température pour la production d'acide citrique, on a mis au point un modèle composite central à l'aide de fermentations par lots en ballon de 200 mL. Nous avons ainsi effectué 31 fermentations en utilisant diverses combinaisons de valeurs faibles et élevées de ces quatre paramètres.

À partir de cette étude, nous avons constaté que les niveaux optimums de chaque paramètre pour la production d'acide citrique étaient de 10 à 12 % pour le volume de concentration initiale de la biomasse, de 10 à 15 % pour le volume de concentration de la n-paraffine, de 10 mg/l pour la concentration de nitrate de fer, et de 26 à 30°C pour la température.

Ces niveaux optimums pour chacun des paramètres ci-dessus ont ensuite été utilisés dans des fermentations à plus grande échelle (7 litres) effectuées dans un fermenteur Chemap de 14L. On a alors étudié les effets de l'aération, de l'agitation (agitation mécanique du fermenteur et d'un agitateur externe) et des systèmes par lots (batch systems) par rapport aux systèmes à écoulement discontinu (fed-batch) sur la biomasse et la production d'acide citrique. Il a été déterminé qu'une augmentation de l'aération de 0,5 à 2,0 vvm dans les systèmes par lots ne modifiait pas la production globale d'acide citrique ni la productivité. Une augmentation du taux d'agitation de 400 à 800 tours/minute dans les systèmes par lots a augmenté la productivité moyenne d'acide citrique de 39 %. Dans les systèmes à écoulement discontinu, l'augmentation de l'agitation de 400 à 1000 tours/minute s'est soldée par une augmentation de la productivité d'acide citrique de 125 %. L'utilisation d'un agitateur externe fonctionnant à 1400 tours/minute (pendant que l'agitateur du fermenteur fonctionnait à 400 tours/minute) a augmenté la productivité d'acide citrique de 75 % par rapport à la productivité du système sans agitateur externe. Mais, une agitation externe de 1600 tours-minute a produit une augmentation de 20 à 30 % de lyse cellulaire. La production d'acide citrique s'est accrue de 100 % lorsque l'on est passé du système par lots au système à écoulement discontinu. Lorsque l'on a mis en oeuvre un système à écoulement discontinu en trois cycles, la production d'acide citrique a augmenté de 100 % dans le système à écoulement discontinu, et de 200 % dans le système par lots.

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Lastly, but certainly not least, I would like to thank my family for their patience and relentless support without which this would not have been possible. I would especially like to thank my mother and father for their continuous guidance and encouragement, and all the ways they have taught me.

Dedication

To my parents, Iolanda and Gaetano Crolla,
the type of people I aspire of becoming.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
CA	Citric acid
CL	Citrate lyase
FDA	Food and Drug Administration
L-LDH	L-lactate dehydrogenase
MDH	Malate dehydrogenase
NADH	Nicoinamide-adenine denucleotid
NRRL	Northern Regional Research Laboratory
RSM	Response surface method
vvm	Volume of air per volume of medium per minute

NOMENCLATURE

BIO_G_L	Biomass concentration, g/L
BIOMASS%	Coded Initial biomass concentration, % volume
CITR_G_L	Citric acid concentration, g/L
E(Y)	Expected value of Y
f	Degrees of Freedom
FE_MG_L	Coded Ferric nitrate concentration, mg/L
HC_%	Coded n-Paraffin concentration, % volume
k	Number of factors being studied
N	Total number of design points
n_0	Number of center points
p	Number of coefficients
R^2	Coefficient of Determination
SS	Sum of squares
TEMP_C	Coded Temperature, °C
X	Coded factor
X_1	Initial biomass concentration, % volume
X_2	n-Paraffin concentration, % volume
X_3	Ferric nitrate concentration, mg/L
X_4	Temperature, °C
y	Observed value

Greek Letters

α	Distance from star point to center
β	Model parameter

CHAPTER 1:
Introduction

Citric acid is widely used in food, pharmaceutical and chemical industries due to its distinctive properties as an acidulent, flavoring agent, antioxidant and chelating agent. Commercially, citric acid is produced from molasses, sucrose, or glucose syrups by *Aspergillus niger* in surface or submerged oxidative fermentation processes. Several yeast have also been shown to produce citric acid from a wide variety of carbon sources, including glucose, n-alkanes, and edible oils, in submerged oxidative fermentation processes. This thesis focuses on the evaluation of producing citric acid through the utilization of yeast and n-alkanes, as a potential alternative to the more traditional use of carbohydrate substrates. The results of the study will be considered for eventual commercialization possibilities by Touchstone Contracting, Management and Engineering Services Ltd.

§ 1.1 Citric Acid Production Worldwide

Citric acid is manufactured in over 20 countries with 1994 production at approximately 550 thousand metric tons with a value at over $750 million (Blair and Staal, 1991; Bradly, 1996). The distribution of worldwide production is shown in Figure 1. Most of this production is used for foods and beverages, with industrial applications (e.g. detergents, metal cleaning) becoming more important on a worldwide basis. The world's largest producing companies are Bayer and ADM (each of which has a 17% market share) followed by Jungbunzlauer, Cargill and Citrique Berge (Bradly, 1996).

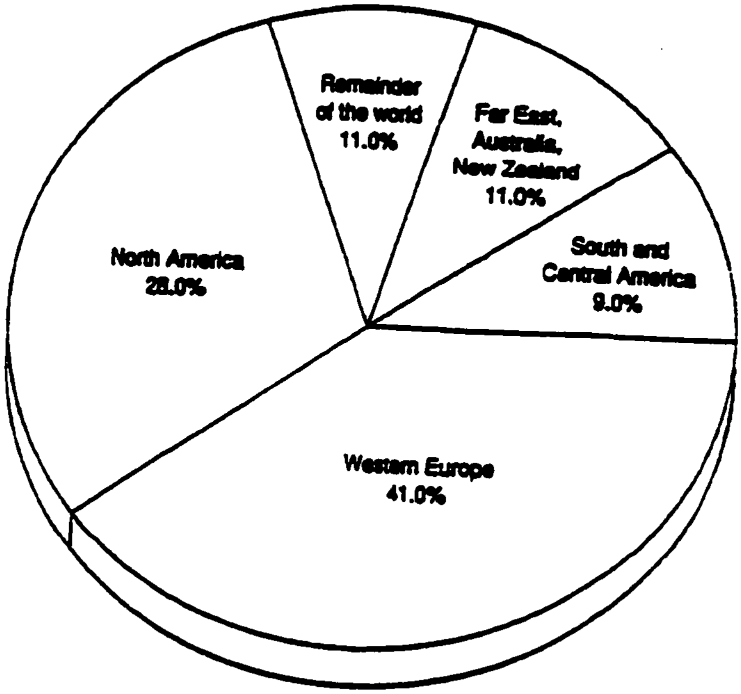


Figure 1: Worldwide Citric Acid Production (Blair and Staal, 1991)

Western Europe produces more citric acid than any other region, while the United States is the runner up. The United States is a net importer of citric acid, while Western Europe is a net exporter. Although domestic consumption volumes in Western Europe and United States in 1994 were roughly equal at 192-200 thousand metric tons, end-use market demand in the two regions varies (Bradly, 1996). For instance, Western European demand for citric acid use in household detergents and cleaners was 30% higher than that in the United States, even though the United States was still transitioning out of phosphate builders for laundry detergent formulations in 1994 (Bradly, 1996). Similarly, the United States utilized more citric acid in food and beverages applications, particularly in beverages, than did Western Europe.

Canada is currently without a citric acid producer, and was estimated in 1995 to have imported 11.9 thousand metric tons of citric acid from the United States and Europe at approximately $2.40/kg (Touchstone Engineering Ltd., 1996). Similarly to the United States, the largest market in Canada for citric acid is as a food acidulent. The U.S. market is relatively mature and is growing at a rate of 3-5% annually. The Canadian food acidulent market for citric acid is said to have experienced measurable growth in recent years; there has been some shifting of the dry drink mixtures business to Canada from the U.S. to take advantage of lower sugar prices (Touchstone Engineering Ltd., 1996). The major citric acid buyers in Canada are food and pharmaceutical companies situated in Toronto and Montreal. For this reason, Touchstone Engineering Ltd. is proposing to establish a Canadian facility in eastern Ontario, to best serve the major consumers of the two regions. Touchstone Engineering Ltd. has proposed the use of hydrocarbons as a cheap fermentation feedstock for the production of citric acid. As there is currently no plant in production utilizing hydrocarbons, the company required an investigation, by the University of Ottawa, into the feasibility of utilizing hydrocarbon feedstocks.

§ 1.2 Objectives and Scope of Research

The overall purpose of the research was to evaluate the technical feasibility of the production of citric acid through a fermentation process utilizing yeast and n-alkanes. Specifically the goals of the research were:

1. To determine a strain of the *Candida lipolytica* species and hydrocarbon feedstock for use in the study of citric acid production;
2. To study the effects of temperature, initial biomass inoculum, initial hydrocarbon concentration and iron salt concentration on the production of citric acid;
3. To observe the effects of aeration and agitation on the production of citric acid, while also comparing the effects of mechanical agitation in the fermentor itself and mechanical agitation externally;
4. To compare citric acid production between batch and fed-batch fermentations.

In selecting the strain of *Candida lipolytica* to be used throughout the study, two strains were obtained from the United States Department of Agriculture, *C. lipolytica NRRL-Y1094* and *C. lipolytica NRRL-Y1095*. The two strains were used in batch fermentations using two hydrocarbon feedstocks, n-paraffin and kerosene. The *Candida lipolytica* strain and feedstock used in the subsequent studies of the thesis was the yeast and hydrocarbon feedstock fermentation mixture with optimum citric acid production; *C. lipolytica NRRL-Y1095* and n-paraffin feedstock.

A central composite design was implemented to determine the effects of four parameters, temperature, initial biomass inoculum, initial hydrocarbon concentration and iron salt concentration, on the production of citric acid. From the design, the level of each parameter for optimal citric acid production was determined. These operating levels for each parameter were then used for the subsequent study of larger scale fermentations.

The effects of aeration and agitation on citric acid production were investigated using a Chemap fermentor. An external agitator was also introduced to the fermentor system and citric yields compared between fermentations under only mechanical agitation from the fermentor and fermentations under mechanical agitation from both the fermentor and external agitator. Citric acid yields were also compared between batch and fed-batch fermentations.

§ 1.3 Layout of Thesis

The thesis has been divided into three sections:

- the selection of a yeast strain and hydrocarbon feedstock;
- the setting of operating values for temperature, initial biomass inoculum, initial hydrocarbon concentration and iron salt concentration;
- the assessment of the technical feasibility of the production of citric acid through hydrocarbon fermentation, under the influences of aeration, mechanical agitation (from both a Chemap fermentor and an external agitator).

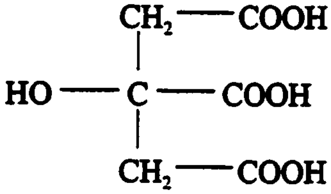
The basis for this approach is presented in Chapter 2. This chapter includes a critical analysis of the development and evolution of citric acid production. Chapter 3 presents the experimental method and analytical basis for *Candida lipolytica* strain selection and the selection of a n-paraffin solvent as the hydrocarbon feedstock. Chapter 4 presents the design and analysis of the central composite design for the selection of operating values of temperature, initial biomass inoculum, initial hydrocarbon concentration and iron salt concentration. Chapter 5 includes the experimental method and comprehensive analysis of the effects of aeration and agitation on citric acid production under the influence of both batch and fed-batch systems. This chapter also focuses on examining the effects of an external agitator, added to the fermentation apparatus, on citric acid production. A summary of the findings from the entire work and recommendations for further research are presented in Chapter 6. References are provided in Chapter 7.

CHAPTER 2:
Production of Citric Acid: A Fermentation Approach

§ 2.1 Citric Acid Background

2.1.1 Physical Properties

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid), is a natural component and common metabolite of plants and animals. It is the most versatile and widely used organic acid in foods, beverages, and pharmaceuticals.



$$\begin{array}{ccccc} & & CH_2 & — & COOH \\ & & | & & \\ HO & — & C & — & COOH \\ & & | & & \\ & & CH_2 & — & COOH \end{array}$$

(Citric Acid, Anhydrous)

Because of its functionality and environmental acceptability, citric acid and its salts (primarily sodium and potassium) are used in many industrial applications for chelation, buffering, pH adjustment, and derivatization. These uses include laundry detergents, shampoos, cosmetics, enhanced oil recovery, and chemical cleaning (Blair and Staal, 1991). Aqueous solutions of citric acid are excellent buffering systems when partially neutralized because citric acid is a weak acid and has three carboxylic groups, hence three pK_a's. At 20°C $pK_1 = 3.15$, $pK_2 = 4.77$, and $pK_3 = 6.39$ (Weast, 1989), while the buffering range for citrate solutions is pH 2.5 to 6.5 (Blair and Staal, 1991).

2.1.2 Chemical Properties

Citric acid undergoes most of the reactions typical of organic hydroxy polycarboxylates:

- *Decomposition*: When heated above 175°C, citric acid decomposes to form aconitic acid, citraconic acid, itaconic acid, acetonedicarboxylic acid, carbon dioxide, and water (Blair and Staal, 1991).
- *Esterification*: Citric acid is easily esterified with many alcohols under azeotropic conditions in the presence of a catalyst such as sulfuric acid, *p*-toluenesulfonic acid, or sulfonic acid-type ion-exchange resin. Alcohols typically used in citric acid esterification are methyl, ethyl, butyl, and allyl alcohols (Blair and Staal, 1991).
- *Oxidation*: Citric acid is easily oxidized by a variety of oxidizing agents such as peroxides, hypochlorite, persulfate, permanganate, periodate, hypobromite, chromate, manganese dioxide, and nitric acid. The products of oxidation are usually acetonedicarboxylic acid, oxalic acid, carbon dioxide, and water (Blair and Staal, 1991).
- *Salt Formation*: Citric acid forms mono-, di-, and tribasic salts with many cations such as alkalies, ammonia, and amines. Salts may be prepared by direct neutralization of a solution of citric acid in water using the appropriate base, or by double decomposition using a citrate salt and a soluble metal salt (Blair and Staal, 1991). Trisodium citrate is more widely used than any of the other salts of citric acid.
- *Chelate Formation*: Citric acid complexes with many multivalent metal ions to form chelates. This important chemical property makes citric acid and citrates useful in

controlling metal contamination that can affect the colour, stability, or appearance of a product or the efficiency of a process (Blair and Staal, 1991).

2.1.3 Occurrence of Citric Acid

Citric acid occurs widely in the plant and animal kingdom. It is found most abundantly in the fruits of the citrus species, but is also present as a free acid or as a salt in the fruit, seeds, or juices of a wide variety of flowers and plants. The citrate ion occurs in all animal tissues and fluids (Blair and Staal, 1991).

Citric acid occurs in the terminal oxidative metabolic system of virtually all organisms. This oxidative metabolic system (Figure 2), variously called the Krebs Cycle (for its discoverer, H. A. Krebs), the tricarboxylic acid cycle, or the citric acid cycle, is a metabolic cycle involving the conversion of carbohydrates, fats, or proteins to carbon dioxide and water. This cycle releases energy necessary for an organism's growth, movement, luminescence, chemosynthesis, and reproduction (Blair and Staal, 1991). The cycle also provides the carbon containing materials from which cells synthesize amino acids and fats. Many yeasts, molds, and bacteria conduct the citric acid cycle, and can be selected for their ability to maximize citric acid production in the process. This is the basis for the commercial fermentation processes used today to produce citric acid.

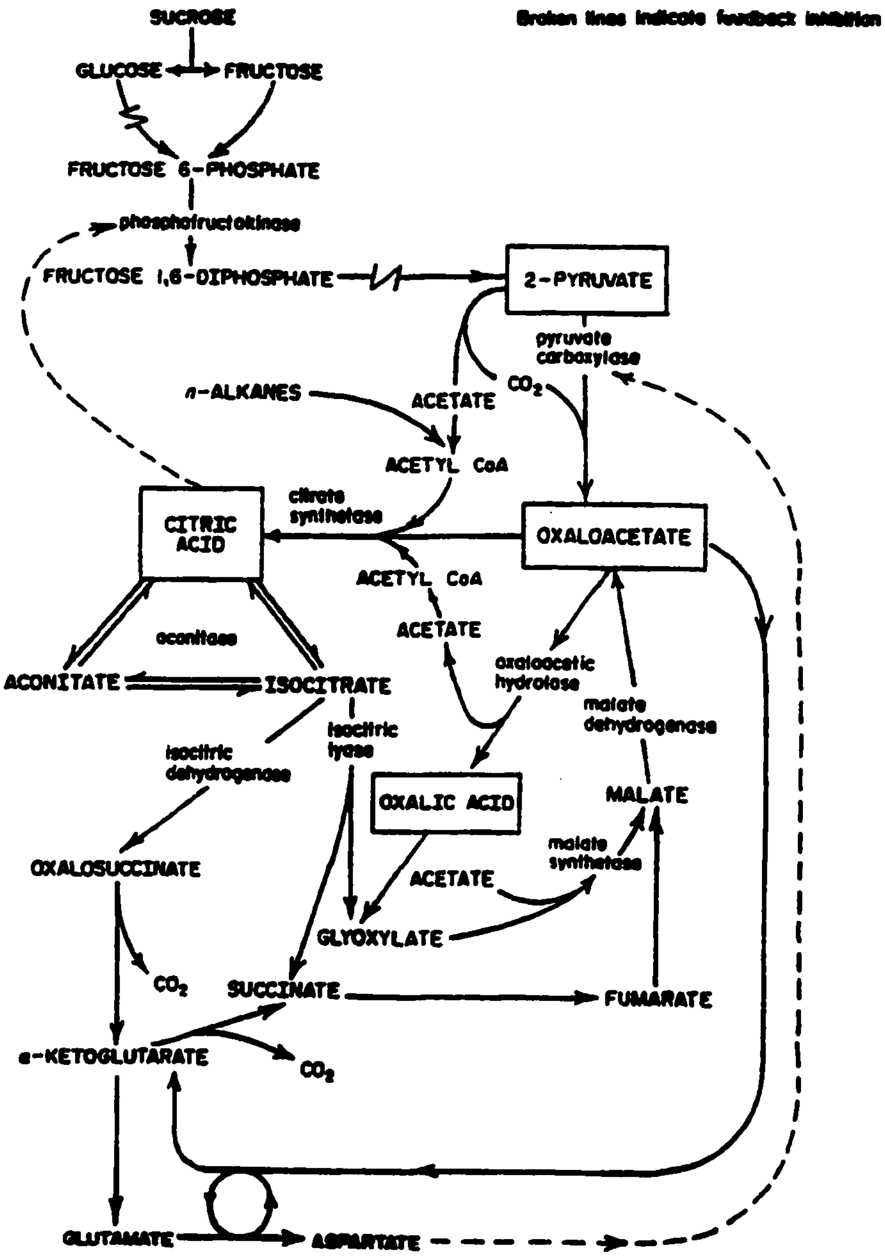


Figure 2: A Simplified Metabolic Scheme Showing Citric Acid Production from Carbohydrates and n-Alkanes (Milsom and Meers, 1985)

§ 2.2 Citric Acid Production Processes: Fermentation

From among the historically used processes for the production of citric acid the following are still important: (Milsom and Meers, 1985)

(a) *Aspergillus niger*

i. Surface fermentation using beet molasses;

ii. Submerged fermentation using beet or cane molasses or glucose syrup.

(b) ***Yeast***

i. Submerged fermentation using beet molasses or glucose syrup;

ii. Submerged fermentation using n-alkanes.

2.2.1 Processes using *Aspergillus niger*

2.2.1.1 Surface Fermentation

The surface fermentation using *A. niger* with beet molasses as raw material is still being used by some manufacturers (Milsom and Meers, 1985). The power requirements, however somewhat labour intensive, are much less than in the submerged fermentation. Because citric acid manufacturers keep their methods a secret, little authoritative material has been published. However it is known that when using beet molasses, an initial pH must be adjusted between 5 to 7 as *A. niger* will not germinate at higher hydrogen ion concentrations. The lack of germination in molasses at low pH is ascribed to the presence of acetic acid, which is a normal constituent of molasses (Fencl and Leopold, 1957). Unionized acetic acid is the species that prevents the germination.

Once the molasses based medium is sterilized and cooled, it is run down into a series of trays supported on racks in a ventilated chamber. The trays, which are usually made of very high purity aluminum, are filled to a depth of between 0.05 and 0.2 m (Milsom and Meers, 1985). Spores of *A. niger* are then distributed over the surface of the medium in the trays. Sterile air is supplied to the fermentation chamber. The air performs the dual function of supplying oxygen and carrying away fermentation heat. A temperature of

30°C is often employed. After a period of 7 to 15 days the trays are emptied, while the mycelium is separated at the same time from the fermentation broth. The fermentation liquor is then pumped forward where the citric acid is recovered.

2.2.1.2 Submerged Fermentation

Although the production of citric acid through surface fermentation using strains *A. niger* is still being used today, it is being replaced by a submerged process known as deep tank fermentation (Blair and Staal, 1991). In submerged fermentation a vegetative inoculum stage is used to allow spores of *A. niger* to germinate in an inoculum medium before being transferred to the main fermentation medium in a larger fermentation vessel. This initial growth stage is of the utmost importance to the success of the fermentation. "The morphology of the mycelium at this point is crucial according to many reports, not only in relation to the shape of the hyphae themselves, but also in the aggregation of the growth into small spherical pellets. Thus the hyphae should be abnormally short, stubby, forked and bulbous." (Snell and Schweiger, 1951; Kisser et al., 1980)

When a separate inoculum stage is employed, a suspension of spores of *A. niger* is introduced into a sterilized medium in the inoculum fermentor. The medium is aerated and agitated at a temperature of about 30°C for a period of 18 to 30 hours (Hustede and Rudy, 1976). At which point the sterilized fermentation medium is transferred to the main fermentor and the grown inoculum incorporated at the ratio 1 L of inoculum to 10 L of fermentation medium. In molasses based inoculum and fermentation media the initial pH is normally in the range of 5 to 7. As previously mentioned, *A. niger* strains do not germinate or grow at lower pH values in this type of medium. However, lower pH values can be tolerated in glucose or sucrose based media, and are often used with advantage (Milsom and Meers, 1985). At lower pH values there is a lesser chance of infection by adventitious organisms.

When the rate of increase in citric acid concentration has reached a point where it is uneconomical to proceed, the fermentation is discontinued and the broth pumped to a filter or centrifuge where the mould is separated from the liquor which is passed forward for citric acid recovery (Parton and Willis; 1990).

2.2.2 Processes using Yeast

2.2.2.1 Submerged Fermentation using Carbohydrates

In 1965 Tabuchi and Abe (1968) filed a patent for the manufacture of citric acid from glucose and molasses in which the organisms used were eight genera of yeasts including *Candida*. The fermentation was carried out at a neutral pH by being buffered by calcium carbonate incorporated in the medium. In 1971, a further patent by Iizuka (1971) reports yields of about 65% from blackstrap molasses containing calcium carbonate using strains of *Candida oleophila*. However, it was soon found that a limitation on citric acid yield was the production of quantities of L-(+)-isocitric acid (Kyowa Hakko Kogyo Ltd., 1972). Kimura et al. (1974) selected a subspecies of *Candida guilliermondii* producing only small quantities of iso-citric acid. A continuous fermentation process for the production of citric acid from blackstrap molasses using *Candida guilliermondii* has been described by Miall and Parker (1975).

An inoculum culture is prepared in a smaller fermentor. When the inoculum is sufficiently grown it is transferred to a fermentation medium in the larger main fermentor. The fermentation is conducted at a temperature between 25 and 37°C and a pH that is not too low (depending on the organism), or yeast growth will be impaired. The pH can subsequently be allowed to fall. When citric acid accumulation becomes uneconomically low, the broth is harvested for citric acid and yeast.

The advantages of using yeasts rather than *A. niger*, is not only that yeasts are more tolerant to infection by adventitious organisms, but are also capable of using very high initial sugar concentrations and have faster fermentation cycles. This combination results in high productivity rates per run.

2.2.2.2 Submerged Fermentation using n-Alkanes

It has been known for some time that some microorganisms are able to grow on hydrocarbon substrates (Bushnell and Haas, 1941; Milsom and Meers, 1985). Johnson (1964) noted that organisms preferred straight chain alkanes to branched compounds and that the preferred chain length depended on the strain. Takeda Chemical Industries Ltd. (1970) first reported the production of citric acid by yeasts from n-alkanes in the patent literature. The patent claims the use of *Candida* species, in particular *C. lipolytica*, *C. tropicalis*, *C. intermedia*, *C. parapsilosis* and *C. guilliermondii*. The patent has a priority date in Japan of June 7, 1967 and seems to be a master patent where the use of *Candida* is concerned in the production of citric acid from n-alkanes (Milsom and Meers, 1985).

n-Alkanes are insoluble in aqueous solutions, therefore it is essential during fermentation to maintain a fine dispersion of the hydrocarbons in the aqueous phase. To aid this dispersion mechanical agitation must be implemented during the fermentation process. The submerged fermentation using n-alkanes, as that using carbohydrates, has an initial inoculum growth phase. Once the inoculum growth is sufficient it is then transferred to the main fermentor containing the fermentation medium. The fermentation is conducted at a temperature between 25 and 37°C and a pH that is not too low, between 4 and 7 (depending on the organism), that yeast growth will be impaired. The pH can subsequently be allowed to fall, anywhere between 2.8 and 4 (again depending on the organism), as citric acid accumulates (Miall and Parker, 1975).

The advantages of using n-alkanes over carbohydrates are lower substrate costs and potentially higher citric acid yields. Starting from a highly reduced form of carbon such as n-alkanes, very high weight yields of citric acid are possible. The 'theoretical' yield is about 250% but allowing for biomass and carbon dioxide production a reasonable value would be 175% (Milsom and Meers, 1985).

§ 2.3 Citric Acid Producing Yeast: *Candida lipolytica*

The ability of some microorganisms, including yeasts, to utilize alkanes as a source of carbon has been known for many years, and during the Second World War these organisms were identified as causative agents in the biodeterioration of oil supplies (Rose, 1987). The interest in alkane-utilizing microorganisms grew in the early 1970s as they were considered to be potential sources of single-cell protein using oil as a feedstock. Research became intense when they were seen as potentially very acceptable food and fodder microorganisms (Rose, 1987). There are many kinds of yeasts capable of producing citric acid from various hydrocarbon sources (Tabuchi and Igoshi., 1978). A historical survey of growth of yeasts on alkanes came from Shennan and Levi in 1974 who reviewed production of yeast biomass from alkanes. At least seven genera of ascomycetous yeasts (*Debaryomyces*, *Lodderomyces*, *Metschikowia*, *Pichia*, *Saccharomycopsis*, *Schwanniomyces* and *Wingea*) contain species that can grow at the expense of hydrocarbons (Rose, 1987). But undoubtedly the most studied yeast genus with species able to grow on hydrocarbons is *Candida*, while the genera Rhodotorula, Selenotila (now considered to be *Candida* species), *Sporidiobolus*, *Sporobolomyces*, *Torulopsis* and *Trichosporon* complete the list of yeast genera with species able to utilize n-alkanes among the Deuteromycetes (Rose, 1987).

A considerable number of members of the genus *Candida* will accumulate citric acid when they are aerobically propagated in a nutrient medium comprising an appropriate carbon source, intimately mixed with an aqueous phase containing essential minerals and growth factors. *Candida lipolytica*, *Candida guilliermondii* and *Candida tropicalis* have successfully shown the ability to assimilate hydrocarbons for the production of citric acid. The three *Candida* species have demonstrated citric acid yields in the range of 46-112g/L (Pfizer Inc., 1975; Takeda Chemical Industries Ltd., 1975). *Candida lipolytica* has shown to generally produce higher citric acid yields than *Candida guilliermondii* (Teranishi et al., 1974; Shimizu et al., 1974), with a Pfizer Inc. patent (1975) illustrating citric acid yields 20 times higher when using *Candida lipolytica*. However, *Candida lipolytica* has not consistently shown higher citric acid yields than *Candida tropicalis*. Takeda Chemical Industries Ltd. demonstrated in its two patents (1975, 1972) 5% higher citric acid yields when using *Candida lipolytica* over *Candida tropicalis*, while Matsumoto et al. (1984) illustrated 25% higher citric acid yields when using *Candida tropicalis* over *Candida lipolytica*. Teranishi et al. (1974) and Shimizu et al. (1974) studied the respiration (oxidation of substrate) behavior of *Candida lipolytica*, *Candida guilliermondii* and *Candida tropicalis* on n-alkane substrates. Consistently they found that despite the fact that *Candida tropicalis* cells showed a higher n-alkane-oxidizing capacity than either *Candida lipolytica* or *Candida guilliermondii*, the cell yield (based on the amount of substrate consumed) was relatively low. In their studies, *Candida tropicalis* demonstrated a significantly greater sensitivity to respiratory inhibitors, cyanide and azide, when grown on n-alkanes where respiratory activities were lowered by about 50%. Teranishi et al. (1974) and Shimizu et al. (1974) concluded from their studies that each *Candida* yeast possess a complex respiratory system that is significantly changeable depending on the growth and fermentation conditions that are applied, like the carbon substrate used or the cell-cycle phase.

From these studies, all three *Candida* species have shown both successful biomass growth and citric acid production rates while assimilating n-alkane substrates, and were considered as potential candidates for the study. However, *Candida guilliermondii* and *Candida tropicalis* are classified as human pathogens (Douglas, 1987; Hurley et al., 1987). While the laboratory used for the fermentation studies in this thesis was not equipped to handle pathogenic cultures, *C. guilliermondii* and *C. tropicalis* could not be considered for the study. Therefore the fermentation studies was conducted using *Candida lipolytica* species.

2.3.1 *Candida lipolytica*-The Organism

The discovery in the late 1960s that high yields of citric acid were produced by *Candida lipolytica* when grown on n-paraffins signaled a potential dramatic change in the fermentation industry because this organic acid had been commercially produced since 1919 by *Aspergillus niger* (Kurtzman, 1988). The findings occurred at a time when sugar substrates were being increasingly diverted to food use and petroleum was still inexpensive. Whether *C. lipolytica* replaces *A. niger* depends upon the price of petroleum.

Food and Drug Administration (FDA) regulations (1995) document *C. lipolytica* as a food additive that may be safely used as the organism for fermentation production of citric acid. The food additive is the enzyme system of the organism *C. lipolytica* and its associated metabolites produced during the fermentation process. *Candida lipolytica* is the only species of the genus *Candida* recognized by the FDA regulations as an additive intended for the use in the fermentation process for the production of citric acid from n-alkanes (FDA Regulations, 1995).

The nonpathogenic *Candida lipolytica* organism is classified as follows (FDA Regulations, 1995):

Class: Deutaromycetes
Order: Moniliales
Family: Cryptococcaceae
Genus: *Candida*
Species: *lipolytica*

2.3.2 Using *Candida lipolytica* in the Production of Citric Acid

Many studies utilizing *Candida lipolytica* in the production of citric acid have been conducted on both carbohydrate and hydrocarbon feedstocks. Recently, Rane and Sims (1996) showed citric acid yields of *Candida lipolytica* (*NRRL Y-1095*) grown on glucose, in a 2 litre mechanically agitated batch system, in the range of 0.38-0.77 g citric acid/g glucose consumed. While in 1994 Rane and Sims (1995) described citric acid yields of *Candida lipolytica* (*NRRL Y-1095*) grown on glucose, in a 2 litre mechanically agitated fed-batch system, in the range of 0.48-0.59 g citric acid/g glucose consumed. Wentworth and Cooper (1996) investigated citric acid production using *Candida lipolytica* (*ATCC 20390*) and glucose in a self-cycling fermentation system. The self-cycling fermentor consisted of a cyclone column fermentor in which the broth is continuously re-circulated. In this case Wentworth and Cooper described citric acid yields in the range of 0.11-0.23 g citric acid/g glucose consumed. A few years early, Briffaud and Engasser (1979-I) also described a citric acid yield for *Candida lipolytica* (*D 1805*) grown on glucose, in a 2 litre mechanically agitated batch system, of 0.75 g citric acid/g glucose consumed. They further described a trickle-flow fermentation (Briffaud and Engasser, 1979-II) with *Candida lipolytica* (D 1805) grown on glucose, in a 3 litre spherical flask, with a slightly lower citric acid yield of 0.63 g citric acid/g glucose consumed. The trickle-flow fermentor consisted of a glass column fixed in a stirred spherical flask. The column was packed with cylindrical wood shavings. The culture medium was pumped from the flask to the top of the column, then it trickled down the wood packing. Air was introduced at the top of the column, and was operated in a co-current trickling mode. Simultaneously in 1979, Aiba and Matsuoka (1979) showed citric acid yields in the range of 0.14-0.33 g citric acid/g glucose consumed in a 4 litre continuous fermentation process using *Candida lipolytica*.

Although research in the area of citric acid production has concentrated on the utilization of carbohydrate feedstocks, there have been numerous studies conducted on the utilization of hydrocarbon feedstocks. Most of the work on hydrocarbon fermentation for citric acid production took place predominantly in the early to mid seventies. Much of the work in hydrocarbon fermentation was commenced by the Japanese, and soon followed by the North Americans. This interest in hydrocarbon utilization was driven by falling petroleum prices, and was quickly abandoned as petroleum prices drastically increased in the late seventies, early eighties. Today with moderate petroleum prices, there is a resurfacing of interest in hydrocarbon utilization for the production of citric acid, and other organic acids.

Much of the initial work in the production of citric acid by yeast growing on hydrocarbons was started by Takeda Chemical Industries Ltd. With several of their patents (Takeda Chemical Industries Ltd., 1970, 1972, 1975), the company developed a process for the production of citric acid using n-paraffins (having 13 to 15 carbon atoms) and *Candida lipolytica* (*ATCC 20114*). The process involves an 8 day batch fermentation resulting in citric acid yields in the range of 0.38-1.2 g citric acid/g n-paraffin consumed. Takeda Chemical Industries Ltd. stated in their patent that the yeast, while able to utilize the hydrocarbons, was not able to consume the citric acid being produced, and isocitric acid was produced in minute quantities (Takeda Chemical Industries Ltd., 1975). In 1974, Pfizer Limited also issued a patent for the production of citric acid using *Candida lipolytica* (*ATCC 20228 and NRRL Y-1094*) grown on a n-paraffin feedstock (having 14 to 16 carbon atoms) (Miall and Parker, 1974; Pfizer Ltd., 1975). This process involves a 6 litre continuous fermentation running for 304 hours (71 hours batch process plus 233 hours continuous process). The patent describes a citric acid yield of 1.27 g citric acid/g n-paraffin consumed. Also in 1974 Hattori et al. illustrated citric acid yields in the range of 0.51-0.86 g citric acid/g n-paraffin with *Candida lipolytica* (*KY6221*) grown on n-paraffins (having 12 to 15 carbon atoms) in a 80 hour 3 litre continuous fermentation process. Gledhill et al. (1973) demonstrated a 7.5 litre semi-continuous cell recycle system utilizing *Candida lipolytica* (*ATCC 8661*) and n-paraffins (containing 12 to 15 carbon atoms). The cell recycle system involved removing a portion of the fermentor broth and separating the yeast cells under non aseptic conditions by use of a centrifuge. Yeast cells from the centrifuged pellet and hydrocarbon layer were recombined, diluted to the original volume in a non sterile recycle medium, and returned to the fermentor. Gledhill et al. (1973) described citric acid yields of 0.75-0.8 g citric acid/g n-paraffin consumed.

Following these earlier works, that set the foundation for hydrocarbon fermentation, Furukawa et al. (1977, 1982) screened mutants of the *Candida lipolytica* parent and found a mutant which they classified as a new species called *Candida citrica*. After conducting 7 day 500 mL batch fermentations on a n-paraffin feedstock (containing 14-17 carbon atoms), they illustrated citric acid yields in the range of 0.77-0.86 g citric acid/g n-paraffin consumed. In 1974 Hattori et al. (1974) also reviewed the citric acid yields of a mutant and wild-type strain of the *Candida lipolytica* parent strain. The wild-type strain was classified as *ATCC 20346* and the mutant strain was classified as *ATCC 20324*. It was demonstrated that the citric acid yield obtained while using *ATCC 20346* and a n-paraffin feedstock hydrocarbon in a 7 day 2 litre fermentation was 0.66 g citric acid/g n-paraffin consumed. When using *ATCC 20324* under the same conditions a 38% higher yield was obtained, 0.89 g citric acid/g n-paraffin. In 1991, Avchieva and Vinarov (1993) also screen the *Candida lipolytica* (*917*) parent strain for a higher citric acid producing mutant. They found mutant *917-20* which produced a citric acid yield of 0.71 g citric acid/g n-paraffin consumed, when allowed to grow on a n-paraffin feedstock for 96 hours in a 750 mL flask fermentation. The 917-20 mutant produced a higher citric acid yield than the parent strain by about 9 folds.

After reviewing the literature survey and a verbal discussion with Dr. Kurtzman, a yeast taxonomist with the U.S. Department of Agriculture, *Candida lipolytica NRRL Y-1094* and *Candida lipolytica NRRL Y-1095* were chosen as the candidates for this study. *NRRL Y-1094* was suggested by the Pfizer Limited patent (Miall and Parker, 1974) as a good culture, and this was confirmed by Dr. Kurtzman. Dr. Kurtzman also suggested *NRRL Y-1095*, as the yeast had a successful reputation when used on glucose for citric acid production and should be as successful on hydrocarbons.

§ 2.4 Composition of Medium

Medium formulation is an essential stage in the design of successful laboratory experiments, pilot-scale development and manufacturing processes. The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite production and there must be an adequate supply of energy for biosynthesis and cell maintenance (Stanbury, 1995). This section will focus on the components of the medium that will be used in this study on citric acid fermentation. These components include, carbon source, nitrogen source, mineral sources, biomass concentration, pH and temperature.

2.4.1 Carbon Source

Energy for growth comes from either the oxidation of medium components or from light. Since most industrial micro-organisms are chemo-oganotrophs (Stanbury, 1995), the most common source of energy is the carbon source. The most common carbon sources are carbohydrates, hydrocarbons, lipids, methanol and proteins.

It is now recognized that the rate at which the carbon source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites. The main product of a fermentation process will often determine the choice of carbon, as alternative carbon sources will affect the yield of product and thus the cost of producing biomass and/or metabolites.

In reviewing the literature survey the most evident feedstock to utilize is n-paraffin (Hattori et al., 1974; Glendhill et al., 1973; Takeda Chemical Industries Ltd., 1970, 1972, 1975; Miall and Parker, 1974; Pfizer Ltd., 1975; Avchieva and Vinarov; 1991). The n-paraffin feedstock of choice should contain 9 to 19 carbon atoms, particularly n-paraffins containing 12 to 16 carbon atoms or 14 to 19 carbon atoms (Miall and Parker, 1974). However, the Pfizer Ltd. (1975) patent suggests utilizing kerosene as a potential feedstock. Although kerosene is a mixture of alkanes and alkenes, its significantly cheaper cost may prove it to be the more economically viable choice. Therefore, the two feedstocks chosen for screening in this study were n-paraffin (Norpar 15) and kerosene (Kero 1-K) solvents obtained from Imperial Oil Ltd., Toronto, Canada. Norpar 15 contains more than 97% mixed normal paraffins composed of predominantly 14 to 17 carbon atoms. Kero 1-K is composed of a 46-53% total paraffins mixture, including both branched (iso) and normal paraffins having predominantly 9 to 13 carbon atoms. Less than half of the total paraffins content is normal paraffins. Approximately 18% of the kerosene solvent is composed of aromatic molecules. The main interest in Kero 1-K is its market value which is half that of Norpar 15. The amount of hydrocarbon feedstocks utilized in the fermentation process of this study will be determined by running batch flask fermentation tests and performing a central composite design on the tests to evaluate hydrocarbon levels for optimum citric acid production. However it has been stated by Takeda Chemical Industries Ltd. (1970, 1972, 1975) and Pfizer Ltd. (Miall and Parker, 1974) that operating hydrocarbon levels should range between 5-20 % by volume, although they do state a lesser or higher amount can be used.

2.4.2 Nitrogen Source

An essential nutrient for biomass reproduction is nitrogen. However citric acid accumulation occurs in a nitrogen limited environment. Most microorganisms can utilize inorganic or organic sources of nitrogen. Inorganic nitrogen may be supplied as ammonia gas, ammonium salts or nitrates. Ammonium salts such as ammonium sulphate will usually produce acid conditions as the ammonium ion is utilized and the free acid is liberated. Organic nitrogen can be supplied as amino acids, protein or urea. In many instances growth will be faster with a supply of organic nitrogen (Stanbury et al., 1995).

The nitrogen source and content in this study has been modeled after the Pfizer Ltd. patent (Miall and Parker, 1974). Urea was chosen as the nitrogen source in the fermentation broth as it can be consumed by the organisms quicker than an inorganic source, therefore reaching a nitrogen limited environment sooner for citric acid initiation. The concentration of urea utilized in the study was 2.0 g/L.

2.4.3 Mineral Sources

All microorganisms require certain mineral elements for growth and metabolism (Parton and Willis, 1990; Stanbury et al., 1995; Miall and Parker, 1974). In many media, magnesium, phosphorus, potassium, sulphur, calcium and chlorine are essential components. Others such as copper, iron, manganese and zinc are essential as trace elements. The mineral components used in the study have been modeled after the Pfizer Ltd. patent (Miall and Parker, 1974). However the iron content is to be determined by batch flask fermentation tests where the iron salt level will be determined for optimal citric acid production.

Iron concentration in the fermentation medium for citric acid production is essential because it determines the ratio of citric acid to isocitric acid being produced. Too high an iron concentration will result in high isocitric acid levels, while too low an iron concentration will result in low citric acid yields (Akiyama et al., 1973). Iron is essential for activating the citrate synthetase enzyme for citric acid production, however high iron levels will activate the aconitate hydratase (aconitase) enzyme for isocitric acid production (Akiyama et al., 1973, Shu and Johnson, 1948). The recommended level of iron salt concentrations is between 10 to 100 mg/L (Stanbury et al., 1995).

2.4.4 pH and Temperature

Traditionally cell culture medium have been buffered with a bicarbonate/carbon dioxide system. The normal starting pH range of the most commonly employed culture medium is between 6.8 and 7.8 (Lavery, 1990). The optimum for each cell type will vary depending on the physiology of the culture. In this study, the pH will be allowed to drop as citric acid is being produced. The pH will be maintained at 4.0 with a 1.0 N NaOH/1.0 N HCl system. Takeda Chemical Industries Ltd. (1970, 1972, 1975), Pfizer Ltd. (Miall and Parker, 1974) state that the pH in the fermentation medium during citric acid production can be maintained between 2.8 and 3.5. They state that the yeast can function in such an acidic environment. They also suggest keeping the pH this low so that contamination can be kept at a minimum. However, Rane and Sims (1993, 1994, 1995) suggested that the pH of the medium be kept at 4.0 when using *Candida lipolytica NRRL Y-1095.*

Maintenance of temperature is crucial throughout a fermentation process, as biomass metabolism is highly variable with changes in temperature. For the production of citric acid, the operating temperature of the fermentation system is to be controlled at a set point in the range of 25-30°C (Miall and Parker, 1974; Furukawa et al., 1977), while Takeda Chemical Industries Ltd. (1970, 1972, 1975) suggests a temperature in the range of 25-37°C. The temperature utilized throughout the fermentation systems in this study is to be determined by batch fermentation flask tests and a central composite design, where the temperature for optimal citric acid production is evaluated.

§ 2.5 Aeration and Agitation

The majority of fermentation processes are aerobic and, therefore, require the provision of oxygen (Stanbury et al., 1995). The oxygen demand of a fermentation process is usually satisfied by aeration and agitation of the fermentation broth.

In hydrocarbon fermentation there are at least two physical problems of engineering interest which are crucial for hydrocarbon fermentations. The first relates to the very high oxygen demand per unit cell mass and the other arises from the limited solubility of hydrocarbon oils in aqueous media (Moo-Young et al., 1971). Stanbury et al. (1995) states that a hydrocarbon fermentation requires three times the amount of oxygen to produce the same amount of biomass from carbohydrates. Furthermore, Johnson (1964) and Aiba et al.(1969), have suggested that dissolved hydrocarbon oil may not be the main source of substrate supply, and that rather uptake occurs by cell attachment to droplets of oil.

Oxygen is supplied to the culture in the form of air sparging through the fermentation media. Stanbury et al. (1995) represented the transfer of oxygen from air to the cell as occurring in a number of steps:

i. The transfer of oxygen from an air bubble into solution.
ii. The transfer of the dissolved oxygen through the fermentation medium to the microbial cell.
iii. The uptake of the dissolved oxygen by the cell.

Both aeration and agitation affect the extent of dissolved oxygen in the medium. Aeration rates serve as the initial volume of air bubbles introduced to the system whereas agitation rates serve to assist oxygen transfer in the following ways (Stanbury et al., 1995):

i. Agitation increases the area available for oxygen transfer by dispersing air in the culture fluid in the form of small bubbles.
ii. Agitation delays the escape of air bubbles from the liquid
iii. Agitation prevents coalescence of air bubbles.
iv. Agitation decreases the thickness of the liquid film at the gas-liquid interface by creating turbulence in the culture fluid.

Agitation in hydrocarbon fermentations is also crucial to the dispersion of oil droplets throughout the medium. Moo-Young et al. (1971) found that smaller oil droplets, the result of higher agitation rates, resulted in greater assimilation of the hydrocarbon source by the yeast. However agitation rates running too high can impose a dangerously large shear stress on cell walls, ultimately decreasing or destroying cell viability.

For the purposes of this study, a Chemap fermentor (Figure 3) will be used in the investigation of the effects of aeration and agitation on citric acid and biomass production. In this fermentor, the air will be introduced into the medium from the bottom of the fermentor to prolong the detention of air in the medium; allowing for a longer surface contact between the medium and air bubbles during oxygen transfer between the two phases.

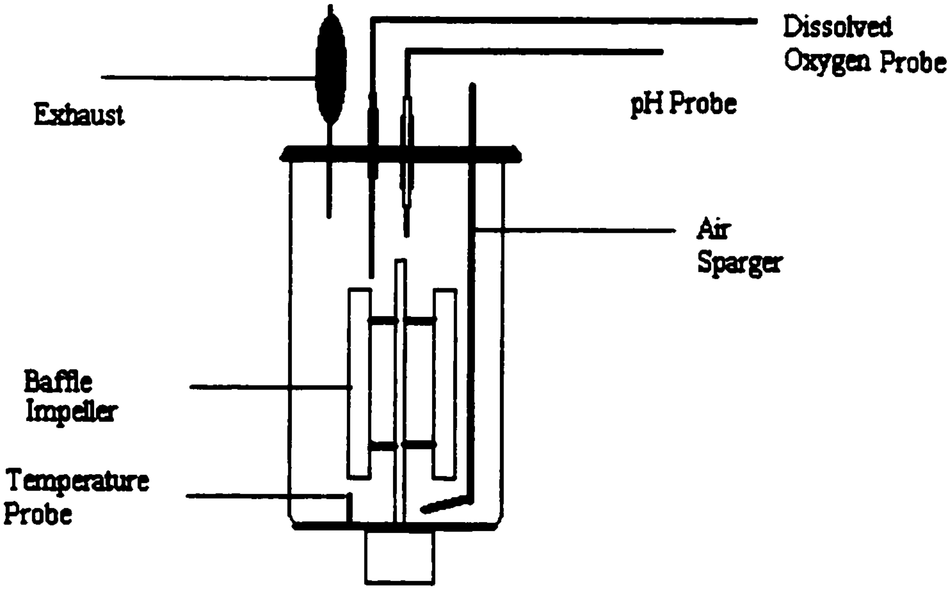


Figure 3: Schematic of Chemap Fermentor Utilized for Fermentation Runs

The air line is fitted with a filter to collect any grease that may be present. The fermentor is equip with 4 stainless steel baffle impellers for agitation. The impellers are driven by a bottom belt drive. A pH probe, temperature sensor and dissolved oxygen probe are also present for analysis and control.

The study will also investigate the potential of utilizing an external agitator in the production of citric acid. Due to the high oxygen demand of the process, an external agitator will be connected in-line with the fermentor (Figure 4).

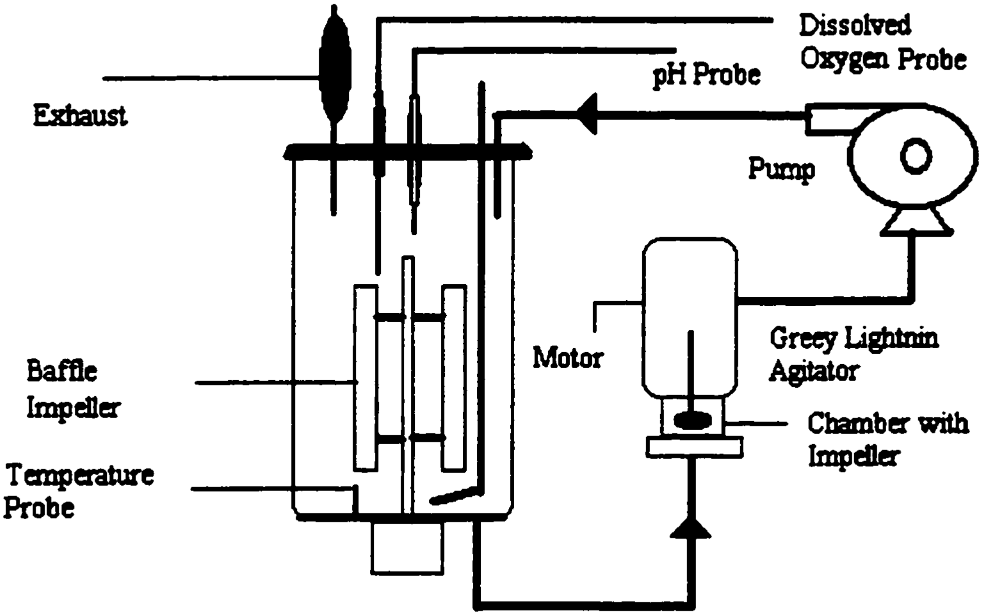


Figure 4: Schematic of Greey Lightnin External Agitator In-line with Fermentor

The fermentation broth will be re-circulated through the external agitator (Greey Lightnin In-Line Agitator) and back into the fermentor. Agitation will be implemented in bpth the external agitator and in the fermentor. The external agitator has the potential of serving three purposes:

i. As the chamber of the agitator is 5 by 3.8 cm, with an impeller span of 2.5 cm, it has the potential of producing smaller, more uniformly dispersed air bubbles throughout the medium. There will be a more uniform interaction between air bubbles (introduced by the sparger in the fermentor) and impellers. These smaller air bubbles increase the surface area for interaction between the gaseous and aqueous phases;

ii. By re-circulating the medium, there will be a longer detention time of air bubbles in the medium, therefore allowing for a larger transfer of oxygen between the two phases.

iii. The smaller, more uniform air bubbles produced by the external agitator will also allow for more surface area for the yeast to adhere to for hydrocarbon consumption.

From the literature review there has not been any mention of utilizing an external agitator in a fermentation process for the production of citric acid from n-alkanes. Therefore this is a novel approach to a solution for the high oxygen demand in citric acid production from hydrocarbons. Since there has not been any work in this area, the agitator will have to be compared to the agitation effectiveness of the fermentor. Several fermentations will be carried out to determine the full potential of an external agitator.

CHAPTER 3:
Selection of Hydrocarbon Feedstock and Yeast Culture

§3.1 Introduction

Many *Candida* species are capable of utilizing hydrocarbons as the principle source of assimilable carbon. *Candida lipolytica* has been shown to metabolize hydrocarbon solvents while accumulating citric acid. Concurring with the U.S. Department of Agriculture, *Candida lipolytica* NRRL-Y-1094 and *Candida lipolytica* NRRL-Y-1095 have demonstrated successful citric acid accumulation (Kurtzman, 1996).

The hydrocarbon forming the principle source of assimilable carbon should consist essentially of one or more straight chain alkanes or 1-alkenes from 9 to 19 carbons, particularly n-alkanes containing from 12 to 16 carbon atoms or from 14 to 19 carbon atoms (Miall and Parker, 1974). From the literature review, as reviewed in Chapter 2, it was possible to narrow feedstock candidates to kerosene and n-paraffin solvents. The interest in kerosene is governed by its market value which is half that of n-paraffin (Imperial Oil, 1995).

This chapter focuses on screening citric acid production with two publicly available strains of *Candida lipolytica* and two industrially available hydrocarbon feedstocks. The selected yeast strain and hydrocarbon feedstock was then used in the subsequent work on the production of citric acid from hydrocarbons, Chapters 4and 5.

§3.2 Materials and Methods

3.2.1 Yeast Culture

The yeast cultures used in this part of the study are *Candida lipolytica NRRL-Y1094* and *Candida lipolytica NRRL-Y1095* described in Appendix A, Section A.1. The lyophilized yeast cells were propagated as described in Appendix A, Section A.2.

3.2.2 Hydrocarbon Feedstock

The hydrocarbon feedstocks used in this part of the study are Kero 1-K and Norpar 15 described in Appendix A, Section A.3.

3.2.3 Yeast Cell Growth and Fermentation Media

The media described in Tables A2, A3 and A4 in Appendix A, Section A.4 were used for the inoculation yeast seed culture, the pre-fermentation stage for yeast biomass growth and the fermentation stage for citric acid production, respectively.

3.2.4 Experimental Protocols

3.2.4.1 Preparation of Yeast Seed Culture

The yeast seed culture was prepared by dispensing 645 mL quantities of the medium described in Table A2 (Appendix A) into two 2.0 litre Erlenmeyer flasks. The medium in each flask was inoculated with one of the two yeast cultures maintained on agar plates; one flask with *Candida lipolytica-Y-1094* and the other with *Candida lipolytica-Y-1095*. These flasks were fitted with foam bungs, to minimize contamination of media, and then incubated at 25 ± 1°C for 48 hours in a Lab-Line Instruments 3597 Environ-Shaker rotary shaker running at 90 rpm.

3.2.4.2 Preparation of Pre-Fermentation Stage-Biomass Growth

At the end of the seed culture propagation, the seed cultures were used as inoculation cultures for both *Candida* culture fermentations. A 1725 mL volume of pre-fermentation stage medium described in Table A3 (Appendix A) was dispensed into each of four 4.0 litre Erlenmeyer flasks. The four flasks were inoculated under aseptic conditions with different yeast culture and hydrocarbon solvent combinations, as described in Table 1, and then fitted with foam bungs.

Table 1: Yeast Culture and Hydrocarbon Feedstock Composition in Pre-Fermentation Stage Biomass Growth Flasks

Bottle Number	Yeast Culture	Hydrocarbon Feedstock
1	*Candida lipolytica-Y-1094*	Kerosene
2	*Candida lipolytica-Y-1094*	n-Paraffin
3	*Candida lipolytica-Y-1095*	Kerosene
4	*Candida lipolytica-Y-1095*	n-Paraffin

The pre-fermentation stage flasks were then incubated at 25 ± 1°C in the same Lab-Line shaker at 50 rpm for 70 hours while being aerated by sparging with filtered air at a rate of 1.0 litres per minute (0.58 volume air/volume medium/minute [vvm]).

3.2.4.3 Preparation of Fermentation Stage-Citric Acid Production

The citric acid producing stage of the fermentation was started by dispensing 1875 mL of the fermentation media described in Table A4 (Appendix A) into each of four 4.0 litre

Erlenmeyer flasks. Each flask contained the same yeast culture and hydrocarbon feedstock combination described in Table 1; the yeast culture inoculations were from the respective pre-fermentation growth. The flasks were fitted with foam bungs and then incubated at 25 ± 1°C for 120 hours in the Lab-Line shaker running at 100 rpm. Using filtered air, each flask was aerated at a rate of 2.5 litres per minute (1.33 vvm). Samples were taken daily for analysis of biomass concentration, hydrocarbon feedstock consumption and citric acid production.

3.2.5 Analytical Methods

The analytical methods used are described in Appendix A, Section A.5. The biomass concentration was analyzed using the method outlined in Section A.5.1. The hydrocarbon concentrations were analyzed using the method outlined in Section A.5.2. The citric acid concentrations were analyzed using the method outlined in Section A.5.3.1.

§3.3 Results and Discussion

All raw data for the following experiments can be found in Appendix B.

3.3.1 Effect of Hydrocarbon Feedstock on Citric Acid Production

3.3.1.1 4 Litre Fermentation with Kerosene Feedstock

Figures 5 and 6 describe citric acid fermentations utilizing kerosene as a feedstock over a 120 hour fermentation period. Figure 5 illustrates the use of *Candida lipolytica-Y-1094* with the kerosene substrate. It was observed that at the completion of the fermentation 14.5 mg/L of citric acid was produced with an overall productivity of 0.12 mg/L-h. Kerosene consumption was 18%. The biomass concentration increased 4.5 folds from initial concentration.

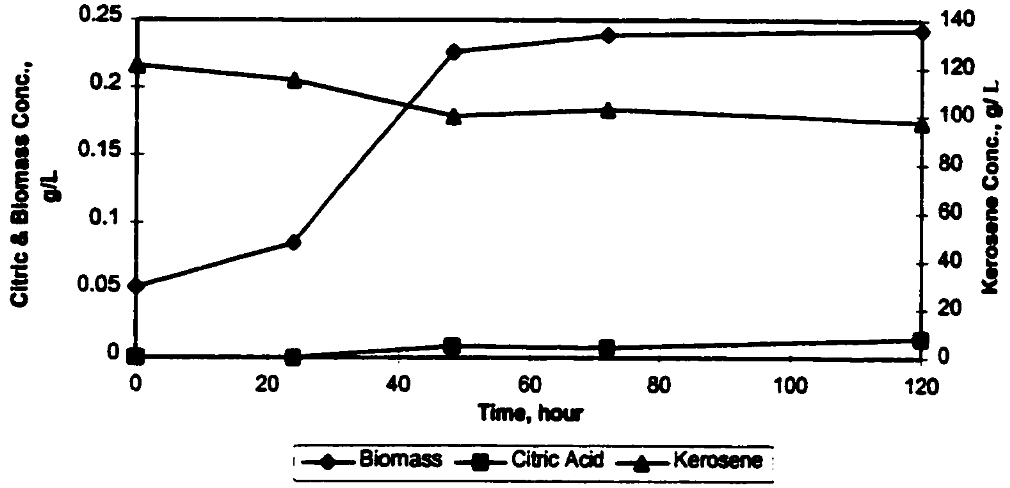


Figure 5: Citric Acid Fermentation using Kerosene and *Candida lipolytica-Y-1094*

In Figure 6, where *Candida lipolytica-Y-1095* was used, citric acid production was 20 mg/L with an overall productivity of 0.17 mg/L-h. Kerosene consumption was 20 %. Biomass concentration resulted in a 5 fold increase from initial concentration.

In comparing Figures 5 and 6, *Candida lipolytica-Y-1095* seems to assimilate the kerosene feedstock more effectively than *Candida lipolytica-Y-1094*, with a 40 % higher citric acid yield.

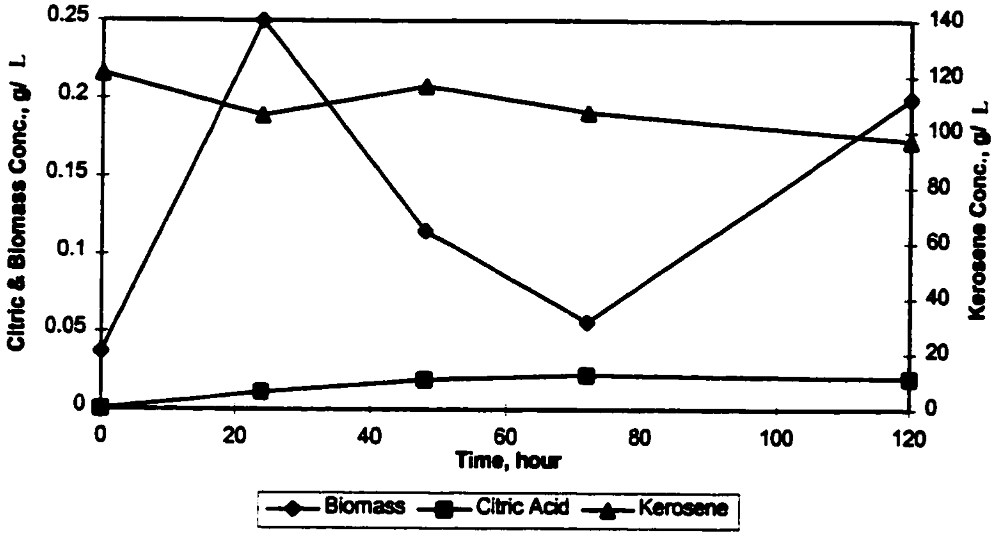


Figure 6: Citric Acid Fermentation using Kerosene and *Candida lipolytica-Y-1095*

However, the final biomass concentration is 20 % higher with *Candida lipolytica-Y-1094* than *Candida lipolytica-Y-1095* using the kerosene substrate. However the production trends portrayed in both fermentations are similar with only slight shifts in data. Both yeast cultures go through a 20 hour lag phase in citric acid production.

3.3.1.2 4 Litre Fermentation with n-Paraffin Feedstock

Figures 7 and 8 illustrate citric acid production utilizing the n-paraffin feedstock over a 120 hour fermentation period. Figure 7 describes the fermentation of n-paraffin with *Candida lipolytica-Y-1094*. In this fermentation the citric acid yield was 2.79 g/L with an overall productivity of 0.023 g/L-h or 23 mg/L-h. n-Paraffin consumption was 35 %. Biomass concentration increased by 86 folds from the initial concentration.

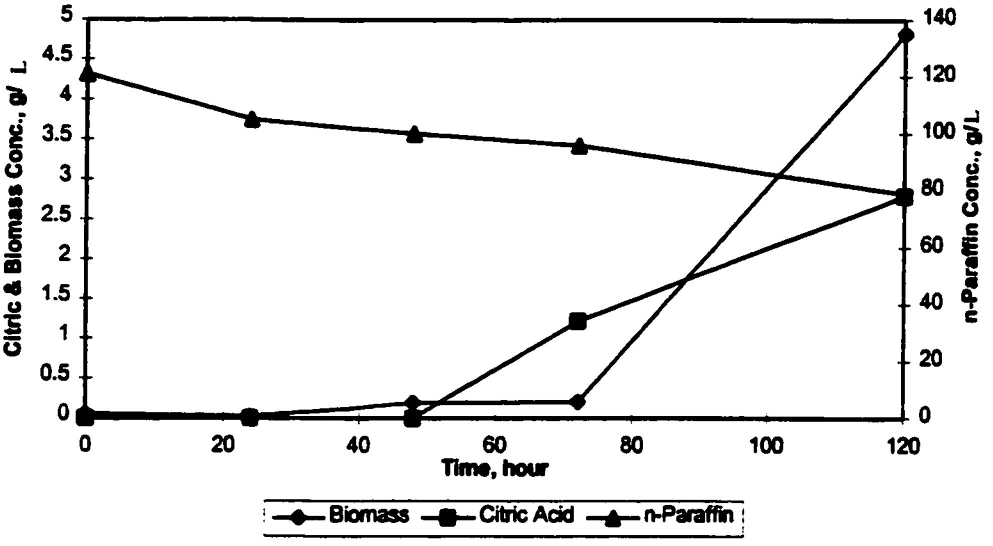


Figure 7: Citric Acid Fermentation using n-Paraffin and *Candida lipolytica-Y-1094*

Figure 8 illustrates the fermentation of n-paraffin with *Candida lipolytica-Y-1095* over a 120 hour fermentation period. Citric acid production was 5.69 g/L with a productivity of 0.047 g/L-h or 47 mg/L-h. n-Paraffin consumption was 43 %. Biomass concentration increased by a 171 fold of initial concentration.

Comparing Figures 7 and 8, *Candida lipolytica-Y-1095* seems to do a better job at assimilating n-paraffin to citric acid than *Candida lipolytica-Y-1094*. *Candida lipolytica-Y-1095* species produced a 104 % higher citric acid yield than the fermentation involving *Candida Y-1094*. The use of *Candida lipolytica-Y-1095* also resulted in a 99 % higher biomass concentration over *Candida lipolytica-Y-1094*. Both figures show the same trend in biomass, citric acid and n-paraffin concentrations.

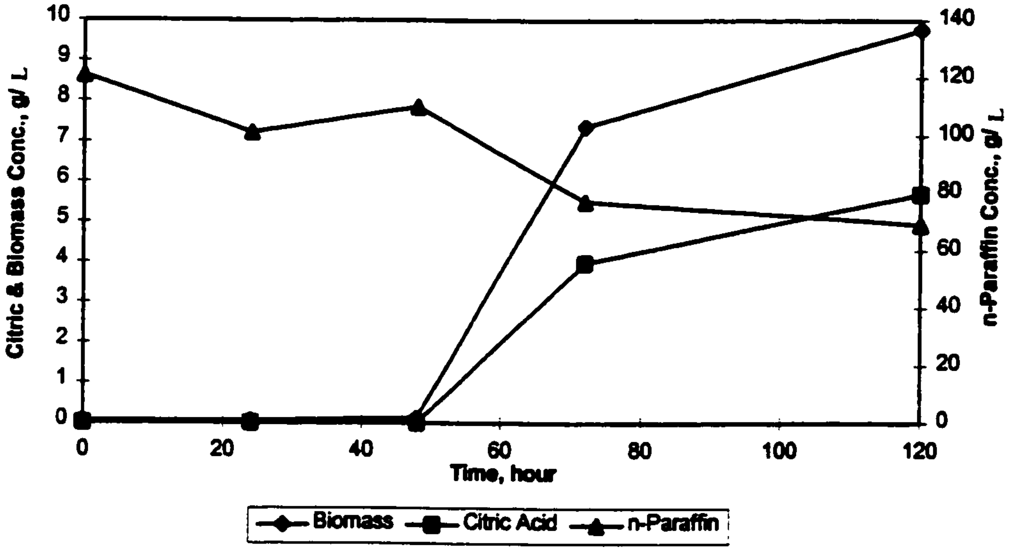


Figure 8: Citric Acid Fermentation using n-Paraffin and *Candida lipolytica-Y-1095*

There is a similar lag phase with no significant acid production until hour 40-50 when citric acid production begins.

In summary, it seems that the n-paraffin feedstock rather than the kerosene is more effectively assimilated by both *Candida* cultures. Higher citric acid and biomass yields were associated with n-paraffin solvent. As the kerosene solvent contains 20% aromatics, which are more soluble in aqueous liquids than long straight chain n-alkanes, and the yeast cannot readily break these aromatic carbon-carbon bonds, the kerosene may have been a toxic element to the yeast during growth and citric acid production.

3.3.2 Effect of Yeast Culture on Citric Acid Production

Figures 5 to 8 describe fermentations utilizing *Candida lipolytica-Y-1094* and *Candida lipolytica-Y-1095* under the influence of kerosene and n-paraffin feedstocks.

3.3.2.1 Fermentation with Candida lipolytica-Y-1094

Figures 5 and 7 show citric acid production using *Candida lipolytica-Y-1094* on kerosene and n-paraffin, respectively. Citric Acid yield with kerosene was 6.2×10^{-4} g citric acid/g kerosene consumed with a biomass yield of 8.2×10^{-3} g biomass/g kerosene consumed. Slightly higher yields were found when using n-paraffin as a feedstock, citric acid yield was 0.07 g citric acid/g n-paraffin consumed and biomass concentration was 0.11 g biomass/g n-paraffin consumed.

3.3.2.2 Fermentation with Candida lipolytica-Y-1095

Figures 6 and 8 describe citric acid production using *Candida lipolytica-Y-1095* on kerosene and n-paraffin, respectively. The kerosene fermentation resulted in a citric acid yield of 8.3×10^{-4} g citric acid/g kerosene consumed and a biomass yield of 6.7×10^{-3} g biomass/g kerosene consumed. The n-paraffin fermentation yielded higher concentrations for both citric acid and biomass, with citric acid yield of 0.11 g citric acid/g n-paraffin consumed and biomass yield of 0.19 g biomass/g n-paraffin consumed.

In summary, *Candida lipolytica-Y-1095* assimilated both hydrocarbon feedstocks more effectively for citric acid production than *Candida lipolytica-Y-1094*.

§3.4 Conclusions

Based on the results discussed in this chapter, it was found that the yeast of choice was *Candida lipolytica-Y1095* and the desired feedstock was the n-paraffin solvent. In particular, it was found that:

1. Fermenting n-paraffin solvent with *Candida lipolytica-Y-1095* resulted in citric acid yield of 0.11 g citric acid/g n-paraffin consumed, biomass yield of 0.19 g biomass/g n-paraffin consumed with a citric acid productivity of 47 mg/L-h;
2. Fermenting n-paraffin solvent with *Candida lipolytica-Y-1094* resulted in citric acid yield of 0.07 g citric acid/g n-paraffin, biomass yield of 0.11 g biomass/g n-paraffin consumed with a citric acid productivity of 23 mg/L-h;
3. Fermenting kerosene solvent with *Candida lipolytica-Y-1095* resulted in a citric acid yield of 8.3×10^{-4} g citric acid/g kerosene consumed, biomass yield of 6.7×10^{-3} g biomass/g kerosene consumed with a citric acid productivity of 0.17 mg/L-h;
4. Fermenting kerosene solvent with *Candida lipolytica-Y-1094* resulted in a citric acid yield of 6.2×10^{-4} g citric acid/g kerosene consumed, biomass yield of 8.2×10^{-3} g biomass/g kerosene consumed with a citric acid productivity of 0.12 mg/L-h.

CHAPTER 4:
Optimization of Biomass Inoculum, n-Paraffin Concentration, Iron Salt Concentration and Temperature for the Production of Citric Acid using *Candida lipolytica Y-1095*

§ 4.1 Introduction

The mechanism by which *Candida lipolytica* produce citric acid in large amount under certain conditions is still a problem of great interest. In general, unlimited growth of *C. lipolytica* in a rich medium results in minimal citric acid production. However, citric acid could be produced in great quantity if the growth of *C. lipolytica* was properly restricted (Kapoor et al., 1982; Milsom, 1987) during the citric acid producing phase of the fermentation cycle.

Growth restriction by carbon, nitrogen or phosphorus for high citric acid accumulation has been the subject of controversy. Some researchers say microorganisms need a fairly high initial concentration of carbon feedstock (15-18%) to induce citric acid accumulation in batch fermentations (Kapoor, et al., 1982). Xu et al. (1989) observed the optimal citric acid concentration at a sugar concentration of 10%. Honecker et al. (1989) reported that an initial sugar concentration of 20% led to maximal citric acid production by free cells. Kubicek and Rohr (1977) have shown citric acid production is possible in the presence of an excess of nitrogen provided that phosphate is limiting. Kristiansen and Sinclair (1978), on the other hand, considered that exhaustion of nitrogen to be a necessary prerequisite for citric acid production in batch fermentation. Kristiansen and

Sinclair (1979) reported that less citric acid was produced during phosphate-limited steady states than was obtained during the comparable nitrogen-limited states in a continuous fermentation.

The temperature of the fermentation process should be kept in the range of 26 to 35°C (Miall and Parker, 1974). The specific temperature is dependent on the organism used and equipment used. An optimal temperature range of ± 1°C should be determined in flask batch fermentations prior to initiating large scale fermentations.

Iron salts are essential to the production of citric acid as it activates the production of Acetyl co-enzyme A, essential for the production of citric acid (Milsom and Meers, 1985). However, an excess of iron will activate the production of aconitate hydratase (aconitase), the enzyme which catalyzes the production of iso-citric acid. Iso-citric acid is a by-product of the fermentation that is not desired, as a an isomer of citric acid, it takes away from potential yield.

Initial biomass inoculum is usually in the range of 10-12% volume (Moo Young et al., 1971; Rane and Sims, 1996; Blair et al., 1991). An excess in the biomass concentration will lead to high yields in biomass concentrations and lower citric acid concentrations (Whitworth and Moo Young, 1973), while a deficiency in biomass concentration will lead to a long lag in citric acid production.

Most of the previous works related to citric acid fermentation were conducted using "one-factor-at-a-time" technique. Unfortunately, it frequently fails to locate the region of optimum response, since the production does not take into account any joint effects of the factors on the response. An alternative approach is to lay out a "matrix" of all interesting combinations of the operating variables. The matrix method has the advantage of thoroughly exploring the experimental surface, but it requires a number of measurements too large to be explored in a realistic amount of time and resources. The response surface method (RSM) is preferred, because it can simultaneously consider several factors at many different levels and corresponding interactions among these factors using a small number of observations. Furthermore, statistical inference techniques can be used to assess the importance of the individual factors, the appropriateness of their functional form, and the sensitivity of the response to each factor (McLean and Burns, 1997). Recently, RSM has been employed to solve the multivariate problems and optimize several responses in many types of experimentation (Maddox and Richert, 1977; Giovanni, 1983; King and Zall, 1992).

In this study the RSM approach is adopted to locate the co-optimum levels of temperature, initial hydrocarbon concentration, initial biomass inoculum and iron salt concentration and to gain an insight of the interactions among these factors during the citric acid fermentation of *Candida lipolytica NRRL-Y1095* on n-paraffin.

§ 4.2 Material and Methods

4.2.1 Yeast Culture

The yeast culture used in this part of the study was *Candida lipolytica NRRL-Y1095* described in Appendix A, Section A.1. The lyophilized yeast was propagated by the method described in Appendix A, Section A.2.

4.2.2 Hydrocarbon Feedstock

The hydrocarbon feedstock used in this part of the study was the Norpar 15 solvent described in Appendix A, Section A.3.

4.2.3 Yeast Cell Growth and Fermentation Media

The media described in Tables A2, A3 and A4 in Appendix A, Section A.4 were used for the inoculation yeast seed culture, the pre-fermentation stage for yeast biomass growth and the fermentative stage for citric acid production, respectively. However, in this part of the study the hydrocarbon feedstock and yeast seed culture growth inoculation concentrations in the medium described in Table A3 (Appendix A) become 77 mL/L and 154 mL/L, respectively. For the medium described in Table A4 (Appendix A), the concentrations of ferric nitrate, hydrocarbon feedstock and pre-fermentation yeast growth inoculation are varied in the central composite design batch fermentations.

4.2.4 Experimental Protocols

4.2.4.1 Preparation of Yeast Seed Culture

A yeast seed culture was prepared by dispensing 600 ml of sterilized medium, as described in Table A2 (Appendix A), into a 2.0 litre Erlenmeyer flask. The medium was inoculated aseptically with three loopfuls, using a sterilized inoculating loop, of yeast culture that was maintained on glucose agar plates. The flask was fitted with foam bungs to minimize contamination of culture. Incubation occurred at 26 ± 1°C for 48 hours in a Lab-Line Instruments 3597 Environ-Shaker rotary shaker running at 120 rpm.

4.2.4.2 Preparation of Pre-Fermentation Stage - Inoculum Biomass Growth

The 48 hour pre-fermentation stage began by dispensing 2.0 litres of sterilized media, described in Table A3 (Appendix A), into a 4.0 L Erlenmeyer flask. Yeast culture inoculation was by way of aseptically dispensing the yeast seed culture growth into the flask. The inoculated medium was agitated at 26 ± 1°C in a Lab-Line Instruments 3597 Environ-Shaker rotary shaker running at 250 rpm and aerated with filtered air at 1.0 vvm.

4.2.4.3 Preparation of Fermentation Stage - Citric Acid Production

Thirty one batch fermentation bottle tests were performed to satisfy a central composite design. The central composite design investigated four parameters, initial biomass inoculum, n-paraffin concentration, ferric nitrate concentration, and temperature. Tables 2 and 3 describe how each parameter was varied in the bottle tests. Fermentation began by dispensing 200 mL of sterilized medium, described in Table A4 (Appendix A) and adjusted as per each parameter level described in Tables 2 and 3, into each of the thirty one 500 mL Erlenmeyer flasks. Yeast culture inoculation was accomplished by aseptically decanting the desired volume of pre-fermentation culture into the yeast-free fermentation medium. The batch fermentations were carried out at an agitation speed of 200 rpm in a Lab-Line Instruments 3597 Environ-Shaker rotary shaker and a New Brunswick Scientific Environmental Incubation Shaker, and at an aeration rate of 1.0 vvm. Fermentations were conducted for the duration of 5 days.

Table 2: Parameter Levels of Central Composite Design (Coded Values)

Bottle Number	Initial Biomass Inoculum	n-Paraffin Concentration	Ferric Nitrate Concentration	Temperature
1	-1	-1	-1	-1
2	+1	-1	-1	-1
3	-1	+1	-1	-1
4	+1	+1	-1	-1
5	-1	-1	+1	-1
6	+1	-1	+1	-1
7	-1	+1	+1	-1
8	+1	+1	+1	-1
9	-1	-1	-1	+1
10	+1	-1	-1	+1
11	-1	+1	-1	+1
12	+1	+1	-1	+1
13	-1	-1	+1	+1
14	+1	-1	+1	+1
15	-1	+1	+1	+1
16	+1	+1	+1	+1
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0
21	0	0	0	0
22	0	0	0	0
23	0	0	0	0
24	+2	0	0	0
25	-2	0	0	0
26	0	+2	0	0
27	0	-2	0	0
28	0	0	+2	0
29	0	0	-2	0
30	0	0	0	+2
31	0	0	0	-2

Table 3: Quantitative Values of the Coded Parameter Levels

Parameter	-2	-1	0	+1	+2
Initial Biomass Inoculum, %volume	5	7.5	10	12.5	15
n-Paraffin Concentration, % volume	5	7.5	10	12.5	15
Ferric Nitrate Concentration, mg/L	2	6.0	10	14	18
Temperature, °C	22	25	28	31	34

Samples were taken for biomass production, n-paraffin consumption and citric acid production analysis.

4.2.5 Analytical Methods

The analytical methods used are described in Appendix A, Section A.5. The biomass concentration was analyzed using the method outlined in Section A.5.1. The hydrocarbon concentrations were analyzed using the method outlined in Section A.5.2. The citric acid concentrations were analyzed using the method outlined in Section A.5.3.2.

§ 4.3 Results and Discussion

The raw data for the following experiments can be found in Appendix B. Details of the design can be found in Appendix C and D. The following discussion focuses on the central composite design and the effects of initial biomass inoculum, n-paraffin concentration, iron nitrate concentration and temperature on citric acid and biomass production.

RSM is a sequential procedure with an initial objective to lead the experimenter rapidly and efficiently to the general vicinity of the optimum. Since the location of the optimum is unknown prior to running the RSM experiment, it is essential to use a design that provides equal precision of estimation in all direction. In other words, rotability is a very important property in the selection of a response surface design. Although two-level factorial experiments will only yield data to fit a limited model, linear in all factors with some product terms, equation [1] (Myers, 1990), it and its functions are the most common initial experiments in the study of response surfaces, because orthoganility of the design minimizes the variance of the regression coefficients and any first-order (two-level) orthogonal design is rotatable (McLean and Burns, 1997; Thompson, 1982). The experimental raw data is presented in Appendix B and the model design in Appendix C.

The data collected from the batch runs were used to develop empirical models describing the experimental system. The models were generated using the method of least squares. The technique involves the estimation of model parameters for second order models of the form:

$$E(Y) = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \sum_{j\geq 1}^{k} \beta_{ij} X_i X_j \qquad [1]$$

where

E(Y) is the expected value of the response variable
β_o, β_i, β_j are the model parameters
X_i and X_j are the coded factors being studied
k is the number of factors being studied

The development of the empirical models for citric acid production and biomass production are presented in Appendix D with sample calculations presented in Appendix F. Table 4 summarizes the parameter estimates obtained by least-squares analysis for citric acid and biomass production.

Table 4: Summary of Empirical Models Developed for Citric Acid and Biomass Production Model Response Parameter Estimates (Quantitative Values)

Parameter	Citric Acid, g/L	Biomass, g/L
β_0	8.85 ± 0.39	4.61 ± 0.11
β_1	0.99 ± 0.21	0.46 ± 0.06
β_2	1.33 ± 0.21	0.48 ± 0.06
β_3	0.49 ± 0.21	0.18 ± 0.06
β_4	0.52 ± 0.21	0.29 ± 0.06
β_{11}	-1.03 ± 0.20	-0.23 ± 0.06
β_{22}	-0.71 ± 0.20	-0.21 ± 0.06
β_{33}	-0.91 ± 0.20	-0.10 ± 0.06
β_{44}	-1.27 ± 0.20	-0.40 ± 0.06
β_{12}	---	-0.18 ± 0.07
β_{23}	---	0.17 ± 0.07
R^2	0.900	0.925
F (regression)	19.1	22.89
$F_{.05,v1,v2}$ (regression)	2.40	2.35
F (lack of fit)	10.29	2.0
$F_{.05,v1,v2}$ (lack of fit)	3.93	3.96

β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} were not considered in the citric acid model since the p-levels for these parameters were greater than 0.05. While β_{13}, β_{14}, β_{24}, β_{34} were not considered in the biomass model since the p-levels were greater than 0.05, see Appendix D for details. Therefore, substituting the corresponding β parameter values found in Table 4 into equation [1], the equations for the citric acid and biomass models become:

Citric Acid Production Model:

$$\begin{aligned} E(Y) = 8.85 + 0.99X_1 + 1.33X_2 + 0.49X_3 + 0.52X_4 \\ - 1.03X_1^2 - 0.71X_2^2 - 0.91X_3^2 - 1.27X_4^2 \end{aligned} \qquad [2]$$

Biomass Production Model:

$$E(Y) = 4.61 + 0.46X_1 + 0.48X_2 + 0.18X_3 + 0.29X_4 - 0.23X_1^2 - 0.21X_2^2 - 0.10X_3^2 - 0.40X_4^2 - 0.18X_1X_2 + 0.17X_2X_3 \quad [3]$$

The responses to equations [2] and [3], at various coded levels of temperature, initial biomass inoculum, initial n-paraffin concentration and ferric nitrate concentration, are described in Figures 9 and 10 for citric acid and biomass production, respectively. Both Figures 9 and 10 show quantitative values for citric acid and biomass production, whereas the biomass inoculum, n-paraffin concentration, ferric nitrate concentration and temperature are represented as their coded values.

Figure 9 indicates that optimal citric acid production occurs at an initial biomass inoculum of 10-12 % volume, an initial n-paraffin concentration of 10-15 % volume, a ferric nitrate concentration of 10 mg/L and a temperature of 26-30 °C. The figure shows how crucial the iron salt (ferric nitrate) is to the production of citric acid. Levels as low as 2 mg/L and as high as 18 mg/L can inhibit the production of citric acid at a low n-paraffin level of 5% volume. However a level of 10 mg/L, at the same level of n-paraffin, can produce approximately 3.4 g/L citric acid.

Figure 10 shows that the optimal level of biomass production occurs at an initial biomass concentration between 12-15 % volume, an initial n-paraffin concentration of 10-15 % volume, a ferric nitrate concentration of 10-18 mg/L and a temperature of 26-30 °C. Here, very low concentrations of n-paraffin and ferric nitrate do not inhibit biomass production as significantly as in citric acid production.

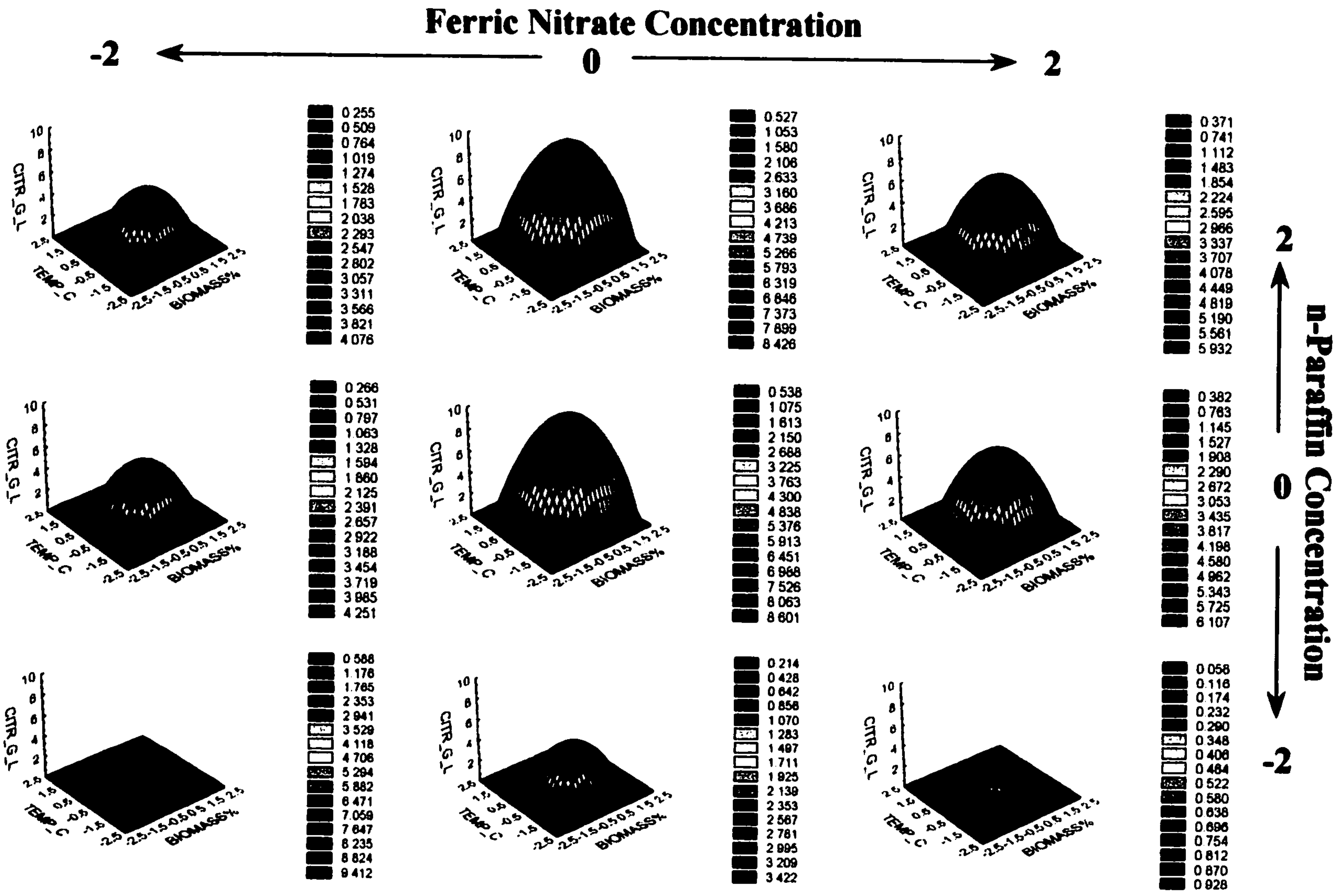


Figure 9: Surface Plots of the Empirical Model of the Effect of Initial Biomass Inoculum, n-Paraffin Concentration, Ferric Nitrate Concentration and Temperature (all parameters are coded values) on the Production of Citric Acid (quantitative value)

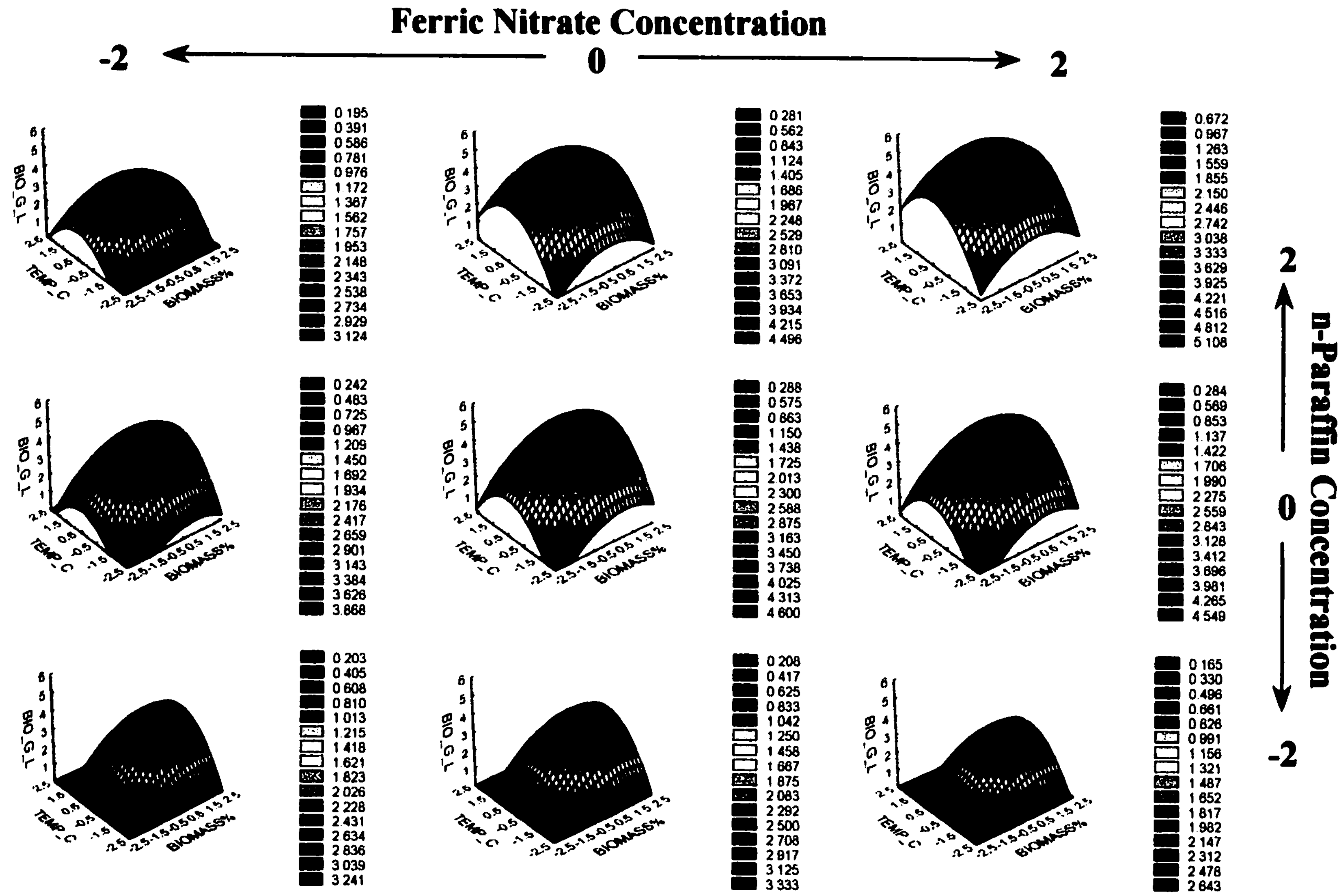


Figure 10: Surface Plots of the Empirical Model of the Effect of Initial Biomass Inoculum, n-Paraffin Concentration, Ferric Nitrate Concentration and Temperature (all parameters are coded values) on the Production of Biomass (quantitative value)

Figures 11 and 12 show normal probability plots for both the citric acid and biomass production models. The residuals for both models appear to follow a normal distribution. The data, in both graphs, follow the line representing a normal distribution.

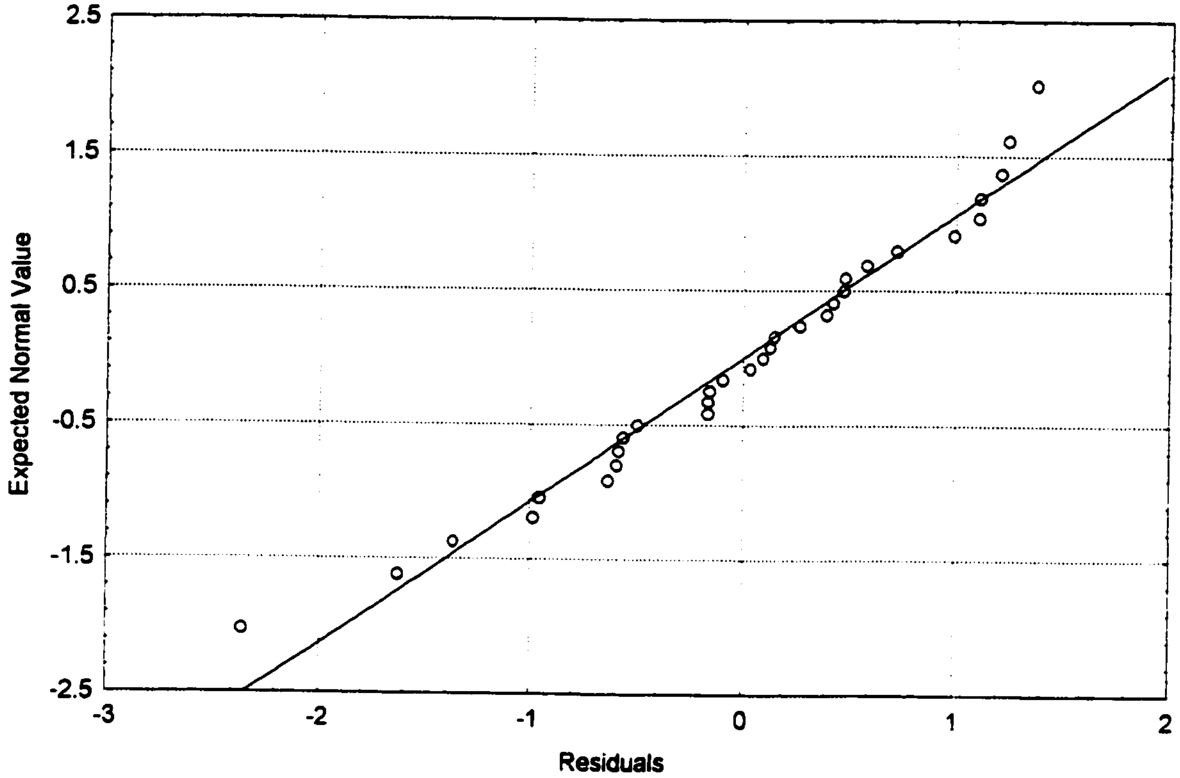


Figure 11: Normal Probability Plot for the Residuals from the Citric Acid Production Model

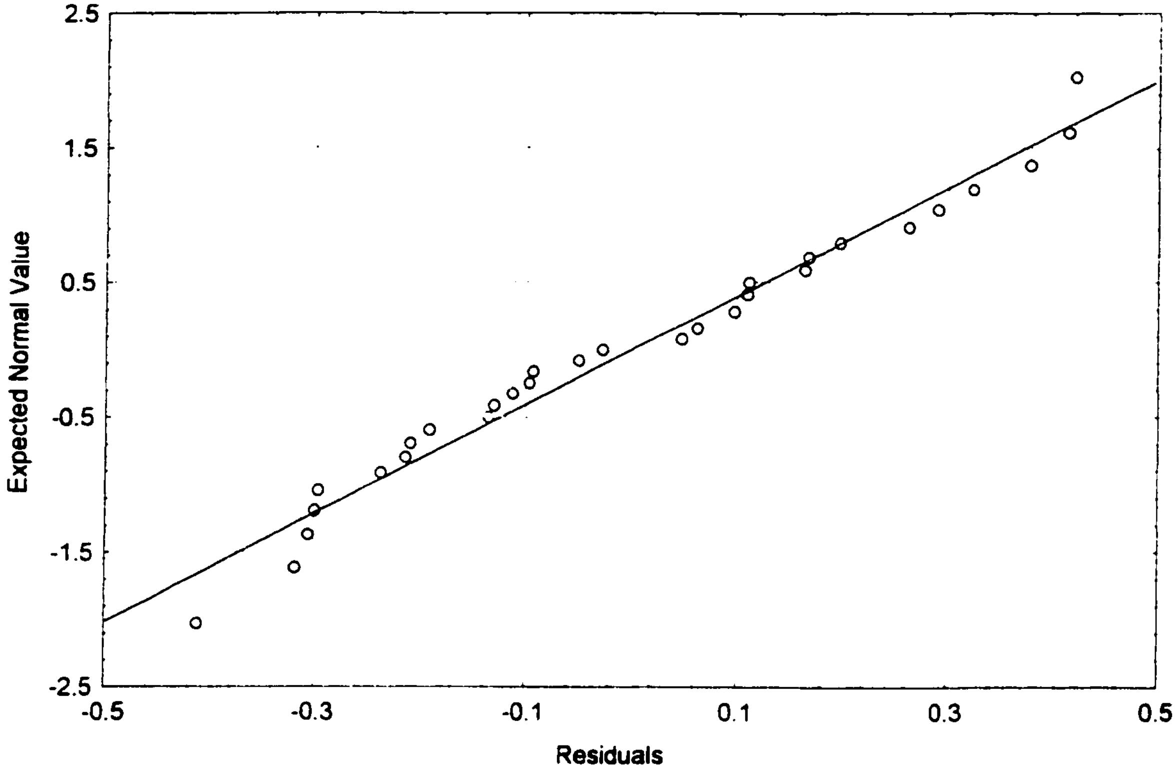


Figure 12: Normal Probability Plot for the Residuals from the Biomass Production Model

Figures 13 and 14 show the residual plots versus the predicted values of the response. These figures indicate a constant variance. The equal scatter of the residual data above and below the x-axis indicates that the variance was independent of the value of the citric acid and biomass production.

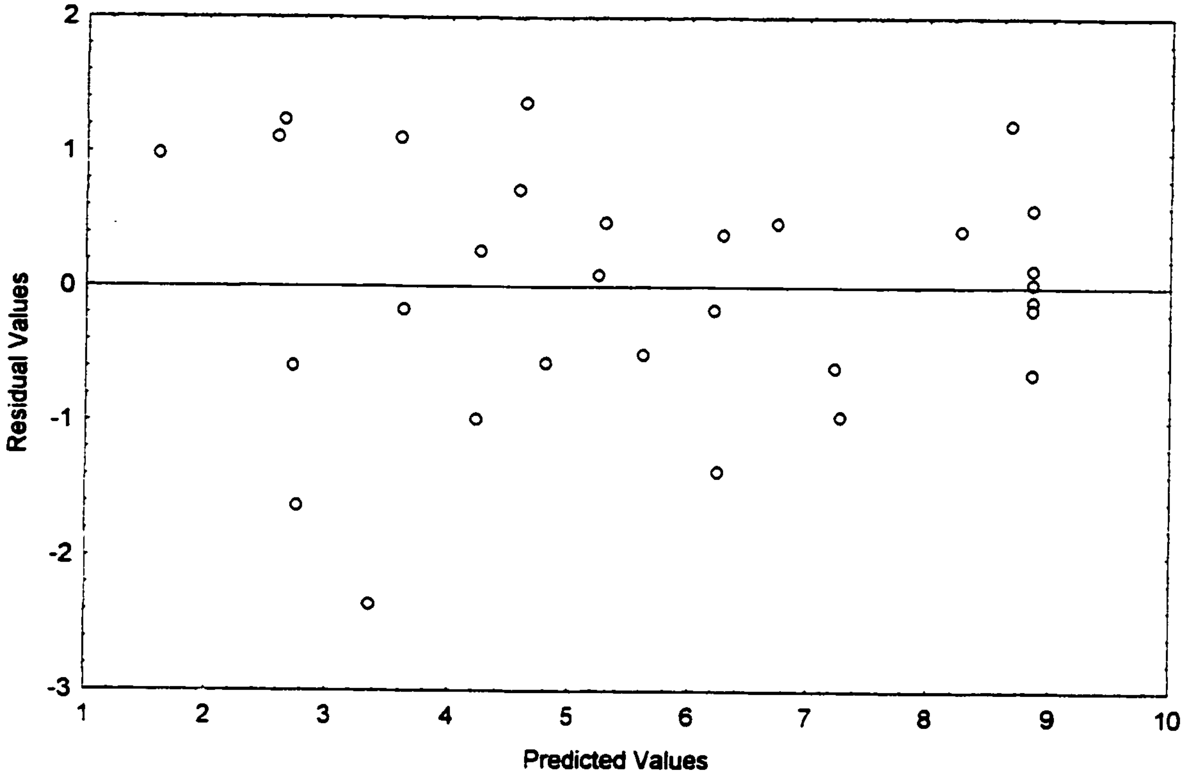


Figure 13: Residual Plot for Citric Acid Production Model

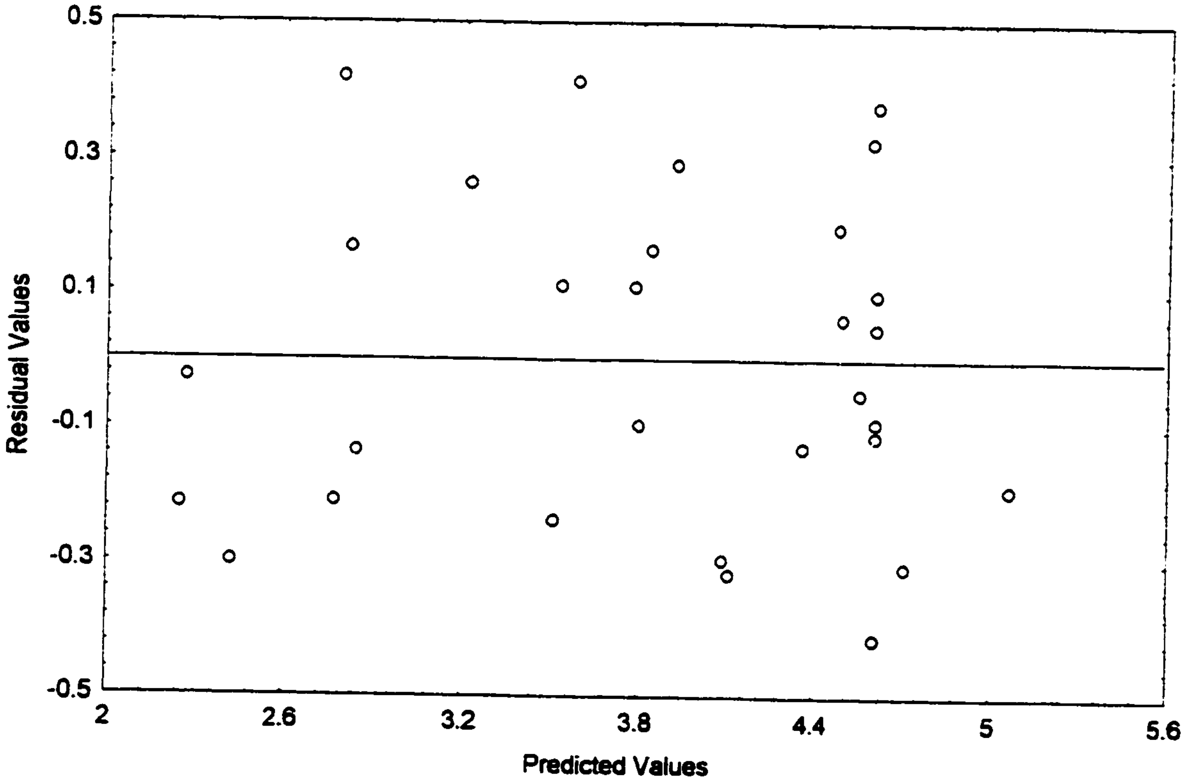


Figure 14: Residual Plot for Biomass Production Model

§ 4.4 Conclusions

From results obtained during this study, it was found that:

1. Ferric nitrate and initial n-paraffin concentrations had significant effects on the production of citric acid;
2. The optimal level of temperature was found to be 26-30°C for both citric acid and biomass models;
3. The optimal level of initial biomass inoculum was found to be 10-12 % volume for citric acid production and 12-15 % volume for biomass production;
4. The optimal level of initial n-paraffin concentration was found to be 10-15 % volume for both models;
5. The optimal level of ferric nitrate concentration was found to be 10 mg/L for citric acid production and 10-18mg/L for biomass production;
6. Both the citric acid and biomass production models have normal distributions and constant variances.

CHAPTER 5:
Citric Acid Production - Investigation of Aeration and Agitation Effects in Batch and Fed-Batch Systems

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§5.1 Introduction

The fermentation pattern for citric acid production demonstrates a biomass growth phase followed by a citric acid accumulation phase, the transition being brought about by microbial nitrogen limitation (Milsom and Meers, 1985). When yeast cells find themselves in a nitrogen limited condition and carbon excess environment, they are forced to store carbon through the Krebs Cycle. The carbon substrate is oxidized to CO_2 via the tricarboxylic acid cycle, Figure 2 in Chapter 2, where citric acid is accumulated. When n-paraffins are used as substrate they are converted by β-oxidation to acetyl coenzyme A which is combined with oxaloacetic acid to produce citric acid by means of citrate synthetase. Many yeasts have the ability to accumulate high concentrations of citric acid during tricarboxylic acid cycle respiration. *Candida lipolytica* is one species known for maximizing citric acid production while assimilating either carbohydrate or hydrocarbon substrates (Milsom and Meers, 1985; Miall and Parker, 1972).

When producing citric acid through n-paraffin fermentation, as in all fermentations, preparation of culture inoculum should not be taken for granted. The development of the inoculum, through various stages, has a definite effect on the subsequent performance of the process. In some cases poor management of the inoculum can result in an almost complete loss of productivity. A fermentation process requires biomass which is highly efficient and predictable in performance (Stanbury et al., 1995; Parton and Willis, 1990).

In a laboratory fermentation the inoculum stages rarely exceed two stages, whereas as many as six may be required for certain production processes (Parton and Willis, 1990). The n-paraffin batch fermentations studied in this chapter employ a two stage inoculum growth. The preparation of the primary inoculum (seed culture) occurs in a shaker flask where it provides a biomass growth which is used to start the secondary inoculum culture. The secondary inoculum (pre-fermentation stage) takes place in a fermentor (same fermentor vessel as in fermentation stage) to provide a fermentor-stirred culture grown under controlled parameters, such as medium composition, agitation speed, aeration rate, temperature and pH. Subsequently the secondary inoculum growth is used to start the fermentation stage, where biomass production is continued but quickly hindered as nitrogen is soon depleted and citric acid production/accumulation begins.

The scaled-up pre-fermentation stage (scaled-up to Chemap fermentor) inoculum preparation serves two purposes; to supply an inoculum of optimum size and to provide a viable inoculum culture with a reproducible and reliable baseline of operating parameters. To satisfy these criteria, a fermentor is implemented in the cultivation of a secondary inoculum as the vessel size and control over operating parameters is significantly greater than shaker flask cultivation. Controlling fermentor parameters in both pre-fermentation and fermentation stages at inoculum transfer assists in reducing the lag phase in the fermentation stage, since culture adaptation under changing fermentor environment can be kept to a minimum. Lincoln (1960) states that the lag phase in a fermentation is minimized by growing the culture in the 'final type' medium. Lincoln's argument is that the inoculum development medium should be sufficiently similar in composition, pH and temperature to the production medium to minimize any period of adaptation of the culture to the production medium. Stanbury et al. (1995) adds that a major difference in pH and osmotic pressures may cause sudden changes in uptake rates which, in turn, may affect viability.

In hydrocarbon fermentation there are at least two physical problems of engineering interest which are crucial for hydrocarbon fermentations. The first relates to the very high oxygen demand per unit cell mass and the other arises from the limited solubility of hydrocarbon oils in aqueous media (Moo-Young et al., 1971). Furthermore, Johnson (1964) and Aiba et al.(1969), have suggested that dissolved hydrocarbon oil may not be the main source of substrate supply, and that rather uptake occurs by cell attachment to droplets of oil.

Oxygen is supplied to the culture in the form of air sparging through the fermentation media. Stanbury et al. (1995) represented the transfer of oxygen from air to the cell as occurring in a three steps:

i. The transfer of oxygen from an air bubble into solution.

ii. The transfer of the dissolved oxygen through the fermentation medium to the microbial cell.

iii. The uptake of the dissolved oxygen by the cell.

Both aeration and agitation affect the extent of dissolved oxygen in the medium. Aeration rates serve as the initial volume of air bubbles introduced to the system whereas agitation rates serve to assist oxygen transfer in the following ways (Stanbury et al., 1995):

i. Agitation increases the area available for oxygen transfer by dispersing air in the culture fluid in the form of small bubbles.
ii. Agitation delays the escape of air bubbles from the liquid
iii. Agitation prevents coalescence of air bubbles.
iv. Agitation decreases the thickness of the liquid film at the gas-liquid interface by creating turbulence in the culture fluid.

Agitation in hydrocarbon fermentations is also crucial to the dispersion of oil droplets throughout the medium. Moo-Young et al. (1971) found that smaller oil droplets, the result of higher agitation rates, resulted in greater assimilation of the hydrocarbon source by the yeast. However agitation rates running too high can impose an dangerously large shear stress on cell walls, ultimately decreasing or destroying cell viability.

The purpose of this study is to determine the effects of aeration and agitation on cell growth and citric acid production. These parameters were experimentally evaluated in a batch and fed-batch systems utilizing n-paraffin as the hydrocarbon feedstock and *Candida lipolytica NRRL-Y-1095* as the organism.. The optimal operating levels determined in Chapter 4 are implemented in these fermentations.

§5.2 Materials and Methods

5.2.1 Yeast Culture

The yeast culture used in this part of the study was *Candida lipolytica NRRL-Y1095* described in Appendix A, Section A.1. The lyophilized yeast was propagated by the method described in Appendix A, Section A.2.

5.2.2 Feedstock

The hydrocarbon feedstock used in this part of the study was the Norpar 15 solvent described in Appendix A, Section A.3.

5.2.3 Yeast Cell Growth and Fermentation Media

5.2.3.1 Batch Systems

The media described in Tables A2, A3 and A4 in Appendix A, Section A.4, were used for the inoculation yeast seed culture, the pre-fermentation stage for yeast biomass growth and the fermentative stage for citric acid production, respectively. However, in this part of the study the hydrocarbon feedstock and yeast seed culture growth inoculation concentrations in Table A3 (Appendix) become 77 mL/L and 154 mL/L, respectively. For the medium described in Table A4 (Appendix A), the concentrations of ferric nitrate, hydrocarbon feedstock and pre-fermentation yeast growth inoculation become 10 mg/L, 143 mL/L and 143 mL/L respecitvely.

5.2.3.2 Fed-Batch Systems

The propagation of a yeast seed culture, serving as the inoculation culture, was conducted using the defined medium described in Table A2 (Appendix A). The media used for the pre-fermentation stage biomass growth and fermentation stage citric acid production are described in Tables A3 and A4, respectively. The medium used for the feeding of the fermentation stage in the 7 day fed-batch systems is described in Table A5 in Appendix A, Section A.4. The medium used for the feeding of the fermentation stage in the 18 day fed-batch systems is described in Table A6 in Appendix A, Section A.4. This medium is similar to that described in Table A5, however a small amount of nitrogen is added to medim to help maintain yeast cell viability throughout the fermentation.

5.2.4 Experimental Protocols

5.2.4.1 Preparation of Yeast Seed Culture for Batch and Fed-Batch Systems

A yeast seed culture was prepared by dispensing 600 ml of sterilized medium, as described in Table A2, into a 2.0 litre Erlenmeyer flask. The medium was inoculated aseptically with three loopfuls, using a sterilized inoculating loop, of yeast culture that was maintained on glucose agar plates. The flask was fitted with foam bungs to minimize contamination of culture. Incubation occurred at 26 ± 1°C for 48 hours in a Lab-Line Instruments 3597 Environ-Shaker rotary shaker running at 120 rpm.

5.2.4.2 Preparation of Pre-Fermentation Stage for Batch and Fed-Batch Systems-Biomass Growth

The 48 hour pre-fermentation stage began by dispensing 3.9 litres of sterilized medium, described in Table A3, into a 14 L Chemap AG Fermentor (24.7 x 31.1 cm cylindrical glass vessel). Yeast culture inoculation was by way of aseptically dispensing the yeast seed culture growth into the fermentor. The inoculated medium was agitated at 28 ± 1°C with a standard four bladed (3.8 x 0.2 x 18.4 cm stainless steel impeller) open turbine at various speeds (400, 800, 1000, 1200 rpm) and aerated with filtered air at various rates (0.5,1.0,2.0 vvm).

5.2.4.3 Preparation of Fermentation Stage for Batch Systems - Citric Acid Production

Fermentation began by dispensing 7 litres of sterilized medium, as described in Table A3, into the 14 L Chemap Fermentor. Yeast culture inoculation was accomplished by aseptically draining 3/4 of the pre-fermentation culture from the fermentor and adding yeast-free fermentation medium. The fermentation was carried out at 28 ± 1°C for 5 days. Agitation, with a four bladed open turbine, and aeration, with filtered air, were applied at various rates, see section 5.2.4.2.

5.2.4.4 Preparation of Fermentation Stage for Fed-Batch Systems - Citric Acid Production

Fermentation began by dispensing 4 litres of sterilized medium, as described in Table A3, into the 14 L Chemap Fermentor. Yeast culture inoculation was accomplished by aseptically draining 3/4 of the pre-fermentation culture from the fermentor and adding yeast-free fermentation medium described in Table A4. The fermentation was carried out at 28 ± 1°C for 5 days. Feeding of the fed-batch fermentation with feeding medium, described in Tables A5 and A6 (Appendix A), was done using a Cole-Parmer peristaltic pump, where the final volume in the fermentor was taken to 7 litres. The 7 day fed-batch system was fed at the 96 hour mark with medium described in Table A5. The 18 day fed-batch system was fed at the 96, 216, 336 hour marks with the medium described in Table A6. The feeding rates varied as 0.75, 1.0 and 1.5 L/day.

Agitation, with a four blade open turbine, was supplied at various rates, 400, 800, 1000, 1200 rpm and aeration supplied with filtered air at 1.0 vvm. When external agitation was implemented into the design, a Greey Lightnin In-Line Agitator was used to agitate the broth being re-circulated from the fermentor, into the agitator, then back into the fermentor, see Figures 3 and 4 in Chapter 2. External agitation was investigated at 1400 and 1600 rpm at a re-circulation rate of 4.0 L/min. Samples were taken every 12 hours for biomass production, n-paraffin consumption and citric acid production analysis.

5.2.5 Analytical Methods for Batch and Fed-Batch Systems

The analytical methods used are described in Appendix A, Section A.5. The biomass concentration was analyzed using the method outlined in Section A.5.1. The hydrocarbon concentrations were analyzed using the method outlined in Section A.5.2. The citric acid concentrations were analyzed using the method outlined in Section A.5.3.2.

§5.3 Results and Discussion

Details of the experiments and associated raw data can be found in Appendix A and B, respectively. All fermentations involved a "growth phase" for biomass growth and "production phase" for citric acid production (as nitrogen is depleted the yeast go into citric acid production). The following discussion focuses on aeration and agitation influences on both phases in batch and fed-batch n-paraffin fermentations. This chapter also demonstrates the effects of agitation, resulting from mechanical agitation from the fermentor and in-line agitator, on biomass and citric acid production in fed-batch systems. It should be noted that "yield" referred to in the discussion is the amount of citric acid produced per n-paraffin consumed at a certain point in time in the fermentation. However "overall yield" referred to in the discussion refers to the total amount of citric acid produced per the total n-paraffin consumed at the end of the fermentation.

5.3.1 Effect of Aeration on Biomass and Citric Acid Production - Batch Systems

Three different aeration rates were investigated, 0.5, 1.0 and 2.0 vvm while maintaining an agitation rate of 400 rpm. Figures 15 and 16 describe the production of biomass and citric acid under the influence of the various aeration rates. Table 5 summarizes overall biomass and citric acid yields and productivity as affected by aeration. Reviewing Figure 15(a), in the first 48 hours (growth phase) aeration of 1.0 vvm produced the highest biomass concentration, 8 g/L. While the subsequent 120 hour citric acid production phase results in the 0.5 vvm aeration producing the highest biomass concentration of 10.9 g/L. Considering biomass yields, Figure 15(b) shows that in the growth phase an aeration of 0.5 vvm resulted in highest biomass production with a biomass yield of 0.5 g biomass/g n-paraffin-consumed. In the citric acid production phase, the aeration of 0.5 vvm generated the highest production with a biomass yield of 0.5 g biomass/g n-paraffin-consumed about 25 hours into the production phase, then dropping to 0.25 g biomass/g n-paraffin-consumed by the end of the phase (hour 168). While overall biomass yield, shown in Table 5, supports that an aeration of 1.0 vvm generated the highest biomass production with an overall biomass yield value of 0.23 g biomass/g n-paraffin-consumed. The 2.0 vvm fermentation has the lowest production of biomass in both growth and production phase. A possible explanation is that if the impeller is unable to disperse the incoming air, then low oxygen transfer rates are achieved due to the impeller becoming 'flooded' (Stanbury et al., 1995). Stanbury et al. describe flooding as the phenomenon where the air-flow dominates the flow pattern and is due to an inappropriate combination of air flow rate and speed of agitation. Even though the dissolved oxygen probe recorded dissolved oxygen values in the range of 74-78%, the probe may have been in the line of the dominating air flow and not recording representative dissolved oxygen values of the dispersed medium.

Moo-Young et al. (1971) reported biomass production in the range of 4.5 to 5.0 g/L under an aeration of 1.0 vvm and agitation of 500 rpm during a 48 hour hydrocarbon fermentation. Biomass production (Figure 15(a)) in this study, at 1.0 vvm and 400 rpm, shows a slightly higher biomass concentration of 8 g/L 48 hours into the production phase of the fermentation. Alternatively Furukawa et al. (1977) reported a biomass concentration of 12.1 g/L, at 0.5 vvm and 600 rpm, during a 93 hour hydrocarbon fermentation, while Figure 15(a) of this study shows a lower biomass production of 10.9 g/L, at 0.5 vvm and 400 rpm 93 hours into the production phase. Although the current study on biomass concentrations during fermentation differ slightly from previous studies the growth profiles are consistent. The production of biomass enters an exponential phase, after a short lag phase, prior to reaching a stabilization in growth.

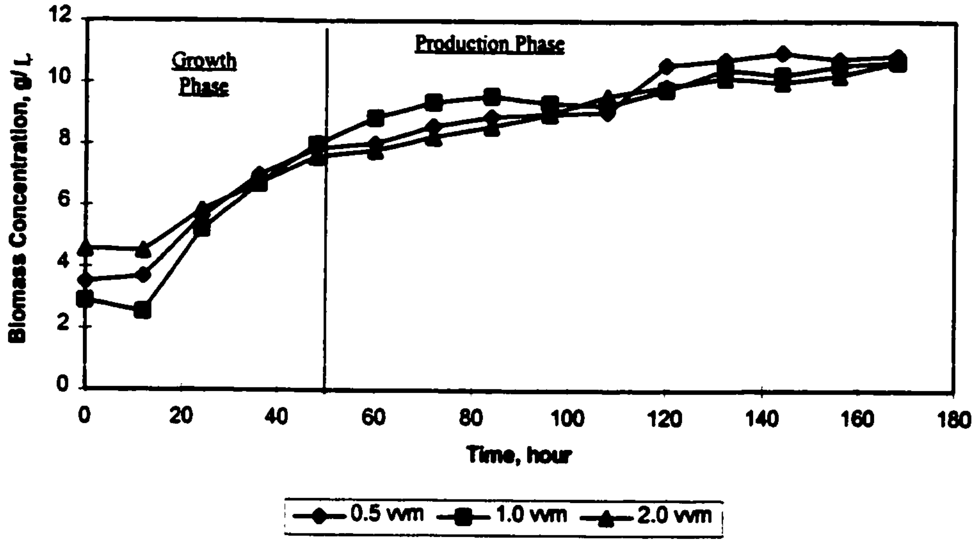
The current data observations of an increase in biomass production during the fermentation stage is consistent with Kristiansen et al. (1978) findings. Kristiansen et al. found that even under nitrogen limited environments (indicated as production phase in this discussion) biomass production continued. He attributed this growth to carbon storing cells and concluded that cells under excess carbon not only store carbon for citric acid production but also for biomass production.

Figure 16(a) shows the production of citric acid during the fermentation stage. No citric acid production occurs in the growth phase. During the initial 92 hours of the production phase the 1.0 vvm aerated fermentation results in the highest citric acid production, 4.25 g/L. However in the final 28 hours the 2.0 vvm aerated fermentation surpasses this to a final citric acid production of 4.8 g/L. Considering citric acid yields, Figure 16(b), the highest citric acid yield of 0.23 g citric acid/g n-paraffin-consumed is produced by the 1.0 vvm aerated fermentation during the initial 48 hours of the production phase, while it ends the fermentation with a lower production yield of 0.15 g citric acid/g n-paraffin-consumed. Increasing aeration from 0.5 to 1.0 vvm resulted in increasing citric acid production. The higher aeration rate increased the rate of oxygen transfer throughout the medium and therefore increased the production of citric acid. The possible 'flooding' of the impeller at an aeration of 2.0 vvm affected citric acid production resulting in the lowest citric acid yield of approximately 0.1 g citric acid/g n-paraffin consumed.

Citric acid concentrations found in this study are lower than some published values. Furukawa et al. (1977) reported citric acid production of 65 g/L while using a mutant strain of the parent *Candida lipolytica* in a n-paraffin fermentation for 93 hours at 0.5 vvm and 600 rpm. Akiyama et al. (1973) shows citric acid production to be 60 g/L using the parent *Candida lipolytica* in a n-paraffin fermentation for 72 hours in a 200 ml shake flask at 200 rpm on a rotary shaker. Whereas in this study the highest citric acid concentration achieved was 4.8 g/L in a 120 hour production phase fermentation. While the magnitude of citric acid production is different from the published values, the production profile is still consistent. The lag phase is virtually non existent, and the exponential phase is sharp followed by a slow stabilization of production.

All the fermentations experience peaking of citric acid yields in the first 24 to 48 hours of the production phase. Table 5 shows the highest overall citric acid yield at an aeration rate of 1.0 vvm with 0.13 g citric acid/g n-paraffin-consumed and an average productivity of 0.04 g/L-h. Theoretically, a peaking citric acid yield should be maintained for more than a 12-24 hour period and not experience such a drastic decline in production yields, as is seen in all the fermentations of the current study.

a) Biomass Production



b) Biomass Yield

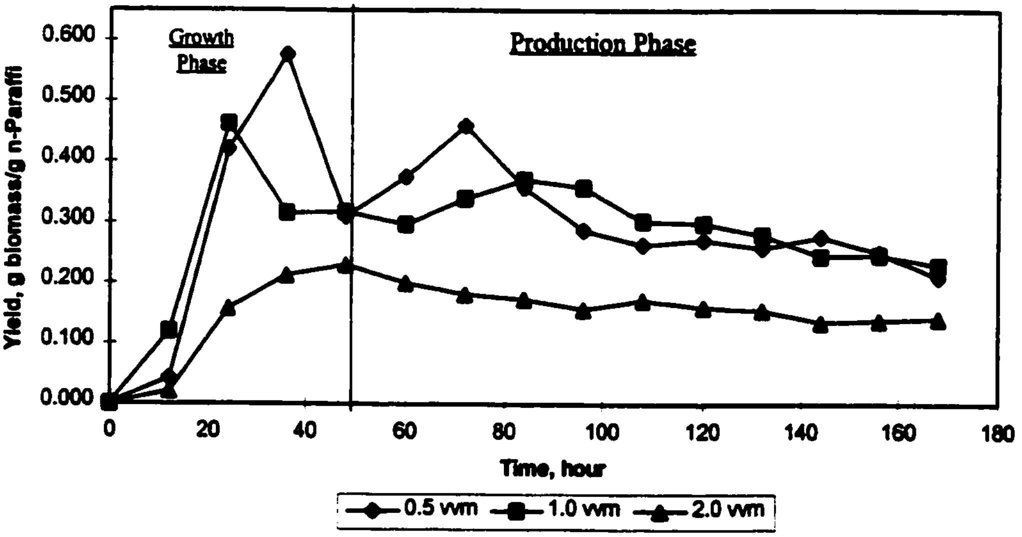
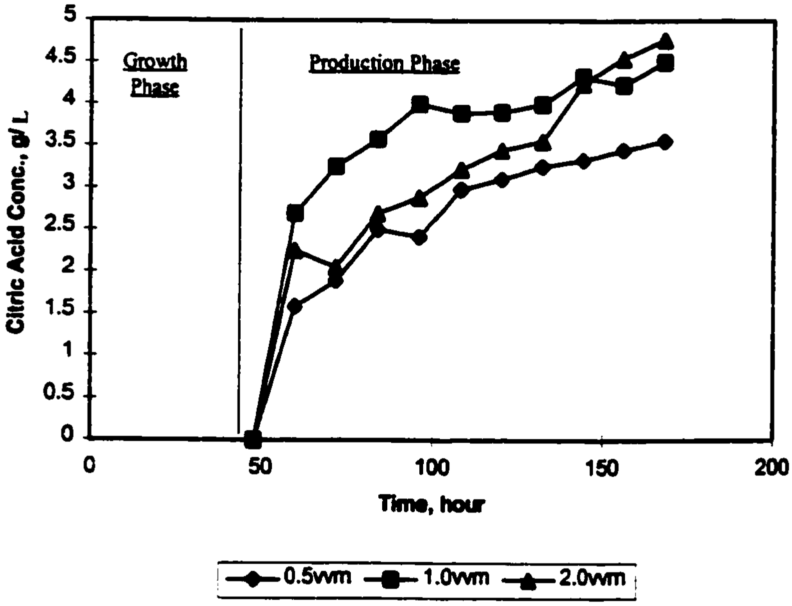


Figure 15: Aeration Effects on Biomass Production in Batch n-Paraffin Fermentation at 400rpm and 28 ± 1°C

a) Citric Acid Production



b) Citric Acid Yield

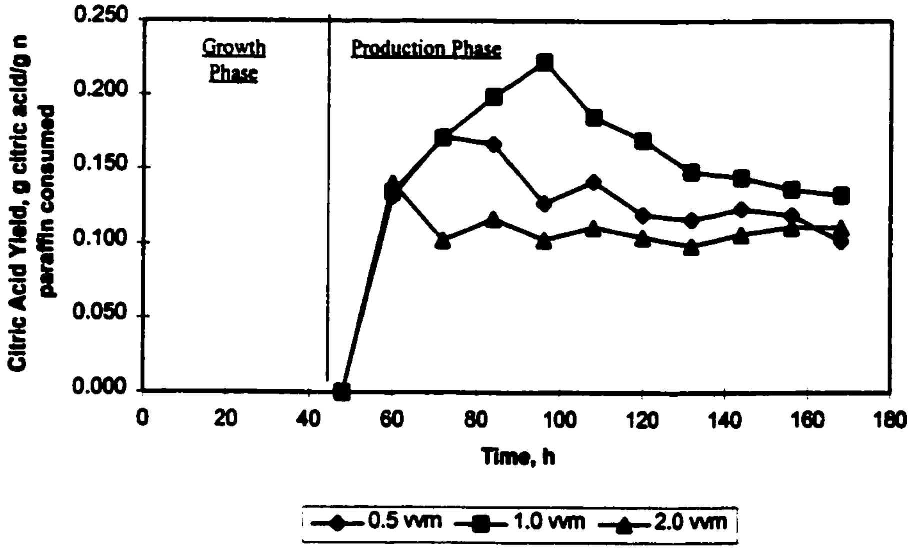


Figure 16: Aeration Effects on Citric Acid Production in Batch n-Paraffin Fermentation at 400 rpm and 28 ± 1°C

Table 5: Summary of Aeration Effects on Biomass and Citric Acid Production in Batch Fermentation (120 hour production phase)

Parameter	0.5 vvm*	1.0 vvm*	2.0 vvm*
Overall Biomass Yield, g biomass/g n-paraffin consumed	0.21	0.23	0.14
Overall Citric Acid Yield, g citric acid/g n-paraffin consumed	0.10	0.13	0.11
Overall Citric Acid Productivity, g/L-h	0.03	0.04	0.04

* All fermentations under agitation rate of 400 rpm

5.3.2 Effect of Agitation on Biomass and Citric Acid Production - Batch Systems

Four agitation rates were investigated, 400, 800, 1000 and 1200 rpm. Figures 17 and 18 describe biomass and citric acid production under the influence of these agitation rates, respectively, while maintaining aeration rates of 1.0 vvm. Table 6 summarizes overall biomass and citric acid yields along with citric acid productivity. Figure 17(a) shows an increase in biomass production with agitation, in the growth phase. Biomass production in the 1000 and 1200 rpm fermentations is highest at 8 g/L at the end of the growth phase. In the initial 48 hours of the production phase the agitation rate is not effecting the production of biomass, while in the final 20 hours of production phase the highly agitated fermentations, 1000 rpm and 1200 rpm, produce the highest biomass concentrations of 14.8 and 14.6 g/L, respectively. Considering biomass yield, all the fermentations have decreasing yields during the growth phase. The 800 and 1000 rpm fermentation produces the highest biomass yield of 0.57 g biomass/g n-paraffin-consumed in the growth phase. All fermentations experience a biomass yield peak in the initial 12 to 48 hours of the production phase with a subsequently decreasing biomass yield. In the production phase, the 800 rpm agitated fermentation produces the highest peaking biomass yield of 0.5 g biomass/g n-paraffin-consumed. The overall biomass yields, Table 19, show consistent values between 0.22-0.27 g biomass/g n-paraffin-consumed. The observed increasing biomass production with agitation can be attributed to the fact that as agitation increases two things occur, oxygen transfer from air bubbles into solution increases as impellers efficiently disperse incoming air and n-paraffin oil droplets are smaller, greater in number and more adequately dispersed throughout the medium. Hence the higher agitation rates allow for higher transfer rates of oxygen and n-paraffin carbon into the cells for biomass production.

Moo-Young et al. (1971) reported biomass production of 4.5 g/L 48 hours into two n-paraffin fermentation at 500 rpm and 1.0 vvm and 800 rpm and 1.0 vvm. However, in the current study biomass production 48 hours into the growth phase of each agitated fermentation is significantly higher; the 400, 1000 and 1200 rpm agitated fermentations demonstrate a biomass concentration of 8.0 g/L, while the 800 rpm agitated fermentation demonstrates a biomass concentration of 7 g/L. Each fermentation in the study goes on to produce even higher biomass concentrations in the production phase, with the 1000 and 1200 rpm conditions having the highest biomass production in the range of 14.6-14.8 g/L. All four fermentation runs seem to be going through a lag in biomass production during the initial 48 hours of the production phase.

Figure 18(a) shows citric acid concentrations increasing with agitation. The 1200 rpm agitated fermentation produces the highest citric acid concentration of 9.5 g/L. Considering citric acid yield, Figure 18(b), the 1200 rpm agitated fermentation results in the highest yield of 0.3 g citric acid/g n-paraffin-consumed. All fermentations experience peaking citric acid yields in the initial 48 to 72 hours of the production phase, followed by a decrease in citric acid yield. The highest overall citric acid yield, Table 6, of 0.21 g citric acid/g n-paraffin-consumed is shared by both the 1000 rpm and 1200 rpm agitated fermentations, respectively. However the 1000 rpm fermentation has the highest overall productivity of 0.07 g/L-h.

Citric acid production is highest at higher agitation levels. As discussed in biomass production high agitation results in greater oxygen and carbon transfer into the yeast cells, hence increasing the rate of respiration (citric acid production). Again citric acid concentrations are lower than published values. Both Furukawa et al.(1977) and Akiyama et al.(1973) report citric acid concentrations of 65g/L (93 hours at 0.5 vvm and 600 rpm) and 60 g/L (72 hours in a 200 mL flask at 200 rpm on a rotary shaker), respectively. While the highest citric acid concentration achieved in this study was 9.5 g/L in a 120 hour production phase fermentation. As discussed in section 5.3.1 the citric acid production profile is consistent with published work, however the magnitudes of production differ. The lag phase is virtually non existent, and the exponential phase is sharp followed by a slow stabilization of production.

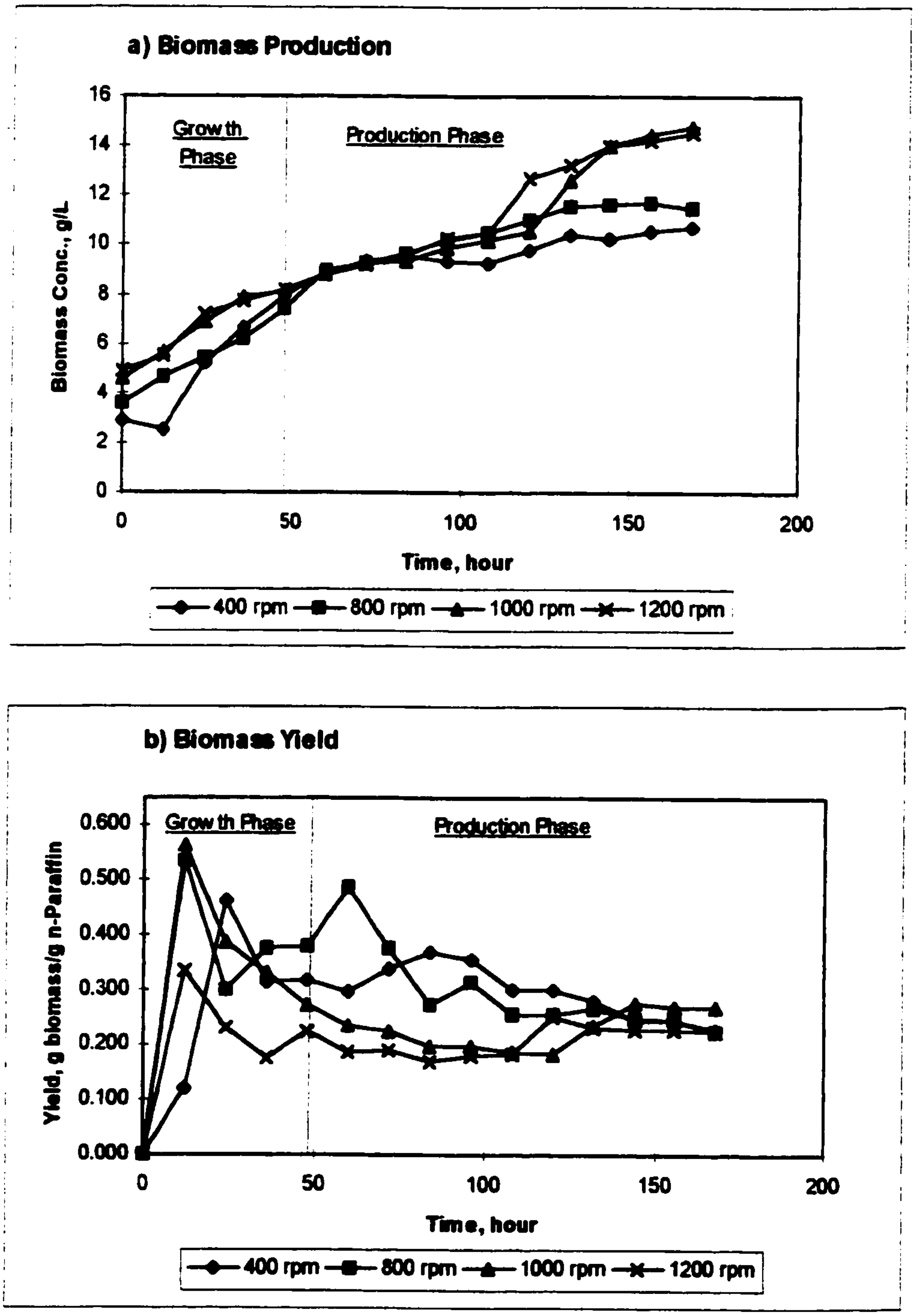
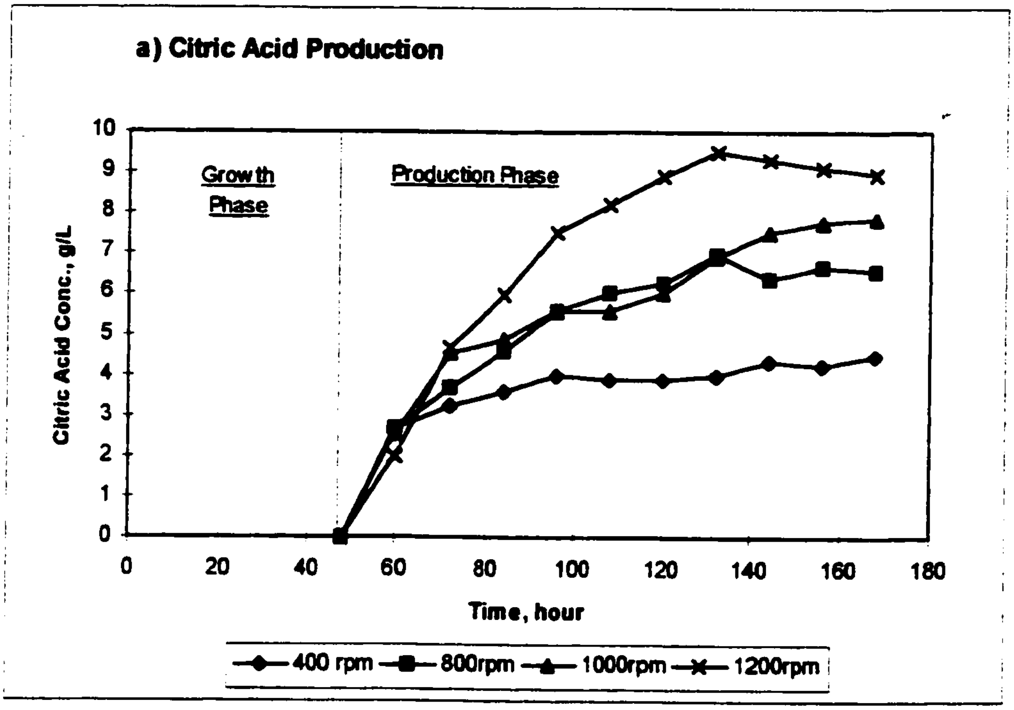


Figure 17: Agitation Effects on Biomass Production in Batch n-Paraffin Fermentation at 1.0 vvm and 28 ± 1°C



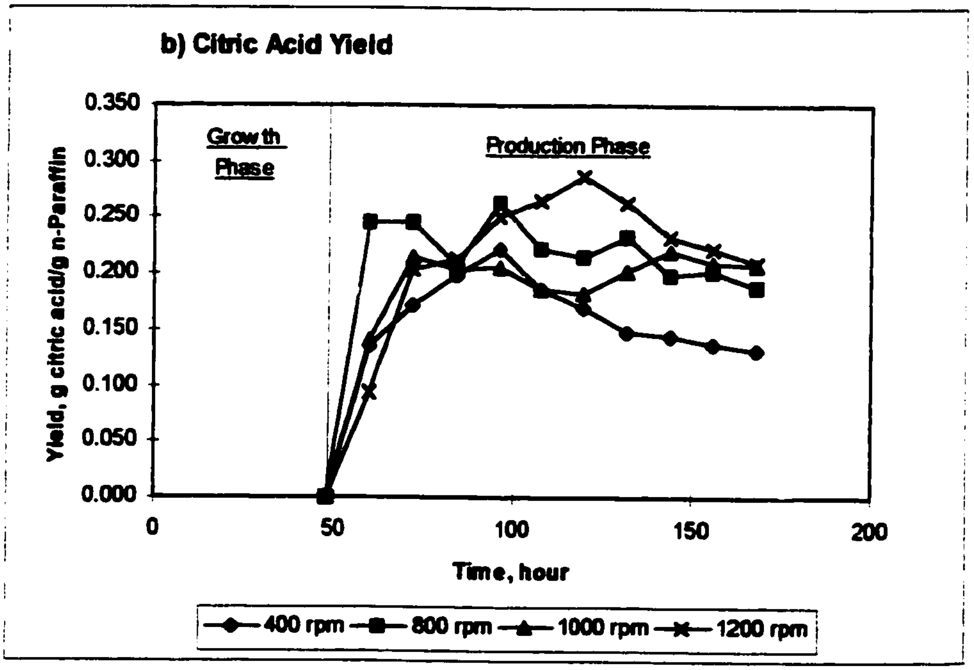


Figure 18: Agitation Effects on Citric Acid Production in Batch n-Paraffin Fermentation at 1.0 vvm and 28 ± 1°C

Table 6: Summary of Agitation Effects on Biomass and Citric Acid Production in Batch n-Paraffin Fermentation (120 hour production phase)

Parameter	400 rpm*	800 rpm*	1000 rpm*	1200 rpm*
Overall Biomass Yield, g biomass/g n-paraffin consumed	0.23	0.22	0.27	0.23
Overall Citric Acid Yield, g citric acid/g n-paraffin consumed	0.13	0.19	0.21	0.21
Overall Citric Acid Productivity, g/L-h	0.04	0.06	0.07	0.05

* All fermentations under aeration rate of 1.0 vvm

5.3.3 Effect of Agitation on Citric Acid Production - Fed-Batch Systems

Four agitation rates were investigated in this study, 400, 800, 1000, 1200 rpm while maintaining an aeration rate of 1.0 vvm.. Figures 19 and 20 describe biomass and citric acid production under these agitation rates, respectively. Table 7 summarizes overall biomass and citric acid yields along with citric acid productivity. Figure 19(a) shows biomass production to be fairly consistent with changing agitation rates, with biomass concentrations in the range of 10.5-11.5 g/L suspended solids. Considering biomass yields, Figure 19(b), all fermentations experience a peak in biomass yield in the growth phase, where the 400 and 800 rpm agitated fermentations experience the highest biomass yield of about 0.625 g biomass/g n-paraffin consumed. While the 1000 and 1200 rpm agitated fermentations have the lowest biomass yield of 0.3 g biomass/g n-paraffin consumed in the growth phase. In the production phase, all fermentations experience increasing biomass yields with the 400 and 800 rpm agitated fermentations being highest with a value of 0.45 g biomass/g n-paraffin consumed, and the 1000 and 1200 rpm agitated fermentations being lowest with a value of 0.30 g biomass/g n-paraffin consumed. The highest overall biomass yield was 0.45 g biomass/g n-paraffin consumed by the 400 rpm agitated fermentation. The observed increasing biomass production with agitation can be attributed to the fact that as agitation increases two things happen, oxygen transfer from air bubbles into solution increases as impellers efficiently disperse incoming air and n-paraffin oil droplets are smaller, greater in number and more adequately dispersed throughout the medium. Hence the higher agitation rates allow for higher transfer rates of oxygen and n-paraffin carbon into the cells for biomass production. This theory has been consistently supported with the experimental data from the fermentation studies conducted.

Moo-Young et al. (1971) reported biomass production of 4.5 g/L 48 hours into two n-paraffin fermentation at 500 rpm and 1.0 vvm and 800 rpm and 1.0 vvm. However, in the current study biomass production 48 hours into the production phase of each agitated fermentation is significantly higher, with the 400, 800 and 1000 rpm agitated fermentations having similar concentrations from 7.0 to 8.0 g/L, while that of the 1200 rpm agitated fermentation is at 5 g/L. Both this fed-batch fermentation and the previous batch fermentations produced higher biomass concentrations than what Moo-Young reported. Each fermentation in the study goes on to produce even higher biomass concentrations, in fact each fermentation has a biomass concentration that stabilizes in the range of 10.5 to 11.5 g/L. The 1200 rpm fermentation seems to be going through a lag in biomass production during the initial 48 hours of the production phase. This could be attributed to the yeast cell adapting to its surroundings of higher shear stresses. If this is the case, the lag in biomass production would also be expected in the 1000 rpm fermentation, however no apparent lag in biomass production is evident in the initial phase of the production phase.

Figure 20(a) shows citric acid concentrations increasing with agitation with the 1000 rpm agitated fermentation having the highest final citric acid concentration of 11 g/L and the 400 rpm agitated fermentation having the lowest final citric acid concentration of 5 g/L. Considering citric acid yields, Figure 20(b), the 800 rpm agitated fermentation has the highest yield of 0.4 g citric acid/g n-paraffin consumed. The highest overall citric acid yield, Table 7, of 0.41 g citric acid/g n-paraffin consumed belongs to the 800 rpm agitated fermentation. However, the 1000 rpm agitated fermentation had the highest citric acid productivity of 0.09 g/L-h.

The citric acid concentrations found in this study are lower than some published values. Furukawa et al.(1977) and Akiyama et al.(1973) report citric acid concentrations of 65g/L (93 hours at 0.5 vvm and 600 rpm) and 60 g/L (72 hours in a 200 ml flask at 200 rpm on a rotary shaker), respectively. While the highest citric acid concentration achieved in this study was 12.0 g/L in a 120 hour fermentation. While the magnitude of citric acid production is not consistent with published values, the production profile was consistent. The lag phase in citric acid production is virtually non existent, and the exponential phase is sharp followed by a slow plateauing of production. Another significant trend observed is the sharp increase in citric acid production after the 96 hour feeding of 1.0 L/day. The 800 and 1000 rpm agitated fermentations experience a 75 % increase in citric acid yield

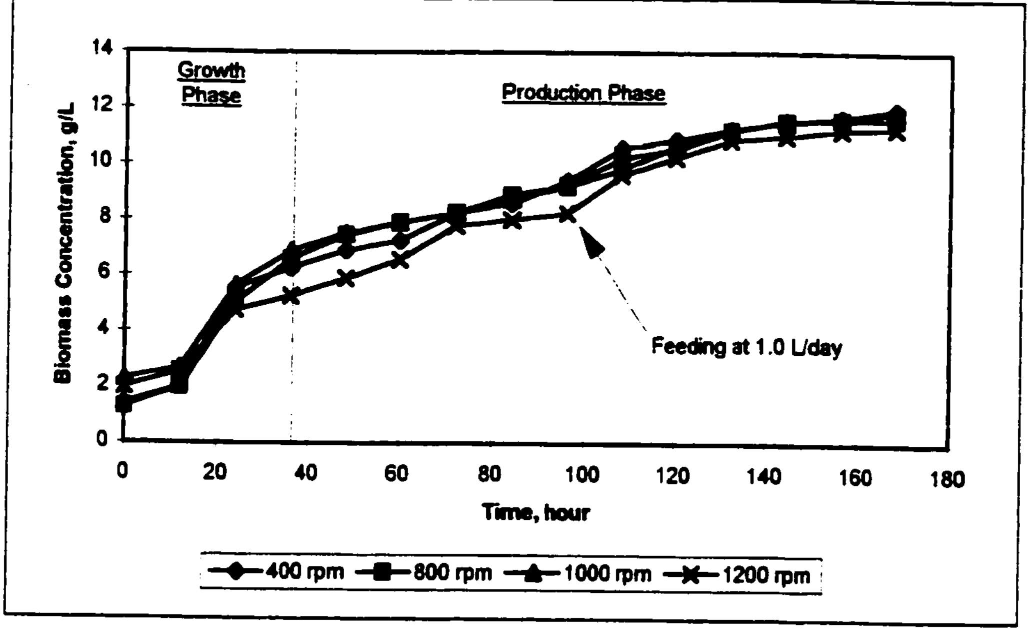
24 hours into the fed-batch feeding. This feeding significantly shifts the yeast from its stationary production phase, prior to the 96 hour feeding, to an exponential production phase following feeding.

Table 7: Summary of Agitation Effects on Biomass and Citric Acid Production in Fed-Batch Fermentation

Parameter	400 rpm*	800 rpm*	1000 rpm*	1200 rpm*
Overall Biomass Yield, g biomass/g n-paraffin consumed	0.45	0.43	0.31	0.32
Overall Citric Acid Yield, g citric acid/g n-paraffin consumed	0.22	0.41	0.37	0.32
Overall Citric Acid Productivity, g/L-h	0.04	0.07	0.09	0.07

* All fermentations under aeration rate of 1.0 vvm

a) Biomass Production



b) Biomass Yield

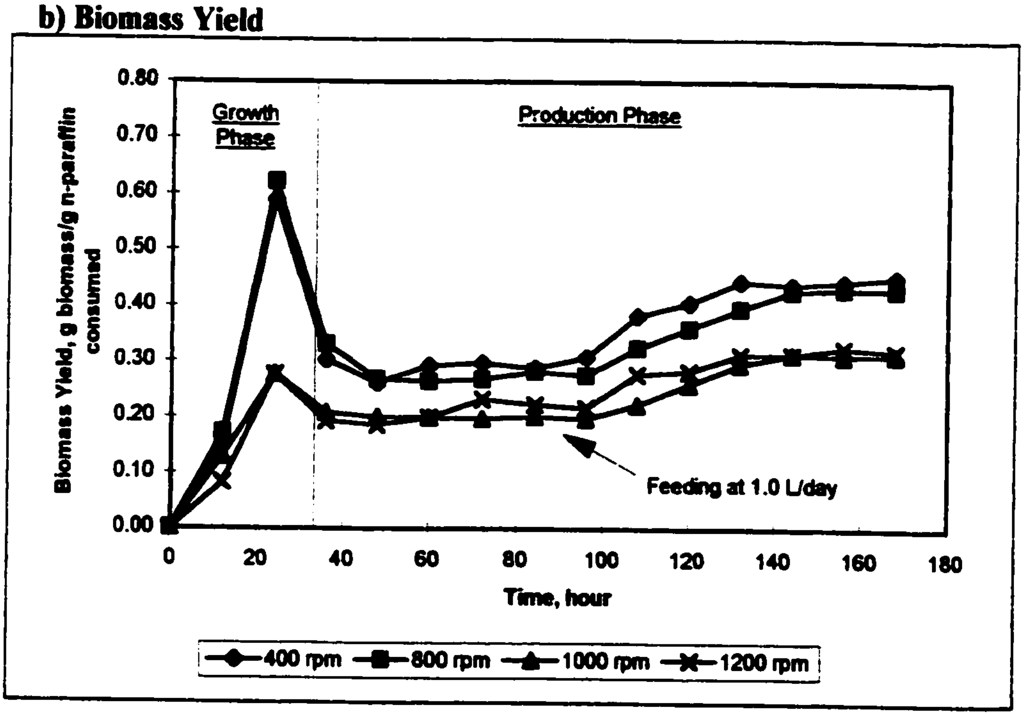
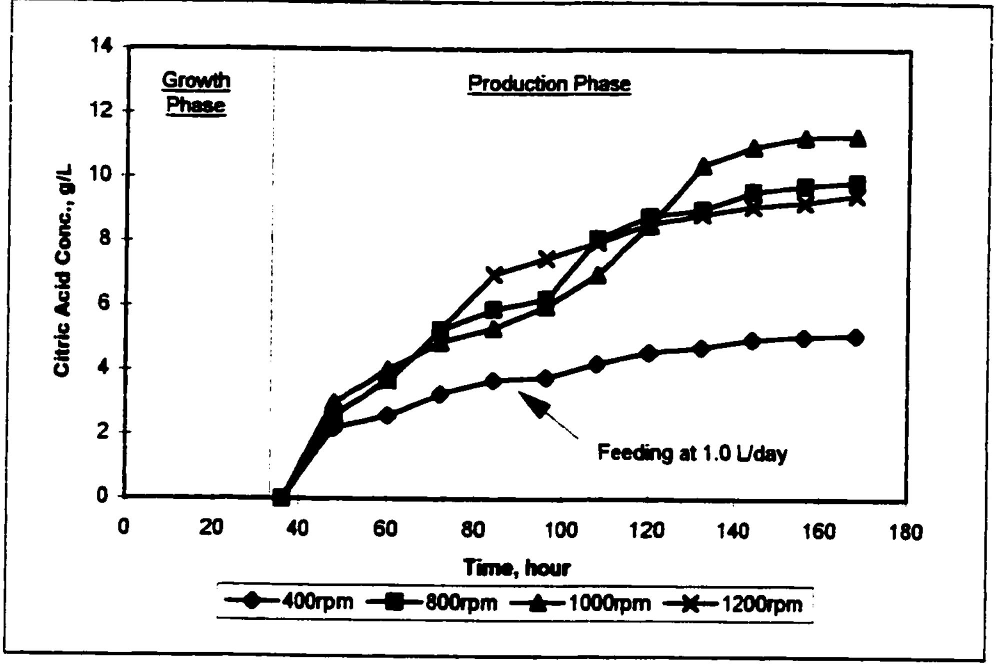


Figure 19: Agitation Effects on Biomass Production in Fed-Batch n-Paraffin Fermentation at 1.0 vvm and 28 ± 1°C

a) Citric Acid Production



b) Citric Acid Yield

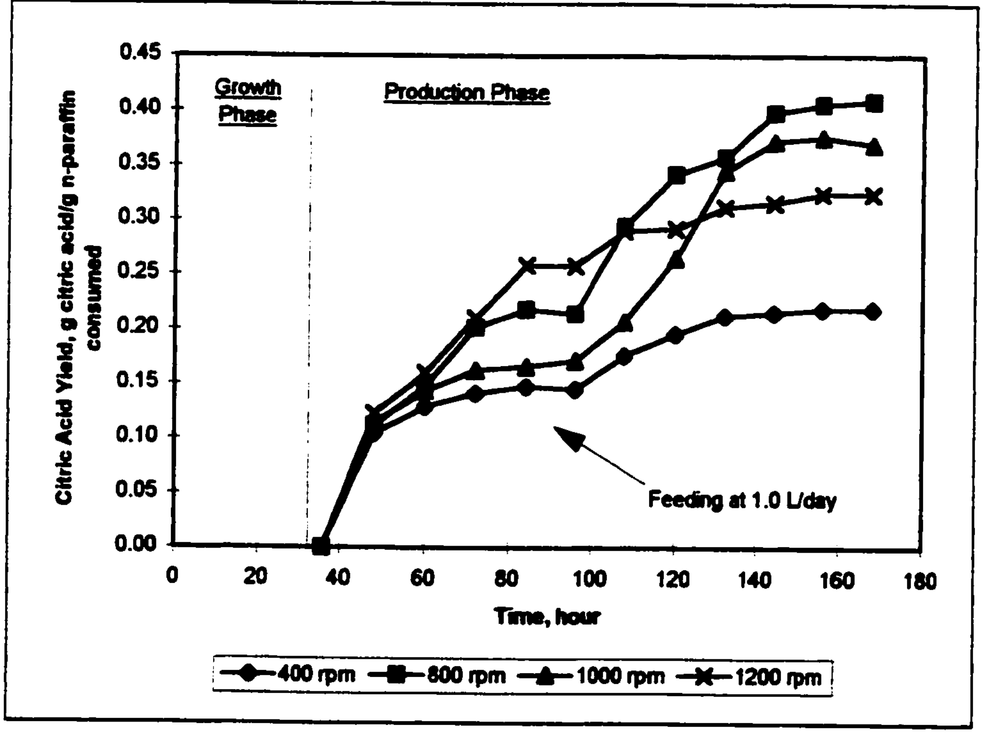


Figure 20: Agitation Effects on Citric Acid Production in Fed-Batch n-Paraffin Fermentation at 1.0 vvm and 28 ± 1°C

5.3.4 Effect of Greey Lightnin In-Line Agitator on Biomass and Citric Acid Production - Fed-Batch Systems

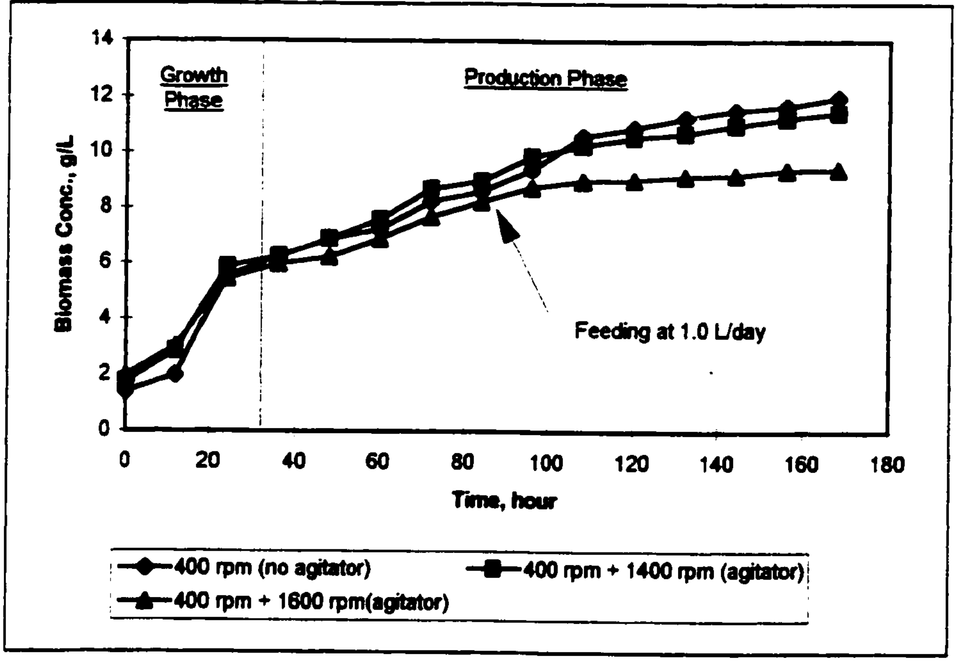
Two different external agitation rates were investigated, 1400 and 1600 rpm. While the external agitator was operating at these speeds the fermentor agitation was maintained at 400 rpm with an aeration of 1.0 vvm. Figure 21 and 22 describe the production of biomass and citric acid under the influence of the various external agitation rates, respectively. Table 8 describes the overall biomass and citric acid yield, along with citric acid productivity. Biomass production, Figure 21(a), shows that the fermentations with no external agitator and the 1400 rpm agitator have fairly consistent biomass concentrations of 11.5 g/L. Biomass yields, Figure 21(b), show the fermentation with no external agitator has the highest yield of 0.45 g biomass/g n-paraffin consumed. The highest overall biomass yield, Table 21, is 0.45 g biomass/g n-paraffin belonging to the fermentation with no external agitation.

Figure 22(a) shows the production of citric acid to be highest with the fermentation with the external agitator running at 1400 rpm, 9.5 g/L. Both the fermentations without the external agitator and with the agitator at 1600 rpm have the same citric acid production of 5 g/L. The highest overall citric acid yield is 0.37 g citric acid/g n-paraffin consumed from the fermentation with an external agitation of 1400 rpm with the highest productivity of 0.07 g/L-h belonging to this same fermentation. The fermentation running with external agitation of 1600 rpm experienced 20-30 % of cell lyses, see Appendix E, and this is the most likely factor attributing to citric acid yields being as low as the control fermentation (without external agitation).

In this study it was found that the external agitator, operating at 1400 rpm, contributed to an increased citric acid production, a 90 % increase from the control fermentation was observed. However, operating the external agitator at a speed of 1600 rpm did result in a significant degree of cell lyses (Appendix E), that resulted in a loss in citric acid production. The increase in external agitation does follow the principle put forward by Moo-Young et al. (1971), that higher levels of agitation result in n-paraffin oil droplets being smaller, greater in number and more adequately dispersed throughout the medium allowing for a more efficient contact with yeast cells to adhere to the droplets for carbon metabolism. While the external agitator may in fact have accomplished this, the physical shear on biomass cells resulted in cell lyses being the limiting factor in citric acid production.

Again citric acid concentrations are lower than published values reported by Furukawa et al.(1977) and Akiyama et al.(1973), citric acid concentrations of 65g/L (93 hours at 0.5 vvm and 600 rpm) and 60 g/L (72 hours in a 200 ml flask at 200 rpm on a rotary shaker), respectively. While the highest citric acid concentration achieved in this study was 9.5 g/L in a 120 hour fermentation with the 1400 rpm external agitated fermentation. As discussed in section 5.3.1 the citric acid production profile is consistent with published work. The lag phase is virtually non existent, and the exponential phase is sharp followed by a slow stabilizing of production and after the 96 hour feeding of 1.0 L/day there is another exponential phase in citric acid production. However this was predominant in the fermentation with the external agitator running at 1400 rpm.

a) Biomass Production



b) Biomass Yield

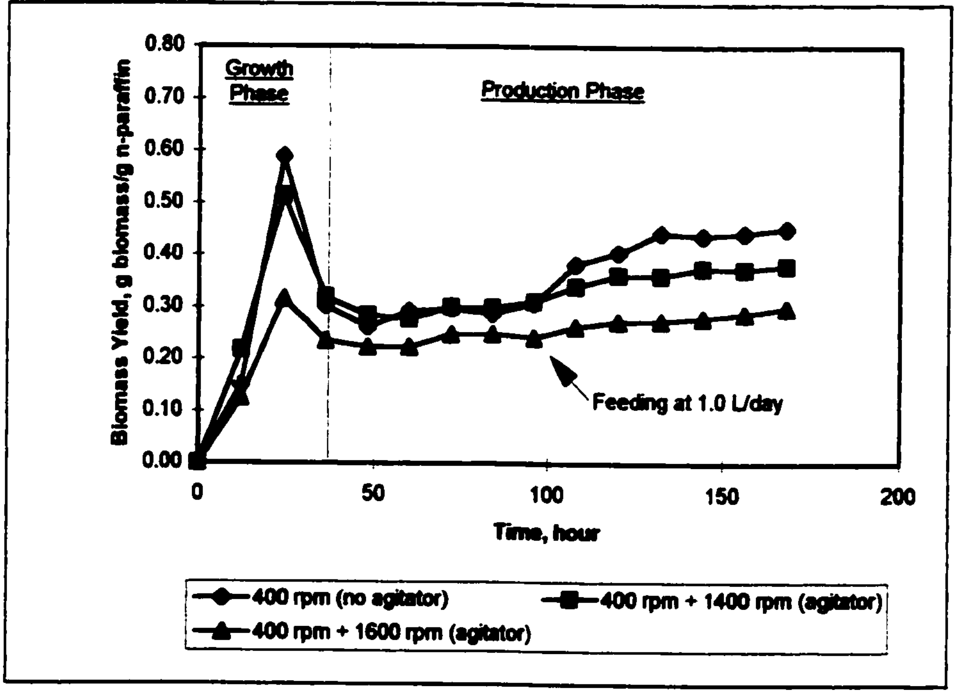
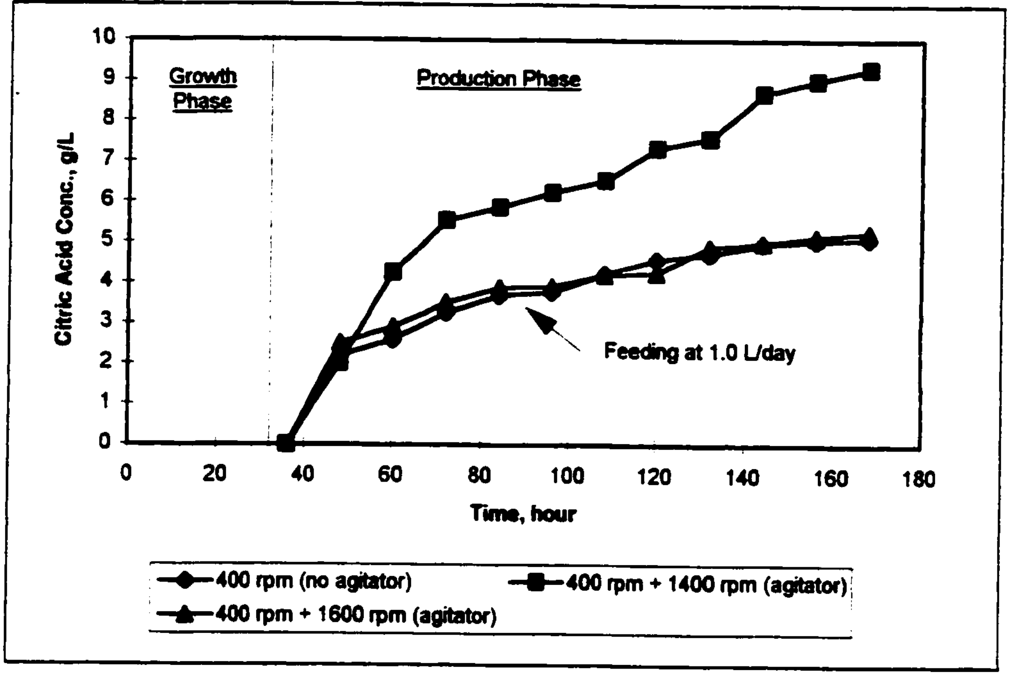


Figure 21: Agitation Effects on Biomass Production in Fed-Batch n-Paraffin Fermentation using Greey Lightnin In-Line Agitator at 1.0 vvm and 28 ± 1°C

a) Citric Acid Production



b) Citric Acid Yield

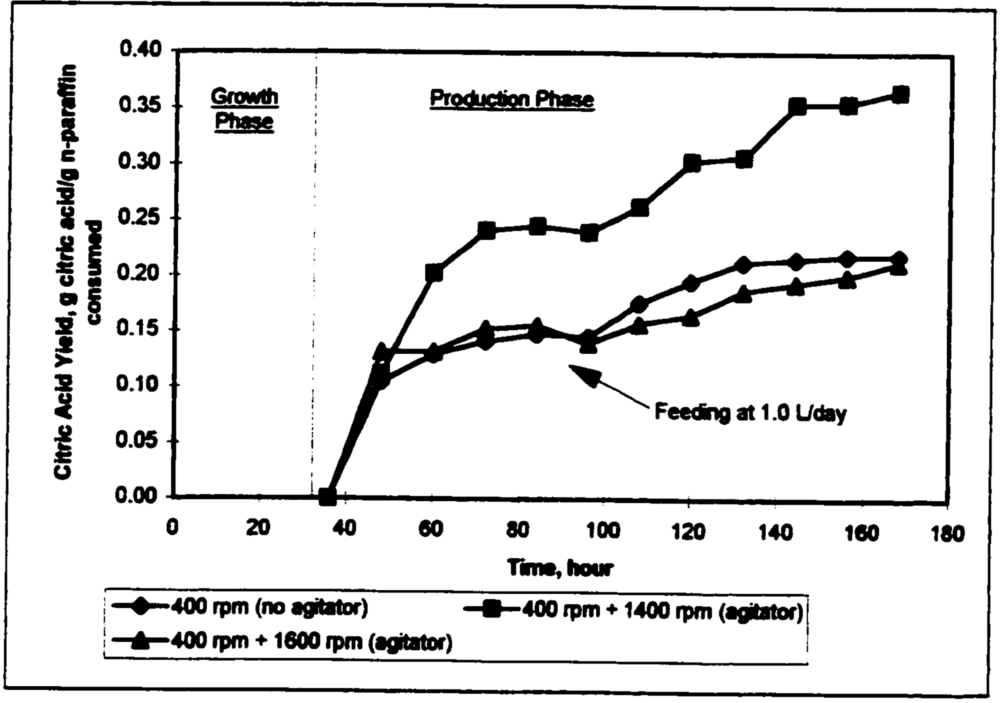


Figure 22: Agitation Effects on Citric Acid Production in Fed-Batch n-Paraffin Fermentation using Greey Lightnin In-Line Agitator at 1.0 vvm and 28 ± 1°C

Table 8: Summary of Agitation Effects with and without Greey Lightnin In-Line Agitator on Biomass and Citric Acid Production in Fed-Batch Fermentation

Parameter	400 rpm (no agitator)*	400 rpm + 1400 rpm (agitator)*	400 rpm + 1600 rpm (agitator)*
Overall Biomass Yield, g biomass/g n-paraffin consumed	0.45	0.38	0.30
Overall Citric Acid Yield, g citric acid/g n-paraffin consumed	0.22	0.37	0.21
Overall Citric Acid Productivity, g/L-h	0.04	0.07	0.04

* All fermentations under aeration rate of 1.0 vvm

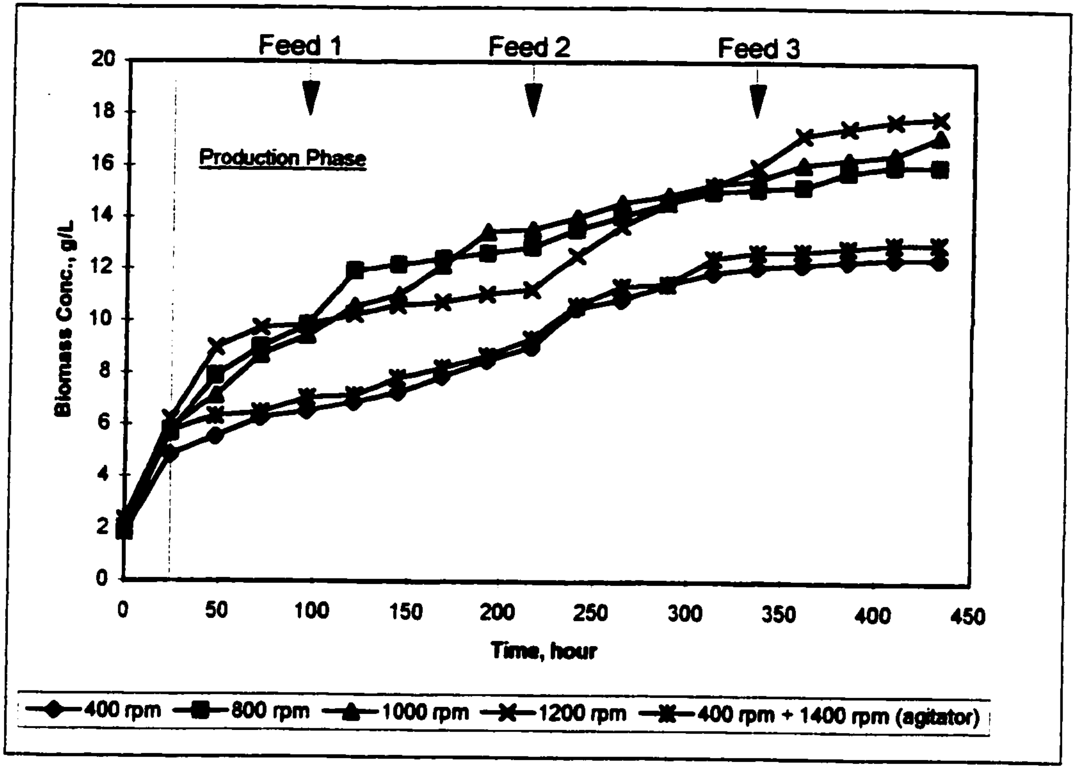
5.3.5 Effect of Agitation and 3 Cycle Fed-Batch Fermentation on Biomass and Citric Acid Yields

From the two previous studies on batch and fed-batch systems, there was a 100% increase in citric acid yield moving from batch to fed-batch fermentations. However, there was no significant improvement in citric acid productivity. With this in mind, the next study investigates the potential of improving citric acid yield and productivity under a 3-cycle fed-batch system. The 3-cycle system involves feeding the fermentation at three different points in the fermentation. Figure 23 and 24 describe biomass and citric acid production, respectively, under the influences of agitation and a 3 cycle fed batch system. Table 9 summarizes the overall biomass and citric acid yields and citric acid productivity. Figure 23(a) describes the highest biomass production to be with the 800, 1000 and 1200 rpm agitated fermentations with biomass concentrations in the range of 16-18 g/L. This is re-iterated in Figure 23(b) with biomass yield values in the range of 0.4 to 0.6 g biomass/ g n-paraffin consumed. The highest overall biomass yield of 0.59 g biomass/g n-paraffin consumed occurs with the 1000 rpm agitated fermentation.

Figure 24(a) shows the highest citric acid concentration occur for the 800, 1000, and 1200 rpm agitated fermentations with citric acid concentrations in the range of 30 to 40 g/L. The lowest citric acid concentration was produced from the 400 rpm agitated fermentation, 9 g/L. Figure 24(b) indicates that the highest citric acid yields of 0.8 to 1.0 g citric acid/g n-paraffin consumed resulted from fermentations at 800, 1000, and 1200 rpm. The highest overall citric acid yield (0.99 g citric acid/g n-paraffin consumed) and highest citric acid productivity (0.1 g/L-h) occurred all while fermenting at 1000 rpm.

The most obvious trend is after each feeding cycle of 1.0 L/day, at 96, 216 and 336 hours, there is an exponential increase in citric acid production. However this sharp increase in citric acid production is most evident after the 96 and 216 hour feedings while after the 336 hour feeding the citric acid production slightly increases but quickly stabilizes. Citric acid yields are still lower than published values. The 3 cycle fed-batch system did increase the magnitude of citric acid concentration but it did not affect the citric acid productivity of the fermentations. Furukawa et al. (1977) and Akiyama et al. (1973) reported citric acid productivities of 0.7 g/L-h and 0.8 g/L-h, respectively. The highest citric acid productivity in this study was 0.1 g/L-h by the 1000 rpm agitated fermentation. Again these differences between published and the reported data can be attributed to various factors such as n-paraffin components not being consistent over the past 20 years and a different *Candida lipolytica* strain and/or mutant of strain was used.

a) Biomass Production



b) Biomass Yield

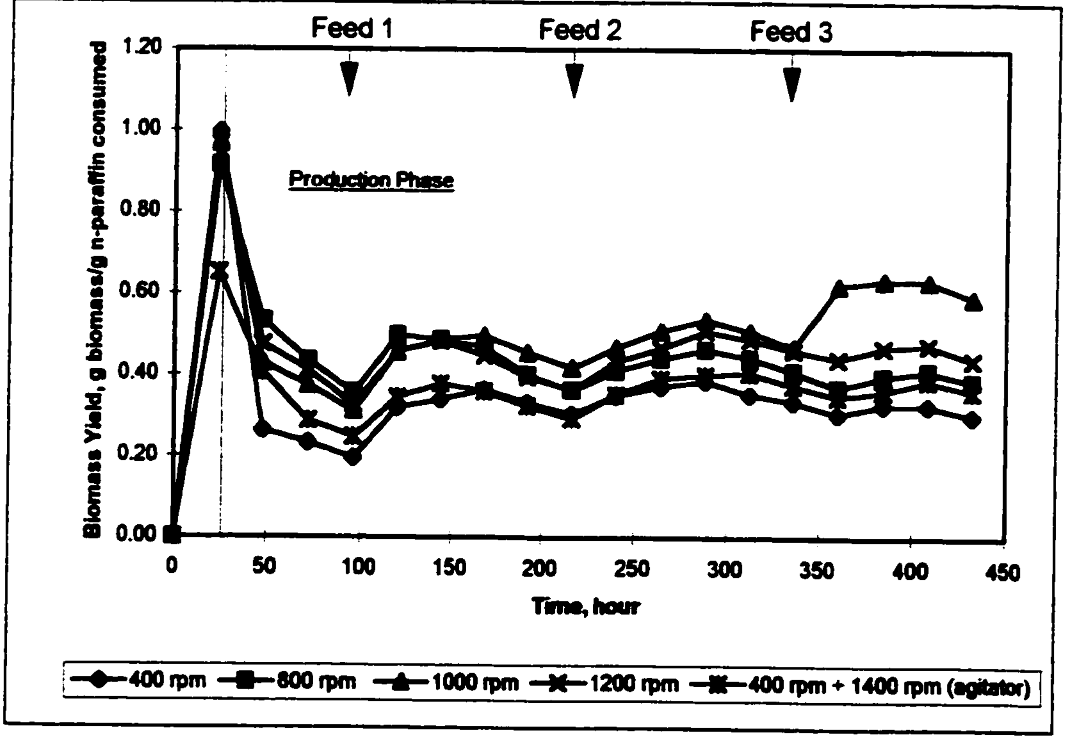
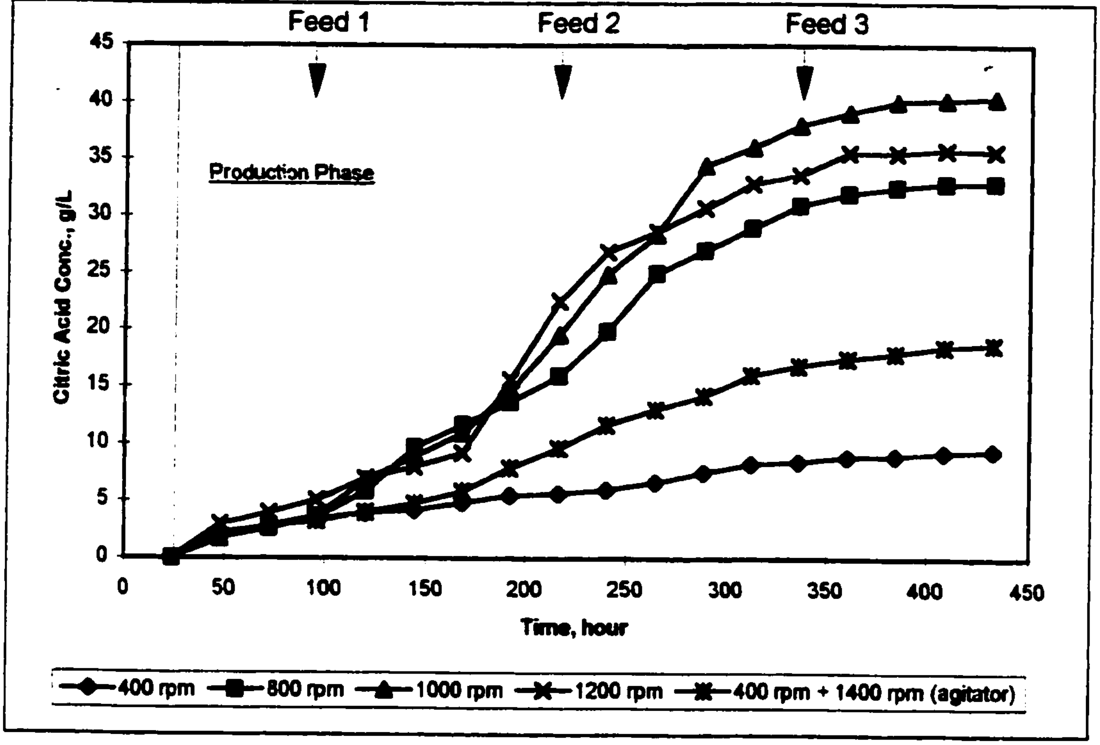


Figure 23: Agitation Effects on Biomass Production in 3 Cycle Fed-Batch n-Paraffin Fermentation with and without Greey Lightnin In-Line Agitator at 1.0 vvm and 28 ± 1°C

a) Citric Acid Production



b) Citric Acid Yield

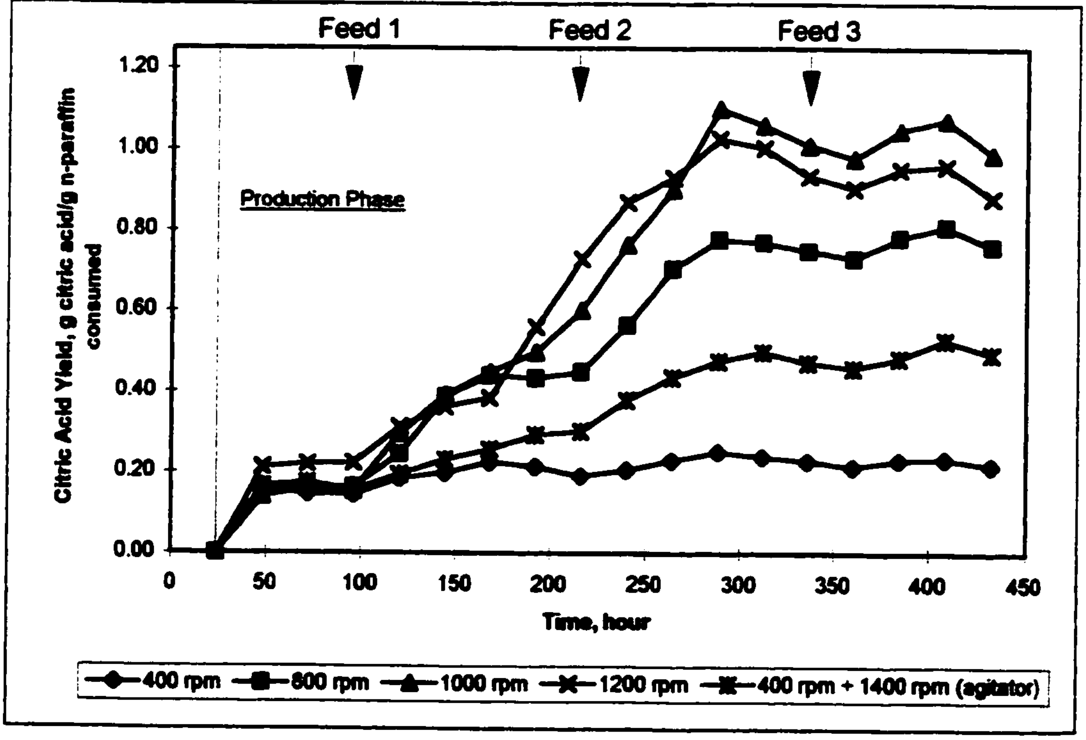


Figure 24: Agitation Effects on Citric Acid Production in 3 Cycle Fed-Batch n-Paraffin Fermentation with and without Greey Lightnin In-Line Agitator at 1.0 vvm and 28 ± 1°C

Table 9: Summary of Agitation Effects with and without Greey Lightnin In-Line Agitator on Biomass and Citric Acid Production in 3 Cycle Fed-Batch Fermentation

Parameter	400 rpm*	400rpm +1400 rpm (agitator)*	800 rpm*	1000 rpm*	1200 rpm*
Overall Biomass Yield, g biomass/g n-paraffin consumed	0.30	0.36	0.39	0.59	0.44
Overall Citric Acid Yield, g citric acid/g n-paraffin consumed	0.22	0.49	0.76	0.99	0.88
Overall Productivity, g/L-h	0.02	0.05	0.08	0.10	0.09

* All fermentations under aeration of 1.0 vvm

The citric acid yields in the 3-cycle fed-batch system where 100% higher than the yields obtained from the single cycle fed-batch system and where 200% higher than the yields obtained from the batch system. Appendix E illustrates the cell viability during the 3-cycle fermentation.

§5.4 Conclusions

Batch Systems

Based on the results discussed in this chapter, it was found that:

1. An aeration rate increase from 0.5 to 2.0 vvm resulted in a 46 % decrease in overall biomass yield, however overall citric acid yield and productivity was not affected;

2. An agitation rate increase from 400 to 800 rpm resulted in a 58% increase in overall citric acid yield and a 39% increase in citric acid productivity, however overall biomass yield was not affected;
3. Optimum citric acid production, under the influence of various aeration rates, was 4.8 g/L at 2.0 vvm and 400 rpm;
4. Optimum citric acid production, under the influence of various agitation rates, was 9.5 g/L at 1200 rpm and 1.0 vvm;
5. Biomass continued to be produced in the nitrogen limited fermentation stage;
6. Citric acid yields peaked in the first 12 to 24 hours of the fermentation stage with subsequently decreasing yields.

Fed-Batch Systems

Based on the results discussed in this chapter, it was found that:

1. An agitation rate increase from 400 to 1200 rpm resulted in a biomass yield decrease of 0.15 g biomass/g n-paraffin;
2. An agitation rate increase from 400 to 1000 rpm resulted in a citric acid yield increase of 0.21 g citric acid/g n-paraffin;
3. The addition of an external agitator (Greey Lightnin In-Line Agitator) running at 1400rpm decreased the biomass yield by 0.07 g biomass/g n-paraffin from the control fermentation of no external agitation;
4. The addition of an external agitator (Greey Lightnin In-Line Agitator) running at 1400 rpm increased the citric acid yield by 0.15 g citric acid/g n-paraffin;

5. The addition of an external agitator (Greey Lightnin In-Line Agitator) running at 1600 rpm experienced 20-30 % of cell lyses;
6. An agitation rate increase of 400 to 1000 rpm resulted in a biomass yield increase of 0.29 g biomass/g n-paraffin;
7. An agitation rate increase of 400 to 1000 rpm resulted in a citric acid yield increase of 0.77 g citric acid/g n-paraffin;
8. The 3 cycle fed-batch fermentations increased citric acid yields by 0.56 g citric acid/g n-paraffin, but the citric acid productivity was not affected;
9. Following fed-batch feedings the citric acid production sharply increased; taking production from a stationary phase to an exponential phase;
10. The 3-cyle fed-batch system increased overall citric acid yields by 100% from those found in the single cycle fed-batch system and increased overall citric acid yields by 200% from those found in the batch system.

CHAPTER 6:
Conclusions and Recommendations

From this thesis study it was found that *Candida lipolytica* can assimilate hydrocarbons for the production of citric acid and biomass. In particular, throughout the study it was found that:

1. *Candida lipolytica NRRL-Y-1095* can assimilated a n-paraffin solvent but cannot utilize kerosene;
2. The optimum levels of the following parameters for citric acid production are:

   Initial Biomass Inoculum = 10-12% volume
   n-Paraffin Concentration = 10-15% volume
   Ferric Nitrate Concentration = 10mg/L
   Temperature = 26-30°C

3. Under Batch Systems:
   1. An aeration rate increase from 0.5 to 2.0 vvm resulted in a 46 % decrease in overall biomass yield, however overall citric acid yield and productivity was not affected;
   2. An agitation rate increase from 400 to 800 rpm resulted in a 58% increase in overall citric acid yield and a 39% increase in citric acid productivity, however overall biomass yield was not affected;
   3. Optimum citric acid production, under the influence of various aeration rates, was 4.8 g/L at 2.0 vvm and 400 rpm;
   4. Optimum citric acid production, under the influence of various agitation rates, was 9.5 g/L at 1200 rpm and 1.0 vvm;

4. Under Fed-Batch Systems:

    i. An agitation rate increase from 400 to 1200 rpm resulted in a biomass yield decrease of 0.15 g biomass/g n-paraffin;
    ii. An agitation rate increase from 400 to 1000 rpm resulted in a citric acid yield increase of 0.21 g citric acid/g n-paraffin;
    iii. The addition of an external agitator (Greey Lightnin In-Line Agitator) running at 1400rpm decreased the biomass yield by 0.07 g biomass/g n-paraffin from the control fermentation of no external agitation;
    iv. The addition of an external agitator (Greey Lightnin In-Line Agitator) running at 1400 rpm increased the citric acid yield by 0.15 g citric acid/g n-paraffin;
    v. The addition of an external agitator (Greey Lightnin In-Line Agitator) running at 1600 rpm experienced 20-30 % of cell lyses;
    vi. An agitation rate increase of 400 to 1000 rpm resulted in a biomass yield increase of 0.29 g biomass/g n-paraffin;
    vii. An agitation rate increase of 400 to 1000 rpm resulted in a citric acid yield increase of 0.77 g citric acid/g n-paraffin;
    viii. The 3 cycle fed-batch fermentations increased citric acid yields by 0.56 g citric acid/g n-paraffin, but the citric acid productivity was not affected;
    ix. The 3-cyle fed-batch system increased overall citric acid yields by 100% from those found in the single cycle fed-batch system and increased overall citric acid yields by 200% from those found in the batch system.

Although the feasibility of utilizing n-alkanes as a feedstock for citric acid production has been demonstrated, further investigations are required:

1. Studies on continuous fermentation systems to fully understand the advantages/disadvantages on citric acid production;
2. Investigate the potential of utilizing a mixture of carbohydrate and hydrocarbon feedstocks to assess how carbohydrates can influence the assimilation of hydrocarbons (lag phase may be shortened significantly);
3. Mutation of the yeast, to isolate higher citric acid producing populations;
4. Investigate the use of surfactants to allow hydrocarbons to be kept in solution longer;
5. Further runs should be established to improve on citric acid yields;
6. Based on present and future studies, conducting a cost/benefit analysis would asses the advantages/disadvantages of utilizing hydrocarbon feedstocks over carbohydrate feedstocks (using the conventional method with *A. niger*) in an industrial scale process in today's market.

CHAPTER 7:
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APPENDIX A:

Analytical Methods

This appendix provides a detailed listing of all analytical methods used throughout the experimental program.

A.1 Yeast Culture

Two yeast cultures were obtained from the Agricultural Research Services Culture Collection Division of the U.S. Department of Agriculture, Peoria, United States, *Candida lipolytica* NRRL-Y-1094 *and Candida lipolytica* NRRL-Y-1095. Yeast colonies were maintained on glucose agar plates and transferred monthly to fresh agar plates to insure cell viability.

A.2 Cultivation of Yeast from Lyophilized Preparation

To cultivate the yeasts from lyophilized preparation, yeast pellets were dissolved in the sterilized nutrient media described in Table A1.

Table A1: Nutrient Medium for the Reconstitution of Yeast Pellets from Lyophilized Preparation (Miall and Parker, 1974)

Components	Concentration (g/L)
Yeast Extract	3.0
Malt Extract	3.0
Peptone	5.0
Glucose	10.0
Hydrocarbon Feedstock (mL/L)	70

Loopfuls of each yeast and nutrient suspension were streaked, using aseptic techniques, on to agar plates composed of the media described in Table 1 with the addition of 10g/L of Bacto agar. The agar plates were incubated at 25°C for 2-3 days or until yeast colonies populated the plates. The yeast plates were stored in a refrigerator held between 4 ± 1°C. These yeast colonies served as parent strains for the inoculation of subsequent fermentations conducted throughout the study. The yeast plates were transferred to fresh agar plates monthly to insure yeast cell viability.

A.3 Hydrocarbon Feedstock

The two hydrocarbon feedstocks used as the principle carbon source were kerosene and normal paraffin solvents. Both feedstock were obtained from Imperial Oil Ltd., Toronto, Canada. The kerosene solvent (Kerol-K) was composed of 80% total paraffins, including both branched (iso), cyclo and normal paraffins having predominantly 9 to 13 carbon atoms. Less than half of this total paraffin content is normal paraffins. Approximately 20% of the kerosene solvent was composed of 9-10 carbon atom aromatic molecules. The second hydrocarbon feedstock was a normal paraffin solvent (Norpar 15), containing more than 97% mixed normal paraffins composed of predominantly 14 to 17 carbon atoms. Specifically, the Norpar 15 solvent (density of 771 g/L) was composed of 32-34% 14 carbon atom molecules, 42-45% of 15 carbon atom molecules, 16-18% of 16 carbon atom molecules, 4-6% of 17 carbon atom molecules, and 1-3% 18 carbon atom molecules.

A.4 Yeast Cell Growth and Fermentation Media

The media described in Tables A2, A3 and A4 were used for the inoculation yeast seed culture, the pre-fermentation stage for yeast biomass growth and the fermentative stage for citric acid production, respectively.

Table A2: Medium for Yeast Seed Culture Growth (Miall and Parker, 1974)

Components	Concentration
Yeast Extract (g/L)	3.0
Malt Extract (g/L)	3.0
Peptone (g/L)	5.0
Glucose (g/L)	10.0
Hydrocarbon Feedstock (mL/L)	70.0

Table A3: Medium for Pre-Fermentation Stage used for Yeast Biomass Growth (Miall and Parker, 1974)

Components	Concentration
Corn Steep Liquor (g/L)	2.6
Ammonium Sulphate (g/L)	4.0
Calcium Carbonate (g/L)	15.0
Hydrocarbon Feedstock (mL/L)	87.0
Yeast Seed Culture Growth Solution (mL/L)	43.0

Table A4: Medium for Fermentation Stage used for Citric Acid Production (Miall and Parker, 1974)

Components	Concentration
Urea (g/L)	2.0
Magnesium Sulphate Heptahydrate (g/L)	0.4
Calcium Carbonate (g/L)	6.0
Potassium Dihydrogen Ortho-Phosphate (g/L)	0.75
Thiamine Hydrochloride (mg/L)	0.25
Ferric Nitrate Nonahydrate (mg/L)	0.65
Zinc Sulphate Heptahydrate (mg/L)	1.2
Copper Sulphate Pentahydrate (mg/L)	0.31
Manganese Sulphate Monohydrate (mg/L)	0.27
Hydrocarbon Feedstock (mL/L)	157.0
Pre-Fermentation Yeast Growth Solution (mL/L)	43.0

Media described in Tables A5 and A6 were used for feeding in fed-batch fermentation systems.

Table A5: Medium for Feeding of Fermentation Stage in 7 Day Fed-Batch Systems - Citric Acid Production (Miall and Parker, 1974)

Components	Concentration
Magnesium sulphate heptahydrate (g/L)	0.4
Potassium dihydrogen ortho-phosphate (g/L)	0.75
Thiamine hydrochloride (mg/L)	0.25
Ferric nitrate nonahydrate (mg/L)	0.65
Zinc sulphate heptahydrate (mg/L)	1.2
Copper sulphate pentahydrate (mg/L)	0.31
Manganese sulphate monohydrate (mg/L)	0.27
n-Paraffin (ml/L)	166

Table A6: Medium for Feeding of Fermentation Stage in 18 day Fed-Batch Systems - Citric Acid Production

Components	Concentration
Urea (g/L)	0.25
Magnesium sulphate heptahydrate (g/L)	0.4
Potassium dihydrogen ortho-phosphate (g/L)	0.75
Thiamine hydrochloride (mg/L)	0.25
Ferric nitrate nonahydrate (mg/L)	0.65
Zinc sulphate heptahydrate (mg/L)	1.2
Copper sulphate pentahydrate (mg/L)	0.31
Manganese sulphate monohydrate (mg/L)	0.27
n-Paraffin (ml/L)	166

All media was sterilized by autoclaving at 121°C and pressure of 15 p.s.i.g. for 20 minutes, followed by a slow exhaust, prior to the addition of the hydrocarbon feedstock and the yeast cell inoculation.

A.5 Analytical Methods

A.5.1 Biomass Concentration

Yeast concentration was determined by dry cell weight analysis. 40 mL samples taken over time of the fermentation broth were centrifuged at 12,000 rpm at 5°C for 15 minutes using a Dupont Sorvall Instrument RC5C centrifuge. Supernatant was carefully removed from the centrifuge vials using Pasteur pipettes, making sure not to disturb the yeast pellet at the base of the vial. The supernatant was set aside for further analysis of citric acid and hydrocarbon concentrations. The yeast pellet was removed from the vial and placed in a pretreated and pre-weighed aluminum dish. (The aluminum dish, prior to being used, had been placed in a muffle furnace at 500 ± 50°C for 1 hour and cooled in a desiccator to remove any volatile residue.) The aluminum dish containing the yeast pellet was placed in a drying oven at 104 ± 1°C for 2 hours. The aluminum dish plus the dried yeast pellet was cooled in a desiccator and then weighed. The cycle of drying, cooling and weighing was repeated until the change in weight was less than 4% of the previous weight or 0.5mg. The dried yeast sample was then ignited by placing the dish in a muffle furnace at 500 ± 50°C for 20 minutes and then cooled in a desiccator and then weighed. Once again, the cycle of ignition, cooling and weighing was repeated until the change in weight was less than 4% of the previous weight or 0.5mg. The concentration of dried yeast cells was calculated by the following equation:

$$Y_{conc} = (m_{bef} - m_{aft}) / V_{sample} \qquad [A1]$$

where:

Y_{conc} = concentration of dried yeast pellet in mg/mL (g/L)
m_{bef} = weight of dried yeast pellet plus dish before ignition, mg

m_{aft} = weight of dried yeast pellet plus dish after ignition, mg
V_{sample} = volume of the fermentation broth sample, mL

This method measures the organic composition of the yeast cells, and does not take into account the trace inorganic compounds in the yeast. It should also be noted that although the fermentation broth also contains calcium carbonate, this inorganic salt is stable up to a temperature of 825°C (Metcalf and Eddy, Inc., 1991) and will not influence the yeast concentration.

A.5.2 Hydrocarbon Concentration

Hydrocarbon concentration was determined by extracting the residual hydrocarbons from the fermentation broth supernatant with hexane as the extraction solvent. The volume of the supernatant was recorded with a graduated cylinder. A known volume of hexane solvent was decanted into a separatory funnel, at least twice the volume of fermentation supernatant, followed by the measured volume of supernatant. The final addition was done slowly to avoid emulsification. The separatory funnel was then shaken gently and placed on a retort stand to allow the organic and aqueous phases to separate. The shaking and settling steps are repeated several times, until there are no visible hydrocarbon droplets left in the aqueous phase liquid. The aqueous phase liquid is decanted through the base port of the separatory funnel. Anhydrous magnesium sulphate was added to the organic phase liquid remaining in the funnel to remove any residuals aqueous material. The magnesium sulphate was then filtered out of the organic phase liquid. The volume of the organic phase liquid was then recorded with a graduated cylinder. The concentration of residual hydrocarbon in the fermentation broth sample can then be measured as follows:

$$HC_{conc} = \frac{V_{org} - V_{hex}}{V_{super}} \times \rho_{HC} \qquad [A2]$$

where:

HC_{conc} = concentration of hydrocarbon feedstock in supernatant of centrifuged fermentation sample, mg/mL (g/L)
V_{org} = volume of organic phase liquid from extraction containing both hexane solvent and hydrocarbon feedstock, mL
V_{hex} = volume of hexane solvent added to extraction process, mL
V_{super} = volume of supernatant of centrifuged fermentation sample, mL
ρ_{HC} = density of hydrocarbon feedstock, mg/mL

A.5.3 Citric Acid Concentration

A.5.3.1 Enzymatic Assay Analysis

Citric acid concentration was determine by enzymatic assay. The enzymatic test kit was obtained from Boehringer Mannheim, Montréal, Canada. The test, as outlined by Boehringer Mannheim (1992), is based on the principle that citric acid (citrate) is converted to oxaloacetate and acetate in the reaction catalyzed by the enzyme citrate lyase (CL) :

$$\text{Citric Acid} \xrightarrow{CL} \text{Oxaloacetate} + \text{Acetate} \qquad [A3]$$

In the presence of the enzyme malate dehyrdrogenase (MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively, by reduced nicotinamide-adenine dinucleotide (NADH):

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+ \qquad [A4]$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + \text{NAD}^+ \qquad [A5]$$

The amount of NADH oxidized in reactions [A4] and [A5] is stoichiometric with the amount of citric acid. NADH is determined by means of its absorbance at 340 nm.

The enzymatic test kit is composed of two solutions, containing NADH and Citrate lyase solution. The NADH was a 12 mL solution composed of 1.4 g of lyophilisate, consisting of: glycylglycine buffer, pH 7.8; malate dehydrogenase, 136 U; L-lactate dehydrogenase, 280 U; NADH, 6.0 mg; stabilizers. The Citrate lyase Solution was a 0.3 mL solution composed of 50 mg of lyophilisate citrate lyase, 12 U. NADH concentration was determined using a Beckman DU-40 spectrophotometer to measure absorbance readings at a wavelength of 340 nm. Glass cuvettes with a 1 cm light path were used with the spectrophotometer.

For each citric acid concentration measurement two absorbance readings need to be taken, one before the oxidation of NADH in the sample solution and the other after the reaction is complete. A blank reading must always be taken to avoid the influence of the absorbance of any contaminants present. Table A7 describes the solutions that need to be prepared.

Table A7: Preparation of Solutions for Citric Acid Assay (Boehringer and Mannheim, 1992)

Pipette into Cuvettes	Blank Solution Cuvette	Sample Solution Cuvette
Mixture 1:		
NADH Solution	1.00 mL	1.00 mL
Distilled Water	2.00 mL	1.80 mL
Fermentation Supernatant *	---	0.20 mL
Mixture 2:		
Mixture 1 + Citrate lyase Solution	0.02 mL	0.02 mL

* Supernatant after hydrocarbons had been extracted

Once Mixture 1 is pipetted into the Blank solution cuvette and the Sample solution cuvette, each cuvette was covered with Parafilm® and mixed by gentle swirling. The absorbance at 340nm of these solutions (A_1) was read after approximately 5 minutes. The reaction in each cuvette was started by addition of the Citrate lyase Solution, as described by Mixture 2. Absorbance at 340nm was then taken on completion of the reaction (A_2) in each cuvette, after 5 minutes. The absorbance differences ($\Delta A = A_1 - A_2$) for both the blank and sample cuvettes were determined. The absorbance difference of the blank was then subtracted from the absorbance difference of the sample, as follows:

$$\Delta(\Delta A) = \Delta A_{sample} - \Delta A_{blank} \qquad [A6]$$

where:

$\Delta A_{sample} = (A_1 - A_2)_{sample}$
$\Delta A_{blank} = (A_1 - A_2)_{blank}$

The citric acid concentration can then be calculated by the following equation:

$$C_{conc} = \frac{V_f \times MW}{\varepsilon \times d \times V_s \times 1000} \times \Delta(\Delta A) \qquad [A7]$$

where:

C_{conc} = citric acid concentration, g/L
V_f = final volume in cuvettes, 3.02 mL
V_s = hydrocarbon-free fermentation supernatant volume, mL

MW = molecular weight of citric acid, g/mol
= 192.1 g/mol
d = light path, cm
= 1.0 cm
ε = absorption coefficient of NADH at 340 nm, L x mmol^{-1} x cm^{-1}
= 6.3 L x mmol^{-1} x cm^{-1}

A.5.3.2 High Performance Liquid Chromatograph (HPLC) Analysis

Citric Acid concentration was determined by isocratic HPLC analysis using a Hewlett Packard 1090 Liquid Chromatograph and Waters μ Bondapak C18 3.9 x 300 mm column. The mobile phase consisted of 0.1 M KH_2PO_4 in distilled deionized water adjusted to a pH of 2.5 with concentrated H_3PO_4. Analysis consisted of a mobile phase flow rate of 0.6 ml/min, ambient column temperature (25°C), and injection volume of 20 μL. 1.0 ml of each n-paraffin free supernatant sample was filtered using 0.2 μm Millipore GV-13 filters prior to injection into column. Absorbance readings were taken at a wavelength of 215 nm and citric acid concentrations determined using a standard curve of absorbance readings at various known citric acid concentrations. However, in this citric acid concentration there is also trace amounts of iso-citric acid. The HPLC retention time cannot differentiate between the two isomers. Therefore to get the actual citric acid values minus the isocitric acid, isocitric acid was determined using an enzymatic assay.

The enzymatic assay kit was obtained by Boehringer and Mannheim, Montréal, Canada. The kit contained solutions of imidazole buffer, lyophilisate (consisting of NADP) and manganese sulphate. The assay involved operations at a wavelength of 340 nm and a 1 cm light path using a Beckman DU-40 spectrophotometer. Once the concentration was determined it was subtracted from the HPLC concentration and citric acid was determined.

The principle behind the test is that D-isocitric acid (D-isocitrate) is oxidatively decarboxylated by nicotinamide-adenine dinucleotide phosphate (NADP) in the presence of the enzyme isocitrate dehydrogenase (ICDH):

$$\text{D-isocitrate} + \text{NADP}^+ \xrightarrow{\text{ICDH}} \text{2-oxoglutarate} + \text{CO}_2 + \text{NADPH} + \text{H}^+ \qquad [\text{A8}]$$

The amount of NADPH formed in reaction [A8] is stoichiometric with the amount of D-isocitrate. NADPH is determined by means of its absorbance at 340 nm. The bound D-isocitric acid (esters, lactones) is determined after alkaline hydrolysis, [A9] and [A10]:

$$\text{D-isocitric acid ester} + \text{H}_2\text{O} \xrightarrow{\text{pH 9-10}} \text{D-isocitrate} + \text{alcohol} \qquad [\text{A9}]$$

$$\text{D-isocitric acid lactone} + \text{H}_2\text{O} \xrightarrow{\text{pH 9-10}} \text{D-isocitrate} \qquad [\text{A10}]$$

APPENDIX B:

Raw Data

This appendix lists all of the raw data collected throughout the experiments presented in this thesis

B.1 Screening of Hydrocarbon Feedstock and Yeast Strain

Table B.1: Citric Acid and Biomass Concentrations When Using Kerosene and *Candida lipolytica Y-1094*

Time (hrs)	Yeast Conc. (g/L)	itric Acid Conc (g/L)	Kerosene Conc. (g/L)
0	0.052	0	121
24	0.085	0	115
48	0.226	0.0085	100
72	0.239	0.00745	103
120	0.243	0.0145	97.9

Table B.2: Citric Acid and Biomass Concentrations When Using n-Paraffin and *Candida lipolytica Y-1094*

Time (hrs)	Yeast Conc. (g/L)	Citric Acid (g/L)	n-Paraffin (g/L)
0	0.056	0	121
24	0.019	0	105
48	0.186	0.001	100
72	0.201	1.22	96
120	4.825	2.79	79

Table B.3: Citric Acid and Biomass Concentrations When Using Kerosene and *Candida lipolytica Y-1095*

Time (hrs)	Yeast Conc. (g/L)	itric Acid Conc (g/L)	Kerosene Conc. (g/L)
0	0.037	0	121
24	0.25	0.011	106
48	0.115	0.019	116.4
72	0.056	0.022	107
120	0.2	0.02	96.98

Table B.4: Citric Acid and Biomass Concentrations When Using n-Paraffin and *Candida lipolytica Y-1095*

Time (hrs)	Yeast (g/L)	Citric Acid (g/L)	n-Paraffin (g/L)
0	0.057	0	121
24	0.059	0.0058	101
48	0.12	0.0042	110
72	7.37	3.97	77
120	9.773	5.69	69

B.2 Raw Data of Central Composite Design Flask Batch Tests

Table B.5: Biomass Model - Raw Data of Flask Batch Tests

BIO_G_L	BIOMASS%	HC_%	FE_MG_L	TEMP_C
2.03	-1.00	-1.00	-1.00	-1.00
3.27	1.00	-1.00	-1.00	-1.00
3.48	-1.00	1.00	-1.00	-1.00
3.89	1.00	1.00	-1.00	-1.00
2.24	-1.00	-1.00	1.00	-1.00
3.64	1.00	-1.00	1.00	-1.00
4.21	-1.00	1.00	1.00	-1.00
4.68	1.00	1.00	1.00	-1.00
2.99	-1.00	-1.00	-1.00	1.00
3.79	1.00	-1.00	-1.00	1.00
3.70	-1.00	1.00	-1.00	1.00
4.23	1.00	1.00	-1.00	1.00
2.71	-1.00	-1.00	1.00	1.00
3.79	1.00	-1.00	1.00	1.00
4.56	-1.00	1.00	1.00	1.00
4.87	1.00	1.00	1.00	1.00
4.50	0.00	0.00	0.00	0.00
4.71	0.00	0.00	0.00	0.00
4.99	0.00	0.00	0.00	0.00
4.66	0.00	0.00	0.00	0.00
4.71	0.00	0.00	0.00	0.00
4.52	0.00	0.00	0.00	0.00
4.20	0.00	0.00	0.00	0.00
4.92	2.00	0.00	0.00	0.00
2.56	-2.00	0.00	0.00	0.00
4.41	0.00	2.00	0.00	0.00
3.21	0.00	-2.00	0.00	0.00
4.51	0.00	0.00	2.00	0.00
4.00	0.00	0.00	-2.00	0.00
3.99	0.00	0.00	0.00	2.00
2.12	0.00	0.00	0.00	-2.00

Table B.6: Citric Acid Model - Raw Data of Flask Batch Tests

CITR_G_L	BIOMASS%	HC_%	FE_MG_L	TEMP_C
2.58	-1.00	-1.00	-1.00	-1.00
4.69	1.00	-1.00	-1.00	-1.00
4.52	-1.00	1.00	-1.00	-1.00
4.87	1.00	1.00	-1.00	-1.00
3.69	-1.00	-1.00	1.00	-1.00
5.29	1.00	-1.00	1.00	-1.00
5.33	-1.00	1.00	1.00	-1.00
6.63	1.00	1.00	1.00	-1.00
3.87	-1.00	-1.00	-1.00	1.00
5.99	1.00	-1.00	-1.00	1.00
5.77	-1.00	1.00	-1.00	1.00
6.32	1.00	1.00	-1.00	1.00
3.45	-1.00	-1.00	1.00	1.00
5.11	1.00	-1.00	1.00	1.00
6.67	-1.00	1.00	1.00	1.00
8.69	1.00	1.00	1.00	1.00
9.43	0.00	0.00	0.00	0.00
8.21	0.00	0.00	0.00	0.00
8.98	0.00	0.00	0.00	0.00
8.88	0.00	0.00	0.00	0.00
8.75	0.00	0.00	0.00	0.00
8.69	0.00	0.00	0.00	0.00
9.00	0.00	0.00	0.00	0.00
7.21	2.00	0.00	0.00	0.00
1.12	-2.00	0.00	0.00	0.00
9.87	0.00	2.00	0.00	0.00
0.99	0.00	-2.00	0.00	0.00
6.03	0.00	0.00	2.00	0.00
3.24	0.00	0.00	-2.00	0.00
4.23	0.00	0.00	0.00	2.00
2.13	0.00	0.00	0.00	-2.00

B.3 Study of the Effects of Aeration on Citric Acid and Biomass Production

Table B.7: Citric Acid and Biomass Production at 0.5 vvm and 400 rpm

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	g CA/ g n-paraffin	g biomass/ g n-paraffin	Productivity g/L-h CA
0		3.52	114		0.000	
12		3.69	110		0.043	
24		5.62	109		0.420	
36		6.98	108		0.577	
48	0.00	7.85	100	0.000	0.309	0.000
60	1.58	8.02	102	0.132	0.375	0.132
72	1.89	8.57	103	0.172	0.459	0.079
84	2.5	8.88	99	0.167	0.357	0.069
96	2.41	8.98	95	0.127	0.287	0.050
108	2.98	9.05	93	0.142	0.263	0.050
120	3.1	10.58	88	0.119	0.272	0.043
132	3.25	10.75	86	0.116	0.258	0.039
144	3.33	10.98	87	0.123	0.276	0.035
156	3.45	10.76	85	0.119	0.250	0.032
168	3.56	10.88	79	0.102	0.210	0.030

Table B.8: Citric Acid and Biomass Production at 1.0 vvm and 400 rpm

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	g CA/ g n-paraffin	g biomass/ g n-paraffin	Productivity g/L-h CA
0		2.9	115		0.000	
12		2.54	112		0.120	
24		5.21	110		0.462	
36		6.69	103		0.316	
48	0.00	7.98	99	0.000	0.318	0.000
60	2.69	8.85	95	0.135	0.298	0.224
72	3.25	9.36	96	0.171	0.340	0.135
84	3.58	9.57	97	0.199	0.371	0.099
96	4	9.33	97	0.222	0.357	0.083
108	3.89	9.25	94	0.185	0.302	0.065
120	3.9	9.78	92	0.170	0.299	0.054
132	4	10.44	88	0.148	0.279	0.048
144	4.33	10.25	85	0.144	0.245	0.045
156	4.23	10.55	84	0.136	0.247	0.039
168	4.5	10.7	81	0.132	0.229	0.038

Table B.9: Citric Acid and Biomass Production at 2.0 vvm and 400 rpm

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	g CA/ g n-paraffin	g biomass/ g n-paraffin	Productivity g/L-h CA
0		4.58	115		0.000	
12		4.52	112		0.020	
24		5.84	107		0.158	
36		6.71	105		0.213	
48	0.00	7.58	102	0.000	0.231	0.000
60	2.25	7.79	99	0.141	0.201	0.188
72	2.05	8.22	95	0.103	0.182	0.085
84	2.69	8.57	92	0.117	0.173	0.075
96	2.88	8.98	87	0.103	0.157	0.060
108	3.22	9.56	86	0.111	0.172	0.054
120	3.45	9.88	82	0.105	0.161	0.048
132	3.56	10.16	79	0.099	0.155	0.042
144	4.25	10.02	75	0.106	0.136	0.044
156	4.55	10.25	74	0.111	0.138	0.042
168	4.77	10.69	72	0.111	0.142	0.040

B.4 Batch Fermentations - Effects of Agitation on Citric Acid and Biomass Production

Table B.10: Citric Acid and Biomass Production at 400 rpm and 1.0 vvm

Time (hour)	Citric Aci (g/L)	Biomass (g/L)	n-Paraffin (g/L)	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		2.9	115		0.000	
12		2.54	112		0.120	
24		5.21	110		0.462	
36		6.69	103		0.316	
48	0.00	7.98	99	0.000	0.318	0.000
60	2.69	8.85	95	0.135	0.298	0.224
72	3.25	9.36	96	0.171	0.340	0.135
84	3.58	9.57	97	0.199	0.371	0.099
96	4	9.33	97	0.222	0.357	0.083
108	3.89	9.25	94	0.185	0.302	0.065
120	3.9	9.78	92	0.170	0.299	0.054
132	4	10.44	88	0.148	0.279	0.048
144	4.33	10.25	85	0.144	0.245	0.045
156	4.23	10.55	84	0.136	0.247	0.039
168	4.5	10.7	81	0.132	0.229	0.038

Table B.11: Citric Acid and Biomass Production at 800 rpm and 1.0 vvm

Time (hour)	Citric Aci (g/L)	Biomass (g/L)	n-Paraffin (g/L)	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		3.6	114		0.000	
12		4.67	112		0.535	
24		5.41	108		0.302	
36		6.23	107		0.376	
48	0.00	7.41	104	0.000	0.381	0.000
60	2.69	8.98	103	0.245	0.489	0.224
72	3.68	9.25	99	0.245	0.377	0.153
84	4.57	9.61	92	0.208	0.273	0.127
96	5.55	10.2	93	0.264	0.314	0.116
108	6.01	10.5	87	0.223	0.256	0.100
120	6.25	11	85	0.216	0.255	0.087
132	6.99	11.58	84	0.233	0.266	0.083
144	6.35	11.6	82	0.198	0.250	0.066
156	6.69	11.75	81	0.203	0.247	0.062
168	6.58	11.45	79	0.188	0.224	0.055

Table B.12: Citric Acid and Biomass Production at 1000 rpm and 1.0 vvm

Time (hour)	Citric Aci (g/L)	Biomass (g/L)	n-Paraffin (g/L)	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		4.56	113		0.000	
12		5.69	111		0.565	
24		6.88	107		0.387	
36		7.89	103		0.333	
48	0	8.12	100	0	0.274	0
60	2.54	8.78	95	0.141	0.234	0.212
72	4.52	9.25	92	0.215	0.223	0.188
84	4.9	9.31	89	0.204	0.198	0.136
96	5.55	9.87	86	0.206	0.197	0.116
108	5.58	10.2	83	0.186	0.188	0.093
120	6	10.6	80	0.182	0.183	0.083
132	6.9	12.6	79	0.203	0.236	0.082
144	7.5	13.99	79	0.221	0.277	0.078
156	7.77	14.5	76	0.210	0.269	0.072
168	7.87	14.78	75	0.207	0.269	0.066

Table B.13: Citric Acid and Biomass Production at 1200 rpm and 1.0 vvm

Time (hour)	Citric Aci (g/L)	Biomass (g/L)	n-Paraffin (g/L)	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		4.87	115		0.000	
12		5.54	113		0.335	
24		7.18	105		0.231	
36		7.71	99		0.178	
48	0.00	8.22	100	0.00	0.223	0.000
60	1.98	8.79	94	0.094	0.187	0.033
72	4.69	9.22	92	0.204	0.189	0.065
84	5.98	9.58	87	0.214	0.168	0.071
96	7.5	10.23	85	0.250	0.179	0.078
108	8.21	10.5	84	0.265	0.182	0.076
120	8.89	12.69	84	0.287	0.252	0.074
132	9.49	13.25	79	0.264	0.233	0.072
144	9.3	13.98	75	0.233	0.228	0.065
156	9.125	14.21	74	0.223	0.228	0.058
168	8.98	14.55	72	0.209	0.225	0.053

B.5 Fed-Batch Fermentations - Effects of Agitation on Citric Acid and Biomass Production

Table B.14: Citric Acid and Biomass Production at 400 rpm and 1.0 vvm at a feeding rate of 1.0L/day

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		1.41	116		0.000	
12		2.01	112		0.150	
24		5.54	109		0.590	
36	0.00	6.25	100	0.000	0.303	0.000
48	2.20	6.89	95	0.105	0.261	0.183
60	2.58	7.25	96	0.129	0.292	0.108
72	3.25	8.25	93	0.141	0.297	0.090
84	3.69	8.59	91	0.148	0.287	0.077
96	3.77	9.41	90	0.145	0.308	0.063
108	4.22	10.58	93	0.176	0.382	0.059
120	4.57	10.89	95	0.195	0.405	0.054
132	4.71	11.25	97	0.212	0.444	0.049
144	4.97	11.53	97	0.215	0.438	0.046
156	5.06	11.68	98	0.219	0.444	0.042
168	5.11	11.99	97	0.219	0.453	0.039

Table B.15: Citric Acid and Biomass Production at 800 rpm and 1.0 vvm at a feeding rate of 1.0L/day

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		1.29	114		0.000	
12		1.98	110		0.173	
24		5.02	108		0.622	
36	0	6.58	98	0.000	0.331	0.000
48	2.58	7.45	91	0.112	0.268	0.215
60	3.69	7.89	89	0.148	0.264	0.154
72	5.24	8.25	88	0.202	0.268	0.146
84	5.89	8.88	87	0.218	0.281	0.123
96	6.21	9.25	85	0.214	0.274	0.104
108	8.11	10.21	88	0.294	0.324	0.113
120	8.79	10.54	91	0.342	0.360	0.105
132	9.01	11.22	93	0.358	0.394	0.094
144	9.56	11.54	95	0.398	0.427	0.089
156	9.75	11.61	97	0.406	0.430	0.081
168	9.87	11.65	97	0.409	0.429	0.075

Table B.16: Citric Acid and Biomass Production at 1000 rpm and 1.0 vvm at a feeding rate of 1.0L/day

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		2.30	115		0.000	
12		2.69	112		0.130	
24		5.63	103		0.278	
36	0.00	6.87	93	0.000	0.208	0.000
48	2.98	7.51	89	0.115	0.200	0.248
60	4.01	7.89	87	0.143	0.200	0.167
72	4.87	8.22	85	0.162	0.197	0.135
84	5.31	8.74	83	0.166	0.201	0.111
96	5.99	9.21	80	0.171	0.197	0.100
108	6.99	9.82	83	0.207	0.223	0.097
120	8.53	10.66	86	0.265	0.260	0.102
132	10.41	11.21	89	0.345	0.295	0.108
144	10.98	11.56	90	0.372	0.314	0.102
156	11.28	11.62	90	0.376	0.311	0.094
168	11.32	11.87	90	0.369	0.312	0.086

Table B.17: Citric Acid and Biomass Production at 1200 rpm and 1.0 vvm at a feeding rate of 1.0L/day

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		1.98	116		0.000	
12		2.56	109		0.083	
24		4.75	106		0.277	
36	0.00	5.25	99	0.000	0.192	0.000
48	2.58	5.89	95	0.123	0.186	0.215
60	3.68	6.57	93	0.160	0.200	0.153
72	5.25	7.78	91	0.210	0.232	0.146
84	6.98	7.99	89	0.259	0.223	0.145
96	7.48	8.25	87	0.258	0.216	0.125
108	7.99	9.61	90	0.290	0.277	0.111
120	8.56	10.25	89	0.292	0.282	0.102
132	8.87	10.88	91	0.312	0.313	0.092
144	9.11	11.02	91	0.316	0.314	0.084
156	9.23	11.23	92	0.324	0.325	0.077
168	9.47	11.31	92	0.324	0.319	0.072

B.6 Fed-Batch Fermentations - Effect of Feeding Rate on Citric Acid and Biomass Production

Table B.18: Effects of Feeding Rate on Citric Acid and Biomass Production

Feeding rate of 0.75 L/day				Feeding rate of 1.0 L/day				Feeding rate of 1.5 L/day			
Time	itric Aci	Biomass	-paraffi	Time	itric Aci	Biomass	-paraffi	Time	Citric Acid	Biomass	-paraffi
hour	g/L	g/L	g/L	hour	g/L	g/L	g/L	hour	g/L	g/L	g/L
0		2.05	113	0		1.29	114	0		1.45	113
12		2.47	108	12		1.98	110	12		2.1	110
24		3.5	106	24		5.02	108	24		5.02	108
36	0	4.12	98	36	0	6.58	98	36	0	6.58	97
48	2.2	5.14	92	48	2.58	7.45	91	48	2.45	7.45	92
60	2.94	5.87	89	60	3.69	7.89	89	60	3.02	7.89	89
72	3.25	6.23	84	72	5.24	8.25	88	72	4.11	8.25	81
84	3.78	6.57	82	84	5.89	8.88	87	84	4.87	8.88	79
96	3.88	6.99	80	96	6.21	9.25	85	96	5.33	9.25	77
108	4.12	7.99	82	108	8.11	10.21	88	108	6.58	10.21	83
120	5.11	8.69	84	120	8.79	10.54	91	120	7.11	10.54	87
132	5.45	8.77	83	132	9.01	11.22	93	132	7.55	11.22	88
144	5.77	8.86	83	144	9.56	11.54	95	144	7.74	11.54	87
156	6.02	9.07	84	156	9.75	11.61	97	156	8.01	11.61	85
168	6.24	9.28	85	168	9.87	11.65	97	168	8.12	11.65	83

B.7 Batch Fermentations - Effect of External Agitator

Table B.19: Effects of External Agitator on Citric Acid and Biomass Production

Agitator at 1400rpm				Agitator at 1600rpm			
Time hours	Citric Acid g/L	Biomass g/L	n-paraffin g/L	Time hours	Citric Acid g/L	Biomass g/L	-paraffin g/L
0		1.49	114	0		1.89	116
12		2.1	110	12		2.5	111
24		4.87	102	24		5.12	99
36	0	6.57	99	36	0	6.69	94
48	1.89	7.25	95	48	1.71	7.25	89
60	2.69	7.89	93	60	2.87	8.25	85
72	2.89	8.01	91	72	3.02	8.89	83
84	3.21	8.25	90	84	3.25	9.31	86
96	3.11	8.44	85	96	3.69	9.56	81
108	3.54	8.69	82	108	4.01	9.78	79
120	3.87	9.5	89	120	4.4	10.11	75
132	4.25	10.06	79	132	4.56	10.35	76
144	4.33	10.25	74	144	4.78	10.45	78
156	4.41	10.55	77	156	4.98	10.52	81
168	4.53	10.69	75	168	5.02	10.63	79

Nore: Fermentor Agitation is kept at 400 rpm throughout fermentations

B.8 Fed-Batch Fermentations - Effect of External Agitator

Table B.20: Citric Acid and Biomass Production at an External Agitation of 1400 rpm, 1.0 vvm and feeding rate of 1.0L/day

Time hour	itric Aci g/L	Biomass g/L	n-paraffin g/L	gCA/ n-paraffi	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		1.78	113		0.000	
12		2.87	108		0.218	
24		5.89	105		0.514	
36	0.00	6.25	99	0.000	0.319	0.000
48	2.01	6.89	95	0.112	0.284	0.168
60	4.25	7.58	92	0.202	0.276	0.177
72	5.54	8.69	90	0.241	0.300	0.154
84	5.87	8.98	89	0.245	0.300	0.122
96	6.23	9.87	87	0.240	0.311	0.104
108	6.54	10.25	90	0.263	0.340	0.091
120	7.34	10.54	92	0.303	0.362	0.087
132	7.59	10.69	92	0.307	0.360	0.079
144	8.69	10.98	93	0.355	0.376	0.080
156	9.02	11.24	93	0.355	0.373	0.075
168	9.31	11.45	94	0.366	0.380	0.071

Note: Fermentor Agitation is kept at 400 rpm throughout fermentations

Table B.21: Citric Acid and Biomass Production at an External Agitation of 1600 rpm, 1.0 vvm and feeding rate of 1.0L/day

Time hour	itric Aci g/L	Biomass g/L	n-paraffin g/L	gCA/ n-paraffi	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		1.99	112		0.000	
12		3.04	103.6		0.125	
24		5.47	101		0.316	
36	0.00	5.99	95	0.000	0.235	0.000
48	2.50	6.23	93	0.132	0.223	0.208
60	2.89	6.89	90	0.131	0.223	0.120
72	3.50	7.69	89	0.152	0.248	0.097
84	3.88	8.22	87	0.155	0.249	0.081
96	3.90	8.75	84	0.139	0.241	0.065
108	4.19	8.97	87	0.157	0.262	0.058
120	4.25	9.01	89	0.165	0.272	0.051
132	4.89	9.15	90	0.187	0.273	0.051
144	5.00	9.22	91	0.194	0.280	0.046
156	5.15	9.38	92	0.200	0.288	0.043
168	5.26	9.42	94	0.212	0.300	0.040

Note: Fermentor Agitation is kept at 400 rpm throughout fermentations

B.9 Fed-Batch Fermentations - Effect of a 3 Cycle Feeding System

Table B.22: Citric Acid and Biomass Production at 400 rpm, 1.0 vvm and feeding rate of 1.0 L/day

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		1.87	115		0.000	
24	0.00	4.86	112	0.000	0.997	0.000
48	2.22	5.55	101	0.159	0.263	0.093
72	2.80	6.28	96	0.147	0.232	0.058
96	3.45	6.55	91	0.144	0.195	0.048
120	3.99	6.88	96	0.185	0.319	0.042
144	4.21	7.24	98	0.197	0.339	0.035
168	4.87	7.87	99	0.224	0.362	0.034
192	5.50	8.50	95	0.214	0.331	0.033
216	5.68	9.01	91	0.191	0.303	0.030
240	5.99	10.50	94	0.206	0.349	0.028
264	6.66	10.84	96	0.230	0.371	0.028
288	7.54	11.45	96	0.251	0.385	0.029
312	8.27	11.87	91	0.239	0.352	0.029
336	8.47	12.11	88	0.228	0.335	0.027
360	8.88	12.21	91	0.213	0.305	0.026
384	9.01	12.36	95	0.231	0.328	0.025
408	9.28	12.44	94	0.234	0.325	0.024
432	9.45	12.45	89	0.218	0.300	0.023

Table B.23: Citric Acid and Biomass Production at 800 rpm, 1.0 vvm and feeding rate of 1.0L/day

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		2.03	111		0.000	
24	0.00	5.69	107	0.000	0.915	0.000
48	1.78	7.89	100	0.162	0.533	0.074
72	2.60	8.99	95	0.163	0.435	0.054
96	3.58	9.87	89	0.163	0.356	0.050
120	5.87	11.94	94	0.245	0.498	0.061
144	9.68	12.16	95	0.387	0.486	0.081
168	11.68	12.41	94	0.440	0.467	0.081
192	13.69	12.63	89	0.434	0.400	0.081
216	15.99	12.88	85	0.450	0.362	0.083
240	19.87	13.57	91	0.565	0.409	0.092
264	25.00	14.05	94	0.705	0.439	0.104
288	27.00	14.59	95	0.777	0.463	0.102
312	29.00	15.02	92	0.770	0.442	0.101
336	31.00	15.11	88	0.749	0.409	0.099
360	32.00	15.23	92	0.730	0.369	0.095
384	32.55	15.80	95	0.782	0.398	0.090
408	32.87	16.04	95	0.809	0.411	0.086
432	32.99	16.02	91	0.761	0.385	0.081

Table B.24: Citric Acid and Biomass Production at 1000 rpm, 1.0 vvm and feeding rate of 1.0L/day

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		1.99	112		0.000	
24	0.00	5.89	108	0.000	0.975	0.000
48	1.68	7.12	100	0.140	0.428	0.070
72	2.87	8.70	94	0.159	0.373	0.060
96	3.69	9.45	88	0.154	0.311	0.051
120	6.87	10.58	92	0.296	0.456	0.072
144	8.88	11.01	95	0.392	0.486	0.074
168	10.99	12.18	95	0.447	0.496	0.076
192	14.69	13.45	90	0.497	0.455	0.087
216	19.58	13.57	87	0.601	0.417	0.102
240	24.87	14.01	93	0.764	0.467	0.115
264	28.44	14.58	96	0.901	0.508	0.119
288	34.51	14.87	98	1.101	0.535	0.131
312	36.10	15.30	95	1.060	0.507	0.125
336	38.04	15.50	91	1.010	0.467	0.122
360	39.11	16.09	94	0.978	0.621	0.116
384	40.11	16.28	96	1.049	0.634	0.111
408	40.28	16.47	96	1.074	0.631	0.105
432	40.47	17.20	91	0.989	0.591	0.099

Table B.25: Citric Acid and Biomass Production at 1200 rpm, 1.0 vvm and feeding rate of 1.0 L/day

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		2.34	113		0.000	
24	0.00	6.21	109	0.000	0.968	0.000
48	2.98	8.98	99	0.213	0.474	0.124
72	3.96	9.74	95	0.220	0.411	0.083
96	5.12	9.84	90	0.223	0.326	0.071
120	6.98	10.25	94	0.312	0.458	0.073
144	7.98	10.61	96	0.363	0.482	0.067
168	9.22	10.73	95	0.384	0.447	0.064
192	15.68	11.04	91	0.560	0.394	0.093
216	22.58	11.23	88	0.728	0.362	0.118
240	26.88	12.55	93	0.869	0.432	0.124
264	28.69	13.69	95	0.931	0.466	0.120
288	30.78	14.57	97	1.027	0.507	0.117
312	32.87	15.24	94	1.004	0.487	0.114
336	33.68	16.00	90	0.935	0.462	0.108
360	35.63	17.22	94	0.904	0.440	0.106
384	35.60	17.51	96	0.952	0.468	0.099
408	35.89	17.78	95	0.960	0.475	0.093
432	35.78	17.91	90	0.881	0.438	0.088

Table B.26: Citric Acid and Biomass Production at an External Agitation of 1400 rpm, 1.0 vvm and feeding rate of 1.0 L/day

Time hour	Citric Acid g/L	Biomass g/L	n-paraffin g/L	gCA/ g n-paraffin	gbiomass/ g n-parffin	Productivity (g/L-h) CA
0		1.87	112		0.000	
24	0.00	5.78	106	0.000	0.652	0.000
48	1.82	6.33	101	0.165	0.405	0.076
72	2.80	6.47	96	0.175	0.288	0.058
96	3.25	7.05	91	0.155	0.247	0.045
120	4.02	7.15	94	0.193	0.344	0.042
144	4.77	7.81	96	0.231	0.378	0.040
168	5.87	8.21	95	0.257	0.359	0.041
192	7.88	8.66	91	0.293	0.322	0.047
216	9.63	9.33	86	0.302	0.293	0.050
240	11.67	10.61	93	0.378	0.351	0.054
264	13.05	11.34	96	0.436	0.390	0.054
288	14.22	11.45	97	0.476	0.401	0.054
312	16.11	12.45	95	0.499	0.405	0.056
336	16.87	12.68	91	0.471	0.374	0.054
360	17.52	12.73	95	0.459	0.346	0.052
384	17.98	12.88	96	0.484	0.361	0.050
408	18.54	13.02	99	0.528	0.387	0.048
432	18.79	13.04	95	0.495	0.359	0.046

Nore: Fermentor Agitation is kept at 400 rpm throughout fermentations

B.10 Standard Curves

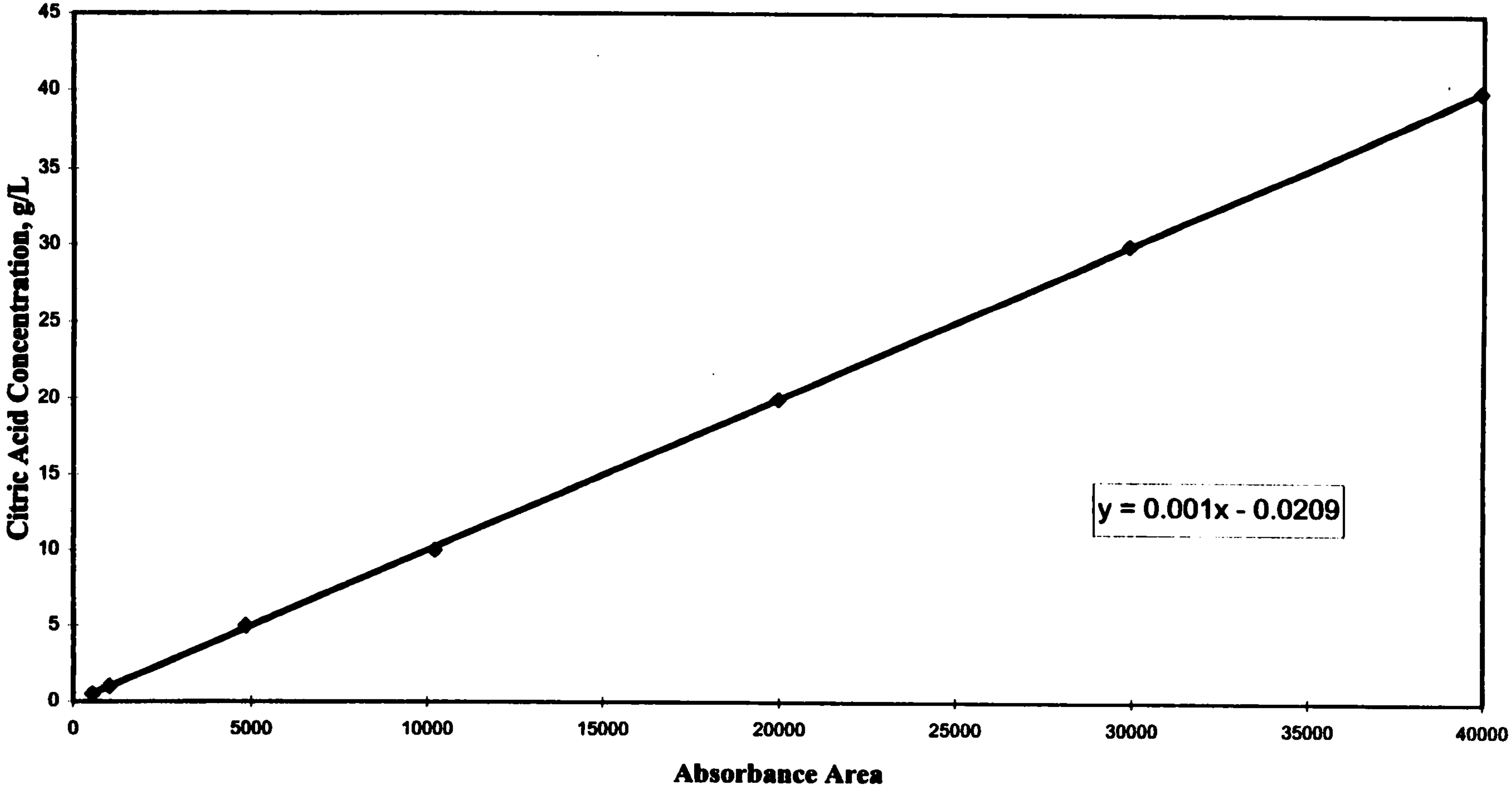

Figure B.1:HPLC Standard Curve for Citric Acid Analysis

APPENDIX C:

Central Composite Experimental Design

This appendix describes the experimental strategy, the central composite design, and the model-building techniques

C.1 Background

A response surface methodology in chemical process development is demonstrated by the work of Thompson (1982). The central composite design for second order designs is as follows:

$$E(Y) = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \sum_{j\geq 1}^{k} \beta_{ij} X_i X_j \qquad [C1]$$

where

$E(Y)$ is the expected value of the response variable
β_o, β_i, β_j are the model parameters
X_i and X_j are the coded factors being studied
k is the number of factors being studied

The design consists of a 2^k factorial or fractional factorial design augmented by 2k axial (star) points at $(\pm\alpha, 0, 0,\ldots, 0)$, $(0, 0, \pm\alpha,\ldots, 0)$,…, $(0, 0, 0,\ldots, \pm\alpha)$ and n_o center points at $(0, 0,\ldots, 0)$, where α is the distance of the star point from the center. The choice of α establishes the rotatability of a central composite design. For a four factor design, α should be set at 2.0, as discussed in McLean et al. (1997), it is the recommended α value for a four factor design.

The central composite design that was used for this study was a four factor design for citric acid production and biomass production. The design used is described as a Box-Wilson central composite circumscribed design for four factors (McLean et al., 1997). The design if further described in section C2.

C.2 Four-Factor Central Composite Design

The proposed model for the four-factor design is :

$$
\begin{aligned}
E(Y) = \beta_0 &+ \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \\
&+ \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 \\
&+ \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4
\end{aligned}
\qquad [C2]
$$

The run conditions used for the batch flask tests for citric acid and biomass production are described in Tables C1.

Table C.1: Run Conditions for the Four-Factor Central Composite Design

Biomass X_1,%	HC X_2,%	FE X_3,mg/L	Temp X_4, °C	Coded X_1	Coded X_2	Coded X_3	Coded X_4
7.5	7.5	6	25	-1	-1	-1	-1
12.5	7.5	6	25	1	-1	-1	-1
7.5	12.5	6	25	-1	1	-1	-1
12.5	12.5	6	25	1	1	-1	-1
7.5	7.5	14	25	-1	-1	1	-1
12.5	7.5	14	25	1	-1	1	-1
7.5	12.5	14	25	-1	1	1	-1
12.5	12.5	14	25	1	1	1	-1
7.5	7.5	6	31	-1	-1	-1	1
12.5	7.5	6	31	1	-1	-1	1
7.5	12.5	6	31	-1	1	-1	1
12.5	12.5	6	31	1	1	-1	1
7.5	7.5	14	31	-1	-1	1	1
12.5	7.5	14	31	1	-1	1	1
7.5	12.5	14	31	-1	1	1	1
12.5	12.5	14	31	1	1	1	1
10	10	10	28	0	0	0	0
10	10	10	28	0	0	0	0
10	10	10	28	0	0	0	0
10	10	10	28	0	0	0	0
10	10	10	28	0	0	0	0
10	10	10	28	0	0	0	0
10	10	10	28	0	0	0	0
15	10	0	28	2	0	0	0
5	10	0	28	-2	0	0	0
10	15	0	28	0	2	0	0
10	5	0	28	0	-2	0	0
10	0	18	28	0	0	2	0
10	0	2	28	0	0	-2	0
10	0	10	34	0	0	0	2
10	0	10	22	0	0	0	-2

C.3 Analysis of Model

Using least squares analysis, four critical assumptions are made: (McLean et al., 1997)

1. The values of the experimental factors are known exactly; the associated variance is zero. In other words, the uncertainty associated with the value of the experimental factor has less of an effect on the response value than the uncertainty associated with the measured value of the response itself.
2. The expected value of the random error is zero.
3. The variance of the random error is constant over the range of the experimental factors that are used to collect the data.
4. There is no association of the random error for any one data point with the random error for any other data point.

It is necessary to perform an analysis of the residual from the model, to determine the adequacy of the least squares fit. Plots of the residuals, both normal probability plots and plots of the residuals versus predicted values of the response variable, are constructed to verify that the random error follows the underlying assumption.

ANOVA (Analysis of Variance) tables are constructed as well. In an ANOVA analysis, the variation is separated into regression and residual variance, as shown in Table C2.

Table C2: ANOVA Table for Multiple Regression Models (Thompson, 1982)

Source Variance	Degrees of Freedom	Sum of Squares	Mean Squares	F
Regression	p	R_eSS	MR_e=ReSS/p	MR_e/MR
Residual	N-p-1	RSS	MR=RSS/N-p-1	
(lack of fit)	N-p-1-(n_0-1)	R_LSS	MR_L=RLSS/ N-p-1-(n_0-1)	MR_L/MR_p
(pure error)	n_0-1	R_pSS	$MR_p=R_pSS/ n_0-1$	
Total	N-1	SS		

In multiple regression models, there are p degrees of freedom for the sum of squares due to regression, because p coefficients, β_1, β_2, …, β_p, must be estimated to obtain RSS. The degrees of freedom for the entire system is defined as the total number of experimental runs minus 1 (N-1).

Therefore,

Sum of Squares

$$SS = \Sigma (y_i - y)^2 \qquad [C3]$$

where SS is the sum of squares of deviations of the observed values from their mean value;

$$RSS = \Sigma (y_i - y_{predi})^2 \qquad [C4]$$

where RSS is the sum of squares of the residuals;

$$R_eSS = SS - RSS \qquad [C5]$$

where R_eSS is the sum of squares due to the regression and,

y_i is the observed value of response variable at design point i

y is the mean value of the observed responses

y_{predi} is the predicted response variable at point i

The lack fit test checks whether the order of the model is correct. The test involves dividing the variability of the residuals into two components, pure error and lack of fit. The pure error sum of squares is calculated using the values of the response variables at the replicate (center) points (Thompson, 1982):

$$R_pSS = \Sigma \left(y_{0i} - y_0\right)^2 \qquad [C6]$$

The corresponding degrees of freedom is the number of center points minus 1 (n_0-1). The lack of fit F-statistic shown in Table B2 provides the basis for the evaluation of lack of fit.

The coefficient of determination, R^2, is another measure of the adequacy of the fit where,

$$R^2 = 1\text{-}RSS/SS = (SS - RSS)/SS = R_eSS/SS \qquad [C7]$$

Values range from 0 to 1, with values closer to 1 indicating high consistencies between the predicted and observed values. R^2 also provides a measure of the fraction of the total variability in the data explained by the regression.

APPENDIX D:

Model Results

This appendix contains the empirical modeling data and results for the control.

D.1 Citric Acid Production

Table D.1: Parameter Estimates for Citric Acid Production Model

	β_0	β_1	β_2	β_3	β_4	β_{11}	β_{22}
Estimate	8.849	0.995	1.329	0.493	0.520	-1.027	-0.711
Std. Err.	0.421	0.227	0.227	0.227	0.227	0.208	0.208
t(16)	21.028	4.380	5.847	2.169	2.286	-4.932	-3.413
p-level	0.000	0.001	0.000	0.046	0.036	0.000	0.004

Table D.1 cont'd : Parameter Estimates for Citric Acid Production Model

	β_{33}	β_{44}	β_{12}	β_{13}	β_{14}	β_{23}	β_{24}	β_{34}
Estimate	-0.909	-1.273	-0.205	0.091	0.061	0.339	0.245	-0.144
Std. Err.	0.208	0.208	0.278	0.278	0.278	0.278	0.278	0.278
t(16)	-4.367	-6.114	-0.734	0.325	0.325	1.219	0.882	-0.518
p-level	0.000	0.000	0.474	0.748	0.748	0.240	0.390	0.611

Variance explained 89.7%   R=0.947

Table D.2: Parameter Estimates for Modified Citric Acid Production Model*

	β_0	β_1	β_2	β_3	β_4	β_{11}	β_{22}
Estimate	8.849	0.995	1.329	0.493	0.520	-1.027	-0.711
Std. Err.	0.393	0.212	0.212	0.212	0.212	0.194	0.195
t(22)	22.492	4.685	6.254	2.32	2.445	-5.275	-3.651
p-level	0.000	0.000	0.000	0.030	0.023	0.000	0.001

Table D.2 cont'd: Parameter Estimates for Modified Citric Acid Production Model *

	β_{33}	β_{44}
Estimate	-0.909	-1.273
Std. Err.	0.194	0.195
t(22)	-4.671	-6.541
p-level	0.000	0.000

Variance explained 87.5%   R= 0.935

* Modified Model has β parameters with p-levels greater than 0.05 in Table D1 removed

Table D.3: ANOVA Table for Citric Acid Production Model

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Squares	F	$F_{.95,v1,v2}$
Regression	8	166.6	20.8	19.1	2.40
Residual	22	24.0	1.09		
(Lack of Fit)	16	23.18	1.44	10.29	3.93
(Pure Error)	6	0.82	0.14		
Total	30	190.6			

Table D.4: Pure Error Variance for Citric Acid Production Model

For Replicate Set	
Mean	5.81
Sum of Residuals Squared	0.82
Degrees of Freedom	6
Pure Error Variance	0.14

Table D.5: Correlation Matrix for Parameter Estimates for Citric Acid Production

	β_0	β_1	β_2	β_3	β_4	β_{11}	β_{22}	β_{33}	β_{44}
β_0	1.00	0.00	0.00	0.00	0.00	-0.51	-0.51	-0.51	-0.51
β_1	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
β_2	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00
β_3	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00
β_4	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00
β_{11}	-0.51	0.00	0.00	0.00	0.00	1.00	0.11	0.11	0.11
β_{22}	-0.51	0.00	0.00	0.00	0.00	0.11	1.00	0.11	0.11
β_{33}	-0.51	0.00	0.00	0.00	0.00	0.11	0.11	1.00	0.11
β_{44}	-0.51	0.00	0.00	0.00	0.00	0.11	0.11	0.11	1.00

D.2 Biomass Production Model

Table D.6: Parameter Estimates for Biomass Production Model

	β_0	β_1	β_2	β_3	β_4	β_{11}	β_{22}
Estimate	4.613	0.457	0.481	0.181	0.289	-0.232	-0.215
Std. Err.	0.116	0.063	0.063	0.063	0.063	0.057	0.057
t(16)	39.474	7.236	7.632	2.865	4.581	-4.019	-3.717
p-level	0.000	0.000	0.000	0.011	0.000	0.000	0.002

Table D.6 cont'd: Parameter Estimates for Biomass Production Model

	β_{33}	β_{44}	β_{12}	β_{13}	β_{14}	β_{23}	β_{24}	β_{34}
Estimate	-0.106	-0.404	-1.175	0.017	-0.050	0.170	-0.062	-0.055
Std. Err.	0.057	0.057	0.077	0.077	0.077	0.078	0.078	0.077
t(16)	-1.792	-6.981	-2.264	0.226	-0.647	2.199	-0.808	-0.712
p-level	0.092	0.000	0.038	0.823	0.526	0.043	0.431	0.487

Variance explained 93.2% R= 0.965

Table D.7: Parameter Estimates for Modified Biomass Production Model*

	β_0	β_1	β_2	β_3	β_4	β_{11}	β_{22}
Estimate	4.61309	0.457	0.481	0.181	0.289	-0.232	-0.215
Std. Err.	0.109	0.059	0.059	0.059	0.059	0.054	0.054
t(20)	42.044	7.706	8.128	3.052	4.880	-4.281	-3.959
p-level	0.000	0.000	0.000	0.006	0.000	0.000	0.001

Table D.7 cont'd: Parameter Estimates for Modified Biomass Production Model*

	β_{33}	β_{44}	β_{12}	β_{23}
Estimate	-0.106	-0.404	-1.175	0.170
Std. Err.	0.054	0.054	0.072	0.072
t(20)	-1.910	-7.436	-2.412	2.343
p-level	0.071	0.000	0.025	0.029

Variance explained 92.5% R= 0.962

* Modified Model has β parameters with p-levels greater than 0.05 in Table D6 removed

Table D.8: ANOVA Table for Biomass Production Model

Source of Variance	**Degrees of Freedom**	**Sum of Squares**	**Mean Squares**	**F**	**$F_{.05,v1,v2}$**
Regression	10	20.64	2.06	22.89	2.35
Residual	20	1.69	0.09		
(Lack of Fit)	14	1.38	0.10	2.0	3.96
(Pure Error)	6	0.31	0.05		
Total	30	22.33			

Table D.9: Pure Error Variance for Biomass Production Model

For Replicate Set	
Mean	3.87
Sum of Residuals Squared	0.31
Degrees of Freedom	6
Pure Error Variance	0.05

Table D.10: Correlation Matrix for Parameter Estimates for Biomass Production

	β_0	β_1	β_2	β_3	β_4	β_{11}	β_{22}	β_{33}	β_{44}
β_0	1.00	0.00	0.00	0.00	0.00	-0.51	-0.51	-0.51	-0.51
β_1	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
β_2	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00
β_3	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00
β_4	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00
β_{11}	-0.51	0.00	0.00	0.00	0.00	1.00	0.11	0.11	0.11
β_{22}	-0.51	0.00	0.00	0.00	0.00	0.11	1.00	0.11	0.11
β_{33}	-0.51	0.00	0.00	0.00	0.00	0.11	0.11	1.00	0.11
β_{44}	-0.51	0.00	0.00	0.00	0.00	0.11	0.11	0.11	1.00
β_{12}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
β_{23}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table D.10 cont'd: Correlation Matrix for Parameter Estimates for Biomass Production

	β_{12}	β_{23}
β_0	0.00	0.00
β_1	0.00	0.00
β_2	0.00	0.00
β_3	0.00	0.00
β_4	0.00	0.00
β_{11}	0.00	0.00
β_{22}	0.00	0.00
β_{33}	0.00	0.00
β_{44}	0.00	0.00
β_{12}	1.00	0.00
β_{23}	0.00	1.00

APPENDIX E

Cell lyses and Cell Viability

This appendix presents the data on the cell lyses and cell viability in fermentation systems

E.1 Cell Lyses

Table E.1 : Cell Lyses during Fermentation* using Greey Lightnin In-Line Agitator

Time, hrs	Cell Lyses at 1400 rpm (% cells)	Cell Lyses at 1600 rpm (% cells)
0	5.4	4.7
42	6.9	10.8
84	7.8	16.9
126	10.5	21.3
168	14.1	28.3

* Analysis was done in a fed-batch system at 1.0 vvm, 400rpm fermentor agitation, 28°C and feeding rate of 1.0 L/day

Measurement of Cell Lyses

Cell lyses was determined using a hemocytometer. The hemocytometer allows for a direct microscopic count of yeast cells. The percentage of lysed cells was determined as the number of cells lysed per the total number of cells. A hemocytometer is a slide with a counting chamber 0.1 mm deep. On the bottom of the counting chamber there is an etched square divided into nine squares, each 1 mm^2. The central square is divided into 16 squares. Therefore, within the central square millimeter there are a total of 400 small squares. To count the number of cells, count only the number of cells in 5 of the squares (central square and the 4 corner squares). Multiply the number of cells counted in the 5 squares by 5 to estimate the number of $cells/mm^2$ of surface.

The procedure involves placing a drop of the yeast suspension in each of the two counting chambers. The hemocytometer is then placed on the microscope stage and the yeast cells are counted using the 40x lens.

E.2 Cell Viability

Table E.2: Cell Viability of Yeast during 3 Cycle Fed-Batch System

Time, hrs	Cell Viability at 800 rpm, %	Cell Viability at 1000 rpm, %	Cell Viability at 1200 rpm, %
0	61	58	65
108	75	69	72
216	82	78	80
324	72	77	76
432	59	63	49

Measurement of cell viability

Cell Viability was measured using a methylene blue staining procedure. Blue cells indicate dead cells. Therefore, cell viability was taken as the number of unstained cells divided by the total number of cells. The cell count follows the hemocytometer procedure described in section E.1

APPENDIX F

Sample Calculations

This appendix illustrates the sample calculations

F.1 Yield and Productivity Calculations

Note: using data from Table B.7

Overall Citric Acid Yield

= total g citric acid produced / total g n-paraffin consumed

= 3.56 / (114-79) = 0.102 g citric acid/g n-paraffin

Overall Biomass Yield

= total g biomass produced / total g n-paraffin consumed

= (10.88-3.52) / (114-79) = 0.210 g biomass / g n-paraffin

Citric Acid Productivity

= citric acid produced / time duration of fermentation

= 3.56 / (168-48) = 0.03 g/L-h

F.2 Central Composite Design Calculations

Note: Biomass Production Model is Considered here

Table F.1 shows the Predicted and Residual values of the model.

Calculating the mean,

Mean = (2.03 + 3.27 + 3.48 + ... + 3.99 + 2.12 + 4.99) / 30
= 3.87

Mean of Replicates = (4.50 + 4.71 + ... + 4.52 + 4.2) / 6
= 5.38

Sum of Residual Squared = $(-0.065)^2 + (-0.153)^2 + \ldots + (-0.097)^2 + (-0.092)^2 + (-0.412)^2$
= 1.69

Sum of squares = $(2.03-4.01)^2 + (3.27-4.01)^2 + \ldots + (2.12-4.01)^2 + (4.99-4.01)^2$
= 22.33

$$\text{Sum of squares of regression} = 22.33 - 1.69$$
$$= 20.64$$

$$\text{Pure Error Variance} = (0.233)^2 + (0.023)^2 + \ldots + (0.412)^2 + (0.092)^2$$
$$= 0.31$$

Table F.1: Predicted and Residual Values for Biomass Production Model

Predicted	Residual
2.095	0.065
3.423	0.153
3.193	0.287
3.822	0.068
2.191	0.048
3.590	0.049
3.969	0.240
4.668	0.011
3.008	0.018
4.127	0.347
3.857	0.156
4.284	0.054
2.884	0.179
4.083	0.293
4.413	0.146
4.912	0.041
4.733	0.233
4.733	0.023
4.597	0.323
2.77	0.21
4.712	0.306
2.79	0.419
4.559	0.049
3.836	0.163
3.576	0.413
2.419	0.299
4.733	0.257
4.733	0.047
4.733	0.097
4.733	0.092
4.733	0.412

Therefore,

$$\text{Sum of Squares for lack of fit} = 1.69 - 0.31 = 1.38$$

Mean Squares for regression = 20.64/10 = 2.06

Mean Squares for residual = 1.69/20 = 0.09

Mean Squares for lack of fit = 1.38/14 = 0.10

Mean Squares for pure error = 0.31/6 = 0.05

F (regression) = 2.06/0.09 = 22.89

F (residual) = 0.1/0.05 = 2.0