# Identification and characterisation of *Yarrowia lipolytica* RP2 growing on tallow

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Doctor of Philosophy

by

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D., is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____

I.D. No.: 96970901

Date: 5-01-04

*'No one will ever know or understand the fun there was; for there was fun and there was laughing- foolish, silly fun and foolish, silly laughing; but what it was all about you can't remember, can you?*

*Just the memory of it- that's all you have now- just the memory; and even now, even so soon, it is being distilled of all its coarseness; and what's left is going to be precious gold...'*

[Philadelphia Here I Come, Brian Friel]

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**ABSTRACT**

The ability of 10 yeasts, isolated from the waste treatment system at a commercial rendering operation, to utilise tallow (20 g $L^{-1}$) as a sole carbon source was investigated. One isolate, identified as *Yarrowia lipolytica*, demonstrated superior fat removal ability and consequently was chosen for further studies in the development of a microbial-based fat removal system. Initially, the influence of temperature, medium pH, agitation and nitrogen source was assessed in shake flask studies. Maximum tallow removal of 75% in 168 hours was achieved under the optimal conditions of 25°C, pH 7.0, 130 rpm and 0.4 g N $L^{-1}$, irrespective of whether ammonium sulphate, urea or peptone was employed as the nitrogen source. Medium pH was controlled using 0.1 M potassium phosphate buffer. Addition of glucose (1.0 and 10.0 g $L^{-1}$) to the medium under optimal environmental conditions did not inhibit tallow removal and there was a concomitant use of both substrates. Acclimation of the yeast inoculum to lipids did not influence fat removal. The fat removal system was scaled-up to 2 L and 10 L fermentation. Under optimum aeration of 1 VVM and agitation of 500 rpm and 1000 rpm, the time required to achieve maximum tallow removal was significantly reduced from 168 to 65 hours in the 2 L and 10 L fermenters, respectively. Potassium was key to optimal fat removal with the requirement of greater than 60 mM $K^+$ in the medium by the yeast. Cellular potassium levels of 80 nmol $K^+$ $(10^6$ cell$)^{-1}$ corresponded with maximum growth and fat removal. Extracellular biosurfactant production was detected under optimal growth conditions, which corresponded with emulsification of the tallow in the growth vessel. *Y. lipolytica* is a dimorphic yeast however, mycelial growth was not considered to play an important role in fat removal by the yeast.

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# 1.0 INTRODUCTION

## 1.1 Yeast

Yeasts have been described as unicellular fungi, which reproduce vegetatively by budding or fission and that form sexual states which are not enclosed in a fruiting body (Boekhout and Kurtzman, 1996). The distinction between yeasts and filamentous fungi has often been uncertain and there has been considerable controversy on their origin. Some have viewed the yeasts as primitive fungi, while others perceived them to be reduced forms of more evolved taxa (Kurtzman and Sugiyama, 2001). Although not as ubiquitous as bacteria, yeasts are widespread in the natural environment, including soil, water, plants and animals and in extreme habitats such as the halotolerant and osmsophilic yeasts (Walker, 1998). Yeast cells lack chlorophyll and are strict chemoorganothrophs and as such require fixed, organic forms of carbon for growth. Sources of carbon are quite diverse and include simple sugars, polyols, organic and fatty acids, aliphatic alcohols, hydrocarbons and various polymeric compounds. The dynamic and diverse activities of yeasts impinge on many areas of science, technology and medicine. Over 700 species of yeast exist, with the continued identification of new strains (Kurtzman and Sugiyama, 2001). Some species play beneficial roles in the production of foods, beverages and pharmaceuticals, while others play a detrimental role as spoilage organisms and agents of human disease.

### 1.1.1 Yeast classification

Yeasts are classified into subdivisions based on their methods of reproduction. Ascomycotina and Basidiomycotina can reproduce sexually, whereas Deuteromycotina cannot (Walker, 1998). The classification of yeast is based on a hierarchical system, as detailed in Table 1.1. The morphological, physiological and genetic characteristics of the yeast facilitate the determination of the lower taxonomic categories. It can be expected that application of molecular identification methods will allow for the detection of numerous new species, leading to a better understanding of yeast biodiversity (Kurtzman and Sugiyama, 2001).

**Table 1.1:** Classification of the yeasts.

| Subdivision | Families/ Subfamilies | Genera[a] | | |
|---|---|---|---|---|
| ***Ascomycotina*** | | | | |
| Hemiascomycetes | | | | |
| Endomycetales | Spermophthoraceae | *Coccidascus (1)* | *Metschnikowia (10)* | *Nematospora (1)* |
| | Saccharomycetaceae | | | |
| | -Schizosaccharomycetoideae | *Schizosaccharomyces (3)* | | |
| | -Nadsonionideae | *Hanseniaspora (6)* | *Saccharomycodes (2)* | |
| | | *Nadsonia (3)* | *Wickerhamia (1)* | |
| | -Lipomycetoideae | *Lipomyces (5)* | | |
| | -Saccharomycetoideae | *Ambrosiozyma (2)* | *Hansenula (1)* | *Sporopachydermia (3)* |
| | | *Arthroascus (4)* | *Issatchenkia (4)* | *Stephanoascus (2)* |
| | | *Arxiozyma (1)* | *Kluveromyces (17)* | *Torulaspora (3)* |
| | | *Citeromyces (1)* | *Lodderomyces (1)* | *Wickerhamiella (1)* |
| | | *Clavispora (2)* | *Pachysolen (1)* | *Williopsis (5)* |
| | | *Cyniclomyces (1)* | *Pichia (87)* | *Yarrowia (1)* |
| | | *Debaromyces (10)* | *Saccharomyces (16)* | *Zygoascus (1)* |
| | | *Dekkera (2)* | *Saccharomycopsis (6)* | *Zygosaccharomyces (9)* |
| | | *Guilliermondella (1)* | *Schwanniomyces (1)* | *Zygozyma (4)* |

[a] the number in brackets refers to the number of species in the genera. Adapted from Kreger-van Rij (1984) and Boekhout and Kurtzman (1996)

**Table 1.1 [contd.]:** Classification of the yeasts.

| Subdivision | Families/ Subfamilies | Genera[a] | | |
|---|---|---|---|---|
| ***Basidiomycotina*** | | | | |
| Ustilaginales | Filobasidiaceae | *Bulleromyces (1)* | *Filobasidiella (1)* | *Udeniomyces (3)* |
| | | *Chinosphaera (1)* | *Filobasidium (5)* | *Xanthophyllomyces (1)* |
| | | *Cystofilobasidium (4)* | *Tilletiaria (1)* | |
| | Teliospore-forming yeasts | *Leucosporidium (3)* | *Rhodosporidium (9)* | *Kondoa (1)* |
| | | *Erythrobasidium (1)* | *Sporidiobolus (3)* | *Mrakia (4)* |
| | Unclassified | *Sterigmatosporidium (1)* | | |
| Tremellales | Sirobasidiaceae / Tremellaceae | *Sirobasidium and Tremella (12)* | | |
| ***Deuteromycotina*** | | | | |
| Blastomycetes | Cryptococcaceae | *Aciculoconidium (1)* | *Kloeckera (1)* | *Saitoella (1)* |
| | | *Arxula (2)* | *Kurtzmanomyces (2)* | *Schizoblastosporion (2)* |
| | | *Brettanomyces (3)* | *Myxozyma (9)* | *Sterigmatomyces (2)* |
| | | *Candida (152)* | *Malassezia (7)* | *Sympodiomyces (1)* |
| | | *Cryptococcus (40)* | *Oosporidium (1)* | *Tilletiopsis (6)* |
| | | *Itersonilia (1)* | *Phaffia (1)* | *Trichosporon (20)* |
| | | *Fellomyces (4)* | *Rhodotorula (37)* | *Trigonopsis (1)* |
| | Sporobolmycetaceae | *Bensingtonia (10)* | *Bullera (14)* | *Sporobolomyces (27)* |

[a] the number in brackets refers to the number of species in the genera. Adapted from Kreger-van Rij (1984) and Boekhout and Kurtzman (1996)

Kreger-van Rij (1987) described the principal distinguishing morphological characteristics of the three main groups of yeasts.

The ascosporogenous yeasts are classified in the Hemiascomycetes, which are Ascomycotina that lack ascocarps (fruiting bodies) and ascogenous hyphae. In the class Hemiascomycetes, the yeast are classified into two families, the Saccharomycetaceae and the Spermophthoraceae of the order Endomycetales. The shape of the ascospores distinguishes the two families. In the Spermophthoraceae the spores are needle-shaped and in the Saccharomycetaceae the spores have a variety of different shapes. The four subfamilies of the Saccharomycetaceae differ based on reproduction and ascospore shape. In the Schizosaccharomycetoideae, vegetative reproduction is exclusively by fission. In the Nadsonionideae, budding is bipolar. In the Lipomycetoideae, there is a typical process of ascus formation, with 2 - 30 amber-coloured spores per ascus. In the Saccharomycetoideae, budding is multilateral.

The Basidiomycotina are classified into two main groups, the Ustilaginales and the Tremellales. These yeasts are the haploid phase in the life cycle of mostly heterothallic basidiomycetes, which form basidiospores. The Tremellaceae may conjugate and produce a dikaryotic mycelium on which a basidiocarp, basidia and basidiospores develop. The Ustilaginales are divided into three families. The Filobasidiaceae are characterised by the formation of slender, non-septate basidia with terminal basidiospores. The Teliospore-forming yeasts form thick-walled cells, called teliospores, on the hyphae. The last group consists of *Sterigmatosporidium polymorphum*, which on dikaryotic mycelium forms two types of chlamydospores, round and oval cylindrical ones.

The Deuteromycotina or the Imperfect yeasts include yeasts that do not form ascospores or basidiospores. Several imperfect yeasts closely resemble perfect species and only differ in the ability to form sexual spores. Generic differentiation of the imperfect yeasts is based on morphological characteristics and on a few physiological characteristics. The Deuteromyctoina are divided into two families, the Sporobolomycetaceae, which form ballistospores and the Cryptococcaceae, which do not.

### 1.1.2 Yeast identification

Yeast identification and characterisation is of the utmost importance in yeast biotechnology. The ability to differentiate between wild and cultured yeasts is essential in industrial processes. This is exemplified in brewing fermentations where the presence of wild yeasts may impart undesirable off-flavours to the product and also in baker's yeast propagations where wild yeasts like *Candida utilis* may out-grow pure baking strains of *Saccharomyces cerevisiae* due to more efficient sugar transport capabilities of the wild yeast (Walker, 1998). Identification of yeast genera can be achieved by both morphological and physiological tests. Keys are required for the identification of yeasts. With the increase in the number of yeast species, the keys have become more complex. A key may follow the taxonomic classification and first lead to genera, and then to the species within the genus or may follow physiological responses of the yeast to lead to identification of both genus and species.

The chief characteristics of yeast include the macroscopic and microscopic appearance of the organism (Barnett *et al.*, 1983). Examination of the macroscopic appearance of yeast can be useful in its identification. If the yeast forms ballistoconidia or ballistospores, the mirror image can be seen in the lid of the Petri dish, which is indicative of the *Sporobolomycetaceae*. Coloured colonies are formed by yeast of certain genera. The presence of red, yellow and orange carotenoid pigments are characteristic of the genera *Rhodotorula*, *Sporobolomyces*, *Phaffia*, *Rhodosporidium* and *Sporodiobolus*, while the production of non-cartenoid pigments is typical of only certain yeasts including *Metschnikowia pulcherrima* (van der Walt and Yarrow, 1984). The majority of yeasts, however, produce growth ranging from white to cream. Slimy colonies are a chief characteristic of genera *Cryptococcus*, *Filobasidiella* and *Filobasidium* (Barnett *et al.*, 1983). Examination of the microscopic appearance of the yeast includes its mode of reproduction and cell morphology. For the ascosporogenous yeasts, this generally means that characteristics of the ascopores are of the utmost importance in their identification. In the imperfect yeasts, the type of vegetative reproduction is often decisive for generic differentiation.

The physiological responses of yeast to a variety of tests, including the assimilation of carbons and nitrate, fermentation of sugars, response to urease, Diazonium Blue B colour test and growth with antibiotics are important tools in the identification of the genus and species of yeast.

It is practicable to distinguish between many yeast species by their differing abilities to assimilate certain organic compounds as the sole carbon source under aerobic conditions. These compounds include sugars, alditols and organic acids (Barnett *et al.*, 1983). In the standard description of the species the result of 18 – 30 compounds is recorded. However, compounds with a low differentiating value and those that are often weakly and slowly assimilated may give inconsistent results. In their standard description, van der Walt and Yarrow (1984) omitted these compounds and their assimilation method comprised just 18 test compounds. The ability of a yeast to use nitrate as the sole source of nitrogen is a valuable aid in identifying yeasts since about one quarter of all species utilise nitrate and this is usually a uniform feature of all the strains in the species (Barnett *et al.*, 1983).

Yeasts vary in their ability to ferment sugars. For example, the genera *Kluveromyces* and *Saccharomyces* are characterised by the vigorous fermentation of at least glucose, other genera such as *Lipomyces* are strictly non-fermentative, while other genera have an entire range from non-fermentative to strongly fermentative species as in the case of *Hansenula* (van der Walt and Yarrow, 1984). A large variety of species can grow well in glucose concentrations of up to 40% by weight, while only a few species are capable of development with sugar concentrations between 50 – 70% (w/v) (van der Walt and Yarrow, 1984). Similarly, only a number of yeasts are capable of growth in high salt concentrations.

Two tests used in the standard description are of importance for the distinction of basidiomycetous yeasts, the Diazonium Blue B (DBB) colour test and the urease test (Kreger-van Rij, 1987). The basidiomycetes produce an intense red colour in the presence of DBB dye, although the mechanism of this method has not been fully elucidated (van der Walt and Yarrow, 1984). So far, only one exception to

the test has been found, the ascomycete *Sporopachydermia quercuum*. Urea can be utilised as a sole source of nitrogen by practically all yeasts, when present in low concentrations. However, in higher concentrations, as in the urease test, the activity is lacking in the ascogenous species and a strong reaction is normally associated with the basidiomycetes, although there are some exceptions.

Whiffen (1948) was the first to report that yeasts varied in their sensitivity towards the antibiotic actidione (cycloheximide). Cycloheximide stops the growth of eukaryotes by inhibiting protein synthesis in the 80 S ribosomes. Yeasts may be divided into three categories on the basis of their sensitivity to the antibiotic: species that are markedly sensitive (inhibited by 1 $\mu g\ ml^{-1}$) such as *Saccharomyces cerevisiae*, those that are moderately sensitive (inhibited by 25 $\mu g\ ml^{-1}$) such as *Schizosaccharomyces pombe* and those that are tolerant to levels of 1000 $\mu g\ ml^{-1}$ such as *Kluveromyces lactis*. Because of the possibility that strains may become adapted to low concentrations of cycloheximide, yeasts are assessed for tolerance in 100 and 1000 ppm concentrations (van der Walt and Yarrow, 1984).

In addition to morphological and physiological characteristics, yeast species classification and strain identification can be performed through immunological and molecular characteristics. Immunological methods include serology, immuno-electrophoresis and immunoflourescence microscopy. Molecular methods include rRNA and rDNA phylogeny, DNA base composition (mol % G + C), karyotype analysis, DNA hybridization and random amplification of polymorphic DNA (RAPD) (Walker, 1998).

### 1.1.3 Medical, industrial and environmental importance

Yeasts are of major economic, social and health significance. Yeast exploitation has been applied to many fermentation industries, including food, chemical and health care and yeast play an important role in environmental technologies and in fundamental biological and biomedical research (Fig. 1.1).

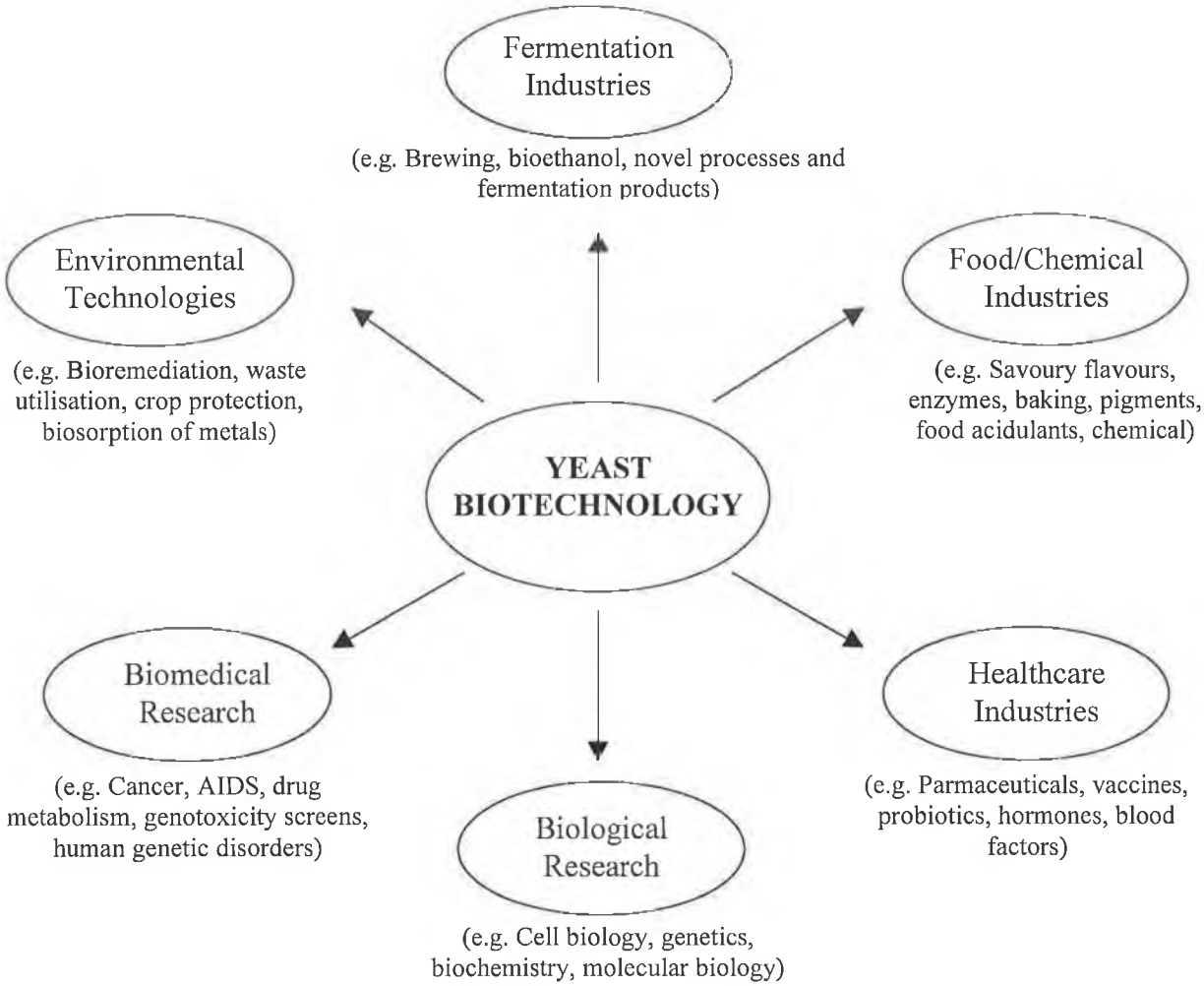



**Figure 1.1**: Diversity of outlets involving yeast biotechnology (Walker, 1998).

Yeasts are important from a medical perspective. The principal yeasts pathogenic for humans are *Candida albicans* and *Filobasidiella (Cryptococcus) neoformans*. *C. albicans* is endogenous in the oral, gastrointestinal and uriogenital tracts of humans and other warm-blooded animals (Jones, 1990). Over 90% of deep yeast infections are attributed to *C. albicans*.

Yeasts are involved in four of the worlds leading industrial fermentation processes, including beer (60 million tons/year), wine (30 million tons/year), single cell protein and fodder (800,000 tons/year) and bakers yeast (600,000 tones/year) (Kurtzman and Sugiyama, 2001). Another important yeast fermentation is the production of riboflavin (vitamin $B_{12}$) by *Eremothecium*

*gossypii*, *E. ashbyii* and more recently, by *Candida famata* (Heefner *et al.*, 1992). Fuel alcohol, ethanol production for motor fuel, has become increasingly important and production is primarily by *Saccharomyces cerevisiae* with hydrolysed corn starch as the substrate (Ma and Hanna, 1999). Yeasts are increasingly exploited for expression of recombinant DNA products. Examples of heterologous genes cloned in *Saccharomyces cerevisiae* and secreted by recombinant yeast include human interferon (Hitzeman *et al.*, 1983) and human epidermal growth factor (Brake *et al.*, 1984).

The spoilage of foods and beverages from growth of contaminating yeasts results in major economic losses worldwide (Fleet, 1990). However, yeasts responsible for food spoilage are not known to cause food poisoning. Products with a high sugar content are commonly spoiled by species of *Zygosaccharomyces*. Certain cheeses and meat products, such as salami may be spoiled by *Debaromyces hansenii* and *Yarrowia lipolytica*. Species of *Brettanomyces*, *Dekkera* and *Pichia* are often responsible for the turbidity and off-flavours in wines, beer and soft drinks (Kurtzman and Sugiyama, 2001).

Yeasts are employed in various environmental technologies, including bioremediation and biosensors. Bioremediation technology capitalises on certain growth characteristics of the yeast, such as assimilation of recalcitrant carbons, biosorption of toxic metals and secretion of metabolites, such as enzymes and biosurfactants. *Yarrowia lipolytica* and *Candida maltosa* have been employed for bioremediation of waste hydrocarbons and crude oils, *Schwanniomyces occidentalis* metabolises starches completely and *Pichia methanolica* can metabolise methanol (Spencer *et al.*, 2002). Spent brewer's yeast can effectively remove heavy metals (Ag, U, Co, Cu, Cd) from industrial effluents (Simmons *et al.*, 1995). *Trichosporon cutaneum* may be useful in biosensor monitoring of biochemical oxygen demand (B.O.D.) of wastewaters, carbohydrate analysis, amperometric determination of ammonium ions and phenol determination (Reiser *et al.*, 1994).

Overall, yeasts are central to many present-day agricultural and industrial processes and with the development of recombinant DNA technologies, as well as new needs by society, the role of yeast in human advancement can be expected to increase (Kurtzman and Sugiyama, 2001). Molecular systematics will have a key role in future uses. Application of molecular methods for strain identification will assist medical diagnostics as well as define industrially and agriculturally important species, including those important in bioremediation.

## 1.2 *Yarrowia lipolytica*

*Yarrowia lipolytica* is one of the most extensively studied non-conventional yeasts as it is quite different from other intensively studied conventional yeasts like *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. The ecological niche for *Y. lipolytica* encompasses lipid-rich food like margarine, olive oil and cheese and in meat or shrimp products and it is also found in sewage and oil plants (Barth and Gaillardin, 1997; Casaregola *et al.*, 2000). The inability of the yeast to survive under anaerobic conditions permits its elimination from dairy products. Other carbon sources utilised by *Y. lipolytica* include glucose, alcohols, acetate and hydrophobic substrates such as fatty acids and alkanes, but not sucrose (Barth and Gaillardin, 1996 & 1997). *Y. lipolytica* is non-pathogenic to humans and has been approved for several GRAS (generally regarded as safe) industrial processes (Casaregola *et al.*, 2000). The unusual biochemical properties of *Y. lipolytica*, its ability to produce several biotechnologically important metabolites, intensive protein secretion, its dimorphism and amenability to molecular techniques have combined to make this a very well studied organism (Barth and Gaillardin, 1996).

### 1.2.1 Nomenclature

*Y. lipolytica* is the only species recognised within the *Yarrowia* genus. It has, however, had an interesting history and has undergone a number of changes of genus in the past 30 years with the advent of improved genetic and classification technology. The species was originally classified as a *Candida*, since no sexual state had been described. The perfect form of the yeast was identified on the

observation of asci formation. Two mating types, called A and B were identified and nearly all other wild type isolates from the species would mate to one of these two types, albeit at low frequency, suggesting that most natural isolates are haploid. The perfect form was reclassified as *Endomycopsis lipolytica* (Barth and Gaillardin, 1997). However, *Endomycopsis* was shown to be an obligate synonym of *Saccharomycopsis* genus and consequently, the yeast was renamed as *Saccharomycopsis lipolytica* (van der Walt and Van Arx, 1980). However, the yeast differed from other species in the genera in its Co-enzyme Q system. *S. lipolytica* also differed from other genera in the structure of its ascospores, which are variable in both size and shape. Similarly, the positive urease reaction and a G+C content of 49.5 - 50.2% reported as high for ascomyceteous yeast provided no clue as to its possible filiation. Consequently, it takes an isolated position in the genus *Yarrowia* (van der Walt and Van Arx, 1980). As a result of this frequent name changing, *Candida* (perfect form), *Endomycopsis* and *Saccharomycopsis lipolytica* are the same organism, *Yarrowia lipolytica.*

### 1.2.2 Dimorphism

*Y. lipolytica* is dimorphic. Yeast dimorphism is the ability of fungal cells to grow in two distinct morphological forms, yeasts and highly elongated, filament-forming mycelia, which are in the form of pseudomycelia or septate or true mycelia (Barth and Gaillardin, 1997; Szabo and Štofaníková, 2002). True mycelium consists of septate hyphae 3 to 5 μm in width and up to several mm in length. Apical cells often exceed 100 μm, whereas segments are 50 to 70 μm long (Walker, 1998). Dimorphism attracts much attention due to a proposed link to virulence of several important fungal pathogens including *Candida albicans* (Klotz, 1989; Madhani and Fink, 1998). *Y. lipolytica* appears to be a useful model for the study of dimorphism in fungi, since in contrast to other dimorphic species, it has a sexual cycle (Wickerham *et al.*, 1970) and can be used as a subject for genetic manipulation and transformation (Gaillardin and Heslot, 1988). However, the proportion of the different cell forms is dependent on the strain of the yeast.

Certain conditions for the preferential formation of either the yeast or mycelium form in *Y. lipolytica* have been reported. Some of these conditions are detailed in Table 1.2. The contradictory influence of glucose on mycelial formation (Rodríguez and Domínguez, 1984; Novotný *et al.*, 1994; Szabo, 1999) underlines the relationship between the strain of the yeast and dimorphism. Olive oil and oleic acid, the fatty acid present in the oil, were reported to induce mycelial growth (Ota *et al.*, 1984). However, not all fatty acids were morphogens and when *Y. lipolytica* was cultivated on stearic and palmitic acid, only yeast-shaped cells were present (Ota *et al.*, 1984).

Physiological changes occurring during yeast-mycelia transition have been studied by many investigators including, Vega and Dominguez (1986), Rodríguez *et al.* (1990) and Guevara-Olvera *et al.* (1993). In the comparison of the composition of yeast and mycelial cells, mycelial cells exhibited a higher content of amino sugars and a reduced content of protein (Vega and Dominguez, 1986). Furthermore, ornithine decarboxylase activity and polyamine cell pools increased in hyphal cells grown on N-acetylglucosamine-containing medium (Guevara-Olvera *et al.*, 1993). A phosphatidylinositol/ phosphatidylcholine transfer protein is required for differentiation from the yeast to mycelial form (Lopez *et al.*, 1994). Although the exact mechanism of the yeast to mycelial transition is not known, a number of genes, including *SEC14*, *GRP1* and the deletion of *XPR5* and *ClA4* genes have strong effects on the transition (Barth and Gaillardin, 1997; Szabo, 2001).

**Table 1.2:** Effect of nutrition and environmental growth conditions on the morphology of *Yarrowia lipolytica*.

| Nutrient/ growth condition | | Cell morphology | Reference |
|---|---|---|---|
| Lipids/ hydro-carbons | olive oil | 77% mycelia | Ota *et al.* (1984) |
| | oleic acid | 79% mycelia | Ota *et al.* (1984) |
| | palmitic acid | no mycelia | Ota *et al.* (1984) |
| | stearic acid | no mycelia | Ota *et al.* (1984) |
| | hexadecane | no mycelia | Rodríguez and Domínguez (1984) |
| Metal ions | 200 μM magnesium | 70% mycelia | Ota *et al.* (1984) |
| | 50 μM iron | 72% mycelia | Ota *et al.* (1984) |
| | calcium | no effect | Ota *et al.* (1984) |
| Sugars | glucose | 68% mycelia | Szabo (1999) |
| | | 40% mycelia | Novotný *et al.* (1994) |
| | | no mycelia | Rodríguez and Domínguez (1984) |
| | maltose | no mycelia | Rodríguez and Domínguez (1984) |
| | fructose | 61% mycelia | Novotný *et al.* (1994) |
| Other carbons | N-acetylglucosamine | mainly mycelia | Kim *et al.* (2000a) |
| | | 72% mycelia | Novotný *et al.* (1994) |
| | | mainly mycelia | Rodríguez and Domínguez (1984) |
| | sodium acetate | mycelia and yeast | Rodríguez and Domínguez (1984) |
| Proteins | calf serum | mainly mycelia | Kim *et al.* (2000a) |
| | bovine milk caesin | 50-70% mycelia | Ota *et al.* (1984) |
| | yeast extract | 5-30% mycelia | Ota *et al.* (1984) |
| | peptone | 5-30% myeclia | Ota *et al.* (1984) |
| | malt extract | no mycelia | Ota *et al.* (1984) |
| Nitrogen | ammonium sulphate[a] | mycelial-shaped (%) | Szabo (1999) |
| | 0 mg $L^{-1}$ | 1.0 ± 0.28 | |
| | 50 mg $L^{-1}$ | 2.8 ± 1.60 | |
| | 250 mg $L^{-1}$ | 4.1 ± 2.30 | |
| | 5000 mg $L^{-1}$ | 6.6 ± 2.10 | |
| pH | no control | yeast shape only | Rodríguez and Domínguez (1984) |
| | pH control from 5.0 to 7.0 | increase in mycelia | Szabo (1999) |

[a]added to a yeast nitrogen broth devoid of any other nitrogen source

### 1.2.3 Biotechnological importance

*Y. lipolytica* is an excellent model organism and is the focus of many molecular studies including determinants of the mating type, alkane and fatty acid metabolism, structure and function of retransposons, glyoxylic pathway and peroxisome biogenesis (Barth and Gaillardin, 1997). Furthermore, fundamental studies are now directed on the structure and functioning of the genome, which is in some aspects very different from that occurring in other yeast, such as the widely studied *Saccharomyces cerevisiae*.

*Y. lipolytica* is not only of interest for fundamental research, but also for biotechnological applications. *Y. lipolytica* is able to utilise several unusual carbon sources including paraffins, various alcohols and acetate. Indeed, interest in this yeast began because of its ability to metabolise paraffin hydrocarbons with consequent production of single cell protein (SCP) (Tsugawa *et al.*, 1969). However, development of SCP industries waned in the 1970's and large-scale production of SCP on crude oils ceased due to the accumulation of a toxic component in the cells, which could not be removed (Ratledge, 1988). However, there has been a recent resurgence in the investigation of potential of SCP production from waste streams (Rhishipal and Philip, 1998; Choi and Park, 1999; Nigam and Kakati, 2002; Paul *et al.*, 2002).

One of the characteristic features of *Y. lipolytica* is its ability to secrete large amounts of various metabolites and enzymes. The secretion of citrate and isocitrate, when cultivated on cheap hydrocarbon wastes was commercialised and proved more successful than SCP production (Barth and Gaillardin, 1996). Indeed, citric acid production is one of the main commercial applications of the yeast. There is a continued interest in this metabolite with an investigation into the potential of glycerol as a substrate for citrate production (Papanikolaou *et al.*, 2002b). *Y. lipolytica* has also been exploited for the production of another commercially important metabolite, lysine (Barth and Gaillardin, 1997). Lipase, which is secreted when grown on lipid substrates, may be of interest for synthesis of 2,4-dimethylglutaric acid monoesters, transesterification of meso-cyclopentane diols (Thiele *et al.*, 1991) or in the leather industry or cheese manufacturing

(Barth and Gaillardin, 1997). Cultivation on a rich medium at pH 6.8 resulted in the secretion of large amounts (1 - 2 g $L^{-1}$) of an alkaline extracellular protease (AEP) and RNase (Barth and Gaillardin, 1996).

*Y. lipolytica* is a potentially useful host for heterologous protein production through a combination of a strong inducible promoter and its ability to secrete large amounts of protein (Casaregola *et al.*, 2000; Kim *et al.*, 2000b). However, despite its potential for commercially valuable protein production, very little research on the development of efficient culture techniques has been performed (Chang *et al.*, 1997 & 1998). Overall, the potential of this important yeast for biotechnological, environmental and commercial applications, with the exception of citric acid production, has not yet been fully investigated and developed.

### 1.3 Lipids

Lipids are biomolecules composed of fatty acids, or closely related structures bound to alcohols (Gunstone, 1996). When the alcohol involved is glycerol, the most common alcohol component in lipids, glycerides, phospholipids and glycosylglycerides are formed. Other alcohols found less commonly in lipids include sterols in sterol esters and long chain alcohols in wax esters. Lipids form an essential part of living cells. Phospholipids, sterol esters, glycosylglycerides and sphingolipids are structural lipids and form cellular and organelle membranes. Lipids also act as energy and storage molecules. Lipids are the most essential nutrient of human and animal diets. They provide the most concentrated energy (9 kcal $g^{-1}$) of any foodstuff, supply essential fatty acids (which are precursors for hormones and prostanoids), serve as carriers for fat soluble vitamins and make food more palatable (Shukla, 1994). The major reserve lipids in living organisms are the glycerides, mainly triglycerides, with mono- and diglycerides present as minor components (Gunstone, 1996). Glyceride lipids are comprised of fats, oils and greases, which is dependent on their source (Table 1.3). Lipids that are solid at room temperature are referred to as fats, those that are liquid are oils and those that are soft, but not liquid comprise the greases (Metcalf and Eddy, 1991).

**Table 1.3:** Source of major oils and fats.

| Category | | Examples |
|---|---|---|
| *Plant sources* | vegetable oils | bran oil, corn oil, cottonseed oil, rapeseed oil, rice oil, soybean oil, sunflower oil |
| | tree crop oils | coconut oil, palm oil, olive oil |
| | industrial oils | castor oil, linseed oil |
| *Animal sources* | land animals | tallow, lard, milk fat |
| | marine animals | fish oil |

Adapted from Gunstone (1996).

The constituent fatty acids determine the chemical and physical characteristics of the lipid. Animal fats consist of mainly saturated fatty acids, or with limited amounts of mono- or polyunsaturated fatty acids. Plant fatty acids may be more complex and contain a variety of other functional groups such as acetylenic bonds, epoxyl, hydroxyl and keto groups. Saturated glycerides pack very well together, resulting in stronger molecular forces, which in turn results in the high melting point of fats. Oils differ from fats in that they contain mainly unsaturated fatty acids. The double bonds in unsaturated fatty acids are usually in the *cis* rather than the *trans* form, which leads to larger molecules that pack poorly together. This leads to weaker forces between molecules and lower melting points (Shukla, 1994). An illustration of the saturated stearic acid and unsaturated oleic acid is presented in Fig. 1.2. The fatty acid composition of the major oils and fats is detailed in Table 1.4.

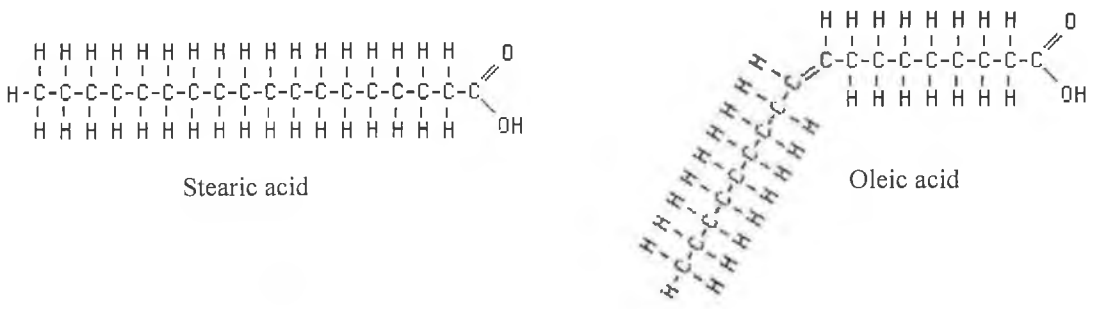



**Figure 1.2:** Structure of stearic acid and oleic acid. Stearic acid is a saturated fatty acid and has a line configuration. Oleic acid is a monounsaturated fatty acid with a *cis* configuration in its double bond.

**Table 1.4:** Fatty acid composition of major oil and fat sources (%).

| Oil or fat | lauric 12:0 | myristic 14:0 | palmitic 16:0 | stearic 18:0 | arachidic 20:0 | palmitoleic 16:1 | oleic 18:1 | gadoleic 20:1 | linoleic 18:2 |
|---|---|---|---|---|---|---|---|---|---|
| *Saturated* | | | | | | | | | |
| Coconut oil | 47 | 18 | 9 | 3 | | | 6 | | 2 |
| Palm oil | | 1 | 45 | 4 | | | 40 | | 10 |
| Milk fat | 3 | 11 | 27 | 12 | | 2 | 29 | | 2 |
| Cocoa butter | | | 26 | 35 | 1 | | 35 | | 3 |
| Beef tallow | | 3 | 24 | 19 | | 4 | 43 | | 3 |
| Mutton tallow | | 6 | 27 | 32 | | 2 | 31 | 1 | 2 |
| Lard | | 2 | 26 | 14 | | 3 | 44 | 1 | 10 |
| *Mono-unsaturated* | | | | | | | | | |
| Olive oil | | | 13 | 3 | 1 | 1 | 71 | | 10 |
| Rapeseed oil | | | 4 | 2 | | | 62 | | 22 |
| *Polyunsaturated* | | | | | | | | | |
| Sunflower oil | | | 7 | 2 | | | 19 | | 68 |
| Soybean oil | | | 11 | 4 | | | 24 | | 54 |
| Corn oil | | | 11 | 2 | | | 28 | | 58 |

Adapted from Shukla (1994).

Fats are usually of animal origin and terrestrial animals are the source of the hard fats such as tallows and lard. These are produced from the processing and rendering of animal carcasses. The hardest fats, as measured by iodine value, are beef and sheep tallows (Grummer, 1992). Natural fats consist mainly of triglycerides, with lower levels of diglycerides, monoglycerides, free fatty acids, sterols, sterol esters, tocopherols and trace amounts of other components (Gunstone, 1996). Non-lipid material, in the form of moisture, impurities and unsaponifiables can also be present at up to 4% (w/w) in tallow, depending on its purity grade (Grummer, 1992). In lard and beef tallow, saturated fatty acids account for approximately 40% of total fatty acids, while in mutton tallow this can be as high as 65%. The most significant saturated fatty acids present are palmitic and stearic acids. Oleic acid is the major unsaturated fatty acid in all the fats. Linoleic acid is present in significant quantity in lard and palmitoleic appears in higher levels in beef tallow.

### 1.4 Waste lipids

Fats, oils and greases, commonly known as FOG, present a major disposal problem in our society. They contribute to the pollution load in wastewater treatment plants and come from a variety of sources including both municipal and industrial wastes. Unprocessed urban domestic wastewaters carry a large amount of organic matter. Lipids make up between 10 and 25% of this organic material with concentrations ranging from 40 to 150 mg $L^{-1}$ (Fong and Tan, 2000; Hammer and Hammer, 2001). Kitchen wastes contain 14 to 36% lipids derived from vegetable oils and animal fats, whereas faeces contain 14 to 23% lipid (Quéméneur and Marty, 1994). In industrial wastes, lipids are present at concentrations of 500 mg $L^{-1}$, or higher (Fong and Tan, 2000). The types of industry that may produce high-lipid wastewater are numerous and some are detailed in Table 1.5.

Fats, oils and greases are not easily decomposed biologically while treatment by other conventional means is hampered because of their consistency (Stoll and Gupta, 1997). Being sticky in nature, these lipids tend to clog drain pipes and

sewer lines, cause an odour nuisance and lead to the corrosion of sewer lines under anaerobic conditions. When reaching the municipal wastewater treatment plant they float as a layer on top of the water and stick to pipes and walls. Consequently, they block strainers and filters and therefore interfere with treatment unit operations in the plant. As they remain persistent during the treatment process, they are present in the sludge at the end of the treatment and make the handling of sludge very difficult (Stoll and Gupta, 1997). This accumulation is more apparent in large sewers and wastewater treatment plants than in smaller ones due to the larger volume of wastewater processed. Also, if lipid material is not recovered from the final effluent, it can interfere with biological activity in receiving waters and is unsightly and aesthetically unpleasant (Metcalf and Eddy, 1991).

**Table 1.5**: Lipid content of wastewater from some lipid related industries

| **Industry** | **Lipid content (mg $L^{-1}$)** | **Reference** |
|---|---|---|
| Bakery wastewater | 1512 | Keenan and Sabelnikov (2000) |
| Dairy | 4000 | Hanaki *et al.* (1981) |
| Meat processing | 2300 | Okuda *et al.* (1991) |
| Restaurant and food processing | 2000 | Stoll and Gupta (1997) |
| Slaughterhouses and abattoirs | 2100 | Borja *et al.* (1998) |
| Vegetable oil production | 16000 | DeFelice *et al.* (1997) |
| Wool scouring | 10000 | Ang and Himawan (1994) |

Legally, all industries are responsible for ensuring the proper treatment of wastes resulting from their operations. In most cities in developing countries, regulations related to lipids in wastewater do not exist or are not enforced (Stoll and Gupta, 1997). Emissions from industries in Ireland are regulated by the Environmental Protection Agency (EPA). The EPA sets maximum levels for fat, oil and grease emissions as a single parameter. This level can vary from industry to industry and is dependent on the nature of the wastewater and the ultimate disposal location of the resulting effluent. The limits set for discharge into rivers are between 10 and 15 mg $L^{-1}$ with emissions to marine environments limited to 30 mg $L^{-1}$. Adhering to these limits is a challenge for these industries, which can produce considerable

amounts of lipid-containing wastewater and underlines the requirement for efficient removal and disposal of waste lipids.

## 1.5 Lipid removal from waste streams

Fats are among the most non-degradable compounds encountered in wastewater treatment and have to be removed by either physico-chemical or biological means. This presents a problem due to their insoluble nature and recalcitrance and in the case of physico-chemical removal, by the large quantities recovered (Ratledge, 1992).

### 1.5.1 Physical and chemical methods of lipid removal

Many wastewater treatment plants employ physico-chemical primary treatment for fat removal prior to passing the waste stream to the biological stage. This can be as simple as a fat trap or more involved such as Dissolved Air Floatation (DAF) system.

Fat traps are a low-technology method of removing fat from waste streams. Wastewater flows into the trap and passes through a series of baffles to reduce turbulence and increase residence time, causing fat to separate from the wastewater. The fat floats to the top of the unit due to its lower density, while the effluent is taken from the clarified liquid. The fat is then physically removed periodically from the top of the trap. These units require constant maintenance and the recovered fat presents an additional disposal problem. Poorly maintained traps result in reduced efficiency in fat removal and contribute to odour and hygiene nuisances. Fat traps are considered to be unsatisfactory for fat removal (Wakelin and Forster, 1997), although they have been employed as part of an overall physico-biological treatment system (de Villiers and Pretorius, 2001). Attempts have been made to improve design with the addition of flocculants and acids improved removal (Ang and Himawan, 1994). However, increased chemical additions to wastewaters can pose further problems for the biological stage in treatment and as such are undesirable.

DAF systems use both physical and chemical means to remove lipids. Their operation comprises both the use of a combination of flocculating chemicals and the saturation of all or part of the waste stream with air under several atmospheres pressure. The flocculating agents, such as ferric chloride and polyelectrolytes, are added to form larger particles, which can trap air bubbles more easily. The fine bubbles float to the top of the tank, carrying the particulate aggregates, where they can be removed and collected in a receiving vessel. The course of the DAF process in treating lipid wastewaters depends mainly on: the volume of air corresponding to the unit mass of removed suspended solids, the doses and types of flocculating agents and the floatation layout (Wasowski, 1995). Although effective with constant streams, these systems are prone to shock overloading and can be ineffective with high concentrations of lipids (Forster, 1992; Chu and Hsu, 1999).

Treatment with physico-chemical technology results in the recovery of lipid, which still presents a disposal problem. Re-use of this waste lipid is often not practical because of its low-grade composition and possible contamination with tissue or soil (Broughton *et al.*, 1998). Disposal of the waste lipids to landfill is coming under increasing opposition and indeed is now banned in many countries (Nakona and Matsumua, 2001). Incineration is another option for disposal. However, incineration can be expensive due to the high water content in the recovered lipid wastes. Moreover, the use of incinerators is controversial due to secondary pollution at elevated temperatures and the production of dioxins. Biological systems, however, are an attractive alternative to physico-chemical methods and their associated disposal problems. Biological systems offer the potential for near complete removal of the waste lipid and their operation at near ambient conditions effectively removes the risk of the generation of harmful substances.

### 1.5.2 Biological removal of lipids

Both anaerobic and aerobic systems have been studied. Biological removal of lipids can be included in conventional anaerobic or activated sludge systems or can be dealt with separately from the main treatment system following removal

with physico-chemical methods. Both anaerobic and aerobic systems face a challenging substrate when fed high concentrations of fats. The limited bioavailability of fats through their formation of hydrophobic masses and the hydrophobicity of many fatty acids can inhibit their breakdown and utilisation. Biodegradation is dependent on the uptake of primary metabolites, such as long chain fatty acids, into the microbial cell. Therefore, hydrolytic organisms must be present and extracellular hydrolytic activity is required.

### 1.5.2.1 Anaerobic biological removal

Anaerobic digesters have been extensively used in food processing industries to reduce the chemical oxygen demand of wastes. However, fats, oils and greases tend to accumulate in anaerobic units, including digesters and lagoons (Huban and Plowman, 1997). The degradation of lipids is not energetically favourable in truly anaerobic environments. This is as a result of the long retention times of 30 to 60 days with these systems, which creates operational problems with the formation of a substantial fat layer on the surface of the liquid. Although an anaerobic lagoon was employed successfully for the treatment of wool-scouring wastewater, large areas of land were required and the resultant emission of odours and its unsightliness were distinct disadvantages (Ang and Himawan, 1994).

Recent developments in anaerobic treatment technology have overcome many of the constraints of conventional systems, such as shock loads and long hydraulic times and have included: phase separation of the anaerobic process, the adoption of upflow anaerobic sludge blanket reactors, the development of fixed-film systems such as anaerobic filters and fluidized-bed reactors (Borja *et al.*, 1998). In these more advanced systems retention times were typically reduced to 15 days or less (Nakona and Matsumura, 2001). However, the presence of highly concentrated lipid waste can seriously inhibit these processes and can be problematic with the production of a high lipid effluent.

High lipid concentration in anaerobic effluent can result in a number of phenomena including the absence of hydrolysis, the accumulation of fatty acids and toxicity of fatty acids. Hydrolysis of triglycerides, especially fats may be

inhibited when applied in large concentrations (Vidal *et al.*, 2000). A long lag phase of 50 days resulted during solid fat removal in a batch anaerobic reactor, with 25% lipid remaining after 300 days (Terashima and Lin, 2000). In laboratory-scale investigations into milk fat degradation, the majority of the lipid was absorbed onto the biomass and only 22% of the initial fat loading was completely metabolised after 27 days (Petruy and Lettinga, 1997).

The products of triglyceride hydrolysis, long chain fatty acids, can also accumulate in anaerobic systems (Hanaki *et al.*, 1981; Broughton *et al.*, 1998). Saturated fatty acids can be more recalcitrant than unsaturated acids and their presence may inhibit their own oxidation. Oleic, linoleic and palmitoleic acids were all readily converted to saturated fatty acids when presented to biomass (Lalman and Bagley, 2000). Stearic acid degradation was very slow with 50% remaining after 50 days. In these cases, the complete oxidation of the fatty acids did not occur. The accumulation of long chain fatty acids can be toxic to the biomass, inhibiting not only their own breakdown but also that of other nutrients in the waste stream. Methanogenic and acetogenic bacteria are especially prone to fatty acid toxicity (Hanaki *et al.*, 1981; Becker *et al.*, 1999). Oleic acid has been found to be responsible for the failure of treatment systems. In anaerobic fixed-bed systems, oleic acid was toxic at concentrations of 80 mg $L^{-1}$ or higher (Alves *et al.*, 2001). In suspended biomass systems, concentrations of oleic acid as low as 30 mg $L^{-1}$ were toxic while concentrations of stearic acid up to 100 mg $L^{-1}$ were not (Lalman and Bagely, 2000). Overall, anaerobic systems have a limited use in the treatment of high lipid wastewaters.

### 1.5.2.2 Aerobic biological removal

Aerobic biological removal of lipids can be considered on a number of levels. These include treatment of waste with conventional activated sludge systems, treatment with defined mixed microbial populations, addition of bioaugmentation products to systems and treatment with pure microbial cultures.

Conventional biological waste treatment plants have been considered capable of successful grease and oil removal from wastewater (Young, 1979). Activated sludge removed up to 80% of a 20 g $L^{-1}$ loading of fast food restaurant grease (Wakelin and Forster, 1998) and up to 70% of a 2 g $L^{-1}$ loading of olive oil waste (Velioğlu *et al.*, 1992). Activated sludge has the ability to adapt itself to variations in the presence and concentrations of lipids in the waste stream. This adaptation, however, sometimes results in elongated periods for acclimatisation to the waste, which in the meantime can lead to operational problems (Franz and Matsché, 1994).

The presence of lipids in activated sludge aeration tanks can lead to the problem of stable foam formation. Filamentous organisms associated with foaming such as *Microthrix parvicella*, can utilise and store lipid which induces hydrophobicity on the cell surfaces (Forster, 1992). *Rhodococcus rubra* is also associated with aeration tank foaming when a lipid substrate is present in the wastewater. Foam formation is problematic as it can float large amounts of activated sludge solids to the surface of treatment units and cause an increase in effluent biochemical oxygen demand (BOD) and suspended solids concentration due to foam escaping from secondary clarifiers (Franz and Matsché, 1994). Spillage of the foam from aeration tanks can also cause odour nuisance during hot weather and problems with surface freezing and mechanical damage of surface aerators during winter. Other organisms in activated sludge may also be affected by lipid waste. Protozoan levels in activated sludge decreased on the addition of a high lipid loading to the system (Hrudey, 1982). In the design of a microbial consortium for lipid degradation in wastewaters, these problems outlined would have to be considered.

An alternative to treatment with activated sludge is the employment of a defined mixed microbial consortium, selected for their lipolytic ability. This would also remove the presence of organisms that aggravate foam formation. A defined mixed culture of 15 bacterial species removed between 24 to 73% of a 100 g $L^{-1}$ loading of various oils after 14 days (Tano-Debrah *et al.*, 1999). The use of a mixed culture of yeast species for lipid removal was reported (Chigusa *et al.*, 1996). The nine yeast isolates, mostly *Candida* species, reduced an oil loading of

10,000 mg $L^{-1}$ in wastewater to 100 mg $L^{-1}$ in the effluent. A Biomaster G system was developed for the handling of fat containing wastewater with 80% removal of up to 2 g $L^{-1}$ lipid in a municipal wastewater (Grulois *et al.*, 1992 & 1993). The system consisted of an aeration basin into which the wastewater was pumped and an inoculum of a defined consortium of microorganisms was added.

Bioaugmentation fortifies biomass with micoorganisms and in some cases biological material, such as enzymes, that have been selectively adapted to degrade specific compounds (Huban and Plowman, 1997). Added to a wastewater treatment system, the microbes enhance the ability of the biomass to respond to certain situations or to tackle contaminants not broken down by their indigenous counterparts. This may result in improved treatment. The use of only enzyme-only preparations for degradation of lipids can be problematic. Enzymes result in the production of fatty acids only whereas microbes can facilitate complete degradation of the lipids. Also, factors in the wastewater environment, such as pH, temperature and salt concentration can accelerate denaturation of the enzymes (Keenan and Sabelnikov, 2000). In contrast, microbial cells are protected from the environment by their cell envelopes and walls and thus have a greater tolerance to extreme environmental changes that may be encountered in wastewaters.

Commercial bioaugmentation products have been formulated for the degradation of animal and vegetable fats and greases at certain locations in the wastewater system, including pumps and sumps. (Biofuture Ltd., 2002). The ability of several commercial bioadditives to biodegrade 50 g $L^{-1}$ emulsified olive oil was investigated (Chappe *et al.*, 1994). However, none of the products were capable of complete fat removal. Although bioaugmentation products are available on the market, tallow and other animal fats still present a significant problem in wastewater treatment. Also, in some cases the degradative ability of these products was similar to mixed microbial activity (Mendoza-Espinosa and Stephenson, 1996). This would indicate that bioaugmentation products would not be a commercially viable option for lipid removal on a large scale.

The use of pure microbial cultures for lipid degradation offers a number of advantages over mixed microbial cultures and bioaugmentation products. The

organism can be selected based on its ability to degrade a high concentration of a specific lipid that may be present in the wastewater, such as tallow, in a relatively short time period. This would be advantageous if the system was prone to shock loads of lipid. Biodegradative ability of conventional systems is significantly reduced when shock loads are applied. The employment of a pure culture would reduce any start-up time or acclimatisation period that would be required for conventional activated sludge systems. Also, the pure culture can be exploited for the production of specific by-products from lipid metabolism, such as biosurfactants. In mixed populations, production and recovery of by-products may be hampered by nutritional requirements of other organisms, degradation of the products by other organisms or indeed, the by-product may be toxic or inhibitory to the other organisms. Although employment of pure cultures is advantageous in the treatment of fats and they form a large segment of lipid biodegradation research, their commercial potential has not yet been exploited.

The employment of bacteria, yeast and to a lesser degree filamentous fungi, for the degradation of oils and greases has been widely documented. Some of these organisms and the lipid source employed are detailed in Table 1.6. The majority of the oils used, olive, soybean, sunflower and fish oils include those commonly found in waste streams of food processing industries.

The hard fats, such as tallow, have not been investigated to the same degree. A *Bacillus* sp. was successfully employed for the removal of tallow, 90% in 24 hours, from meat processing wastewater (Okuda *et al.*, 1991). In a two-phase aqueous-organic system, *Pseudomonas putida* achieved up to 80% hydrolysis of beef tallow (Kim and Rhee, 1993). The yeast *Yarrowia (Saccharomycopsis) lipolytica* featured predominantly in fat degradation studies (Tan and Gill, 1985; Marek and Bednarski, 1996; Papanikolaou *et al.*, 2001, 2002a & 2003). Fat removal values in excess of 70% have been reported for *Y. lipolytica* (Tan and Gill, 1985), although this study was performed in shake flask culture. Three fungi, *Geotrichum candidum*, *Aspergillus niger* and *Mucor miehei* all demonstrated fat degradation ability on poultry and beef tallow, with up to 70% removal of poultry fat by *G. candidum* (Bednarski *et al.*, 1993).

**Table 1.6:** Bacteria, yeast and fungi studied for oil and grease degradation.

| | Organism | Waste lipid source | Reference |
|---|---|---|---|
| Bacteria | *Acinetobacter* sp. | sunflower and olive oil | Chappe *et al.* (1994) |
| | | restaurant grease | Wakelin and Forster (1997) |
| | *Pseudomonas* sp. | olive oil and waste frying oil | Haba *et al.* (2000) |
| | *Pseudomonas aeruginosa* | soybean oil | Shabati (1991) |
| | *Pseudomonas flourescens* | olive oil | Tan and Gill (1987) |
| Yeast | *Candida* sp. | sardine oil | Ota and Kushida (1988) |
| | *Candidia lipolytica* | evening primrose oil | Aggelis *et al.* (1997) |
| | *Candida tropicalis* | evening primrose oil | Aggelis *et al.* (1997) |
| | *Yarrowia lipolytica* | olive oil and lard | Tan and Gill (1984) |
| | | olive oil | DeFelice *et al.* (1997) Scioli and Vollaro (1997) |
| | | fish oil | Hottinger *et al.* (1974 a&b) |
| | | crude oil | Zinjarde and Pant (2000) |
| | | diesel oil | Ashy and Abou-Zeid (1984) Margesin and Schinner (1997) |
| | | rapeseed oil | Marek and Bednarski (1996) |
| | | palm oil | Oswal *et al.* (2002) |
| Fungi | *Mucorales* sp. | sunflower oil | Čertik *et al.* (1997) |
| | *Mucor circinelloides* | sunflower oil | Jeffery *et al.* (1999) |
| | *Mortierella alpina* | fish oil | Shinmen *et al.* (1992) |

## 1.6 Biodegradation of lipid

The biodegradation of glyceride lipids consists of two main steps: lipase catalysed hydrolysis of the glycerides and the metabolism of the resulting fatty acids after their transport into the cell. Different enzyme systems are responsible for each step, so the ability of a microorganism to carry out one stage of the metabolism does not confer on it the ability to carry out the other stage as successfully. The process of solid lipid utilisation, such as the fats, may also include biosurfactant production to emulsify the solid substrate to make it more available for microbial attack. A generalised schematic of the biodegradation of triglyceride by yeast is presented in Fig. 1.3.

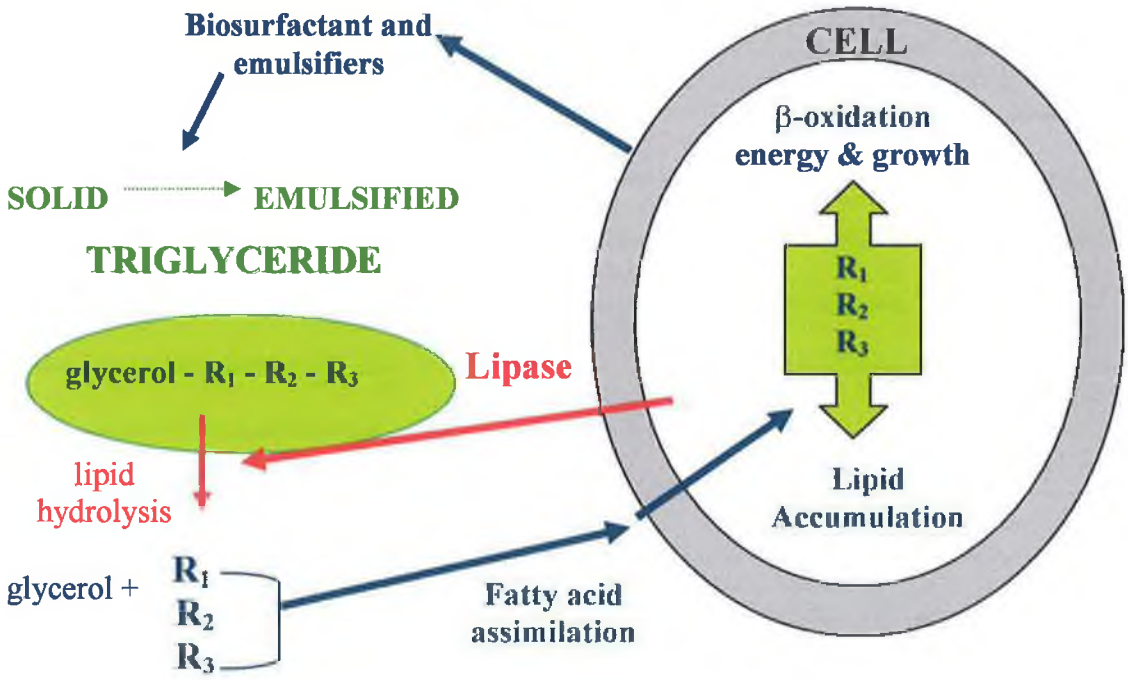



**Figure 1.3:** Generalised schematic of triglyceride utilisation by yeast. $R_1$, $R_2$ and $R_3$ represent the fatty acids in the triglyceride.

### 1.6.1 Lipase catalysed hydrolysis

The biodegradation of lipids begins with their hydrolysis by lipases, releasing free fatty acids and glycerol (Fig. 1.4). In general, this occurs extracellularly as glycerides are high molecular weight molecules, which cannot easily penetrate the cell wall or membrane. This breakdown to the more easily assimilated molecules of fatty acids is necessary before the yeast can utilise the substrate for energy and growth (Hammer and Hammer, 2001).

Microbial lipases may be divided into two groups: non-specific and specific lipases (Sztajer and Zboínska, 1988; Thompson *et al.*, 1999). Enzymes from the first group do not distinguish between the three positions of the glycerol esters. These non-specific lipases bring about a total hydrolysis of triglycerides to fatty acids and glycerol. The specific lipases, or regiospecific lipases, hydrolyse esters in the 1 and 2 positions of glycerides, giving free fatty acids and a mixture of mono- and di-glycerides. The 2-monoglycerides and, to a lesser degree, the 1,2- or 2,3-diglycerides are unstable and therefore the enzymatic hydrolysis is followed by acyl group migrations. This leads to 1-monoglycerides and 1,3-diglycerides. Therefore, the extension of the incubation time may result in a total splitting of triglycerides (Sztajer and Zboínska, 1988). Stereoselectivity of a range of microbial lipases was described by Rogalska *et al.* (1993).

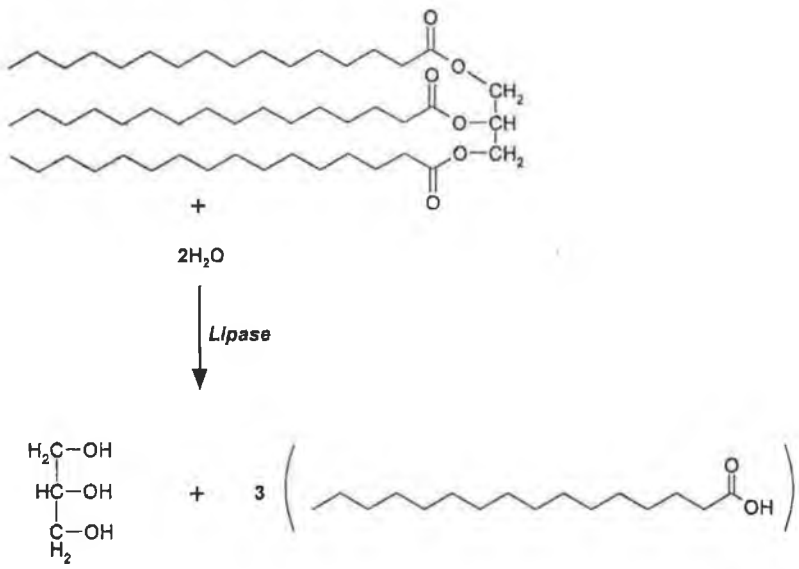



**Figure 1.4:** Schematic of tripalmitin hydrolysis by lipase (Kallel *et al.*, 1994)

Lipases can also be specific to particular fatty acids. Lipase produced by *Geotrichum candidum* exhibits a high specificity to oleic acid and linoleic acid, independent of their positions in the triglyceride (Jensen, 1983). In contrast, lipase produced by *Yarrowia lipolytica* acts preferentially on oleyl residues at the 1, 3 positions of glycerides (Alford *et al.*, 1964). This specificity was not deemed to be limiting to lipid hydrolysis. During the cultivation of *Y. lipolytica* on olive oil, the rapid accumulation of hydrolysis products and the maintenance of high concentrations of preferentially utilised fatty acids indicated the lipase would not limit growth under optimal growth conditions (Tan and Gill, 1984). However, the use of triglycerides with acyl groups refractory to lipase attack and the release of fatty acids less readily metabolised than oleic acid may impose constraints on microbial biodegradation of fats.

Lipase activity is not limited to extracellular production in the growth medium. Lipase activity has been detected on both the cell surface and intracellularly (Ota *et al.*, 1982; Pereira-Meirelles *et al.*, 2000). An extracellular and two cell-bound lipases, corresponding to lipase I (39 kDa) and lipase II (44 kDa) were described for *Yarrowia lipolytica* (Ota *et al.*, 1982). The extracellular lipase required oleic acid as a stabiliser-activator, whereas the cell-bound lipases did not. The rates of production of the extracellular and cell-bound lipases were reported to be dependent on the carbon and nitrogen composition of the medium. The gene which codes for extracellular lipase production in *Y. lipolytica*, *LIP2* gene, was recently elucidated (Pignède *et al.*, 2000). Lipase production was repressed by glucose and induced by olive oil and oleic acid. A pattern of extracellular lipase production was reported for *Y. lipolytica* when cultivated on olive oil (Pereira-Meirelles *et al.*, 2000). Lipase production was induced intracellularly when the yeast was exposed to olive oil. The lipase then moved to the surface of the cell and was released extracellularly.

The synthesis of microbial lipases is affected by growth conditions, including the availability of carbon and nitrogen sources, the presence of activators, stimulators and inhibitors, surfactants, temperature, pH and the source of the inoculum (Hadeball, 1991; Thompson *et al.*, 1999). Oxygen tension can also influence lipase production (Chartrain *et al.*, 1993). Certain lipids in the culture medium

may influence the production and activity of microbial lipases. Generally, the activity of intra- and extracellular lipases increases with increasing lipid concentrations (Marek and Bednarski, 1996). However, when fats are present as substrates, the activity of lipases is a function of both the chain length and the saturation ratio of the fatty acids present (Sztajer and Zboínska, 1988). The lipase reaction rate varies directly with the surface area of substrate available to the enzyme (Hadeball, 1991). Short chain triacylglycerols can be poor substrates for lipase as they have a decreased tendency to form micelles and the surface tension is suboptimal for activity (Kordel *et al.*, 1991). Conversely, long-chain, saturated triacylglycerols are packed very densely because of strong hydrophobic interactions between the hydrocarbon chains which prevents the lipase coming into close contact with the substrate molecules (Thompson *et al.*, 1999). Similarly, unsaturated triglycerides are good inducers of lipase production while saturated triglycerides are not (Ota *et al.*, 1968). The problems associated with physical nature of the lipid may be overcome through emulsification (by action of surfactants or biosurfactants), blending or vigorous shaking. This ensures substrate dispersion and an optimum substrate surface for lipolysis.

Although the primary function of lipases is the hydrolysis of lipids and related molecules, lipases also catalyse other reactions associated with acyl groups. These reactions may be exploited in fat biotechnology and include:

- hydrolysis: reaction of ester with water producing acid and alcohol and with hydrogen peroxide to give peroxy acids.
- esterification: the reverse of hydrolysis- production of ester from acid and alcohol.
- alcoholysis: reaction of an ester with a monohydric alcohol such as ethanol, lauric alcohol or a polyhydric alcohol such as glycerol to produce an ester with a different alkyl group. Similar reaction with amines leads to amides.
- acidolysis: reaction of an ester with an acid leading to acyl groups.
- interesterification: reaction of one ester with another leading to random acyl and alcohol moieties.

These process can be selected by choosing appropriate substrates and reaction conditions (Gunstone, 1999).

### 1.6.2 Lipid emulsification

Microbes that degrade hydrophobic, water-insoluble substrates such as hydrocarbon liquids or solids and fats, oils or greases usually produce surfactant substances or biosurfactants (Gerson, 1992). Insolubility, having a very low saturation concentration in water, limits the available aqueous concentration of these substrates and the rate of lipolysis. Biosurfactants improve the availability of the substrates to cells by facilitating emulsion formation. The terms biosurfactant and bioemulsifier are frequently used interchangeably (Fiechter, 1992). The term bioemulsifier is often used in an application-oriented manner to describe the combination of all the surface-active compounds that constitute the emulsion secreted by the cell to facilitate uptake of an insoluble substrate (Hommel, 1990). Most biosurfactants are lipids and some of the major types and sources are listed in Table 1.7.

**Table 1.7:** Microbial surfactants

| **Surfactants** | **Organisms (genus)** |
|---|---|
| Fatty acids | *Acinetobacter, Aspergillus, Candida, Corynebacterium, Mycococcus, Norcardia, Penicillium, Pseudomonas* |
| Neutral lipids | *Acinetobacter, Arthrobacter, Mycobacterium, Thiobacillus* |
| Phospholipids | *Candida, Corneybacterium, Micrococcus, Thiobaccillus* |
| Glycolipids | |
| Rhamnolipids | *Arthrobacter, Corynebacterium, Norcardia, Pseudomonas* |
| Sophorolipids | *Candida, Torulopsis* |
| Trehalolipids | *Arthrobacter, Brevibacterium, Corynebacterium, Mycobacterium, Norcardia* |
| Lipopeptides | *Bacillus, Candida, Corynebacterium, Mycobacterium, Norcardia, Streptomyces* |
| Glycosyldiglycerides | *Lactobacillus* |

Adapted from Gerson (1992)

Unlike chemically synthesised surfactants, which are classified according to the nature of their polar grouping, biosurfactants are categorised mainly by their chemical composition (Deasi and Banat, 1997). Microbial biosurfactants combine hydrophilic and lipophilic functional groups to form amphiphilic structures (Sekelsky and Shreve, 1998). The hydrophilic moiety consists of amino acids or peptides, anions or cations, mono-, di-, or polysaccharides and the lipophilic moiety consists of unsaturated or saturated fatty acids. Most biosurfactants are either neutral, such as the neutral lipids or negatively charged, such as sophorolipids.

Biosurfactant activities can be determined by measuring the changes in surface tension and by the stabilisation or destabilisation of emulsions. When a surfactant is added to oil/water systems at increasing concentrations, a reduction of the surface tension is observed up to a critical level, above which amphiphilic molecules associate readily to form supramolecular structures like micelles, bilayers and vesicles (Desai and Banat, 1997). This value is known as the critical micelle concentration (CMC). CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase. Biosurfactants may stabilise (emulsifiers) or destabilise (deemulsifiers) the emulsion.

Several bacteria and yeast produce large quantities of fatty acid and phospholipid surfactants during growth on *n*-alkanes (Desai and Banat, 1997). A potent biosurfactant, phosphatidylethanolamine was produced by an *Acinetobacter* sp. which formed optically clear microemulsions of alkanes in water (Käppeli and Finnerty, 1979). Most known biosurfactants are glycolipids and include rhamnolipids, trehalolipids and sophorolipids. The biosurfactant produced by *Yarrowia lipolytica* during cultivation on alkanes was elucidated as a glycolipid and was composed of 5% protein, 20% carbohydrate and 75% lipid (Zinjarde *et al.*, 1997).

Some organisms produce growth-associated extracellular emulsifiers when cultivated on fats/hydrocarbons (Zinjarde and Pant, 2002a) while others produce cell-associated emulsifiers that make the cell surfaces hydrophobic and enhance substrate uptake (Käppeli and Fiechter, 1977). Ionic biosurfactants, such as rhamnolipids or sophorolipids emulsify the substrate, which increases the surface area available for microbial attack (Syldatk *et al.*, 1984). Neutral biosurfactants, such as trehalose lipids render the charged surface of the cell hydrophobic (Ratledge, 1988; Kim *et al.*, 2000d). However, these mechanisms of substrate emulsification are not mutually exclusive. Both cell-associated and extracellular emulsifier production was observed in a marine strain of *Y. lipolytica* (Zinjarde and Pant, 2002a). Moreover, Kim *et al.* (2000d) described the cell surface emulsifier as a polysaccharide fatty acid complex and it was determined to be part of the alkane-binding system. The mechanism of microbial alkane utilisation is similar to that for fatty acids (Kajs and Vanderzant, 1980; Walker, 1998). *Y. lipolytica* exhibited interesting cell surface characteristics when grown on hydrocarbons (Käppeli *et al.*, 1978; Kim *et al.* 2000d). There were radial protrusions on the cell surface and a thickening of the periplasmic layer, which were not observed during cultivation on glucose. These protrusions were associated with alkane binding affinity.

In the biosynthesis of biosurfactants, two primary metabolic pathways are involved, a fatty acid and a carbohydrate pathway for the lipophilic and hydrophilic moieties (Syldatk and Wagner, 1987). A number of possibilities exist for the synthesis of these moieties and include: (i) de novo synthesis of both moieties by independent pathways, (ii) de novo synthesis of the lipophilic moiety with substrate dependent synthesis of the hydrophilic, (iii) de novo synthesis of the hydrophilic moiety with substrate dependent synthesis of the lipophilic and (iv) substrate dependent synthesis of both moieties (Desai and Desai, 1993). A common scheme of possible glycolipid biosynthetic routes is presented in Fig. 1.5.

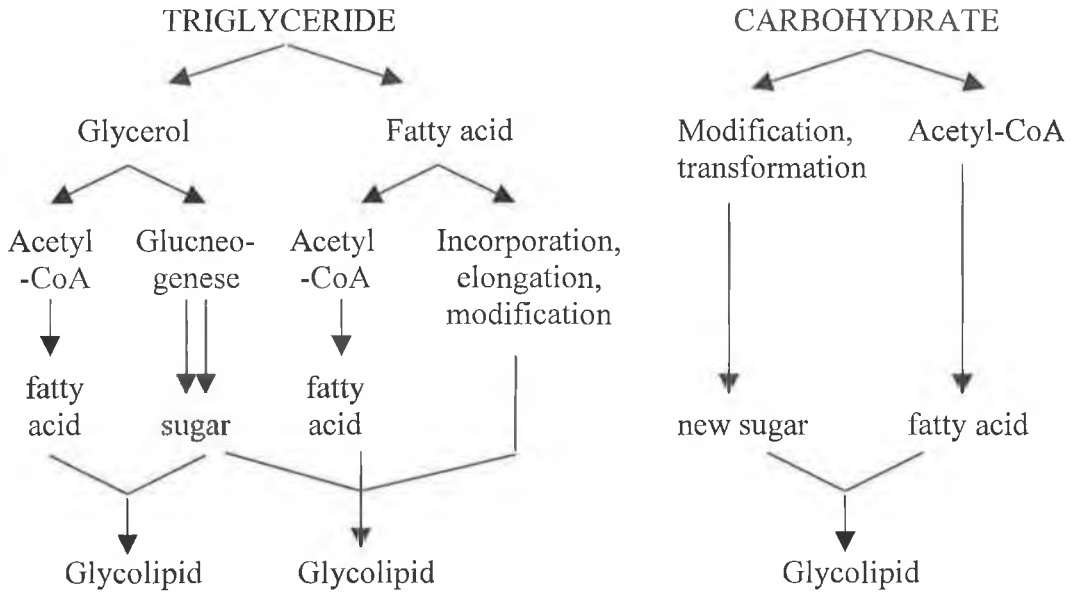



**Figure 1.5**: General scheme of possible routes for the microbial glycolipid synthesis and/or glucose (Lang and Wagner, 1992).

The kinetics of biosurfactant production exhibit many variations among systems but can be generalised as: (i) growth-associated production, with a parallel relationship between growth, substrate utilisation and biosurfactant production, (ii) production under growth-limiting conditions, (iii) production by resting cells and (iv) production with precursor supplementation, such as lipophilic compounds (Desai and Banat, 1997). Three mechanisms operate in the regulation of biosurfactant production and include induction, repression and concentrations of nitrogen and multivalent ions (Lang and Wagner, 1992). The carbon substrate is key to both induction and repression. Lipids and hydrocarbons induce biosurfactant production (Roy *et al.*, 1979; Cirigliano and Carman, 1984; Fiechter, 1992) whereas organic acids and glucose can repress its production (Duvnjak *et al.*, 1983). In the case of nitrogen- or metal ion-dependent regulation, their effect is linked to the organism itself (Guerra-Santos *et al.*, 1984; Mulligan and Gibbs, 1992).

Overall, the production of biosurfactants by microorganims growing on lipids facilitates the dispersion of lipid into the aqueous phase through extracellular and/or cell-associated properties. This in turn facilitates extracellular and/or cell-

associated lipolytic cleavage of triglycerides into fatty acids. These fatty acids are then assimilated into the cell for biodegradation or accumulation.

### 1.6.3 Fatty acid uptake

In yeast, utilisation of extracellular fatty acids requires their transfer from the uptake site to peroxisomes (Dell'Angelica *et al.*, 1992). Peroxisomes are single membraned-delimited organelles in which the fatty acids undergo oxidation (Walker, 1998). Several alternatives have been proposed for the transport of fatty acids across the plasma membrane to organelles including protein-catalysed transfer and diffusion (Sleight, 1987). Fatty acid binding proteins were elucidated in yeast when cultivated on lipids and were not detected during growth on glucose, implicating their role in fatty acid transfer (Dell'Angelica *et al.*, 1992 & 1996). These carrier-mediated fatty acid transport systems account for fatty acid uptake at low substrate concentration, whereas transport is thought to be by diffusion at high substrate concentrations (Kohlwein and Paltauf, 1983). In *Y. lipolytica*, two fatty acid carrier systems have been detected (Kohlwein and Paltauf, 1983). One is specific for $C_{12}$ and $C_{14}$ fatty acids and the other for $C_{16}$ and $C_{18}$ saturated or unsaturated fatty acids.

### 1.6.4 β-oxidation

Once fatty acids are assimilated into the cell they can be oxidised via β-oxidation in peroxisomes for energy and growth or are incorporated into lipid structures or stored lipid, either unchanged or following desaturation or elongation (Čertík *et al.*, 1997; Wakelin and Forster, 1997). Of these, β-oxidation is the pathway to complete degradation of the lipid material.

β-oxidation involves the release of a series of 2-carbon acetyl-CoA units from the fatty acid molecules (Ratledge, 1992). Firstly the free fatty acid is bound to Coenzyme A to form a fatty acyl-CoA. This molecule then undergoes a series of reactions resulting in the release of acetyl CoA and shortening of the fatty acyl-CoA by 2 carbons (Fig. 1.6). This shortened molecule then undergoes another

cycle of reactions, which is repeated until 2 molecules of acetyl CoA are released by the final cycle. This cycle of breakdown is applicable to saturated fatty acids. Unsaturated fatty acids, however, require additional steps for successful oxidation. When the double bond is encountered, isomerisation is the first step to produce a trans-double bond, which is then reduced to eliminate the double bond. The resulting saturated molecule can then undergo the β-oxidation pathway. Some intermediates of the breakdown pathway, in particular saturated fatty acids such as palmitic and myristic acids, may accumulate in the cell due to their inhibition of their own breakdown (Lalman and Bagley, 2000).

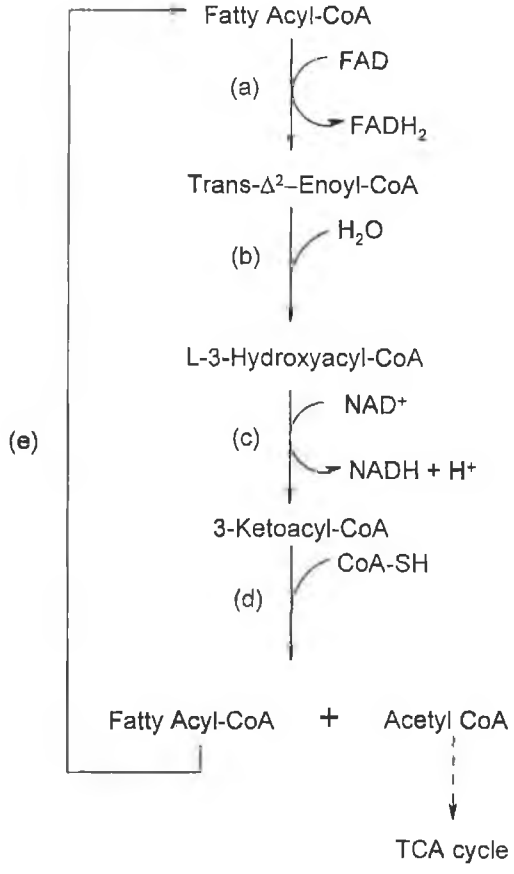



**Figure 1.6:** β-oxidation of a fatty acyl-CoA. The sequence of reactions are (a) dehydrogenation by fatty acyl CoA oxidase; (b) hydration by trans-2,3-enoyl-CoA hydratase; (c) dehydrogenation by L-3-hydroxyacyl-CoA dehyrogenase and (d) thiolytic cleavage by 3-oxoacyl-CoA thiolase; (e) the shortened fatty acyl-CoA undergoes (a-d) again until in the final cycle, 2 molecules of acetyl CoA are released (Mathews and van Holde, 1990).

### 1.6.5 Lipid accumulation

The accumulation of intracellular lipid occurs in a limited number of yeasts. Of the 700 species of yeasts, only 25 or so are known to be able to accumulate more than 20% (w/w) lipid (Ratledge, 1994). Such species are termed oleaginous (Thorpe and Ratledge, 1972). Oleaginous yeasts (Table 1.8) can accumulate up to 70% of biomass weight as lipid (Ratledge, 1994; Walker, 1998; Papanikolaou *et al.*, 2003). The key enzyme that oleaginous organisms posses, which is not present in non-oleaginous ones, is ATP:citrate lyase (Ratledge, 1987). This enzyme catalyses the cleavage of citrate in the presence of CoA and ATP to acetyl-CoA and oxaloacetate. Acetyl-CoA enters a multienzyme complex known as the fatty acid synthase, which results in the synthesis of microbial lipid (Singh *et al.*, 1985). These stored lipids may have interesting properties including high concentrations of saturated fatty acids and fatty acids with potential commercial use, such as γ-linoleic acid (Aggelis *et al.*, 1997). In general, it is considered that oleaginous microorganisms mainly accumulate their lipids as intracellular droplets composed mostly of triglycerides (80-90% w/w of total lipids) (Ykema *et al.*, 1986; Meesters *et al.*, 1996). However, lower but still significant triglyceride percentages (45-70% w/w of total lipids) have been reported (Ratledge and Boulton, 1985; Kendrick and Ratledge, 1996). The relative composition of the fatty acids of the triglycerides generally follows the pattern: oleic (18:1) > palmitic (16:0) > linoleic (18:2) and stearic (18:0) (Walker, 1998).

**Table 1.8:** Some oleaginous yeasts

| Genus | Representative species |
|---|---|
| *Candida* | *C. curvata, C. diddensii* |
| *Cryptococcus* | *C. albidus, C. laurentii* |
| *Geotrichum* | *G. candidum* |
| *Hansenula* | *H. saturnus* |
| *Lipomyces* | *L. lipofer, L. starkeyi* |
| *Rhodotorula* | *R. glutinis, R. graminis, R. mucilaginosa* |
| *Trichosporon* | *T. cutaneum, T. fermentans, T. pullulans* |
| *Yarrowia* | *Y. lipolytica* |

Adapted from Ratledge (1994) and Walker (1998).

Although oleaginous yeast have the capacity to store lipids, they can be grown with lipid contents of 10% (w/w) or less (Ratledge, 1994). The characteristics of intracellular lipid content and composition is dependent on the both the substrate and the growth conditions employed. Although the conversion of sugars to lipids by oleaginous organisms has been studied in detail (Ratledge, 1994; Jackson *et al.*, 1998; Aggelis and Komaitis, 1999, Eroshin *et al.*, 2000), information concerning the biochemistry of these oleaginous organisms growing on fats is limited. The mechanism of lipid accumulation with a lipid substrate differs to that with a non-lipid carbon source. Cultivation of oleaginous yeast on media containing fats as the sole carbon and energy source accumulate reserve lipids during primary anabolic growth, since the accumulation of aliphatic chains is not restricted by the presence of nutrients in the medium (Aggelis and Sourdis, 1997; Papanikolaou *et al.*, 2001; Papanikolaou and Aggelis, 2003). Unsaturated fatty acids, such as oleic acid, are rapidly incorporated into the cell and used for growth needs, whereas saturated fatty acids, such as stearic acid, are incorporated more slowly and are partially accumulated (Papanikolaou *et al.*, 2001). Stearic acid is noted for its recalcitrance in many organisms (Tan and Gill, 1985; Lee, 1992; Papanikolaou *et al.*, 2002a). Accumulation of storage lipid from glucose or another similarly metabolised component is a secondary anabolic activity in oleaginous organisms, occurring after nutrient (mainly nitrogen) exhaustion in the growth medium (Ykema *et al.*, 1986; Ratledge, 1987 & 1994; Walker, 1998).

In the study of microbial lipid accumulation, various oils have been employed as the carbon source. *Mucor heimalis* accumulated up to 26% (w/w) lipid when cultivated on olive oil (Akhtar *et al.*, 1983), whereas accumulation of 45% (w/w) was reported for *Mucor circinelloides* (Jeffery *et al.*, 1999). Various fungi have accumulated between 25 - 50% (w/w) lipid when cultivated on polyunsaturated oils (Kendrick and Ratledge, 1996) and 42 – 66% (w/w) lipid was accumulated when Mucorales fungi were grown on sunflower oil (Čertík *et al.*, 1997). However, intracellular lipid accumulation with fats has received little attention. A number of fungi accumulated up to 70% (w/w) lipid when cultivated on beef and poultry tallow (Bednarski *et al.*, 1993). The degree of accumulation was related to the species of fungus, method of cultivation and the carbon source. The intracellular lipid was also greatly affected by the composition of the substrate.

Accumulation of up to 44% (w/w) was reported for *Yarrowia lipolytica* when cultivated on an industrial fat derivative (Papanikolaou *et al.*, 2001). This accumulated fat was mostly composed of saturated fatty acids and it was proposed that the unsaturated fatty acids were metabolised for growth and structural incorporation of the cell.

The phenomenon of lipid accumulation is of importance in fat waste treatment, particularly in the design of the system. Accumulation of lipid can lower the density of the biomass, thereby decreasing its ability to settle out of the clarifiers. Ideally, the accumulation of lipid in a waste system should be low to avoid this complication. Therefore, the complete utilisation of assimilated fatty acids through β-oxidation pathway would be the preferred fate of assimilated waste lipid.

## 1.7 The role of environmental conditions

Environmental conditions of temperature, pH, aeration and nutrients influence yeast growth and the production of any associated metabolites, such as enzymes or surfactants. These environmental parameters can be manipulated during cultivation of a yeast for a specific purpose. These can include optimisation of substrate removal (Margesin and Schinner, 1997; Fleming, 2002; Zinjarde and Pant, 2002b), optimisation of production of biomass (Lucca *et al.*, 1995; Konlani *et al.*, 1996; Cheng *et al.*, 1999) or optimisation of conditions for metabolite production (Rane and Sims, 1996; Papanikolaou *et al.*, 2002b).

### 1.7.1 Temperature, pH and dissolved oxygen

Temperature is one of the most important physical parameters influencing yeast growth (Walker, 1998). The majority of laboratory and industrial yeasts are mesophiles and generally grow best at 20° - 30°C and the majority of industrial processes, such as waste treatment operate in this temperature range (Nakona and Matsumura, 2001). Exceptions to this temperature range are the psychrophilic

yeast, which grow optimally between 12° - 15°C (Phaff *et al.*, 1978) and thermophilic yeast which grow at 50°C, such as *Kluveromyces marxianus* (Banat *et al.*, 1992). Application of high temperatures can have adverse effects on yeast. Repression of protein synthesis can occur, inhibition of respiration and fermentation and ultimately, there is appreciable cell death at the highest growth temperature of many yeasts (Walker, 1998).

Other physical growth requirements for yeast relate to pH and to dissolved oxygen. Most yeast grow very well between pH 4.5 and 6.5, but nearly all species are able to grow in more acidic or alkaline media (pH 3 or pH 8, respectively) (Walker, 1998). The optimum pH for the growth of yeast is dependent on the substrate and the strain of the organism. In the production of citric acid by *Yarrowia lipolytica* cultivated on glycerol, pH 5 was employed (Papanikolaou *et al.*, 2002b), whereas the biodegradation of animal derived fat by the yeast was at pH 6.0 (Papanikolaou *et al.*, 2002a). *Y. lipolytica* 1096 was cultivated on beef tallow at pH 5 – 6 (Bednarski *et al.*, 1994), whereas *Saccharomycopsis (Yarrowia) lipolytica* ATCC 8661 was cultivated on the beef tallow at pH 7.0 (Tan and Gill, 1985). Yeasts require oxygen not just as the terminal electron acceptor in respiratory growth, but also as an essential growth factor for membrane fatty acids and sterol synthesis (Ratledge, 1994). Oxygen is key for the degradation of lipids and fatty acids through the β-oxidation pathway. The influence of oxygen and sugar availability on yeast carbohydrate metabolism has been categorised under various regulatory phenomena: the Pasteur, Crabtree, Custers and Kluyver effects (Walker, 1998).

### 1.7.2 The role of nutrients

Yeast nutrition refers to how yeast cells feed. More specifically, it refers to how yeasts translocate water and essential organic and inorganic nutrients from their surrounding growth medium through the cell wall, across the cell membrane and into the intracellular milieu. A summary of the organic and inorganic elemental requirements of yeasts is detailed in Table 1.9.

**Table 1.9:** Summary of the major organic and inorganic elemental requirements of yeasts.

| Element | Common sources | Cellular functions |
|---|---|---|
| Carbon | Sugars, fatty acids, hydrocarbons | Major structural element of yeast cells in combination with hydrogen, oxygen and nitrogen. Catabolism of compounds provides energy |
| Hydrogen | Protons from acidic environments | Transmembrane proton-motive force is vital for yeast nutrition. Intracellular acidic pH (around 5-6) necessary for yeast metabolism |
| Oxygen | Air, $O_2$ | Substrate for respiratory and other mixed-function oxidative enzymes. Essential for ergosterol and unsaturated fatty acid synthesis. |
| Nitrogen | $NH_4^+$ salts, urea, amino acids | Structurally and functionally as organic amino nitrogen in proteins and enzymes |
| Phosphorous | phosphates | Energy transduction, nucleic acid and membrane structure. |

Adapted from Walker (1998).

The presence of more than one carbon source for yeast assimilation can lead to the co-metabolism of both carbons. In wastewaters, the carbon present can be from a number of sources including fats and sugars, especially in food processing waste streams (Scioli and Vollaro, 1997). The co-metabolism of fats with carbohydrates has been reported for *Y. lipolytica* (Marek and Bednarski, 1996) and *Candida* sp. (Kostov *et al.*, 1986). In waste streams, xenobiotics, such as phenols can also be present, which may be toxic to organisms present (Heipieper *et al.*, 1991). A strain of *Y. lipolytica* removed olive oil and sugars in the presence of phenol, which was then removed by treatment with *Pseudomonas putida* (DeFelice *et al.*, 1997).

The availability of inorganic nutrients, such as nitrogen and phosphorus, may also affect the biodegradation of compounds in wastewater. Yeast cells have a nitrogen content of around 10% of their dry weight (Walker, 1998). Although yeasts cannot fix molecular nitrogen, simple inorganic sources such as ammonium salts

are widely utilised. Limitation of nitrogen in a system can limit the biodegradation of oils (Szabo and Štofaníková, 2002; Zinjarde and Pant, 2002b). Phosphorous is present in nucleic acids and in phospholipids and therefore essential for all yeasts and accounts for 3 – 5% of dry weight (Walker, 1998). Phosphorous is generally assimilated in the form of orthophosphate, $H_2PO_4^-$. Deficiency of phosphate in wastewater can be remedied through the addition of fertilisers and a carbon, nitrogen, phosphorus ratio of 100:10:1 is a general rule of thumb (Wakelin and Forster, 1998).

Ionic nutrition of yeasts is complex but essential for optimal growth. Optimum levels of several cations present in the medium, which promote yeast growth have been calculated (Table 1.10). Yeast requirements of minerals are similar to that of other cells with a supply of potassium, magnesium and several trace elements required for growth. $K^+$ and $Mg^{2+}$ are regarded as bulk or macroelements, which are required to establish the main metallic cationic environment in the yeast cell (Walker, 1998).

$K^+$ performs four main functions in the cell including (i) osmoregulation; (ii) energy source in the form of the $K^+$ gradient; (iii) activation of protein synthesis, including enzymes associated with phosphate transfer, pyruvate kinase and oxidative phosphorylation and (iv) stabilisation of intracellular structures, for example, ribosomes (Jones and Gadd, 1990). Yeast cellular $K^+$ content varies according to growth conditions, but generally represents 1 – 2% of dry weight (Walker, 1998). $Mg^{2+}$ performs both essential structural and metabolic functions and is present in cells at around 0.3% of the dry weight. Although some halotolerant yeasts, such as *Debaromyces hansenii*, can grow in saline environments, there is no evidence that yeasts require $Na^+$, even at very low concentrations (Walker, 1998). Halotolerant yeasts have adapted special osmoregulatory mechanisms for growth in high concentrations of NaCl. It is, however, very difficult to generalise on ionic requirements due to factors such as strain differences, culture media chelation and ionic interactions.

**Table 1.10:** Optimum concentrations of cations stimulating yeast growth and their cellular function.

| Cation | Concentration | Cellular function |
|---|---|---|
| $K^+$ | 2-4 mM | Ionic balance, enzyme activity |
| $Mg^{2+}$ | 2-4 mM | Enzyme activity, cell and organelle structure |
| $Mn^{2+}$ | 2-4 mM | Enzyme activity |
| $Ca^{2+}$ | < μM | Possible second messenger in signal transduction |
| $Cu^{2+}$ | 1.5 μM | Redox pigments |
| $Fe^{2+}$ | 1-3 μM | Haeme-proteins, cytochromes |
| $Zn^{2+}$ | 4-8 μM | Enzyme activity |
| $Ni^{2+}$ | 10-90 μM | Urease activity |
| $Mo^{2+}$ | 1.5 μM | Nitrate metabolism, vitamin $B_{12}$ |

Adapted from Walker (1998).

## 1.8 Design of lipid biodegradation systems

A number of pilot-scale systems have been designed for the biodegradation of lipids. In these schemes, the vessel for lipid biodegradation was designed to integrate as a part of an existing treatment system or as a separate unit to the conventional activated sludge treatment system.

Lipids in wastewater from a meat processing factory was treated on a pilot-scale in a 'circulation tank' with a *Bacillus* sp. followed by treatment in an aeration tank with activated sludge (Okuda *et al.*, 1991). The circulation tank is based on the theory that cells make good contact with both lipid and air if the culture surface is continuously returned to the bottom of the culture. This was achieved by a pump, which resulted in the circulation of the lipid and air in the wastewater. The majority of the lipid in the waste stream was treated in this circulation phase, with a further reduction in COD in the activated sludge tank. This system facilitated the treatment of lipid wastewater without the need for physical treatment in the water. A pilot-scale aeration tank, with 9 yeast strains, was employed for the

pretreatment of lipids in wastewater from a soybean oil factory (Chigusa *et al.*, 1996). The treated effluent underwent a final treatment in an activated sludge tank prior to discharge.

A novel bioreactor with activated sludge was designed for the replacement for existing technologies, such as grease traps, for the treatment of fast food restaurant wastewater, thereby eliminating grease deposits in pipes and sewers and municipal wastewater (Wakelin and Forster, 1998). The weir tank reactor consisted of two chambers, a weir and a main chamber, with a baffle to separate them which facilitated the cascade or weir effect. This was key to the system as it provided surface aeration to the liquor. A circulation pump ensured that the liquor was completely mixed. The heat generated from the pump aided in the maintenance of the liquor temperature at approximately 30°C. A modified sequencing batch reactor (SBR) process was employed for the treatment of abattoir waste with activated sludge (de Villiers and Pretorius, 2001). The system was defined as an SBR process with an external settler to collect the biomass produced. Prior to entry into the SBR, the wastewater was passed through a screen to remove large solid wastes from the processing of the animals. The SBR was supplied with air.

In general, however, investigations into lipid biodegradation have been performed on laboratory scale in shake flasks or in aerated bench-top fermenters (Ota and Kushida, 1988; DeFelice *et al.*, 1997; Scioli and Vollaro, 1997; Papanikolaou and Aggelis, 2001 & 2002) or in small, 10 - 20 L pilot-scale bioreactors or fermenters (Kajs and Vanderzant, 1980; Bednarski *et al.*, 1994; Wakelin and Forster, 1997). For fermenters with agitation, Rushton impellers were employed in these fermenters. Fermenters, although they may not be most suitable method of system design for large-scale or industrial design of waste lipid biodegradation in economic terms, they are a useful system to study the process of lipid biodegradation.

Initial studies, in general, begin with shake flask cultures. Shake flasks facilitate an examination of the organism and its ability to grow under different

environmental conditions (pH, temperature, and agitation) and indeed any restrictions to its biodegradative properties under batch conditions. After preliminary examination in shake flasks, their cultivation may be scaled-up and examined in fermenters. Cultures can be more closely monitored in fermenters than in shake flasks, so better control over the process is possible. Cultivation in a bench-top fermenter permits the collection of information about oxygen requirements of the organism, foaming, any limits imposed by the fermenter and determination of conditions optimal for activity. Pilot-scale studies examine the response of cells to scale-up (Doran, 1995). Ideally, scale-up should be performed so that conditions in the large vessel are as close as possible to those in the small vessel (Doran, 1995). In biological engineering, the rule of thumb method is commonly used as a scale-up procedure (Hur and Kim, 1999).

In systems designed for substrate biodegradation properties such as the physical nature of the substrate and ultimately mixing are important (Doran, 1995; Solà and Gòdia, 1995). For mixing to be effective, the medium, including cells and substrate, circulated by the agitator must sweep the entire vessel in a reasonable time and the velocity of the fluid must be sufficient to carry material into the most remote parts of the tank. Turbulence must also be developed in the fluid (Doran, 1995). The basis of scale-up should be the property most critical to the performance of the bioprocess. In the case of fats and lipids this property is mixing between cells and the substrate. Effective mixing is required for the formation of a lipid emulsion to facilitate enzymatic hydrolysis and the assimilation of the lipid (Hur and Kim, 1999).

Scale-up to industrial scale often results in both technical and economic compromises (Solà and Gòdia, 1995). When developing a process, it is never possible to achieve a perfect anticipation of its industrial performance. One approach to improving the design procedure is to use scale-down methods (Doran, 1995). The general idea behind scale-down is that small-scale experiments, to determine operating parameters, are performed under conditions that can actually be realised, both physically and economically at an industrial scale.

## 1.9 By-products of lipid biodegradation

The processing of solid waste and waste streams is usually not driven by economics, but instead by regulations and public pressure. As regulatory controls are increasingly tightened, the cost of processing wastes increases. This forces industry to examine the entire process and the consideration of the production of by-products from waste processing. It may be possible to convert waste streams into valuable product by simple separation and recovery, or by biological conversion (Najafpour *et al.*, 1994).

### 1.9.1 Bioconversion of fats

The possibility of changing the composition of waste fats and their properties with no need for chemical catalysts, which are difficult to remove, is the advantage of biological fat modification (Bednarski *et al.*, 1994). Industrially important biological processes include hydrolysis, esterification, interesterification and transesterification (Ratledge, 1987; Koritala *et al.*, 1987). As an alternative to complete oxidation, the bioconversion of long chain fatty acids to more useful fatty acids has been investigated. *Mucor circinelloides* was employed to convert linoleic acid from sunflower oil to γ-linoleic acid (GLA), an acid of particular use in both pharmaceutical and cosmetic industries (Aggelis *et al.*, 1991). Oxidation of fatty acids to give dicarboxylic acids has been exploited commercially on a small scale in Japan (Ratledge, 1992 & 1994). Dicarboxylic acids are employed in the production of plasticisers, adhesives and in the cosmetic industry.

### 1.9.2 Single cell oil

The term single cell oil (SCO) was coined (Ratledge, 1976) and continues to be used as a slight euphemism for microbial oil (Kyle and Ratledge, 1992; Aggelis and Komaitis, 1999). SCO production is of particular interest due to the capacity of oleaginous microorganisms (mainly yeasts and moulds) to convert numerous raw materials into value-added end products (fats and oils) (Ratledge, 1994). The advantages of using yeast as lipid producers are that (1) they produce lipids similar to vegetable oils and fats, (2) they grow well on cheap agro-industrial and

food industrial wastes, (3) their lipids can be produced at a faster rate in bulk in large capacity reactors than the usual time-consuming agricultural practices and (4) most of the potential lipid producers and their products seem to be relatively non-toxic to humans (Jacob, 1993).

Successful exploitation of yeast oils is dependent on very careful identification of market opportunity. Of the various targets of SCO that have been considered, cocoa-butter-like oil has been identified as having commercial potential. Cocoa butter enjoyed a high market price, especially in the mid 1980's, which made it an attractive proposition for SCO research groups (Moreton, 1985; Davies *et al.*, 1990; Ykema *et al.*, 1990). More recently, SCO production by *Y. lipolytica* cultivated on animal derived fats and glycerol as a potential cocoa butter substitute has been investigated (Papanikolaou *et al.*, 2001, 2002a & 2003). The accumulated lipid of *Y. lipolytica* cells comprised a high concentration of saturated fatty acids, such as stearic acid, which was comparable to the saturated fatty acid content of cocoa butter. The production of microbial polyunsaturated fatty acids, with high nutritional value such as eicosapentaenoic and arachidonic acids, has also been investigated (Radwan, 1991; Shirasaka and Shimizu, 1995; Cheng *et al.*, 1999). However, as fermentation is an expensive process, it is perhaps not surprising that at present there is no large-scale process for the production of yeast fat and oil.

### 1.9.3 Single cell protein

In the past 25 years, intensive research has been conducted to find and develop cheaper sources of protein to alleviate the anticipated worldwide problem concerning the supply of food protein. The consumption of microbial protein, commonly referred to as single cell protein (SCP), could be an important alternative to prevent protein energetic malnutrition in developing countries (Konlani *et al.*, 1996). However, animal feedstuffs comprise a high protein content and the potential of SCP as animal feed would increase protein availability for human consumption. Waslien and Steinkraus (1980) recommended feeding microbial cells to animals thereby releasing for human consumption the cereal grains and legumes. Testing of SCP products on pigs and

chickens (Giec and Skupin, 1988) suggested that SCP could replace 10 - 20% of the protein in foodstuffs.

SCP is the manufacture of cell mass using microorganisms, typically fungi, by culturing on abundantly available agricultural and industrial wastes. After fermentation, biomass is harvested and may be subjected to downstream processing steps like washing, cell disruption, protein extraction and purification (Faust, 1987). Considerations for commercial operation include culture conditions, pretreatment of substrates, nutrient supplementation and type of fermentation process (Anupama and Ravindra, 2000). Also SCP, being a novel product, demands extensive sanitation and purification processes before the final product is cleared for consumption as per quality control standards. The US Food and Drug Administration and the Protein Evaluation Group of the United Nations have developed guidelines for the safety evaluation of SCP products in humans and domestic livestock (Litchfield, 1985).

Protein content of yeast biomass ranges between 30 to 70% of the cell weight (Anupama and Ravindra, 2000). Cultivation of yeast on lipid waste (Jacob, 1993; de Villiers and Pretorius, 2001) and crude oil wastes (Ashy and Abou-Zeid, 1982) produced SCP as a by-product of the waste treatment process at laboratory and pilot-scale. Commercial operations were developed across Europe in the 1970's with successful production of SCP with *Yarrowia lipolytica* from waste alkanes, although none are currently in operation (Ratledge, 1994). The similarity in mechanisms of growth of yeasts on alkanes and fatty acids (Ratledge, 1987) would indicate the potential of commercial SCP production on lipid wastes. This potential is underlined by a financial feasibility study on SCP production from slaughterhouse waste which indicated that it would be a profitable process (de Villiers and Pretorius, 2001). Although in this study, activated sludge was employed for biodegradation of the waste and SCP production, a similar process may be developed with yeast.

### 1.9.4 Biosurfactant production

Lipids, including waste oils and animal fats have been employed for the production of biosurfactants (Mercadé *et al.*, 1993; Desphande and Daniels, 1995; Mercadé *et al.*, 1996; Haba *et al.*, 2000). Biosurfactants have gained growing interest due to their unique applications in the pharmaceuticals, cosmetics, food processing and petroleum industries (Hu and Ju, 2001). Over their synthetic counterparts, biosurfactants as a group offer some distinct advantages: specificity, biodegradability, nontoxicty and a broad range of structures (Fiechter, 1992).

The focus of many commercial applications of biosurfactants is in oil and hydrocarbon bioremediation. Rhamnolipid from *Pseudomonas aeruginosa* has removed substantial quantities of oil from contaminated Alaskan gravel from the Exxon Valdez oil spill (Harvey *et al.*, 1990). Multi-biotech, a subsidiary of Geodyne Technology (USA), has commercialised biosurfactants, including those from *Bacillus* sp. for enhanced oil recovery (Desai and Banat, 1997). In many cases the biosurfactants produced from cultivation on lipids and hydrocarbons were not competitive against some commercial detergents such as lauryl sulfates. Nevertheless, they may be competitive against other commercial surfactants, including those involved in the food industry and cosmetic surfactants (Desphande and Daniels, 1995; Muriel *et al.*, 1996). Sophorolipid is commercially used by Kao Co. Ltd. (Japan) as a humectant for cosmetic makeup and finds application in lipstick, moisturiser and hair products (Desai and Banat, 1997). A novel biosurfactant from *Candida utilis* has shown potential in salad dressing (Shepord *et al.*, 1995). Many biosurfactants and their production procedures have been patented, but so far only a few have been commercialised. The economic limitation on commercial biosurfactant production can be overcome through the development of cheaper processes, the use of low-cost raw materials and increased product yields through superactive mutants and genetically engineered bacteria (Desai and Banat, 1997).

## 1.10 Aims of the Project

Given the particular problems associated with the disposal of hard fats generated by the food industry, it was of interest to develop a microbial system to treat waste streams containing tallow. The aims of the project were:

- To identify yeast species isolated from the wastewater treatment plant of a commercial rendering operation.

- To select a yeast with a superior ability to degrade tallow for further study.

- To optimise the growth conditions for the yeast on tallow in shake flask culture.

- To investigate the scale-up of the cultivation of the yeast on tallow using 2 L and 10 L fermenters.

- To determine the potential of the yeast biomass as a by-product of the fermentation.

# 2.0 MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Yeast isolates

The yeast cultures used in this study were previously isolated in the laboratory from a waste treatment system at a commercial rendering operation, Dublin Products Ltd., Dunlavin, Co. Wicklow, Ireland and from unsterilised tallow from the same operation (Fleming, 2002). The isolates were assigned a code beginning with NF, which referred to the non-filamentous appearance of the isolates on solid medium. A list of the organisms is detailed in Table 2.1. The code of the isolate is noted together with the source and the agar employed in its isolation.

**Table 2.1:** Source and code of the yeast isolates.

| Isolate Code | Source | Isolation agar |
|---|---|---|
| NF 9 | Wastewater | Nutrient |
| NF 10 | Wastewater | Nutrient |
| NF 12 | Wastewater | Nutrient |
| NF 27 | Non-sterile tallow | Nutrient |
| NF 32 A | Activated sludge | Olive oil |
| NF 32 B | Activated sludge | Olive oil |
| NF 48 | Activated sludge | Nutrient |
| NF 51 | Non-sterile tallow | Olive oil |
| NF 52 | Activated sludge | Olive oil |
| NF C | Non-sterile tallow | Olive oil |

### 2.1.2 Source of chemicals and materials

Chemicals and various medium components were obtained from a number of sources including BDH, LabScan, Oxoid, Reidel-de-Haen and Sigma-Aldrich. Mixed tallow was obtained from Dublin Products Ltd., Dunlavin, Co. Wicklow, Ireland.

### 2.1.3 Media

Bacteriological agar, malt extract agar and both, nutrient broth and purified agar were all obtained from Oxoid and were autoclaved at 121°C for 15 min, except in the case of malt extract agar and broth, which were autoclaved at 115°C for 10 min.

**Minimal medium**

The ingredients of the medium were adapted from Shikoku-Chem (1994) and were combined in distilled water and the pH adjusted to 7.0 with 2 M HCl. For buffered minimal medium, the components were dissolved in the respective buffer prior to sterilisation.

| Component | g $L^{-1}$ |
|---|---|
| $(NH_4)_2SO_4$ | 2.0 |
| $K_2HPO_4$ | 0.9 |
| $MgSO_4.7H_2O$ | 0.2 |
| Yeast extract | 0.1 |
| $CaCl_2.2H_2O$ | 0.05 |
| $FeSO_4.7H_2O$ | 0.02 |

**Nutrient broth**

The nutrient broth was prepared in accordance with the manufacturer's instructions. 10 ml aliquots of the broth were dispensed into glass universals prior to sterilisation by autoclaving at 121°C for 15 min.

**Olive oil agar**

The agar was composed of the components of the minimal medium, as outlined, which were dissolved in 0.1 M potassium phosphate buffer, pH 7.0. To this, 2% (w/v) olive oil and 2% (w/v) bacteriological agar were added. The agar was dissolved in the medium by heating and sterilised by autoclaving at 121°C for 15 min. Approximately 20 ml aliquots of the agar were dispensed into Petri dishes with constant swirling of the medium to ensure an even dispersion of the olive oil.

**Olive oil broth**

The broth comprised the components of the minimal medium dissolved in 0.1 M potassium phosphate buffer, pH 7.0. Olive oil (0.2 g) was added to 20 ml glass universals into which 10 ml aliquots of the medium was dispensed. The universals were sterilised by autoclaving at 121°C for 15 min.

### 2.1.4 Buffers

**Citrate phosphate buffer**

0.1 M citrate phosphate buffer, 100 ml, was prepared at a range of pHs (3.0 – 8.0). by the addition of $x$ ml of 0.2 M $Na_2HPO_4$ to $(100 - x)$ ml of 0.1 M citrate.

| **pH** | 3.0 | 4.0 | 5.0 | 6.0 | 7.0 | 8.0 |
|---|---|---|---|---|---|---|
| ***x* ml** | 81.1 | 61.9 | 49.0 | 37.5 | 17.8 | 2.8 |

**Potassium phosphate buffer**

0.1 M potassium phosphate buffer, 100 ml, was prepared by the addition of 30 ml of 0.2 M KOH to 50 ml 0.2 M $KH_2PO_4$ with volume correction to 100 ml with distilled water.

Increased concentrations of potassium phosphate buffer, 0.1 – 1.0 M, were prepared at pH 7.0 with the volumes indicated above but at increased concentrations of KOH and $KH_2PO_4$.

Concentrations of buffer and components (M)

| ***buffer*** | ***KOH*** | ***$KH_2PO_4$*** |
|---|---|---|
| 0.1 | 0.2 | 0.2 |
| 0.2 | 0.4 | 0.4 |
| 0.3 | 0.6 | 0.6 |
| 0.5 | 1.0 | 1.0 |
| 1.0 | 2.0 | 2.0 |

**Sodium phosphate buffer, $Na_2HPO_4$-$NaH_2PO_4$**

0.1 M sodium phosphate buffer, pH 7.0, was prepared by the equal additions of 61 ml of 0.2 M $Na_2HPO_4$ and 0.2 M $NaH_2PO_4$, and the resulting volume was corrected to 200 ml with distilled water.

**Sodium phosphate buffer, NaOH-$NaH_2PO_4$**

0.1 M sodium phosphate buffer, pH 7.0, was prepared by the addition of 30 ml 0.2 M NaOH to 50 ml 0.2 M $NaH_2PO_4$ and the resulting volume was corrected to 100 ml with distilled water.

**Sodium phosphate buffer (inoculum preparation)**

Sodium phosphate buffer (0.01 M) was prepared by dissolving $Na_2HPO_4$ (0.01 M) and $NaH_2PO_4$ (0.01 M) in distilled water. The pH of the resulting solution was adjusted to pH 7.0 with 2 M NaOH.

### 2.1.5 Stains

Crystal violet (0.01% w/v), malachite green (5.0 % w/v) and safranin (0.5% w/v) were prepared in distilled water to the required strength.

Methylene blue (0.01% w/v) was prepared by dissolving methylene blue (0.01g) together with tri-sodium citrate (1.0 g) in 10 ml of distilled water. A further 60 - 70 ml of water was added and the solution was thoroughly mixed and filtered through Whatman no.1 filter paper (to remove any undissolved solids). The volume was corrected to 100 ml with distilled water.

## 2.2 Methods

### 2.2.1 Identification of yeast isolates

#### 2.2.1.1 Tests used in yeast identification

All methods were in accordance with van der Walt and Yarrow (1984) unless otherwise stated.

***Ascospore test***

The test yeast was brought to a stage of active growth on malt extract agar for 2 days at 25°C. This plate was then used to inoculate a fresh malt extract agar plate which was incubated at 25°C for 3 days. A slide preparation of the yeast was made, and heat fixed. The slide was flooded with 5% (w/v) aqueous malachite green for 60 seconds, and heated to steaming 4 times. The excess stain was rinsed off with water and the slide was counterstained with 0.5% (w/v) aqueous safranin for 30 seconds. The slide was blotted dry and observed under x40 magnification. The ascospores stained blue-green and the vegetative cells red.

***Carbon assimilation***

Carbon addition

Small amounts of test carbon compound, 1 – 2 mg, were deposited at evenly spaced sites on the carbon assimilation agar with a sterile spatula. The addition sites were marked accordingly on the base of the Petri dish in permanent marker. The 19 carbon compounds tested included galactose, sucrose, maltose, cellobiose, trehalose, lactose, raffinose, soluble starch, xylose, galactitol, arabinose, ribitol, rhamnose, erithritol, ribose, manitol, succinic acid, citric acid and inositol. Each carbon test compound was assessed in triplicate. The plates were incubated at 25°C lid-side down.

Observation of assimilation

Results were observed after 2 days and confirmed after 3 days. Response to the assimilation of the carbon compounds was determined to be:

v = strong utilisation, + = rapid utilisation, +s = slow or latent utilisation, – = no utilisation and +/– or –/+s = inconclusive utilisation.

Carbon assimilation medium

20 ml aliquots of sterile molten purified agar were poured into Petri dishes containing one drop of sterile yeast extract (20% w/v) and 2 ml of yeast suspension. The liquid was thoroughly mixed and allowed to set. After solidification the plate was kept, lid side up, at 25°C for a few hours to obtain a dry agar surface.

20% (w/v) yeast extract

Yeast extract (20 g) was dissolved in 100 ml of distilled water, filter sterilised and 10 ml aliquots were dispensed into sterile plastic universals. The universals were stored at 4°C.

Yeast suspension

The yeast isolates were brought to a stage of active growth on malt extract agar for 2 days at 25°C. A yeast suspension was prepared from the mixing of a loopful of culture into 5 ml of sterile water.

***Cell morphology***

A colony from an actively growing malt extract plate was used to inoculate 30 ml of sterile malt extract broth in 100 ml cotton plugged Erlenmeyer flask. After 3 days incubation at 25°C the culture was examined microscopically. The culture was examined again after 2 weeks. Observations were made on cell size, shape and method of reproduction. Images of the yeast cells were taken with an image analysis system connected to an Olympus microscope under x40 magnification with an Optimas package (Version 6.5, Optimas Corporation).

***Colony morphology***

The test yeast was grown on malt extract agar, as outlined in the ascospore test. The colony morphology was examined after 7 days with a stereomicroscope (Nikon) and pictures of the plates were taken with a digital camera (Nikon) and a QV-link package (Version 2.6E, Casio). Observations were made on colony colour, texture, surface, form, elevation and margin.

***Cycloheximide resistance***

Inoculum preparation

The yeast was brought to a state of active growth by transferral twice onto malt extract agar and grown at 25°C over 2-day intervals. A loopful of the culture was added to 3 ml of sterile water in a test-tube. ¾ mm parallel lines were drawn onto a piece of white cardboard in Indian ink. The inoculum was diluted with sterile water until the lines were visible as dark lines through the test tube.

Inoculation of cycloheximide medium

A 50 µl aliquot of the inoculum was added to the cycloheximide medium (100 and 1000 ppm). The tubes were mixed and incubated at 25°C at 150 rpm for 3 weeks.

Observation of growth

Growth was observed on days 7, 14 and 21. The tubes were thoroughly mixed to disperse yeast growth and viewed against the white cardboard with lines drawn in Indian ink. Where the growth in the tubes completely obliterated the lines, it was recorded as 3+; if the lines appeared as diffuse bands, the growth was rated as 2+; if the bands were distinguishable as such but had indistinct edges, it was recorded as 1+; while the absence of growth is indicated as −.

A 2+ or a 3+ reading on the 7$^{th}$ day of incubation was scored as positive (+). If a 2+ or a 3+ reading was delayed until the 14$^{th}$ or 21$^{st}$ day, the reading was scored as slow (+s). A 1+ reading after 7 days was also determined to be slow (+s).

Cycloheximide basal medium

0.1 g (for 100 ppm) or 1.0 g (for 1000 ppm) of cycloheximide was dissolved in 2.5 ml acetone. The acetone solution was added to 6.7 g yeast nitrogen base and 10 g glucose in 100 ml water. The mixture was thoroughly mixed and filter sterilised. In preparation for use, 0.5 ml aliquots of the concentrated medium were added into 4.5 ml sterile water in test tubes.

***Diazonium Blue B (DBB) colour test***

This test was carried out in accordance with Barnett *et al.* (1983).

A malt extract agar plate was lightly inoculated with the yeast and incubated for 7 days at 25°C. The plates were then incubated at 55°C for several hours and

flooded with ice-cold Diazonium Blue B reagent. If the culture turned dark red within 2 min, this was recorded as a positive result.

Diazonium Blue B reagent

The reagent was prepared freshly by dissolving 15 mg of Diazonium Blue B salt in 15 ml chilled 0.25 M Tris buffer, pH 7.0. The reagent was kept in an ice bath and used within 30 min. If the reagent turned dark yellow, it was discarded and a fresh reagent was prepared.

***Fermentation of carbohydrates***

Inoculation of fermentation medium

1 ml aliquots of the sterile test sugars were added to the tubes of fermentation medium aseptically. The medium was inoculated with 0.1 ml of a yeast suspension. The tubes were incubated at 25°C and observed over a 14-day period.

Observation of fermentation

The tubes were shaken regularly and observations on production of gas in the Durham tubes and for change in the indicator colour were made. The fermentation rating was dependent on the time required for the formation of gas as:

| | |
|---|---|
| + | strong, with gas filling the Durham tube within 1 - 3 days |
| +w | weak, with the Durham only partially filled |
| +vw | very weak, with only a bubble formed inside the Durham tube |
| +s | slow or delayed, but with gas filling the Durham tube |
| - | absent |

Yeast suspension

A loopful of yeast colony from a 48 hour malt extract agar plate was mixed with 4.5 ml of sterile distilled water.

Fermentation basal medium

4.5 g yeast extract together with 7.5 g peptone was dissolved in 1 L distilled water. Bromothymol blue was added until a dense green colour resulted. 2 ml aliquots were added to test-tubes containing inverted Durham tubes. The test-tubes were sterilised by autoclaving at 121°C for 15 min.

Preparation of sugars

6% (w/v) aqueous solutions of the test sugars: glucose, galactose, sucrose, maltose and lactose and a 12% (w/v) solution of raffinose were prepared and filter sterilised. The sterile solutions were kept refrigerated until use.

***Growth at 37°C***

The test yeast was streaked onto a malt extract agar plate and incubated at 37°C for 2 days. In the case of weak growth, a subculture was made and incubated for 2 days at 37°C, where the results of the latter test were taken as decisive.

***Growth in 10% sodium chloride-5% glucose medium***

Inoculum preparation

The yeast was brought to a state of active growth by transferral twice onto malt extract agar and grown at 25°C over 2-day intervals. A loopful of the culture was placed into 3 ml of sterile water in a test-tube. ¾ mm parallel lines were drawn onto a piece of white cardboard in Indian ink. The inoculum was diluted with sterile water until the lines were visible as dark lines through the test tube.

Inoculation of the medium

Yeast inoculum (0.1 ml) was added to tubes containing 10% sodium chloride - 5% glucose medium. The tubes were incubated at 25°C and 150 rpm for 21 days.

Observation of growth

Growth was assessed on day 7 and 21. The tubes were thoroughly mixed to disperse yeast growth and viewed against the white cardboard with lines drawn in Indian ink. After 7 days, where growth completely obliterated the lines, it was recorded as 3+ and growth determined to be vigorous (v); if the lines appeared as diffuse bands, it was recorded as 2+ and cell growth was regarded as good (+); if the bands were distinguishable as such but had indistinct edges, it was recorded as 1+ ; while the absence of growth was indicated as negative (–).

A negative or 1+ reaction after 7 days followed by a 2+ or 3+ reaction after 21 days was regarded as slow (+s).

10% sodium chloride-5% glucose medium

5 g glucose and 10 g sodium chloride were dissolved in 100 ml distilled water. 4.5 ml aliquots were dispensed into test tubes and sterilised by autoclaving at 121°C

for 15 min. When cool, 0.5ml of a filter-sterilised solution of 6.7% (w/v) yeast nitrogen base (Sigma) was added to each tube and the contents were thoroughly mixed.

***Growth in 50% glucose-yeast extract medium***

The 50% glucose-yeast extract agar slants were lightly inoculated from an actively growing culture and incubated at 25°C for 5 days. Growth was observed as vigorous (v), good (+), slow (+s) or negative (–).

50% glucose-yeast extract medium

A 1% (v/v) solution of yeast extract was prepared in 50 ml distilled water. Glucose (50 g) was dissolved in the solution and the final volume was adjusted to 100 ml. Bacteriological agar (3% w/v) was added to the solution and dissolved by heating. 5 ml aliquots were dispensed into test tubes and sterilised by autoclaving at 110°C for 10 min to prevent caramelisation of the glucose. The tubes were slanted and allowed to cool.

***Liquid morphology***

A colony from an actively growing malt extract plate was used to inoculate 30 ml of sterile malt extract broth in 100 ml cotton plugged Erlenmeyer flask. Observations on cultural characteristics were made after 1 week and included the formation of a sediment, pellicle, ring or surface growth.

***Nitrate assimilation***

20 ml aliquots of sterile molten nitrate assimilation agar were poured into Petri dishes containing one drop of vitamin stock solution and 2 ml of yeast suspension. The liquid was thoroughly mixed and allowed to set. A small amount of potassium nitrate was added to the plates and the position was marked on the Petri dish base in permanent marker. The plates were incubated for 3 days at 25°C.

Observation of assimilation

Results were observed after 2 days and confirmed after 3 days. Response to the assimilation of nitrate was determined to be:

v = strong utilisation, + = rapid utilisation, +s = slow or latent utilisation,

– = no utilisation and +/– or –/+s = inconclusive utilisation.

Yeast suspension

The yeast isolates were brought to a stage of active growth on malt extract agar for 2 days at 25°C. A yeast suspension was prepared by mixing a loopful of culture into 5 ml of sterile water.

Nitrate assimilation basal medium

The medium was composed of: 0.1% (w/v) $K_2HPO_4$, 0.05% (w/v) $MgSO_4.7H_2O$ and 2% (w/v) purified agar which were dissolved and autoclaved for 15 min at 121°C. A 2% (w/v) solution of glucose was sterilised separately and added to the basal medium after sterilisation aseptically to prevent caramelisation of the sugar.

Vitamin stock

A stock vitamin solution was prepared composed of the outlined components and filter sterilised. The stock was dispensed into 10 ml aliquots and stored at -10°C.

| Component | mg $L^{-1}$ | Component | mg $L^{-1}$ |
|---|---|---|---|
| Biotin | 0.2 | Niacin | 40 |
| Calcium pantothenate | 40 | *p*-aminobenzoic acid | 20 |
| Folic acid | 0.2 | pyridoxine hydrochloride | 40 |
| Inositol | 200 | Riboflavin | 20 |
| Thiamine | 100 | | |

***Production of ammonia from urea (urease test)***

The test yeast was lightly inoculated onto a urea agar slope from a malt extract plate and incubated at 25°C for 24 hours. A positive reaction resulted from the formation of a deep pink colour.

Urea agar

The following components were dissolved in 1 L distilled water and the pH was adjusted to pH 6.8.

| Component | g $L^{-1}$ |
|---|---|
| Peptone | 1.0 |
| Glucose | 1.0 |
| NaCl | 5.0 |
| $KH_2PO_4$ | 2.0 |
| Phenol red | 0.012 |

Bacteriological agar (2% w/v) was dissolved in the solution. 9 ml aliquots were dispensed into glass universals and sterilised by autoclaving at 121°C for 15 min. A 20% (w/v) solution of urea was prepared and the solution was filter sterilised. 1 ml of the sterile urea solution was added to the agar immediately after autoclaving and mixed. The tubes were then slanted and allowed to set.

#### 2.2.1.2 Identification scheme for yeast isolates

The yeast identification scheme was based on keys devised by Barnett *et al.* (1983) which comprised both physiological and morphological responses of yeasts. The selection of the key for identification was based on the response of the yeast to D-glucose fermentation. Key No. 1 corresponded to all yeasts that did not ferment D-glucose (Appendix A). Key No. 3 corresponded to all yeasts that did ferment D-glucose (Appendix B). The keys consisted of a list of numbered tests where the number referred to a test in the respective key and instructions for a negative or a positive response to that specific test. The response of the isolate to the test was recorded as negative or positive. The instructions in the key for the response gave either the number of the next test in the identification scheme or the identity of the yeast. Where the result of the test was not known or the response was not conclusive (both positive and negative responses), then the scheme was followed for both the negative and positive response. If the scheme resulted in the identification of the yeast as more than one possible organism, the identity of the isolate was based on all the physiological and morphology tests employed. Yeast identification characteristics were compared to those detailed in Barnett *et al.* (1983).

### 2.2.2 Yeast maintenance

The yeast culture(s) was maintained on malt extract agar at 4°C, with routine sub-culturing for 48 h at 25°C every three weeks. In the inoculum preparation investigation, the yeast was also maintained on olive oil agar and sub-culturing on both olive oil and malt extract agars was performed weekly for a period of six weeks prior to the investigation.

### 2.2.3 Inoculum preparation

A loopful of yeast was taken from a malt extract or olive oil agar plate and grown for 24 h at 30°C and 150 rpm in 10 ml nutrient or olive oil broth. After 24 h, the broth(s) was centrifuged at 4000 rpm for 10 min and the pellet was washed with 10 ml of 0.01 M sodium phosphate buffer. The inoculum was centrifuged again at 4000 rpm for 10 min and the supernatant discarded. Where nutrient broth was used to grow the inoculum, the pellet was resuspended in the buffer to give an optical density of 1.000 at 660 nm. Where olive oil broth was employed, the pellet was resuspended in the buffer to give a cell number of 2.5 x $10^7$ cell $ml^{-1}$ (approximately).

### 2.2.4 Measurement of growth

#### 2.2.4.1 Cell number and cell viability

Cell number was reported as the total number of viable cells per ml. Cell viability was determined using the citrate methylene blue staining technique (Pierce, 1971). After a suitable dilution to give a countable number of cells, 0.5 ml cell suspension was added to 0.5 ml 0.01% (w/v) methylene blue, vortexed and allowed to incubate for 5 min prior to counting on a Neubauer haemocytometer. Dead cells stained blue whereas viable cells appeared colourless. A minimum of 250 cells was counted and the number of viable cells to total number counted was expressed as a percentage based on the average of three counts.

#### 2.2.4.2 Cell dry weight

A 20 ml sample was centrifuged at 4000 rpm for 20 min and the supernatant was retained for fat removal analysis. The cell pellet was washed with 0.9% (w/v) NaCl and centrifuged at 4000 rpm for 20 min to remove any fat trapped in the pellet in accordance with Lee and Rhee (1994). The cell pellet was resuspended in water and filtered through a pre-weighed Whatman GF/C filter. The filter paper was rinsed once with *n*-hexane to remove any residual fat trapped on the filter. The filter paper was dried overnight at 80°C. The filter paper was re-weighed and the difference in weight due to the cells was multiplied by a factor of 50 to convert the cell dry weight to g $L^{-1}$.

### 2.2.5 Measurement of mycelium ratio

A drop of 0.01% (w/v) crystal violet was added to 1 ml sample from the growth vessel and the suspension was mixed. The stain was added to improve the definition of the yeast cells under microscopic observation. The cells were viewed under x20 magnification, unless otherwise stated, with an Olympus microscope using an image analysis system with an Optimas package (Version 6.5, Optimas Corporation). Images were taken of 9 fields for each sample and the number of mycelial cells calculated as a percentage of the total number of cells present. Cells were considered as mycelial, where they had a well-defined slender germ tube longer than the large diameter of the mother cell (Guevara-Olvera *et al.*, 1993).

### 2.2.6 Growth studies

The three growth vessels employed in this study, 250ml shake flask, 2 L bench-top fermenter and 10 L pilot-scale fermenter, were inoculated with the yeast culture(s) at a concentration of 2% (v/v). Tallow was added to the medium at a concentration of 2% (w/v) in all investigations. Each batch of tallow was assessed for free fatty acid (FFA) content and only tallow that contained less than 7% FFA was used. Samples were aseptically removed at regular intervals and growth, cell morphology and pH were monitored. Substrate removal was assessed during the growth or at the end of the growth period, as specified for each investigation. Other parameters assessed included intracellular lipid content, biosurfactant analysis including surface tension, emulsification activity and emulsion stability of the medium, medium and intracellular potassium measurements, settled sludge volume, intracellular protein and total nitrogen measurement. These parameters are specified for the appropriate growth conditions investigated. Controls, which were not inoculated, were incubated in parallel in order to monitor any physiological changes in tallow and/or the medium. Photographs of the growth vessels were taken using a digital camera (Nikon) and a QV-link computer package (Version 2.6E, Casio).

### 2.2.6.1 Shake flask studies

Growth studies in shake flask culture were performed in 250 ml Erlenmeyer flasks containing a final culture volume, following inoculation, of 100 ml. The minimal medium and tallow were sterilised by autoclaving at 121°C for 15 min. After inoculation, the flasks were incubated for up to 240 hours at 25°C and 130 rpm in a thermostat controlled room on an orbital shaker (Gallenkamp, Sanyo), except where the effects of altering these environmental conditions was investigated. In such cases, the appropriate environmental conditions were specified. Where growth temperatures of 4°C and 30°C were required, growth studies were performed in thermostat controlled rooms with orbital agitation. Temperature was controlled to 37°C in an orbital incubator (Gallenkamp, Sanyo) and a lidded shaking water bath (Gallenkamp, Sanyo) was employed for temperature control to 55°C. Baffled agitation was achieved through the presence of 4 internal baffles in the Erlenmeyer flask. Surfactants and nitrogen sources were added to the medium prior to sterilisation. Glucose was sterilised by autoclaving at 121°C for 15 min separate to the medium to prevent caramelisation, and then added to the sterile medium prior to inoculation. Where pH was controlled, this was achieved through the addition of 0.1 M potassium phosphate buffer, pH 7.0, except where the buffer, pH and concentration were investigated. In such cases, the conditions were specified and medium contents were dissolved in the respective buffers prior to sterilisation. pH was measured using an Orion Triode™ pH electrode Model 91-57BN connected to an Orion benchtop pH/ISE meter (model 920A). Fat removal was determined either during the course of the fermentation or at the end and is specified for each investigation. Glucose removal was monitored during the fermentation. All shake flask investigations were performed in triplicate.

### 2.2.6.2 2 L bench-top fermenter studies

#### Fermenter configuration

In 2 L fermentations, the yeast was cultivated in a baffled bench-top fermenter (B. Braun Biotech. International), in a working volume of 1 L (maximum working volume of 2 L). Air was supplied to the fermentation medium by an aquarium air pump and was filtered through a sterile air filter (0.22 μm). The air was

introduced by a sparger, centrally positioned, 10 mm below the impeller. Agitation was supplied by a motor (B. Braun Biotech. International), coupled to a drive shaft, which was fitted with two 6-bladed Rushton impellers positioned at the base of the drive shaft. Temperature control to 25°C ± 2°C in the vessel was implemented using a circulating water bath connected to the fermenter. The configuration and dimensions of the 2 L fermenter are presented in Fig. 2.1.

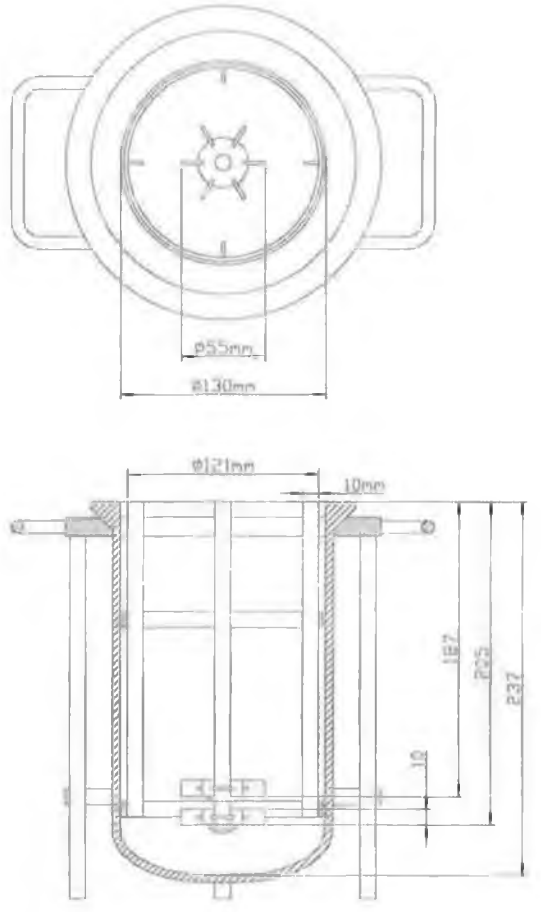



**Figure 2.1:** Schematic of the 2 L fermenter configuration. Dimensions are recorded in mm.

**Fermenter set-up**

The minimal medium and the tallow was sterilised in the fermenter, including associated air filters, by autoclaving at 121°C for 15 min. The fermenter was inoculated through one of the top ports. Agitation was set at 500 rpm and aeration at 1.0 VVM (based on initial medium volume), except where the effects of altering these environmental conditions were being investigated. In such cases, the appropriate environmental conditions were specified. pH was controlled to pH 7.0 with 0.1 M potassium phosphate buffer and medium contents were dissolved in

the buffer prior to sterilisation, except where the method of pH control was investigated. pH control was implemented through the inclusion of phosphate salts, 13.6 g $L^{-1}$ $KH_2PO_4$ or 12.0 g $L^{-1}$ $NaH_2PO_4$, prior to sterilisation of the medium. In both cases, 0.5 M NaOH (approximately 10 ml) was added to the medium prior to inoculation to correct the pH to 7.0, with subsequent periodic addition during the fermentation to maintain neutral pH (approximately 5 ml). The control of medium pH was also implemented through the addition of 0.5 M NaOH, KOH or $Ca(OH)_2$. Where base addition was required for pH control, 500 ml corrective agent bottles were used which were sterilised, together with silicone tubing by autoclaving at 121°C for 15 min. A peristaltic pump was employed for the periodic addition of the base(s) into the fermenter, through one of the top ports, after inoculation. Where pH measurement was *in situ*, the pH probe, Orion Triode™ pH electrode Model 91-57BN connected to an Orion benchtop pH/ISE meter (model 920A), was calibrated externally according to the manufacturer's instructions, rinsed once with IMS, and inserted into the medium after inoculation via one of the top ports. The port was plugged with sterile cotton wool and no contamination of the vessel was observed. Foam formation was controlled through the periodic addition of sterile antifoam (Antifoam A, Sigma). The antifoam was sterilised in a 250 ml corrective agent bottle together with silicone tubing by autoclaving at 121°C for 15 min. Antifoam was pumped into the vessel by a peristaltic pump through one of the top ports. The yeast was cultivated for up to 65 hours, by which time the volume in the vessel had reduced by half to approximately 500 ml. Fat removal was determined at the end of the fermentation. All 2 L fermenter investigations were performed in duplicate.

#### 2.2.6.3 10 L pilot-scale fermenter studies

**Fermenter configuration**

In 10 L fermentations, the yeast was cultivated in a pilot-scale baffled fermenter (Biostat C, B. Braun Biotech. International) in a working volume of 5 L (maximum working volume of 10 L). Air was supplied to the medium through a sterile filter (0.22 μm) at a rate of 1 VVM (based on the initial medium volume) into a sparger centrally positioned below the bottom impeller. Agitation was

supplied at a rate of 1000 rpm, which was scaled up based on constant mixing in the 2 L and 10 L fermenter, as detailed in Section 2.2.6.4. Agitation was provided by a drive shaft fitted with two 6-bladed Rushton impellers, placed 135 mm apart, unless otherwise stated. Temperature was controlled to 25°C ± 1°C via jacketed closed thermostat system. The operation was controlled and the pH and dissolved oxygen levels recorded with a digital control unit in combination with a MFCS/Win software package (B. Braun Biotech., International). The configuration and dimensions of the 10 L fermenter are presented in Fig. 2.2.

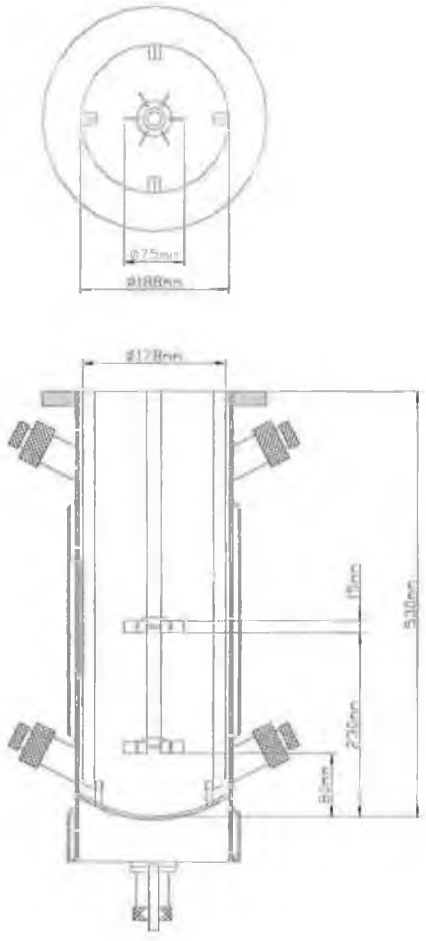


**Figure 2.2:** Schematic of the 10 L fermenter configuration. Dimensions are recorded in mm.

**Fermenter set-up**

Prior to medium addition, the dissolved oxygen probe (Mettler Toledo) was polarised and the pH probe (Mettler Toledo) was calibrated externally according to the manufacturer's instructions and each placed into one of the side ports. Sterilisation of the fermenter, minimal medium and tallow was performed *in situ*

at 121°C for 15 min. pH was controlled to pH 7.0 in all cases. This was achieved by 0.1 M potassium phosphate buffer or through the automated addition of 0.5 M KOH/HCl. In the latter case, only base addition was required to control pH, but HCl was included in the fermenter set-up to counteract any pH overshoot through the addition of the base. Where the addition of KOH/HCl and antifoam or the inoculum was required, 500 ml and 250 ml corrective agent bottles were employed, respectively. These bottles, together with silicone tubing and stab needles, were sterilised by autoclaving at 121°C for 15 min. The inoculum was pumped into the fermenter manually via peristaltic action through an inoculum line, which added the yeast directly into the medium. KOH/HCl were pumped into the fermenter for automated pH control via peristaltic action through an inoculum line, which allowed them to enter the vessel directly into the medium. Antifoam (Antifoam A, Sigma) was pumped in on top of the medium periodically via manual addition, to allow for foam breakdown. The yeast was cultivated for up to 65 hours by which time the volume was reduced to approximately 2.5 L. Fat removal was determined at the end of the fermentation, after 65 h. All 10 L investigations were performed in triplicate.

#### 2.2.6.4 Scale-up of agitation from 2 L to 10 L fermenter

Scale-up of the rate of agitation from the 2 L to the 10 L fermenter was based on constant mixing. $N_i t_m$ is a dimensionless number which represents the number of stirrer rotations required to homogenise the liquid for a baffled tank with a Rushton turbine (Doran, 1995). At high impeller Reynolds number ($Re_i$), above 5 x $10^3$, $N_i t_m$ approaches a constant value which persisted at high $Re_i$. Under these conditions, their relationship is represented by Eqn. [1].

$$N_i t_m = \frac{1.54V}{D_i^3} \text{ at high } Re_i \quad [1]$$

where: $t_m$ = mixing time (m); $D_i$ = impeller diameter (mm); $N_i$ = agitation rate (rpm); $Re_i$ = impeller Reynolds number; V = liquid volume (L)

It was assumed that the flow in the 2 L fermenter was fully turbulent and that the impeller Reynolds number was high. Eqn. [1] was rearranged based on the mixing time to give Eqn. [2]. In the determination of the rate of agitation required in the 10 L fermenter, constant mixing in both fermenters was applied (Eqn. [3]) and the equation was rearranged for agitation in the 10 L fermenter (Eqn.s [4] & [5]). The respective parameters were applied and the agitation rate for scale-up to 10 L fermenter was calculated as approximately 1000 rpm (Eqn. [6]).

$$t_m = \frac{1.54V}{D_i^3 N_i} \qquad [2]$$

$$t_{m2L} = t_{m10L} \qquad [3]$$

$$\frac{1.54V_{2L}}{D_{i2L}^3 N_{i2L}} = \frac{1.54V_{10L}}{D_{i10L}^3 N_{i10L}} \qquad [4]$$

$$N_{i10L} = N_{i2L}\left(\frac{V_{10L}}{V_{2l}}\right)\left(\frac{D_{i2L}}{D_{i10L}}\right)^3 \qquad [5]$$

$$N_{i10L} = 500 \text{ rpm}\left(\frac{5L}{1L}\right)\left(\frac{55mm}{75mm}\right)^3 \qquad [6]$$

$$N_{i10L} \simeq 1000 \text{ rpm}$$

### 2.2.7 Measurement of glucose

Glucose was determined by the DNS method (Miller, 1959). Cells were removed from the samples by centrifugation at 4000 rpm for 10 min before assaying. 1 ml standard or suitably diluted sample and 1 ml distilled water was placed in test tube. 2 ml of DNS reagent was added. The tubes were capped, vortexed and placed in a boiling water bath for 10 min. The tubes were then rapidly cooled and 10 ml distilled water was added and the tubes were vortexed again. Absorbance was read at 540 nm. Glucose (0 - 1.5 mg $ml^{-1}$) was the standard. A sample standard curve for the DNS assay is presented in Fig. 2.3.

DNS reagent: 1 g 3,5 dinitrosalicylic acid
30 g KNa tartrate ( for long term storage)
50 ml distilled water
16 ml 10 % (w/v) NaOH

Warm to dissolve (DO NOT BOIL). When dissolved, cool and make up to 100 ml with distilled water.

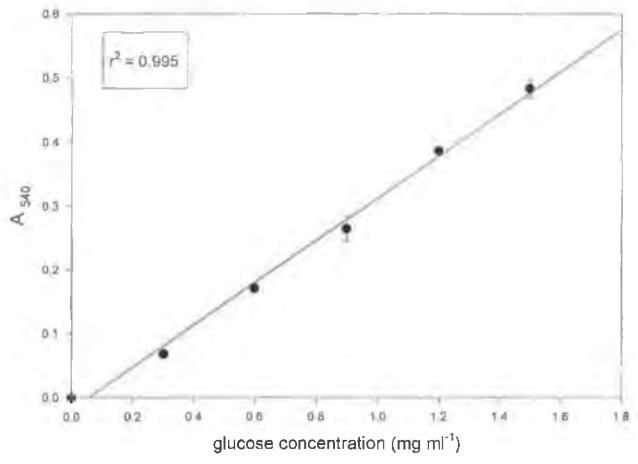



**Figure 2.3:** Standard curve for glucose using the DNS assay.

### 2.2.8 Measurement of percentage free fatty acids in tallow

Tallow (approximately 2 g) was weighed into a 100 ml Erlenmeyer flask and the actual weight added noted. The tallow was warmed in 10 ml IMS. The free fatty acids in the tallow were then titrated against 0.1 M NaOH. Titration end-point was determined with the addition of phenolpthalein indicator and a colour change to pink. The volume of alkali addition was noted. The percentage free fatty acids were calculated based on the equation:

$$\% \text{ free fatty acids} = \frac{100 \text{ x ml of alkali x N x } 282}{1000 \text{ x weight of sample}}$$

where: N = the normality of NaOH, 0.1 mol $L^{-1}$ 1000 = conversion from ml to L
282 = the molecular weight of oleic acid, g $mol^{-1}$ 100 = conversion for percentage

### 2.2.9 Measurement of fat removal

The degree of fat or tallow removal was determined by an extraction-gravimetric method based on that described by Shikoku-Chem (1994) and Koritala *et al.* (1987). The contents of the flask or fermenter (dispensed into 250 ml Erlenmeyer flasks) were acidified to pH 2.0 or lower with 2 M HCl to prevent any further hydrolysis activity and to aid in the fat extraction. The contents were then warmed to melt the tallow and added to a separatory funnel. The flask was rinsed with *n*-hexane and added to the separatory funnel. The contents and washings were extracted twice with equal volumes of *n*-hexane and the solvent layer collected in glass universals. The aqueous layer was collected and extracted again with the solvent. The organic layer was collected and added to the solvent extract. The solvent extract was then centrifuged at 4000 rpm for 8 min to separate the cellular layer, which was then removed. The solvent extract was filtered through anhydrous sodium sulphate into a preweighed 250 ml round-bottomed flask. The solvent was removed through rotary vacuum evaporation (Büchi, R110) at 80°C, and the flask was further dried at 50°C until all the solvent was removed and a constant weight was achieved. When cool, the flask was weighed and the degree of fat removed determined based on the initial concentration of tallow in the medium (20 g $L^{-1}$).

### 2.2.10 Intracellular analysis

#### 2.2.10.1 Preparation of freeze-dried cells

Cell pellets for freeze-drying were prepared in the same way as for dry weight determination. The washed pellet was covered in parafilm and some small puncture holes were made to prevent the parafilm from being removed in the vacuum. The pellet was frozen at –80°C for 1 h prior to being freeze-dried overnight using a vacuum freeze-drier (Labcono).

#### 2.2.10.2 Measurement of intracellular lipid

Intracellular lipid measurement was based on the method of Bligh and Dyer (1959). The freeze-dried yeast pellet was resuscitated in 4 ml water. 10 ml

methanol and 5 ml chloroform were added to the suspension, vortexed and left for 1 hour, after which 5 ml water and 5 ml chloroform were added to complete the extraction. The bottom solvent layer was collected and centrifuged at 2000 rpm for 5 min to separate the chloroform layer from the cellular debris. The top layer was discarded and the bottom layer was filtered through anhydrous sodium sulfate into a preweighed 50 ml round bottomed flask. The solvent was removed from the flask by rotary vacuum evaporation (Büchi, R110) at 60°C and the flask contents were further dried at 60°C for 1 hour. When cool, the flasks were weighed and the differential multiplied by 50 to convert the intracellular lipid to g $L^{-1}$.

#### 2.2.10.3 Cell disruption with glass beads

This method was modified from that described by Pereira-Merielles *et al.* (1997). The reagent for cell disruption comprised 0.1 M MOPS buffer, pH 7.0, with 0.02 M EDTA and 0.05 M mercaptoethanol. The freeze-dried cell pellet was resuscitated in water and washed once with water and once with the buffer solution. The cell pellet was suspended in 1 ml buffer or according to 0.5 ml reagent per 50 mg cell pellet. Glass beads, size 40 mesh, were added to the level of the liquid and the suspension was vortexed for 5 x 1 min cycles. Samples were kept on ice for 1 min between cycles. The suspension was then transferred to a 10 ml polyproplyene column (Pierce) containing a foam sinter and centrifuged at 4000 rpm for 10 min into an Eppendorf tube.

#### 2.2.10.4 Measurement of intracellular protein

The protein content in the disrupted cell suspension was determined by the method of Lowry *et al.* (1951). Reagent A (2.5 ml) was added to 0.5 ml of a suitable diluted sample or standard in an acid washed test-tube. The tubes were left for 10 min in the dark at room temperature. Reagent B (0.125 ml) was then added and the tubes were left for 25 min at room temperature in the dark. The tubes were then mixed by vortexing and left for a further 5 min. The absorbance was read at 600 nm. Standards were prepared with bovine serum albumin (0 – 1 mg $ml^{-1}$). A sample standard curve for the Lowry assay is presented in Fig. 2.4.

Reagent A: 50 ml 0.2% (w/v) $Na_2CO_3$ in 0.1 M NaOH
0.5 ml 1% (w/v) $CuSO_4$
0.5 ml 2% (w/v) NaK tartrate

Reagent B: 50% (v/v) Folin-Ciolcalteau in water

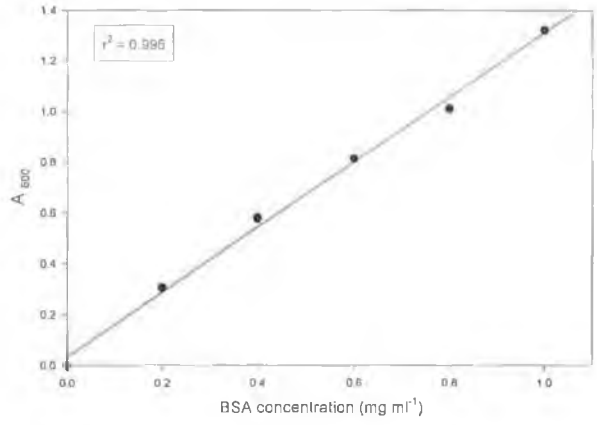



**Figure 2.4:** Standard curve for protein using Lowry assay.

#### 2.2.10.5 Measurement of cellular total nitrogen

The total nitrogen in the cells was determined from a suitable dilution of the disrupted cell suspension on a Dohramm DC190 TOC analyser with TN attachment.

### 2.2.11 Biosurfactant measurement

#### 2.2.11.1 Surface tension measurement

A cell free extract of the growth medium was prepared by filtration of the sample through a 0.2 μm Acrodisc (Pall) filter (Cirigliano and Carman, 1984). The surface tension of the extract and subsequent dilutions in distilled water were determined using a Torsion balance (White Ltd., England) fitted with a platinum ring, 4 cm diameter in accordance with Muriel *et al.* (1996). Measurements were based on a mean of 6 readings. The ring was passed through a flame between readings to remove residual sample. Fresh glassware, which had been washed with tap water, chromic acid and distilled water was used for each sample.

#### 2.2.11.2 Measurement of relative biosurfactant concentration

This method was adapted from that described by Persson and Molin (1987). A serial dilution of the medium (cell free) in distilled water was made and the respective surface tensions of the dilutions measured. Dilution of the medium was based on a fraction of the medium concentration where 1.0 (v/v) represented no dilution of the medium and 0.5 (v/v) represented a 1 in 2 dilution of the medium, *etc.* The critical micelle dilution (CMD) was the dilution fraction of the medium, which did not result in an increase in the minimum surface tension of the neat medium (1.0 v/v). An inverse of this dilution fraction ($CMD^{-1}$), indicated the relative concentration of the biosurfactant in the medium.

#### 2.2.11.3 Emulsification measurement

Emulsification activity measurement was based on that described by Muriel *et al.* (1996). Cell free extract (4 ml) prepared as for surface tension measurements, was added to 1 ml kerosene and vortexed for 2 min. The resultant oil in water emulsion was allowed to sit for 10 min after which its absorbance, through 1 cm pathway, was measured at 540 nm at intervals for up to 1 hour. The absorbance reading after 1 hour indicated the emulsification activity of the (bio)surfactant.

The decay constant, $K_d$, was calculated from the slope of the line of the log of absorbance over 20 – 60 min. The $K_d$ indicated the stability of the emulsion formed between the (bio)surfactant and kersosene and the smaller the $K_d$ value, the greater the stability of the emulsion.

#### 2.2.11.4 Validation of biosurfactant measurement

The validity of the methods of biosurfactant measurement as surface tension, emulsification activity and stability were assessed with commercial surfactants, Triton X-100 and SDS at their critical micelle concentrations (CMC) of 0.18 and 2.31 mg $ml^{-1}$, respectively. The resultant measurements and those reported by Muriel *et al.* (1996), for the two surfactants at the same concentration are detailed in Table 2.2.

**Table 2.2:** Surface tension, emulsification activity and stability values of the surfactants Triton X-100 (0.18 mg $ml^{-1}$) and SDS (2.31 mg $ml^{-1}$).

| Parameter | *Muriel et al. (1996)* | | *This study* | |
|---|---|---|---|---|
| | Triton X-100 | SDS | Triton X-100 | SDS |
| Surface tension (dynes $cm^{-1}$) | 32 | 33 | 32.0 ± 0.3 | 33.0 ± 0.1 |
| Emulsification activity ($A_{540}$) | 0.7 | 0.15 | 0.71 ± 0.03 | 0.16 ± 0.07 |
| Emulsion stability (decay ratio, $K_d \times 10^{-3}$) | -3.75 | -2.68 | -3.77 ± 0.03 | -2.66 ± 0.07 |

### 2.2.12 Metal analysis

Potassium was analysed using a Perkin-Elmer 3100 atomic absorption spectrophotometer, fitted with a 10 cm single slot burner head with an air-acetylene flame. Metal concentrations were determined by reference to appropriate standard metal solutions, prepared from potassium salt (KCl) dissolved in distilled deionised water. Metal concentration in the medium was determined from a suitable dilution in distilled deionised water of a cell free sample. Cellular metal concentration was determined from suitable dilution of supernatant from an acid digest of the cells. 5 ml aliquot of the cell suspension was centrifuged at 4000 rpm for 20 min and washed twice with distilled deionised water. The cell pellet was digested for 1 h at 100°C in 2 ml of 6 M $HNO_3$ with 1 ml water.

### 2.2.13 Sludge Volume Index (SVI)

The sludge volume index (SVI) was determined based on the method outlined in Standard Methods for the Examination of Water and Wastewater (Greenberg *et al.*, 1998). SVI is the volume in ml occupied by 1 g of a suspension after 30min settling. The index is calculated as:

$$SVI = \frac{\text{settled sludge volume (ml } L^{-1})}{\text{suspended solids (g } L^{-1})}$$

1 L of the mixed liquor (after 65 h) was dispensed into a plastic 1 L graduated cylinder. The medium was inverted in the cylinder three times and allowed to settle for 30 min. After this time, the volume of settled sludge was recorded in ml. The suspended solids were determined as outlined in the measurement of dry weight where:

$$\text{g suspended solids } L^{-1} = \frac{(A - B) \times 1000}{\text{sample volume, ml}}$$

where: A = weight of filter and dried residue, g
B = weight of filter, g

### 2.2.14 Data analysis

Calculation of slopes and standard errors were determined using a Sigma Plot package (Version 1.02, Jandel Corporation). Experiments and analyses, unless otherwise stated, were performed in triplicate and the mean values were presented with the standard error mean. Error bars were shown on figures where their size was greater than that of the symbols used.

***Growth rate***

Growth rate ($h^{-1}$), $\mu$, was calculated based on the number of cells or cell dry weight produced per hour during exponential growth as:

$$\mu = \frac{\ln N_2 - \ln N_1}{t}$$

Where $N_2$ = cell density at time 2 (at end of growth period)
$N_1$ = cell density at time 1 (at beginning of growth period)
ln = natural logarithm
t = time interval (time 2 - time 1)

*Specific rate of substrate removal*

The specific rate of substrate removal was calculated and used for the comparison of removal efficiency under differing conditions. Rates of substrate removal were calculated following the lag period and were expressed as g $L^{-1}$ substrate removed per unit dry weight (g $L^{-1}$) per unit time (g $g^{-1}$ $h^{-1}$). The lag period was defined as follows (Fig. 2.5): in a plot of substrate remaining against time, the straight line was extrapolated to the initial substrate level ($S_0$) and the intercept on the time axis was taken to be the length of the lag period (L).

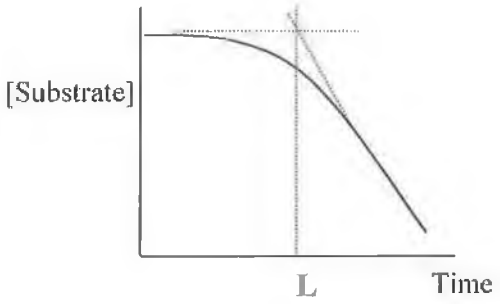



**Figure 2.5:** Definition of lag period, L.

*Yield coefficient*

The determination of the yield coefficient, $Y_{x/s}$ was based on the degree of cell dry weight produced (x) per substrate consumed (s) [(g dry weight)(g substrate consumed)$^{-1}$], unless otherwise stated.

# 3.0 RESULTS

## 3.1 Identification and selection of yeast isolates capable of growth on tallow as the sole carbon source

Previous studies in the laboratory resulted in the isolation of a number of fungi from the waste treatment system of a commercial rendering plant, capable of growth on tallow as the sole carbon source. The fungi comprised both filamentous and non-filamentous organisms. The filamentous fungi were part of a separate investigation, while the non-filamentous organisms were the focus of this study. Ten non-filamentous fungi were chosen for investigation and preliminary examination indicated that all ten were yeasts. The isolates, which were coded as outlined in Section 2.1.1, were first identified and then selected for further study, based on their ability to degrade tallow.

### 3.1.1 Identification of the isolates

Isolate morphology was compared macroscopically on both solid and in liquid medium. A microscopic observation of the isolate morphology was determined from liquid and solid media. The isolates were subjected to a number of physiological tests. Finally, the isolates were identified based on an identification scheme by reference to their respective responses to the different physiological tests and their morphology.

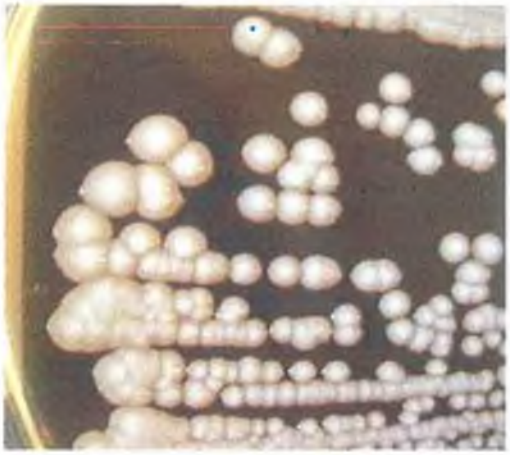
#### 3.1.1.1 Macroscopic appearance of the isolates

The appearance of the yeast on solid medium was examined by observing the colony characteristics of the ten yeast isolates grown on malt extract agar (Table 3.1.1). Colony colour, texture and surface appearance, type of colony form, elevation and margin were assessed. An illustration of the colony morphology of the ten isolates grown on malt extract agar is presented in Fig. 3.1.1 a and 3.1.1 b. All the isolates were cream coloured and butyrous in texture. Two isolates, NF 9 and NF 32 B, had glistening and smooth surfaces. Their form, elevation and margin were circular, pulvinate and entire respectively. The other isolates had a rough surface, irregular form, raised elevation and undulate margin.

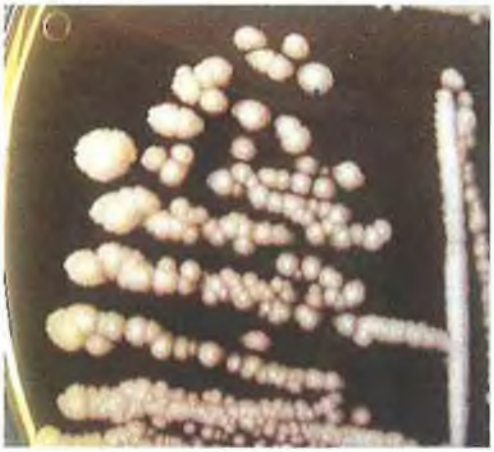
**Table 3.1.1:** Observations of isolate colony morphology after 7 days growth on malt extract agar at 25°C.

| Isolate | Colour and texture | Surface | Form | Elevation | Margin |
|---|---|---|---|---|---|
| NF 9 | Cream and butyrous | Glistening and smooth | Circular | Pulvinate | Entire |
| NF 10 | Cream and butyrous | Rough-crystalline pattern at edges | Irregular | Raised | Undulate |
| NF 12 | Cream and butyrous | Rough-crystalline pattern on surface | Irregular | Raised | Undulate |
| NF 27 | Cream and butyrous | Rough-crystalline pattern on surface | Irregular | Raised | Undulate |
| NF32 A | Cream and butyrous | Rough-crystalline pattern on surface | Irregular | Raised | Undulate |
| NF 32 B | Cream and butyrous | Glistening and smooth | Circular | Pulvinate | Entire |
| NF 48 | Cream and butyrous | Glistening and rough | Irregular | Raised | Undulate |
| NF51 | Cream and butyrous | Rough-crystalline pattern on surface | Irregular | Raised | Undulate |
| NF 52 | Cream and butyrous | Rough | Irregular | Raised | Undulate |
| NF C | Cream and butyrous | Rough-crystalline pattern on surface | Irregular | Raised | Undulate |

NF 9



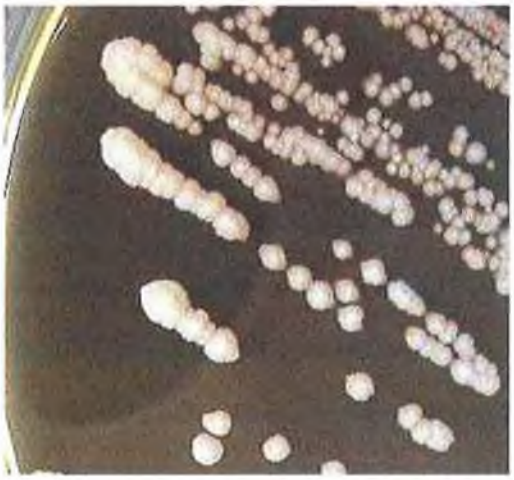
NF 10



NF 12

NF 27

NF 32 A



NF 32 B

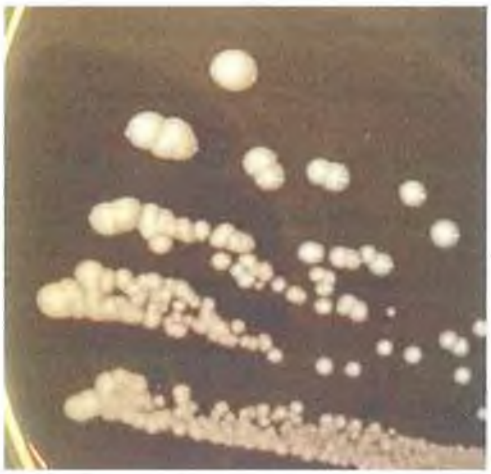


**Figure 3.1.1 a:** Illustration of the colony morphology of yeast isolates after 7 days growth on malt extract agar at 25°C.

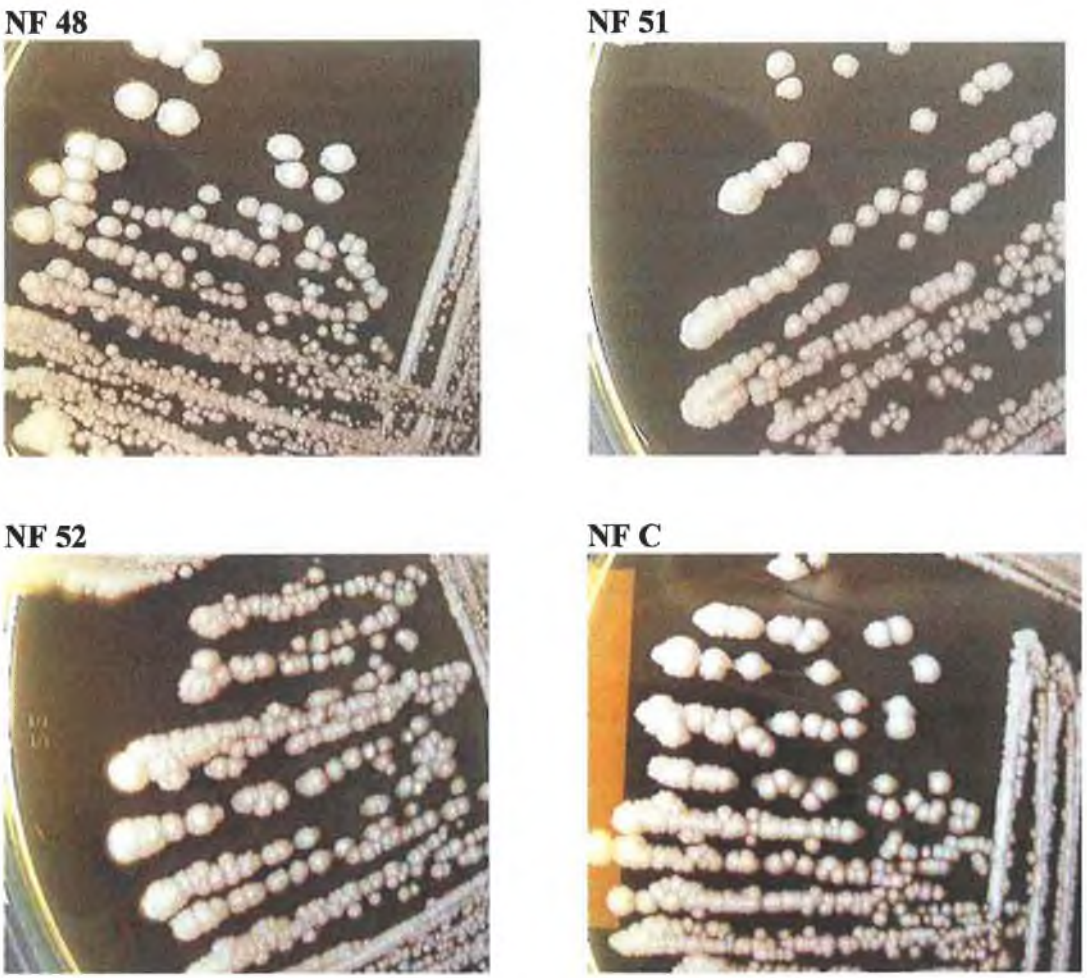



**Figure 3.1.1 b:** Illustration of the colony morphology of yeast isolates after 7 days growth on malt extract agar at 25°C.

The gross morphology of the ten isolates in liquid culture was examined. Growth characteristics of the yeast cultures were observed after 7 days growth in malt extract broth (Table 3.1.2). The isolates formed compact sediment with light surface growth, except in the case of NF 9 and NF 32 B. These two isolates had flocculent sediment and a ring formation was observed for NF 9.

**Table 3.1.2:** Observation of isolate gross morphology after 7 days growth in malt extract broth at 25°C.

| Isolate | Morphology characteristics |
|---|---|
| NF 9 | Flocculent sediment with ring formation |
| NF 10 | Compact sediment with light surface growth |
| NF 12 | Compact sediment with light surface growth |
| NF27 | Compact sediment with light surface growth |
| NF32 A | Compact sediment with light surface growth |
| NF 32 B | Flocculent sediment |
| NF 48 | Compact sediment with light surface growth |
| NF51 | Compact sediment with light surface growth |
| NF 52 | Compact sediment with light surface growth |
| NF C | Compact sediment with light surface growth |

### 3.1.1.2 Microscopic appearance of the isolates

The isolates were examined microscopically after 3 days growth in malt extract broth. Observations were made on cell shape, cell size relative to each isolate, and the presence of budding and mycelium formation (Table 3.1.3). The production of ascospores was assessed after 3 days growth on malt extract agar.

All of the isolates had oval shaped cells with the exception of NF 9, which had ellipsoidal cells. NF 32 B had small cells and NF 9 had medium-sized cells, whereas the rest of the isolates had relatively large cells. Multi-lateral budding was observed for all the isolates. After 3 days growth, pseudomycelium formation was observed by NF 32 B only, which were observed to be in the myco-candida shape. After 2 weeks growth in liquid culture, pseudomycelium were produced by the majority of the isolates, with the exception of NF 9 and NF 48 (Fig.s 3.1.2 a & 3.1.2 b). The isolates produced ascospores after 3 days growth on malt extract agar with the exception of NF 32 B.

**Table 3.1.3:** Observations on isolate cell morphology. The yeast were cultured in malt extract broth for up to 2 weeks or in the case of ascospore formation on malt extract agar for 3 days, all at 25°C. Cells were examined under x40 magnification.

| **Isolate** | ***Observations on cell morphology*** | | | | | |
|---|---|---|---|---|---|---|
| | **Cell shape** | **Relative Cell size** | **Budding** | **Pseudomycelium formation** | | **Ascospore formation** |
| | | | | *3 days* | *2 weeks* | |
| NF 9 | Ellipsoidal | Medium | Multilateral | No | No | Present |
| NF 10 | Oval | Large | Multilateral | No | Yes | Present |
| NF 12 | Oval | Large | Multilateral | No | Yes | Present |
| NF 27 | Oval | Large | Multilateral | No | Yes | Present |
| NF 32 A | Oval | Large | Multilateral | No | Yes | Present |
| NF 32 B | Oval to long-oval | Small | Multilateral | Yes- in myco-candida form | Yes | Absent |
| NF 48 | Oval | Large | Multilateral | No | No | Present |
| NF 51 | Oval | Large | Multilateral | No | Yes | Present |
| NF 52 | Oval | Large | Multilateral | No | Yes | Present |
| NF C | Oval | Large | Multilateral | No | Yes | Present |

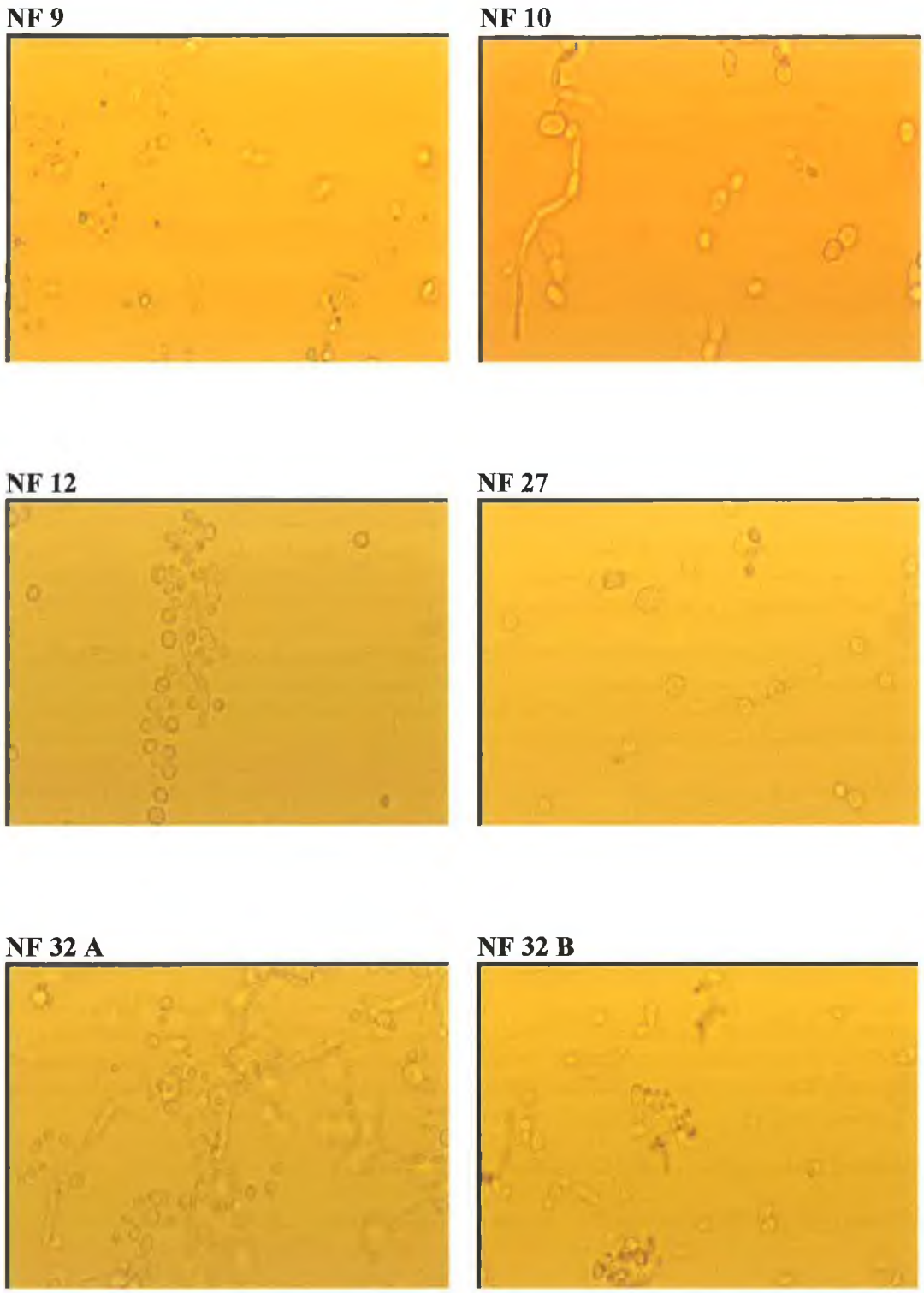



**Figure 3.1.2 a:** Illustration of isolate cell morphology after 2 weeks growth in malt extract broth at 25°C. Cells were examined under x40 magnification.

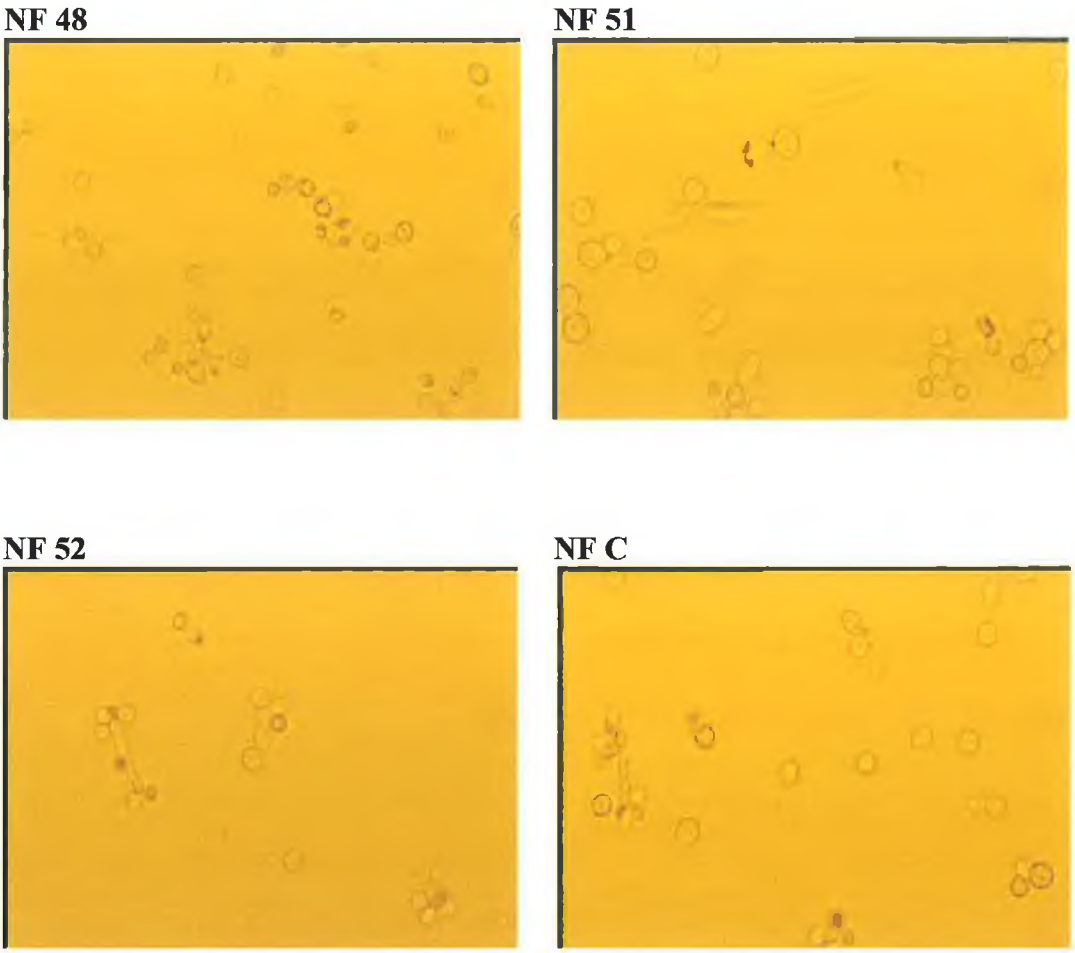



**Figure 3.1.2 b:** Illustration of isolate cell morphology after 2 weeks growth in malt extract broth at 25°C. Cells were examined under x40 magnification.

### 3.1.1.3 Physiological characteristics of the isolates

A number of physiological tests were performed with the ten isolates in the course of their identification. These tests included: the ability of the yeasts to ferment certain sugars, ability to assimilate nitrate as the sole nitrogen source, to grow in high sugar or osmotic medium, to grow at 37°C, to grow in the presence of cycloheximide, to hydrolyse urea, to react with Diazonium Blue B (DBB) and to assimilate certain carbons as the sole carbon source (Tables 3.1.4 - 3.1.6). *Saccharomyces cerevisiae* was included in the tests as a control organism.

Two of the isolates fermented glucose, NF 9 and NF 32 B (Table 3.1.4). However, these two isolates did not ferment the other sugars galactose, sucrose, maltose, lactose or raffinose. In contrast, none of the other isolates demonstrated any fermentative ability.

None of the isolates assimilated nitrate as the sole nitrogen source (Table 3.1.5). All of the isolates demonstrated the ability to grow in high sugar medium (50% glucose-yeast extract). However, only NF 32 B was able to grow under high osmotic pressure (10% NaCl- glucose). The majority of the isolates demonstrated good growth at 37°C, with the exception of NF 32 B, which displayed very little growth, if any at that temperature. The isolates grew in the presence of both 100 ppm and 1000 ppm cycloheximide. The ability to hydrolyse urea was evident in the majority of the isolates, with the exception of NF 9 and NF 32 B. None of the isolates produced a colour reaction with DBB.

The ability of the isolates to assimilate 19 different carbon sources is summarised in Table 3.1.6. A degree of similarity in assimilation of a number of the carbon compounds was observed for the isolates with the exception of NF 9 and NF 32 B. In comparison to the other yeasts, NF 32 B differed in its response to galactose, rhamnose, erythritol and ribose, whereas NF 9 differed in its response to sucrose and lactose. Both isolates differed to the rest of the yeasts in their response to trehalose and ribitol.

**Table 3.1.4:** The presence (+) or absence (–) of the ability of the yeast isolates to ferment the sugars glucose, galactose, sucrose, maltose, lactose and raffinose. *S. cerevisiae* was included as a control organism.

| | **Sugars** | | | | | |
|---|---|---|---|---|---|---|
| **Isolate** | Glucose | Galactose | Sucrose | Maltose | Lactose | Raffinose |
| NF 9 | + | – | – | – | – | – |
| NF 10 | – | – | – | – | – | – |
| NF 12 | – | – | – | – | – | – |
| NF 27 | – | – | – | – | – | – |
| NF 32 A | – | – | – | – | – | – |
| NF 32 B | + | – | – | – | – | – |
| NF 48 | – | – | – | – | – | – |
| NF 51 | – | – | – | – | – | – |
| NF 52 | – | – | – | – | – | – |
| NF C | – | – | – | – | – | – |
| *S. cerevisiae* | + | + | + | + | – | + |

**Table 3.1.5:** Reaction of isolates to the physiological tests: nitrate utilisation, growth on 50% glucose-yeast extract medium, growth in 10% NaCl-glucose medium, growth at 37°C, growth in the presence of 100 and 1000 ppm cycloheximide, presence of urease and reaction to Diazonium Blue B test. *S. cerevisiae* was included as a control organism.

| Isolate | Nitrate Assimilation | 50 % glucose-yeast extract | 10% NaCl-glucose | Growth at 37°C | 100ppm cyclo-heximide | 1000ppm cyclo-heximide | Urease test | Diazonium Blue B test |
|---|---|---|---|---|---|---|---|---|
| NF 9 | – | v | – | v | + | + | – | – |
| NF 10 | – | v | – | + | + | + | + | – |
| NF 12 | – | v | – | + | + | + | + | – |
| NF 27 | – | v | – | + | + | + | + | – |
| NF 32 A | – | v | – | + | + | + | + | – |
| NF 32 B | – | v | + | – (+ s) | + | + | – | – |
| NF 48 | – | v | – | + | + | + | + | – |
| NF 51 | – | v | – | + | + | + | + | – |
| NF 52 | – | v | – | + | + | + | + | – |
| NF C | – | v | – | + | + | + | + | – |
| *S. cerevisiae* | – | v | + | v | – | – | – | – |

**Note:** v = vigorous growth, + = good growth or positive response, +s = slow growth or slow response and – = no growth or negative response

**Table 3.1.6:** Assimilation of various carbon compounds by yeast isolates. *S. cerevisiae* was included as a control organism.

| Isolate | Gal | Suc | Mal | Cell | Tre | Lac | Raff | SolS | Xyl | Glc | Ara | Rib | Rha | Ery | Ribo | Man | Suc.A | Cit. A | Inos |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NF 9 | v | v | v | + | + | v | v | v | + s | +/– | v | – | + | + | + | v | v | + | –/+s |
| NF 10 | + | – | – | + s | – | – | + s | – | v | – | + s | + | + | v | + | + (v) | + | + | –/+s |
| NF 12 | + | – | + s | + s | – | +/– | + s | – | +/– | – | + s | + | + | + | + | v | v | v | –/+s |
| NF 27 | + | +/– | +s | + s | – | – | + | – | +/– | – | –/ +s | + | + | + | + | v | + | v | + s |
| NF 32 A | + | – | +/– | +/– | – | +/– | +/– | – | +/– | – | +/– | + | + | + (v) | + | + (v) | + | + | +/– |
| NF 32 B | – | – | – | – | + | +/– | +/– | + | +/– | – | – | – | – | – | – | + | + | + | – |
| NF 48 | + | +/– | v | + | – | +/– | + | +/– | + | – | + | + | + (v) | + | v | v | + | + s | + s |
| NF 51 | + | +/– | + | + s | – | – | + | + | +/– | – | –/ +s | + | + | + | + | v | + | + | –/+s |
| NF 52 | v | +/– | v | v | – | – | + | + | + | – | + | + | +(v) | + | + | + (v) | v | v | –/+s |
| NF C | + | – | – | + s | – | – | + s | – | +/– | – | –/+s | + | + | + | + | v | + | + | –/+s |
| *S. cerevisiae* | v | v | v | – | v | – | v | v | – | – | – | – | – | – | – | + | +s | – | – |

**Note:** Gal = galactose; Suc = sucrose; Mal = maltose; Cell = cellobiose; Tre = trehalose; Lac = lactose; Raff = Raffinose; SolS = soluble starch; Xyl = xylose; Glc = galactitol; Ara = arabinose; Rib = ribitol; Rha = Rhamnose; Ery = erithritol; Ribo = ribose; Man = manitol; Suc.A = succinic acid; Cit.A = citric acid; and Inos = inositol. **Also:** v = strong utilisation, + = rapid utilisation, + s = slow or latent utilisation, – = no utilisation and +/– or –/+s = inconclusive utilisation.

### 3.1.1.4 Isolate identification schemes

The identification of the isolates was proposed based on the observations and results from morphological and physiological tests for each isolate by reference to the identification keys devised by Barnett *et al.* (1983). Two keys were employed for the identification and the selection of the key was based on the response of the isolate to the fermentation of glucose. Key No.1 (Appendix A) applied to yeasts that did not ferment D-glucose and Key No.3 (Appendix B) applied to yeasts that did ferment D-glucose. In using the keys, the number of the test was specified in brackets before the test and the response, positive (+) or negative (–), was noted after the test. The response indicated the next test in the key or the identity of the yeast. Where the response of the test was not known or inconclusive, both the negative and positive responses were investigated. Where more than one possible identification could be made, the identification of the isolate was made using a more comprehensive interpretation of the physiological and morphological tests.

***Identification of isolates using Key No. 1***

Isolates NF 10, NF 12, NF 27, NF 32 A, NF 48, NF 51, NF 52 and NF C were identified using Key No. 1 and were all identified as *Yarrowia lipolytica*.

The identification scheme for isolate NF 10 (Fig. 3.1.3) and NF C (Fig. 3.1.4) resulted in the one possible identification of the isolates from the key, *Yarrowia lipolytica.* The identification scheme for NF 12 (Fig. 3.1.5) and NF 32 A (Fig. 3.1.6) resulted in two possible identities of the isolates, *Yarrowia lipolytica* and *Trichosporon beigelii. Yarrowia lipolytica* was selected as the proposed identification of NF 12 and NF 32 A. This was based on the negative response to the Diazonium Blue B test by both the isolates (Table 3.1.5) and *Yarrowia lipolytica* and a positive response by *Trichosporon beigelii* (Barnett *et al.*, 1983).

The identification schemes for NF 27, NF 51 and NF 52 resulted in four possible identities of the isolates (Fig.s 3.1.7 – 3.1.9) and nine possible identities of NF 48 (Fig. 3.1.10). Their identification as *Yarrowia lipolytica*, as detailed in Tables 3.1.7 – 3.1.9 and 3.1.10, respectively, was based on the both the ability of the isolates and *Yarrowia lipolytica* to growth in 1000 ppm cycloheximide and their negative response to the Diazonium Blue B test.

(1) Nitrate growth- (–)
↓
(2) Erythritol growth- (+)
↓
(332) Galactitol growth- (–)
↓
(333) Sucrose growth- (–)
↓
(334) Galactose growth- (+)
↓
(351) Trehalose growth- (–)
↓
(352) Urea hydrolysis- (+)
↓
(356) Lactose growth- (–)
↓
***Yarrowia lipolytica***

**Figure 3.1.3:** Identification scheme for yeast isolate NF 10. The identification was based on Key No.1, in accordance with Barnett *et al.* (1983), for yeasts that do not ferment D-glucose. The proposed identification of the yeast, *Yarrowia lipolytica*, is highlighted in grey.

(1) Nitrate growth- (–)
↓
(2) Erythritol growth- (+)
↓
(332) Galactitol growth- (–)
↓
(333) Sucrose growth- (–)
↓
(334) Galactose growth- (+)
↓
(351) Trehalose growth- (–)
↓
(352) Urea hydrolysis- (+)
↓
(356) Lactose growth- (–)
↓
***Yarrowia lipolytica***

**Figure 3.1.4:** Identification scheme for yeast isolate NF C. The identification was based on Key No.1, in accordance with Barnett *et al.* (1983), for yeasts that do not ferment D-glucose. The proposed identification of the yeast, *Yarrowia lipolytica* is highlighted in grey.

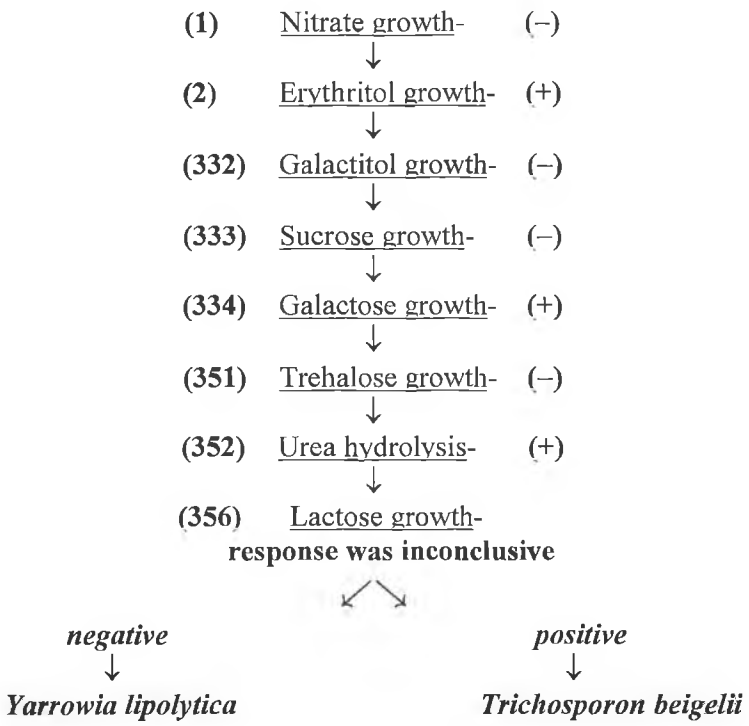



**Figure 3.1.5:** Identification scheme for yeast isolate NF 12. The identification was based on Key No.1, in accordance with Barnett *et al.* (1983), for yeasts that do not ferment D-glucose. The possible identities of the yeast are highlighted in grey.

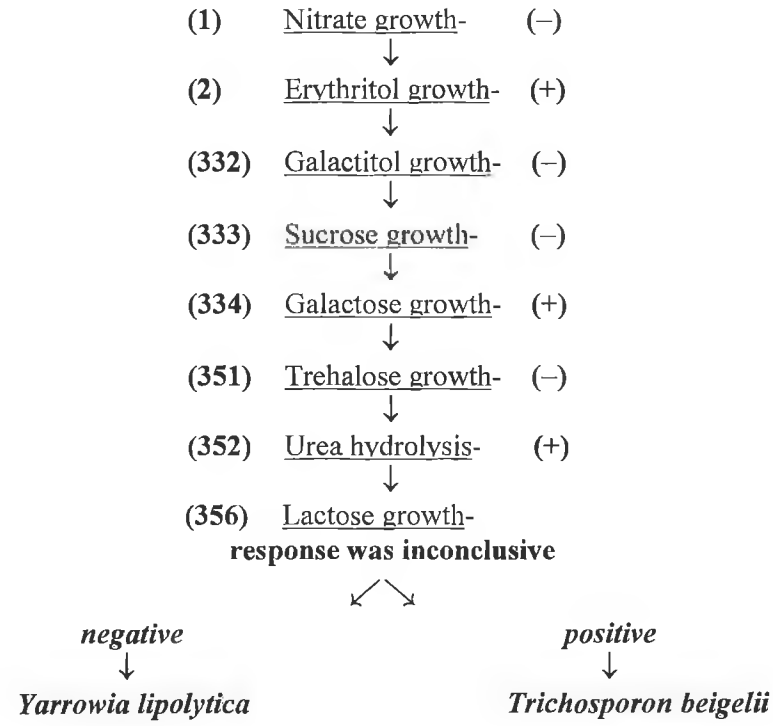



**Figure 3.1.6:** Identification scheme for yeast isolate NF 32 A. The identification was based on Key No.1, in accordance with Barnett *et al.* (1983), for yeasts that do not ferment D-glucose. The possible identities of the yeast are highlighted in grey.

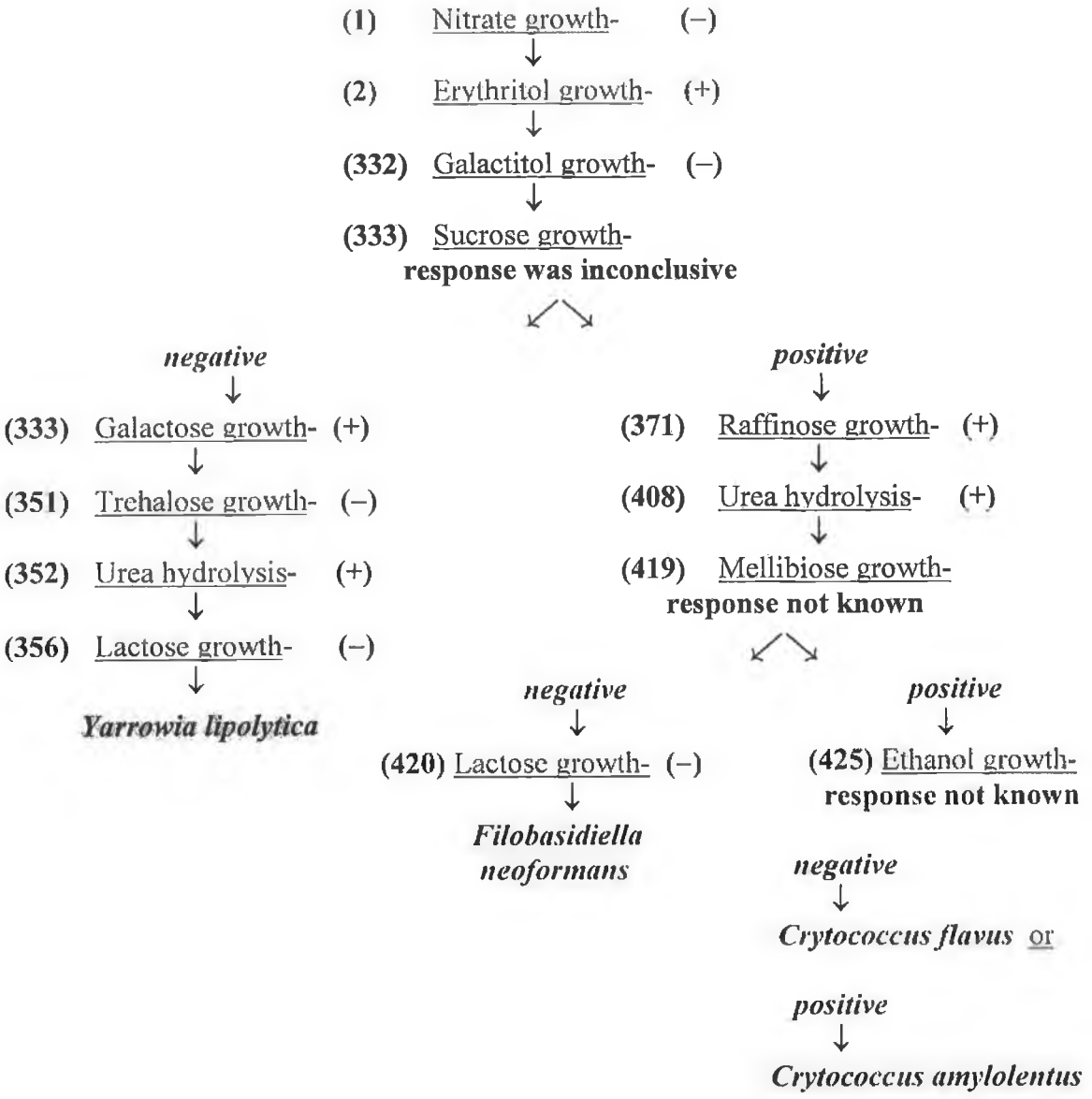



**Figure 3.1.7:** Identification scheme for yeast isolate NF 27. The identification was based on Key No.1, in accordance with Barnett *et al.* (1983), for yeasts that do not ferment D-glucose. The possible identities of NF 27 are highlighted in grey. Further tests for the identification of NF 27 are detailed in Table 3.1.7.

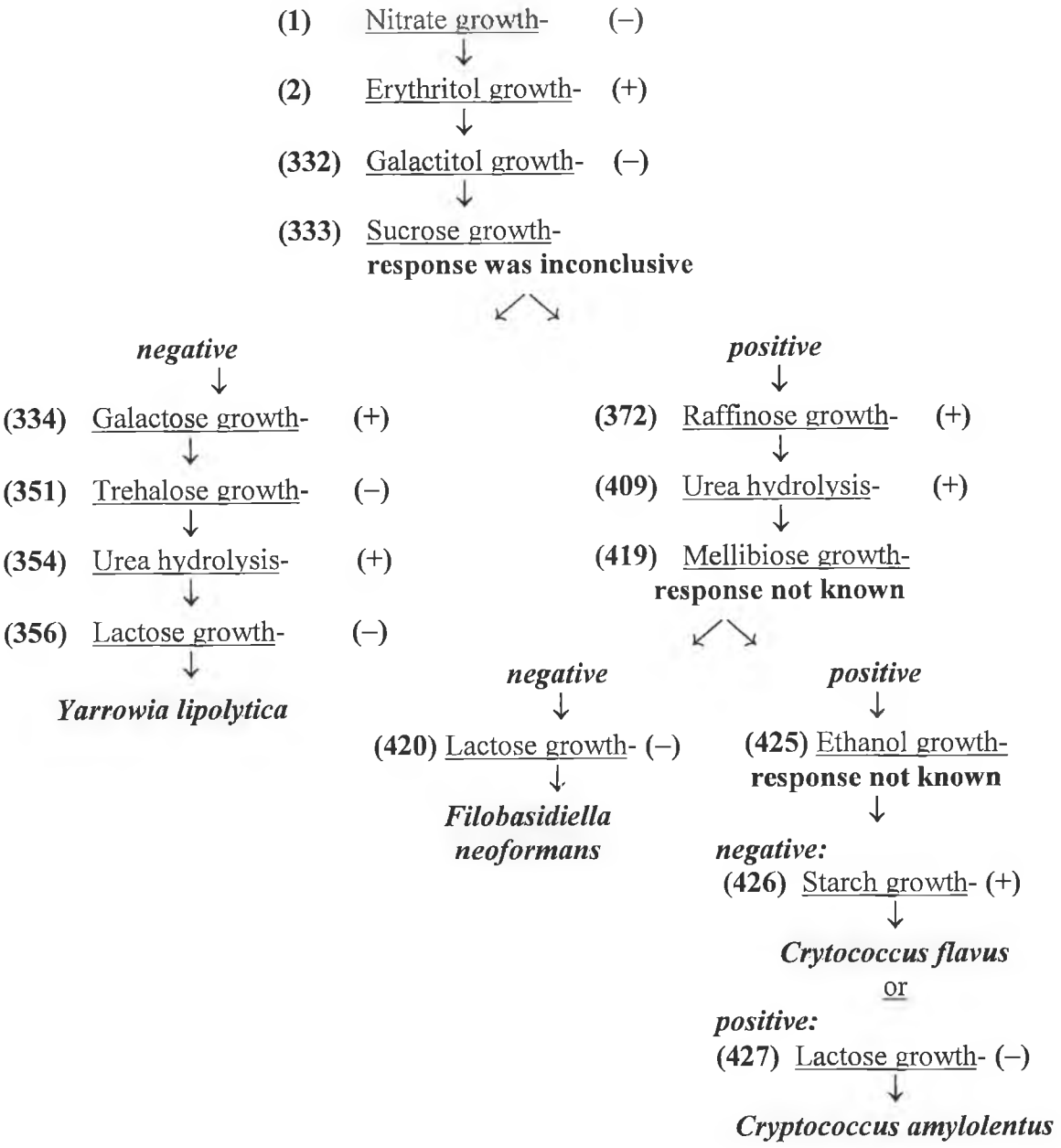



**Figure 3.1.8:** Identification scheme for NF 51. The identification was based on Key No.1, in accordance with Barnett *et al.* (1983), for yeasts that do not ferment D-glucose. The possible identities of NF 51 are highlighted in grey. Further tests for the identification of NF 51 are detailed in Table 3.1.8.

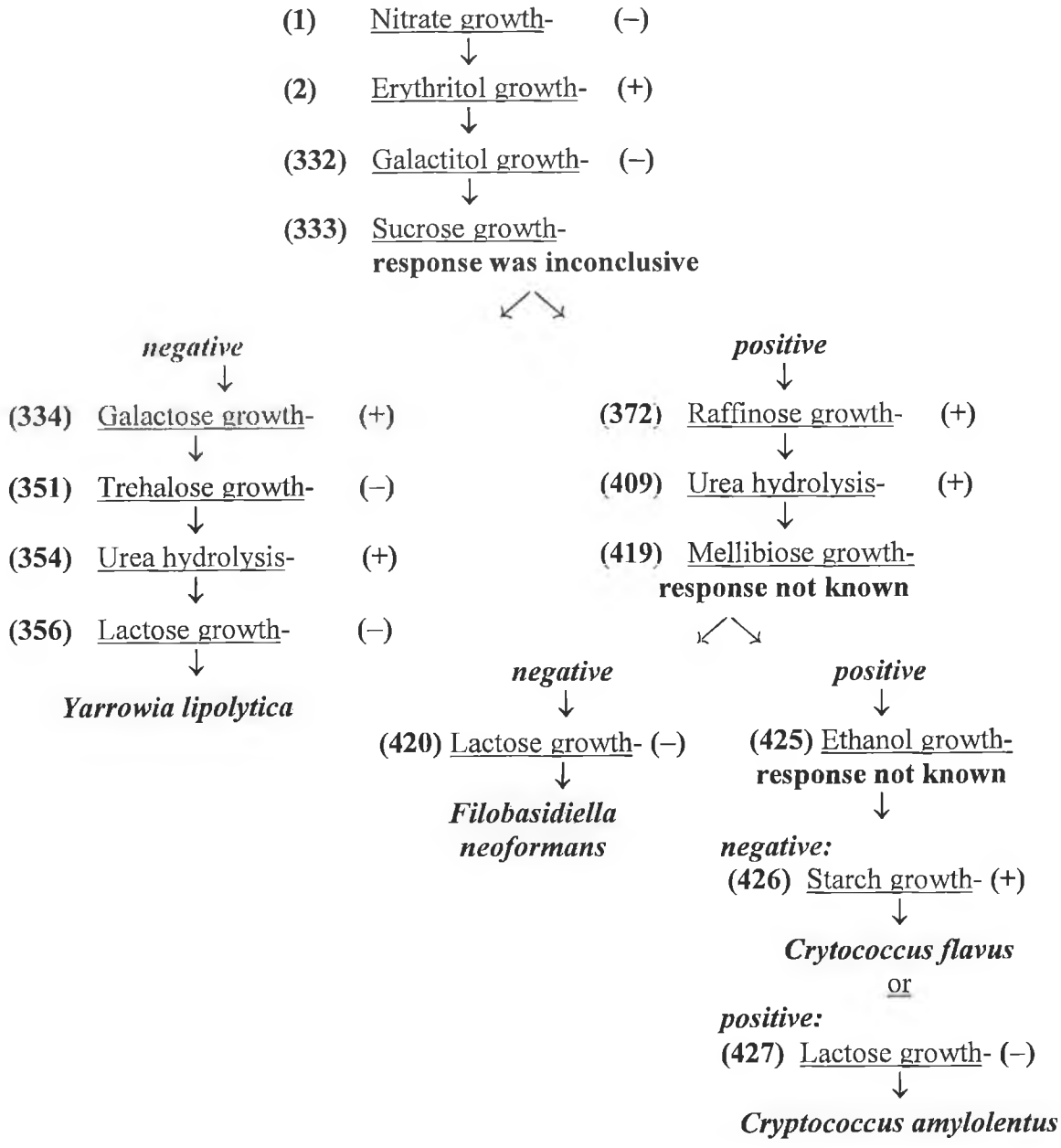



**Figure 3.1.9:** Identification scheme for NF 52. The identification was based on Key No.1, in accordance with Barnett *et al.* (1983), for yeasts that do not ferment D-glucose. The possible identities of NF 52 are highlighted in grey. Further tests for the identification of NF 52 are detailed in Table 3.1.9.

**Table 3.1.7:** Proposed identification of yeast isolate NF 27. NF 27 was identified as *Yarrowia lipolytica* as a result of its growth in 1000 ppm cycloheximide and negative response to the Diazonium Blue B test.

| | 1000 ppm cycloheximide | Diazonium Blue B |
|---|---|---|
| NF 27[a] | + | – |
| *Filobasidiella neoformans*[b] | + | + |
| *Crytococcus flavus*[b] | – | ? |
| *Crytococcus amylolentus*[b] | – | + |
| *Yarrowia lipolytica*[b] | + | – |

[a] test reponsed from this study; [b] test responses from Barnett *et al.* (1983)
note: (+) = positive response and (–) = negative response and (?) = response not known

**Table 3.1.8:** Proposed identification of yeast isolate NF 51. NF 51 was identified as *Yarrowia lipolytica* as a result of its growth in 1000 ppm cycloheximide and negative response to the Diazonium Blue B test.

| | 1000 ppm cycloheximide | Diazonium Blue B |
|---|---|---|
| NF 51[a] | + | – |
| *Filobasidiella neoformans* [b] | – | + |
| *Crytococcus flavus*[b] | – | ? |
| *Cryptococcus amylolentus*[b] | – | + |
| *Yarrowia lipolytica*[b] | + | – |

[a] test responses from this study; [b] test responses from Barnett *et al.* (1983)
note: (+) = positive response; (–) = negative response and (?) = response not known

**Table 3.1.9:** Proposed identification of yeast isolate NF 52. NF 52 was identified as *Yarrowia lipolytica* as a result of its growth in 1000 ppm cycloheximide and negative response to the Diazonium Blue B test.

| | 1000 ppm cycloheximide | Diazonium Blue B |
|---|---|---|
| NF 51[a] | + | – |
| *Filobasidiella neoformans* [b] | – | + |
| *Crytococcus flavus*[b] | – | ? |
| *Cryptococcus amylolentus*[b] | – | + |
| *Yarrowia lipolytica*[b] | + | – |

[a] test responses from this study; [b] test responses from Barnett *et al.* (1983)
note: (+) = positive response; (–) = negative response and (?) = response not known

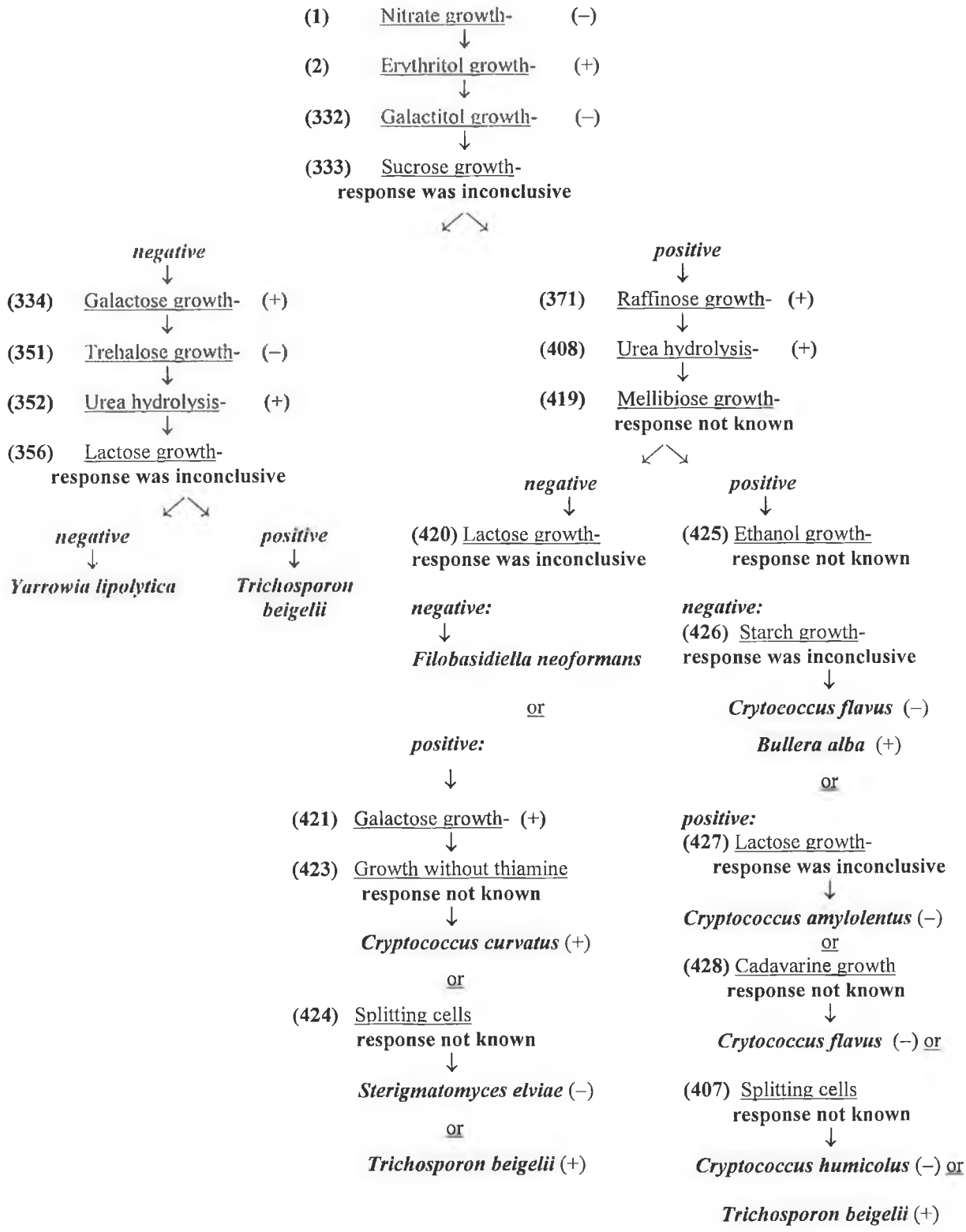



**Figure 3.1.10:** Identification scheme for yeast isolate NF 48. The identification was based on Key No.1, in accordance with Barnett *et al.* (1983), for yeasts that do not ferment D-glucose. The possible identities of NF 48 are highlighted in grey. Further tests for the identification of NF 48 are detailed in Table 3.1.10.

**Table 3.1.10:** Proposed identification of yeast isolate NF 48. NF 48 was identified as *Yarrowia lipolytica* as a result of its growth in 1000 ppm cycloheximide and negative response to the Diazonium Blue B test.

| | 1000 ppm cycloheximide | Diazonium Blue B |
|---|---|---|
| NF 48[a] | + | – |
| *Trichosporon beigelii*[b] | + | + |
| *Filobasidiella neoformans* [b] | – | + |
| *Cryptococcus curvatus*[b] | – | – |
| *Crytococcus flavus*[b] | – | ? |
| *Sterigmatomyces elviae*[b] | – | + |
| *Bullera alba*[b] | – | + |
| *Cryptococcus amylolentus*[b] | – | + |
| *Cryptococcus humicolus*[b] | + | + |
| *Yarrowia lipolytica*[b] | + | – |

[a] test responses from this study; [b] test responses from Barnett *et al.* (1983)
note: (+) = positive response; (–) = negative response and (?) = response not known

***Identification of isolates using Key No. 3***

Isolates NF 9 and NF 32 B were identified using Key No.3 as *Debaromyces hansenii* and *Candida zeylanoides*, respectively.

The identification schemes for isolate NF 9 and NF 32 B resulted in seven and five possible identities of the yeasts, respectively (Fig.s 3.1.11 and 3.1.12). The proposed identification of NF 9, *Debaromyces hansenii*, is detailed in Table 3.1.11. The identification was based on the positive response of the isolate to growth in 1000 ppm cycloheximide (Table 3.1.5), the absence of pseudomycelium formation (Table 3.1.3) and its inability to ferment sucrose (Table 3.1.4) and the identical responses to these tests by *Debaromyces hanseni* (Barnett *et al.*, 1983). *Candida zeylanoides* was selected as the proposed identification of NF 32 B, as detailed in Table 3.1.12. Identification was based on the ability of the isolate to grow in 1000 ppm cycloheximide and in 50% glucose (Table 3.1.5) and the identical response of *Candida zeylanoides* to these tests (Barnett *et al.*, 1983).

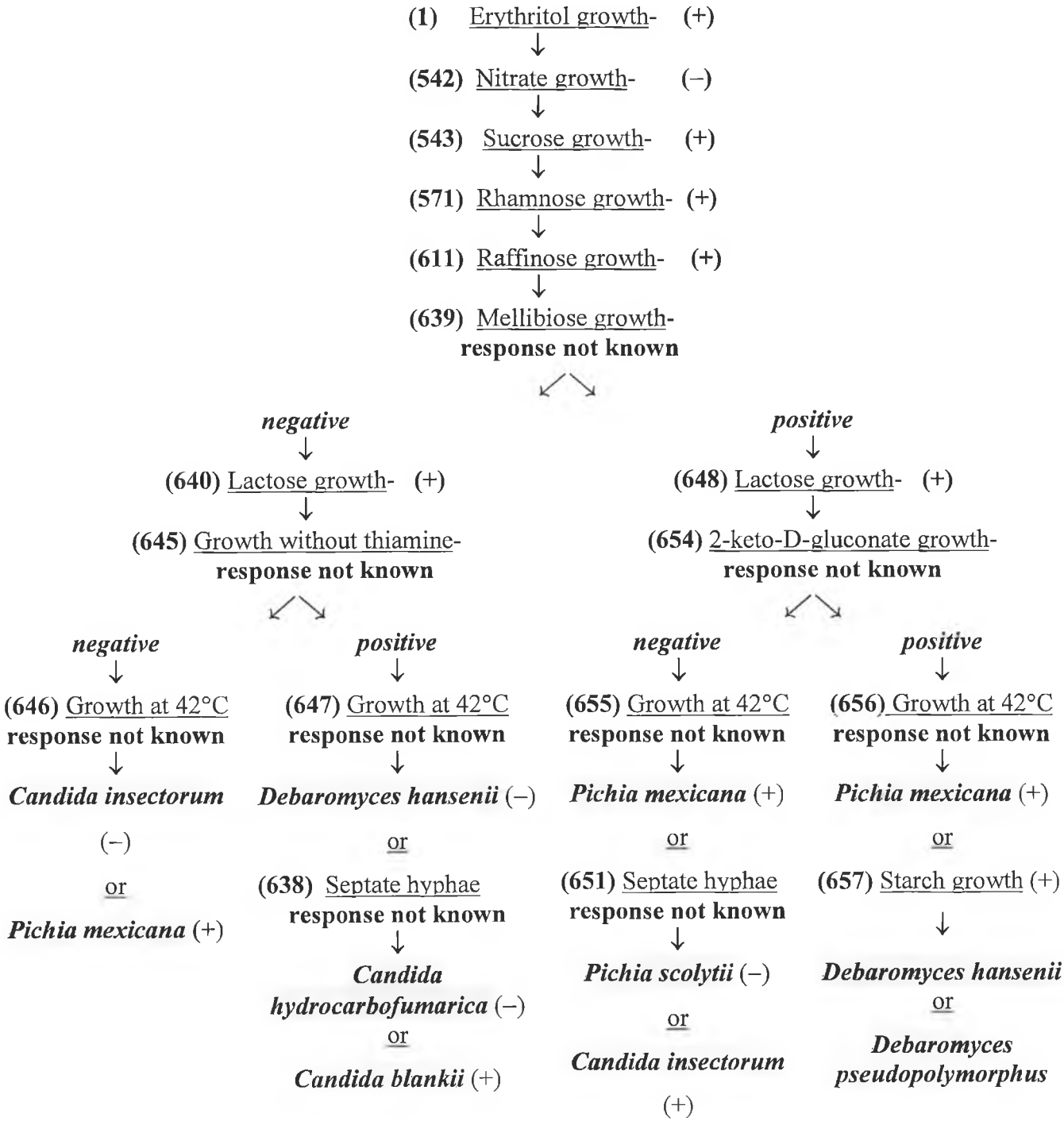



**Figure 3.1.11:** Identification scheme for yeast isolate NF 9. The identification was based on Key No. 3, in accordance with Barnett *et al.* (1983), for yeasts that ferment D-glucose. The possible identities of NF 9 are highlighted in grey. Further tests for the identification of NF 9 are detailed in Table 3.1.11.

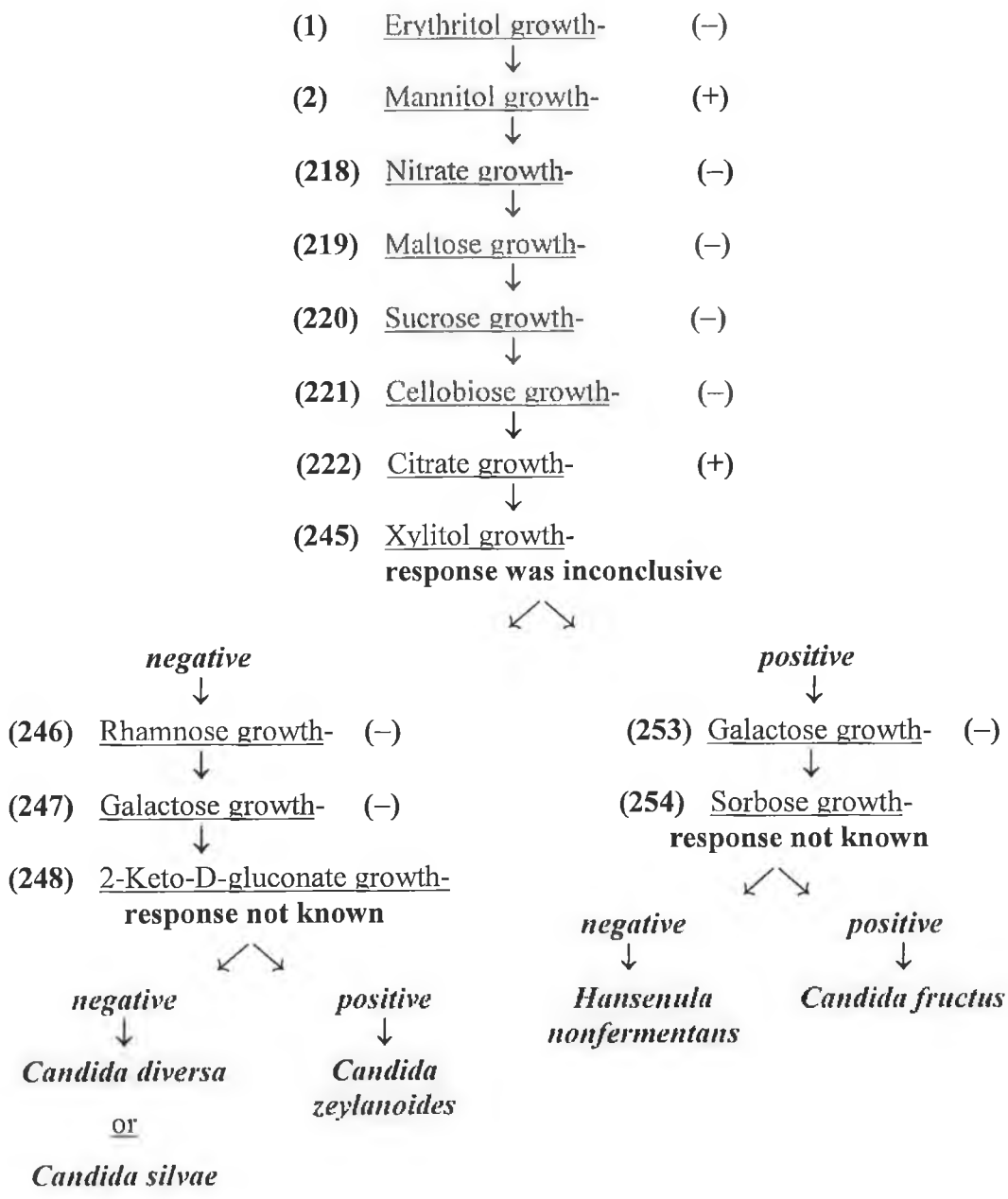



**Figure 3.1.12:** Identification scheme for NF 32 B. The identification was based on Key No.3, in accordance with Barnett *et al.* (1983), for yeasts that ferment D-glucose. The possible identities of NF 32 are highlighted in grey. Further tests for the identification of NF 32 B are detailed in Table 3.1.12.

**Table 3.1.11:** Proposed identification of yeast isolate NF 9. NF 9 was identified as *Debaromyces hansenii* as a result of its growth with 1000 ppm cycloheximide, inability to ferment sucrose or form pseudomycelium.

| | 1000 ppm cycloheximide | Fermentation of sucrose | Pseudomycelium formation |
|---|---|---|---|
| Isolate NF 9[a] | + | – | – |
| *Candida insectorum*[b] | – | + | + |
| *Pichia mexicana*[b] | – | – | + |
| *Candida hydrocarbofumarica*[b] | + | – | + |
| *Pichia scolyti*[b] | – | + | + |
| *Candida blankii*[b] | + | + | + |
| *Debaromyces pseudopolymorphus*[b] | + | + | +/– |
| *Debaromyces hansenii*[b] | + | – | – |

[a] test responses from this study; [b] test responses from Barnett *et al.* (1983)
Note: (+) = poisitive response; (–) = negative response and (+/–) = mixed response.

**Table 3.1.12:** Proposed identification of yeast isolate NF 32 B. NF 32 B was identified as *Candida zeylanoides* as a result of its growth in 1000 ppm cycloheximide and 50% glucose.

| | 1000 ppm cycloheximide | 50% glucose |
|---|---|---|
| NF 32 B[a] | + | + |
| *Candida diversa*[b] | – | – |
| *Candida silvae*[b] | – | – |
| *Hansenula nonfermentans*[b] | + | – |
| *Candida fructus*[b] | – | – |
| *Candida zeylanoides*[b] | + | + |

[a] test responses from this study; [b] test responses from Barnett *et al.* (1983)
note: (+) = positive response and (–) = negative response

A summary of the proposed identification of the 10 isolated yeasts is presented in Table 3.1.13. Three distinct yeasts were identified, isolate NF 9 was identified as *Debaromyces hansenii*, isolate NF 32 B was identified as *Candida zeylanoides* and the remaining isolates were identified as *Yarrowia lipolytica.*

**Table 3.1.13:** Summary of the proposed identification of the yeast isolates.

| Isolate | Proposed Genus | Proposed species |
|---|---|---|
| NF 9 | *Debaromyces* | *hansenii* |
| NF 10, NF 12, NF 32 A, NF 48, NF 51, NF 52, NF C | *Yarrowia* | *lipolytica* |
| NF 32 B | *Candida* | *zeylanoides* |

### 3.1.2 Selection of the isolates

Selection of the isolates for further study in the development of a microbial fat removal system was based on their ability to remove tallow. The ten isolates were grown on 20 g $L^{-1}$ tallow as the sole carbon source in minimal medium for 168 h. Growth temperature was set at room temperature (22° - 25°C) and fat removal assessed with agitation at 130 rpm and under non-agitated growth. The percentage fat removed was determined after 168 h.

The isolates identified as *Yarrowia lipolytica* removed 21 ± 1.5 % fat after 168 h at room temperature and 130 rpm (Fig. 3.1.13). The isolates identified as *Debaromyces hansenii* (NF 9) and *Candida zeylanoides* (NF 32 B) achieved approximately 16 and 17% removal, respectively, under the same conditions. Fat removal was significantly reduced under non-agitated growth, in all cases and all further growth studies were performed with agitation.

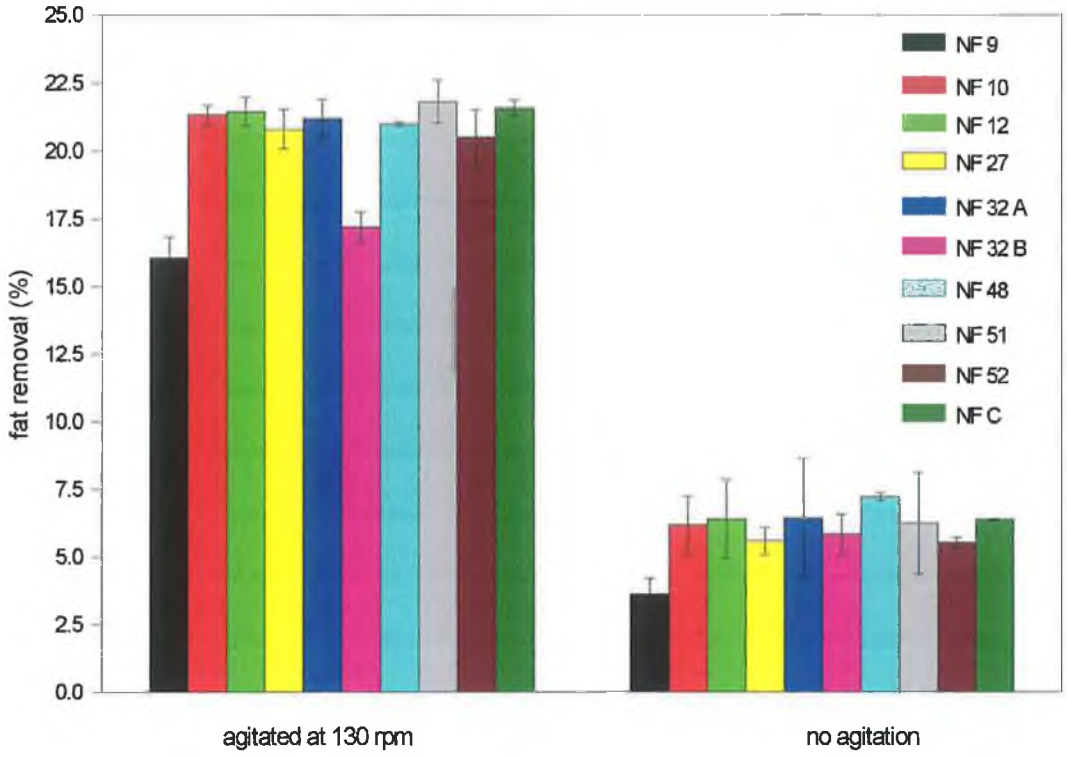



**Figure 3.1.13**: Percentage fat removal by 10 yeast isolates after 168 h growth on tallow (20 g $L^{-1}$) in minimal medium at room temperature (22° - 25°C) with agitation at 130 rpm and with no agitation.

Isolate NF 32 A was chosen as a representative of the *Yarrowia lipolytica* isolates and was selected for further investigation in this study. The identification of the yeast NF 32 A as *Yarrowia lipolytica* was confirmed by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany (Appendix C). The strain isolated in the lab was then designated as *Yarrowia lipolytica* RP2. RP denoted the source of the yeast from a rendering plant and the number 2 denoted that the yeasts were the second group of isolates from the rendering plant (the first were filamentous fungi) to be studied in the laboratory.

## 3.2 Characterisation and optimisation of the environmental conditions for the degradation of tallow (20 g $L^{-1}$) by *Yarrowia lipolytica* RP2 in shake flask culture

The characteristics of the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$), as the sole carbon source, were assessed in shake flask culture. Environmental growth parameters play an important role in the development of a microbial-based fat removal system. Therefore, a study was undertaken to optimise the growth conditions for tallow degradation by the yeast and included growth temperature, inclusion of surfactants, medium pH, agitation and inoculum preparation. The influence of the addition of glucose and nitrogen to the system on fat removal and growth was also investigated.

### 3.2.1 Characteristics of the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in minimal medium

The growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) was assessed in minimal medium over a period of 240 h at room temperature (22° - 25°C) and 130 rpm. The flasks were sampled at regular intervals and fat removal, cell number, dry weight, cell viability, intracellular lipid and pH were monitored. Medium pH was not controlled.

Maximum fat removal, 21%, was achieved after 168 h and corresponded to a decrease in the tallow concentration from 20 g $L^{-1}$ to approximately 15.8 g $L^{-1}$ (Fig. 3.2.1). No further fat removal occurred after 168 h and this time period was selected for fat removal measurements in further shake flask investigations. Both cell number and dry weight were used to monitor growth. Cell number increased to $1.08 \pm 0.04 \times 10^8$ cell $ml^{-1}$ by 168 h. This correlated with the maximum degree of fat removal at that time. After 168 h, the cells entered stationary growth with no increase in cell number at 240 h. Cells were 100% viable after 168 h, with a marginal decrease to 98 ± 1.5% viability after 240 h (results not shown). Cell morphology was observed to be in the yeast shape only during the growth period.

Dry weight production followed a similar pattern to cell number and maximum dry weight, 4.0 ± 0.1 g $L^{-1}$, was achieved after 168 h with no further change after 240 h. Dry weight was used to calculate the growth rate and the specific rate of fat removal, 0.010 $h^{-1}$ and 0.024 g $g^{-1}$ $h^{-1}$, respectively. A decrease in medium pH from pH 7.0 to 2.5 occurred after 72 h, with no further decrease after 240 h. The decrease in pH correlated with fat metabolism and growth of the yeast. Intracellular lipid comprised approximately 0.4 g $g^{-1}$ after 24 h, which was a 30% increase in cellular lipid from the time of inoculation. Lipid content decreased after 24 h to 0.20 ± 0.03 g $g^{-1}$ at 168 h and remained at this level until the end of the fermentation.

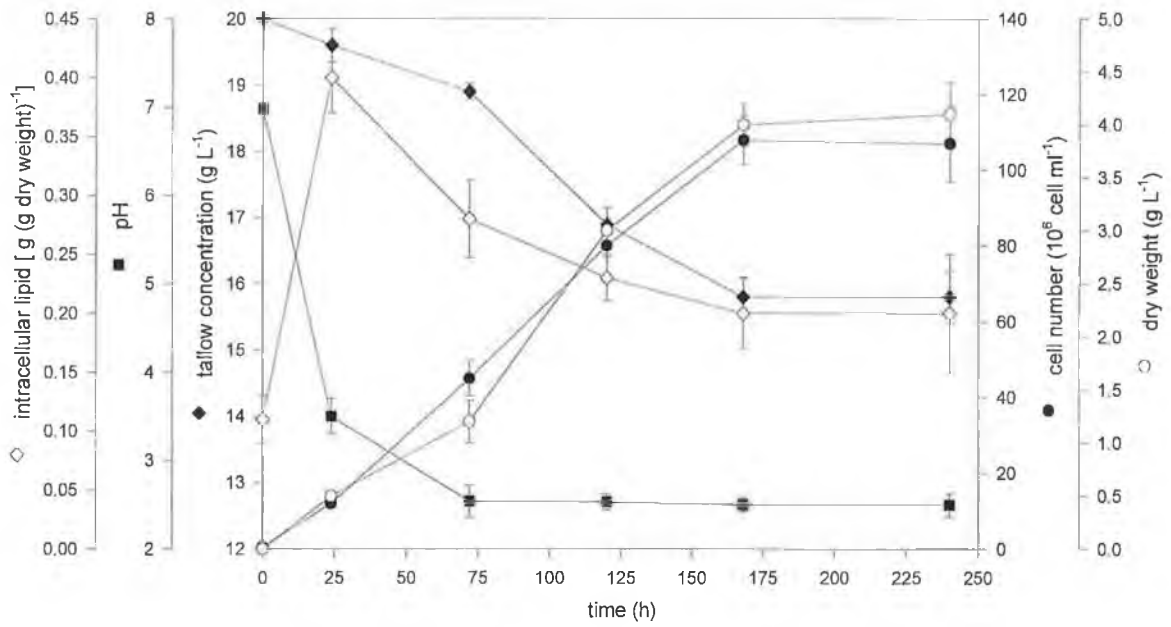



**Figure 3.2.1:** Tallow concentration (◆), cell number (●), dry weight (○), medium pH (■) and intracellular lipid content (◇) during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) for 240 h with no pH control. Temperature and agitation were set to room temperature (22° - 25°C) and 130 rpm, respectively.

The yield coefficient, $Y_{x/s}$ was calculated based on the amount of dry weight (x) produced per tallow removed (s) at the time of maximum fat removal, 168 h, and corresponded to 0.95 g $g^{-1}$. However, the presence of accumulated lipid in the cells implied that some of the extracellular lipid removed was not metabolised.

Consequently, the calculated yield at 168 h may not have given an accurate indication of dry weight or biomass produced per unit substrate. The yield of fat free dry weight on the metabolised lipid was then determined. This was expressed as the yield coefficient, $Y_{xf/sm}$. The metabolised lipid (sm) was the extracellular lipid removed by the yeast, less any accumulation in the biomass:

$$sm = s_{rem} - s_{acc}$$

where: $s_{rem}$ = lipid removed from the supernatant
$s_{acc}$ = lipid accumulated in the biomass

The lipid-free dry weight (xf) was the cellular material of *Y. lipolytica* RP2, exclusive of the lipid content.

$$xf = x - s_{acc}$$

The calculated value of the new yield coefficient, $Y_{xf/sm}$, based on lipid-free dry weight and metabolised fat was 0.94 g $g^{-1}$. The similarity in the yields indicated that the accumulation of lipid intracellularly did not alter the overall yield of biomass of the yeast on tallow. Consequently, all further yield calculations were based on the coefficient $Y_{x/s}$. Similarly, the calculation of the growth rate and specific rate of fat removal based on lipid free dry weight and metabolised tallow were identical to those rates determined based on the dry weight and tallow removed. A comparison of these parameters is presented in Table 3.2.1.

**Table 3.2.1:** Comparison of the yield, growth rate and specific rate of fat removal by *Y. lipolytica* RP2 growth based on both the dry weight (x) and fat removed (s) and the lipid free dry weight (xf) and metabolised fat (sm). The yeast was grown on tallow (20 g $L^{-1}$) for 168 h at room temperature (22 - 25°C) and 130 rpm with no pH control.

| | **Dry weight (x) and tallow removed (s)** | **Lipid free dry weight (xf) and tallow metabolised (sm)** |
|---|---|---|
| Yield (g $g^{-1}$) | 0.95 ± 0.03 | 0.94 ± 0.02 |
| Growth rate ($h^{-1}$) | 0.010 ± 0.001 | 0.010 ± 0.001 |
| Specific rate of fat removal (g $g^{-1}h^{-1}$) | 0.024 ± 0.002 | 0.024 ± 0.002 |

Increased dispersal of the tallow was observed during the growth of the yeast (Fig. 3.2.2). At 24 h, the tallow presented as a 'lump' of fat, which was dispersed into 3 smaller lumps by 120 h. After 168 h, the tallow was dispersed into numerous small lumps of fat, with no further increase in dispersal observed after 240 h (illustration not shown). The tallow in the control flask, which was not inoculated, remained as a single lump of fat throughout the 240 h.

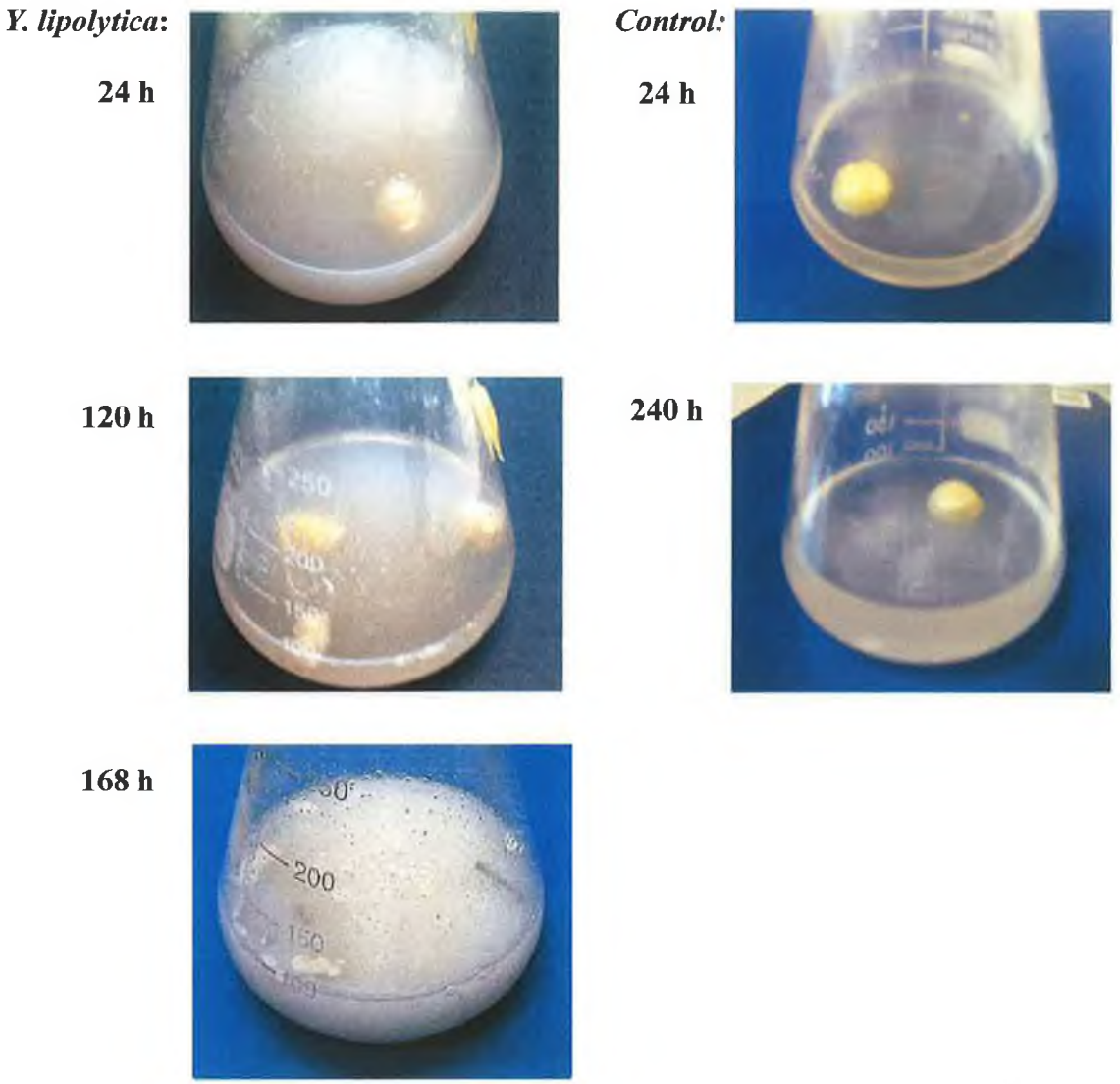



**Figure 3.2.2:** Illustration of the growth flasks of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) with no pH control after 24 h, 120 h and 168 h and control flask (no cells) after 24 h and 240 h. Temperature and agitation were set to room temperature (22° - 25°C) and 130 rpm, respectively.

The parameters of biosurfactant activity were monitored in terms of surface tension and emulsification activity ($A_{540}$) and emulsion stability ($K_d$, decay ratio) with kerosene (Table 3.2.2). The observed dispersal of the tallow corresponded to a reduction in the surface tension from approximately 70 to 62 dynes $cm^{-1}$ by 168 h, with no further decrease at 240 h. Emulsification activity increased during growth to a maximum absorbance of approximately 0.7 during 120 – 168 h, which corresponded to the observed increase in tallow dispersal and increased growth at these times. After 168 h, a decrease in activity occurred. Emulsion stability with kerosene increased during growth also, which corresponded to a decrease in $K_d$ values, to –5.10 at 120 h. However, after 120 h, emulsion stability decreased with increased $K_d$. In the control flask (no cells), surface tension remained at 70 ± 0.5 dynes $cm^{-1}$ throughout the 240 h. No emulsification activity was detected during 240 h and similarly, no change in the decay ratio occurred in the control flask, which correlated with the observed absence of tallow dispersal.

In the growth flask, the reduction in surface tension, detection of emulsification activity and emulsion stability were therefore attributed to the growth of the yeast on tallow and suggested the production of a biosurfactant (bioemulsifier) by the yeast.

**Table 3.2.2:** Surface tension, emulsification ability ($A_{540}$) and emulsion stability (decay ratio, $K_d$) of the medium during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) and the respective control (cell free) for 240 h with no pH control. Temperature and agitation were set to room temperature (22° - 25°C) and 130 rpm, respectively. The medium was filtered through 0.2 μm filter prior to measurement. The absorbance and decay ratios were measured against kerosene.

| Time (h) | *Y. lipolytica RP2* | | | *Control* | | |
|---|---|---|---|---|---|---|
| | **Surface tension (dynes $cm^{-1}$)** | **Emulsification activity ($A_{540}$)** | **Decay ratio ($K_d$ x $10^{-3}$)** | **Surface tension (dynes $cm^{-1}$)** | **Emulsification activity ($A_{540}$)** | **Decay ratio ($K_d$ x $10^{-3}$)** |
| 0 | 70.5 ± 0.50 | 0.05 ± 0.01 | -12.50 ± 0.50 | 70.5 ± 0.50 | 0.05 ± 0.01 | -12.50 ± 0.50 |
| 24 | 64.0 ± 0.33 | 0.45 ± 0.02 | -6.60 ± 0.10 | 70.5 ± 0.33 | 0.07 ± 0.01 | -12.41 ± 0.31 |
| 72 | 63.3 ± 0.33 | 0.65 ± 0.03 | -5.25 ± 0.11 | 70.0 ± 0.13 | 0.07 ± 0.02 | -12.52 ± 0.12 |
| 120 | 62.5 ± 0.25 | 0.70 ± 0.01 | -5.10 ± 0.12 | 70.0 ± 0.67 | 0.06 ± 0.02 | -13.00 ± 0.54 |
| 168 | 62.0 ± 0.33 | 0.69 ± 0.02 | -6.33 ± 0.23 | 69.5 ± 0.33 | 0.06 ± 0.01 | -13.05 ± 0.22 |
| 240 | 62.0 ± 0.13 | 0.55 ± 0.10 | -7.75 ± 0.22 | 70.0 ± 0.67 | 0.06 ± 0.02 | -12.98 ± 0.61 |

### 3.2.2 Optimisation of growth temperature

An investigation into the optimisation of temperature was undertaken with the assessment of growth of *Y. lipolytica* RP2 on tallow at 4°, 25°, 30°, 37° and 55°C at 130 rpm, with no pH control. The percentage fat removal was determined after 168 h and cell number, cell viability and pH were monitored at regular intervals.

Maximum fat removal, 21 ± 0.5%, was achieved at 25°C (Fig. 3.2.3). Reduced fat removal occurred at the higher growth temperatures of 30° and 37°C, at 18% and 10%, respectively, with no fat removed at 55°C. Growth at 4°C resulted in the removal of 2.8 ± 1.0 % fat. The degree of tallow removed from the medium by the yeast corresponded with the degree of growth at the different temperatures. 25°C resulted in the greatest degree of growth, with a final cell number of 1.1 ± 0.06 x $10^8$ cell $ml^{-1}$ after 168 h (Fig. 3.2.4). Growth was reduced with increased temperature, with a final cell number of 9.4 ± 0.4 and 3.8 ± 0.4 x $10^7$ cell $ml^{-1}$ after 168 h for 30° and 37°C, respectively. No growth was detected at 55°C. Growth was also reduced at 4°C, with a final cell count of 1.4 ± 0.4 x $10^7$ cell $ml^{-1}$. Cells were 98 ± 2.0% viable at all temperatures, except at 55°C, where a complete loss in viability occurred. Cell morphology was in the yeast-shape only at all temperatures.

A decrease in medium pH was noted at the various growth temperatures with the exception of 55°C, where the pH remained at pH 7.0. The magnitude of the decrease in pH correlated with the degree of growth observed and corresponded with fat metabolism at the respective temperatures. At 25° and 30°C, pH was reduced to between pH 2.5 – 3.0 after 72 h. This corresponded to the greater level of growth and fat removal at these temperatures. At 168 h, a decrease to pH 3.5 was noted at 37°C while a reduction to pH 5 occurred at 4°C.

Incubation at 55°C resulted in the liquefaction of the tallow (illustration not shown), however, the tallow remained solid at 4°C – 37°C.

25°C was selected as the ideal temperature for fat degradation by *Y. lipolytica* RP2 and all further growth studies were performed at this temperature.

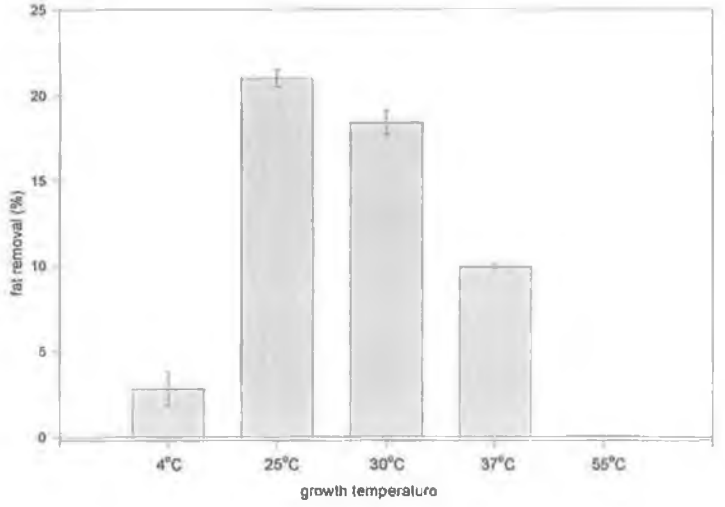



**Figure 3.2.3:** Percentage fat removal by *Y. lipolytica* RP2 after 168 growth on tallow (20 g $L^{-1}$) at 4°, 25°, 30°, 37° and 55°C. Agitation was set to 130 rpm with no pH control.

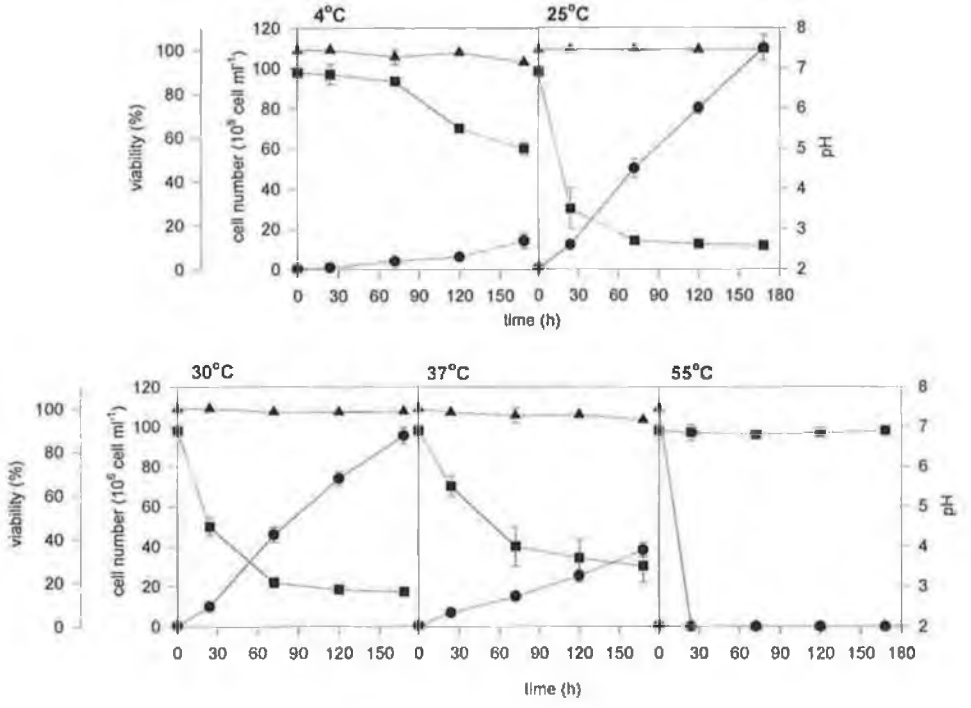



**Figure 3.2.4:** Cell number (●), percentage viability (▲) and medium pH (■) during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) for 168 h at 4°, 25°, 30°, 37° and 55°C. Agitation was set to 130 rpm with no pH control.

### 3.2.3 Effect of chemical surfactant addition on tallow emulsification and biodegradation

The influence of surfactants Triton X-100 and Tween 80, added at concentrations of 0.05, 0.1, 0.25 and 0.5% (w/v), on tallow emulsification, fat removal and growth was investigated. The yeast was cultivated for 168 h on tallow (20 g $L^{-1}$) and fat removal was determined after 168 h. Temperature and agitation were set at 25°C and 130 rpm, respectively. The flasks were sampled at regular intervals and cell number, viability, pH and surfactant activity were monitored.

The greatest degree of fat removal, 21 ± 0.5%, was achieved in the absence of surfactant addition (Fig. 3.2.5). In contrast, the presence of both surfactants, at all concentrations, resulted in a lower degree of fat removal after 168 h. Increased concentrations of Triton X-100, between 0.05 – 0.5% (w/v), resulted in decreased removal to between approximately 10.0 – 6.0%. The addition of Tween 80 resulted in 2.0 – 3.0% fat removal, irrespective of the concentration of surfactant added.

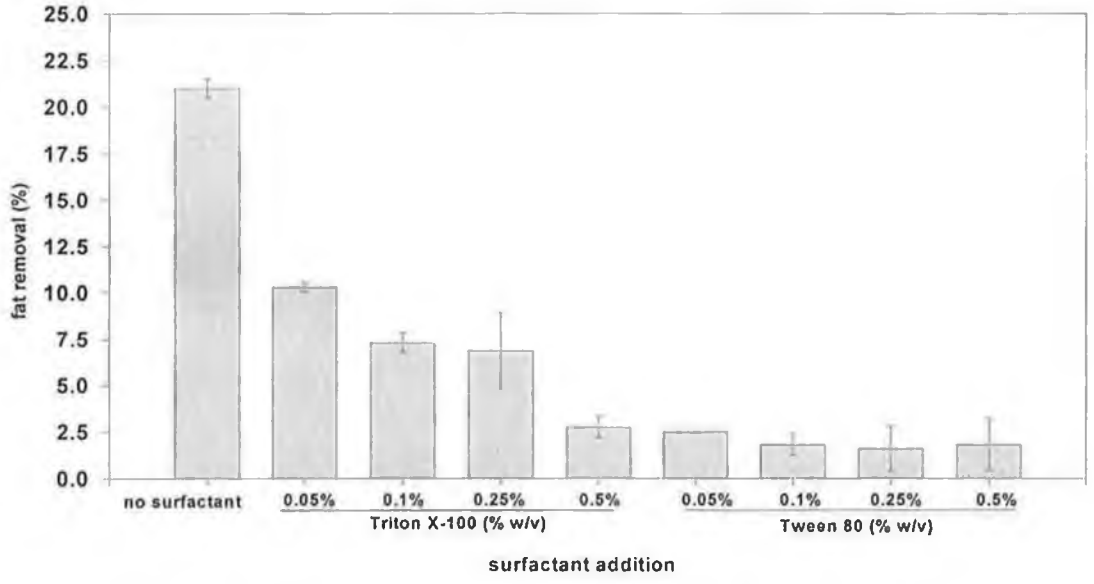



**Figure 3.2.5:** Percentage fat removal by *Y. lipolytica* RP2 after 168 h growth on tallow (20 g $L^{-1}$) in the presence of surfactants Triton X-100 and Tween 80 at concentrations between 0 - 0.5% (w/v). Temperature and agitation were set to 25°C and 130 rpm, respectively with no pH control.

The decrease in fat removal in the presence of the surfactants correlated with a decrease in cell viability and cell growth (Fig. 3.2.6). In the absence of surfactant addition, the cells maintained 100% viability with a final cell number of approximately 1.1 x $10^8$ cell $ml^{-1}$ after 168 h. Increased concentrations of Triton X-100 (0.05 – 0.5% w/v) resulted in a decrease in cell viability after 168 h to between 70 – 50%. Correspondingly, the final cell number decreased to between 6 – 3 x $10^7$ cell $ml^{-1}$ after 168 h. In contrast, the addition of Tween 80 resulted in a similar reduction in viability and growth after 168 h, to approximately 50% viability and 3 x $10^7$ cell $ml^{-1}$, respectively, irrespective of the concentration added. This correlated with the similar degree of fat removal achieved at all Tween 80 concentrations.

A decrease in medium pH was observed in all cases (results not shown). A reduction to pH 2.5 by 168 h was observed in the absence of surfactant addition. Medium pH did not decrease by the same magnitude in the presence of the surfactants, which corresponded to the lower degree of fat removed. A decrease to pH 3.5 – 4.0 and 4.0 – 4.5 by 168 h was noted for Triton X-100 and Tween 80, respectively, irrespective of surfactant concentration. There was no decrease in pH in the control flasks (no cells) irrespective of the addition of either surfactant, at any concentration or where no surfactant was added (results not shown).

The addition of the surfactants influenced the dispersion of the tallow in the medium prior to inoculation, with the exception of 0.05% Tween 80 where no tallow dispersal was noted (Fig. 3.2.7). Increased concentration of both surfactants between 0.05 – 0.5% (w/v) resulted in increased dispersion. However, there was no further change in tallow dispersion during the 168 h. In the control flasks (no cells) with the respective concentrations of the surfactants, an identical degree of tallow dispersal was observed compared to the growth flasks whereas no dispersal was observed in absence of the surfactants (illustrations not shown). Triton X-100 was observed to be a more effective surfactant than Tween 80, with greater tallow dispersal at all concentrations and complete emulsification of the tallow in 0.5% (w/v) Triton X-100. In the growth flasks without the surfactants, tallow dispersion was observed only during growth on tallow, as outlined in Section 3.2.1 (Fig. 3.2.2) and by 168 h was similar to that observed with 0.1% Triton X-100 and 0.25% Tween 80.

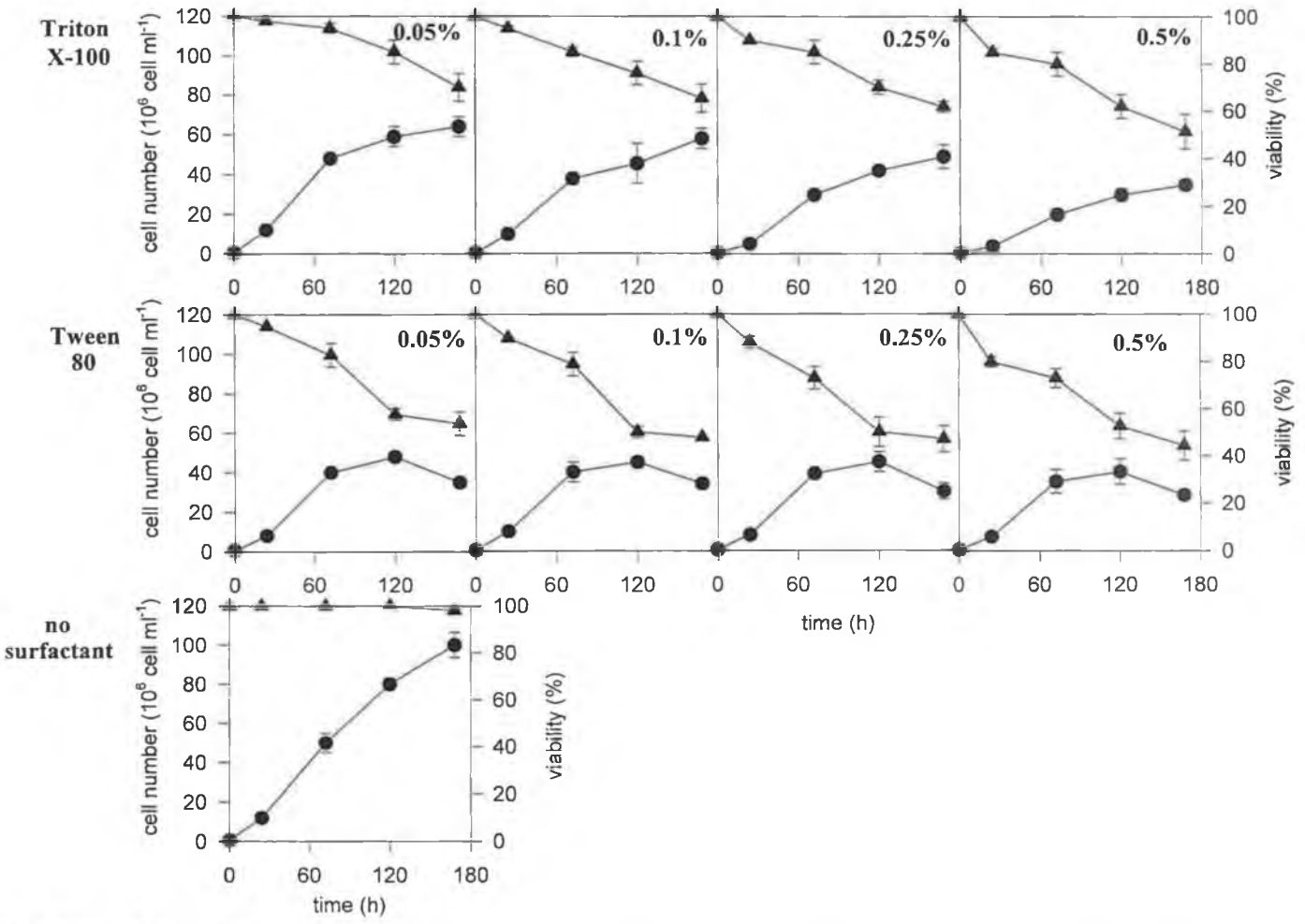



**Figure 3.2.6:** Cell number (●) and cell viability (▲) for *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) for 168 h with surfactants Triton X-100 and Tween 80 at concentrations between 0.0 – 0.5% (w/v). Temperature and agitation were set to 25°C and 130 rpm, respectively, with no pH control.

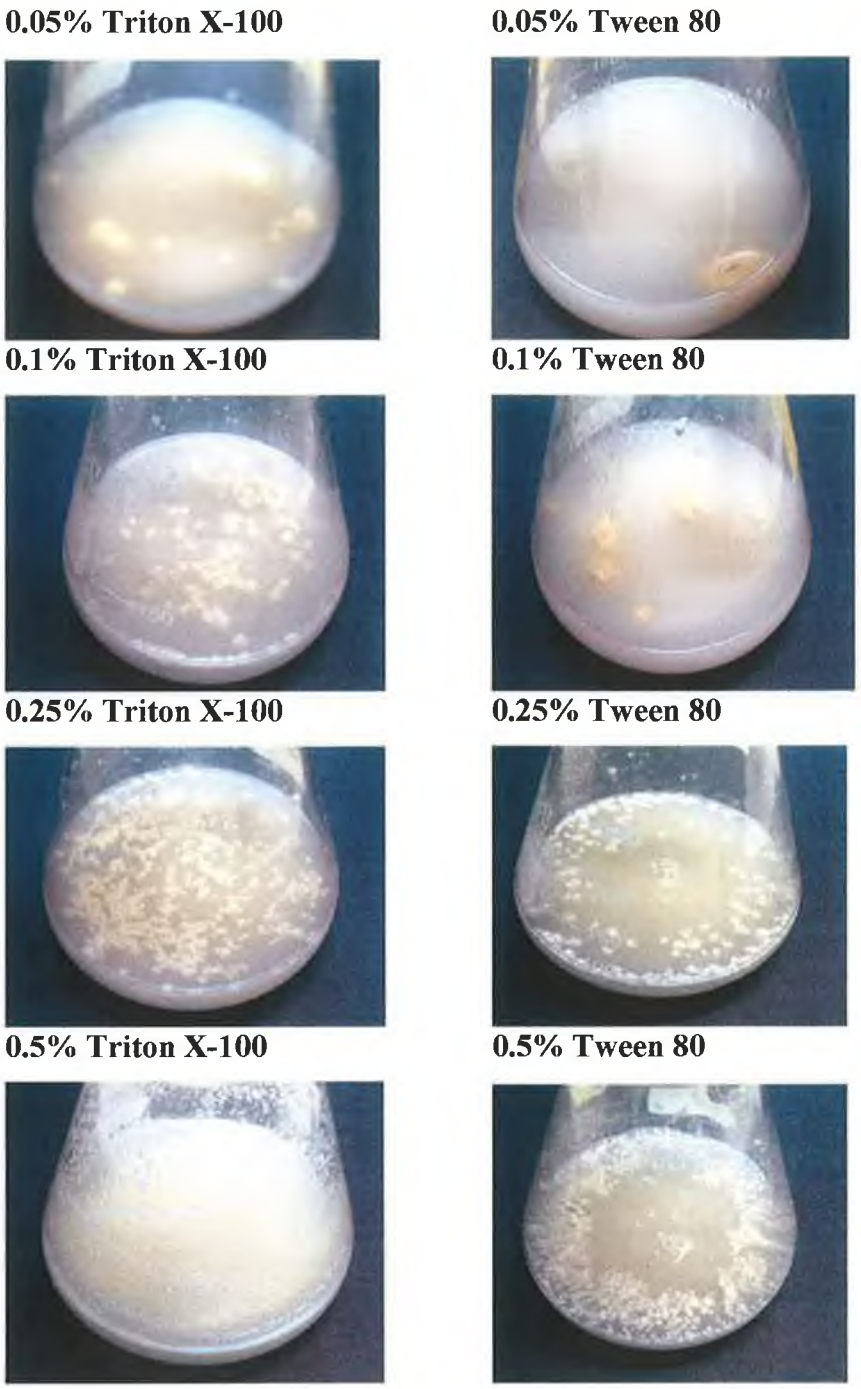



**Figure 3.2.7:** Illustration of tallow (20 g $L^{-1}$) dispersal in the presence of surfactants Triton X-100 and Tween 80 at concentrations between 0.05 - 0.5 % (w/v) prior to yeast inoculation.

The presence of Triton X-100 and Tween 80 reduced the surface tension to approximately 30 - 34 and 42 - 44 dynes $cm^{-1}$, respectively, from the time of their addition with no further change up to 168 h, irrespective of surfactant concentration (Table 3.2.3). In the absence of surfactant addition, surface tension was reduced to 62 dynes $cm^{-1}$ after 168 h as outlined in Section 3.2.1 (Table 3.2.2). Consequently, surface tension was not considered an indication of the degree of tallow dispersion in the medium, as dispersion was observed in the flasks at both a lower (30 - 44 dynes $cm^{-1}$) and higher (62 dynes $cm^{-1}$) surface tension with the surfactants and without the surfactants, respectively. In contrast, emulsification activity ($A_{540}$) correlated with the observed tallow dispersion. Emulsification activity was greater for Triton X-100 compared to Tween 80 at each concentration. Activity increased upon increased concentration of the surfactants up to 0.5% (w/v), with a corresponding absorbance of 1.22 and 1.06 for Triton X-100 and Tween 80, respectively at 0 h. Significantly, an emulsification activity of 0.69 absorbance units was detected with no surfactant addition (Table 3.2.2), which was comparable to that of 0.1% Triton X-100 ($A_{540}$, 0.70) and 0.25% Tween 80 ($A_{540}$, 0.78), after 168 h. This corresponded with the observed similarity in tallow dispersal into numerous small lumps in these flasks. Emulsion stability (decay ratio, $K_d$), was similar for both surfactants at the respective concentrations between –3.3 to –1.8 for 0.05 to 0.5% (w/v). No significant change in either absorbance or the decay ratio occurred during the 168 h. In contrast, the emulsion formed with no surfactant addition was not as stable as that formed with the surfactants, which corresponded to a lower decay ratio (-6.33) at 168 h. The corresponding control flasks (no cells) for both surfactants resulted a similar surface tension, absorbance and decay ratio as the surfactant growth flasks, at all concentrations (results not shown). In contrast, controls with no surfactant addition did not reduce the surface tension and no emulsification activity or stability was detected.

Overall, the addition of the surfactants Triton X-100 and Tween 80 to the medium at concentrations greater than 0.05% (w/v) was found to increase the dispersal of the tallow in the medium. However, the surfactants exerted a toxic effect on the yeast, which resulted in both reduced viability and growth and subsequently reduced fat removal. Therefore, the addition of the surfactants was not found to be beneficial in the optimisation of the growth of *Y. lipolytica* RP2 on tallow.

**Table 3.2.3:** Surface tension, emulsification ability ($A_{540}$) and emulsion stability (decay ratio, $K_d$) of the medium during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) for 168 h with the surfactants Triton X-100 and Tween 80 at concentrations between 0.05 – 0.5% (w/v). Temperature and agitation were set to room temperature 25°C and 130 rpm, respectively with no pH control and the medium was filtered through 0.2 μm filter prior to measurement. The absorbance and decay ratios were measured against kerosene.

| | | Triton X-100 | | | Tween 80 | | |
|---|---|---|---|---|---|---|---|
| **Time (h)** | **Conc. (% w/v)** | **Surface tension (dynes $cm^{-1}$)** | **Emulsification activity ($A_{540}$)** | **Decay ratio ($K_d$ x $10^{-3}$)** | **Surface tension (dynes $cm^{-1}$)** | **Emulsification activity ($A_{540}$)** | **Decay ratio ($K_d$ x $10^{-3}$)** |
| 0 | 0.05 | 33.0 ± 0.5 | 0.50 ± 0.01 | -3.25 ± 0.02 | 44.0 ± 0.3 | 0.21 ± 0.06 | -3.30 ± 0.10 |
| | 0.10 | 33.0 ± 0.3 | 0.65 ± 0.08 | -2.89 ± 0.01 | 43.0 ± 0.7 | 0.44 ± 0.11 | -2.90 ± 0.03 |
| | 0.25 | 31.0 ± 0.1 | 1.05 ± 0.10 | -2.20 ± 0.03 | 43.0 ± 0.1 | 0.82 ± 0.04 | -2.15 ± 0.05 |
| | 0.50 | 31.0 ± 0.1 | 1.22 ± 0.07 | -1.85 ± 0.07 | 42.5 ± 0.1 | 1.06 ± 0.13 | -2.00 ± 0.06 |
| 120 | 0.05 | 33.0 ± 0.1 | 0.50 ± 0.05 | -3.30 ± 0.04 | 44.0 ± 0.7 | 0.21 ± 0.05 | -3.25 ± 0.22 |
| | 0.10 | 33.5 ± 0.1 | 0.71 ± 0.05 | -2.85 ± 0.02 | 43.3 ± 0.3 | 0.43 ± 0.10 | -3.00 ± 0.14 |
| | 0.25 | 31.0 ± 0.1 | 1.04 ± 0.06 | -2.25 ± 0.05 | 43.0 ± 0.1 | 0.80 ± 0.02 | -2.20 ± 0.04 |
| | 0.50 | 31.0 ± 0.2 | 1.20 ± 0.09 | -1.82 ± 0.02 | 42.0 ± 0.2 | 1.10 ± 0.20 | -2.15 ± 0.09 |
| 168 | 0.05 | 33.5 ± 0.1 | 0.49 ± 0.05 | -3.35 ± 0.01 | 44.0 ± 0.7 | 0.20 ± 0.05 | -3.27 ± 0.13 |
| | 0.10 | 33.0 ± 0.3 | 0.70 ± 0.07 | -2.90 ± 0.02 | 43.3 ± 0.3 | 0.40 ± 0.15 | -3.04 ± 0.04 |
| | 0.25 | 31.5 ± 0.2 | 1.01 ± 0.04 | -2.27 ± 0.03 | 43.0 ± 0.1 | 0.78 ± 0.08 | -2.22 ± 0.05 |
| | 0.50 | 31.0 ± 0.3 | 1.17 ± 0.10 | -1.80 ± 0.02 | 42.0 ± 0.2 | 1.11 ± 0.20 | -2.20 ± 0.01 |

### 3.2.4 Role of pH control

Medium pH was reduced from pH 7.0 to approximately pH 2.5 during the growth of *Y. lipolytica* RP2 on tallow at 25°C and 130 rpm in shake flask culture. It was anticipated that control of the medium pH by the inclusion of a buffer would improve fat removal and growth of the yeast. A study was undertaken which included the determination of the optimum pH for fat removal, a comparison of various buffers at optimum pH, determination of optimum buffer concentration and optimum growth temperature with pH control, under these growth conditions. The influence of pH control on yeast morphology and biosurfactant production was also assessed.

#### 3.2.2.1 Optimisation of medium pH between pH 3.0 – 8.0 with 0.1 M citrate phosphate buffer

The medium pH was controlled via the inclusion of 0.1 M citrate phosphate buffer, a wide pH range buffer, between pH 3.0 - 8.0. Temperature and agitation were set to 25°C and 130 rpm, respectively. The degree of fat removal by *Y. lipolytica* RP2 was determined after 168 h. The growth flasks were sampled at regular intervals and pH, cell number, cell viability and yeast morphology were monitored.

Maximum fat removal, 68.6 ± 1.2%, was achieved at pH 7.0 after 168 h (Fig. 3.2.8). This was a dramatic increase in fat removal compared to no pH control which resulted in 21% removal after 168 h. Increased pH resulted in increased fat removal up to pH 7.0 with a marginal decrease at pH 8.0, with 65% fat removed. Medium pH was maintained at the respective pH values by the buffer during the 168 h, with a decrease to pH 2.5 in the absence of pH control (Fig. 3.2.9). The degree of cell growth correlated with the degree of fat removed at each pH. Control to pH 7.0 resulted in the greatest cell number, $1.7 \times 10^8$ cell $ml^{-1}$, after 168 h. Increased pH resulted in increased cell number after 168 h between pH 3.0 - 7.0, with a marginal decrease in cell number to $1.6 \times 10^8$ cell $ml^{-1}$ at pH 8.0. Cell viability was maintained at 100% during 168 h, in all cases.

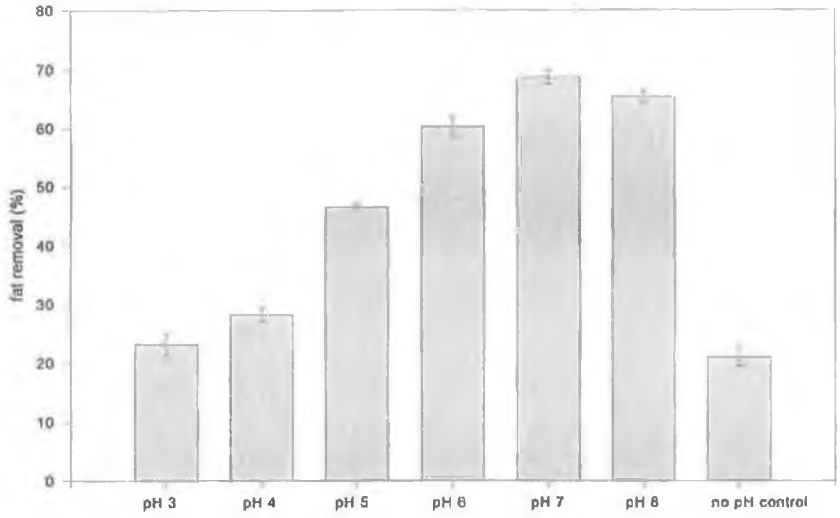



**Figure 3.2.8:** Percentage fat removal by *Y. lipolytica* RP2 after 168 h growth on tallow (20 g $L^{-1}$) with pH control between pH 3.0 – 8.0 by 0.1 M citrate phosphate buffer and with no pH control. Temperature and agitation were set to 25°C and 130 rpm, respectively.

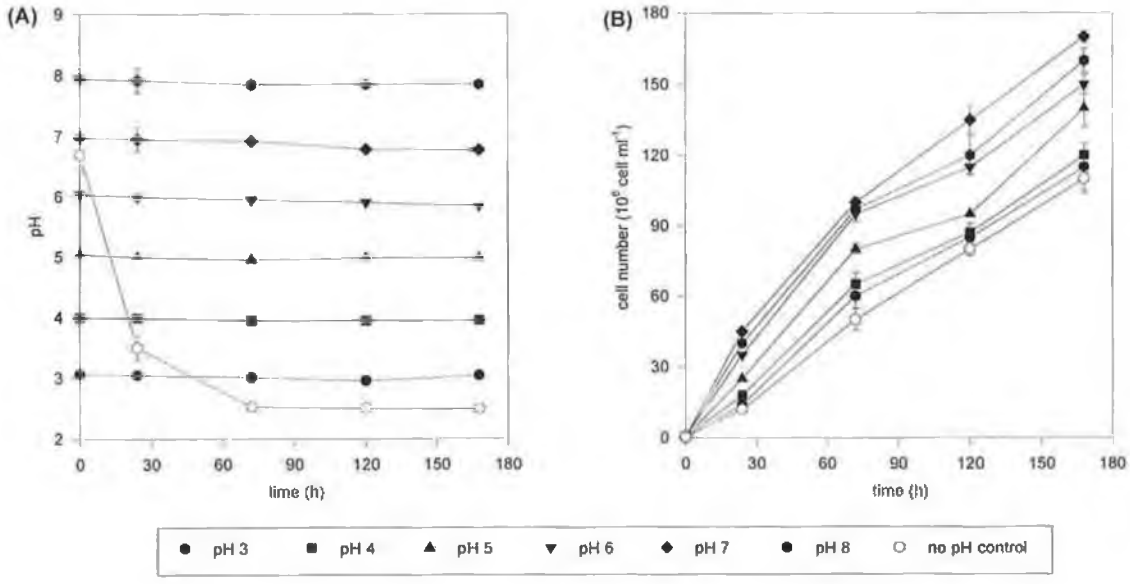



**Figure 3.2.9:** pH (A) and cell number (B) during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) for 168 h with pH control between pH 3.0 – 8.0 by 0.1 M citrate phosphate buffer and with no pH control. Temperature and agitation were set to 25°C and 130 rpm, respectively.

Interestingly, a difference in yeast morphology was observed at the various pH values when grown on tallow. Dimorphic growth, the ability of the yeast to produce both yeast- and mycelial-shaped cells was noted at 24 h growth at pH values 5.0 - 8.0 (Table 3.2.4). An increase in percentage mycelial-shaped cells from 5.0% to 6.3%, based on a ratio of the overall cell number, was observed upon increased pH between pH 5.0 to 8.0. However, after 24 h, a decrease in mycelium formation was observed with less than 1% mycelial-shaped cells present after 168 h at pHs 5.0 to 8.0. In contrast, yeast morphology was in the yeast-shape only during 168 h at pH 3.0 and 4.0 and in the absence of pH control. In the control flasks, which contained yeast and buffer only (no tallow), cell morphology was in the yeast shape only (results not shown). This implied that the dimorphism was as a result of pH control near neutrality with the presence of tallow and not as a result of the buffer itself. An illustration of the dimorphic phenomenon at both 24 and 168 h at pH 7.0 is illustrated in Fig. 3.2.10.

**Table 3.2.4:** Percentage mycelia ratio for *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) for 168 h with pH control between pH 3.0 – 8.0 with 0.1 M citrate phosphate buffer and with no pH control. Temperature and agitation were set to 25°C and 130 rpm, respectively.

| | **Mycelial ratio (%)** | | | | |
|---|---|---|---|---|---|
| **pH** | **0 h** | **24 h** | **72 h** | **120 h** | **168 h** |
| 3.0 | 0 | 0 | 0 | 0 | 0 |
| 4.0 | 0 | 0 | 0 | 0 | 0 |
| 5.0 | 0 | 5.0 ± 1.2 | 1.0 ± 0.5 | 0.6 ± 0.2 | 0.5 ± 0.2 |
| 6.0 | 0 | 5.7 ± 0.8 | 1.0 ± 0.1 | 0.8 ± 0.3 | 0.8 ± 0.1 |
| 7.0 | 0 | 6.0 ± 1.1 | 1.3 ± 1.2 | 0.9 ± 0.5 | 0.8 ± 0.1 |
| 8.0 | 0 | 6.3 ± 0.6 | 1.9 ± 0.6 | 1.5 ± 0.6 | 0.9 ± 0.1 |
| No control | 0 | 0 | 0 | 0 | 0 |

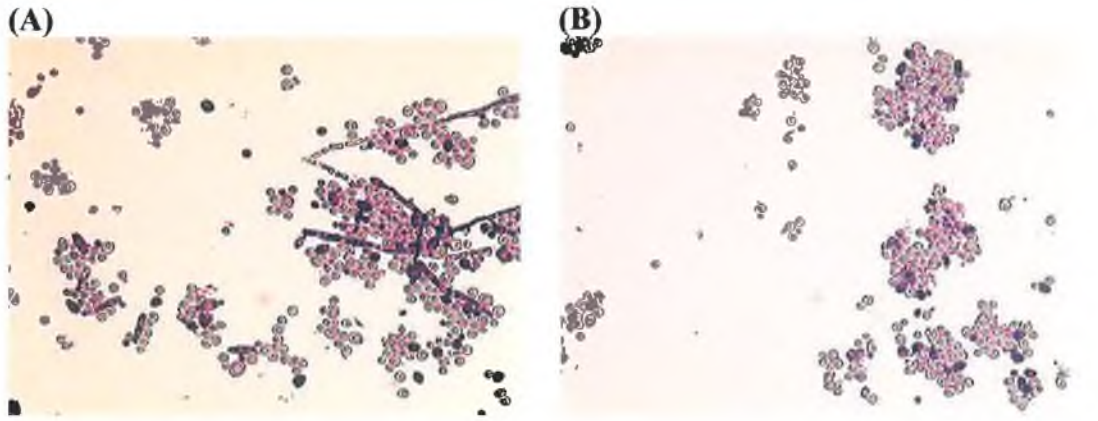



**Figure 3.2.10:** Illustration of *Y. lipolytica* RP2 cell morphology after (A) 24 h and (B) 168 h growth on tallow (20 g $L^{-1}$) with pH control to 7.0 by 0.1 M citrate phosphate buffer. Temperature and agitation were set to 25°C and 130 rpm, respectively. Cells were observed under x20 magnification.

In the control flasks for citrate phosphate buffer at pH 3.0 – 8.0, which contained yeast and buffer only and no tallow, a degree of growth was observed with an increase from 5 x $10^5$ cell $ml^{-1}$ to 1.7 ± 1.0 x $10^7$ cell $ml^{-1}$ after 168 h (results not shown). An increase in pH by 1.5 ± 0.5 pH units at each pH was also noted after 168 h. The rise in pH was attributed to the utilisation of the citrate in the buffer, thereby increasing the concentration of phosphate and subsequently increasing the pH. The pH remained at the respective pH values (3.0 – 8.0) throughout the fermentation when no yeast was added to the buffered medium. The presence of citrate phosphate buffer in the medium provided an additional carbon source with the tallow for the yeast and therefore would not be suitable for investigations with tallow as the sole carbon source.

pH 7.0 was selected for all further growth studies of *Y. lipolytica* RP2 on tallow. The unsuitability of 0.1 M citrate phosphate buffer for pH control necessitated a further investigation into the inclusion of buffers at pH 7.0 on fat removal by the yeast.

### 3.2.4.2 Influence of various buffers (0.1 M, pH 7.0) on tallow biodegradation

A number of buffers at a concentration of 0.1 M and at pH 7.0, were assessed as to their influence on fat removal and growth. The four buffers included citrate phosphate, di-sodium phosphate ($Na_2HPO_4$-$NaH_2PO_4$), potassium phosphate (KOH--$KH_2PO_4$) and sodium phosphate buffer (NaOH-$NaH_2PO_4$). Citrate phosphate buffer was included for comparison purposes, although it was shown to provide an additional carbon source with the tallow in the previous section. Temperature and agitation were set to 25°C and 130 rpm. Fat removal was determined after 168 h. The flasks were sampled at regular intervals and dry weight, cell viability and morphology were monitored.

Potassium phosphate buffer achieved a marginally better degree of fat removal, 75 ± 0.5%, after 168 h compared to the other buffers (Table 3.2.5). Inclusion of citrate and both sodium buffers removed a similar degree of fat removal, approximately 68.5 ± 1.0%. Correspondingly, the growth rate was greater with potassium phosphate buffer, 0.020 $h^{-1}$, compared to other buffers (0.016 $h^{-1}$). Cell viability was maintained at 100% during the 168 h, in all cases.

The presence of citrate as an additional carbon source did not result in increased fat removal or growth compared to the other buffers. In the control flasks, which contained the yeast and buffers only and no tallow, no growth or change in pH was detected with potassium phosphate or both sodium phosphate buffers (results not shown). This indicated that the yeast could not utilise these buffers as a sole carbon source.

Dimorphic growth was observed in the presence of all the buffers and tallow. The presence of approximately 6.0 ± 1.5 % mycelia, as a ratio of the overall cell number, was detected at 24 h in all cases (results not shown). However, after 24 h, a reduction in the number of mycelia occurred with less than 1% mycelial-shaped cells present after 168 h, in all cases.

**Table 3.2.5:** Percentage fat removal and growth rate for *Y. lipolytica* RP2 after 168 h growth on tallow (20 g $L^{-1}$) with pH control to pH 7.0 by citrate phosphate, di-sodium phosphate, potassium phosphate and sodium phosphate buffers. Temperature and agitation were set to 25°C and 130 rpm, respectively.

| Buffer | Fat removal (%) | Growth rate ($h^{-1}$) |
|---|---|---|
| Citrate phosphate | 68.0 ± 1.5 | 0.016 ± 0.001 |
| $Na_2HPO_4$-$NaH_2PO_4$ | 67.9 ± 1.2 | 0.016 ± 0.001 |
| KOH-$KH_2PO_4$ | 74.5 ± 0.5 | 0.020 ± 0.001 |
| NaOH-$NaH_2PO_4$ | 65.7 ± 2.3 | 0.016 ± 0.001 |

Maximum fat removal and growth, 75% and 0.02 $h^{-1}$, respectively, were achieved after 168 h with 0.1 M potassium phosphate buffer, pH 7.0, which resulted in its selection for further studies into the optimisation of growth conditions in shake flask culture.

### 3.2.4.3 Optimisation of the concentration of potassium phosphate buffer, pH 7.0 and growth temperature

In the previous investigation, potassium phosphate buffer was employed at a concentration of 0.1 M. The influence of increased concentrations of potassium phosphate buffer to 0.2, 0.3, 0.5 and 1.0 M, all at pH 7.0, on growth and fat removal by *Y. lipolytica* RP2 was investigated. The influence of temperature on the buffering capacity and on growth of the yeast under pH control was also determined with growth at 4°, 25°, 30° and 37°C. 55°C was not included in the temperature range as a result of the complete loss in viability observed previously at this temperature (Section 3.2.2). Agitation was set to 130 rpm. Fat removal was determined after 168 h. The flasks were sampled regularly and pH, dry weight, cell viability and morphology were monitored.

Maximum fat removal, 75%, was achieved at 25°C with a buffer concentration of 0.1 M and 0.2 M (Table 3.2.6). Increased buffer concentration greater than 0.2 M resulted in decreased fat removal, irrespective of the growth temperature. Also,

increased growth temperature to 30° and 37°C resulted in decreased fat removal compared to 25°C, with only 1 - 6% fat removed at 4°C at the various concentrations. The effect of growth temperature with the buffer at 4°C was similar to that observed previously in Section 3.2.2 (Fig. 3.2.3) where no pH control was employed. Correspondingly, the small degree of growth observed at 4°C, at approximately 0.001 - 0.003 $h^{-1}$ correlated with the reduced degree of fat removed at this temperature. In contrast, pH control with 0.1 and 0.2 M buffer at 30° and 37°C resulted in approximately four times more fat removed compared to no pH control (66% and 44% compared to 18% and 10% for pH control and no control at 30°C and 37°C, respectively).

Maximum growth rate was achieved at 25°C, at a buffer concentration of 0.1 M and 0.2 M, at 0.02 ± 0.001 $h^{-1}$, with decreased growth rates for 30° and 37°C under the same conditions at approximately 0.019 and 0.017 $h^{-1}$, respectively. Increased concentrations of buffer above 0.2 M, resulted in a decreased growth rate at all temperatures, which corresponded to decreased fat removal under these conditions.

The buffer was not found to be toxic to the yeast at concentrations 0.1 – 0.3 M, with 100% viability observed over the 168 h at all temperatures. There was a marginal reduction in cell viability after 168 h for 0.5 and 1.0 M buffer to approximately 98 ± 0.3% and 95 ± 0.5%, respectively, irrespective of the growth temperature (results not shown).

**Table 3.2.6:** Percentage fat removal and growth rate for *Y. lipolytica* RP2 after 168 h growth on tallow (20 g $L^{-1}$) at 130 rpm with pH control by 0.1, 0.2, 0.3, 0.5, and 1.0 M potassium phosphate buffer, pH 7.0, at growth temperatures 4°, 25°, 30° and 37 °C, respectively.

| | **Temperature** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **4°C** | | **25°C** | | **30°C** | | **37°C** | |
| **Buffer concentration (M)** | Fat removal (%) | Growth rate ($h^{-1}$) | Fat removal (%) | Growth rate ($h^{-1}$) | Fat removal (%) | Growth rate ($h^{-1}$) | Fat removal (%) | Growth rate ($h^{-1}$) |
| 0.1 | 5.9 ± 1.4 | 0.003 ± 0.0003 | 74.5 ± 0.5 | 0.020 ± 0.0010 | 66.2 ± 0.4 | 0.019 ± 0.0005 | 43.6 ± 1.3 | 0.017 ± 0.0003 |
| 0.2 | 5.8 ± 0.9 | 0.003 ± 0.0002 | 74.0 ± 2.2 | 0.020 ± 0.0010 | 65.6 ± 0.6 | 0.019 ± 0.0003 | 43.2 ± 1.8 | 0.017 ± 0.0005 |
| 0.3 | 2.5 ± 0.7 | 0.002 ± 0.0002 | 65.0 ± 1.0 | 0.018 ± 0.0005 | 48.2 ± 0.4 | 0.015 ± 0.0002 | 33.9 ± 1.3 | 0.012 ± 0.0004 |
| 0.5 | 1.5 ± 0.7 | 0.001 ± 0.0002 | 44.8 ± 1.5 | 0.014 ± 0.0006 | 40.5 ± 0.4 | 0.014 ± 0.0001 | 28.7 ± 0.8 | 0.011 ± 0.0005 |
| 1.0 | 1.1 ± 0.7 | 0.0009 ± 0.0001 | 20.5 ± 1.7 | 0.005 ± 0.0001 | 17.7 ± 0.3 | 0.004 ± 0.0002 | 10.6 ± 0.6 | 0.004 ± 0.0001 |

Buffer concentration and growth temperature with pH control, were both found to influence yeast morphology after 24 h growth. A similar degree of mycelium production was observed for 0.1 and 0.2 M buffer at approximately 6% for 25°C and 30°C and 2.5% for 37°C (Fig. 3.2.11). Increased concentration of the buffer above 0.2 M resulted in a decrease in the ratio of mycelia at 25°, 30° and 37°C, with less than 1% mycelia present in 1.0 M concentration. After 24 h, a decrease in the mycelia ratio was observed, with less than 1% mycelia present by the end of the growth period. In contrast, no mycelia were detected at 4°C at any stage of growth, irrespective of the buffer concentration, with morphology observed to be in the yeast-shape only. At all buffer concentrations, 25°C and 30°C resulted in similar ratios of mycelium production, but a lower ratio was observed at 37°C.

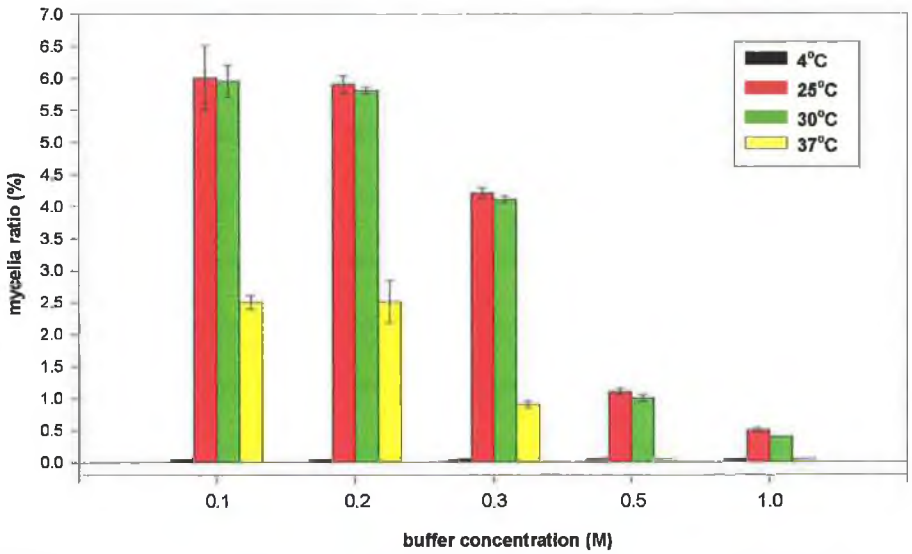



**Figure 3.2.11:** Percentage mycelial ratio after 24 h growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) with pH control by potassium phosphate buffer at concentrations 0.1, 0.2, 0.3, 0.5 and 1.0 M at temperatures 4°, 25°, 30° and 37°C, respectively. Agitation was set to 130 rpm.

The absence of increased growth or fat removal upon the inclusion of increased concentrations of potassium phosphate buffer, coupled with the similar degree of removal and growth observed with 0.1 M and 0.2 M at 25°C, 75% and 0.02 $h^{-1}$, respectively, at 25°C indicated that 0.1 M potassium phosphate buffer, pH 7.0, was ideal for the growth of *Y. lipolytica* RP2 on tallow, under these conditions.

Similarly, the superior growth and fat removal achieved at 25°C indicated that this temperature was best for the growth of the yeast on tallow with pH control and all further growth studies were carried out at 25°C.

### 3.2.4.4 Characteristics of the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) at optimised growth temperature, 25°C and pH, 7.0

Fat removal was determined only after 168 h in the previous temperature and pH investigations. Therefore, the growth characteristics of *Y. lipolytica* RP2 on tallow were assessed in minimal medium for up to 240 h under the optimised conditions of temperature and pH, at 25°C and pH 7.0 with 0.1 M potassium phosphate buffer, respectively. The flasks were sampled at regular intervals and fat removal, cell number, dry weight, cell viability, morphology and intracellular lipid were monitored. The surface tension, emulsification activity and emulsion stability of the growth medium were also measured.

Maximum fat removal, 75%, occurred after 168 h, which corresponded to 5 g $L^{-1}$ tallow in the medium, with no further removal after 240 h (Fig 3.2.12). The time required to achieve this fat removal, 168 h, was identical to that required in the absence of pH control as outlined in Section 3.2.1. Stationary growth was reached at 144 h, which corresponded to 2.2 x $10^8$ cell $ml^{-1}$. Yeast viability was maintained at 100% during the 240 h. Dimorphic growth was observed after 24 h with 6.0 ± 1.0% mycelial-shaped cells produced (results not shown). However, after 24 h, the ratio of mycelial cells decreased with less than 1% present at the end of the fermentation. Dry weight increased up to 15 g $L^{-1}$ at 144 h, with no increase after that time. Dry weight values were used to determine the growth rate, specific rate of fat removal and yield coefficient, 0.020 ± 0.001 $h^{-1}$, 0.035 ± 0.002 g $g^{-1}$ $h^{-1}$ and 1.01 g $g^{-1}$, respectively. These parameters were all greater with pH control to pH 7.0, compared to no pH control in Section 3.2.1.

The intracellular lipid content of the yeast cells changed during growth on tallow. At 24 h, intracellular lipid comprised 0.42 ± 0.20 g $g^{-1}$. After 24 h, intracellular lipid content decreased to approximately 0.10 g $g^{-1}$ by 120 h, and remained at this level until 240 h. This decrease in intracellular lipid was more pronounced and greater in magnitude with pH control compared to no pH control, where intracellular lipid decreased to 0.20 g $g^{-1}$ after 168 h (Fig. 3.2.1). This corresponded to the greater degree of growth and fat removal under pH control compared to no pH control.

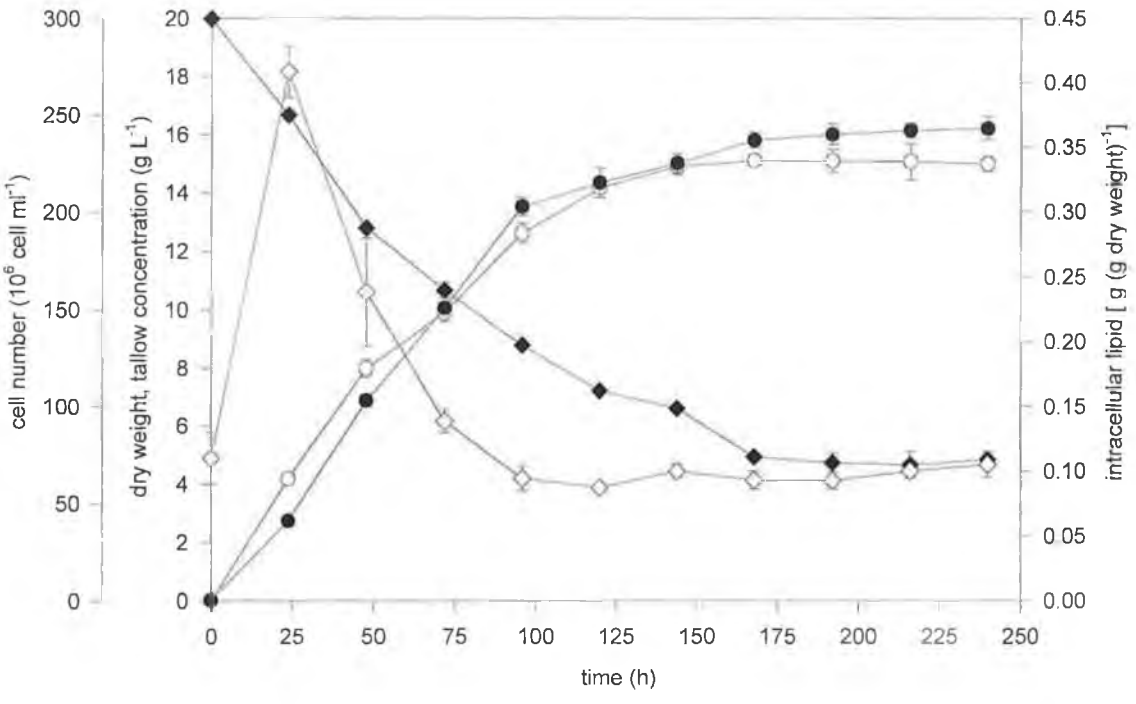



**Figure 3.2.12:** Cell number (●), dry weight (○), tallow concentration (◆) and intracellular lipid (◇) content of *Y. lipolytica* RP2 when grown on tallow (20 g $L^{-1}$) for 240 h with pH control by 0.1 M potassium phosphate buffer, pH 7.0. Temperature and agitation were set to 25°C and 130 rpm.

Tallow dispersion increased over time during growth of the yeast and complete emulsification of the tallow occurred by 168 h (Fig. 3.2.13). No further change in dispersion occurred after 168 h. This corresponded to the time of maximum removal of the tallow by the yeast. The degree of dispersal with pH control was also greater that that observed during growth in the absence of pH control in Section 3.2.1.

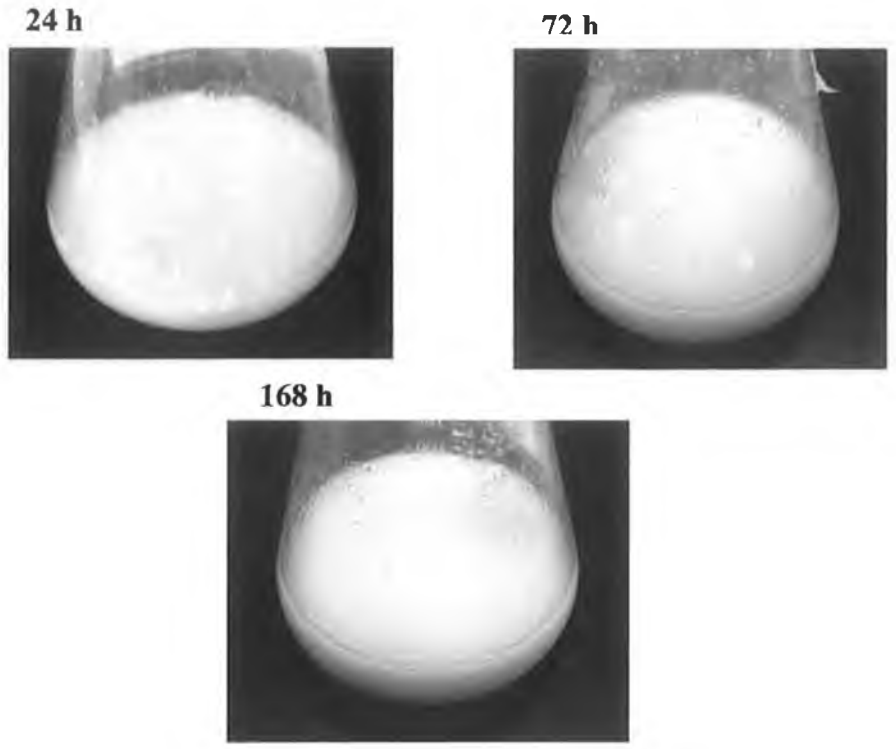



**Figure 3.2.13:** Illustration of the growth flasks of *Y. lipolytica* RP2 on (20 g $L^{-1}$) with 0.1 M potassium phosphate buffer, pH 7.0 after 24, 72 and 168 h. Temperature and agitation were set to 25°C and 130 rpm, respectively.

The increased tallow dispersion achieved with pH control compared to no control was also reflected in greater biosurfactant activity with the buffer (Table 3.2.7). Surface tension was reduced to 56 dynes $cm^{-1}$ after 120 h, with no further reduction after this time. In contrast, surface tension was reduced to 62 dynes $cm^{-1}$ in the absence of pH control, as outlined in Section 3.2.1. Similarly, under pH control to 7.0, emulsification activity ($A_{540}$) was also enhanced. Emulsification activity increased to an absorbance of 1.61 ± 0.09 after 120 h, which was more than double that with no pH control ($A_{540}$, 0.70). The decay ratio under pH control, decreased to –3.80 after 120 h, which demonstrated increased emulsion stabilisation compared to no pH control ($K_d$, –5.10) after the same growth period. A small decrease in the surface tension, from 70 to 65 dynes $cm^{-1}$ occurred in the control flask, which was not inoculated. No emulsification activity or stability was detected in the controls during 240 h.

Maximum fat removal and growth, 75% and 0.02 $h^{-1}$ respectively, were achieved after 168 h growth with pH control by 0.1 M potassium phosphate buffer, pH 7.0. Consequently, all further shake flask studies were performed for 168 h.

**Table 3.2.7:** Surface tension, emulsification ability ($A_{540}$) and emulsion stability (decay ratio, $K_d$) of the medium during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) and the respective control (cell free) for 168 h with pH control by 0.1 M potassium phosphate buffer, pH 7.0. Temperature and agitation were set to 25°C and 130 rpm, respectively. The medium was filtered through 0.2 μm filter prior to measurement. The absorbance and decay ratios were measured against kerosene.

| Time (h) | *Y. lipolytica RP2* | | | *Control* | | |
|---|---|---|---|---|---|---|
| | **Surface tension (dynes $cm^{-1}$)** | **Emulsification activity ($A_{540}$)** | **Decay ratio ($K_d$ x $10^{-3}$)** | **Surface tension (dynes $cm^{-1}$)** | **Emulsification activity ($A_{540}$)** | **Decay ratio ($K_d$ x $10^{-3}$)** |
| 0 | 70.0 ± 1.0 | 0.09 ± 0.02 | -6.35 ± 0.11 | 70.0 ± 1.0 | 0.09 ± 0.02 | -6.35 ± 0.11 |
| 24 | 58.0 ± 0.5 | 0.71 ± 0.05 | -4.25 ± 0.05 | 69.5 ± 0.0 | 0.10 ± 0.02 | -6.40 ± 0.10 |
| 48 | 57.5 ± 0.1 | 1.20 ± 0.11 | -4.04 ± 0.04 | 69.0 ± 0.1 | 0.11 ± 0.03 | -6.35 ± 0.05 |
| 72 | 57.5 ± 0.0 | 1.45 ± 0.06 | -4.00 ± 0.06 | 69.0 ± 0.1 | 0.10 ± 0.02 | -6.25 ± 0.05 |
| 120 | 56.0 ± 0.1 | 1.61 ± 0.09 | -3.80 ± 0.07 | 67.0 ± 0.0 | 0.11 ± 0.04 | -6.30 ± 0.10 |
| 144 | 56.0 ± 0.0 | 1.32 ± 0.08 | -4.21 ± 0.02 | 66.0 ± 0.1 | 0.10 ± 0.04 | -6.35 ± 0.04 |
| 168 | 56.0 ± 0.0 | 1.11 ± 0.10 | -5.50 ± 0.04 | 65.0 ± 0.1 | 0.09 ± 0.01 | -6.33 ± 0.13 |
| 240 | 56.0 ± 0.1 | 1.04 ± 0.06 | -5.81 ± 0.05 | 65.0 ± 0.0 | 0.10 ± 0.02 | -6.35 ± 0.02 |

### 3.2.5 Effect of agitation on growth and fat removal

In the process for the selection of the isolates for further study (Section 3.1.2), fat removal with agitation was significantly greater than with no agitation. However, all previous agitated growth was performed at 130 rpm. Therefore, the effect of increased agitation to 200 rpm and baffled agitation at 130 rpm was investigated. Temperature was set to 25°C and pH was controlled with 0.1 M potassium phosphate buffer, pH 7.0. The flasks were sampled at regular intervals and the degree of fat removal, dry weight, cell viability and morphology were monitored over 168 h.

A similar degree of fat removal and dry weight production was observed for 130 rpm under baffled and non-baffled agitation with approximately 5 g $L^{-1}$ tallow remaining in flask after 168 h, which corresponded to 75% fat removal and 15 g $L^{-1}$ dry weight (Fig. 3.2.14). The specific rate of fat removal and growth rate were similar at approximately 0.035 g $g^{-1}$ $h^{-1}$ and 0.02 $h^{-1}$, respectively (Table 3.2.8). Increased agitation at 200 rpm resulted in lower fat removal, 60% after 168 h, which corresponded to 7.9 g $L^{-1}$ tallow. Similarly, agitation at 200 rpm resulted in a lower degree of growth, with 12 g $L^{-1}$ dry weight after 168 h. This corresponded to a lower rate of specific fat removal and growth compared to agitation at 130 rpm, 0.027 g $g^{-1}$ $h^{-1}$ and 0.018 $h^{-1}$, respectively. Cell viability was maintained at 100% during 168 h growth period, in all cases.

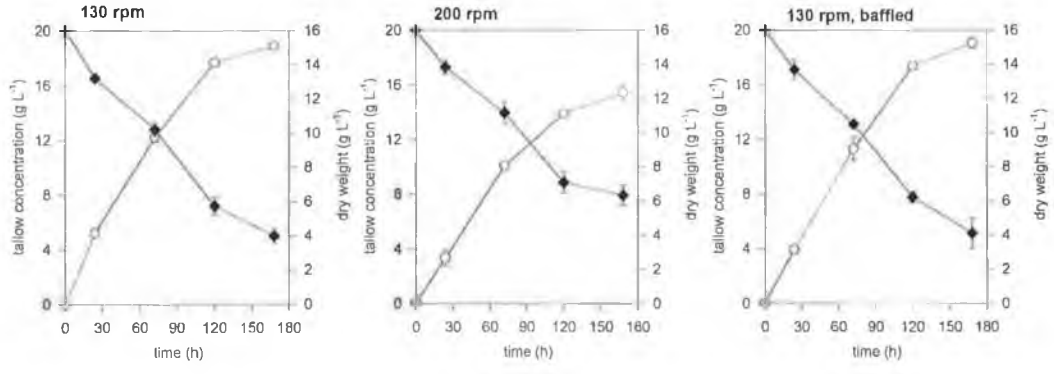



**Figure 3.2.14:** Tallow concentration (◆) and dry weight (○) during growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) for 168 h at agitation rates of 130 rpm, 200 rpm and 130 rpm in baffled flasks. Temperature was set at 25°C with pH control by 0.1 M potassium phosphate buffer, pH 7.0.

**Table 3.2.8:** Percentage fat removal, specific rate of fat removal and growth rate for *Y. lipolytica* RP2 after 168 h growth on tallow (20 g $L^{-1}$) with agitation at 130 rpm, 200 rpm and 130 rpm in baffled flasks. Temperature was set at 25°C with pH control by 0.1 M potassium phosphate buffer, pH 7.0.

| | Agitation rate (rpm) | | |
|---|---|---|---|
| | **130** | **200** | **130, baffled** |
| Fat removal (%) | 74.5 ± 0.5 | 60.0 ± 0.8 | 75.0 ± 1.2 |
| Specific rate of fat removal (g $g^{-1}$ $h^{-1}$) | 0.035 ± 0.002 | 0.027 ± 0.002 | 0.035 ± 0.002 |
| Growth rate ($h^{-1}$) | 0.020 ± 0.001 | 0.018 ± 0.002 | 0.020 ± 0.002 |

The rate and type of agitation influenced yeast morphology at the beginning of the growth period (Table 3.2.9). At 24 h, 6.0 ± 1.0% mycelial-shaped cells were present at 130 rpm compared to approximately 2% mycelium produced at 200 rpm and baffled growth. This corresponded to a marginally higher dry weight at 130 rpm after 24 h, 4 g $L^{-1}$, compared to 200 rpm and baffled agitation, 2.9 and 3.1 g $L^{-1}$, respectively. After 24 h, there was a reversion back to the yeast-shaped cells with less than 1.0% mycelium present after 168 h, in all cases. Consequently, any differences in dry weight between the agitation conditions after 24 h were not as a result of the yeast morphology. Correspondingly, yeast morphology did not influence either the growth rate or the specific rate of fat removal, as illustrated by the identical rates achieved under baffled and non-baffled agitation at 130 rpm.

**Table 3.2.9:** Percentage ratio of mycelial cells of *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) for 168 h at agitation rates of 130 rpm, 200 rpm and 130 rpm in baffled flasks. Temperature was set at 25°C with pH control by 0.1 M potassium phosphate buffer, pH 7.0.

| | Ratio of mycelial cells (%) | | | | |
|---|---|---|---|---|---|
| **Agitation (rpm)** | 0 h | 24 h | 72 h | 120 h | 168 h |
| 130 | 0 | 6.0 ± 1.0 | 1.3 ± 1.2 | 0.9 ± 0.50 | 0.8 ± 0.10 |
| 200 | 0 | 1.9 ± 0.7 | 1.5 ± 0.5 | 0.4 ± 0.10 | 0.1 ± 0.02 |
| 130, baffled | 0 | 2.2 ± 0.6 | 1.0 ± 0.5 | 0.7 ± 0.15 | 0.5 ± 0.10 |

Increased agitation to 200 rpm was found not to be beneficial and indeed, resulted in reduced fat removal ability and growth. Baffled agitation at 130 rpm achieved a similar growth and fat removal rate compared to non-baffled agitation, but foam formation was observed in the baffled flasks. Therefore, agitation at 130 rpm, in non-baffled flasks, was selected as the ideal agitation rate and all further shake flask studies were carried out at this rate of agitation.

### 3.2.6 Influence of the method of inoculum preparation of *Y. lipolytica* RP2

In the previous investigations, *Y. lipolytica* RP2 was maintained on malt extract agar and inoculum for growth studies was prepared by growing the yeast in nutrient broth for 24 h. The influence of maintaining and preparing the yeast in lipid olive oil agar and broth, respectively, on growth and fat removal was determined. The growth flasks were maintained at 25°C and 130 rpm with pH control by 0.1 M potassium phosphate buffer, pH 7.0, for 168 h. The flasks were sampled at regular intervals and dry weight, cell viability and morphology and fat removal were monitored.

The growth rate, specific fat removal rate and yield values were similar, 0.02 $h^{-1}$, 0.035 g $g^{-1}$ $h^{-1}$ and 1.01 g $g^{-1}$, respectively (Table 3.2.10). This was the case irrespective of the agar employed to maintain the yeast. Similarly, no difference in the degree of growth or fat removal was observed when either nutrient broth or olive oil broth was employed to prepare the inoculum.

**Table 3.2.10:** Growth rate, specific rate of fat removal and yield for *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) for 168 h where the yeast inoculum was sourced from either malt extract agar or olive oil agar and prepared in nutrient broth or in olive oil broth. Temperature and agitation were set to 25° C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

| | **Nutrient broth** | | **Olive oil broth** | |
|---|---|---|---|---|
| | ***Malt extract agar*** | ***Olive oil agar*** | ***Malt extract agar*** | ***Olive oil agar*** |
| Growth rate ($h^{-1}$) | 0.020 ± 0.001 | 0.020 ± 0.003 | 0.020 ± 0.002 | 0.020 ± 0.003 |
| Specific rate of fat removal ( $g\ g^{-1}\ h^{-1}$) | 0.035 ± 0.002 | 0.035 ± 0.002 | 0.035 ± 0.003 | 0.035 ± 0.003 |
| Yield [(g dry weight) $(g\ fat\ removed)^{-1}$] | 1.01 ± 0.01 | 1.01 ± 0.02 | 1.00 ± 0.02 | 1.01 ± 0.02 |

The yeast cells were found to be 100% viable in all cases. In the preparation of the inoculum in nutrient broth, yeast morphology was similar, irrespective of the agar employed (results not shown). Yeast cells were in the yeast-shape at the time of inoculation and produced approximately 6% mycelia by 24 h growth, with a reduction to less than 1% mycelia-shape cells by the end of the growth period. In contrast, inoculum preparation in olive oil broth induced mycelium production, with approximately 3.0 ± 1.5% mycelia in the growth flasks at the time of inoculation, irrespective of the agar employed. However, the presence of mycelia in the medium at the time of inoculation did not result in increased fat removal or growth. Mycelia comprised less than 1.0% of overall cell number by the end of the fermentation, in all cases.

Biosurfactant activity during the fermentation was identical under all the parameters (results not shown). At 120 h, surface tension was reduced to 56 dynes $cm^{-1}$, with no further change in tension after that time. Maximum emulsification activity and stability values occurred at 120 h, 1.61 absorbance units and a $K_d$ of –3.8, respectively.

In the absence of improved growth and fat removal by *Y. lipolytica* RP2 when maintained on a lipid medium (olive oil agar) and prepared as an inoculum in a lipid medium (olive oil broth), malt extract agar and nutrient broth were selected for yeast maintenance and inoculum preparation, respectively, for all further growth studies.

### 3.2.7 Influence of glucose as an additional carbon source with tallow and as a sole carbon source

The influence of glucose, as an additional substrate with tallow, on both the fat removal and growth rate was assessed. The yeast was also grown on glucose as the sole carbon source to assess its influence on cell morphology and biosurfactant production. Glucose was added to the medium at a concentration of 1.0 or 10.0 g $L^{-1}$ and tallow at 20 g $L^{-1}$. The growth flasks were sampled regularly and substrate removal, dry weight, intracellular lipid content, cell viability, morphology and biosurfactant activity were monitored.

Inclusion of glucose in the medium as an additional carbon source had little or no effect on fat removal and cell growth (Fig. 3.2.15). After 168 h, tallow concentrations were approximately 5.1, 5.3, and 5.4 g $L^{-1}$ in 0, 1.0 and 10.0 g $L^{-1}$ glucose, respectively, corresponding to 75%, 73% and 71% tallow removal (Table 3.2.11). Similarly, growth rates on tallow were approximately 0.020 – 0.022 $h^{-1}$ with glucose (1.0 and 10.0 g $L^{-1}$) and without glucose, with a corresponding final cell dry weight of approximately 15 g $L^{-1}$. The specific rate of tallow removal was 0.030 - 0.035 g $g^{-1}$ $h^{-1}$, in all cases. The rate of glucose depletion from the medium was reduced considerably in the presence of tallow. Complete glucose removal occurred after 24 h and 72 h for 1.0 and 10.0 g $L^{-1}$ glucose, respectively, where glucose was present as the sole carbon source. In contrast, the glucose concentration was reduced from 1.0 to 0.28 g $L^{-1}$ and 10.0 to 1.3 g $L^{-1}$ after 168 h in the presence of tallow. Yeast viability was maintained at 100% during 168 h, in all cases.

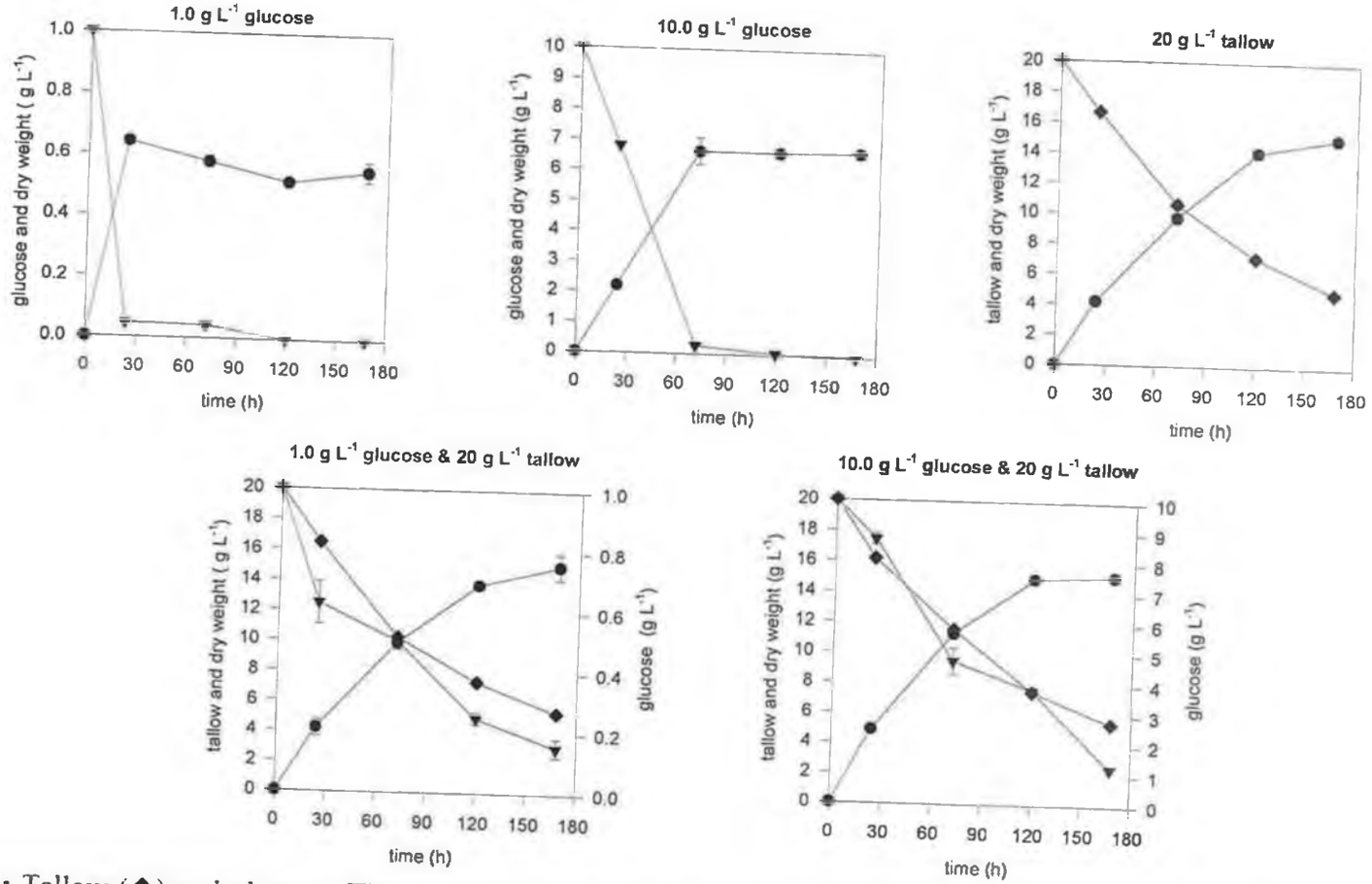



**Figure 3.2.15:** Tallow (◆) and glucose (▼) concentration and dry weight (●) for *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) and/or glucose (1.0 or 10.0 g $L^{-1}$) for 168 h. Temperature and agitation were set to 25°C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

**Table 3.2.11:** Growth rate, percentage substrate removal and the specific substrate removal rate by *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) and/or glucose (1.0 or 10.0 g $L^{-1}$) for 168 h. Temperature and agitation were set at 25°C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

| Substrate | Growth rate ($h^{-1}$) | Substrate removal (%) | | Specific removal rate (g $g^{-1}$ $h^{-1}$) | |
|---|---|---|---|---|---|
| | | *Glucose* | *Tallow* | *Glucose* | *Tallow* |
| 1.0 g $L^{-1}$ glucose | 0.002 ± 0.0005 | 100 | | 0.120 ± 0.011 | |
| 10.0 g $L^{-1}$ glucose | 0.012 ± 0.001 | 100 | | 0.070 ± 0.002 | |
| 1.0 g $L^{-1}$ glucose & 20.0 g $L^{-1}$ tallow | 0.020 ± 0.001 | 84.7 ± 0.4 | 73.4 ± 0.6 | 0.003± 0.001 | 0.035 ± 0.002 |
| 10.0 g $L^{-1}$ glucose & 20.0 g $L^{-1}$ tallow | 0.022 ± 0.002 | 87.5 ± 0.3 | 71.4 ± 1.2 | 0.018 ± 0.002 | 0.030 ± 0.001 |
| 20.0 g $L^{-1}$ tallow | 0.020 ± 0.001 | | 74.5 ± 0.5 | | 0.035 ± 0.002 |

Intracellular lipid content was 0.09 ± 0.01 g $g^{-1}$ after 168 h growth, irrespective of the substrate employed (Fig 3.2.16). A similar pattern of lipid content was observed when tallow was present as the carbon source, irrespective of glucose addition or concentration. An increase to approximately 0.4 g $g^{-1}$ lipid was detected at 24 h, with a subsequent decrease during growth to approximately 0.10 g $g^{-1}$ by 168 h. This indicated that the increased intracellular lipid levels at 24 h was due to the presence of tallow and not glucose in the medium.

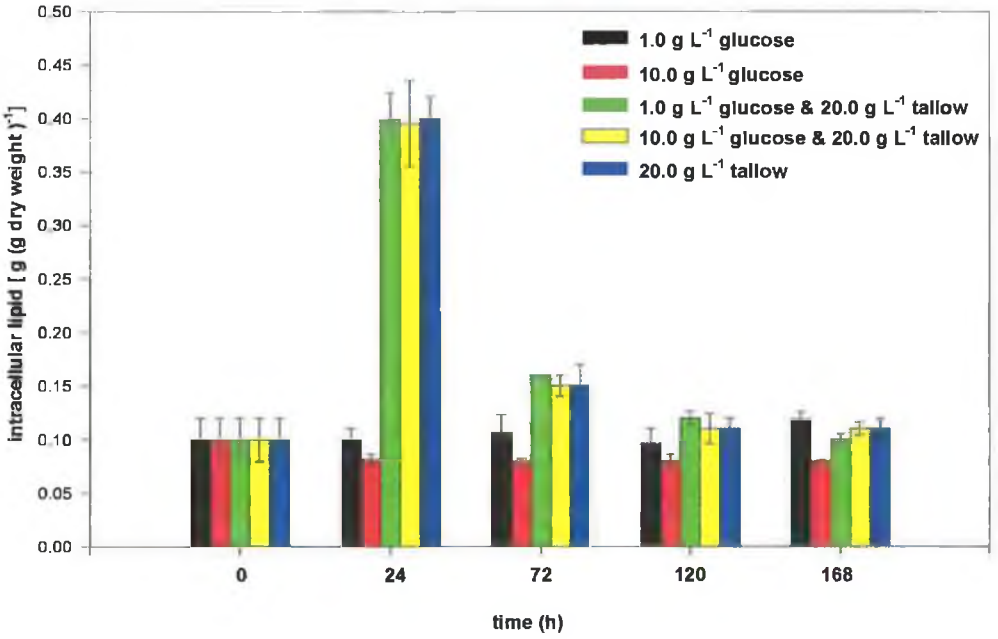



**Figure 3.2.16:** Comparison of intracellular lipid levels in *Y. lipolytica* RP2 cells grown on either tallow (20 g $L^{-1}$) or glucose (1.0 or 10.0 g $L^{-1}$) as sole carbon source or as mixed substrate. Temperature and agitation were set to 25°C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

Growth on glucose as the sole carbon source did not induce mycelium formation, which was only observed when tallow was employed as mixed or as the sole carbon source, at approximately 6% at 24 h (Table 3.2.12). A decrease in mycelium formation over time was observed with less than 1% mycelia present after 168 h. An illustration of the cell morphology for the respective substrates after 24 h growth is presented in Fig. 3.2.17.

**Table 3.2.12:** Percentage mycelial formation by *Y. lipolytica* RP2 when grown on either tallow (20 g $L^{-1}$) or glucose (1.0 or 10.0 g $L^{-1}$) as sole carbon source or as mixed substrate. Temperature and agitation were set to 25°C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

| | Ratio of mycelial cells (%) | | | | |
|---|---|---|---|---|---|
| **Time (h)** | 1.0 g $L^{-1}$ glucose | 10.0 g $L^{-1}$ glucose | 1.0 g $L^{-1}$ glucose & 20 g $L^{-1}$ tallow | 10.0 g $L^{-1}$ glucose & 20 g $L^{-1}$ tallow | 20 g $L^{-1}$ tallow |
| 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 0 | 0 | 5.8 ± 1.2 | 5.5 ± 1.1 | 6.0 ± 1.0 |
| 72 | 0 | 0 | 1.4 ± 0.8 | 1.3 ± 0.7 | 1.3 ± 1.2 |
| 120 | 0 | 0 | 1.0 ± 0.4 | 2.0 ± 0.4 | 0.9 ± 0.5 |
| 168 | 0 | 0 | 0.8 ± 0.3 | 0.8 ± 0.1 | 0.8 ± 0.1 |

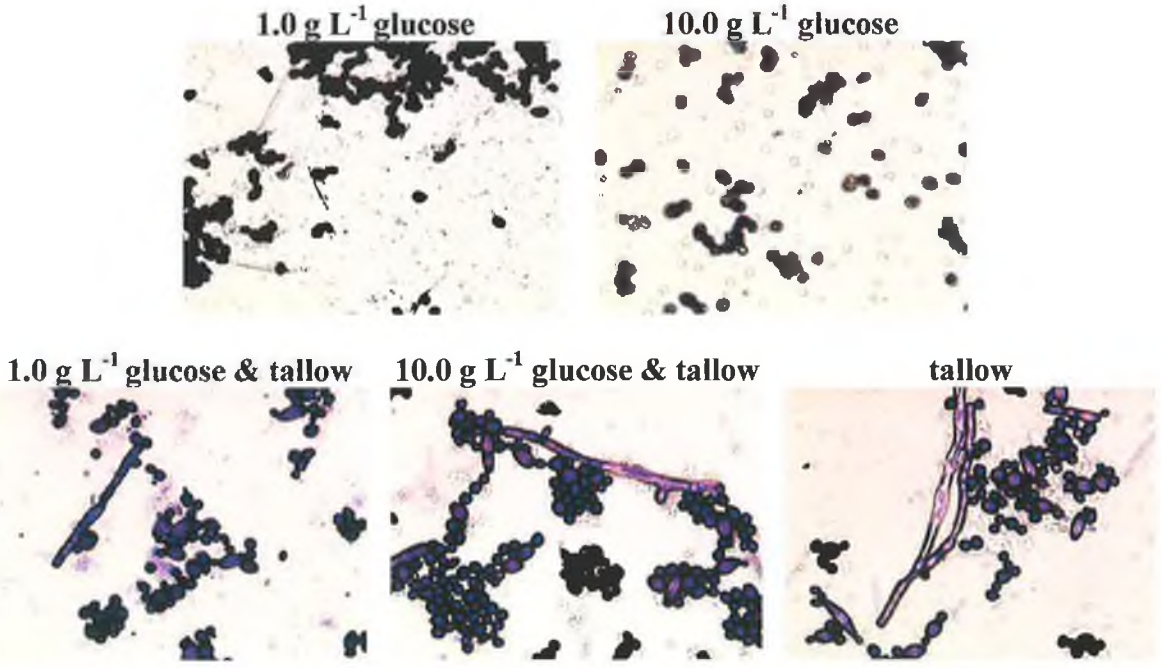



**Figure 3.2.17:** Illustration of *Y. lipolytica* RP2 cell morphology after 24 growth on either tallow (20 g $L^{-1}$) or glucose (1.0 or 10.0 g $L^{-1}$) as sole carbon source or as mixed substrate. Temperature and agitation were set to 25°C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0. Cells were observed under x40 magnification.

Surface tension was reduced from 70 to 56 dynes $cm^{-1}$ after growth on tallow for 168 h, irrespective of the inclusion of glucose in the medium (Table 3.2.13). In contrast, there was no change in surface tension with glucose employed as the sole carbon source. Similarly, emulsification activity ($A_{540}$) and emulsion stability ($K_d$) were detected only when tallow was present in the medium, irrespective of glucose addition. Activity and stability increased after 24 h to a maximum at 120 h, with a corresponding absorbance and $K_d$ of 1.6 and $-3.82 \pm 0.03$, respectively. Activity and stability decreased after this time. In contrast, no activity or stability was detected when glucose was the sole carbon source.

A reduction in surface tension to approximately $67.0 \pm 2.0$ dynes $cm^{-1}$ was detected in the control flasks (no cells) containing either tallow or glucose (both concentrations) as the sole carbon source or with tallow and glucose as mixed substrates. No emulsification activity or emulsion stability was detected in any of the control flasks.

Overall, the inclusion of glucose at 1.0 and 10.0 g $L^{-1}$ had no effect on tallow removal by *Y. lipolytica* RP2. There was a concommitant use of both substrates with a similar degree of fat removal, $73.0 \pm 2.0\%$, achieved after 168 h, irrespective of glucose presence in the medium. In the presence of tallow as the sole carbon source, or mixed with glucose, the yeast displayed dimorphic growth and demonstrated biosurfactant activity. Dimorphism or biosurfactant activity were not detected when glucose provided the sole carbon source.

**Table 3.2.13:** Surface tension, emulsification activity ($A_{540}$) and emulsion stability (decay ratio, $K_d$) during the growth of *Y. lipolytica* RP2 on either tallow (20 g $L^{-1}$) or glucose (1.0 or 10.0 g $L^{-1}$) as sole carbon source or as mixed substrate. Temperature and agitation were set to 25°C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0. The medium was filtered through 0.2 μm filter prior to measurement. The absorbance and decay ratios were measured against kerosene.

***Surface tension (dynes $cm^{-1}$)***

| Time (h) | 1.0 g $L^{-1}$ glucose | 10.0 g $L^{-1}$ glucose | 1.0 g $L^{-1}$ glucose & 20 g $L^{-1}$ tallow | 10.0 g $L^{-1}$ glucose & 20 g $L^{-1}$ tallow | 20 g $L^{-1}$ tallow |
|---|---|---|---|---|---|
| 0 | 70.0 ± 1.0 | 70.0 ± 1.0 | 70.0 ± 1.0 | 70.0 ± 1.0 | 70.0 ± 1.0 |
| 24 | 69.5 ± 0.7 | 70.0 ± 0.3 | 58.3 ± 0.3 | 58.0 ± 0.7 | 58.0 ± 0.5 |
| 72 | 70.0 ± 0.1 | 70.0 ± 0.7 | 57.0 ± 0.3 | 57.5 ±0.1 | 57.5 ± 0.3 |
| 120 | 70.0 ± 0.0 | 70.5 ± 0.5 | 56.0 ± 0.1 | 56.0 ± 0.2 | 56.0 ± 0.1 |
| 168 | 70.0 ± 0.3 | 70.0 ± 0.1 | 56.0 ± 0.0 | 56.0 ± 0.2 | 56.0 ± 0.1 |

***Emulsification activity ($A_{540}$)***

| Time (h) | 1.0 g $L^{-1}$ glucose | 10.0 g $L^{-1}$ glucose | 1.0 g $L^{-1}$ glucose & 20 g $L^{-1}$ tallow | 10.0 g $L^{-1}$ glucose & 20 g $L^{-1}$ tallow | 20 g $L^{-1}$ tallow |
|---|---|---|---|---|---|
| 0 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 |
| 24 | 0.11 ± 0.05 | 0.10 ± 0.01 | 0.70 ± 0.02 | 0.69 ± 0.05 | 0.71 ± 0.05 |
| 72 | 0.11 ± 0.01 | 0.10 ± 0.02 | 1.46 ± 0.07 | 1.44 ± 0.02 | 1.45 ± 0.06 |
| 120 | 0.10 ± 0.02 | 0.11 ± 0.05 | 1.60 ± 0.05 | 1.60 ± 0.08 | 1.61 ± 0.09 |
| 168 | 0.11 ± 0.03 | 0.10 ± 0.04 | 1.13 ± 0.09 | 1.12 ± 0.09 | 1.11 ± 0.10 |

***Decay ratio ($K_d x\ 10^{-3}$)***

| Time (h) | 1.0 g $L^{-1}$ glucose | 10.0 g $L^{-1}$ glucose | 1.0 g $L^{-1}$ glucose & 20 g $L^{-1}$ tallow | 10.0 g $L^{-1}$ glucose & 20 g $L^{-1}$ tallow | 20 g $L^{-1}$ tallow |
|---|---|---|---|---|---|
| 0 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 |
| 24 | -6.30 ± 0.08 | -6.29 ± 0.02 | -4.23 ± 0.03 | -4.27 ± 0.04 | -4.25 ± 0.05 |
| 72 | -6.31 ± 0.09 | -6.28 ± 0.05 | -4.02 ± 0.05 | -4.00 ± 0.02 | -4.00 ± 0.06 |
| 120 | -6.30 ± 0.05 | -6.29 ± 0.04 | -3.79 ± 0.05 | -3.85 ± 0.03 | -3.80 ± 0.07 |
| 168 | -6.30 ± 0.07 | -6.32 ± 0.10 | -5.69 ± 0.10 | -5.75 ± 0.02 | -5.50 ± 0.04 |

### 3.2.8 Influence of nitrogen source and concentration

All previous growth studies in this investigation were performed with 2.0 g $L^{-1}$ ammonium sulphate [$(NH_4)_2SO_4$] as the nitrogen source. The influence of the source and concentration of nitrogen in the medium was investigated with respect to growth and fat removal, intracellular lipid content, biosurfactant production, and cellular morphology of *Y. lipolytica* RP2.

A range of nitrogen concentrations at 0.5, 1.0, 2.0, 3.0 and 5.0 g $L^{-1}$ were assessed, and the source of nitrogen as ammonium sulphate, urea and peptone were compared. The different nature of the three sources, complex chemical and simple and complex organic, meant that a simple comparison with the amount added to the growth flasks could not be made. Therefore, the nitrogen source and concentration were compared based on the total nitrogen (g N $L^{-1}$) added to the media (Table 3.2.14). Yeast extract provided 9.0 mg $L^{-1}$ of total nitrogen in the minimal medium, and this value was not included in the calculation of the amount of total nitrogen in the medium.

**Table 3.2.14:** Comparison of concentration (0.5 - 5.0 g $L^{-1}$) of nitrogen source, as $(NH_4)_2SO_4$, urea and peptone added to the medium, with the total nitrogen concentration in the medium (g N $L^{-1}$).

| | **Total Nitrogen in the medium (g N $L^{-1}$)** | | |
|---|---|---|---|
| **Concentration of nitrogen source added (g $L^{-1}$)** | **$(NH_4)_2SO_4$** | **Urea** | **Peptone** |
| 0.5 | 0.11 | 0.24 | 0.08 |
| 1.0 | 0.21 | 0.47 | 0.15 |
| 2.0 | 0.42 | 0.93 | 0.29 |
| 3.0 | 0.64 | 1.40 | 0.43 |
| 5.0 | 1.10 | 2.33 | 0.71 |

Maximum fat removal, 73 – 75%, was achieved at concentrations between 2 – 5 g $L^{-1}$ $(NH_4)_2SO_4$, 1 – 3 g $L^{-1}$ urea and 3 – 5 g $L^{-1}$ peptone (Fig. 3.2.18). This corresponded to total nitrogen concentration in the medium between 0.42 – 1.40 g N $L^{-1}$. At lower nitrogen concentrations, 0.5 – 1.0 g $L^{-1}$ $(NH_4)_2SO_4$, 0.5 g $L^{-1}$ urea and 0.5 – 2.0 g $L^{-1}$ peptone, fat removal was reduced to 60 - 65%, 65% and 43 – 65%, respectively. Also, fat removal was reduced at high nitrogen levels. Inclusion of 5 g $L^{-1}$ urea, corresponding to 2.33 g N $L^{-1}$, resulted in 52% fat removal.

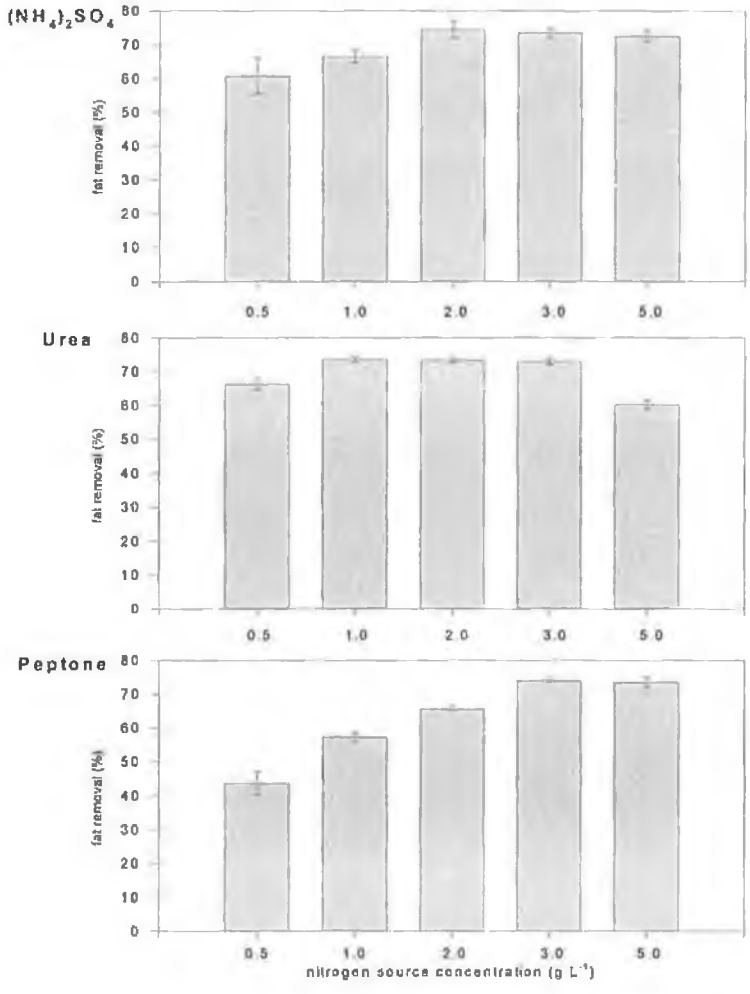



**Figure 3.2.18:** Percentage fat removal by *Y. lipolytica* RP2 after 168 h growth on tallow (20 g $L^{-1}$) with addition of $(NH_4)_2SO_4$, urea and peptone at concentrations of 0.5, 1.0, 2.0, 3.0, and 5.0 g $L^{-1}$. Temperature and agitation were set at 25°C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

Maximum growth rates, 0.020 - 0.021 $h^{-1}$, also occurred at nitrogen levels between 0.42 and 1.40 g N $L^{-1}$ (Table 3.2.15). At lower nitrogen levels, at 0.5 – 1.0 g $L^{-1}$ $(NH_4)_2SO_4$, 0.5 g $L^{-1}$ urea, and 0.5 – 2.0 g $L^{-1}$ peptone, growth rates were reduced to 0.015 – 0.018 $h^{-1}$, 0.018 $h^{-1}$ and 0.008 – 0.018 $h^{-1}$, respectively. Similarly, a lower growth rate was achieved at the highest nitrogen concentration of 5 g $L^{-1}$ urea, at 0.017 $h^{-1}$, which corresponded to the lower degree of fat removal at this concentration. Yeast viability was 100% in all cases.

**Table 3.2.15:** Growth rate for *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) with addition of $(NH_4)_2SO_4$, urea and peptone at concentrations of 0.5, 1.0, 2.0, 3.0, and 5.0 g $L^{-1}$. pH control was by 0.1 M potassium phosphate buffer, pH 7.0 and temperature and agitation were set at 25°C and 130 rpm.

| | **Growth rate ($h^{-1}$)** | | | | |
|---|---|---|---|---|---|
| | Concentration of nitrogen source added to medium (g $L^{-1}$) | | | | |
| **Nitrogen Source** | 0.5 | 1.0 | 2.0 | 3.0 | 5.0 |
| $(NH_4)_2SO_4$ | 0.015 ± 0.002 | 0.018 ± 0.002 | 0.020 ± 0.001 | 0.020 ± 0.002 | 0.020 ± 0.001 |
| Urea | 0.018 ± 0.001 | 0.021 ± 0.001 | 0.020 ± 0.002 | 0.020 ± 0.001 | 0.017 ± 0.001 |
| Peptone | 0.008 ± 0.001 | 0.014 ± 0.002 | 0.018 ± 0.001 | 0.021 ± 0.001 | 0.021 ± 0.001 |

The concentration of nitrogen influenced the amount of intracellular lipid after 168 h (Table 3.2.16). Intracellular lipid content was approximately 0.1 g $g^{-1}$ when nitrogen levels were between 0.42 – 1.40 g N $L^{-1}$, corresponding to a carbon nitrogen ratio (C:N) of 41:1 – 12:1. This corresponded to the total nitrogen concentration range resulting in maximum fat removal and growth rate. At nitrogen levels below 0.42 g N $L^{-1}$, corresponding to C:N ratios above 56:1, lipid accumulation was induced. This was clearly evident for 0.5 g $L^{-1}$ peptone (0.08 g N $L^{-1}$), where intracellular lipid content was 0.35 g $g^{-1}$. This indicated that a high ratio of a tallow carbon source to nitrogen induced lipid accumulation in *Y. lipolytica* RP2, irrespective of the nitrogen source. Similarly, a high C:N ratio (between 206:1 to 56:1) resulted in a lower yield for all nitrogen sources, which

also corresponded to reduced growth and fat removal at these ratios. At C:N ratios below 56:1, yield values at unity were achieved, irrespective of the source of nitrogen. The exception was at 5 g $L^{-1}$ urea, where reduced growth and fat removal resulted in a reduced yield of 0.89 g $g^{-1}$.

**Table 3.2.16:** Comparison of the carbon to nitrogen ratio (C:N) with the amount of intracellular fat and yield of *Y. lipolytica* RP2 cells grown on tallow (20 g $L^{-1}$) with nitrogen addition by $(NH_4)_2SO_4$, urea and peptone at 0.5 - 5.0 g $L^{-1}$.

| Nitrogen Addition | | C:N ratio | Intracellular lipid [g (g dry weight) $^{-1}$] | Yield [(g dry weight) (g fat removed)$^{-1}$] |
|---|---|---|---|---|
| $(NH_4)_2SO_4$ (g $L^{-1}$): | 0.5 | 151:1 | 0.29 ± 0.02 | 0.80 ± 0.01 |
| | 1.0 | 79:1 | 0.18 ± 0.02 | 0.94 ± 0.02 |
| | 2.0 | 41:1 | 0.09 ± 0.02 | 1.01 ± 0.01 |
| | 3.0 | 27:1 | 0.10 ± 0.01 | 1.00 ± 0.02 |
| | 5.0 | 16:1 | 0.09 ± 0.01 | 1.00 ± 0.02 |
| Urea (g $L^{-1}$): | 0.5 | 72:1 | 0.17 ± 0.02 | 0.95 ± 0.02 |
| | 1.0 | 37:1 | 0.10 ± 0.01 | 1.02 ± 0.02 |
| | 2.0 | 19:1 | 0.10 ± 0.02 | 1.02 ± 0.01 |
| | 3.0 | 12:1 | 0.09 ± 0.02 | 1.01 ± 0.01 |
| | 5.0 | 8:1 | 0.09 ± 0.03 | 0.89 ± 0.02 |
| Peptone (g $L^{-1}$): | 0.5 | 206:1 | 0.35 ± 0.03 | 0.64 ± 0.02 |
| | 1.0 | 108:1 | 0.30 ± 0.01 | 0.84 ± 0.02 |
| | 2.0 | 56:1 | 0.15 ± 0.01 | 0.98 ± 0.01 |
| | 3.0 | 38:1 | 0.09 ± 0.03 | 1.01 ± 0.01 |
| | 5.0 | 20:1 | 0.11 ± 0.02 | 1.02 ± 0.02 |

A similar degree of tallow dispersal was observed in the growth flasks, irrespective of the source and concentration of nitrogen. The nitrogen levels in the medium were not found to influence the biosurfactant properties surface tension, emulsification activity or decay ratio during growth (results not shown). Surface tension was reduced from 70 to 56 dynes $cm^{-1}$ after 120 h, in all cases, with no further reduction after this time. Emulsification activity and stability were greatest at 120 h, with an absorbance and $K_d$ of approximately 1.60 and –3.80, irrespective of nitrogen source or concentration. In the control flasks (no cells), the surface tension was reduced to approximately 65 dynes $cm^{-1}$ by 168 h. No emulsification activity or emulsion stability was detected in the control flasks.

Interestingly, both the source and concentration of nitrogen added to the growth medium did have an effect on yeast morphology (Fig. 3.2.19). The ratio of mycelial cells to overall cell number ranged between 20 - 30% between 0.5 and 1.0 g $L^{-1}$ addition for both $(NH_4)_2SO_4$ and urea after 24 h growth. However, mycelium formation was not induced to the same degree for peptone, with only 10 - 15% mycelia present at these concentrations, at 24 h. Increased concentration of nitrogen addition above 1.0 g N $L^{-1}$ resulted in a decrease in the production of mycelial-shape cells, irrespective of the source of nitrogen. After 24 h, the ratio of mycelial cells decreased over the course of the growth period to less than 4% mycelia at the lower concentrations, 0.5 - 1.0 g $L^{-1}$ and to less than 1% at the higher concentrations, for all three sources. An illustration of the degree of mycelium formation for the different concentrations and sources of nitrogen at 24 h growth is shown in Fig.s 3.2.20 a - b.

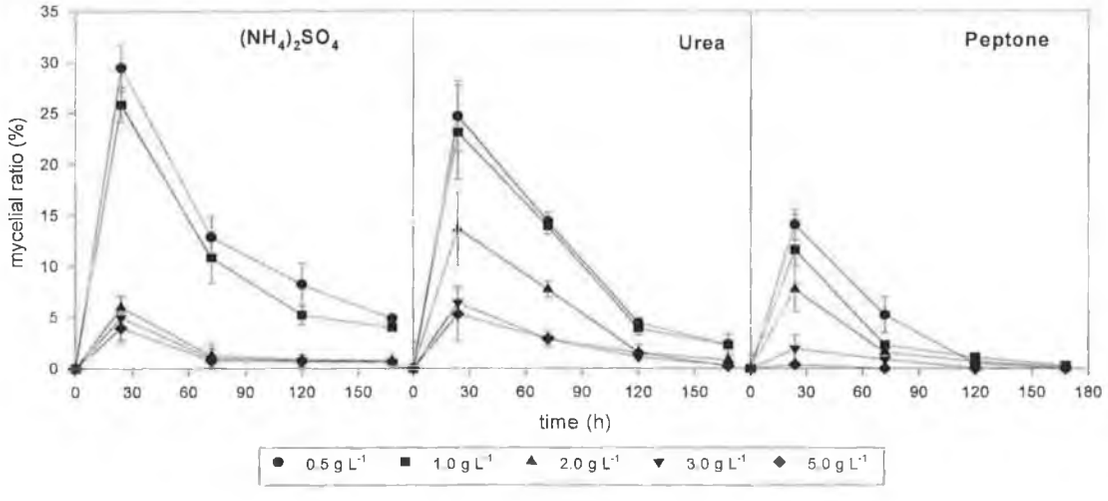



**Figure 3.2.19:** Percentage mycelium formation by *Y. lipolytica* RP2 during 168 h growth on tallow (20 g $L^{-1}$) with the addition of $(NH_4)_2SO_4$, urea and peptone at concentrations of 0.5 to 5.0 g $L^{-1}$. Temperature and agitation were set to 25°C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

Overall, maximum fat removal and growth rates occurred at nitrogen concentrations between 0.42 – 1.40 g N $L^{-1}$, and decreased above and below this concentration. $(NH_4)_2SO_4$, at a concentration of 2 g $L^{-1}$, was chosen as a suitable nitrogen source for all further studies as it provided 0.42 g N $L^{-1}$ in the medium and did not induce mycelium formation to any great extent.

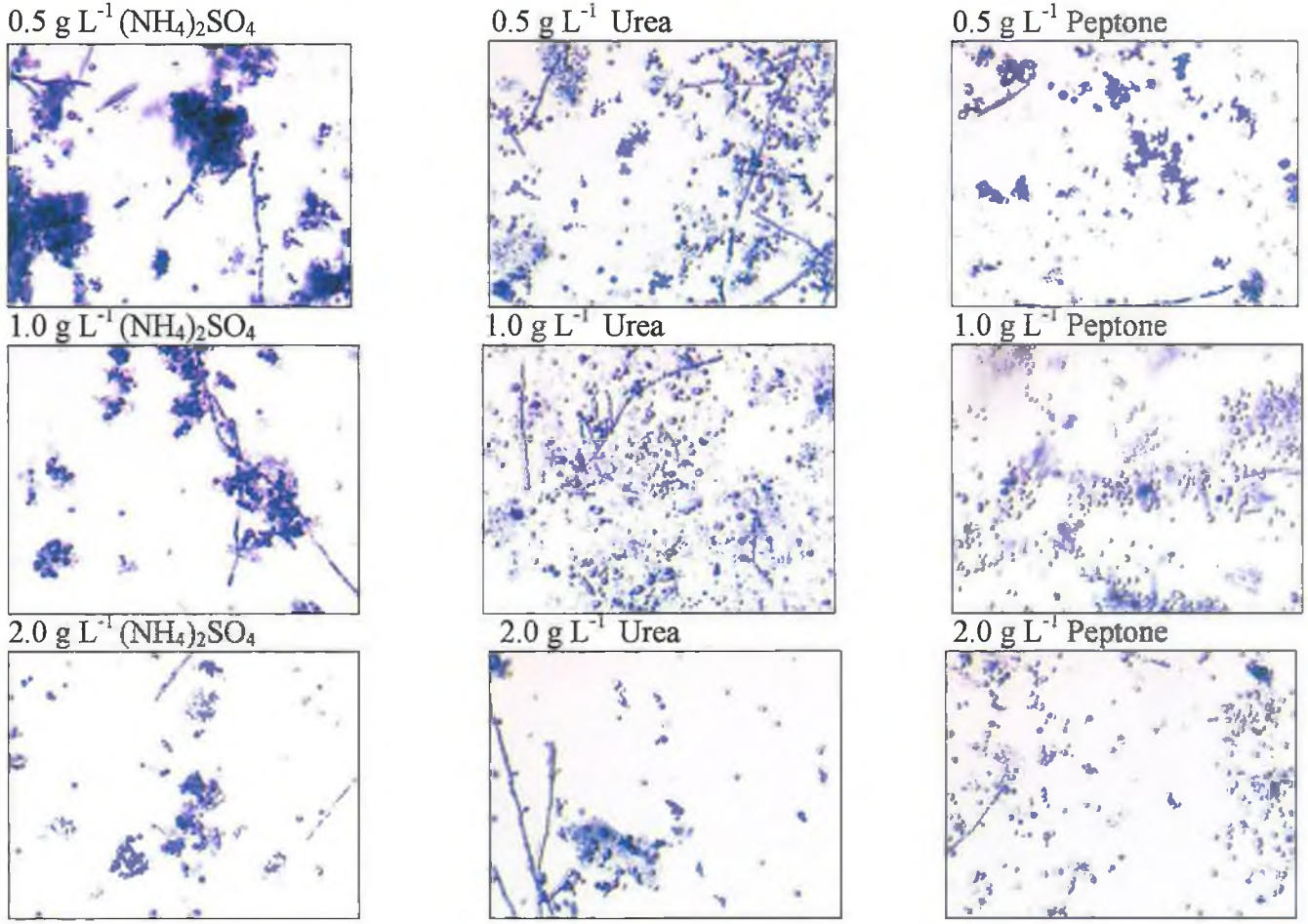



**Figure 3.2.20 a:** Illustration of cell morphology of *Y. lipolytica* RP2 after 24 h growth on tallow (20 g $L^{-1}$) with nitrogen addition at 0.5, 1.0 and 2.0 g $L^{-1}$ as $(NH_4)_2SO_4$, urea and peptone. Temperature and agitation were set to 25°C and 130 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0. Cells were observed under x20 magnification.

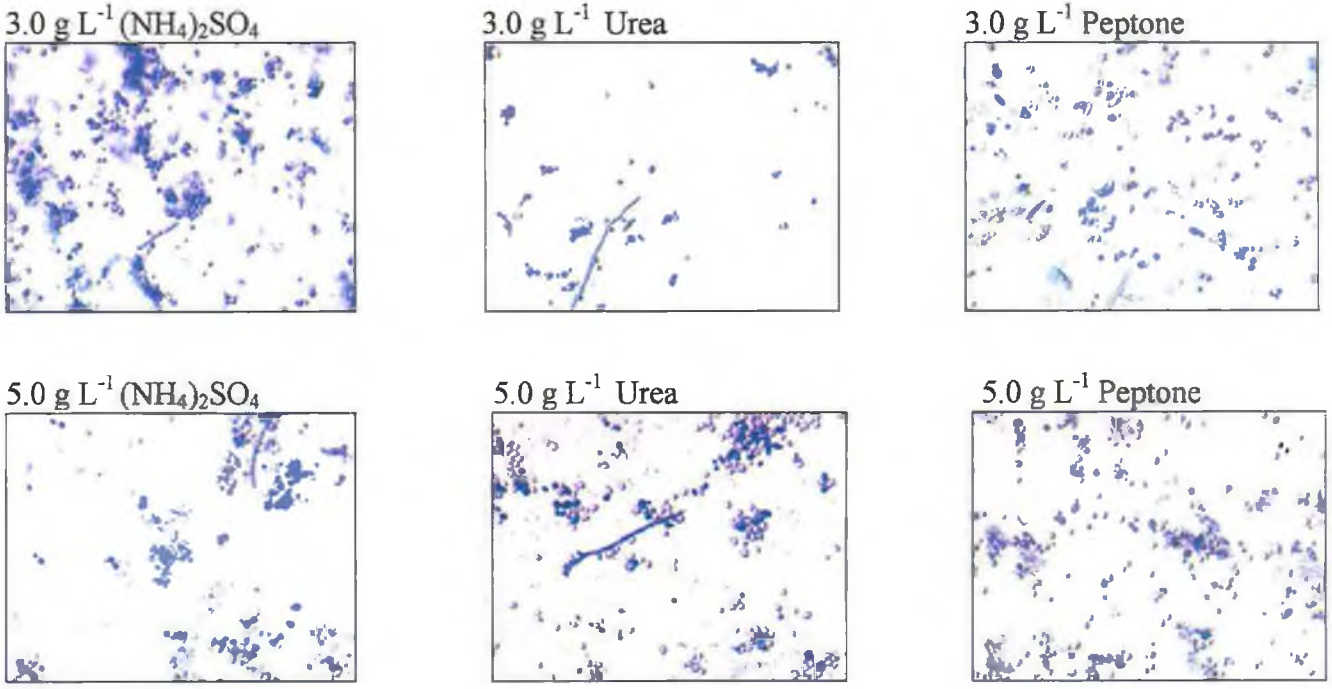



**Figure 3.2.20 b:** Illustration of cell morphology of *Y. lipolytica* RP2 after 24 h growth on tallow (20 g $L^{-1}$) with nitrogen addition at 3.0 and 5.0 g $L^{-1}$ as $(NH_4)_2SO_4$, urea and peptone. Temperature and agitation were set to 25°C and 130 rpm, respectively with 0.1 M potassium phosphate buffer, pH 7.0. Cells were observed under x20 magnification.

### 3.2.9 Summary of the main findings from shake flask studies on the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$)

| | |
|---|---|
| • Growth on tallow | 21% fat removed after 168 h at room temperature and 130 rpm with no pH control. Observed reduction in surface tension and detection of emulsification activity in growth medium indicated biosurfactant production by *Y. lipolytica* RP2. |
| • Growth temperature | 25°C |
| • Surfactant addition | Surfactants Triton X-100 and Tween 80 were toxic to *Y. lipolytica* RP2 and tallow removal was reduced. |
| • pH control | Increased fat removal to 75% after 168 h under pH control to pH 7.0 with 0.1 M potassium phosphate buffer at 25°C. Induction of dimorphic growth with pH control. Increased biosurfactant activity with pH control. |
| • Agitation rate | 130 rpm |
| • Inoculum preparation | Maintenance of the yeast on a lipid agar and preparation of the inoculum for growth on tallow in a lipid medium did not improve growth or fat removal ability. |
| • Additional carbon, glucose | Concommitant use of glucose and tallow as mixed substrate by the yeast. Similar degree of tallow removed when glucose present as an additional carbon source. Glucose did not induce dimorphic growth or biosurfactant production. |
| • Nitrogen source and concentration | Maximum tallow removal with 0.42 – 1.4 g $L^{-1}$ total nitrogen in medium. $(NH_4)_2SO_4$ at 2.0 g $L^{-1}$ selected as superior nitrogen source and concentration, respectively. A high carbon to nitrogen ratio resulted in cellular lipid accumulation. |

## 3.3 Scale-up of the degradation of tallow (20 g $L^{-1}$) by *Yarrowia lipolytica* RP2 in 2 L and 10 L fermenters

An investigation was undertaken to assess the ability of *Y. lipolytica* RP2 to remove tallow on a larger scale. Scale-up studies were initially applied to a 2 L bench-top fermenter and subsequently to a pilot-scale 10 L fermenter.

### 3.3.1 2 L fermenter studies

Scale-up of the growth of *Y. lipolytica* on tallow to a 2 L fermenter was performed under the conditions determined as ideal for fat removal in shake flask investigations and included temperature (25°C), pH (7.0), nitrogen (2 g $L^{-1}$ $(NH_4)_2SO_4$), with inoculum preparation in nutrient broth. The rates of agitation and aeration were optimised for the fermentation and the effect of the method of pH control to 7.0 investigated.

#### 3.3.1.1 Optimisation of agitation

*Y. lipolytica* RP2 was grown on tallow in the 2 L fermenter at agitation rates of 250, 500, 700 and 900 rpm with an aeration rate of 1 VVM with pH control by 0.1 M potassium phosphate buffer, pH 7.0. Fat removal was determined at the end of the fermentation, after 65 h. The fermenter was sampled at regular intervals and dry weight, cell viability, cell morphology, intracellular lipid and biosurfactant activity were monitored.

Fat removal, growth rates and yield were calculated after 65 h (Table 3.3.1). Results were similar at agitation rates of 500, 700 and 900 rpm. After 65 h, approximately 75% of the fat was removed with a corresponding growth rate of 0.072 $h^{-1}$. This was a significant reduction in the time required for fat removal compared to shake flask studies (168 h) and correspondingly, a greater growth rate (0.02 $h^{-1}$). At the lower agitation rate in the fermenter, 250 rpm, less fat was removed, 26.8% and a lower growth rate achieved, 0.03 $h^{-1}$, compared to the higher agitation rates. Similarly, the yield value at 500 – 900 rpm, 0.94 g $g^{-1}$, was

greater than achieved at 250 rpm, 0.86 g $g^{-1}$. In all fermentations, 100% cell viability was maintained during 65 h.

**Table 3.3.1:** Percentage fat removal, growth rate and yield after 65 h growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 2 L fermenter, at agitation rates of 250, 500, 700 and 900 rpm. Aeration and temperature were set to 1 VVM and 25°C, with 0.1 M potassium phosphate buffer, pH 7.0

| | **Agitation rate (rpm)** | | | |
|---|---|---|---|---|
| | 250 | 500 | 700 | 900 |
| Fat removal (%) | 28.6 ± 3.2 | 75.0 ± 1.0 | 74.9 ± 1.5 | 74.5 ± 1.2 |
| Growth rate ($h^{-1}$) | 0.030 ± 0.003 | 0.072 ± 0.002 | 0.072 ± 0.003 | 0.072 ± 0.003 |
| Yield [(g dry weight) (g fat removed)$^{-1}$] | 0.86 ± 0.005 | 0.94 ± 0.002 | 0.94 ± 0.003 | 0.94 ± 0.005 |

Intracellular lipid content followed a similar pattern, irrespective of the agitation rate (Fig. 3.3.1). At 12 h, the cell lipid content increased to approximately 0.5 g $g^{-1}$ after inoculation and then decreased to between 0.10 g $g^{-1}$ and 0.15 g $g^{-1}$ lipid between 12 to 65 h. The initial lipid content of the cells was marginally higher in the fermenter compared to shake flask studies where approximately 0.4 g $g^{-1}$ lipid was present at 12 h (Section 3.2.4.4). However, the final value was similar in the both the fermenter and shake flasks at 65 and 168 h, respectively.

Yeast morphology was also monitored during the fermentations. Agitation in the 2 L fermenter resulted in a lower production of mycelial cells compared to shake flask studies at 130 rpm. Mycelial-shaped cells comprised 1.0 ± 1.0% of the overall cell shape at 12 h in the 2 L fermenter compared to 6.0% at 24 h in shake flask culture. After 12 h in the fermenter, no mycelial-shaped cells were observed and the yeast was in the yeast-shape only. The cell morphology was identical, irrespective of the agitation rate employed. An illustration of the cell morphology at 12 h is provided in Fig. 3.3.2.

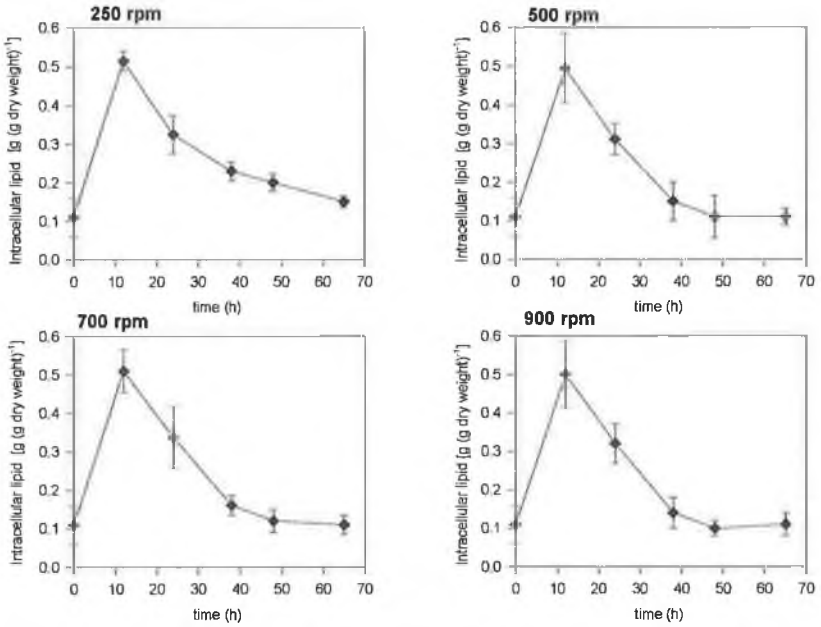



**Figure 3.3.1:** Intracellular lipid content of *Y. lipolytica* RP2 cells when grown on tallow (20 g $L^{-1}$) in 2 L fermenter for 65 h at agitation rates of 250, 500, 700 and 900 rpm. Temperature and aeration were set to 25°C and 1 VVM, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

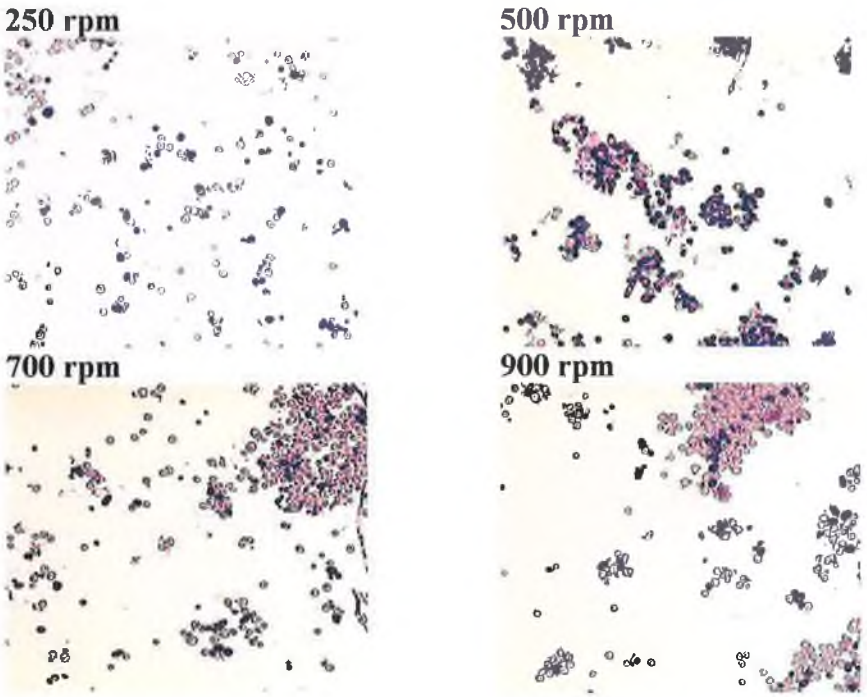



**Fig. 3.3.2:** Illustration of *Y. lipolytica* RP2 cell morphology after 12 h growth on tallow (20 g $L^{-1}$) in 2 L fermenter at agitation rates of 250, 500, 700 and 900 rpm. Aeration and temperature were set to 1 VVM and 25°C, respectively, with 0.1 M potassium phosphate buffer, pH 7.0. Cells were observed under x20 magnification.

A reduction in surface tension was detected during the fermentation at all agitation rates (Table 3.3.2). The greatest reduction was achieved at agitation rates of 500, 700 and 900 rpm, with a final surface tension of approximately 42.5 dynes $cm^{-1}$ after 65 h. This value was lower than that detected in shake flask studies (Section 3.2.4.4), where surface tension was reduced to 56.0 dynes $cm^{-1}$ after 120 h. At 250 rpm, surface tension was not reduced to the same magnitude as that detected at the higher agitation rates, with a tension of 58.0 ± 0.1 dynes $cm^{-1}$ after 65 h.

Maximum emulsification activity was achieved after 24 h at 500 – 900 rpm with an absorbance in the range of 2.03 to 2.08 units. Similarly, maximum emulsion stability was detected at 24 h for 500 – 900 rpm with a $K_d$ (decay ratio) between -2.23 and –2.27. Activity and stability were enhanced in 2 L fermentation compared to shake flask culture at 130 rpm, which achieved a maximum absorbance and $K_d$ of 1.6 and –3.8, respectively, after 120 h (Section 3.2.4.4). In contrast, agitation at 250 rpm in 2 L fermenter resulted in a slower increase to maximum activity and stability with an absorbance and $K_d$ of 1.83 and –2.85, respectively, after 38 h. This correlated with the higher surface tension achieved at 250 rpm.

The surface tension in the controls (no cells) demonstrated only a marginal decrease in surface tension during the fermentation, from 70 dynes $cm^{-1}$ to approximately 65.0 dynes $cm^{-1}$ at 65 h (results not shown), irrespective of the agitation rate employed. Similarly, no emulsification activity or emulsion stability was detected in the controls, irrespective of the agitation rate.

Maximum fat removal and growth, 75% and 0.072 $h^{-1}$, were achieved after 65 h agitation at 500 rpm. No further increase in fat removal, growth or biosurfactant activity occurred at higher agitation rates. Agitation below 500 rpm resulted in reduced fat removal, growth and activity. Consequently, 500 rpm was chosen as the ideal rate of agitation for all further 2 L fermenter studies.

**Table 3.3.2:** Surface tension, emulsification activity ($A_{540}$) and emulsion stability (decay ratio, $K_d$) of growth medium of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 2 L fermenter for 65 h at agitation rates of 250, 500, 700 and 900 rpm. Temperature and aeration were set to 25°C and 1 VVM, respectively, with 0.1 M potassium phosphate buffer, pH 7.0. The medium was filtered through 0.2 μm filter prior to measurement. The absorbance and decay ratios were measured against kerosene.

| ***Surface tension (dynes $cm^{-1}$)*** | | | | |
|---|---|---|---|---|
| **Time (h)** | **250 rpm** | **500 rpm** | **700 rpm** | **900 rpm** |
| 0 | 70.0 ± 1.0 | 70.0 ± 1.0 | 70.0 ± 1.0 | 70.0 ± 1.0 |
| 12 | 66.5 ± 0.1 | 52.5 ± 0.5 | 52.0 ± 0.1 | 52.0 ± 0.1 |
| 24 | 60.0 ± 0.3 | 43.5 ± 0.1 | 43.5 ± 0.0 | 44.0 ± 0.2 |
| 38 | 58.5 ± 0.3 | 43.0 ± 0.1 | 43.0 ± 0.3 | 43.0 ± 0.3 |
| 48 | 58.0 ± 0.6 | 43.0 ± 0.0 | 42.5 ± 0.1 | 43.0 ± 0.6 |
| 65 | 58.0 ± 0.1 | 42.5 ± 0.0 | 42.5 ± 0.1 | 42.5 ± 0.3 |
| ***Emulsification activity ($A_{540}$)*** | | | | |
| **Time (h)** | **250 rpm** | **500 rpm** | **700 rpm** | **900 rpm** |
| 0 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 |
| 12 | 1.55 ± 0.10 | 1.95 ± 0.05 | 1.93 ± 0.07 | 1.97 ± 0.04 |
| 24 | 1.77 ± 0.05 | 2.05 ± 0.11 | 2.08 ± 0.04 | 2.03 ± 0.10 |
| 38 | 1.83 ± 0.12 | 1.90 ± 0.06 | 1.93 ± 0.04 | 1.82 ± 0.03 |
| 48 | 1.50 ± 0.06 | 1.77 ± 0.14 | 1.80 ± 0.06 | 1.75 ± 0.11 |
| 65 | 1.22 ± 0.15 | 1.54 ± 0.07 | 1.55 ± 0.10 | 1.48± 0.05 |
| ***Decay ratio ($K_d$ x $10^{-3}$)*** | | | | |
| **Time (h)** | **250 rpm** | **500 rpm** | **700 rpm** | **900 rpm** |
| 0 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 |
| 12 | -3.30 ± 0.05 | -2.35 ± 0.11 | -2.37 ± 0.02 | -2.31 ± 0.04 |
| 24 | -3.00 ± 0.21 | -2.27 ± 0.04 | -2.23 ± 0.06 | -2.25 ± 0.08 |
| 38 | -2.85 ± 0.07 | -3.80 ± 0.15 | -3.81 ± 0.12 | -3.75 ± 0.05 |
| 48 | -3.05 ± 0.01 | -4.08 ± 0.04 | -3.99 ± 0.21 | -4.01 ± 0.14 |
| 65 | -3.89 ± 0.03 | -4.45 ± 0.02 | -4.40 ± 0.11 | -4.51 ± 0.09 |

### 3.3.1.2 Optimisation of aeration

*Y. lipolytica* RP2 was grown on tallow at aeration rates of 0.5, 1.0, and 3.0 VVM and no aeration. Agitation was set at 500 rpm and pH was controlled by 0.1 M potassium phosphate buffer, pH 7.0. Fat removal was determined at the end of the fermentation, after 65 h. The fermenters were sampled at regular intervals and dry weight, cell viability, cell morphology, intracellular lipid and biosurfactant activity were monitored.

The fat removal, growth rate and yield were calculated after 65 h (Table 3.3.3). Maximum fat removal, 75%, was achieved at aeration rates of 1.0 and 3.0 VVM after 65 h. This corresponded to a maximum growth rate and yield of 0.072 $h^{-1}$ and 0.94 g $g^{-1}$, respectively. Fat removal was lower at 0.5 VVM, with 65% removal. Correspondingly, growth rate and yield were lower, 0.046 $h^{-1}$ and 0.89 g $g^{-1}$, respectively. In the absence of aeration, fat removal was reduced considerably to approximately 5%. Consequently, the growth rate and yield without aeration were also significantly reduced to 0.005 $h^{-1}$ and 0.07 g $g^{-1}$, respectively. Cell viability was 100% during aerated growth. In contrast, in the absence of aeration, cell viability was reduced to approximately 60% after 65 h.

**Table 3.3.3:** Percentage fat removal, growth rate and yield for *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) in 2 L fermenter for 65 h at aeration rates of 0, 0.5, 1.0 and 3.0 VVM. Temperature and agitation were set at 25°C and 500 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

| | **Aeration rate (VVM)** | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 3.0 |
| Fat removal (%) | 5.0 ± 1.0 | 64.5 ± 2.1 | 75.0 ± 1.0 | 74.5 ± 0.9 |
| Growth rate ($h^{-1}$) | 0.005 ± 0.003 | 0.046 ± 0.005 | 0.072 ± 0.002 | 0.072 ± 0.001 |
| Yield [(g dry weight) (g fat removed)$^{-1}$] | 0.07 ± 0.002 | 0.89 ± 0.005 | 0.94 ± 0.002 | 0.94 ± 0.004 |

Intracellular lipid content followed a similar pattern when the fermenter was aerated at 0.5, 1.0 and 3.0 VVM (Fig. 3.3.3). An increase in lipid content occurred at 12 h, to 0.5 g $g^{-1}$, which subsequently decreased during the course of the fermentation to approximately 0.10 g $g^{-1}$ by 65 h. In the absence of aeration, cellular lipid increased to 0.3 g $g^{-1}$ at 12 h and decreased to 0.25 g $g^{-1}$ at 65 h. This accumulation of lipid was attributed to the cessation of growth, which occurred after 12 h in the absence of aeration (results not shown).

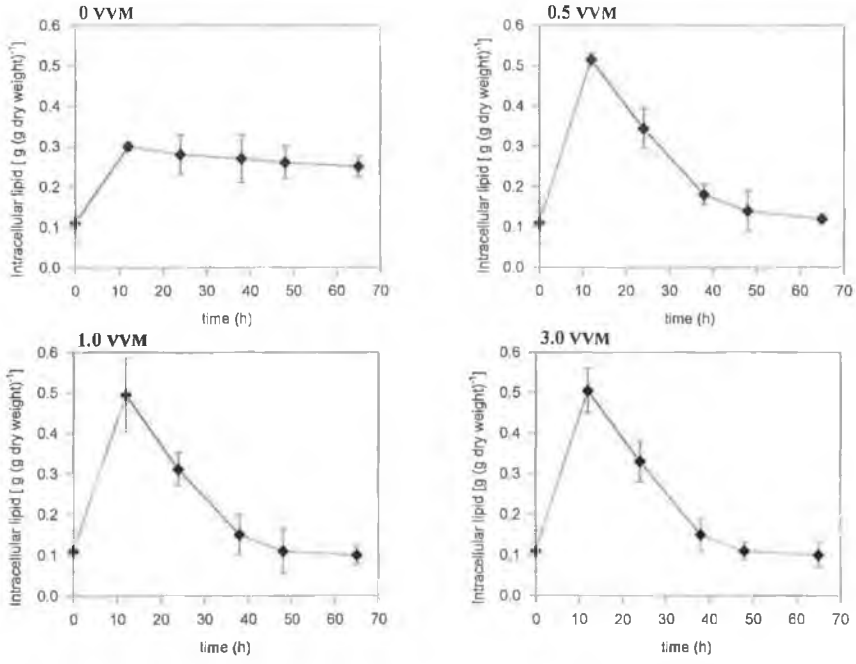



**Figure 3.3.3:** Intracellular lipid content of *Y. lipolytica* RP2 cells grown on tallow (20 g $L^{-1}$) in 2 L fermenter for 65 h at aeration rates of 0, 0.5, 1.0 and 3.0 VVM. Temperature and agitation were set to 25°C and 500 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0.

Yeast morphology was also monitored during the fermentations. A small degree of mycelial cells were detected after 12 h growth, at approximately 1.0 ± 1.0 % of overall cell number, when air was supplied to the fermenter at 0.5, 1.0 and 3.0 VVM (results not shown). After 12 h, no mycelial shape cells were detected. In contrast, the yeast morphology was in the yeast shape only during 65 h in the absence of aeration.

A similar reduction in the surface tension occurred at 0.5, 1.0 and 3.0 VVM, from approximately 70 dynes $cm^{-1}$ at the time of inoculation to 43 – 44 dynes $cm^{-1}$ by 24 h and remained at this tension until the end of the fermentation (Table 3.3.4). In the absence of aeration, there was a marginal reduction in surface tension to approximately 65 dynes $cm^{-1}$.

Maximum emulsification activity ($A_{540}$) and emulsion stability (decay ratio, $K_d$) were achieved after 24 h when the fermenter was aerated at 0.5, 1.0 and 3.0 VVM, with an aborbance in the range of 2.00 to 2.05 units and a $K_d$ between –2.24 and -2.30, respectively. In the absence of aeration, emulsification activity and emulsion stability were significantly reduced with only a marginal increase in absorbance to 0.20 and decrease in $K_d$ to –6.00 after 24 h. This corresponded to the absence of any significant reduction in surface tension.

The surface tension in the controls (no cells) was reduced from 70 to 65.0 dynes $cm^{-1}$ after 65 h (results not shown), irrespective of the aeration rate employed. Similarly, no emulsification activity or emulsion stability occurred in the controls.

Maximum fat removal, 75% and growth, 0.072 $h^{-1}$ were achieved at an aeration rate of 1 VVM, with identical results at 3 VVM. Aeration of the fermenter at rates below 1 VVM resulted in reduced fat removal, growth and yield. Consequently, 1 VVM was chosen as the aeration rate for all further fermenter studies.

**Table 3.3.4:** Surface tension, emulsification activity ($A_{540}$) and emulsion stability (decay ratio, $K_d$) of growth medium of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 2 L fermenter for 65 h at aeration rates of 0, 0.5, 1.0 and 3.0 VVM. Temperature and agitation were set to 25°C and 500 rpm, respectively, with 0.1 M potassium phosphate buffer, pH 7.0. The medium was filtered through 0.2 μm filter prior to measurement. The absorbance and decay ratios were measured against kerosene.

***Surface tension (dynes $cm^{-1}$)***

| Time (h) | 0 VVM | 0.5 VVM | 1.0 VVM | 3.0 VVM |
|---|---|---|---|---|
| 0 | 70.0 ± 1.0 | 70.0 ± 1.0 | 70.0 ± 1.0 | 70.0 ± 1.0 |
| 12 | 65.0 ± 0.2 | 53.0 ± 0.3 | 52.5 ± 0.5 | 53.0 ± 0.1 |
| 24 | 65.0 ± 0.3 | 43.0 ± 0.6 | 43.5 ± 0.1 | 44.0 ± 0.1 |
| 38 | 66.0 ± 0.2 | 43.0 ± 0.6 | 43.0 ± 0.1 | 43.0 ± 0.1 |
| 48 | 64.5 ± 0.5 | 43.0 ± 0.1 | 43.0 ± 0.0 | 43.5 ± 0.3 |
| 65 | 65.0 ± 0.5 | 43.0 ± 0.3 | 42.5 ± 0.0 | 43.0 ± 0.1 |

***Emulsification activity ($A_{540}$)***

| Time (h) | 0 VVM | 0.5 VVM | 1.0 VVM | 3.0 VVM |
|---|---|---|---|---|
| 0 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 |
| 12 | 0.18 ± 0.05 | 1.95 ± 0.02 | 1.95 ± 0.05 | 1.94 ± 0.08 |
| 24 | 0.20 ± 0.02 | 2.03 ± 0.05 | 2.05 ± 0.11 | 2.00 ± 0.05 |
| 38 | 0.12 ± 0.03 | 1.80 ± 0.11 | 1.90 ± 0.06 | 1.89 ± 0.03 |
| 48 | 0.13 ± 0.08 | 1.65 ± 0.07 | 1.77 ± 0.14 | 1.80 ± 0.01 |
| 65 | 0.12 ± 0.07 | 1.40 ± 0.02 | 1.55 ± 0.10 | 1.50± 0.15 |

***Decay ratio ($K_d$ x $10^{-3}$)***

| Time (h) | 0 VVM | 0.5 VVM | 1.0 VVM | 3.0 VVM |
|---|---|---|---|---|
| 0 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 |
| 12 | -6.01 ± 0.04 | -2.45 ± 0.05 | -2.35 ± 0.11 | -2.31 ± 0.04 |
| 24 | -6.00 ± 0.21 | -2.30 ± 0.11 | -2.27 ± 0.04 | -2.24 ± 0.08 |
| 38 | -6.25 ± 0.11 | -3.88 ± 0.02 | -3.80 ± 0.15 | -3.77 ± 0.05 |
| 48 | -6.24 ± 0.04 | -4.17 ± 0.11 | -4.08 ± 0.04 | -4.10 ± 0.14 |
| 65 | -6.11 ± 0.01 | -4.60 ± 0.07 | -4.45 ± 0.02 | -4.51 ± 0.09 |

The operational set-up for the 2 L fermenter after 24 h growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) under agitation and aeration rates of 500 rpm and 1 VVM, respectively, is illustrated in Fig. 3.3.4. Temperature was set to 25°C and pH was controlled to 7.0 by 0.1 M potassium phosphate buffer.

**Figure 3.3.4:** Illustration of the 2 L fermenter set-up after 24 h growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) with pH control to pH 7.0 by 0.1 M potassium phosphate buffer. Agitation and aeration were set at 500 rpm and 1 VVM, respectively. Temperature was controlled to 25°C by a circulating water bath.

### 3.3.1.3 Effect of the method of pH control to pH 7.0 on tallow biodegradation

The effect of various methods to control medium pH to 7.0 in the 2 L fermenter was assessed. The methods included (a) the inclusion of 0.1 M potassium phosphate (KOH-$KH_2PO_4$) buffer in the medium; (b) the inclusion of phosphate in the medium, as either $NaH_2PO_4$ or $KH_2PO_4$, at a concentration of 12.0 and 13.6 g $L^{-1}$, respectively, with pH adjustment to 7.0 with 0.5 M NaOH and (c) manual addition of NaOH, $Ca(OH)_2$ or KOH, at a concentration of 0.5 M when required. Temperature, agitation, and aeration were set at 25°C, 500 rpm and 1 VVM, respectively. Fat removal was determined at the end of the fermentation, after 65 h. The fermenters were sampled at regular intervals and pH, growth, cell viability, morphology, intracellular lipid and biosurfactant activity were monitored.

Percentage fat removal, growth rate and yield for pH control by the various methods were determined (Table 3.3.5). Maximum fat removal, 75%, was achieved after 65 h with pH control by 0.1 M potassium phosphate buffer. pH control with 0.5 M KOH achieved a lower rate of removal at approximately 66%. In contrast, fat removal was greatly reduced with pH control by either $NaH_2PO_4$ and $KH_2PO_4$ or with the addition of 0.5 M NaOH and $Ca(OH)_2$. Growth patterns were similar with the buffer and KOH and a final dry weight of 14 and 13 g $L^{-1}$ was achieved with the buffer and KOH, respectively (Fig. 3.3.5). Maximum growth rate was achieved with the buffer, 0.072 $h^{-1}$ with a lower growth rate with KOH at 0.069 $h^{-1}$. Growth was reduced with the other four methods of pH control, with corresponding lower final dry weights and growth rates. Yield values were similar with all the methods of pH control between 0.094 and 0.096 g $g^{-1}$, with the exception of $Ca(OH)_2$, which achieved a lower yield of 0.75 g $g^{-1}$.

Inclusion of the phosphate salts, $NaH_2PO_4$ and $KH_2PO_4$, required the addition of approximately 10 ml of 0.5 M NaOH at the start of the fermentation to adjust the media to pH 7.0. No further addition was required during the course of the fermentation. Manual pH control with NaOH and $Ca(OH)_2$ resulted in the addition of 25 ml and 5 ml of the bases, respectively, during the initial 24 h of the fermentation. In contrast, the higher growth rate associated with KOH-control,

necessitated the addition of 50 ml over the initial 24 h growth period and a further 5 ml was added during 24 – 68 h.

**Table 3.3.5:** Percentage fat removal, growth rate and yield for *Y. lipolytica* RP2 after 65 h growth on tallow (20 g $L^{-1}$) in 2 L fermenter with manual pH control to pH 7.0 by various methods. Aeration, agitation and temperature were set at 1 VVM, 500 rpm and 25°C, respectively.

| Methods of pH control | Fat removal (%) | Growth rate ($h^{-1}$) | Yield [(g dry weight) (g fat removed)$^{-1}$] |
|---|---|---|---|
| 0.1 M KOH-$KH_2PO_4$ | 75.0 ± 1.0 | 0.072 ± 0.002 | 0.94 ± 0.002 |
| 12 g $L^{-1}$ $NaH_2PO_4$ | 27.4 ± 1.2 | 0.029 ± 0.003 | 0.95 ± 0.090 |
| 13.6 g $L^{-1}$ $KH_2PO_4$ | 35.5 ± 2.5 | 0.033 ± 0.002 | 0.94 ± 0.030 |
| 0.5 M NaOH | 25.3 ± 1.8 | 0.028 ± 0.003 | 0.96 ± 0.050 |
| 0.5 M $Ca(OH)_2$ | 20.3 ± 0.8 | 0.018 ± 0.007 | 0.75 ± 0.050 |
| 0.5 M KOH | 66.3 ± 1.5 | 0.069 ± 0.002 | 0.94 ± 0.020 |

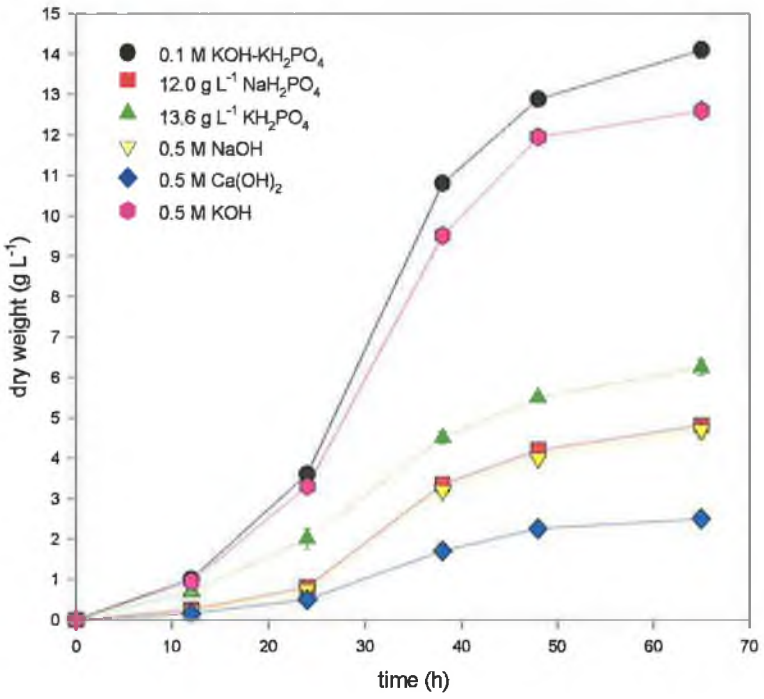



**Figure 3.3.5:** Dry weight of *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) for 65 h in 2 L fermenter with manual pH control to pH 7.0 by various methods. Aeration, agitation and temperature were set at 1 VVM, 500 rpm and 25°C, respectively.

Intracellular lipid increased after 12 h to approximately 0.5 g $g^{-1}$, after which time intracellular lipid decreased to between 0.15 and 0.10 g $g^{-1}$ with the various methods of pH control (Fig. 3.3.6). The exception was with $Ca(OH)_2$, where intracellular lipid increased to 0.38 g $g^{-1}$ at 12 h and was not reduced to the same degree after this time with 0.3 g $g^{-1}$ lipid present at the end of the fermentation.

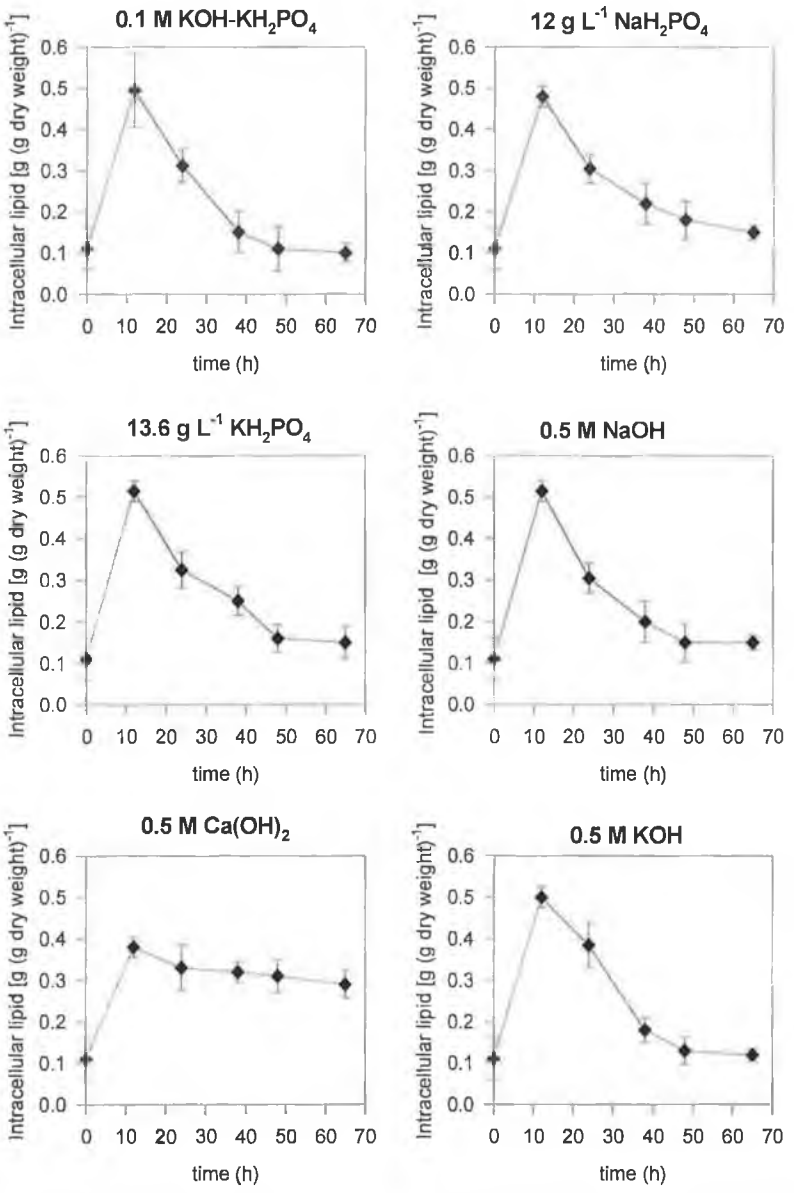



**Figure 3.3.6:** Intracellular lipid content of *Y. lipolytica* RP2 cells grown on tallow (20 g $L^{-1}$) for 65 h in 2 L fermenter with various methods of pH control to pH 7.0. Aeration, agitation and temperature were set 1 VVM, 500 rpm and 25°C, respectively.

Yeast viability was 100% during 65 h for all the fermentations. Dimorphic growth was detected at 12 h growth with approximately 1.0 ± 1.0 % mycelial-shaped cells observed for the different methods of pH control (results not shown). The exception was 0.5 M $Ca(OH)_2$ control, where no mycelial cells were observed. Cells were in the yeast-shape only after 12 h under all conditions and remained in the yeast-shape until the end of the fermentation.

A difference in the degree of tallow dispersal was observed at 24 h with the different methods of pH control, which correlated with the degree of fat removed (Fig. 3.3.7). The tallow was emulsified by 24 h with 0.5 M KOH and 0.1 M potassium phosphate. The tallow was dispersed into very small 'lumps' by 24 h with $KH_2PO_4$, but no further emulsification of the tallow was observed after 24 h. In contrast, the tallow remained as large 'lumps' with pH control by $NaH_2PO_4$, NaOH and $Ca(OH)_2$ control. By the end of the fermentation, the tallow was dispersed into smaller lumps and partially emulsified with $NaH_2PO_4$ and NaOH control, but remained in large lumps with $Ca(OH)_2$. In the controls (no cells), tallow dispersal was not observed with any method of pH control (results not shown).

**0.1 M KOH-$KH_2PO_4$**

**12.0 g $L^{-1}$ $NaH_2PO_4$**

**13.6 g $L^{-1}$ $KH_2PO_4$**

**0.5 M NaOH**

**0.5 M $Ca(OH)_2$**

**0.5 M KOH**

**Figure 3.3.7:** Illustration of the growth medium of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 2 L fermenter after 24 h where pH was controlled to pH 7.0 by various methods. Aeration, agitation and temperature were set to 1 VVM, 500 rpm and 25°C, respectively.

Biosurfactant activity measurements correlated with the observed differences in tallow dispersion between the various methods of pH control (Table 3.3.6). Surface tension was reduced to 42 dynes $cm^{-1}$ by 24 h with pH control by the buffer and 0.5 M KOH and no further change in tension occurred by the end of the fermentation. pH control with $KH_2PO_4$ resulted in a decrease to 45 dynes $cm^{-1}$ after 24 h, with no further change after 65 h. In contrast, a more gradual decrease in the surface tension over the course of the fermentation occurred with pH control by NaOH, $NaH_2PO_4$ and $Ca(OH)_2$, which resulted in a reduction in surface tension to between 50.0 and 58.5 dynes $cm^{-1}$ after 65 h. Correspondingly, tallow dispersion was observed to be lower with these three methods of pH control.

Maximum emulsification activity, which corresponded to an absorbance of between 1.99 and 2.05 units after 24 h, was achieved with the buffer, KOH and $KH_2PO_4$. Activity was reduced with NaOH, $NaH_2PO_4$ and $Ca(OH)_2$. Similarly, maximum emulsion stability was achieved with the buffer, KOH and $KH_2PO_4$ after 24 h, with a corresponding $K_d$ of between –2.27 and –2.33. A significantly higher $K_d$ was achieved after 24 h with NaOH, $NaH_2PO_4$ and $Ca(OH)_2$, which indicated a lower stabilising capability with these methods of pH control.

In the controls (no cells), surface tension was reduced to between 65 and 70 dynes $cm^{-1}$ with the various methods of pH control (results not shown). Emulsification activity or stability were not detected in the controls, irrespective of the method of pH control employed.

**Table 3.3.6:** Surface tension, emulsification activity ($A_{540}$) and emulsion stability (decay ratio, $K_d$) of growth medium of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 2 L fermenter for 65 h with pH control to pH 7.0 by various methods. Temperature, agitation and aeration were set to 25°C, 500 rpm and 1 VVM, respectively. The medium was filtered through 0.2 μm filter prior to measurement. The absorbance and decay ratios were measured against kerosene.

***Surface tension (dynes $cm^{-1}$)***

| Time (h) | 0.1 M KOH-$KH_2PO_4$ | 12.0 g $L^{-1}$ $NaH_2PO_4$ | 13.6 g $L^{-1}$ $KH_2PO_4$ | 0.5 M NaOH | 0.5 M $Ca(OH)_2$ | 0.5 M KOH |
|---|---|---|---|---|---|---|
| 0 | 70.0 ± 1.0 | 70.0 ± 1.0 | 70.0 ± 1.0 | 70.5 ± 0.3 | 70.5 ± 0.3 | 70.5 ± 0.3 |
| 12 | 52.5 ± 0.5 | 63.5 ± 0.7 | 54.0 ± 0.3 | 62.5 ± 0.5 | 65.5 ± 0.2 | 50.5 ± 0.3 |
| 24 | 43.5 ± 0.1 | 60.5 ± 0.1 | 45.0 ± 0.5 | 60.5 ± 0.1 | 63.5 ± 0.3 | 42.5 ± 0.3 |
| 38 | 43.0 ± 0.1 | 56.0 ± 0.3 | 45.0 ± 0.1 | 57.0 ± 0.3 | 60.5 ± 0.3 | 43.0 ± 0.5 |
| 48 | 43.0 ± 0.0 | 54.0 ± 0.5 | 45.5 ± 0.3 | 55.0 ± 0.5 | 59.0 ± 0.5 | 43.0 ± 0.1 |
| 65 | 42.5 ± 0.0 | 50.0 ± 0.3 | 45.0 ± 0.3 | 50.0 ± 0.3 | 58.5 ± 0.1 | 42.0 ± 0.1 |

***Emulsification activity ($A_{540}$)***

| Time (h) | 0.1 M KOH-$KH_2PO_4$ | 12.0 g $L^{-1}$ $NaH_2PO_4$ | 13.6 g $L^{-1}$ $KH_2PO_4$ | 0.5 M NaOH | 0.5 M $Ca(OH)_2$ | 0.5 M KOH |
|---|---|---|---|---|---|---|
| 0 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.02 |
| 12 | 1.95 ± 0.05 | 1.60 ± 0.02 | 1.90 ± 0.04 | 1.56 ± 0.20 | 0.35 ± 0.07 | 1.92 ± 0.02 |
| 24 | 2.05 ± 0.11 | 1.82 ± 0.03 | 1.99 ± 0.01 | 1.80 ± 0.05 | 0.45 ± 0.02 | 2.02 ± 0.05 |
| 38 | 1.90 ± 0.06 | 1.84 ± 0.07 | 1.87 ± 0.02 | 1.82 ± 0.10 | 0.40 ± 0.06 | 1.92 ± 0.06 |
| 48 | 1.77 ± 0.14 | 1.66 ± 0.05 | 1.67 ± 0.05 | 1.65 ± 0.06 | 0.18 ± 0.02 | 1.75 ± 0.02 |
| 65 | 1.55 ± 0.10 | 1.17 ± 0.09 | 1.25 ± 0.10 | 1.20 ± 0.11 | 0.17 ± 0.04 | 1.50 ± 0.06 |

***Decay ratio ($K_d$ x $10^{-3}$)***

| Time (h) | 0.1 M KOH-$KH_2PO_4$ | 12.0 g $L^{-1}$ $NaH_2PO_4$ | 13.6 g $L^{-1}$ $KH_2PO_4$ | 0.5 M NaOH | 0.5 M $Ca(OH)_2$ | 0.5 M KOH |
|---|---|---|---|---|---|---|
| 0 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 | -6.35 ± 0.11 |
| 12 | -2.35 ± 0.11 | -4.30 ± 0.05 | -2.60 ± 0.15 | -3.35 ± 0.05 | -5.85 ± 0.02 | -2.65 ± 0.06 |
| 24 | -2.27 ± 0.04 | -3.41 ± 0.11 | -2.31 ± 0.06 | -3.51 ± 0.11 | -5.50 ± 0.11 | -2.33 ± 0.05 |
| 38 | -3.80 ± 0.15 | -4.10 ± 0.05 | -3.95 ± 0.04 | -4.25 ± 0.05 | -5.95 ± 0.04 | -4.00 ± 0.08 |
| 48 | -4.08 ± 0.04 | -4.44 ± 0.04 | -4.15 ± 0.08 | -4.50 ± 0.04 | -6.00 ± 0.03 | -4.20 ± 0.10 |
| 65 | -4.45 ± 0.02 | -5.80 ± 0.05 | -4.85 ± 0.01 | -5.35 ± 0.05 | -6.00 ± 0.12 | -4.75 ± 0.07 |

Reduced fat removal and growth with NaOH, $NaH_2PO_4$ and $Ca(OH)_2$ control indicated that $Na^+$ and $Ca^{2+}$ ions may be inhibitory to the yeast. Increased phosphate, as either $NaH_2PO_4$ or $KH_2PO_4$, did not result in increased fat removal or growth. The superior degree of growth and fat removal achieved with the buffer (KOH-$KH_2PO_4$) and 0.5 M KOH, together with a higher degree of biosurfactant activity in the presence of $K^+$ (with the buffer, KOH and $KH_2PO_4$), implied a possible role of this ion in the fermentation. To investigate this further, the level of potassium in both the yeast cell and the medium was assessed with the different methods of pH control.

In all cases, the initial $K^+$ concentration in the cells was approximately 70 nmol $K^+$ $(10^6$ cell$)^{-1}$ (Figure 3.3.8). Cellular $K^+$ levels were greatest with pH control by 0.5 M KOH and 0.1 M potassium phosphate buffer and followed a similar pattern during the course of the fermentation. An increase from 70 to 180 nmol $K^+$ $(10^6$ cell$)^{-1}$ occurred in the cells at 12 h, which then decreased to between 80 and 60 nmol $K^+$ $(10^6$ cell$)^{-1}$ by 24 h for the buffer and KOH, respectively. Cellular $K^+$ levels remained at this level until the end of the fermentation. Cells under these two methods of pH control increased in number exponentially, to approximately 3.8 x $10^8$ cell $ml^{-1}$ by the end of the fermentation. Cellular $K^+$ levels for pH control with $KH_2PO_4$ increased to approximately 160 nmol $(10^6$ cell$)^{-1}$ $K^+$ at 12 h. However, after 12 h, cellular $K^+$ continued to decrease to 18 ± 0.5 nmol $K^+$ $(10^6$ cell$)^{-1}$ by the end of the fermentation. Correspondingly, cell growth was not as extensive as achieved with the buffer and KOH, with a final cell count of 2.5 x $10^8$ cell $ml^{-1}$. Where the source of $K^+$ was from the minimal medium only (0.9 g $L^{-1}$ $K_2HPO_4$) as for NaOH, $Ca(OH)_2$ and $NaH_2PO_4$ control, no significant increase in cellular $K^+$ was observed at 12 h and $K^+$ concentration remained at the initial level of 70 nmol $K^+$ $(10^6$ cell$)^{-1}$. After 12 h, $K^+$ levels decreased and less than 10 nmol $K^+$ $(10^6$ cell$)^{-1}$ was detected by 65 h, with these three methods of pH control. This decrease in cellular $K^+$ correlated with a lower degree of growth, with a final cell number of approximately 1.5 x $10^8$ cell $ml^{-1}$ for NaOH and $NaH_2PO_4$ and 0.8 x $10^8$ cell $ml^{-1}$ for $Ca(OH)_2$.

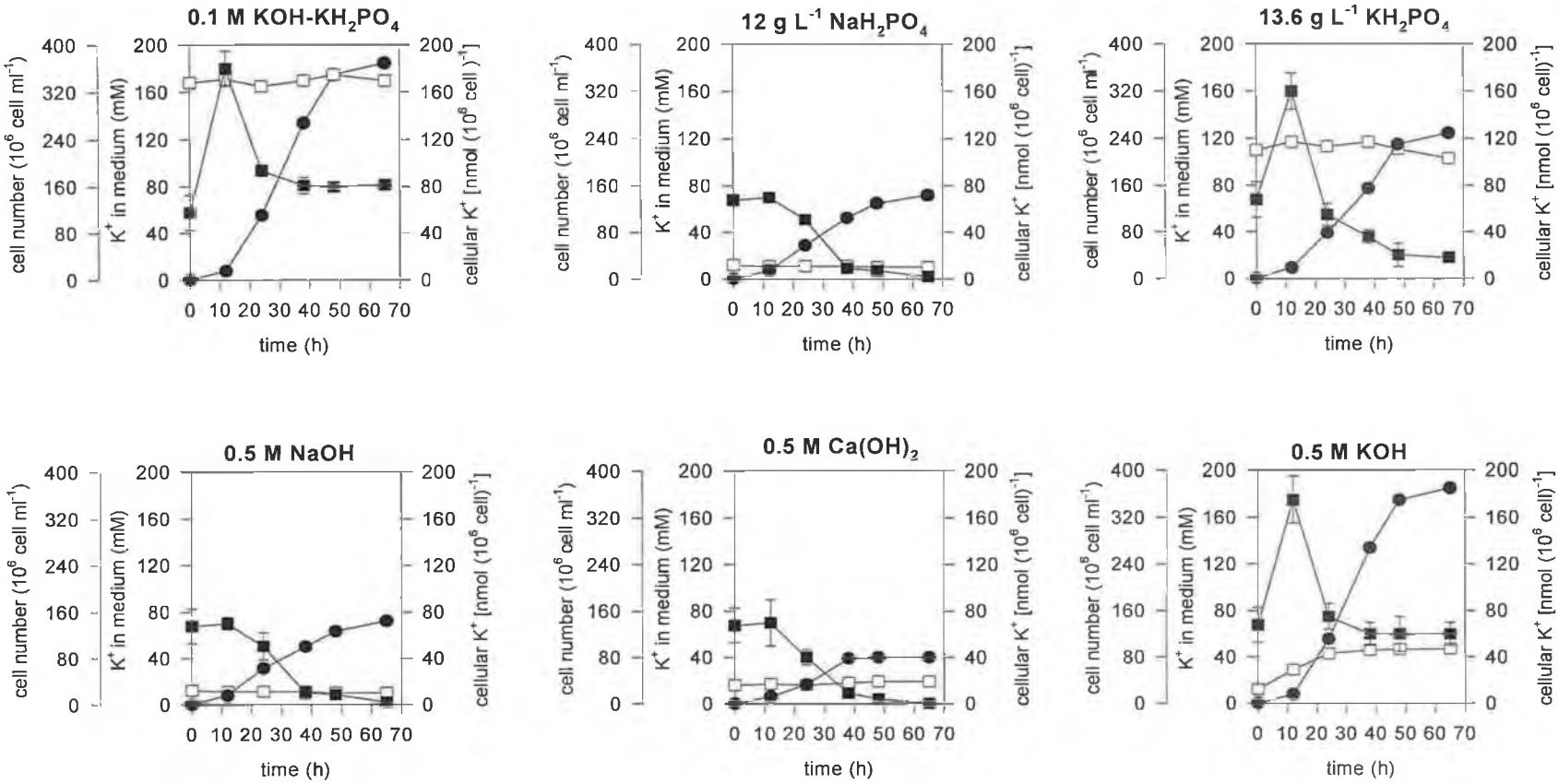



**Figure 3.3.8:** Comparison of cell number (●), intracellular $K^+$ (■) and $K^+$ in the medium (□) during growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 2 L fermenter for 65 h with pH control to pH 7.0 by various methods. Aeration, agitation and temperature were set to 1 VVM, 500 rpm and 25°C.

The extracellular $K^+$ levels remained constant during the fermentations at their respective molarities and included 10 mM $K^+$ addition from the medium itself. The exception was with 0.5 M KOH, where $K^+$ increased during the fermentation from approximately 10 to 50 mM $K^+$ after 24 h, due to the periodic addition of approximately 50 ml of the base to the medium over the initial 24 h to maintain neutral pH. The buffer contributed the greatest amount of $K^+$ to the medium, at approximately 160 mM, through its composition as KOH (60 mM) and $KH_2PO_4$ (100mM). pH control by 13.6 g $L^{-1}$ $KH_2PO_4$ contributed approximately 100 mM $K^+$ (exclusive of the 10 mM contribution from the minimal medium). However, the higher level of $K^+$ in the medium with $KH_2PO_4$ control compared to KOH did not correlate with cellular $K^+$ values or the degree of growth and fat removal.

Maximum growth and fat removal by *Y. lipolytica* RP2 occurred with $K^+$ at a greater concentration than present in the minimal medium, corresponding to intracellular $K^+$ content of approximately 80 nmol $K^+$ $(10^6 \text{ cell})^{-1}$. Maximum $K^+$ addition and pH control was by 0.1 M potassium phosphate buffer or 0.5 M KOH. Consequently, 0.1 M potassium phosphate buffer and 0.5 M KOH were selected for pH control in further fermentation investigations.

### 3.3.2 10 L fermenter studies

The ability of *Y. lipolytica* RP2 to remove tallow was assessed in a larger working volume, in a 10 L fermenter. The effect of agitation in the 10 L fermenter was investigated with respect to impeller position on the agitator with pH control by 0.1 M potassium phosphate buffer, pH 7.0. The effect of pH control to 7.0 by the buffer and 0.5 M KOH/HCl on tallow biodegradation and growth was also compared.

An illustration of the operational set-up for the 10 L fermenter is shown in Fig. 3.3.9, with pH control by 0.5 M KOH/HCl. Where pH control was by potassium phosphate buffer, acid/base reservoirs were not required.

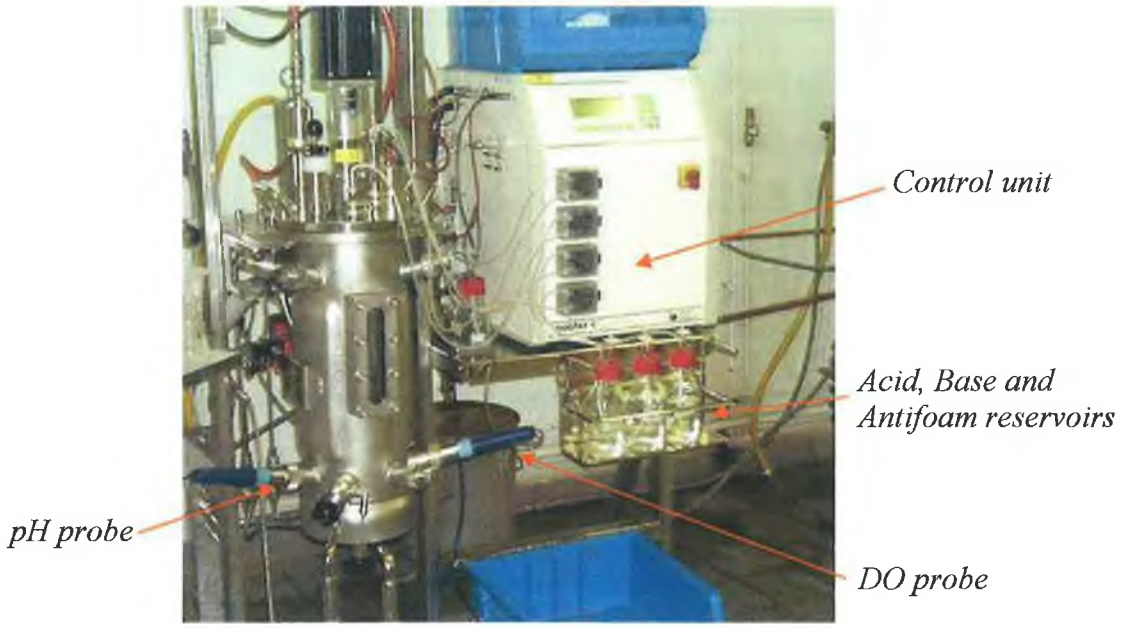



**Figure 3.3.9:** Illustration of the operational set-up of the 10 L fermenter during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) under automated pH control to pH 7.0 with 0.5 M KOH/0.5 M HCl. Temperature, aeration and agitation were controlled to 25°C, 1 VVM and 1000 rpm, respectively.

### 3.3.2.1 Effect of agitation on tallow biodegradation

The influence of the impeller position on the degree of growth and fat removal was assessed during scale-up to the 10 L fermenter. Two impellers were fixed at the base of the agitator in the 2 L fermenter during operation. In the 10 L fermenter, performance was compared with either two impellers positioned at the base of the shaft, similar to 2 L operation, or with one impeller at the base of the shaft and the second positioned 135 mm above it (Fig. 3.3.10). In the latter case, the second impeller was located at the surface of the media at the start of the fermentation.


**Figure 3.3.10:** Illustration of the impeller position on the agitator for the 10 L fermenter. (A) 2 impellers are positioned at the base of the shaft and (B) 1 impeller is positioned at the base of the shaft with the second impeller 135 mm above it.

The 10 L fermenter was operated under the superior conditions of temperature and aeration, as determined in previous investigations, at 25°C and 1 VVM, respectively. pH was controlled with 0.1 M potassium phosphate buffer, pH 7.0. Agitation was increased to 1000 rpm for scale-up from 2 L to 10 L fermentation based on constant mixing as detailed in Section 2.2.6.4. Fat removal was determined at the end of the fermentation, at 65 h. The fermentations were sampled at regular intervals and growth, viability, intracellular lipid, dissolved oxygen, cell morphology and biosurfactant production were monitored. Intracellular and medium $K^+$ levels were also assessed.

Approximately 68% and 64% tallow was removed with 1 and 2 impellers positioned at the base of the agitator shaft, respectively (Table 3.3.7). The placement of 2 impellers at the base of the agitator shaft resulted in high foam formation, which was difficult to control even with the addition of antifoam.

Yeast growth followed a similar pattern, irrespective of impeller position, with exponential growth after 12 h and a similar final dry weight of approximately 14 g $L^{-1}$ (Fig. 3.3.11). This corresponded to a growth rate of 0.072 $h^{-1}$, in both cases. A marginal decrease in dissolved oxygen (DO) during the fermentation indicated increased use of oxygen by the yeast during exponential growth from 12 to 48 h, irrespective of impeller position. Maximum decrease occurred at 24 h, to approximately 86 ± 1% DO, and increased to above 90% DO after 48 h. This indicated that aeration at 1 VVM supplied sufficient oxygen to the vessel. The yield achieved with 2 impellers was marginally higher than that with 1 impeller, as a result of the lower degree of fat removed, at approximately 1.09 and 1.03 g $g^{-1}$, respectively.

**Table 3.3.7:** Percentage fat removal, growth rate and yield after 65 h growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 10 L fermenter where (A) 1 impeller and (B) 2 impellers were fitted to the base of the agitator. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively, with pH control by 0.1 M potassium phosphate buffer, pH 7.0.

| | **No. of impellers fitted to the base of the agitator** | |
|---|---|---|
| | 1 | 2 |
| Fat removal (%) | 68.3 ± 1.5 | 64.4 ± 0.85 |
| Growth rate ($h^{-1}$) | 0.072 ± 0.003 | 0.072 ± 0.001 |
| Yield [(g dry weight) (g fat removed)$^{-1}$] | 1.03 ± 0.02 | 1.09 ± 0.01 |

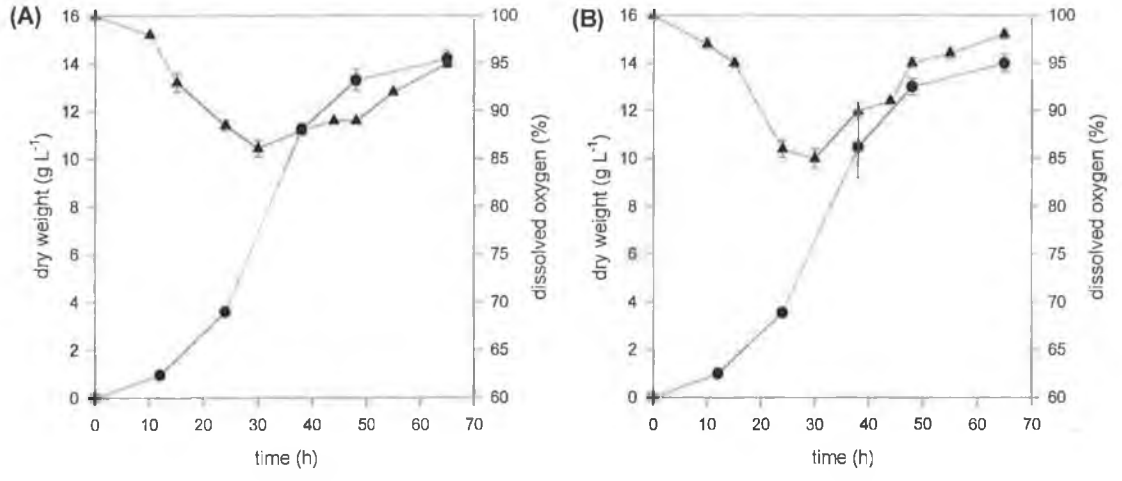



**Figure 3.3.11:** Dry weight (●) and percentage dissolved oxygen (▲) during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h where (A) 1 impeller and (B) 2 impellers were fitted to the base of the agitator. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively, with pH control by 0.1 M potassium phosphate buffer, pH 7.0.

The intracellular lipid content was similar, irrespective of the impeller position (Fig. 3.3.12). Cellular lipid content increased after inoculation up to approximately 0.5 g $g^{-1}$ at 12 h. A decrease to 0.10 ± 0.01 g $g^{-1}$ occurred by 38 h, with no further change in lipid levels by 65 h.

Cell viability was 100% under both agitation conditions. No mycelial cells were observed with cell morphology in the yeast-shape only during the fermentation, irrespective of impeller number at the base of the agitator. An illustration of the yeast morphology is shown in Fig. 3.3.13.

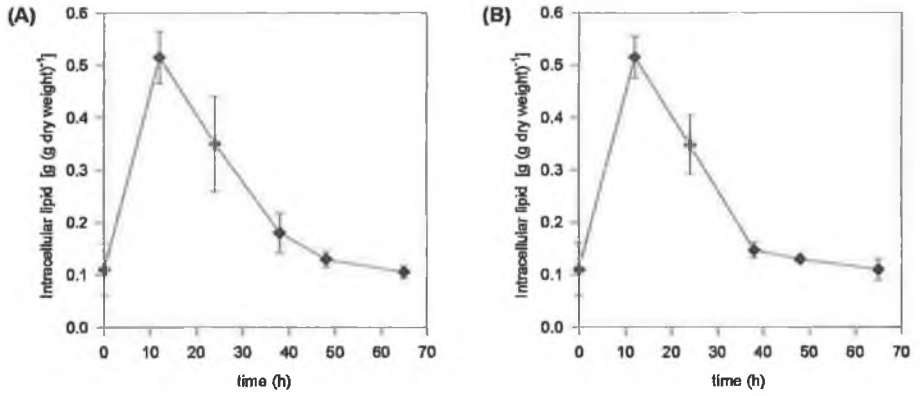



**Figure 3.3.12:** Intracellular lipid content of *Y. lipolytica* RP2 cells grown on tallow (20 g $L^{-1}$) for 65 h in 10 L fermenter where (A) 1 and (B) 2 impellers were fitted to the base of the agitator. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively, with pH control by 0.1 M potassium phosphate buffer, pH 7.0.

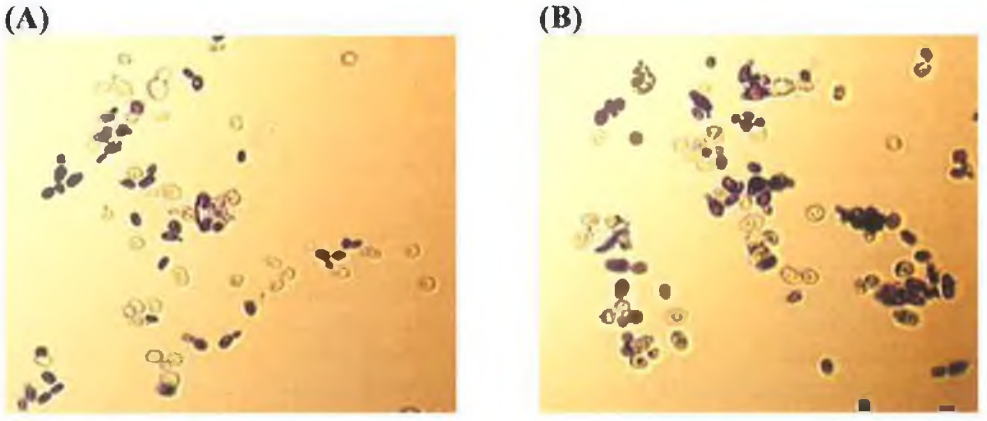



**Figure 3.3.13:** Illustration of *Y. lipolytica* RP2 cell morphology after 12 h growth on tallow (20 g $L^{-1}$) in 10 L fermenter where (A) 1 impeller and (B) 2 impellers were fitted to the base of the shaft. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively, agitator with pH control by 0.1 M potassium phosphate buffer, pH 7.0. Cells were observed under x40 magnification.

Surface tension of the cell free medium was reduced to 42 dynes $cm^{-1}$ after 24 h, irrespective of the impeller position with no further change after this time (Table 3.3.8). In the control (no cells), a decrease in surface tension to approximately 65 dynes $cm^{-1}$ occurred after 65 h (results not shown).

The relative concentration of the biosurfactant in the medium was assessed and was determined as the inverse of the critical micelle dilution, $(CMD)^{-1}$, in accordance to the method detailed in Section 2.2.11.2. The surface tension of a series of dilutions of the cell free medium in distilled water were measured (Fig. 3.3.14). The critical micelle concentration (CMC) of the surfactant corresponded to the maximum dilution of the growth medium at which the decreased surface tension level was maintained.

After 12 h, dilution of the medium resulted in an immediate increase in surface tension, which implied that the biosurfactant was at its CMC in the medium. At 24 to 65 h, the surface tension was retained at 42 dynes $cm^{-1}$ in a dilution of the medium to 0.5 (v/v) and increased at higher dilutions. This indicated an increase in biosurfactant concentration after 12 h, with $CMD^{-1}$ corresponding to a value of 2.0. The positioning of the impellers in the fermenter did not affect surface tension reduction, with identical values under both agitation conditions.

**Table 3.3.8:** Surface tension of growth medium of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h where 1 or 2 impellers were fitted to the base of the agitator. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively, with pH control by 0.1 M potassium phosphate buffer, pH 7.0. Medium was filtered through 0.2 μm filter prior to measurement.

| | **Surface tension (dynes $cm^{-1}$)** | |
|---|---|---|
| | No. of impellers fitted to the base of the agitator | |
| **Time (h)** | 1 | 2 |
| 0 | 70.0 ± 1.00 | 70.0 ± 1.00 |
| 12 | 49.3 ± 0.33 | 48.8 ± 0.33 |
| 24 | 41.8 ± 0.25 | 42.0 ± 0.25 |
| 38 | 42.0 ± 0.00 | 42.0 ± 0.33 |
| 48 | 42.8 ± 0.25 | 42.5 ± 0.50 |
| 65 | 42.0 ± 0.00 | 42.3 ± 0.17 |

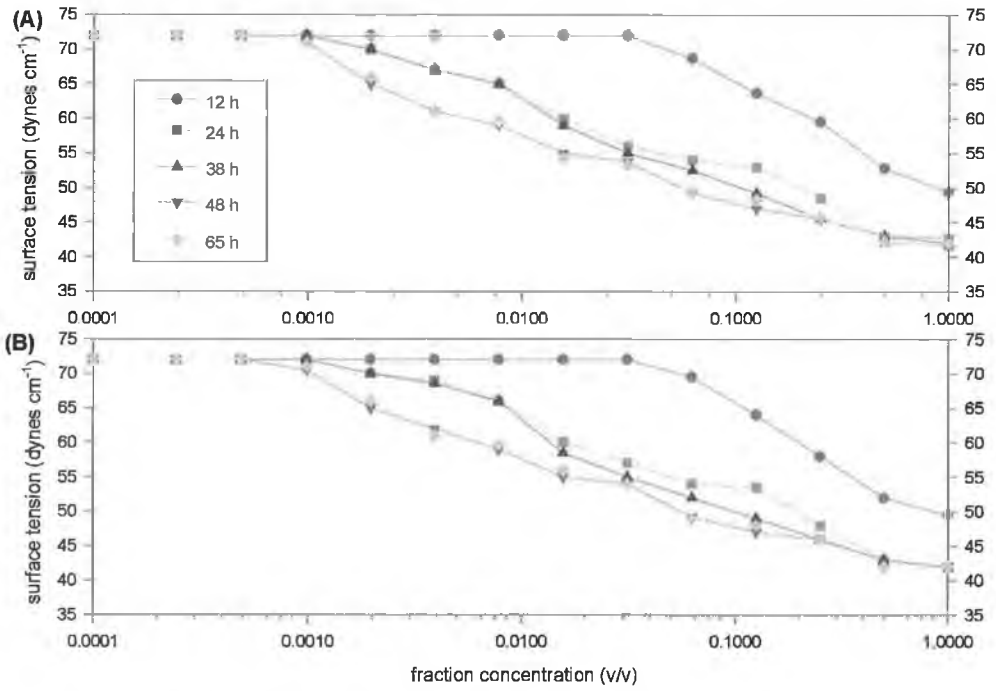



**Figure 3.3.14:** Change in surface tension with medium concentration during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h where (A) 1 or (B) 2 impellers were fitted to the base of the agitator. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively, with pH control by 0.1 M potassium phosphate buffer, pH 7.0.

The emulsification activity of the biosurfactant and the stability of its emulsion formation (decay ratio, $K_d$) with kerosene was determined (Table 3.3.9). As noted in surface tension measurements, impeller positioning did not affect activity or stability with identical values achieved. Maximum emulsification activity and emulsion stability occurred after 24 h and corresponded to an absorbance of between 2.10 and 2.05 units and a $K_d$ of between –2.22 and –2.28 for 1 and 2 impellers, respectively. Although the surface tension remained constant after 24 h, the emulsification activity of the biosurfactant decreased to between 1.58 and 1.55 units by the end of the fermentation for 1 and 2 impellers, respectively. Similarly, the emulsion stability, as indicated by the $K_d$ values decreased after 24 h with a $K_d$ of between –4.40 and –4.44 for 1 and 2 impellers, respectively at 65 h.

**Table 3.3.9:** Emulsification activity and emulsification stability (decay ratio, $K_d$) of growth medium of *Y. lipolytica* RP2 when grown on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h where 1 or 2 impellers were fitted to the base of the agitator. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively, with pH control to 7.0 by 0.1 M potassium phosphate buffer. Medium was filtered through a 0.2 μm filter prior to measurement against kerosene.

| | **Number of impellers fitted to the base of the agitator** | | | |
|---|---|---|---|---|
| | **1** | | **2** | |
| Time (h) | Emulsification activity ($A_{540}$) | Decay ratio ($K_d \times 10^{-3}$) | Emulsification activity ($A_{540}$) | Decay ratio ($K_d \times 10^{-3}$) |
| 0 | 0.09 ± 0.02 | -6.35 ± 0.11 | 0.09 ± 0.02 | -6.35 ± 0.11 |
| 12 | 1.99 ± 0.03 | -2.25 ± 0.01 | 2.00 ± 0.05 | -2.30 ± 0.11 |
| 24 | 2.10 ± 0.05 | -2.22 ± 0.05 | 2.05 ± 0.02 | -2.28 ± 0.09 |
| 38 | 1.89 ± 0.08 | -3.75 ± 0.02 | 1.90 ± 0.01 | -3.80 ± 0.02 |
| 48 | 1.75 ± 0.05 | -3.99 ± 0.07 | 1.73 ± 0.02 | -3.97 ± 0.10 |
| 65 | 1.58 ± 0.04 | -4.40 ± 0.11 | 1.55 ± 0.03 | -4.44 ± 0.05 |

Intracellular $K^+$ increased to approximately 180 nmol $K^+$ $(10^6 \text{ cell})^{-1}$ by 12 h, with a decrease to 80 nmol $K^+$ $(10^6 \text{ cell})^{-1}$ by 24 h with no further change detected after this time, irrespective of impeller positioning (Fig. 3.3.15). This was similar to the variation in $K^+$ levels during 2 L fermentation with the buffer. Cells increased exponentially after 12 h to approximately 3.9 x $10^8$ cell $ml^{-1}$ by the end of the fermentation, which was also similar to 2 L fermentation. The level of $K^+$ in the medium was constant during the fermentation, at approximately 170 mM.

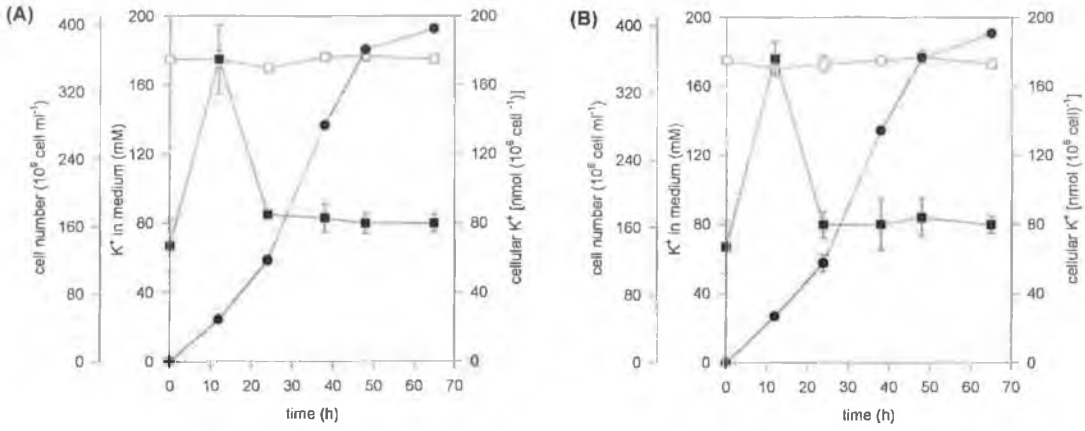



**Figure 3.3.15:** $K^+$ in the medium (□), intracellular $K^+$ (■) and cell number (●) of *Y. lipolytica* RP2 during growth on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h with (A) 1 impeller and (B) 2 impellers were fitted to the base of the agitator. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively, with pH control by 0.1 M potassium phosphate buffer, pH 7.0.

The placement of two impellers fixed to the base of the agitator did not result in increased fat removal or growth but did result in greater foam formation compared to one impeller at the base. Consequently, the positioning of one impeller at the base and the other 135 mm above it on the drive shaft was selected as the preferred method of agitation in the 10 L fermenter and was employed for all further 10 L studies.

### 3.3.2.2 Comparison of pH control to 7.0 with 0.5 M KOH/HCl and 0.1 M potassium phosphate buffer

In the 10 L fermenter, automated pH control to 7.0 with 0.5 M KOH/HCl and with 0.1 M potassium phosphate buffer were compared. Agitation was set at 1000 rpm with one impeller positioned at the base of the agitator shaft and the other 135 mm above it, with aeration and agitation set to 1 VVM and 25°C, respectively. Fat removal was determined after 65 h. The fermenters were sampled at regular intervals and growth, viability, dissolved oxygen, cell morphology, intracellular lipid and biosurfactant production were monitored. Intracellular and medium $K^+$ levels were also assessed.

An identical degree of fat was removed after 65 h, approximately 68%, with pH control by both KOH/HCl and the buffer (Table 3.3.10). Correspondingly, the growth rates and yield values were identical for the two methods of pH control, 0.072 $h^{-1}$ and 1.03 g $g^{-1}$, respectively. Exponential growth occurred after 12 h and dry weight increased to 14 g $L^{-1}$ by the end of the fermentation (Fig. 3.3.16). In both cases, a marginal decrease in the dissolved oxygen between 84 and 86% occurred after 24 h growth and increased to approximately 90% at the end of the fermentation. This indicated that aeration at 1 VVM supplied sufficient oxygen to the vessel.

In terms of scale-up from the 2 L fermenter, automatic pH control with 0.5 M KOH in 10 L fermenter resulted in a similar degree of fat removal compared to manual addition in 2 L fermenter, which removed 66% fat. The growth rate and final dry weight in the 10 L fermenter was marginally higher than that achieved in the 2 L vessel (0.069 $h^{-1}$ and 12.6 g $L^{-1}$, respectively). This was attributed to the automation of base addition in the 10 L fermenter compared to manual addition in the 2 L vessel. The scale-up to 10 L fermentation with pH control by the buffer resulted a marginal decrease in fat removal from 75% to 68%. However, the growth rate and final dry weight values were identical in both 2 L and 10 L fermenters.

**Table 3.3.10:** Percentage fat removal, growth rate and yield after 65 h growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 10 L fermenter with pH control to pH 7.0 by 0.5 M KOH/HCl and 0.1 M potassium phosphate buffer. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm.

| | **0.5 M KOH/ HCl** | **0.1 M potassium phosphate buffer** |
|---|---|---|
| Fat removal (%) | 68.2 ± 1.3 | 68.3 ± 1.5 |
| Growth rate ($h^{-1}$) | 0.072 ± 0.01 | 0.072 ± 0.003 |
| Yield [(g dry weight) (g fat removed)$^{-1}$] | 1.03 ± 0.02 | 1.03 ± 0.02 |

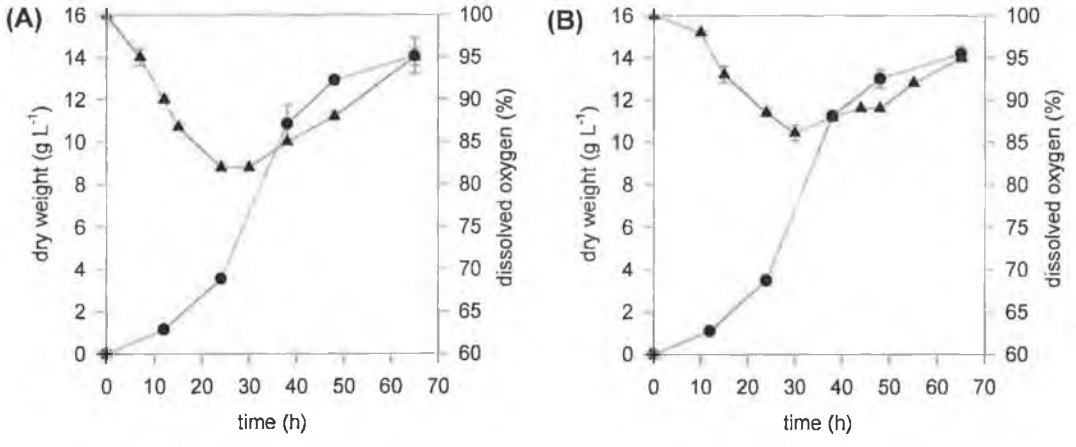



**Figure 3.3.16:** Dry weight (●) and dissolved oxygen (▲) during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) for 65 h in 10 L fermenter with pH control to pH 7.0 by (A) 0.5 M KOH/HCl and (B) 0.1 M potassium phosphate buffer. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively.

Yeast viability was 100% during the fermentation with both methods of pH control. Yeast morphology was observed to be in the yeast-shape only (results not shown) throughout the 65 h, in both cases. In contrast, a small percentage of mycelial cells, 1.0 ± 1.0%, were observed in the 2 L fermentations at 12 h with yeast-shape only cells after this time.

Intracellular lipid content followed an identical pattern, irrespective of the method of pH control employed (Fig 3.3.17). Lipid content in the cells increased after inoculation to approximately 0.5 g $g^{-1}$ at 12 h. After 12 h, the fat content of the cells decreased and by 38 h had reduced to approximately 0.10 g $g^{-1}$ and remained at this level until the end of the fermentation. This was identical to intracellular lipid levels during the course of 2 L fermentation with manual addition of KOH for pH control and the inclusion of the buffer.

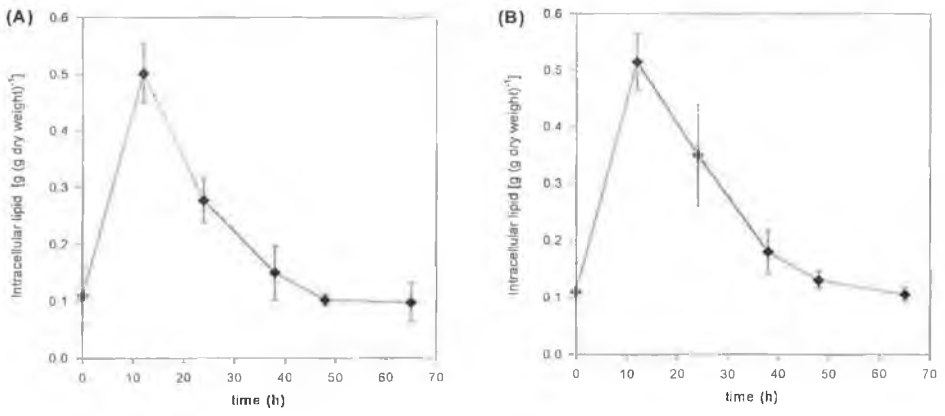



**Figure 3.3.17:** Intracellular lipid content for *Y. lipolytica* RP2 cells grown on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h with pH control to 7.0 by (A) 0.5 M KOH/HCl and (B) 0.1M potassium phosphate buffer. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively.

Surface tension was reduced to approximately 42 dynes $cm^{-1}$ after 24 h growth with no further change after 65 h, irrespective of the method of pH control (Table 3.3.11). This was a similar reduction to that achieved in the 2 L fermenter (Fig. 3.3.6), in both cases. In the controls (no cells), surface tension was reduced marginally during the 65 h to between 65 and 70 dynes $cm^{-1}$ for both methods of pH control.

The surface tension was maintained at 42 dynes $cm^{-1}$ when diluted to 0.5 (v/v) with distilled water after 24 – 65 h (Fig. 3.3.18). This corresponded to a $CMD^{-1}$ of 2.0. In contrast, the surface tension increased when diluted to 0.5 (v/v) after 12 h growth, which indicated that the biosurfactant was not as concentrated at the earlier stage of the fermentation. Increased dilution of the cell free medium greater than 0.5 (v/v) resulted in increased surface tension up to 72 dynes $cm^{-1}$. A similar pattern of surface tension resulted with both buffer and KOH pH control.

**Table 3.3.11:** Surface tension of growth medium of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h with pH control to 7.0 by 0.5 M KOH/HCl and 0.1 M potassium phosphate buffer. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively. Medium was filtered through 0.2 μm filter prior to measurement.

| | Surface tension (dynes $cm^{-1}$) | | | |
|---|---|---|---|---|
| | **0.5 M KOH/HCl** | | **0.1 M potassium phosphate buffer** | |
| **Time (h)** | *Y. lipolytica RP2* | *Control* | *Y. lipolytica RP2* | *Control* |
| 0 | 70.5 ± 0.3 | 70.5 ± 0.3 | 70.0 ± 1.0 | 70.0 ± 1.0 |
| 12 | 45.0 ± 0.2 | 70.0 ± 0.5 | 49.3 ± 0.3 | 69.0 ± 0.5 |
| 24 | 42.0 ± 0.3 | 69.0 ± 0.7 | 41.8 ± 0.3 | 69.0 ± 0.5 |
| 38 | 42.0 ± 0.0 | 69.5 ± 0.0 | 42.0 ± 0.0 | 67.0 ± 0.3 |
| 48 | 42.5 ± 0.3 | 70.0 ± 0.1 | 42.8 ± 0.3 | 65.0 ± 0.1 |
| 65 | 42.0 ± 0.2 | 70.0 ± 0.1 | 42.0 ± 0.0 | 65.0 ± 0.0 |

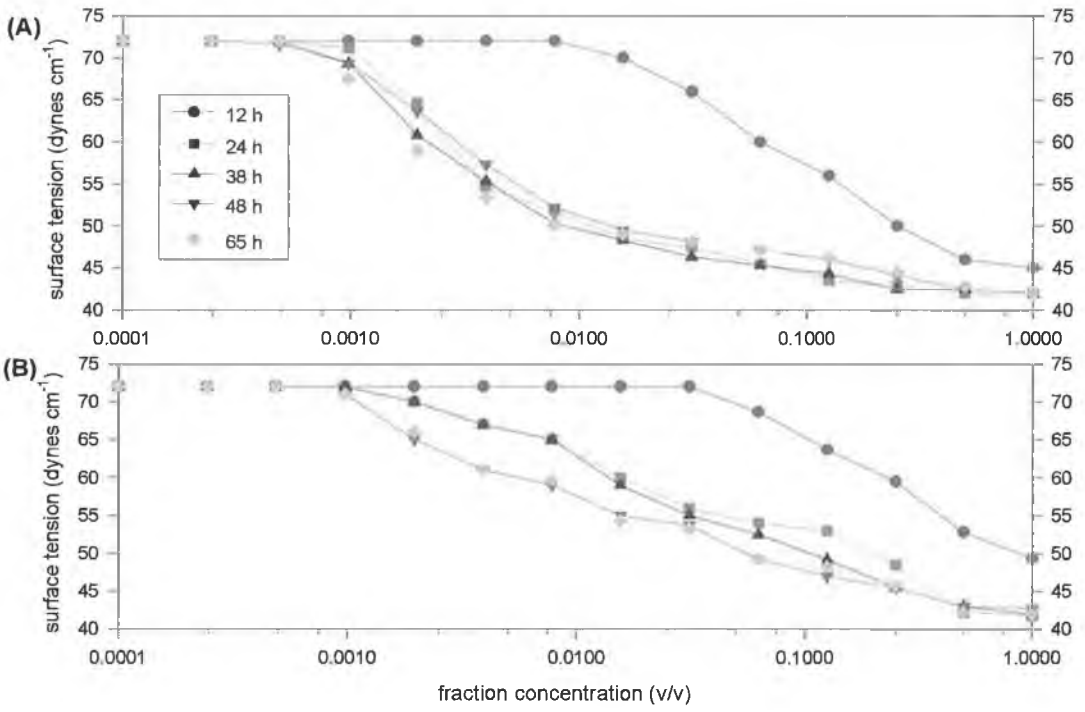



**Figure 3.3.18:** Change in surface tension with medium concentration during the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 with pH control to 7.0 by (A) 0.5 M KOH/ HCl and (B) 0.1 M potassium phosphate buffer. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm.

The emulsification activity ($A_{540}$) and the stability of its emulsion formation (decay ratio, $K_d$) was determined and no difference was detected between pH control by KOH and the buffer (Table 3.3.12). Activity was similar during 12 – 38 h growth period, with a marginally greater activity at 24 h, corresponding to an absorbance of 2.14 and 2.10 for KOH and the buffer, respectively. After 38 h, the activity decreased. Emulsion stability was similar in the 12 - 24 h period with a corresponding $K_d$ in the range of –2.22 to –2.27 for KOH and the buffer. After 24 h, a decrease in emulsion stability occurred. The activity and stability values followed a similar pattern to those achieved under 2 L fermentation after 24 h, for both methods of pH control. No emulsification activity or stability was detected in the controls (no cells), irrespective of the method of pH control (results not shown).

**Table 3.3.12:** Emulsification activity ($A_{540}$) and emulsification stability (decay ratio, $K_d$) of growth medium of *Y. lipolytica* RP2 grown on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h with pH control to 7.0 by 0.5 M KOH/HCl. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively. Medium was filtered through a 0.2 μm filter prior to measurement against kerosene.

| | **0.5 M KOH/HCl** | | **0.1 M potassium phosphate buffer** | |
|---|---|---|---|---|
| **Time (h)** | Emulsification activity ($A_{540}$) | Decay constant ($K_d \times 10^{-3}$) | Emulsification activity ($A_{540}$) | Decay constant ($K_d \times 10^{-3}$) |
| 0 | 0.09 ± 0.02 | -6.35 ± 0.11 | 0.09 ± 0.02 | -6.35 ± 0.11 |
| 12 | 1.95 ± 0.05 | -2.27 ± 0.08 | 1.99 ± 0.03 | -2.25 ± 0.01 |
| 24 | 2.14 ± 0.10 | -2.23 ± 0.13 | 2.10 ± 0.05 | -2.22 ± 0.05 |
| 38 | 1.98 ± 0.08 | -3.64 ± 0.21 | 1.89 ± 0.08 | -3.75 ± 0.02 |
| 48 | 1.67 ± 0.03 | -4.05 ± 0.02 | 1.75 ± 0.05 | -3.99 ± 0.07 |
| 65 | 1.50 ± 0.14 | -4.35 ± 0.07 | 1.58 ± 0.04 | -4.40 ± 0.11 |

Intracellular $K^+$ levels were similar for pH control by both KOH and the buffer (Fig. 3.3.19). Intracellular $K^+$ levels increased after 12 h growth to approximately 180 nmol $K^+$ $(10^6 \text{ cell})^{-1}$ with a subsequent decrease by 24 h to approximately 80 nmol $K^+$ $(10^6 \text{ cell})^{-1}$ with no further change by the end of the fermentation. This overall pattern was similar to that achieved in 2 L fermentation with the buffer and KOH, although $K^+$ levels were marginally lower after 65 h with manual KOH pH control at 60 nmol $K^+$ $(10^6 \text{ cell})^{-1}$ in the 2 L vessel (Fig. 3.3.8). Cell number increased exponentially after 12 h to a final cell count of approximately 3.9 x $10^8$ cell $ml^{-1}$ after 65 h, for both methods of pH control. This was similar to that achieved in 2 L fermentation with both KOH and the buffer, which had a cell number of approximately 3.8 x $10^8$ cell $ml^{-1}$ after 65 h.

$K^+$ in the medium increased over the initial 24 h growth period, from approximately 10 mM to 60 mM with KOH, through the automated addition of 400 ml KOH. After 24 h, no further KOH was required as pH remained at 7.0 and consequently, $K^+$ levels remained constant in the medium. This level of $K^+$ addition was marginally higher compared manual KOH control in the 2 L vessel, which resulted in the addition of 50 ml of the base to the medium (40 mM $K^+$). The $K^+$ level in the medium with buffer pH control remained constant throughout the fermentation at approximately 170 mM. This was identical to that detected in the 2 L fermenter.

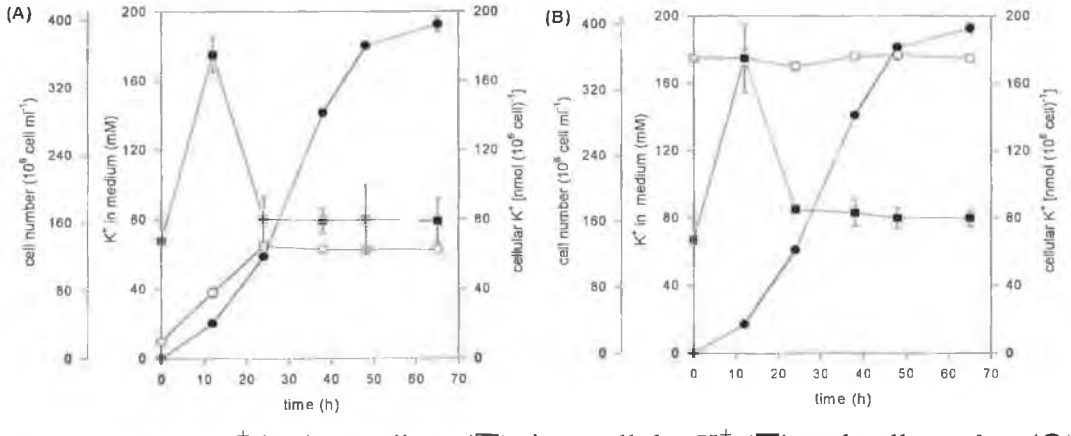



**Figure 3.3.19:** $K^+$ in the medium (□), intracellular $K^+$ (■) and cell number (●) of *Y. lipolytica* RP2 during growth on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h with pH control to 7.0 by (A) 0.5 M KOH/HCl and (B) 0.1 M potassium phosphate buffer. Temperature, aeration and agitation were set to 25°C, 1 VVM and 1000 rpm, respectively.

Overall, 68% fat was removed with a corresponding growth rate of 0.072 $h^{-1}$ during the growth of *Y. lipolytica* RP2 on tallow (2% w/v) in 10 L fermenter with pH control to 7.0 by either 0.5 M KOH/HCl or 0.1 M potassium phosphate buffer. There was no loss in fat removal ability during scale-up from 2 L to 10 L fermentation with pH control by KOH. A marginal loss in fat removal, from 75% to 68%, occurred upon scale-up with 0.1 M potassium phosphate buffer. The addition of KOH was selected as the preferred method of pH control and $K^+$ addition to the medium.

### 3.3.2.3 Further characterisation of the growth of *Yarrowia lipolytica* RP2 on tallow (20 g $L^{-1}$) with pH control to 7.0 by 0.5 M KOH/HCl

During the growth of *Y. lipolytica* RP2 on tallow, information on other parameters associated with growth and fat removal is desirable. These included sludge volume index (SVI), as an indication of yeast settleability, the location of the residual tallow in the system and the lipid, nitrogen and protein content of the yeast cell. These parameters were assessed during the growth of the yeast on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h with pH control to pH 7.0 by 0.5 M KOH/HCl. Aeration, agitation and temperature were set to 1 VVM, 1000 rpm and 25°C, respectively.

The yeast cells at the end of the fermentation, 65 h, settled out of the medium quickly yielding an SVI of 1.0 ± 0.01 ml $g^{-1}$. The bulk of the fat that was not removed during the fermentation by the yeast remained inside the fermenter and approximately 5% of the tallow loading was detected in the biomass, which had settled out of the medium (Table 3.3.13). The waste liquor, or the cell free medium after the yeast cells had settled, contained 2.1% lipid at the end of the fermentation.

The growth of *Y. lipolytica* RP2 on tallow under optimised conditions yielded a high degree of biomass, with a cell dry weight of approximately 14 g $L^{-1}$ after 65 h. The biomass was assessed as to its possible use for single cell protein (SCP).

The total nitrogen and protein content of the cells was determined (Table 3.3.14). In the early stages of fermentation, at 12 h, cells comprised a higher lipid than protein level, approximately 0.5 g $g^{-1}$ and 0.2 g $g^{-1}$, respectively. Nitrogen content in the cells comprised 0.37 g $g^{-1}$. After 12 h, intracellular lipid decreased. In contrast, the level of protein and nitrogen increased. The high protein content at the end of the fermentation, 0.42 g $g^{-1}$ after 65 h, indicated the possible use of the yeast for SCP production. The overall yield of protein per tallow consumed at the end of the fermentation was 0.44 g $g^{-1}$.

**Table 3.3.13:** Location of fat not removed by *Y. lipolytica* RP2 after 65 growth on tallow (20 g $L^{-1}$) in 10 L fermenter. Temperature, agitation and aeration were set at 25°C, 1000 rpm and 1 VVM, respectively. pH was controlled automatically with 0.5 M KOH/HCl.

| Location of fat | Percentage of initial tallow loading |
|---|---|
| Trapped in settled biomass | 5.2 ± 0.8 |
| Remained inside the fermenter | 24.7 ± 1.5 |
| Waste liquor | 2.1 ± 0.7 |

**Table 3.3.14:** Total nitrogen and protein composition of *Y. lipolytica* RP2 cells grown on tallow (20 g $L^{-1}$) in 10 L fermenter for 65 h. Temperature, agitation and aeration were set at 25°C, 1000 rpm and 1 VVM, respectively. pH was controlled automatically with 0.5 M KOH/HCl.

| Time (h) | 0 | 12 | 24 | 38 | 48 | 65 |
|---|---|---|---|---|---|---|
| Total Nitrogen [g (g dry weight)$^{-1}$] | 0.43 ± 0.005 | 0.37 ± 0.008 | 0.55 ± 0.010 | 0.53 ± 0.005 | 0.55 ± 0.006 | 0.51 ± 0.005 |
| Total protein [g (g dry weight)$^{-1}$] | 0.14 ± 0.008 | 0.20 ± 0.009 | 0.45± 0.007 | 0.42 ± 0.002 | 0.43 ± 0.003 | 0.42 ± 0.006 |
| Total lipid [g (g dry weight)$^{-1}$] | 0.11 ± 0.050 | 0.50 ± 0.021 | 0.28 ± 0.040 | 0.15 ± 0.052 | 0.10 ± 0.011 | 0.10 ± 0.013 |

### 3.3.3 Summary of the main findings from 2 L and 10 L fermentation studies on the growth of *Y. lipolytica* RP2 on tallow (20 g $L^{-1}$)

| | |
|---|---|
| • 2 L fermenter | 75% fat removed after 65 h with pH control by 0.1 M potassium phosphate buffer– significant reduction from 168 h required for similar removal in shake flask studies.<br><br>500 rpm and 1 VVM were selected as the rates of agitation and aeration, respectively.<br><br>Increased biosurfactant activity and reduced dimorphism compared to shake flask fermentations under optimised conditions.<br><br>$K^+$ was key for maximum fat removal and growth.<br>~80 nmol $K^+$ $(10^6$ cell$)^{-1}$ correlated with maximum biodegradation.<br><br>The presence of $Na^+$ and increased $Ca^{2+}$ in the medium reduced fat removal. Addition of $H_2PO_4^-$ at a greater concentration than present in the minimal medium had no effect on fat removal. |
| • 10 L fermenter | 68% fat removed after 65 h on scale-up from 2 L fermenter, with pH control by 0.1 M potassium phosphate buffer or 0.5 M KOH/HCl.<br><br>Agitation set at 1000 rpm with one impeller placed at the base of the agitator and the second positioned 135 mm above it.<br><br>No loss in growth or biosurfactant activity during scale-up from 2 L to 10 L fermentation.<br><br>Biosurfactant concentration correlated to a $CMD^{-1}$ of 2.0.<br><br>The biomass generated after 65 h with pH control by KOH/HCl comprised 0.4 g $g^{-1}$ protein, which indicated the potential of *Y. lipolytica* RP2 in SCP production. |

# 4.0 DISCUSSION

Isolation of fat-degrading organisms from the wastewater treatment system of a rendering factory provided a novel source for organisms in this study. The production of tallow and bonemeal in the factory resulted in wastes with high levels of tallow, which in turn could promote the growth of organisms with the particular ability to degrade tallow. Activated sludge and wastewater from the rendering operation were chosen as potential sources of organisms. Tallow itself was also assessed as a potential source of lipid-degraders.

In the isolation of microorganisms from various environments, the use of enrichment culturing is generally applied. In the laboratory, tallow was used as the sole carbon source in the enrichment culture at a concentration of 20 g $L^{-1}$ (2% w/v), to promote the growth and survival of fat-degrading microorganisms only. This concentration was chosen to provide a sufficiently high concentration of tallow for the enrichment and was also employed in all of the growth studies. Interestingly, the use of tallow for isolation and enrichment has not previously been reported. However, a number of concentrations of various oils, including olive, diesel and crude oil have been reported for isolation purposes, ranging from 5 to 20 g $L^{-1}$ (Okuda *et al.*, 1996; Margesin and Schinner, 1997; Zinjarde *et al.*, 1997; Zinjarde and Pant, 2002b).

The minimal medium chosen was based on that described by Shikoku-Chem (1994), where it was previously used for the growth of lipolytic organisms isolated from a food-factory waste treatment plant. In this study, olive oil and nutrient agars were selected as the isolation media for lipolytic organisms. As a result of the enrichment process in the laboratory, a number of both filamentous and non-filamentous organisms were isolated. Activated sludge, wastewater and the tallow itself provided a source of non-filamentous organisms. Of these non-filamentous organisms, 10 of the isolates were yeast and these were selected as part of this investigation, which began with their identification. The filamentous organisms formed part of a separate investigation (Fleming, 2002).

Lipid-containing waste streams and sludges, including those from industrial and food processing industries and domestic systems, are well documented as potential sources of lipolytic microorganisms. Okuda *et al.* (1991) isolated several

lipid-degrading *Bacillus* sp. from wastewater systems of meat processing plants. Shikoku-Chem (1994) isolated a *Pseudomonas* and an *Acinetobacter* species from activated sludge sourced from a food factory. Microbial consortia were isolated from restaurant grease traps and from activated sludge in a domestic wastewater treatment system, which were then applied to the biological treatment of grease and food waste (Wakelin and Forster, 1998; Tano-Debrah *et al.*, 1999; Fong and Tan, 2000). Food derived industrial wastes have provided the source of many lipid degrading yeasts, including *Yarrowia lipolytica* (Chigusa *et al.*, 1996; Deak *et al.*, 2000; Lee *et al.*, 2001). Natural environments, such as water and soil, which were contaminated with waste lipids, have also served as a source of lipid-degrading organisms. Ota and Kushida (1988) recovered a number of fungi and yeast from soil that demonstrated lipolytic ability. A strain of *Yarrowia lipolytica* was isolated from both oil-polluted soil (Margesin and Schinner, 1997) and oil-polluted seawater (Ahrean *et al.*, 1971; Heslot, 1990; Zinjarde *et al.*, 1998; Zinjarde and Pant, 2002a & b). The majority of studies on tallow biodegradation, however, have employed lipolytic microorganisms sourced from culture collections (Kajs and Vanderzant, 1980; Bednarski *et al.*, 1993 & 1994; Tan and Gill, 1984, 1985 & 1987; Koritala *et al.*, 1987; Papanikolaou *et al.*, 2001 & 2002a).

The first stage in the identification of the isolates began with an examination of the gross morphology of yeast cultures on solid medium. Observations on colony surface, colour and texture aid in the identification of the organism and so reduce the number of tests required (Barnett *et al.*, 1983). The isolated yeasts in this study all produced cream-coloured, non-mucoid colonies that lacked ballistospores (Table 3.1.1). Other differentiating characteristics of the colonies included form, elevation and margin. Based on colony morphology, two of the isolates, NF 9 and NF 32 B were distinct from the other yeast cultures.

Although examination of the isolate gross morphology in liquid culture is of diagnostic value, examination of cellular morphology is a more powerful tool and provides information on the shape and mode of reproduction of the isolate. As a rule, the cellular morphology and mode of reproduction of yeast strains are examined in liquid culture (van der Walt and Yarrow, 1984) and for this purpose,

malt extract broth was employed. In many cases, the cell shape may be characteristic of a particular genera or species, for example: triangular cells of *Trigonopsis* or oval cells of *Malassezia* (van der Walt and Yarrow, 1984; Zinjarde and Pant, 2002b). The isolated yeasts in this study all possessed an oval cell shape with the exception of NF 9, which was ellipsoidal (Table 3.1.3). *Malassezia* may be stimulated in the presence of lipid, such as olive oil, however growth is very weak at 25°C which effectively dismissed this species as one of the isolates in this study (Yarrow and Ahearn, 1984).

The mode of yeast reproduction, as either vegetative or sexual, is an important characteristic in its identification. Vegetative reproduction is achieved by either budding or by the formation of cross walls in mycelium or single cells (Kreger-van Rij, 1987). Budding may be referred to in terms of the position of the budding sites and the position, as monopolar, bipolar or multipolar (multilateral) and this characteristic may be useful in differentiating different species of yeasts (Kreger-van Rij, 1984). Multilateral budding was noted for all of the isolates (Table 3.1.3). Similarly, the formation of pseudomycelium and true mycelium aids species identification. Pseudomycelium formation was noted in the majority of the isolates with the exception of NF 9 and NF 48. However, it is worth noting that the morphological features of yeast can vary when cultured on different media (Kreger-van Rij, 1987).

Ascospore formation and shape is an important taxonomic criterion and is the main feature of sexual reproduction in ascosporogenous yeasts. However, a clear view of the ascospore structure cannot always be detected by light microscopy and may require scanning electron microscopy (SEM) or transmission electron microscopy (TEM) (Kreger-van Rij, 1987). Typical features include round, oval, reniform, cylindrical, wartiness and needle-shaped spores. Ascospores were produced by the isolates, with the exception of NF 32 B, but their structure was not determined (Table 3.1.3).

Physiological properties primarily serve to describe, differentiate and identify yeast strains. They also serve to describe, characterise and differentiate species

and to a lesser extent, genera (van der Walt and Yarrow, 1984). The characteristics which have proved most useful for routine identification purposes are those associated with the utilisation of carbon and nitrogen sources, growth at elevated temperatures, growth on media of high sugar and salt content, formation of typical metabolites and the susceptibility of the yeast to antibiotics (cycloheximide) (Barnett *et al.*, 1983; van der Walt and Yarrow, 1984). Consequently, these physiological tests were chosen in this study to identify the isolates (Table 3.1.4 – 3.1.6).

In the physiological tests, NF 9 and NF 32 B were further characterised as distinct from the other isolates as both organisms fermented D-glucose and had a negative urease test. The two organisms also differed in their ability to grow in 10% NaCl. Fermentation of sugars was not achieved by the remaining isolates but all eight had a positive urease test. A positive urease test is an indication of membership of the basidiomycetes (van der Walt and Yarrow, 1984). However, ascospore formation was detected in these isolates, which implied that they were ascomycetous in nature. The urease test follows a general rule of thumb only and a number of ascomycetes are an exception to this, including *Yarrowia lipolytica* and species of the genus *Lipomyces* (van der Walt and Yarrow, 1984). The negative reaction to the Diazonium Blue B test for all the isolates indicated that none were members of the basidiomycetes. All of the isolates demonstrated good growth in 1000 ppm cycloheximide, which proved to be a useful differentiating characteristic in the complete identification of the isolates.

A key was employed, which was devised by Barnett *et al.* (1983) to identify the isolates. This key permitted the identification of yeast based predominantly on its physiological characteristics and did not necessitate a definitive examination of cell morphology and mode of reproduction. The primary key characteristics were the fermentation of D-glucose and the assimilation of nitrate and erythritol (Appendix A & B). The identification methods of Barnett *et al.* (1983) and Kreger-van Rij (1984) have been routinely employed (Ota and Kushida, 1988; Chigusa *et al.*, 1996; Zinjarde and Pant, 2002b). Other identification methods have included karyotyping (Deak *et al.*, 2000), the Biolog identification system

(Lee *et al.*, 2001) and the employment of a yeast identification agency (Ashy and Abou-Zeid, 1982).

Application of the keys resulted in the identification of three distinct yeasts among the ten isolates. The isolates, NF 9 and NF 32 B, were identified as *Debaromyces hansenii* (Fig. 3.1.11, Table 3.1.11) and *Candida zeylanoides* (Fig. 3.1.12, Table 3.1.12), respectively. The remaining isolates were all identified as *Yarrowia lipolytica* (Fig.s 3.1.3 – 3.1.10, Tables 3.1.7 – 3.1.10). These three organisms have also been isolated together from a common dairy source (Peterson *et al.*, 2001).

The characteristic in the key, which leads to identification, does not give a complete description of the species. It should be noted that the correctness of identification should be checked by comparing the features of the strain with those of the complete standard description of a species. This is necessary (i) because the strain may belong to a new, undescribed species and (ii) because an error in observation of one of the tests of one of the key characteristics may lead to an incorrect name (Kreger-van Rij, 1987). In this study, the identification of isolate NF 32 A as *Yarrowia lipolytica* was verified by Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH, Germany (Appendix C).

Screening of the isolates in pure culture on tallow as the sole carbon source permitted the selection of one of the isolated yeasts with superior degradative ability, for further study. Investigations were undertaken in pure culture, as opposed to mixed culture, to allow for a more accurate understanding of the characteristics of the growth of the isolate on tallow and the effect of environmental conditions on tallow degradation and removal. In mixed cultures, interactions between species and potential shifts in the population distribution of species could introduce variability in the systems. The minimal medium employed in the enrichment culturing was also used in the screening process and in all growth studies. Degradative ability of the isolates was assessed after growth for 168 h on 20 g $L^{-1}$ tallow at room temperature (22 - 25°C) with and without agitation at 130 rpm. Tallow, which comprised less than 7% free fatty acids, was employed in all growth studies in this investigation. This ensured that batch to

batch variation of the tallow was kept to a minimum and that triglycerides comprised greater than 90% of the substrate. The concentration of tallow used in this study, 20 g $L^{-1}$, was within the range of levels previously reported. Concentrations of 1 - 8 g $L^{-1}$ of tallow and grease (Tan and Gill, 1985 & 1987; Wakelin and Forster, 1997), 10 g $L^{-1}$ of both tallow (Kajs and Vanderzant, 1980) and animal derived fat (Papanikolaou *et al.*, 2002a) and 30 g $L^{-1}$ of various tallows (Bednarski *et al.* 1993 & 1994; Marek and Bednarski, 1996) have been employed. In the absence of agitation, the fermentative capacity was reduced, for all of the isolates. Agitation of the medium has been reported as an essential parameter in the effective degradation of lipids and hydrocarbons (Tan and Gill, 1984; Zinjarde and Pant, 2002b).

The isolates identified as *Yarrowia lipolytica* demonstrated superior fat removal ability and removed 21% of the tallow after 168 h (Fig. 3.1.13). The lipid-degrading ability of various *Yarrowia lipolytica* strains has been well documented, especially on oils and greases with some investigations specific to its degradation of animal fats (Kajs and Vanderzant, 1981; Tan and Gill, 1985; Bednarski *et al.*, 1994; Papanikolaou *et al.*, 2001 & 2002a). A lower degree of fat removal was achieved by *Debaromyces hansenii* and *Candida zeylanoides*, 16% and 17%, respectively. Although reports on the ability of these two organisms to degrade lipid have not been specifically documented, a number of unidentified *Candida* species isolated from waste streams have demonstrated lipolytic ability (Ota and Kushida, 1988; Chen *et al.*, 1990). However, *Candida zeylanoides* has been reported as a potential pathogen (Levenson *et al.*, 1991; Liao *et al.*, 1993; Whitby *et al.*, 1996). This, coupled with its lower degradative ability and that of *Debaromyces hansenii* ruled out the suitability of these organisms for further investigation.

*Yarrowia lipolytica* is a non-pathogenic yeast and has a recognised GRAS (generally regarded as safe) status, which made it the preferred candidate for further investigation (Barth and Gaillardin, 1997; Casaregola *et al.*, 2000; Pérez-Campo and Domínguez, 2001; Spencer *et al.*, 2002). The yeast was designated as *Yarrowia lipolytica* RP2, where RP referred to its isolation from a rendering plant and 2 indicated that it was the second isolate to be extensively studied in the

laboratory. The first organism studies in the laboratory for its ability to degrade tallow was a filamentous fungus, *Trichoderma harzianum* Rifai RP1 (Fleming, 2002).

The characteristics of the growth of *Yarrowia lipolytica* RP2 on tallow in minimal medium were investigated. Growth studies were scaled-up from shake flask to 2 L and 10 L fermenters. The key growth parameters assessed included inoculum preparation, growth temperature, surfactant addition and biosurfactant production, medium pH, nutrition, agitation and aeration. The effect of these parameters on the yeast cell, including its morphology and lipid composition and ultimately on tallow removal was also investigated.

An inoculum size of 2% (v/v) was employed in all growth studies. Inoculum sizes for growth on lipids vary, ranging from 1% (v/v) (Ashy and Abou-Zeid, 1982; Okuda *et al.*, 1991) to 4 - 5% (v/v) (Wakelin and Forster, 1997; Chen *et al.*, 1999; Rau *et al.*, 2001) and up to 10 – 12 % (v/v) (Tan and Gill, 1984 & 1985; Desphande and Daniels, 1995; Oswal *et al.*, 2002). In general, the majority of studies use a 2% (v/v) inoculum, which was superior for the growth of *Candida lipolytica* on fish oil (Hottinger *et al.*, 1974b). The employment of nutrient broth and olive oil broth as inoculum media was compared. It was hoped that preparation of the inoculum on olive oil, a lipid carbon source, would be advantageous to the cultivation of RP2 on tallow through the stimulation of properties, such as enzymes and emulsifiers, commonly associated with growth on lipid. Olive oil grown inocula for lipid biodegradation has been reported (Tan and Gill, 1985 & 1987; Novotný *et al.*, 1988; DeFelice *et al.*, 1997). However, inocula from both nutrient and olive oil broths achieved identical growth and fat removal (Table 3.2.10). Consequently, nutrient broth was selected as the preferred method of inoculum preparation as it provided not only an effective medium for yeast growth, but also facilitated an easier extraction of the yeast cells.

Cultivation of the inoculum on tallow itself was not performed in this study based on two considerations. Firstly, the stimulation of any extracellular properties associated with growth on tallow would be lost, as the inoculum comprises the addition of the yeast cells only to the growth medium. Addition of the medium

would add additional substrate to the growth flask, which would then have to be accounted for in the determination of fat removal by RP2. Secondly, the absence of a long lag phase coupled with the small number of yeast cells added as the inoculum meant that any activity stimulated in the inoculum cells would be soon surpassed through growth on tallow in the growth vessel itself. The short lag phase for the initiation of growth of *Saccharomycopsis lipolytica* on olive oil with a small inocula of washed, glucose grown cells indicated that substantial changes in cell metabolism was not necessary for effective utilisation of fats by the yeast (Tan and Gill, 1984).

Tallow removal and growth of RP2 were superior at 25°C, with a decrease in biodegradation with increased temperature to 30°C and 37°C and at 4°C (Fig.s 3.2.3 & 3.2.4). RP2 was unable to survive at 55°C and consequently no tallow was removed. However, cell viability was maintained at 4°C, 30°C and 37°C, although growth was reduced at these temperatures. The ability of RP2 to survive outside its ideal growth temperature on tallow is an important characteristic in the development of a fat removal system, where fluctuations in temperature can result from extraneous environmental factors. The ability to withstand periods of non-optimal growth conditions is of importance in the selection of a microbial culture (Wakelin and Forster, 1997).

The influence of temperature on microbial growth on lipid can be considered on a number of levels. Temperature may be optimised with respect to cell growth. In general, *Y. lipolytica* has been grown on lipids in the mesophilic range at 15°-20°C (Margesin and Schinner, 1997), 25°C (Kajs and Vanderzant, 1980; Marek and Bednarski, 1997), 28°C (Papanikolaou *et al.*, 2001, 2002a & b) with the majority of studies performed at 30°C (Tan and Gill, 1985; Chigusa *et al.*, 1996; DeFelice *et al.*, 1997; Scioli and Vollaro, 1997; Kim *et al.*, 1999; Zinjarde and Pant, 2002b).

Optimisation of temperature can also be considered in terms of the substrate itself. The solid nature of the tallow can be overcome through its liquefaction at elevated temperatures. Beef tallow has a melting point of 42°C (Hur and Kim, 1999;

Mason, 2003). In this study, liquefaction of tallow occurred at 55°C, but it also resulted in cell death. The application of elevated temperatures for lipid biodegradation has proved to be both detrimental and beneficial. Removal rates of sheep tallow were lower at 50°C compared to 35°C in an anaerobic system using mesophilic sludge (Broughton *et al.*, 1998). In contrast, effective removal of wool grease was achieved through its melting (Becker *et al.*, 1999) and successful lipid removal with a microbial consortium at 60°C was reported (Nakona and Matsumura, 2001). However, in both these cases, thermophilic organisms were employed. Although the elevation of the temperature would liquefy the fat, the use of a thermophilic organism for the biodegradation of tallow would incur certain disadvantages. Primarily, this would include the required heating of the biodegradation vessel together with subsequent downstream cooling and overall higher capital costs. For this reason, mesophilic temperatures have generally been used for studies on the assimilation of lipid substrates.

Lastly, temperature can also be optimised with respect to enzyme activity, specifically lipase, which would result in optimal hydrolysation of the lipid for microbial uptake. Temperature optima of lipases are as diverse as the sources from which they are obtained with those from fungal sources having activity peaks ranging from 20°C to 50°C (White and White, 1997). Activity peaks for lipases from *Y. lipolytica* are generally at 37°C (Ota *et al.*, 1982; Destain *et al.*, 1997; Corzo and Revah, 1999). Interestingly, in these studies the temperature employed for lipase production is lower, at 29°C to 30°C. This would suggest that the temperature of optimum production does not necessarily correlate with optimum activity. Indeed, Hadeball (1991) states that in general, the optimal temperature for lipase production normally agrees with the optimal conditions for growth. In the case of RP2, this temperature was 25°C and all growth studies were performed at this temperature.

The main restriction to the growth of organisms on the hard fats, such as tallow, is their solid nature in the mesophilic range of growth. As growth at elevated temperatures was not feasible for RP2, increasing the bioavailability of the tallow through the addition of surfactants was investigated. Surfactants have been shown

to be beneficial in various technological applications, especially in terms of biodegradation of hydrocarbon liquids or solids, fats, oils and various organic compounds (West and Harwell, 1992; Finnerty, 1994; Rouse *et al.*, 1995; Banat, 1995; Van der Meeren and Verstraete, 1996). Biodegradation of organic compounds with limited water solubility, such as the hard fats, is slow due to the low availability of these compounds to microbial cells (Zhang and Miller, 1994). The surfactant may improve the availability of the substrate to the microbial cells through emulsification thereby reducing the surface tension at the phase boundary.

Cationic and anionic commercial surfactants can result in damaging effects on cell membranes and for this reason are often disregarded for use in biological systems (Shabati and Gutrick, 1985). However, nonionic surfactants, such as Triton X-100 and Tween 80 are attractive due to their low toxicity and relative insensitivity to pH and electrolyte concentration. Inclusion of Tween 80, at 0.05% (w/v), improved the growth of *Candida intermedia* on the alkane hexadecane (Wang and Ochoa, 1972). Tween 80, included at 0.01% (w/v), was used to increase olive oil dispersal during an investigation into lipid degradation by activated sludge (Hsu *et al.*, 1983). Similarly, Tween 80 (1.0% w/v) in the growth medium of *Y. lipolytica* on animal derived fat was used to increase fat dispersion (Papanikolaou *et al.*, 2002a). In this study, Triton X-100 and Tween 80 were included in the growth medium at various concentrations (0 – 0.5% w/v) and their subsequent effect on fat dispersion and growth investigated.

The surfactants did increase the surface area of the tallow through increased dispersal (Fig. 3.2.7). However, fat removal was dramatically decreased in the presence of the surfactants and they exerted a toxic effect on the yeast (Fig.s 3.2.5 & 3.2.6). This was most evident with Tween 80, at all concentrations, where less than 40% of the yeast cells were viable with only 2 - 3% of the tallow removed. The presence of Triton X-100 also reduced yeast cell viability, albeit with a marginally less toxic effect. Nevertheless, Triton X-100 resulted in decreased biodegradation with only 3 to 10% of the fat removed. Similarly, a number of surfactants were toxic to test bacteria and prevented the biodegradation of hydrocarbons (Roch and Alexander, 1995).

The prevention of tallow removal by the surfactants may be attributed to three factors: (i) their presence was detrimental to cell physiology, which in turn led to cell death, (ii) the surfactants impeded lipid assimilation by interfering with substrate-cell interactions and (iii) the surfactants were incompatible with any surfactant material produced by the yeast itself. In the first case, studies have shown that the presence of non-ionic surfactants in the culture medium can alter the physiological properties of yeast (Vasileva-Tonkova *et al.*, 2001). *Y. lipolytica* cells were permeabilised by 0.1% Triton X-100, which led to the release of 20% protein from the cells and morphological changes including altered cell shape and folded walls (Galabova *et al.*, 1996). Ultrastructural changes in the cell and membrane occurred, weakening the cohesion between proteins and lipids and thereby resulted in cell lysis and an overall decrease in culture viability.

Contact between the cell and the substrate is a prerequisite for lipid uptake. The cell surface aids in the hydrophobic interaction between the cell and the lipid, thereby facilitating its transport into the cell (Käppeli and Fiechter, 1977; Mineki *et al.*, 1984). Reduction in fat removal with surfactants has been attributed to the removal of this attraction between the cell and the substrate. For example, growth of *Candida lipolytica* on fish oil was inhibited by Triton X-100, at a concentration of 0.1% (w/v) (Hottinger *et al.*, 1974b). A reduction in attraction between oil droplets and the microbial cell occurred in the presence of the surfactant. Similarly, efforts to increase the specific surface area of an oil phase by surfactant addition had the undesirable result of inhibiting oil degradation (Hori *et al.*, 2002).

The presence of the surfactants may result in an incompatibility with the natural cell-associated and extracellular emulsifying properties of *Y. lipolytica*. Biosurfactant production by *Y. lipolytica* has been reported (Roy *et al.*, 1979; Zinjarde *et al.*, 1997; Kim *et al.*, 2000d). This incompatibility between chemical surfactants and biosurfactants leads to reduced lipid biodegradation (Zhang and Miller, 1994; Kim *et al.*, 2000d). Ultimately, it is the ionic compatibility of additional surfactants with extracellular components rather than substrate accessibility that is of greater concern (Rouse *et al.*, 1995). Consequently, surfactants were not considered in a fat removal system with *Y. lipolytica* RP2.

During the growth of RP2 on tallow (without pH control), in the absence of chemical surfactant addition, the tallow was dispersed from a solid substrate into discrete balls of fat in shake flasks (Fig. 3.2.2). This suggested the possible production of a biosurfactant by the yeast. Biosurfactant production in *Y. lipolytica* may be induced by the addition of long chain fatty acids, hydrocarbons or glycerides (Pareilleux, 1979; Cirigliano and Carman, 1984 & 1985; Kim *et al.*, 2000d).

The characteristics of a biosurfactant can be difficult to assess (Parkinson, 1985). Many present the properties of the specified preparation, which can be the whole broth, separated broth or an extract, in terms of surface tension and emulsifying properties (Cirigliano and Carman, 1984; Mulligan *et al.*, 1984; Muriel *et al.*, 1996; Kim *et al.*, 2000c). Emulsification is assessed generally by both its activity and its stabilising properties. The activity indicates the ability of the surfactant to form an emulsion through spectrophotometric measurement, where one liquid phase is dispersed into micro-droplets in another liquid phase (Deasi and Banat, 1997). The ability of the surfactant to stabilise this emulsion is determined through the decay ratio, which is assessed by measuring the breakdown time, under gravity, of a suspended hydrocarbon in aqueous surfactant. The most common hydrocarbons employed include kerosene and hexadecane (Cirigliano and Carman, 1984; Muriel *et al.*, 1996; Kim *et al.*, 2000c). In this study, separated broth was used to determine the three aspects of biosurfactant activity, with the use of kerosene to determine both the emulsification activity and emulsion stability.

In shake flask studies, when RP2 was grown on tallow in minimal medium at ambient temperature, surface tension decreased from 70 to 62 dynes $cm^{-1}$ (Table 3.2.2). This reduction was small. However, biosurfactant production by *Y. lipolytica* has more notably been considered in terms of emulsification activity rather than its surface tension reducing properties (Roy *et al.*, 1979; Cirigliano and Carman, 1985; Zinjarde *et al.*, 1997; Kim *et al.*, 2000d; Waché *et al.*, 2000). Emulsification activity of the medium reached a maximum of 0.7 absorbance units which was almost four times that achieved by *Cladosporium resinae* grown on various hydrocarbons (Muriel *et al.*, 1996). Emulsion stability, however was

not as effective compared to the fungus, which had a $K_d$ of –3.68 compared to –5.1 for RP2, under non-optimised growth conditions. This was not surprising, as the two parameters are not mutually dependent (Cirigliano and Carman, 1985; Kim *et al.*, 2000c). Emulsification activity and stability in the medium increased with growth of RP2 to a maximum level at 120 h and then decreased after 168 h, corresponding to the beginning of stationary growth. This is similar to the growth pattern of *Endomycopsis lipolytica* on hydrocarbon, where activity increased during the active growth phase and then decreased with the decline in growth (Roy *et al.*, 1979). Overall, the reduction in surface tension and detection of emulsification activity by *Y. lipolytica* RP2 in the presence of tallow and the subsequent substrate-uptake was taken as indirect proof of biosurfactant production by the yeast.

Environmental factors and growth conditions such as temperature, pH, agitation and oxygen availability affect biosurfactant production through their affects on cellular growth and activity (Desai and Banat, 1997). In this study, pH, agitation and aeration and nutrition were found to influence biosurfactant activity.

Under the optimised conditions of pH and temperature, 25°C and pH 7.0, tallow dispersion was greatly enhanced with an observed emulsification of the tallow compared to the observed 'balls' of fat where pH was not controlled (Fig. 3.2.13). Similarly, a two-phase mixed-liquor occurred during grease degradation with activated sludge when the pH was acidic, resulting in the formation of small balls of grease (Wakelin and Forster, 1998). At or near neutral pH, a single-phase mixed-liquor was observed, with complete dispersion of the lipid. This was considered of importance in the operation of the system.

The increase in tallow dispersion with pH control was reflected in an increase of biosurfactant activity compared to the absence of pH control. Surface tension was further reduced from 62 to 56 dynes $cm^{-1}$ (Table 3.2.7). The pattern of activity and emulsion stabilisation was identical to that observed with no pH control. However, emulsification activity increased to a maximum of 1.6 absorbance units by 120 h, which was more than double that achieved with no pH control.

Similarly, emulsion stability was also increased with pH control. This degree of activity was greater than that detected in this study with the commercial surfactants Triton X-100 and Tween 80 ($A_{540}$ of 1.22 and 1.06, respectively) at a concentration of 0.5% (w/v) (Table 3.2.3). An accurate comparison with activity values for other systems cannot be made as methods differ in both its determination and hydrocarbons used. However, an activity of 0.21 absorbance units was achieved by *Enodmycopsis lipolytica* against *n*-dodecane (Roy *et al.*, 1979), 0.88 absorbance units against hexadecane by *Candida lipolytica* (Cirigliano and Carman, 1984) and 2.51 units against hexadecane by a *Norcardia* sp. (Kim *et al.*, 2000c). In terms of emulsion stabilising properties, a decay ratio, $K_d$, of -3.8 achieved by RP2 with pH control was now comparative to that produced by the fungus *Cladosporium resinae,* with a $K_d$ of -3.7 (Muriel *et al.*, 1996). In their investigation, Muriel *et al.* suggested that the properties of the biosurfactant produced by the fungus were comparative with commercial surfactants.

The increase in biosurfactant activity by RP2 with pH control was attributed to two factors. Firstly, many biosurfactants are pH sensitive. Cultivation of sphorolipid by *Candida bambicola* grown on animal fat demonstrated increased productivity under pH controlled conditions compared to no pH control (Desphande and Daniels, 1995). Similarly, pH sensitivity was noted for *Pseudomonas aeruginosa* (Guerra-Santos *et al.*, 1986; Zhang and Miller, 1992). Secondly, increased activity is related to the increase in cell growth. For growth associated biosurfactant production, parallel relationships exist between growth, substrate utilisation and biosurfactant production (Desai and Banat, 1997). Emulsification is a cell density-dependent phenomenon, that is, the greater the number of cells, the higher the concentration of extracellular product (Ron and Rosenberg, 2001).

Interestingly, surface tension values between 65 and 70 dynes $cm^{-1}$ were detected in the control (no cells) with pH control (Table 3.2.2 & 3.2.7). However, this reduction in tension was not attributed to any surfactant activity as neither emulsification activity or stabilising properties were detected. No reference to reduction in surface tension of a control medium was found in the literature. This

small reduction in tension was therefore not considered of great consequence given the large reduction in tension in the growth vessel.

Increased agitation and aeration under fermenter growth (2 L and 10 L) resulted in a further increase in biosurfactant activity. This was reflected in a decrease in surface tension to a minimum tension of 42 dynes $cm^{-1}$, compared to 56 dynes $cm^{-1}$ in shake flasks (Table 3.3.2, 3.3.4 & 3.3.8). This was comparable to the surface tension of the biosurfactant produced by *Candida lipolytica* when grown on waste olive oil (Haba *et al.*, 2000) and tetradecane (Pareilleux, 1979) and a *Pseudomonas* sp. grown on waste oil (Cooper *et al.*, 1981). In general, a good biosurfactant producer is one that reduces the surface tension to 40 dynes $cm^{-1}$ (Haba *et al.*, 2000). Higher emulsification activity and emulsion stability values with a maximum absorbance and $K_d$ ranges between 2.05 to 2.14 units and –2.27 to –2.22 by 24 h, respectively were detected in the fermenters (Table 3.3.2, 3.3.4 & 3.3.9). These values corresponded with exponential growth of the yeast and observed emulsification of the tallow in the 2 L and 10 L fermenters. In yeast, biosurfactant production has shown to be increased when agitation and aeration rates are increased (Spencer *et al.*, 1979). Moreover, Sheppard and Cooper (1990) concluded that oxygen transfer is one of the key promoters for the process optimisation and scale-up of surfactin production in *Bacillus subtilis*. Consequently, the increased activity by the biosurfactant produced by RP2 when cultivated in the fermenter may be attributed to the aeration of the growth vessel.

Production and yields of biosurfactants vary greatly depending on the nutritional environment of the growing microorganism (Rosenberg, 1986). No reduction in surface tension or presence of emulsification activity was noted when *Y. lipolytica* RP2 was grown on glucose as the sole carbon source. This was in agreement with previous investigations (Pareilleux, 1979; Cirigliano and Carman, 1984). Activity of the biosurfactant produced by RP2 grown on tallow was not influenced by the presence of glucose. This is contrary to a previous report where biosurfactant production by *Candida lipolytica* was repressed upon the addition of glucose (Cirigliano and Carman, 1984). However, glucose has been included in the growth medium of *Candida bambicola* with lipid for biosurfactant production, with no

detrimental effects (Desphande and Daniels, 1995; Casas and Garcíca-Ochoa, 1999).

The source or concentration of nitrogen did not affect the activity of the biosurfactant produced by *Y. lipolytica* RP2. This is inconsistent with other reports on the influence of nitrogen on biosurfactant production. Nitrogen source and C:N ratio influenced rhamnolipid production by *Pseudomonas* 47T2 (Haba *et al.*, 2000). Increased productivity of the *Pseudomonas* biosurfactant occurred with increased nitrogen and a low C:N ratio (8:1) yielded highest production of the biosurfactant. The source of nitrogen had a different effect on different organisms. Ammonium salts and urea were the preferred nitrogen sources for production by *Arthrobacter parafineus* (Duvnjak *et al.*, 1983), whereas nitrate supported maximum surfactant production in *Pseudomonas aeruginosa* (Guerra-Santos *et al.*, 1984). However, the common regulatory principles of biosurfactant synthesis are different for each organism and respond to different kinds of nutrient limitation (Syldatk and Wagner, 1987). It is the concentration of the carbon source that determines the conversion of carbon available to the biosurfactant (Hommel *et al.*, 1987). The presence of 20 g $L^{-1}$ tallow in each flask provided an identical concentration of carbon to the yeast, which would have directly contributed to the identical biosurfactant activity, irrespective of nitrogen addition.

Ionic nutrition, under optimised temperature and pH, was found to influence RP2 biosurfactant activity. The presence of $K^+$, at a concentration of 60 mM resulted in maximum activity (Table 3.3.6). This was achieved through pH control with KOH-$KH_2PO_4$ buffer, KOH and $KH_2PO_4$. $Na^+$ and $Ca^{2+}$ additions to the medium with pH control resulted in decreased activity. Growth of the yeast was also enhanced with $K^+$ and reduced in the presence of $Na^+$ and $Ca^{2+}$. This underlined the relationship between cell growth and biosufactant activity. The presence and concentration of metal ions in the medium can affect the production of biosurfactants (Desai and Banat, 1997). However, the effect of metal ions is related to the organism itself. Iron limitation stimulates biosurfactant production in *Pseudomonas flourescens* (Persson *et al.*, 1990) and *Pseudomonas aeruginosa* (Guerra-Santos *et al.*, 1984 & 1986), whereas addition of iron and manganese salts stimulates biosurfactant production in both *Bacillus subtilis* (Cooper *et al.*,

1981) and *Rhodococcus* sp. (Abu-Ruwaida *et al.*, 1991). Interestingly, the emulsifier produced by *Endomycopsis lipolytica* was characterised as a complex of metal ion(s) with peptide, although the ion(s) involved were not elucidated (Roy *et al.*, 1979).

The critical micelle dilution (CMD) of the growth medium was measured, as its inverse is an indication of the relative concentration of the biosurfactant produced. An appreciable increase in surface tension, above 42 dynes $cm^{-1}$, occurred only when the CMD was more than 0.5 (v/v) (Fig. 3.3.16). The $CMD^{-1}$ and relative concentration of RP2 biosurfactant corresponded to a value of 2.0. A similar biosurfactant concentration was reported for *Rhodococcus rubra* (Khan and Forster, 1988). In contrast, a biosurfactant with a $CMD^{-1}$ in excess of 40 was reported for a *Pseudomonas* sp. (Persson and Molin, 1987) and in excess of 200 for a Gram-negative bacterium (Mulligan *et al.*, 1984). This indicated that RP2 biosurfactant concentration was low and that its potential for production as a by-product of the fermentation would not be economically viable under these conditions. However, under the optimised fermenter conditions for RP2, this concentration of biosurfactant was sufficient to effectively emulsify the tallow and thereby aid in its uptake.

Medium pH was monitored regularly as a small reduction in pH is often an indication of metabolic activity in the yeast. However, a decrease in medium pH occurred, from pH 7.0 to 2.5, in the early stages of the fermentation with RP2 at 25°C (after 75 h) (Fig. 3.2.1). This decline in pH may be attributed to both the metabolism of fatty acids (Tan and Gill, 1984) and the depletion of ammonium sulphate during growth (Zinjarde and Pant, 2002a). Similarly, a decrease in pH during the growth of activated sludge on restaurant grease, which is mainly composed of triglyceride lipids, resulted in a highly acidic effluent from fed-batch cultures (Wakelin and Forster, 1998). Reduction in pH inhibited the growth of yeast on fats (Hottinger *et al.*, 1974a). Cultivation of *Saccharomycopsis lipolytica* and *Candida utilis* on tallow resulted in a significant decrease in medium pH while the use of a pH controlled medium increased the overall yield (Kajs and Vanderzant, 1980).

The production of an acidic environment may be detrimental to the production and activity of lipase by RP2. The cell-bound lipase of *Saccharomycopsis lipolytica* is inhibited at low pH values (Ota *et al.*, 1982). Although a variety of pH values have been employed for growth and lipase production of *Y. lipolytica*, none were reported below pH 4.0. Cultivation of different strains of the yeast has been achieved at low pH values of 4.0 to 5.0 (Novotný *et al.*, 1988; DeFelice *et al.*, 1997; Corzo and Revah, 1999). However, pH 6.0 to 7.0 is the more commonly employed pH range (Ota *et al.*, 1982; Marek and Bednarski, 1996; Destain *et al.*, 1997; Pereira-Meirelles *et al.*, 2000; Papanikolaou *et al.*, 2002a). Although the use of media at pH 5.0 or lower in alkane fermentations can restrict the growth of a contaminant (Litchfield, 1977), such low pH values would seriously reduce growth rates on fats. In this investigation, the effect of pH control on tallow removal by RP2 was investigated. Buffered medium was employed to control the medium pH to minimise the risk of contamination through acid/base addition or pH probes during the fermentation.

The choice of the buffer for a system is dependent on a number of factors including the pKa, which should be close to the required pH, buffer solubility, cost effectiveness and interaction with ions/molecules in the solution. Biological systems can be particularly sensitive to certain buffers. Tris buffers can be cytotoxic, phenolic buffers posses antiseptic properties and imidazole is too sensitive and unstable for a biological buffer (Perrin and Dempsey, 1979). Biologically inactive buffers such as MES and HEPES have the advantage of forming only weak complexes with certain ions. However, these buffers can interfere in biological assays such as the Folin and Lowry protein assays, giving false results (Perrin and Dempsey, 1979) and can also be more expensive than other alternatives.

Phosphate buffers have been traditionally used in biological systems and generally fall within the physiological range of pH 6 to 8 (Perrin and Dempsey, 1979). Although phosphate buffers can be problematic in terms of ion complexation, phosphate provides a source of the required element phosphorous. Phosphate is required in large amounts by yeast cells for nucleic acid and phospholipid production (Walker, 1998). The ability of RP2 to degrade tallow in the presence

of phosphate is of the utmost importance as generally waste streams contain phosphate, which is often present in high concentrations, as a result of food (DeFelice *et al.*, 1997) and animal processing (Borja *et al.*, 1998). The use of various phosphate buffers for pH control or inclusion of phosphates for both nutrient and internal buffering has been widely documented (Moo-Young *et al.*, 1971; Hottinger *et al.*, 1974b; Ashy and Abou-Zeid, 1982; Okuda *et al.*, 1991; Aggelis and Komaitis, 1999; Fléck *et al.*, 2000; Vasileva-Tonkova *et al.*, 2001).

To determine the pH for superior fat removal by RP2, a wide pH range buffer, 0.1 M citrate phosphate, was employed to control the pH from pH 3.0 to 8.0. In agreement with the majority of investigations, pH 7.0 was superior for growth of RP2 (Fig. 3.2.9). pH control to 7.0 greatly increased tallow degradation after 168 h from 21% to 69% in shake flasks (Fig. 3.2.8). Although the pH may not be optimal specifically for lipase or assimilation of lipids, it does represent the value at which the combination of the processes involved worked together most efficiently.

Interestingly, variation from pH 7.0 by ± 1.0 unit resulted in less than 10% reduction in maximum tallow removal and growth, but control below pH 6.0 resulted in a considerable decrease in biodegradation to approximately 23% at pH 3.0. Reduced tallow removal at the more acidic pH values was not related to cell viability, which was maintained at all pH values. However, it may be due to lipase inactivation, as noted previously. Similarly, maximum growth rates for *Saccharomycopsis lipolytica* on olive oil and oleic acid were achieved at pH 7.0, with similar values at pH 8.0 but a decline in growth occurred below pH 6.0 (Tan and Gill, 1984). The stability of RP2 viability over a wide range of pH values coupled with the similarity in degradative ability with small changes in pH is an important characteristic in the development of a fat removal system, where pH fluctuations can occur during operation. Indeed, the pH for superior fat removal by RP2 is comparable to the pH of many industrial and domestic waste streams which, in general, falls between pH 6.5 to 7.5 (Boczar *et al.*, 1992; Chigusa *et al.*, 1996).

In the investigation into pH it was noted that the yeast could utilise the citrate in the buffer as a carbon source. This ruled out the suitability of citrate phosphate buffer for further growth studies as tallow would not be the sole carbon source available to the yeast. The ability of *Y. lipolytica* to assimilate citrate has been noted (Heslot, 1990; Novotný *et al.*, 1994). Biodegradation of tallow was therefore assessed at pH 7.0, in the presence of other phosphate buffers (potassium and sodium) at a concentration of 0.1 M, which do not provide an additional carbon source. Use of potassium phosphate ($KOH\text{-}KH_2PO_4$) buffer resulted in the greatest degree of fat removal, 75%, after 168 h in shake flasks (Table 3.2.5). Interestingly, this was greater than that achieved with citrate phosphate buffer.

Potassium phosphate buffer concentration was varied from 0.1 to 1.0 M and the influence of temperature (between 4° to 37°C) on buffering capacity and on biodegradation was also assessed. The similar degree of fat removed and growth rate with 0.1 and 0.2 M concentrations resulted in the selection of 0.1 M for further growth studies (Table 3.2.6). Temperature profiles with pH control were similar to that noted during growth temperature investigations in absence of pH control with 25°C resulting in superior growth and biodegradation. pH control increased the degree of fat removed at the other mesophilic temperatures, 30°C and 37°C, by the same magnitude (approximately 4) as was noted at 25°C. At 4°C, pH control resulted in the same degree of tallow removal, irrespective of pH control and reduced growth may be attributed to the inability of RP2 to proliferate to any great degree at this temperature.

Various methods of pH control to 7.0 were examined in the 2 L fermenter. These methods included: (i) the inclusion of 0.1M potassium phosphate ($KOH\text{-}KH_2PO_4$) buffer; (ii) the inclusion of either $NaH_2PO_4$ or $KH_2PO_4$ in the medium with an initial adjustment of the pH to 7.0 with 0.5 M NaOH and (iii) the manual addition of 0.5 M NaOH, KOH or $Ca(OH)_2$, where required. The inclusion of 12 g $L^{-1}$ $KH_2PO_4$ in the medium for pH control was reported for the cultivation of *Y. lipolytica*, although 5 M KOH was employed for the pH adjustment, as opposed to 0.5 M NaOH in this study (Papanikolaou *et al.*, 2002b). The three bases assessed

for pH control have been employed for pH control in wastewater treatment and fermentations (Taylor *et al.*, 1994; DeFelice *et al.*, 1997; Kim *et al.*, 2000b; Voss and Steinbüchel, 2001). The concentration employed in this study, 0.5 M, was lower than concentrations reported for pH control during lipid degradation studies, which ranged between 1.0 to 5.0 M (DeFelice *et al.*, 1997; Papanikolaou and Aggelis, 2002; Papanikolaou *et al.*, 2002b). However, 0.5 M was selected to ensure that during its addition to the medium, the cells would not be exposed to damaging large pH oscillations.

pH control with potassium phosphate buffer resulted in maximum fat removal and growth in the 2 L fermenter, which was closely followed by manual KOH control (Table 3.3.5). However, under automated pH control in the 10 L fermenter, both the buffer and KOH resulted in an identical degree of fat removal. In large-scale and industrial systems for treatment of fats, oils and greases, pH control is generally implemented through the addition of a base, rather than the inclusion of a buffer (Keenan and Sabelnikov, 2000; de Villiers and Pretorius, 2001). Control of pH via buffered medium may have a serious financial impact on the development of a fat removal system. Consequently, KOH addition was selected as the preferred method of pH control in the development of a fat removal system.

*Y. lipolytica* RP2 readily assimilated glucose as the sole carbon source, which was reflected in a greater rate of growth and substrate removal with the sugar compared to tallow (Table 3.2.11). Interestingly, when glucose was present as an additional substrate with the tallow, there was a concomitant use of both substrates. Conflicting evidence exists on the pattern of glucose and lipid utilisation when present as mixed substrates. Diauxic growth by *Streptomyces lividans* results where lipid assimilation occurs only after glucose exhaustion (Peacock *et al.*, 2003). In contrast, *Candida bombicola* utilises both glucose and sunflower oil simultaneously (Casas and Garcíca-Ochoa, 1999). Similarly, a mixed culture of *Candida scotii* and a *Candida* sp. effectively assimilates animal fat and sugars from continuous culture (Kostov *et al.*, 1986).

The specific rate of tallow removal by RP2 was not inhibited by the sugar (0.030-0.035 g $g^{-1}$ $h^{-1}$, in all cases) but quite the reverse occurred, with an inhibition of the

rate of glucose removal by the fat. This is contrary to that reported for *Streptomyces lividans* by where the rate of glucose assimilation was identical when present as the sole carbon source or mixed with triolein and the presence of glucose was inhibitory to fatty acid degradation (Peacock *et al.*, 2003). Interestingly, glucose inhibited lipase production by *Candida curvata* (Montet *et al.*, 1985) and by *Candida deformans* (Muderhwa and Ratomahenina, 1985). Similarly, high levels of glucose, at 10 g $L^{-1}$, repressed lipase activity in a strain of *Y. lipolytica* (Marek and Bednarski, 1996). This was attributed to lipase regulation through catabolic repression. For RP2, catabolic repression of lipase was not evident based on the similar degree of tallow removal, even in the presence of 10 g $L^{-1}$ glucose. Instead, glucose assimilation was reduced with unaltered fat removal by RP2. Inhibition of glucose assimilation has been reported. Alkanes inhibited both the transport and the assimilation of glucose by *Candida* 107, which was attributed to the breakdown products formed during alkane assimilation and degradation (Gill and Ratledge, 1973).

Fatty acids, when assimilated by yeast are utilised for lipid synthesis or are degraded completely via the β-oxidation pathway. The absence of lipid accumulation in *Y. lipolytica* RP2 at the end of the fermentation indicated that assimilated lipids underwent β-oxidation (Fig. 3.2.16). During oxidation, acetyl CoA is produced and its build-up can inhibit glucose transport in the yeast. This may be attributed to acetyl CoA exerting allosteric modification to a glucose carrier (Gill and Ratledge, 1973). The build-up of ATP during fatty acid oxidation can inhibit the enzymes of glycolysis, which in turn can result in accumulation of glucose-6-phosphate. Glucose-6-phosphate is an intermediate in the production of new biomass from glucose and its accumulation results in catabolite repression of glucose transport (Azam and Kotyk, 1969; Walker, 1998). In this investigation, glucose inhibition was not complete.

Uptake of glucose may also be physically prevented by alkanes through their association with boundary layers of the yeast cell during assimilation (Gill and Ratledge, 1973). However, in the case of RP2 this was not considered. If the alkanes formed a physical barrier, required elements for proliferation of the yeast

such as nitrogen, phosphates and potassium would also have been prevented from being assimilated, with a subsequent reduction in growth.

Overall, the ability of RP2 to grow on and degrade tallow to the same degree even in the presence of an additional and more readily available substrate, glucose, is an important characteristic of the yeast. Sugars and carbohydrates can often form part of the waste liquor from food processing wastes with fats (Najafpour *et al.*, 1994; DeFelice *et al.*, 1997; Scioli and Vollaro, 1997; Fong and Tan, 2000). In the development of a microbial system to remove waste fats, the selected organism(s) would be required to remove fats, irrespective of any additional carbon composition of the waste. The concomitant use of sugar and lipid by RP2 would theoretically allow for the reduction of two COD contributing wastes.

Nitrogen is an essential requirement for yeast proliferation and specifically for protein (enzyme) production. The metabolism of *Y. lipolytica* is lipid- and protein-oriented and consequently, nitrogen sources may play a critical role in the metabolism of this yeast (Szabo and Štofaníková, 2002). Therefore, the influence of both the source and the concentration of nitrogen on tallow biodegradation by RP2 were assessed. Nitrogen was added to the medium at concentrations between 0.5 and 5.0 g $L^{-1}$ from three sources: the inorganic ammonium sulphate and the organic urea and peptone, the latter of which is a complex compound. These compounds represented the most commonly used nitrogen sources, although concentrations employed varied between 1 and 6 g $L^{-1}$ (Novotný *et al.*, 1988; Mendoza-Espinosa and Stephenson, 1996; Pereira-Meirelles *et al.*, 2000). In industrial fermentation media, available nitrogen is usually in the form of complex mixtures of amino acids. Similarly, commercial fertilisers can supply the required nitrogen (Wakelin and Forster, 1998). Nevertheless, media are often supplemented with inexpensive inorganic nitrogen forms, such as ammonium sulphate (Walker, 1998).

The varying levels of total nitrogen in ammonium sulphate, urea and peptone necessitated a comparison of the three compounds in terms of total nitrogen rather than simply the concentration added to the medium (Table 3.2.14). RP2 required a threshold level of total nitrogen in the system, at 0.42 g N $L^{-1}$ or higher, in order to

achieve 75% removal of the tallow (Fig. 3.2.18). This threshold level was independent of the nitrogen source, which implies an absence of specificity by RP2 for the source of nitrogen provided. This is an important consideration in the development of a system to biodegrade fat, where the type of nitrogen supplied is often dependent on economic factors. Ammonium sulphate, at a concentration of 2 g $L^{-1}$, provided the threshold concentration of nitrogen required and was selected as the nitrogen source for growth of RP2 on tallow. Interestingly, ammonium sulphate also provides a source of sulphates, which is required by yeast for the synthesis of sulphur-containing amino acids (Walker, 1998).

A threshold level of total nitrogen was also required by *Y. lipolytica* for maximum removal of crude oil (Zinjarde and Pant, 2002b). Ammonium sulphate concentrations at 5 g $L^{-1}$ resulted in maximum biodegradation with no further increase in removal upon increased nitrogen addition. In the case of RP2, the requirement for nitrogen was lower, at approximately 2 g $L^{-1}$ ammonium sulphate. Interestingly, at the highest concentration of total nitrogen, 2.3 g N $L^{-1}$ (corresponding to 5 g $L^{-1}$ of urea), there was a decrease in tallow biodegradation with approximately 60% fat removed by RP2. This was as a result of either the elevated concentration of the nitrogen or urea. Evidence for reduced biodegradation with urea as compared to ammonium sulphate by *Y. lipolytica* has been reported (Zinjarde and Pant, 2002b).

An essential but often neglected part of the nutritional requirements of yeast is the ionic constituents of the medium. The bioavailability of these ions and their interactions with each other will influence fermentation (Jones and Gadd, 1990). In the pH investigation in the 2 L fermenter with (i) phosphate buffer; (ii) phosphate salts $NaH_2PO_4$ and $KH_2PO_4$ and (iii) bases NaOH, $Ca(OH)_2$ and KOH, the influence of their ionic components ($K^+$, $H_2PO_4$, $Na^+$ and $Ca^{2+}$) on fat removal were assessed. Enhanced fat biodegradation by RP2 and growth correlated with $K^+$ addition from pH control by the buffer and KOH and to a lesser extent with $KH_2PO_4$. The addition of $H_2PO_4^-$ greater than that present in the minimal medium did not benefit fat removal and the presence of $Na^+$ and $Ca^{2+}$ resulted in reduced fat removal and growth. The minimal medium comprised approximately 5 mM

$H_2PO_4^-$ and a concentration of 2 – 4 mM has been reported as optimal for yeast growth (Jones and Gadd, 1990).

Yeast has an absolute requirement for $K^+$ for growth and it plays a central role in the regulation of growth and fermentation under aerobic and fermentative conditions (Jones and Gadd, 1990; Chelius and Wodzinski, 1994; Walker, 1998). The influence of $K^+$ was examined further and $K^+$ levels in the medium and intracellular $K^+$ levels were measured during the course of the fermentations. Intracellular $K^+$ levels increased during 0 - 12 h from approximately 70 to between 160 and 180 nmoles $K^+$ $(10^6$ cell$)^{-1}$ with pH control by the buffer, KOH and $KH_2PO_4$ (Fig. 3.3.8). After 12 h, intracellular $K^+$ decreased and the $K^+$ level was maintained between 60 and 80 nmoles $K^+$ $(10^6$ cell$)^{-1}$ with the buffer and KOH control, which corresponded to the $K^+$ level of the initial inoculum. Cells were harvested in the exponential phase after growth in nutrient broth and it is apparent that maintenance of such a $K^+$ level in RP2 was required for maximum growth and corresponding maximum fat removal. In metabolising yeast cells, the net uptake of $K^+$ is rapid (Jones and Gadd, 1990). The rapid accumulation of $K^+$ in the earlier stages of growth has been suggested as reflecting the activities and requirements of protein synthesis (Hughes and Poole, 1989). A similar pattern of intracellular $K^+$ was reported for *Saccharomyces cerevisiae*, with an increase in $K^+$ during the first 12 h of growth followed by a decrease to initial $K^+$ level (Perkins and Gadd, 1993). The level of $K^+$ in *S. cerevisiae* was 8-times lower than that detected in RP2, with a constant level of approximately 10 nmoles $K^+$ $(10^6$ cell$)^{-1}$ reported. This indicated a higher requirement for intracellular $K^+$ in RP2 compared to *S. cerevisiae*.

The intracellular $K^+$ level was not maintained to initial inoculum concentration with $KH_2PO_4$ control after 24 h and the $K^+$ level decreased to 18 nmoles $K^+$ $(10^6$ cell$)^{-1}$ by the end of the fermentation (Fig. 3.3.8). The decrease in intracellular $K^+$ associated with $KH_2PO_4$ control did not correspond with the level of $K^+$ available in the medium, as $KH_2PO_4$ contributed approximately 100 mM $K^+$ compared to 40 mM $K^+$ with KOH control. Instead, the presence of $Na^+$, as NaOH was added at approximately 20 mM for initial pH adjustment, was assumed to be detrimental to

$K^+$ uptake and consequently, maintenance of the intracellular $K^+$ levels associated with maximum growth. $Na^+$ and $K^+$ share the same carrier system in yeast (Eilam, 1982) and there is an inter-relationship between $K^+$ and $Na^+$, where the requirement for $K^+$ increases with increased $Na^+$ concentration in the medium (Jones and Gadd, 1990). $Na^+$ may exert toxic effects on cellular functions in yeast cells (Suelter, 1970). The effect of reduced growth rate with $Na^+$ was underlined by agar growth studies with *Saccharomyces cerevisiae* (Camacho *et al.*, 1981). The maximum growth rate attained with $Na^+$ was lower as the $K^+$ concentration was decreased in the agar from 6.6 to 3.4 mM. In this study, the $K^+/Na^+$ ratio in the medium was approximately 5:1 for $KH_2PO_4$ control compared to approximately 10:1 and 30:1 for KOH and buffer control. This implied that a ratio of $K^+/Na^+$ greater than 10:1 in the growth medium is required by RP2 for superior fat removal and growth in the fermenter.

A complete loss in intracellular $K^+$ concentration, to less than 10 nmoles $K^+$ $(10^6$ cell$)^{-1}$ by the end of the fermentation, occurred with pH control by $NaH_2PO_4$, NaOH and $Ca(OH)_2$ (Fig. 3.3.8). Additional $K^+$ was not added to the medium with pH control by $NaH_2PO_4$, NaOH and $Ca(OH)_2$ and 10 mM $K^+$ was provided from the minimal medium itself. This lower concentration of $K^+$ available to RP2 correlated with the lower degree of fat biodegradation and growth with these methods. The high affinity $K^+$ carrier system is expressed when cells are grown in low $K^+$ concentration (Borst-Pauwels, 1981; Jones and Gadd, 1990; Walker, 1998). This system requires energy expenditure by the cell to locate and transport $K^+$ and coupled with competition between $Na^+$ and $K^+$, would, in turn, reduce the overall growth rate of the yeast.

In the case of control with $Ca(OH)_2$, growth of RP2 was greatly restricted. The deleterious effect of $Ca^{2+}$ on the growth of RP2 may be considered at two levels. Firstly, $Ca^{2+}$ may interfere with $K^+$ uptake. $Ca^{2+}$ at 2mM inhibited $K^+$ (5mM) uptake by 40% in yeast (Jones and Gadd, 1990). Secondly, $Ca^{2+}$ may be inhibitory due to antagonism of the uptake of $Mg^{2+}$ (Walker, 1994). $Mg^{2+}$ is required by yeast primarily as an enzyme co-factor (Walker, 1998) and it has also been postulated to co-ordinate cell growth and division (Walker, 1986). pH control by $Ca(OH)_2$ resulted in an overall concentration of 5.3 mM $Ca^{2+}$ in the medium

compared to 0.7 mM $Mg^{2+}$. A 10-times excess of $Ca^{2+}$ over $Mg^{2+}$ prevented cell growth in *Saccharomyces cerevisiae*, while a 3-times excess decreased the growth rate (Walker, 1998). Moreover, the role of $Ca^{2+}$ as a competitor for $Mg^{2+}$ binding to ATP presents one reason for the inhibitory effects of excess $Ca^{2+}$ so commonly encountered in industrial media (Jones and Gadd, 1990).

In shake flask investigations into the selection of a suitable buffer for fat biodegradation by RP2, the antagonistic effect of $Na^+$ on fat removal with sodium phosphate buffers ($Na_2HPO_4$-$NaH_2PO_4$ and NaOH-$NaH_2PO_4$) was not as pronounced as growth in the fermenters. However, fat removal and growth was lower with the sodium phosphate buffers compared to KOH-$KH_2PO_4$ buffer, 68% compared to 75% removal and 0.016 $h^{-1}$ compared to 0.020 $h^{-1}$ after 168 h, respectively (Table 3.2.5). The reduced impact of $Na^+$ on growth in shake flask studies may be attributed to the lower growth rate in the flasks compared to fermenter cultivation. The lower growth rate would reduce the rate of $K^+$ uptake from the medium in the shake flasks. This in turn would have lowered the requirement for a high extracellular $K^+$ concentration. In $K^+$-deficient conditions, $Na^+$ may reduce the minimum concentration of $K^+$ required to prevent cell death (Camacho *et al.*, 1981). This indicated that yeast viability was maintained in the presence of high $Na^+$ and low $K^+$ concentrations. This was the case as viability was 100% during the shake flask investigations with the sodium phosphate buffers. However, yeast vitality would not be maintained, which would result in lower growth and fat removal.

In general, lipid fermentation media comprise both $K^+$ and $Na^+$ and $K^+/Na^+$ ratios in the range of 7:5 (Papanikolaou *et al.*, 2002a; Papanikolaou and Aggelis, 2002), 3:1 (DeFelice *et al.*, 1997) and 5:1 (Chigusa *et al.*, 1996) are reported. However, these fermentations were operated at or below pH 6.0. External pH plays an important role on the regulation of ionic uptake by yeast (Borst-Pauwels, 1981). The deleterious effect of $Na^+$ is decreased, as the pH is decreased below pH 7.0 (Jones and Gadd, 1990) and the effect of $Na^+$ on $K^+$ uptake would not be as pronounced below pH 6.0 in these fermentations.

pH control to 7.0 with potassium phosphate buffer and KOH was also assessed in the 10 L fermenter. Fat removal, growth and intracellular $K^+$ levels were identical with both methods of control (Fig. 3.3.19). The automation of KOH control in the 10 L fermenter resulted in the addition of 60 mM $K^+$ to the medium compared to 50 mM with manual addition in the 2 L vessel. Consequently, intracellular $K^+$ levels increased from 60 nmol $K^+$ $(10^6$ cell$)^{-1}$ under manual KOH addition in the 2 L fermenter (Fig. 3.3.8) to 80 nmol $K^+$ $(10^6$ cell$)^{-1}$ under automated addition in the 10 L fermenter at 65 h (Fig. 3.3.19). This indicated that a threshold level of approximately 60 mM $K^+$ was required to achieve maximum fat removal.

This study underlined the importance and complexity of yeast ionic nutrition during cultivation on lipid. The influence of $K^+$ on yeast cells during lipid or hydrocarbon assimilation has not been outlined directly in the literature. However, increased growth rate and yield of *Candida lipolytica*, cultivated on fish oil at pH 7.5, occurred when the concentration of $KH_2PO_4$ was increased from 6 to 15 g $L^{-1}$ (Hottinger *et al.*, 1974b). Overall, in this system, under ideal growth conditions, a $K^+/Na^+$ ratio in the medium greater than 10:1 resulted in superior growth and fat removal by the yeast. In the development of a fat removal system with RP2, $K^+$ concentration will be a limiting factor in biodegradation if it is not present at the required concentration (> 60 mM). However, any deficiency in $K^+$ can be overcome through pH control with KOH. The addition of $Ca^{2+}$ above that present in the minimal medium was detrimental to growth and biodegradation. In contrast, increased $H_2PO_4^-$ above that present in the minimal medium did not affect fat removal or growth. This is an important consideration in light of the potentially high phosphate content of waste streams (DeFelice *et al.*, 1997; Borja *et al.*, 1998).

*Y. lipolytica* is a dimorphic yeast and the stimulation of mycelial growth is dependent on growth conditions (Barth and Gaillardin, 1997; Walker, 1998). In this study, pH, the carbon and nitrogen source and agitation and aeration were found to be influential on RP2 morphology.

In shake flask investigations, RP2 cell morphology varied with pH control during its cultivation on tallow. In the absence of pH control, yeast morphology was observed to be in the yeast-shape only. Similarly, only yeast-shape morphology was observed during the growth of the yeast on hexadecane in minimal media, where medium pH decreased from pH 6.0 to 3.0 (Rodríguez and Domínguez, 1984). In contrast, when the medium pH for RP2 was controlled with 0.1 M citrate phosphate buffer between pH 5.0 to 8.0, both yeast- and mycelium-shaped cells were observed (Table 3.2.4). At pH 3.0 and 4.0, only yeast-shaped cells were detected. Changes in the pH of the medium have been shown to induce a strong morphogenic response in *Y. lipolytica,* with an increase in mycelial growth parallel to an increase in pH (Szabo, 1999; Ruiz-Herrera and Sentandreu, 2002; Szabo and Štofaníková, 2002). Yeast-shaped cells are the dominant morphological form in an acidic environment, whereas mycelium growth is induced upon the increase of pH to neutral. Dimorphic growth of *Candida lipolytica* occurs on hexadecane when the medium pH is near neutral (Rodríguez and Domínguez, 1984). Similarly, *Y. lipolytica* mycelium formation increases when pH is controlled to pH 6.0 by potassium phosphate buffer (Novotný *et al.*, 1994). Although the morphogenic influence of pH has been known for decades, the mechanism of its action has not yet been elucidated.

The production of approximately 6% mycelial shaped cells after 24 h growth of RP2 on tallow at pH 7.0 also occurred when both sodium and phosphate buffers were used for pH control. The mycelium development was therefore not as a result of citrate, which has been shown to induce dimorphism in some strains of *Y. lipolytica* (Rodríguez and Domínguez, 1984; Novotný *et al.*, 1994; Ruiz-Herrera and Sentandreu, 2002), but attributed to pH and the presence of tallow. Potassium phosphate buffer concentration and temperature also influenced mycelium production during the initial 24 h growth period at pH 7.0. Increased buffer concentration above 0.2 M resulted in a decrease in mycelium production at all temperatures (Fig. 3.2.11). Increased phosphate was reported to induce increased mycelium production in *Y. lipolytica*, but was shown to be strain dependent (Novotný *et al.*, 1994). Mycelia ratios were similar at 25°C and 30°C but reduced at 37°C, at all buffer concentrations. At 4°C, no mycelia were produced,

irrespective of buffer concentration, although all cell proliferation was greatly reduced at this temperature. The influence of temperature on morphology has not been previously documented, with investigations performed solely at optimum growth temperatures (Ota *et al.*, 1984; Szabo, 1999; Kim *et al.*, 2000a).

In shake flasks, increased agitation to 200 rpm and with baffled growth at 130 rpm reduced mycelium formation to approximately 2% at 24 h compared to 6% in non-baffled agitation at 130 rpm (Table 3.2.9). Increased agitation and aeration in the 2 L fermenter also resulted in a reduction in mycelium production. When the fermenter was both agitated and aerated, irrespective of the rate, dimorphism was observed only at 12 h and mycelial-shaped cells comprised only 1% of the overall cell shape. Interestingly, when the fermenter was not aerated, only yeast-shape cells were present. In contrast, anaerobic stress was reported to have induced mycelium formation in *Y. lipolytica* (Ruiz-Herrera and Sentandreu, 2002). In the absence of aeration, however, growth of RP2 cells was restricted and cell viability was reduced to 60% by the end of the fermentation. Similarly, when *Y. lipolytica* cell growth was inhibited through the use of antimycin A, yeast-shaped cells were the dominant morphology (Sazbo, 1999). Scale-up from 2 L to 10 L fermentation resulted in only yeast-shaped cell morphology during the fermentation. This may be attributed to the higher degree of agitation employed in the 10 L vessel compared to 2 L studies in order to achieve constant mixing. The absence of mycelium production in the 10 L fermenter further underlined the absence of its role in tallow biodegradation.

The amount of mycelial-shaped cells after 24 h in shake flasks was reduced in all cases, with less than 1% mycelium present after 168 h. The presence of less than 6% mycelium after 24 h implied that dry weight values reflected the number of cells present and dimorphism did not interfere with its use for the determination of growth and specific removal rates. Yeast shaped cells were the dominant morphology in all growth vessels during growth of the yeast and subsequent fat removal. Similarly, the yeast-shape was observed to be the predominant morphology when *Y. lipolytica* was grown on alkanes, animal derived fats and during emulsifier production (Gutierrez and Erickson, 1977; Rodríguez and Domínguez, 1984; Zinjarde *et al.*, 1997; Papanikolaou *et al.*, 2002b). A transition

from mycelium to yeast-shape has been suggested as a prerequisite for effective alkane degradation (Zinjarde *et al.*, 1998). Preparation of RP2 inoculum in olive oil broth stimulated a small degree of dimorphic growth. Inoculation with mycelial cells did not influence degradation and yeast-shaped cells dominated during growth of RP2 on tallow. Consequently, the induction of dimorphism in RP2 when exposed to lipid at or near neutral pH was an interesting phenomenon, but was not considered of importance in the degradation of tallow.

The presence of glucose as the sole substrate for RP2 did not induce mycelium production and yeast morphology when grown on tallow was not influenced by the presence of the sugar (Table 3.2.12). The morphology of *Saccharomycopsis lipolytica* was in yeast-shape only when glucose was the carbon source (Ota *et al.*, 1984).

The concentration and the source of nitrogen added had an interesting effect on the morphology of RP2, which was independent of the total nitrogen concentration in the medium. Mycelium production was observed to be greater where nitrogen as ammonium sulphate and peptone was limiting compared to nitrogen-rich media (Fig. 3.2.19). Similarly, the growth of *Y. lipolytica* under nitrogen-limited conditions resulted in a predominance of mycelial-shaped cells with some yeast-shaped cells also observed (Aggelis and Komaitis, 1999). This has been interpreted as a foraging strategy by the yeast under limiting conditions (Szabo and Štofaníková, 2002). Under nitrogen-limiting conditions, at less than 0.42 g N $L^{-1}$, ammonium sulphate induced dimorphic growth to a greater extent than peptone, with the detection of between 25 to 30% mycelial cells compared to 7 to 15% at 24 h, respectively. The effect of peptone on the morphology of *Y. lipolytica* has been reported with a reduction in the presence of mycelial cells, in comparison to other nitrogen sources (Szabo and Štofaníková, 2002). In nitrogen-rich media, mycelial cells comprised up to 6% of overall cell shape of RP2 at 24 h with ammonium sulphate and urea. In contrast, increased concentration of ammonium sulphate stimulated rather than inhibited the formation of mycelium by *Y. lipolytica* (Szabo, 1999). Interestingly, the addition of urea induced a higher percentage of mycelial cells at 24 h compared to the other two nitrogen sources where the required nitrogen levels for maximum biodegradation were applied. In

all cases, a reduction in mycelial cells was observed after 24 h and yeast-shaped cells comprised greater than 95% of the overall cell number by the end of the fermentations.

Although mycelial development was only initiated during the lag phase, the induction of a quarter of the cells as mycelial-shaped (as with 1 g $L^{-1}$ urea) may be disadvantageous to fat removal system design and its operation on a large scale. Also, the observed transition from mycelial- to yeast-shaped cells, required for tallow biodegradation, might be retarded if the initial mycelium concentration is high. This in turn may alter the rate of tallow removal when fermentations are operated on a larger scale. Consequently, although dimorphism was found not to play a role in fat removal, a nitrogen source that would both supply the required concentration of nitrogen and not be a strong morphogen would be desirable in the design of the system, such as 2 g $L^{-1}$ ammonium sulphate.

Cell growth was assessed in terms of viable cell number through both methylene blue staining and haemocytometer counts and also by dry weight measurements. Haemocytometer counting was preferred over the more 'traditional' plate count method, as it was a more rapid method of cell enumeration. Also, examination of the cells microscopically permitted a cursory examination of the yeast morphology, which was also monitored. Assessment of the growth of RP2 by the two methods ensured that yeast cells were increasing in number and not just in weight through the accumulation of intracellular lipid. This phenomenon has been previously observed, where microscopic evaluation of individual cells substantiated that cells were getting larger without multiplication (Kajs and Vanderzant, 1980). In this study, the cell number and dry weight followed a similar pattern, which permitted the use of dry weight values in the determination of the growth rate and the specific rate of fat removal by RP2 (Fig. 3.2.1).

The growth rate was influenced by the optimisation of the growth conditions for tallow biodegradation. pH control with 0.1 M potassium phosphate buffer, pH 7.0, increased the growth rate in shake flasks to 0.02 $h^{-1}$, which was double that compared to growth in absence of pH control (Table 3.2.5). Fermenter growth, with increased agitation and aeration and the addition of $\geq$ 60 mM $K^{+}$ though pH

control, resulted in a considerable increase in the growth rate to 0.072 $h^{-1}$ (Table 3.3.1 & Table 3.3.7). The influence of $K^+$ on growth was underlined during scale-up from 2 L to 10 L fermentation. $K^+$ addition increased under automated addition from 50 to 60 mM and the growth rate increased from 0.069 to 0.072 $h^{-1}$, respectively (Table 3.3.5 & Table 3.3.10, respectively). Variation from these optimised conditions resulted in a reduced growth rate.

Stationary growth in shake flasks was not reached until approximately 144 h (Fig. 3.2.12), which contributed to the low growth rate of RP2 compared to other organisms cultivated on lipids. Stationary growth was reached by 50 h during the cultivation of *Y. lipolytica* in shake flasks on hydrolysed oleic acid (Papanikolaou *et al.*, 2001) and by 100 h when grown on raw glycerol (Papanikolaou *et al.*, 2002b). A growth rate of 0.06 $h^{-1}$ was reported for *Phanerochaete chrysosporium* cultivated on olive mill wastewaters (García García *et al.*, 2000). Growth rates between 0.20 $h^{-1}$ and 0.25 $h^{-1}$ were achieved by *Y. lipolytica* and *S. lipolytica* cultivated on glycerol and animal derived fats, respectively (Papanikolaou *et al.*, 2002a & b; Tan and Gill, 1985). Similarly, a growth rate of 0.22 $h^{-1}$ was reported for *Candida lipolytica* grown on fish oil (Hottinger *et al.*, 1974b). However, the relatively slow growth rate for RP2 did not result in a lower biomass production.

Under optimised shake flask conditions, maximum biomass production of RP2, 15 g $L^{-1}$ was achieved after 144 h (Fig. 3.2.12). Under optimised fermenter conditions, 14 g $L^{-1}$ biomass was achieved after 65 h (Fig. 3.3.5 & Fig. 3.3.16). These values were comparable and in some cases exceeded values previously reported. Levels between 11 and 17 g $L^{-1}$ were produced by *Candida lipolytica* grown on fish oil in shake flask cultures (Hottinger *et al.*, 1974b), while the growth of *Y. lipolytica* on 20 g $L^{-1}$ animal derived fats resulted in 10.3 g $L^{-1}$ biomass after approximately 65 h in a bench-top fermenter (Papanikolaou *et al.*, 2002a).

The possible presence of accumulated lipid in RP2 cells when grown on tallow necessitated a comparison of the yield of dry weight per weight of tallow removed ($Y_{x/s}$) to the yield of fat free cell weight on metabolised lipid ($Y_{xf/sm}$). This permitted a more accurate determination of the actual yield. Fat free dry weight

was the weight of cells less accumulated lipid and represented the actual growth of the yeast. However, the similarity in the calculated yields for $Y_{x/s}$ and $Y_{xf/sm}$ indicated that lipid accumulation in the cell did not influence yield coefficient (Table 3.2.1).

Under optimised growth conditions of pH and temperature in shake flasks, the yield was 1.02 g $g^{-1}$. The overall yield in the 2 L fermenter under the optimised conditions, was marginally lower at 0.94 g $g^{-1}$. This marginal decrease in yield was not attributed to increased agitation and aeration directly but to foam formation in the fermenter. Foaming causes the biomass with which it is associated to be lifted into the foam zone (Forster, 1992). Interestingly, this decrease in yield was not detected in 10 L fermentation, which was attributed to better foam management in the larger vessel. The similarity in yields, 0.94 to 0.96 g $g^{-1}$, with the different methods of pH control in the 2 L fermenter indicated that ionic nutrition, with $Na^+$, $K^+$ and $H_2PO_4^-$, did not have an effect on overall yield (Table 3.3.5). The exception was $Ca^{2+}$ with $Ca(OH)_2$ control, where the yield was reduced to 0.75 g $g^{-1}$. This may be attributed to the lower degree of growth of RP2 with $Ca^{2+}$, as noted previously. The C:N ratio influenced the yield of RP2. Where the C:N ratio was greater than 40:1, the yield was reduced. This was especially evident for 0.5 g $L^{-1}$ peptone, where the C:N of 200:1 resulted in a yield of 0.64 g $g^{-1}$ (Table 3.2.16). Yields of approximately 1.0 were achieved, however, when the threshold level of carbon and nitrogen were applied, at 40:1 or lower. The exception was in the case of urea addition at 5 g $L^{-1}$, where the reduced yield of 0.89 g $g^{-1}$ was attributed to the reduction in both growth and tallow removal at this concentration.

Yield coefficients near unity are to be expected for highly reduced substrates like long chain fatty acids. A lower yield might be expected from nitrogen-limited than from carbon-limited culture because of the less efficient use of the abundant carbon source (Tan and Gill, 1984). The near unity yield achieved by RP2 under optimised growth conditions was comparative to values for *Y. lipolytica* grown on animal derived fats (Papanikolaou *et al.*, 2001 & 2002a) but greater than the majority of values reported for growth of yeast on lipids. A value of 0.76 g $g^{-1}$ was

determined for *Saccharomycopsis lipolytica* and *Candida utilis* grown on tallow (Kajs and Vanderzant, 1980). A *Candida* sp. grown on sardine oil had a yield of 0.88 g $g^{-1}$, whereas the growth of *Saccharomycopsis lipolytica* on beef tallow only yielded half this value (Ota and Kushida, 1988). The yield generated with RP2 was also greater than values reported for other organisms grown on lipids. Yields ranging from 0.15 to 0.67 g $g^{-1}$ were reported for growth of *Acinetobacter* sp. on oil (Wakelin and Forster, 1997).

*Y. lipolytica* is classified as an oleaginous yeast and as such can accumulated lipid intracellularly (Ratledge, 1976 & 1994). However, accumulation of lipids in the cell is governed by a number of factors including pH, carbon source and available nitrogen (Papanikolaou *et al.*, 2001). Similarly, accumulation of a high concentration of lipid in *Y. lipolytica* is both strain specific and related to the cultivation process (Papanikolaou *et al.*, 2002a & b; Papanikolaou and Aggelis, 2002). Limitation of nutrients (other than carbon) and growth can promote the accumulation of lipid (Walker, 1998). In this study, pH, carbon source, ionic nutrition, aeration and nitrogen concentration were all found to influence intracellular lipid in RP2.

In all cases, the lipid content of RP2 cells increased from approximately 0.1 g $g^{-1}$ of cell weight at the time of inoculation to between 0.4 and 0.5 g $g^{-1}$ during the early phase of growth on tallow. An initial accumulation in cellular lipid in *Y. lipolytica*, when cultivated on lipids has been reported (Papanikolaou *et al.*, 2001). Both intra- and extracellular lipid degradation occurred during growth. However, the magnitude of the reduction in intracellular lipid was dependent on the growth of the yeast and consequently the growth conditions.

In the absence of pH control in shake flasks, intracellular lipid comprised 0.2 g $g^{-1}$ of cell content after 168 h (Fig. 3.2.16). Control of pH to 7.0 resulted in a decrease in intracellular lipid content to 0.1 g $g^{-1}$ by 100 h, which was the same as the lipid content of inoculum. This indicated that there was no lipid accumulation with pH control. Growth of *Y. lipolytica* LGAM S(7)1 on animal derived fats followed a pattern of substrate removal and intracellular lipid content similar to that of RP2 (Papanikolaou *et al.*, 2001). In the first 50 h (lag phase), accumulated lipid

comprised more than 40% of cell biomass. During 50 to 100 h, there was a simultaneous degradation of cellular lipids and extracellular lipid. In the late fermentation phase, 100 to 240 h, a further decrease of the extracellular lipid occurred whereas intracellular lipid levels remained constant.

RP2 cells grown on either 1.0 or 10.0 g $L^{-1}$ glucose did not accumulate lipid, with less than 10% (w/w) lipid comprising the overall cell weight. Similarly, 5 – 9% (w/w) lipid in dry cellular mass of *Y. lipolytica* grown on glucose was reported (Papanikolaou *et al.*, 2002b). Glucose did not influence intracellular lipid profiles during growth on tallow with a similar pattern of lipid content in the cells as detected with pH control to 7.0. In the 2 L fermenter, ionic nutrition as $K^+$, $Na^+$ and $H_2PO_4^-$, with the various methods of pH control, resulted in a similar pattern of lipid content to pH control, albeit over a shorter time period. Lipid content increased from 0.1 to approximately 0.5 g $g^{-1}$ at 12 h, after which time it decreased to between 0.10 and 0.15 g $g^{-1}$ at the end of the fermentation (Fig. 3.3.6). The exception was $Ca^{2+}$ with $Ca(OH)_2$ control, where lipid accumulation to 0.3 g $g^{-1}$ occurred. As noted for yield production, this accumulation may be attributed to the lower degree of growth of RP2 with $Ca^{2+}$. Limitation of oxygen to RP2 growth resulted in lipid accumulation. Lipid comprised 0.25 g $g^{-1}$ after 65 h when the fermenter was not aerated. This accumulation may be attributed to the absence of growth of RP2 under non-aerated cultivation. Aeration has been noted as an important factor in the degree of lipid content in yeast cells. Lipid synthesis was favoured in *Y. lipolytica* cells when cultivated on animal derived fatty acids in low aerated media, but in highly aerated media noticeable synthesis of fat free material occurred and lipid production was low (Papanikolaou *et al.*, 2002a). Similarly, *Candida lipolytica* 1094 growing on corn oil accumulated 55% (w/w) of lipids at 0% - 5% air saturation, but at 80% saturation, accumulated lipids were reduced (Bati *et al.*, 1984).

The concentration of total nitrogen had an interesting effect on lipid accumulation in the yeast cell at the end of the fermentation in shake flasks. At the lower concentrations of nitrogen, below the threshold level of 0.42 g N $L^{-1}$, RP2 accumulated lipid within the cell. This accumulation was observed to be inversely proportional to the concentration of nitrogen added and was independent of the

nitrogen source. This was most evident for peptone where 0.5 g $L^{-1}$ addition resulted in 0.35 g $g^{-1}$ lipid in the cells compared to just 0.09 g $g^{-1}$ with 3.0 g $L^{-1}$ addition (Table 3.2.16). At and above the threshold nitrogen concentration, lipid content was approximately 10% of the overall cell weight. Similarly, *Y. lipolytica* accumulated intracellular lipid with low nitrogen levels when cultivated on animal derived fatty acids (Papanikolaou *et al.*, 2002a). The greatest degree of lipid was accumulated at 0.5 g $L^{-1}$ ammonium sulphate, with an inverse relationship between intracellular lipid per cell weight and nitrogen concentration noted. It is commonly observed that microorganisms that exhaust their nitrogen source during growth in a carbon rich medium will accumulate storage materials (Kristiansen and Sinclair, 1978). This is attributed to the decrease in the intracellular concentration of AMP as the cultures become depleted of nitrogen (Botham and Ratledge, 1979). This results in the accumulation of oxalate in the cell, which in turn causes the accumulation of lipid via fatty acid biosynthesis.

Lipid accumulation was shown to be strongly dependent on the molar carbon to nitrogen ratio (C:N) of the growth medium (Ykema *et al.*, 1986; Ratledge, 1994). Therefore, application of a C:N of approximately 40:1, or lower, is important in order to achieve both maximum tallow biodegradation by RP2 and to produce low lipid-containing sludge. In general, nitrogen loading is much greater in systems developed for lipid degradation compared to that required by RP2 with C:N ratios ranging from 20:1 to 10:1 (Mendoza-Espinosa and Stephenson, 1996; Chigusa *et al.*, 1996; DeFelice *et al.*, 1997; Wakelin and Forster, 1998). The lower requirement for nitrogen by RP2 may therefore be advantageous in economic terms in the development of a fat removal system.

Although the ability of fungi including *Y. lipolytica* to accumulate lipid has been exploited in a number of investigations for single cell oil production (Koritala *et al.*, 1987; Kendrick and Ratledge, 1996; Čertík *et al.*, 1997; Aggelis *et al.*, 1999; Papanikolaou *et al.*, 2003), the production of fatty cells is not considered desirable in fat removal systems. High lipid sludges can lead to settleability and disposal problems (Stoll and Gupta, 1997). The absence of the accumulation of lipid in RP2 under the growth conditions identified as superior for fat removal is a desirable characteristic in a fat removal system.

During the screening of the original 10 isolates, the importance of agitation was underlined. In all cases, the percentage fat removal was reduced considerably in the absence of agitation compared to agitated growth at 130 rpm (Fig. 3.1.13). In shake flask investigations, under optimised temperature at 25°C and pH control with potassium phosphate buffer, pH 7.0, the inclusion of baffles and increased agitation to 200 rpm was investigated. The presence of baffles during agitation at 130 rpm resulted in an identical degree of fat removal to non-baffled agitation (Table 3.2.8). However, their inclusion was problematic as they resulted in foam formation. Both the degree of fat removal and growth were reduced at 200 rpm. This reduction was not attributed to an alteration in cell viability through increased shear stress as cell viability was maintained at 100% at 200 rpm. Instead the decrease in fat removal may be attributed to a disruption in fat/yeast attraction at the higher agitation rate, which would have resulted in a decrease in the ability of RP2 to assimilate fatty acids. A similar phenomenon occurred during the cultivation of *Acinetobacter* sp. on crude oil where increased agitation resulted in an inhibition of cell adhesion to the oil surface (Hori *et al.*, 2002). This would imply that non-baffled agitation at 130 rpm was the speed where aeration and bioavailability of the tallow were at levels that were ideal for RP2 in shake flask fermentations.

Under the optimised conditions determined in shake flask culture, the biodegradation of tallow by *Y. lipolytica* RP2 was scaled up to cultivation in fermenters. Growth and fat removal were first assessed in a bench-top fermenter and then in a pilot-scale 10 L fermenter.

The fermentation period was 65 h, after which time the volume of the medium in the vessel had been reduced by approximately 50%, as a result of regular sampling of the vessel. Fat removal was only determined after 65 h. Monitoring of the concentration of the tallow during the fermentation was not feasible due to the lack of complete homogeneity between the medium and the lipid. As a result, any sample taken would not have been a true representative of the concentration of lipid present in the fermenter at that time. The absence of assessment of the tallow concentration during the fermentation was a reflection of the solid nature of the

tallow, a problem not associated with other lipid substrates, especially the oils and soft greases. Regular monitoring of oil and grease substrate concentrations during the cultivation of yeast in fermenters has been performed (Chigusa *et al.*, 1996; Mendoza-Espinosa and Stephenson, 1996; Scioli and Vollaro, 1997; Casas and García-Ochoa, 1999). In systems where hard fats are employed, concentration and subsequent degree of fat removal are, generally, assessed only at the end of the fermentation, as in this study (Bednarski *et al.*, 1993; Wakelin and Forster, 1997). The exception is where the fat is dispersed into the aqueous phase to form a homogenous emulsion through the addition of surfactants, which facilitate the monitoring of the fat during the course of the fermentation (Papanikolaou *et al.*, 2002a).

In the fermenters, only half the available operating volume was employed (1 L and 5 L in the 2 L and 10 L fermenters, respectively). This ensured that there was sufficient head-space in the vessel to allow for foam formation and breakdown. Similarly, operating volumes in fermenters, which consisted of approximately half the total volume available, were reported for the cultivation of yeast on lipids (Kajs and Vanderzant, 1980; Ota and Kushida, 1988; Desphande and Daniels, 1995; Papanikolaou and Aggelis, 2002). During the cultivation of RP2 on tallow in the fermenters, foam formation was observed upon aeration of the vessel, which was controlled through the periodic addition of antifoam. Foam formation is a common occurrence during lipid or FOG- fats, oils and greases degradation (Forster, 1992). Foams are easily generated in aerobic microbial fermentations as a result of surface-active materials that could be present initially in the culture medium or excreted by the organism during the process and the intense agitation/aeration required for acceptable oxygen transfer for growth (Brown *et al.*, 2001).

Agitation and aeration rates of 500 rpm and 1 VVM in the 2 L fermenter resulted in 75% tallow removal after 65 h (Tables 3.3.1 & 3.3.3, respectively). At agitation and aeration rates above these rates, an identical degree of tallow was removed. In contrast, at rates below these, fat removal was reduced. Interestingly, fat removal was reduced to 5% in the absence of aeration. This confirmed the highly aerobic nature of the yeast (Barth and Gaillardin, 1997; Spencer *et al.*, 2002) and that lipid

biodegradation is a highly aerobic process (Ratledge, 1992; Wakelin and Forster, 1998; Nakona and Matsumura, 2001). Agitation at 500 rpm in bench-top fermenters has been reported for yeast cultivation on lipid (DeFelice *et al.*, 1997; Papanikolaou and Aggelis, 2002). Agitation below this rate was found in one instance at 300 rpm (Papanikolaou *et al*, 2002a), whereas rates exceeding 500 rpm have been documented (Ota and Kushida, 1988; Aggelis and Komaitis, 1999; Casas and García-Ochoa, 1999). Aeration rates, between 0.5 to 1.8 VVM, were reported for yeast cultivation on lipid in bench-top fermenters (DeFelice *et al.*, 1997; Casas and García-Ochoa, 1999; Papanikolaou and Aggelis, 2002).

The biodegradation of tallow by RP2 was scaled-up to 10 L fermentation to assess the ability of RP2 to biodegrade tallow on a larger scale at 1 VVM aeration. The 10 L fermenter offered a number of advantages over the bench-top 2 L vessel as it permitted the measurement of dissolved oxygen levels and an automated control of the medium pH. Agitation in the 10 L vessel, at 1000 rpm, was scaled-up from the 2 L fermenter based on constant mixing. As mixing is an important function of fermenters, it is desirable to keep the mixing time constant on scale-up (Doran, 1995). Reduced productivity and performance often accompany scale-up of fermentations as a result of lower mixing efficiency and subsequent alteration of the physical environment. However, constant mixing is not generally considered as a normal rule of thumb for scale-up in biological engineering, whereas power per volume ratio, impeller tip speed and oxygen transfer rate are more commonly employed methods (Bailey and Ollis, 1986; Solà and Gòdia, 1995; Doran, 1995). In heterogeneous reactions, such as the hydrolysis of tallow, the emulsion conditions have a large effect on the rate of reaction (Hur and Kim, 1999). Emulsification of the fat is directly related to the mixing in the fermentation. Constant emulsion conditions were achieved through an operation temperature of 42°C in a study by Hur and Kim (1999) which ensured dispersal of the lipid and permitted scale-up of their system based on a working volume per agitator. This was not feasible in this study as 25°C was superior temperature for fat removal by RP2, at which the tallow was solid. Therefore, the scale-up of agitation of the tallow and yeast was based on constant mixing.

In the 2 L fermenter, two impellers were fixed at the base of the drive shaft. In general, however, the impellers would be placed equidistant from each other along the drive shaft (Desphande and Daniels, 1995; Doran, 1995, Hur and Kim, 1999). Consequently, the effect of the position of the impellers on tallow biodegradation was investigated. In the first system, the impellers were placed equidistant from each other along the shaft and in the second, two impellers were employed at the base of the shaft, as in the 2 L design.

Fat removal was 68% with the placement of one impeller at the base of the drive shaft with the second 135 mm above it compared to 64% with two impellers at the base (Table 3.3.7). Impeller positioning did not affect yeast growth, which was identical in both systems. There was a higher degree of foam formation when 2 impellers were employed at the base. This may have resulted in the elevation of the substrate and/or biomass from the medium into the foam zone (Forster, 1992), thereby reducing the degree of fat removal.

Scale-up from the 2 L to 10 L fermentation resulted in a marginal decrease in fat removal ability by RP2 from 75% to 68%, respectively, with pH control by potassium phosphate buffer. This reduction in fat removal was not attributed to decreased growth of the yeast, which was identical in both vessels, but to the larger working volume in the 10 L fermenter. Similarly, utilisation of sucrose by *Rhodococcus opacus* decreased from 67% to 42% on scale-up from 30 L to 500 L fermentation under similar growth conditions (Voss and Steinbüchel, 2001).

In shake flask investigations, fat removal by *Y. lipolytica* RP2 increased from 21% after 168 h under non-optimised growth conditions to a maximum of 75% in 168 h . This was achieved at 25°C with pH control by potassium phosphate buffer, pH 7.0, agitation at 130 rpm and a 2 g $L^{-1}$ ammonium sulphate as the nitrogen source. Scale-up of growth to 2 L and 10 L fermentation achieved 75% and 68% fat removal respectively. Although the degree of tallow biodegradation was not increased in the fermenters, there was a reduction in the time, to 65 h, for maximum fat removal. Fat removal by RP2 was comparable and in many cases superior to other values reported. Various fungi cultivated on beef tallow achieved

fat removal values between 18 to 23% (Bednarski *et al.*, 1993 & 1994). *Norcardia amarae* and *Rhodococcus rubra* removed up 26% of fast food restaurant grease (Wakelin and Forster, 1997). In comparison to the other isolate studied in the laboratory for tallow degradation, the filamentous fungus, *Trichoderma harzianum* Rifai RP1, RP2 removal was superior as the fungus removed only 31% of a 20 g $L^{-1}$ loading of the tallow after 10 days (Fleming, 2002). *Y. lipolytica* 1096 removed 41% of a loading of 30 g $L^{-1}$ poultry tallow after 4 days (Bednarski *et al.*, 1994) and *Y. lipolytica* ACA-DC 50109 achieved 50% removal of a loading of 20 g $L^{-1}$ of animal derived fat after a similar time period (Papanikolaou *et al.*, 2002a). After 4 days in this study (96 h), RP2 had removed 60% of the tallow. *Y. lipolytica* was reported to remove between 50 to 70% of a 15 g $L^{-1}$ loading of animal derived fat after 70 h in a bench-top fermenter (Papanikolaou *et al.*, 2002a). Interestingly, 70% removal of olive oil, added at 8 g $L^{-1}$, was achieved by activated sludge after 4 days cultivation (Wakelin and Forster, 1998). The authors suggested that the use of a mixed culture, such as activated sludge offers the best option for the treatment of lipid-rich wastes and that the a pure culture would not be ideally suited for use in a commercial operation. However, the ability of RP2 to remove a higher and more recalcitrant substrate loading, albeit at a marginally lower rate compared to activated sludge, indicated its potential in the design of a fat removal system.

The specific rate of tallow removal also increased in shake flasks from 0.024 g $g^{-1}$ $h^{-1}$ under non-optimised growth conditions to 0.035 g $g^{-1}$ $h^{-1}$ under optimised growth (Sections 3.2.1 & 3.2.4.4, respectively). The specific rate of fat removal is a more accurate method of comparison between biological fat removal systems as it accounts for the diverse range of substrate loading. However, the majority of investigations, as noted, only report lipid removal in terms of total amount removed. Interestingly, one report by Chigusa *et al.* (1996) did report specific rate of removal of soybean oil with a mixed yeast population, which was lower than achieved by RP2, at approximately 0.021 g $g^{-1}$ $h^{-1}$.

Fat removal rates in excess of 70% have been reported, although at lower substrate loading than applied in this study. A *Bacillus* sp. removed approximately 80% of 5 g $L^{-1}$ beef tallow after 24 hours (Okuda *et al.*, 1991). *Saccharomycopsis*

*lipolytica* achieved 90% removal of 2.2 g $L^{-1}$ beef tallow after just 8 hours (Tan and Gill, 1985). In the latter investigation, however, agitation comprised the addition of stirrer bars to the growth flask and magnetic stirring of the tallow at 1200 rpm with periodic scraping of the fat from the stirrer bar. This method of agitation, although successful in mixing the tallow in the medium, would not be feasible to operate on a larger scale and so was not considered in this study. Also, a relatively high inoculum of the yeast was used, 12% (w/v), compared to 2% (w/v) in this study. Removal rates for oils, particularly waste olive oil, in excess of 70% have also been reported. Complete removal of 16 g $L^{-1}$ olive oil waste was demonstrated by *Y. lipolytica* in 24 h (DeFelice *et al.*, 1997; Scioli and Vollaro, 1997). However, in these cases, olive oil is a more easily degradable substrate for the yeast as it contains mainly oleic residues and is liquid at ambient temperatures. The high saturation and solid nature of tallow makes it a more challenging substrate for biodegradation.

Complete utilisation of tallow was not achieved by RP2 and in excess of 25% of the tallow remained in the growth vessel. An incomplete utilisation of hard fats is widely documented. This was underlined during the scale-up from shake flask to 2 L fermentation where 75% tallow was removed in both vessels. In this study, this was attributed to the discrimination by RP2 against certain saturated fatty acids. In particular, stearic acid is known for its recalcitrance. Stearic acid comprises up to 30% of the fatty acids in tallow (Gunstone, 1996; Broughton *et al.*, 1998; Fleming, 2002). Although a judicious investigation into fatty acid profile of the remaining substrate or assimilated lipids was not performed, stearic acid has proved to be resistant to assimilation by a number of microorganims. These include *Apiotrichum curvatum* (Lee, 1992), *Pseudomonas oleovorans* (Füchtenbusch *et al.*, 2000), *Saccharomycopsis lipolytica* (Tan and Gill, 1985) and *Yarrowia lipolytica* (Papanikolaou *et al.*, 2002a). *Saccharomycopsis lipolytica* could not sustain growth when cultivated solely on stearic acid (Tan and Gill, 1985). It was suggested that even if the residence time was extended indefinitely, a residue of saturated acids is likely to remain for any process involving the growth of microorganisms on animal fats. The amount left would largely be dependent on the physical nature of the fermentation and the nature of the major

triglycerides. Ultimately, although complete removal of tallow is not attainable with RP2, approximately 70% reduction of the solid waste was achieved.

At the end of the fermentation, there are a number of important parameters that can impact on the efficiency of the system. These include the settleability of the yeast sludge and the fate of the residual fat. Furthermore, the potential of the yeast sludge as a by-product of the fermentation would be very advantageous to the system. These parameters were assessed after 10 L fermentation under superior growth conditions, including pH control by 0.5 M KOH/HCl.

RP2 biomass settled very quickly at the end of the fermentation and consequently the SVI was very low, 1.0 ml $g^{-1}$ (Section 3.3.2.3). SVI values less than 120 ml $g^{-1}$ indicate good sludge settleability (Martins *et al.*, 2003). The production of an easy settleable biomass with a low SVI is important where the biomass is destined for further use (de Villiers and Pretorius, 2001). A high percentage of the tallow not biodegraded by RP2, approximately 25% lipid, remained in the fermenter. This fat may not have been exposed to the yeast in the fermenter through foam elevation or stagnant areas in the vessel. Moreover, the residual tallow may correspond to the stearic acid component of the fat. The waste liquor remaining after the yeast had settled contained a small quantity of lipid, approximately 2% of the initial tallow loading (Table 3.3.13). With a high loading of 20 g $L^{-1}$ tallow, this resulted in 400 mg lipid $L^{-1}$ waste liquor. Lipid comprises approximately 10% of domestic wastewater, which is treated by a conventional activated sludge system (Metcalf and Eddy, 1991). Consequently, the liquor produced by RP2 fermentation contained lipid levels that could then be further treated by a traditional biological system, without overloading the system and introducing the problems associated with solid fats. The permitted level of lipid in effluent treated wastewater is 100 mg $L^{-1}$ (Keenan and Sabelnikov, 2000). The successful biological treatment of highly-lipid waste and wastewater and the sequential treatment of the treated waste liquor by a conventional municipal system has been reported (Keenan and Sabelnikov, 2000).

RP2 cells comprised 0.42 g $g^{-1}$ protein after 65 h (Table 3.3.14). Protein was the main component of cells nitrogenous content. The remaining nitrogen content, approximately 0.09 g $g^{-1}$ was attributed to nucleic acid content, which is consistent with yeast nucleic acid content (Anupama and Ravindra, 2000). Although a judicious examination of the amino acid content of the protein of RP2 has not been performed, the commercial value of SCP is linked to its protein content. The protein yield of 0.44 g $g^{-1}$ in RP2 cells cultivated on tallow is notably greater than those reported for other organisms cultivated on a variety of carbon sources (Table 4.1).

**Table 4.1:** Comparison of yield of single cell protein produced by various microorganisms grown on a variety of carbon sources

| **Organism** | **Carbon source** | **Yield (g protein) (g substrate)$^{-1}$** | **Reference** |
|---|---|---|---|
| *Kluveromyces fragilis* | Whey | 0.370 | Paul *et al.* (2002) |
| *Pichia guilliermondii* A9 | Reducing sugars in kimchi wastewater | 0.256 | Choi and Park (1999) |
| *Candida kruseii* SO1<br>*Saccharomyces* sp LK3G | Sorghum hydrolysate | 0.175<br>0.125 | Konlani *et al.* (1996) |
| *Rhodotorula* sp Y-38 | Ethanol | 0.300 | Yeehn (1996) |
| *Candida bombicola* | Glucose and animal fat | 0.110 | Desphande and Daniels (1995) |
| *Saccharomyces diastaticus*<br>*Saccharomycopsis fibuligera* | Soluble starch in DAF waste from poultry processing | 0.260<br>0.275 | Najafpour *et al.* (1994) |
| *Candida lipolytica and Geotrichum candidum* | Fish oil | 0.320 | Hottinger *et al.* (1974b) |
| *Yarrowia lipolytica* RP2 | Tallow | 0.440 | This study |

The high yield of protein with RP2 cultivation on tallow may be of commercial interest. The use of inedible tallow for the potential production of SCP and metabolites by *Saccaromycopsis lipolytica* and *Candida utilis* has been reported (Kajs and Vanderzant, 1980; Tan and Gill, 1984). Similarly, SCP was produced, which contained all amino acids according to FAO standards except methione, by *Candida lipolytica*, *Torulopsis holmi*, *Candidia mesenterica* and *Cryptococcus albidus* cultivated on fat wastes containing olein (Jacob, 1993).

The potential SCP produced by RP2 cultivated on tallow would not be suitable for human consumption. The nucleic acid content would be too high as FAO standards demand a nucleic acid content of between 1 and 3% for human food. Also, the possible association of tallow with tissue, soil or faecal matter does not make for a palatable product. However, these limitations are not enforced for animal feed. SCP produced from the microbial transformation of prawn-shell waste was utilised as aquaculture feed, which contributed to pollution abatement and the recycling of waste back into the food chain (Rhishipal and Philip, 1998). Protein sludge produced from abattoir effluent may be added to carcass meal as a protein supplement and may generate income (de Villiers and Pretorius, 2001). Overall, the production of value-added products from organic waste-processing systems, together with the reduction of the organic load offers a distinct economic and environmental advantage to such systems (van der Westhuizen and Pretorius, 1996; Nigam and Kakati, 2002).

In the development of a fat removal system with *Y. lipolytica* RP2, investigations were performed in batch fermentations only. Waste fats, especially the hard fats like tallow are produced from abattoir or slaughterhouse processing and generally not operated on a continuous basis (de Villiers and Pretorius, 2001). There can be frequent shutdowns in operation, especially at weekends and holidays. Similarly, many high lipid-containing wastes are as a result of batch processing, including grease traps and olive oil mill wastes (DeFelice *et al.*, 1997; Flores *et al.*, 1999). The use of continuous culture on a commercial scale may be uncertain as there are high costs involved in ensuring complete freedom from adventitious contamination with other microrganisms (Ratledge, 1994). Application of a

continuous system would therefore not be practical and a batch system would be a more cost-effective method of waste fat treatment.

The system was also operated as an aerobic process under near ambient conditions. An aerobic organism and fat removal system was selected over anaerobic treatment based on a number of considerations. Solid fat is considered a problem substrate for anaerobic digesters due to its insolubility and the bacteriotoxicity of long chain fatty acids formed during fat hydrolysis (Broughton *et al.*, 1998). Conventional anaerobic digesters have been shown to achieve only low rates of organic matter removal and require long hydraulic retention (Borja *et al.*, 1998). Anaerobic digesters are also very sensitive to shock loads and operation at elevated temperatures can lead to the production of harmful substances such as dioxins.

These problems associated with anaerobic digestion would not be considered in a fat removal system with RP2. The unique emulsifying properties of the yeast can overcome the solid nature of fat, and toxicity of long chain fatty acids was not evident based on the retention of cell viability during the fermentation. RP2 also achieved a high biomass together with high fat removal over a relatively short period of time. The ability of RP2 to biodegrade 20 g $L^{-1}$ tallow would indicate that the system would not be sensitive to shock loads, as the majority of waste streams contain a lower lipid content (Scioli and Vollaro, 1997; Wakelin and Forster, 1998). Also, the employment of current technology to collect fats, oils and greases from the waste stream, with dissolved air floatation (DAF), for degradation by RP2 would facilitate a standard loading of fat. The development of a fat removal system to operate in conjunction with current technology would obviously be economically advantageous. The development of a large-scale system (60 kL) for the pre-treatment of abattoir effluent with activate sludge has been successful in the removal of fats with a retention time of 120 h (de Villiers and Pretorius, 2001). The ability of *Y. lipolytica* RP2 to achieve similar fat removal rates as activated sludge (Wakelin and Forster, 1998) in a similar time period when grown on a recalcitrant fat such as tallow provides the impetus for the development of a fat removal system with this yeast.

# 5.0 CONCLUSIONS

1. 10 yeast isolates from a commercial rendering plant with lipolytic ability were identified as three distinct yeasts, *Yarrowia lipolytica*, *Candida zeylanoides* and *Debaromyces hansenii*. *Yarrowia lipolytica* demonstrated superior fat removal ability and was selected for study into its growth on tallow. *Y. lipolytica* is a GRAS organism. *Y. lipolytica* was assigned the code RP2, which indicated that it was the second organism isolated from the rendering plant investigated in the laboratory.

2. *Y. lipolytica* RP2 removed up to 75% of a 20 g $L^{-1}$ loading of tallow in batch cultures. The scale-up from shake flasks to 2 L and 10 L fermentation reduced the time required to achieve this from 168 h in shake flasks to 65 h in the fermenters. Superior tallow biodegradation occurred at 25°C and pH 7.0. The growth temperature and pH are in the range of those commonly employed for aerobic systems for the treatment of industrial and processing lipid wastes. Rate of agitation was specific to the growth vessel at 130, 500 and 1000 rpm for shake flask, 2 L and 10 L fermentation, respectively.

3. Nitrogen loading and source were key for tallow biodegradation and a threshold of 0.42 g N $L^{-1}$ and a C:N ratio of 40:1 or lower was required to achieve maximum fat removal. This was provided with 2 g $L^{-1}$ ammonium sulphate. Ionic nutrition was influential on tallow biodegradation by *Y. lipolytica* RP2. $K^+$ was required at a threshold concentration of 60 mM in the medium. The presence of 80 nmol $K^+$ $(10^6 \text{ cell})^{-1}$ correlated with maximum growth and fat removal. $Na^+$ was antagonistic to biodegradation but its presence may be overcome with a $K^+/Na^+$ ratio greater than 10:1 in the medium. $Ca^{2+}$ addition at 10 mM was inhibitory to biodegradation. Addition of $H_2PO_4^-$ at 100 mM did not affect biodegradation. The required concentration of $K^+$ was supplied through pH control with 0.5 M KOH or 0.1 M potassium phosphate buffer. KOH was selected as the preferred method of pH control and $K^+$ addition.

4. The presence of glucose, up to 10 g $L^{-1}$, as an additional carbon source with tallow resulted in the concomitant use of both substrates by *Y. lipolytica* RP2.

5. *Y. lipolytica* RP2 produced a biosurfactant during its growth on tallow. Under superior growth conditions, the biosurfactant demonstrated an emulsifying activity superior to commercial surfactants Triton X-100 and Tween 80 at 0.5 % (w/v).

6. *Y. lipolytica* RP2 is a dimorphic yeast and tallow stimulated mycelial growth at or near pH 7.0. However, dimorphism did not influence tallow biodegradation and yeast-shaped morphology dominated during the removal of fat.

7. Tallow biodegradation by *Y. lipolytica* RP2 resulted in the production of 14 g $L^{-1}$ of biomass with a low lipid content, 0.1 g $g^{-1}$ and a high protein yield, 0.44 g $g^{-1}$. This indicated the potential for the biomass for SCP production.

**Further Study**

The ability of *Y. lipolytica* RP2 to biodegrade other high-lipid containing waste, such as food processing, grease-trap and abattoir wastes would indicate the capacity of the yeast to remove a mixture of lipid sources which would not be as recalcitrant as tallow. Furthermore, the bioaugmentation of activated sludge with the yeast could result in a microbial consortium, which readily removes and metabolises fats.

An examination of the amino acid content of the protein in the biomass would indicate its potential for use. The assessment of the involved cost of protein production and recovery from tallow biodegradation would provide an economic insight into the potential production of SCP.

The design of the fat removal system would require some consideration. An investigation into other methods of agitation, including paddles and recirculation pumps may be useful.

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# 7.0 APPENDIX

# Appendix A: Identification Key No.1 (Barnett *et al.*, 1983)

## Key No. 1: All yeasts that do not ferment D-glucose (test no. 1 negative

## Key involving physiological tests and microscopical description

**Yeasts in Key No. 1**

1 *Aciculoconidium aculeatum*
4 *Ambrosiozyma philentoma*
5 *Arthroascus javanensis*
6 *Botryoascus synnaedendrus*
10 *Brettanomyces custersianus*
13 *Brettanomyces naardenensis*
14 *Bullera alba*
15 *Bullera armeniaca*
16 *Bullera crocea*
17 *Bullera dendrophila*
18 *Bullera pirilcola*
19 *Bullera tsugae*
20 *Candida aaseri*
21 *Candida acnia*
24 *Candida antarctica*
25 *Candida apicola*
26 *Candida apis*
27 *Candida aquatica*
28 *Candida atmosphaerica*
29 *Candida auriculariae*
31 *Candida austromarina*
32 *Candida azyma*
33 *Candida bacarum*
34 *Candida beechii*
35 *Candida berthetii*
36 *Candida blankii*
40 *Candida bombicola*
41 *Candida buffonii*
43 *Candida butyri*
46 *Candida carnislignicola*
48 *Candida catenulata*
50 *Candida chiropterarum*
53 *Candida dendrica*
57 *Candida edax*
62 *Candida etchellsii*
63 *Candida ethanolica*
65 *Candida fermentiearens*
66 *Candida fluvialilis*
67 *Candida foliorum*
68 *Cundida fragariorum*
72 *Candida fusiformata*
73 *Candida geochares*
75 *Candida gloebosa*
76 *Candida graminis*
77 *Candida gropengiesseri*
79 *Candida halonitratophila*
81 *Candida hellenica*
84 *Candida hydrocarbofumarica*
85 *Candida hylophila*
86 *Candida incommunis*
87 *Candida inconspicua*
88 *Candida inositophila*
89 *Candida intestalens*
90 *Candida intestinalis*
94 *Candida karawaiewii*
95 *Candida krissii*
101 *Candida marmitofaciens*
102 *Candida maris*
103 *Candida maritima*
105 *Candida melibiosophila*
106 *Candida membranaefaciens*
107 *Candida mesenterica*
112 *Candida mucilaginu*
113 *Candida multi-gemmis*
116 *Candida nemodendra*
117 *Candida nitratophila*
119 *Candida norvegica*
121 *Candida oregonensis*
122 *Candida paludigena*
124 *Candida pararugosa*
126 *Candida philyla*
128 *Candida pinus*
129 *Candida polymorpha*
131 *Candida psychrophila*
132 *Candida pustula*
134 *Candida quercuum*
137 *Candida rugata*
141 *Candida savonica*
142 *Candida schatavii*
143 *Candida silvae*
145 *Candida silvatica*
148 *Candida sonckii*
150 *Candida sorbophila*
151 *Candida sorboxylosa*
156 *Candida suecica*
158 *Candida tenuis*
159 *Candida tepae*
161 *Candida torresii*
163 *Candida tsukubaensis*
164 *Candida valdiviana*
165 *Candida vanderwaltii*
169 *Candida vinaria*
170 *Candida vini*
173 *Candida xestoba*
174 *Candida zeylanoides*
177 *Cryptococcus albidus*
178 *Cryptococcus amylolentus*
179 *Cryptococcus ater*
180 *Cryptococcus bhutanensis*
181 *Cryptococcus curvatus*
182 *Cryptococcus dimennae*
183 *Cryptococcus elinovii*
184 *Cryptococcus flavus*
185 *Cryptococcus gastricus*
186 *Cryptococcus heveanensis*
187 *Cryptococcus himalayensis*
188 *Cryptococcus humicolus*
189 *Cryptococcus hungaricus*
190 *Cryptococcus kuetzingii*
191 *Cryptococcus laurentii*
192 *Cryptococcus lupi*
193 *Cryptococcus luteolus*
194 *Cryptococcus macerans*
195 *Cryptococcus magnus*
196 *Cryptococcus marinus*
197 *Cryptococcus podzolicus*
198 *Cryptococcus skinneri*
199 *Cryptococcus terreus*
200 *Cryptococcus vishniacii*
202 *Debaryomyces coudertii*
203 *Debaryomyces hansenii*
204 *Debaryomyces marama*
205 *Debaryomyces melissophilus*
206 *Debaryomyces nepalensis*
207 *Debaryomyces polymorphus*
209 *Debaryomyces tamarii*
210 *Debaryomyces vanrijiae*
211 *Debaryomyces yarrowii*
213 *Dekkera bruxellensis*
216 *Endomyces fibuliger*
217 *Endomycopsella crataegensis*
218 *Endomycopsella vini*
219 *Filobasidiella neoformans*
220 *Filobasidium capsuligenum*
221 *Filobasidium floriforme*
222 *Filobasidium uniguttulatum*
223 *Geotrichum armillariae*
224 *Geotrichum candidum*
225 *Geotrichum capitatum*
226 *Geotrichum eriense*
228 *Geotrichum fici*
229 *Geotrichum fragrans*
230 *Geotrichum klebahnii*
231 *Geotrichum penicillatum*
232 *Geotrichum terrestre*
233 *Guilliermondella selenospora*
241 *Hansenula alni*
243 *Hansenula beckii*
244 *Hansenula bimundalis*
245 *Hansenula canadensis*
247 *Hansenula ciferrii*
248 *Hansenula dryadoides*
250 *Hansenula glucozyma*
251 *Hansenula henricii*
258 *Hansenula nonfermentans*
259 *Hansenula ofunaensis*
260 *Hansenula petersonii*
261 *Hansenula philodendra*
263 *Hansenula populi*
267 *Hansenula wickerhamii*
268 *Hormoascus platypodis*
269 *Hyphopichia burtonii*
273 *Issatchenkia terricola*
280 *Kluyveromyces marxianus*
286 *Leucosporidium antarcticum*
287 *Leucosporidium frigidum*
288 *Leucosporidium gelidum*
289 *Leucosporidium rivalis*
290 *Leucosporidium scottii*
291 *Leucosporidium stokesii*
292 *Lipomyces anomalus*
293 *Lipomyces kononenkoae*
294 *Lipomyces lipofer*
295 *Lipomyces starkeyi*
296 *Lipomyces tetrasporus*
298 *Mastigomyces philippovii*
299 *Metschnikowia bicuspidata*
300 *Metschnikowia krissii*
301 *Metschnikowia lunata*
305 *Nadsonia commutata*
311 *Phaffia rhodozyma*
312 *Pichia abadiae*
314 *Pichia ambrosiae*
315 *Pichia amethionina*
320 *Pichia cactophila*
321 *Pichia carsonii*
322 *Pichia castillae*
323 *Pichia chambardii*
324 *Pichia delftensis*
326 *Pichia etchellsii*
327 *Pichia farinosa*
329 *Pichia fluxuum*
330 *Pichia guilliermondii*
331 *Pichia haplophila*
332 *Pichia heedii*
334 *Pichia humboldtii*
335 *Pichia inositovora*
338 *Pichia lindneri*
339 *Pichia media*
340 *Pichia membranaefaciens*
348 *Pichia nakazawae*
349 *Pichia norvegensis*
352 *Pichia opuntiae*
354 *Pichia philogaea*
356 *Pichia pini*
357 *Pichia quercuum*
361 *Pichia salictaria*
369 *Pichia tannicola*
373 *Pichia wickerhamii*
374 *Rhodosporidium bisporidius*
375 *Rhodosporidium capitatum*
376 *Rhodosporidium dacryoidum*
377 *Rhodosporidium diobovatum*
378 *Rhodosporidium infirmo-miniatum*
379 *Rhodosporidium malvinellum*
380 *Rhodosporidium paludigenum*
381 *Rhodosporidium sphaerocarpum*
382 *Rhodosporidium toruloides*
383 *Rhodotorula acheniorum*
384 *Rhodotorula araucariae*
385 *Rhodotorula aurantiaca*
386 *Rhodotorula bogoriensis*
387 *Rhodotorula diffluens*
388 *Rhodotorula fujisanense*
389 *Rhodotorula glutinis*
390 *Rhodotorula graminis*
391 *Rhodotorula ingeniosa*
392 *Rhodotorula javanica*
393 *Rhodotorula lactosa*
394 *Rhodotorula marina*
395 *Rhodotorula minuta*
396 *Rhodotorula mucilaginosa*
397 *Rhodotorula muscorum*
398 *Rhodotorula pallida*
399 *Rhodotorula pilatii*
400 *Rhodotorula pilimanae*
410 *Saccharomycopsis capsularis*
411 *Saccharomycopsis malanga*
412 *Sarcinosporon inkin*
413 *Schizoblastosporion starkeyi-henricii*
419 *Sporidiobolus johnsonii*
420 *Sporidiobolus microsporus*
421 *Sporidiobolus pararoseus*
422 *Sporidiobolus ruinenii*
423 *Sporidiobolus salmonicolor*
424 *Sporobolomyces albo-rubescens*
425 *Sporobolomyces gracilis*
426 *Sporobolomyces holsaticus*
427 *Sporobolomyces puniceus*
428 *Sporobolomyces roseus*
429 *Sporobolomyces singularis*
430 *Sporopachydermia cereana*
431 *Sporopachydermia lactativora*
432 *Sporopachydermia quercuum*
433 *Stephanoascus ciferrii*
434 *Sterigmatomyces elviae*
435 *Sterigmatomyces halophilus*
436 *Sterigmatomyces indicus*
437 *Sterigmatomyces nectairii*
438 *Sterigmatomyces penicillatus*
439 *Sterigmatomyces polyborus*
440 *Sympodiomyces parvus*
444 *Trichosporon aquatile*
445 *Trichosporon beemeri*
446 *Trichosporon beigelii*
447 *Trichosporon brassicae*
448 *Trichosporon dulcitum*
449 *Trichosporon loubieri*
450 *Trichosporon lutetiae*
451 *Trichosporon pullulans*
452 *Trigonopsis variabilis*
454 *Wickerhamiella domercqiae*
459 *Williopsis saturnus*
461 *Yarrowia lipolytica*

**Tests in Key No. 1**

14 D-Galactose growth
15 L-Serbose growth
16 D-Glucosamine growth
19 L-Arabinose growth
20 D-Arabinose growth
22 Sucrose growth
23 Maltose growth
24 α,α-Trehalose growth
25 Me α-D-glucoside growth
26 Cellobiose growth
29 Melibiose growth
30 Lactose growth
31 Raffinose growth
32 Melezitose growth
34 Starch growth
36 Erythritol growth
38 Xylitol growth
41 D-Mannitol growth
42 Galactitol growth
44 D-Glucono-1,5-lactone growth
45 2-Keto-D-gluconate growth
49 DL-Lactate growth
51 Citrate growth
53 Ethanol growth
54 Nitrate growth
56 Ethylamine growth
58 Cadaverine growth
64 Growth without Biotin
65 Growth without Thiamin
67 Growth without Pyridoxine
70 Growth without PABA
71 Growth at 25°C
72 Growth at 30°C
74 Growth at 37°C
75 Growth at 42°C
76 0.01% Cycloheximide growth
78 50% D-Glucose growth
80 Starch formation
82 Urea hydrolysis
84 Pink colonies
87 Splitting cells
88 Filamentous
89 Pseudohyphae
90 Septate hyphae
92 Ballistoconidia

number of different tests 45

## Key No. 1

| | Negative | Positive |
|---|---|---|
| 1 Nitrate growth | 2 | 476 |
| 2 Erythritol growth | 3 | 332 |
| 3 Sucrose growth | 4 | 190 |
| 4 D-Mannitol growth | 5 | 69 |
| 5 D-Galactose growth | 6 | 32 |
| 6 Citrate growth | 7 | 27 |
| 7 0.01% Cycloheximide growth | 8 | 17 |
| 8 Cadaverine growth | 9 | 13 |
| 9 Ethanol growth | 10 | 12 |
| 10 Growth at 25°C | *Nadsonia commutata* | 11 |
| 11 Ethylamine growth | *Lipomyces anomalus* | *Schizoblastosporion starkeyi-henricii* |
| 12 DL-Lactate growth | *Nadsonia commutata* | *Pichia amethionina* |
| 13 Growth without Pyridoxine | 14 | 15 |

| | | Negative | Positive |
|---|---|---|---|
| 14 | D-Gluconsamine growth | *Pichia opuntiae* | *Pichia norvegensis* |
| 15 | Growth without Biotin | *Pichia heedii* | 16 |
| | | *Pichia membranaefaciens* | |
| 16 | Growth without Thiamin | *Candida rugosa* | *Candida ethanolica* |
| | | *Pichia membranaefaciens* | *Pichia membranaefaciens* |
| 17 | Growth at 37°C | 18 | 23 |
| 18 | 50% D-Glucose growth | 19 | 22 |
| 19 | Ethanol growth | *Lipomyces anomalus* | 20 |
| 20 | Cellobiose growth | 21 | *Brettanomyces naardenensis* |
| 21 | D-Glucono-1,5-lactone growth | *Arthrobacus jdvanensis* | *Endomycopsella crataegensis* |
| 22 | L-Sorbose growth | *Saccharomycopsis capsularis* | *Endomycopsella vini* |
| 23 | Xylitol growth | 24 | 26 |
| 24 | Growth without Biotin | *Brettanomyces custersianus* | 25 |
| 25 | Growth without Thiamin | *Geotrichum capitatum* | *Pichia humboldtii* |
| 26 | Growth at 42°C | *Issatchenkia quercuum* | *Sporopachydermia lactativora* |
| 27 | Cellobiose growth | 28 | 33 |
| 28 | D-Glucosamine growth | 29 | *Candida inconspicua* |
| | | | *Pichia caciophila* |
| 29 | Growth without Pyridoxine | 30 | 31 |
| 30 | Xylitol growth | *Pichia opuntiae* | *Candida sorboxylosa* |
| 31 | L-Arabinose growth | 32 | *Trichosporon lutetiae* |
| 32 | Growth without Biotin | *Pichia heedii* | *Candida rugosa* |
| | | | *Issatchenkia terricola* |
| 33 | DL-Lactate growth | 34 | 35 |
| 34 | Galactitol growth | *Saccharomycopsis capsularis* | *Rhodotorula fujisanense* |
| 35 | D-Glucosamine growth | 36 | *Pichia norvegensis* |
| 36 | L-Arabinose growth | 37 | *Trichosporon lutetiae* |
| 37 | D-Glucono-1,5-lactone growth | *Pichia opuntiae* | *Candida dendrica* |
| 38 | Xylitol growth | 39 | 58 |
| 39 | DL-Lactate growth | 40 | 47 |
| 40 | Ethanol growth | 41 | 43 |
| 41 | Ethylamine growth | 42 | *Schizoblastosporion starkeyi-henricii* |
| 42 | Growth without Thiamin | *Candida austromarina* | *Lipomyces anomalus* |
| 43 | Cellobiose growth | 44 | 46 |
| 44 | Starch growth | 45 | *Guilliermondella selenospora* |
| 45 | Ethylamine growth | *Candida austromarina* | *Candida rugosa* |
| 46 | L-Arabinose growth | *Brettanomyces naardenensis* | *Cryptococcus gastricus* |
| 47 | α,α-Trehalose growth | 48 | 55 |
| 48 | Cellobiose growth | 49 | 54 |
| 49 | Growth without Thiamin | 50 | 52 |
| 50 | Growth without Biotin | *Geotrichum fici* | 51 |
| 51 | Growth at 42°C | *Candida rugosa* | *Geotrichum capitatum* |
| 52 | Growth at 30°C | *Geotrichum klebahnii* | 53 |
| 53 | Growth at 37°C | *Geotrichum penicillatum* | *Pichia humboldtii* |
| 54 | Lactose growth | *Pichia chambardii* | *Trichosporon beigelii* |
| 55 | Ethanol growth | *Pichia obeidae* | 56 |
| 56 | Cellobiose growth | 57 | 58 |
| 57 | L-Sorbose growth | *Pichia tannicola* | *Trichosporon brassicae* |
| 58 | Lactose growth | *Brettanomyces naardenensis* | *Trichosporon beigelii* |
| 59 | Cellobiose growth | 60 | 63 |
| 60 | α,α-Trehalose growth | 61 | *Trigonopsis variabilis* |
| 61 | Starch growth | 62 | *Guilliermondella selenospora* |
| 62 | Cadaverine growth | *Candida vinaria* | *Candida rugosa* |
| 63 | α,α-Trehalose growth | 64 | 67 |
| 64 | Urea hydrolysis | 65 | 66 |
| 65 | Melibiose growth | *Candida mucilagina* | *Candida melibiosophila* |
| 66 | Lactose growth | *Rhodotorula fujisanense* | *Trichosporon beigelii* |
| 67 | Urea hydrolysis | *Brettanomyces naardensis* | 68 |
| 68 | DL-Lactate growth | *Cryptococcus gastricus* | *Trichosporon beigelii* |
| 69 | Xylitol growth | 70 | 141 |
| 70 | α,α-Trehalose growth | 71 | 116 |
| 71 | Cellobiose growth | 72 | 103 |
| 72 | D-Galactose growth | 73 | 94 |
| 73 | Ethanol growth | 74 | 77 |
| 74 | 2-Keto-D-gluconate growth | 75 | *Candida karawaiewii* |
| 75 | Cadaverine growth | *Schizoblastosporion starkeyi-henricii* | 76 |
| 76 | D-Glucono-1,5-lactone growth | *Geotrichum armillariae* | *Candida apis* |
| 77 | Ethylamine growth | 78 | 81 |

| | | Negative | Positive |
|---|---|---|---|
| 78 | Growth without Biotin | 79 | *Candida hylophila* |
| 79 | Growth at 37°C | 80 | *Pichia amethionina* |
| 80 | 50% D-Glucose growth | *Endomycopsella vini* | *Endomycopsella vini* |
| 81 | DL-Lactate growth | 82 | 91 |
| 82 | Citrate growth | 83 | 90 |
| 83 | D-Glucono-1,5-lactone growth | 84 | 85 |
| 84 | Growth at 37°C | *Candida vini* | *Candida rugosa* |
| | | *Pichia delftensis* | |
| | | *Pichia fluxuum* | |
| 85 | Growth without Thiamin | 86 | 89 |
| 86 | Growth without Pyridoxine | 87 | 88 |
| 87 | 50% D-Glucose growth | *Candida silvae* | *Candida apis* |
| 88 | Growth at 37°C | *Candida apis* | *Candida rugosa* |
| 89 | 50% D-Glucose growth | *Pichia delftensis* | *Candida apis* |
| 90 | 2-Keto-D-gluconate growth | 86 | *Candida krissii* |
| 91 | Growth without Pyridoxine | 92 | 93 |
| 92 | D-Glucono-1,5-lactone growth | *Pichia opuntiae* | *Candida silvae* |
| 93 | Growth at 37°C | *Candida vini* | *Candida rugosa* |
| 94 | DL-Lactate growth | 95 | 98 |
| 95 | 0.01% Cycloheximide growth | 96 | 97 |
| 96 | Cadaverine growth | *Schizoblastosporion starkeyi-henricii* | 99 |
| 97 | Growth without Thiamin | *Candida sorbophila* | *Geotrichum armillariae* |
| 98 | Growth without Thiamin | 99 | 101 |
| 99 | Growth without Biotin | 100 | *Candida rugosa* |
| 100 | L-Sorbose growth | *Candida cariosilata* | *Geotrichum fragrans* |
| 101 | Ethanol growth | *Geotrichum armillariae* | 102 |
| 102 | D-Glucono-1,5-lactone growth | *Geotrichum klebahnii* | *Oosporium candidum* |
| 103 | DL-Lactate growth | 104 | 111 |
| 104 | 2-Keto-D-gluconate growth | 105 | 107 |
| 105 | Ethanol growth | *Geotrichum armillariae* | 106 |
| 106 | L-Sorbose growth | *Pichia quercuum* | *Endomycopsella vini* |
| 107 | Lactose growth | 108 | *Sporobolomyces singularis* |
| 108 | Starch growth | 109 | 110 |
| 109 | Citrate growth | *Endomycopsella vini* | *Candida krissii* |
| 110 | Growth at 37°C | *Saccharomycopsis capsularis* | *Candida parapsilosis malitosa* |
| 111 | Lactose growth | 112 | 115 |
| 112 | Ethanol growth | *Geotrichum armillariae* | 113 |
| 113 | D-Glucono-1,5-lactone growth | 114 | *Pichia salicaria* |
| 114 | Growth without Pyridoxine | *Pichia opuntiae* | *Geotrichum erictor* |
| 115 | D-Galactose growth | *Sporobolomyces singularis* | *Trichosporon beigelii* |
| 116 | Citrate growth | 117 | 128 |
| 117 | Cellobiose growth | 118 | 122 |
| 118 | Ethylamine growth | 119 | 120 |
| 119 | Growth without Biotin | 85 | *Rhodotorula pallida* |
| 120 | Ethanol growth | *Schizoblastosporion starkeyi-henricii* | 121 |
| 121 | Growth without Thiamin | *Candida silvatica* | *Candida intermedia* |
| 122 | Urea hydrolysis | 123 | 126 |
| 123 | L-Sorbose growth | 124 | *Endomycopsella vini* |
| 124 | Ethylamine growth | *Saccharomycopsis capsularis* | 125 |
| 125 | Growth without Thiamin | *Bretianomyces naardenensis* | *Candida intermedia* |
| 126 | Growth without PABA | 127 | 68 |
| 127 | Ethylamine growth | *Rhodotorula pallida* | *Rhodosporidium dacryoidum* |
| 128 | L-Arabinose growth | 129 | 136 |
| 129 | Cellobiose growth | 130 | 132 |
| 130 | DL-Lactate growth | 131 | *Candida carnulata* |
| 131 | 0.01% Cycloheximide growth | *Candida philyla* | *Candida zeylanoides* |
| 132 | Growth without Thiamin | 133 | 135 |
| 133 | Lactose growth | 134 | *Trichosporon beigelii* |
| 134 | Starch growth | *Rhodosporidium dacryoidum* | *Saccharomycopsis capsularis* |
| 135 | Maltose growth | *Candida beechii* | *Candida intermedia* |
| | | *Candida zeylanoides* | |
| 136 | Lactose growth | 137 | 140 |
| 137 | D-Glucosamine growth | 138 | 57 |
| 138 | Maltose growth | 139 | *Saccharomycopsis capsularis* |
| 139 | D-Arabinose growth | *Trichosporon huetiae* | *Cryptococcus skinneri* |
| 140 | Ethanol growth | *Pichia abadiae* | 115 |
| 141 | Cellobiose growth | 142 | 157 |
| 142 | D-Galactose growth | 143 | 149 |

| | Negative | Positive |
|---|---|---|
| 143 α,α-Trehalose growth | 144 | 147 |
| 144 Ethylamine growth | 79 | 145 |
| 145 0.01% Cycloheximide growth | 93 | 146 |
| 146 L-Sorbose growth | *Candida maris* | *Sporopachydermia lactativora* |
| 147 Citrate growth | 119 | 148 |
| 148 D-Glucosamine growth | *Hansenula nonfermentans* | *Candida philyla* |
| 149 α,α-Trehalose growth | 150 | 154 |
| 150 Growth without Pyridoxine | 151 | 152 |
| 151 0.01% Cycloheximide growth | *Candida pelliculosa* | *Candida sorbophila* |
| 152 Cadaverine growth | *Candida vinaria* | 153 |
| 153 Growth without Biotin | *Candida catenulata* | *Candida rugosa* |
| 154 Galactitol growth | 155 | *Hansenula ofunaensis* |
| 155 Citrate growth | *Rhodotorula pallida* | 156 |
| 156 L-Sorbose growth | *Candida catenulata* | *Trigonopsis variabilis* |
| 157 L-Arabinose growth | 158 | 177 |
| 158 α,α-Trehalose growth | 159 | 165 |
| 159 DL-Lactate growth | 160 | 163 |
| 160 Ethanol growth | *Sporobolomyces gracilis* | 161 |
| 161 L-Sorbose growth | 162 | *Endomycopsella vini* |
| 162 2-Keto-D-gluconate growth | *Pichia quercuum* | 130 |
| 163 Ethanol growth | *Sporobolomyces gracilis* | 164 |
| 164 D-Galactose growth | *Pichia solictaria* | *Trichosporon beigelii* |
| 165 2-Keto-D-gluconate growth | 166 | 171 |
| 166 Citrate growth | 167 | 169 |
| 167 Ethanol growth | *Sporobolomyces gracilis* | 168 |
| 168 L-Sorbose growth | *Brettanomyces naardenensis* | *Endomycopsella vini* |
| 169 Ethanol growth | *Sporobolomyces gracilis* | 170 |
| 170 D-Glucosamine growth | *Hansenula nonfermentans* | *Filobasidium capsuligenum* |
| | *Pichia lindneri* | |
| 171 Urea hydrolysis | 172 | 176 |
| 172 Ethylamine growth | 22 | 173 |
| 173 Growth without Thiamin | 174 | 193 |
| 174 0.01% Cycloheximide growth | *Candida torresii* | *Brettanomyces naardenensis* |
| 175 D-Galactose growth | *Candida beechii* | *Candida savonica* |
| 176 Lactose growth | 127 | *Trichosporon beigelii* |
| 177 Melezitose growth | 178 | 186 |
| 178 Lactose growth | 179 | 184 |
| 179 Maltose growth | 180 | 183 |
| 180 α,α-Trehalose growth | 181 | 182 |
| 181 Galactitol growth | *Saccharomycopsis malanga* | *Rhodotorula fujisanense* |
| 182 Citrate growth | *Rhodotorula pallida* | *Cryptococcus skinneri* |
| 183 D-Glucosamine growth | 110 | *Filobasidium capsuligenum* |
| 184 D-Galactose growth | *Sporobolomyces singularis* | 185 |
| 185 Urea hydrolysis | *Candida melibiosophila* | *Trichosporon beigelii* |
| 186 α,α-Trehalose growth | 187 | 188 |
| 187 Urea hydrolysis | *Candida mucilagina* | *Trichosporon beigelii* |
| 188 DL-Lactate growth | 189 | *Trichosporon beigelii* |
| 189 Citrate growth | *Cryptococcus gastricus* | *Rhodotorula hoguorienis* |
| 190 Raffinose growth | 191 | 254 |
| 191 Citrate growth | 192 | 218 |
| 192 Cellobiose growth | 193 | 201 |
| 193 D-Mannitol growth | 194 | 196 |
| 194 L-Sorbose growth | *Dekkera bruxellensis* | 195 |
| 195 50% D-Glucose growth | *Arthroascus javanensis* | *Endomycopsella vini* |
| 196 Melezitose growth | 197 | 200 |
| 197 50% D-Glucose growth | 198 | 199 |
| 198 Maltose growth | *Rhodotorula pallida* | *Candida suecica* |
| 199 Ethylamine growth | *Endomycopsella vini* | *Candida bombicola* |
| 200 Xylitol growth | *Candida auriculariae* | *Candida azyma* |
| 201 Urea hydrolysis | 202 | 209 |
| 202 D-Galactose growth | 203 | 206 |
| 203 Melezitose growth | 204 | *Saccharomyces kluyveri* |
| 204 DL-Lactate growth | 205 | *Candida tepae* |
| 205 Growth without Thiamin | *Saccharomyces vini* | *Candida suecica* |
| 206 Maltose growth | *Debaryomyces hansenii* | 207 |
| 207 DL-Lactate growth | 208 | *Candida tepae* |
| 208 Growth at 37°C | *Hansenula henricipiana* | *Metschnikowia lunata* |
| 209 Maltose growth | 210 | 214 |

| | | Negative | Positive |
|---|---|---|---|
| 210 | Lactose growth | 211 | 213 |
| 211 | L-Arabinose growth | 127 | 212 |
| 212 | Melezitose growth | *Rhodotorula pallida* | *Rhodotorula minuta* |
| 213 | Pink colonies | *Trichosporon brigelii* | *Rhodotorula minuta* |
| 214 | Lactose growth | 215 | 217 |
| 215 | D-Gluconо-1,5-lactone growth | *Trichosporon aquatile* | 216 |
| 216 | Starch growth | *Rhodosporidium dacryoidum* | *Sporidiobolus pararoseus* |
| 217 | Ethanol growth | *Rhodotorula marina* | *Trichosporon aquatile* |
| | | | *Trichosporon brigelii* |
| 218 | Urea hydrolysis | 219 | 242 |
| 219 | L-Sorbose growth | 220 | 229 |
| 220 | D-Galactose growth | 221 | 226 |
| 221 | DL-Lactate growth | 222 | 224 |
| 222 | Growth without Biotin | 223 | *Candida maritima* |
| 223 | Growth without Thiamin | *Aciculoconidium aculeatum* | *Candida oregonensis* |
| 224 | Growth without Biotin | 225 | *Candida maritima* |
| 225 | Growth at 37°C | *Candida quercuum* | *Pichia vickerhamii* |
| 226 | D-Arabinose growth | 227 | *Candida tenuis* |
| 227 | Maltose growth | *Debaryomyces tamarii* | 228 |
| 228 | Lactose growth | *Metschnikowia lunata* | *Candida fluviatilis* |
| 229 | Xylitol growth | 230 | 234 |
| 230 | Ethanol growth | *Candida geochares* | 231 |
| 231 | D-Arabinose growth | 232 | *Candida tenuis* |
| 232 | D-Glucosamine growth | 233 | *Candida valdiviana* |
| 233 | Melezitose growth | *Candida suecica* | *Pichia silvicola* |
| 234 | D-Mannitol growth | 235 | 237 |
| 235 | Ethanol growth | *Candida multis-gemmis* | 236 |
| 236 | D-Glucosamine growth | *Candida mucilagina* | *Candida xestobii* |
| 237 | α,α-Trehalose growth | 238 | 239 |
| 238 | Ethanol growth | *Candida geochares* | *Candida mucilagina* |
| 239 | Growth at 37°C | 240 | 241 |
| 240 | D-Arabinose growth | *Pichia carsonii* | *Candida tenuis* |
| 241 | DL-Lactate growth | *Metschnikowia lunata* | *Pichia etchellsii* |
| 242 | Ethanol growth | 243 | 246 |
| 243 | Galactitol growth | 216 | 244 |
| 244 | D-Glucosamine growth | 245 | *Saccharomycopsis gemmifera* |
| 245 | L-Arabinose growth | *Rhodotorula marina* | *Galactomyces geotrichum* |
| 246 | Lactose growth | 247 | 253 |
| 247 | D-Glucosamine growth | 248 | 251 |
| 248 | D-Gluconо-1,5-lactone growth | *Trichosporon mucoides* | 249 |
| 249 | Starch growth | *Rhodosporidium dacryoidum* | 250 |
| 250 | Pink colonies | *Filobasidiella neoformans* | *Sporidiobolus pararoseus* |
| 251 | Me α-D-glucoside growth | *Trichosporon brassicae* | 252 |
| 252 | Melezitose growth | *Phaeococcomyces catenatus* | *Filobasidiella neoformans* |
| 253 | Cadaverine growth | *Guehomyces pullulans* | *Trichosporon aquatile* |
| | | | *Trichosporon brigelii* |
| 254 | Lactose growth | 255 | 300 |
| 255 | Urea hydrolysis | 256 | 284 |
| 256 | Melezitose growth | 257 | 267 |
| 257 | Ethanol growth | 258 | 259 |
| 258 | Growth without Thiamin | *Candida apicola* | *Candida gropengiesseri* |
| 259 | Galactitol growth | 260 | *Candida hellenica* |
| 260 | D-Mannitol growth | 261 | 263 |
| 261 | D-Galactose growth | *Endomycopsella vini* | 262 |
| 262 | D-Glucosamine growth | *Kluyveromyces marxianus* | *Candida xestobii* |
| 263 | D-Galactose growth | 264 | 265 |
| 264 | Ethylamine growth | *Endomycopsella vini* | *Candida apicola* |
| | | | *Candida bombicola* |
| 265 | DL-Lactate growth | 266 | *Kluyveromyces marxianus* |
| 266 | α,α-Trehalose growth | *Candida bombicola* | *Debaryomyces tamarii* |
| 267 | Citrate growth | 268 | 273 |
| 268 | Galactitol growth | 269 | 271 |
| 269 | D-Glucosamine growth | 270 | *Candida valdiviana* |
| 270 | DL-Lactate growth | *Debaryomyces yarrowii* | *Kluyveromyces marxianus* |
| 271 | Melibiose growth | 272 | *Debaryomyces vanrijiae* |
| 272 | Growth without Biotin | *Candida hellenica* | *Candida tropicalis* |
| 273 | D-Glucosamine growth | 274 | 280 |
| 274 | Galactitol growth | 275 | 279 |

| | | Negative | Positive |
|---|---|---|---|
| 275 | D-Galactose growth | *Candida maritima* | 276 |
| 276 | 2-Keto-D-gluconate growth | *Kluyveromyces marxianus* | 277 |
| 277 | Growth without Thiamin | *Pichia carsonii* | 278 |
| 278 | 50% D-Glucose growth | *Lipomyces kononenkoae* | *Debaryomyces hansenii* |
| 279 | 50% D-Glucose growth | *Lipomyces kononenkoae* | *Debaryomyces hansenii* *Debaryomyces vanrijiae* |
| 280 | D-Galactose growth | *Candida maritima* | 281 |
| 281 | D-Mannitol growth | 282 | 283 |
| 282 | D-Arabinose growth | *Candida zeylanoides* | *Pichia guilliermondii* |
| 283 | D-Arabinose growth | *Debaryomyces hansenii* *Debaryomyces vanrijiae* | *Debaryomyces hansenii* *Pichia guilliermondii* |
| 284 | Galactitol growth | 285 | 293 |
| 285 | Starch growth | 286 | 289 |
| 286 | Maltose growth | *Rhodotorula pilimanae* | 287 |
| 287 | D-Glucono-1,5-lactone growth | *Filobasidium uniguttulatum* | 288 |
| 288 | Ballistoconidia | *Rhodotorula mucilaginosa* | *Sporobolomyces roseus* |
| 289 | Cadaverine growth | 290 | 291 |
| 290 | 2-Keto-D-gluconate growth | *Sporobolomyces roseus* | *Filobasidiella neoformans* |
| 291 | D-Arabinose growth | *Filobasidium uniguttulatum* | 250 |
| 292 | Growth without Biotin | *Phaffia rhodozyma* | 297 |
| 293 | Growth at 30°C | 294 | 296 |
| 294 | Ballistoconidia | 295 | *Cryptococcus hungaricus* |
| 295 | Pink colonies | *Sterigmatomyces penicillatus* | *Bullera alba* |
| 296 | Melibiose growth | *Bullera alba* | 299 |
| 297 | Ethanol growth | 298 | *Sporobolomyces roseus* |
| 298 | D-Glucosamine growth | *Rhodotorula mucilaginosa* | *Filobasidiella neoformans* |
| 299 | Starch growth | *Rhodotorula mucilaginosa* | 313 |
| 300 | Ethanol growth | 301 | 300 |
| 301 | Melibiose growth | 302 | 307 |
| 302 | D-Glucosamine growth | 303 | 304 |
| 303 | 2-Keto-D-gluconate growth | *Rhodotorula marina* | 306 |
| 304 | Growth at 30°C | 305 | *Cryptococcus hungaricus* |
| 305 | Galactitol growth | *Cryptococcus magnus* | *Cryptococcus curvatus* |
| 306 | Growth without Thiamin | *Cryptococcus dimennae* | 308 |
| 307 | Ethylamine growth | 295 | *Cryptococcus curvatus* |
| 308 | Growth without Thiamin | *Cryptococcus ater* | 312 |
| 309 | Ballistoconidia | 310 | 311 |
| 310 | Urea hydrolysis | *Lipomyces lipofer* | *Cryptococcus hungaricus* *Cryptococcus laurentii* |
| 311 | Pink colonies | *Cryptococcus laurentii* *Sterigmatomyces penicillatus* | *Bullera alba* |
| 312 | Me α-D-glucoside growth | *Bullera piricola* | 319 |
| 313 | Melezitose growth | 314 | 317 |
| 314 | Urea hydrolysis | 315 | 316 |
| 315 | DL-Lactate growth | *Debaryomyces tamarii* | *Candida glaebosa* |
| 316 | Melibiose growth | *Kluyveromyces marxianus* | *Cryptococcus curvatus* |
| 317 | Growth without Thiamin | 318 | *Trichosporon beigelii* *Trichosporon loubieri* |
| 318 | Splitting cells | *Cryptococcus dimennae* | 326 |
| 319 | Melibiose growth | 320 | 323 |
| 320 | Urea hydrolysis | 321 | 322 |
| 321 | 2-Keto-D-gluconate growth | *Kluyveromyces marxianus* | *Debaryomyces hansenii* |
| 322 | Xylitol growth | *Candida paludigena* | 324 |
| 323 | D-Galactose growth | *Trichosporon dulcitum* | 325 |
| 324 | Cadaverine growth | *Cryptococcus hungaricus* | *Cryptococcus curvatus* |
| 325 | Growth without Thiamin | *Trichosporon beigelii* | 328 |
| 326 | Urea hydrolysis | 327 | *Debaryomyces hansenii* |
| 327 | 50% D-Glucose growth | *Lipomyces lipofer* | 330 |
| 328 | D-Mannitol growth | 329 | *Cryptococcus podzolicus* |
| 329 | Growth without Thiamin | *Trichosporon beigelii* | *Trichosporon beigelii* |
| 330 | Splitting cells | 331 | *Bullera piricola* |
| 331 | Ballistoconidia | *Cryptococcus hungaricus* *Cryptococcus laurentii* | 429 |
| 332 | Galactitol growth | 333 | 371 |
| 333 | Sucrose growth | 334 | 351 |
| 334 | D-Galactose growth | 335 | 347 |
| 335 | Maltose growth | 336 | 342 |
| 336 | α,α-Trehalose growth | 337 | 340 |
| 337 | 2-Keto-D-gluconate growth | 338 | |

| | Negative | Positive |
|---|---|---|
| 338 Cadaverine growth | *Botryoascus synnaedendrus* | 339 |
| 339 Xylitol growth | *Yarrowia lipolytica* | *Candida fermenticarens* |
| 340 Cadaverine growth | 341 | *Yarrowia lipolytica* |
| 341 Ethylamine growth | *Saccharomycopsis malanga* | *Sterigmatomyces indicus* |
| 342 Cellobiose growth | 343 | 345 |
| 343 DL-Lactate growth | 344 | *Botryoascus synnaedendrus* |
| 344 Citrate growth | *Cryptococcus marinus* | *Candida pinu* |
| 345 D-Glucono-1,5-lactone growth | *Sporobolomyces cereana* | 346 |
| 346 Growth at 30°C | *Cryptococcus marinus* | *Pichia pini* |
| 347 Cellobiose growth | *Botryoascus synnaedendrus* | 348 |
| 348 Growth at 37°C | 349 | 350 |
| 349 2-Keto-D-gluconate growth | *Pichia media* | *Saccharomycopsis capsularis* |
| 350 Me α-D-glucoside growth | *Saccharomycopsis malanga* | *Endomyces fibuliger* |
| 351 α,α-Trehalose growth | 352 | 357 |
| 352 Urea hydrolysis | 353 | 356 |
| 353 50% D-Glucose growth | 354 | 355 |
| 354 Cellobiose growth | *Pichia kapsophila* | *Symplodiomyces parvus* |
| 355 Growth without Biotin | *Candida fermenticarens* | *Pichia farinosa* |
| 356 Lactose growth | *Yarrowia lipolytica* | *Trichosporon beigelii* |
| 357 Cellobiose growth | 358 | 363 |
| 358 Citrate growth | *Candida psychrophila* | 359 |
| 359 L-Arabinose growth | 360 | 361 |
| 360 Growth at 42°C | *Candida schatavi* | *Pichia farinosa* |
| 361 Growth at 30°C | *Debaryomyces coudertii* | 362 |
| 362 Growth at 42°C | *Pichia castillae* | *Pichia farinosa* |
| 363 2-Keto-D-gluconate growth | 364 | 368 |
| 364 50% D-Glucose growth | 365 | 367 |
| 365 Ethanol growth | *Symplodiomyces parvus* | 366 |
| 366 0.01% Cycloheximide growth | *Pichia castillae* | *Pichia pini* |
| 367 Maltose growth | *Pichia farinosa* | *Pichia media* |
| 368 DL-Lactate growth | 369 | *Trichosporon beigelii* |
| 369 Growth at 30°C | *Debaryomyces coudertii* | 370 |
| 370 Growth at 42°C | *Pichia pini* | *Pichia farinosa* |
| 371 Raffinose growth | 372 | 408 |
| 372 DL-Lactate growth | 373 | 398 |
| 373 D-Galactose growth | 374 | 378 |
| 374 Starch growth | 375 | 376 |
| 375 L-Sorbose growth | *Debaryomyces melissophilus* | *Candida rugosa* |
| 376 Growth without Biotin | 377 | *Filobasidiella neoformans* |
| 377 Growth at 37°C | *Pichia ambrosiae* | *Endomyces fibuliger* |
| 378 D-Arabinose growth | 379 | 391 |
| 379 Ethanol growth | 380 | 382 |
| 380 Melezitose growth | *Symplodiomyces parvus* | 381 |
| 381 2-Keto-D-gluconate growth | *Candida atmosphaerica* | *Pichia philogaea* |
| 382 D-Glucosamine growth | 383 | 389 |
| 383 Xylitol growth | 384 | 385 |
| 384 Maltose growth | *Pichia farinosa* | *Debaryomyces melissophilus* |
| 385 Maltose growth | *Pichia farinosa* | 386 |
| 386 2-Keto-D-gluconate growth | 387 | 393 |
| 387 D-Glucono-1,5-lactone growth | *Candida polymorpha* | *Candida ravenii* |
| 388 Filamentous | *Debaryomyces marama* | *Myxophichia burtonii* |
| 389 2-Keto-D-gluconate growth | *Candida atmosphaerica* | 390 |
| 390 Growth without Thiamin | *Pichia nakazawae* | *Myxophichia burtonii* |
| 391 Ethanol growth | 380 | 392 |
| 392 2-Keto-D-gluconate growth | 393 | 395 |
| 393 Lactose growth | 394 | *Leucosporidium scottii* |
| 394 Growth at 37°C | *Candida atmosphaerica* | *Candida butyri* |
| 395 Urea hydrolysis | 396 | *Filobasidiella neoformans* |
| 396 Pseudohyphae | *Myxophichia burtonii* | 397 |
| 397 Septate hyphae | *Candida tenuis* | *Pichia nakazawae* |
| 398 D-Arabinose growth | 399 | 404 |
| 399 Xylitol growth | 400 | 401 |
| 400 Growth without Thiamin | *Trichosporon aquatile* | *Sarcosporon inkin* |
| | *Trichosporon beigelii* | |
| 401 D-Galactose growth | 402 | 403 |
| 402 Starch growth | *Leucosporidium phlebotomus* | *Pichia imbaxaca* |
| 403 Growth without Thiamin | *Trichosporon beigelii* | *Debaryomyces marama* |
| 404 Growth without Biotin | 405 | 406 |

| | Negative | Positive |
|---|---|---|
| 405 Urea hydrolysis | *Candida tenuis* | *Trichosporon aquatile*<br>*Trichosporon beigelii* |
| 406 Lactose growth | *Filobasidiella neoformans* | 407 |
| 407 Splitting cells | *Cryptococcus humicolus* | *Trichosporon beigelii* |
| 408 Urea hydrolysis | 409 | 419 |
| 409 D-Galactose growth | 410 | 411 |
| 410 L-Sorbose growth | *Endomyces fibuliger* | *Lipomyces tetrasporus* |
| 411 50% D-Glucose growth | *Lipomyces starkeyi*<br>*Lipomyces tetrasporus* | 412 |
| 412 D-Glucosamine growth | 413 | 417 |
| 413 Growth without Biotin | 414 | 416 |
| 414 Filamentation | *Debaryomyces hansenii*<br>*Debaryomyces marama* | 415 |
| 415 Septate hyphae | *Debaryomyces hansenii* | *Hyphopichia burtonii* |
| 416 Septate hyphae | *Debaryomyces hansenii*<br>*Debaryomyces polymorphus* | *Hyphopichia burtonii* |
| 417 Melibiose growth | 416 | 418 |
| 418 Growth without Biotin | *Debaryomyces hansenii*<br>*Debaryomyces nepalensis* | *Debaryomyces hansenii*<br>*Debaryomyces polymorphus* |
| 419 Melibiose growth | 420 | 425 |
| 420 Lactose growth | *Filobasidiella neoformans* | 421 |
| 421 D-Galactose growth | 422 | 423 |
| 422 α,α-Trehalose growth | *Candida acuta* | *Sterigmatomyces elviae* |
| 423 Growth without Thiamin | 424 | *Cryptococcus curvatus* |
| 424 Splitting cells | *Sterigmatomyces elviae* | *Trichosporon beigelii* |
| 425 Ethanol growth | 426 | 427 |
| 426 Starch growth | *Bullera alba* | *Cryptococcus flavus* |
| 427 Lactose growth | *Cryptococcus amylolentus* | 428 |
| 428 Cadaverine growth | *Cryptococcus flavus* | 407 |
| 429 Lactose growth | 430 | 454 |
| 430 Melezitose growth | 431 | 441 |
| 431 α,α-Trehalose growth | 432 | 454 |
| 432 Cellobiose growth | 433 | *Sympodiomyces parvus* |
| 433 Citrate growth | *Pichia burtonii* | *Candida fermenticarens* |
| 434 Sucrose growth | 435 | 439 |
| 435 Ethanol growth | 436 | 437 |
| 436 Ethylamine growth | *Sympodiomyces parvus* | *Pichia media* |
| 437 Citrate growth | *Candida nemodendra* | 438 |
| 438 Growth at 37°C | *Pichia media* | *Pichia castillae* |
| 439 L-Sorbose growth | *Sympodiomyces parvus* | 440 |
| 440 Xylitol growth | *Candida chiropterorum* | *Torulaspora delbrii* |
| 441 Urea hydrolysis | 442 | 447 |
| 442 50% D-Glucose growth | *Lipomyces starkeyi*<br>*Lipomyces tetrasporus* | 443 |
| 443 Xylitol growth | *Candida chiropterorum* | 444 |
| 444 D-Arabinose growth | 445 | 446 |
| 445 Growth without Biotin | *Debaryomyces hansenii*<br>*Debaryomyces marama* | *Debaryomyces hansenii*<br>*Debaryomyces polymorphus*<br>*Debaryomyces vanrijiae* |
| 446 Septate hyphae | *Debaryomyces hansenii*<br>*Debaryomyces polymorphus* | *Candida membranaefaciens* |
| 447 Ethanol growth | 448 | 449 |
| 448 Ethylamine growth | 295 | *Cryptococcus luteolus* |
| 449 Melibiose growth | 450 | 453 |
| 450 Starch growth | 451 | 452 |
| 451 Ethylamine growth | *Cryptococcus hungaricus* | *Cryptococcus luteolus* |
| 452 Growth at 30°C | *Cryptococcus hungaricus* | *Filobasidiella neoformans* |
| 453 Starch formation | *Cryptococcus amylolentus* | 451 |
| 454 Urea hydrolysis | 455 | 461 |
| 455 50% D-Glucose growth | 456 | 459 |
| 456 Melibiose growth | 457 | *Lipomyces lipofer*<br>*Lipomyces starkeyi*<br>*Lipomyces tetrasporus* |
| 457 Growth at 37°C | *Lipomyces tetrasporus* | *Candida blankii* |
| 458 Growth at 42°C | 459 | 460 |
| 459 Growth without Biotin | *Debaryomyces hansenii*<br>*Debaryomyces marama* | *Debaryomyces hansenii*<br>*Debaryomyces polymorphus* |
| 460 Septate hyphae | *Candida hydrocarbofumarica* | *Candida blankii* |

| | | Negative | Positive |
|---|---|---|---|
| 461 | Ethanol growth | 462 | 460 |
| 462 | Coneverine growth | 463 | 467 |
| 463 | D-Glucosamine growth | 464 | 465 |
| 464 | Starch formation | *Cryptococcus flavus* | *Cryptococcus hungaricus* |
| 465 | **Pink colonies** | 466 | *Cryptococcus hungaricus* |
| 466 | **Filamentous** | *Cryptococcus laurentii* | *Cryptococcus laurentii* |
| | | *Sterigmatomyces polyborus* | *Sterigmatomyces penicillatus* |
| 467 | Melibiose growth | *Cryptococcus curvatus* | 468 |
| 468 | **Ballistoconidia** | *Cryptococcus laurentii* | *Bullera alba* |
| 469 | Melibiose growth | 470 | 474 |
| 470 | Maltose growth | 471 | 471 |
| 471 | Cadaverine growth | *Cryptococcus hungaricus* | 472 |
| 472 | Growth without Thiamin | 473 | *Cryptococcus curvatus* |
| 473 | **Splitting cells** | *Cryptococcus terrestris* | *Trichosporon beigelii* |
| 474 | **Cadaverine growth** | 464 | 475 |
| 475 | Splitting cells | *Cryptococcus humicolus* | *Trichosporon beemeri* |
| | | *Cryptococcus laurentii* | *Trichosporon beigelii* |
| 476 | Erythritol growth | 477 | 393 |
| 477 | Melezitose growth | 478 | 535 |
| 478 | Cellobiose growth | 479 | 496 |
| 479 | α,α-Trehalose growth | 480 | 485 |
| 480 | Urea hydrolysis | 481 | 483 |
| 481 | **Maltose growth** | 482 | *Candida etchellsii* |
| 482 | Citrate growth | *Candida halonitratophila* | *Wickerhamiella domercqiae* |
| 483 | Cadaverine growth | 484 | *Leucosporidium antarcticum* |
| 484 | **Growth without Thiamin** | *Rhodotorula aurantiaca* | *Sporidiobolus salmonicolor* |
| 485 | Xylitol growth | 486 | 491 |
| 486 | **2-Keto-D-gluconate growth** | 487 | 489 |
| 487 | Growth without Thiamin | 488 | *Sporidiobolus salmonicolor* |
| 488 | Growth without PABA | *Sterigmatomyces nectairii* | *Rhodotorula aurantiaca* |
| 489 | Growth without Thiamin | 490 | *Rhodocerula araucariae* |
| 490 | Pink colonies | *Candida sonckii* | *Rhodotorula aurantiaca* |
| 491 | D-Glucosamine growth | 492 | 494 |
| 492 | Cadaverine growth | 484 | 493 |
| 493 | Ethanol growth | *Candida vanderwaltii* | *Hansenula nonfermentans* |
| 494 | **Galactitol growth** | 495 | *Hansenula nonfermentans* |
| 495 | Cadaverine growth | *Sporidiobolus salmonicolor* | *Candida nitratophila* |
| 496 | **Sucrose growth** | 497 | 520 |
| 497 | DL-Lactate growth | 498 | 511 |
| 498 | Galactitol growth | 499 | 509 |
| 499 | 2-Keto-D-gluconate growth | 500 | 505 |
| 500 | Growth at 37°C | 501 | 503 |
| 501 | Growth without Thiamin | 502 | *Sporidiobolus salmonicolor* |
| 502 | Growth without PABA | *Rhodosporidium malvinellum* | *Rhodotorula aurantiaca* |
| 503 | Growth at 42°C | *Hansenula nonfermentans* | 504 |
| 504 | Growth without Thiamin | *Hansenula glucozyma* | *Hansenula henricii* |
| 505 | Lactose growth | 506 | 508 |
| 506 | Pink colonies | 507 | *Rhodotorula aurantiaca* |
| 507 | L-Sorbose growth | *Candida foliorum* | *Candida pustula* |
| 508 | Growth at 30°C | *Candida foliorum* | *Cryptococcus terreus* |
| 509 | Lactose growth | 510 | *Cryptococcus himalayensis* |
| | | | *Cryptococcus terreus* |
| 510 | Melibiose growth | *Rhodosporidium malvinellum* | *Rhodotorula javanica* |
| 511 | α,α-Trehalose growth | 512 | 517 |
| 512 | 0.01% Cycloheximide growth | 513 | 515 |
| 513 | Growth without Thiamin | *Hansenula popnii* | 514 |
| 514 | Growth without Pyridoxine | *Candida norvegica* | *Williopsis saturnus* |
| 515 | Cadaverine growth | *Sporidiobolus salmonicolor* | 516 |
| 516 | Growth at 42°C | *Hansenula dryadoides* | *Candida berthetii* |
| 517 | 2-Keto-D-gluconate growth | 518 | *Candida foliorum* |
| 518 | Growth without Thiamin | *Rhodosporidium malvinellum* | 519 |
| 519 | Cadaverine growth | *Sporidiobolus salmonicolor* | *Williopsis saturnus* |
| 520 | Maltose growth | 521 | 526 |
| 521 | 2-Keto-D-gluconate growth | 522 | 524 |
| 522 | 0.01% Cycloheximide growth | 523 | *Williopsis saturnus* |
| 523 | DL-Lactate growth | *Rhodotorula aurantiaca* | |

Negative

524 Septate hyphae — *Rhodotorula graminis*
525 Galactitol growth — *Sporidiobolus salmonicolor*
526 Raffinose growth — *Rhodotorula aurantiaca*
527 D-Galactose growth — *Cryptococcus kuetzingii*
528 Ethylamine growth — *Leucosporidium frigidum*
*Leucosporidium nivalis*

529 Growth without Thiamin — 530
530 Me α-D-glucoside growth — 531
531 Growth without PABA — *Rhodosporidium malvinellum*
532 Cadaverine growth — *Rhodotorula aurantiaca*
533 Ballistoconidia — *Rhodosporidium paludigenum*
*Rhodotorula graminis*

534 Galactitol growth — *Sporidiobolus salmonicolor*
535 Raffinose growth — 536
536 Sucrose growth — 537
537 Lactose growth — 538

538 Growth without Biotin — *Candida buffoni*
539 Urea hydrolysis — 540
540 Cellobiose growth — *Dekkera bruxellensis*
541 Septate hyphae — 542
542 Growth at 37°C — *Hansenula alni*
*Hansenula bimundalis*

543 2-Keto-D-gluconate growth — 544
544 Ballistoconidia — 545
545 Growth at 30°C — *Rhodotorula aurantiaca*

546 Growth at 37°C — 547
547 Pseudohyphae — *Sporobolomyces holsaticus*
548 Pink colonies — 549
549 L-Arabinose growth — 550
550 Growth without Thiamin — *Bullera tsugae*
551 Growth at 30°C — *Rhodotorula aurantiaca*
552 Melibiose growth — 553
553 2-Keto-D-gluconate growth — 554
554 D-Glucono-1,5-lactone growth — 555
555 Growth at 25°C — *Cryptococcus hupi*
*Cryptococcus vishniacii*

556 Filamentous — *Rhodotorula glutinis*
557 Cadaverine growth — 558
558 Ballistoconidia — *Rhodosporidium sphaerocarpum*
*Rhodotorula glutinis*

559 Growth without Biotin — *Hansenula petersonii*

560 D-Arabinose growth — 561
561 D-Mannitol growth — 562
562 Starch growth — *Rhodotorula glutinis*

563 L-Arabinose growth — 564
564 Ethanol growth — *Rhodotorula ingeniosa*
565 Growth without Biotin — *Sporobolomyces puniceus*
566 Growth at 30°C — *Leucosporidium scottii*
*Rhodotorula muscorum*

567 D-Glucosamine growth — 568
568 Starch formation — 569
569 Pink colonies — *Cryptococcus albidus*
*Leucosporidium scottii*

570 Growth at 25°C — *Sporobolomyces puniceus*

571 Me α-D-glucoside growth — 572
572 Starch growth — *Cryptococcus albidus*
*Leucosporidium scottii*

573 Growth at 25°C — *Leucosporidium stokesii*
574 Growth without Thiamin — *Cryptococcus albidus*
*Leucosporidium scottii*

575 Starch growth — 576
576 Growth at 30°C — 577

Positive

525
*Sporidiobolus microsporus*
527
528
*Rhodotorula graminis*
533
*Candida valdiviana*
532
*Candida mannitofaciens*
534
*Sporidiobolus ruinenii*
552
539
*Cryptococcus elinovii*
*Cryptococcus terreus*
484
543
541
*Hansenula beckii*
*Hansenula binundalis*
*Hansenula canadensis*
548
546
*Rhodosporidium toruloides*
*Rhodotorula glutinis*
*Sporidiobolus johnsonii*
*Sporidiobolus salmonicolor*
551
*Cryptococcus albidus*
*Rhodotorula pilatii*
*Rhodotorula glutinis*
585
560
557
556
*Sporidiobolus salmonicolor*
599
*Sporidiobolus salmonicolor*
*Rhodosporidium toruloides*
*Rhodotorula glutinis*
575
563
*Cryptococcus luqi*
*Cryptococcus vishniacii*
567
565
566
*Rhodotorula glutinis*
571
570
*Rhodotorula glutinis*
*Cryptococcus albidus*
*Cryptococcus bhutanensis*
574
573
*Cryptococcus albidus*
*Candida fragariorum*
*Leucosporidium scottii*
579
578

| | | Negative | Positive |
|---|---|---|---|
| 577 | L-Arabinose growth | *Leucosporidium scottii* | 574 |
| | | *Rhodotorula mucosum* | |
| 578 | Pink colonies | *Cryptococcus albidus* | *Rhodosporidium diobovatum* |
| | | | *Rhodotorula glutinis* |
| 579 | Me α-D-glucoside growth | 580 | 582 |
| 580 | Growth at 25°C | *Rhodosporidium capitatum* | 581 |
| | | *Rhodosporidium infirmo-miniatum* | |
| 581 | Pink colonies | *Cryptococcus albidus* | *Rhodosporidium bisporidii* |
| | | | *Rhodosporidium infirmo-miniatum* |
| 582 | D-Glucosamine growth | 583 | 584 |
| 583 | Pink colonies | *Cryptococcus albidus* | *Rhodosporidium infirmo-miniatum* |
| | | *Cryptococcus bhutanensis* | |
| 584 | Filamentous | *Cryptococcus albidus* | *Rhodosporidium fluviale* |
| 585 | Growth at 25°C | 586 | 587 |
| 586 | Septate hyphae | *Candida aquatica* | *Leucosporidium scottii* |
| 587 | Me α-D-glucoside growth | 588 | 590 |
| 588 | Lactose growth | 589 | *Rhodosporidium bisporidii* |
| 589 | Pink colonies | *Cryptococcus albidus* | 591 |
| 590 | Growth without PABA | *Rhodotorula lactosa* | *Bullera armeniaca* |
| 591 | Filamentous | *Cryptococcus albidus* | *Candida valdiviana* |
| 592 | 0.01% Cycloheximide growth | *Cryptococcus albidus* | 603 |
| 593 | Raffinose growth | 594 | 598 |
| 594 | Sucrose growth | 595 | 597 |
| 595 | Cellobiose growth | 596 | *Nematozyma wickerhamii* |
| 596 | D-Arabinose growth | *Hansenula philodendra* | *Sterigmatomyces halophilus* |
| 597 | 2-Keto-D-gluconate growth | 804 | 601 |
| 598 | Xylitol growth | 599 | *Cryptococcus albidus* |
| 599 | L-Arabinose growth | 600 | *Rhodotorula diffluens* |
| 600 | Galactitol growth | *Candida gramina* | *Cryptococcus albidus* |
| 601 | L-Arabinose growth | 602 | *Candida incommunis* |
| 602 | L-Sorbose growth | *Hormonema platypodis* | 612 |
| 603 | Melibiose growth | 604 | 606 |
| 604 | Melezitose growth | 605 | *Geotrichum terricola* |
| 605 | Galactitol growth | *Candida carnsilignicola* | 608 |
| 606 | 2-Keto-D-gluconate growth | 607 | *Hansenula ciferrii* |
| 607 | D-Galactose growth | *Candida bacarum* | 609 |
| 608 | D-Mannitol growth | *Candida tsukubaensis* | *Candida fusiformata* |
| 609 | Growth without Thiamin | 610 | 611 |
| 610 | Pink colonies | *Cryptococcus albidus* | *Rhodosporidium infirmo-miniatum* |
| 611 | Ethylamine growth | *Cryptococcus macerans* | 617 |
| 612 | DL-Lactate growth | 613 | 616 |
| 613 | Galactitol growth | 614 | *Cryptococcus macerans* |
| 614 | Pink colonies | 615 | *Trichosporon pullulans* |
| 615 | Splitting cells | *Cryptococcus albidus* | *Candida edax* |
| 616 | Growth without Thiamin | *Cryptococcus albidus* | 620 |
| 617 | Lactose growth | 618 | *Candida fusiformata* |
| 618 | Growth without Thiamin | 619 | *Cryptococcus macerans* |
| 619 | Pink colonies | *Cryptococcus albidus* | 623 |
| 620 | Me α-D-glucoside growth | 621 | 622 |
| 621 | Pink colonies | *Cryptococcus albidus* | *Cryptococcus macerans* |
| 622 | D-Glucono-1,5-lactone growth | *Rhodotorula acheniorum* | *Candida antarctica* |
| 623 | Filamentous | 624 | *Rhodotorula acheniorum* |
| 624 | Pink colonies | *Cryptococcus albidus* | |

# Appendix B: Identification Key No.3 (Barnett *et al.*, 1993)

## Key No. 3: All yeasts that ferment D-glucose (test no. 1 positive)

## Key involving physiological tests and microscopical description

Yeasts in Key No. 3

1 *Aciculoconidium aculeatum*
2 *Ambrosiozyma cicatricosa*
3 *Ambrosiozyma monospora*
4 *Ambrosiozyma philentoma*
7 *Brettanomyces abstinens*
8 *Brettanomyces anomalus*
9 *Brettanomyces clausenii*
10 *Brettanomyces custersianus*
11 *Brettanomyces custersii*
12 *Brettanomyces lambicus*
13 *Brettanomyces naardenensis*
22 *Candida albicans*
23 *Candida anatomiae*
25 *Candida apicola*
27 *Candida aquatica*
28 *Candida atmosphaerica*
30 *Candida auringiensis*
31 *Candida austromarina*
34 *Candida beechii*
35 *Candida berthelii*
36 *Candida blankii*
37 *Candida boidinii*
38 *Candida boleticola*
39 *Candida bombi*
40 *Candida bombicola*
42 *Candida buinensis*
43 *Candida butyri*
44 *Candida cacaoi*
45 *Candida cantarellii*
46 *Candida cariosilignicola*
47 *Candida castellii*
48 *Candida catenulata*
49 *Candida chilensis*
51 *Candida conglobata*
52 *Candida curiosa*
53 *Candida dendrica*
54 *Candida dendronema*
55 *Candida diddensiae*
56 *Candida diversa*
58 *Candida entomaea*
59 *Candida entomophila*
60 *Candida ergasiensis*
61 *Candida ernobii*
62 *Candida etchellsii*
63 *Candida ethanolica*
64 *Candida fennica*
66 *Candida fluviatilis*
69 *Candida freyschussii*
70 *Candida friedrichii*
71 *Candida fructus*
73 *Candida geochares*
74 *Candida glabrata*
77 *Candida gropengiesseri*
78 *Candida haemulonii*
79 *Candida holmiitratophila*
80 *Candida halophila*
81 *Candida hellenica*
82 *Candida homilentoma*
83 *Candida humilis*
84 *Candida hydrocarbofumarica*
86 *Candida incommunis*
88 *Candida insectophila*
90 *Candida insectamans*
91 *Candida insectorum*
92 *Candida intermedia*
93 *Candida ishiwadae*
94 *Candida karawaiewii*
96 *Candida krusei*
97 *Candida lactis-condensi*
98 *Candida lodderae*
99 *Candida magnaliae*
100 *Candida maltosa*
101 *Candida mannitofaciens*
103 *Candida maritima*
104 *Candida melibiosica*
106 *Candida membranaefaciens*
107 *Candida mesenterica*
108 *Candida methanosorbosa*
109 *Candida methylica*
110 *Candida milleri*
111 *Candida magii*
113 *Candida multis-gemmis*
114 *Candida musae*
115 *Candida naeodendra*
117 *Candida nitratophila*
118 *Candida nodaensis*
119 *Candida norvegica*
120 *Candida oleophila*
121 *Candida oregonensis*
122 *Candida paludigena*
123 *Candida parapsilosis*
125 *Candida peltata*
127 *Candida pignaliae*
128 *Candida pinus*
129 *Candida polymorpha*
130 *Candida pseudointermedia*
133 *Candida quercitrusa*
134 *Candida quercuum*
135 *Candida rhagii*
136 *Candida ragopelliculosa*
138 *Candida sake*
139 *Candida salmanticensis*
140 *Candida santamariae*
141 *Candida savonica*
142 *Candida schatavii*
143 *Candida silvae*
144 *Candida silvanorum*
146 *Candida silvicultrix*
147 *Candida solani*
149 *Candida sonorensis*
151 *Candida sorboxylosa*
152 *Candida spandovensis*
153 *Candida steatolytica*
154 *Candida stellata*
155 *Candida succiphila*
156 *Candida succica*
157 *Candida tannotolerans*
158 *Candida tenuis*
160 *Candida terebra*
161 *Candida torresii*
162 *Candida tropicalis*
164 *Candida valdiviana*
166 *Candida vartiovaarat*
167 *Candida veronae*
168 *Candida versatilis*
170 *Candida vini*
171 *Candida viswanathii*
172 *Candida wickerhamii*
173 *Candida xestobii*
174 *Candida zeylanoides*
175 *Citeromyces matritensis*
176 *Clavispora lusitaniae*
201 *Debaryomyces castellii*
203 *Debaryomyces hansenii*
206 *Debaryomyces nepalensis*
207 *Debaryomyces polymorphus*
208 *Debaryomyces pseudopolymorphus*
209 *Debaryomyces tamaru*
210 *Debaryomyces vanrijiae*
212 *Debaryozyma yamadae*
213 *Dekkera bruxellensis*
214 *Dekkera intermedia*
215 *Eeniella nana*
216 *Endomyces fibuliger*
217 *Endomycopsella crataegensis*
218 *Endomycopsella vini*
220 *Fibobasidium capsuligenum*
224 *Geotrichum candidum*
226 *Geotrichum eriense*
227 *Geotrichum fermentans*
228 *Geotrichum fici*
229 *Geotrichum fragrans*
230 *Geotrichum klebahnii*
231 *Geotrichum penicillatum*
233 *Guilliermondella selenospora*
234 *Hanseniaspora guilliermondii*
235 *Hanseniaspora nodistigri*
236 *Hanseniaspora occidentalis*
237 *Hanseniaspora osmophila*
238 *Hanseniaspora uvarum*
239 *Hanseniaspora valbyensis*
240 *Hanseniaspora vineae*
242 *Hansenula anomala*
243 *Hansenula beckii*
244 *Hansenula bimundalis*
246 *Hansenula capsulata*
247 *Hansenula ciferrii*
249 *Hansenula fabianii*
250 *Hansenula glucozyma*
251 *Hansenula henricii*
252 *Hansenula holstii*
253 *Hansenula jadinii*
254 *Hansenula lynferdii*
255 *Hansenula minuta*
256 *Hansenula mrakii*
257 *Hansenula muscicola*
258 *Hansenula nonfermentans*
260 *Hansenula petersonii*
262 *Hansenula polymorpha*
264 *Hansenula silvicola*
265 *Hansenula subpelliculosa*
266 *Hansenula sydowiorum*
268 *Hormoascus platypodis*
269 *Hyphopichia burtonii*
270 *Issatchenkia occidentalis*
271 *Issatchenkia orientalis*
272 *Issatchenkia scutulata*
273 *Issatchenkia terricola*
274 *Kloeckera lindneri*
275 *Kluyveromyces aestuarii*
276 *Kluyveromyces africanus*
277 *Kluyveromyces blattae*
278 *Kluyveromyces delphensis*
279 *Kluyveromyces lodderae*
280 *Kluyveromyces marxianus*
281 *Kluyveromyces phaffii*
282 *Kluyveromyces polysporus*
283 *Kluyveromyces thermotolerans*
284 *Kluyveromyces waltii*
285 *Kluyveromyces wickerhamii*
287 *Leucosporidium frigidum*
288 *Leucosporidium gelidum*
289 *Leucosporidium nivalis*
291 *Leucosporidium scotii*
297 *Lodderomyces elongisporus*
298 *Mastigomyces philippovii*
299 *Metschnikowia hicuspidata*
301 *Metschnikowia lunata*
302 *Metschnikowia pulcherrima*
303 *Metschnikowia reukaufii*
304 *Metschnikowia zobellii*
306 *Nadsonia elongata*
307 *Nadsonia fulvescens*
308 *Nematospora coryli*
309 *Pachysolen tannophilus*
310 *Pachytichospora transvaalensis*
311 *Phaffia rhodozyma*
312 *Pichia abadiae*
313 *Pichia acaciae*
314 *Pichia ambrosiae*
316 *Pichia amylophila*
317 *Pichia angophorae*
318 *Pichia besseyi*
319 *Pichia bovis*
320 *Pichia cactophila*
324 *Pichia delftensis*
325 *Pichia dispora*
326 *Pichia etchellsii*
327 *Pichia farinosa*
328 *Pichia fermentans*
329 *Pichia fluxuum*
330 *Pichia guilliermondii*
331 *Pichia haplophila*
332 *Pichia heedii*
333 *Pichia heimii*
336 *Pichia kluyveri*
337 *Pichia kodamae*
338 *Pichia lindneri*
340 *Pichia membranaefaciens*
341 *Pichia methanolica*
342 *Pichia mexicana*
343 *Pichia meyerae*
344 *Pichia mitsiisippiensis*
345 *Pichia mucosa*
346 *Pichia naganishii*
347 *Pichia nakasei*
348 *Pichia sakazakii*
349 *Pichia norvegensis*
350 *Pichia ohmeri*
351 *Pichia onychis*
353 *Pichia pastoris*
354 *Pichia philogaea*
355 *Pichia pijperi*
356 *Pichia pini*
357 *Pichia quercuum*
358 *Pichia rabadensis*
359 *Pichia rhodanensis*
360 *Pichia salicl*
362 *Pichia sargentensis*
363 *Pichia scolyti*
364 *Pichia segobiensis*
365 *Pichia sorbitophila*
366 *Pichia spartinae*
367 *Pichia stipitis*
368 *Pichia strasburgensis*
369 *Pichia tannicola*
370 *Pichia toletana*
371 *Pichia trehalophila*
372 *Pichia veronae*
373 *Pichia wickerhamii*
401 *Saccharomyces cerevisiae*
402 *Saccharomyces dairensis*
403 *Saccharomyces exiguus*
404 *Saccharomyces kluyveri*
405 *Saccharomyces servazzii*
406 *Saccharomyces telluris*
407 *Saccharomyces unisporus*
408 *Saccharomycodes ludwigii*
409 *Saccharomycodes sinensis*
410 *Saccharomycopsis capsularis*
411 *Saccharomycopsis malanga*
414 *Schizosaccharomyces japonicus*
415 *Schizosaccharomyces malidevorans*
416 *Schizosaccharomyces octosporus*
417 *Schizosaccharomyces pombe*
418 *Schwanniomyces occidentalis*
432 *Sporopachydermia quercuum*
441 *Torulaspora delbrueckii*
442 *Torulaspora globosa*
443 *Torulaspora pretoriensis*
453 *Wickerhamia fluorescens*
455 *Williopsis beiferinckii*
456 *Williopsis californica*
457 *Williopsis mrakii*
458 *Williopsis pratensis*
459 *Williopsis saturnus*
460 *Wingea robertsiae*
462 *Zygosaccharomyces bailii*
463 *Zygosaccharomyces bisporus*
464 *Zygosaccharomyces cidri*
465 *Zygosaccharomyces fermentati*
466 *Zygosaccharomyces florentinus*
467 *Zygosaccharomyces microellipsoides*
468 *Zygosaccharomyces mrakii*
469 *Zygosaccharomyces rouxii*

Tests in Key No. 3

9 Cellobiose fermentation
12 Inulin fermentation
14 D-Galactose growth
15 L-Sorbose growth
19 L-Arabinose growth
20 D-Arabinose growth
21 L-Rhamnose growth
22 Sucrose growth
23 Maltose growth
25 Me α-D-glucoside growth
26 Cellobiose growth
29 Melibiose growth
30 Lactose growth
31 Raffinose growth
34 Starch growth
35 Glycerol growth
36 Erythritol growth
38 Xylitol growth
41 D-Mannitol growth
44 D-Glucono-1,5-lactone growth
45 2-Keto-D-gluconate growth
47 D-Gluconate growth
49 DL-Lactate growth
50 Succinate growth
51 Citrate growth
54 Nitrate growth
57 L-Lysine growth
58 Cadaverine growth
62 Growth without *myo*-Inositol
64 Growth without Biotin
65 Growth without Thiamin
67 Growth without Pyridoxine
68 Growth without Niacin
72 Growth at 30°C
73 Growth at 35°C
74 Growth at 37°C
75 Growth at 42°C
76 0.01% Cycloheximide growth
78 50% D-Glucose growth
79 60% D-Glucose growth
82 Urea hydrolysis
88 Filamentous
90 Septate hyphae

number of different tests 43

## Key No. 3

| | Negative | Positive |
|---|---|---|
| 1 Erythritol growth | 2 | 542 |
| 2 D-Mannitol growth | 3 | 218 |
| 3 Sucrose growth | 4 | 135 |
| 4 D-Galactose growth | 5 | 100 |
| 5 Cellobiose growth | 6 | 75 |

| | | Negative | Positive |
|---|---|---|---|
| 6 | Succinate growth | 7 | 7 |
| 7 | Growth without Pyridoxine | 8 | 8 |
| 8 | L-Sorbose growth | 9 | 9 |
| 9 | Glycerol growth | 10 | 10 |
| 10 | D-Gluconate growth | 11 | 11 |
| 11 | Growth at 35°C | 12 | 12 |
| 12 | L-Lysine growth | 13 | *Kloeckera lindner* |
| 13 | Growth without Niacin | *Saccharomycodes ludwigii* | *Saccharomyces cerevisia* |
| 14 | Urea hydrolysis | 15 | *Schizosaccharomyces octosporu* |
| 15 | Growth without Niacin | *Saccharomyces telluris* | *Saccharomyces cerevisia* |
| 16 | Growth without *myo*-Inositol | 17 | *Candida glabrat* |
| 17 | L-Lysine growth | *Schizosaccharomyces octosporus* | *Kloeckera lindner* |
| 18 | D-Gluconate growth | 19 | *Candida glabrat* |
| 19 | Growth without Niacin | *Kluyveromyces delphensis* | *Saccharomyces cerevisia* |
| 20 | L-Lysine growth | *Endomycopsella vini* | *Nadsonia elongat* |
| 21 | DL-Lactate growth | 22 | 32 |
| 22 | 2-Keto-D-gluconate growth | 23 | 28 |
| 23 | Nitrate growth | 24 | 29 |
| 24 | 0.01% Cycloheximide growth | 25 | 27 |
| 25 | L-Lysine growth | 26 | *Pichia membranaefacien* |
| | | | *Zygosaccharomyces baili* |
| | | | *Zygosaccharomyces bisporu* |
| 26 | Urea hydrolysis | *Saccharomyces cerevisiae* | *Schizosaccharomyces octosporus* |
| 27 | Cellobiose fermentation | *Brettanomyces custersianus* | *Brettanomyces clausseni* |
| 28 | Cellobiose fermentation | *Candida halonitratophila* | *Brettanomyces clausseni* |
| 29 | Nitrate growth | 30 | *Candida intermedia acanthea* |
| 30 | L-Rhamnose growth | 31 | *Candida anatomiae* |
| 31 | Cadaverine growth | 32 | *Zygosaccharomyces baili* |
| | | | *Zygosaccharomyces bisporus* |
| 32 | Growth without *myo*-Inositol | *Candida castellii* | *Torulaspora delbrueckii* |
| 33 | 0.01% Cycloheximide growth | 34 | 37 |
| 34 | 2-Keto-D-gluconate growth | 35 | *Torulaspora delbrueckii* |
| 35 | L-Lysine growth | *Saccharomyces cerevisiae* | 36 |
| 36 | 50% D-Glucose growth | *Issatchenkia occidentalis* | *Issatchenkia scutulata* |
| | | *Pichia membranaefaciens* | *Pichia membranaefaciens* |
| 37 | L-Sorbose growth | 38 | 65 |
| 38 | Growth without Thiamin | 39 | 54 |
| 39 | Citrate growth | 40 | 42 |
| 40 | 0.01% Cycloheximide growth | 41 | 44 |
| 41 | L-Lysine growth | 15 | 43 |
| 42 | Nitrate growth | 43 | *Candida etchellsii* |
| 43 | DL-Lactate growth | *Pichia membranaefaciens* | *Pichia terricola* |
| | | *Pichia nakasei* | *Pichia membranaefaciens* |
| 44 | L-Rhamnose growth | 45 | *Candida anatomiae* |
| 45 | Cellobiose fermentation | 46 | *Brettanomyces clausseni* |
| 46 | D-Gluconate growth | *Brettanomyces custersianus* | *Endomycopsella crataegensis* |
| 47 | DL-Lactate growth | 48 | 51 |
| 48 | Glycerol growth | *Candida anatomiae* | 49 |
| 49 | Nitrate growth | 50 | *Candida etchellsii* |
| 50 | Growth without Biotin | *Pichia nakasei* | *Issatchenkia terricola* |
| 51 | Nitrate growth | 52 | *Candida etchellsii* |
| 52 | Growth without Biotin | 53 | *Pichia fermentans* |
| 53 | Growth without Pyridoxine | *Pichia cactophila* | *Pichia heedii* |
| 54 | Maltose growth | 55 | 64 |
| 55 | Cadaverine growth | 56 | 57 |
| 56 | 2-Keto-D-gluconate growth | *Saccharomyces cerevisiae* | *Torulaspora delbrueckii* |
| 57 | Glycerol growth | 58 | 67 |
| 58 | D-Glucono-1,5-lactone growth | 59 | 61 |
| 59 | Growth at 42°C | 60 | *Candida ethanolica* |
| | | | *Issatchenkia occidentalis* |
| 60 | 50% D-Glucose growth | *Issatchenkia occidentalis* | *Pichia membranaefaciens* |
| | | *Issatchenkia orientalis* | *Issatchenkia orientalis* |
| | | *Pichia membranaefaciens* | *Issatchenkia scutulata* |
| 61 | Growth at 42°C | *Candida rugopelliculosa* | *Pichia membranaefaciens* |
| | | *Issatchenkia orientalis* | *Candida ethanolica* |
| | | *Pichia membranaefaciens* | *Pichia membranaefaciens* |
| 62 | L-Rhamnose growth | 63 | *Pichia pastoris* |

| | | Negative | Positive |
|---|---|---|---|
| 63 | Growth without Pyridoxine | *Pichia kluyveri* | 59 |
| 64 | Nitrate growth | 56 | *Candida etchellsii* |
| 65 | Growth without Pyridoxine | 66 | 70 |
| 66 | Citrate growth | 67 | 69 |
| 67 | L-Lysine growth | 68 | *Nadsonia elongata* |
| 68 | 50% D-Glucose growth | *Endomycopsella ernaegensis* | *Endomycopsella vini* |
| 69 | DL-Lactate growth | *Candida fructus* | *Candida sorboxylosa* |
| 70 | Nitrate growth | 71 | *Candida etchellsii* |
| 71 | Xylitol growth | 72 | 73 |
| 72 | 2-Keto-D-gluconate growth | 36 | *Torulaspora delbrueckii* |
| 73 | 2-Keto-D-gluconate growth | 74 | *Torulaspora delbrueckii* |
| 74 | 0.01% Cycloheximide growth | *Pichia membranaefaciens* | *Sporopachydermia quercuum* |
| 75 | Glycerol growth | 76 | 39 |
| 76 | 2-Keto-D-gluconate growth | 77 | 84 |
| 77 | Growth without *myo*-Inositol | 78 | 81 |
| 78 | **Growth without Thiamin** | 79 | 80 |
| 79 | 0.01% Cycloheximide growth | *Hanseniaspora osmophila* | *Kloeckera lindneri* |
| | | *Hanseniaspora vineae* | |
| 80 | Growth without Biotin | *Hanseniaspora valbyensis* | *Hanseniaspora uvarum* |
| 81 | L-Sorbose growth | 82 | *Endomycopsella vini* |
| 82 | **Growth at 35°C** | *Brettanomyces naardenensis* | 83 |
| 83 | Septate hyphae | *Dekkera intermedia* | *Brettanomyces claussenii* |
| 84 | L-Lysine growth | 85 | 86 |
| 85 | L-Sorbose growth | *Saccharomycopsis capsularis* | *Endomycopsella vini* |
| 86 | Growth without *myo*-Inositol | 87 | 88 |
| 87 | Growth at 35°C | *Hanseniaspora uvarum* | *Hanseniaspora guilliermondii* |
| 88 | D-Gluconate growth | *Brettanomyces naardenensis* | *Candida amazoniae* |
| 89 | DL-Lactate growth | 90 | 93 |
| 90 | L-Sorbose growth | 91 | *Endomycopsella vini* |
| 91 | 2-Keto-D-gluconate growth | 92 | *Saccharomycopsis malanga* |
| 92 | Growth at 35°C | *Candida ernobii* | 83 |
| 93 | D-Gluconate growth | 94 | 98 |
| 94 | L-Rhamnose growth | 95 | *Pichia sargentensis* |
| 95 | Succinate growth | 83 | 96 |
| 96 | Growth without Thiamin | 97 | *Candida berthetii* |
| 97 | Growth without Pyridoxine | *Pichia norvegensis* | *Brettanomyces claussenii* |
| 98 | Cadaverine growth | *Candida dendrica* | 99 |
| 99 | Growth without Thiamin | *Brettanomyces claussenii* | *Williopsis saturnus* |
| 100 | Succinate growth | 101 | 122 |
| 101 | Growth without Nicotinic | 102 | 110 |
| 102 | **0.01% Cycloheximide growth** | 103 | 107 |
| 103 | Glycerol growth | 104 | 106 |
| 104 | Growth at 37°C | 105 | *Pachytichaspora transvaalensis* |
| 105 | Growth without *myo*-Inositol | *Kluyveromyces africanus* | *Saccharomyces dairensis* |
| 106 | 2-Keto-D-gluconate growth | *Zygolatamyces dairensis* | *Kluyveromyces blattae* |
| 107 | Cadaverine growth | 108 | 109 |
| 108 | Growth at 37°C | *Saccharomyces dairensis* | *Saccharomyces servazzii* |
| 109 | Growth without *myo*-Inositol | *Eremella nana* | *Saccharomyces unisporus* |
| 110 | Nitrate growth | 111 | 119 |
| 111 | L-Lysine growth | 112 | 117 |
| 112 | Glycerol growth | 113 | 114 |
| 113 | 0.01% Cycloheximide growth | *Saccharomyces cerevisiae* | *Guilliermondella selenospora* |
| 114 | DL-Lactate growth | 115 | 116 |
| 115 | Growth at 30°C | *Candida tannotolerans* | *Kluyveromyces phaffii* |
| | | | *Saccharomyces cerevisiae* |
| 116 | 0.01% Cycloheximide growth | *Saccharomyces cerevisiae* | *Candida humilis* |
| 117 | 0.01% Cycloheximide growth | 118 | 83 |
| 118 | Cadaverine growth | *Torulaspora delbrueckii* | *Zygosaccharomyces bailii* |
| | | | *Zygosaccharomyces bisporus* |
| 119 | Cellobiose growth | 28 | 120 |
| 120 | Growth at 42°C | 121 | *Brettanomyces abstinens* |
| 121 | Septate hyphae | *Dekkera intermedia* | *Brettanomyces anomalus* |
| | | | *Brettanomyces claussenii* |
| 122 | L-Sorbose growth | 123 | 130 |
| 123 | Nitrate growth | 124 | 129 |
| 124 | L-Lysine growth | 125 | 126 |
| 125 | Growth at 30°C | *Candida wickerhamii* | 113 |
| 126 | L-Arabitose growth | 127 | *Pichia tannicola* |

| | | Negative | Positive |
|---|---|---|---|
| 127 | Cadaverine growth | *Torulaspora delbrueckii* | 128 |
| 128 | Growth at 35°C | *Brettanomyces naardenensis* | *Brettanomyces clausenii* |
| 129 | 0.01% Cycloheximide growth | *Candida etchellsii* | *Brettanomyces anomalus*<br>*Brettanomyces claussenii* |
| 130 | 0.01% Cycloheximide growth | 131 | 132 |
| 131 | Nitrate growth | *Torulaspora delbrueckii* | *Candida etchellsii* |
| 132 | L-Arabinose growth | 133 | *Pichia wickerhamii* |
| 133 | D-Glucono-1,5-lactone growth | 134 | *Geotrichum fici* |
| 134 | Growth at 30°C | *Geotrichum klebahnii* | *Geotrichum penicillatum* |
| 135 | **Xylitol growth** | 136 | 189 |
| 136 | **Cellobiose** growth | 137 | 172 |
| 137 | D-Galactose growth | 138 | 159 |
| 138 | L-Sorbose growth | 139 | 155 |
| 139 | 2-Keto-D-gluconate growth | 140 | 149 |
| 140 | Cadaverine growth | 141 | 146 |
| 141 | Urea hydrolysis | 142 | 145 |
| 142 | Nitrate growth | 143 | *Candida lactis-condensi* |
| 143 | L-Lysine growth | 144 | *Candida stellata* |
| 144 | 0.01% Cycloheximide growth | *Saccharomyces cerevisiae* | *Nematospora coryli* |
| 145 | 50% D-Glucose growth | *Schizosaccharomyces japonicus* | *Schizosaccharomyces pombe* |
| 146 | Cellobiose fermentation | 147 | *Brettanomyces claussenii* |
| 147 | Maltose growth | *Zygosaccharomyces bailii* | 148 |
| 148 | Raffinose growth | *Dekkera bruxellensis* | *Schizosaccharomyces pombe* |
| 149 | **Maltose growth** | 150 | 153 |
| 150 | Cadaverine growth | 151 | 152 |
| 151 | 0.01% Cycloheximide growth | *Torulaspora delbrueckii* | *Torulaspora globosa* |
| 152 | Growth without *myo*-Inositol | *Schizosaccharomyces malidevorans* | *Zygosaccharomyces bailii* |
| 153 | Growth without *myo*-Inositol | *Schizosaccharomyces pombe* | 154 |
| 154 | Cadaverine growth | *Torulaspora delbrueckii* | *Dekkera bruxellensis* |
| 155 | Cadaverine growth | 156 | 157 |
| 156 | L-Lysine growth | *Endomycopsella vini* | *Torulaspora delbrueckii* |
| 157 | Me α-D-glucoside growth | 158 | *Zygosaccharomyces florentinus* |
| 158 | Succinate growth | *Zygosaccharomyces bailii* | *Candida bombi* |
| 159 | Melibiose growth | 160 | 169 |
| 160 | Cadaverine growth | 161 | 163 |
| 161 | Citrate growth | 162 | *Kluyveromyces polysporus* |
| 162 | 2-Keto-D-gluconate growth | *Candida milleri*<br>*Saccharomyces cerevisiae*<br>*Saccharomyces exiguus* | *Torulaspora delbrueckii* |
| 163 | Succinate growth | 164 | 167 |
| 164 | Cellobiose fermentation | 165 | *Brettanomyces clausenii* |
| 165 | **Maltose growth** | 166 | *Brettanomyces lambicus* |
| 166 | DL-Lactate growth | *Zygosaccharomyces bailii* | *Kluyveromyces lodderae* |
| 167 | 2-Keto-D-gluconate growth | 168 | *Candida quercuum* |
| 168 | Growth without Niacin | *Kluyveromyces marxianus* | *Brettanomyces clausenii* |
| 169 | Cadaverine growth | 56 | 170 |
| 170 | Me α-D-glucoside growth | *Zygosaccharomyces microellipsoides* | 171 |
| 171 | Growth at 37°C | *Zygosaccharomyces florentinus* | *Saccharomyces kluyveri* |
| 172 | Nitrate growth | 173 | 186 |
| 173 | D-Galactose growth | 174 | 178 |
| 174 | Growth without Pyridoxine | 175 | 92 |
| 175 | L-Sorbose growth | 176 | *Endomycopsella vini* |
| 176 | Growth without *myo*-Inositol | 177 | *Saccharomycodes ludwigii* |
| 177 | 50% D-Glucose growth | *Hanseniaspora occidentalis* | *Hanseniaspora vineae* |
| 178 | L-Arabinose growth | 179 | 183 |
| 179 | Growth without Niacin | 180 | 181 |
| 180 | Growth at 35°C | *Kluyveromyces aestuarii* | *Kluyveromyces marxianus* |
| 181 | Growth without Thiamin | 83 | 182 |
| 182 | Starch growth | *Saccharomyces kluyveri* | *Schwanniomyces occidentalis* |
| 183 | DL-Lactate growth | 184 | 185 |
| 184 | Glycerol growth | *Candida paludigena* | *Schwanniomyces occidentalis* |
| 185 | L-Rhamnose growth | *Kluyveromyces marxianus* | *Pichia strasburgensis* |
| 186 | Growth without Thiamin | 187 | 188 |
| 187 | 2-Keto-D-gluconate growth | 121 | *Brettanomyces custersii* |
| 188 | **Maltose growth** | *Willopsis saturnus* | *Hansenula jadinii* |
| 189 | Nitrate growth | 190 | 213 |
| 190 | Citrate growth | 191 | 206 |
| 191 | Melibiose growth | 192 | 200 |

| | | Negative | Positive |
|---|---|---|---|
| 192 | DL-Lactate growth | 193 | 195 |
| 193 | Cellobiose growth | 194 | 196 |
| 194 | Cadaverine growth | 156 | 195 |
| 195 | Maltose growth | *Zygosaccharomyces bailii* | *Kluyveromyces thermotolerans* |
| 196 | D-Galactose growth | 197 | *Schwanniomyces occidentalis* |
| 197 | 0.01% Cycloheximide growth | *Candida famata* | *Endomycopsella vini* |
| 198 | 2-Keto-D-gluconate growth | 199 | *Torulaspora delbrueckii* |
| 199 | Raffinose growth | *Kluyveromyces wickerhamii* | *Kluyveromyces marxianus* |
| 200 | Cellobiose growth | 201 | 204 |
| 201 | Me α-D-glucoside growth | 202 | 203 |
| 202 | Cadaverine growth | *Torulaspora delbrueckii* | *Zygosaccharomyces microellipsoides* |
| 203 | Cadaverine growth | *Torulaspora delbrueckii* | 171 |
| 204 | Starch growth | 205 | *Schwanniomyces occidentalis* |
| 205 | D-Arabinose growth | *Saccharomyces kluyveri* | *Candida salmanticensis* |
| 206 | Raffinose growth | 207 | 210 |
| 207 | L-Arabinose growth | 208 | 209 |
| 208 | D-Galactose growth | *Pichia meyerae* | *Candida quercitrusa* |
| 209 | Me α-D-glucoside growth | *Candida multi-gemmis* | *Candida xestobii* |
| 210 | DL-Lactate growth | 211 | 185 |
| 211 | Starch growth | 212 | *Schwanniomyces occidentalis* |
| 212 | D-Arabinose growth | *Candida xestobii* | *Pichia guilliermondii* |
| 213 | D-Galactose growth | 214 | 217 |
| 214 | Maltose growth | *Williopsis saturnus* | 215 |
| 215 | L-Rhamnose growth | 216 | *Hansenula lochii* |
| 216 | Citrate growth | *Williopsis beijerinckii* | *Hansenula jadinii* |
| 217 | Melibiose growth | *Hansenula silvicola* | *Leucosporidium scidum* |
| 218 | Nitrate growth | 219 | 489 |
| 219 | Maltose growth | 220 | 314 |
| 220 | Sucrose growth | 221 | 281 |
| 221 | Cellobiose growth | 222 | 255 |
| 222 | Citrate growth | 223 | 245 |
| 223 | 0.01% Cycloheximide growth | 224 | 238 |
| 224 | Succinate growth | 225 | 220 |
| 225 | L-Lysine growth | 226 | 227 |
| 226 | 2-Keto-D-gluconate growth | *Saccharomyces cerevisiae* | *Candida karawaiewii* |
| 227 | Cadaverine growth | *Torulaspora delbrueckii* | 228 |
| 228 | 50% D-Glucose growth | *Pichia delftensis* | *Zygosaccharomyces bailii*<br>*Zygosaccharomyces bisporus*<br>*Zygosaccharomyces rouxii* |
| 229 | 2-Keto-D-gluconate growth | 230 | 235 |
| 230 | Growth without Pyridoxine | 231 | 234 |
| 231 | L-Lysine growth | *Saccharomyces cerevisiae* | 232 |
| 232 | Growth at 35°C | *Ficsia dispora* | 233 |
| 233 | D-Gluconо-1,5-lactone growth | *Pichia silvae* | *Candida silvae* |
| 234 | L-Lysine growth | *Saccharomyces cerevisiae* | *Candida vini*<br>*Pichia delftensis*<br>*Pichia fluxuum* |
| 235 | Growth without Pyridoxine | 236 | 237 |
| 236 | DL-Lactate growth | *Pichia dispora* | *Ficsia beaveri* |
| 237 | Cadaverine growth | *Torulaspora delbrueckii* | *Candida vini* |
| 238 | Glycerol growth | 239 | 241 |
| 239 | D-Galactose growth | 240 | *Bevilla annae* |
| 240 | L-Sorbose growth | *Candida anatomiae* | *Endomycopsella vini* |
| 241 | D-Galactose growth | 242 | 243 |
| 242 | L-Rhamnose growth | 66 | *Pichia pastoris* |
| 243 | Growth without Biotin | *Oosporidium fragrans* | 244 |
| 244 | D-Glucono-1,5-lactone growth | *Geotrichum klebahnii* | *Saccharomyces candidum* |
| 245 | Xylitol growth | 246 | 253 |
| 246 | L-Rhamnose growth | 247 | 251 |
| 247 | D-Galactose growth | 248 | 249 |
| 248 | 2-Keto-D-gluconate growth | *Candida diversa*<br>*Candida silvae* | *Candida zeylanoides* |
| 249 | DL-Lactate growth | *Candida zeylanoides* | 250 |
| 250 | L-Sorbose growth | *Candida catenulata* | *Geotrichum candidum* |
| 251 | D-Galactose growth | *Candida anatomiae* | 252 |
| 252 | L-Sorbose growth | *Pichia tannicola* | *Pichia abadiae* |
| 253 | D-Galactose growth | 254 | *Candida catenulata* |
| 254 | L-Sorbose growth | *Hansenula nonfermentans* | *Candida fructus* |

| | | Negative | Positive |
|---|---|---|---|
| 255 | Glycerol growth | 256 | 261 |
| 256 | Growth without Pyridoxine | 257 | 258 |
| 257 | D-Galactose growth | *Endomycopsella vini* | *Saccharomyces* [illegible] |
| 258 | L-Arabinose growth | 259 | *Candida sonorensis* |
| 259 | D-Gluconate growth | 260 | *Candida anatomiae* |
| 260 | Citrate growth | *Brettanomyces naardenensis* | *Hansenula nonfermentans* |
| 261 | DL-Lactate growth | 262 | 275 |
| 262 | 2-Keto-D-gluconate growth | 263 | 264 |
| 263 | Citrate growth | 264 | 266 |
| 264 | L-Sorbose growth | 265 | *Endomycopsella vini* |
| 265 | Xylitol growth | *Candida ernobii* | *Candida sonorensis* |
| 266 | Xylitol growth | 267 | 268 |
| 267 | Growth without Biotin | *Candida ernobii* | *Pichia quercuum* |
| 268 | Growth without Biotin | *Hansenula nonfermentans*<br>*Pichia lindneri* | *Pichia quercuum* |
| 269 | Growth without Thiamin | 270 | 272 |
| 270 | Starch growth | 271 | *Saccharomyces* [illegible] |
| 271 | L-Lysine growth | *Endomycopsella vini* | *Candida torresii* |
| 272 | Septate hyphae | 273 | *Candida tanzawaica* |
| 273 | Xylitol growth | *Candida beechii*<br>*Candida zeylanoides*<br>*Candida* [illegible] | 274 |
| 274 | D-Galactose growth | | *Candida tenuis* |
| 275 | Citrate growth | 276 | 278 |
| 276 | L-Arabinose growth | 277 | *Candida sonorensis* |
| 277 | L-Rhamnose growth | *Geotrichum vrienii* | *Pichia sargentensis* |
| 278 | D-Gluconо-1,5-lactone growth | *Geotrichum vrienii* | 279 |
| 279 | 2-Keto-D-gluconate growth | 280 | *Candida tanzawaica* |
| 280 | D-Galactose growth | *Pichia piperi* | *Geotrichum fermentans* |
| 281 | L-Sorbose growth | 282 | 295 |
| 282 | Cellobiose growth | 283 | 293 |
| 283 | Melibiose growth | 284 | 291 |
| 284 | Cadaverine growth | 285 | 286 |
| 285 | 2-Keto-D-gluconate growth | *Saccharomyces cerevisiae* | 151 |
| 286 | Succinate growth | 287 | 289 |
| 287 | Raffinose growth | *Candida bombicola*<br>*Zygosaccharomyces bailii*<br>*Zygosaccharomyces rouxii* | 288 |
| 288 | Growth without Thiamin | *Candida apicola*<br>*Candida bombicola* | *Candida bombicola*<br>*Zygosaccharomyces bailii* |
| 289 | 60% D-Glucose growth | 290 | *Candida* [illegible]<br>*Candida bombicola* |
| 290 | Growth without Niacin | *Kluyveromyces marxianus* | *Wickerhamia fluorescens* |
| 291 | L-Lysine growth | *Saccharomyces cerevisiae* | 292 |
| 292 | 0.01% Cycloheximide growth | 202 | *Zygosaccharomyces mrakii* |
| 293 | DL-Lactate growth | 294 | 180 |
| 294 | D-Galactose growth | *Candida ernobii* | *Debaryomyces tamarii* |
| 295 | Melibiose growth | 296 | 312 |
| 296 | Raffinose growth | 297 | 299 |
| 297 | Cadaverine growth | 156 | 298 |
| 298 | Citrate growth | *Candida bombicola*<br>*Zygosaccharomyces bailii*<br>*Zygosaccharomyces rouxii* | *Candida geochares* |
| 299 | D-Galactose growth | 300 | 308 |
| 300 | Growth without Thiamin | 301 | 303 |
| 301 | L-Lysine growth | *Endomycopsella vini* | 302 |
| 302 | Growth at 37°C | *Candida apicola*<br>*Candida bombicola* | *Candida bombi* |
| 303 | Glycerol growth | 304 | 306 |
| 304 | Cadaverine growth | *Torulaspora delbrueckii* | 305 |
| 305 | Growth without *myo*-Inositol | *Kluyveromyces waltii* | *Zygosaccharomyces bailii* |
| 306 | Citrate growth | 307 | *Candida tropicalis* |
| 307 | Cadaverine growth | *Torulaspora delbrueckii* | *Candida bombicola*<br>*Zygosaccharomyces bailii* |
| 308 | L-Arabinose growth | 309 | 311 |
| 309 | DL-Lactate growth | 306 | 310 |
| 310 | 2-Keto-D-gluconate growth | *Kluyveromyces marxianus* | *Torulaspora delbrueckii* |
| 311 | Growth without Pyridoxine | *Candida spandovensis* | *Kluyveromyces marxianus* |
| 312 | 0.01% Cycloheximide growth | 202 | 313 |

| | | Negative | Positive |
|---|---|---|---|
| 313 | Growth without *myo*-Inositol | *Zygosaccharomyces eidri* | *Zygosaccharomyces florentinus* |
| 314 | D-Galactose growth | 315 | 315 |
| 315 | L-Sorbose growth | 316 | 361 |
| 316 | 2-Keto-D-gluconate growth | 317 | 346 |
| 317 | L-Rhamnose growth | 318 | 337 |
| 318 | L-Arabinose growth | 319 | 332 |
| 319 | Xylitol growth | 320 | 326 |
| 320 | Cellobiose growth | 321 | 322 |
| 321 | L-Lysine growth | *Saccharomyces cerevisiae* | *Zygosaccharomyces rouxii* |
| 322 | Glycerol growth | 323 | 324 |
| 323 | Sucrose growth | *Brettanomyces naardenensis* | *Pichia spartinae* |
| 324 | Growth without Thiamin | 325 | *Pichia spartinae* |
| 325 | 0.01% Cycloheximide growth | *Pichia toletana* | *Candida ernobii* |
| 326 | Citrate growth | 327 | 328 |
| 327 | Cellobiose growth | *Zygosaccharomyces rouxii* | *Brettanomyces naardenensis* |
| 328 | Glycerol growth | 329 | 330 |
| 329 | Growth without Thiamin | *Pichia angusta* | *Pichia spartinae* |
| 330 | Starch growth | 331 | *Filobasidium capsuligenum* |
| 331 | Growth without Thiamin | *Candida guilliermondii* | *Pichia spartinae* |
| 332 | Raffinose growth | 333 | 336 |
| 333 | Xylitol growth | *Candida ernobii* | 334 |
| 334 | DL-Lactate growth | *Filobasidium capsuligenum* | 335 |
| 335 | Growth at 37°C | *Pichia bovis* | *Pichia mississippiensis* |
| 336 | Growth at 42°C | *Pichia onychis* | *Pichia rabaulensis* |
| 337 | Growth at 42°C | 338 | 342 |
| 338 | D-Gluconate growth | 339 | 341 |
| 339 | Glycerol growth | *Brettanomyces naardenensis* | 340 |
| 340 | Growth without Pyridoxine | *Pichia angusta* | 375 |
| 341 | Growth without Biotin | *Pichia ernobii* *Pichia wickerhamii* | *Candida maritima* |
| 342 | L-Arabinose growth | 343 | 345 |
| 343 | Growth without *myo*-Inositol | *Pichia angusta* | 344 |
| 344 | Growth without Pyridoxine | *Pichia rhodanensis* | *Candida guilliermondii* |
| 345 | Raffinose growth | *Pichia mississippiensis* | *Pichia rabaulensis* |
| 346 | Sucrose growth | 347 | 352 |
| 347 | Cellobiose growth | 348 | 349 |
| 348 | Cadaverine growth | *Torulaspora delbrueckii* | *Zygosaccharomyces rouxii* |
| 349 | L-Lysine growth | 350 | 351 |
| 350 | Growth at 35°C | *Saccharomycopsis capsularis* | *Zygosaccharomyces mellonga* |
| 351 | Citrate growth | *Brettanomyces naardenensis* | *Candida intermedia* |
| 352 | Cellobiose growth | 353 | 354 |
| 353 | Citrate growth | 348 | *Candida haemulonii* |
| 354 | L-Rhamnose growth | 355 | 358 |
| 355 | Raffinose growth | 356 | *Phaffia rhodozyma* |
| 356 | Citrate growth | *Candida tolani* | 357 |
| 357 | Growth without Thiamin | *Acidodendridium acidotherum* | *Pichia spartinae* |
| 358 | Growth without Thiamin | 359 | 360 |
| 359 | Growth without Biotin | *Pichia wickerhamii* *Candida oregonensis* | *Candida maritima* |
| 360 | Raffinose growth | | *Phaffia rhodozyma* |
| 361 | Cellobiose growth | 362 | 374 |
| 362 | Citrate growth | 363 | 371 |
| 363 | Raffinose growth | 364 | 369 |
| 364 | Cadaverine growth | 365 | 367 |
| 365 | L-Lysine growth | 366 | *Torulaspora delbrueckii* |
| 366 | Growth without Thiamin | *Endomycopsella vini* | *Candida succica* |
| 367 | L-Rhamnose growth | 368 | *Clavispora lusitaniae* |
| 368 | Me α-D-glucoside growth | *Zygosaccharomyces rouxii* | *Nadsonia fulvescens* |
| 369 | Cadaverine growth | 156 | 370 |
| 370 | Melibiose growth | *Kluyveromyces thermotolerans* | *Zygosaccharomyces florentinus* |
| 371 | Sucrose growth | *Candida succica* | 372 |
| 372 | Growth without Thiamin | *Clavispora lusitaniae* | 373 |
| 373 | Growth without Pyridoxine | *Candida musae* | *Candida haemulonii* |
| 374 | DL-Lactate growth | 375 | 378 |
| 375 | Cadaverine growth | 376 | 377 |
| 376 | Growth without Thiamin | 197 | *Candida succica* |
| 377 | L-Rhamnose growth | *Metschnikowia reukaufii* *Pichia spartinae* | *Clavispora lusitaniae* |
| 378 | L-Arabinose growth | 379 | 381 |

| | | Negative | Positive |
|---|---|---|---|
| 379 | L-Rhamnose growth | 380 | *Clavispora lusitaniae* |
| 380 | Citrate growth | *Candida solani* | *Pichia sparsa* |
| 381 | Starch growth | *Pichia mississippiensis* | *Pichia anomala* |
| 382 | Raffinose growth | 383 | 433 |
| 383 | Cellobiose growth | 384 | 405 |
| 384 | Citrate growth | 385 | 395 |
| 385 | Melibiose growth | 386 | 393 |
| 386 | 2-Keto-D-gluconate growth | 321 | 387 |
| 387 | Growth without Thiamin | 388 | 389 |
| 388 | Xylitol growth | *Hanseniaspora fabianii* | *Clavispora lusitaniae* |
| 389 | 0.01% Cycloheximide growth | 390 | 392 |
| 390 | Cadaverine growth | *Torulaspora delbrueckii* | 391 |
| 391 | 60% D-Glucose growth | *Candida tolerans* | *Zygosaccharomyces rouxii* |
| 392 | Starch growth | *Candida maltosa* | *Candida tropicalis* |
| 393 | L-Lysine growth | *Saccharomyces cerevisiae* | 394 |
| 394 | Cadaverine growth | *Torulaspora delbrueckii* | *Saccharomyces kluyveri* |
| 395 | Growth without Thiamin | 396 | 398 |
| 396 | D-Sorbose growth | *Candida castellata* | 397 |
| 397 | L-Rhamnose growth | *Candida glabrata* | *Clavispora lusitaniae* |
| 398 | Growth at 37°C | 399 | 400 |
| 399 | Growth at 35°C | *Candida tolerans* | *Candida quercitrusa* |
| 400 | L-Arabinose growth | 401 | 403 |
| 401 | Growth at 42°C | 402 | *Candida albicans* |
| | | | *Candida tropicalis* |
| 402 | 60% D-Glucose growth | *Candida maltosa* | *Candida haemulonii* |
| 403 | DL-Lactate growth | 404 | *Candida albicans* |
| 404 | 60% D-Glucose growth | *Candida parapsilosis* | *Candida haemulonii* |
| 405 | Growth at 37°C | 406 | 422 |
| 406 | Citrate growth | 407 | 412 |
| 407 | L-Sorbose growth | 408 | 410 |
| 408 | Sucrose growth | *Brettanomyces naardenensis* | 409 |
| 409 | Lactose growth | *Metschnikowia bicuspidata* | *Kluyveromyces wickerhamii* |
| 410 | Growth without Thiamin | 411 | *Candida sake* |
| 411 | DL-Lactate growth | *Metschnikowia bicuspidata* | *Metschnikowia zobellii* |
| 412 | L-Rhamnose growth | 413 | 420 |
| 413 | 2-Keto-D-gluconate growth | 414 | 415 |
| 414 | L-Sorbose growth | *Filobasidium capsuligenum* | *Candida bullnensis* |
| 415 | 0.01% Cycloheximide growth | *Candida sake* | 416 |
| | | *Metschnikowia pulcherrima* | |
| 416 | Glycerol growth | *Candida palmioleophila* | 417 |
| 417 | Growth without Biotin | 418 | *Candida krusei* |
| 418 | Growth at 35°C | 419 | *Pichia anomala* |
| 419 | 50% D-Glucose growth | *Pichia segobiensis* | *Candida ethanolica* |
| 420 | Growth at 35°C | 421 | *Pichia stipitis* |
| 421 | 50% D-Glucose growth | *Candida tenuis* | *Debaryozyma yamadae* |
| 422 | L-Rhamnose growth | 423 | 431 |
| 423 | 0.01% Cycloheximide growth | 424 | 428 |
| 424 | Melibiose growth | 425 | 427 |
| 425 | DL-Lactate growth | 426 | *Pichia etchellsii* |
| 426 | Growth without Thiamin | *Metschnikowia lunata* | *Metschnikowia reukaufii* |
| 427 | Citrate growth | *Saccharomyces kluyveri* | *Candida melibiosica* |
| 428 | Lactose growth | 429 | *Candida fluviatilis* |
| 429 | Starch growth | *Candida maltosa* | 430 |
| 430 | Septate arthros | *Candida boidinae* | *Candida tropicalis* |
| | | | *Candida viswanathii* |
| 431 | L-Arabinose growth | *Clavispora lusitaniae* | 432 |
| 432 | D-Arabinose growth | *Candida sheatae* | *Candida peltata* |
| 433 | Citrate growth | 434 | 454 |
| 434 | Cellobiose growth | 435 | 443 |
| 435 | Melibiose growth | 436 | 440 |
| 436 | 2-Keto-D-gluconate growth | 437 | 439 |
| 437 | L-Lysine growth | *Saccharomyces cerevisiae* | 438 |
| 438 | DL-Lactate growth | *Kluyveromyces thermotolerans* | *Khuyveromyces marxianus* |
| 439 | Cadaverine growth | *Torulaspora delbrueckii* | *Kluyveromyces thermotolerans* |
| | | *Torulaspora pretoriensis* | |
| 440 | 0.01% Cycloheximide growth | 391 | 441 |
| 441 | Growth without *myo*-Inositol | *Zygosaccharomyces eldri* | 442 |
| 442 | Growth at 37°C | *Zygosaccharomyces florentinus* | *Debaryomyces vanrijiae* |

| | | Negative | Positive |
|---|---|---|---|
| 443 | Melibiose growth | 444 | 450 |
| 444 | DL-Lactate growth | 445 | 448 |
| 445 | 2-Keto-D-gluconate growth | 446 | 447 |
| 446 | Growth without Biotin | *Candida hellenica* | *Candida inositophila* |
| 447 | Starch growth | *Candida rhagii* | *Schwanniomyces occidentalis* |
| 448 | Growth without Niacin | 180 | 449 |
| 449 | Growth without *myo*-Inositol | *Zygosaccharomyces fermentati* | *Candida rhagii* |
| 450 | D-Arabinose growth | 451 | 453 |
| 451 | 0.01% Cycloheximide growth | *Saccharomyces kluyveri* | 452 |
| 452 | Growth without Biotin | *Schwanniomyces occidentalis* | *Debaryomyces vanrijiae* |
| 453 | Glycerol growth | *Candida salmanticensis* | *Schwanniomyces occidentalis* |
| 454 | Xylitol growth | 455 | 461 |
| 455 | DL-Lactate growth | 456 | 185 |
| 456 | Glycerol growth | 457 | 458 |
| 457 | Growth without Thiamin | *Candida paludigena* | *Candida intermedia* |
| 458 | Cellobiose growth | *Candida haemulonii* | 459 |
| 459 | Filamentous | *Schwanniomyces occidentalis* | 460 |
| 460 | Lactose growth | *Pichia silvicola* | *Candida intermedia* |
| 461 | Melibiose growth | 462 | 484 |
| 462 | Cellobiose growth | 463 | 465 |
| 463 | 2-Keto-D-gluconate growth | 464 | *Candida haemulonii* |
| | | | *Debaryomyces hansenii* |
| 464 | DL-Lactate growth | *Candida magnoliae* | *Kluyveromyces marxianus* |
| 465 | Glycerol growth | 466 | 469 |
| 466 | DL-Lactate growth | 467 | *Kluyveromyces marxianus* |
| 467 | Lactose growth | 468 | *Candida intermedia* |
| 468 | L-Arabinose growth | *Candida pseudointermedia* | *Pichia guilliermondii* |
| 469 | DL-Lactate growth | 470 | 480 |
| 470 | L-Rhamnose growth | 471 | 476 |
| 471 | Lactose growth | 472 | 474 |
| 472 | Starch growth | *Debaryomyces hansenii* | 473 |
| | | *Pichia guilliermondii* | |
| 473 | 50% D-Glucose growth | *Schwanniomyces occidentalis* | *Debaryomyces hansenii* |
| 474 | 50% D-Glucose growth | 475 | *Candida intermedia* |
| | | | *Debaryomyces hansenii* |
| 475 | Filamentous | *Schwanniomyces occidentalis* | *Candida intermedia* |
| 476 | Lactose growth | 477 | 478 |
| 477 | 2-Keto-D-gluconate growth | *Candida steatolytica* | *Debaryomyces hansenii* |
| | | | *Pichia guilliermondii* |
| 478 | 2-Keto-D-gluconate growth | *Candida steatolytica* | 479 |
| 479 | Filamentous | *Debaryomyces hansenii* | *Candida intermedia* |
| | | *Debaryotyma yamadae* | *Debaryomyces hansenii* |
| 480 | L-Rhamnose growth | 481 | 482 |
| 481 | 2-Keto-D-gluconate growth | *Kluyveromyces marxianus* | *Debaryomyces hansenii* |
| 482 | Growth without Thiamin | 483 | *Debaryomyces hansenii* |
| 483 | L-Sorbose growth | *Pichia strasburgensis* | *Candida steatolytica* |
| 484 | D-Arabinose growth | 485 | 472 |
| 485 | L-Rhamnose growth | 486 | 488 |
| 486 | 0.01% Cycloheximide growth | *Candida membranifaciens* | 487 |
| | | *Debaryomyces hansenii* | |
| 487 | 50% D-Glucose growth | *Schwanniomyces occidentalis* | *Debaryomyces hansenii* |
| | | | *Debaryomyces vanrijiae* |
| 488 | Inulin fermentation | *Debaryomyces hansenii* | *Debaryomyces castellii* |
| | | *Debaryomyces vanrijiae* | |
| 489 | Maltose growth | 490 | 514 |
| 490 | D-Galactose growth | 491 | 503 |
| 491 | DL-Lactate growth | 492 | 499 |
| 492 | L-Rhamnose growth | 493 | 497 |
| 493 | 50% D-Glucose growth | 494 | 496 |
| 494 | L-Arabinose growth | 495 | *Candida pignaliae* |
| 495 | Growth at 37°C | *Hansenula anomala* | *Hansenula nonfermentans* |
| 496 | Growth at 35°C | *Candida halonitratophila* | *Candida magnoliae* |
| | | *Candida sodaensis* | |
| 497 | Growth at 42°C | *Hansenula nonfermentans* | 498 |
| 498 | Growth without Thiamin | *Hansenula glucozyma* | *Hansenula henricii* |
| 499 | Growth without Pyridoxine | 500 | 501 |
| 500 | Growth without Thiamin | *Hansenula minneapolisensis* | *Candida norvegica* |
| 501 | Me α-D-glucoside growth | 502 | *Williopsis californica* |

| | | Negative | Positive |
|---|---|---|---|
| 502 | D-Gluconate growth | *Candida berthetii* | *Williopsis mrakii*<br>*Williopsis saturnus* |
| 503 | L-Rhamnose growth | 504 | 510 |
| 504 | L-Arabinose growth | 505 | 507 |
| 505 | Cellobiose growth | 496 | 506 |
| 506 | D-Glucono-1,5-lactone growth | *Candida halophila* | *Candida magnoliae* |
| 507 | Raffinose growth | 508 | 509 |
| 508 | Citrate growth | *Pachysolen tannophilus* | *Candida halophila* |
| 509 | Citrate growth | *Candida curiosa* | *Leucosporidium frigidum*<br>*Leucosporidium nivalis* |
| 510 | Sucrose growth | 511 | *Leucosporidium frigidum*<br>*Leucosporidium nivalis* |
| 511 | DL-Lactate growth | 512 | 513 |
| 512 | Cellobiose growth | *Candida nitratophila* | *Candida wickerhamii* |
| 513 | L-Sorbose growth | *Hansenula mucicola* | *Candida methanosorbosa* |
| 514 | Raffinose growth | 515 | 520 |
| 515 | L-Rhamnose growth | 516 | 524 |
| 516 | Me α-D-glucoside growth | 517 | 520 |
| 517 | Cellobiose growth | 518 | 519 |
| 518 | Succinate growth | *Candida oddsiensis* | *Candida etchellsii* |
| 519 | Growth at 30°C | *Candida mannitofaciens* | *Candida versatilis* |
| 520 | Glycerol growth | *Williopsis pratensis* | 521 |
| 521 | L-Sorbose growth | 522 | *Williopsis californica* |
| 522 | 2-Keto-D-gluconate growth | 523 | *Candida versatilis* |
| 523 | Starch growth | *Candida vartiovaarai* | *Hansenula anomala* |
| 524 | D-Galactose growth | 525 | 528 |
| 525 | Growth without Pyridoxine | 526 | 527 |
| 526 | Septate hyphae | *Hansenula bimundalis* | *Hansenula beckii* |
| 527 | L-Arabinose growth | *Williopsis californica* | *Hansenula holstii* |
| 528 | D-Arabinose growth | 529 | *Hansenula silvicola* |
| 529 | Sucrose growth | *Hansenula mucicola* | 536 |
| 530 | D-Galactose growth | 531 | 534 |
| 531 | Growth at 42°C | 532 | *Citeromyces matritensis* |
| 532 | L-Sorbose growth | 533 | *Hansenula anomala* |
| 533 | Citrate growth | *Williopsis beijerinckii* | *Hansenula petersonii* |
| 534 | L-Rhamnose growth | 535 | *Hansenula fabianii* |
| 535 | Starch growth | *Hansenula jadinii* | 539 |
| 536 | L-Sorbose growth | 537 | 538 |
| 537 | 2-Keto-D-gluconate growth | *Hansenula anomala* | *Leucosporidium scotii* |
| 538 | Starch growth | 539 | 540 |
| 539 | Xylitol growth | *Candida valdiviana* | 541 |
| 540 | Melibiose growth | *Leucosporidium scotii* | *Leucosporidium gelidum* |
| 541 | L-Rhamnose growth | *Candida aquatica* | 542 |
| 542 | Nitrate growth | 543 | 571 |
| 543 | Sucrose growth | 544 | 555 |
| 544 | D-Galactose growth | 545 | 548 |
| 545 | Cellobiose growth | 546 | 548 |
| 546 | L-Sorbose growth | 547 | *Pichia kodamae* |
| 547 | Succinate growth | *Candida pinus* | *Pichia trehalophila* |
| 548 | L-Arabinose growth | *Candida canturelii* | 553 |
| 549 | Maltose growth | 550 | 552 |
| 550 | 2-Keto-D-gluconate growth | 551 | *Pichia methanolica* |
| 551 | Growth without Thiamin | *Pichia kodamae*<br>*Pichia pini* | *Pichia pini* |
| 552 | Starch growth | *Pichia pini* | *Saccharomycopsis malanga* |
| 553 | Growth at 35°C | *Saccharomycopsis capsularis* | 554 |
| 554 | Me α-D-glucoside growth | *Saccharomycopsis malanga* | *Endomyces fibuliger* |
| 555 | Cellobiose growth | 556 | 558 |
| 556 | Succinate growth | 557 | *Pichia farinosa* |
| 557 | Citrate growth | *Pichia haplophila* | 559 |
| 558 | Cadaverine growth | *Candida canturelii* | 560 |
| 559 | Growth without Biotin | *Candida boleticola* | *Pichia farinosa* |
| 560 | Growth at 42°C | *Candida schatavii* | 566 |
| 561 | 2-Keto-D-gluconate growth | 562 | 565 |
| 562 | Maltose growth | 563 | *Candida cacaoi*<br>*Pichia farinosa* |
| 563 | Growth at 42°C | 564 | |

| | | Negative | Positive |
|---|---|---|---|
| 564 | 0.01% Cycloheximide growth | *Candida conglobata* | *Pichia membranaefaciens* |
| | | | *Pichia pini* |
| 565 | Growth without Biotin | *Pichia acaciae* | *Pichia sorbitophila* |
| 566 | Citrate growth | 567 | 569 |
| 567 | Lactose growth | 568 | *Candida auringiensis* |
| 568 | DL-Lactate growth | *Pichia pini* | *Candida succiphila* |
| 569 | Growth at 42°C | 570 | *Pichia farinosa* |
| 570 | 50% D-Glucose growth | *Pichia pini* | *Candida boleticola* |
| 571 | L-Rhamnose growth | 572 | 611 |
| 572 | Raffinose growth | 573 | 589 |
| 573 | D-Galactose growth | 574 | 578 |
| 574 | L-Arabinose growth | 575 | 577 |
| 575 | L-Sorbose growth | 576 | *Candida mesenterica* |
| 576 | Growth at 35°C | *Pichia ambrosiae* | *Endomyces fibuliger* |
| 577 | 2-Keto-D-gluconate growth | *Ambrosiozyma monospora* | *Ambrosiozyma cicatricosa* |
| 578 | 2-Keto-D-gluconate growth | 579 | 583 |
| 579 | Growth at 35°C | 580 | 581 |
| 580 | Lactose growth | *Candida atmosphaerica* | *Candida ergatensis* |
| 581 | Maltose growth | *Pichia farinosa* | 582 |
| 582 | Starch growth | *Candida butyri* | *Candida naeodendra* |
| | | *Candida diddensiae* | |
| 583 | Starch growth | 584 | 587 |
| 584 | Maltose growth | *Pichia farinosa* | 585 |
| 585 | L-Lysine growth | *Pichia philogaea* | 586 |
| 586 | Growth without Biotin | *Candida diddensiae* | *Pichia heimii* |
| 587 | Growth without Thiamin | *Pichia nakazawae* | 588 |
| 588 | 0.01% Cycloheximide growth | *Candida fennica* | *Pichia stipitis* |
| | | *Hyphopichia burtonii* | |
| 589 | Melibiose growth | 590 | 595 |
| 590 | Starch growth | 591 | 593 |
| 591 | Citrate growth | *Candida rhagii* | 592 |
| 592 | Septate hyphae | *Debaryomyces hansenii* | *Pichia heimii* |
| 593 | D-Galactose growth | *Endomyces fibuliger* | 594 |
| 594 | Septate hyphae | *Debaryomyces hansenii* | *Candida fennica* |
| | | *Debaryomyces polymorphus* | *Hyphopichia burtonii* |
| 595 | D-Arabinose growth | 596 | 604 |
| 596 | Lactose growth | 597 | 602 |
| 597 | Growth without Biotin | 598 | 601 |
| 598 | Starch growth | *Candida friedrichii* | 599 |
| | | *Debaryomyces hansenii* | |
| 599 | Growth at 37°C | 600 | *Debaryomyces hansenii* |
| | | | *Debaryomyces nepalensis* |
| 600 | Septate hyphae | *Debaryomyces hansenii* | *Candida fennica* |
| 601 | 0.01% Cycloheximide growth | 600 | *Debaryomyces hansenii* |
| | | | *Debaryomyces polymorphus* |
| | | | *Debaryomyces vanrijiae* |
| 602 | Growth without Thiamin | *Candida entomophila* | 603 |
| 603 | Septate hyphae | *Debaryomyces hansenii* | *Candida fennica* |
| | | *Debaryomyces polymorphus* | |
| 604 | Lactose growth | 605 | 610 |
| 605 | L-Sorbose growth | 606 | 608 |
| 606 | 2-Keto-D-gluconate growth | *Candida silvicultrix* | 607 |
| 607 | Septate hyphae | *Debaryomyces hansenii* | *Candida membranaefaciens* |
| 608 | Growth without Biotin | 609 | *Debaryomyces hansenii* |
| | | | *Debaryomyces polymorphus* |
| 609 | Septate hyphae | *Candida friedrichii* | *Candida membranaefaciens* |
| | | *Debaryomyces hansenii* | |
| 610 | Growth without Thiamin | *Candida entomophila* | *Debaryomyces hansenii* |
| | | | *Debaryomyces polymorphus* |
| 611 | Raffinose growth | 612 | 639 |
| 612 | Lactose growth | 613 | 631 |
| 613 | 2-Keto-D-gluconate growth | 614 | 625 |
| 614 | Me α-D-glucoside growth | 615 | 617 |
| 615 | Maltose growth | *Pichia farinosa* | 616 |
| 616 | D-Gluconate growth | *Pichia naganishii* | *Candida veronae* |
| 617 | D-Glucono-1,5-lactone growth | 618 | 620 |
| 618 | Growth at 42°C | 619 | *Candida naeodendra* |
| 619 | D-Arabinose growth | *Candida polymorpha* | *Candida dendronema* |

620 D-Galactose growth
621 D-Gluconate growth
622 Septate hyphae
623 50% D-Glucose growth
624 60% D-Glucose growth
625 Starch growth
626 Growth without Biotin
627 Growth at 35°C
628 Septate hyphae
629 Growth at 35°C
630 Maltose growth
631 Growth at 42°C
632 2-Keto-D-gluconate growth
633 Growth without Thiamin
634 L-Sorbose growth
635 Maltose growth
636 50% D-Glucose growth
637 Maltose growth
638 Septate hyphae
639 Melibiose growth
640 Lactose growth
641 Citrate growth
642 2-Keto-D-gluconate growth
643 Starch growth
644 L-Sorbose growth

645 Growth without Thiamin
646 Growth at 42°C
647 Growth at 42°C
648 Lactose growth
649 2-Keto-D-gluconate growth
650 Starch growth
651 Septate hyphae
652 Growth without Biotin

653 Septate hyphae

654 2-Keto-D-gluconate growth
655 Growth at 42°C
656 Growth at 42°C
657 Starch growth

658 Sucrose growth
659 DL-Lactate growth
660 Maltose growth
661 D-Galactose growth
662 L-Rhamnose growth
663 Raffinose growth
664 D-Arabinose growth
665 2-Keto-D-gluconate growth
666 Growth without Biotin
667 Growth without Thiamin
668 Lactose growth
669 Starch growth
670 Growth at 37°C
671 Xylitol growth
672 Starch growth
673 L-Rhamnose growth
674 Growth without Thiamin
675 Septate hyphae
676 D-Galactose growth
677 Septate hyphae

Negative

Candida pelliculosa
Candida pelliculosa
Candida pelliculosa
Candida veronae
Candida insectorum
625
626
627
Candida tenuis
629
Candida tenuis
Pichia farinosa
632
633
Candida
635
Pichia farinosa
Pichia mexicana
Pichia farinosa
Candida hydrocarbofumarica
640
641
Candida rhagii
643
Candida insectorum
Debaryomyces hansenii
646
Candida insectorum
Debaryomyces hansenii
649
650
651
Pichia scalyi
653
Debaryomyces hansenii
Pichia scalyi
655
631
657
Debaryomyces hansenii
Pichia scalyi
659
660
496
662
Candida boidinii
664
665
666
667
Hormoascus platypodis
669
Hansenula polymorpha
Candida ishiwadae
672
Hansenula lynferdii
674
Hansenula subpelliculosa
Hansenula anomala
Candida carassilignicola
Hansenula subpelliculosa

Positive

621
622
623
Candida terebra
630
628
Pichia heimii
Candida terebra
Pichia nakazawae
Pichia stipitis
Candida homilentoma
634
629
Mastigomyces philippovii
637
636
Candida entomaea
638
Candida tanzawaensis
648
645
642
644
Wingea robertsiae
Debaryomyces hansenii
Wingea robertsiae
647
Pichia mexicana
639
654
652
Candida silvanorum
Candida insectorum
Debaryomyces hansenii
Debaryomyces vanrijiae
Candida membranaefaciens
656
Pichia mexicana
Pichia mexicana
Debaryomyces hansenii
Debaryomyces pseudopolymorphus
663
661
Hansenula capsulata
Hansenula nonfermentans
Candida methylica
671
668
Candida incommunis
Hansenula anomala
Hansenula polymorpha
Candida chilensis
670
Hansenula holstii
673
Hansenula anomala
676
675
Hansenula ciferrii
677
Hansenula ciferrii

## Appendix C: Identification report of yeast NF 32 A as *Yarrowia lipolytica.* Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany.

Strain designation YA was employed for yeast isolate NF 32 A for identification purposes

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

**Identification of Yeast strains**

**Sent by:** Dublin City University

**Strain designation:** YA

**Substrate:** activated sludge

**1. Morphology**

Colony on potato dextrose agar butyrous, smooth, creme coloured. Blastospores ellipsoidal. Pseudomycelium and true mycelium present; no sexual reproduction detected. Good growth at 37°C

**2. Utilization of C- and N-sources**

anaerobic: Glucose -

aerobic:

| | | | |
|---|---|---|---|
| Glucose | + | α-methylglycoside | - |
| Galactose | + | Salicin | - |
| Sorbose | + | Cellobiose | - |
| Rhamnose | - | Maltose | - |
| Dulcit | - | Lactose | - |
| Inositol | - | Melibiose | - |
| Mannitol | + | Sucrose | - |
| Sorbitol | + | Trehalose | - |
| Glycerol | + | Inulin | - |
| Erythritol | + | Melezitose | - |
| D-Arabinose | - | Raffinose | - |
| L-Arabinose | - | Starch | - |
| Ribose | + | Xylitol | - |
| D-Xylose | - | Gluconate | weak |
| L-Xylose | - | 2-keto-Gluconate | + |
| Adonitol | - | 5-keto-Gluconate | - |

Nitrate -

3. Identification:

*Yarrowia lipolytica* van der Walt & von Arx

DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Braunschweig, 3.11.98