Impact of malonate on the metabolism and fatty acid synthesis of genetically engineered *saccharomyces cerevisiae*

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**IMPACT OF MALONATE ON THE METABOLISM AND
FATTY ACID SYNTHESIS OF GENETICALLY
ENGINEERED *SACCHAROMYCES CEREVISIAE***

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Publications

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Abbreviations

ACC	Acetyl-CoA carboxylase
CoA	Coenzyme A
BCCP	Biotin carboxyl carrier protein
BC	Biotin-carboxylase
CT	Carboxyl-transferase
FAS	Fatty acid synthase
HPLC	High-performance liquid chromatography
GC	Gas chromatography
LC-MS	Liquid chromatography–mass spectrometry
THF	Tetrahydrofuran
ESI	Electrospray ionisation
ACPI	Atmospheric pressure chemical ionisation
APPI	Atmospheric pressure photo-ionisation
TOF	Time-of-flight
ICAT	Isotope-coded affinity tags

TMT	Tandem mass tags
iTRAQ	Isobaric tags for relative and absolute quantitation
SILAC	Stable isotope labelling with amino acids in cell culture
PCR	Polymerase chain reaction
RT-PCR	Reverse Transcriptase polymerase chain reaction
HRP	Horseradish peroxidase
BSA	Bovine serum albumin
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
DTT	Dithiothreitol
PVDF	Hybond-P Polyvinylidene Fluoride
TCA	Tricarboxylic acid cycle
OD ₆₀₀	Optical density at wavelength 600nm

Summary

In view of the increasing global energy usage, biological fuel production has proved to be able to serve as a sustainable, carbon-neutral energy source compatible with current engine technology. Biofuels include fuels derived from biomass conversion, as well as solid biomass, liquid fuels and various biogases. The current range of biofuels consists primarily of microbially derived fatty acids, ethanol and plant-based biodiesel. The use of microbial systems for the production of industrially relevant compounds has been popular in the past years as a direct result of the genomics revolution. Further advances in gene regulation, protein engineering, pathway portability, synthetic biology and metabolic engineering have propelled the development of cost-efficient systems for biofuel production.

Malonyl-CoA plays an important role in the synthesis and elongation of fatty acids in yeast *Saccharomyces cerevisiae*. It is one of the main components for the initiation of the fatty acid synthesis and also acts as a building block for the elongation of fatty acid after every round of fatty acid synthesis. However, Malonyl-CoA is at a low concentration inside the cell and it is produced mainly from Acetyl-CoA through the actions of the enzyme acetyl-CoA carboxylase (ACC). As a result, it would be beneficial to find an alternative source of Malonyl-CoA and thus increasing its intracellular concentration. By doing so, the overall synthesis of the fatty acids inside the yeast should increase as well.

MatB gene from the bacteria, *Rhizobium leguminosarium* bv *trifolii* encodes for a malonyl-CoA synthetase which is able to catalyze the formation of the Malonyl-CoA directly from malonate and CoA with the hydrolysis of ATP. However, results from HPLC proved that *Saccharomyces cerevisiae* itself does not contain enough cytoplasmic malonate within them and is not able to uptake exogenously supplied malonate in the form of malonic acid.

As such, a gene known as the *mae1* gene from another species of yeast, *Schizosaccharomyces pombe* had been successfully cloned and transformed inside the target yeast, *Saccharomyces cerevisiae*. This gene encodes a dicarboxylic acid plasma membrane transporter which enables the cells to uptake exogenous malonic acid. Yeast immunofluorescence was used to detect the presence and localization of the expressed proteins in the target cells. The results had convincingly showed that the *mae1* gene is successfully expressed and the expressed dicarboxylic acid transporter proteins were localized to the plasma membrane of the cells as intended. Furthermore, HPLC and LC-MS were also able to provide substantial results to show the existence of the encoded protein, which is the plasma membrane dicarboxylic acid transporter. With the correct negative controls within HPLC and LC-MS, the functional activities of the protein could also be demonstrated and verified. Therefore, the positive results from HPLC and LC-MS, together with the positive results from RT-PCR and yeast immunofluorescences, the plasma membrane dicarboxylic acid transporter was verified to be successfully expressed and functioning as intended as malonic acid was

detected inside the transformed cells and having a significant impact on the proteomics of the cells as demonstrated by the LC-MS results.

Being an inhibitor to the succinate dehydrogenase of the citric acid cycle in the mitochondria, malonic acid, after being transported into the yeast cells, seem to have a certain degree of toxicity displayed towards the cells. From the LC-MS results, most of the up-regulated proteins were those that were involved one way or another in the metabolism of carbohydrates to produce energy. It is also known that when the citric acid cycle was impaired due to post-mitotic aging or a result of activity from inhibitors such as malonate, alternative mechanism would be triggered to continue supply energy for the survivability of the cells. In this case, the glyoxylate cycle is activated. This is evident from the LC-MS results as the enzymes involved in the glyoxylate cycle were shown to be significantly up-regulated.

Among those proteins that were down-regulated, 6-phosphogluconate dehydrogenase was decreased by around 40%. This dehydrogenase catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO₂, with concomitant reduction of NADP to NADPH in the pentose phosphate. Furthermore, inositol-3-phosphate synthase, which catalyzes the chemical reaction of converting D-glucose 6-phosphate to 1D-myo-inositol 3-phosphate to form phospholipids, was also decreased by around 60%. This hinted at an energy deprived state of the cells where carbohydrates such as glucose seem to be channelled away from the other pathways and was used to increase the rate of glycolysis.

Next, the *MatB* gene from the bacteria, *Rhizobium leguminosarium* bv *trifolii* was cloned and expressed in the yeast cells with the *mae1* gene. When grown in medium containing malonic acid, the yeast cells, containing the 2 genes, were able to grow at a normal rate as compared to the wild type yeast cells. Furthermore, the toxicity due to the intake of malonate exhibited by the cells with only the *mae1* gene seemed to be eliminated when growth curves were compared. Results also showed that yeast cells that contained the 2 genes were also taking in more malonate from the medium as compared to cells that only contained the *mae1* gene. The increased uptake of malonate and the reduced toxicity exhibited by the cells showed that the malonate transported in were utilized and not accumulated to inhibit the citric acid cycle. Results from HPLC showed that the amount of malonate present in the cells were indeed much lower than those present in cells with only the *mae1* gene. Fatty acid profiling also showed a significant increase in the amount of fatty acids produced by the cells with 2 genes as compared with wild type yeast cells and yeast cells with only the *mae1* gene. Fatty acids that were typically produced by the *Saccharomyces cerevisiae* cells such as palmitic acid, palmitoleic acid, stearic acid, oleic acid and linoleic acid were significantly increased and accumulated. This verified the functional expression of the *matB* gene and the ability of the encoded malonyl-CoA synthetase to increase the overall amount of fatty acids produced.

1. Introduction

1.1 Production of biofuels

In view of the increasing global energy usage, biological fuel production has proved to be able to serve as a sustainable, carbon-neutral energy source compatible with current engine technology [1]. Biofuels include fuels derived from biomass conversion, as well as solid biomass, liquid fuels and various biogases. The current range of biofuels consists primarily of microbially derived fatty acids, ethanol and plant-based biodiesel [2].

In 2010, worldwide biofuel production reached 105 billion liters (28 billion gallons US), up 17% from 2009, and biofuels provided 2.7% of the world's fuels for road transport, a contribution largely made up of ethanol and biodiesel [3]. Global ethanol fuel production reached 86 billion liters (23 billion gallons US) in 2010, with the United States and Brazil as the world's top producers, accounting together for 90% of global production. The world's largest biodiesel producer is the European Union, accounting for 53% of all biodiesel production in 2010 [3]. As of 2011, mandates for blending biofuels exist in 31 countries at the national level and in 29 states/provinces [4]. According to the International Energy Agency, biofuels have the potential to meet more than a quarter of world demand for transportation fuels by 2050 [5]. Although biodiesel is favored in several European countries, ethanol dominates the majority of the world biofuel market, including that of the United States [6].

The use of microbial systems for the production of industrially relevant compounds has seen substantial gains in the past years as a direct result of the genomics revolution. Further advances in gene regulation, protein engineering, pathway portability, synthetic biology and metabolic engineering will propel the development of cost-efficient systems for biofuel production [6].

In this project, the yeast *Saccharomyces cerevisiae*, has been proposed as a suitable candidate for such production of biofuels. *Saccharomyces cerevisiae* offers quite a lot of advantages for lipidomics due to its high accessibility of its molecular and classical genetics, the ease of cultivation and its short generation time. Furthermore, it had served as the prime model organism for studying the molecular organization and regulatory circuitry of eukaryotic lipidomes [7]. Therefore, it is a good candidate to generate precursor for biofuels such as fatty acids through genetic and metabolic engineering.

Malonyl-CoA plays an important role in the synthesis and elongation of fatty acids in yeast *Saccharomyces cerevisiae*. It is one of the main components for the initiation of the fatty acid synthesis and also acts as a building block for the elongation of fatty acid after every round of fatty acid synthesis. However, Malonyl-CoA is at a low concentration inside the cell and it is produced mainly from Acetyl-CoA through the actions of the enzyme acetyl-CoA carboxylase (ACC) [8].

As such, the aim of the project is to find alternative source of Malonyl-CoA and thus increasing its intracellular concentration. By doing so, the overall synthesis of the fatty acids inside the yeast was expected to increase as well.

1.2 Fatty acid synthesis in yeast

Fatty acid is one of the most important precursors for biofuels. Moreover it is also an essential compound in the cell serving multiple functions [8]. The accumulation spectrum of fatty acids in yeast cells such as *Saccharomyces cerevisiae* consists mainly of fatty acids with 16 carbons and 18 carbons. Due to a reaction usually catalysed essentially by desaturases, Ole1, 80% of yeast fatty acids are usually monounsaturated. Minor species include fatty acids with 14 carbons and 26 carbons. These fatty acids play essential functions in modifying proteins and also act as components of sphingolipids and GPI-anchors [8].

Intracellular fatty acids are usually derived from three different sources such as endogenous lipid and protein turnover, de novo synthesis and external sources as shown in Figure 1.

Figure 1: Schematic representation of fatty acid metabolism [9]. (Permission from ref.9 was obtained from publisher to use this figure.)

However, yeast cells grown in environment such as laboratories do not usually get their needed fatty acids from the culture medium. As such, they gained their fatty acids through de novo synthesis. On the other hand, if the culture medium does indeed contain fatty acids, they can be readily absorbed by the yeast cells and incorporated into lipids. This is usually what happens in yeast cell's natural habitat. During lipolysis or when adjustments of specific acyl-compositions of membrane phospholipids are required, neutral and phospholipids usually go through fast turnover [10]. Such reactions usually produced a significant amount of toxic fatty acids and removals of such fatty acids require the activation of coenzyme A by fatty acid activation enzymes. Furthermore, nearly all organelles inside a cell structure are one way or another involved in fatty acid metabolism. As such, the regulation and maintenance of fatty acid homeostasis require multiple regulation mechanisms [8].

Although the enzymes involved in the fatty acid synthesis as well as their molecular structures are quite different among the different species, the reaction mechanisms are usually the same in all these different types of cells as shown in Figure 2.

Figure 2: Reaction schemes of fatty acid synthesis and elongation [8]. (Permission from ref.8 was obtained from publisher to use this figure.)

In an initial step, acetyl-CoA is carboxylated by the addition of CO₂ to malonyl-CoA, by the enzyme acetyl-CoA carboxylase (ACC; encoded by ACC1 and HFA1 in yeast). Biotin is an essential cofactor in this reaction, and is covalently attached to the ACC apoprotein, by the enzyme biotin: apoprotein ligase (encoded by BPL1/ACC2 in yeast). ACC is a trifunctional enzyme, harboring a biotin carboxyl carrier protein (BCCP) domain, a biotin-carboxylase (BC) domain, and a carboxyl-transferase (CT) domain. In most bacteria, these domains are expressed as individual polypeptides and assembled into a heteromeric complex. In contrast, eukaryotic ACC, including mitochondrial ACC variants (Hfa1 in yeast) harbor these functions on a single polypeptide. Malonyl-CoA produced by ACC serves as a two carbon donor in a cyclic series of reactions catalyzed by fatty acid synthase (FAS) and elongases [8].

In most bacteria but also in mitochondria or in chloroplasts of eukaryotic cells, the reactions associated with saturated fatty acid synthesis are catalyzed by dissociated, individual gene products (type II FAS systems), similarly to the initial ACC reaction. In contrast, in mammals or in yeast, the individual functions involved in cytosolic fatty acid synthesis are represented as discrete domains on a single or on two different polypeptide chains, respectively. Yeast cytosolic fatty acid synthase is composed of two subunits, Fas1 (β subunit) and Fas2 (α subunit) which are organized as a hexameric $\alpha_6\beta_6$ complex [11]. Fas1 harbors acetyl transferase, enoyl reductase, dehydratase, malonyl-palmitoyl transferase activities; Fas2 contains acyl carrier protein, 3-ketoreductase, 3-ketosynthase and the phosphopantetheine transferase activities [11].

Acetyl-CoA is the C2-carbon donor for fatty acid synthesis and elongation, which is also typically initiated by the attachment of acetyl-CoA to the FAS complex. However, propionyl-CoA or longer chain fatty acids may also initiate fatty acid synthesis, potentially giving rise to odd acyl-chain numbers. Carbon dioxide is required for the carboxylation of acetyl-CoA to malonyl-CoA in the ATP-dependent reaction catalyzed by acetyl-CoA carboxylase. However, since the condensation reaction of FAS or elongases releases carbon dioxide, there is no net requirement for carbon dioxide in fatty acid synthesis and elongation. NADPH which is required for two reduction steps in the fatty acid elongation cycle is mainly produced by malic enzyme (decarboxylating malate dehydrogenase), and the pentose phosphate pathway (glucose 6-phosphate dehydrogenase and decarboxylating P-gluconate dehydrogenase). NADP⁺ may also be formed by NAD kinase, and NAD(P) transhydrogenases may be involved in establishing NAD/NADP ratios, depending on the cellular energy status. Quite remarkably, although the redox potentials for NAD⁺/NADH and NADP⁺/NADPH are quite similar ($E^{\circ} = -320$ mV and -324 mV, respectively), most cellular NAD is present in the oxidized form, and NADP in its reduced form. Obviously, fatty acid synthesis is restricted to conditions of high energy load of the cells, indicated by increased ATP/AMP ratio, elevated reduction equivalents and elevated acetyl-CoA pool. Thus, fatty acid synthesis may also be considered an efficient means to control cellular acetyl-CoA and NAD(P)H levels [8].

1.2.1 Malonyl-CoA

Malonyl-CoA plays an important role in the synthesis and elongation of fatty acids in yeast *Saccharomyces cerevisiae*. It is one of the main components for the initiation of the fatty acid synthesis and also acts as a building block for the elongation of fatty acid

after every round of fatty acid synthesis. However, Malonyl-CoA is at a low concentration inside the cell and it is produced mainly from Acetyl-CoA through the actions of the enzyme acetyl-CoA carboxylase (ACC) [8].

Malonyl-CoA synthetase is an enzyme that catalyzes the formation of malonyl-CoA directly from malonate and CoA with the hydrolysis of ATP into AMP and PPi in the presence of Mg²⁺ as shown as the reaction below [12].

This enzyme was first discovered in the bacteroids, *Bradyrhizobium japonicum*, of soybean nodules. The malonate-specific enzyme has long been expected to exist in nodules since free malonate is known to occur in legumes, and its level increases under symbiotic conditions. It was also reported that in the symbiotic host plant cell, malonate is passively transported into bacteroids. However, nothing is known about the fate of malonate in bacteroids. This enzyme was first purified from the symbiotic bacteria *B. japonicum* that is grown on a GYP medium, and later from *Rhizobium leguminosarium* bv *trifolii*, which has symbiosis with clover. The high substrate specificity of malonate, CoA and ATP, has been revealed, but Mn²⁺ could be substituted for Mg²⁺ with no difference in activity. Also, during the catalysis, malonyl-AMP is formed as a reaction intermediate [12].

The *Mat* operon in *R. leguminosarium* bv *trifolii* consists of 4 genes that encodes malonyl-CoA decarboxylase (*matA*), malonyl-CoA synthetase (*matB*), a putative malonate carrier protein (*matC*), and a regulatory protein (*matR*). A gene cluster that

consists of three consecutive genes, *matABC*, was first isolated using a probe that was prepared from the amino acid sequence information of malonyl-CoA synthetase, and was subsequently sequenced. The *matA* and *matB* sequences were overlapped by four base pairs; whereas, the intergenic region between *matB* and *matC* had 95 base pairs. The ribosome binding sites were found 7 to 12 base pairs upstream of each gene. The *MatA* gene encoded a polypeptide of 462 amino acid residues with a deduced molecular mass of 51,414 Da. It was confirmed to be a malonyl-CoA decarboxylase. *MatB* encoded a polypeptide of 504 amino acid residues with a deduced molecular mass of 54,612 Da. This gene was expressed in *E. coli* and characterized to be essentially identical to the native malonyl-CoA synthetase. *MatC* encoded a 46,453 Da protein with a high content of hydrophobic residues. It showed similarities to the dicarboxylate carrier protein, indicating that it might be a malonate carrier protein. These results strongly suggest that the gene cluster encodes proteins that are involved in the malonate-metabolizing system, where exogenous malonate is transported into the cells and is used to produce malonyl-CoA and acetyl-CoA in *R. leguminosarium* bv *trifolii*. Also, the metabolic pathway in the malonate-rich clover nodule might play an important role in symbiosis [12].

In addition to *matABC*, a novel gene (coined *matR*) was discovered on the upstream region of *R. leguminosarium* bv *trifolii* *mat* operon. The *matR* gene product (MatR) interacts specifically with the DNA fragment that contains the upstream region of the promoter. MatR has an N-terminal DNA-binding domain that employs a helix-trun-helix motif and the C-terminal domain that is involved in malonate binding. The addition of malonate increased the association of MatR and the DNA fragment [12].

As such, the *MatB* gene which encodes for the malonyl-CoA synthetase can be utilized through genetic engineering to enable our target yeast cell, *Saccharomyces cerevisiae* to increase the production of malonyl-CoA using exogenous malonate. With the enhanced production of malonyl-CoA, the target of increasing the overall fatty acids production can be achieved and this forms the basis of the project.

1.2.2 Dicarboxylic acid transporter

Malonic acid, which malonate is derived from is a dicarboxylic acid. However, there is no dicarboxylic acid transporter reported to be present on the plasma membrane of *Saccharomyces cerevisiae* [13]. Thus, *Saccharomyces cerevisiae* lacks the ability to take in exogenous dicarboxylic acid such as malonic acid.

Another species of yeast known as *Schizosaccharomyces pombe* was reported to have such dicarboxylic acid transporter present on its plasma membrane. *Mae1* was identified as the gene responsible for the coding of this plasma membrane dicarboxylic acid transporter. It corresponds to a 49-kDa protein with 10 transmembrane predicted segments that has been classified in the TDT family of telurite and dicarboxylate transporters. The *Mae1* gene encodes a permease for malate and other C4 dicarboxylic acids, including malonic acid and behaves as a proton symporter not subjected to glucose repression [13].

Cloning and expression of this *maeI* gene in *Saccharomyces cerevisiae* had been performed before elsewhere [14] and the physiological characterization of the *S. cerevisiae* strain transformed with the *S. pombe maeI* gene showed that the monoanionic form of malic acid, together with other dicarboxylic acid such as malonic acid is actively transported [14]. The transport mechanism is reversible, accumulative and dependent both on the transmembrane gradient of the substrate. Maleic, oxaloacetic, malonic, succinic and fumaric acids inhibit malate transport, suggesting that these compounds share the same carrier [14].

1.3 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC), is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture. HPLC is also considered an instrumentation technique of analytical chemistry, instead of a gravimetric technique. HPLC has many uses including medical, legal, research and manufacturing.

HPLC relies on the pressure of mechanical pumps on a liquid solvent to load a sample mixture onto a separation column, in which the separation occurs. A HPLC separation column is filled with solid particles such as silica, polymers, or sorbents, and the sample mixture is separated into compounds as it interacts with the column particles. HPLC separation is influenced by the liquid solvent's condition like pressure, temperature, chemical interactions between the sample mixture and the

liquid solvent and chemical interactions between the sample mixture and the solid particles packed inside of the separation column.

HPLC is distinguished from ordinary liquid chromatography because the pressure of HPLC is relatively high, while ordinary liquid chromatography typically relies on the force of gravity to provide pressure. Due to the higher pressure separation conditions of HPLC, HPLC columns have relatively small internal diameter, are short, and packed more densely with smaller particles, which helps achieve finer separations of a sample mixture than ordinary liquid chromatography can. This gives HPLC superior resolving power when separating mixtures, which is why it is a popular chromatographic technique.

The schematic of an HPLC instrument typically includes a sampler by which the sample mixture is injected into the HPLC, one or more mechanical pumps for pushing liquid through a tubing system, a separation column, a digital analyte detector such as a UV detector for qualitative or quantitative analysis of the separation, and a digital microprocessor for controlling the HPLC components and user software.

HPLC has been used as an efficient and thorough method in analysing the presence and content of organic acid present in the various coffee beans [15]. The methodology of extracting, detecting and analysing of organic acids using HPLC has thus been well established and is a reliable method to detect organic acids including malonic acid.

1.3.1 Fatty acid analysis using HPLC

Analysis of common fatty acids (with one straight chain and one acid group) is usually carried out by gas chromatography (GC) but in special cases it may be necessary to process HPLC separations. The greatest value of HPLC is for volatile components (short chain fatty acids), for preparative scale separations or for studying isotopically labelled fatty acids. A simple and rapid method for determination of short-chain fatty acids by HPLC with ultraviolet detection has been reported [16]. For some samples, these short-chain fatty acids may be previously concentrated by ultrafiltration [17]. A headspace solid-phase microextraction procedure for the determination of free volatile fatty acids in waste waters has been reported [18].

Positional and conformational isomers are more easily separated by HPLC than GC. All kinds of detectors may be used but separations of derivatized fatty acids are usually monitored with UV spectrophotometer or by fluorimetry. Sometimes, fatty acids are separated without any derivatization either for quantitative estimation or for preparative purposes. A reversed-phased HPLC separation of underivatized fatty acids from oils and animal tissues was proposed after low temperature saponification [19]. A simple HPLC system allowing the separation of short, medium, and long chain fatty acids has also been described [20]. However, a more sophisticated and precise method combining HPLC and mass spectrometry was developed to measure short-chain fatty acids in blood [21]. A precise and facile analysis of short-chain fatty acids using 4-nitrophenol as derivatization reagent has also been proposed [22].

Efficient purification and analysis procedures of polyunsaturated methyl esters have been described using reversed-phase HPLC and light-scattering detection [23]. A similar method has also been developed for the separation and quantitative analysis of fatty acid methyl esters in three vegetal oils (soybean, rice bran, pumpkin seed), response factors being accurately determined [24]. A HPLC method with an evaporative light-scattering detector has been developed for the separation and quantitative analysis of four underivatized long chain fatty acids present in vegetable oils (camellia oil, olive oil, *Brucea javanica* oil and sesame oil) [24].

A very sensitive fluorescence method for the direct determination of free fatty acids was proposed using the reagent DBD-PZ from Tokyo Chemical Industry Co, Product N° A5555. A new BODIPY-based carboxyl-reactive fluorescent labeling reagent, TMBB-EDAN has been developed for the sensitive fluorimetric determination of fatty acids with HPLC [25]. The derivatization of TMBB-EDAN with fatty acids can be performed at room temperature. The detection limits range from 0.2 to 0.4 nM, which are lower than most of the derivatization-based HPLC methods for fatty acids.

The coupling of HPLC on a normal phase coupled with an ozonolysis reactor and a mass spectrometer has been used for the direct determination of double bond position in fatty acid mixtures [26].

1.4 Proteomics study using liquid chromatography–mass spectrometry (LC-MS)

Liquid chromatography–mass spectrometry (LC-MS) is a chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally its application is oriented towards the general detection and potential identification of chemicals in the presence of other chemicals like in a complex mixture. Preparative LC-MS system can be used for fast and mass directed purification of natural products extracts and new molecular entities important to food, pharmaceutical, agrochemical and other industries.

Proteomics was first proposed and defined as the large scale characterization of the total protein components of a tissue, an organism or a cell line. The proteomics functions as a bridge between the genomic function and complex cellular structure and behaviour [27].

The method LC-MS is a useful tool for proteomics analysis. The LC part is used for the components separation and the MS part is used for the components identification. Normally, the total LC-MS system consists of chromatography columns, which can separate different peptide mixtures based on their physicochemical properties difference; the ionization source such as electrospray ionization or matrix-assisted laser desorption/ionization, used to build charges on eluted peptides for identification;

mass analyser, used to separate ions on the basis of m/z ratios and finally, a detector, which can detect the relative abundance of ions at discrete m/z .

In most cases, a design of tandem MS can overcome the problem of ambiguous results limitation. For the first MS step, the precursor ion, which represents the intact peptide, is detected and in the following step, the precursor ion is isolated from other peptide ions and then dissociated into fragments, and then, the mass of the peptides from the second step is determined and form a MS/MS spectrum. By software analysis and comparison with databases, the quantitation results can be obtained.

The established methods which are used in relative quantitative of proteins should undergo isotope labeling by amino acids in cell extracts, chemical labeling and label-free quantification. Commonly, the Isobaric tag for relative and absolute quantitation, known as the iTRAQ, is usually used as one of the chemical labeling. In this method, up to four protein samples can be analyzed simultaneously with the same operation [28]. According to the provided process, the proteins are digested to peptides and each peptide is labeled, and they then appear at the same mass and then isolated and identified [29].

1.4.1 General Proteomics

Generally, proteomics is the study of the whole set of proteins produced by a cell. These proteins are expressed by the genome of that particular cell and include post transcriptional modifications such as phosphorylation and glycosylation. Since

proteins are responsible for almost every metabolisms and reactions inside the cell, a study of their functions and expressions will provide insight into the state of the cell. As such, it is more significant and informative to study protein expression and functional levels as compared to expression level of genes alone.

In proteomics, the proteins needed to be separated and resolved first. This is usually achieved via two methods, gel-based proteomics and chromatography-based proteomics. 2-D gel electrophoresis which is coupled to mass spectrometry is the common setup employed in gel-based proteomics. In 2-D gel electrophoresis, proteins are separated based on two dimensions with the first dimension being their pH and the second dimension being their molecular weight. After the gel electrophoresis is done, the proteins are usually stained and by comparing such stained gels of different samples, proteins may be isolated and identified. Furthermore, proteins trapped in the gel may be enzymatically digested with trypsin and then sent for peptide sequence analysis using mass spectrometry. However, one of the most significant disadvantages of such method is the inability to detect proteins that are low in abundance during the gel staining stage.

Chromatography-based proteomics, on the other hand, removes the need of gel staining and peptide extraction. Moreover, proteins that are low in abundance can also be reliably detected using this method. This is possible as the whole sample can be pre-purified using liquid chromatography and then sent for mass spectrometry analysis. Common liquid chromatography methods used in this application includes HPLC and capillary electrophoresis. As this method proves to be more efficient in resolving

proteins with higher degree of complexity and its ability to detect low abundance proteins, it has largely replaced gel-based proteomics in protein study.

1.4.2 Liquid Chromatography

In LC-MS, the sample is first resolved by the liquid chromatography component. Many different types of columns are available for use in liquid chromatography and each column has different physical properties which allow the columns to separate specific samples accordingly. Table 1 shows a list of the different types of columns commonly used.

Table 1: Different types of columns.

Type of Column	Separation Mode
Normal-Phase	Polarity. Polar bound phase with nonpolar mobile phase
Reversed-Phase	Polarity. Nonpolar bound phase with a polar mobile phase
Ion-Exchange	Net charge. Retained ionized material eluted by different salt and salt gradients
Size Separation	Size (ie. Stokes radius).
Bonded-Phase Silica columns	Structure (eg. Enantiomeric separation).

Two of the most common columns used are the ion-exchange and reversed-phase columns. In ion-exchange chromatography, the stationary phase surface displays ionic

functional groups that interact with analyte ions of opposite charge. Ions of similar charge get eluted while oppositely charged ions are retained on the stationary phase of the column and later eluted by increasing the concentration of a similarly charged species that will displace the analyte ions from the stationary phase. This is an excellent way of separating proteins because proteins have many charged functional groups. By varying the pH and ionic concentration of the mobile phase, especially the pH, the proteins will be eluted out of the column as its net charge changes from one sign to another.

In reversed-phase chromatography, a hydrophobic stationary phase and a polar mobile phase column is used. As a result, hydrophobic molecules in the polar mobile phase adsorb onto the hydrophobic stationary phase, and hydrophilic molecules in the mobile phase will pass through the column and get eluted first. Mixtures of water or aqueous buffers and organic solvents are used to elute the analytes from the reversed-phase column. The solvents must be miscible with water, and the most common organic solvents used are acetonitrile, methanol, and tetrahydrofuran (THF). Other solvents can include ethanol or 2-propanol (isopropyl alcohol). Elution may be performed isocratically or by using a solution gradient.

Two reasons why LC is encouraged prior to MS are because firstly, MS alone is unable to distinguish isomers due to their same mass. Many biological chemicals exist as isomers, with the same molecular mass but different structures. Hence, an additional step of LC would aid in differentiating between two isomers. Secondly, LC may be able to help avoid or at least alleviate ion suppression, a situation where molecules that

are low in abundance or poorly ionised are undetected by MS due to the presence of other highly expressed compounds. Pre-purification of the ionisation mixture can separate these components from each other so that the masking effects are minimized.

1.4.3 Mass Spectrometry

After separating the sample within the LC columns, the samples are next prepared for detection and identification in the MS. While the LC separates the components, it does not identify a compound. Therefore, MS coupled to LC performs this task of identifying the compounds present after some pre-purification. Mass spectrometers convert analyte molecules into an ionised state, and subsequently analyse them (and any fragment ions produced in the ionization process) based on the mass to charge ratio (m/z). One common method used to form ions from the analytes is electrospray ionisation (ESI). This method works well with moderately polar molecules and therefore is suitable in the study of peptides, metabolites and xenobiotics. Little fragmentation occurs under normal circumstances. The liquid sample is pumped and charged through a metal capillary, forming a fine spray of charged droplets. Heat and dry nitrogen dries the droplets by evaporating the liquid, and any electrical charge is transferred onto the analytes. The ionised analytes are next charged through a vacuum, through a series of small apertures and focusing voltages, and finally detected. Small molecules with a single charge-carrying functional group tend to carry a single charge while larger molecules with multiple charge-carrying functional groups (ie. Peptides and proteins) can carry multiple charges. This difference in ion charges within a sample can be used to determine analytes up to 100kDa. This is the basic working principle of ESI in MS. Many variations of ESI have been developed to improve on the quality of detection.

While ESI is useful for ionising biological molecules, neutral and low polarity molecules may not be efficiently ionised by this method. Instead, atmospheric pressure chemical ionisation (APCI) may be a better option. In this method, gas and solvent that have been ionised in the ion source react with the analyte and transfers their charge to it. Alternatively, atmospheric pressure photo-ionisation (APPI) uses photons to excite and ionise molecules. These options are useful for small, thermally stable molecules not easily ionised by ESI.

Following ionisation, the ions are accelerated through a mass analyser. The quadrupole analyser is the component in a MS responsible for filtering sample ions based on their m/z value. This is achieved by using a combination of constant and varying voltages, resulting in a mass spectrum. Stepping voltages may be used to focus the detection of a range of ions of a certain m/z value. While the ionisation process itself produces little or no fragmentation, ions may be made to fragment by passing them through a collision cell. In the collision cell, the ions collide with an inert gas such as nitrogen or argon. A collision cell may be placed between two mass analysers, also known as a triple quadrupole mass spectrometer. One main benefit of using a tandem MS is the increased specificity in its detection. The product ion scans contain both structural information about the analyte and confirms its identity with greater certainty [30]. Tandem MS is frequently used in LC-MS applications.

Another popular mode of analyser is the time-of-flight (TOF). Ions are accelerated through a high voltage and reach the detector at different times, depending on their m/z value. Ion trap analysers introduce an inert gas into the trap and ions are fragmented several times before the final mass spectrum is obtained. Hybrid analysers combine the different analysers in the MS. When the third quadrupole of a triple quadrupole MS is replaced by a TOF analyser, a hybrid MS (QTOF) is produced. QTOF is widely used in proteomics. If an ion trap analyser is replaced for the third quadrupole, a QTrap MS is formed. QTOF MS has a high sensitivity, high resolution and mass accuracy. Q1 in a QTOF MS is operated in the mass filter mode to transmit only the parent ion of interest. These ions are accelerated before they enter the collision cell Q2, where they get fragmented due to collision with inert gas molecules. If no collision is desired, a single mass spectrum can be obtained by setting the collision energy to below 10eV. The fragmented ions are cooled, re-focused and re-accelerated into the ion modulator of the TOF analyser. A pulsed electric field applied across the modulator gap changes the direction of the ions to a path perpendicular to that of its original direction, where they accelerate in the accelerating column and mass separation occurs. Ions reach the ion mirror and get deflected to the TOF detector where the mass spectra are recorded [31]. Figure 3 shows the trajectory of ions in a typical QTOF MS.

Figure 3: Schematic diagram of a tandem QTOF MS [20]. Ions are accelerated and collided with inert gas molecules to form daughter ions in Q1 and Q2 of the QTOF. The fragmented ions are re-accelerated in the ion-modulator and a subsequent electric pulse applied such that it changes the direction of the ions perpendicularly, where they then accelerate and separate. They are finally deflected into the TOF detector where mass spectra are recorded. (Permission from ref.20 was obtained from publisher to use this figure.)

1.4.4 LC/MS Software

Data analysis software is employed to extract and interpret information from MS datasets. Molecules detected by MS are next identified through a MS database search. At present, the standard libraries of mass spectral data that are commonly used include Swiss-prot, NIST and Wiley et al. Current limitations of the LC-MS technique lie primarily in the separation speed, peak resolution, data analysis and cost.

1.4.5 Applications of LC-MS/MS

The LC-MS/MS technology may be used in a variety of applications. Millington et al. utilised this technology in the screening of neonatal dried blood spots for errors of

metabolism. Dried blood spots are extracted and derivatised and scanned for a number of marker amino acids and acyl carnitines. This may also be applied to screening other conditions, such as sickle cell anaemia, galactosaemia, lysosomal disorders, disorders of porphyrin, purine and pyrimidine, peroxisomal and bile acid metabolism. Also, instead of measuring the levels of metabolites, the amounts of enzymes may be measured instead.

Apart from the biochemical screening for genetic disorders, LC-MS may also be applied in therapeutic drug monitoring and toxicology. The study of drug therapy and their variable cross-reactivity with metabolites have been improved with the tandem use of the LC-MS. LC-MS can be used not only to confirm the structure of the final metabolite product and its impurities, but also to study the precursor purity, intermediate compounds in the synthesis pathway, and the completeness of the drug conversion. It has been used to assay multiple drugs at the same time, due to the capacity to multiplex LC-MS assays, making it a more convenient assay as compared to immunoassays.

Many other types of studies may be performed with the LC-MS. Vitamins, steroid hormones and proteins are a few of them that may be studied. Some studies use LC-MS for the analysis of specific proteins from complex biological samples. Chang group developed a LC-MS/MS method for the quantitation of a large peptide, T-20 and its metabolite in human plasma. The method was developed and used for analysing pharmacokinetic profiles and metabolite of samples treated by the HIV fusion inhibitor peptide drug [32]. Lin described a LC-MS/MS method for the

determination of levovirin in rat and Cynomolgus monkey plasma, and the assay was validated and used in pharmacokinetic studies in rats and monkeys [33]. Feng et al. [34] has shown the feasibility of using this method of protein profiling by applying the iTRAQ-coupled 2-D LC-MS/MS analysis to reveal and quantify the differences of protein expression levels of normal HepG2 cells and those transfected with HBx of three different genotypes (A, B and C). Their results showed that HBx alters the expression levels of proteins involved in metabolic enzymes, signalling pathway and cytoskeleton regulation. Proteins regulating cell migration were also successfully identified via this comparative proteomics approach. The same group did another study [35] using this approach in the identification of secreted proteins in their cell-based HBV replication system to establish potential biomarkers of liver disease development. Zhang et al. [36] identified enzymes associated with angiogenesis in HBV replicating RPHs and HepG2 cells by 2-D LC-MS/MS analysis. The identified proteins may lead to a novel anti-angiogenic HCC therapy based on tumour vascular targeting.

These studies highlight the significance of the LC-MS/MS approach in protein profiling, as it is able to identifying novel markers indicative of diseases as well as explaining the mechanisms involved in disease development.

1.4.6 Quantitative Proteomics

As mentioned earlier, the coupling of LC to MS enables the detection and identification of unknown compounds like drugs, proteins, etc. Proteomics refers to the entire complement of proteins expressed in a given cell, tissue or organism. While it is useful to identify the proteins present in the samples, a quantitative proteomics

approach is able to yield the difference in protein levels of different samples. MS itself is not inherently quantitative; inaccuracies may occur due to the differences in ionisation efficiencies, and the peaks obtained in a mass spectrum is not a good indicator of the amount of analyte in a sample. Relative quantitation is still possible using MS alone, but may be less sensitive to experimental bias. Moreover, only one sample may be analysed in a single run, making it a relatively inconvenient method to study larger sample sizes.

One way to circumvent these problems would be to incorporate stable isotope labels, such as isotopic tags, to the samples. What this does is to cause a mass shift of a labelled protein or peptide in the mass spectrum. Differentially labelled samples are combined and analysed together, and the differences in the peak intensities of the isotope pairs accurately reflect the difference in the abundance of their corresponding proteins. Known concentrations of labels may be added to samples for absolute quantification of target proteins. Many types of labels are available, including isotope-coded affinity tags (ICAT), tandem mass tags (TMT), isobaric tags for relative and absolute quantitation (iTRAQ), metal-coded tags, and stable isotope labelling with amino acids in cell culture (SILAC). Table 2 shows the principles of some of these labelling methods.

Table 2: Working mechanisms of different types of labels.

Different types of labels	Mechanisms
ICAT	Two-sample simultaneous quantitation. One sample is labelled with light hydrogen while the other, with a heavier version (ie. Deuterium).
iTRAQ	Up to eight samples may be studied simultaneously. Samples are labelled with reagents as in Figure 4.
Metal-coded tags	A macrocyclic metal chelate complex loaded with different lanthanides (metal (III) ions) forms the essential part of the tag.
SILAC	Two-sample simultaneous quantitation. Labelling occurs at cell culture level. Cells of one sample is fed with growth medium containing normal amino acids while cells of the other sample is fed with growth medium containing amino acids labelled with stable (non-radioactive) heavy isotopes.

There are three major types of labelling: 1) Metabolic labelling; 2) Protein labelling; 3) Peptide labelling. Peptide labelling has the advantage over protein labelling by increasing the specificity and accuracy of proteins identified.

Of all the developed stable isotope-based quantification methods, iTRAQ has gained much popularity as it allows up to eight samples to be examined within one experiment. The reagents are composed of an amino reactive NHS group coupled to a balancer and reporter group. Using iTRAQ 4-plex to illustrate, up to four samples can

be done in a single experiment, with four different reporter groups (MW: 114Da, 115Da, 116Da, 117Da). Accordingly, the molecular weights of the balancers are: 31Da, 30Da, 29Da, 28Da. Each reporter group is linked to a balancer, contributing to a total molecular weight of 145. The NHS group labels all peptides at the 22 lysine side chain. At the first MS, the same peptides (from different samples) will elute at the same retention time as they have the same molecular weight. At the second MS (MS/MS), the balancer is lost and the label dissociates and releases the reporter group as a single charged ion of masses 114Da, 115Da, 116Da, or 117Da, respectively. The relative peak areas of the reporter groups indicate the contribution of each sample to the total peptide present, providing a measure of relative abundance. The principle of iTRAQ labelling is shown in Figure 4 and Figure 5. Briefly, sample proteins are extracted and digested into their peptides and labelled with iTRAQ reagents. Different samples are labelled with different iTRAQ reagents, each with a different reporter group. The underlying principle is that, the mass difference resulting from the introduction of the individual stable isotope provides a ratio for the reporters, and this directly corresponds to the ratio of the analytes. The samples are then pooled and separated sequentially on the multi-dimensional columns of the LC based on charge or hydrophobicity of the ionized analytes and eluted into the MS for identification and quantification. On-line libraries of information and sequence structures of polypeptides are available to aid in quickly identifying the peptide sequence, and a bottom-up approach is taken to identify the original protein. Generally, two or more unique peptides are usually sufficient to recognize a protein. In our study, an iTRAQ LC-MS/MS was applied in studying the metabolic state of the yeast cells.

Figure 4: iTRAQ reagents and their chemical structures. Up to 8 samples may be labelled per experiment (Applied Biosystems). The labelling reagent consists of a quantification (reporter) group (N-methylpiperazine), a balance group (carbonyl), and a hydroxyl succinimide ester group that reacts with the N-terminal amino groups of peptides and the amino groups of lysine. (Adapted from <http://www.creative-proteomics.com/iTRAQ.htm>)

Figure 5: Summary of iTRAQ-based LC-MS. Proteins from each sample are denatured, reduced and digested into peptides, and labelled with an iTRAQ reagent. Samples are pooled and sent for LC-MS analysis, where the peptides are identified and quantified simultaneously. The signal intensity ratios of the reporter groups indicate the ratios of the peptide quantities. The MS/MS spectra of the individual peptides show signals reflecting amino acid sequences and also show reporter ions reflecting the protein contents of the samples. A database search is performed using fragmentation data to identify the labelled peptides and hence the corresponding proteins whilst the iTRAQ mass reporter ion relatively quantifies the peptides. (Adapted from <http://www.creative-proteomics.com/iTRAQ.htm>)

2. Materials and methods

2.1 Yeast strain

The yeast strain used in this project is *Saccharomyces cerevisiae* BY4741 (*MAT* Δ , *his3* Δ , *leu2* Δ , *met15* Δ , *ura3* Δ). The cells were grown at 30°C, 250rpm in YPD medium (1% yeast extract, 2% peptone, 2% dextrose). They were also stored at -80°C as glycerol stocks after the addition of 20% glycerol.

2.2 Enzymes and chemicals

The restriction enzymes used in this project were from New England Biolabs. T4 ligase was purchased from Fermentas while Taq DNA polymerase was from Promega. Oligonucleotide primers were synthesized at 1st BASE Pte Ltd. Antibiotics and other chemicals used in this study were purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade and used without further purification.

2.3 Cloning of *mae1* gene and *matB* gene

pYES2/CT, from Invitrogen was the vector chosen as the cloning vector to clone the *mae1* gene from *Schizosaccharomyces pombe* into our target yeast cells, *Saccharomyces cerevisiae*. It is a 6kb vector designed for inducible expression of recombinant proteins in *Saccharomyces cerevisiae*. *GAL1* promoter on the vector allows inducible protein expression in yeast by galactose and repression by glucose.

URA3 gene on the vector allows the selection of transformed yeast cells with the *ura3* genotype. This made sure that only cells with the transformed vector, thus containing our recombinant gene can survive and grow.

The gene of interest, *mae1* gene was cloned and ligated into the multiple cloning site of pYES2/CT. The resulting plasmids were transformed into competent *E. coli* cells and the transformed *E. coli* cells were selected on LB plates containing 100µg/ml ampicillin. The positive colonies were then confirmed by DNA gel electrophoresis after the double digestion of the extracted plasmids. The plasmids were then transformed into *Saccharomyces cerevisiae*. Transformed yeast cells were selected by uracil prototrophy by growing them on SC minimal medium deficient in uracil. Presence of the gene *mae1* in the yeast cells were confirmed by colony polymerase chain reaction (PCR) and the protein expression of the dicarboxylic acid transporter encoded by *mae1* was confirmed by Reverse Transcriptase PCR (RT-PCR) and immunofluorescence.

MatB gene from *Rhizobium leguminosarium* bv *trifolii*, on the other hand, was cloned into *Saccharomyces cerevisiae* cells which already contained the previously cloned *mae1* gene. *MatB* gene was cloned using the pESC-LEU vector from Agilent following protocols as described above. Transformed yeast cells were selected by leucine prototrophy by growing them on SC minimal medium deficient in uracil and leucine. Presence of the gene *matB* in the yeast cells were confirmed by colony PCR and the protein expression of the malonyl-CoA synthetase encoded by *matB* was confirmed by western blot via a C-terminal 6xHis epitope tag.

2.4 Reverse Transcriptase PCR (RT-PCR)

Once the *mae1* gene has been successfully transformed into the yeast cells via the pYES2/CT vector, Reverse Transcriptase PCR (RT-PCR) technique was chosen to prove the functional existence of the target gene in our target yeast cells. Upon being successfully cloned inside the cells, the expression of the *mae1* gene would be triggered by the addition of galactose due to the presence of the GAL1 promoter. During expression, the gene would be transcribed into messenger RNA (mRNA) and then later translated by ribosomes to produce the encoded protein, plasma membrane dicarboxylic acid transporter. The purpose of RT-PCR would then to capture these mRNAs before translation and reverse transcribe them into DNA sequences through the action of an enzyme known as reverse transcriptase. After that, the DNA sequences thus obtained would be put through polymerase chain reaction (PCR) to amplify the amount of such identical DNA sequences, which can be collected and further analyzed downstream.

Before RT-PCR could be performed, total RNA extraction needs to be performed on the yeast cells samples. These yeast cells were those with the *mae1* gene transformed into them via the pYES2/CT vector and wild type yeast cells were also used as a negative control. RNeasy Mini Kit for total RNA extraction from yeast cell from Qiagen was used.

Yeast cells were harvested during the log phase and were lyzed using mechanical disruption. Equal volume of glass beads (425-600 μm) from Sigma were added to each sample and were placed in an agitator machine to completely lyse the yeast cells.

Subsequent steps to obtain the total RNA from the yeast cells were as described in the protocol manual supplied with the RNeasy Mini Kit.

After the total RNA had been obtained, iScript™ One-Step RT-PCR Kit from Bio-Rad was used to perform the RT-PCR on the total RNA. Content of each PCR mixture was shown below by Table 3 according to the protocol from the kit.

Table 3: Contents of each PCR mixture for RT-PCR

Component	Volume per reaction (50 µl)
RT-PCR Reaction Mix	25 µl
Forward primer (10 µM)	1.5 µl
Reverse primer (10 µM)	1.5 µl
iScript Reverse Transcriptase for One-Step RT-PCR	1 µl
RNA template (1 pg to 100 ng total RNA)	added accordingly
Nuclease-free H₂O	make up to 50 µl

The forward and reverse primers used were 25 nucleotides long with a GC content of less than 60% and were synthesised by 1st BASE Pte. Ltd. The PCR mixtures were then placed in a thermal cycler for the PCR to take place. The temperature settings and duration were as according to the protocol from the kit. Finally, the final PCR products were then analyzed using DNA gel electrophoresis.

2.5 Yeast immunofluorescence

In order to further prove the successful expression of the *mae1* gene in our target yeast cells, immunofluorescence was carried out to detect the presence of the dicarboxylic acid transporter expressed by the *mae1* gene. The cloning vector, pYES2/CT contains a C-terminal V5 epitope tag at the end of its multiple cloning site. As a result, the dicarboxylic acid transporter protein expressed by the *mae1* gene cloned into this vector will contain the 14 amino acid V5 epitope tag. With this tag in place, a primary antibody could be used to bind specifically to the V5 tag. After that, a secondary antibody conjugated with the enzyme horseradish peroxidase (HRP) would be added to bind to the primary antibody to amplify the signal and to enable detection through a microscope due to the chemiluminescence properties of HRP.

The transformed yeast cells were first grown to log phase under galactose induction. After that, cells were fixed for 2 hours by adding 1/10 volume of 37% formaldehyde to the cell culture. The cells were then pelleted and resuspended in 0.5 ml of spheroplasting buffer containing 2% of 1.42 M β -ME and 2.5% of 5mg/ml zymolase enzyme to digest the yeast cell walls. The culture was then incubated in 30°C for 60 min. The chamber glass slides were prepared by first coating each well with 1mg/ml polylysine. The fixed and spheroplasted cells were then added to each well. To enhance the adhesion of the cells to the surface of the slides, the wells were first immersed in methanol for 5min at -20°C and then immersed in acetone at -20°C for 30 seconds. Next, the cells were incubated for 30 min in a blocking solution containing PBS with 1mg/ml of BSA. This is to prevent any nonspecific binding of the antibodies onto the empty surface of the glass slides and hence giving an inaccurate result at the

end. V5 mouse monoclonal antibody purchased from Life Technologies was used as the primary antibody and was added to the wells after the blocking solution was aspirated off. The cells were then incubated in this diluted primary antibody solution for 1 hour at room temperature. Goat anti-mouse antibody conjugated with the HRP enzyme purchased from Life Technologies was used as the secondary antibody to amplify the signals. As such, after the primary antibody solution was removed, the cells were then incubated in the diluted secondary antibody solution for 1 hour in darkness in order to preserve the chemiluminescence strength of the HRP enzyme. After incubation with the secondary antibody, 1 µg/ml DAPI in PBS was added to each well. Finally, mounting solution was added to each well and a glass coverslip was put on the slides. The slides were then observed under microscope to detect the fluorescent signals given out by the HRP-fused antibodies.

2.6 HPLC sample preparation

2.6.1 Samples for malonic acid detection

In order to induce expression of the protein of interest from the *GALI* promoter, galactose needs to be added to the medium. For cells that have been maintained in raffinose, recombinant protein can be detected in as little as 2 hours after galactose induction.

Yeast cells were inoculated and maintained in SC-U medium (SC minimal medium with uracil dropout) containing 2% raffinose as the carbon source. After reaching stationary growth, the cells were pelleted using centrifugation and the supernatant was

discarded. After that, the cell pellet was resuspended in induction medium containing 2% galactose. After 3 hours of induction, 1% malonic acid is added into the medium and samples were collected 2 hours after the addition of malonic acid and every 2 hours thereafter. The samples were centrifuged and the cell pellets were collected. The pellets were washed twice with distilled water and once with 70% methanol. Finally, the cells in 70% methanol were lysed using Sigma's acid-washed glass beads with a diameter of approximately around 425-600 μm . The glass beads and cell debris were then allowed to settle and the cell lysate collected. The tubes containing the cell lysate were placed on a 50 °C heating block to evaporate away the methanol. Finally, distilled water was added and the samples were ready to run HPLC.

2.6.2 Samples for fatty acids detection

Same amounts of cells from culture medium of *Saccharomyces cerevisiae* of the various strains were collected from the specific growth phase and centrifuged at 12,000 rpm for 5 min. The supernatants were discarded and the cell pellets were washed twice with distilled water and then re-dissolved in 800 μl pure methanol. 10 μl ribitol (2 mg/ml) and 10 μl heptadecanoic acid ($\text{C}_{17}\text{H}_{34}\text{O}_2$) (2 mg/ml) were added into the sample tubes as internal control, to correct the metabolites concentration deviation caused during the cell lysis operation. The cell lysis was carried out in an ultrasonic processor. The ultra-sonication procedure was as carried out as following: the amplitude was set at 50%; the time of one cycle was set at 0.3 s. A total of 60 cycles were performed on each sample. After that, the cell samples were kept in liquid nitrogen for ten minutes and then immediately transferred to room temperature for five minutes. A second running of sonication was conducted on the cell samples, to make sure the complete breakage of the *S. cerevisiae* cells. 500 μl ddH₂O and 300 μl chloroform were added into

the cell samples after sonication and the sample tubes were vibrated for 2 h at room temperature. After methanol/chloroform/ddH₂O extraction, the sample tubes were centrifuged for 10 min and a two-phase mix was formed. The chloroform phase was separated from the methanol/ddH₂O phase and collected. After which, 300 µl chloroform was added into the remaining mix again and vibrated for 1 h at room temperature. Finally, the chloroform phase including the lipid contents and the methanol/ddH₂O phase including the soluble metabolites were collected, respectively and they were evaporated to complete dryness for the analysis of soluble fatty acid.

After the fatty acids had been obtained, they were dissolved in methanol and neutralized with the KOH solution (with the help of phenolphthalein as an indicator). Next, the mixture was evaporated under nitrogen. After that, 0.1 ml of 2mM 18-crown-6 in acetonitrile and 0.1 ml 4-bromophenacyl bromide were added to the residue. Lastly, the mixture was heated at 80°C for 15 min with mixing gently several times. Finally, the vial was cooled and diluted to a larger volume with acetonitrile.

The HPLC separation is made using a C18 column and a gradient of acetonitrile in acetonitrile/water (1/1, v/v) at 1ml/min. UV detection was set at 242 nm. The gradient shape is adapted to the fatty acid mixture and the column type but it can be recommended at the beginning to make a linear gradient portion for 60 min followed by an isocratic step (at about 80% acetonitrile) for 30-40 min.

2.7 High-performance liquid chromatography (HPLC)

In order to test the existence and function of the plasma membrane dicarboxylic acid transporter encoded by the cloned gene *mae1*, High-performance liquid chromatography (HPLC) was performed to test the presence of malonic acid or malonate inside the cells after incubation in medium containing malonic acid.

Samples were run on a HPLC machine from Agilent using Ultra Aqueous C18 column from RESTEK. As suggested from RESTEK's protocol [37], HPLC-grade water was used as solvent with a solution of 50mM potassium phosphate pH 2.5 and acetonitrile with a ratio of 99:1 as the buffer. Samples were injected at 10 μ L each and ran for 30 min and the flow through the column was set at 1.5 mL/min at a temperature of 25 °C. Finally the results were detected by a UV detector with a wavelength of 210nm.

2.8 LC-MS sample preparation

Similar to HPLC sample preparation, yeast cells were first inoculated and maintained in SC-U medium (SC minimal medium with uracil dropout) containing 2% raffinose as the carbon source. After reaching stationary growth, the cells were pelleted using centrifugation and the supernatant was discarded. After that, the cell pellet was resuspended in induction medium containing 2% galactose. After 3 hours of induction, 1% malonic acid is added into the medium and samples were collected 2 hours after the addition of malonic acid and every 2 hours thereafter. The samples were centrifuged and the cell pellets were collected. The pellets were then washed twice with distilled water.

The crude protein from the cells were extracted using a lysis buffer (50mM HEPES, 5% glycerol, 1mM DTT, 1Mm PMSF, 1mM EDTA) with equal volume of glass beads (425-600 μ m) from Sigma. After vortexing, the cleared supenatant was collected by centrifugation and the protein concentration was determined using the 2D-quant kit from GE Healthcare. A standard curve was made using BSA as a control. A total of 100 μ g of each sample was precipitated by the addition of 4 times the sample volume of cold (-20 °C) acetone to the tube for 2 h and carefully decanting the supernatant. The protein pellets were then dissolved in the solution buffer and denatured, and cysteines were blocked as described in the iTRAQ protocol from Applied Biosystems. Each sample was then digested with 20 μ l of 0.25 μ g/ μ l sequencing grade modified trypsin solution from Promega at 37 °C overnight and labeled with the iTRAQ tags [38-40]. Wild type *Saccharomyces cerevisiae* cells were labelled with iTRAQ 114 and transformed *Saccharomyces cerevisiae* cells with the *mae1* gene were labelled with iTRAQ 115. The labeled samples were then pooled before analysis.

2.9 Liquid chromatography–mass spectrometry (LC-MS)

The samples were then used for an online 2D Nano-LC-MS/MS Analysis. The analysis was performed on an Agilent 1200 nanoflow LC system (Agilent Technologies) interfaced with a QSTAR XL mass spectrometer from Applied Biosystems/MDS Sciex. In the first dimension 3 μ l of the combined peptide mixture was loaded onto the PolySulfoethyl A SCX column (0.32 x 50 mm, 5 μ m) and was eluted stepwise by injecting salt plugs of 10 different molar concentrations of 10, 20, 30, 40, 50, 60, 80, 100, 300, and 500 mM KCl solution. Figure 7 below illustrates the flow diagram through a 10-port valve for an online 2D Nano-LC. At the “A” position, the 10-port valve was in position 1 which bypassed the SCX column and where the

enrichment column 1 was washed while peptides from enrichment column 2 were analyzed [41].

Figure 7: Flow diagram through a 10-port valve for an online 2D Nano-LC [41]. (Permission from ref.41 was obtained from publisher to use this figure.)

In the second dimension, while the 10-port valve was in position 1, the sequentially eluted peptides from the SCX column were trapped onto the Zorbax 300SB C18 enrichment column 1 (0.3 x 5 mm, 5 μ m) and washed isocratically with buffer A (5%

acetonitrile and 0.1% formic acid) at 0.005 ml/min for 100 min to remove any excess reagent. Meanwhile the peptides bound on Zorbax 300SB C18 enrichment column 2 during the previous run were eluted with buffer B (0.1% formic acid) and buffer C (a nanoflow gradient of 5–80% acetonitrile with 0.1% formic acid) at a flow rate of 500 nl/min. Further separation was achieved onto the analytical Zorbax 300SB C18 reversed-phase column (75 μ m x 50 mm, 3.5 μ m). In the next run, while the 10-port valve was switched to position 2, column 1 was switched into the solvent path of the nanopump, and column 2 was used to trap the newly eluted peptides from SCX. Below is a figure illustrating the flow paths position 1 and position 2 [41].

Altogether 11 runs were performed to finish one experiment. For MS/MS analysis, survey scans were acquired from m/z 300 to 1500 with up to two precursors selected for MS/MS from m/z 100 to 2000 using dynamic exclusion, and the rolling collision energy was used to promote fragmentation [41].

2.10 LC-MS data analysis

Relative abundance quantitation and peptide and protein identification were performed using ProteinPilot Software 4.2 from Applied Biosystems. Each MS/MS spectrum was searched for species of *Saccharomyces cerevisiae* against the UniProt database. The searches were run using the following parameters: fixed modification of methylmethanethiosulfate- labeled cysteine, fixed iTRAQ modification of free amine in the amino terminus and lysine, variable iTRAQ modifications of tyrosine, and allowing serine and threonine residues undergoing side reaction with the iTRAQ reagent. Other parameters such as tryptic cleavage specificity, precursor ion mass

accuracy, and fragment ion mass accuracy are built-in functions of ProteinPilot software. Relative quantification of proteins in the case of iTRAQ was performed on the MS/MS scans and was the ratio of the areas under the peaks at 114 and 115 Da, which were the masses of the tags that correspond to the iTRAQ reagents. The relative amount of a peptide in each sample was calculated by dividing the peak areas observed at 115.1 m/z by that observed at 114.1 m/z . The calculated peak area ratios were corrected for overlapping isotopic contributions and were used to estimate the relative abundances of a particular peptide. The unused protein score is ProteinPilot's measurement of protein identification confidence taking into account all peptide evidence for a protein, excluding any evidence that is better explained by a higher ranking protein. Sequence coverage was calculated by dividing the number of amino acids observed by the protein amino acid length. Error factor (EF) is a statistic that has been created for reporting errors in ratios and expresses the 95% confidence interval for an average ratio ($EF = 10^{95\% \text{ confidence interval}}$ where $95\% \text{ confidence interval} = (\text{ratio} \times EF) - (\text{ratio}/EF)$). The following criteria were required to consider a protein for further statistical analysis: two or more high confidence (>95%) unique peptides had to be identified, the p value in the Protein Quant had to be $p < 0.05$, and the -fold difference had to be greater than 1.2. The candidate proteins were carefully examined in the Protein ID of the ProteinPilot software. The peptides without any modification of free amine in the amino terminus or without iTRAQ modification of free amine in the lysine were excluded from calculation of the protein ratios. To account for small differences in protein loading, all protein ratios were normalized using the overall ratios for all proteins in the sample as recommended by Applied Biosystems [41].

2.11 Western blot verification of the expression of malonyl-CoA synthetase and validation of LC-MS/MS Results

2.11.1 Protein Quantification

The Bradford dye-binding assay was used to measure the total protein concentration in the cell lysates. Bovine serum albumin (BSA) (Sigma) (1mg/ml) was used to prepare protein standards. 0, 5, 10, 15, 20, 25 and 30µl of BSA were individually pipetted into 1ml disposable plastic PLASTIBRAND[®] cuvettes (Brand). The volume was topped up to 1ml with Bradford reagent and mixed by pipetting gently till a homogeneous solution is formed. Absorbance at 595nm was measured using a spectrometer. The absorbance values were used to plot a BSA standard curve.

For measurement of sample protein concentration, 10µl of protein sample was diluted in 990µl of Bradford reagent. 1ml of Bradford reagent without proteins added was used as the blank control. The OD was read at 595nm and protein concentration was determined against the BSA standard curve.

2.11.2 Gel Electrophoresis

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the Bio-Rad mini-protean electrophoresis system. Each 1mm thick gel consists of a stacking gel and separating gel. Separating gels were prepared by mixing appropriate amounts of 40% acrylamide/bisacrylamide solution (37.5:1, acrylamide/bis; Bio-rad) (1.875 ml for 7.5% gel, 3.125 ml for 12.5% gel and 3.75 ml for 15% gel) with 2.5 ml of 1.5 M Tris·Cl (pH 8.8), 100µl of 10% SDS, and 100µl of 10% fresh ammonium persulfate (APS) (Bio-rad) and H₂O to a total volume of 10ml.

The volumes can be adjusted accordingly. 5µl of N, N, N', N'-tetramethylethylenediamine (TEMED) (Bio-rad) was added just prior to pouring the gel into the gel apparatus setup. 100µl pure ethanol was immediately placed over the separating gel to rid of bubbles. After polymerization, the ethanol was decanted, and a 5% stacking gel was poured on top of the separating gel. 4ml of 5% stacking gel was prepared by mixing 0.5ml of 40% acrylamide/bisacrylamide solution with 0.5ml 1.0 M Tris·Cl (pH 6.8), 40µl of 10% SDS, 40µl of 10% APS, 4µl TEMED and H₂O. A comb was inserted at the top and the gel and it was allowed to polymerize completely before preparing for gel electrophoresis.

30µg protein per sample was mixed with 1 x loading buffer (62.5 mM Tris·Cl pH6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue), and 100 mM dithiothreitol (DTT) (Sigma) and denatured at 90°C for 5min. For non-reducing condition, the DTT was omitted. After loading the samples into the wells, the gel was run under constant current (20mA) condition until satisfactory protein separation was observed. 10µl Novex Sharp Protein Standard (Life Technologies) was loaded per lane to verify protein size.

2.11.3 Gel transfer

The separated proteins were electrophoretically transferred to a Hybond-P Polyvinylidene Fluoride (PVDF) membrane (Amersham-Pharmacia) using Mini-PROTEAN 3 Electrophoresis System (Bio-rad). The polyacrylamide gel obtained after electrophoresis was rinsed in water, placed in-between layers of Whatman paper and membrane, cut to exact size of the gel, and inserted with 8 layer filter papers soaked in

transfer buffer (0.3% Tris, 1.45% glycine and 20% methanol), with the membrane facing the anode. The bubbles were carefully and completely removed by rolling across a glass spreading rod. The transfer was performed in 1x transfer buffer at 21V for 50min. The blotted membrane was marked and washed with 1x Tris-buffered saline (TBS) (20mM Tris·Cl pH 7.4, 150mM NaCl) buffer and stored at 4°C if not used immediately.

2.11.4 Immunoprobng

The antibodies used in this study include anti-6X His tag antibody (ab18184, abcam), anti-alcohol dehydrogenase antibody (ab20994, abcam), malate dehydrogenase antibody (HRP) from GeneTex, anti-beta actin antibody (ab170325, abcam), anti-goat IgG HRP-conjugated secondary antibody from Thermo Scientific, anti-mouse IgG HRP-conjugated secondary antibody from Thermo Scientific and anti-rabbit IgG HRP-conjugated secondary antibody from Thermo Scientific. Bands were analysed and quantified by G:BOX machine and software from Syngene.

The immunoprobng was carried out following the standard protocol. Briefly, the membrane was wetted with 1X TBS buffer before being blocked with 5% nonfat milk powder in 0.1% TBST (0.1% Tween-20 in 1x TBS) for 1h at room temperature. Following blocking, the membrane was incubated with the recommended amount of primary antibody in 5% nonfat milk powder in 0.1% TBST overnight at 4°C on a roller. After that, it was washed three times with 0.1% TBST for 10min each time. After the washings, the membrane was incubated in anti-goat, anti-mouse or anti-rabbit IgG HRP-conjugated secondary antibody (Thermo) in 5% nonfat milk powder

in 0.1% TBST for 1h at room temperature on a roller. The membrane was then washed three times as before and incubated in the SuperSignal West Pico Substrate solution (Pierce) by mixing equal volumes of the two reagents for 3min and then exposing to Hyperfilm X-ray films (Amersham-Pharmacia) for the desired amount of time and then G:BOX from Syngene for further analysis.

2.12 Statistical Analysis

All data are presented as mean $\pm$ SD. 2-tailed Student's *t*-test was used for all analyses where two experimental groups of values are being compared. Statistical significance was accepted at $p < 0.05$. Where the means of three or more groups were being compared for statistical difference, ANOVA was used. The value of *n* refers to the number of independent batch experiments performed for analysis.

3. Verification of the functional expression of the dicarboxylic acid transporter encoded by *mae1* gene

3.1 Introduction

Malonic acid, which malonate is derived from is a dicarboxylic acid. However, there is no dicarboxylic acid transporter reported to be present on the plasma membrane of *Saccharomyces cerevisiae* [13]. Thus, *Saccharomyces cerevisiae* lacks the ability to take in exogenous dicarboxylic acid such as malonic acid.

Another species of yeast known as *Schizosaccharomyces pombe* was reported to have such dicarboxylic acid transporter present on its plasma membrane. *Mae1* was identified as the gene responsible for the coding of this plasma membrane dicarboxylic acid transporter. It corresponds to a 49-kDa protein with 10 transmembrane predicted segments that has been classified in the TDT family of telurite and dicarboxylate transporters. The *Mae1* gene encodes a permease for malate and other C4 dicarboxylic acids, including malonic acid and behaves as a proton symporter not subjected to glucose repression [13].

3.2 Results and discussion

3.2.1 Cloning and expression of the *mae1* gene

Gene sequencing results on the plasmid extracts from *E. coli* cells transformed with the final recombinant vector showed there is no site mutation along the pYES2/CT vector and the gene of interest, *mae1* gene. As a result, the vector was introduced into the final target host, *Saccharomyces cerevisiae*.

Saccharomyces cerevisiae cells that were transformed with the pYES2/CT plasmid containing the *mae1* gene were grown and selected as described above. After that, the cells were harvested and colony PCR was performed to verify whether the cells from the selected colonies contain the target gene *mae1*. Primers used in colony PCR were all around 25 nucleotides long and synthesized by 1st BASE Pte. Ltd. Figure 8 below shows the DNA gel electrophoresis result of the colony PCR.

Figure 8: DNA gel electrophoresis of colony PCR on transformed yeast colonies

The ladder used is the 1kb standard DNA ladder from Fermentas. Lane 1 contained the colony PCR result of a wild type *Saccharomyces cerevisiae* with no plasmid transformed into it. This served as a negative control for the experiment. Lane 2 to lane 4 each contained the colony PCR results of a transformed *Saccharomyces cerevisiae* cell colony grown and selected as described above. These 3 colonies were separated from each other and were randomly picked from the SC minimal medium plate.

From the DNA electrophoresis result above, it was evident that wild type *Saccharomyces cerevisiae* itself does not contain the *mae1* gene as shown by the negative control in lane 1 of the result above. In lane 2 to lane 4 of the result above, there was a discreet band in the 1.3kb region for each of those lanes which agreed with the base pair length of the *mae1* gene. As such, the *mae1* gene was successfully cloned and transformed into the target yeast cells as indicated by this result.

3.2.2 Functional verification of the *mae1* gene by reverse transcriptase PCR (RT-PCR)

After the transformed cells were grown in induction medium containing galactose, the cells were harvested and total RNA extraction was performed as described above. After which, RT-PCR would be performed on the total RNA extracts. The cells used in this step were from the colonies verified to contain the gene of interest, *mae1* gene via colony PCR. Figure 9 below shows the result of the DNA gel electrophoresis following the RT-PCR.

Figure 9: DNA gel electrophoresis of RT-PCR on transformed yeast colonies

The ladder used is the same 1kb standard DNA ladder from Fermentas. Similar to the previous setup as described in the colony PCR section, lane 1 contained the RT-PCR result of wild type *Saccharomyces cerevisiae* with no plasmid transformed into it. This served as a negative control for the experiment. Lane 2 to lane 4 each contained the RT-PCR results of a transformed *Saccharomyces cerevisiae* cell colony grown and

selected as described above. These 3 colonies were separated from each other and were randomly picked from the SC minimal medium plate.

Unlike the primers used in the colony PCR as described in the previous section, the primers used in RT-PCR were designed to amplify only a short section of around 400 bp from the entire *mae1* gene which is around 1.3kb long. This was to improve the specificity and accuracy of the results as RT-PCR is very sensitive to contamination from genomic and plasmid DNA that may still be present after the total RNA extraction.

Once again from the DNA gel electrophoresis result above, it was evident that wild type *Saccharomyces cerevisiae* itself does not contain the *mae1* gene and thus no respective mRNA was detected as shown by the negative control in lane 1 of the result above. In lane 2 to lane 4 of the result above, there was a discreet band in the 400 bp region for each of those lanes. This agreed with the length of gene fragment that the specially designed primers were meant to amplify from the entire length of the *mae1* gene. As such, it was concluded that the respective mRNA, which was derived from the original *mae1* gene during transcription, was present which proved that the *mae1* gene is undergoing protein expression to express the plasma membrane dicarboxylic acid transporter which was encoded by the *mae1* gene.

3.2.3 Expression and localization of the dicarboxylic acid transporter demonstrated by yeast immunofluorescence

Samples were prepared from a single colony of transformed *Saccharomyces cerevisiae* and also from another single colony of wild type *Saccharomyces cerevisiae* to serve as control. Samples were prepared as described above. Samples were observed under normal phase contrast microscopy and under conditions to detect the fluorescent signals resulting from the HRP enzymes.

Figure 10: Immunofluorescence images taken at 60x magnification. (a) Phase contrast image of wild type yeast cells; (b) HRP fluorescent image of wild type yeast cells; (c) Phase contrast image of transformed yeast cells; (d) HRP fluorescent image of transformed yeast cells.

Figure 10 showed the images taken from the prepared samples, both wild type and transformed yeast cells, at 60x magnification with the same amount of exposure time. Figure 10(a) and (b) were taken with the same group of cells in focus. This is also true for Figure 10(c) and (d).

From Figure 10(a) and (c), cells could be seen clearly adhering to the surface of the glass slides which proved that the sample preparation was successful at capturing cells onto the glass slides. Furthermore, from the HRP fluorescent image of the transformed yeast cells as shown in Figure 10(d), strong fluorescence signals were shown indicating the presence of HRP enzymes and as such, the presence of the dicarboxylic acid transporter proteins expressed by the *mael* gene. In contrast, from the HRP fluorescent image of the wild type yeast cells as shown in Figure 10(b), the fluorescence signals were very weak and few as compared to the amount of cells captured in its corresponding phase contrast image. This formed a strong contrast with Figure 10(d) which indicated an absence of the same dicarboxylic acid transporter proteins which triggered the strong bright signals as shown. Moreover, the weak signal of Figure 10(b) was due to some inevitable nonspecific bindings of the antibodies onto the wild type cell surfaces.

Figure 11: Immunofluorescence images taken at 100x magnification. (a) Phase contrast image of wild type yeast cells; (b) HRP fluorescent image of wild type yeast cells; (c) Phase contrast image of transformed yeast cells; (d) HRP fluorescent image of transformed yeast cells.

Similar to Figure 10, images from Figure 11 were taken with the same amount of exposure time but at 100x magnification. Images were taken at the same group of cells for Figure 11(a) and (b) and for Figure 11(c) and (d) respectively.

The results as shown here in Figure 11 were similar to that exhibited by Figure 10. Once again from the HRP fluorescent images, it is evident that there is a presence of dicarboxylic acid transporter proteins in the transformed yeast cells and absent from the wild type yeast cells. Moreover, it is also important to note that in Figure 11(d), the distribution of the fluorescence signals tend to localize at the circumference of the

cells. This further proved the functional expression of the dicarboxylic acid transporter proteins which were intended for the plasma membrane of the cells and this localization of signals became more apparent at higher magnification. This goes to show that the *mae1* gene is not only successfully cloned into the target yeast cells, but is also actively and correctly expressing the dicarboxylic acid transporter proteins as intended.

3.3 Section conclusion

From the results above, it was evident that wild type *Saccharomyces cerevisiae* itself does not contain the *mae1* gene as shown by the negative controls in the DNA gel electrophoresis performed in Chapter 3.2.1 and 3.2.2. It had also shown that the 1.3kb long *mae1* gene had been successfully cloned and transformed into the yeast cells. The functional expression of the dicarboxylic acid transporter as encoded by this *mae1* gene was also shown by the subsequent RT-PCR and immunofluorescence.

RT-PCR results showed that the respective mRNA, which was derived from the original *mae1* gene during transcription, was present which proved that the *mae1* gene is undergoing protein expression to express the plasma membrane dicarboxylic acid transporter.

Furthermore, the results from the immunofluorescence experiments further proved the functional expression of the dicarboxylic acid transporter proteins which were intended for the plasma membrane of the cells and this localization of signals became

more apparent at higher magnification. This goes to show that the *mae1* gene is not only successfully cloned into the target yeast cells, but is also actively and correctly expressing the dicarboxylic acid transporter proteins as intended.

4. Effects of exogenously absorbed malonate on the metabolic state of *Saccharomyces cerevisiae*

4.1 Introduction

Malonic acid or malonate is a well-known inhibitor to the succinate dehydrogenase of the citric acid cycle in the mitochondria of yeast cells [42]. Furthermore, according to a research on the compensatory role of succinate oxidation to aging *Saccharomyces cerevisiae* with impaired mitochondria activity [43], it was found out that when the citric acid cycle was impaired due to post-mitotic aging or a result of activity from inhibitors such as malonate, alternative mechanism would be triggered to continue supply energy for the survivability of the cells. In this case, the glyoxylate cycle is activated. The glyoxylate cycle, a variation of the citric acid cycle, is an anabolic metabolic pathway occurring in plants, bacteria, protists, and fungi. The glyoxylate cycle centers on the conversion of acetyl-CoA to succinate for the synthesis of carbohydrates and for energy production [44]. In microorganisms, the glyoxylate cycle allows cells to utilize simple carbon compounds as a carbon source when complex sources such as glucose are not available [45]. As a result, glyoxylate cycle, through the production of succinate, is a reliable alternative pathway to produce energy while TCA cycle in the mitochondria is impaired.

As such, it is vital to find out how the exogenously supplied malonate will affect the growth, survival and metabolism of our target yeast cells, *Saccharomyces cerevisiae*. Only when protein and metabolic profiles were studied can we anticipate how the yeast cells will react and thus make preparations for further genetic engineering to increase the overall production of fatty acids.

4.2 Results and discussion

4.2.1 Detection of exogenously absorbed malonate using high-performance liquid chromatography (HPLC)

In an effort to find out the cytoplasmic concentration of malonate of wild type *Saccharomyces cerevisiae*, the cells, with no plasmid transformed into them, were grown in induction medium containing galactose and malonic acid to prepare them for HPLC as described in Chapter 2.6. Besides determining the intracellular malonate concentration of the yeast cells, the aim was also to determine whether the wild type yeast cells are able to uptake exogenously supplied malonate.

Figure 12: HPLC result of 1%malonic acid standard

Figure 13: HPLC result of wild type *Saccharomyces cerevisiae* cells in a medium with no malonic acid

In Figure 12, the chromatogram belongs to a standard solution of 1% malonic acid and the 2nd peak (circled in red) corresponds to the presence of malonate. In Figure 13, the chromatogram belongs to a yeast culture that is inoculated in normal YPD culture for 4 hours. From both chromatograms, they both contain a peak at the same retention time (circled in blue) and although one may look taller than the other, they both actually have the same intensity (y-axis value) and as such this 1st peak or signal corresponds to the signal caused by the mobile phase used in the chromatography. This signal is commonly known as the solvent front. Moreover, the malonate signal is missing from the Figure 13 and as such, it can be concluded that the yeast cell itself contains little to no free intracellular malonate.

Following the previous tests to determine the presence of intracellular malonate, another set of experiments were performed to see whether wild type yeast cells can uptake exogenously supplied malonate on its own.

Figure 14: HPLC result of wild type *Saccharomyces cerevisiae* cells in a medium containing 1% malonic acid

In Figure 14, the chromatogram belongs to a wild type yeast culture that is inoculated in YPD mixed with 1% malonic acid for 4 hours. The first peak corresponds to the signal caused by the mobile phase of the chromatography similar to the previous results. Moreover, the malonate signal that is present in the standard solution from Figure 12 is still missing from that in this chromatogram.

Therefore, in order for the Malonyl-CoA synthetase encoded by the *MatB* gene, as discussed, to function properly inside the yeast cells, a way must be found first to transport malonate into the cytoplasm of the yeast cells.

HPLC was then done on *Saccharomyces cerevisiae* cells that were transformed with the *mae1* gene as detailed in Chapter 3 to determine whether the yeast cells can now uptake exogenously supplied malonate with the expression of the dicarboxylic acid transporter encoded by the *mae1* gene.

Figure 15: HPLC result of 1%malonic acid standard (for calibration)

Figure 15 shows a chromatogram of a 1% malonic acid standard solution similar to Figure 12. The elution time and intensity of both the solvent front and the malonic acid signals are both very similar from these 2 chromatograms.

Figure 16: HPLC result of *Saccharomyces cerevisiae* cells with the *mae1* gene in a medium containing 1% malonic acid

Figure 16 shows the chromatogram of *Saccharomyces cerevisiae* cells that were transformed with the *mae1* gene and expressing the dicarboxylic acid transporter on its plasma membrane. From the chromatogram, a signal peak was detected at the same elution time as the malonate signal from the standard solution chromatograms as shown in Figure 12 and Figure 15. This proved that the cells were able to uptake exogenous malonate that was supplied in the medium into their cytoplasm. However,

the intensity of this peak is half of those in the standard solution chromatograms which suggested that the transport of malonate across the plasma membrane as mediated by the dicarboxylic acid transporter encoded by the *mae1* gene was not 100% efficient. It was also interesting to note that there was another significant peak at the elution time of around 5.5 min. The compound that caused this peak is still unknown, but it only appeared after the transformation and expression of the *mae1* gene.

4.2.2 Toxicity of malonate on cell growth of *Saccharomyces cerevisiae*

Yeast cells transformed with the *mae1* gene as detailed in Chapter 3, were cultivated in growth medium containing different concentrations of malonate, 1%, 3%, 6% and 9%. Since malonate is an inhibitor of the citric acid cycle of tricarboxylic acid cycle (TCA cycle), certain degree of toxicity from the exogenously absorbed malonate is to be expected. Therefore, by cultivating the transformed yeast cells with varying concentration of malonate, the degree of toxicity from malonate on the growth and survival of the yeast cells can be monitored. Optical density at wavelength 600nm (OD_{600}) was used to determine the concentration of the yeast cells at the different time points. 1 value of OD_{600} corresponds to approximately 3×10^7 cells/ml.

Figure 17: Growth curve of yeast cells transformed with *mae1* gene with 1% malonate added at different time

Figure 17 showed the growth curve of 3 different cultures of yeast cells transformed with *mae1* gene with no malonate added, 1% malonate added at the 6th hour and 1% malonate added at the 12th hour respectively. From the curve it can be seen that, the 3 cultures grew as a relatively similar rate initially. However, the cultures with malonate added, started to grow as a slightly slower rate at their respectively time point when malonate was added. Finally, the 3 cultures reached stationary phase at around the 30th hour with each of their ending OD₆₀₀ values as shown below in Table 4.

Table 4: Stationary phase OD₆₀₀ values of yeast cells transformed with *mae1* gene with 1% malonate added at different time

Yeast culture	Stationary phase OD ₆₀₀ value
No malonate added	12.35
1% malonate added at 6th hour	9.37
1% malonate added at 12th hour	10.25

Similarly, Figure 18 to Figure 20 showed the growth curves of different sets of 3 cultures of yeast cells transformed with *mae1* gene with 3%, 6% and 9% malonate added at different time points respectively. Table 5 to Table 7 showed the stationary phase OD₆₀₀ values of those yeast cells with 3%, 6% and 9% malonate added at different time points respectively.

Figure 18: Growth curve of yeast cells transformed with *mae1* gene with 3% malonate added at different time

Table 5: Stationary phase OD₆₀₀ values of yeast cells transformed with *mae1* gene with 3% malonate added at different time

Yeast culture	Stationary phase OD ₆₀₀ value
No malonate added	12.4
3% malonate added at 6th hour	7.12
3% malonate added at 12th hour	8.74

Figure 19: Growth curve of yeast cells transformed with *mae1* gene with 6% malonate added at different time

Table 6: Stationary phase OD₆₀₀ values of yeast cells transformed with *mae1* gene with 6% malonate added at different time

Yeast culture	Stationary phase OD ₆₀₀ value
No malonate added	12.3
6% malonate added at 6th hour	2.36
6% malonate added at 12th hour	4.42

Figure 20: Growth curve of yeast cells transformed with *mae1* gene with 9% malonate added at different time

Table 7: Stationary phase OD₆₀₀ values of yeast cells transformed with *mae1* gene with 9% malonate added at different time

Yeast culture	Stationary phase OD ₆₀₀ value
No malonate added	12.3
9% malonate added at 6th hour	1.37
9% malonate added at 12th hour	4.21

From these results, it is evident that malonate does indeed possess a certain degree of toxicity to the overall growth and survival of the yeast cells. As shown in Figure 21, whenever malonate was introduced into the culture medium, the cells inevitably grew at a slower rate and attained a lower stationary phase OD₆₀₀ value as compared to cells that were growing in a malonate free environment.

However, yeast cells were still able to grow relatively well when 1% and 3% malonate was added with a relatively high stationary phase OD₆₀₀ values. Interestingly, the growth curves for the 3% malonate cultures, Figure 18, showed that the cells seemed to go through an intermittent stationary phase after malonate was added into the culture medium. This kind of intermittent stationary phase happens when the cells are adapting to changes to their surroundings. During this period, cell growth will usually halt while the cells access the changes and adapt accordingly. If the conditions are still favourable to growth, the cells will then continue to reproduce and grow to the true stationary phase. Such intermittent stationary phase, however, seemed to be absent in the cultures growing in 1% malonate. This may be due to the fact that the amount of malonate is too minute to totally halt growth but still significant enough to lower the final stationary phase OD₆₀₀ value.

Figure 21: Stationary phase OD₆₀₀ values of the different yeast cultures where varying concentrations of malonate (1%, 3%, 6% and 9%) were added at different time points

In contrast, the toxicity of malonate seemed to be more significant at 6% and 9% concentrations as shown by the results. From Figure 21, there is a drastic drop in stationary phase OD₆₀₀ values for cell cultures growing in 6% and 9% malonate concentration. As such, the optimal working concentration for malonate for subsequent experiments should be kept between 1% and 3%.

4.2.3 Proteomics study of the effects of malonate on the metabolic state of *Saccharomyces cerevisiae* using liquid chromatography–mass spectrometry (LC-MS)

LC-MS, being a powerful proteomics analyzing tool, was used to perform protein profiling experiments on wild type *Saccharomyces cerevisiae* cells and those that were transformed with the *mae1* gene. Samples were prepared as described. Wild type yeast cells were labelled with the iTRAQ tag 114 and those with the *mae1* gene and were expressing plasma membrane dicarboxylic acid transporter were labelled with iTRAQ tag 115. Results were analyzed with ProteinPilot Software 4.2 from Applied Biosystems. Relative abundances of each protein from the database were compared with the tag 114 forming the basis.

Similar experiments were repeated multiple times with 1% malonate, each time with freshly prepared samples. This is to ensure the accuracy and consistency of the results thus obtained. Two of the runs (Test 1 and Test 2) were used to do the final analysis. Around 170 proteins were detected from both Test 1 and Test 2. After comparing the 2

results, 32 proteins were identified as having significantly up-regulated or down-regulated.

Table 8 shows the proteins that were up-regulated when malonic acid was transported into the cells via the dicarboxylic acid transporter encoded by the *mae1* gene, as compared to wild type yeast cells with no dicarboxylic acid transporter. On the other hand, Table 9 shows the proteins that were down-regulated.

Table 8: Table of up-regulated proteins

No.	Gene Nomenclature	Name	Function	Test 1 (115:114) ratio	Test 2 (115:114) ratio
1	HSP12	12 kDa heat shock protein	Increases cell thermo resistance under heat shock or stress.	2.0137	2.3335
2	RS28A	40S ribosomal protein S28-A	Subunit of ribosomal protein.	8.3946	11.272
3	RL26B	60S ribosomal protein L26-B	Subunit of ribosomal protein.	1.3062	1.406
4	RL7B	60S ribosomal protein L7-B	Subunit of ribosomal protein.	2.4434	2.5823
5	ACBP	Acyl-CoA-binding protein	Binds medium- and long-chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters.	1.4588	1.5417
6	ATPB	ATP synthase subunit beta	Translocation of protons across the mitochondrial membrane.	1.5136	1.6144
7	CIS3	Cell wall mannoprotein CIS3	Component of the outer cell wall layer. Required for stability of the cell wall and for optimal growth. Required for resistance against several antifungal and cell wall-perturbing agents.	1.6749	1.8197
8	CIT1	Citrate synthase, mitochondrial	Catalyzes the production of citrate and CoA. Acetyl-CoA + H ₂ O + oxaloacetate = citrate + CoA.	1.803	2.0324

9	EF1A	Elongation factor 1-alpha	Responsible for the selection and binding of the cognate aminoacyl-tRNA to the A-site (acceptor site) of the ribosome. Elongation factors are responsible for achieving accuracy of translation.	1.783	1.967
10	GLRX2	Glutaredoxin-2, mitochondrial	A family of glutathione-dependent hydrogen donors that participate in a variety of cellular redox reactions	1.6904	1.7865
11	PMA1	Plasma membrane ATPase 1	A hydrogen ion pump. The proton gradient it generates drives the active transport of nutrients by H ⁺ -symport.	2.5586	2.5119
12	ACO1	Aconitate hydratase, mitochondrial	Catalyzes the isomerization of citrate to isocitrate via cis-aconitate, a step in the citric acid cycle.	2.208	2.2491
13	IPB2	Protease B inhibitors 2 and 1	Regulate protease B (yscB) activity during limited proteolysis. PBI2 is a componnet of the LMA1 complex, which is involved in the facilitation of vesicle fusion such as homotypic vacuole and ER-derived COPII vesicle fusion with the Golgi.	2.214	2.225
14	MDH1	Malate dehydrogenase, mitochondrial	Catalyzes the reaction: (S)-malate + NAD ⁺ = oxaloacetate + NADH	1.8197	1.9588
15	ZEO1	Protein ZEO1	Acts antagonistically to MID2 in signaling cell wall stress to the PKC1-MPK1 cell integrity pathway	1.873	2.051

16	KPYK1	Pyruvate kinase 1	An enzyme involved in glycolysis. It catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of ATP.	1.4454	1.5849
17	MLS1	Malate synthase 1, glyoxysomal	This isozyme is necessary for growth on acetate as sole C-source. (S)-malate from isocitrate	1.4997	1.5849
18	SDO1L	Restriction of telomere capping protein 3	Plays a role in RNA metabolism, rRNA-processing, and in a process influencing telomere capping.	1.516	1.5387
19	TRX1	Thioredoxin-1	Participates in various redox reactions through the reversible oxidation of its active center dithiol to a disulfide and catalyzes dithiol-disulfide exchange reactions.	2.6303	2.7797
20	ICL1	Isocitrate lyase	Catalyzes the formation of succinate and glyoxylate from isocitrate, a key step of the glyoxylate cycle, which operates as an anaplerotic route for replenishing the tricarboxylic acid cycle.	1.4191	1.5136

21	TRX2	Thioredoxin-2	<p>Participates as a hydrogen donor in redox reactions. It is involved in many cellular processes, including deoxyribonucleotide synthesis, repair of oxidatively damaged proteins, protein folding, sulfur metabolism, and redox homeostasis. Thioredoxin is also involved in protection against reducing stress. As part of the LMA1 complex, it is involved in the facilitation of vesicle fusion such as homotypic vacuole and ER-derived COPII vesicle fusion with the Golgi.</p>	3.5975	3.6644
22	YN034	Uncharacterized protein YNR034W-A	Occurs in the cytoplasm.	3.0761	3.3113
23	YP225	UPF0368 protein YPL225W	Occurs in the cytoplasm.	1.5235	1.5923

Table 9: Table of down-regulated proteins

No.	Gene Nomenclature	Name	Function	Test 1 (115:114) ratio	Test 2 (115:114) ratio
1	6PGD1	6-phosphogluconate dehydrogenase, decarboxylating 1	Catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO ₂ , with concomitant reduction of NADP to NADPH in pentose phosphate pathway.	0.6668	0.7112
2	SAHH	Adenosylhomocysteinase	May play a key role in the control of methylations via regulation of the intracellular concentration of adenosylhomocysteine.	0.6918	0.7311
3	ADH1	Alcohol dehydrogenase 1	This isozyme preferentially catalyzes the conversion of primary unbranched alcohols to their corresponding aldehydes. Also also shows activity toward secondary alcohols.	0.6982	0.5734
4	GAL1	Galactokinase	An enzyme in the the Leloir pathway to catalyzes the conversion of galactose to glucose.	0.1888	0.5861
5	HSP71	Heat shock protein SSA1	May play a role in the transport of polypeptides both across the mitochondrial membranes and into the endoplasmic reticulum. Can also participate in the ATP-dependent disassembly of clathrin-coated vesicles.	0.4055	0.4325

6	HXKB	Hexokinase-2	Main glucose phosphorylating enzyme. May play a regulatory role in both induction and repression of gene expression by glucose.	0.6668	0.7047
7	INO1	Inositol-3-phosphate synthase	Catalyzes the chemical reaction of converting D-glucose 6-phosphate to 1D-myo-inositol 3-phosphate. Needed to form phospholipids and its expression is sensitive to the availability of phospholipid precursors as well as growth phase.	0.3733	0.4093
8	PGM2	Phosphoglucomutase-2	This enzyme participates in both the breakdown and synthesis of glucose. Can also act on mannose.	0.6194	0.597
9	YNN4	Uncharacterized protein YNL134C	Occurs in the cytoplasm	0.3251	0.3436

From the tables, most of the up-regulated proteins were those that were involved one way or another in the metabolism of carbohydrates to produce energy. Among those proteins that were down-regulated, 6-phosphogluconate dehydrogenase was decreased by around 40%. This dehydrogenase catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO₂, with concomitant reduction of NADP to NADPH in the pentose phosphate. Furthermore, inositol-3-phosphate synthase, which catalyzes the chemical reaction of converting D-glucose 6-phosphate to 1D-myo-inositol 3-phosphate to form phospholipids, was also decreased by around 60%. This hinted at an energy deprived state of the cells where carbohydrates such as glucose seem to be channelled away from the other pathways and was used to increase the rate of glycolysis. Interestingly, proteins involved in the expression of proteins

from genes such as ribosomal proteins and elongation factors were increased on an average of 2-fold.

Another important protein that was up-regulated by around 3-fold was the plasma membrane ATPase. The plasma membrane ATPase of plants and fungi is a hydrogen ion pump. The proton gradient it generates drives the active transport of nutrients by H^+ - symport. Furthermore, the resulting external acidification and/or internal alkalinization may mediate growth responses. Such significant increase in a plasma membrane hydrogen pump signified an increased activity of plasma membrane transport of nutrients that use the proton symport mechanism. The plasma membrane dicarboxylic acid transporter encoded by the *mae1* gene that was successfully cloned and transformed into the target yeast cells also uses the proton symport mechanism [13]. Therefore, an increase in the proton symport activity not only proved the physical existence of dicarboxylic acid transporter on the plasma membrane of the yeast cells, but also proved that the transporter was performing its intended functions as a proton symporter to transport malonic acid across the plasma membrane of *Saccharomyces cerevisiae*.

Furthermore, due to the presence of malonate, the target yeast cells would have an impaired TCA cycle activity. From Table 8 and Table 9, there seemed to have no significant decrease in the concentration of enzymes of the TCA cycle. Interestingly, some of the enzymes involved in the TCA cycle namely, malate dehydrogenase, citrate synthase and aconitate hydratase increased by around 87%, 90% and 120% respectively as shown in Table 8. This is due to the fact that in an environment where

its TCA cycle activity is impaired, *Saccharomyces cerevisiae* will trigger an alternative mechanism known as the glyoxylate cycle to continue the supply of energy for the maintenance and survivability of the cells [43]. The glyoxylate cycle is a variation of the TCA cycle and is an anabolic metabolic pathway which focuses on the conversion of acetyl-CoA to succinate for the synthesis of carbohydrates and for energy production [44].

Figure 22: Pathway showing the TCA cycle. Enzymes in red signified an increase in concentration as shown in Table 8

Figure 23: Pathway showing the glyoxylate cycle. Enzymes in red signified an increase in concentration as shown in Table 8

Moreover, the glyoxylate cycle shares parts of the TCA cycle as shown in Figure 22 and Figure 23. Therefore, an increase in only three enzymes, malate dehydrogenase, citrate synthase and aconitate hydratase and not the other TCA cycle enzymes signified that the increase was not due to an increase in TCA cycle activity, but due to the increase in glyoxylate cycle as shown in Figure 22 and Figure 23. It can also be seen from Table 8 that there was an increase in the enzymes malate synthase by around 55% and isocitrate lyase by around 50%. Malate synthase and isocitrate lyase are key enzymes involved in the glyoxylate cycle, therefore an increase in these two enzymes alongside malate dehydrogenase, citrate synthase and aconitate hydratase further proved the onset of the glyoxylate cycle as shown in Figure 23.

4.2.4 Western blot validation of proteins identified by LC-MS

From the proteins that were significantly up-regulated and down-regulated when exogenous malonate was transported into the target *Saccharomyces cerevisiae* cells as shown in Chapter 4.2.3, the up-regulated malate dehydrogenase (65kDa) and the down-regulated alcohol dehydrogenase (80kDa) were chosen to perform quantitative western blot on as to validate the proteins identified by LC-MS in Chapter 4.2.3. Malate dehydrogenase was up-regulated by around 87% and the alcohol dehydrogenase was down-regulated by around 37%. As such, a quantitative western blot, where the intensity of the bands will be measured and quantified by the G:BOX machine from Syngene, would provide similar comparison as those provided by the LC-MS.

Actin (42kDa) was chosen to act as an internal control for this part of the study. As actin is essential for cell survival, it has been postulated that the quantity of actin is under such tight control at a cellular level that it can be assumed that its transcription and translation are practically constant and independent of experimental conditions. Therefore, it is common practice in protein quantification studies as such western blot and transcription studies such as quantitative PCR to carry out the quantification of the gene of interest and also the quantification of a reference gene such as the one that codes for actin. By dividing the quantity of the protein of interest by that of the actin protein it is possible to obtain a relative quantity that can be compared between different experiments whenever the expression of the latter is constant [46].

Proteins contents were compared between wild type *Saccharomyces cerevisiae* cells grown in culture medium containing 1% malonate (Lane 1), *Saccharomyces cerevisiae* cells transformed with *mae1* gene grown in culture medium containing no malonate (Lane 2) and *Saccharomyces cerevisiae* cells transformed with *mae1* gene grown in culture medium containing 1% malonate (Lane 3). Total proteins were extracted from these 3 different cell cultures as described in Chapter 2. Western blot procedures and analytical methods were described in Chapter 2 as well.

Figure 24: Western blot for the detection of alcohol dehydrogenase, malate dehydrogenase and actin (internal control) Lane 1 contained proteins from wild type *Saccharomyces cerevisiae* cells grown in culture medium containing 1% malonate; lane 2 contained proteins from *Saccharomyces cerevisiae* cells transformed with *mae1* gene grown in culture medium containing no malonate and lane 3 contained proteins from *Saccharomyces cerevisiae* cells transformed with *mae1* gene grown in culture medium containing 1% malonate

Figure 24 showed the western blots for the detection of actin, malate dehydrogenase and alcohol dehydrogenase. (*Individual western blots can be found in the Appendix*). As expected, the intensities of the 3 cell cultures in the actin western blot as shown in Figure 24 were very similar, signifying very similar level of concentration in the 3 cell cultures. This proved that actin is adequate as an internal control for this study and formed a basis of study for the comparison of the concentration levels of malate dehydrogenase and alcohol dehydrogenase among the 3 cell cultures.

From Figure 24, it is also evident that the intensities of the alcohol dehydrogenase bands for lane 1 and lane 2 are much brighter than that of lane 3. This sufficiently proved that alcohol dehydrogenase was down-regulated when *Saccharomyces cerevisiae* cells transformed with *mae1* gene were grown in culture medium containing 1% malonate. As for malate dehydrogenase, the intensity of the malate dehydrogenase band for lane 3 is much brighter than those of lane 1 and lane 2. Once again, this sufficiently proved that malate dehydrogenase was up-regulated when *Saccharomyces cerevisiae* cells transformed with *mae1* gene were grown in culture medium containing 1% malonate.

The intensity of each band in the malate dehydrogenase and alcohol dehydrogenase western blots would then be quantified by the imager machine and then divided by the intensity of the corresponding actin band to measure their respective relative abundance in the cell culture. Similar experiments were repeated multiple times, each time with freshly prepared samples. This is to ensure the accuracy and consistency of the results thus obtained.

Figure 25: Relative abundance of both malate dehydrogenase and alcohol dehydrogenase in the 3 cell cultures

Figure 25 showed the relative abundance normalized to the actin concentration of both malate dehydrogenase and alcohol dehydrogenase in the 3 different cell cultures. Evidently, by comparing the relative abundance of Lane 3 to Lane 1, it seemed that the concentration of malate dehydrogenase did indeed increase by around 87% and that the concentration of alcohol dehydrogenase did indeed decrease by around 37%. This result aligned with the results obtained by LC-MS in Chapter 4.2.3. Therefore, this western blots study validated the phenomenon whereby malate dehydrogenase was up-regulated by around 87% when intracellular malonate concentration was increased and that alcohol dehydrogenase was down-regulated by around 37% at the same growth conditions. Furthermore, Lane 2, which contained proteins from *Saccharomyces cerevisiae* cells transformed with *mae1* gene grown in culture medium containing no malonate, provided additional insight to the workings of the *mae1* gene when no malonate was added to the growth medium. Since Lane 2 generally has the same relative abundance as Lane 1, it seemed that with the absence of malonate, the

dicarboxylic acid transporter encoded by the *mae1* gene did not provide any additional stress or changes to the cells' metabolic state and the cells generally behaved like wild type cells.

4.3 Section conclusion

In summary, this section showed that wild type *Saccharomyces cerevisiae* generally has little to no intracellular malonate and that wild type yeast cells are unable to transport in exogenously supplied malonate in the culture medium. As such, the dicarboxylic acid transporter encoded by the transformed *mae1* gene proved successful in transporting in malonate from the culture medium.

However, being an inhibitor to the TCA cycle, malonate exhibited a certain degree of toxicity towards the growth and survival of the yeast cells when their intracellular concentration was increased. From the LC-MS results, most of the up-regulated proteins were those that were involved one way or another in the metabolism of carbohydrates to produce energy. Among those proteins that were down-regulated, 6-phosphogluconate dehydrogenase was decreased by around 40%. This dehydrogenase catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO₂, with concomitant reduction of NADP to NADPH in the pentose phosphate. Furthermore, inositol-3-phosphate synthase, which catalyzes the chemical reaction of converting D-glucose 6-phosphate to 1D-myo-inositol 3-phosphate to form phospholipids, was also decreased by around 60%. This hinted at an energy deprived state of the cells where carbohydrates such as glucose seem to be channeled away from the other pathways and was used to increase the rate of glycolysis.

Furthermore, from the proteins that were significantly up-regulated and down-regulated, proteins that were involved in the glyoxylate cycle were the most prominent. All five proteins that were involved in the glyoxylate cycle were significantly up-regulated as demonstrated in Chapter 4.2.3. Thus, this phenomenon provided an interesting insight into the metabolic state of the yeast cells when intracellular concentration of malonate was increased. This group of glyoxylate proteins was the most iconic among the proteins that were significantly up-regulated and down-regulated.

5. Expression of the malonyl-CoA synthetase encoded by the *matB* gene and the impact of the enzyme on the overall production of fatty acids

5.1 Introduction

Malonyl-CoA plays an important role in the synthesis and elongation of fatty acids in yeast *Saccharomyces cerevisiae*. It is one of the main components for the initiation of the fatty acid synthesis and also acts as a building block for the elongation of fatty acid after every round fatty acid synthesis. However, Malonyl-CoA is at a low concentration inside the cell and it is produced mainly from Acetyl-CoA through the actions of the enzyme acetyl-CoA carboxylase (ACC) [8].

Malonyl-CoA synthetase is an enzyme that catalyzes the formation of malonyl-CoA directly from malonate and CoA with the hydrolysis of ATP into AMP and PPi in the presence of Mg²⁺ as shown as the reaction below [12].

This enzyme was first discovered in the bacteroids, *Bradyrhizobium japonicum*, of soybean nodules. The malonate-specific enzyme has long been expected to exist in nodules since free malonate is known to occur in legumes, and its level increases under symbiotic conditions. It was also reported that in the symbiotic hostplant cell,

malonate is passively transported into bacteroids. However, nothing is known about the fate of malonate in bacteroids. This enzyme was first purified from the symbiotic bacteria *B. japonicum* that is grown on a GYP medium, and later from *Rhizobium leguminosarium* bv *trifolii*, which has symbiosis with clover. The high substrate specificity of malonate, CoA and ATP, has been revealed, but Mn^{2+} could be substituted for Mg^{2+} with no difference in activity. Also, during the catalysis, malonyl-AMP is formed as a reaction intermediate [12].

MatB gene encoded a polypeptide of 504 amino acid residues with a deduced molecular mass of 54,612 Da. This gene was expressed in *E. coli* and characterized to be essentially identical to the native malonyl-CoA synthetase. *MatC* encoded a 46,453Da protein with a high content of hydrophobic residues. It showed similarities to the dicarboxylate carrier protein, indicating that it might be a malonate carrier protein. These results strongly suggest that the gene cluster encodes proteins that are involved in the malonate-metabolizing system, where exogenous malonate is transported into the cells and is used to produce malonyl-CoA and acetyl-CoA in *R. leguminosarium* bv *trifolii*. Also, the metabolic pathway in the malonate-rich clover nodule might play an important role in symbiosis [12].

As such, the *matB* gene which encodes for the malonyl-CoA synthetase can be utilized through genetic engineering to enable our target yeast cell, *Saccharomyces cerevisiae* to increase the production of malonyl-CoA using exogenous malonate. With the enhanced production of malonyl-CoA, the target of increasing the overall fatty acids production can be achieved and this forms the basis of the project.

5.2 Results and discussions

5.2.1 Western blot validation of the expression of the malonyl-CoA synthetase encoded by the *matB* gene

Gene sequencing results on the plasmid extracts from *E. coli* cells transformed with the final recombinant vector showed there is no site mutation along the pESC-LEU vector and the gene of interest, *matB* gene. As a result, the vector was introduced into the final target host, *Saccharomyces cerevisiae* that already had *mae1* gene, which codes for the dicarboxylic acid transporter, transformed into it.

Saccharomyces cerevisiae cells with *mae1* gene were further transformed with the pESC-LEU plasmid containing the *matB* gene and were grown and selected as described in Chapter 2. After that, the cells were harvested and colony PCR was performed to verify whether the cells from the selected colonies contain the target gene *mae1* and *matB*. Primers used in colony PCR were all around 25 nucleotides long and synthesized by 1st BASE Pte. Ltd. Furthermore, the presence of malonyl-CoA synthetase encoded by *matB* was confirmed by western blot via a C-terminal 6xHis epitope tag.

Figure 26: Western blot using anti 6xHis antibody. Lane 1 contained proteins from wild type *Saccharomyces cerevisiae* cell culture; lane 2 contained proteins from *Saccharomyces cerevisiae* transformed with the *mae1* gene and lane 3 contained proteins from *Saccharomyces cerevisiae* transformed with both the *mae1* gene and the *matB* gene

As shown in Figure 26, lane 1 and lane 2 served as negative control in this western blot. Lane 1 contained proteins from wild type *Saccharomyces cerevisiae* cell culture; lane 2 contained proteins from *Saccharomyces cerevisiae* transformed with only the *mae1* gene and lane 3 contained proteins from *Saccharomyces cerevisiae* transformed with both the *mae1* gene and the *matB* gene. Therefore, this showed that the *matB* gene had been successfully cloned inside the target yeast cells and the expression of the malonyl-CoA synthetase was verified.

5.2.2 Utilization and toxicity of malonate inside genetically engineered *Saccharomyces cerevisiae* with the presence of malonyl-CoA synthetase.

In Chapter 4, the degree of toxicity of malonate inside target *Saccharomyces cerevisiae* cells transformed with only the *mae1* gene was explored and demonstrated. However, when *matB* gene was transformed into the same yeast cells used in Chapter 4, it will encode for a malonyl-CoA synthetase to utilize the intracellular malonate to produce malonyl-CoA. The intracellular malonate was transported into the cells

cytoplasm from the surrounding culture medium through the expression of a dicarboxylic acid transporter encoded by the *mae1* gene previously cloned inside the same cells. As such, the toxicity demonstrated by the cells with only the *mae1* gene should decrease when *matB* gene is cloned into the same cells.

Figure 27: Growth curves comparison between yeast cells transformed with *mae1* gene only (1 gene) and yeast cells transformed with both *mae1* and *matB* gene (2 genes). 1% malonate was added at different time points for each of the data.

Figure 28: Stationary phase OD₆₀₀ values comparison between yeast cells transformed with *maeI* gene only (1 gene) and yeast cells transformed with both *maeI* and *matB* gene (2 genes). 1% malonate was added at different time points for each of the data. ($p < 0.05$)

Figure 27 and Figure 28 clearly showed that the yeast cells with 2 genes (*maeI* and *matB*) had a better growth rate and stationary phase OD₆₀₀ values as compared to the yeast cells with only 1 gene (*maeI* gene) when 1% malonate was added to the various time points. Moreover, it was interesting to note that when the malonate was added at a later time point, the cells growth was more and more similar to that when no malonate is added and the stationary phase OD₆₀₀ values became closer in value. This clearly indicated a decrease in toxicity of the intracellular malonate. The effect of such decrease in toxicity is more evident as the concentration of malonate added was increased to 3% as shown in Figure 29 and Figure 30. This may indicate that when the amount of malonate taken in by the cells increases, the amount of malonate utilized by the malonyl-CoA encoded by the *matB* gene also increases and thus the decrease in toxicity becomes more prominent.

Figure 29: Growth curves comparison between yeast cells transformed with *maeI* gene only (1 gene) and yeast cells transformed with both *maeI* and *matB* gene (2 genes). 3% malonate was added at different time points for each of the data.

Figure 30: Stationary phase OD₆₀₀ values comparison between yeast cells transformed with *maeI* gene only (1 gene) and yeast cells transformed with both *maeI* and *matB* gene (2 genes). 3% malonate was added at different time points for each of the data. ($p < 0.05$)

Figure 31: Growth curves comparison between yeast cells transformed with *maeI* gene only (1 gene) and yeast cells transformed with both *maeI* and *matB* gene (2 genes). 6% malonate was added at different time points for each of the data.

Figure 32: Stationary phase OD₆₀₀ values comparison between yeast cells transformed with *maeI* gene only (1 gene) and yeast cells transformed with both *maeI* and *matB* gene (2 genes). 6% malonate was added at different time points for each of the data. ($p < 0.05$)

Figure 33: Growth curves comparison between yeast cells transformed with *maeI* gene only (1 gene) and yeast cells transformed with both *maeI* and *matB* gene (2 genes). 9% malonate was added at different time points for each of the data.

Figure 34: Stationary phase OD₆₀₀ values comparison between yeast cells transformed with *maeI* gene only (1 gene) and yeast cells transformed with both *maeI* and *matB* gene (2 genes). 9% malonate was added at different time points for each of the data. ($p < 0.05$)

However, when the malonate concentration added was increased to 6% and 9%, the yeast cells with 2 genes exhibited the same growth patterns as those yeast cells with only 1 gene in Chapter 4. This is shown in the results obtained as shown from Figure 31 to Figure 34. This further reinforced the observation that 6% and more malonate concentration is the threshold value for the yeast cells to grow healthily even in the presence of malonyl-CoA synthetase. This may be due to the fact that the rate of uptake and accumulation of intracellular malonate is much faster than the rate of utilization of the same malonate by the malonyl-CoA synthetase to produce malonyl-CoA. It may also mean that the rate of utilization of malonyl-CoA itself for fatty acid synthesis and other metabolic activities is slower and this induced a negative feedback to the whole metabolism and slowed down the production of malonyl-CoA. This is further reinforced by Figure 35. (*individual growth curves with the yeast cells with both genes can be found at the Appendix.*)

Figure 35: Stationary phase OD₆₀₀ values of the different yeast cultures with 2 genes (*mae1* and *matB*) where varying concentration of malonate (1%, 3%, 6% and 9%) were added at different time points

As compared to a similar profile of yeast cultures with only 1 gene transformed (*mae1* gene) as shown in Figure 21, Figure 35 showed a much larger drop in the stationary OD₆₀₀ values when the concentration of malonate was increased to 6% and 9%. This establishes the point that the threshold value is around 6% malonate concentration and the optimal working concentration for malonate is around 1% to 3%.

5.2.3 Fatty acid detections and quantifications using HPLC

The expression of dicarboxylic acid transporter as well as the malonyl-CoA synthetase as encoded by the *mae1* gene and *matB* gene respectively in the target *Saccharomyces cerevisiae* cells decreased the toxicity of intracellular malonate as long as the concentration of malonate in the culture medium does not reach 6% and more as demonstrated in Chapter 5.2.2. This proved that the malonate transported in from the exogenous culture medium is being utilized and removed from the cytoplasm to reduce its toxicity. In this case, malonyl-CoA synthetase encoded by the *matB* gene will utilize the intracellular malonate and convert it into malonyl-CoA which will be further utilized by the cells to act as a building block for the elongation of the various fatty acids.

Fatty acid samples were prepared from wild type yeast cells and yeast cells with both the *mae1* gene and the *matB* gene as described in Chapter 2. After the fatty acids were obtained, the samples were analysed and quantified by HPLC to detect the various

fatty acids present in the different yeast cells. The procedures were described in Chapter 2.

Figure 36: Fatty acids detected by HPLC from samples prepared from wild type *Saccharomyces cerevisiae* cells.

Figure 37: Fatty acids detected by HPLC from samples prepared from *Saccharomyces cerevisiae* transformed with the *mae1* gene only and grown in 1% malonate.

Figure 38: Fatty acids detected by HPLC from samples prepared from *Saccharomyces cerevisiae* transformed with both the *mae1* gene and the *matB* gene and grown in 1% malonate.

Figure 36 showed the HPLC graph of the various fatty acid species detected from samples prepared from wild type *Saccharomyces cerevisiae* cells. Figure 37 showed the HPLC graph of the various fatty acid species detected from samples prepared from *Saccharomyces cerevisiae* transformed with the *mae1* gene only and grown in 1% malonate. Figure 38 showed the HPLC graph from samples prepared from *Saccharomyces cerevisiae* transformed with both the *mae1* gene and the *matB* gene and grown in 1% malonate.

From the graphs, it is evident that there is an overall increase in the accumulation of fatty acids inside the yeast cells when both the *mae1* gene and *matB* gene were cloned inside the target yeast cells grown in 1% malonate. However, interestingly, there seemed to be a decrease in higher carbon chain fatty acids especially oleic acid, stearic acid and linoleic acid when only the *mae1* gene was cloned inside the target yeast cells grown in 1% malonate.

Figure 39: Quantified fatty acids profile from the HPLC results. ($p < 0.05$)

Figure 39 showed the quantified fatty acids profile obtained from the HPLC for the 3 cell cultures. There is a clear increase in the overall concentration of the various fatty acids species inside the engineered yeast cells with both the *maeI* gene and the *matB* gene cloned inside as compared to a wild type *Saccharomyces cerevisiae* cells. Although some of the increase is not as significant but the biggest increase is as high as 50% as shown by the stearic acid (18:0). Moreover, there was a significant decrease in fatty acids such as oleic acid, stearic acid and linoleic acid when only the *maeI* gene was cloned inside. This is evident that the toxicity of malonate without the presence of a malonyl-CoA synthetase was affecting the usual fatty acid accumulation of the yeast cells. Interestingly, only the higher carbon chains fatty acids were affected, which meant that the fatty acid synthesis was still functioning relatively normally in the initial stages however, the elongation of longer chain fatty acids became induced due to the toxicity of the intracellular malonate.

This is a very significant results which also showed that the malonyl-CoA synthetase expressed in the engineered yeast cells are exhibiting its function as intended and utilizing the intracellular malonate to produce malonyl-CoA which in turns decrease the toxicity in the cells and increased the overall production of the fatty acids inside the cells.

5.3 Section conclusion

In summary, the introduction of a malonyl-CoA synthetase as encoded by the cloned *matB* gene decreases the overall toxicity of the intracellular malonate transported in from the culture medium by the dicarboxylic acid transporter encoded by the previously cloned *mae1* gene. This hinted that the exogenously absorbed malonate is being utilized to form other substances thus decreasing its toxicity. However, the threshold malonate concentration for the cells to continue to grow healthily remained at 6% and above regardless of the presence of the malonyl-CoA synthetase.

Furthermore, HPLC results had shown that the overall concentration of fatty acids inside the yeast cells had increased in yeast cells transformed with both the *mae1* gene and the *matB* gene. Together with the data showing the decrease in toxicity of intracellular malonate, this provided proof that the functional expression of the malonyl-CoA synthetase does indeed utilize the transported in malonate to form malonyl-CoA which in turns contributed to the overall increase in the fatty acids concentration inside the cells. This fulfilled the overall aim of this project.

6. Conclusion

From the results of the experiments thus far, it could be concluded that the *mae1* gene from another species of yeast, *Schizosaccharomyces pombe* had been successfully cloned and transformed inside the target yeast, *Saccharomyces cerevisiae* via the vector, pYES2/CT. Prior to transforming into the yeast cells, the recombinant vector was cloned inside *E. coli* first. Sequencing results from the plasmids extracted from the *E. coli* cells proved that there was no mutation in the *mae1* gene inside the recombinant construct.

The *mae1* gene encodes a plasma membrane dicarboxylic acid transporter which has the ability to transport exogenous dicarboxylic acid such as malonic acid into the cell's cytoplasm. Various difficulties were encountered when trying to verify the expression of *mae1* gene or the existence of the dicarboxylic acid transporter inside the yeast cells. Western blot technology is the well-established and recognized method to verify, identify and quantify the presence of the proteins of interest. However, due to the nature of membrane proteins, problems were encountered when trying to extract significant amount of the target membrane proteins to be used in the Western blot.

As such, RT-PCR was used instead of Western blot in hopes to prove the existence of the target protein inside the cells. However, RT-PCR could not be used as a concrete proof of the existence of the dicarboxylic acid transporter on the cell membrane. Due

to the nature of RT-PCR, its results were only substantial to prove that there was expression of the *mae1* gene that encodes for the dicarboxylic acid. However, it could not be used to prove the actual existence of the encoded functional protein as the newly translated protein from ribosomes still have to go through several steps, such as folding to become the final functional form. Therefore, yeast immunofluorescence was used to detect the presence and localization of the expressed proteins in the target cells. The results had convincingly showed that the *mae1* gene is successfully expressed and the expressed dicarboxylic acid transporter proteins were localized to the plasma membrane of the cells as intended.

In view of this, HPLC and LC-MS were also able to provide enough results to prove the existence of the encoded protein, which is the plasma membrane dicarboxylic acid transporter. With the correct negative controls within HPLC and LC-MS, the functional activities of the protein could also be demonstrated and verified. Therefore, the positive results from HPLC and LC-MS, together with the positive results from RT-PCR, the plasma membrane dicarboxylic acid transporter was verified to be successfully expressed and functioning as intended as malonic acid was detected inside the transformed cells and having an significant impact on the proteomics of the cells as demonstrated by the LC-MS results.

Being an inhibitor to the succinate dehydrogenase of the citric acid cycle in the mitochondria [42], malonic acid, after being transported into the yeast cells, seem to have a certain degree of toxicity displayed towards the cells. From the LC-MS results, most of the up-regulated proteins were those that were involved one way or another in

the metabolism of carbohydrates to produce energy. Among those proteins that were down-regulated, 6-phosphogluconate dehydrogenase was decreased by around 40%. This dehydrogenase catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO₂, with concomitant reduction of NADP to NADPH in the pentose phosphate. Furthermore, inositol-3-phosphate synthase, which catalyzes the chemical reaction of converting D-glucose 6-phosphate to 1D-myo-inositol 3-phosphate to form phospholipids, was also decreased by around 60%. This hinted at an energy deprived state of the cells where carbohydrates such as glucose seem to be channeled away from the other pathways and was used to increase the rate of glycolysis.

Furthermore, due to the presence of malonate, the target yeast cells would have an impaired TCA cycle activity. From Table 8 and Table 9, there seemed to have no significant decrease in the concentration of enzymes of the TCA cycle. Interestingly, some of the enzymes involved in the TCA cycle namely, malate dehydrogenase, citrate synthase and aconitate hydratase increased by around 87%, 90% and 120% respectively as shown in Table 8. This is due to the fact that in an environment where its TCA cycle activity is impaired, *Saccharomyces cerevisiae* will trigger an alternative mechanism known as the glyoxylate cycle to continue the supply of energy for the maintenance and survivability of the cells [43]. The glyoxylate cycle is a variation of the TCA cycle and is an anabolic metabolic pathway which focuses on the conversion of acetyl-CoA to succinate for the synthesis of carbohydrates and for energy production [44].

Moreover, the glyoxylate cycle shares parts of the TCA cycle as shown in Figure 22 and Figure 23. Therefore, an increase in only three enzymes, malate dehydrogenase, citrate synthase and aconitate hydratase and not the other TCA cycle enzymes signified that the increase was not due to an increase in TCA cycle activity, but due to the increase in glyoxylate cycle as shown in Figure 22 and Figure 23. It can also be seen from Table 8 that there was an increase in the enzymes malate synthase by around 55% and isocitrate lyase by around 50%. Malate synthase and isocitrate lyase are key enzymes involved in the glyoxylate cycle, therefore an increase in these two enzymes alongside malate dehydrogenase, citrate synthase and aconitate hydratase further proved the onset of the glyoxylate cycle as shown in Figure 23.

According to a research on the compensatory role of succinate oxidation to aging *Saccharomyces cerevisiae* with impaired mitochondria activity [43], it was found out that when the critic acid cycle was impaired due to post-mitotic aging or a result of activity from inhibitors such as malonate, alternative mechanism would be triggered to continue supply energy for the survivability of the cells. In this case, the glyoxylate cycle is activated. The glyoxylate cycle, a variation of the critic acid cycle, is an anabolic metabolic pathway occurring in plants, bacteria, protists, and fungi. The glyoxylate cycle centers on the conversion of acetyl-CoA to succinate for the synthesis of carbohydrates and for energy production [44]. In microorganisms, the glyoxylate cycle allows cells to utilize simple carbon compounds as a carbon source when complex sources such as glucose are not available [45]. As a result, glyoxylate cycle, through the production of succinate, is a reliable alternative pathway to produce energy while TCA cycle in the mitochondria is impaired as demonstrated by the

results from the LC-MS. On the other hand, malonate, while displaying certain degree of toxicity to the yeast cells, is not totally lethal to the cells.

Figure 40: Impact of malonate accumulated on *Saccharomyces cerevisiae* cells cloned with both *mae1* gene and the *matB* gene and grown in culture medium containing malonate.

Figure 40 showed the overall impact of exogenously absorbed malonate on *Saccharomyces cerevisiae* cells cloned with both *mae1* gene and the *matB* gene and grown in culture medium containing malonate. Furthermore, inhibition of malonate on the TCA cycle leads to the accumulation of succinate and acetyl-CoA. This indirectly leads to the inhibition of the fatty acid beta oxidation and pyruvate decarboxylation. Furthermore, an excess of acetyl-CoA will lead to an increase in the conversion to malonyl-CoA through the action of ACC enzyme which in turn, further increasing the overall synthesis of fatty acids. Excess acetyl-CoA will also start to react with itself to

form acetoacetyl-CoA. Acetoacetyl-CoA is relatively unstable and will eventually break down into acetoacetate and CoA. This in turn leads to an accumulation of free CoA. This increased pool of free CoA will further enhance the validity of my project by providing CoA for my cloned enzyme, malonyl-CoA synthetase to produce more malonyl-CoA, further increasing the rate of fatty acid formation and potential accumulation of specific C8 fatty acids [47].

The introduction of a malonyl-CoA synthetase as encoded by the cloned *matB* gene decreases the overall toxicity of the intracellular malonate transported in from the culture medium by the dicarboxylic acid transporter encoded by the previously cloned *maeI* gene. This hinted that the exogenously absorbed malonate are being utilized to form other substances thus decreasing its toxicity. However, the threshold malonate concentration for the cells to continue to grow healthily remained at 6% and above regardless of the presence of the malonyl-CoA synthetase.

In conclusion, HPLC results had shown that the overall concentration of fatty acids inside the yeast cells had increased in yeast cells transformed with both the *maeI* gene and the *matB* gene. Together with the data showing the decrease in toxicity of intracellular malonate, this provided proof that the functional expression of the malonyl-CoA synthetase does indeed utilize the transported in malonate to form malonyl-CoA which in turns contributed to the overall increase in the fatty acids concentration inside the cells. As such, this fulfilled the overall aim of this project.

7. Future work

Rhizobium leguminosarium bv *trifolii*, where its *matB* gene encodes for malonyl-CoA synthetase (which is the focus of this project), also has another gene, *matC* gene which encodes for another plasma membrane dicarboxylic acid capable to transporting malonate across the membrane. As a result, *matC* gene could be studied and be cloned and transformed separately into the *Saccharomyces cerevisiae*, and a comparison study will be done to compare the efficiency of these 2 dicarboxylic acid transporters at uptaking exogenous malonic acid. Tests will be run to verify the presence of these cloned genes and to verify their intended functions inside the cells and toxicity caused to the cells if any. Once all verification tests are done, an overall increase in the production in Malonyl-CoA by exogenously supplying malonate would be expected. Following which, an overall increase in the production of fatty acid within the cells would then be expected again.

There are multiple sources of malonate commercially available now. As such, economical sources should be explored in order to serve as cheap feedstock to produce the desired fatty acids and ultimately, biofuels. A classical preparation of malonic acid starts from chloroacetic acid and chloroacetic acid is commercially available which makes it an attractive candidate to serve as feedstock for the project. Moreover, the calcium salt of malonic acid occurs naturally in beetroot, but the acid itself is also usually prepared by hydrolyzing diethyl malonate. Diethyl malonate, also known

as DEM, is the diethyl ester of malonic acid. It occurs naturally in the oil of nuts and in fruit juices such as grapes and strawberries juice with a fruity smell. As such, these alternatives feedstock to malonate should be studied together with the current project to test the viability of the cells when fed with such cheaper feedstock. This will serve as a basis for a scale-up operation in the future to produce biofuels.

The accumulated fatty acids from our results could also be used as precursors for biofuel production. However, *Saccharomyces cerevisiae* itself cannot efficiently transform the accumulated fatty acids to useful fuels. The strategies to produce biofuels from these precursors need to be carried out in the *Saccharomyces cerevisiae* cells.

Inspired from our research work, the destination of fatty acids in the yeast cells can be regulated. The designed pathway to turn TAGs storage forms to fatty acyl-CoAs activated forms will be combined with the enhanced fatty acid production pathways so as to efficiently convert the fatty acids into the useful fatty acid-derived fuels and chemicals.

The carboxylic acid reductase has also been discovered as a versatile enzyme, which can directly convert the fatty acids into the corresponding fatty aldehydes [48]. Together with the enzymes of aldehyde reductase or aldehyde decarbonylase, the intermediates can be finally converted to fatty alcohols or alkanes. This kind of

enzyme system is powerful and they will be constructed in the yeast cells to serve as a biofuel production platform.

Finally, the ultimate goal of the research project is to realize the industrial microbial production of biofuels. Hence, the effects of culture condition such as the nutrient concentration, temperature, pH and oxygen content on the scale up fermentation capacity and productivity of the engineered *Saccharomyces cerevisiae* need to be studied. The yields of the industrial production of biofuels are expected to be increased by achieving the optimal production conditions.

Moreover, the downstream separation and purification process also act as a dominant role in the industrial production of chemicals and fuels. The studies to find out efficient strategies for the separation and purification of biofuels from the industrial fermentation process need to be carried out. The way to realize the production and application of biofuels will be shorted based on persevere research works.

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Appendix

Western blot for the detection of Actin:

Western blot for the detection of alcohol dehydrogenase:

Western blot for the detection of malate dehydrogenase:

Growth curve of *Saccharomyces cerevisiae* cells with 1% malonate added at different time points:

Growth curve of *Saccharomyces cerevisiae* cells with 3% malonate added at different time points:

Growth curve of *Saccharomyces cerevisiae* cells with 6% malonate added at different time points:

Growth curve of *Saccharomyces cerevisiae* cells with 9% malonate added at different time points: