UNIVERSITI PUTRA MALAYSIA

CITRIC ACID PRODUCTION BY *SACCHAROMYCOPSIS LIPOLYTICA* USING BATCH CULTURE: STUDY ON OXYGEN REQUIREMENT

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BY

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THIS THESIS IS DEDICATED; TO MY LATE FATHER AND GRANDMA

TO MY SON, FAROUQ AND MY DAUGHTER, AFREENA WHO WERE BORN DURING MY GRADUATE STUDIES YEARS

AND ESPECIALLY TO MY HUBBY AND MUM, THANK YOU FOR YOUR SUPPORT AND ENCOURAGEMENT

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Chairman: Dr Baharuddin Abdul Ghani

Faculty: Food Science and Biotechnology

A citric acid producing yeast strain *Saccharomycopsis lipolytic* 5054 formerly known as *Candida lipolytica* was used throughout the study. The strain was obtained from Thailand Institute of Scientific and Technological Research (TISTR). A preliminary study on the development for the optimization of the fermentation process was conducted. The experiments

included the effect of medium contents such as $CaCO_3$ and initial glucose concentrations, inoculum size, agitation rates, K_La (volumetric oxygen transfer coefficient) and dissolved oxygen concentrations.

From the preliminary experiments, Medium I (10% glucose, 0.1% yeast extract, 0.1% $MgSO_4.7H_2O$, 0.2% NH_4Cl, 0.1% KH_2PO, 1% $CaCO_3$, pH 6.6) was selected as the fermentation medium throughout the study. The optimum growth temperature and inoculum size was 30°C and 10%, respectively. 1% $CaCO_3$ (w/v) was selected as the optimum concentration. The highest K_La obtained was 29.6 $hour^{-1}$ at the agitation rate of 700 rpm using the Rushton turbine impeller. This impeller speed was employed during the studies on optimum dissolved oxygen concentration, where the highest citric acid production obtained was 11.6 g/L during fermentation at 50% (20 mg/L) dissolved oxygen concentration. The optimum fermentation period was 50 hours where the exponential phase ended and the stationary phase started.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains.

PENGHASILAN ASID SITRIK OLEH *SACCHAROMYCOPSIS LIPOLYTICA* : KAJIAN TERHADAP PENGGUNAAN OKSIGEN

OLEH

ROSMAWATI OSMAN

OKTOBER 1994

Pengerusi: Dr. Baharuddin Abdul Ghani

Fakulti: Sains Makanan dan Bioteknologi

Satu strain penghasil asid sitrik, *Saccharomycopsis lipolytica* TISTR 505 yang nama dahulunya *Candida lipolytica* telah digunakan di sepanjang projek ini. Strain tersebut telah diperolehi dari Thailand Institute of Scientific and Technological Research. Kajian awal untuk memilih bahan-bahan yang paling sesuai untuk kaldu seperti saiz inokulum dan kelajuan pengaduk telah dilakukan sebelum proses fermentasi. Semasa proses fermentasi dengan menggunakan fermenter makmal 1.5L, K_La (angkali pemindahan oksigen

terisipadu) dan kepekatan oksigen terlarut telah dikaji. Daripada sampel, analisis telah dilakukan terhadap biojisim sel, profil pH, asid sitrik dan kepekatan glukosa.

Daripada kajian awal, Medium I (10% glukosa, 0.1 ekstrak yis, 0.1 $MgSO_4.7H_2O$, 0.2% NH_4CL, 0.1% KH_2PO_4, 1% $CaCO_3$, pH 6.6) telah dipilih sebagai kaldu fermentasi untuk kajian seterusnya. Suhu optima dan saiz inokulum didapati masing-masing sebagai 30°C and 10%. Nilai K_La tertinggi ialah 29.6 j^{-1} dengan kelajuan pengaduk 700 putaran per minit. Kelajuan pengaduk ini telah digunakan di dalam kajian kepekatan oksigen terlarut. Keputusannya, penghasilan asid sitrik tertinggi ialah 11.6 g/L yang telah diperolehi pada tahap 50% (20 mg/L) kepekatan oksigen terlarut bila sistem fermentasi menggunakan oksigen tulin. Jangkamasa fermentasi optima ialah 50 jam di mana fasa eksponen tamat dan fasa pegun bermula.

CHAPTER I

INTRODUCTION

Citric acid or 2-hydroxy-1,2,3-propanetricarboxylic acid (77-92-9), one of the fruit acids, is relatively widespread in nature (Figure 1). The widespread presence of citric acid in animal, plant and in the breast milk of a nursing mothers assured its non-toxic nature and has long been used as an acidulant in soft drinks, jams and other confectionaries. Furthermore, citric is kind to the environment for it is biodegradable (Pfizer Inc. handbook, 1978).

Scheele reported the first isolation and crystalization of the sour constituent of the lemon juice in 1784 (Lockwood, 1978). Citric acid crystalises from water in two forms, anhydrous and monohydrate. Crystalization at the temperature of more than 36.6°C, anhydrous is formed. At lower temperatures, monohydrate is produced (Milsom and Meers, 1985).

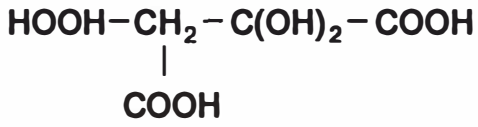

Figure 1. Structure of Citric Acid

In 1826, John and Edmund Sturge produced the first commercial citric acid derived from lemon juice. Up until about 1920, all commercial citric acid was obtained from the juices of lemons or limes (Milsom, 1987). Due to supply difficulties and increasing prices led to the development of fermentation processes for citric acid

production. Now almost all of the citric acid produced in the world are by fermentation processes using the microbial cells as the inoculum although a small amount estimated to be less than 1% is recovered from the citrus fruits in South America and Mexico. The first commercial processes employed *Aspergillus niger* growing on the surface of a medium consisting of sucrose and inorganic salts. The development of submerged citric acid fermentation only began in the last fourty years utilising glucose or beet or cane molasses as the substrate.

More recently, certain yeasts have been used instead of *A.niger* (Milsom, 1987). Almost all of the citric acid produced before 1968, used *Aspergillus niger* or a few other fungi as the inoculum. Now, it is known that various types of yeasts could also accumulate citric acid in the growth media. They include the species of *Candida (Saccharomycopsis), Hansenula, Pichia, Debaromyces, Torulopsis and Saccharomyces.* The *Candida* species are the ones which are widely used for studies on citric acid production. A few of the species are *C.lipolytica, C.tropicalis, C.zeylanoides, C.citrica, C.gulliermondii C.oleophila* and *C.sucrosa.*

According to Abou Zeid *et al.*(1984), yeasts of the candida strains seem to hold most promise for future production of citric acid. There are a few advantages of using yeasts over fungi:-

1. Yeasts are easily handled because they have no spores or mycelia which will interfere with the Oxygen transfer in the liquid media. They grow as a homogeneous suspension.

2. Yeasts were reported to have the ability to utilize a wide range of carbon sources such as hydrocarbons including n-paraffins, n-alkanes and n-hexadecane also molasses, alcohols, fatty acids and natural oils.

3. Once citric acid is produced in a large scale using a yeast, the biomass of the yeasts could be used for animal nutrition (Milsom, 1987).

4. Yeast-based processes have higher productivity meaning that the time period taken to consume all the substrate is half the time taken by fungi.

5. When grown in a fermentor, yeasts do not attach to probes or block ports (Roberts-Thomson and Maddox, 1993).

6. They do not require a metal ion deficiency, thus eliminating substrate pre-treatment step (Roberts-Thomson and Maddox, 1993).

Fermentation process is always under aerobic conditions and a temperature between 20°C-33°C should be employed for growth and citric acid production. However, along with the citric acid, the undesirable isocitric acid is also produced by the yeasts. Therefore, yeast strains which could produce high level of citric acid and very low level or none isocitric acid are desirable.

Since aconitase is the enzyme required to convert citric acid to isocitric acid, the enyzme activity could be inhibited either by chelating the Fe^{2+} ions (cofactor) using ferrocyanide or by adding fluoroacetate that will be converted to fluorocitrate which is the enzyme inhibitor.

The yeasts are highly oxidative, so a considerable amount of aeration for growth and metabolism is needed. Therefore, the citric acid fermentation using yeasts must be vigorously agitated.

Due to its flavour and other chemical properties, citric acid faces a very high demand. Approximately 75% of the citric acid produced is used in food and food related industries (Milsom, 1987). It is used as flavour enhancer, preservative, antioxidant and food additive for the production of soft drinks, wines, candies, jellies, jams and others. Citric acid is non-toxic, very digestible and tasty. In pharmaceutical and medical industries, citric acid acts as an anticoagulant, a solvent and a flavouring agent. Other uses are in cosmetics as an antioxidant and synergist for the production of astringent lotions and shampoos (Table 1).

Table 1

Application of Citric Acid

Industries	Properties	Example of Uses
Foods and Beverages	Sharpened flavours	Carbonated beverage
	Taste enhancer	"Still" drinks Candies Beverage syrups Toppings Jellies and jams
	Smooth texture	Cheese products
	Preservative	Seafood products
	Antioxidant	Processed fruits and vegetables
	Retard rancidity	Frozen fish
	pH control and chelating	Meat products
	Acidity adjuster	Wines

(Cont'd)

Industries	Properties	Example of Uses
Pharmaceutical and Cosmetics	Add effervescence	Anticids, analgesics Dentures cleaning tablets Bath salts
	Buffering agent Preservatives Flavour enhancer	Liquid products (cough syrups, aspirins)
	Buffering agent Antixidant	Shampoos, Lotions, cream and toothpastes
Other Indusries	Metal-ion sequestrant (metal cleaning processes)	Boilers Superheated tubings Nuclear reactors Stainless steel components
	"Unplug deposites"	Oil wells
	Neutralizing agent pH adjuster	Detergents Soaps
	Buffering agent	Textile

Source: Pfizer Incorporation Handbook, 1978)

The level of citric acid in food and beverages depends on the tartness desired. An example of high-acid drink is lemonade where 4-5 grams of acid used per quart of drinks. Orange, punch and cherry are medium-acid drinks which contain 2.5-3.5 grams acid per quart and low-acid drink like strawberry, black cherry and grapes consist of only 1.5-2.0 grams citric acid per quart of drinks. The sequestering action of citric acid used in the pharmaceutical field is able to stabilize ascorbic acid. The effervescent effect produced when combined with carbonates and bicarbonates are useful in the preparations of antacids and soluble aspirins. The metallic salt of trisodium citrate is used as a blood preservative where it prevents clotting by complexing calcium. In other industries, due to the citric acid ability to complex heavy metals such as iron and copper, it is used as stabilizer of oils and

fats where oxidation process catalysed by these metals are reduced. Its low degree attack on special steels combined with the ability to complex metals allows citric acid to be used in cleaning of power station boilers.

In this study, the strain *Saccharomycopsis lipolytica* formerly known as *Candida lipolytica* (Davenport, 1980) was used due to its ability to produce citric acid in a relatively high amount (Thailand Institute of Scientific and Technological Research). Although the concentration of citric acid produced is not as much as those produced by *Aspergillus niger, Candida* strains give the most promising future in citric acid production industry (Abou Zeid *et al.*, 1984). A number of studies on strain development has been done to obtain a more productive strain (Hamissa *et al.*, 1982; Good *et al.*, 1985; Akiyama *et al.*, 1972).

This project was done with the objective of learning the dissolved oxygen level at which citric acid is being produced efficiently. The knowledge gained in this study would further contribute to the utilization of yeast especially the *Candida* strains to produce citric acid.

CHAPTER II

LITERATURE REVIEW

Biosynthetic Pathway

In the biosynthesis of citric acid, both Embden-Meyerhoff glycolysis and Tricarboxylic acid (Kreb) cycle are involved. In the normal way, one molecule of acetate is condenced with a molecule of citrate. The citrate is further metabolized via the TCA cycle to regenerate oxaloacetate. If the TCA cycle is interrupted at the citric acid, no oxaloacetate is generated to condense acetyl-CoA to form citrate. Therefore oxaloacetate is being produced via anapleuratic reaction where the pyruvate is converted to oxaloacetate by the enzyme pyruvate carboxylase (Figure 2).

Eversince the year 1904, the theories of how citric acid accumulated in a microbial cell system had been published but none of these proposed theories has satisfactorily explained all the known observations. Various theories had been viewed by Abou Zeid *et al* (1984) in a review paper on acid production. According to the review, Maze and Perrier (1904) suggested that citric acid arose as a product of an incomplete metabolism, whereas Raistrick and Clark (1919) suggested that glucose is broken down to α–ω–diketoadipic acid which was hydrolized to acetic acid and oxaloacetic acid. However, the review also reported that Wehmer (1917)

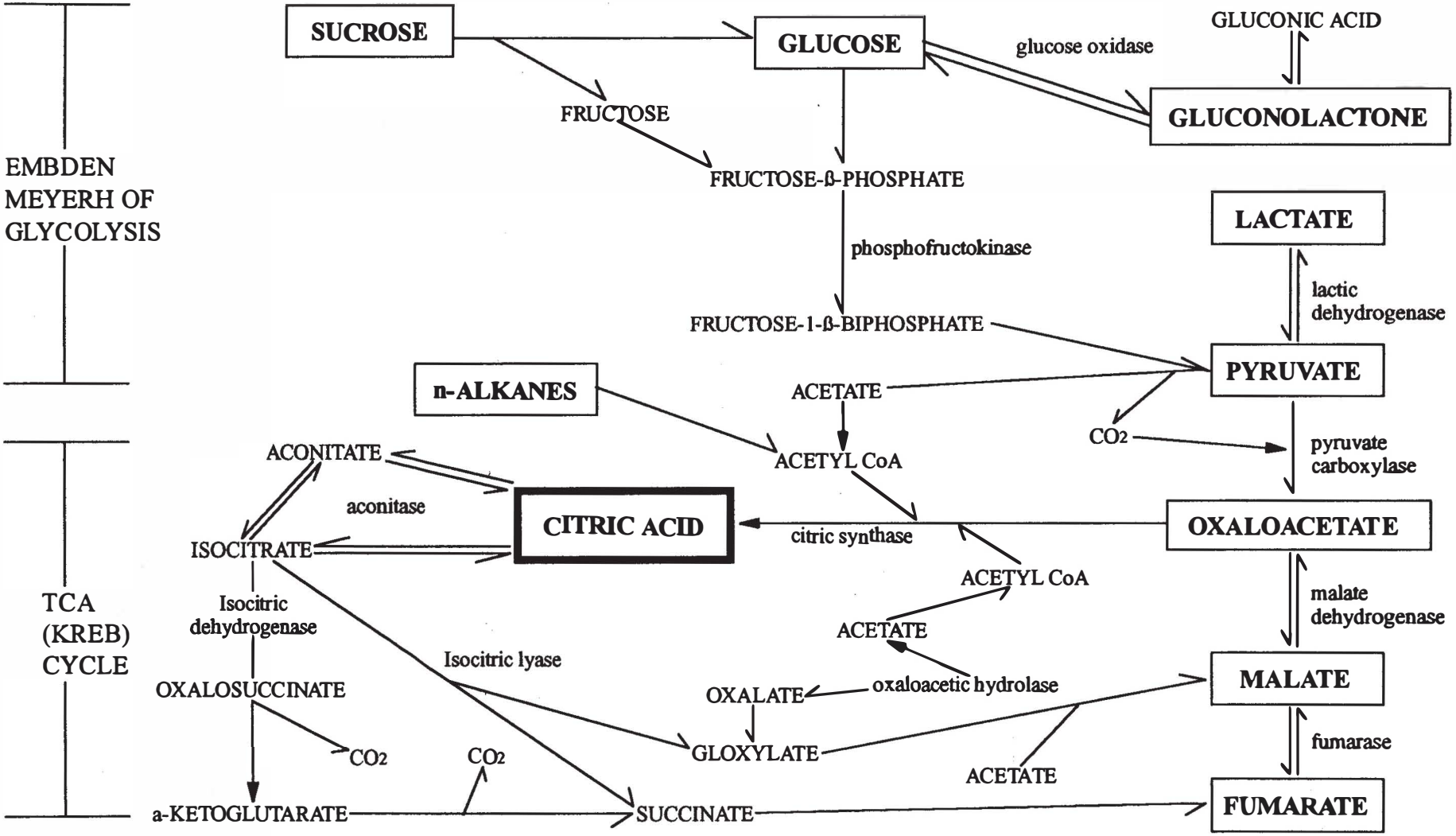

Figure 2: Biosynthetic Pathway of Citric Acid Accumulation Using Carbohydrate of n-Alkanes

suggested the possibility of glucose being oxidized to gluconic acid and the gluconic acid is then oxidized to citric acid (Abou Zeid *et al.*, 1984).

Ramakrishnan and Martin (1955) reported that *Aspergillus niger* possesses the condencing enzyme citrate synthase which catalize the reaction of condencing oxaloacetate with Acetyl-CoA to produce citric acid. The enzyme is very specific for acetyl-CoA and oxaloacetate with fluoroacetyl-CoA and fluoroacetate as the only known alternative substrates. During the citric acid accumulation, specific activity of the condencing enzyme increased whereas isocitrate dehydrogenase and aconitase activities decreased. The acccumulated citric acid was shown to inhibit isocitrate dehydrogenase (Abou Zeid *et al.*, 1984). Akiyama *et al.* (1973) stated that monofluoroacetate is enzymatically converted to monofluorocitrate which causes the inhibition of aconitase activity. Thus, the conversion of citric acid to isocitric acid is inhibited resulting in the accumulation of citric acid.

Aconitase requires iron as a cofactor. When ferrocyanide or other chelating agents of the ions were present, the action of aconitase will be inhibited and the TCA cycle is interrupted at the citric acid. Therefore, in the fermentation media, either fluoroacetate or ferrocyanide could be added to prevent the conversion of citric acid to isocitric acid and thus accumulated the citric acid produced during the TCA cycle (Riviere, 1977).