The production of citric acid by *Yarrowia lipolytica* when cultivated on edible and waste fats.

by

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Contents

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Key words

Yarrowia lipolytica

Citric acid

Sunflower

Lipids

Oils

Fats

Edible oil waste

Polymerized triglycerides

Glyoxylate cycle

Acetate

Kernwoorde

Yarrowia lipolytica

Sitroensuur

Sonneblom

Lipiede

Olies

Vette

Eetbare vet afval

Gepolimeriseerde trigliseriede

Gliöksilaat siklus

Asetaat

Chapter 1

Introduction

1.1 Motivation

Large amounts of edible oil waste (approx. 100 000 tons p.a.) is generated in South Africa when edible oil, mainly sunflower oil, is used in frying processes. However, part of this waste may be toxic. When oils and fats are overexposed to heat, especially during repeated use in frying processes, toxic breakdown products not fit for human consumption are formed. These potentially harmful wastes can only be used under carefully controlled conditions by oleochemical industries. It is important to realize that another part of these fat wastes (approx. 50%) are still fit for human consumption and has been discarded by frying establishments while within regulatory limits. Consequently, these wastes have the potential to be processed to safe usable foodstuffs (Kock *et al.*, 2002).

In South Africa, an organic acid i.e. citric acid is extensively imported by various industries where it is used mainly in the food and pharmaceutical industries (Chem-expo, 1998). Currently citric acid is produced (Demain & Davies, 1999) by the fungus *Aspergillus niger*. This process was optimized with *Aspergillus niger* converting glucose to citric acid (Roehr *et al*., 1996).

Interestingly, Good *et al*. (1985) noted that *Yarrowia lipolytica* (yeast) have the capability to convert monounsaturated oils (such as canola oil) to citric acid.

This leads to the question, whether a process could be developed to convert edible oil waste (within regulatory limits) to a useful food product such as citric acid. In addition, Jeffery *et al*. (1999) reported the large enhancing effect of acetate on biomass production, oil utilization and high value lipid production when acetate was added to a sunflower oil containing medium on which several fungi were cultivated.

Consequently, the aim of this dissertation became to explore the possibility of using *Yarrowia lipolytica* as a bioconversion agent to convert used edible oil waste (still fit for human consumption) to a more valuable product, citric acid, in the presence of acetate.

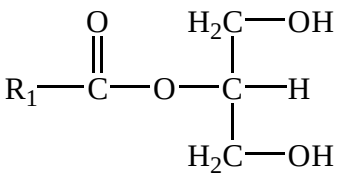
1.2 Fats

1.2.1 Composition

Though fats and oils have the same basic structure, *fats* are solid at room temperature (21°C) while *oils* are liquid. Nevertheless, fats and oils have the same basic structure (Ebbing, 1996). Both consist mainly of triacylglycerols (TAGs) with small amounts of monoacylglycerols (MAGs), diacylglycerols (DAGs), phospholipids (PLs), and free fatty acids (FFAs) (Fig. 1). Edible oils and fats (from

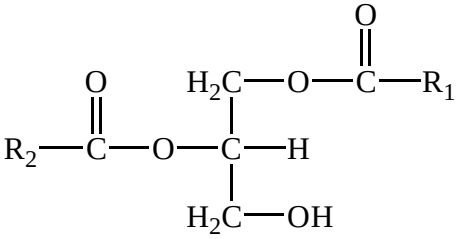
now on referred to as fats) are generally characterized as non-polar compounds indicating that they are only soluble in non-polar solvents e.g. ether, chloroform, alcohols and acetone (Ratledge & Wilkinson, 1988). According to Badenhorst (1998) most edible fats consumed in South Africa contain a considerable amount of polyunsaturated (two or more double bonds in the carbon chain) fatty acids (PUFAs) such as linoleic acid (18:2) (Fig. 1). These PUFAs are rapidly oxidized (section 1.2.2) yielding toxic compounds. These compounds include polymers, cyclic monomers, free radicals, dimers, trimers, aldehydes, hydroperoxides, alcohols and low molecular weight products such as malondialdehyde and 4-hydroxyalkenals (Chow & Gupta, 1994; Kock *et al.*, 1995).

Monoacylglycerol



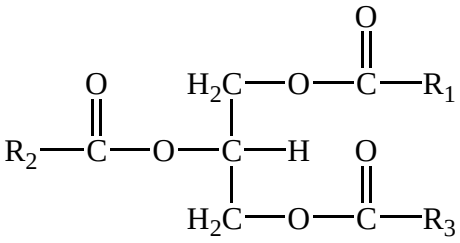
1-Acyl-sn-glycerol

Diacylglycerol



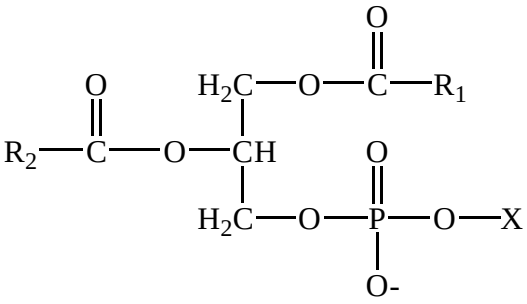
1,2-Diacyl-sn-glycerol

Triacylglycerol



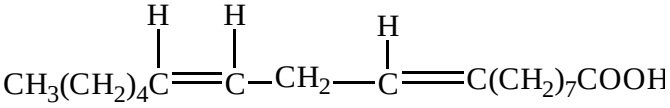
1,2,3-Triacyl-*sn*-glycerol

Phospholipid



Phosphatidic acid

Free fatty acid



Linoleic acid (C18:2)

Figure 1. Fatty acid derivatives mainly present in fats. R_1 CO-, R_2 CO-, R_3 CO- represent fatty acyl groups (Ratledge & Wilkinson, 1988). X = different ligands can be esterified at this point i.e. hydrogen, choline, serine, etc.

1.2.2 Autoxidation of fats

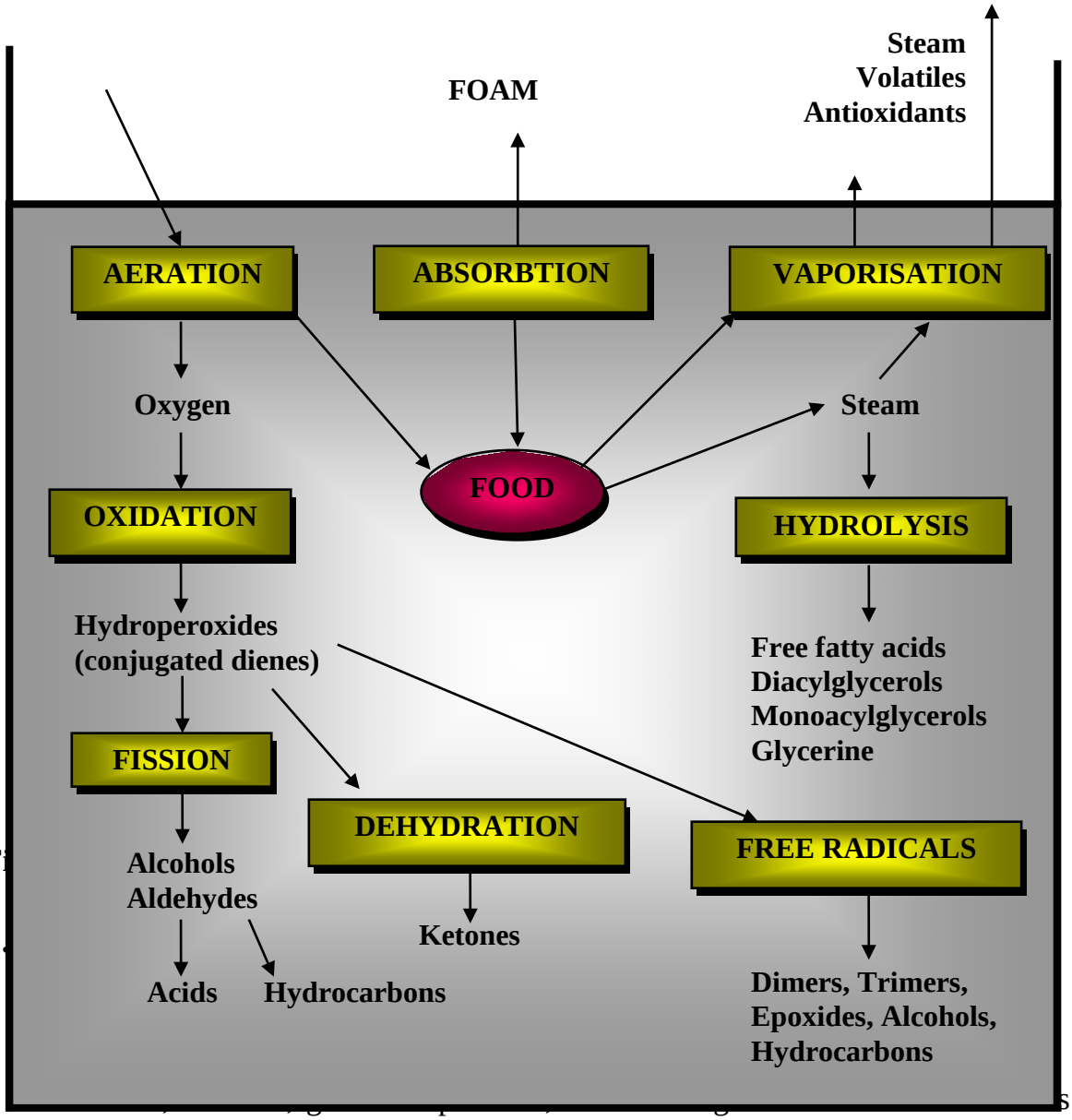
Oxidation of fatty acids (FAs) especially PUFAs is caused by repeated use and prolonged exposure to elevated temperatures (above 200ºC) in the presence of

moisture (Chow & Gupta, 1994). This results in the darkening of oil, unacceptable odours and taste (rancid), excessive foaming and an increase in oil viscosity (Kock *et al*., 1997).

Fritsch (1981) clearly demonstrated the changes that occur within fats during the frying process (Fig. 2). As noted, severe heating of frying fats yields dimers and cyclic compounds, which may be toxic and also destroy antioxidants. Fractions of water present in food are vaporized together with the water-soluble antioxidants and other volatiles present in fat. The resulting steam is responsible for hydrolyzing the ester bonds of the TAGs. This in turn leads to the formation of DAGs, MAGs, FFAs and glycerol. The composition of the food fried will also influence the products that form. Spicy food for instance often contains heavy metals, which can transform FFAs to soap-like compounds causing foam at the surface. This process consequently increases oil aeration and oxidation. Oxidation yields hydroperoxides, which in turn produce free radicals to form dimers, trimers, epoxides, alcohols and hydrocarbons. These compounds can also be dehydrated to ketones and may undergo fission yielding alcohols and aldehydes (Fritsch, 1981; Frankel, 1998).

The extend to which fat has been broken down, can be determined by measuring the levels of polar compounds i.e. all the breakdown products of the TAGs (Official Methods of Analysis of the AOAC, 2000) or the levels of polymerized triglycerides i.e. dimers, trimers and polymers (Beljaars *et al.*, 1994).

Polymerized triglycerides include products formed by carbon to carbon and/or carbon to oxygen linkage within triglyceride-bound fatty acids to produce dimers or polymers (Beljaars *et al.*, 1994; Frankel, 1998; Anelich *et al.*, 2001). It is interesting to note that the more the fats are broken down, the more is absorbed by the food and the more is eventually consumed (Kock *et al.*, 2002).



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are some of the diseases that may be caused when humans are continuously exposed

through ingestion or inhalation to over-oxidised fats (Chow & Gupta, 1994; STOA Report, 2000). For this reason legislation was proclaimed in 1996 in South Africa prohibiting the use of overused frying fats in food preparation (Kock *et al*., 1997; Kock *et al*., 1999; Kock, 2001). According to The Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act no. 54 of 1972), published on 16 August 1996, the legal limit for polymerized triglycerides in frying fats must be below 16% and that for polar compounds below 25% - if above these levels, these fats may be harmful to human health (Second National Symposium On Abused Cooking Oils, 1996).

As a result of these regulations, large quantities (approx. 100 000 tons p.a.) of these fats accumulate in South Africa (Kock *et al*., 2002). Of these fats, approx. 50% is still fit for human consumption and can be regarded as an excellent energy source to produce value added products such as lipids e.g. gamma-linoleic acid (Badenhorst, 1998), animal feed (Kock *et al.*, 1997), biodiesel fuel (Fukuda *et al.*, 2001) and possibly citric acid.

1.3 Citric acid

1.3.1 Structure

Citric acid (2 – hydroxy - 1, 2, 3 - propanetricarboxylic acid) (Fig. 3a) is an intermediate in the citric acid (also known as the Tricarboxylic Acid / Krebs cycle) and glyoxylate cycles. Citric acid and isocitric acid consist of three concomitant carbon atoms chained together, with three carboxyl groups attached at each carbon. The only difference between the two acids is the position of the hydroxyl group. In yeasts both citrate and isocitrate (Fig. 3b) are excreted as metabolic by-products into the extracellular environment. The ratio of citrate to isocitrate varies yielding in many cases an unfavourable end-product composition. In fact, up to 50% of the total acid produced can be isocitrate (Roehr *et al.*, 1996).

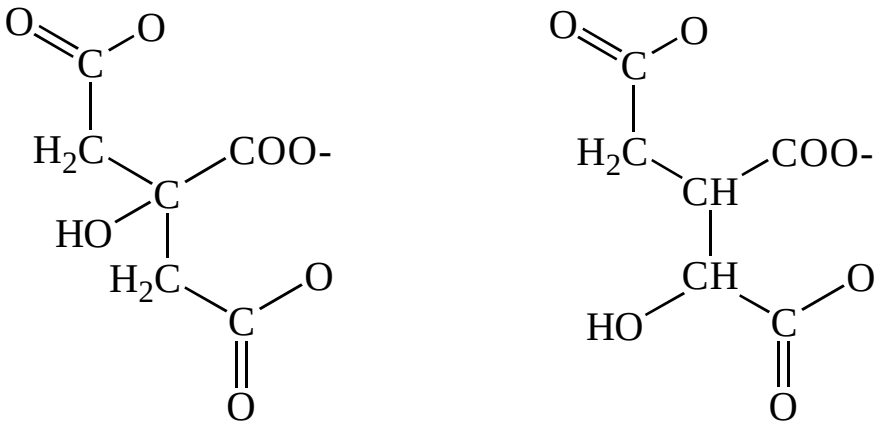

Figure 3. The chemical structures of (a) citrate and (b) isocitrate.

1.3.2 Brief history

Scheele, a Swedish Chemist, first obtained citric acid from lemon juice as early as 1784 (Grewal & Kalra, 1995). Short after that, in 1920, citric acid was

produced by the sedimentation of hot lemon juice by using calcium carbonate (Asian and Pacific Centre for Transfer of Technology [APCTT], 2002). In 1917, Currie led the way for successful industrial production of citric acid by mould fermentation, using *Aspergillus niger* (Roehr *et al.*, 1996). Two years later, a Belgium manufacturer succeeded with the shallow pan fermentation process using *Aspergillus niger* (APCTT, 2002). In 1952, the America Miles company, USA, successfully produced citric acid on a large scale by deep-level fermentation and today they are still the leading producers of this product (Roehr *et al.*, 1996; APCTT, 2002).

1.3.3 Production

The mycelial fungus, *Aspergillus niger*, is the traditional producer of citric acid with other yeasts like *Candida tropicalis, Rhodotorula* spp. and *Yarrowia lipolytica* not far behind. The main carbon source, used by *Aspergillus niger* for the commercial production of citric acid, is glucose (Roehr *et al.*, 1996). With glucose as substrate, *Aspergillus niger* produced 172.8 g/l citric acid with a maximum productivity of 0.8 g/l/h (Good *et al.*, 1985). Molasses, sucrose and other carbohydrate compounds i.e. hexadecane and edible fats are equally functional as

carbon sources for citric acid production by yeast (Rane & Sims, 1993; Good *et al.*, 1985).

In some commercial processes, *Aspergillus niger* is cultured on potato starch residue for six to seven days at 30 - 40ºC. The classical process for the manufacturing of citric acid takes place in shallow pans (Demain & Davies, 1999) which generates a large contact area between the liquid phase, mycelium and the surrounding atmosphere providing the oxygen required. However, the disadvantage with this type of operation is the large infrastructure required and the high probability of contamination (Grewal & Kalra, 1995).

The submerged fermentation process is an alternative system applied in the production of citric acid. This process, being the choice for industrial scale manufacturers yields more than 80% of the world's citric acid annually. Amongst others, the advantage of this process includes elevated yields, high productivity and low labour cost. However, this process suffers the disadvantage of microorganisms being extremely sensitive to fermenter construction materials containing traces of metal ions, especially iron and manganese (Grewal & Kalra, 1995). Finally the recovery of citric acid from fermentation broths is generally accomplish by three basic procedures including precipitation, extraction and adsorption (mainly using ion exchange resins) (Roehr *et al.*, 1996).

1.3.4 Market

Citric acid is a well-known product in the food, pharmaceutical, cosmetics, and other industries (Fig. 4; Demain & Davies, 1999; Arzumanov *et al.*, 2000). According to Chem-expo (1998) 70% of the total citric acid is used for food and beverage; 18% for detergents and cleaners; 6% for pharmaceuticals and cosmetics and 6% for industrial and chemical processing. According to Esker et al. (1999), the world market for citric acid in the late '90s was approximately 880 000 tons p.a. and was still increasing. Recent statistics show that the annual production of citric acid is around one million tons of which most is obtained by fermentation using the filamentous fungus *Aspergillus niger* (Guebel & Darias, 2001). The market price for citric acid is relatively stable at 1.5 U.S. $ per kg citric acid monohydrate (Schneider & Steinmüller, 1996).

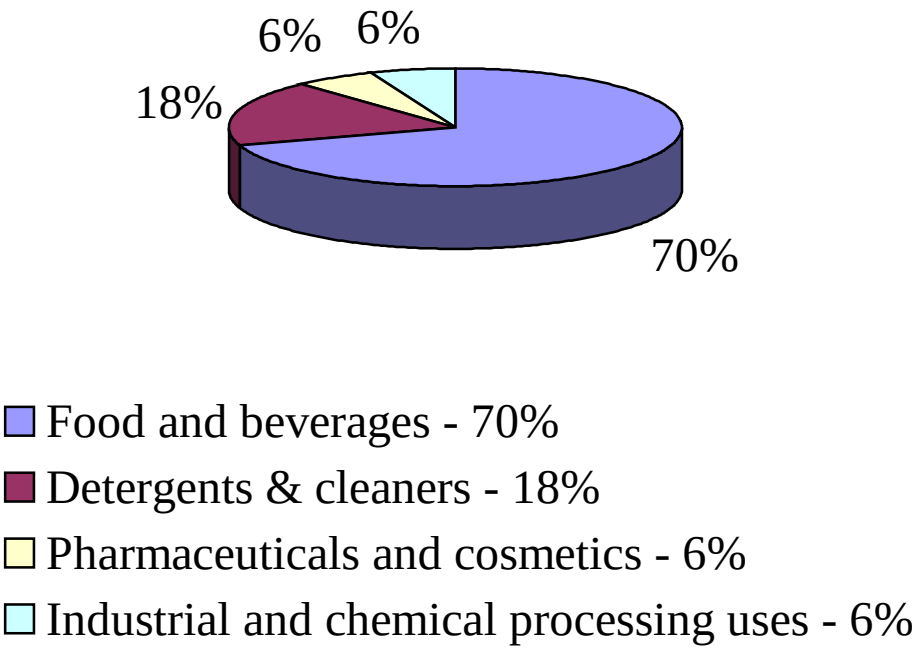

Figure 4. Uses of citric acid. (Chem-expo, 1998).

Citric acid is accepted as a GRAS (generally regarded as safe) product. The main advantages of citric acid as an edible acidifier, is its high solubility, non-toxicity, strong chelating power and its pleasant taste (APCTT, 2002). It finds its use as a condiment, preservative, pH adjustor and an antioxidant when acting with ascorbic acid. In the pharmaceutical industry, it produces frothing effects in many medicines when acid carbonate is to be made. In the chemical industry, citric esters can be applied as nontoxic plasticizers for the production of plastic film (Roehr *et al.*, 1996). To meet the requirements of environment protection, as a detergent

ingredient, citric acid is increasingly used to replace phosphorus, which negatively influences the environment (Schneider & Steinmüller, 1996).

Although *Aspergillus niger* is today the fungus of choice for the production of citric acid, the focus progressed over the past 30 years to the use of yeasts as citric acid producers. The possible advantages of using yeasts instead of *Aspergillus niger*, include: (1) greater tolerance to high substrate concentrations, (2) higher yields, (3) greater productivity, (4) their insensitivity to metal ions and (5) better process control due to the unicellular nature (Rane & Sims, 1993). Concequently the use of *Yarrowia lipolytica* (previously known as *Candida lipolytica*, *Endomycopsis lipolytica*, *Saccharomycopsis lipolytica*) as an alternative citric acid producer is intensely researched at present (Arzumanov *et al*., 2000; Finogenova *et al*., 2002; Kamzolova *et al*., 2003).

1.4 The utilization of fats by fungi

A different metabolic pathway is followed when fungi utilize fats as carbon source, instead of glucose. Also different enzymes are responsible for the conversion of fats to citric acid, compared to those used for citric acid production from glucose.

When fats are present in the medium as sole carbon source, lipases become activated and hydrolyze the TAGs to yield DAGs, MAGs, FFAs and glycerol. Once hydrolyzed, FFAs enter the cells through simple or facilitated diffusion (McKee & McKee, 1999). Inside the cytoplasm, the FFAs are transformed to acyl-S-CoA esters by acyl-S-CoA synthetases, before it is included in TAGs or mobilized for other bioreactions (Finnerty, 1989).

1.4.1 Transport and β-oxidation of free fatty acids

Fatty acid acyl-S-CoA esters in the cytoplasm, may enter the mitochondria through carnitine carriers (Ratledge, 1989). Here acyl-S-CoA reacts with carnitine to form an acylcarnitine derivative, which is catalyzed by carnitine acyltransferase l. Acylcarnitine is then transported across the inner membrane by the carrier protein and is subsequently reconverted to carnitine and acyl-S-CoA by carnitine acyltransferase ll (Fig. 5) (McKee & McKee, 1999).

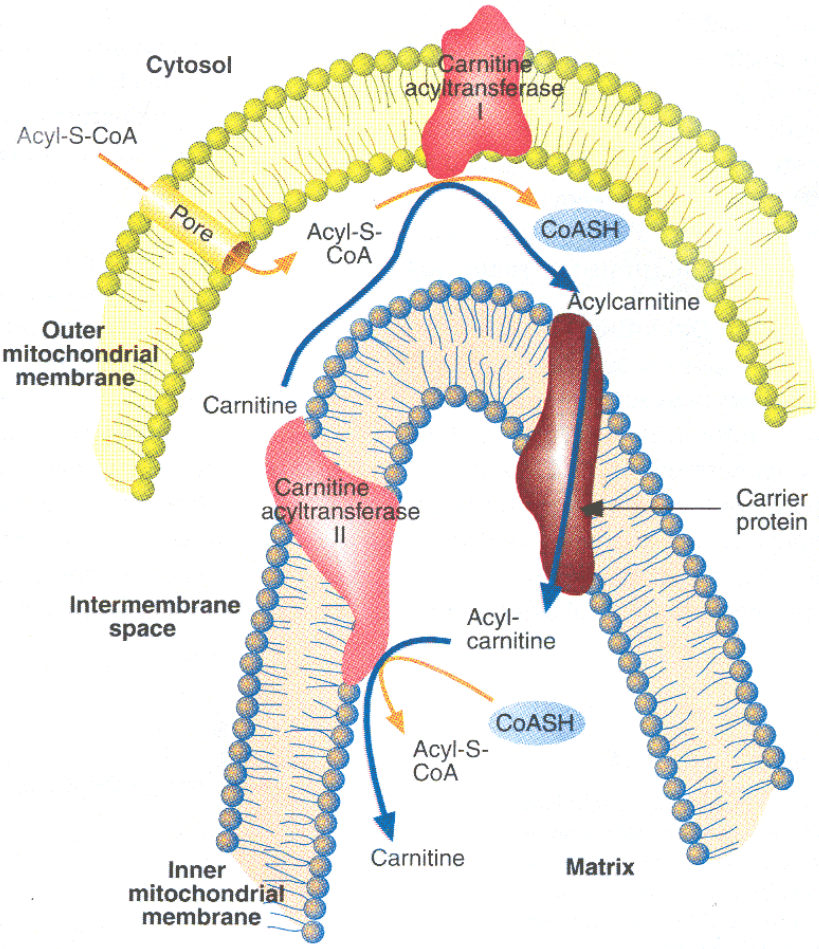

Figure 5. Acyl-S-CoA transport into the mitochondrion (McKee & McKee, 1999).

The acyl-S-CoA molecule is now ready to be ß-oxidized. The complete ß-oxidation pathway (in general) is shown in Fig. 6. In short, ß-oxidation basically consists of 4 steps: (1) oxidation-reduction reaction, (2) hydration reaction, (3) a dehydrogenation reaction and finally (4) a thiolase reaction. In this last reaction an acetyl-S-CoA molecule and an acyl-S-CoA are released. The acyl-S-CoA molecule

is re-cycled back to the oxidative pathway to be completely broken down to acetyl-S-CoA (i.e. C2 units) and further oxidized to CO_2, water and energy (Mathews & Van Holde, 1990).

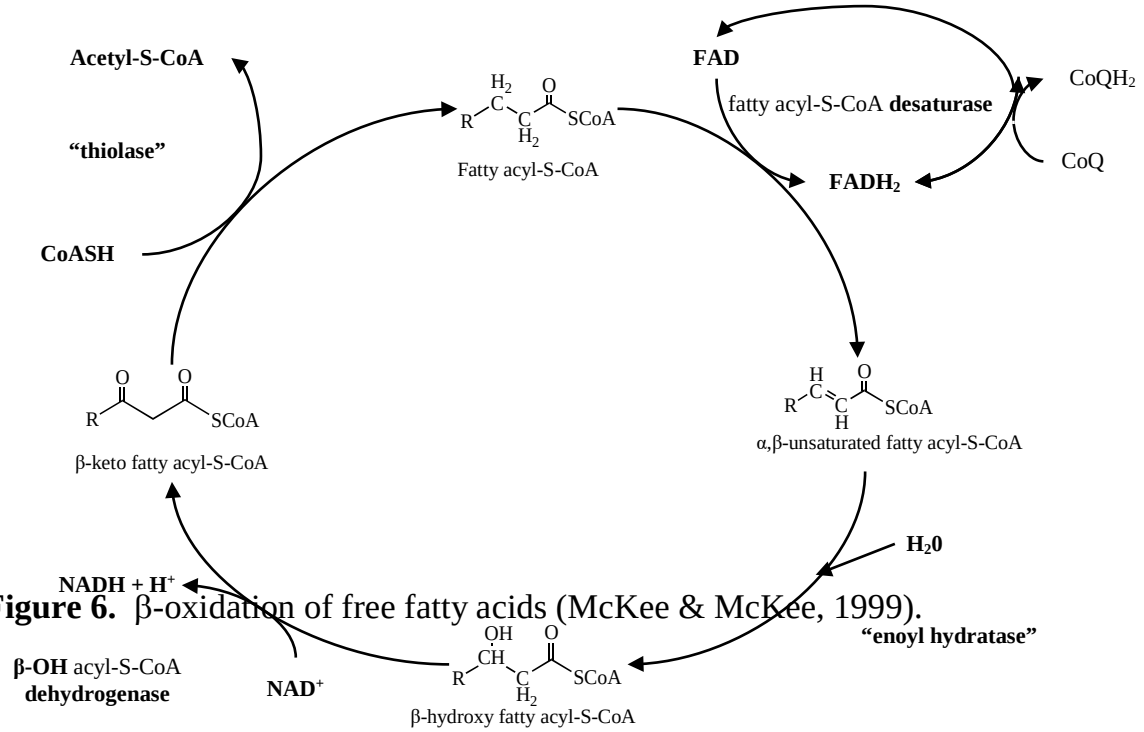

Figure 6. β-oxidation of free fatty acids (McKee & McKee, 1999).

1.4.2 Formation of citric acid

The acetyl-S-CoA molecules (originated from ß-oxidation where fat was used as carbon source or from pyruvate where glucose was used as carbon source) can enter one of two systems to be converted to citric acid. The first is the citric acid cycle (Fig. 7), where acetyl-S-CoA can only be converted to citrate if oxaloacetate is available. This reaction is catalyzed by citrate synthase (Mathews & van Holde, 1990). It has been reported that this system frequently fails to produce sufficient

oxaloacetate, due to the formation of many by-products such as α-Ketoglutarate, Succinyl-S-CoA, Succinate and Fumarate (McKee & McKee, 1999).

In *Yarrowia lipolytica* a synergistic cycle, the glyoxylate cycle (Fig. 8), solves this problem by incorporating additional acetyl-S-CoA directly to malate, which is consequently converted to oxaloacetate, needed for citrate production (Madigan *et al*., 1997). This cycle is activated when two carbon acids (such as acetate) are utilized by the organism and can only continue to operate if the acceptor molecule, oxaloacetate, is regenerated at each turn of the cycle.

This cycle is composed of most of the citric acid cycle reactions plus two additional enzymes: isocitrate lyase, which splits isocitrate to succinate and glyoxylate, and malate synthase, which converts glyoxylate and acetyl-S-CoA to malate.

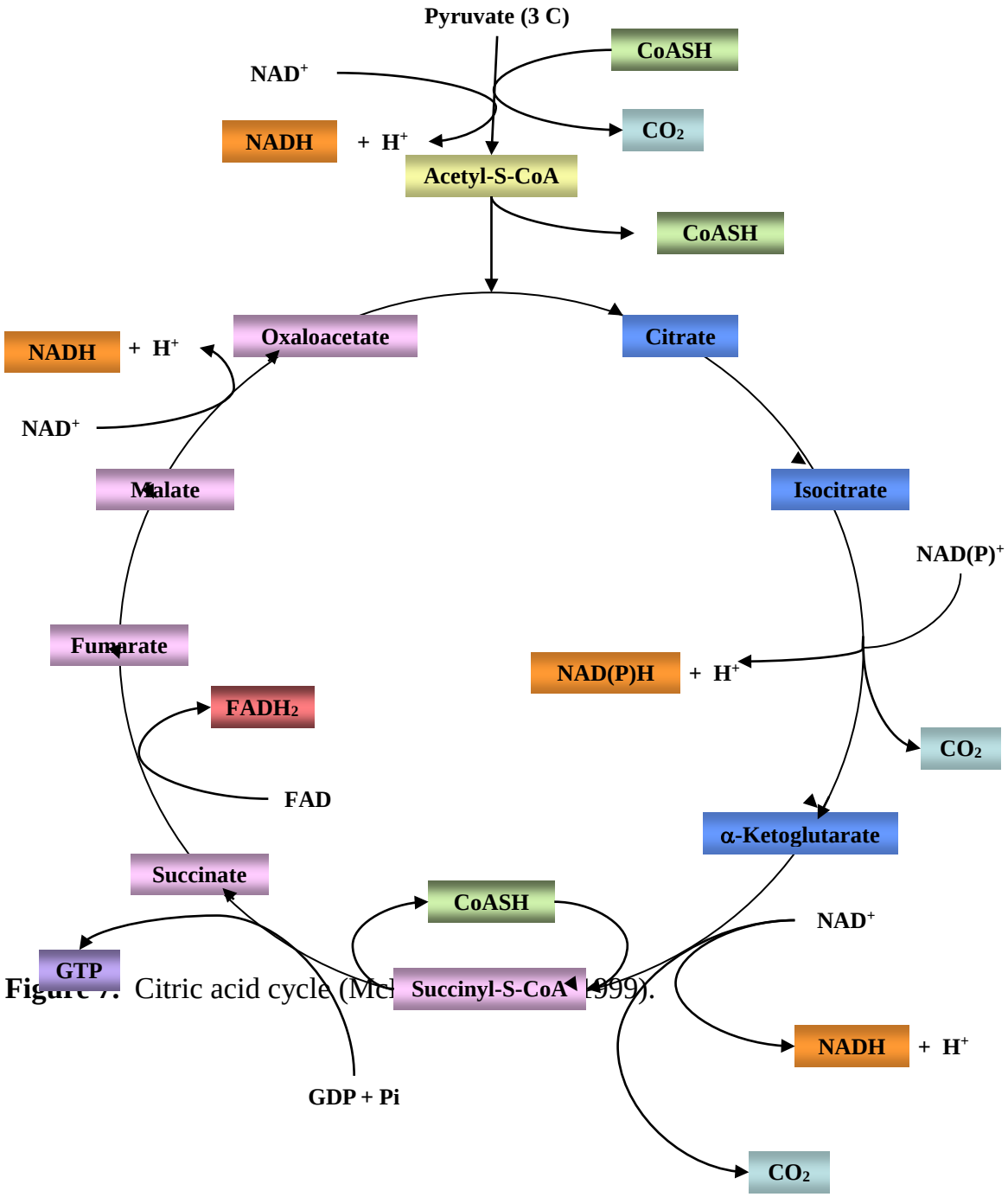

Figure 7. Citric acid cycle (Mc[illegible]1999).

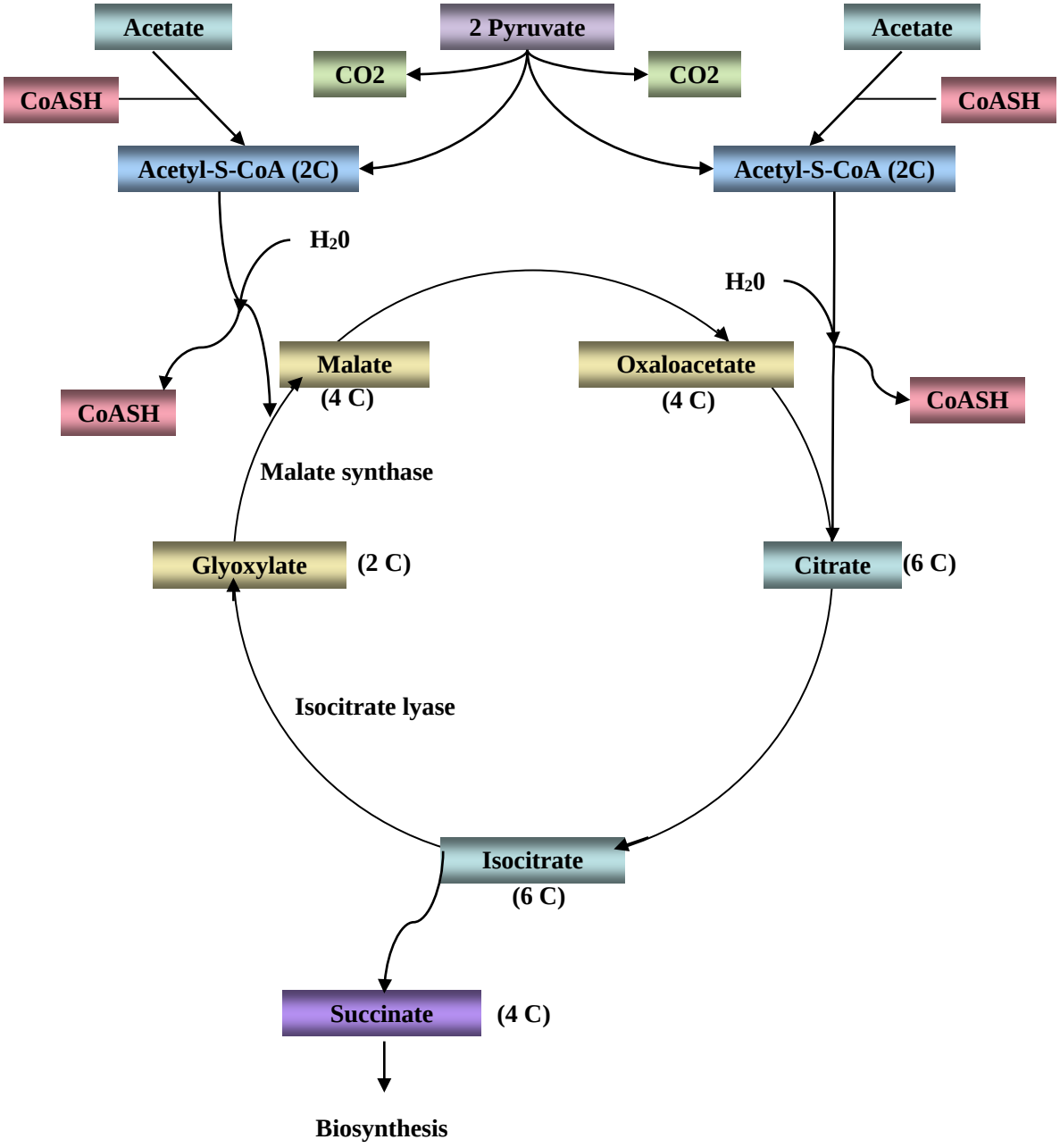

Figure 8. Glyoxylate cycle (Madigan *et al.*, 1997).

A question to be answered concerns the fate of acetate (converted in the cytoplasm to acetyl-S-CoA) when added to the growth medium of *Yarrowia lipolytica* containing fats as carbon source. Especially since it is known that *Yarrowia lipolytica* can follow the glyoxylate cycle when converting fats to citric acid (Finogenova *et al*., 1986). Will the addition of acetate to the medium containing sunflower fats enhance citric acid production?

Jeffery *et al*. (1999) as well as Bareetseng (2000) reported an increase in biomass and lipid utilisation of both *Mucor* and *Yarrowia lipolytica* respectively after the addition of acetate to the growth medium containing sunflower fat.

1.5 Purpose of research

With this as background it became the aim of this study to:

1) explore the possibility of the yeast *Yarrowia lipolytica* to convert edible fat waste (still fit for human consumption) to citric acid thereby adding value to this relatively cheap substrate (i.e. zero cost),
2) determine the influence of fat breakdown products on citric acid production by this yeast and

3) assess the effects of acetate (when added to a medium containing edible oil waste) on citric acid production through the glyoxylate cycle – as found in *Yarrowia lipolytica* (Finogenova *et al*., 1986).

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Chapter 2

The effect of acetate on citric acid production by Yarrowia lipolytica when cultivated on sunflower fat

This chapter has been accepted for publication in Systematic and Applied Microbiology

PATENT UNDER EVALUATION

2.1 Abstract

Eighteen strains of *Yarrowia lipolytica* were grown for 6 days on a medium consisting of sunflower fat in the presence and absence of 10 g/l acetate. It was discovered that the addition of acetate caused a drastic increase in citric acid production by many strains of *Yarrowia lipolytica.* Strikingly *Yarrowia lipolytica* UOFS Y-1701 produced increased amounts of citric acid in the presence of acetate i.e. 0.5 g/l in the absence of acetate to 18.7 g/l in the presence of acetate. Similarly, the ratio of citric acid : isocitric acid increased significantly from 1.7:1 in the absence of acetate to 3.7:1 in the presence of acetate after 240 h of growth. During the growth period the acetate as well as the 30 g/l fat was almost completely utilized (100% and 99.3% respectively).

2.2 Introduction

Citric acid is widely used in the food, chemical and pharmaceutical industries due to its properties as an acidifier, preservative, pH adjuster, antioxidant and sequestrant (Rane & Sims, 1993). The annual global production of citric acid is estimated at 880 000 metric tons which is currently being produced by the submerged fermentation process applying the mycelial fungus, *Aspergillus niger* (Esker *et al*., 1999).

According to Good *et al*. (1985) and Rane and Sims (1993) yeasts like *Candida tropicalis*, some *Rhodotorula* spp. and *Yarrowia lipolytica* also have the ability to produce citric acid from various carbon sources, which include edible fats. One disadvantage though is the significant amounts of isocitric acid produced during fermentation, which can reach levels of up to 50% of the total acid production thereby influencing citric acid yields negatively. It was shown by Roehr *et al*. (1996) that isocitric acid is influenced by the type of organism, the carbon source and the micronutrient concentration available to the yeast.

In the past, several groups have attempted to alter the growth media in order to increase the production of citric acid (Finogenova *et al*., 1986; Good *et al*., 1985; Rane & Sims, 1993). In this paper we report on a novel finding where the addition of acetate to a medium containing sunflower fat as sole carbon substrate, significantly increased citric acid production by *Y. lipolytica* UOFS Y-1701.

2.3 Materials & Methods

2.3.1 Selection of strains

Strains used. Eighteen strains (Table 1) of *Yarrowia lipolytica* (all obtained from the University of the Free State, Bloemfontein, South Africa) were screened for the best

producer of citric acid in both the presence and absence of acetate. Strains were cultivated for six days at 26°C. Biomass, pH, citric acid and isocitric acid determinations were performed on all eighteen strains as describe in 2.3.2.

2.3.2 Lipid turnover and citric acid production

Strain used. *Yarrowia lipolytica* UOFS Y-1701 (the best citric acid producer in 2.3.1) was used in further experiments to determine its lipid turnover and citric acid production.

Cultivation and harvesting of cells. Yarrowia lipolytica UOFS Y-1701 was cultivated in 36x250 ml conical flasks at 26ºC (shaken at 160 rpm) for ten days. Each flask contained 50 ml sterile growth medium (pH 5.8). The medium consisted of the following (in g/l): sunflower fat, 30; sodium acetate, 10; yeast extract, 0.1; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 10; $CaCl_2.2H_2O$, 0.05; NH_4Cl, 1.28. Tap water was used or trace elements were added to the following final concentration (g/l): $FeSO_4.7H_2O$, 0.035; $MnSO_4.4H_2O$, 0.007; $ZnSO_4.7H_2O$, 0.011; $CuSO_4.5H_2O$, 0.001; $CoCl_2.6H_2O$, 0.002; $Na_2MoO_4.2H_2O$, 0.0013; H_3BO_3, 0.002; KI, 0.00035; $Al_2(SO_4)_3$, 0.0005. As a control experiment, the same medium as above was used with the exception that the sodium acetate (10 g/l) was omitted and 40 g/l sunflower fat included as sole carbon source. At every time interval, the cells were harvested by centrifugation at 8000 g for 15 min i.e. after all residual extracellular fat in the culture was extracted with *n*-hexane (see Lipid extraction section). Cultures in

flasks were harvested as follows: four pooled flasks each after 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h and 240 h respectively. After harvesting, cells were immediately frozen, freeze-dried and then weighed. In addition, the pH was determined for each flask at regular intervals (Fig. 2) over the growth cycle. All experiments were performed at least in triplicate.

Lipid extraction. This was performed according to the methods described by Kock *et al*. (1997). In short, extracellular lipids (ECL) present in the corresponding supernatant (pH < 3) from each flask mentioned above were immediately extracted after harvesting with *n*-hexane until almost no extracellular lipids could be detected. Intracellular lipids (ICL) were extracted from the freeze-dried cells using chloroform/methanol (2:1, by vol) as described by Folch *et al*. (1957). The lipids were dissolved in diethyl ether and transferred to preweighed vials. The samples were dried to a constant weight in a vacuum oven at 50°C over P_2O_5.

Fatty acid analysis. Trans-esterification of extracellular and intracellular lipids were performed by the respective addition of trimethylsulphonium hydroxide (TMSOH) according to the method of Butte (1983). The fatty acid methyl esters were determined by gas chromatography (Hewlett Packard Model 5830A GC equipped with a dual flame-ionization detector) using a Supelcowax 10 column (30 m X 0.75 mm) with nitrogen as carrier gas. The initial column temperature of 145ºC was increased by 3ºC/min to 225ºC and, following a 10 min isothermal period, was then

increased to 240ºC, respectively. Nitrogen was used as carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards.

Citric acid, isocitric acid and acetic acid analysis. Citric-, isocitric and acetic acid contents in the supernatant were determined by high-performance liquid chromatography (HPLC) (Shimadzu SPD-10A VP with UV detector). The medium (1 ml; pH < 3) was filtered through a 0.45 μm filter (LCR non-sterile, Millex) prior to injection. Citric-, isocitric- and acetic acids were well separated using a Synergi 4μ Hydro-RP 80A (Phenomenex) column and these components were detected at 220 nm. The mobile phase consisted of 1% acetonitrile 190/UV UL to 20 mM KH_2PO_4 set to pH 2.5 and was pumped at a flow rate of 0.8 ml/min. Chromatographic data were quantitated using a Shimadzu C-R6A Chromatopac integrator. These organic acids were identified and quantified with reference to authentic standards.

Chemicals. All organic chemicals and solvents used were of analytical reagent grade and obtained from major retailers. All standards were obtained from Sigma.

2.4 Results & Discussion

There was a drastic increase in the production of citric acid by almost all strains when acetate was added to the growth medium (Table 1) with the exception of strains UOFS Y-0829, UOFS Y-1138, UOFS Y-1570, UOFS Y-2110 and UOFS Y-2160. In the presence of acetate, the pH of all the strains was higher than in its absence. The pH however did not rise to pH 8 as was experienced by Jeffery *et al*. (1999) after the addition of acetate to the growth medium wherein *Mucor* was cultivated. This is most probably due to the production of citric acid that decreased the pH. No real pattern concerning the biomass production and the ratio of citric acid : isocitric acid (CA:ICA) in the presence and absence of acetate was observed. Strain UOFS Y-0809 produced the highest amount of citric acid of all strains screened, but the ratio between the two acids was low. Strain UOFS Y-1701 was thus selected for further evaluation not only because of its high citric acid concentration of almost 18 g/l, but also because of its high ratio CA:ICA of 3.9:1.

When strain UOFS Y-1701 was cultivated on sunflower fat as sole carbon source (Fig. 1A), it reached maximum biomass after only 48 h. Interestingly, the biomass decreased after 72 h i.e. from 14.1 g/l to 4.2 g/l after 240 h. The intracellular lipids followed a similar pattern and may have contributed to the decrease in biomass experienced during the growth cycle. The fate of the intracellular lipids cannot be explained at present. During growth, this yeast actively

utilized the extracellular lipids i.e. sunflower fat within the first 48 h. After 48 h extracellular lipid concentration remained more or less constant at about 9.3 g/l. During growth the pH dropped sharply within the first 24 h from pH 5.8 to pH 2.4 and then decreased steadily to pH 2.0 (Fig. 2). This may be ascribed to the production of organic acids. After 120 h citric acid concentrations leveled off at an average of 0.5 g/l with a citric acid : isocitric acid ratio of 1.7:1 (Fig. 1A).

When cultivated in a mixed medium containing both sunflower fat and acetate, similar patterns regarding biomass production, extracellular lipid (sunflower fat) utilization and intracellular lipid turnover was experienced (Fig. 1B). Maximum biomass was reached after 48 h and followed an immediate sharp decrease after that. A similar pattern was again experienced concerning the intracellular lipid turnover and it is concluded that the decrease in intracellular lipids contributed to the decrease in biomass. Both acetate and sunflower fat was almost completely utilized within 48 h (Figs 1B and 2) resulting in the cessation of active growth. Strikingly, the citric acid production increased sharply up to 120 h of growth to eventually reach a value of 18.7 g/l with a citric acid : isocitric acid ratio of 3.7:1 after 240 h of growth. In this case the pH decreased from 5.8 to 2.7. The reason for the drastic increase in citric acid production is still unclear and is under investigation. In previous studies we found similar stimulatory effects for acetate on biomass and γ-linolenic acid production by *Mucor* when grown in 30 g/l sunflower fat and 10 g/l acetate as carbon sources (Jeffery *et al*., 1999). In the *Mucor* studies the increase in

production could be attributed to a large difference in pH (i.e. about 2.2 and 8.0) when cultivated in the absence and presence of acetate respectively.

The ECL fractions analyzed from experiments in the presence and absence of acetate were characterized by the presence of 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid) and 18:2 (linoleic acid). The relative amounts of these FAs over the growth cycle in the absence of acetate were similar to that of sunflower fat implying a lack of preference to any particular FAs during growth (Table 2).

However, in the presence of acetate, the organism seems to have developed a preference towards the utilization of unsaturated FAs i.e. and 18:2 (Table 3) as this FA decreased after especially 72 h of growth. At the same time an increase in 18:0 was found reaching a maximum (45.3%) also after 120 h of growth. This latter phenomenon can not be explained at present.

The ICL fractions of cells grown in the presence and absence of acetate contained as expected also 16:0, 16:1, 18:0, 18:1 and 18:2 FAs. In the absence and presence of acetate the FA profiles at time 0 h were significantly different from that of sunflower fat due to the pre-preparation of these cells as inoculum in a complex medium devoid of sunflower fat. In the absence of acetate (Table 2) the sunflower fat FA profile was more or less restored after 72h of growth after which it remained

more or less the same. This phenomenon was also reported previously in literature (Kendrick, 1991). After 240 h of growth the ICL FA profile was almost identical to that of the sunflower fat fed to the medium.

In the presence of acetate (Table 3) the FA profile of sunflower fat was again restored to a certain extent after 72 h after which a decrease in 18:2 and concomitant increase in 18:0 occurred. Interestingly the 18:1 remained more or less constant. This interesting trend can not be explained at present. Is the decrease in 18:2 a result of the increased utilization of this FA to produce amongst others citric acid? If so, why did the 18:1 content then remained similar?

In the present study it was discovered that the addition of 10 g/l acetate to a medium containing 30 g/l sunflower fat caused a drastic increase in citric acid production by *Y. lipolytica* UOFS Y-1701 while the ratio of citric acid : isocitric acid increased significantly. This new unexplained phenomenon and its general validity in other strains of *Y. lipolytica* (with some exceptions) showed the same trend. The effect of acetate in media containing other types of carbon sources should also be assessed. In addition, radio labeled acetate should be included to follow its metabolizable route, and clarify its enhancing effects.

2.5 Acknowledgements

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Table 1. Iso- and citric acid production by different strains of *Yarrowia lipolytica* (six days; 26ºC; 160 rpm)

Strain no.	pH	Citric acid (g/l)	Isocitric acid (g/l)	Biomass (g/l)	Ratio CA:ICA
UOFS Y-1703					
+ Acetate	3.1	13.3	7.2	12.4	1.8:1
- Acetate	2.6	0.1	0.2	16.0	0.5:1
UOFS Y-1700					
+ Acetate	3.0	15.4	9.2	16.3	1.7:1
- Acetate	2.4	3.3	1.9	27.6	1.7:1
UOFS Y-1701					
+ Acetate	2.7	17.8	4.6	7.1	3.9:1
- Acetate	2.0	0.4	0.3	6.4	1.3:1
UOFS Y-0829					
+ Acetate	7.5	0.1	0.0	16.2	-
- Acetate	2.3	0.1	0.1	14.2	1.:1
UOFS Y-1698					
+ Acetate	3.1	15.5	4.6	11.1	3.4:1
- Acetate	2.6	0.7	0.3	15.0	2.3:1
UOFS Y-0164					
+ Acetate	3.7	8.2	2.6	18.3	3.2:1
- Acetate	2.6	0.1	0.1	20.3	1:1
UOFS Y-0097					
+ Acetate	3.7	7.3	3.1	15.1	2.4:1
- Acetate	2.5	0.4	0.2	25.8	2:1
UOFS Y-1065					
+ Acetate	2.9	14.6	13.6	11.6	1.1:1
- Acetate	2.4	2.8	1.7	13.0	1.6:1
UOFS Y-1138					
+ Acetate	6.7	0.8	0.2	15.3	4:1
- Acetate	2.7	0.4	0.1	8.2	4:1

Organism	pH	Citric acid (g/l)	Isocitric acid (g/l)	Biomass (g/l)	Ratio CA:ICA
UOFS Y-0809					
+ Acetate	2.8	20.0	12.8	12.3	1.6:1
- Acetate	2.2	10.5	6.8	14.4	1.5:1
UOFS Y-1570					
+ Acetate	5.4	0.7	49.0	2.9	0.01:1
- Acetate	2.5	0.5	10.3	14.5	0.1:1
UOFS Y-1568					
+ Acetate	3.6	8.5	2.4	17.1	3.5:1
- Acetate	2.5	2.2	1.7	13.7	1.3:1
UOFS Y-1699					
+ Acetate	3.2	10.5	10.0	9.6	1.1:1
- Acetate	2.6	1.0	0.8	14.1	1.3:1
UOFS Y-2110					
+ Acetate	6.6	0.1	0.2	3.2	0.5:1
- Acetate	5.5	0.1	0.1	10.2	1:1
UOFS Y-1569					
+ Acetate	3.2	12.0	9.0	7.0	1.3:1
- Acetate	2.5	0.4	0.5	14.9	0.8:1
CBS 6124T					
+ Acetate	2.9	12.8	19.0	11.8	0.7:1
- Acetate	2.4	2.5	3.2	14.5	0.8:1
UOFS Y-1571					
+ Acetate	3.3	10.0	8.0	13.0	1.3:1
- Acetate	2.5	0.3	0.2	15.5	1.5:1
UOFS Y-2160					
+ Acetate	7.2	0.0	0.1	15.6	-
- Acetate	2.3	2.3	4.0	10.8	0.6:1

This data was reproducible and in all cases the standard error was less than 10%. CA = citric acid; ICA = isocitric acid.

Table 2. Lipid turnover when *Yarrowia lipolytica* was grown on sunflower fat in the absence of acetate for 240 h.

Time (h)	Type of lipid	FA composition				
		16:0	**16:1**	**18:0**	**18:1**	**18:2**
	ECL					
0		7.5	0.1	0.01	27.5	59.7
72		6.5	0.1	4.7	24.7	57.5
96		6.6	0.1	4.8	23.8	57.3
120		6.7	0.1	4.5	23.5	57.9
144		6.6	0.1	4.5	23.9	57.8
168		6.8	0.1	0.0	27.7	57.8
240		6.8	0.1	4.0	23.6	58.0
	ICL					
0		12.2	0.1	2.1	32.3	30.4
72		6.5	0.8	3.1	23.4	59.8
96		6.3	0.7	3.2	23.6	57.9
120		6.4	0.6	3.8	23.2	57.4
144		6.1	0.6	3.6	23.8	57.7
168		6.7	0.5	3.8	23.1	56.7
240		6.8	0.3	0.0	27.0	58.6
	Sunflower fat	**6.6**	**0.1**	**0.01**	**29.0**	**59.0**

FA = fatty acid; ECL = extracellular lipids; ICL = intracellular lipids. Similar patterns were found when experiment was repeated in at least triplicate. 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid.

Table 3. Lipid turnover when *Yarrowia lipolytica* was grown on sunflower fat in the presence of acetate for 240 h.

Time (h)	Type of lipid	FA composition				
		16:0	**16:1**	**18:0**	**18:1**	**18:2**
	ECL					
0		7.5	0.1	0.01	27.4	59.5
72		5.6	0.1	9.4	33.0	46.7
96		6.7	0.2	22.1	33.1	32.4
120		5.2	0.04	45.3	23.0	20.3
144		7.5	0.1	11.8	22.9	48.8
168		5.3	0.1	17.7	25.8	33.9
240		7.3	0.0	18.6	23.3	33.1
	ICL					
0		12.2	0.1	2.1	32.3	30.4
72		5.1	2.7	4.3	23.5	59.8
96		4.7	2.8	7.4	21.4	53.6
120		4.2	3.4	6.9	22.6	50.9
144		3.7	2.6	15.4	24.6	48.7
168		4.0	2.2	20.5	25.9	40.2
240		4.4	2.2	22.9	23.4	38.7
	Sunflower fat	**6.6**	**0.1**	**0.01**	**29.0**	**59.0**

FA = fatty acid; ECL = extracellular lipids; ICL = intracellular lipids. Similar patterns were found when this experiment was repeated in at least triplicate. 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid.

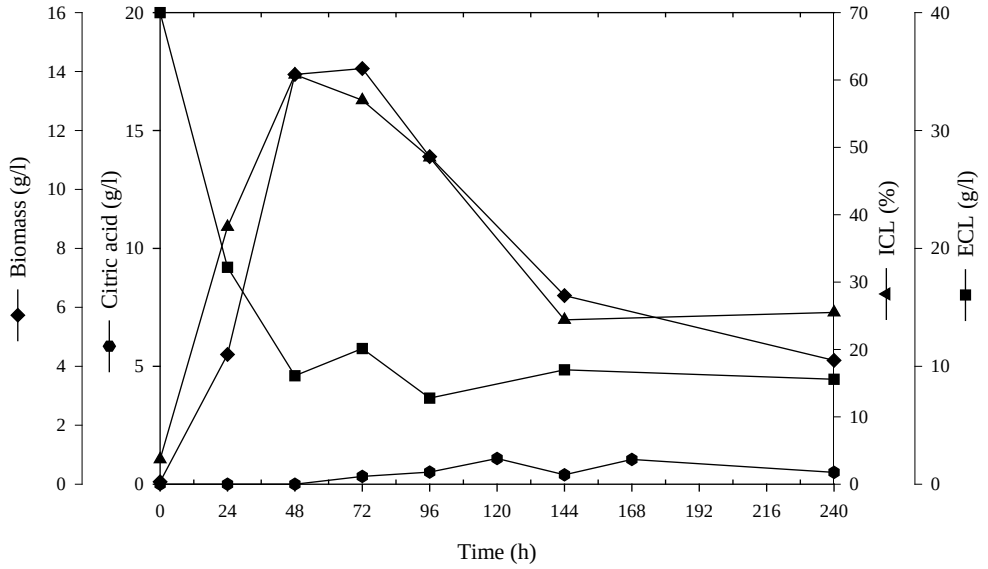

Figure 1A. Biomass-, citric acid production, intracellular lipid content and extracellular lipids of cells grown on substrate with sunflower fat as sole carbon source. ICL = Intracellular lipids. ECL = Extracellular lipids. Similar patterns were observed when this experiment was repeated in triplicate.

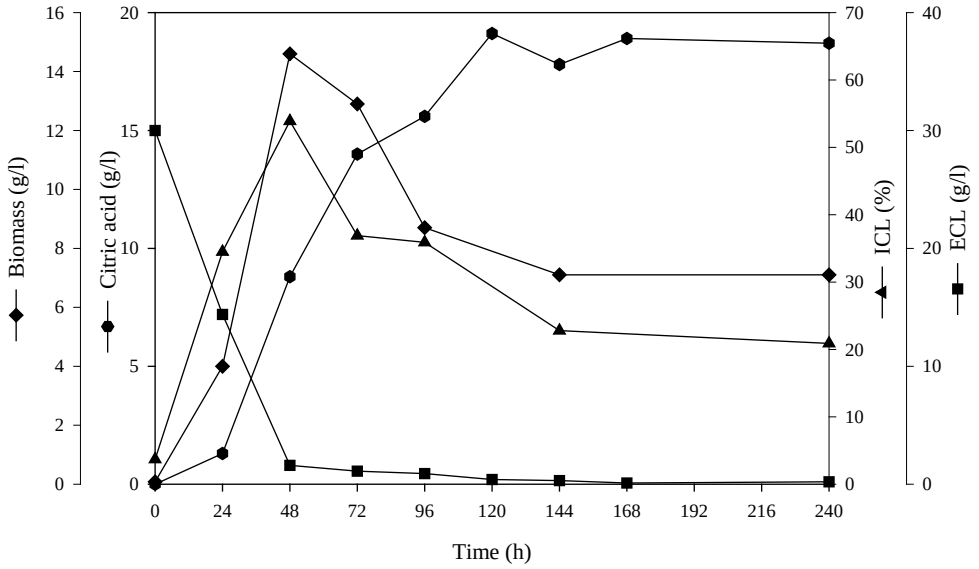

Figure 1B. Biomass-, citric acid production, extracellular lipids in medium and intracellular lipid content of cells grown on medium containing acetate and sunflower fat. ICL = Intracellular lipids. ECL = Extracellular lipids. Similar patterns were observed when this experiment was repeated in triplicate.

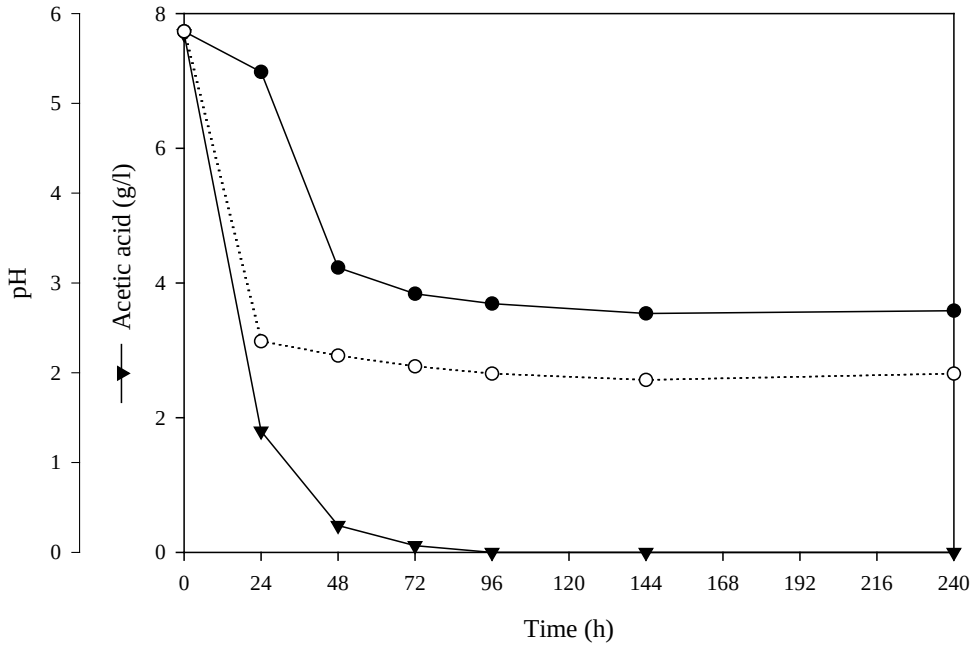

Figure 2. Change in acetic acid concentration (τ) and pH of the mixed substrate (•——•) and only sunflower fat (o·····o). Similar patterns were observed when this experiment was repeated in triplicate.

Chapter 3

Citric acid production by Yarrowia lipolytica

when cultivated on edible fat waste

3.1 Abstract

Simulated sunflower fat waste (30 g/l and 40 g/l) containing 11% (m/m) polymerized triglycerides (PTGs) was utilized only to a limited extend, even in the presence of 10 g/l acetate by the yeast *Yarrowia lipolytica*. Furthermore, only small amounts of citric acid was produced i.e. 0.3 g/l maximum in the absence of acetate and 1.0 g/l maximum in the presence of acetate. This may be ascribed to the presence of toxic breakdown products such as PTGs. Strikingly, this yeast was capable of utilizing most of the PTGs after 144 h in the presence of acetate.

3.2 Introduction

When fats are exposed to heat e.g. during frying, various changes occur. These changes include amongst others the destruction of antioxidants, hydrolysis of the triglycerides, increased oxidation of the fatty acids and the formation of polymers (Fritsch, 1981). Many of these compounds may influence human health adversely (STOA Report, 2000; Kock *et al*., 2002).

In South Africa approx. 100 000 tons of fat are discarded annually by frying establishments of which about 50 000 tons are still safe for human consumption i.e. containing less than the S.A. safety regulatory limit of 16% polymerized

triglycerides (PTGs). Consequently, these wastes are available to be used as high-energy substrates for the production of biotechnological products such as citric acid.

Previously, we have reported on the enhanced utilization of fresh and unused sunflower fat in the presence of acetate and the subsequent production of increased amounts of citric acid (Venter *et al*., 2003). Since fat wastes in South Africa can be considered a low cost substrate, it became the aim of this study to investigate the transformation of these fat wastes, containing 1% and 11% PTGs respectively, to citric acid in the presence and absence of acetate.

3.3 Materials & Methods

Strain used. Yarrowia lipolytica UOFS Y-1701 (proven high citric acid producer – see Chapter 2) used in this study was obtained from the culture collection of the University of the Free State in Bloemfontein, South Africa.

Cultivation and harvesting of cells. Yarrowia lipolytica UOFS Y-1701 was first cultivated in 50 ml complex medium (1% glucose m/m and 3 g/l yeast malt extract) present in 250 ml conical flasks for 24 h while shaking at 160 rpm. This was used to inoculate (to 10 Klett units) into 32x250 ml conical flasks, which were then grown at 26ºC (shaken at 160 rpm) for seven days. Each flask contained 50 ml sterile

growth medium and the initial pH was adjusted to pH 5.8 with 1M HCl. The medium consisted of the following (in g/l): simulated sunflower fat waste (PTG conc. at 11% m/m after autoclaving), 30; sodium acetate, 10; yeast extract, 0.1; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 10; $CaCl_2.2H_2O$, 0.05; NH_4Cl, 1.28. Tap water was used or trace elements were added to the following final concentration (g/l): $FeSO_4.7H_2O$, 0.035; $MnSO_4.4H_2O$, 0.007; $ZnSO_4.7H_2O$, 0.011; $CuSO_4.5H_2O$, 0.001; $CoCl_2.6H_2O$, 0.002; $Na_2MoO_4.2H_2O$, 0.0013; H_3BO_3, 0.002; KI, 0.00035; $Al_2(SO_4)_3$, 0.0005. As a control experiment, the same medium as above was used with the exception that sodium acetate (10 g/l) was omitted and 40 g/l simulated sunflower fat waste was included as sole carbon source. At every time interval, the cells were harvested by centrifugation for 15 min at 8000 g i.e. after all residual extracellular oil in the culture was extracted with *n*-hexane (see Lipid extraction section). Cultures were harvested from four pooled flasks each after 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h respectively. After harvesting, cells were immediately frozen, freeze-dried and then weighed. In addition, the pH was determined for each flask at regular time intervals (Fig. 2) over the growth cycle. All experiments were performed at least in duplicate.

Lipid extraction. This was performed according to the methods described by Kock *et al*. (1997). In short, extracellular lipids (ECL) present in the corresponding supernatant (pH < 3) from each flask mentioned above were immediately extracted after harvesting with n-hexane until almost no extracellular lipids could be detected.

Intracellular lipids (ICL) were extracted from the freeze-dried cells using chloroform/methanol (2:1, v/v) as described by Folch *et al*. (1957), followed by two washes with distilled water and final evaporation of the organic phase under vacuum. The lipids were dissolved in diethyl ether and transferred to preweighed vials. Before lipids were weighed, they were dried to constant weight in a vacuum oven over P_2O_5 at 55°C.

Citric- and isocitric acid analysis. Citric- and isocitric acid content in the supernatant were determined by high-performance liquid chromatography (HPLC) (Shimadzu SPD-10A VP with UV detector). The medium (1 ml; pH < 3) was filtered through a 0.45 μm filter (LCR non-sterile, Millex) prior to injection. Citric- and isocitric acid were well separated using a Synergi 4μ Hydro-RP 80A (Phenomenex) column and these components were detected at 220 nm (wavelength). The mobile phase consisted of 1% acetonitrile 190/UV UL to 20 mM KH_2PO_4 set to pH 2.5 and was pumped at a flow rate of 0.8 ml/min. Chromatographic data were quantitated using a Shimadzu C-R6A Chromatopac integrator. These organic acids were identified and quantified with reference to authentic standards.

Acetic acid analysis. Residual acetic acid present in the supernatants of all the flasks harvested were determined by gas chromatography (GC) as described by Du Preez (1980).

Fatty acid analysis. The fatty acid composition was determined after transesterification by the addition of trimethylsulphonium hydroxide (TMSOH) as described by Butte (1983). The fatty acid methyl esters were analyzed by GC (Hewlett Packard Model 5830A GC equipped with a dual flame-ionization detector) and a Supelcowax 10 column (30 m x 0.75 mm). Nitrogen was used as carrier gas at 5 ml/min. The initial column temperature (145°C) was increased by 3°C/min to 225°C and, following a 10 min isothermal period, was then increased to 240°C at the same rate. The inlet and detector temperatures were 170°C and 250°C respectively. Peaks were identified by reference to authentic standards.

Preparation of fat waste. Unused sunflower fat (PTG = 1.1% m/m) was heated at a temperature of 200ºC with continuous stirring and aeration to simulate the frying process using a Millipore vacuum pump XF54 230 50 until the PTG level of 5% m/m was obtained. After autoclaving, the PTG level rose to 11% m/m.

Polymer analysis. In short, both intracellular and extracellular lipids as well as prepared fat waste were dissolved in tetra hydro furan (THF) and polymers, which included products formed by carbon to carbon and/or carbon to oxygen linkages between tri-, mono and diglyceride-bound fatty acids, as well as free fatty acids, to

produce dimeric or higher polymeric compounds, were determined by gel permeation chromatography as described by Beljaars *et al.* (1994).

Chemicals. All organic chemicals and solvents used were of analytical reagent grade and obtained from major retailers. All standards were obtained from Sigma.

3.4 Results & Discussion

When *Y. lipolytica* strain UOFS Y-1701 was grown on simulated sunflower fat waste containing 11% (m/m) PTGs as only carbon source (Fig. 1A), it reached maximum growth after only 72 h i.e. much later compared to when cultivated on fresh unused sunflower fat i.e. after 48 h. Again the biomass decreased – this time after 120 h (from 3.0 g/l to 2.0 g/l after 168 h) of growth compared to 72 h on unused sunflower fat (i.e. from 14.1 g/l to 4.2 g/l after 240 h of growth - Chapter 2; Fig. 1A, p. 46). Here the intracellular lipids (ICL) increased from 3.7% (0 h) to 32.6% m/m biomass after 120 h when it reached a maximum after which it decreased together with the biomass to 15.8% m/m biomass after 168 h. We conclude that this drop in ICL may have contributed to the decrease in biomass experienced after 120 h of growth probably through the utilization of this stored energy source after utilization of the acetate and the lower levels of edible fat waste left in the medium (Figs 1A and 2). The extracellular lipid (ECL) concentration also

decreased at a much slower rate to reach a minimum of 11.9 g/l only after 144 h compared to 48 h when grown on fresh, unused sunflower fat and reaching a value of 9.3 g/l (Chapter 2; Fig. 1A, p. 46). During growth the pH dropped (Fig. 2) sharply but at a much slower pace compared to when grown on unused fat (Chapter 2; Fig. 2, p. 48) to reach a minimum of pH 2.0 after 72 h after which it remained more or less the same. When grown on fresh fat, the pH dropped sharply within 24 h from pH 5.8 to pH 2.4. This difference may be ascribed to the slower growth on fat waste and probably slower production of organic acids responsible for the drop in pH. Extremely low citric acid concentrations were recorded over the growth cycle i.e. ranging from 0 g/l to 0.3 g/l which is lower compared to pervious experiments (Chapter 2; Fig. 1A, p. 46) where cells were grown on fresh unused sunflower fat as sole carbon source and produced up to 0.5 g/l citric acid after 120 h of growth.

This poorer performance may be ascribed to the presence of breakdown products such as PTGs, which may have affected cell growth and citric acid production adversely. In the presence of acetate 82% of PTGs was utilized, while in its absence, only 37% of PTGs was utilized over 168 h.

When cultivated in a mixed medium containing both simulated sunflower fat waste and acetate, a similar pattern (compared to Chapter 2 results; Fig. 1B, p. 47) regarding biomass production, extracellular lipid (simulated sunflower fat waste)

utilization and lipid turnover was experienced, only this time at a much slower pace and level (Fig. 1B). The maximum biomass production of 1.9 g/l was only reached after 144 h of growth compared to 14.6 g/l within 48 h when grown on unused sunflower fat (Chapter 2; Fig. 1B, p. 47). The acetate as well as waste sunflower fat (ECL) was also utilized at a much slower pace compared to when it was grown on unused sunflower fat (ECL) in the presence of acetate (Chapter 2; Fig. 1B, p. 47). After 168 h, 24.8 g/l of the waste sunflower fat (ECL) and almost all the acetic acid were utilized (Figs 1B and 2). When unused sunflower fat in the presence of acetate were utilized, most of the sunflower fat (ECL) (28.4 g/l) and acetic acid were utilized already within 48 h (Chapter 2; Figs 1B and 2, p. 47-48). No remarkable increase in citric acid production was observed during growth in the presence of used fat and acetate (Fig. 1B). The production of citric acid increased very slowly during growth and reach a value of 0.8 g/l after 168 h with a citric acid : isocitric acid ratio of 0.7:1 (Fig. 1B). Only this time a gradual decrease in pH was observed (from pH 5.8 to pH 4.1) over 168 h of growth (Fig. 2) and not a sharp decrease as experienced when the yeast was cultivated in unused sunflower fat in the presence of acetate (from pH 5.8 to pH 2.7 within 144 h – Chapter 2; Fig. 2, p. 48). A possible reason for the slower drop in pH in the presence of acetate can be ascribed to the poor production of citric acid.

It was found that the simulated sunflower fat waste contained much higher proportions of 16:0 and 18:0 and lower proportions of 18:1 and 18:2 compared to

untreated sunflower fat (Table 1). This is probably due to the polymerization of the latter two FAs during preparation to form polymers and consequently results in increased proportions of 16:0 and 18:0 (Frankel, 1998). The ECL fractions studied from experiments performed in the presence and absence of acetate was characterized by the presence of 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid) and 18:2 (linoleic acid). The relative amounts of these FAs over the growth cycle in the absence of acetate showed no pattern with possibly the exception of 18:1 that gradually increased implying in general a lack of preference to particular FAs during growth (Table 1). In the presence of acetate an increase in saturated FAs i.e. 16:0 (from 20.5% to 29.8%) and 18:0 (from 3.2% to 7.8%) as well as 18:1 (from 16.5% to 21.5%) occurred with a general decrease (from 49.4% to 39.9%) in polyunsaturated FAs i.e. 18:2 after 168 h of growth (Table 2). This difference in patterns cannot be explained at present.

In the presence and absence of acetate the ICL fractions were as expected characterized by the presence of 16:0, 16:1, 18:0, 18:1 and 18:2. In the presence and absence of acetate the FA profiles at time 0 h were significantly different from that of the simulated sunflower fat waste due to the pre-preparation of these cells as inoculum in a complex medium devoid of simulated sunflower fat waste. In the absence of acetate the simulated sunflower fat waste FA profile was partially restored already after 48 h of growth (Table 1). This phenomenon was also previously reported (Kendrick, 1991).

In the presence of acetate, the FA profile of the simulated sunflower fat waste was more or less restored after about 96 h of growth (Table 2) although the 18:1 and 18:2 content was somewhat higher. Interestingly, the 16:0 and 18:0 showed a drastic increase after 24 h of growth (reaching 36.7% and 12.7% respectively mainly at the expense of 18:1 and 18:2 – i.e. 19.5% and 7.4% respectively). This pattern cannot explain at present.

In this study it was discovered that the addition of 10 g/l acetate to a medium containing 30 g/l simulated sunflower fat waste caused an enhanced utilization of PTGs (82%). This cannot be explained at present and is further research.

Unfortunately simulated sunflower fat waste containing 11% (m/m) PTGs was utilized only to a limited extend, even in the presence of acetate. Furthermore, only small amounts of citric acid was produced i.e. 0.3 g/l maximum (in the absence of acetate) and 1.0 g/l maximum (presence of acetate). This may probably be ascribed to the presence of toxic breakdown products such as PTGs (STOA Report, 2000). These results indicate that low cost used frying fat waste is probably not a good substrate for citric acid or biomass production.

3.5 Acknowledgements

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Table 1. Lipid turnover when *Yarrowia lipolytica* was grown on simulated waste fat (PTG = 11% m/m) in the absence of acetate for 168 h.

Time (h)	Type of lipid	FA composition				
		16:0	**16:1**	**18:0**	**18:1**	**18:2**
	ECL					
0		20.4	0.4	3.0	16.5	49.4
24		20.2	0.4	3.7	16.5	47.6
48		20.3	0.4	3.4	16.9	44.6
72		18.7	0.4	3.1	17.7	47.5
96		19.7	0.4	4.1	16.9	43.8
120		21.2	0.4	4.2	21.4	46.1
144		20.1	0.4	4.2	19.7	50.6
168		17.5	0.4	3.4	19.8	52.9
	ICL					
0		12.2	0.1	2.1	32.2	30.4
24		17.7	1.1	4.8	12.8	50.3
48		18.9	0.8	2.6	17.6	51.4
72		19.4	0.6	4.4	19.6	52.7
96		15.6	0.6	4.3	19.9	56.1
120		16.8	0.5	2.9	21.4	53.4
144		22.6	0.7	3.8	17.4	48.7
168		15.8	0.6	3.5	21.5	52.7
	Simulated sunflower fat waste	**20.5**	**0.4**	**3.2**	**16.5**	**49.4**
	Sunflower fat	**6.6**	**0.1**	**0.01**	**29.0**	**59.0**

FA = fatty acid; ECL = extracellular lipids; ICL = intracellular lipids; 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid. Similar patterns were found when this experiment was repeated in at least triplicate.

Table 2. Lipid turnover when *Yarrowia lipolytica* was grown on simulated waste fat (PTG = 11% m/m) in the presence of acetate for 168 h.

Time (h)	Type of lipid	FA composition				
		16:0	**16:1**	**18:0**	**18:1**	**18:2**
	ECL					
0		20.5	0.4	3.2	16.5	49.4
24		21.3	0.5	4.2	16.7	43.7
48		22.8	0.4	3.8	17.8	43.6
72		24.5	0.5	3.9	18.8	-
96		20.6	0.4	5.0	18.9	45.3
120		26.8	-	7.1	21.0	45.2
144		26.7	-	5.5	19.2	-
168		29.8	-	7.8	21.5	39.9
	ICL					
0		12.2	0.1	2.1	32.2	30.4
24		36.7	0.0	12.7	19.5	7.4
72		30.8	1.1	6.4	20.4	24.8
96		19.0	0.3	3.5	20.0	53.2
120		18.0	1.5	3.2	21.7	52.4
144		15.9	1.5	3.3	18.9	53.2
168		21.1	1.3	3.8	18.3	46.6
	Simulated sunflower fat waste	**20.5**	**0.4**	**3.2**	**16.5**	**49.4**
	Sunflower fat	**6.6.**	**0.1**	**0.01**	**29.0**	**59.0**

FA = fatty acid; ECL = extracellular lipids; ICL = intracellular lipid; 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid. Similar patterns were found when this experiment was repeated in at least triplicate.

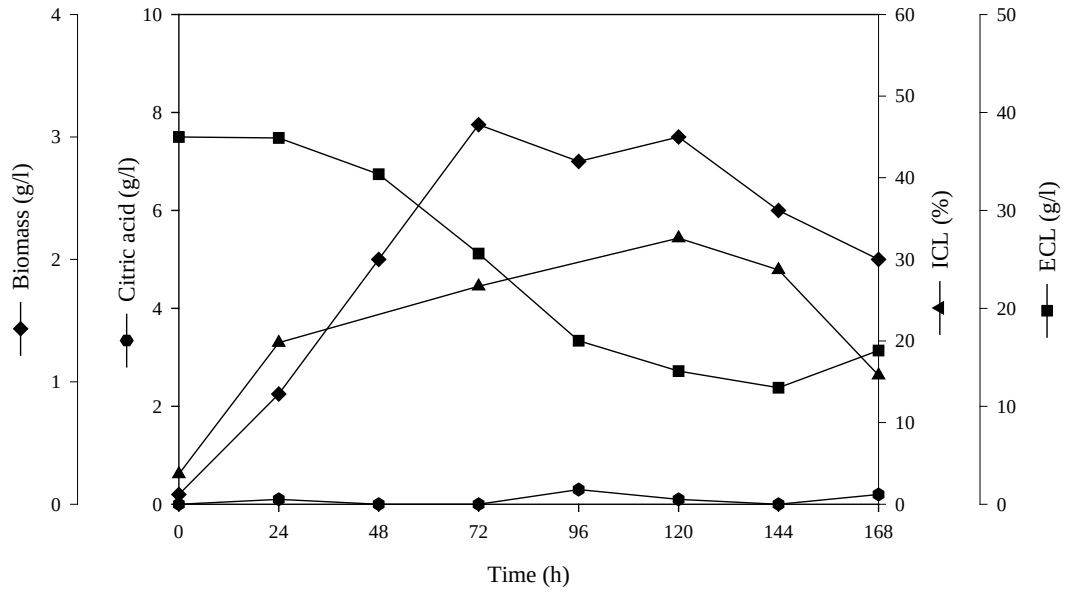

Figure 1A. Biomass production, extracellular lipid utilization, percentage intracellular lipids and concentration citric acid of cells grown on simulated sunflower fat waste as sole carbon source. ICL = Intracellular lipids. ECL = Extracellular lipids. Similar patterns were observed when these experiments were repeated in triplicate.

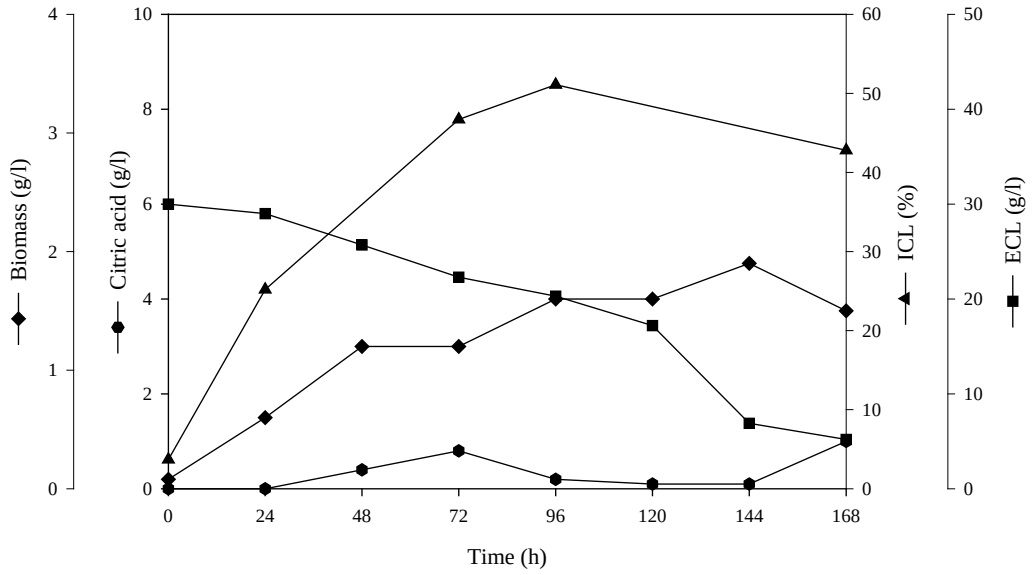

Figure 1B. Biomass production, extracellular lipid utilization, percentage intracellular lipids and concentration citric acid of cells grown on the mixed substrate containing simulated sunflower fat waste and acetate. ICL = Intracellular lipids. ECL = Extracellular lipids. Similar patterns were observed when these experiments were repeated in triplicate.

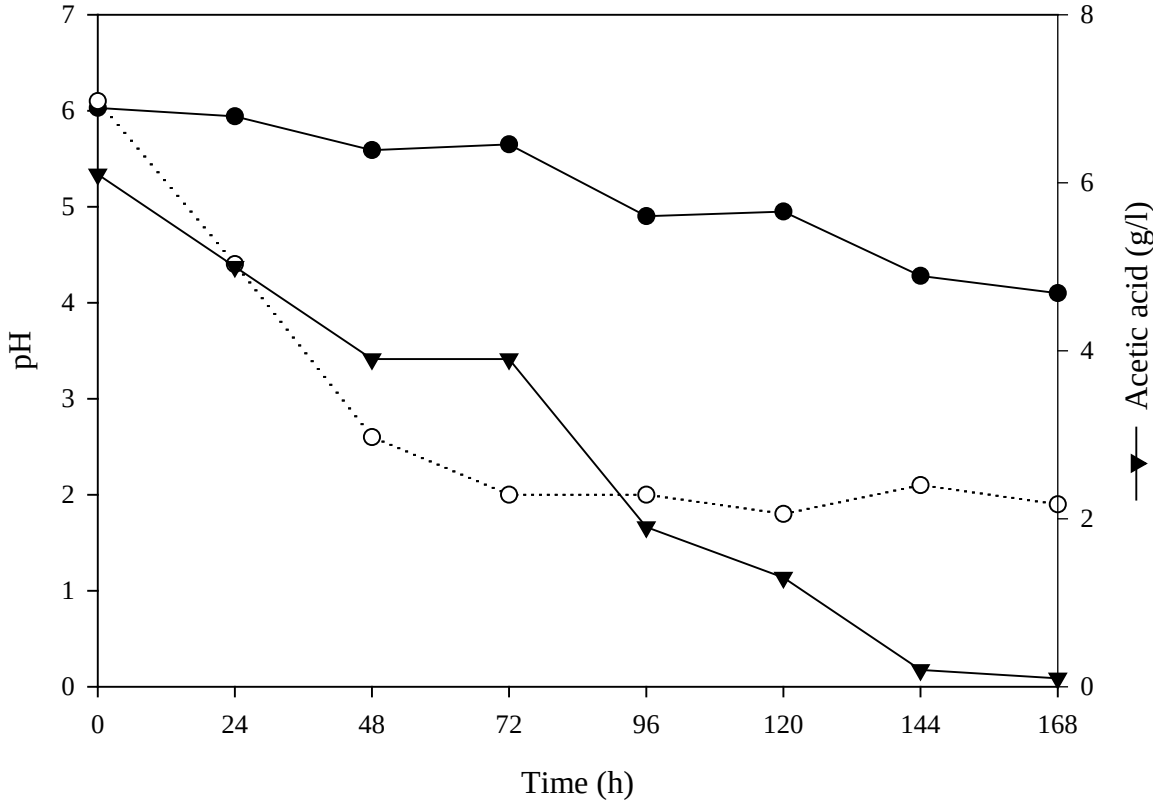

Figure 2. Changes in acetic acid concentration (τ) and pH of the mixed substrate (●——●) and only simulated sunflower fat waste (o·····o). Similar patterns were observed when this experiment was repeated in triplicate.

Chapter 4

Overall conclusions

4.1 Introduction

Experiments performed in Chapters 2 and 3 were designed according to a 2^2 factorial design thereby calculating the effects of the addition of acetate to unused sunflower fat as well as simulated sunflower fat waste. In addition, this experimental design also shows the effect of increased levels of polymerized triglycerides (PTGs) to these fat substrates.

4.2 Statistical analysis (Box and Draper, 1969)

The experimental layout (including results from Chapters 2 and 3) was based on a 2^2 factorial design as shown in Table 1 to yield four separate experiments for each of the responses analyzed (i.e. citric acid, biomass, extracellular lipids, intracellular lipids, pH). The average values over all four experimental combinations for each of the five responses were calculated and are shown in Table 2. The effects of each variable i.e. acetate and polymerized triglycerides (PTGs) are shown in Table 3.

4.3 Overall conclusions

When comparing the results in Chapter 2 and Chapter 3 on the basis of a 2^2 factorial design, the following interesting conclusions can be drawn (Table 3):

1) The addition of 10 g/l acetate showed a large average increase in citric acid production i.e. +4.7 units over the conditions used. This effect was cancelled (-4.7 units) when the PTG levels increased by 10% (m/m). This is probably contributed to the toxicity of these polymers (STOA Report, 2000).
2) The addition of 10 g/l acetate lead to an increase in biomass production (+4.1 units) while an increase in PTGs decreased its production by –2.3 units over the conditions used.
3) When 10 g/l acetate was added, a large increase in extracellular lipids (i.e. sunflower fat and waste) utilization occurred (+12.0 units) while the opposite was (ECL decreased by –7.6 units) true when the PTG level increased by 10% (m/m). This is probably due to the toxic effect of polymers as previously reported in animal experiments (STOA Report, 2000; Pelesane *et al.*, 2001).
4) Interestingly, the intracellular lipids increased in cells when both acetate (+6.0 units) and PTG levels (+2.9 units) were increased. This cannot be explained at present.
5) Furthermore, the pH increased when both acetate (+0.7 units) and PTG levels (+0.3 units) were raised. This phenomenon is further researched at present.

These results indicated that low cost used frying fat waste is probably not a good substrate for citric acid production.

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Table 1. A 2^2 factorial design showing four experiments for each of the responses studied i.e. citric acid, biomass, % extracellular lipids (ECL) utilization, % intracellular lipids (ICL) and pH.

Chapter *	Experimental number	Acetic acid (0 g/l and 10 g/l)	PTG (1% and 11%)
2	1	0	1
2	2	10	1
3	3	0	11
3	4	10	11

*Experimental results from both Chapters (2 and 3) were used for calculations.

Table 2. Lipid turnover as well as citric acid and biomass production by *Yarrowia lipolytica* after 168 h when grown on sunflower fat and simulated sunflower fat waste in the presence and absence of acetate.

Responses measured	Sunflower fat (1% PTG)		Waste fat (11% PTG)	
	- Ac	+ Ac	- Ac	+ Ac
Citric acid (g/l)	1.1	18.9	0.2	1.0
Biomass (g/l)	5.7	7.1	2.0	1.5
ECL (% utilization)	73.5	99.7	60.8	82.7
ICL (%)	25.0	21.9	15.8	42.8
pH	2.0	2.7	1.9	4.1

ECL = extracellular lipids; ICL = intracellular lipids; PTG = polymerized triglycerides; +Ac = presence of acetate; -Ac = absence of acetate.

Table 3. The effect of acetate and polymerized triglycerides on various responses when *Yarrowia lipolytica* was grown for 168 h on sunflower fat (containing 1% PTGs) and simulated sunflower fat waste (containing 11% PTGs) in the presence (+Ac) and absence (-Ac) of acetate (Box and Draper, 1969).

Response	***Effects**	
	Acetate (0 g/l and 10 g/l)	**PTGs (1% and 11%)**
Citric acid (g/l)	+ 4.7	- 4.7
Biomass (g/l)	+ 4.1	- 2.3
ECL utilization (%)	+ 12.0	- 7.6
ICL (%)	+ 6.0	+ 2.9
pH	+ 0.7	+ 0.3

ECL = extracellular lipids; ICL = intracellular lipids; PTGs = polymerized triglycerides.
*Effects of increases in acetate and PTG levels on different responses have been calculated according to the method of Box and Draper (1969) when treated as a 2^2 Factorial Design.

Summary

Large amounts of edible fat waste (approx. 100 000 tons p.a.) are generated in South Africa when edible fats, mainly sunflower fat, are used in frying processes. When these fats are overexposed to heat a part of this fat waste becomes toxic to humans. Another part of these fat waste (approx. 50%) are however still fit for human consumption and has been discarded by frying establishments while within regulatory limits. Consequently these wastes have the potential to be processed to safe usable foodstuffs such as citric acid which is mainly imported to South Africa. Since it was shown that *Yarrowia lipolytica* can convert monounsaturated fats (such as canola fat) to citric acid, the aim of this dissertation became to explore the possibility of this yeast to convert the low cost waste product i.e. used edible fat waste (still fit for human consumption) to a more valuable product such as citric acid in the presence of acetate. In order to achieve this, eighteen strains of *Yarrowia lipolytica* were grown for 6 days on a medium consisting of 30 g/l sunflower fat in the presence and absence of 10 g/l acetate. It was discovered that the addition of acetate caused a drastic increase in citric acid production by many strains of *Yarrowia lipolytica.* Strikingly *Yarrowia lipolytica* UOFS Y-1701 produced increased amounts of citric acid in the presence of acetate i.e. 0.5 g/l in the absence of acetate to 18.7 g/l in the presence of acetate. Similarly, the ratio of citric acid : isocitric acid increased significantly from 1.7:1 in the absence of acetate to 3.7:1 in the presence of acetate after 240 h of growth. During the growth period the acetate as well as the 30 g/l fat was almost completely utilized (100% and 99.3% respectively). This same experiment was repeated, but this time with 30 g/l

simulated sunflower fat waste (PTGs = 11%) together with 10 g/l acetate, and as control, 40 g/l simulated sunflower fat waste without acetate. Very low citric acid concentrations were obtained when *Yarrowia lipolytica* was cultivated in simulated sunflower fat waste in the presence (maximum of 1.0 g/l) and the absence (maximum of 0.3 g/l) of acetate. When the effects of increased levels of acetate and PTGs were calculated on the basis of a 2^2 factorial design and by interpreting responses in both Chapters 2 and 3, the following were found: (1) The addition of 10 g/l acetate showed a large increase of +4.7 units in citric acid production. This effect was cancelled (citric acid production dropped by –4.7 units) when the PTG levels increased by 10% (m/m). (2) The addition of 10 g/l acetate lead to an increase (+4.1 units) in biomass production while the increase in PTGs decreased its production by –2.3 units. (3) When 10 g/l acetate was added, a large increase in extracellular lipids (i.e. sunflower fat and waste) utilization occurred (+12.0 units) while the opposite was true (extracellular lipids decreased by –7.6 units) when the PTG level increased by 10% (m/m). (4) Interestingly, the intracellular lipids increased in cells when both acetate and PTG levels were increased (i.e. increased by +6.0 and +2.9 units respectively). (5) Furthermore, the pH increased when both acetate (by +0.7 units) and PTG levels (by +0.3 units) were raised. These results indicate that low cost used frying fat waste is probably not a good substrate for citric acid production.

Opsomming

In Suid-Afrika word daar jaarliks ‘n groot hoeveelheid eetbare afval vet gegenereer vanaf verskeie braai-prosesse (omtrent 100 000 ton p.j.). Wanneer hierdie vet (meestal sonneblom vet) uitermatig blootgestel word aan hitte, word ‘n gedeelte hiervan giftig vir menslike gebruik. Die oorblywende gedeelte (omtrent 50%) van hierdie afval vet is steeds aanvaarbaar vir menslike gebruik en word soms onnodig deur braai-instellings weggegooi, al voldoen dit steeds aan wetlike vereistes. Díe afval vet het die potensiaal om na veilige, bruikbare voedsel-produkte omgeskakel te word, bv. sitroensuur (wat meestal ingevoer word na Suid-Afrika). Dis reeds bewys dat *Yarrowia lipolytica* mono-onversadigde vette (soos kanola vet) na sitroensuur kan omskakel. Juis om hierdie rede het die doel van hierdie dissertasie die volgende geword: om die moontlikheid van *Yarrowia lipolytica* te bestudeer om vanuit ‘n lae-koste afval produk soos eetbare afval vet (steeds aanvaarbaar vir menslike gebruik), ‘n waardevolle produk soos sitroensuur, te vervaardig. Om dit te bereik, het ons begin deur agtien stamme van *Yarrowia lipolytica* vir ses dae te groei op ‘n medium bestaande uit 30 g/l sonneblom olie in die teenwoordigheid en afwesigheid van 10 g/l asetaat. Gevolglik het die toediening van asetaat, ‘n drastiese toename in sitroensuur produksie getoon by die meeste van die *Yarrowia lipolytica* stamme. Veral *Yarrowia lipolytica* UOFS Y-1701 het uitsonderlik hoë konsentrasies sitroensuur opgelwer in die teenwoordigheid van asetaat. Soortgelyke toenames in die verhouding van sitroensuur : isositroensuur is opgemerk na 24 h van groei: van 1.7:1 in die afwesigheid van asetaat, tot 3.7:1 in die teenwoordigheid van asetaat. Die asetaat, asook die 30 g/l vet is byna heeltemal benut tydens die 240 h groei

periode (100% en 99.3% respektiewelik). Dieselfde eksperiment is herhaal, maar díe keer met 30 g/l gesimuleerde sonneblom afval vet (gepolimeriseerde trigliseriede (PTGs) = 11%) saam met 10 g/l asetaat en, as ‘n kontrole, slegs 40 g/l gesimuleerde sonneblom afval vet sonder asetaat se toediening. Baie lae sitroensuur konsentrasies was verkry toe *Yarrowia lipolytica* op gesimuleerde sonneblom afval vet gegroei is: ‘n maksimum van 1.0 g/l in die teenwoordigheid van asetaat en 0.3 g/l in die afwesigheid van asetaat. Toe die effekte van die verhoogde asetaat en PTG vlakke bereken is volgens ‘n 2^2 faktoriale ontwerp en die response van beide hoofstukke 2 en 3 geïnterpeteer was, is die volgende gevind: (1) Die toediening van 10 g/l asetaat het gelei tot ‘n geweldige toename van +4.7 eenhede in sitroensuurproduksie. Hiedie effek is wel gekanselleer (sitroensuur produksie daal met –4.7 eenhede) toe PTG vlakke met 10% (m/m) verhoog is. (2) Die toevoeging van 10 g/l asetaat het gelei tot ‘n toename (+4.1 eenhede) in biomassa produksie, terwyl die toename in PTGs gelei het tot ‘n afname in díe produksie (-2.3 eenhede). (3) Wanneer 10 g/l asetaat bygevoeg word, is daar ‘n groot toename in die verbruik van die ekstrasellulêre lipiede (sonneblom vet en afval) van +12 eenhede, terwyl die teenoorgestelde opgelet is (ekstrasellulêre lipiede neem af met –7.6 eenhede) toe die PTG vlak verhoog is met 10% (m/m). (4) Dit is interessant om te sien dat die intrasellulêre lipiede toegeneem het in die selle wanneer beide asetaat en PTG vlakke verhoog is (bv. verhoog met +6.0 en +2.9 eenhede respektiewelik). (5) Verder het die pH ook verhoog toe beide asetaat (met +0.7 eenhede) en PTG vlakke (met +0.3 eenhede) toegeneem het. Hierdie resultate is ‘n aanduiding dat lae koste

gebruikte braai-vet afval nie ‘n goeie substraat is vir die produksie van sitroensuur nie.