Mutation of *Eremothecium gossypii* and Statistical Media Optimization to Increase Riboflavin Production

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I hereby declare that this dissertation represents my own work. It has not been submitted for any diploma/degree or examination at any other Technikon/University. It is being submitted for the Degree of Master of Technology: Biotechnology, in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa.

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I hereby approve the final submission of the following dissertation.

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This _____day of _____, 2010, at Durban University of Technology.

Dedication

Pranams at the lotus feet of my Most Merciful Creator
Bhagawan Shri Sathya Sai Baba,
My Nurturing Mother and Encouraging Sister

The mind is like a lake. When the water is calm,

the rays of the sun are reflected on the surface of water, like those on a mirror.

But if the water is disturbed because the wind is blowing, then there is very little reflection.

Man must learn to control his mind. Man is Divine, and if only he could remember that, he would see life so very differently;

he would cease to be affected by so much of what goes on in the world.

SHRI SATHYA SAI BABA…

Acknowledgements

"We all have obstacles to overcome. I have come to understand also that there are many people willing to accept and help you, if only you first accept and help yourself. It's true. If you are an angry person, the hostility drives people away from you, but if you are at peace and have confidence in yourself, people are drawn to you". Stedman Graham

My humble salutations to **My Creator**, without whom nothing in this universe is possible. ***"To whom much is given, much is expected"*** Baba

The dream begins with a teacher who believes in you, who tugs and pushes and leads you to the next plateau, sometimes poking you with a sharp stick called "truth." Dan Rather

I most sincerely thank ***Dr. Feroz M. Swalaha and Prof. K. Permaul***, for their inspiration and motivation to always strive for the best that you can do.

To my ***mother***, I thank you for all the sacrifices you have made to give me the best that you could, without which I would not be where I am today. **"*Mothers love is the fuel that enables a normal human being to do the impossible."*** Marion C. Garretty

For their continuous support, encouragement and making me believe in myself, I honour my loving ***sister Julie, niece Lavashnee and nephew Denolan***. "*People become really quite remarkable when they start thinking that they can do things. When they believe in themselves they have the first secret of success."* Norman Vincent

My since gratitude goes to my loving and caring friends ***Rena Brijal and Bilkis Kazi*** for all their words of encouragement and motivation that made the completion of this dissertation possible, friends like these should always be treasured***. "No man is an island, entire of itself; every man is a piece of the continent, a part of the main."*** Kristen Ison

For his undying love, support and inspiration during the trying times of carrying out this research and the insane times of doing the writing up, I want to say thank you to my best friend ***Raymond Ntshanagse. "Good humor is tonic for mind and body. It is the best antidote for anxiety and depression. It is a business asset. It attracts and keeps friends. It lightens human burdens. It is the direct route to serenity and contentment."*** Grenville Kleiser

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ABSTRACT

Eremothecium gossypii has the ability to utilize vegetable oils as a carbon source to produce riboflavin. This organism has been known to produce as much as 40 000 times more riboflavin than it requires after genetic modification on simple sugars. Adaptation of this organism to various oil substrates for riboflavin production has been poorly investigated. The aim of this research was thus to investigate the production of riboflavin by *Eremothecium gossypii,* on various oils and to improve production by mutating the organism and optimising media components using Design of Experiments (DOE). Nine overproducing mutants were obtained after mutating with various concentrations of ethylmethane sulphonate (EMS), *n*-methyl-*n*'-nitro-*n*-nitrosoguanidine (MNNG) and Ultraviolet light. Riboflavin overproducing mutants were screened on an itaconate-containing medium; the colonies appeared yellow instead of white in the case of the wild-type. The itaconate screening medium isolated mutants with an isocitrate lyase that was insensitive to feedback inhibition. Mutations performed using EMS increased the ability of *E. gossypii* to produce riboflavin by 611% (7-fold) compared to the wild-type. This was achieved with soybean oil as a carbon source and was better than the other five oils used. Using DOE, fractional factorial experiments were carried out to optimise media components for riboflavin production on soybean oil. The total riboflavin produced by *E. gossypii* mutant EMS30/1 increased from 59.30 mg l^{-1} on a standard O&K medium using soybean oil as a carbon source to 100.03 mg l^{-1} on a DOE improved O&K medium, a 69% increase. The final optimised growth medium was determined from a central composite design using response surface plots together with a mathematical point-prediction tool and consisted of 5.0 g l^{-1} peptone, 5.0 g l^{-1} malt extract, 5.1 g l^{-1} yeast extract, 0.64 g l^{-1} K_2HPO_4, 0.6 g l^{-1} $MgSO_4$ and 20 g l^{-1} soybean oil. Fractional factorial and central composite media optimization designs increased riboflavin production by several fold over their iterations. There was an overall increase of 1099% (12-fold) in riboflavin production by the mutant grown in an optimized medium compared to the initial riboflavin produced by the wild-type.

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1.0 LITERATURE REVIEW

1.1 INTRODUCTION

Malnutrition and famine threatens more than three quarters of the world's population. To fight nutritional deficiencies, vitamins are added to foods to fortify them. One of these vitamins is riboflavin, which plays an important role in the transfer of electrons in redox reactions. Humans are unable to produce riboflavin and must therefore obtain it from their diet. Due to the decrease in fresh food high in vitamins and increase in the intake of highly processed food and an increasing population there is a need to provide these essential vitamins in the form of supplements (Massey, 2000).

Riboflavin is produced in two ways; the first is a multi-stepped chemical process and the second, a single-step biological process. The chemical production process is an expensive and time-consuming method that was widely used, but is fast becoming less attractive when compared to the inexpensive, shorter and less raw materials consuming biological process. The biological method uses microorganisms to produce this valuable vitamin. Some of these known riboflavin-producing organisms included two fungi belonging to the ascomycetes class of organisms, *Eremothecium gossypii* and *Eremothecium ashbyi* (Burgess *et al.*, 2006).

A commonly used fungus for the production of riboflavin is *Eremothecium gossypii,* a filamentous phytopathogenic fungus that produces 40 000 times more riboflavin than it needs (Stahmann, *et al.,* 2001).

This natural overproducer was manipulated to increase its riboflavin yields for industrial applications. *Eremothecium gossypii* is preferred over other bacteria and fungi (*Bacillus subtilis*, *E. ashbyi, Corynebacterium ammoniagenes)* for the production of riboflavin because of its genetic stability. It has been recorded to produce maximum yields of 15 g l^{-1} riboflavin using glucose (Stahmann *et al.*, 2001). In 2003, Lim *et al.,* reported a riboflavin concentration of 2.5 g l^{-1} in a medium containing adsorbed soybean oil, which was 1.6-fold higher than the riboflavin produced in the medium without soybean oil. Over the years, many substrates have been used as carbon sources to produce riboflavin. Some of these substrates included whey, peanut seed cake, vegetable oil and molasses (Lim *et al.,* 2003, Stahmann *et al.,* 2001, Kalingan, 1998)

Eremothecium gossypii is an oil-utilising microorganism which catabolises oil as a carbon source by cleaving the lipids and using the liberated fatty acids for its storage lipid formation. This research attempted to improve riboflavin production by *E. gossypii* by physical mutagenesis using ultraviolet light and chemical mutagenesis using *n*-methyl-*n*'-nitro-*n*-nitrosoguanidine and ethylmethane sulphonate

(Gagliard, 2003). Once a successful mutant was obtained, it was grown on six different oil substrates to allow for the increase in riboflavin production. These oils were selected from literature as well as two oils (Coconut and mustard oil) that were not used before for riboflavin production. Soybean oil showed the highest production of riboflavin by the mutant EMS30/1 and was therefore used in optimization of media components using design of experiments.

1.1.1 Vitamins

1.1.1.1 Flavins as vitamins

A flavin is a tricyclic heteronuclear organic ring whose biochemical source is the vitamin riboflavin. Flavins have been documented as being capable of both one and two-electron transfers and play a fundamental role in coupling the two- electron oxidation of most organic substrates to the one-electron transfers of the respiratory chain (Massey, 2000). They are commonly referred to as versatile compounds that can have the multi-function of an electrophile and nucleophiles. Flavins have been used by bioluminescent bacteria for the production of light and are closely associated with light-initiated reactions such as plant phototropism and nucleic acid repair (Massey, 2000).

1.1.1.2 History of flavins

In 1887, an English chemist Wynter Blyth discovered flavins during his research on the components of milk. He isolated a yellow-green pigment, which he called lactochrome, which was later shown to be the compound we now refer to as riboflavin. Following his work, rapid development took place and interest in the yellow pigment with a bright greenish fluorescence grew. At this point, it was being isolated from a wide range of sources (dairy products, poultry, fish and meats).

Research intensified when this yellow pigment was recognized as being a component of the vitamin B complex. Kuhn from Heidelberg in 1938 and Karrer in 1937 from Zurich were the foremost scientists engaged in the race to determine the structure of lactochrome and produce it by chemical synthesis (Massey, 2000). Both scientists succeeded simultaneously and the compound was named riboflavin to replace the many previous names, some being lactoflavin and ovoflavin, which made reference to the sources of its isolation. The name was derived from its ribityl side chain and the yellow colour of the conjugated ring system (Massey, 2000).

1.2 Riboflavin

Riboflavin is a yellow water-soluble vitamin that is produced by both plants and microorganisms. However, higher animals lack this biosynthetic capability and must therefore obtain it from their diet (Burgess, 2006). Riboflavin (Figure 1.1) is the precursor of the enzyme cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) which are essential for most of the body's enzymatic functions, one of which is the transfer of electrons in oxidation-reduction reactions (Burgess *et al.*, 2006).

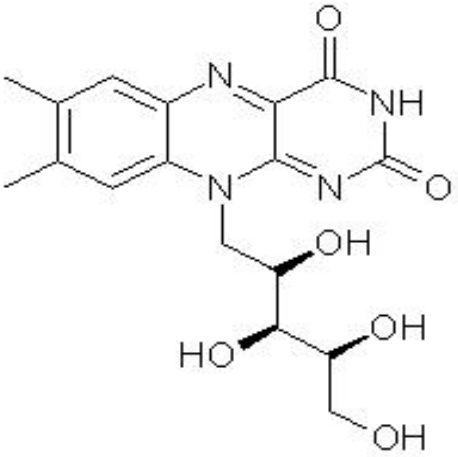

Figure 1.1 Chemical structure of riboflavin, showing the elements that make up this vitamin (Marx *et al.,* 2008).

1.2.1 Riboflavin dosage and deficiency

Riboflavin is crucial to energy production and metabolism and is one of the most important vitamins in the body. Due to it being water soluble, the body cannot store sufficient amounts and it must be replenished on a daily basis. A deficiency in riboflavin in humans leads to symptoms such as trembling, dizziness, poor concentration, memory loss and blood-shot, red, tired or gritty eyes a condition commonly referred to as ariboflavinosis (Survase *et al.*, 2006). In order to maintain health and prevent the above symptoms, the recommended dietary allowance for riboflavin must be adhered to (Table 1.1).

The U.S. Dietary Reference Intake (Recommended Dietary Allowance (RDA) for riboflavin is listed in Table 1.1

Table 1.1 Recommended dietary allowance of riboflavin for different age groups (Schoenen *et al*, 1998)

AGE GROUP	RECOMMENDED DIETARY ALLOWANCE (mg)
Infants 0–5 months	0.3
6–11 months	0.4
Children 1–3 years	0.5
4–8 years	0.6
9–13 years	0.9
Males 14 years and older	1.3
Females, 14–18 years	1.0
19 years and older	1.1
Pregnant women	1.4
Nursing women	1.6

There is, to date, no know toxic effects of riboflavin and any excess is expelled in the urine which may be a bright yellow colour. Riboflavin is light-sensitive and should therefore be kept in the dark and away from direct light. However, it is not sensitive to heat up to 290^{o}C or air and cannot be damaged by over-heating or cooking (Survase *et al.*, 2006).

1.2.2 Benefits of Riboflavin

Riboflavin is commonly referred to as a “nutraceutical”, referring to a food compound that supplements the diet and aids in the prevention and/or treatment of diseases and/or disorders (Burgess *et al.*, 2006). Riboflavin plays a key role in maintaining health. Some of its major health benefits are:

- **Production of energy:** by assisting in the metabolism of fats, carbohydrates, and proteins (Adnani, 2008).
- **Production of red blood cells:** essential for the formation of red blood cells and antibodies in humans (Burgess *et al.*, 2006).
- **Adjustment of thyroid activity:** Vitamin B_2 can regulate thyroid activity (Adnani, 2008).
- **Prevention of disease conditions:** helps to prevent many common diseases conditions such as migraine headaches, cataracts, acne, dermatitis, rheumatoid arthritis, and eczema (Adnani, 2008).

- **Prevention and control of acne:** improves the mucous secretion of skin and cleans up the skin pustules (Adnani, 2008).
- **Promotion of increased immunity:** enhances the natural immunity by strengthening the antibody reserves and by reinforcing the defense system against infections (Burgess *et al.*, 2006).
- **Repair of tissues:** repair of tissues, healing of wounds and other injuries, ensuring healthy cornea and vision (Adnani, 2008).
- **Protection of the nervous system:** treats nervous system conditions such as numbness, Alzheimer's disease, multiple sclerosis, anxiety and epilepsy among others. It is thought that riboflavin when used along with vitamin B_6 is effective to Carpal Tunnel syndrome (Adnani, 2008).
- **Slowing the progression of AIDS:** Studies have shown that riboflavin can slow down the progression of AIDS. Currently, in the field of AIDS research riboflavin is being used to treat nucleoside analogue-induced type B lactic acid acidosis, which occurs as a result of intensive AIDS drug treatment (Dalton and Rahimi, 2001).
- **Absorption of minerals:** It helps in the absorption of minerals such as iron, folic acid and other vitamins such as B_1, B_3 and B_6 (Adnani, 2008).

1.3 Biological production of vitamins

Biological production of vitamins has many advantages compared to the chemical process. The biological route involves a single step fermentation while the chemical production is a multi-step process. The products formed from chemical production methods are often mixtures and require a wide range of extraction processes to obtain the final purified vitamin this increases the time it takes to produce the desirable amount of vitamin (Survase *et al.*, 2006). Table 1.2 contains a list of biologically produced fat and water soluble vitamins, as well as the microorganisms used to produce them and the carbon sources utilized. These biologically-produced vitamins are now well established industrial fermentations.

Table 1.2 Summary of the vitamins being produced biologically (Survase *et al.*, 2006)

VITAMIN	ENZYME/ORGANISM USED	METHOD OF FERMENTATION AND CARBON SOURCE
Fat-soluble vitamins		
Vitamin E (α-tocopherol)	Freshwater microalgae *Euglena gracillis*	Fermentative conversion of glucose
Vitamin K_2	Mutated strains of *Bacillus subtilis*	Fermented using soybean extract
Water-soluble vitamins		
Vitamin C (ascorbic acid)	2,5-diketo-D-gluconic acid reductase *Cyanobacterium* sp.	Fermentative process to 2-keto-D-gluconic acid followed by chemical conversion to D-ascorbic acid
Vitamin B_7	Fermentation *Serratia marcescens*	Fermented by a genetically modified stain
	Multiple enzyme system (*Bacillus sphaericus)*	Conversion from diaminopimelic acid using the biotin biosynthetic enzyme system of a mutant of *Bacillus sphaericus*
Vitamin B_2	Fermentation (*Eremothecium ashbyi, Ashbya gossypii, Bacillus* sp.)	Fermentative conversion of glucose
Vitamin B_{12}	Fermentation (*Propionibacterium shermanii,*)	Fermentative conversion of glucose

1.4 Production of riboflavin using fermentation

In the 1980's, the world's riboflavin consumption was estimated to be around 1250 tons for both human and animals (Vandamme, 1992), in 2008 this increased to 6500 tons per year and 7000 tons in 2010. Riboflavin production is mainly a chemical process, but interest in the well-established biological process is increasing rapidly (Marx *et al.,* 2008).

Biological production of riboflavin is an excellent example of a success story where a well established chemical process is replaced with a more productive biological one. When biological riboflavin production entered the market in 1990, it contributed to about 5% of the global vitamin B_2 market. Seventy five percent of the vitamin B_2 market is produced via the cultivation of microorganisms (Karos *et al.*, 2004).

Fermentation of riboflavin is carried out using submerged cultures. Factors such as carbon source, minerals, pH and microbial strains contribute to the amounts of riboflavin produced. All of the above have to be at optimum levels for maximum riboflavin production. Many researchers have optimized these factors to yield high amounts of riboflavin (Ozbas and Kutsal, 1986 Stahmann *et al.,* 1994 and 1996, Stahmann *et al.,* 2001, Survase *et al.*, 2006,).

1.5 Industrial production of riboflavin

One of the first industrial producers of riboflavin was Badische Anilin- und Soda-Fabrik (Baden Aniline and Soda Factory) (BASF) a German chemical company, the largest chemical company in the world. Riboflavin was primarily produced to be added into animal feed to keep the livestock healthy and free from disease. In 1990, BASF replaced the chemical production process with a biotechnological one. The organism used was *E. gossypii* (Figure 1.2) and it is still being used today (Thayer and Houston, 2001).

Figure 1.2 Plate culture of *Eremothecium gossypii* used by BASF in their production of riboflavin (Thayer and Houston, 2001).

The fungus, *E. gossypii* has six enzymes (GTP cyclohydrolase II, 2,5-diamino-6-ribosylamino-4-(3H)-pyrimidinone-5'-phosphate reductase, 2,5-diamino-6-ribitylamino-4-(3H)-pyrimidinone-5'-phosphate deaminase, 3,4-dihydroxy-2-butanone-4-phosphate synthase,

6,7-dimethyl-8-ribityllumazine synthase and riboflavin synthetase that helps in the production of vitamin B_2 (Marx *et al.,* 2008).

Production depends on the growth of the fungus and the quality of its enzymes. Using this crucial information, BASF could increase riboflavin production exponentially by manipulating these enzymes. In 2008 BASF produced 1000 metric tons of riboflavin a year and currently holds 16 percent of the world market share for riboflavin production (Marx *et al.,* 2008).

1.6 Recovery of riboflavin

Downstream recovery of riboflavin was achieved by heating the production media (autoclaving at 121^{o}C for 30 min.) to inactivate the riboflavin-producing microorganisms and lyse the cells to release the riboflavin present inside the cells. Acetic acid (0.2 M) was then added to dissolve the riboflavin. The broth was then centrifuged using differential centrifugation to pellet cells. The supernatant was pasteurized (63^{o}C for 15 min) to kill any remaining viable cells. Riboflavin was finally extracted from the clear cell-free broth by evaporation and vacuum drying (Survase *et al.*, 2006). Inventers Kilbride *et al* in 1991 patented a method for spray drying of riboflavin to produce a granulated product. This was achieved by creating a mixture of the riboflavin together with a binder and water, which was then homogenized to allow the granules to be sprayed (Kilbride *et al.,* 1991)

1.7 Producers of riboflavin

Numerous valuable polymers that are used by both human and animals are synthesized by microorganisms. Vitamins are an example of these polymers. Higher organisms have lost this ability to produce these essential vitamins. However, lower life forms have retained the ability and are therefore used in vitamin synthesis (Lim *et al*., 2001).

Bacteria such as *Clostridium* spp and the yeast *Candida* spp are, according to literature, “good producers”, but the closely related ascomycete fungi *Eremothecium ashbyi* and *Eremothecium gossypii* are considered the best producers of riboflavin. Natural overproducers of riboflavin have been termed flavinogenic organisms (Marx *et al.,* 2008), Ferrous ions have been known to inhibit riboflavin production in both *Clostridium* and *Candida* but *E. ashbyi* and *E. gossypii* have the ability to repress the adverse effect of iron, therefore overproducing riboflavin (Survase *et al.*, 2006).

1.7.1 *Bacillus subtilis*

To obtain riboflavin from a gram-positive prokaryotic bacterium requires the deregulation of purine synthesis and mutation in a flavokinase/FAD synthetase, which is common in all organisms that produce riboflavin, producing the recognisable yellow-coloured colonies (Stahmann *et al.*, 2000). *Bacillus subtilis* has the ablity to produce riboflavin since it has a

cluster of five non-overlapping genes encoding for the enzymes needed to calalyse riboflavin production starting at GTP (Solovieva *et al.,* 1999). Bacteria frequently first consume their preferred substrate in a batch growth mixture of carbon substrates. Consumption of any other substrate/s occurs only when the preferred one is depleted. This, in turn, leads to a diauxic pattern of growth (Stahmann *et al.,* 2000).

1.7.2 *Eremothecium ashbyi*

Guilliermond discovered this filamentous ascomycete in the early 1900s and much work has been done to date on this natural riboflavin overproducer. Early media development produced 200 mg l^{-1} of riboflavin, later tween-80, protein and glycine was added and the maximum production was increased to 1.4 g l^{-1}. In 2001 Lim *et al.* produced between 1.5 to 2.5 g l^{-1} using the same organism (Lim *et al.,* 2001). Mutation of this organism and the addition of 8-azaguanine to the medium by Madia *et al,* in 2003 resulted in a further increase to about two to four times more than the original strain.

1.7.3 *Eremothecium gossypii*

Eremothecium gossypii is commonly known as Ashbya gossypii and is a haploid hemiascomycete that was first described in 1926 as cotton pathogen transmitted by sucking insects and caused dry-rot in fruit. This hemiascomycete has the smallest genome yet to be characterized amongst living eukaryotes. *Eremothecium gossypii* is known as one of

the best industrial producers of riboflavin. Therefore, its biosynthesis, regulation and production methods have been widely studied, which in turn has resulted in an established fermentation process which to date has produced a recorded maximum yield of 15 g l^{-1} (Forster, 2001).

Eremothecium gossypii has natural enzymes that it uses to produce riboflavin. The amount produced depends on the quantity of enzyme and the growth conditions. This organism has the ability to use vegetable oils to produce high amounts of riboflavin as well (Park *et al.,* 2007). Park and his team of scientists in 2007 used oil waste to produce riboflavin they succeeded in producing 8.7 g l^{-1} in a period of 5 days.

Two studies have reported that the duplication of the genome of *E. gossypii* more that 100 million years ago gave rise to the common baker's yeast. Research has shown that more than 90 percent of the *E. gossypii* genes show homology with *S. cerevisiae*, investigation of these patterns revealed 300 inversions and translocations that have occurred. This provides evidence that the evolution of *S. cerevisiae* included a whole genome duplication or fusion of the two related species (Stahmann, *et al.,* 2000).

1.8 *Eremothecium gossypii* for riboflavin production

Eremothecium gossypii is preferred over other microorganisms (*Bacillus subtilis, E. ashbyi, and C. ammoniagenes*) for the production of riboflavin because of its genetic stability (Stahmann *et al.,* 1996). When *E. gossypii* is used in fermentation processes to produce riboflavin, oils are used as substrates instead of glucose. The use of triglycerides (oils) by *E. gossypii* increased productivity of riboflavin (Stahmann, *et al.,* 1994). It was reported that the addition of sunflower oil had increased the production of riboflavin (Ozbas and Kutsal, 1986) using the riboflavin producers, *E. gossypii* and *E. ashbyi*. Numerous efforts have been made to improve riboflavin production using edible vegetable oil as a substrate and oil-utilising microorganisms like *E. gossypii* and *E. ashbyi* (Ozbas and Kutsal, 1986, Stahmann *et al*., 1994, Kalingan 1998, Stahmann *et al.,* 2001,).

1.9 Riboflavin production

1.9.1 The riboflavin production pathway

To form one molecule of riboflavin, one molecule of GTP and two molecules of ribulose 5-phosphate are required. There are six RIB genes that are responsible for the overall production of riboflavin in the pathway (Figure 1.3), with small differences in bacteria and fungi. Each gene controls its own enzyme production and action (Marx *et al.,* 2008).

There are six RIB genes and their corresponding enzymes in the riboflavin production pathway: *RIB1*: GTP cyclohydrolase II, *RIB2*: 2,5-

diamino-6-ribitylamino-4-(3H)-pyrimidinone-5'-phosphate deaminase, *RIB3*: 3,4-dihydroxy-2-butanone-4-phosphate synthase, *RIB4*: 6,7-dimethyl-8-ribityllumazine synthase, *RIB5*: riboflavin synthetase, *RIB7*: 2,5-diamino-6-ribosylamino-4-(3H)-pyrimidinone-5'-phosphate reductase

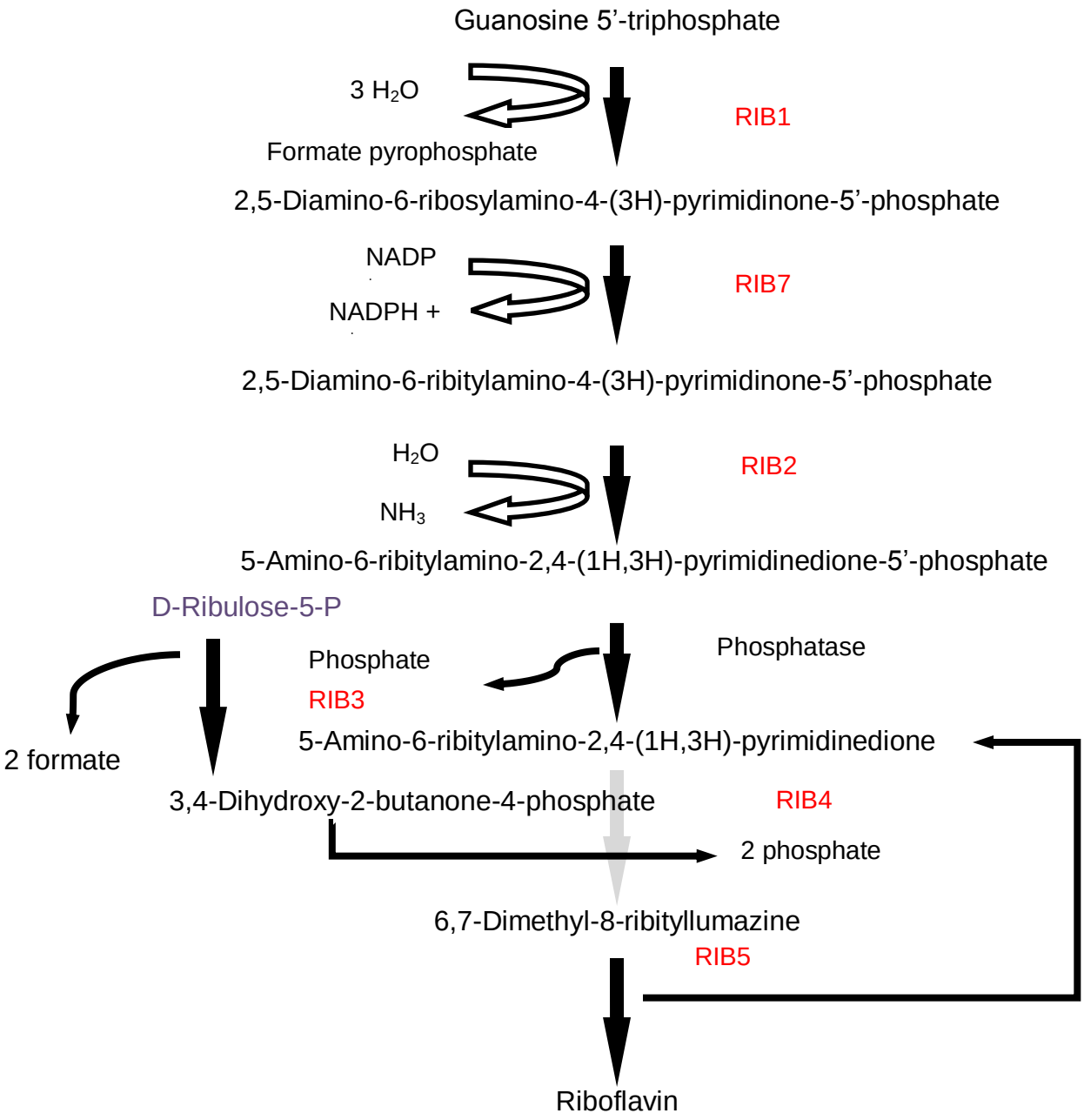

Figure 1.3 Schematic representation of the metabolic pathway of riboflavin biosynthesis in *Pichia pastoris* (Marx *et al.*, 2008).

1.9.2 Production rates and amounts

Riboflavin production rates and amounts differ from one species to another depending on their utilization of a suitable carbon source. In 2003, Sung *et al.* reported a riboflavin concentration of 2.5 g l^{-1} in the medium containing adsorbed soybean oil, which was 1.6 fold higher than the riboflavin produced in the medium without the soybean oil. Riboflavin is not produced in the early growth phase. When all the glucose in the medium was consumed riboflavin production began in the late growth phase (Stahmann *et al.*, 2001). Wu *et al.,* in 2007, used a recombinant strain of *Bacillus subtilis* and statistical designs to obtain a maximum riboflavin concentration of 6.65 mg l^{-1}, which 76.4% higher than it produced on a standard medium. With the aid of recombinant strains researchers have been able to vastly increase riboflavin production rates. Using a recombinant strain of *Bacillus subtilis* Perkins *et al.,* 1999 produced 15 g l^{-1} of riboflavin in 56 h in a fed-batch system (Perkins *et al.,* 1999).

1.10 Mutation to improve riboflavin production yields

There are two basic forms of mutation one is a loss of function (when the gene products lose the ability to perform their function) and the other is a gain of function (when there are additions to the genes characteristics). The first type allows the geneticist to examine what a cell does without a specific gene that is if there is only one present. If

more are part of the gene product it is possible to mutate one and monitor the other. In the second type of mutation, by studying the function in a cell that is exaggerated, that function that the gene product has can be inferred (Trun and Trempy, 2004).

Microlesions are mutations that involve a single nucleotide change, whereas macrolesions are those that affect multiple nucleotide bases. Single nucleotide (point) mutations are examples of microlesions. These occur when there is a base substitution. In this type of mutation, a purine can substitute a purine or a pyrimidine for a pyrimidine. This type of mutation is known as a transition. Point mutations that substitute a purine for a pyrimidine or a pyrimidine for a purine are called transversions. A frameshift mutation is also another form of microlesions, in the addition or deletion of a single base pair in a gene sequence. There is an alteration in the reading frame. This causes a change in the mRNA sequence and therefore a change in the protein product formed. If a frameshift results from an addition of a single base it is referred to as a +1 frameshift and if there is a deletion it is called a -1 frameshift (Trun and Trempy, 2004).

1.11 Mutagens

Chemical or physical mutagens can increase the mutation rate of DNA. Mutagens are grouped into four main types; chemicals that behave like normal bases (base analogues); chemicals that react with bases (base modifiers); chemicals that bind DNA (intercalators); and agents that physically change DNA (Trun and Trempy, 2004).

1.11.1 Physical mutagenesis

1.11.1.1 *Ultraviolet light*

Ultraviolet radiation, also referred to as non-ionizing radiation, mainly affects the cytosine and the thymine of the DNA strand, changing its properties. This type of mutation falls under the category of induced mutations. The damage of DNA by ultraviolet light (UV) started investigations on the repair of DNA and the exposure of the cells to UV irradiation. Ultraviolet irradiation damages DNA at a wavelength of 260 nm, which can be available from a normal germicidal lamp. DNA has an absorption peak of about 254 nm and wavelengths above this causes DNA damage (Friedberg *et al.,* 1995). Ultraviolet light causes the formation of pyrimidine dimers. These can block DNA replication. Also, dimers interfere with base-pairing between the two DNA polymers (Otto *et al*., 1981). Ultraviolet light also generates free radicals, which creates different kinds of genetic damage causing mutations in bacteria and other cells (Friedberg *et al*., 1995).

1.11.2. Chemical Mutagenesis

Chemical mutagens are compounds that increase the frequency of some types of mutations. Varied in their potency, these reflect their ability to enter the cell, their reactivity with DNA, their general toxicity and the type of chemical changes the DNA goes through will determine what type of repair will be introduced (Friedberg *et al.,* 1995).

1.11.2.1 *N-methyl-n'-nitro-n-nitrosoguanidine (MNNG)*

N-methyl-*n*'-nitro-*n*-nitrosoguanidine reacts with various nucleophiles, especially with amines and thiols at acid pH. The chemical slowly releases nitrous acid when dissolved in these solutions. Diazomethane, a toxic gas, is produced when MNNG comes into contact with alkali hydroxide (Ul-haq *et al*., 2008). *N*-methyl-*n*'-nitro-*n*-nitrosoguanidine has been used as a popular mutagen to induce mutants that exhibit potential improvement production properties (Ohnishi *et al*., 2008). In 2009, Tajima *et al.* used this mutagen to increase riboflavin production from activated bleaching earth using *E. gossypii.* The mutation was successful as riboflavin concentration was increased by 10 fold (Tajima *et al*., 2009).

1.11.2.2 *Ethylmethane sulphonate (EMS)*

Ethylmethane sulphonate ($C_3H_8O_3S$) is a base-altering mutagen, which chemically alters normal bases in DNA and causes increased frequency of mispairing. It is known to cause transitions by methylation of G residues in microorganisms but can produce a number of other

mutations (Moses, 2004). This mutagen can be used in both forward and reverse mutagenesis (Kim *et al*., 2006). Ethylmethane sulphonate is an alkylating agent which has a cytotoxic effect via. the transfer of alkyl groups. DNA cross-linking is a primary role of these agents. Replicating cells are most susceptible and alkylating agents are not cell-cycle specific (Friedberg *et al*., 1995). In most cases (99% of the time) EMS induces C to T transitions thus resulting in a C/G to T/A base replacement. In low frequency, EMS generates G/C to C/G or G/C to T/A transversions using 7-ethylguanine hydrolysis or A/T to G/C transition by 3-ethyladenine. This is a pairing error (Kim *et al*., 2006).

This mutagen was used by Ul-haq, *et al*. (2008) to randomly mutate *S. cerevisiae* to enhance its production of D-fructofuranosidase (FFase). This mutagen produced a mutant that produced 25.56 U ml^{-1} of FFase when compared to the wild-type which produced 1.10 U ml^{-1}. Research has been conducted where mainly random mutagenesis and screening with metabolites have been used, with large emphasis being placed on the precursors upstream of riboflavin synthesis (Stahmann *et al.,* 2001 and 1996).

1.12 Screening for mutants

1.12.1 Itaconic Acid

When produced by microorganisms riboflavin displays a characteristic yellow colour, which can be used in the screening of mutants. Itaconate, an antimetabolite that inhibits isocitrate lyase, which is a peroxismal enzyme that causes mutants of *Eremothecium gossypii* to produce yellow colonies instead of white ones (wild-type). Isocitrate lyase directs the carbon flux into a carbon-conserving pathway. This makes isocitrate lyase an indicator of growth and riboflavin synthesis. The antimetabolite is introduced into the growth medium and if the colonies produce a yellow colour then the mutant is a successful riboflavin producer. This antimetabolite was used by Stahmann *et al.* (2000) in their research on three biotechnical processes using *A. gossypii, C. famata,* and *B. subtilis* to compete with chemical riboflavin production, to screen for improved riboflavin producers. These overproducer were screened based on their colour (yellow – overproducer).

1.13 Substrates used in riboflavin production

Various substrates have been used in the production of riboflavin. These include compounds like whey, peanut seed cake, a range of vegetable oils, molasses, rape seed oils and spent oils (Park and Ming, 2003). Lizama *et al.* in 2007 produced 225 mg l^{-1} of riboflavin from orange rind. *Eremothecium gossypii* is an oil-utilising microorganism

which uses oil as a carbon source by cleaving the lipids and uses the liberated fatty acids for its storage lipid formation, therefore oils/lipids would be the substrate of choice to produce riboflavin using *E. gossypii* (Lizama *et al*., 2007). Below is a list of the potential oil substrates that can be used (Table 1.3).

1.13.1 Fat Composition of Oils

Oils contain saturated, monounsaturated and polyunsaturated fats. The fat compositions of oils are listed in Table 1.3.

Table 1.3 Fat composition of various Oils (Gunstone, 2002)

Type of Oil or Fat	Saturated	Monounsaturated	Polyunsaturated
Mustard Oil	1%	76%	23%
Canola Oil	6%	62%	32%
Almond Oil	8%	73%	19%
Sunflower Oil	11%	20%	69%
Grape Seed Oil	12%	17%	71%
Olive Oil	14%	77%	9%
Sesame Oil	14%	40%	46%
Walnut Oil	14%	19%	67%
Soybean Oil	15%	24%	61%
Peanut Oil	18%	49%	33%
Margarine (Soft)	20%	47%	33%
Cottonseed Oil	24%	26%	50%
Palm Oil	52%	38%	10%
Butter	66%	30%	4%
Margarine (Hard)	80%	14%	16%
Palm Kernel Oil	86%	12%	2%
Coconut Oil	92%	6%	2%

There are two main commodities from vegetables, oil and a protein rich meal. Two extraction procedures are carried out to obtain these components, namely pressing and/or solvent extraction. Oil is obtained in different concentrations from these seeds and fruit. In January 2002, the average oil yields were as represented in Figure 1.4 (Gunstone, 2002).

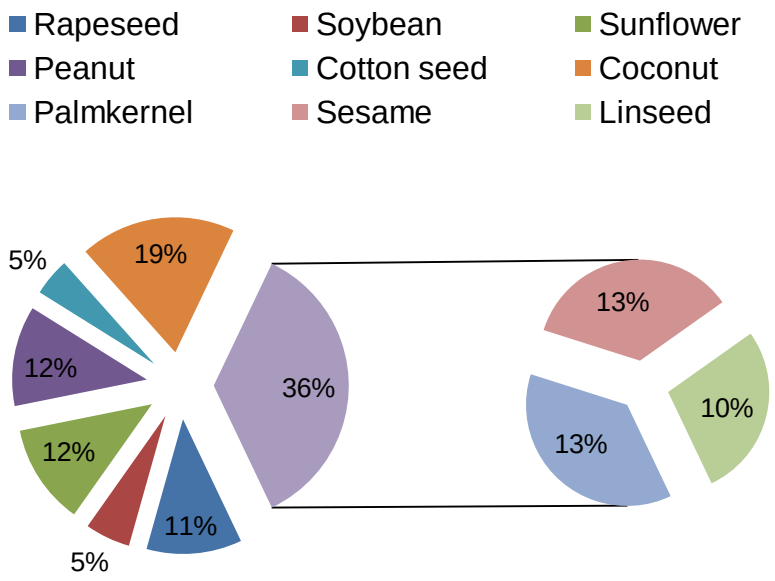

Figure 1.4 World average oil yields (Gunstone, 2002).

About 117 million tons per year comes from vegetable and animal sources. Of the total world production of oils and fats, 80% is used for food additives, 6% in animal feeds and 14% is used in the oleochemical industry (Gunstone, 2002).

Vegetable oil can be broken down into three types:

- Byproducts: cotton is grown primarily for fibre and corn for cereal. The oil is a byproduct of these. Soybean falls into this category, for it gives two products an oil and a meal.
- Tree crops: these oils are harvested from mature trees grown primarily for oils production. Examples of these crops are palm, coconut and olive.
- Annual crop: this is planted yearly and decisions on which crop to plant are critical, as the climate plays a large role in the harvesting. Annual crops include grape, sunflower and linseed (Gunstone, 2002).

1.13.2 Mustard oil

Mustard refers to several *Brassica* species that are valued for their spicy and pungent dried seeds. Reaching a height of one metre, these plants have branching stems, yellow flowers, brown fruit, and brown-black seeds (Tinoi *et al.*, 2004). The seeds are the source of rapeseed or colza oil and are used as industrial lubricating oil and edible salad oil. The enzymatic action of myrosin on the glucoside sinigrin in black and brown mustard or on sinalbin in white mustard releases the mustard oil, which consists principally of allyl isothiocyanate in black and brown mustards and p-hydroxybenzyl isothiocyanate in white mustard, the

compounds responsible for the pungency (Simon *et al.*, 1984). Tinoi *et al.* 2004 used mustard meal as a substrate for the growth of *Xanthophyllomyces dendrorhous* (yeast) and production of astaxanthin. A maximum yield of 6.25 g l^{-1} dry cell weight was obtained with 3.83 mg l^{-1} astaxanthin (Tinoi *et al.*, 2004).

1.13.3 Peanut oil

The ground nut plant (*Arachis hyogaea)* is a legume native to South America and has been cultivated since 3000 BC. Peanut seeds contain 40-50% oil and are thus considered an excellent source of oil (Sanders, 2002). Peanut oil contains small traces of nonglyceride, its fatty-acid composition is complicated; including saturated fatty-acids covering a wide range of molecular weights. Linolenic acid appears to be entirely absent. Traces of eicosenoic acid have been found. The unsaponifiable portion of peanut oil includes tocopherols and other antioxidants, sterols, squalene, and other hydrocarbons (Shi *et al*., 1998).

Peanut seed cake was used in 1998 by Kalingan, to investigate the kinetics of riboflavin secretion by *E. ashbyi* NRRL 1363. This carbon and nitrogen source favoured the growth of this riboflavin producer as the mycelium grew strongly and its growth was at an optimum within 3 to 4 days.

1.13.4 Sesame oil

Sesame (*Sesamum indicum)* seeds are used whole, or processed for oil and meal, these seeds have high oil content (about 50%) but crops are heavily variable. *Sesamum* species is the oldest oilseed crop known to man (Kochhar, 2002). The oil contains approximately 10% palmitic acid, 4% stearic acid, 47% oleic acid and 39% linoleic acid, is expensive and used for culinary purposes mainly in countries of its production. Its good conservation capacity is due to the presence of a potent antioxidant, sesamol that is released from sesaminol upon hydrolysis by storage. Sesaminol plays an active role in the physiological suppression of lipid per-oxidation.

The oil is also used as a solvent or carrier for medicines and cosmetics. 'Tila or Til' as it is called in India is widely used in domestic Ayurvedic medicines (Cunha, 2005). Seed colour may vary from a white to different shades of brown, violet and gold, together with its colours and characteristic flavour is widely used in the food industry as a flavour additive or a garnish (Kochhar, 2002).

These plants are annual crops and grow in hot dry conditions, the seed are drought-resistant and can adapt well to these regions (Cunha, 2005). Depending on the species and the region in which it is grown the sesame seed plant can be ready for harvesting from between 80-140

days in India, 80 days in the USA and Mexico, to 60 days in Sudan (Kochhar, 2002).

Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using coconut and sesame oilcakes as a substrate was carried out by Ramachandra *et al.* 2005. It was shown that a combination of coconut oil cake and sesame oil cake produced a two-fold increase at optimal conditions, producing 64 U/gds phytase when compared to 30.1 U/gds produced by coconut oil cake alone (Ramachandra *et al.,* 2005).

Sesame seed oil cake was used in 2000 by Pujari and Chandra in their study to increase riboflavin production using statistical optimization of medium components. They produced 1070 µg ml^{-1} using this substrate in conjunction with molasses, NaCl, yeast extract and tween 80 (Pujari and Chandra, 2000).

1.13.5 Coconut oil

Copra, which is the white, dried, fleshy meat of the coconut, is the component from which the oil is derived. *Cocos nucifera* species of coconut palm grows well in humid areas. Figure 1.5 refers to the makeup of a typical coconut.

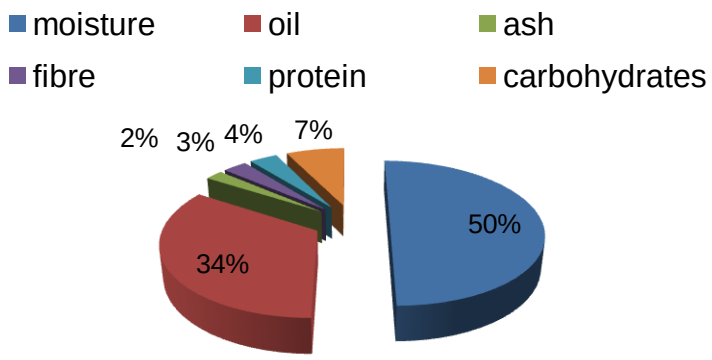

Figure 1.5 Percentage make up of a typical coconut (Pantzaris and Basiron, 2002).

Lauric acid is most predominantly found in coconut oil. Lauric acid is a medium-chain fatty acid, which has the additional beneficial function of being formed into monolaurin in the human or animal body. Monolaurin is the antiviral, antibacterial, and antiprotozoal monoglyceride used by the human or animal to destroy lipid-coated viruses such as herpes, cytomegalovirus, influenza, various pathogenic bacteria, including *Listeria monocytogenes* and *Helicobacter pylori*, and protozoa such as *Giardia lamblia* (Pantzaris and Basiron, 2002).

Besides triacylglycerols and free fatty-acids, unrefined coconut oil is made up of 0.5% unsaponifiable matter, which consists mainly of sterols, tocols, squalene, colour compounds, carbohydrates and odour components like lactones. The characteristic smell and odour of coconut oil is due to the *lactones* (Pantzaris and Basiron, 2002).

Coconut oil cake was used in 2005 by Ramachandra *et al.* in their research on mixed substrate fermentation to produce phytases. *Rhizopus oryzae* NRRL 1891 using coconut oil cake produced 30.1 U/gds of phytases. This substrate was the most preferred compared to sesame oil cake, palm kernel cake, groundnut oil cake, cottonseed oil cake and olive oil cake (Ramachandra *et al.,* 2005).

1.13.6 Sunflower oil

Sunflower oil is mainly composed of linoleic acid in triglyceride form. However, it does consist of many other components such as 4-9% palmitic acid, 1-7% stearic acid, 14-40% oleic acid, 48-74% linoleic acid, lecithin, tocopherols, carotenoids and waxes. Sunflower oil is produced from sunflower seeds, which are high in vitamin E and a combination of mono-unsaturated and polyunsaturated fats (Gupta, 2002)

Linoleic oil is sunflower oil that is most commonly found in foods. It has a high essential oil content and is rich in vitamin E. Oleic oil has a high monounsaturated acid concentration, which makes it appropriate for use in restaurants and cosmetic industries. In restaurants, sunflower oil is often used because it can withstand high temperatures, which makes it appropriate for cooking and frying. Sunflower oil is used in cosmetics due to its ability to retain the skin's moisture (Gupta, 2002). Vegetable oil was used in 2009 by Tajima *et al.* in their research on increasing

riboflavin production from activated bleaching earth by a mutant of *E. gossypii* (Tajima, *et al.,* 2009). They showed that when vegetable oil was used as a sole carbon source, the glyoxylate cycle played a vital role in the mediation of cell growth and riboflavin production. Isocitrate lyase (ICL) is an enzyme that converts isocitrate into glyoxylate, by measuring this conversion, they showed that their MNNG mutant produced 3.7 g l^{-1} riboflavin compared to the wild-type which produced 0.8 g l^{-1} (Tajima, *et al.,* 2009).

1.13.7 Soybean oil

Soybeans (*Glycine max*) are usually oval shaped when they are mature and their size depends on the variety of cultivar. This high protein bean is made up of three parts: the hull/seed coat, cotyledon and the hypocotyls/germ. The oil is extracted using solvent extraction using hexane or mechanical pressing. The oil obtained from this process is referred to as crude extract and needs to be refined before it can be used (Wang, 2002). The primary lipids found in soybean oil are triacylglycerols.

This oil is produced in large quantities by the USA (28%), Brazil (12%), Argentina, China (13%) and the Europe-15 (7%). Argentina is amongst the biggest exporters of soybean oil (43% of its total production) and in 2000 India imported roughly 1.2 million tons of this widely-used oil

(Gunstone, 2002). Soybean oil makes up over half of all oil seed worldwide (Wang, 2002).

Using *E. gossypii* and the fermentation of soybean oil, Schmidt *et al.* in 1996 showed that there was a correlation in the isocitrate lyase activity and riboflavin production. They also demonstrated that when a itaconate-resistant mutant was grown on soybean oil it had a 25-fold increase compared to the wild-type (Schmidt *et al.,* 1996).

Peanut, sunflower, soybean and sesame seed oils have been used in previous studies by *E. gossypii* to produce riboflavin however; coconut oil and mustard seed oils have not been used before.

1.14 Statistical media optimization

Numerous statistical methods have been used in media optimization. These methods of experimentation have led to reducing the number of experiments and quickly finding main factors that play a role in the optimization process (Cai *et al.,* 2009).

1.14.1 Design of Experiments

Design of experiments (DOE) is a statistical problem-solving approach to running experiments that uses fewer well-planned small matrix-type experiments compared to the one-at-a-time approach. The reason behind doing a series of smaller experiments is that as the study continues, the type of experiments can change in order to show the changing objectives (Haaland, 1989).

1.14.2 History of Design of Experiments

The one-factor-at-a-time (OFAT) method was called the “scientific” approach attributed to the 17^{th} century by Francis Bacon, but its origins can be found as far back as the Greeks in 1600 BC. Ronald Fisher, working in the agricultural field at the time developed a new approach to experiments called the two-level factorial design. Mathematically, he showed it was 2^k, where k represented the number of experiments. His experimental designs overcame the year-long growing cycles by introducing many factors in parallel using matrix-based test plans (Anderson, 2008).

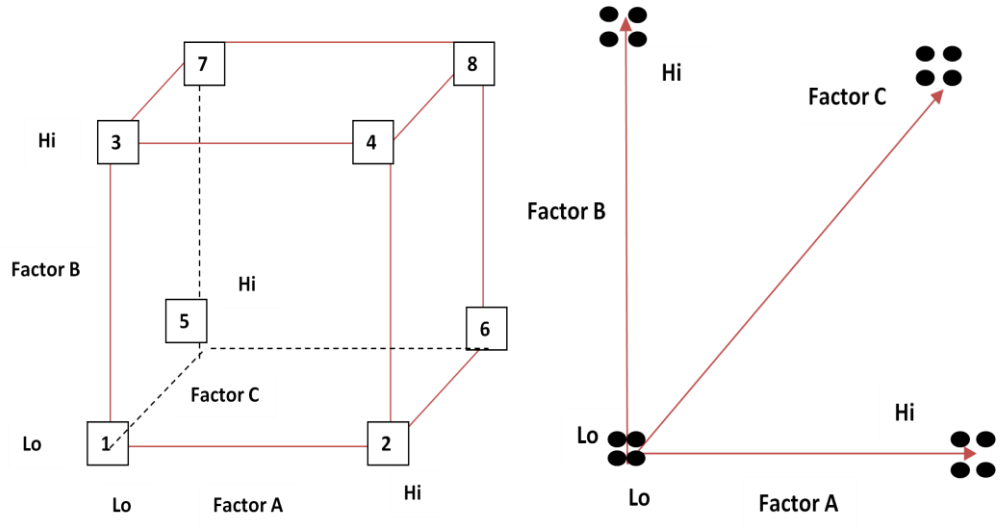

Figure 1.6 Comparison of the two level factorial designs (left) versus one factor at a time (right).

The two-level factorial designs (Figure 1.6) offer four runs at the high level (A) and the same for the low level. Likewise factors B and C have four runs at high and low levels each. If the OFAT method had to provide the same level of replication, its experiments would total 16 runs compared to the two-level design of 8 runs. Due to this the 2^k, DOE is advantageous and this becomes more evident as the number of factors increases (Anderson, 2008). Design of experiments allows scientists to become more productive and efficient, using a form of multivariable testing to the OFAT method, thus gathering knowledge faster.

1.14.3 Data analysis of the factional factorial optimization

1.14.3.1 *Analyses of variance (ANOVA)*

ANOVA compares the hypothesis of difference means of two or more factors, provided the means are normally distributed. $P<0.05$ and below indicate that there is a significant difference in the means of the selected factors with a confidence of 95% (Anthony, 2003). This analysis method is used to determine if the model used to predict the riboflavin concentration is significant or not, thus determining if all the data points fit the model. Analysis of variance was used in this study to determine if the dependant and the independent variables had a linear relationship (Tobias, 2003).

1.14.3.2 *Pareto Charts*

This chart ranks the effects of the media components according to their results in the final evaluation. The chart shows two different *t*-values, one being the Bonferroni limit and the other a standard *t*-value. Those effects that fall above the Bonferroni limits are significant and those that are above the *t*-value possibly significant and therefore, if not selected should be.

The Pareto principle also known as the 80-20 rule, demonstrates that for many events about 80% of the effects come from 20% of the causes. This vital principle was named after Vilfredo Pareto, who noted that 80% of the land in Italy was owned by 20% of the people. In mathematics

when something is shared among a large number of factors, there will be a number *k* between 50 and 100 such that *k*% is taken by (100-*k*) % of the factors, but *k* may vary from 50 (in cases of equal distribution) to nearly 100 with a small number of factors account for almost all the resources (Anderson, 2008).

1.14.3.*3 Box-Cox normality plots*

Several statistical test and analysis methods are based on the hypothesis of normality, however many data sets are not approximately normal. Due to this hypothesis an appropriate transformation of the data set yields values that follow a normal distribution. Using theses transformation methods increases the applicability and usefulness of statistical techniques. The Box-Cox power transformation is one such technique that can be used to transform response data to normality and is defined as follows:

$$T(Y) = (Y^{\lambda} - 1)/\lambda$$

Where Y is the response variable and the λ is the transformation parameter.

When λ= 0, natural log is used instead of the above equation (Tobias, 2003).

In figure 1.7 the green line is the best model fit, blue line is the current transformation and the red line indicates the confidence interval. If the blue line fall between the red lines, then no transformation is required.

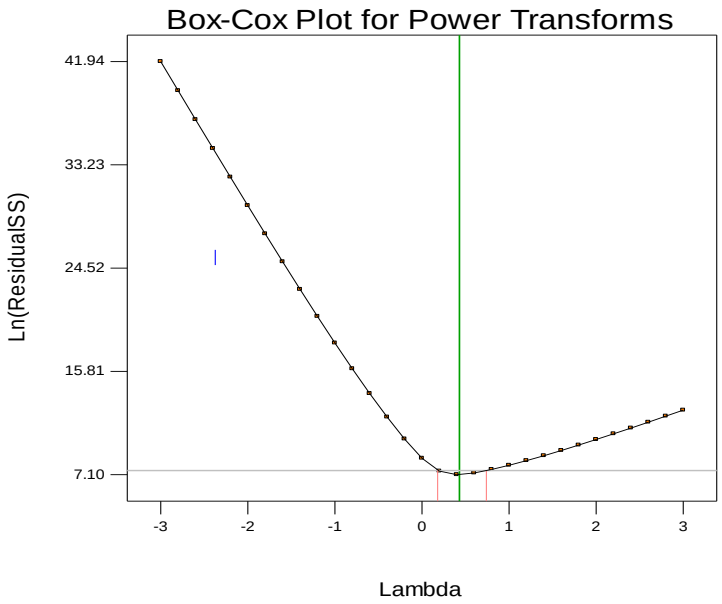

Figure 1.7 Box Cox plot of power transformation, indicating the position of lambda (Tobias, 2003).

1.14.3.4 *Cube plots*

These indicate three factor effects at a time. This plot shows predicted values from the model for combinations of both upper and lower levels of any three effects (factors) selected. These plots are used to ascertain the steepest ascent or descent of an optimization experiment (Anthony, 2003). The cube plot was used in this research to further investigate the effects that were determined to be important to increase riboflavin production.

1.14.3.5 *Contour plot*

These show the relationship between three factors represented as two dimensional, two factors for the X and Y axes, while factor Z is the contour lines, which are plotted as curves and the areas between them are coloured coded to represent interpolated values (using know values to determine the unknown) (Anderson, 2008). These contour plots give a two dimensional view of selected factors. They helped predict the areas of highest riboflavin production in screening and optimization experiments.

1.15 Summary

A range of essential vitamins cannot be manufactured by the body and must therefore be obtained from the foods we eat. There is a huge market for the production of these vitamins. Most of the production processes are chemically-based, expensive and are not environmentally-friendly, therefore, much research has gone into developing a biological based, inexpensive and environmentally-friendly process. These biological processes have been widely implemented in many industries worldwide and have proven to be very productive in the amounts of vitamins produced. They have also increased the profit margin, due to the process being considerably less expensive.

Biological production uses a number of different microorganisms (*E. ashbyi, E. gossypii, Bacillus* sp*.)* to produce vitamins. One such biologically produced vitamin is riboflavin. Many researchers have shown that mutation of a riboflavin producing organism may cause the mutated strain to increase the amount of riboflavin being produced. This research deals with physical mutations using ultraviolet light and chemical using MNNG and EMS. After mutation the wild-type and the mutant were compared to determine if the mutant produced more riboflavin than the wild-type, followed by using the resulting mutant to determining which oil carbon source would yield the most riboflavin. Six oils were selected for this study, four of which were from previous research and two new oils that have not yet been used for riboflavin

production. Statistical media optimization methods (DOE) were then used to further increase the riboflavin production rate for that mutant.

1.16 Aim

To investigate the production of riboflavin by *Eremothecium gossypii* on various organic oils and to improve riboflavin production on the highest yielding oil by both chemical and physical mutation together with optimisation of media components using Design of Experiments.

Objectives

i) To formulate an optimal medium for the growth of *Eremothecium gossypii* wild- type, using supplements at varying concentrations.

ii) To measure riboflavin produced by the growth of the wild-type in order to determine baseline growth and riboflavin production.

iii) To subject the wild-type *Eremothecium gossypii* to physical and chemical mutagens and to screen for over-producers of riboflavin using itaconate as the screening pressure.

iv) To compare the amount of growth and riboflavin produced by both the wild-type and mutants growing on an optimized medium, in order to determine the most successful mutation method and isolate the best riboflavin-producer.

v) To grow the wild-type and the mutant on an optimized medium containing different organic oils, (coconut, sesame, peanut, mustard, sunflower and soybean oils), to determine which oil is a suitable carbon source for riboflavin production.

vi) To increase riboflavin production of an *Eremothecium gossypii* mutant by optimizing media supplements using Design of Experiments.

2. MATERIALS AND METHODS

2.1 *Eremothecium gossypii* strain maintenance

Eremothecium gossypii wild-type strain (ATCC 10895) was obtained from Peter Stahmann, Institute of Biotechnology, Forschungszentrum, Germany. The wild-type strain was stored as long-term freeze-dried cultures on malt extract agar (5.0 g l^{-1} gelatin peptone, 2.5 g l^{-1} dextrin, 13 g l^{-1} malt extract, 15 g l^{-1} agar) (Merck). Weekly working stocks of the wild-type strain were cultured on malt extract agar and incubated at 30^{o}C for four days.

2.2 Construction of a riboflavin standard curve for determination of riboflavin concentrations

One hundred milligram per litre stock solution of riboflavin was made using USP grade riboflavin (Sigma) in 0.02 M acetic acid (Merck). This stock was diluted to make up riboflavin concentrations from 0.1 to 100 mg l^{-1} (Horwitz, 2000). All riboflavin experiments were conducted in the dark to prevent the degradation of light sensitive riboflavin. A wavelength scan was run on the 100 mg l^{-1} stock solution using a WPA Lightwave S2000 diode array spectrophotometer (Labotec). Significant riboflavin peaks were detected between 440-445 nm. The standard curve used in this study was constructed using a wavelength of 445 nm, and all concentrations of riboflavin prepared previously with a R^2 value of 0.9945. Supernatants obtained from the filtration was analysed at a

wavelength of 445 nm using a WPA Lightwave S2000 diode array spectrophotometer (Labotec), and results were converted to mg l^{-1} using the constructed standard curve (Figure 3.1) (Kalingan, 1998).

2.3 Growth profile of *Eremothecium gossypii* on standard Ozbas and Kutsal medium

Determining the growth profile of an organism is essential in assessing when the sampling points would be and the point at which inoculations should take place. It is crucial to know when the culture is at its mid-log phase so that the starter inoculum can be transferred into experiments, when they are at their most viable. If the starter inoculum is transferred too late, then the organism being used would already be in the death phase and most of the inoculum used would be non-viable. Similarly, if inoculated too early the organism would spend too much time trying to adapt and would use up most of the supplements long before it was actively growing. For these reasons, it was essential to map the growth curve of *E. gossypii* used in this study. This growth curve was used as the baseline for all subsequent experiments.

A growth curve was constructed to determine the mid-log phase of an *E. gossypii* starter culture for the inoculation of subsequent experiments. Thirty millilitres (10%) of a four-day-old *E. gossypii* culture was inoculated into 270 ml O&K medium (peptone 5.0 g l^{-1}; yeast extract 5.0 g l^{-1}; malt extract 5.0 g l^{-1}; potassium hydrogen phosphate (K_2HPO_4)

0.2 g l^{-1}; magnesium sulphate ($MgSO_4.7H_2O$) 0.2 g l^{-1} (Ozbas and Kutsal, 1986), in a 500 ml Erlenmeyer flask. Glucose was used as a carbon source. Flasks were incubated at 30^{o}C on a rotary shaking incubator (Infors, Polychem) at a speed of 120 rpm. Five millilitre samples were taken every 24 hours and analysed as described below.

2.3.1 Biomass monitoring

Samples were filtered through pre-weighed Whatman No 1 filter paper and dried at 105^{o}C overnight and weighed. The results obtained were represented graphically and specific growth rates (μ) were calculated using the formula:

$$\mu = \frac{2.3\,(log\,X - log\,Xo)}{t} \qquad (3.1)$$

Where X = biomass, Xo = initial biomass and t = time. Doubling time (Td) was determined as given in equation 3.2.

$$Td = \frac{ln2}{\mu} \qquad (3.2)$$

2.4 Assessment of glucose and sunflower oil as carbon sources for growth of and riboflavin production by *Eremothecium gossypii*

These experiments were carried out to determine riboflavin production rates by the wild-type strain using different carbon sources (glucose and sunflower oil). A growth medium used was made up of the following components: peptone 5.0 g l^{-1}; yeast extract 5.0 g l^{-1}; malt extract 5.0 g l^{-1}; potassium hydrogen phosphate (K_2HPO_4) 0.2 g l^{-1}; magnesium sulphate ($MgSO_4.7H_2O$) 0.2 g l^{-1} (Ozbas and Kutsal, 1986) to which glucose and sunflower oil was added at concentrations of 5, 10, 15 and 20 g l^{-1}. Controls for these experiments were set up using the same media but without the addition of a carbon source. All chemicals used were supplied by Merck. Samples were removed at constant intervals of 24 h. Biomass was measured gravimetrically and riboflavin, spectrophotometrically at 445 nm.

Following this set of experiments with sunflower oil, it was determined that *E. gossypii* could utilize this oil as a carbon source and produce riboflavin. Based on these results and research conducted by other scientists (Stahmann *et al.,* 2000, Ozbas and Kutsal, 1986), other oils were chosen as possible carbon sources to determine which oil supported maximum riboflavin production. These oils were coconut, mustard, sunflower, sesame seed, soybean and peanut, at concentrations of 5, 10, 15 and 20 g l^{-1}. Oils were substituted for glucose as the carbon source in the O&K medium listed previously.

2.5 Treatment of samples containing oil: Liquid-liquid extraction

Oils added to the experiment as the carbon source, adhered to the microorganism and had to be removed before riboflavin analysis. Samples containing oils had to be treated with a liquid-liquid extraction process using the non-polar solvent hexane-isopropanol.

2.5.1 Hexane-isopropanol extraction

A mixture of 3:2 hexane (Merck) to isopropanol (Merck) was used as the extraction solvent. The extraction was performed using a 1:1 ratio of the extraction mixture to the sample (Stahmann *et al.,* 1994) After the two solutions were added together into a 250 ml separating funnel, the mixture was shaken rapidly for two minutes and allowed to stand for five minutes to allow the aqueous and the oil layers to separate (top organic layer and the bottom aqueous layer). The aqueous layer was filtered through pre-weighed filter paper which was dried overnight at 105^{o}C to determine biomass gravimetrically. The organic layer containing the oil was discarded. The filtrate was used to determine riboflavin concentration as described in section 2.2.

2.6 Mutation of *Eremothecium gossypii*

Eremothecium gossypii is a filamentous fungus, causing problems in the cotton plantations as a pathogen that destroys crops. However, this naturally-producing riboflavin fungus is an asset to the world of biological production of vitamins. Scientists have manipulated this riboflavin-

producing characteristic and developed mutants that have increase their production rates. The physical and chemical mutations carried out to increase riboflavin production yields of *E. gossypii* are described in the following sections.

2.6.1 Physical mutagenesis using *Ultraviolet (UV) Light mutation (250-300 nm)*

Eremothecium gossypii was inoculated onto solid O&K medium and were incubated at 30^{o}C for four days in order to obtain an actively-dividing culture. The UV light source used for the mutations was switched on twenty minutes before it was required. *Eremothecium gossypii* was exposed to UV light at wavelengths of between 250-300 nm at a distance of 30 cm, for exposure times of 10, 20 and 30 minutes (Barichievich and Calza, 1996). Mutants were then screened as described in 2.7.1.

2.6.2 Chemical mutagenesis

One hundred millilitres of O&K liquid medium (pH 6) in 250 ml flasks were inoculated with 1 cm^{3} of a four-day-old *E. gossypii* culture. Flasks were then incubated at 30^{o}C on a rotary shaker at 120 rpm for four days, after which aliquots of 10 ml were exposed to chemical mutagens as described in the following two sections.

2.6.2.1 N-methyl-n'-nitro-n-nitrosoguanidine

Fifty microgram per millilitre *n*-methyl-*n*'-nitro-*n*-nitrosoguanidine (Sigma) was dissolved in 95% ethanol (Barichievich and Calza, 1996). Ten ml of liquid culture described in 2.6.2 was added to 100 ml stock of 50 μg ml^{-1} MNNG for exposure times of 10, 20 and 30 minutes.

2.6.2.2 Ethylmethane sulphonate

Freshly prepared 50 µg ml^{-1} EMS (Sigma) was diluted in 0.05 M potassium phosphate buffer (pH 7) (8.0 g l^{-1} sodium chloride, 1.21 g l^{-1} di-potassium hydrogen phosphate, 0.34 g l^{-1} potassium di-hydrogen phosphate) (Moses, 2004). Ten ml of liquid culture described in 2.6.2 was added to 100 ml freshly prepared 50 µg ml^{-1} EMS for exposure times of 10, 20 and 30 minutes. After each exposure time interval (10, 20 and 30 min) samples were removed and screened as described in 2.7.2.

2.7 Screening for mutants

2.7.1 Physical mutagenesis screening

One cm^3 of the physically mutated culture was inoculated into a liquid screening medium containing 50 mM itaconate, 10 g l^{-1} yeast extract and 0.05% (v/v) oleic acid (Stahmann *et al.*, 1996). This was incubated for four days, after which 0.1 ml was plated out onto a solid itaconate-containing screening medium (10 g l^{-1} yeast extract, 0.05% (v/v) oleic

acid, 50 mM itaconate and 20 g l^{-1} agar). Plates were incubated at 30^{o}C for four days.

2.7.2 Chemical mutagenesis screening

Aliquots of 0.1 ml samples of the liquid culture were removed and spread over the surface of a solid screening medium containing 50 mM itaconate (Stahmann *et al.*, 1996). Plates were incubated at 30^{o}C for four days. The control was 0.1 ml unmutated *E. gossypii* spread over the screening medium.

2.8 Analysis of the screened mutants

Successful riboflavin-producing mutants (yellow colonies), were grown on the growth medium described in section 2.10 and riboflavin analysis was carried out as described in section 2.2. After the screening process was complete and a successful mutant was chosen, based on its riboflavin production, growth kinetics of that mutant (*E. gossypii* EMS30/1) was determined in order to evaluate growth rates and sampling points.

2.9 Growth profile of *E. gossypii* EMS30/1 (mutant)

A growth curve was constructed to determine the mid-log phase of the mutant *E. gossypii* EMS30/1 starter culture for the inoculation of experiments. One cm^3 of a four-day old mutant *E. gossypii* EMS30/1 was inoculated into 300 ml O&K medium in a 500 ml Erlenmeyer flask.

Glucose was used as the carbon source. All flasks were incubated at 30^{o}C on a rotary shaking incubator at a speed of 120 rpm. Five millilitre samples were taken every three hours and analysed for biomass production as in section 2.3.1 and riboflavin production according to section 2.2. A comparison of the colony morphology of *E. gossypii* wild-type and *E. gossypii* EMS30/1 was conducted, by inoculation both on O&K media for four days, after which a wet mount using lactophenol blue was prepared to investigate its microscopic characteristics. *Eremothecium gossypii* has the ability to store lipids in its vacuoles for use in adverse conditions. To demonstrate this culture's lipid storage ability *E. gossypii* EMS30/1 was stained with nile red (Vijayalakshmi *et al.*, 2003) to view the points at which lipids were stored in fungal calls.

2.10 Screening for the best carbon source for riboflavin production

A growth medium was made up as described in 2.4, with glucose being substituted with either sunflower, mustard, coconut, peanut, soybean or sesame seed oils as the carbon sources. The oils were added at concentrations of 5, 10, 15 and 20 g l^{-1}, respectively. Controls contained no carbon source. Cultures used in these experiments were *E. gossypii* wild-type and *E. gossypii* EMS30/1. (All chemicals were supplied by Merck).

All liquid cultures were incubated at 30^{o}C on a rotary shaker at 120 rpm for four days. Ten millilitre samples were removed at intervals of 24 h

and samples containing oils were treated as per section 2.5.1, analyzed gravimetrically for biomass and spectrophotometricaly for riboflavin production. Results obtained were represented graphically.

2.11 Measurement of growth and spectrophotometric analysis of riboflavin

A hexane-isopropanol extraction described in 2.5.1 was carried out to remove oil from oil-containing samples; the aqueous layer was filtered through pre-weighed Whatman No.1 filter paper which was weighed after drying overnight at 105^{o}C to determine biomass gravimetrically. The filtrate was used to determine riboflavin concentration at a wavelength of 445 nm, using a WPA Lightwave S2000 diode array spectrophotometer (Labotec).

The data obtained was then analyzed to determine whether there was an increase in growth and riboflavin production by the resulting mutants on the various carbon sources.

2.12 Optimization of growth medium components

2.12.1 Media optimization using fractional factorial

Once an oil was selected as a carbon source DOE was used to screen five nutrient factors to determine the best combinations and amounts to produce maximum riboflavin. Based on the first-order model equation obtained from the design (Equation 1), a series of screening experiments (FF0516 and FF0416) were performed in the direction of the steepest ascent. The most significant nutrient factors affecting riboflavin production were determined from the Pareto chart. A cube plot was generated to determine the effects of three components and their interaction on production rates. Relative effects from two factors were identified from contour plots. The program used in this study to run experimental designs was Design Expert® version 7.1.6 (Statease, Minneapolis, USA).

A fractional factorial experiment FF0516 was used as a primary screening experiment (Table 2.1), which determined the most important media components, among peptone, yeast extract, malt extract, K_2HPO_4 and $MgSO_4$ (components of O&K medium). Values represented in tables 3.4, 3.6, 3.8 and 3.10 were calculated using Design Expert 7.1.6. Each factor was set at a high (+1) and a low (-1) value with 3 center points (0) per experiment (Table 2.2). The same was carried out for secondary screening (Tables 2.3).

(FF0516: The first two numbers (**05**) show the factors used and the second two numbers (**16**) indicate the number of runs excluding the three center points carried out.)

Table 2.1 Levels of nutrient parameters used in experiment FF0516, a primary screening experiment

Factors		**Levels**		
Variables	**Nutrients (g l^{-1})**	**-1**	**0**	**+1**
X_1	Peptone	0	2.5	5.0
X_2	Yeast extract	0	2.5	5.0
X_3	Malt extract	0	2.5	5.0
X_4	$MgSO_4$	0	0.1	0.2
X_5	K_2HPO_4	0	0.1	0.2

Tables 2.2, 2.4 and 2.6 refer to the design matrix for media components to increase riboflavin production. The tables showed them in their coded levels, these codes were then substituted with the corresponding amount of media components for the actual experiment together with 20 g l^{-1} soybean oil.

Table 2.2 Experimental design matrix for the primary screening of media components (in coded levels) for riboflavin production by *E. gossypii* EMS30/1. Both standard and randomized run orders are shown

Std	Runs	Peptone (X_1)	Yeast extract (X_2)	Malt extract (X_3)	$MgSO_4$ (X_4)	K_2HPO_4 (X_5)
1	5	**-1**	**-1**	**-1**	**-1**	**+1**
2	15	**+1**	**-1**	**-1**	**-1**	**-1**
3	16	**-1**	**+1**	**-1**	**-1**	**-1**
4	19	**+1**	**+1**	**-1**	**-1**	**+1**
5	10	**-1**	**-1**	**+1**	**-1**	**-1**
6	18	**+1**	**-1**	**+1**	**-1**	**+1**
7	14	**-1**	**+1**	**+1**	**-1**	**+1**
8	8	**+1**	**+1**	**+1**	**-1**	**-1**
9	12	**-1**	**-1**	**-1**	**+1**	**-1**
10	11	**+1**	**-1**	**-1**	**+1**	**+1**
11	13	**-1**	**+1**	**-1**	**+1**	**+1**
12	2	**+1**	**+1**	**-1**	**+1**	**-1**
13	6	**-1**	**-1**	**+1**	**+1**	**+1**
14	1	**+1**	**-1**	**+1**	**+1**	**-1**
15	7	**-1**	**+1**	**+1**	**+1**	**-1**
16	17	**+1**	**+1**	**+1**	**+1**	**+1**
17	9	**0**	**0**	**0**	**0**	**0**
18	4	**0**	**0**	**0**	**0**	**0**
19	3	**0**	**0**	**0**	**0**	**0**

A second (FF0416) (Table 2.3) fractional factorial experiment was conducted using the information gathered from the analysis of FF0516 to further optimize the number of components. Factors were designated X_1, X_2, X_3, X_4 and X_5 to represent the medium components.

Magnesium sulphate (X_4), based on the primary screen (FF0516) was shown to be not significant in the production of riboflavin and was set at a low of 0.6 g l^{-1}

Table 2.3 Levels of nutrient parameters used in experiment FF0416, a secondary screening experiment

Factors		**Levels**		
Variables	**Nutrients (g l^{-1})**	**-1**	**0**	**+1**
X_1	Peptone	5.0	6.65	8.30
X_2	Yeast extract	5.0	6.65	8.30
X_3	Malt extract	5.0	6.65	8.30
X_5	K_2HPO_4	0.2	0.30	0.40

Table 2.4 Experimental design matrix for the secondary screening of media components (in coded levels) for riboflavin production by *E. gossypii* EMS30/1. Both standard and randomized run orders are shown

Std	Runs	Peptone (X_1)	Yeast extract (X_2)	Malt extract (X_3)	K_2HPO_4 (X_5)
1	18	**-1**	**-1**	**-1**	**-1**
2	10	**+1**	**-1**	**-1**	**-1**
3	3	**-1**	**+1**	**-1**	**-1**
4	17	**+1**	**+1**	**-1**	**-1**
5	12	**-1**	**-1**	**+1**	**-1**
6	5	**+1**	**-1**	**+1**	**-1**
7	14	**-1**	**+1**	**+1**	**-1**
8	6	**+1**	**+1**	**+1**	**-1**
9	11	**-1**	**-1**	**-1**	**+1**
10	19	**+1**	**-1**	**-1**	**+1**
11	7	**-1**	**+1**	**-1**	**+1**
12	2	**+1**	**+1**	**-1**	**+1**
13	1	**-1**	**-1**	**+1**	**+1**
14	16	**+1**	**-1**	**+1**	**+1**
15	9	**-1**	**+1**	**+1**	**+1**
16	15	**+1**	**+1**	**+1**	**+1**
17	4	**0**	**0**	**0**	**0**
18	13	**0**	**0**	**0**	**0**
19	8	**0**	**0**	**0**	**0**

X_1 and X_3 were set at a low of 5.0 g l^{-1} based on the secondary screen (FF0416). It was determined that increasing or decreasing these two factors did not have a significant effect on riboflavin production.

2.13 Central composite experiment (CC0208)

To fit the empiric second order model, a central composite design was executed. The quadratic model for predicting the optimal riboflavin concentration was carried out. An optimum value for the nutrient factors for maximum riboflavin production was determined by point prediction tools (Table 3.10).

This experiment was carried out with 13 flasks and 2 factors (X_2 and X_5). The central composite experiment was conducted to obtain a predicted maximum riboflavin concentration using the optimised factors obtained from experiment FF0416.

Table 2.5 Levels of nutrient parameters used in a central composite experiment to predict maximum riboflavin production

Factors		**Levels**				
Variables	**Nutrients (g l^{-1})**	**-2**	**-1**	**0**	**+1**	**+2**
X_2	Yeast extract	1.55	3.0	6.50	10.0	11.45
X_5	K_2HPO_4	0.28	0.4	0.70	1.00	1.12

The result of the central composite was used to set up a confirmation experiment. Nutrient parameters were set up as per Table 2.5.

Table 2.6 Experimental design matrix for the central composite of media components (in coded levels) for riboflavin production by *E. gossypii* EMS30/1. Both standard and randomized run orders are shown

Std	Runs	Yeast extract (X_2)	K_2HPO_4 (X_5)
1	12	**-1**	**-1**
2	11	**+1**	**-1**
3	13	**-1**	**+1**
4	3	**+1**	**+1**
5	4	**-2**	**0**
6	1	**+2**	**0**
7	2	**0**	**-2**
8	10	**0**	**+2**
9	6	**0**	**0**
10	8	**0**	**0**
11	9	**0**	**0**
12	5	**0**	**0**
13	7	**0**	**0**

3.0 RESULTS

3.1 Riboflavin standard curve

Riboflavin analysis was carried out spectrophotometrically, a standard curve was drawn up to determine the riboflavin concentrations (Figure 3.1). A wavelength scan of the 100 mg l^{-1} solution of riboflavin showed the highest peak at 445 nm, this was used as the wavelength to measure the riboflavin concentration. The standard curve had an R^2 of 0.997, the formula

$$\boldsymbol{y = mx + c}$$

Where y = riboflavin absorbance, m = x co-efficient, x = riboflavin concentration, c = constant, was used to calculate the riboflavin concentration.

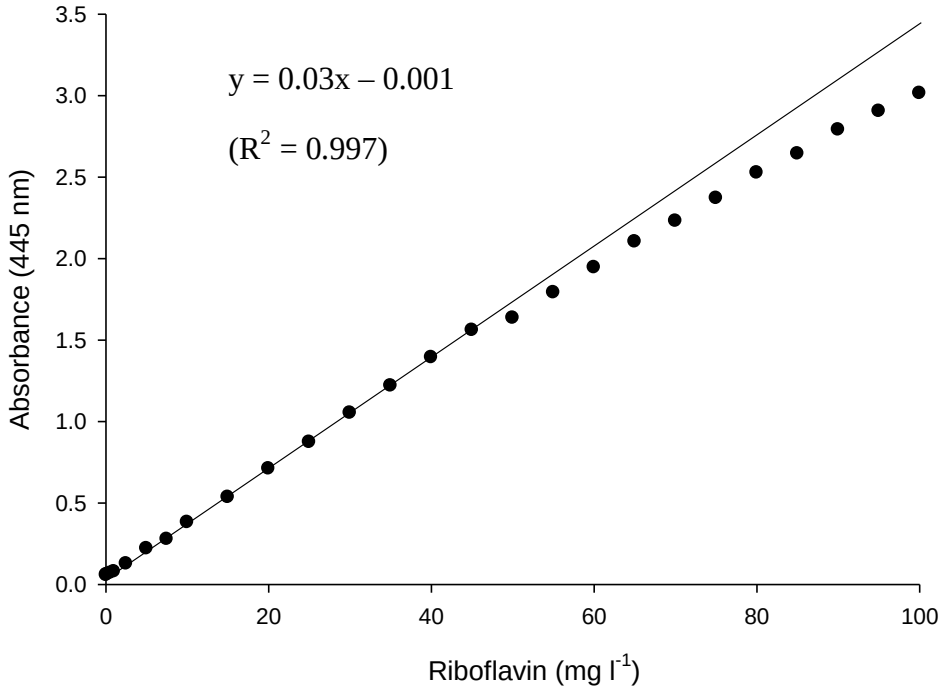

Figure 3.1 Riboflavin standard curve in 0.02 M acetic acid, with an R^2 of 0.997 was used to calculate riboflavin concentrations.

3.2 Growth curve of *Eremothecium gossypii*

Ozbas and Kutsal medium was inoculated with 0.06 g l^{-1} of *E.gossypii* at time zero. *Eremothecium gossypii* (wild-type) had a maximum specific growth rate of 0.02 mg l^{-1} and a doubling time of 23.38 h (Table 3.1). There was no lag phase noted, the organism adapted to the medium and log phase was maintained up to 72 h. After which the organism remained at stationary phase (72-120 h). Using this growth curve experiment (Figure 3.2) the mid-log phase was determined to be 36 h and was the duration for which the starter cultures was grown, before inoculation into subsequent experiments.

Table 3.1 Maximum specific growth rate and doubling time of *Eremothecium gossypii* wild-type

Organisium	Maximum specific growth rate (μ max) h^{-1}	Doubling time (Td) h
Eremothecium gossypii	0.02	23.38

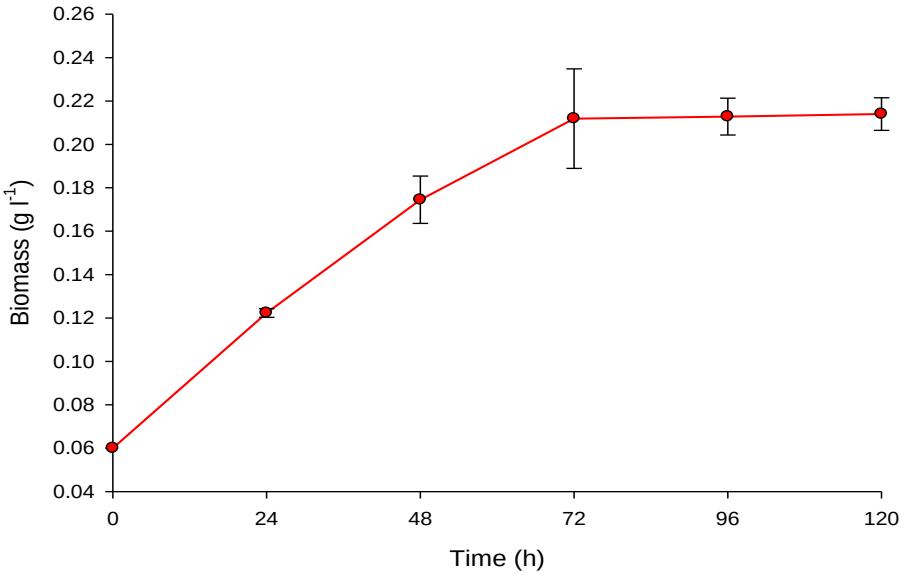

Figure 3.2 Growth curve of *Eremothecium gossypii* wild-type growing on Ozbas and Kutsal medium over 120 h. Error bars indicate the standard deviations.

3.3 Growth of *Eremothecium gossypii* wid-type on different carbon sources (glucose and sunflower oil)

Eremothecium gossypii was grown on O&K medium with glucose as a carbon source to verify that the strain being used in this research was a strain that could produce riboflavin. Glucose is a simple carbon source and was therefore easy for the fungus to break down. Results obtained were then used as baseline amounts for the wild-type which was

compared to the final concentrations of riboflavin produced by the mutants.

3.3.1 Riboflavin produced using glucose as a carbon source

Eremothecium gossypii produced little or no riboflavin for the first 24 h, indicated by low values (Figure 3.3). After 48 h small amounts of riboflavin were produced. A maximum concentration of 31.64 mg l^{-1} riboflavin was reached at 72 h using 10 g l^{-1} glucose. After 72 h riboflavin production started to decline, this was observed for all concentration of glucose. This experiment (Figure 3.3) determined the concentration of glucose to use in order to obtain maximum riboflavin concentrations (10 g l^{-1} glucose). The control contained no carbon source.

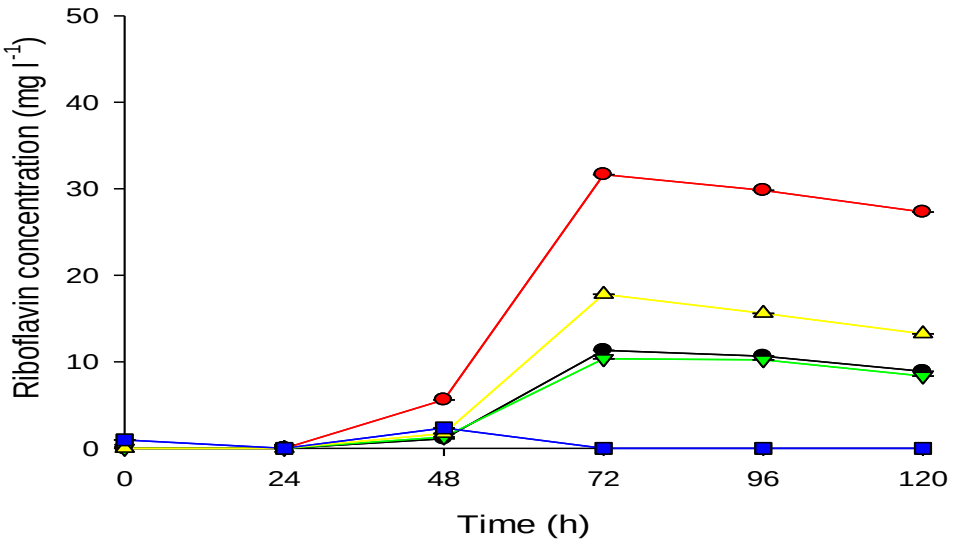

Figure 3.3 Riboflavin produced by *Eremothecium gossypii* wild-type grown on Ozbas and Kutsal media using different concentrations of glucose as the sole carbon source. (●) 5 g l^{-1}, (●) 10 g l^{-1}, (▼) 15 g l^{-1} and (△) 20 g l^{-1} and (■) control.

3.3.2 Riboflavin produced using sunflower oil as the carbon source

Eremothecium gossypii breaks down these complex fats into simpler carbon sources that it can use, it also is able to store large amounts of lipids in its vacuoles. These stored lipids are then used when there are no nutrients available in the medium (Stahmann *et al.,* 1994). Growing *E. gossypii* on the sunflower oil determine if the strain being used was able to utilize vegetable oil to produce riboflavin. In the first 24 h there was little or no riboflavin produced (lag phase). There was an exponential increase in riboflavin production for the next 24 h, using 15 and 20 g l^{-1} oil. Stationary phase was observed for the time intervals between 72 and 120 h. Using 20 g l^{-1} sunflower oil *E. gossypii* was able to produce a maximum of 31.05 mg l^{-1} of riboflavin in 48 h (Figure 3.4). The control, which contained no carbon source, produced negligible amounts of riboflavin. The experiment represented in Figure 3.4 showed that this organism can grow and produce riboflavin in the presence of vegetable oil.

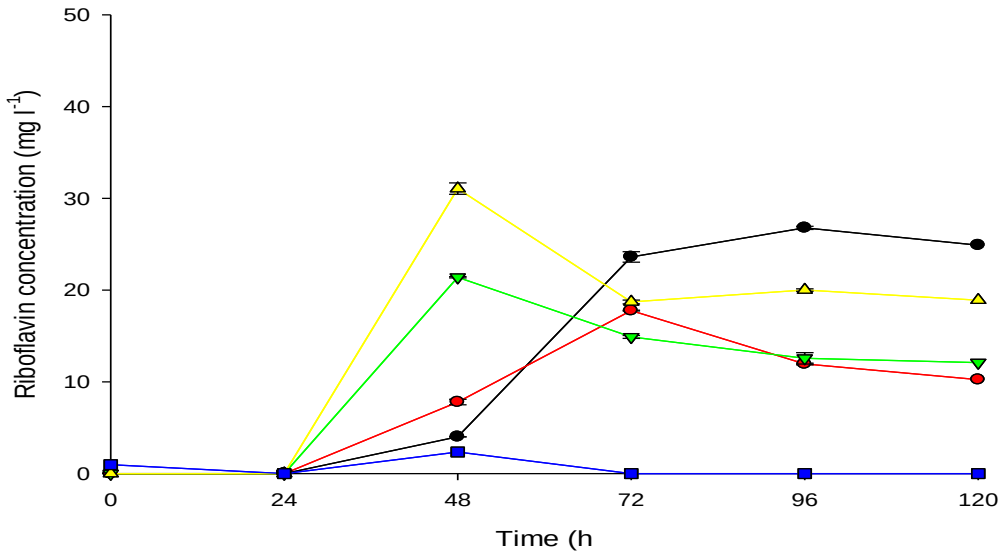

Figure 3.4 Riboflavin produced by *Eremothecium gossypii* using different concentrations of sunflower oil as the sole carbon source. (●) 5 g l^{-1}, (●) 10 g l^{-1}, (▼) 15 g l^{-1} and (△) 20 g l^{-1} and (■) control.

After establishing that the wild-type was able to utilize sunflower oil and glucose as a carbon source, mutation was carried out with the following results.

There were eighteen colonies (yellow) screened, three highest producers for each mutagen were chosen to be further screened on a medium containing a glucose carbon source, three induced by UV, EMS and MNNG were screened by growing them on an itaconate containing screening medium. Those that grew yellow were isolated and inoculated onto growth media, their riboflavin concentrations were compared. The one with the highest concentration of riboflavin was selected to be used in all subsequent experiments, of the nine colonies screened EMS30/1 was the most successful.

3.4 Physical mutagenesis using ultraviolet light

Three mutants were isolated from the UV mutation and screening on an itaconate containing medium; these were *E. gossypii* UV1, *E. gossypii* UV2 and *E. gossypii* UV3. *Eremothecium gossypii* UV1 produced a maximum of 54.50 mg l^{-1} riboflavin in a period of 72 hours (Figure 3.5), whereas *E. gossypii* UV2 and *E. gossypii* UV3 produced 25.98 mg l^{-1} and 41.32 mg l^{-1} respectively in the same period. The control (*E. gossypii* wild-type) produced a maximum of 31.64 mg l^{-1} riboflavin. This was more than that produced by mutant UV2. *Eremothecium gossypii* UV1 and *E. gossypii* UV3 produced 22.86 and 9.68 mg l^{-1} more riboflavin than the wild-type, which was an increase of 72 and 31 percent respectively. All the mutants as well as the wild-type peaked at 72 h after which production rates started to decline.

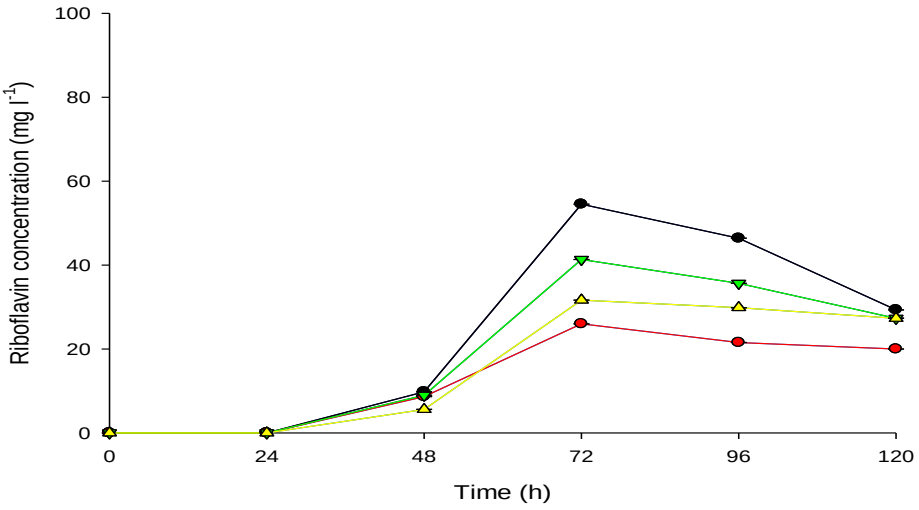

Figure 3.5 Riboflavin produced by three Ultraviolet light induced mutants of *Eremothecium gossypii*, grown on Ozbas and Kutsal medium. (●) mutant UV1, (●) mutant UV2, (▼) mutant UV3 and (△) control.

3.5 Mutagenesis using ethylmethane sulphonate

Ethylmethane sulphonate induced six riboflavin producing mutants, of which three of the highest produces were chosen for further investigation. *Eremothecium gossypii* EMS20/1 and *E. gossypii* EMS20/2 produced a concentration of 65.20 mg l^{-1} and 56.42 mg l^{-1} riboflavin respectively at 72 h, and *E. gossypii* EMS30/1 produced a maximum of 68.45 mg l^{-1} (Figure 3.6). The control containing *E. gossypii* wild-type produced 31.64 mg l^{-1} riboflavin. The mutant produced 36.81 mg l^{-1} more than the wild-type, which was an increase of 117%. There as a decrease in riboflavin production after 72 h, by both the mutants and the control.

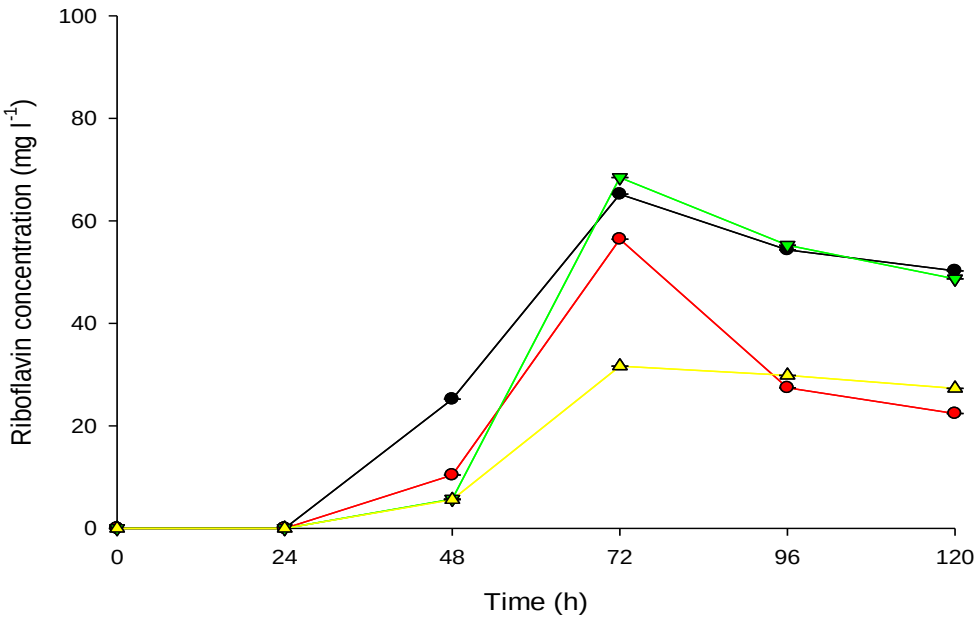

Figure 3.6 Riboflavin produced by three ethylmethane sulphonate (EMS) induced mutants of *Eremothecium gossypii*, grown on Ozbas and Kutsal medium. (●) mutant EMS20/1, (●) mutant EMS20/2, (▼) mutant EMS30/1 and (△) control.

3.6 Mutagenesis using *n*-methyl-*n*'-nitro-*n*-nitrosoguanidine

Mutants *E. gossypii* MNNG20/2, *E. gossypii* MNNG30/2 and *E. gossypii* MNNG10/2 induced by MNNG were the three mutants that produced the highest amount of riboflavin of the nine isolated. *E. gossypii* MNNG20/2 and *E. gossypii* MNNG30/2 produced a maximum of 16.84 mg l^{-1} and 16.96 mg l^{-1} riboflavin in 72 h. *Eremothecium gossypii* MNNG10/2 produced 28.34 mg l^{-1} in the same time (Figure 3.7). The control (*Eremothecum gossypii* wild-type) produced 31.56 mg l^{-1} riboflavin in the same time. *E. gossypii* MNNG10/2 produced 3.22 mg l^{-1} less than the wild-type, which was a decrease of 11.36%. At 120 h riboflavin concentration was depleted to zero.

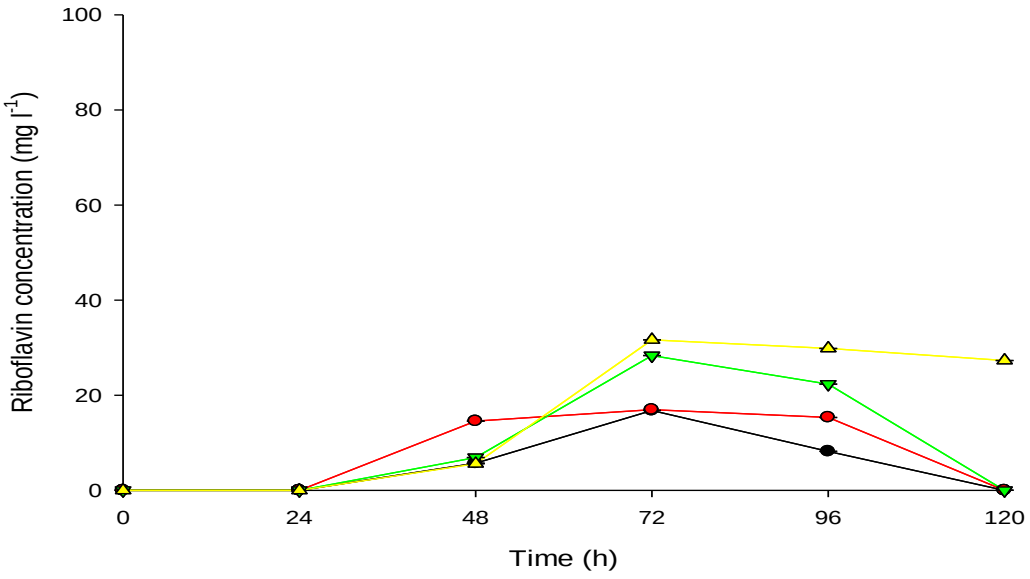

Figure 3.7 **Riboflavin produced by three *n*-methyl-*n*'-nitro-*n*-nitrosoguanidine induced mutants of *Eremothecium gossypii*, grown on Ozbas and Kutsal medium. (●) mutant MNNG20/2, (●) mutant MNNG30/2, (▼) mutant MNNG10/2 and (△) control.**

3.7 Colony morphology and microscopic examination of the *E. gossypii* wild-type and *E. gossypii* EMS30/1

Eremothecium gossypii EMS30/1 when compared to the wild-type showed an increase in growth and the yellowing around the growth indicated an increase in riboflavin production (Figure 3.8c and Figure 3.8d). In figure 3.8a and 3.8b the wild-type showed compact and tight mycelia, while the *E. gossypii* EMS30/1 grew loosely packed mycelia. This is also evident from the growth on plates. Needle-shaped ascospores (indicated by the arrows) could be seen in both stains which was typical of this species (Figure 3.8a and Figure 3.8b). *Eremothecium gossypii* EMS30/1 (Table 3.2) colony sizes were $2^1/_2$ times bigger than the wild-type. Colony size of the wild-type was 10 mm (Figure 3.8c) compared to that of *E. gossypii* EMS30/1 which was 25 mm (Figure 3.8d) after 96 h.

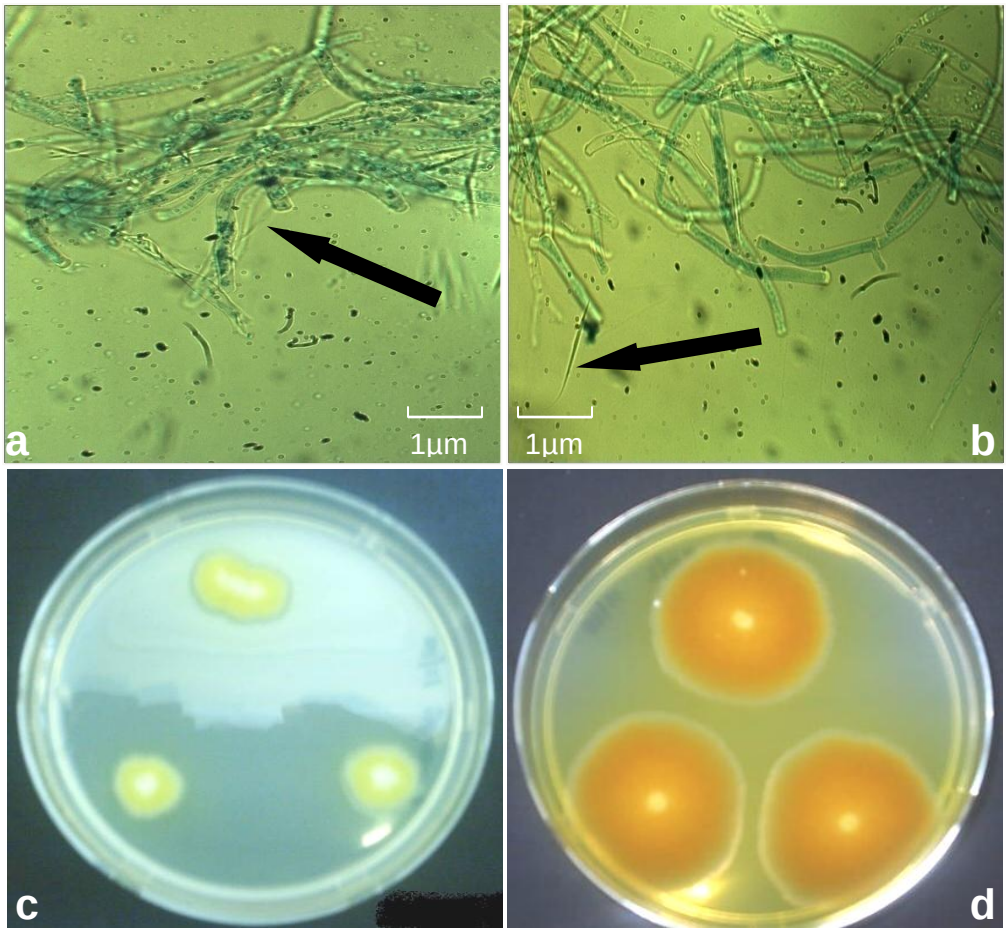

Figure 3.8 **Microscopic analysis of a four-day-old culture of (a) *Eremothecium gossypii* wild-type (b) *Eremothecium gossypii* EMS30/1 with the corresponding plate cultures, growing on Ozbas and Kutsal medium (c and d).**

Table 3.2 Description of the colony and cellular morphology of *Eremothecium gossypii* wild-type and *Eremothecium gossypii* EMS30/1

	***Eremothecium gossypii* wild-type**	***Eremothecium gossypii* EMS30/1**
Colony		
Colony size (96 h)	10 mm	25 mm
Edge	Filamentous	Filamentous
Margin	Curled	Curled
Elevation	Raised	Flat
Colour	White to Yellow	Yellow to Orange
Cellular		
Mycelium arrangement	Compact and tight	Loose and proliferated
Spores	Needle-shaped ascospores	Needle-shaped ascospores
Inclusions	Large vacuoles	Large vacuoles

Cultures were grown in an oil-containing medium before staining. Micro droplets of oil (orange/yellow) was seen in the hyphae (Figure 3.9b and 3.9c), the organism uses its large vacuoles (circular structures) as storage spaces (Figure 3.9a). It was capable of storing neutral lipids as energy reserves. This ability allows these fungi to survive in adverse conditions (Stahmann *et al.,* 1994).

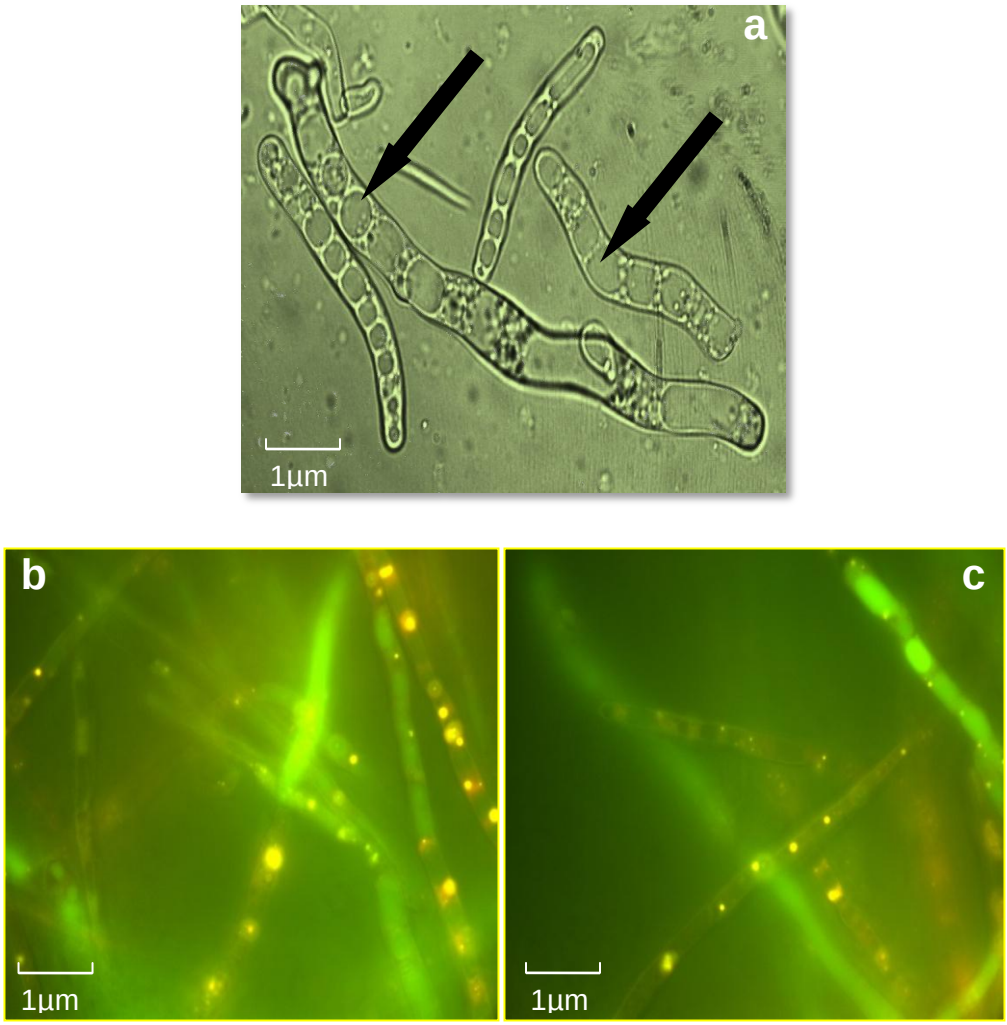

Figure 3.9 **Micrographs (a) of *E. gossypii* EMS30/1 using light microscopy, showing large vacuoles present, indicated by the arrows (1000x). The stain used was lactophenol blue. (b and c) 400x magnification of *E. gossypii* EMS30/1 using fluorescent microscopy stained with nile blue showing lipids trapped in vacuoles (yellow spots).**

3.8 Riboflavin production and growth curve of *E. gossypii* wild-type and *E. gossypii* EMS30/1

Eremothecium gossypii EMS30/1 (mutant) had a maximum specific growth rate of 0.04 h^{-1} and a doubling time of 16.98 h, while the wild-type had a maximum specific growth rate of 0.02 h^{-1} and a doubling time of 23.38 h (Figure 3.10). Although both the mutant and the wild-types growth pattern followd the same tend, the mutant produced more biomass than the wild-type, the same can be seen for the riboflavin production (Figure 3.11). The two growth curves were significantly different, $p<0.003$ was obtained when a *t-test* was performed, for the time interval of between 48 and 120 hours indicated by the shaded area on the graph.

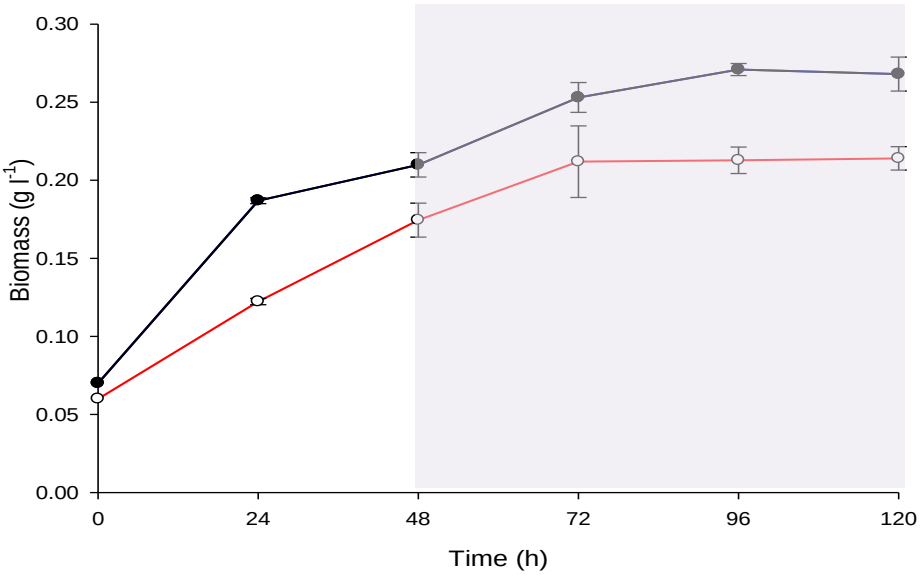

Figure 3.10 Growth curve of *E. gossypii* wild-type (○) and *E. gossypii* EMS30/1 (●) growing on O&K media, a *t-test* was performed to compare the shaded region between 48 and 120 h.

Riboflavin produced by the wild-type and the mutant were significantly different as they had a $p>0.04$ (Figure 3.11), when a *t-test* was performed for the interval between 72-120 h.

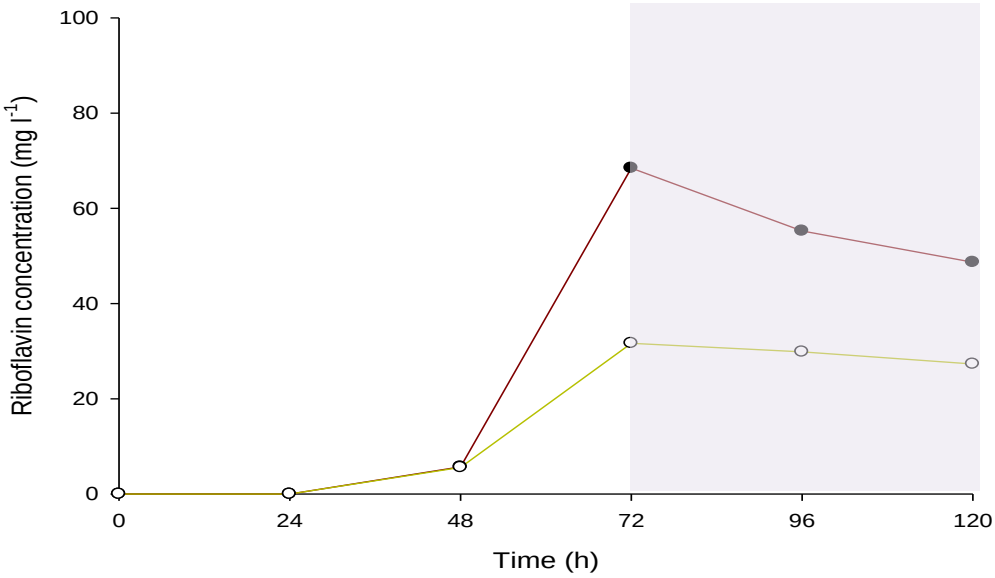

Figure 3.11 Riboflavin produced by *E. gossypii* wild-type (○) and *E. gossypii* EMS30/1 (●) growing on O&K media containing 10 g l^{-1} glucose, a *t-test* was performed to compare the shaded region between 72 and 120 h.

3.9 Comparison of riboflavin produced by mutant EMS and wild-type *E. gossypii* using different concentrations of organic oils.

Maximum riboflavin produced by the wild-type was 21.30 mg l^{-1} in 72 h using 5.0 g l^{-1} of coconut oil (Figure 3.12a), the *E. gossypii* EMS30/1 produced 15.21 mg l^{-1} in 96 hours using 5.0 g l^{-1} of the same oil (Figure 3.12b). The wild-type produced 6.09 mg l^{-1} more than the *E. gossypii* EMS30/1 using the same concentraction of oil. A *t-test* of the riboflavin produced by the wild-type and *E. gossypii* EMS30/1 growing on 5.0 g l^{-1} of coconut oil was performed for the time interval of between 48 to 120 hours (shaded region) had a $p>0.64$, therefore the two lines were not significantly different.

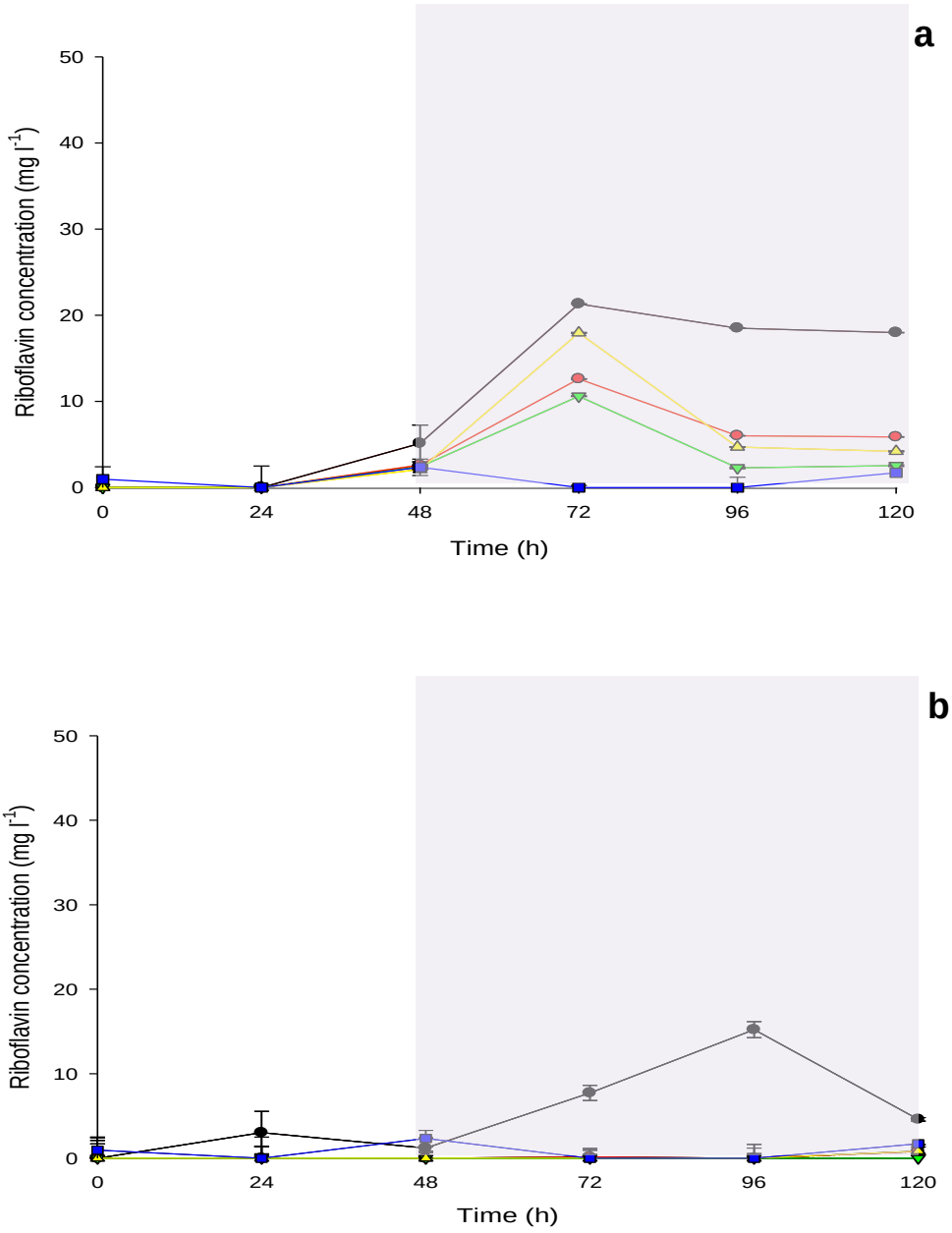

Figure 3.12 Riboflavin produced by *E. gossypii* wild-type (a) and *E. gossypii* EMS30/1(b) both growing on O&K media with coconut oil substituted for glucose as carbon source. (●) 5 g l^{-1}, (○) 10 g l^{-1}, (▼) 15 g l^{-1} and (△) 20 g l^{-1} and (■) control.

The wild-type produced 28.67 mg l^{-1} of riboflavin in 72 h using 5.0 g l^{-1} of mustard oil (Figure 3.13a), *E. gossypii* EMS30/1 produced 6.45 mg l^{-1} in 48 h using the same amount of mustard oil (Figure 3.13b). The wild-type produced 22.22 mg l^{-1} (40%) more riboflavin than *E. gossypii* EMS30/1, however it took 24 h more to do so. A *t-test* of the riboflavin produced by the wild-type and *E. gossypii* EMS30/1 growing on 5.0 g l^{-1} of mustard oil was performed for the time interval of between 72 to 120 hours (indicated by the shaded region) a $p<0.05$ was obtained, indicating the two lines were significantly different.

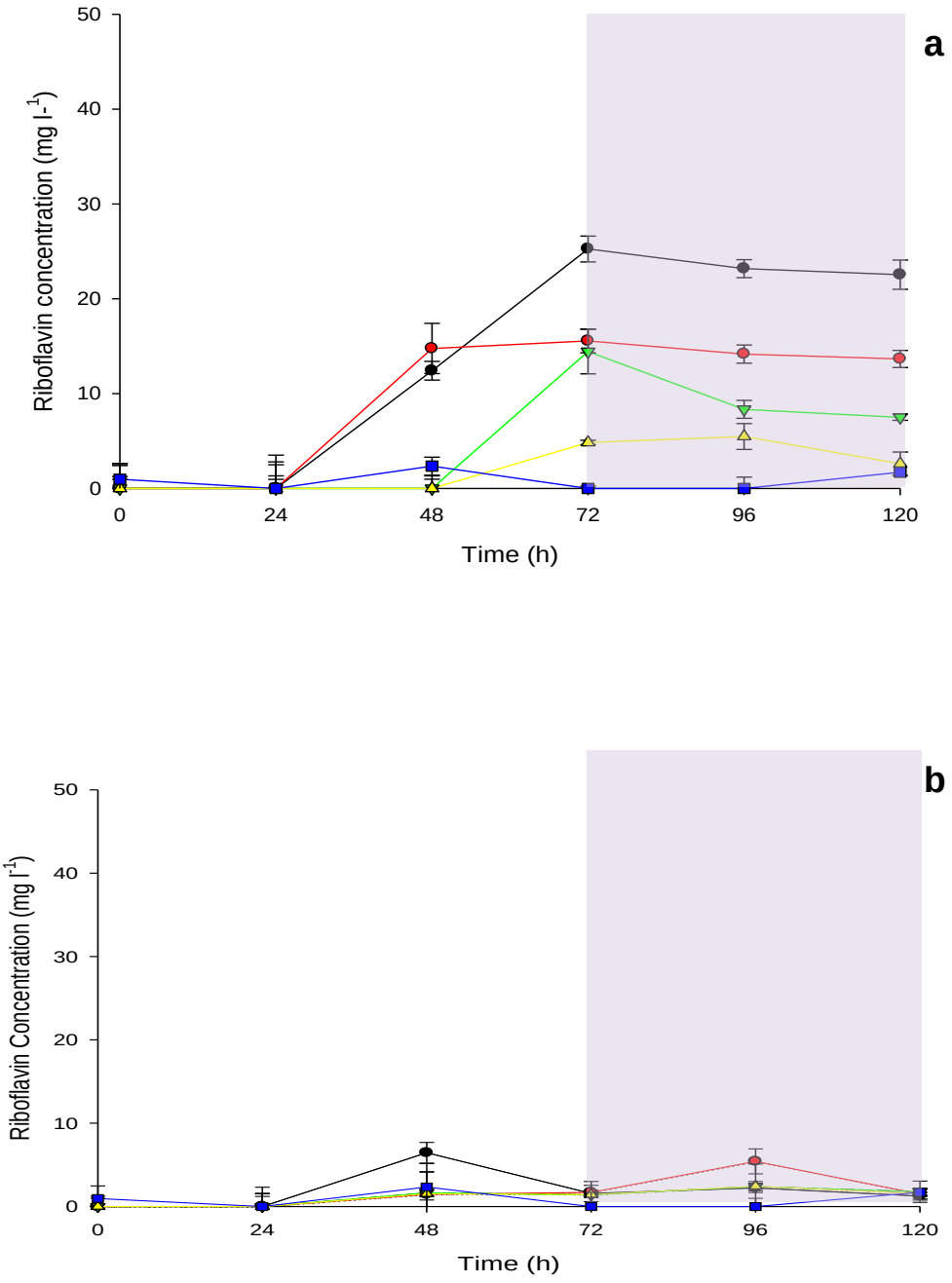

Figure 3.13 **Riboflavin produced by *E. gossypii* wild-type (a) and *E. gossypii* EMS30/1(b) both growing on O&K media with mustard oil substituted for glucose as carbon source. (●) 5 g l^{-1}, (○) 10 g l^{-1}, (▼) 15 g l^{-1} and (△) 20 g l^{-1} and (■) control.**

Using 15 g l^{-1} of peanut oil (Figure 3.14a) 8.64 mg l^{-1} of riboflavin was produced in 96 h whereas *E. gossypii* EMS30/1 produced 27.34 mg l^{-1} in 96 hrs using 5.0 g l^{-1} of oil (Figure 3.14b). A *t-test* of the riboflavin produced by the wild-type growing on 15 g l^{-1} of peanut oil and the mutant growing on 5.0 g l^{-1} of the same oil was performed for the time interval of between 72 to 120 hours (shaded region) and it had a $p<0.04$, therefore the two lines were significantly different. The wild-type did not produce much riboflavin using peanut oil all concentration produced by the wild-type were below 10 mg l^{-1} , was for the entire growth period of 120h. *Eremothecium gossypii* EMS30/1 produced 238% more riboflavin from this oil, in comparison to the wild-type.

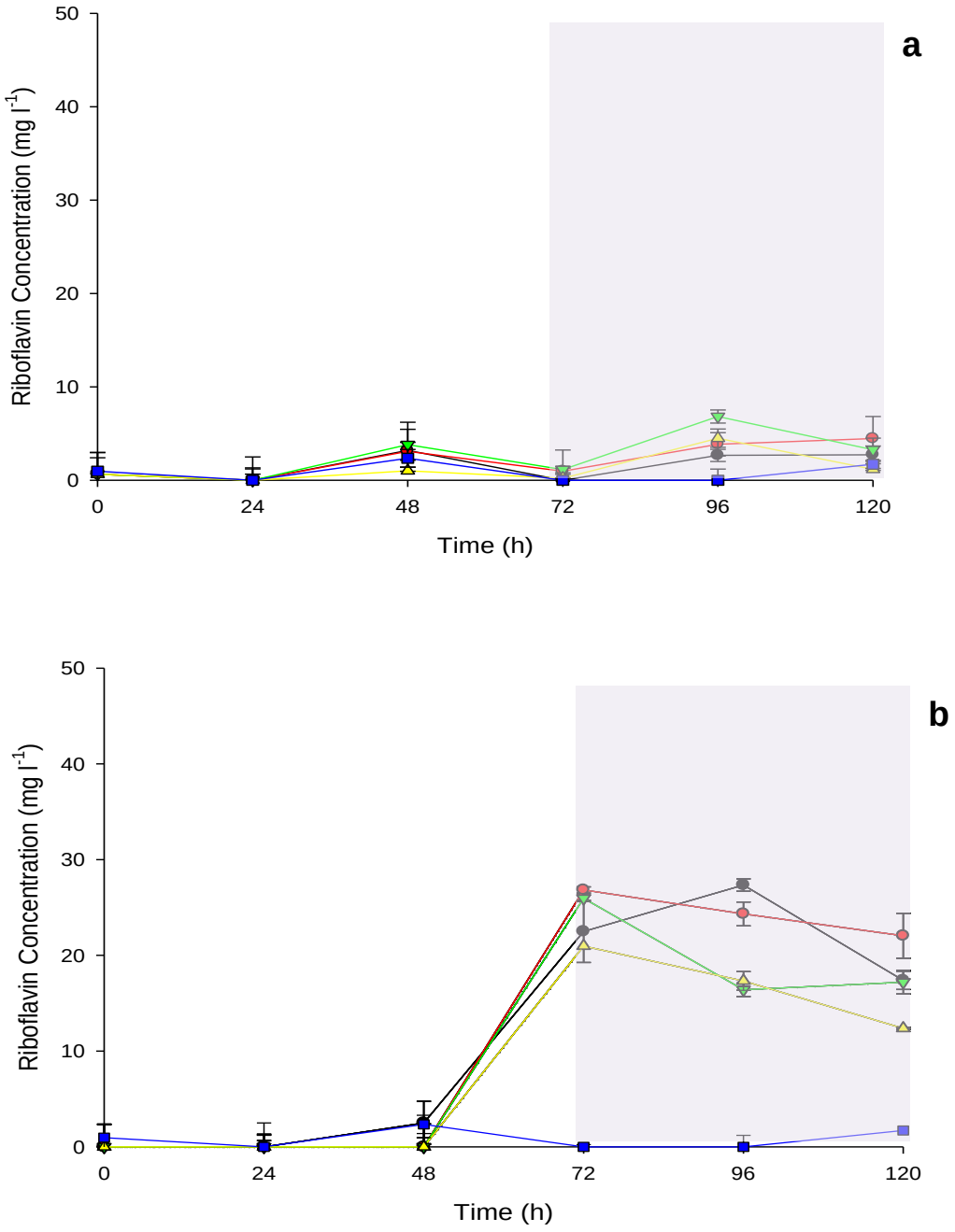

Figure 3.14 **Riboflavin produced by *E. gossypii* wild-type (a) and *E. gossypii* EMS30/1 (b) both growing on O&K media with peanut oil substituted for glucose as carbon source. (●) 5 g l^{-1}, (○) 10 g l^{-1}, (▼) 15 g l^{-1} and (△) 20 g l^{-1} and (■) control.**

The experiments carried out using sesame oil showed that the wild-type could utilize sesame oil; however it could not produce high amounts of riboflavin. It produced a maximum riboflavin concentration of 7.04 mg l^{-1} in 120 h using 10 g l^{-1} of seame oil (Figure 3.15a), whereas *E. gossypii* EMS30/1 produced 21.54 mg l^{-1} in 96 h using 20 g l^{-1} of oil (Figure 3.15b). A *t-test* of the riboflavin produced by the wild-type growing on 10 g l^{-1} of sesame oil and the mutant growing on 20 g l^{-1} of the same oil was performed for the time interval (shaded region) of between 72 to 120 hours and it had a $p<0.02$, therefore the two lines were significantly different. With the use of this oil *E. gossypii* EMS30/1 at its maximum, was able to produce 206% more riboflavin than the wild-type.

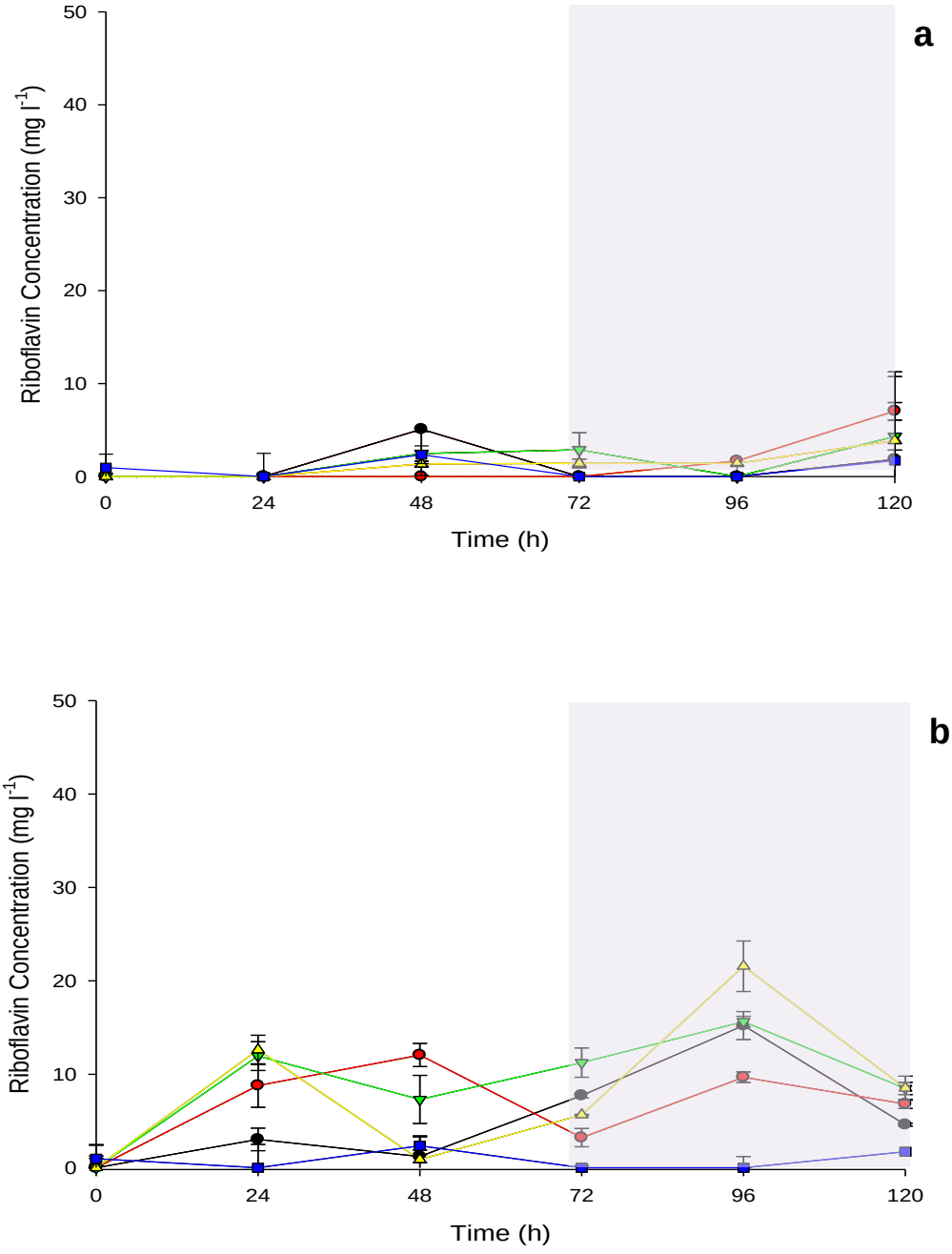

Figure 3.15 **Riboflavin produced by *E. gossypii* wild-type (a) and *E. gossypii* EMS30/1 (b) both growing on O&K media with sesame oil substituted for glucose as carbon source. (●) 5 g l^{-1}, (○) 10 g l^{-1}, (▼) 15 g l^{-1} and (△) 20 g l^{-1} and (■) control.**

The wild-type produced a maximum riboflavin concentration of 31.05 mg l^{-1} in 48 h using 20 g l^{-1} of sunflower oil (Figure 3.16a), whereas *E. gossypii* EMS30/ produced 10.90 mg l^{-1} in 120 h using 10 g l^{-1} of oil (Figure 3.16b). Statistical evaluation (t-test) was carried out on the riboflavin produced by the wild-type growing on 20 g l^{-1} of sunflower oil and the mutant growing on 10 g l^{-1} of the same oil was performed for the time interval of between 48 to 120 hours (shades region) and a $p<0.03$ was obtained, therefore indicating the two lines were significantly different. *Eremothecium gossypii* EMS30/1 produced 26% less riboflavin compared to the wild-type using this oil. This experiment showed that the wild-type using sunflower oil could produce more riboflavin than the mutant.

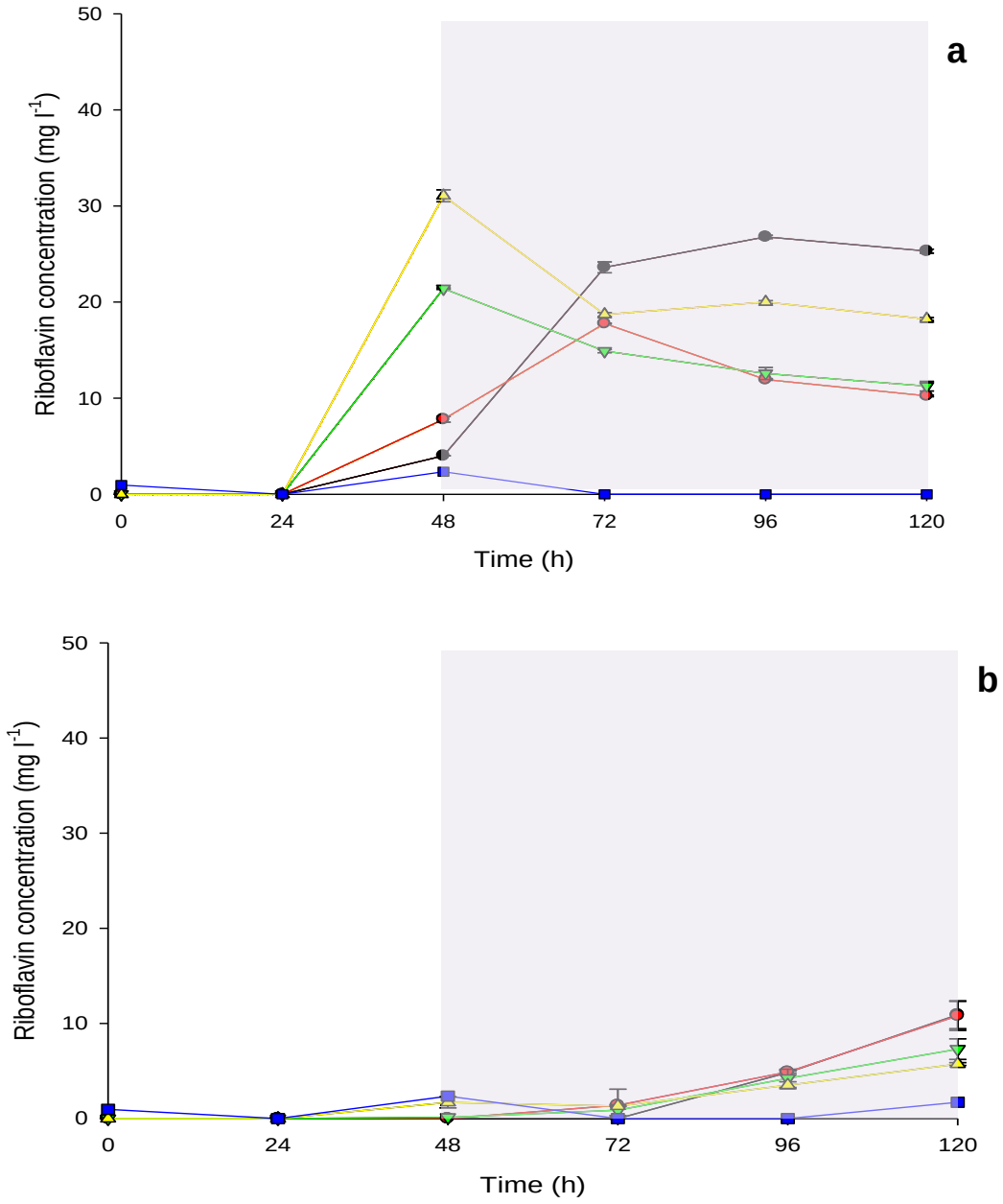

Figure 3.16 **Riboflavin produced by *E. gossypii* wild-type (a) and *E. gossypii* EMS30/1 (b) both growing on O&K media with sunflower oil substituted for glucose as carbon source. (●) 5 g l^{-1}, (○) 10 g l^{-1}, (▼) 15 g l^{-1} and (△) 20 g l^{-1} and (■) control.**

The wild-type produced a maximum riboflavin concentration of 8.34 mg l^{-1} in 24 h using 20 g l^{-1} of soybean oil (Figure 3.17a), whereas *E. gossypii* EMS30/1 produced 59.30 mg l^{-1} (611% more) in 96 h using 20 g l^{-1} of oil (Figure 3.17b). A *t-test* to determine significant difference of the riboflavin produced by the wild-type growing on 20 g l^{-1} of soybean oil and *E. gossypii* EMS30/1 growing on 20 g l^{-1} of the same oil was performed for the time interval of between 48 to 120 hours and it had a $p<0.001$ (shaded region), therefore the two lines were significantly different. The wild-type did not use the soybean oil positively as its production rates were very low.

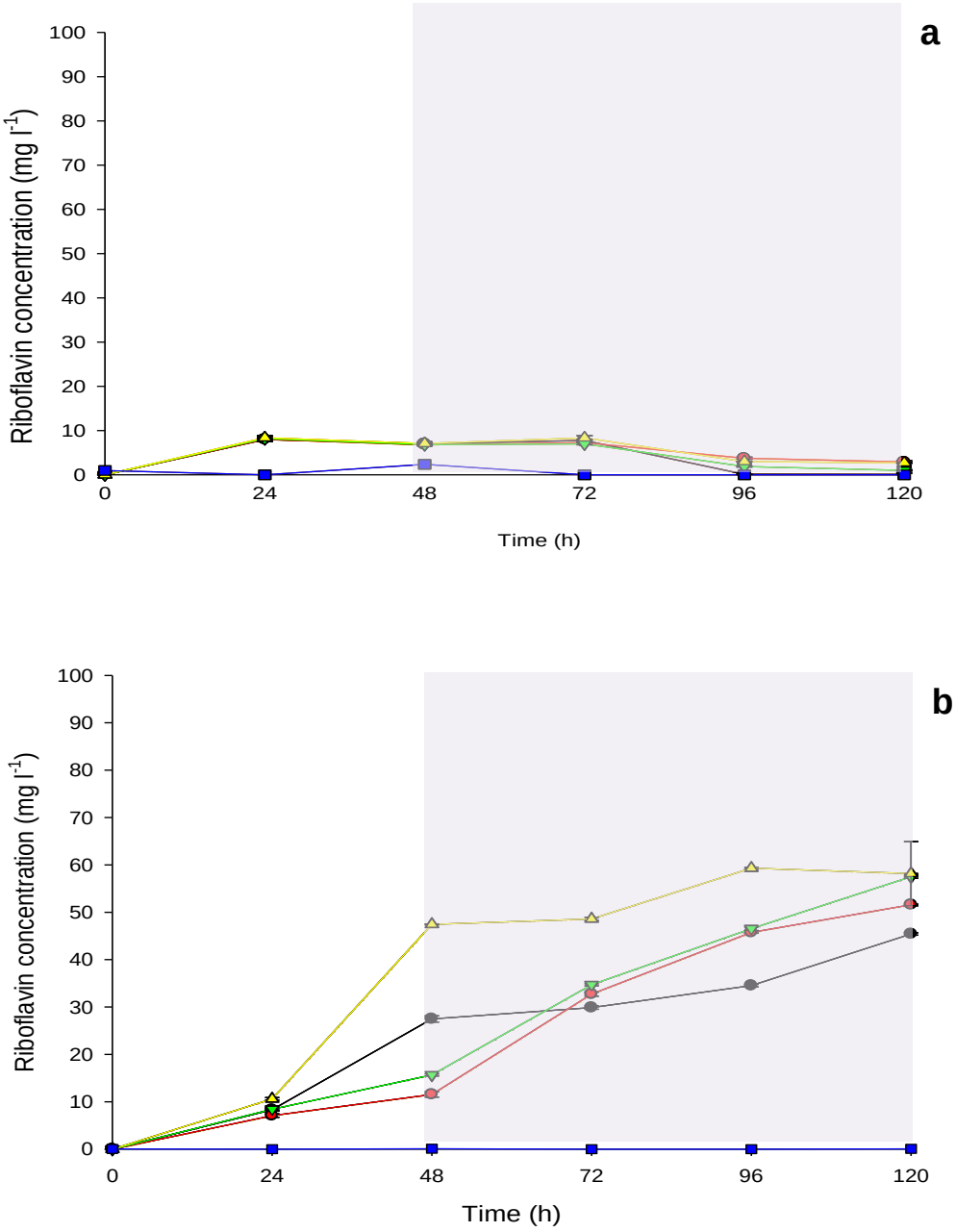

Figure 3.17 **Riboflavin produced by *E. gossypii* wild-type (a) and *E. gossypii* EMS30/1 (b) both growing on O&K media with soybean oil substituted for glucose as carbon source. (●) 5 g l^{-1}, (○) 10 g l^{-1}, (▼) 15 g l^{-1} and (△) 20 g l^{-1} and (■) control.**

A comparison of maximum riboflavin concentration produced by the wild-type and *E. gossypii* EMS30/1 growing on the different oil carbon sources is shown in Table 3.3. The third column indicated the increase or decrease in riboflavin production by the mutant compared to that of the wild-type. The oil that was the most suitable for the production of riboflavin was soybean oil and *E. gossypii* EMS30/1produced 611% more riboflavin than the wild-type on this oil. Table 3.3 shows that, using sunflower oil, the wild-type produced more riboflavin (31.56 mg l^{-1}) than the mutant (23.47 mg l^{-1}).

Table 3.3 Comparison of maximum riboflavin produced by the wild-type and *E. gossypii* EMS30/1 at different times and concentrations of carbon source

Oils	*E. gossypii* wild-type (mg l^{-1})	*E. gossypii* EMS30/1 (mg l^{-1})	Increase/decrease in riboflavin production (%)
Coconut	21.30	15.21	- 40.03
Mustard	28.67	6.45	-77.50
Peanut	8.64	29.26	238.65
Sesame	7.04	21.56	206.25
Sunflower	31.56	23.47	- 25.63
Soybean	8.34	59.30	611.03

3.10 Statistical optimization of medium composition for *Eremothecium gossypii* mutant EMS

The first screen to optimize growth media components was a five-factor sixteen run experiment (FF0516) with three center point (Table 3.4) – Five-factors X_1: peptone (high-5.0, low-0 g l^{-1}), X_2: yeast extract (high-5.0, low-0 g l^{-1}), X_3: malt extract (high-5.0, low-0 g l^{-1}), X_4: $MgSO_4$ (high-0.2, low-0 g l^{-1}), X_5: K_2HPO_4 (high-0.2, low-0 g l^{-1}). The design was a resolution V design, it confounded main effects with 4-factor interactions and 2-factor interactions with 3-factor interaction. The model for a resolution V design can resolve all the main factors and 2-factor interactions and this was the reason it was used. According to previous experiments (Table 3.2), it was determined that soybean oil allowed for the highest production of riboflavin, therefore it was used as the sole source of carbon at 20 g l^{-1}.

3.11 Primary screening experiment

Table 3.4 indicated the high and low values of the components that made up a growth medium in order to determine the best components and their effect on riboflavin production. The predicted values were calculated using the mathematical equation (equation 1) and the residual is the difference between the actual and the predicted values. The maximum riboflavin produced in FF0516 was 86.13 mg l^{-1} which was 45% more than *E. gossypii* EMS30/1 produced on the standard O&K medium. This amount of riboflavin was achieved in standard six,

run eighteen using 5.0 g l^{-1} peptone, 5.0 g l^{-1} malt extract, 0.2 g l^{-1} K_2HPO_4 and in the absence of $MgSO_4$ and yeast extract.

Table 3.4 Primary screening experiment FF0516 with three center points together with the actual, predicted and residual riboflavin yields after 120 hours

Order		X_1	X_2	X_3	X_4	X_5	Riboflavin mg l^{-1}		
Std	Runs						Actual	Predicted	Residual
1	5	0.0	0.0	0.0	0.0	0.2	3.17	18.54	-15.37
2	15	5.0	0.0	0.0	0.0	0.0	33.99	26.58	7.41
3	16	0.0	5.0	0.0	0.0	0.0	40.57	35.90	4.68
4	19	5.0	5.0	0.0	0.0	0.2	83.86	80.59	3.27
5	10	0.0	0.0	5.0	0.0	0.0	0.02	11.18	-11.15
6	18	5.0	0.0	5.0	0.0	0.2	86.13	75.16	10.98
7	14	0.0	5.0	5.0	0.0	0.2	52.82	51.70	1.12
8	8	5.0	5.0	5.0	0.0	0.0	31.60	32.54	-0.94
9	12	0.0	0.0	0.0	0.2	0.0	3.11	4.05	-0.94
10	11	5.0	0.0	0.0	0.2	0.2	51.60	50.49	1.12
11	13	0.0	5.0	0.0	0.2	0.2	38.01	27.04	10.98
12	2	5.0	5.0	0.0	0.2	0.0	14.25	25.41	-11.15
13	6	0.0	0.0	5.0	0.2	0.2	25.93	22.66	3.27
14	1	5.0	0.0	5.0	0.2	0.0	6.43	1.75	4.68
15	7	0.0	5.0	5.0	0.2	0.0	18.47	11.07	7.41
16	17	5.0	5.0	5.0	0.2	0.2	69.34	84.71	-15.37
17	9	2.5	2.5	2.5	0.1	0.1	35.00	34.50	0.51
18	4	2.5	2.5	2.5	0.1	0.1	37.25	34.50	2.76
19	3	2.5	2.5	2.5	0.1	0.1	31.23	34.50	-3.26

3.11.1 Evaluation of the Model Effects for FF0516

A Pareto chart expresses effects of individual as well as combinations of components and ranks them according to the largest effect based on the producton of riboflavin. Di-potassium hydrogen phosphate (X_5) has the largest positive effect, followed by peptone and yeast extract. K_2HPO_4 (X_5) and peptone (X_1) fell above the *Bonferroni limit* and were therefore significant, yeast extract (X_2) and a combination of peptone and yeast extract (X_1X_2) were above the *t*-value limit and were significant, although the combination of peptone and yeast extract (X_1X_2) had a negative effect (Figure 3.18).

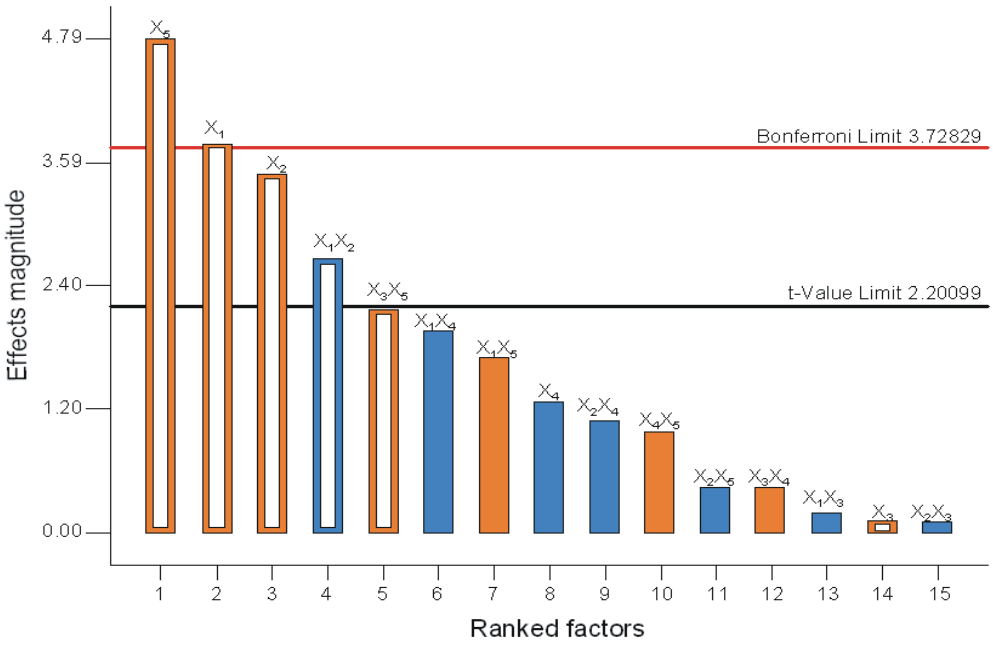

Figure 3.18 Pareto chart indicating the most significant factors of FF0516. *X_1, X_2, X_3, X_4, and X_5*. *Bonferroni limit* (Red line) 3.7 and *t-value* (Blue line) 2.2, selected factors are indicated by the clear bars. The orange bars represent the positive effect and the blue bar the negative effects.

3.11.2 Validation of the Model for FF0516

The Box Cox plot helps to determine the power transformation needed; the blue line showed the current transformation, the green line was the best model and the red lines represent the confidence intervals. A lambda value of 1 indicated that no transformation was required. This is indicated in the plot, when the blue line fell between the two red lines. In the plot shown below (Figure 3.19) the blue line (0.43) fell between the two red lines (95% confidence intervals) and 0.07 from the best fit model (0.5), no transformation was required so the linear model was used for data description.

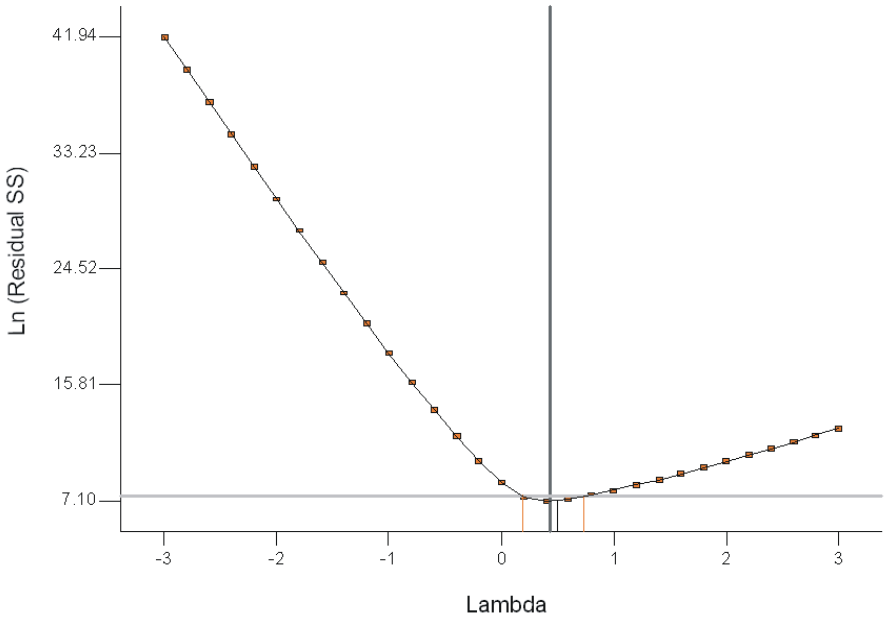

Figure 3.19 Box-Cox for power transformation for FF0516, indicating the position of lambda.

The analysis of variance table showed that only malt extract did not significantly affect riboflavin production (Table 3.5). All the other factors and their interactions were significant. This was indicated by the 'Prob > F" values, figures less than 0.05 indicated the model terms were significant (X_1, X_2, X_5 and X_1X_2) and values greater than 0.1 were not significant (X_3). This model fitted the experimental data to a precision of 99.94%. Due to the model having an F value of 10.17; there is only 0.06% chance that a model with such a large F value could occur due to noise, the model is significant indicating that all the data fit the model. The lack of fit (p<0.03) is significant indicating that the variation in the mean of the replicates are less than that of the design points. This meant the runs replicated well and their variances were negligible, or the model did not predict well or a combination of both.

Table 3.5 Analysis of variance for selected factorial model (partial sum of squares – Type III) for FF0516

Source	Sum of Squares	Df	Mean Square	F Value	P-value Prob > F	Significance Level
Model	94.84	6	15.80	10.16	0.01	Significant
X_1-peptone	22.09	1	22.09	14.21	0.01	Significant
X_2-yeast ext	18.80	1	18.80	12.09	0.01	Significant
X_3-malt ext	0.019	1	0.01	0.01	0.91	
X_5- K_2HPO_4	35.67	1	35.67	22.95	0.01	Significant
$X_1 X_2$	10.96	1	10.96	7.05	0.02	Significant
$X_3 X_5$	7.28	1	7.28	4.68	0.05	Significant
Curvature	0.85	1	0.85	0.54	0.47	
Residual	17.09	11	1.55			
Lack of Fit	16.96	9	1.88	27.76	0.03	Significant
Pure Error	0.13	2	0.06			
Cor Total	112.79	18				

Df – Degrees of freedom

Final equation in terms of coded factors

$$\text{Riboflavin (mg l}^{-1}) = 5.29 + 1.18\ X_1 + 1.08\ X_2 + 0.035\ X_3 + 1.49\ X_5 - 0.83\ X_1 X_2 + 0.67\ X_3 X_5 \quad \textbf{(Equation 1)}$$

3.11.3 Main effects of FF0516

The three factors represented in the cube plot had a significant impact on the production of riboflavin. A maximum of 79.63 mg l^{-1} riboflavin was predicted when these components are used viz, X_1 (peptone) at a high (5.0 g l^{-1}), X_2 (yeast extract) at a high (5.0 g l^{-1}) and X_3 (malt extract) at a high (5.0 g l^{-1}) (Figure 3.20). The production of riboflavin was negatively affected when X_1, X_2 and X_3 were absent from the medium.

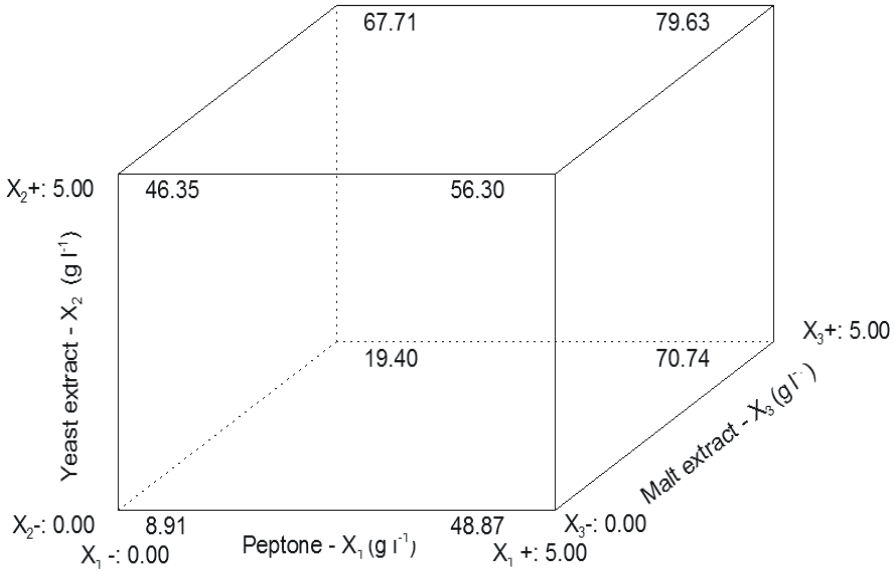

Figure 3.20 Cube plot showing effects of the three most significant factors from the first screening experiment X_1 (peptone), X_2 (yeast extract) and X_3 (malt extract).

3.11.4 Three-dimensional and contour plots of FF0516

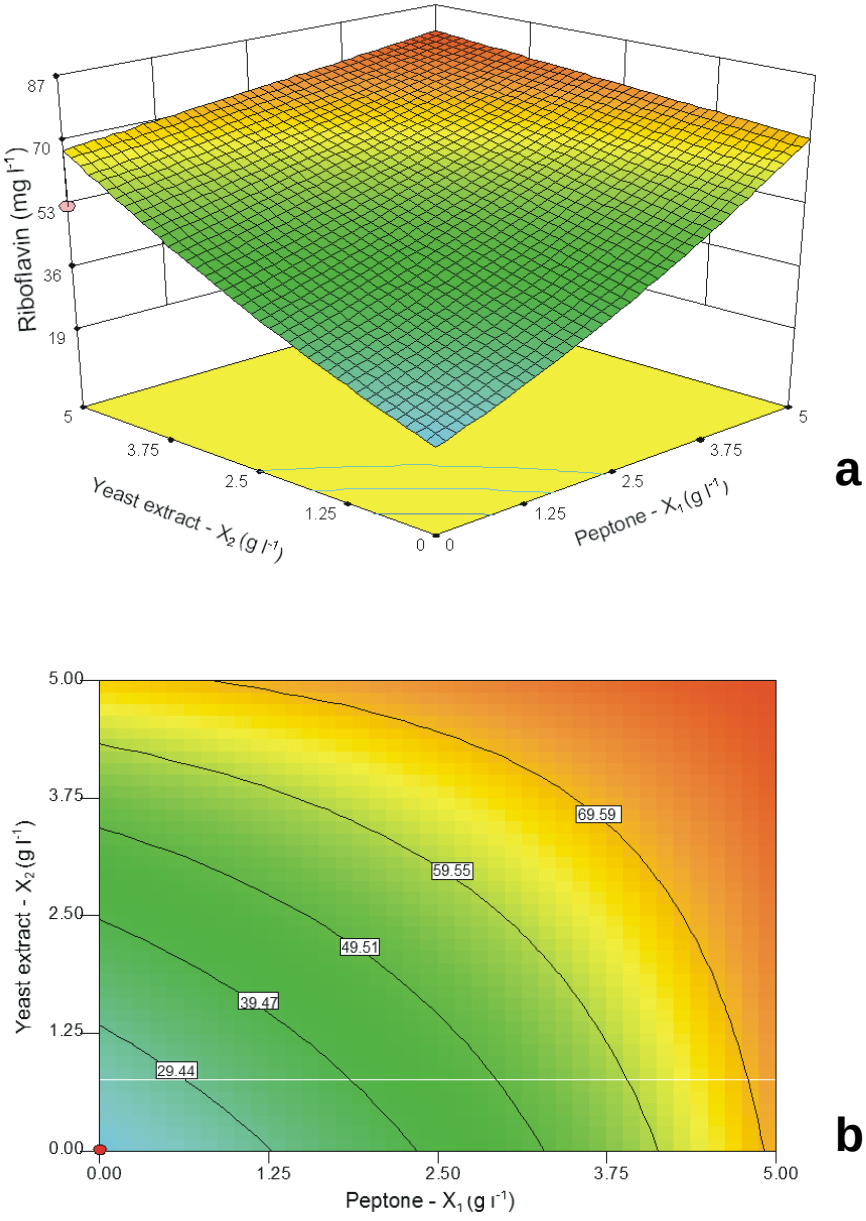

Figure 3.21 Three-dimensional (a) and contour plots (b) of riboflavin production by *E. gossypii* EMS30/1 showing the effects of two variables, peptone (X_1) and yeast extract (X_2).

Based on the above three-dimensional (Figure 3.21a) and contour plots (Figure 3.21b) it was determined that peptone, yeast extract and malt extract had to be increased and set at a low of 5.0 g l^{-1} and a high of 8.3 g l^{-1} with center points set at 6.65 g l^{-1}, K_2HPO_4 was to be set at a low of 0.2 and a high of 0.4 g l^{-1} with center points at 0.30 g l^{-1}. A maximum of 69.59 mg l^{-1} riboflavin was produced using 3.75 g l^{-1} of peptone and yeast extract (Figure 3.21a). Screen one showed that $MgSO_4$ did not make a significant difference to the production rate of riboflavin, the increase or decrease in the amount of $MgSO_4$ did not affect the predicted riboflavin concentration. Therefore, $MgSO_4$ was finally set at a low of 0.6 g l^{-1} for all remaining experiments. FF0416 was set up as follows: peptone, yeast extract and malt extract had a range of 5-8.3 g l^{-1}, K_2HPO_4 a range of 0.2-0.4 g l^{-1}, $MgSO_4$ was set at 0.6 g l^{-1} and soybean oil at 20 g l^{-1}.

3.12 Secondary screening experiment FF0416

The secondary screen to optimize growth media components was made up of four factors with three center point. The four factors were as follows: X_1: peptone (high-8.3, low-5 g l^{-1}), X_2: yeast extract (high-8.3, low-5 g l^{-1}), X_3: malt extract (high-8.3, low-5 g l^{-1}), X_5: K_2HPO_4 (high-0.4, low-0.2 g l^{-1}). The design was a resolution V design. Table 3.6 indicates the high and low values of the chemicals that made up a growth medium in order to determine the best components and its effect on riboflavin

production. The predicted values were calculated using the model (Equation 2) and the residual was the difference between the actual and the predicted values. In screen two with four factors sixteen runs and three center points, *E. gossypii* EMS30/1 produced 96.43 mg l^{-1} (Table 3.6) of riboflavin which was 62% more than it did on the standard O&K medium.

Table 3.6 Secondary screening experiment with actual, predicted and residual riboflavin yields after 120 hours (FF0416)

Order		Peptone	Yeast extract	Malt extract	K_2HPO_4	Riboflavin (mg l^{-1})		
		g l^{-1}	g l^{-1}	g l^{-1}	g l^{-1}			
Std	Runs	(X_1)	(X_2)	(X_3)	(X_5)	Actual	Predicted	Residual
1	18	5.00	5.00	5.00	0.20	69.41	69.58	-0.17
2	10	8.30	5.00	5.00	0.20	84.34	84.17	0.17
3	3	5.00	8.30	5.00	0.20	95.53	95.36	0.17
4	17	8.30	8.30	5.00	0.20	96.43	96.60	-0.17
5	12	5.00	5.00	8.30	0.20	85.72	85.55	0.17
6	5	8.30	5.00	8.30	0.20	84.16	84.33	-0.17
7	14	5.00	8.30	8.30	0.20	90.47	90.64	-0.17
8	6	8.30	8.30	8.30	0.20	86.95	86.78	0.17
9	11	5.00	5.00	5.00	0.40	96.10	95.93	0.17
10	19	8.30	5.00	5.00	0.40	89.66	89.83	-0.17
11	7	5.00	8.30	5.00	0.40	92.76	92.93	-0.17
12	2	8.30	8.30	5.00	0.40	87.62	87.45	0.17
13	1	5.00	5.00	8.30	0.40	95.35	95.52	-0.17
14	16	8.30	5.00	8.30	0.40	84.10	83.93	0.17
15	9	5.00	8.30	8.30	0.40	89.33	89.16	0.17
16	15	8.30	8.30	8.30	0.40	88.76	88.93	-0.17
17	4	6.65	6.65	6.65	0.30	95.24	95.42	-0.18
18	13	6.65	6.65	6.65	0.30	95.33	95.42	-0.09
19	8	6.65	6.65	6.65	0.30	95.68	95.42	0.26

3.12.1 Evaluation of the Model Effects for FF0416

Yeast extract (X_2) had the largest positive effect (Figure 3.22), followed by the interaction of yeast extract (X_2), malt extract (X_3) and K_2HPO_4 (X_5). Although yeast extract and K_2HPO_4 as individual factors had a positive effect, as a combination they had a negative effect. All the three-factor interactions in this experiment had positive effects. Factors above the *Bonferroni limit* were significant and those that fell above the *t-value* were possibly significant in the production of riboflavin and were taken into consideration when choosing factors. The only factors that were not significant and fell below both the *Bonferroni limit* and the *t-value* were the interactions between X_1X_2 and $X_1X_2X_3X_5$, they also had negative effects on the production of riboflavin.

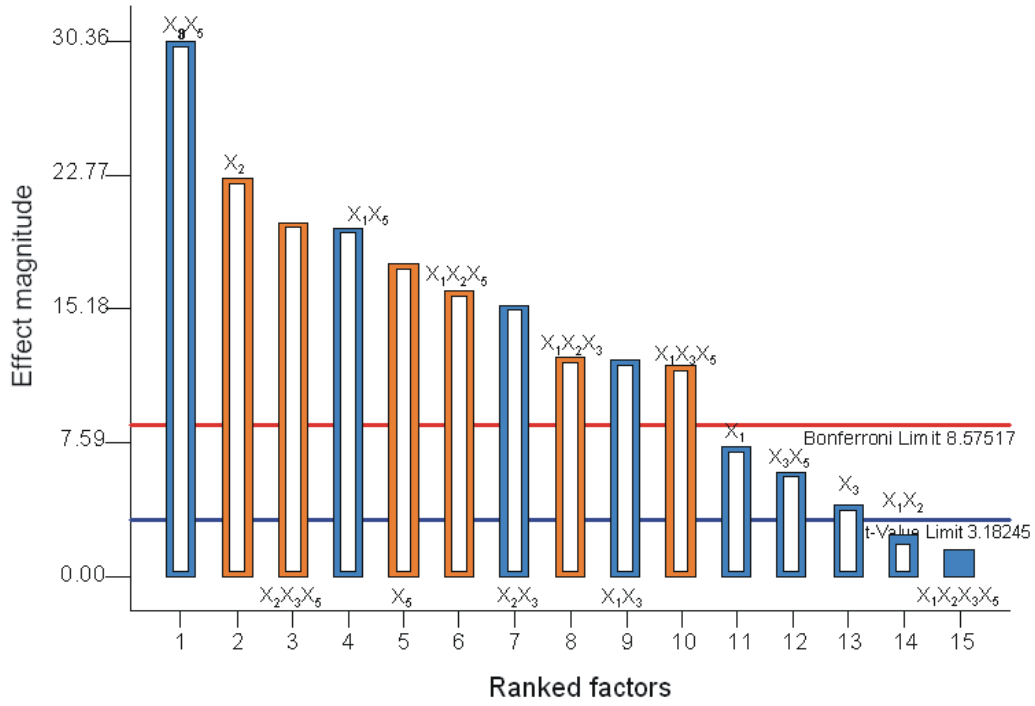

Figure 3.22 Pareto chart indicating the most significant factors. X_1: peptone, X_2: yeast extract, X_3: malt extract, X_5: K_2HPO_4. The selected factors are indicated by the clear bars, *Bonferroni limit* (Red line) 8.57 and *t-value* (Blue line) 3.18. Orange bars represent positive effects and blue bars, negative effects

3.12.2 Validation of the Model for FF0416

The analysis of variance (Table 3.7) showed that only the interaction between peptone (X_1) and yeast extract (X_2) was not significant. All the other factors and their interactions were significant in the production of riboflavin. This was indicated by the 'Prob > F" values. Figures less than 0.05 indicated the model terms were significant and values greater than 0.1 were not significant. This model fitted the experimental data to a precision of 99.96%, due to it having an F value of 256.79.

There was only 0.04% chance that a model with such a large F value could occur due to noise. Lack of fit was not significant with an F value of 0.101, this meant that all the data points fell within the design space.

The final equation in terms of coded factors was:

Riboflavin (mg l^{-1}) = 88.54 - 0.79 X_1 + 2.44 X_2 -0.44 X_3 + 1.92 X_5 - 0.25 X_1 X_2 - 1.32 X_1X_3 - 2.13 X_1 X_5 - 1.67 X_2 X_3 - 3.28 X_2 X_5 - 0.64 X_3 X_5 + 1.34 X_1 X_2 X_3 + 1.75 X_1 X_2 X_5 + 1.29 X_1 X_3 X_5 + 2.17 X_2 X_3 X_5

(Equation 2)

Where X_1: peptone, X_2: yeast extract, X_3: malt extract, X_5: K_2HPO_4.

Table 3.7 Analysis of variance for selected factorial (partial sum of squares – Type III) model for FF0416

Source	Sum of Squares	df	Mean Square	F Value	P-value Prob > F	Significance level
Model	671.47	14	47.96	256.79	0.0004	Significant
X_1-peptone	10.00	1	10.00	53.54	0.0053	Significant
X_2-yeast ext	95.11	1	95.11	509.23	0.0002	Significant
X_3-malt ext	3.07	1	3.07	16.44	0.027	Significant
X_5-K_2HPO_4	58.79	1	58.79	314.76	0.0004	Significant
$X_1 X_2$	1.01	1	1.01	5.38	0.1031	
$X_1 X_3$	27.96	1	27.96	149.68	0.0012	Significant
$X_1 X_5$	72.89	1	72.89	390.25	0.0003	Significant
$X_2 X_3$	44.39	1	44.39	237.66	0.0006	Significant
$X_2 X_5$	172.20	1	172.20	921.96	< 0.0001	Significant
$X_3 X_5$	6.49	1	6.49	34.74	0.0097	Significant
$X_1 X_2 X_3$	28.76	1	28.76	153.96	0.0011	Significant
$X_1 X_2 X_5$	48.90	1	48.90	261.78	0.0005	Significant
$X_1 X_3 X_5$	26.70	1	26.70	142.97	0.0013	Significant
$X_2 X_3 X_5$	75.21	1	75.21	402.69	0.0003	Significant
Curvature	119.36	1	119.36	639.04	0.0001	Significant
Residual	0.56	3	0.19			
Lack of Fit	0.45	1	0.45	8.36	0.1016	
Pure Error	0.11	2	0.05			
Cor Total	791.39	18				

3.12.3 Main Effects of FF0416

Three factors represented in the cube plot (Figure 3.23) have a significant impact on the production of riboflavin, a maximum of 95.93 mg l^{-1} riboflavin was predicted when these salts are used viz, X_2: yeast extract at a low (5.0 g l^{-1}), X_3: malt extract at a low (5.0 g l^{-1}) and X_5: K_2HPO_4 at a high (0.4 g l^{-1}). The production of riboflavin was negatively affected when yeast extract, malt extracts and K_2HPO_4 were set at low values.

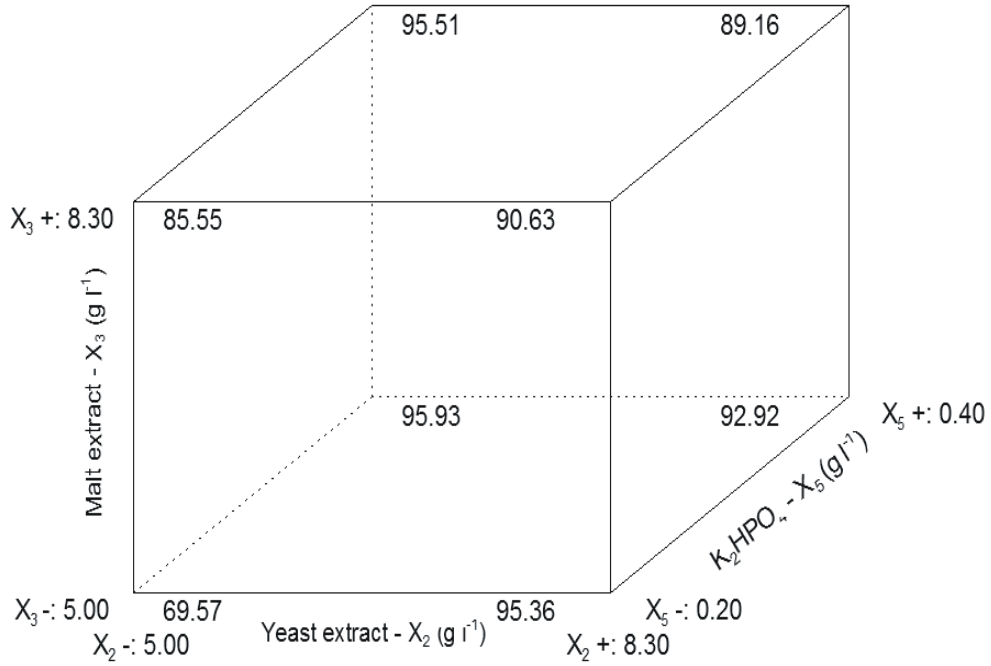

Figure 3.23 Cube plot showing effects of X_2 (yeast extract), X_3 (malt extract) and X_5 (K_2HPO_4) on riboflavin production.

3.12.4 Three-dimensional plot of FF0416

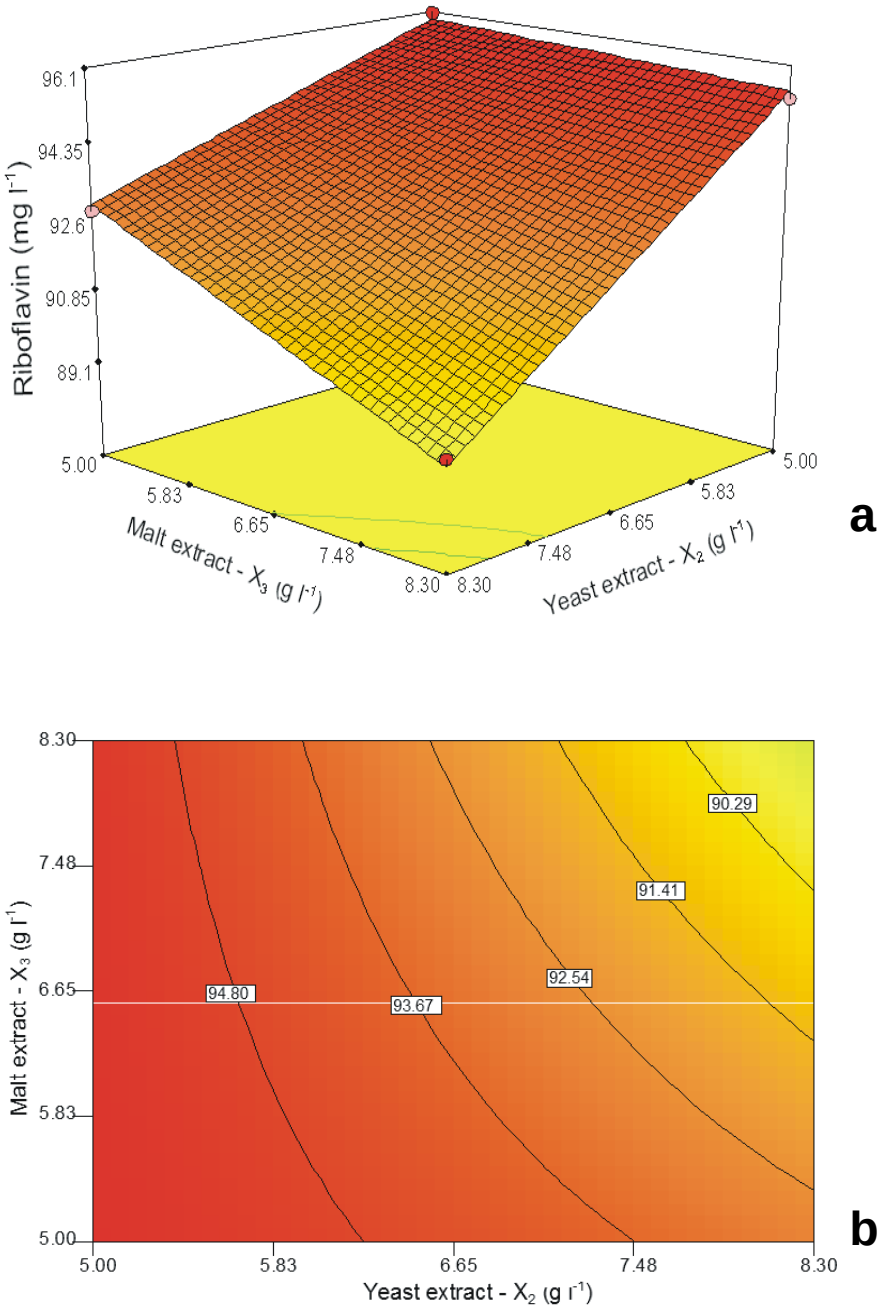

Figure 3.24 Three-dimensional plot (a) and contour plot (b) of riboflavin production by *E. gossypii* EMS30/1 showing the effects of two variables, yeast extract and malt extract.

A maximum of 94.80 mg l^{-1} riboflavin was produced using 5.83 g l^{-1} of yeast extract and 6.65 g l^{-1} malt extract after the second screening experiment. Using the three-dimensional (Figure 3.24a) and contour plots (Figure 3.24b) it was determined that increasing or decreasing the amount of peptone and malt extract did not have a significant impact on the concentration of riboflavin, therefore these factors were set at a low of 5.0 g l^{-1}.

3.13 Central composite experiment (CC0208) with five center points

The central composite matrix was set up using yeast extract (X_2) and K_2HPO_4 (X_5) (Table 3.8). This central composite experiment with two factors eight runs and five center points produced a maximum concentration of 97.12 mg l^{-1} riboflavin using 6.50 g l^{-1} of yeast extract and 0.70 g l^{-1} K_2HPO_4. The predicted value was 96.38 mg l^{-1} which was calculated using the model using the quadratic equation (Equation 4). *Eremothecium gossypii* EMS30/1 produced 0.74 mg l^{-1} more riboflavin than predicted.

Table 3.8 Central composite experimental design matrix for the prediction of maximum riboflavin production (CC0208)

Order		Yeast extract g l^{-1}	K_2HPO_4 g l^{-1}	Riboflavin (mg l^{-1})		
Std	Runs	(X_2)	(X_5)	Actual	Predicted	Residual
1	12	3.00	0.40	91.44	97.22	-5.78
2	11	10.00	0.40	62.68	56.70	5.99
3	13	3.00	1.00	88.25	91.92	-3.67
4	3	10.00	1.00	68.64	60.53	8.11
5	4	1.55	0.70	93.42	86.26	7.16
6	1	11.45	0.70	25.92	35.41	-9.49
7	2	6.50	0.28	93.21	92.87	0.34
8	10	6.50	1.12	89.18	91.84	-2.66
9	6	6.50	0.70	97.12	96.38	0.75
10	8	6.50	0.70	94.05	96.38	-2.32
11	9	6.50	0.70	96.46	96.38	0.084
12	5	6.50	0.70	97.12	96.38	0.75
13	7	6.50	0.70	97.12	96.38	0.75

Standard orders runs 1 and 6 were ignore in the ANOVA calculation due to them exceeding the limits, when diagnostics case statistics (Appendix 2 and 3) were carried out.

3.13.1 Validation of the Model for CC0208

The model for CC0208 had an F-value of 22.13 (Table 3.9) indicating the model was significant. With Prob>F less than 0.05, X_2 and X_2^2 were shown to be significant factors to the production of riboflavin. Values greater than 0.1 indicated that the factors were not significant, these included X_5, X_5^2 and a combination of X_2 X_5. The lack of fit was significant denoting that not all the data points fitted the design space, this means

the runs replicate well and their variances are negligible, or the model does not predict well or a combination of both.

Table 3.9 Analysis of variance Response Surface Quadratic Model (partial sum of squares – Type III) for CC0208

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance level
Model	1414.338	5	282.87	146.41	< 0.0001	Significant
X_2-yeast ext	872.8957	1	872.9	451.82	< 0.0001	Significant
X_5- K_2HPO_4	21.26645	1	21.266	11.008	0.0211	Significant
$X_2 X_5$	67.59034	1	67.59	34.985	0.0020	Significant
X_2^2	656.9433	1	656.94	340.04	< 0.0001	Significant
X_5^2	44.31185	1	44.312	22.936	0.0049	Significant
Residual	9.659814	5	1.932			
Lack of Fit	2.591028	1	2.591	1.4662	0.2926	
Pure Error	7.068786	4	1.7672			
Cor Total	1423.998	10				

Quadratic equation

Final equation in terms of coded factors:

Riboflavin (mg l^{-1}) = 96.38 - 17.98 X_2 - 0.37 X_5 + 2.29 $X_2 X_5$ - 17.77 X_2^2 - 2.01 X_5^2

(Equation 4)

3.13.2 Main effects of CC0208

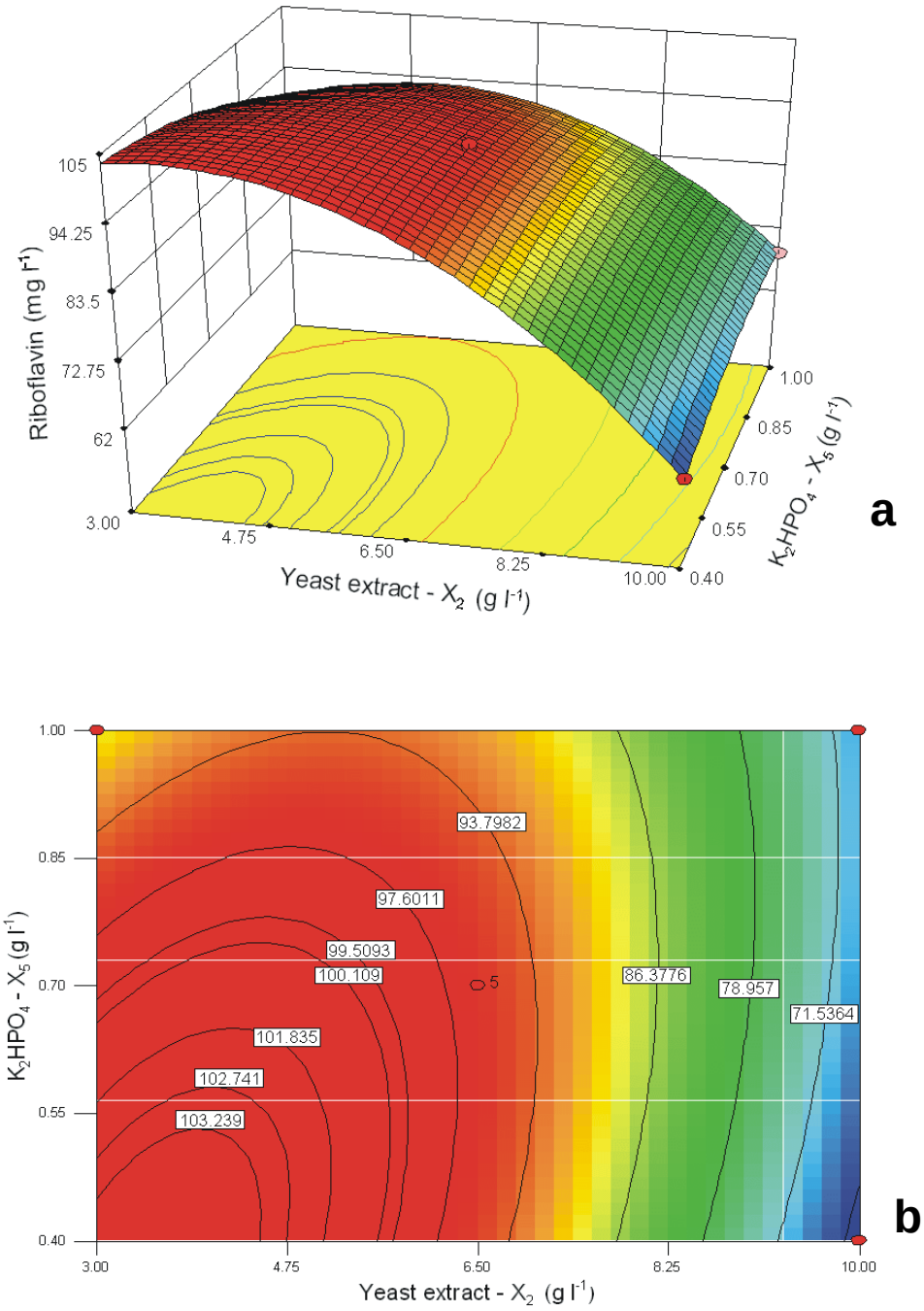

Figure 3.25 Three-dimensional plot (a) and contour plot (b) of riboflavin production by *E. gossypii* EMS30/1 showing the effects of two variables, yeast extract and K_2HPO_4. A maximum of 97.12 mg l^{-1} riboflavin was produce using 6.5 g l^{-1} yeast extract and 0.70 g l^{-1} K_2HPO_4.

In the central composite experiment, the three-dimensional and contour graph (Figure 3.25) showed that X_2 amounts higher that 8.25 g l^{-1} caused a decrease in the riboflavin production and indicated clear areas were maximum riboflavin could be achieved.

Using the point predictions in Table 3.10, confirmation experiments were set up and run. The maximum riboflavin concentration produced from the confirmation experiment was 100.03 mg l^{-1} using 5.0 g l^{-1} peptone, 5.0 g l^{-1} malt extract, 3.7 g l^{-1} yeast extract, 0.46 g l^{-1} K_2HPO_4, 0.6 g l^{-1} $MgSO_4$ and 20 g l^{-1} soybean oil. This was compared to that produced by the wild-type growing on the same carbon source (soybean oil) (Figure 3.26).

Table 3.10 Central composite point prediction for maximum riboflavin production using yeast extract and K_2HPO_4

	Prediction (mg l^{-1})	SE Mean	95% CI low	95% CI high	Yeast extract (X_2) (g l^{-1})	K_2HPO_4 (X_5) (g l^{-1})
Response						
Riboflavin	103.75	1.32	100.37	107.14	3.7	0.46
	100.16	2.87	93.36	106.96	5.1	0.79
	97.89	3.39	89.88	105.91	4.0	0.91
	100.93	2.87	94.13	107.73	5.1	0.61

3.14 Comparison of riboflavin production by the *E. gossypii* wild-type and *E. gossypii* EMS30/1using soybean oil as a carbon source.

At the start of this research, the wild-type *E. gossypii* strain using soybean oil as a carbon source only produced 8.34 mg l^{-1}, after the mutation and optimization there was an increase in production of 100.03 mg l^{-1} (Figure 3.26). This was an overall increase of 1099% (12-fold). This increase could be obtained using 5.0 g l^{-1} peptone, 5.0 g l^{-1} malt extract, 3.7 g l^{-1} yeast extract, 0.46 g l^{-1} K_2HPO_4, 0.6 g l^{-1} $MgSO_4$ and 20 g l^{-1} soybean oil. Figure 3.26 shows the increase of riboflavin over the different experiments, (A) shows mutant EMS30/1, (B) is after the primary screening, (C) amount of riboflavin produced after the second screening, (D) is following the central composite experiment.

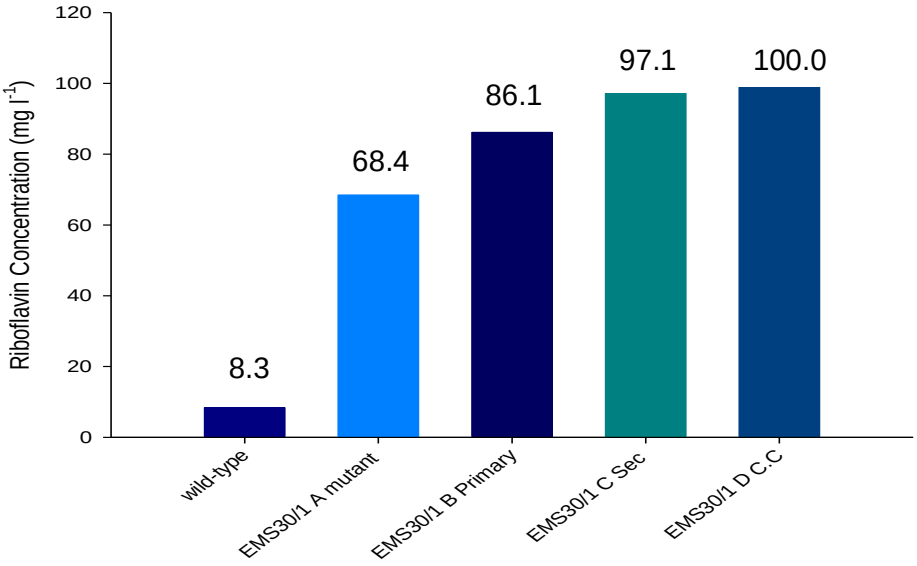

Figure 3.26 Comparison of riboflavin production by the *E. gossypii* wild-type and *E. gossypii* EMS30/1using soybean oil. Mutant EMS30/1 (A), primary screening (B), secondary screening (C) and central composite (D).

4.0 DISCUSSION

Presently, both chemical and biological production of riboflavin is being done on a large scale. The biological process uses both the wild-type and mutants of many organisms (Marx *et al.,* 2008). One of the most commonly used microorganisms for riboflavin production is *E. gossypii*, in this study we attempted to create a mutant of *E. gossypii* that could produce large amounts of riboflavin, using an oil carbon source. The mutagens used were chemical (MNNG and EMS) and physical (UV light). Six different oils were used in these experiments; sunflower, soybean, coconut, mustard, peanut and sesame oils.

Previous mutation protocols on *Eremothecium ashbya* and *Eremothecium gossypii* have been very successful in producing large amounts of riboflavin. A maximum of 15 g l^{-1} was obtained using glucose as a carbon source in 2000 by Stahmann *et al* (Stahmann *et al.*, 2000).

There are various ways in which to measure riboflavin, these include the use of the HPLC (Schmidt *et al*., 1996) and spectrophotometric methods (Kalingan, 1998). In the latter, a standard curve was constructed using known concentrations of a riboflavin solution (Figure 3.1); the standard curve had an R^2 of 0.997. In this study a riboflavin wavelength scan showed the highest peak at 445 nm. This wavelength was used in all the

riboflavin analyses in this research. This wavelength has been used by Kalingan in 1998 together with Marx *et al,* in 2008.

Eremothecium gossypii wild-type had a maximum specific growth rate of 0.02 h^{-1} and a doubling time of 23.38 h when using glucose as a carbon source (Table 3.1). Biomass rates increased exponentially between 0 to 48 hours, after which it remained at stationary phase (Figure 3.2). The exponential growth was due to the organism having a readily available carbon source that it could use. When the carbon source started to deplete, the organism progressed to stationary phase where it remained untill all the glucose was consumed after which it descend into the death phase.

Kapralek in 1962, showed in his research on the physiology of the riboflavin producer *E. ashbyi*, that during fermentation in a submerged culture, riboflavin-producing strains showed three phases of growth. The initial phase, being the exponential growth of mycelium and oxidation of glucose with a decrease in the pH, due to the increase of pyruvic acid. This stage was then followed by rapid cell-bound riboflavin synthesis, together with an increase in catalase activity and decrease in pyruvate. This resulted in ammonia build-up and an alkaline pH. The final phase was when there was autolysis of mycelium and the release of riboflavin (Kapralek, 1962). This corresponds with the experiments in this research; riboflavin was produced in the last to final phases of growth.

Glucose is a relatively simple carbon source and is therefore easy for the microorganism to break down and utilize rapidly, producing large amounts of riboflavin in a shorter time. *Eremothecium gossypii* did not produce riboflavin in the early stages of fermentation using glucose as a carbon source in this study (Figure 3.3); production started after all or most of the carbon source has been utilized and this agreed with the results of Mickelson, (1950) and Schlosser *et al,* (2001) who showed that riboflavin increased as the growth rate started to drop and that most of the riboflavin was produced in the stationary phase/production phase. Four different concentrations of glucose were used together with components of the O&K medium to determine which would be the best concentration to obtain the highest amount of riboflavin. Little or no riboflavin was produced in the first 24 hours, followed by small amounts being produced at 48 hours. However, there was a sharp increase after 48 hours, with a peak at 72 hours. *Eremothecium gossypii* using glucose as the sole carbon source produced a maximum of 31.64 mg l^{-1} in the first 72 hours with 10 g l^{-1} glucose, followed by 20, 15 and 5 g l^{-1} (Figure 3.3) after which there was a gradual decrease in riboflavin concentration. The control containing no glucose produced no riboflavin. Using the above experiment, it was determined that 10 g l^{-1} glucose was the concentration that would be used in all further experiments that used glucose as a carbon source for the growth of *E. gossypii*. This amount (10 g l^{-1}) was used due to it being the lowest amount of carbon source to product maximum riboflavin. In the medium containing 15 and 20 g l^{-1}

glucose, there was a drop in the pH and acid conditions were not favorable for the production of riboflavin.

Stahmann *et al.* (1994) in their research with *E. gossypii* determined that this organism had the unique ability to break down complex mono-unsaturated and polyunsaturated fats into simpler molecules that it could use to produce riboflavin. These fungi have many vacuoles that are used as storage ports for lipids (Figure 3.9a). These stored lipids are then used when nutrients become unavailable in adverse conditions (Stahmann *et al.,* 1994). Trial experiments were first conducted using sunflower oil as sole carbon source, to determine if the strain being used in this study was able to utilise lipids to produce riboflavin. *Eremothecium gossypii* wild-type had the ability grow and produce riboflavin using the oil as a carbon source (Figure 3.4). A maximum of 31.05 mg l^{-1} of riboflavin was produced in 48 hours using 10 g l^{-1} sunflower oil. No riboflavin was produce in the first 24 hours. This occurs due to the organism breaking down the complex fats to simpler molecules to be used in the next 24 hours (Stahmann *et al.,* 1994).

The aims of the subsequent experiments were to increase the amount of riboflavin being produced by mutating the wild-type *E. gossypii* using physical and chemical mutagenesis. Itaconate, an anti-metabolite which inhibits isocitrate lyase, was used to screen for *E. gossypii* mutants. Itaconate did not affect the growth of this fungus, only riboflavin

production. Successful mutants were able to overcome the inhibition of isocitrate lyase and produce riboflavin on this screening medium. Mutants grew yellow while the unmutated organisms grew white producing no or little riboflavin (Figure 3.8).

Ultraviolet radiation damage often causes random mutations in the DNA strand (Otto *et al*., 1981). After mutation with Ultraviolet three successful mutants were selected for further experimentation (UV1, UV2 and UV3). These three ultraviolet-induced mutants of *E. gossypii* were grown on an O&K medium with a glucose carbon source. *Eremothecium gossypii* UV1 produced 3.33% more than the wild-type (Figure 3.5). These results indicated that UV light was able to mutate *E. gossypii,* however the mutant produced only a small increase in the riboflavin production compared to the wild-type.

Chemical mutagenesis involved the use of two chemical mutagens (*N*-methyl-*n*'-nitro-*n*-nitrosoguanidine and ethylmethane sulphonate). *Eremothecium gossypii* EMS30/1 mutant produced a maximum riboflavin concentration of 68.45 mg l^{-1} (Figure 3.6) whereas the control (*Eremothecum gossypii* wild-type) produced 31.56 mg l^{-1}. The mutant *Eremothecium gossypii* EMS30/1 produced 36.89 mg l^{-1} more riboflavin than the wild-type, which was an increase of 117% over the wild-type. *Eremothecium gossypii* MNNG10/2 produced 3.22 mg l^{-1} less than the

wild-type (Figure 3.7), which was a decrease of 10%. This mutagen was therefore not successful in inducing a mutant that could produce more riboflavin than the wild-type.

When comparing the percentage increase of the riboflavin produced by all the mutants to that of the wild-type, ethylmethane sulphonate produced mutant EMS30/1 that had the highest percentage increase of 117% compared to the wild-type. When a *t-test* was performed between *E. gossypii* EMS30/1 and the wild-type (Figure 3.11), for the time interval of between 72 and 120 hours, it showed that the two were significantly different ($p<0.04$). *Eremothecium gossypii* EMS30/1 was used in all further experiments, to determine the best oil carbon source that would produce more than 117% increase in the concentration of riboflavin.

Macroscopic and microscopic analysis of both the *E. gossypii* EMS30/1 and the wild-type revealed that there was a difference in the growth of the mutant compared to the wild-type. *Eremothecium gossypii* EMS30/1 showed an increase in growth and production of riboflavin when compared to the wild-type (indicated by the difference in the diameter of the colonies and the yellowing around the area of growth) (Figure 3.8c and 3.8d). Microscopic examination at 400X (Figure 3.8a and 3.8b) showed the wild-type to have more tightly packed mycelia compared to

the mutant. Figures 3.10 and 3.11 indicated that the growth curves and the riboflavin produced by both the wild-type and *E. gossypii* EMS30/1 were directly proportional to each other. Lim *et al,* 2003 showed that the growth of *E. gossypii* affects the riboflavin concentration in their work on improvement of riboflavin production using mineral support in the culture of *Ashbya gossypii* (Lim *et al*., 2003). They found that when they increased the mineral support there was an increase in mycelium production thereby improving riboflavin production. Similarly with *E. gossypii* EMS30/1 and *E. gossypii* wild-type an increase in the growth rates, improved riboflavin production.

Eremothecium gossypii is capable of using plant oils to produce the valuable vitamin riboflavin. Lim *et al* (2003) showed that the accumulation of triglycerides in the mycelia of *E. gossypii* can be seen by using the nile red staining method. Nile red is strongly fluorescent; the dye does not react with any tissue present but is very soluble in lipids with the use of this stain the oil droplets appear orange/yellow (Figure 3.9 a and b). *Eremothecium gossypii* uses its large vacuoles as storage vessels for the lipid molecule (Figure 3.9), which serves as energy reserves for metabolic processes (Stahmann *et al.,* 1994). In conjunction with riboflavin production rates Lim *et al.* (2003) showed that as the concentration of riboflavin increased the amount of stored lipids decreased. After two days of incubation, lipid droplets were noted, from

the third day onwards the degradation of lipids began and riboflavin production increased (Lim *et al*., 2003).

Ozbas and Kutsal in 1986 showed that *E. gossypii* could use sunflower oil to produce high amounts of riboflavin. In our experiments carried out using the six organic oils, sunflower oils allowed for a maximum of 31.05 mg l^{-1} (Figure 3.16) by the wild-type (Table 3.3) and peanut oil produced a maximum of 29.26 mg l^{-1} (Figure 3.14) with a maximum riboflavin yield of 0.025 mg g^{-1} biomass which was the second higest produce by *E. gossypii* EMS30/1. Gas chromatography/mass spectrometry analysis carried out on the oils (Appendix 1, Table 6.1, Figure 6.1 and Figure 6.2) showed a presence of 10-demethylsqualene in both the peanut and sunflower oils (Tables 6.2 and 6.3), which was not presence in any other oils. Concentrations of riboflavin obtained may be due to the presences of the same compound as in the peanut oil and the ability of *E. gossypii* EMS30/1 to utilise this substrate.

When comparing riboflavin production of *E. gossypii* EMS30/1 to the wild-type using different organic oils as a carbon source, (Table 3.3 and Figure 3.12 to 3.17) the wild-type produced 8.34 mg l^{-1} on soybean oil while the *E. gossypii* EMS30/1 produced a maximum of 59.03 mg l^{-1} (Figure 3.17). This was a 611% increase from the wild-type. Lim *et al*, 2003 showed that *E. gossypii* could utilize soybean oil absorbed onto a

mineral support to produce increased amounts of riboflavin. The organism was able to grow thicker and faster on a mineral support with soybean oil than on one that had no soybean oil (Lim *et al*., 2003) this corresponds with the results obtained in this research.

Eremothecium gossypii EMS30/1 produced lower amounts of riboflavin than the wild-type when it was grown on sunflower (Figure 3.16), mustard (Figure 3.13) and coconut oils (Figure 3.12). This could be due to the mutant not being able to use the components of those oils compared to soybean oil. When grown on sesame oil the mutant produced more riboflavin (21.56 mg l^{-1}) than the wild-type (7.04 mg l^{-1}) (Figure 3.15), however this was less than the mutant produced using soybean oil (59.03 mg l^{-1}).

Pharmaceutical and biotechnological companies in the competitive world of vitamin production are forced to increase productivity of already well established production methods to meet the ever increasing market for vitamins. In the industrial production processes, even increases of a small percent, affect the final product scale and the profit margin vastly. These minor changes are often obtained by media optimization (Kovar *et al.,* 2000).

The EMS induced mutant, which could produce 611% more riboflavin that the wild-type using soybean oil as a sole carbon source, was investigated to increase riboflavin production with the help of statistical optimization tools. Design of experiments was used to optimize media components developed by Ozbas and Kutsal in 1986. The medium was made up of five factors peptone, yeast extract, malt extract, $MgSO_4$, K_2HPO_4 and 20 g l^{-1} soybean oil was substituted for glucose as the carbon source.

The optimization process was started by running a screening fractional factorial experiment, the first of which was a primary screening (FF0516) experiment including three center points to allow for enhanced estimation of the experimental error and provide information on the yields in the experimental area (Haaland, 1989) (Table 3.4). *Eremothecium gossypii* EMS30/1 produced the highest concentration of riboflavin, 86.13 mg l^{-1} using 5.0 g l^{-1} peptone, no yeast extract, 5.0 g l^{-1} malt extract, no $MgSO_4$ and 0.2 g l^{-1} K_2HPO_4. The Pareto chart, indicated the most significant factors and rank them based on the greatest effect on riboflavin production. In FF0516 di-potassium hydrogen phosphate had the largest positive effect (Figure 3.18). Di-potassium hydrogen phosphate provides the phosphorus component in the growth medium and is an energy source for cell growth and product formation (Wu *et al.,* 2007).

The Box Cox plot indicated that the predicted model fell between the 95% confidence interval and therefore a transformation was not required (Figure 3.19). An ANOVA ran on the FF0516 experiment (Table 3.5) revealed that the model significantly fit the data ($p<0.05$). Peptone, yeast extract and K_2HPO_4 were the significant factors; malt extract with a $p>0.91$ was not significant. Three factors represented in the cube plot (Figure 3.20) had a significant impact on the production of riboflavin, a maximum of 79.63 mg l^{-1} riboflavin was predicted when peptone at a high (5.0 g l^{-1}), yeast extract at a high (5.0 g l^{-1}) and malt extract at a high (5.0 g l^{-1}) was used. These three components provide nutrients for *E. gossypii.* Peptone provides a source of proteins and amino acid, yeast extract is the source of nitrogen and K_2HPO_4 acts as the buffering agent. The production of riboflavin was negatively affected (dropped from 79.63 to 8.91 mg l^{-1}) when peptone, yeast extract and malt extracts were absent from the medium. The mathematical model (equation 1) was used to calculate the predicted values.

Using the three dimensional and contours plots of FF0516 (Figure 3.21) it was determined that peptone, yeast extract and malt extract had to be increased and set at a low of 5 g l^{-1} and a high of 8.3 g l^{-1} with center points set at 6.65 g l^{-1}, K_2HPO_4 was to be set at a low of 0.2 g l^{-1} and a high of 0.4 g l^{-1} with center points at 0.30 g l^{-1}. The primary screen showed that $MgSO_4$ did not make a significant difference to the

production rate of riboflavin, the increase or decrease in the amount of $MgSO_4$ did not affect the riboflavin concentration. This could be due to the fact that $MgSO_4$ serves as a good source of magnesium however not all fungi are able to utilize sulphates as a source of sulphur. Magnesium sulphate was therefore set at a low of 0.6 g l^{-1}.

The second screening experiment was a fractional factorial experiment (0416) which was run to reduce the number of factors from five to four required to produce higher amounts of riboflavin. Table 3.6 shows the design for FF0416, the composition and amounts of each chemical component in runs one to nineteen. Run 17, standard 4, produced (96.43 mg l^{-1}) the highest amount of riboflavin in this set of experiments. This maximum was produced using 8.30 g l^{-1} peptone, 8.30 g l^{-1} yeast extract, 5.0 g l^{-1} malt extract, 0.20 g l^{-1} K_2HPO_4, 0.6 g l^{-1} $MgSO_4$ and 20 g l^{-1} soybean oil, which was 62% more than it did on the standard O&K medium.

Yeast extract in the Pareto chart had the largest positive effect, followed by the interaction of yeast extract, malt extract and K_2HPO_4. Although yeast extract and K_2HPO_4 as individual factors had a positive effect, as a combination they had a negative effect (Figure 3.22). All the three-factor interactions in this experiment had a positive effect on riboflavin production.

Analysis of variance (Table 3.7) showed that the model was significant and indicated that all the individual factors as well as their interaction with the exception of the combination of peptone and yeast extract had a positive effect on riboflavin production. Yeast extract, malt extract and K_2HPO_4 were the three main factors represented in the cube plot (Figure 3.25). The production of riboflavin was negatively affected (dropped from 95.93 to 69.57 mg l^{-1}) when yeast extract, malt extracts and K_2HPO_4 were set at a low concentration, but increased when yeast extract and malt extract were at low concentrations with K_2HPO_4 at a high concentration.

The three-dimensional plot together with the contour plot (Figure 3.24a and 3.24b) obtained from FF0416 were used to understand the interactions between the growth medium components and determine the optimum range of factors. It was revealed that increasing or decreasing the amount of peptone and malt extract did not have a significant impact on the concentration of riboflavin (Figure 3.23), However peptone was a significant factor (p<0.0053), the use of peptone in riboflavin production is important. It is added as a nitrogen source in the production medium. In 1950 Dulaney and Grutter, showed that *E. ashbyi* did not grow in media that had no source of peptones. Riboflavin production was only observed when peptones were added, with the model showing that peptones were significant, therefore it was set at a high of 5.0 g l^{-1}.

The range of yeast extract was increase from between 5 and 8.3 g l^{-1} to being set at 3 g l^{-1} for the low and 10 g l^{-1} for the high. K_2HPO_4 range was between 0.4 to 1 g l^{-1} with 0.7 g l^{-1} as a center point.

Using the primary (FF0516) and secondary (FF0416) screening experiments, a central composite experiment was designed in which peptone and K_2HPO_4 were optimized. An optimization experiment is aimed at producing a response surface that curves upwards (Figure 3.25), indicating that the optimal response will be on the interior region. Peptone ranged from 1.55 – 11.45 g l^{-1} and K_2HPO_4 from 0.28 – 1.12 g l^{-1}. The resulting model developed was statistically significant (p<0.004).

A central composite experiment (CC0208) (Table 3.8) produced a maxium concentration of 97.12 mg l^{-1} riboflavin using 6.50 g l^{-1} of yeast extract and 0.70 g l^{-1} K_2HPO_4. Di-potassium hydrogen phosphate provides phosphorus to the growth medium, which is essential for cellular growth and product formation. This compound has also been known to double as a buffer for pH in culture media (Wu *et al.*, 2007). The predicted value was 103.75 mg l^{-1}. This was predicted using the quadratic second order equation (4).

The ANOVA conducted (Table 3.9) on the CC0208 design originally showed that the two-factor interaction between yeast extract and K_2HPO_4 was not significant. These two-factors needed to be significant because they were the key factors used in this central composite design. The diagnostics case statistics (Appendix 2, Figure 6.4 and Appendix 3, Figure 6.5) for the central composite showed that standard order runs 1 and 6 had exceeded the set limits for the external studentized residual, influence on the fitted value and the Cook's distance. Cook's distance is often used to determine the influence of data points in statistical regression analysis, based on these results standard order runs 1 and 6 were ignored, allowing for all remaining points to fit the model significantly ($P<0.0001$). The two-factor interaction between yeast extract and K_2HPO_4 was now significant ($P<0.002$).

Using the mathematical model developed from the CC0208 design it was predicted that the maximum riboflavin produced could be 103.75 mg l^{-1} (SE means 1.32). This amount could be obtained using a predicted 5.0 g l^{-1} peptone, 5.0 g l^{-1} malt extract, 3.7 g l^{-1} yeast extract, 0.46 g l^{-1} K_2HPO_4, 0.6 g l^{-1} $MgSO_4$ and 20 g l^{-1} soybean oil.

Confirmation experiments (Table 3.10) were carried out and 100.03 mg l^{-1} of riboflavin (Figure 3.36) was obtained. This was 68% more than *E. gossypii* EMS30/1 produced growing on a standard O&K

medium using soybean oil as a carbon source and 1090% more than the wild-type growing on a standard O&K medium using glucose as a carbon source.

4.1 CONCLUSIONS

- This research showed that a chemical mutation using ethylmethane sulphonate of *E. gossypii* successfully produced a mutant that was capable of producing high amounts of riboflavin.
- Mutants induced by ultraviolet light and *N*-methyl-*n*'-nitro-*n*-nitrosoguanidine were not successful at producing larger increases in riboflavin production.
- Mutant *Eremotheium gossypii* EMS30/1 produced the most riboflavin and demonstrated colony and microscopic differences when compared to the wild-type.
- Soybean oil supported the growth of both *E. gossypii* EMS30/1 and the wild-type and *E. gossypii* EMS30/1 produced 611% more riboflavin than the wild-type.
- Fractional factorial and central composite designs increased riboflavin production by several fold over their iterations.
- There was an overall increase of 1099% (12-fold) in riboflavin production by the *E. gossypii* EMS30/1 (100.03 mg l^{-1}) compared to the initial riboflavin produced by the wild-type (8.34 mg l^{-1}) using 20 g l^{-1} soybean oil.

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6.0 APPENDICES

Appendix 1

Oil Sample Preparation for GCMS

The oil samples (one drop each) were dissolved in methylene chloride (1 mL) and analyzed.

One microlitre of the above sample was injected into the HP 6890 series Gas Chromatograph interfaced to an HP 5973 Mass Selective Detector (MSD) and controlled by HP Chemstation software (version b.02.05, 1989-1997). The chromatographic separation was achieved using a DB-5 MS capillary column (30.0 m x 250 µm x 0.25 µm). The column stationary phase comprised of 5%-diphenyl-95% dimethylpolysiloxane.

Table 6.1 GCMS parameters for the analysis of oil samples were set as follows:

GAS CHROMATOGRAPH CONDITIONS:	
Oven Temperature Programme:	
Initial Temp:	50°C
Initial Time:	2 minutes
Ramp Rate:	10°C/min
Final Temp:	300°C
Final time:	3 minutes
Injector Conditions:	
Injection mode:	Splitless
Injector Temp:	250°C
Injector volume:	1 µL

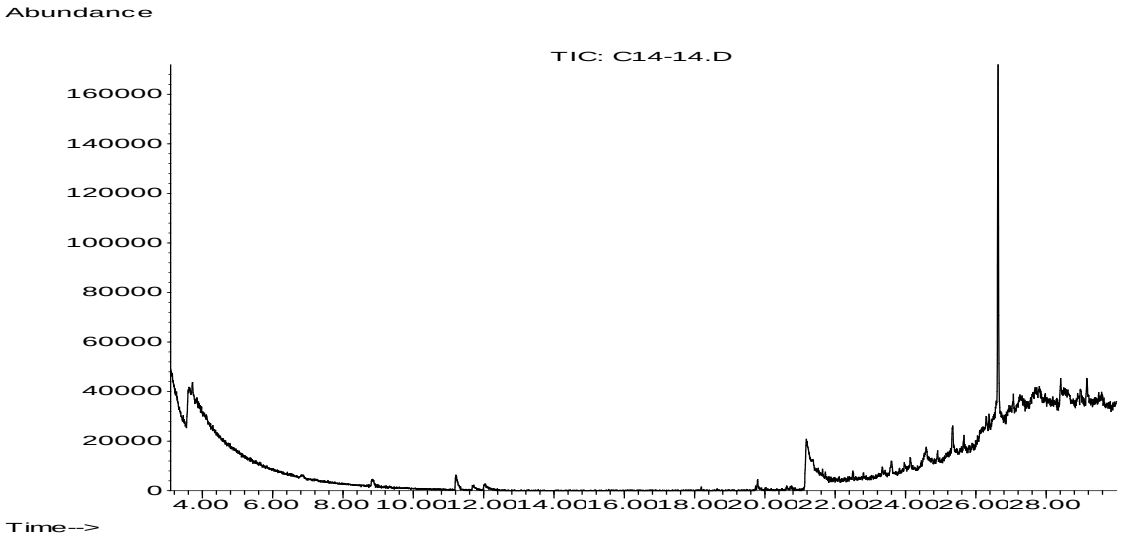

Figure 6.1 Chromatogram of Peanut oil sample

Table 6.2 Compounds identified in Peanut oil

Compounds	Library Match Quality (%)	Retention time
10-Demethylsqualene	90	26.63
Vitamin E	81	29.16

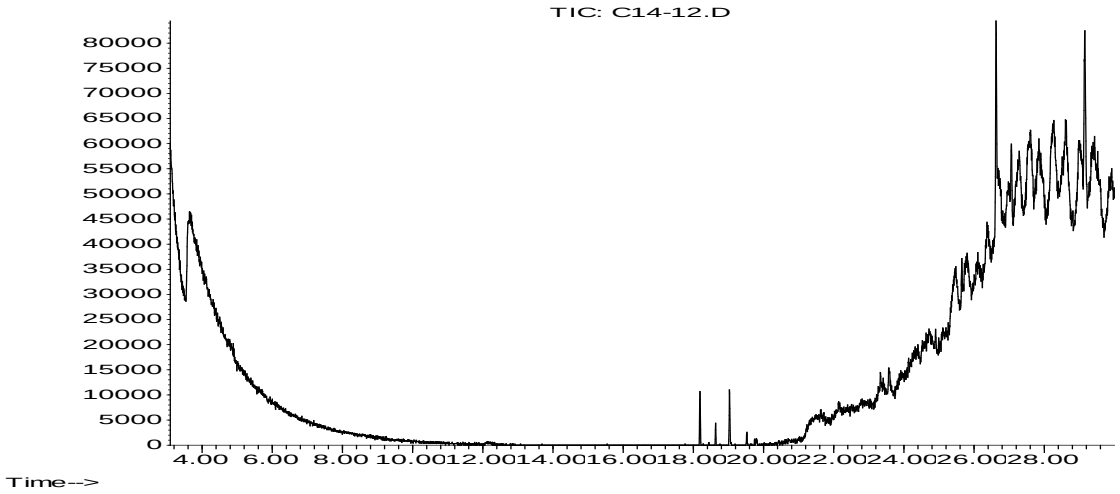

Figure 6.2 Chromatogram of sunflower oil sample

Table 6.3 Compounds identified in Sunflower oil

Compounds	Library Match Quality (%)	Retention time
10-Demethylsqualene	87	26.63
Vitamin E	94	29.16

Appendix 2

Table 6.4 First diagnostics case statistics for the Central composite

Diagnostics Case Statistics

Std Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residual	Externally Studentized Residual	Influence on Fitted Value DFFITS	Cook's Distance	Run Order
1	91.44	97.22	-5.78	0.63	-1.43	-1.58	* -2.04	0.57	12
2	62.68	56.70	5.99	0.63	1.48	1.66	* 2.14	0.61	11
3	88.25	91.92	-3.67	0.63	-0.91	-0.90	-1.16	0.23	13
4	68.64	60.53	8.11	0.63	2.01	2.86	* 3.69	* 1.12	3
5	93.42	86.26	7.16	0.63	1.78	2.22	* 2.86	0.88	4
6	25.92	35.41	-9.49	0.63	-2.35	** -4.74	* -6.12	* 1.53	1
7	93.21	92.87	0.34	0.63	0.08	0.08	0.10	0.00	2
8	89.18	91.84	-2.66	0.63	-0.66	-0.63	-0.81	0.12	10
9	97.13	96.38	0.75	0.20	0.13	0.12	0.06	0.00	6
10	94.06	96.38	-2.32	0.20	-0.39	-0.37	-0.18	0.01	8
11	96.46	96.38	0.08	0.20	0.01	0.01	0.01	0.00	9
12	97.13	96.38	0.75	0.20	0.13	0.12	0.06	0.00	5
13	97.13	96.38	0.75	0.20	0.13	0.12	0.06	0.00	7

** Case(s) with |External Stud. Residuals| > 4.24
* Exceeds limits

Appendix 3

Table 6.5 Second diagnostics case statistics for the Central composite

Diagnostics Case Statistics									
Std Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residual	Externally Studentized Residual	Influence on Fitted Value DFFITS	Cook's Distance	Run Order
1	91.44	95.91	-4.47	0.63	-2.26	** -5.31	* -6.96	* 1.46	12
2	62.68	64.33	-1.64	0.87	-1.38	-1.53	* -3.92	* 2.10	11
3	88.25	90.61	-2.36	0.63	-1.19	-1.24	-1.63	0.41	13
4	68.64	68.17	0.47	0.87	0.40	0.37	0.94	0.17	3
5	93.42	89.42	4.00	0.67	2.12	3.87	* 5.48	* 1.50	4
7	93.21	89.71	3.50	0.67	1.85	2.59	* 3.66	* 1.15	2
8	89.18	88.68	0.50	0.67	0.27	0.25	0.35	0.02	10
9	97.13	96.38	0.75	0.20	0.26	0.23	0.12	0.00	6
10	94.06	96.38	-2.32	0.20	-0.79	-0.77	-0.38	0.03	8
11	96.46	96.38	0.08	0.20	0.03	0.03	0.01	0.00	9
12	97.13	96.38	0.75	0.20	0.26	0.23	0.12	0.00	5
13	97.13	96.38	0.75	0.20	0.26	0.23	0.12	0.00	7

** Case(s) with |External Stud. Residuals| > 4.29
* Exceeds limits