DURBAN UNIVERSITY of TECHNOLOGY

Bioproduction of Riboflavin by Fungi using Spent Industrial Oils

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DECLARATION

I declare that the work done in this dissertation was carried out in accordance with the Regulations of the Durban University of Technology. The work is original, except where indicated by special reference in the text, and part of the dissertation has been submitted for publication.

Signed: ..... Date: .....

Supervisor: ..... Date: .....

Co-supervisor..... Date: .....

PREFACE

Title of Publication: Spent Motor Oil as a Substrate for Riboflavin Production using a mutant of *Eremothecium gossypii*

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ABSTRACT

Riboflavin (vitamin B_2), an essential water-soluble vitamin is commercially produced because it cannot be synthesized by vertebrates. Although this vitamin is produced chemically, bioproduction is a better option since it is more economical, requires less energy, produces less waste and can use renewable sources. In this study we investigated spent oil from the food and motor industries as alternative cheap carbon sources for the bioproduction of this vitamin. Commercial fungal strains namely; *Eremothecium gossypii* ATCC 10895, *Eremothecium gossypii* CBS 109.51, *Eremothecium ashbyi* CBS 206.58 and the yeast, *Candida famata* ATCC 20850, as well as a laboratory mutated *Eremothecium gossypii* EMS 30/1 strain were used. Statistical experimental design using a series of fractional factorial experimental designs was used to optimize the effect of yeast extract, peptone, malt extract, K_2HPO_4 and $MgSO_4.7H_2O$ to supplement the used oils for optimum riboflavin production. Response surface methodology based on central composite experimental designs was then applied and together with the point predictions made, production media for both substrates were further optimized. The optimized conditions were then tested with laboratory experiments. Results showed that mutant *E. gossypii* EMS 30/1 produced the most riboflavin in spent motor oil (20.45 $mg.l^{-1}$) while *Candida famata* ATCC 20850 produced the highest concentration (16.99 $mg.l^{-1}$) in spent vegetable oil. With these strains and using the experimental designs from the fractional factorial experiments, supplemented spent motor and spent vegetable oils produced 66.27 $mg.l^{-1}$ and 72.50 $mg.l^{-1}$ riboflavin, respectively. The central composite optimization results showed that 0.18 $g.l^{-1}$ and 0.45 $g.l^{-1}$ K_2HPO_4 and 12.5 $g.l^{-1}$ malt extract increased the production to 91.88 $mg.l^{-1}$ and 78.68 $mg.l^{-1}$ in spent vegetable oil and motor oil respectively. A point prediction from the response surface methodology was used to validate these and it was found that 103.59 $mg.l^{-1}$ riboflavin was produced by mutant *E. gossypii* EMS 30/1 using 2.5 $g.l^{-1}$ yeast extract, 0.5 $g.l^{-1}$ peptone, 12.5 $g.l^{-1}$ malt extract, 0.18 $g.l^{-1}$ K_2HPO_4 and 0.3 $g.l^{-1}$ $MgSO_4.7H_2O$. After optimizing K_2HPO_4 in a one-factor-at-a-time experiment, 82.75 $mg.l^{-1}$ riboflavin was produced by *C. famata*on

SVO using 6.5 $g.l^{-1}$ peptone, 12.5 $g.l^{-1}$ malt extract 0.15 $g.l^{-1}$ K_2HPO_4 and 1.75 $g.l^{-1}$ $MgSO_4.7H_2O$. This is a 5.08 and 4.87 fold increase respectively when compared to spent oil prior to optimization. This shows that spent motor oil and mutant *E. gossypii* produces 103.59 $mg.l^{-1}$ riboflavin while spent vegetable oil and *C. famata* produces 82.75 $mg.l^{-1}$ riboflavin. Hence, *E. gossypii* can be used to generate riboflavin using spent motor oil and *C. famata*, using spent vegetable oil.

DEDICATION

This thesis is dedicated to my late parents Sumdhara and Roopnarain Ramphal, my loving husband, Feiaz Mohamed Hassan Ally, and my children Farhana, Zaheer, Mohamed Zameer and Sumaya Hassan Ally.

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Sincere thanks to my family who gave me the courage and continuous support that I required, to return to study, after a dormant period of twelve years.

LIST OF ABBREVIATIONS

ANOVA - Analysis of Variance

ARS – Agricultural Research Service

ATCC – American Type Culture Collection

CBS – Centraalbureau voor Schimmelcultures

DOE - Design of Experiments

EMS – ethylmethane sulphonate

FAD – flavin adenine dinucleotide

FMN – flavin mononucleotide

NRRL – Northern Regional Research Laboratory

O and K – Ozbas and Kutsal

OFAT - One-factor-at-a-time

rpm - revolutions per minute

SMO – spent motor oil

SPC - statistical process control

SVO – spent vegetable oil

T_d - doubling time

λ - lambda - A lambda value of 1 indicates that no transformation is required ie. The model lies within the 95% confidence interval.

μ - specific growth rates

μ_{max} - maximum specific growth rate

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CHAPTER ONE

1.0 INTRODUCTION

Riboflavin, commonly known as vitamin B_2, is a precursor of coenzymes flavin mono nucleotide (FMN) and flavin adenine dinucleotide (FAD) and is therefore considered an essential component of basic metabolism (Wu *et al.*, 2007). It plays a major role in maintaining good health as it participates in the production of energy during the metabolism of carbohydrates, proteins and lipids. The constant loss of this vitamin by the body, due mainly to its water-soluble property, emphasises the importance of daily riboflavin replenishment, obtained orally, either from a healthy diet, or from supplements. Presently, more than 6,500 tonnes of riboflavin is produced annually to accommodate this increasing worldwide demand (Yuanyuan, 2008). Consequently, riboflavin is produced at a commercial level, either chemically, biologically or by a combination of both methods (Peterson and Kiener, 1998).

Chemical synthesis of riboflavin, which involves numerous steps, produces riboflavin of two qualities: a 96% chemical purity for the animal feed market and 98% chemical purity for human consumption and the pharmaceutical market (Bretzel *et al.*, 1999). The alternative method, a one-step biological synthesis process requires less energy, uses renewable sources, generates less wastes and most importantly, is less costly (Sung *et al.*, 2003), when compared to the multi-step chemical synthesis. Consequently, biological synthesis of riboflavin is preferred, and is gradually replacing chemical synthesis (Karos *et al.*, 2004).

Improvement of the biological production of riboflavin has been a focus of research for many years. Attempts to improve production were made either by genetic engineering or modification of cultivation media. This bioprocess is notably determined by the type and initial concentration of carbon and nitrogen sources (Kalingan and Krishnan, 1997).

Initially, research directed towards developing a low-cost fermentative method for the production of this vitamin, was conducted by manipulating fermentative conditions in order to rapidly produce riboflavin and to increase yields using glucose-based media (Perkins *et al.*, 1999). Numerous efforts were made using various vegetable oils (soybean oil, peanut oil, sunflower oil) as carbon sources for the production of this vitamin (Ozbas and Kutsal, 1986), but spent oils as a source of carbon are yet to be investigated. Hence, with the intention of minimizing production costs, inexpensive spent industrial oils were selected, as substrates, for this study.

Spent industrial oils selected for this study were investigated for their availability which led to the selection of spent motor oil from the motor industry and spent vegetable oil from the food industry, as carbon substrates for the bioproduction of riboflavin. A glucose medium supplemented with nutrients yeast extract, peptone, malt extract, di-potassium hydrogen phosphate and magnesium phosphate hepta-hydrate that supported fungal growth and riboflavin production, comprised the standard cultivation medium (Ozbas and Kutsal, 1986), that was modified by substituting glucose with spent industrial oil, to produce riboflavin. Statistical experimental design was used to establish the effects of these incorporated nutrients on riboflavin production using either spent motor or vegetable oils as a carbon source.

Previous studies (Goodwin and Pendlington, 1954) have shown that the yeast *Candida famata* and two filamentous ascomycetes, *Eremothecium ashbyi* and *Eremothecium gossypii* synthesise and secrete riboflavin into culture media. *Eremothecium gossypii* is preferred over *E. ashbyi* due to its genetic stability (Bretzel *et al*, 1999). However, for this study, *E. gossypii* (wild type), *E. gossypii* (strain 109.51), a mutant strain *E. gossypii* EMS 30/1 derived by using the chemical mutagen ethyl-methyl sulphonate), *E. ashbyi* (strain 206.58) and *C. famata* (strain 20850) were selected for the bioproduction of riboflavin.

This study is considered unique in that although numerous patents for riboflavin production have been recorded, no reports for the utilisation of spent industrial oils for the production of this vitamin have been recorded.

1.2 AIM:

The aim was to produce riboflavin from spent industrial oils using fungi and to optimize this production using statistical experimental designs.

1.3 OBJECTIVES:

The objectives were thus to:

1. Investigate the availability and composition of spent industrial oils.

2. Select riboflavin-producing microorganisms that can utilise spent oils for riboflavin production.

3. Screen nutrient supplements that affect riboflavin production by selected microorganisms on spent oils using fractional factorial experimental designs.

4. Optimize nutrient supplements required for riboflavin production on selected substrates using response surface methodology based on central composite experimental designs.

5. Verify optimized parameters for riboflavin production by comparing concentrations produced using the optimized media with the predicted concentrations.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 VITAMINS

Vitamins, described as essential micronutrients, are required in small amounts for cellular metabolism in all living organisms. Despite their importance, mammals are unable to synthesise vitamins, which are produced by microorganisms and plants. Consequently, the body acquires these micronutrients through dietary supplementation. Vitamins may be categorized as either fat-soluble or water-soluble. The former category includes vitamins A, D, E and K, while the latter includes B_1 (thiamine), B_2 (riboflavin), B_3 (niacin), folic acid, B_6 (pyridoxine), B_{12}, pantothenic acid, biotin and vitamin C (Survase *et al.*, 2006).

2.1.1 Production of Vitamins

Vitamins are widely introduced to the body as food/feed supplements, medical therapeutic agents, health supplements and as technical aids where they perform numerous functions. Vitamin production may be accomplished chemically, biologically or by a combination of both processes. Chemical methods are considered disadvantageous as they require vast amounts of energy as well as excess costs to dispose of the waste generated (Survase *et al.*, 2006).

Biological production of riboflavin is now replacing chemical processes, the manner in which this vitamin was initially mainly produced. Many industries produce vitamins by fermentation using enzymes and micro-organisms. Some of the micro-organisms used for the production of vitamins are shown in Table 2.1 (Prescott *et al.*, 2005).

Table 2.1 Microorganisms that produce vitamins (Prescott *et al.*, 2005)

Vitamin	Microbial Producers
Vitamin B_{12}	*Streptomyces, Propionibacterium, Pseudomonas*
Vitamin C	*Gluconobacter, Erwinia, Corynebacterium*
Vitamin D	*Saccharomyces*
Vitamin B_2	*Clostridium, Candida, Eremothecium, Ashbya, Bacillus*

Initially, the bacterium *Clostridium acetobutylicum* was used, followed by the yeast *Candida* and filamentous fungi *Eremothecium ashbyi* and *Eremothecium gossypii* (Stahmann, 2002).

2.2 RIBOFLAVIN

Riboflavin was discovered shortly after the isolation of thiamine from yeast concentrates. In 1879, this vitamin was initially isolated and characterized after the recognition of its growth promoting activity in young rats (Yee Aw *et al.*, 1983).

The free form of riboflavin is present only in whey, urine and the retina. In all other living cells it is found as a coenzyme. Trace quantities of this vitamin are necessary for metabolism in all living organisms. Constant replenishment of riboflavin is important due to the inability of the body to produce it and the constant loss of this vitamin via excretion due to its water-soluble property (Survase *et al.*, 2006).

2.2.1 Sources of Riboflavin

Foods such as milk, yeast, cheese, oily fish, eggs and dark-green leafy vegetables contain large amounts of riboflavin. Flour and cereals are frequently fortified with riboflavin during the manufacturing processes. Although riboflavin is stable at high temperatures, it leaches into the cooking water because it is water-soluble. Since this vitamin is denatured in the presence of light, foods containing riboflavin should not be stored in clear glass containers (Rutherford, 2007).

An important role of riboflavin in human nutrition is that of a colourant (E - 101) present in soft drinks and yoghurt. Riboflavin in a highly pure form is required for multivitamin juices, colouring foods or pharmaceutical applications. A less pure form that makes up for more than 80% of commercially produced riboflavin is used as animal feed (Stahmann *et al.*, 2000).

2.2.2 Structure and Properties of Riboflavin

The chemical structure of riboflavin was determined in 1935, as having two distinct parts: a ribose sugar unit and a three-ring flavin structure, known as lumichrome (Figure 2.1). The term riboflavin was derived from the sugar alcohol moiety (ribitol) and the ring structure flavin that is responsible for the characteristic yellow colour (Smith, 2007).

The riboflavin moiety is joined either to phosphate or to ADP to form the most commonly-known biochemically active coenzymes FMN (Figure 2.2a) or FAD (Figure 2.2b), respectively (Stahmann, 2000). Flavin mononucleotide and FAD, also called flavin coenzymes, participate in the transfer of electrons and hydrogen in processes catalysed by a variety of enzymes. They are also important components of the mitochondrial electron transport chain (Garraway and Evans, 1984).

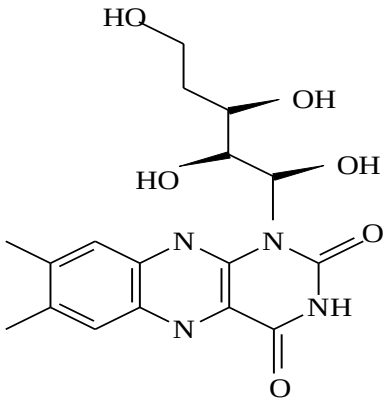
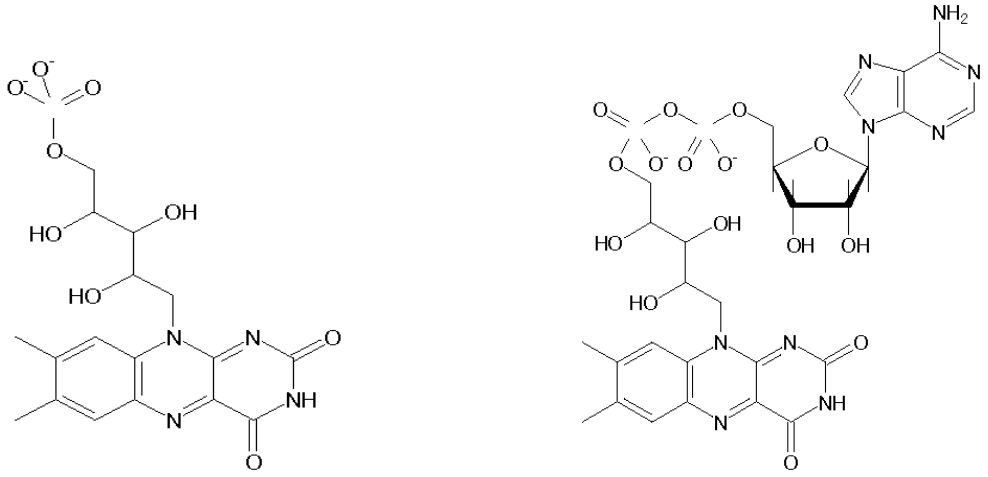


Figure 2.1 Chemical structure of riboflavin (Lehninger, 1975).



(a) (b)

Figure 2.2 Structures of (a) FMN and (b) FAD (Lehninger, 1975).

Riboflavin has an empirical formula $C_{17}H_{20}N_4O_6$ and is also known as 7,8-dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxypentyl)-isoalloxazine as well as 7,8-dimethyl-10-ribitylisoalloxazine. Riboflavin has a molar mass of 376.37 grams and decomposes between 278 – 282^{o}C. It darkens in colour at 240^{o}C. This vitamin is odourless and has a bitter taste. It is soluble in water to the extent of 10 - 13 mg in 100 ml water at 25 - 27.5^{o}C, 19 mg in 100 ml water at 40^{o}C and 230 mg in 100 ml water at 100^{o}C. In addition to this vitamin being heat-stable and easily degradable in the presence of light,

vitamin B_2 is also hygroscopic, forming lumps when moisture is absorbed (Vandamme, 1989).

Riboflavin in its pure form exists as yellowish-orange crystals, which are known to exhibit a yellowish-green fluorescence. Absorption peaks appear at wavelengths 475, 446, 359-375, 268 and 223 nm of the absorption spectrum. Absorption in the visible region of the spectrum is used for quantitative determination of this vitamin. Neutral solutions display intense fluorescence, with a maximum at 565 nm, which can be used for quantitative analysis. The fluorescence diminishes with the addition of acid or alkali. However, maximum fluorescence occurs between pH 3 to pH 8 (Vandamme, 1989).

In food, riboflavin stability is affected by heat, light and reactions that occur in cells during storage. Destruction during cooking is negligible because riboflavin is heat-stable, but it decomposes rapidly in alkali solutions. Excess heating at 120^{o}C for a period of six hours results in slight destruction. However, neutral riboflavin solutions may be sterilised by autoclaving for a short time. Eighty-five percent of riboflavin in milk is destroyed when milk is exposed to sunlight for a period of two hours. This indicates the rapid rate at which riboflavin degrades in the presence of light (Vandamme, 1989).

2.3 SYNTHESIS OF RIBOFLAVIN

In the early 1980s, the world consumption of riboflavin was reported to be 1250 tonnes for human and animal use. Presently, the amount of riboflavin production is speculated to be more than 6,500 tonnes per annum (Yuanyuan, 2008).

Chemical, microbiological or combinations of both processes are ways in which riboflavin is commercially produced in order to accommodate the increasing demand of this vitamin. The one-step microbiological process is preferred as it is less costly, when compared to multi-step chemical methods (Stahmann *et al*., 2000).

2.3.1 Chemical Synthesis

Chemical riboflavin production still prevails in industry. This process starts with the reaction of D-ribose 3,4-xylidine in methanol. The resulting riboside is hydrogenated to give *N*-(3,4-dimethylphenyl)-D-1'-ribamine, which couples with a phenyl diazonium halogenide, giving rise to an azo compound that is used in the cyclocondensation with barbituric acid to form riboflavin. In this step, aniline is eliminated; however, traces of this compound may still be present in chemically synthesized riboflavin (Stahmann, *et al.*, 2000).

Chemical riboflavin synthesis is considered disadvantageous as a yield of only 60% is produced, thereby generating large amounts of waste. It also requires organic solvents, which increases production cost and requires 25% more energy than the biological process that uses the bacterium, *Bacillus*. It is for these reasons that the chemical method, although successful for many decades, is being replaced by microbial processes (Stahmann, *et al.*, 2000).

2.3.2 Biological Synthesis

The first biological production of riboflavin was established using *Clostridium acetobutylicum*, grown on grain mashes or on whey. In 1940, this process was replaced using the fungus *Eremothecium ashbyi*, which was succeeded in 1946 with the use of the closely related *Ashbya gossypii*, now known as *Eremothecium gossypii* (Tanner *et al.*, 1949). These ascomycetes, when grown in glucose, produce ethanol until the glucose is depleted. During this time, lipid droplets accumulate within the cells. Following glucose depletion, both lipid and ethanol are metabolised by the fungi, to produce riboflavin. This introduced the idea of increasing lipid levels in the production medium, in order to improve production of this vitamin (Lee and Kun, 2003).

In 1965, three chemical companies Commercial Solvents, Grain Processing Corporation and Premier Malt Products, produced riboflavin by fermentation. Three years later, these

companies were shut down because they were unable to compete with the chemical production of riboflavin. In 1974, when the cost of commercially producing riboflavin became a concern, the process of fermentation was reinstated as the microbial route proved to be less costly, which resulted in the biological processes becoming increasingly attractive. In 1990, the chemical company BASF (Badische Anilin und Soda Fabrik), ran a fermentation plant parallel with a chemical plant, but was shut down in 1996. This method replaced the seven-step chemical synthesis of riboflavin illustrated in Figure 2.3 (BASF group fermentations, 2005).

The chemical company ADM (Archer Daniels Midland) improved the production of riboflavin using the yeast *Candida famata.* In 1998, the company Roche proposed a production plant in Grenzach, Germany, that could produce 3000 t/annum riboflavin, thus saving half the calculated cost in comparison to the chemical process. In 2000, Roche replaced chemical production with microbial production using the Gram-positive bacterium *Bacillus subtilis* (Stahmann *et al.*, 2000).

In February 2001, BASF claimed to have significantly optimized the biotechnological production of vitamin B_2. Certain enzymes enabled *E. gossypii* to live on vegetable oils and to produce vitamin B_2 by fermentation (BASF group fermentations, 2005).

Generally, riboflavin is still synthesized chemically for medical formulations, whereas feed concentrates for poultry and livestock are synthesized biochemically by fermentation processes using these fungi. However, fermented concentrates may also be upgraded from feed to pharmaceutical standards by repeated downstream processing and purification in accordance with the FDA (Food and Drug Administration) guidelines published in 2000 (Kalingan, 2002).

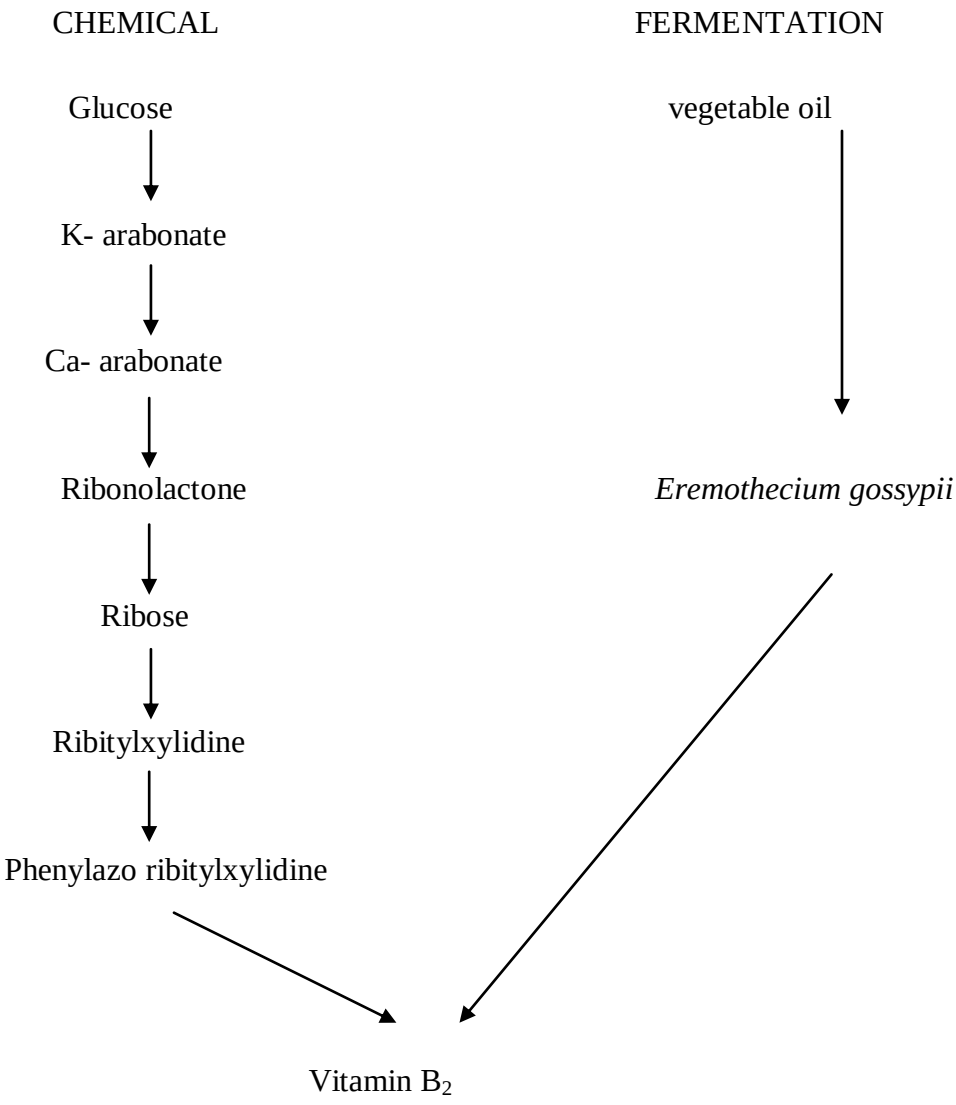


Figure 2.3 Chemical and fermentation processes of riboflavin production adopted by BASF (BASF group fermentations, 2005).

2.3.3 Biosynthesis of Riboflavin

The biosynthetic pathway of riboflavin is illustrated in Figure 2.4. The imidazole ring in GTP is opened hydrolytically together with the release of formate accompanied by the release of pyrophosphate, which is catalyzed by the enzyme GTP cyclohydrolase II. This reaction occurs in bacteria, fungi and yeasts (Karos *et al.*, 2004). Variation in

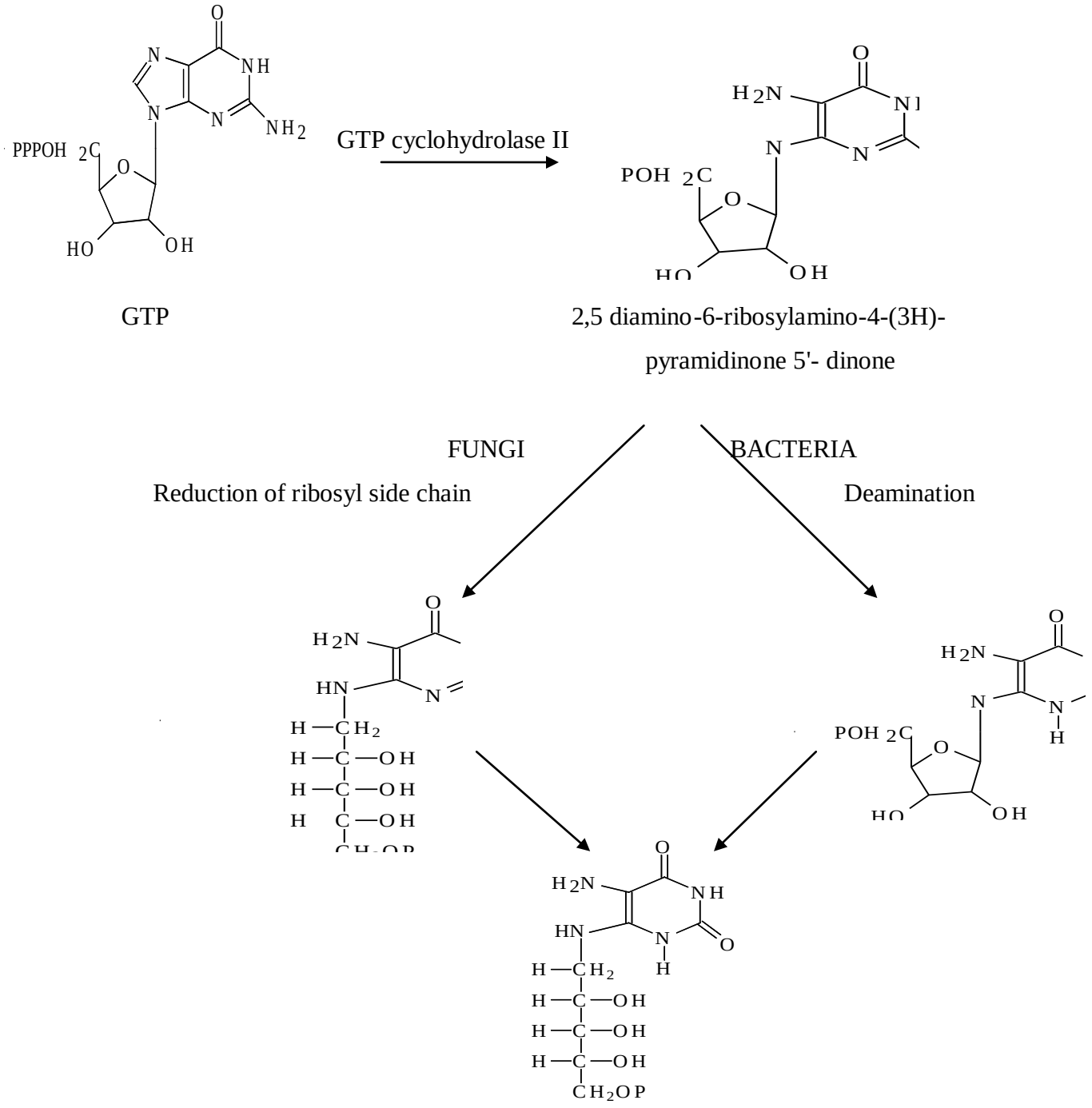


Figure 2.4 Second and third steps of riboflavin bioproduction that occurs in fungi and bacteria (Bacher *et al.*, 2000, Fischer *et al.*, 2004).

biosynthesis in eubacteria and fungi exists in the second step where 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate by deamination of the ring followed by reduction of the side chain in fungi, and reduction of the side chain followed by deamination of the ring in eubacteria. Biosynthesis in plants follows the same pattern as in eubacteria (Fischer *et al.*, 2004).

In fungi, the 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5-phosphate is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimdinedione 5-phosphate by a two-reaction step; the hydrolytic cleavage of the position 2 amino group of the heterocyclic ring and the reduction of the ribosyl side chain generating the side chain of riboflavin. The sequence of these steps varies in microorganisms. In eubacteria, the deamination precedes the side chain reduction, while in fungi, the reduction precedes the deamination (Bacher *et al.*, 2000).

However, this 5-phosphate structure in 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimdinedione 5-phosphate cannot serve as a substrate for the enzyme 6,7-dimethyl-8-ribityllumazine synthase. Hence this compound had to be dephosphorylated prior to further conversion. The dephosphorylated 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimdinedione is condensed with 3,4-dihydroxybutanone 4-phosphate by the enzyme 6,7-dimethyl-8ribityllumazine synthase. The dismutation of the 6,7-dimethyl-8-ribityllumazine, catalyzed by the enzyme riboflavin synthase, forms the final step of the biosynthetic synthesis of riboflavin (Bacher *et al*., 2000). This enzyme has been found in many microorganisms and plants. In yeasts it was purified and it’s substrate-specificity together with it’s stereospecific mode of action was studied in detail in order to understand the biosynthetic synthesis of riboflavin (Bacher and Mailander, 1978).

2.4 RIBOFLAVIN-PRODUCING MICROORGANISMS

The natural occurrence of microorganisms that produce riboflavin in concentrations exceeding their metabolic requirements, had led to their use in the fermentation processes of riboflavin production. These include bacteria, fungi, yeasts and molds.

2.4.1 Bacteria

The first microorganism employed primarily for the production of riboflavin was the butyl alcohol-producing bacterium, *Clostridium acetobutylicum.* Carbohydrate mashes were fermented in a manner that maintained low concentrations of metals iron, nickel, cobalt, copper, lead and zinc, as these metals had an inhibitory effect on riboflavin production. The mash was treated with solvents such as butyl alcohol and acetone, prior to inoculation. Experiments using various mash concentrations and levels of metal were carried out. A maximum riboflavin concentration of 30 $\mu g.ml^{-1}$ was recorded (Hickey and Haute, 1945). Numerous efforts using genetic engineering as a tool for producing a bacterial strain that can produce high riboflavin yields in a short fermentation cycle, were reported. Dry residues containing 4 - 5 mg riboflavin per gram biomass, was reported (Hans-Peter *et al.*, 2001). In 2001, a maximum concentration of 100 $mg.l^{-1}$ was reported by researchers (Lim *et al.,* 2001). According to the United States Patent 6322995, attempts to select a recombinant Gram-positive bacterium from the group of *Corynebactrium, Streptococcus, Staphylococcus, Lactococcus* and *Bacillus* that had one or more DNA sequences transformed, were recorded and results indicated that *B. subtilis* was the predominant bacterium used in industry (Hans-Peter *et al.,* 2001).

The chemical company Hoffmann-La Roche in Switzerland uses a recombinant *Bacillus subtilis* for the production of riboflavin, reaching concentrations of over 10 $g.l^{-1}$ with glucose as a carbon source (Peterson and Kiener, 1998, Buhler, 2003). There are many reasons as to why the bacterium *Bacillus subtilis* was selected for the bioproduction of riboflavin. Many useful compounds produced by this microorganism are classified as GRAS (generally regarded as safe) as they are successfully used in the food and feed

industries. This bacterium is known to produce large amounts of inosine and guanosine, and since GTP is a precursor for riboflavin production, this organism could be engineered to direct high carbon levels towards riboflavin production. Finally, the riboflavin biosynthetic genes of this microorganism are found in a single *rib*-operon and introduction of a plasmid containing the *rib*-operon into an appropriate strain of *B. subtilis* resulted in increased riboflavin production. Furthermore, advanced molecular and genetic engineering technology were available to apply to this strain (Perkins *et al.*, 1998).

Inventors Perkins, Sloma and Pero reported their modification of *B. subtilis* involving the nucleotide sequence of its *rib*-operon and its open-reading frames, and the recombinant bacteria that contain the *rib*-operon. Their study involved bacteria that were mutated so that their production of riboflavin and/or purines was deregulated, and strains that had copies of the *rib* operon inserted and amplified within their chromosomal DNA. A mutant of *B. subtilis* 1A382, RB50::›pRF8$_{60}$ (Ade^{+}) was one of the recombinant bacteria used, producing more than 5 g.l^{-1} riboflavin after 48 hours of fermentation in a 14-litre vessel. Other strains, when grown in suitable media, were able to produce more than 10 g.l^{-1} riboflavin (Perkins *et al.*, 1998).

Hoffmann-La Roche in the year 2000, proposed to replace chemical synthesis with bioproduction using this Gram-positive bacterium. A strain genetically engineered by Omnigene in the USA, was used to run tests in a 35 m^3 pilot plant which led to the production of 3 000 tonnes of riboflavin in a plant in Grenzach, Germany. A reduction in cost by 50% compared to chemical production was expected by Hoffmann-La Roche as the multi-step chemical process would have required much more energy, generated lower yields and would have encountered more cost to deal with generated waste products (Stahmann *et al.*, 2000).

On the basis of engineering microorganisms with osmopressure resistance which may obtain culture products in high concentration and high yield, Lee *et al.* (2002) claimed that by inducing mutation on *B. subtilis* AS5 and introducing proline analogue resistance to it, riboflavin production was increased. The mutant *B. subtilis* (strain KC CM – 10445) produced 8 $g.l^{-1}$ riboflavin which was 11% higher than that produced by the parent strain *B. subtilis* AS5 in a flask culture. In a five litre fermenter 26.8 $g.l^{-1}$ riboflavin, which is 19.6% higher than that of the parent strain was obtained (Lee *et al*., 2002).

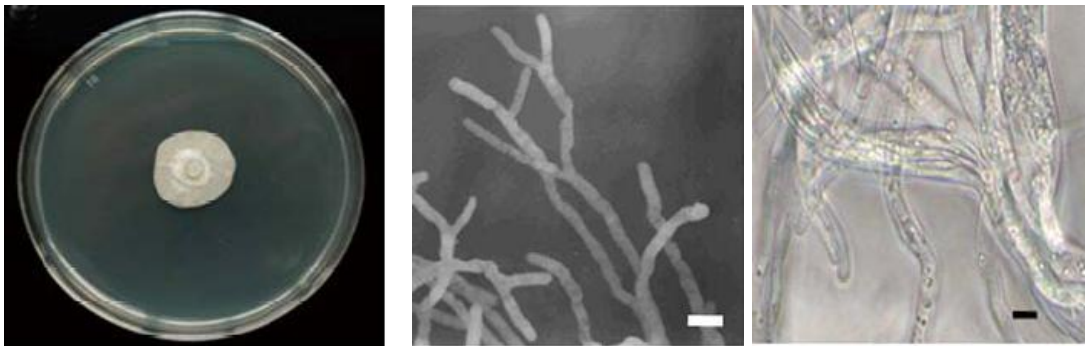
2.4.2 Filamentous Fungi

The use of the mutation-selection and fermentation development approach, improved the production of riboflavin by fungi (Goodwin and Pendlington, 1954). Among the many fungi known to synthesize riboflavin, three are considered as riboflavin over-producers. These are ascomycetes *Eremothecium ashbyi*, *Eremothecium gossypii* and the unicellular yeast, *Candida famata*. These organisms are known to release riboflavin into the growth medium. Both these filamentous fungi are closely related hemiascomycetes, thus showing a high homology in ribosomal genes to the yeast *Saccharomyces cerevisiae*. However, *E. gossypii* is preferred over *E. ashbyi* because of its genetic stability (Stahmann *et al.*, 2000).

In 1940, the very first riboflavin production process that used *Clostridium acetobutylicum* was succeeded by the fermentation process using the fungus *E. ashbyi* which produced riboflavin concentrations of up to 2 $mg.ml^{-1}$. In 1946, processes using *E. gossypii* was introduced (Perlman, 1979). In 1974, the chemical company Merck, used *E. gossypii* for the production of riboflavin. Most experimental processes used mutants of *E. ashbyi* and *E. gossypii*, producing riboflavin concentrations of up to 15 $g.l^{-1}$. In 1986, Ozbas and Kutsal reported an increase in riboflavin production by these two fungi when vegetable oil was introduced into the medium (Ozbas and Kutsal, 1986).

2.4.2.1. *Eremothecium ashbyi*

The yellow pigment with a flavin nature, produced by *E. ashbyi*, was spectrographically identified as riboflavin. A morphological study was conducted by Kimura *et al.* (2008) of *E. ashbyi* isolated from a stink bug and its characteristic features are illustrated in Figure 2.5.



(a) (b) (c)

Figure 2.5 Typical features of *Eremothecium ashbyi* isolated from a stink bug *Riptortus clavatus*. (a) a seven-day old colony grown on yeast malt agar (YMA), (b) dichotomously branched hyphae and (c) ascus and ascospores viewed with light microscopy after fourteen days growth on YMA. Bar = 10 µm (Kimura *et al.*, 2008).

Kapralek (1962) observed three phases during the growth of *E. ashbyi* in submerged batch culture. The first involved rapid mycelial growth, rapid utilisation and oxidation of glucose with a decrease in medium pH due to the accumulation of pyruvic acid. This phase ended with the depletion of glucose and cessation of growth. The second phase began with sporulation and production of intracellular riboflavin. A rapid increase in catalase activity and decrease in pyruvate and acetoin levels were simultaneously observed. Ammonia accumulated in the medium and an increase in pH was observed. The third phase was characterised by autolysis of mycelia which led to the release of riboflavin and a decrease in enzymatic activities.

Figure 2.6 indicated a typical relationship between growth and riboflavin production by *E. ashbyi* grown in a basal medium containing peptone. Growth was complete after five days but riboflavin production increased almost at the same rate as growth for the first five days after inoculation, and continued for eight days. Riboflavin production reached a maximum at the late growth phase, when growth stopped (Goodwin and Pendlington, 1954).

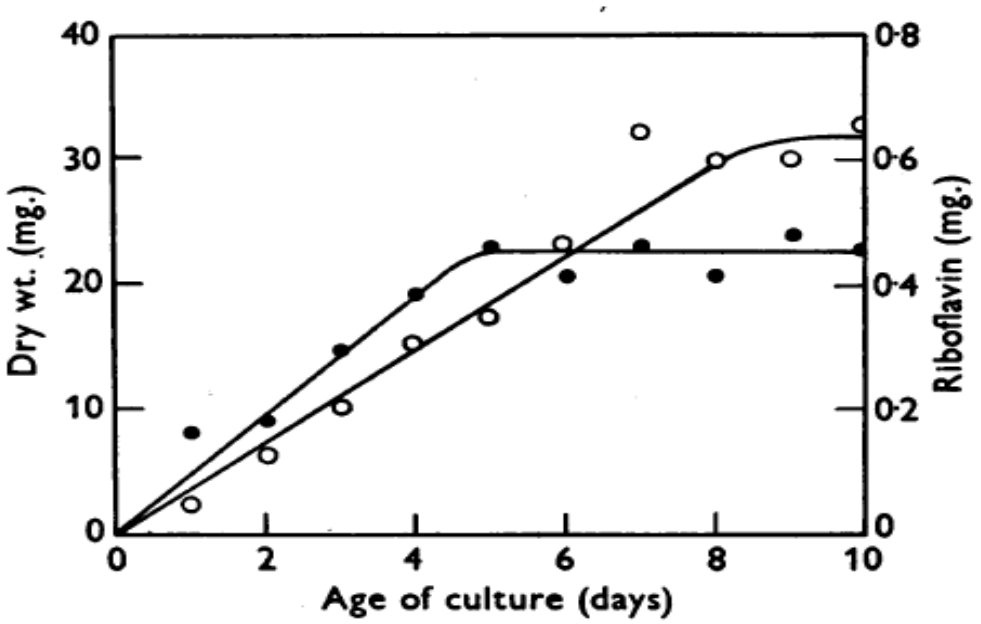


Figure 2.6 Relationship between growth and riboflavin production in *E. ashbyi* (Goodwin and Pendlingon, 1954). Growth is indicated by filled in circles while riboflavin production is indicated by empty circles.

In the early 1950s, a riboflavin yield of 200 $mg.l^{-1}$ produced by *E. ashbyi* was recorded. Incorporation of the surfactant Tween 80 and the proteins casein or glycine increased production yields, reaching concentrations of 1.4 $g.l^{-1}$. The use of raw materials in media increased production concentrations between 1.5 to 2.5 $g.l^{-1}$. The use of molasses and peanut seed cake as carbon and nitrogen sources, respectively, produced a concentration of 3.3 $g.l^{-1}$ riboflavin by the wild type *E. ashbyi* (Lim *et al.*, 2001).

Colonies of size 8 – 10 mm in diameter after 96 hours growth were considered appropriate for riboflavin production by Lim *et al.* (2001). These colonies were of intense yellow colour, possessed a smooth surface and were covered with mycelia. Maximum riboflavin production was produced at an initial pH of 6.5. Maintaining constant pH at 6.5 resulted in gradual increase of swollen hyphal cells and the formation of ascospores. In experiments allowing for change in pH, maximum riboflavin productivity was observed at pH 4.5 to 5.5, and microscopic examination resulted in asci and ascospores outnumbering other forms (Lim *et al.*, 2001)

2.4.2.2 *Eremothecium gossypii*

Eremothecium gossypii, described as a phytopathogenic fungus, was isolated from cultivated cotton plants (*Gossypium hirsutum)* in the West Indies (Bretzel *et al*., 1999). It caused severe economic losses to the country's textile industry by infecting plantations with a disease that was mechanically transmitted to new host plants on the mouth of feeding insects belonging to the order *Hemiptera*. The ability of this microorganism to overproduce riboflavin was discovered in 1946. It has the ability to produce 40 000 times more vitamin than is required for cellular growth (Survase *et al.*, 2006).

Hyphae of *E. gossypii* are hyaline, often vacuolated or containing granular material and numerous hyaline droplets, initially non-septate and later becoming septate at maturity. Dichotomous branching is another characteristic of this fungus (Kurtzman and Fell, 1998). A comparison of hyphal growth between the wild type (ATCC 10895) and a derived mutant, conducted by Dunkler *et al.* (2008) indicated tip-splitting and septation in both strains. Regular septation was evident in both strains, however tip splitting occurred more frequently in the mutant than the wild type (Figure 2.7).

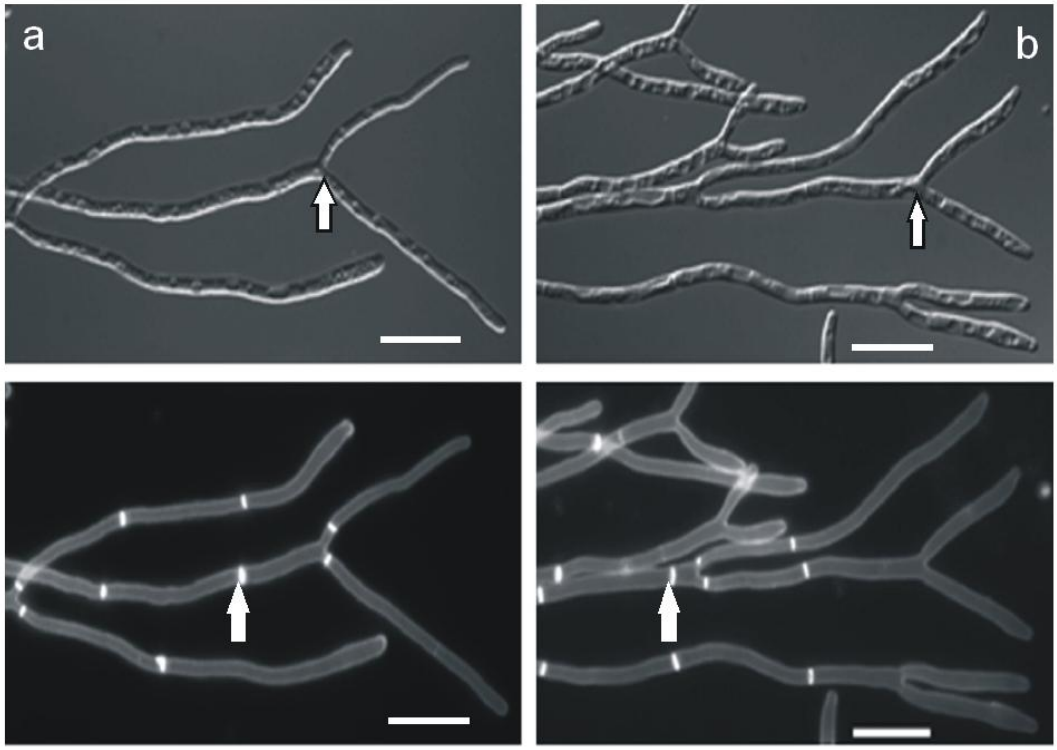


Figure 2.7 Comparison of hyphal growth between *E. gossypii* wild type (a) and a mutant (b) showing evidence of dichotomous tip splitting and regular septation brightfield (top) and fluorescence microscopy (bottom). Scale bar is 40 µm. (Dunkler *et al.*, 2008).

This organism is known to over-produce riboflavin during the late growth phase, irrespective of the type of carbon substrate used, as indicated in Figure 2.8 (Stahmann, 2002). Growth of *E. gossypii* reached a maximum at 72 hours while riboflavin production peaked at 120 hours, when biomass decreased. The fermentation process of riboflavin production in *E. gossypii* occurs in four phases. The first phase is characterised by the rapid growth of this fungus. During this phase glucose is utilised and pyruvic acid accumulated. The pH level decreases due to the increase in acidity while glucose decreases in the medium. This phase ends when growth ceases. The second phase, also known as the production phase, begins with sporulation, and the level of pyruvate decreases, while ammonia accumulates due to deaminase activity. This

results in an increase in pH as alkalinity increases. Phase three involves the synthesis of cell bound riboflavin in the form of FAD and FMN, while the last phase is characterised by the autolysis of cells, which releases riboflavin into the medium (Pharmaceutical Biotechnology and Diagnostic Techniques Handbook – [Accessed on line], 2009).

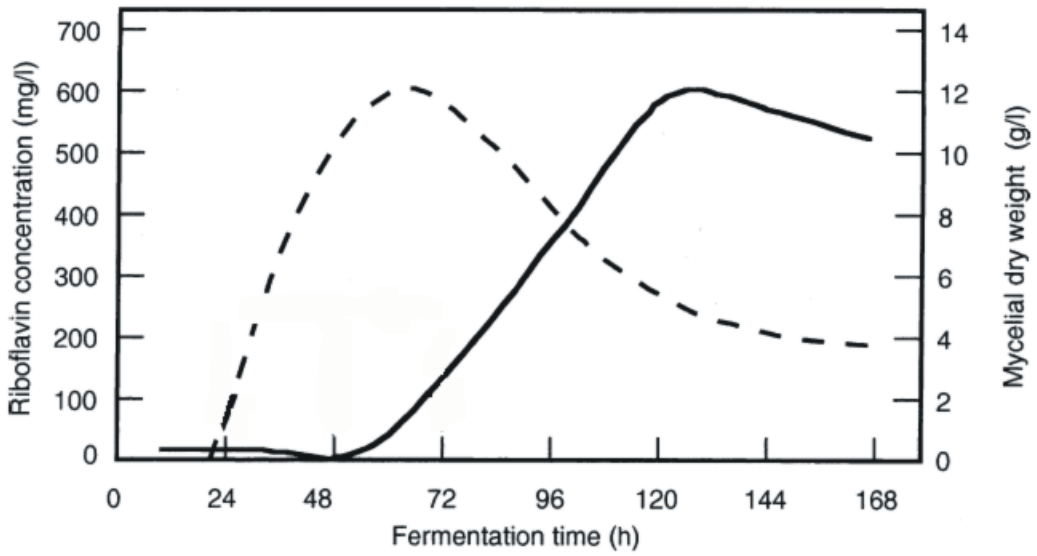


Figure 2.8 Typical relationship between riboflavin production (solid line) and growth (dashed line), calculated by mycelia dry weight of *E. gossypii* (Karos *et al.*, 2004).

Several attempts were made to genetically engineer this fungus in order to improve riboflavin yields since its importance increased in the industrial production of this vitamin. Some mutants were found to produce more riboflavin (10 – 15 $g.l^{-1}$) than *E. ashbyi*, however possibilities of degeneration and back-mutation had to be considered (Kalingan and Liao, 2002). This fungus was first used in industry in 1968 (Lim *et al.*, 2001). Improved producer strains of *E. gossypii* were used commercially, producing concentrations of up to 15 $g.l^{-1}$ riboflavin (Monschau *et al.*, 1998). However, BASF, a German chemical company used genetically modified *E. gossypii* reaching yields of over 10 $g.l^{-1}$ riboflavin (Peterson and Kiener, 1998).

In early studies, *E. gossypii* produced 370 mg.l^{-1} riboflavin in a defined medium that included Tween 80 and purine. Thereafter media components were altered in order to improve riboflavin production. In another medium consisting of glucose, vitamins, amino acids and mineral salts, *E. gossypii* produced 1 g.l^{-1} riboflavin. Lim *et al.* (2001) reported that a complex medium that included soybean oil as a source of carbon, and collagenous protein and corn steep liquor as nitrogen sources, produced a riboflavin concentration of 5 g.l^{-1}. Other media that included 3% bone fat, 2% soybean oil supplemented with additional soybean oil after 72 hours and 120 hours, also produced 5 g.l^{-1} riboflavin. Another medium containing 3% skin glue (medical adhesive that brings edges of wounds together, while healing occurs below) as a source of nitrogen and 4% gelatin lard as a carbon source, to which 2.5% gelatin lard was added after 72 and 120 hours, produced riboflavin concentrations of more than 5.5 g.l^{-1} (Lim *et al*., 2001).

Among the many fungi that are able to produce neutral lipid and utilise it (mainly during spore germination), *E. gossypii* accumulates extremely high levels of lipid. This implies that this fungus has greater potential in metabolising lipids compared to other lipid-producing fungi. Furthermore, improvements in riboflavin production by *E. gossypii* as a result of using plant oil as a carbon source, was reported in early studies. Microscopic observations of *E. gossypii* stained with Nile red revealed microdroplets within the hyphae (Figure 2.9). Lipid content is minimal and evidence of empty cytosols resulting from excretion of riboflavin into the medium may be observed on the fifth day. This contributes towards the decrease in dry biomass on this day (Lim *et al.*, 2003). These lipid bodies store neutral lipid as energy reserves, a fact that was confirmed in two separate experiments. In the first experiment using soybean oil as a carbon source, the levels of neutral lipid reached 22% of the mycelia dry weight, while in the second experiment, using glucose as a source of carbon, neutral lipid levels reached 12%, then decreased to 3–4% as glucose was utilized (Lim *et al*., 2001).

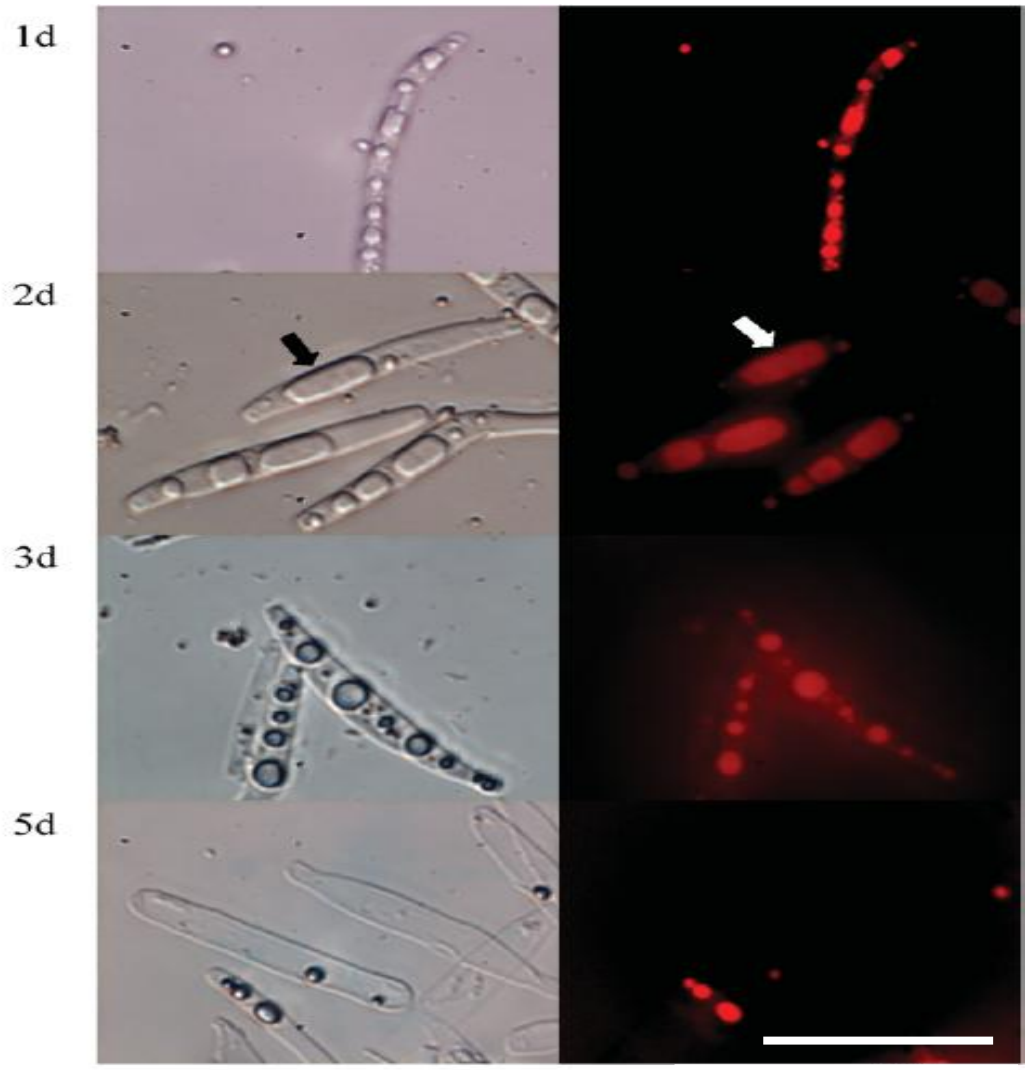


Figure 2.9 Accumulation of intracellular lipid droplets in *E. gossypii* ATCC 10895 during five days of growth in the presence of soybean oil using light microscopy (left) and fluorescence microscopy (right). Bar is 50 µm. (Lim *et al.* 2003).

2.4.2.3 Other fungal riboflavin producers

Other fungi were also investigated for riboflavin production. *Mycobacterium phlei* produced 0.10 $g.l^{-1}$ riboflavin in a medium consisting of beet molasses as a carbon source and peptone as a nitrogen source after six days. *Aspergillus terreus*, a filamentous fungus isolated from crude oil, produced 1 $g.l^{-1}$ riboflavin after 16 days, using beet

molasses as a carbon source, peptone as a nitrogen source, and supplemented with asparagines (Lim *et al.*, 2001). In a medium consisting of glucose, peptone, yeast extract, KH_2PO_4, $MgSO_4$.7H2O and corn steep liquor, a strain of *A. terreus* obtained from the Centre of Cultures of the Microbial and Natural Products Chemistry Department, National Research Centre, in Cairo, Egypt, produced 1.05 $g.l^{-1}$ after 8 days (El-Refai *et al.*, 2009).

2.4.3 Yeasts

Yeasts such as *Saccharomyces cerevisae*, *Candida guillermondi* as well as *Candida flareri*, also known as *Candida famata*, are known to produce riboflavin. Reports of experiments with native strains of *C. guillermondi* had indicated very low riboflavin yields (220 $mg.l^{-1}$) after 3 days, using liquid brewery waste, supplemented with biotin (Lim *et al.*, 2001). DNA engineering was not applied in order to over-express the riboflavin genes in this microorganism (Babyak *et al*., 2002). Amongst the yeasts that produce riboflavin, only *C. famata* was considered an industrial microorganism. This fungus was genetically improved by the company Coors in the USA, and was later used by the chemical company Archer Daniels Midland (ADM), also located in the USA (Stahmann *et al.*, 2000).

Heefner *et al.* (1992) reported in United States Patent No. 5,164,303 that mutant strains of *C. famata* were able to produce 10 $g.l^{-1}$ riboflavin in six days. Riboflavin yields of more than 20 $g.l^{-1}$ in 200 hours were achieved by the same strains and were identified by ATCC (American Type Culture Collection) accession numbers 20849 and 20850 (Heefner *et al.*,1992).

2.5 BIOLOGICAL RIBOFLAVIN PRODUCTION

Numerous efforts were made focusing on optimization of riboflavin production by understanding the metabolic pathways and then controlling the pathways obtained by medium supplementation and alteration of process conditions (Stahmann *et al.*, 2000).

Mutation and genetic engineering was also applied in order to improve production of this vitamin. Stahmann *et al.* (2000) reported attempts to improve riboflavin production by means of mutant selection, precursor supplementation and metabolic design in *E. gossypii*, and *C. famata.*

2.5.1 *Eremothecium gossypii*

In *E. gossypii* a peroxisomal enzyme that is essential for the metabolism of oleic acid may be transcriptionally regulated. A mutant that is resistant to itaconate, inhibits the activity of this enzyme thereby increasing riboflavin production producing yields much higher than parent strains. Therefore, the medium used to screen for successful *E. gossypii* mutants contained itaconate (Stahmann *et al.*, 2000).

Riboflavin production by wild type *E. gossypii* may be improved by medium supplementation. Precursors of GTP, a metabolite of riboflavin biosynthesis increased productivity (Figure 2.10). In 1976, Investigation of riboflavin production by *E. gossypii* by researcher Hanson, confirmed that the amino acid glycine is a limiting precursor in GTP synthesis (Stahmann and Weber, 2003). It was also concluded that the easiest way to overcome this limitation would be to supplement the cultivation medium. The medium supplemented with glycine indicated no change in growth but a significant increase in riboflavin production. Consequently, attempts aimed at enhancing glycine biosynthesis within the cell were conducted in order to avoid glycine supplementation into the medium.

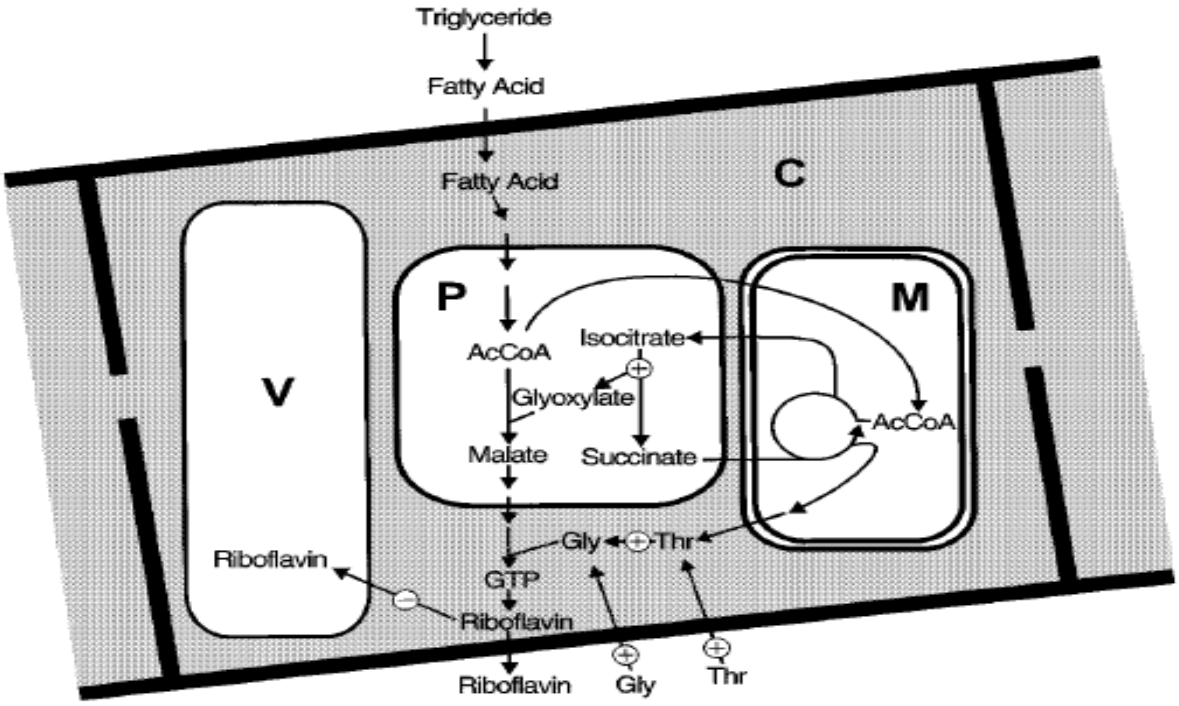


Figure 2.10 Metabolic model of riboflavin production in *E.gossypii* when plant oil is used as a source of carbon. Subcellular compartments are C – cytoplasm, V- vacuole, P – peroxisome and M – mitochondrium. Θ and ⊕ indicates decreased or increased fluxes or activities, respectively, both having a positive effect on riboflavin production (Stahmann *et al.*, 2000).

In *E. gossypii* glycine may be produced in three ways illustrated in Figure 2.11 (Stahmann *et al.*, 2000). This concept was supported by the increasing specific activity of threonine aldolase, an enzyme that catalyses the conversion of threonine to glycine (Figure 2.12). Overexpression of *GLY1* under the control of a constitutive *TEF* promoter and terminator led to the increase of the enzyme's activity by ten-fold and a nine-fold increase in riboflavin production medium when the cultivation medium was supplemented with threonine. The resulting increase in riboflavin production was not achieved with the supplementation of glycine alone, but also with the increased uptake of threonine and its intracellular conversion to glycine, a reaction catalysed by threonine aldolase (Monschau *et al.*, 1998).

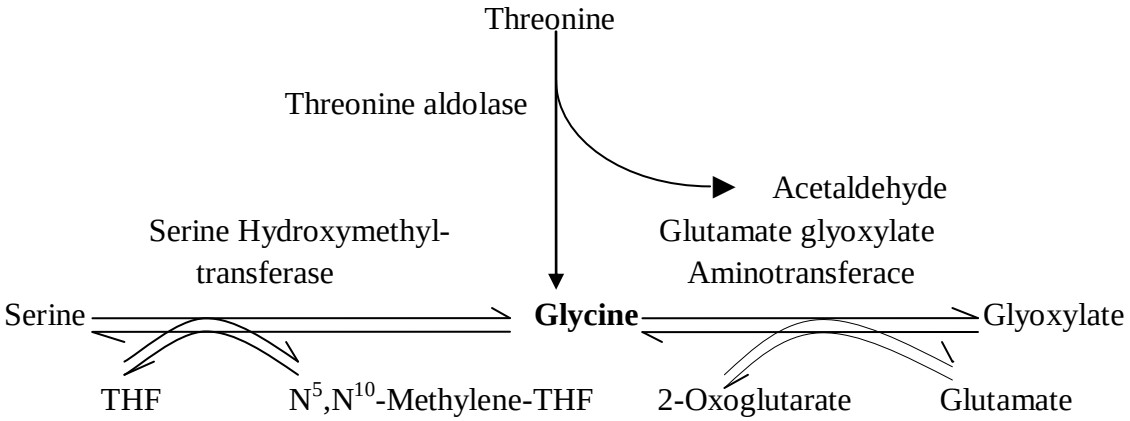


Figure 2.11 Production of glycine in *Eremothecium gossypii* (Stahmann *et al.*, 2000).

Injection of a second copy of the *ICL*1-gene, responsible for coding of isocitrate lyase in *E.gossypii*, improved production when soybean oil was used as a carbon substrate. Transport of riboflavin into the vacuoles slowed production, therefore, the vacuolar ATPase subunit gene, the VMA 1-gene was inactivated, thereby promoting the secretion of the entire production yield into the medium (Lee and Kun, 2003).

2.5.2 *Candida famata*

Riboflavin production by *C. famata* is negatively affected by iron and improved production was observed in mutants resistant to increased iron concentrations (Figure 2.12). Therefore iron sulphate and cobalt sulphate were included in screening media used for mutant selection. Deregulation of purine synthesis in mutants selected for resistance to analogues of purine was reported for this yeast. This approach was considered plausible as GTP is a precursor of riboflavin. Successful mutants were selected using screening media that consisted of tubercidin. Higher concentrations were observed when medium supplementation using glycine was conducted (Stahmann *et al.*, 2000).

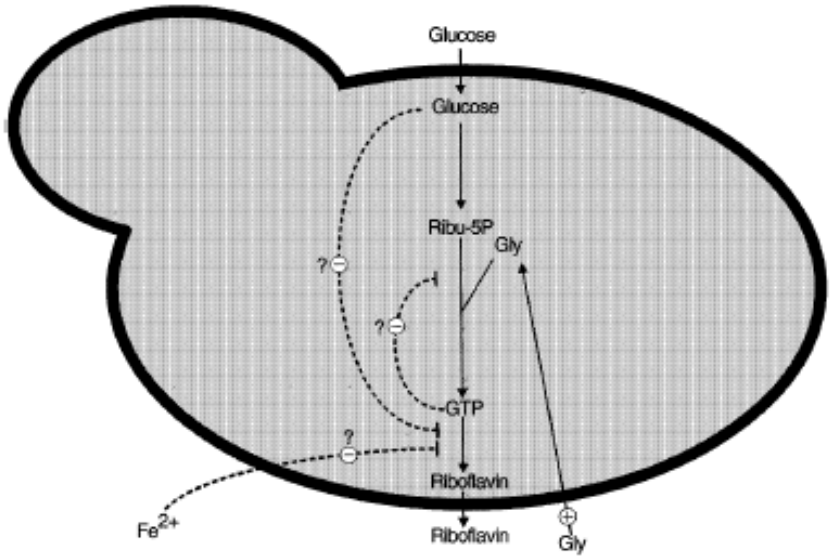


Figure 2.12 Metabolic model of riboflavin production in *Candida famata*. Regulative effects, which are thought to be lost in antimetabolite resistant mutants, are indicated by broken lines. Θ indicates changes in proposed regulations resulting in increased riboflavin production while ⊕ indicates increased flux, also with positive riboflavin yields (Stahmann *et al.*, 2000).

2.6 MEDIUM COMPOSITION FOR RIBOFLAVIN PRODUCTION

The production of riboflavin is significantly determined by type as well as the initial concentrations of the carbon and the nitrogen sources used in the production. Flavinogenic stimulants may also affect the production of this vitamin. Several attempts had been made to formulate an inexpensive industrial medium for the production of riboflavin that could be commercialized. Areas investigated in order to formulate inexpensive industrial media for the bioproduction of riboflavin included culture conditions, inocula development, sterilisation procedures and low-cost media (Tanner *et al.*, 1949).

Bacteria and fungi synthesize riboflavin from various substrates, ranging from crude paraffin (C_{10} to C_{15}) to purified dextrose. As a result, riboflavin yields vary under the different bioprocess conditions (Kalingan, 1998). Numerous efforts using inexpensive carbon and nitrogen sources and optimized fermentation conditions, were made in view of the exorbitant cost of riboflavin production and its significantly increasing consumption (Kalingan and Krishnan, 1997).

Initial studies showed that substantial growth occurred in a medium containing glucose, corn-steep liquor, animal-stick liquor, tankage or meat scraps. Further improvements resulted when enzymatically degraded collagen and lipids were used as energy sources together with other factors present in corn steep liquor, distiller's solubles and brewer's yeast producing riboflavin concentrations of up to 4.2 $g.l^{-1}$. Lipid used as an energy source was proposed earlier for fermentation using *E. ashbyi,* and this also worked well for those processes using *E. gossypii* (Perlman, 1979).

Sabry *et al.* (1989) reported attempts to improve riboflavin production by *Candida guilliermondii* using various emulsifying agents, oil additives, amino acids, vitamins, purines and purine-related compounds and some trace elements. Results indicated that the use of tweens 20, 40 and 80 inhibited growth and retarded riboflavin production by this yeast. Experimental data using oil additives, corn oil, cotton seed oil, olive oil, caproic acid, lauric acid, stearic acid and oleic acid for riboflavin production indicated that corn oil exhibited maximum biomass (717 $mg.l^{-1}$) and riboflavin production (10.68 $mg.l^{-1}$), followed by oleic acid with a biomass of 688 $mg.l^{-1}$ and a riboflavin concentration of 10.40 $mg.l^{-1}$. Among the numerous amino acids (argininge-HCl, phenyl alanine, serine, aspartic acid, valine, asparagines, glycine, isoleucine, proline, glutamic acid, methionine, alanine) used, arginine-HCl produced maximum riboflavin at a concentration of 12.06 $mg.l^{-1}$, and alanine promoted most growth, with a maximum biomass of 566 $mg.l^{-1}$. The incorporation of nicotinic acid and biotin had no effect on riboflavin production by this yeast, but Ca pantothenate, folic acid, inositol, Para-

aminobenzoic acid, thiamine HCl and pyridoxine HCl had inhibitory effects on riboflavin production. Results using adenosine, adenine, xanthine and xanthone indicate that the use of xanthine slightly improved riboflavin production (11.49 $mg.l^{-1}$) compared to the control (10.32 $mg.l^{-1}$). The effects of the three trace elements iron, cobalt and manganese, at various concentrations, were tested on riboflavin production. $CoSO_4 \cdot 7H_2O$ at a concentration of $1mg.l^{-1}$ produced maximum biomass (912 $mg.l^{-1}$) and riboflavin (10.98 $mg.l^{-1}$) (Sabry *et al*., 1989).

In India, a country that ranked first for the production of molasses and peanuts, an industrial riboflavin-producing, fermentation medium was designed using molasses as the carbon source and peanut seed cake as the nitrogen source. The ascomycete *E. ashbyi* was used for this study. Molasses at an optimum concentration of 50 $g.l^{-1}$ produced maximum biomass of 14.16 $g.l^{-1}$ and a riboflavin yield of 2.85 $g.l^{-1}$ while peanut seed cake at a concentration of 50 $g.l^{-1}$, produced maximum biomass (13.70 $g.l^{-1}$) and riboflavin (2.45 $g.l^{-1}$). The effects of the seeds were investigated in the presence of 50 $g.l^{-1}$ molasses as a carbon source and 50 $g.l^{-1}$ peanut seed cake as a nitrogen source (Kalingan and Krishnan, 1997).

India was known as the second largest producer of fruit in the world in 1994, therefore, the some of the seeds of edible fruit were selected as flavinogenic stimulants for riboflavin production. Results proved that the flavinogenicity increased with increased concentrations for all seeds that were investigated. Twenty five grams *Achras sapota* seeds per litre medium yielded a 47% increase in riboflavin production while *C. papaya* seeds and *A. sqamosa* resulted in a 35% and 12% increase in the rate of riboflavin production, respectively. Cotton seeds *E. anfractuosum* increased the rate of production by 10%, when compared to the control (Kalingan and Krishnan, 1997).

Ertrk *et al.* (1998), reported the use of whey for the production of riboflavin in Turkey, a country where whey is produced in large quantities (2 million tons per annum) by the

dairy industry, often becoming an environmental threat. As a result, numerous processes using whey and microorganisms was suggested, one of them being the production of riboflavin using *E. gossypii* NRRL Y-1056 producing a yield of 29.2 $mg.l^{-1}$. Further studies involving supplementation with bran, soybean flour, glycine and peptone, sucrose, glycine, yeast extract, peptone and soybean oil improved production yields, producing 389.5, 120.7, 87.5, 78.3, 68.4, 23.2 and 17.5 $mg.l^{-1}$ riboflavin, respectively (Ertrk *et al.*, 1998).

Kalingan and Liao (2002) investigated the effects of low cost organic wastes, beef extract, hog casings and blood meal or fish meal on the production of riboflavin by *E. ashbyi* NRRL 1363. They concluded that hog casings with beef extract improved riboflavin production using the optimized fermentation medium developed by Kalingan and Krishnan (1997).

Sung *et al*.(2003) 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 (Sung *et al.*, 2003). Optimisation of riboflavin production using a byproduct from vegetable oil refining process as a main substrate and *C. guilliermondii* DM 644 by fractional factorial experimental designs and the evaluation of the effects of the oil substrate concentration, nitrogen source, pH, orbital agitation velocity, phosphate source and yeast extract, was conducted by Pessoa *et al*. (2003). A maximum riboflavin concentration of 19.12 $g.l^{-1}$ was recorded with the most important factors being oil substrate concentration and nitrogen source, while phosphate source and yeast extract was not significant. The best conditions for this process were 10 $g.l^{-1}$ oil effluent, 2.5 $g.l^{-1}$ urea and a medium pH of 5.0 (Pessoa *et al.*, 2003).

Waste activated bleaching earth discharged by an oil refinery plant containing 40% palm oil was added to a culture of *E. gossypii*. After four days 80% of the palm oil was utilised, and after 10 days, a maximum riboflavin concentration of 2.1 $g.l^{-1}$ was obtained

which led to the conclusion that waste palm oil was suitable for use as a raw material for the production of this vitamin (Park and Ming, 2004).

Park *et al.* (2007) improved riboflavin yields three-fold using *E. gossypii* spores mutated by UV light exposure and a mutant ZP4 strain. Addition of activated bleaching earth containing 75 $g.l^{-1}$ rapeseed oil and oxygen enriched air to a ZP4 culture, increased riboflavin concentrations to 8.7 $g.l^{-1}$ after 5 days (Park *et al.*, 2007).

The incorporation of oils within the medium was to increase lipid levels found within the cells as enhanced supplementation of the culture medium with vegetable oil and free fatty acids, is known to increase riboflavin production. In addition, a neutral pH and high oxygen levels may also support riboflavin production. It is during this process that lipid is catabolised via beta-oxidation in the peroxisomes to acetyl units that are transported to the mitochondria (Lee and Kun, 2003).

2.7 INDUSTRIAL OILS

In South Africa, the uses of oils are predominant in the food and motor industries. In the food industry, vegetable oil is used for frying of foods, mostly potato chips, while in the motor industry, oil is used as a lubricant for efficient operation of the engine.

2.7.1 Vegetable Oil

Vegetable oils are substances derived from plants that are composed of triglyceride (a glycerol esterified with three fatty acids), thus referred to as triacylglycerol (TAG). The chain lengths of the fatty acids in naturally occurring triglycerides can be of varying lengths, but 16, 18 and 20 carbons are the most common. Triglycerides are the main constituents of fats and oils, and have lower densities than water. At room temperature, these triglycerides may be solid (fats) or liquids (oils) (Zamora, 2005).

2.7.1.1 Types of plant oils

Many different parts of the plant including seeds may yield oil, and are therefore used in the commercial production of plant oils. These include almond, avocado, canola, coconut, corn, cotton seed and sunflower oils. Edible, sunflower oil is the most widely used oil in the food industry and may consist of fatty acids in percentages illustrated in Table 2.2 (Riera, 2000).

Table 2.2 Fatty acid composition of sunflower oil (Riera, 2000)

Fatty Acid	Percentage
polyunsaturated acids larger than C-18	1
stearic acid (saturated C-18)	2
palmitic acid (saturated C-16)	6
oleic acid (mono-saturated C-16)	25
polyunsaturated acids C-18	66

2.7.1.2 Spent vegetable oils

Vegetable oil is used for the process of frying, which is one of the most commonly used methods for food preparation in a home or food industry. Frying is a process in which cooking oil is heated to temperatures of 170 – 220^{o}C. During this process the oil undergoes chemical reactions such as hydrolysis, oxidation and polymerisation. Oxidation occurs through a free radical mechanism, initially characterised by an emergence of a sweetish and unpleasant odour, becoming progressively worse until a characteristic smell of rancid fat develops, resulting from the decomposition of hydroxides and peroxides in low molecular weight acids and aldehydes (Souza *et al.*, 2004). Degradation products such as free fatty acids hydroperoxides and polymerized triglycerides may be formed. In addition, the viscosity of the cooking oil increases and the colour darkens (Quiles *et al.*, 2002).

The quantity of degradation products increases with the duration of the heating of the oil at high temperatures. Some of these products may be used to assess the level of degradation of the cooking oil. It is the toxicity of these degradation products that is of

health concern, therefore frequent visits from health inspectors are expected in food outlets. Polycyclic aromatic hydrocarbons (PAHs) are contaminants that may be found in spent oil as a result of prolonged heating. These compounds may also be found in the smoke from the heating process. Some PAHs are potentially carcinogenic (Quiles *et al.*, 2002).

Acrylamide, a neurotoxic potential carcinogen, is formed when carbohydrate-rich foods are fried, grilled or toasted. The formation of this product is closely linked to the Maillard reaction, which is the non-enzymatic browning reaction. The free amino acid asparagine and reducing sugars are considered as main precursors of acrylamide. Fried foodstuffs, such as potatoes are susceptible to acrylamide formation because they contain these compounds in relatively high amounts (Mestdagh *et al.*, 2007).

2.7.2 Motor Oils

Motor oils are lubricants which are fluids that are filled into engines, gearboxes and hydraulic systems. Semi-fluid lubricants such as greases are filled into rolling element bearings and into small industrial gearboxes. Much of the used oil that is generated nationally is as a result of routine replacement of deteriorated lubricating oils or what can be classified as viscosity breakdown. Frequently, the lubricants are replaced because they no longer meet their performance. In South Africa, three types of lubricating oils, turbine, hydraulic and engine oils, are most frequently used (Lochen, 2005).

2.7.2.1 Types of motor oils

Turbine oils are lubricating oils specialized for use in gas and steam turbines. They are made from special base oils and specialised additive packages. Turbine oils are made to last for long periods of time - typically 45 000 hours and longer. They must cool and lubricate working parts as well as prevent corrosion of turbine parts (Lochen, 2005).

Hydraulic oils are also very specialized lubricants which, after extensive use, are drained to become part of the used oil pool. Hydraulic oils must be formulated to allow rapid shedding of water, quick release of entrained air and must also prevent rusting of hydraulic system parts (Lochen, 2005).

2.7.2.2 Chemical composition of motor oil

Motor oil is made up of two basic components, a base stock or material, and additives comprising up to 20% of the volume. These additives greatly influence the specific performance of the finished product which eventually becomes a trademark of various lubricants. Typical additives include colour stabilizer, viscosity improvers, corrosion inhibitors, rust inhibitors and detergents. Detergents and dispersants assist in keeping the engine clean by reducing sludge accumulation. Alkaline additives are included to neutralize products from acidic oxidation of motor oil. Zincdialkylthiophosphate is incorporated to minimize oil degradation to protect contacting metal surfaces with zinc during metal to metal contact. Molybdenum disulfide is added to reduce friction, bond to metal or have anti-wear properties. All these additives contain specific metal and chemical compounds which result in specific performance standards (Holmes *et al.*, 1993).

These lubricants are typically manufactured from chemical feedstocks, mainly petroleum or petroleum products which are derived from crude oil. They contain a wide assortment of hydrocarbon in addition to chemical compounds such as sulphonates and sulphur, chlorine and nitrogen compounds; they also contain metals such as barium, zinc and chromium as a result of additives. Basically this complex mixture of hydrocarbons and other organic compounds, including some other organometallic constituents is used to lubricate the parts of an automobile engine, in order to facilitate smooth engine motion and the most important characteristic of this industrial oil is its viscosity. Unspent motor oil contains a higher percentage of fresh and lighter (often more volatile and water soluble) hydrocarbons that would be toxic to organisms. Spent motor oil

contains more metals and heavy PAHs that contribute to chronic hazards including mutagenicity and carcinogenicity (Mandri and Lin, 2007).

Oils from the motor industry are used as lubricants, aiding the efficiency of the motors and may be hazardous when they leak from cars onto the roads, into waterways from boats and they seep from old cars and motor-driven machinery. Because of its non-polar nature, it is not soluble in water and is less dense than water. Therefore it concentrates on the top of ground water and may accumulate on lakes, rivers and oceans (Mandri and Lin, 2007).

2.7.2.3 Spent motor oils

Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer. In addition, PAHs have a widespread occurrence in various ecosystems that contribute to the persistence of these compounds in the environment. The illegal dumping of spent motor oil is an environmental hazard with global ramifications and hence the release of this oil into the environment causes environmental concern and attracts public attention (Mandri and Lin, 2007).

2.8 USE OF STATISTICAL ANALYSIS FOR OPTIMISATION OF RIBOFLAVIN PRODUCTION

Numerous efforts using various carbon sources like palm oil, corn steep liquor, glucose, molasses and whey have been made to improve riboflavin production. Park and Ming (2004) investigated industrial waste material like activated bleaching earth and agroindustrial byproducts as substrates for riboflavin production while the effects of low-cost organic wastes such as flavinogenic factors were also researched, but no research with the use of spent oil was reported. The nutritional requirements for riboflavin production are constantly changing with the intention of optimizing production media. The task of having to select the best medium for optimum production

of a particular metabolite by a specific microorganism was considered laborious and time-consuming, therefore statistical design of experiments was applied in order to scientifically approach experiments in order to optimize production. In 2005, Rodrigues and Iemma described the use of statistical design as a very useful tool in optimizing metabolite production as simultaneous variation of factors are allowed, resulting in improved analytical methodology that decreases the number of experiments to be conducted (Suzuki *et al.*,2009).

Experimentation may be approaced in three ways, the one-at-a-time approach, the matrix method and the statistical design approach. The one-at-a-time approach investigates each parameter individually resulting large amounts of data, and the experimental space explored may still miss the solution. The matrix method is effective, but is considered to be inefficient because it requires too many measurements. The statistical design approach is efficient and effective because it provides good coverage of the experimental space incorporating few measurements (Anderson and Whitcomb, 2000).

2.8.1 History of Experimental Design

Design of experiments (DOE) is defined as the method used for determining cause and effect relationships, and may be applied to any process with input and output measurements. This method was originally developed for agricultural purposes during World War II, and thereafter used for quality improvement, together with statistical process control (SPC). Until 1980, engineers in chemical, food and pharmaceutical industries exploited DOE when manipulating factors such as time, temperature, pressure and flow rate to improve product quality (Haaland, 1989).

2.8.2 Statistical Analysis of Data

The process performance for most bioprocess technologies cannot be explained using theoretical models, which makes the solutions of problems difficult to accomplish. Consequently, successful problem solving was achieved using empirical problem

solving techniques, which were governed by limitations on time and resources. It is for this reason that statistical problem solving, which provides solutions to maximise the efficiency and productivity of empirical problem solving techniques, was adopted by researchers (Anderson and Whitcomb, 2000).

These processes generate large quantities of data that are managed by the use of computers, and since problem solving is controlled by limited time and resources to understand this data, it is important for this data to be information-rich. Statistical experimental design is one way to increase the amount of information-rich data of the process performance. The statistical problem solving approach consists of a series of experiments that explore the experimental space while investigating many factors using few observations. An important characteristic of this method is that it is iterative, which implies that a series of experiments are conducted to solve problems. Knowledge acquired by analysing data for the initial experiment is applied in the planning of the next experiment and this iterative nature of experimentation refines and improves problem solving by taking advantage of what is known. Further experiments are conducted until optimisation of the process is established (Haaland, 1989).

Experimentation is characterised by three stages which are, screening, optimisation and verification. Screening experiments are used early in the investigation to narrow the focus of the problem by identifying the most important factors affecting the process performance. Such experiments are conducted when the researcher identifies numerous factors that may affect the process, but is unaware of the settings that may produce optimal or nearly optimal responses (Anderson and Whitcomb, 2000). Therefore their optimum settings are investigated at this stage. Optimisation experiments are used to build a predictive mathematical model which may be used to provide specific information about the solution. These experiments which include fewer experimental factors than the screening experiments, have the objective of producing specific optimal values for the experimental factors. A verification experiment confirms the findings,

thus concluding the investigation. Experiments may be designed using statistical software (Anderson and Whitcomb, 2000).

2.8.3 Design Expert® Version 7.1.6

This program offers combined, mixture, response surface and factorial experimental designs. Selection from an array of two-level full and fractional factorial designs that provide an effective means for screening through numerous factors to find those that have the largest effect may be made when designing experiments. The total number of runs for a two-level full factorial design is calculated as 2^k runs, k being the number of factors chosen. Therfore, the total number of runs for a two-level, full factorial design with five factors will have 2^5 (32) runs. Full factorial designs are powerful because they provide information about all the main effects, two-factor interactions as well as higher order interactions. Basically, all possible interactions can be estimated. The number of runs for a two-level fractional factorial may be calculated as 2^{k-p}, where p designates the fraction of the design, making this design more efficient because of it's reduced run size. Hence, a two-level fractional factorial represents only one-half, one-fourth, one-eighth, one-sixteenth, etc. of the total possible combinations of a two-level full factorial design (Haaland, 1989).

Experimental designs that are resolution III fractional factorials allow main effects to be confounded with two-factor interactions but do not allow main effects to be confounded with each other. Resolution IV fractional factorial designs allow confounding among two-factor interactions but do not allow any two-factor interaction to be confounded with any main effect. Resolution V experimental designs allows all the main effects and two-factor interactions to be independantly estimated, which is non-confounding and is therefore considered appropriate for biological use (Haaland, 1989).

Design Expert 7.1.6 is able to generate various experimental designs provided factor settings are integrated into the program. Two to 21 factors may be investigated at the

same time, while resolutions are colour-coded and may be selected prior to generating the final design. The number of replicates as well as center points that are required may be incorporated at the beginning of designing an expriment. Once the response data is applied to the response column, statistical analysis may be conducted using the program. The ratio of maximum to minimum response will establish whether a transformation of the data is required or not. A transformation is generally suggested if the ratio exceeds 10. A choice from no transformation, a square root, natural log, base 10 log, inverse square root, inverse, power, logit and ArcSin square root transformations may be made followed by the effects mode which allows one to choose significant effects to be included in the model. The model is made hierarchical should the selection of factors, using a list (effects) or graphs (Half-Normal, Normal or Pareto), not produce a hierarchical model. A model is termed hierarchical only when the individual factors of interactions (between two or more factors), whether significant or not, are included.

The selected model is statistically analysed and the results viewed in an ANOVA table, where the results are described and the mathematical equation given. The model fit and choice of transformation is evaluated using the graphs (Box-Cox plot) in diagnostics while the model graphs are used to interpret and evaluate the model. Factor interactions may be investigated using one-factor, two-factor interations, contour, 3-D or cube plots, depending on whether the effects of 1, 2 or 3 factors on the response is to be investigated. Optimisation of the response may be accomplished numerically, graphically or by point-prediction (Two-Level Factorial Tutorial, 2006).

Statistical analysis was used to optimize medium components for riboflavin production by a UV-mutant of *E. ashbyi* (Pujari and Chandra, 2000). The effects of the six nutrient factors molasses, sesame seed cake, yeast extract, KH_2PO_4, NaCl and $MgSO_4.7H_2O$ on riboflavin production by this mutant was investigated using Plackett-Burman experimental designs and optimization of the four factors molasses, sesame seed cake, KH_2PO_4 and $MgSO_4.7H_2O$ using a 2^4 full factorial central composite experiment. The

optimized medium produced a maximum concentration of 1756 µg.ml^{-1}. Suzuki *et al.* (2009) used fractional factorial designs to evaluate the effects of various carbon and nitrogen sources (sucrose, KH_2PO_4, $ZnSO_4.7H_2O$, flaxseed oil and yeast extract Prodax Lac) on riboflavin production by the wild type yeast *Candida* sp. LEB 130. The first supplement screening experiment resulted in a maximum of 36.9 µg.ml^{-1} riboflavin. The central composite that followed included the variables sucrose, KH_2PO_4, $ZnSO_4.7H_2O$ and flaxseed oil resulting in an optimized medium that produced 105.66 µg.ml^{-1} riboflavin. Similarly, statistical analysis was adopted to optimize riboflavin-producing media using spent industrial oils.

CHAPTER THREE

MATERIALS AND METHODS

3.0 STUDY DESIGN

This chapter outlines methods used to accomplish each objective for this study. A description of the two selected substrates and optimization of riboflavin production using these oils is reported. Details of materials and methods of experiments conducted in this project are discussed herein.

Spent oils from motor vehicles and chip-fryers in food outlets, were selected as carbon sources for the production of riboflavin, by selected riboflavin-producing fungi. A standard medium consisting of yeast extract, peptone, malt extract, dipotassium hydrogen phosphate, magnesium sulphate hepta hydrate and the carbon source glucose, was chosen from literature as the selected ascomycetes had grown successfully in this medium (Ozbas and Kutsal, 1986). This medium, referred to as Ozbas and Kutsal medium (O and K medium) was used to determine the growth phases of *E. gossypii* ATCC 10895, *E. gossypii* CBS 109.51, *E. ashbyi* CBS 206.58, *C. famata* ATCC 20850 and mutant *E. gossypii* EMS 30/1. The mid-log point or the time at which each fungus is most rapidly growing, was established from its respective growth curve. This O and K medium was modified by incorporating both glucose and either one of the spent oil substrates to confirm growth and riboflavin production. The five microorganisms were then grown in another modified medium in which the entire 20 $g.l^{-1}$ glucose of the O and K medium was substituted with 20 $g.l^{-1}$ of either spent oil. Riboflavin concentrations produced were then compared in order to establish which fungus produced the most riboflavin in spent motor and vegetable oils.

A series of fractional factorial experiments that involved the five nutrient supplements of the O and K medium, yeast extract (X_1), peptone (X_2), malt extract (X_3), dipotassium

hydrogen phosphate (X_4) and magnesium sulphate-hepta-hydrate (X_5) were designed using the statistical software, Design Expert 7.1.6 (Statease, USA). This program statistically analysed the experimental data, generating a mathematical model, for each experiment. The models, described with mathematical equations, were used to guide the optimization in the experiments that followed. These experiments allowed for screening of significant factors affecting riboflavin production in each substrate (fractional factorial experimental designs). Response surface methodology, based on central composite experimental designs, was applied to optimize riboflavin production. The optimized media developed for each oil substrate were verified in confirmatory experiments. To prevent degradation of riboflavin, all experiments were conducted in flasks covered in aluminium foil.

3.1 CARBON SUBSTRATES USED

Spent oils from the food and motor industries were selected as carbon substrates for riboflavin production. Spent motor oil, collected during oil changes when servicing various diesel trucks, was collected, mixed and stored in a single twenty litre plastic container, at room temperature until used. Oil, from potato chip fryers after being used repeatedly for several frying processes by numerous food vendors, were collected, combined, mixed and also stored in a single twenty litre plastic container and stored at room temperature.

3.2 STRAIN SELECTION

Microorganisms that utilize lipids as an energy source for growth and riboflavin production were selected from literature for this study. Riboflavin over-producers; namely wild type *Eremothecium gossypii* ATCC 10895, *Eremothecium gossypii* CBS 109.51, *Eremothecium ashbyi* CBS 206.58 and *Candida famata* ATCC 20850 were chosen. In addition, a mutant derived by chemical mutagenesis from the wild type *E. gossypii* ATCC 10895 (Govender, 2011) was also selected.

3.2.1 *Eremothecium gossypii* (wild type) American Type Culture Collection (ATCC) strain10895

Riboflavin over-production by this fungus was discovered long before the commercial production of this vitamin. Numerous attempts to genetically engineer this organism with the aim of isolating mutants that have abilities to produce riboflavin at concentrations greater than the parent strain, were made. Mutational studies using this wild type ascomycete were conducted by Govender (2011) in order to derive successful riboflavin over-producing mutants. *Eremothecium gossypii* ATCC 10895 was obtained from Peter Stahmann (Institute of Biotechnology in Julich, Germany).

3.2.2 *Eremothecium gossypii* Centraalbureau voor Schimmelcultures (CBS) strain 109.51

This strain was described as a high riboflavin-yielding mutant that can grow on potato dextrose agar and malt extract agar (CBS Fungal Database, http://www.cbs.knaw.nl/databases/). This strain was deposited by NRRL (Northern Regional Research Laboratory) also known as the Agricultural Research Service (ARS) Culture Collection (Illinois, USA). This organism is known to produce riboflavin concentrations of up to 15 $g.l^{-1}$ (Forster *et al.,* 1999) and was purchased from the CBS fungal biodiversity centre (Utrech, Netherlands).

3.2.3 *Eremothecium ashbyi* (CBS 206.58)

This highly flavinogenic fungus, known to secrete riboflavin in amounts of up to 2.5 $g.l^{-1}$ isolated by Goodwin (Liverpool University) and deposited by Robsin (2006) into the CBS Fungal Database. This microorganism can grow on potato dextrose agar and malt extract agar. Its derived mutants are known to produce up to 15 $g.l^{-1}$ riboflavin (Stahmann *et al.*, 2000). This strain was purchased from CBS.

3.2.4 *Candida famata* (ATCC 20850)

This unicellular fungus was improved for riboflavin production by a North American company, Coors (USA). The chemical company Archer Daniels Midland (ADM, USA), well known for the production of vitamins, useS this microorganism for the production of riboflavin. This yeast, also known as *Candida flareri* according to the ATCC classification, is known to produce more than 20 $g.l^{-1}$ riboflavin (Stahmann *et al.*, 2000). This strain was derived from strain ATCC 20849 and was purchased from the ATCC (University Boulevard, Manassas, USA.

3.2.5 Mutant Strain Derivation from Wild-type *Eremothecium gossypii* (ATCC 10895)

Wild type *E. gossypii* (ATCC 10895) was mutated by Govender (2011) using the chemical mutagen ethylmethane sulphonate (Moses, 2004) at the Department of Biotechnology, Durban University of Technology, Durban.

Fragmented hyphae of *Eremothecium gossypii* (ATCC 10895) was grown on the O and K medium consisting of yeast extract, peptone, malt extract, magnesium sulphate hepta hydrate, dipotassium hydrogen phosphate and glucose, as a carbon source, at concentrations listed in Table 3.1, for 24 hours, at 30^{o}C on a rotary shaker at 130 rpm. Five milliliters of this culture was then added to the mutagen ethylmethane sulphonate (EMS), diluted with potassium-phosphate buffer (0.05 M), pH 7 (Moses, 2004), with an exposure time of 30 minutes. After the 30-minute exposure period, a 0.1 ml aliquot was removed and used to inoculate the screening medium that contained itaconate (Moses, 2004).

Successful riboflavin-producing mutants were again grown in the O and K medium (Table 3.1) and the riboflavin yields compared with that produced by *E. gossypii* (ATCC 10895). The selected strain was referred to as mutant *Eremothecium gossypii* EMS 30/1 (Govender, 2011).

Table 3.1 Components of the O and K medium, with glucose as the carbon substrate (Ozbas and Kutsal, 1986)

Nutrient Supplement	Concentration ($g.l^{-1}$)
Glucose	20
Yeast extract	5
Peptone	5
Malt extract	5
K_2HPO_4	0.2
$MgSO_4.7H_2O$	0.2

pH was adjusted to 6.5 with 1 M NaOH

3.3 MORPHOLOGICAL STUDIES

Inspection of colony morphologies of the five selected fungi were conducted after an incubation period of seven days, at 30^{o}C in the absence of light, on solid O and K medium in order to identify similarities and differences between the chosen strains, with respect to colony morphology.

Microscopic views of stained, wet-mount preparations of the selected fungal strains (after a seven-day incubation on solid O and K medium) were investigated using light, dark phase and fluorescence microscopy. Lactophenol blue was used to stain filamentous fungi while a drop of crystal violet was applied to the yeasts. Dispersion of lipid droplets within the cells was observed by staining with Nile red (Lim *et al.*, 2003) and viewing under 1000 x magnification, using a fluorescence microscope.

3.4 CULTURE GROWTH, INOCULA PREPARATION AND MAINTENANCE

3.4.1 Ozbas and Kutsal Growth Medium

From literature, a medium consisting of nutrient supplements yeast extract, peptone, malt extract, magnesium sulphate hepta hydrate, di-potassium hydrogen phosphate and glucose as a carbon source, in which *E. gossypii* and *E. ashbyi* successfully grew (Ozbas and Kutsal, 1986), was selected as the Ozbas and Kutsal (O and K) medium for growth

and maintenance of the selected fungi. Concentrations of the nutrient supplements are listed in Table 3.1.

3.4.2 Growth and Preparation of Fungal inocula

Three hundred millilitres of O and K medium (Table 3.1) in 500 ml flasks were inoculated either with fungal mycelia (one cm^2 of the filamentous fungus per 100 ml medium), or yeast colonies (two colonies per 100 ml medium) that were grown on solid O and K medium.

Fungal inocula were grown in O and K medium until their mid-log points were reached. Fifteen millilitres of this medium containing fungal mycelia or the yeast cells were aseptically removed and transferred into sterile 15 ml plastic centrifuge tubes. The tubes were centrifuged at 11627.2 x *g* for 5 minutes, the supernatant discarded and the fungal pellet resuspended in 15 ml sterile distilled water. This washing process was repeated thrice in order to ensure complete removal of the O and K medium, as glucose from this medium would have been used for riboflavin production resulting in inaccurate data which would not have been a true reflection of riboflavin produced by the spent oil in the production medium. These inocula were used immediately to inoculate the experimental media.

3.4.3 Storage of Cultures

To preserve the original genetic information of each fungal strain throughout this study, the organisms were stored using various methods.

3.4.3.1 Short term storage

Working stock cultures of the five fungi were maintained on solid O and K medium in petri plates. Plates were prepared by adding 12 $g.l^{-1}$ agar to the O and K medium (Table 3.1) prior to sterilisation. This medium was autoclaved at 121^oC for 15 minutes, then cooled, poured into 90 mm Petri plates (Merck, Pty, Ltd) and stored at 4^oC until

required. Subculturing was conducted on a weekly basis. Filamentous fungi were subcultured using the three-point inoculation technique while the streak-plate technique was used for the yeast. Plates were sealed using parafilm to avoid contamination, and incubated in a Labcon incubator at 30^{o}C for one week, before subculturing. These plates we stored at 4^{o}C formed short-term stock cultures.

3.4.3.2 Long term storage

Four cubes (1 cm^2 surface area) of agar on which fungal growth was still attached, were aseptically removed from the peripheral growth region of fully grown filamentous fungi and aseptically placed in 20 ml sterile distilled water in universal bottles. Similarly, agar cubes with yeast colonies were also placed in universal bottles containing sterile distilled water. Universal bottles containing the fungi were stored at 4^{o}C for 12 months and resuscitated by re-plating squares upside-down on solid O and K medium when required.

3.5 QUANTITATIVE DETERMINATIONS

Fifteen milliliter samples were aseptically removed at regular intervals in order to monitor growth and riboflavin production. The pH of the samples removed was measured using a Beckman PHI 50 pH meter. Samples containing oil were treated with an equal volume of hexane:isopropanol (3:2 v/v) (Stahmann *et al.*, 1994) to extract the spent oil present in the medium as illustrated in Figure 3.1. The upper layer contained the dissolved oil while riboflavin and biomass were in the lower aqueous layer.

Figure 3.1 Removal of the top, organic, blackish hexane layer containing the dissolved SMO with a Pasteur pipette. The aqueous layer below contained riboflavin and biomass.

3.5.1 Biomass Measurement and Fungal Growth Kinetics

Growth curves of the fungi were determined by measuring the change in biomass with time in order to establish the various growth phases. Biomass was measured gravimetrically for filamentous fungi and spectrophotometrically for the yeast. These experiments were done in triplicate.

3.5.1.1 Growth kinetics for filamentous fungi

Three 500 ml Erlenmeyer flasks, each containing 300 ml O and K medium (Table 3.1), were autoclaved at 121^{o}C for 15 minutes and inoculated with 3 cm^{2} fungal mycelia grown on solid O and K medium. These flasks were incubated at 30^{o}C, on an orbital shaker at 130 rpm for 120 hours (5 days). Three times a day at three hourly intervals (beginning at 0 hours), 10 ml aliquots were aseptically removed to monitor biomass gravimetrically using pre-weighed Whatman No.1 filter paper, through which the sample was filtered, and re-weighed after drying overnight in an oven at 105^{o}C. The calculated change in biomass was plotted against time to illustrate growth curves of each

organism. Specific growth rates, doubling time as well as maximum specific growth rates were calculated.

Specific growth rate may be calculated using the equation below:

$$\mu = K' = \frac{\ln(m_{t_2}/m_{t_1})}{t_2 - t_1}; \quad t_2 > t_1$$

where $m_{t.}$ are biomasses at the different time points (t_1 and t_2).

Doubling time (t_d) is t_2 - t_1 when $m_{t2}/m_{t1} = 2$

Absorbance at 442 nm was measured using a Beckman DU 640 spectrophotometer, to monitor the amount of riboflavin present in the medium. The change in pH was also monitored.

3.5.1.2 Growth kinetics for *Candida famata*

Six yeast colonies were aseptically transferred to each of three 500 ml Erlenmeyer flasks, each bearing 300 ml O and K medium (Table 3.1). Flasks were incubated as for filamentous fungi, and samples removed three times a day at three hourly intervals to measure cell density (600 nm) and the amount of riboflavin produced using the Beckman DU 640 Spectrophotometer. Biomass of this yeast was established using an optical density versus cell mass standard curve for *C. famata*.

3.5.2 Analysis of Riboflavin

Following the extraction of oil from samples that were removed during each experiment (Stahmann *et al.*, 1994), the amount of riboflavin produced was monitored spectrophotometrically at 442 nm. The concentration of riboflavin present was calculated from a riboflavin standard curve (Appendix 1).

3.6 SUBSTRATE ANALYSIS

Spent oils from the food and motor industries were analysed by Gas Chromatography-Mass Spectrometry (GC-MS) before and after fungal growth to establish the major organic compounds present. These tests were conducted by the laboratory services unit of, Umgeni Water, located in Pietermaritzburg, Kwa - Zulu Natal. Samples (one drop each) were dissolved in methylene chloride (1 ml) and analysed.

Instrumentation:

One microlitre of the sample was injected into an 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). 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. The instrument parameters were set as outlined in Table 3.2.

Table 3.2 Gas chromatography conditions used to analyse spent motor and vegetable oils

Oven Temperature Programme	
Initial Temperature	50^{o}C
Initial Time	2 minutes
Ramp Rate	10^{o}C/minute
Final Temperature	300^{o}C
Final Time	3 minutes
Injector Conditions	
Injector Mode	Splitless
Injector Temperature	250^{o}C
Injector Volume	1 μl

Chromatograms produced indicated chemical compounds that were identified using the Wiley 275 spectra library.

3.7 PRELIMINARY GROWTH IN SPENT OILS AND SELECTION OF RIBOFLAVIN-PRODUCING FUNGI

The ability of the selected fungi to grow using the spent oils as a carbon source was accomplished by growing the microorganisms in the O and K medium (Table 3.1) that was modified by substituting the 20 $g.l^{-1}$ carbon source with both 15 $g.l^{-1}$ spent oil and 5 $g.l^{-1}$ glucose. Glucose was incorporated to initiate fungal growth.

The modified O and K media were prepared and dispensed in volumes of 285 ml in 500 ml Erlenmeyer flasks, after which they were autoclaved at 121^{o}C for 15 minutes. Each flask was inoculated with a 5% mid-log, pre-washed inoculum (15 ml), and incubated at 30^{o}C, on a rotary shaker at 130 rpm, for 120 hours. Every 24 hours (starting at time = 0 hours), 10 ml samples were aseptically removed from which oil was extracted using an equal volume of hexane:isopropanol (3:2 v/v) solution (Stahmann *et al.*, 1994). Biomass was gravimetrically monitored in the case of the filamentous fungi, and cell density measured at 600 nm for the yeast. Glucose reduction was measured using the dinitrosalicylic colorimetric method (Wang, 2006) as described in Appendix 2.

3.7.1 Selection of Maximum Riboflavin-producing Fungi for Each Substrate

Results of the preliminary growth experiments confirmed that all five fungi grew successfully in autoclaved spent oils even after the depletion of glucose. Therefore, to determine which of the five fungi, produced the most riboflavin in each substrate, the O and K medium (Table 3.1) was modified by substituting the 20 $g.l^{-1}$ glucose with either 20 $g.l^{-1}$ spent motor oil or 20 $g.l^{-1}$ spent vegetable oil.

The modified media were prepared, dispensed and inoculated as outlined in section 3.5. Samples were similarly analysed for riboflavin and biomass. Concentrations of riboflavin produced in each spent oil were compared in order to determine the organism that produced most riboflavin in each substrate.

3.8 RIBOFLAVIN PRODUCTION BY MUTANT *Eremothecium gossypii* EMS 30/1 IN SPENT MOTOR OIL

The comparison of riboflavin yields by the five selected fungi in supplemented spent motor oil confirmed that mutant *E. gossypii* EMS 30/1 produced the most riboflavin, and was therefore selected as the most appropriate riboflavin over-producer for this substrate. Consequently, all future experiments with this carbon source used mutant *E. gossypii* EMS 30/1 for the bioproduction of this vitamin.

3.8.1 Screening for Effects of Nutrient Supplements using Fractional Factorial Experimental Designs

The effects of the five nutrient supplements were screened using fractional factorial designs generated by Design Expert Version 7.1.6 (Statease, USA), in order to determine the two most significant factors affecting riboflavin production by mutant *E. gossypii* EMS 30/1 in supplemented spent motor oil. Nutrient supplements were supplied by Merck Laboratory Suppliers.

3.8.1.1 First supplement screening for riboflavin production by mutant *E. gossypii* EMS 30/1 in SMO

Effects of the five nutrient supplements yeast extract (X_1), peptone (X_2), malt extract (X_3), di-potassium hydrogen phosphate (X_4), and magnesium sulphate-hepta-hydrate (X_5) incorporated in the O and K medium (Ozbas and Kutsal, 1986), were screened using a five-factor, two-level, resolution V, fractional factorial experimental design. Factor concentrations were set at 50% higher and 50% lower than that of the O and K medium (Table 3.3). This experimental design constituted 19 runs, 3 of which were center points. The 20 $g.l^{-1}$ glucose of the O and K medium was substituted with 20 $g.l^{-1}$ SMO (spent motor oil).

Table 3.3 A two-level, resolution V, 2^{5-1} fractional factorial experimental design for the first supplement screening experiment for riboflavin production by *E. gossypii*. The concentration of SMO in all runs was 20 $g.l^{-1}$

Run	Yeast extract (X_1) ($g.l^{-1}$)	Peptone (X_2) ($g.l^{-1}$)	Malt extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	$MgSO_4.7H_2O$ (X_5) ($g.l^{-1}$)
1	2.5	2.5	2.5	0.1	0.3
2	7.5	2.5	2.5	0.1	0.1
3	2.5	7.5	2.5	0.1	0.1
4	7.5	7.5	2.5	0.1	0.3
5	2.5	2.5	7.5	0.1	0.1
6	7.5	2.5	7.5	0.1	0.3
7	2.5	7.5	7.5	0.1	0.3
8	7.5	7.5	7.5	0.1	0.1
9	2.5	2.5	2.5	0.3	0.1
10	7.5	2.5	2.5	0.3	0.3
11	2.5	7.5	2.5	0.3	0.3
12	7.5	7.5	2.5	0.3	0.1
13	2.5	2.5	7.5	0.3	0.3
14	7.5	2.5	7.5	0.3	0.1
15	2.5	7.5	7.5	0.3	0.1
16	7.5	7.5	7.5	0.3	0.3
17	5	5	5	0.2	0.2
18	5	5	5	0.2	0.2
19	5	5	5	0.2	0.2

Each run from the above table was prepared in distilled water in volumes of 300 ml in 500 ml Erlenmeyer flasks. Fifteen milliliters of each medium was removed and stored in universal bottles to be used as blanks during riboflavin analysis after appropriate dilution. All media were autoclaved for 15 minutes, and the Erlenmeyer flasks were inoculated with a 5% mid log phase, pre-washed inoculum (15 ml), and incubated at 30^{o} C, on a rotary shaker at 130 rpm, for 120 hours. Every 24 hours (starting time at 0 hours), a 10 ml sample from each flask was aseptically removed. The oil from the sample was extracted using the same volume of hexane:isopropanol (3:2 parts in volume) solution (Stahmann *et al.*, 1994). The change in biomass was gravimetrically monitored and riboflavin, spectrophotometrically measured at 442 nm.

3.8.1.2 Second supplement screening for riboflavin production by mutant *E. gossypii* EMS 30/1 in SMO

Results from the first supplement screening experiment, indicated that the concentrations of yeast extract, peptone, malt extract and K_2HPO_4 had to be decreased, while the concentration of $MgSO_4.7H_2O$ had to be increased, in order to improve riboflavin production by mutant *E. gossypii* EMS 30/1. Therefore, the second screening experiment was designed accordingly (Table 3.4).

Table 3.4 A two-level, resolution V, 2^{5-1} fractional factorial experimental design for the second supplement screening experiment for riboflavin production by *E. gossypii*. The concentration of SMO in all runs was 20 $g.l^{-1}$

Runs	Yeast Extract (X_1) ($g.l^{-1}$)	Peptone (X_2) ($g.l^{-1}$)	Malt Extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	$MgSO_4.7H_2O$ (X_5) ($g.l^{-1}$)
1	0.5	0.5	0.5	0.02	0.5
2	2.5	0.5	0.5	0.02	0.3
3	0.5	2.5	0.5	0.02	0.3
4	2.5	2.5	0.5	0.02	0.5
5	0.5	0.5	2.5	0.02	0.3
6	2.5	0.5	2.5	0.02	0.3
7	0.5	2.5	2.5	0.02	0.5
8	2.5	2.5	2.5	0.02	0.3
9	0.5	0.5	0.5	0.1	0.3
10	2.5	0.5	0.5	0.1	0.5
11	0.5	2.5	0.5	0.1	0.5
12	2.5	2.5	0.5	0.1	0.3
13	0.5	0.5	2.5	0.1	0.5
14	2.5	0.5	2.5	0.1	0.3
15	0.5	2.5	2.5	0.1	0.3
16	2.5	2.5	2.5	0.1	0.5
17	1.5	1.5	1.5	0.06	0.4
18	1.5	1.5	1.5	0.06	0.4
19	1.5	1.5	1.5	0.06	0.4

Each run was prepared and samples analysed as outlined in 3.8.1.1.

3.8.1.3 Third supplement screening for riboflavin production by mutant *E. gossypii* EMS 30/1 in supplemented SMO

Statistical analysis of data from the second supplement screening experiment resulted in a mathematical model which predicted improvement of riboflavin yields by mutant *E.* gossypii EMS 30/1 in supplemented spent motor oil increasing the concentrations of yeast extract, malt extract and K_2HPO_4. The concentration of peptone and $MgSO_4.7H_2O$ were set at the low levels, thereby reducing the number of factors for investigation, from five to three. Hence, the third supplemented screening experiment was designed (Table 3.5).

Table 3.5 A two-level, resolution V, 2^{3-1} fractional factorial experimental design for the third supplement screening experiment for riboflavin production by *E. gossypii*. The concentration of SMO in all runs was 20 $g.l^{-1}$

Run	Yeast extract (X_1) ($g.l^{-1}$)	Malt extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)
1	2.5	2.5	0.1
2	5	2.5	0.1
3	2.5	5	0.1
4	5	5	0.1
5	2.5	2.5	0.2
6	5	2.5	0.2
7	2.5	5	0.2
8	5	5	0.2
9	3.75	3.75	0.15
10	3.75	3.75	0.15
11	3.75	3.75	0.15

Each run was prepared and samples analysed as outlined in 3.8.1.1.

3.8.2 Optimization of Riboflavin Yields by Response Surface Methodology

Results of the third supplement screening experiment indicated that the two most influential nutrient factors for riboflavin production by mutant *E. gossypii* EMS 30/1 in SMO, were malt extract and K_2HPO_4. Optimization of these factors were accomplished using response surface methodology, based on a central composite experimental design.

3.8.2.1 Central composite experiment

A two-factor central composite experiment of thirteen runs, five of which were center points was designed using Design Expert 7.1.6. The low and high concentration levels for malt extract and K_2HPO_4 were set at 1.0 to 5.0 $g.l^{-1}$ and 0.1 to 0.18 $g.l^{-1}$, respectively. Concentrations of yeast extract (X_1), peptone (X_2) and $MgSO_4.7H_2O$ (X_5) for all media were maintained at 2.5 $g.l^{-1}$, 0.5 $g.l^{-1}$ and 0.3 $g.l^{-1}$, respectively. Spent motor oil, at a concentration of 20 $g.l^{-1}$ was used in each run (Table 3.6).

Table 3.6 Central composite experimental design for mutant *E. gossypii* EMS 30/1 in supplemented SMO

Run	Malt Extract (X_2) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)
1	1.00	0.10
2	5.00	0.10
3	1.00	0.18
4	5.00	0.18
5	0.17	0.14
6	5.83	0.14
7	3.00	0.18
8	3.00	0.20
9	3.00	0.14
10	3.00	0.14
11	3.00	0.14
12	3.00	0.14
13	3.00	0.14

Each run was prepared and samples analysed as outlined in 3.8.1.1.

3.8.3 Verification Experiment for *E. gossypii* strain EMS 1 in Spent Motor Oil

The verification experiment confirmed maximum riboflavin production by mutant *E. gossypii* EMS 30/1 in supplemented SMO with the optimized medium (Table 3.7). This confirmatory experiment, in which all factors were set at their optimum concentrations, was conducted in triplicate.

Table 3.7 Optimized riboflavin-producing medium for mutant *E. gossypii* EMS 30/1 in supplemented SMO

Chemical	Code	Concentration ($g.l^{-1}$)
Yeast extract	(X_1)	2.5
Peptone	(X_2)	0.5
Malt extract	(X_3)	12.5
K_2HPO_4	(X_4)	0.18
$MgSO_4.7H_2O$	(X_5)	0.3
Spent motor oil		20

The medium was prepared and analysed as in 3.8.1.1. The organic layer in which the oil was dissolved was analysed by GC-MS by Umgeni Water, in Pietermaritzburg. Change in biomass was gravimetrically monitored and riboflavin spectrophotometrically measured, at 442 nm.

3.9 RIBOFLAVIN PRODUCTION BY *Candida famata* IN SPENT VEGETABLE OIL

The comparison of riboflavin yields by all five selected fungi in supplemented spent vegetable oil (SVO) as a carbon source indicated that the yeast *C. famata* ATCC 20850 produced the most vitamin B_2 and was therefore considered the superior riboflavin-producing fungus. Four fractional factorial experimental designs were generated by the statistical software Design Expert 7.1.6 (Statease, USA) in order to select from the five nutrient supplements of the O and K medium, the two factors that influenced riboflavin production the most.

3.9.1 Screening for Most Effective Nutrient Supplements by Fractional Factorial Designs

Four two-level, resolution five, fractional factorial screening experiments, from which inferences and predictions were made, were conducted in order to identify the two most influential nutrient supplements from the five nutrient supplements of the O and K medium (Table 3.1).

3.9.1.1 First supplement screening for riboflavin production by *C. famata* ATCC 20850 in supplemented SVO

The five nutrient supplements yeast extract (X_1), peptone (X_2), malt extract (X_3), di-potassium hydrogen phosphate (X_4), magnesium sulphate-hepta-hydrate (X_5) were set at two levels (each concentration set at a 50% higher and 50% lower than the concentration of the O and K medium) in a five-factor fractional factorial design (Table 3.8). Twenty $g.l^{-1}$ glucose spent vegetable oil was added to each experimental run.

Table 3.8 A two-level, resolution V, 2^{5-1} fractional factorial experimental design for the first supplement screening experiment for riboflavin production by *C. famata.* The concentration of SVO in all runs was 20 $g.l^{-1}$

Run	Yeast extract (X_1) ($g.l^{-1}$)	Peptone (X_2) ($g.l^{-1}$)	Malt extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	$MgSO_4.7H_2O$ (X_5) ($g.l^{-1}$)
1	2.5	2.5	2.5	0.1	0.3
2	7.5	2.5	2.5	0.1	0.1
3	2.5	7.5	2.5	0.1	0.1
4	7.5	7.5	2.5	0.1	0.3
5	2.5	2.5	7.5	0.1	0.1
6	7.5	2.5	7.5	0.1	0.3
7	2.5	7.5	7.5	0.1	0.3
8	7.5	7.5	7.5	0.1	0.1
9	2.5	2.5	2.5	0.3	0.1
10	7.5	2.5	2.5	0.3	0.3
11	2.5	7.5	2.5	0.3	0.3
12	7.5	7.5	2.5	0.3	0.1
13	2.5	2.5	7.5	0.3	0.3
14	7.5	2.5	7.5	0.3	0.1
15	2.5	7.5	7.5	0.3	0.1
16	7.5	7.5	7.5	0.3	0.3
17	5	5	5	0.2	0.2
18	5	5	5	0.2	0.2
19	5	5	5	0.2	0.2

Each run was prepared and samples analysed as outlined in 3.8.1.1.

3.9.1.2 Second supplement screening for riboflavin production by *C. famata* in supplemented SVO.

The model developed with the data from the first supplement screening experiment predicted that the concentrations of factors; peptone (X_2), malt extract (X_3) and K_2HPO_4 (X_5) should be increased, while that of yeast extract (X_1), and $MgSO_4.7H_2O$ (X_5) decreased in order to improve production of riboflavin. The second screening experiment was designed as in Table 3.9.

Table 3.9 A two-level, resolution V, 2^{5-1} fractional factorial experimental design for the second supplement screening experiment for riboflavin production by *C. famata.* The concentration of SVO in all runs was 20 $g.l^{-1}$

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Run	Yeast extract (X_1) ($g.l^{-1}$)	Peptone (X_2) ($g.l^{-1}$)	Malt extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_5) ($g.l^{-1}$)	$MgSO_4.7H_2O$ (X_4) ($g.l^{-1}$)
1	0.5	7.5	7.5	0.3	0.1
2	2.5	7.5	7.5	0.3	0.02
3	0.5	12.5	7.5	0.3	0.02
4	2.5	12.5	7.5	0.3	0.1
5	0.5	7.5	12.5	0.3	0.02
6	2.5	7.5	12.5	0.3	0.1
7	0.5	12.5	12.5	0.3	0.1
8	2.5	12.5	12.5	0.3	0.02
9	0.5	7.5	7.5	0.5	0.02
10	2.5	7.5	7.5	0.5	0.1
11	0.5	12.5	7.5	0.5	0.1
12	2.5	12.5	7.5	0.5	0.02
13	0.5	7.5	12.5	0.5	0.1
14	2.5	7.5	12.5	0.5	0.02
15	0.5	12.5	12.5	0.5	0.02
16	2.5	12.5	12.5	0.5	0.1
17	1.5	10	10	0.4	0.06
18	1.5	10	10	0.4	0.06
19	1.5	10	10	0.4	0.06

Each run was prepared and samples analysed as outlined in 3.8.1.1.

3.9.1.3 Third supplement screening for riboflavin production significant by *C. famata* in supplemented SVO.

Statistical analysis of the data recorded for the second supplement screening experiment led to the development of a mathematical model from which, with the use of a mathematical equation, a prediction to improve riboflavin production, by decreasing the concentrations of yeast extract (X_1), peptone (X_2), and malt extract (X_3) and K_2HPO_4 (X_4), and increasing that of $MgSO_4.7H_2O$ (X_5), was made. The experimental design is illustrated in Table 3.10.

Table 3.10 A two-level, resolution V, 2^{5-1} fractional factorial experimental design for the third supplement screening experiment for riboflavin production by *C. famata.* The concentration of SVO in all runs was 20 $g.l^{-1}$

Run	Yeast extract (X_1) ($g.l^{-1}$)	Peptone (X_2) ($g.l^{-1}$)	Malt extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	$MgSO_4.7H_2O$ (X_5) ($g.l^{-1}$)
1	0	5.5	6.5	0.25	0.15
2	0.5	6.5	6.5	0.25	0.05
3	0	8.5	6.5	0.25	0.05
34	0.5	8.5	6.5	0.25	0.15
5	0	6.5	8.5	0.25	0.05
6	0.5	6.5	8.5	0.25	0.15
7	0	8.5	8.5	0.25	0.15
8	0.5	8.5	8.5	0.25	0.05
9	0	6.5	6.5	0.35	0.05
10	2.5	6.5	6.5	0.35	0.15
11	0	8.5	6.5	0.35	0.15
12	0.5	8.5	6.5	0.35	0.05
13	0	6.5	8.5	0.35	0.15
14	0.5	6.5	8.5	0.35	0.05
15	0	8.5	8.5	0.35	0.05
16	0.5	8.5	8.5	0.35	0.15
17	0.25	7.5	7.5	0.3	0.1
18	0.25	7.5	7.5	0.3	0.1
19	0.25	7.5	7.5	0.3	0.1

Each run was prepared and samples analysed as outlined in 3.8.1.1.

3.9.1.4 Fourth supplement screening for riboflavin production by *C. famata* in supplemented SVO

The mathematical model obtained from statistically analysing data recorded for the previous screening experiment predicted an increase in riboflavin production with a decrease in yeast extract (X_1) and peptone (X_2) concentrations, while that of malt extract (X_3) and K_2HPO_4 (X_4) was to be increased. Since the change in $MgSO_4.7H_2O$ (X_5) concentration had no effect on riboflavin production, its concentration was set at its minimum (0.1 $g.l^{-1}$) in further experimentation and since the minimum concentration of yeast extract (X_1) was 0 $g.l^{-1}$, to decrease its concentration was to omit this factor in

future experiments. A three-factor, fractional factorial experiment was thereafter designed, based on the concentration changes that supported improvement in riboflavin production (Table 3.11).

Table 3.11 A two-level, resolution V, 2^{3-1} fractional factorial experimental design for the fourth supplement screening experiment for riboflavin production by *C. famata*. The concentration of SVO in all runs was 20 $g.l^{-1}$

Run	Peptone (X_2) ($g.l^{-1}$)	Malt extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)
1	5.5	7.5	0.35
2	6.5	7.5	0.35
3	5.5	10	0.35
4	6.5	10	0.35
5	5.5	7.5	0.45
6	6.5	7.5	0.45
7	5.5	10	0.45
8	6.5	10	0.45
9	6	8.75	0.4
10	6	8.75	0.4
11	6	8.75	0.4

Each run was prepared and samples analysed as outlined in 3.8.1.1.

3.9.2 Optimization of Riboflavin Production by Response Surface Methodology (RSM)

Results from the fourth supplement screening experiment identified malt extract and K_2HPO_4 as the two most significant nutrient factors that affected riboflavin production by *C. famata* in supplemented spent vegetable oil. These factors were optimized using response surface methodology based on the commonly used central composite experimental design (Table 3.12).

Table 3.12 Central composite experimental design for *C. famata* in supplemented spent vegetable oil in which concentrations of peptone (X_2) and $MgSO_4.7H_2O$ (X_5) in all media below were set at 6.5 $g.l^{-1}$ and 0.15 $g.l^{-1}$, respectively

Run	Malt extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)
1	0.45	10
2	0.65	10
3	0.45	14
4	0.65	14
5	0.41	12
6	0.69	12
7	0.55	9.17
8	0.55	14.83
9	0.55	12
10	0.55	12
11	0.55	12
12	0.55	12
13	0.55	12

Each run was prepared and samples analysed as outlined in 3.8.1.1.

3.9.2.1 One-Factor-At-Time (OFAT) Experiment

The optimized concentration of malt extract only was obtained from the results obtained for this central composite experiment. Therefore, an OFAT experiment, with varying K_2HPO_4 concentrations of 0.65 to 2.00 $g.l^{-1}$ and the optimized malt extract concentration (12.5 $g.l^{-1}$) was conducted. The experimental design is summarized in Table 3.13.

Table 3.13 One-factor-at-a-time experimental design with varying K_2HPO_4 concentrations for riboflavin production by *C. famata* in supplemented SVO. Concentrations of peptone (X_2) and $MgSO_4.7H_2O$ (X_5) in all media below were set at 6.5 $g.l^{-1}$ and 0.15 $g.l^{-1}$, respectively

.

Run	Malt extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)
1	12.5	0.65
2	12.5	0.75
3	12.5	0.85
4	12.5	1
5	12.5	1.25
6	12.5	1.5
7	12.5	1.75
8	12.5	2

The runs were prepared and samples analysed as outlined in 3.8.1.1.

3.9.3 Verification Experiment for Riboflavin Production by *C. famata* in SVO

To verify the optimized medium for riboflavin production by *C. famata* in supplemented spent vegetable oil, a confirmatory experiment, consisting of all factors set at optimum levels (Table 3.14), was conducted in triplicate.

Table 3.14 Optimized riboflavin-producing medium for *C. famata* in supplemented SVO

Chemical	Code	Concentration ($g.l^{-1}$)
Peptone	(X_2)	6.5
Malt extract	(X_3)	12.5
K_2HPO_4	(X_4)	0.15
$MgSO_4.7H_2O$	(X_5)	1.8
Spent vegetable oil		20

.

The runs were prepared and samples analysed as outlined in 3.8.1.1. The organic hexane layer that contained dissolved spent vegetable oil was analysed by GC-MS at Umgeni Water, in Pietermaritzburg, Kwa – Zulu Natal at the end of this experiment.

CHAPTER FOUR

4.0 RESULTS

This chapter reports data obtained during the experiments designed for each proposed objective in this study. Literature indicated that the selected fungi grew successfully in a medium that consisted of glucose as a carbon source (Ozbas and Kutsal, 1986) and was referred to as the O and K medium. The ability of these strains to utilize each spent oil for growth and riboflavin production was tested by introducing these fungi to modified O and K media which the glucose was partially substituted with either spent oil. Thereafter, glucose was totally omitted and substituted with spent oil. Riboflavin produced by the fungi in each substrate was compared in order to establish the strain that was most successful in either medium. The nutrient supplements of the O and K medium that enabled growth and riboflavin production in the spent oils were then optimized using statistical experimental design to maximize the bioproduction of this vitamin using spent industrial oils.

4.1 MICROORGANISMS USED

The colony morphology of the five, selected fungal strains were observed after growing the strains on solid O and K medium for a period of seven days. Microscopic studies using stains were conducted in order to view each strain closely with respect to size, shape and structure, as well as the dispersion of oil droplets within each fungus.

4.1.1 Morphological Studies

Morphological studies of the selected fungi revealed information concerning growth of these microorganisms. Fungal morphology was investigated by inspection of colony growth on solid standard media with respect to colony colour, surface, elevation and margin.

4.1.1.1 Culture characteristics

Agar plates showing growth of the fungal isolates are illustrated in Figure 4.1 and that of the yeast *C. famata* in Figure 4.2. Descriptions of colony growth for the riboflavin-producing fungi *E. gossypii* ATCC 10895, *E. gossypii* CBS 109.51, *E. ashbyi* CBS 208.58 and mutant *E. gossypii* EMS 30/1 with respect to shape, elevation, margin, surface and colour are summarised in Table 4.1.

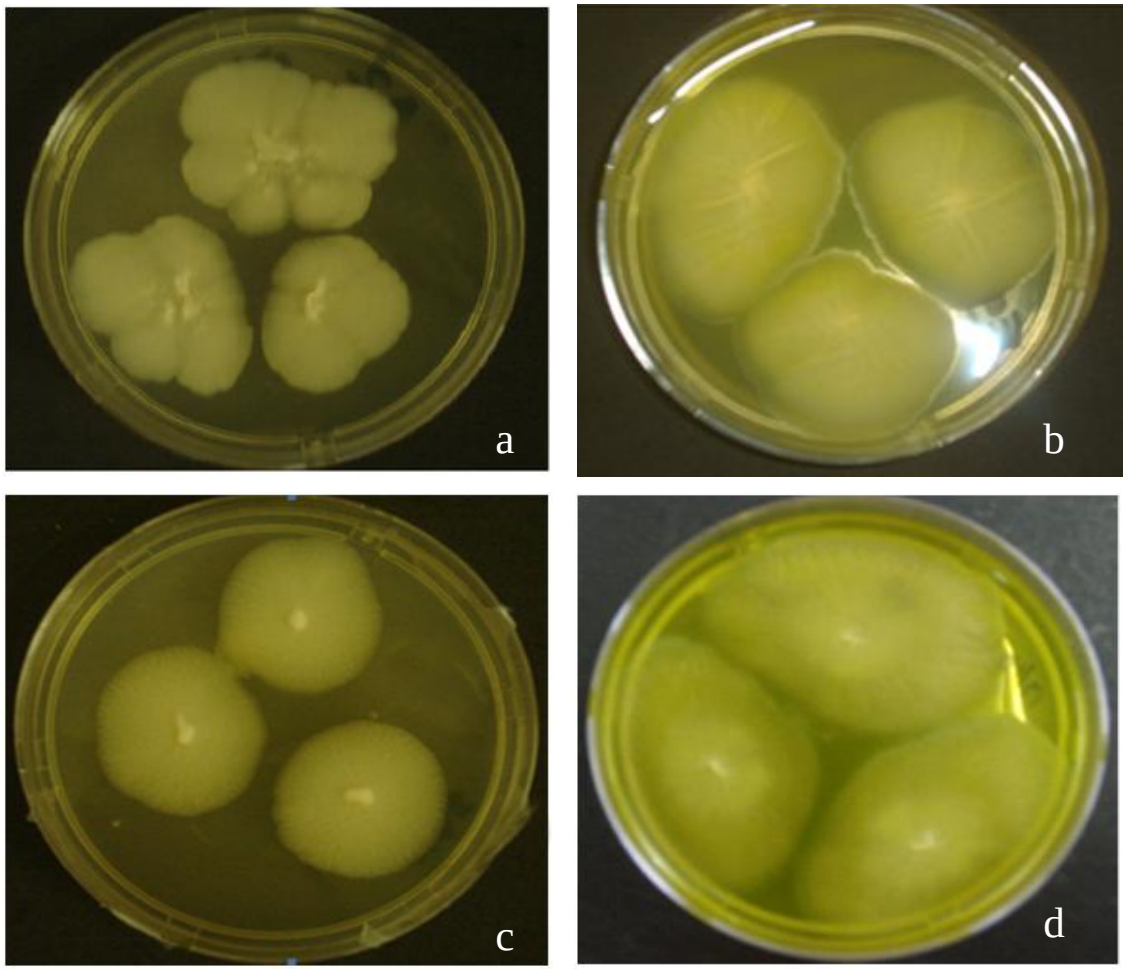


Figure 4.1 Growth of filamentous fungi a) *E. gossypii* ATCC 10895, b) *E. gossypii* 109.51, c) *E. ashbyi* CBS 208.58 and d) mutant *E. gossypii* on solid O and K medium after seven days incubation at 30^{o}C.

Figure 4.2 Growth of *C. famata* ATCC 20850 on solid O and K medium after seven days inoculation at 30°C.

Table 4.1 Colony morphology of *E. gossypii* ATCC 10895, *E. gossypii* (CBS), *E. ashbyi* CBS), *C. famata* ATCC 20850 and mutant *E. gossypii* EMS 30/1 on solid O and K medium after seven days of incubation at 30°C

	E. gossypii ATCC 10895	*E. gossypii* CBS 109.51	*E. ashbyi* CBS 208.58	Mutant *E. gossypii* EMS 30/1	*C. famata* ATCC 20850
Growth	Thick carpet-like growth	Thin carpet-like growth	Thin carpet-like growth	Thin carpet-like growth	Bacterial-like colonial growth
Shape	Irregular	Filamentous	Filamentous	Filamentous	Circular
Elevation	Umbonate	Umbonate	Umbonate	Umbonate	Convex
Margin	Undulate	Filiform	Circular, filiform	Filiform	Entire
Surface	Rough with radial lines extending from centre	Rough and rugose	Rough	Rough and rugose	Glossy
Colour	Cream	Orange	Light orange	Dark orange	Cream

Table 4.1 indicates that the only similarity between (Figure 4.1a) *E. gossypii* ATCC 10895 and the other filamentous fungi was the umbonate elevation of the colonies, which remained cream in colour, like the yeast *C. famata.* However, the colour of the growth medium for *E. gossypii* ATCC 10895 did not change to orange as happened for the yeast *C. famata*, which implied that no riboflavin was secreted into the growth medium. *Eremothecium ashbyi* showed variation in overall colony shape compared to the mutants of *E. gossypii* (Figures 4.1b and d) as it was circular and filiform. Both mutants *E. gossypii* CBS 109.51 and mutant *E. gossypii* EMS 30/1 showed similar colony growth except for size and colour with colonies of the latter microorganism being larger and more orange, which indicated greater concentrations of riboflavin being produced. *Candida famata* (Figure 4.2) showed typical yeast colony growth.

4.1.1.2 Morphological characteristics of the selected fungi

Microscopic analysis of ascomycetes *E. gossypii* (wild type), *E. gossypii* CBS 109.51 and *E. ashbyi* CBS 206.58 showed their filamentous nature as shown in Figures 4.3 a, b and c respectively. The general characteristic of budding in the yeast *C. famata* was observed in Figure 4.3d. Mutant *E. gossypii* EMS 30/1 was microscopically inspected after 72, 96 and 120 hours in e, f and g, respectively. Apical dichotomous branching of mutant *E. gossypii* EMS 30/1exhibited the characteristic polar growth at 72 hours (Figure 4.3e), twirling of hyphae after 96 hours (Figure 4.3e) and the production of apical asci after 120 hours (Figure 4.3f). An apical ascospore at the end of a mutant *E. gossypii* EMS 30/1 hypha, stained with Lactophenol blue under 1000 x magnification, is shown in Figure 4.3h.

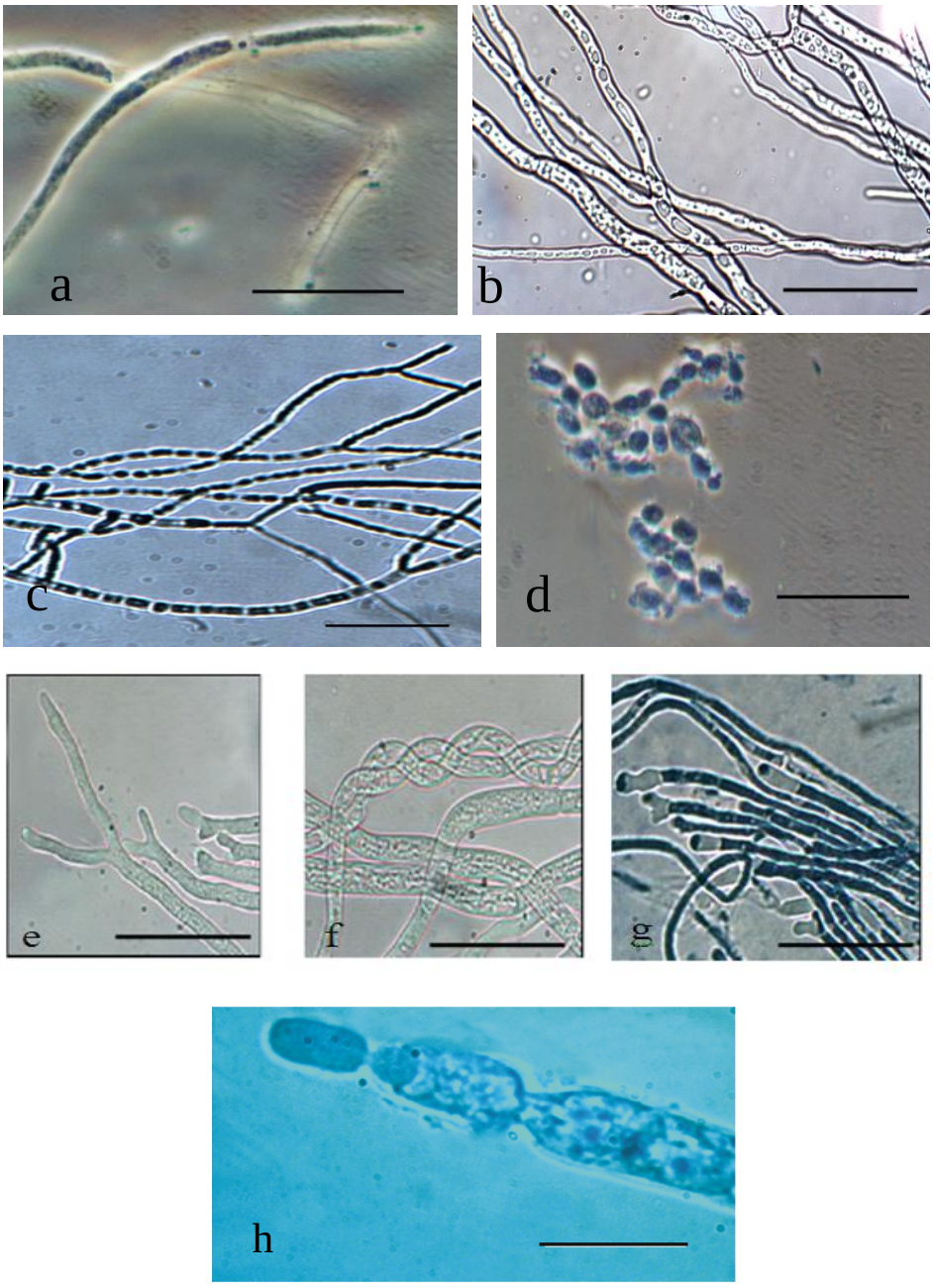


Figure 4.3 Microscopic views of *E. gossypii* ATCC 10895 (a), *E. gossypii* CBS 109.51(b), *E. ashbyi* CBS 208.58 (c), and *C. famata* ATCC 20850 (d). Microscopic images of mutant *E. gossypii* EMS 30/1 shows tip-splitting after 72 hours (e), twirling after 96 hours (f) and apical ascospores after 120 hours (g). Apical ascus of mutant *E. gossypii* EMS 30/1 stained with lactophenol blue is indicated in h. All images were under 1000 x magnification using light microscopy. Bars = 10 µm.

Distribution of the oil droplets in the fungal hyphae were viewed under a fluorescent microscope and are shown in Figure 4.4. Figure 4.4a shows lipid dispersion within a mycelium of *E. gossypii* ATCC 10895 while figures 4.4 b and c shows intracellular oil dispersion and oil dispersion within spindle-shaped ascospores of *E. gossypii* (CBS),

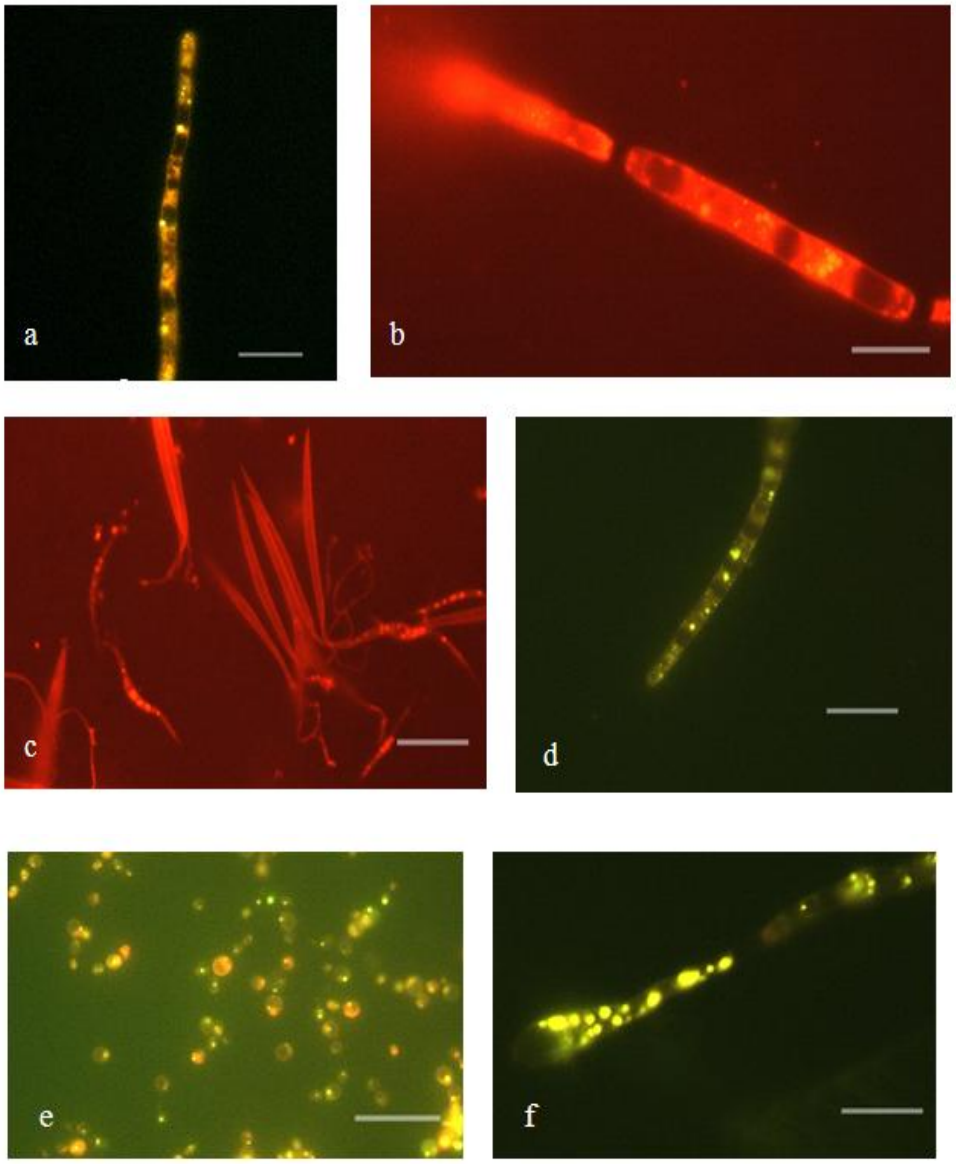


Figure 4.4 Microscopic images of *E. gossypii* ATCC 10895 (a), *E. gossypii* CBS 109.51(b) and it's spindle shaped spores (c), *E. ashbyi* CBS 208.58 (d), and *C. famata* ATCC 20850 (e) and apex of mutant *E. gossypii* EMS 30/1 stained with Nile red, at fluorescence light, at 1000 x magnification showing intracellular oil dispersion of the selected fungi. Bars = 10 µm.

respectively. Figure 4.4d illustrates oil dispersion within a filament of *E. ashbyi* (CBS), and Figure 4.4e shows oil dispersion within the yeast, *C. famata* ATCC 20850. Figure 4.4f illustrates oil dispersion at the hyphal apex of mutant *E. gossypii* EMS 30/1.

4.2 QUANTITATIVE DETERMINATIONS

4.2.1 Growth Curves of the Selected Riboflavin-producing Fungi

The growth curve of *E. gossypii* ATCC 10895 (Figure 4.5a) shown in black, indicated a lag phase during the first 21 hours of incubation, followed by an exponential growth phase, extending from 21 to 97 hours. At the centre of this phase lies the mid-log point (60 hours), indicated by the arrows. This point indicates the time at which this fungus most vigorously grew and was used to determine the growth duration of the experimental inocula. A stationary phase during which growth tapered off, occurred after 97 hours incubation.

The growth curve of *E. gossypii* CBS 109.51 (Figure 4.5a) shown in red indicated a shorter lag phase than that of the wild type, that extended for the first 6 hours of incubation. The exponential growth phase occurred between 6 and 40 hours, the centre of which the mid-log point (26 hours) lay (indicated by the arrow). At this point in time this microorganism most actively grew and inocula were grown for this duration.

The growth curve of *E. ashbyi* CBS 206.58 (Figure 4.5a) illustrated in green, indicated a lag phase of 6 hours, followed by the exponential growth phase extending from 17 to 54 hours incubation time, and the centre of which lay the organism's mid-log point (35.5 hours) indicated arrows. This phase was followed by the stationary phase during which growth tapers off, and extends until 103 hours, after which, the death phase, indicated by a steep decrease in biomass, is evident.

The growth curve of mutant *E. gossypii* EMS 30/1 (Figure 4.5a) indicated in yellow showed a lag phase of 6 hours, followed by the exponential growth phase that extended until 48 hours. The intervening point (25 hours) marked with arrows showed the mid-log point of this filamentous fungus and this point marked the most active growth of this mutant. Hence, experimental inocula were grown for 25 hours prior to harvesting cells. The growth curve of *Candida famata* ATCC 20850 (Figure 4.5b), measured as absorbance at 600 nm, indicated a lag phase that extended to 21 hours, after which the exponential growth phase extended to 120 hours. The intermediate of this phase, (72 hours) marked by arrows indicated the yeast's mid-log point, which was the time at which most vigorous growth was recorded. This was the time for which the *C. famata* experimental inocula were grown.

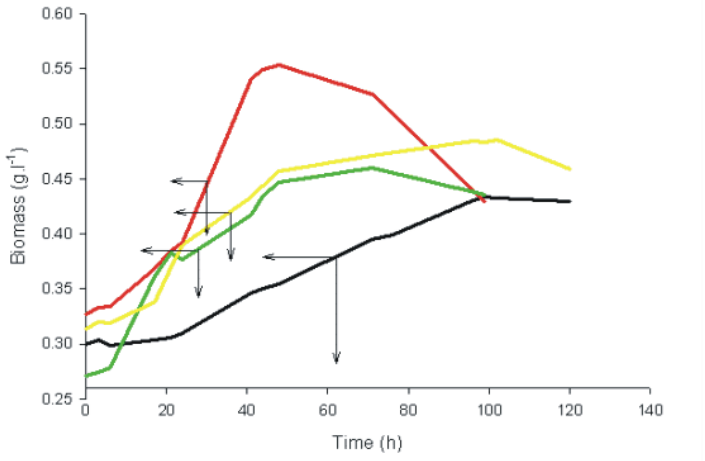


Figure 4.5a Growth curves of *E. gossypii* ATCC 10895 (black), *E. gossypii* CBS 109.51 (red), *E. ashbyi* CBS 208.58 (green) and mutant *E. gossypii* EMS 30/1 (yellow) with their mid-log points indicated by arrows.

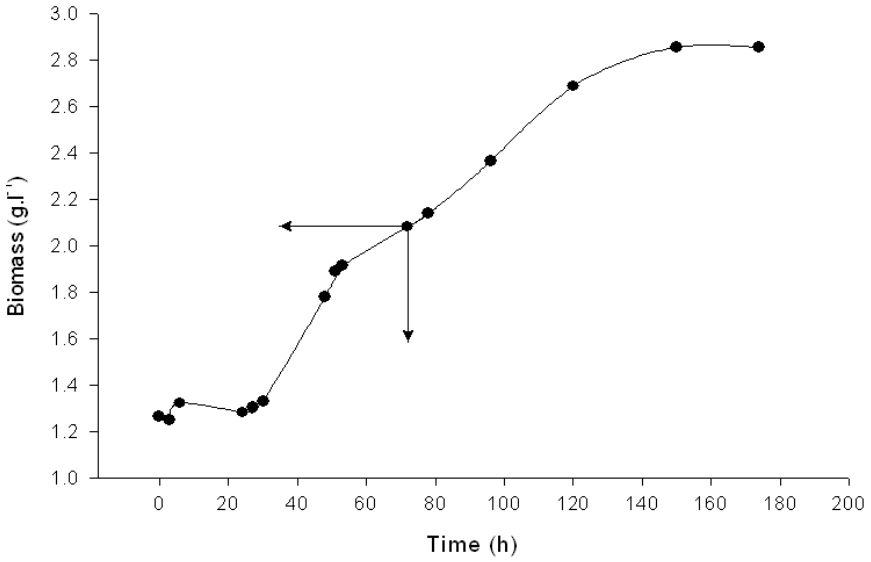


Figure 4.5b Growth curve of *C. famata.* The mid-log point is shown with arrows.

Maximum specific growth rates and doubling time for each of the selected fungi are shown in Table 4.2.

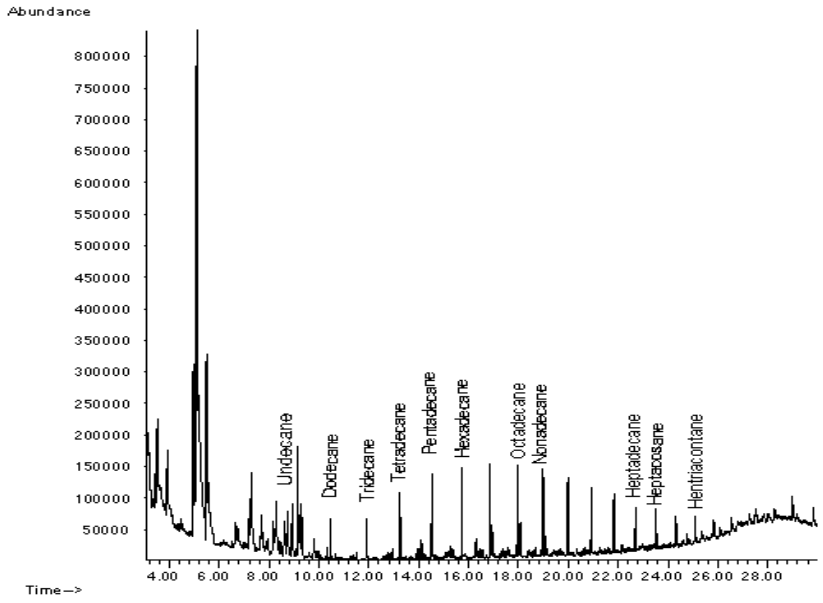
Table 4.2 Maximum specific growth rates and doubling time for each fungus

Time (h)	Maximum specific growth rate (μ_{max})	Doubling Time (T_d)
E. gossypii ATCC 10895	0.0251 h^{-1}	27.55 h
E. gossypii CBS 109.51	0.0055 h^{-1}	12.47 h
E. ashbyi CBS 208.58	0.0219 h^{-1}	31.60 h
Mutant *E. gossypii* EMS 30/1	0.0238 h^{-1}	29.11 h
C. famata ATCC 20850	0.0647 h^{-1}	1.017 h

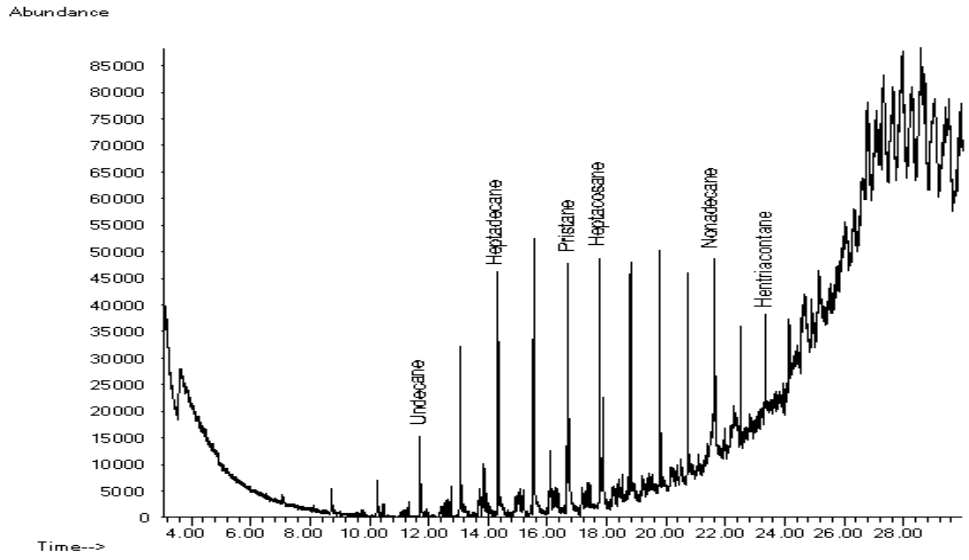
4.3 ANALYSIS OF SPENT MOTOR AND VEGETABLE OILS

GC analyses of the spent oils before and after fungal growth are shown in Figures 4.6a and 4.6b for SMO and in 4.7a and 4.7b respectively for SVO and the predominant compounds are listed in Tables 4.3 – 4.6. Compounds with longer carbon chains (C_{17} to C_{31}) were present before and after fungal growth, while those with shorter carbon chains (C_{12} to C_{15}) were not detected after growth. Furthermore, the chromatograms

indicated that those compounds present after fungal growth were of a much lower concentration than before. The magnitude of the peaks in Figure 4.6b show that these compounds decreased to a tenth of the initial concentrations.



(a)



(b)

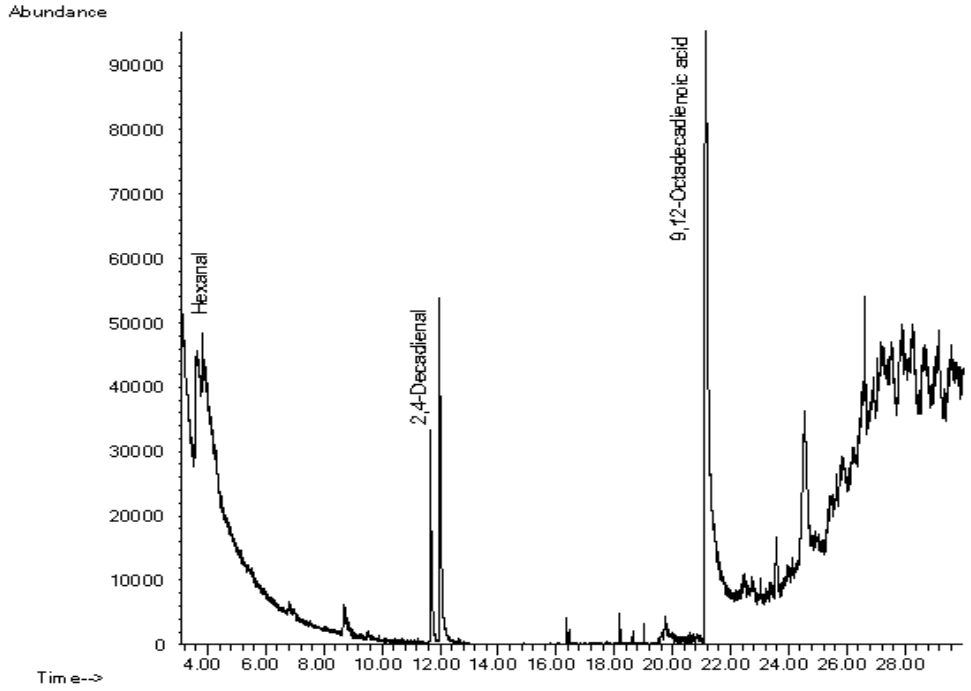
Figure 4.6 Chromatogram of SMO before (a) and after (b) growth of mutant *E.gossypii* EMS 30/1.

Table 4.3 List of compounds identified in SMO prior to fungal growth

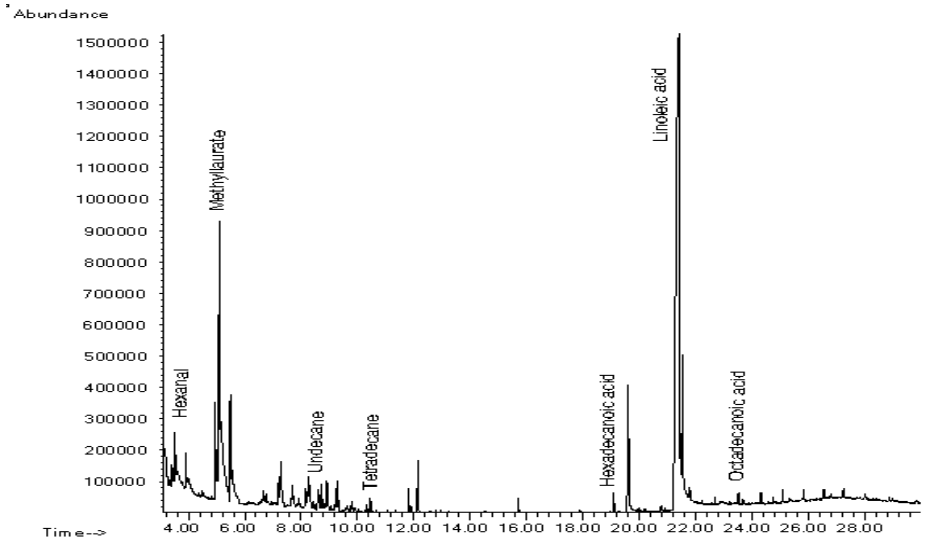
Compounds	Library Match Quality (%)	Retention time
Undecane ($C_{11}H_{24}$)	97	8.92
Heptadecane ($C_{17}H_{36}$)	91	22.69
Heptacosane ($C_{27}H_{56}$)	91	23.53
Nonadecane ($C_{19}H_{40}$)	98	18.99
Hentriacontane ($C_{31}H_{64}$)	87	25.1
Naphthalene ($C_{10}H_{8}$)	95	10.31
Dodecane ($C_{12}H_{28}$)	94	10.46
Tridecane ($C_{13}H_{28}$)	97	11.90
Tetradecane ($C_{14}H_{30}$)	97	13.25
Pentadecane ($C_{15}H_{32}$)	96	14.53
10-Methylnonadecane	80	15.30
Hexadecane ($C_{16}H_{34}$)	94	15.73
Tridecane	81	16.46
Heptadecane ($C_{17}H_{36}$)	98	16.88
2,6,10-Trimethyl-Pentadecane	90	16.95
Octadecane ($C_{18}H_{38}$)	98	17.96
2,6,10,14-Tetramethyl-hexadecane	91	18.07
Nonadecane ($C_{19}H_{40}$)	98	18.99
Eicosane ($C_{20}H_{42}$)	97	19.98
Octacosane ($C_{28}H_{58}$)	91	20.93
Octadecane ($C_{18}H_{37}$)	91	21.83
Heptadecane ($C_{17}H_{36}$)	91	22.69
Heneicosane ($C_{21}H_{44}$)	91	24.33
Dotriacontane ($C_{4}H_{6}O_{2}$)	86	25.84

Table 4.4 Compounds identified in SMO after fungal growth using the Wiley 275 spectra library

Compounds	Library Match Quality (%)	Retention time
Undecane ($C_{11}H_{24}$)	83	11.71
Heptadecane ($C_{17}H_{36}$)	91	14.33
Pristane ($C_{19}H_{40}$)	90	16.74
Heptacosane ($C_{27}H_{56}$)	90	17.77
Nonadecane ($C_{19}H_{40}$)	90	21.64
Hentriacontane ($C_{31}H_{64}$)	80	23.34



(a)



(b)

Figure 4.7 Chromatograms of SVO before (a) and after (b) growth of *C. famata*.

Table 4.5 Compounds identified in SVO before fungal growth

Compounds	Library Match Quality (%)	Retention time
2-Methyl-heptane (C_7H_{16})	91	3.42
Toulene (C_7H_8)	94	3.54
Octane (C_4H_9)	91	3.92
Hexanal ($C_6H_{12}O$)	80	3.99
2,3-Dimethyl-heptane	83	4.83
Methyllaurate	95	4.96
Undecane ($C_{11}H_{36}$)	93	8.92
Tetradecane ($C_{14}H_{30}$)	90	10.46
2,4-Decadienal	93	11.83
Hexadecanoic acid	99	19.62
Linoleic acid ($C_{18}H_{30}O_2$)	99	21.37
9-Octadecenoic acid	94	21.4
Octadecanoic acid	99	21.54
Heneicosane ($C_{21}H_{44}$)	87	23.52
Hexadecane ($C_{16}H_{35}$)	91	24.32
Tetracontane ($C_{46}H_{82}$)	91	25.09
Heptacosane ($C_{27}H_{56}$)	87	25.84
Hexacosane ($C_{18}H_{36}N_2O_6$)	91	26.56
Triacontane ($C_{30}H_{62}$)	87	27.25
Eicosane ($C_{20}H_{42}$)	93	28.01

Table 4.6 Compounds identified in SVO after fungal growth

Compounds	Library Match Quality (%)	Retention time
Hexanal ($C_6H_{12}O$)	83	3.82
2,4-Decadienal ($C_{10}H_{16}O$)	91	11.67
9,12-Octadecadienoic acid ($C_{18}H_{32}O_2$)	98	21.16

4.4 PRELIMINARY GROWTH IN SPENT OILS AND SELECTION OF SUPERIOR RIBOFLAVIN-PRODUCING FUNGI

4.4.1 Spent Motor Oil as Carbon Source for Riboflavin production

All five fungi were grown in media consisting of both glucose and spent motor oil as carbon sources. Figure 4.8 indicated complete depletion of glucose after 20 hours of incubation during growth of the five selected fungi in the modified O and K medium that consisted of both glucose and spent motor oil.

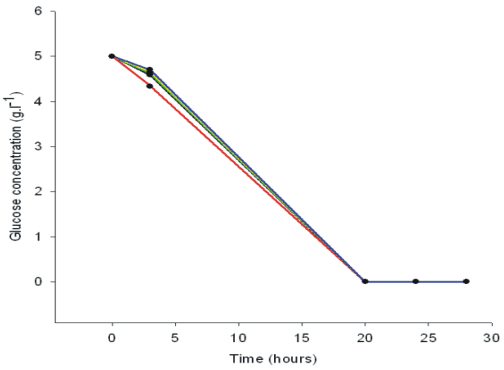


Figure 4.8 Glucose depletion during the preliminary growth experiments of mutant *E. gossypii* EMS 30/1 (black), *E. gossypii* CBS (red), *E. gossypii* wild type (green), *E. ashbyi* (yellow) and *C. famata* (blue) using glucose and SMO as a carbon sources.

The increase in biomass after twenty hours of incubation (Figure 4.9) illustrated an increase the growth of the fungi after glucose depletion. This suggested that the fungi grew using spent motor oil as carbon source when glucose was completely depleted.

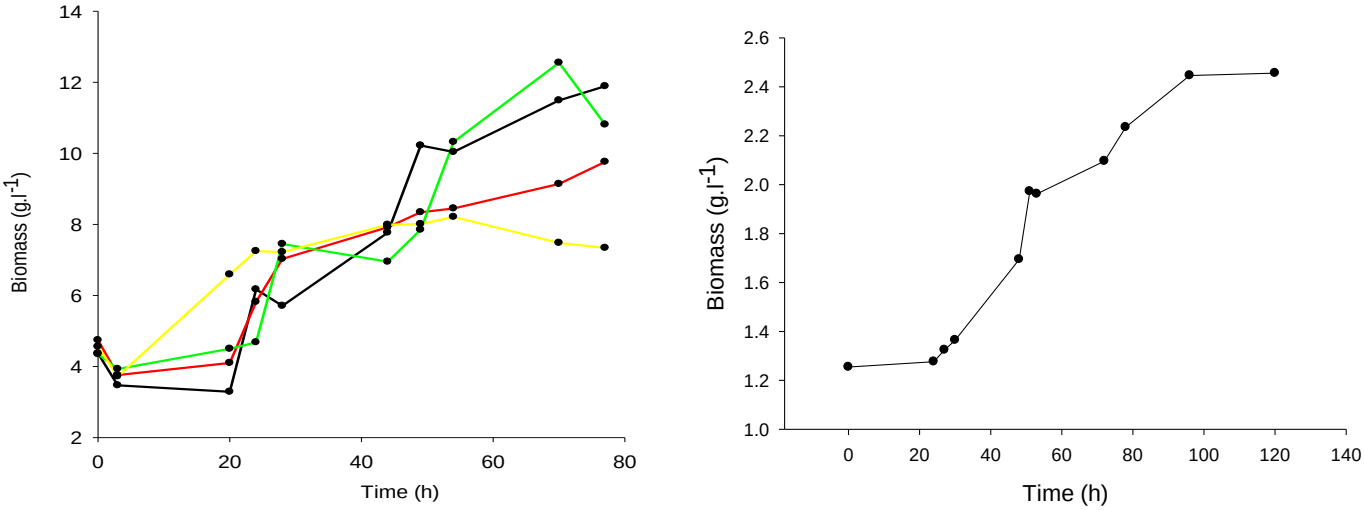


Figure 4.9 Change in biomass of (a)mutant *E. gossypii* EMS 30/1 (black), *E. gossypii* (CBS) (red), *E. gossypii* wild type (green) and *E. ashbyi* (yellow) during preliminary growth experiments in glucose and SMO and (b) *C. famata.*

Evidence of riboflavin production by the selected fungi when grown in both SMO and glucose was observed after fifty hours, when growth was beginning to slow or taper off (Figure 4.10) and after glucose had diminished. This suggests that glucose that might have been stored and SMO could have contributed towards riboflavin production.

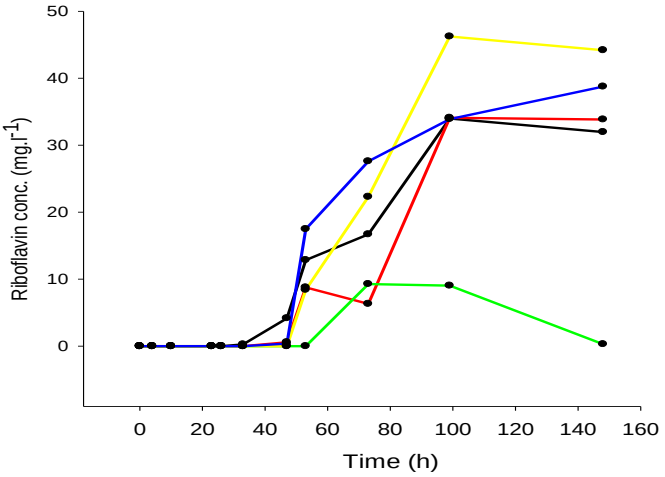


Figure 4.10 Production of riboflavin by the five selected fungi, mutant *E. gossypii* EMS 30/1(black), *E. gossypii* (CBS) (red), *E. gossypii* wild type (green), *E. ashbyi* (yellow) and *C. famata* (blue) in preliminary growth experiments using both glucose and SMO as carbon sources.

4.4.2 Spent Vegetable Oil as a Carbon Source for Riboflavin Production

The five selected riboflavin-producers were grown in both glucose and SVO as carbon sources. Glucose was almost completely depleted after ten hours (Figure 4.11). At about twenty hours, this carbon source was completely utilised by the selected fungi, however, glucose depletion recorded was different when compared to the data recorded when spent motor oil was used (Figure 4.8).

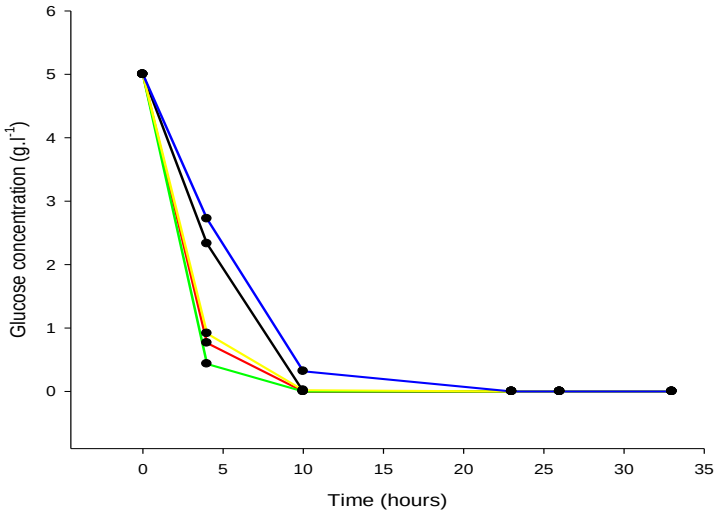
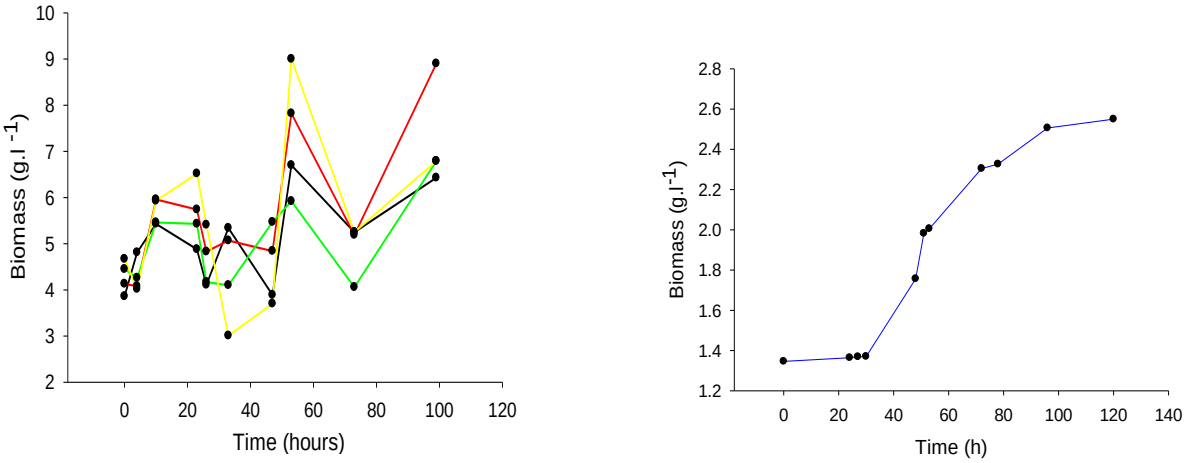


Figure 4.11 Glucose depletion by mutant *E. gossypii* EMS 30/1 (black), *E. gossypii* (CBS) (red), *E. gossypii* wild type (green), *E. ashbyi* (yellow) and *C. famata* (blue) in the preliminary growth experiments in which both glucose and SVO were utilized as carbon sources.

Increasing biomass in the initial twenty hours for the filamentous fungi (Figure 4.12a) and after thirty hours for *C. famata* (Figure 4.12b) suggests successful fungal growth of in the presence of both glucose and SVO. This increase indicates the use of spent vegetable oil for growth since glucose was completely depleted by this point. Change in biomass in this experiment indicated a difference in growth when compared to SMO.



(a) (b)

Figure 4.12 Change in biomass of (a) mutant *E. gossypii* EMS 30/1 (black), *E. gossypii* (CBS) (red), *E. gossypii* wild type (green) and *E. ashbyi* (yellow) and (b) *C. famata* in preliminary growth experiments using both glucose and SVO as carbon sources.

Riboflavin production by *C. famata* and *E. gossypii* (CBS) increased rapidly after fifty hours, when fungal growth had decreased (Figure 4.13). This confirmed the use of SVO as a carbon source for riboflavin production as production yields increased despite complete depletion of glucose.

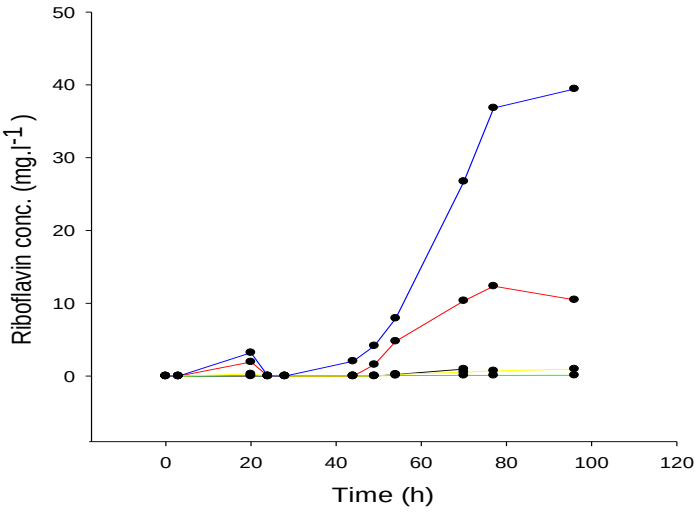


Figure 4.13 Production of riboflavin by mutant *E.* gossypii EMS 30/1 (black), *E. gossypii* (CBS) (red), *E. gossypii* (green), *E. ashbyi* (yellow) and *C. famata* (blue) in preliminary growth experiments that used both glucose and vegetable oil as carbon sources.

4.4.3 Selection of Fungi for Riboflavin Production Using SMO and SVO

The five selected fungi were grown in the modified O and K media in which glucose was completely substituted with either 20 $g.l^{-1}$ SMO or 20 $g.l^{-1}$ SVO. Riboflavin production by each organism in SMO and SVO was compared in Figures 4.14 and 4.15, respectively in order to select those fungi which produced the highest riboflavin concentrations when grown in SMO and SVO. The superior riboflavin-producing fungi for these spent oils were mutant *E. gossypii* EMS 30/1 and (20.44 $mg.l^{-1}$) and *C. famata* (16.99 $mg.l^{-1}$) since they had the highest production of riboflavin in SMO and SVO, respectively.

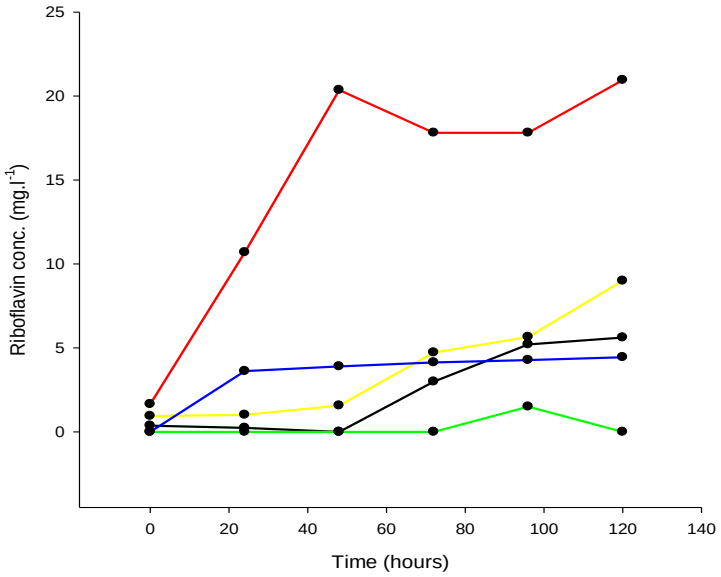


Figure 4.14 Comparison of riboflavin production by the five selected fungi, *E. ashbyi* (black), mutant *E. gossypii* EMS 30/1(red), *E. gossypii* wild type (green), *E. gossypii* (CBS) (yellow) and *C. famata* (blue) in the modified O and K medium in which 20 g.$^{-1}$l glucose was substituted with 20 g.l^{-1} spent motor oil.

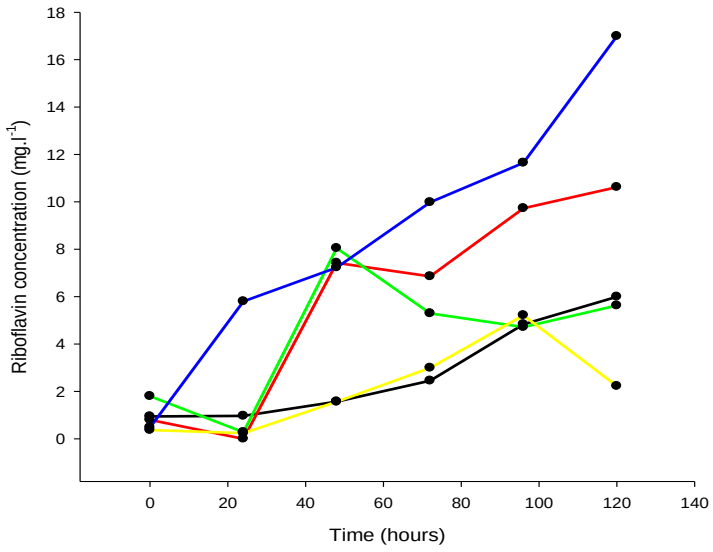


Figure 4.15 Riboflavin produced by *E. ashbyi* (CBS) (black), mutant *E. gossypii* EMS 30/1 (red), *E. gossypii* ATCC 10895 (green), *E. gossypii* CBS (yellow) and *C. famata* (ATCC 20850) (blue) using only SVO as a carbon source.

4.5 MUTANT *Eremothecium gossypii* EMS 30/1 IN SPENT MOTOR OIL

4.5.1 Screening for Effects of Nutrient Supplements by Fractional Factorial Experimental design

4.5.1.1 First Supplement Screening for Riboflavin Production by mutant *E. gossypii* EMS 30/1 in SMO

The first screening fractional factorial experiment was a five-factor, two-level, resolution V design. In this experiment the high and low concentration levels of the nutrient supplements were set at 50% higher and 50% lower than that of the O and K medium (Table 3.1). A comparison of riboflavin production for all runs indicated that production yield was optimum at 120 hours. Therefore, riboflavin production was analysed at this time for all future experiments.

Table 4.7 shows the experimental design for the first supplement screening experiment for mutant *E. gossypii* EMS 30/1 in supplemented SMO together with the riboflavin production recorded at 120 hours, the time at which most riboflavin was produced by this microorganism. The response ranges from 11.77 $mg.l^{-1}$ to 61.42 $mg.l^{-1}$, resulting in the ratio of maximum to minimum concentrations equivalent to 5.22. Since this ratio is less than 10, no transformation of the data was necessary.

The Box-Cox plot for power transformation (Figure 4.16) showed that no transformation was required as Lambda = 1, and was positioned 1.03 away from the best transformation (green line) which lay at -0.03.

Table 4.7 Riboflavin production in the first supplement screening experiment for mutant *E. gossypii* EMS 30/1 in supplemented SMO

Run	Yeast Extract (X_1) ($g.l^{-1}$)	Peptone (X_2) ($g.l^{-1}$)	Malt Extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	$MgSO_4.7H_2O$ (X_5) ($g.l^{-1}$)	[Ribo] ($g.l^{-1}$)
1	2.5	2.5	2.5	0.1	0.3	61.42
2	7.5	2.5	2.5	0.1	0.1	19.74
3	2.5	7.5	2.5	0.1	0.1	25.27
4	7.5	7.5	2.5	0.1	0.3	11.77
5	2.5	2.5	7.5	0.1	0.1	48.31
6	7.5	2.5	7.5	0.1	0.3	22.80
7	2.5	7.5	7.5	0.1	0.3	42.85
8	7.5	7.5	7.5	0.1	0.1	20.15
9	2.5	2.5	2.5	0.3	0.1	24.68
10	7.5	2.5	2.5	0.3	0.3	18.22
11	2.5	7.5	2.5	0.3	0.3	28.83
12	7.5	7.5	2.5	0.3	0.1	18.81
13	2.5	2.5	7.5	0.3	0.3	20.86
14	7.5	2.5	7.5	0.3	0.1	22.23
15	2.5	7.5	7.5	0.3	0.1	14.14
16	7.5	7.5	7.5	0.3	0.3	14.27
17	5	5	5	0.2	0.2	24.04
18	5	5	5	0.2	0.2	22.29
19	5	5	5	0.2	0.2	16.50

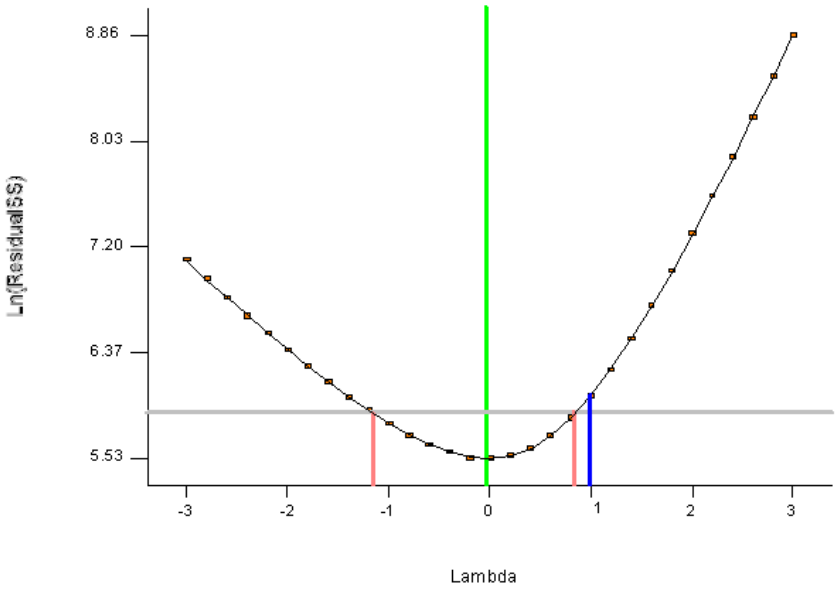


Figure 4.16 Box-Cox plot for power transformations for the first supplement screening experiment. The blue line indicates the current transformation of the model for this experiment while the green line indicates the best transformation.

The Half-Normal Plot (Figure 4.17) indicated factors that were selected to be included in the mathematical model. Factors on the right were more significant than those on the left, hence they were selected first. Therefore, selection was made from the top right corner to the left bottom, with the aim of obtaining a significant model.

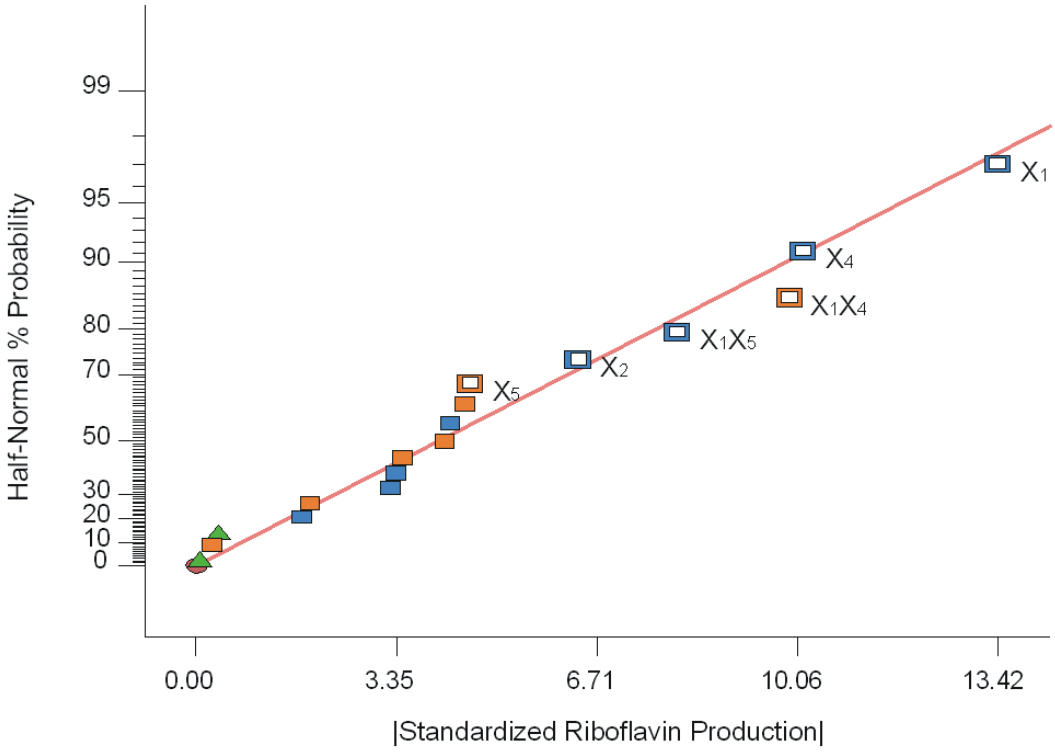


Figure 4.17 Half-Normal plot illustrating selection of factors (X_1- yeast extract, X_2- peptone, X_5 - magnesium heptahydrate and X_4 - di-potassium hydrogen phosphate) (empty blocks) to be included in the model for the first screening experiment for mutant *E. gossypii* EMS 30/1 in supplemented spent motor oil. Positive effects appear in red while the negative effects are shown in blue. Triangles are center points.

Design Expert 7.1.6 also allows for the selection of factors to be made using the Pareto Chart, which is a graphic illustration of the t-value of effects, which is more informative than the Half-Normal plot. The individual factors in order of significance, that affected the production of riboflavin by mutant *E. gossypii* EMS 30/1 in the first supplement screening experiment, were expressed in Figure 4.18. Yeast extract, which had the largest effect on riboflavin production, lay above the Bonferroni limit and was therefore considered as definitely significant. Individual factors K_2HPO_4 (X_4), and mixture of components yeast extract-K_2HPO_4 (X_1X_4) and yeast extract-

$MgSO_4.7H_2O(X_1X_5)$ were possibly significant as they lay above the t-value limit. In addition to these significant factors, non-significant factors peptone (X_2) and $MgSO_4.7H_2O(X_5)$ were also selected by the software to obtain a hierarchical model. Amongst the significant factors X_1, X_4, X_1X_4 and X_1X_5, only X_1X_4 had a positive effect on the production of riboflavin in this screening experiment (Figure 4.18).

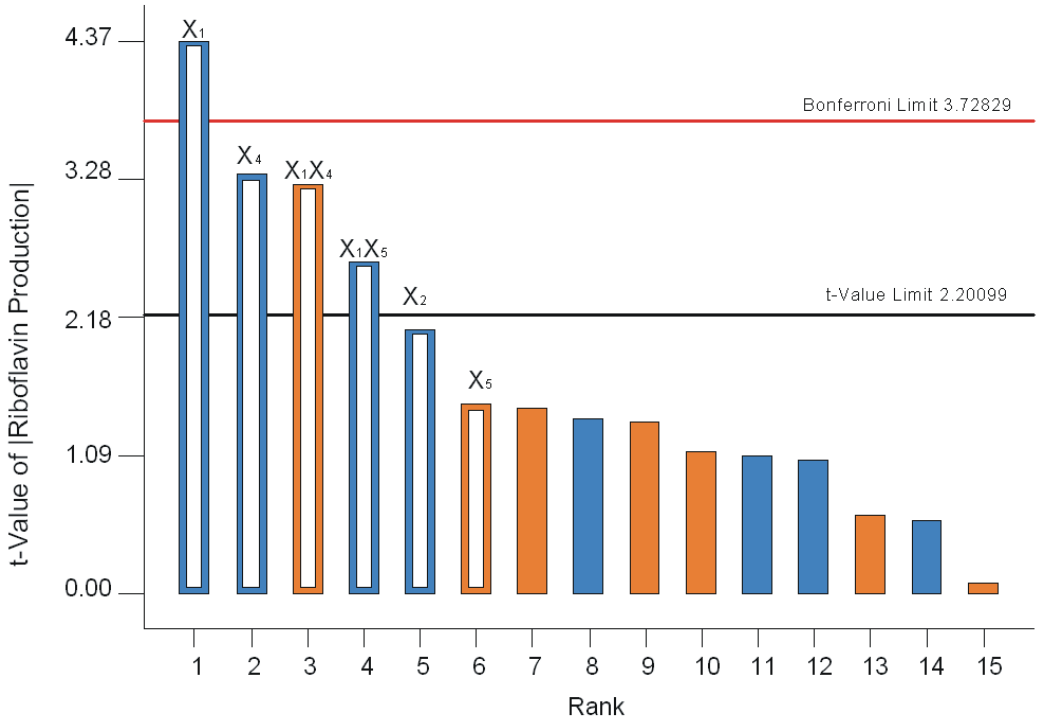


Figure 4.18 Pareto Chart showing the effects of yeast extract (X_1), peptone (X_2), K_2HPO_4 (X_4) and $MgSO_4.7H_2O$ (X_5). Positive effects are shown in red and negative effects are shown in blue. Factors included in the model are represented in open boxes.

The developed model for the data was statistically significant (Table 4.8). Significance was determined by an F-value of 8.98 which implied that the model fitted the design appropriately with only a 0.1% chance that an F-value this large could have occurred

due to noise. All inferences from the graphs were made with a 99.90% probability. Individual factors with *p*-values less than 0.05 were considered to have a significant effect on riboflavin production by mutant *E. gossypii* EMS 30/1 in this experiment. In this case, individual factors X_1 and X_4 were significant. Mixture of components with p-values less than 0.05, in this case factors X_1X_4 and X_1X_5, also significantly affected riboflavin production by this fungus in supplemented spent motor oil.

Furthermore, a 'Lack of Fit-value' of 2.73 implied that the Lack of Fit was not significant relative to the pure error. There was a 29.65% chance that a 'Lack of Fit-value' this large could have occurred due to noise. Noise is the response within one standard deviation. An insignificant Lack of Fit implies that the data fits the model which is significant.

Table 4.8 Analysis of variance table for the first screening experiment for mutant *E. gossypii* EMS 30/1 in supplemented spent motor oil

Source	Sum of Squares	df	Mean Square	F Value	p-valueProb > F	Significance
Model	2033.97	6	338.99	8.98	0.001	significant
X_1-Yeast Extract	720.37	1	720.37	19.08	0.001	significant
X_2 -Peptone	163.58	1	163.58	4.33	0.0615	
X_4 -K_2HPO_4	412.58	1	412.58	10.93	0.007	significant
X_5 -$MgSO_4.7H_2O$	84.07	1	84.07	2.23	0.1638	
X_1X_4	393.97	1	393.97	10.43	0.008	significant
X_1X_5	259.39	1	259.39	6.87	0.0238	significant
Lack of Fit	384.1	9	42.68	2.73	0.2965	not significant

The derived mathematical model for the production of riboflavin by mutant *E. gossypii* EMS 30/1 in this experiment was described by the following equation:

The final equation in terms of coded factors:

$$\text{Riboflavin concentration} = +39.59 - 7.17 * X_1 + 7.92 * X_2 + 8.51 * X_3 + 3.21 * X_1 * X_2 + 4.43 * X_1 * X_3 + 4.93 * X_2 * X_3 + 4.86 * X_1 * X_2 * X_3 \quad \text{.....4.1}$$

where X_1 = yeast extract, X_2 = peptone, $X_4 = K_2HPO_4$ and $X_5 = MgSO_4.7H_2O$.

The model was based on individual effects and combinations of factors that are given by equation 4.1. Cube plots based on the model were drawn and the equation was used to predict riboflavin yields based on the mathematical model. The cube plot (Figure 4.19) illustrated the effect of the three most significant factors X_1, X_4 and X_5 that affected riboflavin production by *E. gossypii* EMS 30/1 in supplemented SMO in the first screening experiment. Although X_5 ($MgSO_4.7H_2O$) on its own was not significant, it was selected because when interacting with yeast extract (X_1), it produced a significant effect. Maximum riboflavin production was obtained at minimum concentrations of factors X_1 and X_4, and maximum concentration of X_5. An increase in concentration from 48.28 to 51.48 $mg.l^{-1}$ riboflavin was obtained by decreasing the concentrations of concentrations of both factors X_2 (peptone) and X_3 (malt extract) from 5.00 $g.l^{-1}$ to 2.5 $g.l^{-1}$.

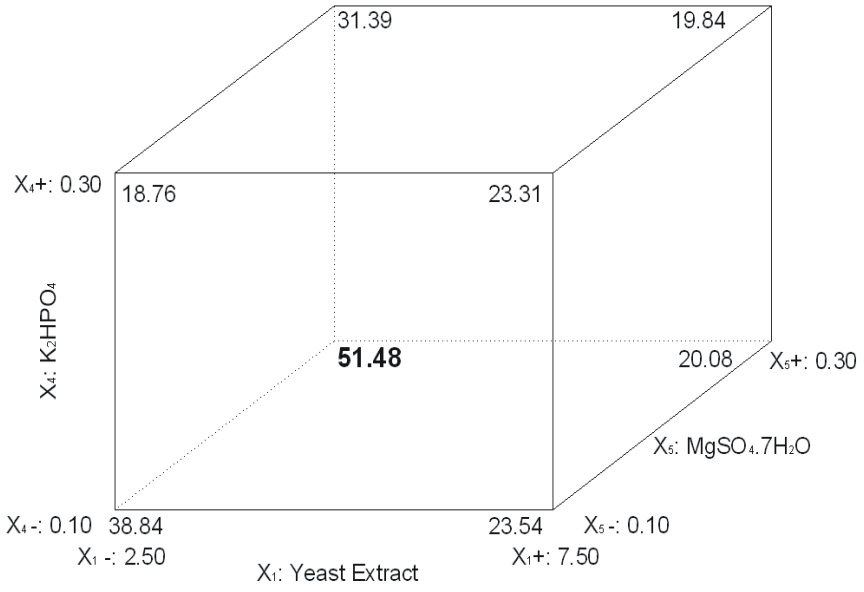


Figure 4.19 Cube plot illustrating the effects of the three most significant factors X_1 (yeast extract), X_4 (K_2HPO_4) and X_5 ($MgSO_4.7H_2O$). Concentrations of both peptone and malt extract were 2.50 $g.l^{-1}$. Maximum riboflavin concentration produced is shown in bold.

4.5.1.2 Second supplement screening for riboflavin production by mutant *E. gossypii* EMS 30/1 in supplemented SMO

Analysis of the results of the first supplement screening experiment was used to design the second supplement screening experiment. The design together with the riboflavin yields recorded at 120 hours is listed in Table 4.9. The response recorded for run 17 was ignored as this run was contaminated. Response ranges from minimum and maximum concentrations of 6.33 $mg.l^{-1}$ and 34.25 $mg.l^{-1}$ respectively, which resulted in the ratio of maximum to minimum concentrations equivalent to of 5.40. This implied that no transformation of data was necessary.

The model was evaluated using the Box-Cox plot (Figure 4.20) which suggested that no transformation was required as Lambda = 1.

Table 4.9 Riboflavin production in the second supplement screening experiment for mutant *E. gossypii* EMS 30/1 in supplemented spent motor oil

	Yeast Extract (X_1) ($g.l^{-1}$)	Peptone (X_2) ($g.l^{-1}$)	Malt Extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	$MgSO_4.7H_2O$ (X_5) ($g.l^{-1}$)	[Ribo] ($mg.l^{-1}$)
1	0.5	0.5	0.5	0.02	0.5	6.33
2	2.5	0.5	0.5	0.02	0.3	6.79
3	0.5	2.5	0.5	0.02	0.3	7.04
4	2.5	2.5	0.5	0.02	0.5	9.41
5	0.5	0.5	2.5	0.02	0.3	8.72
6	2.5	0.5	2.5	0.02	0.5	19.24
7	0.5	2.5	2.5	0.02	0.5	15.42
8	2.5	2.5	2.5	0.02	0.3	28.50
9	0.5	0.5	0.5	0.1	0.3	7.812
10	2.5	0.5	0.5	0.1	0.5	19.00
11	0.5	2.5	0.5	0.1	0.3	7.54
12	2.5	2.5	0.5	0.1	0.3	21.62
13	0.5	0.5	2.5	0.1	0.5	34.25
14	2.5	0.5	2.5	0.1	0.3	29.98
15	0.5	2.5	2.5	0.1	0.3	32.95
16	2.5	2.5	2.5	0.1	0.5	28.56
17	1.5	1.5	1.5	0.06	0.4	42.11
18	1.5	1.5	1.5	0.06	0.4	19.65
19	1.5	1.5	1.5	0.06	0.4	16.77

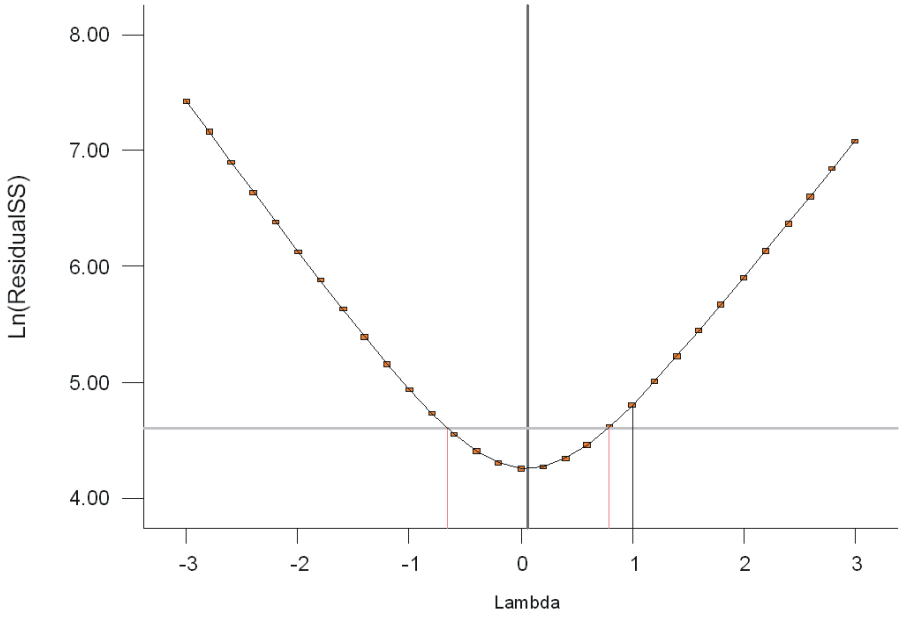


Figure 4.20 Box-Cox plot for power transformations for the second supplement screening experiment. The blue line indicates the current transformation of the model for this experiment while the green line indicates the best transformation.

Selection of factors to be included in the model that described riboflavin production is illustrated in the Half-Normal Probability plot (Figure 4.21). Significant factors located away from the line factors malt extract (X_3), K_2HPO_4 (X_4), peptone-$MgSO_4.7H_2O$ (X_2X_5) and X_1 (yeast extract) were initially chosen, while non-significant factors peptone (X_2) and $MgSO_4.7H_2O$ (X_5) were selected by the software to make the model hierarchical.

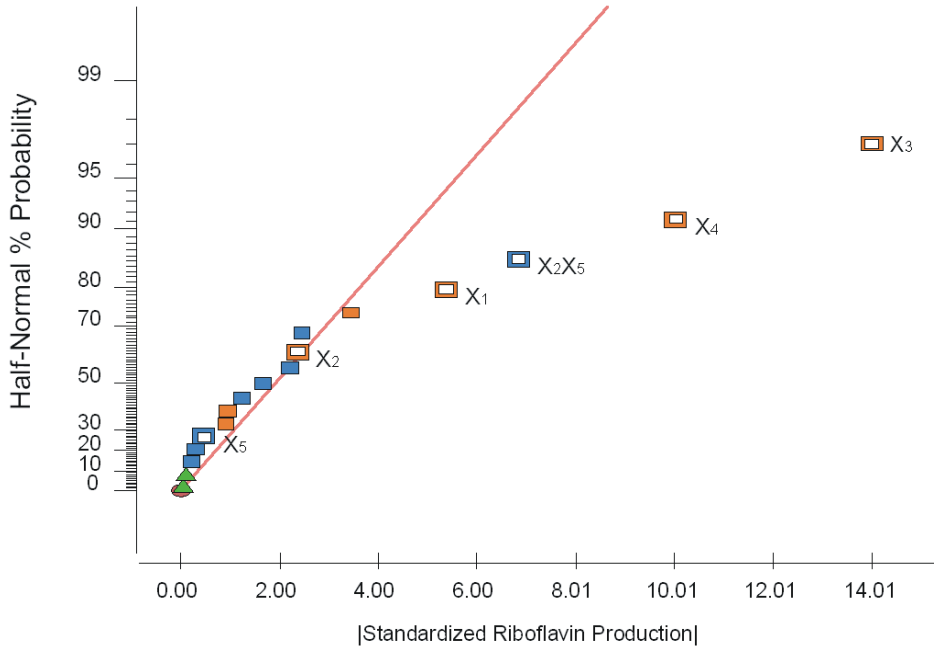


Figure 4.21 Half-Normal Plot indicating selection of factors X_1 (yeast extract), X_2 (peptone), X_3 (malt extract), X_4 (K_2HPO_4) and $MgSO_4.7H_2O$ (X_5) to be included in the mode that described riboflavin production in the second screening experiment with mutant *E. gossypii* EMS 30/1 in supplemented spent motor oil.

Effects of individual factors and mixture of components, ranked in order of significance were graphically illustrated in the Pareto Chart (Figure 4.22). Malt extract (X_3), followed by K_2HPO_4 (X_4) and peptone-$MgSO_4.7H_2O$ (X_2X_5) were considered definitely significant as they lay above the Bonferroni limit (red line). Amongst these factors, only (X_2X_5) peptone-$MgSO_4.7H_2O$ had a negative effect on the production of

riboflavin by mutant *E. gossypii* EMS 30/1 in this screening experiment. Yeast extract (X_1) significantly affected riboflavin production as it lay above the t-value limit.

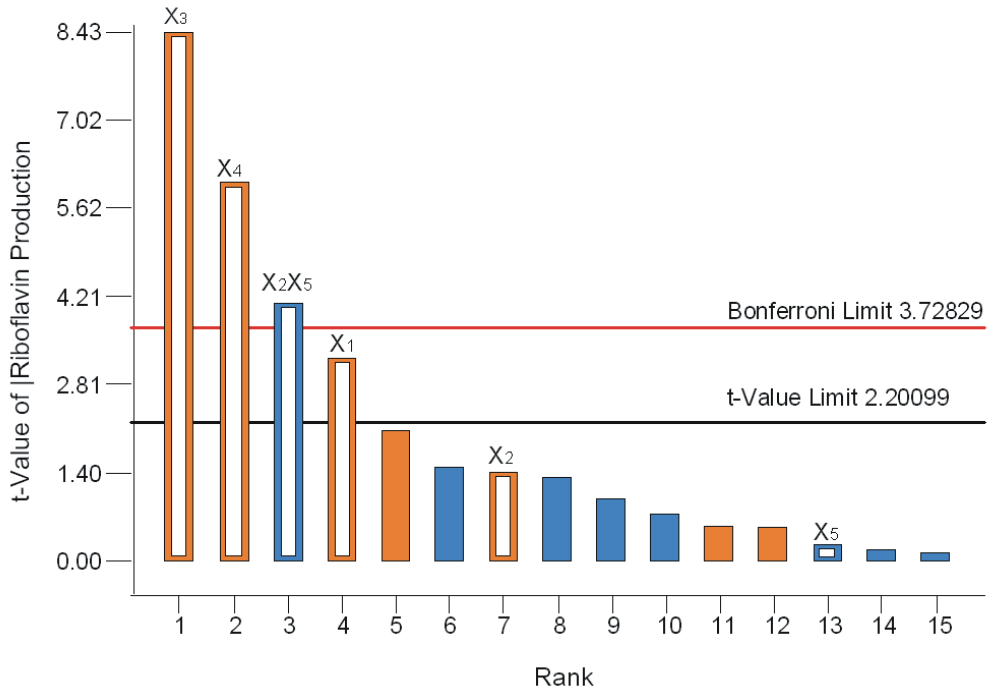


Figure 4.22 Pareto chart showing selection of factors (open boxes) X_1 (yeast extract), X_2 (peptone), X_3 (malt extract), X_4 (K_2HPO_4) and $MgSO_4.7H_2O$ (X_5) with their effects according to ranking in the second screening experiment for mutant *E. gossypii* EMS 30/1 in supplemented SMO. Positive effects are indicated in red while negative effects are shown in blue.

The model that was statistically significant to the data was determined by the F-value which was 22.81 (Table 4.10). This implied that there was only a 0.01% chance that a F-value this large could have occurred due to noise. All inferences from graphs were made with a 99.99% probability. Individual factors with *p*-values less than 0.05 were considered to have a statistically significant effect on riboflavin production in this experiment (in this case, the factors were malt extract (X_3), K_2HPO_4 (X_4) and (X_1) yeast extract). Mixtures of components with *p*-values less than 0.05, (X_2X_5; which is peptone-$MgSO_4.7H_2O$) also significantly affected riboflavin production by mutant *E.*

gossypii EMS 30/1 in supplemented SMO in this screening experiment. A 'Lack of Fit - value' of 3.91 implied that the Lack of Fit was not significant relative to pure error. There was a 41.64% chance that a 'Lack of Fit - value' this large could have occurred due to noise. A non-significant Lack of Fit meant that the model fitted the data.

Table 4.10 Analysis of Variance Table for second supplement screening for riboflavin production by *E. gossypii* EMS 30/1 in supplemented SMO

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance
Model	1513.5	6	252.25	22.81	<0.0001	significant
X_1-Yeast Extract	115.76	1	115.76	10.74	0.0079	significant
X_2-Peptone	22.34	1	22.34	2.02	0.1830	
X_3- Malt Extract	785.29	1	785.29	71.01	<0.0001	significant
X_4-K_2HPO_4	402.22	1	402.22	36.37	<0.0001	significant
X_5-$MgSO_4.7H_2O$	0.83	1	0.83	0.075	0.7892	
X_2X_5	187.06	1	187.06	16.92	0.0017	significant
Lack of Fit	115.1	9	12.79	3.91	0.2202	not significant

The model was based on individual effects and combinations of factors that were expressed by equation (4.2) and the derived mathematical model that described the second supplement screening experiment may be expressed by the equation that follows:

Final equation in terms of coded factors:

$$\text{Riboflavin concentration} = +17.70 + 2.69 * X_1 + 1.18 * X_2 + 7.01 * X_3 + 5.01 * X_4 - 0.23 X_5 - 3.42 * X_2 * X_5$$

where X_1 = yeast extract, X_2 = peptone, X_3 = malt extract, X_4 = K_2HPO_4 and X_5 = $MgSO_4.7H_2O$……..(4.2)

The main effects of the three most significant factors i.e. malt extract, K_2HPO_4 and yeast extract that contribute positively to riboflavin production by mutant *E. gossypii*

EMS 30/1 in supplemented spent motor oil during the second screening experiment are shown in Figure 4.23. The Cube plot illustrate maximum riboflavin production in the presence of maximum concentrations of all three factors and an improvement in production from 32.41 to 37.24 mg.l^{-1} was obtained by decreasing the concentrations of factors X_2 (peptone) and X_5 ($MgSO_4.7H_2O$) during statistical analysis using Design Expert 7.1.6. Since alteration of $MgSO_4.7H_2O$ had no effect on riboflavin production and appeared not significant in the Pareto chart, its concentration was set at its minimum (0.3 g.l^{-1}) in all future experimentation. Peptone (X_2) was not significant as it appeared below the t-value limit and was therefore set at its maximum concentration of 2.5 g.l^{-1}.

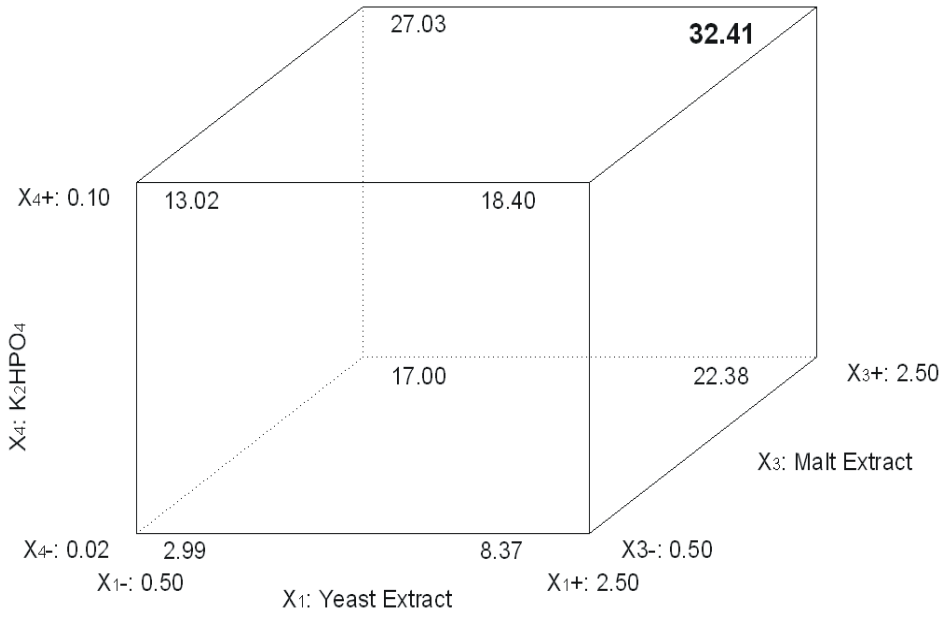


Figure 4.23 Interactions of the effects of the three most significant factors affecting riboflavin production by mutant *E. gossypii* EMS 30/1 in the second screening experiment. Concentration of peptone is 2.5 g.l^{-1} while that of $MgSO_4.7H_2O$ was 0.3 g.l^{-1}. The highest riboflavin concentration is shown in bold.

4.5.1.3 Third screening experiment for mutant *E. gossypii* EMS 30/1 in supplemented SMO

Concentrations of factors X_1 (yeast extract), X_3 (malt extract) and X_4 (K_2HPO_4) were increased as shown in the cube plot in Figure 4.25. They were increased from 0.5 - 2.5 $g.l^{-1}$ to 2.5 - 5.0 $g.l^{-1}$ while the concentration of factor X_4, was increased from 0.025 - 1.0 $g.l^{-1}$ to 0.1 - 0.2 $g.l^{-1}$ in this third screening experiment. Concentrations of peptone and $MgSO_4.7H_2O$ were set at concentrations of 2.5 $g.l^{-1}$ and 0.3 $g.l^{-1}$, respectively, as they were both not significant in the production of riboflavin in the second screening experiment. The three-factor, fractional factorial experimental design used for this third screening experiment, together with the recorded response at 120 hours, is summarised in Table 4.11.

Table 4.11 Riboflavin production in the third supplement screening, fractional factorial experiment for mutant *E. gossypii* EMS 30/1 using supplemented spent motor oil

Run	Yeast extract (X_1) ($g.l^{-1}$)	Malt extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	[Ribo] ($mg.l^{-1}$)
1	2.5	2.5	0.1	38.04
2	5	2.5	0.1	18.13
3	2.5	5	0.1	47.32
4	5	5	0.1	20.81
5	2.5	2.5	0.2	46.07
6	5	2.5	0.2	24.43
7	2.5	5	0.2	55.60
8	5	5	0.2	66.27
9	3.75	3.75	0.15	24.42
10	3.75	3.75	0.15	26.40
11	3.75	3.75	0.15	24.05

The response ranges from 18.13 $mg.l^{-1}$ to 66.27 $mg.l^{-1}$ which resulted in the ratio of maximum to minimum yields of 3.65487. This implied that no transformation of data was required. All individual components and combinations of components were

selected to be included in the model as they were all positioned away from the line in the Half-Normal plot (Figure 4.24).

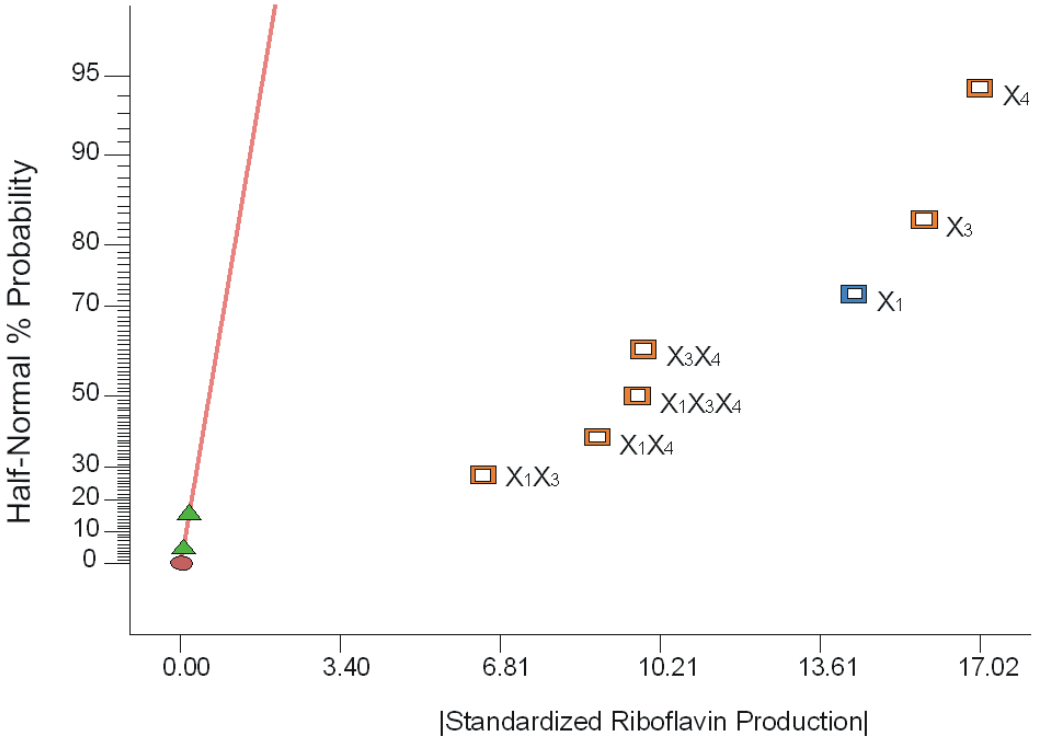


Figure 4.24 Selection of factors included in the model that described the third screening experiment for mutant *E. gossypii* EMS 30/1 in supplemented SMO.

A graphic illustration of standardised riboflavin production of the individual and combined factors on riboflavin production in this third screening experiment were shown in the Pareto chart (Figure 4.25). The Pareto chart which ranked the individual factors and combined factors according to the largest effect on the production of riboflavin by mutant *E. gossypii* in the third screening experiment assessed effects of combined factors thereby enabling one to make better decisions about their effects on each other. K_2HPO_4 (X_4), followed by malt extract (X_3) then yeast extract (X_1), were definitely significant as they lay above the Bonferroni limit, while, combined factors X_3X_4, $X_1X_3X_4$, X_1X_4 and X_1X_3, were also significant as they lay above the t-value limit.

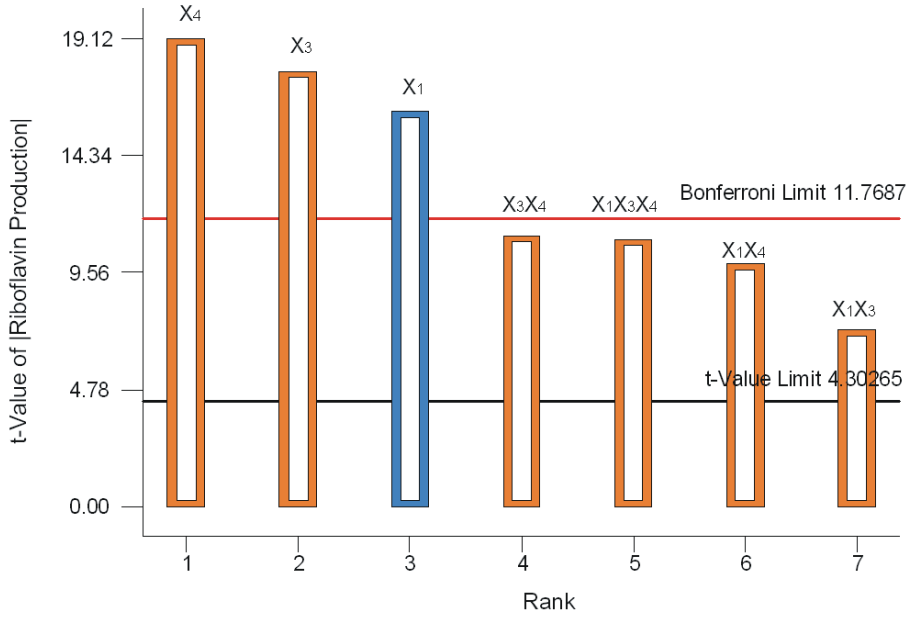


Figure 4.25 Pareto Chart for the third screening experiment. Factors X_1, X_3 and X_4 represents yeast extract, malt extract and K_2HPO_4, respectively, with positive effects in red and negative effects in blue.

A model with an F-value of 190.71, was based on the effects of individual and combination of components described by the mathematical equation below. There was only a 0.52% chance that an F-value this large could have occurred due to noise (Table 4.12). Factors with Prob>F -values less than 0.05 indicated model terms that were significant, (X_4, X_3, X_1, X_3X_4, $X_1X_3X_4$, X_1X_4 and X_1X_3). The derived equation was statistically significant and all inferences made from the graphs drawn, were significant to 99.48% probability.

Table 4.12 Analysis of variance table for the third screening experiment

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	Significance
Model	3.384E + 005	7	3.384E + 0.005	190.72	0.0052	significant
Yeast extract (X_1)	6.587E + 005	1	6.587E + 0.005	259.84	0.0038	significant
Malt extract (X_3)	8.021E + 005	1	8.021E + 0.005	316.42	0.0031	significant
K_2HPO_4 (X_4)	9.266E + 005	1	9.266E + 0.005	365.53	0.0027	significant
X_1X_3	1.323E + 005	1	1.323E + 0.005	52.19	0.0186	significant
X_1X_4	2.513E + 005	1	2.513E + 0.005	99.14	0.0091	significant
X_3X_4	3.105E + 005	1	3.105E + 0.005	122.51	0.0081	significant
$X_1X_3X_4$	3.027E + 005	1	3.027E + 0.005	119.4	0.0083	significant

Final equation in terms of coded factors:

Riboflavin concentration = + 39.59 - 7.17 * X_1 + 7.92 * X_3 + 8.51 * X_4 + 3.21 * X_1 * X_3 + 4.43 *X_1* X_4+ 4.93 * X_3 * X_4+ 4.86 * X_1 * X_3* X_4

where X_1= yeast extract, X_3= malt extract and X_4 = K_2HPO_4……(4.3)

Effects of factors yeast extract, malt extract and K_2HPO_4 are illustrated in the cube plot (Figure 4.26). Maximum riboflavin yields of 66.27 mg.l^{-1} were obtained in the presence of 5.0 g.l^{-1} yeast extract, 0.20 g.l^{-1} K_2HPO_4 and 5.0 g.l^{-1} malt extract. Among the three factors, yeast extract had the smallest effect which was negative on riboflavin production in this third screening experiment and was therefore set at 5.0 g.l^{-1}. The other factors were then considered the two most significant factors affecting riboflavin production by mutant *E. gossypii* EMS 30/1 in supplemented SMO and were optimized using response surface methodology, based on a central composite design.

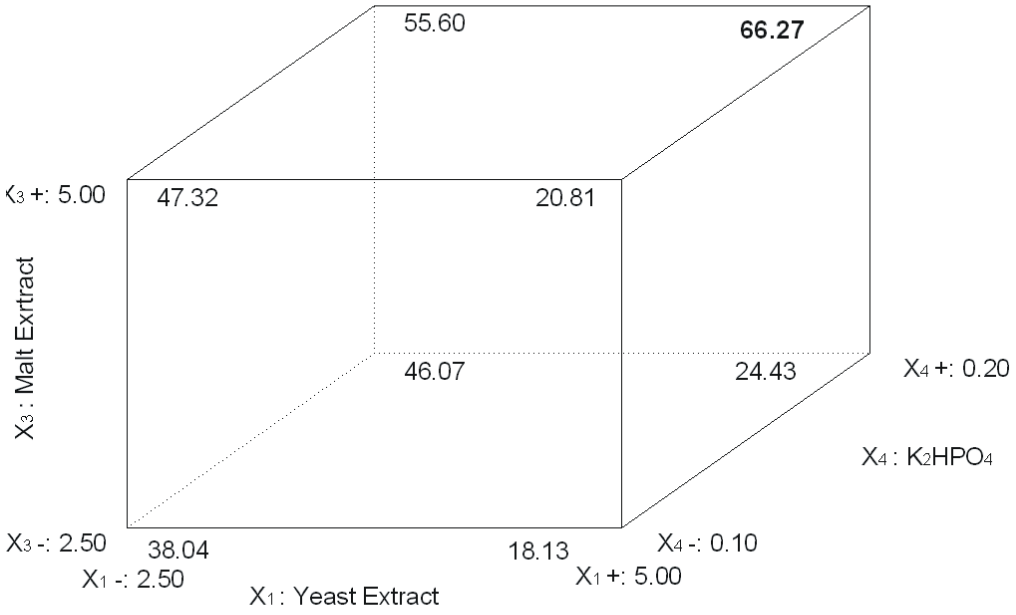


Figure 4.26 Interaction of effects of factors yeast extract, malt extract and K_2HPO_4 in the third screening experiment for mutant *E. gossypii* EMS 30/1 in supplemented SMO. Maximum riboflavin concentration is shown in bold.

4.5.2 Optimization of Riboflavin Production by Response Surface Methodology

The two most significant nutrient factors malt extract and K_2HPO_4 were optimized in the central composite experiment in order to develop an optimized medium for riboflavin production by mutant *E. gossypii* EMS 30/1 in supplemented SMO.

4.5.2.1 Optimization Experiment for Riboflavin Production by mutant *E. gossypii* EMS 30/1 in Supplemented SMO

This experiment was designed in order to optimize malt extract and K_2HPO_4 nutrient supplements which were investigated at concentration ranges between 1.0 to 5.0 $g.l^{-1}$ and 0.1 to 0.18 $g.l^{-1}$, respectively. The response ranged from 12.04 $mg.l^{-1}$ to 97.44 $mg.l^{-1}$ riboflavin which resulted in a maximum is to minimum ratio of 8.09128. This implied

that no transformation of data was required in developing a model to describe this process. Analysis of Variance (ANOVA) table below indicates a significant model with a *p*-value of <0.0001.

Table 4.13 Riboflavin produced by mutant *E. gossypii* EMS 30/1 in supplemented SMO in the central composite experiment recorded at 120 hours

Run	Malt Extract (X_3) ($mg.l^{-1}$)	K_2HPO_4 (X_4) ($mg.l^{-1}$)	[Ribo] ($mg.l^{-1}$)
1	1	0.1	45.57
2	5	0.1	72.05
3	1	0.18	30.97
4	5	0.18	97.44
5	0.17	0.14	12.04
6	5.83	0.14	71.44
7	3	0.18	74.94
8	3	0.2	69.21
9	3	0.14	72.48
10	3	0.14	80.98
11	3	0.14	81.89
12	3	0.14	81.53
13	3	0.14	83.82

The developed model for the data with an F-value of 52.69 (Table 4.14) was statistically significant with only a 0.01% chance that a 'Model F-value' this large could have occurred due to noise. Values of 'Prob>F' less than 0.05 indicate model terms are significant and in this case malt extract (X_3), X_3X_4 and X_3^2 were significant. The 'Lack of Fit-value' of 1.66 implied that Lack of Fit was not significant due to pure error. There was a 31.09% chance that a 'Lack of Fit' this large could have occurred due to noise.

Table 4.14 Analysis of variance table for the central composite experiment for mutant *E. gossypii* EMS 30/1 in SMO

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	Significance
model	6583.64	5	1316.73	52.69	<0.0001	significant
X_3- Malt extract	3914.41	1	3914.41	156.64	<0.0001	significant
X_4 -K_2HPO_4	0.9	1	0.9	0.036	0.8548	
X_3X_4	399.69	1	399.69	15.99	0.0052	significant
X_3^2	2266.65	1	2266.65	90.7	<0.0001	significant
X_4^2	57.87	1	57.87	2.32	0.1719	
Lack of Fit	97.03	3	32.34	1.66	0.3109	not significant

The final equation in terms of coded factors:

$$\text{Riboflavin concentration} = +80.15 + 22.12 * X_3 + 0.34 * X_4 + 10.00 * X_3 * X_4 - 18.05 * X_4^2 - 2.88 * X_4^2$$

where X_3 = malt extract and X_4 = K_2HPO_4……(4.5)

The equation may be used to predict riboflavin concentrations by mutant *E. gossypii* EMS 30/1 in supplemented SMO at a predicted confidence of 99.99%. Data from the central composite experiment was analysed to develop the optimized riboflavin producing medium for mutant *E. gossypii* EMS 30/1 using SMO as a carbon source. The interaction between these two nutrient supplements may be viewed in the response surface plot in Figure 4.27.

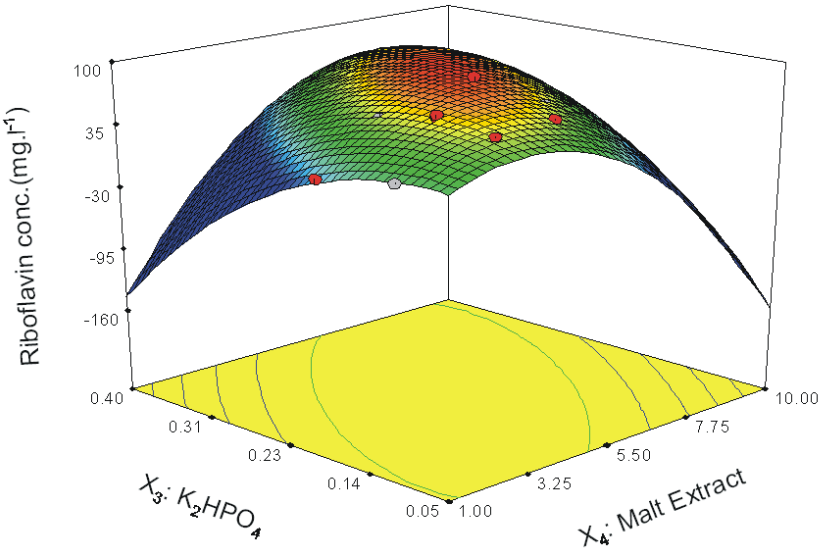


Figure 4.27 Response surface plot showing the relative effect of the two most important nutrient supplements (K_2HPO_4 and malt extract) affecting riboflavin production by mutant *E. gossypii* EMS 30/1, while keeping other supplement at constant levels in the central composite experiment.

The point at the centre of Figure 4.27 was calculated using a Design Expert optimization algorithm on the model given in equation 4.5. Four solutions were found and the solution giving the highest riboflavin concentration together with the other optimal media components was used (Table 4.15)

Table 4.15 Central composite point prediction for maximum riboflavin production using yeast extract and K_2HPO_4

Response	SE Mean	95% CI low	95% CI high	Malt Extract	K_2HPO_4
91.88	3.62	83.30	100.46	4.78	0.18
91.88	3.66	83.22	100.54	4.80	0.18
91.86	3.55	83.47	100.27	4.72	0.18
91.84	3.75	82.95	100.73	4.87	0.18

4.5.3 Verification Experiment for Riboflavin Production by Mutant *E. gossypii* 30/1 in Supplemented SMO

A confirmatory experiment using the optimized riboflavin-producing medium was conducted in triplicate in order to verify the results obtained. The results were confirmed in the verification experiment in which the maximum riboflavin yield was 103.59 $mg.l^{-1}$ that was obtained after 120 h (Figure 4.28), resulting in an increase of 406% in production yield, in comparison to yields obtained prior to the application of statistical experimental designs (20.44 $mg.l^{-1}$).

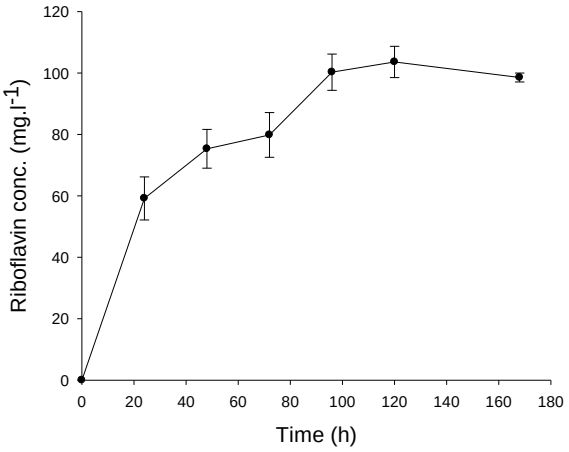


Figure 4.28 Riboflavin produced by mutant *E. gossypii* EMS 30/1 using the optimized supplemented SMO medium in the verification experiment.

4.6 *Candida famata* IN SUPPLEMENTED SPENT VEGETABLE OIL

Comparison of riboflavin yields by the five selected fungi in supplemented spent vegetable oil (SVO) in Figure 4.17 clearly indicated that *C. famata* produced the highest concentration of vitamin B_2 in supplemented vegetable oil. An attempt to optimize riboflavin production was accomplished by investigating the effects of the five nutrient supplements included in the O and K medium by applying statistical

experimental design with the aid of the statistical software, Design Expert Version 7.1.6 (Statease, USA). Four screening fractional factorial experiments were conducted, at the end of which the two most significant factors affecting riboflavin production were identified. These factors were optimized using response surface methodology based on central composite experiments. The optimized medium was confirmed in a verification experiment.

4.6.1 Screening for Effects of Nutrient Supplements by Fractional Factorial Experimental design

4.6.1.1 First supplement screening of riboflavin production by *C. famata* in SVO

Spent vegetable oil, alone as a medium, was insufficient for riboflavin production and was therefore supplemented with nutrients present in the O and K medium. The first screening experiment was designed with the high and low concentration levels being set at 50% higher and 50% lower than that of the O and K medium components. Comparison of riboflavin yields by *C. famata* in the first supplement screening experiment indicated that production of this vitamin was maximal after 120 hours. Therefore, data recorded at this time interval was used for all future experiments. The response recorded at 120 hours (Table 4.16) was statistically analysed using Design Expert 7.1.6 (Statease, USA).

The ratio of maximum to minimum yield was calculated and since a denominator of 0 is undefined, the minimum concentration of 0 $mg.l^{-1}$ recorded for run 12 was substituted with a negligible response of 0.1 $mg.l^{-1}$ to enable mathematical division. Data recorded at 120 hours for run 17 was ignored as it appeared incorrect in comparison to runs 18 and 19 all of which were replicated experiments (center points). A Gram stain showed that this run was contaminated with an unknown bacterial organism. Response ranges from 0.1 $mg.l^{-1}$ to 87.31 $mg.l^{-1}$ riboflavin, resulting in a ratio of maximum to minimum yields of 873.199, which implied that transformation of

the data was necessary. Therefore a square-root transformation was applied to the data in order to fit a model with significantly high significance to draw conclusions.

Table 4.16 Riboflavin production in the first supplement screening experiment for *C. famata* in supplemented SVO

Run	Yeast Extract (X_1) ($g.l^{-1}$)	Peptone (X_2) ($g.l^{-1}$)	Malt Extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	$MgSO_4.7H_2O$ (X_5) ($g.l^{-1}$)	[Ribo] ($mg.l^{-1}$)
1	2.5	2.5	2.5	0.1	0.3	20.42
2	7.5	2.5	2.5	0.1	0.1	0.1
3	2.5	7.5	2.5	0.1	0.1	12.07
4	7.5	7.5	2.5	0.1	0.3	26.07
5	2.5	2.5	7.5	0.1	0.1	34.36
6	7.5	2.5	7.5	0.1	0.3	34.97
7	2.5	7.5	7.5	0.1	0.3	41.54
8	7.5	7.5	7.5	0.1	0.1	64.99
9	2.5	2.5	2.5	0.3	0.1	11.55
10	7.5	2.5	2.5	0.3	0.3	22.70
11	2.5	7.5	2.5	0.3	0.3	10.89
12	7.5	7.5	2.5	0.3	0.1	0.1
13	2.5	2.5	7.5	0.3	0.3	10.94
14	7.5	2.5	7.5	0.3	0.1	41.18
15	2.5	7.5	7.5	0.3	0.1	87.31
16	7.5	7.5	7.5	0.3	0.3	41.54
17	5	5	5	0.2	0.2	2.961
18	5	5	5	0.2	0.2	20.26
19	5	5	5	0.2	0.2	20.99

Half-Normal plot (Figure 4.29) illustrates the selection of factors to be included in the model. Factors malt extract (X_3), malt extract-$MgSO_4.7H_2O$ (X_3X_5), yeast extract-$MgSO_4.7H_2O$ (X_1X_5), peptone-malt extract (X_2X_3) and peptone (X_2) were initially selected. Factors X_5, X_1 and a combination of X_1X_3, were selected to make the model hierarchical (Figure 4.29).

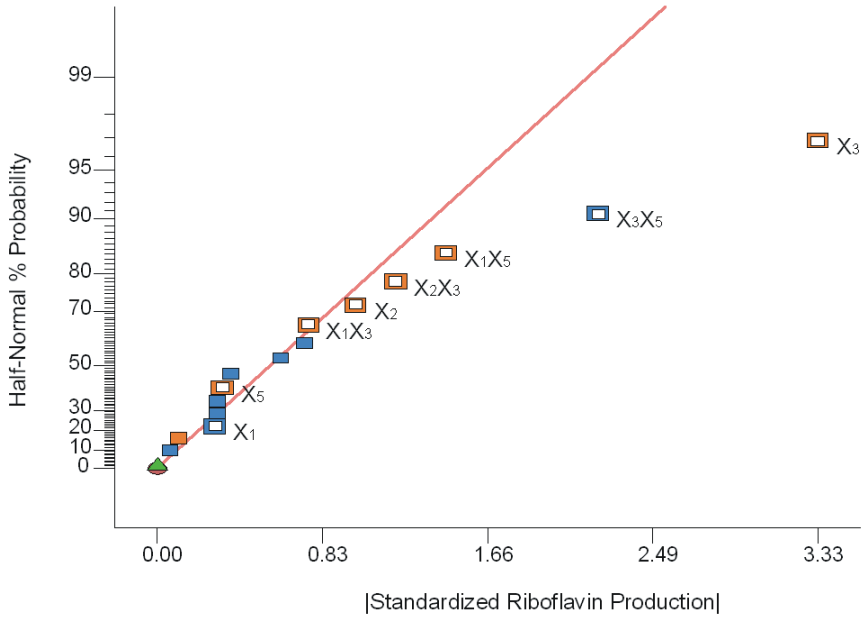


Figure 4.29 Selection of factors that was included in the mathematical model that described riboflavin production by *C. famata* in spent vegetable oil in the first supplement screening experiment.

A more detailed illustration of factor selection may be viewed in the Pareto chart (Figure 4.30) that illustrated the effects of individual factors and combined factors affecting riboflavin production in this experiment. Those factors positioned above the Bonferroni limit, in this case malt extract (X_3) and malt extract-$MgSO_4.7H_2O$(X_3X_5), were definitely significant. Combination of factors yeast extract-$MgSO_4.7H_2O$ (X_1X_5) and peptone-malt extract (X_2X_3) factors and yeast extract on its own were significant as they lay above the *t*-value limit. They were all included in the model with non-significant X_1X_3, X_5 and X_1 selected to make the model hierarchical.

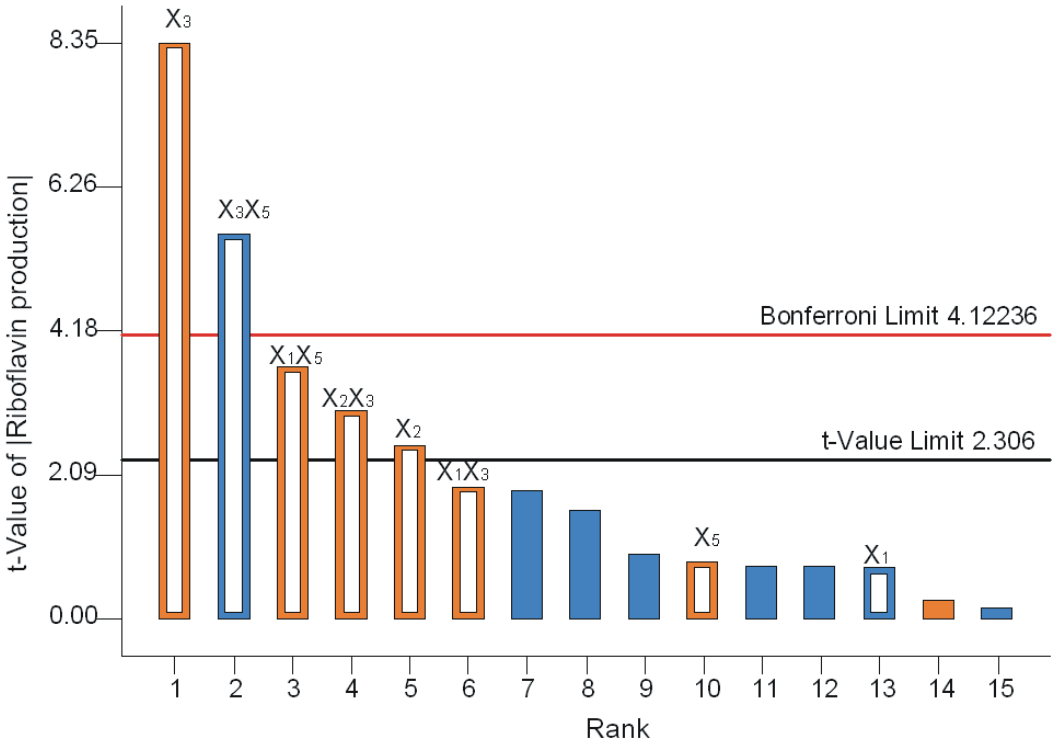


Figure 4.30 Pareto chart graphically indicting effects of the factors affecting riboflavin production by *C. famata* in SVO for the first screening experiment, in order of significance. Factors included in the model are shown as open boxes. Positive effects are in red while negative effects are in blue. X_1, X_2, X_3, and X_5 represent yeast extract, peptone, malt extract and $MgSO_4.7H_2O$, respectively.

The Box-Cox Plot for power transformation (Figure 4.31) suggested that the current square-root transformation (blue line) set on Lambda was positioned at 0.5 which was 0.05 away from the best transformation indicated by the green line. Furthermore, the transformation also lay within the optimal zone, which is between the 95% confidence interval limits positioned between 0.23 and 0.88. The model was therefore considered satisfactory for further analysis and hypothesis testing.

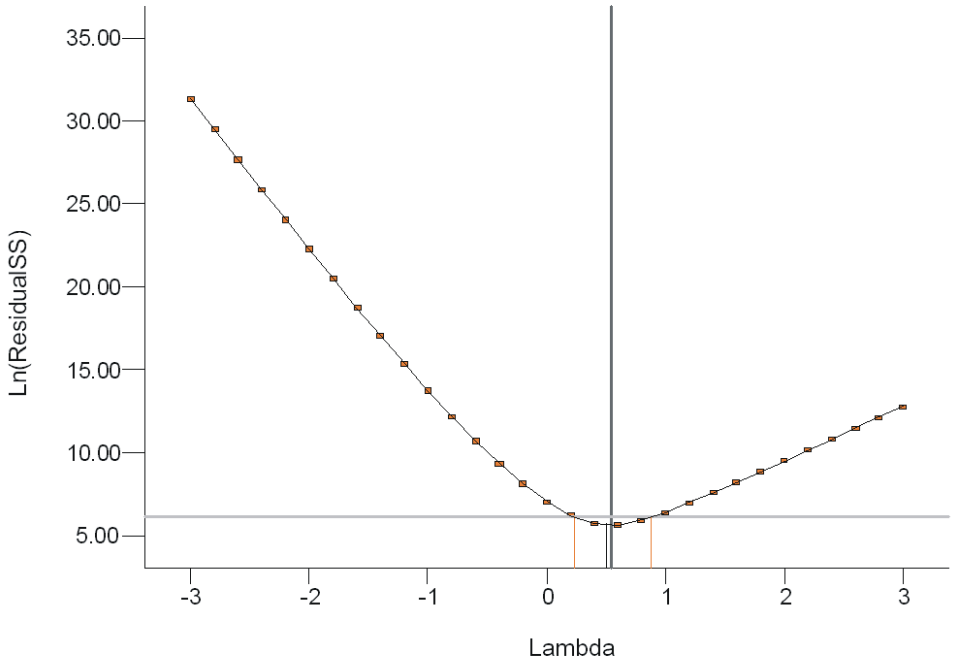


Figure 4.31 Box-Cox plot that evaluates the model for the first screening experiment for the production of riboflavin by *C. famata* in SVO. The blue line indicates the current transformation of the model.

The model F-Value of 16.80 implied that the model was statistically significant (Table 4.17). There was only a 0.03% chance that an F-value this large could have occurred due to noise. Factors with values of 'Prob>F' less than 0.05, in this case X_2, X_3, X_1X_5, X_2X_3 and X_3X_5, indicate the model terms that are significant. Individual factor X_5 ($MgSO_4.7H_2O$) with a p-value of 0.4357 was not significant but was selected because it was present in two significant interactions, X_1X_5 and X_3X_5. The 'Lack of Fit' of 220.09 implied that there was a 5.19% chance that a Lack of Fit this large could have occurred due to noise. A non-significant Lack of Fit implies that the model fits the data.

Table 4.17 Analysis of variance table for the first screening experiment for *C. famata* in SVO

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	Significance
Model	85.19	8	10.65	16.8	0.0003	significant
X_1-Yeast Extract	0.33	1	0.33	0.53	0.4885	
X_2-Peptone	4	1	4	6.3	0.0364	significant
X_3- Malt Extract	44.23	1	44.23	69.76	<0.0001	significant
X_5-$MgSO_4{\cdot}7{\cdot}H_2O$	0.43	1	0.43	0.67	0.4357	
X_1X_3	2.3	1	2.3	3.63	0.0932	significant
X_1X_5	8.44	1	8.44	13.32	0.0065	significant
X_2X_3	5.75	1	5.75	9.07	0.0168	significant
X_3X_5	19.71	1	19.71	31.09	0.001	significant
Lack of Fit	5.07	7	0.72	220.09	0.0519	not significant

The final equation in terms of coded factors:

Square root (Ribo. Conc) = +4.81 - 0.14 *X_1 + 0.50 *X_2 + 1.66 * X_3 + 0.16 *X_5 + 0.73

*X_1*X_5 + 0.60 *X_2*X_3 - 1.11 *X_3*X_5……(4.6)

where X_1 = yeast extract, X_2 = peptone, X_3 = malt extract and X_5 = $MgSO_4{\cdot}7{\cdot}H_2O$

The cube plot (Figure 4.32) indicated that maximum riboflavin was produced in the presence of maximum concentrations of peptone and malt extract and a minimum concentration of $MgSO_4.7H_2O$. A maximum riboflavin concentration of 72.60 $mg.l^{-1}$ was produced in the presence of these conditions and that of yeast extract and K_2HPO_4 which were 5.00 and 0.2 $g.l^{-1}$, respectively. An improvement in product yield from 72.60 to 81.23 $mg.l^{-1}$ was observed by decreasing the concentrations of both yeast extract and K_2HPO_4 to 2.50 and 0.1 $g.l^{-1}$, respectively.

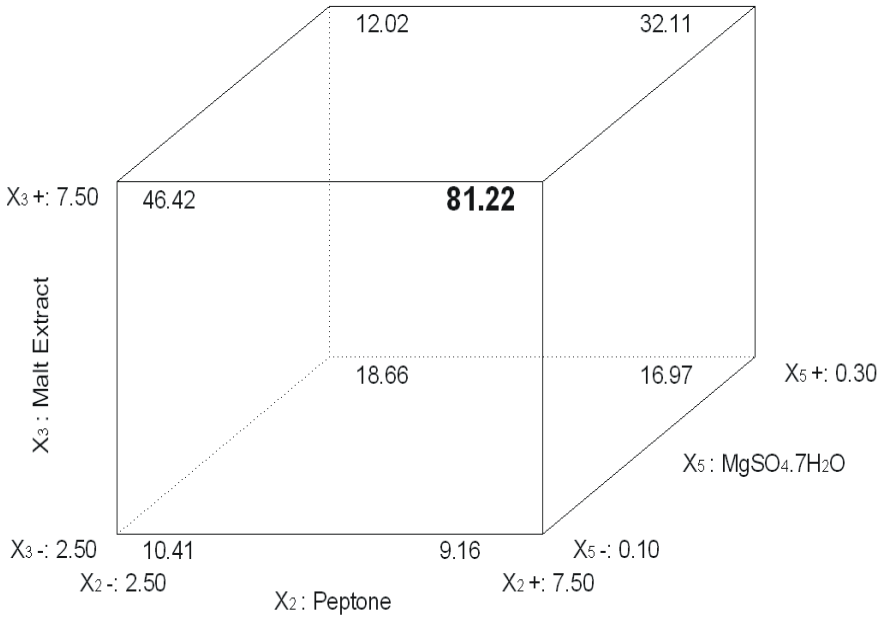


Figure 4.32 Interactions of the three most significant factors affecting riboflavin production in the first screening experiment. Maximum riboflavin production is shown in bold.

4.6.1.2 Second supplement screening for riboflavin production by *C. famata* in Supplemented SVO

Following the directions in which concentration changes should be made to improve riboflavin production by *C. famata* in SVO, the second supplement screening experiment was designed. The concentration of yeast extract (X_1) was decreased from 2.5 - 7.5 g.l^{-1} to 0.5 - 2.5 g.l^{-1} and that of peptone (X_2) and malt extract (X_3) were increased from 2.5 - 7.5 g.l^{-1} to 7.5 - 12.5 g.l^{-1}. The concentration of K_2HPO_4 (X_4) was also increased from 0.1 - 0.3 g.l^{-1} to 0.3 - 0.5 g.l^{-1} and that of $MgSO_4.7H_2O$ (X_5) was decreased from 0.1- 0.3 g.l^{-1} to 0.025 - 0.1 g.l^{-1}. The response recorded at 120 hours (Table 4.18) was statistically analysed. The response ranges from 0.1 mg.l^{-1} to 29.0949 mg.l^{-1}, resulting in a ratio of maximum to minimum yields of 290.949, which implied

that a transformation of the data was required; therefore a square-root transformation applied to the data in order to develop a sufficiently significant model from which conclusions could be made.

Table 4.18 Riboflavin production by *C. famata* in the second supplement screening experiment in supplemented SVO

Run	Yeast Extract (X_1) ($g.l^{-1}$)	Peptone (X_2) ($g.l^{-1}$)	Malt Extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	$MgSO_4.7H_2O$ (X_5) ($g.l^{-1}$)	[Ribo] ($mg.l^{-1}$)
1	0.5	7.5	7.5	0.3	0.1	29.09
2	2.5	7.5	7.5	0.3	0.02	3.54
3	0.5	12.5	7.5	0.3	0.02	6.48
4	2.5	12.5	7.5	0.3	0.1	4.47
5	0.5	7.5	12.5	0.3	0.02	9.90
6	2.5	7.5	12.5	0.3	0.1	8.22
7	0.5	12.5	12.5	0.3	0.1	4.15
8	2.5	12.5	12.5	0.3	0.02	5.60
9	0.5	7.5	7.5	0.5	0.02	0.67
10	2.5	7.5	7.5	0.5	0.1	23.62
11	0.5	12.5	7.5	0.5	0.1	0.1
12	2.5	12.5	7.5	0.5	0.02	6.32
13	0.5	7.5	12.5	0.5	0.1	8.23
14	2.5	7.5	12.5	0.5	0.02	9.27
15	0.5	12.5	12.5	0.5	0.02	8.52
16	2.5	12.5	12.5	0.5	0.1	8.18
17	1.5	10	10	0.4	0.06	8.40
18	1.5	10	10	0.4	0.06	9.71
19	1.5	10	10	0.4	0.06	11.25

Selection of factors to be included in the model was illustrated in the Half-Normal plot (Figure 4.33). A combination of factors yeast extract-K_2HPO_4 (X_1X_4), peptone-$MgSO_4.7H_2O$ (X_2X_5) and individual factor peptone (X_2) were included in the model.

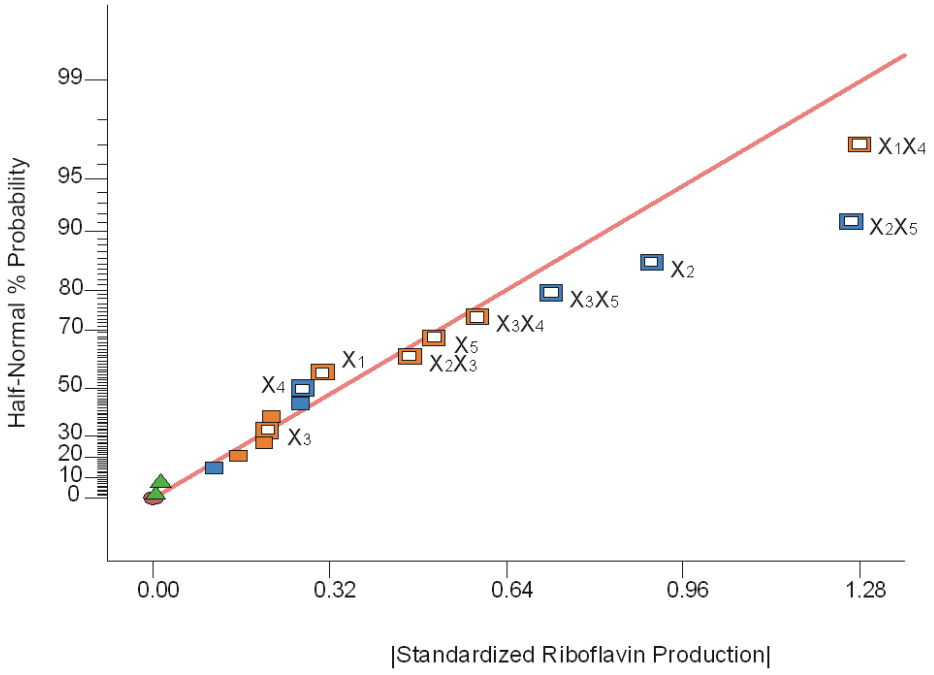


Figure 4.33 Half-Normal plot illustrating the selection of factors to be included in the model for the second screening experiment for *C. famata* in SVO.

The Box-Cox plot (Figure 4.34) for power transformations suggested that the developed model with the square-root transformation being set on 'Lambda' (blue line) positioned at 0.5, only 0.26 away from the best transformation (green line), was significant, and was therefore considered satisfactory for analysis and forthcoming hypothesis testing. Furthermore, this transformation lay within the optimal zone which lay between the 95% confidence interval limit represented by the red lines positioned at 0.35 and 1.23.

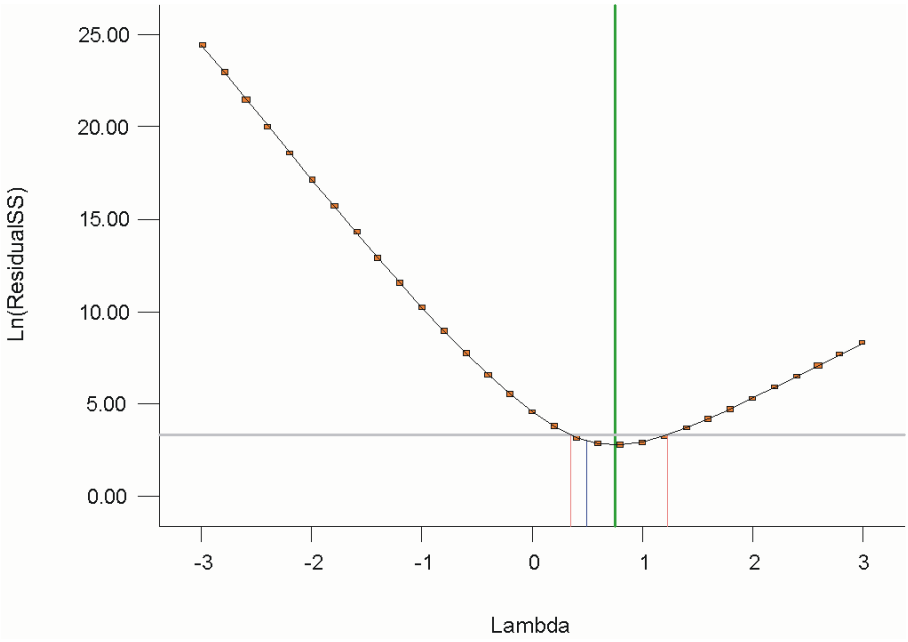


Figure 4.34 Box-Cox plot used to evaluate the model that described the production of riboflavin by *C. famata* in SVO in the second supplement screening experiment.

The effects of individual and combined factors were expressed in the Pareto chart (Figure 4.35). These effects are ranked in order of significance. The factors located above the Bonferroni limit are definitely significant, in this case yeast extract-K_2HPO_4 (X_1X_4), peptone-$MgSO_4.7H_2O$ (X_2X_5) and peptone itself (X_2), while those lying above the *t*-value Limit, are possibly significant, and should be included in the model malt extract-$MgSO_4.7H_2O$ (X_3X_5), malt extract- K_2HPO_4(X_3X_4), $MgSO_4.7H_2O$ and (X_2X_3) peptone-malt extract.

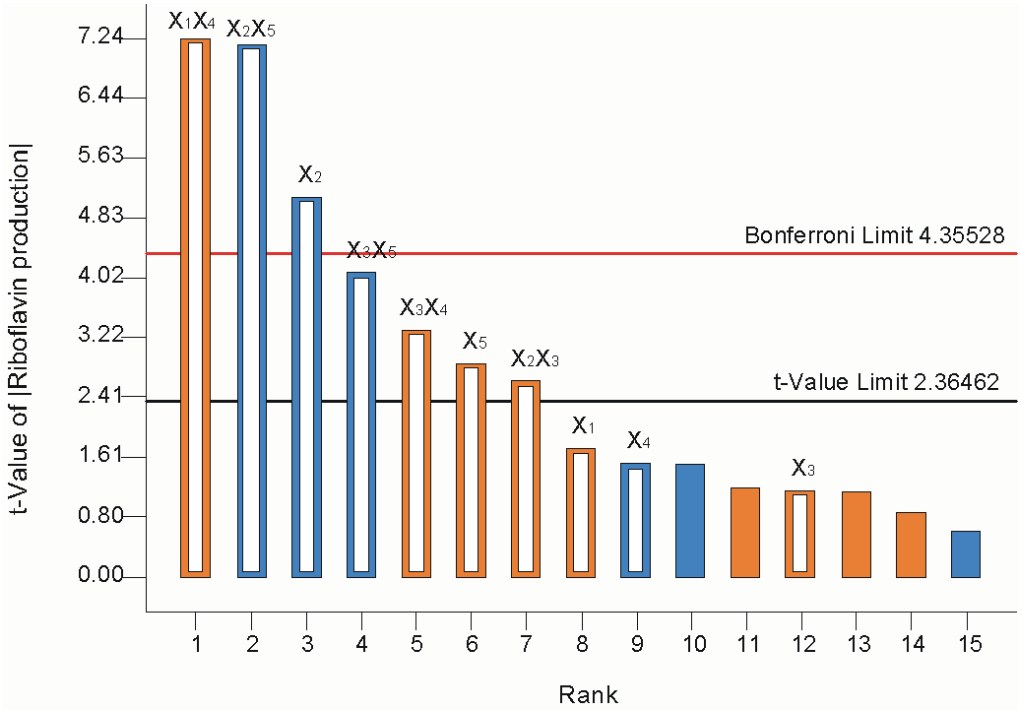


Figure 4.35 Pareto chart illustrating the effects of individual and combination of factors on riboflavin production by *C. famata* in SVO in the second screening experiment. Factors with positive effects are indicated in red while those with negative effects are shown in blue. Selected factors that are included in the model are represented by open boxes.

The model's F-value of 17.95 was statistically significant (Table 4.19). There was only a 0.05% chance that a model this large could have occurred due to noise. Factors with *p*-values less than 0.05 indicate model terms that are significant. In this case, individual factors peptone (X_2), $MgSO_4.7H_2O$ (X_5) and combination of factors peptone-malt extract (X_2X_3), peptone-$MgSO_4.7H_2O$ (X_2X_5), malt extract-K_2HPO_4 (X_3X_4) and malt extract- $MgSO_4.7H_2O$ (X_3X_5) were selected as they were significant model terms. Yeast extract (X_1) with a *p*-value of 0.1269 was not significant to riboflavin production in this experiment, but the interaction with X_4, produced the largest positive effect, therefore, it was selected. A model with a "Lack of Fit" value of 2.96 implied that the Lack of Fit was not significant due to pure error. There was a 27.18% chance that a Lack of Fit this large could have occurred due to noise. A not significant model Lack of Fit implies that the model fits the data.

Table 4.19 Analysis of variance table for the second supplement screening experiment for *C. famata* in supplemented SVO

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	Significance
Model	22.4	10	2.24	17.95	0.001	significant
X_1-Yeast Extract	0.37	1	0.37	3	0.1269	
X_2-Peptone	3.26	1	3.26	26.12	0.0014	significant
X_3- Malt Extract	0.17	1	0.17	1.37	0.2799	
X_4- K_2HPO_4	0.29		0.29	2.34	0.1702	
X_5-$MgSO_4.7H_2O$	1.03	1	1.03	8.29	0.0237	significant
X_1X_4	6.55	1	6.55	52.47	0	significant
X_2X_3	0.86	1	0.86	6.91	0.034	significant
X_2X_5	6.4	1	6.4	51.28	0	significant
X_3X_4	1.37	1	1.37	11.02	0.0128	significant
X_3X_5	2.08	1	2.08	16.67	0.0047	significant
Lack of Fit	0.77	5	0.15	2.96	0.2718	not significant

The mathematical equation that describes this experiment may be written as follows:

$$\text{Square root (Riboflavin concentration)} = +2.66 + 0.15*X_1 - 0.35*X_2 + 0.10*X_3 - 0.13*X_4 + 0.25*X_5 + 0.64*X_1*X_4 + 0.23*X_2*X_3 - 0.63*X_2*X_5 + 0.29*X_3*X_4 - 0.36*X_3*X_5$$

where X_1 = yeast extract, X_2 = peptone, X_3 = malt extract, X_4 = K_2HPO_4 and X_5 = $MgSO_4.7H_2O$ ….....…….....…….(4.7)

Equation 4.7 significantly fits the data and all inferences and predictions made from the graphs drawn based on this model are significant to 99.95%. Maximum riboflavin production of 14.21 $mg.l^{-1}$ was produced in the presence of maximum concentration $MgSO_4.7H_2O$, and minimum concentrations of peptone and yeast extract while the concentrations of malt extract and K_2HPO_4 were set at 10.0 $g.l^{-1}$ and 0.4 $g.l^{-1}$, respectively. An increase in product yield from 14.21 to 29.19 $mg.l^{-1}$ was observed when the concentrations of malt extract and K_2HPO_4 were decreased to 7.5 $g.l^{-1}$ and 0.3

g.l^{-1}, respectively (Figure 4.36). These conclusions contributed towards changes made when the third supplement screening experiment was designed.

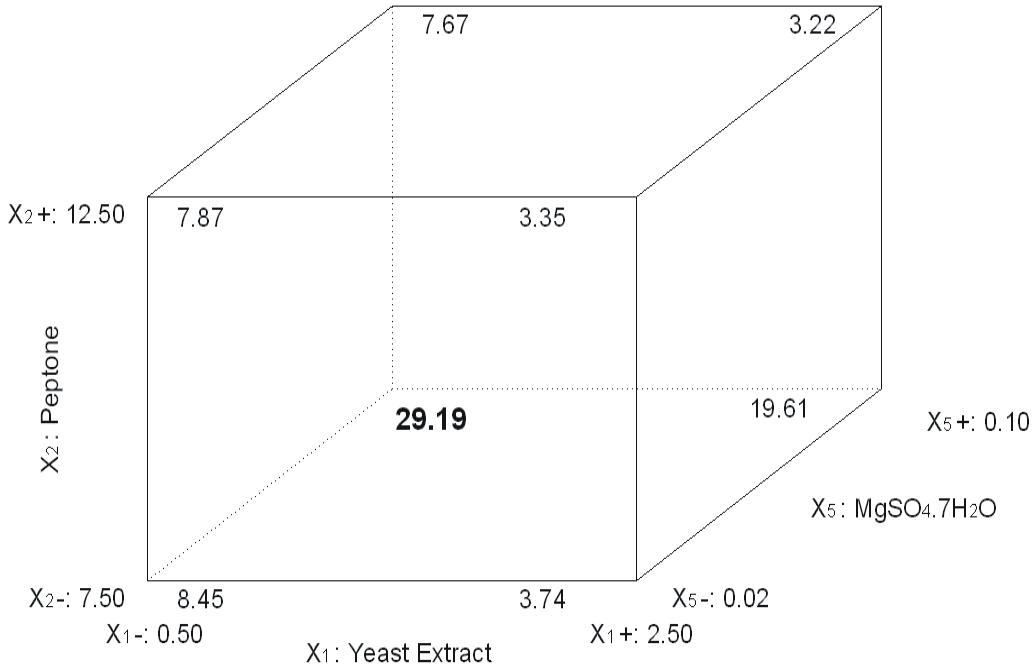


Figure 4.36 Effects of the three most significant factors affecting riboflavin production by *C. famata* in SVO in the second supplement screening experiment. Maximum riboflavin concentration is shown in bold.

4.6.1.3 Third supplement screening for riboflavin production by *C. famata* in Supplemented SVO

The third screening experiment was designed according to predictions made from equation (4.7) that described the second supplement screening experiment. The concentration settings of yeast extract, peptone, malt extract and K_2HPO_4 were decreased from 0.5- 2.5 g.l^{-1} to 0- 0.5 g.l^{-1} and 7.5 - 12.5 to 6.5 - 8.5 g.l^{-1}, 7.5 - 12.5 to

6.5- 8.5 $g.l^{-1}$ and 0.3 - 0.5 to 0.25 - 0.35 $g.l^{-1}$, respectively. The concentration of factor X_5 ($MgSO_4.7H_2O$) was increased from 0.02 - 0.1 $g.l^{-1}$ to 0.05 - 0.15 $g.l^{-1}$. The experimental design together with response data recorded at 120 hours is summarised in Table 4.20. Runs 3 and 13 were ignored during the analysis as they were contaminated. The response ranged from 1.83 $mg.l^{-1}$ to 15.82 $mg.l^{-1}$, which resulted in a ratio of maximum to minimum equivalent of 8.62093. Since this ratio was less than 10, no transformation of the data was required.

Table 4.20 Riboflavin production by *C famata* in the third screening experiment in supplemented SVO

Run	Yeast Extract (X_1) ($g.^{-1}l$)	Peptone (X_2) ($g.^{-1}l$)	Malt Extract (X_3) ($g.^{-1}l$)	K_2HPO_4 (X_4) ($g.^{-1}l$)	$MgSO_4.7H_2O$ (X_5) ($g.^{-1}l$)	[Ribo] ($mg.^{-1}l$)
1	0	6.5	6.5	0.25	0.15	4.41
2	0.5	6.5	6.5	0.25	0.05	4.07
3	0	8.5	6.5	0.25	0.05	0
4	0.5	8.5	6.5	0.25	0.15	3.21
5	0	6.5	8.5	0.25	0.05	2.70
6	0.5	6.5	8.5	0.25	0.15	3.53
7	0	8.5	8.5	0.25	0.15	11.87
8	0.5	8.5	8.5	0.25	0.05	8.51
9	0	6.5	6.5	0.35	0.05	3.64
10	0.5	6.5	6.5	0.35	0.15	4.34
11	0	8.5	6.5	0.35	0.15	2.08
12	0.5	8.5	6.5	0.35	0.05	1.83
13	0	6.5	8.5	0.35	0.15	0
14	0.5	6.5	8.5	0.35	0.05	15.82
15	0	8.5	8.5	0.35	0.05	4.11
16	0.5	8.5	8.5	0.35	0.15	3.94
17	0.25	7.5	7.5	0.3	0.1	4.15
18	0.25	7.5	7.5	0.3	0.1	2.51
19	0.25	7.5	7.5	0.3	0.1	4.27

Selection of factors that was included in the model is shown in the Half-Normal plot (Figure 4.37). Factors X_3, X_2X_4, X_1X_5, X_2, X_4, X_5 and X_1, in order of significance were selected to be included in the mathematical model that described riboflavin production by *C. famata* in the third supplement screening experiment. Factors X_3, X_2X_4 and X_1X_5

were definitely significant as they lay away from the line. Peptone (X_2), was possibly significant, while factors X_4, X_5 and X_1 were selected in order to develop a hierarchical model.

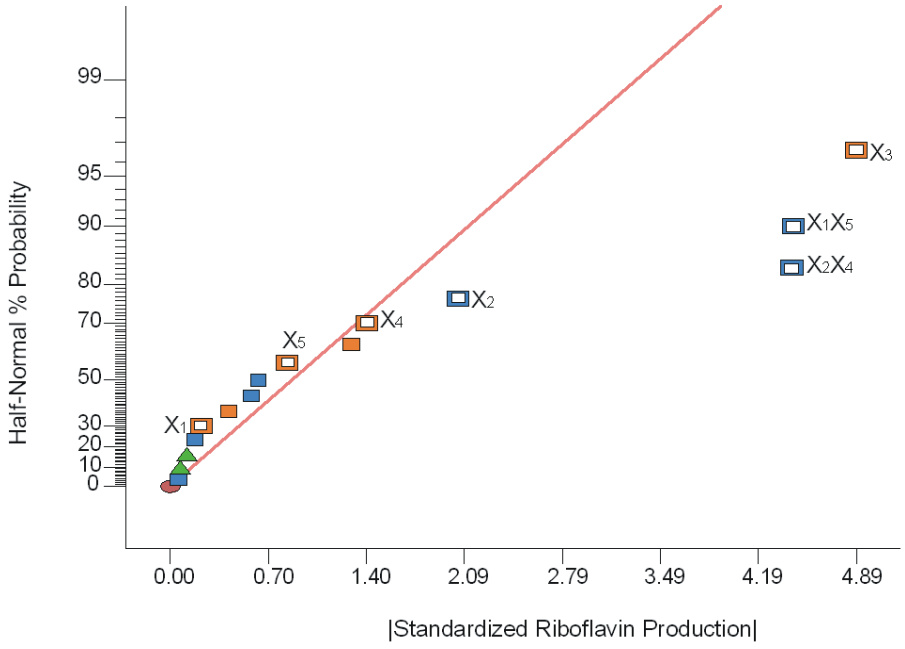


Figure 4.37 Selected factors to be included in the model that shall describe riboflavin production by *C. famata* in SVO in the third supplement screening experiment.

The Box-Cox plot (Figure 4.38) was used to evaluate this model. Lambda was equivalent to 1 and was represented by the blue line, which lay between the 95% confidence interval (low and high confidence intervals of -0.68 and 1.06, respectively).

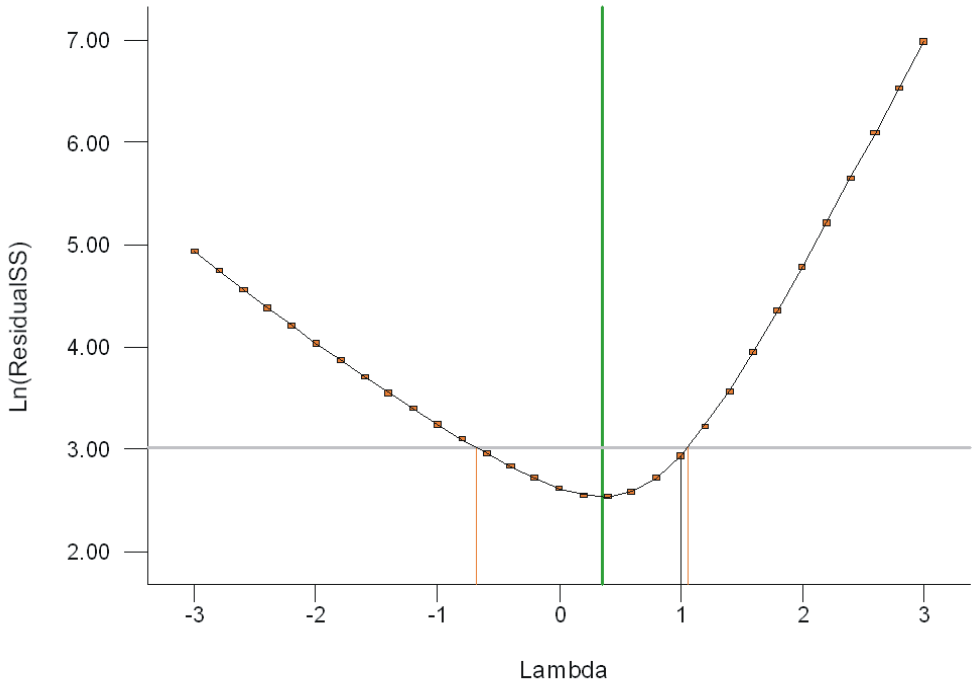


Figure 4.38 The Box-Cox plot used to evaluate the model for the third supplement screening experiment for *C. famata* in SVO. The blue line indicated the current transformation of the model while the green line indicates the best transformation.

A more detailed selection of factors is illustrated in the Pareto chart (Figure 4.39). Factors malt extract (X_3), peptone-K_2HPO_4 (X_2X_4) and yeast extract-$MgSO_4.7H_2O$ (X_1X_5) were definitely significant as they lay above the Bonferroni limit, while factor X_2 (peptone) was possibly significant as it lay above the t-value limit. Individual factors K_2HPO_4 (X_4), $MgSO_4.7H_2O$ (X_5) and yeast extract (X_1) were selected despite having little significance, to make the developed model hierarchical.

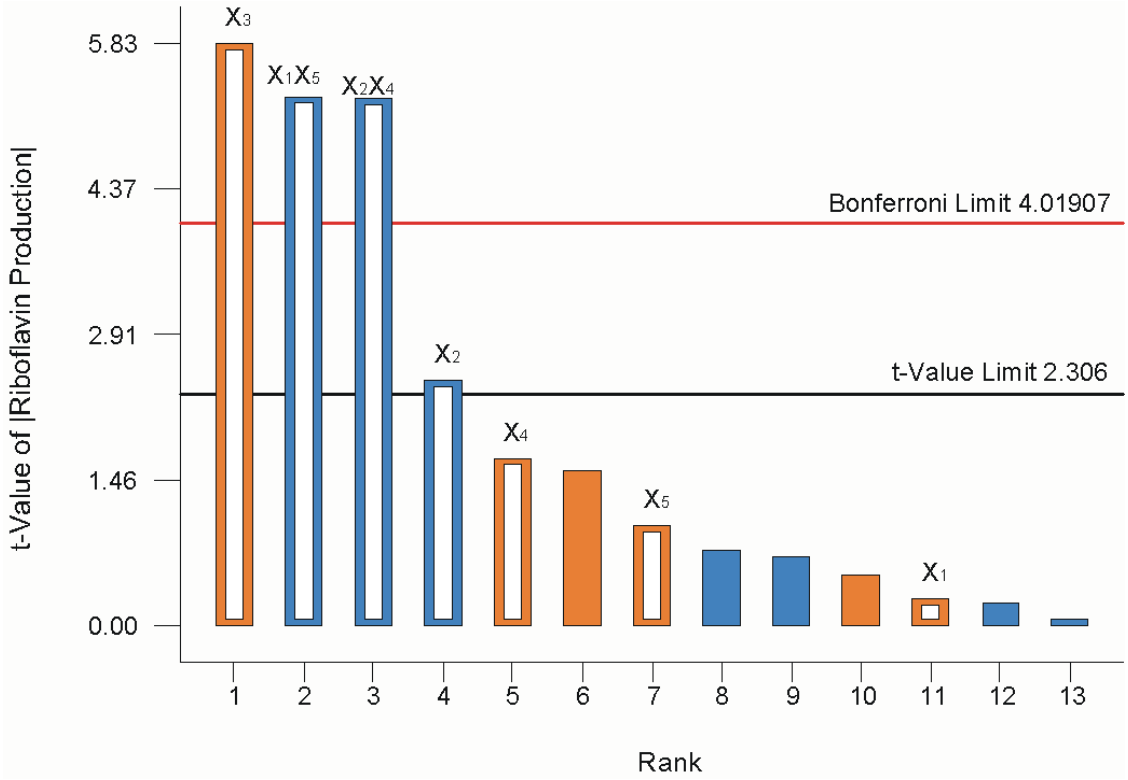


Figure 4.39 Pareto Chart indicating the importance of the factors in order of significance for the third supplement screening experiment. Positive effects are indicated in red bars while negative effects are shown in blue. Selected factors that are incorporated in the model is represented by open bars.

The ANOVA table (Table 4.21) showed a model with a *p*-value of 0.0013 indicating that the derived model was significant and that factors X_3, X_1X_5 followed by X_2X_4 with *p*-values of 0.0004, 0.0007 and 0.0008, respectively, were definitely significant in the third screening experiment for *C. famata* in SVO.

Table 4.21 Analysis of variance table for the third screening experiment for *C. famata* in SVO

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	Significance
Model	190.4	7	27.2	11.55	0.0013	significant
X_1-Yeast Extract	0.031	1	0.031	0.013	0.9112	
X_2-Peptone	14.1	1	14.1	5.98	0.0402	significant
X_3- Malt Extract	79.95	1	79.95	33.94	0.0004	significant
X_4- K_2HPO_4	6.6	1	6.6	2.8	0.1328	
X_5- $MgSO_4.7H_2O$	6.6	1	6.6	0.99	0.3478	
X_1X_5	65.93	1	65.93	27.99	0.0007	significant
X_2X_4	68.88	1	68.88	29.24	0.0006	significant
Lack of Fit	16.92	6	2.82	2.92	0.2767	not significant

The model that described riboflavin production for the third supplement screening experiment for *C. famata* in spent vegetable oil may be defined by the following final equation in terms of coded factors:

$$\text{Riboflavin concentration} = + 5.61 + 0.048 *X_1 - 1.08 *X_2 + 2.58 *X_3 + 0.74 *X_4 + 0.44*X_5 - 2.34 *X_1*X_5 - 2.27 *X_2*X_4 \quad \text{.....(4.8)}$$

where X_1 = yeast extract, X_2 = peptone, X_3= malt extract, X_4= K_2HPO_4 and X_5 = $MgSO_4.7H_2O$.

A Cube plot (Figure 4.40) illustrated the effects of the three most important factors peptone, malt extract and K_2HPO_4 showing a maximum riboflavin yield of 12.29 $mg.l^{-1}$ in the presence of maximum K_2HPO_4 and malt extract concentrations, with peptone at its minimum concentration. The concentrations of yeast extract and $MgSO_4.7H_2O$ were at 0.25 and 0.1 $g.l^{-1}$, respectively. An increase in product concentration from 12.29 to 15.03 $mg.l^{-1}$ riboflavin was observed by decreasing the concentration of yeast extract to 0 $g.l^{-1}$ and increased that of MgSO4.7H_2O to 0.15 $g.l^{-1}$.

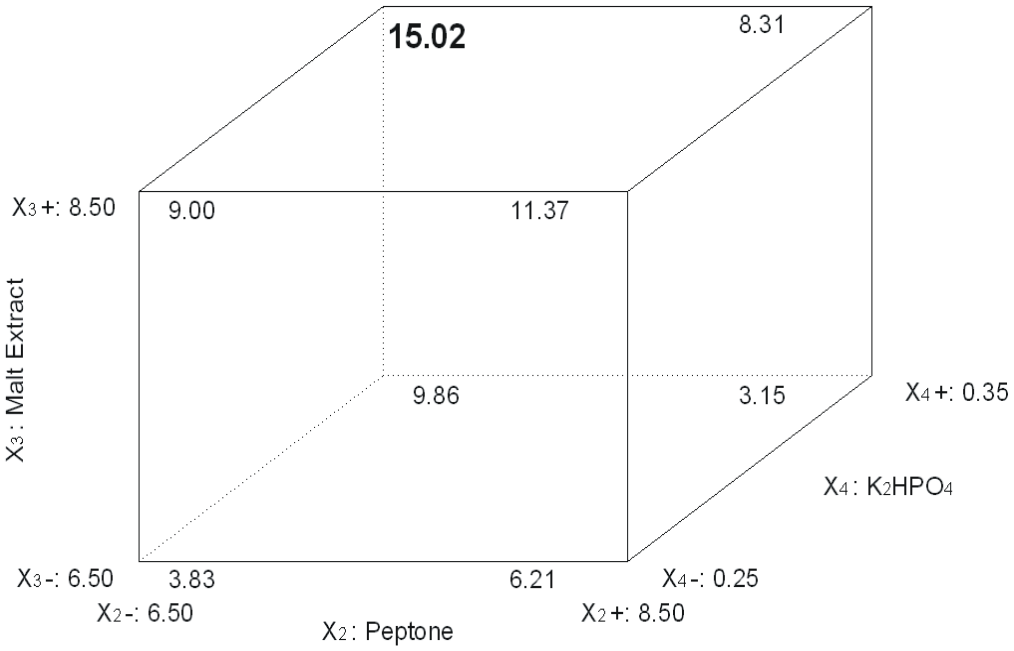


Figure 4.40 Effects of the three most significant factors affecting riboflavin production by *C. famata* in SVO in the third screening experiment. Maximum concentration is shown in bold.

4.6.1.4 Fourth supplement screening for riboflavin production by *C.famata* in SVO

The fourth screening experiment was designed according to conclusions made from analysing the data from the previous supplement screening experiment. The concentration of peptone (X_2) was decreased, while those of malt extract (X_3) and K_2HPO_4 (X_4) were increased as indicated in the cube plot in Figure 4.40. The minimum concentration of yeast extract in the previous experiment was 0 $g.l^{-1}$, and since its concentration was to be decreased to improve riboflavin yields, this nutrient supplement was omitted in all further experiments. The concentration of $MgSO_4.7H_2O$ was set at its minimum as alterations in its concentration had no effect on the riboflavin production in the third screening experiment. Subsequently, a three-factor, two-level,

resolution five experiment was designed for the fourth screening experiment, the design of which together with the response is summarised in Table 4.22.

Table 4.22 Riboflavin produced by *C. famata* in the fourth supplement screening experiment in supplemented SVO

Run	Peptone (X_2) ($g.l^{-1}$)	Malt Extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	[Ribo] ($mg.l^{-1}$)
1	5.5	7.5	0.35	69.01
2	6.5	7.5	0.35	9.15
3	5.5	10	0.35	63.61
4	6.5	10	0.35	55.85
5	5.5	7.5	0.45	58.16
6	6.5	7.5	0.45	52.76
7	5.5	10	0.45	57.12
8	6.5	10	0.45	72.50
9	6	8.75	0.4	63.27
10	6	8.75	0.4	57.68
11	6	8.75	0.4	50.09

The response ranged from a minimum concentration of 9.15 $mg.l^{-1}$ riboflavin to a maximum of 72.50 $mg.l^{-1}$, resulting in a ratio of maximum to minimum of 7.92. This implied that no transformation was required as this ratio was less than 10. Selection of factors that was included in the model is illustrated in the Half-Normal plot (Figure 4.41).

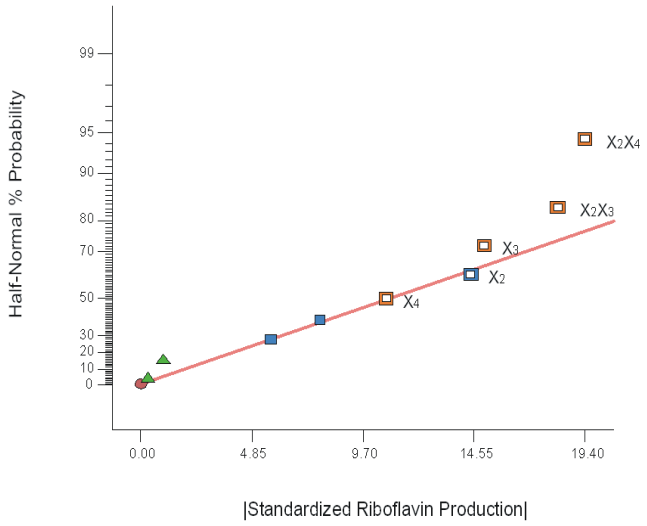


Figure 4.41 Selection of factors to be included in the model for the fourth screening experiment for *C. famata* in SVO.

The Box-Cox plot (Figure 4.42) for power transformations suggested that no transformation of the model was required as the current transformation was set on 'Lambda' = 1 and positioned only 1.09 away from the best transformation, positioned at 2.09. Furthermore, the model lay within the optimal zone situated between 0.87 and 3.92.

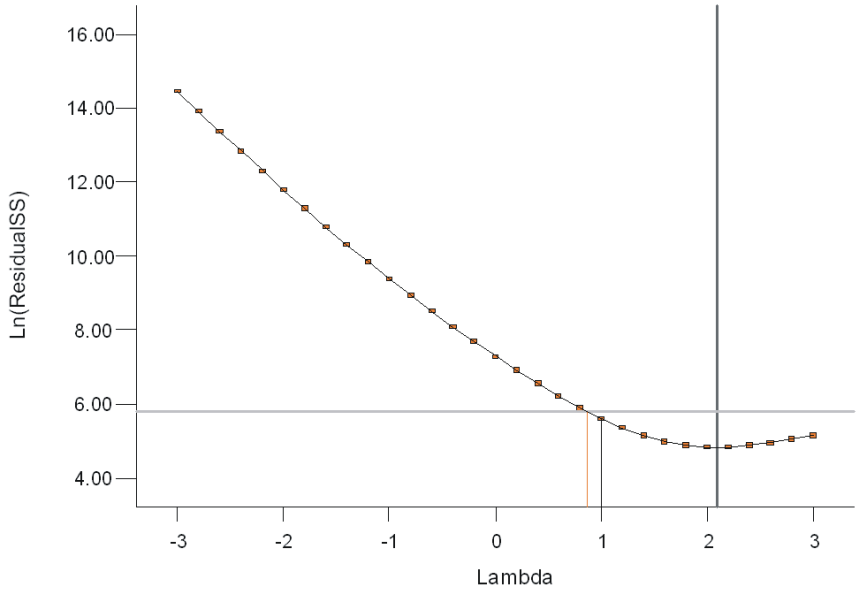


Figure 4.42 Box-Cox plot for power transformations for the fourth supplement screening experiment for *C. famata* in SVO. The blue line indicates the current transformation of the model while the green line represents the best transformation.

A graphic illustration of the effects of the factors on riboflavin production in order of significance is shown in the Pareto chart (Figure 4.43). Factors X_2X_4, X_2X_3, X_3, X_2 then X_4, in order of significance were selected to be included in the model. Factors X_2X_4 and X_2X_3 were possibly significant as they lie above the t-value limit.

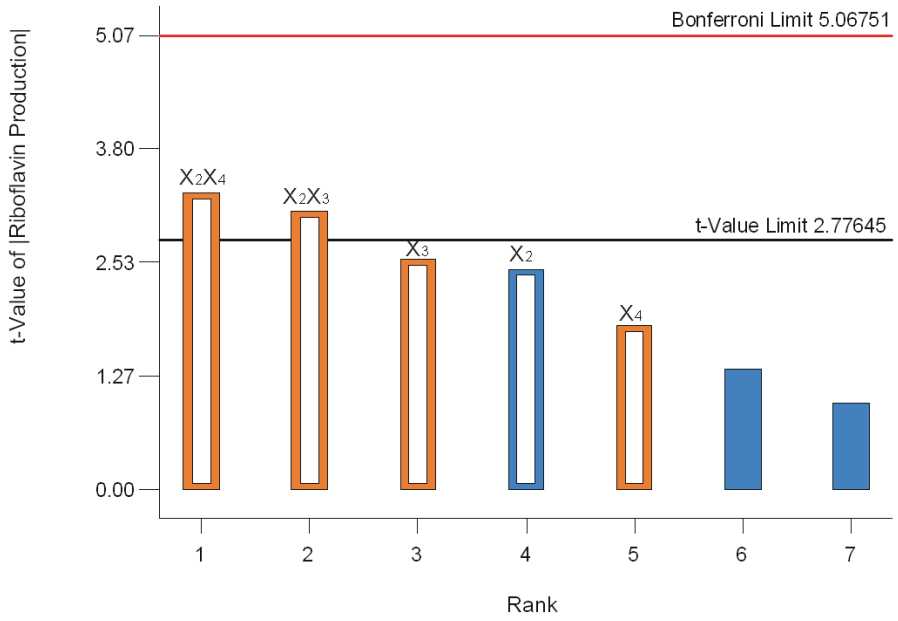


Figure 4.43 Pareto chart showing the effects of individual and combination of factors on riboflavin production by *C. famata* in SVO in the fourth supplement screening experiment. Positive effects are indicated in red while negative effects are shown in blue. X_2, X_3 and X_4 represent peptone, malt extract and K_2HPO_4, respectively. Open bars represent factors included in the model.

Factors X_3 and X_4 had a positive effect on riboflavin production by *C. famata* in the fourth screening experiment, while factor X_2 had a negative effect. The *p*-values which determine the significance of the model and individual factors, and the 'Lack of Fit' value are tabulated in Table 4.23.

Table 4.23 Analysis of variance table for the fourth supplement screening experiment for *C.famata* in SVO

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	Significance
Model	2512.04	5	502.41	7.33	0.0383	significant
X_2- Peptone	415.26	1	415.26	6.06	0.0696	
X_3- Malt extract	449.96	1	449.96	6.57	0.0625	
X_4- K_2HPO_4	230.15	1	230.15	3.36	0.1408	
X_2X_3	663.78	1	663.78	9.69	0.0358	significant
X_2X_4	752.9	1	752.9	10.99	0.0295	significant
Lack of Fit	186.59	2	93.29	2.13	0.3193	not significant

The final equation in terms of coded factors that described riboflavin production by *C. famata* in spent vegetable oil in the fourth screening experiment:

$$\text{Riboflavin concentration} = +54.78 - 7.20 * X_2 + 7.50 * X_3 + 5.36 * X_4 + 9.11 * X_2 * X3 + 9.70 * X_2 * X_4 \quad (4.9)$$

where X_2= peptone, X_3 = malt extract and $X_4 = K_2HPO_4$.

The cube plot (Figure 4.44) indicated that the maximum riboflavin concentration of 79.24 $mg.l^{-1}$ was produced by *C. famata* in the presence of the maximum concentrations of all three factors in this fourth screening experiment.

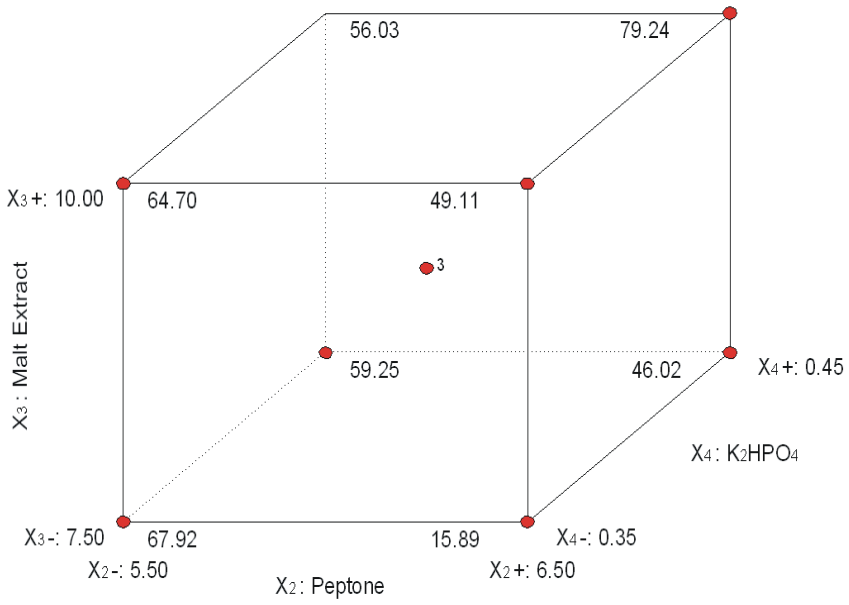


Figure 4.44 Effects of the three factors affecting riboflavin production by *C. famata* in SVO in the fourth screening experiment.

4.6.2 Optimization by Response Surface Methodology and OFAT Experiments

The Pareto Chart of the fourth screening experiment indicated that both factors X_3 (malt extract) and X_4 (K_2HPO_4) had positive effects on riboflavin production by *C. famata* in supplemented SVO. Peptone (factor X_2) was the only individual factor that had a negative effect on this process, therefore its concentration was set at its minimum of 5.5 $g.l^{-1}$. Consequently, factors K_2HPO_4 and malt extract were considered as the two most significant factors affecting riboflavin production and were optimized in a central composite and a OFAT experiment, respectively.

4.6.2.1 Optimization experiment for *C. famata* in SVO using central composite experiemental design

A central composite experiment with thirteen runs, five of which were center points was conducted in order to optimize the two most significant nutrients malt extract (X_3) and K_2HPO_4 (X_4). The design together with the response is shown in Table 4.24.

Table 4.24 Central composite experimental design for *C.famata* in SVO

Run	Malt Extract (X_3) ($g.l^{-1}$)	K_2HPO_4 (X_4) ($g.l^{-1}$)	[Ribo] ($mg.l^{-1}$)
1	10	0.45	55.30
2	10	0.65	17.56
3	14	0.45	56.92
4	14	0.65	23.86
5	12	0.41	43.65
6	12	0.69	72.36
7	9.17	0.55	47.73
8	14.83	0.55	45.48
9	12	0.55	47.95
10	12	0.55	57.49
11	12	0.55	52.15
12	12	0.55	61.09
13	12	0.55	57.31

The response ranged from 17.56 $mg.l^{-1}$ to 72.36 $mg.l^{-1}$ riboflavin which resulted in a ratio of maximum to minimum of 4.11905, which implied that no transformation of the data was required.

The Box-Cox plot (Figure 4.45) for power transformations indicated that no transformation of the data was required as the 95% confidence interval (indicated by the red line) around the best transformation (indicated by the green line positioned at 2.1) includes 1. The current transformation set at 'Lambda'= 1 was positioned 1.1 away from the best transformation. In addition, the model lay within the optimal zone that extended from 0.42 to 3.93.

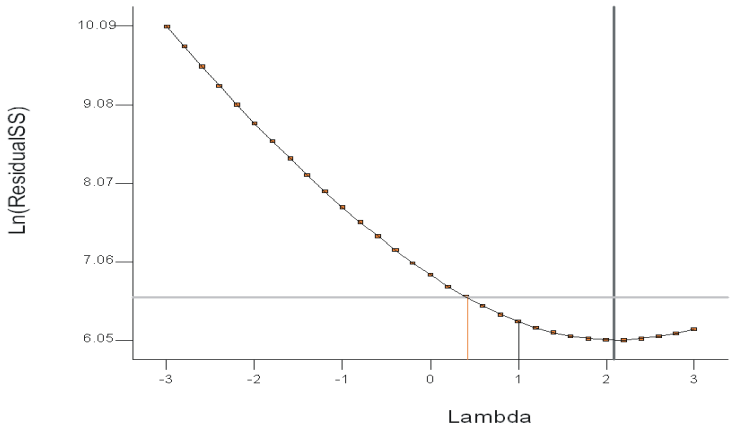


Figure 4.45 The Box-Cox plot used to evaluate the power transformations for central composite experiment. The blue line indicates the current transformation for the model while the green line indicates the best transformation.

The ANOVA table (Table 4.25) showed a model with a *p*-value of 0.0237 to be statistically significant.

Table 4.25 Analysis of variance table for central composite experiment for *C. famata* in SVO

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F	Significance
Model	2071.73	5	414.35	5.4	0.0237	significant
X_4-K_2HPO_4	412.01	1	412.01	5.37	0.0537	
X_3- Malt Extract	2.8	1	2.8	0.037	0.8539	
X_3^2	398.11	1	398.11	5.13	0.0569	
$X_4X_3^2$	1551.29	1	1551.29	20.21	0.0029	significant
Lack of Fit	431.28	3	143.76	5.42	0.0681	not significant

The derived final equation, in terms of coded factors is expressed below:

Riboflavin concentration = + 53.76 + 10.15 *X_4 + 0.59 *X_3+ 1.17 *X_4*X_3 - 7.50 *X_3^2 -

27.85 X_4*X_3^2…….(4.10)

where X_3 = malt extract and X_4 = K_2HPO_4.

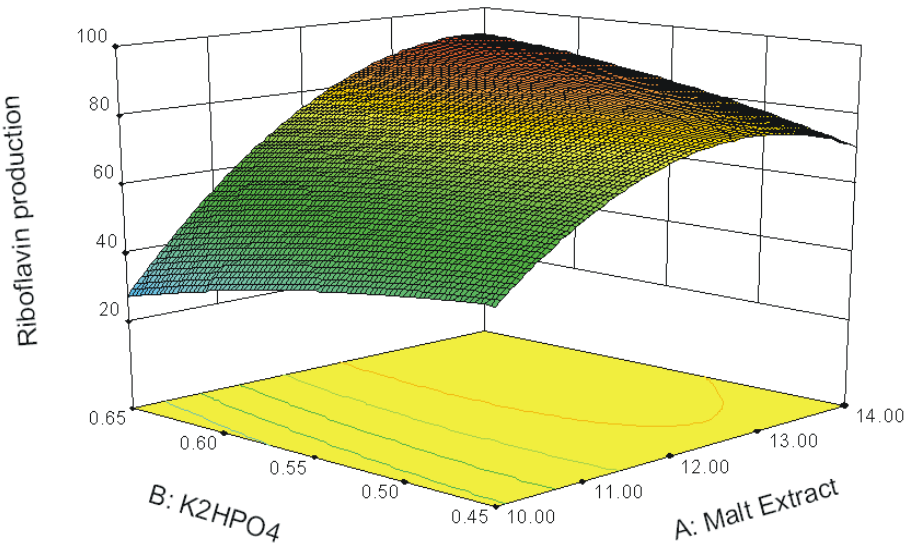


Figure 4.46 Effects of K_2HPO_4 and malt extract on riboflavin production by *C. famata* in SVO in the central composite experiment.

The 3-dimensional plot above showed the effect of K_2HPO_4 and malt extract on riboflavin production by *C. famata* in SVO (Figure 4.46). The optimum concentration for malt extract was established as 12.05 $g.l^{-1}$ by conferring with the statistical analysis of the experimental data. The optimum concentration for K_2HPO_4 was later established in a one-factor-a-time (OFAT) experiment with varying K_2HPO_4 concentrations (0.65 to 2.0 $g.l^{-1}$).

The optimum point in Figure 4.46 where most riboflavin was produced was calculated using a Design Expert optimization algorithm on the model described in equation 4.5. The four solutions offered (Table 4.26) were inspected and it was observed that 12.5 $g.l^{-1}$ malt extract would generate the highest riboflavin production.

Table 4.26 Central composite point prediction for maximum riboflavin production using yeast extract and K_2HPO_4

Response	SE Mean	95% CI low	95% CI high	Malt Extract	K_2HPO_4
64.53	7.45	62.15	82.15	12.5	0.45
63.93	5.40	51.15	76.71	12.5	0.55
63.92	5.40	51.15	76.69	14.00	0.55
63.76	5.40	50.98	76.53	14.00	0.55

4.6.2.2 One factor at a time (OFAT) experiment for *C. famata* in spent vegetable oil.

Riboflavin production maximized for all experiments after 60 hours of incubation which is in the late growth phase, despite the concentration of K_2HPO_4 present in the medium. The response recorded at 120 hours was compared in order to establish the optimum level for maximum riboflavin production. Figure 4.47 shows that maximum riboflavin was produced in the presence of 1.8 $g.l^{-1}$ K_2HPO_4.

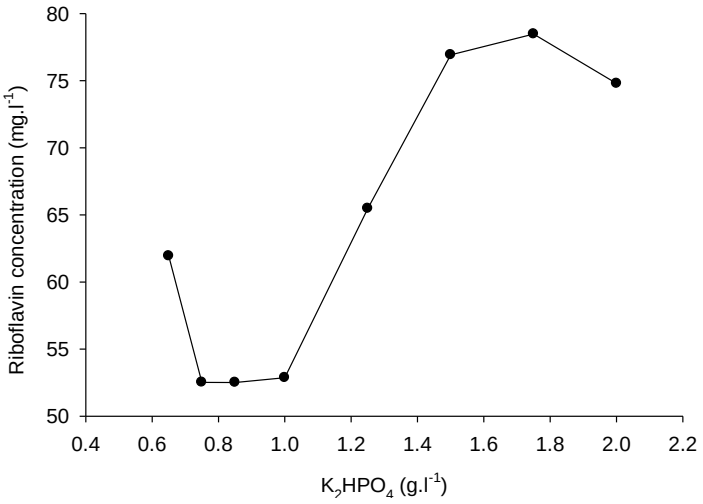


Figure 4.47 Comparison of riboflavin produced at 120 hours at various K_2HPO_4 concentrations by *C. famata* in the OFAT experiment.

4.6.3 Verification Experiment for *C. famata* in SVO

All five nutrient supplements were set at their optimum forming the optimized riboflavin producing medium for *C. famata* using SVO as a carbon source. This medium was verified in a confirmatory test conducted in triplicate in which after an incubation period of 120 hours, a maximum riboflavin yield of 80.11 $mg.l^{-1}$ was produced (Figure 4.48). The concentration obtained was much higher than the predicted value (64.53 $mg.l^{-1}$), however, it lies within the 95% confidence interval (from 62.15 $mg.l^{-1}$ to 82.15 $mg.l^{-1}$), thereby making it acceptable.

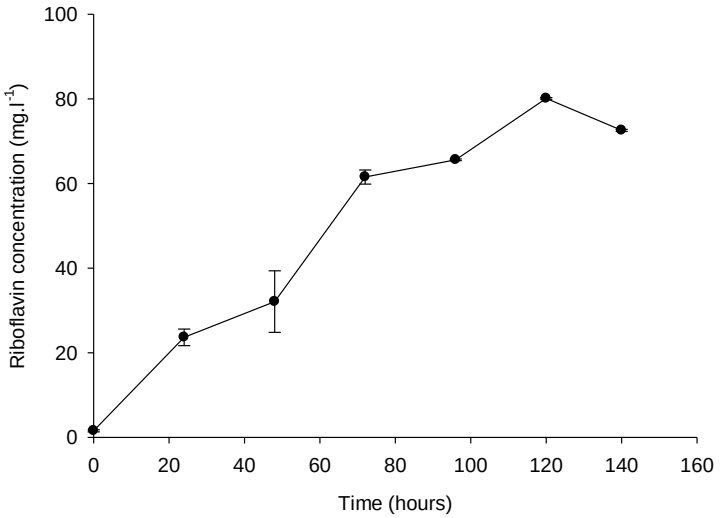


Figure 4.48 Riboflavin produced by *C. famata* in the confirmatory experiment.

CHAPTER FIVE

5.0 DISCUSSION

Bioproduction of riboflavin is an appropriate example of a successful, industrial bioprocess that has competed and almost entirely replaced the chemical process (Karos *et al.*, 2004). This study investigated the possibility of using spent industrial oils as carbon sources for the bioproduction of riboflavin by fungi.

Four fungi *E. gossypii* ATCC 10895, *E. gossypii* CBS 109.51, *E. ashbyi* CBS 206.58 and *C. famata* ATCC 20850 selected from literature and a derived mutant *E. gossypii* EMS 30/1 were used in this study. They were grown on solid O and K medium, consisting of 20 $g.l^{-1}$ glucose as a carbon source, in order to confirm their morphological characteristics with that from literature. The observed morphology characteristics of the colonies were summarized in Table 4.1. The four filamentous fungi belonging to the genus *Eremothecium* were grown on solid medium using the three-point inoculation technique which is considered an acceptable standard technique used for the cultivation of fungal cultures such as Aspergilli and Penicilli that allows for the lateral spreading of mycelia (Fischer and Dott, 2002). *Candida famata* was streaked onto solid media and its growth thereon resembled that of bacteria. The wild type *E. gossypii* showed no evidence of riboflavin production (Figure 4.1a) as it remained cream in colour and this supported the choice of *E. gossypii* CBS 109.51 by Centraalbureau voor Schimmelcultures as a better riboflavin producer. Radial lines extending towards the periphery were observed only for the wild type and this resembled the radial furrows that Kurtzman and Fell (1998) reported when they grew the closely related ascomycete *Eremothecium ashbyi* on a solid medium consisting of 4% malt extract and 0.5% yeast extract at 20-22^{o}C. Such furrows did not occur when *E. ashbyi* CBS 206.58 was grown on the solid O and K medium (Figure 4.1c).

Eremothecium gossypii CBS 109.51 grew differently compared to the wild type (Figure 4.1b), but similar to mutant *E.gossypii* EMS 30/1, with no formation of radial lines or furrows. Evidence of riboflavin production was clearly visible along the periphery (colour change from cream to yellow). This strain that genetically differed from the wild type, was recommended by Centraalbureau voor Schimmelcultures for riboflavin production, and was therefore purchased from this biological resource centre.

Colony diameters of *E. ashbyi* CBS 208.58 differed from reports made by Kurtzman and Fell (1998) which stated that after ten days of growth in the presence of 4% malt extract and 0.5% yeast extract, at 20-22^{o}C, *E. ashbyi* colonies had diameters of 5 mm. Figure 4.1c shows colonies with average diameters of 16 mm. Furthermore, their report included that the colour of the colonies had changed from tan to yellow, due to riboflavin production, dry, initially smooth, but later developed deep radial furrows with sharp lobed margins. Figure 4.1c showed no evidence of radial furrows which were observations made when wild type *E. gossypii* was grown on the solid O and K medium (Figure 4.1a). This variation in growth patterns is probably due to different medium components used or variation in growth conditions.

Kimura *et al.* (2008) investigated the colonial morphologies of sixteen *E. ashbyi* isolates grown in yeast malt agar (YMA) at 25^{o}C and after seven days, they also, observed white pellicles with colony diameters of approximately 5 mm for all isolates. All sixteen strains produced colonies that were moist, initially smooth, but later developed sharp lobed margins, similar to observations made by Kurtzman and Fell (1998). The increased diameters of *E. ashbyi* CBS 208.58 was probably due to variation in growth medium or conditions, or as a result of genetic difference.

Mutant *E. gossypii* EMS 30/1 indicated similar colonial growth (Figure 4.1d) to *E. gossypii* CBS 109.51. However, colonies were larger and more orange in colour which

implied that the mutant produced more vitamin B_2 than the wild type or *E. gossypii* CBS 109.51.

The yeast *C. famata* ATCC 20850 appeared glossy with smooth margins initially light cream in colour, then turned grayish-white after a month (Figure 4.2). Kurtzman and Fell (1998) reported similar characteristics of a month-old streak culture of *C. famata* on yeast malt agar which changed from grayish-white to yellowish, soft, shiny or dull, smooth or entirely wrinkled. These characteristics were also in accordance with the colonial morphological description of *C. famata* in the Bioresource Collection and Research Center (BCRC) catalogue, which appeared as "white, butyrous, smooth, glistening and convex with entire margin when grown in glucose-yeast extract-peptone agar at 25^{o}C" [BCRC Catalogue, Accessed on line, 7 October 2009).

The filamentous nature of the ascomycetes was clearly evident under the light microscope. Compartmentalisation of the hyphae of *E. gossypii* by a septum, which in young parts appear as rings was seen in (Figure 4.3a). In older filaments, these septa appeared as closed discs (World Lingo Translations, 2009). The characteristic lateral branching of *E. gossypii* may be viewed in this microscopic image. *Eremothecium ashbyi* known for its dichotomously, branching hyphae (Kimura *et al.*, 2008) and its sparingly septate charcteristic (Kurtzman and Fell, 1998) was observed in Figure 4.3c. Spheroidal to short-ovoidal shaped cells, characteristic of the yeast *C. famata* observed in Figure 4.3 d. Cells were arranged in short chains, but may also occur singly or in pairs (Kurtzman and Fell, 1998). Evidence of budding was also found.

Mutant *E. gossypii* EMS 30/1 was inspected at various time intervals to show detailed growth on the solid O and K medium. At 72 hours, apical tip-splitting was evident in all mycelia (Figure 4.3e), which probably contributed towards the rapid increase in colonial diameter, compared to the *E. gossypii* CBS 109.51. After 96 hours of incubation,

twirling of the mycelia was observed (Figure 4.3f). Upon nutrient deprivation, this fungus is known to sporulate, leading to contraction of the septum, followed by cytokinesis, and abscission thereby forming sporangia, that contains up to eight haploid spores (World Lingo Translations, 2009). Apical ascospores were indeed seen while constriction of septa forming an apical ascospore was evident (Figure 4.3h).

Glucose, when used as a carbon source for riboflavin production is converted to ethanol. During this time the dry mass of the cells may increase up to 20% and formation of oil droplets occurs within the cell (Yuan Kun, 2006). Nile red is a stain used to determine oil dispersion within fungi under UV light and may also be used for intracellular lipid determination. Its fluorescence characteristic may be used to quantify lipids within a cell, but since its fluorescence depends on the location of the lipid, its use is not so common (Kimura *et al.,* 2008). The selected riboflavin-producers were therefore stained with Nile red and viewed under UV fluorescence microscopy. Figures 4.4a and b indicate lipid accumulation throughout the cell in *E. gossypii* ATCC 10895 and CBS 109.51, respectively. Figure 4.5c showed the characteristic fluorescent spindle-shaped ascospores that tapered on both ends, with appendages that coil to keep ascospores together. Leeuw (2006) mapped the presence of intracellular lipid in *E. gossypii* using oxylipin specific antibodies and immunofluorescence microscopy. He also reported complete fluorescence of the ascospore and terminal appendages that hold spores together. Stahmann *et al.* (1994) investigated *E. gossypii* grown in submerged culture and stained with Nile red. Granular and hyaline droplets that absorbed the stain resulted in intense yellow fluorescence of the oil droplets when viewed under UV light, as shown in the microscopic images (Figure 4.4). Figure 4.4d shows oil dispersion throughout the filament which is similar to observations made of *E. gossypii* ATCC 10895 (Figure 4.4a). Single fluorescent oil droplets were evident in the cells of *C. famata* in Figure 4.4e while the accumulation of lipid droplets at apices in mutant *E. gossypii* EMS 30/1 could be seen in Figure 4.4f.

Ozbas and Kutsal (1986) investigated the effect of pH on riboflavin production within the ranges of 6.0 to 8.0 for *E. ashbyi* and 5.5 - 7.0 for *E. gossypii* (Vandamme, 1989). They confirmed that optimal specific growth rate and riboflavin production for both ascomycetes were at pH 6.5 (Kolonne *et al.,* 1994). The initial pH for all experiments were therefore adjusted to 6.5 using NaOH.

Ozbas and Kutsal (1986) claimed to have obtained maximum riboflavin production at an initial glucose concentration of 20 $g.l^{-1}$ (Ozbas and Kutsal, 1986-cited by Vandamme, 1989), and therefore, the O and K medium used to grow the selected fungi consisted of 20 $g.l^{-1}$ glucose as a carbon source. Tanner *et al.* (1949) investigated the effect of the age of *E. gossypii* inoculum on riboflavin production. Cultures of 24, 72, 96 and 120 hours were used to inoculate the production medium. Results indicated that riboflavin concentrations of 0.5 $mg.l^{-1}$, 0.4 $mg.l^{-1}$, 0.25 $mg.l^{-1}$ and 0.2 $mg.l^{-1}$ were produced by the 24, 72, 96 and 120 hour cultures, respectively. This clearly indicated that the youngest culture of 24 hours was most appropriate for this process. Furthermore, monitoring of riboflavin yields over 7 to 10 days revealed that the young vigorously growing cells displayed the fastest and most prolonged period of riboflavin production, which also indicated that experimental inocula should be in its mid-log point, when cells are most actively growing. Consequently, growth profiles for each selected fungus were established in order to ascertain the mid-log point of each microorganism.

A comparison of the lag phases for *E. gossypii* strains indicated that the wild type (Figure 4.5a) had an extended lag phase of approximately 21 hours while the other two strains, CBS 109.51 and the mutant *E. gossypii* 30/1, had lag phases of 3 hours. In a graphic illustration of typical growth of *E. gossypii* ATCC 10895, measured as mycelial dry weight, a lag phase indicating no growth for the first 20 hours was observed, followed by a rapid increase in biomass until 72 hours. Thereafter, biomass decreased until 120 hours, after which stationary phase occurred (Karos *et al.*, 2004). Jimenez *et al.*

(2008) investigated the growth curve of wild type *E. gossypii* using a rich nutrient liquid medium called MA2 medium. *Eremothecium gossypii* ATCC 10985 indicated immediate increase in biomass, showing no evidence of a lag phase. The nutrient composition of this medium or perhaps the growth conditions affected fungal growth, resulting in the absence of a lag phase.

The growth curve of *E. ashbyi* indicated a lag phase of 6 hours, which was described by Kapralek (1962) as the initial phase of riboflavin production characterised by rapid growth of mycelia, rapid utilisation and oxidation of glucose, and a decrease in pH due to the accumulation of pyruvic acid. Kolonne *et al.* (1994) established growth curves at various constant pH levels for this organism by measuring biomass on a daily basis for seven days. The lag phase at pH levels 4.5, 5.5, 6.5 and 7.5 occurred for the first 12 hours, after which a rapid increase in biomass to 48 hours was shown, followed by a gentle decrease for the next 24 hours. Thereafter, biomass rapidly decreased. At pH levels 3.5 and 8.5, the lag phase extended to 72 hours. At pH 8.5, biomass increased for the next 24 hours, after which it rapidly decreased. At pH 3.5, biomass rapidly increased until 144 hours, showing signs of tapering. These results opposed that of *E. ashbyi* CBS 206.58 as the lag phase observed at pH 6.5 was much shorter (half the time) (Kolonne *et al.*, 1994) which was probably due to genetic or medium variation.

Figure 4.5b shows the growth curve of a *Candida famata* ATCC 20850 with a lag phase of approximately 21 hours, which was similar to the lag phase of wild type *E.gossypii* ATCC 10895. A *C. famata* strain isolated from Moroccan sourdough using a soluble starch medium, supplemented with KH_2PO_4, $(NH)_4SO_4$, $MgSO_4$ and yeast extract was investigated by Mohamed *et al.* (2007). Biomass was monitored spectrophotometrically at 600 nm, and results showed no signs of a lag phase, but immediate, rapid increase in biomass for the first 24 hours, with growth rate decreasing gradually until 72 hours, after which stationary phase was obtained (Mohamed *et al.*, 2007). This variation was

probably due to strain difference or variation in medium composition. Organisms with longer lag phases probably require more time to adapt to new growth conditions before rapid growth occurs. In this study, all five selected fungi were exposed to different growth conditions (from a solid to a liquid growth medium), followed by agitation. This could have contributed towards the occurrence of lag phases.

This phase was followed by the exponential growth phase which was characterised by increasing specific growth rates. It is in this phase that the mid-log for each fungus was graphically calculated. However, the maximum specific growth rate does not necessarily occur at the mid-log point. The maximum specific growth rates for *E. gossypii* ATCC 10895, *E. gossypii* CBS 109.51, *E. ashbyi* CBS 206.58, *C. famata* ATCC 20850 and mutant *E.gossypii* EMS 30/1 were calculated as 0.0025, 0.0555, 0.0219, 0.0238 and 0.6427 h^{-1}, respectively (Table 4.2). Specific growth rates of filamentous fungi in submerged cultures are affected by the branching frequency of the microorganism. As nutrients deplete in the medium, filamentous fungi do not branch out as frequently, but increases their mycelial length, in order to seek nutrition, resulting in a decrease in growth rate (Gibbs *et al.*, 2000).

The transfer of culture fluid together with inocula was reported to have decreased the lag phase of bacteria during experiments and known to affect fungal germination tests when the growth medium adhered to spores. Consequently, the washing of cells was adopted to prevent such shortcomings (Norris and Ribbons, 1972). Therefore, experimental inocula was washed thrice with sterile distilled water to remove adhering culture medium that contained glucose which may have affected experimentation.

Spent motor and vegetable oils were the two most commonly discarded oils in South Africa, and were therefore selected as carbon substrates for this study. Chemical analysis of these oils were conducted before and after fungal growth to establish compounds that

possibly contributed towards riboflavin production. The eleven carbon compound undecane decreased in concentration after fungal growth. Low carbon chain compounds(C_{12} to C_{15}) dodedecane, tridecane, tetradecane and hexadecane are compounds that were completely used by the fungus while compounds with longer carbon chains (C_{19} to C_{31}) were decreased in concentration (by approximately 90%). This suggests the lower carbon chain compounds are more easily utilized by mutant *E. gossypii* EMS 30/1. Pristane, a 19-carbon, branched alkane occurred only after fungal growth (Figure 4.6b). *Eremothecium gossypii* is known to cleave the lipid source extracellularly prior to taking in the liberated fatty acids to form its storage lipid (Stahmann *et al.*, 1994). Pristane is probably a product of this breaking down process. This chemical is used in the cleaning operation of oil spills as it was used as a dispersant in the study that investigated the degradation of long-chain alkanes released during shipping activities and oil spills (Yamada-Onodera *et al.*, 2002).

The possible use of spent motor oil for the bioproduction of riboflavin is advantageous as it contains compounds that are hazardous to the environment. It is considered as a toxic environmental contaminant (Dominguez-Rosado and Pichtel., 2004). The compound undecane present in fuels and solvents is known to pollute air and water, and is considered an environmental pollutant because of its highly carcinogenic property that promotes the formation of tumors (Summary of data for chemical selection, Accessed on 12 May 2009). Cotton *et al.* (1977) chemically analysed 30 used motor oils collected from throughout the United States in order to establish the effectiveness of reclaiming this oil. The data characterised the degree of contamination in terms of wear metals, metals derived from additive compounds and lead that accumulated in the motor vehicles crankcase as the oil became contaminated with leaded fuel and combustion residue. Chemical compounds that belonged to the group of monoaromatics, diaromatics and polyaromatic were found in all the oils but in various quantities. In addition, wear and metallic contaminants included aluminium, chromium, copper, iron, potassium,

manganese, silicon and tin were also identified in all the spent oils. This study was considered rare as it is unusual to use a toxic substance like spent motor oil to produce a nutrient compound like riboflavin.

Analysis of SVO before and after fungal growth resulted in the presence of numerous long chain carbon compounds (C_{20} to C_{46}) prior to the growth of *C. famata* (Table 4.5) which were not detected after growth. Table 4.6 shows the presence of hexanal, 2,4-decadienal and 9,12-octadecadienoic acid which are shorter carbon chain chemicals, after fungal growth. This suggested that the yeast had the potential to utilize these long carbon chain chemicals for growth and riboflavin production. Vijayalakshmi *et al.*, (2010) investigated the effects of lipid accumulation and membrane fluidity on mycelial stability and riboflavin production by the ascomycete *E. ashbyi*. Octadecadienoic acid was a compound that contributed towards the riboflavin production phase which supported the presence of this chemical before and after growth of *C. famata* in SVO. Oils of plant origin are either cleaved liberating fatty acids (linolenic, linoleic, oleic, stearic, palmitoleic and palmitic acids) or taken up as triacylglycerides for growth and riboflavin production by *E. gossypii* (Stahmann *et al.*, 1994). Similarly, the fatty acid linoleic acid was utilised by this riboflavin-producing fungus growth and riboflavin production.

The use of other low cost organic wastes such as hog casings and fish meal for the production of riboflavin by ascomycete *E. ashbyi* was investigated by Kalingan and Liao (2002). The former substrate possessed fatty acids that consisted mainly of stearic and linoleic acid while fish meal consisted of linolenic acid. These fatty acids were considered as strong flavinogenic stimulators that promoted the production of this vitamin. Hog casings in association with beef extract was also investigated as substrates for the production of riboflavin. Results indicated that stearic and palmitic acids present in these substrates supported maximum riboflavin production by *E. ashbyi* (3.5 $g.l^{-1}$).

Oil alone, as a medium, lacked the necessary nutrients required for riboflavin production, therefore, supplementation using the nutrients that supplemented glucose in the O and K medium, was conducted. The nutrients were yeast extract, malt extract, peptone, K_2HPO_4 and $MgSO_4.7H_2O$. Oil has the ability to alter surface tension, decrease of oxygen transfer in this liquid substrate and to increase permeability of the cell membrane which could be the rationale behind the positive effect of oil in riboflavin production (Sabry *et al.*, 1989).

The O and K medium was modified by incorporating both glucose and spent oil (Table 3.3) as carbon sources in order to investigate growth and riboflavin production by each fungus using either of the two selected substrates. The addition of glucose was to initiate fungal growth and their concentrations (glucose and spent oil) was determined from literature that reported the production of riboflavin production by the ascomycete *E. ashbyi* using both glucose and sunflower oil as carbon substrates (Ozbas and Kutsal, 1986). Sunflower oil was combined with various concentrations of glucose, which together equated to 20 $g.l^{-1}$, initial carbon source. Results indicated maximum specific organism growth and vitamin production in the medium consisting of 5.0 $g.l^{-1}$ glucose and 15.0 $g.l^{-1}$ sunflower oil. Consequently, the same concentrations of glucose and oil were incorporated, forming the modified O and K medium that was designed to test for preliminary growth of each fungus in either spent oil.

Figure 4.8 indicated complete depletion of glucose after 20 hours for all five fungi grown in supplemented spent motor oil, which implied that glucose successfully initiated growth of all the selected microorganisms. This fact was corroborated in Figure 4.11 that showed rapid increase in biomass after 20 hours of incubation. Thereafter, biomass continued to increase until approximately fifty hours, when growth began to diminish. It is during this late phase of growth that riboflavin was rapidly produced (after 50 hours) (Figure 4.10), which was typical of riboflavin production by the

ascomycetes (Esser and Bennet, 2002). The observation that growth and riboflavin production increased after glucose depletion strongly suggested the successful utilisation of spent motor oil as a carbon source for both these activities of all five, selected fungi.

Glucose analysis in Figure 4.11 showed a rapid decrease, in the first 10 hours, indicating rapid utilisation of glucose in the presence of spent vegetable oil, in comparison to the presence of spent motor oil. It is possible that the incorporation of the toxic spent motor oil had changed the production medium so as to hinder glucose utilisation for growth, in its presence. The steep inclination in fungal biomass (Figure 4.12) for the first 10 hours also confirmed utilisation of glucose to initiate fungal growth, followed by slowing of the growth rate, when glucose was completely depleted. Riboflavin production increased rapidly after 50 hours (Figure 4.13) which was during the late growth phase. The production of riboflavin in the late growth phase was synonymous with results that were reported by researchers Esser and Bennet (2002).

The variation in biomass with time probably occurred as a result of agitation, occasionally causing the fungi to clump, forming pellets, or possibly, there was error in removing samples. It is possible that there was no consistency in sampling as the filamentous fungi were not evenly distributed in the production media. Nonetheless, symptoms of increasing biomass and riboflavin production after glucose depletion indicated successful utilisation of spent vegetable oil by all five riboflavin over-producers used in this study.

The five selected riboflavin-producing fungi were grown in the O and K medium that was modified by substituting 20 $g.l^{-1}$ glucose (carbon source) with 20 $g.l^{-1}$ spent motor or vegetable oil (Table 3.4). Figure 4.14 illustrated the riboflavin yields by the selected fungi in supplemented SMO and a comparison of the riboflavin yields led to the selection of mutant *E. gossypii* EMS 30/1 as the preeminent riboflavin-producing fungus

in SMO as it produced the maximum concentration of 20.44 $mg.l^{-1}$. This suggested that among the five selected fungi, mutant *E. gossypii* EMS 30/1 was most capable of cleaving SMO into fatty acids that was easily taken up to form storage lipid that was later used to produce riboflavin. Similarly, all five fungi were grown in supplemented SVO and the comparison of riboflavin production by each fungus (Figure 4.15) led to the selection of *C. famata* as the most suitable fungus for this substrate because it produced the most riboflavin (16.99 $mg.l^{1}$) These results suggests that *C. famata* had the most potential in cleaving this oil into fatty acids that were most efficiently taken up into the yeast as storage lipid, that was later transformed to riboflavin.

The first step taken towards improving riboflavin production by mutant *E. gossypii* EMS 30/1 in SMO was to screen the effects of nutrient supplements yeast extract (X_1), peptone (X_2), malt extract (X_3), K_2HPO_4 (X_4) and $MgSO_4.7H_2O$ (X_5) on this process. A comparison of riboflavin produced in the first supplement screening experiment confirmed that production of this vitamin was at its maximum at 120 hours. Consequently, the response at this point in time was analysed for all experiments that followed.

The first screening experiment was set at 50% higher and 50% lower than factor concentrations of the O and K medium in order to encompass within the ranges of each variable the supplement concentrations stipulated in literature. The experimental design was summarised together with response data recorded at 120 hours in Table 4.7. The ratio of maximum to minimum (5.22) concentrations is 5.22 fold which indicated that no transformation of data was required. A ratio greater than ten generally indicates that a transformation of the data is necessary. A statistical model which included all the main and two-factor interactions was fitted to the data. The Box-Cox plot for power transformation (Figure 4.16), which is colour coded to assist with interpretation, was used to validate the developed model given in Table 4.8. This plot suggested that no

transformation of the data was required as the current transformation represented by a blue line was set on Lambda (blue vertical line) = 1. It was positioned 1.03 away from the best transformation (green vertical line) set at -0.03. This evaluated the model as satisfactory for further analysis and hypothesis testing.

The absolute values of all the effects were plotted as squared values in the Half-Normal Plot which was colour coded to indicate positive (orange) and negative effects (blue). Figure 4.17 illustrated the selection of factors yeast extract (X_1), peptone (X_2), yeast extract-K_2HPO_4 (X_1X_4), yeast extract-$MgSO_4.7H_2O$ (X_1X_5), peptone (X_2) and $MgSO_4.7H_2O$ (X_5) to be included in the mathematical model. Factors from left to right were ranked in order of significance. Effects of individual factors and combination of factors were ranked according to significance in the Pareto chart (Figure 4.18). The vertical axis shows the t-value of the absolute effects. Yeast extract (X_1) had the largest negative effect on the production of riboflavin in this experiment, and was considered most significant as it lay above the Bonferroni limit. K_2HPO_4 (X_4) also had a negative effect, followed by the interaction of yeast extract and K_2HPO_4 (X_1X_4), which together had a positive effect on this process as these factors, together with the combined effect of yeast extract and $MgSO_4.7H_2O$ (X_1X_5), lay above the t-value limit.

The ANOVA (Table 4.8) indicated that the model with an F-value of 8.98 was statistically significant with a *p*-value of 0.0010. This implied that there was only a 0.1% chance that a Model F-value this large could have occurred due to noise. Factors yeast extract (X_1) followed by K_2HPO_4 (X_4), yeast extract and K_2HPO_4 (X_1X_4) then yeast extract and $MgSO_4.7H_2O$ (X_1X_5) with *p*-values 0.0011, 0.0070 and 0.0238, respectively were significant contributes to the model (Table 4.8). Equation (4.1) accompanying the Table 4.13 was statistically significant to the data and all inferences as well as predictions made from the graphs drawn, based on the mathematical model, were significant to 99.90%.

A cube plot (Figure 4.19) illustrated the interaction of the three most significant factors yeast extract (X_1), K_2HPO_4 (X_4) and $MgSO_4.7H_2O$ (X_5) affecting riboflavin production by mutant *E. gossypii* EMS 30/1 in the first supplement screening experiment. A maximum riboflavin yield of 48.28 $mg.l^{-1}$ was recorded in the presence of minimum yeast extract (2.5 $g.l^{-1}$), minimum K_2HPO_4 (0.1 $g.l^{-1}$), maximum $MgSO_4.7H_2O$ (0.3 $g.l^{-1}$), 5.0 $g.l^{-1}$ peptone and 5.0 $g.l^{-1}$ malt extract. An improvement in riboflavin yield from 48.28 $mg.l^{-1}$ to 51.48 $mg.l^{-1}$ was obtained by decreasing the concentrations of peptone and malt extract shown in Figure 4.20. This indicated a 6.63% increase in product yield from the previous experiment using the O and K medium which was modified by supplementing the glucose with 20 $g.l^{-1}$ SMO.

The second screening experiment for riboflavin production by mutant *E. gossypii* EMS 30/1 in SMO was designed according to inferences made in the statistical analysis of the data obtained in the first screening experiment. Cconcentrations of factors yeast extract, peptone, malt extract and K_2HPO_4 had to be decreased, and that of $MgSO_4.7H_2O$ increased in order to improve riboflavin yields. This fractional factorial experimental design together with response data recorded at 120 hours was summarised in Table 4.9.
The Box-Cox plot (Figure 4.20) for power transformations was used to evaluate the derived model and no transformation was required as lambda was equal to 1. The Half-Normal Plot (Figure 4.21) illustrated selection of the factors malt extract (X_3), K_2HPO_4 (X_4) peptone-$MgSO_4.7H_2O$ (X_2X_5) and yeast extract (X_1) to be included in the model. These factors were significant and positioned away from the red line. Despite the poor significance of peptone (X_2) and $MgSO_4.7H_2O$ (X_5) to riboflavin production in this experiment, they were also selected in order to make the model hierarchical.

Analysis of individual factors and mixture of components were graphically illustrated in the Pareto Chart (Figure 4.22) and were ranked according to the greatest effect on the production of riboflavin by mutant *E. gossypii* EMS 30/1. Malt extract (X_3), K_2HPO_4

(X_4) and the peptone-$MgSO_4.7H_2O$ (X_2X_5) combination were definitely significant to this bioprocess as they were positioned above the Bonferroni limit. Factors X_3 and X_4 as well as yeast extract, positively contributed towards the production of riboflavin in this experiment while peptone-$MgSO_4.7H_2O$ (X_2X_5) had a negative effect and could not be disregarded as it lay above the Bonferroni limit, hence it was considered significant.

A mathematical model based on the effects of individual factors and mixture of factors fitted the data for this second supplement screening experiment and was described with equation (4.2) accompanying the ANOVA Table 4.10. Statistical evaluation of the model was done using Fisher's statistical test for ANOVA, the results of which indicates that the model significantly fitted the data (F-value of 21.16 and a low *p*-value <0.0001. Furthermore, factors with *p*-values less than 0.05 implies that they significantly affects riboflavin production in this experiment. Therefore, individual factors malt extract (X_3) and K_2HPO_4 (X_4), and combined factors peptone-$MgSO_4.7H_2O$ (X_2X_5) and yeast extract (X_1), with *p*-values of <0.0001, 0.0002, 0.0027 and 0.011 respectively, were statistically significant. The mathematical equation 4.2 that described the model was therefore significant and all inferences drawn based on this model were significant to 99.99%.
The main effects of the three most significant factors malt extract (X_3), K_2HPO_4 (X_4) and yeast extract (X_1) on riboflavin production in this experiment, were illustrated in the cube plot (Figure 4.23) which showed maximum riboflavin (32.41 $mg.l^{-1}$) produced in the presence of 2.5 $g.l^{-1}$ malt extract, 0.1 $g.l^{-1}$ K_2HPO_4, 2.5 $g.l^{-1}$ yeast extract, 1.5 $g.l^{-1}$ peptone and 0.40 $g.l^{-1}$ $MgSO_4.7H_2O$. This yield was lower than the maximum concentration produced in the previous experiment as the changes in factor concentrations were too extreme.

Increasing the concentrations of factors X_2 (peptone) resulted in an increase in riboflavin production by 1.18 $mg.l^{-1}$, which was negligible. A decrease in X_5 ($MgSO_4.7H_2O$) predicted an increase in production yield to 37.24 $mg.l^{-1}$. It was established at this point

that instead of the riboflavin yield increasing from 41.28 $mg.l^{-1}$ in the previous experiment, it decreased to 32.41 $mg.l^{-1}$. Furthermore, the concentrations of factors yeast extract (X_1), malt extract (X_3) and K_2HPO_4 (X_4), were decreased from the previous experiment according to inferences made using equation 4.1 and results recorded in the second screening experiment, showed improvement of riboflavin yields, by increasing the concentrations of the same three factors, which was opposite to changes made to nutrient concentrations when designing the second screening experiment. This implied that the magnitude of alteration in concentration settings from the previous experiment for this experiment, were too large.

The third screening experiment was designed according to inferences made from equation (2) that described the second screening experiment. Concentrations of factors yeast extract (X_1) and malt extract(X_3) was increased from 0.5 - 2.5 $g.l^{-1}$ to 2.5 - 5.0 $g.l^{-1}$ and factor X_4, was increased from 0.025 - 1.0 $g.l^{-1}$ to 0.1 - 0.2 $g.l^{-1}$ in the third screening experiment. The concentrations of peptone and $MgSO_4.7H_2O$ were set at its minimum 0.5 $g.l^{-1}$ and 0.3 $g.l^{-1}$, respectively as they both were non-significant in the production of riboflavin in this experiment.

Riboflavin concentrations recorded at 120 hours in the third screening experiment was analysed using Design Expert 7.1.6. The design together with the response were summarised in Table 4.11. The ratio of maximum to minimum yields was 3.65, and because it is lower than 10, no transformation of the data was required. The Half-Normal Plot (Figure 4.24) indicated factors chosen to be included within the model. Factors malt extract (X_3), peptone (X_2), yeast extract (X_1), peptone-malt extract (X_2X_3), yeast extract-peptone-malt extract ($X_1X_2X_3$), yeast extract-malt extract (X_1X_3) as well as yeast extract-peptone (X_1X_2) were selected as they all were positioned away from the line. A significant model having a *p*-value of 0.0052 was developed using Design Expert. Factors yeast extract (X_1), peptone (X_2) and malt extract (X_3) were significant, with p-

values of 0.0038, 0.0031 and 0.0027, respectively as shown in the ANOVA table (Table 4.12). Effects of individual and combination of components were expressed in the Pareto chart (Figure 4.25). The order of positive effects was:

K_2HPO_4 > malt extract > malt extract-K_2HPO_4 >yeast extract-malt extract-K_2HPO_4>yeast extract-K_2HPO_4 > yeast extract-malt extract

Yeast extract (X_1) which ranked third in order of significance had a negative effect in this experiment while K_2HPO_4 and malt extract had larger positive effects on riboflavin production as could be seen in runs 7 and 8 (Table 4.7), where 55.60 $mg.l^{-1}$ and 66.27 $mg.l^{-1}$ riboflavin, respectively, were produced. In the first screening experiment at concentration range 2.5 to7.5 $g.l^{-1}$, yeast extract had the largest effect on riboflavin production which was also negative. In the second supplement screening experiment, yeast extract at a concentration range of 0.5 to 2.5 $g.l^{-1}$ was the third effective nutrient supplement which had a positive effect. Despite its negative effect on riboflavin production, yeast extract was considered significant as it lay above the Bonferroni limit. A statistical model based on the effects of individual and combination of factors given by the equation accompanying Table 4.17, fitted the data. A *p*-value of 0.0052 which is less than 0.05 indicates that the model is statistically significant and may be described by equation 4.3. Furthermore, all inferences and predictions made from the graphs drawn were significant to 99.48%.

The cube plot (Figure 4.26) illustrated the effects of all three factors and it confirmed the production of maximum riboflavin (66.27 $mg.l^{-1}$) produced in the presence of 0.2 $g.l^{-1}$ K_2HPO_4, 5.0 $g.l^{-1}$ yeast extract and 5.0 $g.l^{-1}$ malt extract. Since yeast extract was least significant and had a negative effect, its concentration was set at 5.0 $g.l^{-1}$ (where maximum riboflavin yield was obtained) while the other two factors were optimized using response surface methodology, based on the central composite design.

A central composite experiment was designed at concentration ranges of 1.0 to 5.0 $g.l^{-1}$ and 0.1 to 0.18 $g.l^{-1}$ for malt extract and K_2HPO_4, respectively. Data recorded at 120 hours together with the experimental design was listed in Table 4.13. The ratio of maximum to minimum (8.09) indicated that no transformation was required. A model with an F-value of 52.69 was significant. The ANOVA (Table 4.14) indicated that there was only a 0.01% chance that a model F-value this large could have occurred due to noise. This model with a *p*-value of <0.0001was significant with only individual factor X_3 (malt extract), being significant. According to the non significant Lack of Fit, the model fitted the data. All inferences and predictions made from graphs drawn were at a predicted confidence of 99.99%. The response surface plot in Figure 4.27 illustrated that the crest in the design space was covered, and according to solutions in numerical optimisation of the software program, the first solution with a desirability of 0.935 producing 91.88 $mg.l^{-1}$ riboflavin was obtained in the presence of 4.78 $g.l^{-1}$ malt extract and 0.18 $g.l^{-1}$ K_2HPO_4 (Table 4.15). Yeast extract was set at 2.5 $g.l^{-1}$, $MgSO_4 \cdot 7H_2O$ at 0.3 $g.l^{-1}$ and peptone at 0.5 $g.l^{-1}$.

To confirm the optimized medium for riboflavin production by mutant *E. gossypii* EMS 30/1 in SMO, a verification experiment with concentration settings for yeast extract, peptone, malt extract, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ at 5.0 $g.l^{-1}$, 0.5 $g.l^{-1}$, 12.5 $g.l^{-1}$, 0.18 $g.l^{-1}$ and 0.3 $g.l^{-1}$, respectively, was conducted in triplicate. A maximum mean concentration of 109.59 $mg.l^{-1}$ riboflavin was produced by mutant *E. gossypii* EMS 30/1 in SMO at 120 hours (Figure 4.28).

A comparison of riboflavin produced by the five riboflavin producing fungi *Eremothecium gossypii* (wild type) (ATCC 10895), *Eremothecium gossypii* (CBS 109.51), *Eremothecium ashbyi* (CBS 206.58), mutant *Eremothecium gossypii* EMS 30/1 and *Candida famata* (ATCC 20850) in the modified O and K medium in which glucose was substituted with spent vegetable oil, *C. famata* produced the most riboflavin (16.99

$mg.l^{-1}$) and was considered the microorganism of choice for this substrate (Figure 4.15). Four screening experiments followed to identify the two most significant factors affecting riboflavin production by this yeast, in SVO.

The first screening experiment was set at 50% higher and 50% lower than the factor concentrations of the standard and the design was summarised together with response data at 120 hours, in Table 4.16. Riboflavin produced at the various time intervals for five days was compared and results indicated that maximum riboflavin was produced at 120 hours. Therefore, the response recorded at this time interval was analysed for all future experiments. The ratio of maximum to minimum concentrations produced recorded in the first supplement screening experiment was 873.19 which indicated that a transformation of the data was required. Hence, a square-root transformation was applied to the data in order to develop a sufficiently significant model from which conclusions could be made. Selection of factors that was included in the model was illustrated in the Half-Normal plot in Figure 4.29, which indicated that factors X_3 (malt extract), X_3X_5 (malt extract-$MgSO_4.7H_2O$), X_1X_5 (yeast extract-$MgSO_4.7H_2O$), X_2X_3 (peptone-malt extract) and X_2 (peptone) that lay away from the line, were chosen. To make the model hierarchical, factors X_1X_3 (yeast extract-malt extract), X_5 ($MgSO_4.7H_2O$) and X_1 (yeast extract), were also selected despite having little significance to riboflavin production in this experiment.

Effects of individual and combinations of factors were graphically illustrated in the Pareto chart (Figure 4.30). Definitely significant factors that lay above the Bonferroni limit, were X_3 (malt extract) which had a positive effect and X_3X_5 (malt extract-$MgSO_4.7H_2O$), which had a negative effect. Combination of factors X_1X_5 (yeast extract-$MgSO_4.7H_2O$), X_2X_3 (peptone-malt extract) and individual factor X_2 (peptone) were significant as they lay above the t-value limit, and all had positive effects on riboflavin production by *C. famata* in SVO, in this first supplement screening experiment. This

chart also showed the inclusion of $X_5(MgSO_4.7H_2O)$ and yeast extract (X_1) which had little significance only to make the mathematical model hierarchical.

The developed model was evaluated using the Box-Cox plot for power transformations (Figure 4.31). The square-root transformation positioned at 0.5, was only 0.05 away from the best transformation was represented by a green line. Furthermore, the model lay within the 95% confidence interval, represented by the red line. This plot validated the model as satisfactory for further analysis and hypothesis testing.

The Pareto chart in Figure 4.32 indicated the positive effects of individual factors malt extract (X_3), peptone (X_2) and $MgSO_4.7H_2O$ (X_5), which were also illustrated in the cube plot in Figure 4.32. This was also confirmed with a maximum riboflavin concentration of 81.22 $mg.l^{-1}$ in the presence of 7.50 $g.l^{-1}$ peptone and 7.50 $g.l^{-1}$ malt extract, 0.1 $g.l^{-1}$ $MgSO_4.7H_2O$, 0.30 $g.l^{-1}$ K_2HPO_4 and 2.50 $g.l^{-1}$ yeast extract. An increase of 8.62 $mg.l^{-1}$ was obtained by decreasing the concentrations of yeast extract (X_1) and increasing that of K_2HPO_4 (X_4).

The model's F-value of 16.80 implied that the model was statistically significant to the data (Table 4.17). There was only a 0.03% chance that an F-value this large could have occurred due to noise. Factors with values of 'Prob>F' less than 0.05, in this case X_2, X_3, X_1X_5, X_2X_3 and X_3X_5, indicate the model terms that are significant. The 'Lack of Fit' of 220.09 implied that there was a 5.19% chance that a Lack of Fit this large could have occurred due to noise. A non-significant Lack of Fit implies that the model fits the data.

A second supplement screening experiment was designed making reference to inferences made using equation (4.6) that mathematically described the first screening experiment. The low and high concentration settings were altered for yeast extract from 2.5 - 7.5 to 0.5 - 2.5 $mg.l^{-1}$, for peptone, from 2.5 - 7.5 to 7.5 - 12.5 mg.l-1, for malt extract, from 2.5 - 7.5 to 7.5 - 12.5 $mg.l^{-1}$, for K_2HPO_4, from 0.1 - 0.3 to 0.3 - 0.5 $mg.l^{-1}$

and for $MgSO_4.7H_2O$ from 0.1 - 0.3 to 0.02 - 0.1 $mg.l^{-1}$. The experimental design together with the recorded response at 120 hours was summarised in Table 4.18.

A square root transformation was applied to the data because the ratio of maximum to minimum riboflavin yields was equivalent to 290.95. The Half Normal Plot (Figure 4.33) which indicated factors chosen to be incorporated into the model which was evaluated by the Box-Cox plot of power transforms in Figure 4.34. This plot suggested that the square-root transformation being set on 'Lambda' (blue line) positioned only 0.26 away from the best transformation (green line), was significant and considered the model satisfactory for future analysis and forthcoming hypothesis testing. Furthermore, this transformation lay within the optimal zone which was within the 95% confidence interval limit.

An assessment of the effects of single and combined factors allowed one to make better decisions about their effects on riboflavin production. The Pareto chart (Figure 4.35) indicated the effects of factors in order of significance. The interaction of yeast extract and K_2HPO_4 had the largest positive effect on the production of riboflavin by *C. famata* in SVO in this screening experiment. The interaction of peptone and $MgSO_4.7H_2O$, peptone by itself followed by the interaction of malt extract and $MgSO_4.7H_2O$, had negative effects on production yields. Malt extract- K_2HPO_4 (X_3X_4), $MgSO_4.7H_2O$ (X_5) and the interaction of peptone-malt extract had positive effects. The individual factors yeast extract, K_2HPO_4 and malt extract although possessed little significance were only selected to develop a model that was hierarchical.

The ANOVA in Table 4.19 indicated that the Model with an F-value of 17.95 was statistically significant with a *p*-value of 0.0005. This implied that there was only a 0.05% chance that a model with an F-value this large could have occurred due to noise. Model terms peptone (X_2), $MgSO_4.7H_2O$ (X_5), X_1X_4, peptone-malt extract (X_2X_3),

peptone-$MgSO_4.7H_2O$ (X_2X_5), malt extract-K_2HPO_4 (X_3X_4) and malt extract-$MgSO_4.7H_2O$ (X_3X_5) were statistically significant with *p*-values of 0.0014, 0.0237, 0.0002, 0.034, 0.0002, 0.0128 and 0.0047, respectively. This implied that the mathematical equation (4.7) accompanying Table 4.19 was significant and all inferences and predictions made from graphs drawn based on the model were significant.

A cube plot (Figure 4.36) was used to assess the interaction of the three factors yeast extract, peptone and $MgSO_4.7H_2O$. A maximum riboflavin yield 23.24 $mg.l^{-1}$ was produced in the presence of 2.50 $g.l^{-1}$ yeast extract, 7.50 $g.l^{-1}$ peptone, 0.1 $g.l^{-1}$ $MgSO_4.7H_2O$, 10 $g.l^{-1}$ malt extract and 0.4 $g.l^{-1}$ K_2HPO_4 and an increase in yield to 23.543 $mg.l^{-1}$ was observed by decreasing the concentration of malt extract and increasing that of K_2HPO_4.

The third supplement screening experimental design together with response data recorded at 120 hours was summarised in Table 4.20. A minimum yield of 1.83 $mg.l^{-1}$ was obtained in the presence of 0.5 $g.l^{-1}$ yeast extract, 8.5 $g.l^{-1}$ peptone, 6.5 $g.l^{-1}$ malt extract, 0.35 $g.l^{-1}$ K_2HPO_4 and 0.05 $g.l^{-1}$ $MgSO_4.7H_2O$ while a maximum yield of 15.82 $mg.l^{-1}$ was produced in the presence of 0.5 $g.l^{-1}$ yeast extract, 6.5 $g.l^{-1}$ peptone, 8.5 $g.l^{-1}$ malt extract, 0.35 $g.l^{-1}$ K_2HPO_4 and 0.05 $g.l^{-1}$ $MgSO_4.7H_2O$. A comparison of the media components showed difference in peptone (X_2) and malt extract (X_3) concentrations. A decrease in peptone concentration by 2.0 $g.l^{-1}$ and an increase in malt extract concentration by 2.0 $g.l^{-1}$, improved riboflavin yield by 13.99 $mg.l^{-1}$. The ratio of maximum is to minimum riboflavin yields was equivalent to 8.6209, which implied that no transformation of the data was required (Table 4.20). The Half-Normal Plot (Figure 4.39) illustrated factors chosen, to be included in the mathematical model. Factors X_3 (malt extract), peptone-K_2HPO_4 (X_2X_4), yeast extract- $MgSO_4.7H_2O$ (X_1X_5) and peptone (X_2) were selected as they appeared significant because of their position, away from the

line. Factors K_2HPO_4 (X_4), $MgSO_4.7H_2O$ (X_5) and yeast extract (X_1) were included to develop a hierarchical model (Figure 4.37).

The Box-Cox Plot for power transformations (Figure 4.38) was used to evaluate the developed model given in Table 4.20. The plot suggested that no transformation of the data was required as the transformation being set on Lambda (blue vertical line) = 1, positioned 0.65 away from the best transformation (green vertical line) set at 0.35. Furthermore, the model lay within the optimal confidence interval demarcated with red lines positioned at -0.68 and 1.06. This evaluated the model as satisfactory for future analysis and hypothesis testing (Figure 4.38).

The developed model was based on the effects of individual and combination of components graphically illustrated in the Pareto chart in Figure 4.39. Individual factor X_3 (malt extract) together with combination of factors peptone-K_2HPO_4 (X_2X_4) and yeast extract- $MgSO_4.7H_2O$ (X_1X_5) lay above the Bonferroni limit, making them definitely significant while peptone (X_2) was positioned above the t-value limit and was possibly significant, and was therefore included in the model. Individual factors K_2HPO_4 (X_4), $MgSO_4.7H_2O$ (X_5) and yeast extract (X_1) with little significance as they lay below the t-value limit, were included in order to develop a hierarchical model (Figure 4.39).

By fitting the data to a statistical model, it was confirmed that malt extract (X_3), followed by peptone-K_2HPO_4 (X_2X_4) then yeast extract- $MgSO_4.7H_2O$ (X_1X_5), with 'Prob>F'-values of 0.0004, 0.0006 and 0.0007, respectively, were significant contributes to riboflavin production. This model with an F-value of 11.55 was statistically significant as indicated by a *p*-value of 0.0013. The derived model with a "Lack of Fit" value of 2.92 implied that the Lack of Fit was not significant due to pure error. There was a 27.67% chance that a Lack of Fit this large could have occurred due to noise. A

non-significant model implied that the model fitted the data, which was the aim when developing the model. The mathematical equation (4.8) accompanying the Table 4.21 was significant and all inferences as well as predictions made from the graphs drawn, based on the mathematical model, were significant to 99.87%.

A cube plot (Figure 4.40) was used to assess the interaction of the three factors malt extract (X_3), peptone (X_2) and K_2HPO_4 (X_4). A maximum riboflavin concentration of 12.29 $mg.l^{-1}$ was produced in the presence of 8.50 $g.l^{-1}$ malt extract, 6.50 $g.l^{-1}$ peptone, 0.35 $g.l^{-1}$ K_2HPO_4. An increase in yield to 12.34 $mg.l^{-1}$ riboflavin was obtained by decreasing the concentration of yeast extract and to 15.03 $mg.l^{-1}$, and by increasing the concentration of $MgSO_4.7H_2O$. Since the minimum concentration for yeast extract was 0 $g.l^{-1}$, this factor was omitted in further experiments. An increase by 2.34 $mg.l^{-1}$ riboflavin was considered negligible by increasing the concentration of non-significant MgSO4.7H2O (*p*-value of 0.3478) led to the decision of setting its concentration at 0.15 $g.l^{-1}$ in all future experiments, leaving only three factors peptone, malt extract and K_2HPO_4 to be investigated in the fourth supplement screening experiment.

The experimental design for the fourth supplement screening experiment was summarised in Table 4.22. A minimum riboflavin yield of 9.15 $mg.l^{-1}$ was produced in the presence of 6.5 $g.l^{-1}$ peptone, 7.5 $g.l^{-1}$ malt extract and 0.35 $g.l^{-1}$ K_2HPO_4 (X_4) and a maximum yield of 69.01 $mg.l^{-1}$, in the presence of 5.5 $g.l^{-1}$ peptone, 7.5 $g.l^{-1}$ malt extract and 0.35 $g.l^{-1}$ K_2HPO_4. The ratio of maximum to minimum concentrations produced (7.92) indicated that no transformation of data was required.

The Half-Normal plot in Figure 4.41 showed factors that were included in the model. Combinations of factors peptone-K_2HPO_4 (X_1X_4) and peptone-malt extract (X_1X_2) positioned away from the line were selected as they were significant while individual

factors peptone (X_1), malt extract (X_2) and K_2HPO_4 (X_4) with little significance was also included to obtain a hierarchical model.

The Box-Cox Plot for power transformations (Figure 4.42) was used to evaluate the developed model given in Table 4.23. The plot suggested that no transformation of the data was required as the transformation being set on Lambda (blue vertical line) = 1, positioned 1.09 away from the best transformation (green vertical line) was set at 2.09. Furthermore, the model lay within the optimal confidence interval positioned between at 0.87 and 3.92. This evaluated the model as satisfactory for further analysis and hypothesis testing (Figure 4.42).

Effects of individual and combination of factors were graphically illustrated in the Pareto chart (Figure 4.43) which showed combined components X_1X_4 and X_1X_2 to be significant as they lay above the t-Value limit. Individual factors yeast extract (X_1), peptone (X_2) and K_2HPO_4 (X_4) with little significance were also included in the model to make it hierarchical.

By fitting the data to a statistical model with an F-value of 7.33 and a *p*-value of 0.0383 (Table 4.22), it was confirmed that combined factors yeast extract-peptone (X_1X_2) and yeast extract-K_2HPO_4 (X_1X_4), were significant nutrient factors in the production of riboflavin by *C. famata* in supplemented SVO. A model with a "Lack of Fit" value of 2.13 implied that the Lack of Fit was not significant due to pure error. There was a 31.93% chance that a Lack of Fit this large could have occurred due to noise. A non-significant model implied that the model fitted the data. The mathematical equation 4.9 accompanying the Table 4.23 was significant and all inferences as well as predictions made from the graphs drawn, based on the mathematical model, were significant to 96.17%.

The effects of peptone, malt extract and K_2HPO_4 were assessed using the cube plot in Figure 4.44. A maximum yield of 79.24 $mg.l^{-1}$ was obtained in the presence of 6.5 $g.l^{-1}$ peptone, 10 $g.l^{-1}$ malt extract and 0.45 $g.l^{-1}K_2HPO_4$. Malt extract had a greater positive effect than K_2HPO_4 while peptone (X_2) had a negative effect. The concentration of peptone was then set at 6.5 $g.l^{-1}$, resulting in malt extract and K_2HPO_4, to be investigated in the central composite experiment that followed.

A two-level, two-factor, resolution five, central composite experimental design that consisted of 13 runs, 5 of which were center points was designed using Design Expert 7.1.6. Only malt extract was optimized in this experiment. The ratio of maximum to minimum concentrations produced was 4.12 which implied that no transformation of the data was required.

The Box-Cox Plot for power transformations (Figure 4.45) was used to evaluate the developed model given in Table 4.24 which listed the design together with recorded data at 120 hours. The plot suggests that no transformation of the data was required as the transformation being set on Lambda (blue vertical line) = 1, positioned 1.1 away from the best transformation (green vertical line) set at 2.1. Furthermore, the model lay within the optimal confidence interval positioned between at 0.42 and 3.93. This evaluated the model as satisfactory for future analysis and hypothesis testing (Figure 4.45).

ANOVA within the model indicated that the model had an F-value of 5.40 and was statistically significant with a *p*-value of 0.0237 (Table 4.25). This implied that there was only a 2.37% chance that a Model F-value this large could have occurred due to noise. Those factors with 'Prob>F'-values less than 0.05 indicate model terms that are significant. In this case, only $(X_3\text{-}X_4)^2$ with a 'Prob>F'-value of 0.0028 significantly contributed towards the mathematical model. A model with a "Lack of Fit" value of 5.42 implied the Lack of Fit was not significant due to pure error. There was a 6.81% chance

that a Lack of Fit this large could have occurred due to noise. A non-significant model implied that the model fitted the data, which was the intention when developing a model. The mathematical equation (4.10) accompanying the Table 4.25 was significant to the data and all inferences as well as predictions made from the graphs drawn, based on the mathematical model, were significant to 96.73%.

The response surface plot (Figure 4.46) showed the effect of the two factors on riboflavin production by *C. famata* in the central composite experiment in which the optimum value of only malt extract was determined (12.5 $g.l^{-1}$). The optimum concentration of K_2HPO_4 was established in a one-factor-at-a-time (OFAT) experiment set at various K_2HPO_4 concentrations ranging from 0.65 to 2.0 $g.l^{-1}$. This experiment consisted of 8 runs set a different K_2HPO_4 concentrations. A comparison of riboflavin production at 120 hours by *C. famata* in supplemented SVO at the various K_2HPO_4 concentrations clearly showed the optimum concentration of K_2HPO_4 to be 1.75 $g.l^{-1}$ (Figure 4.47).

The optima for all five nutrient factors formed the optimized riboflavin production medium for *C. famata* in supplemented SVO (Table 3.18). A confirmatory test using this medium was conducted to verify the results obtained. A maximum of 80.10 $mg.l^{-1}$ was produced after 120 hours (Figure 4.48).

CONCLUSIONS

- Spent motor oil and SVO, the two most easily available spent oils and five selected riboflavin-producing fungi were selected for this study and all five fungi were found to grow on the two selected oil types.
- A comparison of riboflavin production in each oil resulted in mutant *E. gossypii* EMS 30/1 and *C. famata* being considered as superior fungi as they produced the highest concentrations in SMO (20.45 $mg.l^{-1}$) and SVO (16.99 $mg.l^{-1}$), respectively.
- Screening using fractional factorial experimental design for the most important nutrients affecting riboflavin production resulted in malt extract and K_2HPO_4 having the largest effect, in both bioprocesses.
- Both these nutrients were optimized using central composite experimental design resulting in media for SMO and SVO that produced 103.59 $mg.l^{-1}$ and 82.75 $mg.l^{-1}$ riboflavin by mutant *E. gossypii* EMS 30/1 and *C. famata*.
- Riboflavin increased by 406% and 387% in supplemented SMO and SVO respectively after optimization of media components.

FUTURE WORK

To optimize this process even further, abiotic factors such as pH, temperature and agitation speed shall be investigated and optimized using statistical experimental design.

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APPENDICES

Appendix 1

Riboflavin Standard Curve (Horwitz, 2000)

Riboflavin stock:

100 mg (1.0 g) USP riboflavin was dissolved in 500ml of 0.02M CH_3COOH using a 500 ml volumetric flask. Flasks were wrapped in foil to prevent degradation of the riboflavin in the presence of light.

Diluent:

Since riboflavin was to be measured after growing fungi in oil-containing O and K medium, the diluent used was prepared as follows:

One and a half litres modified O and K medium (glucose substituted with spent oil) was prepared. The oil was extracted with 1.5 l hexane/isopropanol solution (Stahmann *et al.*, 1994).

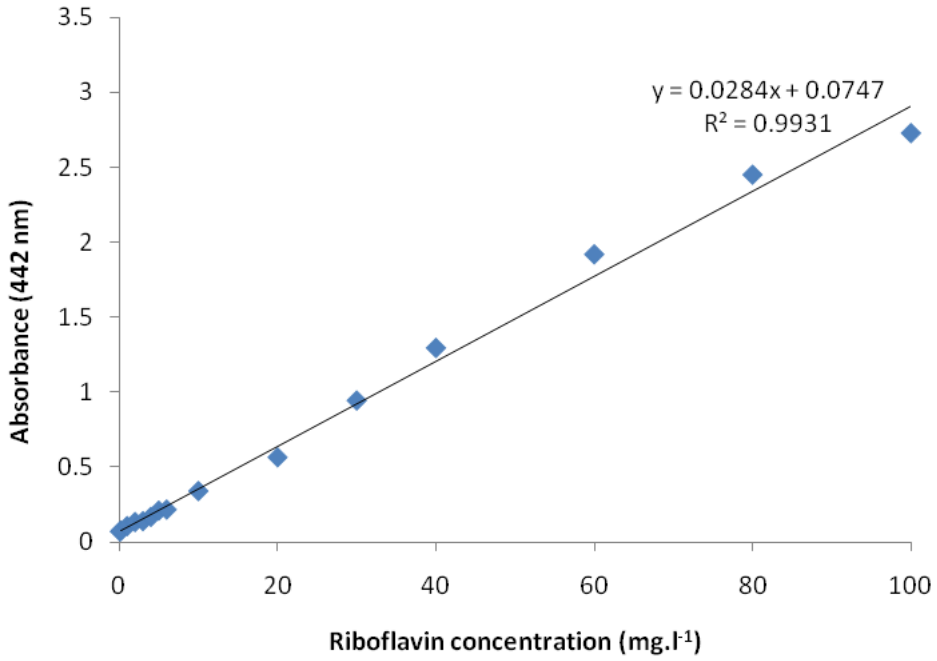
The following solutions were prepared using Beckman P100, P200, P1000 and P5000 micropippetes.

Solutions were stored darkness to prevent denaturation of the riboflavin.

Table 3.3 Dilutions prepared for the riboflavin standard curve (Horwitz, 2000)

	Riboflavin	Diluent (ml)	DF	[riboflavin] $mg.l^{-1}$	Absorbance (442 nm)
1	50 ul	99.95	2000	0.1	0.0716
2	100 ul	99.9	1000	0.2	0.0785
3	500 ul	99.5	200	1	0.1083
4	1000 ul	99	100	2	0.1353
5	1500 ul	98.5	66.67	3	0.1407
6	2000 ul	98	50	4	0.1697
7	2500 ul	97.5	40	5	0.2109
8	3 ml	97	33.33	6	0.2183
9	5 ml	95	20	10	0.3417
10	10 ml	90	10	20	0.56616
11	15 ml	85	6.66	30	0.9446
12	20 ml	80	5	40	1.295
13	30 ml	70	3.33	60	1.9177
14	40 ml	60	2.5	80	2.4484
15	50 ml	50	2	100	2.7253

The absorbance at 442 nm for each solution was measured to establish the riboflavin standard curve below which was used to extrapolate sample concentrations.



Riboflavin standard curve in acetic acid solution.

Appendix 2

Glucose Assay (Wang, 2006)

Reagents:

Dinitrosalicylic Acid Reagent Solution, 1%

Dinitrosalicylic acid: 10 g

Phenol: 2 g (optional, see Note 1)

Sodium sulfite: 0.5 g

Sodium hydroxide: 10 g

Add water to: 1 liter

Potassium sodium tartrate solution, 40%

Method

1. Three millilitres of DNS reagent was added to 3 ml of sample in a lightly capped test tube. (To avoid the loss of liquid due to evaporation, the test tubes were covered with a piece of paraffin film)
2. The mixtures were heated in a 90ºC water bath for 5-15 minutes to develop the red-brown color.
3. One millilitre 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the color.
4. After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer at 575 nm.