INTRODUCTION

CHAPTER 1

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1.1 Introduction

Citric acid was first isolated from lemon juice by Sceele in 1784. By 1922, more then 90 percent of citric acid came from Italian lemon industry, and commanding high prices. A German botanist, Carl Wehmer in 1893 (Milsom and Meers, 1985) discovered citric acid production through fungal fermentation. He demonstrated that several species of *Penicillium* could produce citric acid. Large-scale production using this mould yielded low level citric acid eventhough incubation periods were up to 60 days (Rose, 1961). In 1916, Thom and Currie (Rose, 1961; Milsom and Meers, 1985) discovered that a mould *Aspergillus niger* was able to accumulate better yields of citric acid under certain condition of mineral nutrient deficiency.

The exact conditions required to induce citric acid accumulation by mould have been closely studied for many years and this has been reviewed by Rohr *et al.* (1983). The discovery of Krebs cycle (also known as citric acid or tricarboxylic acid cycle) as one of the major pathway in the aerobic dissimilation of sugars by living cells, has a biochemical explanation for citric acid production using microorganism. Citric acid is a primary metabolite and formed by the condensation of acetyl coenzyme A and oxaloacetic acid under the influence of citrate synthase (Milsom

and Meers, 1985). Species of *Candida* such as *Candida lipolytica* or its sexual form *Yarrowia lipolytica* (Figure 1) used for citric acid on an experimental scale and has not achieved industrial production until 1965 (Milsom and Meers, 1985). In 1967, a Japanese Firm, Takeda, reported that this yeast was able to produce significant quantities of citric acid in media containing hydrocarbons and carbohydrate (Bigelis, 1989). To date there is no commercial production of citric acid with yeast.

Production of citric acid by fermentation using the yeast has several advantages. The rate was faster compared to *A. niger*, with very high initial sugar utilisation. Its acid resistance with easy handling on large scale production and fulfilled the important characteristics of microorganism employed for fermentation such as high cell viability in repeated recycling, temperature tolerance and genetic stability. However, a distinct disadvantage is that significant amount of undesirable isocitric acid is also produced (Rohr *et al.*, 1983). In order to attain economical production, the strain used has to be modified to improve the proportion of citric acid.

Several techniques have been employed to achieve this target. Traditional techniques of using mutagenesis had been successful in several instances. For example, Akiyama *et al.* (1972) reported that fluoroacetate-sensitive mutant induced from *C. lipolytica* showed very high citric acid and low isocitric acid production from n-paraffin. They employed two successive N-methyl-N'-nitro-N-nitrosoguanidine treatment (NTG) to obtain the strains (K-20 and S-22) in which the aconitate hydratase activity was reduced as compared to its parent strain (ATCC 20111). The new strain (S-22) excreted a mixture of 97% citric acid and 3% isocitric acid, thus showing a mark improvement over the initial performances of the

Figure 1: Growth of *Yarrowia lipolytica* on YEPD medium.

wild type (citric, 60%: isocitric, 40%). The increase of petroleum prices and sceptical consumer acceptance of food products from petroleum, has made n-paraffin a very expensive and unsuitable substrate, which lead researchers to explore other potential substrates. These include: beverage waste, pulp waste, vegetable oil, whey permeate (McKay *et al.*, 1990) and palm oil (Ikeno *et al.*, 1975).

Palm oil from *Elaeis guineensis* (Figure 2) is the second largest vegetable oil in world production where Malaysia is the largest producer and exporter. By 1990, palm oil has shot up to become the second most important edible vegetable oil after soybean oil, taking up 18% of the world's vegetable oil production. Within a year, from 1991 to 1992, crude palm oil production was 6.4 million tonnes in 1992 increased by 3.8%. And this has contributed to Malaysia regaining its position as the world main producer in 1992, followed by Indonesia (24.5%) and Nigeria (5.2%) (Bank Negara Malaysia Annual Report, 1992). The increase was justified by the increased in most of the planted area coming into maturity (Table 1).

Palm plantations in Malaysia are largely based on the estate management system and organised smallholders scheme. The total planted areas of palm oil in Malaysia are projected to reach 2.63 million hectares in year 2000 (Ibrahim and Ahmad, 1993). Table 2 indicates the forecasted world palm oil needs and Malaysia's contribution to this need. Therefore, efforts are being made to diversify and increase its usage through research and development. One such attempt is the production of citric acid using palm oil as carbon source (Ajam *et al.*, 1991). Palm oil is a cheap renewable resource. Its availability in large volume and of standardised consistent quality is an attractive substrate for fermentation process.

Figure 2: Palm oil tree (*Elaeis guineensis*)

Table 1: Oil palm: Area, production and yield in Malaysia.

	1991	1992p
Area ('000 hectares)		
Planted	2,093	2,142
Mature	1,826	1,897
Production ('000 tonnes)		
Palm oil	6,141	6,373
Palm kernel oil	782	812
Yield (kg /mature hectare)		
Palm oil	3,363	3,360
Palm kernel oil	428	428

p: preliminary
(Source: Bank Negara Malaysia Annual Report, 1992).

Table 2. World's requirement and Malaysian production.

Year	Total requirement ('000,000 tonnes)	World palm oil production ('000,000 tonnes)	Malaysian palm oil production ('000,000 tonnes)
1988	74.27	8.885	4.920
1989	76.62	10.374	5.219
1990	79.08	10.649	5.494
1995	86.92	13.878	6.825
2000	95.48	17.498	8.103

(Source: PORLA, 1991).

Preliminary studies to assess the ability of the yeast to grown on palm oil as a carbon source was conducted by Ikeno *et al.* (1975). They showed that a mutant of *Candida lipolytica* could produce 102 g/l of citric acid utilising 7% of palm oil. This indicated the feasibility of high level of citric acid production using palm oil as a carbon source. However, this result was not repeatable. Ajam *et al.* (1991) was only able to show a yield 8 g/l of citric acid utilising 2% of palm olein as the carbon source. Higher concentration tend to inhibit growth and production.

It is predicted that the commercial utilisation of citric acid will increase from year to year because of its multipurpose nature. Citric acid is one of the most widely used acidulants in the food and pharmaceutical industries. Its lemon flavour has resulted in large quantities of the acid being used in the manufacture of carbonated beverages, jelly-powder, jams and the confectionery industries (Rohr *et al.*, 1983; Milsom and Meers, 1985). In recent years, increasing amount of citric acid in the form of citrate ester have been employed as plasticisers and also as chelating and sequestering agents. For example in pharmaceutical industry citric acid was used as stabiliser in ascorbic acid. Citric acid is used as preservative for stored blood, tablets, ointments and in cosmetic preparation (Rose, 1961; Milsom and Meers, 1985; Bigelis and Arora, 1992). In the areas where there are restrictions on phosphates in the detergents, trisodium citrate is replacing phosphates in speciality cleaners and heavy-duty liquids. Smaller amounts are used in dying, silvering mirrors and preparing inks (Bigelis and Arora, 1992). 100,000 to 200,000 tonnes per year of citric acid was produced in the form of trisodium citrate which is equivalent to roughly 50% of the total estimated world production (Rohr *et al.*,

1983; Bigelis, 1989). Malaysia imported of citric acid amounted to RM 3,603,196 in 1977 and increased to RM 7,955,570 in 1984 (Joyce, 1990).

1.2 *Yarrowia lipolytica*

Y. lipolytica was formerly known as *Mycotorula lipolytica* (Kreger-van Rij, 1984). It was originally isolated from spoiled margarine in 1921. In 1942, Diddens and Lodder (cited by Kreger-van Rij, 1984), restudied the original strain and changed to the genus *Candida* and it was known as *Candida lipolytica*. In 1970, Wickerham *et al.* (1970) discovered the sexual state and they rename it as *Endomycopsis lipolytica*. Yarrow (1972) renamed the strain as *Saccharomycopsis lipolytica*. The unique structure of the ascospores, its coenzymes Q-9 systems and its relatively high nuclear guanine + cytosine (G + C) content of 49.5-50.2 placed the ascigenous teleomorph of *S. lipolytica* in a new genus, *Yarrowia*, type species *Yarrowia lipolytica* (Kreger-van Rij, 1984).

1.2.1 Morphology and life cycle

Y. lipolytica is a dimorphic yeast (Ogrydziak *et al.*, 1978). Vegetative cells rmultiply by budding and under appropriate conditions, pseudomycelium with sparse formation of blastoconidia on septate mycelium is formed (Wickerham *et al.*, 1970). The cells are short-oval to elongated (2-4.5) X (4-22) μm. The colony of *Y. lipolytica* will then appear rough and wrinkles. Smooth and soft colonies showed mostly yeast-phase cells. Sexual reproduction is accomplished when complementary mating types designated as A and B mated. The heterozygous diploid undergoes meiosis and formed four ascospores (Figure 3).

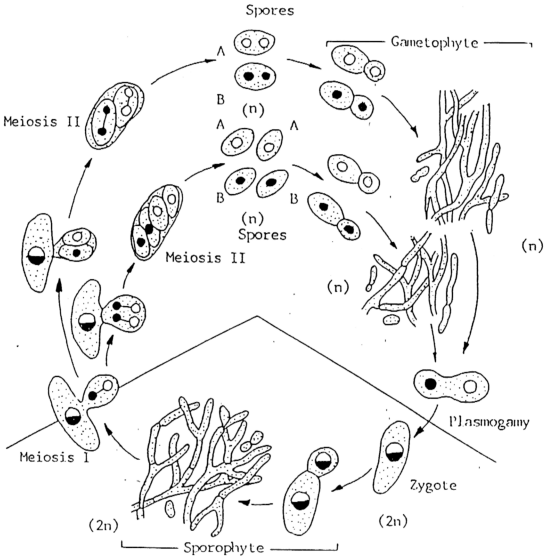

Figure 3: Life cycle of *Yarrowia* (*Saccharomycopsis*) *lipolytica*. The white and black nuclei represent the genetic difference by mating type alleles A/B. (Adapted from: Esser and Stahl, 1976).

1.2.2 Biochemical properties

An important biochemical property of *Y. lipolytica* is its ability to produce lipases for lipid metabolism. Lipases catalyse the hydrolysis of esters of long chain aliphatic fatty acids, saturated or unsaturated with twelve or more carbon atoms, mostly triglycerides (Figure 4 and 5) (Brokerhoff and Jensen, 1974). *Y. lipolytica* produces two types of lipases one extracellular and two cell-bound lipases, lipase I and II (Ota *et al.*, 1973, 1978 and 1982). It has been demonstrated that the enzymes are mainly present in the cell wall on the outer surface of the cytoplasmic membrane (Zvyagintseva *et al.*, 1980). However, Ratledge and Tan (1990) reported that there are three forms of extracellular lipase activities that have been identified: a constitutive cell-associated activity, an inducible cell-associated activity and an inducible cell-free activity. The constitutive lipase activity is weak and ineffective in the hydrolysis of naturally occurring triacylglycerols (Kelle *et al.*, 1972; Suguira *et al.*, 1976). This type of lipase serves as a physiological function that is unrelated to hydrolysis of extracellular function. The inducible extracellular lipases of *Y. lipolytica* have a clear extracellular function. An inducer is required for the enzymes to be formed. Inducers include natural or synthetic triacylglycerol oils for example by growing the yeast in the presence of olive oil, 3-hyroxymyristic acid or unsaturated long-chain fatty acids (Suguira *et al.*, 1975). The induced extracellular lipase activity initially is wholly cell-associated. As growth of induced cells proceeds, cell-free lipase activity appears to coincident with a decrease in cell-associated lipase activity, suggesting translocation of cell-associated lipase to the cell free-form (Ratledge and Tan, 1990; Ruschen and Winkler, 1982).

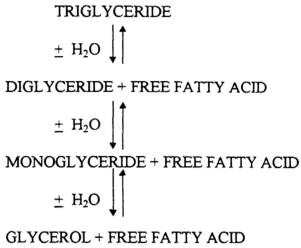

Figure 4: The lipase reaction

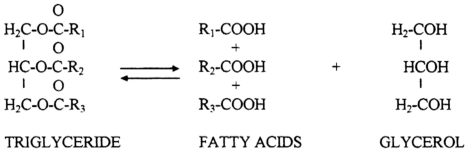

Figure 5: The net lipase reaction.

The cell-associated lipase activity is present in the cell wall fraction of fragmented cells. By direct visualisation of intact cells, it has been shown that the activity is in fact localised at the outer surface of the cytoplasmic membrane.

Ruschen and Winkler (1982) reported that the addition of potassium hyaluronate (a linear polymer of repeating disaccharide unit) to an induced cell suspension of *Y. lipolytica* caused an immediate increases in extracellular lipases activity although the chemical compound supplied cannot be utilised by the cells. This finding was called the 'detachment hypothesis' which refer to the exolipases-stimulatory effects of several polysaccharides which promote the detachment (solubilisation) of preexisting lipase molecules from the surface of the yeast cells.

Another important biochemical property of *Y. lipolytica* is its ability to utilise n-alkane for growth. It was observed a large number of specific organelle, peroxisomes or microbodies play an essential role in the degradation the of fatty acids (Kockova-Kratochvilova, 1990). Such organelles can only rarely be observed in glucose-grown cells. The development of peroxisome in yeast is closely correlated with the increase in catalase activity and assimilation of alkanes or higher fatty acid metabolism (Kockova-Kratochvilova, 1990).

Y. lipolytica also produces higher levels of extracellular proteases than most other yeast species (Meyers and Ahern, 1977). Different strain *Y. lipolytica* will produce various combinations of extracellular protease (Yamada and Ogrydziak, 1983). This includes the extracellular alkaline, acid and neutral proteases.

1.2.3 Genetics

The progress toward developing procedures and establishing the basic genetics of Y. *lipolytica* were initiated after Wickerham *et al.* (1969) discovered sexuality in a strain of the dimorphic hydrocarbon utilising yeast isolated from a corn processing plant. As mentioned earlier the yeast exists in two mating types designated as A and B (Bassel and Mortimer, 1973). However, the application on genetics analysis in this yeast has faced a few problems. Strains isolated from various natural sources usually had low spore viability and the percentage of four-spored asci were very low. They also exhibited low mating frequencies (Herman,1971; Bassel and Mortimer, 1973). However, an extensive program of inbreeding has led to greatly increased spore viability from 15% to 85% as well as an increase of four-spored asci (Ogrydziak *et al.* 1973). These improvements have made tetrad analysis and the construction of the genetic map of *Y. lipolytica* possible by Gaillardin *et al.* (1978). Ogrydziak *et al.* (1978) detected 22 cases of linkage among 278 gene pairs and developed a genetic map consisting of six linkage fragments. However, the number of chromosomes in *Y. lipolytica* is still not certain.

As in *Saccharomyces cerevisiae*, gene transformation was also attempted in this yeast. Gaillardin *et al.* (1985) and Davidow *et al.* (1985) have successfully developed DNA-mediated transformation systems for *Y. lipolytica* where it acted as host for secretion of foreign protein. Gaillardin *et al.* (1985) adapted the standard procedure for transformation method that was based on the lithium acetate method developed by Ito *et al.* (1983) on *S. cerevisiae*. The electroporation method where DNA vectors were forced into yeast cells for the same purpose was introduced to this yeast by Nuttley *et al.* (1993).

Three genes were explored employing the DNA recombinant techniques. The *LYS5* gene which encode saccharopine dehydrogenase (Xuan *et al.*, 1988), the *XPR2* gene which resulted in reduced ability to produce extracellular protease (Davidow *et al.*, 1987b; Ogrydziak and Mortimer, 1977) and the isopropylmalate dehydrogenase (*LEU2*) gene (Gaillardin and Ribet, 1987). These were followed by the cloning of a lipase gene (Ng, 1989) and isocitrate lyase gene (*ICL1*) (Barth and Scheuber, 1993).

1.3 Genetic application in obtaining high producers of citric acid

The possibility of introducing foreign gene into a useful microorganism could lead to several applied benefits. These include extending the range of utilisation of carbon sources, over production of certain metabolites and extracellular protein (Gaillardin and Heslot, 1988). However, application of this procedure to increase citric acid production was not studied. Bigelis (1989) reported that there was no strain of microorganisms able to produce enhanced quantity of citric acid that has been obtained *via* recombinant DNA techniques. This is despite the availability of transformation technology in several filamentous fungi such as *A. niger*. A reason for this lack of progress is primarily the incompleteness of information on the genes, enzymes, limiting steps, regulatory factors and specific mechanism related to citric acid over production (Kubicek and Rohr, 1986).

The main technique employed for genetic improvement of citric acid producing fungi has been the age-old technique of mutation and selection. Banik (1975) has reported on a mutant of *A. niger* by sequential mutagenesis using UV,

which has the ability to produce 11.6 mg/ml of citric acid. Rugsaseel *et al.* (1993) has derived 8 mutants from *A. niger* WU-2223L, among them, a mutant 2M-43, produced 48.0 g/l of citric acid from 120 g/l of soluble starch. Its parent strain, WU-2223L produced only 35.1 g/l of citric acid. Besides induction by using various mutagens, strain improvement in *A. niger* by somatic recombination has also been attempted since this fungus lacks a sexual cycle. Sarangbin *et al.* (1994) hybridised different strains of *A. niger* by protoplast fusion and obtained new strain with enhanced citric acid production. A representative autodiploid strain L-d1, yielded more citric acid than the parental strain WU-2223. L-dl produced 67.2 g/l of citric acid from 120 g/l of glucose, an increased of 1.4 times production over its parent.

Mutants of *Y. lipolytica* derived by this method have shown increased yield of citric acid production concomitant with significant amount of isocitric acid. The development of mutants that accumulate both citric acid and isocitric acid has been attempted by Akiyama *et al.* (1972) and Ikeno *et al.* (1975). They have studied various *Candida* mutants derived by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis in various substrates. Similarly, Finogenova *et al.* (1986) and Wojtatowicz *et al.* (1993) have isolated a set of *Y. lipolytica* mutants by using the same techniques, which produced higher amount citric acid than the parent strain. McKay *et al.* (1990) have also reported using UV radiation to obtain mutant strains for the same purpose.

1.3.1 Mutation

A mutation is an alteration in the nucleotide sequence of a DNA molecule (Freifelder, 1987). When it occur within a gene it may alter the gene product and

generate an observable change in the phenotype of the organism. Two chemical mutagens that are used extensively for isolating mutants such as high producer strains of citric acid are alkylating agents; EMS (Ethylmethane sulphonate) and NTG (N-methyl-N'-nitro-N-nitrosoguanidine) (Figure 6).

a. CH_3-CH_2-O-SO_2-CH_3

b. HN=C-N-NO_2
   |
   O=N-N-CH_3

Figure 6: Structure of alkylating agents.
a. Ethylmethane sulphonate
b. N-methyl-N'-nitro-N-nitrosoguanidine
(Source: Crueger, 1993).

EMS-induced mutations are distributed more or less randomly throughout the genome (Guerola and Cerda-Olmedo, 1975). In contrast, NTG acts preferentially at the replicating fork inducing clusters of mutations in closely linked genes, a phenomenon referred to as (localised) comutation. An explanation for such mutagenic events is that NTG can interact with the replication machinery and induce error-prone replication over limited regions of the genome (Cerda-Olmedo and Ruiz-Vazquez, 1979). In *Escherichia coli* 40% comutation are concentrated in a region of about 50,000 base pairs (about $^1/_{60}$ of the genome). In *Streptomyces coelicolor* the mutation region is about twice as large. It has been observed that the major EMS and NTG targets are almost exclusively at G.C sites (Kohalmi and Kunz, 1988). In *in vivo* studies, this should lead to G.C to A.T transitions.

NTG also has a low killing rate. In *E. coli* 10% auxotrophs are obtained at a survival rate of 1% whereas in *Schizosaccharomyces pombe* 8% auxotrophs at a survival level of the 20% (Sinha and Chatto, 1975).

1.3.2 Cloning in yeast

The ability to transform yeast cells with DNA and development of versatile series of cloning vectors have extended recombinant DNA technology to include yeast as a recipient host for gene transfer studies. These developments have revolutionised molecular biology and placed yeast as the model eukaryote for biological investigations (Struhl, 1983). In theory, it is now possible to isolate any yeast gene by phenotypic complementation of mutant strain which generally involved: the regeneration of specific or random DNA fragments of suitable size, ligation of these fragments into appropriate vectors, propagation and selection of recombinant DNA and selection of appropriate transformants (Dahl *et al.*, 1982; Beggs, 1982; Maniatis *et al.*, 1982).

1.3.2.1 Genetic markers for yeast transformation

A number of yeast genes were initially isolated by virtue of their ability to complement *E. coli* mutants defective in pathways required for amino acid and nucleotide biosynthesis. The first gene cloned in this manner was *HIS3* by complementation *his*B mutation of *E. coli* (Struhl *et al.*, 1976). The yeast *LEU2* and *HIS1* of *Y. lipolytica* were also isolated by similar strategy (Davidow *et al.*, 1985; Gaillardin and Heslot, 1988). These genes constitute useful genetic markers for yeast transformation: that is when they are present in a cloning vector their

expression in recipient auxotrophic mutant strain permits the identification of transformed yeast cells.

Some bacterial antibiotic resistance genes are also used as selectable markers for yeast transformation but this could only be used with sensitive yeast strain. Most of the yeast are naturally resistant to most antibiotics commonly used such as chloramphenicol or G 418 (Cohen *et al.*, 1980). G 418 is an aminoglycoside antibiotic, which inhibits the growth of a wide range of prokaryotic and eukaryotic organisms (Davis and Jamenez, 1980; Webster and Dickson, 1983).

1.3.2.2 Selection of enhanced producer strains of citric acid

One of the difficulties in screening mutants for better citric acid productions is the lack of a precise method of identification by which high producing strains can be easily selected. The isolation of lipase gene in *Y. lipolytica* (Ng, 1989) was successfully by its ability to secrete lipase, hydrolyse the substrate and formed halo around the colony which form the basis of selection for lipase-producing transformants.

Working on the same principle, McKay *et al.* (1990) used calcium carbonate plate for selection of higher citric acid producer strains of *Y. lipolytica* after UV irradiation. Choudruri (1972) used 1% bromocresol green to isolate citric acid producing strains of *A. niger* . The high yielding strains would form large diameter of the acid zone. Therefore it was proposed that initially similar approach be attempted to isolate mutants of *Y. lipolytica* which show high level production of citric acid.

1.3.2.3 Yeast cloning vectors

All vectors for cloning of yeast gene rely on *E. coli* for replication and amplification. Most of these vectors consist of *E. coli*-derived plasmids such as pBR322 (containing a replication origin and markers for dominant selection in *E. coli*) as well as one of the yeast selectable markers such as *URA*3, *LEU*2, *TRP*1, *TRP*5 and *ARG*4. This facilitate selection in *E. coli* (for amplification) and in yeasts where recombinant sequences are expressed allowing the shuttling of these plasmids between the two hosts (hence the term shuttle vectors).

There are several types of cloning vector that can be utilised in yeast, as described below:

1. Yeast-integrating plasmids (YIp). Plasmid that can be used to integrate DNA fragments are called yeast-integrating plasmids (YIp). YIp is basically bacterial plasmid carrying a yeast gene and lack an origin of replication. An example of YIp is pBRR322 with an inserted *URA*3 gene which encodes orotidine-5'-phosphate decarboxylase in uracil biosynthetic pathway. This enzyme converts 5-fluororotic acid to 5-fluorouracil, which is toxic. 5-fluorouracil therefore kills *URA3* cells but *ura*3 cells are not. Loss of gene function can be selected by growth on 5-fluorouracil. Another example of YIp is pBR322 with an inserted *LEU*2 gene of *Y. lipolytica* such a pINA62 (Figure 7). (Gaillardin and Ribet, 1987). Uncut vector yields typically between 1 to 100 transformants per μg of input DNA. Linearisation of vector with restriction enzyme results in 1,000 fold increased of the transformation efficiency (Gaillardin and Heslot, 1988). Orr-Weaver *et al.* (1981)

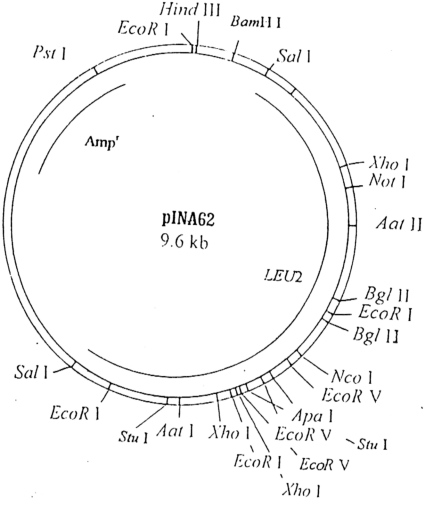

Figure 7: Plasmid pINA62 with *LEU2* marker from *Yarrowia lipolytica*. (Adapted from: Gaillardin and Ribet, 1987).

reported that linearisation or formation of gaps in yeast plasmids within the yeast DNA sequences increased the frequency of integrative transformation. This observation gave impetus to the development of model of genetic recombination and gene conversion in yeast based on formation of double-stranded breaks in chromosomal DNA (Orr-Weaver and Szostak, 1983).

2. Yeast episomal plasmids (YEp). A yeast-integrating plasmid cannot replicate on its own in the yeast cell. Beggs (1978) resolved this problem by inserting the replication origin of endogenous yeast plasmid 2 μm into the transformation vector. The resulting circular vector no longer integrated into the chromosome but replicates on its own (yeast episomal plasmid, YEp).

3. Yeast-replicating plasmid (YRp). The 2 μm origin can be replaced by autonomously replicating sequence (ARS) forming to a yeast-replicating plasmid (YRp) (Iserentant, 1990). Yeast replicating plasmids (YRp) replicate efficiently and can generate as many as 30 copies per cell on average (Orr-Weaver *et al.*, 1981). ARS sequences presumably act as origins of replication in yeast chromosomes or is heterologous DNA sequences with similar activity. YRp vectors transform yeast with high frequency but comparatively unstable as compared to that of YEp vectors.

4. Yeast-centromic plasmid (YCp) and yeast linear plasmids (YIp). The stability of YRp plasmids can be improved by the addition of yeast centromere plasmid, YCp (Clarke and Carbon, 1980). The transformation efficiency of this kind of plasmid remains high but the copy number drops to approximately one per cell. YRp plasmids can be linearised by addition of the telomeres originating, for example from yeast (Oertal, 1984) or from other organism such as *Tetrahymena* (Szostak and

Blackburn, 1982). The resulting plasmid (Yeast linear, Ylp) is generally more stable than the corresponding YRp.

When the Ylp contains a centromere sequence in addition to the ARS and the telomeres the plasmid behaved like an artificial chromosome. The stability of such a construction is relatively high and seems to increase when the length of the plasmid is increased.

5. **Yeast-artificial chromosome**. The final type of yeast cloning vector to be considered is YAC, which stands for yeast artificial chromosome. An important use of these vectors is to obtain intact clones of very long genes. Brown (1990) described the utilisation of YAC vectors in order to produce gene libraries. For example a human gene library in a cosmid vector will comprise over a quarter million clones. With YAC vector this number can be reduced immensely to just 60,000 clones if 150 kb fragments are used to make the library.

1.3.2.4 Transformation of yeast cells

Two procedures are commonly used for the transformation of yeast cells. The first is applicable to all available yeast vectors. It involved the formation of yeast spheroplasts, which are incubated with transforming DNA in the presence of polyethylene glycol (PEG) and calcium chloride. Treated spheroplasts are then plated under the conditions that allow selection of transformants as well as regeneration of the cell wall (Figure 8). However, transformation using this method suffers from three disadvantages; firstly, PEG causes cell fusion and diploid and polyploids may result, secondly, the transformant colonies are

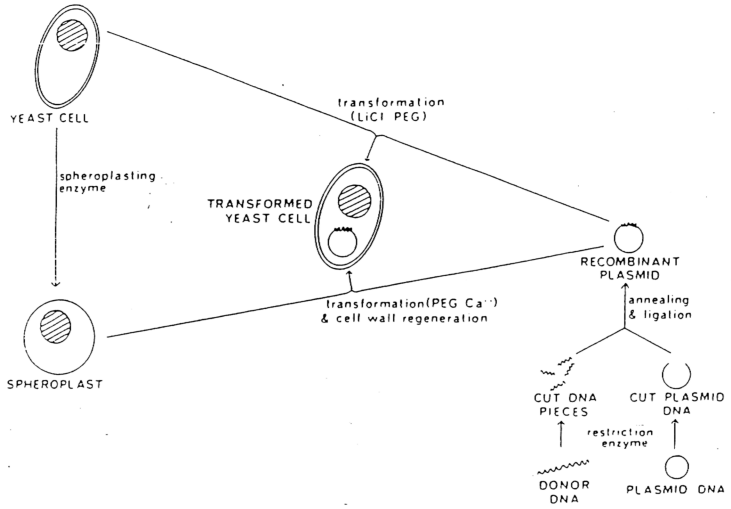

Figure 8 : Plasmid transformation of yeast using either spheroplasts or lithium-treated cells.

(Source: Tubb and Hammond, 1987).

embedded in the agar overlay which makes subsequent screening difficult and thirdly, it is both laborious and time-consuming.

Ito et al. (1983) has developed an alternative transformation procedure, which has been successfully conducted on *Y. lipolytica*. The cells of *Y. lipolytica* were treated with lithium acetate followed by incubation in PEG where plasmid DNA was added. After a brief heat shock the cells are plated on selective medium. Electroporation method has also been successfully employed for transform yeast cells (Nuttley *et al.*, 1993). Both these procedures are more convenient than the protoplast method. The transformants colonies grow on the surface of agar plate, which make selection more amenable.

1.4 Palm oil - its composition

Palm oil is extracted from the mesocarp of the oil palm fruit. The species of palm oil tree which are grown in Malaysia is *Elaeis guineensis* mainly the hybrid Tenera (Figure 9). A large portion of the palm oil is composed of triglycerides (Table 3). Triglycerides or so-called neutral fats are esters of the alcohol glycerol and fatty acids. The fatty acid residue at the three ester positions can either be all the same or mixed, for example tripalmitin (three molecules of palmitic acid attached to the glycerol molecule) or oleo-dipalmitin (one molecule of oleic acid and two molecules of palmitic acid attached to glycerol) or oleo-palmitostearin (one molecule of each of oleic acid, palmitic acid and stearic acid attached to glycerol). The two major components of triglycerides in palm oil are the unsaturated-dipalmitic (C50) and palmitodiunsaturated (C52).

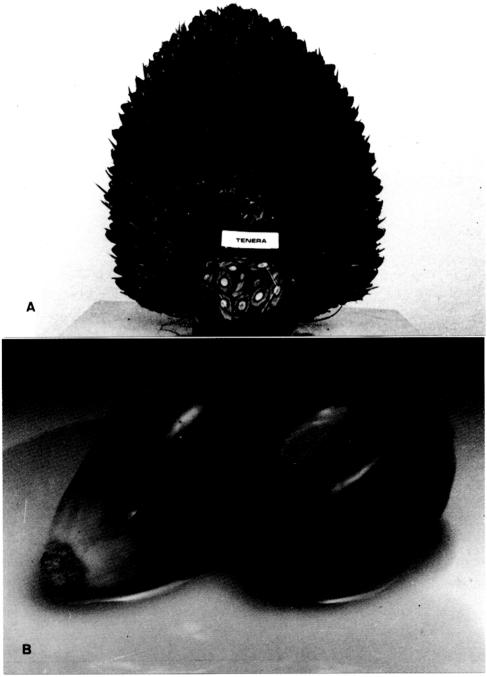

Figure 9: A. *Elaeis guineensis* hybrid *Tenera*. Hybrid Tenera is a hybrid between *Dura* and *Pisifera*. The *Dura* is homozygous and dominant for shell while *Pisifera* form is homozygous and recessive for shell. The hybrid between these two fruit forms gives an intermediate shell type known as *Tenera* or D x P (Rajanaidu, 1993).
B. Ripe palm oil fruits.

Table 3: Composition of palm oil.

Major Constituents	**> 99%**
Triglycerides	
Partial Glycerides	5-8
Free Fatty Acids	2-5
Minor Constituents	**< 1%**
Carotenoids	0.05-0.07
Tocopherol and Tocotrienols	0.06-0.10
Sterols	< 0.10
Triterpenes	
Phospholipids	
Aliphatic Hydrocarbons	

(Source: Goh, 1991).

Breakdown products of triglycerides such as diglycerides, monoglycerides and free fatty acids are also present in the palm oil due to the action of lipases from endogenous or microbial sources. Carotenoids, tocopherols, sterols and phosphatides are grouped under the unsaponifiable (non-glyceride) matter of the palm oil. Carotenoids, namely the α-carotene and β-carotene, are natural pigments that give Crude Palm Oil (CPO) its deep orange-red colour. Carotenes are precursors of vitamin A. Tocopherols and tocotrienols have anti-oxidising property which contributes to the stability of the oil and they are also a good source of vitamin E (Goh, 1991). The fatty acids in palm oil present consist of almost equal amounts of unsaturated fatty acids (39% of mono-unsaturated oleic acid) and saturated ones (45% of saturated palmitic acid) (Table 4). Saturated fatty acids are those with only single bonds between the carbon atoms. Unsaturated fatty acids have double bonds, besides single bonds between the carbon atoms and they are differentiated by the number of double bonds present as well as their positions on the carbon chain. The unique composition of fatty acids in the palm oil contributes

to its unique physical properties, which are taken advantage of, in many food applications.

Table 4: Fatty acid composition of palm oil.

Fatty acid	Symbol	Mean (wt. %)	Range (wt. %)
Saturated Acids			
Lauric	C12:0	0.2	0.1-0.3
Myristic	C14:0	1.2	1.0-1.3
Palmitic	C16:0	44.7	43.9-46.0
Stearic	C18:0	4.2	3.9-4.4
Arachidic	C20:0	0.4	0.3-0.7
Mono-unsaturated Acids			
Palmitoleic	C16:1	0.1	0-0.1
Oleic	C18:1	39.2	38.0-40.6
Poly-unsaturated Acids			
Linoleic	C18:2	10.0	9.2-10.5
Linolenic	C18:3	0.3	0.3-0.6

(Source: Goh, 1991).

1.4.1 NBD palm olein

Refining crude palm oil usually involves several steps such as fractionation; crystallisation, neutralisation, bleaching and deodorisation before refined oil can be obtained (Figure 10). Fractionation will produce various fractions of palm oil including NBD palm olein, which has been used in this study. The process of fractionation takes advantage of the fact that palm oil contains triglyceride with fatty acids of different chain lengths unsaturation and melting point. NBD Palm olein is a pale yellow liquid at ambient temperature 28°C (Figure 11). It also characterised by a low cloud point and a higher iodine value than the unfractionated oil (Tan and Oh, 1981).

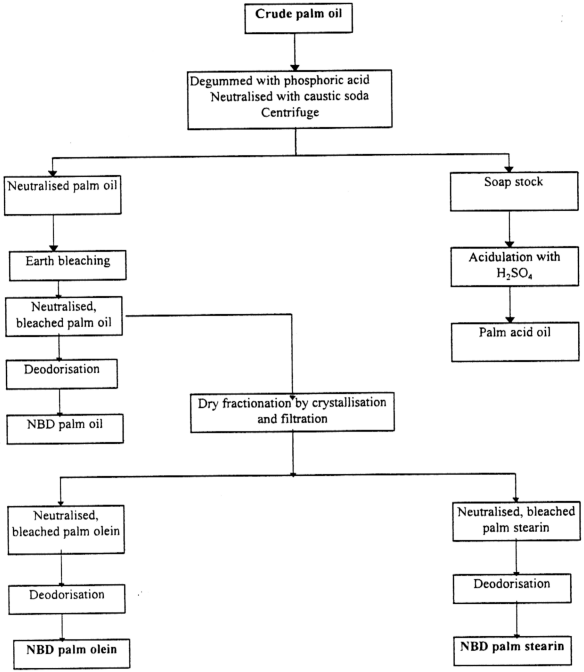

Figure 10 : Production of various refined fractions from crude palm oil. (Source: United Plantations Berhad, Teluk Intan, Perak. (UNITATA), 1991.)

The compositions of fatty acids in NBD palm olein are shown in Table 5. Oleic acid (C 18) is the major unsaturated acid component (47.2%) followed by, 10.5% of linoleic acid (C 18:2) and 0.1% of linolenic acids. Whereas, saturated fatty acids accounts for about 41.2% of the NBD palm olein.

Table 5: Fatty acid components of NBD palm olein.

	No. of C atoms to no. of double bonds	NBD palm olein
Iodine value		57.1
Fatty acids		
Lauric	C12:0	0.1
Myristic	C14:0	0.6
Palmitic	C16:0	39.1
Stearic	C18:0	2.3
Oleic	C18:1	47.2
Linoleic	C18:2	10.5
Linolenic	C18:3	0.1

(Source: United Plantations Berhad, Teluk Intan, Perak. (UNITATA), 1991).

1.4.2 NBD palm olein as the carbon source

Studies on the citric acid production by *Y. lipolytica* utilising palm oil and fatty acids as the carbon source has been conducted by Ikeno *et al.* (1975). There were several other findings which indicated that palm oil was usable as a raw material for the production of various products such as a single-cell protein (Koh *et al.*, 1985) and antibiotics (Ho *et al.*, 1984). Palm oil is also a cheap renewable resource. Bader *et al.* (1984) described some of the key technical points favouring the choice of an oil as fermentation feedstock as compared with a carbohydrate.

Figure 11: NBD palm olein at 28°C.

Energetically, a typical oil contains about 2.4 times the energy of glucose on a per weight basis. Oils are also preferable to carbohydrates on a volume basis. For example, it takes 1.24 litres of soyabean oil to add 10 k cal of energy to fermenter, whereas it takes over 5 litres of glucose or sucrose to add the same amount of energy, assuming that the latter are added as 50 % (w/v) solutions. Since Malaysia represents the largest producer of palm oil in the world, every effort is geared towards exploiting palm oil in fermentation processes, one that shows promise is NBD palm olein in citric acid production (Ajam *et al.*, 1991).

NBD palm oil is a non-toxic substrate. Its physical properties play an important role in enhancing yeast cell growth, small droplets of oil were more easily utilised than large ones, thus, surfactants must be added to disperse the oils or fats in the broth. Koh *et al.* (1983) reported the addition of surfactants in the medium containing fats, not only stimulated yeast growth but also increased the aeration of fermentation medium. Good growth will also increase the production of the citric acid.

1.5 Citric acid fermentation

1.5.1 Effects of fermentation medium on citric acid production

It is generally agreed that in order to achieve an abundant excretion of citric acid, the fermentation medium should be deficient in one or more essential elements (Rohr *et al.*, 1983). Typical media for citric acid production by the yeast *Y. lipolytica* growing on natural oils is shown in Table 6.

The nitrogen requirement for citric acid production is generally supplied by the addition of organic nitrogen sources in the form of the yeast extract or ammonium salt (sulfate, phosphate, nitrate).

Table 6: Composition of citric acid producing media by *Yarrowia lipolytica* utilising coconut and olive oil as the carbon source.

	Olive oil (g/l)[a]	Coconut oil (g/l)[b]
Na_2HPO_4	4.3	-
$(NH_4)_2SO_4$	1.0-1.5	2.0
$(NH_4)Fe(SO_4)_2$	0.005	-
Yeast extract	0.1	-
Corn steep liquor	-	2.6
KH_2PO_4	2.6	0.125
$MgSO_4.7H_2O$	0.02	0.1
$CaCO_3$	-	5.0
Antifoam	-	5.0
Thiamine hydrochloride	1 mg/l	0.1
Olive oil	2.7	-
Coconut oil	-	50.0

([a] Source: Tan and Gill, 1984 and 1985).
([b] Source: Ikeno *et al.*,1975).

According Koh *et al.* (1983) ammonium sulfate was most effective as nitrogen source compared to nitrogen of nitrate group which were more difficult to assimilate. An addition of NH_4^+ has no influence on the growth of citric acid producer, *A. niger* (Rohr *et al.*, 1983). Ammonium compounds are also generally preferred because of its consumption during the growth will decrease the pH which is a prequisite of citric acid accumulation (Koh *et al.*, 1983). According Aiba (1986) *Y. lipolytica* will begin to accumulate the acid when nitrogen becomes almost exhausted.

Besides nitrogen, phosphorus is also important as a macronutrient for synthesising nucleotides, phospholipids, phosphoprotein and other phosphorylated compounds (Kockova-Kratochvilova, 1990). Phosphorus is added to nutrient media

in the form of potassium, ammonium or sodium phosphates. The most common salt is KH_2PO_4 since it buffers the media as a suitable pH.

Joyce (1990) reported that high level of phosphate stimulates more growth but less acid production in *Y. lipolytica*. Martin and Steel (1955) (cited by Rohr *et al.*, 1983) have also obtained the same observation, however they found the accumulation of the citric acid is also concomitant with the formation of certain sugar acids in *A. niger*. Therefore, it is generally agreed that citric acid overflow occurs as a results of phosphate deficiency (Best, 1985; Rohr *et al.*, 1983). Rohr *et al.* (1983) reported in *A. niger* low concentration of phosphate could only be used when the trace metals content could not be effectively controlled. Under rigorous metal limitation phosphate does not need to be limiting (Best, 1985).

Appropriate levels of trace elements such as zinc, iron, magnesium, copper and manganese in the medium has also been considered in the production of citric acid in *A. niger* (Perlman *et al.*, 1946 cited by Rohr *et al.*, 1983). The optimum concentration of Fe^{2+} is also required for maximal citric acid production in *A. niger* and *Y. lipolytica*. Ferrous is an essential factor for the enzyme activity of aconitate hydratase, which played an important role in determining the ratio of citric acid to isocitric acid (Akiyama *et al.*, 1972). According to Tabuchi and Hara (1974) high concentration of ferrous iron results in high activity of aconitate hydratase and thus reduce citric acid production. The addition 400 mg $FeSO_4.7H_2O$ per liter will reduce the productivity of *Y. lipolytica* by 30% (Marchal *et al.*,1973 cited by Rohr *et al.*, 1983). Schweigner (1961, cited by Joyce 1990) reported the traditional fermentation of molasses using a strain of *A. niger* was severely affected by the presence of iron concentration as low as 0.2 ppm. However, the addition of copper

at 0.1-0.5 ppm at the time inoculation or during the first 50 hours of fermentation was found to counteract the deleterious effect of iron.

Another trace element, which is essential for a variety of enzymatic reactions, is magnesium. This metal ion is a stimulator of many enzyme-catalysed processes and phosphatase (Kockova-Kratochvilova, 1990). The optimum concentration of $MgSO_4$ for maximum citric acid production in *A. niger* varies from 0.02 to 0.0025%. Some reports also indicated that zinc has a critical role to play in determining the efficiency of fermentation (Joyce, 1990). At the concentration of 1-2 μM of zinc allows continuation of *A. niger* growth phase but restricted the growth at concentration less than 1 μM. Excess of Zn^{2+} in the medium will reverse the acid production phase. This suggested that Zn^{2+} have an important role in regulating growth in relation to citric acid production. It deficiency during growth apparently signals the transition from growth phase to the acid production phase (Wold and Suzuki, 1976 cited by Joyce 1990) have also suggested that zinc has an indirect role in the functioning of cyclic AMP. Addition of CAMP during the production phase will enhance citric acid production while the addition of zinc will retard the production of citric acid. Other trace elements such as Mn^{2+}, Ba^{2+} and Al^{2+} have been reported to have an effect on fungal morphology and citric acid production at concentration that generally do not inhibit growth (Kumamoto and Okamura, 1976; Tondon and Snivatava, 1971, both cited by Joyce, 1990).

The addition of thiamine at appropriate concentration is also important in yeast fermentation. Tabuchi and Hara (1974) reported that in thiamine restricted medium, *Y. lipolytica* produced a large amount of α-ketoglutarate compared to citrate. Whereas, in medium with sufficient thiamine a large amount of citric acid

was produced. However, Rohr *et al.*(1983) described that *Y. lipolytica* fermentation medium where n-alkane was the carbon source, lack of thiamine would accumulate oxoacids such as α-oxoglutarate and reduced in citric acid production. The effect was not clearly understood but might be related to the fact that n-alkane degradation involved oxidative decarboxylation which require thiamine-pyrophosphate as co-enzyme.

1.5.2 pH

The maintenance of favourable pH is essential for growth and production of citric acid. The initial pH required depends on the carbon source used. For example, when sucrose, glucose or clarified molasses or other relatively pure materials are used in the fermentation of *A. niger*, pH lower than 3.0 is desirable. A low pH has the main advantage of preventing contamination, suppressing oxalic acid fermentation and making the sterilisation operation more efficient. Whereas, if the initial pH is high, accumulation of oxalic acid (Joyce, 1990) and gluconic acids tend to occur (Rohr *et al.*, 1983).

Unlike *A. niger*, *Y. lipolytica* needs pH above 5 for citric acid production. Tabuchi and Hara (1975) observed that *Y. lipolytica* grown on n-paraffin with pH lower than 5 resulted in the production polyhydroxy compound such as erythritol and arabitol. For citric acid production of *Y. lipolytica* growing on fats and natural oils, pH 5-7 was suggested (Ikeno *et al.*, 1975; Tan and Gill, 1984).

1.5.3 Aeration and agitation

The citric acid fermentation is an aerobic fermentation process. To ensure an appropriate oxygen supply, aeration and agitation are employed to maintain proper mixing conditions and prevent contamination by providing a positive pressure inside the fermenter (Joyce, 1990).

There are three ways in which aeration is accomplished; surface liquid cultivation where oxygen diffuses through the surface which is maintained in static condition, submerged aeration in which sterile air or oxygen is pumped from beneath the surface of the culture (Milsom and Meers, 1989) and shake culture technique where both surface as well as submerged aeration are being employed (Tan and Gill, 1985), however this process is not feasible on an industrial scale.

Citric acid fermentation by surface culture is carried out in shallow pans of high grade aluminium or stainless steel containers. The growth of the culture is on the surface of the liquid medium converting sugar to acid occurs intracellularly before it is excreted. If the surface culture was agitated citric acid production was retarded (Rohr *et al.*, 1983). In the submerged culture a limited supply of oxygen will also reduce citric acid production in which citric acid is remetabolised by the organism (Rohr *et al.*, 1983).

1.5.4 Temperature and duration of incubation

The correct temperature is necessary for the optimum citric acid production. *A. niger* and other fungi used in citric acid fermentation have an optimum temperature between 25°C to 30°C (Rohr *et al.*, 1983; and Tan and Gill, 1984). Studies by Ajam *et al.* (1991) on the growth of *Y. lipolytica* on NBD palm olein

indicated incubation at 32°C seems to be the optimum for cell growth and citric acid production. However, to use *Y. lipolytica* as an industrial microorganism in the production of citric acid utilising oil, heat generation must be considered. The yeast cells which have been grown on oil obviously liberated heat due to higher oxygen demand when compared to cells grown on glucose. Furthermore, as the process is presumably aimed at operating in Malaysia then the yeast that can only grow maximally at 32°C is going to be of very little practical use where the ambient temperature may be 30-34°C. A considerable amount of energy would therefore be expended in cooling a large fermenter both to counter the excess heat production as well as high local temperature (Ratledge, personal communication).

The fermentation of *Y. lipolytica* on NBD palm olein for citric acid production usually completed in 4-6 days while *A. niger* took 7-10 days.

1.6 Citric acid production from lipid as the carbon source

1.6.1 Utilisation of lipid

A lipid source is composed predominantly of tryglycerides. These are compounds that have a glycerol core to which are attached three molecules of fatty acids. Besides, tryglycerides a lipid source may also contain a small amount of free fatty acids, monoglycerides and diglycerides. The breakdown products of tryglycerides are free fatty acids, monoglycerides and diglycerides, which resulted from the action of lipases from endogenous and microbial sources (Brockerhoff and Jensen, 1974).

In mamalian system, the fatty acids formed are dismembered through the β-oxidation of fatty acyl-CoA (Voet and Voet, 1991). The fatty acyl-CoA has to be

transported across into the inner mitochondrial membrane where it undergoes oxidation and resulted in the formation of acetyl-CoA.

In yeast, assimilation of fatty acids or alkane is related with the existence of specific organelle calls peroxisomes or microbodies. The appearance of conspicuous numbers of peroxisomes in yeast cells grew in higher fatty acids or alkanes is accompanied by a remarkable increase in the cellular catalase activity. The changes in activity of this enzyme were rarely observed in the cells grown on ethanol, acetate and glucose (Osumi, *et al.*, 1974). In the study of ultrastructure of *C. lipolytica* mutants and their parent strain grown on glucose or hexadecane by Ermakova et *al.* (1986), it has been shown that the number of peroxisomes in yeast cells grown in glucose was not exceeding from 4 organelles per slice. Whereas, in cells grown on n-alkanes the number of these organelles increased approximately 4 to 5 folds. Several authors have concluded that growing yeasts on higher fatty acids or alkanes induces the development of the peroxisomes (Teranishi *et al.*, 1974; Veenhuis *et al.*, 1987; Kokckova-Kratochvilova, 1990).

The peroxisomes isolated by differential and sucrose density-gradient centrifugations contain various enzymes; acyl-coA synthase, a fatty acid β-oxidation system, catalase, carnitine acetyltransferase, NAD-linked glycerol-3-phosphate dehydrogenase, isocitrale lyase, malate synthase, NAD-linked isocitrate dehyrogenase, uricase and D-amino acid oxidase (Fukui and Tanaka, 1979). By contrast, the alkane hydroxylation system and enzymes of the TCA cycle are not located in the organelles.

These results indicate that the peroxisomes participate in the degradation of the alkane skeleton into C_2-units and in the synthesis of C_4-compounds from the C_2-

units (Fukui and Tanaka, 1979). Conversion of alkanes to the corresponding higher alcohols, the first essential step in alkane metabolism catalysed by cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase, does not take place in the peroxisomes but may occur in the microsomes (Figure 12). In microsomes, higher alcohols derived from alkanes by hydroxylation, are oxidised to the corresponding fatty acids *via* aldehydes (Figure 13A) (Fukui and Tanaka, 1981).

The fatty acids undergo β-oxidation system in yeast peroxisomes as indicated in Figure 13B. Acyl-CoA is oxidised by acy-CoA oxidase, a FAD-containing enzyme to enoyl-CoA with concomitant consumption of molecular oxygen and formation of hydrogen peroxide. Catalase induced by alkanes participates in the degradation of the hydrogen peroxide thus formed. Enoyl-CoA is further metabolised to yield acetyl-CoA in the presence of NAD and CoA.

NADH formed at the stage of β-hydroxyacyl-CoA dehydrogenation seems to be reoxidised to NAD by the glycerol-3-phosphate/dihyroxyacetone phosphate shuttle involving NAD-dependent glycerol-3-phosphate dehydrogenase in peroxisomes and FAD-dependent glycerol-3-phosphate dehydrogenase in mitochondria (Figure 13B) (Fukui and Tanaka, 1981). The reducing power transferred to FAD from NADH may be used to yield energy (Fukui and Tanaka, 1981). Since fatty acid β-oxidation activity could not be detected in mitochondria of yeast when it grown on higher fatty acids or alkanes, acetyl-CoA required for the citrate synthesis must be transported to mitochondria from peroxisomes. Carnitine acetyltransferase in peroxisomes and mitochondria might be responsible for this transportation (Fukui and Tanaka, 1981). Figure 12 illustrates possible roles of

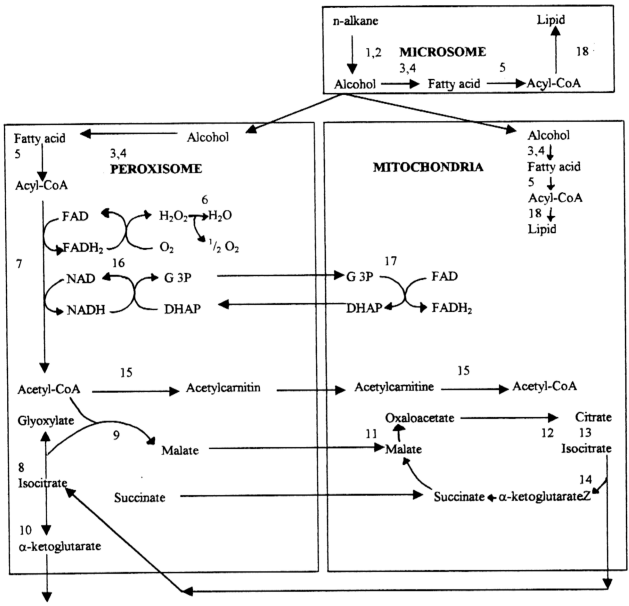

Figure 12: The possible roles of peroxisomes in connection with those of mitochondria and microsomes in alkane assimilation by yeasts. DHAP= dihydroxyacetone phosphate; G 3P= glycerol-3-phosphate;Enzymes: 1= cytochrome P-450;2= NADPH-cytochrome P-450 (cytochrome c) reductase; 3= alcohol dehydrogenase; 4= aldehyde dehydrogenase; 5= acyl-CoA synthetase; 6= catalase; 7= β-oxidation system; 8= isocitrate lyase; 9= malate synthase; 10= NADP-dependent isocitrate dehydrogenase; 11= malate dehydrogenase; 12= citrate synthase; 13= aconite hydratase; 14= NAD-dependent isocitrate dehydrogenase; 15= carnitine acetyltransferase; 16= NAD-dependent glycerol-3-phosphate dehydrogenase; 17= FAD-dependent glycerol-3-phosphate dehydrogenase; 18= glycerophosphate acyltransferase.
(Fukui and Tanaka, 1981).

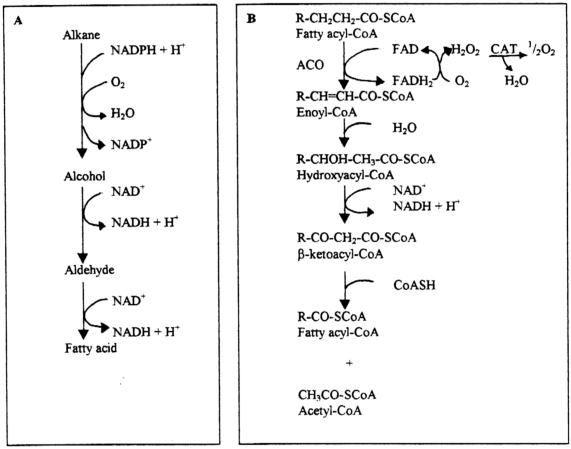

Figure 13: A. Oxidation pathway of alkanes to corresponding fatty acids.
B. Fatty acid β-oxidation system in yeast peroxisomes.
(Fukui and Tanaka, 1981).

microsomes, mitochondria and peroxisomes in alkane-utilising yeasts. Thus, the metabolic significance of yeast peroxisomes in alkane or fatty acid assimilation will be easily understood on the basis of currently available knowledge.

1.6.2 Tricarboxylic acid cycle and citric acid formation

As mentioned earlier in *Y. lipolytica*, the peroxisomes were not formed in a large number when the cells were grown on glucose compared to cells grown on alkane as reported by Ermakova *et al.* (1986). Under this condition, glyoxylate cycle was not actively functioned. The accumulation of citric acid in *Y. lipolytica* was only relying on TCA cycles in mitochondria. The glucose undergoes glycolysis or Embden-Meyerhof pathway to produce two pyruvate molecules. Pyruvate is further oxidised through oxidative decarboxylation to form acetyl-CoA by multienzymes complex pyruvate dehydrogenase. The acetyl-CoA is the substrate which enter the tricarboxylic acid cycle (Figure 14). The TCA cycle continues its operation by the condensation of acetyl-CoA and four carbon acid oxaloacetate to yield citric acid. Citric acid occupies a key position in the intermediary metabolism of all living cells where it has a multifunctional role in regulating both anabolic and catabolic pathways in the cell (Evan and Ratledge, 1985). However, this acid does not normally accumulate in appreciable quantity in living cells. Exceptions to this generalisation are found among citrus fruits, pineapples and gooseberries that contain large amounts of citric acid.

Two enzymes that have been examined in detail in relation to citric acid fermentation are aconitate hydratase and isocitrate dehydrogenase. The activities of these enzymes have been shown to decrease to very low levels during period of

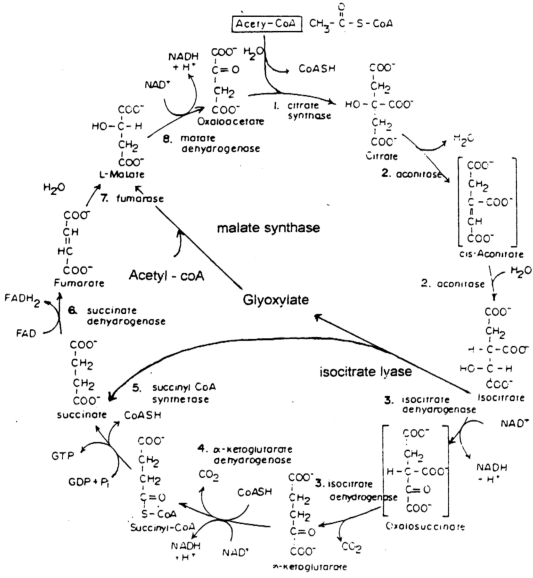

Figure 14: The Citric acid and glyoxylate cycles.
(Adapted from: Voet and Voet, 1994).

citric acid accumulation while the activity of isocitrate dehydrogenase has been found to increase (Tabuchi and Hara, 1973).

Aconitate hydratase which catalyses the conversion of citric acid to isocitric acid, is sensitive to high concentration of the metallic ions as Fe^{2+}. In *A. niger* addition of Fe^{2+} increases the aconitate hydratase activity which decreases citrate and increases isocitrate accumulation.

The enzyme functions reversibly with bias toward a citrate formation and this is a regulatory mechanism incorporated into the cycle to prevent excessive substrate congesting the following step and to ensure efficient cycling of the TCA cycle. The present of chemical such as monofluoroacetate (MFA) will deprive iron and accumulate citric acid in the cells. MFA administered to animals is an enzymatically converted to monofluorocitrate (MFC) *in vivo* which causes a competitive inhibition of aconitate hydratase activity (Akiyama *et al.*, 1972; Gribble, 1973). Base on this facts Akiyama *et al.* (1972) have successfully isolated strain of *Y. lipolytica* which was sensitive to MFA and low in aconitate hydratase activity as a citric acid producer strain.

The activity of isocitrate dehydrogenase in *Y. lipolytica* also determines the amount of isocitrate and citrate produced. Addition of potassium ferrocyanide or thiamine deficiency in a glucose medium leads to decrease in isocitrate dehydrogenase activity and to an increased citrate accumulation (Tabuchi and Hara, 1973)

1.7 The objectives of the research project

The objective of the research is to explore the possibility of improving citric acid production by *Y. lipolytica*. The choice of the microorganism is due to its prominent characteristics, which was mentioned earlier. To fulfil the objective of the research a plan of work will be conducted as follows:

1. Production of citric acid using NBD palm olein as the main carbon source.
2. Isolation of high yielding strain of *Y. lipolytica* by NTG treatment.
3. Detail biochemical and genetic study of available mutants.
4. Improved of the strain using recombinant DNA techniques.
5. Upscaling the production of citric acid using strain obtained from 2.