Neutral lipid production by the yeast *Debaryomyces hansenii* NCYC102 under different stress conditions

By

Zeena H O Alwan

MSc. University of Babylon, Iraq

MPhil, University of Sheffield, England

Thesis submitted for the degree of Doctor of Philosophy (PhD)

Department of Molecular Biology and Biotechnology
The University of Sheffield, UK

November 2017

Abstract

Oleaginous yeasts are very efficient in the accumulation of triacylglycerol, and are expected to be one of the most important feedstocks for the biofuel industry in the future. Lipid content can be enhanced through physiological stress or genetic manipulation. *Debaryomyces hansenii* NCYC102 was selected from three different yeast species (also including *Yarrowia lipolytica* NCYC476 and *Cryptococcus curvatus* NCYC2904) due to the highest neutral lipid content. The growth rate, osmolytes and neutral lipids were measured in cells grown under different concentrations (0, 0.8, 1.6 M) of NaCl. The maximum content of total osmolytes was found in 0.8 M NaCl YM medium. However, the highest level of glycerol was measured in 1.6 M NaCl grown cells. The main osmolytes identified by ^{1}H NMR spectroscopy were glycerol, arabitol, glucose and trehalose. *Debaryomyces. hansenii* cells were grown in minimal medium with different carbon/nitrogen ratios using either glucose or glycerol as the sole carbon source along with ammonium sulphate as nitrogen source. Maximal neutral lipid production was observed in 48:0.5 glucose/ammonium sulphate ratio which achieved 1.4-fold increase compared with glycerol-based medium (8 glycerol: 0.25 ammonium sulphate). GC-MS analysis of the transesterified fatty acids showed that palmitic, oleic and stearic acids were the main fatty acids present, under normal and stress conditions (high salt and limited nitrogen source). Deletion of the *GUT2* encoding for G3P dehydrogenase increased neutral lipid production up to a 1.4-fold compared to wild type strains. The mutant strains displayed slightly higher cell densities in medium with glucose when compared with wild type strains, while they failed to grow on glycerol as a sole carbon source.

Collectively, these results indicate that *D. hansenii* is a good organism to produce biofuels as it has an intrinsic ability to accumulate neutral lipids and this can be further enhanced by genetic and metabolic engineering.

Acknowledgment

First and foremost, my deep thanks and gratitude to Almighty Allah who blessed me with countless great kindness which enabled me to perform practical researches and writing up this thesis. To those people in my country Iraq who sacrifices themselves to protect the families and keep us continue study, there is no word can express how much I am grateful for your sacrifices.

I would like to express my sincere gratitude to my great supervisors Dr. Jim Gilmour and Dr. Ewald Hettema for their support, guidance, patience, and valuable criticism during this study. I would like to thank many staff members and former colleagues which have helped me, including Professor Mike Williamson for processing NMR samples, Dr. Donald J Watts for assistance in molecular techniques, Prof Peter Sudbery for providing pFA SAT and knowledge about *Candida albicans*, and Simon Thorpe (Chemistry) for assistance in GC-MS analysis. Many thanks to the great staff members in particular Linda Harris, Mark Johnson, Martin Price, Ruth Roberts, Dean Swift, and Glennis Swift for their support. Special thanks to the Prof Alastair Goldman for his support.

I would like to thank the Ministry of higher education in Iraq and Iraqi Cultural Attaché to provide me this great opportunity to complete my PhD study in the University of Sheffield and to facilitate all the difficulties.

I would like to express the deepest appreciation to the many project students who aided me in term of knowledge, mentorship and tuition experience. Most notable amongst them are Richard Smith, Murtakab Y Alhejjaj, Lakhan Ekal, Nadal A Al Saryi, Georgia Hulmes, Ghazay F. Alotaibi and Tom Burns.

My special and deep thanks to my best friends Lina Albayati and her husband Dr. Yassir Ubaidi, Dr. Shleir Kassim, Dr. Bushra Juhi, Marzieh Radi for their kindness and support especially during the tough times. Many thanks to Dr. O P Purohit, Dr. L Maraqa, and Dr. A Morritt for their help and care.

My deep gratitude to my sisters, brothers and their families. They were always supporting me and encouraging me with their best wishes.

Abbreviations

ACL	ATP-citrate lyase
ACC	acetyl-CoA carboxylase
AMPK	AMP kinase
AMP-deaminase	adenosine monophosphate- deaminase
ATP	adenosine triphosphate
bp	base pair
CDM	cell dry material
C/N	carbon/ nitrogen ratio
C. curvatus	*Cryptococcus curvatus*
D. hansenii	*Debaryomyces hansenii*
dH_2O	deionised water
DHAP	dihydroxyacetone phosphate
DMSO	dimethyl sulfoxide
D_2O	deuterium oxide
DAG	diacylglycerol
DTT	dithiothreiol
EDTA	ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
FAs	fatty acids
FAME	fatty acid methyl ester
FDA	food and Drug Administration
GC-MS	gas chromatography -mass spectrometry
G3P	glycerol-3-phosphate

GPD	glycerol 3-phosphate dehydrogenase
GRAS	generally regarded as safe
GUT2	gene encoding for G3P dehydrogenase
g/L	gram/ litter
HCL	hydrochloric acid
HPLC	high performance liquid chromatography
HOG	hyper osmotic glycerol
IEA	international Energy Agency
IMP	inosine monophosphate
KB	kilobase
kDa	kilodalton
LPA	lysophosphatidic acid
LB	lipid bodies
M	molar
ME	malic enzyme
MDH	malate dehydrogenase
NaCl	sodium chloride
NCYC	national Collection of Yeast Cultures
NADPH	nicotinamide adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
NMR	nuclear magnetic resonance
OAA	oxaloacetate
OD	optical density
PA	phosphatidic acid, PA
PAD1	gene encoding for Phenylacrylic acid decarboxylase
PC	pyruvate carboxylase

PCR	polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
SAP	stress-activated protein
SAT1	gene encoding for streptothricin acyltransferase
SCO	single cell oil
SE	steryl-esters
TAG	triacyleglycerol
TLC	thin layer chromatography
TCA	trichloroacetic
TE buffer	tris EDTA buffer
URA3	gene encoding for orotine-5-monophosphate decarboxylase
(w/w).	weight/ weight
w/v)	weight/ volume
Y. lipolytica	*Yarrowia lipolytica*

Table of contents

List of figures

List of tables

Chapter One Literature Review

1. Literature Review

1.1. Introduction:

With the expected deterioration of oil supplies around the world and rapid growth in the price of crude oil, due to population growth and industrialization, there is greater understanding of the environmental effects of fossil fuels. In recent years, this recognition has attracted researchers' interested in transportation biofuels, e.g. (Soriano et al., 2006, Tsigie et al., 2013). Moreover, emissions of fossil fuel are thought to be a major contributor to global warming (IEA, 2008). To resolve these serious problems, such as the deteriorating situation of the energy supply around the world, considerable attention has been directed towards alternative renewable biofuels (Hill et al., 2006).

In the last 20 years, metabolic engineering has developed as a major approach to enhance cell factories by genetic engineering. Traditionally, the improvement of product yield, product range, and substrate utilization with the aim to modify the metabolic pathways in the host microorganism, was the purpose of metabolic engineering (Christensen et al., 2000). For production of transportation fuels, 55% of crude oil was used in 2008, (OPEC: World Oil Outlook, 2011). Other exhaustible sources that can be converted into transportation fuels, such as shale oil, shale gas, tar sands, natural gas (gas-to liquid) and coal (coal-to-liquid), cannot substitute traditional oil due to their limited reserves ((Buijs et al., 2013) and OPEC: World Oil Outlook, 2011) or problems with large green-house gas emissions associated with the process of conversion (Caspeta et al., 2013). With the need for developing a more sustainable society there is willingness to change from fossil fuels to biofuels, by using the concept of biorefineries (de Jong et al., 2012).

The current energy crisis is expected to be relieved by the development of different biofuels as alternative, sustainable fuels (Schubert, 2006). Currently, bioethanol and biodiesel have been considered as the most widely biofuels used in industries. However, bioethanol is not considered as the perfect biofuel in the future because of its

incompatibility with the existing fuel infrastructure and low energy density (Stephanopoulos, 2007, Atsumi et al., 2008). Thus, there is great interest to replace this biofuel with alternatives, for example fatty acid esters i.e. biodiesel, that are compatible with the existing infrastructure and have been extensively tested as an alternative fuel on the market. In combustion properties, biodiesel is similar to petro-diesel, allowing it to work well in traditional diesel engines and making it fit into the existing fuel infrastructure (Demirbaş, 2002). In addition, in several ways biodiesel is better than petro-diesel, such as environmental friendliness, renewability, reduced emissions, improved lubricity, higher combustion efficiency, better safety, etc. (Demirbas, 2007). In light of these demands, the production of biodiesel has been increasing continuously, with a 16 fold rise over the last 10 years, and was estimated to reach to around 4 billion gallons in 2009, mainly produced in the European Union and the USA (Brown, 2009). In 2006, it was reported that the overall biodiesel production in EU rose from 3.2 million tons in 2005 to about 4.9 million tons. This represents a 54% yearly increase for biodiesel production in EU, which follows a 65% record high growth in the previous year 2005. For biofuel production and consumption in EU Member States, it was reported that "between 2010 and 2011 the consumption increased by 3%, which translates into 13.6 million tonnes of oil equivalent (toe) used in 2011 compared to 13.2 million tonnes in 2010 (European Technology and Innovation Platform (ETIP Bioenergy) 2016). Biodiesel production in the United States has also increased significantly in the last few years (Carriquiry, 2007). In order to replace the 2007 consumption of all transport fuels in the US, it was estimated that it would require the production of 0.53 billion m^3 of biodiesel annually (Chisti, 2007). Consequently several large chemical and fuel companies have exhibited a high level of commercial interest in the production of advanced biofuels using cell factories, which is an indicator of the great commercial trust in successful and fast advanced biofuel production in the near future (de Jong et al., 2012). Studies by international organisations, biofuels associations, and independent consultants which made for global economic growth of biofuels production to 2020, indicated that US is currently considered the world

leader in biofuel investments with around $1.5bn in 2012 (European Technology and Innovation Platform (ETIP Bioenergy), 2017).

Biodiesel, a non-petroleum based diesel fuel, is defined as alkyl esters of long chain fatty acids. In the presence of a suitable catalyst and alcohols, lipids (mainly triglycerides/triacylglycerols, TAGs) are converted into fatty acid methyl/ethyl esters (FAME/FAEE) which is the principal process of biodiesel production and is also known as transesterification. These catalysts may be either enzymatic or chemical (Bajpai and Tyagi, 2006, Demirbas, 2008). For biodiesel production, major feedstocks used are based on either plant or animal materials. Both types face the problem of continuous availability in sufficient quantity for long periods. To solve this problem, we need to find a feedstock with higher lipid content and sufficient quantity. One possible solution, is to use microorganisms; in particular oleaginous species, due to their similar composition of fatty acids with plant and animal feedstock along with higher lipid content (Gohel et al., 2013).

1.2. Oleaginous yeast as biodiesel producer

As mentioned before, higher cost and lesser availability are the major obstacles with plant and animal based sources of biofuel. Comparatively cheaper feedstocks could solve the cost problem like waste oils and greases but again availability in sufficient amounts is the main problem (Vasudevan and Briggs, 2008). Due to their significant roles and specific characteristics, microbial oils, also called single cell oils (SCO), are lipids produced by oleaginous microorganisms that have been of potential interest to many researchers during the last decades (Ratledge, 1991). In fact, the typical and intensive source of TAGs is the biomass of oleaginous microorganisms which show similar composition of FAs and energy value of plant oils. Lipid produced by oleaginous microorganisms have many characteristic features (short life cycle, less influence by venue, season and climate, and easy to scale-up) that can resolve many difficulties with plant oils, but can very importantly be produced from low cost feedstocks with high amount and productivity (Azócar et al., 2010, Liu and Zhao, 2007, Rossi et al., 2011). Compared to petroleum diesel,

this kind of biodiesel also exhibited less sulphur as well as CO in its content and it also lacks poly aromatic hydrocarbons (Bajpai and Tyagi, 2006).

Oleaginous species are defined as; microorganisms that can accumulate lipid to higher than 20% of their biomass (Li et al., 2008). Several species of yeasts are considered as oleaginous, since they are able to synthesize and accumulate large amounts (up to 70%) of intracellular TAG, of their biomass weight. The study of oleaginous yeasts has a long history: their ability to accumulate lipids has been known from the 1970s, but research into the exploitation of SCO for biodiesel production has been focused on only in the last few years. They are widely distributed in all-natural ecosystems and represent a part of the microbiota, such as soils, freshwater and marine waters, as well as extreme environments, such as low temperatures, low oxygen availabilities, and oligotrophic oceanic waters (Butinar et al., 2007). The oleaginous yeast *Cryptococcus curvatus* (previously known as *Apiotrichum curvatum* ATCC 20509 and *Candida curvata*) was discovered at Iowa State University in 1978 (Moon and Hammond, 1978). This yeast was mentioned as an effective oil producer and easy to grow with minimum nutritional requirements (Zhang et al., 2011). An important characteristic of *C. curvatus*, is the ability to utilize a large group of substrates such as glycerol and convert them into lipids that are stored as intracellular TAGs (accumulating up to 60% of its cellular dry weight) (Lee et al., 1992, Meesters et al., 1996).

Basidiomycetous yeasts strongly dominate among oleaginous yeasts, including most of the strains identified as lipid producers, although some important oleaginous species have been identified within Ascomycota as well (Ageitos et al., 2011). Certain Ascomycetous yeast, like species of the genus *Lipomyces* accumulate significant amounts of lipids (above 50% of CDM) (Ratledge and Wynn, 2002). The high ability of *Debaryomyces hansenii* (anamorph: *Candida famata*) to synthesize, accumulate and store lipids (up to 75% (w/w)) could be beneficial for both natural and artificial products through biotechnological production (Petersen and Jespersen, 2004). All *D. hansenii* species are perfect, haploid yeasts, asexual reproduction by multilateral budding, whereas they reproduce sexually by heterogamous conjugation. A pseudomycelium is

absent, primitive or sometimes well developed (Nakase et al., 1998). It is widespread in nature and common in cheeses (Borelli et al., 2006, Cosentino et al., 2001, Vasdinyei and Deak, 2003) and high salt food products (Del Bove (Del Bove et al., 2009, Prista et al., 2005). This yeast also shows a broad spectrum in assimilation of carbon sources (Yadav and Loper, 1999). *Yarrowia lipolytica* is related to the family Dipodascaceae (Kurtzman and Piškur, 2006), and has great potential for biofuel production (Beopoulos et al., 2009a, Beopoulos et al., 2009b). This organism has been well studied and is an obligate aerobe having the ability to produce key metabolites, which can often be secreted from the cells. These characteristics justify its use in industry, in molecular biology and in genetics studies. It is non-pathogenic and the Food and Drug Administration (FDA, USA) classified this organism as generally regarded as safe (GRAS). Besides, *Y. lipolytica* has been used in yeast dimorphism studies, due to the presence of an efficient transformation system for genetic engineering, and easy differentiation between its morphotypes (Coelho et al., 2010).

1.3. Biochemistry of lipid accumulation

Lipid biosynthesis pathways do not differ significantly among oleaginous and non-oleaginous fungi, compared to other eukaryotic organisms. The ability to synthesise and accumulate lipid mostly depends on the regulation of the biochemical pathways as well as the availability of essential precursors such as acetyl-CoA and malonyl-CoA, and reducing power in the form of NADPH. Most information on the biochemistry of lipid synthesis is from the yeast *Saccharomyces cerevisiae* (Kohlwein, 2010), that is not a lipid accumulator, and *Y. lipolytica*, which is a model species for biodiesel production, and is amenable to genetic manipulation (Beopoulos et al., 2009b). *Y. lipolytica* can normally accumulate up to 40% lipids in the form of intracellular lipid bodies, which are mostly composed of TAGs. The accumulation of TAG may take place either from exogenous oil uptake or *de novo* biosynthesis from sugar substrates. Lipid accumulation is induced by nutrient limitation in the presence of excess carbon source (Tai, 2012). The oleaginous yeast *C. curvatus* can accumulate >60% storage lipid in terms of dry weight, with 44% saturated fatty acid, when it is grown under nitrogen-limiting conditions (Ratledge, 1982).

During the growth phase, nitrogen is crucial for protein and nucleic acid synthesis, while the carbon flux is divided between energetic and anabolic processes resulting in the synthesis of carbohydrates, lipids, nucleic acids and proteins. When nitrogen is exhausted, the growth rate slows down and the synthesis of proteins and nucleic acids is inhibited. The excess of carbon remains unutilized or converted into storage polysaccharides in non-oleaginous species. In oleaginous species, it is preferentially channelled toward lipid synthesis, leading to the accumulation of TAG inside lipid bodies (Ratledge and Wynn, 2002). Both organic and inorganic nitrogen sources can be used for yeast cultivation with varied impact on oil accumulation (Liu et al., 1999, Shi et al., 1997).

Fatty acid biosynthesis pathway initially requires a constant supply of acetyl-CoA unit while at each elongation step it requires two carbons molecules supplied by malonyl-CoA unit. In oleaginous fungi, lipid accumulation does not take place under balanced nutrient conditions (Ratledge, 2002). Nitrogen exhaustion stimulates AMP-deaminase (Ratledge and Wynn, 2002), responsible to provide ammonium to the nitrogen-stressed cell. As a result, low concentrations of AMP in the mitochondria inhibit isocitrate dehydrogenase, which depends on the content of AMP (Figure 1.1) (Evans et al., 1983). The suppression of the isocitrate dehydrogenase slows down or blocks the conversion of isocitrate to α-ketoglutarate. In the mitochondrion, the isocitrate is rapidly converted into citrate by aconitase, and low concentrations of isocitrate are found (Evans et al., 1983). As a consequence of increasing citrate content inside mitochondria, citrate enters the cytosol interchangeably with malate by citrate/malate translocase. In the cytosol, citrate is split via ATP-citrate lyase (ACL) to acetyl-CoA and oxaloacetate (OAA) (Ratledge, 2004). ACL represents one of the key enzymes found specifically in oleaginous yeasts. This enzyme is formed from two subunits, encoded by *ACL1* and *ACL2,* and is negatively regulated by exogenous FA. Acetyl-CoA carboxylase (ACC) then catalyses the first committed step of lipid biosynthesis, it produces malonyl-CoA by a condensation reaction of an acetyl-CoA with bicarbonate:

$$\text{Acetyl-CoA} + HCO^{-3} + \text{ATP} \rightleftharpoons \text{malonyl-CoA} + \text{ADP} + \text{Pi} \quad \text{Equation no. 1}$$

ACC is also a key enzyme in *de novo* FA synthesis, since mutant strains with *ACC1* gene maintain low levels of ACC activity or became FA auxotrophs strain (Tehlivets et al., 2007). Control of *ACC1* is by allosteric activation with citrate (Figure 1.1). The figure demonstrated that two molecules of NADPH are required for each elongation step of the acyl chain. The main sources are the pentose phosphate pathway and the transhydrogenase cycle, which converts NADH into NADPH through multiple reactions catalyse by pyruvate carboxylase (PC), malate dehydrogenase (MDH), and malic enzyme (ME) as follows.

$$\text{Pyruvate} + CO_2 + \text{ATP} \rightleftharpoons \text{oxaloacetate} + \text{ADP} + \text{Pi (PC)}$$

$$\text{Oxaloacetate} + \text{NADH} \rightleftharpoons \text{Malate} + NAD^+ \text{ (MDH)}$$

$$\text{Malate} + NADP^+ \rightleftharpoons \text{pyruvate} + CO_2 + \text{NADPH (ME)}$$

$$\text{NADH} + NADP^+ \rightleftharpoons \text{NADPH} + NAD^+$$

Malic enzyme was also considered as key enzyme in lipid accumulation. This enzymes has been discovered in several oleaginous fungi (Ratledge, 2002). In *Mortierella circinelloides*, overexpression of ME improved lipid accumulation (Zhang et al., 2011), whereas overexpression of the homologous ME in *Y. lipolytica* had no effect.

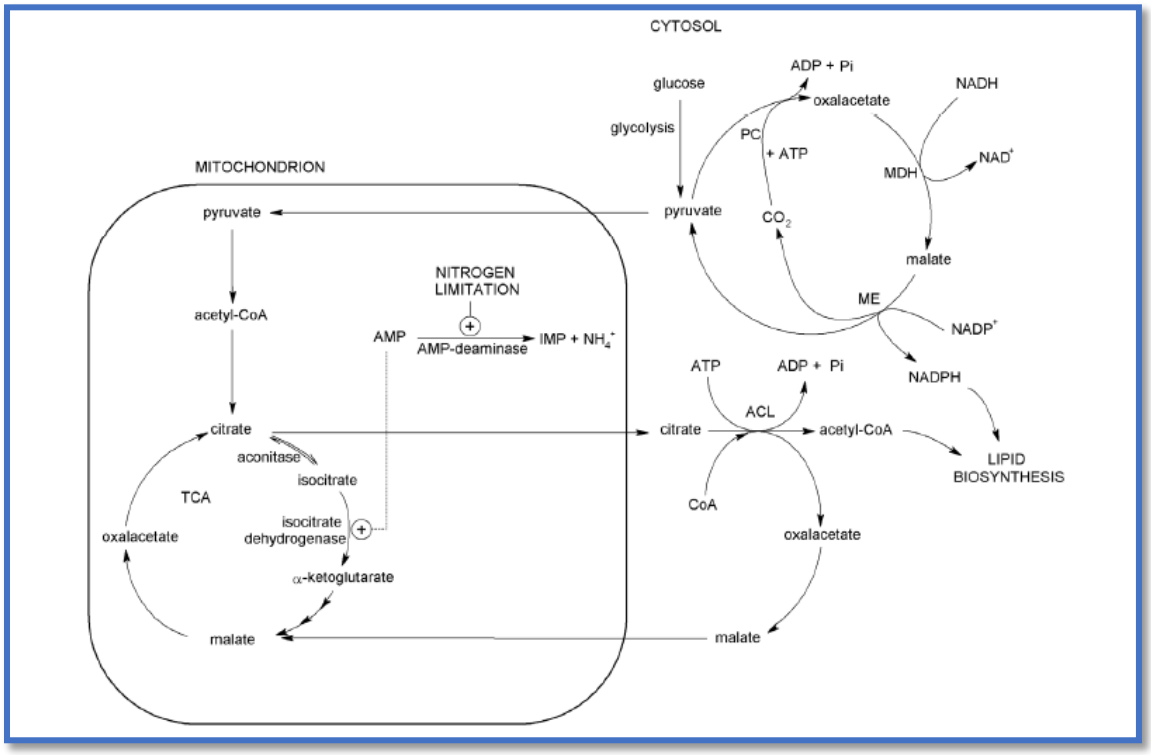


Figure 1.1. The biosynthesis of lipid as a consequence of nitrogen limitation and excess of carbon source. Adapted from (Ratledge, 2004).

The main fatty acids found in yeast oils are myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid, which are the final products of fatty acids (FAs). With the catalysis by lipase or chemical catalyst with glycerol or sterols, such yeast oils can be used as oil feedstocks to produce triacylglycerol (TAG) and steryl-esters (SE), respectively (Li et al., 2007a, Liu and Zhao, 2007). The neutral lipids SE and TAG, formed in oleaginous fungi, are stored inside the lipid bodies (LB). TAGs are predominantly formed by successive acylation of glycerol-3-phosphate (G3P), carried out through acyl transferases (Figure 1.2). Glycerol-3-phosphate (G3P) is synthesized from glycerol via glycerol kinase or from dihydroxyacetone phosphate (DHAP) via G3P dehydrogenase, in a reversible reaction. Both G3P and DHAP can be utilized as acyl-group acceptors by *S. cerevisiae*. In

most oleaginous yeasts, neutral lipid of lipid bodies (LBs) mostly consist of TAG, while a small fraction is made up of steryl esters.

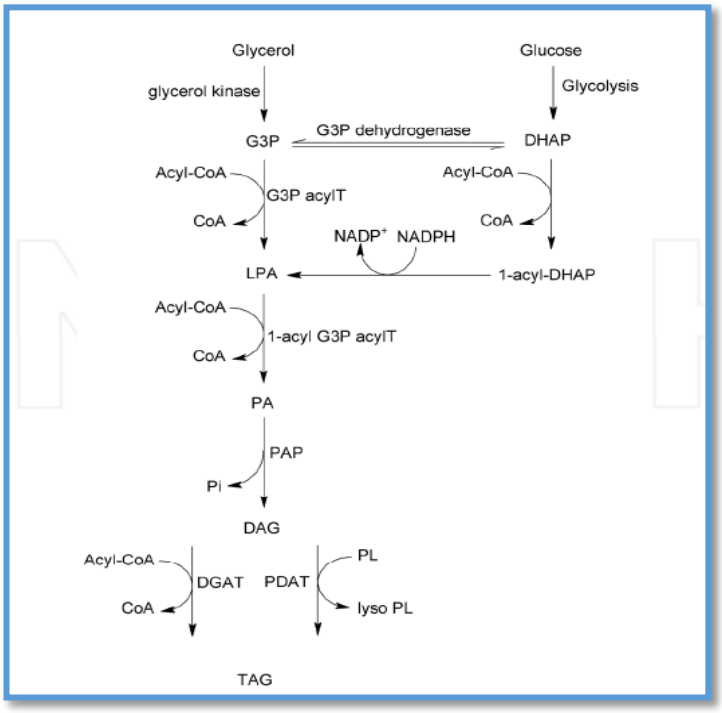


Figure 1.2. Triacylglycerol synthesis Pathway, adapted from (Czabany et al., 2007): Glycerol-3- phosphate (G3P) is either synthesis from glycerol via glycerol kinase or from dihydroxyacetone phosphate (DHAP) via G3P dehydrogenase. Lysophosphatidic acid (LPA), also named 1-acyl G3P is formed by the addition of first acyl group or by reduction of acyl-DHAP via the activity of a NADPH dependent reductase. Following step, 1, 2-diacyl G3P (phosphatidic acid, PA) compound formed after addition of another acyl group catalysed by a second acyltransferase. The next compound in TAG synthesis is diacylglycerol (DAG) formed after removing phosphate from PA by the activity of phosphatidate phosphatase. In the last step DAG can be either precursor of TAG or directed toward the formation of phospholipids

Neutral lipids are in the core of LB, surrounded by a phospholipid monolayer in which several proteins are embedded (see Figure 1.3). These proteins exert a main role in lipid biosynthesis and trafficking of substrate. When required, storage lipids are mobilized via triacylglycerol lipases and steryl ester hydrolases, from this compartment. The

degradation products serve as energy sources and/or building units for membrane formation. FA hydrolyzed from TAG or SE is either channelled to the peroxisome, where β-oxidation takes place, or to the synthesis of phospholipid (Rossi et al., 2011).

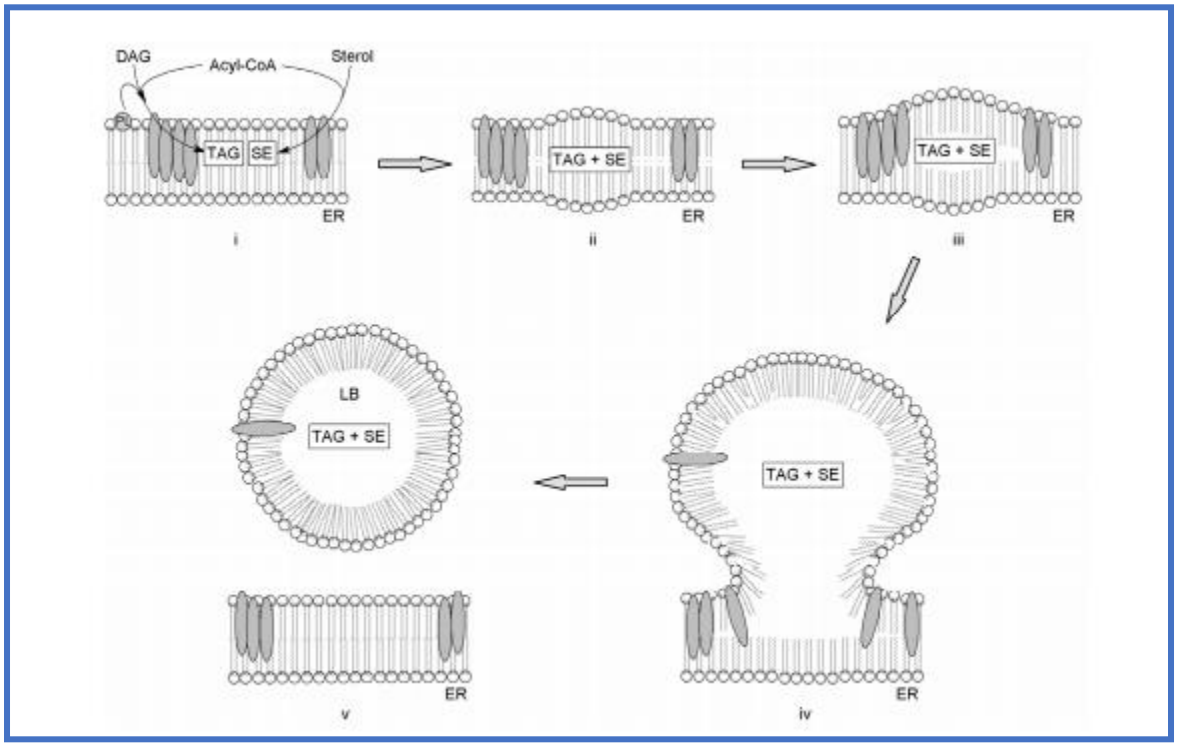


Figure 1.3. Schematic diagram of lipid bodies biogenesis from the membrane of the endoplasmic reticulum (ER) adapted from (Czabany et al., 2007). TAG and SE accumulate between the two leaflets of the phospholipid bilayer (i to iii). The micro-droplet generated (iii, iv) evolve to lipid bodies (v).

1.4. Adaptation to salt stress in salt- tolerant strains of yeast

Natural ecosystems that are identified by human standards as extreme environments (e.g., high or low acidic, alkaline, temperature, and salinity), are predominant with simple forms of life such as microorganisms, which have the capability to survive in these ecological regions (Galinski and Trüper, 1994). In industries, yeast fermenters can be effected by a number of a biotic and non-biotic stress conditions that may interfere with

their functions and thus the progress of fermentation (Albertyn et al., 1994). Salinity is a major environmental parameter that attracted more researchers, due to the protective strategies that are used by the cells for salt tolerance, which may have important commercial applications (Galinski and Trüper, 1994). Kushner (1978) classified organisms into three groups depending on the range of the salinity: Slight halophiles (with optimal growth at about 3% w/v NaCl, such as marine organisms), moderate halophiles (between 3-15% w/v salt), extreme halophiles (grow in condition higher than 12% w/v salt) (Kushner, 1978). According to this classification, the yeast *D. hansenii* is described as moderately halophilic, because the optimal growth of this species was at 3-5% w/v salt (Breuer and Harms, 2006).

Under osmotic stress a number of physiological changes take place within yeast cells, including: efflux of intracellular H_2O and cell volume reduction, (Albertyn et al., 1994), temporary increases in glycolytic intermediates (Allison et al., 1999), accumulation of cytosolic glycerol, and stimulation of the hyper osmotic glycerol (HOG) signalling pathway (Bellinger and Larher, 1988). Concerning osmotic stress such as salt stress, a microorganism like the yeast *S. cerevisiae* develops systems to face this effect (Andreishcheva et al., 1999), Salt stress results in two different phenomena which are ion toxicity and osmotic stress (Mager and Siderius, 2002). Defence responses to salt stress are based on osmotic regulation through the synthesis of osmolytes and cation transport systems for sodium exclusion (Yancey, 2005). The exposing of yeast cells to hyper osmotic environment results in rapid dehydration and inhibition of cell growth (Yale and Bohnert, 2001). Under these conditions, cellular reprogramming or adaptation, that includes the accumulation of intracellular osmolytes to balance the internal osmotic pressure with the external environment, represents the major defence against these factors (Gacto et al., 2003). In yeast cells certain osmolytes act as compatible solutes e.g. polyols (particularly glycerol), amino acids (e.g. proline), trehalose and glycogen are accumulated intracellularly (Butinar et al., 2005). They might make up 25% of the dry cell mass depending on environmental conditions (Herdeiro et al., 2006). Due to these osmolytes,

the osmolarity is increased in the cells, which enables them to retrieve water from the medium in an efficient way. Most microorganisms tend to use one major compatible solute, but may also accumulate other compatible solutes to a lesser degree. For instance, the yeast *S. cerevisiae* produces mainly glycerol but under different levels of stress may also produce glycogen and trehalose (Tamás and Hohmann, 2003). Sodium chloride (NaCl) is the most common compound used in experiments to create hyperosmotic stress. It has been found that the glycerol concentration inside the cell increases in parallel with the external concentration of NaCl. In general, the increase in intracellular concentration of glycerol is due to increasing glycerol production, increasing the retention by cytoplasmic membranes, decreasing the dissimilation of glycerol or taking up glycerol from the environment (Parrou et al., 1997, Posas et al., 2000)

During glycolysis, glycerol is formed by the reduction of dihydroxyacetone phosphate to G3P via glycerol 3-phosphate dehydrogenase (GPD) (Reed et al., 1987, Parrou et al., 1997, Posas et al., 2000). Under osmotic stress, increasing the level of glycerol takes place due to the rise in the GPD activity. Higher osmotic stress also induces the H^+-ATPase in plasma membrane (Nishi and yagi, 1993, Watanabe et al., 1993), and the Na^+/H^+ antiporter that uses the proton electrochemical gradient on the plasma membrane as the driving force to remove the excessive intracellular Na^+ ions (Jia et al., 1992, Nishi and Yagi, 1995).

Y. lipolytica is a yeast species known for its ability to grow in the presence of high concentration of NaCl, it responds to salt stress by using the same principles of osmoregulation, which have been outlined above. In this species, the higher salinity of the growth medium (9% NaCl = 1.5 M NaCl) enhanced the accumulation of intracellular glycerol and a small but reproducible increase in the intracellular Na^+ concentration. Furthermore, rapid accumulation of free amino acids also accompanied the salt stress (Andreishcheva et al., 1999). On the other hand, the yeast species *C. curvatus* which is frequently found in marine water (Atlas and Bartha, 1981), accumulated trehalose in

response to osmotic stress when the yeast cells were grown in the presence of 1.5 M NaCl (Tekolo, 2007).

1.5. The role of glycerol in osmotic regulation

Glycerol is an industrial alcoholic, slightly sweet compound that is used in different applications in the food, beverage, chemical and pharmaceutical industries. This nontoxic trihydroxy alcohol is soluble in polar solvents such as water, whereas it is insoluble in non-polar organic solutions. In S. *cerevisiae* studies demonstrated that glycerol has effective roles in some physiological processes such as resistance towards osmotic stress, regulating the level of cytosolic phosphate and maintaining the NAD^+/NADH intracellular redox balance (Blomberg and Adler, 1992, Scanes et al., 1998a, Hohmann, 1997). The manipulation of *S. cerevisiae* to increase or decrease the glycerol production can be achieved by either optimizing the growth conditions (nutrients and other environmental conditions) which can regulate the yield of glycerol for example the nitrogen source, temperature, as well as the osmotic stress. Alternatively, this can be achieved by genetic modification approaches. Osmotic stress is the most common environmental factor that can affect yeast cells, therefore it is essential for the cell to develop mechanisms to withstand osmotic stress conditions (Scanes et al., 1998a). The osmotolerant yeast *D. hansenii* is commonly found in natural saline habitats ranging from sea-water (Norkrans, 1966) to concentrated brines (ŌNishi, 1963). Research noted that yeast cells displayed the ability to regulate their internal solute concentrations, as the water potential of the extracellular environment fluctuated greatly, allowing the maintenance of turgor pressure and metabolic functions. Under osmotic stress conditions, surplus sodium ions are excluded whereas potassium ions are accumulated inside the cell (Norkrans and Kylin, 1969, Hobot and Jennings, 1981). In high salinity, the Na^+/K^+ content is not enough to maintain osmotic equilibrium inside the growing cell *visa à vies* the environment. Several research papers connect the survival of the cells under such stress conditions with the accumulation of an organic solute, particularly glycerol (Gustafsson and Norkrans, 1976, Gustafsson, 1979, Adler and Gustafsson, 1980, Adler et al., 1985, Nobre and Costa, 1985). Glycerol works as a compatible solute (Brown, 1978, Yancey et al., 1982) to raise the

internal osmotic pressure without interfering with the structure and function of intracellular macromolecules. (Lowry and Zitomer, 1984) reported that in medium with glucose as a carbon source, the aerobic production of glycerol take place resulting in respirofermentative growth. This means the cell requires glycerol production to maintain the redox balance when respiration is inhibited by glucose and oxygen limitation. Interestingly some research reported that the production of glycerol was higher in minimal medium than in enriched medium, as intracellular formation of amino acids from ammonia and glucose leads to an increased the accumulation of NADH which is then re-oxidised to NAD^+ through the formation of glycerol (Albers et al., 1996) (Figure 1.4). This observation is correlated with the maintenance of the redox balance and has been supported by the inability of mutant cells to synthesise glycerol when grown in an anaerobic environment as well as by the intracellular accumulation of NADH (Ansell et al., 1997).

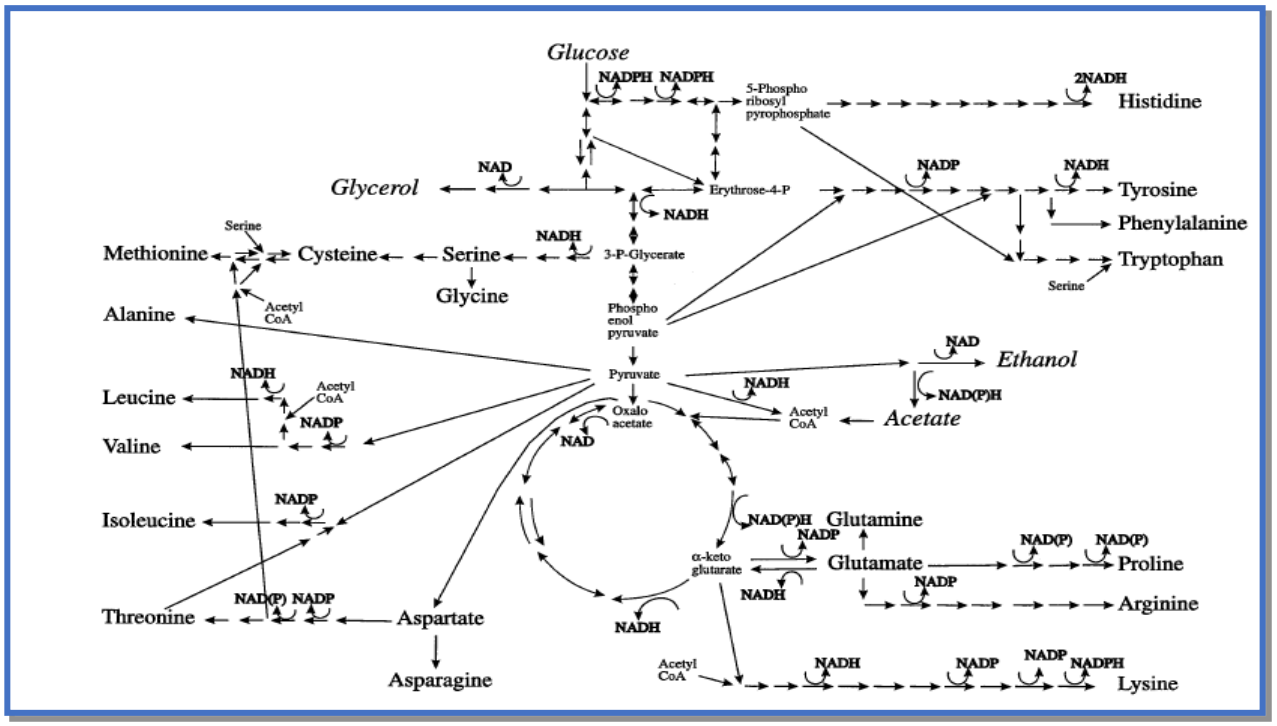


Figure 1.4. Simplified scheme for the formation of reducing equivalents in the biosynthesis of amino acids Adapted from (Jones and Fink, 1982, Gancedo and Serrano, 1989)

Radler and Schütz (1982) noted that in media with some single amino acids such as asparagine, alanine, valine, and serine the level of glycerol decreased compared with mixture of amino acids (Radler and Schütz, 1982).

The regulation of the assimilation of glycerol involves both its transport and catabolism. The first step of glycerol assimilation is transport from the fermentation medium into the cells. This transportation is achieved by some unique transport mechanisms that include passive diffusion (Romano, 1986), and facilitated diffusion (mediated by specific carriers located in cell membranes, and called facilitator proteins). Glycerol facilitator proteins are regulated via membrane lipid composition, thus the alteration in the lipid composition can interfere with the transport rate of glycerol (Truniger and Boos, 1993, Sutherland et al., 1997). Under hyperosmotic stress condition, the glycerol facilitator Fps1 closed rapidly to prevent the glycerol outflow. In the absence of hyperosmotic stress, the surplus of glycerol leaks out freely via glycerol facilitator Fps1 (Luyten et al., 1995, Tamás et al., 1999). It was reported that the *S. cerevisiae* yeast exploits glycerol as compatible solute for osmoregulation (Figure 1.5).

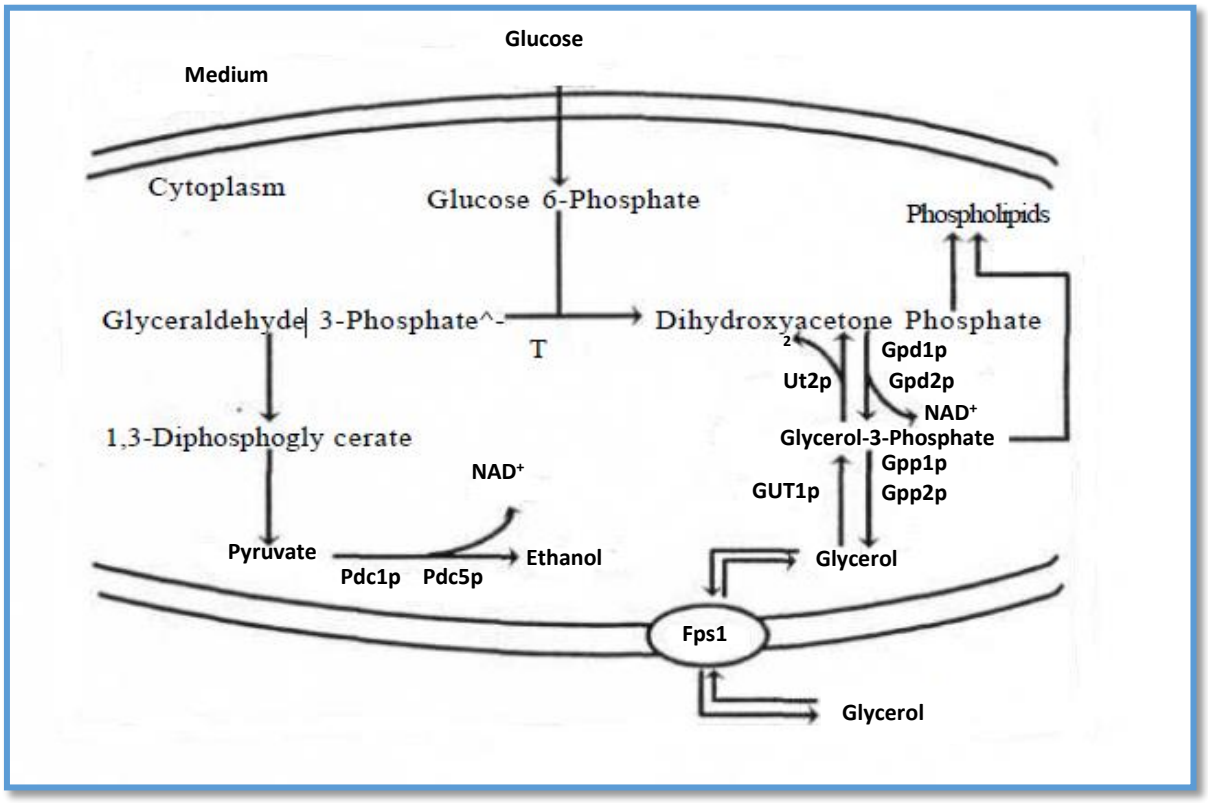


Figure 1.5. Glycerol synthesis in *S. cerevisiae.* Reduction of dihydroxyacetone phosphate to glycerol-3- phosphate and NADH oxidation to NAD^+ leads to the formation of glycerol, adapted from (Scanes et al., 1998b).

The High Osmolarity Glycerol (HOG) pathway is induced under hyper osmotic shock, and subsequently leading to the phosphorylation of stress-activated protein (SAP) kinase Hog1. This phosphoregulation of Hog1 induces the expression of genes encoding enzymes that are essential for the production and uptake of glycerol (Ansell et al., 1997, Yancey, 2005). Glycerol synthesis pathway starts with the reduction of the glycolytic intermediate DHAP into G3P which is catalyzed via the NAD^+-dependent G3P dehydrogenase. This enzyme is encoded by two isogenes, while osmostress induction is required for expression of *GPD1* which then enhanced the production of glycerol, the cellular redox potential is

controlled by the expression of GPD2 (Albertyn et al., 1994, Ansell et al., 1997, Påhlman et al., 2001, Valadi et al., 2004). Subsequently, G3P is converted to glycerol via G3P phosphatases Gpp1/Rhr2 as well as Gpp2/Hor2 (Albertyn et al., 1994, Ansell et al., 1997, Påhlman et al., 2001, Norbeck et al., 1996). In budding yeast, this pathway is essential for cell growth in a number of contexts. The first committed step of glycerol assimilation in most fungi is the phosphorylation by glycerol kinase, which is expressed by *GUT1* gene (Pavlik et al., 1993). This gene was investigated by (Sprague and Cronan, 1977) who selected *GUT1* mutants that were defective in glycerol assimilation. (Pavlik et al., 1993) showed that the disruption of the open reading frame of *GUT1* produced mutants that are not able to synthesize glycerol kinase and hence lost the ability to grow on glycerol. The regulation of *GUT1* expression is mediated by activation and repression mechanisms and depends on the carbon source. Expression of *GUT1* is repressed when the cells grow on glucose whereas it is derepressed in media with non-fermentable carbon sources, for instance glycerol and ethanol (Grauslund et al., 1999) The second step of the phosphorylation pathway includes the mitochondrial glycerol-3-phosphate dehydrogenase (the product of *GUT2* gene) (Ronnow and Kielland-Brandt, 1993). *GUT2* was also originally investigated by (Sprague and Cronan, 1977). The regulatory properties of *GUT2* are very much similar to those of *GUT1*. *GUT2* is inhibited through growing on glucose and activated under non-fermentable carbon sources, such as glycerol, ethanol, and lactate (Grauslund and Rønnow, 2000).

1.6. Regulation of Lipid Biosynthesis

In order to balance cell growth with energy storage, the pathway of lipid synthesis is highly regulated in organisms. In an oleaginous yeast, cell metabolism is geared towards the activation and up regulation of lipogenic machinery. In eukaryotic cells, a number of key regulators in lipid synthesis have been found, including energy regulation and signalling pathways, as well as transcriptional and allosteric regulation of rate limiting steps (Hardie and Pan, 2002). The controlling steps are described in detail below:

1. Lipid accumulation is triggered by cells exhausting nitrogen from the medium, but an excess of carbon is still assimilated by the cells and converted into TAGs. As a consequence activity of isocitrate dehydrogenase within the mitochondria slows or even ceases due to the shortage of AMP within the cells (Ratledge, 2002).
2. Two critically regulated enzymes, ATP/citrate lyase (ACL) and malic enzyme (ME), have key effects on lipid accumulation. In yeasts, fungi and other oleaginous microorganisms, there is a strong relation between the presence of ACL activity and the ability to accumulate lipid. However, it is not the only important factor (Adams et al., 2002). The range of lipid accumulation is thought to be regulated through the activity of malic enzyme (ME), which is the sole source of NADPH for fatty acid synthase (FAS). Lipid accumulation is slowed down, if the activity of ME is inhibited, or genetically disabled. Therefore, the stability of ME is crucial, and it is suggested that FAS is physically attached to ME as part of the lipogenic pathway. In two filamentous fungi, *Mucor circinelloides* and *Mortierella alpine,* ME has a close relationship with lipid accumulation. When ME activity is inhibited, lipid accumulation also stops. No other enzyme has the same correlation (Ratledge, 2002). The situation is less clear for *Y. lipolytica*, because the evidence suggests that the source of NADPH is the pentose phosphate pathway rather than through malic enzyme (Blank et al., 2005).
3. The energy state of the cells is monitored by the enzyme AMP kinase (AMPK) which responds with the phosphorylation of a broad array of enzymes to control the anabolic and catabolic pathways for the consumption and storage of energy.

Since lipid synthesis is highly correlated with storage and utilization of energy, it is strongly regulated by this enzyme. In a low energy state (low ATP/ high AMP content) or cell stress, pathways toward regeneration of ATP such as fatty acid β-oxidation and glycolysis are upregulated whereas pathways that are ATP consuming are inhibited (e.g. synthesis of triglyceride and cholesterol). The opposite occurs in a state of higher energy, with AMPK inducing lipogenesis for the storage of excess energy (Hardie and Pan, 2002). This enzyme is expressed through the SNF gene family in yeast cells. The deletion of *snf2* in *S. cerevisiae* resulted in an oleaginous phenotype, accumulating more than 20% lipid concentration (Kamisaka et al., 2007).

4. Several lines of evidence indicate that the acetyl-CoA carboxylase (ACC) represents an entrance for carbon leading towards cellular lipids. It catalyzes the conversion of acetyl-CoA to malonyl-CoA. ACC is directly regulated via AMPK; the phosphorylation of ACC suppresses enzymatic activity (Figure 1.6). Furthermore, ACC is allosterically regulated by a number of metabolites: citrate induces the enzyme whereas acyl-CoA (typically palmityl-CoA) inhibits it (Ohlrogge and Jaworski, 1997).

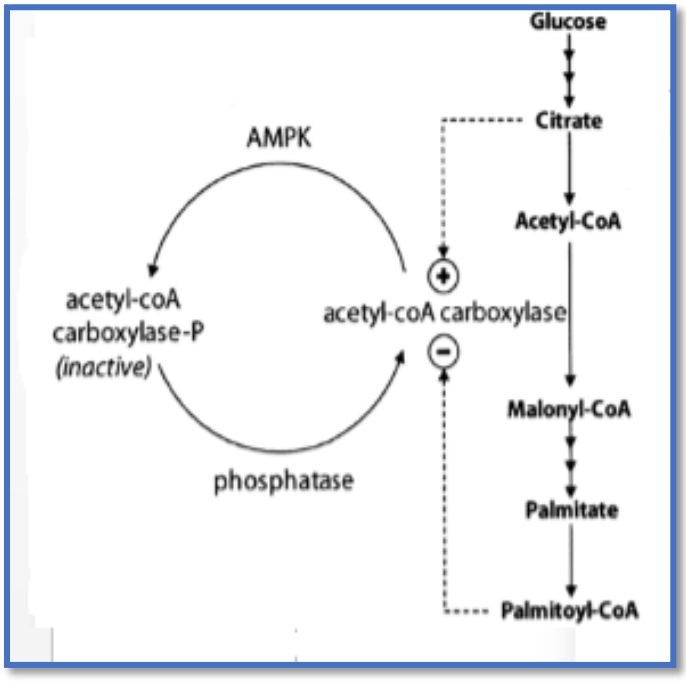


Figure 1.6. Feedback regulation of acetyl Co-A carboxylase by two metabolites; citrate activates ACC while palmitoyl Co-A repress it. ACC is also induced and inhibited by phosphorylation by the global energy regulator, AMPK (Tai, 2012).

5. In *Y. lipolytica*, TAG synthesis from glycerol-3-phosphate (G3P) has been investigated (Beopoulos et al., 2008b). G3P is either synthesized from glycerol through the activity of glycerol kinase, result of the transcription of *GUT1* gene, or it is formed from DHAP through G3P dehydrogenase, encoded by *GPD1*. The counter reaction, that produces DHAP from G3P, is performed by G3P dehydrogenase, the second isoform, encoded by *GUT2*. In order to direct the conversion of DHAP into G3P, the gene *GUT2* was deleted while the gene *GPD1* was over-expressed (Rossi et al., 2011).
6. In order to accumulate lipid, a diverse strategy was based on the inhibition of the β-oxidative metabolism. The POX genes encode acetyl-coenzyme oxidases, which are localised in the peroxisome. Deletion of the all 6 *POX* genes (*POX1* to *POX6*)

led to an increase in lipid as a percentage of dry biomass (Mlí et al., 2004, Mlíčková et al., 2004, Beopoulos et al., 2008b). It was also found useful to couple an engineered increase in the level of G3P with inhibition of the β-oxidation pathway (Dulermo and Nicaud, 2011).

7. Metabolic engineering, using heterologous gene expression, has been utilized to increase the range of substrates consumed by oleaginous fungi. It has been found that inulin (β-2,1-linked D-fructofuranose with a terminal glucose residue) (Sheng et al., 2007) is a good substrate for bio-manufacturing (Chi et al., 2009). In order to induce *Y. lipolytica* to accumulate lipids on inulin containing substrates, the gene for exo-inulinase from *Kluyveromyces marxianus* (*INU1*) was heterologously expressed on a high copy plasmid. The inulinase was effectively produced in *Y. lipolytica*, and inulin was successfully transformed into TAG (Zhao et al., 2010).

1.7. Optimization of lipid enriched biomass from oleaginous yeast in response to culture conditions

Cultivation conditions such as C/N ratio, carbon and nitrogen sources, temperature, pH, incubation period, inoculum volume, and concentration of inorganic salts have been shown to influence oil accumulation (Johnson et al., 1992, Johnson et al., 1995). To a variable extent, the trace metal ions also affect oil accumulation (Hassan et al., 1996, Wang et al., 2005, Yong-Hong et al., 2006). (Yong-Hong et al., 2006) found that, by the optimization of Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, and Ca^{2+} concentrations, the biomass and oil content could be enhanced significantly. Other cultivation parameters such as the concentration of dissolved oxygen in the culture medium has a positive correlation with oil accumulation (Yan and Cnen, 2003, Liang et al., 2006, Yi and Zheng, 2006).

Hence, optimization of medium ingredients coupled with statistical design approach is inevitable to understand the effects of various factors and their interactions with oil accumulation (Khuri and Cornell, 1996). Oleaginous microorganisms are expected to be a

good substitute as a feedstock for biodiesel production. Thus, more research become essential to investigate the effect of cultivation conditions, in particular medium ingredients on lipid accumulation of oleaginous yeasts prior to their large-scale production (Jadhav et al., 2012). Optimization of media is usually performed by keeping one parameter constant while changing the quantity of others (Khuri and Cornell, 1996).

1.7.1. Nitrogen source

The quantity and concentration of nitrogen source have an effective role on the microbial oil synthesis. Numerous studies have been reported the impact of nitrogen starvation on the activity of yeast cells such as the capacity of substrate uptake, slowdown of cell poliferation (Jørgensen et al., 2002, Sainz et al., 2003), and fermentation activity (Varela et al., 2004). Under nitrogen limitation the assimilation of carbon sources continues in both oleaginous and non-oleaginous strains, whereas the ATP/AMP ratio increases only in oleaginous organisms and lipid accumulation occurs. The size of these cells increases along with the volume of lipid particles that become larger (Fidler et al., 1999, Wynn and Ratledge, 2005). In such conditions, protein and nucleic acid synthesis ceases, at the same time the excess of carbon source continues to be metabolized into lipid or chitin accumulation (structural polysaccharide of fungal cell walls [$(C_8H_{13}O_5N)n$] as reported in the batch cultures of *Rhodotorula glutinis* (Berthe et al., 1981). Thus when undergoing nitrogen limitation, carbon fluxes could be distributed between lipid and carbohydrate storage (Pan et al., 1986). Botham and Ratledge (1979) proposed a hypothesis that explains lipid accumulation in yeasts. This states that intra-mitochondrial citrate primarily accumulates as a result of the decrease in intracellular AMP concentration, which then leads to a decline in activity of the AMP-dependent NAD^+ isocitrate dehydrogenase in mitochondria (Botham and Ratledge, 1979) (Figure 1.7).

Previous studies showed that some nitrogen sources may be more preferable in oil production than others, for example the red yeast *Rhodosporidium toruloides* preferred organic glutamate in lipid production more than inorganic ammonium (NH_4^+), thus oil

content was about 50% compared with 18% per dry biomass respectively (Evans and Ratledge, 1984a). (Huang et al., 1997) reported that inorganic nitrogen sources were not suitable for oil production but are useful for cell growth, whereas organic nitrogen sources like peptone were good for oil production but not beneficial for cell growth (Huang et al., 1997). The increase in lipid accumulation was hypothesized by the authors to be a result of the intracellular presence of NH_4^+ synthesized from the deamination of complex nitrogen sources. Another study by (Evans and Ratledge, 1984b) concluded that a certain threshold of intracellular NH_4^+ pools seems to be required to initiate the regulatory mechanism that induces citrate accumulation and the formation of fatty acids. NH_4^+ pool inside the cell resulted from nitrogen sources which might be needs more deamination reaction: for example, glutamate dehydrogenase for glutamate and urease for urea. Thus, enzymes catalysing the initial deamination step to release NH_4^+ may play an essential role in regulating lipid accumulation. In *Rhodotorulla* 110, among different nitrogen sources, yeast extract plus ammonium sulphate showed lipid content of 40%, 36%, and 30% along with glucose, xylose, and bran as carbon source respectively (Enshaeieh et al., 2012). One of the most important factors in TAG accumulation is the carbon/nitrogen (C: N, mol/mol) ratio. This ratio is an essential factor in optimizing SCO production (Beopoulos et al., 2009a).

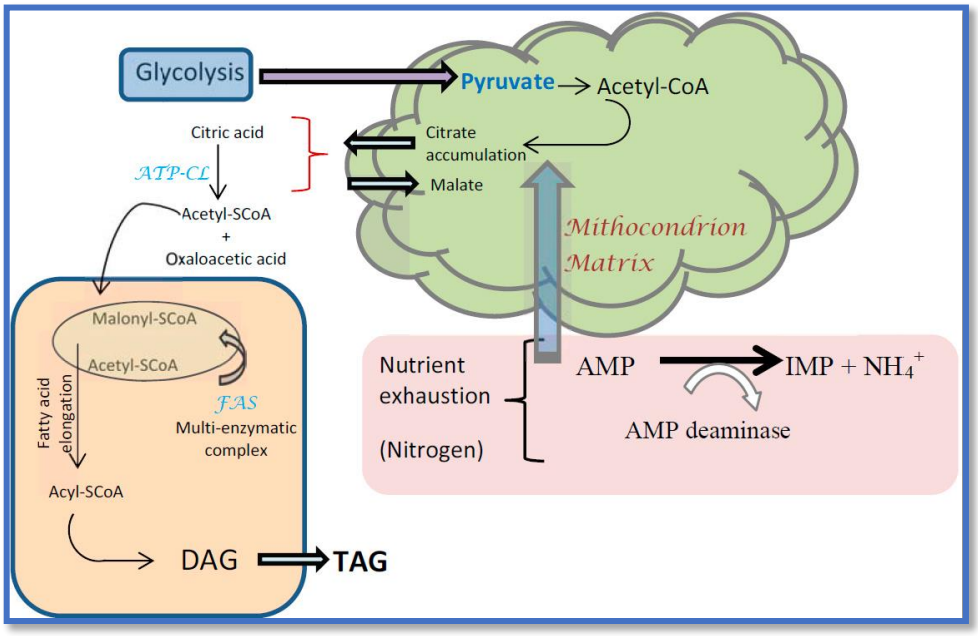


Figure 1.7. Schematic pathway of *de novo* lipid biosynthesis and formation of TAG under exhaustion of nitrogen source (adapted from Ratledge, 2004 and Xu *et al.*, 2013). IMP: Inosine monophosphate; DAG: Diacylglycerol; TAG: Triacylglycerol

Study of *Kodamaea ohmeri* and *Trichosporonoides spathulata* showed that addition of yeast extract and peptone (1:1) C/N gave the highest biomass and maximum lipid production. In respect to the economic cost, a less expensive inorganic nitrogen source (ammonium sulphate) was selected and gave similar, but slightly lower, levels of biomass and lipid accumulation for *K. ohmeri*. However, for *T. spathulata*, both biomass and the maximum lipid production were significantly lower when ammonium sulphate was used as the nitrogen source (Kitcha and Cheirsilp, 2011).

1.7.2. Carbon source

Many species of fungi can accumulate high level of lipids (up to 70% of their biomass) (Rattray et al., 1975) when grown with excess of carbon and a limiting amount of nitrogen. When the nitrogen runs out, the carbon flow is directed towards citric acid which acts as

an acetyl-CoA donor in the cytoplasm. Citrate moves out of the mitochondria into the cytoplasm, where it is split by the enzyme ATP: citrate lyase to give oxaloacetate and acetyl-CoA, the precursor of fatty acid biosynthesis. Non oleaginous organisms (e.g. *Candida utilis*) do not accumulate more than 7 to 8% of its biomass as lipid under nitrogen limited conditions (Babij et al., 1969, Thorpe and Ratledge, 1972). Many oleaginous micro-organisms are able to utilize a wide range of carbon substrates for biofuel synthesis, including glucose, xylose and glycerol (Papanikolaou et al., 2010, Fei et al., 2011). These carbons are dissimilated/assimilated by a range of pathways before finally entering the TAG synthesis pathway (Flores et al., 2000, Ratledge and Wynn, 2002). Waste materials rich in carbohydrates or hydrocarbons can be utilized by oleaginous yeast and converted into neutral lipids (Liu et al., 2009, Makri et al., 2010, Michely et al., 2013). Therefore, attempts have been made to investigate the effects of a wide range of carbon sources on cell growth as well as lipid synthesis. For instance, lipids may be produced in lower amounts when cells are grown on polymers like starch compared with yields obtained using glucose as the sole source of carbon (Papanikolaou et al., 2007). (Evans and Ratledge, 1983) studied *Candida curvata* growing on glucose (C6) and xylose (C5) in a continuous culture (average cell density of 14 g l^{-1}) and obtained lipid production rates of 0.16 g l^{-1} h^{-1} for glucose and 0.27 g l^{-1} h^{-1} for xylose. (Hassan et al., 1993) optimized the growth of *Apiotrichum curvatum* in a continuous fermentation set up using glucose as the only carbon source and reached a lipid production rate of 0.42 g l^{-1} h^{-1} and a high lipid content of 31.9% (w/w). Not only the carbon source has a significant impact on microbial oil accumulation, carbon concentration also has a great influence in the same culture conditions. Increasing the glucose concentration from 0.6 % to 20 % in cultures of *Candida parapsilosis* was reported to increase the concentrations of several enzymes; in particular the contents of glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase and pyruvate decarboxylase increased four- to tenfold (Hommes, 1966). (Babij et al., 1969) showed that increasing the glucose concentration in cultures of *Candida utilis* from 0.1 % to 4.5 % also increased the total fatty-acid content of this yeast from 2 % to 10 % dry weight.

1.8. Fatty acids

Fatty acids are basic elements of lipid compounds. Their role can be either incorporated into phospholipids and sphingolipids or considered as an energy reservoir in specific metabolites such as TAG and SE that are stored in lipid bodies. Their functions as transcriptional regulators and signaling molecules, and involvement in post-translational modification of proteins were also reported (Klug and Daum, 2014). Free FAs can be defined as carboxylic acid molecules with hydrocarbon chains varying in length and in degree of (un)saturation, which lead to the large variety of fatty acid molecules, and then to the formation of different compounds of lipid (Tehlivets et al., 2007). Yeast cells can obtain their FA by *de novo* synthesis, hydrolysis of complex lipids and delipidation of proteins, and by uptake from the external environment (Tehlivets et al., 2007). Intracellular synthesis of fatty acids takes place in the cytosol and in mitochondria while the elongation and desaturation steps occur in the endoplasmic reticulum. The first step of fatty acids synthesis is catalyzed by acetyl-CoA-carboxylase (Hasslacher et al., 1993). Through this reaction, acetyl-CoA, that is derived either from the degradation of citrate or acetate, is carboxylated to form the two carbon molecule malonyl-CoA which serves as a backbone in the following FA formation (Hoja et al., 2004). These high energy molecules, saturated fatty acids, which have been considered as reduced form of carbon, are able to be stored for long periods without significant degradation (Burr and Burr, 1929, Burr et al., 1932, Innis, 1991). By undergoing β -oxidation, fatty acids are converted into two-carbon acyl-coenzyme A (acetyl-CoA) molecules, which are then used for energy production via citric acid cycle (Eaton et al., 1996). The most common structure of fatty acid species in yeast consist of saturated 18:0 (stearic acid) and 16:0 (palmitic acid), the monounsaturated 18:1 (oleic acid) and 16:1 (palmitoleic acid), the diunsaturated 18:2 (linoleic acid) and the polyunsaturated 18:3 (linolenic acid) (see Figure 1.8). In *S. cerevisiae* the major fatty acyl groups are restricted into saturated 16:0 and 18:0 and monounsaturated 16:1 and 18:1 (de Kroon et al., 2013). Interestingly, along with fatty acids, the intracellular and extracellular metabolites are usually evaluated in order to visualise the cellular biochemical changes in microorganism such as genetic modification,

metabolic response towards environmental stress conditions, and growth state of cells (Chen and Chen, 2014). Different techniques of fatty acids analyses have been reported by (Christie, 2003). These techniques include the major analytical methods such as thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and analysis by GC coupled with mass spectrometry (GC–MS). The latter technique displayed high sensitivity, excellent separation of fatty acyl chains in different samples and accurate mass measurements that allowing for precise identification of fatty acids composition in yeast and different organisms. Fatty acids composition was reported to be variable between organisms when growing in different environments, for example *Candida. lipolytica* produced around 98-99% of new-chain fatty acids after growing on n-pentadecane or n-heptadecane at 30°C. Whereas, very low amount of these fatty acids were detected in yeast cells after growth on glucose as a sole carbon source (Mishina et al., 1973, Tanaka et al., 1978). Furthermore the influence of other factors such as salt stress on lipid structure and membrane fluidity has been evaluated in bacterial cells (Russell et al., 1995), as well as in yeasts, in particular salt-sensitive *S. cerevisiae* (Tunblad-Johansson and Adler, 1987, Sharma et al., 1996). In halotolerant yeasts and halophilic/halotolerant melanized yeast-like fungi the lipid composition and properties have also been studied. Under salt stress these organisms displayed different responses, for example *Zygosaccharomyces rouxii* cells exhibited increased content of free (non-esterified) ergosterol, decreased amount of unsaturated fatty acids, as well as decreased membrane fluidity when grown in medium with 15% NaCl (w/v) compared with their response in medium without NaCl (Hosono, 1992, Yoshikawa et al., 1995). However, *Candida. membranefaciens* produced increased fatty acid unsaturation and phosphatidylinositol (PI) and phosphatidylethanolamine (PE), and higher membrane fluidity when grown at higher concentration of NaCl (Khaware et al., 1995).

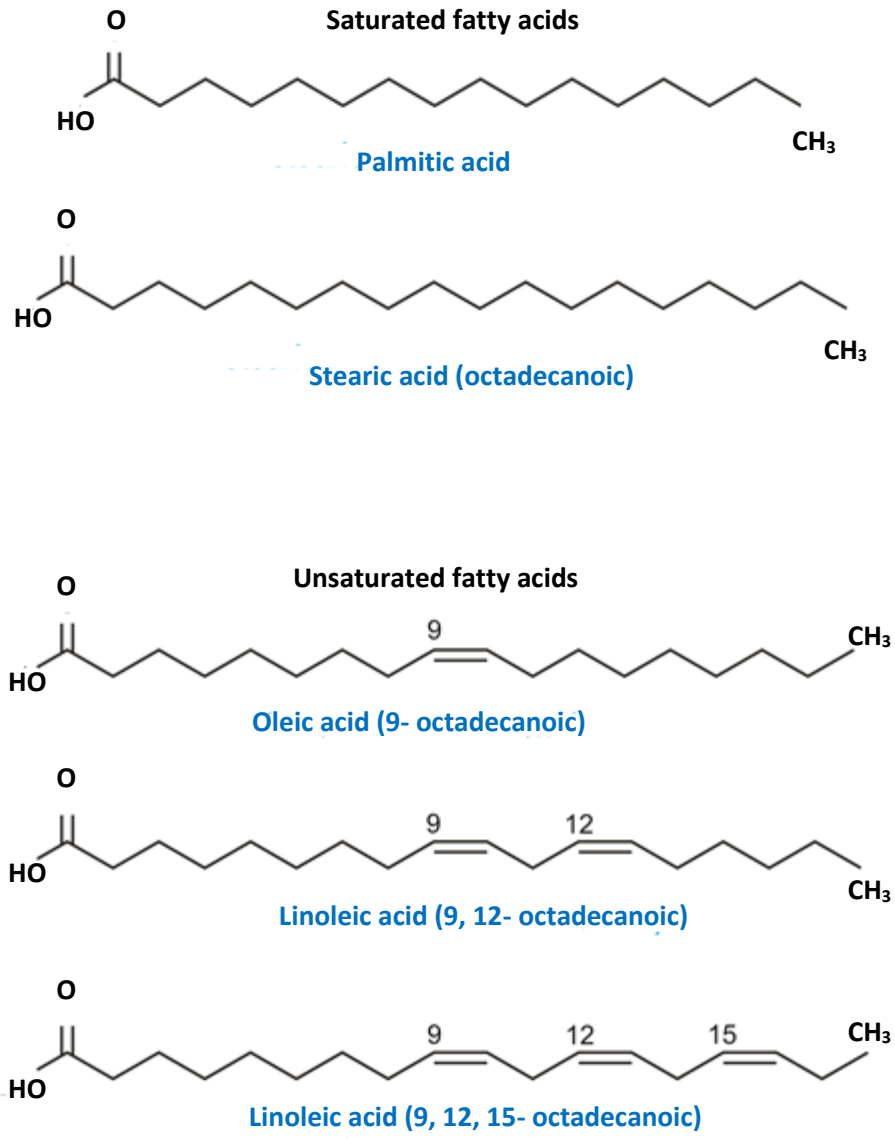


Figure 1.8. Diagram of some saturated and unsaturated fatty acids adapted by CHEMISTRY GLOSSARY web.

1.9. Genetic modification for biodiesel yield improvement

To enhance lipid accumulation, numerous studies have been performed by using metabolic engineering strategies in different species. These strategies included different approaches such as: overexpressing of enzymes that improve TAG biosynthesis pathway, regulation of related TAG biosynthesis approaches, multi-gene transgenic procedure, and repressing of the competing pathways of other metabolites (Liang and Jiang, 2013). In the oleaginous yeast *Y. lipolytica*, (Beopoulos et al., 2008a), reported that deletion of *GUT2* gene resulted in a threefold rise in overall neutral lipid yield compared with wild type strain. The *GUT2* gene encodes for the mitochondrial glycerol-3-phosphate dehydrogenase that oxidizes glycerol-3- phosphate (G3P) into dihydroxyacetone phosphate (DHAP), which is the competing pathway of triacylglycerol production. Studies showed that in *S. cerevisiae* the *GUT2* gene was found in chromosome IX (predicted molecular mass of the polypeptide expressed from *GUT2* gene is about 68.8 kDa and consists of 615 amino acids in length) (Rønnow and Kielland-Brandt, 1993). This gene displays identity of about 57% with the *D. hansenni GUT2* gene (DEHA2E08624g), located on the chromosome E. (NCBI BLAST). Extensive research has shown that *D. hansenii* is a lipid accumulating, osmotolerant, haploid yeast that reproduces by multilateral budding (Forrest et al., 1987), and contains two varieties: *D. hansenii* var. famata and *D. hansenii* var. fabryi (John and Spangenberg, 2005). The knowledge about this yeast is fairly limited even though the genomic sequence was fully analysed by the Genolevures consortium in 2004 (Dujon et al., 2004). Thus, research groups have considered this species as 'Cinderella' in the microorganism world since studies into its several remarkable aspects has not been sufficiently investigated. Besides that, this yeast was not reported as clinically important, therefore the investment on *D. hansenii* research was slowed down. (Desnos-Ollivier et al., 2008).

1.9.1. Gene deletion by homologous recombination

DNA double strand repair mechanism was considered as the basic concept of genetic manipulation in fungi. Thus, the exogenous DNA is integrated into the genomic DNA by one of two mechanisms such as homologous recombination (HR) or by nonhomologous end joining (NHEJ). Homologous recombination takes place between flanking regions of the targeted gene. These flanks are cloned in the disruption DNA cassette that contained the selectable marker (Richard et al., 2005). Thus, the compatible sequences in the genome will allow the replacement of the targeted gene of one of the wild-type alleles in the chromosomal DNA with the selectable marker. Targeted gene inactivation is an essential way to investigate gene function. Gene disruptions (knockouts) are often carried out in auxotrophic strains which display defects in particular biosynthetic pathways of yeast cells (for example histidine, arginine, and uracil). Gene repairing carried out through the complementation with the corresponding intact genes (e.g. *HIS1*, *ARG4,* or *URA3*) as selectable markers (Noble and Johnson, 2007). In *D. hansenii* the low-efficiency transformation system used in earlier studies basically depended on the *URA3* mutant and an autonomous replication sequence ARS, which were isolated in *S. cerevisiae* via functional screening procedure (Ricaurte and Govind, 1999). In recent research the transformation system in *Candida famata*: an anamorph of *D. hansenii*, has been developed (Voronovsky et al., 2002), nevertheless, the functional studies of the *D*. *hansenii* genes are continually based on complementation of *S. cerevisiae* mutants. To fill this gap in *D*. *hansenii* species, an efficient transformation system has been developed by Minhas (2009), depending on a histidine auxotrophic recipient strain and the *DhHIS4* gene as the selectable marker (Minhas et al., 2009). The most commonly used protocols for the *C*. *albicans* transformation are based either on electroporation or lithium acetate procedure and were also applied in *Saccharomyces cerevisiae*. For successful transformation, Schaub (2006) developed an arsenal of pFA-modules, that contain the heterologous marker genes *LEU2* from *C. maltose* as well as *HIS1* from *C. dubliniensis* (Noble and Johnson, 2005, Schaub et al., 2006) and *caSAT1* as the dominant selectable marker(Reuß et al., 2004). The construct pFA-*SAT1* consists of 1.956bp including the

newly selectable marker, *caSAT1,* that offered resistance to nourseothricin antibiotic (Reuß et al., 2004). Nourseothricin is related to streptothricin group, and produced by *Streptomyces noursei* (Krügel et al., 1988). The *sat-1* gene from the bacterial transposon Tn *1825* encodes for the streptothricin acetyltransferase polypeptide, that introduces resistance to nourseothricin via inhibition of the activity of antibiotic (Heim et al., 1989, Joshi et al., 1995). This gene was modified by changing the single CTG codon in the *SAT1* ORF, which would be mistranslated as serine instead of leucine due to the noncanonical codon usage of *C. albicans* (Santos and Tuite, 1995), to the leucine-specific codon CTC. This modification led to functional expression in *C. albicans*, resulting in *Candida*- adapted *caSAT1* gene that allowed the selection of resistant transformant strain after genomic integration (Reuß et al., 2004).

1.10. Aims of the project

This study aimed to investigate the optimum species, cultivation conditions, and molecular techniques to improve the TAG production by using single cell oil from yeast as a feedstock for biodiesel production. Primary experiments were aimed to screen three different yeast strains (*Debaryomyces hansenii* NCYC 102, *Cryptococcus curvatus* NCYC 476, *Yarrowia lipolytica* NCYC 2904) based on their ability to grow and produce neutral lipid under different concentrations of NaCl augmented in YM medium. In the second part of project *D. hansenii* was selected for further experiments and the main goals are:

1. Study the effect of high concentration of NaCl, augmented in cultivation media, on the accumulation of osmolytes, particularly glycerol, and their correlation with neutral lipid production compared with biomass production.
2. Improve the cultivation conditions to optimize the production of neutral lipids by using different concentrations of carbon/nitrogen (C/N) ratios in the minimal medium composition.
3. Study the effect of high salinity and different C/N ratios on the composition of the transesterified fatty acids.

4. Investigate the possibility of using homologous recombination technique in *D. hansenii* to knockout the *GUT2* gene, in order to disrupt the conversion of glycerol 3-phosphate into dihyroxyacetone phosphate via G3P dehydrogenase to increase TAG production.

Chapter Two: Materials and Methods

2. Materials and Methods

This chapter describes the procedures used to carry out my experiments.

2.1. Growth and sterile techniques

2.1.1. Microorganisms

Debaryomyces hansenii NCYC 102, *Cryptococcus curvatus* NCYC 476, *Yarrowia lipolytica* NCYC 2904 were obtained from National Collection of Yeast Cultures.

2.1.2. Preparation of inoculum

Using a sterile Pasteur pipette, 0.5 ml of YM medium (0.3% g yeast extract, 0.3% g malt extract, 0.5% g peptone, and 1% g glucose) (see section 2.1.5.1) was added to the dried material of each strain, gently re-suspended and transferred to 10 ml YM medium. After one day of incubation at 25°C on a rotary shaker at 120 rpm, 200 µl of inoculum was transferred to 250 ml conical flasks containing 50 ml of YM medium, and incubated under the same conditions. Other inocula were cultivated at 25°C in YM Agar medium with pouring and streaking methods.

2.1.3 Culture maintenance and storage

Strains were routinely maintained on YM liquid and solid medium and incubated at 25˚C for 24 hours. After which they were maintained at 4˚C.

2.1.4. Growth curve determination

Cell growth was monitored turbidimetrically by measuring the optical density regularly (every two hours) at 600 nm by using a spectrophotometer (Helios α). The blank was set using the growth medium with the same components and concentrations. The growth curve was plotted using the absorbance readings and time of incubation.

2.1.5. Effect of adaptation to different salinity concentrations on yeast growth

Debaryomyces hansenii NCYC 102, *C. curvatus* NCYC 476, *Y. lipolytica* NCYC 2904 were adapted to different concentrations of NaCl (0, 0.4, 0.8, 1.6, 2, 2.4, 2.8, and 3.2 M) augmented in YM broth. Flasks with 50 ml of the different salinity media were inoculated with each strain and incubated under shaking (120 rpm) at 25°C. Cell growth was monitored turbidimetrically by measuring the optical density at 600 nm using a spectrophotometer (Helios α).

2.1.5.1. YM Medium

YM medium was prepared by dissolving 3 g yeast extract, 3 g malt extract, 5 g peptone (Oxide), and 10 g glucose (Fisher Scientific) in 1 litre of distilled water. For solid medium 15 g of bacteriological agar No.1 (Oxoid) were added per litre. To examine the effect of salinity on yeast growth, NaCl (Fisher Scientific) was added to this medium at different concentrations (0.4, 0.8, 1.2, 1.6, 2, 2.4, 2.8, and 3.2 M) before autoclaving.

2.1.5.2. Minimal medium

Minimal medium was prepared by dissolving 5 g ammonium sulphate (Fisher Scientific), and 20 g glucose in 900 ml of distilled water. Sodium chloride was added to this medium at different concentrations (0, 0.8, and 1.6 M) before autoclaving at 121°C to examine the effect of salinity. A 10x stock solution of YNB (Yeast Nitrogen Base) was prepared by suspending 1.7 g of yeast nitrogen base without amino acids and ammonium sulfate in 100 ml of cold distilled water. Warmed slightly to solubilize and sterilized by filtration. Then the solution was diluted 1:10 with autoclaved medium under aseptic conditions.

2.1.6. Cleaning and sterile technique

All culture equipment was autoclaved at 121°C and dried in oven at 70 °C before using in experiments to avoid contamination. Inoculation and sub-culturing were performed with

a flame after surface sterilization of the bench by 70% ethanol or 1% Virkon disinfectant solution. Moreover, the glassware was soaked for 2 hours in 1% Virkon disinfectant solution to ensure that all contaminants were removed.

2.1.7. Biomass preparation of *D. hansenii* cells

Thirty ml from a *D. hansenii* culture growing in minimal or YM medium under different cultivation conditions was taken at the end of incubation period and centrifuged for 10 min at 3000 *g* in a bench centrifuge. The supernatant was discarded, and the pellets re-suspended in 1 ml distilled water. Fresh Eppendorf tubes were prepared by cutting off the lids from other Eppendorf tubes, and making a hole in these lids with a needle. The lids with a hole were attached to a complete Eppendorf tube with its own lid, which had been weighed. Subsequently, the concentrated culture was transferred to these Eppendorf tubes with two lids and put in -80˚C freezer overnight. The final step was to freeze dry (lyophilise) the samples, after removing them from the -80˚C freezer (without allowing them to thaw) and putting in the freeze-dryer. The samples were left for about 72 hours in the freeze dryer, after that the lids with the holes were removed and the other lids used to seal the Eppendorf tubes, and then all samples were re-weighed.

2.1.8. Glycerol stock for culture storage

Yeast and bacterial strains were stored long term in 15% glycerol as the cryoprotectant. 500 µl of liquid culture was added to 500 µl 30% (v/v) glycerol in a Nunc cryovial tube. After vortexing, tubes were stored at -80°C. To rescue a frozen stock, cells were collected using a sterile inoculating loop and streaked onto a 2TY (1.6% Bacto Tryptone, 1% yeast extract, and 0.5% sodium chloride) (see section 2.8.1.2.4.1.) or YM agar plate. Individual colonies were then inoculated into overnight liquid cultures.

2.2. Identification and quantification of the total osmolytes and glycerol

2.2.1. Effect of adaptation to different salinity concentrations on compatible solutes accumulated in three yeast strains

Due to previous studies that referred to the accumulation of osmolytes in yeast cells in response to the high external salinity, experiments were carried out to determine the total osmolytes synthesised based on the procedure adopted by (Ben-Amotz and Avron, 1978). Yeast strains were grown in 50 ml of YM and minimal media augmented with different concentrations of NaCl (0, 0.8, and 1.6 M). After adaptation, cultures were incubated under shaking at 120 rpm and 25˚C for 24 hours, and the total osmolytes quantified as described below.

2.2.1.1. Glycerol standard curve

1- Different concentrations of glycerol were prepared (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 µg glycerol μl^{-1}) from the stock solution 1.25 µg glycerol μl^{1} (Fisher Scientific). ten tubes were set up as follows:

Concentration (µg/100 µl)	Blank	10	20	30	40	50	60	70	80	90	100
Distilled water (µl)	100	_	_	_	_	_	_	_	_	_	_
glycerol solutions (µl) (different concentrations	_	100	100	100	100	100	100	100	100	100	100
30% TCA (Trichloroacetic acid) (µl)	10	10	10	10	10	10	10	10	10	10	10

2- 1 ml of the periodate reagent was added to all tubes. Mixed well and waited for 5 min.

3- Added 2.5 ml of acetylacetone reagent to all ten tubes. Mixed well and placed them in water bath at 45°C for 15 min.

4- Tubes were removed from water bath and allowed to cool for a few minutes at room temperature. The optical density (OD) was read at 410 nm in spectrophotometer. Following measurement, the standard curve was plotted between the concentration and absorbance (see Figure 2.1) and a standard deviation was performed for each sample.

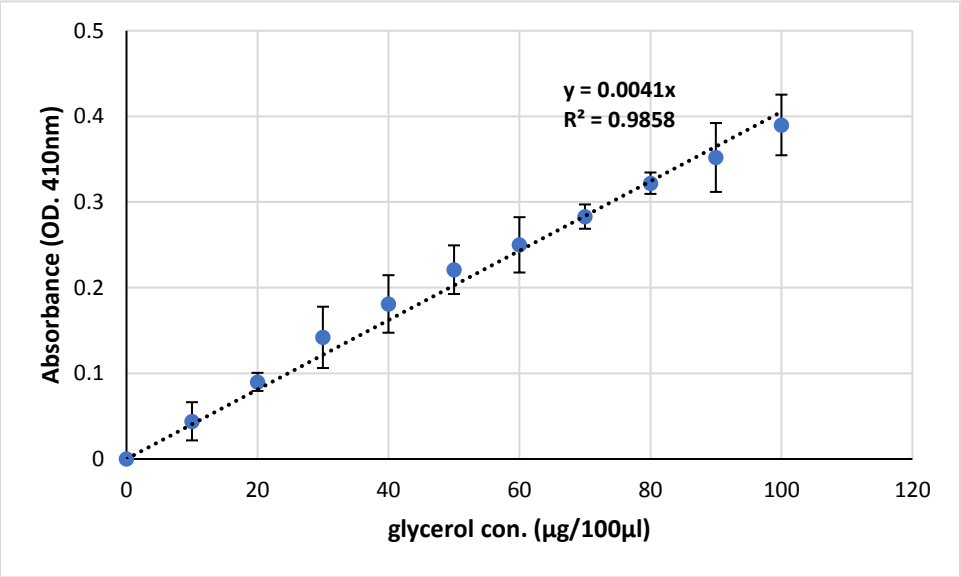


Figure 2.1. Standard curve for glycerol, OD. at 410. Experiment was done with three replicates, Bars represent standard deviation.

2.2.1.1.1. Periodate reagent

To prepare this reagent 130 mg sodium periodate (Fisher Scientific) was dissolved in 180 ml 2% acetic acid (Fisher Scientific) containing 15.4 g ammonium acetate (Sigma), and when fully dissolved 20 ml glacial acetic acid was added.

2.2.1.1.2. Acetylacetone reagent

Acetylacetone (2.5 ml) (Fisher Scientific) was added to approximately 200 ml of isopropanol and made up to a final volume of 250 ml with isopropanol. The solution was stored in dark bottle away from light.

2.2.1.2. Osmolytes determination in all three yeast species

After a suitable glycerol concentration curve was obtained, the amount of total osmolytes of each yeast was determined by taking 1 ml aliquots from each sample growing in YM medium and put in Eppendorf tubes. Centrifuged for 10 minutes at 3000 g, then suspended again in 1 ml distilled water. One hundred µl of 30% trichloroacetic acid (TCA) were added to each 1 ml aliquot, whirlimixed and centrifuged in microfuge for 5 minutes at full speed. One hundred and ten µl of clear supernatants were taken into separate glass test tubes. The blank was set up containing 100 µl distilled water and 10 µl 30% TCA, then periodate and acetylacetone reagents were added as described above (2.2.1.1) and OD was read at 410 nm against the blank.

2.2.1.3. Osmolytes determination in *D. hansenii* cells growing in different media

The same procedure was performed (as described in section 2.2.1.2) for measurement the total osmolytes except the results were compared with the biomass (µg glycerol / mg biomass) which was measured in the same way for all samples (see section 2.1.7.).

All readings were multiplied by 10 to be consistent with glycerol values that were measured using free glycerol reagent in *D. hansenii*.

2.2.2. Glycerol determination using free glycerol reagent (Sigma F6428)

In this test, free endogenous glycerol was tested by using Free Glycerol Reagent via two enzyme reactions without the initial lipase hydrolysis. Glycerol is phosphorylated into glycerol-1-phosphate (G-1-P) and adenosine- 5'-diphosphate (ADP) by adenosine-5'-

triphosphate (ATP) in the reaction catalyzed by glycerol kinase (GK). Subsequently, Glycerol-1-phosphate oxidized via glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Peroxidase (POD) then catalyzes the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance maximum at 540 nm. Free glycerol concentration of the sample is directly proportional to the increase in absorbance at 540 nm.

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol- 1- phosphate + ADP} \qquad \textbf{Equation 2.1}$$

$$\text{Glycerol- 1- phosphate + } O_2 \xrightarrow{\text{GPO}} \text{DAP +2 } H_2O_2 \qquad \textbf{Equation 2.2}$$

$$2\ H_2O_2 + \text{4- AAP+ESPA} \xrightarrow{\text{POD}} \text{Quinoneimine Dye + } H_2O \qquad \textbf{Equation 2.3}$$

2.2.2.1. Glycerol concentration curve

1. The free glycerol reagent was taken from the fridge and allowed to reach room temperature. The spectrophotometer was set to wavelength 540 nm.
2. To six cuvettes 0.8 ml free glycerol reagent was added to all cuvettes containing different volumes of standard glycerol and distilled water as follow:
   A. 25 µl distilled water plus 0 µl glycerol standard (Blank)
   B. 20 µl distilled water plus 5 µl glycerol standard (1.3 µg glycerol)
   C. 15 µl distilled water plus 10 µl glycerol standard (2.6 µg glycerol)
   D. 10 µl distilled water plus 15 µl glycerol standard (3.9 µg glycerol)
   E. 5 µl distilled water plus 20 µl glycerol standard (5.2 µg glycerol)
   F. 0 µl distilled water plus 25 µl glycerol standard (6.5 µg glycerol).

All cuvettes were incubated on the bench for 15 minutes and the optical density read at 540 nm using the blank to zero the spectrophotometer. The standard curve was plotted from which glycerol concentration for each sample can be determined (see Figure 2.2)

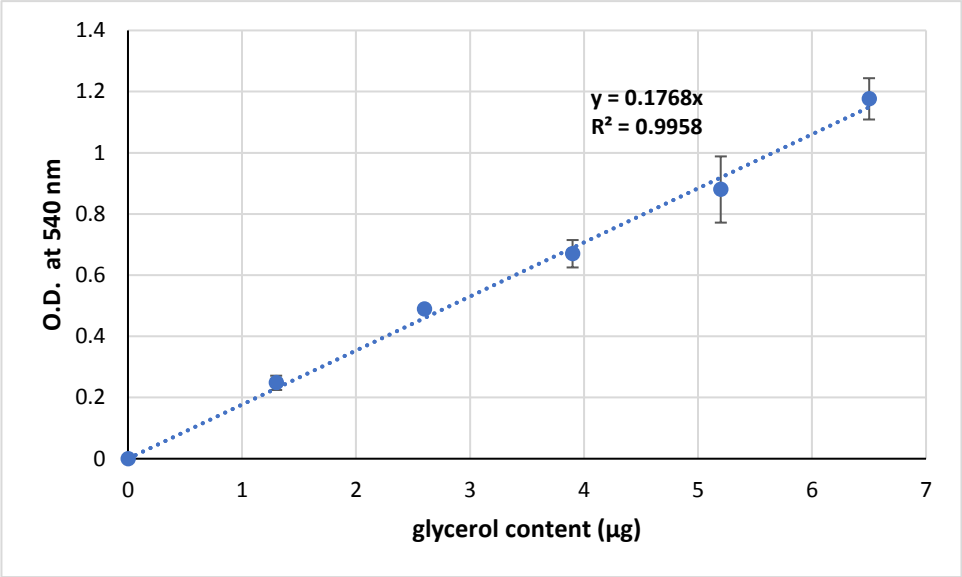


Figure 2.2. Standard curve for glycerol, OD at 540 nm versus µg glycerol, Experiment was done with three replicates, Bars represent standard deviation.

2.2.2.1.1. Free glycerol reagent

Follow the instruction of Sigma Chemical Company, the free glycerol reagent was prepared by adding 40 ml of deionised water to the glycerol reagent powder, covered with aluminium foil to protect from light, and mixed by inverting a few times without shaking. The reconstituted Free Glycerol Reagent is stable for two months when stored at 2–8°C.

2.2.2.2. Glycerol determination in *D. hansenii* cells using free glycerol reagent (Sigma F6428)

After preparing the sample in the same way that was done for osmolytes determination in *D. hansenii* in both minimal and YM cultures (section 2.2.1.2 until centrifugation). After centrifugation, 25µl of the clear supernatants were taken into separate glass test tubes to be consistent with the same volume of blank, which containing only 25µl distilled water, then 0.8 ml free glycerol reagent was added to all cuvettes, and readings were taken at 540 nm using the blank to zero the spectrophotometer. All the results were compared with the biomass (µg glycerol / mg biomass) which was measured in the same conditions for all samples. All the readings were multiplied with 40 to be consistent with the values of total osmolytes measurement in *D. hansenii*.

2.3 Nuclear Magnetic Resonance (NMR) analysis of compatible solutes

2.3.1. NMR analysis of compatible osmolytes in all three yeast strains growing in different concentrations of NaCl

Twenty ml from each yeast culture growing in a different concentration of NaCl (0, 0.8, and 1.6 M) was taken after 24 hours of incubation at 25˚C and centrifuged for 10 min at 3000 *g* in a bench centrifuge. The supernatant was discarded, and the pellets resuspended in 1 ml distilled water. Then the samples were sonicated in 50 ml Falcon tube on ice 3 x 20 second with 15 second cooling periods between sonication steps. The sonicated samples were transferred to Eppendorf tubes and centrifuged at 13000 *g* in microfuge for 5 min. Fresh Eppendorf tubes were prepared by cutting off the lids from other Eppendorf tubes, and making a hole in these lids with scissors or a needle. The lids with a hole were attached to a complete Eppendorf with its own lid, which had been weighed. Then the supernatant transferred to these Eppendorf tubes with two lids and put in -80˚C freezer overnight. The final step was to freeze dry (lyophilise) the samples, after removing them from the -80˚C freezer (without allowed them to thaw) and putting in freeze-dryer. The samples were left for about 72 hours in the freeze dryer, and then the lids with the holes

were removed and the other lids used to seal the Eppendorf tubes, then all samples were re-weighed (Derome, 2013). Freeze-dried samples were sent to Prof M P Williamson in the MBB department for NMR analysis. Samples were dissolved in 530 µl of D_2O plus 5 µl of 100 mM trimethylsilyl propionate (TSP) to act as a chemical shift reference (0 ppm) and standard. Spectra were acquired using pre-saturation of the residual solvent signal for 1.5 s.

2.3.2. NMR analysis of compatible solutes in *D. hansenii* cells growing in different concentrations of NaCl

To identify the osmolytes in *D. hansenii* cells, the same procedure was followed as described above, except for the step of cell wall disruption. In order to be sure that the sonication technique didn't affect the intracellular osmolytes, a bead beating technique was used in addition and the results compared between the two techniques. The samples of all cultures were divided into two groups. For the first group, the cells were disrupted by bead beating for about 4 x 60 second at 4000 rpm with 60 second cooling intervals. For the second group cells were broken by sonication with 3 x 20 second with 15 second cooling periods between sonication steps. After centrifugation, the samples were freeze-dried as described above (section 2.3.1) before NMR analysis.

2.4. Triacylglycerol (neutral lipid) quantification and microscopic imaging of the lipid bodies.

2.4.1. Optimization of Nile Red technique conditions to determine neutral lipid in yeast cells

2.4.1.1. Cell concentration

To determine the content of neutral lipid in yeast cells, at least 10 ml of fresh culture (in the late log phase) was collected and centrifuged for 5 min at 3000 *g*. After centrifugation, the pellet was re-suspended with an equivalent volume of distilled water, and the OD was adjusted to 5.5 at 600 nm by using the appropriate amount of distilled water which was

also used as blank. From this concentration, serial dilutions of yeast cells were prepared with the final volume of 2ml in Eppendorf tubes as follows (Table 2.1):

Table 2-1. Serial dilutions of yeast cells

Percentage	100	87.5	75	62.5	50	37.5	25	12.5	Total (ml)
Culture (µl)	2000	1750	1500	1250	1000	750	500	250	9
D. W. (µl)	0	250	500	750	1000	1250	1500	1750	7

2.4.1.1.1. Cell Count Calibration

To perform cell counting calibration curve, numbers of cells counting were plotted against the optical density at OD_{600} nm based on the method from Guillard and Sieracki (Guillard and Sieracki, 2005). 50ml of *D. hansenii* YM and minimal culture were centrifuged and adjusted aseptically with sterilized distilled water to an OD of 5.5 at 600 nm using a 1 ml plastic cuvette and spectrophotometer. We used the dilutions in Table 2.1 for cell counting by combining the adjusted culture with sterilized distilled water in 2 ml Eppendorf tubes. The samples were whirlmixed, the 20 µl aliquots were transferred into 0.2 mm depth haemocytometer with Thoma ruling (Z30000 Helber Counting Chamber, Hawkley). The heavy slide cover was carefully placed on top. Cells were allowed to settle for approximately 2 minutes on the slide before counting in a phase contrast illumination microscope with an achromatic lens of 40 x (Labophot, Nikon). The count was repeated three times for each sample, and the number of cells in the original culture was calculated using the mean number of cells per small square based on the following equations:

1- $Volume\ of\ one\ grid\ element$

- $Depth\ of\ counting\ chamber\ = 0.02\ mm$
- $Area\ of\ small\ square\ = \frac{1}{400}\ mm^2 = 2.5\ x\ 10^{-3}\ mm^2$ $\boldsymbol{Equation\ 2.4}$

- $Volume\ of\ small\ square = 2.5\ x\ 10^{-3}\ mm^2\ x\ 0.02\ mm$ **Equation 2.5**
- $Volume\ of\ culture\ per\ small\ square = 5.0\ x\ 10^{-5}\ mm^3$

  $= 5.0\ x10^{-8}\ cm^3\ (\equiv\ ml)$ **Equation 2.6**

2- $Cells\ in\ 1\ ml\ sample\ (if\ counted\ using\ 400\ squares)$:

- $Cell\ no.per\ small\ square = cell\ count/\ 400$ **Equation 2.7**
- $Cell\ no.per\ small\ square\ x\ (\frac{volume\ of\ 1\ ml}{volume\ of\ culture\ per\ small\ square}) =$

  $\frac{1\ ml^{-1}}{5\ x\ 10^{-8}\ ml^{-1}} = cell\ ml^{-1}\ sample$ **Equation 2.8**
- $Cell\ ml^{-1}\ original\ culture = cell\ ml^{-1}\ sample\ x\ DF$ **Equation 2.9**

  DF is the dilution factor

2.4.1.2. Stain concentration

In order to test the effect of different concentrations of Nile red dye on the lipid measurement, the procedure was repeated with different concentrations (0.025, 0.05, 0.1, 0.2, 0.4, and 0.6 µmole/ml) of stain using the optimal concentration of yeast cells.

2.4.1.2.1. Primary stock of Nile red dye

Ten mg of Nile red (Sigma) was added to 10 ml of dimethyl sulfoxide (DMSO), making the concentration 3.14 mmole/L (or 3.14 µmole/ml). From this concentration, secondary stocks of Nile red dye were prepared with a final volume of 1000 µl as shown in Table 2.2.

Table 2-2. Secondary concentrations of Nile red dye

NR µMol/ml	From primary (µl)	DMSO (µl)
0.025	8	992
0.05	16	984
0.1	32	968
0.2	64	936
0.4	128	872
0.6	192	808

2.4.1.3. Plate reader settings

Before carrying out the procedure, the plate reader was checked to ensure that the correct filters for excitation and emission wavelengths, 485 nm and 580 nm respectively, were installed (Almutairi, 2015).

2.4.1.4. Procedure

After preparing the samples of yeast cells at the optimum cell concentration, all Eppendorf tubes were whirlimixed, and 1 ml from each sample was transferred to a row of the reagent reservoir. Then 4 x 200 µl from each row were collected, and added to the 96 well plate as the unstained cells at the relevant concentration using a multichannel pipette. Next, 20 µl of Nile red solution was added to all Eppendorf tubes and the timer was started. All samples were whirlimixed, and transferred to a row of the reagent reservoir. Four x 200 µl of stained samples were transferred to appropriate wells. After removing the cover, the plate was placed in plate reader machine to start the NR KB- Peak finder, and the readings were taken at a regular time intervals (7, 15, 30, 45, and 60 min) (Chen et al., 2009).

2.4.2. Spectroflurometric quantification of neutral lipid in three yeast strains growing in YM medium with different concentrations of NaCl

Neutral lipid accumulation was determined in all three yeast strains growing under different osmotic stress condition. The experiment was carried out by growing all strains in 3x250 ml flasks containing 50 ml YM medium augmented with different concentrations (0, 0.8, 1.6 M) of NaCl under shaking at 25°C. Readings were taken after different times of incubation (at the logarithmic and stationary phase) for each yeast strain.

2.4.3. Spectroflurometric quantification of neutral lipid in *D. hansenii* cells under different stress conditions using Nile red dye

2.4.3.1. NaCl stress

To determine the neutral lipid production in *D. hansenii* strain, the same procedure was followed as described in section 2.4.2. First, the Nile red technique was optimized for *D. hansenii* growing in minimal medium. Then the lipid content was compared with biomass (mg lipid/ mg biomass) in both minimal and YM media with different concentrations (0, 0.8, 1.6 M) of NaCl.

2.4.3.2. Effect of different carbon/nitrogen ratios on neutral lipid production

To visualize the effect of carbon/nitrogen ratios in relation to the type of carbon source used (glucose and glycerol) on neutral lipid production in *D. hansenii*, experiments were performed using 3x250 ml shaking flasks containing 50 ml minimal medium with different carbon/nitrogen (ammonium sulfate) ratios. The experiments were carried out by using glucose or glycerol as the only carbon source in minimal medium. First, the nitrogen source content was fixed at the original concentration in minimal medium and the concentration of carbon source was changed (8:1, 16:1, 32:1, and 48:1 C/N). Then the selected concentration of carbon source was used with lower concentrations of ammonium sulphate (48:1, 48:0.5, 48:0.25, and 48:0 C/N). Each culture was adapted to the same concentration of carbon/nitrogen before measuring the neutral lipid inside the

cells after 40 hours of incubation, and the OD was set initially at 0.2 in all cultures. After centrifugation at *3000 g* for 5 minutes, yeast cells were re- suspended in distilled water, the OD was set at 4.72 ±, and the lipid content was compared with biomass (mg lipid/ mg biomass).

Following optimization, the C/N ratio, an experiment was performed to compare between 48:0.5 glucose/ ammonium sulphate with 8:0.25 glycerol/ ammonium sulphate ratios on neutral lipid production. The readings were taken for 3x50 ml liquid media after 40 hours of incubation, then compared with the biomass of each culture.

2.4.3.3. Changing the cells environment from high salt to the 48:0.5 glucose/ ammonium sulphate medium

In all previous experiments the cultures were inoculated with cells pre-adapted three times in the same culture conditions before measuring the neutral lipids. This experiment was carried out by using inoculum from pre-adapted cells to high salt environment. After 40 hours of incubation in 1.6 M NaCl minimal medium, 3X 50ml cultures were centrifuged at 3000 g for 5 minutes then re-suspended under aseptic conditions in 3X 50ml 48:0.5 glucose/ammonium sulphate medium at different times (2, 4, 8, 24, and 40 hours) of incubation. Initially three replicates of 1.6 M minimal medium were performed for each time of incubation in 48:0.5 glucose/ammonium sulphate medium (3X 50ml). The OD was started at 0.2 for all cultures and the readings of neutral lipid and total osmlytes were taken and compared with the biomass (mg).

2.4.4. Triolein Calibration Curve

Standard curve of triolein was created to allow quantification of the total neutral lipid inside the cell. Based on the work of (Bangert, 2014) and modified in this thesis, neutral lipid calibration curve was carried out in a 96 well plate format. A series of eight lipid concentrations were created using the ratio 1:20 polar (PC-phosphatidylcholine (L-

lecithin Type XVI-E, from fresh egg yolk ~99%)) to neutral (TO- Triolein) lipids, isopropanol and distilled water. A primary solution was prepared by adding 500 µl (2500 µg) of TO to 12.5 µl (125 µg) of PC (with chloroform) in O-ring sealed 1.5 ml Eppendorf to make 512.5 µl of stabilized triolein. Then eight lipid secondary standards were made by combining isopropanol with different volumes of primary standard and distilled water (Table 2.3) in 2 ml Eppendorf tubes.

Table 2-3. Eight triolein secondary standards

Triolein (mg/ml)	**0.1**	**0.08**	**0.06**	**0.04**	**0.02**	**0.01**	**0**
Distilled water	1959.5	1959.5	1959.5	1959.5	1959.5	1959.5	1959.5
1:20 Triolein (µl)	40.5	32.8	24.6	16.4	8.2	4.05	0
Isopropanol (µl)	0	7.7	15.9	24.1	32.3	36.4	40.5

The standards then sealed, kept on ice and in the dark to avoid degradation. One ml from each tube was transferred to reservoir and 4 x 200 µl collected, and added to the 96 well plate as the unstained cells. Another 4 x 200 µl was added to plate as stained cells after staining with Nile red for about 7 minutes. All wells were read using the same conditions (an excitation wavelength of 485 nm and emission of 580 nm with gain 35), and the standard curve made between the fluorescent intensity and lipid concentration (see Figure 2.3).

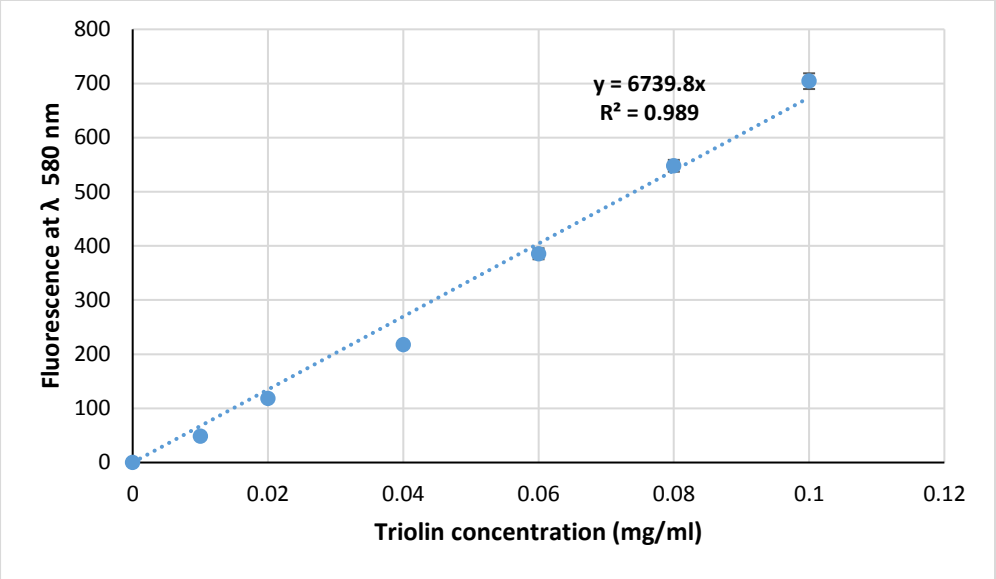


Figure 2.3. Standard curve of Triolein concentration versus Nile red fluorescence intensity. Experiment was done with four replicates, Bars represent standard deviation.

2. 5. Imaging of neutral lipid bodies within the cells by using Fluorescence Microscopy

2.5.1. Preparing of the cells

Neutral lipid body accumulation within *D. hansenii* cells was observed by using Fluorescence Microscopy. Cultures from both minimal and YM media were grown until the late logarithmic phase. After centrifugation, the pellet was re-suspended in distilled water and the optical density was set at 4.72 ±. Then 1 ml from each culture was added to 1.5 ml Eppendorf tube along with 20 µl of Nile red dye at 0.4 and 0.2 µmol/ml for minimal and YM cultures respectively, and mixed well with monitoring the time of staining. Ten µl of the sample was transferred into a clean microscope slide and the coverslip was added carefully. The edges of the coverslip were sealed with nail varnish to

stop evaporation, and the slide was ready to view once the varnish had dried and after at least 7 minutes of staining.

2.5.2. Fluorescence microscopy

Images were captured on a Axiovert 200M (Zeiss) fluorescence microscope equipped with Exfo X-cite 120 excitation light source, band-pass filters (Zeiss and Chroma), an alpha plan-Fluar 100x/1.45, A-plan-pochromat 63x /1.40 oil immersion lens, or A-plan 40x/0.65 NA Ph2 objective lens (Zeiss) and Hamamatsu Orca ER digital camera. Filters (525 and 700 nm) were used for green Channel and Red Channel respectively. Image acquisition was performed on Volocity software (Perkin Elmer). Live cells were imaged in non-fluorescent media at room temperature. Fluorescence images were collected as 0.5 µm z-stack, merged into one plane after contrast enhancing in Openlab (Improvision) software and the figures were prepared using Photoshop. Brightfield images were collected in one plane and added into the blue channel in Photoshop (Adobe). The level of the brightfield images was modified, and the image was blurred and sharpened before further level adjustment (Al Saryi, 2016).

2.6. Fatty acid analysis of the neutral lipid

2.6.1. Fatty acids transesterification and analysis by GC-MS technique in *D. hansenii*

Fatty acid structure in *D. hansenii* cells was identified under different stress conditions (NaCl stress and low amount of nitrogen source). Fifty ml of pre-adapted YM and minimal cultures (with different concentrations of NaCl: 0, 0.8, and 1.6 M; as well as different C/N ratios: 48:0.5 and 8:0.25 glucose/ ammonium sulphate and glycerol/ ammonium sulphate respectively) were collected after incubation for the same time as used for neutral lipid measurement for each medium (Section 2.4). Cultures were centrifuged and then re-suspended in distilled water, the OD was set at 4.72 ± for all cultures, and 30 ml from each culture were freeze dried and then weighed. In 1.5 ml GC vials (9301-1388, Agilent) 5-10 mg aliquots of freeze dried biomass was weighed and the hot plate was preheated to 85 ˚C before adding the transesterification solutions. To each vial 300 µl of 0.6 M HCl:

methanol was added, then 200 µl of chloroform: methanol (2:1 v/v) and 20µl of tridecanoic acid methyl ester (C13: ME, 10 mg ml^{-1} as internal recovery standard that does not naturally occur in yeast and was used to correct the variability of FAME extraction and solvent evaporation in the samples (Smith, 2016). Gas-tight syringes (250, 100 µl and 20 µl, Hamilton) were used and all vials were quickly sealed with PTFE/silicone/PFTE septa crimp caps (5181-1211, Agilent). Once the working solutions addition was completed, all the sealed vials were placed in the pre-heated hot plate at 85°C for one hour to allow the transesterification reaction to occur. In this procedure the whole lipids in freeze dried biomass including phospholipids and galactoglycerolipids were transesterified by acid catalyzed reaction to fatty acid methyl esters (FAMEs) according to method described by (Van Wychen and Laurens, 2013b). After incubation, the samples were cooled down at room temperature for at least 15 minutes, but no more than one hour. When samples became cold, 1 ml of HPLC grade hexane was added to each vial by using a plastic syringe with hollow core needles (25 mm, BD Microlance) without removing the cap. This was achieved by putting another needle (to equalise internal pressure) in the direction away from the first needle of plastic syringe. In this step, the FAME were extracted from the polar methanol phase, and leaving the polar compounds such as glycerol, or phosphatidic acid behind. The vial was vortexed for 10 seconds, then left to stand without disturbance for at least 60 minutes, but no more than 4 hours, to allow the separation of phases. At this point the samples can stored overnight at - 20 ˚C, but should be left for no more than 24 hours to avoid co-extraction of unwanted compounds, (longer storage up to one week can be done after transferring the hexane layer to new 1.5 ml GC vial (after allowing for complete extraction of at least 1 hour), and store in a freezer at -20°C) (Van Wychen and Laurens, 2013b). One hundred µl from the upper phase of the samples were diluted with 400 µl HPLC grade hexane and transferred to a 1.5 ml GC vial for precise quantification of the expected high lipid values. In 300 µl GC vials (9301-1388, Agilent) 200 µl from the diluted sample was added plus 5 µl of pentadecane (1 mg ml^{-1} which was used as an internal standard to correct the error between instruments and solvent evaporation during FAME analysis). Then the new vials were capped immediately. According to the

revised version of (Van Wychen and Laurens, 2013b) in 2015, the standards (section 2.6.1.1) and working solutions can be stored for up to 6 months at -20˚C before analyzing by gas chromatography mass spectrometry (GC-MS) technique. Fatty acid methyl esters (FAMEs) were analyzed by GC/MS using an Autosystem XL Gas Chromatograph (CHM-100-790, Perkin Elmer), which combined with a TurboMass Mass Spectrometer software (13657, Perkin Elmer). The GC was fitted with a Zebron™ ZB-5ms, 30m x 0.25 mm ID x 0.25 µm FT (7HG-G010-11, Phenomenex) GC capillary column. Samples were injected (5 µl volume) via an auto-sampler onto the column and eluted at an injection temperature of 250°C with a 100:1 split ratio and a He constant carrier flow (1 ml min-1).Sample peaks was identified by using Turbomass software (Ver 5.2.4, Perkin Elmer) and the National Institute of Standard Technology (NIST) spectral database. A response was calculated for each dilution point of each FAME component of interest by using the FAME standard dilution series, depending on the following formula of Turbomass software:

$$Response = Area_{FAME} \times \frac{con_{Is}}{Area_{Is}} \qquad \textbf{Equation 2.10}$$

$Area_{FAME}$ is the peak area (height and width) of each FAME component, $Area_{Is}$ is the peak area of the internal standard (pentadecane), and $Conc_{Is}$ is the known concentration of internal standard.

A linear regression was then carried out with the response and relative FAME component concentration. Due to the number of components within the samples, the standard concentrations were set to relative concentrations of 500, 250, 100, 30 and 10, which represent the fractions of the original 10 mg ml^{-1} FAME standard multiplied by 1000 to increase resolution (software rounds to 2 decimal places). Subsequently the software calculated the relative concentration of each FAME component within the sample by using the following equation:

$$Con_{rel} = Area_{FAME} \times RF \qquad \textbf{Equation 2.11}$$

Con_{rel} is relative concentration, and RF is the response factor calculated by linear regression of FAME standard response.

(Van Wychen and Laurens, 2013b, Smith, 2016).

2.6.1.1. FAME standard preparation

Separately, FAME standards were prepared by creating a dilution series from 10 mg ml^{-1} C4:0 – C24:0 FAME mix (18919-AMP, 37 Component Mix, Supelco) and HPLC grade hexane (500:500, 250:750, 100:900, 30:970, 10:990 µl, respectively). In addition, a calibration verification standard was created with 90 µl FAME mix and 910 µl hexane. The standards were prepared and mixed with the internal standard using the same method as the samples (described in Section 2.6.1). Vials were stored upright at -20°C until required.

2.7. Phylogenetic identification of *D. hansenii* strain

2.7.1. Genomic DNA Extraction and Purification

Aliquots (1.5 ml) of *D. hansenii* cultures (growing in 30 ml YM or minimal media at 25˚C, 120 rpm shaking) were harvested and spun down at 1000 *g* for less than 2 minutes. After centrifugation, the supernatant was removed completely and gDNA from the cells was extracted following the YeaStar™ Genomic DNA KIT (40 preps, Zymo Research). The extracted gDNA was then re- suspended in 1xTE buffer (10 x TE; 10mM Tris, 0.1 mM EDTA, pH8, see section 2.8.6.1) and stored at -20˚C. Before storage, 10 µl samples + 2µl loading dye for each sample were run on a 1% Agarose gel (80 V, 45 minutes) along with 6 µl 10 Kb ladder (1µl ladder + 1 µl loading dye + 5µl deionized water) to confirm the presence of gDNA.

2.7.2. Agarose Gel Electrophoresis

Agarose gels were made in the following steps; 1.2 ml of 50x TAE buffer, [50x TAE buffer (2 M Trizma® base, 50 mM EDTA-Na_2, 1 M glacial acetic acid, pH 8.5) (242 g Tris Methylamine, 18.61 g EDTA and 57.1ml Acetic Acid at pH 8.5, per litre)] were added to a standard 250 ml conical flask and filled up to 60 ml with distilled water. 0.6 g of agarose were then added to make a 1% gel. The solution was then microwaved (Matsui M185T 850W) for 90 seconds at full power, stirred, then microwaved again for 30 seconds. Five µl of Gel Red™ (Nucleic Acid Gel Stain 10,000X in Water) after dilution to 3x with water, were then added and the mixture stirred again. The molten gel was then poured into the gel tank (Bio-Rad Laboratories Inc), and allowed to cool. A 1x TAE solution (1.5 litres) was then prepared and added to the tank. The samples stained by mixing 2 µl of 6x DNA loading dye (Fermentas life sciences, #R0611) with 5 µl of PCR product on Parafilm M (Bemis Flexible Packaging). Seven µl of Hyper Ladder I (Bioline H1K5- 1006) were loaded in between. The tank was then connected to the power supply (Bio-Rad Laboratories Inc, Power PAC 300) and run at 80 volts for 45 minutes. The bands of DNA were visualised using an ultraviolet transilluminator imaging system and a picture recorded digitally using Gene snap software (SynGene).

2.7.3. Polymerase Chain Reaction (PCR) (Amplification of *PAD1* gene encoding Phenylacrylic acid decarboxylase

After gDNA extraction PCR amplifications were performed by using the forward primer DhPadF (5’ GCGACTATGAACAGGTTTCC AACGA 3’) and reverse primer DhPadR (5’CCTTCAATGTAACATCAGCGGCCC 3’) that selects for the sequence 101 to 125 and 479 to 502 respectively based on the *S. cerevisiae* PAD1/YDR538W gene sequence (http://www.yeastgenome.orgin (Wrent et al., 2015). PCR was set by using Taq DNA polymerase Master Mix (5 PRIME) under the following conditions: (denaturation for 5 min at 98°C, annealing at 55°C for 30 seconds, and extension at 72°C for 1.5 minutes, 30 cycles), along with initial 5 minutes denaturation at 95°C and a final 7 minutes extension at 72°C.

2.7.4. Purification of PCR Products

PCR products were purified by using PCR clean up procedure for Nucleic Acid Extraction Kit, Key Prep, to remove any compounds that might affect the purity of gDNA. After purification gDNA samples were eluted in 30 µl 1xTE buffer (see section 2.8.1.6.1), and the concentration measured by using a Nanodrop device. Blank was set by using 1 µl of 1xTE buffer. Following quantification, the samples then run on 1% Agarose gel electrophoresis and the band sizes were analysed compared with 10 Kb ladder.

2.7.5. Sequencing of PCR Products

For DNA sequencing the PCR products for both YM and minimal media cultures were diluted to the required volume and concentrations (15 µl, 10 ng μl^{-1}) by 1xTE buffer. At the same time, the primer pair used to amplify the PAD1 gene were prepared to 30 µl at 10 pmol μl^{-1}. The solutions were then sent to Eurofins Genomics for sequencing. The DNA samples were sequenced in forward and reverse directions using cycle sequencing technology (modified Sanger sequencing) on an ABI 3730XL sequencing machine (Smith, 2016).

2.7.6. Phylogenetic analysis

The obtained sequence was searched by using the Basic Local Alignment Search Tool (BLAST) online at the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) website. (Johnson et al., 2008).

2.8. Genetic modification of *D. hansenii* strain

2.8.1. Gene deletion and construct design for *D. hansenii* transformation

2.8.1.1. Yeast knockout construction by homologous recombination

Figure 2.4 demonstrates the proposed plan to delete the *GUT2* gene (encode for the mitochondrial glycerol 3-phosphate dehydrogenase), which catalysizes the conversion of G3P into DHAP) via homologous recombination. To do this a plasmid construct was designed containing the *SAT1* marker (gene encoding for streptothricin acyltransferase),

flanked by 500 bp upstream and 500 bp downstream of the *GUT2* ORF. At the same time, another construct was designed with the same marker flanked by 500 bp upstream and 500 bp downstream of the *URA3* ORF (see Figure 2.4). This was used as a standard to evaluate the frequency of gene deletion by homologous recombination in transformed cells of *D. hansenii*. Yeast cells containing the integrated cassette were identified using selective medium (YM containing 1.5 or 5 µg/ml of nourseothricin antibiotic). The selected colonies (transformed with *URA3* flanks construct) were subsequently transferred to Minimal medium lacking uracil by replica plating.

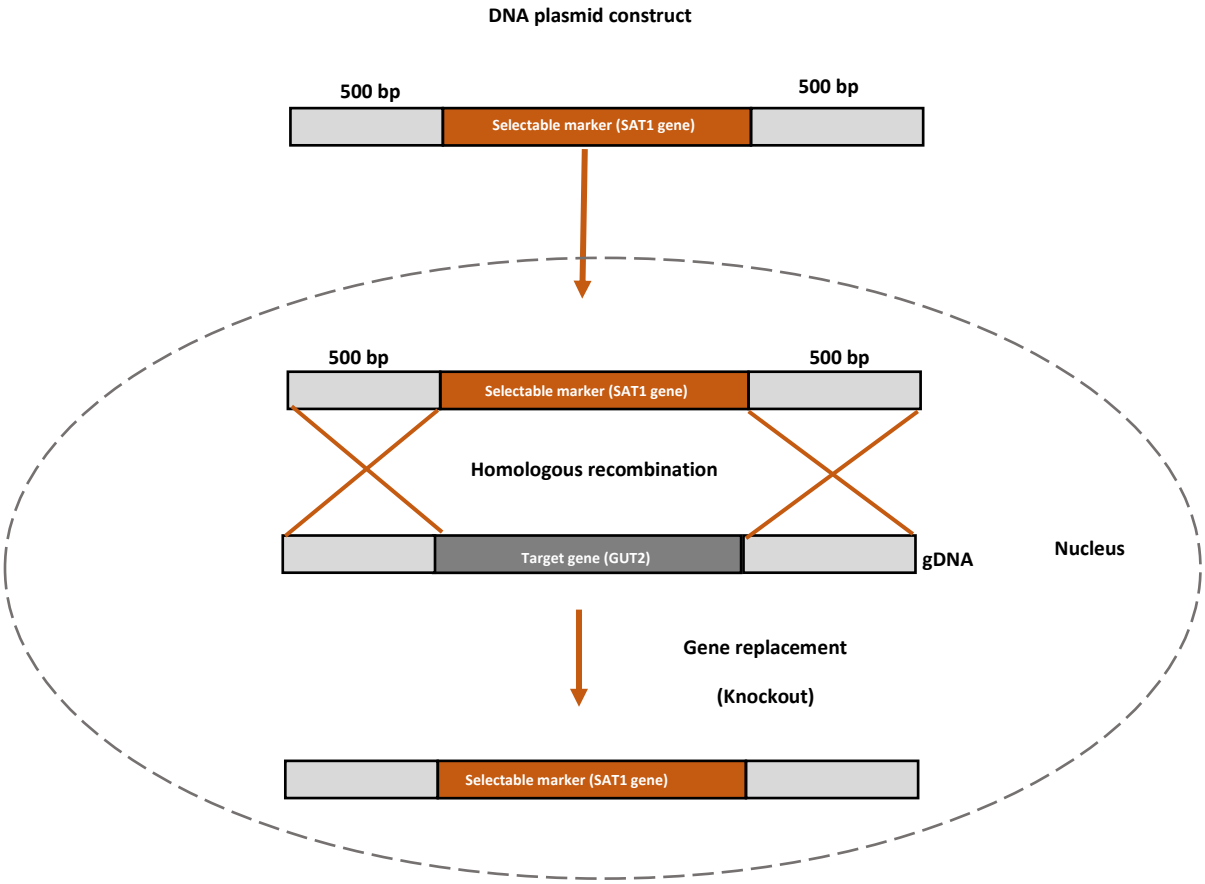


Figure 2.4. Construction of gene knockouts by homologous recombination. Schematic of gene knockout

2.8.1.2. Cloning the DNA plasmid construct using chemically competent *E. coli* DH5α cells

2.8.1.2.1. Growth and maintenance of *E. coli* DH5α cells

E. coli DH5α cells from frozen stock (stored in 15% glycerol at -80°C) were streaked onto a 2TY agar plate (see section 2.8.1.2.4.1) without antibiotic and then incubated overnight at 37°C. The plate was wrapped in Parafilm and stored in refrigerator at 4°C for use within one week.

2.8.1.2.2. Making chemically competent cells

Five ml of 2TY broth without antibiotic was inoculated with cells from DH5α *E. coli* 2TY agar culture, and incubated overnight at 37°C with shaking. Next day, the OD was measured at 600 nm after zeroing the spectrophotometer with 2TY broth. Fifty µl from the culture were diluted with 950 µl 2TY medium in a 1 ml cuvette, and the absorbance reading given was then multiplied by 20 to give the density of the culture. A sufficient amount from this culture was added to 200 ml 2TY broth to make the starting culture with initial OD 0.05, then the bacterial cells were incubated with shaking at 37°C and the OD was checked regularly. Once the OD reached 0.5-0.6, the culture was immediately removed and placed on ice to chill cells. The sample was divided into 4x50 ml Falcon tubes and all tubes centrifuged for 10 minutes at 3000 rpm and 4°C. The supernatant was poured off and the pellet was re-suspended into two tubes with 35 ml ice cold RF1 solution (100 mM rubidium chloride, 50 mM manganese chloride, 30 mM potassium acetate, 10 mM calcium chloride, 15% w/v glycerol, pH 5.8) in each tube (re-suspended in small volume and mixed each two together without vortexing), and left on ice for 20 minutes. The suspended cells were centrifuged again for 10 minutes at 3000 rpm and 4°C. The bacterial cells were collected after resuspension in one tube containing 16 ml of ice cold RF2 solution (10 mM MOPS, 10 mM rubidium chloride, 75 mM calcium chloride, 15% w/v glycerol, pH 6.8). Aliquots (200 µl) were added into 25 pre-cooled Eppendorf tubes,

as well as 400 µl samples were added to another 25 pre-cooled Eppendorf tubes. All tubes were frozen in liquid nitrogen and stored at -80°C.

2.8.1.2.3. Transformation of chemically competent *E. coli* DH5α cells.

In order to test the DH5α *E. coli* competent cells, 400 µl of the cells were taken from -80°C and thawed immediately on ice. After thawing the cells were divided into 4 Eppendorf tubes each one has 100 µl of cells. One µl of pFA *SAT1*, pBluescript SK^+ (Stratagene), and 1ng/µl DNA of plasmids was added separately to each chilled Eppendorf tube, except the fourth tube was left with only cells as the negative control. All tubes were mixed gently by pipette tip, and then left on ice for 20 minutes. Before the end of the 20 minutes an ice water bath was prepared. For 90 seconds, all tubes were put at 42°C then immediately transferred to the ice water for about 5 minutes. 2TY broth without antibiotic (750 µl) were added to all tubes, and then incubated at 37°C for 45 minutes. All tubes were centrifuged for 1 minute at 13000 rpm, and the supernatant poured away leaving just 50 µl to re-suspend the cells. Then the bacterial cells were spread on 2TY-ampicillin agar plates until the medium had dried. All plates were incubated overnight (approximately 16 hours) at 37°C. After incubation, the results were read by counting the number of cells growing in 2TY-ampicillin medium.

2.8.1.2.4. Growth and preparation of *E. coli* DH5α cells for isolation of high copy plasmid DNA

Before starting the isolation of high-copy plasmids (pFA *SAT1* and pBluescript SK^+), 50 ml of 2TY plus ampicillin broth medium was inoculated with *E. coli* cells (cells with different plasmid were inoculated separately) and incubated overnight for about 16 hours at 37°C under shaking. After incubation, each culture was centrifuged for about one minute at 13000 rpm at room temperature. The supernatant was discarded and the whole dried pellet collected in 1.5 ml Eppendorf tube. Plasmid DNA isolation from *E. coli* overnight cultures was carried out using the Sigma Aldrich mini prep kit following manufacturer's instructions.

2.8.1.2.4.1. 2TY medium

This medium was prepared by dissolving 1.6% Bacto Tryptone, 1% yeast extract, and 0.5% sodium chloride in a suitable amount of distilled water. Following autoclaving at 121°C, antibiotics were added to *E. coli* medium (75 µg/ml ampicillin final concentration) when required. Solid media was supplemented with 2% (w/v) agar.

2.8.1.3. DNA construct designed to knockout the *GUT2* and standard *URA3* gene

2.8.1.3.1 The pBluescript SK$^+$ vector and *SAT1* marker

The *SAT1* marker was used to disrupt the target gene, according to the study of Reuß *et al*. (Reuß et al., 2004) that relied on the use of nourseothricin resistance marker (ca*SAT1*) for the selection of integrative transformants in *C. albicans* wild-type strains. DNA plasmid construct with selectable marker was produced using pBluescript SK+ plasmid, which has an ampicillin gene as a selectable marker, to transform *E. coli* DH5α cells. The *SAT1* gene (1871 bp), which provides resistance to nourseothricin (ClonNAT), was used for the selection of recombinant transformants of *D. hansenii*. *SAT1* was amplified from pFA*SAT1* plasmid in a PCR reaction with the primers vip3286 and vip3287 using Accuzyme DNA polymerase (30 cycles, and 2 minutes for extension time). The primers were designed to have the restriction sites *Pst*I in the forward primer, and *BamH*I site in the reverse primer. Since the reverse primer annealed on part of the plasmid that contains a *Pst*I site, this sequence was mutated to avoid the action of the *Pst*I restriction enzyme at that end of the PCR product.

2.8.1.3.2. First attempt at designing DNA plasmid construct

The *SAT1* PCR product was digested with the restriction enzymes *Pst*I and *BamH*I, then ligated using T4 DNA ligase (see ligation reaction in section 2.8.9) into the pBluescript SK$^+$ plasmid vector which was linearized by the same enzymes. This construct (pZA1) was modified to contain two flanks around the *SAT1* gene (Figure 2.5). The flanks consisted of 500 bp amplified from the sequence upstream and downstream of the gene coding for

uracil prototrophy i.e. ortidine-5´-phosphate decarboxylase (*URA3* gene). PCR was performed using primer pairs vip3319-vip3320 (extended with the restriction sites *Sac*I and *Xba*I) for the upstream flank, and vip3321- vip3322, having *Pst*1 and *Xho*1 sites for the downstream flank. The products were cloned into the multi cloning site of the pBluescript SK^+ vector containing the *SAT1* cassette and inserted in *Sac*1-*Xba*1 and *Pst*I-*Xho*I sites to form the pZA5 DNA construct (Figure 2.5.B).

To design pZA3 plasmid construct (Figure 2.5.A), fragments with 500 bp (sequence upstream and downstream of *GUT2* open reading frame) was amplified by using primer pair vip3317-vip3318. This PCR product was digested by *EcoR*I and *Xho*I before insertion into the *EcoR*I- *Sal*I site of pZA1 plasmid DNA. The second fragment was amplified with primers vip3315-vip3316 and inserted into the *Sac*I-*Xba*I site. The PCR product for each flank was produced using Velocity DNA polymerase (35 cycles and 30 seconds extension time).

2.8.1.3.3. Second attempt at designing DNA plasmid construct

Based on the results of DNA sequencing for the pZA3 and pZA5 construct, the orientation of the open reading frame of the *SAT1* gene was in opposite direction with respect to flanks in both constructs. This might affect the transcription of the *SAT1* gene by RNA polymerase II, so it was decided to flip the orientation of *SAT1*. Firstly, the *SAT1* gene region was amplified by using primer pair (*SAT1* For. and *SAT1* Rev.), introducing the restriction site *Xba*I at the 5´ end, and a *Pst*I site in 3´end of the *SAT1* ORF. At the same time, the *BamH*I site was removed from the 3´end of *SAT1* gene. Then the PCR product along with pZA3, and pZA5 vectors were digested by *Xba*I-*Pst*I for ligation. After ligation and transformation of the new construct into *E. coli* DH5α cells, the plasmid DNA was isolated and named as pZA6 and pZA7 designed to replace the URA3 and *GUT2* gene with the *SAT1* gene, respectively (Figure 2.5.C and D). Then the orientation of the cloning fragment was examined. To do that, two digestion reactions (*Xho*I-*BamH*I and *Pst*I-*Xba*I) were set up to differentiate between the orientation of the *SAT1* gene in pZA5 and pZA6.

To distinguish between pZA3 and pZA7 constructs, two digestion reactions were set up by using *Sac*I-*Sal*I and *Sal*I-*Kpn*I. These last two tests depend on the size of the fragments resulting from the digestion by using each reaction

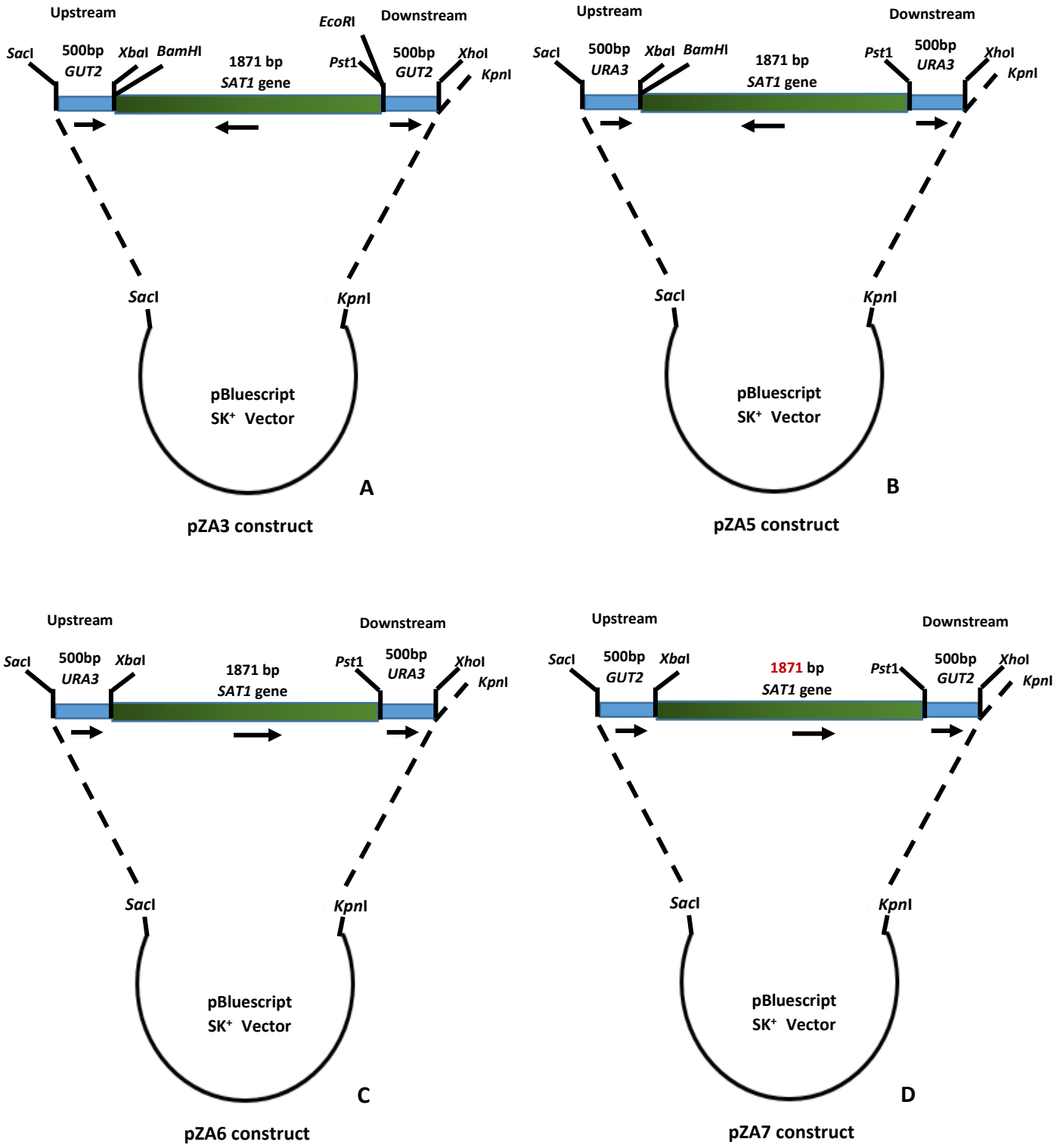


Figure 2.5. Schematic diagram of pZA3, pZA5, pZA6, and pZA7 construct

2.8.1.4. Isolation of high copy plasmid DNA by using Sigma Aldrich mini prep kit

Resuspension buffer (P1, 250 µl) was added to each Eppendorf tube and mixed with stirring by pipette tip or by vortexing. Lysis buffer (250 µl) was added and mixed by inverting 6-8 times, and the samples incubated for about 5 minutes at room temperature. After incubation, 300 µl neutralization buffer (P3) was added, and mixed thoroughly by inverting tube 6-8 times. The tubes were centrifuged for about 5 minutes at 12,470 x g in room temperature, then 750 µl of the supernatant was transferred to the Plasmid Mini Spin Column and centrifuged at 11,000 g for one minute at room temperature. The flow through was discarded and 500 µl of preheated (at 50°C) Wash buffer (PW1) was added. It was then centrifuged for 1 minute at 12,470 x g twice to remove all of the ethanol. Then the Isolate II Plasmid Mini Spin Column was placed in a 1.5 ml microcentrifuge tube and 50 µl of Elution buffer (P) was added directly onto the centre of the silica membrane, and centrifuged at 12,470 x g for one minute. After that the column was removed and the sample kept in the microcentrifuge tube. The plasmid DNA concentration was measured by using the Nanodrop device.

2.8.1.5. Polymerase chain reaction (PCR)

To amplify certain region of DNA, PCR was performed using either Accuzyme, Velocity polymerase, or My Taq DNA polymerase. Velocity and Accuzyme possesses 5'-3' and 3'-5' proofreading enzyme activities and provides high fidelity for all cloning work. The following tables (Table 2.4 and 2.5) show the PCR reaction components and conditions for the polymerase enzymes used.

Table 2-4. PCR reaction components

Component	My Taq polymerase	Accuzyme polymerase	Velocity polymerase
Forward primer (5 µM)	5µl of 5µM	5µl of 5µM	10µl of 5µM
Reverse primer (5 µM)	5µl of 5µM	5µl of 5µM	10µl of 5µM
Reaction buffer	10µl 5x My Taq buffer	5µl 10x Accuzyme buffer	10 µl of 5x Hi Fi buffer
dNTPS	-	6 µl of 2.5 mM	5 µl of 2.5 mM
DNA template (10 ng)	As required	As required	As required
DNA polymerase	0.25 µl of 5 U/µl	1µl of 2.5 U/µl	1µl of 2 U/µl
dH_2O	Up to 50 µl	Up to 50 µl	Up to 50 µl
Total volume	50 µl	50 µl	50 µl

Table 2-5. PCR conditions for different polymerase enzymes

Step	My Taq polymerase	Accuzyme polymerase	Velocity polymerase
1. initial denaturation of DNA	2 mins. at 95 °C	1 mins. at 95 °C	2 mins. at 98 °C
2. Denaturation of DNA	30 sec. at 95 °C	15 sec. at 95 °C	30 sec. at 98 °C
3. Annealing of the primers	30 sec. at 55 °C	15 sec. at 55 °C	30 sec. at 57 °C
4. Extension	10 sec. at 72 °C/ 1 Kb	1.5 - 2 min/kb at 72 C°	30 sec. at 72 °C/ 1 Kb
5. Final extension	10 mins. at 72 °C	10 mins. at 72 °C	10 mins. at 72 °C
2-4 steps repeating	repeated for 35 cycles	repeated for 30 cycles	repeated for 30 cycles

2.8.1.6. Oligonucleotide primers design

All primers were ordered from Sigma and are shown in Table 2.6. These primers were optimized to include a unique sequence, and their composition designed to contain around 20-25 base pairs in length and to anneal at both ends of the chosen DNA sequence. Moreover, all primers contained either a C or G base at the 5' and 3' end to control miss-priming. Forward and reverse primers used for cloning each contained a restriction site to allow directional cloning. However, primers with a GC content of 45-55% were desired. Additionally, sets of primers were designed to have similar melting temperatures (Tm), close to 60°C, to allow proper annealing of primers. The following formula was used to calculate Tm.

$$Tm = 2°C\,(A + T) + 4°C\,(G + C) \qquad \textbf{Equation 2.16}$$

On delivery from the supplier all primers were dissolving in required volume of TE buffer following the company instructions. The working stock for each primer was prepared at 5 µM concentration.

Table 2-6. Primers used in this study

Name	Description	Sequence (5'-3')	Source
VIP049	To amplify the construct	**GTTTTCCCAGTCACGACG**	Dr. Hettema lab. Molecular Biology and Biotechnology department (university of Sheffield
VIP050	To amplify the construct	**GGAAACAGCTATGACCATG**	Dr. Hettema lab. Molecular Biology and Biotechnology department (university of Sheffield
VIP3286	To amplify *SAT1*	**TGAAGCTTCGTACGCTGCAG**	This study
VIP3287	To amplify *SAT1*	**GCTGGATCCATGCAGGACCACCTTTGATTG**	This study
VIP3315	To amplify the 500bp upstream of *GUT2*	**GACGAGCTCTACCTATCCGATATATCCTTGC**	This study
VIP3316	To amplify the 500bp upstream of *GUT2*	**GACTCTAGACTTTCTAGGGTGTTCTTACTGTC**	This study
VIP3317	To amplify the 500bp downstream of *GUT2*	**GACGAATTCGATGTTGAAGATTTCATCGTCC**	This study
VIP3318	To amplify the 500bp downstream of *GUT2*	**GACGTCGACTGTCGGTGCTACTGGAATCAAC**	This study
VIP3319	To amplify the 500bp upstream of *URA3*	**GACGAGCTCTAGCAAGACTGAATTATGGAAAAC**	This study
VIP3320	To amplify the 500bp upstream of *URA3*	**GACTCTAGACGCTAATATGGGATTTGTTAATTG**	This study
VIP3321	To amplify the 500bp downstream of *URA3*	**GACCTGCAGAGATGCAGGTTGGAATGC**	This study
VIP3322	To amplify the 500bp downstream of *URA3*	**GACCTCGAGAACAATCTGATGAACAAGTCCTC**	This study
SAT1 For	To amplify the flipping orientation of *SAT1*	**CTGTCTAGAGCAGGTCGAGCGTCAAAAC**	This study
SAT1 Rev	To amplify the flipping orientation of *SAT1*	**GCTCTGCAGATGCAGGACCACCTTTGATTG**	This study
VIP3374	To check knockout by pZA6 construct	**GTCCCAGTTTGATCTGGAAG**	This study
VIP3375	To check knockout by pZA7 construct	**CGCCTAACATATGTGAAGTG**	This study
VIP3396	To check knockout by pZA7 construct	**CAGACTACTGGCAGAGAAATTG**	This study
VIP3396	To check knockout by pZA7 construct	**GCATTGTCTCGCTGATGAAC**	This study
VIP3397	To check knockout by pZA7 construct	**TAGCACACACCCACAACAAC**	This study
VIP3408	To check knockout by pZA7 construct	**GAATTGATGGTCACGGAGAAGG**	This study
VIP3410	To check the *GUT2* gene	**CGTACCAATCGTTTGACGAG**	This study

2.8.1.6.1. TE buffer

10X of TE buffer (10mM Tris, 0.1 mM EDTA) was prepared by dissolving 0.788 g Tris Cl plus 0.0186 g of EDTA in 40 ml dH_2O water, after dissolving the pH was adjusted with 0.1 M HCl to 8). Ten ml from this concentrated buffer was diluted with 90 ml dH_2O water to make the working stock that was used to dilute the PCR product and the primers).

2.8.1.7. Agarose gel electrophoresis

For each step of molecular experiments, DNA samples were analysed by electrophoresis with 0.7% agarose gel [prepared by dissolving 0.35 g agarose in 50 ml of 1x TBE buffer (90 mM Tris-Borate, 1 mM EDTA, pH 8.5) and adding ethidium bromide to a final concentration of 0.5 µg/ml], except for small size DNA fragments when 1% agarose was used. The samples were loaded after mixing with DNA loading buffer at 1x final concentration (6x loading buffer: 0.25% bromophenol blue w/v), then run at 90 V for 45 min in 0.5 x TBE running buffer. Gel images were visualized on a UV transilluminator and DNA size was determined by comparison to a DNA 10kb ladder.

2.8.1.8. Digestion reaction

The digestion reactions were set as follow:

PCR product digest tube	Amount (µl)
PCR product	5 µl
10x CUT SMART buffer	4µl
First restriction enzyme (20 units / µl)	1µl
Second restriction enzyme (20 units/ µl)	1µl
dH_2O	9 µl
Total volume	20 µl

The digestion reaction was used to examine the ligation in some colonies after colony PCR, or to cut the insert before ligation, the same enzymes were used with the same concentrations. The total volume of each reaction was 20 µl. Also, we set up a positive control containing DNA without restriction enzymes. Different enzymes were used for the digestion of the PCR product which depended on the specific sequence of the restriction site put in the PCR primers. For all these enzymes, the same concentration was used as in the previous reactions and the total volume was 20 µl for each reaction. The digests were incubated overnight at 37°C for most enzymes.

2.8.1.9. Ligation

To ligate the PCR product with linearized plasmid DNA, the reaction was set to contain 1 µl 10x ligase buffer, 0.5 µl T4 DNA ligase (Promega), the purified insert volume was based on the molar ratio 3:1 (insert DNA/vector), 2 µl linearised plasmid (20ng/µl) in a final volume of 10µl. The ligation mixture was incubated for 2 hours at 18°C. For the negative control, double cut plasmid without insert was used in the ligation reaction.

2.8.1.10. DNA gel extraction

All DNA samples were extracted from gels using a Qiagen gel extraction kit and the manufacturer's instructions were followed as outlined below.

The DNA fragment from the agarose gel was excised with a clean, sharp scalpel, and then the gel slice was weighed in a colourless tube. Three volumes of QG buffer were added to 1 volume of gel and incubated at 50°C for 10 minutes or until the gel slice has completely dissolved. The tube was vortexed every 2-3 minutes to help dissolve the gel. If the colour is orange or violet then 10µl sodium acetate, pH 5.0 will be added and mixed, after that the mixture should turn yellow. Next, 1 volume of isopropanol was added to the sample, mixed and placed in a QIAquick spin column with a 2ml collection tube. Centrifuged for 1 minute at 17,900 x g, then the flow through was discarded and the QIAquick column was placed back in the same tube. Buffer PE (750 µl) was added to QIAquick column for washing and the column was left to stand for 2-5 minutes. Centrifuged for 1 minute at

17,900 xg, then the flow through was discarded and the QIAquick column was placed back in the same tube. Centrifuge again for 1 minute at 17,900 xg to remove residual wash buffer. Finally, the QIAquick column was placed into a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 50 µl Buffer EB to the center of the QIAquick membrane and the column was centrifuged for 1 minute at the same previous speed (if necessary to increase DNA concentration only 30 µl Buffer EB was added to the center of the QIAquick membrane).

2.8.1.11. Sequencing

All cloned plasmids were sequenced by Beckman Coulter Genomics. pDNA was prepared to the required volume and concentration (5 µl, 10ng/µl) and (5µl, 3.5 pmol/µl) for primers.

2.8.1.12. Nourseothricin antibiotic assay

The growth sensitivity of the wild type *D*. *hansenii* was evaluated towards a wide range of nourseothricin antibiotic concentration (300 to 0.1 µg/ml) augmented to YM agar medium (antibiotic was added to the medium after cooling and mixed well). The number of colonies was counted after 5 days of incubation at 25˚C.

2.8.1.13. Transformation of *D. hansenii* cells by electroporation

Two days before: Fifty ml of YM medium were inoculated with *D. hansenii* cells and incubated overnight at 25°C with shaking at 120 rpm.

One day before: Re-inoculated 50 ml of YM medium at A_{600} = 0.025 and 0.0125 and grow overnight until the A_{600} reaches 2.6-2.8.

The following day: When the OD of the *D. hansenii* cultures reached 2.6-2.8, the cells were transferred into a sterile 50 mL Falcon tube and centrifuged for 5 min at 3,300

g. The supernatant was discarded, and the pellet was re-suspended in 6 ml of 50 mM sodium phosphate buffer, pH 7.5, containing 25 mM DTT. Cells were then incubated at 30°C for 15 minutes and centrifuged for 5 min at 3,300 *g*. In 40 ml sterile cold (4°C) double-distilled water, cells were re-suspended again, and centrifuged as previously. The supernatant was discarded, the cell pellets were re-suspended with 1 mL sterile ice cold 1 M sorbitol, and centrifuged as before. The supernatant was discarded again, and the cells were re-suspended in the remaining liquid to obtain a dense suspension (ca. 200 µl). Forty µl of the cell suspension was mixed with 1 µl of 2.0 and 3.4 µg purified amplified product of pZA6 and pZA7 constructs respectively, (see Figure 2.6) respectively and placed in a precooled electroporation 0.2 cm cuvette. A mock electroporation without DNA was also included. Subsequently the cuvettes were incubated with the cells for 5 min on ice, then the electroporation cuvettes were put into the Equibio electroporator (a BioRad Gene Pulser) and electroporated at 11.5 $kVcm^{-1}$ and placed back on ice. Following electroporation 1 mL YM medium containing 0.1M sorbitol was added to the cells, mixed, and the cell suspension transferred to a 2 mL Eppendorf tube. Then all the samples were incubated for 4 hours at 30°C with shaking at 120 rpm. After incubation, the cells were centrifuged as before, the supernatant was discarded, and the cells were re-suspended in the remaining liquid to obtain a dense suspension (ca. 100 µl), An aliquot of the cell suspension (50 µl) was spread on YM plates containing 1.5 and 5 µg/mL nourseothricin, and incubated at 25°C for 3-4 days. When colonies appeared after 3 days of incubation, single colonies without background (small new colonies) were selected, and grown on YM plates containing 5 µg/mL nourseothricin.

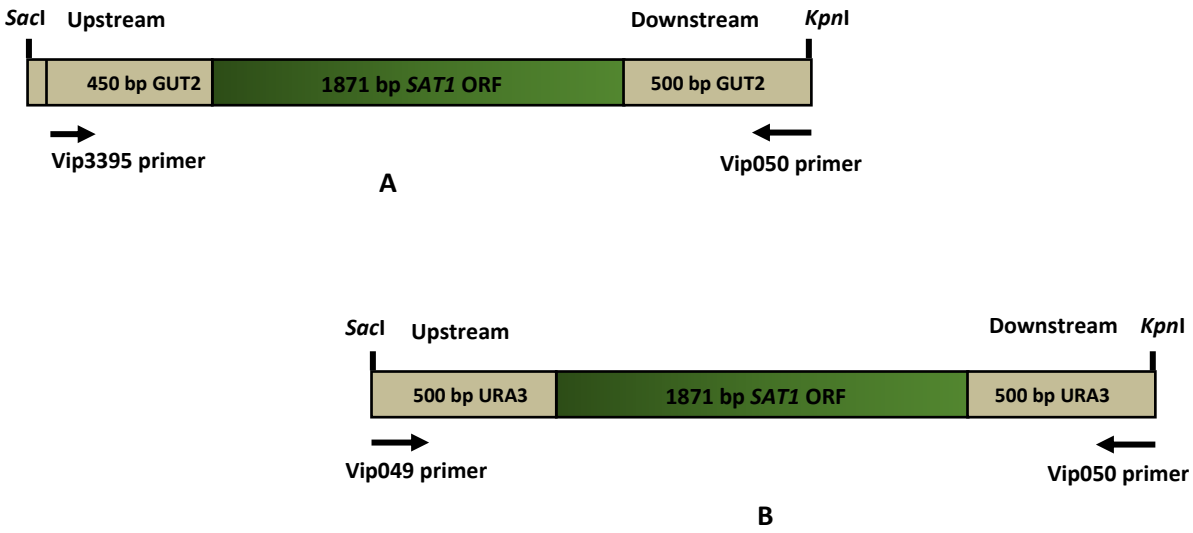


Figure 2.6. Schematic diagram representing different PCR reactions designed to amplify fragment for A- *GUT2* gene deletion and B- *URA3* deletion. Dark green rectangle refers to either *SAT1* or *URA3* ORF, while the light boxes represent the flanking chromosomal region. Arrows indicate the positions of the primers used for PCR.

Following the selection of colonies grown in YM agar medium with 5 µg/mL nourseothricin, replica plate technique (using minimal medium without amino acids) was used to check the knockout of *URA3* gene. The growth of these colonies was compared with their growth in minimal medium containing uracil (0.1 g/L). A control was set by growing wild type colonies in the same conditions. The replica plating technique was also used to distinguish Δ*ura3* colonies in 5-FOA plate (0.17% yeast nitrogen base, 0.1% 5-FOA, 0.005% uracil, 0.5% ammonium sulphate, 2% glucose, 2% bacto-agar with amino acids added as required after autoclaving). Cells were grown in 5-FOA plate in order to be sure about the result of the transformed colonies with pZA6 construct.

2.8.1.13.1. 50mM sodium phosphate buffer (pH 7.5), containing 25 mM DTT

This buffer was prepared freshly by mixing 500 µl of 1 M sodium phosphate buffer (pH 7.5) with 250 µl of 1 M DTT, and 9.25 ml distilled water.

2.8.1.13.2. 1 M sodium phosphate buffer (pH 7.5)

The buffer was prepared by mixing 2 ml of NaH_2PO_4 (1 M) with 8 ml from Na_2HPO_4 (1 M). The total volume was 10 ml.

2.8.1.14. Genomic DNA isolation

To extract the gDNA from the *D. hansenii* cells, the transformed cells were streaked in YM agar augmented with 5 µg/ml nourseothricin antibiotic as a selective marker for the transformed yeast cells. After 24 hours of incubation, cells were scraped from the selective plate and 200 µl of TENTS solution (20 mM Tris/HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 2% (v/v) Triton X-100, 1% (w/v) SDS) plus 200 µl of glass beads and 200 µl of phenol: chloroform: isoamyl Alcohol (25:24:1) were added to the samples which they underwent bead beating at high speed for about 45 seconds. After cell disruption, all samples were centrifuged at 13,201 x g for 30 seconds before adding another 200 µl of TENTS solution. The mixture was then vortexed and centrifuged at 13,201 x g for further 5 minutes, and 350 µl from the resulting supernatant was transferred to fresh Eppendorf tubes. Subsequently 200 µl of phenol: chloroform: isoamyl Alcohol (25:24:1) was added, vortexed and then centrifuged as before. From each sample 300 µl of the supernatant was removed and 30 µl (1/10 volume) of 3 M sodium acetate pH 5.2 and 750 µl (2.5x volume) of 100% ethanol were added to precipitate the gDNA. Precipitation reactions were left on ice or at -20°C for about 1 hour, followed by centrifugation at 13,201 x g for 15 minutes (Sigma 1-14K, rotor 12094). The resultant pellets were washed in 70% ethanol, centrifuged as previously described, then re- suspended in 200 µl of 1xTE pH 8.0 containing 5 µg/ml RNAse and left at room temperature for 10 minutes. After that DNA was precipitated by incubating samples at -20°C for 1 hour and DNA was pelleted by

centrifugation as before. Pellets were washed in 70% ethanol, dried at 50˚C, followed by resuspension in 50 µl 1xTE pH 8.0.

2.8.2. Evaluation of neutral lipid production after *GUT2* deletion

2.8.2.1. Comparing between *GUT2* mutant and wild type strains of *D. hansenii* NCYC102 for neutral lipid production

Experiments were carried out to evaluate the effect of the *GUT2* deletion of mutant cells on neutral lipid production. Three Δ*gut2* and three wild type strains of *D. hansenii* were grown in 3x500 ml flasks containing 100 ml of 48: 0.5 Glucose/ ammonium sulphate minimal medium. Cells were grown in this medium due to the highest amount of TAG yield in previous experiments with wild type cells. Another set of mutant and wild type strains were grown in glycerol based minimal medium (8 glycerol: 0.25 ammonium sulphate). The same procedure was used as described in section (2.4.3.2) and the readings were taken after 40 hours of incubation, then compared with the biomass of each culture.

2.8.2.2. Neutral lipid production in Δ*gut2* and wild type strains of *D. hansenii* NCYC102 grown under different C/N ratios

in order to optimise neutral lipid production in Δ*gut2* mutant strain compared with wild type strain, one single colony from both preadapted wild type and mutant stains were grown in 3x250 ml flasks containing 60 ml minimal medium with different carbon source and C/N ratios: 48:0.5 and 8:0.5 glucose: ammonium sulphate as well as 4:4:0.5 and 8:8:0.5 glucose: glycerol: ammonium sulphate. Readings were taken after 40 hours of incubation, then compared with the biomass of each culture as in section 2.4.3.2.

2.8.3. Spot growth assay

Experiments were carried out to evaluate the growth of the wild type and *gut*2Δ mutant strains of *D. hansenii* in different growth conditions [minimal medium containing glucose or glycerol as a sole carbon source. Glucose based medium was prepared containing different concentrations (0, 0.8, 1.6 M) of NaCl]. Ten ml of overnight cultures of wild type

and mutant strains of *D. hansenii* grown in minimal medium were centrifuged at *3000 g* for 5 minutes, then the cells were washed and adjusted to OD 4.6- 4.65 using distilled water. 200 µl of each culture was put into the second row of a 96 well plate (the first row and column was left empty) and three serial dilutions were made in the three rows below by transferring 20 µl of culture from the previous row into 180 µl distilled water in each well. A Pinning device was immersed for one minute in the microtiter plate and then spotted onto different types of media. After 2 days of incubation plates containing glucose without NaCl were photographed. Other plates were photographed after 6 days of incubation at 25 ^{o}C.

2.9. Statistical analysis

The statistical analysis of most experiments was carried out using one-way analysis of variances (ANOVA) and t-test. Experiments were carried out in triplicate and error bars represent standard deviation (±) of the means. Analysis was performed using GraphPad Prism7 and Excel software.

Chapter Three: Screening between three yeast strains for best accumulation of neutral lipid

3. Screening between three yeast strains for best accumulation of neutral lipid

3.1. Introduction

The production of second generation biodiesel from microbial cells is currently receiving significant attention with the aim to replace the existing technologies that are based on oil crops and plants. As their production capacity and rate of production are limited, these agricultural materials are inadequate for large scale biodiesel production. Alternative fuels can be obtained from oleaginous microorganisms that under certain cultivation conditions produce and accumulate lipids up to the 60% of the total cell dry weight (Li et al., 2008). Unicellular yeasts have high growth rate and can accumulate lipid in separate lipid bodies (Drucken, 2008, Li et al., 2008, Mullner and Daum, 2004, Mlíčková et al., 2004). They can also grow on low cost fermentation substrates for example waste material of agricultural and industrial products (Amaretti et al., 2010).

In yeast cells lipids are stored in the form of triacylglycerol (TAG) so different types of fatty acids are the major target for improving the biotechnological products. Studies have described about 1500 species of yeast arranged into over 100 genera (Satyanarayana and Kunze, 2010). The derivatives of long-chain fatty acid methyl esters and alkanes in TAGs have been explored as raw materials for biofuels(Li et al., 2010, Madsen et al., 2011). As the oleaginous yeasts are found in different genera, thus microbial oils are different in fatty acid compositions (Easterling et al., 2009). TAG is mostly formed by consecutive acylation of glycerol-3-phosphate (G3P), catalysis by diverse acyl transferases. G3P is synthesize from glycerol by glycerol kinase or can be synthesized from dihydroxyacetone phosphate (DHAP) in reversible reaction catalysis by G3P dehydrogenase (GPD). Under osmotic stress, increasing the level of glycerol takes place due to the rise in the GPD activity (Rossi et al., 2011).

The main objective of this chapter is to monitor the neutral lipid production between three yeast strains, in order to select the best producer for further experiments to enhance lipid production. As the main component of TAG, glycerol and other osmolytes

were also evaluated under salt stress condition (0, 0.8, and 1.6 M NaCl) as it was thought that salt will increase the intracellular accumulation of glycerol, which will then exploit into TAG synthesis inside the cells. After quantification, these polyols were identified by NMR analysis. Neutral lipid production was measured in cell suspension using 96-well plate format by Spectroflurometric quantification with fluorescent Nile red dye instead of the time and solvent consuming gas chromatographically analysis.

3.2. Results and discussion

3.2.1. Effect of adaptation to different salinity concentrations on yeast growth

All yeast strains were adapted to different concentrations of NaCl augmented to YM medium (Table 3.1). Firstly, all strains were cultured in medium with 0.4 M NaCl, and the readings of spectrophotometer were set at wavelength 600 nm. For the next higher concentration of NaCl, the inoculum was taken from the previous concentration, after adaptation during the incubation period. The results showed that all strains grew well in the first set of NaCl concentrations (0.4, 0.8, 1.2, and 1.6 M) comparing with control cultures which were incubated in YM medium without salt. The highest growth rates were clearly observed in *Cryptococcus curvatus* followed by *Debaryomyces hansenii*. In the second set of salinities (2, 2.4, 2.8, and 3.2) the growth rates started to decrease in all strains, especially for *Yarrowia lipolytica* which was inhibited at 2 M NaCl, followed by *D. hansenii* which showed great reduction in growth rates on 2.4 M NaCl when compared with no salt culture. However, the growth rate of *C. curvatus* only showed a gradual decrease with increasing salt concentrations and was only significantly inhibited at 3.2 M NaCl. Previous study by (Almalki, 2012) mention that when the NaCl concentration was increased to 3 M, *D. hansenii* was struggling to grow at all pH values while little or no growth was observed in 3.5 M NaCl. Another study by (Andreishcheva et al., 1999) showed that *Y. lipolytica* can grow over a wide range of pH in the presence of 12% (2 M) NaCl. When yeast cells subjected to high levels of inorganic electrolytes particularly sodium chloride, several parameters of yeast activity have been reported to be effected

such as cell growth and multiplication, the number of viable yeast cells per unit volume of liquid growth medium, culture biomass and the length of the lag phase, ,as well as changes in the concentration of metabolic products such as a decrease in the production of ethanol and increasing the concentration of other fermented products (e.g. glycerol, acetaldehyde, etc.) (Wei et al., 1982).

Table 3.1. The effect of adaptation of three strains of yeast: *D. hansenii* (102), *C. curvatus* (476), and *Y. lipolytica* (2904) to different concentrations of NaCl, incubated under shaking at 120 rpm and 25°C. Each number represent the average of three replicates with ± standard deviation. Blank cells of the table were not determined (n.d.)

Salt con. (Molar)	Strain	OD. after 24 hr.		OD. after 48 hr.		OD. after 72 hr.		OD. after 96 hr.		OD. after 120 hr.	
		control	salt	control	salt	control	salt	control	salt	control	salt
0.4 M	102	8.6 ±0.001	4.4 ±0.003	12.5±0.002	11 ±0.005	n.d.	n.d.	n.d.	n.d.	15.3 ±0.001	15.3 ±0.002
	476	8.4 ±0.012	6.4 ±0.005	10.8 ±0.008	11.4 ±0.004	n.d.	n.d.	n.d.	n.d.	16.3±0.002	15.3 ±0.0015
	2904	5.4 ±0.015	4.4 ±0.002	13.42±0.012	8.5 ±0.003	n.d.	n.d.	n.d.	n.d.	13.4 ±0.005	12.9 ±0.002
0.8 M	102	8.2 ±0.002	3.1 ±0.003	12.46±0.001	11 ±0.002	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	476	6.9 ±0.004	5.8 ±0.018	10.84±0.005	11.4 ±0.008	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2904	6 ±0.001	3 ±0.004	13.42±0.001	8.5 ±0.005	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1.2 M	102	n.d.	n.d.	n.d.	n.d.	15.1 ±0.005	9.8 ±0.014	14.8 ±0.0035	11.3 ±0.002	13.4 ±0.002	12.5 ±0.003
	476	n.d.	n.d.	n.d.	n.d.	16.5 ±0.007	12.7 ±0.021	15.5±0.004	12.8 ±0.024	14.9 ±0.003	13.7 ±0.23
	2904	n.d.	n.d.	n.d.	n.d.	13.4 ±0.024	11.9 ±0.02	13.4±0.015	10.4 ±0.008	14.7 ±0.007	9.6 ±0.009
1.6 M	102	4.9 ±0.008	0.8 ±0.012	10 ±0.007	6.8 ±0.007	n.d.	n.d.	n.d.	n.d.	11.8 ±0.008	11.6 ±0.004
	476	5.3 ±0.002	1.8 ±0.005	11.1 ±0.001	9 ±0.003	n.d.	n.d.	n.d.	n.d.	13.4 ±0.002	14.4±0.003
	2904	5.6 ±0.015	0.3 ±0.002	12.3 ±0.01	3.2 ±0.001	n.d.	n.d.	n.d.	n.d.	12.9 ±0.006	9.1±0.001
2 M	102	4.9 ±0.002	0.5 ±0.012	10.1 ±0.002	3.2 ±0.009	n.d.	n.d.	n.d.	n.d.	11.8 ±0.003	10.1 ±0.008
	476	5.3 ±0.001	0.8 ±0.008	11.9 ±0.002	5.7 ±0.009	n.d.	n.d.	n.d.	n.d.	13.4 ±0.001	11.2±0.009
	2904	5.6 ±0.01	0.2 ±0.004	12.3 ±0.03	0.2 ±0.04	n.d.	n.d.	n.d.	n.d.	12.9 ±0.025	1.2 ±0.003
2.4	102	7 ±0.024	0.2 ±0.01	18.5 ±0.006	0.6 ±0.004	13.4 ±0.003	0.5 ±0.002	12.5 ±0.0025	1.4 ±0.001	n.d.	n.d.
	476	6 ±0.015	0.4 ±0.008	14.9 ±0.002	2.2 ±0.005	12.1 ±0.001	4.6 ±0.001	14.4 ±0.001	8.6 ±0.001	n.d.	n.d.
2.8	102	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14.5 ±0.008	0.16 ±0.013	13.9 ±0.007	0.4 ±0.009
	476	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14.5 ±0.002	6.1 ±0.008	13.8 ±0.003	7.2 ±0.005
3.2	476	7.5 ±0.028	0.2 ±0.005	n.d.	n.d.	n.d.	n.d.	15.4±0.02	1.5 ±0.003	n.d.	n.d.

3.2.2. The effect of adaptation to different concentrations (0, 0.8, and 1.6 M) of NaCl on the total osmolytes accumulation in yeast cells

From the results obtained (Table 3.2) it is obvious that NaCl had a positive effect on the total osmolytes accumulation in all yeasts strains after 24 hours of incubation in saline media at 25°C comparing with cultures without salt. Among the different yeast strains *C. curvatus* was found to be most efficient for solutes accumulation in different concentrations (0, 0.8, and 1.6 M) of NaCl, and the highest amount was found in 0.8 M NaCl. Similarly, the 0.8 M NaCl induced the highest effect on osmolytes accumulation in all yeast strains. As the concentration of NaCl increased more than 0.8 M the production of osmolytes decreased in all yeast strains especially in *Y. lipolytica,* which showed the lowest amount in all concentrations of salt. Under osmotic stress, cells are be able to restore their volume and turgor pressure by accumulating intracellular low molecular weight solutes that increase the internal osmolarity (Le Rudulier et al., 1984, Higgins et al., 1987). In general, the high concentration of intracellular glycerol can take place as a result of increasing the level of glycerol production, and retention by cytoplasmic membranes, or decreasing of the dissimilation or uptake of glycerol from the medium. During glycolysis glycerol is synthesize by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate via glycerol 3-phosphate dehydrogenase (GPD) (Reed et al., 1987, Parrou et al., 1997, Posas et al., 2000). Under osmotic stress, increased levels of glycerol take place due to the increase of the activity of GPD. The acetylacetone test is not specific for glycerol and will also measure other polyols such as sorbitol or arabitol. For this reason, NMR studies were depended to find out whether glycerol was the major compatible solute accumulated in all three yeast strains (see next section).

Table 3-2. The effect of adaptation to different salinity concentration (0, 0.8, and 1.6 M) NaCl on total osmolytes accumulation (µg glycerol/ml culture) ± standard deviation, in three yeast species measured at OD. 410 nm.

Species	Without salt	0.8 M NaCl	1.6 M NaCl
D. hansenii	527.5 ±17.8	776.1 ±5.9	614.5 ±20.1
C. curvatus	430.7 ±4.9	860.6 ±13.7	778.6 ±5.5
Y. lipolytica	344.6 ±4.9	610.4 ±15.5	430.7 ±19.7

3.2.3. NMR analysis of compatible solutes in yeast cells growing under high salt stress condition

From the results obtained (Figure 3.1) the NMR profiles of yeasts grown under NaCl stress were generally found to have notable peaks when compared with cells grown without stress. Identification of the main peaks as detected by ^{1}H NMR spectroscopy (Figure 3.1) revealed that these osmolytes was accumulated by yeast in response to high salt stress. In *D. hansenii* the result showed an increasing in the amount of osmolytes in cultures with 0.8 and 1.6 M of NaCl. Glycerol was the main osmolyte accumulated in response to high salt, however there was no obvious products in culture without NaCl. When exposed to osmotic stress yeast accumulated polyols such as glycerol, D-arabitol, D-mannitol and meso-erythritol (Spencer and Spencer, 1978) , which are compatible with metabolic activity (known as compatible solutes ; (Brown, 1978)). In high salt, the intracellular accumulation of osmolytes in yeast cells is related to the species, growth phase (Nobre and Costa, 1985) as well as the carbon source used in medium (Van Eck et al., 1989). For example, xylitol, is produced when xylose is used as a carbon source while *Pichia farinosa* produces glycerol and arabitol when grown on glucose, and mannose as carbon sources (Spencer and Spencer, 1978). However, a large number of yeast species produce glycerol intracellularly as the main osmolyte in response to hyperosmotic stress (Ansell et al., 1997). *C. curvatus* strain was found to accumulate less glycerol in 1.6 M than in 0.8 M of

NaCl, and that might be happened because the cells were struggling to grow at high osmolarity. In contrast, the accumulated osmolyte in *Y. lipolytica* grown at 0.8 M NaCl was identified as arabitol, but in 1.6 M it was entirely switched to glycerol (Figure 3.1C). In spite of the great diversity in the degrees of salt-tolerance among different yeast including *Saccharomyces cerevisiae* (André et al., 1988, Andre et al., 1991), *Zygosaccharomyces rouxii* (Hosono, 1992, Ohshiro and yagi, 1996), *D. hansenii* (Lucas et al., 1990, Larsson and Gustafsson, 1993), *Pichia* sorbitophilla (Lages and Lucas, 1995, Oliveira et al., 1996), and *Y. lipolytica*, these yeasts display higher intracellular accumulation of osmolytes, in particular glycerol (André et al., 1988, Nishi and yagi, 1993, Lages and Lucas, 1995) and compatible ions when subjected to high concentration of NaCl. Yagi (1991) revealed that in *Zygosaccharomyces rouxii* the intracellular contents of glycerol increased for up to 6 hours in media supplemented with 1 M and 2 M NaCl but did not increase when concentration become 3 M. In such a high concentration of salt, the synthesis and intracellular accumulation of glycerol are repressed, and Na^+ and Cl^- ions are taken into the cells for osmotic balance. (Yagi, 1991, Yagi and Nishi, 1993)

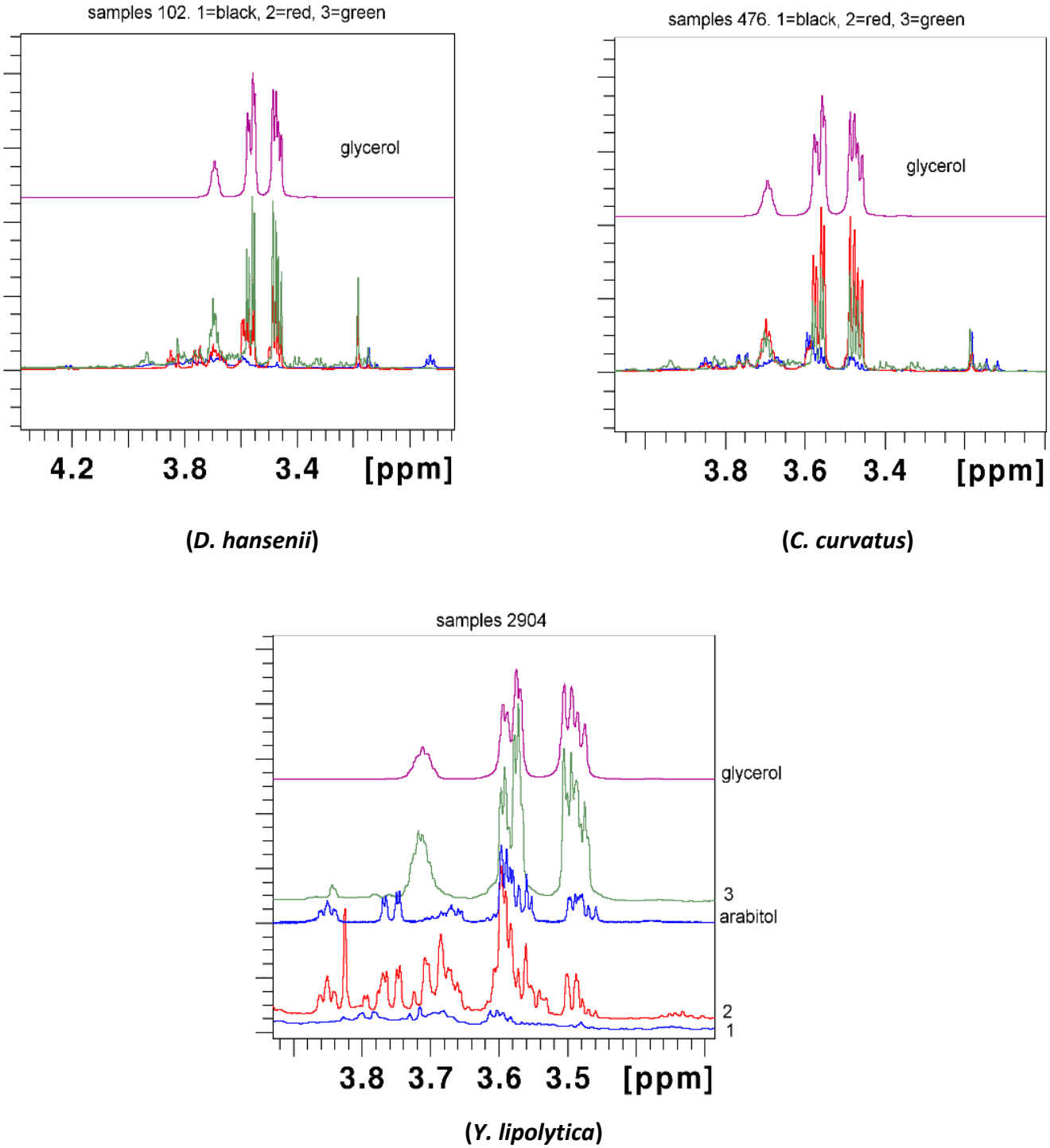


Figure 3.1. The compounds identified by ^{1}H NMR spectroscopy from *D. hansenii*, *C. curvatus*, and *Y. lipolytica* when exposed to different concentrations of NaCl. blue line peak represents no salt, red represents 0.8 M NaCl and green line 1.6 M NaCl. The spectra for glycerol and arabitol standard are shown where indicated

3.2.4. Growth curve determination

3.2.4.1 Growth curve determination of pre-adapted cells for different concentrations of NaCl

For growth curve determination, each yeast strain was grown in 250 ml shake flask containing 50 ml YM medium with different concentrations (0, 0.8, and 1.6 M) of NaCl at 25°C. Pre-adapted fresh cells at different concentrations of salt were used as inoculum for culturing in the same concentration of NaCl. The optical density was measured with the spectrophotometer at given intervals (usually 2 hours) at wavelength 600 nm, and the absorbance of each flask was set initially at 0.05. According to the results (Figure 3.2) it is obvious that all yeast strains grew quickly in media without salt with short lag phase, and the cells reached the stationary phase after 14 hours of incubation. For 0.8 M NaCl the logarithmic phase extended between the period 6 to 18 hours for all strains cultured in this medium, whereas in media with 1.6 M NaCl the logarithmic growth phase was extended over an even longer period (up to 24 hours). From the logarithmic part of the growth curves appropriate log2 OD readings were used to calculate the generation time of each yeast strain culturing in different concentrations of NaCl. From these curves, we found that the generation time was around 2 and 3 hours for cultures without salt and 0.8 M NaCl respectively. However, in 1.6 M NaCl the generation time was about 4, 4, and 5 hours for *C. curvatus*, *D. hansenii*, and *Y. lipolytica* respectively (see Table 3.3).

Table 3.3. Presented the doubling time of *D. hansenii*, C. curvatus, and Y. lipolytica cells growing in YM media with different concentrations of NaCl

Strain	0 M NaCl	0.8 M NaCl	1.6 M NaCl
D. hansenii	2 hrs. and 21 mins. ± 0.06	3 hrs. and 6 min. ± 0.06	4 hrs. and 39 inm ± 0.2
C. curvatus	2 hrs. and 32 mins. ± 0.02	2 hrs. and 54 min ± 0.07	4 hrs. and 57 min ± 0.2
Y. lipolytica	2 hrs. and 32 mins. ± 0.07	2 hrs. and 50 mins. ± 0.3	4 hrs. and 57 min ± 0.7

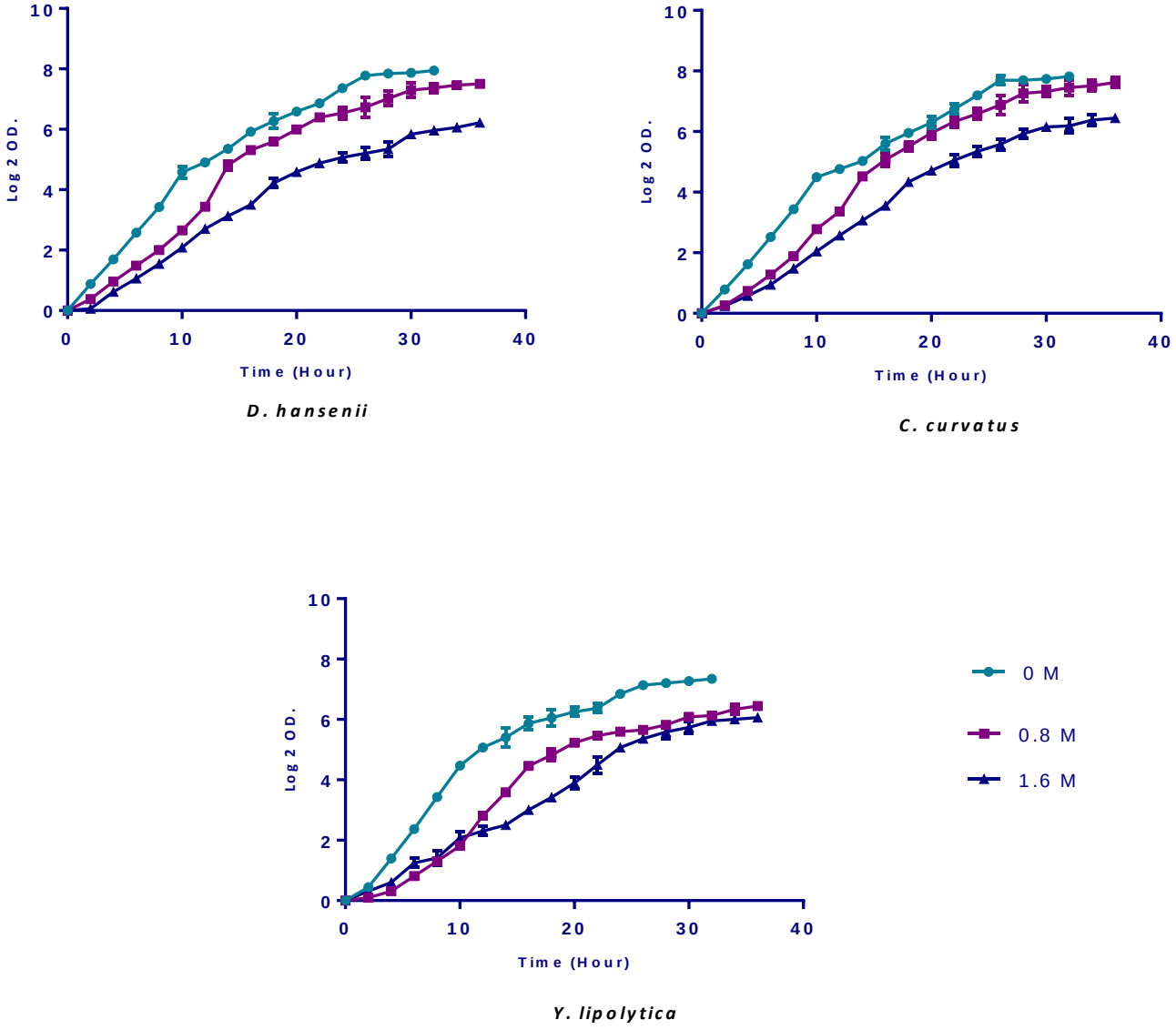


Figure 3.2. Logarithmic growth curve for pre-adapted strains of *D. hansenii*, *C. curvatus*, and *Y. lipolytica* which growing in media with different concentrations (0, 0.8, and 1.6 M) NaCl. Bars represent standard deviation.

3.2.5. Spectrofluorometric quantification measurement of neutral lipid in yeast using Nile red dye

Triacylglycerol (neutral lipid) production was measured in all yeast strains, growing in YM media with different concentrations of NaCl (0, 0.8, and 1.6 M) by using Nile red technique. In traditional methods the evaluation of lipid involves solvent extraction and gravimetric determination (Bligh and Dyer, 1959), and in case of further quantification, separation of the crude extractions and quantification by thin-layer chromatography (TLC), HPLC or gas chromatography (GC) are also required (Eltgroth et al., 2005). The major disadvantages of these traditional methods are time- and labour- intensive, thus, increasing attention has focused on the direct method of the lipid quantification depending on Nile red technique (Cooksey et al., 1987, Izard and Limberger, 2003). Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one), a lipid-soluble fluorescent dye, mainly used to quantify the amount of lipid in some animal organisms such as mammalian cells (Genicot et al., 2005), as well as microorganisms: bacteria (Izard and Limberger, 2003), yeasts (Evans et al., 1985, Kimura et al., 2004), zooplankton (Kamisaka et al., 1999), and microalgae (McGinnis et al., 1997, Eltgroth et al., 2005, Elsey et al., 2007). After experimentally choosing DMSO as the best solvent for Nile red dye (it enhances staining efficiency compared to acetone), we tested the best conditions for neutral lipid measurement including cell concentration, time of staining, and the dye concentration. The optimum concentration of yeast suspension in all three strains was at OD. 2.6 (refers to 50 dilution in the graphs of Figure 3.3). Kimura (2004) demonstrated that the fluorescence intensity of neutral lipid increased with an increase in culture broth amount up to 100 µl, whereas higher than this amount, the intensity of suspension did not linearly go up with the lipid content (Kimura et al., 2004).

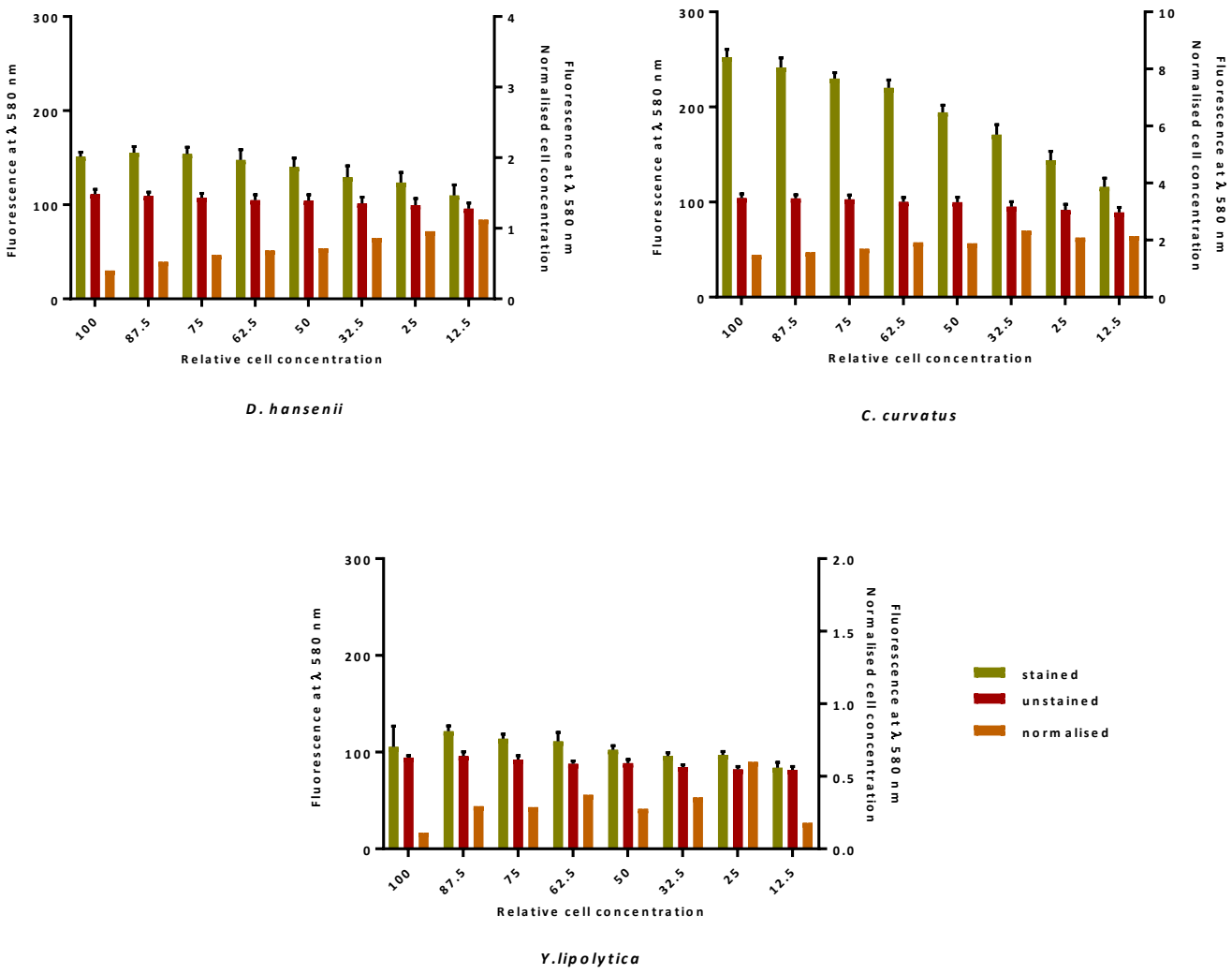


Figure 3.3. The fluorescence of neutral lipid with different cell concentrations in *D. hansenii*, *C. curvatus*, and *Y. lipolytica:* The graphs show the normalised value which depended on the stained and unstained average with their standard deviation represented by bars.

The best fluorescence of different cells concentration was chosen based on higher normalised value and the smallest standard error value of stain average in order to avoid the overlapping between the stained and unstained average value, as shown in the figure 3.3. The best time of staining was after 7 minutes for *D. hansenii* and *C. curvatus.* This

time was also adopted for *Y. lipolytica* as well, since at higher salt concentrations 7 minutes was the optimum time of staining (Figure 3.4). According to the data related to different Nile red concentrations (Figure 3.5), 0.2 µmol/ml concentration was selected to stain both *D. hansenii* and *Y. lipolytica* on the basis of the value being very close to the optimum at 0.4 µmol/ml and there was less chance of self-shading at the lower concentration than 0.4 µmol/ml. For *C. curvatus* the optimum concentration was 0.1 µmol/ml in media with and without salt. To some extent, the conditions of neutral lipid measurement in our study agreed with (Kimura et al., 2004) who demonstrated that the fluorescence fading was observed at concentration above 2.3 µg/ml of Nile red dye after five minutes of staining. High concentration of Nile red can amplify the fluorescence intensity, but this amplification will not linearly increase with the concentration of the Nile red.

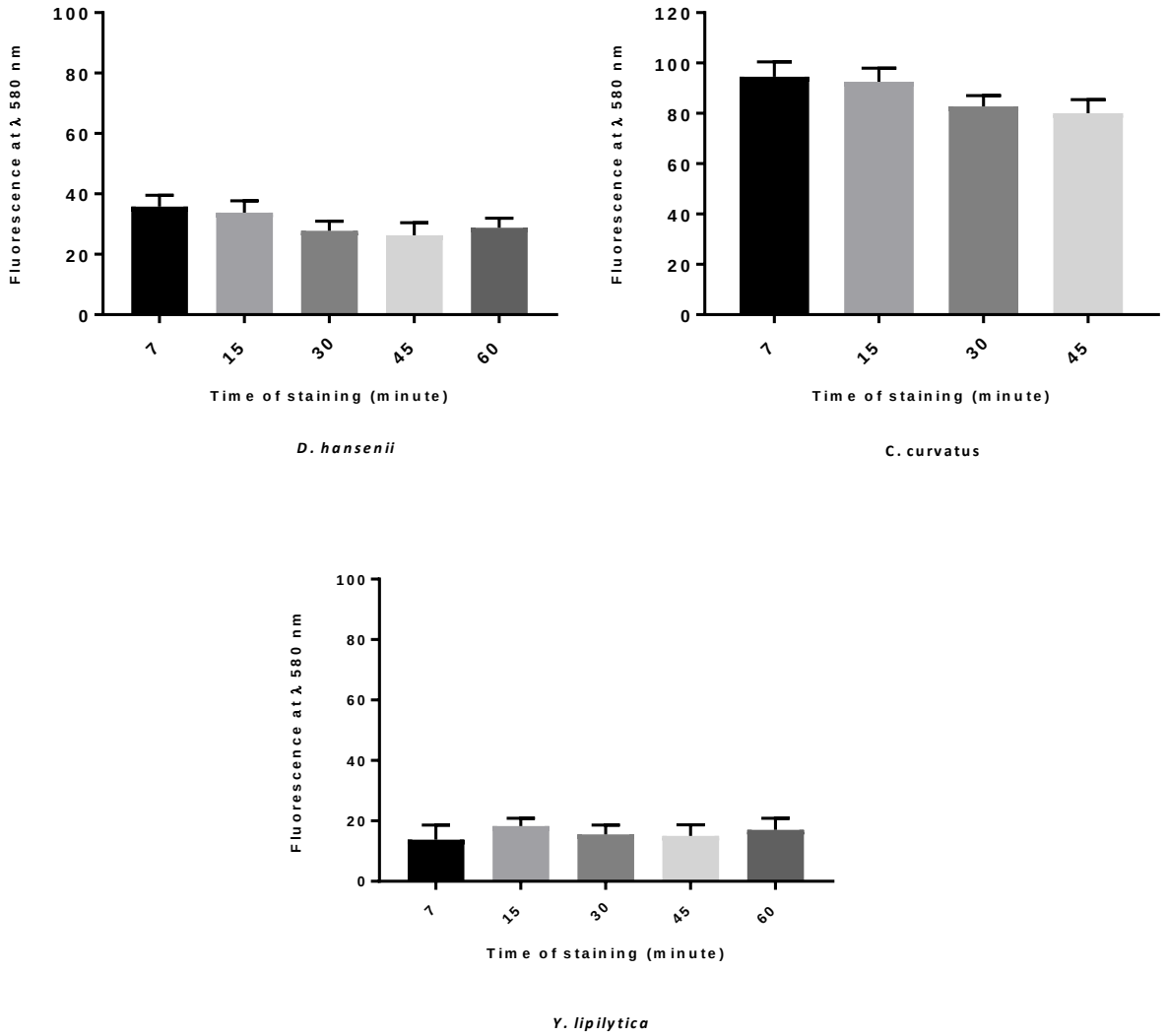


Figure 3.4. The fluorescence intensity of neutral lipid after staining with 0.2 µmol/ ml Nile red dye in *D. hansenii*, *C. curvatus*, and *Y. lipolytica* with increasing the time of staining. Cells were grown in YM medium without salt. Similar experiments were carried out at 0.8 and 1.6 M NaCl (data not shown). Bars represent standard deviation.

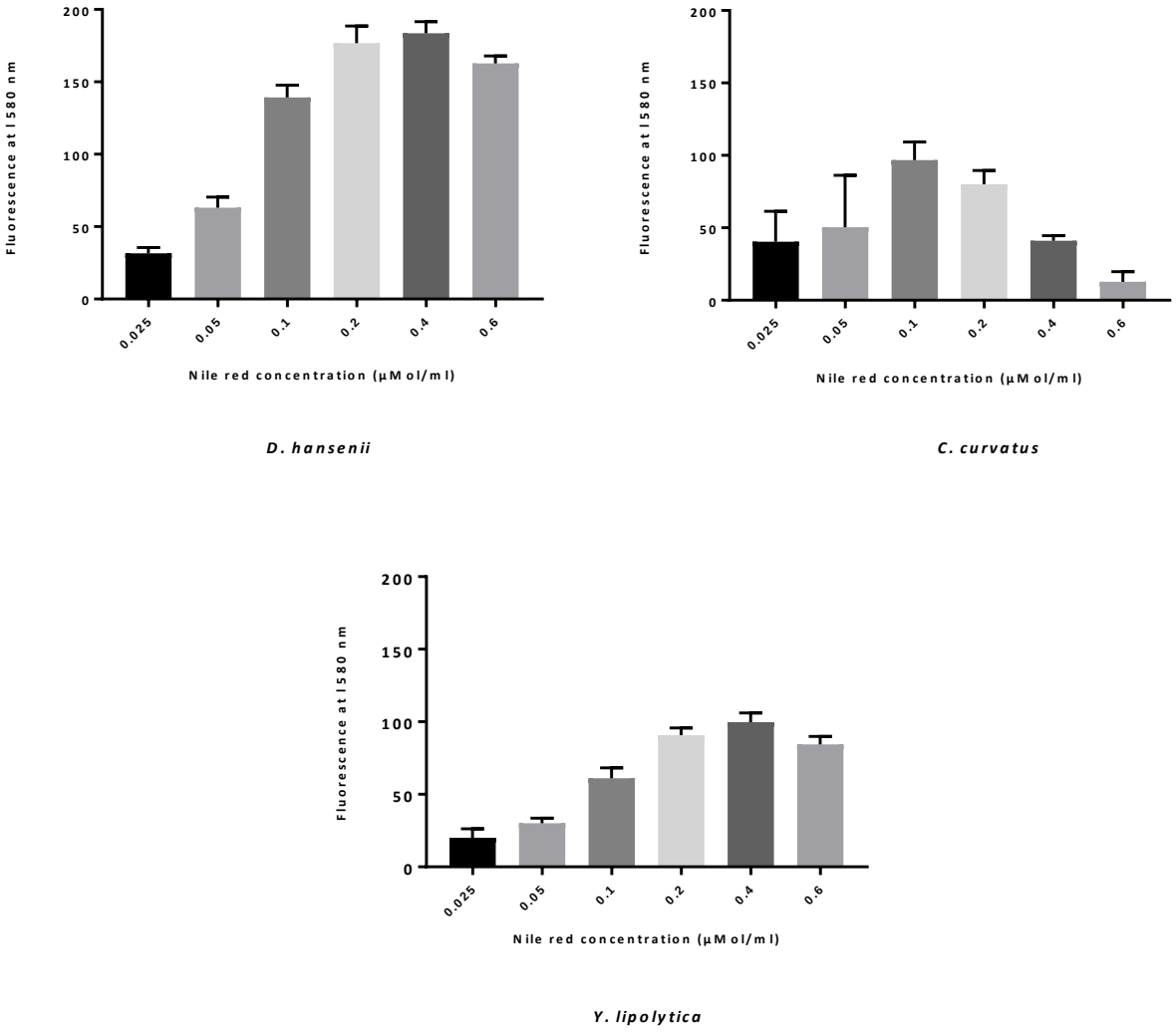


Figure 3.5. The fluorescence intensity of neutral lipid in: *D. hansenii*, *C. curvatus*, and *Y. lipolytica* measured by using different concentrations of Nile red dye. The staining time was 7 minutes Cells were grown in YM medium without salt. Similar experiments were carried out at 0.8 and 1.6 M NaCl (data not shown). Bars represent standard deviation.

After optimization, the fluorescence of neutral lipid was measured in the logarithmic and stationary phase for all yeast strains. As shown in Figure 3.6, the fluorescence intensity of the Nile red in the logarithmic phase was higher than in stationary phase. Neutral lipid evaluation in the late exponential phase has been adopted by (Castelli et al., 1969) for *S. ccerveciae* and by (Dawson and Craig, 1966) for *Candida utilis* which they showed an increasing amount of lipid through the late logarithmic phase , while these amount were noticeably decreased in the stationary phase. In *C. curvatus* and *Y. lipolytica*, we found that the highest accumulation of neutral lipids was in the absence of salt, whereas in *D. hansenii*, the highest accumulation was at 0.8 M NaCl, (Figure 3.6). These results do not agree with the findings of Castanha (2014) who reported that increasing amounts of inorganic salts and yeast extract in the growth medium led to a higher yield of neutral lipids in *Cryptococcus laurentii (Castanha et al., 2014).*

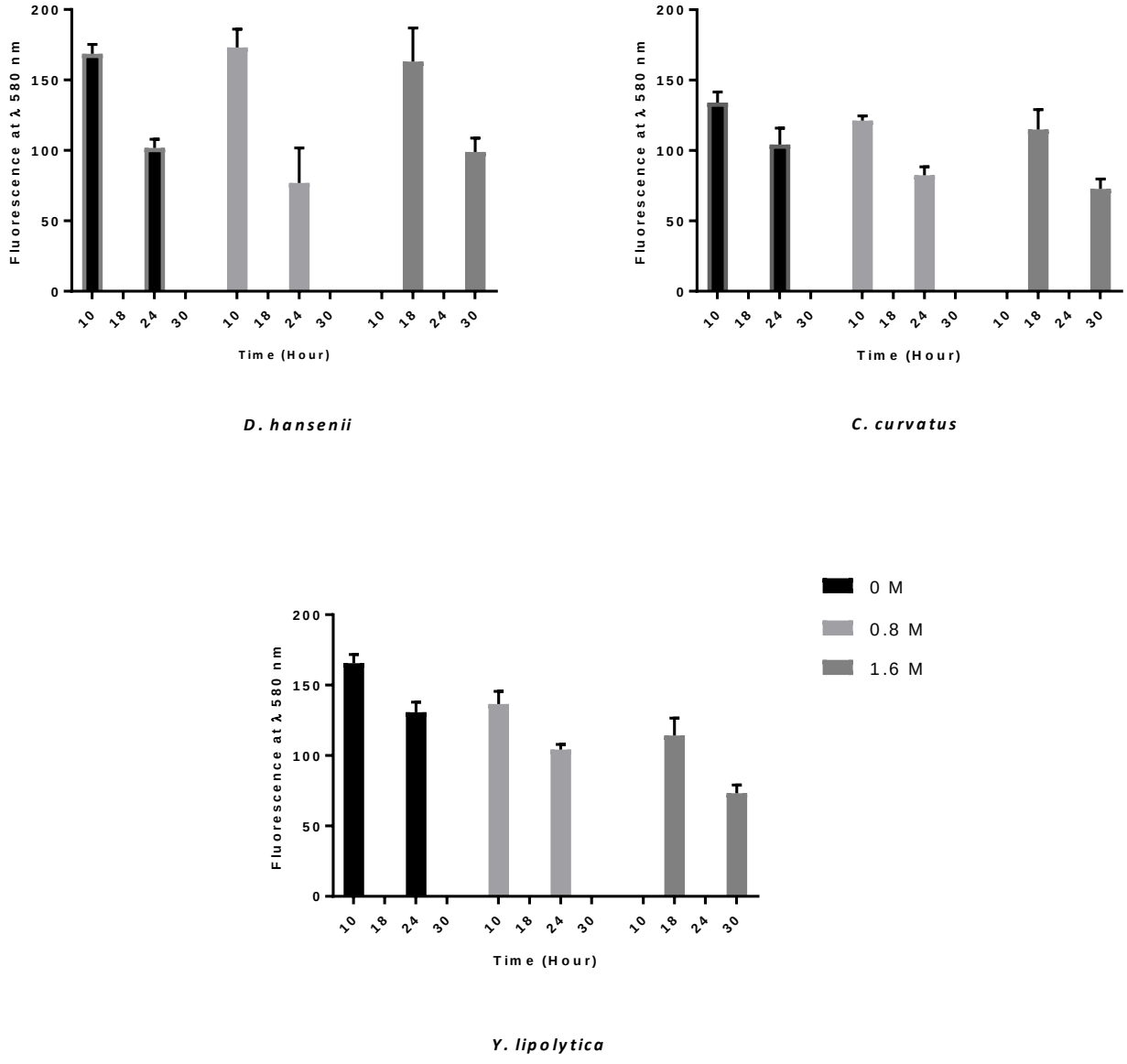


Figure 3.6 The fluorescence intensity of neutral lipid in both log and stationary phase of: *D. hansenii*, *C. curvatus*, and *Y. lipolytica* in media with different concentrations (0, 0.8, 1.6 M) of NaCl. Bars represent standard deviation.

3.3. Conclusion

Without doubt, screening for a high-level lipid producer among oleaginous yeast strains could be the way to find a breakthrough in the biodiesel research area. For that reason, three yeast strains (*D. hansenii*, *C. curvatus*, and *Y. lipolytica)* have been screened for their ability to grow at high salinity and to produce TAG under this condition. Our findings clearly showed that all three strains grew well in the media containing salt between 0.4 to 1.6 M, with highest growth rates in the genus *D. hansenii* and *C. curvatus* respectively. Their growth at high concentrations of NaCl were accompanied with increasing the intracellular concentration of osmolytes in all yeast strains, particularly at 0.8 M NaCl. From NMR analysis, the main osmolyte accumulated in response to high salinity was glycerol in *D. hansenii* and *C. curvatus*, whereas in *Y. lipolytica* arabitol was the main osmolytes, but it was entirely switched to glycerol at 1.6 M concentration of NaCl. Neutral lipid production was evaluated by using Nile red technique at 485 nm and 580 nm for excitation and emission wavelengths respectively. The fluorescence intensity of neutral lipid in the logarithmic phase was higher than in stationary phase for all these microorganisms. Compared with the other two strains the highest fluorescence of neutral lipid was found in *D. hansenii* cells grown at 0.8 M NaCl. Depending on the results of neutral lipid and the ability to grow at high concentration of NaCl, by accumulating glycerol as the main compatible solute, *D*. *hansenii* was selected for further investigation to increase lipid under stress condition. Additionally, from the molecular point, there was limited progress in the molecular tools for *D. hansenii* strain and most research metabolic engineering was focused on *Y. lipolytica* strain. For that reason, it will be worth to investigate the mechanisms that facilitate genetic modification in *D. hansenii* strain, and exploit it for the improvement of biodiesel production.

Chapter Four: Phylogenetic identification of *Debaryomyces hansenii* strain

4. Phylogenetic identification of *D. hansenii* strain

4.1 Introduction

According to the last revision of (Nakase et al., 1998) the genus *Debaryomyces* comprises 15 species. Many of them can be isolated from different natural environments such as air, soil, pollen, fruits, tree exudates, plants, insects, and gut and faeces of vertebrates (Barnett et al., 1983). Nine of these species (*D. hansenii*, *D. carsonii*, *D. etchellsii*, *D. melissophilus*, *D. maramus*, *D. robertsiae*, *D. pseudopolymorphus*, *D. polymorphus*, and *D. vanrijiae*), have been identified in a variety of processed foods, such as soft drinks and fruit juices, bakery products, wine, beer, sugary products, dairy products and meat or processed meats (Deak and Beuchat, 1996, Kurtzman et al., 2003). In recent year's microorganism's identification by traditional methods are more and more frequently substituted by molecular techniques which mainly depend on Polymerase Chain Reaction (PCR) to amplify specific fragments of genomic or mitochondrial DNA (Esteve-Zarzoso et al., 1999, Van den Tempel and Jakobsen, 2000, Hall et al., 2004). This technique is much more accurate, affordable, and quick compared with morphological and physiological criteria which are little used nowadays (Kurtzman et al., 2011). Depending on nucleotide sequence of the large subunit (26S) rRNA, PCR primers have been designed for the identification of *D. hansenii* (and its anamorph *Candida famata*) in a clinical setting (Nishikawa et al., 1997, Nishikawa et al., 1999). However, the usefulness of using only the 26S rRNA sequence is limited because it did not differentiate *D. hansenii* from other similar species that are commonly present in foods. This is due to the difficulty in finding species-specific signature nucleotides and sequence analysis of the 26S rRNA gene has confirmed that *Debaryomyces* species are closely related to each other, making their differentiation difficult (Kurtzman and Robnett, 1991, Yamada et al., 1991). Analysis of the small-subunit (18S) rRNA gene came to the same conclusions (Yamada et al., 1991, Cai et al., 1996). For these reasons, a specific PCR assay for *D. hansenii* strains was carried out by the amplification of a putative homologous *PAD1* region (encodes for phenylacrylic acid decarboxylase) (729 bp) present in this yeast species as a target sequence. The amplification was performed with primer pair (DhPADF/DhPADR) and it is a specific, rapid,

and an affordable method enabling identification of *D. hansenii* from other yeast strains (Wrent et al., 2015).

4.2. Results

4.2.1. Extraction of genomic DNA

Following extraction of the genomic DNA from overnight growth of *D. hansenii* cells in YM and minimal liquid media without NaCl, the obtained gDNA was re-suspended in 50 µl 1xTE buffer, before being run on a 1% Agarose gel, to check for the presence of gDNA (Figure 4.1). DNA extraction involves cell wall digestion, centrifugation to remove cell debris and then nucleic acid precipitation and purification, (Almalki, 2012). The electrophoresis photo showed clear bands for gDNA isolated from each sample, and demonstrates the efficiency of the YeaStar Genomic DNA kit.

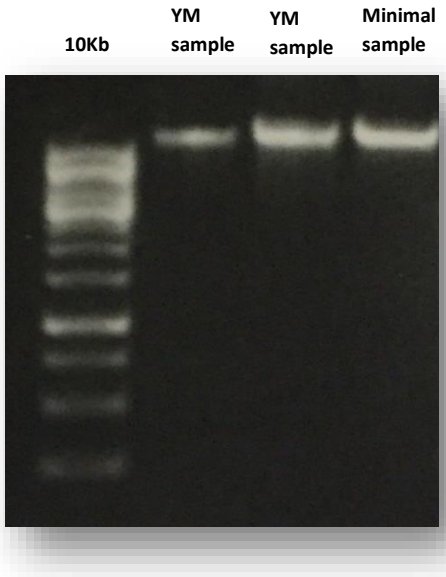


Figure 4.1. Gel electrophoresis photo of *D. hansenii* genomic DNA extracted from YM and minimal cultures after running on 1% Agarose gel for 45 minutes and 90 V.

4.2.2. Genomic DNA amplification by PCR

DNA amplification was carried out to the putative *PAD1* homologous region (729 bp) present in *D. hansenii* according to (Wrent et al., 2015). The sequences were obtained from NCBI. The forward DhPadF, 5′ GCGACTATGAACAGGTTTCCAACGA 3′, and reverse DhPadR, 5′CCTTCAATGTAACATCAGCGGCCC 3′ primers designed for *D. hansenii* were based on the *S. cerevisiae* PAD1/YDR538W gene sequence (http://www.yeastgenome.org), Clustal Omega (McWilliam et al., 2013). The *PAD1* gene encodes for phenylacrylic acid decarboxylase in *S. cerevisiae*, which provides the resistance to cinnamic acid (Clausen et al., 1994). Other putative *PAD1* homologues have been distinguished in some yeast species such as *Candida albicans*, *C. dubliniensis*, D*. hansenii* and *Wickerhamomyces anomalus* (Stratford et al., 2007). A recent study has been distinguished a putative homologous region of the *PAD1* gene in *D. hansenii* with significant differences to the homologous regions with *S. cerevisiae*, so that this sequence could be a good target for the *D. hansenii* specific identification (Wrent et al., 2015). Figure (4.2) shows the success of the amplification process of putative *PAD1* homologous region (729 bp) from genomic DNA for both samples with band size of approximately 400 base pairs which is similar to the band size obtained by (Wrent et al., 2015) (the target sites of the primers are sequences flanking a putative homologous region of the *PAD1* gene).

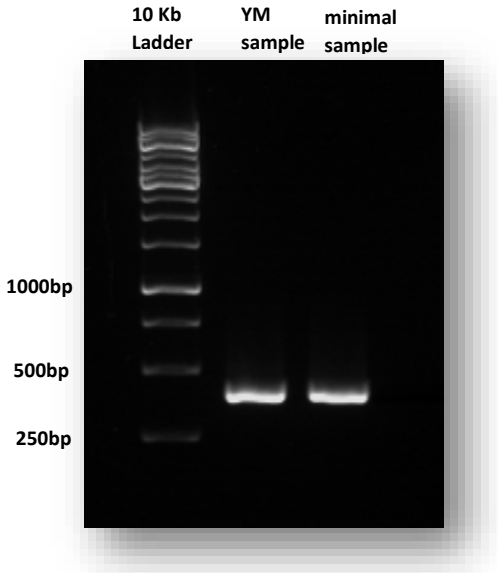


Figure 4.2. Gel electrophoresis of *PAD1* gene PCR product of YM and minimal cultures With DhPadF and DhPadR primers. Samples run in 1% Agarose gel for 45 minutes and 90 V. The size of the bands was around 400bp

PCR product was purified by using PCR clean up procedure for Nucleic Acid Extraction Kit, Key Prep, to remove any possibility of the presence of compounds that might affected the purity of gDNA which was used for sequencing.

For sequencing, the PCR purified product was send to Eurofins MWG, and the results were as follows:

PAD1 gene sequence of *D. hansenii* cells growing in minimal medium

Forward sequence (377 letters)

AATGTATCTTACAAGACCTAAGAGGATAGTCGTGGCAATAACTGGGGCTACAGGTATTGC
AATCGGTGTAAGGGTATTGGAATTATTAAAGCAATGTAAAGTTGAGACACATTTAATTAT
GTCCAAATGGGGTATGGCAACAATGAAATATGAAACAGATTATCATATGGACGACATAAT
GGCACTTGCGTCAAAGGTGTACACTGCCAGAGACGTGAGTGCGCCGATTTCGTCAGGATC
TTTCCAACACGATGGTATGATTGTCGTGCCATGTTCGATGAAGACATTGGCTGGGATTAG
GATGGGATTCACAGAGGATCTTATCGTAAGGGCCGCTGATGTTACATTGAAGGAAGG.

Reverse sequence (376 letters)

TGATCCCATCCTAATCCCAGCCAATGTCTTCATCGAACATGGCACGACAATCATACCATC
GTGTTGGAAAGATCCTGACGAAATCGGCGCACTCACGTCTCTGGCAGTGTACACCTTTGA
CGCAAGTGCCATTATGTCGTCCATATGATAATCTGTTTCATATTTCATTGTTGCCATACC
CCATTTGGACATAATTAAATGTGTCTCAACTTTACATTGCTTTAATAATTCCAATACCCT
TACACCGATTGCAATACCTGTAGCCCCAGTTATTGCCACGACTATCCTCTTAGGTCTTGT
AAGATACAATCCTGTTGACTGATTCTGGTATATATCGTTGGAAACCTGTTCATAGTCGCA
A

PAD1 gene sequence of *D. hansenii* cells growing in YM medium

Forward sequence (382 letters)

GATTGTATCTTACAAGACCTAAGAGGATAGTCGTGGCAATAACTGGGGCTACAGGTATTG
CAATCGGTGTAAGGGTATTGGAATTATTAAAGCAATGTAAAGTTGAGACACATTTAATTA
TGTCCAAATGGGGTATGGCAACAATGAAATATGAAACAGATTATCATATGGACGACATAA
TGGCACTTGCGTCAAAGGTGTACACTGCCAGAGACGTGAGTGCGCCGATTTCGTCAGGAT
CTTTCCAACACGATGGTATGATTGTCGTGCCATGTTCGATGAAGACATTGGCTGGGATTA
GGATGGGATTCACAGAGGATCTTATCGTAAGGGCCGCTGATGTTACATTGAAGGAAGATC
TTA.

Reverse sequence (379 letters)

TCTCTGTGATCCCATCCTAATCCCAGCCAATGTCTTCATCGAACATGGCACGACAATCAT
ACCATCGTGTTGGAAAGATCCTGACGAAATCGGCGCACTCACGTCTCTGGCAGTGTACAC
CTTTGACGCAAGTGCCATTATGTCGTCCATATGATAATCTGTTTCATATTTCATTGTTGC
CATACCCCATTTGGACATAATTAAATGTGTCTCAACTTTACATTGCTTTAATAATTCCAA
TACCCTTACACCGATTGCAATACCTGTAGCCCCAGTTATTGCCACGACTATCCTCTTAGG
TCTTGTAAGATACAATCCTGTTGACTGATTCTGGTATATATCGTTGGAAACCTGTTCATA
ATCGCAAGATCTC.

The obtained sequences were analysed using BLAST algorithm (Altschul et al., 1990) and the NCBI server (National Centre for Biotechnology Information) website, and was found to be 99% identical to *D. hansenii* (Figure 4.3)

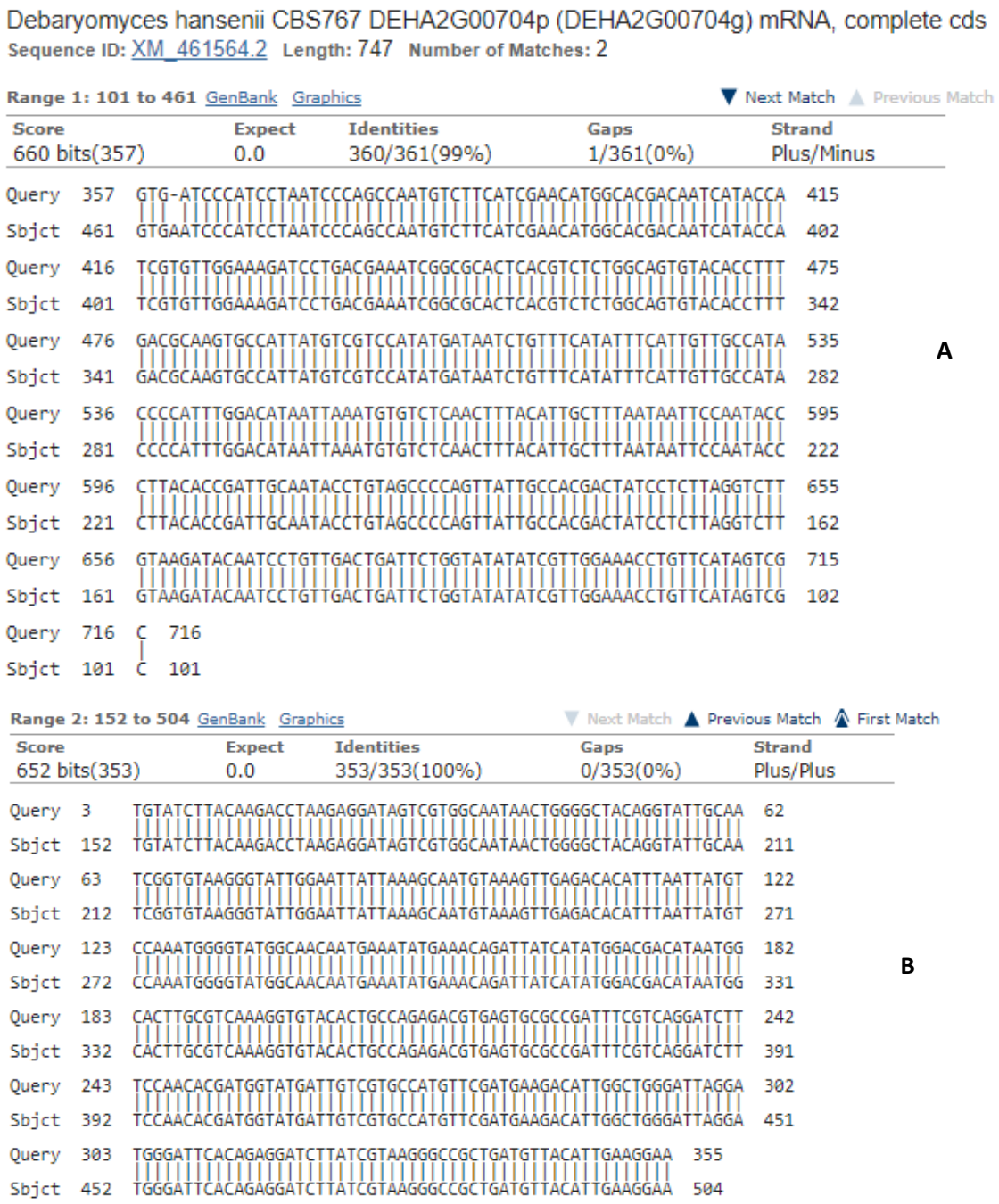


Figure 4.3. Blast of PCR product for A- forward and B-reverse primers of *PAD1* gene of *D. hansenii* cells growing in minimal medium with the existing information sequence of National Centre for Biotechnology Information (NCBI) website

4.3. Conclusion

In conclusion, our data shows that the primers DhPADF/ DhPADR used in this assay produced a clear single fragment of 400 bp of putative *PAD1* homologous region in both YM and minimal cultures samples for *D. hansenii* strains. No false negatives were detected in this assay, and provided high confidence matches when combined with the existing sequence information in the National Centre for Biotechnology Information (NCBI) website, indicating the organism was *D. hansenii*. The primers developed in this work can be used directly on colonies with 100% success rate, and this could save users considerable time. The assay here proposed is a rapid and affordable method that enables the identification of *D. hansenii*. Moreover, individual or institutional culture collections might depend on this assay to achieve a rapid confirmation or re-identification of *D. hansenii* strains.

Chapter Five: Biotechnological methods to optimize neutral lipid production in *Debaryomyces hansenii* cells

5. Biotechnological methods to optimize neutral lipid production in *Debaryomyces hansenii* cells

5.1. Introduction

Our selected hemiascomycetous yeast *D. hansenii* (also identified as *Candida famata*, (Dmytruk and Sibirny, 2012), is reported to possess many characteristics, which allow it to be an important yeast for biotechnological work. It is a heterogeneous species, has the ability to grow in extreme environments, such as, high concentration of NaCl (Norkrans, 1966, Gunde-Cimerman et al., 2009), which correlates with its importance for manufacturing salty foods like different types of cheeses and meat products (Eliskases-Lechner and Ginzinger, 1995, Jessen, 1995, Gunde-Cimerman et al., 2009). When exposed to high salinity this osmotolerant species *D. hansenii*, produces and accumulates glycerol as the main compatible solute (Gustafsson and Norkrans, 1976, Adler and Gustafsson, 1980). At high salinity, the intracellular content of Na^+/K^+ in the growing cells is not sufficient to equilibrate the osmotic level *visa vise* the external environment. Several studies demonstrated that glycerol and an organic solute accumulation, is due to growth at increased salt stress (Gustafsson and Norkrans, 1976, Gustafsson, 1979, Adler and Gustafsson, 1980, Adler et al., 1985, Nobre and Costa, 1985). Glycerol works as a compatible solute (Brown, 1978, Yancey et al., 1982) which raises the internal osmotic pressure of the cells without interfering with the macromolecular function and structure. In addition these low molecular weight compounds such as glycerol and arabitol work to protect the intracellular enzymes and structural proteins from the inhibition, inactivation and denaturation problems caused by low water activity (Brown, 1978). Environmental factors such as aeration, temperature, inorganic salts, pH, incubation period, inoculum size and the microorganism itself affect the lipid content and the fatty acid composition (Moreton, 1988, Subramaniam et al., 2010). The basic physiology of lipid accumulation has been well investigated in microorganisms. Lipid production requires medium with an excess of carbon source (e.g., sugar, glycerol, polysaccharides, etc.) and limiting amount of another nutrient such as nitrogen. So that, the lipid potential of oleaginous yeast is

critically affected by the carbon to nitrogen (C/N) ratio of the culture and other factors such as the inorganic salt, aeration, etc. (Moreton, 1988). In this chapter, we investigated the possible correlation between the NaCl stress conditions that induce glycerol accumulation with the level of intracellular neutral lipid production, due to lack of studies discussing the relationship between these two metabolites under high salt. On the other hand, the composition of fatty acids and neutral lipid production were also evaluated in *D. hansenii* yeast cells when growing in media with different stress conditions (high salt, low nitrogen and high level of carbon source).

5.2. Results

5.2.1. Growth curve determination of pre-adapted cells of *D. hansenii* in YM and minimal medium with different concentrations of NaCl

Growth curves of *D. hansenii* was monitored and compared in both minimal and YM media with different concentrations of NaCl (0, 0.8, and 1.6 M) incubated at 25°C under shaking at 120 rpm. The optical density was measured with the spectrophotometer at given intervals (usually 2 hours) at wavelength 600 nm against a medium blank with the same concentration of NaCl. The results in Figure 5.1.A and B show that *D. hansenii* cells grow well in both media under given concentrations of NaCl. In 0 M cultures, the logarithmic phase started earlier than other concentrations of NaCl in both minimal and YM cultures, and slowed down after 22 and 20 hours of incubation respectively. However in response to hyperosmotic stress in 0.8 and 1.6 M cultures the yeast cells enter stationary phase after 26 hours of incubation particularly 1.6 M in both Minimal and YM media. (Almalki, 2012) reported that *D. hansenii* showed higher growth rates and adapted to grow and thrive in higher salinity concentrations and low pH when grown in rich YPD medium compared with M9 minimal medium. During the exponential phase, the generation time was calculated in both minimal and YM cultures with different concentrations of NaCl (Almalki, 2012). Depending on the results displayed in the Table 5.1 it, was obvious that the doubling times in minimal medium were longer than in YM

medium. Along with the type of medium the doubling times were also affected with increasing the concentration of NaCl, especially in 1.6 M Minimal medium cultures where it was reached up to 5 hours and 48 minutes of incubation (see Table 5.1).

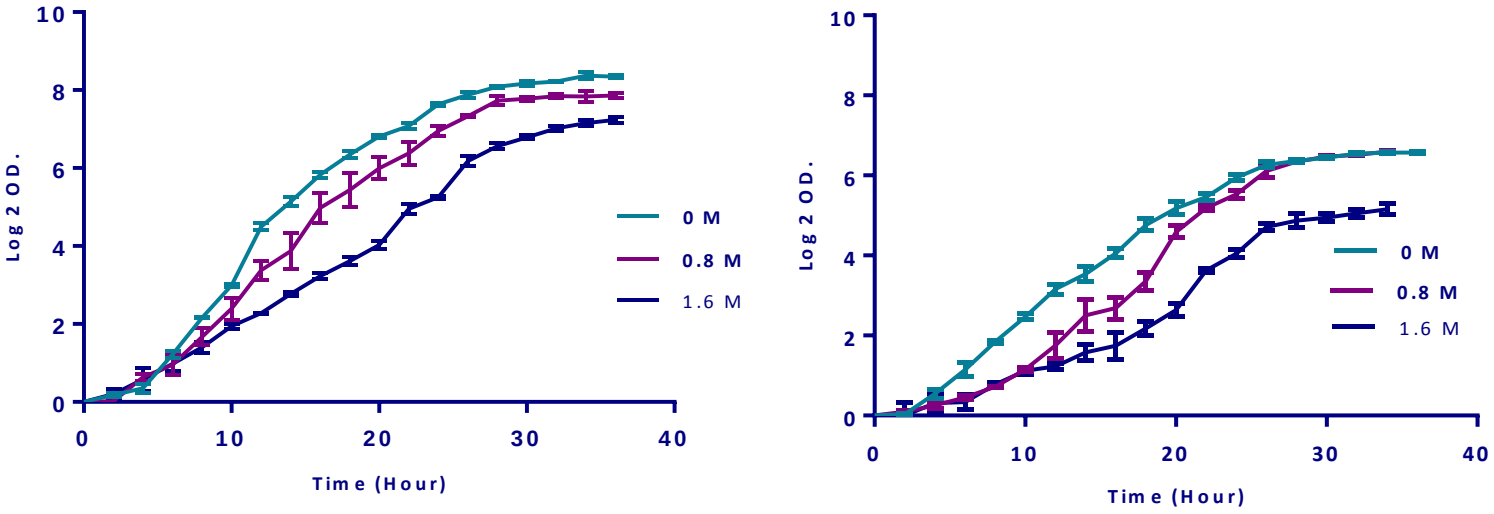


Figure 5.1. Logarithmic growth curves for pre-adapted strains of *D. hansenii* growing at 25 °C under shaking at 150 rpm in: A- YM medium and B- Minimal medium with different concentrations (0, 0.8, and 1.6 M) of NaCl. Bars represent standard deviations.

Table 5.1. The doubling times of *D. hansenii* cells ± standard deviation. Cells growing in minimal and YM media with different concentrations of NaCl

Sample	Doubling time
0 M Minimal cultures	3 hours and 14 minutes ± 0.04
0.8 M Minimal cultures	4 hours and 13 minutes ± 0.4
1.6 M Minimal cultures	5 hours and 48 minutes ± 0.3
0 M YM cultures	2 hours and 40 minutes ± 0.04
0.8 M YM cultures	3 hours ± 0.1
1.6 M YM cultures	4 hours and 36 minutes ± 0.5

5.2.2. The effect of adaptation to different salinity concentrations on the total osmolytes and glycerol accumulation in *D. hansenii* growing in both minimal and YM media

The evaluation of total osmolytes and glycerol was carried out using different methods (acetyl acetone and free glycerol reagent procedure respectively). Cells were grown in 3x50 ml shaking flasks at 25°C with different concentrations of NaCl (0, 0.8, 1.6 M) augmented in both minimal and YM media (Figure 5.2). The production of each type (total osmolytes and glycerol) was compared with biomass (mg) yield at the same growing conditions. The results showed low level of glycerol compared with high content of total osmolytes in both media. The accumulation amounts of both types in YM cultures were higher than those in minimal medium, and it was significant in 0.8 M NaCl cultures (for total osmolytes and glycerol the P value was ≤ 0.003 and 0.0004 respectively). In 1.6 M NaCl there was less significant differences (P value ≤ 0.0482) between glycerol values in YM and minimal cultures. Interestingly the current results were different from the hypothesis presented by (Albers et al., 1996). The author mention that the production of glycerol was higher in the minimal medium than in enriched medium, as intracellular formation of amino acids from ammonia and glucose leads to an increased the accumulation of NADH which is then re-oxidised to NAD^+ through the formation of glycerol (Albers *et al,* 1996). The maximum content of total osmolytes was in 0.8 M NaCl, especially in YM cultures which reached up to 1430.4 µg osmolytes/ mg biomass (figure 5.2). However, the highest level of glycerol achieved in 1.6 M NaCl, which was about 69.5 µg glycerol/ mg biomass in YM cultures. Our results agreed with previous studies about the positive correlation between the level of intracellular glycerol in *D. hansenii* cells and the salinity of the surrounding medium (Adler and Gustafsson, 1980, Adler et al., 1985, André et al., 1988, Larsson et al., 1990) findings who demonstrated that the internal concentration of solutes which were essentially independent of growth rate, were directly proportional to the salinity of the medium.

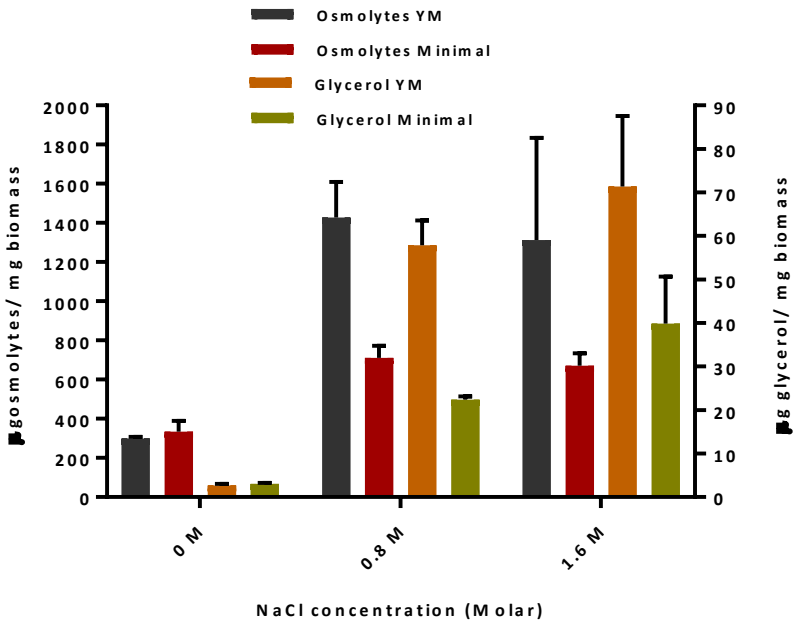
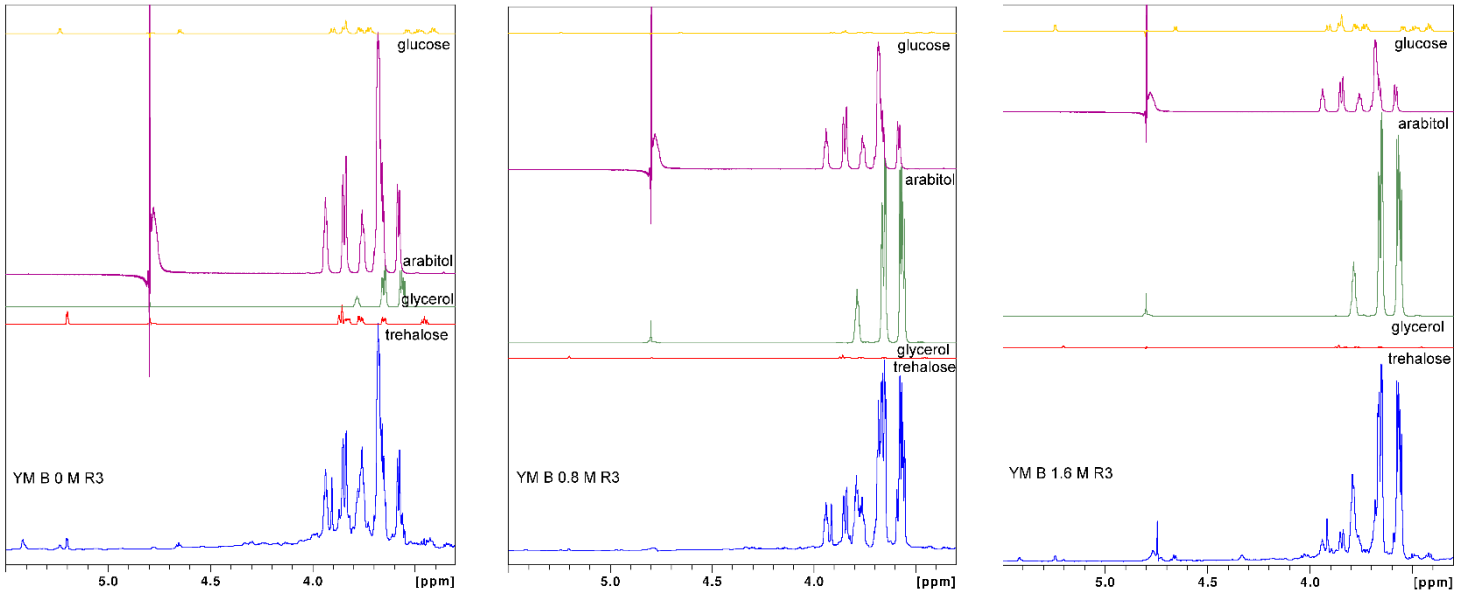


Figure 5.2. The amount of total osmolytes and glycerol µg/ mg biomass in *D. hansenii* minimal and YM cultures with different concentrations (0, 0.8, and 1.6 M) NaCl. Left scale plotted for total osmolytes, while the right scale used for glycerol. Bars represent standard deviations.

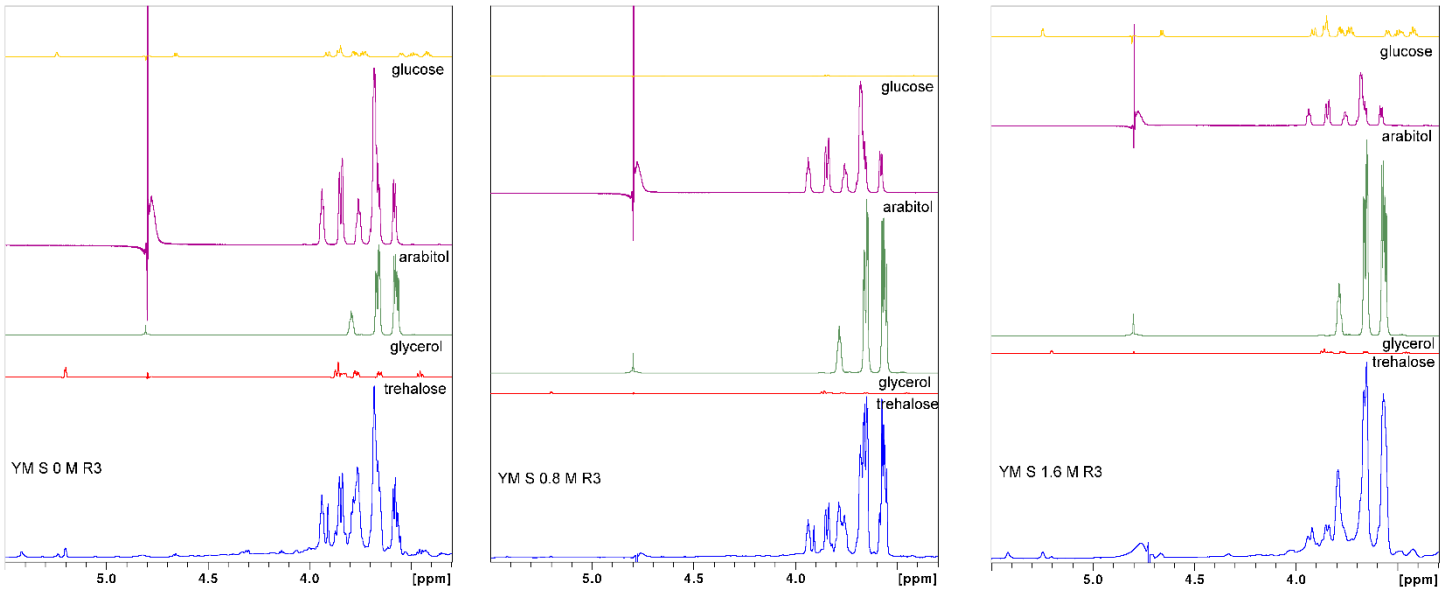
5.2.3. NMR analysis of compatible solutes in *D. hansenii*

^{1}H NMR spectrograph was used as an indication of the change in concentrations or absence/presence of most metabolites inside the cell. For the best cell disruption, different methods were used to yield the intracellular polyols. The samples of all cultures were divided into two groups. In the first group, the cells were disrupted by bead beating for about 4 x 60 second at 4000 rpm with 60 second cooling intervals. In the second group cells were broken by sonication for 3 x 20 second with 15 second cooling periods between sonication steps. The profiles of *D. hansenii* yeasts grown under NaCl stress were generally found to have fewer notable peaks when compared to yeast cells grown without stress (Figure 5.3 and 5.4). In parallel the accumulation patterns of total osmolytes and glycerol were also changed in response to the salinity. Identification of the main peaks revealed that the main osmolytes identified by NMR spectroscopy were glycerol, arabitol,

glucose and trehalose. From the data shown it was obvious that in both groups of cell disruption technique the osmolytes have similar peaks in each sample (Figures 5.3 and 5.4), therefore both groups were combined when measuring the percentage of each metabolites. The peak height was measured rather than peak area, to avoid overlapping between peaks. Variations in the percentages of glucose, trehalose, arabitol, and glycerol between different concentrations were observed. In the minimal medium the percentages of each osmolyte revealed: glucose is roughly the same at 0 and 0.8 M and then increases at 1.6 M; trehalose is reasonably at high concentration in 0 M but then decreases to zero by 1.6 M; arabitol is fairly similar at 0 and 0.8 M but gets quite a bit less at 1.6 M; and glycerol is present at 0 M but gets steadily more as the salt concentration goes up (Figure 5.5. B). In the YM cultures the results showed that glucose and trehalose were present in much lower concentration than they were in minimal medium. However, there was more glycerol produced in media with high salt which reached up to 80 and 79% in 0.8 and 1.6 M YM cultures respectively whereas these percentages decreased up to 26% in 0.8 M and 35% in 1.6 M minimal cultures (Figure 5.5.A and B). Our results were agreed with (Almalki, 2012) who reported that glycerol was detected as the main osmolytes in all NMR spectra of *D*. *hansenii* cells growing in high salinity for both M9 minimal and YPD media followed by arabitol as the next important solute.

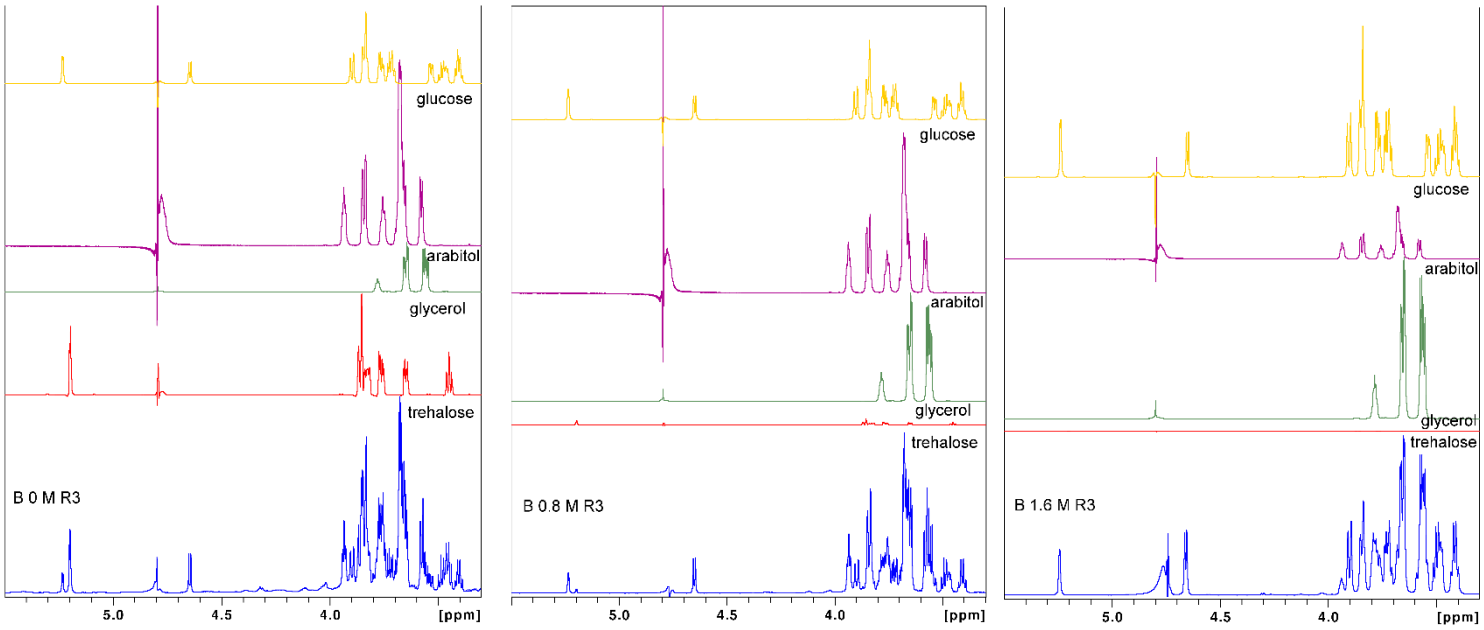


A

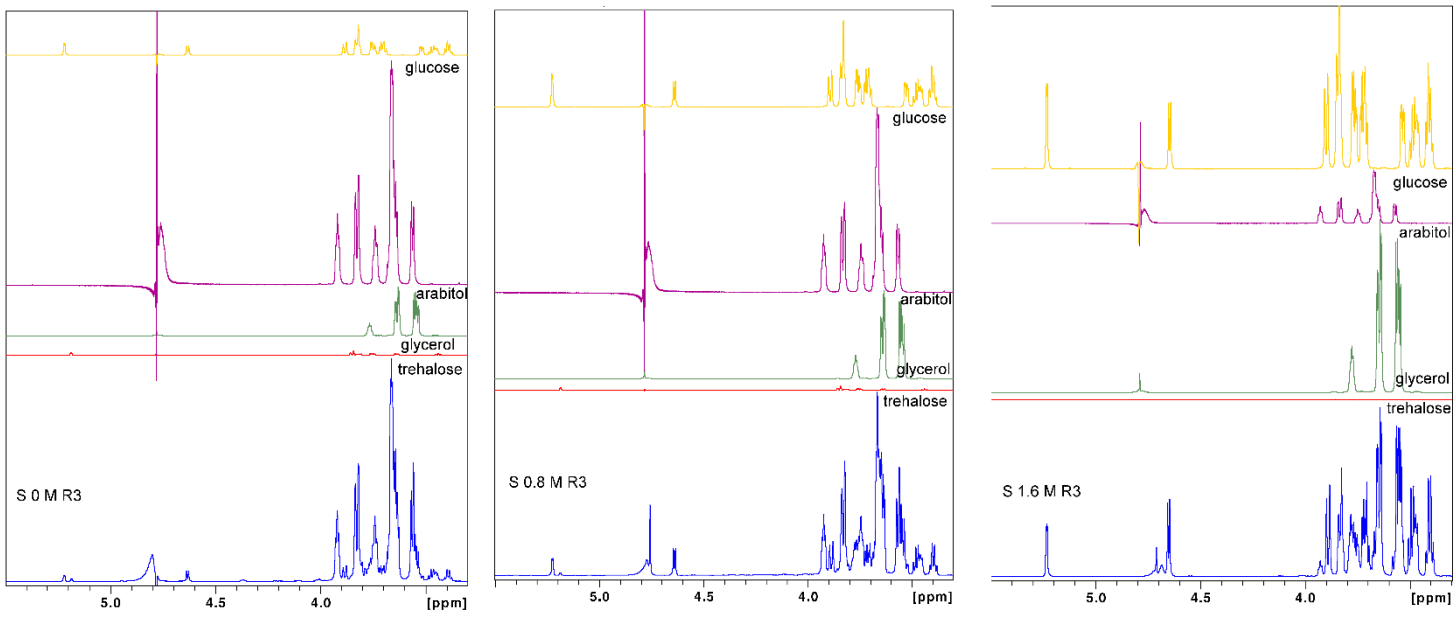


B

Figure 5.3. Represents the ^{1}H NMR analysis of the osmolyte of *D. hansenii* strain growing in YM broth with different concentrations (0, 0.8, and 1.6 M) of NaCl. Yeast cells disrupted by: A- beads beating and B- sonication before lyophilisation.



A



B

Figure 5.4. Represents the ^{1}H NMR analysis of the osmolyte of *D. hansenii* strain growing in minimal medium broth with different concentrations (0, 0.8, and 1.6 M) of NaCl. Yeast cells disrupted by: A- bead beating and B- sonication before lyophilisation

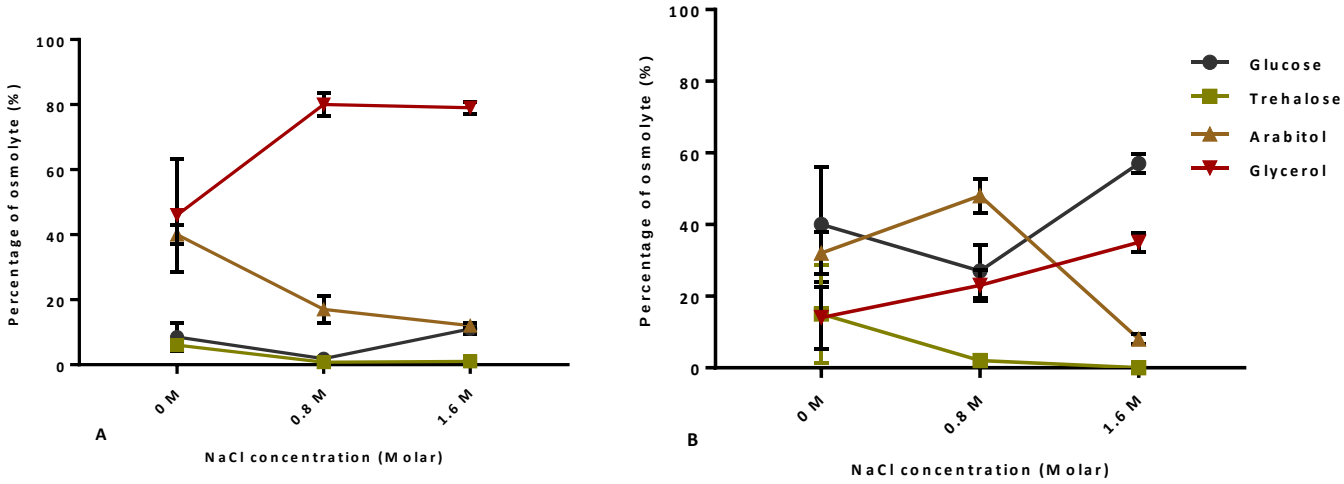


Figure 5.5. The Percentage of osmolytes in A- YM, and B- Minimal cultures with different concentrations (0, 0.8, and 1.6 M) of NaCl when measuring the peak height of ^{1}H NMR of these samples. Bars represent standard deviations.

5.2.4. Spectrofluorometric quantification measurement of neutral lipid in *D. hansenii* using Nile red dye

In order to investigate the effect of hyperosmotic and limited nutrient stress on neutral lipid content (mg neutral lipid / mg biomass) in *D. hansenii* cells, experiments were carried out using minimal and YM medium with different stress conditions. Initially, serial experiments were performed to optimize the measurement conditions (cell concentration, time of staining, and stain concentrations) using Nile red dye for minimal medium. For YM medium we depend on the optimum measurement conditions that are shown in section 3.2.5 for the following experiments. As shown in Figure 5.6 the best cell concentration was chosen based on a high normalised value and smaller standard error value of stain average in order to avoid overlapping between stained and unstained average values. The best cell concentration for *D. hansenii* cells was at the relative cell concentration of 87.5% which equates to an OD. of about 4.72. In addition, 7 minutes was

chosen as the best time of staining and 0.4 µmol/ml was chosen as the optimal concentration of Nile red dye (see Figure 5.7.A and B).

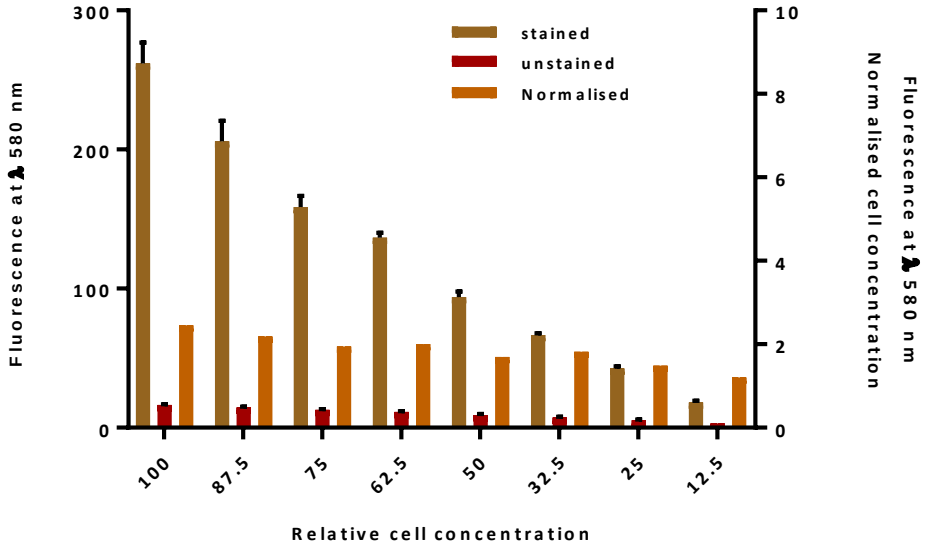


Figure 5.6. The fluorescence of neutral lipid with different cell concentrations in *D. hansenii* after 24 hours of incubation in minimal medium without salt. Bars represent standard deviations.

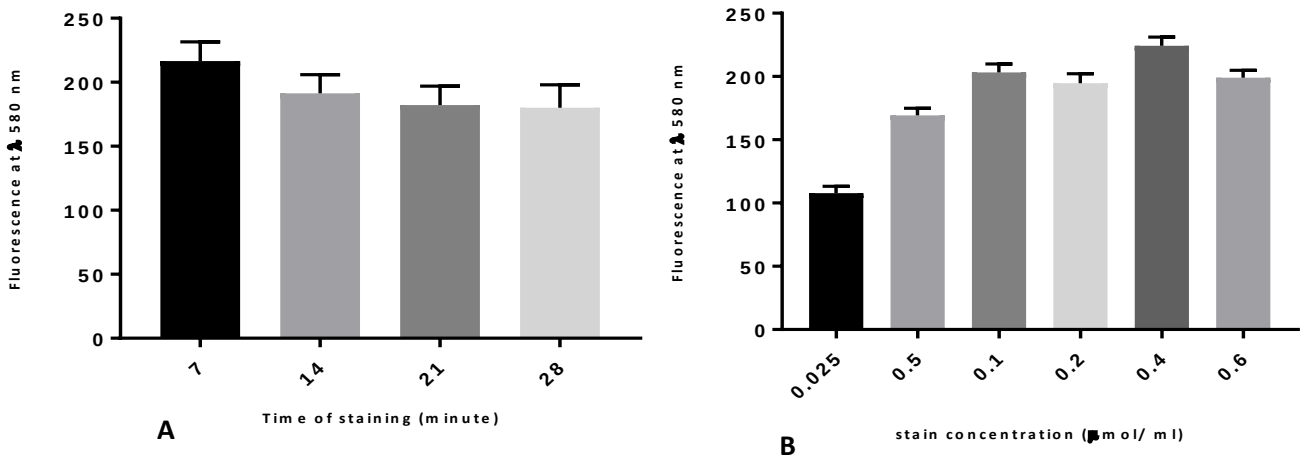


Figure 5.7. The fluorescence of neutral lipid with: A- Increasing time of staining with 0.2 (µmol/ml) Nile red and B- different concentrations of Nile red for *D. hansenii* after 24 hours of cultivation in minimal medium without salt. Bars represent standard deviations.

5.2.4.1. Cell count calibration

D. hansenii cells were counted in different cultures dilutions (shown in Table 2.1). Data in Figure 5.8 showed increasing cell countings in parallel with increasing the cells concentrations for all dilutions made for YM and minimal cultures. Cell number for yeast growing in minimal medium was higher than YM medium. We thought this might be due to the cell size in YM was higher than in minimal medium which was clearly seen in a standard optical microscope with an x 40 objective magnification. Cell number in minimal medium at OD 4.7 (best OD of cell concentration in neutral lipid evaluation in minimal medium) was about 33 x $10^{7,}$ while at OD 2.59 of YM cultures (best OD of cell concentration for neutral lipid evaluation in YM medium) was 5.75 x 10^{7}.

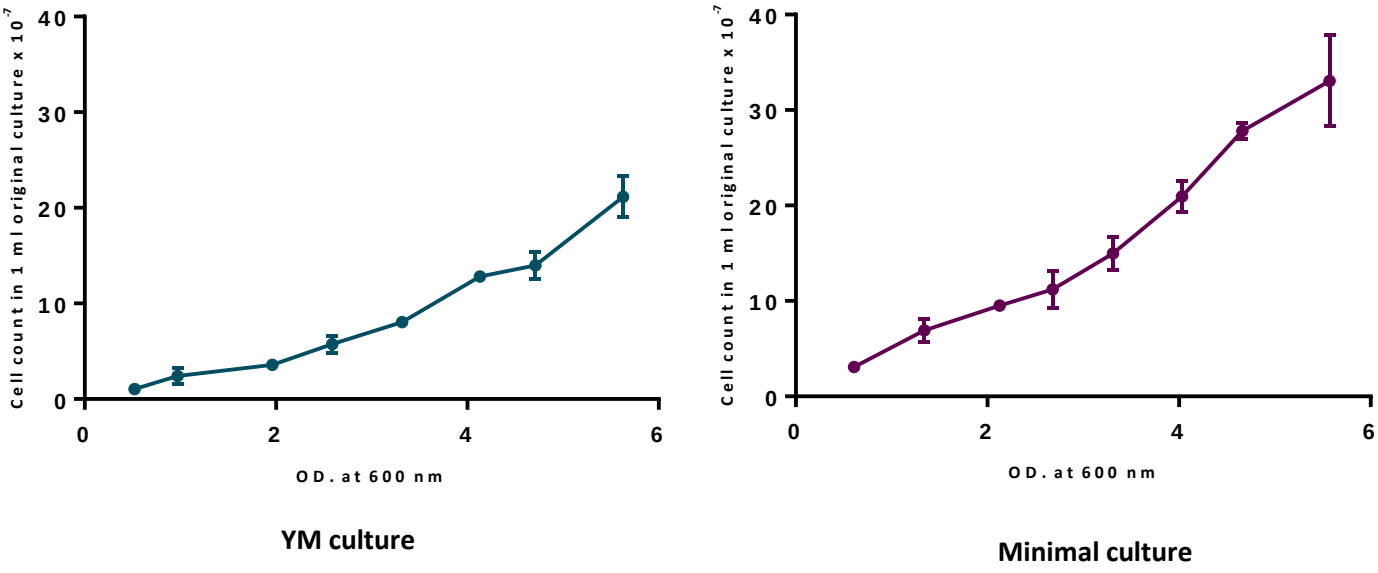


Figure 5.8. Cell number versus OD at 600 nm of serial dilutions of *D. hansenii* cells growing in: YM and minimal medium. Cell number was counted under x40 objective magnification. Readings in Y axis were multiplied with x 10^{-7}. Bars represent standard deviations.

5.2.4.2. Effect of growth phase and hyperosmotic stress on neutral lipid production

Neutral lipids were monitored during the growth phases, the readings were taken through the early logarithmic phase as well as in early and the late stationary phase. The results showed that the highest accumulation was after 24, 16, and 24 hours for 0, 0.8, and 1.6 M minimal medium cultures respectively, which are all in the early stationary phase for *D. hansenii* (Figure 5.9.B). In the same pattern, the highest levels of neutral lipid were detected after 16 hours of incubation for the cultures without salt and after 20 hours for both 0.8 and 1.6 M YM cultures (Figure 5.9.A). Lipid accumulation is a dynamic process that depends on different factors such as the growth conditions, microorganism, and the growth phase. It is started when the nutrients supply is depleted, particularly nitrogen source, but when an excessive amount of carbon is still available to the cells. Due to nitrogen limitation the cell proliferation slower, and the synthesized lipid has to be stored

within the existing cells in the form of lipid droplets (LDs), which can no longer assimilated (Ratledge and Wynn, 2002, Markgraf et al., 2014) reported that through the early stationary phase in *S. cerevisiae* the diacylglycerol acyl-transferase Dga1p moves from the endoplasmic reticulum to LDs which is essential in TAG synthesis from diacylglycerol. The number and size of LDs increase when yeast cells reach the early stationary phase. Other work by (Uzuka et al., 1985) also demonstrated that the accumulation rate in *Lipomyces starkeyi* yeast reached the highest level in the second stage of the retardation phase (early stationary phase), and slowed down after that (Figure 5.10). Based on previous studies and our results that agreed with their findings, cells were harvested in the late logarithmic phase in the following experiments to compare lipid production at different concentrations of NaCl in both minimal and YM media.

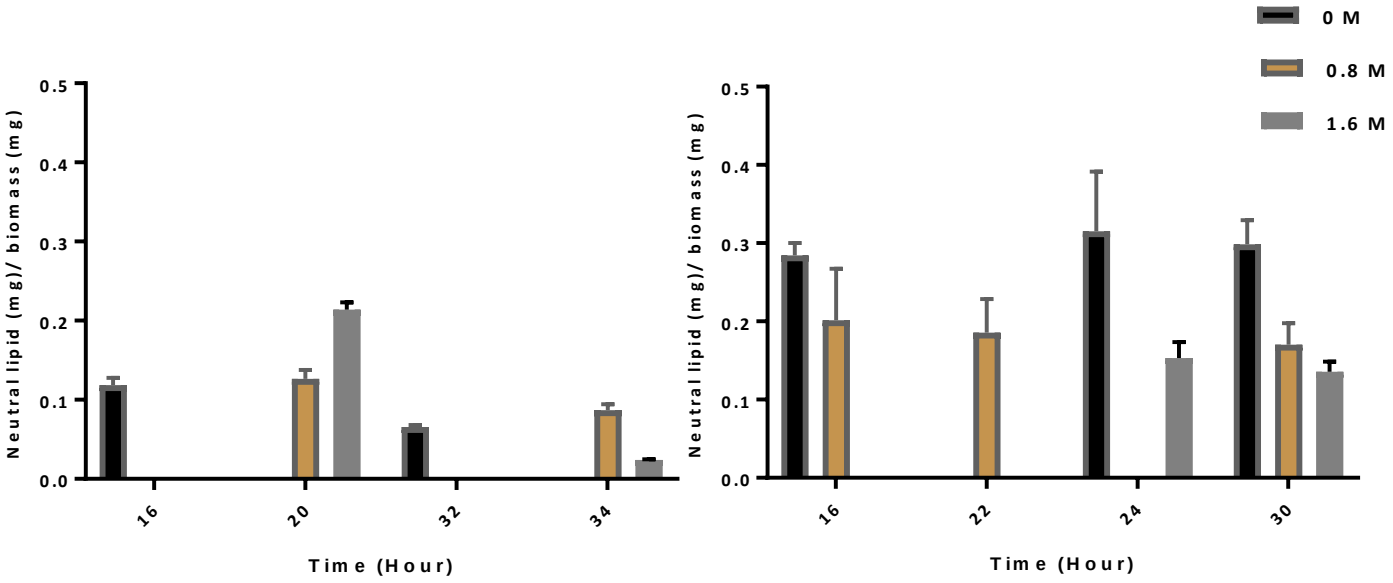


Figure 5.9. Neutral lipid concentration in both log and stationary phase (early and late) for *D. hansenii* growing in: YM medium, minimal medium with different concentrations of NaCl (0, 0.8 and 1.6 M). Bars represent standard deviations.

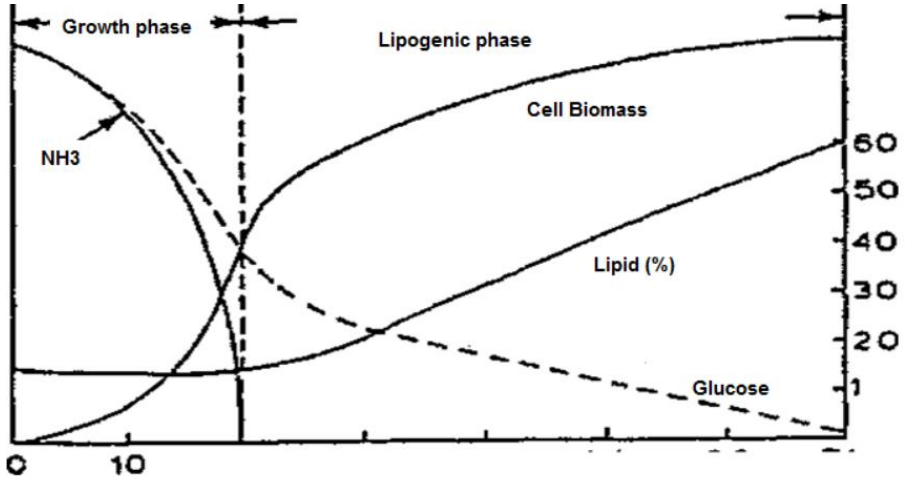


Figure 5.10. The typical pattern of lipid accumulation and biomass production in oleaginous microorganisms. Adopted from (Akpinar-Bayizit, 2014)

Interestingly neutral lipid production is found to be significantly higher in minimal medium than in enriched YM medium with different concentrations of NaCl especially for 0 M concentration which displayed highly significant (P value ≤ 0.001) differences when compared with other salt concentrations of both media. However, there was nonsignificant variation in neutral lipid values between cultures grown in YM and minimal media at 1.6 M NaCl. The highest lipid accumulation was for 0 M minimal medium in which cells accumulated about 0.152 mg neutral lipid / mg biomass, while the lowest content was for 0.8 M YM culture (Figure 5.11). Several studies also examined the effect of NaCl stress on neutral lipid production in yeast which to some extent agreed with our study. For example In *Saccharomyces rouxii* the content of TAG was decreased when the NaCl concentration rose from 0 to 2 M in the medium, whereas further addition of NaCl up to 3M stimulated an increasing amount of TAG (Andreishcheva et al., 1999). Watanabe and Takakuwa (1984) reported that the pool of free fatty acids increased in *Yarowia lypolytica* cells due to less active utilization for TAG synthesis in the presence of 9% NaCl,

thus the amounts of TAG and sterol esters decreased in response to this salt stress (Watanabe and Takakuwa, 1984).

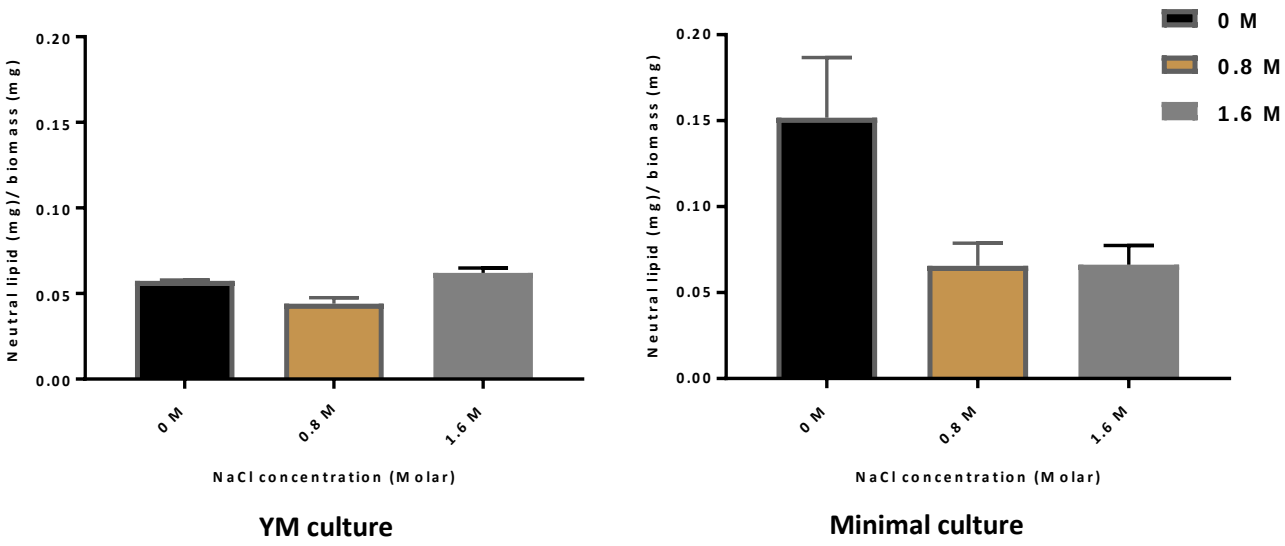


Figure 5.11. Neutral lipid concentration (mg) / (mg) biomass for *D. hansenii* growing in: YM medium, minimal medium with different concentrations of NaCl (0, 0.8 and 1.6 M) at late logarithmic phase. Bars represent standard deviations

The cell density was also studied in minimal and YM cultures with different salt concentrations, at the same time with neutral lipid measurements (Figure 5.12). The average of OD was 2.8 ± 0.01, 3.2 ± 0.02, and 0.8 ± 0.088 in 0, 0.8, and 1.6 M YM cultures respectively at late logarithmic phase. However, in minimal cultures the OD average of the samples in different concentrations of NaCl were 2.7 ± 0.025, 2 ± 0.056, and 0.8 ± 0.02 in 0, 0.8, and 1.6 M respectively at the late logarithmic phase.

5.2.4.3. Effect of different carbon/ nitrogen ratios on neutral lipid production

To determine the effect of carbon/ nitrogen ratios alongside the type of carbon source (glucose and glycerol) used on neutral lipid production in *D. hansenii*, experiments were performed using 3 x 250 ml shaking flasks containing 50 ml minimal medium with different carbon/nitrogen (ammonium sulphate) ratios.

5.2.4.3.1. Glucose/ Ammonium sulphate ratios

The data in Figure 5.13.A shown that after adaptation, the neutral lipid accumulation was raised significantly (P value ≤ 0.0003) inside the cell with increasing carbon concentration up to 48:1 C/N, along with one limited concentration of nitrogen source (ammonium sulphate). There was a slight decrease in the lipid accumulation at the 16:1 concentration, comparing with 8:1 C/N ratio. This decline might be because the yeast cells were adapted longer to the first concentration than the other ones. The increase in neutral lipid production was less significant (P value ≤ 0.0102) between the last two higher concentrations of glucose, and the maximum lipid accumulation was observed in 48:1 ratio which accumulated about 0.389 mg neutral lipid/ mg biomass (Figure 5.13.A).

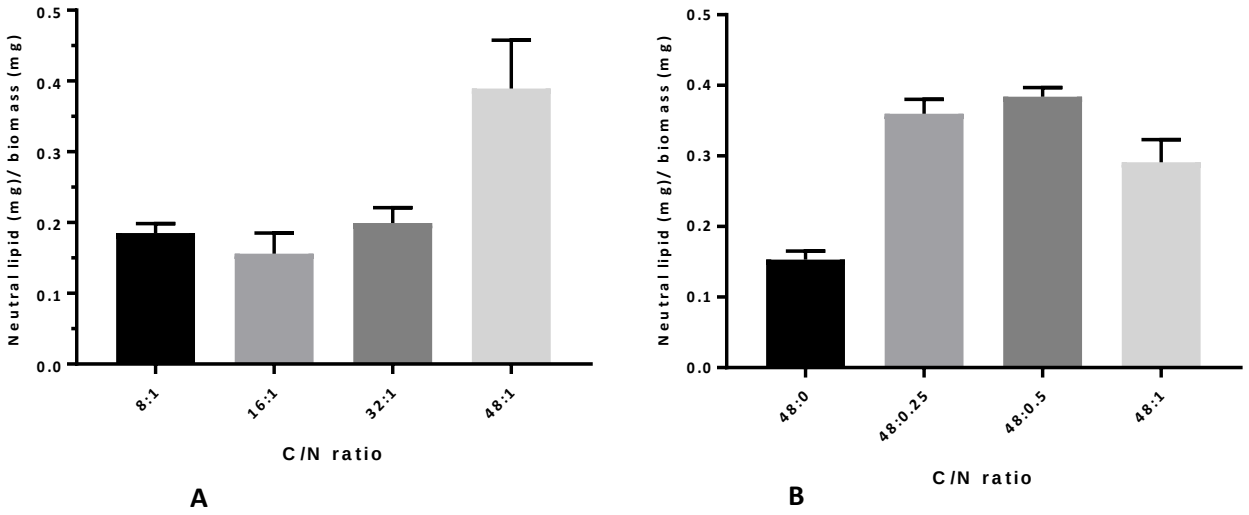


Figure 5.12. The effect of different concentrations of A - glucose and B - ammonium sulphate on neutral lipid production (mg)/ (mg) biomass of *D. hansenii* growing on minimal medium after 40 hours of incubation at 25°C under shaking. Bars represent standard deviation.

To monitor the effect of nitrogen concentration, cultures were prepared by using the optimum concentration of glucose with low amounts (48:1, 48:0.5, 48:0.25, and 48:0 C/N) of ammonium sulphate, due to previous research which referred to the positive effect of lower nitrogen concentration on neutral lipid production in yeast cells (Hall and Ratledge, 1977, Wynn et al., 2001). In Figure 5.13.B the data show that the 48:0.5 C/N ratio presented the highest amount of neutral lipid comparing with biomass, and it was slightly decreased at the 48:0.25 C/N ratio (non-significant variation). However, in the absence of nitrogen source, the amount of lipid was highly decreased in these cultures.

5.2.4.3.1.1. Changing the cells environment from high salt to the 48:0.5 glucose/ ammonium sulphate medium

Based on the results obtained, another method was attempted to optimize neutral lipid production by inoculating the 48:0.5 glucose/ ammonium sulphate medium with pre-adapted cells of *D. hansenii* grown in 1.6 M minimal medium. The hypothesis of this procedure is to exploit the storage glycerol produced in high salinity stressed cells into neutral lipids production after changing the growth environment directly from high salt into 48:0.5 glucose/ ammonium sulphate medium. After growing in new environment, the cells were harvested at different time of incubation in individual 3 x 50 ml 48:0.5 glucose/ ammonium sulphate medium. The results showed (Figure 5.14) that the neutral lipids production was significantly (P value ≤ 0.0001) increased through the time of incubation up to 0.4 mg lipid/ mg biomass after 24 hours of incubation in 48:0.5 glucose/ ammonium sulphate medium. On the other hand, the values of total osmolytes was 0 µg/ mg biomass directly after transferring the cells to the new environment, whereas it was about 518.4 µg osmolytes/ mg biomass when measured in 1.6 M NaCl culture. For that reason, and along with the non-highly significant amount of neutral lipids achieved in this long procedure, it was decided not to use the pre- stressed cells in high concentration of NaCl in the inoculation.

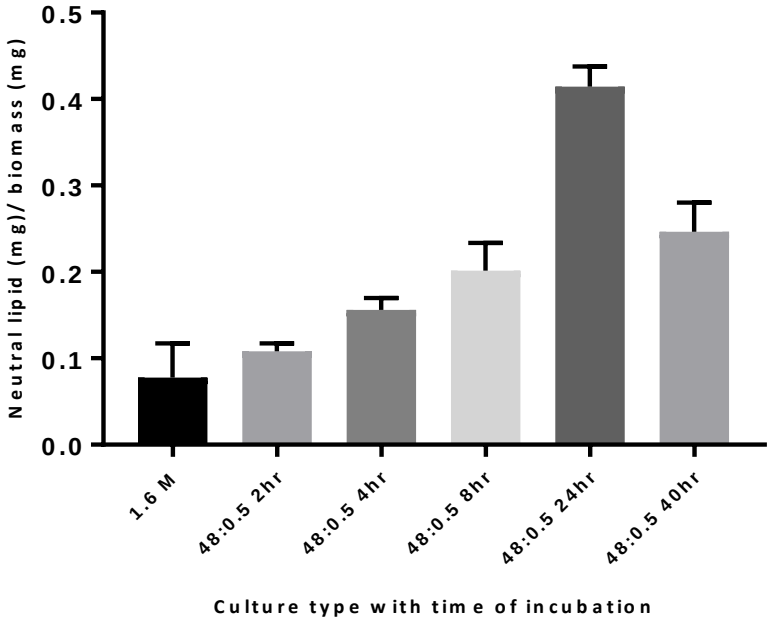


Figure 5.13. Neutral lipid production in *D. hansenii* cells after transferring the cells from 1.6 M NaCl minimal culture to the 48:0.5 glucose/ ammonium sulphate medium. The readings were taking after different time of incubation. Bars represent standard deviation.

.

5.2.4.3.2. Glycerol/ Ammonium sulphate ratios

From the data shown in Figure 5.15.A, the production of neutral lipid displayed significant (P value ≤ 0.0045) decreases with increasing amounts of glycerol as the only carbon source in the minimal medium. Lipid production patterens are shown to be different when compared with the cultures using glucose as the only carbon source (figure 5.13.A) with limited amount of ammonium sulphate. The maximum lipid production was observed at the lowest concentration examined for glycerol, giving the efficiency of *D. hansenii* cultures to accumulate neutral lipid at about 0.468 mg lipid/mg biomass.

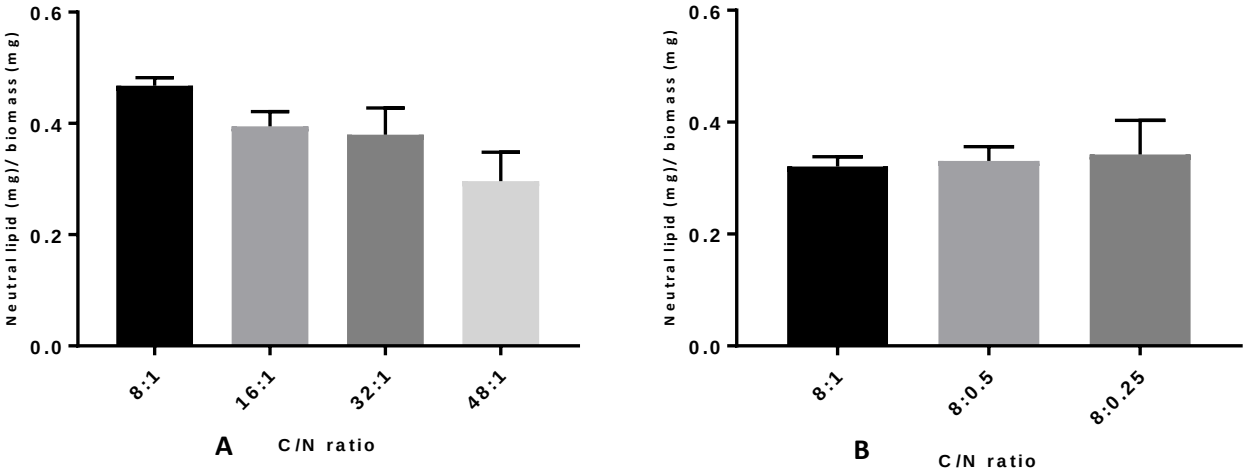


Figure 5.14. The effect of different concentrations of A- glycerol and B- ammonium sulphate on neutral lipid production (mg)/ (mg) biomass of *D. hansenii* growing on minimal medium after 40 hours of incubation at 25°C under shaking. Bars represent standard deviation

However, there was non-significant increase of neutral lipid compared with biomass, as the concentration of ammonium sulphate decreased in minimal cultures. The highest lipid accumulation was at 8:0.25 C/N ratio (Figure 5.15.B). In the medium with no nitrogen present, along with optimum concentration of glycerol, the yeast cells didn't grow, so we couldn't evaluate the lipid content, and the biomass in these cultures.

5.2.4.3.3. Comparing between the effect of 48:0.5 Glucose/ ammonium sulphate with 8:0.25 Glycerol/ ammonium sulphate ratio on neutral lipid production of *D. hansenii* cells.

The comparative experiment of neutral lipid accumulation under different cultivation conditions was conducted when the cells were cultured in minimal medium with 48:0.5 and 8:0.25 glucose/ ammonium sulphate and glycerol/ ammonium sulphate ratios respectively. After 40 hours incubation, lipid production was significantly (p value <0.0001) higher in the 48:0.5 glucose/ ammonium sulphate cultures which accumulated 0.33 ± 0.01 mg neutral lipid/ mg biomass (Figure 5.16). This value comprises 1.4-fold increase compared with 0.23 ± 0.006 mg lipid/ mg biomass produced in 8:0.25 glycerol/ ammonium sulphate ratio. In respect with the cell density it was found that there were similar values of culture absorbance at OD_{600} after 40 hours of incubation in both media. Consequently, there were no differences in biomass produced from both 48: 0.5 and 8:0.25 glucose/ ammonium sulphate and glycerol/ ammonium sulphate ratios after 40 hours of incubation respectively (Figure 5.17).

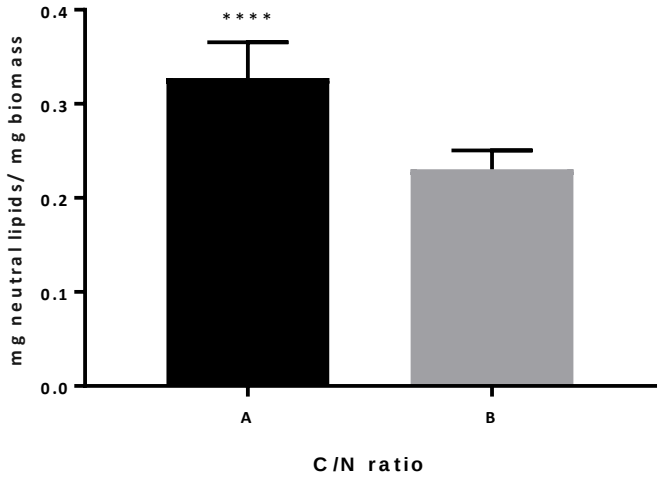


Figure 5.15. Neutral lipid production (mg) in *D. hansenii* cells comparing with biomass (mg) when the cells were grown in: A- 48:0.5 glucose/ ammonium sulphate ratio and B- 8:0.25 glycerol/ ammonium sulphate ratio of Minimal medium. Bars represent standard deviation.

Among numerous previous works on lipid production (Boulton and Ratledge, 1984, Hansson and Dostálek, 1986, Pan et al., 1986, Heredia and Ratledge, 1988, Jacob, 1991, Jacob, 1992, Johnson et al., 1992, Hassan et al., 1993, Saxena et al., 1998, Ratledge, 2004, Rau et al., 2005, Li et al., 2007b, Zhao et al., 2008) glucose is considered as the most commonly used carbon source for growth and lipid production in oleaginous fungi. High glucose concentrations improve the carbon flow that is directed toward the production of TAG, thus increasing lipid production in several yeasts (Li et al., 2007b).

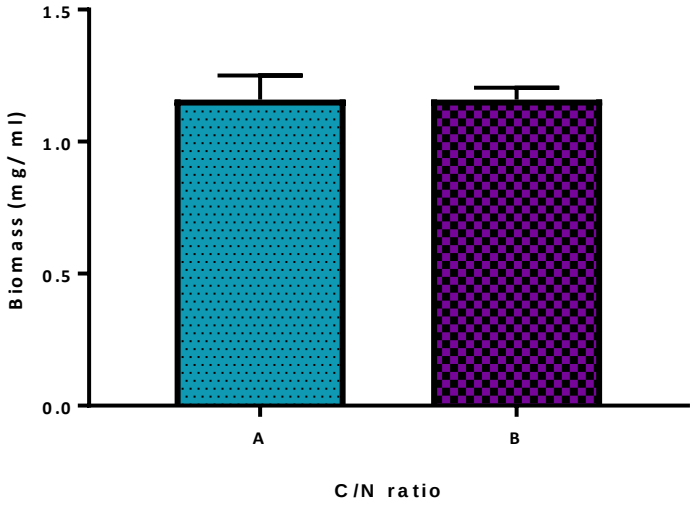


Figure 5.16. Biomass (mg) of *D. hansenii* cells growing in: A- 48:0.5 glucose/ ammonium sulphate ratio and B- 8:0.25 glycerol/ ammonium sulphate ratio of minimal medium. The cells harvested after 40 hours of incubation in each medium at 25 °C under 120 rpm shaking. Bars represent standard deviation.

5.2.5. Imaging of neutral lipid bodies within the cells by using fluorescence microscopy

Neutral lipid bodies have been studied using light and fluorescence microscopy to locate their position and intensity in some selected high lipid content *D. hansenii* cells growing in 1.6 M NaCl YM medium, 0 M NaCl minimal medium, 48:0.5 glucose/ammonium sulphate, and 8:0.25 glycerol/ ammonium sulphate ratios in minimal media. Based on the previous results that showed the highest content of neutral lipid was in the early

stationary phase, therefore lipid was visualised after 24 and 20 hours of incubation for 0 M minimal and 1.6 M YM cultures respectively. However, the rest of the images were taken after 40 hours of incubation. The cells were stained using the same conditions employed in the quantification method of neutral lipid production. The presented cells were selected from the photos captured by using an Axiovert 200M (Zeiss) fluorescence microscope.

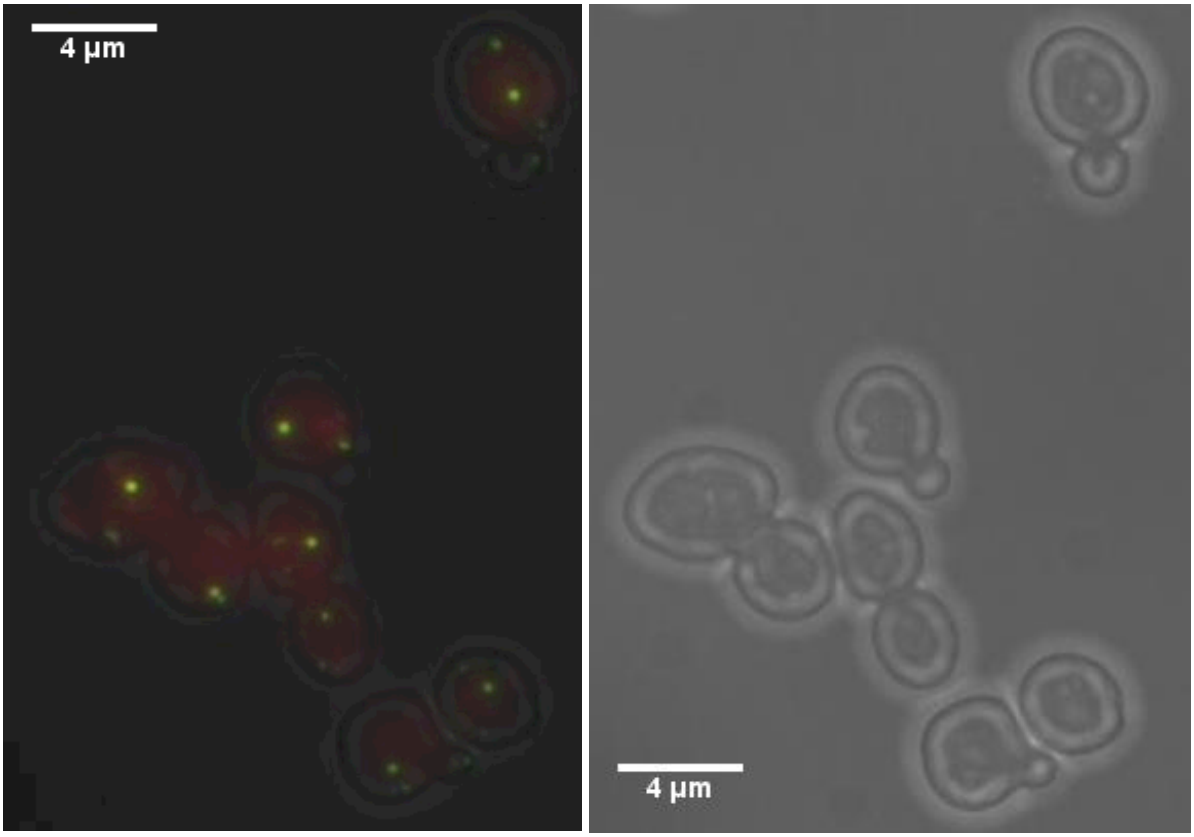


Figure 5.17. Fluorescence microscopy images of *D. hansenii* cells stained with Nile Red dye, the figures show cytoplasmic lipid droplets (yellow) from cells growing in 1.6 M YM medium after 20 hours of incubation at 25°C under shaking. Some cells contained two neutral lipid bodies.

The images showed that the highly lipophilic benzophenoxazone dye Nile red intercalated in lipid droplets and stained neutral lipids within the yeast cells. Among several dyes, Nile red seems preferable for determination of the intracellular lipid and its fluorescence

characteristics vary depending on the type of lipids present (Greenspan and Fowler, 1985). When examined following staining, neutral lipid bodies emit gold-yellow and green light after excitation at 450- 490 and 525 nm and 700 nm respectively. In some cells, the neutral lipids appeared to have a central location in the cytoplasm while in others they integrated within the cell membrane especially in the budding cells (Figures 5.18. and 5.19). This pattern of fluorescence was similar across the population of yeast cells indicated and observed for the presence of intracellular lipid bodies. In *S*. *cerevisiae* and *S*. *pombe*, (Rostron et al., 2015) reported that cells exhibited discrete fluorescent bodies, of varying sizes, within the cytoplasm when stained by Nile red dye and viewed under green fluorescence (EGFP filter Ex. 488/Em. 509 nm) at the stationary phase. In 0 M Minimal culture cells, neutral lipid bodies were visually larger than lipid bodies for cells growing in different conditions (Figure 5.19). In general, each conical to round shaped cell of *D. hansenii* has a single cytoplasmic yellow/ green neutral lipid body detected inside each cell. However, for the high salt concentration (1.6 M) YM cultures there were two lipid bodies inside a few cells growing in this medium (Figure 5.18). In oleaginous yeasts, the total lipid concentration and fluorescence intensity increased with an increase in the dry cell weight, following the glucose consumption from the culture broth, and appeared with different shapes and development, which depended on the species and culture condition (Kimura et al., 2004).

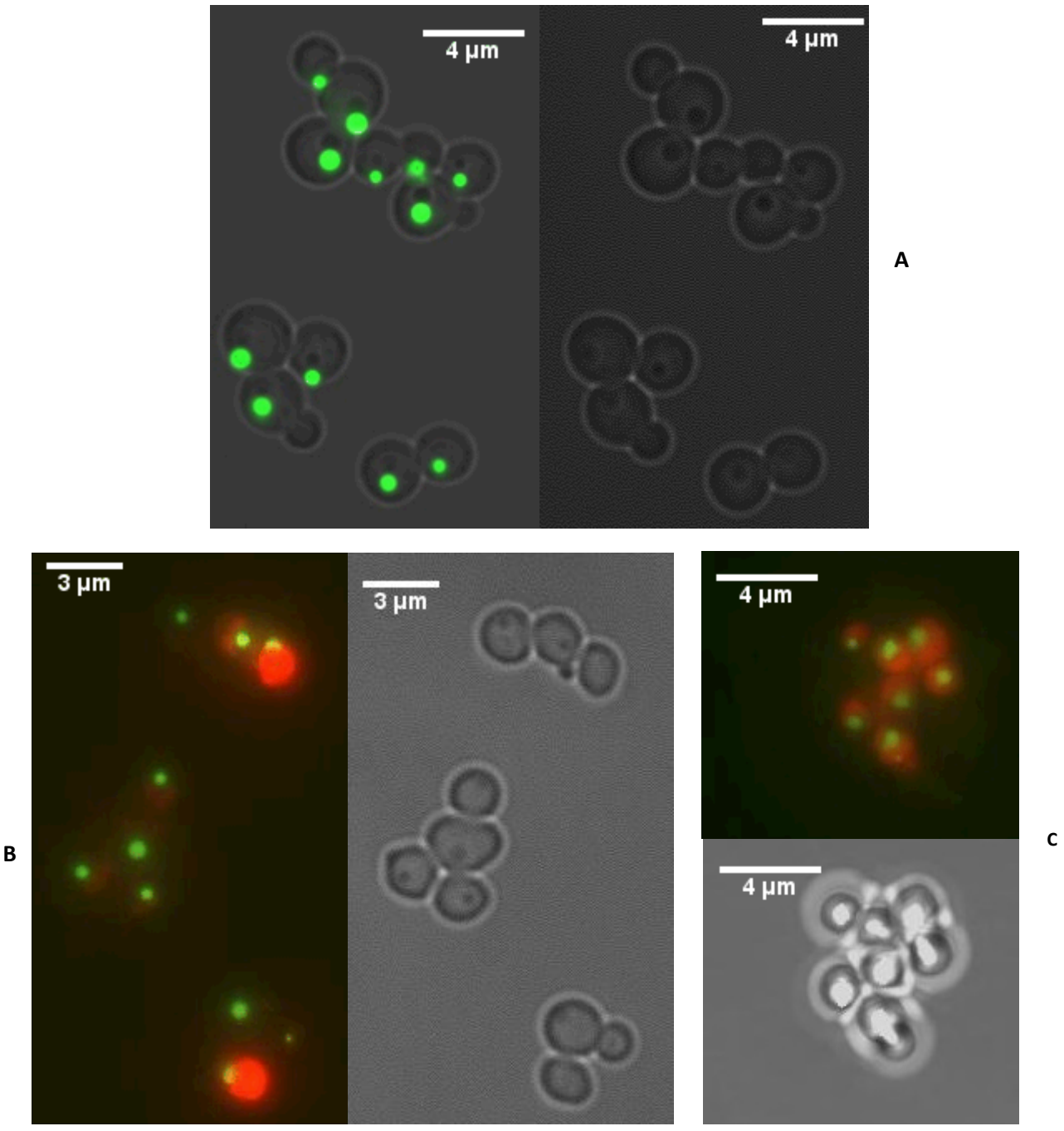


Figure 5.18. Fluorescence microscopy images of *D. hansenii* cells stained with Nile Red dye, growing in A-0 M NaCl, B- 8:0.25 glycerol/ ammonium sulphate, and C- 48:0.5 glucose/ammonium sulphate ratios Minimal media after 24, 40, and 40 hours of incubation respectively at 25°C under shaking. the figures show cytoplasmic lipid droplets (yellow). In 0 M NaCl Minimal medium cells appear with big neutral lipid bodies comparing with cells growing in another condition. In all three conditions cells introduce single and mostly centric lipid bodies.

5.2.6. Determination of total lipids as fatty acid methyl ester (FAME) in *D. hansenii* yeast under different stress conditions

To identify the fatty acid structure and content in *D. hansenii* cells under different stress conditions (NaCl stress and low amount of nitrogen source). 5 to 10 mg of freeze dried samples were transesterified by acid catalyzed reaction to fatty acid methyl ester (FAME) according to method described by (Van Wychen and Laurens, 2013a). Our study is the first report on the effect of stress conditions on fatty acids profile of *D. hansenii* strain depending on biochemical data. Fatty acid methyl esters (FAMEs) were analyzed by GC/MS using an Autosystem XL Gas Chromatograph (CHM-100-790, Perkin Elmer), which combined with a TurboMass Mass Spectrometer software (13657, Perkin Elmer). During the procedure, a mixture of chloroform and methanol was used to extract hydrophobic small molecules such as fatty acids, which are readily soluble in the organic phase. The addition of a methyl group work to decrease the boiling point and polarity of the molecules, so facilitating the analysis by GC-MS. FAMEs were identified by comparson with different concentrations of FAMEs standard (37 Component FAME Mix) and quantified by the internal standard methyl tridecanoate (C13:0 ME). Different fatty acids were positively identified, along with standards from this analysis, matching retention times of known standards and fragmentation patterns in the NIST database. GC peaks were labeled as follows: tetradecanoic acid (C13:0) at 8.4 min, tridecanoic acid (C13:0) at 12.5 min, palmitolic acid (C16:1) at 25.5 min, oleic acid (C18:1) at 36 min, and stearic acid (C18:0) at 37.6 min retention time (Figures 5.20, 5.21, and 5.22). The oil accumulated in yeast cells predominantly consists of oleic, linoleic (C18:2), stearic, palmitic or palmitoleic acids (C16:1) (Meng et al., 2009). These fatty acids were observed in all cultures with or without salinity/nutrient stress, based on their nearly similar retention time (Figures 5.20, 5.21, and 5.22).

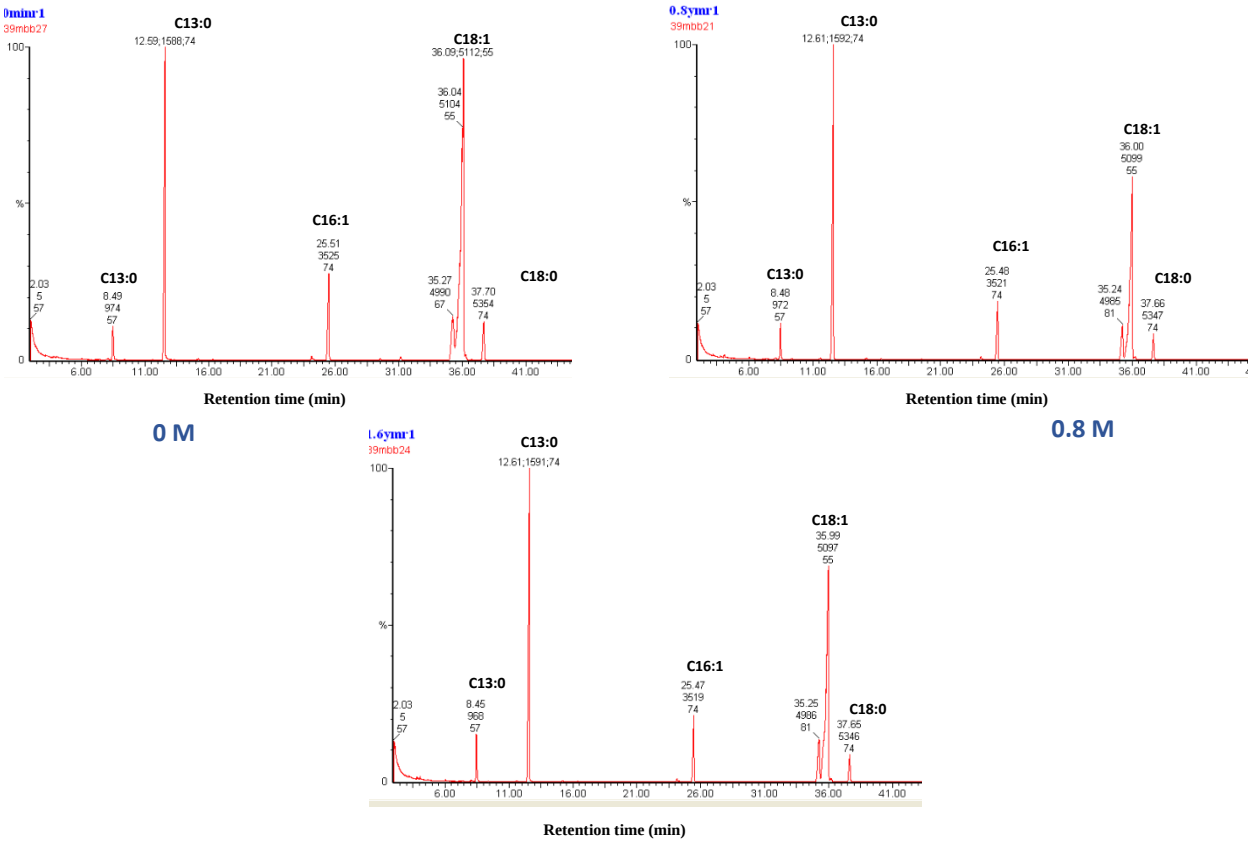


Figure 5.19. GC- MS analysis of fatty acids methyl ester (FAME) from *D. hansenii* freeze dried samples yielded from YM cultures with different concentration (0, 0.8, and 1.6 M) of NaCl. The samples collected from their cultures at different times depending on incubation periods for neutral lipid measurement. Each sample identified with three replicates.

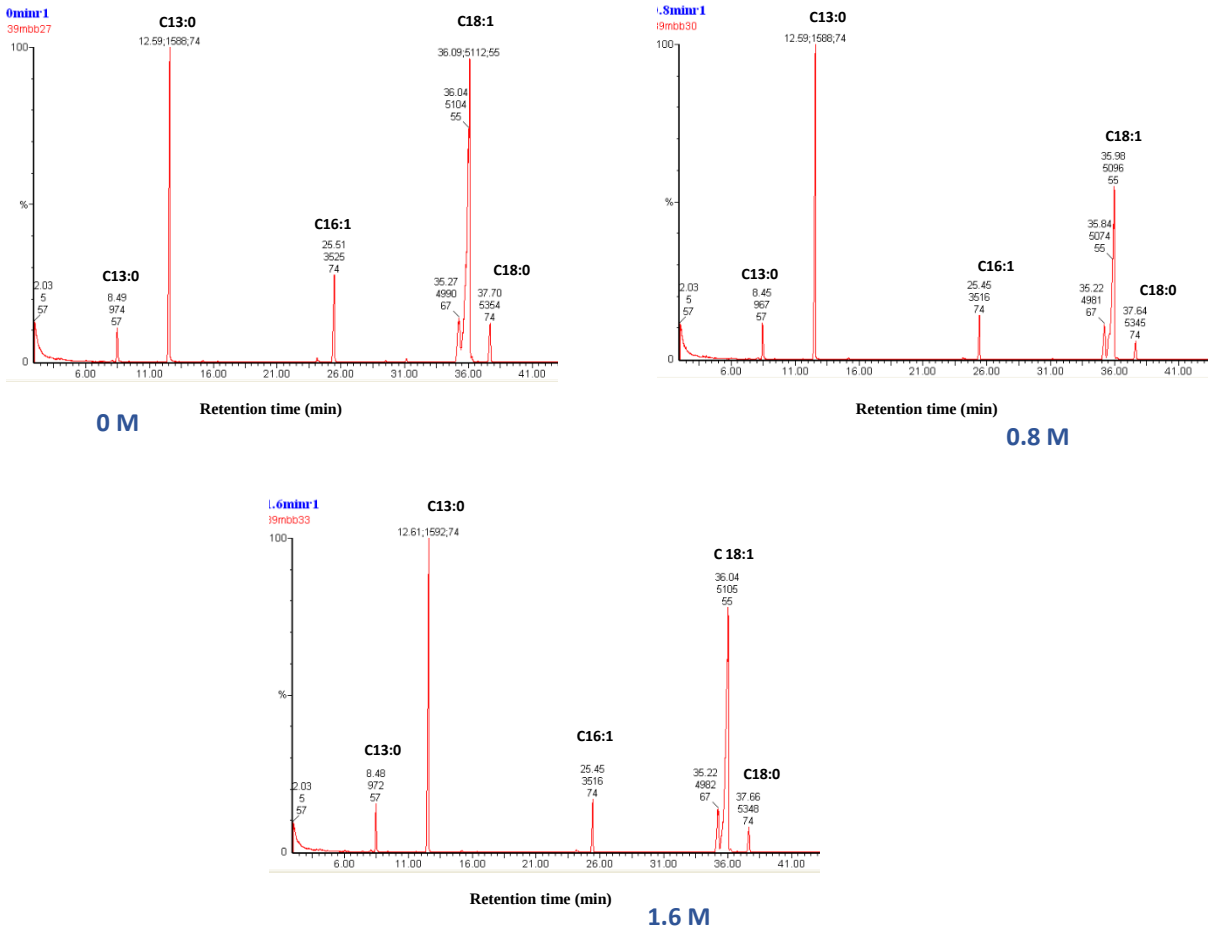


Figure 5.20 . GC- MS analysis of fatty acids methyl ester (FAME) from *D. hansenii* freeze dried samples yielded from minimal cultures with different concentration (0, 0.8, and 1.6 M) of NaCl. The samples collected from their cultures at different times depending on incubation periods for neutral lipid measurement. Each sample identified with three replicates.

Based on GC-MS chromatographs, the C18:0 peak height with retention time 37.6 min, appeared noticeably to vary between different samples. These variations could reflect the differences in the amount of fatty acids in each sample, especially for 0.8 and 1.6 M cultures which appeared smaller than other samples. Our results agreed with Ratledge and Hall (1979) who reported that the composition of fatty acids of total lipids didn't

change in response to nitrogen limited conditions, while slight changes were identified in carbon limited conditions (Ratledge and Hall, 1979). In contrast another study (Yoon and Rhee, 1983) mention that the fatty acid composition changed as the growth rate altered in response to nitrogen limited conditions. High salinity does not induce significant changes in the unsaturation of fatty acids in *Yarrowia lipolytica* (Andreishcheva et al., 1999), whereas decrease the content of sterol and phospholipid.

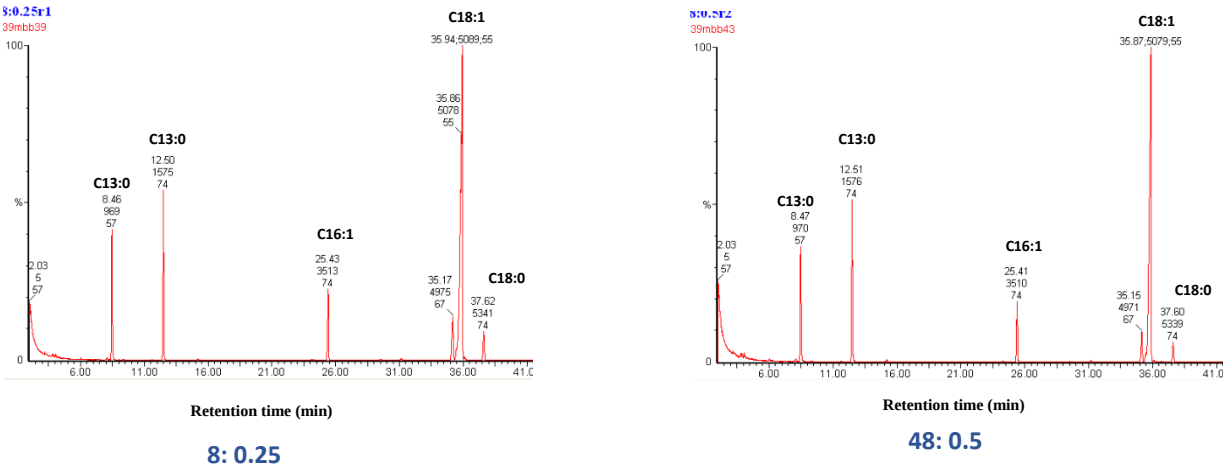


Figure 5.21. GC- MS analysis of fatty acids methyl ester (FAME) from *D. hansenii* freeze dried samples yielded from minimal cultures with different C/N ratios. 8:0.25 glycerol/ ammonium sulphate and 48:0.5 glucose/ ammonium sulphate. The samples collected from their cultures at different times depending on incubation periods for neutral lipid measurement. Each sample identified with three replicates.

Fatty acids are usually present as esters of glycerol (as in phospholipids and triacylglycerols) or sterol esters and the concentration and composition varies depending on yeast strain and alterations in the cultivation conditions. However, it is not surprising if there is low variation when different strains are grown under the same conditions in the same laboratory (Ratledge, 1989). Higher lipid yield and cellular lipid content were observed when inorganic nitrogen sources were used instead of organic sources.

Moreover the oleaginous yeast *Cryptococcus albidus* var. *albidus* CBS 4517 was able to accumulate lipid under limited and excess-nitrogen conditions, and the highest capacity was observed in nitrogen-limited medium (Hansson and Dostálek, 1986). The literature also refers to the increase in the unsaturation level of fatty acids in *Candida membranefaciens* (Khaware et al., 1995) as well as in the halophilic *Hortaea werneckii* and halotolerant *A. pullulans* when grown at high concentration of NaCl, whereas a slight decrease was observed in halophilic *Phaeotheca triangularis* (Turk et al., 2004) under salt stress condition.

5.3. Conclusion

In conclusion, after monitoring the growth curves of the selected strain *D*. *hansenii* in both minimal and YM media with different concentrations (0, 0.8, and 1.6 M) of NaCl, our results revealed that the total intracellular osmolytes increased due to osmotic stress condition. Their accumulation in YM cultures were higher than in minimal cultures. Under these conditions the main identified osmolytes in ^{1}H NMR analysis were glycerol, arabitol, glucose and trehalose. From the peak height for each polyol, the percentage of glycerol was notably increased in media with high concentration of NaCl, and it was higher in YM cultures than minimal cultures. From the obtained results, we concluded that the effect of high salinity on osmolytes accumulation in YM medium was much higher than in minimal medium. Along with intracellular osmolytes, neutral lipid production was also evaluated. The cells were harvested at the late logarithmic phase, which was found to be the optimal phase of TAG production. Neutral lipid production was found to be significantly higher in minimal medium than in enriched YM medium in different concentrations of NaCl. Whereas the highest intracellular polyols were in media with higher amount of salt, the maximum amount of neutral lipid appeared in minimal cultures without salt (0.152 mg neutral lipid / mg biomass). That means higher concentration of salt didn't enhanced TAG production even though it induced maximium amount of glycerol inside the cells. The production of TAG was optimized in minimal medium when *D. hansenii* cells grown in different carbon/ nitrogen ratios. The optimization was based

on the type and the economic value of the medium composition. Glucose and glycerol were selected as a fermentable and a non-fermentable carbon sources along with ammonium sulphate as nitrogen source. We achieved the best yield of neutral lipids when cells were cultured in medium consisting of 48:0.5 and 8:0.25 glucose/ ammonium sulphate and glycerol/ ammonium sulphate ratios respectively. However, neutral lipid production (mg / mg biomass) in medium with 48:0.5 glucose/ ammonium sulphate was significantly (p value <0.0001) higher than medium with 8:0.25 glycerol/ ammonium sulphate ratio. In all these stress conditions (high NaCl and limited nitrogen sources) we identified the fatty acids in freeze dried *D. hansenii* cells after transestrification with acid catalysis and methanol. The FAME GC- MS identification revealed that palmitic (C16:1), oleic (C18:1), and stearic acid (C18:0) were the main fatty acids found in FAME analysis, under normal and different stress conditions (high salt and limited nitrogen source).

Chapter Six: Genetic modification of *Debaryomyces hansenii* cells by deletion of the *GUT2* gene via homologous recombination

6. Genetic modification of *D. hansenii* cells by deletion of the *GUT2* gene via homologous recombination

6.1. Introduction

Recently metabolic and genetic engineering has established itself as applied technologies for biofuel development. Both tools have the ability to modify microorganisms with the aim to enhance their natural features, productivity, and even introduce new characteristics (Liang and Jiang, 2013). Most metabolic engineering basically depends on the introduction of a physiological stress such as nutrient-limitation, in particular nitrogen- or phosphorous-limitation, to channel metabolic fluxes to lipid accumulation in a microorganism (Beopoulos et al., 2009a). In contrast, genetic engineering approaches in oleaginous yeast have been largely confined to the well characterized oleaginous yeast *Yarrowia lipolytica* (Barth and Gaillardin, 1997) for which good genetic tools are available (Chen et al., 1997, Fickers et al., 2003, Yamane et al., 2008, Blazeck et al., 2011, Wang et al., 2011, Blazeck et al., 2013, Liu et al., 2014). Improvements in lipid production by *Y. lipolytica* have been achieved through genetic engineering. Dulermo and Nicaud (2011) increased lipid production two-fold by over-expressing the *GPD1* gene (encoding for glycerol-3-P dehydrogenase) and deleting the gene (*GUT2*) for the second isoform of this enzyme in a strain deficient in β-oxidation (*Δpox1-6*) (Beopoulos et al., 2008a, Dulermo and Nicaud, 2011). Tai and Stephanopoulos (2013) employed a "push-pull" strategy by over-expressing the first (acetyl-CoA carboxylase, ACC1) and last (diacylglycerol acyltransferase, DGA1) enzymes in the neutral lipid biosynthesis pathway, which increased lipid production five fold (Tai and Stephanopoulos, 2013). Only a few studies report genetic modification in *D. hansenii*. These studies made use of auxotrophic markers (Minhas et al., 2009). The work described in this chapter parallels similar efforts by extending the genetic toolbox in *D. hansenii* yeast. We are using the wild type NCYC102 strain, which lack any auxotrophic markers. Instead of using auxotrophic markers, we decided to use a dominant selectable marker that gives rise to resistance to an antibiotic. More specifically, a modified version of the bacterial transposon Tn1825 *SAT1* gene which

encodes streptothricin acetyltransferase was used. This enzyme inactivates the antibiotic nourseothricin. We replaced the *GUT2* gene via homologous recombination using the *SAT1* marker flanked by regions upstream and downstream of the *GUT2* gene. With this approach, we tried to increase the accumulation of triacyl glycerol by repressing the conversion of glycerol-3-phosphate into dihyroxyacetone phosphate via glycerol-3-phosphate dehydrogenase.

6.2. Results

6.2.1. *Debaryomyces hansenii* NCYC102 transformation

6.2.1.1. Nourseothricin antibiotic assay

The *SAT1* gene is a suitable marker for selection of recombinant transformants in a variety of yeast species (Reuß et al., 2004, Ding and Butler, 2007, Ueno et al., 2007, Millerioux et al., 2011, Kunigo et al., 2013). Since *D. hansenii* mistranslates CUG codons into serine, we need a version of the *SAT1* gene where the CUG codon was replaced by CUC. For this we used a *SAT1* cassette designed for genetic modification of *Candida albicans*. This cassette consists of the *SAT1* ORF and is controlled by the *CaACT1* promoter and first 15 codons including intron. The *SAT1* ORF is followed by the *CaURA3* terminator (Reuß et al., 2004)). This cassette has previously been shown to function in *C. glabrata* as well (Ueno et al., 2007).

In order to design a gene replacement cassette using *CaSAT1* as a dominant selectable marker, we first needed to test whether *D. hansenii* is sensitive to nourseothricin. 100 µl of exponentially growing WT cultures (OD 4.66) were plated onto agar plates containing a wide range of nourseothricin concentrations (0.1 to 300 µg/ml) and incubated at 25 °C for 5 days. There was no growth on the YM agar plates containing 1.5 µg/ml or higher. Based on these results (Figure 6.1) we initially selected 1.5 and 5 µg/ml as the selective condition for the growth of transformed yeast in YM medium. In previous studies that used the *SAT1* gene as a selectable marker, the concentration of nourseothicin used to select transformed colonies ranged from 10 -200 µg/ml depending on the organism (Reuß

et al., 2004, Ding and Butler, 2007, Ueno et al., 2007, Millerioux et al., 2011, Kunigo et al., 2013).

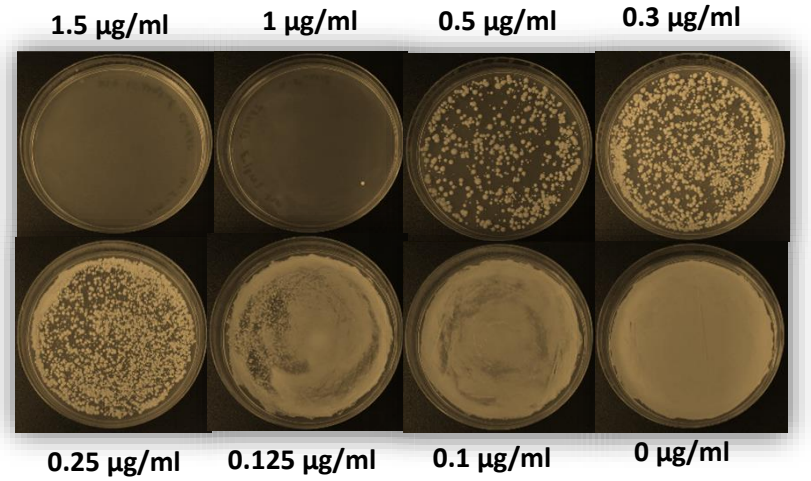


Figure 6.1 Effect of different concentrations of nourseothricin on the growth of *D. hansenii* after 5 days of incubation in YM medium plus antibiotic at 25 ˚C. Photos of higher than 1.5 µg/ml are not included due to no growth in these plates.

6.2.1.2. Development of gene replacement constructs for *DhURA3* and *DhGUT2*

After establishing that nourseothricin is a suitable antibiotic in *D. hansenii*, constructs were designed to disrupt the *URA3* and *GUT2* gene as described in materials and methods section 2.8.3.1 and 2.8.3.2). The DNA sequence of the insert of the pZA3 (for *URA3*) and pZA5 (for *GUT2*) construct were analyzed to confirm their design and were found to be correct. However, we decided that, since the orientation of the transcription of the *SAT1* gene was in the opposite direction to that of the target genes, we would also construct plasmids where the orientation of the *SAT1* gene was in the same direction to that of the target genes. For the design of these plasmids (see materials and methods section 2.8.3.2). This resulted in the plasmids pZA6 (for *URA3*) and pZA7 (for *GUT2*) (see Figure

2.5). To confirm the orientation of the *SAT1* cassette, two digestion reactions (*Xho*I-*BamH*I and *Pst*I-*Xba*I) were performed to differentiate between pZA5 and pZA6 (Figure 6.2). Agarose gel photo showed there was one band in the first two samples representing pZA6, while there were two bands (around 2500 and 3000bp) produced from the pZA5 sample, when it was digested with *Xho*1-*BamH*1 enzymes. This is exactly as we predicted for these constructs. For *Pst*1-*Xba*1 enzymes, both plasmids showed two bands as expected (Figure 6.2).

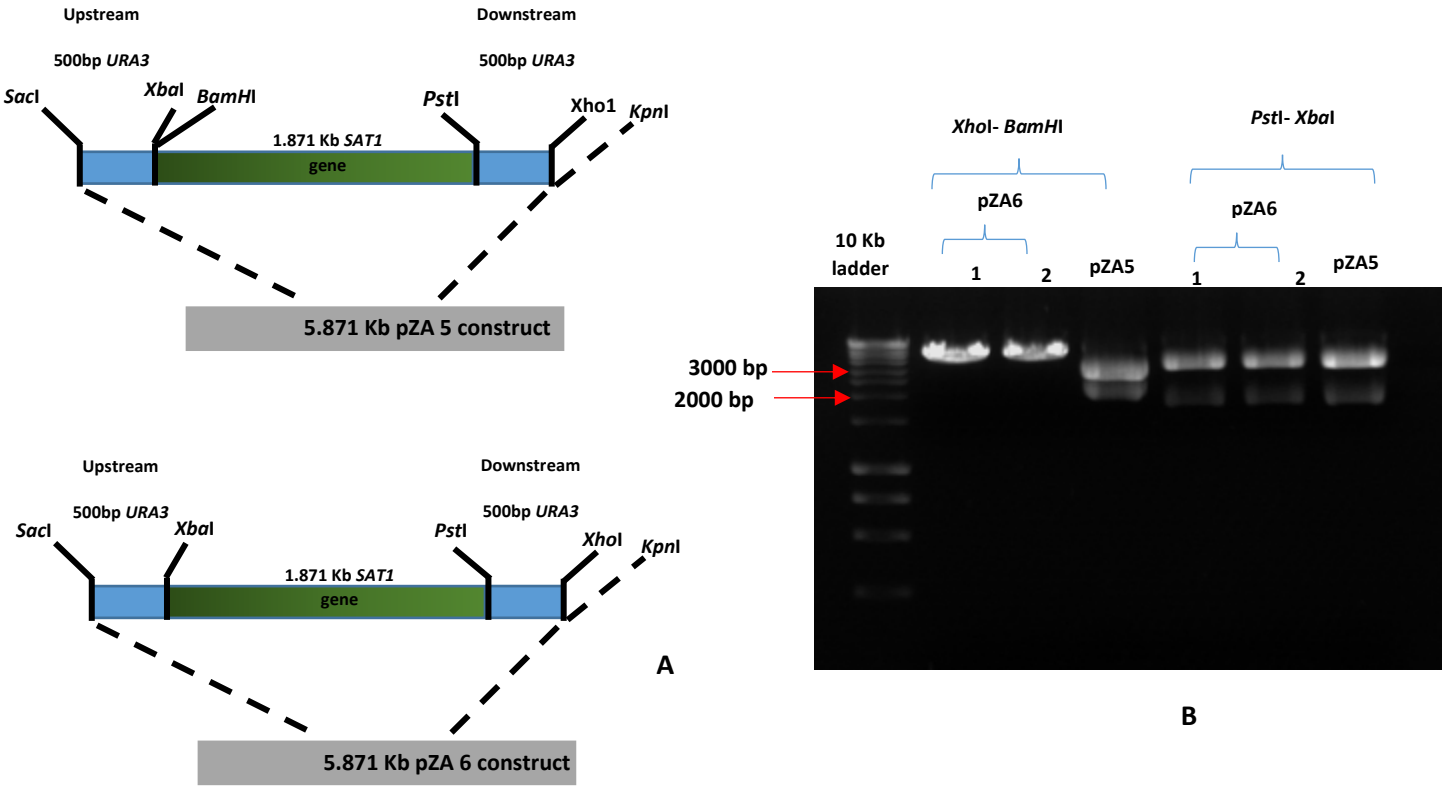


Figure 6.2 Analysis of *URA3* gene replacement cassette containing plasmids. A) Schematic representation of plasmids pZA5 and pZA6. B) 0.7% Agarose gel electrophoretic separation of plasmid fragments generated by digestion with the restriction enzymes *Xho*I- *BamH*I and *Pst*I- *Xba*I

To distinguish between pZA3 and pZA7 constructs, two double digestion reactions were set up by using the restriction enzymes *Sac*I-*Sal*I and *Sal*I-*Kpn*I. For the pZA7 construct, we expected to get insert bands of around 1200 bp when digested with *Sac*I-*Sal*I, and 1700 bp band with *Sal*I-*Kpn*I reaction. When pZA3 is digested with *Sac*I-*Sal*I and *Sal*I-*Kpn*I, the insert bands will be 1700 bp and 1200 bp, respectively as the orientation of the *SAT1* cassette is reversed in pZA3 compared to pZA7 (Figure 6.3).

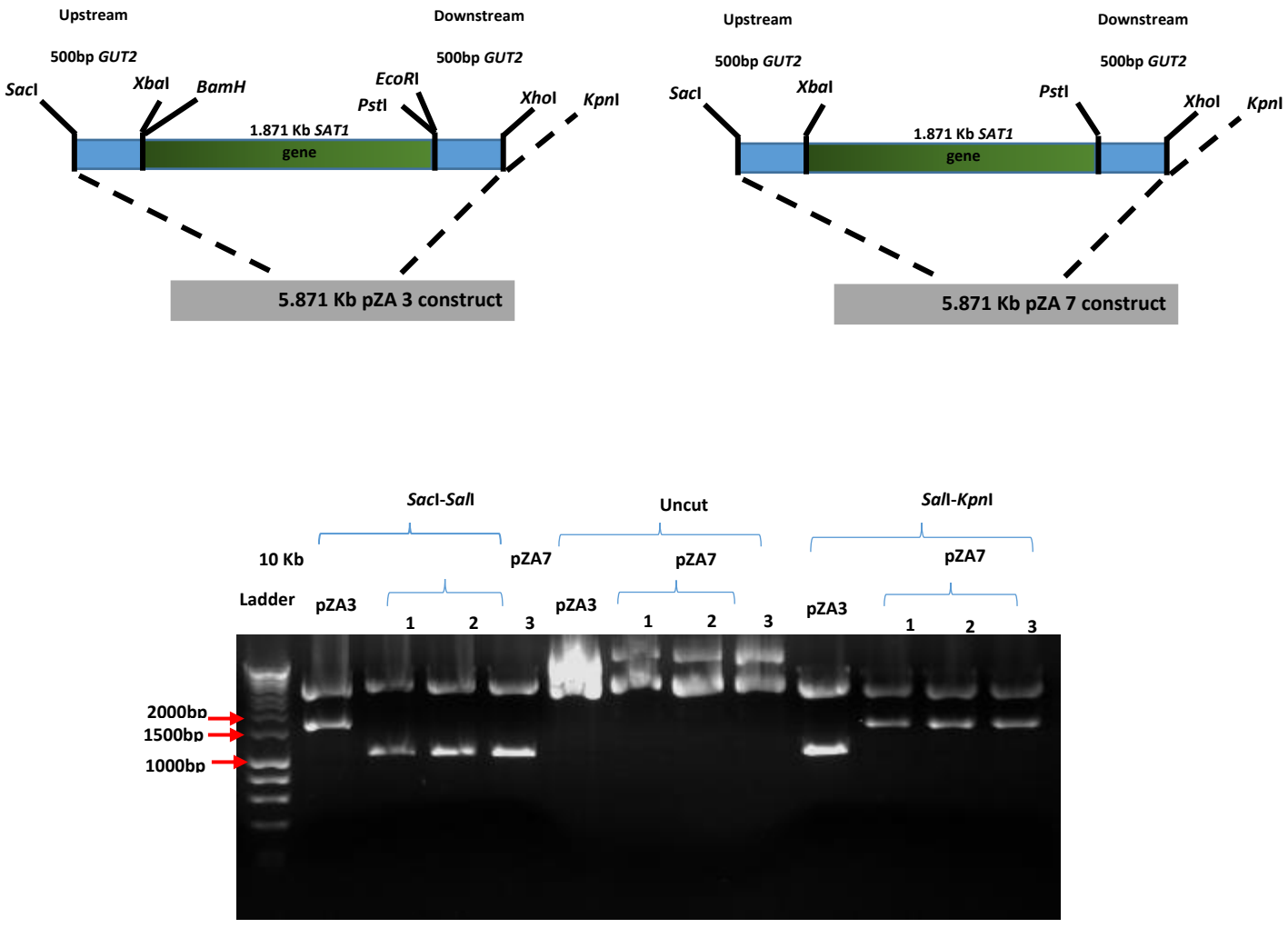


Figure 6.3 . Analysis of *GUT2* gene replacement cassette containing plasmids. Top- schematic diagrams of plasmids pZA3 and pZA7 with the sites of restriction enzymes used in the digestion reactions. Bottom- Gel electrophoresis was used to represent distinguish between pZA3 and pZA7 constructs depending on two digestion reactions comparing with uncut samples. The size of the bands in the 0.7% Agarose exchanged between those two constructs when used *Sac*I-Sal1 and *Sal*I-*Kpn*I enzymes.

6.2.1.3. *Debaryomyces hansenii* transformation by electroporation

In order to transform cells with the gene replacement cassettes, the cassettes were first amplified by PCR (see materials and methods Figure 2.6). Yeast cells were transformed with 2.0 and 3.4 µg PCR product for *URA3* and *GUT2*, respectively. Following electroporation, cells were recovered in YM medium augmented with 1 M sorbitol as osmotic stabiliser instead of sucrose, according to previously published protocols (Minhas et al., 2009). Electroporation of the *URA3* and *GUT2* gene replacement cassettes resulted in high numbers of transformants (>1000), on agar plates with 1.5 and 5 µg/ml nourseothricin whereas no colonies could be seen in control electroporated without DNA. To check whether *URA3* was deficient in any of selected colonies, cells were plated onto minimal medium without uracil by using replica plating. The result showed that all colonies grew well in this medium after 48 hours of incubation at 25˚C, which indicates that the *URA3* gene is still available in these cells. To confirm this result, cells were plated on medium containing 5-Fluoro-orotic acid (5-FOA), and none of the selected colonies grew. This indicates the *URA3* gene product (orotine-5-monophosphate decarboxylase) is still active in these transformants as they still produce the potent inhibitor of thymidylate synthase, fluorodeoxyuridine, and therefore fail to grow. The failure of obtaining URA3 deficient cells may be because there is more than one copy of *URA3* gene, or the gene replacement cassette inserted randomly inside the genomic DNA. For cells with a GUT2 gene deletion as a consequence of transformation with *GUT2* gene replacement cassette, we expect no clear phenotype. One reliable way to test for the replacement of the *GUT2* gene by *SAT1* is via PCR on genomic DNA (Figure 6.4) of selected cells. First, we tested for disruption of *GUT2* by using a PCR with a primer inside the *SAT1* ORF and a primer that anneals 200 bp outside the cloned regions flanking the *GUT2* gene (Figure 6.4.1.A). An amplified product of 816 bp for the 5' side and 853 bp for the 3' side will be seen only in the case of the *GUT2* gene is disrupted by *SAT1* marker. Subsequently, another PCR reaction was set up that uses primers that anneal inside the transformed construct (Figure 6.5.1.B). This latter PCR confirms the presence of the SAT1 gene. Eight single selected colonies along with wild type *D. hansenii* cells were tested as described above. In three

out of the eight selected colonies, the *GUT2* gene has been replaced by the *SAT1* cassette. Agarose gel photos revealed that, there were clear bands of the expected size in three transformants with PCR A and B (Figure 6.4.2).

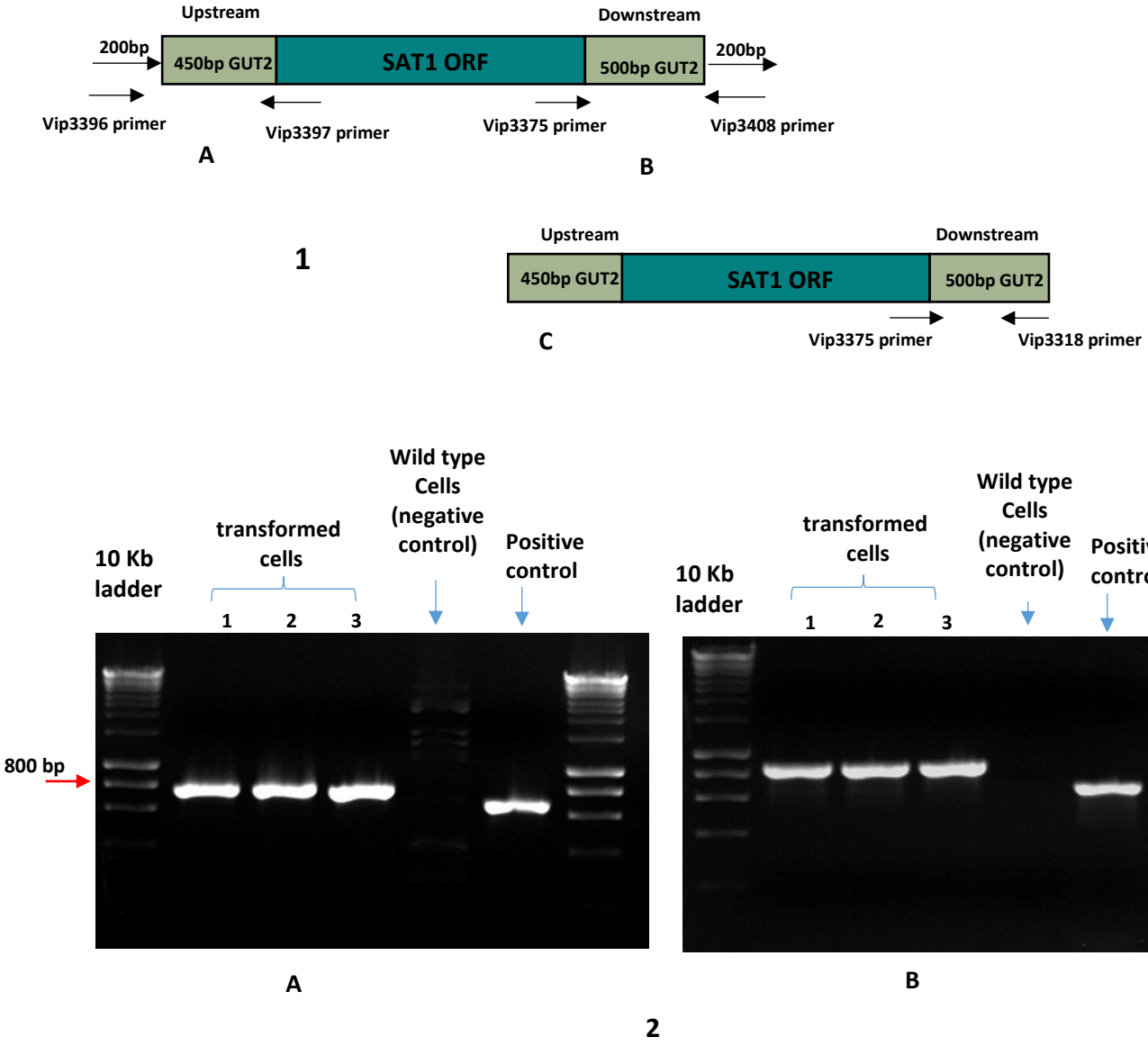


Figure 6.4 Diagnosis of the replacement of *GUT2* gene with SAT1 construct via homologous recombination. 1: Schematic diagram of A and B PCR reactions inside and outside the upstream and downstream *SAT1* cassette respectively. 2: Gel electrophoresis images of A and B PCR reactions. Three clear bands with 816 bp and 853 bp produced from the PCR reaction A and B respectively. No band present from the PCR of wild type strain (negative control), and the positive control was performed inside the construct (reaction C of PCR).

To confirm the replacement of the *GUT2* gene, another PCR was performed to test for the absence of *GUT2* ORF in those selected cells (see Figure 6. 5. A). This PCR was also carried out in the genomic DNA of the wild type cells as a control. Gel electrophoresis image (Figure 6. 5. B) shows no bands at the expected length (987 bp) in transformants. These results convincingly indicate that the *GUT2* gene has been replaced by homologous recombination with the *SAT1* cassette.

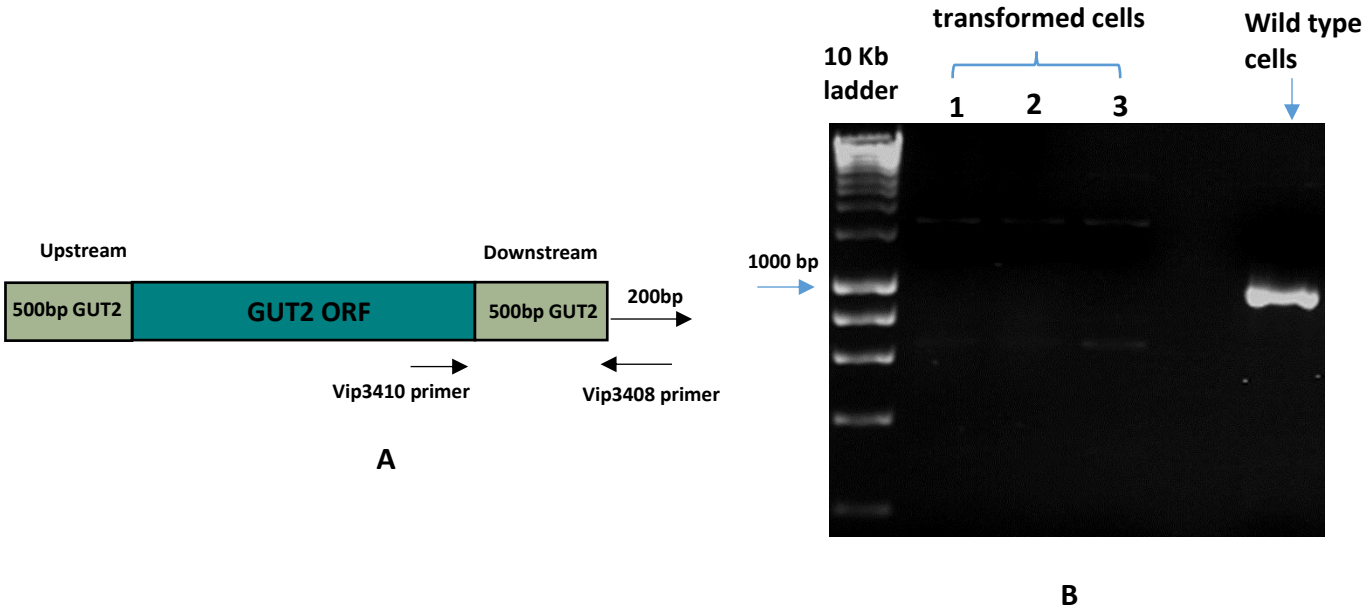


Figure 6.5 . Diagnosis of the availability of *GUT2* gene in three selected single colonies transformed with *SAT1* construct. A: schematic diagram of PCR inside and outside the 700 bp downstream the *GUT2* gene. B: Gel electrophoresis image of PCR reactions confirmed *GUT2* knockout in the gDNA of three transformed cells of *D. hansenii*. The images revealed no bands for PCR in transformed cells at 987 bp when using primers inside and outside the *GUT2* compared with wild type cells which have clear band at that size.

6.2.2. Lipid accumulation is affected in a *GUT2* gene deletion mutant

Subsequently, we investigated the effect of a *DhGUT2* deletion on the level of neutral lipid production. Both wild type (clone 1, 2, and 3) and *gut2*Δ cells (*gut2*Δ*.1-3*) were grown in minimal medium with 48 glucose: 0.5 ammonium sulphate ratio (C/N). Another set of mutant and wild type cells were grown in minimal medium with 8 glycerol: 0.25 ammonium sulphate (C/ N) ratio. The values of neutral lipid of the mutant cells were compared with the values of the wild type *D*. *hansenii* cells. The mutant strains failed to grow in minimal medium with glycerol as the only carbon source, as gluconeogenesis is disrupted due to a block in the conversion of glycerol 3-phosphate into dihydroxyacetone phosphate (see Figure 6.6) although growth was unaffected on minimal medium with glucose as sole carbon source in the presence of high salt (0.8M, 1.6M NaCl).

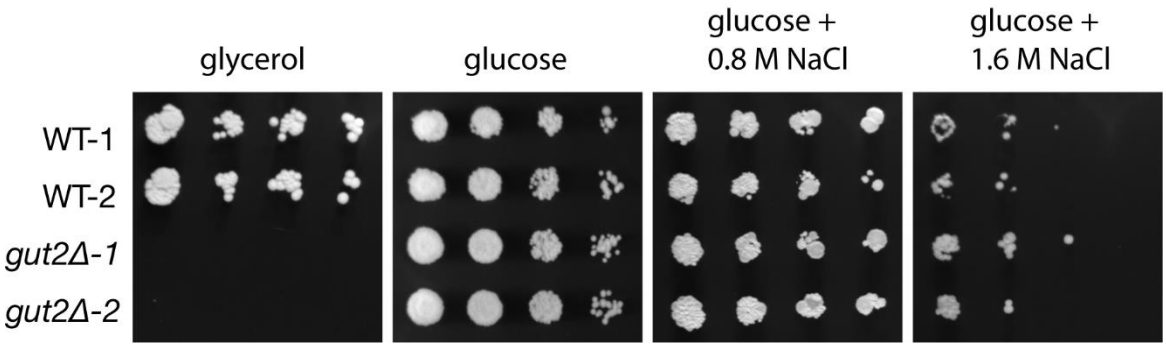


Figure 6.6 Images of the spot growth of different dilutions of wild types and mutant strains after 6 days of incubation on normal minimal medium that containing glucose as a sole carbon source along with different concentrations (0, 0.8, 1.6 M) of NaCl and glycerol based minimal medium. For the 0 M glucose based medium the photo was taken after 2 days of cultivation at 25 ^{o}C.

For the neutral lipid production, the results (Figure 6.7) showed that it was significantly (P value ≤ 0.0001) higher in all three transformed cells compared with wild type strains. The maximum quantity of neutral lipid was observed in the *gut2*Δ (1) strain which produced 0.46 mg neutral lipid/ mg biomass after 40 hours of incubation at 25 ^{o}C under shaking. The average of neutral lipid production of all three mutant strains was 44.3% ± 0.01271 of the cellular dry weight (DW) compared to 32.2% ± 0.008 of the three wild type clones of *D. hansenii* NCYC102, which correspond to a 1.4-fold increase in the mutant cells (Figure 6.8). With respect to cell growth, the Δ*gut2* strains grew to a slightly higher density in stationary phase. The OD_{600} of mutant cells reached up to 3 ± 0.14 in comparison to 2.7 ± 0.11 of the wild type strains after 40 hours of incubation in the same medium at 25 ^{o}C under shaking. This difference is non-significant (P<0.2). Our results were slightly higher than the values of previous work by Beopoulos (2008). This work achieved 8.7% lipid accumulation in *gut2*Δ cells of *Y. lipolytica* (JMY1202) compare to 7.0% for the wild type strain (JMY330) of the cell DW after 11 hours of incubation in glucose based medium (YNBD). This corresponds to 1.2-fold increase (Beopoulos et al., 2008a). As in *D. hansenii*, the *Y. lipolytica gut2*Δ strain showed a slightly higher density of cells after 11 h (late logarithmic phase) of growth compared to the control strain.

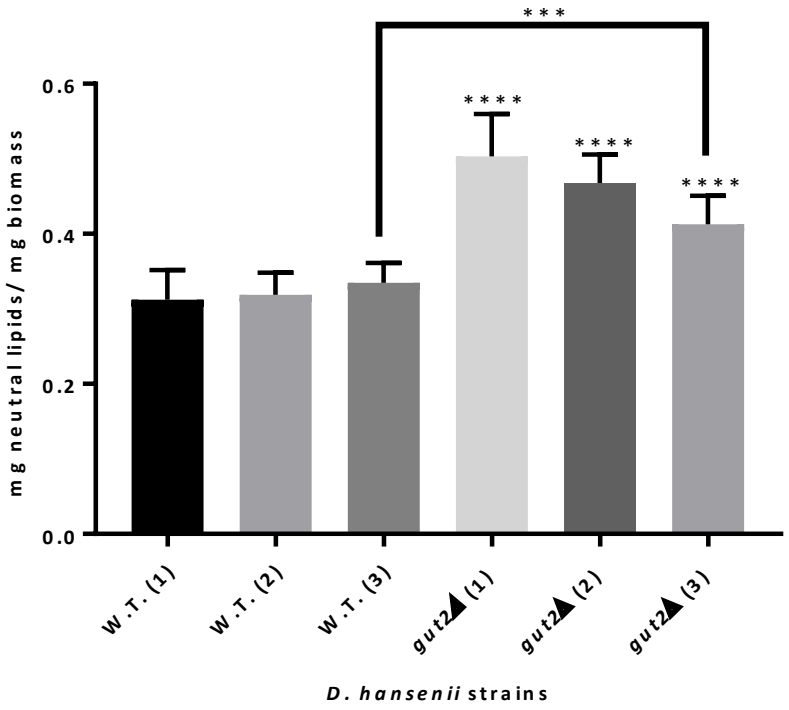


Figure 6.7 Neutral lipid production (mg) / biomass (mg) in three single mutant strains: *gut2*Δ (1), *gut2*Δ (2), and *gut2*Δ (3) of *D. hansenii* growing in minimal medium at 48 glucose: 0.5 ammonium sulphate ratio. The value of each mutant strain was compared with the value of each three single wild type strains of *D. hansenii* growing in the same conditions after 40 hours of incubation at 25 ^{o}C under shaking. The figure shows highly significant differences of neutral lipid production between the mutant and wild type strains. There are less significant differences between *gut2*Δ (3) mutant strain and number 3 wild type strain.

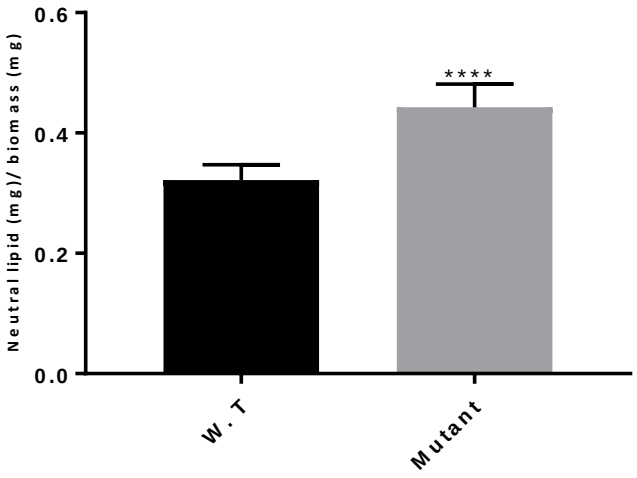


Figure 6.8 The average of neutral lipid production (mg) / biomass (mg) of three wild types and three mutant strains for *D. hansenii* growing in minimal medium at 48 glucose: 0.5 ammonium sulphate ratio, after 40 hours of incubation at 25 C under shaking.

For more clarification of the cell growth after genetic modification, we measured the OD_{600} of the wild type and mutant cell cultures at different time of incubation in minimal medium at 25 ^{o}C under shaking. From the figure below (Figure 6.9) the mutant cells started with a slightly longer lag phase while it appears to grow slightly faster after 10 hours of incubation compared with the wild type strain.

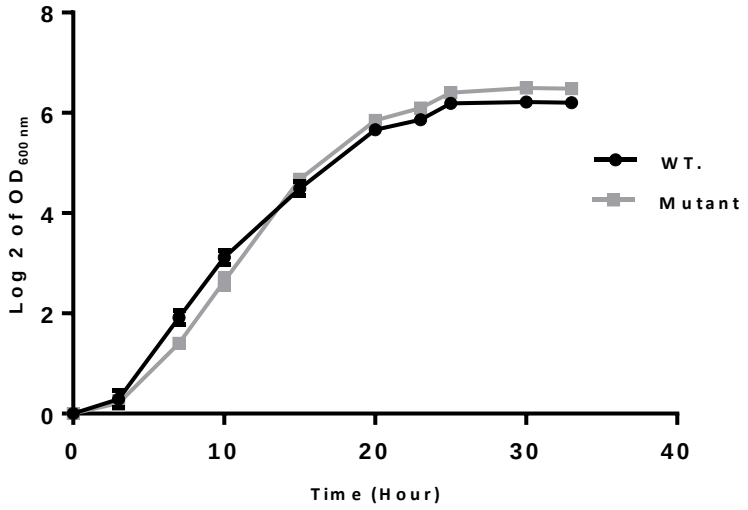


Figure 6.9 Growth of wild type and mutant stains of *D. hansenii* cells growing in minimal medium at 25 °C under shaking at 150 rpm.

6.2.2.1. Neutral lipid production in Δ*gut2* and wild type strains of *D. hansenii* NCYC102 grown under different C/N ratios

According to the previous results (Section 6.2.2) that confirmed the inability of *gut2*Δ strains to grow in glycerol as a sole carbon source, while their densities were slightly increased in glucose base medium, an experiment was performed to investigate the effect of using medium containing both glucose and glycerol on neutral lipid production. Using glucose in media containing specific amount of glycerol was to support cell growth of Δ*gut2* strain due to the disruption of gluconeogenesis in these cells. Lipid accumulation was investigated in medium containing different C/ N ratios of glucose: glycerol: ammonium sulphate (4:4:0.5 and 8:8:0.5) and compared with 48: 0.5 as well as 8: 0.5 ratios of glucose/ ammonium sulphate media. The results showed (Figure 6.10) that neutral lipid production in *GUT2* mutant strain was significantly higher than wild type strain in media containing glucose as the sole carbon source. However, there was only slightly increase in minimal medium mutant cultures containing glucose and glycerol

compared with wild type strain. According to these results, it's obvious that glucose presented higher amount of lipids when used as a sole carbon source for *gut2*Δ cells, although there was no significant variation between 8:0.5 and 4:4:0.5 glucose: ammonium sulphate and glucose: glycerol: ammonium sulphate ratios respectively related to this strain. On the other hands, in the wild type strain neutral lipid production in glucose and glycerol based media were higher than media consisting of glucose as a sole carbon source. The increase in lipid accumulation was significant in 4:4:0.5 glucose: glycerol: ammonium sulphate medium compared with 48:0.5 (P value= 0.002) and 8:0.5 (P value= 0.0043) glucose: ammonium sulphate ratios. In terms of cell density, the *gut2*Δ strain grew slightly higher than wild type strain in all types of media and there were no significant differences in the cell densities for the individual strain among different types of media investigated in this experiment.

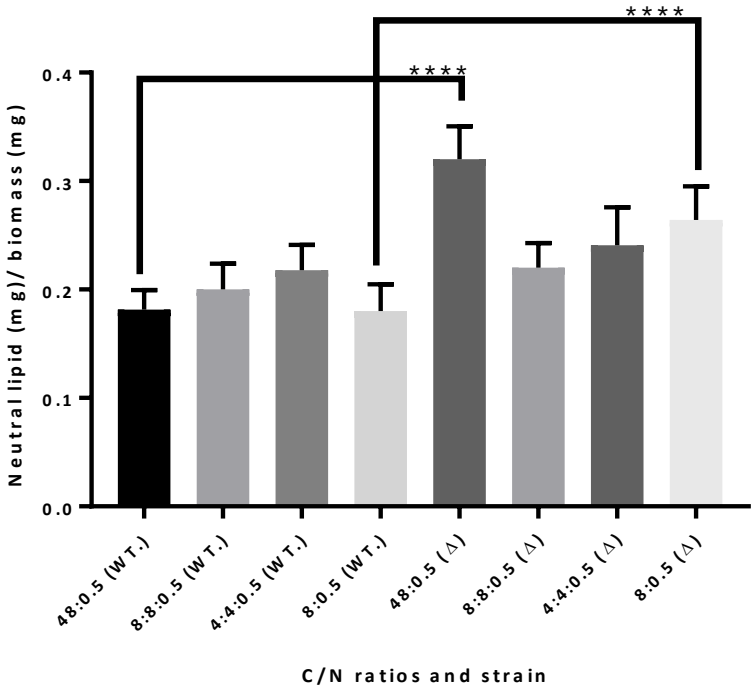


Figure 6.10 Neutral lipid production (mg) / biomass (mg) in Δ*gut2* and wild type strains of *D. hansenii* NCYC102 growing in C/ N ratios of glucose: glycerol: ammonium sulphate (4:4:0.5 and 8:8:0.5) and compared with 48: 0.5 as well as 8: 0.5 ratios of glucose/ ammonium medium. the results were taking after 40 hours of incubation at 25 C under shaking.

6.3. Conclusions

In this chapter, we aimed to increase lipid accumulation by deletion of the *GUT2* gene that encodes the FAD-dependent G3P dehydrogenase that catalyzes the conversion of G3P to DHAP. We hypothesized that a block in G3P oxidation, would increase the availability of G3P for TAG biosynthesis. We attempted to replace the *GUT2* gene with the *SAT1* selectable marker through homologous recombination. Our results indicate that *D. hansenii* strain (NCYC 102) is highly sensitive to the nourseothricin antibiotic when compared with the sensitivity of other yeast strains (*Candida guilliermondii* (Millerioux et al., 2011) and C. *albicans* (Reuß et al., 2004) grown on the same antibiotic. Based on our results 1.5 and 5 µg /ml concentrations of nourseothricin were chosen as a selectable marker to test the growth of transformed yeast cells in YM agar plate. The *D. hansenii* NCYC 102 strain was successfully mutated by the mechanism of homologous recombination using the *SAT1* marker flanked by fragments comprising 450 bp upstream and 500 bp downstream of *GUT2* ORF. The *GUT2* gene deletion construct was transformed by electroporation by adapting a previous protocol (Minhas et al., 2009). Since the *gut2*Δ mutant strains were unable to grow on glycerol as the sole carbon source, we assume that the reduced conversion of G3P into DHAP under these physiological conditions results in a block in gluconeogenesis ((Beopoulos et al., 2008a) and this work). Compared to the wild type strains, the *gut2*Δ mutant strains demonstrated a 1.4-fold increase in the accumulation of neutral lipids after 40 hours of incubation in minimal medium with a 48:0.5 glucose/ ammonium sulphate ratio. This increase was highly significant. In addition, growth of the *gut2*Δ mutant strain in the same medium reached a higher cell density compared to the wild type strain. Therefore, we conclude that the *gut2*Δ mutant has an elevated production of neutral lipids both per biomass and per volume culture. The results also suggest that glucose as a sole carbon source induced more neutral lipid production in the mutant strain compared with media including both glucose and glycerol in their components.

Chapter Seven: General conclusions and future work

7. General conclusions and future work

7.1. General conclusions

In conclusion experiments presented in this thesis demonstrated that the yeast *Debaromyces hansenii* NCYC102 was able to grow in medium with high concentration of NaCl reaching up to 2.4 M, and with limited growth at 2.8 M. From the ^{1}H NMR spectrograph, the osmolyte profiles of *D. hansenii* cells grown under NaCl stress were generally found to have fewer notable peaks when compared to yeasts grown without stress. Identification of the main peaks revealed that the main osmolytes identified by NMR spectroscopy were glycerol, arabitol, glucose and trehalose. These osmolytes worked to adjust the intracellular osmotic balance in the cells growing in high salt with the external environment (Gacto et al., 2003). The percentage of glycerol produced in media with high salt reached up to 80% and 79% in 0.8 and 1.6 M YM cultures respectively. Although, the glycerol quantity was increased inside the cell in high salinity, neutral lipid amount (mg/ mg biomass) in 0 M minimal medium was significantly higher than in medium with high salt (0.8 M and 1.6 M NaCl) when the readings were taken in the late logarithmic phase. In such conditions, the cell might be kept these solutes in particular glycerol to maintain the osmotic balance inside the cells rather than exploit it in neutral lipid synthesis.

Further experiments to enhance neutral lipid accumulation were achieved through optimizing the carbon/ nitrogen ratios in minimal medium with glucose or glycerol (commercially cheap carbon source) as the sole carbon source along with ammonium sulphate as nitrogen source. Medium with 48 glucose: 0.5 ammonium sulphate produced the maximum amount of neutral lipid that constitutes a 1.4 fold increase compared with the neutral lipid yield in medium with cheaper compound of carbon source (8 glycerol: 0.25 ammonium sulphate). So, with regard to economic cost we can consider glycerol as a potentially suitable carbon source to increase TAG accumulation in *D. hansenii* cells. In the present study, the effect of growth conditions (high salt and limited nitrogen source) on the fatty acid composition of TAG, were also investigated. GC-MS analysis revealed

that the profile of all these fatty acids were similar in all samples even they grown under different stress conditions. From all the results obtained it was found that *D. hansenii* could be one of the most preferable and viable sources for biodiesel production. In agreement with previous studies C16 and C18 long chain fatty acids, were shown to be the major lipid component in yeast cells, and would produce similar composition of biodiesel to that produced from either plant or animal based feedstock (Gill et al., 1977, Hall and Ratledge, 1977, Blagovic et al., 2001). In medium with limited nitrogen source, the quantity of these two fatty acids increased under normal cultivation conditions (Brown and Rose, 1969).

In respect of molecular modification to improve neutral lipid production in *D. hansenii* NCYC102, research in this yeast has not been sufficiently boosted, due to the limited molecular tools available to manipulate this yeast (Desnos-Ollivier et al., 2008). A previous study of *D. hansenii* by Minhas (2009) in gene disruption by homologous recombination was based on a histidine auxotrophic recipient strain (Minhas et al., 2009), along with protocols of gene deletion of the closely related wild type strain *Candida albicans* using *SAT1* flipping strategy (Sasse and Morschhäuser, 2012), this approach have been successfully utilised in this study to knockout the *GUT2* gene via homologous recombination. In order to increase TAG production, the conversion of G3P into DHAP was disrupted by deletion of *GUT2* gene encoding to mitochondrial glycerol-3-phosphate dehydrogenase responsible to catalyse this reaction, which is a competing step of TAG production. Gene deletion was achieved in 3 from 8 selected single colonies via replacement of *GUT2* gene with *SAT1* marker flanked with 450 bp upstream and 500 bp downstream sequences of *GUT2* ORF. As a result, the mutant strains were failed to grow in minimal medium with glycerol as the sole carbon source. However, the growth of mutant strain in minimal medium with 48 glucose: 0.5 ammonium sulphate was slightly higher than the growth of wild type strains. Additionally, the average of neutral lipid production increased 1.4-fold in *gut2*Δ strain compared with wild type strain after 40 hours of incubation in this medium at 25 ^{o}C under shaking. To some extent this results were compatible with the findings of the previous study of *GUT2* deletion in *Yarrowia*

lipolytica by Beopulos (2008) that achieved a 1.2 fold increase in neutral lipid production after 11 hours of incubation in glucose based medium (YNBD) (Beopoulos et al., 2008a).

7.2. Future work

Although this work has presented biotechnological and molecular tools for engineering the halotolerant *D. hansenii* NCYC102 cells that significantly improved lipid overproduction, there are still a number of interesting opportunities for further investigation. One of the most important strategies is to extend the utilization of low cost alternative substrates such as bioproduct waste material as yeast substrate, to be compared with the percentages of neutral lipid production achieved in this study. Additionally, further identification of fatty acid profile and quantification are strongly recommended for *gut2*Δ strains compared with wild type strains in the cultivation conditions that induced the improvement of TAG accumulation. Fluorescence microscopy and Environmental Scanning Electron Microscope (ESEM) are also recommended to examine the intracellular lipid bodies after genetic modification in *D. hansenii* cells along with an investigation of any significant changes happening to microbial cells growing under different stress conditions. As we followed one of the most important strategies of neutral lipid improvement achieved for *Yarrowia lipolytica* (1029) strain in particular *GUT2* deletion, additional deletion of β- oxidation POX1 to POX6 genes (that was adopted in *Y. lipolytica* strain and led to a fourfold increase in lipid content) could be useful for *D. hansenii* NCYC102 strain to further enhance lipid production. We can perform these approaches in *Y. lipolytica* NCYC476 strain already used in the third chapter of this study along with *gut2*Δ *D. hansenii* strain to compare how efficiently molecular tools can be used to increase neutral lipid yield in the yeast strains under the most favorable cultivation conditions that we can achieve in future optimization experiments. To block β- oxidation, we could delete any of the core machinery. However, the genome of *D. hansenii* appears to encode three different acyl-CoA oxidases (Pox1),

three 3-keto-acyl-CoA thiolases (Pot1 or Fox3). However, a single gene encodes the 3 hydroxy acyl-CoA dehydrogenase/hydratase (fox2).

Chapter Eight: References

8. References

ADAMS, I. P., DACK, S., DICKINSON, F. M. & RATLEDGE, C. 2002. The distinctiveness of ATP: citrate lyase from Aspergillus nidulans. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology,* 1597**,** 36-41.

ADLER, L., BLOMBERG, A. & NILSSON, A. 1985. Glycerol metabolism and osmoregulation in the salt-tolerant yeast Debaryomyces hansenii. *Journal of Bacteriology,* 162**,** 300-306.

ADLER, L. & GUSTAFSSON, L. 1980. Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of the yeast Debaryomyces hansenii. *Archives of Microbiology,* 124**,** 123-130.

AGEITOS, J. M., VALLEJO, J. A., VEIGA-CRESPO, P. & VILLA, T. G. 2011. Oily yeasts as oleaginous cell factories. *Applied microbiology and biotechnology,* 90**,** 1219-1227.

AKPINAR-BAYIZIT, A. 2014. Fungal lipids: The biochemistry of lipid accumulation. *International Journal of Chemical Engineering and Applications,* 5**,** 409.

AL SARYI, N. 2016. *Molecular studies of peroxisome biogenesis in Saccharomyces cerevisiae.* University of Sheffield.

ALBERS, E., LARSSON, C., LIDÉN, G., NIKLASSON, C. & GUSTAFSSON, L. 1996. Influence of the nitrogen source on Saccharomyces cerevisiae anaerobic growth and product formation. *Applied and Environmental Microbiology,* 62**,** 3187-3195.

ALBERTYN, J., HOHMANN, S. & PRIOR, B. A. 1994. Characterization of the osmotic-stress response inSaccharomyces cerevisiae: osmotic stress and glucose repression regulate glycerol-3-phosphate dehydrogenase independently. *Current genetics,* 25**,** 12-18.

ALLISON, S. D., CHANG, B., RANDOLPH, T. W. & CARPENTER, J. F. 1999. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. *Archives of Biochemistry and Biophysics,* 365**,** 289-298.

ALMALKI, M. 2012. *Molecular Identification and Characterisation of Acid Tolerant Microorganisms Isolated from Rivelin and Limb Valleys.* University of Sheffield.

ALMUTAIRI, A. 2015. *Production of biofuels from the green alga Tetraselmis.* University of Sheffield.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology,* 215**,** 403-410.

AMARETTI, A., RAIMONDI, S., SALA, M., RONCAGLIA, L., DE LUCIA, M., LEONARDI, A. & ROSSI, M. 2010. Production of single cell oils by the cold-adapted oleaginous yeast Rhodotorula glacialis AS 4.7: effects of the growth temperature and the C: N ratio. *CHEMICAL ENGINEERING,* 20.

ANDRE, L., HEMMING, A. & ADLER, L. 1991. Osmoregulation in Saccharomyces cerevisiae studies on the osmotic induction of glycerol production and glycerol 3-phosphate dehydrogenase (NAD+). *FEBS letters,* 286**,** 13-17.

ANDRÉ, L., NILSSON, A. & ADLER, L. 1988. The role of glycerol in osmotolerance of the yeast Debaryomyces hansenii. *Microbiology,* 134**,** 669-677.

ANDREISHCHEVA, E., ISAKOVA, E., SIDOROV, N., ABRAMOVA, N., USHAKOVA, N., SHAPOSHNIKOV, G., SOARES, M. & ZVYAGILSKAYA, R. 1999. Adaptation to salt stress in a salt-tolerant strain of the yeast Yarrowia lipolytica. *BIOCHEMISTRY C/C OF BIOKHIMIIA,* 64**,** 1061-1067.

ANSELL, R., GRANATH, K., HOHMANN, S., THEVELEIN, J. M. & ADLER, L. 1997. The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded

by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. *The EMBO journal,* 16**,** 2179-2187.
ATLAS, M. & BARTHA, R. 1981. A habitat approach to microbial ecology. *Microbial Ecology: Fundamentals and Applications. Addison-Wesley, Philippines*.
ATSUMI, S., HANAI, T. & LIAO, J. C. 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *nature,* 451**,** 86.
AZÓCAR, L., HEIPIEPER, H. J. & NAVIA, R. 2010. Biotechnological processes for biodiesel production using alternative oils. *Applied microbiology and biotechnology,* 88**,** 621-636.
BABIJ, T., MOSS, F. & RALPH, B. 1969. Effects of oxygen and glucose levels on lipid composition of yeast Candida utilis grown in continuous culture. *Biotechnology and bioengineering,* 11**,** 593-603.
BAJPAI, D. & TYAGI, V. 2006. Biodiesel: source, production, composition, properties and its benefits. *Journal of Oleo Science,* 55**,** 487-502.
BANGERT, K. 2014. *Photo-Bioreactor Modelling And Development Of Methods For The Optimisation Of Micro-algal Biodiesel Production.* University of Sheffield.
BARNETT, J. A., PAYNE, R. W. & YARROW, D. 1983. *Yeasts: Characteristics and identification*, Cambridge University Press.
BARTH, G. & GAILLARDIN, C. 1997. Physiology and genetics of the dimorphic fungus Yarrowia lipolytica. *FEMS microbiology reviews,* 19**,** 219-237.
BELLINGER, Y. & LARHER, F. 1988. A 13C comparative nuclear magnetic resonance study of organic solute production and excretion by the yeasts Hansenula anomala and Saccharomyces cerevisiae in saline media. *Canadian journal of microbiology,* 34**,** 605-612.
BEN-AMOTZ, A. & AVRON, M. 1978. On the mechanism of osmoregulation in Dunaliella. *Developments in halophilic microorganisms*.
BEOPOULOS, A., CESCUT, J., HADDOUCHE, R., URIBELARREA, J.-L., MOLINA-JOUVE, C. & NICAUD, J.-M. 2009a. Yarrowia lipolytica as a model for bio-oil production. *Progress in lipid research,* 48**,** 375-387.
BEOPOULOS, A., CHARDOT, T. & NICAUD, J.-M. 2009b. Yarrowia lipolytica: A model and a tool to understand the mechanisms implicated in lipid accumulation. *Biochimie,* 91**,** 692-696.
BEOPOULOS, A., MROZOVA, Z., THEVENIEAU, F., LE DALL, M.-T., HAPALA, I., PAPANIKOLAOU, S., CHARDOT, T. & NICAUD, J.-M. 2008a. Control of lipid accumulation in the yeast Yarrowia lipolytica. *Applied and environmental microbiology,* 74**,** 7779-7789.
BEOPOULOS, A., MROZOVA, Z., THEVENIEAU, F., LE DALL, M., HAPALA, I., PAPANIKOLAOU, S., CHARDOT, T. & NICAUD, J. M. 2008b. Control of Lipid Accumulation in the Yeast Yarrowia lipolytica. *Appl Environ Microbiol,* 74**,** 7779-89.
BERTHE, M., CHARPENTIER, C., LEMATRE, J. & BONALY, R. 1981. Glucosamine and chitin accumulation in cell walls of the yeast Rhodotorula glutinis CBS 3044. Influence of culture conditions. *Biochemical and biophysical research communications,* 100**,** 1504-1514.
BLAGOVIC, B., RUPCIC, J., MESARIC, M., GEORGIÚ, K. & MARIC, V. 2001. Lipid composition of brewer's yeast. *Food Technology and Biotechnology,* 39**,** 175-182.
BLANK, L. M., LEHMBECK, F. & SAUER, U. 2005. Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. *FEMS yeast research,* 5**,** 545-558.
BLAZECK, J., LIU, L., REDDEN, H. & ALPER, H. 2011. Tuning gene expression in Yarrowia lipolytica by a hybrid promoter approach. *Applied and environmental microbiology,* 77**,** 7905-7914.

BLAZECK, J., REED, B., GARG, R., GERSTNER, R., PAN, A., AGARWALA, V. & ALPER, H. S. 2013. Generalizing a hybrid synthetic promoter approach in Yarrowia lipolytica. *Applied microbiology and biotechnology,* 97**,** 3037-3052.

BLIGH, E. G. & DYER, W. J. 1959. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology,* 37**,** 911-917.

BLOMBERG, A. & ADLER, L. 1992. Physiology of osmotolerance in fungi. *Advances in microbial physiology,* 33**,** 145-212.

BORELLI, B. M., FERREIRA, E. G., LACERDA, I. C., FRANCO, G. R. & ROSA, C. A. 2006. Yeast populations associated with the artisanal cheese produced in the region of Serra da Canastra, Brazil. *World Journal of Microbiology and Biotechnology,* 22**,** 1115-1119.

BOTHAM, P. A. & RATLEDGE, C. 1979. A biochemical explanation for lipid accumulation in Candida 107 and other oleaginous micro-organisms. *Microbiology,* 114**,** 361-375.

BOULTON, C. A. & RATLEDGE, C. 1984. Cryptococcus terricolus, an oleaginous yeast re-appraised. *Applied microbiology and biotechnology,* 20**,** 72-76.

BREUER, U. & HARMS, H. 2006. Debaryomyces hansenii—an extremophilic yeast with biotechnological potential. *Yeast,* 23**,** 415-437.

BROWN, A. 1978. Compatible solutes and extreme water stress in eukaryotic micro-organisms. *Advances in microbial physiology,* 17**,** 181-242.

BROWN, C. & ROSE, A. 1969. Fatty-acid composition of Candida utilis as affected by growth temperature and dissolved-oxygen tension. *Journal of Bacteriology,* 99**,** 371-378.

BROWN, L. 2009. Plan B 4.0: Mobilizing to civilization. New York: WW. Norton & Co.

BUIJS, N. A., SIEWERS, V. & NIELSEN, J. 2013. Advanced biofuel production by the yeast Saccharomyces cerevisiae. *Current opinion in chemical biology,* 17**,** 480-488.

BURR, G. O. & BURR, M. M. 1929. A new deficiency disease produced by the rigid exclusion of fat from the diet. *Journal of Biological Chemistry,* 82**,** 345-367.

BURR, G. O., BURR, M. M. & MILLER, E. S. 1932. On the fatty acids essential in nutrition. 3. *Journal of Biological Chemistry,* 97**,** 1-9.

BUTINAR, L., SANTOS, S., SPENCER-MARTINS, I., OREN, A., GUNDE-CIMERMAN, N., NECOCHEA, R., VALDERRAMA, B., DÍAZ-SANDOVAL, S., FOLCH-MALLOL, J. L. & VÁZQUEZ-DUHALT, R. 2005. 2. Yeast diversity in hypersaline habitats• SHORT COMMUNICATION. *FEMS MICROBIOLOGY LETTERS,* 244.

BUTINAR, L., SPENCER-MARTINS, I. & GUNDE-CIMERMAN, N. 2007. Yeasts in high Arctic glaciers: the discovery of a new habitat for eukaryotic microorganisms. *Antonie van Leeuwenhoek,* 91**,** 277-289.

CAI, J., ROBERTS, I. N. & COLLINS, M. D. 1996. Phylogenetic relationships among members of the ascomycetous yeast genera Brettanomyces, Debaryomyces, Dekkera, and Kluyveromyces deduced by small-subunit rRNA gene sequences. *International Journal of Systematic and Evolutionary Microbiology,* 46**,** 542-549.

CARRIQUIRY, M. 2007. US biodiesel production: recent developments and prospects. *Iowa Ag Review,* 13**,** 8-11.

CASPETA, L., BUIJS, N. A. & NIELSEN, J. 2013. The role of biofuels in the future energy supply. *Energy & Environmental Science,* 6**,** 1077-1082.

CASTANHA, R. F., MARIANO, A. P., MORAIS, L. A. S. D., SCRAMIN, S. & MONTEIRO, R. T. R. 2014. Optimization of lipids production by Cryptococcus laurentii 11 using cheese whey with molasses. *Brazilian Journal of Microbiology,* 45**,** 379-387.

CASTELLI, A., BARBARESI, G., BERTOLI, E. & ORLANDO, P. 1969. Fatty acids of subcellular fractions of Saccharomyces cerevisiae during the phases of growth. *The Italian journal of biochemistry,* 18**,** 78.

CHEN, D.-C., BECKERICH, J.-M. & GAILLARDIN, C. 1997. One-step transformation of the dimorphic yeast Yarrowia lipolytica. *Applied microbiology and biotechnology,* 48**,** 232-235.

CHEN, L. & CHEN, W. N. 2014. Metabolite and Fatty Acid Analysis of Yeast Cells and Culture Supernatants. *Bio-protocol,* 4**,** e1219.

CHEN, W., ZHANG, C., SONG, L., SOMMERFELD, M. & HU, Q. 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of microbiological methods,* 77**,** 41-47.

CHI, Z., CHI, Z., ZHANG, T., LIU, G. & YUE, L. 2009. Inulinase-expressing microorganisms and applications of inulinases. *Applied Microbiology and Biotechnology,* 82**,** 211-220.

CHISTI, Y. 2007. Biodiesel from microalgae. *Biotechnology advances,* 25**,** 294-306.

CHRISTENSEN, B., THYKAER, J. & NIELSEN, J. 2000. Metabolic characterization of high- and low-yielding strains of Penicillium chrysogenum. *Appl Microbiol Biotechnol,* 54**,** 212-7.

CHRISTIE, W. 2003. Lipid analysis. Isolation, separation, identification and structural analysis of lipids. Bridgwater. The Oily Press.(In the text).

CLAUSEN, M., LAMB, C. J., MEGNET, R. & DOERNER, P. W. 1994. PAD1 encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in Saccharomyces cerevisiae. *Gene,* 142**,** 107-112.

COELHO, M., AMARAL, P. & BELO, I. 2010. Yarrowia lipolytica: an industrial workhorse. *Current research, technology and education topics in applied microbiology and microbial biotechnology,* 2**,** 930-940.

COOKSEY, K. E., GUCKERT, J. B., WILLIAMS, S. A. & CALLIS, P. R. 1987. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. *Journal of microbiological methods,* 6**,** 333-345.

COSENTINO, S., FADDA, M., DEPLANO, M., MULARGIA, A. & PALMAS, F. 2001. Yeasts associated with Sardinian ewe's dairy products. *International journal of food microbiology,* 69**,** 53-58.

CZABANY, T., ATHENSTAEDT, K. & DAUM, G. 2007. Synthesis, storage and degradation of neutral lipids in yeast. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids,* 1771**,** 299-309.

DAWSON, P. & CRAIG, B. 1966. Lipids of Candida utilis: changes with growth. *Canadian journal of microbiology,* 12**,** 775-785.

DE JONG, B., SIEWERS, V. & NIELSEN, J. 2012. Systems biology of yeast: enabling technology for development of cell factories for production of advanced biofuels. *Current opinion in biotechnology,* 23**,** 624-630.

DE KROON, A. I., RIJKEN, P. J. & DE SMET, C. H. 2013. Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective. *Progress in lipid research,* 52**,** 374-394.

DEAK, T. & BEUCHAT, L. 1996. Handbook of food spoilage yeasts, CRC series in contemporary food science. *CRC, Boca Raton*.

DEL BOVE, M., LATTANZI, M., RELLINI, P., PELLICCIA, C., FATICHENTI, F. & CARDINALI, G. 2009. Comparison of molecular and metabolomic methods as characterization tools of Debaryomyces hansenii cheese isolates. *Food microbiology,* 26**,** 453-459.

DEMIRBAS, A. 2007. Importance of biodiesel as transportation fuel. *Energy policy,* 35**,** 4661-4670.

DEMIRBAS, A. 2008. Comparison of transesterification methods for production of biodiesel from vegetable oils and fats. *Energy Conversion and Management,* 49**,** 125-130.

DEMIRBAŞ, A. 2002. Diesel fuel from vegetable oil via transesterification and soap pyrolysis. *Energy Sources,* 24**,** 835-841.
DEROME, A. E. 2013. *Modern NMR techniques for chemistry research*, Elsevier.
DESNOS-OLLIVIER, M., RAGON, M., ROBERT, V., RAOUX, D., GANTIER, J.-C. & DROMER, F. 2008. Debaryomyces hansenii (Candida famata), a rare human fungal pathogen often misidentified as Pichia guilliermondii (Candida guilliermondii). *Journal of clinical microbiology,* 46**,** 3237-3242.
DING, C. & BUTLER, G. 2007. Development of a gene knockout system in Candida parapsilosis reveals a conserved role for BCR1 in biofilm formation. *Eukaryotic cell,* 6**,** 1310-1319.
DMYTRUK, K. V. & SIBIRNY, A. A. 2012. Candida famata (Candida flareri). *Yeast,* 29**,** 453-458.
DRUCKEN, Z. 2008. Triacylglycerol synthesis in the oleaginous yeast Yarrowia lipolytica. *Bioresource Technology,* 90**,** 309-319.
DUJON, B., SHERMAN, D., FISCHER, G., DURRENS, P., CASAREGOLA, S., LAFONTAINE, I., DE MONTIGNY, J., MARCK, C., NEUVÉGLISE, C. & TALLA, E. 2004. Genome evolution in yeasts. *Nature,* 430**,** 35-44.
DULERMO, T. & NICAUD, J.-M. 2011. Involvement of the G3P shuttle and β-oxidation pathway in the control of TAG synthesis and lipid accumulation in Yarrowia lipolytica. *Metabolic engineering,* 13**,** 482-491.
EASTERLING, E. R., FRENCH, W. T., HERNANDEZ, R. & LICHA, M. 2009. The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of Rhodotorula glutinis. *Bioresource Technology,* 100**,** 356-361.
EATON, S., BARTLETT, K. B. & POURFARZAM, M. 1996. Mammalian mitochondrial β-oxidation. *Biochemical Journal,* 320**,** 345-357.
ELISKASES-LECHNER, F. & GINZINGER, W. 1995. The yeast flora of surface-ripened cheeses. *Milchwissenschaft (Germany)*.
ELSEY, D., JAMESON, D., RALEIGH, B. & COONEY, M. J. 2007. Fluorescent measurement of microalgal neutral lipids. *Journal of microbiological methods,* 68**,** 639-642.
ELTGROTH, M. L., WATWOOD, R. L. & WOLFE, G. V. 2005. PRODUCTION AND CELLULAR LOCALIZATION OF NEUTRAL LONG-CHAIN LIPIDS IN THE HAPTOPHYTE ALGAE ISOCHRYSIS GALBANA AND EMILIANIA HUXLEYI1. *Journal of Phycology,* 41**,** 1000-1009.
ENSHAEIEH, M., ABDOLI, A., NAHVI, I. & MADANI, M. 2012. Bioconversion of different carbon sources into microbial oil and biodiesel using oleaginous yeasts. *Journal of Biology and Today's World,* 1**,** 42-47.
ESTEVE-ZARZOSO, B., BELLOCH, C., URUBURU, F. & QUEROL, A. 1999. Identification of yeasts by RFLP analysis of the 5.8 S rRNA gene and the two ribosomal internal transcribed spacers. *International Journal of Systematic and Evolutionary Microbiology,* 49**,** 329-337.
EVANS, C. T. & RATLEDGE, C. 1983. A comparison of the oleaginous yeast, Candida curvata, grown on different carbon sources in continuous and batch culture. *Lipids,* 18**,** 623-629.
EVANS, C. T. & RATLEDGE, C. 1984a. Effect of nitrogen source on lipid accumulation in oleaginous yeasts. *Microbiology,* 130**,** 1693-1704.
EVANS, C. T. & RATLEDGE, C. 1984b. Influence of nitrogen metabolism on lipid accumulation by Rhodosporidium toruloides CBS 14. *Microbiology,* 130**,** 1705-1710.
EVANS, C. T., RATLEDGE, C. & GILBERT, S. C. 1985. A rapid screening method for lipid-accumulating yeast using a replica-printing technique. *Journal of Microbiological Methods,* 4**,** 203-210.
EVANS, C. T., SCRAGG, A. H. & RATLEDGE, C. 1983. A comparative study of citrate efflux from mitochondria of oleaginous and non-oleaginous yeasts. *European journal of biochemistry,* 130**,** 195-204.

FEI, Q., CHANG, H. N., SHANG, L. & CHOI, J.-D.-R. 2011. Exploring low-cost carbon sources for microbial lipids production by fed-batch cultivation of Cryptococcus albidus. *Biotechnology and Bioprocess Engineering,* 16**,** 482-487.

FICKERS, P., LE DALL, M., GAILLARDIN, C., THONART, P. & NICAUD, J. 2003. New disruption cassettes for rapid gene disruption and marker rescue in the yeast Yarrowia lipolytica. *Journal of microbiological methods,* 55**,** 727-737.

FIDLER, N., KOLETZKO, B. & SAUERWALD, T. U. 1999. Single cell oils production and application. *Zb. Biotehniške fak. Univ. v Ljubljani. Kmetijstvo. Zootehnika,* 74**,** 37-45.

FLORES, C.-L., RODRÍGUEZ, C., PETIT, T. & GANCEDO, C. 2000. Carbohydrate and energy-yielding metabolism in non-conventional yeasts. *FEMS microbiology reviews,* 24**,** 507-529.

FORREST, S. I., ROBINOW, C. F. & LACHANCE, M.-A. 1987. Nuclear behaviour accompanying ascus formation in Debaryomyces polymorphus. *Canadian journal of microbiology,* 33**,** 967-970.

GACTO, M., SOTO, T., VICENTE-SOLER, J., VILLA, T. G. & CANSADO, J. 2003. Learning from yeasts: intracellular sensing of stress conditions. *International Microbiology,* 6**,** 211-219.

GALINSKI, E. A. & TRÜPER, H. G. 1994. Microbial behaviour in salt-stressed ecosystems. *FEMS Microbiology Reviews,* 15**,** 95-108.

GANCEDO, C. & SERRANO, R. 1989. Energy-yielding metabolism. *The yeasts,* 3**,** 205-259.

GENICOT, G., LEROY, J., VAN SOOM, A. & DONNAY, I. 2005. The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. *Theriogenology,* 63**,** 1181-1194.

GILL, C., HALL, M. & RATLEDGE, C. 1977. Lipid accumulation in an oleaginous yeast (Candida 107) growing on glucose in single-stage continuous culture. *Applied and environmental microbiology,* 33**,** 231-239.

GOHEL, H., GHOSH, S. & BRAGANZA, V. J. 2013. Yeast as a viable and prolonged feedstock for biodiesel production. *International Journal of Renewable Energy Research (IJRER),* 3**,** 126-131.

GRAUSLUND, M., LOPES, J. M. & RØNNOW, B. 1999. Expression of GUT1, which encodes glycerol kinase in Saccharomyces cerevisiae, is controlled by the positive regulators Adr1p, Ino2p and Ino4p and the negative regulator Opi1p in a carbon source-dependent fashion. *Nucleic acids research,* 27**,** 4391-4398.

GRAUSLUND, M. & RØNNOW, B. 2000. Carbon source-dependent transcriptional regulation of the mitochondrial glycerol-3-phosphate dehydrogenase gene, GUT2, from Saccharomyces cerevisiae. *Canadian journal of microbiology,* 46**,** 1096-1100.

GREENSPAN, P. & FOWLER, S. D. 1985. Spectrofluorometric studies of the lipid probe, nile red. *Journal of lipid research,* 26**,** 781-789.

GUILLARD, R. R. & SIERACKI, M. S. 2005. Counting cells in cultures with the light microscope. *Algal culturing techniques***,** 239-252.

GUNDE-CIMERMAN, N., RAMOS, J. & PLEMENITAŠ, A. 2009. Halotolerant and halophilic fungi. *Mycological research,* 113**,** 1231-1241.

GUSTAFSSON, L. 1979. The ATP pool in relation to the production of glycerol and heat during growth of the halotolerant yeast Debaryomyces hansenii. *Archives of Microbiology,* 120**,** 15-23.

GUSTAFSSON, L. & NORKRANS, B. 1976. On the mechanism of salt tolerance. *Archives of microbiology,* 110**,** 177-183.

HALL, L., WOHLFIEL, S. & ROBERTS, G. D. 2004. Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of filamentous fungi encountered in the clinical laboratory. *Journal of clinical microbiology,* 42**,** 622-626.

HALL, M. J. & RATLEDGE, C. 1977. Lipid accumulation in an oleaginous yeast (Candida 107) growing on glucose under various conditions in a one-and two-stage continuous culture. *Applied and Environmental Microbiology,* 33**,** 577-584.

HANSSON, L. & DOSTÁLEK, M. 1986. Influence of cultivation conditions on lipid production by Cryptococcus albidus. *Applied microbiology and biotechnology,* 24**,** 12-18.

HARDIE, D. & PAN, D. 2002. Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. Portland Press Limited.

HASSAN, M., BLANC, P. J., GRANGER, L.-M., PAREILLEUX, A. & GOMA, G. 1993. Lipid production by an unsaturated fatty acid auxotroph of the oleaginous yeast Apiotrichum curvatum grown in single-stage continuous culture. *Applied microbiology and biotechnology,* 40**,** 483-488.

HASSAN, M., BLANC, P. J., GRANGER, L.-M., PAREILLEUX, A. & GOMA, G. 1996. Influence of nitrogen and iron limitations on lipid production by Cryptococcus curvatus grown in batch and fed-batch culture. *Process Biochemistry,* 31**,** 355-361.

HASSLACHER, M., IVESSA, A., PALTAUF, F. & KOHLWEIN, S. 1993. Acetyl-CoA carboxylase from yeast is an essential enzyme and is regulated by factors that control phospholipid metabolism. *Journal of Biological Chemistry,* 268**,** 10946-10952.

HEIM, U., TIETZE, E., WESCHKE, W., TSCHÄPE, H. & WOBUS, U. 1989. Nucleotide sequence of a plasmid born streptothricin-acetyl-transferase gene (sat-1). *Nucleic acids research,* 17**,** 7103.

HERDEIRO, R., PEREIRA, M., PANEK, A. & ELEUTHERIO, E. 2006. Trehalose protects Saccharomyces cerevisiae from lipid peroxidation during oxidative stress. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1760**,** 340-346.

HEREDIA, L. & RATLEDGE, C. 1988. Simultaneous utilization of glucose and xylose byCandida curvata D in continuous culture. *Biotechnology letters,* 10**,** 25-30.

HIGGINS, C. F., CAIRNEY, J., STIRLING, D. A., SUTHERLAND, L. & BOOTH, I. R. 1987. Osmotic regulation of gene expression: ionic strength as an intracellular signal? *Trends in Biochemical Sciences,* 12**,** 339-344.

HILL, J., NELSON, E., TILMAN, D., POLASKY, S. & TIFFANY, D. 2006. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceedings of the National Academy of sciences,* 103**,** 11206-11210.

HOBOT, J. A. & JENNINGS, D. H. 1981. Growth ofDebaryomyces hansenii andSaccharomyces cerevisiae in relation to pH and salinity. *Experimental Mycology,* 5**,** 217-228.

HOHMANN, S. 1997. Shaping up: the response of yeast to osmotic stress. *Yeast stress responses***,** 101-146.

HOJA, U., MARTHOL, S., HOFMANN, J., STEGNER, S., SCHULZ, R., MEIER, S., GREINER, E. & SCHWEIZER, E. 2004. HFA1 encoding an organelle-specific acetyl-CoA carboxylase controls mitochondrial fatty acid synthesis in Saccharomyces cerevisiae. *Journal of Biological Chemistry,* 279**,** 21779-21786.

HOMMES, F. 1966. Effect of glucose on the level of glycolytic enzymes in yeast. *Archives of biochemistry and biophysics,* 114**,** 231-233.

HOSONO, K. 1992. Effect of salt stress on lipid composition and membrane fluidity of the salttolerant yeast Zygosaccharomyces rouxii. *Microbiology,* 138**,** 91-96.

HUANG, J., SHI, Q., ZHOU, X., LIN, Y., XIE, B. & WU, S. 1997. Studies on the breeding of Mortierella isabellina mutant high producing lipid and its fermentation conditions. *Wei sheng wu xue tong bao,* 25**,** 187-191.

IEA 2008. World Energy Outlook, OECD *Publication Service, Paris*.

INNIS, S. M. 1991. Essential fatty acids in growth and development. *Progress in lipid research,* 30**,** 39-103.
IZARD, J. & LIMBERGER, R. J. 2003. Rapid screening method for quantitation of bacterial cell lipids from whole cells. *Journal of microbiological methods,* 55**,** 411-418.
JACOB, Z. 1991. Enrichment of wheat bran byRhodotorula gracilis through solid-state fermentation. *Folia microbiologica,* 36**,** 86-91.
JACOB, Z. 1992. Linear growth and lipid synthesis in the oleaginous yeastRhodotorula gracilis. *Folia microbiologica,* 37**,** 117-121.
JADHAV, V. V., SALUNKHE, D. S. & BHADEKAR, R. K. 2012. Effect of alterations in conventional medium on lipid accumulation and fatty acid content in oleaginous yeasts. *International Journal of Pharmacy and Biological Sciences B,* 3**,** 757-769.
JESSEN, B. 1995. Starter cultures for meat fermentations. *Fermented meats.* Springer.
JIA, Z., MCCULLOUGH, N., MARTEL, R., HEMMINGSEN, S. & YOUNG, P. 1992. Gene amplification at a locus encoding a putative Na+/H+ antiporter confers sodium and lithium tolerance in fission yeast. *The EMBO journal,* 11**,** 1631.
JOHN, U. P. & SPANGENBERG, G. C. 2005. Xenogenomics: genomic bioprospecting in indigenous and exotic plants through EST discovery, cDNA microarray-based expression profiling and functional genomics. *Comparative and functional genomics,* 6**,** 230-235.
JOHNSON, M., ZARETSKAYA, I., RAYTSELIS, Y., MEREZHUK, Y., MCGINNIS, S. & MADDEN, T. L. 2008. NCBI BLAST: a better web interface. *Nucleic acids research,* 36**,** W5-W9.
JOHNSON, V., SINGH, M., SAINI, V., SISTA, V. & YADAV, N. 1992. Effect of pH on lipid accumulation by an oleaginous yeast: Rhodotorula glutinis IIP-30. *World Journal of Microbiology and Biotechnology,* 8**,** 382-384.
JOHNSON, V. W., SINGH, M., SAINI, V. S., ADHIKARI, D. K., SISTA, V. & YADAV, N. K. 1995. Utilization of molasses for the production of fat by an oleaginous yeast, Rhodotorula glutinis IIP-30. *Journal of Industrial Microbiology & Biotechnology,* 14**,** 1-4.
JONES, E. W. & FINK, G. R. 1982. Regulation of amino acid and nucleotide biosynthesis in yeast. *Cold Spring Harbor Monograph Archive,* 11**,** 181-299.
JØRGENSEN, H., OLSSON, L., RØNNOW, B. & PALMQVIST, E. 2002. Fed-batch cultivation of baker's yeast followed by nitrogen or carbon starvation: effects on fermentative capacity and content of trehalose and glycogen. *Applied microbiology and biotechnology,* 59**,** 310-317.
JOSHI, P. B., WEBB, J. R., DAVIES, J. E. & MCMASTER, W. R. 1995. The gene encoding streptothricin acetyltransferase (sat) as a selectable marker for Leishmania expression vectors. *Gene,* 156**,** 145-149.
KAMISAKA, Y., NODA, N., SAKAI, T. & KAWASAKI, K. 1999. Lipid bodies and lipid body formation in an oleaginous fungus, Mortierella ramanniana var. angulispora. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids,* 1438**,** 185-198.
KAMISAKA, Y., TOMITA, N., KIMURA, K., KAINOU, K. & UEMURA, H. 2007. DGA1 (diacylglycerol acyltransferase gene) overexpression and leucine biosynthesis significantly increase lipid accumulation in the Δsnf2 disruptant of Saccharomyces cerevisiae. *Biochemical Journal,* 408**,** 61-68.
KHAWARE, R. K., KOUL, A. & PRASAD, R. 1995. High membrane fluidity is related to NaCl stress in Candida membranefaciens. *Biochemistry and molecular biology international,* 35**,** 875-880.
KHURI, A. I. & CORNELL, J. A. 1996. *Response surfaces: designs and analyses*, CRC press.
KIMURA, K., YAMAOKA, M. & KAMISAKA, Y. 2004. Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. *Journal of Microbiological Methods,* 56**,** 331-338.

KITCHA, S. & CHEIRSILP, B. 2011. Screening of oleaginous yeasts and optimization for lipid production using crude glycerol as a carbon source. *Energy Procedia,* 9**,** 274-282.
KLUG, L. & DAUM, G. 2014. Yeast lipid metabolism at a glance. *FEMS yeast research,* 14**,** 369-388.
KOHLWEIN, S. D. 2010. Triacylglycerol homeostasis: insights from yeast. *Journal of Biological Chemistry,* 285**,** 15663-15667.
KRÜGEL, H., FIEDLER, G., HAUPT, I., SARFERT, E. & SIMON, H. 1988. Analysis of the nourseothricin-resistance gene (nat) of Streptomyces noursei. *Gene,* 62**,** 209-217.
KUNIGO, M., BUERTH, C., TIELKER, D. & ERNST, J. F. 2013. Heterologous protein secretion by Candida utilis. *Applied microbiology and biotechnology,* 97**,** 7357-7368.
KURTZMAN, C., BOEKHOUT, T., ROBERT, V., FELL, J. & DEAK, T. 2003. Methods to identify yeasts. *Yeasts in Food, Beneficial and Detrimental Aspects***,** 69-121.
KURTZMAN, C., FELL, J. W. & BOEKHOUT, T. 2011. *The yeasts: a taxonomic study*, Elsevier.
KURTZMAN, C. & PIŠKUR, J. 2006. Taxonomy and phylogenetic diversity among the yeasts. *Comparative Genomics***,** 29-46.
KURTZMAN, C. P. & ROBNETT, C. J. 1991. Phylogenetic relationships among species of Saccharomyces, Schizosaccharomyces, Debaryomyces and Schwanniomyces determined from partial ribosomal RNA sequences. *Yeast,* 7**,** 61-72.
KUSHNER, D. J. 1978. Life in high salt and solute concentrations: halophilic bacteria. *Microbial life in extreme environments***,** 317-368.
LAGES, F. & LUCAS, C. 1995. Characterization of a glycerol/H+ symport in the halotolerant yeast Pichia sorbitophila. *Yeast,* 11**,** 111-119.
LARSSON, C. & GUSTAFSSON, L. 1993. The role of physiological state in osmotolerance of the salt-tolerant yeast Debaryomyces hansenii. *Canadian journal of microbiology,* 39**,** 603-609.
LARSSON, C., MORALES, C., GUSTAFSSON, L. & ADLER, L. 1990. Osmoregulation of the salt-tolerant yeast Debaryomyces hansenii grown in a chemostat at different salinities. *Journal of bacteriology,* 172**,** 1769-1774.
LE RUDULIER, D., STROM, A., DANDEKAR, A., SMITH, L. & VALENTINE, R. 1984. Molecular biology of osmoregulation. *Science,* 224**,** 1064-1069.
LEE, I., HAMMOND, E. & GLATZ, B. 1992. Triacylglycerol assembly from lipid substrates by Apiotrichum curvatum. *Industrial applications of single cell oils. AOCS Press, Champaign***,** 139-155.
LI, H., CANN, A. F. & LIAO, J. C. 2010. Biofuels: biomolecular engineering fundamentals and advances. *Annual review of chemical and biomolecular engineering,* 1**,** 19-36.
LI, Q., DU, W. & LIU, D. 2008. Perspectives of microbial oils for biodiesel production. *Applied microbiology and biotechnology,* 80**,** 749-756.
LI, W., DU, W., LI, Y.-H., LIU, D.-H. & ZHAO, Z.-B. 2007a. Enzymatic transesterification of yeast oil for biodiesel fuel production. *Chinese Journal of Process Engineering,* 7**,** 133.
LI, Y., ZHAO, Z. K. & BAI, F. 2007b. High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture. *Enzyme and microbial technology,* 41**,** 312-317.
LIANG, M.-H. & JIANG, J.-G. 2013. Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. *Progress in lipid research,* 52**,** 395-408.
LIANG, X., DONG, W., MIAO, X. & DAI, C. 2006. Production technology and influencing factors of microorganism grease. *Food Res Dev,* 27**,** 46-47.
LIU, B. & ZHAO, Z. K. 2007. Biodiesel production by direct methanolysis of oleaginous microbial biomass. *Journal of Chemical Technology and Biotechnology,* 82**,** 775-780.

LIU, H., ZHAO, X., WANG, F., LI, Y., JIANG, X., YE, M., ZHAO, Z. K. & ZOU, H. 2009. Comparative proteomic analysis of Rhodosporidium toruloides during lipid accumulation. *Yeast,* 26**,** 553-566.

LIU, L., OTOUPAL, P., PAN, A. & ALPER, H. S. 2014. Increasing expression level and copy number of a Yarrowia lipolytica plasmid through regulated centromere function. *FEMS yeast research,* 14**,** 1124-1127.

LIU, S., YANG, W. & SHI, A. 1999. Screeing of the high lipid production strains and studies on its flask culture conditions. *Wei sheng wu xue tong bao,* 27**,** 93-97.

LOWRY, C. V. & ZITOMER, R. S. 1984. Oxygen regulation of anaerobic and aerobic genes mediated by a common factor in yeast. *Proceedings of the National Academy of Sciences,* 81**,** 6129-6133.

LUCAS, C., DA COSTA, M. & VAN UDEN, N. 1990. Osmoregulatory active sodium-glycerol co-transport in the halotolerant yeast Debaryomyces hansenii. *Yeast,* 6**,** 187-191.

LUYTEN, K., ALBERTYN, J., SKIBBE, W. F., PRIOR, B., RAMOS, J., THEVELEIN, J. & HOHMANN, S. 1995. Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. *The EMBO Journal,* 14**,** 1360.

MADSEN, A. T., CHRISTENSEN, C. H., FEHRMANN, R. & RIISAGER, A. 2011. Hydrodeoxygenation of waste fat for diesel production: Study on model feed with Pt/alumina catalyst. *Fuel,* 90**,** 3433-3438.

MAGER, W. H. & SIDERIUS, M. 2002. Novel insights into the osmotic stress response of yeast. *FEMS yeast research,* 2**,** 251-257.

MAKRI, A., FAKAS, S. & AGGELIS, G. 2010. Metabolic activities of biotechnological interest in Yarrowia lipolytica grown on glycerol in repeated batch cultures. *Bioresource technology,* 101**,** 2351-2358.

MARKGRAF, D. F., KLEMM, R. W., JUNKER, M., HANNIBAL-BACH, H. K., EJSING, C. S. & RAPOPORT, T. A. 2014. An ER protein functionally couples neutral lipid metabolism on lipid droplets to membrane lipid synthesis in the ER. *Cell reports,* 6**,** 44-55.

MCGINNIS, K., DEMPSTER, T. & SOMMERFELD, M. 1997. Characterization of the growth and lipid content of the diatom Chaetoceros muelleri. *Journal of Applied Phycology,* 9**,** 19-24.

MCWILLIAM, H., LI, W., ULUDAG, M., SQUIZZATO, S., PARK, Y. M., BUSO, N., COWLEY, A. P. & LOPEZ, R. 2013. Analysis tool web services from the EMBL-EBI. *Nucleic acids research,* 41**,** W597-W600.

MEESTERS, P., HUIJBERTS, G. & EGGINK, G. 1996. High-cell-density cultivation of the lipid accumulating yeast Cryptococcus curvatus using glycerol as a carbon source. *Applied microbiology and biotechnology,* 45**,** 575-579.

MENG, X., YANG, J., XU, X., ZHANG, L., NIE, Q. & XIAN, M. 2009. Biodiesel production from oleaginous microorganisms. *Renewable energy,* 34**,** 1-5.

MICHELY, S., GAILLARDIN, C., NICAUD, J.-M. & NEUVÉGLISE, C. 2013. Comparative physiology of oleaginous species from the Yarrowia clade. *PLoS one,* 8**,** e63356.

MILLERIOUX, Y., CLASTRE, M., SIMKIN, A. J., MARAIS, E., SIBIRNY, A. A., NOËL, T., CRÈCHE, J., GIGLIOLI-GUIVARC'H, N. & PAPON, N. 2011. Development of a URA5 integrative cassette for gene disruption in the Candida guilliermondii ATCC 6260 strain. *Journal of microbiological methods,* 84**,** 355-358.

MINHAS, A., BISWAS, D. & MONDAL, A. K. 2009. Development of host and vector for high-efficiency transformation and gene disruption in Debaryomyces hansenii. *FEMS yeast research,* 9**,** 95-102.

MISHINA, M., YANAGAWA, S., TANAKA, A. & FUKUI, S. 1973. Effects of chain-length of alkane substrate on fatty acid composition and biosynthetic pathway in some Candida yeasts. *Agricultural and Biological Chemistry,* 37**,** 863-870.

MLí, K., LUO, Y., D'ANDREA, S., PEČ, P., CHARDOT, T. & NICAUD, J.-M. 2004. Acyl-CoA oxidase, a key step for lipid accumulation in the yeast Yarrowia lipolytica. *Journal of Molecular Catalysis B: Enzymatic,* 28**,** 81-85.

MLÍČKOVÁ, K., ROUX, E., ATHENSTAEDT, K., D'ANDREA, S., DAUM, G., CHARDOT, T. & NICAUD, J.-M. 2004. Lipid accumulation, lipid body formation, and acyl coenzyme A oxidases of the yeast Yarrowia lipolytica. *Applied and environmental microbiology,* 70**,** 3918-3924.

MOON, N. J. & HAMMOND, E. 1978. Oil production by fermentation of lactose and the effect of temperature on the fatty acid composition1. *Journal of the American Oil Chemists' Society,* 55**,** 683-688.

MORETON, R. 1988. *Single cell oil*, Longman Scientific and Technical.

MULLNER, H. & DAUM, G. 2004. Dynamics of neutral lipid storage in yeast. *ACTA BIOCHIMICA POLONICA-ENGLISH EDITION-,* 51**,** 323-348.

NAKASE, T., SUZUKI, M., PHAFF, H. & KURTZMAN, C. 1998. DebaryomycesLodder & Kreger-van Rij Nom. Cons-26.

NISHI, T. & YAGI, T. 1993. PARTICIPATION OF cAMP IN THE INDUCTION OF THE SYNTHESIS OF GLYCEROL FOR THE OSMOREGULATORY RESPONSE IN THE SALT-TOLERANT YEAST ZYCOSACCHAROMYCES ROUXII. *The Journal of General and Applied Microbiology,* 39**,** 493-503.

NISHI, T. & YAGI, T. 1995. Efflux of sodium ions by a Na+/H+-antiporter during salt stress in the salt-tolerant yeast Zygosaccharomyces rouxii. *The Journal of General and Applied Microbiology,* 41**,** 87-97.

NISHIKAWA, A., SUGITA, T. & SHINODA, T. 1997. Differentiation between Debaryomyces hansenii/Candida famata complex and Candida guilliermondii by polymerase chain reaction. *FEMS Immunology & Medical Microbiology,* 19**,** 125-129.

NISHIKAWA, A., SUGITA, T. & SHINODA, T. 1999. Rapid identification of Debaryomyces hansenii/Candida famata by polymerase chain reaction. *Medical mycology,* 37**,** 101-104.

NOBLE, S. M. & JOHNSON, A. D. 2005. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen Candida albicans. *Eukaryotic cell,* 4**,** 298-309.

NOBLE, S. M. & JOHNSON, A. D. 2007. Genetics of Candida albicans, a diploid human fungal pathogen. *Annu. Rev. Genet.,* 41**,** 193-211.

NOBRE, M. F. & COSTA, M. S. D. 1985. The accumulation of polyols by the yeast Debaryomyces hansenii in response to water stress. *Canadian journal of microbiology,* 31**,** 1061-1064.

NORBECK, J., PÅHLMAN, A.-K., AKHTAR, N., BLOMBERG, A. & ADLER, L. 1996. Purification and characterization of two isoenzymes of dl-glycerol-3-phosphatase from Saccharomyces cerevisiae identification of the corresponding GPP1 and GPP2 genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. *Journal of Biological Chemistry,* 271**,** 13875-13881.

NORKRANS, B. 1966. Studies on marine occurring yeasts: growth related to pH, NaCl concentration and temperature. *Archiv für Mikrobiologie,* 54**,** 374-392.

NORKRANS, B. & KYLIN, A. 1969. Regulation of the potassium to sodium ratio and of the osmotic potential in relation to salt tolerance in yeasts. *Journal of Bacteriology,* 100**,** 836-845.

OHLROGGE, J. B. & JAWORSKI, J. G. 1997. Regulation of fatty acid synthesis. *Annual review of plant biology,* 48**,** 109-136.

OHSHIRO, K. & YAGI, T. 1996. REGULATION OF INTRACELLULAR OSMOTIC PRESSURE AND SOME FACTORS THAT INFLUENCE THE PROMOTION OF GLYCEROL SYNTHESIS IN A

RESPIRATION-DEFICIENT MUTANT OF THE SALT-TOLERANT YEAST ZYGOSACCHAROMYCES ROUXH DURING SALT STRESS. *The Journal of General and Applied Microbiology,* 42**,** 201-212.

OLIVEIRA, R. P., LAGES, F. & LUCAS, C. 1996. Isolation and characterisation of mutants from the halotolerant yeast Pichia sorbitophila defective in H+/glycerol symport activity. *FEMS microbiology letters,* 142**,** 147-153.

ŌNISHI, H. 1963. Osmophilic yeasts. *Advances in food research,* 12**,** 53-94.

PÅHLMAN, A.-K., GRANATH, K., ANSELL, R., HOHMANN, S. & ADLER, L. 2001. The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress. *Journal of Biological Chemistry,* 276**,** 3555-3563.

PAN, J. G., KWAK, M. Y. & RHEE, J. S. 1986. High density cell culture ofRhodotorulaglutinis using oxygen-enriched air. *Biotechnology letters,* 8**,** 715-718.

PAPANIKOLAOU, S., DIAMANTOPOULOU, P., CHATZIFRAGKOU, A., PHILIPPOUSSIS, A. & AGGELIS, G. 2010. Suitability of low-cost sugars as substrates for lipid production by the fungus Thamnidium elegans. *Energy & Fuels,* 24**,** 4078-4086.

PAPANIKOLAOU, S., GALIOTOU-PANAYOTOU, M., FAKAS, S., KOMAITIS, M. & AGGELIS, G. 2007. Lipid production by oleaginous Mucorales cultivated on renewable carbon sources. *European Journal of Lipid Science and Technology,* 109**,** 1060-1070.

PARROU, J. L., TESTE, M.-A. & FRANÇOIS, J. 1997. Effects of various types of stress on the metabolism of reserve carbohydrates in Saccharomyces cerevisiae: genetic evidence for a stress-induced recycling of glycogen and trehalose. *Microbiology,* 143**,** 1891-1900.

PAVLIK, P., SIMON, M., SCHUSTER, T. & RUIS, H. 1993. The glycerol kinase (GUT1) gene of Saccharomyces cerevisiae: cloning and characterization. *Current genetics,* 24**,** 21-25.

PETERSEN, K. & JESPERSEN, L. 2004. Genetic diversity of the species Debaryomyces hansenii and the use of chromosome polymorphism for typing of strains isolated from surface-ripened cheeses. *Journal of applied microbiology,* 97**,** 205-213.

POSAS, F., CHAMBERS, J. R., HEYMAN, J. A., HOEFFLER, J. P., DE NADAL, E. & ARIÑO, J. N. 2000. The transcriptional response of yeast to saline stress. *Journal of Biological Chemistry,* 275**,** 17249-17255.

PRISTA, C., LOUREIRO-DIAS, M. C., MONTIEL, V., GARCÍA, R. & RAMOS, J. 2005. Mechanisms underlying the halotolerant way of Debaryomyces hansenii. *FEMS yeast research,* 5**,** 693-701.

RADLER, F. & SCHÜTZ, H. 1982. Glycerol production of various strains of Saccharomyces. *American Journal of Enology and Viticulture,* 33**,** 36-40.

RATLEDGE, C. 1982. Microbial oils and fats: an assessment of their commercial potential [Algae, yeasts, fungi]. *Progress in industrial microbiology*.

RATLEDGE, C. 1989. Lipids and their metabolism. *The yeasts: metabolism and physiology of yeasts,* 3**,** 367-455.

RATLEDGE, C. 1991. Microorganisms for lipids. *Acta Biotechnol,* 11**,** 429–438.

RATLEDGE, C. 2002. Regulation of lipid accumulation in oleaginous micro-organisms. Portland Press Limited.

RATLEDGE, C. 2004. Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie,* 86**,** 807-815.

RATLEDGE, C. & HALL, M. J. 1979. Accumulation of lipid byRhodotorula glutinis in continuous culture. *Biotechnology Letters,* 1**,** 115-120.

RATLEDGE, C. & WYNN, J. P. 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Advances in applied microbiology,* 51**,** 1-52.

RATTRAY, J., SCHIBECI, A. & KIDBY, D. K. 1975. Lipids of yeasts. *Bacteriological Reviews,* 39**,** 197.
RAU, U., NGUYEN, L., ROEPER, H., KOCH, H. & LANG, S. 2005. Fed-batch bioreactor production of mannosylerythritol lipids secreted by Pseudozyma aphidis. *Applied microbiology and biotechnology,* 68**,** 607-613.
REED, R. H., CHUDEK, J., FOSTER, R. & GADD, G. 1987. Osmotic significance of glycerol accumulation in exponentially growing yeasts. *Applied and Environmental Microbiology,* 53**,** 2119-2123.
REUß, O., VIK, Å., KOLTER, R. & MORSCHHÄUSER, J. 2004. The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. *Gene,* 341**,** 119-127.
RICAURTE, M. L. & GOVIND, N. S. 1999. Construction of plasmid vectors and transformation of the marine yeast Debaryomyces hansenii. *Marine Biotechnology,* 1**,** 15-19.
RICHARD, G.-F., KERREST, A., LAFONTAINE, I. & DUJON, B. 2005. Comparative genomics of hemiascomycete yeasts: genes involved in DNA replication, repair, and recombination. *Molecular biology and evolution,* 22**,** 1011-1023.
ROMANO, A. H. 1986. Microbial sugar transport systems and their importance in biotechnology. *Trends in biotechnology,* 4**,** 207-213.
RØNNOW, B. & KIELLAND-BRANDT, M. C. 1993. GUT2, a gene for mitochondrial glycerol 3-phosphate dehydrogenase of Saccharomyces cerevisiae. *Yeast,* 9**,** 1121-1130.
ROSSI, M., AMARETTI, A., RAIMONDI, S. & LEONARDI, A. 2011. Getting lipids for biodiesel production from oleaginous fungi. *Biodiesel-Feedstocks and Processing Technologies,* 1**,** 72-74.
ROSTRON, K. A., ROLPH, C. E. & LAWRENCE, C. L. 2015. Nile red fluorescence screening facilitating neutral lipid phenotype determination in budding yeast, Saccharomyces cerevisiae, and the fission yeast Schizosaccharomyces pombe. *Antonie Van Leeuwenhoek,* 108**,** 97-106.
RUSSELL, N., EVANS, R., TER STEEG, P., HELLEMONS, J., VERHEUL, A. & ABEE, T. 1995. Membranes as a target for stress adaptation. *International journal of food microbiology,* 28**,** 255-261.
SAINZ, J., PIZARRO, F., PÉREZ-CORREA, J. R. & AGOSIN, E. 2003. Modeling of yeast metabolism and process dynamics in batch fermentation. *Biotechnology and Bioengineering,* 81**,** 818-828.
SANTOS, M. A. & TUITE, M. F. 1995. The CUG codon is decoded in vivo as serine and not leucine in Candida albicans. *Nucleic Acids Research,* 23**,** 1481-1486.
SASSE, C. & MORSCHHÄUSER, J. 2012. Gene deletion in Candida albicans wild-type strains using the SAT1-flipping strategy. *Host-Fungus Interactions: Methods and Protocols***,** 3-17.
SATYANARAYANA, T. & KUNZE, G. 2010. Gatersleben. *Germany (Eds.) Yeast Biotechnology: Diversity and Applications,* 746.
SAXENA, V., SHARMA, C., BHAGAT, S., SAINI, V. & ADHIKARI, D. 1998. Lipid and fatty acid biosynthesis by Rhodotorula minuta. *Journal of the American Oil Chemists' Society,* 75**,** 501-505.
SCANES, K., HOHMANN, S. & PRIOR, B. 1998a. Glycerol production by the yeast Saccharomyces cerevisiae and its relevance to wine: a review. *South African Journal for Enology and Viticulture,* 19**,** 17-24.
SCANES, K., HOHRNANN, S. & PRIOR, B. 1998b. Glycerol production by the yeast Saccharomyces cerevisiae and its relevance to wine: a review. *South African Journal of Enology and Viticulture,* 19**,** 17-24.

SCHAUB, Y., DÜNKLER, A., WALTHER, A. & WENDLAND, J. 2006. New pFA-cassettes for PCR-based gene manipulation in Candida albicans. *Journal of basic microbiology,* 46**,** 416-429.

SCHUBERT, C. 2006. Can biofuels finally take center stage? *Nature biotechnology,* 24**,** 777.

SHARMA, S. C., RAJ, D., FOROUZANDEH, M. & BANSAL, M. P. 1996. Salt-induced changes in lipid composition and ethanol tolerance inSaccharomyces cerevisiae. *Applied biochemistry and biotechnology,* 56**,** 189-195.

SHENG, J., CHI, Z., LI, J., GAO, L. & GONG, F. 2007. Inulinase production by the marine yeast Cryptococcus aureus G7a and inulin hydrolysis by the crude inulinase. *Process Biochemistry,* 42**,** 805-811.

SHI, A., GU, J., LIU, S. & MA, Y. 1997. Screening high oil yield yeast strains, fermentation conditions optimization and fat composition analysis. *China Brewing,* 4**,** 10-13.

SMITH, R. 2016. *The influence of organic carbon supplementation on the carbon metabolism of green algae.* University of Sheffield.

SORIANO, N. U., MIGO, V. P. & MATSUMURA, M. 2006. Ozonized vegetable oil as pour point depressant for neat biodiesel. *Fuel,* 85**,** 25-31.

SPENCER, J. & SPENCER, D. 1978. Production of polyhydroxy alcohols by osmotolerant yeasts. *Economic microbiology*.

SPRAGUE, G. & CRONAN, J. E. 1977. Isolation and characterization of Saccharomyces cerevisiae mutants defective in glycerol catabolism. *Journal of Bacteriology,* 129**,** 1335-1342.

STEPHANOPOULOS, G. 2007. Challenges in engineering microbes for biofuels production. *Science,* 315**,** 801-804.

STRATFORD, M., PLUMRIDGE, A. & ARCHER, D. B. 2007. Decarboxylation of sorbic acid by spoilage yeasts is associated with the PAD1 gene. *Applied and environmental microbiology,* 73**,** 6534-6542.

SUBRAMANIAM, R., DUFRECHE, S., ZAPPI, M. & BAJPAI, R. 2010. Microbial lipids from renewable resources: production and characterization. *Journal of industrial microbiology & biotechnology,* 37**,** 1271-1287.

SUTHERLAND, F., LAGES, F., LUCAS, C., LUYTEN, K., ALBERTYN, J., HOHMANN, S., PRIOR, B. & KILIAN, S. 1997. Characteristics of Fps1-dependent and-independent glycerol transport in Saccharomyces cerevisiae. *Journal of bacteriology,* 179**,** 7790-7795.

TAI, M. 2012. *Metabolic engineering of oleaginous yeast for the production of biofuels.* Massachusetts Institute of Technology.

TAI, M. & STEPHANOPOULOS, G. 2013. Engineering the push and pull of lipid biosynthesis in oleaginous yeast Yarrowia lipolytica for biofuel production. *Metabolic engineering,* 15**,** 1-9.

TAMÁS, M. J. & HOHMANN, S. 2003. The osmotic stress response of Saccharomyces cerevisiae. *Yeast stress responses.* Springer.

TAMÁS, M. J., LUYTEN, K., SUTHERLAND, F. C. W., HERNANDEZ, A., ALBERTYN, J., VALADI, H., LI, H., PRIOR, B. A., KILIAN, S. G. & RAMOS, J. 1999. Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Molecular microbiology,* 31**,** 1087-1104.

TANAKA, A., HAGIHARA, T., KAMIRYO, T., MISHINA, M., TASHIRO, S.-I., NUMA, S. & FUKUI, S. 1978. Candida lipolytica mutants defective in an acyl-CoA synthetase. *European journal of applied microbiology and biotechnology,* 5**,** 79-86.

TEHLIVETS, O., SCHEURINGER, K. & KOHLWEIN, S. D. 2007. Fatty acid synthesis and elongation in yeast. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids,* 1771**,** 255-270.

TEKOLO, O. M. 2007. *The osmotic stress response of basidiomycetous yeasts.* Stellenbosch University.
THORPE, R. & RATLEDGE, C. 1972. Fatty acid distribution in triglycerides of yeasts grown on glucose or n-alkanes. *Microbiology,* 72**,** 151-163.
TRUNIGER, V. & BOOS, W. 1993. Glycerol uptake in Escherichia coli is sensitive to membrane lipid composition. *Research in microbiology,* 144**,** 565-574.
TSIGIE, Y. A., WU, C.-H., HUYNH, L. H., ISMADJI, S. & JU, Y.-H. 2013. Bioethanol production from Yarrowia lipolytica Po1g biomass. *Bioresource technology,* 145**,** 210-216.
TUNBLAD-JOHANSSON, I. & ADLER, L. 1987. Effects of sodium chloride concentration on phospholipid fatty acid composition of yeasts differing in osmotolerance. *FEMS microbiology letters,* 43**,** 275-278.
TURK, M., MEJANELLE, L., ŠENTJURC, M., GRIMALT, J. O., GUNDE-CIMERMAN, N. & PLEMENITAŠ, A. 2004. Salt-induced changes in lipid composition and membrane fluidity of halophilic yeast-like melanized fungi. *Extremophiles,* 8**,** 53-61.
UENO, K., UNO, J., NAKAYAMA, H., SASAMOTO, K., MIKAMI, Y. & CHIBANA, H. 2007. Development of a highly efficient gene targeting system induced by transient repression of YKU80 expression in Candida glabrata. *Eukaryotic cell,* 6**,** 1239-1247.
UZUKA, Y., NAGANUMA, T., TANAKA, K. & SUZUKI, K. 1985. Relation between neutral lipid accumulation and the growth phase in the yeast, Lipomyces starkeyi, a fat producing yeast. *Agricultural and biological chemistry,* 49**,** 851-852.
VALADI, Å., GRANATH, K., GUSTAFSSON, L. & ADLER, L. 2004. Distinct intracellular localization of Gpd1p and Gpd2p, the two yeast isoforms of NAD+-dependent glycerol-3-phosphate dehydrogenase, explains their different contributions to redox-driven glycerol production. *Journal of Biological Chemistry,* 279**,** 39677-39685.
VAN DEN TEMPEL, T. & JAKOBSEN, M. 2000. The technological characteristics of Debaryomyces hansenii and Yarrowia lipolytica and their potential as starter cultures for production of Danablu. *International dairy journal,* 10**,** 263-270.
VAN ECK, J. H., PRIOR, B. A. & BRANDT, E. V. 1989. Accumulation of polyhydroxy alcohols by Hansenula anomala in response to water stress. *Microbiology,* 135**,** 3505-3513.
VAN WYCHEN, S. & LAURENS, L. 2013a. Determination of total lipids as fatty acid methyl esters (FAME) by in situ transesterification. *Contract,* 303**,** 375-300.
VAN WYCHEN, S. & LAURENS, L. 2013b. Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by in situ Transesterification: Laboratory Analytical Procedure (LAP). National Renewable Energy Laboratory (NREL), Golden, CO.
VARELA, C., PIZARRO, F. & AGOSIN, E. 2004. Biomass content governs fermentation rate in nitrogen-deficient wine musts. *Applied and Environmental Microbiology,* 70**,** 3392-3400.
VASDINYEI, R. & DEAK, T. 2003. Characterization of yeast isolates originating from Hungarian dairy products using traditional and molecular identification techniques. *International journal of food microbiology,* 86**,** 123-130.
VASUDEVAN, P. T. & BRIGGS, M. 2008. Biodiesel production—current state of the art and challenges. *Journal of Industrial Microbiology & Biotechnology,* 35**,** 421.
VORONOVSKY, A. A., ABBAS, C. A., FAYURA, L. R., KSHANOVSKA, B. V., DMYTRUK, K. V., SYBIRNA, K. A. & SIBIRNY, A. A. 2002. Development of a transformation system for the flavinogenic yeast Candida famata. *FEMS yeast research,* 2**,** 381-388.
WANG, J.-H., HUNG, W. & TSAI, S.-H. 2011. High efficiency transformation by electroporation of Yarrowia lipolytica. *The Journal of Microbiology,* 49**,** 469-472.

WANG, L., SUN, Y., WANG, P. & ZHAO, Z. 2005. Effects of metal ions on lipid production by fermentation with Trichosporon fermentans. *JOURNAL-DALIAN INSTITUTE OF LIGHT INDUSTRY,* 24**,** 259.

WATANABE, Y., SANEMITSU, Y. & TAMAI, Y. 1993. Expression of plasma membrane proton-ATPase gene in salt-tolerant yeast Zygosaccharomyces rouxii is induced by sodium chloride. *FEMS microbiology letters,* 114**,** 105-108.

WATANABE, Y. & TAKAKUWA, M. 1984. Effect of sodium chloride on lipid composition of Saccharomyces rouxii. *Agricultural and biological chemistry,* 48**,** 2415-2422.

WEI, C.-J., TANNER, R. D. & MALANEY, G. W. 1982. Effect of sodium chloride on bakers' yeast growing in gelatin. *Applied and environmental microbiology,* 43**,** 757-763.

WRENT, P., RIVAS, E.-M., DE PRADO, E. G., PEINADO, J. M. & DE SILÓNIZ, M.-I. 2015. Development of species-specific primers for rapid identification of Debaryomyces hansenii. *International journal of food microbiology,* 193**,** 109-113.

WYNN, J. P., HAMID, A. A., LI, Y. & RATLEDGE, C. 2001. Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi Mucor circinelloides and Mortierella alpina. *Microbiology,* 147**,** 2857-2864.

WYNN, J. P. & RATLEDGE, C. 2005. Oils from microorganisms. *Bailey's Industrial Oil and Fat Products*.

YADAV, J. S. & LOPER, J. C. 1999. Multiple P450alk (cytochrome P450 alkane hydroxylase) genes from the halotolerant yeast Debaryomyces hansenii. *Gene,* 226**,** 139-146.

YAGI, T. 1991. Regulation of intracellular osmotic pressure during the initial stages of salt stress in a salt-tolerant yeast, Zygosaccharomyces rouxii. *Microbios,* 70**,** 93-102.

YAGI, T. & NISHI, T. 1993. Regulation of intracellular osmotic pressure and changes in intracellular proteins during the initial stages of salt stress in Zygosaccharomyces strains exhibiting differences in salt-tolerance. *Microbios,* 74**,** 155-166.

YALE, J. & BOHNERT, H. J. 2001. Transcript Expression in Saccharomyces cerevisiae at high salinty. *Journal of Biological Chemistry*.

YAMADA, Y., NAGAHAMA, T. & BANNO, I. 1991. The molecular phylogeny of the Q9-equipped ascomycetous teleomorphic yeast genus Debaryomyces Lodder et Kreger-van Rij based on the partial sequences of 18S and 26S ribosomal ribonucleic acids. *The Journal of General and Applied Microbiology,* 37**,** 277-288.

YAMANE, T., SAKAI, H., NAGAHAMA, K., OGAWA, T. & MATSUOKA, M. 2008. Dissection of centromeric DNA from yeast Yarrowia lipolytica and identification of protein-binding site required for plasmid transmission. *Journal of bioscience and bioengineering,* 105**,** 571-578.

YAN, Z. & CNEN, J. 2003. Research advance on microbial oils and their exploitation and utilization. *Journal of Cereals & Oils,* 7**,** 13-15.

YANCEY, P. H. 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology,* 208**,** 2819-2830.

YANCEY, P. H., CLARK, M. E., HAND, S. C., BOWLUS, R. D. & SOMERO, G. N. 1982. Living with water stress: evolution of osmolyte systems. *Science,* 217**,** 1214-1222.

YI, S. & ZHENG, Y. 2006. Research and application of oleaginous microorganism. *China Foreign Energy,* 11**,** 90-94.

YONG-HONG, L., BO, L., ZONG-BAO, Z. & FENG-WU, B. 2006. Optimization of culture conditions for lipid production by Rhodosporidium toruloides. *Chinese Journal of Biotechnology,* 22**,** 650-656.

YOON, S. & RHEE, J. 1983. Lipid from yeast fermentation: effects of cultural conditions on lipid production and its characteristics of Rhodotorula glutinis. *Journal of the American Oil Chemists' Society,* 60**,** 1281-1286.

YOSHIKAWA, S., MITSUI, N., CHIKARA, K.-I., HASHIMOTO, H., SHIMOSAKA, M. & OKAZAKI, M. 1995. Effect of salt stress on plasma membrane permeability and lipid saturation in the salt-tolerant yeast Zygosaccharomyces rouxii. *Journal of fermentation and bioengineering,* 80**,** 131-135.

ZHANG, J., FANG, X., ZHU, X.-L., LI, Y., XU, H.-P., ZHAO, B.-F., CHEN, L. & ZHANG, X.-D. 2011. Microbial lipid production by the oleaginous yeast Cryptococcus curvatus O3 grown in fed-batch culture. *biomass and bioenergy,* 35**,** 1906-1911.

ZHAO, C.-H., CUI, W., LIU, X.-Y., CHI, Z.-M. & MADZAK, C. 2010. Expression of inulinase gene in the oleaginous yeast Yarrowia lipolytica and single cell oil production from inulin-containingmaterials. *Metabolic engineering,* 12**,** 510-517.

ZHAO, X., KONG, X., HUA, Y., FENG, B. & ZHAO, Z. K. 2008. Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast Lipomyces starkeyi. *European Journal of Lipid Science and Technology,* 110**,** 405-412.